CELL CYCLE MEDIATED REGULATION OF RETINAL DEVELOPMENT: INSIGHTS FROM D-CYCLIN KNOCKOUT MICE

by

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ABSTRACT

During nervous system development, progenitor cells multiply under the control of the cell cycle pathway. When they are poised to differentiate, they withdraw from the cell cycle to form appropriate neuronal cell types. Cell cycle regulation is therefore closely intertwined with progenitor proliferation and neurogenesis in the developing nervous system and often, common factors and pathways are utilized in these processes. Two properties of neuronal progenitors can be critical for proper nervous system development: the time they take to complete one cell cycle, and the exact timing of their withdrawal/exit from the cell cycle to form neurons. Understanding how these properties can be manipulated to influence progenitor cell proliferation and neurogenesis can be invaluable for devising therapeutic strategies involving neuronal stem/progenitor cells. Retina, the primary tissue for vision, is an excellent model system for studying nervous system development and neuronal progenitor cell biology. To gain potential insights into the issues described above, this dissertation focuses on the role of the cell cycle regulators the D-cyclins, *Cyclin D1* (*Ccnd1*) and *Cyclin D3* (*Ccnd3*), during retinal development and characterizes the retinal phenotypes of *Ccnd1* and *Ccnd3* knockout mice.

Chapter 1 is an introduction to retinal development and sets up the relevant questions addressed here.

Chapter 2 is a reprint of a published journal article titled, “Cyclin D1 fine-tunes

the neurogenic output of embryonic retinal progenitor cells." The study shows that during mouse embryonic development, CCND1 expression in retinal progenitor cells (RPCs) is critical for maintenance of their cell cycle time and also for their timing of exit from the cell cycle. Further, CCND1 ensures that the correct complements of early-born retinal neurons are generated from progenitors.

Chapter 3 deals with the role of D-cyclins during postnatal retina development. The study shows that CCND1 also influences the production rate of late-born retinal cell types. Unexpectedly, although *Ccnd1* null retinas experience progenitor cell depletion during development, proliferation, and neurogenesis persist well beyond the normal period of retinal histogenesis in mutant retinas. Further, *Ccnd3* is unable to compensate for *Ccnd1's* role in regulation of cell cycle time and cell cycle withdrawal.

Chapter 4 discusses the implications and relevance of the above studies. Future directions for these studies are also outlined.

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CHAPTER 1

INTRODUCTION

Chapter Overview

This chapter is a primer on retina development from a cell biological perspective. It begins with a short introduction to retinal histogenesis. Subsequently, relevant properties of retinal cell types are discussed. Proliferation of progenitor cells in the retina and differentiation of retinal neurons from these progenitors is intricately associated with the cell cycle pathway. The time a progenitor takes to complete one cell cycle and the timing of its withdrawal/exit from the cell cycle are both critical factors for proper development of retinal form and function. Cell cycle regulators play a prominent role in retinal development. D-type cyclins are positive regulators of cell cycle progression. *CyclinD1* (*Ccnd1*) is highly expressed in all retinal progenitors, but *Cyclin D3* (*Ccnd3)* is normally expressed in glial cells of the retina. Towards the end of this chapter, a rationale for studying the role of CCND1 and CCND3 during retinal histogenesis is presented.

Overview of retina development

The basic pattern of vertebrate ocular and retinal development, outlined in Fig. 1.1, is conserved across species (Chow and Lang, 2001; Gehring, 2004; Lamb et al., 2007). Eye formation begins when a population of cells in the anterior neural plate, later to be the forebrain, is specified as the eye field (Fig. 1.1A). With the neural plate growing upward and inwards towards the midline (Fig. 1.1B), the optic grooves, derived from the eye field, evaginate towards the surface ectoderm (Fig. 1.1C and D). With the progress of development, the lips of the neural fold meet at the midline and pinch off from the surface ectoderm to form the neural tube (Fig. 1.1E). In addition, by this time, the optic grooves are in close contact with the non-neural surface ectoderm and form the optic

vesicles (Fig. 1.1 E). Signaling and patterning events at this stage begin to demarcate the presumptive neural retina, retinal-pigmented epithelium (RPE), and optic stalk domains (Yang, 2004). Interaction between the optic vesicle and the surface ectoderm induces the former to invaginate and the latter to differentiate into the lens placode (Fig. 1.1E). Invagination of the optic vesicle forms the optic cup structure, which consists of both the retina and the RPE in close apposition (Fig. 1.1F). At this stage, the progenitor cells in the retina domain have a greater rate of proliferation than RPE progenitors, making the retina much thicker than the single layered RPE (Bharti et al., 2006). The optic cups stay connected to the forebrain through the optic stalks (Fig. 1.1F). The lens placode also starts to proliferate and invaginate during this time; soon, it will pinch off from the surface ectoderm and form the lens (Fig. 1.1G). The optic cup eventually grows circumferentially to enclose the choroid fissure and neurogenesis initiates in the central optic cup, producing ganglion cells, whose axons form the optic nerve (Fig. 1.1G). This begins retinal histogenesis, a period of intense progenitor proliferation and the simultaneous production of all retinal cell classes (Agathocleous and Harris, 2009; Livesey, R. and Cepko, C., 2001).

Retinal cell types

Retinal progenitor cells (RPCs)

Restricted potential

Dividing neuronal progenitor cells that populate the retina from its specification up to the termination of its histogenesis can be called retinal progenitor cells (RPCs). All cell types in the retina ultimately arise from the RPCs. Unlike pluripotent stem cells

RPCs are more restricted in their potential. They are capable of producing only retinal neurons. Therefore, both embryonic stem cells (ES) and induced pluripotent stem cells (iPS) are capable of generating RPCs and retinal neurons. To do so, they require specific growth factor/small molecule treatment or misexpression of the so-called 'eye field transcription factors' (EFTFs) (Dong et al., 2003; Ikeda et al., 2005; Lamba et al., 2006; Lamba et al., 2010; Osakada et al., 2009a; Osakada et al., 2009b; Osakada et al., 2008; Viczian et al., 2009; Zuber et al., 2003).

Pre-neurogenic versus neurogenic RPCs

Up to a certain point in retinal development, RPC are 'pre-neurogenic', in that they undergo division to produce only proliferating daughter RPCs (exponential division). Subsequently, RPCs are ‘neurogenic’ and become capable of producing immature neuronal precursor cells (precursors) that withdraw/exit from the cell cycle. The initiation of neurogenesis from neurogenic RPCs begins in the central optic cup (McCabe et al., 1999; Prada et al., 1991; Reese and Colello, 1992). Subsequently, a wave of neurogenesis spreads out towards the peripheral retinal regions. Recent studies in the chicken retina revealed distinct patterns of gene expression that distinguish between pre-neurogenic and neurogenic RPCs. Pre-neurogenic RPCs express the transcription factor PAX6 and the notch receptor pathway ligand DELTA1. As these RPCs transition to a neurogenic state, they decrease PAX6 expression, extinguish DELTA1 expression, and begin to express E2A, a known binding partner for pro-neural BHLH factors (see below) (Hsieh and Yang, 2009; Yang et al., 2009). The DELTA1 to E2A switch happens *en masse* in a group of neighboring RPCs and leads the outward-spreading wave of neurogenesis (Yang et al.,

2009). In regions through which the neurogenesis wave has passed, RPCs divide to produce either two daughter RPCs, a combination of an RPC and a precursor or two precursor cells. As retinal development progresses, the frequency of RPC-producing divisions decreases and neuron-producing divisions increases. Eventually, when the balance shifts to exclusively precursor-producing terminal divisions, retinal histogenesis terminates (Livesey, F. J. and Cepko, C. L., 2001). This dynamic pattern of progenitor cell division is also seen in other parts of the developing nervous system including the developing cortex (Chenn and McConnell, 1995).

Diversity in RPC expression profile

Being either pre-neurogenic or neurogenic is not the only parameter of variability amongst RPCs. RNA expression profiling of mouse RPCs at various stages of development revealed great diversity amongst these cells, even when they were isolated from the same stage of development (Trimarchi et al., 2008). Dynamic expression of transcription factors in RPCs accounted for a major part of this diversity (Trimarchi et al., 2008). Expression differences amongst multipotent RPCs point towards diversity in their intrinsic identity, capacity to respond to their environment, and competence to produce different precursor cell types over their developmental history.

Temporal changes in RPC competence

Several lines of evidence support the idea that RPCs change their 'competence' over developmental time. Competence is defined as the ability of an RPC to respond to

its environment and make certain types of precursors or acquire a strong bias towards making these precursors.

It was observed that precursor cell types were produced from RPCs in an evolutionarily conserved temporal sequence (Young, 1985a; Young, 1985b). Further, when 'early' RPCs were co-cultured with 'late' RPCs, or were transplanted into a developmentally advanced retina, they still generated the early-born cell types that they normally would in spite of being in a 'late' environment. Conversely, when late RPCs were co-cultured with excess of early RPCs, they did not generate early-born cell types (Belliveau and Cepko, 1999; Belliveau et al., 2000; Rapaport et al., 2001; Watanabe and Raff, 1990). This suggests that RPCs have intrinsic 'competence states' that undergo unidirectional changes over time. Such competence stages were also observed in the developing cortex (Desai and McConnell, 2000). The competence model was nicely demonstrated in Drosophila neuroblasts (NBs), where transient and sequential expression of the four transcription factors Hunchback (*Hb*), Krüppel (*Kr*), *Pdm,* and *Castor* conferred temporal competence upon NBs to produce a conserved order of distinct progeny neurons. These factors retained their expression in the neurons produced from NBs during their expression period (Isshiki et al., 2001). Factors like HB, KR, PDM, and CASTOR were both necessary and sufficient for production of the cell types that retained the expression of these factors (Grosskortenhaus et al., 2006; Isshiki et al., 2001; Pearson and Doe, 2003). Thus, when HB or KR was overexpressed, they induced NBs to produce more of the cell types that express these factors, at the expense of the later-born cell types. Conversely, when HB or KR was removed, the NBs skipped the production of the earlier-born cell types that expressed factors but produced later-born cell types (Isshiki et al.,

2001; Livesey, R. and Cepko, C., 2001). However, mere expression of these factors was not always enough to induce NBs to produce neurons that retained their expression. Although misexpression of HB in older NBs was sufficient to induce production of early-born cell types, this ability declined with increasing age of the NBs (Pearson and Doe, 2003).

Recent studies have lent more credence to this model in the mouse retina. Expression of IKAROS (IK), the mouse ortholog of HB, was seen in early embryonic RPCs, but its expression faded from late postnatal RPCs, many of which were derived from IKAROS-expressing RPCs (Elliott et al., 2008). IKAROS expression was also observed exclusively in early-born retinal ganglion, horizontal, and amacrine cells. In $Ik^{-/-}$ mice, production of the above-mentioned cell types were significantly reduced, not eliminated, but production of late-born cell types was normal. Forced expression of *Ik* in later stage retinas (postnatal day one mice), significantly increased the expression of horizontal and amacrine cells. *Ik* misexpression did not induce the production of ganglion cell, the earliest born retinal cells, *in vivo*. However, *Ik* misexpression did induce ganglion cell production from RPCs in low-density cultures of late retinal cells (Elliot 2008). Interestingly, loss of IKAROS expression was required for production of Müller glia cells and misexpression of IKAROS prevented glial cell formation.

In another study, conditional deletion of the RNAse III enzyme *Dicer* in the mouse retina, important for microRNA (miRNA) maturation, extended the period of ganglion cell genesis well beyond birth (Georgi and Reh, 2010). Further, differentiation of late-born cell types like glia or bipolar cells was severely impaired in retinal areas lacking *Dicer*. This indicates that *Dicer* activity or specific miRNAs are required by

RPCs to progress from an early ganglion cell producing competence state to late-born cell type producing competence state (Georgi and Reh, 2010). Incidentally, most cell types except ganglion cells failed to survive in mature *Dicer* deficient retinas, underlining the extensive requirement for DICER in retinal development and function.

The above studies highlight the importance of both intrinsic state and external environment in defining competence. For testing whether a factor truly induces a competence state for production of certain cell types, misexpression of the factor is not adequate. The misexpressing cells must be placed in a permissive environment for true test of a competence state and a competence-inducing factor.

Retinal neuronal precursors and neurons

Defined order of cell type genesis

If a progeny cell of an RPC division is fated to become a neuron, it exits or withdraws from the cell cycle pathway to form an immature precursor cell. The time of exit of a precursor from the cell cycle is the 'birth date' of that cell. With the progress of retinal development, precursor cells express markers of maturation appropriate to their particular neuronal lineage. Subsequently, they migrate to their appropriate laminar location in the retina and integrate into the emerging visual circuit. Retinal precursors are 'born' in a defined but overlapping order (La Vail et al., 1991; Rapaport et al., 2004; Wong and Rapaport, 2009). In general, the less abundant cell types are born earlier and the more abundant cell types are born later during retinal development (Farah and Easter, 2005; Finlay, 2008; Jeon et al., 1998). Thus, retinal ganglion cells (RGCs) are the first cell types to be born, followed closely, in an overlapping manner, by horizontal cells and

cone photoreceptor cells. Amacrine and rod photoreceptor cell production follows, with the genesis period of the rod cells, the most abundant cell type in the murine retina (Jeon et al., 1998), straddling both embryonic and postnatal stages. Finally, the last cell types to be generated are the bipolar neurons and Müller glia cells. This order of neuronal cell production is highly conserved amongst various species. The only known subtle exception to this temporal sequence is rod photoreceptor cell generation. Nocturnal animals have relatively more rods for facilitating low light vision. The peak of rod photoreceptor production in these animals moves towards later development. This has been proposed to be part of an evolutionary strategy, where the 'envelope' of cell cycle exit, and the production of more abundant cell types, is shifted towards later development (Dyer et al., 2009; Finlay, 2008). Therefore, in *Xenopus*, rod generation closely follows cone generation (Wong and Rapaport, 2009). However, in the nocturnal mouse retina, the period of rod genesis is pushed more toward the end of retinal histogenesis, away from cone genesis. Temporal orders of birth also exist among subclasses of cells within a major class. For example, cone bipolar cells are born before rod bipolar cells, GABAergic amacrine cells are produced ahead of glycinergic amacrine cells, and axon-bearing horizontal cells are born before axon-less horizontal cells in chick (Cherry et al., 2009; Edqvist et al., 2008; Morrow et al., 2008; Voinescu et al., 2009).

The cell cycle

Overview

The cell cycle is the one-way molecular pathway through which a progenitor undergoes cell division. In course of the cell cycle, a progenitor cell doubles its diploid

genomic content, and during mitosis, its DNA is equally distributed between daughter cells. In somatic tissues, the cell cycle is also associated with cellular growth (Levine, 2004), and besides duplicating and dividing its genome, a dividing cell also undertakes 'cytokinesis', whereby it distributes its cytoplasm amongst its daughter cells.

The most common somatic cell cycle, coupled to growth and occurring in the developing retina, has four major phases (G1, S, G2, and M). It also has major 'checkpoints' to prevent erroneous cell cycle progression and a ‘restriction-point’ towards the end of G1 beyond which an RPC is irreversibly committed to cell division (Hartwell and Weinert, 1989; Kastan and Bartek, 2004; Pardee, 1974). Progression through the cell cycle is mainly ensured by the Cyclin and Cyclin dependent Kinase family (CDK) of proteins (Malumbres and Barbacid, 2009). Cyclin dependent kinase inhibitors (CDKIs) are negative regulators of cell cycle progression that provide balance to the process (Sherr and Roberts, 1999).

Cell cycle time and neurogenic output

It is intuitive to comprehend that the time an RPC takes to go around one complete cell cycle can affect the overall cell number of a tissue. A faster cell cycle may lead to increased proliferation and growth. A slower cell cycle can result in a tissue with low cell number. Usually as development progresses, the average cell cycle time of the RPC population increases (Alexiades and Cepko, 1996). This correlates with an increase in precursor producing cell divisions and a decrease in progenitor producing divisions. As we shall see in subsequent chapters, a change in cell cycle time regulation can result in

altered proliferation, lower total cell number, and perhaps even an altered period of retinal histogenesis (Chapters 2 and 3).

The rate and timing of cell cycle exit of the RPC population during development can be critical for proper growth and the final size of a tissue. In a developing tissue, cell cycle exit is the primary process that initially apportions precursor cells to each cell class. At any given point during retinal development, the rate of precursor generation from RPCs by virtue of cell cycle exit can be defined as the neurogenic output of RPCs. Neurogenic output, or the rate of cell cycle exit, is amenable to direct measurement by simple assays.

Historically, neurogenic output is similar to measurements of the quiescent (Q) fraction, which is the fraction of progenitor cells exiting the cell cycle during a single cell cycle (Takahashi et al., 1996; Takahashi et al., 1997). The complementary measurement is the proliferative (P = 1-Q) fraction, which is the fraction of progenitors that continue to proliferate. Chapters 2 and 3 of this dissertation will highlight instances where cell cycle exit decisions ultimately affect retinal cell fate decisions and cellular composition of the retina.

Cell cycle phases

Gap 1 (G1) phase: cell cycle progression versus cell cycle exit

The G1 phase or the first 'gap' phase is so termed because it is considered a gap between the mitosis (M) and the DNA synthesis (S) phase. This phase of the cell cycle is critical during the development of a tissue because a progenitor cell makes the decision either to remain in the cell cycle or to withdraw from the cell cycle at G1. To stay in the

cell cycle, a progenitor cell has to pass the 'restriction point', near the end of the G1 phase of the cell cycle (Blagosklonny and Pardee, 2002). Once past this point, a progenitor enters the DNA synthesis phase and is almost sure to complete cell division.

The molecular pathway that is central to regulation of G1 phase progression or withdrawal from the cell cycle is the retinoblastoma (RB) pathway (Sun et al., 2007). The retinoblastoma protein family (*Rb1*, *Rbl1*/*p107*, and *Rbl2*/p130) is important for regulation of G1 progression, cell cycle exit, and maintenance of the precursor state after cell cycle exit. Active or under-phosphorylated forms of RB proteins prevent a progenitor from progressing past the restriction point and seem to keep exited cells from re-entering the cell cycle. Deletion of RB in the retina results in ectopic proliferation of neuronal precursor cells (Chen et al., 2004; Dyer and Bremner, 2005; Zhang et al., 2004). This is strikingly demonstrated in the 'single copy *p107*' retina, where only a single genomic copy of the *p107* gene is expressed in the retina. Horizontal cells in the retina of these animals re-enter the cell cycle and proliferate prodigiously. However, at the same time, they retain characteristics of mature horizontal cells (Ajioka et al., 2007). Therefore, Rb family members are critical for connecting cell cycle state with cellular differentiation state, so that neuronal maturation only occurs in cells that have exited the cell cycle.

D and E type cyclins are G1 phase specific (Sherr, 1993). Activity of D-cyclins is required in early G1 phase. They bind to and partner with CDK4/6 to perform two important functions that lead to phosphorylation and thus inactivation of RB proteins. Firstly, they directly phosphorylate RB proteins through the kinase activity of CDKs. Secondly, they sequester P27KIP1, an inhibitor of Cyclin E-CDK2 complex, and enable the complex to further phosphorylate retinoblastoma proteins at a different site (Kozar

and Sicinski, 2005; Levine and Green, 2004; Musgrove, 2006). Cyclin E expression is itself repressed by RB proteins through their inhibitory effect on E2F transcription factors (Chen, H. Z. et al., 2009; Sherr, 1995a). Thus, activation of Cyclin E-CDK2 complex initiates a positive feedback loop that leads to full activation of Cyclin E. The phosphorylation-mediated inactivation of RB proteins ultimately leads to overcoming the restriction point. E2Fs are instrumental downstream of RB proteins in this process and promotes entry into S-phase by activating targets like Cyclin E, Cyclin A, and DNA polymerases for DNA replication (Lundberg and Weinberg, 1998; Sherr, 1993; Sherr and Roberts, 1999).

Given that the retinoblastoma pathway is central to the regulation of cell cycle progression versus exit, CDK inhibitors that keep RB proteins in an active state tend to promote cell cycle exit. Loss of *p27Kip1*, *p57Kip2*, and *p19Ink4d*, the three CDKIs expressed in the retina, leads to ectopic proliferation (Dyer and Cepko, 2000a; Dyer and Cepko, 2001; Levine and Green, 2004; Levine et al., 2000). Interestingly, combined deletion of *p27Kip1* and *p19Ink4d* led to synergistic increase in ectopic proliferation and cell cycle re-entry of horizontal and amacrine cells (Cunningham et al., 2002).

DNA synthesis (S) phase

A progenitor cell doubles its 2N DNA content to 4N through DNA replication in the S phase. DNA replication is initiated at multiple replication origin points in the genome and the action of replication complexes that include DNA helicases and polymerases ensure duplication of the genome. Cyclin A-Cdk2 complex promotes S-phase progression by activating existing replication complexes (Coverley et al., 2002). It

also inhibits assembly of new replication complexes during the same period, ensuring that origins do not fire again until the next S phase (Coverley et al., 2002; Machida et al., 2005). Passage through S phase involves unwinding and rewinding of the genome. During this process, chromatin-remodeling complexes can potentially change the configuration of the genome in progenitor cells. Further, this can provide access for cell fate determinants to previously inaccessible parts of the genome and so influence cell fate determination (Fichelson et al., 2005; Holtzer et al., 1975; Ohnuma and Harris, 2003).

Gap 2 (G2) phase

The second 'gap' phase lies between the S phase and the M phase. In G2, progenitor cells prepare for mitosis. Checkpoints at the junction of G2/M phase ensure that entry into mitosis is halted if DNA replication is incomplete or defective (Johnson and Walker, 1999). The Cyclin A-Cdk2 complex is still the active driver of G2 and as its level builds up, it promotes enough Cyclin B1 expression for initiating mitosis (Fung and Poon, 2005; Lindqvist et al., 2009). It is postulated that the G2 phase is important for receiving signals that induce cell cycle exit and cell fate specification (Livesey, F. J. and Cepko, C. L., 2001). In the developing cortex, progenitor cells can be influenced in their cell fate decisions in late S/G2 phase (McConnell and Kaznowski, 1991). In the retina, it seems likely that amacrine cell fate specification occurs at G2 (Belliveau and Cepko, 1999). Further, certain fate specification factors like OTX2 and cell cycle exit regulators as P27KIP1 also upregulated their expression in G2 (Dyer and Cepko, 2001; Trimarchi et al., 2008). This indicates that events in G2 may be critical for cell cycle exit and cell fate.

Mitosis (M) phase

Mitosis sees the equal distribution of genetic material of a progenitor cell into two of its daughter cells. The Cyclin B-Cdk1 complex is the driving force of mitosis, forming maturation promoting factor or mitosis promoting factor (MPF), first studied in frog oocytes as a factor that initiated mitosis (Gerhart et al., 1984; Lohka et al., 1988; Masui, 2001; Masui and Markert, 1971). Cyclin B-Cdk1 drives progression of M phase up to the point where sister chromosomes are lined up at the centre, with the mitotic spindles pulling on them (Sullivan and Morgan, 2007). Beyond this point, the anaphase promoting complex (APC), an E3 ubiquitin ligase, promotes progression of the cell to G1 by mediating separation of sister chromatids and degradation of Cyclin B (Fung and Poon, 2005; Sullivan and Morgan, 2007). Division of the cytoplasm during M phase is not an equal distribution of cellular content. Amongst factors that are asymmetrically distributed between daughter cells are cell fate determinants. The consequences of asymmetrical cell division in the nervous system/retina and cell fate specification are succinctly outlined in the following reviews (Cayouette and Raff, 2002; Cayouette and Raff, 2003; Cayouette et al., 2006; Gotz and Huttner, 2005; Malicki, 2004; Miyata, 2007; Zhong and Chia, 2008).

Factors regulating RPC proliferation and precursor cell fate

Regulation of RPC proliferation and determination of cell fate is critical for proper retinal histogenesis. The cell cycle is intimately associated with both of these processes. This section offers an overview of the major classes of determinants that influence retinal proliferation and precursor cell fate. Not surprisingly, G1 phase regulators feature prominently among these factors.

Homeobox genes

Homeobox genes get their name from a highly conserved stretch of DNA, 180 base pairs long (excluding introns). The homeobox encodes for a DNA binding motif, the homeodomain (McGinnis et al., 1984). Homeobox proteins bind DNA and usually code for transcription factors (Gehring et al., 1990; Gehring et al., 1994). They were first identified in the early 1980s in Drosophila homeotic genes, which are responsible for laying out the body plan during development (McGinnis et al., 1984; Scott and Weiner, 1984). Since then, highly conserved homologs have been identified in many species where they perform similar functions. Homeobox genes are often classified by the presence of other conserved functional DNA binding motifs. Many homeobox genes influence cell fate specification in RPCs and precursors (Del Bene and Wittbrodt, 2005; Levine and Green, 2004; Wang and Harris, 2005).

Several homeobox genes are expressed in RPCs from the earliest stages of eye development. Genes as *Rx1*, *Lhx2*, and *Pax6* are important for early eye field specification, optic vesicle patterning, and maintenance of RPC proliferation. Some of these genes and their functions are discussed below.

Rx1 is important for eye field specification. In *Rx1* null mice, optic vesicles fails to form (Mathers et al., 1997). Misexpression of *Rx*, along with other factors, in pluripotent animal pole cells from Xenopus blastula induces formation of retinal cell types (Viczian et al., 2009). This indicates that *Rx1* is also important for a retinal fate.

Lhx2, another homeobox gene, is essential for eye formation. Deletion of the *Lhx2* arrests eye development at the optic vesicle stage, leading to anopthalmia (Porter et al., 1997; Tetreault et al., 2009; Yun et al., 2009). *Lhx2* has been deemed crucial to link

intrinsic and extrinsic pathways of optic cup formation in early eye development. Germline deletion of *Lhx2* leads to precocious neurogenesis. Conditional deletion of *Lhx2* later during development results in loss of RPCs and overproduction of stage specific precursors (Yun et al., 2009). This indicates a possible role of *Lhx2* in maintenance of RPC identity (Yun and Levine, unpublished).

Pax6 is a much studied homeobox gene. Targeted expression of the *Pax6* Drosophila homolog *eyeless* induces ectopic eye formation in flies, as does the Xenopus homolog of *Pax6* in frogs (Chow et al., 1999; Halder et al., 1995). Loss of the *Pax6* gene in mouse allows eye field determination but leads to developmental arrest of optic vesicles, premature neurogenesis, and loss of RPC multipotency (Baumer et al., 2002; Philips et al., 2005).

Two other homeobox genes, *Vsx2* and *Sox2*, are also important for maintenance of RPC identity, proliferation, and multipotency. *Vsx2* expression is critical for the specification of retinal cells from RPE cells during early eye development (Horsford et al., 2005; Rowan and Cepko, 2004). RPC proliferation is severely reduced in *Vsx2* mutants, in part due to its role in cell cycle time regulation. Further, the onset of neurogenesis is delayed in the *Vsx2* null retinas, indicating that the transition from pre-neurogenic to neurogenic RPC state may be delayed (Bone-Larson et al., 2000; Green et al., 2003; Sigulinsky et al., 2008). The requirement for *Sox2* function in the retina was demonstrated in the *Sox2* mutants. RPC proliferation is reduced in *Sox2* null retinas and their capacity to produce neurons is severely impaired (Taranova et al., 2006).

Homeodomain proteins exert their influence at most cell cycle phases. *Prox1* expression, required for horizontal cell specification in the retina, is seen at its highest

level in select RPCs at G2 (Dyer et al., 2003). This suggests PROX1 influences horizontal cell fate by functioning in G2. *Six3* promotes RPC proliferation by repressing GEMININ, an inhibitor of S phase DNA replication, by direct binding (Del Bene et al., 2004).

Several homeobox genes that are expressed in the RPCs also maintain their expression in precursor cells. For example, VSX2 retains its expression in post-mitotic bipolar cells (Burmeister et al., 1996). Deletion of VSX2 results in complete elimination of bipolar cells (Green et al., 2003). *Sox9* is expressed in RPCs during development and it maintains its expression in mature Müller glia cells. It is necessary for glia specification in the retina (Poche et al., 2008). Deletion of *Sox9* severely reduces or eliminates Müller glia cells from the mouse retina (Muto et al., 2009; Poche et al., 2008). *Sox2* is also expressed in a subset of amacrine cells and Müller glia cells. Forced expression of SOX2 promotes the differentiation of these cell types (Lin et al., 2009).

Other homeobox genes are expressed exclusively in post-mitotic retinal cells and are important for differentiation, cell fate specification, and maintenance of neurons (Ohsawa and Kageyama, 2008). A few examples are cited below.

Pou4f2 (*Brn3b)* is expressed by almost 80% of newly born ganglion cell precursors. It is essential for maintenance of ganglion cell identity and ganglion cell survival (Badea et al., 2009; Gan et al., 1999; Qiu et al., 2008).

Crx or the cone rod homeobox gene is expressed in photoreceptor precursors. *Crx* plays a critical role in photoreceptor precursor differentiation, identity, and maintenance (Freund et al., 1997; Furukawa et al., 1997; Morrow et al., 1998).

Otx2 is another homeodomain transcription factor expressed in photoreceptors and bipolar precursor cells. It is important for maintenance of photoreceptor and bipolar cell fates (Koike et al., 2007; Nishida et al., 2003).

The above examples highlight the diversity in expression and function of the homeobox genes during development in all retinal cell types. A thorough appreciation of their function is essential for understanding retinal development.

BHLH genes

Another important group of retinogenesis determinants are the basic helix-loop-helix (BHLH) family of transcription factors that are related to the Drosophila *atonal* and *achaete-schute* pro-neural genes (Vetter and Brown, 2001). BHLH factors can be broadly divided into two categories based on function. Repressor BHLH proteins maintain a RPC's proliferative status and inhibit precursor formation. Activator or pro-neural BHLH factors promotes specific precursor cell fate and function (Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008). Repressor BHLH factors such as the *Hes* genes prevent differentiation of RPCs by repressing the pro-neural genes (Ishibashi et al., 1995; Van Doren et al., 1994).

Hes1 and *Hes5* belong to the *Hes* family of genes that are homologs of Drosophila *Hairy* and *Enhancer of split* (Sasai et al., 1992). *Hes1* is expressed in RPCs during development in a cell cycle phase specific pattern (Das et al., 2009; Tomita et al., 1996) (Fig. 2.14). Germline deletion of *Hes1* leads to loss of RPCs, premature differentiation of precursors, and a microphthalmic eye (Tomita et al., 1996). *Hes5* is also expressed in the developing retina. Although *Hes5* null retinas look normal, Müller glia population is

decreased, suggesting a role for Hes5 in glia specification. This notion is further supported by the fact that misexpression of *Hes5* enhances Müller glia production (Hojo et al., 2000). *Hes1*, *Hes5* double mutant embryos completely lack optic vesicles, indicating that these genes are able to compensate for each others' loss (Hatakeyama et al., 2004). *Hes1* and *Hes5* are downstream targets of the *Notch* signaling pathway, which is itself required for maintenance of proliferative state in RPCs (Livesey, F. J. and Cepko, C. L., 2001; Perron and Harris, 2000). A recent study suggests that a combination of Notch receptors and downstream targets like *Hes1* or *Hes5* regulates production of retinal cell types (Riesenberg et al., 2009).

Activator or pro-neural BHLH factors are associated with all cell classes in the retina and play a role in their genesis. A few of them are discussed below.

Math5 is essential for ganglion cell formation and is expressed in a subset of RPCs that are about to exit from the cell cycle. Deletion of *Math5* or its homologs leads to loss of ganglion cells in multiple species (Brown et al., 2001; Brown et al., 1998; Kanekar et al., 1997; Kay et al., 2001).

Math3 and *NeuroD* are expressed in RPCs and regulate amacrine cell genesis. Combined deletion of *Math3* and *NeuroD* results in complete ablation of amacrine cells (Hatakeyama et al., 2001; Inoue et al., 2002).

Ptf1a is a BHLH transcription factor that is expressed in postmitotic horizontal and amacrine precursor cells. It is crucial for horizontal and amacrine cell specification (Fujitani et al., 2006; Nakhai et al., 2007). Deletion of *Ptf1a* results in a fate switch of horizontal/amacrine cells to ganglion cells (Fujitani et al., 2006; Nakhai et al., 2007). In

Xenopus retinas, *Ptf1a* promotes GABAergic neuronal cell fate over glycinergic cell fate (Dullin et al., 2007).

Horizontal and amacrine cell fate specification pathways are closely related, and involve the action of *Math3*, *NeuroD*, *Ptf1a* along with genes like *Prox1* and *Foxn4* (Ohsawa and Kageyama, 2008). Other BHLH genes like *Barhl2* and *Bhlhb5* are also expressed in amacrine precursors and are known to promote glycinergic and GABAergic amacrine cell formation, respectively (Feng et al., 2006; Mo et al., 2004).

Mash1 and *Math3* are expressed in bipolar precursor cells and are critical for bipolar cell specification (Jasoni and Reh, 1996; Takebayashi et al., 1997). Combined deletion of both genes leads to complete absence of bipolar cells (Tomita et al., 2000). Another factor *Bhlhb4* is crucial for rod bipolar cell survival but not specification. Rod bipolar cells are generated in the absence of *Bhlhb4*. However, they fail to thrive and eventually undergo apoptosis (Bramblett et al., 2004).

Typical BHLH factors such as those mentioned here usually act together with homeobox factors and other transcription factors in determination of RPC proliferation or precursor cell fate (Ohsawa and Kageyama, 2008). Thus, a complex network of signaling pathways and multiple classes of transcription factors control retinal histogenesis.

Cell cycle regulators

Cell cycle regulators are perfectly poised to influence proliferation and neurogenesis during development. Although regulators in all cell cycle phases can influence developmental processes in the retina, G1 phase regulators have received the

most attention. Therefore, this section will focus on G1 regulators, especially those in the retinoblastoma (RB) pathway.

CDKI, RB, and E2F factors

E2F1, E2F2, and E2F3 are transcriptional activators that are crucial for G1/S progression of progenitor cells. Although *E2f1*$^{-/-}$, *E2f2*$^{-/-}$, *E2f3*$^{-/-}$ triple null mouse embryonic fibroblasts (MEFs) are unable to progress into S phase, *E2f1*$^{-/-}$, *E2f2*$^{-/-}$, *E2f3*$^{-/-}$ mouse RPCs still proliferate due to compensation by the BHLH gene *Nmyc* (Chen, D. et al., 2009; Wu et al., 2001). In the retina, E2Fs mainly act downstream of RB proteins to prevent ectopic division of precursor cells due to cell cycle re-entry (Chen, H. Z. et al., 2009). An isoform of E2F3, E2F3a, acts downstream of RB to affect starburst amacrine cell differentiation via a cell cycle independent mechanism (Chen et al., 2007).

As mentioned before, the retinoblastoma protein family (*Rb1*, *Rbl1*/*p107*, and *Rbl2*/p130) regulates G1/S progression versus cell cycle exit. The loss of RB proteins in the retina results in ectopic proliferation and cell death (Chen et al., 2004; Dyer and Bremner, 2005). *Rb1* is also involved in differentiation of rod photoreceptor cells (Zhang et al., 2004).

The three CDKIs (*p27Kip1*, *p57Kip2*, and *p19*^{Ink4d}) have a crucial role in promoting cell cycle exit. They are generally inhibitors of proliferation and their deletion leads to prolonged proliferation in the retina (Levine and Green, 2004). P27KIP1 and p57KIP2 are negative regulators of proliferation and are expressed in distinct populations of RPCs (Dyer and Cepko, 2000a; Dyer and Cepko, 2001; Levine et al., 2000). The

Xenopus homolog of KIP1, *p27^{Xic1}*, is necessary and sufficient for Müller glia cell fate specification. This function is independent of its cell fate role (Ohnuma et al., 1999).

D-type cyclins

D-cyclins are small (33-34 kDa), evolutionarily conserved proteins that are the principal drivers of G1 (Sherr, 1995b). In humans, the three D-cyclins, Cyclin D1, D2, and D3, share high identity (~78%) within the CDK-binding 'cyclin-box' motif. However, outside the cyclin-box, identity is only at ~58%, suggesting significant divergence of functions amongst the D-cyclins (Xiong et al., 1992). It is believed that D-cyclins connect extracellular signaling pathways with the core G1 progression machinery (Matsushime et al., 1991; Paavonen et al., 1996; Pagano and Jackson, 2004; Sherr, 1994). No direct cell fate instructive roles for D-cyclins have been identified yet in the retina. However, G1 regulation is intricately related to cell cycle exit and differentiation of precursor cells.

Besides their better-known role in the cell cycle through CDK interaction, D-cyclins have cell cycle-independent roles and can even act as a transcription factors (Coqueret, 2002; Fu et al., 2004; Li et al., 2006). Therefore, D-cyclins can potentially influence cell fate through both cell cycle dependent and independent mechanisms.

In mice, the three D-cyclins are expressed in tissues-specific, dynamic, and often mutually exclusive patterns. Deletion of individual D-cyclins yields phenotypes reflecting their tissue-specific requirement (Ciemerych and Sicinski, 2005; Ciemerych et al., 2002; Pagano and Jackson, 2004; Sherr and Roberts, 2004). It was observed that development progressed relatively normally in most cellular lineages of mouse embryos lacking all D-cyclins up to E16.5, at which point the embryos died due to severe anemia and heart

defects (Kozar et al., 2004). Despite reduced proliferation in triple knockout tissues, including the retina, D-cyclins seem mostly expendable for cell cycle progression (Kozar et al., 2004).

Rationale for studies

Cyclin D1 (*Ccnd1*) expression is strongest in the retina during mouse embryonic development; it is also significantly expressed in brain and other restricted tissue compartments (Sicinski et al., 1995). In the developing retina, CCND1 is expressed in RPCs but not in post-mitotic precursors and mature neurons (Barton and Levine, 2008; Trimarchi et al., 2008). *Ccnd1* deletion or knockdown resulted in hypocellular retinas in mouse and zebrafish (Duffy et al., 2005; Fantl et al., 1995; Sicinski et al., 1995). Another study of *Ccnd1*$^{-/-}$ retinas demonstrated a focal pattern of photoreceptor cell death, initiating at around postnatal day 5 (Ma et al., 1998). Further, by [^{3}H]Thymidine labeling of P0 retinas, it was demonstrated that proliferation was reduced in the *Ccnd1*$^{-/-}$ retinas compared to wild type/heterozygous control retinas (Ma et al., 1998). Reduction in proliferation was most likely responsible for almost a threefold decrease in total cell number of mutant retinas (Ma et al., 1998). Overall, CCND1 loss was detrimental for retina development, and electroretinogram (ERG) readings were essentially flat in the mutant mice, implying almost no visual function (Geng et al., 1999; Sicinski et al., 1995).

It was clear from the hypo-proliferative and hypocellular P0 *Ccnd1*$^{-/-}$ retinas that CCND1 was required for proper retina development during the embryonic period. However, none of the studies elucidated the precise role of CCND1 during embryonic retinal development.

Therefore, we wanted to elucidate CCND1's function in RPCs from the earliest possible time point. One important issue was whether cell death in the absence of CCND1 contributed to the smaller size of the mutant retina. Further, we could not formally rule out a cell cycle-independent role for CCND1 during retinal development. However, given the expression of CCND1 in dividing progenitors and the general role of D-cyclins, it was almost certain that CCND1 had cell cycle-specific functions in the retina. We wanted to know what these functions were and how the cell cycle of RPCs was affected in the absence of CCND1. Finally, given that D-cyclins usually promote G1/S progression, we were curious whether cell cycle progression and complementarily, differentiation/neurogenesis were affected in the *Ccnd1* mutant retinas. The study described in Chapter 2 of this dissertation addressed the above issues during retina development in *Ccnd1* null mouse embryos.

It was observed that proliferation was not entirely eliminated from *Ccnd1* null retinas at birth. Further, in spite of a smaller retina, a fair number of retinal neurons were generated in the mutant (Ma et al., 1998) (Chapter 2). Potentially, this could be due to compensation from the other two D-cyclins during development (Ciemerych et al., 2002; Satyanarayana and Kaldis, 2009). CCND2 is not normally expressed in the developing retina and CCND3 is expressed only in Müller glia cells (Dyer and Cepko, 2000b; Geng et al., 1999). However, a previous study demonstrated that CCND3 levels were prematurely elevated in *Ccnd1* null neonatal retinas (Tong and Pollard, 2001). To address potential compensation by CCND3 in the *Ccnd1* mutant retinas and further, to verify predictions arising from our first study (Chapter 2), we carried out the experiments described in Chapter 3 of this dissertation.

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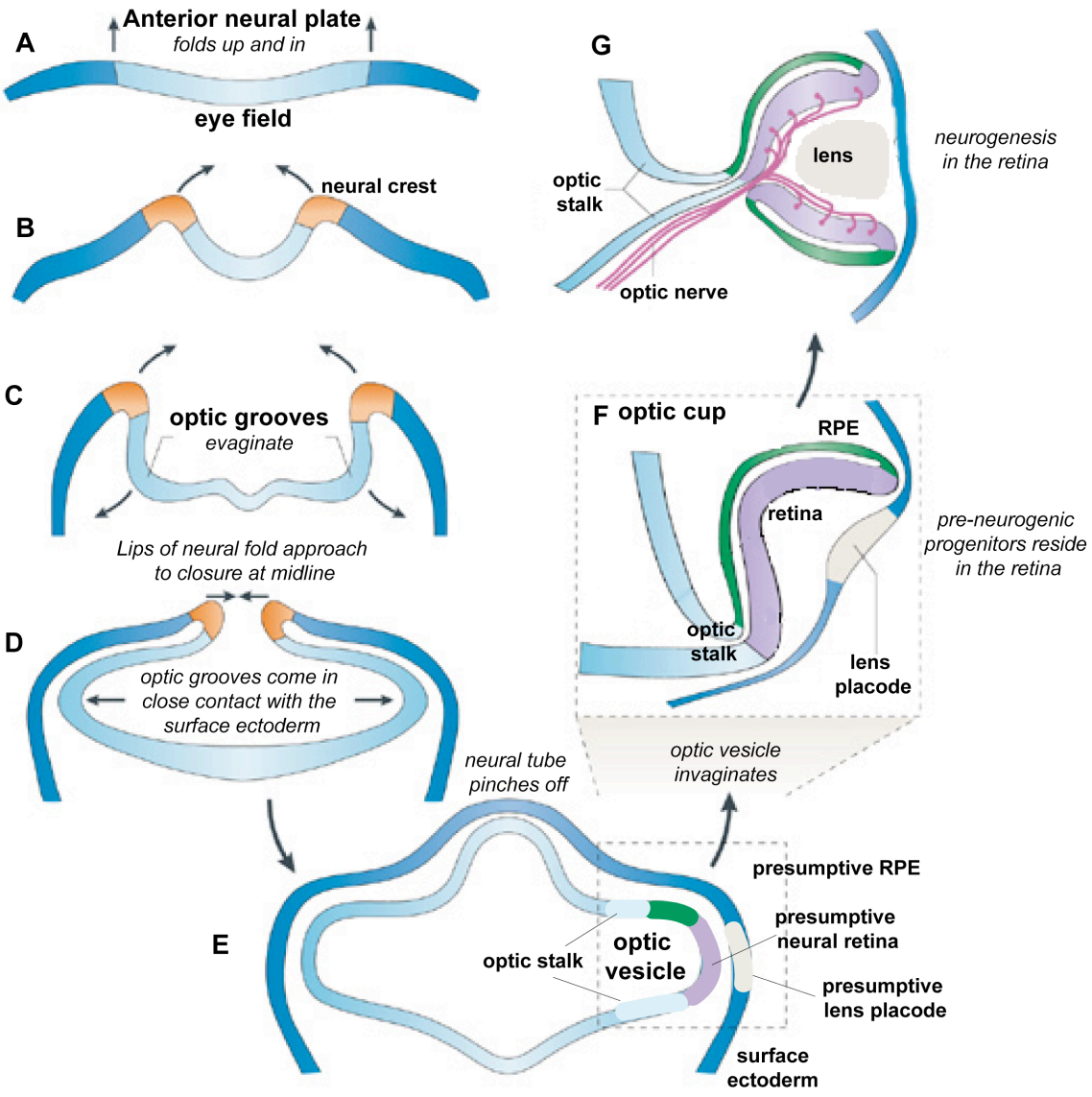
Figure 1.1: Overview of retina development. Cross section of anterior neural plate showing the eye field (A). The neural plate folds upward and in toward the midline (B). The two optic grooves evaginates and moves toward the surface ectoderm (C). The neural optic grooves are now in close contact with the non-neural surface ectoderm and cross talk ensues (D). The neural folds merge, the neural tube pinches off, and the presumptive retina, retinal pigmented epithelium (RPE), and optic stalk regions are patterned (E). The presumptive lens placode is also patterned from the surface ectoderm (E). The optic vesicle invaginates, bringing the retina in close apposition to the RPE and forms the optic cup. The lens placode also grows and invaginates. Retinal progenitor cells are yet to produce neurons and are pre-neurogenic (F). The optic cups grows circumferentially around the choroid fissure. Ganglion cells generated from retinal progenitors send out axons to form the optic nerve, which exits the eye through the choroid fissure. By this time, the lens has pinched off from the surface ectoderm (G). This structure now resembles the adult eye, retinal histogenesis is underway, and eventually other retinal neurons are also produced. Modified by permission from Macmillan Publishers Ltd: [Nature Reviews Neuroscience] (Evolution of the vertebrate eye: opsins, photoreceptors, retina and eye cup. Lamb TD, Collin SP, Pugh EN Jr. 2007 Dec;8(12):960-76. Review.), copyright (2007).


A
Anterior neural plate
folds up and in
eye field
B
neural crest
C
optic grooves
evaginate
Lips of neural fold approach
to closure at midline
D
optic grooves come in
close contact with the
surface ectoderm
neural tube
pinches off
E
optic stalk
optic
vesicle
presumptive RPE
presumptive
neural retina
presumptive
lens placode
surface
ectoderm
optic vesicle
invaginates
F optic cup
RPE
retina
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stalk
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in the retina
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neurogenesis
in the retina

CHAPTER 2

CYCLIN D1 FINE-TUNES THE NEUROGENIC OUTPUT OF EMBRYONIC RETINAL PROGENITOR CELLS

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Cyclin D1 fine-tunes the neurogenic output of embryonic retinal progenitor cells

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Abstract

Background: Maintaining the correct balance of proliferation versus differentiation in retinal progenitor cells (RPCs) is essential for proper development of the retina. The cell cycle regulator cyclin D1 is expressed in RPCs, and mice with a targeted null allele at the cyclin D1 locus (*Ccnd1*$^{-/-}$) have microphthalmia and hypocellular retinas, the latter phenotype attributed to reduced RPC proliferation and increased photoreceptor cell death during the postnatal period. How cyclin D1 influences RPC behavior, especially during the embryonic period, is unclear.

Results: In this study, we show that embryonic RPCs lacking cyclin D1 progress through the cell cycle at a slower rate and exit the cell cycle at a faster rate. Consistent with enhanced cell cycle exit, the relative proportions of cell types born in the embryonic period, such as retinal ganglion cells and photoreceptor cells, are increased. Unexpectedly, cyclin D1 deficiency decreases the proportions of other early born retinal neurons, namely horizontal cells and specific amacrine cell types. We also found that the laminar positioning of horizontal cells and other cell types is altered in the absence of cyclin D1. Genetically replacing cyclin D1 with cyclin D2 is not efficient at correcting the phenotypes due to the cyclin D1 deficiency, which suggests the D-cyclins are not fully redundant. Replacement with cyclin E or inactivation of cyclin-dependent kinase inhibitor p27Kip1 restores the balance of RPCs and retinal cell types to more normal distributions, which suggests that regulation of the retinoblastoma pathway is an important function for cyclin D1 during embryonic retinal development.

Conclusion: Our findings show that cyclin D1 has important roles in RPC cell cycle regulation and retinal histogenesis. The reduction in the RPC population due to a longer cell cycle time and to an enhanced rate of cell cycle exit are likely to be the primary factors driving retinal hypocellularity and altered output of precursor populations in the embryonic *Ccnd1*$^{-/-}$ retina.

Background

The vertebrate retina is composed of seven major cell classes that arise from a common source, the retinal progenitor cell (RPC) population. Although RPCs at any given stage are largely multipotential, they are constrained such that each cell class is generated in a temporal, albeit

overlapping order. Production of retinal ganglion cells (RGCs), horizontal cells, and cone photoreceptors is initiated at the earliest stage of retinal neurogenesis, followed by amacrine cells and rod photoreceptors, which is then followed by bipolar cells and Müller glia. The relative proportion of cells in each class differs widely. For example, cones account for approximately 3% and rods approximately 97% of the photoreceptors in the mouse retina, and rod photoreceptors are the most abundant cell class accounting for approximately 70% of all retinal cells [1]. In general, the early-born cell classes constitute a much smaller percentage of the retina than do the late-born cell classes [1,3]. While cell death contributes to the final cell distribution of the adult retina [4,6], the initial allocation of precursor cells (that is, RPCs that exit the cell cycle) to each class is a predominant factor in setting their relative proportions.

In addition to generating the different cell classes, RPCs need to proliferate in order to produce enough cells to populate the retina. In the rat retina, RPC proliferation drives an approximately 400-fold expansion of total cell number in a 17-day period between embryonic day (E)14 and postnatal day (P)8 [7]. This interval also corresponds to when the bulk of the RPC population exits the cell cycle to generate precursors [2]. Thus, RPCs are exposed to competing forces that either influence them to stay in the cell cycle in order to produce enough cells or to exit the cell cycle at the appropriate time in order to generate the correct proportion of cells corresponding to each cell class.

It is generally accepted that multiple cell-extrinsic and -intrinsic factors play important roles in establishing the correct balance between RPC proliferation and precursor generation during development [8-10]. While it is important to understand how these different factors are integrated into networks, an understanding of the molecular mechanisms used to exit the cell cycle during the transition from RPC to precursor is also needed.

D-type cyclins promote progression from G1 to S phase in dividing cells by activating cyclin-dependent kinases 4 or 6 (CDK4/6) and by sequestering cyclin-dependent kinase inhibitors such as cyclin-dependent kinase inhibitor 1B (CDKN1B, henceforth referred to as P27KIP1) [11]. The net result is enhanced CDK2 activity, inactivation of retinoblastoma proteins, and activation of DNA replication. D-cyclins are also downstream of various signaling pathways and, thus, are well positioned to co-ordinate cell cycle progression with the extracellular environment [11,12]. Mice have three D-cyclin genes: cyclin D1 (*Ccnd1*), cyclin D2 (*Ccnd2*) and cyclin D3 (*Ccnd3*). The expression and requirement of the D-cyclins during development is tissue specific [13]. Surprisingly, mouse embryos lacking all three D-cyclins develop until E16.5, when they die due to heart abnormalities combined with severe anemia [14]. Although developmental defects are apparent in these mice prior to E16.5, proliferation of many tissues, including the retina, still occurs, indicating that the D-cyclins are not absolutely required for cell cycle progression.

Ccnd1 is the predominant D-cyclin in the developing retina and is highly expressed in RPCs but absent from exited precursors and differentiated cells [15,18] (this study). Zebrafish embryos treated with a *Ccnd1* morpholino exhibit small eye [19] and mice lacking *Ccnd1* have small eyes and hypocellular retinas due to reduced RPC proliferation and postnatal retinal cell death [17,20,21]. However, the impact of *Ccnd1* on embryonic retinal development has not been directly assessed.

In this study, we characterized the embryonic retinal phenotype in *Ccnd1*$^{-/-}$ mice. We found that the cell cycle rate of the *Ccnd1*$^{-/-}$ RPC population is slower than normal and this population undergoes a faster rate of depletion due to an increased rate of cell cycle exit. Consistent with this, RGCs and photoreceptors are overrepresented. Surprisingly, other early-born embryonic cell classes in the retina, namely horizontal and amacrine cells, are underrepresented in the absence of *Ccnd1*. Analysis of retinas from newborn mice in which *Ccnd1* is replaced by *Ccnd2* reveal that the proportions of at least some cell types remain altered, suggesting a unique requirement for *Ccnd1* in RPCs. We also analyzed the retinas of newborn mice in which *Ccnd1* is replaced by human Cyclin E (*hCcne*) or in *Ccnd1*$^{-/-}$, *p27Kip1*$^{-/-}$ double mutants and found that the proportions of cell types approach a more normal distribution. These findings led us to propose that *Ccnd1* controls the timing of cell cycle exit in embryonic RPCs and, by doing so, contributes to the appropriate allocation of precursor cells to each cell class. We also propose that *Ccnd1* contributes to the correct proliferative expansion of the retina by influencing the time it takes for RPCs to transit through the cell cycle and by maintaining a sufficient number of RPCs during the period of neurogenesis.

Materials and methods

Animals

Ccnd1$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Drs Matthew Fero and James Roberts (Fred Hutchinson Cancer Center, Seattle, WA) kindly provided the *p27Kip1*$^{-/-}$ mice. The mouse strains containing the *Ccnd2* cDNA targeted to the *Ccnd1* locus (*Ccnd1*$^{D2/D2}$) and human *Ccne* cDNA targeted to the *Ccnd1* locus (*Ccnd1*$^{hE/hE}$) were maintained in the Sicinski laboratory. The noon of the day a vaginal plug was observed was designated E0.5. Genotyping was done as previously described [17,22-24]. All animal use and care was conducted in accordance with protocols approved by the University of Utah Institutional Animal Care and Use

Committee and set forth in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals. Efforts were made to minimize discomfort to animals and, when possible, the number of animals needed per analysis was kept to a minimum.

Immunohistochemistry

Tissue preparation and immunohistochemistry were done as previously described [25]. Radial cryosections through the retina were cut at a thickness of 10 μm. Primary antibodies are listed in Table 1. Antigen unmasking (0.18 mM citric acid, 77 μM sodium citrate, pH 6.0, 15 minutes, 90–95°C) was performed prior to incubation with the proliferating cell nuclear antigen (PCNA) antibody. Hydrochloric acid treatment (2N HCl, 30 minutes, room temperature) was performed prior to incubation with the bromodeoxyuridine (BrdU) antibody.

Image analysis

Sections were analyzed by epi-fluorescence using a Nikon E-600 microscope and images captured in gray scale mode with a Spot-RT slider CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). Confocal images were scanned using an Olympus Fluoview 1000 microscope. Color (RGB) images were assembled from individual monochrome channels using Photoshop CS (Adobe Systems Inc., San Jose, CA, USA). The levels function was used to adjust the digital images to be consistent with visual observations.

Marker quantification and statistical analysis

The relative proportions, lineal densities, or areal densities of marker-positive (+) or -negative (-) cells were quantified at E12, E14.5 and P0. For each genotype, a minimum of three animals from at least two litters was sampled. For each animal, three different non-adjacent central-retina sections were used for cell counting.

At E12, epi-fluorescence images of whole retinal sections were captured. Cell populations were quantified over the total area of the sections (marker$^+$ cells/mm^2 retina). The exception was for PCNA$^+$ cells, which were quantified as a percentage of the total cell population ((PCNA$^+$ cells/DAPI$^+$ cells) × 100). PCNA$^+$ population was sampled from the dorsal retina, where neurogenesis initiates. At E14.5, marker$^+$ cells were calculated as a percentage of total cells (marker$^+$ cells/DAPI$^+$ cells) from 400×-magnified confocal images (1,600 × 1,600 resolution), captured at comparable dorsal-medial regions. At P0, marker$^+$ cells were quantified as a percentage of total cells from confocal images of medial-central retina, within 200 μm of the optic nerve head. Neurofilament medium (NEFM)$^+$ horizontal and SRY-box containing gene 2 (SOX2)$^+$ amacrine cells were quantified as a ratio of the unit length of apical surface of the retina (marker$^+$ cells/mm retina) because of their sparse, linear distribution. The entire peripheral-central-peripheral extent of individual sections was used for these measurements. All cell counts, area, and length measurements were done using Adobe Photoshop CS and ImageJ (NIH). Students' *t*-test was performed using Kaleidagraph statistical and graphing software (Synergy Software, Reading, PA, USA) to determine statistical significance in the marker$^+$ cell population between mutant and control samples. In all graphs, numbers inside bars indicate the number of samples analyzed. Error bars indicate standard deviation.

Window-labeling using thymidine analogs to measure cell cycle times

Retinas with lens attached were cultured for 2.5 hours and sequentially exposed to two thymidine analogs for defined intervals. At P0, 5-iodo-2'-deoxy-uridine (IdU) was added to the culture medium for the first 2 hours and replaced with 5-bromo-2'-deoxy-uridine (BrdU) for the final 30 minutes. At E14.5, BrdU was added to the culture medium for the first 2 hours and replaced with 5-ethynyl-2'-deoxy-uridine (EdU) for the final 30 minutes. As previously described [26-28], a combination of mouse anti-BrdU (clone B44; BD Biosciences, San Jose, CA, USA) and rat anti-BrdU (clone BU1/75; Serotec, Raleigh, NC, USA) were used to detect the analogs at P0. For the E14.5 samples, the mouse anti-BrdU antibody was used to detect BrdU (EdU is also detected), and EdU was specifically detected using the Click-it Reaction (Molecular Probes, Carlsbad, CA, USA) [29]. PCNA was used at both ages to identify RPCs in all phases of the cell cycle [15]. The length of the cell cycle (T_c) in hours was calculated by the formulae:

$$(T_c) = 2\ \text{h} \times [\text{PCNA}^+\ \text{cells/IdU}^+\ \text{only cells}]\ \text{at P0}$$

or

$$(T_c) = 2\ \text{h} \times [\text{PCNA}^+\ \text{cells/BrdU}^+\ \text{only cells}]\ \text{at E14.5}$$

and the length of the S-phase (T_s) in hours was calculated by the formulae:

$$(T_s) = 2\ \text{h} \times [\text{BrdU}^+\ \text{cells/IdU}^+\ \text{only cells}]\ \text{at P0}$$

or

$$(T_s) = 2\ \text{h} \times [\text{EdU}^+\ \text{cells/BrdU}^+\ \text{only cells}]\ \text{at E14.5}$$

At E14.5 cell counts were done from a single central field on each section (at least three sections per animal), generally on the same side. At P0, cell counts were done on six fields spanning an entire section (at least two sections per animal). Dorsal-ventral orientation was lost upon dis-

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Table 1: Primary antibodies

Antigen	Host	Target (relevant to this study)	Dilution factor	Source
BHLHB5	Goat	amacrine precursors[1]	1000	Santa Cruz (sc-6045)
BrdU	Mouse	cells that have uptaken BrdU in S-phase	100	BD biosciences (clone B44)
BrdU	Rat	cells that have uptaken BrdU in S-phase	50-200	Serotec (clone BU1/75)
POU4F2	Goat	RGC precursors	50	Santa Cruz (sc-6026)
CASPASE-3	Rabbit	dying cells	500	BD biosciences (clone C92-605)
VSX2	Sheep	RPCs	400	Exalpha Biologicals (X1180P)
CCND1	Rabbit	RPCs	400	Lab Vision (RB-212)
CCND1	Mouse	RPCs	400	Santa Cruz (clone 72-13G)
ISL1	Mouse	amacrine cells [1] and RGCs [1]	50	DSHB (clone 39.4D5)
HES1	Rabbit	RPCs	800	Nadean Brown
MITF	Mouse	RPE	500	Exalpha Biologicals (clone C5)
NEFM	Rabbit	RGC and horizontal cells	1000	Chemicon (AB1987)
NR2E3	Rabbit	rod precursors	100	Anand Swaroop
OTX2	Rabbit	photoreceptor and amacrine precursors[1]	1000	Chemicon (AB9566)
OTX2	Goat	photoreceptor and amacrine precursors[1]	400	Santa Cruz (sc-30659)
PAX6	Mouse	RPCs	10	DSHB (clone P3U1)
PCNA	Mouse	RPCs	500	DAKO (clone PC10)
PCNA	Rabbit	RPCs	100	Santa Cruz (sc-7907)
pHH3	Rabbit	mitotic cells	500	Upstate Biotechnology (06-570)
PTF1A	Rabbit	amacrine[1] and horizontal precursors	800	Helena Edlund
RCVRN	Rabbit	photoreceptor cells	4000	Chemicon (AB5585)
RXRγ	Rabbit	cone and RGC precursors	200	Santa Cruz (sc-555)
SOX2	Rabbit	RPCs and amacrine cells [1]	400	Abcam (ab15830)
acTUBB3	Rabbit	neuronal precursors	4000	Covance (PRB-435P)
acTUBB3	Mouse	neuronal precursors	1000	Covance (clone TUJ1)

[1]marks a subset of cells in this class

secting eyes out. A more detailed analysis of this assay will appear in a forthcoming manuscript (GD and EML).

Cell cycle exit assay and RGC birthdating

Pregnant mice were injected once with a dose of BrdU (10 mg/ml stock in 0.1 M Tris (pH 7): 100 μg/gm of body weight injected) at E13.5 or E18.5 and sacrificed 24 hours later at E14.5 and P0.5, respectively. Sections were co-labeled with antibodies against BrdU and PCNA and imaged by confocal microscopy. The cell cycle exit index was calculated as the percentage of $BrdU^+$ cells that were $PCNA^-$ (($BrdU^+$, $PCNA^-$ cells/Total $BrdU^+$ cells) × 100).

To measure the production of RGCs from RPCs, sections from the same animals used for the cell cycle exit index were co-labeled with antibodies against BrdU and POU domain, class 4, transcription factor 2 (POU4F2; formerly BRN3B). The index for RGC production was calculated as the percentage of $BrdU^+$ cells that were $POU4F2^+$ (($BrdU^+$, $POU4F2^+$ cells/Total $BrdU^+$ cells) × 100). Cell counts were done from a single dorsal-central field per section (at least two sections per animal) retina at E14.5. For P0 samples, counts were done from two peripheral fields at opposite ends per section (at least two sections per animal).

Results

CCND1 expression pattern during the early stages of retinal development

In the mouse retina, CCND1 protein is expressed as early as E11 [17]. However, a systematic analysis of its expression pattern during early retinal development has not been done. Therefore, we examined CCND1 expression from E9.5 to E14.5, the period of optic cup formation and onset of retinal neurogenesis (Figure 1). CCND1 protein is expressed as early as E9.5 in several tissues that give rise to the eye, including the optic vesicle and surface ectoderm, as well as in the adjacent diencephalic neuroepithelium (Figure 1A). At E11, CCND1 expression is strongest in the central region of the neural retina and in the lens vesicle (Figure 1B), and the high level of CCND1 expression in the neural retina spreads outward by E12 and reaches the peripheral retina by approximately E14.5 (Figure 1C, D). This dynamic pattern is reminiscent of the wave of neurogenesis. To examine this relationship further, these sections were also labeled with the Tuj1 antibody (Figure 1E–H), which detects the acetylated form of class III beta-Tubulin (acTUBB3) and reveals the initial formation of the differentiated cell layer (DCL) [30,31]. A direct comparison of the CCND1 (dashed lines) and acTUBB3 expression patterns (Figure 1I–L) indicates that the high level of CCND1 expression in the neuroblast

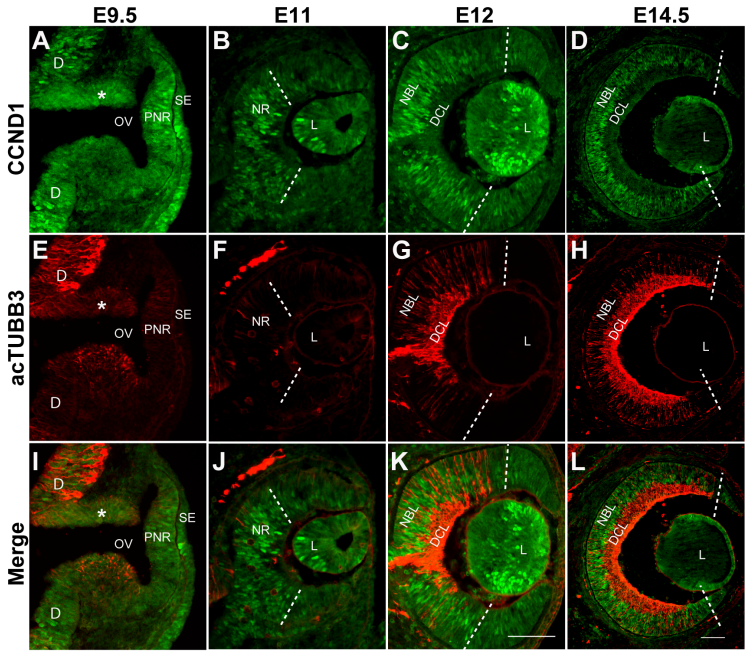

Figure 1
Expression patterns of CCND1 and acTUBB3 during early retinal development. Wild-type retinas were double-labeled with antibodies against **(A-D)** CCND1 and **(E-H)** acTUBB3. **(I-L)** Merged images. Dashed lines indicate the peripheral extent of strong CCND1$^+$ cells in retinas from E11 (B, F, J), E12 (C, G, K), and E14.5 (D, H, L) embryos. Asterisks in (A, E, I) indicate that this region of the neuroepithelium is folded over in the section. Abbreviations: D, diencephalon; DCL, differentiated cell layer; L, lens; NBL, neuroblast layer; NR, neural retina; OV, optic vesicle; PNR, presumptive neural retina; SE, surface ectoderm. Scale bars: 100 μm; (K) is representative for (A-C, E-G, I-K); (L) is representative for (D, H, L).

layer (NBL) precedes the wave of acTUBB3 expression, but their relative timing and similar patterns suggest they are linked.

Patterning and apoptosis are unaltered by Ccnd1 *inactivation at embryonic ages*

Since *Ccnd1* is expressed during optic cup formation, the hypocellularity of the *Ccnd1*$^{-/-}$ retina could be due to altered regional patterning. However, analysis of several markers of optic vesicle and cup patterning did not reveal differences in the establishment or size of the neural retinal domain (Additional file 1). Likewise, we did not observe obvious differences in apoptosis at any of the embryonic ages analyzed as revealed by activated caspase 3 (CASP3) immunoreactivity (Additional file 2) or by TUNEL assay (data not shown).

Cell cycle time is longer in the Ccnd1$^{-/-}$ *RPC population*

Having ruled out major changes in retinal domain formation and cell death, we measured other parameters that could cause the hypocellularity observed in the *Ccnd1*$^{-/-}$ retina. At birth, *Ccnd1*$^{-/-}$ retinas show a three-fold decrease in total cells and a concomitant three-fold decrease in cells that incorporate tritiated thymidine [21]. While these findings suggest reduced RPC proliferation prior to P0, we directly analyzed proliferative activity during the embryonic period, first by detection of phosphorylated histone H3 (pHH3), a marker of RPCs in M-phase (Additional file 3) [15]. Fewer pHH3$^+$ cells are evident by E14.5, confirming that RPC proliferation is reduced in the embryonic *Ccnd1*$^{-/-}$ retina.

To get an estimate of the cell cycle time and related measures, we adapted a window-labeling paradigm that utilizes two thymidine analogs that can be differentially detected [27] (manuscript in preparation). Frozen sections from P0 retinas were triple-labeled with a mouse anti-BrdU antibody identifying both IdU and BrdU (Figure 2A, D), a rat anti-BrdU antibody identifying only BrdU (Figure 2B, E) and an anti-PCNA antibody for labeling the complete RPC cohort (Figure 2C, F) [15]. BrdU and EdU on E14.5 sections were detected as described (see Materials and methods).

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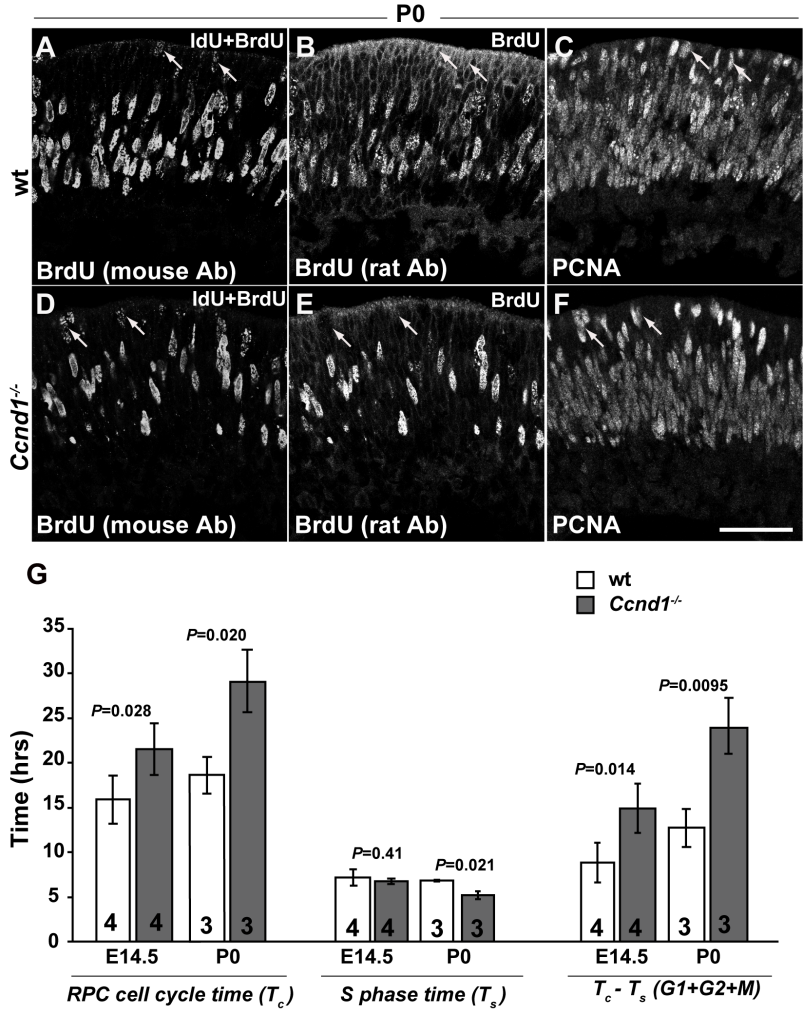

Figure 2

Retinal progenitor cell (RPC) cell cycle is lengthened in the *Ccnd1*$^{-/-}$ retina. P0 wild-type and *Ccnd1*$^{-/-}$ retinas, cultured successively in iododeoxyuridine (IdU) for 2 hours and bromodeoxyuridine (BrdU) for 30 minutes, were triple-stained with mouse **(A, D)** anti-BrdU antibody (Ab) recognizing both IdU and BrdU, **(B, E)** rat anti-BrdU antibody recognizing only BrdU, and **(C, F)** with an antibody against PCNA marking RPCs. Arrows in (A-C) mark IdU$^+$ only RPCs (IdU$^+$, BrdU$^-$; positive signal in (A, C) but not (B)) in wild-type retina that have moved up to the apical surface during the labeling period. Arrows in (D-F) mark IdU$^+$ only RPCs (positive signal in (D, F) but not (E)) in the *Ccnd1*$^{-/-}$ retina during the same period. **(G)** Quantification of average RPC cell cycle time (T_c), S phase time (T_s) and G1 + G2 + M phase time (T_c - T_s) in wild-type (wt) and *Ccnd1*$^{-/-}$ retinas at E14.5 and P0. Scale bar: 50 μm; (F) is representative for (A-F).

We observed that the cell cycle time (T_c) of the *Ccnd1*$^{-/-}$ RPC population was increased relative to that of the wild-type RPC population at E14.5 and P0 (Figure 2G). Although S phase time (T_s) did not vary between the two genotypes at E14.5, there was a decrease in T_s for the *Ccnd1*$^{-/-}$ RPC population at P0 (Figure 2G). We then subtracted the S-phase time from cell cycle time (T_c - T_s), which yields an estimate of the cumulative time spent in G1, G2, and M phases, and found that the T_c - T_s value of the *Ccnd1*$^{-/-}$ RPC population is significantly increased compared to the wild-type RPC population at both ages (Figure 2G). Since the function of *Ccnd1* in the cell cycle

is thought to be specific to the G1 phase, this suggested that the increase in T_c was due to a longer G1 phase, although we cannot exclude potential changes in G2 or M phases. In sum, these findings demonstrate that *Ccnd1* is required to ensure an appropriate rate of passage through the cell cycle and that the slower rate of proliferation in the absence of *Ccnd1* is likely to contribute to the hypocellularity of the *Ccnd1*$^{-/-}$ retina.

Increased cell cycle exit in the Ccnd1$^{-/-}$ *retina reduces the relative size of the RPC population*

In addition to a slower cell cycle rate, a reduction in size of the RPC population due to enhanced cell cycle exit could also contribute to the proliferation problems associated with the hypocellularity of the *Ccnd1*$^{-/-}$ retina. To assess this, we first examined the expression pattern of PCNA and measured the proportion of PCNA$^+$ cells relative to the total cell population at E12, E14.5 and P0 (Figure 3A–G). PCNA is expressed in the vast majority of RPCs during development [15] and its expression characteristics are not altered relative to other RPC markers in the *Ccnd1*$^{-/-}$ retina (Additional file 4). While the distribution of PCNA$^+$ cells appears unchanged at E12 (Figure 3A, D), the NBL is visibly thinner in the *Ccnd1*$^{-/-}$ retina at E14.5 (Figure 3B, E) and this is confirmed upon quantification (Figure 3G). At this stage, the thinning of the NBL layer occurs at the expense of the DCL. At P0, the thinning of the NBL is more pronounced (Figure 3C, F) and the proportion of PCNA$^+$ cells is reduced further (Figure 3G). In contrast to E14.5, a gap in PCNA immunoreactivity is observed at the apical side of the retina (brackets in Figure 3F), which is filled by orthodenticle homolog 2 (OTX2)$^+$ cells (Additional file 5A–F), indicating that these cells are predominantly post-mitotic photoreceptor precursors (see below). A similar pattern of PCNA and OTX2 expression was observed at E17.5 (data not shown).

These observations suggest that RPCs in the *Ccnd1*$^{-/-}$ retina are exiting the cell cycle at a comparably more rapid rate than normal. To assess this more directly, a cell cycle exit index was measured for the interval between E13.5 to E14.5, when RPC proliferation is robust and neurogenesis is well underway, and later between E18.5 and P0.5 (Figure 3H–O; see Materials and methods). We observed that a significantly greater proportion of BrdU$^+$ RPCs exit the cell cycle in the *Ccnd1*$^{-/-}$ retina compared to wild type at both ages (Figure 3O), thereby indicating that increased cell cycle exit is a primary cause for the reduction in RPCs and is also a contributing factor in causing the hypocellularity observed by birth in the *Ccnd1*$^{-/-}$ retina.

Enhanced cell cycle exit in the Ccnd1$^{-/-}$ *retina leads to increased proportions of RGCs and photoreceptors, but not an earlier onset of neurogenesis*

Enhanced cell cycle exit can lead to two non-mutually exclusive changes in neurogenesis: an earlier onset or enhanced neuron production from prematurely exiting RPCs after onset. Importantly, either change could increase the proportions of early born neuronal populations such as RGCs. To determine if neurogenesis initiates earlier than normal in the *Ccnd1*$^{-/-}$ retina, we examined the expression of acTUBB3 and the transcription factor POU4F2, a marker of RGC precursors [32,33]. Cells positive for either marker were not observed at E11 or earlier regardless of genotype (data not shown; Figure 4A, E). Therefore, it is not likely that neurogenesis initiates early in the *Ccnd1*$^{-/-}$ retina.

To determine whether *Ccnd1* inactivation increases the proportion of early-born neurons, we examined the expression pattern of POU4F2 after the onset of neurogenesis (Figure 4B–D, F–H) and measured the percentage of POU4F2$^+$ cells relative to the total cell population at E12, E14.5, and P0 (Figure 4I). Although the spatial patterns of POU4F2$^+$ cells in the *Ccnd1*$^{-/-}$ retina are similar to wild type at E12 (Figure 4B, F) and E14.5 (Figure 4C, G), their relative proportions are significantly higher in the mutant at these ages and at P0 (Figure 4J). Additionally, most wild-type RGCs are located below the inner plexiform layer (IPL; Figure 4D) at P0, but the mutant has an extra layer of POU4F2$^+$ cells positioned on the apical side of the IPL (Figure 4H, arrows). A similar pattern of mislocalized POU4F2$^+$ cells was also observed at E17.5 (data not shown).

To confirm that the greater proportion of RGCs is correlated with enhanced RPC cell cycle exit, we directly measured the proportion of RPCs that were becoming POU4F2$^+$ RGCs in a given time period. Using the same samples as for the cell cycle exit assay described above, we calculated the percentage of BrdU$^+$ cells that express POU4F2$^+$. By this approach, we found that a significantly higher percentage of BrdU$^+$ RPCs exit the cell cycle and form RGCs in the *Ccnd1*$^{-/-}$ retina from E13.5 to E14.5 (Figure 4J), confirming that enhanced cell cycle exit of *Ccnd1*$^{-/-}$ RPCs leads to increased proportions of early born neurons.

As described above, the apical 'gap' of PCNA expression in the *Ccnd1*$^{-/-}$ retina is filled with OTX2$^+$ cells (Additional file 5D–F). Since OTX2$^+$ cells include both cone and rod precursors, we examined the P0 expression patterns of retinoid × receptor gamma (RXRγ) and nuclear receptor subfamily 2, group E, member 3 (NR2E3), which mark cone and rod precursors respectively [34-36]. Consistent with the increase in OTX2$^+$ cells, the proportions of RXRγ$^+$

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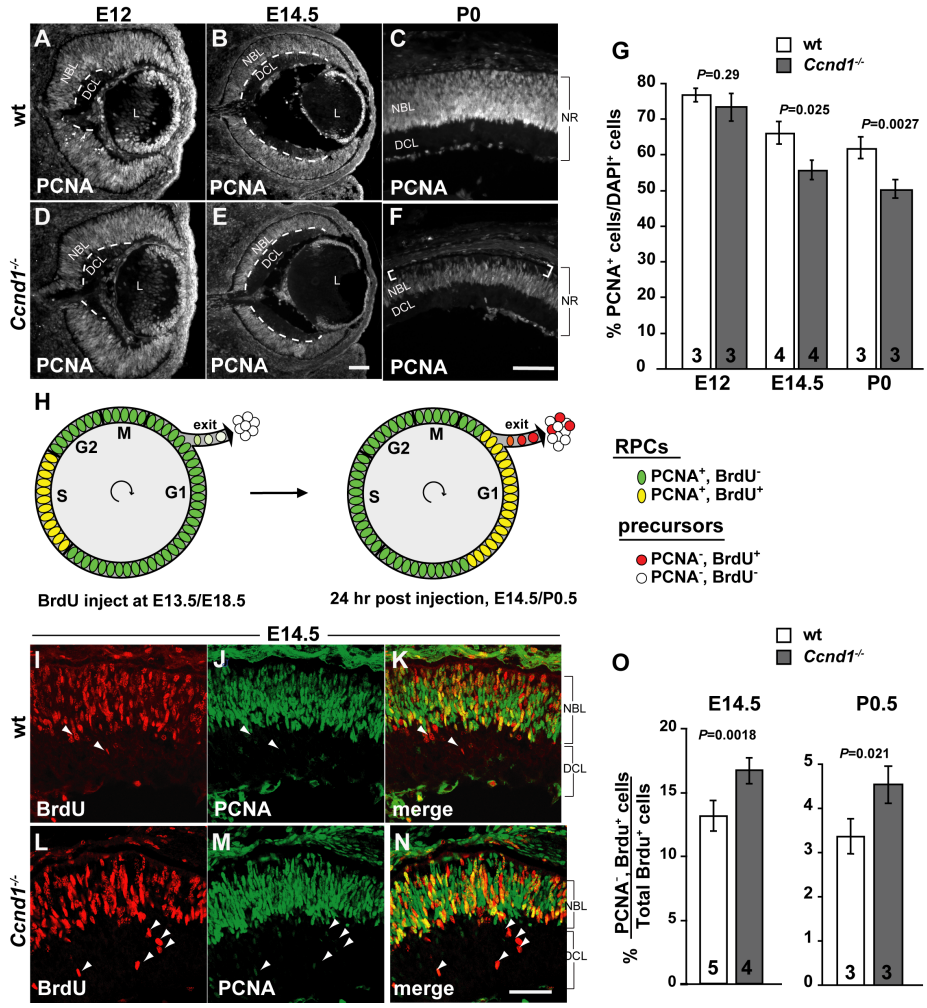

Figure 3

Gradual depletion of retinal progenitor cell (RPC) population in the *Ccnd1*$^{-/-}$ retina is caused by enhanced cell cycle exit. (A-F) Wild-type (wt) and *Ccnd1*$^{-/-}$ retinas were labeled with an antibody against PCNA from E12 to P0. Dashed lines in (A, B, D, E) demarcate the differentiated cell layer (DCL) from the neuroblast layer (NBL). Brackets in (F) show the 'apical gap' in the P0 mutant retina. **(G)** Quantification of PCNA$^+$ cells from E12, E14.5 and P0 retinas. **(H)** Schematic representation of cell cycle exit assay. **(I-N)** Wild-type and *Ccnd1*$^{-/-}$ retina samples, collected at 24 h following a single bromodeoxyuridine (BrdU) injection at E13.5, were co-labeled with antibodies against PCNA and BrdU to measure rate of cell cycle exit, as outlined in (H). Arrowheads in (I-N) indicate cells that had exited the cell cycle in the last 24 h. **(O)** Quantification of exited cells (BrdU$^+$, PCNA$^-$) as a percentage of BrdU$^+$ cells at E14 and P0.5. Abbreviations: DCL, differentiated cell layer; L, lens; NBL; neuroblast layer NR; neural retina. Scale bars: 100 μm; (E) is representative for (B, E); (F) for (A, C, D, F); (N) for (I-N).

cells (Figure 5A, D, G) and NR2E3$^+$ cells (Figure 5B, E, H) are increased in the *Ccnd1*$^{-/-}$ retina. The increased proportion of photoreceptors is confirmed by the expression of recoverin (RCVRN; Figure 5C, F, I), a calcium-binding protein expressed in photoreceptors at perinatal ages [37]. Further, an apparent increase in cells expressing blue cone opsin (OPN1SW) at P0 and rhodopsin at P4 (data not shown) support our conclusion that cones and rods contribute to the relative increase in photoreceptor production.

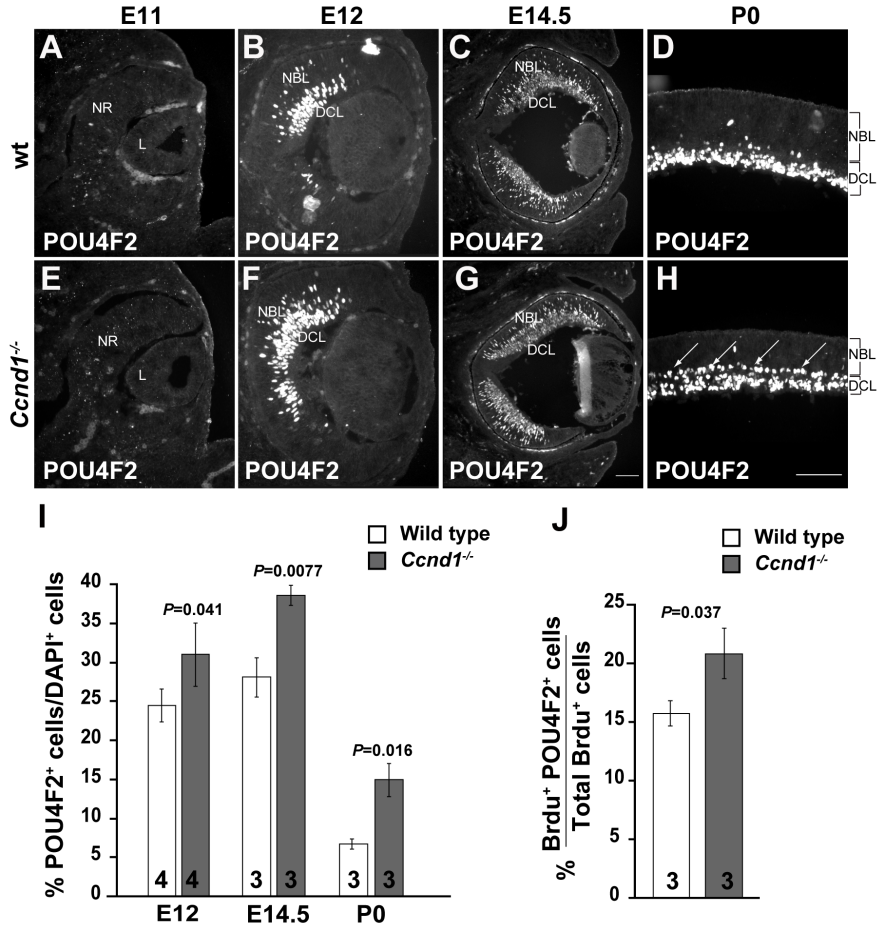

Figure 4

Retinal ganglion cells (RGCs) are overproduced in the *Ccnd1*$^{-/-}$ retina. (A-H) Wild-type (wt) and *Ccnd1*$^{-/-}$ retinas were stained with an antibody against POU4F2, which marks a majority of RGCs, at E11, E12, E14.5 and P0. Arrows in (H) mark the extra layer of RGCs in the *Ccnd1*$^{-/-}$ retina at P0. **(I)** Quantification of relative proportions of POU4F2$^+$ RGCs. **(J)** Quantification of relative rate of POU4F2$^+$ RGC production between E13.5 to E14.5. Abbreviations: DCL, differentiated cell layer; L, lens; NBL, neuroblast layer; NR, neural retina. Scale bars: 100 μm; (G) is representative for (C, G); (H) for (A, B, D, E, F, H).

The proportions of horizontal and amacrine cells are reduced in the Ccnd1$^{-/-}$ *retina, despite increased cell cycle exit*

Since the decrease in the relative proportion of the RPC population correlates with increased neurogenesis in the embryonic *Ccnd1*$^{-/-}$ retina, it stands to reason that the proportions of other early-born cell types, such as horizontal and amacrine cells, would also be increased. We found, however, that these cell types are in fact underrepresented at E17.5 (data not shown) and P0 (Figure 6). Horizontal cells, which express NEFM, are positioned in a single line towards the outer part of the NBL (Figure 6A, arrows). In the *Ccnd1*$^{-/-}$ retina, these cells are spaced further apart, and displaced toward the IPL (Figure 6C, arrows). This reduction in horizontal cells is clearly indicated in retinal whole mounts (Figure 6B, D), by quantification of their lineal density on retinal sections (Figure 6E), and with other markers of horizontal cells (aquaporin 4, prox1, and calbindin; data not shown). We also observed a distinct reduction in a subpopulation of amacrine cells marked by SOX2 and islet1 (ISL1) co-expression (Figure 6F–L) [38]. Whereas SOX2$^+$, ISL1$^+$ cells appear as an orderly bi-layer on both sides of the IPL (Figure 6F–H), the cells positioned below the IPL are mostly absent in the *Ccnd1*$^{-/-}$ ret-

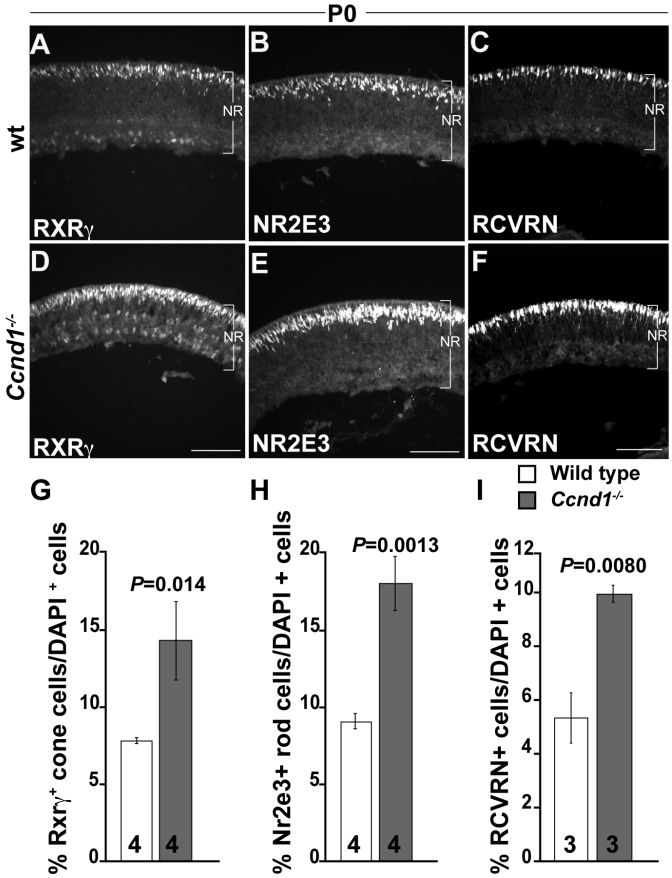

Figure 5

Proportion of photoreceptor cells is increased in the *Ccnd1*$^{-/-}$ retina. Expression pattern of cone precursor marker **(A, D)** RXRγ, **(B, E)** rod precursor marker NR2E3, and **(C, F)** general photoreceptor marker RCVRN at P0. **(G-I)** Quantification of relative proportions of RXRγ^{+}, NR2E3^{+} RCVRN^{+} cells, respectively, at P0. Abbreviations: NR, neural retina. Scale bars: 100 μm (D) is representative for (A, D); (E) for (B, E); (F) for (C, F).

ina and the remaining cells are spaced further apart (Figure 6I–K).

Ccnd1 *deficiency has different effects on distinct precursor populations*

Although our analysis of apoptosis (Additional file 2 and data not shown) suggests that cell death is not contributing to the embryonic phenotype, we cannot entirely rule it out as a factor in causing the reduction in horizontal cells and SOX2^{+}, ISL1^{+} amacrine cells as these cell types are normally in low abundance. Furthermore, as it is hard to discern NEFM^{+} horizontal cells during early neurogenesis and SOX2 expression is indicative of advanced stages of maturation, the reductions in these markers could also be due to delayed differentiation. This is unlikely, however, as cells expressing these markers continue to appear reduced at later ages (data not shown). Another possibility is that the *Ccnd1* deficiency is causing an underproduction in the post-mitotic precursors from which these particular cell types arise. To address this, we examined retinas at earlier stages of development using markers expressed in newly generated precursors of horizontal, amacrine, and photoreceptor cells (Figure 7). Pancreas specific transcription factor, 1a (*Ptf1a*) encodes a basic helix-loop-helix transcription factor expressed in horizontal cell precursors and a subset of amacrine cell precursors [39-41]. Basic helix-loop-helix family, member e22 (*Bhlhe22*, henceforth referred to as *Bhlhb5*) is another

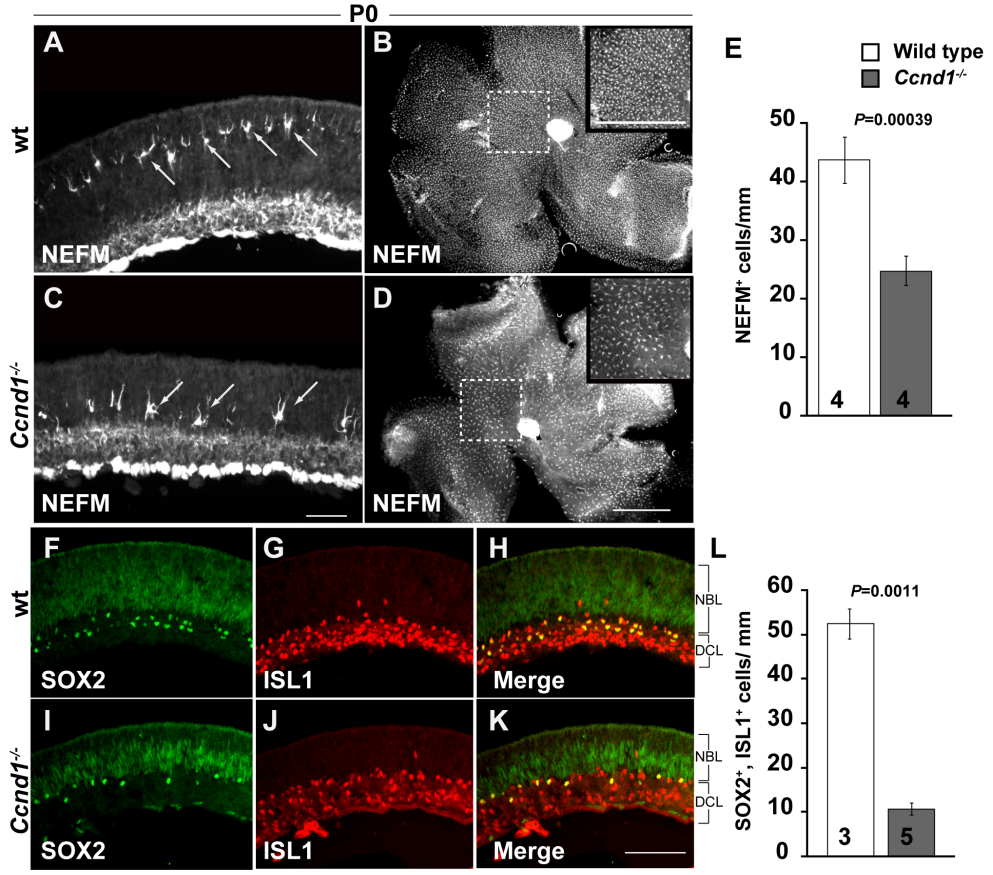

Figure 6

Reduced densities of horizontal and amacrine cells in the *Ccnd1*$^{-/-}$ retina. **(A, C)** Expression pattern of NEFM at P0 is shown. Arrows point to representative horizontal cells. Bright staining in the differentiated cell layer (DCL) is due to NEFM expression in retinal ganglion cells (RGCs). **(B, D)** Retinal whole mounts stained with NEFM antibody reveal differences in horizontal cell density across retina. Tissues were imaged from basal surface to reduce interference from NEFM immunoreactivity in RGCs. Insets show boxed regions. **(E)** Quantification of NEFM$^+$ horizontal cells at P0. **(F-K)** Expression patterns of SOX2 (F, I) and ISL1 (G, J) at P0 (merged images in (H, K)) are shown. **(L)** Quantification of SOX2$^+$, ISL1B$^+$ amacrine cells at P0. Abbreviations: DCL, differentiated cell layer; NBL; neuroblast layer. Scale bars: 100 μm; (C) is representative for (A, C); (D) for (B, D).

basic helix-loop-helix factor expressed in embryonic precursors that give rise to GABAergic and displaced amacrine cells [42]. *Otx2* is predominantly expressed in photoreceptor precursors, although a subset of RGC and amacrine precursors transiently express *Otx2* at the start of their differentiation [43-45].

Whereas a few cells in the E12 wild-type dorsal retina express PTF1A, significantly fewer PTF1A$^+$ cells are detected in the *Ccnd1*$^{-/-}$ retina (Figure 7A, D, G). In contrast, BHLHB5$^+$ cells, which are more abundant at this age, do not differ in their relative proportions between the wild-type and *Ccnd1*$^{-/-}$ retina (Figure 7B, E, G). OTX2 expression is evident in the retinal pigmented epithelium, peripheral retina, and isolated cells in the NBL (Figure 7C, F) and quantification of OTX2$^+$ cells in the NBL reveals a decrease in their proportion in the *Ccnd1*$^{-/-}$ retina (Figure 7G). This decrease is also reflected in RXRγ immunoreactivity (data not shown), which suggests a drop in cone precursor production at this age. At E14.5, the general trends for each marker are similar to that found at E12 (Figure 7H–N), but it appears that the proportion of OTX2$^+$ cells is catching up in the mutant (Figure 7N). At P0, the proportion of PTF1A$^+$ cells remains reduced in the

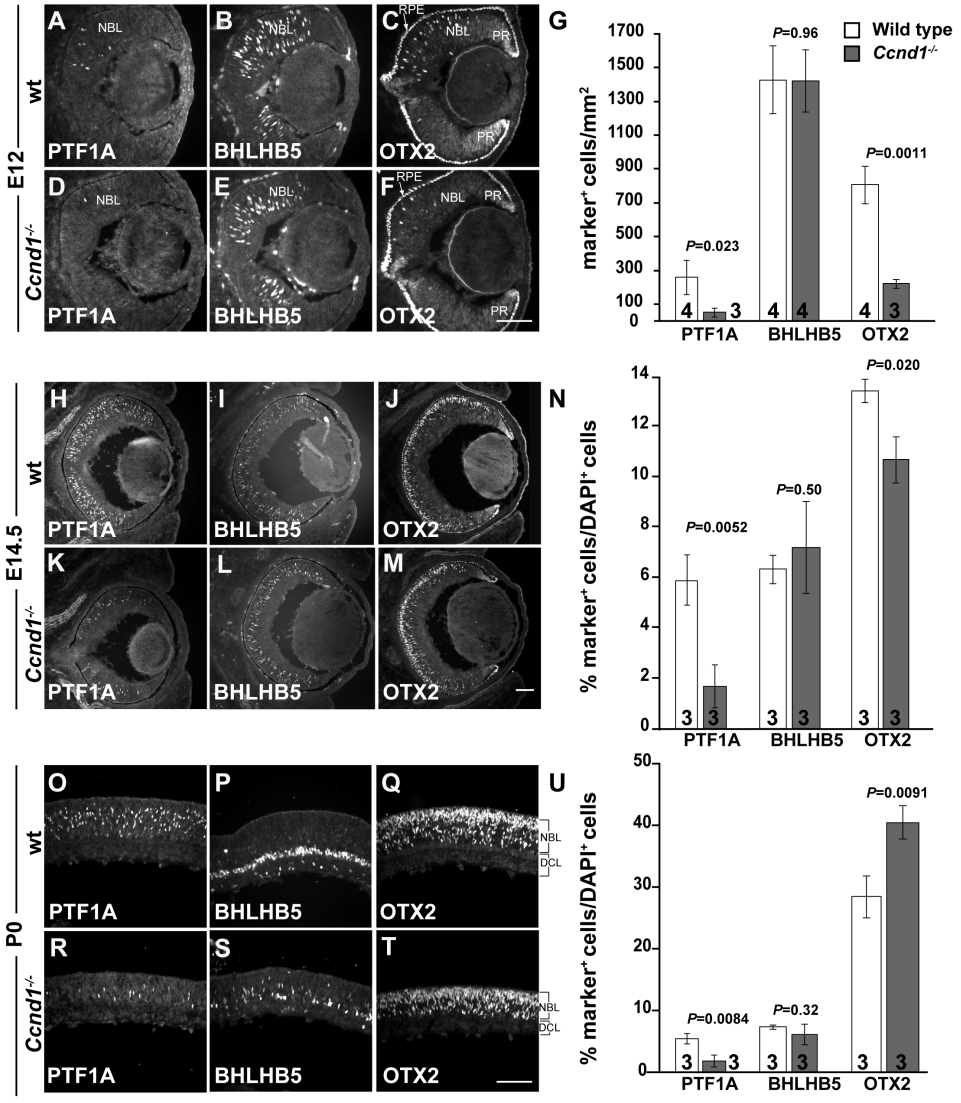

Figure 7

***Ccnd1*-deficiency causes alterations in the proportions of precursor cell populations**. Expression patterns of **(A, D, H, K, O, R)** PTF1A, **(B, C, I, L, P, S)** BHLHB5 and **(C, F, J, M, Q, T)** OTX2 at E12 (A-F), E14.5 (H-M), and P0 (O-T) are shown. **(G, N, U)** Quantification of marker$^+$ cells at E12 (G), E14.5 (N), and P0 (U). Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer; PR, peripheral retina; RPE, retinal pigmented epithelium. Scale bar: 100 μm; (F) is representative for (A-F); (M) for (H-M); (T) for (O-T).

Ccnd1$^{-/-}$ retina (Figure 7O, R, U) and the relative proportion of Bhlhb5$^+$ cells does not differ between the wild-type and *Ccnd1*$^{-/-}$ retina (Figure 7U), although their distribution is altered (Figure 7P, U). The relative proportion of OTX2$^+$ cells in the *Ccnd1*$^{-/-}$ retina is greater than in wild-type at P0 (Figure 7Q, T, U) and the larger proportions of RCVRN$^+$, NR2E3$^+$, and RXRγ$^+$ cells (Figure 5) collectively support the idea that, by P0, rod and cone precursor production is enhanced in the absence of *Ccnd1*.

To gain insight into the potential relationships between these precursor populations, we directly compared the

expression patterns of PTF1A, BHLHB5, and OTX2 at E12, E14.5 and P0 (Figure 8). Regardless of age, PTF1A is not expressed in the same cells as BHLHB5 or OTX2, which suggests that PTF1A$^+$ precursors are distinct from BHLHB5$^+$ precursors (Figure 8A, D, G, J, M, P) and OTX2$^+$ precursors (Figure 8B, E, H, K, N, Q). In contrast, OTX2 and BHLHB5 are co-expressed in a subset of cells from both populations (Figure 8C, F, I, L, O, R, arrowheads). At E12, cells co-expressing OTX2 and BHLHB5 persist in the *Ccnd1*$^{-/-}$ retina even though OTX2$^+$ cells are fewer (Figure 8C, F). At E14.5 and P0, the majority of cells co-expressing BHLHB5 and OTX2 are found in the NBL and not in the apical layer of OTX2$^+$ cells (Figure 8I, O), which are instead marked by RXRγ or NR2E3 (data not shown), and these relationships are maintained in the *Ccnd1*$^{-/-}$ retina (Figure 8L, R; data not shown). These observations suggest that the combinatorial expression of OTX2 and BHLHB5 marks multiple precursor populations. In sum, although we cannot definitively rule out apoptosis or altered differentiation as contributing factors, these data strongly suggest that *Ccnd1* inactivation alters the production of specific cell populations from the earliest times after onset of neurogenesis by altering the relative output of precursor cells from RPCs.

Ccnd2 *cannot completely rescue the* Ccnd1$^{-/-}$ *retinal phenotype*

Genetic replacement of *Ccnd1* by *Ccnd2* in *Ccnd1*$^{D2/D2}$ knock-in mice restores the histological appearance of the adult retina and electroretinographic response of photoreceptors [22]. In this model, the *Ccnd2* cDNA sequence is inserted into the *Ccnd1* locus and regulated by the *Ccnd1* promoter and enhancer elements. We examined the P0 retina in these mice to determine if *Ccnd2* is sufficient to correct the developmental changes due to *Ccnd1* deficiency (Figure 9). PCNA immunolabeling reveals that, similar to the *Ccnd1*$^{-/-}$ retina, the RPC layer is thinner in the *Ccnd1*$^{D2/D2}$ retina compared to its wild-type control, with a 'gap' at the apical surface (Figure 9A, B; brackets in B) that is filled with OTX2$^+$ cells (Additional file 5J–L). Scoring of PCNA$^+$ cells reveals that their proportion is significantly reduced (Figure 9E). Unlike the *Ccnd1*$^{-/-}$ retina, POU4F2$^+$ cells are not mis-positioned on the apical side of the IPL layer (Additional file 6E). Quantification of NEFM$^+$ horizontal cells revealed a significant decrease in their numbers (Figure 9F, G, J) although not to the same magnitude as in the *Ccnd1*$^{-/-}$ retina (Figure 6E). In agreement with this trend, there appear to be fewer PTF1A$^+$, SOX2$^+$, and BHLHB5$^+$ cells in the *Ccnd1*$^{D2/D2}$ retina compared to its wild-type control (Additional file 6B–D, F–H). These findings indicate that *Ccnd2* is not sufficient to completely compensate for *Ccnd1* in retinal cell production.

Genetic manipulation of downstream cell cycle regulators minimizes the impact of the Ccnd1 *deficiency on embryonic retinal development*

Genetic and biochemical evidence suggests that the rate-limiting function of *Ccnd1* in promoting cell cycle progression is to stimulate *Ccne* activity [23]. Based on this model, the altered cell production in the *Ccnd1*$^{-/-}$ RPC population could be due to limited Ccne activity. To address this, we analyzed the newborn retina in a mouse strain in which the human *Ccne* cDNA is inserted into the *Ccnd1* locus. In this strain, referred to as *Ccnd1*$^{hE/hE}$, human *Ccne* is expressed in place of *Ccnd1*. Similar to the *Ccnd1*$^{D2/D2}$ mouse, the adult retina in this model appears histologically normal and electrophysiological properties are better than in the *Ccnd1*$^{-/-}$ retina [23].

Our initial analysis revealed that the *Ccnd1*$^{hE/hE}$ retina is thinner than its wild-type counterpart, which may be due to an increase in apoptosis, especially in the NBL (Figure 9K–N). In contrast to the *Ccnd1*$^{-/-}$ and *Ccnd1*$^{D2/D2}$ retinas, PCNA staining shows that the RPC layer extends all the way to the apical edge, similar to the wild-type control (Figure 9C, D). Whereas the proportions of PCNA$^+$ and NEFM$^+$ cell populations are not significantly different from wild type, they exhibit downward trends (Figure 9E, H, I, J). The relative proportions and positions of cells expressing POU4F2, PTF1A, SOX2, and BHLHB5 appear to be similar between the *Ccnd1*$^{hE/hE}$ and its wild-type control retina (Additional file 6I–P). These findings suggest that *Ccne* is more efficient than *Ccnd2* in replacing *Ccnd1* to control the balance of retinal cell types produced.

The sequestration of P27KIP1 by CCND1 protein is one mechanism by which CCND1 is predicted to enhance CCNE activity and promote cell cycle progression [11]. Consistent with this, genetic inactivation of *p27Kip1* alleviates many of the phenotypes seen in the *Ccnd1*$^{-/-}$ mouse [46,47]. Furthermore, ectopic proliferation occurs in the *p27Kip1*$^{-/-}$ retina and its overexpression inhibits RPC proliferation [48,50]. To test whether the removal of *p27Kip1* restores the balance of cell types in the absence of *Ccnd1*, we analyzed *Ccnd1*$^{-/-}$, *p27Kip1*$^{-/-}$ double mutant retinas at P0 (Additional file 7). We found that the expression pattern of PCNA in the double mutant retina is more similar to the control retina (*Ccnd1*$^{+/-}$) than to the *Ccnd1*$^{-/-}$ retina, which is indicated by the absence of an 'apical gap' in staining (Additional file 7A, G, S). The cellular distributions of POU4F2$^+$ RGCs, NEFM$^+$ horizontal cells as well as other cell populations expressing PTF1A, SOX2, and BHLHB5 in the double mutant also appear more similar to the control patterns than those in the *Ccnd1*$^{-/-}$ retina (Additional file 7B–F, H–L, T–X). For comparative purposes, the expression patterns for these markers in the *p27Kip1*$^{-/-}$ retina are shown in Additional file 7M–R. The sum of our observations from the *Ccnd1*$^{hE/hE}$ and *Ccnd1*$^{-/-}$,

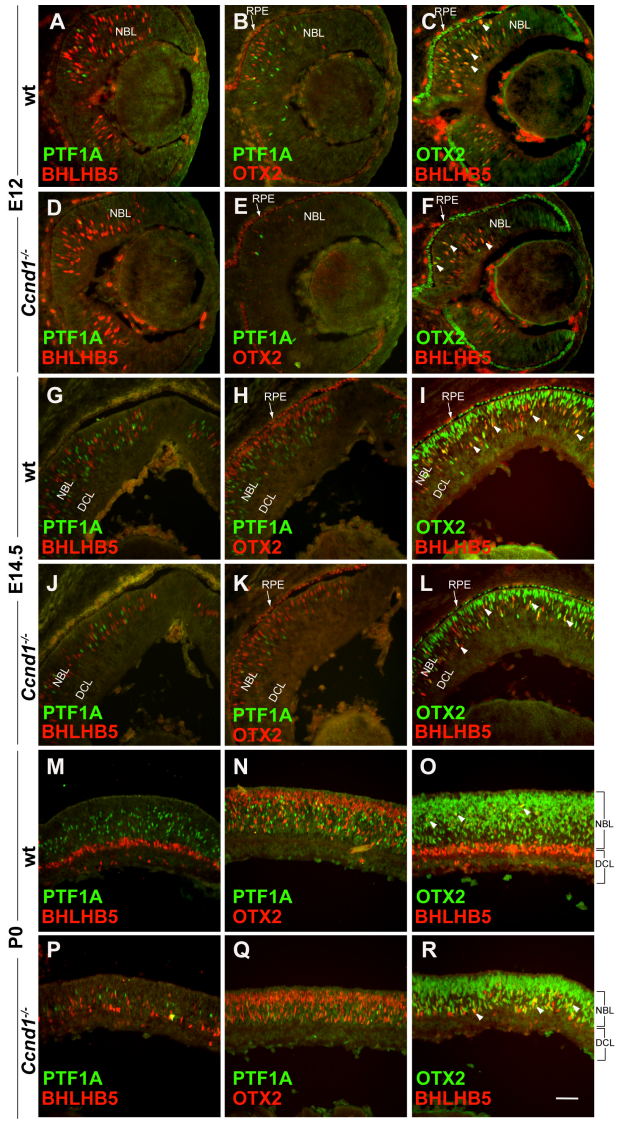

Figure 8
Relationship between PTF1A⁺, BHLHB5⁺, and OTX2⁺ precursors. Retinal sections at **(A-F)** E12, **(G-L)** E14.5, and **(M-R)** P0 were double-labeled with combinations of antibodies against PTF1A, BHLHB5 and OTX2. Arrowheads in (C, F, I, L, O, R) show examples of cell co-expressing OTX2 and BHLHB5. Arrows in (B, C, E, F, H, I, K, L) point to the retinal pigmented epithelium (RPE). Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer; RPE, retinal pigmented epithelium; wt, wild type. Scale bar: 100 μm; (R) is representative for all panels.

p27Kip1$^{-/-}$ mice suggest that *Ccnd1*'s influence on precursor cell output is dependent on its role in regulating *Ccne* and *p27Kip1*.

Discussion

We report here that *Ccnd1* has important functions in regulating embryonic retinal histogenesis. In addition to hypocellularity due to changes in proliferation, the relative proportions of multiple post-mitotic precursor popu-

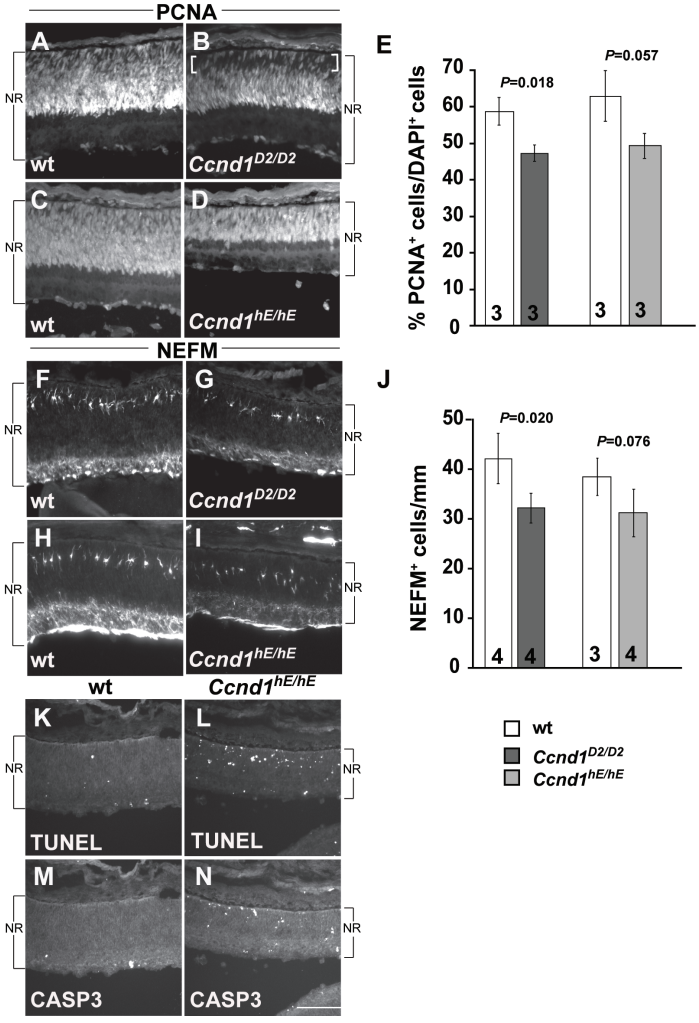

Figure 9

Analysis of *Ccnd1*$^{D2/D2}$ and *Ccnd1*$^{hE/hE}$ retinas at P0. **(A-D)** Expression pattern of PCNA) in *Ccnd1*$^{D2/D2}$ retina (B), *Ccnd1*$^{hE/hE}$ retina (D) and their respective wild-type (wt) controls (A, C) is shown. **(E)** Quantification of proportions of PCNA^{+} cells. **(F-I)** Expression pattern of NEFM in *Ccnd1*$^{D2/D2}$ retina (G), *Ccnd1*$^{hE/hE}$ retina (I) and their respective wild-type controls (F, H) is shown. **(J)** Quantification of NEFM^{+} cells. **(K, L)** TUNEL labeling in wild-type (K) and *Ccnd1*$^{hE/hE}$ retina (L). **(M, N)** Activated CASP3 immunoreactivity in wild-type (M) and *Ccnd1*$^{hE/hE}$ retina (N). Brackets in (B) show the 'apical gap' in the P0 *Ccnd1*$^{D2/D2}$ retina. Abbreviations: NR, neural retina. Scale bar: 100 μm (N is representative for all panels).

lations are altered in the *Ccnd1*$^{-/-}$ retina. RGC precursors are overrepresented, and horizontal cell and a subset of amacrine cell precursors are underrepresented in the *Ccnd1*$^{-/-}$ retina relative to wild type. Photoreceptor precursors, while underrepresented early on, are overrepresented later. Since overall cell number is lower in the mutant retina [21], our data suggest that the initial reduction in photoreceptor precursors and the apparent permanent reduction in horizontal and amacrine cell precursors reveals a true reduction in their numbers compared to wild type. While it is not known if the absolute number of RGCs and photoreceptors differs from wild type, our data show that their relative contributions to the cell composition of the *Ccnd1*$^{-/-}$ retina is greater than in wild type.

The changes outlined above are likely to be the result of *Ccnd1*'s roles in the cell cycle. Since cell cycle transit time is longer and the relative rate of cell cycle exit is enhanced in the absence of *Ccnd1*, it is possible that these changes in proliferation are linked. While longer cell cycle times are predictive of increased cell cycle exit in the brain [51,52], this does not appear to be the case in the zebrafish retina [53,54]. So whether the lengthening of the cell cycle directly causes enhanced cell cycle exit in the *Ccnd1*$^{-/-}$ retina is not clear. Regardless, we propose that *Ccnd1* is required for establishing the proper balance of cell types produced during embryonic retinal development by mediating cell cycle exit, or in other words, the rate of precursor output from RPCs.

Ccnd1 *regulates the timing of cell cycle exit in a limited manner after the onset of neurogenesis*

Interestingly, not all *Ccnd1*$^{-/-}$ RPCs exit prematurely, and the extent of RPC depletion, while significant, is not severe. A reasonable percentage of *Ccnd1*$^{-/-}$ RPCs remain in the cell cycle even as neurogenesis progresses. This is not due to restricted expression as *Ccnd1* is widely expressed in RPCs and throughout most of the cell cycle (this study) [15,18]. Rather, D-cyclins are not absolutely required for proliferation in embryonic RPCs since proliferation still occurs in mice deficient in *Ccnd1*, *Ccnd2*, and *Ccnd3* [14]. We also found no evidence that *Ccnd1*$^{-/-}$ RPCs exit the cell cycle before the normal onset of neurogenesis even though *Ccnd1* is abundantly expressed as early as E9.5. This is not because RPCs are inherently unable to initiate neurogenesis early since precocious neurons are produced in the paired box gene 6 (*Pax6*$^{-/-}$) and hairy and enhancer of split 1 (*Hes1*$^{-/-}$) retinas [55,57]. Furthermore, the spreading wave of neurogenesis is not altered in the *Ccnd1*$^{-/-}$ retina, even though wild-type RPCs increase their level of CCND1 expression just ahead of the neurogenic wave. These observations led us to propose a model stating that once neurogenesis begins, a limited number of RPCs become *Ccnd1*-dependent and their timing of cell cycle exit is determined by their level of CCND1 expression or activity (Figure 10A). We also predict that *Ccnd1*-dependent RPCs are generated continuously during retinal development and have limited proliferative potential. Otherwise, *Ccnd1* deficiency should have caused a more discontinuous or severe decline in the RPC population. It appears then that downregulation of *Ccnd1* is an important step in the transition of RPCs to post-mitotic precursors. Consistent with this, forced expression of CCND1 in photoreceptor precursors causes unscheduled proliferation, differentiation defects, and apoptosis [58].

Mechanism of Ccnd1*-mediated cell cycle exit*

D-cyclins regulate the retinoblastoma pathway by binding to and activating CDK4/6 and by sequestering CDK2 inhibitors such as P27KIP1 [11]. Both mechanisms ultimately lead to inactivation of retinoblastoma proteins by CDK2/4/6-mediated phosphorylation, allowing cells to progress from G1 to S phase and undergo DNA replication. Although the importance of the retinoblastoma pathway in continuously cycling cells is not clear, it is critical in many cell lineages for differentiation [59-61]. In the mouse retina, genetic deletion of the retinoblastoma proteins (*Rb1*, *Rbl1*/*p107*, or *Rbl2*/p130) uncouples cell cycle exit and differentiation, resulting in ectopic proliferating cells that express markers of multiple precursor cell types in the retina [62,65]. Further evidence of this decoupling is seen in the postnatal *p107* single copy mutant retina where mature horizontal cells proliferate extensively, all the while retaining their differentiated characteristics [66]. This suggests that retinoblastoma pathway activity regulates cell cycle exit of RPCs and controls the post-mitotic state for some period of time after cell cycle exit. Additional evidence to this effect comes from genetic studies of molecular regulators in this pathway: inactivation of cyclin-dependent kinase inhibitors such as *p27Kip1*, *p57Kip2*, and *p19Ink4d* cause ectopic proliferation [9,48,50,67]. Forced expression of *Ccnd1*, the large T-antigen from simian virus 40 or the human papillomavirus type 16 (HPV-16) E7 protein (negative regulators of retinoblastoma proteins) in post-mitotic photoreceptor precursors causes inappropriate cell cycle re-entry and subsequent cell death or tumorigenesis depending on the nature of the transgene construct [58,68-72]. A similar phenomenon is also observed for other retinal cell types [73,75]. Furthermore, *Rb1* phosphorylation is greatly diminished in the *Ccnd1*$^{-/-}$ retina, probably due to reductions in CDK2 and CDK4 activities [23,46,47,76]. Since the retinoblastoma proteins are expressed in dynamic and temporal patterns in mouse RPCs [60,64,77], their expression levels in individual RPCs may determine the timing of *Ccnd1*-dependence (Figure 10A). However, other mechanisms such as extracellular signaling are also likely to contribute to tempering retinoblastoma protein activity in continuously cycling RPCs [12].

Our results indicate that *Ccnd2* may not influence RPC cell cycle exit in the same manner as *Ccnd1*. Although the retina in the *Ccnd1*$^{D2/D2}$ mouse is not as severely affected as in the *Ccnd1*$^{-/-}$ mouse, cell production is not restored to normal proportions. Limited *Ccnd2* expression is not the likely reason for this [22]. Rather, molecular analyses indicate that CCND2 activity is not identical to CCND1 [22,46,47,78]. The newborn *Ccnd1*$^{hE/hE}$ retina also has a more normal cellular composition than the *Ccnd1*$^{-/-}$ retina and may surpass the extent of rescue in the *Ccnd1*$^{D2/D2}$ retina. Interpretation of the phenotype is complicated by enhanced cell death, which is not observed in the newborn *Ccnd1*$^{-/-}$ or *Ccnd1*$^{D2/D2}$ retinas. This is probably due to high *hCcne* expression as endogenous *Ccne* is normally expressed at low levels [18] (unpublished observations).

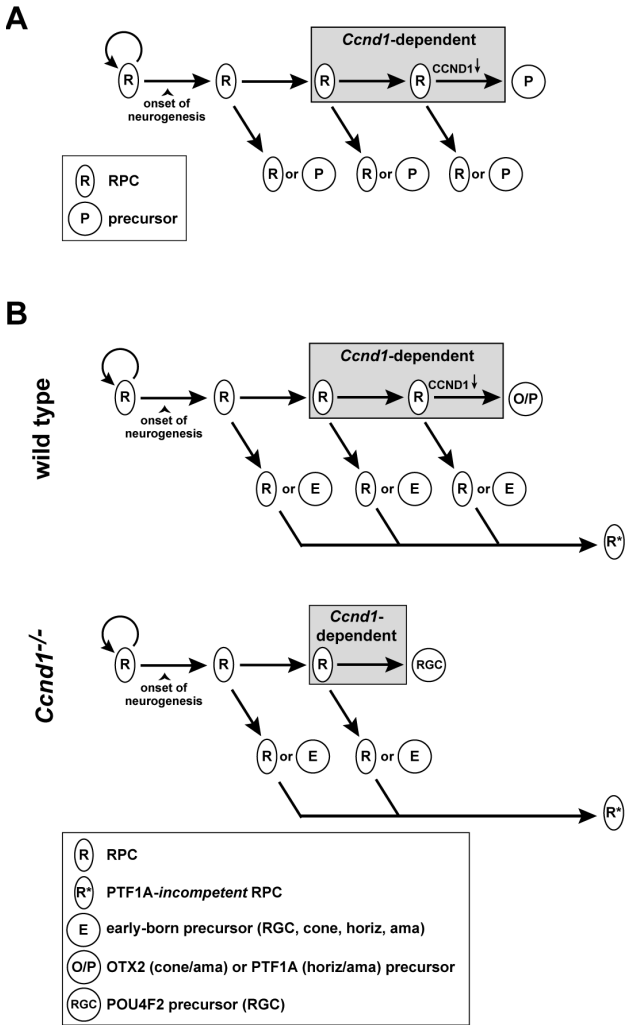

Figure 10

Models of *Ccnd1* function in the retina. **(A)** General model of *Ccnd1*-dependence in retinal progenitor cells (RPCs). Most, if not all RPCs express CCND1. At least one division before cell cycle exit, RPCs become dependent on CCND1 to remain in the cell cycle (RPCs in gray box). Those that retain sufficiently high Ccnd1 levels or activity continue to divide whereas those that drop below a threshold will produce at least one post-mitotic precursor cell (P). It is not known if the other daughters of each division are *Ccnd1*-dependent, nor is the mode of division known for *Ccnd1*-dependent RPCs. It is presumed that at least some of the RPCs that persist contribute to the RPC population at later stages. **(B)** Model of how cell production is altered in the absence of *Ccnd1* during early retinal development. In the wild-type retina, a proportion of CCND1-dependent RPCs will produce precursors that differentiate into cone, horizontal, or amacrine cells (O/P). In the *Ccnd1*$^{-/-}$ retina, *Ccnd1*-dependent RPCs exit at least one division sooner, resulting in a gradual reduction in the size of the RPC population and an enhancement in the relative production of retinal ganglion cell (RGC) precursors at the expense of other precursor types. This could be due to an instructive role for *Ccnd1* in cell fate specification or to a consequence of RPC competence and/or altered environment at the time of exit. The inability of RPCs to replenish the PTF1A precursor population (as it does for the OTX2 precursor population) suggests that most RPCs lose their competence to make PTF1A precursors (R*). Similar mechanisms may influence the output of other precursor populations.

While *hCcne* may rescue premature cell cycle exit due to the *Ccnd1* deficiency, it could also activate apoptosis by causing an incompatible activation of proliferation and differentiation pathways in precursor cells. Nevertheless, instead of functionally replacing *Ccnd1*, ectopically expressed hCCNE bypasses the retinoblastoma proteins [23], by partnering with CDK2 to induce S-phase entry without sufficient RB1 phosphorylation [79]. A similar bypass mechanism appears to be operating in the *Ccnd1*$^{-/-}$, *p27Kip1*$^{-/-}$ retina [46,47] and the more normal distribution of cell types in the newborn *Ccnd1*$^{-/-}$, *p27Kip1*$^{-/-}$ retina at P0 supports the idea that *p27Kip1* is downstream of *Ccnd1* in regulating the production of precursor populations. The sum of these findings agrees with the model that *Ccnd1*-mediated regulation of the retinoblastoma pathway is an important mechanism for controlling the timing of cell cycle exit in embryonic RPCs.

Ccnd1 *influences the production of precursor cells allocated to multiple cell types*

In multipotential progenitor cell lineages, enhanced rates of cell cycle exit tend to cause reductions in late-born cell types that may or may not be accompanied by increases in the production of early-born cell types [55,57,80-83]. Interestingly, the changes in cell production that occur in the embryonic *Ccnd1*$^{-/-}$ retina diverge from this general rule. RGC production is enhanced whereas unexpectedly, production of other early-born cell types, namely horizontal cells, SOX2^{+}, ISL1^{+} amacrine cells, and cones (initially), is reduced, and these types of alterations are indicative of changes in cell fate specification (Figure 10B). Since *Ccnd1* is expressed in RPCs and not in postmitotic precursors, how might *Ccnd1* inactivation produce these changes?

One possibility is that *Ccnd1* has an instructive role in retinal cell fate determination, similar to *Ccne* in the thoracic NB6-4 neuroblast lineage in *Drosophila* [84]. *Ccnd1* may prevent a subset of early neurogenic RPCs from becoming RGCs by directing them toward horizontal, amacrine, or cone cell fates. Indeed, production of PTF1A^{+} precursors is reduced in the *Ccnd1*$^{-/-}$retina, and *Ptf1a* inactivation results in a cell fate switch from horizontal and amacrine cells to RGCs [39-41]. Although OT*x2*$^{+}$ (and RXRγ^{+}) precursors are also underrepresented in the *Ccnd1*$^{-/-}$ retina at E12 and E14.5, it is unclear how *Ccnd1* could instruct photoreceptor fate since inactivation of *OTx2* in photoreceptor precursors causes conspicuous amacrine cell overproduction and apoptosis by P0 [45], two changes not observed in the *Ccnd1*$^{-/-}$ retina. Regardless, if *Ccnd1* is instructive for cell fate, we predict that the mechanism involved could operate independently of its role in timing RPC cell cycle exit since altering precursor cell fates does not necessarily involve changes in proliferation.

Another possibility is that *Ccnd1* deficiency could produce cell fate changes that are linked to the altered timing of cell cycle exit (Figure 10B). In this scenario, an early neurogenic, *Ccnd1*-dependent RPC is competent to become an RGC, but is prevented from doing so because it expresses CCND1 and stays in the cell cycle. As CCND1 levels drop below a threshold in a subsequent cell cycle, the RPC exits and differentiates into the other early-born cell types (that is, horizontal, amacrine, cone; O/P precursor in Figure 10B) because of changes in its competence and/or in its surrounding environmental milieu. In the absence of CCND1, the *Ccnd1*-dependent RPC exits at least one cell cycle sooner and differentiates into an RGC at the expense of other early-born cell types (Figure 10B). Attractive features of this model are that it incorporates current ideas on retinal development: that RPCs are multipotential; that temporal shifts in RPC competence occur as development progresses; and that the concerted actions of cell-extrinsic and -intrinsic pathways mediate cell fates [85]. Importantly, it doesn't invoke a function for *Ccnd1* beyond controlling the timing of cell cycle exit.

An unresolved issue, however, is that while this model accounts for enhanced RGC production early and photoreceptor production late, it fails to explain the persistent underproduction of other early-born cell types in the mutant. If RPCs are multipotential and premature cell cycle exit is a continuous and ongoing process in the *Ccnd1*$^{-/-}$ retina, then the RPCs that exit subsequently should compensate for the earlier exited RPCs and produce the precursors that are initially underproduced. While this is observed for the OTX2^{+}, RXRγ^{+} precursors (cones), production of PTF1A^{+} precursors (horizontal cells and some amacrine cells) fails to 'catch up'. One possibility is that most RPCs lose their competence to produce PTF1A^{+} precursors (R* in Figure 10B). In the *Ccnd1* mutant, the PTF1A-incompent RPCs are unable to compensate for the early underproduction of PTF1A^{+} precursors; thereby resulting in a permanent deficit in these precursors and the cell types they give rise to.

The BHLHB5^{+} cell population is unique in that its proportion does not vary between the wild type in the *Ccnd1*$^{-/-}$ retina, at least up to P0. Given the idea that subsets of RPCs may utilize different proteins to control cell cycle exit [18], BHLHB5^{+} precursors may not require *Ccnd1* to regulate the number of RPCs needed for their production. The fact that the proportion of BHLHB5^{+} precursors remains consistent may also be an indication that production of this cell population is dependent on non-cell autonomous feedback signaling [86-88].

As mentioned at the start of this section, a more rapid rate of RPC depletion due to enhanced neurogenesis should cause a reduction or absence in the last generated cell

types. Interestingly, rods, bipolar cells, and Müller glia are present in the postnatal *Ccnd1*$^{-/-}$ retina as are PCNA^{+} cells [21] (unpublished observations), which indicates that RPCs persist until the last stages of normal histogenesis. This could occur if our model of *Ccnd1*-dependence in embryonic RPCs also holds for postnatal RPCs. If true, then the rate of RPC decline may not be steep enough to deplete the population prior to production of the last-born cell types, although again, we would expect a drop in their numbers. Our observation of an increased proportion of rod precursors at P0 suggests that they are being produced at the expense of bipolar cells and Müller glia, similar to what may be happening for RGC precursors and the other early-generated precursor populations. Assessing this is difficult, however, because of the extensive cell death in the postnatal *Ccnd1*$^{-/-}$ retina, when bipolar cells and Müller glia are being produced [21,89]. Alternatively, RPCs in the postnatal period may not require *Ccnd1* to control timing of cell cycle exit, and one possible explanation is that *Ccnd3* takes over, a scenario analogous to D-cyclin utilization in cerebellar granule precursors, which depend on *Ccnd1* early and *Ccnd2* late, to produce the correct number of granule cells [82,90]. *Ccnd3* is normally expressed in Müller glia and possibly in RPCs at the end of histogenesis (that is, P5 and older). Importantly, CCND3 expression is upregulated by P0 in the *Ccnd1*$^{-/-}$ retina (unpublished observation) [47], which suggests a possible compensatory mechanism for maintaining postnatal RPCs.

Does Ccnd1 *regulate laminar positioning of retinal cells?*

Retinal cells occupy distinct locations in the retina and cells of the same cell type generally occupy the same laminar position. Unexpectedly, we found that the locations of cells belonging to several different classes are altered in the *Ccnd1*$^{-/-}$ retina. For example, RGCs are distributed on both sides of the IPL and horizontal cells are positioned closer than normal to the IPL. Why this occurs is not clear, but *Ccnd1* can influence cell migration via the ROCK pathway [91,92]. Important to note, however, is that horizontal cells briefly reside in this position during their normal course of differentiation [93,94]. Whether *Ccnd1* has a direct role in regulating precursor cell positioning/migration or if these changes are due to indirect effects of altered differentiation or because of compromised cell-cell interactions due to the changes in the proportions of retinal cell types awaits further analysis.

Conclusion

This study elucidates the roles of *Ccnd1* in embryonic retinal development. We show that *Ccnd1* is expressed globally in RPCs and contributes to two aspects of proliferation control – the rate of cell cycle progression and the timing of cell cycle exit. *Ccnd1* is also required to ensure that precursor populations are produced in their appropriate proportions. We propose that *Ccnd1* does this through its control of cell cycle exit and that the permanent reduction in the PTF1A^{+} precursor population in the *Ccnd1*$^{-/-}$ retina is the result of a temporal shift in RPC competence. More studies are needed to address whether *Ccnd1* also has a direct role in regulating precursor fates and, if so, whether *p27Kip1* or other cell cycle regulators that are downstream of *Ccnd1* are involved. This work provides further evidence for the model that cell cycle regulators contribute to the neurogenic output of multipotential progenitor populations.

Abbreviations

BrdU: 5-bromo-2'-deoxy-uridine; DCL: differentiated cell layer; E: embryonic day; EdU: 5-ethynyl-2'-deoxy-uridine; IdU: 5-iodo-2'-deoxy-uridine; IPL: inner plexiform layer; NBL: neuroblast layer; P: postnatal day; RGC: retinal ganglion cell; RPC: retinal progenitor cell.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EML and GD conceived the study and contributed to the experimental design, interpretation of data, and preparation of the figures and manuscript. GD contributed to the experimental design, collected and analyzed the data, and prepared the figures and manuscript. YC bred and genotyped the knock-in mouse lines, and prepared the eyes for analysis. EML and PS oversaw the work done in their respective laboratories. All authors read and approved the manuscript.

Additional material

Additional file 1

Expression domains of neural retina and retinal pigmented epithelium markers are not altered in the* Ccnd1$^{-/-}$ *eye prior to the onset of neurogenesis. *Wild-type and* Ccnd1$^{-/-}$ *retinas at E9.5 and E11 were stained with antibodies against* ***(A, B, I, J)*** *PAX6,* ***(C, D, K, L)*** *SOX2,* ***(E, F, M, N)*** *VSX2 and* ***(K, L, O, P)*** *MITF. The asterisk in (D) indicates that this region of the neuroepithelium is folded over in the section. Abbreviations: NR, neural retina; OV, optic vesicle; PNR, presumptive neural retina; PRPE, presumptive retinal pigmented epithelium; RPE, retinal pigmented epithelium. Scale bar: 100 μm; (P) is representative for (A-P).*

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Additional file 2

Cell death is not altered in the* Ccnd1$^{-/-}$ *retina during embryonic development. *Sections from* **(A-D)** *wild-type (wt) and* **(E-H)** Ccnd1$^{-/-}$ *retinas were stained with an antibody against activated-CASPASE 3, a marker of dying cells. No differences were observed in the pattern or number of immunoreactive cells at E12, E14.5, or E17.5. At P0,* Ccnd1$^{-/-}$ *retinas showed a slight increase in the number of activated CASP3$^+$ cells. Bright dots in (D) are non-specific background staining. Scale bars: 100 μm; (G) is representative for (A, C, E, G); (H) for (B, D, F, H).*
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Additional file 3

Phosphorylated histone H3 immunoreactivity. *Expression patterns of pHH3 at* **(A, D)** *E12,* **(B, E)** *E14.5, and* **(C, F)** *P0 in wild-type (wt) (A-C) and* Ccnd1$^{-/-}$ *retinas (D-F) are shown. Scale bars: 100 μm; (D) is representative for (A, D); (F) for (B, C, E, F).*
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Additional file 4

Co-expression patterns of retinal progenitor cell markers are maintained in the* Ccnd1$^{-/-}$ *retina. **(A-D)** *Expression patterns of VSX2 and HES1 at E12 (A, B) and P0 (C, D) in wild-type (wt) retinas are shown.* **(E-H)** *Expression patterns of VSX2 and HES1 at E12 (E, F) and P0 (G, H) in* Ccnd1$^{-/-}$ *retinas are shown.* **(I-N)** *Co-expression patterns of PCNA and VSX2 at P0 in wild-type (I-K) and* Ccnd1$^{-/-}$ *retinas (L-N) are shown.* **(O-T)** *Co-expression patterns of PCNA and HES1 at P0 in wild-type (O-Q) and* Ccnd1$^{-/-}$ *retinas (R-T) are shown. Note that in all cases the co-expression relationships are maintained, indicating that the altered expression patterns in the* Ccnd1$^{-/-}$ *retina are due to the decrease in retinal progenitor cell numbers and not to direct regulation of the marker proteins. Abbreviations: NR, neural retina. Scale bars: 100 μm; (H) is representative for (A-H); (T) for (I-T).*
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Additional file 5

Co-expression patterns of PCNA and OTX2 in* Ccnd1$^{-/-}$, Ccnd1$^{D2/D2}$, *and* Ccnd1$^{hE/hE}$ *retinas at P0. **(A-R)** Ccnd1$^{-/-}$ *(D-F),* Ccnd1$^{D2/D2}$ *(J-L) and* Ccnd1$^{hE/hE}$ *(P-R) retinas and their respective wild type controls ((A-C), (G-I), and (M-O), respectively) at P0 were double-labeled with antibodies against PCNA and OTX2 Merged images show that OTX2-expressing cells completely fill the PCNA$^-$ 'gap' in the* Ccnd1$^{-/-}$ *and* Ccnd1$^{D2/D2}$ *retinas (F, L). Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer; PRL, photoreceptor cell layer; RPE, retinal pigmented epithelium. Scale bar: 100 μm; (R) is representative for all panels.*
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Additional file 6

Expression patterns of POU4F2, PTF1A, SOX2, and BHLHB5 in* Ccnd1$^{D2/D2}$ *and* Ccnd1$^{hE/hE}$ *retinas at P0. *POU4F2 expression in* **(A, I)** *wild-type (wt) controls,* **(E)** Ccnd1$^{D2/D2}$ *and* **(M)** Ccnd1$^{hE/hE}$ *retina at P0, is shown. PTF1A expression in* **(B, J)** *wild-type controls,* **(F)** Ccnd1$^{D2/D2}$ *and* **(N)** Ccnd1$^{hE/hE}$ *retina at P0 is shown. SOX2 expression in* **(C, K)** *wild-type controls,* **(G)** Ccnd1$^{D2/D2}$ *and* **(O)** Ccnd1$^{hE/hE}$ *retina at P0 is shown. BHLHB5 expression in* **(D, L)** *wild-type controls,* **(H)** Ccnd1$^{D2/D2}$ *and* **(P)** Ccnd1$^{hE/hE}$ *retina at P0 is also shown. Abbreviations: NR, neural retina. Scale bar: 100 μm; (P) is representative for all panels.*
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Additional file 7

Expression patterns of PCNA, POU4F2, NEFM, PTF1A, SOX2, and BHLHB5 in wild type (*Ccnd1$^{+/-}$), Ccnd1$^{-/-}$ *single null,* p27Kip1$^{-/-}$ *single null, and* Ccnd1$^{-/-}$, p27Kip1$^{-/-}$ **double null retinas** *at P0***. **(A, G, M, S)** *PCNA expression in retinal progenitor cells, showing absence of a significant apical gap in the* Ccnd1$^{-/-}$, p27Kip1$^{-/-}$ *double null (S) compared to* Ccnd1$^{-/-}$ *(G). The distributions of* **(B, H, N, T)** *POU4F2$^+$ retinal ganglion cells,* **(C, I, O, U)** *NEFM$^+$ horizontal cells in the outer neuroblast layer,* **(D, J, P, V)** *PTF1A$^+$,* **(E, K, Q, W)** *SOX2$^+$ and* **(F, L, R, X)** *BHLHB5$^+$ precursors in the double null (bottom row) are more similar to wild type (top row) than to the* Ccnd1$^{-/-}$ *(second row). Expression patterns for each of the markers in the* p27Kip1$^{-/-}$ *retina (third row) are shown for comparison. Scale bar: 100 μm; (X) is representative for all panels.*
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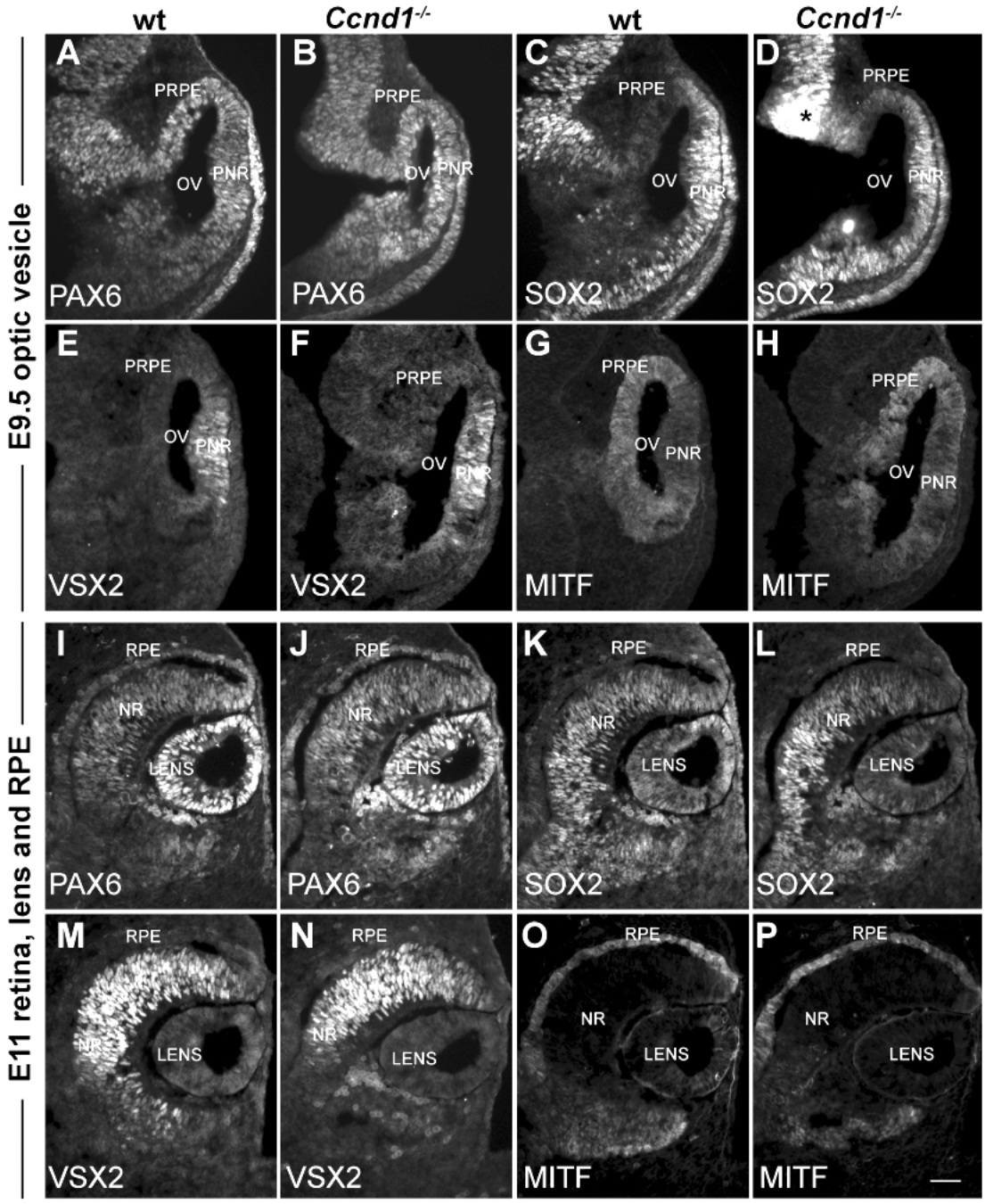
Figure 2.11. (Additional file 1) Expression domains of neural retina and retinal pigmented epithelium markers are not altered in the *Ccnd1*$^{-/-}$ eye prior to the onset of neurogenesis. Wild-type and *Ccnd1*$^{-/-}$ retinas at E9.5 and E11 were stained with antibodies against **(A, B, I, J)** PAX6, **(C, D, K, L)** SOX2, **(E, F, M, N)** VSX2 and **(K, L, O, P)** MITF. The asterisk in (D) indicates that this region of the neuroepithelium is folded over in the section. Abbreviations: NR, neural retina; OV, optic vesicle; PNR, presumptive neural retina; PRPE, presumptive retinal pigmented epithelium; RPE, retinal pigmented epithelium. Scale bar: 100 μm; (P) is representative for (A-P).


wt
Ccnd1-/-
wt
Ccnd1-/-
E9.5 optic vesicle
E11 retina, lens and RPE
A
PRPE
OV
PNR
PAX6
B
PRPE
OV
PNR
PAX6
C
PRPE
OV
PNR
SOX2
D
PRPE
*
OV
PNR
SOX2
E
PRPE
OV
PNR
VSX2
F
PRPE
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MITF
H
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MITF
I
RPE
NR
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PAX6
J
RPE
NR
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K
RPE
NR
LENS
SOX2
L
RPE
NR
LENS
SOX2
M
RPE
NR
LENS
VSX2
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NR
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NR
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MITF

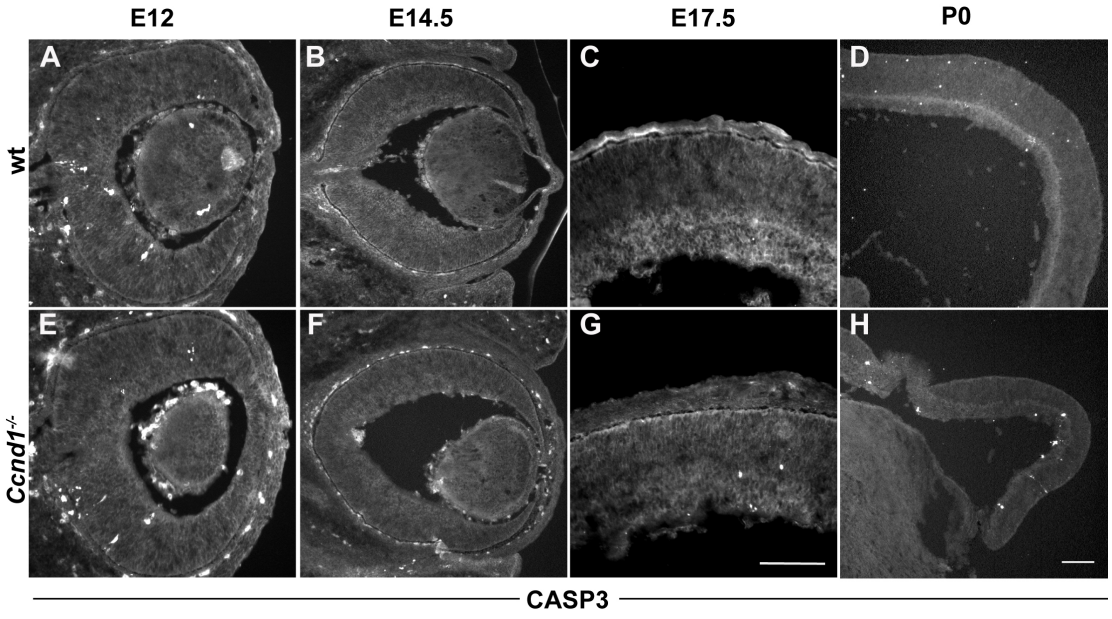


Figure 2.12. (Additional file 2) Cell death is not altered in the *Ccnd1*$^{-/-}$ retina during embryonic development. Sections from **(A-D)** wild-type (wt) and **(E-H)** *Ccnd1*$^{-/-}$ retinas were stained with an antibody against activated-CASPASE 3, a marker of dying cells. No differences were observed in the pattern or number of immunoreactive cells at E12, E14.5, or E17.5. At P0, *Ccnd1*$^{-/-}$ retinas showed a slight increase in the number of activated CASP3^{+} cells. Bright dots in (D) are non-specific background staining. Scale bars: 100 μm; (G) is representative for (A, C, E, G); (H) for (B, D, F, H).

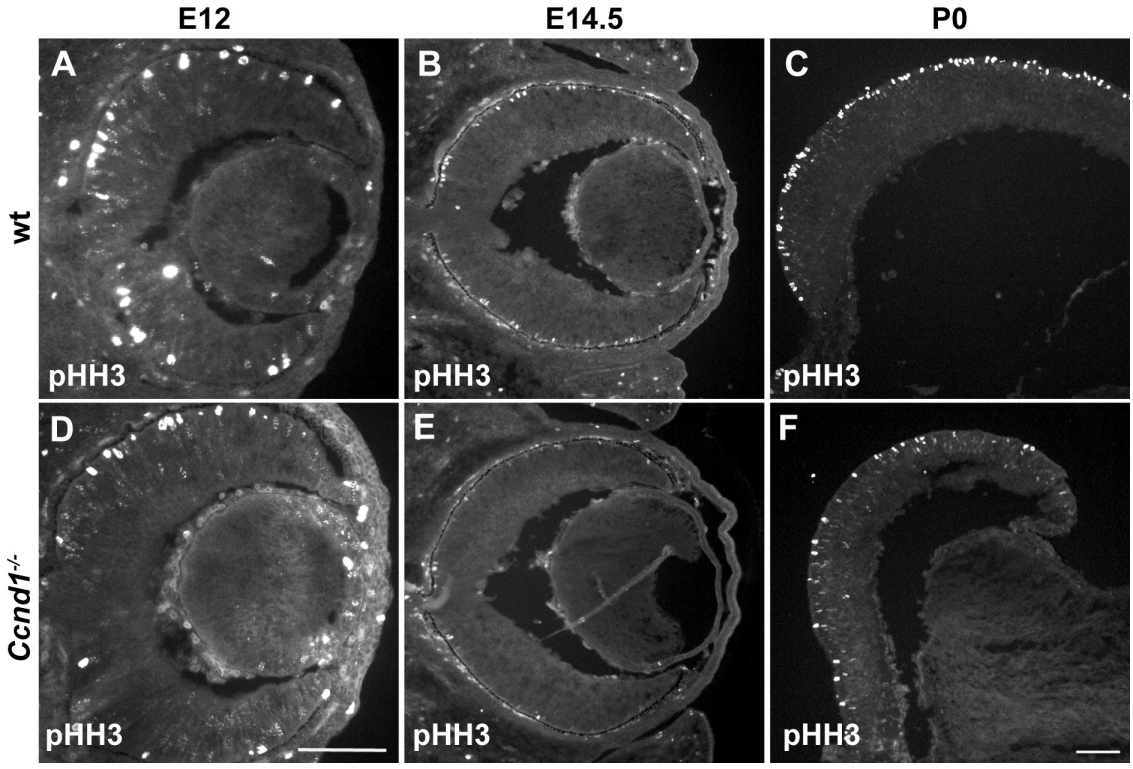


Figure 2.13. (Additional file 3) Phosphorylated histone H3 immunoreactivity. Expression patterns of pHH3 at **(A, D)** E12, **(B, E)** E14.5, and **(C, F)** P0 in wild-type (wt) (A-C) and *Ccnd1*$^{-/-}$ retinas (D-F) are shown. Scale bars: 100 μm; (D) is representative for (A, D); (F) for (B, C, E, F).

Figure 2.14. (Additional file 4) Co-expression patterns of retinal progenitor cell markers are maintained in the *Ccnd1*$^{-/-}$ retina. **(A-D)** Expression patterns of VSX2 and HES1 at E12 (A, B) and P0 (C, D) in wild-type (wt) retinas are shown. **(E-H)** Expression patterns of VSX2 and HES1 at E12 (E, F) and P0 (G, H) in *Ccnd1*$^{-/-}$ retinas are shown. **(I-N)** Co-expression patterns of PCNA and VSX2 at P0 in wild-type (I-K) and *Ccnd1*$^{-/-}$ retinas (L-N) are shown. **(O-T)** Co-expression patterns of PCNA and HES1 at P0 in wild-type (O-Q) and *Ccnd1*$^{-/-}$ retinas (R-T) are shown. Note that in all cases the co-expression relationships are maintained, indicating that the altered expression patterns in the *Ccnd1*$^{-/-}$ retina are due to the decrease in retinal progenitor cell numbers and not to direct regulation of the marker proteins. Abbreviations: NR, neural retina. Scale bars: 100 μm; (H) is representative for (A-H); (T) for (I-T).

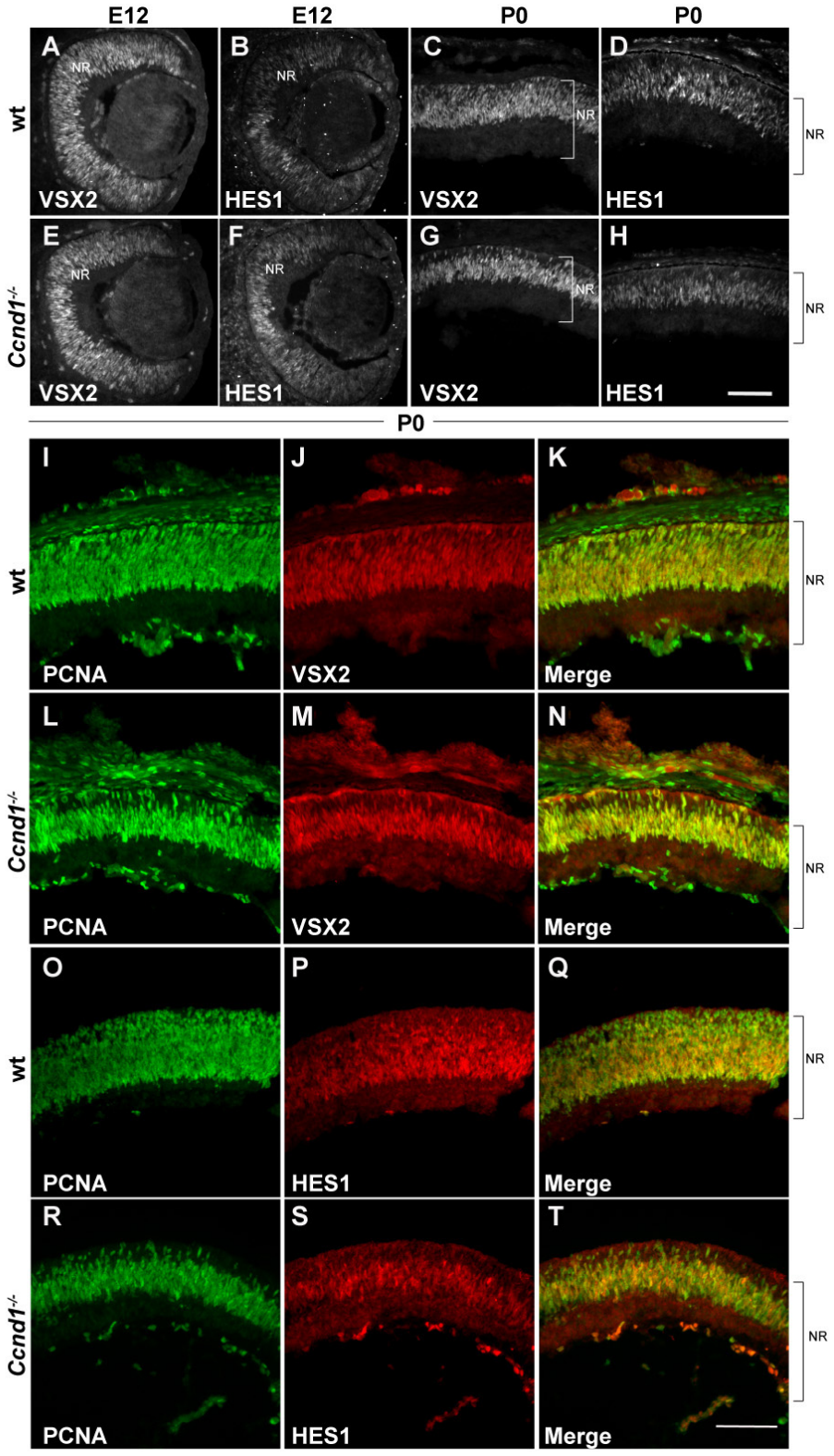
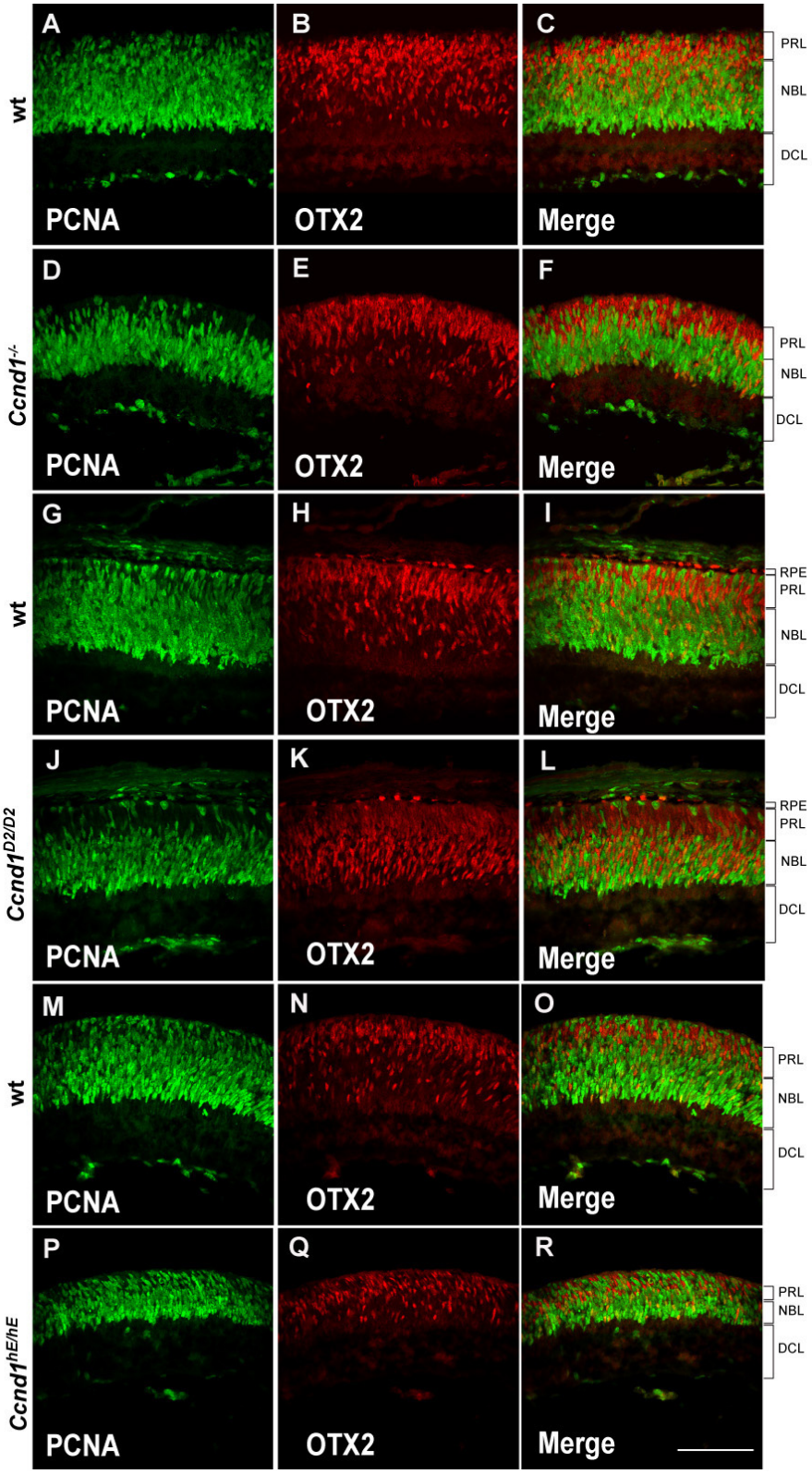

E12
E12
P0
P0
wt
Ccnd1-/-
A
B
C
D
E
F
G
H
NR
VSX2
HES1
P0
I
J
K
L
M
N
O
P
Q
R
S
T
PCNA
Merge

Figure 2.15 (Additional file 5) Co-expression patterns of PCNA and OTX2 in *Ccnd1*$^{-/-}$, *Ccnd1D*$^{2/}$*D*^{2}, and *Ccnd1hE*$^{/}$*hE* retinas at P0. **(A-R)** *Ccnd1*$^{-/-}$ (D-F), *Ccnd1D*$^{2/}$*D*^{2} (J-L) and *Ccnd1hE*$^{/}$*hE* (P-R) retinas and their respective wild type controls ((A-C), (G-I), and (M-O), respectively) at P0 were double-labeled with antibodies against PCNA and OTX2 Merged images show that OTX2-expressing cells completely fill the PCNA^{-} 'gap' in the *Ccnd1*$^{-/-}$ and *Ccnd1D*$^{2/}$*D*^{2} retinas (F, L). Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer; PRL, photoreceptor cell layer; RPE, retinal pigmented epithelium. Scale bar: 100 μm; (R) is representative for all panels.


A
B
C
wt
PCNA
OTX2
Merge
PRL
NBL
DCL
D
E
F
Ccnd1-/-
PCNA
OTX2
Merge
PRL
NBL
DCL
G
H
I
wt
PCNA
OTX2
Merge
RPE
PRL
NBL
DCL
J
K
L
Ccnd1D2/D2
PCNA
OTX2
Merge
RPE
PRL
NBL
DCL
M
N
O
wt
PCNA
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PCNA
OTX2
Merge
PRL
NBL
DCL

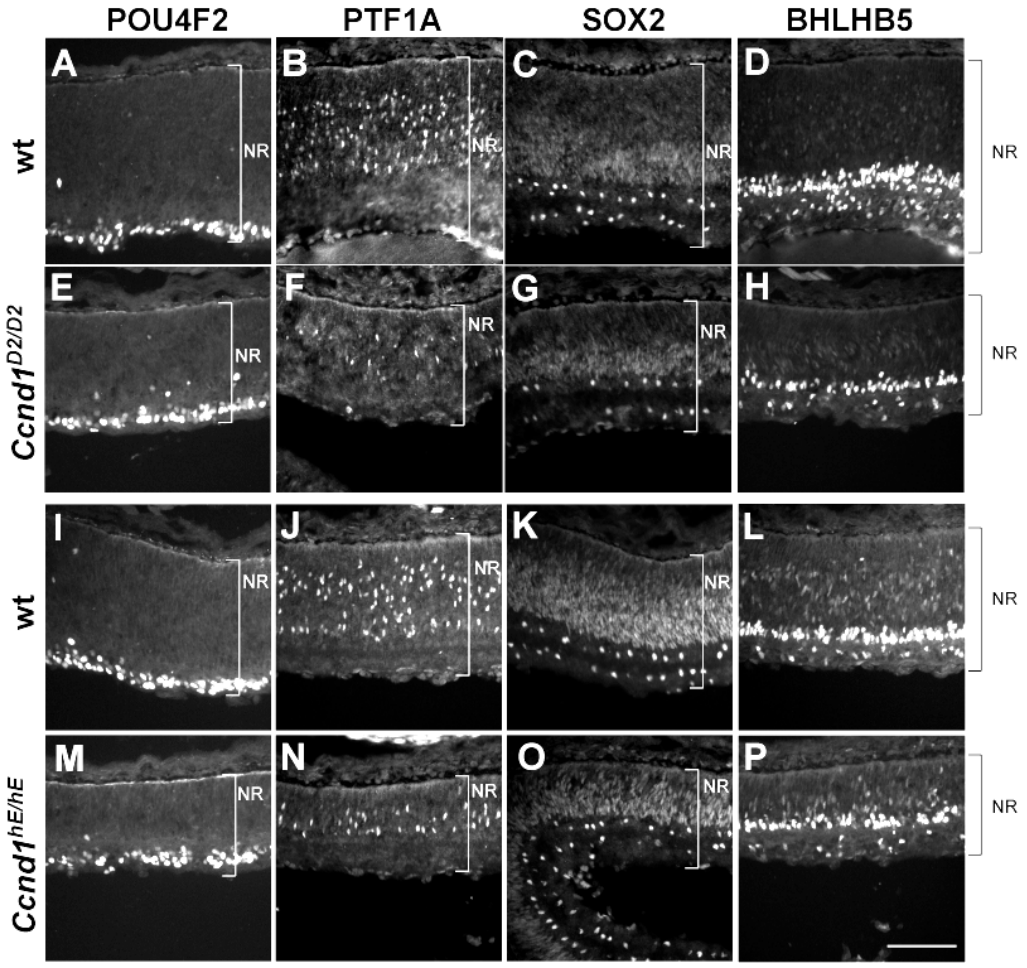
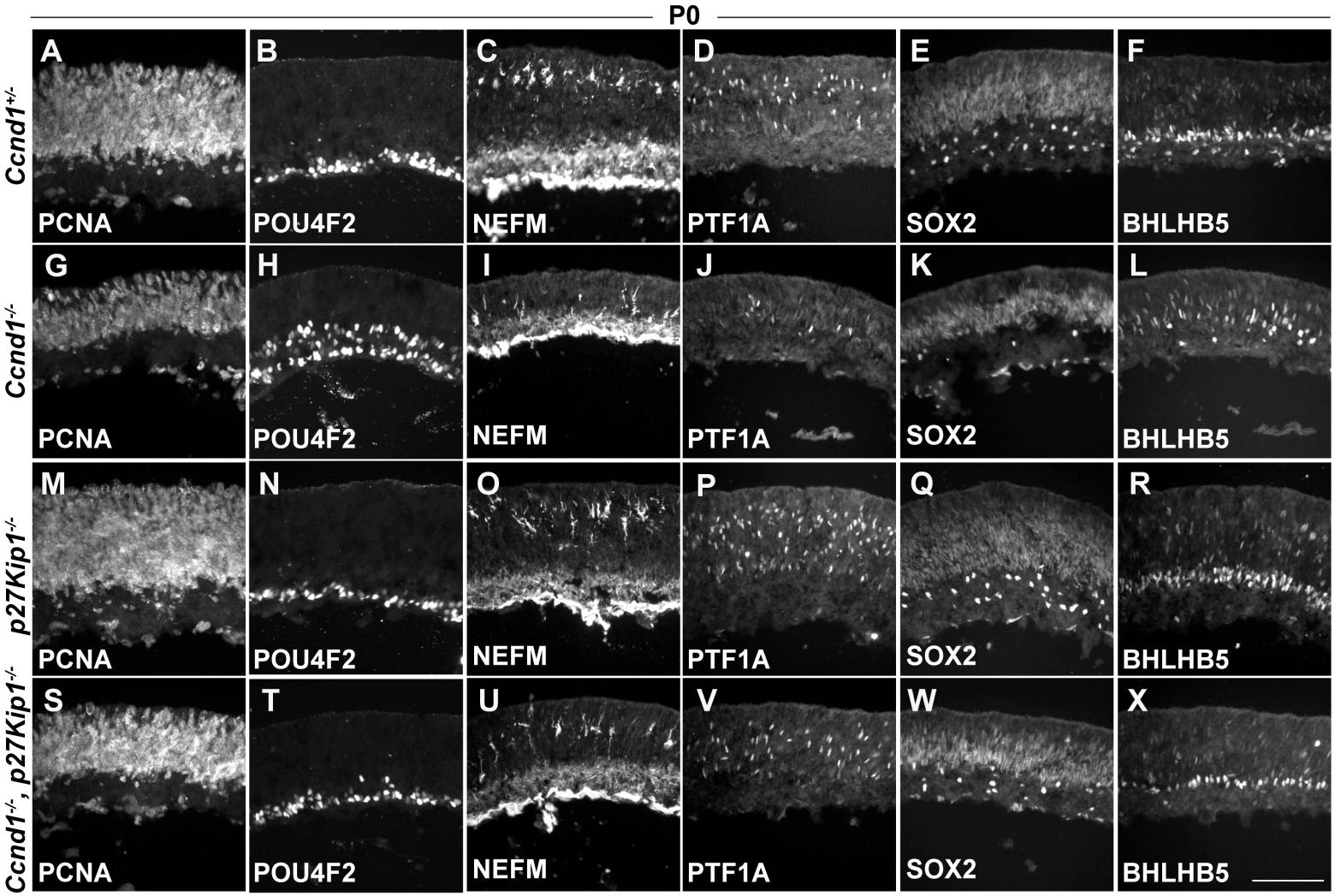


Figure 2.16. (Additional file 6) Expression patterns of POU4F2, PTF1A, SOX2, and BHLHB5 in *Ccnd1D*$^{2/}$*D*^{2} and *Ccnd1hE*$^{/}$*hE* retinas at P0. POU4F2 expression in **(A, I)** wild-type (wt) controls, **(E)** *Ccnd1D*$^{2/}$*D*^{2} and **(M)** *Ccnd1hE*$^{/}$*hE* retina at P0, is shown. PTF1A expression in **(B, J)** wild-type controls, **(F)** *Ccnd1D*$^{2/}$*D*^{2} and **(N)** *Ccnd1hE*$^{/}$*hE* retina at P0 is shown. SOX2 expression in **(C, K)** wild-type controls, **(G)** *Ccnd1D*$^{2/}$*D*^{2} and **(O)** *Ccnd1hE*$^{/}$*hE* retina at P0 is shown. BHLHB5 expression in **(D, L)** wild-type controls, **(H)** *Ccnd1D*$^{2/}$*D*^{2} and **(P)** *Ccnd1hE*$^{/}$*hE* retina at P0 is also shown. Abbreviations: NR, neural retina. Scale bar: 100 μm; (P) is representative for all panels.

Figure 2.17. (Additional file 7) Expression patterns of PCNA, POU4F2, NEFM, PTF1A, SOX2, and BHLHB5 in wild type (*Ccnd1*$^{+/-}$), *Ccnd1*$^{-/-}$ single null, *p27Kip1*$^{-/-}$ single null, and *Ccnd1*$^{-/-}$, *p27Kip1*$^{-/-}$*double null retinas* at P0. **(A, G, M, S)** PCNA expression in retinal progenitor cells, showing absence of a significant apical gap in the *Ccnd1*$^{-/-}$, *p27Kip1*$^{-/-}$ double null (S) compared to *Ccnd1*$^{-/-}$ (G). The distributions of **(B, H, N, T)** POU4F2^{+} retinal ganglion cells, **(C, I, O, U)** NEFM^{+} horizontal cells in the outer neuroblast layer, **(D, J, P, V)** PTF1A^{+}, **(E, K, Q, W)** SOX2^{+} and **(F, L, R, X)** BHLHB5^{+} precursors in the double null (bottom row) are more similar to wild type (top row) than to the *Ccnd1*$^{-/-}$(second row). Expression patterns for each of the markers in the *p27Kip1*$^{-/-}$ retina (third row) are shown for comparison. Scale bar: 100 μm; (X) is representative for all panels.


P0
Ccnd1+/-
Ccnd1-/-
p27Kip1-/-
Ccnd1-/-, p27Kip1-/-
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
PCNA
POU4F2
NEFM
PTF1A
SOX2
BHLHB5

CHAPTER 3

CYCLIN D1 and CYCLIN D3 HAVE NONOVERLAPPING REQUIREMENTS IN MOUSE RETINAL DEVELOPMENT

Abstract

The cell cycle regulator Cyclin D1 (*Ccnd1*) is expressed in embryonic retinal progenitor cells (RPCs) and regulates their cell cycle kinetics and neurogenic output. In this study, we report that *Ccnd1* also has important functions in postnatal retinal histogenesis. Early production of Müller glia and bipolar cells was enhanced in *Ccnd1*$^{-/-}$ retinas, indicating a role for *Ccnd1* in controlling the timing of generation of these late born cell types. Surprisingly, despite a steeper rate of depletion of the RPC population throughout the neurogenic interval, *Ccnd1*$^{-/-}$ retinas exhibited an extended window of proliferation, neurogenesis, and gliogenesis. Cyclin D3 (*Ccnd3*), normally seen in Müller glia, was prematurely expressed in *Ccnd1*$^{-/-}$ RPCs. However, despite this early expression, *Ccnd3* did not compensate for *Ccnd1*'s role in regulating cell cycle kinetics or neurogenic output. The data presented in this study, along with our previous finding that Cyclin D2 was unable to fully compensate for *Ccnd1,* indicate that *Ccnd1* has essential functions in controlling retinal histogenesis that are not shared by the other D-cyclins.

Introduction

During the course of mammalian retinal histogenesis, retinal progenitor cells (RPCs) proliferate extensively and concomitantly give rise to the six major classes of retinal neurons and Müller glia in a temporal order (Livesey and Cepko, 2001; Rapaport et al., 2004). Controlled progression through the cell cycle is central for this to occur (Bilitou and Ohnuma, 2010; Ohnuma and Harris, 2003). Once committed to undergo cell division, an RPC can produce two RPCs, two postmitotic precursor cells, or an RPC and a precursor. Since several cell types (i.e. rod photoreceptors, bipolar interneurons, and

Müller glia) are produced during the latter half of the histogenic interval, proper regulation of the RPC or precursor fate choice in each cell cycle is essential to retain enough RPCs for generating the later born cell types. A combination of extrinsic signaling pathways and intrinsic fate-determining networks are likely to influence the cell cycle machinery, inducing cell cycle reentry (S phase commitment) for RPC-fated cells and cell cycle exit for neuronal or glial precursor-fated cells (Andreazzoli, 2009; Cayouette et al., 2006; Livesey and Cepko, 2001; Ohnuma and Harris, 2003).

The three D-type cyclins, Cyclin D1, D2, and D3, are important for the transmission of extracellular growth signals to the core cell cycle machinery, facilitating progression from G1 into S (Sherr, 1995a; Sherr, 1995b). Previously considered universally important for proper development and survival, D-cyclins were found to be dispensable for many aspects of development (Kozar et al., 2004; Sherr and Roberts, 2004). This is in part due to their selective and dynamic expression in specific tissue compartments, and to functional redundancy and compensation in some instances (Ciemerych et al., 2002; Kozar and Sicinski, 2005; Kozar et al., 2004). However, cell proliferation and a good portion of embryogenesis still occurs in animals lacking all three D-cyclins (Kozar et al., 2004). A similar phenomenon has also been observed in mice lacking other key cell cycle regulators such as the E-cyclins and most impressively in CDK4, CDK6, CDK2 triple knockouts (Geng et al., 2003; Santamaria et al., 2007). A current model based on these observations is that molecular adaptation takes place (i.e. CDK1 substitutes for the missing Cdks in the triple knockout) **(**Santamaria et al., 2007**)**. In light of these findings, it is reasonable to question the complexity of the mammalian cell cycle and what advantages are conferred upon the organism by retaining it, especially

when many cell cycle proteins are oncogenes or tumor suppressors, and drive tumor formation when mutated or misregulated. One potential way to get at this question is to better understand how inactivation of these cell cycle genes alter histogenesis in tissues like the retina, where proliferation must be balanced with precursor production.

Ccnd1 is the predominant D-cyclin expressed in RPCs during retinal histogenesis and *Ccnd1* knockout (*Ccnd1*$^{-/-}$) mice have hypocellular retinas (Das et al., 2009; Fantl et al., 1995; Ma et al., 1998; Sicinski et al., 1995). We recently demonstrated that *Ccnd1* inactivation increases the cell cycle time in embryonic and neonatal RPCs and enhances their rate of cell cycle exit (Das et al., 2009). As a consequence, the RPC population undergoes a faster rate of depletion. We also found that, in the absence of *Ccnd1*, the proportions of neuronal precursor populations were changed due to their altered production, underlining the importance of CCND1 in generating the correct complement of retinal neurons (Das et al., 2009).

Besides CCND1, the other D-cyclin expressed in the retina is CCND3 (Dyer and Cepko, 2000). CCND3 is not usually expressed in RPCs during development. Rather, towards the end of histogenesis, it is expressed in newly generated Müller glia precursors and possibly in some RPC that give rise to glial cells ((Dyer and Cepko, 2000); see below). Western blot analysis of P1 *Ccnd1*$^{-/-}$ retinas revealed that CCND3 is precociously expressed in the mutant, raising the possibility that CCND3 could be compensating for CCND1 (Tong and Pollard, 2001).

In this study, we characterize the effects of *Ccnd1* inactivation on postnatal retinal histogenesis and determine whether CCND3 is compensatory for CCND1. In spite of the smaller RPC population at birth in *Ccnd1*$^{-/-}$ retinas, generation of postnatal cell types still

occurred, although with altered timing. Unexpectedly, the histogenic period is extended in *Ccnd1*$^{-/-}$ retinas, further underscoring the importance of CCND1 in regulating RPC proliferation dynamics. We also found that CCND3 does not compensate for CCND1 loss, at least up until birth. We propose that while D-cyclins and other cell cycle regulators are not absolutely required for cell proliferation or tissue histogenesis, the diversity of cell cycle proteins and complexity of the cell cycle is maintained in part because of nonoverlapping functions of specific cell cycle proteins and a dependence of progenitor populations on these proteins to properly time and coordinate proliferation with precursor production.

Results

Proliferation persists late in *Ccnd1*$^{-/-}$ retina development

Due to premature cell cycle exit and a lengthening of cell cycle time, RPCs undergo a steeper rate of depletion compared to their wild type counterparts (Das et al., 2009). Therefore, we expected proliferation and neurogenesis to terminate prematurely in *Ccnd1*$^{-/-}$ retinas during postnatal development. Before verifying if this was true, we first established the normal expression of CCND1 protein from postnatal day zero (P0) to P14 (Fig 3.1A-D) in wild type retinas. During this period, CCND1 was expressed in RPCs and its pattern matched the expected RPC depletion pattern associated with the completion of histogenesis. By P6, CCND1 expression was restricted to almost a single row of cells in the central retina (arrows; Fig 1C). Due to the central to peripheral progression of retinal histogenesis, many CCND1 expressing cells were still seen in the

peripheral retina (data not shown). By P14, CCND1 expressing cells were no longer observed in the central (Fig 3.1D) or peripheral retina (data not shown).

To determine if the RPC population was depleted earlier in *Ccnd1*$^{-/-}$ retinas, we analyzed wild type and *Ccnd1*$^{-/-}$ retinas at P6 for PCNA expression, a comprehensive marker of RPCs (Barton and Levine, 2008). At this age, a large number of PCNA expressing cells were present in the peripheral wild type retina (Fig 3.1E). In comparable areas of the mutant retina, the PCNA expressing RPC layer was thinner (Fig 3.1I). That proliferation was overall reduced in peripheral regions of *Ccnd1*$^{-/-}$ retinas was further corroborated by the presence of fewer PCNA^{+} cells at the apical surface where mitosis occurs (arrows; Fig 3.1E and I) and fewer EdU^{+} cells (Fig 3.1F and J). In the wild type central retina, the few cells that expressed PCNA did so at reduced levels (Fig 3.1G). Since EdU labeled cells were not detected in this region (Fig 3.1H), the low level of PCNA expression (Fig 3.1G) is not likely to correspond to active proliferation. Surprisingly, bright PCNA expressing cells were detected in the *Ccnd1*$^{-/-}$ central retina with some PCNA^{+} cells undergoing mitosis (Fig 3.1K; arrows point to mitotic figures). EdU labeled cells were also detected in this region (arrowheads in Fig 3.1L).

In sum, although there are fewer proliferating cells in the peripheral regions of the mutant retina consistent with a faster rate of RPC depletion, there also exists a population of proliferative cells that persists beyond the normal histogenic interval in the central retina. This unexpected change in proliferation dynamics is further demonstrated in low power view (Fig 3.1M and N).

CCND3 expression initiates earlier in the *Ccnd1*$^{-/-}$ retina in RPCs, but does not affect cell cycle rate or precursor cell type output

In other tissues, loss of a D-cyclin can be compensated by ectopic or precocious expression of the other D-cyclins (Ciemerych et al., 2002; Cooper et al., 2006; Glickstein et al., 2007; Lam et al., 2000; Solvason et al., 2000). In neonatal *Ccnd1*$^{-/-}$ retinas, western blots showed that CCND3 protein was precociously expressed (Tong and Pollard, 2001). Thus, it seemed possible that CCND3 may compensate for the absence of CCND1 in promoting proliferation.

First, we determined the expression pattern of CCND3 at P0, P4, P6, and P14 (Fig 3.2A-H). In wild type retinas, CCND3 expression was not observed until P6 (Fig 3.2A-C), where it was principally seen in the INL and in scattered cells in the ONL (Fig 3.2C; arrowheads). CCND3 expression was absent from the peripheral retina, indicating that its onset of expression occurs in a central to peripheral fashion and is likely the result of the wave of Muller glia genesis . Interestingly, the central to peripheral pattern of CCND3 expression is coincident with the disappearance of CCND1 along the same gradient, with a few cells expressing both proteins at the overlapping expression boundaries (data not shown). Together, this suggested a developmental change in utilization of D-cyclins from CCND1 to CCND3. At P14, CCND3 is expressed in a single row of cells in the INL (Fig 3.2D), which was previously shown to be Müller glia (Dyer and Cepko, 2000; Vazquez-Chona et al., 2009).

In contrast, we detected CCND3 expression in the neuroblast layer of *Ccnd1*$^{-/-}$ retinas from E17.5 onward (Fig 3.2E,F; data not shown). Colocalization with VSX2 (Belecky-Adams et al., 1997; Chen and Cepko, 2000; Green et al., 2003; Liu et al., 1994)

suggested that CCND3 was expressed in *Ccnd1*$^{-/-}$ RPCs but not in all phases of the cell cycle as indicated by the lack of CCND3 and pHH3 co-labeling (data not shown). By P6, CCND3 was expressed strongly in the ONL and INL in a bilayer type pattern. The CCND3^{+} ONL cells were often seen displaced towards dysplastic regions of the ONL that were associated with extensive photoreceptor cell death (Ma et al., 1998) (arrow; Fig 3.2G). Unlike wild type, in the mutant retina, CCND3 was expressed uniformly from center to periphery at P6, with the bilayer appearance evident centrally (data not shown). By P14, the bilayer pattern of CCND3+ cells extended peripherally and these cells co-labeled with markers of mature Müller glia (Fig 3.2H; data not shown).

To determine whether CCND3 was compensating for the loss of CCND1, we generated *Ccnd1*$^{-/-}$, *Ccnd3*$^{-/-}$ double knockout (*DKO*) animals by crossing the two single null strains (Fantl et al., 1995; Sicinska et al., 2003; Sicinski et al., 1995). Due to the high mortality rate of *DKO* neonates (Ciemerych et al., 2002), we analyzed the potential compensatory effect of CCND3 on RPC proliferation and precursor output at P0.

We previously reported that the average cell cycle time of *Ccnd1*$^{-/-}$ RPCs was extended (Das et al., 2009). Therefore, if CCND3 had a role in regulating the cell cycle rate of *Ccnd1*$^{-/-}$ RPCs, the average cell cycle time of *DKO* RPCs would be further increased. To test this hypothesis, we sequentially cultured retinas with BrdU and EdU from P0 *Ccnd1*$^{-/-}$ and *DKO* animals (see Methods). Frozen sections from these cultured retinas were labeled with anti-PCNA to identify RPCs, anti-BrdU antibody to detect BrdU (and EdU), and with a fluroscently tagged-azide to detect EdU through click-iT reaction. The PCNA expression pattern was identical in *DKO* and *Ccnd1*$^{-/-}$ retinas (Fig 3.3A and D) and quantification of PCNA^{+} cells showed that the proportion of RPCs did

not change (data not shown). Determination of average cell cycle time (T_c) of the *Ccnd1*$^{-/-}$ and *DKO* RPC population did not show any significant difference between the two groups either (Fig 3G). Similar results were obtained for S-phase time (T_s), cumulative G1+G2 +M phase time (T_c-T_s), and percent S-phase (T_s/T_c) measurements (Fig 3.3G). These data indicate that CCND3 did not influence RPC cell cycle rate, despite its precocious expression in *Ccnd1*$^{-/-}$ retinas.

Next, we wanted to determine if CCND3 influenced the proportions of neuronal precursor cells in *Ccnd1*$^{-/-}$ retinas. Because CCND3 expression appeared in *Ccnd1*$^{-/-}$ retinas sometime between E14.5 and E17.5 (data not shown), we did not anticipate changes in early retinal cell type precursors in the *DKO* retina. Indeed, expression of retinal ganglion cell precursor marker POU4F2 (Gan et al., 1999; Qiu et al., 2008) (Fig 3.4A and E), horizontal/amacrine cell precursor marker PTF1A (Fujitani et al., 2006; Nakhai et al., 2007) (Fig 3.4B and F), and cone photoreceptor cell precursor marker RXRγ (Mori et al., 2001) (Fig 4C and G) were not different between the two mutants. Further, we did not detect changes in the expression of NR2E3, a precursor marker for rod photoreceptors (Bumsted O'Brien et al., 2004; Chen et al., 2005) (Fig 3.4D and H). Thus, CCND3 does not appear to regulate early or late precursor production from *Ccnd1*$^{-/-}$ RPCs, at least up to P0.

CCND1 inactivation alters the production of Müller glia and bipolar cells

Since CCND3 is normally expressed in Müller glia, the early expression of CCND3 in *Ccnd1*$^{-/-}$ retinas could signify an earlier onset or enhancement in gliogenesis. Having observed a robust number of Müller glia in adult *Ccnd1*$^{-/-}$ retinas (Fig 3.2H) (Ma

et al., 1998), we performed birthdating assays to determine if Müller glial cells were born earlier. Pregnant dams were injected at E18.5 with EdU and retinas were harvested from pups at P14 and examined for evidence of EdU^{+} Müller glia (schema; Fig 3.5). RPCs that exited the cell cycle soon after EdU incorporation are predicted to retain most of the label and appear as the 'brightest' EdU^{+} cells (EdU^{high}). In both wild type and *Ccnd1*$^{-/-}$ retina sections, we observed EdU^{+} cells of varying brightness (Fig 3.5A and D). In the central wild type retina, most of the EdU^{high} cells were in the ONL, consistent with rod photoreceptor production. A few EdU^{high}, $SOX9^{+}$ (Poche et al., 2008) double labeled cells were detected in the INL (normal arrow; Fig 3.5A-C, inset box C' in Fig 3.5C), but most of the double labeled cells exhibited weak EdU labeling (EdU^{low}), suggesting that more than one cell cycle passed before exit (hollow-head arrows in Fig 3.5A-C, inset box C" in Fig 3.5C). All $SOX9^{+}$ cells, regardless of the intensity of EdU detection, expressed Glutamine Synthetase (GS), a marker of Müller glia (Vardimon et al., 1986) (normal arrow in Fig 3.5C' and hollow-head arrows in Fig 3.5C"). In contrast, *Ccnd1*$^{-/-}$ retinas showed a higher occurrence of EdU^{high}, $SOX9^{+}$ cells (normal arrow in Fig 3.5D-F, inset boxes F' and F" in Fig 3.5F) and a lower incidence of EdU^{low}, $SOX9^{+}$ cells (hollow-head arrow in Fig 3.5D-F, inset box F" in Fig 3.5F). As in the wild type, all the double labeled cells expressed GS, confirming their identity as Müller glia (normal arrows in Fig 3.5F' and F" and hollow-head arrow in Fig 3.5F"). These data suggest that the rate of Müller glia production was enhanced in the absence of CCND1.

We also birthdated bipolar cells at the same age using the identical protocol and observed more EdU^{high} cells expressing VSX2 (Burmeister et al., 1996; Green et al., 2003) or PKCalpha (Greferath et al., 1990) in the mutant retinas than in comparable areas

of wild type (data not shown). These data suggest that the general program of late retinal histogenesis was accelerated in the *Ccnd1*$^{-/-}$ mutant.

Late proliferating cells in *Ccnd1*$^{-/-}$ retinas exhibit RPC-like properties

The persistent proliferation in the postnatal *Ccnd1*$^{-/-}$ central retina was unexpected because of the increased rate of RPC depletion. However, since apoptosis is increased postnatally and especially in the ONL (Ma et al., 1998), it is possible that the Müller glia re-entered the cell cycle, a phenomenon observed in several instances of retinal injury and disease (Dyer and Cepko, 2000; Fischer and Reh, 2003; Karl et al., 2008; Kohno et al., 2006; Tackenberg et al., 2009; Thummel et al., 2008). It is also possible that the proliferating cells were RPCs that persisted longer, potentially due to the slower rate of cell cycle progression. Distinguishing between Müller glia and RPCs is difficult because their gene expression profiles are remarkably similar (Roesch et al., 2008). However, proliferative Müller glia typically adopt a reactive phenotype, which among other criteria, is associated with elevated GFAP expression, a protein not found in RPCs (Humphrey et al., 1997; Sahel et al., 1990; Taomoto et al., 1998). Furthermore, RPCs would be expected to produce both neurons and glia given their mulitpotential character, a property not normally observed in mammalian Müller glia (Jadhav et al., 2009; Karl and Reh, 2010).

Based on these operational criteria, we sought to determine the identity of the proliferating cells by examining GFAP expression and by a birthdating assay in which P6 pups were injected with EdU and analyzed for neuronal and glial marker expression at P14 (schema; Fig 3.6). We did not observe GFAP expression at P6 or any marker for glia

maturation (data not shown) at P8. At P14, GFAP expression was mostly confined to regions of focal photoreceptor degeneration (data not shown)**.** In contrast to the wild type retina, which contained EdU^+ cells only in the peripheral retina, EdU^+ cells were found in both central and peripheral mutant retina (Fig 3.6A and B). Importantly, PCNA was not expressed centrally, indicating that the EdU^+ cells were no longer proliferating (data not shown). Some EdU^+ cells were found to be $SOX9^+$ and GS^+, identifying them as Müller glia (Fig 3.6C-F; open arrows) whereas others were SOX9 and GS negative (Fig 3.6C-F; closed arrows). Consistent with the latter cohort, some EdU^+ cells were $VSX2^+$, $PKCA^-$, which are presumptive cone bipolar cells (Fig 3.6G-J; double headed arrow), $VSX2^+$, $PKCA^+$, identifying them as rod bipolar cells (Fig 6G-J; arrowhead). Rare EdU^+ cells were also $RCVRN^+$ in the ONL indicative of photoreceptors (Fig 3.6K-M; open arrow). These observations suggested that the late proliferating cells in *Ccnd1*$^{-/-}$ retinas, being capable of both neurogenesis and gliogenesis, were more likely to be RPCs or cells retaining RPC-like properties.

Discussion

Altered histogenesis in *Ccnd1*$^{-/-}$ retinas

A novel finding of our study was that CCND1 inactivation resulted in the persistence of proliferating cells beyond the normal period of RPC proliferation. This was unexpected because the *Ccnd1*$^{-/-}$ RPC population has a steeper rate of depletion during embryonic development (Das et al., 2009). Indeed, in the postnatal peripheral retina, where proliferation was still ongoing in wild type, comparable areas of the *Ccnd1*$^{-/-}$ retinas were visibly deficient in RPCs and proliferation, consistent with premature cell

cycle exit. But in the central retina, histogenesis, although terminated in the wild type, was still evident in the mutant.

One explanation for the latter observation is that CCND1, in reversal to its' embryonic role, promotes cell cycle exit during postnatal histogenesis. This is highly unlikely, however, given that it is an oncogene and its expression generally promotes cell cycle progression (Liao et al., 2007; Motokura and Arnold, 1993; Sherr, 1995a). For example, CCND1 overexpression in the developing telencephalon enhanced proliferation, due in large part to shortening the duration of G1, and CCND1 RNAi produced the opposite result (Lange et al., 2009; Pilaz et al., 2009). Rather, a more plausible explanation is that RPCs persist in the mutant because of the slower cell cycle rate, and that they continue to proliferate until they reach a certain number of cell cycles or until the extracellular environment no longer supports their proliferation (Fig 3.7). This may appear to be at odds with our previous findings that *Ccnd1*$^{-/-}$ RPCs undergo premature cell cycle exit and that CCND1 is expressed in the vast majority of RPCs (Barton and Levine, 2008; Das et al., 2009). But since the faster depletion rate in the mutant is not severe, it appears that CCND1 influences the timing of cell cycle exit in a small subset of RPCs with limited proliferative potential at any given stage of histogenesis (Das et al., 2009). This restricted mode of exit control, combined with the slower cell cycle rate, could allow for an extended proliferative period. Whether all RPCs ultimately pass through a CCND1-dependent phase to control their timing of cell cycle exit is still an open question.

Our P6 birthdating experiments showed that the late proliferating cells were capable of producing both late-born retinal neurons and Müller glia and suggest that these

cells could be bona-fide RPCs, consistent with the idea of extended histogenesis. However, we can not exclude the possibilities that proliferating Müller glia are intermingled with RPCs or that the dividing cells are not true RPCs but are neurogenic Müller glia, of which there is evidence in other vertebrates and under certain experimental conditions (Fischer and Reh, 2003; Jadhav et al., 2009; Karl and Reh, 2010). The likelihood that some of these cells are proliferating Müller glia is low because we did not detect GFAP expression until several days after P6 and many studies indicate that proliferating Müller glia express GFAP as part of their progression into a reactive state (Humphrey et al., 1997; Sahel et al., 1990; Taomoto et al., 1998). Distinguishing between neurogenic Müller glia and RPCs is difficult because there may be little difference between them other than the cell of origin (Roesch et al., 2008). However, if these neurogenic progenitors do arise from Müller glia, it suggests that preventing the reactivity in otherwise proliferative Müller glia may enhance their neurogenic potential.

We also observed that CCND3 express came on in the *Ccnd1*$^{-/-}$ RPCs prematurely. Interestingly, this early expression of CCND3 was abrogated in mice models where loss of Ccnd1 was rescued to various degrees. Thus, CCND3 expression was not observed in P0 retinas from *Ccnd1*$^{-/-}$, *p27Kip1*$^{-/-}$ double mutant (Geng et al., 2001; Tong and Pollard, 2001) and mouse strains containing the *Ccnd2* cDNA targeted to the *Ccnd1* locus (*Ccnd1*$^{D2/D2}$) (Carthon et al., 2005) and human *Ccne* cDNA targeted to the *Ccnd1* locus (*Ccnd1*$^{hE/hE}$) (Geng et al., 1999) (data not shown). This argues against a direct transcriptional or post-translational control of CCND3 by CCND1, and instead suggests that premature CCND3 expression may be a manifestation of an accelerated developmental program in *Ccnd1*$^{-/-}$ retinas. This is somewhat supported by our current

observations that the initial production of Müller glia, a cell type associated with CCND3 expression, and bipolar cells is heightened in the mutant retina (Fig 3.5 and data not shown). Further, it seems that photoreceptor precursor cells mature faster in *Ccnd1*$^{-/-}$ retinas and show earlier than normal expression of RCVRN, a photoreceptor maturity marker (Sharma et al., 2003), at E17.5 (G.D and E.M.L unpublished observations).

However, adult *Ccnd3*$^{-/-}$ retinas look perfectly identical to wild type, with normal numbers of glia cells. Also, CCND3 does not seem to be required for Müller glia response in certain retinal injury models (G.D, E.M.L, and F.V.Z unpublished observations). This indicates that CCND3 is not required for glia formation or function, at least at our level of analysis. It further suggests that the increased production of glia and bipolar cells observed in *Ccnd1*$^{-/-}$ retinas may not be a consequence of the early activation of CCND3, but rather a direct result of *Ccnd1* deficiency.

Lack of compensation among D-cyclins in retinal development

In other developing tissues, loss of one D-cyclin often results in the upregulation of another compensating D-cyclin (Ciemerych et al., 2002; Cooper et al., 2006; Glickstein et al., 2007; Lam et al., 2000; Satyanarayana and Kaldis, 2009). Although CCND3 is precociously expressed in *Ccnd1*$^{-/-}$ retinas, it did not have any measureable effects on RPC cell cycle rate or precursor production, suggesting that CCND3 does not compensate for CCND1. Further, reports indicate that *Ccnd1*$^{-/-}$, *Ccnd3*$^{-/-}$ knockout RPCs do not see upregulation of *Ccnd2* and that proliferation of *Ccnd1*$^{-/-}$, *Ccnd2*$^{-/-}$, *Ccnd3*$^{-/-}$ triple knockout RPCs were similar to *Ccnd1*$^{-/-}$ null RPCs (Ciemerych et al., 2002; Kozar

et al., 2004). These observations argue against potential compensation by CCND2 in *Ccnd1*$^{-/-}$, *Ccnd3*$^{-/-}$ mutants.

While CCND3 is expressed in neonatal RPCs, it differs from CCND1 in that it is not expressed in all RPCs. This could explain CCND3's failure to compensate for CCND1. However, we also found that CCND2 did not completely restore retinal development when expressed from the CCND1 locus (Das et al., 2009). It is therefore possible that in addition to potential differences in expression, the failure of CCND3 and CCND2 to compensate for the CCND1 deficiency is because CCND1 has functions not shared by the other D-cyclins. However, it would require knocking-in of *Ccnd3* to replace *Ccnd1* expression from its' genomic locus, to conclusively validate whether CCND3 could functionally replace CCND1.

Summary and significance

We studied the role of D-cyclins during postnatal retina development. We found that CCND1 was also required for proper postnatal development of the mouse retina. Unexpectedly, we showed that proliferation persisted in *Ccnd1*$^{-/-}$ retinas even after normal termination of proliferation in wild type. One possibility we outline is that the extended proliferation is a result of gradually extended cell cycle time in mutant RPCs (Fig 3.7). Potentially, injecting cell cycle slowing factors to wild type retinas or cell cycle accelerating factors to postnatal *Ccnd1*$^{-/-}$ retinas can determine whether regulation of cell cycle time is responsible for this phenomenon. Along with our earlier study on CCND1's role during embryonic retinogenesis (Das et al., 2009), this current study highlights two

roles for CCND1 during mammalian retina development: a) to prevent cell cycle exit in a limited set of RPCs and b) to regulate proper cell cycle length in RPCs.

Our work supports the idea that although a D-cyclin like CCND1 may be expressed globally in a tissue compartment, its requirement is restricted to a currently undefined subset of proliferating cells. Our model predicts that, at least in the developing retina and maybe even more globally, this limited subset of proliferating cells is restricted in their proliferative potential, producing upon mitosis a daughter progenitor cell that requires CCND1 to commit to at least another round of mitosis before terminal division.

Materials and methods

Animals

Ccnd1$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *Ccnd3*$^{-/-}$ mice were kindly provided by Dr. Iannis Aifantis (New York University Medical Center, NY) and *p27Kip1*$^{-/-}$ mice by Drs. James Roberts and Matthew Fero (Fred Hutchinson Cancer Center, Seattle, WA). The noon of the day a vaginal plug was observed was designated E0.5. Genotyping was done as previously described (Fero et al., 1996; Sicinska et al., 2003; Sicinski et al., 1995). All animal use and care was conducted in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee and set forth in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals. Efforts were made to minimize discomfort to animals and, when possible, the number of animals needed per analysis was kept to a minimum.

Immunohistochemistry and image analysis

Tissue preparation and immunohistochemistry were done as previously described (Clark et al., 2008; Das et al., 2009). Radial cryosections through the retina were cut at a thickness of 10 μm. Primary antibodies are listed in Table 3.1. Sections were analyzed by epi-fluorescence using a Nikon E-600 microscope and images captured in gray scale mode with a Spot-RT slider CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). Confocal images were scanned using an Olympus Fluoview 1000 microscope. Color (RGB) images were assembled from individual monochrome channels using Photoshop CS (Adobe Systems Inc., San Jose, CA, USA). The levels function was used to adjust the digital images to be consistent with visual observations.

Marker quantification and statistical analysis

The relative proportions of marker-positive (+) cells were quantified at P0. For each genotype, a minimum of three animals from at least two litters was sampled. For each animal, generally three different areas from non-adjacent central-retina sections were used for cell counting. Alternatively, two areas from each section, from opposite sides of the optic nerve head from at least two nonadjacent sections, were counted. All cell counts were done using Adobe Photoshop CS and ImageJ (NIH). Students' t-test was performed using Kaleidagraph statistical and graphing software (Synergy Software, Reading, PA, USA) to determine statistical significance in the marker+ cell population between mutant and control samples. Numbers inside bars on all graphs indicate the number of samples

analyzed. Error bars represent standard deviation. For further details of cell counting protocols refer to (Das et al., 2009).

Window-labeling using BrdU and EdU to measure cell cycle times

Retinas with lens attached were cultured for 2.5 hours and sequentially exposed to two thymidine analogs for defined intervals. At P0, BrdU was added to the culture medium for the first 2 hours and replaced with 5-ethynyl-2′-deoxy-uridine (EdU) for the final 30 minutes. A mouse anti-BrdU antibody(clone B44; BD Biosciences, San Jose, CA, USA) or rat anti-BrdU antibody(clone BU1/75; Serotec, Raleigh, NC, USA) were used to detect the analogs at P0. EdU was specifically detected using the Click-iT Reaction (Molecular Probes, Carlsbad, CA, USA) (Buck et al., 2008). PCNA was used to identify RPCs in all phases of the cell cycle (Barton and Levine, 2008). For further details on how to derive the various parameters of the cell cycle refer to (Das et al., 2009). Cell counts were done as described in the previous section. A more detailed analysis of this assay will appear in a forthcoming manuscript.

Neuronal birthdating assay

Retinal neurons were birthdated by EdU injections at appropriate ages. For late embryonic birthdating (E16.5-E18.5), pregnant mice were injected twice, 4 hours apart with doses of EdU (10 mM stock in H20: 25 mg/gm of body weight injected; equates to 10mL/gm body weight to inject). For postnatal birthdating (P6-P10), each individual animal was injected with a single dose of EdU (10 mM stock in H20: 50 mg/gm of body weight injected; equates to 20mL/gm body weight to inject). All animals were sacrificed and retina tissue was harvested at P14.

To identify a particular class or type of cell born, soon after the EdU injections, sections were co-labeled with Click-iT reaction for EdU detection and antibodies against cell type specific marker(s). A cell co-positive for specific marker(s) and EdU (bright cells), in all likelihood exited the cell cycle or was born, after a single mitotic division of its EdU labeled parent RPC. Because of the importance of identifying varying levels of EdU by fluorescent detection (brightness of the EdU label), all imaging of EdU within a particular experimental and control groups, was performed with identical settings and subsequently, image adjustments were also performed identically on these samples.

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Table 3.1: List of primary antibodies used in this study.

Antigen	Host	Target cells (relevant to this study)	Dilution factor	Source
BrdU	Mouse	Cells that have uptaken BrdU in S-phase	100	BD biosciences
BrdU	Rat	Cells that have uptaken BrdU in S-phase	50-200	Serotec
CCND1	Rabbit	RPCs	400	Lab Vision
CCND3	Rabbit	Müller Glia and *Ccnd1*$^{-/-}$ RPCs	400	Santa Cruz
GS	Mouse	Müller Glia	1000	BD Transduction Laboratories
NR2E3	Rabbit	Rod precursors	100	Anand Swaroop
PCNA	Mouse	RPCs	500	DAKO
pHH3	Rabbit	Mitotic cells	500	Upstate
PKCalpha	Rabbit	Rod Bipolar cells	10,000	Sigma
POU4F2	Goat	RGC precursors	50	Santa Cruz
PTF1A	Rabbit	Amacrine[1] and horizontal precursors subset	800	Helena Edlund
RCVRN	Rabbit	Photoreceptor cells	4000	Chemicon
RXR	Rabbit	Cone precursors and RGCs	200	Santa Cruz
SOX9	Rabbit	RPCs and Müller Glia	400	Alejandro Sanchez
VSX2	Sheep	RPCs and all Bipolar cells	400	Exalpha Biologicals

Figure 3.1: Late, unexpected proliferation in developing *Ccnd1*$^{-/-}$ retinas.
(A-D) Wild-type retinas were labeled with antibody against CCND1 from P0 to P14. CCND1 was expressed in retinal progenitor cells (RPCs) and expressed in the NBL at P0 and P4 (A and B). Eventually, by P6, expression was restricted to the INL, in more central parts of the retina (C). By P14, retinal proliferation was exhausted, reflected in the absence of RPCs, and thus CCND1 expression in the retina (D). **(E-L)** P6 wild type and *Ccnd1*$^{-/-}$ retinas were labeled with antibody against the RPC marker PCNA and were pulse-labeled with EdU, 2 1/2 hours before tissue fixation. Representative field from peripheral (E, I), and central (G, K) wild type and *Ccnd1*$^{-/-}$ retinas. Arrows in E, I, and K point to PCNA expressing mitotic figures. EdU labeled cells in the peripheral (F, J) and central (H, L) wild type and *Ccnd1*$^{-/-}$ retinas. Arrowheads in (L) point to EdU labeled cells in the central mutant retina. **(M-N)** low power view of peripheral-to-central extent of wild type (M) and *Ccnd1*$^{-/-}$ retinas (N), stained with PCNA antibody. Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 100 цm; (D) is representative for (A-D); (K) for (E, G, I and K), (L) for (F, H, J, and L) and (N) for (M, N).

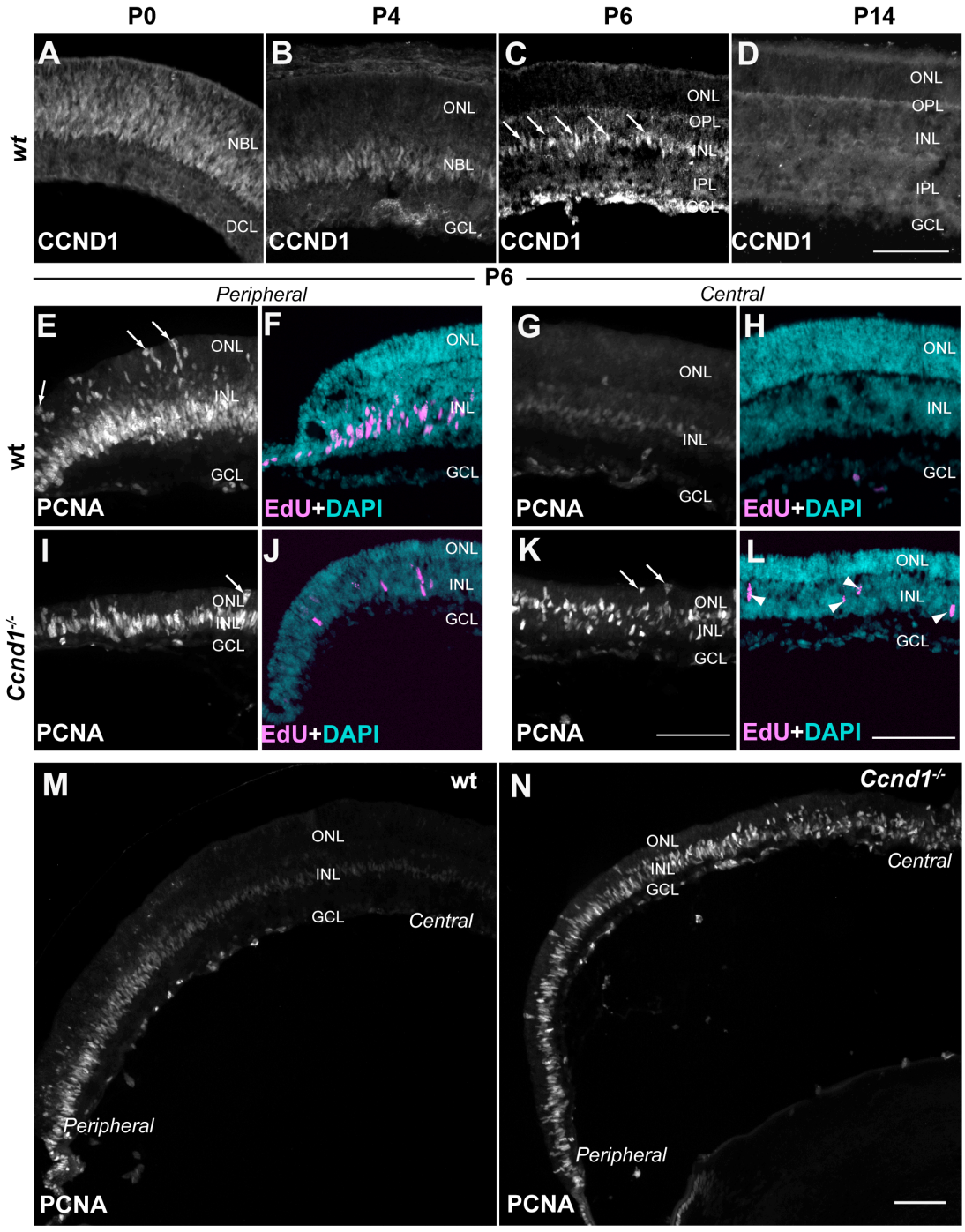

P0
P4
P6
P14
A
B
C
D
wt
NBL
DCL
ONL
NBL
GCL
ONL
OPL
INL
IPL
GCL
CCND1
CCND1
CCND1
CCND1
P6
Peripheral
Central
E
F
G
H
wt
ONL
INL
GCL
ONL
INL
GCL
ONL
INL
GCL
ONL
INL
GCL
PCNA
EdU+DAPI
PCNA
EdU+DAPI
I
J
K
L
Ccnd1-/-
ONL
INL
GCL
ONL
INL
GCL
ONL
INL
GCL
ONL
INL
GCL
PCNA
EdU+DAPI
PCNA
EdU+DAPI
M
wt
N
Ccnd1-/-
ONL
INL
GCL
Central
ONL
INL
GCL
Central
Peripheral
Peripheral
PCNA
PCNA

Figure 3.2: CCND3 was expressed in *Ccnd1*$^{-/-}$ RPCs early in development, in addition to usual latter Müller glia expression.
(**A-H**) Wild type and *Ccnd1*$^{-/-}$ retinas from P0 to P14 were stained with an antibody against CCND3. In wild type retinas, CCND3 expression did not occur until P6 (A-C). CCND3 expression was restricted to the INL at P6 and beyond (C-D). Arrowheads in (C) points to cells expressing low levels of CCND3 in the ONL. CCND3 is expressed earlier in *Ccnd1*$^{-/-}$ retinas (E-F), mainly in the neuroblast layer (NBL). At later stages (G-H; P6 and P14), CCND3 expressing cells are also found in the ONL of *Ccnd1*$^{-/-}$ retinas. Arrow in G points to an area of focal cell death in the mutant retina. Asterisks in (C, D, G, and H) point to blood vessels stained non-specifically by anti-mouse secondary antibody. Scale bars: 100 цm; (D) is representative for (A-D); (H) for (E-H).

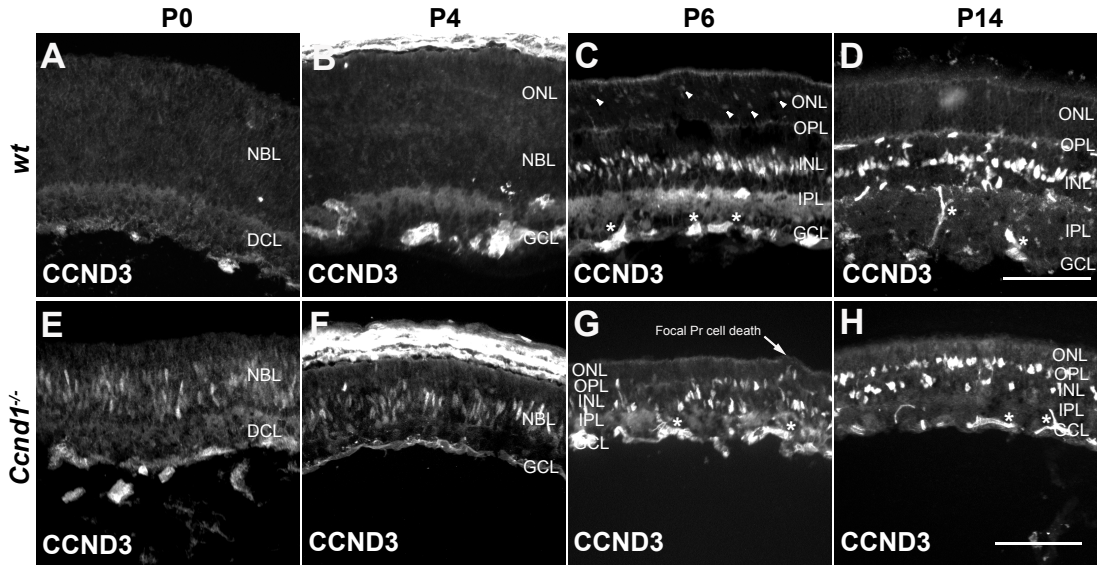

P0
P4
P6
P14
wt
Ccnd1-/-
A
B
C
D
E
F
G
H
NBL
DCL
ONL
OPL
INL
IPL
GCL
Focal Pr cell death
CCND3

Figure 3.3: *Ccnd3* did not regulate cell cycle time of RPCs in *Ccnd1*$^{-/-}$ retinas.
(**A-F**) P0 *Ccnd1*$^{-/-}$ and *Ccnd1*$^{-/-}$, *Ccnd3*$^{-/-}$ retinas cultured successively in Bromodeoxyuridine (BrdU) for 2 hours and 5'-ethynyl-2' deoxyuridine (EdU) for 30 minutes. Sections were triple-labeled with an antibody against PCNA marking RPCs (**A** and **D**), anti-BrdU antibody (Ab) recognizing both BrdU and EdU (**B** and **E**) and an Alexa dye conjugated azide via click-it reaction, for EdU detection (**C** and **F**). Arrows in (A-C) mark $BrdU^+$ only RPCs ($PCNA^+$, $BrdU^+$, EdU^- ; positive signal in A and B but not C) in wild-type retina. Arrows in (D-F) mark similar $BrdU^+$ only RPCs (positive signal in D and E but not F) in the *Ccnd1*$^{-/-}$ retina. **(G)** Quantification of average RPC cell cycle time (T_c), S phase time (T_s), G1 + G2 + M phase time (T_c - T_s) and percent S phase (T_s/ T_c *100) in *Ccnd1*$^{-/-}$ and *Ccnd1*$^{-/-}$, *Ccnd3*$^{-/-}$ retinas at P0. Numbers inside bars represent number of animals analyzed. Scale bar: 100 цm; (F) is representative for (A-F). Error bars represent standard deviation of the mean.

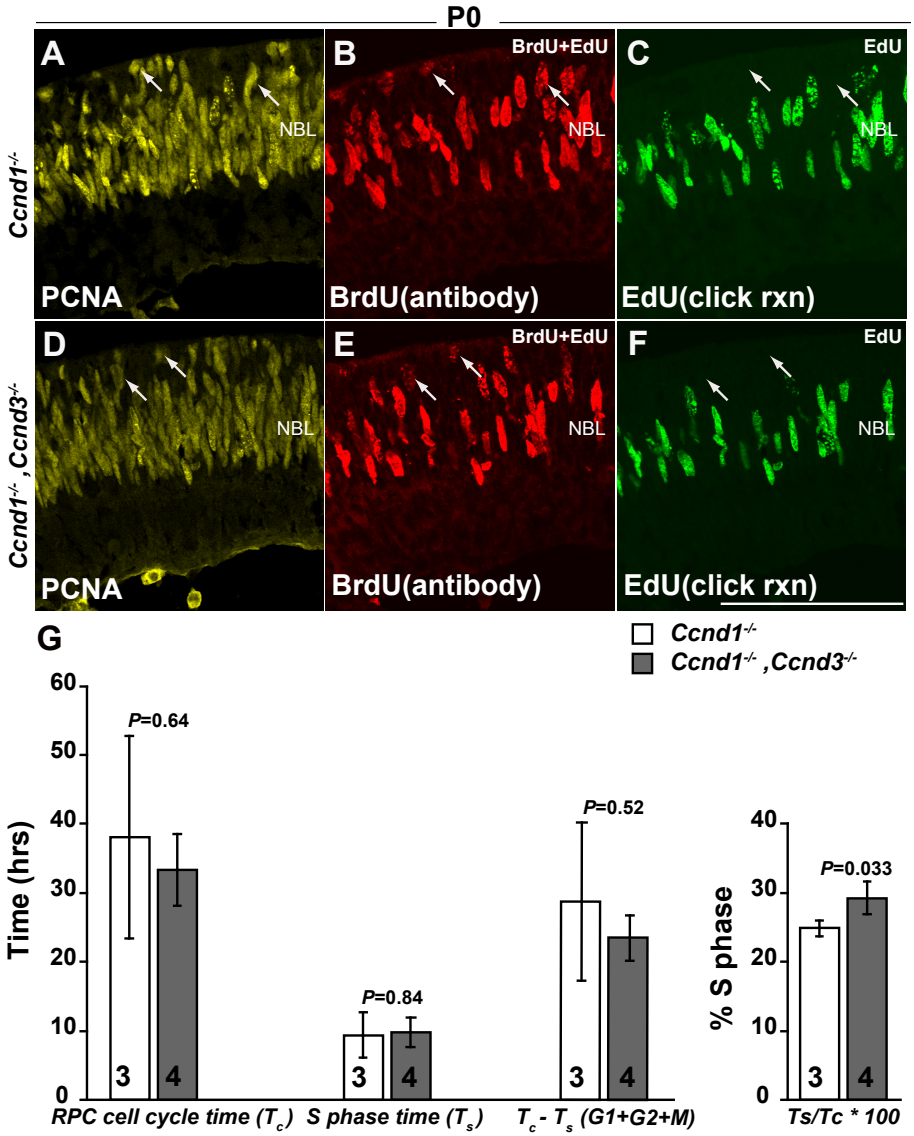

P0
A
B
BrdU+EdU
C
EdU
NBL
PCNA
BrdU(antibody)
EdU(click rxn)
Ccnd1-/-
D
E
F
Ccnd1-/- ,Ccnd3-/-
G
Ccnd1-/-
Ccnd1-/- ,Ccnd3-/-
Time (hrs)
P=0.64
P=0.84
P=0.52
% S phase
P=0.033
RPC cell cycle time (Tc)
S phase time (Ts)
Tc - Ts (G1+G2+M)
Ts/Tc * 100

Figure 3.4: ***Ccnd3*** **did not influence precursor cell type output from RPCs in** ***Ccnd1*** $^{-/-}$ **retinas.**
(A-H) P0 *Ccnd1* $^{-/-}$ and *Ccnd1* $^{-/-}$, *Ccnd3* $^{-/-}$ retinas were stained with antibodies against RGC precursor marker POU4F2 (A and E), horizontal –amacrine precursor marker PTF1A (B and F), cone photoreceptor precursor marker RXRɣ (C and G), and rod photoreceptor precursor marker NR2E3 (D and H). Abbreviation: NBL, neuroblast layer. Scale bar: 100 μm; (H) is representative for (A-H).

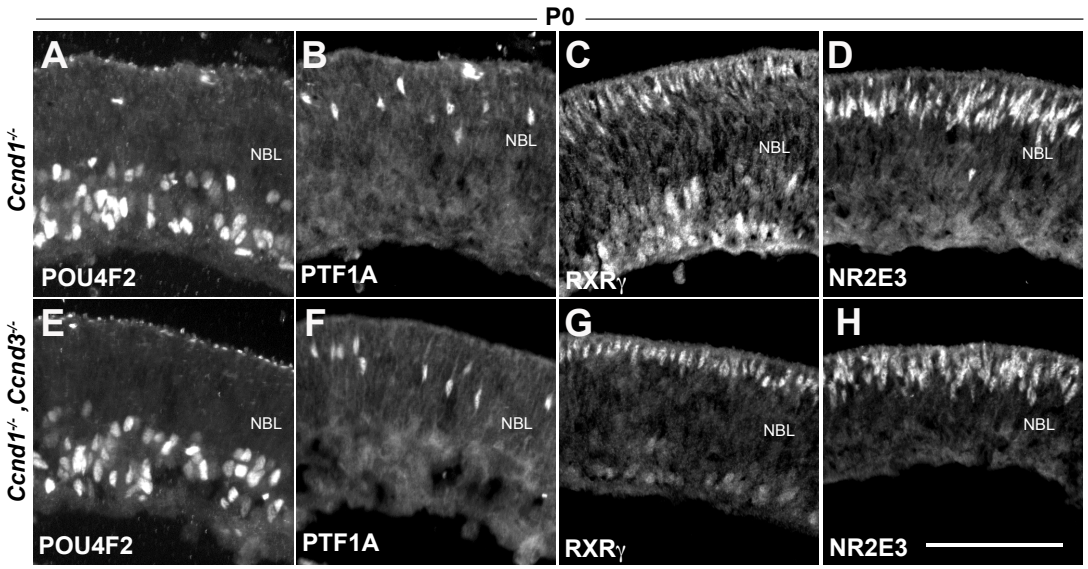

P0
A
B
C
D
NBL
POU4F2
PTF1A
RXRγ
NR2E3
Ccnd1-/-
E
F
G
H
Ccnd1-/-,Ccnd3-/-

Figure 3.5:**Early production of Müller glia cells was enhanced in *Ccnd1*$^{-/-}$ retinas.** Retinal cells were 'birth-dated' at E18.5 by injecting pregnant animals with 2 doses of EdU, 4Hrs apart and retinas were collected at P14 from the litter. (**A-F''**) P14 wild type and *Ccnd1*$^{-/-}$ retina sections were triple labeled with click-it reaction for EdU (A and D), antibody against Müller glia nuclei marker SOX9 (B and E) and antibody against glia maturation marker GS. (C and F) are merged image for EdU and SOX9, and (C', C'', F' and F'') merged images for EdU, SOX9 and GS. Normal head arrows point to 'bright' EdU^{+}, SOX9^{+}, GS^{+} triple labeled cells; these cells, most likely, had exited the cell cycle a cell division or two after incorporating the EdU label (E18.5), to form glial cells. Hollow head arrows point to 'dim' EdU^{+}, SOX9^{+}, GS^{+} triple labeled cells; these cells probably had more than two cell divisions between EdU incorporation and cell cycle exit. Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar in (F) is 100 um and representative for (A-C and D-F); Scale bar in (F'') is 20 um and representative for (C'-C'' and F'-F'').

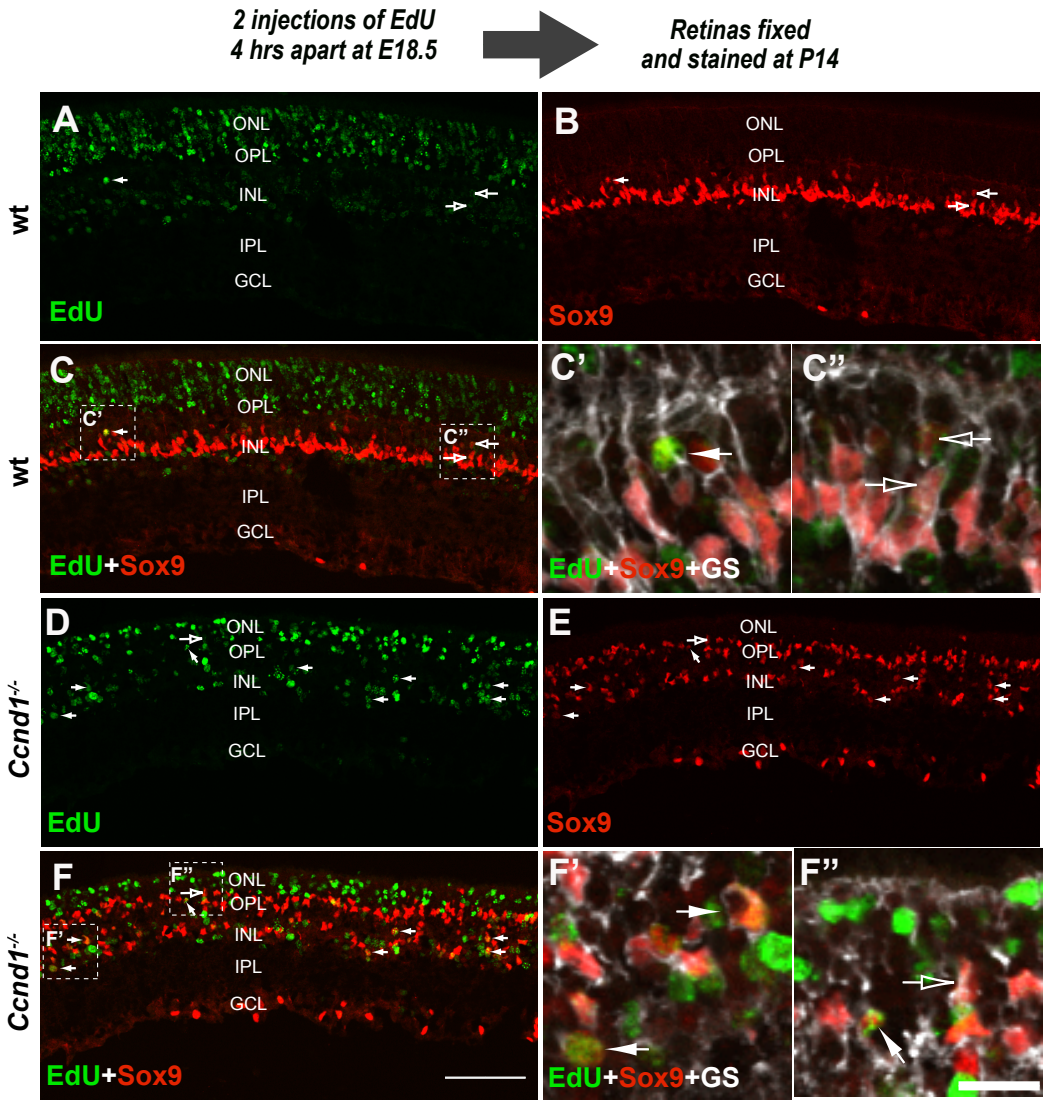

2 injections of EdU
4 hrs apart at E18.5
Retinas fixed
and stained at P14
wt
wt
Ccnd1-/-
Ccnd1-/-
A
B
C
C'
C''
D
E
F
F'
F''
ONL
OPL
INL
IPL
GCL
EdU
Sox9
EdU+Sox9
EdU+Sox9+GS

Figure 3.6: **Late proliferating cells in *Ccnd1*$^{-/-}$ retinas were capable of producing both Müller glia and retinal neurons.**
Retinal cells were 'birthdated' at P6 by a single EdU injection and retinas were collected at P14. (**A-B**) Low power images of P14 wild type and *Ccnd1*$^{-/-}$ retina sections showing EdU labeled cells. (**C-F**) Representative central regions of *Ccnd1*$^{-/-}$ retina sections triple labeled for EdU, SOX9 and GS, for identification of Müller glia cells 'born' after the P6 injection. (F) is a merged image for all three labels. Normal head arrow in (C-F) points to a birthdated non-glia cell (EdU^{+}, SOX9^{-}, GS^{-}). Open head arrows in (C-F) point to a birthdated Müller glia cell (EdU^{+}, SOX9^{+}, GS^{+}). (**G-J**) Representative central regions of *Ccnd1*$^{-/-}$ retina sections tripled labeled for EdU detection, pan-bipolar marker VSX2 and rod bipolar marker PKCA, for identification of cone and rod bipolar cells 'born' after the injection. (J) is a merged image. Double head arrows in (G-J) point to a birthdated cone bipolar cell (EdU^{+}, VSX2^{+}, PKCA^{-}). Arrow heads in (G-J) point to a birthdated rod bipolar cell (EdU^{+}, VSX2^{+}, PKCA^{+}). (**K-M**) P14 central retina section double-labeled for EdU and general photoreceptor cell marker RCVRN, for birthdating photoreceptor cells 'born' after the injection at P6. Hollow head arrows in (K-M) point to a birthdated photoreceptor cell (EdU^{+}, RCVRN^{+}). Abbreviations: DCL, differentiated cell layer; NBL, neuroblast layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar in (B) is 100 um and representative for (A-B); Scale bars in (J and M) are 20 um; (J) is representative for (C-J) and (M) is representative for (K-M).

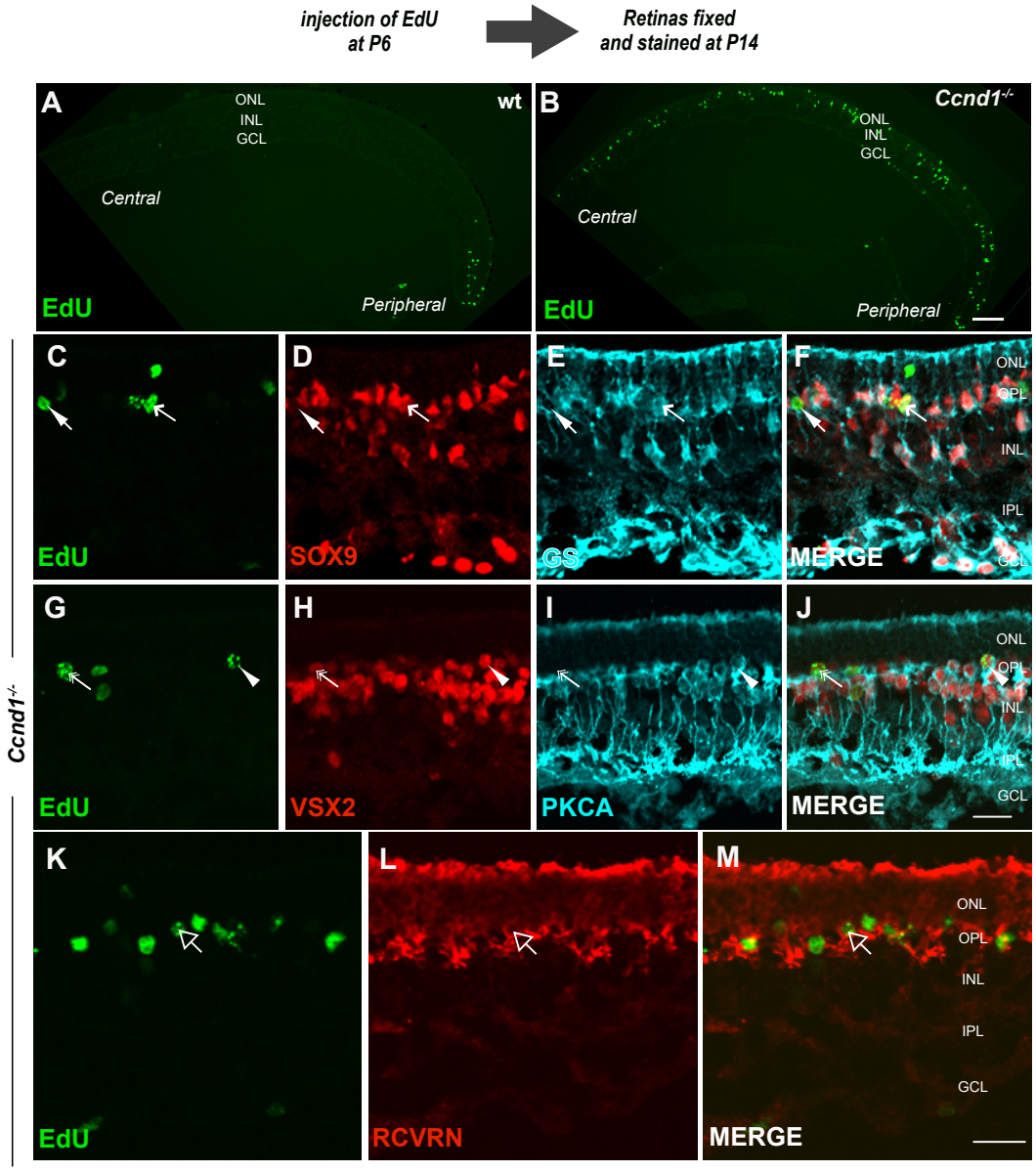
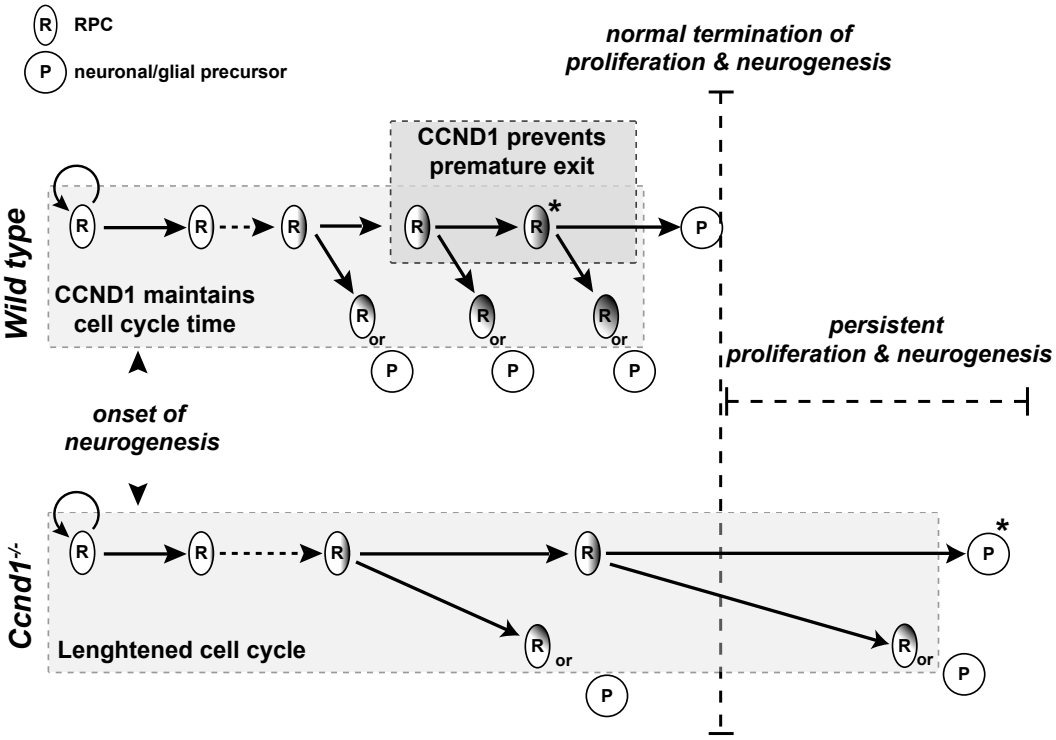

injection of EdU at P6
Retinas fixed and stained at P14
A
wt
ONL
INL
GCL
Central
Peripheral
EdU
B
Ccnd1-/-
ONL
INL
GCL
Central
Peripheral
EdU
C
EdU
D
SOX9
E
GS
F
ONL
OPL
INL
IPL
GCL
MERGE
Ccnd1-/-
G
EdU
H
VSX2
I
PKCA
J
ONL
OPL
INL
IPL
GCL
MERGE
K
EdU
L
RCVRN
M
ONL
OPL
INL
IPL
GCL
MERGE

Figure 3.7: **Model: lengthening cell cycle can lead to extended proliferation, neurogenesis and gliogenesis in *Ccnd1*$^{-/-}$ retinas**

A speculative model as to how increased cell cycle time in *Ccnd1*$^{-/-}$ retinas can lead to extended retinal histogenesis. A set of RPCs restricted in their proliferative potential depends on CCND1 to stay in the cell cycle (dark gray box in wild type). Without it, a *Ccnd1* null RPC that would normally divide again, now prematurely exits from the cell cycle to adopt a precursor fate (asterisk marked cells in wild type and *Ccnd1*$^{-/-}$). A larger set of RPCs is probably dependent on CCND1 to maintain cell cycle time (light gray boxes in wild type and *Ccnd1*$^{-/-}$). Without CCND1, the cell cycle time of mutant RPCs, becomes inappropriately lengthened. These RPCs, not being dependent on CCND1 to stay in the cell cycle, spend a longer time in the cell cycle, and as a result linger in the mutant retina. They eventually exit from the cell cycle to make neurons and glia, even after neurogenesis has ceased in the wild type retina.


R RPC
P neuronal/glial precursor
normal termination of proliferation & neurogenesis
CCND1 prevents premature exit
Wild type
CCND1 maintains cell cycle time
R or
P
persistent proliferation & neurogenesis
onset of neurogenesis
Ccnd1-/-
Lenghtened cell cycle

CHAPTER 4

DISCUSSION

CCND1 regulates cell cycle time of RPCs

Our studies find that without CCND1, the cell cycle time of mutant RPCs is lengthened compared to that of wild type controls and this is probably caused by an increase in G1 phase time. A role for CCND1 and other D-cyclins in controlling the length or time of G1 is supported by studies in other systems as well. Proliferating mouse embryonic fibroblast (MEF) cells from *Ccnd1*$^{-/-}$, *Ccnd2*$^{-/-}$, *Ccnd3*$^{-/-}$ triple knockout mice exhibited an increased proportion of cells in G1, and a decreased proportion of cells in S-phase, indicating that cells spend a longer time in G1 (Kozar et al., 2004). Conversely, overexpression of D-cyclins shortened G1 phase time in MEF cells (Quelle et al., 1993). During mouse cortical neurogenesis, *Ccnd1* knockdown in neural progenitors by *in vivo* RNAi resulted in an elongated G1 phase (Lange et al., 2009; Pilaz et al., 2009). Conversely, overexpression of CCND1 in these progenitors led to a shortened G1 (Lange et al., 2009; Pilaz et al., 2009). In cancer models, oncogenes elevate CCND1 levels to reduce G1 phase time, and in the process, increase S-phase entry and proliferation (Westerheide et al., 2001; Yata et al., 2001).

The length of G1 also varies significantly during embryonic development. The cell cycle during early embryonic development in many organisms have short or almost absent G1 phases (Budirahardja and Gonczy, 2009; Ciemerych and Sicinski, 2005). These early cell cycles consist only of an S phase for DNA replication and an M phase for cell division. They lack both gap phases. Later in murine embryonic development, various cell lineages have varying cell cycle kinetics. This variation is caused by divergent G1 lengths (Ciemerych and Sicinski, 2005). For example, during gastrulation in rat embryos, proliferating cells in the embryonic mesoderm exhibited a cell cycle time of

approximately 7 to 7.5 hours. During the same period, proliferating cells in the primitive streak underwent mitosis every 3-3.5 hours, and had dramatically reduced G1 and G2 phases (Mac Auley et al., 1993). Later in development, during organogenesis (for instance, during retina formation), the cell cycle structure is similar to the typical M-G1-S-G2 organization. Further, with the progression of organogenesis, the overall cell cycle is gradually lengthened. This results mainly from an increase in G1 phase, although in the rat retina, an increase in S phase is also responsible (Alexiades and Cepko, 1996; Caviness et al., 1995).

A parallel situation is observed in murine embryonic stem cells (ESCs), where the length of G1 is much shorter than in somatic progenitor cells (Neganova and Lako, 2008; Savatier et al., 1994). As these ESCs differentiate into specific lineages, the length of the G1 phase undergoes a significant increase (White et al., 2005).

The change in G1 length with development/differentiation has a direct correlation with the status of the retinoblastoma (RB) pathway. In mouse pre-implantation embryos, RB expression was initially observed from immature oocytes to the two-cell stage (Iwamori et al., 2002; Moore et al., 1996). Subsequently, RB transcript and protein expression rapidly disappeared. Expression of RB was re-initiated with the onset of differentiation and implantation of the embryo in the late blastocyst stage (Iwamori et al., 2002). It was observed that the period when RB was absent correlated well with a substantially shortened G1 (Moore et al., 1996; Smith and Johnson, 1986).

In ESCs, RB proteins were held inactive by cell cycle independent kinase activity. With subsequent lineage restriction of ESCs, RB functionality was restored (Stead et al., 2002; White et al., 2005). Together, with the above description of cell cycle kinetics in

ESCs (Neganova and Lako, 2008), this implies that absence or inactivity of RB correlates with an absent/short G1 phase and that establishment of RB expression or function parallels a substantial/longer G1 phase.

In this light, the mechanism of CCND1 mediated control of G1 phase time becomes clearer. CCND1 is an inhibitor of RB function, and a promoter of G1 to S progression. In rapidly dividing cells, such as mouse ESCs, CCND1 plays no role in cell cycle time maintenance because RB is held inactive by D-cyclin independent mechanisms (Stead et al., 2002) and G1 is almost nonexistent. With subsequent activation of the RB pathway during lineage restriction, a D-cyclin like CCND1 is required to ensure progression into S, and to keep G1 from inappropriately elongating by counteracting RB function. In somatic tissues, the central role of the RB pathway in G1 length/time control was highlighted by the finding that pharmacological inhibition of the CyclinE1/Cdk2 complex, another negative regulator of RB activity, resulted in elongated G1 (Calegari and Huttner, 2003).

It is tempting to speculate that CCND1 mediated cell cycle length control may have a crucial role in regulating the window of retinal histogenesis. In Chapter 3, it was reported that in spite of RPC depletion - due to an increase in their rate of cell cycle exit - the period of retinal proliferation and neurogenesis was extended in the *Ccnd1* mutant. An interesting future experiment would be to speed up RPC cell cycle, for instance, by means of CCND1 overexpression, and observe whether retinal histogenesis then terminates earlier. Interestingly, retinas deficient for the CDKI, *p27Kip1*, also had an extended proliferative period (Cunningham et al., 2002; Levine et al., 2000). P27KIP1 acts antagonistically to CCND1 in the retina. Deletion of *p27Kip1* rescued many of the

defects observed in *Ccnd1* null retinas (Das et al., 2009; Geng et al., 2001; Tong and Pollard, 2001). Determination of RPC cell cycle kinetics and the period of retinal histogenesis, in *p27kip1* null and *Ccnd1*, *p27Kip1* double null retinas, can reveal the relationship between these two phenomenons.

CCND1 prevents premature cell cycle exit of RPCs

As reported in Chapter 2, *Ccnd1* controls the timing of cell cycle exit in RPCs. Therefore without CCND1, although a majority of RPCs still undergo cell division, a small but significant number of them prematurely withdraw from the cell cycle. This increases the rate of RPC cell cycle exit during embryonic development. Findings in other experimental systems also support the role of CCND1 in preventing cell cycle exit and some of them are briefly described below.

In C2C12 myoblast cell line, a positive mediator of cell growth NF-kb inhibited myogenic differentiation and induced myoblast proliferation through direct transcriptional activation of *Ccnd1*. Inhibition of NF-kb function led to reduced myoblast proliferation and increased cell cycle exit (Guttridge et al., 1999). A protein called JUMONJI (*jmj*) inhibited *Ccnd1* by direct transcriptional repression in both cardiac myocytes and the hindbrain ventricular zone (VZ) during development. Loss of *jmj* led to hyperproliferation of cardiac myocytes and ectopic proliferation in the hindbrain (Takahashi et al., 2007; Toyoda et al., 2003). Deletion of *Ccnd1* rescued the aberrant phenotypes in both scenarios, indicating a role for *Ccnd1* in regulating cell cycle exit downstream of *jmj* (Takahashi et al., 2007; Toyoda et al., 2003). The orphan nuclear receptor *Tlx* is expressed in periventricular neural progenitor cells during embryonic

mouse development. It regulates cell cycle kinetics and exit by acting on *Ccnd1*. In the absence of *Tlx*, CCND1 expression decreased. This resulted in prolonged cell cycle times and increased cell cycle exit in the embryonic brain (Toyoda et al., 2003). It becomes clear from the above examples that CCND1 is critical for preventing cell cycle exit in dividing cells and as discussed in Chapter 2, it likely does so by regulating the Retinoblastoma (RB) pathway.

Although CCND1 is expressed in all RPCs during development, why are only small fractions of them sensitive to *Ccnd1* loss and exit from the cell cycle prematurely? Results from Chapter 3 indicate that this is not due to compensation by *Ccnd3*; CCND3 does not seem to be expressed in *Ccnd1* null RPCs in the early embryonic retina. CCND3 was not expressed in *Ccnd1* null retinas at E14.5 or before (data not shown). Its expression was first observed at E17.5 in the mutant retina. Even at P0 when *Ccnd3* expression was widespread in *Ccnd1*$^{-/-}$ RPCs, it did not seem to affect either cell cycle length or exit (Fig 3.3 and 3.4).

Before E14.5, CCND1 was expressed robustly in both pre-neurogenic and neurogenic RPCs. Although cell cycle kinetics was not directly measured, no apparent proliferation defects were observed in the *Ccnd1* null pre-neurogenic RPC population. After the onset of neurogenesis, premature cell cycle exit of the mutant RPCs led to overproduction of ganglion cells (Chapter 2).

As *Ccnd1* is a major regulator of cell cycle progression in G1 and an important player in the RB pathway, its requirement may be tied to the setting up of RB protein expression and function in retinal cells. Three RB family members, *RB1*, *p107*, and *p130* are expressed in the developing mouse retina in a dynamic pattern (Donovan et al., 2006;

Spencer et al., 2005). An expression study in the mouse retina reported that none of the RB family members were detected by immunohistochemistry in the undifferentiated neuroblast layer of the embryonic retina (Spencer et al., 2005). The specific stage, however, was not reported and needs to be verified. Nevertheless, this strongly suggests that RB factors are not expressed in pre-neurogenic RPCs. This can be a potential explanation for why CCND1 is not required in pre-neurogenic RPCs to prevent premature cell cycle exit. At E14, P107 is the primary RB protein expressed in approximately 60-77% of RPCs (Donovan et al., 2006). These RPCs extinguished P107 expression as they exited from the cell cycle. However, a short period of P107 expression in newly postmitotic precursor was not ruled out from the study (Donovan-Dyer 2004). Therefore, the expression of CCND1 in embryonic RPCs most likely overlaps with P107 expression. CCND1 is probably required to counteract P107 function in cell cycle exit.

It can be argued that P107 expressing RPCs represent too large a population to be the 'limited RPC-subset' that depends on CCND1 to prevent premature cell cycle exit. However, besides marking the above CCND1-dependent limited subset, the P107 expression pattern may also reflect its role in regulating cell cycle rate/length in RPCs. CCND1 expression may also be required in some or all of these P107 expressing RPCs to maintain proper cell cycle kinetics.

In postnatal stages, RB1 is the primary RB protein expressed in RPCs. Its expression persisted in most postmitotic precursors (Spencer et al., 2005; Zhang et al., 2004). At P5, RB1 expression was seen in most cells of the retina, including a small band of cell in the INL, which can be late RPCs (Spencer et al., 2005). RB1 expression also persisted in Müller glia cells that arise from the same area in the INL (Spencer et al.,

2005; Zhang et al., 2004) (Fig 3.2). This pattern of RB expression correlates nicely with CCND1 expression at the same age (Fig 3.1) and provides possible justification for its requirement during postnatal retina development.

Are G1 length and rate of cell cycle exit related?

CCND1 is required in the G1 phase of RPCs to maintain proper cell cycle time and to prevent premature cell cycle exit. Both requirements seem to be linked to its role as a negative regulator of RB activity. Is it possible that these two aspects of RPC behavior, namely the time they spend in G1 and their terminal exit from the cell cycle at G1, are correlated or even causally related? That is, can an increase in G1 time result in an increased rate of cell cycle exit or vice versa? Although our studies (Chapter 2) show that both of these attributes are increased in the *Ccnd1* null retina, a causal relationship between them was not addressed.

Inhibition of *Ccnd1* or *CcnE* in progenitor cells during cortical development led to longer cell cycle times; additionally, it also led to a larger proportion of these progenitors leaving the cell cycle to enhance neurogenesis (Lange et al., 2009; Pilaz et al., 2009). Conversely, siRNA-mediated knockdown of either *Ccnd1, Ccnd1-Cdk4* complex, or *CcnE*, led to lengthening of G1and depletion of cortical progenitors through enhanced cell cycle exit, thus causing increased neurogenesis. This phenotype is similar to the *Ccnd1* null retina (Lange et al., 2009; Pilaz et al., 2009) (Chapter 2). Further, treatment of whole mouse embryos in culture with olomoucine (a CDK inhibitor) lengthened the cell cycle and led to premature neurogenesis (Calegari and Huttner, 2003). In general, it appears that ‘anti-proliferative’ genes or growth factors that slow the cell cycle increase

neurogenesis. Conversely, when such genes are deleted or when growth factors that accelerate the cell cycle are applied, neurogenesis is reduced (Salomoni and Calegari, 2010).

These observations led to the formation of the cell cycle length hypothesis. This hypothesis predicts that G1 length itself is a determinant of proliferation versus neurogenesis/differentiation decisions (Calegari and Huttner, 2003). It proposes that any increase in G1 time opens a longer time window for a cell fate determinant to act and influence the cell to adopt a different available fate (Calegari and Huttner, 2003; Salomoni and Calegari, 2010). Indeed, as seen by the examples cited earlier, a positive correlation between increased G1 length (cell cycle time) and differentiation is also seen in other stem/progenitor cell systems beside neuroepithelial cells. ESCs usually divide rapidly with a very short G1 phase, but with lineage restriction or differentiation, their G1 and overall cell cycle time is increased (Fluckiger et al., 2006; Neganova and Lako, 2008). Even in a rat model of ischemia or stroke, where adult neurogenesis is promoted, the rapidly dividing proliferating cells initially have a short G1 and low rate of neurogenesis. Later, G1 lengthens, neurogenesis levels climb and eventually reaches pre-stroke normal levels (Zhang et al., 2008; Zhang et al., 2006).

It is important to remember that G1 ‘time’ is probably a reflection of molecular interaction kinetics between positive and negative regulators of G1 progression like CCND1 and RB. It would require further investigation to prove whether the ‘cell cycle length hypothesis’ holds true in the retina. In fact, a couple of studies in the retina suggest the opposite. In the zebrafish mutant *disarrayed*, cell cycle time is greatly increased, but neurogenesis is decreased (Baye and Link, 2007a). In some ways, the *disarrayed* mutant

is similar to the *Vsx2* mutant mouse: they both have hypocellular retinas, lengthened cell cycle time of RPCs, and reduced or unchanged rate of neurogenesis (Levine E.M, unpublished data). Therefore, it is possible that in the developing retina, the 'cell cycle time hypothesis' does not hold true.

However, both the *disarrayed* and *Vsx2* mutant RPCs experience prodigious increases in cell cycle time. In the *disarrayed* mutant, G1 is increased almost one-and-one-half fold, S-phase is increased almost two fold, and overall cell cycle is increased more than two fold (Baye and Link, 2007a). In the *Vsx2* mutant, the increase in cell cycle time is equally large, if not more (Levine E. M; unpublished data). In comparison, the overall increase in cell cycle time is a modest ~50% in *Ccnd1* null RPCs (Fig 2.2). It can be argued that cell cycle rates in *disarrayed* or *Vsx2* mutant retinas are well beyond normal levels during development. Therefore, a wild type RPC at P0 may have cell cycle times that are close to what a *Ccnd1* null RPC had at E14.5 (Fig 2.2). However, wild type RPCs probably never experiences cell cycle times comparable to the *disarrayed* or the *Vsx2* mutant. In fact, the shortest RPC cell cycle in the *disarrayed* mutant (> 16 hours) is longer than the longest RPC cell cycle in the wild type (14.8 hours) (Baye and Link, 2007a). The possibility that the 'cell cycle length' hypothesis in the retina only holds when cell cycle times are within the usual developmental range cannot be formally ruled out without further studies.

CCND1 and cell fate specification in the retina

In the absence of CCND1, changes in the production and number of retinal precursor cells indicate a cellular fate switch phenomenon. So for instance, the excess of

ganglion cell production observed in the early mutant retina could very well be at the expense of cones or PTF1A expressing horizontal/amacrine cell precursors. In Chapter 2, we propose a model where such apparent fate changes could be an indirect effect of mistimed cell cycle exit in the absence of CCND1 (Fig 2.10). Could CCND1 have a direct effect on cell fate? There is ample evidence that CCND1 has cell cycle independent or CDK binding-independent roles. Among other things, CCND1 can repress the transcription of pro-neural or pro-differentiation genes (Coqueret, 2002; Fu et al., 2004). So for instance, CCND1-CDK complexes can inhibit skeletal muscle differentiation by both RB-dependent and independent pathways (Skapek et al., 1996). In epithelial cells of the small intestine, CCND1 acts in the proliferative crypt compartment to transcriptionally inhibit the BHLH factor BETA2/NEUROD in association with the co-activator p300. NEUROD promoted cell cycle withdrawal of epithelial cells to form the differentiated villus compartment (Naya et al., 1995; Ratineau et al., 2002). In the retina, NEUROD is important for amacrine and photoreceptor cell fate specification (Ohsawa and Kageyama, 2008). Modest levels of *NeuroD* mRNA and protein upregulation were observed in *Ccnd1*$^{-/-}$ retinas (Bienvenu et al., 2010; G.D and E.M.L unpublished observations). Whereas *NeuroD* overexpression favored amacrine cell production (Morrow et al., 1999), amacrine cells were selectively reduced in *Ccnd1* mutant retinas (Fig 2.7). However, in both the cases, rod photoreceptor production was enhanced (Morrow et al., 1999). Therefore, it would require careful analysis to determine whether NEUROD actually plays a significant role as a transcriptional target of CCND1 during retina development.

Restricted cellular lineages in the retina

Studies outlined in Chapter 2 convincingly demonstrate that the early increase in ganglion cell production is due to a relative increase in cell cycle exit. However, the loss of other early-born cell types in *Ccnd1* null retinas is harder to explain. It may not be due to increased apoptosis in the mutant retina (Fig 2.12). Two different, not mutually exclusive, possibilities involving roles for CCND1 in cell cycle exit timing and in cell fate was offered (Fig 2.10 and previous section). A third possibility is that horizontal cells and some amacrine cell types arise from lineage-restricted, biased RPCs that are prematurely depleted in the mutant. The situation would be analogous to the loss of cerebellar granule cells or cortical PV^{+}, GABAergic interneurons in $Ccnd1^{-/-}$ or $Ccnd2^{-/-}$ mice (Ciemerych et al., 2002; Glickstein et al., 2007; Huard et al., 1999; Pogoriler et al., 2006). In this scenario, a subset of RPCs with limited cell division and cell fate potential co-exist with the early multipotential RPC population. When these lineage-restricted RPCs prematurely exit the cell cycle, the cell types they were biased to produce are depleted. Further, the complementary branches of multipotential RPC population fail to compensate for the lost cell types.

Existence of such biased or lineage –restricted RPC population, associated with horizontal and amacrine cells, has been shown in multiple species (Alexiades and Cepko, 1997; Godinho et al., 2007; Li et al., 2004; Moody et al., 2000; Rompani and Cepko, 2008). Other studies have shown the presence of rod-committed RPCs that are biased to produce rod photoreceptors (Cayouette et al., 2003; Cohen et al., 2010; Turner and Cepko, 1987; Turner et al., 1990).

Future directions

This work has led to some important questions about the basic mechanisms of retina development and the role of *Ccnd1* in retinal histogenesis. Some potential future directions are briefly discussed below.

Not much is known about cell cycle dynamics during retina development. A relatively simple way to set out in this direction would be to investigate cell cycle kinetics in pre-neurogenic RPCs. Existing literature indicates that these cells express CCND1 (Das et al., 2009) (Fig 1.1) but not RB factors (Spencer et al., 2005). A part of our current hypothesis is that CCND1 is only required in RB expressing RPCs to maintain cell cycle time. Therefore, the prediction would be that the cell cycle time of these RPCs should not change upon deletion of CCND1.

After the onset of neurogenesis, not all RPCs express RB proteins (Donovan et al., 2006; Spencer et al., 2005; Zhang et al., 2004). Another part of our current hypothesis is that RB expression also defines a subset of CCND1-dependent RPCs that exit the cell cycle prematurely in the absence of *Ccnd1*.

Put together, we think that RB expression defines sets of RPCs that rely on CCND1 for proper regulation of cell cycle time and cell cycle exit. Our hypothesis posits that at least two major populations of RPCs exist in the developing retina with significant differences in G1/cell cycle time, based on the status of RB expression. We had also proposed in Chapter 3 (Fig 3.7) that the set of RPCs that depend on CCND1 for cell cycle time regulation is larger than the set of RPCs that require CCND1 to prevent exit. It is possible that besides RB expression, which is common to both sets, expression and utilization of other factors further define these subsets. Therefore, CCND1 may be

required more globally for cell cycle time regulation, but RPCs can utilize other molecules besides CCND1 to regulate cell cycle exit ((Trimarchi et al., 2008). This explains why some RPCs may not exit from the cell cycle prematurely, but may have longer cell cycle times without CCND1.

Live time-lapse imaging in zebrafish retina revealed a large range of cell cycle times of 4 hours to over 11 hours (Baye and Link, 2007b). Likewise, live imaging of the mouse retina will be a direct approach to analyze cell cycle times during development. Conventional modes of cell cycle measurements in fixed tissue using labeled nucleotide analogs may also be able to identify discrete sets of RPCs based on their cell cycle time. Analysis of appropriate genetic models of cell cycle regulators using the above-stated approaches would provide valuable insights on the workings of progenitor cells in the retina.

What are the molecular mechanisms of CCND1's function in the retina? A recent study used a molecularly tagged version of CCND1 to catalog its binding partners and transcriptional targets in the neonatal mouse retina (Bienvenu et al., 2010). *Notch1* was a prominent transcriptional target validated in this study. Its transcript and proteins levels were reduced in the *Ccnd1*$^{-/-}$ mutant (Bienvenu et al., 2010). Previous studies showed that the *Notch1* deletion phenotype in the retina was somewhat similar to the *Ccnd1*$^{-/-}$ retinal phenotype, although the former was much severe than the latter (Das et al., 2009; Jadhav et al., 2006). This indicates that CCND1 mediated regulation of *Notch 1* is somewhat limited. It is probably restricted to a subset of 'exit-prone' RPCs, similar to the CCND1-dependent set described earlier. Additionally, there is plenty of evidence in the literature placing Notch signaling upstream of *Ccnd1* in a wide variety of scenarios ranging from

cancer to development (Campa et al., 2008; Cohen et al., 2009; Ronchini and Capobianco, 2001; Rowan et al., 2008; Tanaka et al., 2009). Therefore, the possibility remains that Notch signaling acts upstream of *Ccnd1* in a cellular context-dependent manner. Understanding of the molecular mechanisms of CCND1 function would require validation of putative binding and transcriptional targets in defined retinal cell populations.

Does CCND1 affect symmetry of division of RPCs? Symmetry of cell division in the retina may play a major role in cell fate and overall histogenesis in the retina (Malicki, 2004). It is not difficult to anticipate a change in symmetry of division without CCND1. Premature cell cycle exit implies that a cell that was destined to stay in the cell cycle now withdraws from it. In the vast majority of cases, this invariably results in a change of division symmetry. A division that would normally produce an RPC and a precursor, in the absence of CCND1, would produce two precursors due to premature cell cycle exit. A recent study combining live imaging of cultured rat RPCs with computational prediction algorithms was able to predict with high accuracy (> 80%) the cellular outcome of RPC division (Cohen et al., 2010). The algorithm 'learned' to associate various visually tracked dynamic characteristics of the pre-mitotic RPC, -like cell shape, size and displacement- with the outcome of division. Ultimately, the algorithm was able to predict the progeny of a live RPC in culture before it divided (Cohen et al., 2010). A similar study with mutant RPCs, lacking CCND1 or other factors, would be able to directly observed progenitor behavior *in vitro,* or in the future, *in vivo*.

Finally, and not of the least importance, would be to study the effect of CCND1 loss in specific retinal lineages. Unfortunately, some of the tools required for such

experiments are not available yet. Foremost amongst them is a conditional mouse allele of *Ccnd1* that can facilitate deletion of *Ccnd1*, from specific retinal cell populations by crossing with retinal lineage specific *Cre* mouse lines (Schweers- Dyer 2005). With a conditional deletion, allele of *Ccnd1* specific questions regarding its role in retinal development can be addressed. This would include temporal deletion of CCND1 during mouse development to study its late role in retina development and degeneration (Ma et al., 1998). *Ccnd1*$^{-/-}$ retinas have defects in retinal vasculature (G.D and E.M.L, unpublished observations). Conditional deletion of *Ccnd1* from endothelial cells or in the retina can potentially address the reasons behind these changes.

Conclusions

Studies in this dissertation were aimed at elucidating the role of D-cyclins in retinal progenitor cell proliferation and neuron production. Further, the goal was to understand how D-cyclins influence these processes by regulation of cell cycle parameters like the duration of RPC cell cycle or the timing of exit of RPCs from the cell cycle. The two major functions for CCND1 that emerges from these studies are to prevent inappropriate increase in RPC cell cycle time and to prevent RPCs from prematurely exiting the cell cycle. CCND1 also regulates the composition of retinal cell populations by altering their output during histogenesis.

These studies were important because they offered insights into mechanisms of retinal development and highlighted the importance of cell cycle regulation during this process. Further, they shed light on fundamental cell cycle properties of dividing stem/progenitor cells. Future studies addressing direct mechanistic connections between

cell cycle kinetics and retinal proliferation/neurogenesis would be both critical and interesting. It may provide clues to producing a desired complement of differentiated cells from a pool of stem/progenitor cells, for specific basic science or therapeutic applications.

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