DEFINING THE RELATIONSHIP BETWEEN THE HOMEOBOX GENE *VSX2* AND EXTRINSIC SIGNALING IN THE REGULATION OF RETINAL PROGENITOR CELL PROPERTIES

by

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ABSTRACT

Development of the neural retina is a complex process requiring step-wise induction of specified retinal progenitor cells (RPCs) from the neural ectoderm and their coordinated proliferative expansion and differentiation into the mature neurons and glia of the adult retina. The homeobox gene Vsx2 is expressed in RPCs and required for proper execution of this retinal program. In the absence of Vsx2 function, maintenance of retinal identity, RPC proliferation, and retinal neurogenesis are disrupted, with serious consequences on overall ocular development and visual function. Despite the obvious importance of Vsx2, an understanding of the molecular mechanisms by which Vsx2 regulates these processes is lacking and few direct targets have been identified. To further define the role of Vsx2, we sought to determine the relationship between Vsx2 and the extrinsic signaling pathways regulating RPC properties. Through the analysis of genetic chimeras and candidate signaling pathways, we evaluated the contribution of Vsx2 to the regulation of extrinsic signaling pathways involved in the cellular processes of retinal development. We find that Vsx2 mediates the response of RPCs to the signals driving retinal specification and maintenance, largely through cell autonomous repression of the RPE determinant Mitf. We also find that Vsx2-deficient RPCs exhibit a robust cell autonomous delay in the initiation of retinal neurogenesis, revealing an essential role for Vsx2 in the temporal regulation of neurogenic competence. In contrast, we find that regulation of RPC proliferation by Vsx2 involves significant cell nonautonomous

contributions, suggesting an important role for Vsx2 in regulating the availability of retinal proliferation signals. Analysis of the retinal mitogen Sonic Hedgehog (Shh) and its signaling pathway in Vsx2-deficient retinas further supported this role, demonstrating that Vsx2 is required to ensure sufficient availability of Shh. We also describe a potential role for Vsx2 in the regulation of RPC responsiveness to Hedgehog (Hh) pathway stimulation. Taken together, the data presented in this dissertation demonstrate the requirement for Vsx2 in promoting both the reception and availability of the extrinsic signals necessary for the regulation of RPC properties, thereby ensuring the proper growth and differentiation of this important sensory tissue.

This dissertation is dedicated to my loving husband and family.

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CHAPTER 1

INTRODUCTION

Functional anatomy of the retina

The retina is the sensory neural tissue of the eye responsible for mediating the initial steps in vision. In the vertebrate eye, the retina lines the inner surface of the posterior aspect of the eyeball (Figure 1.1A). The retina is a laminated tissue composed of six major classes of retinal neurons, which include retinal ganglion cells, amacrine cells, horizontal cells, bipolar cells, and cone and rod photoreceptors (Figure 1.1B). These neurons are organized into three cellular layers separated by two neuropil layers where synaptic contacts are made between cell types. An important glial class in the retina is a type of radial glia known as Müller glia, whose processes span the three cell layers and provide important support functions for adjacent neurons. Microglia and astrocytes are also dispersed throughout the retina, migrating in from extraretinal sources. Basally, the retina bounds the vitreous body, an aqueous chamber filling the main cavity of the eyeball. Apically, the retina abuts the retinal pigmented epithelium (RPE), a pigmented epithelial monolayer providing important support functions for the retina.

The neural retina is a sensory tissue specialized for the detection, transduction, and transmission of visual information. Light entering the eye through the pupil is focused by the lens onto the retina (Figure 1.1A). Photons enter the retina from the vitreal side and pass through its transparent inner layers to the light-detecting cells in the outer nuclear layer (Figure 1.1B). Photoreceptors located in the outer nuclear layer are photosensitive by virtue of visual pigments in their outer segments that undergo a conformational change in response to the absorption of light photons. Photoreceptors transduce these light signals into electrical signals, which they then transmit to ganglion cells in the ganglion cell layer via electrical and chemical synapses with the horizontal,

bipolar, and amacrine interneurons of the inner nuclear layer. The functional circuits created by specific combinations of connections with these interneurons also provide initial processing of the visual image during this transmission. The resultant visual information is transmitted by ganglion cells, the projection neurons of the retina, via their axons, which exit the eye at the optic disc and travel through the optic nerve to visual processing centers of the brain. The structure and organization of the retina and its component parts are essential for their proper function. Thus, disruptions in the development of this tissue have severe consequences on visual function.

Vertebrate retinal development

Development of the neural retina is a complex process requiring precise regulation of many cellular processes and gene functions. This process can be divided into two main developmental periods. In the first, the retinal fate is established through a step-wise induction process and associated with dramatic morphological changes. During the subsequent period of retinal histogenesis, this tissue undergoes extensive growth and maturation.

Step-wise induction of retinal fate

Eye field specification. Induction of the retinal fate initiates early in vertebrate embryonic development, prior to overt morphological evidence of eye formation, with specification of the eye field. Early embryological studies in amphibian and chick embryos identified the existence of an eye anlagen, or eye field, in the anterior neuroepithelium of neurula stage embryos, which exhibits eye forming potential (Figure

1.2A) [reviewed in (Adelmann, 1936a, b)]. Studies in *Xenopus* identified a group of transcription factors whose overlapping expression patterns define the eye field (Zuber et al., 2003). Collectively, these genes are known as the vertebrate eye field transcription factors (EFTFs) and include *ET*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *tll*, and *Optx2*. These genes form a self-regulating transcriptional network that specifies the eye field (Zuber et al., 2003). Coordinated overexpression of these EFTFs together is sufficient to induce ectopic eyes even in nonneural tissues of the *Xenopus* embryo (Zuber et al., 2003) and to direct pluripotent cells from *Xenopus* animal caps to generate ectopic eye-like structures when transplanted into nonneural regions, as well as morphologically and functionally normal eyes when transplanted in place of the host eye field (Viczian et al., 2009). Specification of the eye field by EFTFs is highly conserved. A similar model of coordinated gene expression driving eye field specification has been proposed in *Drosophila*, involving homologs of the vertebrate EFTFs (Kumar, 2009). Many of these EFTFs are also critical for eye formation in the mouse. *Rx*, *Pax6*, *Six3*, and *Lhx2* mouse mutants exhibit anophthalmia (absence of eyes) (Hill et al., 1991; Hogan et al., 1986; Lagutin et al., 2003; Mathers et al., 1997; Porter et al., 1997; Tucker et al., 2001). Detailed analyses of eye formation in these mice reveal that *Pax6* and *Lhx2* null mouse mutants progress past eye field specification and arrest in subsequent stages of retinal development (Baumer et al., 2003; Hill et al., 1991; Porter et al., 1997; Yun et al., 2009). Failure of *Rx* and *Six3* null mouse mutants to also progress into subsequent stages of retinal development suggests that Rx and Six3 may be the key mediators of eye field specification. In support of such a role for Rx, *Rx* null cells are excluded from the eye field in genetic chimeras, suggesting that they lack eye field identity (Medina-Martinez et al., 2009). Despite early expression

in the eye field, *Optx2*/*Six6* and *Tll* are also not required for early specification of the eye field, as eye formation occurs in *Six6* and *Tll* null mouse mutants (Li et al., 2002; Yu et al., 2000).

Many extrinsic signals have been implicated in the induction of the eye field. Most are known neuralizing signals, involved in directing anterior neural fate. Misexpression of the secreted BMP inhibitors *noggin* and *chordin* (in the presence of *Otx2*) induce expression of EFTFs in pluripotent cells of *Xenopus* animal caps (Zuber et al., 2003). Furthermore, Noggin also directs these cells to a retinal fate and eye formation (Lan et al., 2009; Viczian et al., 2009). Multiple members of Wnt/Fz signaling have also been implicated. Signaling through the Wnt receptor Frizzled 3 (Fz3) is both necessary and sufficient to promote expression of EFTFs and eye formation in *Xenopus* (Rasmussen et al., 2001). Similarly, Wnt11/Fz5 signaling through the noncanonical pathway also promotes expression of EFTFs and eye formation. In contrast, Wnt8b/Fz5 signaling through the canonical Wnt/β-catenin pathway antagonizes eye field induction and promotes caudal diencephalon fate (Cavodeassi et al., 2005). Notch is an important eye-inducing signal in *Drosophila* (Kurata et al., 2000). In vertebrates, constitutively active Notch induces expression of EFTFs in animal cap assays and promotes duplication of eye structures in *Xenopus* (Onuma et al., 2002). Insulin-like growth factors (IGFs) also promote ectopic eye formation in neural tissues (Eivers et al., 2004; Pera et al., 2003; Pera et al., 2001; Richard-Parpaillon et al., 2002). In many cases it is not clear whether these signals contribute to the induction of an eye field or simply promote anterior neural fates. Thus, the precise role of these signals in the specification of the eye field is

currently poorly defined. Moreover, how these signals integrate together to effectively promote eye field specification will require future attention.

Following specification, the single central eye field is subsequently resolved into bilateral eye primordial (Figure 1.2B). Lineage tracing experiments in *Xenopus* revealed that bifurcation occurs due to suppression of eye field potential in the medial domain, rather than by lateral migration of eye field cells (Li et al., 1997). This suppression is signaled from the underlying prechordal plate (Li et al., 1997). Removal of the prechordal plate prevents bifurcation of the eye field and promotes cyclopia (Adelmann, 1936b; Li et al., 1997). Current evidence suggests that the prechordal plate-derived bifurcation signal is sonic hedgehog (Shh) (Chiang et al., 1996; Furimsky and Wallace, 2006; Geng et al., 2008; Rorick et al., 2007), consistent with findings of cyclopia in Shh and hedgehog (Hh) pathway mutants (Chiang et al., 1996; Ekker et al., 1995; Macdonald et al., 1995; Zhang et al., 2001).

Patterning of the optic neuroepithelium. Further induction of the retinal fate within the EFTF-defined neuroepithelium of these bilateral eye fields results from patterning. This patterning involves interactions with neighboring tissues and complex morphological changes of the neuroepithelium. From the bilateral eye fields, evagination of the EFTF-defined neuroepithelium forms the bilateral optic vesicles (Figure 1.2C). In mammals, this is immediately preceded by evagination of the presumptive forebrain, forming the optic pit. Contact of the evaginating optic vesicle with the overlying surface ectoderm induces formation of the lens placode in the adjacent surface ectoderm (Figure 1.2D). Subsequent invagination of the lens placode and distal optic vesicle generates the lens vesicle and bilayered optic cup (Figure 1.2E). The inner neuroepithelial layer of the

optic cup further develops into the neural retina, while the outer neuroepithelial layer forms the RPE. Cells located in the peripheral optic cup, at the border between the retina and RPE, eventually give rise to parts of the iris and ciliary body. The optic stalk derives from the ventral proximal aspect of the optic vesicle. The morphogenetic process of invagination that forms the optic cup continues ventrally into the proximal aspect of the optic vesicle, forming the choroid fissure along the length of the optic stalk. Blood vessels and projecting axons traverse through this fissure, which eventually gives rise to the optic nerve.

Establishment of the retinal domain within the EFTF-defined optic neuroepithelium results from patterning of the optic vesicle. By the late optic vesicle stage, regional patterning establishes restricted expression of several transcription factors, which mark the presumptive domains of the retina, RPE, and optic stalk (Figure 1.2 D). Expression of the paired-like homeobox gene Vsx2 is induced in the distal optic vesicle and marks the presumptive retina (Liu et al., 1994). Expression of Mitf, a basic helix-loop-helix gene, in the dorsal proximal region of the optic vesicle marks the presumptive RPE. In mouse, Mitf is initially broadly expressed throughout the optic vesicle, but expression becomes restricted by the late optic vesicle stage (Bora et al., 1998; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). Downregulation of Mitf in the distal domain coincides with Vsx2 upregulation (Horsford et al., 2005), consistent with reports that Mitf transcription is directly repressed by Vsx2 (Bharti et al., 2008). Moreover, Mitf expression persists throughout the presumptive retina in *Vsx2* null mouse mutants (Horsford et al., 2005; Rowan et al., 2004). Whether Mitf exerts reciprocal transcriptional repression of Vsx2 to further define the boundary between the retina and

RPE is still unclear, but appears unlikely as misexpression of Mitf in the presumptive retinal domain failed to alter retinal identity (Horsford et al., 2005). In chick, the existence of an initially broad Mitf expression domain remains controversial (Fuhrmann et al., 2000; Muller et al., 2007). In the ventral proximal region of the optic vesicle, the presumptive optic stalk domain is marked by expression of the ventral determinant and paired homeobox gene, Pax2, and absence of Vsx2 (Baumer et al., 2003; Nornes et al., 1990; Schwarz et al., 2000). These region-specific factors not only mark their respective domains but also facilitate maintenance of their respective regional identities (Horsford et al., 2005; Rowan et al., 2004; Schwarz et al., 2000).

Axial patterning of the optic neuroepithelium also begins during optic vesicle stages. The most prominent and well characterized is dorsal-ventral patterning. Like Pax2, the homeobox genes Vax1 and Vax2 also exhibit restricted expression to the ventral optic vesicle (Mui et al., 2005; Ohsaki et al., 1999; Take-uchi et al., 2003). In contrast, Pax6, another paired homeobox gene and EFTF, and Tbx5, a T-box gene, exhibit dorsal-specific expression in the optic vesicle (Baumer et al., 2003; Behesti et al., 2006; Schwarz et al., 2000; Sowden et al., 2001; Walther and Gruss, 1991). Initially, Pax6 and Pax2 are co-expressed throughout the early optic vesicle, but later exhibit complementary expression along the dorsal-ventral axis (Baumer et al., 2003). Pax6 and Pax2 transcriptionally repress each other and their respective expression domains expand in the absence of the other (Schwarz et al., 2000). Vax1 and Vax2 also transcriptionally repress Pax6 (Mui et al., 2005). Thus, the boundary between the retina and optic stalk is established by reciprocal transcriptional repression of Pax2 and Pax6, facilitated in part by Vax1 and Vax2. Nasal-temporal patterning also occurs within the optic vesicle, but is

not as well characterized. Some factors exhibit patterning along both axes. For example, the ventral determinant Vax1 also exhibits differential naso-temporal expression, with expression restricted nasally (Mui et al., 2005).

Regional patterning of the optic vesicle occurs primarily along the dorsal-ventral axis, and therefore, it is perhaps not surprising that these patterning events are linked. For example, the ventral determinants Pax2, Vax1, and Vax2 are all required for proper development of the optic stalk (Mui et al., 2005; Schwarz et al., 2000; Take-uchi et al., 2003). Furthermore, disruption of dorsal-ventral patterning by inverting the polarity of the optic vesicle during the critical period in which this patterning is established blocks retina and RPE formation. Inversion before or after this critical period resulted in normal or inverted expression, respectively, of both dorsal and ventral gene expression, which in turn permitted subsequent determination of the retinal and RPE domains (Uemonsa et al., 2002). Together, these findings demonstrate that regional fate determination within the optic vesicle is tightly coupled to dorsal-ventral patterning.

Regional and axial patterning of the optic vesicle is driven by inductive signals from neighboring tissues and the optic vesicle itself. Despite much effort, induction of the retinal fate within the optic neuroepithelium remains incompletely defined. Removal of the overlying surface ectoderm blocks induction of Vsx2 expression and subsequent retina formation (Hyer et al., 1998; Nguyen and Arnheiter, 2000), suggesting that signals from the surface ectoderm direct specification of the presumptive retinal domain within the optic vesicle. These surface ectoderm-derived signals have been proposed to be members of the FGF family due to the unique ability of members of this signaling class to direct the presumptive RPE towards a retinal fate (Nguyen and Arnheiter, 2000;

Pittack et al., 1997; Vogel-Hopker et al., 2000; Zhao et al., 2001; Zhao and Overbeek, 1999). FGF1 and FGF2 are expressed in the surface ectoderm overlying the distal optic vesicle (Nguyen and Arnheiter, 2000; Pittack et al., 1997), and experimental manipulations supplying these signals are sufficient to restore Vsx2 expression and retina formation in the absence of surface ectoderm (Hyer et al., 1998; Nguyen and Arnheiter, 2000). Additionally, FGF2 neutralizing antibodies block retina formation (Pittack et al., 1997). However, *Fgf1/2* double knockout mice lack obvious eye defects, suggesting that FGF1 and FGF2 are not the retina-inducing signals in the surface ectoderm. In addition to FGF1 and FGF2, several other FGF family members are also sufficient to direct the presumptive RPE towards a retinal fate. Thus, it is possible that other FGF family members present in the surface ectoderm normally mediate this function or are able to compensate for the loss of FGF1 and FGF2.

Ras-dependent MAPK signaling within the optic neuroepithelium is both necessary and sufficient for retina formation. The protein tyrosine phosphatase Shp2 is essential for full activation of MAPK signaling in response to growth factor stimulation of receptor tyrosine kinases (Dance et al., 2008). Conditional inactivation of *Shp2* in the optic vesicle resulted in cell autonomous loss of Vsx2 expression and disrupted retinal development in the inactivated area. Furthermore, activated Ras, a downstream component of the pathway, restored Vsx2 expression and retinal development in Shp2 mutants (Cai et al., 2010). Activated Ras is also sufficient to direct the presumptive RPE towards a retinal fate (Zhao et al., 2001).

FGF receptors are receptor tyrosine kinases capable of signaling through the Ras-dependent MAPK pathway (Szebenyi and Fallon, 1999) and the ability of at least FGF2

to direct the presumptive RPE towards a retinal fate requires Shp2 (Cai et al., 2010). Thus, an attractive model for retinal induction is one in which FGF signals from the overlying surface ectoderm activate Ras-dependent MAPK signaling in the distal domain of the optic vesicle. Other receptor tyrosine kinases also activate the MAPK pathway. Thus, the surface ectoderm-derived signal for retinal specification may actually be a non-FGF ligand that activates Ras-dependent MAPK signaling. Consistent with this, exogenous EGF is also sufficient to restore Vsx2 induction and retina formation in the absence of surface ectoderm (Nguyen and Arnheiter, 2000). Interestingly, EGF is not sufficient to direct the presumptive RPE towards a retina fate (Nguyen and Arnheiter, 2000), possibly due to absence of the appropriate receptors within this domain. However, a model in which non-FGF signals induce the retinal fate fails to account for the ability of FGF2 neutralizing antibodies to block retina formation. An intriguing addition to these models, and one that helps account for the activity of FGF2 neutralizing antibodies, is the possibility that surface ectoderm signals (FGF or others) establish an FGF-expressing domain within the optic vesicle itself that in turn induces Vsx2 and retinal fate through autocrine signaling (Chow and Lang, 2001; Horsford et al., 2005; Nguyen and Arnheiter, 2000). In support of this model, expression of FGF15 initiates within the presumptive retina around the same time as Vsx2 (McWhirter et al., 1997; Nguyen and Arnheiter, 2000) and this expression is also lost when Ras-MAPK signaling is disrupted in *Shp2* conditional mutants (Cai et al., 2010). However, ocular defects have not been reported in *Fgf15* knockout mice (Vincentz et al., 2005), suggesting that FGF15 may not be an essential factor for induction of the retinal domain. FGF8 and FGF9 also exhibit expression within the optic vesicle. FGF9 is expressed in the distal optic vesicle and both

targeted and transient misexpression within the proximal optic vesicle directs the presumptive RPE toward a retinal fate (Zhao et al., 2001; Zhao and Overbeek, 1999). However, evaluation of ocular development in *Fgf9* knockout mice reveals only minor expansion of the RPE into the peripheral retina, suggesting that FGF9 is also not required for initial specification of the retinal fate; rather, FGF9 is important for defining the boundary between the retina and adjacent RPE (Zhao et al., 2001). In chick, FGF8 expression in the distal optic vesicle initiates soon after contact with the surface ectoderm (Vogel-Hopker et al., 2000). However, ocular defects have not been reported in mice carrying targeted *Fgf8* hypomorphic or null alleles (Frank et al., 2002; Meyers et al., 1998). Although these loss-of-function studies fail to implicate a specific FGF signal in the induction of the retinal fate, it is possible that other FGFs compensate for their loss. Thus, identification of the retinal-inducing signal and its tissue source requires further research.

Additional inductive signals from adjacent tissues direct specification of the RPE and optic stalk and mediate dorsal-ventral patterning within the optic neuroepithelium. Extraocular tissues from the dorsal head region are both necessary and sufficient to specify the RPE fate in the chick optic vesicle (Kagiyama et al., 2005). Additional studies demonstrated that extraocular mesenchyme, but not surface ectoderm, is required for RPE specification (Fuhrmann et al., 2000). The ability of activin to restore RPE development in the absence of extraocular tissues suggests that activin or other activin-like signals from the extraocular mesenchyme specify the RPE fate (Fuhrmann et al., 2000). Shh emanating from the midline of the ventral forebrain ventralizes the optic vesicle by inducing the ventral determinants Pax2, Vax1, and Vax2 to promote

subsequent development of the optic stalk (Chiang et al., 1996; Ekker et al., 1995; Furimsky and Wallace, 2006; Macdonald et al., 1995; Take-uchi et al., 2003). BMP4 signals from the optic vesicle itself are both necessary and sufficient to induce dorsal fates (Behesti et al., 2006; Murali et al., 2005). Canonical Wnt signaling through β-catenin is both necessary and sufficient to direct specification of peripheral fates (iris and ciliary body) from the optic neuroepithelium (Cho and Cepko, 2006). This patterning may initiate as early as the optic vesicle stage via Wnt2b signals from the dorsal surface ectoderm (Cho and Cepko, 2006). While these signals do not directly induce the retinal fate, disruptions of these signals or their downstream regional and axial determinants often indirectly affect retinal specification.

Regional and axial patterning of the optic neuroepithelium requires both inductive signals and an underlying competence to respond to these signals. The LIM homeobox gene and EFTF, Lhx2, is an essential link between these inductive signals and their regional and axial determinants. In *Lhx2* null mice, eye field specification occurs, but development arrests at the optic vesicle stage and the optic cup and lens never form, resulting in anophthalmia (Porter et al., 1997). Both regional and axial patterning of the optic vesicle fails in the absence of Lhx2, as expression of regional and dorsal-ventral determinants either fails to initiate or is not maintained (Yun et al., 2009). Lhx2 mediates these patterning events through both cell autonomous and cell nonautonomous regulation, in part through regulation of BMP signaling (Yun et al., 2009).

Retinal histogenesis

Once the presumptive retinal domain is specified, further development is required for the formation of a mature and functional retina. Although the retinal fate is initially specified in these retinal progenitor cells (RPCs), this identity requires active maintenance. Furthermore, the small population of RPCs specified in the optic vesicle undergoes robust proliferative expansion to generate the necessary cell numbers for population of the adult retina. From these proliferating RPCs, retinal neurogenesis produces the six classes of retinal neurons and one of the three types of glia present in the mature retina. Collectively, these developmental processes define the period of retinal histogenesis.

Maintenance of retinal identity. Experimental manipulations in the mouse indicate that initial specification of retinal identity in optic neuroepithelial cells of the distal optic vesicle is not sufficient for subsequent retinal development. Removal of the overlying surface ectoderm after initial induction of Vsx2 and FGF15 in the presumptive retinal domain abrogated neural retina formation (Nguyen and Arnheiter, 2000). Initial expression of Vsx2 was lost in the distal optic vesicle resulting in maintained Mitf expression and subsequent pigmentation, suggesting differentiation into RPE. The ability of FGFs and EGF to prevent these changes in the absence of surface ectoderm suggests that many of the same signals or signaling pathways implicated in the specification of retinal identity are also involved in its maintenance. The intrinsic factor Vsx2 is also required for maintenance of retinal identity, particularly for the prevention of aberrant gene expression programs and promoting RPC properties (Horsford et al., 2005; Rowan et al., 2004).

The requirement for active maintenance of retinal identity may be transient. Impairment of MAPK signaling (activated by FGFs and other tyrosine kinase receptors) through conditional inactivation of *Shp2* in the retina revealed that only early inhibition of the pathway during the optic vesicle stage disrupted retinal development. Effective blockade of MAPK signaling at later optic cup stages failed to disrupt retinal development, suggesting that retinal identity may no longer require active maintenance at later stages (Cai et al., 2010). Alternatively, another pathway or intrinsic factor may fulfill such a role at later ages. Conditional inactivation of *Vsx2*, for example, at later stages has not been performed.

RPC proliferation. Extensive proliferative expansion of the RPC population is the principal mechanism of retinal growth. Total cell number increases dramatically during retinal histogenesis. Quantification of this increase in the rat retina revealed that over a period of approximately 17 days, beginning at embryonic day 14 (E14), total retinal cell number increased 400-fold, from 62000 to nearly 25 million (Alexiades and Cepko, 1996). RPC proliferation largely accounts for the increase in retinal cell number. Astrocytes, microglia and vascular-associated cells migrate into the retina via the hyaloid artery and choroid fissure of the optic stalk during this period and partially contribute to the increase in retinal cell number. However, the influx of astrocytes in the rat retina involves a relatively small number of cells and begins after the peak rate of increase in total cell number (Watanabe and Raff, 1988). Microglial migration occurs early, during the period of rapid cell number increase, but analysis of retinal microglia reveals a relatively small contribution to total retinal cell number in the rat (Ashwell et al., 1989). The striking increase in cell number in the developing retina is also region specific. The

RPE develops from a similarly sized region of the optic vesicle immediately adjacent to the presumptive retinal domain and maintains close proximity with the developing neural retina; however, cell number differs greatly between these tissues throughout development.

Regulation of proliferation ultimately must influence progression through the cell cycle (Figure 1.3A). The decision to reenter the cell cycle occurs during the G1 phase at the restriction point and is tightly regulated [reviewed in (Giacinti and Giordano, 2006; Levine and Green, 2004; Lundberg and Weinberg, 1999)]. Failure to progress past this critical G1 checkpoint forces cells into G0 and cell cycle exit. Progression through G1 past the restriction point and into S phase is driven by the activation of the G1 cyclin-dependent kinases (CDKs) Cdk4/6 and Cdk2 and inhibition of cyclin-dependent kinase inhibitors (CDKIs), such as KIP and INK family members (Figure 1.3B). These regulatory activities are mediated by the G1 cyclins (D- and E-cyclins). The resulting inactivation of retinoblastoma proteins (RB) through step-wise CDK-mediated phosphorylation releases RB-mediated inhibition of the E2F transcription factors. E2Fs activate transcription of target genes necessary for progression into S-phase. E2F targets include E-cyclins, establishing a positive feedback loop that ensures progression into S phase. Expression analyses and manipulation of many of these G1 components suggests that this regulatory pathway is active in RPC proliferation (Levine and Green, 2004).

Mitogens are essential extrinsic regulators of proliferation. Mitogens are required in G1 prior to the restriction point for progression through the cell cycle (Figure 1.3A). Beyond the restriction point, further exposure to mitogens is no longer necessary as the cell is intrinsically committed to progress through the remaining phases of the cell cycle

and divide. Mitogen signaling in G1 induces rapid upregulation of D-cyclins to initiate the regulatory cascade driving the G1-to-S phase transition (Figure 1.3B). Consistent with this role, inhibition of mitogen signaling in the absence of RB proteins fails to block G1 progression dependent on those mitogens [reviewed in (Giacinti and Giordano, 2006; Levine and Green, 2004; Lundberg and Weinberg, 1999)]. In the retina, many extracellular signals are mitogenic for RPCs, including Shh (Jensen and Wallace, 1997; Sakagami et al., 2009; Wang et al., 2005), TGFα (Anchan et al., 1991; Lillien and Cepko, 1992), TGFβ3 (Anchan and Reh, 1995), FGF2 and FGF1 (Lillien and Cepko, 1992), EGF (Anchan et al., 1991; Lillien and Cepko, 1992), VEGF (Hashimoto et al., 2006), NT-3/Trk C signaling (Das et al., 2000), and Wnts (Kubo et al., 2003, 2005; Sanchez-Sanchez et al., 2010; Van Raay et al., 2005).

Cyclin D1 (Ccnd1) is a critical intrinsic regulator of RPC proliferation in the developing retina. As mentioned, D-cyclins are an integral part of the cell cycle machinery, driving the regulatory cascade that promotes S phase entry (Figure 1.3B). *Ccnd1* null mice exhibit hypocellular retinas as a result of impaired RPC proliferation (Das et al., 2009; Fantl et al., 1995; Ma et al., 1998; Sicinski et al., 1995). Kinetics analysis revealed a lengthened cell cycle in *Ccnd1* null retinas that was largely independent of changes in S phase time, suggesting that *Ccnd1* is required to ensure the proper rate of progression of RPCs through the cell cycle (Das et al., 2009). Cyclin D3 (Ccnd3) is also expressed in the retina and prematurely upregulated in the absence of Ccnd1 (Das et al., 2009; Das et al., 2012). However, genetic deletion of *Ccnd3* in *Ccnd1* null retinas had little effect on the cell cycle progression of RPCs, indicating that the

upregulation of Ccnd3 in RPCs was unable to compensate for the loss of Ccnd1 in the regulation of RPC proliferation, at least at P0 (Das et al., 2012).

In addition to Ccnd1 and components of the cell cycle machinery, many other intrinsic factors have also been implicated in the regulation of RPC proliferation. The propensity of RPCs to proliferate appears to be tightly linked with their specification. Most of the EFTFs are reported to promote RPC proliferation, including Rx, Pax6, Six6, Six3, and Tll. Furthermore, many of these have been shown to regulate components of the cell cycle machinery [reviewed in (Agathocleous and Harris, 2009; Levine and Green, 2004)]. Sox2 is also expressed throughout retinal development and its restricted expression in the neural retina is essential for RPC proliferation. Conditional inactivation of *Sox2* in RPCs dramatically reduced the number of cycling RPCs (Taranova et al., 2006). Additionally, the retina-specific marker Vsx2 is also critical for proper regulation of RPC proliferation. *Vsx2*-deficient mice exhibit aberrant retinal expression of cell cycle components, severely reduced RPC proliferation, and hypocellular retinas (Burmeister et al., 1996; Green et al., 2003; Levine and Green, 2004).

Regulation of RPC proliferation is dynamic. Expression of the intrinsic regulators and extrinsic mitogens changes both temporally and spatially during the proliferative period. For example, the EFTF Six6 promotes RPC proliferation in mouse, at least in part through its interaction with Dach2 to transcriptionally repress p27/Kip1, and Six6 null mice exhibit severely hypolplastic retinas (Li et al., 2002). However, Six6 expression is progressively downregulated in embryonic RPCs during optic cup stages, suggesting that it may not participate in the regulation of postnatal RPC proliferation (Li et al., 2002). A second example is the retinal mitogen Shh, which is first expressed by ventral midline

cells and then later by retinal ganglions cells soon after their differentiation. Expression of Gli1, a target gene whose expression is solely dependent upon active Hh signaling, reveals dynamic activation of this signaling pathway in RPCs in response to these changes in the source of Shh. Expression of Gli1 in the optic vesicle reveals active signaling in response to midline Shh; however, this signaling is transient and Gli1 is dramatically downregulated in the early optic cup (Furimsky and Wallace, 2006). As ganglion cell differentiation proceeds in a central-to-peripheral wave across the optic cup, upregulation of Gli1 in adjacent RPCs closely follows, also expanding in a central-to-peripheral manner, but lagging behind the leading edge of ganglion cell differentiation (Wang et al., 2005). Thus, RPCs experience both temporal and spatial changes in their exposure to the potentially mitogenic effects of Shh. Retinal neurons also produce anti-mitogenic signals. TGFβ1 and TGFβ2 inhibit RPC proliferation in vitro and are expressed in the postnatal retina together with their receptors (Close et al., 2005). Furthermore, inhibition of TGFβ signaling blocks the anti-mitogenic effect of retinal neurons in in vitro cultures and increases and extends postnatal RPC proliferation in vivo (Close et al., 2005). These findings suggest that as retinal neurons accumulate in the postnatal retina, the increased activation of TGFβ signaling, likely in response to accumulating expression of retinal neuron-derived TGFβ2 or TGFβ1, drives the postnatal decline in RPC proliferation.

In addition to developmental changes in the expression of mitogen signals themselves, the responsiveness of RPCs to these mitogens also changes over time. For example, TGFα, FGF2, and FGF1 are mitogenic for RPCs; however, between E15 and P0 (~7 days) the responsiveness of RPCs to both FGF2 and FGF1 decreased, while

responsiveness to TGFα increased (Lillien and Cepko, 1992). The increased responsiveness of RPCs to TGFα correlated with a dramatic increase in the expression of EGF receptors (which mediate TGFα signaling) over the same time period. Increasing EGF receptor number using retroviral infection at the early time points increased RPC responsiveness to endogenous signals, suggesting that changes in EGF receptor expression likely underlie the observed changes in RPC responsiveness to TGFα and may be relevant to regulation of RPC proliferation in vivo (Lillien and Wancio, 1998).

Retinal neurogenesis. Retinal neurogenesis produces the six classes of retinal neurons (ganglion cells, amacrine cells, bipolar cells, horizontal cells, and rod and cone photoreceptors) and one glial cell type (Müller glia) from a common pool of multipotent RPCs (Turner et al., 1990). Generation of the seven classes of retinal cell types occurs according to an evolutionarily conserved sequence such that each cell type is produced during a limited, but overlapping period (Figure 1.4A). Ganglion cells are born first, followed by cones, horizontal cells, amacrine cells, rods, bipolar cells, and, lastly, Müller glia (Young, 1985). Retinal neurogenesis is tightly regulated and proceeds in a specific spatio-temporal pattern. Neurogenesis initiates at different times in different parts of the retina. In the mouse, initiation occurs in the central retina, dorsal to the optic stalk, and progresses in a peripherally-spreading wave (Figure 1.4B) (Hufnagel et al., 2010). Thus, development in the peripheral retina lags behind that of the central retina. In other vertebrates, location of initiation and direction of spread may differ slightly, but all show a wave of neurogenic progression that generally proceeds in a central-to-peripheral fashion (Kay et al., 2005; McCabe et al., 1999). Following specification and migration

into the appropriate retinal layer, these retinal neurons and glia undergo further maturation, send out processes and establish synaptic connections.

Retinal cell fate determination is largely mediated by members of the homeobox, bHLH, and forkhead transcription factor families [reviewed in (Ohsawa and Kageyama, 2008)]. Many of these genes are required for retinal cell type differentiation, and in their absence, the relevant cell type is not produced. However, many of these genes appear to only bias RPCs toward a particular cell fate, as not all cells expressing these genes will go on to differentiate into that cell type, which has been described for ath5 and ganglion cell determination (Mu and Klein, 2004; Yang et al., 2003). Furthermore, many of these genes are inefficient at promoting the relevant cell types on their own. Instead, specific combinations of these factors are required. An intriguing hypothesis that will require further evaluation is that homeobox genes specify the nuclear layer, while bHLH genes specify the particular cell type within that layer (Ohsawa and Kageyama, 2008). Additional levels of regulation are required, however, because factors specifying different cell types are often coexpressed within the same cell. Thus, in addition to transcriptional regulation of these factors, posttranslational regulation through phosphorylation and SUMOylation or posttranscriptional regulation through the regulation of mRNA stability or translation are also important for cell fate determination [reviewed in (Andreazzoli, 2009)].

Extrinsic signals also participate in the regulation of cell fate decisions. In chick and mouse, Shh, Gdf11, and VEGF inhibit the ganglion cell fate, while TGFβ inhibits amacrine cell differentiation. These signals are secreted by the cell types they inhibit, providing a negative feedback loop to locally control cell type generation (Hashimoto et

al., 2006; Kim et al., 2005; Ma et al., 2007; Wallace, 2008; Wang et al., 2005). Shh has also been implicated in promoting Müller glia and possibly bipolar cell fate, independent of its effects on proliferation (Wallace, 2008). Notch signaling also appears to regulate cell fate decisions, with specific ligand and receptor combinations promoting ganglion cell or cone fates (Jadhav et al., 2006a; Jadhav et al., 2006b; Riesenberg et al., 2009; Rocha et al., 2009; Yaron et al., 2006).

Retinal neurogenesis produces the seven retinal cell types from a common pool of multipotent RPCs. However, the observation of single clones comprised entirely of rod photoreceptors, even from early progenitors, provides some evidence for a subpopulation of lineage-biased progenitors (Turner et al., 1990). Conditional ablation of *Pax6* restricts RPCs to the amacrine cell fate, suggesting that Pax6 controls much of the multipotency of RPCs (Marquardt et al., 2001). Consistent with a role in mediating RPC multipotency, Pax6 transcriptionally activates several bHLH factors involved in biasing RPCs towards particular retinal cell fates, with the notable exception of NeuroD, which promotes the amacrine cell fate (Marquardt et al., 2001). Although RPCs are multipotent, their potential for generating the different retinal cell types changes over time. The prevailing model for this temporal change in developmental potential argues that as development proceeds, RPCs progress unidirectionally through competence states that restrict the subsets of retinal cell types RPCs can generate during a given period. Regulation of these competence states appears largely intrinsic. Early progenitors only generate early cell types, while late progenitors only generate late cell types, even in heterochronic environments [reviewed in (Cepko et al., 1996; Livesey and Cepko, 2001)]. In mouse, ikaros was recently identified as a critical intrinsic factor conferring competence for the

generation of early born cell types. The effects of ikaros loss of function and misexpression on cell type generation suggest the presence of at least three different temporal competence states through which RPCs pass (Elliott et al., 2008). However, the precise number of temporal competence states in mouse is not known, nor are the remaining intrinsic factors defining these competence states and controlling their temporal progression. Although extrinsic signals have little contribution to the definition of these temporal competence states, signaling may act to refine their temporal progression. The observation that RPCs are intrinsically competent to generate ganglion cells for a period following the normal termination of ganglion cell genesis has led to the suggestion that feedback inhibition blocks cell type generation until intrinsic changes in competence are established (Kim et al., 2005; Wallace, 2011).

The initiation of neurogenesis is regulated both temporally and spatially by intrinsic and extrinsic factors. Delta-Notch signaling is a central regulator of neurogenesis in the CNS. In the retina, inhibition of Notch signaling in loss of function mutants for Notch pathway components promotes precocious neurogenesis, while constitutive activation of Notch signaling delays neurogenesis (Bao and Cepko, 1997; Riesenberg et al., 2009; Tomita et al., 1996; Yaron et al., 2006). In retinas of *Hes1* null mutants, precocious neurogenesis began days before normal initiation in wild type retinas, while still retaining, in large part, the normal temporal progression of cell type determinants (Lee et al., 2005). These findings suggest that Hes1, possibly in its role as a transcriptional effector of Notch signaling, mediates the temporal onset of retinal neurogenesis. Sox2 is required to confer neurogenic competence. Conditional ablation of *Sox2* not only blocks RPC proliferation but also cell autonomously prevents

differentiation of RPCs. The role for Sox2 in promoting neurogenic competence in RPCs is mediated in large part through Sox2-dependent regulation of Notch1 expression (Taranova et al., 2006).

The initiation of neurogenesis in the central retina may be induced by FGF signals from the optic stalk or central retina. In zebrafish, signals from the distal optic stalk tissue are both necessary and sufficient for the induction of the initial patch of ath5 expression and ganglion cell differentiation in the ventro-nasal retina (Masai et al., 2000). FGF8 was sufficient to restore the initiation of ganglion cell differentiation in the absence of specified optic stalk tissue. Furthermore, the ventro-nasal expression of ath5 is prevented in *fgf3/fgf8* double mutants or through pharmacological inhibition of FGFs (Martinez-Morales et al., 2005). In the chick, FGF3 and FGF8 are expressed in overlapping patterns in the central retina and FGF8 is additionally expressed in the optic stalk (Martinez-Morales et al., 2005). The ability of FGF8 to induce secondary ectopic sites of ganglion cell differentiation and pharmacological inhibition to block normal differentiation (Martinez-Morales et al., 2005) suggests that the role of a central FGF signaling center in the induction of the initial patch of ganglion cell differentiation may be conserved.

Regulation of the subsequent progression of neurogenesis from the initial central patch is controversial. The sequential induction model proposes that signals, likely Hh, from nascent neurons induce neighboring RPCs to differentiate, thereby propagating a wave of ganglion cell differentiation across the retina (Neumann and Nuesslein-Volhard, 2000). However, expression of the ganglion cell determinant ath5 progresses across the retina in the absence of differentiated ganglion cells in zebrafish lakritz mutants (Kay et al., 2005). The peripherally-spreading wave of ganglion cell differentiation also still

occurs in explants of peripheral chick RPCs even when isolated from differentiating central cells well before the wave front reaches the central most extent of the explant (McCabe et al., 1999). Additionally, naïve zebrafish RPCs eventually expressed ath5 and differentiated into ganglion cells and photoreceptors even when transplanted into nonretinal tissues (Kay et al., 2005). These findings argue against the sequential induction model and suggest that the progression of neurogenesis depends largely on intrinsic regulation, based on a preprogrammed intrinsic timer. Furthermore, this timer appears to be sensitive to positional identity. Transplanted zebrafish RPCs express ath5 at times consistent with ath5 expression in their original retinal position, independent of the location into which they are transplanted (Kay et al., 2005). Studies in zebrafish suggest that this timer may be preprogrammed by midline-derived Shh signals (Kay et al., 2005). Neurog2/Ngn2 is an important intrinsic component regulating the spatial progression of neurogenesis. In mouse, a peripherally-spreading wave of Neurog2 expression precedes that of ath5. In the absence of Neurog2, initial progression of the neurogenic wave front stalls, but is rescued at later time points. Induction of the initial patch of ath5 and ganglion cell differentiation is unaffected in Neurog2 null retinas, revealing that initiation and subsequent progression of the neurogenic wave are genetically separable events (Hufnagel et al., 2010).

Although intrinsic regulation is critical for progression of the neurogenic wave front, extrinsic signals are sufficient to alter progression. In zebrafish, Shh promotes progression of the wave front (Neumann and Nuesslein-Volhard, 2000). Inhibition of FGF signaling in chick slowed or blocked progression of the neurogenic wave front, as indicated by the extent of ganglion cell differentiation, while exogenous FGF1, but not

FGF8, promoted progression (McCabe et al., 1999). However, FGF, Shh, and Neurog2 all regulate ath5 and, therefore, ganglion cell differentiation, making it difficult to separate the role of these factors in ganglion cell differentiation from a potential role in the spatial progression of neurogenesis within the progenitor population.

Perspectives. During development, the specification and maintenance of retinal identity, proliferation of RPCs, and retinal neurogenesis are tightly regulated cellular processes involving many intrinsic and extrinsic factors. Much progress has been made in the identification of genes and signaling pathways involved in the regulation and execution of these various cellular processes, revealing the importance of both intrinsic and extrinsic factors to their regulation. However, an understanding of how these extracellular signals and intrinsic factors are combined into efficient regulatory networks is largely lacking.

Further complicating the study of these regulatory networks is the fact that these developmental processes are not entirely separable, nor is their regulation. Regulatory mechanisms driving retinal specification and maintenance, proliferation and neurogenesis all exert their influences on the same cell population, the RPCs. Consequently, perturbations in the regulation of one process often elicit secondary changes in another process and vice versa. Furthermore, factors and signaling pathways often participate in the regulation of multiple processes and even in multiple aspects of a given cellular process. Thus, it will be important in the future to understand the regulation of each of these developmental processes in the context of retinal histogenesis as a whole and to define the mechanisms through which these competing processes are coordinated within RPCs to ensure the orderly and efficient execution of the retinal program.

Vsx2 in retinal development

Vsx2 is a paired-like CVC homeobox gene

The *visual system homeobox* gene Vsx2 was originally named Chx10 to reflect its similarity to Ceh-10, a *C. elegans* homeodomain protein (Liu et al., 1994). Vsx2 contains several conserved regions with other homeobox proteins (Figure 1.5). Vsx2 belongs to the paired-like class of homeodomain-containing transcription factors based on similarity of the homeodomain with that of the *Drosophila paired* gene, absence of a paired domain, and a glutamine at position 50 within the homeodomain (Galliot et al., 1999; Liu et al., 1994). Like many paired class proteins, Vsx2 also contains an OAR domain, named after Otp, Aristaless, and Rax, the homeodomain proteins in which this domain was first identified (Ferda Percin et al., 2000; Galliot et al., 1999). Vsx2 contains an octapeptide sequence (FGIQEILG) located N-terminal to the homeodomain, as observed in several classes of homeodomain proteins; but, like many other paired-like class proteins, Vsx2 contains a phenylalanine in the first position (Ferda Percin et al., 2000; Galliot et al., 1999; Liu et al., 1994). Vsx2 also contains a CVC domain, named for the three homeodomain proteins from which it was identified, Chx10, Vsx1, and Ceh-10. The CVC domain is located immediately C-terminal to the homeodomain and ultimately defines this family of paired-like homeodomain proteins (Ferda Percin et al., 2000; Galliot et al., 1999; Svendsen and McGhee, 1995). Vsx2 and its orthologs (Alx, Chx10, and Ceh-10) are highly conserved from humans to nematodes (Barabino et al., 1997; Belecky-Adams et al., 1997; Chen and Cepko, 2000; Ferda Percin et al., 2000; Levine et al., 1997a; Liu et al., 1994; Passini et al., 1997; Passini et al., 1998; Strickler et al., 2002; Svendsen and McGhee, 1995). Conservation is particularly strong within the

homeodomain and CVC domains, which are identical in human, mouse, goldfish, and cavefish Vsx2 sequences. Together, these two domains mediate the transcriptional activity of Vsx2. Vsx2 functions as a strong general repressor, but also exhibits context-dependent weak activator activity (Dorval et al., 2005). Several point mutations in the Vsx2 homeodomain ablate DNA binding affinity and indicate that the arginine at position 200 and asparagine at position 198 are critical for this function (Dorval et al., 2005; Ferda Percin et al., 2000; Zou and Levine, 2012). Consistent with disruption of DNA binding, these mutations also abolish repressor activity. Although the homeodomain on its own is sufficient to mediate DNA binding (Dorval et al., 2005), mutations in or deletion of the CVC domain weaken DNA binding (Dorval et al., 2005; Zou and Levine, 2012), suggesting that one function of the CVC domain is to enhance the DNA binding affinity of the homeodomain. The CVC domain also appears to mediate the repressor activity of Vsx2, as deletion of the CVC domain abolished transcriptional repression, despite only slightly weakened DNA binding (Dorval et al., 2005). The importance of this repressor activity in vivo is revealed by the fact that several missense mutations identified in the CVC domain of human patients produce phenotypes that are strikingly similar to those produced by mutations predicted to generate a functional null (Bar-Yosef et al., 2004; Iseri et al., 2010; Reis et al., 2011).

Ocular expression of Vsx2

Vsx2 is the earliest and most specific marker of the presumptive retina and, therefore, of specified RPCs. As previously described, Vsx2 expression initiates within the presumptive neural retina during the late optic vesicle stage in response to inductive

signals from the overlying surface ectoderm that activate Ras-dependent MAPK signaling in the distal optic vesicle and establish the presumptive retinal domain. As development proceeds, ocular expression of Vsx2 is primarily restricted to RPCs of the neural retina, with weak expression also reported in the adjacent presumptive ciliary body epithelium. Expression in RPCs is maintained throughout retinal development, but terminated in all postmitotic retinal cells, except bipolar cells and a subset of Müller glia. Thus, as retinal neurogenesis proceeds, Vsx2 expression is restricted to the outer region of the inner nuclear layer where these cell types are located (Barabino et al., 1997; Belecky-Adams et al., 1997; Chen and Cepko, 2000; Ferda Percin et al., 2000; Levine et al., 1997a; Liu et al., 1994; Passini et al., 1997; Passini et al., 1998; Rowan and Cepko, 2004; Strickler et al., 2002). A possible exception is the zebrafish ortholog Alx, whose expression has not been reported in postmitotic neurons (Barabino et al., 1997). However, this expression analysis may not have extended to the period of bipolar cell genesis. Expression analysis in zebrafish using a goldfish Vsx2 probe did reveal expression in the outer aspect of the inner nuclear layer (Passini et al., 1997), suggesting that Alx may indeed be expressed in bipolar cells in zebrafish. Vsx2 expression also persists in the developing and mature germinal zone (or ciliary marginal zone) of retinas from several species of teleosts, including zebrafish, goldfish and several cavefish (Barabino et al., 1997; Levine et al., 1997a; Passini et al., 1997; Passini et al., 1998; Strickler et al., 2002). This peripheral region in the adult retina is present in many teleosts and contains mitotically active retinal progenitors that permit continued generation of mature retinal cells throughout life. Thus, expression of Vsx2 is consistently associated with retinal-specific progenitor cells across vertebrate species.

Mutations in Vsx2 disrupt retinal development

Human patients with mutations in *Vsx2* present clinically with autosomal recessive nonsyndromic congenital microphthalmia (small eye), with or without associated ocular anomalies (Bar-Yosef et al., 2004; Burkitt Wright et al., 2010; Faiyaz-Ul-Haque et al., 2007; Ferda Percin et al., 2000; Iseri et al., 2010; Reis et al., 2011). The associated ocular anomalies vary, but include colobomas, iris anomalies, cataracts, and retinal detachments. Although at least one patient exhibited some light perception, all patients lacked functional vision. The identified mutations in these patients (Figure 1.5) are predicted to impair Vsx2 function through disruption of the homeodomain and/or CVC domain, or through production of an abnormal or truncated protein. Two mouse lines carrying spontaneous recessive mutations in the *Vsx2* gene, *ocular retardation* (*or*) and *ocular retardation J* (*orJ*), also exhibit microphthalmia, cataractous lenses, coloboma, and disrupted retinal lamination, as well as failure to form the optic nerve (Bone-Larson et al., 2000; Burmeister et al., 1996; Robb et al., 1978; Truslove, 1962). Identification of the mutation in *orJ* mice revealed a single point mutation creating a premature stop codon within the homeodomain (Figure 1.5). Absence of detectable Vsx2 protein in these mice suggests that this allele is a functional null (Burmeister et al., 1996). Knockdown of the zebrafish homolog, Alx, with antisense oligonucleotides also promotes reduced eye size and disrupted eye development (Barabino et al., 1997). Studies in the defined Vsx2-null mouse mutant, *orJ*, reveal that these defects in ocular development arise from multiple disruptions in the execution of the retinal program (Figure 1.6), consistent with retinal-specific expression of Vsx2 during ocular development. Specifically, *orJ* retinas exhibit compromised retinal identity, severely

reduced RPC proliferation, delayed neurogenesis, and absence of bipolar cells (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003; Horsford et al., 2005; Rowan et al., 2004).

Several lines of evidence suggest that retinal specification occurs in the absence of Vsx2 function. First, expression of Vsx2 transcript is unaffected in *orJ* retinas (Rutherford et al., 2004; Sigulinsky et al., 2008), indicating that inductive signals have established regional patterning within the optic vesicle. Furthermore, *orJ* retinal cells express several additional neural retina-specific markers and generate mature retinal cell types (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003; Horsford et al., 2005; Rowan et al., 2004; Rutherford et al., 2004), revealing that execution of the retinal program persists in *orJ* retinas, albeit disrupted. Methods used to restore retinal formation in the absence of surface ectoderm and to direct the presumptive RPE towards a retinal fate, including exposure to FGFs and activation of MAPK signaling, are often associated with upregulation of Vsx2 (Cai et al., 2010; Nguyen and Arnheiter, 2000; Zhao et al., 2001). However, misexpression of Vsx2 on its own in the presumptive RPE appears insufficient to direct these cells towards a retinal fate, although one study demonstrated that Vsx2 was sufficient to downregulate several RPE genes (Horsford et al., 2005; Rowan et al., 2004). These findings reveal that Vsx2 is neither necessary nor sufficient to specify the retinal fate and that FGFs and MAPK signaling activate additional mechanisms for specification of the retinal fate. Instead, Vsx2 is required in the maintenance of retinal identity, namely, to prevent aberrant gene expression programs (Figure 1.6). Several genes with RPE-restricted expression, including the RPE determinant Mitf, exhibit ectopic or expanded expression throughout all or part of the *orJ*

retina (Horsford et al., 2005; Rowan et al., 2004). Expression of these genes likely contributes to the hyperpigmentation observed in the periphery of *orJ* retinas as ocular development progresses (Figure 1.6) (Green et al., 2003; Horsford et al., 2005; Rowan et al., 2004; Truslove, 1962). Exacerbating this aberrant gene expression program promotes further activation of the pigmentation program and increased disruption of the retinal program. Forced overexpression of Mitf in *orJ* retinas enhanced the pigmentation phenotype and further impaired neurogenesis (Horsford et al., 2005). Similar phenotypes were observed in the retinas of homozygous $Vsx2^{R227W}$ knock-in mice and correlated with markedly elevated expression of the RPE determinants Mitf and Otx (Zou and Levine, 2012). Conversely, genetic reduction of Mitf improved retinal development in all *Vsx2* mutant backgrounds (Horsford et al., 2005; Konyukhov and Sazhina, 1966; Zou and Levine, 2012). These findings reveal that the aberrant RPE-like gene expression program and in particular, maintained expression of Mitf, contributes significantly to *Vsx2* mutant phenotypes. These findings also suggest that a major function of Vsx2 is to prevent activation of such aberrant gene expression programs. Acquisition and maintenance of retinal identity involve both extrinsic and intrinsic regulators. While studies have implicated Vsx2 downstream of the extrinsic signals and upstream of many intrinsic factors involved in the acquisition and maintenance of retinal identity (Horsford et al., 2005; Nguyen and Arnheiter, 2000; Rowan et al., 2004), it is not clear from these studies whether Vsx2 also influences the extrinsic signals required for maintenance of retinal identity.

Ocular tissues of *orJ* mice develop normally through the initial formation of the optic cup. However, as development proceeds, *orJ* eyes are increasingly smaller than

those of wild type littermates and develop thin, hypocellular retinas. Decreased retinal volume and cell number are detected as early as E11.5 (Bone-Larson et al., 2000; Burmeister et al., 1996). By P0, *orJ* retinas exhibit a striking 19-fold reduction in cell number (Green et al., 2003). The severe hypocellularity of the *orJ* retina results primarily from defective RPC proliferation (Figure 1.6) (Bone-Larson et al., 2000; Burmeister et al., 1996; Dhomen et al., 2006; Green et al., 2003; Konyukhov and Sazhina, 1971). The slowed rate of cell cycle progression in the *orJ* retina appears, in large part, due to aberrant accumulation of the cell cycle inhibitor p27/KIP1. Genetic deletion of p27/KIP1 in the *orJ* retina largely restores retinal cell number without influencing neurogenesis or apoptosis. Regulation of p27/KIP1 by Vsx2 is indirect and involves post-transcriptional mechanisms largely mediated by Ccnd1 (Green et al., 2003). Additionally, genetic removal of the RPE determinant, Mitf, in *orJ* retinas also improves retinal size and RPC proliferation (Horsford et al., 2005; Konyukhov and Sazhina, 1966). In melanocytes and melanoma cell lines, MITF directly promotes p27/KIP1, as well as a related CIP/KIP family member, p21/CIP (Carreira et al., 2005; Lekmine et al., 2007). Recently, Mitf was also implicated in transcriptional activation of p27/KIP1 in the chick optic vesicle (Tsukiji et al., 2009), suggesting that Vsx2-mediated regulation of p27/KIP1 may also be partially Mitf dependent. Mitogens are also key extrinsic regulators of cell cycle progression. Mitogen signals are required in early G1 to promote G1 progression through upregulation of D-cyclins [reviewed in (Levine and Green, 2004)]. In the *orJ* retina, expression of Ccnd1, a G1 phase D-cyclin, is reduced (Green et al., 2003), but it is not known whether this results from direct transcriptional regulation by Vsx2 or indirectly through Vsx2-mediated regulation of mitogen signals or their signaling pathways.

Vsx2 regulates multiple aspects of retinal neurogenesis. Vsx2 is required for proper temporal regulation of the initiation of neurogenesis. While the general principles of neurogenesis appear maintained in the *orJ* retina, including the central to peripheral wave of neuron production and temporal birth order, initiation is delayed (Figure 1.6) (Bone-Larson et al., 2000; Robb et al., 1978; Rutherford et al., 2004). However, it is not clear whether this delay reflects an inability to respond to the neurogenic signal(s) or absence of the necessary signal(s). Vsx2 also participates in the regulation of cell fate. Lineage analysis in zebrafish reveals that Vsx2 RPCs are multipotent, eventually giving rise to all retinal cell types over the course of retinal neurogenesis (Vitorino et al., 2009). However, in contrast to Pax6 (Marquardt et al., 2001), Vsx2 transcriptionally represses several factors which normally bias RPCs towards specific cell fates, including ath5 and Vsx1, while Vsx2 itself promotes a subclass of bipolar cell fates (Clark et al., 2008; Vitorino et al., 2009). Thus, selective downregulation of Vsx2 in RPCs during development results in derepression of bias factors and subsequent restriction of lineage potential (Vitorino et al., 2009). Bipolar cells are uniquely absent in retinas of *orJ* mice (Figure 1.6) (Bone-Larson et al., 2000; Burmeister et al., 1996) and their generation is not rescued in *orJ*, p27/KIP1 double mutants (Green et al., 2003), suggesting their absence is due to a specific requirement for Vsx2 in their specification or maturation, rather than a secondary effect of insufficient proliferative expansion for this late-born cell type. This is further supported by a number of studies showing that Vsx2 promotes the bipolar fate, typically at the expense of rod photoreceptors (Belecky-Adams et al., 1997; Dorval et al., 2006; Hatakeyama et al., 2001; Livne-Bar et al., 2006; Rowan and Cepko, 2004; Rutherford et al., 2004; Toy et al., 2002; Vitorino et al., 2009). Additionally, Müller glia,

another late-born retinal cell type, are generated in the *orJ* retina (Burmeister et al., 1996), which also argues against the possibility that the abnormally small progenitor pool is depleted prior to bipolar cell generation. Excluding bipolar cells, all retinal cell types are generated in the *orJ* retina, but their organization into the retina's stereotyped laminar architecture fails (Bone-Larson et al., 2000). This disruption in retinal lamination appears to be a secondary effect of defective RPC proliferation, as it is largely rescued by significant restoration of cell number in *orJ*, p27/KIP1 double mutants (Green et al., 2003).

Active research examining the affected cellular processes in *orJ* retinas has revealed altered expression of many intrinsic factors, which undoubtedly contributes to the defective regulation of RPC properties and, consequently, the phenotypes observed in *orJ* retinas. However, a critical unanswered question is to what extent are the *orJ* phenotypes dependent on changes in the extrinsic regulation of RPC behavior? Furthermore, to what extent do these changes in extracellular signaling account for the aberrant regulation of intrinsic factors? There are multiple ways to address these questions. One way is to take a candidate approach and examine the impact of Vsx2 deficiency on a pathway with known roles in regulating processes also affected in *orJ* retinas. Using this approach, retinal Shh signaling was examined to determine whether changes in this signaling pathway contributed to the defects in RPC proliferation in the *orJ* retina. Expression of Ccnd1, an established target of mitogen signaling that promotes cell cycle progression, is reduced in the *orJ* retina, suggesting that reduced mitogen input may contribute to slowed RPC proliferation. Shh is an important retinal mitogen and preliminary data indicated that this signaling pathway was sensitive to loss of Vsx2

function in the retina. A second approach is to determine the contribution of altered extracellular signals to the disruption of cellular processes by evaluating the ability of a wild type environment to rescue the *orJ* phenotypes. An established and powerful tool for addressing such questions is the genetic chimera.

Shh signaling in the regulation of RPC proliferation

Shh and the Hh signaling pathway

Sonic hedgehog (Shh) is a member of the Hedgehog (Hh) family of secreted signaling molecules. In mouse, this family also includes Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh) [reviewed in (Ingham and McMahon, 2001)]. Shh is produced as a precursor protein that undergoes several processing steps [reviewed in (Mann and Beachy, 2004; Ryan and Chiang, 2012)]. The catalytically active carboxy-terminal domain of the precursor protein mediates linked autoprocessing events. Autoproteolytic cleavage generates the biologically active amino-terminal fragment (SHH-N). During cleavage, a cholesteryl moiety is covalently added to the carboxy-terminus of the resulting SHH-N polypeptide. The carboxy terminal fragment (SHH-C) is then ubiquitinated and degraded by the proteosome. Subsequent palmitoylation of SHH-N results in covalent addition of palmitate to the amino-terminal cysteine. These lipid modifications are important for modulating the multimerization and spatial distribution of SHH-N (cholesteryl moiety) and for increasing its potency (palmitate) [reviewed in (Mann and Beachy, 2004; Nybakken and Perrimon, 2002; Ryan and Chiang, 2012)]. Release of processed SHH-N (referred to hereafter as SHH) from the producing cell is facilitated by Dispatched (Disp) (Ma et al., 2002). SHH exhibits both short- and long-

range signaling activity (Ingham and McMahon, 2001), and a number of molecules have been implicated in facilitating the movement of SHH and regulating its range of activity [reviewed in (Cohen, 2003; Ingham and McMahon, 2001; Nybakken and Perrimon, 2002; Varjosalo and Taipale, 2008)].

Much of Shh function is mediated by transcriptional regulation of Hh target genes through activity of the Hh signaling pathway. However, some functions of Shh, particularly those of migration and axon guidance, are elicited independent of this transcriptional activity (Riobo and Manning, 2007; Yam et al., 2009). Despite significant conservation from Drosophila to vertebrates, marked differences exist in the mechanisms of Hh signal transduction [reviewed in (Huangfu and Anderson, 2006; Varjosalo and Taipale, 2008)]. Here, only the vertebrate pathway will be described. [For further review see (Huangfu and Anderson, 2006; Ingham and McMahon, 2001; Jiang, 2006; Nybakken and Perrimon, 2002; Riobo and Manning, 2007; Rohatgi and Scott, 2007; Ryan and Chiang, 2012; Varjosalo and Taipale, 2008; Wallace, 2008)].

The Hh pathway is a series of repressive interactions that ultimately regulates the balance of activator and repressor forms of transcriptional effectors (Figure 1.7). The Hh receptor is Patched homolog 1 (Ptch1), a 12-transmembrane domain protein. However, three additional Hh-binding proteins, CDO, BOC, and Gas1, function as essential coreceptors in vertebrates. These proteins form constitutive complexes with Ptch1 and are required for induction of Shh-dependent signaling (Allen et al., 2011; Izzi et al., 2011). Patched homolog 2 (Ptch2) and Hh interacting protein (Hhip) also bind Hh ligands, but lack downstream signaling (Carpenter et al., 1998; Chuang et al., 2003; Chuang and McMahon, 1999; Rahnama et al., 2004). Thus, Ptch2 and Hhip function as negative

regulators, limiting the level and possibly the range of Hh signaling. In the absence of Hh ligand, Ptch1 inhibits Smoothened (Smo), a 7-transmembrane protein related to G-protein-coupled receptors, responsible for transduction of the Hh signal. The mechanism by which Ptch1 inhibits Smo is still unclear, but direct binding is not favored. Binding and expression studies typically fail to support a physical interaction between the two proteins and Ptch1-mediated inhibition of Smo is nonstoichiometric, which would not be expected in a direct binding model. A number of small molecules have been discovered to act as Smo agonists and antagonists, and Ptch1 shares structural similarity with the bacterial Resistance, Nodulation, Division (RND) family of small molecule pumps, suggesting that Ptch1 may inhibit Smo activity through the regulation of local concentrations of small molecules. However, a relevant endogenous small molecule has yet to be identified (Rohatgi and Scott, 2007; Ryan and Chiang, 2012). Hh binding represses Ptch1 function, which relieves the Ptch1-mediated inhibition of Smo and allows Smo activation. Activation of Smo involves a conformational change induced by phosphorylation of the carboxy-terminal tail by GRK2 and CK1α. Smo activity ultimately regulates the processing and localization of the Gli proteins. The Gli proteins are a family of zinc finger transcription factors that function as the principal effectors of the Hh pathway and activate or repress target gene expression depending on the presence or absence of Hh ligand, respectively.

In the absence of Hh ligand, and therefore absence of Smo activity, full-length Gli proteins (Gli-FL) are proteolytically processed, resulting in a truncated transcriptional repressor form (Gli-R) or complete degradation. Suppressor of Fused (SuFu) binds Gli-FL and sequesters it in the cytosol, preventing nuclear localization and activation.

Phosphorylation of carboxy-terminal residues by PKA primes Gli-FL for further phosphorylation by GSK3β, and CK1α. Hyperphosphorylated Gli-FL is recognized by the βTrCP E3 ubiquitin ligase, resulting in ubiquitination and degradation by the proteasome. Limited degradation of only carboxy-terminal peptides results in the truncated amino-terminal form that serves as a transcriptional repressor (Jiang, 2006; Riobo and Manning, 2007; Ryan and Chiang, 2012). SuFu binding promotes the processing of Gli-FL (Humke et al., 2010), possibly by recruiting GSK3β (Kise et al., 2009) and βTrCP (Tempe et al., 2006) to Gli-FL and mediating their interaction.

In the presence of Hh, activated Smo inhibits Gli processing and promotes Gli localization to the nucleus and activation. The mechanisms linking Smo to Gli regulation are still poorly understood, but may center on the inhibition of SuFu and PKA. Activated Smo promotes the disassembly of SuFu-Gli complexes (Humke et al., 2010; Tukachinsky et al., 2010), which likely serves to limit proteolytic processing and release of Gli-FL for nuclear localization and activation. PKA is a potent negative regulator of Hh signaling, promoting the proteolytic processing of Gli while preventing (in a dominant fashion) Hh- and Smo-induced disassembly of SuFu-Gli complexes and subsequent nuclear translocation and formation of Gli-A complexes (Humke et al., 2010; Riobo and Manning, 2007; Tempe et al., 2006; Tukachinsky et al., 2010). Activated Smo, possibly signaling through G-proteins, represses PKA activity using two potential mechanisms. In the first, PKA inhibition is mediated by activation of the PKA inhibitors PI3K and Akt. In the second, PKA activity is reduced by inhibiting adenylyl cyclase, a potent activator of PKA activity (Riobo and Manning, 2007). Following dissociation from SuFu, Gli-FL translocates to the nucleus, a process that involves microtubules. Conversion of Gli-FL to

its activator form (Gli-A) appears to require further processing within the nucleus. The nature of and mechanisms driving such processing are still unclear, but may involve phosphorylation, deacetylation, or other yet unidentified processes. Complicating the study of Gli-A formation is Gli degradation promoted by SPOP-Cul3 E3 ligase, which also occurs in the nucleus. Thus, changes in Gli-FL within the nucleus could be associated with activation, degradation, or both (Humke et al., 2010; Jiang, 2006; Ruiz i Altaba, 1999; Ryan and Chiang, 2012).

The Gli family consists of three members, Gli1-3, that exhibit differential proteolytic processing. Gli3 is efficiently processed into the truncated repressor form and is the primary transcriptional repressor in the absence of Hh ligand. Gli3 also functions as a transcriptional activator in the presence of Hh. Gli2 functions principally as a transcriptional activator. In the absence of Hh, Gli2 is mostly degraded; however, a small, but significant fraction is proteolytically processed into a repressor form, consistent with in vivo findings that Gli2 also exhibits context-specific repressor activity. Gli1 appears to exist solely as a transcriptional activator; proteolytic processing completely degrades Gli-FL with no evidence of a repressor form. Gli1 is a potent transcriptional activator whose expression is completely dependent upon active Hh signaling; however, its function in mouse appears dispensable in the presence of Gli2 and Gli3 (Ingham and McMahon, 2001; Riobo and Manning, 2007; Ryan and Chiang, 2012; Wallace, 2008). These three Gli proteins also differ in the binding affinities of their zinc finger domains for the various Gli DNA binding sites (Nakashima et al., 2002). Thus, transcriptional activation and repression of Hh target genes is determined by both the relative abundance of activator versus repressor forms and relative binding affinity for Gli consensus sites.

Growing evidence supports a role for primary cilia in vertebrate Hh signaling. Primary cilia are solitary, nonmotile, and present on most vertebrate cells, except during cell division. Most Hh pathway components are enriched in primary cilia and exhibit dynamic, Hh-dependent trafficking. Furthermore, mutations in genes required for ciliogenesis disrupt localization of Hh pathway components and Hh signal transduction. In particular, genes involved in intraflagellar transport (IFT), the bidirectional trafficking mechanism required for the construction and maintenance of cilia and basal body formation, are key regulators of Hh signaling (Goetz and Anderson, 2010; Huangfu and Anderson, 2006; Rohatgi and Scott, 2007; Ryan and Chiang, 2012).

Shh-mediated regulation of RPC proliferation

Shh participates in multiple steps of eye and retinal development, including the stimulation of RPC proliferation [reviewed in (Amato et al., 2004; Wallace, 2008)]. In the mouse, RPCs express the necessary signaling components of the Hh pathway and exhibit Hh-dependent target gene expression during retinal development (Dakubo et al., 2003; Jensen and Wallace, 1997; Mu et al., 2004; Nakashima et al., 2002; Wang et al., 2005) and reviewed in (Amato et al., 2004; Wallace, 2008). During the proliferative period, the principal source of Shh ligand in the retina is differentiated ganglion cells. Retinal ganglion cells produce Shh soon after differentiation (Wang et al., 2005). Consistent with this, Shh is a direct transcriptional target of Pou4f2, a required differentiation factor for ganglion cells (Mu et al., 2004). Moreover, Shh expression is severely reduced in *Pou4f2* null retinas (Mu et al., 2004) or upon ganglion cell death (Mu et al., 2005; Wang et al., 2002). Hh-induced target gene expression in RPCs of the

neuroblast layer closely follows the central to peripheral wave of ganglion cell differentiation and Shh production (Wang et al., 2005). Depletion of retinal ganglion cells (Mu et al., 2004; Wang et al., 2002) or conditional ablation of retinal *Shh* results in loss of Hh target gene expression in adjacent RPCs. Shh expression is also detected in the inner nuclear layer, possibly in amacrine cells or melanopsin-expressing ganglion cells (Jensen and Wallace, 1997; Wallace, 2008).

Extraretinal sources of Shh include the RPE and ventral midline. Ihh is expressed in the RPE during this period, but is not sufficient to induce Hh target gene expression in RPCs in the absence of ganglion cell-derived Shh (Dakubo et al., 2003). Midline-derived Shh may contribute early during optic vesicle stages, as optic vesicle outgrowth was reduced in *Gli1*,*Gli2* double mutants (Furimsky and Wallace, 2006). However, proliferation in the optic cup at these stages was not analyzed and any role for midline-derived Shh in RPC proliferation would be transient as Hh-dependent target gene expression is downregulated in the optic cup prior to the initiation of retinal neurogenesis (Furimsky and Wallace, 2006).

Shh and active Hh signaling are both necessary and sufficient for RPC proliferation. In rodents, recombinant, pre-processed SHH-N stimulates RPC proliferation and increases total cell number in vitro (Jensen and Wallace, 1997; Levine et al., 1997b). Furthermore, constitutive activation of the Hh pathway in vivo, through Ptch1 heterozygosity or retroviral infection of a Ptch1-insensitive activated Smo allele (Smo-M2), promoted increased RPC proliferation in mouse (Black et al., 2003; Moshiri and Reh, 2004; Yu et al., 2006). Blocking endogenous Shh and Hh signaling, through treatment with neutralizing anti-SHH antibodies (Wallace and Raff, 1999), conditional

ablation of retinal Shh (Wang et al., 2005), or conditional ablation of Smo in RPCs (Sakagami et al., 2009), reduced RPC proliferation. Consistent with defective RPC proliferation, mice with conditional ablation of Shh or Smo also exhibit a reduced progenitor pool, decreased clone size, and/or microphthalmia (Sakagami et al., 2009; Wang et al., 2005; Wang et al., 2002). Toxin-induced death of ganglion cells also reduced RPC proliferation, suggesting that ganglion-cell derived Shh is the principal source of mitogenic Shh in the retina during the proliferative period (Mu et al., 2005).

Shh and activation of Hh signaling promotes RPC proliferation by influencing cell cycle progression. Loss of Hh signaling through conditional ablation of *Smo* in the mouse resulted in altered distribution of RPCs in cell cycle phases, namely an increased G_1 population and decreased S and G_2/M populations. Further analysis revealed that Hh signaling is critical for promoting the G_1/S phase transition (Sakagami et al., 2009). These changes in the cell cycle correlate with Hh-dependent changes in expression of cell cycle components. Retinal ganglion cell-derived Shh is required for cyclin D1 expression, but not Myc expression (Mu et al., 2005; Wang et al., 2005). Constitutively active Smo induced increased cyclin D1 expression (Yu et al., 2006), while conditional ablation reduced expression of a number of cyclins and E2F1 required for G1 and G2 progression in the cell cycle, and an increased number of cells expressing the cell cycle inhibitor p27/KIP1 (Sakagami et al., 2009). These findings in the mouse are consistent with studies in *Xenopus* and zebrafish, which demonstrated that manipulation of Hh signaling altered the expression of cell cycle components and promoted corresponding changes in the cell cycle (Locker et al., 2006).

Genetic mouse chimeras and dissection of complex gene function

The chimeras of ancient mythology were fantastical imaginary creatures that combined physical elements of multiple animals. Today, chimeras more commonly refer to individuals whose cells derived from more than one zygote, and typically differ in genotype. These modern genetic chimeras are powerful research tools for developmental biologists, providing fine scale resolution of studies addressing cell lineage, patterns of tissue growth and associated cellular behaviors, and gene function.

Generation of genetic mouse chimeras

Genetic mouse chimeras were first introduced in the 1960s. Multiple methods are now available for their generation, differing in the relative contribution of the host cell populations to the embryonic tissues and/or the source of the genotypically distinct cell populations (Figure 1.8) (Nagy and Rossant, 2001; Rossant and Spence, 1998; Tam and Rossant, 2003). Diploid embryos at the eight-cell stage exhibit the greatest developmental potential and their aggregation generates chimeras with the potential for mosaic contribution to all tissues of the embryo. A variation on this method, using a tetraploid embryo, biases the contribution of the diploid embryo towards the epiblast-derived embryo proper, while the tetraploid embryo predominantly contributes to the extraembryonic primitive endoderm and trophectoderm. Such lineage restriction facilitates functional testing of gene requirements in extraembryonic versus embryonic tissues. With the advent of targeted gene mutation in pluripotent embryonic stem (ES) cells, the use of chimeras was no longer restricted to studying spontaneous mutants. ES

cells exhibit more limited lineage potential than diploid eight-cell embryos or inner cell mass (ICM) cells and contribute solely to the embryo proper lineages of the epiblast. Chimeras can be generated either through aggregation of ES cells with diploid or tetraploid embryos or through injection of ES cells into blastocyst embryos. The use of ES cells therefore overcomes several limitations associated with creating chimeras from homozygous lethal mutants. Resulting chimeric blastocysts from all methods are surgically returned to the uterus of a pseudopregnant female for subsequent embryonic development.

Regardless of the method used for the generation of genetic mouse chimeras, a means of distinguishing the two distinct cell populations is crucial for interpreting the resulting cellular behaviors and phenotypes. Such markers must exhibit ubiquitous, cell autonomous expression that can be detected in mosaic tissues and ideally is inert to the expressing cells (Nagy and Rossant, 2001; Rossant and Spence, 1998; Tam and Rossant, 2003). A lack of markers providing the necessary spatial resolution limited the early use of genetic chimeras. Electrophorectic variants of the housekeeping gene GPI, and strain- or mutant-specific DNA polymorphisms, provided the necessary expression profile, but lacked spatial resolution in situ. In the eye, many early chimera studies took advantage of pigment markers to distinguish between the cells deriving from pigmented versus albino embryos. However, the spatial resolution of pigmentation is restricted to the pigmented RPE in the eye and only at later stages of ocular development once pigmentation becomes apparent. Species- and strain-specific DNA satellite markers provided spatial resolution in all embryonic tissues, but were technically difficult. The development of ubiquitously expressed, easily detectable transgenic markers has greatly enhanced the use

of genetic mouse chimeras. The most common of these is the *E. coli*-derived β-galactosidase gene. The use of jellyfish-derived green fluorescent protein (GFP) and its spectral variants are quickly growing in popularity, especially since they enable evaluation of cell distribution in living organisms.

Use of genetic mouse chimeras in the study of development and gene function

Genetic chimeras have multiple uses in the study of developmental biology. Patterns of tissue growth and the underlying cellular behaviors are important for understanding tissue histogenesis and organogenesis. Genetic chimeras provide a unique means to addressing these issues in vivo. In the eye, analyses in chimeras have contributed to the discovery that growth in the RPE becomes progressively restricted to the distal edge and centripetal migration of limbal stem cell progeny continually renews dying cells in the corneal epithelium [reviewed in (Collinson et al., 2004)]. In the retina, analysis of the patterns of chimerism revealed the growth of radial clones and contributed to the realization of the multipotency of RPCs [reviewed in (Collinson et al., 2004)]. Chimera analyses were also instrumental in the identification of tangential dispersion as an active process directing the orderly spacing of retinal subtypes. In particular, chimeras were used to characterize the distance of tangential dispersion for retinal cell types (Reese et al., 1999) and reviewed in (Reese and Galli-Resta, 2002).

Genetic chimeras provide a powerful and unbiased approach to phenotypic analysis. They are particularly useful in defining the roles of genes with complex functions and phenotypes by enabling analyses of lineage potential, autonomy, and cell

behavior in the absence of gene function. Reduced contribution or exclusion of mutant cells from a particular lineage, in the absence of cell death and changes in proliferation, reveals a role for the gene of interest in the specification or differentiation of that lineage (Rossant and Spence, 1998; Tam and Rossant, 2003). In *Rx* null mice, the retina, RPE and optic stalk fail to form due to developmental arrest prior to optic vesicle formation (Mathers et al., 1997). The use of Rx chimeras revealed that this phenotype is the result of failed specification, rather than deficient proliferation, as *Rx* null cells are excluded from the eye field optic neuroepithelium and its subsequent lineages (Medina-Martinez et al., 2009). In complex phenotypes, chimera analyses of lineage potential can be used to evaluate multiple lineages in the same animal to determine the primary site of action of a gene. For example, chimeras demonstrated that the retina degeneration genes *rd* (*Pde6b*) and *Rds* act within the neural retina to promote photoreceptor degeneration, while *rdy* acts in the RPE to promote photoreceptor degeneration in the adjacent neural retina (LaVail and Mullen, 1976; Mullen and LaVail, 1976; Sanyal et al., 1986). *Pax6* null mutants fail to form eyes due to developmental arrest at the optic vesicle stage and lack lenses (Baumer et al., 2003; Hill et al., 1991). However, Pax6 is expressed in many tissues associated with eye development, including the eye field, throughout the optic vesicle, the retina and RPE of the optic cup, facial epithelium, lens placode, and lens (Walther and Gruss, 1991). Thus, defining the individual roles of Pax6 in ocular development using null mutants proved difficult. Analysis of genetic chimeras revealed that the absence of lenses in *Pax6* null embryos resulted from a requirement of Pax6 in the surface ectoderm for lens formation, rather than failure of the *Pax6* null optic vesicle to stimulate lens induction (Collinson et al., 2000; Collinson et al., 2003; Li et al., 2007;

Quinn et al., 1996). These analyses also revealed that retinal formation can proceed even in the absence of a lens when Pax6 was present in the optic vesicle (inferred from the predominance of wild type cells in the retina and RPE), suggesting that developmental arrest in the optic vesicle results from a requirement for Pax6 gene function in the optic vesicle rather than a failure of Pax6 null embryos to generate the lens (Li et al., 2007). In chimeras, *Pax6* null cells were also excluded from the corneal epithelium and displayed reduced contribution to the corneal stroma and endothelium, revealing a previously unappreciated role for Pax6 in cornea development (Collinson et al., 2003; Li et al., 2007). Use of tissue specific conditional knockouts has both confirmed and extended the analysis of Pax6 function in ocular development [reviewed in (Ashery-Padan and Gruss, 2001; Collinson et al., 2004)].

Genetic chimeras also provide the gold standard test for autonomy of gene function (Figure 1.9A). A gene functions cell autonomously if the original mutant phenotype manifests exclusively in genotypically mutant cells, irrespective of mutant:wild type proportions (Figure 1.9A). More commonly, exclusion of mutant cells from a particular tissue or lineage reveals cell autonomous gene function (Rossant and Spence, 1998; Tam and Rossant, 2003). As previously described, the exclusion of *Rx* null cells from the eye field optic neuroepithelium and its resulting lineages in chimeras reveals a cell autonomous role for Rx in the specification of the optic neuroepithelium (Medina-Martinez et al., 2009). *Pax6* null cells are largely excluded from the developing neural retina in Pax6 chimeras, and those that do contribute to the retina die perinatally, leaving only Pax6-deficient microglia, pericytes, astrocytes and vascular endothelial cells, which derive from extra-retinal sources and migrate into the developing retina (Li

et al., 2007). Cell nonautonomous gene function is revealed by the ability of surrounding wild type cells to rescue the mutant phenotype in genotypically mutant cells (Figure 1.9A). Cell nonautonomous gene function is also revealed by the converse; the ability of mutant cells to induce a mutant phenotype on genotypically wild type cells, and typically occurs when mutant cell contribution is high (Rossant and Spence, 1998; Tam and Rossant, 2003). Misexpression of a mutant rhodopsin gene in transgenic mice causes retinal degeneration. Generation of chimeras with these mice revealed that the mutant gene transgene induced degeneration in a cell nonautonomous fashion, as both wild type and mutant patches exhibited equal levels of degeneration. However, the degree of degeneration depended on the contribution of wild type cells; a greater contribution of wild type cells promoted reduced levels of degeneration (Huang et al., 1993). It is important to note that a gene may exhibit cell autonomous and cell nonautonomous functions, depending on the readout. For example, a transcription factor responsible for expression of a cell surface or secreted signaling molecule would exhibit a cell autonomous function if the readout was the expression of the signaling molecule, but cell nonautonomous if the readout was a change in cellular behavior, provided the signal acted in a paracrine fashion. Chimera analysis provides not only an unbiased approach for evaluating the contribution of cell nonautonomous mechanisms to mutant phenotypes, but also a powerful tool for placing gene function in the context of known signaling pathways regulating specific processes. Determining the autonomy of gene function in a cellular behavior enables researchers to determine whether the gene functions to regulate cell surface or secreted signaling molecules or acts on intrinsic factors involved in the regulation of cell behavior (Figure 1.9B). Further analysis of intrinsic factors in chimeras

can then be used to further refine the role of genes involved in the regulation of intrinsic factors.

Chimeras also facilitate detection of defects in cell behavior, which often become more apparent in the competitive environment of chimeras. Such behaviors include cell adhesion and migration. Cell adhesion differences are thought to underlie the segregation of *Pax6* null and wild type cells in Pax6 chimeras and apparent physical exclusion of mutant cells from the presumptive retina into ectopic vesicles (Collinson et al., 2000; Collinson et al., 2003; Li et al., 2007; Quinn et al., 1996). Differences in cell surface properties drove segregation of wild type and *Lhx2* null cells in aggregation assays and likely promoted the clustering of *Lhx2* null and wild type cells into discrete patches in the telencephalon of Lhx2 chimeras (Mangale et al., 2008). In Rx chimeras, *Rx* null cells contributed to the proximal optic stalk region, but segregated from wild type cells creating alternating columns of wild type and *Rx* null cells. These columns exhibited different thicknesses, suggesting that unlike adjacent wild type cells, *Rx* null cells fail to participate in convergent extension (Medina-Martinez et al., 2009).

Genetic mosaics provide an additional approach to answering these same questions (Rossant and Spence, 1998). Together, genetic chimeras and mosaics provide a diverse array of research tools for the study of multiple aspects of development. Each provides its own unique contribution to developmental studies and complements the other. Together and on their own, these techniques provide a powerful means of dissecting complex gene function and embryogenesis.

Aggregation chimeras were reported previously for the *or* allele of Vsx2 (Kindiakov and Koniukhov, 1986; Osipov and Vakhrusheva, 1982, 1984). *or* is allelic to

orJ utilized in the present studies. In these chimeras, both retinal structure and eye size were improved compared to homozygous *or* mice, although microphthalmia was still noted in *or* chimeras. Reduction in eye size generally correlated with the contribution of *or* cells, as indicated by analysis of chimerism in the adjacent RPE. Additionally, eye size and retinal structure both improved with age. The inability to distinguish *or* and wild type cells in the chimeric retina in these studies precluded interpretation of these results. Thus, it is not clear whether improved retinal development resulted from rescued *or* cell behavior or compensation by wild type cells. To distinguish between these possibilities and evaluate Vsx2 function using a defined *Vsx2* null allele, we sought to re-evaluate chimeras in the present study.

Summary and goals

Retinal development is a complex process involving stepwise induction from neural ectoderm and subsequent coordination of competing processes to ensure proper growth and differentiation during retinal histogenesis. The homeobox gene Vsx2 is an essential regulator of multiple aspects of retinal histogenesis. Maintenance of retinal identity, RPC proliferation and retinal neurogenesis still occur to some extent in the *orJ* retina, but the absence of Vsx2 function severely disrupts their execution. This not only impedes retinal development, but also has serious consequences on overall ocular development and visual function. Despite the obvious importance of Vsx2 in these cellular processes and retinal histogenesis, the molecular mechanisms underlying the regulation of these processes by Vsx2 is lacking and few direct targets have been identified. Given the importance of both extracellular cues and intrinsic factors in these

processes and the central role for Vsx2 in the regulation of RPC properties, it is possible that Vsx2 may regulate both extrinsic and intrinsic factors. Thus, the major focus of this work was to determine the degree to which Vsx2 regulates the extracellular signals involved in the various cellular processes of retinal histogenesis. Both candidate and unbiased approaches were undertaken to address this question. In the former, the known retinal mitogen Shh and its signaling pathway were evaluated in *orJ* retinas to determine whether altered Shh production or Hh signaling contributed to the defective proliferation observed in the *orJ* retina (Chapters 2 and 3). An unbiased approach was also undertaken to evaluate the contribution of altered extracellular signals to the disruption of cellular processes during retinal histogenesis in the absence of Vsx2. This was accomplished by determining the autonomy of Vsx2 functions using genetic mouse chimeras (Chapter 4).

These studies will facilitate the dissection of Vsx2 function and help place Vsx2 in the context of known signaling pathways that regulate the processes driving retinal histogenesis. They will also address fundamental questions regarding the mechanisms regulating RPC properties and provide insight into the coordination and integration of extracellular cues and intrinsic factors in the regulation of these properties. Advancing our understanding of normal retinal development and the necessary factors regulating RPC properties will facilitate development of techniques to control stem cells for therapeutic purposes in the treatment of retinal disorders and degenerative diseases. Many of the developmental principles driving retinal histogenesis, including multipotent progenitors, progenitor proliferation, and ordered differentiation, also underlie the development of other central nervous system structures, including the cortex and cerebellum (Donovan and Dyer, 2005). Thus, advances in the understanding of regulation

of RPC properties, integration of extrinsic signals and intrinsic factors, and coordination of progenitor properties will also contribute to a general understanding of the principles of central nervous system development.

Figure 1.1. Basic anatomy of the eye and retina. (A) Schematized cross section of the vertebrate eye illustrating the location of the neural retina and associated ocular tissues. Yellow arrow shows the path of light through the eye. (B) Schematic diagram illustrating the cytoarchitecture of the adult vertebrate retina. Apical is oriented to the top and basal to the bottom. Gray arrows indicate the direction of the light path and neural information flow. Abbreviations: A, amacrine cell; B, bipolar cell; C, cone photoreceptor; CB, ciliary body; Ch, choroid; Co, cornea; G, ganglion cell; GCL, ganglion cell layer; H, horizontal cell; I, iris; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; IS, photoreceptor inner segments; L, lens; M, Müller glia; NF, nerve fiber layer; NR, neural retina; OLM, outer limiting membrane; ON, optic nerve; ONH, optic nerve head; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, photoreceptor outer segments; R, rod photoreceptor; RPE, retinal pigmented epithelium; S, sclera; V, vitreous.

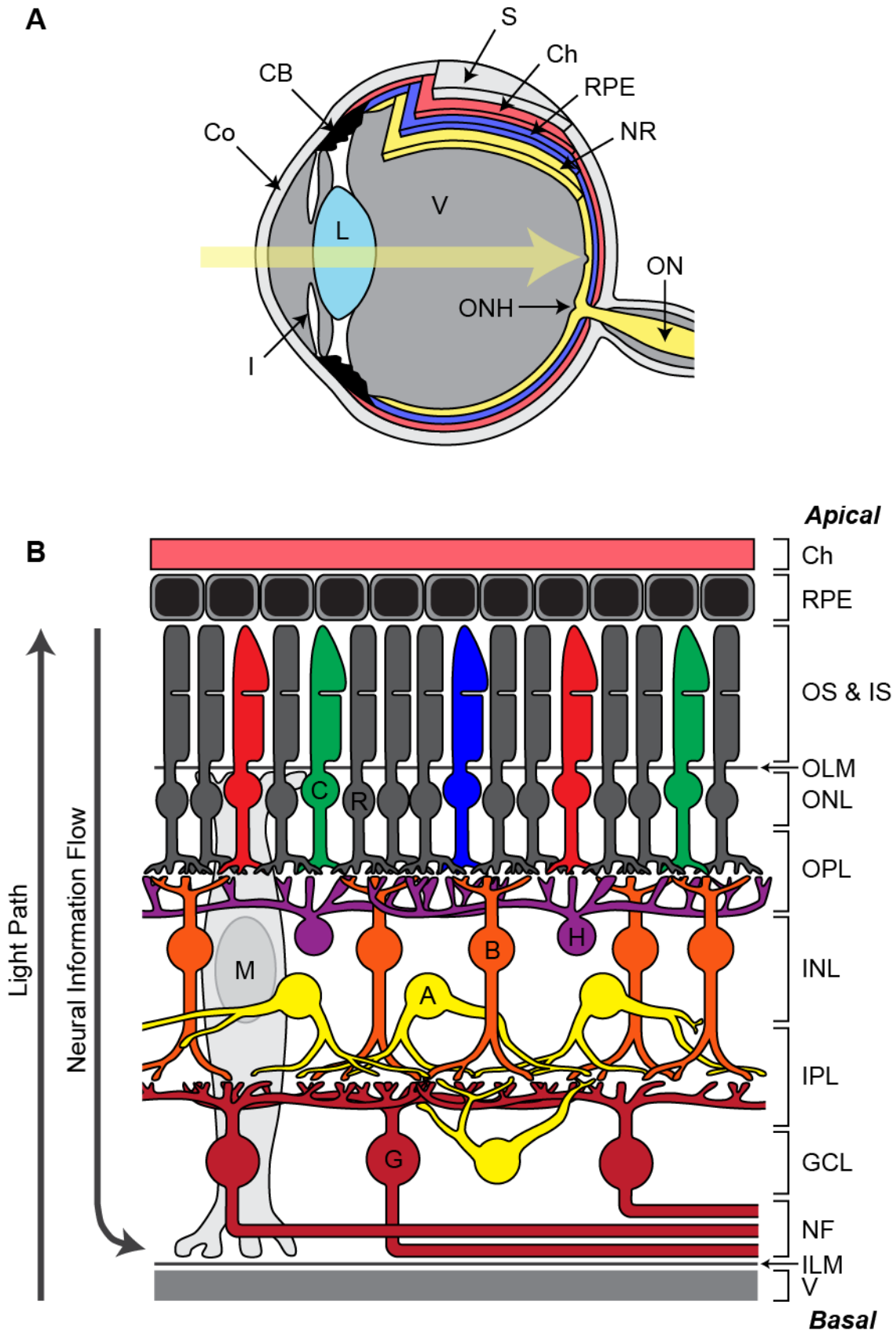
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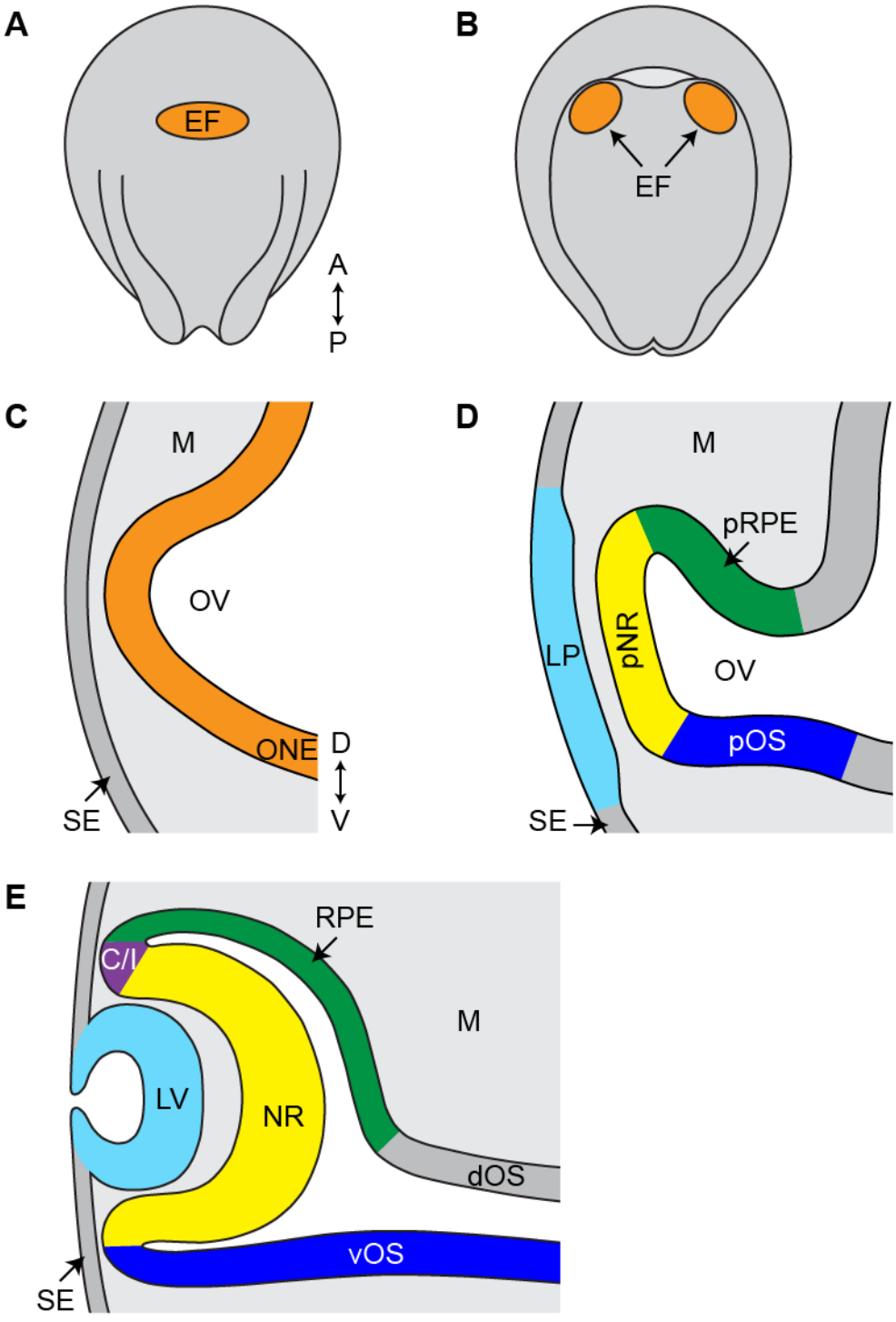
Figure 1.2. Overview of vertebrate retinal development. (A) The eye field (orange) is specified as single territory in the anterior neuroepithelium during the early neurula stage. (B) This single eye field is then resolved into the bilateral eye primordial. (C) Evagination of the neuroepithelium within each eye primordium generates the optic vesicles. (D) Patterning of the optic vesicle divides the optic neuroepithelium into the presumptive domains of the neural retina, retinal pigmented epithelium (RPE), and optic stalk. Early contact of the optic vesicle with the overlying surface ectoderm induces formation of the lens placode. (E) Invagination of the lens placode and distal optic vesicle generates the lens vesicle and bilayered optic cup, establishing the overall structure of the eye and positioning of ocular tissues. The optic neuroepithelium positioned at the border between the retina and RPE eventually contributes to components of the ciliary body and iris (C/I). In A and B, anterior is up and posterior is down; while dorsal is up and ventral is down in C-E. Abbreviations: dOS, dorsal optic stalk; EF, eye field; LP, lens placode; LV, lens vesicle; M, mesenchyme; ONE, optic neuroepithelium; OV, optic vesicle; NR, neural retina; pNR, presumptive neural retina; pOS, presumptive optic stalk; pRPE, presumptive retinal pigmented epithelium; SE, surface ectoderm; vOS, ventral optic stalk.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SE

Figure 1.3. The cell cycle and regulation of G1 progression. (A) Cells progress through four distinct phases of the cell cycle. Mitogen signals are required in early G1 to promote G1 progression past the restriction point for entry into S phase. (B) The regulatory cascade driving the G1-to-S phase transition involves a positive feedback loop and initiation by mitogen signals. Abbreviations: CDKI, cyclin-dependent kinase inhibitor; CycD, D-cyclins; CycE, E-cyclins; (p), phosphorylation; R, restriction point; RB, retinoblastoma proteins.

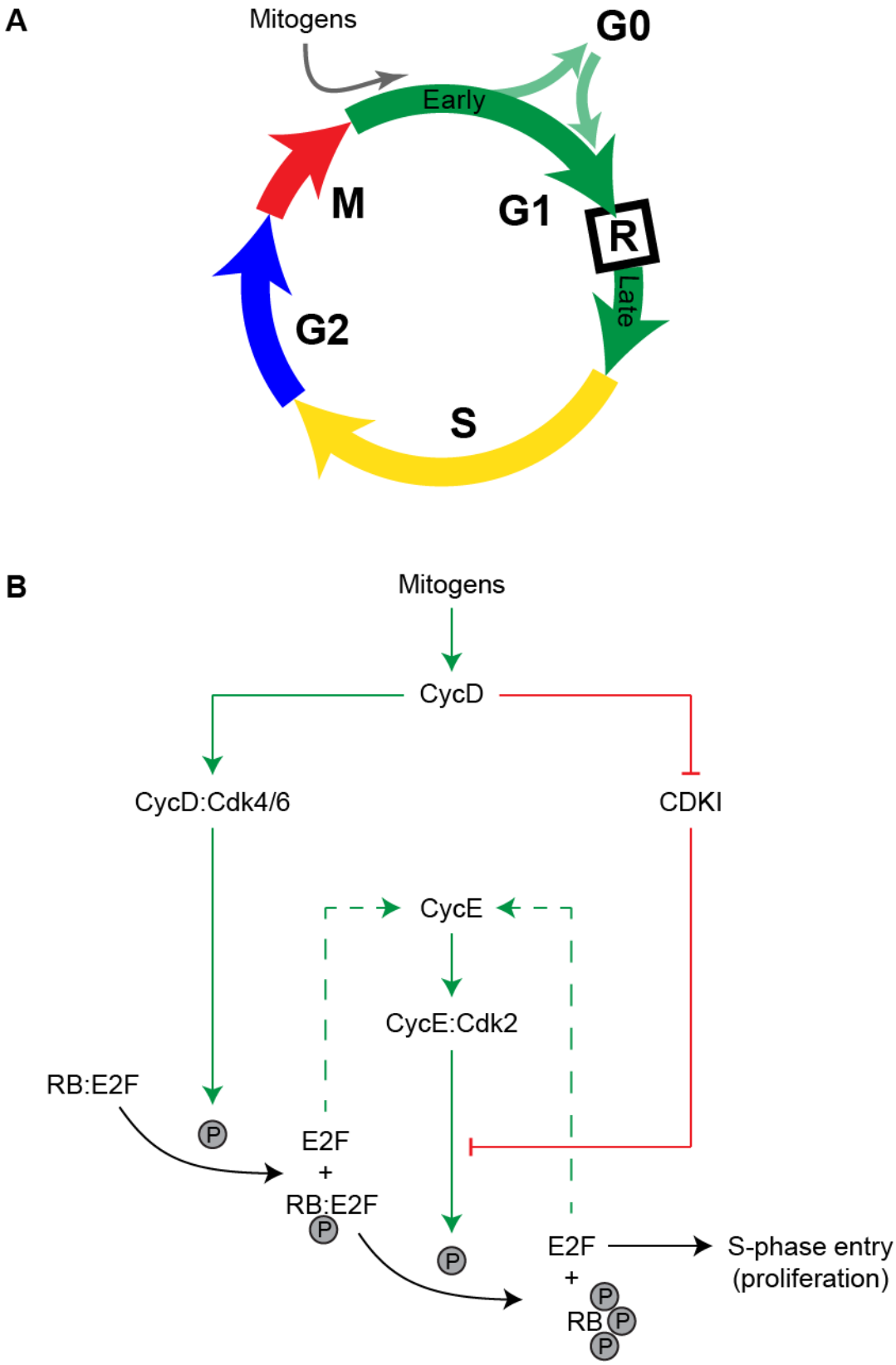

A
Mitogens
G0
Early
M
G1
R
Late
G2
S
B
Mitogens
CycD
CycD:Cdk4/6
CDKI
CycE
CycE:Cdk2
RB:E2F
P
E2F
+
RB:E2F
P
P
E2F
+
P
RB P
P
S-phase entry
(proliferation)

Figure 1.4. Temporal and spatial progression of retinal neurogenesis. (A) The seven major classes of retinal cell types are generated during limited but overlapping periods and according to an evolutionarily conserved birth order. In the mouse, retinal neurogenesis begins with ganglion cell generation around embryonic day 11 (E11) and is completed around postnatal day 12 (P12). (B) Retinal neurogenesis proceeds in a specific spatio-temporal pattern across the neural retina. In the mouse, neurogenesis initiates in a small central patch, dorsal to the optic stalk. Over time, neurogenesis progresses in a peripherally-spreading wave. Dorsal is up and posterior is left. Abbreviations: L, lens; NR, neural retina; OS, optic stalk.

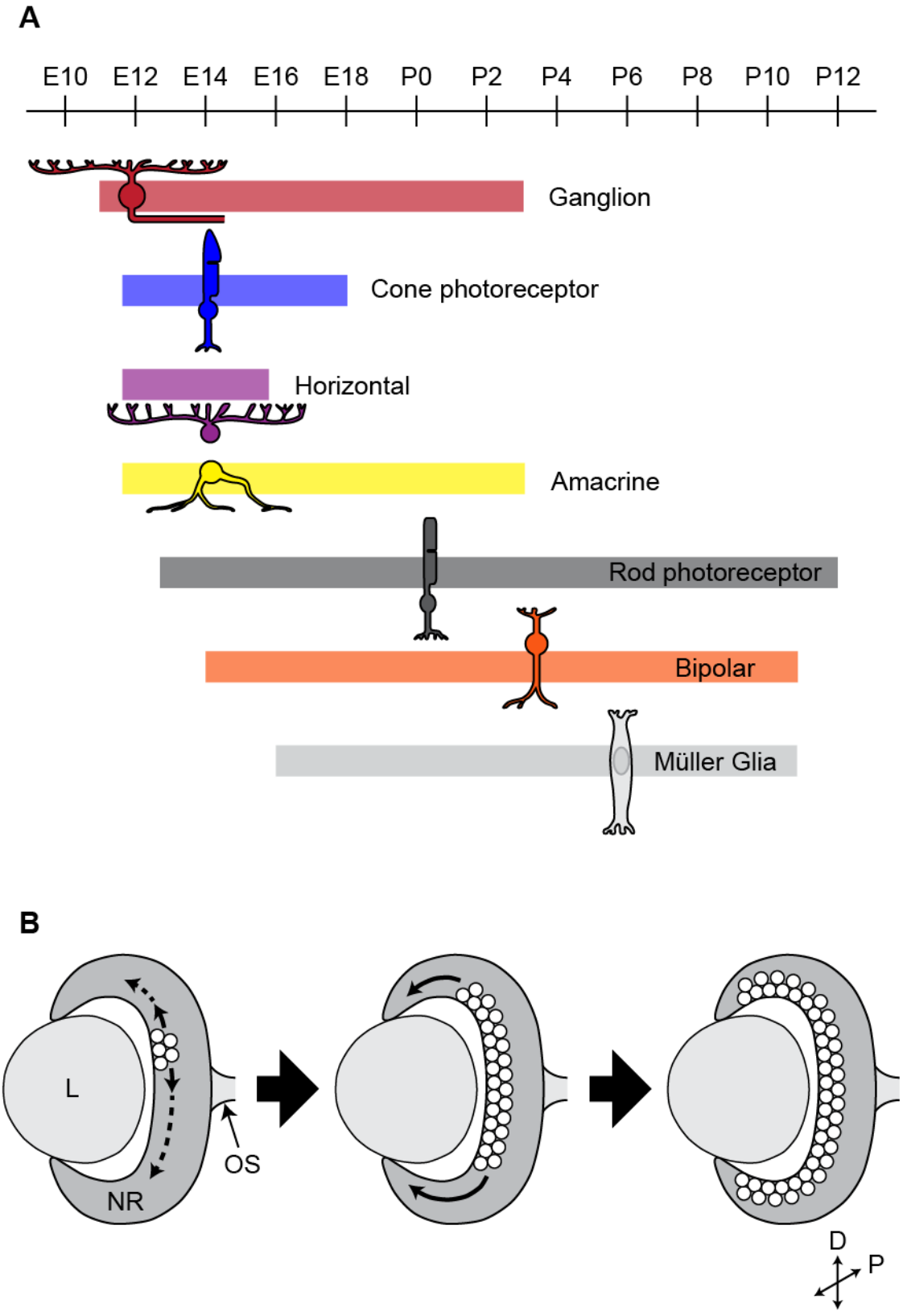

A
E10
E12
E14
E16
E18
P0
P2
P4
P6
P8
P10
P12
Ganglion
Cone photoreceptor
Horizontal
Amacrine
Rod photoreceptor
Bipolar
Müller Glia
B
L
OS
NR
D
P

Figure 1.5. Structure of the Vsx2 gene and protein. The genomic structure of the *Vsx2* gene and its relation to the domain structure of the Vsx2 protein is illustrated. The *Vsx2* gene contains five known exons, represented as numbered boxes. Coding regions of each exon in the *Vsx2* gene are shown in gray, noncoding regions in white. Protein regions encoded by each exon are indicated by dotted lines. Conserved protein domains are indicated as follows: the octapeptide sequence is depicted in blue, the homeodomain in green, the CVC domain in red, and the OAR domain in yellow. Locations of mutations identified in humans are indicated by blue arrows, while the *orJ* mutation in mouse is indicated by the magenta arrow. Abbreviations: HMD, homeodomain; OP, octapeptide sequence.

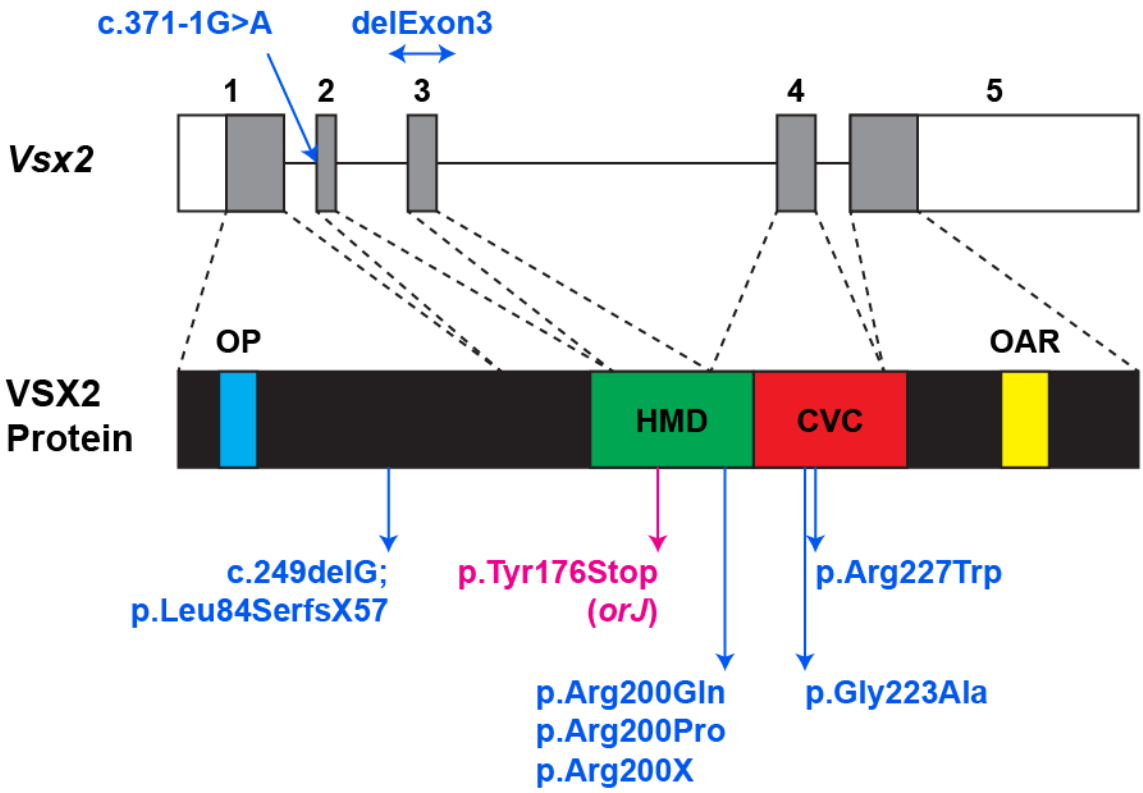

c.371-1G>A
delExon3
1
2
3
4
5
Vsx2
OP
OAR
VSX2
Protein
HMD
CVC
c.249delG;
p.Leu84SerfsX57
p.Tyr176Stop
(orJ)
p.Arg227Trp
p.Arg200Gln
p.Arg200Pro
p.Arg200X
p.Gly223Ala

Figure 1.6. Model of disrupted retinal development in the *orJ* retina. Patterning of the optic vesicle directs optic neuroepithelial cells towards one of three ocular identities, resulting in the specification of progenitors of the neural retina, RPE, and optic stalk. Each of these progenitor populations undergoes proliferative expansion and differentiation into their mature cell types. In the absence of Vsx2 function in the *orJ* retina, these processes are disrupted. Failure to downregulate the RPE determinant Mitf in specified RPCs indicates compromised maintenance of retinal identity (1a). Aberrant expression of Mitf and other genes typically associated with the RPE gene expression program promote activation of a pigmentation program, often leading to hyperpigmentation of retinal cells at later developmental ages (1b). Relative to RPE and optic stalk progenitors, RPCs exhibit robust proliferative expansion that is severely reduced in the absence of Vsx2 function (2). Multiple aspects of retinal neurogenesis are also disrupted. Although the temporal birth order of retinal cell types is maintained, initiation of this process is delayed approximately 2 days in the *orJ* retina (3). Lastly, *orJ* RPCs fail to generate bipolar cells, likely due to a specific requirement for Vsx2 in their specification or maturation (4). Abbreviations: AC, amacrine cell; BC, bipolar cell; Cone, cone photoreceptor; GC, ganglion cell; HC, horizontal cell; MG, Müller glia cell; ONC, optic neuroepithelial cell; OS, optic stalk; OSP, optic stalk progenitor; Prolif, proliferation; RPC, retinal progenitor cell; RPE, retinal pigmented epithelium; RPEP, RPE progenitor cell; Rod, rod photoreceptor.

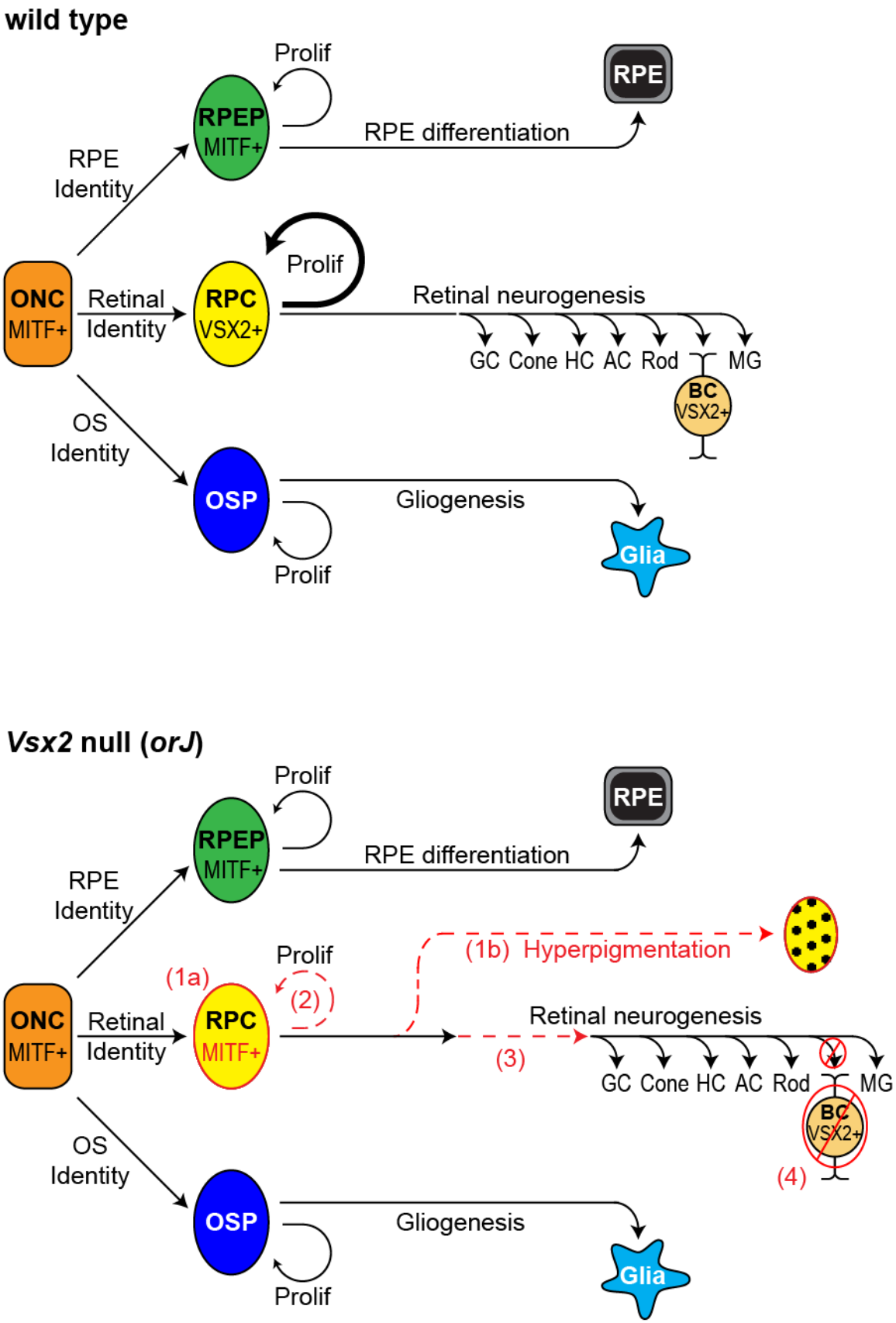

wild type
Prolif
RPEP
MITF+
RPE differentiation
RPE
RPE
Identity
ONC
MITF+
Retinal
Identity
RPC
VSX2+
Prolif
Retinal neurogenesis
GC Cone HC AC Rod MG
BC
VSX2+
OS
Identity
OSP
Gliogenesis
Glia
Prolif
Vsx2 null (orJ)
Prolif
RPEP
MITF+
RPE differentiation
RPE
RPE
Identity
(1b) Hyperpigmentation
Prolif
(1a)
(2)
ONC
MITF+
Retinal
Identity
RPC
MITF+
Retinal neurogenesis
(3)
GC Cone HC AC Rod MG
BC
VSX2+
(4)
OS
Identity
OSP
Gliogenesis
Glia
Prolif

Figure 1.7. Detailed overview of the vertebrate Hh signaling pathway. Transduction of Hh signals in receptive cells alters transcriptional regulation of Hh target genes in the nucleus. Refer to the text for a detailed description of ligand reception and signal transduction mechanisms. Abbreviations: Gli-A, activator form of Gli proteins; Gli-R, Gli repressor form of Gli proteins; Gli-FL, full-length Gli proteins; (p), phosphorylation; Smo-A, activated Smo; Ub-, ubiquitinated form.

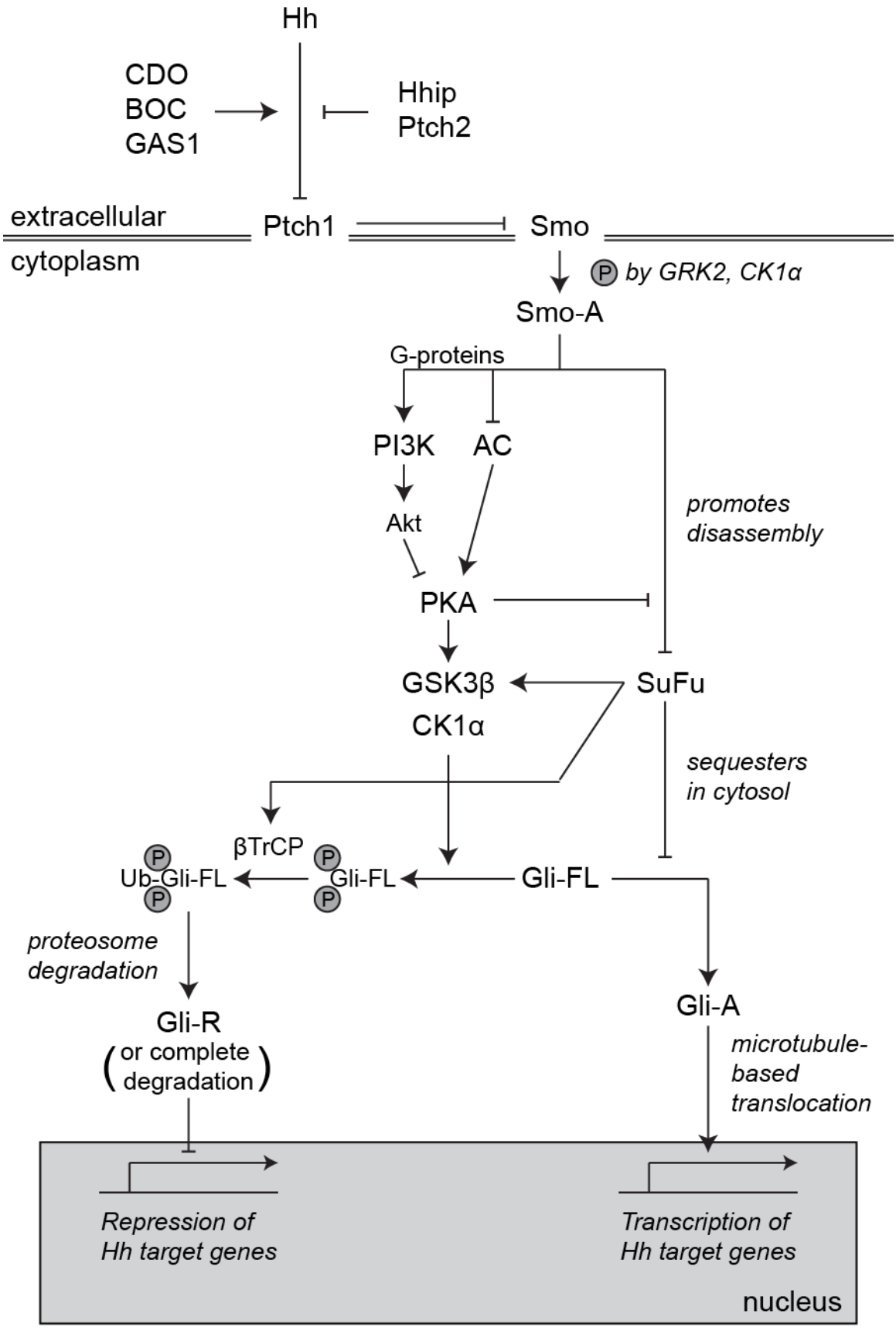

Hh
CDO
BOC
GAS1
Hhip
Ptch2
extracellular
cytoplasm
Ptch1
Smo
P by GRK2, CK1α
Smo-A
G-proteins
PI3K
AC
Akt
promotes disassembly
PKA
GSK3β
CK1α
SuFu
sequesters in cytosol
βTrCP
Ub-Gli-FL
Gli-FL
Gli-FL
proteosome degradation
Gli-R
(or complete degradation)
Gli-A
microtubule-based translocation
Repression of Hh target genes
Transcription of Hh target genes
nucleus

Figure 1.8. Methods of chimera generation and resultant lineage contribution of cell populations. Genotypically distinct cell populations for use in the generation of chimeric blastocysts can be obtained from a variety of sources. Generation of chimeric blastocysts is accomplished either through aggregation of the cell populations or through injection of ES or TS cells into blastocyst stage embryos. Contribution of the distinct cell populations to the chimeric blastocyst and subsequent embryonic tissues varies and depends upon the developmental potential of the source of each cell population. The three lineages in blastocyst stage embryos have distinct fates. The trophectoderm contributes exclusively to the trophoblast layer of the placenta. The primitive endoderm contributes to the yolk sac endoderm. The epiblast contributes to both the embryo proper and extraembryonic cells. Embryo:embryo combinations are shown in green, ES cell:embryo combinations in magenta, and TS cell:embryo combinations in blue. Solid colors indicate nonmosaic contribution while patterns indicate mosaic contribution. Segregation of cell populations in tetraploid:embryo combinations are not as complete as in tetraploid:ES cell combinations. Abbreviations: ep, epiblast; ES, embryonic stem cells; ICM, inner cell mass; P, placenta; pE, primitive endoderm; tr, trophectoderm; TS, trophoblast stem cells; Y, yolk sac.

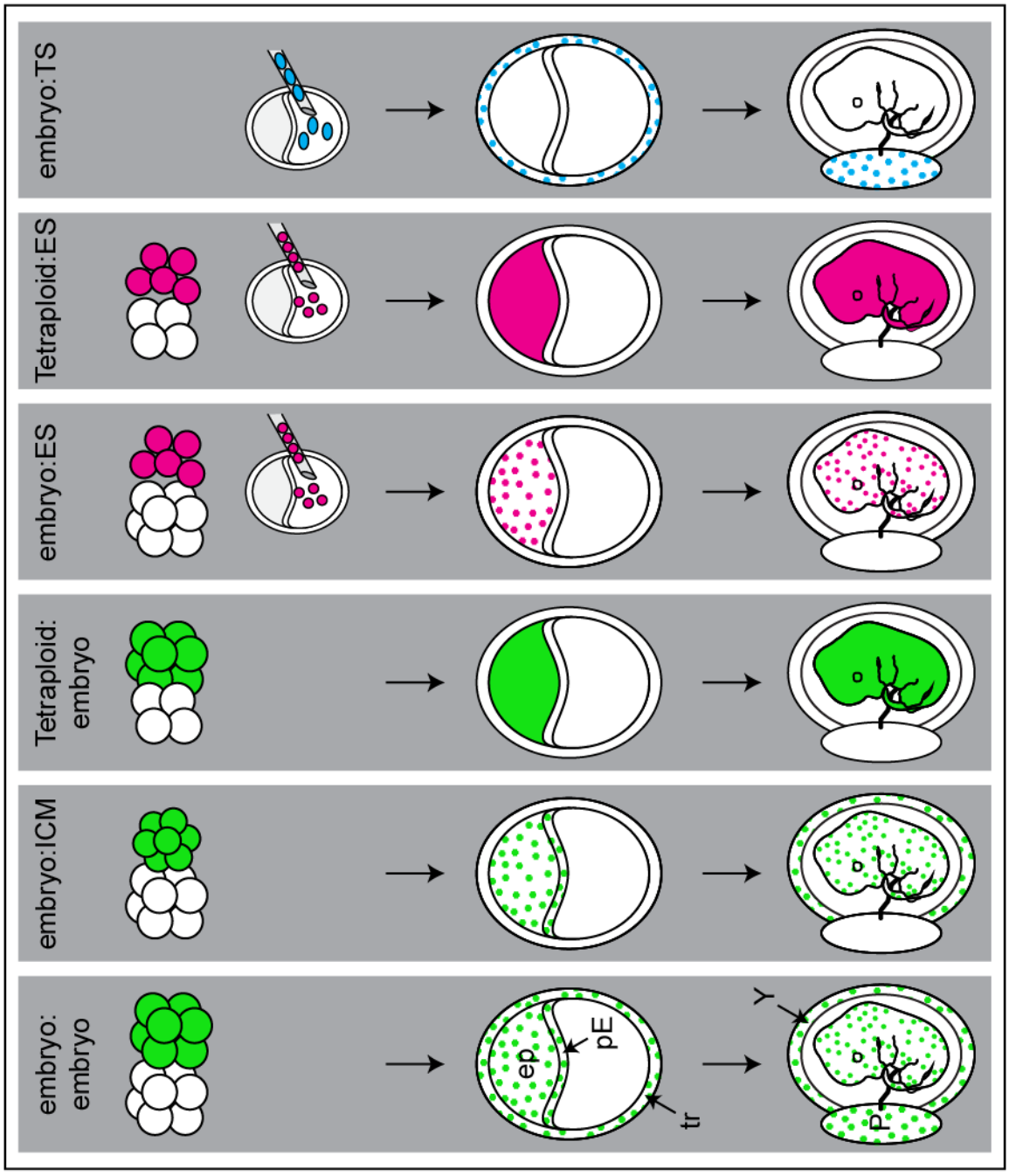
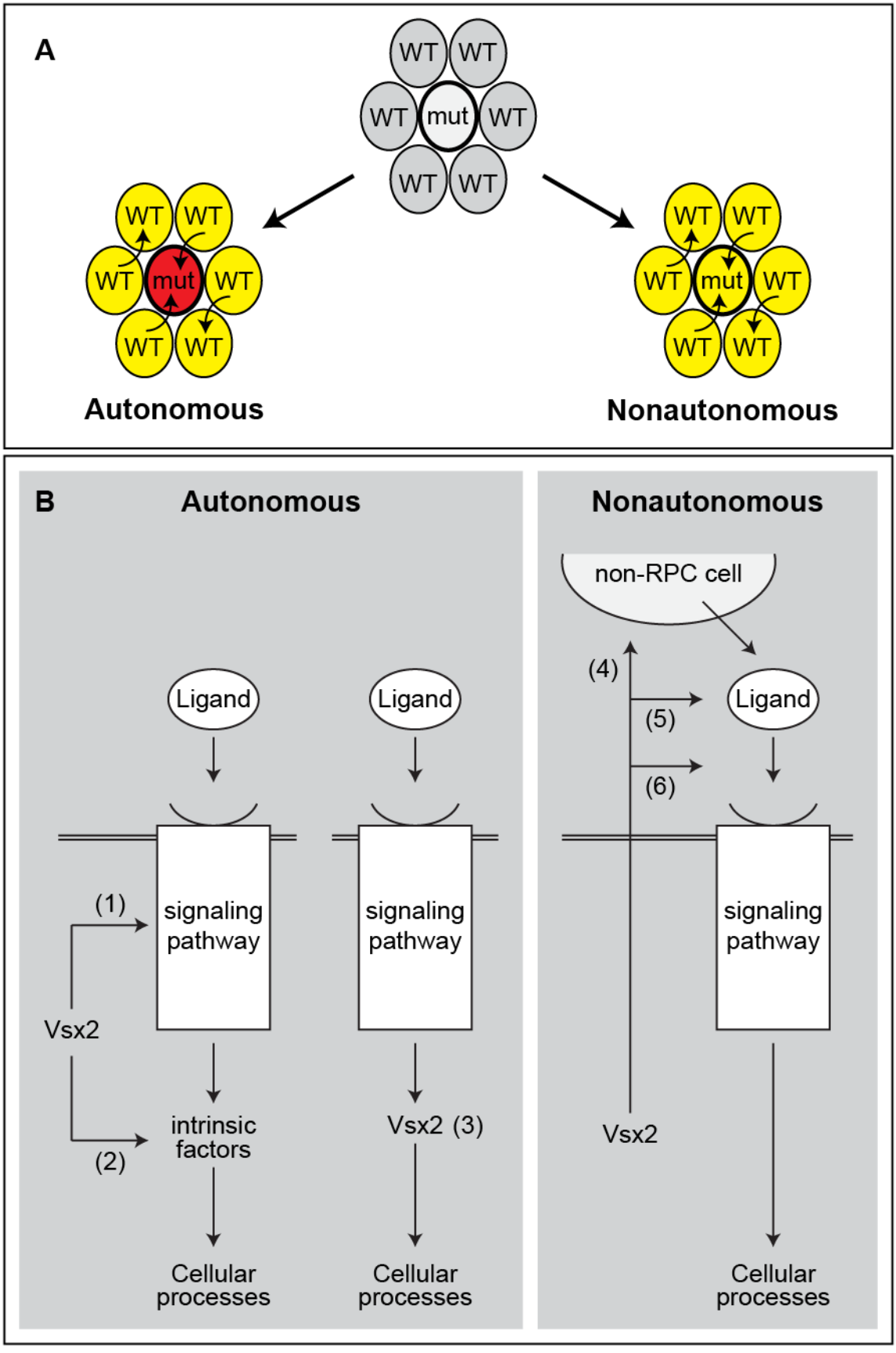

embryo:
embryo
embryo:ICM
Tetraploid:
embryo
embryo:ES
Tetraploid:ES
embryo:TS
ep
pE
tr
Y
P

Figure 1.9. Relationship between autonomy and gene function. (A) Schematic illustrating phenotypic outcomes for autonomous and nonautonomous gene function using genetic chimeras. Cell autonomous gene function is revealed when the mutant cell retains its mutant phenotype, irrespective of wild type cell contribution to the tissue, indicating that the mutant cell is refractory to extrinsic signals provided by adjacent wild type cells. Cell nonautonomous gene function is revealed when the mutant phenotype is rescued in genotypically mutant cells by extrinsic signals provided by wild type cells. (B) Possible autonomous and nonautonomous modes of Vsx2 gene function in the regulation of cellular processes, based on the biochemical function of Vsx2 as a transcription factor and restricted expression of Vsx2 to the cell population exhibiting the mutant phenotypes. The following possible types of Vsx2-mediated regulation would manifest as cell autonomous functions in chimeras: Vsx2 regulates intrinsic components of the regulatory signaling pathway (1) and/or downstream intrinsic factors involved in the execution of the cellular process (2), but Vsx2 itself is not an intrinsic component; Vsx2 mediates the effects of the signaling pathway on the cellular process (3). The following possible types of Vsx2-mediated regulation would manifest as cell nonautonomous functions in chimeras: Vsx2 regulates availability of the ligand indirectly by affecting development of non-RPC cell types responsible for ligand production (4) or directly through transcriptional regulation of the ligand in RPCs (5); Vsx2 regulates extrinsic factors required for ligand presentation, reception, or dispersion (6).


A
WT
mut
Autonomous
Nonautonomous
B
Autonomous
Nonautonomous
non-RPC cell
Ligand
signaling pathway
(1)
(2)
(3)
(4)
(5)
(6)
Vsx2
intrinsic factors
Cellular processes

References

Adelmann, H., 1936a. The problem of cyclopia. Part I. Q. Rev. Biol. 11, 161.

Adelmann, H., 1936b. The problem of cyclopia. Part II. Q. Rev. Biol. 11, 284.

Agathocleous, M., Harris, W.A., 2009. From progenitors to differentiated cells in the vertebrate retina. Annu. Rev. Cell Dev. Biol. 25, 45-69.

Alexiades, M.R., Cepko, C., 1996. Quantitative analysis of proliferation and cell cycle length during development of the rat retina. Dev. Dyn. 205, 293-307.

Allen, B.L., Song, J.Y., Izzi, L., Althaus, I.W., Kang, J.S., Charron, F., Krauss, R.S., McMahon, A.P., 2011. Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. Dev. Cell 20, 775-787.

Amato, M.A., Boy, S., Perron, M., 2004. Hedgehog signaling in vertebrate eye development: a growing puzzle. Cell. Mol. Life Sci. 61, 899-910.

Anchan, R.M., Reh, T.A., 1995. Transforming growth factor-beta-3 is mitogenic for rat retinal progenitor cells in vitro. J. Neurobiol. 28, 133-145.

Anchan, R.M., Reh, T.A., Angello, J., Balliet, A., Walker, M., 1991. EGF and TGF-alpha stimulate retinal neuroepithelial cell proliferation in vitro. Neuron 6, 923-936.

Andreazzoli, M., 2009. Molecular regulation of vertebrate retina cell fate. Birth Defects Res., Part C 87, 284-295.

Ashery-Padan, R., Gruss, P., 2001. Pax6 lights-up the way for eye development. Curr. Opin. Cell Biol. 13, 706-714.

Ashwell, K.W., Hollander, H., Streit, W., Stone, J., 1989. The appearance and distribution of microglia in the developing retina of the rat. Vis. Neurosci. 2, 437-448.

Bao, Z.Z., Cepko, C.L., 1997. The expression and function of Notch pathway genes in the developing rat eye. J. Neurosci. 17, 1425-1434.

Bar-Yosef, U., Abuelaish, I., Harel, T., Hendler, N., Ofir, R., Birk, O.S., 2004. CHX10 mutations cause non-syndromic microphthalmia/ anophthalmia in Arab and Jewish kindreds. Hum. Genet. 115, 302-309.

Barabino, S.M., Spada, F., Cotelli, F., Boncinelli, E., 1997. Inactivation of the zebrafish homologue of Chx10 by antisense oligonucleotides causes eye malformations similar to the ocular retardation phenotype. Mech. Dev. 63, 133-143.

Baumer, N., Marquardt, T., Stoykova, A., Spieler, D., Treichel, D., Ashery-Padan, R., Gruss, P., 2003. Retinal pigmented epithelium determination requires the redundant activities of Pax2 and Pax6. Development 130, 2903-2915.

Behesti, H., Holt, J.K., Sowden, J.C., 2006. The level of BMP4 signaling is critical for the regulation of distinct T-box gene expression domains and growth along the dorso-ventral axis of the optic cup. BMC Dev. Biol. 6, 62.

Belecky-Adams, T., Tomarev, S., Li, H.S., Ploder, L., McInnes, R.R., Sundin, O., Adler, R., 1997. Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. Invest. Ophthalmol. Vis. Sci. 38, 1293-1303.

Bharti, K., Liu, W., Csermely, T., Bertuzzi, S., Arnheiter, H., 2008. Alternative promoter use in eye development: the complex role and regulation of the transcription factor MITF. Development 135, 1169-1178.

Black, G.C., Mazerolle, C.J., Wang, Y., Campsall, K.D., Petrin, D., Leonard, B.C., Damji, K.F., Evans, D.G., McLeod, D., Wallace, V.A., 2003. Abnormalities of the vitreoretinal interface caused by dysregulated Hedgehog signaling during retinal development. Hum. Mol. Genet. 12, 3269-3276.

Bone-Larson, C., Basu, S., Radel, J.D., Liang, M., Perozek, T., Kapousta-Bruneau, N., Green, D.G., Burmeister, M., Hankin, M.H., 2000. Partial rescue of the ocular retardation phenotype by genetic modifiers. J. Neurobiol. 42, 232-247.

Bora, N., Conway, S.J., Liang, H., Smith, S.B., 1998. Transient overexpression of the Microphthalmia gene in the eyes of Microphthalmia vitiligo mutant mice. Dev. Dyn. 213, 283-292.

Burkitt Wright, E.M., Perveen, R., Bowers, N., Ramsden, S., McCann, E., O'Driscoll, M., Lloyd, I.C., Clayton-Smith, J., Black, G.C., 2010. VSX2 in microphthalmia: a novel splice site mutation producing a severe microphthalmia phenotype. Br. J. Ophthalmol. 94, 386-388.

Burmeister, M., Novak, J., Liang, M.Y., Basu, S., Ploder, L., Hawes, N.L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V.I., Roderick, T.H., Taylor, B.A., Hankin, M.H., McInnes, R.R., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. 12, 376-384.

Cai, Z., Feng, G.S., Zhang, X., 2010. Temporal requirement of the protein tyrosine phosphatase Shp2 in establishing the neuronal fate in early retinal development. J. Neurosci. 30, 4110-4119.

Carpenter, D., Stone, D.M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A., de Sauvage, F.J., 1998. Characterization of two patched receptors for the vertebrate hedgehog protein family. Proc. Natl. Acad. Sci. U. S. A. 95, 13630-13634.

Carreira, S., Goodall, J., Aksan, I., La Rocca, S.A., Galibert, M.D., Denat, L., Larue, L., Goding, C.R., 2005. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature 433, 764-769.

Cavodeassi, F., Carreira-Barbosa, F., Young, R.M., Concha, M.L., Allende, M.L., Houart, C., Tada, M., Wilson, S.W., 2005. Early stages of zebrafish eye formation require the coordinated activity of Wnt11, Fz5, and the Wnt/beta-catenin pathway. Neuron 47, 43-56.

Cepko, C.L., Austin, C.P., Yang, X., Alexiades, M., Ezzeddine, D., 1996. Cell fate determination in the vertebrate retina. Proc. Natl. Acad. Sci. U. S. A. 93, 589-595.

Chen, C.M., Cepko, C.L., 2000. Expression of Chx10 and Chx10-1 in the developing chicken retina. Mech. Dev. 90, 293-297.

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383, 407-413.

Cho, S.H., Cepko, C.L., 2006. Wnt2b/beta-catenin-mediated canonical Wnt signaling determines the peripheral fates of the chick eye. Development 133, 3167-3177.

Chow, R.L., Lang, R.A., 2001. Early eye development in vertebrates. Annu. Rev. Cell Dev. Biol. 17, 255-296.

Chuang, P.T., Kawcak, T., McMahon, A.P., 2003. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. Genes Dev. 17, 342-347.

Chuang, P.T., McMahon, A.P., 1999. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature 397, 617-621.

Clark, A.M., Yun, S., Veien, E.S., Wu, Y.Y., Chow, R.L., Dorsky, R.I., Levine, E.M., 2008. Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina. Brain Res. 1192, 99-113.

Close, J.L., Gumuscu, B., Reh, T.A., 2005. Retinal neurons regulate proliferation of postnatal progenitors and Muller glia in the rat retina via TGF beta signaling. Development 132, 3015-3026.

Cohen, M.M., Jr., 2003. The hedgehog signaling network. Am. J. Med. Genet. A 123A, 5-28.

Collinson, J.M., Hill, R.E., West, J.D., 2000. Different roles for Pax6 in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye. Development 127, 945-956.

Collinson, J.M., Hill, R.E., West, J.D., 2004. Analysis of mouse eye development with chimeras and mosaics. Int. J. Dev. Biol. 48, 793-804.

Collinson, J.M., Quinn, J.C., Hill, R.E., West, J.D., 2003. The roles of Pax6 in the cornea, retina, and olfactory epithelium of the developing mouse embryo. Dev. Biol. 255, 303-312.

Dakubo, G.D., Wang, Y.P., Mazerolle, C., Campsall, K., McMahon, A.P., Wallace, V.A., 2003. Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development. Development 130, 2967-2980.

Dance, M., Montagner, A., Salles, J.P., Yart, A., Raynal, P., 2008. The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway. Cell. Signal. 20, 453-459.

Das, G., Choi, Y., Sicinski, P., Levine, E.M., 2009. Cyclin D1 fine-tunes the neurogenic output of embryonic retinal progenitor cells. Neural Dev. 4, 15.

Das, G., Clark, A.M., Levine, E.M., 2012. Cyclin D1 inactivation extends proliferation and alters histogenesis in the postnatal mouse retina. Dev. Dyn. 241, 941-952.

Das, I., Sparrow, J.R., Lin, M.I., Shih, E., Mikawa, T., Hempstead, B.L., 2000. Trk C signaling is required for retinal progenitor cell proliferation. J. Neurosci. 20, 2887-2895.

Dhomen, N.S., Balaggan, K.S., Pearson, R.A., Bainbridge, J.W., Levine, E.M., Ali, R.R., Sowden, J.C., 2006. Absence of chx10 causes neural progenitors to persist in the adult retina. Invest. Ophthal. Vis. Sci. 47, 386-396.

Donovan, S.L., Dyer, M.A., 2005. Regulation of proliferation during central nervous system development. Semin. Cell Dev. Biol. 16, 407-421.

Dorval, K.M., Bobechko, B.P., Ahmad, K.F., Bremner, R., 2005. Transcriptional activity of the paired-like homeodomain proteins CHX10 and VSX1. J Biol. Chem. 280, 10100-10108.

Dorval, K.M., Bobechko, B.P., Fujieda, H., Chen, S., Zack, D.J., Bremner, R., 2006. CHX10 targets a subset of photoreceptor genes. J. Biol. Chem. 281, 744-751.

Eivers, E., McCarthy, K., Glynn, C., Nolan, C.M., Byrnes, L., 2004. Insulin-like growth factor (IGF) signalling is required for early dorso-anterior development of the zebrafish embryo. Int. J. Dev. Biol. 48, 1131-1140.

Ekker, S.C., Ungar, A.R., Greenstein, P., von Kessler, D.P., Porter, J.A., Moon, R.T., Beachy, P.A., 1995. Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. Curr. Biol. 5, 944-955.

Elliott, J., Jolicoeur, C., Ramamurthy, V., Cayouette, M., 2008. Ikaros confers early temporal competence to mouse retinal progenitor cells. Neuron 60, 26-39.

Faiyaz-Ul-Haque, M., Zaidi, S.H., Al-Mureikhi, M.S., Peltekova, I., Tsui, L.C., Teebi, A.S., 2007. Mutations in the CHX10 gene in non-syndromic microphthalmia/anophthalmia patients from Qatar. Clin. Genet. 72, 164-166.

Fantl, V., Stamp, G., Andrews, A., Rosewell, I., Dickson, C., 1995. Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. 9, 2364-2372.

Ferda Percin, E., Ploder, L.A., Yu, J.J., Arici, K., Horsford, D.J., Rutherford, A., Bapat, B., Cox, D.W., Duncan, A.M., Kalnins, V.I., Kocak-Altintas, A., Sowden, J.C., Traboulsi, E., Sarfarazi, M., McInnes, R.R., 2000. Human microphthalmia associated with mutations in the retinal homeobox gene CHX10. Nat. Genet. 25, 397-401.

Frank, D.U., Fotheringham, L.K., Brewer, J.A., Muglia, L.J., Tristani-Firouzi, M., Capecchi, M.R., Moon, A.M., 2002. An Fgf8 mouse mutant phenocopies human 22q11 deletion syndrome. Development 129, 4591-4603.

Fuhrmann, S., Levine, E.M., Reh, T.A., 2000. Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. Development 127, 4599-4609.

Furimsky, M., Wallace, V.A., 2006. Complementary Gli activity mediates early patterning of the mouse visual system. Dev. Dyn. 235, 594-605.

Galliot, B., de Vargas, C., Miller, D., 1999. Evolution of homeobox genes: Q50 Paired-like genes founded the Paired class. Dev. Genes Evol. 209, 186-197.

Geng, X., Speirs, C., Lagutin, O., Inbal, A., Liu, W., Solnica-Krezel, L., Jeong, Y., Epstein, D.J., Oliver, G., 2008. Haploinsufficiency of Six3 fails to activate Sonic hedgehog expression in the ventral forebrain and causes holoprosencephaly. Dev. Cell 15, 236-247.

Giacinti, C., Giordano, A., 2006. RB and cell cycle progression. Oncogene 25, 5220-5227.

Goetz, S.C., Anderson, K.V., 2010. The primary cilium: a signalling centre during vertebrate development. Nat. Rev. Genet. 11, 331-344.

Green, E.S., Stubbs, J.L., Levine, E.M., 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development 130, 539-552.

Hashimoto, T., Zhang, X.M., Chen, B.Y., Yang, X.J., 2006. VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation. Development 133, 2201-2210.

Hatakeyama, J., Tomita, K., Inoue, T., Kageyama, R., 2001. Roles of homeobox and bHLH genes in specification of a retinal cell type. Development 128, 1313-1322.

Hill, R.E., Favor, J., Hogan, B.L., Ton, C.C., Saunders, G.F., Hanson, I.M., Prosser, J., Jordan, T., Hastie, N.D., van Heyningen, V., 1991. Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature 354, 522-525.

Hogan, B.L., Horsburgh, G., Cohen, J., Hetherington, C.M., Fisher, G., Lyon, M.F., 1986. Small eyes (Sey): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. J. Embryol. Exp. Morphol. 97, 95-110.

Horsford, D.J., Nguyen, M.T., Sellar, G.C., Kothary, R., Arnheiter, H., McInnes, R.R., 2005. Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. Development 132, 177-187.

Huang, P.C., Gaitan, A.E., Hao, Y., Petters, R.M., Wong, F., 1993. Cellular interactions implicated in the mechanism of photoreceptor degeneration in transgenic mice expressing a mutant rhodopsin gene. Proc. Natl. Acad. Sci. U. S. A. 90, 8484-8488.

Huangfu, D., Anderson, K.V., 2006. Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. Development 133, 3-14.

Hufnagel, R.B., Le, T.T., Riesenberg, A.L., Brown, N.L., 2010. Neurog2 controls the leading edge of neurogenesis in the mammalian retina. Dev. Biol. 340, 490-503.

Humke, E.W., Dorn, K.V., Milenkovic, L., Scott, M.P., Rohatgi, R., 2010. The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes Dev. 24, 670-682.

Hyer, J., Mima, T., Mikawa, T., 1998. FGF1 patterns the optic vesicle by directing the placement of the neural retina domain. Development 125, 869-877.

Ingham, P.W., McMahon, A.P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15, 3059-3087.

Iseri, S.U., Wyatt, A.W., Nurnberg, G., Kluck, C., Nurnberg, P., Holder, G.E., Blair, E., Salt, A., Ragge, N.K., 2010. Use of genome-wide SNP homozygosity mapping in small pedigrees to identify new mutations in VSX2 causing recessive microphthalmia and a semidominant inner retinal dystrophy. Hum. genet. 128, 51-60.

Izzi, L., Levesque, M., Morin, S., Laniel, D., Wilkes, B.C., Mille, F., Krauss, R.S., McMahon, A.P., Allen, B.L., Charron, F., 2011. Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation. Dev. Cell 20, 788-801.

Jadhav, A.P., Cho, S.H., Cepko, C.L., 2006a. Notch activity permits retinal cells to progress through multiple progenitor states and acquire a stem cell property. Proc. Natl. Acad. Sci. U. S. A. 103, 18998-19003.

Jadhav, A.P., Mason, H.A., Cepko, C.L., 2006b. Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development 133, 913-923.

Jensen, A.M., Wallace, V.A., 1997. Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development 124, 363-371.

Jiang, J., 2006. Regulation of Hh/Gli signaling by dual ubiquitin pathways. Cell Cycle 5, 2457-2463.

Kagiyama, Y., Gotouda, N., Sakagami, K., Yasuda, K., Mochii, M., Araki, M., 2005. Extraocular dorsal signal affects the developmental fate of the optic vesicle and patterns the optic neuroepithelium. Dev. Growth Differ. 47, 523-536.

Kay, J.N., Link, B.A., Baier, H., 2005. Staggered cell-intrinsic timing of ath5 expression underlies the wave of ganglion cell neurogenesis in the zebrafish retina. Development 132, 2573-2585.

Kim, J., Wu, H.H., Lander, A.D., Lyons, K.M., Matzuk, M.M., Calof, A.L., 2005. GDF11 controls the timing of progenitor cell competence in developing retina. Science 308, 1927-1930.

Kindiakov, B.N., Koniukhov, B.V., 1986. [Mutant gene expression in murine aggregation chimeras. 5. The ocular retardation and fidget genes]. Ontogenez 17, 47-55.

Kise, Y., Morinaka, A., Teglund, S., Miki, H., 2009. Sufu recruits GSK3beta for efficient processing of Gli3. Biochem. Biophys. Res. Commun. 387, 569-574.

Konyukhov, B.V., Sazhina, M.V., 1966. Interaction of the genes of ocular retardation and microphthalmia in mice. Folia Biol. 12, 116-123.

Konyukhov, B.V., Sazhina, M.V., 1971. Genetic control over the duration of G 1 phase. Experientia 27, 970-971.

Kubo, F., Takeichi, M., Nakagawa, S., 2003. Wnt2b controls retinal cell differentiation at the ciliary marginal zone. Development 130, 587-598.

Kubo, F., Takeichi, M., Nakagawa, S., 2005. Wnt2b inhibits differentiation of retinal progenitor cells in the absence of Notch activity by downregulating the expression of proneural genes. Development 132, 2759-2770.

Kumar, J.P., 2009. The molecular circuitry governing retinal determination. Biochim. Biophys. Acta 1789, 306-314.

Kurata, S., Go, M.J., Artavanis-Tsakonas, S., Gehring, W.J., 2000. Notch signaling and the determination of appendage identity. Proc. Natl Acad. Sci. U. S. A. 97, 2117-2122.

Lagutin, O.V., Zhu, C.C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., Russell, H.R., McKinnon, P.J., Solnica-Krezel, L., Oliver, G., 2003. Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev. 17, 368-379.

Lan, L., Vitobello, A., Bertacchi, M., Cremisi, F., Vignali, R., Andreazzoli, M., Demontis, G.C., Barsacchi, G., Casarosa, S., 2009. Noggin elicits retinal fate in Xenopus animal cap embryonic stem cells. Stem Cells 27, 2146-2152.

LaVail, M.M., Mullen, R.J., 1976. Role of the pigment epithelium in inherited retinal degeneration analyzed with experimental mouse chimeras. Exp. Eye Res. 23, 227-245.

Lee, H.Y., Wroblewski, E., Philips, G.T., Stair, C.N., Conley, K., Reedy, M., Mastick, G.S., Brown, N.L., 2005. Multiple requirements for Hes 1 during early eye formation. Dev. Biol. 284, 464-478.

Lekmine, F., Chang, C.K., Sethakorn, N., Das Gupta, T.K., Salti, G.I., 2007. Role of microphthalmia transcription factor (Mitf) in melanoma differentiation. Biochem. Biophys. Res. Commun. 354, 830-835.

Levine, E.M., Green, E.S., 2004. Cell-intrinsic regulators of proliferation in vertebrate retinal progenitors. Semin. Cell Dev. Biol. 15, 63-74.

Levine, E.M., Passini, M., Hitchcock, P.F., Glasgow, E., Schechter, N., 1997a. Vsx-1 and Vsx-2: two Chx10-like homeobox genes expressed in overlapping domains in the adult goldfish retina. Semin. Cell Dev. Biol 387, 439-448.

Levine, E.M., Roelink, H., Turner, J., Reh, T.A., 1997b. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J. Neurosci. 17, 6277-6288.

Li, H., Tierney, C., Wen, L., Wu, J.Y., Rao, Y., 1997. A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. Development 124, 603-615.

Li, S., Goldowitz, D., Swanson, D.J., 2007. The requirement of pax6 for postnatal eye development: evidence from experimental mouse chimeras. Invest. Ophthalmol. Vis. Sci. 48, 3292-3300.

Li, X., Perissi, V., Liu, F., Rose, D.W., Rosenfeld, M.G., 2002. Tissue-specific regulation of retinal and pituitary precursor cell proliferation. Science 297, 1180-1183.

Lillien, L., Cepko, C., 1992. Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha. Development 115, 253-266.

Lillien, L., Wancio, D., 1998. Changes in epidermal growth factor receptor expression and competence to generate glia regulate timing and choice of differentiation in the retina. Mol. Cell. Neurosci. 10, 296-308.

Liu, I.S., Chen, J.D., Ploder, L., Vidgen, D., van der Kooy, D., Kalnins, V.I., McInnes, R.R., 1994. Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. Neuron 13, 377-393.

Livesey, F.J., Cepko, C.L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev. Neurosci. 2, 109-118.

Livne-Bar, I., Pacal, M., Cheung, M.C., Hankin, M., Trogadis, J., Chen, D., Dorval, K.M., Bremner, R., 2006. Chx10 is required to block photoreceptor differentiation but is dispensable for progenitor proliferation in the postnatal retina. Proc. Natl Acad. Sci. U. S. A. 103, 4988-4993.

Locker, M., Agathocleous, M., Amato, M.A., Parain, K., Harris, W.A., Perron, M., 2006. Hedgehog signaling and the retina: insights into the mechanisms controlling the proliferative properties of neural precursors. Genes Dev. 20, 3036-3048.

Lundberg, A.S., Weinberg, R.A., 1999. Control of the cell cycle and apoptosis. Eur. J. Cancer 35, 531-539.

Ma, C., Papermaster, D., Cepko, C.L., 1998. A unique pattern of photoreceptor degeneration in cyclin D1 mutant mice. Proc. Natl Acad. Sci. U. S. A. 95, 9938-9943.

Ma, L., Cantrup, R., Varrault, A., Colak, D., Klenin, N., Gotz, M., McFarlane, S., Journot, L., Schuurmans, C., 2007. Zac1 functions through TGFbetaII to negatively regulate cell number in the developing retina. Neural Dev. 2, 11.

Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K., Beachy, P.A., 2002. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. Cell 111, 63-75.

Macdonald, R., Barth, K.A., Xu, Q., Holder, N., Mikkola, I., Wilson, S.W., 1995. Midline signalling is required for Pax gene regulation and patterning of the eyes. Development 121, 3267-3278.

Mangale, V.S., Hirokawa, K.E., Satyaki, P.R., Gokulchandran, N., Chikbire, S., Subramanian, L., Shetty, A.S., Martynoga, B., Paul, J., Mai, M.V., Li, Y., Flanagan, L.A., Tole, S., Monuki, E.S., 2008. Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. Science 319, 304-309.

Mann, R.K., Beachy, P.A., 2004. Novel lipid modifications of secreted protein signals. Annu. Rev. Biochem. 73, 891-923.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., Gruss, P., 2001. Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43-55.

Martinez-Morales, J.R., Del Bene, F., Nica, G., Hammerschmidt, M., Bovolenta, P., Wittbrodt, J., 2005. Differentiation of the vertebrate retina is coordinated by an FGF signaling center. Dev. Cell 8, 565-574.

Masai, I., Stemple, D.L., Okamoto, H., Wilson, S.W., 2000. Midline signals regulate retinal neurogenesis in zebrafish. Neuron 27, 251-263.

Mathers, P.H., Grinberg, A., Mahon, K.A., Jamrich, M., 1997. The Rx homeobox gene is essential for vertebrate eye development. Nature 387, 603-607.

McCabe, K.L., Gunther, E.C., Reh, T.A., 1999. The development of the pattern of retinal ganglion cells in the chick retina: mechanisms that control differentiation. Development 126, 5713-5724.

McWhirter, J.R., Goulding, M., Weiner, J.A., Chun, J., Murre, C., 1997. A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. Development 124, 3221-3232.

Medina-Martinez, O., Amaya-Manzanares, F., Liu, C., Mendoza, M., Shah, R., Zhang, L., Behringer, R.R., Mahon, K.A., Jamrich, M., 2009. Cell-autonomous requirement for rx function in the mammalian retina and posterior pituitary. PLoS One 4, e4513.

Meyers, E.N., Lewandoski, M., Martin, G.R., 1998. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat. Genet. 18, 136-141.

Moshiri, A., Reh, T.A., 2004. Persistent progenitors at the retinal margin of ptc+/- mice. J. Neurosci. 24, 229-237.

Mu, X., Beremand, P.D., Zhao, S., Pershad, R., Sun, H., Scarpa, A., Liang, S., Thomas, T.L., Klein, W.H., 2004. Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. Development 131, 1197-1210.

Mu, X., Fu, X., Sun, H., Liang, S., Maeda, H., Frishman, L.J., Klein, W.H., 2005. Ganglion cells are required for normal progenitor- cell proliferation but not cell-fate determination or patterning in the developing mouse retina. Curr. Biol. 15, 525-530.

Mu, X., Klein, W.H., 2004. A gene regulatory hierarchy for retinal ganglion cell specification and differentiation. Semin. Cell Dev. Biol. 15, 115-123.

Mui, S.H., Kim, J.W., Lemke, G., Bertuzzi, S., 2005. Vax genes ventralize the embryonic eye. Genes Dev. 19, 1249-1259.

Mullen, R.J., LaVail, M.M., 1976. Inherited retinal dystrophy: primary defect in pigment epithelium determined with experimental rat chimeras. Science 192, 799-801.

Muller, F., Rohrer, H., Vogel-Hopker, A., 2007. Bone morphogenetic proteins specify the retinal pigment epithelium in the chick embryo. Development 134, 3483-3493.

Murali, D., Yoshikawa, S., Corrigan, R.R., Plas, D.J., Crair, M.C., Oliver, G., Lyons, K.M., Mishina, Y., Furuta, Y., 2005. Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. Development 132, 913-923.

Nagy, A., Rossant, J., 2001. Chimaeras and mosaics for dissecting complex mutant phenotypes. Int. J. Dev. Biol. 45, 577-582.

Nakashima, M., Tanese, N., Ito, M., Auerbach, W., Bai, C., Furukawa, T., Toyono, T., Akamine, A., Joyner, A.L., 2002. A novel gene, GliH1, with homology to the Gli zinc finger domain not required for mouse development. Mech. Dev. 119, 21-34.

Nakayama, A., Nguyen, M.T., Chen, C.C., Opdecamp, K., Hodgkinson, C.A., Arnheiter, H., 1998. Mutations in microphthalmia, the mouse homolog of the human deafness gene MITF, affect neuroepithelial and neural crest-derived melanocytes differently. Mech. Dev. 70, 155-166.

Neumann, C.J., Nuesslein-Volhard, C., 2000. Patterning of the zebrafish retina by a wave of sonic hedgehog activity. Science 289, 2137-2139.

Nguyen, M., Arnheiter, H., 2000. Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. Development 127, 3581-3591.

Nornes, H.O., Dressler, G.R., Knapik, E.W., Deutsch, U., Gruss, P., 1990. Spatially and temporally restricted expression of Pax2 during murine neurogenesis. Development 109, 797-809.

Nybakken, K., Perrimon, N., 2002. Hedgehog signal transduction: recent findings. Curr. Opin. Genet. Dev. 12, 503-511.

Ohsaki, K., Morimitsu, T., Ishida, Y., Kominami, R., Takahashi, N., 1999. Expression of the Vax family homeobox genes suggests multiple roles in eye development. Genes Cells 4, 267-276.

Ohsawa, R., Kageyama, R., 2008. Regulation of retinal cell fate specification by multiple transcription factors. Brain Res. 1192, 90-98.

Onuma, Y., Takahashi, S., Asashima, M., Kurata, S., Gehring, W.J., 2002. Conservation of Pax 6 function and upstream activation by Notch signaling in eye development of frogs and flies. Proc. Natl Acad. Sci. U. S. A. 99, 2020-2025.

Osipov, V.V., Vakhrusheva, M.P., 1982. [Coat pigmentation and effect of the ocular retardation gene in the eye of chimeras between or/or and AKR mice]. Biull. Eksp. Biol. Med. 93, 84-86.

Osipov, V.V., Vakhrusheva, M.P., 1984. [Clonal analysis of the development of the pigment epithelium of the eye in chimeric or/or----AKR mice]. Ontogenez 15, 73-80.

Passini, M.A., Levine, E.M., Canger, A.K., Raymond, P.A., Schechter, N., 1997. Vsx-1 and Vsx-2: differential expression of two paired-like homeobox genes during zebrafish and goldfish retinogenesis. J. Comp. Neurol. 388, 495-505.

Passini, M.A., Raymond, P.A., Schechter, N., 1998. Vsx-2, a gene encoding a paired-type homeodomain, is expressed in the retina, hindbrain, and spinal cord during goldfish embryogenesis. Brain Res. Dev. Brain Res. 109, 129-135.

Pera, E.M., Ikeda, A., Eivers, E., De Robertis, E.M., 2003. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev. 17, 3023-3028.

Pera, E.M., Wessely, O., Li, S.Y., De Robertis, E.M., 2001. Neural and head induction by insulin-like growth factor signals. Dev. Cell 1, 655-665.

Pittack, C., Grunwald, G.B., Reh, T.A., 1997. Fibroblast growth factors are necessary for neural retina but not pigmented epithelium differentiation in chick embryos. Development 124, 805-816.

Porter, F.D., Drago, J., Xu, Y., Cheema, S.S., Wassif, C., Huang, S.P., Lee, E., Grinberg, A., Massalas, J.S., Bodine, D., Alt, F., Westphal, H., 1997. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. Development 124, 2935-2944.

Quinn, J.C., West, J.D., Hill, R.E., 1996. Multiple functions for Pax6 in mouse eye and nasal development. Genes Dev. 10, 435-446.

Rahnama, F., Toftgard, R., Zaphiropoulos, P.G., 2004. Distinct roles of PTCH2 splice variants in Hedgehog signalling. Biochem. J. 378, 325-334.

Rasmussen, J.T., Deardorff, M.A., Tan, C., Rao, M.S., Klein, P.S., Vetter, M.L., 2001. Regulation of eye development by frizzled signaling in Xenopus. Proc. Natl Acad. Sci. U. S. A. 98, 3861-3866.

Reese, B.E., Galli-Resta, L., 2002. The role of tangential dispersion in retinal mosaic formation. Prog. Retin. Eye Res. 21, 153-168.

Reese, B.E., Necessary, B.D., Tam, P.P., Faulkner-Jones, B., Tan, S.S., 1999. Clonal expansion and cell dispersion in the developing mouse retina. Eur. J. Neurosci. 11, 2965-2978.

Reis, L.M., Khan, A., Kariminejad, A., Ebadi, F., Tyler, R.C., Semina, E.V., 2011. VSX2 mutations in autosomal recessive microphthalmia. Mol. Vis. 17, 2527-2532.

Richard-Parpaillon, L., Heligon, C., Chesnel, F., Boujard, D., Philpott, A., 2002. The IGF pathway regulates head formation by inhibiting Wnt signaling in Xenopus. Dev. Biol. 244, 407-417.

Riesenberg, A.N., Liu, Z., Kopan, R., Brown, N.L., 2009. Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J. Neurosci. 29, 12865-12877.

Riobo, N.A., Manning, D.R., 2007. Pathways of signal transduction employed by vertebrate Hedgehogs. Biochem. J. 403, 369-379.

Robb, R.M., Silver, J., Sullivan, R.T., 1978. Ocular retardation (or) in the mouse. Invest. Ophthalmol. Vis. Sci. 17, 468-473.

Rocha, S.F., Lopes, S.S., Gossler, A., Henrique, D., 2009. Dll1 and Dll4 function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity. Dev. Biol. 328, 54-65.

Rohatgi, R., Scott, M.P., 2007. Patching the gaps in Hedgehog signalling. Nat. Cell Biol. 9, 1005-1009.

Rorick, A.M., Mei, W., Liette, N.L., Phiel, C., El-Hodiri, H.M., Yang, J., 2007. PP2A:B56epsilon is required for eye induction and eye field separation. Dev. Biol. 302, 477-493.

Rossant, J., Spence, A., 1998. Chimeras and mosaics in mouse mutant analysis. Trends Genet. 14, 358-363.

Rowan, S., Cepko, C.L., 2004. Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. Dev. Biol. 271, 388-402.

Rowan, S., Chen, C.M., Young, T.L., Fisher, D.E., Cepko, C.L., 2004. Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. Development 131, 5139-5152.

Ruiz i Altaba, A., 1999. Gli proteins encode context-dependent positive and negative functions: implications for development and disease. Development 126, 3205-3216.

Rutherford, A.D., Dhomen, N., Smith, H.K., Sowden, J.C., 2004. Delayed expression of the Crx gene and photoreceptor development in the Chx10-deficient retina. Invest. Ophthalmol. Vis. Sci. 45, 375-384.

Ryan, K.E., Chiang, C., 2012. Hedgehog secretion and signal transduction in vertebrates. J. Biol. Chem. 287, 17905-17913.

Sakagami, K., Gan, L., Yang, X.J., 2009. Distinct effects of Hedgehog signaling on neuronal fate specification and cell cycle progression in the embryonic mouse retina. J. Neurosci. 29, 6932-6944.

Sanchez-Sanchez, A.V., Camp, E., Leal-Tassias, A., Mullor, J.L., 2010. Wnt signaling has different temporal roles during retinal development. Dev. Dyn. 239, 297-310.

Sanyal, S., Dees, C., Zeilmaker, G.H., 1986. Development and degeneration of retina in rds mutant mice: observations in chimaeras of heterozygous mutant and normal genotype. J. Embryol. Exp. Morphol. 98, 111-121.

Schwarz, M., Cecconi, F., Bernier, G., Andrejewski, N., Kammandel, B., Wagner, M., Gruss, P., 2000. Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. Development 127, 4325-4334.

Sicinski, P., Donaher, J.L., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J., Weinberg, R.A., 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell 82, 621-630.

Sigulinsky, C.L., Green, E.S., Clark, A.M., Levine, E.M., 2008. Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina. Dev. Biol. 317, 560-575.

Sowden, J.C., Holt, J.K., Meins, M., Smith, H.K., Bhattacharya, S.S., 2001. Expression of Drosophila omb-related T-box genes in the developing human and mouse neural retina. Invest. Ophthalmol. Vis. Sci. 42, 3095-3102.

Strickler, A.G., Famuditimi, K., Jeffery, W.R., 2002. Retinal homeobox genes and the role of cell proliferation in cavefish eye degeneration. Int. J. Dev. Biol. 46, 285-294.

Svendsen, P.C., McGhee, J.D., 1995. The C. elegans neuronally expressed homeobox gene ceh-10 is closely related to genes expressed in the vertebrate eye. Development 121, 1253-1262.

Szebenyi, G., Fallon, J.F., 1999. Fibroblast growth factors as multifunctional signaling factors. Int. Rev. Cytol. 185, 45-106.

Take-uchi, M., Clarke, J.D., Wilson, S.W., 2003. Hedgehog signalling maintains the optic stalk-retinal interface through the regulation of Vax gene activity. Development 130, 955-968.

Tam, P.P., Rossant, J., 2003. Mouse embryonic chimeras: tools for studying mammalian development. Development 130, 6155-6163.

Taranova, O.V., Magness, S.T., Fagan, B.M., Wu, Y., Surzenko, N., Hutton, S.R., Pevny, L.H., 2006. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev. 20, 1187-1202.

Tempe, D., Casas, M., Karaz, S., Blanchet-Tournier, M.F., Concordet, J.P., 2006. Multisite protein kinase A and glycogen synthase kinase 3beta phosphorylation leads to Gli3 ubiquitination by SCFbetaTrCP. Mol. Cell. Biol. 26, 4316-4326.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F., Kageyama, R., 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16, 723-734.

Toy, J., Norton, J.S., Jibodh, S.R., Adler, R., 2002. Effects of homeobox genes on the differentiation of photoreceptor and nonphotoreceptor neurons. Invest. Ophthalmol. Vis. Sci. 43, 3522-3529.

Truslove, G.M., 1962. A gene causing ocular retardation in the mouse. J. Embryol. Exp. Morphol. 10, 652-660.

Tsukiji, N., Nishihara, D., Yajima, I., Takeda, K., Shibahara, S., Yamamoto, H., 2009. Mitf functions as an in ovo regulator for cell differentiation and proliferation during development of the chick RPE. Dev Biol. 326, 335-346.

Tucker, P., Laemle, L., Munson, A., Kanekar, S., Oliver, E.R., Brown, N., Schlecht, H., Vetter, M., Glaser, T., 2001. The eyeless mouse mutation (ey1) removes an alternative start codon from the Rx/rax homeobox gene. Genesis 31, 43-53.

Tukachinsky, H., Lopez, L.V., Salic, A., 2010. A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. J. Cell Biol. 191, 415-428.

Turner, D.L., Snyder, E.Y., Cepko, C.L., 1990. Lineage-independent determination of cell type in the embryonic mouse retina. Neuron 4, 833-845.

Uemonsa, T., Sakagami, K., Yasuda, K., Araki, M., 2002. Development of dorsal-ventral polarity in the optic vesicle and its presumptive role in eye morphogenesis as shown by embryonic transplantation and in ovo explant culturing. Dev. Biol. 248, 319-330.

Van Raay, T.J., Moore, K.B., Iordanova, I., Steele, M., Jamrich, M., Harris, W.A., Vetter, M.L., 2005. Frizzled 5 signaling governs the neural potential of progenitors in the developing Xenopus retina. Neuron 46, 23-36.

Varjosalo, M., Taipale, J., 2008. Hedgehog: functions and mechanisms. Genes Dev. 22, 2454-2472.

Viczian, A.S., Solessio, E.C., Lyou, Y., Zuber, M.E., 2009. Generation of functional eyes from pluripotent cells. PLoS biol. 7, e1000174.

Vincentz, J.W., McWhirter, J.R., Murre, C., Baldini, A., Furuta, Y., 2005. Fgf15 is required for proper morphogenesis of the mouse cardiac outflow tract. Genesis 41, 192-201.

Vitorino, M., Jusuf, P.R., Maurus, D., Kimura, Y., Higashijima, S., Harris, W.A., 2009. Vsx2 in the zebrafish retina: restricted lineages through derepression. Neural Dev. 4, 14.

Vogel-Hopker, A., Momose, T., Rohrer, H., Yasuda, K., Ishihara, L., Rapaport, D.H., 2000. Multiple functions of fibroblast growth factor-8 (FGF-8) in chick eye development. Mech. Dev. 94, 25-36.

Wallace, V.A., 2008. Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. Brain Res. 1192, 61-75.

Wallace, V.A., 2011. Concise review: making a retina--from the building blocks to clinical applications. Stem Cells 29, 412-417.

Wallace, V.A., Raff, M.C., 1999. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126, 2901-2909.

Walther, C., Gruss, P., 1991. Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 113, 1435-1449.

Wang, Y., Dakubo, G.D., Thurig, S., Mazerolle, C.J., Wallace, V.A., 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development 132, 5103-5113.

Wang, Y.P., Dakubo, G., Howley, P., Campsall, K.D., Mazarolle, C.J., Shiga, S.A., Lewis, P.M., McMahon, A.P., Wallace, V.A., 2002. Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells. Nat. Neurosci. 5, 831-832.

Watanabe, T., Raff, M.C., 1988. Retinal astrocytes are immigrants from the optic nerve. Nature 332, 834-837.

Yam, P.T., Langlois, S.D., Morin, S., Charron, F., 2009. Sonic hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. Neuron 62, 349-362.

Yang, Z., Ding, K., Pan, L., Deng, M., Gan, L., 2003. Math5 determines the competence state of retinal ganglion cell progenitors. Dev. Biol. 264, 240-254.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M., Ashery-Padan, R., 2006. Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development 133, 1367-1378.

Young, R.W., 1985. Cell differentiation in the retina of the mouse. Anat. Rec. 212, 199-205.

Yu, C., Mazerolle, C.J., Thurig, S., Wang, Y., Pacal, M., Bremner, R., Wallace, V.A., 2006. Direct and indirect effects of hedgehog pathway activation in the mammalian retina. Mol. Cell. Neurosci. 32, 274-282.

Yu, R.T., Chiang, M.Y., Tanabe, T., Kobayashi, M., Yasuda, K., Evans, R.M., Umesono, K., 2000. The orphan nuclear receptor Tlx regulates Pax2 and is essential for vision. Proc. Natl Acad. Sci. U. S. A. 97, 2621-2625.

Yun, S., Saijoh, Y., Hirokawa, K.E., Kopinke, D., Murtaugh, L.C., Monuki, E.S., Levine, E.M., 2009. Lhx2 links the intrinsic and extrinsic factors that control optic cup formation. Development 136, 3895-3906.

Zhang, X.M., Ramalho-Santos, M., McMahon, A.P., 2001. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 105, 781-792.

Zhao, S., Hung, F.C., Colvin, J.S., White, A., Dai, W., Lovicu, F.J., Ornitz, D.M., Overbeek, P.A., 2001. Patterning the optic neuroepithelium by FGF signaling and Ras activation. Development 128, 5051-5060.

Zhao, S., Overbeek, P.A., 1999. Tyrosinase-related protein 2 promoter targets transgene expression to ocular and neural crest-derived tissues. Dev. Biol. 216, 154-163.

Zou, C., Levine, E.M., 2012. Vsx2 controls eye organogenesis and retinal progenitor identity via homeodomain and non-homeodomain residues required for high affinity DNA binding. PLoS Genet. 8, e1002924.

Zuber, M.E., Gestri, G., Viczian, A.S., Barsacchi, G., Harris, W.A., 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development 130, 5155-5167.

CHAPTER 2

VSX2/CHX10 ENSURES THE CORRECT TIMING AND MAGNITUDE OF HEDGEHOG SIGNALING IN THE MOUNSE RETINA

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Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina

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ABSTRACT

Vertebrate retinal progenitor cells (RPCs) undergo a robust proliferative expansion to produce enough cells for the retina to form appropriately. Vsx2 (formerly Chx10), a homeodomain protein expressed in RPCs, is required for sufficient proliferation to occur. Sonic Hedgehog protein (SHH), secreted by retinal ganglion cells (RGCs), activates Hedgehog (Hh) signaling in RPCs and is also required for sufficient proliferation to occur. Therefore, we sought to determine if reduced Hh signaling is a contributing factor to the proliferation changes that occur in the absence of Vsx2. To do this, we examined Shh expression and Hh signaling activity in the homozygous *ocular retardation J* (*orJ*) mouse, which harbors a recessive null allele in the *Vsx2* gene. We found that Shh expression and Hh signaling activity are delayed during early retinal development in *orJ* mice and this correlates with a delay in the onset of RGC differentiation. At birth, reduced expression of genes regulated by Hh signaling was observed despite the production of SHH ligand. *orJ* RPCs respond to pre-processed recombinant SHH ligand (SHH-N) in explant culture as evidenced by increased proliferation and expression of Hh target genes. Interestingly, proliferation in the *orJ* retina is further inhibited by cyclopamine, an antagonist of Hh signaling. Our results suggest that reduced Hh signaling contributes to the reduced level of RPC proliferation in the *orJ* retina, thereby revealing a role for Vsx2 in mediating mitogen signaling.

Introduction

Mammalian retinal progenitor cells (RPCs) undergo multiple rounds of proliferative cell division to generate the millions of cells necessary to comprise a typical retina. In mice, RPC proliferation begins at embryonic day 10 (E10) and lasts 3 weeks, until post-natal day 10 (P10; all times approximate). From the beginning of this period, some of the generated cells exit the cell cycle and differentiate (Young, 1985), producing all seven major retinal cell classes in a highly specific and sequential, but overlapping, order (Livesey and Cepko, 2001). Therefore, RPC proliferation is crucial not only for generating the necessary number of cells, but also for ensuring sufficient progenitors are available for the production of all retinal cell types. Disruptions of this process in either direction can lead to alterations in retinal structure and function, with severe disruptions resulting in microphthalmia or retinoblastoma. Thus, normal retinal development requires precise regulation of RPC proliferation (reviewed in Donovan and Dyer, 2005; Levine and Green, 2004).

Proliferation in the retina, as in other tissues, is a process influenced by a number of intrinsic and extrinsic factors. Homeodomain and cell cycle proteins are cell-intrinsic factors necessary for proper RPC proliferation (reviewed in Del Bene and Wittbrodt, 2005; Levine and Green, 2004). Additionally, cell-extrinsic factors, such as mitogens, are also important in promoting RPC proliferation (reviewed in Dakubo and Wallace, 2004; Yang, 2004). However, little is known regarding if and how these different factors coordinate their activities to drive RPC proliferation during retinal development.

The *Vsx2* gene (formerly *Chx10*; Mouse Genome Database (URL: http://www.informatics.jax.org)) encodes a homeodomain-containing transcription factor that is expressed by RPCs from the beginning of retinal development, during optic cup formation. RPCs continue to express Vsx2 throughout their proliferative period, but as they differentiate, Vsx2 expression is lost in all postmitotic retinal cell types, except bipolar interneurons and a subset of Muller glia. Mice and humans with mutations in Vsx2/VSX2 exhibit microphthalmia, or small eyes, a condition resulting in part from a profound defect in RPC proliferation that leads to a severe reduction in retinal cell number (Bar-Yosef et al., 2004; Bone-Larson et al., 2000; Burmeister et al., 1996; Dhomen et al., 2006; Ferda Percin et al., 2000; Green et al., 2003; Konyukhov and Sazhina, 1971; Livne-Bar et al., 2006). Interestingly, Vsx2 apparently utilizes multiple

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mechanisms to control RPC proliferation. For example, Vsx2 regulates cell cycle progression by preventing accumulation of the cyclin-dependent kinase inhibitor protein $p27^{Kip1}$ via a CyclinD1-dependent mechanism (Ccnd1; formerly referred to as CycD1) (Green et al., 2003). Vsx2 also prevents expression of the transcription factor Mitf. Mitf expression in *orJ* RPCs biases them towards a pigmented cell fate, which appears to include a more restricted proliferative expansion (Horsford et al., 2005; Rowan et al., 2004). Later in retinal development, Vsx2 has additional roles in specifying neural cell fate: inhibiting rod photoreceptor generation, and promoting bipolar cell generation (Belecky-Adams et al., 1997; Dorval et al., 2006; Hatakeyama et al., 2001; Livne-Bar et al., 2006; Rowan and Cepko, 2004; Rutherford et al., 2004; Toy et al., 2002). However, these roles appear to be largely independent of its roles in promoting proliferation (Green et al., 2003; Livne-Bar et al., 2006). Despite these advances in understanding the roles of Vsx2 in retinal development, the full extent of its activity is not yet known.

Sonic Hedgehog (*Shh*) is a member of the Hedgehog (Hh) family of genes encoding secreted signaling molecules. Like other Hh family members (Indian Hedgehog (IHH) and Desert Hedgehog (DHH) in mammals), SHH activates the Hh signaling network (Cohen, 2003; Ingham and McMahon, 2001; Nybakken and Perrimon, 2002; Varjosalo and Taipale, 2007). Binding of Hh ligand to the transmembrane receptor, Patched homolog 1 (Ptc1), relieves Ptc1-mediated inhibition of Smoothened (Smo) activity, leading to activation of the Gli family of transcriptional regulators, which ultimately alters gene expression in the target cell. Transcriptional targets of the Hh signaling pathway (referred to hereafter as Hh target genes) include both positive and negative regulators of the Hh signaling network, cell cycle regulators, extrinsic signaling molecules, cell specification and differentiation genes (Ho and Scott, 2002; Oliver et al., 2003). Transcription of these target genes is often context-dependent, which is not surprising given the diverse roles attributed to Hh signaling (Cohen, 2003; Ingham and McMahon, 2001).

SHH is both a well-established and well-characterized retinal mitogen. In the mouse retina, production and secretion of SHH by retinal ganglion cells (RGCs) closely follows the central-to-peripheral wave of RGC differentiation (Wang et al., 2005; Dakubo et al., 2003), which begins around E11.5. RGC-derived SHH activates Hh signaling in adjacent RPCs, as evidenced by changes in Hh target gene expression (Wang et al., 2005). Active Hh signaling in the murine retina overlaps the principal period of proliferative expansion and is both necessary and sufficient for RPC proliferation. Treatment with recombinant, pre-processed SHH protein (SHH-N) (Jensen and Wallace, 1997; Levine et al., 1997) or constitutive activation of Hh signaling (Moshiri and Reh, 2004) increases murine RPC proliferation, while conditional ablation of *Shh* (Wang et al., 2005, 2002) or treatment with anti-SHH antibodies (Wallace and Raff, 1999) decreases proliferation. Recent work in *Xenopus* and zebrafish suggests that the effects of SHH on proliferation are accomplished by acceleration of the G1 and G2 phases of the cell cycle (Locker et al., 2006).

In addition to the fact that both Vsx2 and Shh are critical regulators of RPC proliferation, *Shh* conditional knockout mice exhibit ocular phenotypes similar to *orJ* mice, including disrupted retinal lamination and microphthalmia (Dakubo et al., 2003; Wang et al., 2002). In this study, we investigated whether other facets of proliferative control mediated by Vsx2 involve Hh signaling.

Methods

Nomenclature

Many commonly used gene names and symbols are changing to conform to nomenclature rules implemented by the Mouse Genome Nomenclature Committee and HUGO Gene Nomenclature Committee. Relevant to this study are: Vsx2 for Chx10; Pou4f2 for Brn3b; Ccnd1 for Cyclin D1/CycD1.

Animals

orJ mice were obtained from Jackson labs (Bar Harbor, ME). The *orJ* and + (wild type) alleles were determined by PCR and restriction digests with mouse tail DNA (Burmeister et al., 1996). All mice were on a 129/Sv background. Animals were housed and cared for according to IACUC guidelines. Previous work ((Rowan and Cepko, 2004) and our unpublished observations) suggests that eye development in heterozygous *orJ (orJ/+)* mice is phenotypically indistinguishable from that in wild type (+/+) mice. Therefore, mice with these genotypes were considered equivalent and are referred to hereafter as wild type. Homozygous *orJ* (*orJ/orJ*) mice are referred to as *orJ*.

In situ hybridization

After dissection in Hank's buffered saline solution (HBSS), embryonic heads and P0 eyes were fixed overnight at 4 °C with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.5) or 4% formaldehyde in PBS/2 mM EGTA, followed by cryoprotection and storage at −80 °C. Sections (12 μm) were cut and stored at −20 °C until use. *In situ* hybridization was performed as previously described (Green et al., 2003; Schaeren-Wiemers and Gerfin-Moser, 1993). Probes used in this study were digoxigenin-labeled anti-sense probes against *Shh*, *Gli1*, *Ptc1*, *Ptc2*, *Hedgehog interacting protein* (*Hhip*), and *Vsx2*.

Immunohistochemistry

After dissection in HBSS, embryonic head or retinal tissue was fixed in 4% PFA in PBS for 1 to 3 h at room temperature or overnight at 4 °C. Tissue fixation was followed by cryoprotection and storage at −80 °C until sectioning. Sections (10 μm for E12.5 cultured retinal explants and 12 μm for all other samples) were cut and stored at −20 °C until use.

Sections were pre-treated for 30 min in block buffer (2% normal goat or donkey serum, 0.15% TritonX-100, and 0.01% sodium azide in PBS). Sections were then labeled with primary antibodies diluted in block buffer. The primary antibodies used in this study include rabbit anti-acetylated Class III beta-Tubulin (acTUBB3; Tuj1 antibody; Covance, Richmond, CA); rat anti-bromodeoxyuridine (BrdU; clone ICR1; Accurate Chemical and Scientific Corporation, New York, NY); goat anti-POU4F2 (Brn3b antibody; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phosphorylated Histone H3 (pHH3; Upstate Biotechnology, Lake Placid, NY), mouse anti-PCNA (clone PC10; Dako, Carpinteria, CA) and rabbit anti-SHH (Santa Cruz Biotechnology, Santa Cruz, CA). Hydrochloric acid treatment (2N HCl; 1 h at room temperature) was performed prior to incubation with the BrdU antibody. Antigen unmasking (0.18 mM Citric Acid, 77 μM Sodium Citrate, final working concentrations, pH 6.0, 15 min at 90–95 °C) was performed prior to incubation with the PCNA antibody. Sections were then labeled with species-specific secondary antibodies conjugated to Alexafluor 488 or 568 (Invitrogen, Carlsbad, CA). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Fluka, Switzerland), and slides were mounted with glass coverslips for microscopy.

When immunohistochemistry was performed after *in situ* hybridization on the same sections, pre-treatment with block buffer was omitted and antibodies were diluted in *in situ* buffer B1 (0.1 M Tris, pH 7.5, 0.15 M NaCl) plus 1% goat serum. *In situ* buffer B1 was used in place of PBS for rinse steps.

Semi-quantitative RT-PCR (sqRT-PCR) and analysis

Dissected retinal tissue was rapidly frozen by submersion into liquid nitrogen and stored at −80 °C until use. Total RNA was isolated using the RNeasy® Mini or RNeasy® Micro Spin Protocol for isolation of total RNA from animal cells (Qiagen, Valencia, CA). cDNA for use in RT-PCR was synthesized from 100 ng total RNA using the ReactionReady First Strand cDNA Synthesis kit (SuperArray Bioscience Corporation, Frederick, MD). Analysis of relative gene expression was performed according to the RT^2 End-Point PCR Assay Protocol using RT^2 PCR Primer Sets for each gene of interest (SuperArray Bioscience Corporation, Frederick, MD). The PCR conditions for all genes were as follows: preheat, 95 °C for 15 min; cycling, 95 °C for 30 s followed by 55 °C for 30 s and 72 °C for 30 s. The number of PCR cycles for each gene were as follows: 25 cycles, *beta-Actin* (*Actb*); 30 cycles, *Ccnd1*, *Gli3*, and *Smo*; 33 cycles, *Pou4f2*, *Gli1*, *Gli2*, *Hhip*, *Ptc1*, *Ptc2*, and *Shh*. Cycle number was determined based on optimization curves generated for each primer set (Supplemental Fig. S1). *Actb* served as a control for RNA input for each sample run. Gels were analyzed using ImageJ software (NIH). Three to seven samples (pooled or unpooled) were analyzed. Student's unpaired *t*-test or Welch's Two Sample *t*-test were performed to determine statistical significance as appropriate (based on results of the *F*-test of Variances).

Western blots

Sample preparation and western blots were performed as previously described (Clark et al., 2008). Primary antibodies used included: rabbit anti-SHH; mouse anti-ACTB (Chemicon, Temecula, CA); mouse anti-CCND1 (Santa Cruz Biotechnology). Enhanced chemiluminescent detection of horseradish peroxidase was performed using SuperSignal West Dura (Pierce Biotechnology, Rockford, IL), and captured using a

ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). Specificity controls for SHH immunoreactivity are presented in Supplemental Fig. S2.

Retinal explant cultures

Whole retinal tissue with the lens and vitreal chamber intact was dissected away from surrounding tissues in HBSS and placed into cell culture plates. For P0 samples, retinal explants were cultured in 24-well cell culture plates containing 1–2 ml of culture medium (DMEM/F12; Invitrogen), 0.6% glucose, 0.1125% $NaHCO_3$, 5 mM Hepes, 1% FBS, 1.5 μM thymidine, Glutamax (0.5×, Invitrogen), 25 μg/ml insulin (Sigma, St. Louis, MO), 100 μg/ml transferrin (Sigma), 60 μM putrescine (Sigma), 30 nM selenium (Sigma), 20 nM progesterone (Sigma), and Penicillin/Streptomycin (1×, Invitrogen). E12.5 retinal explants were cultured in 48-well cell culture plates containing 450 μl of culture medium.

To test the effects of SHH treatment, one explant from each mouse was cultured in the absence of SHH-N, while the explant from the contralateral eye was cultured in the presence of 108 ng/ml SHH-N (Levine et al., 1997). Cultures were incubated at 37 °C and 5% CO_2, with nutating to ensure good perfusion of solutions throughout the retina. For proliferation assays, explants were cultured for 24 h, with BrdU present at a final concentration of 10 μg/ml for the last 2 h for P0 samples and 30 min for the E12.5 samples. Explants from a minimum of 4 mice were analyzed for each age, genotype and condition. For western blots, a minimum of three explants from separate mice were cultured for 8 h. At the end of the culture period, lens and vascular tissues were removed and discarded. The isolated retinas were pooled according to genotype and condition prior to protein extraction. For gene expression measurements by sqRT-PCR, explants were cultured for 8 h. Lens and vascular tissues were separated from retinal tissue prior to RNA isolation. Explants from at least four mice were analyzed for each genotype and condition.

To test the effects of cyclopamine, one explant per mouse was cultured with 0.41% (v/v) DMSO added to the media as a vehicle control, while the explant from the contralateral eye was cultured in the presence of 10 μM cyclopamine (Toronto Research Chemicals, North York, Ontario) in 0.41% (v/v) DMSO. For *in situ* analyses, explants were cultured for 16 h. For proliferation analyses, explants were cultured for 24 h with BrdU present at a final concentration of 10 μg/ml for the last 2 h. Explants from a minimum of four mice were analyzed for each condition.

Marker quantification and statistical analyses

Quantification of $BrdU^+$, $pHH3^+$, and $PCNA^+$ cells was accomplished by direct counting in retinal sections. For wild type samples, images of the regions between the central and peripheral domains were analyzed. For *orJ* samples, the entire retinal cross section was analyzed. Images for BrdU and PCNA were collected on an Olympus Fluoview 1000

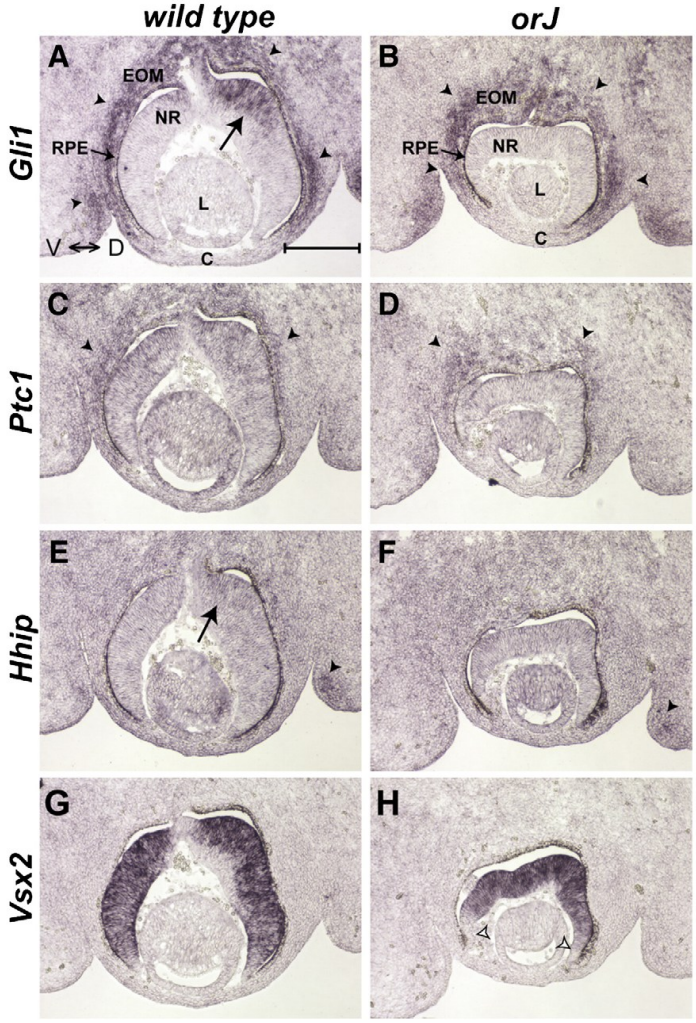


Fig. 1. Expression patterns of Hh target genes at E12.5. *Gli1* (A, B), *Ptc1* (C, D) and *Hhip* (E, F) mRNA expression was detected in wild type retinas, but not in *orJ* retinas. Arrows in (A) and (E) demarcate Gli1 and Hhip expression in the dorsal retina, while closed arrowheads indicate areas of signal outside the retina. *In situ* hybridization for *Vsx2* mRNA (G, H) serves as a control. Open arrowheads in (H) indicate reduced expression of Vsx2 in the extreme periphery of *orJ* retinas. Ventral is to the left in all panels. Scale bar: 200 μm. C, cornea; EOM, extra-ocular mesenchyme; L, lens; NR, neural retina; RPE, retinal pigmented epithelium; D, dorsal; V, ventral.

confocal microscope. Images for pHH3 were collected on a Nikon E600 epifluorescence microscope. Cell counts were done in Photoshop (Adobe) and Image J (NIH). Retinal area was calculated by determining the pixel area using ImageJ and converting to area in mm^2. The apical/ventricular surface distance was calculated by determining the length of this surface in pixels using ImageJ and converting to length in mm. Multiple regions counted from the same retinal section were summed while multiple regions from the same retina but different sections were averaged. Student's paired *t*-test was performed to determine the statistical significance of the effects of SHH-N or cyclopamine treatment between paired samples of each genotype. Tabulated cell counts for the P0 explant experiments are shown in Supplemental Tables 1–3.

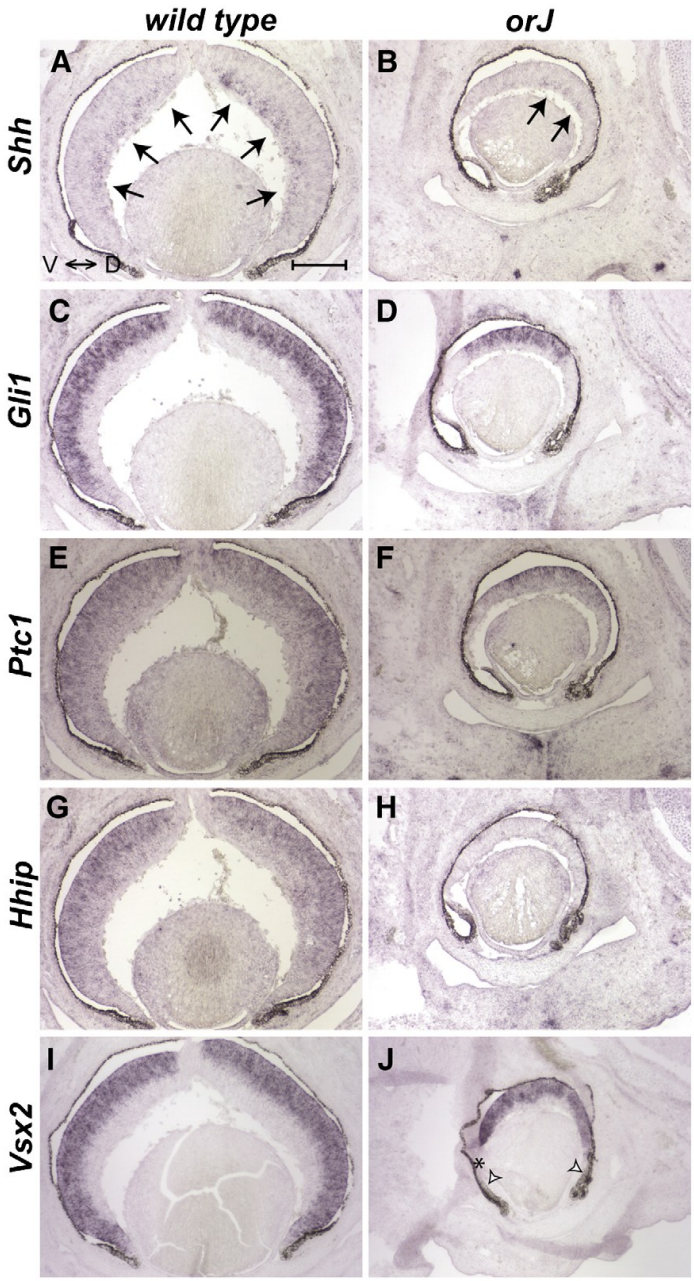


Fig. 2. Expression patterns of *Shh* and Hh target genes at E15.5. *Shh* mRNA (A, B) expression extends to the peripheral retina in wild type eyes, but is restricted to the central region in *orJ* retinas. Areas of retinal expression indicated with arrows. *Gli1* (C) and *Ptc1* (E) transcripts were also detected in wild type retinas, with expression extending to the peripheral retina. However, their expression in the *orJ* retina (D, F) was centrally restricted. *Hhip* mRNA expression was detected in wild type retinas (G), but not in *orJ* retinas (H). *In situ* hybridization for *Vsx2* mRNA (I, J) serves as a control. Open arrowheads in (J) indicate regions of presumptive transdifferentiated retina. Asterisk in (J) indicates that lack of *Vsx2* expression is an artifact due to tissue folding. Ventral is to the left in all panels. Scale bar: 200 μm.

Results

Altered Hh signaling during embryonic stages in the orJ retina coincides with a delay in Shh expression

To determine whether Vsx2 is necessary for Hh signaling in the developing embryonic retina, we compared the spatial and temporal expression patterns of *Shh* and Hh target genes in wild type and *orJ* retinas. For this purpose, we have chosen general transcriptional targets of Hh signaling whose expression is upregulated in response to elevated levels of Hh signaling, including: *Gli1*, *Ptc1*, *Ptc2*, *Hedgehog Interacting Protein* (*Hhip*), and *Cyclin D1* (*Ccnd1*). Since SHH production and Hh target gene upregulation are tightly correlated to the central-to-peripheral wave of RGC differentiation, multiple developmental ages were examined.

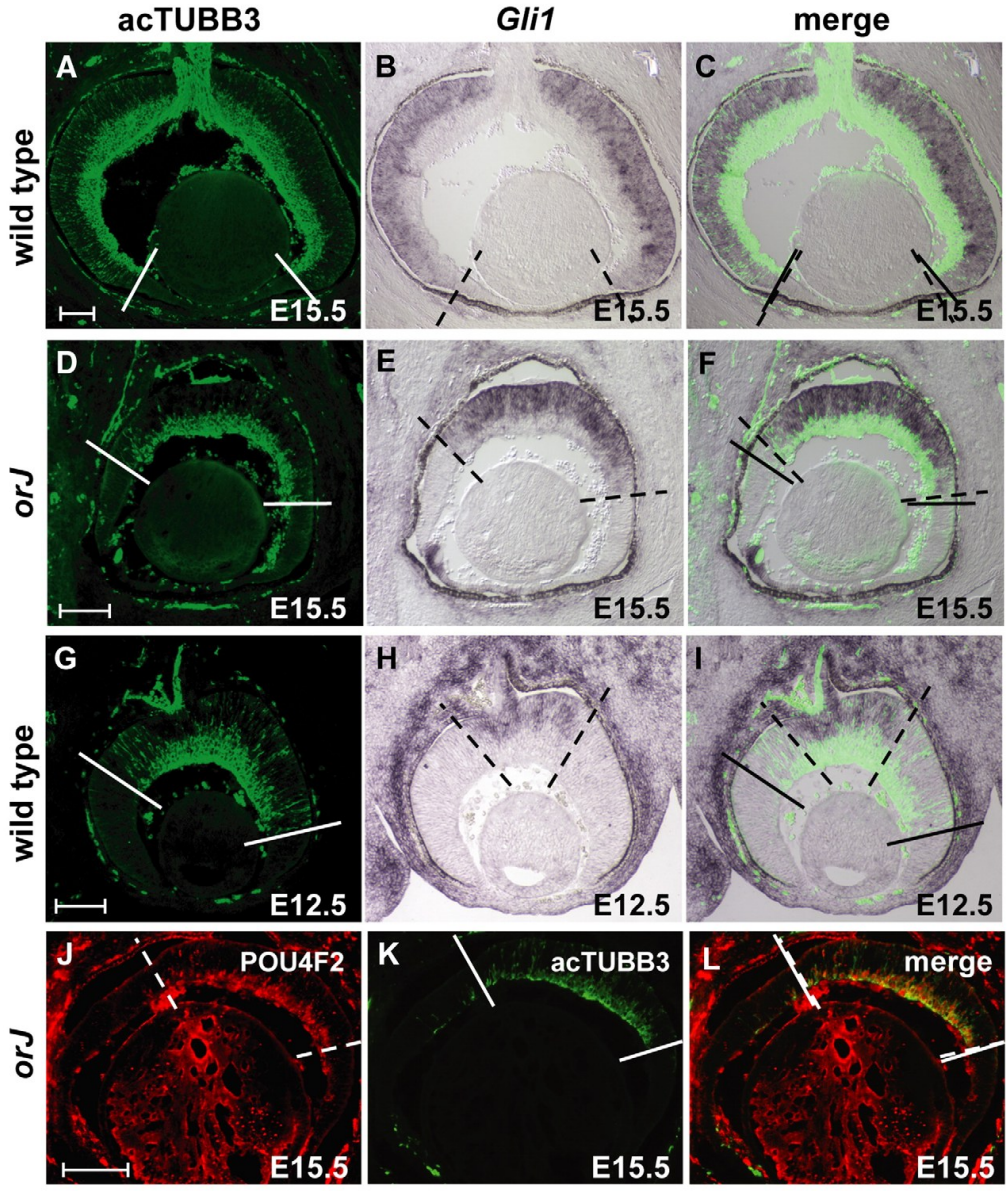


Fig. 3. Hh signaling activity correlates with RGC differentiation in both wild type and *orJ* retinas. (A–I) Solid and dashed lines indicate the extent of acTUBB3 immunoreactivity and Gli1 mRNA expression, respectively. (A–C) In E15.5 wild type retinas, both acTUBB3 immunoreactivity and *Gli1* expression extend to the peripheral retina. (D–F) In E15.5 *orJ* retinas, both acTUBB3 signal and *Gli1* expression are more centrally restricted. Note that acTUBB3-positive cells peripheral to the dashed line in (D) are not located in the retinal neuroepithelium. (G–I) Patterns of acTUBB3 and *Gli1* in E12.5 wild type retinas are centrally restricted. (J–L) Expression of POU4F2 in the E15.5 *orJ* retina correlates well with acTUBB3 staining, indicating that many of the acTUBB3-positive cells are RGCs. Ventral is to the left in all panels. Scale bars: 200 μm for (A–I); 100 μm for (J–L).

We first examined gene expression patterns at E12.5 (Fig. 1), an early stage of retinal development at which RGCs have begun to differentiate in the central retina of wild type mice. We find clear evidence of Hh signaling activity in wild type retinas, in the form of *Gli1* mRNA expression in RPCs. This expression is restricted to the central retina and stronger on the dorsal side (large arrow, Fig. 1A). In contrast, we fail to detect *Gli1* mRNA in *orJ* retinas at this age (Fig. 1B). However, we find strong *Gli1* expression in extra-retinal tissue of *orJ* mice, as in wild type mice, presumably due to extra-retinal sources of Hh ligands (arrowheads, Figs. 1A,B; Dakubo et al., 2003).

The Hh target genes *Ptc1* and *Hhip* are also expressed, albeit weakly, in the central retina of wild type mice, but were not detected in *orJ* retinas (Figs. 1C–F). *Shh* and *Ptc2* mRNAs were difficult to detect at this age in both genotypes (not shown). Since *Vsx2* mRNA is expressed strongly in the *orJ* retina, we used it as both a positive control for the *orJ* retinal tissue and a marker of RPCs (Figs. 1G,H). Open arrowheads in Fig. 1H point to reduced *Vsx2* expression in the extreme retinal periphery (see below). Taken together, our data indicate Hh signaling is aberrantly absent during this early period of retinal development in *orJ* mice.

We next examined gene expression patterns at E15.5, a stage of retinal development at which the wave of RGC differentiation has reached the peripheral retina in wild type mice. *In situ* hybridization reveals *Shh* expression in the nascent RGC layer, extending to the peripheral retina of wild type mice (arrows, Fig. 2A). However, *Shh* expression is only observed in the central retina of *orJ* mice at this age (arrows, Fig. 2B). We also observed a corresponding restriction of Hh target gene expression territories. In wild type mice, expression of the Hh target genes *Gli1* and *Ptc1* in RPCs extends to the peripheral retina (Figs. 2C,E), similar to the extent of *Shh* expression. In *orJ* retinas, however, expression of *Gli1* and *Ptc1*

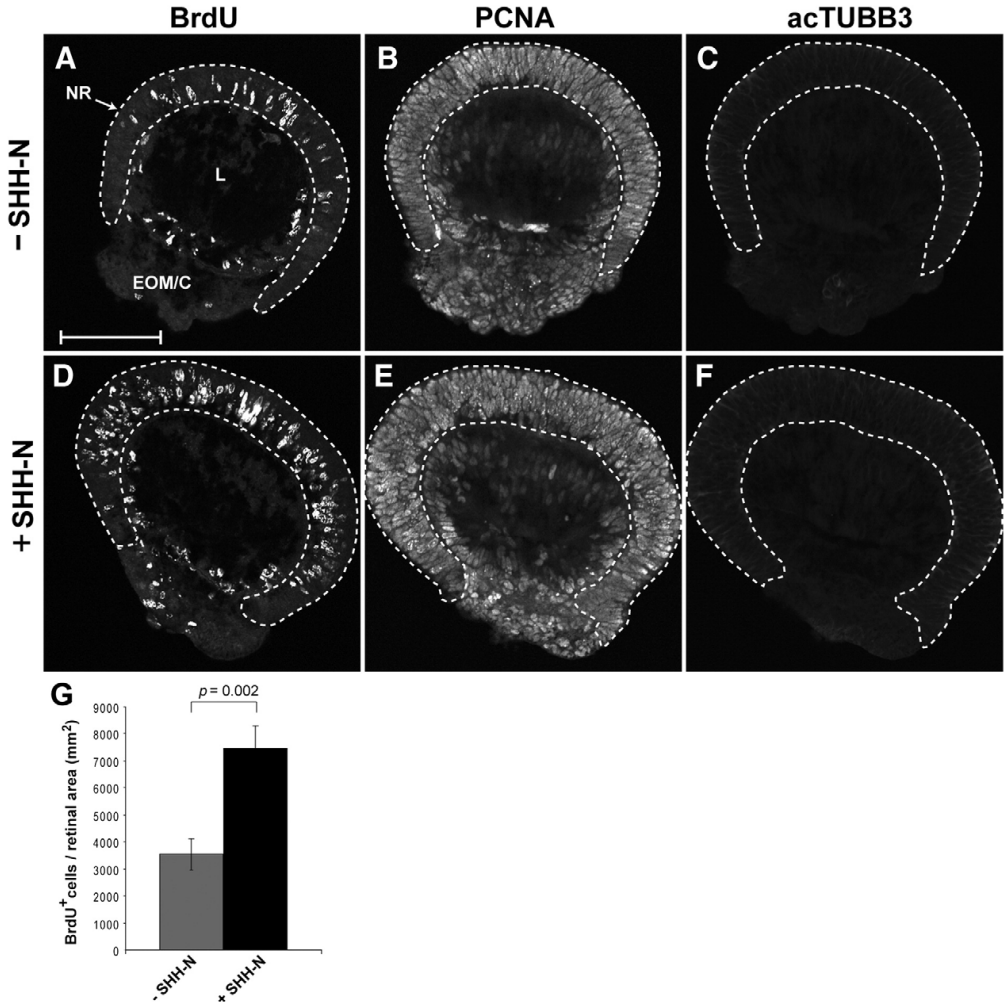


Fig. 4. SHH-N treatment promotes proliferation in embryonic organotypic retinal explant cultures from *orJ* mice. E12.5 explants were cultured in the absence (A–C) or presence (D–F) of SHH-N for 24 h with BrdU added for the last 30 min. (A, D) BrdU incorporation. (B, E) PCNA immunoreactivity. (C, F) acTUBB3 immunoreactivity. Retinal tissue is contained within the dashed lines. Scale bar: 100 μm. (G) Quantification of $BrdU^+$ cells as a function of retinal area (mm^2). Each bar represents the mean ± standard error of the mean (S.E.M.). *p*-values calculated using Student's paired *t*-test.

is centrally restricted (Figs. 2D,F). *Ptc2* expression was again difficult to detect in wild type and *orJ* retinas at this age (data not shown). These gene expression patterns indicate that in *orJ* mice, unlike wild type mice of the same age, Hh signaling is absent in the peripheral E15.5 retina. *In situ* hybridization for *Vsx2* mRNA (Figs. 2I,J) shows that the absence of peripheral Hh target gene

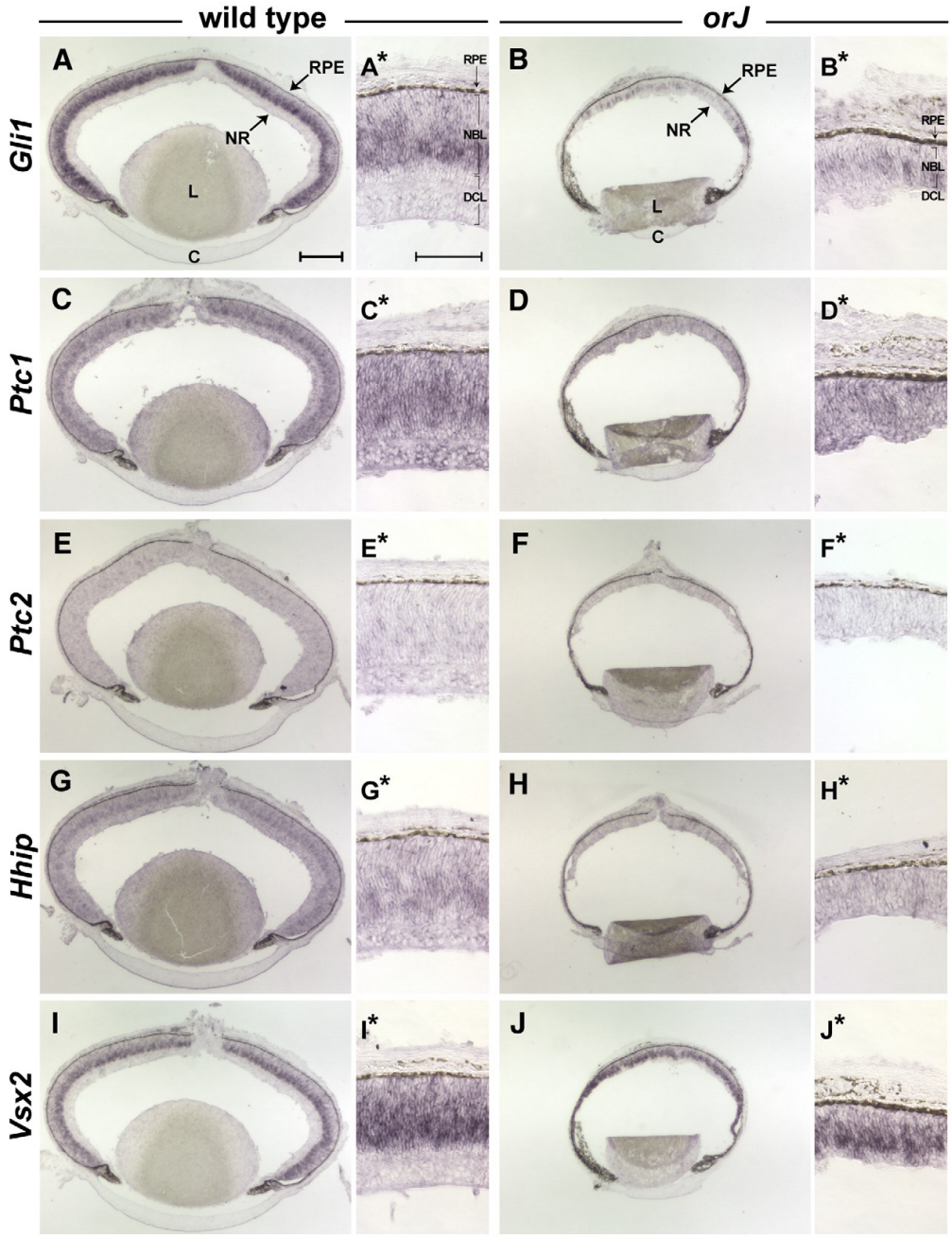


Fig. 5. Expression patterns of Hh target genes at P0. *In situ* hybridization for the Hh target genes *Gli1* (A(*), B(*)) and *Ptc1* (C(*), D(*)) reveals reduced expression for both genes in *orJ* retinas at P0. (E(*), F(*)) *Ptc2* mRNA expression is undetectable in either genotype. (G(*), H(*)) *Hhip* transcript is expressed in wild type retinas, but absent in *orJ* retinas. (I(*), J(*)) *In situ* hybridization for Vsx2 mRNA serves as a control. Scale bars: 250 μm (A–J); 40 μm (A*–J*). NBL, neuroblast layer; DCL, differentiated cell layer.

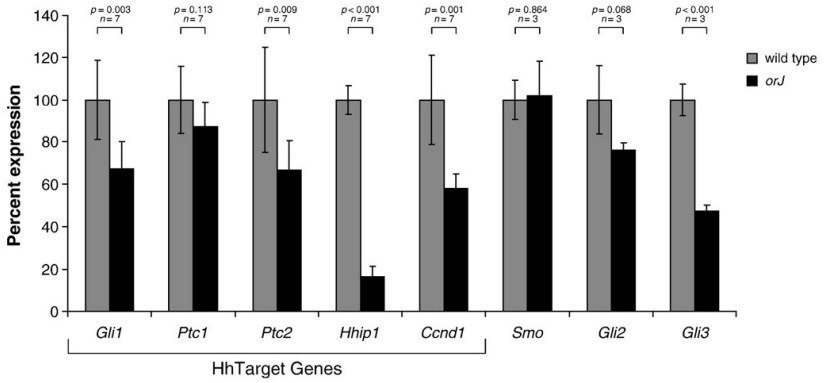


Fig. 6. Quantification of relative expression levels for Hh network components in P0 wild type and *orJ* retinas by sqRT-PCR. The mean wild type expression level for each gene was set at 100% with the *orJ* level presented as a percent of wild type expression. Hh target genes are those genes whose expression levels are generally considered as indicators of pathway activity. Bars represent the mean ± standard deviation. *n* refers to the number of independently isolated RNA samples. *p*-values calculated by Student's unpaired *t*-test or Welch's two sample *t*-test, as appropriate (based on results of an F test of variances). See Supplemental Fig. S1 for sqRT-PCR optimization curves.

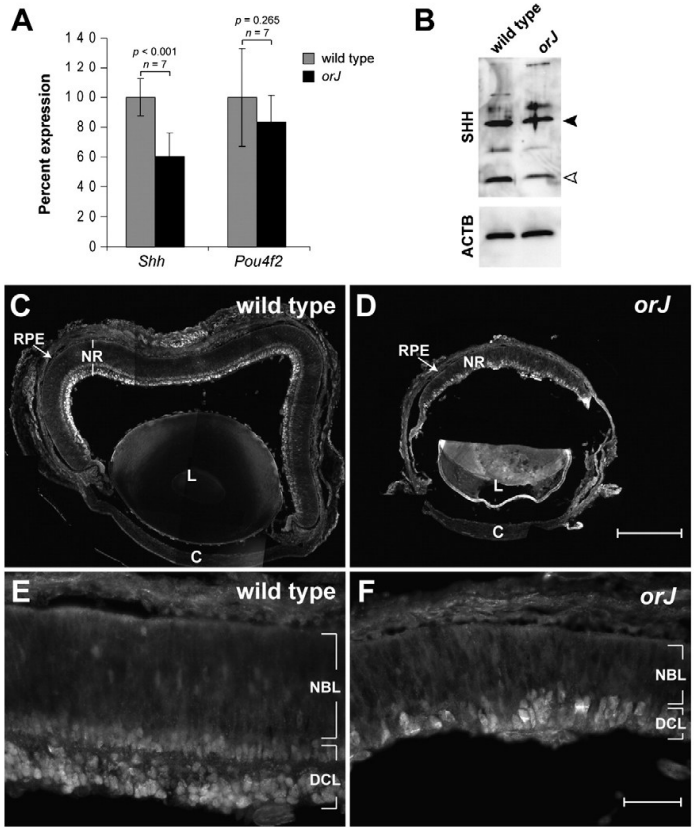
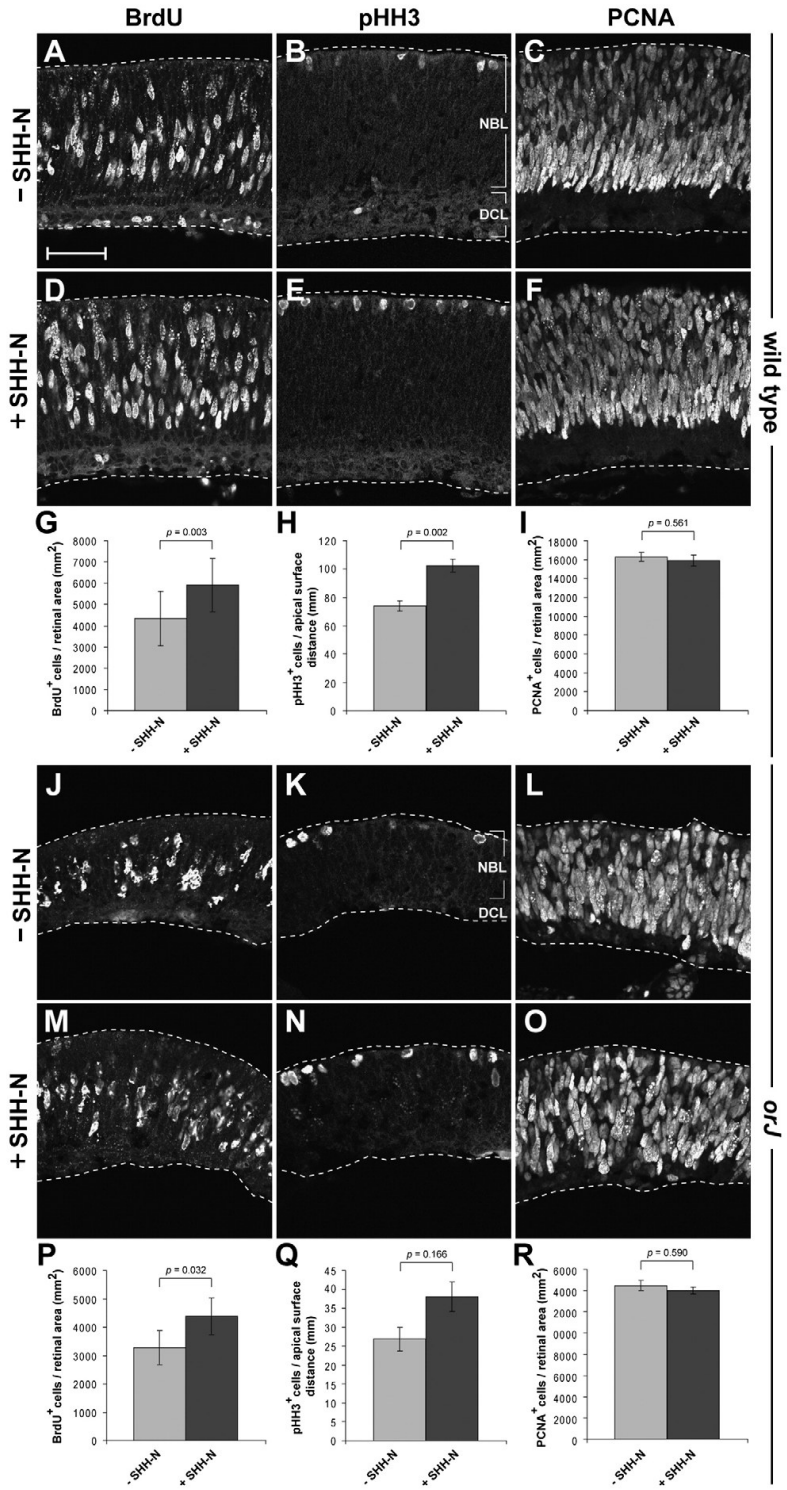


Fig. 7. Shh expression at P0. (A) Relative expression levels of *Shh* and *Pou4f2* mRNAs in wild type and *orJ* retinas as determined by sqRT-PCR. Bars represent the mean ± standard deviation. *n* and *p*-values are defined as described in Fig. 6. (B) Relative expression of SHH protein in wild type and *orJ* retinal protein lysates. Closed arrowhead points to band consistent with size of full-length SHH and open arrowhead points to band consistent with size of the N-terminal fragment of SHH. Blot was reprobed to show ACTB expression as an independent measure for protein loading. (C–F) Spatial expression of SHH protein in wild type and *orJ* eyes. Scale bars: 200 μm for (C, D); 50 μm for (E, F). See Supplemental Fig. S2 for anti-SHH immunoreactivity controls.


BrdU
pHH3
PCNA
A
B
C
NBL
DCL
– SHH-N
D
E
F
+ SHH-N
wild type
G
H
I
p = 0.003
p = 0.002
p = 0.561
BrdU+ cells / retinal area (mm2)
pHH3+ cells / apical surface distance (mm)
PCNA+ cells / retinal area (mm2)
- SHH-N
+ SHH-N
J
K
L
M
N
O
orJ
P
Q
R
p = 0.032
p = 0.166
p = 0.590

expression is not due to an absence of RPCs with the possible exception of the extreme retinal periphery, where a reduction of *Vsx2* expression is correlated with a propensity toward transdifferentiation (open arrowheads in Figs. 1H and 2J; Horsford et al., 2005; Rowan et al., 2004). Interestingly, these centrally restricted expression territories for *Shh* and the Hh target genes are reminiscent of those observed for wild type retinas at earlier stages of development (Fig. 1; Dakubo et al., 2003; Wang et al., 2005). Taken together with the E12.5 expression patterns, these findings suggest a delay, both in the expression of *Shh* and activation of Hh signaling. Consistent with this, *Gli1* mRNA and SHH protein expression extend to the peripheral retina in *orJ* animals by P0 (Figs. 5 and 7).

While *Gli1* and *Ptc1* exhibit altered expression territories in the *orJ* retina, their levels appear normal where expressed (Figs. 2D,F compared to C,E). This indicates that even in the absence of Vsx2, RPCs retain some ability to respond to SHH ligand. However, the expression of another Hh target gene, *Hhip*, suggests that the response of RPCs to SHH is altered to some extent, even in the central retina of *orJ* mice. While the pattern of *Hhip* expression is comparable to those of *Gli1* and *Ptc1* in the wild type retina, *Hhip* mRNA is undetectable in *orJ* retinas (Figs. 2G,H).

The delay in Hh signaling activity correlates with delayed RGC production

Since RPCs have been shown to only respond to RGC-derived SHH in the mouse (Dakubo et al., 2003; and our data) and RGC differentiation is delayed in the *orJ* retina (Bone-Larson et al., 2000), we sought to determine if Hh signaling activity in the *orJ* retina correlates with the extent to which the peripherally-spreading wave of RGC differentiation has progressed.

To begin to address this question, retinal sections were examined for *Gli1* mRNA expression by *in situ* hybridization and acetylated class III beta-Tubulin (acTUBB3) by immunohistochemistry on the same sections. *Gli1* expression is an indicator of active Hh signaling and acTUBB3 serves as a marker of postmitotic neurons. While acTUBB3$^+$ cells and *Gli1* expression extend to the peripheral retina of wild type mice at E15.5 (Figs. 3A–C), *orJ* retinas at this age exhibit a centrally restricted domain of acTUBB3$^+$ cells (Figs. 3D–F), consistent with delayed neurogenesis in the *orJ* retina (note: acTUBB3$^+$ cells more peripheral than the solid lines are located within the vitreal space, not the neural retina). Despite this delay, the spatial boundary of *Gli1* expression in the *orJ* retina aligns with, or is slightly more centrally restricted than, the boundary for acTUBB3 immunoreactivity (Figs. 3E,F). These expression patterns are similar to what is observed in the E12.5 wild type retina (Figs. 3G–I), a stage where the initial wave of neurogenesis is still in the process of progressing towards the periphery. We also found that many of the acTUBB3$^+$ cells also expressed POU4F2 (formerly BRN3B), a marker of a major subset of RGCs (Figs. 3J–L). These data indicate that the relationship between activation of the Hh signaling network and RGC differentiation shown for normal retinal development (Wang et al., 2005) is maintained in the *orJ* retina.

To address this further, we sought to determine whether *orJ* RPCs are competent to respond to SHH ligand prior to the onset of neurogenesis and the appearance of endogenous RGC-derived SHH. To test this, organotypic retinal explants from E12.5 *orJ* mice were maintained in the presence or absence of recombinant, N-terminal SHH protein (SHH-N) for 24 h and examined for proliferation and neurogenesis. Proliferation was assessed by BrdU incorporation and neurogenesis was assessed by acTUBB3 immunoreactivity. PCNA was used to assess the relative size of the RPC population since it is a comprehensive marker of RPCs (Barton and Levine, 2008). We found that *orJ* RPCs exhibited a significant increase in BrdU$^+$ cells in response to SHH-N treatment without any apparent changes in the proportion of PCNA$^+$ or acTUBB3$^+$ cells (Fig. 4). These observations indicate that naive *orJ* RPCs are able to increase proliferation in response to SHH ligand and that SHH-N is acting directly on the RPCs as neurogenesis was not initiated. In sum, our data indicate that the delay in Hh signaling activity negatively impacts proliferation and the delay is primarily the consequence of limited SHH production due to the delay of RGC differentiation in the embryonic *orJ* retina.

Shh signaling is altered in the orJ retina at birth despite the presence of SHH ligand

Reduced proliferation persists in the *orJ* retina at perinatal ages (Dhomen et al., 2006; Green et al., 2003; Livne-Bar et al., 2006). We therefore wished to determine if Hh signaling is altered at P0. To do this, we examined the expression patterns of Hh target genes by *in situ* hybridization and determined their relative expression levels by sqRT-PCR.

In situ hybridization shows that *Gli1* mRNA is abundantly expressed in RPCs of wild type P0 retinas (Fig. 5A$^{(*)}$). However, in the *orJ* retina, *Gli1* expression is reduced, patchy and more variable (Fig. 5B$^{(*)}$). *Ptc1* is expressed in the wild type retina, but appears reduced in the *orJ* retina (Fig. 5C$^{(*)}$,D$^{(*)}$). *Ptc2* mRNA was undetectable in both cases (Fig. 5E$^{(*)}$,F$^{(*)}$). As at the earlier ages, *Hhip* expression is obvious in the wild type retina but undetectable in the *orJ* retina (Figs. 5G$^{(*)}$,H$^{(*)}$). *In situ* hybridization for *Vsx2* mRNA (Figs. 5I$^{(*)}$,J$^{(*)}$) serves as a control and reveals strong expression, regardless of genotype.

To determine the relative expression levels of the Hh target genes, we performed sqRT-PCR on equivalent amounts of total RNA from P0 wild type and *orJ* retinas (Fig. 6). The relative expression levels of *Gli1*, *Ptc2*, *Hhip*, and *Ccnd1* were significantly lower in the *orJ* retina compared to wild type. *Ptc1* was the only target gene that did not show a significant change in expression level.

We also examined the expression of *Smo*, *Gli2*, and *Gli3*, essential components of the signaling pathway (Fig. 6; Supplemental Fig. S1). Only *Gli3* was significantly downregulated in the *orJ* retina, although *Gli2* exhibited a similar trend. These observations demonstrate that Hh signaling, as measured by the level of target gene expression, is reduced in the newborn *orJ* retina, and at least one component of the cell-intrinsic portion of the pathway, *Gli3*, is significantly reduced.

In addition to Hh target genes and important signaling components, we assessed the production and availability of SHH in the retina at P0. We determined the relative levels of *Shh* mRNA expression by sqRT-PCR and found that its expression is reduced in the newborn *orJ* retina (Fig. 7A). *Pou4f2*, which is believed to regulate *Shh* expression in RGCs (Mu et al., 2004), is expressed at levels that are not significantly different from wild type (Fig. 7A). Western blots show strong SHH expression in the *orJ* retinas, although it may be expressed at reduced levels compared to wild type (Fig. 7B; Supplemental Fig. S2). Immunohistochemistry reveals that SHH protein is localized to the nascent RGC layer, with

Fig. 8. SHH-N treatment promotes proliferation in neonatal explants within 24 h. BrdU (A, D), pHH3 (B, E), and PCNA (C, F) immunoreactivity in wild type explants after 24 h in culture. Quantification of BrdU$^+$ (G), pHH3$^+$ (H), and PCNA$^+$ (I) cells in wild type explants as a function of retinal area (G, I) and length of the apical surface of the retina (H). BrdU (J, M), pHH3 (K, N), and PCNA (L, O) immunoreactivity in *orJ* explants after 24 h in culture. Quantification of BrdU$^+$ (P), pHH3$^+$ (Q), and PCNA$^+$ (R) cells in wild type explants as a function of retinal area (P, R) and length of the apical surface of the retina (Q). Bars represent mean ± S.E.M. *p*-values calculated using Student's paired *t*-test. Scale bar: 50 μm.

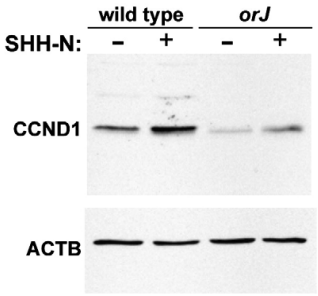


Fig. 9. CCND1 expression in retinal explants after 8 h of culture. Western blot of total retinal protein isolated from retinal explants cultured in the presence (+) or absence (−) of SHH-N and probed with anti-CCND1. Blot was reprobed with anti-ACTB.

expression extending to the peripheral retina (Figs. 7C–F; Supplemental Fig. S2). Together, these experiments show that SHH ligand is expressed in the newborn *orJ* retina in a manner similar to wild type, although the RT-PCR and western blot data indicate a reduced level of expression.

Neonatal orJ RPCs respond to exogenous and endogenous SHH in explant cultures

To determine if neonatal *orJ* RPCs are able to respond to exogenous SHH, we treated P0 retinal explants with SHH-N for 24 h and assayed for proliferation. We observed significant increases in $BrdU^+$ and $pHH3^+$ cells in SHH-N treated explants compared to untreated explants from both wild type (Figs. 8A,B,D,E,G,H; Supplemental Table 1) and *orJ* mice (Figs. 8J,K,M,N,P,Q; Supplemental Table 2). Similarly, western blot analyses revealed that an 8 h exposure of SHH-N was also sufficient to increase CCND1 protein levels, regardless of genotype (Fig. 9). These observations demonstrate that despite evidence of attenuated endogenous Hh signaling in vivo, *orJ* RPCs can increase proliferation in response to exogenous SHH. Interestingly, the relative proportion of $PCNA^+$ cells did not change in either genotype (Figs. 8C,F,I,L,O,R; Supplemental Tables 1 and 2), suggesting that SHH-N stimulates cell cycle

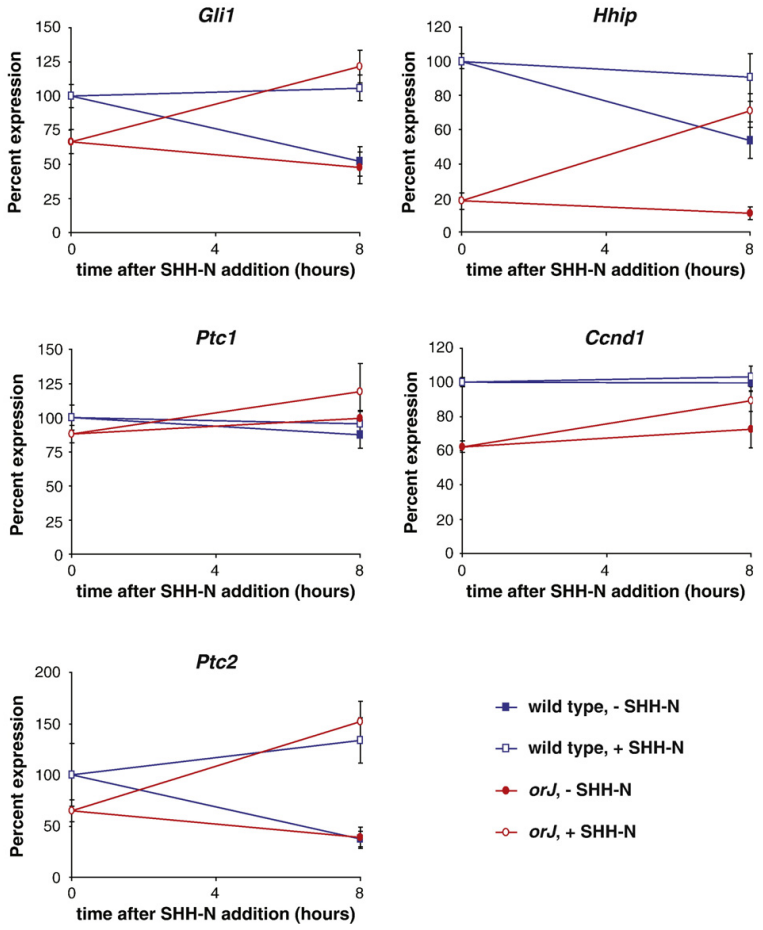


Fig. 10. SHH-N treatment stimulates expression of Hh target genes in explant culture. Relative expression levels of Hh target genes from retinal explants after 8 h in culture. All data points are relative to the wild type expression level at 0 h (set at 100%). 0 h is defined as isolated retinal tissue frozen immediately after dissection. The expression level for each genotype at 0 h is referred to in the text as the baseline expression level. Data points represent the mean±standard deviation. *p*-values are provided in Tables 1 and 2.

Table 1
Expression level changes due to SHH-N treatment at 8 h transcript

Transcript	Genotype	Mean difference±S.D.[a]	p-value[b]
Ccnd1	Wild type	249.75±279.59	0.172
	orJ	1058.07±302.72	0.006
Gli1	Wild type	1876.94±447.19	0.003
	orJ	2593.01±691.73	0.005
Ptc1	Wild type	412.62±222.30	0.034
	orJ	994.47±1215.65	0.20
Ptc2	Wild type	780.06±115.85	0.001
	orJ	918.50±239.46	0.005
Hhip	Wild type	876.37±208.11	0.003
	orJ	1435.12±187.14	0.0006

[a] Mean is calculated for the differences in pixel area measurements of ethidium bromide stained PCR products run through agarose for SHH-N treated versus untreated explants of each genotype. Samples are paired ($n=4$).
[b] p-values calculated by paired Student's t-test.

progression in RPCs rather than changing the rate of cell cycle exit (see Discussion).

To assess whether the SHH-N mediated proliferative response correlated with stimulation of the Hh signaling pathway, we examined the expression of Hh target genes after culturing P0 retinal explants in the presence or absence of SHH-N for 8 h by sqRT-PCR (Fig. 10; Tables 1 and 2). Regardless of genotype, we observed that SHH-N treatment generally resulted in elevated expression levels of Hh target genes when compared to untreated explants (Fig. 10; Table 1). When compared to the baseline expression levels ($t=0$), however, wild type and *orJ* explants exhibited different behaviors. In wild type explants, SHH-N treatment did not cause gene expression levels to increase in a significant manner over baseline levels, whereas in explants not exposed to SHH-N, gene expression levels declined (Fig. 10; Table 2). In contrast, *orJ* explants treated with SHH-N showed elevated gene expression whereas untreated explants showed little change in expression when compared to baseline levels (Fig. 10; Table 2). In sum, these observations show that SHH-N stimulates the Hh signaling pathway in the *orJ* retina, although the modulations of target gene expression differ between genotypes.

While these explant experiments revealed that *orJ* RPCs are able to respond to exogenous ligand, it remains that Hh target gene expression is substantially reduced in vivo even though endogenous ligand is expressed. Thus, we sought to confirm that *orJ* RPCs are in fact responding to the endogenous, RGC-derived SHH ligand in vivo by performing two independent analyses. First, we directly compared the P0 patterns of *Gli1* mRNA and acTUBB3 on the same retinal sections. As at E15.5 we found the highest levels of *Gli1* expression adjacent to acTUBB3-positive cells in both wild type and *orJ* retinas (data not shown), which is consistent with the idea that signaling is most robust near the cells that produce the ligand. Second, we performed explant cultures of P0 *orJ* retinas in the presence or absence of cyclopamine, a pharmacological inhibitor of Hh signaling (Chen et al., 2002; Cooper et al., 1998; Frank-Kamenetsky et al., 2002; Incardona et al., 1998; Taipale et al., 2000). After a 16 h culture period, we found *Gli1* expression was reduced in response to cyclopamine treatment (Figs. 11A,C), while *Vsx2* expression remained unchanged (Figs. 11B, D). Thus, endogenous Hh signaling activity in the *orJ* retina is sufficient to promote some level of Hh target gene expression. Furthermore, cyclopamine-treated explants exhibited a reduction in proliferation as indicated by significant reductions in the numbers of $BrdU^+$, $pHH3^+$, and $PCNA^+$ cells as compared to explants treated with vehicle alone (Figs. 11E–M; Supplemental Table 3). These data indicate *orJ* RPCs are responding to endogenous, RGC-derived SHH, at the levels of target gene expression and proliferation.

Discussion

In this study, we have elucidated some key elements in the relationship between Vsx2 and Shh in promoting RPC proliferation in the developing retina. At early embryonic stages, a delay in Hh signaling in the *orJ* retina correlates with a delay in RGC development. At birth, SHH ligand is produced by RGCs and RPCs respond to it, but Hh signaling in the *orJ* retina occurs at a reduced level compared to wild type. Our observations lead us to propose that Vsx2 has important roles in ensuring the correct timing and magnitude of Hh signaling in the retina, which ultimately contributes to proper execution of the histogenetic program.

Retinal Hh target genes

Our analyses of Hh target gene expression in the mouse retina have revealed several features of their expression and regulation in this tissue. We have presented the first documented expression of *Hhip* in the retina, as well as information regarding its transcriptional regulation in RPCs in response to SHH, thereby revealing its usefulness as an indicator of Hh signaling activity in the mouse retina. *Gli1*, *Ptc2*, and *Hhip* are all highly responsive Hh target genes in the retina. High levels of their expression are dependent upon active Hh signaling, as evidenced by the large expression difference between SHH-N treated and untreated explants within a short culture period. Although *Ptc1* expression is a useful indicator of Hh signaling activity in other systems, we found *Ptc1* to be relatively

Table 2
Relative expression levels at baseline ($t=0$ h) and 8 h in culture

Transcript	Genotype	Baseline[a]	−SHH-N[a]	p-value[b,c]	+SHH-N[a]	p-value[b,d]
Ccnd1	Wild type	100±2.70	99.58±5.03	0.89	103.50±6.06	0.33
	orJ	62.40±3.50	72.49±10.50	0.12	89.11±6.14	0.0003
Gli1	Wild type	100±8.36	52.24±10.99	0.0004	105.84±9.25	0.38
	orJ	66.75±8.71	47.60±11.55	0.038	121.65±11.93	0.0003
Ptc1	Wild type	100±8.99	87.32±9.92	0.11	95.41±8.97	0.50
	orJ	87.91±6.11	99.40±5.69	0.033	118.89±20.84	0.029
Ptc2	Wild type	100±30.30	37.26±7.85	0.007	133.53±21.99	0.12
	orJ	65.15±10.66	38.74±10.30	0.012	152.10±19.57	0.0002
Hhip	Wild type	100±4.45	53.74±10.90	0.0002	90.52±13.88	0.24
	orJ	18.14±4.88	10.95±3.49	0.053	71.17±9.73	6.7e−05

[a] For each transcript, values±S.D. are normalized to the wild type baseline measurement set at 100 ($n=4$). Values in −SHH-N and +SHH-N are after 8 hr in culture.
[b] Unpaired Student's t-tests were done to determine if the values derived from samples taken after 8 h in culture are statistically. Significant from the baseline values. For each statistical comparison, the 8 h measurements were compared to the baseline value of the same genotype ($p<0.05$ reveals a statistically significant difference).
[c] p-value for comparison between baseline and −SHH-N.
[d] p-value for comparison between baseline and +SHH-N.

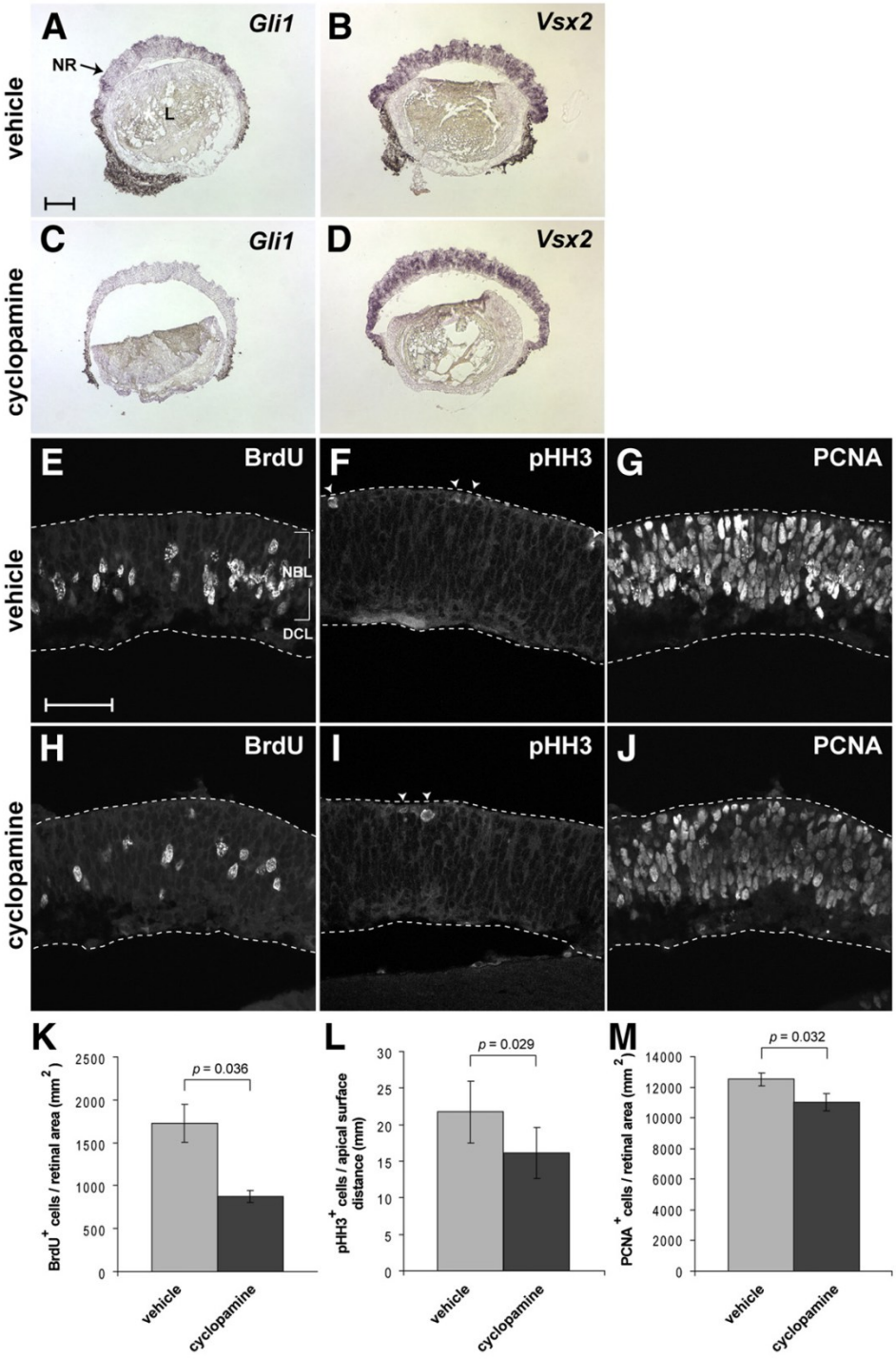


Fig. 11. Endogenous SHH promotes proliferation in P0 *orJ* retinas. *orJ* retinal explants were cultured in vehicle alone (0.41% v/v DMSO) or 10 μM cyclopamine (in 0.41% v/v DMSO) for 16 h (A–D) or 24 h (E–J). (A, C) *Gli1* mRNA expression. (B, D) *Vsx2* mRNA expression. BrdU (E, H), pHH3 (F, I), and PCNA (G, J) immunoreactivity. Closed arrowheads in (F, I) indicate $pHH3^+$ cells at the apical surface of the retina. Quantification of $BrdU^+$ (K), $pHH3^+$ (L), and $PCNA^+$ (M) cells as a function of retinal area (K, M) and length of the apical surface of the retina (L). Bars represent mean ± S.E.M. *p*-values calculated using the Student's paired *t*-test. Scale bar: 120 μm (A–D), 50 μm (E–J).

unresponsive to changes in the level of retinal Hh signaling activity. *Ptc1* exhibits little to no change in expression after explant culturing in the presence or absence of SHH-N. Furthermore, while reduced expression of the other analyzed Hh target genes indicates reduced Hh signaling in the *orJ* retina, *Ptc1* expression is not significantly different across genotypes. Our findings are consistent with observations made in other retinal explant cultures (Wang et al., 2002) and in the *Pou4f2*$^{-/-}$ retina, which exhibits reduced *Shh* expression (Mu et al., 2004).

The delays in Shh expression and Hh signaling activity in the orJ retina are a consequence of delayed RGC differentiation during embryonic development

Our gene expression studies of components of the Hh signaling network revealed drastically altered mRNA expression territories for *Shh* and the Hh target genes *Gli1*, *Ptc1*, and *Hhip* in the *orJ* retina during embryonic stages. The lack of expression at E12.5 and centrally restricted expression patterns observed in *orJ* retinas at E15.5 are reminiscent of the wild type expression patterns observed for earlier ages (our data; Dakubo et al., 2003; Wang et al., 2005). However, at birth, the expression territories (most notably SHH and *Gli1*) extend to the peripheral retina, similar to wild type. Based on these observations, we conclude that the central-to-peripheral wave of Shh expression and Hh signaling still occurs in the *orJ* retina, but is delayed.

RGC differentiation is also delayed in the *orJ* retina (Bone-Larson et al., 2000), and our data are consistent with central-to-peripheral waves of Shh and Hh target gene expression following a peripherally-spreading wave of RGC differentiation (Wang et al., 2005). It appears then, that the relationship between Hh signaling and RGC differentiation is maintained in the *orJ* retina. Thus, the limited spatial extent of Hh signaling activity during embryonic retinal development may be due, in large part, to the altered availability of SHH ligand, owing to a developmental delay in its cellular source, the RGCs.

Extra-retinal sources of Hh ligands, such as Ihh (Dakubo et al., 2003; Levine et al., 1997), are expressed during the early embryonic period, and the high level of *Gli1* expression in the periocular mesenchyme of the *orJ* mouse suggests they are still present. Even so, they do not activate Hh signaling in the retina. This is consistent with previous findings that the only source of Hh ligand to which RPCs respond is RGC-derived Shh (Dakubo et al., 2003).

This delay in RGC differentiation limits the exposure of RPCs to SHH, which could contribute to the proliferation deficiency characteristic of *orJ* RPCs, since proliferation is an established response of RPCs to Hh signaling. Consistent with this idea is our finding that naïve *orJ* RPCs are competent to respond to stimulation of the Hh pathway. Additionally, mouse models in which RGCs either fail to mature (Brown et al., 2001; Le et al., 2006; Mu et al., 2004), are ablated with toxin (Mu et al., 2005), or do not express Shh (Wang et al., 2005, 2002) exhibit decreased RPC proliferation. The sum of these observations supports a model in which the proliferative expansion of the embryonic retina is mechanistically dependent on the production of RGCs (Mu et al., 2005) and perhaps more generally, on the production of postmitotic cells. The delay in RGC differentiation in the *orJ* retina may also limit the availability of other mitogens, such as GDF8/myostatin (Mu et al., 2004) and VEGF (Hashimoto et al., 2006; Yang and Cepko, 1996). How Vsx2 contributes to the correct timing for the onset of RGC production is not known.

Reduced Hh signaling and proliferative expansion in the neonatal orJ retina

In addition to the changes observed in the embryonic retina, our analysis of several Hh target genes reveals that Hh signaling remains reduced in the neonatal *orJ* retina, even though signaling is activated by E15.5 and extends out to the peripheral retina by birth. Since the proportions of the wild type and *orJ* RPC populations relative to their total cell populations do not differ at P0 (Green et al., 2003), the reductions in Hh target gene expression in the *orJ* retina are likely due to changes in gene regulation rather than to changes in cell composition. This conclusion is supported by our observation that SHH-N treatment significantly enhances Hh target gene expression within 8 h.

How might Hh signaling influence *orJ* RPCs? Our explant experiments indicate that the role of Hh signaling is complex. SHH-N treatment increased the relative proportions of cells in S and M phases (as measured by BrdU and pHH3 immunoreactivity) without influencing the relative proportion of RPCs as indicated by PCNA immunoreactivity (Barton and Levine, 2008). These observations suggest that the primary influence of SHH-N is to shorten the time of *orJ* RPCs spend in G1 and/or G2. While cyclopamine treatment had the opposite effect on the relative proportions of BrdU$^+$ and pHH3$^+$ cells, it also caused a reduction in the *orJ* RPC population as inferred by the decrease in PCNA$^+$ cells. In this case, the reduced proliferation could be due to prolongation of the G1 and/or G2 phases in addition to enhanced cell cycle exit. Taken together, it appears then, that a high level of Hh signaling primarily influences cell cycle progression in *orJ* RPCs whereas a low level of Hh signaling may help to maintain the *orJ* RPC population. Consistent with these ideas are the findings that the *orJ* RPC population is at the correct proportion at birth (Green et al., 2003; unpublished observations), that the cell cycle time of *orJ* RPCs is longer than wild type and is primarily due to an increase in G1 time (Konyukhov and Sazhina, 1971; unpublished observations), and Hh signaling is active, but at a reduced level in the *orJ* retina (this paper). In sum, we propose that Hh signaling is likely a limiting factor for the proliferative expansion of *orJ* RPCs. Thus, in addition to any direct roles Vsx2 has in regulating cell cycle progression and other aspects of RPC behavior, we propose that reduced signaling in at least one growth factor pathway also contributes to the proliferation problems characteristic of the *orJ* retina.

Hh signaling is tightly controlled at almost every step of the pathway, from ligand production to activity of the transcriptional effectors. Changes in any of these processes impact the level of signaling activity (Cohen, 2003; Ingham, 1998; Ingham and McMahon, 2001; Nybakken and Perrimon, 2002; Varjosalo and Taipale, 2007). Our expression data revealed that the expression of Shh mRNA and protein are reduced in the *orJ* retina at P0. Whether these drops in expression are sufficient to completely account for the level of reduced signaling is unclear. Interestingly, *orJ* cells that expressed SHH appeared to do so in a robust manner. Since processing and secretion of HH ligands are under complex control and defects in these mechanisms could also contribute to reduced signaling levels, it is possible that problems of this nature are further limiting the exposure of SHH to *orJ* RPCs. Importantly, alterations in Hh signaling and proliferation due to reduced expression or to problems in processing or secretion indicate that non-cell autonomous changes may contribute to the *orJ* phenotype since Vsx2 is not expressed in RGCs.

Although reduced ligand production likely contributes to the diminished levels of Hh signaling in the *orJ* retina, there is also ample precedence for the involvement of extracellular molecules in modulating the magnitude of Hh signaling through their influence on ligand dispersion and/or reception (Cohen, 2003; Ingham, 1998; Ingham and McMahon, 2001; Nybakken and Perrimon, 2002; Varjosalo and Taipale, 2007). In addition to being transcriptional targets, Hhip and Ptc2 are also extracellular regulators of Hh signaling. Their reduced expression in the *orJ* retina, however, is not likely to account for the changes in Hh signaling levels. Hhip functions as a negative regulator resulting in attenuated Hh signaling (Chuang et al., 2003; Chuang and McMahon, 1999). Thus, its expression change is counter to Hhip being a mechanistic explanation of reduced Hh

signaling in the *orJ* retina. Although Ptc2 exhibits high binding affinity for Hh ligands (Carpenter et al., 1998), it lacks the ability to mediate SHH-stimulated signaling and inhibits SHH function (Rahnama et al., 2004). Based on these findings, Ptc2 also functions as a negative regulator. Thus, although it appears that reduced Hhip and Ptc2 expression cannot explain the reduced levels of Hh signaling in the *orJ* retina, they do provide strong evidence that a change in Hh signaling is occurring. Gas1, a GPI-anchored membrane protein, is negatively regulated by Hh signaling (Allen et al., 2007) and is upregulated in the embryonic *orJ* retina (Rowan et al., 2004). Whether this elevated expression is the result of reduced Hh signaling or an unrelated change caused by the absence of Vsx2 is not known. Additionally, since Gas1 can act as a positive (Allen et al., 2007; Lee et al., 2001b; Liu et al., 2002, 2001) or negative (Cobourne et al., 2004; Lee et al., 2001a) regulator of Hh signaling, it is difficult to predict the consequences of its change in expression.

Changes in the intracellular portion of the pathway could also alter Hh signaling, but whether these types of changes are causal factors for reduced signaling and proliferation is unclear. We found that cyclopamine treatment further reduced *Gli1* expression and proliferation, and SHH-N treatment stimulated target gene expression and proliferation. While these findings demonstrate that the intracellular portion of the Hh pathway is functional in the *orJ* retina, we cannot conclude that the response observed in the *orJ* explants is equivalent to a 'wild type response'. This is because the behaviors of *orJ* and wild type explants are qualitatively different. Wild type explants treated with SHH-N exhibit maintenance of their baseline expression levels for most target genes, indicating that wild type RPCs are responding maximally. Furthermore, target gene expression in wild type explants not exposed to SHH-N drop below baseline levels, indicating that continuous exposure to ligand is necessary to sustain expression levels. In contrast, *orJ* explants exposed to SHH-N show stimulation above baseline levels and most target genes do not drop below baseline levels in untreated explants. Furthermore, since the baseline levels of Hh regulators and cell intrinsic effectors differ in the *orJ* retina compared to wild type (e.g. *Gli1*, *Ptc2*, *Hhip*, *Gli3*), a direct comparison of the quality of response to SHH-N between the *orJ* and wild type RPCs is not easily accomplished. Thus, while our data shows that the intrinsic portion of the Hh pathway is functioning in *orJ* RPCs, it is possible that alterations in this part of the pathway are also causing changes in RPC behavior.

In sum, our data provide evidence for a model in which Vsx2 promotes RPC proliferation in a non-cell autonomous manner by ensuring proper RGC differentiation and Hh signaling during embryonic retinal development, which is in addition to its cell autonomous functions in RPCs. Furthermore, we propose that Hh signaling is a limiting factor for RPC proliferation in the absence of Vsx2. While Shh expression is reduced in the *orJ* retina, the loss of Vsx2 may also impact the Hh pathway downstream of ligand expression, by altering the expression of components of the Hh signaling network, or by maintaining an environment in which RPCs can respond appropriately to SHH ligand. While more work is needed to identify the molecular mechanisms by which Vsx2 regulates RPC proliferation, our work highlights the complex interactions between the tissue-restricted cell-intrinsic factor Vsx2 and Hh signaling, in their contributions to the proliferative expansion of cell number during retinal histogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.02.055.

References

Allen, B.L., et al., 2007. The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. Genes Dev. 21, 1244–1257.
Barton, K.M., Levine, E.M., 2008. Expression patterns and cell cycle profiles of PCNA, MCM6, Cyclin D1, Cyclin A2, Cyclin B1, and phosphorylated Histone H3 in the developing mouse retina. Dev. Dyn. 237, 672–682.
Bar-Yosef, U., et al., 2004. CHX10 mutations cause non-syndromic microphthalmia/anophthalmia in Arab and Jewish kindreds. Hum. Genet. 115, 302–309.
Belecky-Adams, T., et al., 1997. Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. Invest. Ophthalmol. Vis. Sci. 38, 1293–1303.
Bone-Larson, C., et al., 2000. Partial rescue of the ocular retardation phenotype by genetic modifiers. J. Neurobiol. 42, 232–247.
Brown, N.L., et al., 2001. Math5 is required for retinal ganglion cell and optic nerve formation. Development 128, 2497–2508.
Burmeister, M., et al., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. 12, 376–384.
Carpenter, D., et al., 1998. Characterization of two patched receptors for the vertebrate hedgehog protein family. Proc. Natl. Acad. Sci. U. S. A. 95, 13630–13634.
Chen, J.K., et al., 2002. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. 16, 2743–2748.
Chuang, P.T., McMahon, A.P., 1999. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature 397, 617–621.
Chuang, P.T., et al., 2003. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. Genes Dev. 17, 342–347.
Clark, A.M., et al., 2008. Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina. Brain Res. 1192, 99–113.
Cobourne, M.T., et al., 2004. Restriction of sonic hedgehog signalling during early tooth development. Development 131, 2875–2885.
Cohen Jr., M.M., 2003. The hedgehog signaling network. Am. J. Med. Genet. A 123, 5–28.
Cooper, M.K., et al., 1998. Teratogen-mediated inhibition of target tissue response to Shh signaling. Science 280, 1603–1607.
Dakubo, G.D., Wallace, V.A., 2004. Hedgehogs and retinal ganglion cells: organizers of the mammalian retina. NeuroReport 15, 479–482.
Dakubo, G.D., et al., 2003. Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development. Development 130, 2967–2980.
Del Bene, F., Wittbrodt, J., 2005. Cell cycle control by homeobox genes in development and disease. Semin. Cell Dev. Biol. 16, 449–460.
Dhomen, N.S., et al., 2006. Absence of chx10 causes neural progenitors to persist in the adult retina. Invest. Ophthalmol. Vis. Sci. 47, 386–396.
Donovan, S.L., Dyer, M.A., 2005. Regulation of proliferation during central nervous system development. Semin. Cell Dev. Biol. 16, 407–421.
Dorval, K.M., et al., 2006. CHX10 targets a subset of photoreceptor genes. J. Biol. Chem. 281, 744–751.
Ferda Percin, E., et al., 2000. Human microphthalmia associated with mutations in the retinal homeobox gene CHX10. Nat. Genet. 25, 397–401.
Frank-Kamenetsky, M., et al., 2002. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. J. Biol. 1, 10.
Green, E.S., et al., 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development 130, 539–552.
Hashimoto, T., et al., 2006. VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation. Development 133, 2201–2210.
Hatakeyama, J., et al., 2001. Roles of homeobox and bHLH genes in specification of a retinal cell type. Development 128, 1313–1322.
Ho, K.S., Scott, M.P., 2002. Sonic hedgehog in the nervous system: functions, modifications and mechanisms. Curr. Opin. Neurobiol. 12, 57–63.
Horsford, D.J., et al., 2005. Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. Development 132, 177–187.
Incardona, J.P., et al., 1998. The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. Development 125, 3553–3562.
Ingham, P.W., 1998. Transducing Hedgehog: the story so far. EMBO J. 17, 3505–3511.

Ingham, P.W., McMahon, A.P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15, 3059–3087.
Jensen, A.M., Wallace, V.A., 1997. Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development 124, 363–371.
Konyukhov, B.V., Sazhina, M.V., 1971. Genetic control over the duration of G 1 phase. Experientia 27, 970–971.
Le, T.T., et al., 2006. Math5 is required for both early retinal neuron differentiation and cell cycle progression. Dev. Biol. 295, 764–778.
Lee, C.S., et al., 2001a. Evidence that the WNT-inducible growth arrest-specific gene 1 encodes an antagonist of sonic hedgehog signaling in the somite. Proc. Natl. Acad. Sci. U. S. A. 98, 11347–11352.
Lee, C.S., et al., 2001b. Transdifferentiation of the ventral retinal pigmented epithelium to neural retina in the growth arrest specific gene 1 mutant. Dev. Biol. 236, 17–29.
Levine, E.M., Green, E.S., 2004. Cell-intrinsic regulators of proliferation in vertebrate retinal progenitors. Semin. Cell Dev. Biol. 15, 63–74.
Levine, E.M., et al., 1997. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J. Neurosci. 17, 6277–6288.
Liu, Y., et al., 2001. Growth arrest specific gene 1 is a positive growth regulator for the cerebellum. Dev. Biol. 236, 30–45.
Liu, Y., et al., 2002. Growth arrest specific gene 1 acts as a region-specific mediator of the Fgf10/Fgfr3 regulatory loop in the limb. Development 129, 5289–5300.
Livesey, F.J., Cepko, C.L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev., Neurosci. 2, 109–118.
Livne-Bar, I., et al., 2006. Chx10 is required to block photoreceptor differentiation but is dispensable for progenitor proliferation in the postnatal retina. Proc. Natl. Acad. Sci. U. S. A. 103, 4988–4993.
Locker, M., et al., 2006. Hedgehog signaling and the retina: insights into the mechanisms controlling the proliferative properties of neural precursors. Genes Dev. 20, 3036–3048.
Moshiri, A., Reh, T.A., 2004. Persistent progenitors at the retinal margin of ptc+/− mice. J. Neurosci. 24, 229–237.
Mu, X., et al., 2004. Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4F2 for their expression in the mouse embryonic retina. Development 131, 1197–1210.
Mu, X., et al., 2005. Ganglion cells are required for normal progenitor-cell proliferation but not cell-fate determination or patterning in the developing mouse retina. Curr. Biol. 15, 525–530.
Nybakken, K., Perrimon, N., 2002. Hedgehog signal transduction: recent findings. Curr. Opin. Genet. Dev. 12, 503–511.
Oliver, T.G., et al., 2003. Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. Proc. Natl. Acad. Sci. U. S. A. 100, 7331–7336.
Rahnama, F., et al., 2004. Distinct roles of PTCH2 splice variants in Hedgehog signalling. Biochem. J. 378, 325–334.
Rowan, S., Cepko, C.L., 2004. Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. Dev. Biol. 271, 388–402.
Rowan, S., et al., 2004. Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. Development 131, 5139–5152.
Rutherford, A.D., et al., 2004. Delayed expression of the Crx gene and photoreceptor development in the Chx10-deficient retina. Invest. Ophthalmol. Vis. Sci. 45, 375–384.
Schaeren-Wiemers, N., Gerfin-Moser, A., 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry 100, 431–440.
Taipale, J., et al., 2000. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature 406, 1005–1009.
Toy, J., et al., 2002. Effects of homeobox genes on the differentiation of photoreceptor and nonphotoreceptor neurons. Invest. Ophthalmol. Vis. Sci. 43, 3522–3529.
Varjosalo, M., Taipale, J., 2007. Hedgehog signaling. J. Cell. Sci. 120, 3–6.
Wallace, V.A., Raff, M.C., 1999. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126, 2901–2909.
Wang, Y.P., et al., 2002. Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells. Nat. Neurosci. 5, 831–832.
Wang, Y., et al., 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development 132, 5103–5113.
Yang, X.J., 2004. Roles of cell-extrinsic growth factors in vertebrate eye pattern formation and retinogenesis. Semin. Cell Dev. Biol. 15, 91–103.
Yang, K., Cepko, C.L., 1996. Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. J. Neurosci. 16, 6089–6099.
Young, R.W., 1985. Cell differentiation in the retina of the mouse. Anat. Rec. 212, 199–205.

CHAPTER 3

ALTERED EXTRACELLULAR ENVIRONMENT IN THE *VSX2* NULL RETINA CHANGES THE DYNAMICS OF HEDGEHOG SIGNALING IN RETINAL PROGENITOR CELLS

Introduction

Proliferative expansion of retinal progenitor cells (RPCs) is required for the proper growth and development of the neural retina, ensuring sufficient generation of both the appropriate number and types of differentiated retinal cells. The homeobox gene Vsx2 is expressed in RPCs and an essential regulator of their proliferation. Mutations in *Vsx2* cause microphthalmia (small eye) in both humans and mice (Bar-Yosef et al., 2004; Bone-Larson et al., 2000; Burkitt Wright et al., 2010; Burmeister et al., 1996; Faiyaz-Ul-Haque et al., 2007; Ferda Percin et al., 2000; Iseri et al., 2010; Reis et al., 2011; Robb et al., 1978). This condition arises primarily from a profound defect in RPC proliferation that produces a severely hypocellular retina (Bone-Larson et al., 2000; Burmeister et al., 1996; Dhomen et al., 2006; Ferda Percin et al., 2000; Green et al., 2003; Konyukhov and Sazhina, 1971).

The slowed rate of cell cycle progression observed in RPCs in the absence of Vsx2 function correlated with aberrant expression of G1 phase cell cycle components, including accumulation of the cell cycle inhibitor p27/KIP1 and downregulation of the cell cycle promoter cyclin D1 (Ccnd1) (Green et al., 2003; Sigulinsky et al., 2008). Genetic deletion of p27/KIP1 in Vsx2-deficient retinas largely restored retinal cell number without influencing neurogenesis or apoptosis (Green et al., 2003). Furthermore, Ccnd1 is an important mediator in the prevention of p27/KIP1 accumulation by Vsx2 in RPCs (Green et al., 2003), suggesting that promoting high levels of Ccnd1 expression is a major function of Vsx2 in its regulation of RPC proliferation. Mitogens are also essential regulators of cell cycle progression, upregulating D-cyclins to promote G1 progression [reviewed in (Giacinti and Giordano, 2006; Lundberg and Weinberg, 1999)]. Thus, it was

not clear whether decreased expression of Ccnd1 resulted from direct transcriptional regulation by Vsx2 or indirectly through Vsx2-mediated regulation of mitogen signals or their signaling pathways.

Sonic hedgehog (Shh) acts as a mitogen in a number of developing tissues. Shh is a secreted glycoprotein that activates the Hedgehog (Hh) pathway. Shh binds to and inhibits the Hh receptor, Patched homolog 1 (Ptch1), relieving Ptch1-mediated inhibition of Smoothened (Smo). Activated Smo both inhibits proteolytic processing of the Gli family of transcriptional effectors into truncated repressors and promotes their activation and nuclear localization to elicit transcriptional activation and derepression of Hh target genes [reviewed in (Ryan and Chiang, 2012)]. In the retina, Shh is expressed by retinal ganglion cells soon after their differentiation. RPCs express Hh pathway components and upregulate Hh target gene expression in response to this ganglion cell-derived Shh production (Wallace, 2008). Shh is a well-established retinal mitogen and required for sufficient RPC proliferation [for review, see (Wallace, 2008)]. Treatment with exogenous Shh or increased signaling activity of the Hedgehog (Hh) pathway through manipulation of Hh pathway components stimulates RPC proliferation and increases retinal cell number (Jensen and Wallace, 1997; Levine et al., 1997; Moshiri and Reh, 2004; Yu et al., 2006). Conversely, attenuation of endogenous Hh signaling through neutralization of endogenous Shh or genetic ablation of *Shh* or Hh pathway components decreases RPC proliferation, often leading to a reduced progenitor pool, reduced clone size, or microphthalmia (Sakagami et al., 2009; Wallace and Raff, 1999; Wang et al., 2005). Shh and activation of the Hh pathway stimulates RPC proliferation by promoting G1 progression (Sakagami et al., 2009). Similar to loss of Vsx2 function, disruption of Hh

signaling results in aberrant expression of cell cycle components, including an increase in the number of p27/KIP1-expressing cells and downregulation of Ccnd1 (Sakagami et al., 2009).

Thus, we examined the potential role for Shh signaling in mediating Vsx2-dependent regulation of RPC proliferation. Previously, we reported reduced Hh signaling in the retinas of *ocular retardation J* (*orJ*) mice, which carry a recessive null allele of *Vsx2* (Sigulinsky et al., 2008). Embryonically, altered activation of Hh signaling in the *orJ* retina correlated with delayed generation of retinal ganglion cells, the relevant source of endogenous retinal Shh. Neonatally, reduced Hh signaling activity persisted in the *orJ* retina and was associated with reduced levels of *Shh* mRNA and protein. Furthermore, addition of exogenous Shh stimulated upregulation of Hh target genes and increased RPC proliferation at both ages. These findings suggested that reduced availability of Shh ligand is a major cause of reduced Hh signaling activity in vivo and likely contributes to the defective proliferation of *orJ* RPCs.

In the present work, we report that upon dissociation, *orJ* cells exhibit greatly diminished responsiveness to treatment with a recombinant, active form of Shh protein (SHH-N). Reduced responsiveness of *orJ* RPCs to Hh pathway stimulation is not mutually exclusive with our previous finding of reduced ligand availability in the *orJ* retina. Thus, we sought to investigate the nature of the change underlying this impaired responsiveness, as this change may be relevant in vivo and would contribute to our understanding of Vsx2-mediated regulation of Shh signaling and RPC proliferation. Here, we detail the diminished responsiveness of *orJ* RPCs to SHH-N in dissociated cell culture and our progress towards the localization of the disruption within the Hh pathway.

Methods

Mice

orJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Wild type (+) and *orJ* alleles were determined by PCR and restriction digest, as previously described (Burmeister et al., 1996) from adult ear clips or neonatal tail samples. Black Swiss mice were purchased from Taconic Farms, Inc. (Hudson, NY, USA). The *orJ* allele was introduced into the Black Swiss background by mating *orJ* (129/Sv) mice with Black Swiss mice and selecting for the *orJ* allele. Except where noted in the text, experiments were performed using mice maintained on the 129/Sv background. Previous analyses and our own unpublished observations suggest that eye development (Rowan et al., 2004) and expression of Hh target genes (this study, data not shown) is indistinguishable between heterozygous *orJ* (+/*orJ*) and wild type (+/+) mice. Thus, mice with these genotypes were considered equivalent and referred to as wild type in the text. Homozygous *orJ* (*orJ*/*orJ*) mice are referred to as *orJ*. The day of birth was considered postnatal day 0 (P0). Animal use and care was conducted in accordance with IACUC guidelines.

Dissociated cell culture

P0 retinal tissue was dissected from surrounding ocular tissues in Hank's buffered saline solution and the lens and inner vasculature removed. Retinal tissue was incubated in 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) in Ca^{2+}- and Mg^{2+}-free HBSS, triturated into single-cell suspension, and resuspended in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen), containing 0.6% glucose,

0.1125% $NaHCO_3$ (Invitrogen), 5 mM HEPES (Invitrogen), 1% FBS (Invitrogen), 1.5 μM thymidine (Invitrogen), GlutaMAX (0.5X, Invitrogen), 25 μg/ml insulin (Sigma, St. Louis, MO, USA); 100 μg/ml transferrin (Sigma), 60 μM putrescine (Sigma), 30 nM selenium (Sigma), 20 nM progesterone (Sigma), and Penicillin/Streptomycin (1X, Invitrogen). Cell suspensions were plated at a density of 100,000 cells/well using 24-well cell culture plates and maintained in 1 ml of the supplemented culture medium. For proliferation assays, dissociated retinal cells were plated onto sterilized and UV-treated cover slips precoated with poly-D-lysine (40 μg/ml; Sigma) and Matrigel (0.01X). For assays examining gene expression, dissociated retinal cells were plated directly onto the bottoms of precoated (40 μg/ml poly-D-lysine and 0.01X Matrigel) wells. Plates were briefly spun and cells allowed to settle for 1 hour at 37 °C and 5% CO_2 to promote adherence to the plate or coverslips prior to addition of growth factors or pharmacological agents. Cultures were incubated at 37 °C and 5% CO_2 with gentle nutating for the remaining culture period. Due to the severe hypocellularity of the *orJ* retina, retinas from multiple animals were pooled per sample. Distinct poolings were each counted as a separate *n*. Wild type retinas were not pooled, each animal providing a separate *n*.

Retinal explant culture

Retinal explant cultures were performed as previously described (Sigulinsky et al., 2008). Briefly, P0 retinal tissue with the lens and vitreal chamber intact was dissected from surrounding ocular tissues in HBSS and cultured in 1 ml of the supplemented culture medium. Growth factors or pharmacological agents were diluted directly in the

culture medium immediately prior to culturing. Cultures were incubated at 37 °C and 5% CO_2 with gentle nutating.

Growth factor and pharmacological challenge

To test responsiveness to Hh ligand stimulation, dissociated retinal cells or retinal explants were cultured in the presence or absence of 108 ng/ml SHH-N (Levine et al., 1997). Purified baculovirus-derived SHH-N was a kind gift from H. Roelink. To test responsiveness to Smo activation, the Smo agonist purmorphamine (EMD Chemicals, Philadelphia, PA, USA) was added to cultures of dissociated retinal cells and retinal explants. Unless otherwise noted, purmorphamine was used at a final concentration of 1 μM (0.4% DMSO). DMSO (0.4%, v/v) served as a vehicle control. To inhibit PKA activity, the isoquinolinesulfonamide H89 (10 μM; Sigma) was added to cultures of dissociated retinal cells. For retinal explants, one explant from each mouse was cultured in the presence of factors or pharmacological agents, while the contralateral explant served as a control, cultured in the absence of factors and pharmacological agents or in the presence of a vehicle control.

For proliferation assays, dividing cells in S phase were labeled by incorporation of the thymidine analog bromodeoxyuridine (BrdU; Sigma). Dissociated retinal cells were cultured for 24 or 48 hours with BrdU present during the final 2 or 4 hours, respectively, at a final concentration of 10 μg/ml. For gene expression measurements, dissociated retinal cells and retinal explants were cultured for 8 hours, except where noted in the text. For retinal explants, the lens and inner vasculature were removed from the retinal tissue immediately following the culture period, prior to RNA isolation. This

was performed on ice to limit changes in gene expression. In the postdissociation recovery experiments, dissociated *orJ* retinal cells were cultured for 24 hours in the presence of nonmitogenic concentrations of FGF-2 (0.1 ng/ml; C. Zou, personal communication; R&D Systems, Minneapolis, MN, UAS) to promote cell survival. FGF-2 was added to the culture medium one hour after plating (0 hour). After 24 hours, purmorphamine (1 μM) or DMSO alone (0.4%) was added to the culture medium by replacing half the existing culture medium with fresh media containing two times the final concentrations of FGF-2 and purmorphamine or DMSO.

Semiquantitative RT-PCR (sqRT-PCR) and analysis

Gene expression was measured by sqRT-PCR and analyzed as previously described (Sigulinsky et al., 2008). Student's unpaired *t*-test or Welch's two sample *t*-test was performed to determine statistical significance, as appropriate (based on results of the *F*-test of Variances), using Jmp 7.0 (SAS Institute, Inc., Cary, NC, USA).

Immunocytochemistry and marker analysis

At the end of the culture period, dissociated retinal cells plated on cover slips were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.5) for 30 minutes. Coverslips with adherent cells were either stained immediately or washed with PBS and stored at 4 °C in PBS containing 0.01% sodium azide (NaN_3) until staining. Staining was performed with adherent cells on cover slips in 24-well culture plates. Adherent cells were washed in PBS and pretreated with blocking buffer (2% normal goat serum, 0.15% TritonX-100, and 0.01% NaN_3 in PBS) for 30 minutes. Cells

were then incubated overnight at 4 °C with the following primary antibodies diluted in blocking buffer: mouse anti-BrdU (1:100; clone B44, Cat# 347580, BD Biosciences, San Jose, CA, USA); mouse anti-γ-tubulin (1:1000; clone GTU-88, Cat# T6557, Sigma); mouse anti-acetylated α-tubulin (1:10,000; clone 6-11B-1, Cat# T6793, Sigma); and rabbit anti-Arl13b (1:4500; gift of Tamara Caspary). Primary antibodies were followed by PBS washes and incubation for 1 hour at room temperature with appropriate species-specific secondary antibodies conjugated to AlexaFluor488 or 568 (Invitrogen-Molecular Probes, Eugene, OR, USA) diluted 1:1000 in blocking buffer. Hydrochloric acid treatment (2N HCl, 30 minutes at room temperature) was performed prior to incubation with the BrdU antibody. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Fluka, Switzerland). After washing in PBS, the coverslips were mounted onto glass slides in Fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

Visualization and image capture for proliferation and cell death assays was performed using an Eclipse E600 epi-fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a Spot-RT slider CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Visualization and image capture for evaluation of primary cilia was performed using an Olympus FluoView FV1000 confocal laser scanning microscope and FV10-ASW software (Olympus America Inc., Center Valley, PA, USA). Images were processed using Adobe Photoshop CS5 Extended (Adobe Systems Inc., San Jose, CA, USA). FluoView confocal files were first converted using the Bio-Formats Importer Plugin (LOCI, University of Wisconsin-Madison, Madison, WI, USA) and ImageJ (NIH).

Proliferation of dissociated retinal cells was assayed by quantification of BrdU incorporation. The percentage of BrdU-labeled cells was calculated by determining the number of BrdU-positive cells in the total cell population (DAPI-positive) per cover slip. A minimum of 1000 cells were counted by random field analysis on each cover slip. Counts were performed blinded to treatment condition and genotype. To determine the statistical significance of the effects of SHH-N on proliferation, Student's unpaired *t*-test or Welch's two sample *t*-test was performed as appropriate (based on results of the *F*-test of Variances). Statistical significance for the dose response curves of purmorphamine treatment on proliferation was determined by ANOVA, followed by Tukey-Kramer HSD post hoc multiple comparison tests. All statistical analyses performed using Jmp 7.0 (SAS Institute, Inc., Cary, NC, USA).

Results

Culturing of acutely-dissociated mammalian retinal cells is a well-established experimental paradigm, resulting in the generation of clones of similar size and composition to those generated in vivo (Cayouette et al., 2003). Such cultures are routinely used in the study of mitogen responsiveness and other developmental behaviors of mammalian retinal cells. Previously, we demonstrated that SHH-N addition to P0 *orJ* retinal explants was sufficient to enhance RPC proliferation and the expression of Hh pathway target genes (Sigulinsky et al., 2008). Expecting similar responses in dissociated P0 *orJ* retinal cells, we were surprised to observe that SHH-N addition failed to elicit a proliferative response (Figure 3.1). Furthermore, expression of the Hh target genes *Ccnd1*, *Gli1*, *Hhip*, *Ptch1* and *Ptch2* were only minimally enhanced, if at all, in response

to SHH-N (Figure 3.2A), indicating impaired responsiveness of *orJ* cells to SHH-N stimulation. These findings are in stark contrast to the robust proliferative and transcriptional responses observed in *orJ* retinal explants within similar or shorter culture periods (Sigulinsky et al., 2008). Dissociated P0 wild type cells responded robustly to SHH-N treatment both at the level of proliferation (Figure 3.1) and Hh target gene expression (Figure 3.2A) under identical culture conditions, suggesting that the dissociation procedure and culture conditions are compatible with SHH-N responsiveness. To address whether differential effects of SHH-N addition on the RPC population underlie the difference in responsiveness, we examined expression of *Vsx2* and *Smo*. The mutation in the *orJ* allele creates a premature stop codon in the *Vsx2* gene and the resulting truncated protein is not detected, but *Vsx2* mRNA is still strongly expressed and provides a reliable marker of RPCs. Smo is required for transduction of Hh signals, but is not a transcriptional target of Hh signaling nor is it affected by loss of Vsx2 function (Sigulinsky et al., 2008), providing a reliable marker of Hh-responsive RPCs. Expression levels of *Vsx2* and *Smo* were unchanged in response to SHH-N addition and not significantly different between wild type and *orJ* dissociated retinal cells (Figure 3.2B), suggesting that the difference in SHH-N responsiveness between wild type and *orJ* cells was not likely due to SHH-N-dependent changes in the RPC population or Smo expression.

To better understand the dynamics of Hh responsiveness in dissociated cell culture, we examined the temporal profile of Hh target gene expression in dissociated wild type cells (Figure 3.3). RNA was collected from freshly dissected retina (tissue), from dissociated retinal cells at the time of plating (-1 hour), at the time of SHH-N

addition (0 hour), and 4, 8, and 12 hours after SHH-N addition. Expression levels were normalized to the expression levels in dissected wild type retinal tissue, which was set at 100% and served as the reference expression level. In general, Hh target gene expression declined in the period between tissue isolation and SHH-N addition, but expression levels stabilized in the cultures exposed to SHH-N. In contrast, target gene expression in the cultures without SHH-N (control) continued to decline. These observations suggest SHH-N treatment did not enhance the magnitude of Hh signaling in wild type cells, but prevented its further decline.

We then compared the gene expression profiles between dissociated wild type and *orJ* cells. Because of the severe hypocellularity of the *orJ* retina and, therefore, the limited availability of *orJ* cells (Green et al., 2003), we restricted our analyses to a single time point, 8 hours after SHH-N addition. By this time, gene expression levels had stabilized in both the control and SHH-N treated wild type cultures (Figure 3.3). Comparison of the wild type and *orJ* control cultures 8 hours after SHH-N addition (Figure 3.4) revealed that they reached an equivalent, low level of Hh target gene expression, consistent with minimal Hh signaling. However, the levels of Hh target gene expression differed considerably between wild type and *orJ* cultures treated with SHH-N, with levels in *orJ* cultures much reduced. The expression levels of *Gli1* and *Ptch2* were modestly elevated in the SHH-N treated *orJ* cultures compared to the *orJ* control, suggesting a limited, but detectable responsiveness to SHH-N, which is more easily seen by calculating their relative fold change in expression (Figure 3.2A). Comparison of *Vsx2* expression 8 hours after SHH-N addition revealed reduced levels in control wild type cultures and both control and SHH-N treated *orJ* cultures, while *Smo* expression declined

similarly in all conditions and genotypes (Figure 3.5). Reduced levels of *Vsx2* and *Smo* are suggestive of a decline in the progenitor population in these cultures. However, the lack of consistent differences between control and SHH-N treated cultures and the presence of a significant progenitor population at the end of culture suggests that the change or lack of change in Hh target gene expression between control and SHH-N treated cultures likely reflects the responsiveness of wild type and *orJ* retinal cells to SHH-N addition.

The diminished response of dissociated *orJ* retinal cells to SHH-N, relative to dissociated wild type cells, is consistent with a change in the Hh pathway that impairs the responsiveness of *orJ* retinal cells to Hh pathway stimulation. As mentioned above, this is in stark contrast to explant cultures where *orJ* cells respond robustly to SHH-N treatment, even surpassing the levels of Hh target gene expression observed in the *orJ* retina in vivo (Sigulinsky et al., 2008). It was therefore unclear what hindered the ability of dissociated *orJ* RPCs to respond to SHH-N. To determine whether disruption occurred at the level of Hh ligand reception or within the intracellular portion of the pathway, we sought to stimulate Hh signaling with purmorphamine, a Hh pathway agonist that binds to and activates Smo in a ligand- and receptor-independent manner (Sinha and Chen, 2006; Wu et al., 2004). Overall, purmorphamine elicited responses similar to SHH-N. In explant cultures of wild type P0 retina, the expression levels of Hh target genes were maintained relative to the in vivo expression level in response to purmorphamine addition, and in P0 *orJ* explants, purmorphamine stimulated increased expression of Hh target genes, surpassing the in vivo expression levels of the *orJ* retina (Figure 3.6). In dissociated cell cultures, purmorphamine enhanced proliferation in a dose-dependent

fashion over control levels in P0 wild type cells, but P0 *orJ* cells failed to exhibit a proliferative response (Figure 3.7). The response of dissociated P0 wild type cells to purmorphamine addition limited the decline or maintained expression of Hh target genes, while P0 *orJ* cells failed to respond or exhibited only a modest transcriptional response to purmorphamine treatment compared to control cultures (Figure 3.8).

The diminished response of dissociated *orJ* cells to purmorphamine suggested that a disruption in the Hh pathway exists at the level of, or downstream of, Smo. Because growing evidence suggests that Smo activity is restricted to the primary cilium in mammalian cells (Goetz and Anderson, 2010; Huangfu and Anderson, 2006; Rohatgi and Scott, 2007; Ryan and Chiang, 2012), we asked whether dissociation caused a deficit of primary cilia in *orJ* cells. To assess this, we stained dissociated cells with antibodies against γ-tubulin and either acetylated α-tubulin or Arl13b one hour after plating (0 hour). γ-tubulin marks the basal body, the site of nucleation and base of the primary cilium (Han et al., 2009). Acetylated α-tubulin is enriched in the ciliary axoneme and commonly used as a marker of the primary cilium in both neural and nonneural cell types (Han et al., 2009; Milenkovic et al., 2009; Rohatgi et al., 2007). However, in dissociated retinal cells, acetylated α-tubulin often stained the entire cell and its processes (data not shown), resulting in limited usefulness for the identification of a cilia deficit in dissociated *orJ* cells. Arl13b is a small GTPase belonging to the Arf/Arl family that is required for ciliogenesis and specifically enriched in the ciliary axoneme, colocalizing with acetylated α-tubulin and adjacent to γ-tubulin in the primary cilium of diverse cell types (Caspary et al., 2007; Hori et al., 2008). Using these markers, we still observed staining patterns that were consistent with the presence of primary cilium on both wild type and *orJ* cells

following dissociation (Figure 3.9). These results suggest that the primary cilium was not uniquely sensitive to the dissociation paradigm in the *orJ* cells. However, presence of the primary cilium in *orJ* cells does not rule out a problem with Smo regulation or activity.

It also remains possible that disruption of Hh signaling occurs further downstream of Smo. cAMP-dependent protein kinase A (PKA) is a potent inhibitor of Hh signaling, promoting proteolytic processing of the Gli proteins into transcriptional repressors and blocking (in a dominant fashion) Hh- and Smo-induced disassembly of SuFu-Gli complexes and subsequent nuclear translocation and formation of Gli-A complexes (Humke et al., 2010; Riobo and Manning, 2007; Tempe et al., 2006; Tukachinsky et al., 2010). Consistent with a dominant role for PKA-mediated inhibition of Hh signaling, increasing PKA activity blocks SHH-N-induced patterning of somites and proliferation of RPCs and purmorphamine-induced osteogenesis in multipotent mesenchymal progenitors (Fan et al., 1995; Jensen and Wallace, 1997; Wu et al., 2004). Additionally, a dominant negative form of PKA mimics the effects of ectopic Shh on patterning of the neural tube (Epstein et al., 1996), indicating that PKA-mediated inhibition is important for modulating Hh signaling activity in vivo. We therefore asked whether Hh signaling in dissociated *orJ* retinal cells could be enhanced by inhibiting PKA activity. To test this, we treated dissociated P0 *orJ* cells with purmorphamine in combination with H89, an isoquinolinesulfonamide that is highly selective for PKA and blocks its kinase activity by competing with ATP for the ATP binding pocket (Engh et al., 1996). In the presence of H89, Hh signaling was enhanced as revealed by approximately 2.5 fold increases in *Gli1* and *Hhip* expression compared to *orJ* cells treated with purmorphamine alone. Furthermore, these enhanced expression levels were roughly equivalent to that observed

in vivo in the *orJ* retina (Figure 3.10A-C). This observation suggests that Gli protein activation and nuclear translocation events are largely intact in *orJ* cells. Preliminary data indicate that H89 elicits only a minimal transcriptional response on its own, well below the level of target gene induction stimulated by the combination of H89 and purmorphamine (Figure 3.10D). Although still a preliminary finding, this suggests that the observed transcriptional response largely depends on Hh pathway stimulation through purmorphamine or SHH-N, and not H89 alone. Confirmation of these results would suggest that dissociated *orJ* cells respond, at least to some degree, to Hh pathway activation, even below Smo. Although such a finding implicates disruption of the Hh pathway at the level of PKA regulation or upstream of PKA input, it does not exclude a problem with Smo regulation or impairment of the pathway between Smo and PKA, as reduced pathway activation coupled with reduced PKA inhibition could also explain the current observations if resting levels of PKA activity are sufficient to largely counteract a low level of pathway activation. Distinguishing between these possibilities will require additional experimentation.

Because the dissociation paradigm is not expected to exert differential effects on wild type and *orJ* cells, we reasoned that perhaps the difference in responsiveness occurred as a result of delayed restoration of a limiting factor that was diminished upon dissociation, possibly due to a general lower rate of biosynthesis in *orJ* cells. To test this possibility, we allowed *orJ* cells to recover for 24 hours following dissociation before stimulating the Hh pathway with purmorphamine. During this postdissociation recovery period, *orJ* cells were cultured only in the presence of nonmitogenic concentrations of FGF-2 to promote survival. Purmorphamine was then added at 24 hours to stimulate the

Hh pathway. Although purmorphamine treated cultures exhibited a 10 fold increase in *Gli1* expression relative to controls, comparison of expression levels at the end of culture to in vivo tissue levels revealed that Hh target gene expression had continued to decline, despite the addition of purmorphamine (Figure 3.11; compare to Figure 3.8). Thus, the recovery period failed to restore the competence of *orJ* cells to respond to Hh pathway activation.

To begin to evaluate the potential contribution of cell death to our findings, we measured the extent of apoptosis in dissociated cultures using an early apoptosis marker, caspase-3. Preliminary findings reveal an elevated frequency of apoptosis in *orJ* cultures relative to wild cultures at 24 hours irrespective of Hh pathway stimulation, as indicated by an increased proportion of caspase-3-positive cells in all treatment groups (Figure 3.12A). However, only minimal levels (<1%) of apoptosis were observed in dissociated *orJ* cultures at 8 hours (Figure 3.12B).

Interpretation and future directions

Here, we show that dissociation results in severely diminished responsiveness of *orJ* retinal cells to Hh pathway stimulation. Dissociated *orJ* retinal cells failed to exhibit a proliferative response to treatment with recombinant Shh ligand or Smo agonist, which correlated with weak or absent maintenance of Hh target gene expression. This is in contrast to wild type retinal cells, which retain responsiveness to Hh stimulation after dissociation, exhibiting transcription of Hh target genes and proliferation of RPCs in response to recombinant Shh ligand and Smo agonist.

The robust responsiveness of *orJ* cells to Hh pathway stimulation in retinal explant cultures [(Sigulinsky et al., 2008) and this study] reveals that the core signal transduction cascade is largely intact in the absence of Vsx2 function. This finding raises concern regarding whether the loss of responsiveness in dissociated *orJ* cells reflects a specific role for Vsx2 in the regulation of Hh pathway activity versus a secondary or nonspecific effect of the dissociation procedure on *orJ* retinal cells. Restoration of Hh responsiveness by transfection of full-length Vsx2 would suggest that absence of Vsx2 is responsible for the altered responsiveness and rule out possible effects of the dissociation procedure that are not Vsx2-specific. However, this finding does not address whether Vsx2 function is directly required for regulation of Hh pathway activity, as roles in processes that influence the RPC population or metabolic activity would indirectly affect Hh responsiveness. Because a postdissociation recovery period of 24 hours failed to restore the competence of *orJ* retinal cells to respond to Hh pathway stimulation, it is unlikely that a role in promoting metabolic activity fully explains the diminished responsiveness in dissociated *orJ* retinal cells.

Reduction of the Hh-responsive cell population (RPCs) during the culture period could mimic loss of Hh responsiveness. Two obvious mechanisms that could cause reductions in the RPC population are precocious differentiation into neurons or cell death. The former possibility is particularly unlikely, since *orJ* cells exhibit delayed neurogenesis in vivo, and exposure to Shh, at least at earlier stages, fails to promote premature initiation of neurogenesis (Bone-Larson et al., 2000; Robb et al., 1978; Rutherford et al., 2004; Sigulinsky et al., 2008). Furthermore, in wild type retinas, precocious cell cycle exit is associated with reduced Shh (Wang et al., 2005). A role for

Vsx2 in promoting RPC survival is lacking. Although one of the earliest phenotypes reported in the *orJ* retina was the absence of a normal early burst of morphogenetic cell death (Theiler et al., 1976), this was later shown to be simply delayed (Robb et al., 1978), consistent with the overall delayed development in the *orJ* retina (Bone-Larson et al., 2000; Robb et al., 1978; Rutherford et al., 2004; Sigulinsky et al., 2008). Furthermore, at P0, *orJ* retinal cells do not exhibit elevated levels of cell death (Green et al., 2003). Despite a lack of propensity towards increased cell death in vivo, preliminary data suggest that *orJ* cells, as a population, are more susceptible to apoptotic cell death in dissociated cell cultures. Cell death is not an unexpected finding in dissociated retinal cell cultures. In the absence of target-derived trophic factors, ganglion cells survive poorly in culture, even in retinal explants, with significant reductions observed within the first 24 hours (Jensen and Wallace, 1997; Wang et al., 2002). Thus, it is not clear whether the elevated levels of apoptotic cell death in *orJ* cultures is indicative of reductions in the RPC population. Further analysis of the apoptotic population with cell type- and progenitor-specific markers will be required to assess this possibility.

The relative contribution of cell death to diminished Hh responsiveness could be assessed through experimental manipulations that promote RPC survival. Extrinsic signals may act as trophic factors at nonmitogenic concentrations (Reh et al., 1996). In the present study, nonmitogenic concentrations of FGF-2 were added in the recovery experiments to promote survival, but resulted in little to no effect on the restoration of Hh responsiveness in dissociated *orJ* retinal cells. However, cell death was not assayed in these cultures to confirm whether survival was indeed enhanced. Alternatively, the effects of blocking apoptosis on restoration of Hh responsiveness in dissociated *orJ*

retinal cells could be tested. Genetic deletion of p53 in mice blocks normal developmental apoptotic neuronal loss in the hippocampus (Murase et al., 2011) and protects against apoptotic neuronal loss triggered by kainic acid-induced epileptic seizures, allowing recovery from damage that normally promotes death (Kinoshita et al., 2012).

In an effort to directly assess the RPC population, we examined expression of the RPC markers Vsx2 and Smo. Neither is a known target of Hh signaling, and their expression was not significantly different between SHH-N treated and control cultures (Figure 3.2B). Differences in *Vsx2* and *Smo* expression were not detected between wild type and *orJ* retinas (Sigulinsky et al., 2008), suggesting that they are also not targets of Vsx2 transcriptional regulation. Thus, changes in *Vsx2* and *Smo* levels reflect changes in the relative contribution of RPCs to the total cell population. Although *Vsx2* exhibited reduced expression in control wild type and both control and SHH-N treated *orJ* cultures, *Smo* expression declined similarly in both genotypes and across all treatment conditions. Furthermore, the elevated cell death observed in dissociated *orJ* retinal cells at 24 hours was independent of purmorphamine concentration or the presence of DMSO. Thus, it appears that neither Vsx2 nor Hh signaling promoted changes in the proportions of the RPC population, consistent with previous findings that SHH-N and Vsx2 and do not promote cell survival or death, respectively (Green et al., 2003; Levine et al., 1997). These findings also argue against the possibility that specific changes in the RPC population account for the observed differences in Hh responsiveness. In the absence of altered proportions of responsive cells, a global loss of *orJ* cells in culture, relative to wild type, could also mimic loss of responsiveness. However, such a loss was not

obvious upon visual inspection of the cultures, and similar numbers of total cells (DAPI-positive) were counted per field of view in wild type and *orJ* cultures at 24 hours when random field analysis was used to quantify proliferation (data not shown). Given the progenitor proportions, as indicated by *Vsx2* and *Smo* expression, a significant progenitor population is present, even at the end of the culture period. Together, these findings argue that despite elevated apoptosis in *orJ* cultures, changes in the RPC population are unlikely to fully account for the diminished Hh responsiveness observed for dissociated *orJ* retinal cells.

Assuming the diminished responsiveness of dissociated *orJ* cells reflects a specific role for Vsx2 in the regulation of Hh pathway activity, where does this regulation intersect with the Hh pathway? Using pharmacological agents to manipulate Hh signaling at different levels in the pathway, we have narrowed the localization of this disruption to an intracellular region of the Hh pathway. Although some findings are preliminary and will require further confirmation, we believe that the primary disruption occurs at or between the levels of Smo activity and PKA regulation. It is important to note, however, that because purmorphamine activates Smo in the absence of Hh ligand and independently of the Hh receptor Ptch1 (Sinha and Chen, 2006; Wu et al., 2004), we are currently unable to rule out any additional deficits in Hh ligand reception. Additionally, our observation that the diminished responsiveness manifests upon perturbation of the extracellular environment suggests misregulation of an environmentally-sensitive component of the Hh pathway. Based on these observations, the most likely candidates within the implicated region of the Hh pathway are PKA and Smo.

PKA is a critical and dominant negative regulator of the Hh pathway. Increased activation is sufficient to block the effects of Hh pathway activation when the activation is ligand or Smo based (Fan et al., 1995; Jensen and Wallace, 1997; Wu et al., 2004). Additionally, activity of PKA is both positively and negatively influenced by extrinsic signals. PKA activity is negatively regulated by PI3K through Akt, and activation of the PI3K/Akt pathway by growth factor signaling sensitizes cells to Hh pathway stimulation (Riobo et al., 2006b). Conversely, PKA activity is potentiated by increased cyclic AMP (cAMP) levels in response to increased production by adenylyl cyclase. Adenylyl cyclase is a common target of G-protein coupled receptor signaling activated by intercellular signals, such as hormones.

Is PKA overactive in dissociated *orJ* retinal cells? Consistent with this possibility, inhibition of PKA activity in dissociated *orJ* cells greatly enhanced their transcriptional response to Smo activation. Our preliminary findings that PKA inhibition alone had little to no effect on Hh signaling activity contradicts observations in zebrafish where a dominant negative form of PKA rescued Hh loss of function phenotypes in *Smo* mutants (Barresi et al., 2000), suggesting that perhaps PKA is not overactive in dissociated *orJ* retinal cells, as PKA inhibition alone was not sufficient to promote Hh target gene upregulation in dissociated *orJ* cells. However, it is unclear whether Hh pathway activation is truly absent in zebrafish *Smo* mutants. Although cloning and functional analyses suggest that the mutations in zebrafish *Smo* mutants are null mutations, maternal *Smo* contribution or duplication of the *Smo* gene (similar to the *Shh* gene duplication in zebrafish) has not been ruled out and may mediate the observed effects of PKA inhibition in *Smo* mutants and account for the milder phenotypes of these mutants compared to *Shh*

null mutations in mouse (Varga et al., 2001). In LIGHT cells, inhibition of PKA activity through IGF-1 activation of the PI3K/Akt pathway was not sufficient to promote Gli reporter activity (Riobo et al., 2006b), suggesting that derepression alone is insufficient to activate Hh signaling-dependent transcription and that pathway stimulation is required, at least in this cell line. H89 inhibits PKA kinase activity by competing with ATP for the ATP binding pocket (Engh et al., 1996), suggesting that the effects of H89 should be dose dependent; therefore, it is also possible only minimal inhibition was achieved in our cultures. Thus, future analyses should seek to confirm whether PKA is indeed overactive in dissociated *orJ* retinal cells. This could be assessed by comparing the dose-dependency of Hh target gene expression to H89 at constant purmorphamine or SHH-N concentration between dissociated wild type and *orJ* retinal cells and between dissociated and explant cultures of *orJ* retinal cells. Alternatively, PKA activity from tissue lysates could be measured directly (Goueli et al., 2001; Goueli et al., 1995) and several commercially available kits are available.

Smo also remains an attractive candidate for the site of pathway disruption in dissociated *orJ* cells. Reduced Smo activity, as a result of reduced levels or impaired function, would impair responsiveness of cells to Hh pathway activation. Reduced Smo levels resulting from altered transcriptional regulation is unlikely to mediate the disruption in dissociated *orJ* cells. Our previous analyses did not reveal any significant differences in either *Smo* expression or the relative proportions of Smo-expressing (RPC) and non-expressing (neuron) cell populations between wild type and *orJ* retinas (Green et al., 2003; Sigulinsky et al., 2008) suggesting that Smo transcription is unaffected by loss of Vsx2. Our current analyses also argue against transcriptional regulation, as changes in

Smo expression largely correlate with changes in the RPC population (as indicated by *Vsx2* expression) during culture across genotypes and treatment groups. Because Smo is a transmembrane protein, it is possible that incubation with the trypsin protease during the dissociation procedure limited the levels of functional Smo. However, Smo is present on the plasma membrane of both stimulated and unstimulated cells and then trafficked via lateral transport to the primary cilium (Milenkovic et al., 2009), making it unlikely that Smo would be differentially exposed to trypsin between *orJ* and wild type cells. Furthermore, dissociated wild type cells respond robustly to SHH-N, which requires the Hh receptor Ptch1, also a transmembrane protein, in addition to functional Smo, suggesting that it is unlikely trypsin treatment has a significant impact on the function of transmembrane proteins and the resulting responsiveness of cells to Hh stimulation. This could be confirmed by using gentler proteases during dissociation. For example, papain was found to be less damaging than trypsin and several other proteases for the dissociation of retinal cells from turtles (Lam, 1972). Alternatively, Smo protein integrity could be analyzed by ESI-MS to determine if the protein sequence is different than expected due to truncation or degradation by the protease. ESI-MS is particularly amenable to detailed profiling of transmembrane proteins (Souda et al., 2011).

A more likely scenario is reduced Smo function resulting from misregulation of Smo activation. Activation of Smo is tightly regulated and still poorly understood, but potentially sensitive to environmental perturbations. Inhibition of Smo activity by the Hh receptor Ptch1and its release by ligand binding to Ptch1 is not well defined but may involve local regulation of the concentrations of small molecules through Ptch1 acting as a pump (Rohatgi and Scott, 2007), but this has yet to be confirmed. Furthermore,

activation of Smo involves a conformational change induced via phosphorylation of its C-terminal tail by CK1α and GRK2 (Chen et al., 2011), which may also be targets of extrinsic signals, much like PKA. This phosphorylation also promotes localization of activated Smo to the primary cilium. Although the precise mechanism is still poorly defined, it involves lateral transport and dependence on β-arrestin and the kinesin motor Kif3b (Chen et al., 2011; Milenkovic et al., 2009). Because Smo activity correlates with its level of phosphorylation (Chen et al., 2011) and subcellular localization, these could be used to evaluate activation of endogenous Smo in dissociated retinal cells. Using an anti-Smo antibody (anti-SmoC, gift of M.P. Scott) that allows visualization of endogenous Smo in cultured mouse fibroblasts (Rohatgi et al., 2007), we have successfully observed endogenous Smo accumulation in acetylated α-tubulin-positive cilia of wild type retinas (data not shown). Using this antibody, activation of endogenous Smo could be evaluated based on localization in dissociated cell cultures. However, ciliary trafficking of Smo in response to purmorphamine has not been reported and we have not ruled out a deficit in Hh reception in dissociated retinal cells. Thus, it may be necessary to use an alternative Smo agonist that is known to promote both phosphorylation and ciliary localization of Smo, such as SAG (Chen et al., 2011) if ciliary localization is not observed in purmorphamine treated cultures of dissociated wild type cells. Endogenous Smo activation, could also be assessed by comparing the level of Smo phosphorylation in dissociated wild type and *orJ* retinal cells using Phos-tag PAGE (Chen et al., 2011) or ESI-MS (Souda et al., 2011).

If activation of endogenous Smo is insufficient to overcome the resting levels of PKA inhibition due to reduced levels or misregulation in dissociated *orJ* retinal cells,

then transfection with a constitutively active form of Smo should stimulate Hh signaling and Hh target gene expression. Because PKA regulation can be dominant, it will be important to first determine whether PKA is overactive in dissociated *orJ* cells, or include PKA inhibitors in these cultures. Smo-M2 is an oncogenic form that exhibits the active conformation, cilia localization, and activating phosphorylation in a Shh-independent fashion (Chen et al., 2011). Although Smo-M2 activity does not require Shh, it does require CK1α and GRK2 phosphorylation (Chen et al., 2011). Thus, if Smo-M2 transfection fails to stimulate Hh signaling in dissociated *orJ* retinal cells, this may indicate a deficit in the phosphorylation activity of CK1α and GRK2, and the phosphorylation state of Smo should be assessed. If purmorphamine stimulates the active Smo conformation in dissociated *orJ* retinal cells, but phosphorylation is deficient, then co-transfection of CK1α and GRK2 should restore Hh signaling (Chen et al., 2011).

Establishing the nature of the disruption occurring in dissociated *orJ* cells will provide significant direction for future efforts aimed at identifying the mechanism of Vsx2-mediated regulation of Hh responsiveness. Evaluation of PKA activity levels and the ability of constitutively active Smo to restore Hh responsiveness in *orJ* cells will be important first steps in evaluating potential targets of this regulation. However, a critical unanswered question remains: Why does this disruption only manifest upon dissociation? The contrasting responsiveness of *orJ* retinal cells to Hh pathway stimulation in dissociated versus explant cultures suggests that loss of responsiveness in dissociated *orJ* cells is linked to the disruption of intercellular interactions. Currently, an attractive model to account for this observation is that extrinsic signals mediating intercellular interactions important for Shh-induced retinal proliferation become limiting

upon dissociation and *orJ* cells are more sensitive as a result of Vsx2-dependent changes in the production of or responsiveness to these signals. In retinal explants, dilution of extrinsic signals is minimal and a limited impairment of Hh responsiveness in *orJ* cells may be masked by dominating sensitization of *orJ* cells to Hh stimulation that results from prolonged exposure to reduced ligand availability. Feedback inhibition is a prominent feature of the Hh signaling pathway and several negative regulators of Hh signaling that are also Hh target genes, exhibit reduced expression in the *orJ* retina (Sigulinsky et al., 2008), which may underlie this sensitization. Proliferation in retinal cells is particularly sensitive to perturbations of the extracellular environment, setting precedence for such a model mechanism. Retinal cells cultured as intact explants exhibited significantly greater BrdU incorporation than when cultured as low density monolayers (Lillien and Cepko, 1992). BrdU incorporation could be increased in these monolayer cultures simply by increasing plating density, suggesting that intercellular interactions are important for proliferation of retinal cells. Moreover, the proliferative response of retinal cells to SHH-N treatment is also sensitive to intercellular interactions. SHH-N was a more potent retinal mitogen when dissociated retinal cells were re-aggregated into pellet cultures rather than cultured as monolayers (Jensen and Wallace, 1997). Although it is not clear whether these intercellular interactions are required for optimal Hh signaling activity in retinal cells or simply to promote proliferation downstream of the Hh pathway, comparison of the transcriptional response in *orJ* retinal cells to Hh pathway stimulation in pellet versus low density monolayer culture would allow evaluation of the above-mentioned model by providing insight into whether the diminished responsiveness of dissociated *orJ* retinal cells results from disrupted

intracellular interactions. Alternatively, the ability of increased plating density to improve the transcriptional response of dissociated *orJ* retinal cells in our low density monolayer cultures could also be examined.

As previously mentioned, both Smo and PKA are regulated by mechanisms that are themselves downstream of extrinsic signals, including the PI3K/Akt and adenylyl cyclase/cAMP pathways. An important addition to this list is the MEK/ERK (MAPK) pathway. Activation of MAPK signaling sensitizes NIH 3T3 cells to Hh pathway stimulation, while inhibition renders these cells unresponsive (Riobo et al., 2006a). MAPK-mediated regulation of Hh responsiveness in NIH 3T3 cells was initiated indirectly by activation of PKCδ or directly through activation of MEK-1, suggesting that a number of extrinsic signals would have the potential to exert such regulation through their activation of G-protein coupled receptors or receptor tyrosine kinases and demonstrated by the ability of FGF2 to stimulate Gli reporter activity in NIH 3T3 cells in a MEK-1-dependent fashion (Riobo et al., 2006a). The mechanism by which MAPK signaling regulates Hh responsiveness is still unclear, but promotes Gli transcriptional activity. Recent evaluation of the relationship between Hh signaling and the MAPK pathway in gastric cancer revealed that MAPK signaling promoted Hh signaling without affecting the level of Shh expression (Seto et al., 2009). Furthermore, MAPK-induced sensitization could be blocked by overexpression of SuFu, but not cyclopamine-mediated inhibition of Smo, suggesting that MAPK signaling intersects with the Hh pathway between Smo and SuFu. This localization of MAPK activity is consistent with the current localization of the disruption observed in dissociated *orJ* retinal cells. Thus, an intriguing possibility is that the diminished responsiveness of dissociated *orJ* retinal cells occurs as

a result of reduced MAPK signaling. Evaluation of readouts for MAPK signaling, such as phosphorylation of ERK, by immunocytochemistry or western blotting, could confirm reduced MAPK signaling in dissociated *orJ* retinal cells (Fogarty et al., 2007). The ability of activated MAPK signaling to restore Hh responsiveness in dissociated *orJ* retinal cells could be tested using a number of methods, including transfection of activated MEK-1, KRAS (constitutively active Ras) or BRAF, activation of PKCδ by phorbol esters, or stimulation with growth factors (Riobo et al., 2006a; Seto et al., 2009). Interestingly, in granule cell precursors of the cerebellum, FGF2 blocks Hh signaling through activation of MAPK signaling (Fogarty et al., 2007). Thus, it will be important to determine whether MAPK activation promotes or inhibits Hh signaling in retinal cells and the relevant extrinsic signals mediating this activation.

The data presented here suggests a potential role for Vsx2 in the regulation of RPC responsiveness to Hh pathway stimulation. Whether this role is relevant in vivo remains to be determined, as reduced responsiveness of *orJ* cells to Hh pathway stimulation is only revealed upon dissociation. Currently, it is unclear why *orJ* cells are more sensitive to the dissociation paradigm, but an intriguing possibility is that it may reflect alterations in extrinsic signals required for the proliferative response of retinal cells to Shh. Thus, although the principal limiting factor for Hh signaling in vivo may be reduced ligand availability, determining the nature of the disruption in dissociated *orJ* cells may still provide insight not only into Vsx2 function, by identifying candidate transcriptional targets, but also into factors required for promoting Hh signaling and proliferation in retinal cells.

Figure 3.1. Diminished proliferative response of *orJ* RPCs to SHH-N in dissociated cell cultures. Dissociated P0 retinal cells from wild type (A-D) and *orJ* (E-H) mice (maintained on the Black Swiss background) were cultured in the absence (A,B,E,F) or presence (C,D,G,H) of SHH-N for 48 hours with BrdU present during the last 4 hours. (A,C,E,G) BrdU immunoreactivity. (B,D,F,H) DAPI counterstain. (I) Quantification of the proportion of BrdU-labeled cells. Each bar represents the mean ± standard error of the mean (SEM). *p*-values calculated using Student's unpaired *t*-test. $n = 3$ for each genotype.

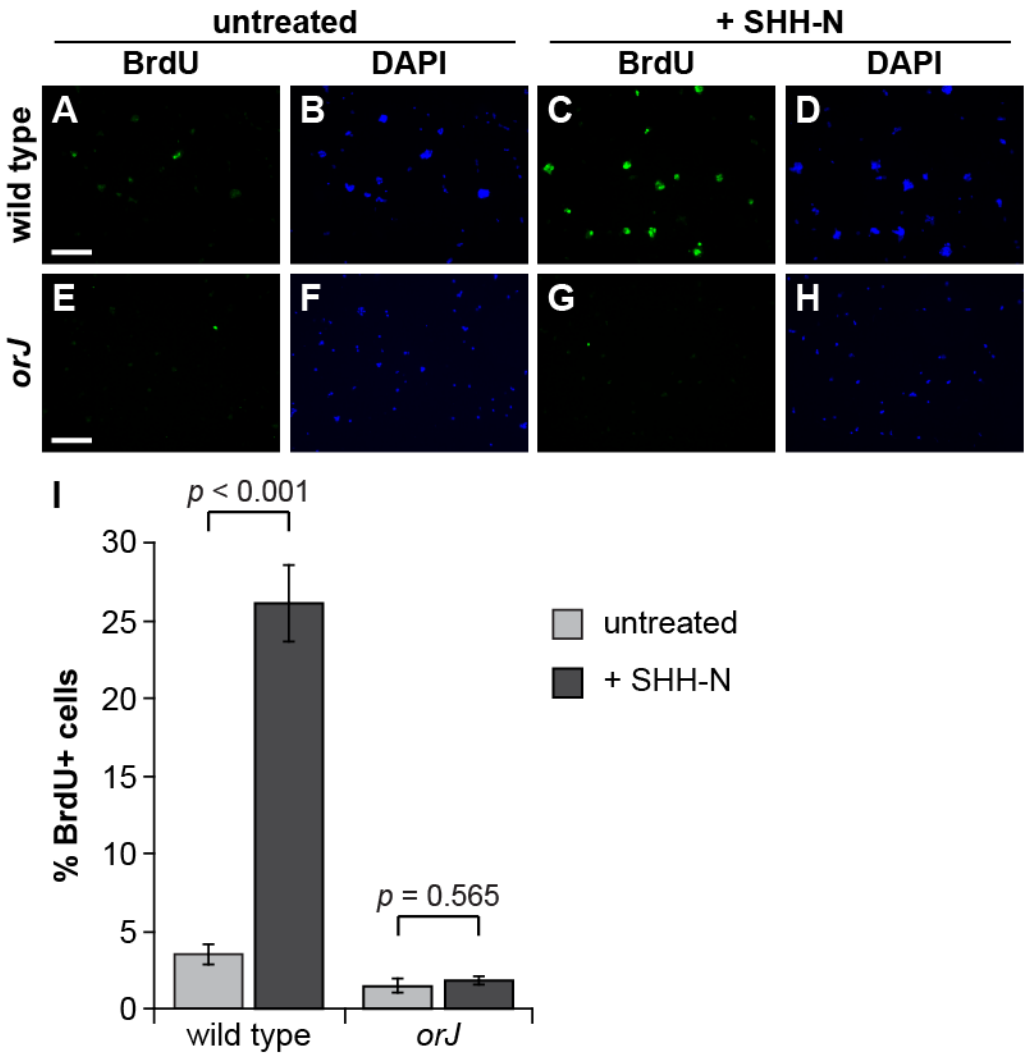

untreated
+ SHH-N
BrdU
DAPI
BrdU
DAPI
wild type
orJ
A
B
C
D
E
F
G
H
I
p < 0.001
p = 0.565
% BrdU+ cells
30
25
20
15
10
5
0
wild type
orJ
untreated
+ SHH-N

Figure 3.2. Diminished transcriptional response of *orJ* RPCs to SHH-N in dissociated cell cultures. P0 dissociated retinal cells were cultured in the presence or absence of SHH-N for 8 hours. (A) Fold change in Hh target gene expression due to SHH-N treatment. (B) Fold change in control gene (progenitor markers) expression following SHH-N treatment. Each bar represents the mean ± standard deviation (SD) in (A) and the mean ± SEM in (B). *p*-values calculated using Student's unpaired *t*-test. $n = 3$ for each genotype.

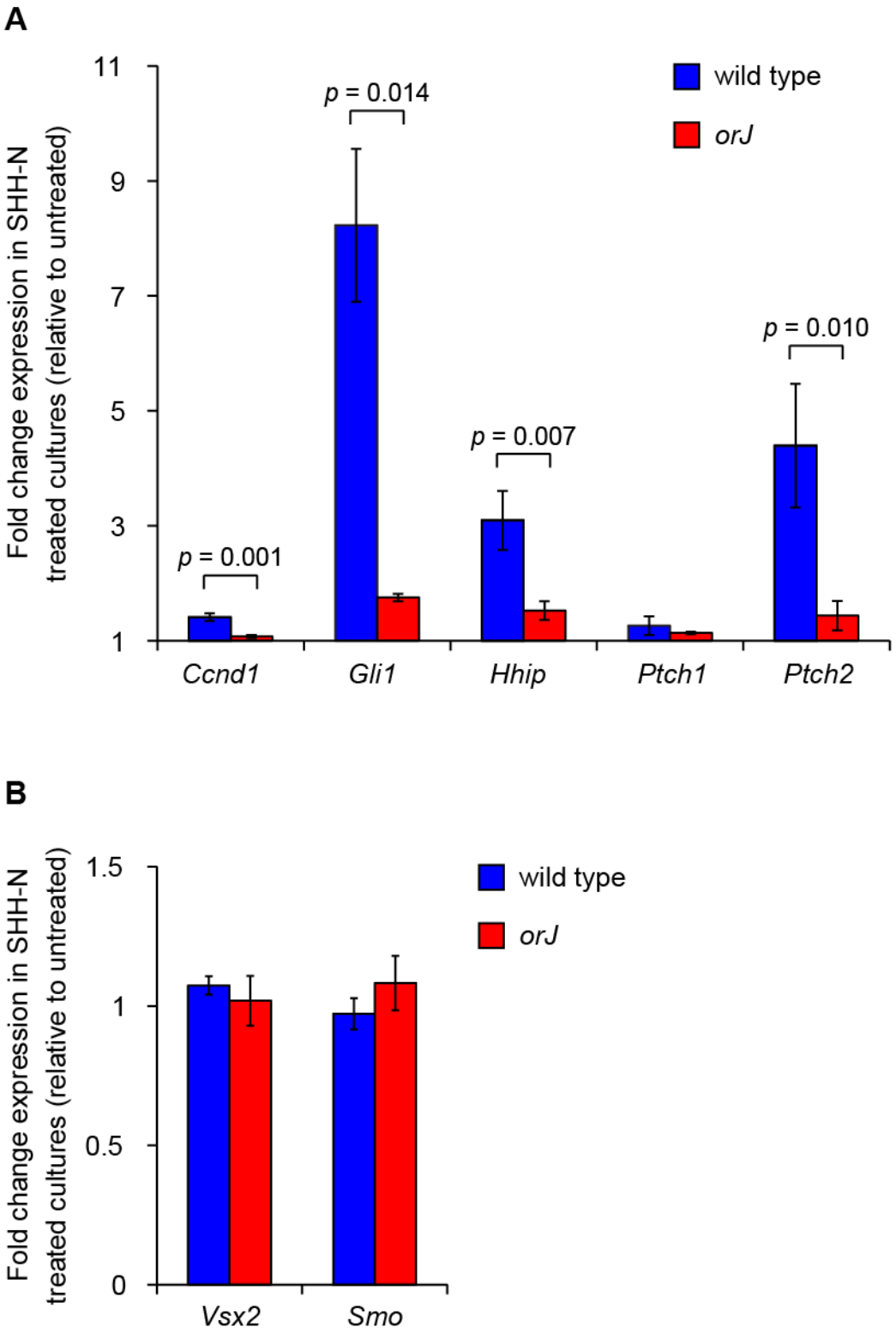

A
Fold change expression in SHH-N treated cultures (relative to untreated)
11
9
7
5
3
1
wild type
orJ
p = 0.014
p = 0.001
p = 0.007
p = 0.010
Ccnd1
Gli1
Hhip
Ptch1
Ptch2
B
Fold change expression in SHH-N treated cultures (relative to untreated)
1.5
1
0.5
0
wild type
orJ
Vsx2
Smo

Figure 3.3. Temporal profile of Hh target gene expression in dissociated wild type retinal cells. Relative expression levels of Hh target genes in P0 wild type retinal cells under the following conditions: freshly dissected retina (tissue), dissociated retinal cells at the time of plating (t = -1 hour), at the time of SHH-N addition (0 hour) and 4, 8, and 12 hours after SHH-N addition. Expression levels were normalized to the expression levels in freshly dissected retinal tissue, which was set at 100% and served as the reference expression level. Each point represents the mean ± SEM. *n* = 7 (tissue) or 3 (all other data points). Abbreviations: WT, wild type.

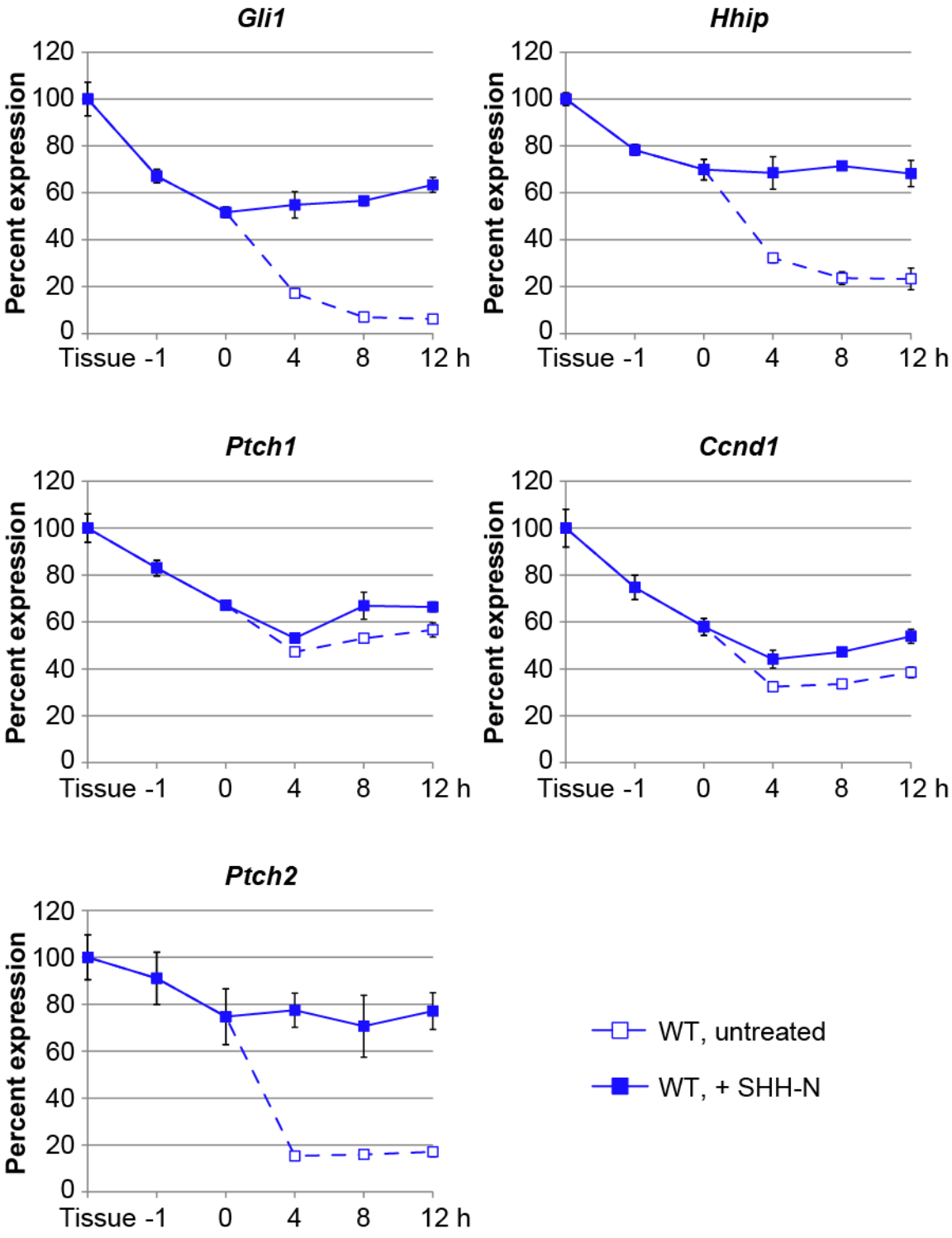

Gli1
Hhip
Ptch1
Ccnd1
Ptch2
Percent expression
120
100
80
60
40
20
0
Tissue -1
0
4
8
12 h
WT, untreated
WT, + SHH-N

Figure 3.4. Reduced ability of *orJ* RPCs to maintain Hh target gene expression in response to SHH-N in dissociated cell cultures. Comparison of the relative expression levels of Hh target genes in P0 wild type and *orJ* retinal cells 8 hours after SHH-N addition in dissociated cell cultures. Expression levels were normalized to the expression level in freshly dissected wild type retinal tissue, which was set at 100% and served as the reference expression level. Each point represents the mean ± SEM. $n = 7$ (tissue) or 3 (8 hours). Abbreviations: WT, wild type.

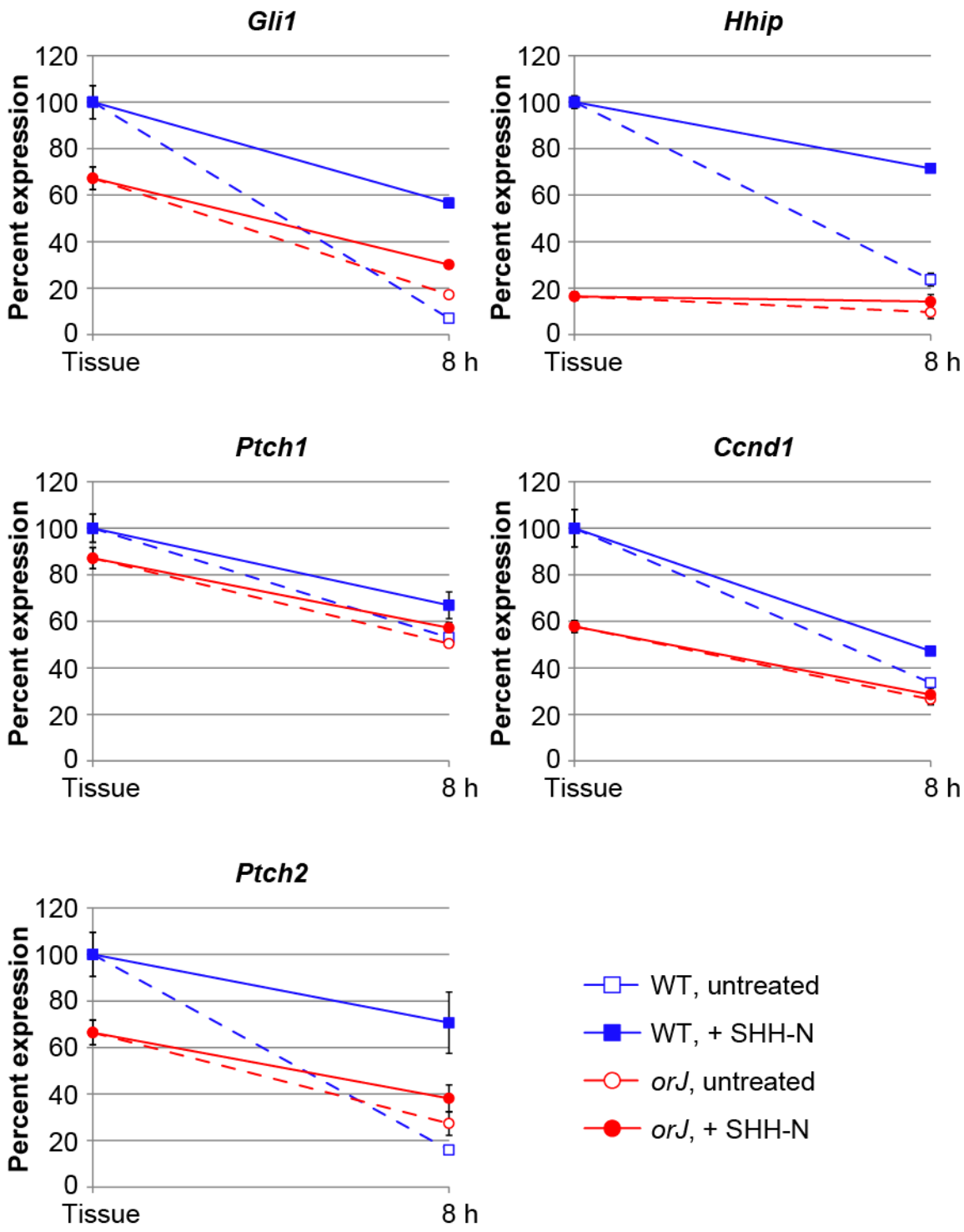

Gli1
Hhip
Ptch1
Ccnd1
Ptch2
Percent expression
120
100
80
60
40
20
0
Tissue
8 h
WT, untreated
WT, + SHH-N
orJ, untreated
orJ, + SHH-N

Figure 3.5. Minimal effects of SHH-N treatment or genotype on progenitor marker expression in dissociated cell cultures. Comparison of the relative expression levels of control genes in P0 wild type and *orJ* retinal cells 8 hours after SHH-N addition in dissociated cell cultures. Expression levels were normalized to the expression level in freshly dissected wild type retinal tissue, which was set at 100% and served as the reference expression level. Each point represents the mean ± SEM. $n = 4$ (tissue) or 3 (8 hours). Abbreviations: WT, wild type.

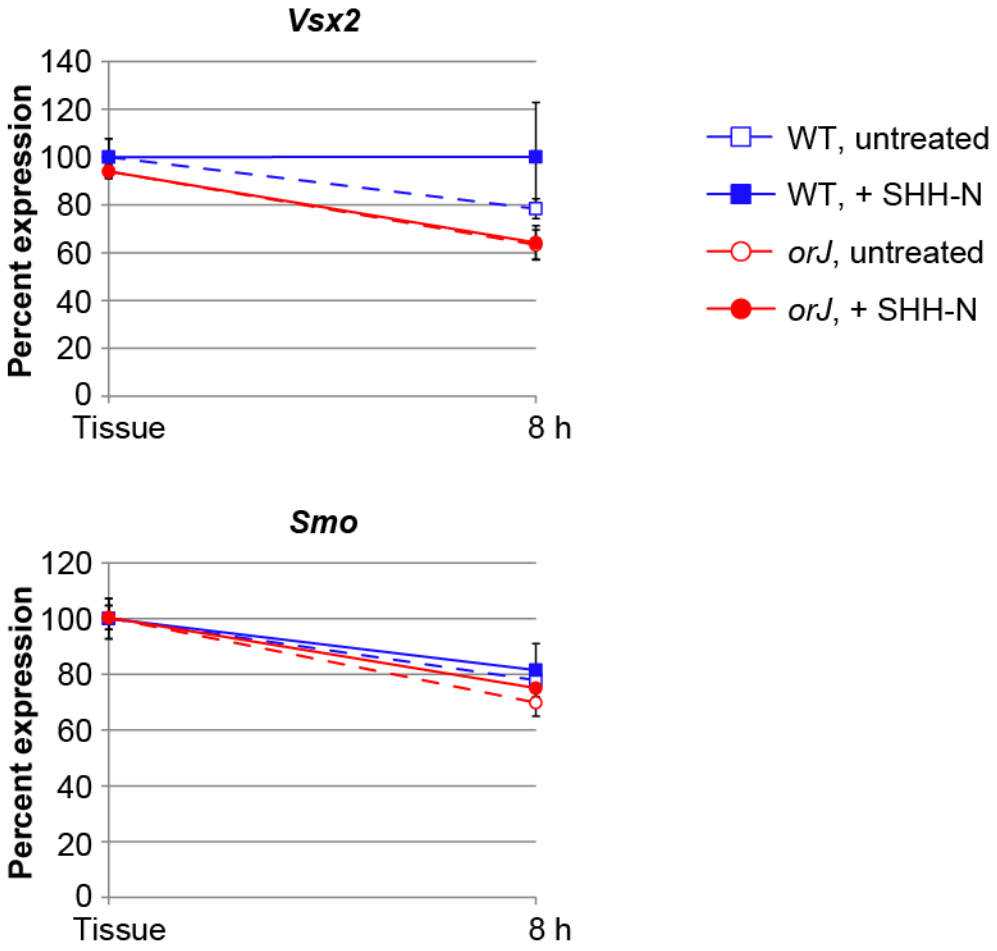

Vsx2
Percent expression
140
120
100
80
60
40
20
0
Tissue
8 h
WT, untreated
WT, + SHH-N
orJ, untreated
orJ, + SHH-N
Smo
Percent expression
120
100
80
60
40
20
0
Tissue
8 h

Figure 3.6. Purmorphamine stimulates robust expression of Hh target genes in retinal explant cultures. Comparison of the relative expression levels of Hh target genes (*Gli1* and *Hhip*) and control genes (*Smo*) in P0 wild type and *orJ* retinal cells 8 hours after purmorphamine addition in explant cultures. Expression levels were normalized to the expression level in freshly dissected wild type retinal tissue, which was set at 100% and served as the reference expression level. Each point represents the mean ± SEM. $n = 7$ (tissue, Hh target genes), 4 (tissue, *Smo*) or 3 (8 hours). Abbreviations: WT, wild type.

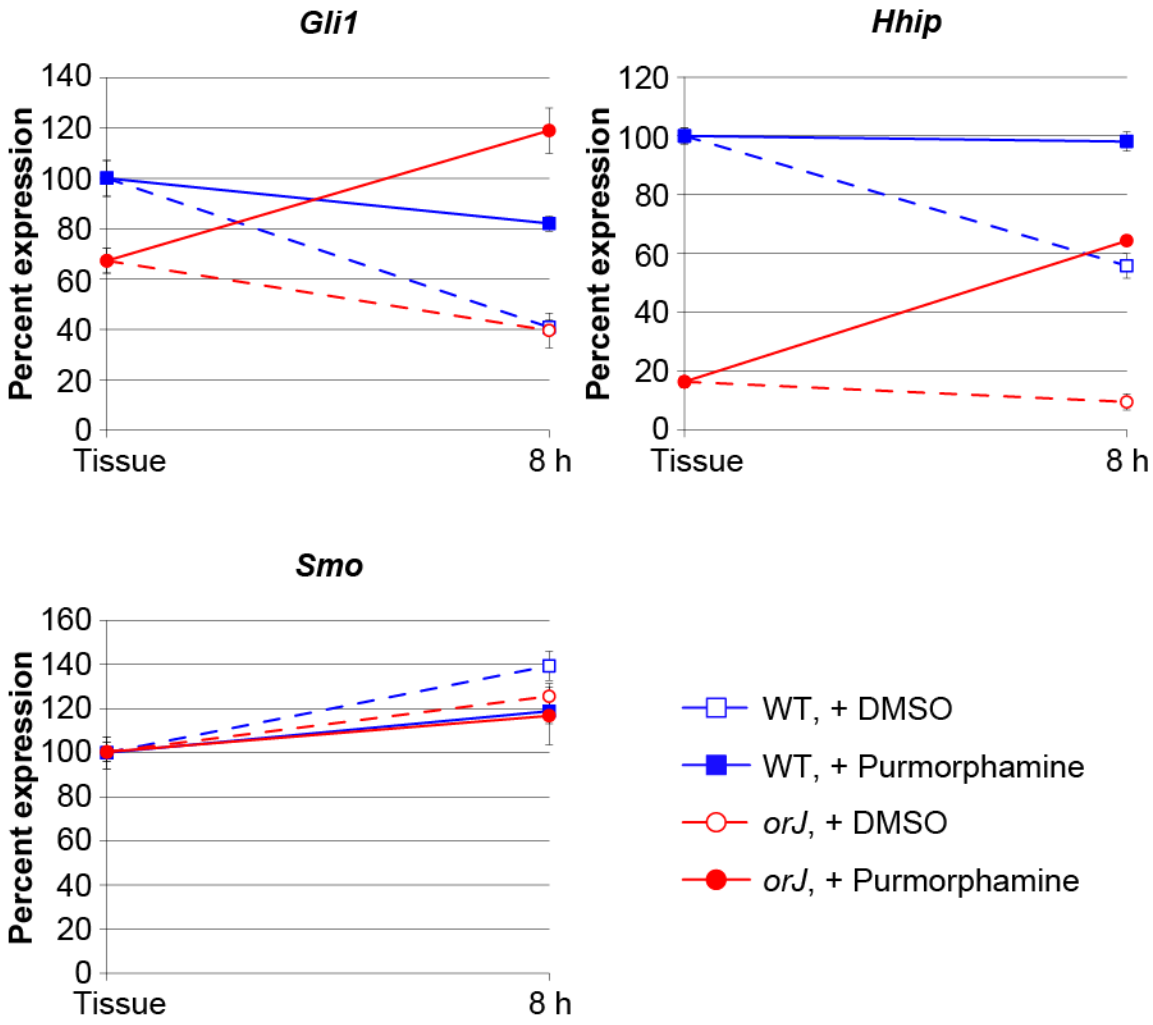

Gli1
Percent expression
140
120
100
80
60
40
20
0
Tissue
8 h
Hhip
Percent expression
120
100
80
60
40
20
0
Tissue
8 h
Smo
Percent expression
160
140
120
100
80
60
40
20
0
Tissue
8 h
WT, + DMSO
WT, + Purmorphamine
orJ, + DMSO
orJ, + Purmorphamine

Figure 3.7. Diminished proliferative response of *orJ* RPCs to purmorphamine in dissociated cell cultures. P0 dissociated retinal cells were cultured in the presence of purmorphamine (0.01, 0.1, 1, or 10 μM and 0.4% DMSO), DMSO alone (0.4%), or the absence of both purmorphamine and DMSO (untreated) for 24 hours with BrdU present for the last 2 hours. Graph represents quantification of the proportion of BrdU-labeled cells. Each bar represents the mean ± SEM. Statistical significance of purmorphamine effect on wild type cells determined by ANOVA, followed by Tukey-Kramer HSD post hoc multiple comparison tests. $n = 3$ (wild type), or as indicated (*orJ*).

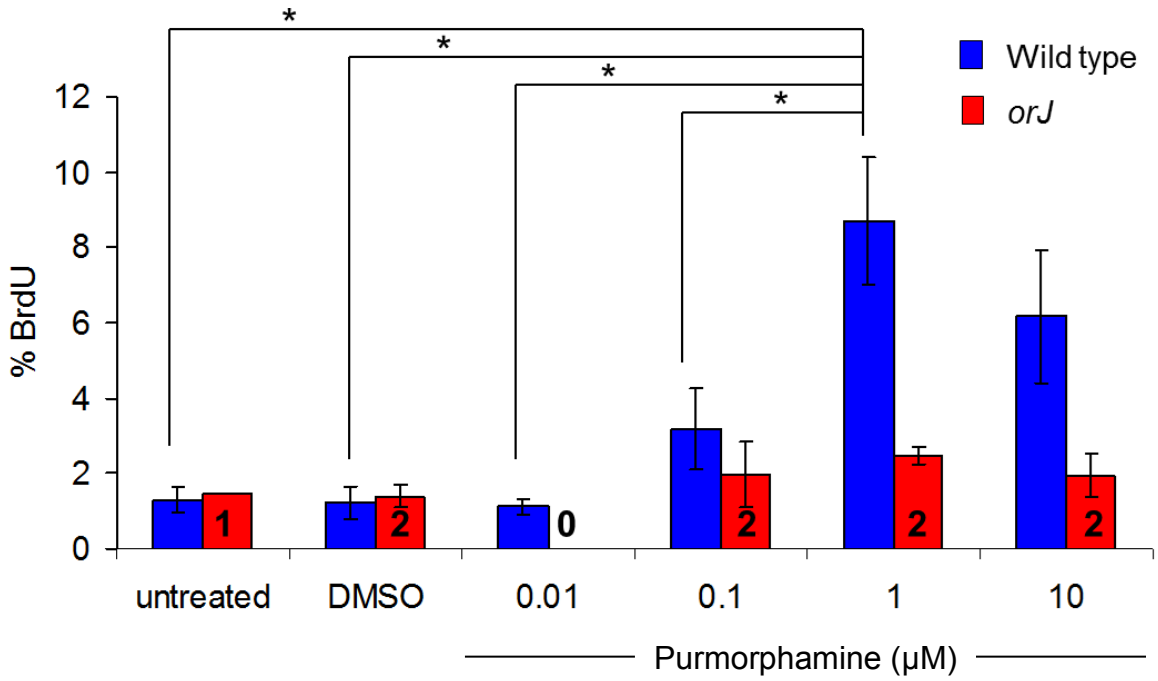

*
*
*
*
Wild type
orJ
12
10
8
6
4
2
0
% BrdU
1
2
0
2
2
2
untreated
DMSO
0.01
0.1
1
10
Purmorphamine (μM)

Figure 3.8. Reduced ability of *orJ* RPCs to maintain Hh target gene expression in response to purmorphamine in dissociated cell cultures. Comparison of the relative expression levels of Hh target genes (*Gli1* and *Hhip*) and control genes (*Smo*) in P0 wild type and *orJ* retinal cells 8 hours after purmorphamine addition (1 μM in 0.4% DMSO) in dissociated cell cultures. DMSO (0.4%) served as a vehicle control. Expression levels were normalized to the expression level in freshly dissected wild type retinal tissue, which was set at 100% and served as the reference expression level. Each point represents the mean ± SEM. $n = 7$ (tissue, Hh target genes), 4 (tissue, *Smo*) or 3 (8 hours). Abbreviations: WT, wild type.

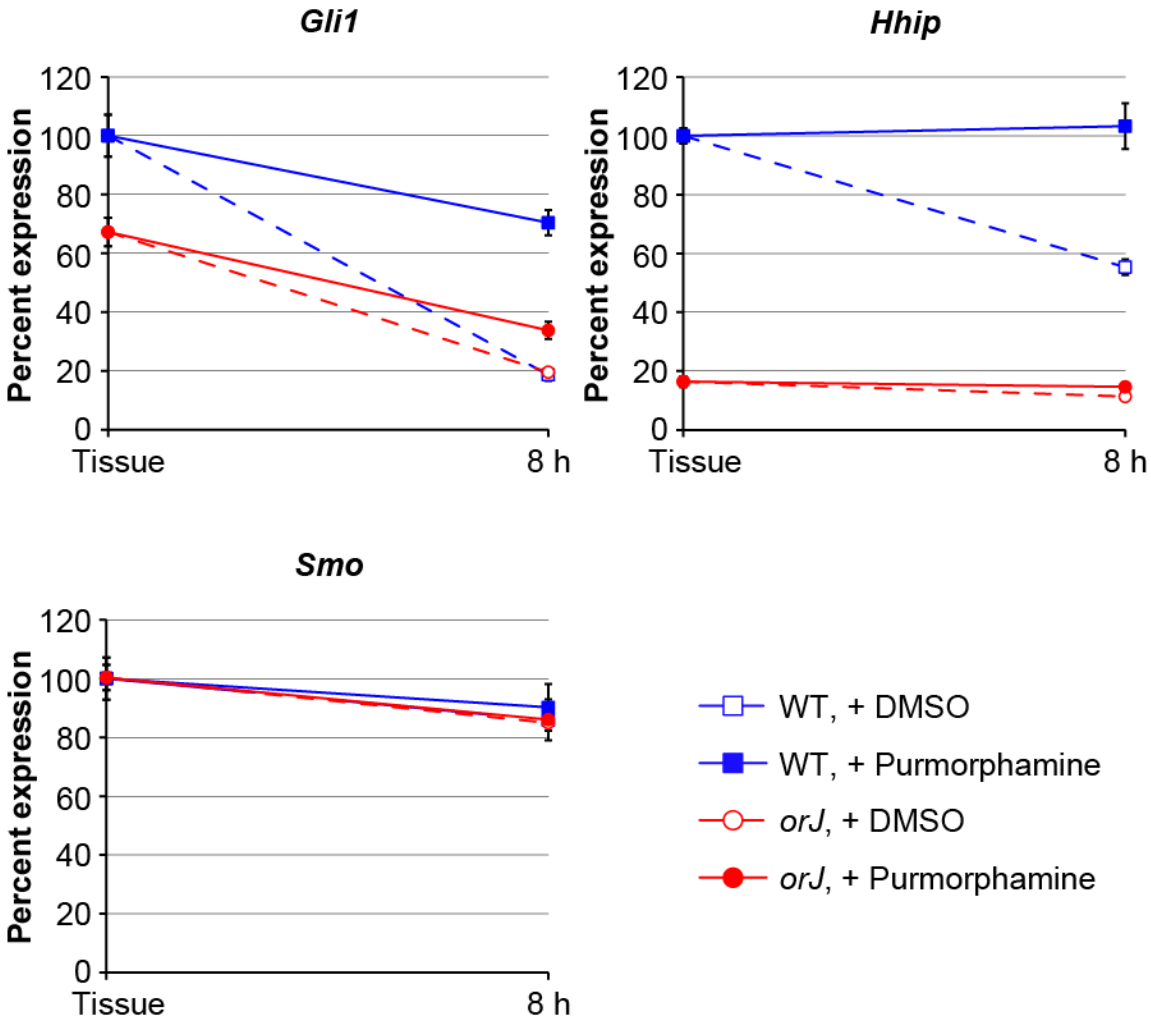

Gli1
Hhip
Smo
Percent expression
120
100
80
60
40
20
0
Tissue
8 h
WT, + DMSO
WT, + Purmorphamine
orJ, + DMSO
orJ, + Purmorphamine

Figure 3.9. Primary cilia are present on dissociated retinal cells. P0 retinal cells were dissociated, plated onto coverslips, and allowed to adhere for 1 hour prior to fixation. Visualization of the primary cilium in dissociated wild type (A,B) and *orJ* (C,D,E) retinal cells at 0 hours was accomplished using the cilia marker ARL13B and a marker of the basal body, γTUB. Cells were counterstained for DAPI to visualize the nuclei. As seen in A-D, primary cilia are associated with basal body markers. Consistent with previous reports, the mitotic cell in (E) lacks a primary cilium.

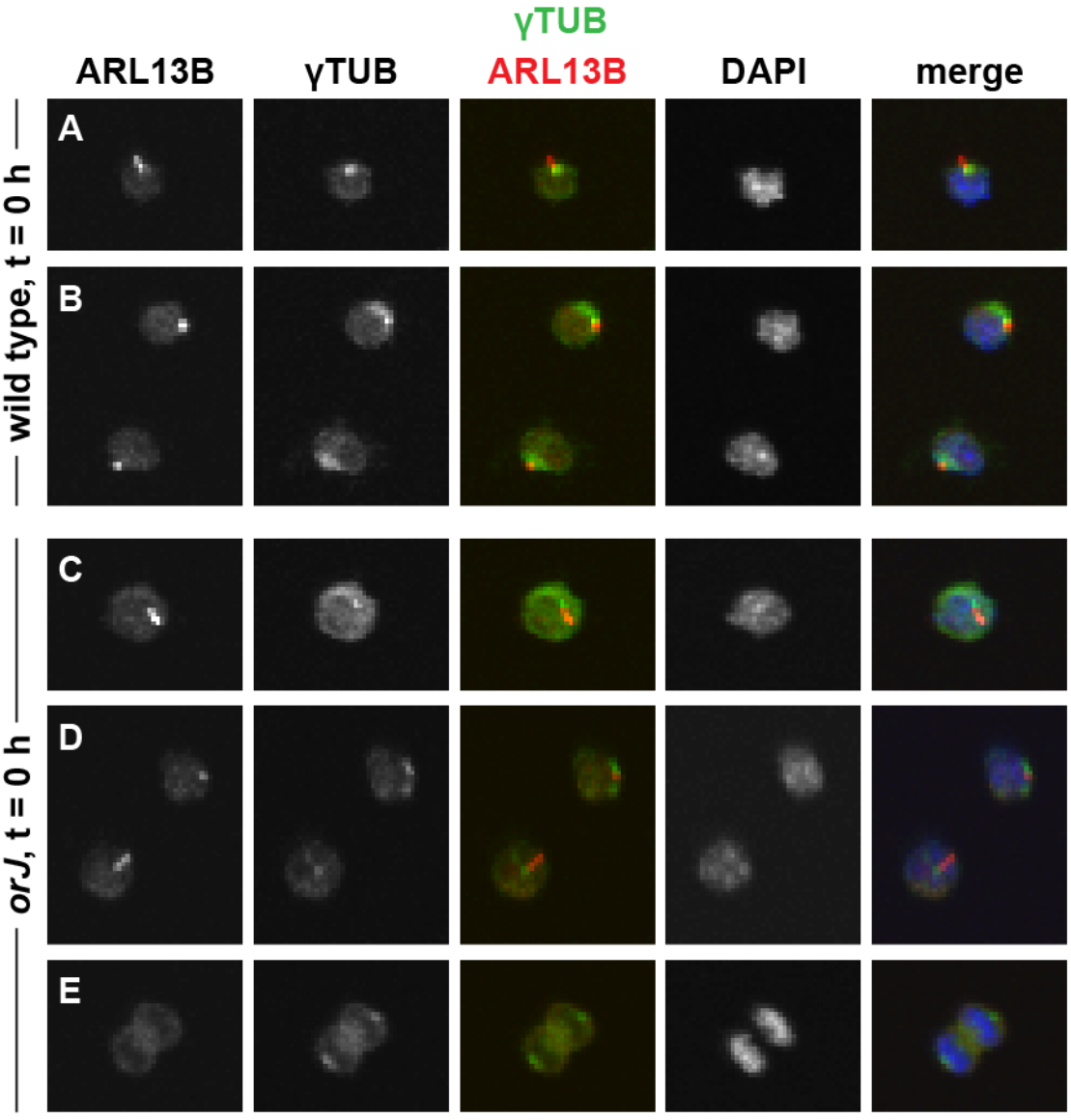
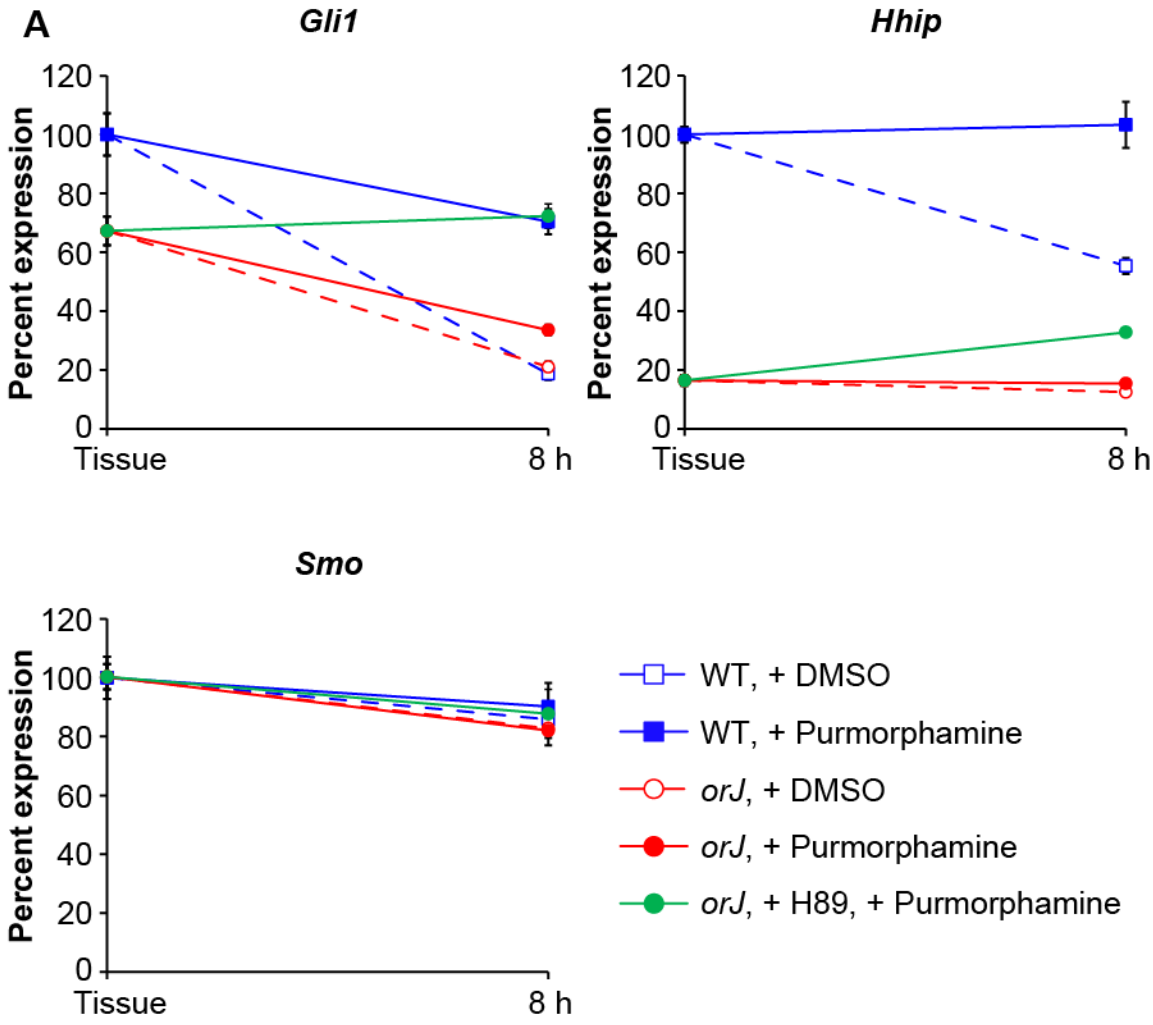

γTUB
ARL13B
ARL13B
γTUB
DAPI
merge
A
B
C
D
E
wild type, t = 0 h
orJ, t = 0 h

Figure 3.10. H89 enhances the ability of *orJ* RPCs to maintain Hh target gene expression in response to purmorphamine in dissociated cell cultures. (A-C) Comparison of the relative expression levels of Hh target genes (*Gli1* and *Hhip*) and control genes (*Smo*) in P0 wild type and *orJ* retinal cells 8 hours after purmorphamine addition (1 μM in 0.4% DMSO), in the presence or absence of H89 (10 μM) in dissociated cell cultures. DMSO (0.4%) served as a vehicle control. (A) *Gli1* expression. (B) *Hhip* expression. (C) *Smo* expression. Expression levels were normalized to the expression level in freshly dissected wild type retinal tissue, which was set at 100% and served as the reference expression level. Note that purmorphamine and DMSO data are from Figure 3.8. Each point represents the mean ± SEM. $n = 7$ (tissue, Hh target genes), 4 (tissue, *Smo*) >3 (8 hours, DMSO, purmorphamine), 2 (*orJ*, purmorphamine + H89). (D) Effects of H89 treatment (10 μM) in the presence and absence of purmorphamine (1 μM, 0.4% DMSO) on Hh target gene expression in *orJ* retinal cells at 8 hours in dissociated cell cultures. Expression levels were normalized to the expression levels observed in control (DMSO) cultures, which was set at 100% and served as the reference expression level. Bars represent a single value for $n = 1$. Abbreviations: WT, wild type.


A
Gli1
Hhip
Smo
Percent expression
120
100
80
60
40
20
0
Tissue
8 h
WT, + DMSO
WT, + Purmorphamine
orJ, + DMSO
orJ, + Purmorphamine
orJ, + H89, + Purmorphamine

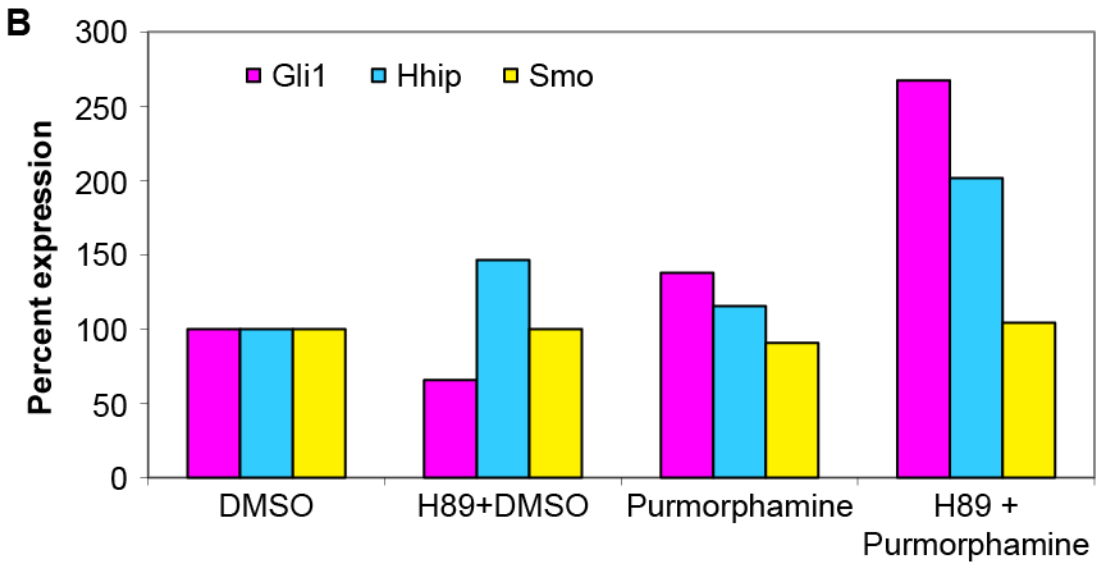

B
Percent expression
300
250
200
150
100
50
0
Gli1
Hhip
Smo
DMSO
H89+DMSO
Purmorphamine
H89 + Purmorphamine

Figure 3.11. Postdissociation recovery fails to restore responsiveness of *orJ* retinal cells to purmorphamine. Comparison of the relative expression levels of Hh target genes (*Gli1* and *Hhip*) and control genes (*Smo*) in P0 *orJ* retinal cells 8 hours after purmorphamine addition (32 total hours in culture) in dissociated cell cultures following an initial 24 hour postdissociation recovery period. DMSO served as a vehicle control. Expression levels were normalized to the expression level in freshly dissected wild type retinal tissue, which was set at 100% and served as the reference expression level. Data points for tissue represent the mean ± SEM. $n = 7$ (tissue, Hh target genes) or 4 (tissue, *Smo*). Data points at 32 hours represent the average of an $n = 2$ (*orJ* only). Abbreviations: WT, wild type.

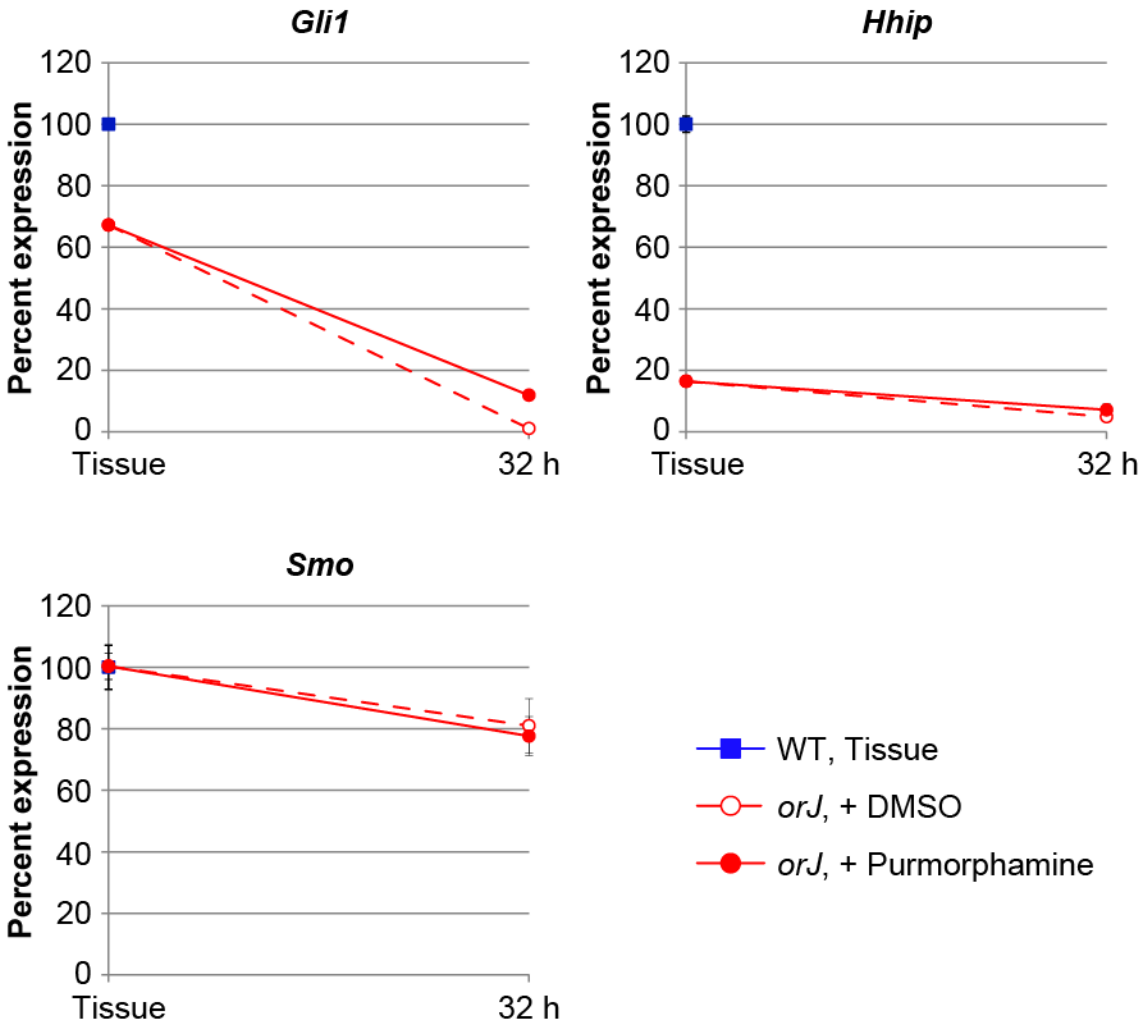
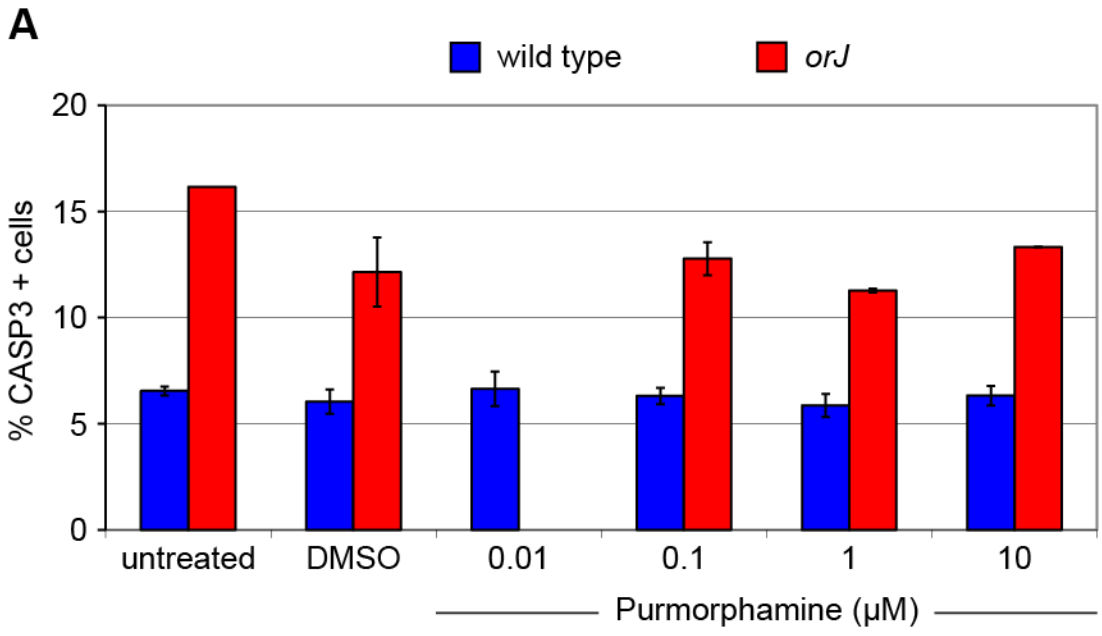
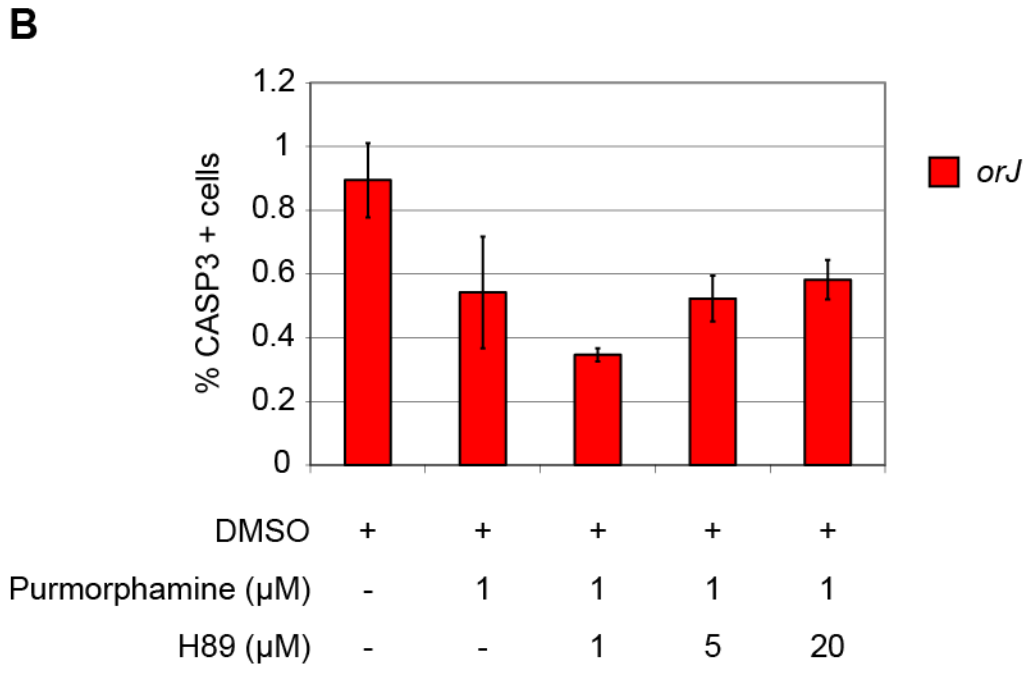

Gli1
Hhip
Smo
Percent expression
120
100
80
60
40
20
0
Tissue
32 h
WT, Tissue
orJ, + DMSO
orJ, + Purmorphamine

Figure 3.12. Cell death in dissociated cell cultures. Quantification of the proportion of Caspase-3-labeled cells in dissociated cultures of wild type and *orJ* retinal cells. (A) P0 dissociated retinal cells were cultured in the presence or absence of purmorphamine for 24 hours. Each bar represents the mean ± SEM, where appropriate. $n = 3$ (wild type) or as indicated (*orJ*). (B) P0 dissociated *orJ* retinal cells were cultured in the presence or absence of purmorphamine and H89 for 8 hours. Each bar represents the mean ± SEM, for a single $n = 1$ (*orJ* only) performed in triplicate.


A
wild type
orJ
20
15
10
5
0
% CASP3 + cells
untreated
DMSO
0.01
0.1
1
10
Purmorphamine (μM)


B
1.2
1
0.8
0.6
0.4
0.2
0
% CASP3 + cells
orJ
DMSO + + + + +
Purmorphamine (μM) - 1 1 1 1
H89 (μM) - - 1 5 20

References

Bar-Yosef, U., Abuelaish, I., Harel, T., Hendler, N., Ofir, R., Birk, O.S., 2004. CHX10 mutations cause non-syndromic microphthalmia/ anophthalmia in Arab and Jewish kindreds. Hum. Genet. 115, 302-309.

Barresi, M.J., Stickney, H.L., Devoto, S.H., 2000. The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity. Development 127, 2189-2199.

Bone-Larson, C., Basu, S., Radel, J.D., Liang, M., Perozek, T., Kapousta-Bruneau, N., Green, D.G., Burmeister, M., Hankin, M.H., 2000. Partial rescue of the ocular retardation phenotype by genetic modifiers. J. Neurobiol. 42, 232-247.

Burkitt Wright, E.M., Perveen, R., Bowers, N., Ramsden, S., McCann, E., O'Driscoll, M., Lloyd, I.C., Clayton-Smith, J., Black, G.C., 2010. VSX2 in microphthalmia: a novel splice site mutation producing a severe microphthalmia phenotype. Br. J. Ophthalmol. 94, 386-388.

Burmeister, M., Novak, J., Liang, M.Y., Basu, S., Ploder, L., Hawes, N.L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V.I., Roderick, T.H., Taylor, B.A., Hankin, M.H., McInnes, R.R., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. 12, 376-384.

Caspary, T., Larkins, C.E., Anderson, K.V., 2007. The graded response to Sonic Hedgehog depends on cilia architecture. Dev. Cell 12, 767-778.

Cayouette, M., Barres, B.A., Raff, M., 2003. Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron 40, 897-904.

Chen, Y., Sasai, N., Ma, G., Yue, T., Jia, J., Briscoe, J., Jiang, J., 2011. Sonic Hedgehog dependent phosphorylation by CK1alpha and GRK2 is required for ciliary accumulation and activation of smoothened. PLoS Biol. 9, e1001083.

Dhomen, N.S., Balaggan, K.S., Pearson, R.A., Bainbridge, J.W., Levine, E.M., Ali, R.R., Sowden, J.C., 2006. Absence of chx10 causes neural progenitors to persist in the adult retina. Invest. Ophthalmol. Vis. Sci. 47, 386-396.

Engh, R.A., Girod, A., Kinzel, V., Huber, R., Bossemeyer, D., 1996. Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl protein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. J. Biol. Chem. 271, 26157-26164.

Epstein, D.J., Marti, E., Scott, M.P., McMahon, A.P., 1996. Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. Development 122, 2885-2894.

Faiyaz-Ul-Haque, M., Zaidi, S.H., Al-Mureikhi, M.S., Peltekova, I., Tsui, L.C., Teebi, A.S., 2007. Mutations in the CHX10 gene in non-syndromic microphthalmia/anophthalmia patients from Qatar. Clin. Genet. 72, 164-166.

Fan, C.M., Porter, J.A., Chiang, C., Chang, D.T., Beachy, P.A., Tessier-Lavigne, M., 1995. Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. Cell 81, 457-465.

Ferda Percin, E., Ploder, L.A., Yu, J.J., Arici, K., Horsford, D.J., Rutherford, A., Bapat, B., Cox, D.W., Duncan, A.M., Kalnins, V.I., Kocak-Altintas, A., Sowden, J.C., Traboulsi, E., Sarfarazi, M., McInnes, R.R., 2000. Human microphthalmia associated with mutations in the retinal homeobox gene CHX10. Nat. Genet. 25, 397-401.

Fogarty, M.P., Emmenegger, B.A., Grasfeder, L.L., Oliver, T.G., Wechsler-Reya, R.J., 2007. Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells. Proc. Natl. Acad. Sci. U. S. A. 104, 2973-2978.

Giacinti, C., Giordano, A., 2006. RB and cell cycle progression. Oncogene 25, 5220-5227.

Goetz, S.C., Anderson, K.V., 2010. The primary cilium: a signalling centre during vertebrate development. Nat. Rev. Genet. 11, 331-344.

Goueli, B.S., Hsiao, K., Goueli, S.A., 2001. A novel and simple method to assay the activity of individual protein kinases in a crude tissue extract. Methods Mol. Med. 39, 633-644.

Goueli, B.S., Hsiao, K., Tereba, A., Goueli, S.A., 1995. A novel and simple method to assay the activity of individual protein kinases in a crude tissue extract. Anal. Biochem. 225, 10-17.

Green, E.S., Stubbs, J.L., Levine, E.M., 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development 130, 539-552.

Han, Y.G., Kim, H.J., Dlugosz, A.A., Ellison, D.W., Gilbertson, R.J., Alvarez-Buylla, A., 2009. Dual and opposing roles of primary cilia in medulloblastoma development. Nat. Med. 15, 1062-1065.

Hori, Y., Kobayashi, T., Kikko, Y., Kontani, K., Katada, T., 2008. Domain architecture of the atypical Arf-family GTPase Arl13b involved in cilia formation. Biochem. Biophys. Res. Commun. 373, 119-124.

Huangfu, D., Anderson, K.V., 2006. Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. Development 133, 3-14.

Humke, E.W., Dorn, K.V., Milenkovic, L., Scott, M.P., Rohatgi, R., 2010. The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes Dev. 24, 670-682.

Iseri, S.U., Wyatt, A.W., Nurnberg, G., Kluck, C., Nurnberg, P., Holder, G.E., Blair, E., Salt, A., Ragge, N.K., 2010. Use of genome-wide SNP homozygosity mapping in small pedigrees to identify new mutations in VSX2 causing recessive microphthalmia and a semidominant inner retinal dystrophy. Hum. Genet. 128, 51-60.

Jensen, A.M., Wallace, V.A., 1997. Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development 124, 363-371.

Kinoshita, Y., Jurgen Wenzel, H., Kinoshita, C., Schwartzkroin, P.A., Morrison, R.S., 2012. Acute, but reversible, kainic acid-induced DNA damage in hippocampal CA1 pyramidal cells of p53-deficient mice. Epilepsia 53 Suppl 1, 125-133.

Konyukhov, B.V., Sazhina, M.V., 1971. Genetic control over the duration of G 1 phase. Experientia 27, 970-971.

Lam, D.M., 1972. Biosynthesis of acetylcholine in turtle photoreceptors. Proc. Natl. Acad. Sci. U. S. A. 69, 1987-1991.

Levine, E.M., Roelink, H., Turner, J., Reh, T.A., 1997. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J. Neurosci. 17, 6277-6288.

Lillien, L., Cepko, C., 1992. Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha. Development 115, 253-266.

Lundberg, A.S., Weinberg, R.A., 1999. Control of the cell cycle and apoptosis. Eur. J. Cancer 35, 1886-1894.

Milenkovic, L., Scott, M.P., Rohatgi, R., 2009. Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium. J. Cell Biol. 187, 365-374.

Moshiri, A., Reh, T.A., 2004. Persistent progenitors at the retinal margin of ptc+/- mice. J. Neurosci. 24, 229-237.

Murase, S., Poser, S.W., Joseph, J., McKay, R.D., 2011. p53 controls neuronal death in the CA3 region of the newborn mouse hippocampus. Eur. J. Neurosci. 34, 374-381.

Reh, T.A., McCabe, K., Kelley, M.W., Bermingham-McDonogh, O., 1996. Growth factors in the treatment of degenerative retinal disorders. Ciba Found. Symp. 196, 120-131; discussion 131-124.

Reis, L.M., Khan, A., Kariminejad, A., Ebadi, F., Tyler, R.C., Semina, E.V., 2011. VSX2 mutations in autosomal recessive microphthalmia. Mol. Vis. 17, 2527-2532.

Riobo, N.A., Haines, G.M., Emerson, C.P., Jr., 2006a. Protein kinase C-delta and mitogen-activated protein/extracellular signal-regulated kinase-1 control GLI activation in hedgehog signaling. Cancer Res. 66, 839-845.

Riobo, N.A., Lu, K., Ai, X., Haines, G.M., Emerson, C.P., Jr., 2006b. Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. Proc. Natl Acad. Sci. U. S. A. 103, 4505-4510.

Riobo, N.A., Manning, D.R., 2007. Pathways of signal transduction employed by vertebrate Hedgehogs. Biochem. J. 403, 369-379.

Robb, R.M., Silver, J., Sullivan, R.T., 1978. Ocular retardation (or) in the mouse. Investigative ophthalmology & visual science 17, 468-473.

Rohatgi, R., Milenkovic, L., Scott, M.P., 2007. Patched1 regulates hedgehog signaling at the primary cilium. Science 317, 372-376.

Rohatgi, R., Scott, M.P., 2007. Patching the gaps in Hedgehog signalling. Nature cell biology 9, 1005-1009.

Rowan, S., Chen, C.M., Young, T.L., Fisher, D.E., Cepko, C.L., 2004. Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. Development 131, 5139-5152.

Rutherford, A.D., Dhomen, N., Smith, H.K., Sowden, J.C., 2004. Delayed expression of the Crx gene and photoreceptor development in the Chx10-deficient retina. Invest. Ophthalmol. Vis. Sci. 45, 375-384.

Ryan, K.E., Chiang, C., 2012. Hedgehog secretion and signal transduction in vertebrates. J. Biol. Chem 287, 17905-17913.

Sakagami, K., Gan, L., Yang, X.J., 2009. Distinct effects of Hedgehog signaling on neuronal fate specification and cell cycle progression in the embryonic mouse retina. J. Neurosci. 29, 6932-6944.

Seto, M., Ohta, M., Asaoka, Y., Ikenoue, T., Tada, M., Miyabayashi, K., Mohri, D., Tanaka, Y., Ijichi, H., Tateishi, K., Kanai, F., Kawabe, T., Omata, M., 2009. Regulation of the hedgehog signaling by the mitogen-activated protein kinase cascade in gastric cancer. Mol. Carcinog. 48, 703-712.

Sigulinsky, C.L., Green, E.S., Clark, A.M., Levine, E.M., 2008. Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina. Dev. Biol. 317, 560-575.

Sinha, S., Chen, J.K., 2006. Purmorphamine activates the Hedgehog pathway by targeting Smoothened. Nat. Chem. Biol. 2, 29-30.

Souda, P., Ryan, C.M., Cramer, W.A., Whitelegge, J., 2011. Profiling of integral membrane proteins and their post translational modifications using high-resolution mass spectrometry. Methods 55, 330-336.

Tempe, D., Casas, M., Karaz, S., Blanchet-Tournier, M.F., Concordet, J.P., 2006. Multisite protein kinase A and glycogen synthase kinase 3beta phosphorylation leads to Gli3 ubiquitination by SCFbetaTrCP. Mol. Cell. Biol. 26, 4316-4326.

Theiler, K., Varnum, D.S., Nadeau, J.H., Stevens, L.C., Cagianut, B., 1976. A new allele of ocular retardation: early development and morphogenetic cell death. Anat. Embryol. 150, 85-97.

Tukachinsky, H., Lopez, L.V., Salic, A., 2010. A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. J. Cell Biol. 191, 415-428.

Varga, Z.M., Amores, A., Lewis, K.E., Yan, Y.L., Postlethwait, J.H., Eisen, J.S., Westerfield, M., 2001. Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. Development 128, 3497-3509.

Wallace, V.A., 2008. Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. Brain Res. 1192, 61-75.

Wallace, V.A., Raff, M.C., 1999. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126, 2901-2909.

Wang, S.W., Mu, X., Bowers, W.J., Klein, W.H., 2002. Retinal ganglion cell differentiation in cultured mouse retinal explants. Methods 28, 448-456.

Wang, Y., Dakubo, G.D., Thurig, S., Mazerolle, C.J., Wallace, V.A., 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development 132, 5103-5113.

Wu, X., Walker, J., Zhang, J., Ding, S., Schultz, P.G., 2004. Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. Chem. Biol. 11, 1229-1238.

Yu, C., Mazerolle, C.J., Thurig, S., Wang, Y., Pacal, M., Bremner, R., Wallace, V.A., 2006. Direct and indirect effects of hedgehog pathway activation in the mammalian retina. Mol. Cell. Neurosci. 32, 274-282.

CHAPTER 4

CELL AUTONOMOUS AND CELL NONAUTONOMOUS REQUIREMENTS FOR VSX2 IN THE REGULATION OF PROGENITOR PROPERTIES DURING EMBRYONIC RETINAL DEVELOPMENT

Abstract

Vertebrate retinal development requires specification and maintenance of retinal identity, proliferative expansion of retinal progenitor cells (RPCs) and differentiation of mature retinal neurons and glia. The homeobox gene Vsx2 is expressed in RPCs and required for proper execution of this retinal program. To further define the requirement for Vsx2 in the regulation of RPC properties, we generated chimeric mouse embryos comprised of wild type and Vsx2-deficient cells. We found that Vsx2 maintains retinal identity in part through the cell autonomous repression of the RPE determinant Mitf and that Lhx2 is required cell autonomously for the ectopic Mitf expression in Vsx2-deficient retinas. We also found significant cell nonautonomous contributions to Vsx2-mediated regulation of RPC proliferation, indicating that Vsx2 has an important role(s) in establishing mitogen signals, or their tissue source, during retinal development. This analysis also revealed a previously unappreciated role for regional variation in the extrinsic regulation of RPC proliferation. Chimera analysis further demonstrated a cell autonomous requirement for Vsx2 in the initiation of neurogenesis, indicating that Vsx2 is an important regulator of neurogenic competence. Our findings reiterate the importance of Vsx2 in retinal development and demonstrate that Vsx2 utilizes both cell autonomous and nonautonomous mechanisms to regulate progenitor properties in the embryonic retina.

Introduction

The vertebrate retina is one of three ocular tissues that develop from the optic vesicle, an evagination of the neuroectoderm at the level of the diencephalon. Extrinsic signals pattern the optic vesicle into three distinct domains, thereby specifying the

identities of the presumptive retina, RPE, and optic stalk. Growing evidence reveals that at least for the RPE and retina, initial specification alone is insufficient for proper developmental progression; rather, these identities require active maintenance and suppression of aberrant gene expression programs (Horsford et al., 2005; Nguyen and Arnheiter, 2000; Rowan et al., 2004; Zou and Levine, 2012). Further development of the retina requires coordinated proliferation and differentiation. An initially small population of specified retinal progenitor cells (RPCs) undergoes extensive proliferative expansion to generate sufficient cell numbers for the formation of a functional retina (Alexiades and Cepko, 1996). During this proliferative period, many of these multipotent RPCs initiate differentiation to generate retinal neurons and glia (Young, 1985). This occurs according to an evolutionarily conserved sequence such that the six classes of retinal neurons and a single glial type are each produced during a limited, yet overlapping, interval (Livesey and Cepko, 2001). Disruptions in any of these processes impair proper development of the retina and visual function.

The homeobox gene Vsx2 is an essential regulator of retinal development. Vsx2 expression in the distal optic vesicle initiates around E9.5 (Liu et al., 1994) and is the earliest specific marker of specified RPCs. Expression is maintained in RPCs throughout retinal development, but terminated in all postmitotic retinal cells, except bipolar cells and a subset of Müller glial cells (Liu et al., 1994; Rowan and Cepko, 2004). Human patients with mutations in Vsx2 present clinically with microphthalmia, iris colobomas, cataracts, and congenital blindness (Bar-Yosef et al., 2004; Ferda Percin et al., 2000). Two mouse lines carrying spontaneous recessive mutations in the Vsx2 gene, *ocular retardation* (*or*) and *ocular retardation J* (*orJ*), also exhibit microphthalmia, cataractous

lenses, and coloboma, as well as failure to form the optic nerve (Bone-Larson et al., 2000; Burmeister et al., 1996; Robb et al., 1978; Truslove, 1962). Knockdown of the zebrafish homolog Alx with antisense oligonucleotides also promotes reduced eye size and disrupted eye development (Barabino et al., 1997). Studies in the defined *Vsx2*-null mouse mutant, *orJ*, reveal that these defects in ocular development arise from disruptions in the execution of the retinal program, including compromised retinal identity, severely reduced RPC proliferation, delayed neurogenesis, and absence of bipolar cells (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003; Horsford et al., 2005; Rowan et al., 2004).

Although Vsx2 expression is used to identify the presumptive retina and loss of this expression is often associated with the failure of retinal development in many studies, evidence suggests that Vsx2 is neither necessary nor sufficient to specify the retinal fate. First, retinal specification occurs in the absence of Vsx2 function. Expression of Vsx2 transcript is unaffected in *orJ* retinas (Rutherford et al., 2004; Sigulinsky et al., 2008) and *orJ* retinal cells express several additional neural retina-specific markers and generate mature retinal cell types (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003; Horsford et al., 2005; Rowan et al., 2004; Rutherford et al., 2004), indicating that retinal identity is initially specified and execution of the retinal program persists, albeit disrupted, in the absence of Vsx2 gene function. Additionally, misexpression of Vsx2 on its own in the presumptive RPE appears insufficient to direct these cells towards a retinal fate, although one study demonstrated that Vsx2 was sufficient to downregulate several RPE genes (Horsford et al., 2005; Rowan et al., 2004). Instead, Vsx2 is required in the maintenance of retinal identity, primarily to prevent activation of nonretinal gene

expression programs. Several genes with RPE-restricted expression exhibit ectopic or expanded expression throughout all or part of the *orJ* retina, (Horsford et al., 2005; Rowan et al., 2004), which likely contributes to the hyperpigmentation observed in *orJ* retinas as ocular development progresses (Green et al., 2003; Horsford et al., 2005; Rowan et al., 2004; Truslove, 1962). Manipulations exacerbating this aberrant gene expression program further enhance this hyperpigmentation and disruption of retinal development, while manipulations alleviating aberrant gene expression improve retinal development and reduce ectopic pigmentation (Horsford et al., 2005; Konyukhov and Sazhina, 1966; Zou and Levine, 2012). Acquisition and maintenance of retinal identity involve both extrinsic and intrinsic regulators. While studies have implicated Vsx2 downstream of extrinsic signals and upstream of many intrinsic factors involved in retinal acquisition or maintenance (Horsford et al., 2005; Nguyen and Arnheiter, 2000; Rowan et al., 2004; Zou and Levine, 2012), it is not clear from these studies whether Vsx2 also influences extrinsic signals required for maintenance of this identity.

Ocular tissues of *orJ* mice develop normally through the initial formation of the optic cup. However, as development proceeds, *orJ* eyes become increasingly smaller than wild type littermates and have thin, hypocellular retinas. Decreased retinal volume and cell number are detected as early as E11.5 (Bone-Larson et al., 2000; Burmeister et al., 1996). By P0, *orJ* retinas exhibit a striking 19-fold reduction in cell number (Green et al., 2003). The severe hypocellularity of the *orJ* retina results primarily from defective RPC proliferation (Bone-Larson et al., 2000; Burmeister et al., 1996; Dhomen et al., 2006; Green et al., 2003; Konyukhov and Sazhina, 1971). The slowed rate of cell cycle progression in the *orJ* retina appears, in large part, due to aberrant accumulation of the

cell cycle inhibitor p27/KIP1. Genetic deletion of p27/KIP1 in the *orJ* retina largely restores retinal cell number without influencing neurogenesis or apoptosis. Regulation of p27/KIP1 by Vsx2 is indirect and involves posttranscriptional mechanisms largely mediated by Ccnd1 (Green et al., 2003). Additionally, genetic removal of the RPE determinant, Mitf, in *orJ* retinas also improves retinal size and RPC proliferation (Horsford et al., 2005; Konyukhov and Sazhina, 1966). In melanocytes and melanoma cell lines, MITF directly promotes p27/KIP1, as well as a related CIP/KIP family member, p21/CIP (Carreira et al., 2005; Lekmine et al., 2007). Recently, Mitf was also implicated in transcriptional activation of p27/KIP1 in the chick optic vesicle (Tsukiji et al., 2009), suggesting that Vsx2-mediated regulation of p27/KIP1 may also be partially Mitf dependent. Mitogens are also key extrinsic regulators of cell cycle progression. Mitogen signals are required in early G1 to promote G1 progression through upregulation of D-cyclins [reviewed in (Levine and Green, 2004)]. Ccnd1 expression is reduced in *orJ* retinas (Green et al., 2003), but it is not known whether this results from direct transcriptional regulation by Vsx2 or indirectly through Vsx2-mediated regulation of mitogen signals or their signaling pathways. We previously reported evidence supporting a role for Vsx2 in the regulation of mitogen signaling (Sigulinsky et al., 2008). Sonic hedgehog (Shh), through activation of the Hedgehog (Hh) signaling pathway, is an important retinal mitogen in mouse (Jensen and Wallace, 1997; Levine et al., 1997; Moshiri and Reh, 2004; Mu et al., 2004; Wallace and Raff, 1999; Wang et al., 2005; Wang et al., 2002). In the *orJ* retina, Hh signaling activity is reduced during retinal development (Sigulinsky et al., 2008). During embryonic stages, delayed activation of Hh signaling correlated with delayed RGC production, the cellular source of Shh in the

retina. During perinatal stages, reduced Hh signaling activity persisted and correlated with reduced Shh expression, despite evidence suggesting overall progenitor:neuron ratios and RGC proportions were unaffected at this age (Green et al., 2003; Sigulinsky et al., 2008). Furthermore, we demonstrated that the reduced activity of the Hh pathway likely contributes to defective RPC proliferation in the *orJ* retina at these ages (Sigulinsky et al., 2008).

Vsx2 also regulates multiple aspects of retinal neurogenesis. Early, Vsx2 is required for proper temporal regulation of the initiation of neurogenesis. While the general principles of neurogenesis appear maintained in the *orJ* retina, including the central to peripheral wave of neuron production and temporal birth order, initiation is delayed by approximately two days (Bone-Larson et al., 2000; Robb et al., 1978; Rutherford et al., 2004; Sigulinsky et al., 2008). However, it is not clear whether this delay reflects an inability to respond to the neurogenic signal(s) or absence of the necessary signal(s). Later, Vsx2 also participates in the regulation of cell fate. Bipolar cells are not present in *orJ* retinas (Bone-Larson et al., 2000; Burmeister et al., 1996) and their generation is not rescued in *orJ*, p27/KIP1 double mutants (Green et al., 2003), suggesting their absence is due to a specific requirement for Vsx2 in their specification or maturation, rather than a secondary effect of insufficient proliferative expansion. This is further supported by a number of studies showing that Vsx2 promotes the bipolar fate at the expense of rod photoreceptors (Belecky-Adams et al., 1997; Dorval et al., 2006; Hatakeyama et al., 2001; Livne-Bar et al., 2006; Rowan and Cepko, 2004; Rutherford et al., 2004; Toy et al., 2002).

Our understanding of the molecular mechanisms by which Vsx2 regulates these developmental processes is lacking and few direct targets have been identified. To further investigate the multiple roles of Vsx2 in retinal development, we generated *orJ* mutant chimeras. Genetic chimeras are particularly useful in examining complex gene function, enabling analyses of lineage-specific gene function, competition behavior, and autonomy of gene function. Aggregation chimeras for *or* mutants were previously reported (Kindiakov and Koniukhov, 1986; Osipov and Vakhrusheva, 1982, 1984). These studies revealed improved eye size and retinal structure in mutant chimeras; however, it is not clear whether this resulted from rescued *orJ* cell behavior or compensation by wild type cells. In the present study, we focused our analyses on the embryonic regulation of RPC properties by Vsx2, including maintenance of retinal identity, RPC proliferation and initiation of neurogenesis.

Methods

Mice

orJ mice on a 129S/Sv background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice carrying a floxed allele of *Lhx2* (*Lhx2*^{f}) were kindly provided by Edwin Monuki (University of California, Irvine). The *α-Cre* transgenic mice (Marquardt et al., 2001) were kindly provided by Dr. Valerie Wallace (Ottawa Health Research Institute, Canada). Genotyping for the *orJ* and *Lhx2*^{f} alleles and *α-Cre* transgene was performed by PCR and subsequent restriction digest [*orJ* allele, as previously described (Burmeister et al., 1996)] using embryonic tail samples or adult ear clips. Tg(CAG-EYFP)7AC5Nagy mice were produced and maintained by the Transgenic

& Gene Targeting Mouse Core at the University of Utah. Briefly, 7AC5/EYFP ES cells (ATCC, Manassus, VA, USA) were injected into C57BL/6J blastocysts. The 7AC5/EYFP ES cells carry the Tg(CAG-EYFP)7AC5Nagy transgene in which EYFP is driven by a CMV immediate early enhancer coupled to the chicken β-actin promoter and first intron. Chimeric mice were intercrossed to generate homozygotes and the transgene was maintained on (129X1/Svj x 129S1/Sv) x C57BL/6 mixed background. Mice were bred overnight and noon the day a vaginal plug was observed was considered embryonic day 0.5 (E0.5). Animal use and care was conducted in accordance with IACUC guidelines.

Generation of aggregation chimeras

Chimeric mice were generated by the Transgenic & Gene Targeting Mouse Core at the University of Utah using morula aggregation techniques (Figure 4.1). Briefly, eight-cell embryos were obtained from three independent homozygous crosses of superovulated females to males of the appropriate strain. The resulting embryos were either homozygous $Vsx2^{orJ/orJ}$, homozygous $Vsx2^{+/+}$, or homozygous Tg(CAG-EYFP)7AC5Nagy/Tg(CAG-EYFP)7AC5Nagy, referred to hereafter as *orJ*, wild type, or EYFP mice, respectively. Mutant chimeras were generated by aggregating homozygous *orJ* embryos with homozygous EYFP embryos. Control chimeras were generated by aggregating homozygous wild type embryos with homozygous EYFP embryos. Most chimeras in this study were generated by aggregating two embryos together. However, in order to increase the contribution of *orJ* cells in the resulting mutant chimeras, some chimeras were generated by aggregation of three embryos (i.e., two *orJ* embryos with one

EYFP embryo). Successfully aggregated chimeric blastocysts were surgically transferred into the uterine horn of E2.5 or oviducts of E0.5 pseudopregnant C57BL/6J x FVB F1 females and allowed to develop to the desired stage. Embryo development was timed according to the pseudopregnancy of the recipient female.

EdU pulse labeling and detection

Pulse labeling of control and chimeric retinas was performed in retinal explant cultures. Retinas were dissected from surrounding tissues in Hank's buffered saline solution (HBSS), leaving the lens and vitreal chamber intact. Retinal explants were cultured for 1 hour in in HBSS containing 33.3 μM 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen-Molecular Probes, Eugene, OR, USA). Cultures were incubated at 37 °C and 5% CO_2, with nutating. Explants were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.5) for 30 minutes at room temperature, cryoprotected, and stored at -80 °C until sectioning. Sections (10-12 μm) were cut and stored at -20 °C until use. EdU incorporation was specifically detected in cryosections using AlexaFluor568 azide and the Click-iT Cell Reaction (Invitrogen-Molecular Probes, Eugene, OR, USA).

Immunohistochemistry

Whole eyes or isolated retinas of control and chimeric mice were dissected in HBSS. Whole eyes for use in MITF expression analyses were fixed in 4% PFA in PBS for 2 hours at 4 °C. Isolated retinas with lenses intact, with or without EdU labeling, were fixed in 4% PFA in PBS for 30 minutes at room temperature. Following fixation, tissue

was cryoprotected and stored at -80 °C until sectioning. Sections (10 µm for E12.5 samples and 12 µm for E15.5 samples) were cut and stored at -20 °C until staining.

Frozen sections were rehydrated in PBS and pretreated with blocking buffer (2% normal goat or donkey serum, 0.15% TritonX-100, and 0.01% sodium azide in PBS) for 30 minutes. Primary antibodies are listed in Table 4.1. Primary antibodies were diluted in the appropriate blocking buffer and incubated overnight at 4 °C. Antigen unmasking with 1% sodium dodecyl sulfate (SDS) in PBS was performed prior to blocking pretreatment for the MITF antibody. Primary antibodies were detected using species-specific secondary antibodies conjugated to AlexaFluor568 or 647 (Invitrogen-Molecular Probes, Eugene, OR, USA). Endogenous EYFP signal was visualized without antibody staining. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Fluka, Switzerland) or TOPRO\u00AE-3 iodide (TOPRO-3; Invitrogen-Molecular Probes, Eugene, OR, USA). Sections were mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) and covered with glass coverslips for fluorescence microscopy.

Image capture and processing

All immunofluorescence images were captured on an Olympus Fluoview 1000 confocal microscope (Olympus America Inc., Center Valley, PA, USA). Images were prepared for quantification and publication using Adobe Photoshop CS5 Extended (Adobe Systems Inc., San Jose, CA, USA). Olympus Fluoview confocal files were first converted using the Bio-Formats Importer Plugin (LOCI, University of Wisconsin-Madison, Madison, WI, USA) and ImageJ (NIH).

Marker quantification and analysis

Proliferation was assayed by regional quantification of EdU incorporation in E12.5 retinas. Single-slice confocal images of retinal sections were divided into six bins (central, intermediate, and peripheral in both retinal hemispheres) using ImageJ and Adobe Photoshop CS5 extended. In retinal sections containing an optic nerve head, retinal hemispheres were divided according to the position of the optic nerve head. Each retinal hemisphere was then further subdivided into three bins (central, intermediate, and peripheral). This was accomplished by first drawing a line from the center of the optic nerve head to the peripheral tip of the retina, which split the retina's width at the apical-basal midpoint. This line was then divided into three equal segments and a perpendicular extended to both apical and basal edges. In retinal sections lacking an obvious optic nerve head, the line drawn at the apical-basal midpoint was drawn from one peripheral tip to the other and divided into six equal segments. Cell counts were performed in Photoshop CS5 Extended. EdU-labeled *orJ* cells (EYFP-negative, EdU-positive) and total *orJ* cells (EYFP-negative, DAPI-positive) were counted in central and peripheral retinal bins of *orJ* and mutant chimeras. At least three sections per retina and four animals per condition were analyzed. Counts were summed within regions across sections from the same animal. The proliferating *orJ* population was calculated as a percentage of the total *orJ* population and compared in corresponding bins of *orJ* and mutant chimeras. Statistical significance was determined using Student's unpaired *t*-test or Welch's two-sample *t*-test, as appropriate (based on results of an F-test for equal variance) using Jmp Pro 9.0 (SAS Institute, Inc., Cary, NC, USA).

Neurogenic output of wild type cells in mutant chimeras was assayed by quantification of POU4F2-positive and OTX2-positive wild type cells in *orJ* versus wild type patches of E15.5 mutant chimeras. Wild type and *orJ* patches in single-slice confocal images were masked by hand in Adobe Photoshop CS5 Extended. Regions of high *orJ* contribution were classified as *orJ* patches and defined by extending perpendiculars on either side at the outermost contiguous *orJ* cell. Regions of *orJ* cells were split into separate *orJ* patches if 3 or more wild type cell widths spanned the retina to divide adjacent groups of *orJ* cells. Wild type patches contained few or no *orJ* cells and were defined by extending perpendiculars on either side at least 3 cell widths from the nearest *orJ* patch. Cell counts were performed in Photoshop CS5 Extended. Differentiated wild type cells (marker-positive, EYFP-positive) and total wild type cells (EYFP-positive) were counted in all masked patches of mutant chimeras. Differentiation of the wild type population was calculated as a percentage of the total wild type cells and compared across patch type. Statistical significance was determined using Student's unpaired *t*-test or Welch's two-sample *t*-test, as appropriate (based on results of an F-test for equal variance) using Jmp Pro 9.0 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Production of chimeras

For our analyses of Vsx2 gene function, we generated both control and mutant chimeras using morula aggregation techniques. To distinguish between the composite cell populations in the resulting chimeric embryos, EYFP embryos were used as the wild type component in both control and mutant chimeras. Table 4.2 describes our efforts to

generate these chimeras. Of the 14 control chimeras generated, 9 were E12.5, 3 were E14.5 and 2 were E15.5. Of the 21 mutant chimeras generated, 15 were E12.5, 3 were E14.5, and 3 were E15.5.

In control chimeras, the degree of contribution and pattern of EYFP-expressing cells was largely consistent across tissues within individual pups (Figure 4.2). Similar results were observed in mutant chimeras (Figure 4.2); however, the pattern of chimerism in retinas of control and mutant chimeras differed slightly (Figure 4.2B-D). This difference is unique to the retina, suggesting that it is likely a consequence of loss of Vsx2 function in the regulation of retinal progenitor cell properties. Importantly, both EYFP-expressing and nonexpressing cells were observed in the retinas of mutant chimeras (Figure 4.2D), indicating mosaic contribution of *orJ* cells to our tissue of interest.

Vsx2-mediated regulation of retinal identity involves cell autonomous repression of MITF

To evaluate retinal identity in *orJ* cells of mutant chimeras, we examined expression of the RPE determinant gene, Mitf. Mitf is required for driving and maintaining RPE identity in the eye (Bumsted and Barnstable, 2000; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). Its ectopic expression in Vsx2-deficient retinas reveals compromised retinal identity (Bharti et al., 2008; Horsford et al., 2005; Rowan et al., 2004). At E12.5, MITF expression in wild type eyes was restricted to the developing RPE and the presumptive ciliary margin (Figure 4.3A). However, in *orJ* animals, MITF expression was not restricted to these regions; rather expression extended ectopically

throughout the retina (Figure 4.3B). In the retinas of E12.5 mutant chimeras, ectopic MITF expression was detected in EYFP-negative *orJ* cells, but not adjacent EYFP-positive wild type cells (Figure 4.3D, E). This is in contrast to control chimeras where both EYFP-negative and EYFP-positive wild type cells lacked MITF expression in the retina (Figure 4.3C). Furthermore, even isolated EYFP-negative *orJ* retinal cells also ectopically expressed MITF (Figure 4.3E). The failure of *orJ* retinal cells to downregulate MITF expression in mutant chimeras demonstrates a cell autonomous requirement for Vsx2 in the repression of Mitf. Furthermore, these findings illustrate that compromised retinal identity persists in *orJ* cells of mutant chimeras due to continued aberrant expression of an RPE-like gene expression program.

Ectopic MITF expression in the *orJ* retina is dependent upon cell autonomous regulation by Lhx2

The LIM homeobox gene Lhx2 is required cell autonomously to induce or maintain expression of regional identity genes in the optic vesicle, including Vsx2 and Mitf (Yun et al., 2009). Strong LHX2 expression was observed in the retina of *orJ* mice at E12.5 (Figure 4.4A, B), suggesting that ectopic expression of MITF in the *orJ* retina may be dependent upon Lhx2. To test this possibility, we conditionally inactivated *Lhx2* in the retina of *orJ* mice using a floxed allele of *Lhx2* and the α-Cre transgene, in which Cre is driven by the retina-specific Pax6 regulatory element. Successful inactivation of *Lhx2* by Cre recombinase is indicated by expression of β-galactosidase (β-gal). At E12.5, in regions where *Lhx2* was conditionally inactivated in the retina (β-gal-positive), MITF expression was absent or downregulated (Figure 4.4C-C”). In contrast, adjacent

noninactivated regions (β-gal-negative) retained ectopic MITF expression (Figure 4.4C-C"), indicating that Lhx2 is required cell autonomously for ectopic expression of MITF in *orJ* retinas.

Vsx2-mediated regulation of RPC proliferation involves significant cell nonautonomous regulation

To examine proliferation of *orJ* cells in mutant chimeras, we quantified incorporation of the thymidine analog, EdU, following a short labeling pulse at E12.5, and compared regional proliferative activity to age-matched *orJ* retinas. In *orJ* retinas, we observed a striking reduction in EdU labeling in the periphery compared to wild type retinas (Figure 4.5A, B), consistent with previous reports of severely reduced proliferative activity in peripheral regions of Vsx2-deficient retinas (Bone-Larson et al., 2000; Burmeister et al., 1996). In mutant chimeras, EYFP-negative *orJ* cells in the peripheral region showed a sevenfold increase in EdU labeling compared to peripheral *orJ* cells of the germline mutant retina (Figure 4.5C, E) (mutant chimeras, 28 ± 8%, n = 922 EYFP-negative *orJ* cells from 6 eyes; *orJ*, 4 ± 2%, n = 1781 *orJ* cells from 4 eyes; $p < 0.001$). Unexpectedly, we observed a 1.5-fold reduction in EdU labeling of EYFP-negative *orJ* cells in the central region of mutant chimeras compared to central *orJ* retinas (Figure 4.5D, E) (mutant chimeras, 19 ± 6%, n = 830 EYFP-negative *orJ* cells from 6 eyes; *orJ*, 30 ± 3%, n = 2406 *orJ* cells from 4 eyes; $p = 0.009$). This may be a slight underrepresentation of *orJ* proliferation in central regions of mutant chimeras because the analysis included *orJ* cells immediately surrounding the optic nerve head, which show significantly reduced proliferative activity. Optic nerves fail to form in *orJ* retinas,

resulting in small or absent optic discs (Bone-Larson et al., 2000; Burmeister et al., 1996; Robb et al., 1978; Truslove, 1962). Thus, *orJ* retinas do not show a similar reduction in proliferative activity in central regions. However, inclusion of *orJ* cells from these areas is unlikely to significantly alter our findings because they represented only a small fraction of the *orJ* population analyzed. The observed changes in the proliferative activity of *orJ* cells in the context of the mutant chimera establish cell nonautonomous mechanisms as critical contributors to Vsx2-mediated regulation of RPC proliferation.

Vsx2 cell autonomously promotes initiation of neurogenesis

Retinal neurogenesis in the mouse initiates at approximately E11 in the central retina, dorsal to the optic stalk, and continues in a peripherally-spreading wave (Hufnagel et al., 2010). By E12.5, neurogenesis is active throughout the central retina of wild type mice but has yet to initiate in *orJ* retinas (Robb et al., 1978; Sigulinsky et al., 2008). This provides a critical time point at which to evaluate the ability of a wild type environment to restore neurogenesis in *orJ* cells at a time when *orJ* cells aberrantly fail to differentiate.

We first examined the location of *orJ* cells in chimeric retinas, as apical-basal location within the retina reflects a cell's differentiation status during the neurogenic period. Behind the neurogenic wave front, nascent postmitotic cells migrate basally to establish a distinct differentiated cell layer, leaving progenitors in an overlying apical neuroblast layer (Figure 4.6A). In the preneurogenic peripheral retina of E12.5 mutant chimeras, EYFP-negative *orJ* cells occupied various positions along the apical-basal axis (Figure 4.6B). However, within the neurogenic (central) region of mutant chimeras, EYFP-positive wild type cells dominated the basal differentiated cell layer, while EYFP-

negative *orJ* cells appeared to be restricted apically, within the neuroblast layer (Figure 4.6B, a). In contrast, EYFP-negative wild type cells in control chimeras readily populated the differentiated cell layer, in addition to the neuroblast layer (Figure 4.6C, b). These findings suggest that in mutant chimeras, *orJ* retinal cells have not participated in neurogenesis by E12.5 and remain undifferentiated.

To determine if *orJ* cells differentiate in mutant chimeras, but fail to localize to the differentiated cell layer, we evaluated the differentiation status of *orJ* cells in chimeric retinas. Retinal neurogenesis produces all seven retinal cell types in a specific sequence, beginning with retinal ganglion cells (RGCs). By E12.5, RGCs were abundant in the central regions of wild type retinas, as indicated by expression of the RGC marker, POU4F2 (Figure 4.7A) (Erkman et al., 1996; Gan et al., 1999; Gan et al., 1996; Qiu et al., 2008; Xiang et al., 1993). However, POU4F2-positive RGCs were absent in *orJ* retinas at this age, clearly revealing the delay in the initiation of neurogenesis (Figure 4.7B). In mutant chimeras, many POU4F2-positive RGCs were present in the central retina, but we rarely observed EYFP-negative *orJ* cells contributing to this population (Figure 4.7D, b). This is in stark contrast to control chimeras, where the POU4F2-positive RGC population was primarily composed of EYFP-negative wild type cells (Figure 4.7C, a).

To rule out the possibility that *orJ* cells differentiate in the mutant chimera, but skip the RGC fate, we also evaluated the expression of precursor markers for the other early-born retinal cell types: cone photoreceptors (PR), horizontal cells (HC), and amacrine cells (AC). In addition to the RGC marker POU4F2, we examined ISL1 (RGC, AC) (Elshatory et al., 2007a; Elshatory et al., 2007b), PTF1A (HC, AC), BHLHB5 (AC), and OTX2 (Cone PR, migrating RGC and AC) [see references in (Das et al., 2009)]. To

limit the possibility of missing differentiated *orJ* cells due to low mutant cell contribution in chimeras, staining for POU4F2, ISL1, PTF1A, and BHLHB5 was performed in combination. The OTX2 antibody also recognizes OTX1, which is upregulated in progenitors in the *orJ* retina, but still distinguishable from postmitotic OTX2 expression. Thus, OTX2 was stained separately to prevent misinterpretation of OTX1 upregulation in *orJ* cells as evidence of their differentiation in chimeras. Although wild type retinas exhibited differentiation of these early-born cell types at E12.5 (Figure 4.8A, E), *orJ* retinas did not (Figure 4.8B, F). Critically, EYFP-negative *orJ* cells in retinas of E12.5 mutant chimeras also lacked expression of these retinal cell markers (Figure 4.8D, b, H, d). Neuron Class III β-tubulin (TUBB3) is a specific marker of postmitotic neurons (Brittis et al., 1995; Lee et al., 1990) that reliably reflects the progression of retinal neurogenesis (Das et al., 2009; Hufnagel et al., 2010; Sigulinsky et al., 2008). Consistent with the absence of cell type specific marker expression, we also typically failed to detect TUBB3 expression in EYFP-negative *orJ* cells in mutant chimeras, even when located within the differentiated cell layer (Figure 4.7H, d). Together, these findings demonstrate that *orJ* retinal cells persist in mutant chimeras as progenitors and fail to participate in retinal neurogenesis.

Because *orJ* cells eventually differentiate in the germline mutant retina (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003; Rutherford et al., 2004; Sigulinsky et al., 2008), we predicted that *orJ* cells would also differentiate in chimeras, but at later developmental stages. In the *orJ* retina, all of the early-born retinal cell types are detected by E15.5 (Figure 4.9A-E). As predicted, in the retinas of E15.5 mutant chimeras, many EYFP-negative *orJ* cells expressed the neuron-specific marker TUBB3

and contributed to the expanding differentiated cell layer (Figure 4.10A). Furthermore, similar to wild type cells in control chimeras (Figure 4.11A-E), *orJ* cells in mutant chimeras contributed to all of the early-born retinal cell types at E15.5, as indicated by expression of POU4F2 (RGC), OTX2 (Cone PR, migrating RGC and AC), PTF1A (HC, AC), and BHLHB5 (AC) in EYFP-negative *orJ* cells of mutant chimeras (Figure 4.10A-E).

Despite active neurogenesis in *orJ* retinas at E15.5, the extent of differentiation was more centrally restricted than in wild type retinas (Figure 4.9, compare A-E to F-J), consistent with delayed initiation of the central-to-peripheral wave of neurogenesis (Sigulinsky et al., 2008). Thus, peripheral *orJ* cells remain undifferentiated progenitors in the germline mutant. Similarly, in retinas of E15.5 mutant chimeras, peripheral patches of EYFP-negative *orJ* cells coincided with gaps in the neuronal marker TUBB3, revealing their delayed differentiation relative to both adjacent EYFP-positive wild type cells (Figure 4.12A,B) and central EYFP-negative *orJ* cells (Figure 4.10A). Cell type-specific markers also showed a similar trend, but it was less obvious due to the sparse nature of their patterns at the leading edge and random positioning of *orJ* patches. To confirm the lag in differentiation of peripheral *orJ* cells relative to adjacent wild type cells in mutant chimeras, we examined an earlier age that exhibited a more pronounced difference in the peripheral extent of neurogenesis between wild type and *orJ* cells. In an E14.5 mutant chimera, a number of EYFP-negative *orJ* cells located within the central retina expressed POU4F2, indicating differentiation as RGCs (arrows, Figure 4.12a). In contrast, a patch of EYFP-negative *orJ* cells in a mid-retina region lacked expression of both POU4F2 and TUBB3, despite expression of both markers in more peripheral EYFP-positive wild type

cells (Figure 4.12b), revealing the failure of these *orJ* cells to participate in the wild type progression of retinal neurogenesis. Interestingly, in the central retina, we only detected a few EYFP-negative *orJ* cells expressing POU4F2 in the differentiated cell layer (red arrows, Figure 4.12a); most were still localized to the neuroblast layer (white arrows, Figure 4.8), consistent with a more recent birthdate. Together, these analyses at E12.5 and E15.5 reveal a cell autonomous delay in neurogenesis of *orJ* cells in retinas of mutant chimeras that is consistent with the delayed progression of neurogenesis in the *orJ* retina.

Cell nonautonomous regulation of neurogenic output
in wild type cells

Our examination of E15.5 mutant chimeras also revealed an unexpected change in the neurogenic output of wild type cells when exposed to an *orJ* environment. In areas of high EYFP-negative *orJ* contribution, EYFP-positive wild type cells were predominantly found in the differentiated cell layer or scattered along the apical edge of the neuroblast layer where Otx2-expressing cone precursors are typically localized; very few EYFP-positive wild type cells were observed in the intervening progenitor zone of mutant chimeras (Figure 4.13A, B). Only in areas of low EYFP-negative *orJ* contribution were patches of EYFP-positive wild type cells found to span the entire apical-basal width of the retina and populate the progenitor zone (Figure 4.13A, B). Furthermore, in EYFP-negative *orJ* environments, most EYFP-positive wild type cells expressed the neuronal marker TUBB3 (Figure 4.10A), suggesting that wild type cells are unable to maintain a progenitor population in mutant surroundings.

A possible explanation for this observation is that the *orJ* environment promoted the death of enclosed wild type progenitors. This is unlikely, because a very low frequency of cell death was detected in the retinas of mutant chimeras at E12.5, E14.5, and E15.5, using the apoptosis marker activated caspase-3 (CASP3, Figure 4.14D-G, J, K). An elevated frequency of apoptosis was observed in the peripheral retina of an E12.5 mutant chimera with very high EYFP-negative *orJ* contribution (Figure 4.14F), but a similar level of apoptosis was also observed in E12.5 *orJ* retinas (Figure 4.14 B) and was not specific to EYFP-positive wild type cells in mutant chimeras (Figure 4.14F).

An alternative possibility is that highly *orJ* environments in mutant chimeras promote precocious differentiation of enclosed wild type cells, resulting in the striking loss of wild type progenitors by E15.5. To address this, we calculated the percentages of EYFP-positive wild type cells that expressed POU4F2 or OTX2 in wild type and *orJ* patches of mutant chimeras (see Methods). In wild type patches with little *orJ* influence, 33 ± 4% (n = 10) of EYFP-positive wild type cells were POU4F2-positive RGCs (Figure 4.13A, C). In contrast, we discovered a significant overrepresentation of POU4F2-positive wild type RGCs in *orJ* environments (50% ± 10%, n = 19; $p < 0.000001$) (Figure 4.13A, C). Somewhat surprisingly, there was no difference in the mean production of OTX2-positive cone PR precursors by EYFP-positive wild type cells between the two environments (16% ± 9%, n = 18 in *orJ* environments, 16% ± 4%, n = 12 in wild type environments; p = 0.83) (Figure 4.13B, D). However, we observed greater variation (both over- and under-representation) in individual *orJ* environments. We did not quantify Ptf1a-positive and BHLHB-positive wild type cell populations due to the smaller size of these populations and reduced probability of finding EYFP-positive wild type cells to

evaluate in the progenitor zone where these markers are localized. This striking overrepresentation of EYFP-positive wild type RGCs suggests that the *orJ* environment promotes altered neurogenic output of the enclosed EYFP-positive wild type cells in mutant chimeras.

It is interesting to note that the peripheral extent of *orJ* and wild type differentiation in chimeric retinas does not appear to match that of control *orJ* and wild type retinas. Specifically, the mutant neurogenic region in chimeric retinas appeared slightly expanded compared to *orJ* germline retinas, while wild type neurogenic region in mutant chimeras with a high *orJ* contribution appeared more centrally restricted than in wild type controls. Unfortunately, confirming this has been difficult, largely due to the random positioning of mutant patches within chimeric retinas and low yield of chimeric retinas with high mutant cell contribution.

Discussion

Vsx2 has multiple key roles in retinal development, but the molecular mechanisms by which Vsx2 regulates these diverse and often competing processes is still poorly understood. Each of these developmental processes is tightly regulated through the coordinated activity of multiple signaling pathways and intrinsic factors, resulting in a large number of candidate mediators. Chimera analysis provides a powerful, unbiased approach to determine the extent of extrinsic influence Vsx2 exerts in its regulation of RPC properties. In the present study, we determined the autonomy of Vsx2 function during the embryonic stages of retinal development to help place Vsx2 in the context of

known regulatory pathways driving maintenance of retinal identity, RPC proliferation, and initiation of neurogenesis.

Retinal identity

A primary role for Vsx2 in the maintenance of retinal identity appears to be preventing aberrant expression of an RPE-like gene expression program through repression of the RPE determinant Mitf (Horsford et al., 2005; Rowan et al., 2004). Genetic removal of Mitf in *orJ* retinas improves retinal development, while genetically increasing Mitf dosage in *orJ* retinas further exacerbates the pigmentation program, suggesting that aberrant Mitf expression is a major contributor to the *orJ* phenotype (Horsford et al., 2005; Konyukhov and Sazhina, 1966). Mitf expression in the eye is also regulated by extrinsic signals, including FGF, Wnt-β-catenin and the TGFβ family member, activin (Fu et al., 2006; Fuhrmann et al., 2000; Nguyen and Arnheiter, 2000). It was therefore possible that Vsx2 influenced extrinsic signals to repress Mitf. In the present study, we show that *orJ* cells fail to downregulate MITF expression in mutant chimeras, demonstrating their inability to fully respond to extrinsic signals driving retinal specification and maintenance and revealing a critical cell autonomous role for Vsx2 in mediating this response. This cell autonomous repression of MITF expression by Vsx2 is consistent with reports that MITF is a direct transcriptional target of Vsx2 (Bharti et al., 2008).

FGF signaling is a critical regulator of Vsx2 and Mitf expression within the developing eye. Surface ectoderm-derived FGF signals are important for promoting Vsx2 expression in the presumptive retina (Nguyen and Arnheiter, 2000). FGFs are also

sufficient to repress Mitf expression and promote an RPE to retina switch in identity (Horsford et al., 2005; Nguyen and Arnheiter, 2000). Failure of FGFs to repress Mitf and induce an ectopic retina in *orJ* retinal explants reveals that Vsx2 mediates much of these functions (Horsford et al., 2005). This is further supported by FGF loss of function studies in which removal of the FGF source or inhibition of FGF signaling results in concomitant loss of Vsx2 expression and ectopic Mitf expression (Cai et al., 2010; Nguyen and Arnheiter, 2000). In the *orJ* retina, maintained expression of Vsx2 transcript (Rutherford et al., 2004; Sigulinsky et al., 2008) indicates FGF signaling is still active. Given the cell autonomous requirement for Vsx2 in the repression of MITF, as demonstrated in the present study, absence of Vsx2 protein in *orJ* cells explains the failure to downregulate MITF expression despite active FGF signaling. However, it is not clear why MITF expression persists. What underlies the competence of the retina to permit such expression? In the present study, we found that Lhx2 is required cell autonomously for the ectopic expression of MITF in *orJ* retinas, as it is in the developing RPE. Because Lhx2 is also required cell autonomously for Vsx2 expression in the retina (Yun et al., 2009), we propose a model in which Lhx2 is necessary for expression of both Vsx2 and Mitf in the retina. FGF signaling from the surface ectoderm promotes Vsx2 in the presumptive retina, which in turn, represses Mitf. A reciprocal inhibitory regulation/repression does not appear to be present, at least in the context of the retina, as Vsx2 transcript expression is maintained in the *orJ* retina, despite ectopic MITF expression. Thus, Lhx2 is an intrinsic factor necessary to allow ectopic MITF expression in the absence of Vsx2.

Maintained ectopic expression of MITF in *orJ* retinas is not sufficient to cause a switch in fate from retina to RPE. While MITF expression may promote a tendency toward pigmentation and reduced proliferative expansion characteristic of the RPE, *orJ* cells still express retinal markers and generate retinal neurons (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003; Horsford et al., 2005; Rowan et al., 2004; Rutherford et al., 2004). Rather, it appears that *orJ* cells exhibit a mixed identity, containing elements of retina, RPE and perhaps ciliary margin identities. Despite this mixed identity, *orJ* cells readily contributed to the developing retina of mutant chimeras. While this is consistent with the execution of a retinal program, albeit disrupted, in the germline mutant, it is somewhat surprising given the competition afforded by wild type cells within chimeras. Competition with wild type cells in chimeras can reveal disrupted developmental processes not detected in germline mutants. In the case of PDGFR mutants, muscle cell lineages lack overt developmental defects; however, when PDGFR mutant cells were placed in competition with wild type cells in mutant chimeras, researchers observed a dramatic reduction in the contribution of PDGFR mutant cells to muscle cell lineages compared to wild type PDGFR cells in control chimeras (Crosby et al., 1998). Although it is possible that *orJ* cells are impaired in their ability to contribute to the developing retina due to this mixed identity, it is difficult to ascertain at the stages examined in this study due to changes in RPC proliferation, which also influence *orJ* cell number in mutant chimeras. However, the presence of substantial numbers of EYFP-negative *orJ* cells in the retinas of E15.5 mutant chimeras suggests that they retain sufficient retinal identity to contribute to the retina and even differentiate into retinal neurons. This is in stark contrast to mutants with fundamental defects in ocular tissue

identity. In Rx chimeras, Rx-deficient cells fail to contribute to the retina due to early exclusion from the presumptive retinal region of the optic vesicle (Medina-Martinez et al., 2009). In Pax6 chimeras, very few Pax6-deficient cells are detected in the retina; most are excluded from the presumptive retina in ectopic vesicles (Collinson et al., 2000; Collinson et al., 2003; Quinn et al., 1996). Those that do contribute to the retina die perinatally, leaving only Pax6-deficient microglia, pericytes, astrocytes and vascular endothelial cells, which derive from extra-retinal sources and migrate into the developing retina (Li et al., 2007).

Although *orJ* cells readily contributed to the developing retina in mutant chimeras, they tended to cluster with other *orJ* cells. This became more apparent at E15.5, where most *orJ* cells were present in fairly *orJ*-dominant patches. In contrast, wild type cells of both EYFP-negative and EYFP-positive lineages were highly intermingled in control chimeras. One possibility is that the initial failure of *orJ* cells to participate in neurogenesis and subsequent proliferative expansion of these *orJ* progenitors may have been sufficient to promote the formation of such patches in mutant chimeras. Consistent with this, wild type cells were often found intermixed, especially within the differentiated cell layer. However, we cannot rule out the possibility that some degree of cell sorting contributes. Differential adhesion can be a powerful sorting mechanism, highlighted by numerous studies in drosophila (see review by (Irvine and Rauskolb, 2001). Pax6 regulates several cell adhesion molecules (Simpson and Price, 2002) and differential cell adhesion has been proposed to underlie the strict segregation of mutant and wild type cells in Pax6 chimeras and physical exclusion of these mutant cells from the presumptive retina into ectopic vesicles (Collinson et al., 2000; Collinson et al., 2003; Li et al., 2007;

Quinn et al., 1996). The segregation of *orJ* and wild type cells in mutant chimeras was not as strict as seen in Pax6 chimeras and lacked evidence of physical exclusion. Culturing chick retinal tissue with antibodies against neural specific cell adhesion molecule, NCAM, can significantly disrupt retinal lamination (Buskirk et al., 1980). Furthermore, mutations in cell polarity genes (Fu et al., 2006; Georgiadis et al., 2010; Sottocornola et al., 2010; Wei et al., 2004) or genes regulating the establishment of cell polarity, such as Notch pathway components (Jadhav et al., 2006; Riesenberg et al., 2009; Tomita et al., 1996), exhibit severely disrupted lamination and retinal rosettes. Retinal lamination is clearly disrupted in the *orJ* retina, but rescued by significant restoration of cell number (Green et al., 2003). Furthermore, in mutant chimeras, we did not detect disruptions in lamination or the presence of retinal rosettes, suggesting that cellular adhesion and polarity was largely intact. The segregation of *orJ* cells in mutant chimeras could still be driven by homophilic affinity preferences. Such affinity differences are likely intimately related to the compromised identity in *orJ* cells. Numerous cell adhesion molecules are expressed in the eye and many exhibit differential expression between ocular tissues (Daniele et al., 2007; Faulkner-Jones et al., 1999; Honjo et al., 2000; Neill and Barnstable, 1990; Strunnikova et al., 2010; Wohrn et al., 1998; Xu et al., 2002). Tissue-specific genes that establish or maintain tissue identity, such as Vsx2 and Mitf, are ideal candidates for establishing regionalization of cell adhesion molecules. Mitf regulates the expression of several cell adhesion molecules in mast cells (Ito et al., 2003; Kim et al., 1998; Shahlaee et al., 2007), suggesting that ectopic Mitf expression, and perhaps other elements of the mixed identity exhibited by *orJ* cells, may promote sorting of *orJ* and wild type cells in chimeras based on hemophilic affinity preferences.

Proliferation

In the present study, significant changes in the proliferative activity of *orJ* cells in mutant chimeras reveals a strong reliance of Vsx2-mediated regulation of RPC proliferation on cell nonautonomous mechanisms. In the peripheral retina, *orJ* cells exhibited a sevenfold increase in EdU incorporation in retinas of mutant chimeras compared to germline mutants, revealing at least a partial rescue of RPC proliferation by extrinsic regulation in this region. This finding indicates that either expression of a retinal mitogen normally present in the wild type eye is restored in the eyes of mutant chimeras, or production of an inhibitory proliferation signal aberrantly present in *orJ* eyes is prevented. In contrast, *orJ* cells in the central retina exhibited a 1.5-fold reduction in EdU incorporation in mutant chimeras compared to germline mutants. This finding suggests the presence of an inhibitory signal in the mutant chimeras that is not typically present in the *orJ* eye or the loss of a mitogen signal ectopically present in the *orJ* eye, neither of which have been suggested by previous studies in *orJ* retinas.

While a cell nonautonomous contribution to central RPC proliferation was predicted, the direction of the proliferation change was surprising. We previously reported evidence for reduced Hh signaling activity in the *orJ* retina, which correlated with delayed RGC differentiation, the retinal source of SHH (Sigulinsky et al., 2008). Numerous studies in the mouse reveal Shh is an important retinal mitogen (Jensen and Wallace, 1997; Levine et al., 1997; Moshiri and Reh, 2004; Mu et al., 2004; Wallace and Raff, 1999; Wang et al., 2005; Wang et al., 2002). Furthermore, we demonstrated that the absence of Hh pathway activation during this period likely contributed to the proliferative defect of the *orJ* retina (Sigulinsky et al., 2008). Thus, we predicted that restoration of

endogenous SHH through RGC production by wild type cells in mutant chimeras would produce a cell nonautonomous increase in proliferation of *orJ* RPCs. However, we observed reduced, rather than increased, *orJ* proliferation in mutant chimeras, suggesting that either the endogenous SHH signal is not sufficient to increase RPC proliferation in *orJ* cells, or that this ability is masked.

Together, these cell nonautonomous effects on RPC proliferation suggest that Vsx2 is critical for establishing retinal proliferation signals in the eye. The identity and source of these signals could not be determined from the chimera analyses. However, candidate sources include retinal neurons or progenitors within the retina, or adjacent tissues whose development may have been deregulated in the *orJ* retina as a result of disrupted tissue-tissue interactions, which have been shown to be critical for proper development of ocular tissues (Fuhrmann et al., 2000; Nguyen and Arnheiter, 2000). Interestingly, our observation that changes in proliferation in the central and peripheral regions occur in opposite directions, argues that regional differences in extrinsic regulation must exist. Whether these regional differences reflect variation in availability of the extrinsic signals or intrinsic variation concerning the response to such signals will be interesting to address.

Neurogenesis

The cell autonomous delay in neurogenesis exhibited by *orJ* cells in mutant chimeras was consistent with the delayed neurogenic program observed in the germline mutant retina. The failure of *orJ* cells to differentiate, despite active neurogenesis in neighboring wild type cells, demonstrates the inherent, although temporary, inability of

orJ cells to respond to neurogenic signal(s). Thus, the observed delay in the onset of neurogenesis in *orJ* retinas results from impaired neurogenic competence, as opposed to altered environmental signals. The intrinsic mechanism underlying Vsx2-mediated competence is not known, but may involve MITF repression.

Intriguingly, *orJ* cells in mutant chimeras maintained the central to peripheral wave of neurogenesis, despite its delayed onset. Peripheral *orJ* cells failed to differentiate despite active neurogenesis in adjacent wild type cells and more centrally located *orJ* cells in mutant chimeras. Thus, two independent waves of neurogenesis were seen in the mutant chimeras: first, the normal central to peripheral wave of neurogenesis in wild type cells, followed by a second central to peripheral wave of neurogenesis in *orJ* cells. According to the sequential induction model, the central to peripheral wave of neurogenesis results from signaling by nascent retinal neurons that induces neighboring RPCs to differentiate. Consistent with this, both Hh and FGF signals can induce premature retinal neurogenesis and influence progression of the neurogenic wave (Martinez-Morales et al., 2005; McCabe et al., 1999; Neumann and Nuesslein-Volhard, 2000). Growing evidence has begun to challenge this model. Peripheral RPCs differentiate despite early physical separation from the central retina in chick (McCabe et al., 1999) and RGC differentiation could occur even when naïve RPCs were transplanted into nonretinal regions of the zebrafish embryo (Kay et al., 2005). In the present study, we found that *orJ* cells at different central to peripheral retinal positions within mutant chimeras do not gain competence all at once, as would be expected for the sequential induction model involving a signal that had already progressed throughout the retina. Furthermore, the ability of more peripheral wild type cells to differentiate beyond an

undifferentiated patch of mutant cells suggests that RPCs do not require direct contact with nascent neurons to initiate neurogenesis. This has also been shown in mouse retinas with mosaic conditional inactivation of Shp2, an important FGF signaling pathway component (Cai et al., 2010), although the range of this neurogenic signal could be far enough to allow transference of this signal across mutant patches without interruption. An alternative model argues for cell autonomous control of neurogenesis, suggesting that RPCs differentiate based on a preprogrammed, intrinsic timer. The underlying source of this cell autonomous "clock" has remained elusive. The proliferative defect in the *orJ* retina could support a model where this clock was tied to cell divisions; however, we found significant improvement in the proliferation of peripheral *orJ* cells in mutant chimeras, yet these cells still remained delayed with respect to neurogenesis. The presence of a second central to peripheral wave of neurogenesis in *orJ* cells of chimeric retinas suggests that there is a strong cell autonomous component driving this gradient of neurogenesis across the retina. The source of this cell autonomous control may largely be based on positional identity. This is consistent with studies in which transplanted zebrafish RPCs expressed ath5 (RGC determinant) according to their original retinal position, independent of the location into which they are transplanted (Kay et al., 2005).

Our investigation of neurogenesis in the present study revealed an unexpected cell nonautonomous effect on neurogenic output of wild type cells in mutant chimeras. In regions with high *orJ* contribution, wild type cells exhibited an impaired ability to maintain a progenitor population and precocious differentiation of RGCs. We failed to detect significant levels of apoptosis in mutant chimeras at several ages, suggesting that the absence of wild type progenitors is not due to their death. Furthermore, if cell death

contributed to the loss of wild type cells in mutant chimeras, we would not predict such a significant population of differentiated wild type cells to remain. Unfortunately, we are unable to watch neurogenesis occur in mutant chimeras; therefore, we do not know for certain the starting location of differentiated wild type cells observed in *orJ* patches. Thus, we cannot rule out the formal possibility that differentiated ganglion cells originating in adjacent wild type patches slide in under *orJ* patches to fill in the ganglion cell layer via tangential migration. Reese and colleagues (Reese et al., 1999) elegantly demonstrated that virtually every ganglion cell participates in tangential migration. Migration distances were variable, but extended up to 144 μm. While tangential migration of RGCs was observed as early as E15.5 (Reese et al., 1999), it is not clear to what extent it has occurred by this age. However, previous chimera studies indicate that clone size at this age (Reese et al., 1999) is smaller than the observed *orJ* patches in our mutant chimeras (also supported by control chimeras in the present study), making it possible that the differentiated wild type cells derived from wild type progenitors located within the *orJ* patch. Additionally, many OTX2-positive wild type cells were detected within *orJ* patches, and OTX2-positive cones do not undergo tangential migration until their maturation in postnatal ages (Reese et al., 1999). Although it is possible that a lack of cone differentiation in *orJ* patches exhibiting delayed neurogenesis could trigger premature tangential migration of adjacent wild type cone precursors, this has not been shown. Therefore, it is likely that OTX2-positive wild type cells in *orJ* patches derived from wild type progenitors within that patch. Thus, while tangential migration likely contributes, it is unlikely to fully account for our observations.

The simplest explanation for our findings is precocious differentiation of wild type cells in regions of high mutant cell contribution. Somewhat surprisingly, there was no change in the mean production of OTX2-positive cone and amacrine precursors by wild type cells in *orJ* environments. However, RGCs are the earliest born cell type in the retina, with cone, horizontal, and amacrine cells born slightly later, but still overlapping RGC production. Thus, depletion of wild type progenitors through precocious differentiation into the earliest born cell fate (RGCs) may have precluded an overrepresentation of these and later born cell types. This is consistent with the observation that few PTF1A (AC, HC) and BHLHB5 (AC)-positive wild types cells were detected in *orJ* environments of mutant chimeras.

What underlies this cell nonautonomous effect on wild type cells? One possibility is that precocious differentiation of wild type cells in *orJ* environments is a secondary effect of the cell autonomous delay in neurogenesis of *orJ* cells. Absence of differentiating *orJ* neurons early in mutant chimeras could result in non-limiting neurogenic signal(s) driving continued differentiation of wild type cells because *orJ* cells were incompetent. Alternatively, reduced neuron production early may result in reduced negative feedback and precocious differentiation of competent cells. Differentiated cells have been shown to produce signals that inhibit neurogenesis in adjacent RPCs, such as Shh (Neumann and Nuesslein-Volhard, 2000) and VEGF (Hashimoto et al., 2006). It is not clear, however, whether these signals cause exiting cells to undergo a cell fate switch or simply promote the progenitor state. An intriguing possibility is that in addition to regulating neurogenic competence, Vsx2 may also regulate availability of RPC-derived

neurogenic or progenitor signals. Vsx2 also may be required indirectly by ensuring proper development of the source tissue.

The period of retinal neurogenesis significantly overlaps that of proliferative expansion in the retina. Inability to maintain a progenitor population for the production of late-born retinal cells types would be detrimental to retinal function and vision. Therefore, mechanisms must be in place to either actively regulate or intrinsically determine the competence of progenitors to differentiate during retinal development. Although both intrinsic factors and extrinsic signaling pathways have been implicated, how progenitors persist once neurogenesis is initiated is still poorly understood. In the present study, the extent of precocious differentiation of wild type cells in mutant chimeras appears to substantially deplete the wild type progenitor population in *orJ* environments. This finding argues that progenitor maintenance is under strong cell nonautonomous regulation. Thus, many more progenitors are competent to differentiate at any given stage than usually observed because of strong extrinsic regulation preventing depletion of this progenitor population to ensure later cell type production.

Conclusion

Retinal development requires coordinated regulation of RPC behavior. Vsx2 is a critical component of this regulation, essential during the embryonic stages of retinal development for proper maintenance of retinal identity, proliferative expansion of the RPC pool, and initiation of neurogenesis. Chimera analysis provides a powerful and insightful approach to further define the roles of Vsx2 in these different processes. In the present study, determining the autonomy of Vsx2 actions through the use of genetic

chimeras has helped to define the primary level of Vsx2 regulation, not readily obvious from studies in the *orJ* retina, which will be valuable for directing future mechanistic studies. The analysis of *orJ* chimeras also provided insight into aspects of retinal development not specifically restricted to Vsx2 function. Here, we provide evidence suggesting that extrinsic regulation of RPC proliferation is regionalized, with potentially different mitogens important at varying degrees in the central versus peripheral retina to drive proliferative expansion. We also provide evidence suggesting that retinal progenitors are generally competent to participate in neurogenesis, but strong extrinsic regulation driving progenitor maintenance normally restricts their participation. Together, the use of genetic chimeras has advanced our understanding of both Vsx2 function and principles of retinal development. The continued analysis of chimeras in the future will likely prove a valuable tool in further defining the mechanisms of Vsx2 function. We have focused on the embryonic roles of Vsx2 in retinal development, but Vsx2 is also required in the postnatal production of bipolar cells. Analysis of *orJ* chimeras at later stages would help distinguish between a role for Vsx2 in the response to bipolar differentiation signals or the generation of such signals.

Table 4.1. Primary antibodies.

Antibody	**Host**	**Dilution Factor**	**Source**
MITF	Mouse	400	Exalpha Biologicals (X1405M)
LHX2	Rabbit	50	Edwin Monuki
B-gal	Rat	1000	Nadean Brown
POU4F2	Goat	50	Santa Cruz (sc-6026)
ISL1	Mouse	100	DSHB (clone 39.4D5)
OTX2/1	Rabbit	15,000	Chemicon (ab9566)
PTF1A	Guinea Pig	5000	Jane Johnson
BHLHB5	Goat	1000	Santa Cruz (sc-6045)
TUBB3	Rabbit	4000	Covance (PRB-435P)
CASP3	Rabbit	750	BD Biosciences (clone C92-605)

Table 4.2. Generation of chimeras by morula aggregation.

Aggregation Type	No. of morula aggregations attempted	No. of embryos			
		Implanted[a]	Recovered	Evaluated for chimerism	Identified as chimeric[b]
1:1 morula aggregations					
WT:EYFP	58	44	8[c]	5	3
orJ:EYFP	77	60	19[d]	13	11
2:1 morula aggregations					
WT:EYFP	120	131[e]	23[f]	21	11
orJ:EYFP	128	128[g]	13[h]	12	10

[a]All successfully aggregated and partially aggregated (successful aggregation of 2 of the 3 morulas during 2:1 morula aggregations) embryos were implanted into pseudopregnant females.

[b]Chimeras identified by evaluation of EYFP contribution in eye or limb sections.

[c]3 embryos exhibited light or absent eye pigmentation and were excluded from further analysis.

[d]5 embryos exhibited light or absent eye pigmentation and were excluded from further analysis.

[e]Includes 102 successfully aggregated embryos, 18 partially aggregated embryos, and 11 nonaggregated embryos implanted as fillers.

[f]1 embryo was grossly underdeveloped and 1 embryo lacked eye pigmentation. Both embryos were excluded from further analysis.

[g]Includes 112 successfully aggregated embryos and 16 partially aggregated embryos.

[h]1 embryo exhibited abnormal gross morphology and was excluded from further analysis.

Figure 4.1. Experimental strategy for the generation of chimeras by morula aggregation.

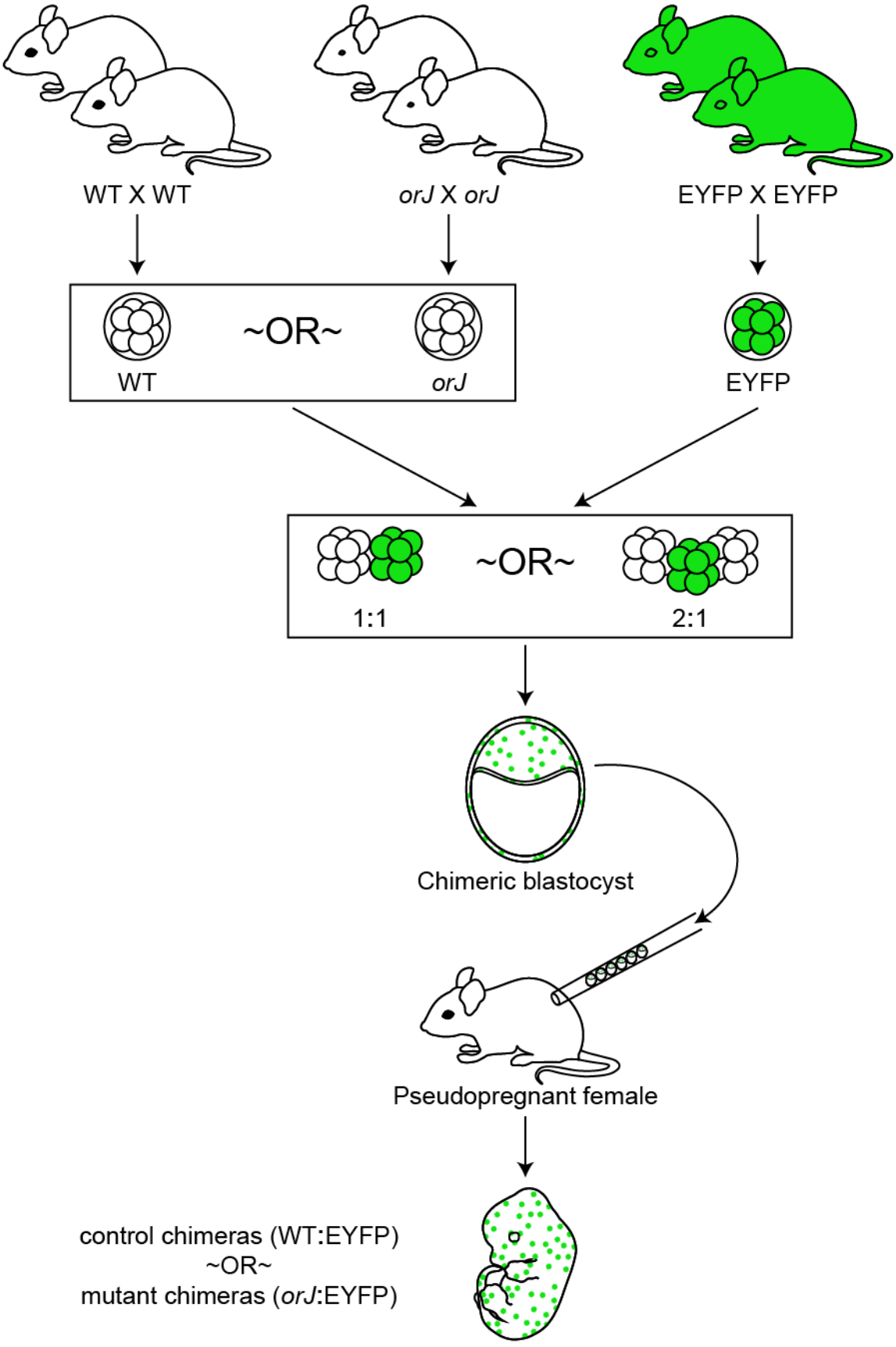

WT X WT
orJ X orJ
EYFP X EYFP
~OR~
WT
orJ
EYFP
~OR~
1:1
2:1
Chimeric blastocyst
Pseudopregnant female
control chimeras (WT:EYFP)
~OR~
mutant chimeras (orJ:EYFP)

Figure 4.2. Comparison of chimerism in control and mutant chimeras across tissues. EYFP signal in the retina (B-D), cortical epithelium (F-H), nasal epithelium (J-L), and limb tissues (N-P) of control and mutant chimeras. Diagrams illustrating the morphology of the developing retina (A), cortical epithelium (E), nasal epithelium (I), and limb (M), along with their associated tissues. Control chimera in (B, F, J, N) exhibits low EYFP contribution, while control chimera in (C, G, K, O) and the mutant chimera in (D, H, L, P) exhibit medium EYFP contribution. Scalebars: 200 μm. Abbreviations: b, cartilage primordium of turbinate bone (nasal capsule) or phalangeal and metacarpal bones (limb); ge, ganglionic eminence (striatum); iz, intermediate zone of telencephalon; L, lens; lv, anterior horn of lateral ventricle; nc, nasal cavity; np, nasopharynx; npc, neopallial cortex; nr, neural retina; ns, cartilage primordium of nasal septum; oep; olfactory epithelium; onh, optic nerve head; v, vitreous; vz, ventricular zone of telencephalon.

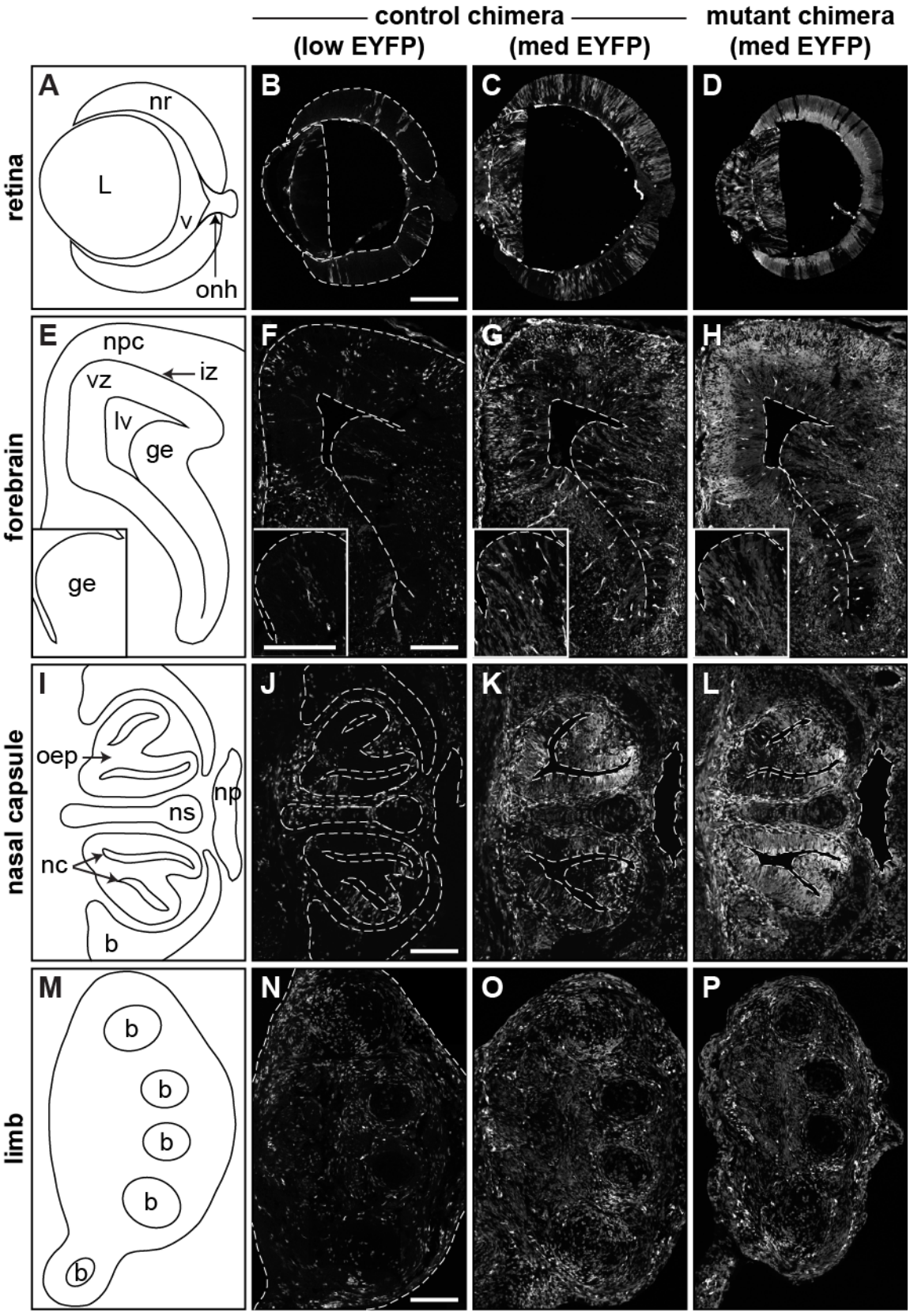

control chimera
(low EYFP)
(med EYFP)
mutant chimera
(med EYFP)
retina
forebrain
nasal capsule
limb
A
nr
L
v
onh
B
C
D
E
npc
vz
iz
lv
ge
ge
F
G
H
I
oep
np
ns
nc
b
J
K
L
M
b
b
b
b
b
N
O
P

Figure 4.3. Cell autonomous regulation of MITF expression by Vsx2. (A-B) Control MITF expression in E12.5 eyes of wild type and *orJ* mice. Nonspecific staining occurs along the vitreal edges of the lens and retina, in the developing corneal epithelium and extraocular mesenchyme, but not in the RPE or retina. (C-E) EYFP and MITF expression in E12.5 retinas of control and mutant chimeras. Scale bars: 100 μm (A, B, C); 40 μm (A', B'). Abbreviations: L, lens; NR, neural retina; RPE, retinal pigmented epithelium.

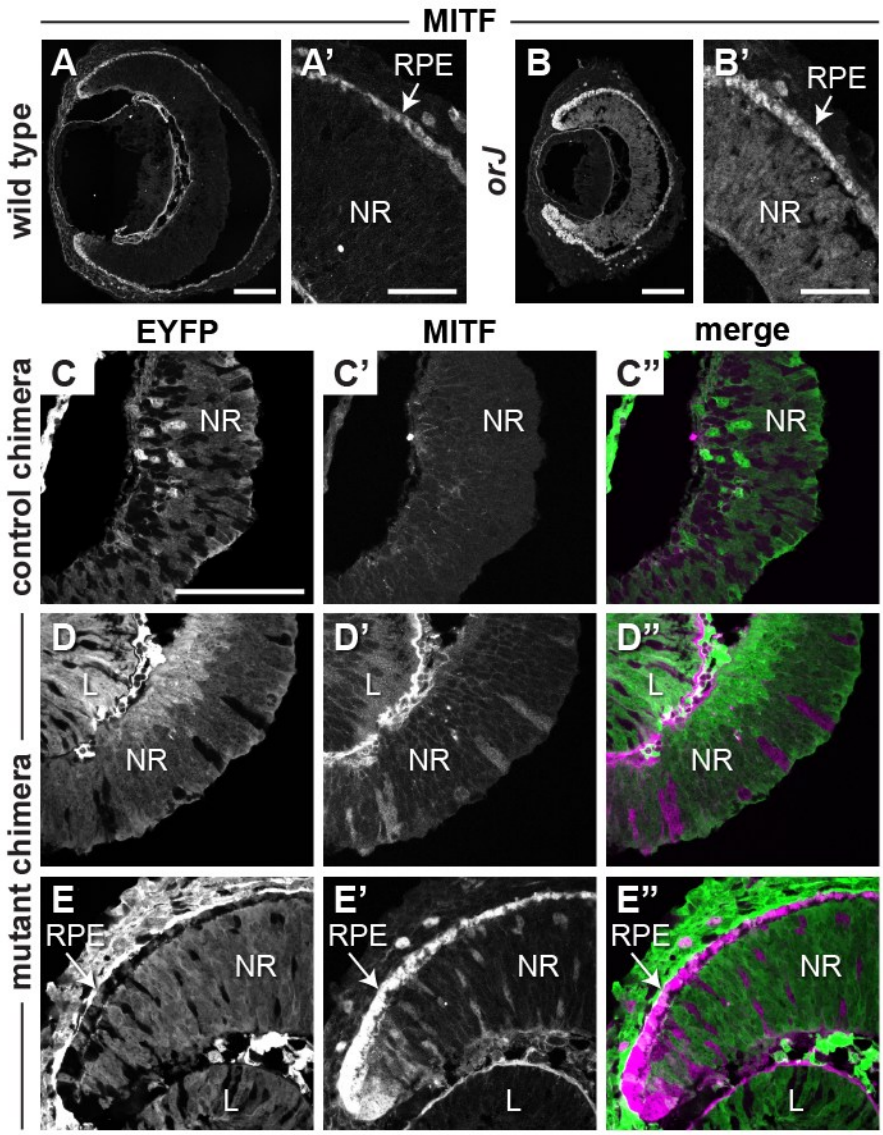

MITF
wild type
A
A'
RPE
NR
orJ
B
B'
RPE
NR
EYFP
MITF
merge
control chimera
C
NR
C'
NR
C''
NR
mutant chimera
D
L
NR
D'
L
NR
D''
L
NR
E
RPE
NR
L
E'
RPE
NR
L
E''
RPE
NR
L

Figure 4.4. Cell autonomous regulation of MITF expression in *orJ* retinas by Lhx2. (A-B) LHX2 expression in wild type and *orJ* eyes at E12.5. β-gal (C) and MITF (C') expression in E12.5 eyes of *orJ* mice with conditional inactivation of *Lhx2* in the retina by α-Cre. β-gal expression marks areas of successful *Lhx2* inactivation. Dashed lines in C-C" demarcate the border of the neural retina. Scale bars: 100 μm (A, B, C); 40 μm (A', B'). Abbreviations: CKO, conditional knockout; NR, neural retina; RPE, retinal pigmented epithelium.

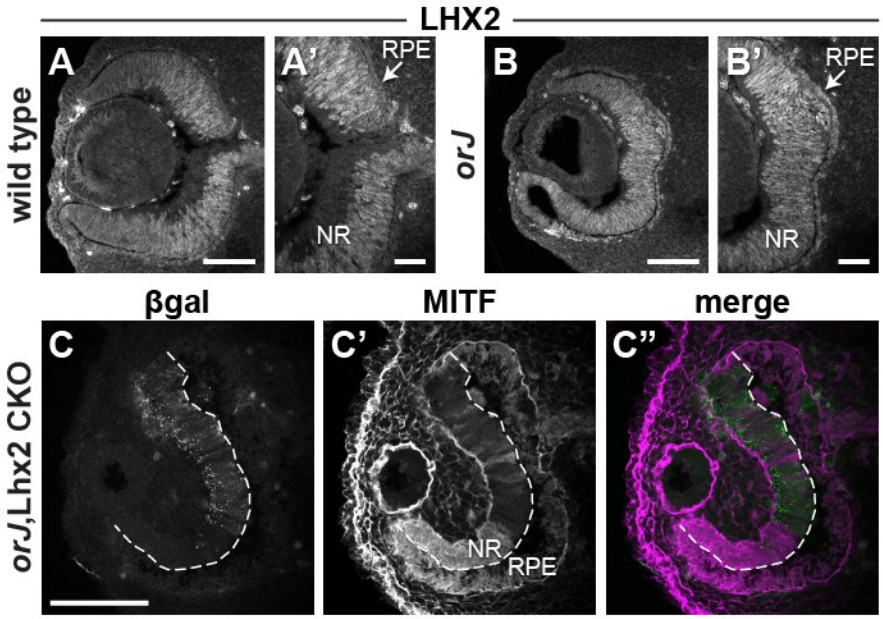

LHX2
wild type
A
A'
RPE
NR
orJ
B
B'
RPE
NR
βgal
MITF
merge
orJ,Lhx2 CKO
C
C'
NR
RPE
C''

Figure 4.5. Cell nonautonomous regulation of RPC proliferation by Vsx2. (A, B) EdU incorporation in E12.5 retinas of wild type and *orJ* mice. EYFP expression (C, D) and EdU incorporation (C', D') in retinas of mutant chimeras in peripheral (C) and central (D) regions. White arrows indicate EdU-positive, EYFP-negative *orJ* cells in mutant chimeras. Red arrowheads in C demarcate the peripheral region from the adjacent intermediate region in this retina. (E) Quantification of EdU-positive *orJ* cells at E12.5 in peripheral and central regions. Bars represent mean ± standard deviation. *p*–values calculated by Student's unpaired *t*-test or Welch's two sample *t*-test, as appropriate (based on results of an F-test of variances). Scale bars: 100 μm.

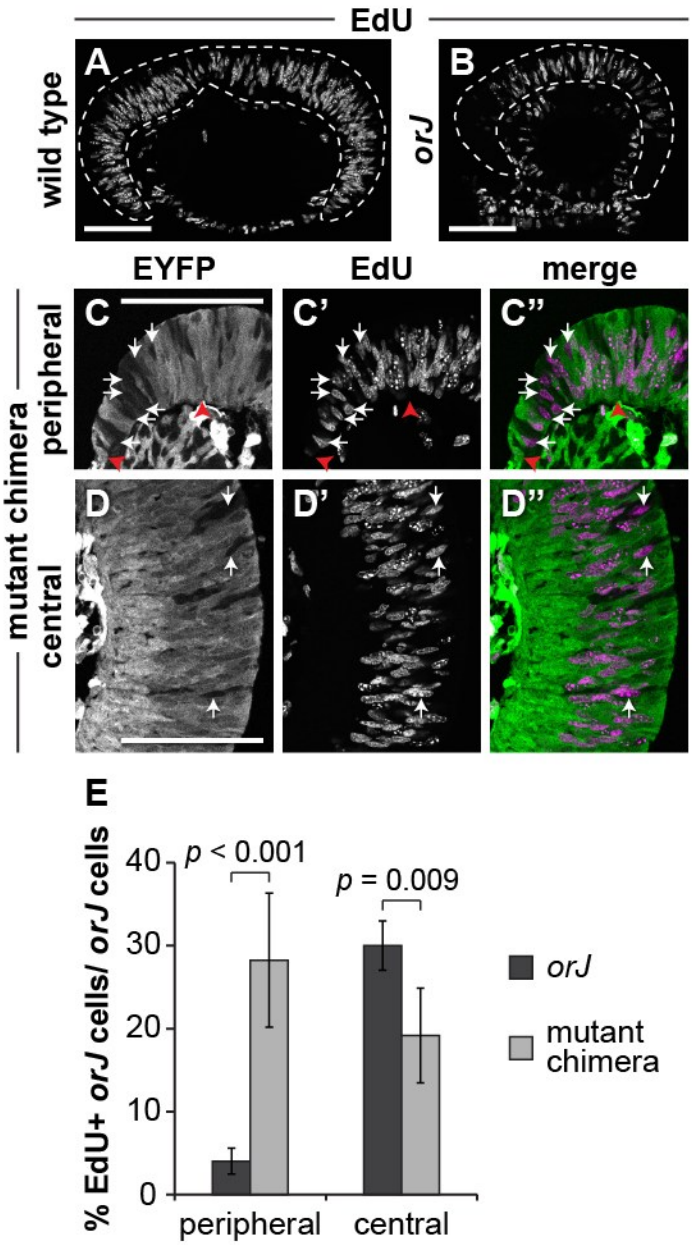

EdU
wild type
A
B
orJ
EYFP
EdU
merge
mutant chimera
peripheral
central
C
C'
C''
D
D'
D''
E
% EdU+ orJ cells/ orJ cells
p < 0.001
p = 0.009
40
30
20
10
0
peripheral
central
orJ
mutant chimera

Figure 4.6. *orJ* cells are rarely located in the differentiated cell layer of mutant chimeras, unlike wild type cells in control chimeras. (A) Diagram illustrating the relationship between differentiation status and apical-basal position within the retina. Nascent postmitotic cells (gray cells at apical surface) migrate basally to establish a distinct differentiated cell layer. (B, C) Distribution of EYFP-positive and EYFP-negative cells in E12.5 retinas of mutant (B) and control (C) chimeras. Boxed area in (B, C) shown at higher magnification to right (a, b). Scale bars: 100 μm. Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer.

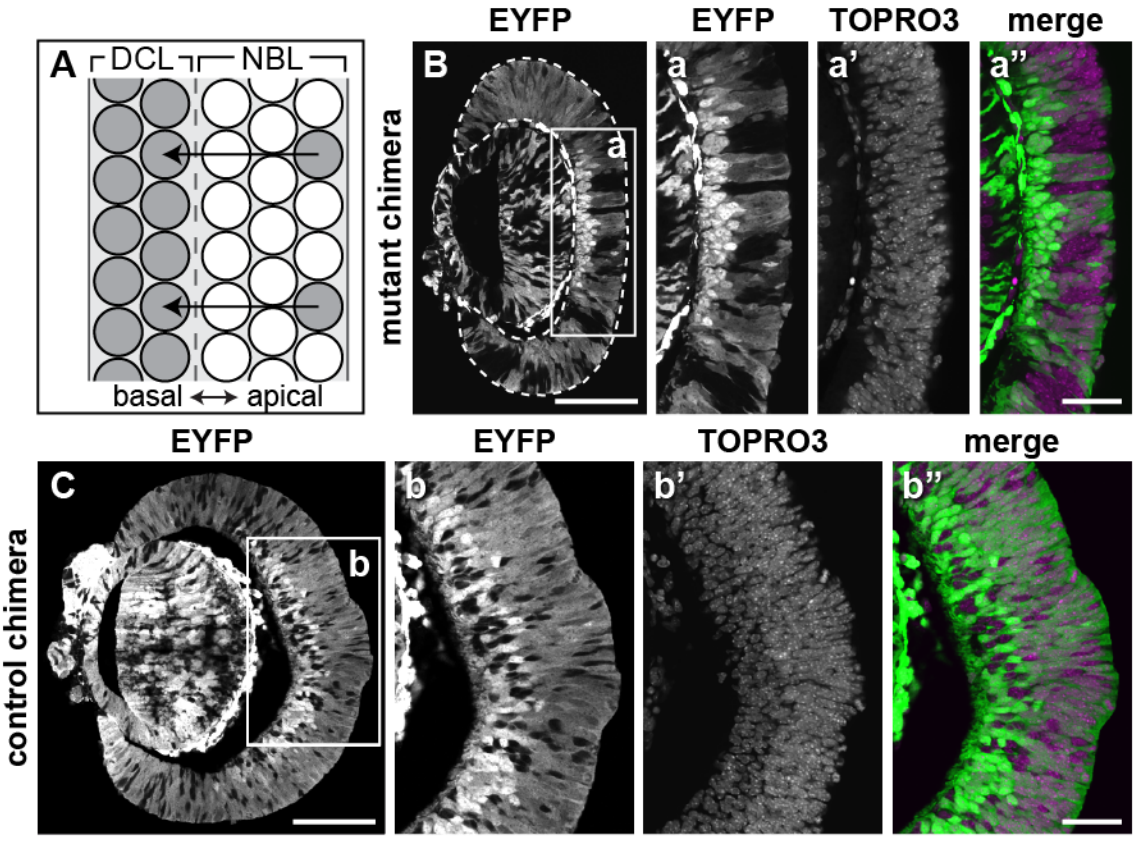

A
DCL
NBL
basal ↔ apical
EYFP
EYFP
TOPRO3
merge
B
a
a'
a''
mutant chimera
EYFP
EYFP
TOPRO3
merge
C
b
b'
b''
control chimera

Figure 4.7. *orJ* cells fail to express differentiation markers at E12.5. Expression of POU4F2 (A-D) and TUBB3 (E-H) in retinas of wild type (A, E), *orJ* (B, F), control chimeras (C, G), and mutant chimeras (D, H) at E12.5. Boxed areas in (C, D, G, H) shown at higher magnification to right (a, b, c, d). Dashed line in B delineates the retina from adjacent lens tissue. Dashed line in E and F delineates the boundary of retinal tissue. All images are maximum Z-projections of confocal scans. Scale bars: 100 μm (A, C, E, G); 40 μm (a, c).

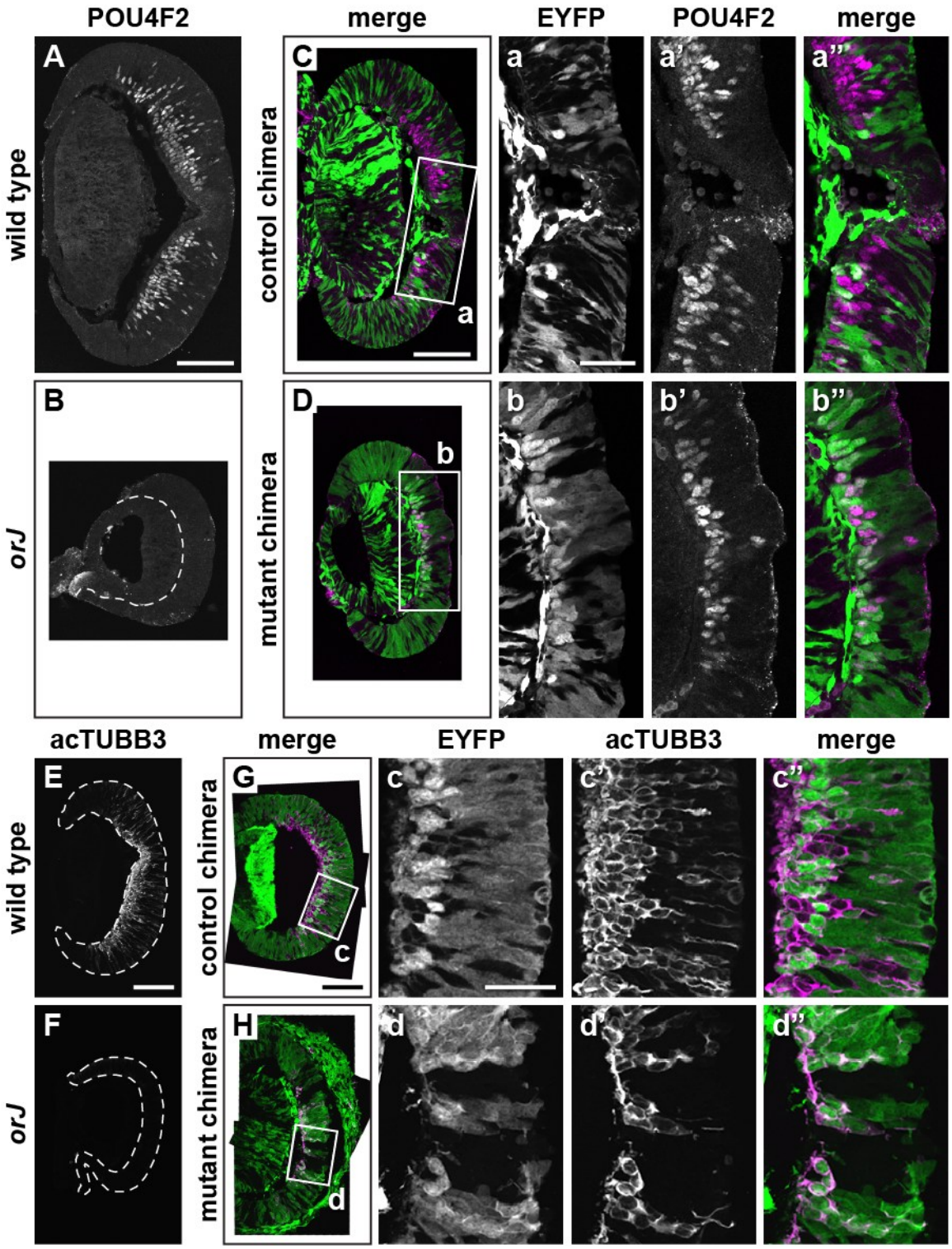

POU4F2
merge
EYFP
POU4F2
merge
wild type
control chimera
A
C
a
a'
a''
orJ
mutant chimera
B
D
b
b
b'
b''
acTUBB3
merge
EYFP
acTUBB3
merge
wild type
control chimera
E
G
c
c
c'
c''
orJ
mutant chimera
F
H
d
d
d'
d''

Figure 4.8. *orJ* cells fail to express differentiation markers at E12.5. Expression of the combination marker stain (Combo; A-D) and OTX2 (E-H) in retinas of wild type (A, E), *orJ* (B, F), control chimeras (C, G), and mutant chimeras (D, H) at E12.5. Boxed areas in (C, D, G, H) shown at higher magnification to right (a, b, c, d). Combo stain represents simultaneous staining for ISL1, POU4F2, PTF1A, and BHLHB5. All images are maximum Z-projections of confocal scans. Scale bars: 100 μm (A, C, E, G, I, H); 40 μm (C', G', H').

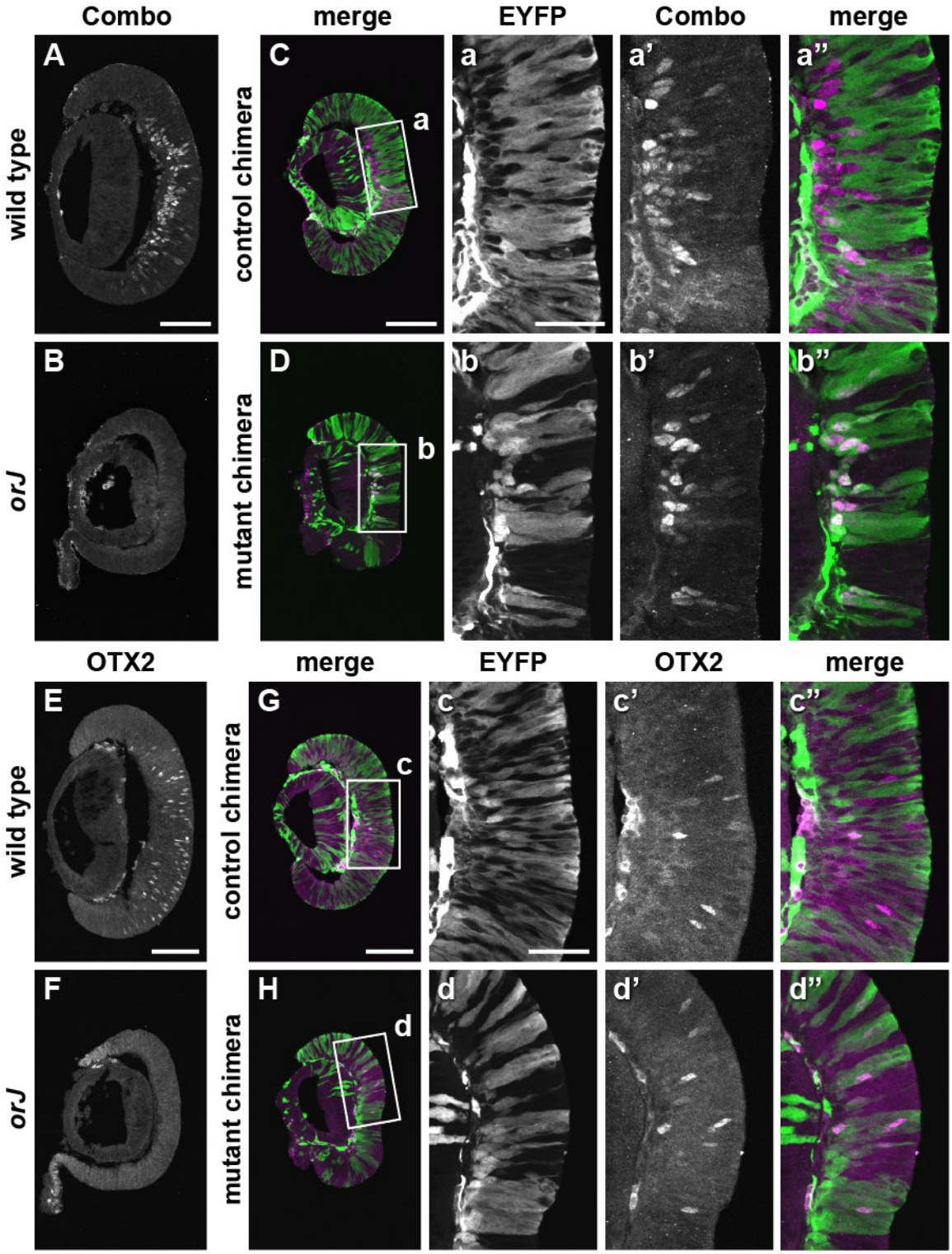

Combo
merge
EYFP
Combo
merge
A
C
a
a'
a''
wild type
control chimera
B
D
b
b'
b''
orJ
mutant chimera
OTX2
merge
EYFP
OTX2
merge
E
G
c
c'
c''
wild type
control chimera
F
H
d
d'
d''
orJ
mutant chimera

Figure 4.9. Early-born retinal cell types are generated in *orJ* retinas by E15.5, but the extent of differentiation is more centrally restricted than in wild type retinas. Expression of TUBB3 (A, F), POU4F2 (B, G), OTX2 (C, H), PTF1A (D, I), and BHLHB5 (E, J) in *orJ* (A-E) and wild type (F-J) retinas at E15.5. White bars in G and J consequence of nonoverlapping fields of view during image capture. Scale bars: 100 μm (A, F). Note size difference in scale bars.

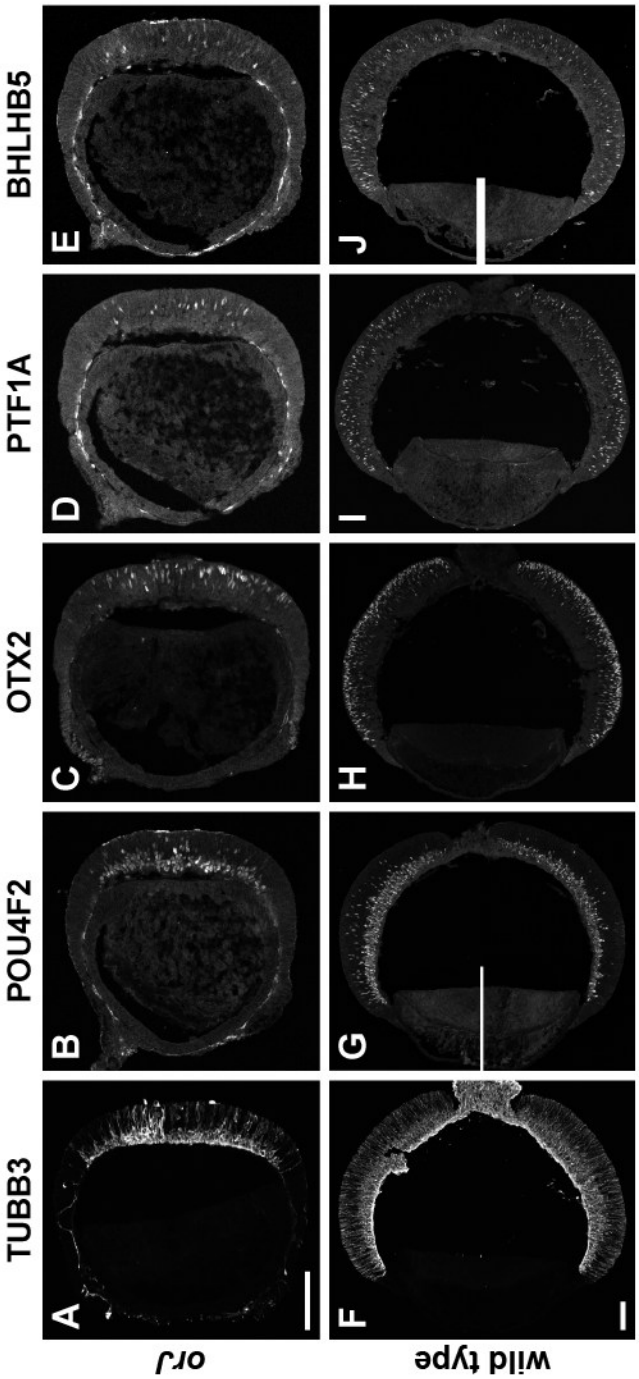

TUBB3
POU4F2
OTX2
PTF1A
BHLHB5
A
B
C
D
E
F
G
H
I
J
orJ
wild type

Figure 4.10. At E15.5, *orJ* cells contribute to all early-born retinal cell types in the retinas of mutant chimeras. Expression of TUBB3 (A), POU4F2 (B), OTX2 (C), PTF1A (D) and BHLHB5 (E) in retinas of mutant chimeras at E15.5. Scale bars: 100 μm.

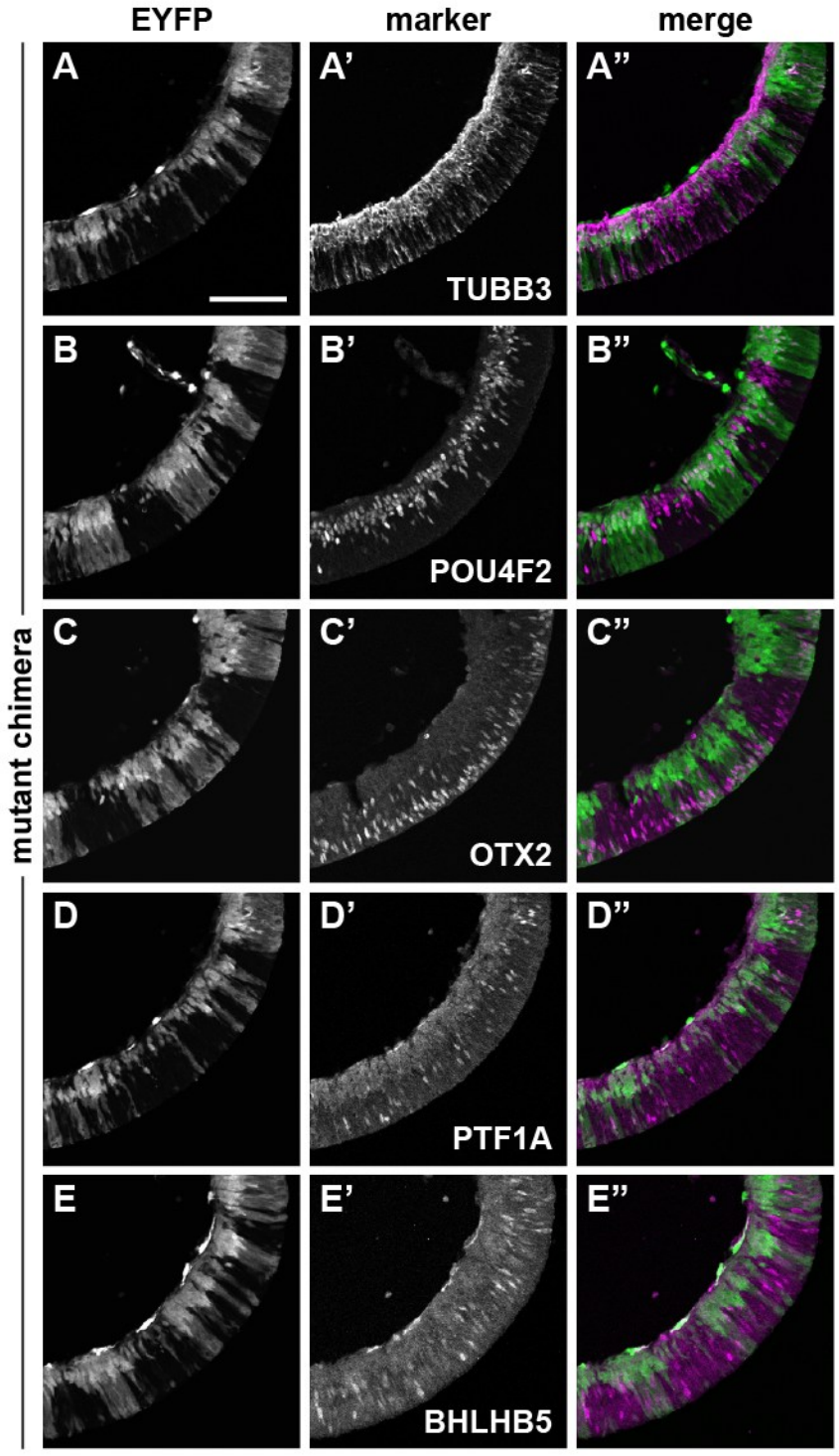

EYFP
marker
merge
mutant chimera
A
A'
A''
TUBB3
B
B'
B''
POU4F2
C
C'
C''
OTX2
D
D'
D''
PTF1A
E
E'
E''
BHLHB5

Figure 4.11. At E15.5, wild type cells contribute to all early-born retinal cell types in the retinas of control chimeras. Expression of TUBB3 (A), POU4F2 (B), OTX2 (C), PTF1A (D) and BHLHB5 (E) in retinas of control chimeras at E15.5. Scale bars: 100 μm.

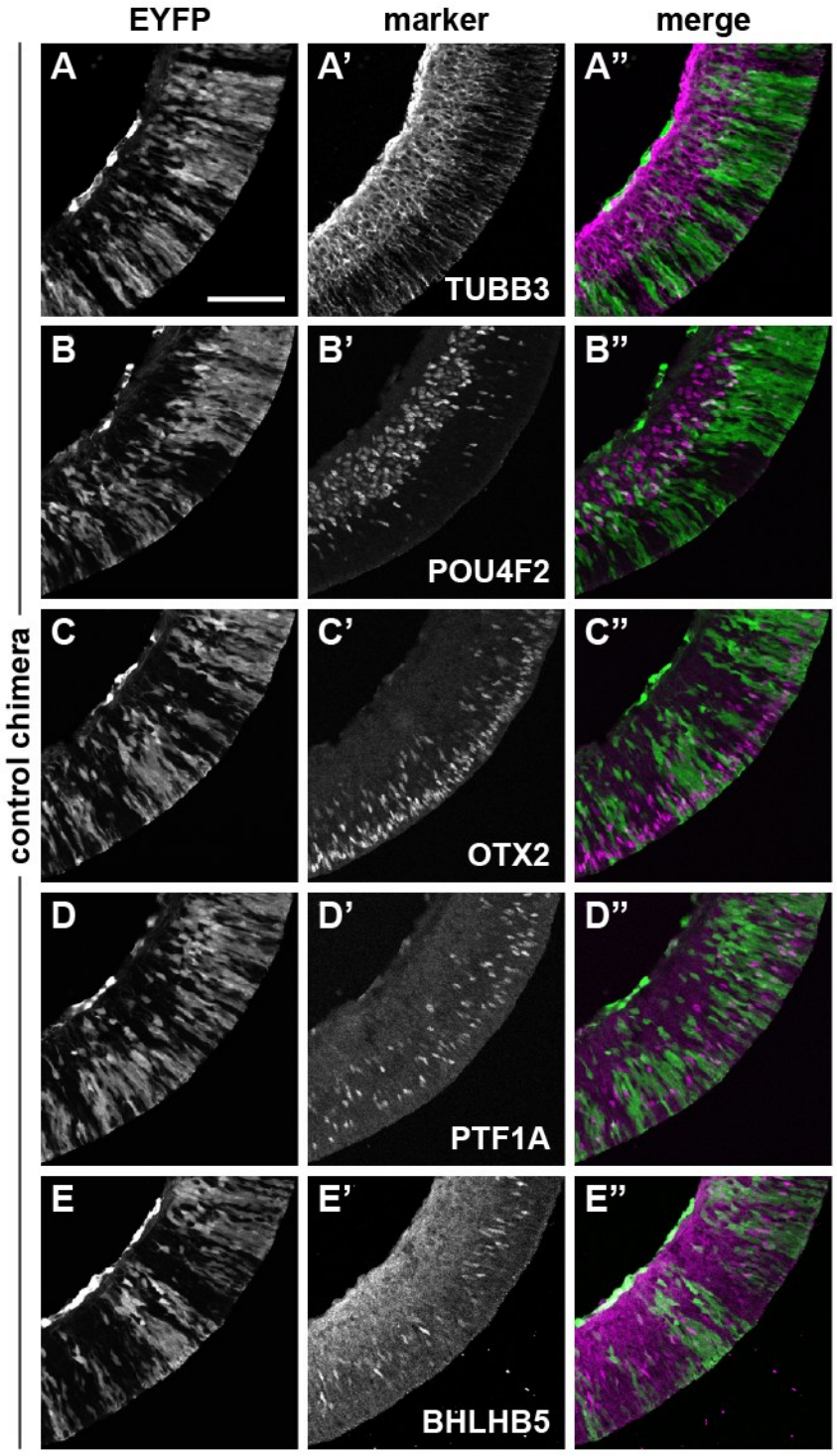

EYFP
marker
merge
A
A'
A''
TUBB3
B
B'
B''
POU4F2
C
C'
C''
OTX2
D
D'
D''
PTF1A
E
E'
E''
BHLHB5
control chimera

Figure 4.12. Delayed differentiation of *orJ* cells persists in the periphery of mutant chimeras. (A-B) EYFP and TUBB3 expression in the peripheral retina of control and mutant chimeras at E15.5. DAPI expression reveals the presence of cells in areas lacking EYFP and TUBB3 expression (red brackets in B). (C) EYFP (green) and POU4F2 (magenta) expression in retina of a mutant chimera at E14.5. Expression of EYFP, POU4F2 and TUBB3 for boxed regions in D are shown at higher magnification in (a, b). Retina in C was co-immunolabeled for POU4F2 and TUBB3 on the same retinal expression. Red brackets in b indicate region of *orJ* cells. Arrows in a indicate differentiated *orJ* cells in the neuroblast (white arrows) or differentiated (red arrows) cell layers. Scale bar: 100 μm.

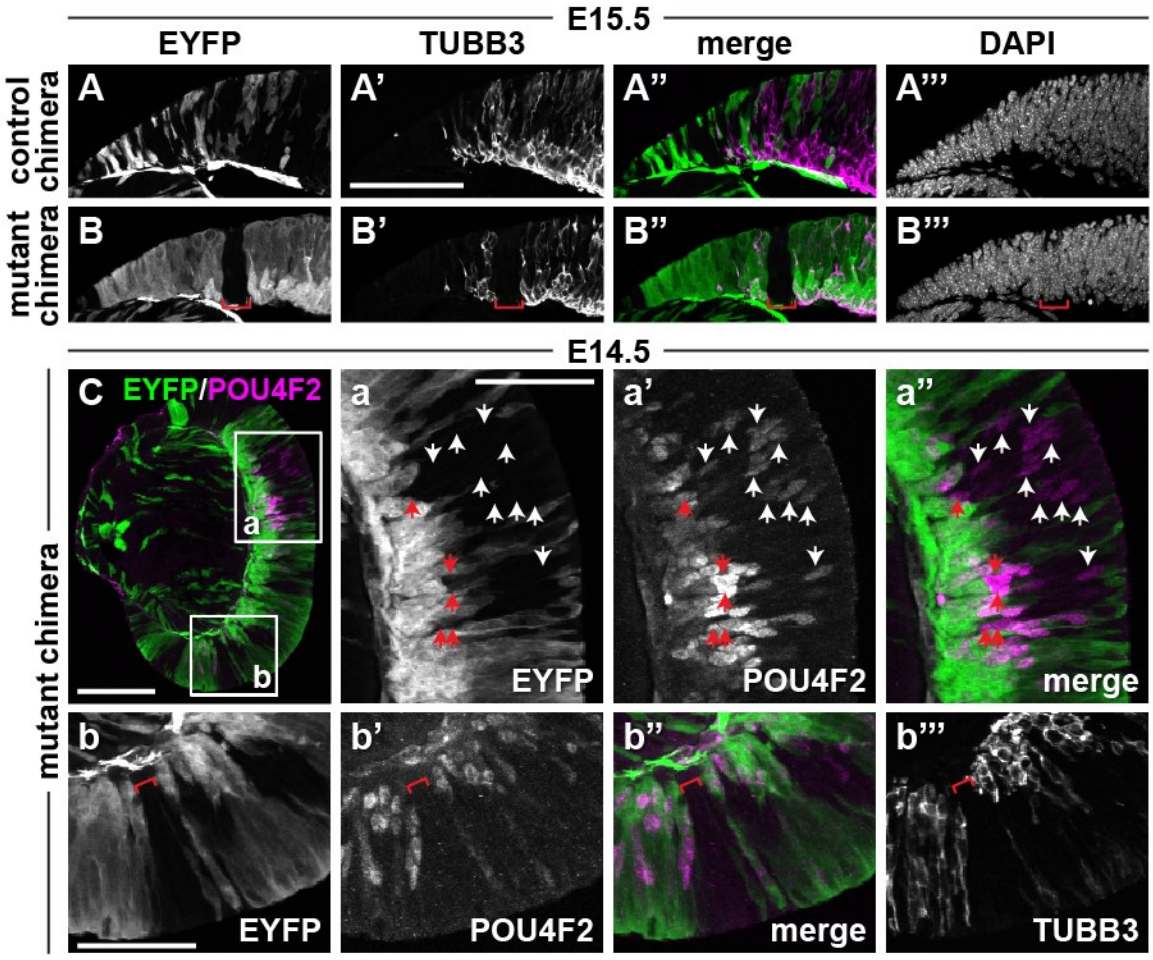

E15.5
EYFP
TUBB3
merge
DAPI
control chimera
mutant chimera
A
A'
A''
A'''
B
B'
B''
B'''
E14.5
mutant chimera
C EYFP/POU4F2
a
b
a
a'
a''
EYFP
POU4F2
merge
b
b'
b''
b'''
EYFP
POU4F2
merge
TUBB3

Figure 4.13. Precocious differentiation of wild type cells in regions of high *orJ* contribution in mutant chimeras. Expression of POU4F2 (A) and OTX2 (B) in retinas of mutant chimeras at E15.5. Quantification of POU4F2-positive (C) and OTX2-positive (D) wild type cells as a function of patch type in retinas of mutant chimeras at E15.5. Black diamonds represent individual patch values and illustrate the variation within patches of the same type. Red squares represent mean ± standard deviation. *p*-values calculated by Student's unpaired *t*-test or Welch's two sample *t*-test, as appropriate (based on results of an F-test of variances). Scale bars: 100 μm.

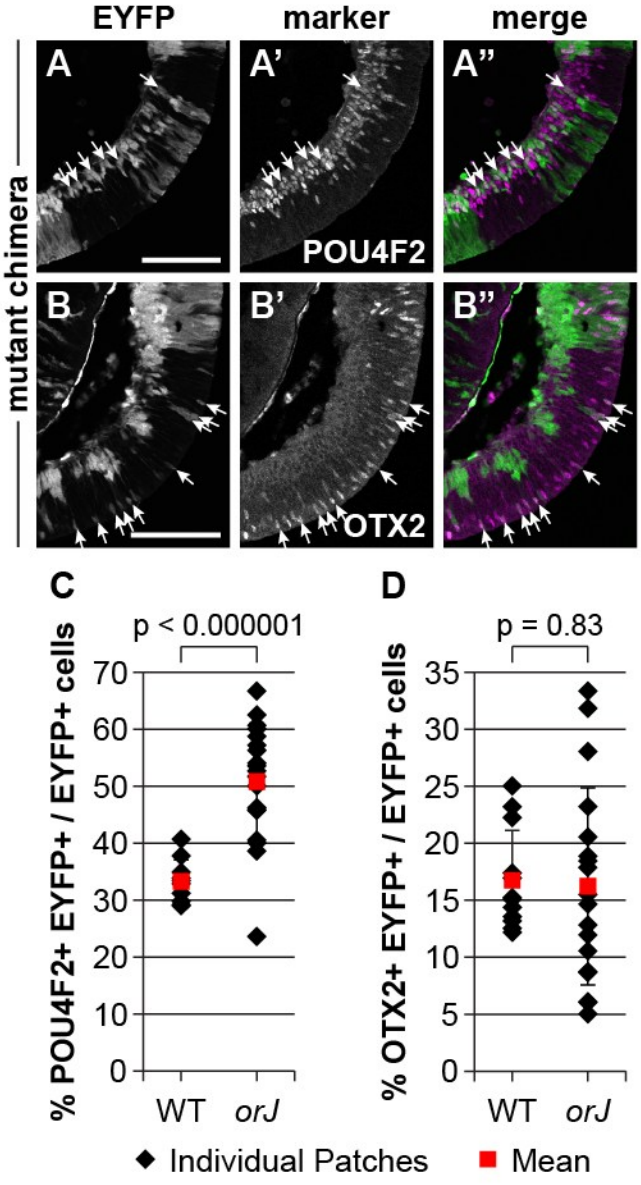
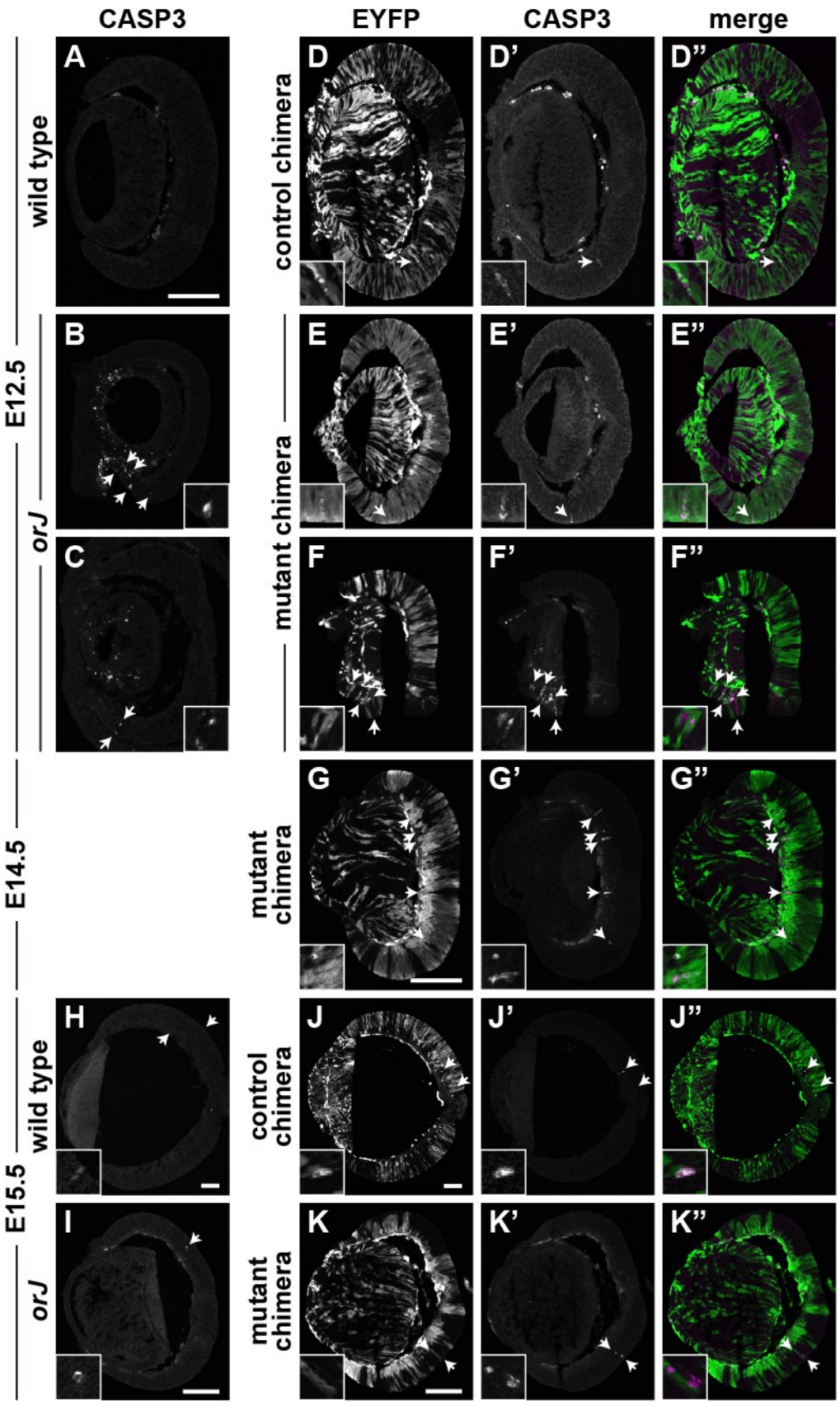

EYFP
marker
merge
mutant chimera
A
A'
A''
POU4F2
B
B'
B''
OTX2
C
p < 0.000001
% POU4F2+ EYFP+ / EYFP+ cells
70
60
50
40
30
20
10
0
WT
orJ
D
p = 0.83
% OTX2+ EYFP+ / EYFP+ cells
35
30
25
20
15
10
5
0
WT
orJ
Individual Patches
Mean

Figure 4.14. Low levels of cell death are detected in control and chimeric retinas. Expression of activated caspase-3 (CASP3) in retinas of wild type, *orJ*, control chimeras, and mutant chimeras at E12.5 (A-F), E14.5 (G), and E15.5 (H-K). Insets show retinal cells stained for activated caspase-3 at higher magnification. Arrows point to retinal cells expressing activated caspase-3. Scale bars: 100 µm. Note size difference in scale bars (A, G, H, I, J, K).


CASP3
EYFP
CASP3
merge
E12.5
wild type
orJ
E14.5
E15.5
wild type
orJ
control chimera
mutant chimera
mutant chimera
control chimera
mutant chimera
A
B
C
D
D'
D''
E
E'
E''
F
F'
F''
G
G'
G''
H
I
J
J'
J''
K
K'
K''

References

Alexiades, M.R., Cepko, C., 1996. Quantitative analysis of proliferation and cell cycle length during development of the rat retina. Dev. Dyn. 205, 293-307.

Ashery-Padan, R., Marquardt, T., Zhou, X., Gruss, P., 2000. Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. Genes Dev. 14, 2701-2711.

Bar-Yosef, U., Abuelaish, I., Harel, T., Hendler, N., Ofir, R., Birk, O.S., 2004. CHX10 mutations cause non-syndromic microphthalmia/ anophthalmia in Arab and Jewish kindreds. Hum. Genet. 115, 302-309.

Barabino, S.M., Spada, F., Cotelli, F., Boncinelli, E., 1997. Inactivation of the zebrafish homologue of Chx10 by antisense oligonucleotides causes eye malformations similar to the ocular retardation phenotype. Mech. Dev. 63, 133-143.

Belecky-Adams, T., Tomarev, S., Li, H.S., Ploder, L., McInnes, R.R., Sundin, O., Adler, R., 1997. Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. Invest. Ophthalmol. Vis. Sci. 38, 1293-1303.

Bharti, K., Liu, W., Csermely, T., Bertuzzi, S., Arnheiter, H., 2008. Alternative promoter use in eye development: the complex role and regulation of the transcription factor MITF. Development 135, 1169-1178.

Bone-Larson, C., Basu, S., Radel, J.D., Liang, M., Perozek, T., Kapousta-Bruneau, N., Green, D.G., Burmeister, M., Hankin, M.H., 2000. Partial rescue of the ocular retardation phenotype by genetic modifiers. J. Neurobiol. 42, 232-247.

Brittis, P.A., Meiri, K., Dent, E., Silver, J., 1995. The earliest patterns of neuronal differentiation and migration in the mammalian central nervous system. Exp. Neurol. 134, 1-12.

Bumsted, K.M., Barnstable, C.J., 2000. Dorsal retinal pigment epithelium differentiates as neural retina in the microphthalmia (mi/mi) mouse. Invest. Ophthalmol. Vis. Sci. 41, 903-908.

Burmeister, M., Novak, J., Liang, M.Y., Basu, S., Ploder, L., Hawes, N.L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V.I., Roderick, T.H., Taylor, B.A., Hankin, M.H., McInnes, R.R., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. 12, 376-384.

Buskirk, D.R., Thiery, J.P., Rutishauser, U., Edelman, G.M., 1980. Antibodies to a neural cell adhesion molecule disrupt histogenesis in cultured chick retinae. Nature 285, 488-489.

Cai, Z., Feng, G.S., Zhang, X., 2010. Temporal requirement of the protein tyrosine phosphatase Shp2 in establishing the neuronal fate in early retinal development. J. Neurosci. 30, 4110-4119.

Carreira, S., Goodall, J., Aksan, I., La Rocca, S.A., Galibert, M.D., Denat, L., Larue, L., Goding, C.R., 2005. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature 433, 764-769.

Collinson, J.M., Hill, R.E., West, J.D., 2000. Different roles for Pax6 in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye. Development 127, 945-956.

Collinson, J.M., Quinn, J.C., Hill, R.E., West, J.D., 2003. The roles of Pax6 in the cornea, retina, and olfactory epithelium of the developing mouse embryo. Dev. Biol. 255, 303-312.

Crosby, J.R., Seifert, R.A., Soriano, P., Bowen-Pope, D.F., 1998. Chimaeric analysis reveals role of Pdgf receptors in all muscle lineages. Nat. Genet. 18, 385-388.

Daniele, L.L., Adams, R.H., Durante, D.E., Pugh, E.N., Jr., Philp, N.J., 2007. Novel distribution of junctional adhesion molecule-C in the neural retina and retinal pigment epithelium. J. Comp. Neurol. 505, 166-176.

Das, G., Choi, Y., Sicinski, P., Levine, E.M., 2009. Cyclin D1 fine-tunes the neurogenic output of embryonic retinal progenitor cells. Neural Dev. 4, 15.

Dhomen, N.S., Balaggan, K.S., Pearson, R.A., Bainbridge, J.W., Levine, E.M., Ali, R.R., Sowden, J.C., 2006. Absence of chx10 causes neural progenitors to persist in the adult retina. Invest. Ophthalmol. Vis. Sci. 47, 386-396.

Dorval, K.M., Bobechko, B.P., Fujieda, H., Chen, S., Zack, D.J., Bremner, R., 2006. CHX10 targets a subset of photoreceptor genes. J. Biol. Chem. 281, 744-751.

Elshatory, Y., Deng, M., Xie, X., Gan, L., 2007a. Expression of the LIM-homeodomain protein Isl1 in the developing and mature mouse retina. J. Comp. Neurol. 503, 182-197.

Elshatory, Y., Everhart, D., Deng, M., Xie, X., Barlow, R.B., Gan, L., 2007b. Islet-1 controls the differentiation of retinal bipolar and cholinergic amacrine cells. J. Neurosci. 27, 12707-12720.

Erkman, L., McEvilly, R.J., Luo, L., Ryan, A.K., Hooshmand, F., O'Connell, S.M., Keithley, E.M., Rapaport, D.H., Ryan, A.F., Rosenfeld, M.G., 1996. Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature 381, 603-606.

Faulkner-Jones, B.E., Godinho, L.N., Tan, S.S., 1999. Multiple cadherin mRNA expression and developmental regulation of a novel cadherin in the developing mouse eye. Exp. Neurol. 156, 316-325.

Ferda Percin, E., Ploder, L.A., Yu, J.J., Arici, K., Horsford, D.J., Rutherford, A., Bapat, B., Cox, D.W., Duncan, A.M., Kalnins, V.I., Kocak-Altintas, A., Sowden, J.C., Traboulsi, E., Sarfarazi, M., McInnes, R.R., 2000. Human microphthalmia associated with mutations in the retinal homeobox gene CHX10. Nat. Genet. 25, 397-401.

Fu, X., Sun, H., Klein, W.H., Mu, X., 2006. Beta-catenin is essential for lamination but not neurogenesis in mouse retinal development. Dev. Biol. 299, 424-437.

Fuhrmann, S., Levine, E.M., Reh, T.A., 2000. Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. Development 127, 4599-4609.

Gan, L., Wang, S.W., Huang, Z., Klein, W.H., 1999. POU domain factor Brn-3b is essential for retinal ganglion cell differentiation and survival but not for initial cell fate specification. Dev. Biol. 210, 469-480.

Gan, L., Xiang, M., Zhou, L., Wagner, D.S., Klein, W.H., Nathans, J., 1996. POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. Proc. Natl. Acad. Sci. U. S. A. 93, 3920-3925.

Georgiadis, A., Tschernutter, M., Bainbridge, J.W., Balaggan, K.S., Mowat, F., West, E.L., Munro, P.M., Thrasher, A.J., Matter, K., Balda, M.S., Ali, R.R., 2010. The tight junction associated signalling proteins ZO-1 and ZONAB regulate retinal pigment epithelium homeostasis in mice. PLoS One 5, e15730.

Green, E.S., Stubbs, J.L., Levine, E.M., 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development 130, 539-552.

Hashimoto, T., Zhang, X.M., Chen, B.Y., Yang, X.J., 2006. VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation. Development 133, 2201-2210.

Hatakeyama, J., Tomita, K., Inoue, T., Kageyama, R., 2001. Roles of homeobox and bHLH genes in specification of a retinal cell type. Development 128, 1313-1322.

Honjo, M., Tanihara, H., Suzuki, S., Tanaka, T., Honda, Y., Takeichi, M., 2000. Differential expression of cadherin adhesion receptors in neural retina of the postnatal mouse. Invest. Ophthalmol. Vis. Sci. 41, 546-551.

Horsford, D.J., Nguyen, M.T., Sellar, G.C., Kothary, R., Arnheiter, H., McInnes, R.R., 2005. Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. Development 132, 177-187.

Hufnagel, R.B., Le, T.T., Riesenberg, A.L., Brown, N.L., 2010. Neurog2 controls the leading edge of neurogenesis in the mammalian retina. Dev. Biol. 340, 490-503.

Irvine, K.D., Rauskolb, C., 2001. Boundaries in development: formation and function. Annu. Rev. Cell Dev. Biol. 17, 189-214.

Ito, A., Jippo, T., Wakayama, T., Morii, E., Koma, Y., Onda, H., Nojima, H., Iseki, S., Kitamura, Y., 2003. SgIGSF: a new mast-cell adhesion molecule used for attachment to fibroblasts and transcriptionally regulated by MITF. Blood 101, 2601-2608.

Jadhav, A.P., Mason, H.A., Cepko, C.L., 2006. Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development 133, 913-923.

Jensen, A.M., Wallace, V.A., 1997. Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development 124, 363-371.

Kay, J.N., Link, B.A., Baier, H., 2005. Staggered cell-intrinsic timing of ath5 expression underlies the wave of ganglion cell neurogenesis in the zebrafish retina. Development 132, 2573-2585.

Kim, D.K., Morii, E., Ogihara, H., Hashimoto, K., Oritani, K., Lee, Y.M., Jippo, T., Adachi, S., Kanakura, Y., Kitamura, Y., 1998. Impaired expression of integrin alpha-4 subunit in cultured mast cells derived from mutant mice of mi/mi genotype. Blood 92, 1973-1980.

Kindiakov, B.N., Koniukhov, B.V., 1986. [Mutant gene expression in murine aggregation chimeras. 5. The ocular retardation and fidget genes]. Ontogenez 17, 47-55.

Konyukhov, B.V., Sazhina, M.V., 1966. Interaction of the genes of ocular retardation and microphthalmia in mice. Folia Biol. 12, 116-123.

Konyukhov, B.V., Sazhina, M.V., 1971. Genetic control over the duration of G 1 phase. Experientia 27, 970-971.

Lee, M.K., Tuttle, J.B., Rebhun, L.I., Cleveland, D.W., Frankfurter, A., 1990. The expression and posttranslational modification of a neuron-specific beta-tubulin isotype during chick embryogenesis. Cell Motil. Cytoskeleton 17, 118-132.

Lekmine, F., Chang, C.K., Sethakorn, N., Das Gupta, T.K., Salti, G.I., 2007. Role of microphthalmia transcription factor (Mitf) in melanoma differentiation. Biochem. Biophys. Res. Commun. 354, 830-835.

Levine, E.M., Green, E.S., 2004. Cell-intrinsic regulators of proliferation in vertebrate retinal progenitors. Semin. Cell Dev. Biol. 15, 63-74.

Levine, E.M., Roelink, H., Turner, J., Reh, T.A., 1997. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J. Neurosci. 17, 6277-6288.

Li, S., Goldowitz, D., Swanson, D.J., 2007. The requirement of pax6 for postnatal eye development: evidence from experimental mouse chimeras. Invest. Ophthalmol. Vis. Sci. 48, 3292-3300.

Liu, I.S., Chen, J.D., Ploder, L., Vidgen, D., van der Kooy, D., Kalnins, V.I., McInnes, R.R., 1994. Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. Neuron 13, 377-393.

Livesey, F.J., Cepko, C.L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev. Neurosci. 2, 109-118.

Livne-Bar, I., Pacal, M., Cheung, M.C., Hankin, M., Trogadis, J., Chen, D., Dorval, K.M., Bremner, R., 2006. Chx10 is required to block photoreceptor differentiation but is dispensable for progenitor proliferation in the postnatal retina. Proc. Natl. Acad. Sci. U. S. A. 103, 4988-4993.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., Gruss, P., 2001. Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43-55.

Martinez-Morales, J.R., Del Bene, F., Nica, G., Hammerschmidt, M., Bovolenta, P., Wittbrodt, J., 2005. Differentiation of the vertebrate retina is coordinated by an FGF signaling center. Dev. Cell 8, 565-574.

McCabe, K.L., Gunther, E.C., Reh, T.A., 1999. The development of the pattern of retinal ganglion cells in the chick retina: mechanisms that control differentiation. Development 126, 5713-5724.

Medina-Martinez, O., Amaya-Manzanares, F., Liu, C., Mendoza, M., Shah, R., Zhang, L., Behringer, R.R., Mahon, K.A., Jamrich, M., 2009. Cell-autonomous requirement for rx function in the mammalian retina and posterior pituitary. PLoS One 4, e4513.

Moshiri, A., Reh, T.A., 2004. Persistent progenitors at the retinal margin of ptc+/- mice. J. Neurosci. 24, 229-237.

Mu, X., Beremand, P.D., Zhao, S., Pershad, R., Sun, H., Scarpa, A., Liang, S., Thomas, T.L., Klein, W.H., 2004. Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. Development 131, 1197-1210.

Nakayama, A., Nguyen, M.T., Chen, C.C., Opdecamp, K., Hodgkinson, C.A., Arnheiter, H., 1998. Mutations in microphthalmia, the mouse homolog of the human deafness gene MITF, affect neuroepithelial and neural crest-derived melanocytes differently. Mech. Dev. 70, 155-166.

Neill, J.M., Barnstable, C.J., 1990. Expression of the cell surface antigens RET-PE2 and N-CAM by rat retinal pigment epithelial cells during development and in tissue culture. Exp. Eye Res. 51, 573-583.

Neumann, C.J., Nuesslein-Volhard, C., 2000. Patterning of the zebrafish retina by a wave of sonic hedgehog activity. Science 289, 2137-2139.

Nguyen, M., Arnheiter, H., 2000. Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. Development 127, 3581-3591.

Osipov, V.V., Vakhrusheva, M.P., 1982. [Coat pigmentation and effect of the ocular retardation gene in the eye of chimeras between or/or and AKR mice]. Biull. Eksp. Biol. Med. 93, 84-86.

Osipov, V.V., Vakhrusheva, M.P., 1984. [Clonal analysis of the development of the pigment epithelium of the eye in chimeric or/or----AKR mice]. Ontogenez 15, 73-80.

Qiu, F., Jiang, H., Xiang, M., 2008. A comprehensive negative regulatory program controlled by Brn3b to ensure ganglion cell specification from multipotential retinal precursors. J. Neurosci. 28, 3392-3403.

Quinn, J.C., West, J.D., Hill, R.E., 1996. Multiple functions for Pax6 in mouse eye and nasal development. Genes Dev. 10, 435-446.

Reese, B.E., Necessary, B.D., Tam, P.P., Faulkner-Jones, B., Tan, S.S., 1999. Clonal expansion and cell dispersion in the developing mouse retina. Eur. J. Neurosci. 11, 2965-2978.

Riesenberg, A.N., Liu, Z., Kopan, R., Brown, N.L., 2009. Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J. Neurosci. 29, 12865-12877.

Robb, R.M., Silver, J., Sullivan, R.T., 1978. Ocular retardation (or) in the mouse. Invest. Ophthalmol. Vis. Sci. 17, 468-473.

Rowan, S., Cepko, C.L., 2004. Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. Dev.Biol. 271, 388-402.

Rowan, S., Chen, C.M., Young, T.L., Fisher, D.E., Cepko, C.L., 2004. Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. Development 131, 5139-5152.

Rutherford, A.D., Dhomen, N., Smith, H.K., Sowden, J.C., 2004. Delayed expression of the Crx gene and photoreceptor development in the Chx10-deficient retina. Invest. Ophthalmol. Vis. Sci. 45, 375-384.

Shahlaee, A.H., Brandal, S., Lee, Y.N., Jie, C., Takemoto, C.M., 2007. Distinct and shared transcriptomes are regulated by microphthalmia-associated transcription factor isoforms in mast cells. J. Immunol. 178, 378-388.

Sigulinsky, C.L., Green, E.S., Clark, A.M., Levine, E.M., 2008. Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina. Dev. Biol. 317, 560-575.

Simpson, T.I., Price, D.J., 2002. Pax6; a pleiotropic player in development. BioEssays 24, 1041-1051.

Sottocornola, R., Royer, C., Vives, V., Tordella, L., Zhong, S., Wang, Y., Ratnayaka, I., Shipman, M., Cheung, A., Gaston-Massuet, C., Ferretti, P., Molnar, Z., Lu, X., 2010. ASPP2 binds Par-3 and controls the polarity and proliferation of neural progenitors during CNS development. Dev. Cell 19, 126-137.

Strunnikova, N.V., Maminishkis, A., Barb, J.J., Wang, F., Zhi, C., Sergeev, Y., Chen, W., Edwards, A.O., Stambolian, D., Abecasis, G., Swaroop, A., Munson, P.J., Miller, S.S., 2010. Transcriptome analysis and molecular signature of human retinal pigment epithelium. Hum. Mol. Genet. 19, 2468-2486.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F., Kageyama, R., 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16, 723-734.

Toy, J., Norton, J.S., Jibodh, S.R., Adler, R., 2002. Effects of homeobox genes on the differentiation of photoreceptor and nonphotoreceptor neurons. Invest. Ophthalmol. Vis. Sci. 43, 3522-3529.

Truslove, G.M., 1962. A gene causing ocular retardation in the mouse. J. Embryol. Exp. Morphol. 10, 652-660.

Tsukiji, N., Nishihara, D., Yajima, I., Takeda, K., Shibahara, S., Yamamoto, H., 2009. Mitf functions as an in ovo regulator for cell differentiation and proliferation during development of the chick RPE. Dev. Biol. 326, 335-346.

Wallace, V.A., Raff, M.C., 1999. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126, 2901-2909.

Wang, Y., Dakubo, G.D., Thurig, S., Mazerolle, C.J., Wallace, V.A., 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development 132, 5103-5113.

Wang, Y.P., Dakubo, G., Howley, P., Campsall, K.D., Mazarolle, C.J., Shiga, S.A., Lewis, P.M., McMahon, A.P., Wallace, V.A., 2002. Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells. Nat. Neurosci. 5, 831-832.

Wei, X., Cheng, Y., Luo, Y., Shi, X., Nelson, S., Hyde, D.R., 2004. The zebrafish Pard3 ortholog is required for separation of the eye fields and retinal lamination. Dev. Biol. 269, 286-301.

Wohrn, J.C., Puelles, L., Nakagawa, S., Takeichi, M., Redies, C., 1998. Cadherin expression in the retina and retinofugal pathways of the chicken embryo. J. Comp. Neurol. 396, 20-38.

Xiang, M., Zhou, L., Peng, Y.W., Eddy, R.L., Shows, T.B., Nathans, J., 1993. Brn-3b: a POU domain gene expressed in a subset of retinal ganglion cells. Neuron 11, 689-701.

Xu, L., Overbeek, P.A., Reneker, L.W., 2002. Systematic analysis of E-, N- and P-cadherin expression in mouse eye development. Exp. Eye Res. 74, 753-760.

Young, R.W., 1985. Cell differentiation in the retina of the mouse. Anat. Rec. 212, 199-205.

Yun, S., Saijoh, Y., Hirokawa, K.E., Kopinke, D., Murtaugh, L.C., Monuki, E.S., Levine, E.M., 2009. Lhx2 links the intrinsic and extrinsic factors that control optic cup formation. Development 136, 3895-3906.

Zou, C., Levine, E.M., 2012. Vsx2 controls eye organogenesis and retinal progenitor identity via homeodomain and non-homeodomain residues required for high affinity DNA binding. PLoS Genet. 8, e1002924.

CHAPTER 5

DISCUSSION

Summary and interpretation

In the present work, we sought to further define the roles of Vsx2 in the regulation of retinal histogenesis. The cellular processes involved in the development of the retina during this period are tightly regulated by both intrinsic factors and extrinsic signals. Vsx2 is an important intrinsic factor in this regulation, but how Vsx2 function integrates with other known regulators is only beginning to be unraveled and few direct targets have been identified. In particular, an understanding of whetherVsx2-mediated regulation integrates with that of extrinsic signals was lacking. Thus, in the present work, we addressed this question by examining the relationship between Vsx2 and the extracellular signals and signaling pathways regulating retinal progenitor cell (RPC) properties during retinal histogenesis, using multiple approaches. Using a candidate approach, we examined the known retinal mitogen, sonic hedgehog (Shh), and its signaling pathway in *orJ* retinas to determine whether changes in this pathway contributed to the disrupted RPC proliferation. In a more unbiased approach, we evaluated the contribution of Vsx2-dependent changes in extrinsic signals to the disruption of retinal histogenesis by determining the autonomy of Vsx2 gene function in genetic mouse chimeras. Together, these approaches have advanced our knowledge of Vsx2-mediated regulation of retinal identity maintenance, RPC proliferation, and neurogenesis, and have provided insight into how this regulation may integrate with that of extrinsic signals.

Retinal identity

Despite its use as one of the earliest, most specific markers of the neural retina domain, the role of Vsx2 in retinal identity appears to be one of maintenance, rather than

specification. In this role, a primary function of Vsx2 is to ensure efficient execution of the retinal program by suppressing competing nonretinal gene expression programs. Much of this regulation involves repression of the RPE determinant, Mitf (Horsford et al., 2005; Rowan et al., 2004). Mitf is a key indicator of an aberrant RPE-like gene expression program, as many of the genes exhibiting altered expression in *orJ* retinas are involved in the pigmentation pathway, downstream of Mitf (Rowan et al., 2004). In the present work, we demonstrate that repression of Mitf by Vsx2 is a cell autonomous function of Vsx2. This finding argues that Vsx2 does not utilize regulation of extrinsic signals in its repression of Mitf, which is consistent with previous reports that Mitf transcription is directly repressed by Vsx2 (Bharti et al., 2008) and recent reports that Vsx2 may repress Mitf function through direct protein-protein interaction (Zou and Levine, 2012). This finding also argues that any extrinsic signals involved in preventing non-retinal gene expression programs, at least with respect to repression of Mitf, are mediated by Vsx2, as restoration of extrinsic signals using genetic chimeras failed to repress Mitf in the absence of functional Vsx2. Furthermore, continued ectopic expression of Mitf in *orJ* cells of mutant chimeras illustrates the persistence of compromised retinal identity, demonstrating that *orJ* cells are unable to fully respond to extrinsic signals driving retinal specification and maintenance, thereby revealing a critical cell autonomous role for Vsx2 in mediating this response. We also demonstrate that the ectopic expression of Mitf in the *orJ* retina is mediated by cell autonomous activity of Lhx2, as in its absence, Mitf expression is not maintained in the *orJ* retina.

Our observation that *orJ* cells readily contribute to the developing retina in genetic chimeras, both in their integration into the retinal domain and their generation of

retinal neurons, reveals that despite aberrant expression of non-retinal elements, *orJ* cells are predominantly retinal in their identity. While this is consistent with the execution of a retinal program, albeit disrupted, in *orJ* retinas, it is somewhat surprising to find in chimeras. The eye field transcription factors Rx and Pax6 are both required for early steps in the specification of retinal identity. In chimeras, cells deficient for either of these genes, and therefore exhibiting fundamental defects in retinal identity, were largely absent in the retina, even at early stages. Those that did contribute, failed to persist, removed by cell death or physical extrusion (Collinson et al., 2000; Collinson et al., 2003; Li et al., 2007; Medina-Martinez et al., 2009; Quinn et al., 1996). Because of the additional competition afforded by wild type cells within chimeras, even developmental defects undetectable in germline mutants are often revealed, as was described for PDGFR mutants in muscle cell lineages (Crosby et al., 1998). Currently, we cannot rule out that this mixed identity exhibited by *orJ* cells impairs their ability to contribute to the retinal lineage because the resulting effect on *orJ* cell numbers in the retina of mutant chimeras is not separable from the effects of Vsx2 on RPC proliferation, which also influences *orJ* cell numbers. However, the substantial contribution of *orJ* cells to chimeric retinas and their differentiation into retinal neurons suggests that aberrant expression of non-retinal gene expression programs has little impact on their apparent identity. Thus, it appears that expression of nonretinal gene expression programs primarily interferes with the efficient execution of a retinal program, as opposed to actually promoting a switch in identity. To what extent other *orJ* phenotypes are linked to these aberrant gene expression programs is beginning to be addressed, including the present work (see below).

RPC proliferation

Vsx2 is essential for the proliferative expansion of RPCs. In the *orJ* mouse, eyes are microphthalmic and the neural retina is extremely hypocellular, exhibiting a nearly 20-fold reduction in cell number by birth (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003). Previous studies implicated the misregulation of several intrinsic factors to the defective RPC proliferation observed in the *orJ* retina, namely, ectopic expression of the RPE determinant Mitf and downregulation of Cyclin D1 (Ccnd1), a G1 phase cyclin critical for initiating the regulatory cascade driving G1 progression. In support of the former, genetic deletion of Mitf in the *orJ* retina largely restored retinal cell number (Horsford et al., 2005; Konyukhov and Sazhina, 1966). However, in the present work, we demonstrate that proliferation of *orJ* RPCs is significantly rescued, at least in the peripheral retina of E12.5 genetic chimeras, despite the presence of maintained, high levels of Mitf expression. Currently, we cannot rule out some level of contribution to regulation of RPC proliferation by ectopic Mitf, as the rescue of RPC proliferation in the peripheral retina, qualitatively, does not appear complete. However, our findings strongly argue that it is unlikely that ectopic Mitf expression alone fully accounts for the severe disruption in RPC proliferation, at least during early embryonic stages in the *orJ* retina. Furthermore, Mitf in several cell types, including optic neuroepithelial cells, directly activates transcription of several cell cycle inhibitors, including p27/KIP1 (Carreira et al., 2005; Lekmine et al., 2007; Tsukiji et al., 2009), which inhibit G1 progression downstream of Ccnd1. Thus, ectopic Mitf may not account for the reduced levels of Ccnd1 expression in the *orJ* retina, whose function tightly correlated with the observed changes in cell cycle regulation (Green et al., 2003).

Ccnd1 is not a reported transcriptional target of Vsx2. Furthermore, the downregulation of Ccnd1 expression in the *orJ* retina observed in the present study and reported previously (Green et al., 2003), would suggest that Vsx2 transcriptionally activates Ccnd1, but Vsx2 transcriptional activity is primarily repressive (Dorval et al., 2005). Although Vsx2 exhibits weak transcriptional activator function in some in vitro contexts, much of Vsx2-dependent regulation of retinal development is mediated by this repressive activity, including prevention of nonretinal gene expression programs through repression of Mitf (Bharti et al., 2008), maintaining multipotent progenitors through repression of lineage-restricting factors such as ath5 and Vsx1 (Clark et al., 2008; Vitorino et al., 2009), and promoting bipolar cell production through repression of yet unidentified targets (Livne-Bar et al., 2006). More likely, reduced Ccnd1 expression is an indirect effect of the loss of Vsx2 function.

Ccnd1 is, however, a well-established target of mitogen signaling pathways (Giacinti and Giordano, 2006; Levine and Green, 2004; Lundberg and Weinberg, 1999). Thus, we hypothesized that the reduced Ccnd1 expression and RPC proliferation observed in the *orJ* retina may be a consequence of reduced mitogen signaling. In support of this mechanism, we demonstrate in the present work that there is a large cell non-autonomous contribution to the regulation of RPC proliferation by Vsx2, as proliferation of *orJ* RPCs was significantly altered in chimeric retinas. Although *orJ* RPCs exhibited differences in their proliferative response to restored environmental signals in central versus peripheral regions, these findings are consistent with significant changes in the availability of retinal mitogens or anti-proliferative signals in the *orJ* retina. Further support was revealed by our examination of Hh signaling in the *orJ* retina. Shh is an

essential retinal mitogen, with numerous studies demonstrating that Shh and activation of the Hh signaling pathway are both necessary and sufficient for the proliferative expansion of RPCs [reviewed in (Amato et al., 2004; Wallace, 2008)]. In the present work, we report evidence of reduced Hh signaling in the *orJ* retina. Further examination revealed that this was primarily the result of delayed or reduced Shh ligand production during early embryonic and neonatal time points, respectively (Figure 5.1). Addition of exogenous Shh enhanced Hh signaling and RPC proliferation at both time points, suggesting that the limited availability of Shh ligand likely contributed to reduced RPC proliferation in *orJ* retinas. Furthermore, Ccnd1 exhibited Hh signaling-dependent changes in the *orJ* retina, suggesting that reduced Ccnd1 expression in the *orJ* retina may indeed reflect deficits in mitogen signaling. We also discovered that dissociated *orJ* retinal cells responded weakly, if at all, to Hh pathway stimulation, suggesting that Vsx2 may also regulate the responsiveness of RPCs to Hh pathway stimulation. Because *orJ* retinal cells respond robustly to exogenous Shh in explant culture and actively respond to endogenous Shh in vivo, it is not clear whether this regulation is relevant in vivo and will require further evaluation.

Together, these findings reveal a significant contribution of altered extrinsic signals to Vsx2-mediated regulation of RPC proliferation, in contrast to a limited contribution of ectopic Mitf, at least at early embryonic stages. Importantly, the observed defects in Hh signaling occur upstream of, and in addition to, any direct regulation Vsx2 or ectopic Mitf may exert on the cell cycle, thereby exposing a previously unrecognized role for Vsx2 in mitogen signaling. How does one reconcile these findings with the observation that deletion of Mitf produces a significant rescue? One possibility is that

mitogen signaling, acting through upregulation of Ccnd1 and potentiated by the strong feed forward regulation of this pathway, is sufficient to inhibit the activity of cell cycle inhibitors, despite their upregulation by Mitf; but, in the context of reduced mitogens (or presence of anti-proliferative signals), the upregulation of cell cycle inhibitors by Mitf goes unchecked and is sufficient to overcome mitogen-mediated G1 progression, resulting in slowed RPC proliferation.

Retinal neurogenesis

Vsx2 is also required to ensure proper temporal initiation of neurogenesis. In the absence of Vsx2 function, onset of the neurogenic program is delayed by approximately two days, but largely intact (Bone-Larson et al., 2000; Robb et al., 1978; Rutherford et al., 2004). In the present work, we show that this delayed neurogenic program is retained by *orJ* cells in the context of genetic chimeras, despite normal progression of neurogenesis in adjacent wild type cells (Figure 5.2), indicating strong cell autonomous regulation of the temporal initiation of neurogenesis by Vsx2. Our observation that *orJ* cells did not regain neurogenic competence all at once, even in the context of the genetic chimeras, but rather retained the stereotyped central-to-peripheral progression of the wave front, argues that the intrinsic regulation of this progression is largely unaffected in by the loss of Vsx2 function. These findings reveal that Vsx2 primarily functions in the temporal regulation of neurogenic competence.

Previous studies show that initiation and subsequent progression of the neurogenic wave are genetically separable events, but only in that progression can be blocked independent of initiation (Hufnagel et al., 2010). Progression does appear to

require initiation, since manipulations blocking initiation also appear to completely prevent neuronal differentiation (Martinez-Morales et al., 2005; Masai et al., 2000). Thus, it was unclear from the *orJ* retina whether the lack of peripheral neurogenesis was simply a result of delayed initiation in the central retina. Our observation that neurogenesis in *orJ* cells remained delayed with respect to their wild type neighbors, indicates that initiation in wild type cells is not sufficient to promote progression in *orJ* cells. This finding argues that progression is under strong intrinsic regulation and that this regulation may be intrinsically linked within individual cells to the initiation mechanism to ensure that these events occur in a coordinated fashion.

Previous studies suggested that progression of the neurogenic wave front is based on a preprogrammed intrinsic timer (Agathocleous and Harris, 2009). Our observation of two independent waves of neurogenesis in *orJ* chimeras: the first reflecting the normal progression of the neurogenic wave front in wild type cells, followed by a second, delayed wave front in *orJ* cells (Figure 5.2), supports this notion of a cell autonomous timer and further implicates positional identity as a critical component in the regulation of this clock. Additionally, peripheral *orJ* cells still maintained their delayed participation in neurogenesis relative to adjacent wild type cells in chimeras, despite significant improvement in the proliferation of these peripheral *orJ* cells, arguing against a model in which this clock is strictly tied to cell divisions.

Our analysis of neurogenesis in Vsx2 chimeras also provided insight into additional regulatory aspects of neurogenesis not mediated by Vsx2. Our unexpected observation of precocious neurogenesis of wild type cells in mutant chimeras, reveals a strong cell non-autonomous effect on the regulation of the progenitor state. This suggests

that typically, many more progenitors are competent to participate in neurogenesis at any given stage than is usually observed because of strong extrinsic regulation preventing the depletion of this progenitor population to ensure production of later cell types.

In summary, the present work has helped further define Vsx2 gene function in the processes of retinal identity maintenance, RPC proliferation, and neurogenesis, by examining the relationship between Vsx2-mediated regulation and that of extracellular signals and their signaling pathways. Our analysis of autonomy in Vsx2 chimeras has helped to define the primary level of Vsx2 regulation in these processes, which was not readily obvious from studies in the *orJ* retina. Specifically, the autonomy analysis identified roles for Vsx2 in establishing the competence in RPCs to fully and appropriately respond to signals and factors important for the maintenance of retinal identity and initiation of neurogenesis (Figure 5.3). Additionally, it also identified a role for Vsx2 in ensuring the availability of extrinsic signals necessary for the proper regulation of RPC proliferation (Figure 5.3). This provides significant insight into the types of potential mechanismsVsx2 may utilize in its regulation of these processes (Figure 5.3), which will in turn provide valuable and needed direction for future studies aimed at identifying the mechanisms through which Vsx2 exerts its regulation. Furthermore, our targeted analysis of Shh signaling as a candidate mediator of Vsx2 regulation in RPC proliferation revealed that Vsx2 is required to ensure proper availability of mitogenic Shh ligand during the proliferative period (Figure 5.3). Although determination of the precise mechanism for this regulation will require further study, this finding provides a specific example of Vsx2-dependent regulation of extrinsic signals, which was proposed for the regulation of RPC proliferation by the autonomy

experiments. Although altered Shh signaling likely contributes to the defective RPC proliferation observed in the *orJ* retina, our observations of *orJ* RPC proliferation in mutant chimeras suggests that additional signals are also involved.

Open questions

Despite considerable effort, our understanding of how Vsx2 integrates into the coordinated regulation of retinal histogenesis is poor. The work presented here contributes to our understanding of Vsx2 function in this regulation, but also raises new questions and refines existing ones.

The importance of Mitf in RPE development and the complementary relationship with Vsx2 in the developing optic cup are well defined. Thus, many studies interpret the ectopic expression of Mitf and pigmentation of retinal cells in the *orJ* retina as evidence of an RPE-like identity. However, Mitf, pigmentation, and many of the other genes exhibiting upregulation in the *orJ* retina are characteristic of the optic neuroepithelium at the RPE/retina border in the optic cup, which eventually gives rise to components of two peripheral fates, the iris and ciliary body. Furthermore, many of these genes are not expressed throughout the entire *orJ* retina, exhibiting instead, only expanded peripheral expression, consistent with the expansion of these peripheral fates (Rowan et al., 2004). Recent evidence suggests that these peripheral fates may be induced as early as the optic vesicle stage (Cho and Cepko, 2006). Furthermore, experimental manipulations promoting peripheral fate in the presumptive neural retina were associated with strong downregulation of Vsx2 expression (Cho and Cepko, 2006). As a result, it has been proposed that peripheral fates are expanded in the *orJ* retina, and that Vsx2 also prevents

peripheral fate identity. However, Vsx2 is weakly expressed in the presumptive ciliary body epithelium (Rowan and Cepko, 2004) suggesting that Vsx2 or the combined expression of Vsx2 and Mitf may be important for defining peripheral fate. Further evaluation with more specific peripheral fate markers will be required to define the role of Vsx2 in peripheral fate identity.

In the present work, we demonstrate that Vsx2 is critical for the proper establishment of retinal proliferation signals in the eye. These could be mitogenic or inhibitory proliferation signals, as extrinsic signals exerting negative effects on retinal proliferation have been identified, in addition to a large number of positive signals. An understanding of Vsx2-mediated regulation of RPC proliferation will require identification of these signals and their sources. Although Vsx2 expression is restricted to RPCs, the source of the affected signals may not be similarly restricted. Retinal mitogens and anti-proliferative signals are produced by RPCs, retinal neurons, the surrounding RPE and other ocular tissues (Anchan et al., 1991; Cho and Cepko, 2006; Close et al., 2005; Das et al., 2000; Hashimoto et al., 2006; Jensen and Wallace, 1997; Levine et al., 1997; Lillien and Cepko, 1992). Because proper development of ocular tissues involves complicated inter-tissue interactions, disrupted development of one tissue can impact development of other tissues. For example, expansion of markers consistent with characterization of peripheral fates (iris and ciliary body), suggests this region is expanded in the absence of Vsx2 function (Rowan et al., 2004), which could alter the expression of mitogens or anti-proliferative signals by these tissues. Our analysis of proliferation in *orJ* chimeras suggests that regional differences in the extrinsic regulation of RPC proliferation exist. Thus, it is likely that the availability of multiple signals will

be regulated by Vsx2. It will be interesting to determine the degree to which Vsx2-mediated regulation contributes to these regional differences.

Our analysis of the role of Vsx2 in the regulation of retinal proliferation signals was limited to E12.5 in genetic chimeras. Regulation of RPC proliferation is dynamic, owing to temporal and spatial changes in the expression of extrinsic signals (Close et al., 2005; Wang et al., 2005) and intrinsic changes that impact the responsiveness of RPCs to these signals during development (Jensen and Wallace, 1997; Lillien and Cepko, 1992; Lillien and Wancio, 1998). Vsx2-dependent regulation of RPC proliferation also changes over time, as manipulations of Vsx2 expression in wild type retinal cells at postnatal ages does not affect RPC proliferation (Livne-Bar et al., 2006). Thus, it will be important to determine how Vsx2-mediated regulation of retinal proliferation signals also changes during development.

In our evaluation of the retinal mitogen Shh and its signaling pathway in *orJ* retinas, we found that activation of Hh signaling was delayed at E12.5, which correlated with the delayed generation of retinal ganglion cells, the relevant source of endogenous retinal Shh ligand (Figure 5.1). Because addition of exogenous SHH-N activated Hh signaling and increased proliferation, we believed it likely that the delayed availability of Shh contributed to the low levels of RPC proliferation in the *orJ* retina. However, in genetic chimeras, where Shh is provided by wild type ganglion cells at the appropriate time, proliferation was not improved in the central region where ganglion cell differentiation had occurred. In fact, proliferation was actually reduced in this region. Based on Hh target gene expression, Shh acts as a short-range mitogen in the retina, suggesting that the rescue in peripheral RPC proliferation does not reflect restoration of

Shh signals. The reason for the lack of improved RPC proliferation in the central retina is not clear. It is unlikely that endogenous Shh fails to activate Hh signaling in *orJ* cells at this age for two reasons. First, *orJ* cells at this age are competent to respond to exogenous SHH-N. Second, *orJ* cells at later ages actively respond to endogenous Shh. However, this could be confirmed by determining whether *orJ* cells in E12.5 chimeras express *Gli1*, a reliable readout of active Hh signaling. We also suggest that the responsiveness of *orJ* cells to Hh pathway stimulation may be impaired, but this requires further evaluation, and could also be examined by analysis of *Gli1* expression. If *orJ* cells do indeed respond to the endogenous Shh provided by wild type ganglion cells in mutant chimeras, it is possible that the effects are masked by the loss of other retinal mitogens, or overcome by the presence of anti-proliferative signals. Alternatively, restoration of endogenous levels of Shh may not be achieved in chimeras or may only promote small changes in RPC proliferation. Consistent with the latter, the mitogenic potency of Shh changes as development progresses, and is stronger at later ages (Jensen and Wallace, 1997; Sakagami et al., 2009; Wang et al., 2005).

By birth, Shh is produced in the *orJ* retina, but levels appear reduced at both the transcript and protein levels. This occurs in the absence of detectable changes in the proportion of Shh-expressing cells, as significant differences were not detected between wild type and *orJ* retinas in the progenitor-to-neuron ratio (Green et al., 2003), nor in the expression of *Pou4f2* (this work), a retinal ganglion cell marker important for the maturation of this cell type and direct activator of Shh expression (Mu et al., 2004). These findings suggest that Shh production is reduced on a cell-by-cell basis. However, Vsx2 is not expressed in differentiating ganglion cells during the period of Shh

expression. Thus, it is not clear why or how this change in Shh production occurs. Vsx2 is expressed in the progenitors from which ganglion cells develop, suggesting that changes incurred during its time as a progenitor may impact its later activities. Recently, conditional inactivation of Dicer, an enzyme required for the production of microRNAs, was reported to promote a dramatic increase in ganglion cell number, but Hh target gene expression was markedly reduced (Davis et al., 2011; Georgi and Reh, 2010). Expression of Shh was not assessed in these studies, so it is possible that reduced Hh target gene expression resulted from the loss of microRNAs in RPCs, rather than reduced production of Shh by ganglion cells. But, it is an intriguing possibility that perhaps Vsx2 is important for the regulation of microRNAs that ultimately influence the ability of ganglion cells to produce Shh. Consistent with this possibility, ganglion cells in Dicer conditional knockouts fail to fully mature (Georgi and Reh, 2011).

In the present work, we demonstrate that Vsx2 is an essential component in the temporal regulation of the initiation of neurogenesis through its role in promoting neurogenic competence. Interestingly, Vsx2 has previously been implicated as a temporal regulator of cell fate, but through inhibition of neurogenic competence. Analysis of Vsx2 function in zebrafish suggests that Vsx2 maintains the multipotency of RPCs through repression of lineage-restricting bias factors, such as ath5 and Vsx1 (Clark et al., 2008; Vitorino et al., 2009). As a result of temporal differences in the production of Vsx2-negative RPCs over the course of retinal neurogenesis, the resulting derepression of these bias factors allows for temporally relevant cell fate restriction (Vitorino et al., 2009). Repression of Vsx1 is a conserved function of Vsx2 in mouse (Clark et al., 2008), suggesting that this model may also be conserved in mouse. However, despite

derepression of these lineage-restricting bias factors in the *orJ* retina, neurogenesis remains delayed, suggesting that *orJ* cells lack the necessary competence factors to promote transcriptional activation of bias factors. This could be confirmed by evaluating expression of these early lineage-restricting bias factors in *orJ* or chimeric retinas.

The intrinsic mechanism by which Vsx2 promotes neurogenic competence is unknown, but our observation that this is a cell autonomous function of Vsx2 suggests that Vsx2 regulates an intrinsic component. Sox2 and Pax6 are strong candidates due to their critical roles in conferring neurogenic competence. However, conditional inactivation of *Sox2* in the mouse blocks neurogenesis (Taranova et al., 2006), and conditional inactivation of Pax6 in the mouse restricts retinal cell production to the amacrine fate (Marquardt et al., 2001). Because acquisition of neurogenic competence is delayed but not completely blocked in the *orJ* retina and most retinal cell types are eventually produced, only moderately reduced expression of these factors would be expected in the *orJ* retina, suggesting thatVsx2 may only modulate the baseline expression level of these candidates. Hes1 exhibits intriguing temporal regulation of the initiation of neurogenesis in the mouse. In *Hes1* null retinas, precocious neurogenesis was observed days before normal initiation in wild type retinas, but the normal progression of cell type determinants was largely retained (Lee et al., 2005). Hes1 is known target of Delta-Notch signaling and manipulations of this pathway also promote temporal changes in neurogenesis (Bao and Cepko, 1997; Riesenberg et al., 2009; Tomita et al., 1996; Yaron et al., 2006). Furthermore, much of Sox2 function in regulating neurogenic competence is mediated by its role in promoting Notch1 expression (Taranova et al., 2006). Thus, autonomous misregulation of Delta-Notch signaling could

also mediate the neurogenic delay observed in the absence of Vsx2 function. However, for most of these candidate factors, expression in RPCs is required to promote competence or initiation of neurogenesis. Although Vsx2 may exhibit activator functions, it is more likely that any regulation of these factors by Vsx2 is indirect. Pax2 is a known transcriptional repressor of Pax6 in the retina and is downregulated prior to the initiation of neurogenesis (Schwarz et al., 2000). Thus, a potential direct target of Vsx2 repression in the regulation of neurogenic competence is Pax2, as its ectopic expression could mediate the necessary repression, at least of Pax6.

In mutant chimeras, the neurogenic delay exhibited by *orJ* cells could not be separated from their ectopic expression of Mitf. Thus, delayed initiation of neurogenesis in *orJ* cells may be, in part, Mitf-dependent. In support of this, genetic deletion of Mitf in the *orJ* retina shortens the delay in the onset of neurogenesis (C. Zou, personal communication). Because Mitf-dependent regulation is also expected to affect neurogenic competence, but separate from Vsx2-mediated regulation, it will be important to determine where Mitf intersects with the retinal differentiation program. However, the Mitf-mediated rescue is not complete, and neurogenesis remains delayed by at least a day in the double mutants (C. Zou, personal communication), suggesting that Mitf regulation alone cannot fully account for the delay in *orJ* retinas. Thus, it will also be important to determine the relative contributions of ectopic Mitf-dependent regulation and loss of Vsx2-dependent regulation to the neurogenic delay.

In *orJ* chimeras, we observed two waves of neurogenesis: the first in wild type cells according to normal neurogenic timing, and a second, delayed wave in the *orJ* cell population. What drives this second, delayed wave of neurogenesis in *orJ* cells?

Progression of the neurogenic wave front is largely controlled by intrinsic factors according to a preprogrammed intrinsic timer. This would suggest that downstream intrinsic regulators, such as Neurog2, would also exhibit cell autonomous delays. Thus, it would be informative to determine whether Neurog2 also exhibits separate waves in wild type and *orJ* cells of chimeric retinas. Alternatively, Neurog2 may proceed across the chimeric retina, irrespective of genotype. In Neurog2 null retinas, the neurogenic wave front initially stalls, but is rescued at later time points, presumably by a subsequent wave of Ascl1 that propagates across the retina shortly after Neurog2, although this latter point requires further confirmation (Hufnagel et al., 2010). While an attractive model, it is unlikely that Ascl1 drives the delayed wave of neurogenesis in *orJ* cells of genetic chimeras. In Neurog2 null retinas, progression of the wave is indistinguishable from that in wild type retinas by E13.5 (Hufnagel et al., 2010); however, in *orJ* chimeras, a delay is still evident in peripheral *orJ* cells relative to their adjacent wild type neighbors at E15.5. While it is possible that another factor mediates the second wave of neurogenesis in *orJ* cells of chimeras, the most likely explanation is that Neurog2 exhibits a cell-autonomous delay in *orJ* cells, but this remains to be tested.

Shh signaling has been proposed to establish the preprogrammed intrinsic timer in RPCs. In the absence of Shh or when Hh signaling is blocked, neurogenesis initiates and progresses in a central-to-peripheral wave, but on a delayed schedule (Kay et al., 2005), not unlike the delayed wave in the *orJ* retina. Furthermore, this timer appears to be established by midline Shh signals during the period of dorsal-ventral patterning in the optic vesicle (Kay et al., 2005). This timing closely coincides with the initiation of Vsx2

expression, making it possible that Vsx2 mediates entrainment of the intrinsic clock in RPCs.

Vsx2 is also required for generation of bipolar cells. Previous studies demonstrate that their absence in *orJ* retinas is due to a specific requirement for Vsx2 in their specification or maturation, rather than a secondary effect of insufficient proliferative expansion of RPCs for the generation of this late-born cell type (Bone-Larson et al., 2000; Burmeister et al., 1996; Green et al., 2003). Numerous studies suggest that Vsx2 may function as a lineage-restricting bias factor for the bipolar cell fate, or at least a subpopulation of bipolar cells in. In zebrafish, all retinal cell types, with the exception of several bipolar cell types, derive from Vsx2-negative progenitors, while progenitors maintaining Vsx2 expression generate only subclasses of bipolar cells (Vitorino et al., 2009). Furthermore, misexpression studies show that Vsx2 promotes the bipolar fate, typically at the expense of rod photoreceptors (Hatakeyama et al., 2001; Livne-Bar et al., 2006; Vitorino et al., 2009). However, evidence for immature bipolar cells was reported in *orJ* mice with genetic modifiers, suggesting that Vsx2 may be required for the maturation, rather than specification of bipolar cells (Bone-Larson et al., 2000). In dissociated cell culture of wild type retinal cells, bipolar cell specification occurs, but subsequent maturation fails, suggesting that extrinsic factors may be important for bipolar cell maturation. While the role of Vsx2 in bipolar cell fate specification versus maturation requires further evaluation, the use of *orJ* chimeras has the potential for providing insight into the role of Vsx2 in bipolar cell production, distinguishing between a requirement for Vsx2 in the response to bipolar differentiation signals or the generation of such signals.

Perspectives

The phenotypes exhibited by *orJ* retinal cells are consistent with the disruption of key properties associated with the retinal progenitor state; however, they are also reminiscent of unspecified optic neuroepithelial cells in the early optic vesicle. In particular, both *orJ* cells and unspecified optic neuroepithelial cells exhibit expression of Mitf, a relatively low rate of proliferation, and lack of neurogenic competence. Thus, it appears that many of the key features of *orJ* cells are shared with early optic neuroepithelial cells. Furthermore, initiation of Vsx2 expression is often associated with temporal changes in these features. For example, prior to initiation of Vsx2 expression, Mitf is expressed throughout the optic vesicle (Horsford et al., 2005; Nguyen and Arnheiter, 2000). Additionally, the earliest precocious initiation of retinal neurogenesis occurs in Hes1 null mutants, around E9.5 (Lee et al., 2005), which coincides with the onset of Vsx2 expression. An intriguing possibility, then, is that Vsx2 is important for transitioning optic neuroepithelial cells into the highly proliferative, neurogenically competent state characteristic of retinal progenitor cells, and in its absence, this transition is impaired.

Retinal histogenesis is a complex process requiring the coordinated activity of many genes and multiple developmental processes. Both extrinsic signals and intrinsic factors have important roles in the regulation of these processes and require coordinated integration into efficient regulatory networks that ensure appropriate execution in the changing environment of the developing embryo. Additionally, these developmental processes are not entirely separable, nor are their regulation. A major reason for this is that while RPCs actively maintain their identity, the competing processes of proliferation

and differentiation also exert their influences on this same cell population. Consequently, perturbations in one process often elicit secondary changes in the others, and vice versa. Moreover, many extracellular signals and intrinsic factors are reused during development, contributing to the regulation of multiple processes. Thus, an additional level of coordination is required within RPCs to ensure the orderly and efficient execution of these cellular processes during retinal histogenesis. Because Vsx2 is expressed in multipotent proliferating RPCs, it is expressed at the right time and in the right place to provide such coordination. Furthermore, maintenance of retinal identity, RPC proliferation, and retinal neurogenesis are severely disrupted in the absence of Vsx2, but they still occur to some extent, suggesting that Vsx2 promotes their efficient execution rather than being strictly required. Thus, Vsx2 also appears to exhibit the regulatory ability necessary for such coordination during retinal histogenesis. Establishment of such a role for Vsx2 in the coordination of these diverse cellular processes would also provide a mechanism through which Vsx2 could act to promote the transition of optic neuroepithelial cells into rapidly proliferating and neurogenically competent RPCs. It will be interesting to see whether these predictions hold as the mechanisms of Vsx2 function are defined.

In addition to complex regulation within retinal development, the importance of inter-tissue interactions and reuse of intrinsic and extrinsic factors at different times, in different places, and even in different ways during ocular development, has made the study of complex gene function difficult in retinal development. However, genetic chimeras have proven powerful and insightful tools in a number of studies, including the present, [(Li et al., 2007; Medina-Martinez et al., 2009) and for review, see (Collinson et

al., 2004)]. However, most previous studies used chimeras for assessing lineage requirements and patterns of tissue growth. Use of chimeras in assessing cellular phenotypes has been limited, but as we demonstrate here, can provide valuable insight into gene function. Additionally, chimeras also provided insight into aspects of retinal development not specifically regulated by Vsx2, including the regionalization of extrinsic regulation controlling RPC proliferation and the importance of extrinsic regulation in driving progenitor maintenance. The relationships between intrinsic factors and extrinsic signals involved in regulating the cellular processes of retinal histogenesis are only beginning to be defined. Continued use of genetic chimeras in these endeavors will be beneficial, particularly if combined with evaluation of the activity state of signaling pathways in the composite cell populations.

While genetic chimeras are powerful tools, other methods could have been used to address the questions of autonomy presented here. For example, although in vitro co-culture techniques lack the spatial information provided by chimera analyses, they too can address questions of autonomy, with the added benefit of determining primary versus secondary effects, since the time of interaction between wild type and mutant cell populations is defined. Co-cultures are also particularly useful in addressing questions of cell behavior, including those regarding cell adhesion, which will be important to address in future studies of Vsx2 function. Generation of genetic mosaics through Cre-loxP technology can also address questions of autonomy with similar spatial resolution as chimeras, but are limited by the expression pattern of the Cre driver. Additionally, chimeras tend to provide more random and variable patterns of chimerism, which permit a greater range of questions. However, because of the spatial and temporal control

afforded by these Cre drivers, genetic mosaics are particularly useful in circumventing early cell autonomous requirements for a gene of interest that prevent analyses of gene function in chimeras, such as exclusion of mutant cells from tissues in which they may exhibit later functions. In the study of Vsx2 gene function, temporal control of Vsx2 inactivation may help separate the contribution of ectopic Mitf function in the regulation of RPC proliferation and neurogenesis, by inactivating Vsx2 after Mitf is downregulated. Furthermore, such temporal control will aid assessment of temporal changes in Vsx2-dependent regulation of retinal histogenesis. These methods, in combination with continued use of chimera analyses, will be necessary in the future to fully analyze Vsx2 function.

Conclusions

In the present work, we illustrate the important relationship between the retinal homeobox gene Vsx2 and extrinsic signaling pathways in the regulation of retinal histogenesis, by demonstrating a requirement for Vsx2 in promoting both the reception and availability of extrinsic signals necessary for the regulation of RPC properties. We show that the use of genetic chimeras can advance our understanding of both Vsx2 function and principles of retinal development, and their continued use will undoubtedly prove a valuable tool in further defining the mechanisms of Vsx2 function and other genes in the regulation of retinal histogenesis. The present work provides direction for future studies that will improve our understanding of progenitor regulation and retinal development, which will facilitate the development of therapeutic techniques for the treatment of retinal disorders and degenerative diseases.

Figure 5.1. Model of disrupted Shh availability and its effects on RPC proliferation in the *orJ* retina. During the period of retinal histogenesis analyzed in the present work, retinal ganglion cells are the relevant source of Shh ligand in the retina, and their production of Shh ligand is both necessary and sufficient to activate Hh signaling in RPCs and enhance their proliferation. In the early embryo, ganglion cells initiate their production of Shh soon after their differentiation, which promotes proliferation of adjacent RPCs. In the orJ retina, differentiation of retinal ganglion cells is delayed. During this delay, the resulting absence of mitogenic Shh contributes to a slower rate of RPC proliferation. Once ganglion cell differentiation initiates in the *orJ* retina, Shh production follows. By birth, the ganglion cell population in wild type retinas produces high levels of Shh ligand that in turn promotes robust proliferation of RPCs. Although the proportion of ganglion cells appears unaffected in the *orJ* retina at birth, production of Shh is reduced. This limited availability of Shh ligand at neonatal ages in the *orJ* retina then contributes to a slower rate of RPC proliferation. Abbreviations: GC, ganglion cell; RPC, retinal progenitor cell; SHH, sonic hedgehog.

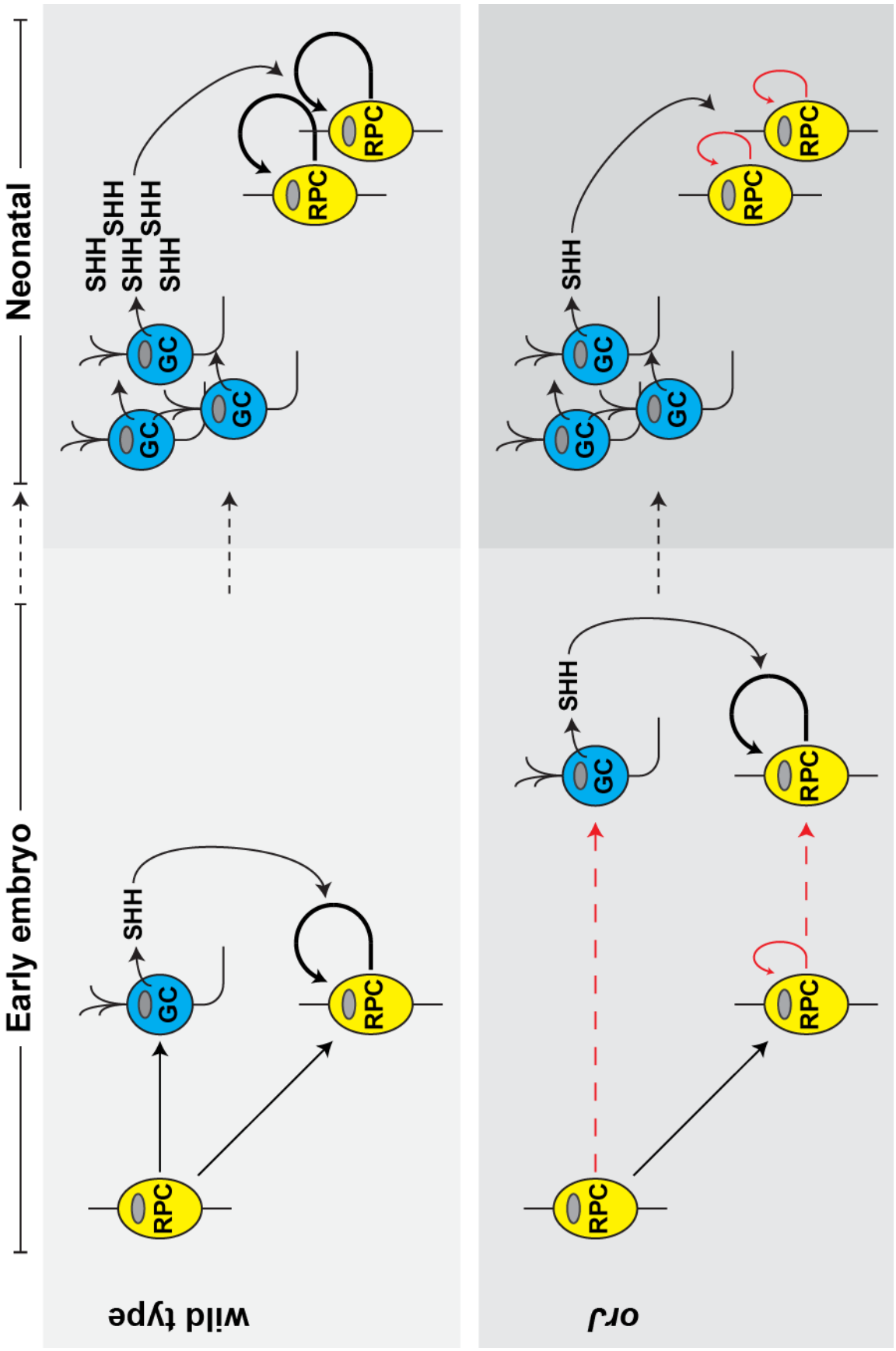

Neonatal
Early embryo
wild type
orJ
RPC
GC
SHH

Figure 5.2. Model of the progression of neurogenesis in the retinas of wild type, *orJ*, and mutant chimeras. In wild type retinas, neurogenesis has progressed throughout the central retina by E12.5 and continues in a peripherally-spreading wave that reaches the periphery by E15.5. In contrast, neurogenesis has yet to initiate at E12.5 in the *orJ* retina. By E15.5, neurogenesis has initiated and progressed throughout the central retina, but has yet to reach the periphery. In mutant chimeras, the patterns of neurogenesis observed for wild type and *orJ* cell populations at E12.5 and E15.5 match that observed in the wild type and *orJ* retinas, respectively, indicating that *orJ* cells retain their delayed neurogenic program despite the normal progression of neurogenesis in adjacent wild type cells. Thus, two independent peripherally-spreading waves of neurogenesis propagate across the retina in mutant chimeras, one in each cell population. Arrows indicate the spatial extent of neurogenesis in the different cell populations. Green represents wild type cell populations and magenta represents *orJ* cell populations. Dorsal is up and posterior is to the left. Abbreviations: L, lens; NR, neural retina; OS, optic stalk.

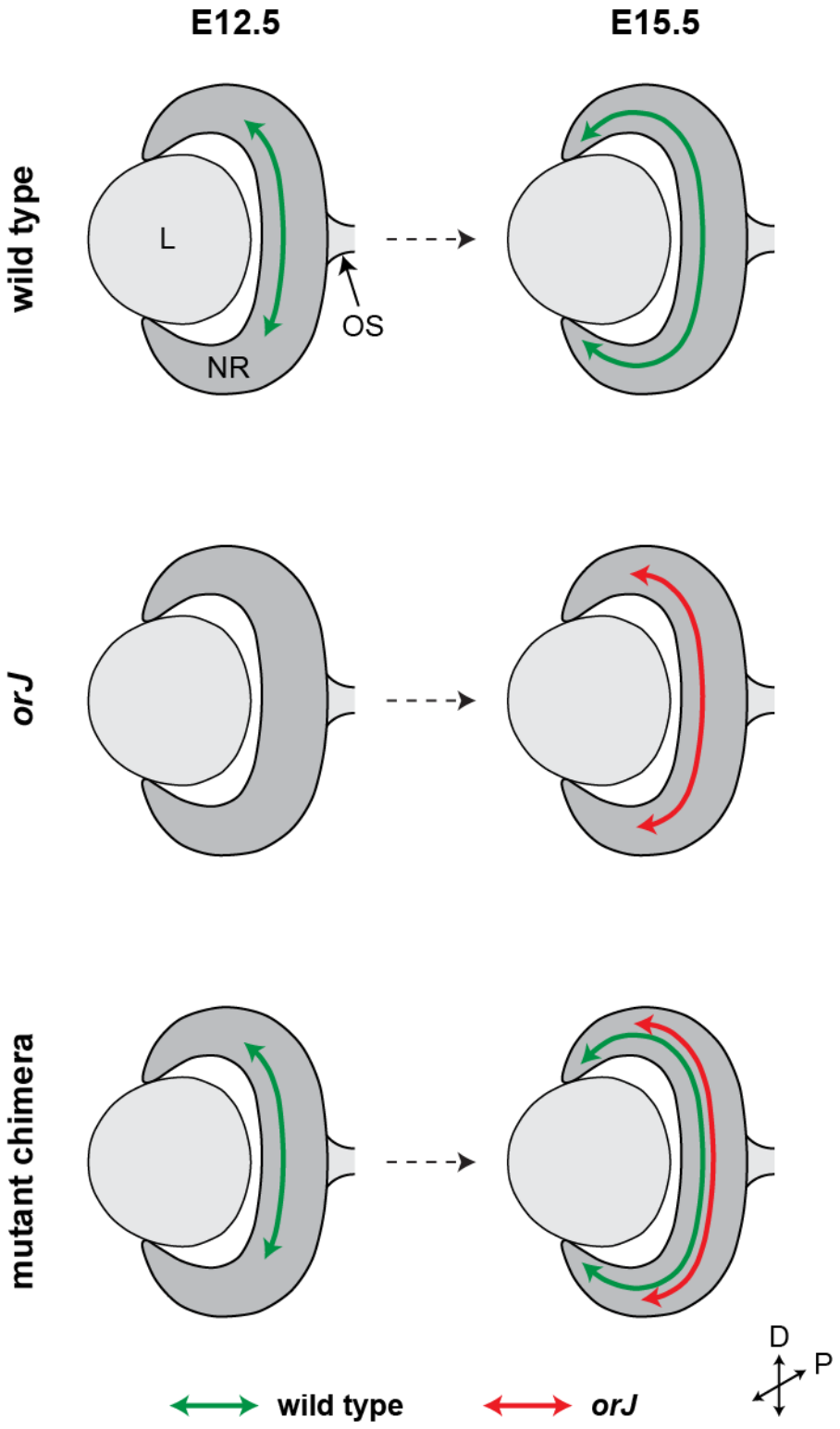
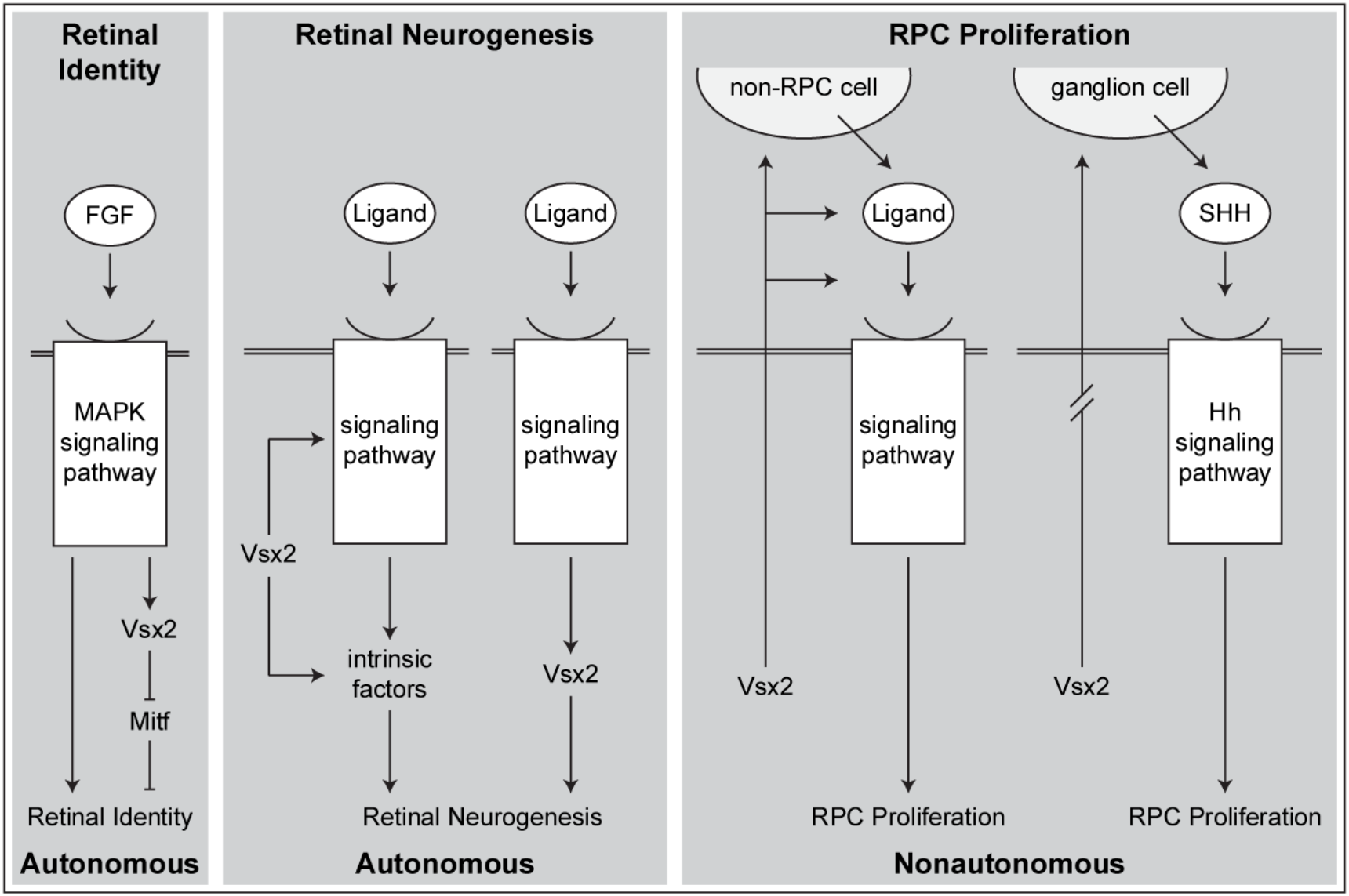

E12.5
E15.5
wild type
L
OS
NR
orJ
mutant chimera
D
P
wild type
orJ

Figure 5.3. Autonomy of Vsx2 functions in the regulation of retinal progenitor properties and potential mechanisms of action. Illustrated are the proposed relationships between Vsx2 and the intrinsic and extrinsic factors regulating the maintenance of retinal identity, RPC proliferation, and retinal neurogenesis, as revealed by the present work.


Retinal Identity
FGF
MAPK signaling pathway
Vsx2
Mitf
Retinal Identity
Autonomous
Retinal Neurogenesis
Ligand
Ligand
signaling pathway
signaling pathway
Vsx2
intrinsic factors
Vsx2
Retinal Neurogenesis
Autonomous
RPC Proliferation
non-RPC cell
ganglion cell
Ligand
SHH
signaling pathway
Hh signaling pathway
Vsx2
Vsx2
RPC Proliferation
RPC Proliferation
Nonautonomous

References

Agathocleous, M., Harris, W.A., 2009. From progenitors to differentiated cells in the vertebrate retina. Annu. Rev. Cell Dev. Biol. 25, 45-69.

Amato, M.A., Boy, S., Perron, M., 2004. Hedgehog signaling in vertebrate eye development: a growing puzzle. Cell. Mol. Life Sci. 61, 899-910.

Anchan, R.M., Reh, T.A., Angello, J., Balliet, A., Walker, M., 1991. EGF and TGF-alpha stimulate retinal neuroepithelial cell proliferation in vitro. Neuron 6, 923-936.

Bao, Z.Z., Cepko, C.L., 1997. The expression and function of Notch pathway genes in the developing rat eye. J. Neurosci. 17, 1425-1434.

Bharti, K., Liu, W., Csermely, T., Bertuzzi, S., Arnheiter, H., 2008. Alternative promoter use in eye development: the complex role and regulation of the transcription factor MITF. Development 135, 1169-1178.

Bone-Larson, C., Basu, S., Radel, J.D., Liang, M., Perozek, T., Kapousta-Bruneau, N., Green, D.G., Burmeister, M., Hankin, M.H., 2000. Partial rescue of the ocular retardation phenotype by genetic modifiers. J. Neurobiol. 42, 232-247.

Burmeister, M., Novak, J., Liang, M.Y., Basu, S., Ploder, L., Hawes, N.L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V.I., Roderick, T.H., Taylor, B.A., Hankin, M.H., McInnes, R.R., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. 12, 376-384.

Carreira, S., Goodall, J., Aksan, I., La Rocca, S.A., Galibert, M.D., Denat, L., Larue, L., Goding, C.R., 2005. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature 433, 764-769.

Cho, S.H., Cepko, C.L., 2006. Wnt2b/beta-catenin-mediated canonical Wnt signaling determines the peripheral fates of the chick eye. Development 133, 3167-3177.

Clark, A.M., Yun, S., Veien, E.S., Wu, Y.Y., Chow, R.L., Dorsky, R.I., Levine, E.M., 2008. Negative regulation of Vsx1 by its paralog Chx10/Vsx2 is conserved in the vertebrate retina. Brain Res. 1192, 99-113.

Close, J.L., Gumuscu, B., Reh, T.A., 2005. Retinal neurons regulate proliferation of postnatal progenitors and Muller glia in the rat retina via TGF beta signaling. Development 132, 3015-3026.

Collinson, J.M., Hill, R.E., West, J.D., 2000. Different roles for Pax6 in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye. Development 127, 945-956.

Collinson, J.M., Quinn, J.C., Hill, R.E., West, J.D., 2003. The roles of Pax6 in the cornea, retina, and olfactory epithelium of the developing mouse embryo. Dev. Biol. 255, 303-312.

Crosby, J.R., Seifert, R.A., Soriano, P., Bowen-Pope, D.F., 1998. Chimaeric analysis reveals role of Pdgf receptors in all muscle lineages. Nat. Genet. 18, 385-388.

Das, I., Sparrow, J.R., Lin, M.I., Shih, E., Mikawa, T., Hempstead, B.L., 2000. Trk C signaling is required for retinal progenitor cell proliferation. J. Neurosci. 20, 2887-2895.

Davis, N., Mor, E., Ashery-Padan, R., 2011. Roles for Dicer1 in the patterning and differentiation of the optic cup neuroepithelium. Development 138, 127-138.

Dorval, K.M., Bobechko, B.P., Ahmad, K.F., Bremner, R., 2005. Transcriptional activity of the paired-like homeodomain proteins CHX10 and VSX1. J Biol. Chem. 280, 10100-10108.

Georgi, S.A., Reh, T.A., 2010. Dicer is required for the transition from early to late progenitor state in the developing mouse retina. J. Neurosci. 30, 4048-4061.

Georgi, S.A., Reh, T.A., 2011. Dicer is required for the maintenance of notch signaling and gliogenic competence during mouse retinal development. Dev. Neurobiol. 71, 1153-1169.

Giacinti, C., Giordano, A., 2006. RB and cell cycle progression. Oncogene 25, 5220-5227.

Green, E.S., Stubbs, J.L., Levine, E.M., 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development 130, 539-552.

Hashimoto, T., Zhang, X.M., Chen, B.Y., Yang, X.J., 2006. VEGF activates divergent intracellular signaling components to regulate retinal progenitor cell proliferation and neuronal differentiation. Development 133, 2201-2210.

Hatakeyama, J., Tomita, K., Inoue, T., Kageyama, R., 2001. Roles of homeobox and bHLH genes in specification of a retinal cell type. Development 128, 1313-1322.

Horsford, D.J., Nguyen, M.T., Sellar, G.C., Kothary, R., Arnheiter, H., McInnes, R.R., 2005. Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. Development 132, 177-187.

Hufnagel, R.B., Le, T.T., Riesenberg, A.L., Brown, N.L., 2010. Neurog2 controls the leading edge of neurogenesis in the mammalian retina. Dev. Biol. 340, 490-503.

Jensen, A.M., Wallace, V.A., 1997. Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. Development 124, 363-371.

Kay, J.N., Link, B.A., Baier, H., 2005. Staggered cell-intrinsic timing of ath5 expression underlies the wave of ganglion cell neurogenesis in the zebrafish retina. Development 132, 2573-2585.

Konyukhov, B.V., Sazhina, M.V., 1966. Interaction of the genes of ocular retardation and microphthalmia in mice. Folia Biol. 12, 116-123.

Lee, H.Y., Wroblewski, E., Philips, G.T., Stair, C.N., Conley, K., Reedy, M., Mastick, G.S., Brown, N.L., 2005. Multiple requirements for Hes 1 during early eye formation. Dev. Biol. 284, 464-478.

Lekmine, F., Chang, C.K., Sethakorn, N., Das Gupta, T.K., Salti, G.I., 2007. Role of microphthalmia transcription factor (Mitf) in melanoma differentiation. Biochem. Biophys. Res. Commun. 354, 830-835.

Levine, E.M., Green, E.S., 2004. Cell-intrinsic regulators of proliferation in vertebrate retinal progenitors. Semin. Cell Dev. Biol. 15, 63-74.

Levine, E.M., Roelink, H., Turner, J., Reh, T.A., 1997. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J. Neurosci. 17, 6277-6288.

Li, S., Goldowitz, D., Swanson, D.J., 2007. The requirement of pax6 for postnatal eye development: evidence from experimental mouse chimeras. Invest. Ophthalmol. Vis. Sci. 48, 3292-3300.

Lillien, L., Cepko, C., 1992. Control of proliferation in the retina: temporal changes in responsiveness to FGF and TGF alpha. Development 115, 253-266.

Lillien, L., Wancio, D., 1998. Changes in epidermal growth factor receptor expression and competence to generate glia regulate timing and choice of differentiation in the retina. Mol. Cell. Neurosci. 10, 296-308.

Livne-Bar, I., Pacal, M., Cheung, M.C., Hankin, M., Trogadis, J., Chen, D., Dorval, K.M., Bremner, R., 2006. Chx10 is required to block photoreceptor differentiation but is dispensable for progenitor proliferation in the postnatal retina. Proc. Natl Acad. Sci. U. S. A. 103, 4988-4993.

Lundberg, A.S., Weinberg, R.A., 1999. Control of the cell cycle and apoptosis. Eur. J. Cancer 35, 531-539.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., Gruss, P., 2001. Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43-55.

Martinez-Morales, J.R., Del Bene, F., Nica, G., Hammerschmidt, M., Bovolenta, P., Wittbrodt, J., 2005. Differentiation of the vertebrate retina is coordinated by an FGF signaling center. Dev. Cell 8, 565-574.

Masai, I., Stemple, D.L., Okamoto, H., Wilson, S.W., 2000. Midline signals regulate retinal neurogenesis in zebrafish. Neuron 27, 251-263.

Medina-Martinez, O., Amaya-Manzanares, F., Liu, C., Mendoza, M., Shah, R., Zhang, L., Behringer, R.R., Mahon, K.A., Jamrich, M., 2009. Cell-autonomous requirement for rx function in the mammalian retina and posterior pituitary. PLoS One 4, e4513.

Mu, X., Beremand, P.D., Zhao, S., Pershad, R., Sun, H., Scarpa, A., Liang, S., Thomas, T.L., Klein, W.H., 2004. Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. Development 131, 1197-1210.

Nguyen, M., Arnheiter, H., 2000. Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. Development 127, 3581-3591.

Quinn, J.C., West, J.D., Hill, R.E., 1996. Multiple functions for Pax6 in mouse eye and nasal development. Genes Dev. 10, 435-446.

Riesenberg, A.N., Liu, Z., Kopan, R., Brown, N.L., 2009. Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J. Neurosci. 29, 12865-12877.

Robb, R.M., Silver, J., Sullivan, R.T., 1978. Ocular retardation (or) in the mouse. Invest. Ophthalmol. Vis. Sci. 17, 468-473.

Rowan, S., Cepko, C.L., 2004. Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. Dev. Biol. 271, 388-402.

Rowan, S., Chen, C.M., Young, T.L., Fisher, D.E., Cepko, C.L., 2004. Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. Development 131, 5139-5152.

Rutherford, A.D., Dhomen, N., Smith, H.K., Sowden, J.C., 2004. Delayed expression of the Crx gene and photoreceptor development in the Chx10-deficient retina. Invest. Ophthalmol. Vis. Sci. 45, 375-384.

Sakagami, K., Gan, L., Yang, X.J., 2009. Distinct effects of Hedgehog signaling on neuronal fate specification and cell cycle progression in the embryonic mouse retina. J. Neurosci. 29, 6932-6944.

Schwarz, M., Cecconi, F., Bernier, G., Andrejewski, N., Kammandel, B., Wagner, M., Gruss, P., 2000. Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. Development 127, 4325-4334.

Taranova, O.V., Magness, S.T., Fagan, B.M., Wu, Y., Surzenko, N., Hutton, S.R., Pevny, L.H., 2006. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev. 20, 1187-1202.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F., Kageyama, R., 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16, 723-734.

Tsukiji, N., Nishihara, D., Yajima, I., Takeda, K., Shibahara, S., Yamamoto, H., 2009. Mitf functions as an in ovo regulator for cell differentiation and proliferation during development of the chick RPE. Dev. Biol. 326, 335-346.

Vitorino, M., Jusuf, P.R., Maurus, D., Kimura, Y., Higashijima, S., Harris, W.A., 2009. Vsx2 in the zebrafish retina: restricted lineages through derepression. Neural Dev. 4, 14.

Wallace, V.A., 2008. Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. Brain Res. 1192, 61-75.

Wang, Y., Dakubo, G.D., Thurig, S., Mazerolle, C.J., Wallace, V.A., 2005. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. Development 132, 5103-5113.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M., Ashery-Padan, R., 2006. Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development 133, 1367-1378.

Zou, C., Levine, E.M., 2012. Vsx2 controls eye organogenesis and retinal progenitor identity via homeodomain and non-homeodomain residues required for high affinity DNA binding. PLoS Genet. 8, e1002924.

APPENDIX

EXPRESSION ANALYSIS OF *SHH* AND HEDGEHOG PATHWAY COMPONENTS IN THE DEVELOPING MOUSE EMBRYO: RELATIONSHIPS BETWEEN REGULATORS OF FEEDBACK INHIBITION AND HH PATHWAY ACTIVATION

Introduction

Sonic Hedgehog (Shh) has emerged as a fundamental signaling molecule in vertebrate development. Throughout embryogenesis, Shh is utilized repeatedly to mediate a diverse array of developmental processes. Shh regulates proliferation, differentiation, fate determination, migration, polarity, and survival in multiple cell types, ultimately directing the patterning, growth and morphogenesis of numerous tissues and organs. Shh also plays important roles in the establishment of left-right asymmetry and axonal guidance (Ingham and McMahon, 2001; Varjosalo and Taipale, 2008). Despite such diversity, Shh often controls multiple processes within a single tissue, even in a temporally overlapping fashion (Amato et al., 2004; Wallace, 2008).

Shh is a secreted glycoprotein belonging to the Hedgehog (Hh) family of intercellular signaling molecules, which in mammals also includes Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh). Much of Shh function is mediated by transcriptional regulation of Hh target genes through activity of the Hh signaling pathway (Riobo and Manning, 2007; Ryan and Chiang, 2012; Varjosalo and Taipale, 2008). Binding of Shh or other Hh ligands to the Hh receptor, Patched homolog 1 (Ptch1), relieves Ptch1-mediated inhibition of Smoothened (Smo). Activated Smo inhibits proteolytic processing of the Gli transcriptional effectors into truncated repressor forms and promotes the nuclear localization and activation of full-length Gli proteins. The resulting reduction of Gli repressor forms and accumulation of Gli activator forms in the nucleus upon pathway activation promotes both the derepression and activation of Hh target genes.

Transcriptional targets of the Hh pathway not only mediate downstream signaling and cellular responses to Hh ligands, but also participate in feedback loops that further

regulate Hh signaling. Ptch1, Ptch2, and Hhip participate in negative feedback loops that act at the level of Hh reception. The Hh receptor, Ptch1, negatively regulates the Hh pathway through inhibition of Smo activity. Ptch1 is upregulated in response to Hh signaling (Chiang et al., 1996; Goodrich et al., 1996). Evaluation of phenotypes and Hh pathway activity in mice heterozygous for Ptch1 suggests that Hh activity is sensitive to Ptch1 gene dosage (Goodrich et al., 1997). In Drosophila, cellular responses to Hh ligand were determined by the ratio of bound to unbound Ptc (Casali and Struhl, 2004). Furthermore, overexpression of Ptch1 was sufficient to reduce Shh-stimulated upregulation of Hh target gene expression and induce phenotypes consistent with reduced Shh signaling (Goodrich et al., 1999). In Drosophila, high levels of Ptc sequester Hh ligands and limit their spread within the embryo (Chen and Struhl, 1996). Thus, upregulation of Ptc/Ptch1 likely serves to increase sequestration of Hh ligands and desensitize the cell to Hh signal, thereby limiting the level and possibly the spatial extent of Hh signaling. Patched homolog 2 (Ptch2) shares sequence homology with Ptch1 and is also upregulated in response to Hh signaling, although this upregulation may be context dependent (Carpenter et al., 1998; Motoyama et al., 1998b; Rahnama et al., 2004). Like Ptch1, Ptch2 also binds Hh ligands with high affinity (Carpenter et al., 1998) and inhibits Shh-induced changes in gene expression (Rahnama et al., 2004). However, unlike Ptch1, Ptch2 fails to block changes in gene expression induced by a constitutively active form of Smo and is unable to replace Ptch1 function in basal carcinoma cells or Ptch1 null cells (Rahnama et al., 2004; Zaphiropoulos et al., 1999). This inability to mediate Shh-stimulated signaling suggests that Ptch2 negatively regulates Hh signaling by binding and sequestering Hh ligands. Hedgehog-interacting protein (Hhip) also participates in

feedback inhibition. Hhip expression is upregulated in response to Hh signaling. Hhip binds Hh ligands with similar affinity as Ptch1 and is both necessary and sufficient to attenuate Hh signaling during embryonic development (Chuang et al., 2003; Chuang and McMahon, 1999). Thus, like Ptch1 and Ptch2, Hhip also negatively regulates the level of Hh ligands to which the responding cell is exposed. The principal positive feedback loop in the Hh pathway involves the transcriptional effector Gli1. Transcription of Gli1 is activated in response to Gli2- and Gli3-mediated transduction of Hh signals. Proteolytic processing completely degrades Gli1 with no evidence of a repressor form. This, together with its potent activator function, suggests Gli1 serves as a strong positive feedback mechanism to increase signaling levels within responding cells while retaining dependence on active Hh signaling. In the present study, the dependence of Gli1 expression on active Hh signaling makes Gli1 an excellent indicator of active Hh reception.

A balance of Hh activator and repressor functions is required for proper embryonic development. As a result, Hh signaling is tightly regulated, both externally through regulation of Hh ligands and pathway components and internally through positive and negative feedback mechanisms. Consistent with the latter is the finding that manipulations of these positive and negative regulators often fail to cause dramatic phenotypes, suggesting that the pathway is largely resistant to subtle changes in regulatory components (Bai et al., 2002; Goodrich et al., 1999; Nieuwenhuis et al., 2006). This resistance is likely a result of compensation through feedback mechanisms altering expression of other regulatory components. The present study details the expression of the regulatory feedback components Ptch1, Ptch2, Hhip, and Gli1 with respect to Shh-

expressing tissues in the developing mouse to evaluate how expression of negative regulators relate to pathway activation.

Methods

Animals

Mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice used in this study were on a 129/Sv background. Some mice were heterozygous for the *ocular retardation J* (*orJ*) allele. The *orJ* allele is a recessive null mutation in the Vsx2 gene (Burmeister et al., 1996). Wild type (+) and *orJ* alleles were determined by PCR and restriction digest, as previously described (Burmeister et al., 1996), from embryonic tail samples or adult ear clips. Phenotypes of homozygous *orJ*/*orJ* mice are restricted to the eye. Previous studies ((Rowan et al., 2004; Sigulinsky et al., 2008) and our unpublished observations) suggest that development of the eye and other Vsx2-expressing tissues in heterozygous (*orJ*/+) mice is phenotypically indistinguishable from wild type (+/+) mice. Thus, both heterozygous *orJ* and homozygous wild type mice were considered equivalent and referred to as wild type in the text. Mice were bred overnight and noon on the day of vaginal plug was considered embryonic day 0.5 (E0.5). All animal use and care was conducted in accordance with protocols approved by the University of Utah IACUC.

In situ hybridization

Following dissection in Hank's buffered saline solution (HBSS), embryonic heads were fixed overnight at 4 °C in either 4% paraformaldehyde (PFA) in phosphate buffered

saline (PBS, pH 7.5) or 4% formaldehyde in PBS/2 mM EGTA. Fixed tissue was cryoprotected by sequential immersion in 5%, 10%, and 20% sucrose/PBS, then embedded and frozen in OCT and stored at -80 °C. Serial sections (12 μm) were cut, placed on separate slides, and stored at -20 °C until use. Adjacent serial sections from the same mouse were stained with digoxigenin-labeled anti-sense probes for *Shh*, *Ptch1*, *Ptch2*, *Gli1*, and *Hhip*. Section in situ hybridization was performed as previously described (Green et al., 2003; Schaeren-Wiemers and Gerfin-Moser, 1993).

Image capture and analysis

Embryonic head sections were captured as mosaicked tiles of 8-bit, 1388 pixel 1036 line frames under voltage-regulated tungsten halogen flux with a variation of 1.2 ± 0.6%/min (mean ± sd). Mosaic image tiles were acquired with automated image capture using with a Peltier-cooled QImaging Fast 1394 QICAM (QImaging, Burnaby, BC, Canada) and automated Scan 100x100 stage (Märzhäuser Wetzlar GmbH, Wetzlar Germany). Mosaic image tiles were autotiled using a Syncroscan montaging system (Synoptics Inc, Frederick, MD, USA). Images were acquired at 10X magnification on a Leica DMR upright microscope. Hair follicles were reimaged at 20X magnification with DIC under brightfield illumination on a Nikon E-600 epifluorescence microscope equipped with a Spot-RT slider CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Due to their small size, hair follicles could not be analyzed for all probes on adjacent serial sections. Thus, similar positions within representative stage-matched follicles were imaged. Images of adjacent serial sections were aligned by hand

and prepared for publication using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA).

Results

To evaluate the relationship between regulators of feedback inhibition and Hh pathway activation, we compared expression of *Ptch1*, *Ptch2*, and *Hhip* with that of *Shh* and *Gli1* in the developing molars, hair follicles, palate, eyelids, and eyes of the E15.5 mouse (Figure A.1). *Shh* expression identified structures with active sources of Hh signaling. Gli1 served as an indicator of Hh-responsive tissues. Expression was examined on adjacent serial sections containing all of the structures of interest. This method provides two advantages: 1) expression levels of a single transcript can be directly compared across multiple structures, and 2) expression patterns of multiple transcripts can be compared within a given structure at relatively similar positions. The following sections detail the expression of *Shh*, *Gli1*, *Ptch1*, *Ptch2*, and *Hhip* in each structure.

Molars

Odontogenesis of molars involves the budding of thickened oral epithelium and subsequent interactions with condensing neural-crest-derived mesenchymal cells (ectomesenchyme). By E15.5, the upper and lower molars have reached the late cap stage (Figure A.2A,G). The oral epithelium has undergone drastic morphological changes and histodifferentiation, giving rise to the internal and external dental epithelium, intermediate stratum, and stellate reticulum, which connect to the oral epithelium via the pedicle. Condensation of the ectomesenchymal cells is the process of establishing the

dental papilla and dental follicle. Expression of Hh pathway genes has been described previously (reviewed in (Cobourne and Sharpe, 2005)) and is consistent with what is reported here.

Shh expression in the late cap stage is restricted to the epithelial components of both the upper and lower molars (Figure A.2B,H). Strong expression is observed within the internal dental epithelium and adjacent intermediate stratum. *Shh* expression also extends into varying portions of the stellate reticulum. In the upper molars, expression into the stellate reticulum is extensive, but in the lower molars is largely restricted to the intermediate stratum. Interestingly, expression in these epithelial tissues is more extensive on the lingual (tongue) side of both the upper and lower molars. Similar to *Shh*, *Ptch2* transcripts are detected throughout the internal and external dental epithelium (Figure A.2.C,I). Strongest expression is observed within the internal dental epithelium and extends beyond the range of *Shh* expression, particularly on the buccal (cheek) side. In contrast, expression within the external dental epithelium is weak. Weak *Ptch2* signal is also observed within the intermediate stratum and stellate reticulum. This expression is not uniform and extends beyond the range of Shh expression. Contrary to previous reports (reviewed in Cobourne and Sharpe, 2005), *Ptch2* is also weakly detected within the condensing ectomesenchyme of the forming dental papilla and follicle. In the upper and lower molars, *Ptch1* is expressed throughout both epithelial and mesenchymal components (Figure A.2D,J). Strongest expression is observed in the condensing ectomesenchyme, including that forming the dental papilla and follicle, and in the most peripheral stellate reticulum and pedicle. Robust expression is also found in the internal and external dental epithelium, intermediate stratum, and distal stellate reticulum. Strong

expression of *Hhip* transcripts is restricted to a narrow band of peripheral mesenchyme, including the dental follicle, which encompasses the developing molars, many cell diameters from *Shh*-expressing cells (Figure A.2E,K). Faint *Hhip* expression is also detected throughout the remaining mesenchymal and epithelial components of the developing upper and lower molars. *Gli1* expression (Figure A.2F,L) mirrors that of *Ptch1*. Interestingly, *Gli1* expression fails to extend into the oral mesenchyme much beyond the extent of strongest *Hhip* expression in the forming dental follicle. Because *Gli1* provides a reliable readout of active Hh signaling, expression of *Gli1* transcripts reveals Hh pathway activation throughout the epithelial and mesenchymal tissues of the developing molars.

Hair follicles

Like molars, hair follicles (HF) arise through a series of interactions between the epidermis and underlying mesenchyme. The basal layer of the epidermis thickens and grows downward toward condensing dermal mesenchyme. By E15.5, most tylotrich pelage hair follicles (Hardy, 1969; Mann, 1962) have reached stage 3 of hair follicle morphogenesis (based on morphological classification described in (Hardy, 1969, 1992; Paus et al., 1999)). At this stage, the follicle, now termed a hair peg, consists of a solid column of epidermally-derived epithelial cells with a concave end that partially or wholly encompasses the dermal condensate, which will form the future dermal papilla (Figure A.2M).

Expression of *Shh* within the stage 3 hair follicle is restricted to the distal end of the epithelial downgrowth, within the concave basal border cells that contact the dermal

condensate (Figure A.2N). *Ptch2* transcripts are similarly expressed in the distal epithelium of the hair peg (Figure A.2O), overlapping the region of *Shh* expression. However, weaker *Ptch2* expression extends beyond the *Shh*-expressing region, up the outer walls of the hair peg. *Ptch1* is expressed in both the epithelial and mesenchymal components of the developing hair follicle (Figure A.2P). Strongest expression is observed in the distal half of the epithelially-derived hair peg, both in the outer layer cells and cells of the interior, and uniformly through the dermal condensate. The most proximal aspect of the hair peg only weakly expresses *Ptch1*. Weak *Ptch1* expression also extends slightly into the surrounding noncondensed dermal mesenchyme directly adjacent to the hair peg and dermal condensate. Expression of *Hhip* is strongest in a narrow region of the noncondensing dermal mesenchyme surrounding the dermal condensate and hair peg (Figure A.2Q). Weak expression is also observed in the peripheral cells of the dermal condensate and inner portions of the hair peg. *Gli1* expression (Figure A.2R) again mimics that of *Ptch1* and is largely restricted within the border of high *Hhip* expression. *Gli1* expression reveals active Hh reception and signaling in both the mesenchymal and epithelial components.

Expression of *Shh* and *Ptch2* at stage 3 (Figure A.2N,O) is consistent with reports of expression patterns reported for other stages (stages 0-2 and stages 4-5; (St-Jacques et al., 1998). While the expression of *Ptch1* and *Gli1* at stage 3 (Figure A.2P,R) is also consistent with reports for earlier stages (0-2), the expression observed at stage 3 is much broader than that reported for Stages 4-5 (St-Jacques et al., 1998).

Palatal rugae

Palate development is a complex process involving elevation, midline contact and eventual fusing of the palatal shelves above the tongue (Rice et al., 2006). In the murine palate at E15.5, fusion of the palatal shelves is mostly complete and the medial edge epithelia seam is undetectable (Figure A.3A). At this rather late stage of palate development, expression of Hh pathway components in the palatal tissues is largely restricted to the developing rugae (Rice et al., 2006). However, some pathway components are also expressed in the developing palatine bones in response to known Ihh signals in this structure (Levi et al., 2011; Nelson et al., 2011; Rice et al., 2006).

Expression of *Shh* transcripts in the palate is restricted to the small, thickened regions of palatal oral epithelium known as rugae (Figure A.3B). Unlike the other epithelial/mesenchymal organs described here, a clear *Ptch2* signal is undetectable in the palatal oral epithelium (Figure A.3C), consistent with previous reports (Rice et al., 2006). *Ptch2* expression was also not observed in the palatal mesenchyme. In previous reports, *Ptch2* was weakly detected only in anterior palatal mesenchyme (Rice et al., 2006). *Ptch1* is strongly expressed by the thickened palatal oral epithelium in a region that overlaps with but extends beyond that of *Shh* expression (Figure A.3D). Robust *Ptch1* expression is also observed extending into the palatal mesenchyme immediately adjacent to the palatal oral epithelium in a gradient fashion. *Hhip* is strongly expressed in the palatal mesenchyme adjacent to the palatal oral epithelium of the developing rugae (Figure A.3E), in a large region that overlaps and extends beyond that of *Gli1* and *Ptch1* expression. This mesenchymal expression is also graded in nature, with the strongest expression closest to the oral epithelium. *Gli1* transcripts are again expressed in a pattern

similar to that observed for *Ptch1* in rugae of the palatal oral epithelium and adjacent mesenchyme, although slightly more restricted in range and less uniform within the mesenchymal component (Figure A.3F). Thus, epithelial-derived Shh activates Hh signaling in both the palatal epithelium and adjacent mesenchyme.

Eyelid

During eyelid development, the lid primordia emerge as folds from the epidermis and dermis surrounding the eye, which then grow to extend over the corneal surface of the eye until they meet and fuse, only to reopen later. By E15.5 in the mouse, the upper and lower eyelids have extended over the corneal surface and fused at approximately the center of the eye (Figure A.3G). Expression patterns of *Shh*, *Ptch1* and *Ptch2* have been reported in the eyelid prior to fusion, during the extension phase (Motoyama et al., 1998a), and appear to be restricted to the basal layer of the eyelid epithelium.

Following fusion, expression of *Shh*, *Ptch1* and *Ptch2* continues in the basal layer of the eyelid epithelium originating from both the upper and lower lid primordial (Figure A.3H-J). *Shh* expression is restricted to a small patch of basal layer eyelid epithelium on the corneal side (Figure A.3H). *Ptch2* expression is similarly restricted to the basal layer eyelid epithelium but exhibits a broader expression territory that overlaps and extends beyond the region of *Shh* expression (Figure A.3I). *Ptch1* is also strongly expressed within a similar range of basal layer eyelid epithelium (Figure A.3J). However, unlike the epithelium-restricted expression observed in the extension stage (Motoyama et al., 1998a), *Ptch1* expression clearly extends into the adjacent mesenchyme of the eyelid tip, although at weaker levels. *Hhip* is only faintly detected in the mesenchyme of the eyelid

tip (Figure A.3K). *Gli1* is expressed in both the basal layer eyelid epithelium and adjacent mesenchyme of the eyelid tip (Figure A.3L) in a range comparable to that of *Ptch1* and reveals active Hh signaling in these tissues.

Ocular tissues

Eye development involves a series of complicated morphogenic processes in which the retina, retinal pigmented epithelium (RPE), and optic stalk derive from the neuroectoderm and come to surround the surface ectoderm-derived lens capsule in a cup-like fashion. By E15.5, the lens vesicle has detached from the overlying surface ectoderm and the remaining surface ectodermal cells together with migrating mesenchymal cells have condensed to form the layers of the corneal epithelium. In the optic cup, the RPE monolayer surrounding the neural retina has become pigmented (Figure A.4A). Furthermore, by this stage of retinal development, retinal neurogenesis is incomplete. Thus, the E15.5 neural retinal contains two distinct layers: a basal differentiated cell layer containing nascent neurons and an apical neuroblast layer containing progenitors (Figure A.4A').

Expression of Hh pathway components in the eye at E15.5 is restricted to the developing retina, cells of the developing choroid and sclera surrounding the RPE, and differentiating stromal cells of the iris and ciliary body (Figure A.4B-F). In the neural retina, *Shh* expression is restricted to the differentiated cell layer (Figure A.4B,B'), consistent with its production by differentiated retinal ganglion cells (Wallace, 2008). In striking contrast to the previously described structures, *Ptch2* is only faintly detectable in the *Shh*-expressing cells of the differentiated cell layer (Figure A.4C,C'). Expression is

also faintly detected in the adjacent neuroblast layer at a level indistinguishable from that of the differentiated cell layer. Expression of *Ptch1* extends throughout both retinal layers, with slightly stronger expression observed in the neuroblast layer (Figure A.4D,D'). Interestingly, *Ptch1* is expressed at relatively low levels in the neural retina, at least compared to *Ptch1* expression in other organ structures (compare Figure A.4D,D' to Figures A.2D,J,P and A.3D,J). *Hhip* is detected throughout the neural retina, also with slightly stronger expression observed in the neuroblast layer (Figure A.4E,E'). In contrast, expression of *Gli1* is restricted to the neuroblast layer (Figure A.4F,F') and consistent with the established roles of Shh and Hh signaling in the proliferation and cell fate decisions of retinal progenitor cells (Wallace, 2008).

A narrow band of *Ptch1*, *Gli1* and *Hhip* expression is also observed outside the retina in the scleral condensation of the periocular mesenchyme (Figure A.4D-F). This periocular expression is a response to IHH signals produced by endothelial cells of the developing choroidal vasculature, a layer of cells situated between the RPE and scleral condensation (Dakubo et al., 2008; Dakubo et al., 2003; Wallace and Raff, 1999). Analyses of Hh target gene expression in ocular tissues of Ihh null mice and conditional Shh mutants reveal that the range of neuron-derived Shh action is restricted to the neural retina, while the range of Ihh action is restricted to the RPE and periocular mesenchyme (Dakubo et al., 2008; Dakubo et al., 2003). In addition, *Gli1* and weak *Ptch1* expression are also detected in the stroma of the iris and ciliary body (Figure A.4D,F). The lack of detectable *Shh* expression (Figure A.4B) and proximity to the RPE suggests this expression may also be a response to IHH signals.

Discussion

In the present study, we evaluated the relationship between regulators of feedback inhibition and Shh-induced activation of Hh signaling by comparing the expression of *Ptch1*, *Ptch2*, and *Hhip* with that of *Shh* and *Gli1* in a variety of developing organs, including the molars, hair follicles, palatal rugae, eyelids, and neural retina. Organogenesis of most of these structures involves epithelial-mesenchymal interactions and Hh pathway components are expressed in both compartments. The neural retina is unique in that it is of neural epithelial origin and Hh pathway components exhibit variable expression within defined regions of this epithelial tissue.

This expression analysis revealed several common patterns of expression among organs and relationships between Hh pathway components. *Shh* expression was restricted to epithelial tissues within the molars, hair follicles, palatal rugae and eyelids, and to the differentiated neurons of the retina. Ptch2 expression was similarly restricted to epithelial tissues. Although staining was performed on separate sections, *Ptch2* expression appears to overlap that of *Shh* in these epithelial tissues, but more broadly, and is consistent with earlier reports (Motoyama et al., 1998a; Motoyama et al., 1998b). In contrast, *Ptch2* expression was not detected in the epithelium of the palatal rugae. In the neural ectoderm of the retina, *Ptch2* expression was extremely faint, but appeared to be uniform across the layers of the retina, in both *Shh*-expressing and nonexpressing layers. *Ptch1* and *Gli1* exhibited largely overlapping patterns of expression. Both were expressed throughout epithelial and mesenchymal tissues. Interestingly, mesenchymal tissues stained more strongly for *Ptch1* and *Gli1* in the molars and hair follicles, while epithelial staining was stronger in the palate and eyelids. In the neural retina, *Gli1* expression overlapped with

that of *Ptch1* in the neuroblast layer, but was absent from the differentiated cell layer, despite *Ptch1* expression in this layer. Faint *Hhip* expression was detected throughout both epithelial and mesenchymal tissues in most organs. A strong, narrow band of expression was observed in the mesenchyme surrounding both molars and hair follicles at the outer edge of *Ptch1* and *Gli1* expression, at a distance from *Shh*-expressing cells. In contrast, the graded *Hhip* expression within the palate was strongest immediately adjacent to the *Shh*-expressing epithelium. This expression appeared to extend beyond *Ptch1* and *Gli1* territories, but such extensive expression is unlikely since *Hhip* depends on Hh signaling for upregulation. A possible explanation is that *Ptch1* and *Gli1* are expressed, but below the level of detection. However, it is more likely that these differences in the extent of expression reflect slightly different positions along the rugae in the different sections. Double labeling will be required to resolve this issue.

Upregulation of the feedback inhibitors generally correlated with levels of *Shh* expression. Shh expression was weakest in the neural retina and was associated with lower expression of *Ptch1*, *Ptch2*, and *Hhip* compared to other organs. This relationship is consistent with their roles in feedback inhibition to attenuate or limit the extent of Hh signaling. However, how the highest levels of *Hhip* expression are accomplished at the outer extent of pathway activation in molars and hair follicles is unclear. Interestingly, *Gli1*, a positive feedback signal, exhibited similar expression levels across organs, irrespective of *Shh* levels. It is tempting to speculate that the upregulation of feedback inhibitors in the presence of high Hh ligand and upregulation of positive feedback components when Hh ligand is low is required to achieve a certain threshold of Hh signaling activity. However, Hh signals are important morphogens whose graded

signaling determines specific cellular outcomes. How such a morphogen gradient is established in the context of these positive and negative feedback signals is not clear but may suggest a critical role for complementary gradients by an opposing morphogen.

The dependence of *Gli1* expression on cell autonomous Hh signal transduction makes it a reliable indicator of active Hh reception and signaling within cells. This feature also provides insight into paracrine versus autocrine signaling in Hh responsive cells. Although expression was analyzed on separate sections, expression of *Gli1* was both broad and uniform throughout the epithelial tissues exhibiting *Shh* expression, suggesting that coexpression is likely. Thus, activation of autocrine signaling may be an important aspect of Shh signaling during organogenesis. Interestingly, in the neural epithelium of the retina, *Gli1* expression is undetectable in the *Shh*-expressing neurons of the differentiated cell layer, suggesting that autocrine signaling does not occur in this tissue. However, Shh directs commissural axon guidance in vivo, independent of transcription, through activation of Src family kinases (Yam et al., 2009). Shh-expressing ganglion cells are the projection neurons of the retina. Thus, it is possible that autocrine signaling also occurs in these cells, but in a transcription-independent fashion. Shh also activates paracrine signaling in epithelial tissues since *Shh* expression was often restricted to a defined subregion. In contrast, paracrine signaling is responsible for activation of Hh signaling in mesenchymal tissues.

Interestingly, negative regulators involved in feedback inhibition of the Hh pathway differentially associated with these modes of signaling. Strong upregulation of *Ptch1* and *Hhip* was principally associated with activation of paracrine signaling in

mesenchymal tissues. In contrast, *Ptch2* expression was principally associated with autocrine signaling within *Shh*-expressing epithelial cells.

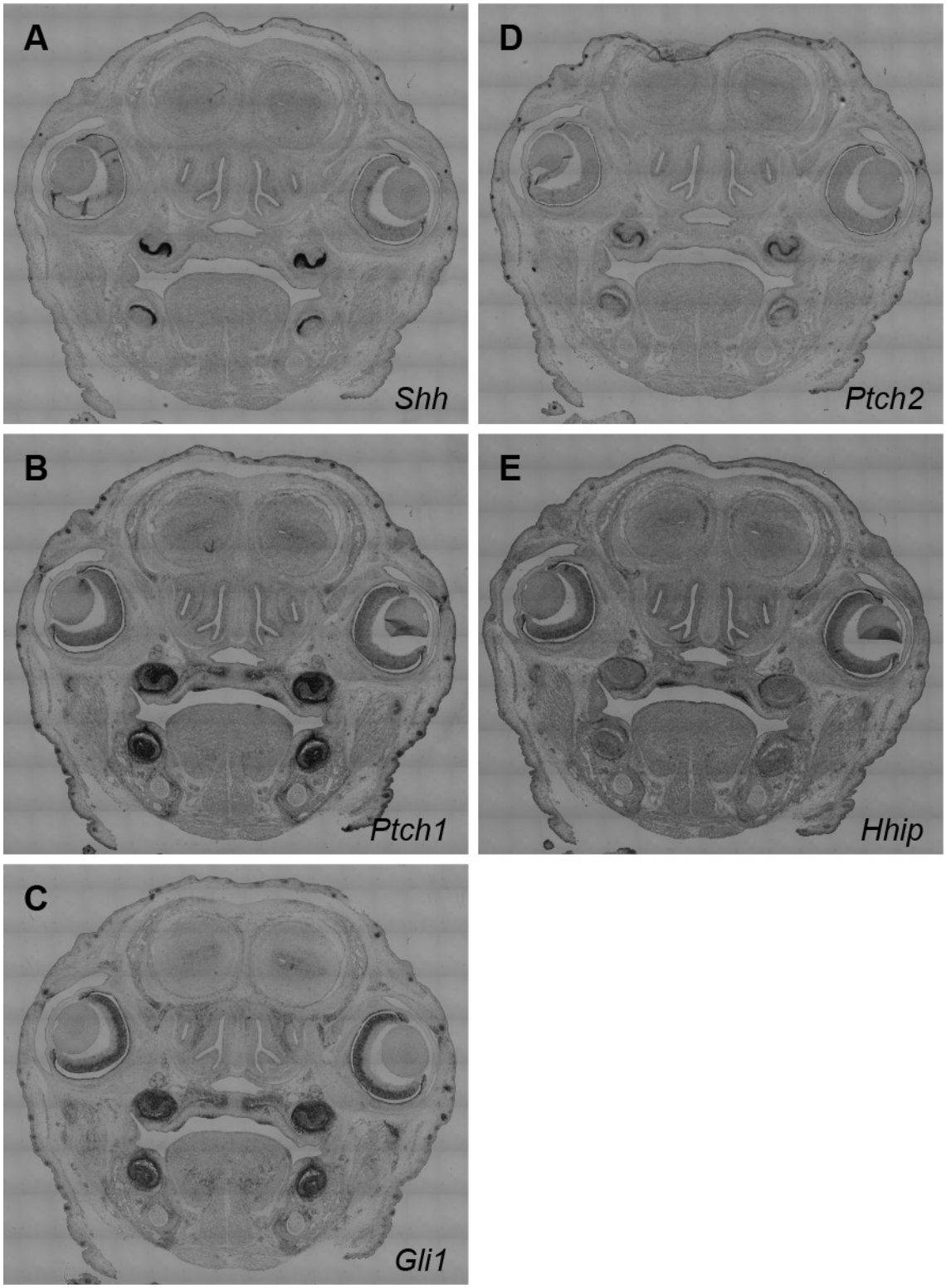
Active Hh signaling was also observed in the stroma of the developing iris and ciliary body. Proximity of these stromal cells to the endothelial cells of the developing choroid, a known source of Ihh (Dakubo et al., 2008; Dakubo et al., 2003; Wallace and Raff, 1999), suggests this signaling may be in response to Ihh. In a previous analysis of wild type and Ihh null mice, *Gli1* expression was not reported in the stroma of the iris or ciliary body (Dakubo et al., 2008; Dakubo et al., 2003). However, differentiation of neural crest and/or mesenchymal cells to generate the stromal cells of the iris and ciliary body initiates around the time that *Gli1* expression was examined in Ihh null mice (Cvekl and Tamm, 2004). Mutations in Shh are associated with iris coloboma (Schimmenti et al., 2003); however, this may simply reflect the role of Shh in early patterning events required for proper morphogenesis of the optic cup and closure of the choroid fissure. Consistent with this idea, ablation of Pax2, a downstream target of midline-derived Shh, in mice also results in coloboma (Schwarz et al., 2000). Although Shh was undetectable near the peripheral optic cup in the present study, weak expression may have been masked by pigmentation of the RPE or iris and ciliary body. *Dhh* has also been detected in the developing postnatal RPE and adult iris of the mouse (Levine et al., 1997; Takabatake et al., 1997), but its role in eye development has not been examined. *Ihh*, but not *Shh*, was also detected in the adult iris of mouse and newt, together with *Ptch1* and *Ptch2* (Takabatake et al., 1997). Thus, the identity and source of the Hh ligand responsible for Hh pathway activation in the stroma of the iris and ciliary body will require future evaluation. Iris and ciliary body formation is principally regulated by

BMP/TGFβ and Wnt signaling (Cvekl and Tamm, 2004; Davis-Silberman and Ashery-Padan, 2008). How Hh signaling contributes to the development of these structures is unclear.

Conclusion

Expression analysis of the Hh pathway components examined in this study reveal that participation of negative regulators involved in feedback inhibition is a common theme of Hh signaling across organs. Although the individual expression patterns of *Ptch1*, *Ptch2* and *Hhip* differed greatly, patterns were often similar across different organs, suggesting that the relationships between these negative regulators and activation of the Hh pathway may be fairly consistent. Furthermore, activation of both paracrine and autocrine signaling by Shh was associated with the expression of these negative regulators. Lastly, the significance of active hedgehog signaling in the developing stroma of the iris and ciliary body is unknown. Further analysis is required to determine the identity and source of the Hh ligand responsible for this activity and its role in the development of these tissues.

Figure A.1. Expression patterns for Shh and Hh pathway components in developing organs of the embryonic murine head. In situ hybridation for *Shh* (A), *Ptch1* (B), *Gli1* (C), *Ptch2* (D), and *Hhip* (E) in adjacent coronal sections of the mouse at E15.5.


A
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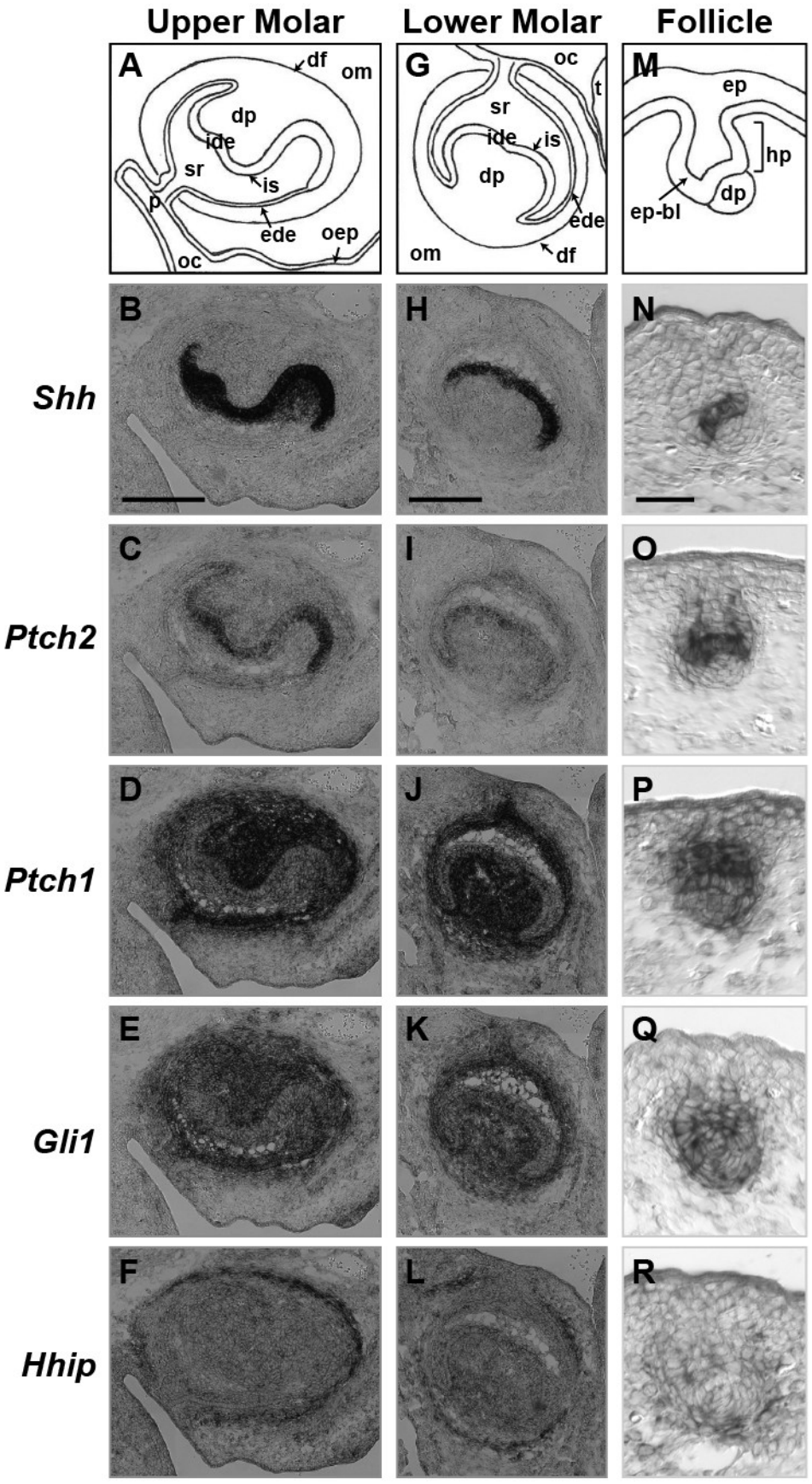
Gli1

Figure A.2. Comparison of expression patterns for Shh and Hh pathway components in the molars and hair follicles. Expression of *Shh* (B, H, N), *Ptch2* (C, I, O), *Ptch1* (D, J, P), *Hhip* (E, K, Q), and *Gli1* (F, L, R) in the upper molars (B-F), lower molars (H-L), and hair follicles (N-R). Diagrams illustrating the morphology of the developing upper (A) and lower (G) molars are shown with buccal to the left, lingual to the right. ide, internal dental epithelium; ede, external dental epithelium; is, intermediate stratum; sr, stellate reticulum; dp, dental papilla; df, dental follicle; p, pedicle; oc, oral cavity; om, oral mesenchyme; oep, oral epithelium; t, tongue. Diagram depicting the morphology of stage 3 hair follicles is shown in (M). ep, epidermis; ep-bl, basal layer of epidermis; hp, hair peg; dp, dermal papilla; der, dermis. Scale bar: 200 μm (B, H), 40 μm (N).


Upper Molar
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Ptch2
C
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Ptch1
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Gli1
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Hhip
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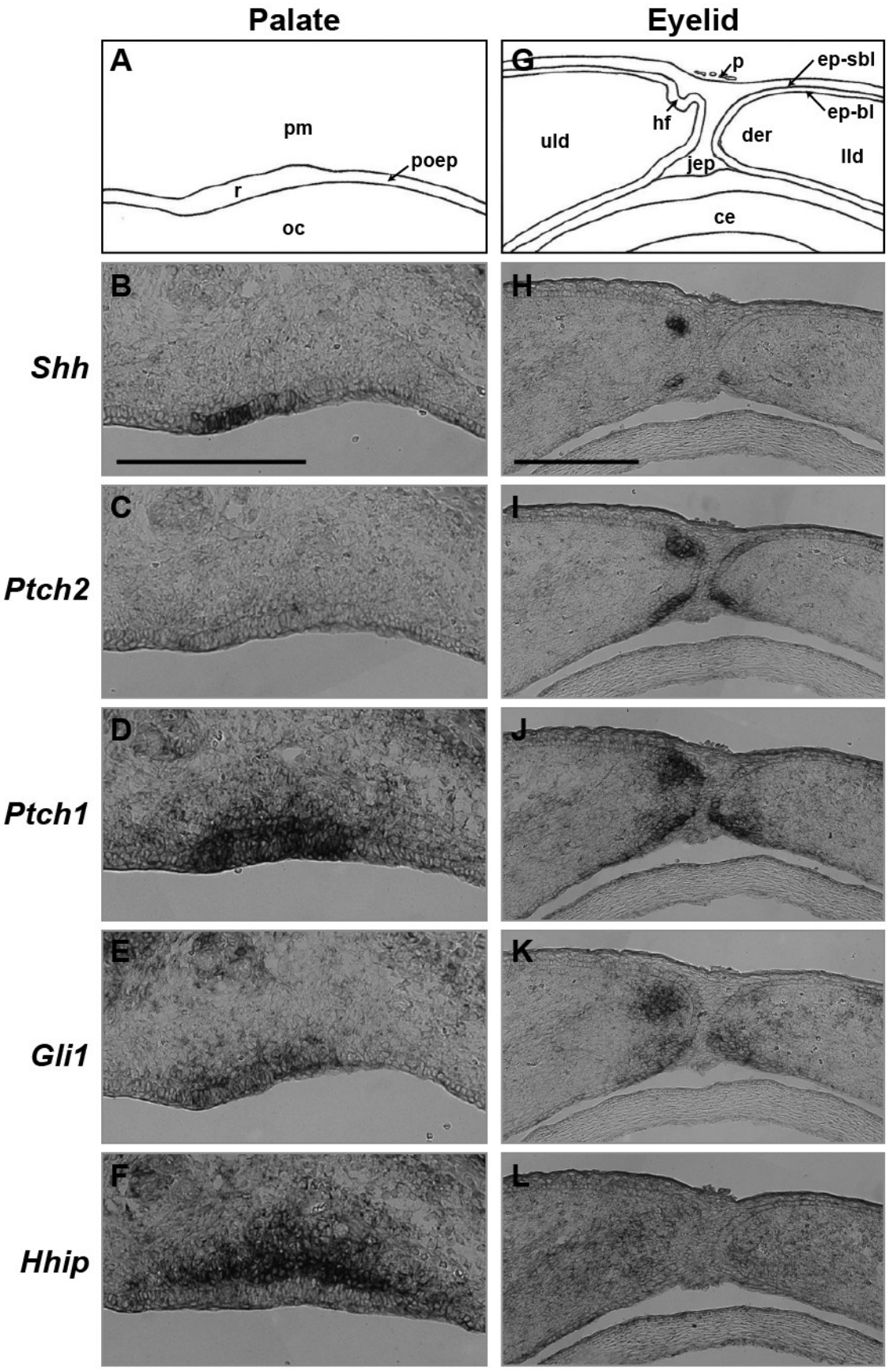
Figure A.3. Comparison of expression patterns for Shh and Hh pathway components in the palate and eyelids. Expression of *Shh* (B, H), *Ptch2* (C, I), *Ptch1* (D, J), *Hhip* (E, K), and *Gli1* (F, L) in the palate (B-F) and eyelids (H-L). Diagram depicting the morphology of the developing palate is shown in (A). r, palatal rugae; poep, palatal oral epithelium; pm, palatal mesenchyme; oc, oral cavity. Diagram depicting morphology of the developing eyelids is shown in (G). uld, upper lid; lld, lower lid; ep-sbl, suprabasal layer of epidermis; ep-bl, basal layer of epidermis; der, dermis; jep; junctional epithelium; p, residual periderm; hf, hair follicle; ce, corneal epithelium. Scale bar: 200 μm (B, H).


Palate
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Shh
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Ptch2
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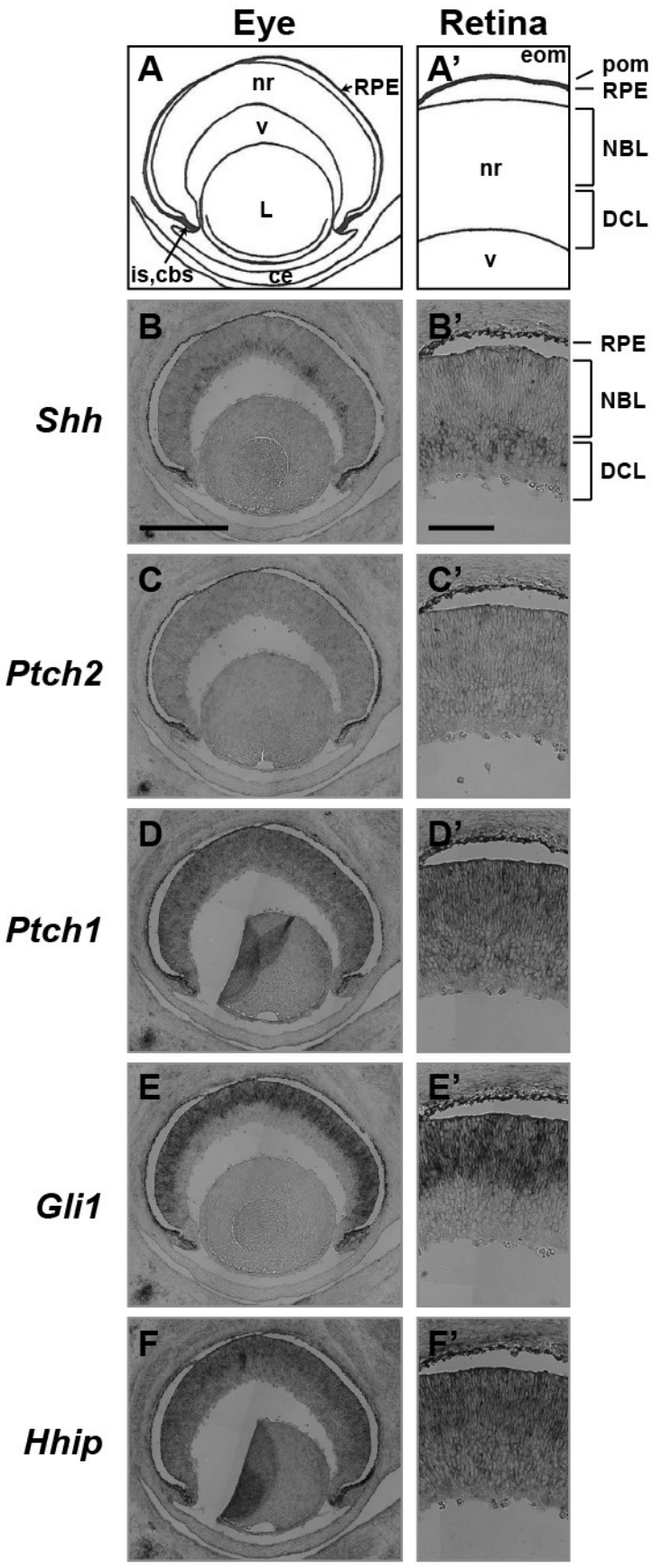
Ptch1
E
K
Gli1
F
L
Hhip

Figure A.4. Comparison of expression patterns for *Shh* and Hh pathway components in the eye and surrounding tissues. Expression of *Shh* (B, B'), *Ptch2* (C, C'), *Ptch1* (D, D'), *Hhip* (E, E'), and *Gli1* (F, F') in the eye (B-F) and retina (B'-F'). Diagrams depicting the morphology of the developing eye (A) and retina (A') are shown. nr, neural retina; RPE, retinal pigmented epithelium; v, vitreous; L, lens; ce, corneal epithelium; pom, periocular mesenchyme; is, iris stroma; cbs, ciliary body stroma; NBL, neuroblast layer; DCL, differentiated cell layer. Scale bar: 400 μm (B), 100 μm (B').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B'
Shh
RPE
NBL
DCL
C
C'
Ptch2
D
D'
Ptch1
E
E'
Gli1
F
F'
Hhip

References

Amato, M.A., Boy, S., Perron, M., 2004. Hedgehog signaling in vertebrate eye development: a growing puzzle. Cell. Mol. Life Sci. 61, 899-910.

Bai, C.B., Auerbach, W., Lee, J.S., Stephen, D., Joyner, A.L., 2002. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development 129, 4753-4761.

Burmeister, M., Novak, J., Liang, M.Y., Basu, S., Ploder, L., Hawes, N.L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V.I., Roderick, T.H., Taylor, B.A., Hankin, M.H., McInnes, R.R., 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. 12, 376-384.

Carpenter, D., Stone, D.M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A., de Sauvage, F.J., 1998. Characterization of two patched receptors for the vertebrate hedgehog protein family. Proc. Natl. Acad. Sci. U. S. A. 95, 13630-13634.

Casali, A., Struhl, G., 2004. Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein. Nature 431, 76-80.

Chen, Y., Struhl, G., 1996. Dual roles for patched in sequestering and transducing Hedgehog. Cell 87, 553-563.

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383, 407-413.

Chuang, P.T., Kawcak, T., McMahon, A.P., 2003. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. Genes Dev. 17, 342-347.

Chuang, P.T., McMahon, A.P., 1999. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature 397, 617-621.

Cobourne, M.T., Sharpe, P.T., 2005. Sonic hedgehog signaling and the developing tooth. Curr. Top. Dev. Biol. 65, 255-287.

Cvekl, A., Tamm, E.R., 2004. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. BioEssays 26, 374-386.

Dakubo, G.D., Mazerolle, C., Furimsky, M., Yu, C., St-Jacques, B., McMahon, A.P., Wallace, V.A., 2008. Indian hedgehog signaling from endothelial cells is required for sclera and retinal pigment epithelium development in the mouse eye. Dev. Biol. 320, 242-255.

Dakubo, G.D., Wang, Y.P., Mazerolle, C., Campsall, K., McMahon, A.P., Wallace, V.A., 2003. Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development. Development 130, 2967-2980.

Davis-Silberman, N., Ashery-Padan, R., 2008. Iris development in vertebrates; genetic and molecular considerations. Brain Res. 1192, 17-28.

Goodrich, L.V., Johnson, R.L., Milenkovic, L., McMahon, J.A., Scott, M.P., 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes Dev. 10, 301-312.

Goodrich, L.V., Jung, D., Higgins, K.M., Scott, M.P., 1999. Overexpression of ptc1 inhibits induction of Shh target genes and prevents normal patterning in the neural tube. Dev. Biol. 211, 323-334.

Goodrich, L.V., Milenkovic, L., Higgins, K.M., Scott, M.P., 1997. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science 277, 1109-1113.

Green, E.S., Stubbs, J.L., Levine, E.M., 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development 130, 539-552.

Hardy, M.H., 1969. The differentiation of hair follicles and hairs in organ culture. Adv. Biol. Skin 9, 35-60.

Hardy, M.H., 1992. The secret life of the hair follicle. Trends Genet. 8, 55-61.

Ingham, P.W., McMahon, A.P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15, 3059-3087.

Levi, B., James, A.W., Nelson, E.R., Brugmann, S.A., Sorkin, M., Manu, A., Longaker, M.T., 2011. Role of Indian hedgehog signaling in palatal osteogenesis. Plast. Reconstr. Surg. 127, 1182-1190.

Levine, E.M., Roelink, H., Turner, J., Reh, T.A., 1997. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J. Neurosci. 17, 6277-6288.

Mann, S.J., 1962. Prenatal formation of hair follicle types. Anat. Rec. 144, 135-141.

Motoyama, J., Heng, H., Crackower, M.A., Takabatake, T., Takeshima, K., Tsui, L.C., Hui, C., 1998a. Overlapping and non-overlapping Ptch2 expression with Shh during mouse embryogenesis. Mech. Dev. 78, 81-84.

Motoyama, J., Takabatake, T., Takeshima, K., Hui, C., 1998b. Ptch2, a second mouse Patched gene is co-expressed with Sonic hedgehog. Nat. Genet. 18, 104-106.

Nelson, E.R., Levi, B., Sorkin, M., James, A.W., Liu, K.J., Quarto, N., Longaker, M.T., 2011. Role of GSK-3beta in the osteogenic differentiation of palatal mesenchyme. PLoS One 6, e25847.

Nieuwenhuis, E., Motoyama, J., Barnfield, P.C., Yoshikawa, Y., Zhang, X., Mo, R., Crackower, M.A., Hui, C.C., 2006. Mice with a targeted mutation of patched2 are viable but develop alopecia and epidermal hyperplasia. Mol. Cell. Biol. 26, 6609-6622.

Paus, R., Muller-Rover, S., Van Der Veen, C., Maurer, M., Eichmuller, S., Ling, G., Hofmann, U., Foitzik, K., Mecklenburg, L., Handjiski, B., 1999. A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. J. Invest. Dermatol. 113, 523-532.

Rahnama, F., Toftgard, R., Zaphiropoulos, P.G., 2004. Distinct roles of PTCH2 splice variants in Hedgehog signalling. Biochem. J. 378, 325-334.

Rice, R., Connor, E., Rice, D.P., 2006. Expression patterns of Hedgehog signalling pathway members during mouse palate development. Gene Expr. Patterns 6, 206-212.

Riobo, N.A., Manning, D.R., 2007. Pathways of signal transduction employed by vertebrate Hedgehogs. Biochem. J. 403, 369-379.

Rowan, S., Chen, C.M., Young, T.L., Fisher, D.E., Cepko, C.L., 2004. Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. Development 131, 5139-5152.

Ryan, K.E., Chiang, C., 2012. Hedgehog secretion and signal transduction in vertebrates. J. Biol. Chem. 287, 17905-17913.

Schaeren-Wiemers, N., Gerfin-Moser, A., 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry 100, 431-440.

Schimmenti, L.A., de la Cruz, J., Lewis, R.A., Karkera, J.D., Manligas, G.S., Roessler, E., Muenke, M., 2003. Novel mutation in sonic hedgehog in non-syndromic colobomatous microphthalmia. Am. J. Med. Genet. A 116A, 215-221.

Schwarz, M., Cecconi, F., Bernier, G., Andrejewski, N., Kammandel, B., Wagner, M., Gruss, P., 2000. Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. Development 127, 4325-4334.

Sigulinsky, C.L., Green, E.S., Clark, A.M., Levine, E.M., 2008. Vsx2/Chx10 ensures the correct timing and magnitude of Hedgehog signaling in the mouse retina. Dev. Biol. 317, 560-575.

St-Jacques, B., Dassule, H.R., Karavanova, I., Botchkarev, V.A., Li, J., Danielian, P.S., McMahon, J.A., Lewis, P.M., Paus, R., McMahon, A.P., 1998. Sonic hedgehog signaling is essential for hair development. Curr. Biol. 8, 1058-1068.

Takabatake, T., Ogawa, M., Takahashi, T.C., Mizuno, M., Okamoto, M., Takeshima, K., 1997. Hedgehog and patched gene expression in adult ocular tissues. FEBS Lett. 410, 485-489.

Varjosalo, M., Taipale, J., 2008. Hedgehog: functions and mechanisms. Genes Dev. 22, 2454-2472.

Wallace, V.A., 2008. Proliferative and cell fate effects of Hedgehog signaling in the vertebrate retina. Brain Res. 1192, 61-75.

Wallace, V.A., Raff, M.C., 1999. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126, 2901-2909.

Yam, P.T., Langlois, S.D., Morin, S., Charron, F., 2009. Sonic hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. Neuron 62, 349-362.

Zaphiropoulos, P.G., Unden, A.B., Rahnama, F., Hollingsworth, R.E., Toftgard, R., 1999. PTCH2, a novel human patched gene, undergoing alternative splicing and up-regulated in basal cell carcinomas. Cancer Res. 59, 787-792.