ROLES OF THE HISTONE METHYLTRANSFERASE

COMPLEX PRC2 DURING RETINAL

DEVELOPMENT IN *XENOPUS*

by

Issam Abdulghani Al Diri

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

Department of Neurobiology and Anatomy

The University of Utah

August 2011

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Issam Abdulghani Al Diri**

has been approved by the following supervisory committee members:

Monica Vetter	, Chair	**03/28/2011**	Date Approved
Alejandro Sanchez Alvarado	, Member	**03/28/2011**	Date Approved
Edward Levine	, Member	**03/28/2011**	Date Approved
Tatjana Piotrowski	, Member	**03/28/2011**	Date Approved
Aloisia Schmid	, Member	**03/28/2011**	Date Approved

and by **Monica Vetter** , Chair of

the Department of **Neurobiology and Anatomy**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

Much has been done to define and characterize the mechanisms that control the
fate of multipotent retinal progenitors during eye development, but our understanding of
this process is still nascent. The histone methyltransferase complex PRC2 is a key
regulator of differentiation during the development of organs such as skin and cortex, but
its roles in vertebrate retinogenesis have not been explored. My work focused on
investigating the possible involvement of PRC2 in the progression of retinal progenitors
from proliferation to differentiation during eye development in *Xenopus laevis* embryos.

In the first chapter, I report the cloning of *Xenopus Suz12*, and determine its
expression pattern during development. *Xsuz12* is provided maternally and its expression
persists as development progresses, particularly in the developing central nervous system.
Comparative analysis of the PRC2 core subunits *Xez*, *Xeed, Xsuz12* and *Xrbbp4* suggests
that their expression largely overlaps in the nervous system.

In the second chapter, I characterize in detail the retinal expression of the PRC2
core subunits, and explore its potential roles during development using a loss of function
approach. I show that the transcripts of the PRC2 core subunits are coincidently
expressed in retinal progenitors and are downregulated upon retinal differentiation.
Surprisingly, I found that the levels of the H3K27me3 mark that is catalyzed by PRC2
greatly increase in terminally differentiated cells. Loss of PRC2 function led to a marked
decrease in H3K27me3 in retinal cell types. Blocking the translation of the core subunits

Xez or *Xsuz12* caused a reduction in eye size, inhibition of differentiation genes and a bias toward generation of late born cell types. ChIP-seq analysis on whole embryos revealed that H3K27me3 transiently and selectively decorates a subset of genes expressed in the eye, some of which are known negative regulators of retinal differentiation.

In the third chapter, I characterize the expression pattern of the newly identified binding partner of PRC2, *Xjarid2*, in the developing nervous system of *Xenopus* and found that it is particularly expressed in differentiated cells. Preliminary loss of function analysis suggests that *Xjarid2* is required for neural differentiation, in agreement with data on PRC2 core subunits. Taken together, my data indicate that the PRC2 complex is an important regulator of retinal neurogenesis in *Xenopus* and highlights the contribution of histone methylation to the regulation of retinal proliferation and differentiation.

To my mom, the greatest fan of science

TABLE OF CONTENTS

ix

ACKNOWLEDGMENTS

My training years at the Department of Neurobiology and Anatomy, University of Utah have been a rewarding educational experience that I will never forget. Making a progress during those years would have been impossible without the help and support of many people.

First and foremost I would like to thank my mentor, Dr. Monica Vetter, for giving me the opportunity to work under her supervision. It has been an honor to learn from her how science works, and I will always admire her modesty and generosity. Additionally, I am grateful to my committee members, Dr. Alejandro Sanchez Alvarado, Dr. Tatjana Piotrowski, Dr. Aloisia Schmid and Dr. Edward Levine, for their helpful suggestions and thoughtful comments, which helped my project to move forward.

Many thanks to the past and present members of the Vetter lab. In particular I really appreciate the help of Dr. Jianmin Zhang with whom I shared lab space with. It was always a gratifying experience to discuss scientific experiments and life experience, in its broadest sense, with him.

Of note, I would like to thank my family for their support during my academic endeavour at the University of Utah. My brothers, Yaser and Hisham, and my sister, Nesreen, have been always encouraging and thoughtful. Above all, I am grateful to my mom who is a fan of science and whose prayers helped me to pursue and finish graduate education.

Finally, I would like to thank my lovely wife, Nora, for her unconditional love and support. She always tried to make life easier around me so that I can focus on my thesis work. During the ups and downs of research work, she has been the shoulder that I cried on. Besides, she gave me the most precious gift of my life: Yara, my daughter.

CHAPTER 1

INTRODUCTION

Overview

During development, neural tissues expand in size and eventually acquire consistent ratios of neurons and glia with dazzling diversity in morphology and function. These cells arise from pools of progenitors as a result of orchestrated developmental events under the control of numerous gene regulatory and signaling processes. Among the different components of the central nervous system, the eye presents an ideal system to study this process as it provides a simple model where the transition from proliferation to differentiation can be easily observed, temporally and spatially (Perron and Harris, 2000). As retinal development progresses, progenitor cells exit the cell cycle and undergo precise execution of a differentiation program through extensive genetic reprogramming that involves the silencing of proliferation genes and the activation of differentiation genes in a step-wise temporal manner.

Recent years have witnessed expansion in our understanding of the genetic networks that contribute to neuron generation and maturation in the retina, as this is a crucial step toward advancing regenerative therapy research for many ocular disorders. Yet, the involvement of known epigenetic mechanisms in the coordination of retinal growth and cell fate acquisition is poorly understood. Data from embryonic stem cell (ESC) studies suggest that the recruitment of chromatin remodelers that work in complexes to mediate global repression or activation of gene expression, and dynamically regulate the structure of the genome and accessibility to the DNA, is essential in regulating the transcription switch from pluripotency/ multipotency to the acquisition of cell type specific features. Hence, it is possible that epigenetic mechanisms are utilized during tissue organogenesis at later stages of development to mediate similar functions.

Indeed, recent studies have demonstrated that the polycomb repressive complex 2 (PRC2), a chromatin remodeling complex that mediates silencing of gene expression, is essential for the proper differentiation of skin, pancreas and vertebrate cortex tissues but its role in eye development remains completely unknown. This work examines the contribution of PRC2 in controlling the neural potential of retinal progenitors and how it may regulate the transition from loss of multipotency to acquiring cell type-specific characteristics during retinal development of *Xenopus laevis*.

Eye Development in Vertebrates

Ocular tissue development is initiated shortly after gastrulation as a result of induction of several key transcription factors that mark the area of the presumptive forebrain (Fig. 1A). These eye-field transcription factors (EFTFs) comprise a network of genes, which includes *Pax6, Rx, Six3, Six6* and *Lhx2*, that have been shown to be important in promoting retinoblast proliferation and subsequent expression of factors essential for proper eye development, such as the proneural basic helix loop helix (bHLH) factors (Marquardt et al., 2001; Agathocleous and Harris, 2009; Willardsen et al., 2009; Bilitou and Ohnuma, 2010). The importance of this network of EFTFs is demonstrated by their ability to induce ectopic eye structures when collectively overexpressed in *Xenopus* embryos (Zuber et al., 2003). As the neural plate starts to fold to form the neural tube, the presumptive eye domain is bilaterally separated into two eye primordia that will emerge from the lateral walls of the developing forebrain at the level of the diencephalon, forming the optic vesicles (Fig. 1A, B). As eye morphogenesis progresses, the optic vesicle further invaginates to form the optic cup. The optic cup

A. Eye field formation and separation in two optic primordia

Eye field

Stage 14   Stage 15   Stage 18
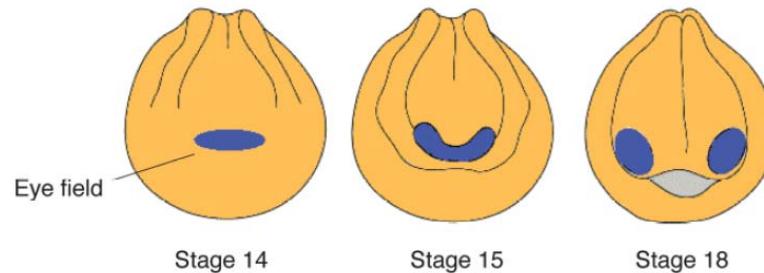
B. Optic vesicle formation and regionalisation of the retina

Ectoderm

Optic vesicle

Optic stalk

Optic cup

Lens vesicle

Neural retina

RPE

Optic nerve

Lens
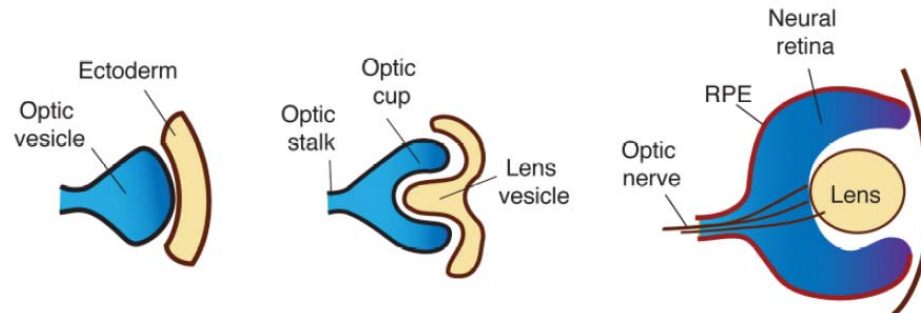
C. Cell fate determination and proliferation

CMZ

stage 15   stage 20   stage 25

stage 35

stage 41

stem cells   actively dividing retinoblasts   retinoblasts at final division   neurons and glial cells
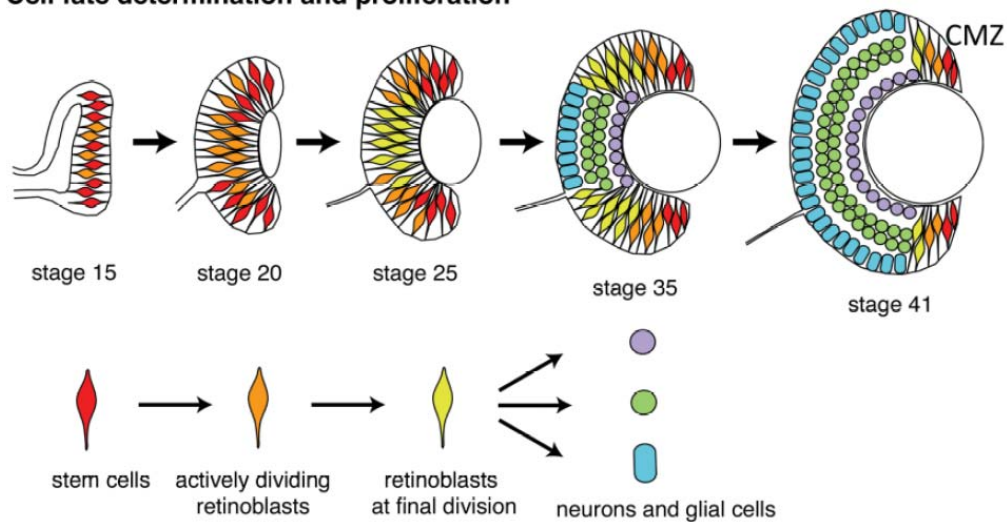
Figure 1. Retinal development. The three major developmental processes in *Xenopus* ocular development are shown. **A**: Eye-field formation and its separation into two optic primordia. **B**: Optic vesicle formation and ocular specification. **C**: Cell-fate determination and cell-cycle regulation in retinal histogenesis. Reprinted with permission from Developmental Dynamics, Bilitou and Ohnuma, 2010, 727-736 volume 239, © 2010, by Wiley-Liss, Inc.

will eventually give rise to the neural retina while the lens is originated from the non-neural ectoderm, the lens placode, which faces the presumptive neural retina due to inductive signals from the retinal neural ectoderm (Chow and Lang, 2001).

Generally, retinal development advances through four major steps: proliferation, cell cycle exit, commitment and differentiation. I will discuses in brief the current understanding of the main aspects of proliferation and differentiation and the major regulatory mechanisms that control these processes.

Proliferation of Retinal Progenitor Cells

Retinal proliferation is essential to expand the tissue and generate sufficient number of cells that will subsequently contribute to different lineages. In humans, reduction of retinal proliferation has been linked to ocular diseases such as microphthalmia and, in severe cases, anophthalmia (Ferda Percin et al., 2000; Fantes et al., 2003; Taranova et al., 2006). Studies on several vertebrates have shown that the speed of proliferation in retinal progenitors changes as development proceeds. Data suggest that initially retinoblasts divide at a lower pace to generate progeny of transit amplifying cells, then division is accelerated around the onset of neurogenesis, producing a mixture of mitotic and postmitotic cells (Alexiades and Cepko, 1996; Harris and Hartenstein, 1991). Finally, proliferation slows down and ceases in differentiating cells as the retina matures (Agathocleous and Harris, 2009; Alexiades and Cepko, 1996).

The regulation of proliferation in ocular tissues has been studied extensively, and has been shown to involve many transcription factors, cell cycle activators (i.e., cyclins and CDKs) and signaling pathways (i.e., Shh and Notch pathways) (Agathocleous et al.,

2007; Barton and Levine, 2008; Ohnuma et al., 2002; Wall et al., 2009). Not surprisingly, EFTFs, which are important for retinal tissue specification, are also regulators of retinal cell division. For instance, overexpression of the transcription factor *Rx* in *Xenopus* enhances retinoblast proliferation and delays retinal neurogenesis while inhibition of its activities leads to a severe reduction in eye size (Andreazzoli et al., 1999; Casarosa et al., 2003). EFTFs mutually activate the expression of each other, and promote proliferation by enhancing the expression of positive cell cycle regulators and by preventing the expression of cell cycle inhibitor genes (Andreazzoli, 2009; Gestri et al., 2005). For instance knockout of *Vsx2* (Chx10) causes microphthalmia due to an abnormal expression of the cell cycle inhibitor *p27kip1* in retinal progenitors (Burmeister et al., 1996; Green et al., 2003). Similarly, overexpression of *Six3* in *Xenopus* inhibits the expression of *p27Xic1* and enhances the expression of the cell cycle activator CyclinD1 (Gestri et al., 2005).

Generation of Rtinal Neurons During Development

As retinal differentiation begins progenitor cells drop out of the cell cycle and acquire the characteristics of retinal-specific cell types. Fully differentiated retinal cell types are functionally and morphologically distinct, and are classified into neurons (ganglion cells, amacrine cells, bipolar cells, horizontal cells, rods and cones) and glia (Müller glia) (Yan et al., 2005). The birth date of these cell types is not random but rather follows a tightly controlled temporal order in a manner that is generally conserved among vertebrates: the first cell type to be born is ganglion cells, followed by horizontal cells, cone photoreceptors and amacrine cells while rods, bipolar cells and Müller glia are

formed later (Livesey and Cepko, 2001; Ohsawa and Kageyama, 2008). Unlike development in the mammalian cortex, different retinal neuronal cell types are born in waves during which the periods of neuron generation overlap considerably (Wang et al., 2002). Hence, retinal cell types are often classified into early born cell types (ganglion cells, cones, amacrine and horizontal cells) and late born cell types (rods, bipolar cells and Müller glia) (Ohsawa and Kageyama, 2008).

Because of its obvious implications in regenerative medicine, the ability of retinal progenitors to generate different retinal cell types has been under intensive study. By examining clones derived from retinoblasts, it has been shown that progenitors can produce all retinal cell types and are thus multipotent (Holt et al., 1988; Turner and Cepko, 1987; Wetts and Fraser, 1988). Experimental evidence suggests that the ability of retinal progenitors to produce different cell types changes as development progresses: early progenitors generate early born cell types while late progenitors produce late born cell types (Andreazzoli, 2009; Livesey and Cepko, 2001). The mechanism that drives this temporal change in retinoblast competence is not well understood but evidence suggests that both environmental cues and intrinsic factors (i.e., transcription factors) contribute significantly to the regulation of this process (Ahmad et al., 1998; Kanekar et al., 1997; Levine et al., 1997). For example, a mouse mutant in the secreted factor GDF11 displays increased number of retinal ganglion cells (RGCs) as a result of prolonged and increased expression of *Math5* in progenitor cells (Kim et al., 2005). *Math5* is an essential transcription factor for the production of ganglion cells (Brown et al., 1998).Thus it is likely that at a given time point during development a progenitor's competence is defined by the cross talk between intrinsic factors temporally expressed by retinoblasts and

signals from the retinal environment that are active at this time point (Livesey and Cepko, 2001; Ohsawa and Kageyama, 2008).

Regulation of Retinal Cell Fate in Vertebrates

Cell fate acquisition is a complex process and involves many intrinsic and extrinsic factors (Levine and Green, 2004). In particular, the bHLH gene family has been shown to play a conserved key role in the regulation of retinal cell type specification. The bHLH group contains members that work as activators to positively regulate neural differentiation (proneural bHLH factors) as well as repressor-type members that inhibit differentiation and promote non-neural cell fate, such as Hes1 and Hes5 (Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008). One of the best studied bHLH molecules is *Ath5*, which is expressed transiently in retinal precursors and positively directs the generation of ganglion cells (Kanekar et al., 1997; Kay et al., 2001; Ohsawa and Kageyama, 2008; Agathocleous and Harris, 2009). Targeted expression of *Ath5* in retinal progenitors promotes ganglion cell formation while loss of *Ath5* results in severe reduction in ganglion cell number and an increase in the number of amacrine cells (Brown et al., 1998; Kanekar et al., 1997; Kay et al., 2001). Interestingly, many members of the proneural bHLH group participate with additional factors to drive the production of particular retina cell types (Akagi et al., 2004; Ohsawa and Kageyama, 2008). For instance, amacrine cells can be generated by the combinatorial expression of *Math3*, *NeuroD* and the homeobox gene *Pax6* while forced expression of *Math3* or *NeuroD* alone promotes rod cell genesis (Hatakeyama et al., 2001; Inoue et al., 2002). Additionally, single knockout of *Math3* does not affect cell fate. However, double

knockout of *Math3* and *Mash1* completely abolish the production of bipolar cells (Tomita et al., 2000). Thus, proneural bHLH factors function through a complex network of transcription factors that drives the genesis of particular cell fates based on the spatial and temporal expression of unique combinations of factors.

Adult Retina in Fish and Amphibians

The mature vertebrate retina contains three distinct layers: the retinal ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer ONL. As the name suggests, the GCL contains RGCs and displaced amacrine cells. The INL is occupied by amacrine cells, bipolar cells, horizontal cells and Müller glia while the ONL contains rod and cone photoreceptors (Ohsawa and Kageyama, 2008). Retinal neurogenesis in amphibians and fish is unique because it persists throughout adulthood, adding more differentiated cells to the retina from its peripheral edge, termed the Ciliary Marginal Zone (CMZ). In *Xenopus* for example, at stage 41 of embryonic development (tadpole stage) differentiated cells occupy the central retina and uncommitted progenitors are maintained at the CMZ as illustrated in Figure 1 (Bilitou and Ohnuma, 2010). Studies have shown that in the CMZ different types of dividing cells are organized in a spatially restricted manner that reflects the sequence of embryonic retinal development: the most peripheral part of the CMZ is occupied by retinal stem cells while proliferative neuroblasts and their progeny are located more centrally (Perron et al., 1998; Agathocleous and Harris, 2009). Thus the mature retina in *Xenopus* provides an advantage to study the expression pattern of genes involved in embryonic retinal

development as it sheds light on temporal changes that occurred in the expression pattern of those genes during development.

Chromatin Structure and Histone Modifications

Nuclear DNA is wrapped around a disc of highly conserved proteins called histones to form the nucleosome, the basic unit of chromatin. Histones are classified into core histones, which contains four members: H2A, H2B, H3 and H4, and are the principle components of the nucleosome, and linker histones (H1), which bind the nucleosome at the cross point of DNA entry/exit sites (Luger et al., 1997; Vignali and Workman, 1998) . Structurally, every nucleosome is an octamer that is composed of two H2A-H2B dimers and a H3-H4 tetramer (Luger et al., 1997).

The state of chromatin structure is essential for regulation of gene expression since it determines accessibility to DNA. Chromatin exists in two dynamic forms: a condensed structure (heterochromatin) that prevents regulators of transcription from accessing and binding DNA, and a loosened form (euchromatin) that permits physical contacts with DNA sequence (Riccio, 2010). These changes in the structure of chromatin are governed in part by post-translational modifications (PTMs) of histones, processes that are mediated by complexes that bind and covalently modify the amino acid side chains of histone tails that are exposed over the surface of the nucleosome. Histone modifications are diverse in nature and include, acetylation of lysines, methylation of arginines and lysines, and phosphorylation of serines and threonines, among others (Berger, 2007). Mechanistically, a histone tail may simultaneously harbor several modifications that collectively form a unique docking site that promotes the recruitment

of distinct protein complexes that subsequently affect chromatin structure and gene expression (Berger, 2007; Turner, 2007).

The correlation between histone modifications and transcription states has been particularly investigated. The current view is that under certain signaling conditions positive- or negative-acting histone PTMs are established on gene promoters which in turn can facilitate recruitment of activators or repressors of gene expression, respectively (Berger, 2007). For instance, a common feature of transcriptionally active promoters is that they are enriched with histone acetylation (Kouzarides, 2007; Turner, 2007). Similarly, trimethylation of lysine 4 in histone 3 (H3K4me3) is enriched on the 5' end of open reading frames and correlates well with transcription activation and is thus considered an activating mark (Berger, 2007; Chi et al., 2010; Turner, 2007). On the other hand, enrichment in modifications such as H3K9me3 and H3K27me3 label silenced genes (Boyer et al., 2006; Lee et al., 2006; Snowden et al., 2002). However, how well histone PTMs can be predictive of the state of transcription remains unclear.

Chromatin Remodeling Enzymes

Histone modifications are catalyzed by highly specialized enzymes that function in multimeric complexes which are specifically recruited to their target sites by DNA binding factors (Riccio, 2010). Examples of these enzymes include histone acetyltransferases (HATs) and histone deacetylases (HDACs) which catalyze the addition and removal of acetyl group to histone lysine residues, respectively. Several enzymes have been identified that promote histone methylation, including the polycomb group protein EZH2 that catalyzes the deposition of H3K27me3 (will be discussed later in

detail), and SUV3-9H1 which is responsible for H3K9me3 addition (Kuzmichev et al., 2002; Zardo et al., 2008). Interestingly, it was initially thought that histone methylation is an irreversible mark (Pedersen and Helin, 2010). However, highly specialized histone demethylases have been recently identified. For instance, the Jumonji C-containing domain proteins UTX and JMJD3 have been shown to specifically and effectively erase H3K27me3 in zebrafish and HeLa cells, respectively (Lan et al., 2007; Xiang et al., 2007). The discovery that H3K27me3 can be actively removed is significant because it implies that this mark can be involved in transient control of gene expression during development, and thus has the potential to play important roles in embryogenesis (Lan et al., 2007).

Polycomb Genes and Development

The polycomb group (PcG) genes are highly conserved factors that were initially identified in Drosophila as repressors of Hox genes during developmental patterning (Sparmann and van Lohuizen, 2006). Mutations in PcG members in Drosophila embryos disrupt the correct spatial and temporal expression pattern of Hox genes in body segmentation, leading to embryonic posteriorization (Ringrose and Paro, 2004). Interestingly, this function is also conserved in vertebrates: mutations in several polycomb factors lead to skeletal malformations as a result of disruption of Hox gene expression (Akasaka et al., 1996; del Mar Lorente et al., 2000). Recent studies have demonstrated that PcGs can mediate silencing of a broader range of genes, and are associated with important biological contexts such as maintenance and differentiation of ESCs, and cancer progression (Boyer et al., 2006; Lee et al., 2006; Schwartz et al., 2006).

How PcGs repress genes is still under intensive study but evidence suggests that they work in complexes that antagonize the function of ATP-dependent nucleosome remodeling by the SWI/SNF complex and by directly preventing transcription initiation (Dellino et al., 2004; Francis and Kingston, 2001). Several highly conserved biochemically and functionally distinct complexes, termed Polycomb Repressive Complexes (PRCs) have been purified including, PRC1 and PRC2 (Martinez and Cavalli, 2006; Margueron and Reinberg, 2011). PRC1 catalyzes the monoubiquitylation of lysine 119 of histone H2A (H2A119ub) while PRC2 has methyltransferase activities and is responsible for H3K27me3 deposition (Kuzmichev et al., 2002; Sawarkar and Paro, 2010). Interestingly, PRC1 binds the PRC2-mediated mark H3K27me3, providing a functional link between both complexes (Fischle et al., 2003). Indeed, PRC1 and PRC2 co-occupy many target genes and both are essential for gene repression (Ringrose, 2007; Margueron and Reinberg, 2011). Evidence suggests that PRC2-mediated trimethylation of K27 facilitates the recruitment of PRC1 to the methylated region, promoting compaction of the chromatin and gene repression (Cao et al., 2005; Spivakov and Fisher, 2007). However this particular recruitment order (PRC2 then PRC1) has not been firmly established (Margueron and Reinberg, 2011). It is noteworthy to mention that the function of PcGs involves catalytic and noncatalytic activities, both of which are required for gene repression (Surface et al., 2010). For the purpose of this thesis further discussion will be limited to PRC2.

The Polycomb Repressive Complex PRC2

The polycomb repressive complex PRC2 consists of four core subunits: SUZ12 (the mammalian orthologue of Suppressor of Zeste Su (z) 12), EZH2 (the mammalian orthologue of Enhancer of Zeste (E (z)), EED (Embryonic Ectoderm Development; the mammalian orthologue of Extra Sex Combs ESC) and Retinoblastoma associated Protein RbAP46/48 (also known as RBBP7/4; the mammalian orthologue of P55) (Fig. 2; Kuzmichev et al., 2002; Margueron and Reinberg, 2011). As mentioned before, EZH2 bears histone methyltransferase activity through its SET domain: mutations in the SET domain cause loss of H3K27 me3 in Drosophila as well as in mammals (Fig. 3; Kuzmichev et al., 2002; Muller et al., 2002; Su et al., 2003). Interestingly, recent studies have identified a version of PRC2 that contains EZH1 instead of its homolog EZH2, and mediates methylation of H3K27 as well (Margueron et al., 2008; Shen et al., 2008). EED is a WD-40 repeat protein that interacts with EZH2 and is required for the EZH2 methyltransferase activity (Ketel et al., 2005; Kuzmichev et al., 2005). It was shown recently that the C-terminal domain of EED interacts with H3K27me3 and is required for further EZH2-mediated methyltransferase activities, providing a mechanism for maintenance and propagation of H3K27me3 (Margueron et al., 2009). SUZ12 contains a zinc finger motif and is also required for EZH2 catalytic activities (Pasini et al., 2004). Together, EZH2, EED and SUZ12 constitute the minimal number of PRC2 subunits required for catalytic activity and subsequent initiation of gene repression (Ketel et al., 2005; Sparmann and van Lohuizen, 2006). Recently, more subunits of PRC2 have been identified including AEBP2 , PCL and JARID2, and have been shown to occupy most of
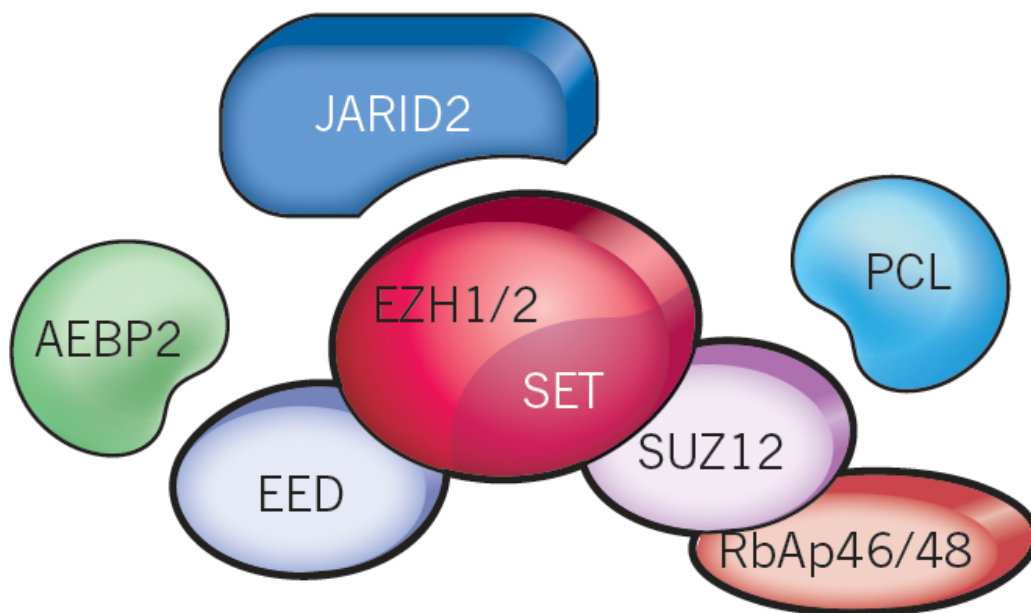
Figure 2. Components of the polycomb complex PRC2. The core components are EZH1/2, SUZ12, EED and RbAp46/48. Other auxiliary partners include PCL, JARID2 and AEBP2. Modified from Margueron and Reinberg, 2011.

PRC2 target genes (Nekrasov et al., 2007; Peng et al., 2009; Shen et al., 2009; Margueron and Reinberg, 2011). The exact function of these components is not well understood, but evidence suggests that while they are not essential for PRC2-mediated catalytic function, their presence optimizes PRC2 enzymatic activities (Margueron and Reinberg, 2011).

Roles of PRC2 and H3K27me3 in Differentiation and Cell Fate Commitment

There has been a great interest in the developmental roles of PRC2 in recent years. Single mouse knockouts of *Suz12, Ezh2* and *Eed* have been generated and found to be lethal at early postimplantation stages, displaying severe developmental and proliferative defects (Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004). Although these mutations demonstrate how essential PRC2 is for development, they prevent further understanding of the tissue-specific roles that PRC2 might play in differentiation and cell fate acquisition.

Important insights into the roles of PRC2 in development came from studies on ESCs. Genome wide analysis of PRC2 targets in ESCs revealed that PRC2 and its mark H3K27me3 occupy the promoters of key developmental regulators that are repressed in ESCs, suggesting a role in the maintenance of ESC pluripotency (Boyer et al., 2006; Lee et al., 2006). However, this role has been questioned in more recent studies (Chamberlain et al., 2008; Surface et al., 2010). ESCs derived from the *Suz12* knockout can be established, but their ability to differentiate in culture is severely compromised, indicating that PRC2 is not strictly required for the maintenance of stem cells

Figure 3. The polycomb complex PRC2 functions. PRC2 recruitment to gene promoters leads to deposition of H3K27me3, which is associated with gene repression. PRC2 is also linked to chromatin compaction.
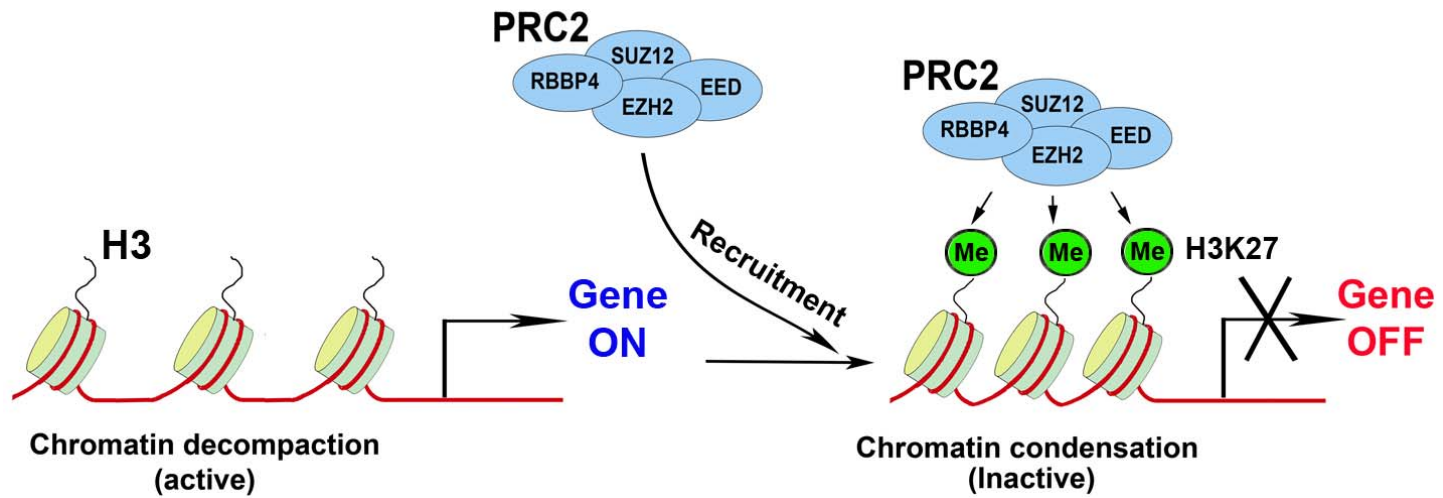
(Pasini et al., 2007). Alternatively, it is proposed that PRC2 has a prominent role in the proper stepwise differentiation of ESCs (Pietersen and van Lohuizen, 2008). In ESCs many ofthe genes involved in differentiation are co-occupied by PRC2 and its repressive mark H3K27me3 and the activating mark H3K4me3, forming a unique status of "bivalent domain" (Bernstein et al., 2006). Upon differentiation, PRC2 occupancy is lost and H3K27me3 is removed while H3K4me3 is maintained, allowing expression of differentiation genes. Thus, the PRC2-mediated repression of the developmental gene promoters occupied with the bivalent domain is transient and seems to prime ESCs for subsequent lineage commitment and cell fate decisions rather than maintaining pluripotency (Landeira et al., 2010; Pietersen and van Lohuizen, 2008; Surface et al., 2010).

Based on published data from ESC differentiation assays, it is predicted that tissue-specific inactivation of PRC2 core components during development should lead to suppression of differentiation and cell fate acquisition (Margueron and Reinberg, 2011). However, observed outcomes from studying the effect of PRC2 mutations on tissue development suggest that PRC2 function is context-specific and depends on the genes being targeted for repression. In blood development for example, a mutation in *Eed* causes a partial block in thymocyte differentiation (Richie et al., 2002). Similarly, inactivation of *Ezh2* in adipose tissue impairs adipocyte differentiation because of an abnormal activation of canonical Wnt signaling, a major inhibitor of adipogenesis (Wang et al., 2010). In contrast, inhibition of *Ezh2* function in the mouse epidermal progenitors results in accelerated skin development due to premature activation of the transcription factor AP-l, which directs the initiation of the late epidermal terminal differentiation

program (Ezhkova et al., 2009; Pirrotta, 2009). In many cases a marked reduction in

tissue cell proliferation is also observed, suggesting an additional role of PRC2 in

controlling the balance between proliferation and differentiation (Chen et al., 2009;

Ezhkova et al., 2009). This is supported by data from cancer studies where upregulation

of *Ezh2* has been linked to different types of malignancies, and is used as a marker for

aggressive prostate and breast cancer (Varambally et al., 2002; Kleer et al., 2003;

Margueron and Reinberg, 2011).

The Roles of PRC2 and H3K27me3 in Neurogenesis

The first glimpse of possible functions of PRC2 in neural differentiation came

from ESCs studies where it has been shown that many of the genes involved in

neurogenesis are targets for PRC2-mediated deposition of H3K27me3 in ESCs (Boyer et

al., 2006; Lee et al., 2006). In line with these data, a null mutation in *Suz12* blocks ESC

ability to differentiate into neurons in culture under certian differentiation conditions

(Pasini et al., 2007). Interestingly, sustained maintenance of H3K27me3 by knocking

down of the H3K27me3-specific demethylase *Jmjd3* is also detrimental to ESC neural

differentiation, further suggesting that dynamic regulation of H3K27me3 deposition is

essential for the proper execution of the differentiation program (Burgold et al., 2008;

Sen et al., 2008).

As data from ESC culture assays were promising, several studies have

investigated the roles of PRC2 in the differentiation of neural progenitors in vivo and in

vitro. In line with studies on non-neural tissue development, the function of PRC2 during

neural development is heavily context-dependent. For instance, in the mammalian cortex,

Ezh2 conditional inactivation results in increased production of neurons at the expense of astrocytes. In this model *Ezh2* restricts the ability of neural progenitors to generate neurons by repressing the expression of the proneural bHLH factor neurogenin1 during the late phase of neocortical development when the time is proper for astrocyte production. Thus *Ezh2* plays a direct role in controlling cell fate switch from neurons to astrocytes (Hirabayashi and Gotoh, 2010; Hirabayashi et al., 2009). A similar model was proposed in postnatal olfactory bulb neurogenesis where H3K27me3 suppresses the expression of neurogenic genes in neural stem cells, thus promoting gliogenesis (Lim et al., 2009). This role is markedly different from *Ezh2* function in differentiation of the mouse embryonic neural stem cells isolated from the telencephalon at E.14 where *Ezh2* appears to control the cell fate choice between oligodendrocytes and astrocytes (Hirabayashi and Gotoh, 2010). Here, while *Ezh2* expression is maintained in oligodendrocytes, downregulation of *Ezh2* expression is essential to promote the production of astrocytes (Sher et al., 2008).

Finally, the roles of PRC2 during eye development have been poorly investigated. A preliminary analysis of the expression pattern of *Ezh2* in the mouse retina suggests that it is expressed during retinal development, mainly in the GCL, and downregulated postnatally (Rao et al., 2010). High levels of H3K27me3 were also observed in the GCL in embryonic retina. However, unlike *Ezh2*, H3K27me3 persists in RGCs during the adult life. In *Xenopus*, *Ezh2* and *Eed* are expressed in the developing CNS, including the eyes, but further investigation of their function during eye organogenesis was not reported (Barnett et al., 2001; Satijn et al., 2001; Showell and Cunliffe, 2002).

The goal of this work was to examine how PRC2 may participate in eye development using *Xenopus laevis* as a model system. In Chapter 2, I describe the cloning of *XSuz12* and investigate its expression pattern, along with the other PRC2 core subunits, in *Xenopus* embryos. The principle conclusion here is that all the major components of PRC2 are coincidently expressed in retinal progenitors during eye development. In Chapter 3, I examine the functional contribution of *Ezh2* and *Suz12* to different aspects of eye development using a loss of function strategy. Additionally, in collaboration with Dr. Gert Veenstra's group, I identify potential targets for the histone methylation mark H3K27me in the retina. The data demonstrate that PRC2 is required for the proper transition from proliferation to differentiation, and is involved in the regulation of important regulators of retinal development, including homeodomain containing genes. In Chapter 4, I explore the expression and the function of the newly identified component of PRC2, *Jarid2* during retinal neurogenesis. The data revealed that *Jarid2* has a distinct expression pattern, as it is abundant in postmitotic retinal neurons rather than progenitors, and is important in the regulation of retinal neurogenesis. Collectively, these findings establish PRC2 as a major player in retinal neurogenesis and suggest that it may have multiple roles during eye development.

References

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

Agathocleous M, Locker M, Harris WA, Perron M. 2007. A general role of hedgehog in the regulation of proliferation. Cell Cycle 6:156-159.

Ahmad I, Dooley CM, Afiat S. 1998. Involvement of Mash1 in EGF-mediated regulation of differentiation in the vertebrate retina. Dev Biol 194:86-98.

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

Andreazzoli M. 2009. Molecular regulation of vertebrate retina cell fate. Birth Defects Res C Embryo Today 87:284-295.

Andreazzoli M, Gestri G, Angeloni D, Menna E, Barsacchi G. 1999. Role of Xrx1 in Xenopus eye and anterior brain development. Development 126:2451-2460.

Barnett MW, Seville RA, Nijjar S, Old RW, Jones EA. 2001. Xenopus Enhancer of Zeste (XEZ); an anteriorly restricted polycomb gene with a role in neural patterning. Mech Dev 102:157-167.

Barton KM, Levine EM. 2008. Expression patterns and cell cycle profiles of PCNA, MCM6, cyclin D1, cyclin A2, cyclin B1, and phosphorylated histone H3 in the developing mouse retina. Dev Dyn 237:672-682.

Berger SL. 2007. The complex language of chromatin regulation during transcription. Nature 447:407-412.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315-326.

Bilitou A, Ohnuma S. 2010. The role of cell cycle in retinal development: cyclin-dependent kinase inhibitors co-ordinate cell-cycle inhibition, cell-fate determination and differentiation in the developing retina. Dev Dyn 239:727-736.

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441:349-353.

Brown NL, Kanekar S, Vetter ML, Tucker PK, Gemza DL, Glaser T. 1998. Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development 125:4821-4833.

Burgold T, Spreafico F, De Santa F, Totaro MG, Prosperini E, Natoli G, Testa G. 2008. The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. PLoS One 3:e3034.

Burmeister M, Novak J, Liang MY, Basu S, Ploder L, Hawes NL, Vidgen D, Hoover F, Goldman D, Kalnins VI, Roderick TH, Taylor BA, Hankin MH, McInnes RR. 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat Genet 12:376-384.

Cao R, Tsukada Y, Zhang Y. 2005. Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol Cell 20:845-854.

Casarosa S, Amato MA, Andreazzoli M, Gestri G, Barsacchi G, Cremisi F. 2003. Xrx1 controls proliferation and multipotency of retinal progenitors. Mol Cell Neurosci 22:25-36.

Chamberlain SJ, Yee D, Magnuson T. 2008. Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells 26:1496-1505.

Chen H, Gu X, Su IH, Bottino R, Contreras JL, Tarakhovsky A, Kim SK. 2009. Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. Genes Dev 23:975-985.

Chi P, Allis CD, Wang GG. 2010. Covalent histone modifications--miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer 10:457-469.

Chow RL, Lang RA. 2001. Early eye development in vertebrates. Annu Rev Cell Dev Biol 17:255-296.

Dellino GI, Schwartz YB, Farkas G, McCabe D, Elgin SC, Pirrotta V. 2004. Polycomb silencing blocks transcription initiation. Mol Cell 13:887-893.

Ezhkova E, Pasolli HA, Parker JS, Stokes N, Su IH, Hannon G, Tarakhovsky A, Fuchs E. 2009. Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell 136:1122-1135.

Fischle W, Wang Y, Jacobs SA, Kim Y, Allis CD, Khorasanizadeh S. 2003. Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev 17:1870-1881.

Francis NJ, Kingston RE. 2001. Mechanisms of transcriptional memory. Nat Rev Mol Cell Biol 2:409-421.

Gestri G, Carl M, Appolloni I, Wilson SW, Barsacchi G, Andreazzoli M. 2005. Six3 functions in anterior neural plate specification by promoting cell proliferation and inhibiting Bmp4 expression. Development 132:2401-2413.

Green ES, Stubbs JL, Levine EM. 2003. Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. Development 130:539-552.

Harris WA, Hartenstein V. 1991. Neuronal determination without cell division in Xenopus embryos. Neuron 6:499-515.

Hirabayashi Y, Gotoh Y. 2010. Epigenetic control of neural precursor cell fate during development. Nat Rev Neurosci 11:377-388.

Hirabayashi Y, Suzki N, Tsuboi M, Endo TA, Toyoda T, Shinga J, Koseki H, Vidal M, Gotoh Y. 2009. Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. Neuron 63:600-613.

Holt CE, Bertsch TW, Ellis HM, Harris WA. 1988. Cellular determination in the Xenopus retina is independent of lineage and birth date. Neuron 1:15-26.

Kanekar S, Perron M, Dorsky R, Harris WA, Jan LY, Jan YN, Vetter ML. 1997. Xath5 participates in a network of bHLH genes in the developing Xenopus retina. Neuron 19:981-994.

Kay JN, Finger-Baier KC, Roeser T, Staub W, Baier H. 2001. Retinal ganglion cell genesis requires lakritz, a Zebrafish atonal Homolog. Neuron 30:725-736.

Kim J, Wu HH, Lander AD, Lyons KM, Matzuk MM, Calof AL. 2005. GDF11 controls the timing of progenitor cell competence in developing retina. Science 308:1927-1930.

Kouzarides T. 2007. Chromatin modifications and their function. Cell 128:693-705.

Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. 2002. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev 16:2893-2905.

Lan F, Bayliss PE, Rinn JL, Whetstine JR, Wang JK, Chen S, Iwase S, Alpatov R, Issaeva I, Canaani E, Roberts TM, Chang HY, Shi Y. 2007. A histone H3 lysine 27 demethylase regulates animal posterior development. Nature 449:689-694.

Landeira D, Sauer S, Poot R, Dvorkina M, Mazzarella L, Jorgensen HF, Pereira CF, Leleu M, Piccolo FM, Spivakov M, Brookes E, Pombo A, Fisher C, Skarnes WC, Snoek T, Bezstarosti K, Demmers J, Klose RJ, Casanova M, Tavares L, Brockdorff N, Merkenschlager M, Fisher AG. 2010. Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. Nat Cell Biol 12:618-624.

Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, Young RA. 2006. Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125:301-313.

Levine EM, Roelink H, Turner J, Reh TA. 1997. Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells in vitro. J Neurosci 17:6277-6288.

Livesey FJ, Cepko CL. 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci 2:109-118.

Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251-260.

Margueron R, Li G, Sarma K, Blais A, Zavadil J, Woodcock CL, Dynlacht BD, Reinberg D. 2008. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Mol Cell 32:503-518.

Margueron R, Reinberg D. 2011. The Polycomb complex PRC2 and its mark in life. Nature 469:343-349.

Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA. 2002. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111:197-208.

Ohnuma S, Hopper S, Wang KC, Philpott A, Harris WA. 2002. Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the Xenopus retina. Development 129:2435-2446.

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

Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. 2007. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol 27:3769-3779.

Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E, Helin K. 2004. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J 23:4061-4071.

Pedersen MT, Helin K. 2010. Histone demethylases in development and disease. Trends Cell Biol 20:662-671.

Pietersen AM, van Lohuizen M. 2008. Stem cell regulation by polycomb repressors: postponing commitment. Curr Opin Cell Biol 20:201-207.

Pirrotta V. 2009. Polycomb repression under the skin. Cell 136:992-994.

Riccio A. 2010. Dynamic epigenetic regulation in neurons: enzymes, stimuli and signaling pathways. Nat Neurosci 13:1330-1337.

Richie ER, Schumacher A, Angel JM, Holloway M, Rinchik EM, Magnuson T. 2002. The Polycomb-group gene eed regulates thymocyte differentiation and suppresses the development of carcinogen-induced T-cell lymphomas. Oncogene 21:299-306.

Ringrose L, Paro R. 2004. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu Rev Genet 38:413-443.

Satijn DP, Hamer KM, den Blaauwen J, Otte AP. 2001. The polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in Xenopus embryos. Mol Cell Biol 21:1360-1369.

Sawarkar R, Paro R. 2010. Interpretation of developmental signaling at chromatin: the Polycomb perspective. Dev Cell 19:651-661.

Schwartz YB, Kahn TG, Nix DA, Li XY, Bourgon R, Biggin M, Pirrotta V. 2006. Genome-wide analysis of Polycomb targets in Drosophila melanogaster. Nat Genet 38:700-705.

Sen GL, Webster DE, Barragan DI, Chang HY, Khavari PA. 2008. Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. Genes Dev 22:1865-1870.

Shen X, Liu Y, Hsu YJ, Fujiwara Y, Kim J, Mao X, Yuan GC, Orkin SH. 2008. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell 32:491-502.

Sher F, Rossler R, Brouwer N, Balasubramaniyan V, Boddeke E, Copray S. 2008. Differentiation of neural stem cells into oligodendrocytes: involvement of the polycomb group protein Ezh2. Stem Cells 26:2875-2883.

Showell C, Cunliffe VT. 2002. Identification of putative interaction partners for the Xenopus Polycomb-group protein Xeed. Gene 291:95-104.

Snowden AW, Gregory PD, Case CC, Pabo CO. 2002. Gene-specific targeting of H3K9 methylation is sufficient for initiating repression in vivo. Curr Biol 12:2159-2166.

Sparmann A, van Lohuizen M. 2006. Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846-856.

Spivakov M, Fisher AG. 2007. Epigenetic signatures of stem-cell identity. Nat Rev Genet 8:263-271.

Su IH, Basavaraj A, Krutchinsky AN, Hobert O, Ullrich A, Chait BT, Tarakhovsky A. 2003. Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. Nat Immunol 4:124-131.

Surface LE, Thornton SR, Boyer LA. 2010. Polycomb group proteins set the stage for early lineage commitment. Cell Stem Cell 7:288-298.

Turner BM. 2007. Defining an epigenetic code. Nat Cell Biol 9:2-6.

Turner DL, Cepko CL. 1987. A common progenitor for neurons and glia persists in rat retina late in development. Nature 328:131-136.

Wall DS, Mears AJ, McNeill B, Mazerolle C, Thurig S, Wang Y, Kageyama R, Wallace VA. 2009. Progenitor cell proliferation in the retina is dependent on Notch-independent Sonic hedgehog/Hes1 activity. J Cell Biol 184:101-112.

Wang SW, Mu X, Bowers WJ, Klein WH. 2002. Retinal ganglion cell differentiation in cultured mouse retinal explants. Methods 28:448-456.

Wetts R, Fraser SE. 1988. Multipotent precursors can give rise to all major cell types of the frog retina. Science 239:1142-1145.

Xiang Y, Zhu Z, Han G, Lin H, Xu L, Chen CD. 2007. JMJD3 is a histone H3K27 demethylase. Cell Res 17:850-857.

Yan RT, Ma W, Liang L, Wang SZ. 2005. bHLH genes and retinal cell fate specification. Mol Neurobiol 32:157-171.

Zardo G, Cimino G, Nervi C. 2008. Epigenetic plasticity of chromatin in embryonic and hematopoietic stem/progenitor cells: therapeutic potential of cell reprogramming. Leukemia 22:1503-1518.

Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development 130:5155-5167.

CHAPTER 2

CHARACTERIZATION OF THE EXPRESSION PATTERN OF THE

PRC2 CORE SUBUNIT *SUZ12* DURING EMBRYONIC

DEVELOPMENT OF *XENOPUS LAEVIS*

Abstract

The Polycomb repressive complex 2 is a multimeric aggregate that mediates silencing of a broad range of genes, and is associated with important biological contexts such as stem cell maintenance and cancer progression. PRC2 mainly trimethylates lysine 27 of histone H3 and is composed of three essential core subunits: EZH2, EED and SUZ12. The *Xenopus* orthologs of PRC2 subunits *Ezh2* and *Eed* have been described but *Suz12* remained unidentified. Here, we report the cloning of the *Xenopus Suz12*, and determine its spatiotemporal expression during development. *Xsuz12* transcript is provided maternally and continues to be expressed throughout development, particularly in the anterior part of the developing central nervous system. Importantly, comparative analysis of the expression of the PRC2 subunits *Xez*, *Xeed* and *Xrbbp4* indicates that their expression largely coincides with *Xsuz12* in the nervous system, suggesting that PRC2 may have unexplored functions in the development of the frog central nervous system.

Introduction

The polycomb group (PcG) genes are highly conserved factors that were initially identified in *Drosophila* as repressors of Hox genes during developmental patterning (Sparmann and van Lohuizen, 2006). Studies have demonstrated that PcG-mediated repression is not restricted to Hox genes, and has been implicated in the biology of embryonic stem cells and cancer (Boyer et al., 2006; Lee et al., 2006; Schwartz et al., 2006). How PcGs repress gene expression is not well understood but evidence suggests that they work in complexes that can directly interfere with transcription initiation or antagonize the function of the chromatin remodeling complex SWI/SNF ((Dellino et al.,

2004; Francis and Kingston, 2001). Three complexes with distinct biochemical and functional properties, termed Polycomb Repressive Complexes (PRCs), have been purified thus far: PRC1, PRC2 and PHORC ((Saurin et al., 2001; Shao et al., 1999; Sparmann and van Lohuizen, 2006).

The polycomb repressive complex PRC2 consists of three core subunits: *Suz12* (the mammalian ortholog of the *Drosophila* Suppressor of Zest *Su(z)12*), *Ehz1/2* (the mammalian orthologs of the *Drosophila* Enhancer of zeste *E(z)*) and *Eed* (the mammalian ortholog of the *Drosophila* Extra Sex Combs *Esc*) (Kuzmichev et al., 2002). EZH2 bears a histone methyltransferase (HMTase) activity through its SET domain that catalyzes the trimethylation of H3K27 in vitro as well as in vivo (Kuzmichev et al., 2002; Muller et al., 2002; Su et al., 2005). H3K27 is a histone mark that is associated with the maintenance of gene repression in multiple developmental processes (Barski et al., 2007; Boyer et al., 2006; Schuettengruber et al., 2007). EZH2 can also interact with DNA methyltransferases thus directly controlling DNA methylation (Vire et al., 2006). The exact functions of SUZ12 and EED are largely unknown, but both proteins are required for the EZH1/2 methyltransferase activity (Ketel et al., 2005; Kuzmichev et al., 2005; Margueron et al., 2008; Pasini et al., 2004; Shen et al., 2008). EZH2, EED and SUZ12 constitute the minimal PRC2 subunits required for HMTase activity and subsequent initiation of gene repression(Ketel et al., 2005; Sparmann and van Lohuizen, 2006). PRC2 also contains other subunits that have been shown to be important for its function. For instance, the *Drosophila* NURF55 and its vertebrate ortholog RbAP48 (also named RBBP4) enhances the catalytic activity of PRC2 and is required for its nucleosome binding (Cao and Zhang, 2004; Nekrasov et al., 2005).

The roles of SUZ12 in development have been poorly explored in part because the mouse knockout of *Suz12* suffers severe developmental abnormalities and dies shortly after gastrulation (Pasini et al., 2004). One of the emerging roles of *Suz12* is its involvement in the mammalian embryonic stem (ES) cell pluripotency. In ES cells SUZ12 mediates repression of a large set of developmental genes that are implicated in differentiation and cell fate decisions (Boyer et al., 2006; Lee et al., 2006). In agreement with these findings, ES cells derived from mouse *Suz12* knockout display a loss of H3K27me3 mark and upregulation of differentiation-specific genes, impairing ES cell differentiation in culture (Pasini et al., 2007).

To study the possible roles of PRC2 in *X. laevis* development it is necessary to characterize all subunits that are important for the complex to function properly. To date only two of the core subunits (*Xez* and *Xeed*) have been cloned in *X. laevis* and their expression patterns have been only partially explored. Thus, cloning the *Suz12* gene and comparing its expression pattern to other PRC2 subunits in *Xenopus* is essential for understanding how this complex may function in proliferation, tissue specification and/or subsequent differentiation throughout the frog development. In this study we report the cloning of the X. *laevis* ortholog of *Suz12* (named *Xsuz12* hereafter) and characterize its spatiotemporal expression during development.

Methods and Materials

Cloning of *Xsuz12*

Primers were designed based on available *X. laevis Suz12* ESTs found in the NCBI database (BU907606.1 and CA791061.1). The following primers were used to

amplify *Suz12* from a cDNA library prepared from whole embryos at stage 17-18:

forward, 5'- TAATTACCCCGTATGGCC- CCTCAGAAGCAC-3', reverse, 3'-

ACACAGCAAAAAGCAGAAGCCCTGAAGG-5'. PCR was performed using Pfu turbo

polymerase (Stratagene), and a band of 2.1 kb, corresponding to the predicted molecular

weight of *XSuz12* full length cDNA (as compared to the molecular weight of its *X.*

tropicalis orthologue) was isolated and sequenced by the University of Utah Sequencing

Core Facility. Final *Xsuz12* sequence was submitted to GenBank (accession number

FJ905047). qRT-PCR and data analyses were performed as was previously described

(Logan et al., 2005b).

Alignment and phylogenetic analyses were performed using MacVector and CLC

Sequence Viewer software (http://www.clcbio.com/index.php?id=28), respectively.

Neighbor-joining algorithm was used to construct SUZ12 phylogenetic tree, and

reproducibility of branching points was determined by performing 100 bootstrap

reiterations. EF1α was used as a loading control for PCR, using the following primers,

forward, 5ʹ-CAGATTGGTGCTGGATA- TGC-3ʹ; reverse, 5ʹ-

ACTGCCTTGATGACTCCTAG-3ʹ.

In situ hybridization

Digoxigenin-labeled antisense probes that span the first 700 bp and 744 bp of

Xsuz12 and *Xrbbp4* cDNA, respectively, were transcribed using T7 RNA polymerase

after plasmid linearization. *Xrbbp4* probe was synthesized from a full length cDNA clone

(accession number BC077257) that was purchased from Open Biosystems. *Xez and Xeed*

probes were generous gifts from Dr Elizabeth A. Jones and Dr Vincent T. Cunliffe,

respectively. Embryos were fixed with 4% paraformaldehyde (PFA) in phosphate buffer

solution (PBS). In situ hybridization was performed as previously described (Hutcheson

and Vetter, 2001).

Results and Discussion

Cloning *Xsuz12*

To isolate the full length cDNA of Xs*uz12*, we first identified *Xenopus* EST

sequences in the NCBI database that showed high similarities to the mammalian *Suz12*.

We designed forward and reverse primers based on two of these EST sequences,

BU907606.1 and CA791061.1, respectively, and used them to amplify the full length

Xsu*z12* from a cDNA library prepared from whole embryos at stage 17/18 using PCR.

We obtained a band of 2.1 kb, corresponding to the predicted molecular weight of *Xsuz12*

full length cDNA as compared to the molecular weight of its *X. tropicalis* ortholog. The

band was sequenced and found to encode for a protein that is composed of 696 amino

acids. Sequence alignment shows that the predicted protein is highly similar to its

vertebrate orthologs, and contains two well conserved domains: a zinc finger motif and a

VEFS box, which are characteristics of the SUZ12 protein, spanning amino acids 405-

428 and 501-638, respectively (See supplementary data). Evidence suggests that both

domains are important for SUZ12 function. For instance, the VEFS box of SUZ12 is

required for SUZ12-EZH2 interaction (Yamamoto et al., 2004). Construction of a

phylogenetic tree confirmed that *Xsuz12* is closely related to its vertebrate orthologs (see

supplementary data).

Expression pattern of *Xsuz12*

We first examined temporal expression of *Xsuz12* by performing reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA extracted from different developmental stages up to stage 41 (tadpole stage). We detected X*suz12* expression at all stages examined, starting as early as fertilized egg (see supplementary data). This indicates that *Xsuz12* transcript is maternally supplied then zygotic expression is initiated later on, in agreement with the expression data of the PRC2 subunits, *Xeed and Xez* (Satijn et al., 2001; Showell and Cunliffe, 2002). We confirmed these results by real time quantitative RT-PCR (qRT-PCR), which also showed robust *Xsuz12* expression after neural induction in neurula and tail bud stage embryos (see supplementary data).

To define tissues where *Xsuz12* is expressed we performed whole-mount in situ hybridization analysis. Generally, *Xsuz12* was mainly detectable in the developing central nervous system along the anterior-posterior axis with a particularly strong signal in the anterior part of the embryo (Fig. 4). Expression starts to become apparent in the open neural plate at around stage 15 (Fig. 4A, B). By stage 20-22 *Xsuz12* transcript is seen in the developing spinal cord and in the head region of the embryo including the developing optic vesicles (Fig. 4C-F). From stage 24 onward, *Xsuz12* expression is well defined in the head region, including developing eye, branchial arches, otic vesicles and in the forebrain (Fig. 4G-L). We also noticed the presence of a recognizable signal in the tail region that is obvious in the tail bud stages (Fig. 4I, K). Further, we performed in situ hybridization analysis on several cross sections taken along the anterior posterior axis of embryos at stage 41 (tadpole stage). We found that *Xsuz12* expression was particularly robust in areas surrounding the ventricular zones in the brain (Fig. 4M, N) and in the

spinal cord (Fig. 4O). Brain and spinal cord expression of Xsuz12 is also detected in cross sections made at tailbud stages (data not shown). In situ hybridization signal cannot be detected when a sense probe is used (Fig. 4K inset).

Expression of *Xeed, Xez and Xrbbp4*

If the PRC2 complex is active and plays important roles in the developing nervous system in *Xenopus*, then we should expect that the expression pattern of its subunits largely, if not fully, coincide. Accordingly, we first sought to compare the spatial expression of *Xsuz12* to those of *Xez* and *Xeed*, as both genes have been previously cloned but their expression was not fully characterized (Barnett et al., 2001; Satijn et al., 2001; Showell and Cunliffe, 2002). *Xez* and *Xeed* expression can be clearly seen at stage 16 in the anterior open neural plate (Fig. 5A, C). We found that *Xez* is expressed along the anterior-posterior axis and is not restricted to the anterior embryo as was previously reported (Fig.5, B, E; Barnett et al, 2001). At stage 19, *Xez* and *Xeed* transcripts are obvious in the presumptive spinal cord and by stage 24 both transcripts are expressed in the head region, including the emerging optic vesicle, as well as in the developing spinal cord (Fig. 5B-H). In the tail bud stages, *Xeed and Xez* expression persists anteriorly in the branchial arches, the otic vesicle, the forebrain and the developing eyes (Fig. 5I-Q). As the case of *Xsuz12* a weak but detectable staining can be seen in the tail region (Fig. 5I, K, M and P). Finally, we sought to characterize the expression of *Xrbbp4*, a vertebrate ortholog of the *Drosophila Nurf55*. This subunit has been purified from the PRC2 complex in *Drosophila* embryos and from HeLa cells, and is required for H3 binding (Cao et al., 2002; Czermin et al., 2002; Nekrasov et al., 2005).
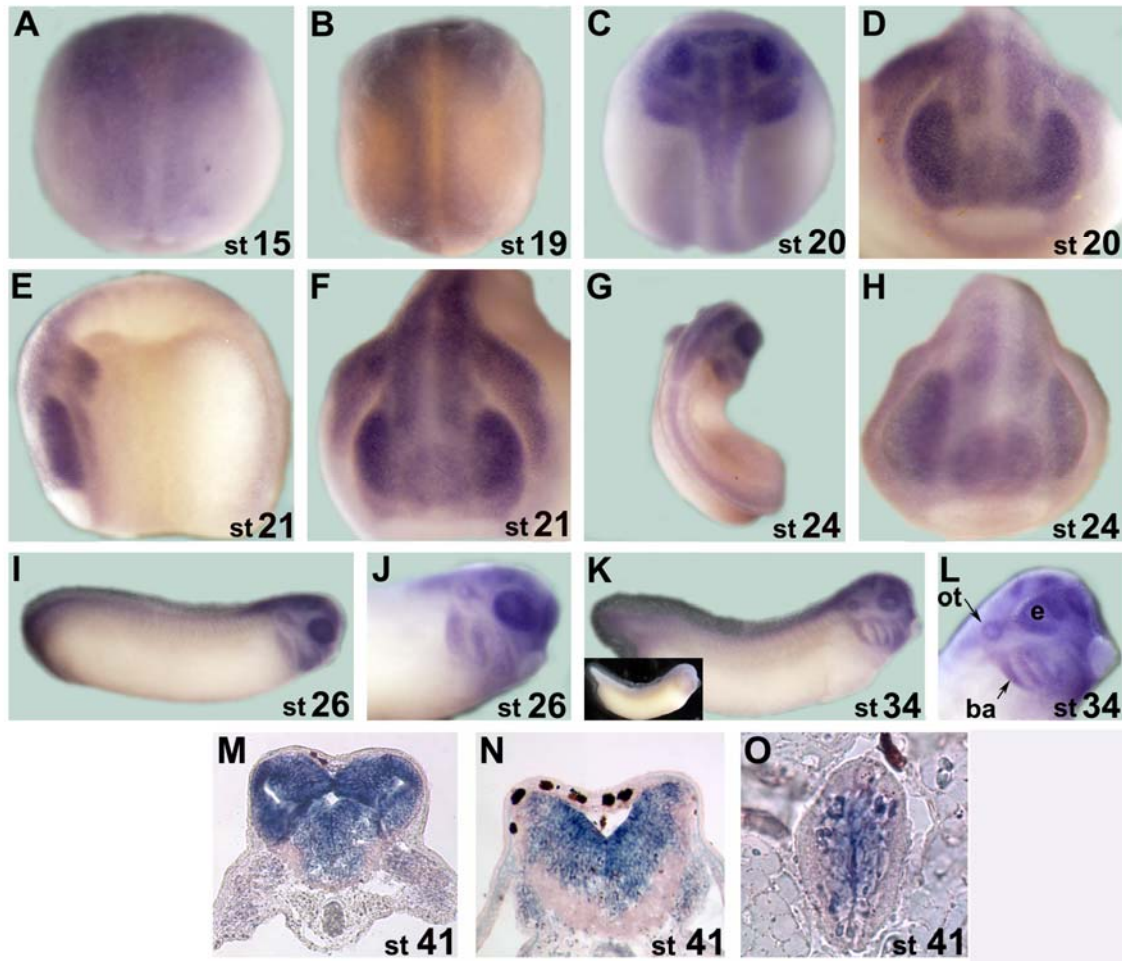
Figure 4. (A-L) Whole mount in situ hybridization analysis of X*suz12* expression starting from stage 15 (neurula). A, B, C and G are dorsal views. D, F and H are anterior views. E, I, G, K and L are lateral views. G and L are magnifications of the head region as viewed laterally at stage 26 and 34, respectively. Inset in K shows an example of a whole mount in situ hybridization that was performed with *Xsuz12* sense probe. (M-O) In situ hybridization analysis performed on traverse sections from the head region at stage 41. M and N sections are at the levels of forebrain and hindbrain, respectively. O is a traverse section in the spinal cord. Stages are indicated at the lower right corner of each image. ot,

Figure 5. Comparison of *Xez* and *Xeed* spatial expression during frog development as assessed by whole mount in situ hybridization. A B, C, D, E and G are dorsal views. F and H are anterior views. I, K, M and P are lateral views. J, L, N and O are magnifications of the head region in I, K, M and P, respectively. ot, otic vesicle; ba, branchial arches; e, eye.

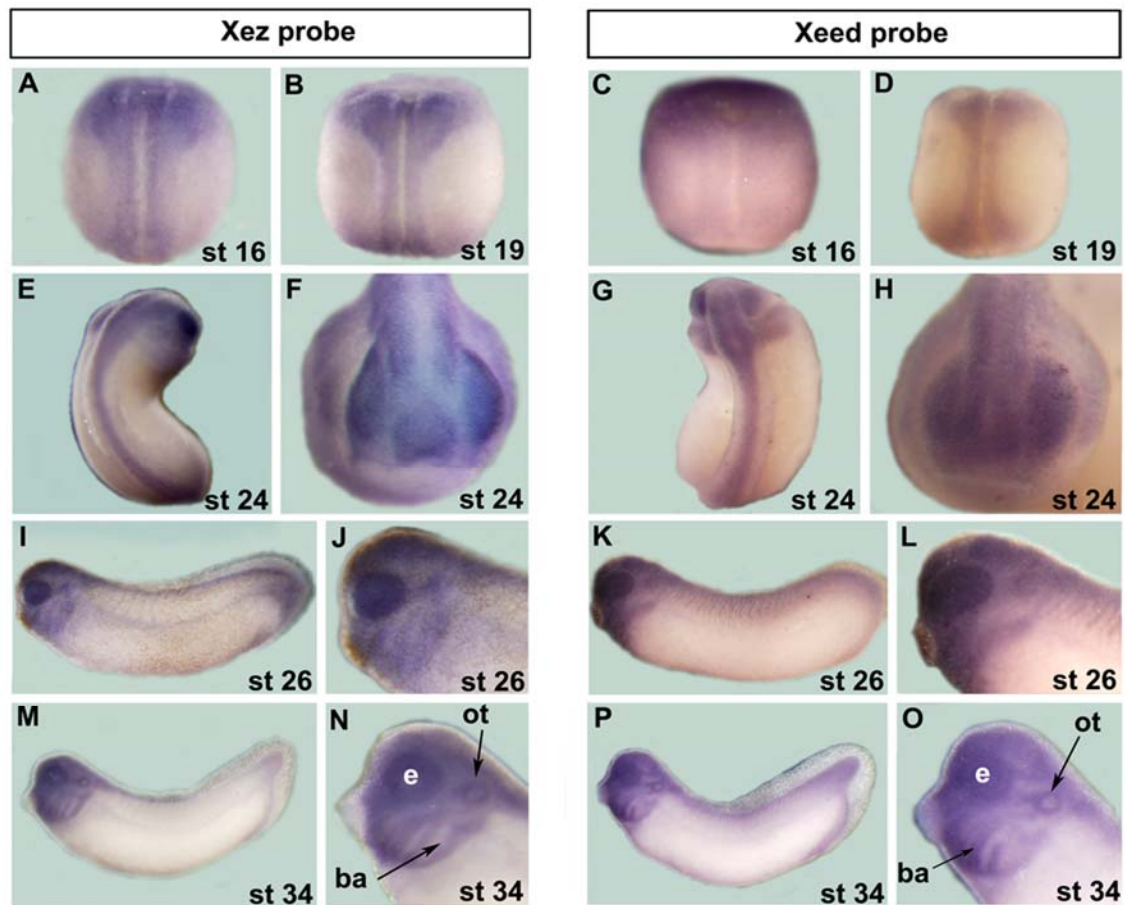RBBP4 is also known to interact with other protein complexes as well, such as the N-CoR complex (Jones et al., 2001). *Xrbbp4* has been cloned but its expression pattern in the frog was not determined (Vermaak et al., 1999). Therefore, we performed a whole mount in situ hybridization analysis on different embryonic stages of *Xenopus* and found that *Xrbbp4* expression in the developing nervous system is virtually identical to that of *Xsuz12, Xeed and Xez. Xrbbp4* is expressed in the developing eyes, branchial arches, otic vesicles and in the brain (Fig. 6A-L). Taken together we conclude that PRC2 principle components are expressed in the developing nervous system, suggesting an important role in neural tissue proliferation and/or differentiation.

Because polycomb genes are highly conserved among species, we speculate that some of the physiological roles of PRC2, at least in certain developmental processes, may also be conserved. To date, the developmental expression pattern of mammalian PRC2 genes has not been thoroughly investigated but available data suggest that they may be expressed in neural tissues in a pattern similar to that of the frog *Xenopus*. For instance, mouse *Ezh2* is expressed during embryogenesis and is detected in the neural tube, optic vesicle, branchial arches and in the developing brain in general (Caretti et al., 2004; Laible et al., 1997).This may indicate that at least some of the biological functions of PRC2 in neural tissues are evolutionarily conserved.
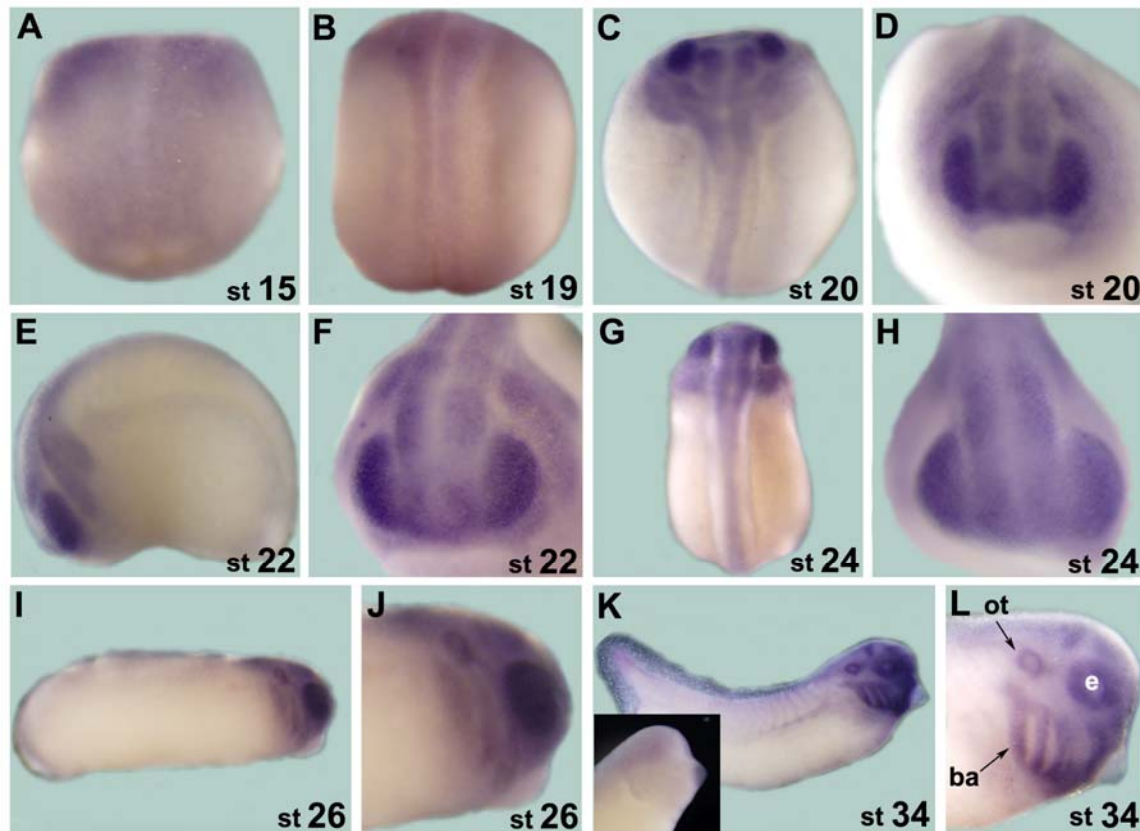
Figure 6. **Expression of X*rbbp4* by whole mount in situ hybridization analysis**.
A B, C and G are dorsal views. D, F and H are anterior views. I, K, M and P are lateral
views. Whole mount in situ hybridization performed with a sense probe is shown as
inset in 3K. ot, otic vesicle; ba, branchial arches; e, eye.

of Yangsook Green Song and Michael Steele with the qRT-PCR analysis. This work was

funded by an NIH grant (EY012274) to MLV.

References

Barnett MW, Seville RA, Nijjar S, Old RW, Jones EA. 2001. Xenopus Enhancer of Zeste (XEZ); an anteriorly restricted polycomb gene with a role in neural patterning. Mech Dev 102:157-167.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-resolution profiling of histone methylations in the human genome. Cell 129:823-837.

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441:349-353.

Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y. 2002. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298:1039-1043.

Cao R, Zhang Y. 2004. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 14:155-164.

Caretti G, Di Padova M, Micales B, Lyons GE, Sartorelli V. 2004. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. Genes Dev 18:2627-2638.

Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V. 2002. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111:185-196.

Dellino GI, Schwartz YB, Farkas G, McCabe D, Elgin SC, Pirrotta V. 2004. Polycomb silencing blocks transcription initiation. Mol Cell 13:887-893.

Francis NJ, Kingston RE. 2001. Mechanisms of transcriptional memory. Nat Rev Mol Cell Biol 2:409-421.

Hutcheson DA, Vetter ML. 2001. The bHLH factors Xath5 and XNeuroD can upregulate the expression of XBrn3d, a POU-homeodomain transcription factor. Dev Biol 232:327-338.

Jones PL, Sachs LM, Rouse N, Wade PA, Shi YB. 2001. Multiple N-CoR complexes contain distinct histone deacetylases. J Biol Chem 276:8807-8811.

Ketel CS, Andersen EF, Vargas ML, Suh J, Strome S, Simon JA. 2005. Subunit contributions to histone methyltransferase activities of fly and worm polycomb group complexes. Mol Cell Biol 25:6857-6868.

Kuzmichev A, Margueron R, Vaquero A, Preissner TS, Scher M, Kirmizis A, Ouyang X, Brockdorff N, Abate-Shen C, Farnham P, Reinberg D. 2005. Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. Proc Natl Acad Sci U S A 102:1859-1864.

Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. 2002. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev 16:2893-2905.

Laible G, Wolf A, Dorn R, Reuter G, Nislow C, Lebersorger A, Popkin D, Pillus L, Jenuwein T. 1997. Mammalian homologues of the Polycomb-group gene Enhancer of zeste mediate gene silencing in Drosophila heterochromatin and at S. cerevisiae telomeres. Embo J 16:3219-3232.

Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, Young RA. 2006. Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125:301-313.

Logan MA, Steele MR, Vetter ML. 2005. Expression of synaptic vesicle two-related protein SVOP in the developing nervous system of Xenopus laevis. Dev Dyn 234:802-807.

Margueron R, Li G, Sarma K, Blais A, Zavadil J, Woodcock CL, Dynlacht BD, Reinberg D. 2008. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. Mol Cell 32:503-518.

Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA. 2002. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111:197-208.

Nekrasov M, Wild B, Muller J. 2005. Nucleosome binding and histone methyltransferase activity of Drosophila PRC2. EMBO Rep 6:348-353.

Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. 2007. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol 27:3769-3779.

Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E, Helin K. 2004. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J 23:4061-4071.

Satijn DP, Hamer KM, den Blaauwen J, Otte AP. 2001. The polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in Xenopus embryos. Mol Cell Biol 21:1360-1369.

Saurin AJ, Shao Z, Erdjument-Bromage H, Tempst P, Kingston RE. 2001. A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. Nature 412:655-660.

Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. 2007. Genome regulation by polycomb and trithorax proteins. Cell 128:735-745.

Schwartz YB, Kahn TG, Nix DA, Li XY, Bourgon R, Biggin M, Pirrotta V. 2006. Genome-wide analysis of Polycomb targets in Drosophila melanogaster. Nat Genet 38:700-705.

Shao Z, Raible F, Mollaaghababa R, Guyon JR, Wu CT, Bender W, Kingston RE. 1999. Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell 98:37-46.

Shen X, Liu Y, Hsu YJ, Fujiwara Y, Kim J, Mao X, Yuan GC, Orkin SH. 2008. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell 32:491-502.

Showell C, Cunliffe VT. 2002. Identification of putative interaction partners for the Xenopus Polycomb-group protein Xeed. Gene 291:95-104.

Sparmann A, van Lohuizen M. 2006. Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846-856.

Su IH, Dobenecker MW, Dickinson E, Oser M, Basavaraj A, Marqueron R, Viale A, Reinberg D, Wulfing C, Tarakhovsky A. 2005. Polycomb group protein ezh2 controls actin polymerization and cell signaling. Cell 121:425-436.

Vermaak D, Wade PA, Jones PL, Shi YB, Wolffe AP. 1999. Functional analysis of the SIN3-histone deacetylase RPD3-RbAp48-histone H4 connection in the Xenopus oocyte. Mol Cell Biol 19:5847-5860.

Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, Fuks F. 2006. The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439:871-874.

Yamamoto K, Sonoda M, Inokuchi J, Shirasawa S, Sasazuki T. 2004. Polycomb group suppressor of zeste 12 links heterochromatin protein 1alpha and enhancer of zeste 2. J Biol Chem 279:401-406.

CHAPTER 3

THE POLYCOMB REPRESSIVE COMPLEX PRC2 REGULATES

RETINAL DIFFERENTIATION IN *XENOPUS*

Abstract

The mechanisms that govern the transition of retinal progenitors from proliferation to differentiation are not fully understood. Recent studies have established that the chromatin remodeling complex PRC2 is a key switch required for dividing cells to execute correct genetic reprogramming as they exit the cell cycle and undergo cellular differentiation in a variety of biological contexts, including in embryonic stem cells and during cortex development. PRC2 is involved in the initiation of gene repression mainly by trimethylating lysine 27 of histone 3 tail (H3K27 me3), a known histone mark that is associated with chromatin compaction. However, the role of PRC2 and its catalytic activities in vertebrate eye development has not been investigated.

Here we report the involvement of PRC2 in regulating the transition from proliferation to differentiation during eye development. Using in situ hybridization analysis and BrdU labeling, we show that the transcripts of the core components of PRC2 are coincidently expressed in retinal progenitors and are downregulated concomitant with retinal differentiation. Surprisingly, the levels of the methylation mark H3K27me3 that is catalyzed by PRC2 greatly increase in terminally differentiated cells. Inhibition of *Xez*, the catalytic subunit of PRC2 (the *Xenopus* homologue of *Ezh2*) using a translation blocking morpholino leads to a marked decrease in H3K27me3 in retinal cell types, indicating that PRC2 is required for this modification in *Xenopus* retina. Blocking *Xez* causes a reduction in eye size and inhibition of retinal differentiation genes. Importantly, targeted knockdown of *Xez* in retinal progenitors biases cell fate toward late born cell types, suggesting that retinal differentiation is delayed or inhibited. In line with these data, H3K27me3 specifically decorates a subset of important regulators of retinal

development, some of which are known negative regulators of retinal differentiation. Taken together, our data establishes PRC2 as a major player in retinal neurogenesis and suggests that it may have multiple roles in eye development, including regulation of retinal proliferation and/or differentiation.

Introduction

In vertebrate retina, differentiation results in the formation of seven major cell types that are born in a highly conserved order (Ohsawa and Kageyama, 2008). Evidence suggests that retinal cell fate decisions are largely independent of cell lineage, but how multipotent retinal progenitor cells lose their multipotency and become committed to give rise to fully differentiated retinal cells remains unclear (Livesey and Cepko, 2001). The transition from proliferation to differentiation is a highly coordinated process, and involves downregulation of cell cycle genes, and activation of the retinal cell fate specification machinery (Agathocleous and Harris, 2009; Agathocleous et al., 2009). This switch from a genetic program that sustains proliferation to another that initiates neural differentiation is regulated by cross talk between transcription factors and signaling pathways, and requires extensive chromatin reorganization and general changes in gene expression (Hsieh and Gage, 2004; Ohsawa and Kageyama, 2008). Thus it is likely that mechanisms that regulate chromatin structure and global gene expression orchestrate critical transitional steps during neural development, including retinal differentiation (Lessard and Crabtree, 2010; Yamaguchi et al., 2005).

Chromatin remodelers are complexes that dynamically regulate the condensation state of chromatin by governing posttranslational modifications of certain amino acids on

the N-termini of histones associated with the DNA (Turner, 2010). This is a critical function because transcription factors can only access DNA when chromatin is decondensed.

The Polycomb group proteins have been shown to form distinct chromatin remodeling complexes that play fundamental roles during development (Lessard and Crabtree, 2010; Margueron and Reinberg, 2011). In particular, the function of PRC2 is essential in regulating the balance between proliferation and differentiation in a variety of contexts (Ezhkova et al., 2009; Yu et al., 2007). PRC2 mediates gene repression mainly through its methyltransferase activity that catalyzes the addition of H3K27 methylation to specific genomic loci, which act as docking sites for recruiting additional repressive complexes (Fischle et al., 2003; Kuzmichev et al., 2002; Rajasekhar and Begemann, 2007). PRC2 consists of four core subunits: EZH2, SUZ12, EED and RBBP4/7, in which the catalytic activity is conferred by EZH2 (Margueron and Reinberg, 2011; Pietersen and van Lohuizen, 2008). RBBP4 is a homologue of the Drosophila Nurf55 which is required for nucleosome binding (Nekrasov et al., 2005). The exact functions of SUZ12 and EED remain poorly understood, but both proteins are required for the EZH2 enzymatic function (Pasini et al., 2007).

The roles of PRC2 in retinal development have not been explored because single mouse knockouts of the core PRC2 subunits are lethal at early postimplantation stages, and eye-specific conditional mutants have not been reported (Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004). In embryonic stem cells (ESCs), the PRC2-mediated mark H3K27me3 decorates many developmental regulators and cell fate genes, which contributes to their repression and primes the cells for subsequent lineage differentiation

and cell fate choices (Boyer et al., 2006; Lee et al., 2006; Pietersen and van Lohuizen, 2008). Indeed, ESCs derived from the *Suz12* mouse knockout fail to differentiate into neurons in culture conditions (Pasini et al., 2007). In the developing cortex, PRC2 suppresses expression of the proneural bHLH gene *Ngn1* in neural precursor cells to promote formation of astrocytes (nonneural cell fate) when the time is appropriate (Hirabayashi et al., 2009). PRC2 is also important for maintaining cell division in progenitors during skin development, and supports proliferation of metastatic cells in prostate cancer (Ezhkova et al., 2009; Varambally et al., 2002).

Whether PRC2 and its components are required for eye development and underlying cell fate decisions is entirely unknown. A recent study has demonstrated that *Ezh2* and its mark H3K27me3 are expressed during mouse retinal development but the significance of this finding has not been explored (Rao et al., 2010). We hypothesize that PRC2 functions in retinoblasts to regulate the transition from proliferation to differentiation. Exploring the functions of PRC2 in vertebrate eye development may provide insight into regulation of the genetic network that controls the neural potential of retinoblasts and how it leads to the generation of neurons.

Materials and Methods

Microinjections of mRNAs

Capped mRNAs were synthesized in vitro using Message Machine kit (Ambion). mRNAs were injected alone or in combination with antisense morpholino (MO) in one animal dorsal blastomere of 8 cell stage *Xenopus* embryos (Huang and Moody, 1993). The following mRNAs were used in injections: GFP (500 pg), β-galactosidase (β-gal;

200 pg); Bcl-xl (450 pg). Embryos were collected at the appropriate developmental stages (according to Nieuwkoop and Faber, 1965), fixed and selected for expression of GFP or presence of X-gal staining in the anterior central nervous system. X-gal staining was performed on β-gal injected embryos as previously described (Turner and Weintraub, 1994).

In situ hybridization analysis

In situ hybridization was performed on whole embryos and retinal sections as previously described (Hutcheson and Vetter, 2001). The following Digoxigenin (DIG)-labeled riboprobes were used for the analysis: Xash1(Ferreiro et al., 1993), Xath5 (Kanekar et al., 1997), Xfz5 (Sumanas and Ekker, 2001), CyclinD1 (Vernon and Philpott, 2003), Xngnr-1 (Ma et al., 1996), Xash3 (Zimmerman et al., 1993), Vsx1 (D'Autilia et al., 2006), Sox2 (Mizuseki et al., 1998), Pax6 (Hirsch and Harris, 1997), NeuroD (Lee et al., 1995), Six3 (Zhou et al., 2000), Xrx1 (Mathers et al., 1997), En-2 (Hemmati-Brivanlou et al., 1991), Hermes (Patterson et al., 2000) and Sbt1 (Logan et al., 2005a).

Morpholinos

Translation blocking morpholinos were engineered and purchased from Gene Tool LLC (Philomath, OR). The following MO sequences and their amounts were used in the eight cell injections:

Xez ATG MO: 5'- CAGATTTCTTCCCCGTCTGGCCCAT-3' (5ng)

Xez UTR MO: 5'- TATCCAAAGGATGAATGGTCGCTCA-3' (20-25ng)

Xez control MO (scrambled sequence of Xez ATG MO):

5'- CGAATTCTTCTCCGCTTCGCGCACT-3' (5ng)

Suz12 MO: 5'- CCATGCGGGATACTACGAGTGATAA-3' (15ng)

Immunohistochemistry, TUNEL analysis and BrdU labeling

Immunostaining was performed using previously described methods. In brief, embryos were grown to stage 41 in 0.1X MMR, fixed in 4% PFA for 45 minutes, embedded in OCT and cryosectioned at 14 microns. After blocking (5% goat serum and 0.1% Triton in PBS) for 30 minutes, sections were incubated with primary rabbit anti-H3K27me3 antibody (Millipore, 1:100) overnight at 4 °C. Then, slides were washed and secondary Alexa Fluor 568-conjugated goat anti-rabbit antibody (Molecular Probes, 1:2000) was added for 2 hours at room temperature. Sections were counterstained with Hoechst (1:15000) to visualize nuclei. BrdU and TUNEL labeling were performed as previously described (Agathocleous et al., 2009; Hensey and Gautier, 1998). Quantification in the TUNEL analysis was performed by dividing the number of TUNEL positive cells in the eye field of the injected side on those in the uninjected eye of the same embryo.

Retinal analysis

Morpholinos were injected in one dorsal blastomere of 32 cell stage embryos along with 300 pg of GFP mRNA (Huang and Moody, 1993). Embryos were grown to stage 41 and then fixed with 4% PFA for 45 minute. After sectioning, GFP positive cells were scored for cell type based on cell position and morphology as previously described (Moore et al., 2002).

Detection of H3K27me3 enriched regions

ChIP-seq was performed as described for the stages indicated (Akkers et al., 2009). Sequencing libraries were based on three biological replicate ChIP samples with similar recoveries and enrichment. Only sequence reads that map to unique positions in the genome were included for analysis. To compare developmental stages, for each data type/antibody the data were normalized for the number of sequence reads by randomly deleting reads from tracks with more sequence reads. To identify chromatin modifications around the 5' ends of genes of interest, a custom python script was used to determine read counts in 40 bins in a region of -10 to +10 kb surrounding the annotated transcription start sites of curated Xenbase genes. The *X. tropicalis* expression database was downloaded from Xenbase (xenbase.org), and genes expressed in the eye were selected for analysis. Clustering was performed using TMEV4.5 (tmev.org).

Results

PRC2 components are abundantly expressed in retinal progenitors

Several studies have shown that multiple polycomb group genes are broadly expressed in the developing central nervous of *Xenopus* embryos, including the eyes (Reijnen et al., 1995; Showell and Cunliffe, 2002; Yoshitake et al., 1999). Indeed, expression of the PRC2 components is detected in the earliest period of eye development in the region of the eye field in the anterior neural plate, and persists during optic vesicle and optic cup stages (Aldiri and Vetter, 2009). We further characterized the expression patterns of *Xez* and other PRC2 core components in the mature eye at stage 41 (tadpole stage; Fig. 7C) using in situ hybridization analysis. At this stage, the differentiated retinal
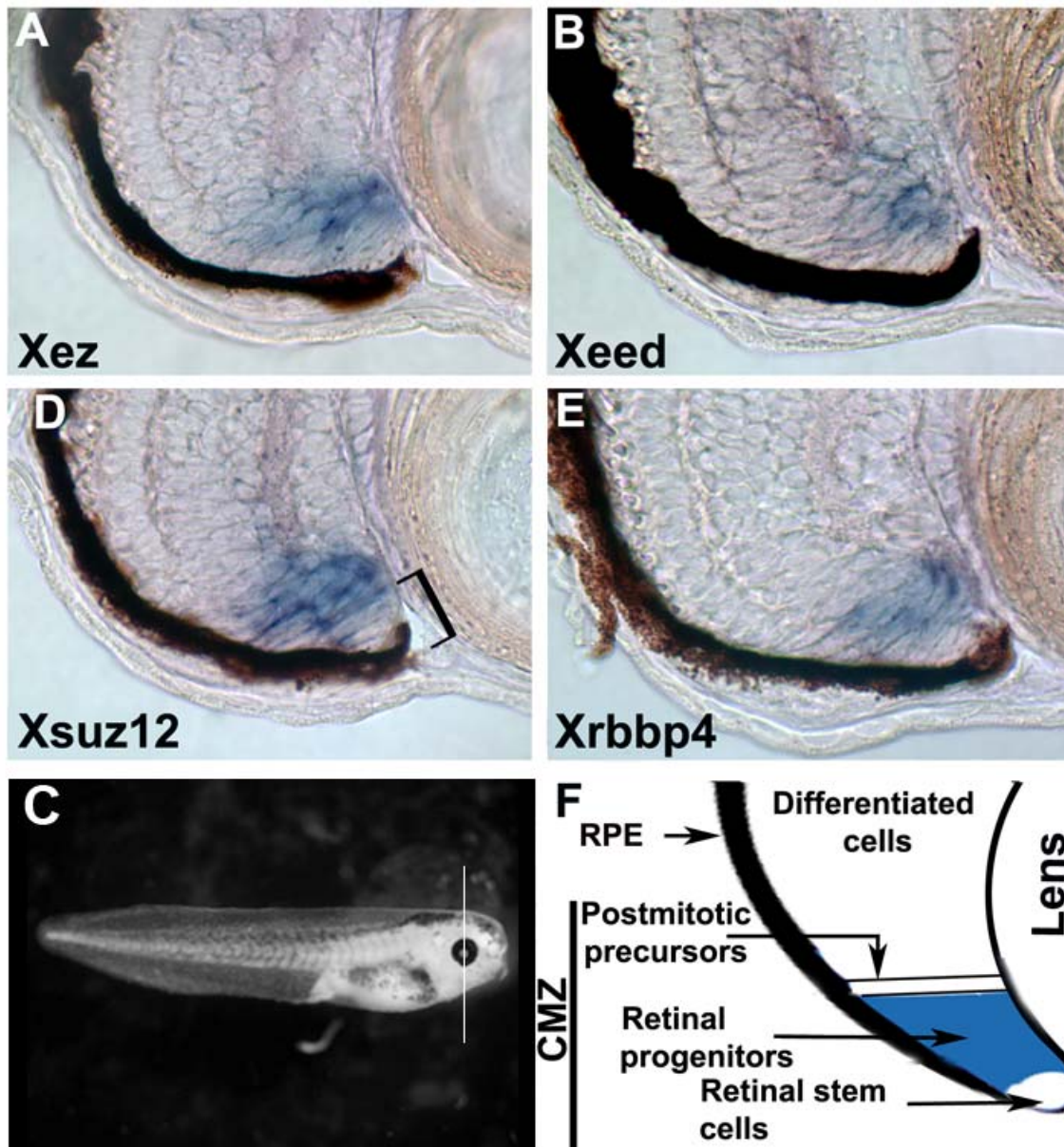
Figure 7. The PRC2 core components are abundant in the CMZ region of mature retina at stage 41. (A, B, D and E) Expression of the PRC2 core subunits Xez, Xeed, Xsuz12 and Xrbbp4 in retinal sections by in situ hybridization. Notice that retinal stem cell domain is negative for staining (bracket in D as an example). (C) Stage 41 frog embryo with the section plain shown as a line. (F) schematic representation of different domains in the frog Xenopus eye at stage 41. CMZ: Ciliary Marginal Zone. RPE: Retinal Pigmented Epithelium.

cells occupy the central part of the retina, while undifferentiated proliferating cells reside

in the peripheral region, termed the Ciliary Marginal Zone (CMZ) (Perron et al., 1998).

Several studies have shown that the temporal sequence of embryonic events during

retinal development are reflected in the spatial distribution of cells in the CMZ region:

the peripheral edge of the CMZ contains a small population of retinal stem cells while

proliferating retinoblasts are located more centrally (Agathocleous and Harris, 2009). The

most central part of the CMZ harbors differentiating retinal precursors (Fig.7F). Notably,

all transcripts of PRC2 core subunits are abundantly expressed in the CMZ region, and

are not detected in terminally differentiated cells (Fig. 7A-D). Interestingly, the

expression of PRC2 seems to be excluded from the retinal stem cell compartment (Fig.

7D).

To confirm that PRC2 components are expressed in actively dividing cells we

pulsed stage 41 embryos with BrdU for 30 minutes then fixed for 1 hour and performed

BrdU immunostaining and in situ hybridization analysis for *Xez, XSuz12* or *Rbbp4* on

processed retinal sections. Indeed, most of the BrdU-labeled cells are also positive for

PRC2 transcripts (Fig. 8). In summary, these data demonstrate that the expression of the

PRC2 components is transient, and predominantly restricted to the retinal progenitor

cells.

H3K27me3 levels are elevated in postmitotic cells

Since PRC2 catalyzes the addition of H3K27me3, a highly conserved function

across species, we examined the distribution pattern of H3K27me3 by immunostaining of
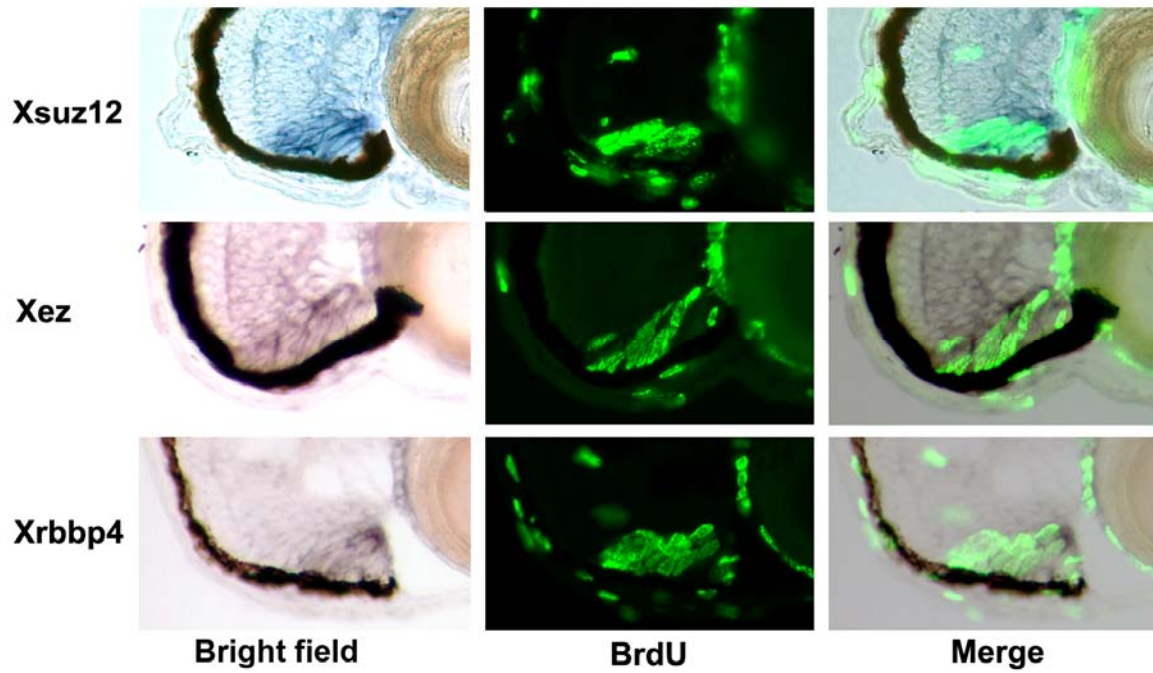
Figure 8. The expression of the PRC2 components is abundant in retinal progenitors. The mRNA level of Xez, Xsuz12 and Xrbbp4 was assayed by in situ hybridization analysis on retinal sections at the tadpole stage (stage 41). Then immunostaining for BrdU was performed. Notice that the in situ hybridization signals largely coincide with BrdU staining in the CMZ region.

the retina at stage 41. Surprisingly, we found that global H3K27me3 levels correlate positively with retinal differentiation as H3K27me3 levels are clearly enriched in postmitotic retinal cells, in direct contrast to the expression pattern of PRC2 core genes (Fig. 9). It is possible that while PRC2 core subunits are actively transcribed in progenitor cells, their transcription is shut down or greatly reduced as progenitors progress toward differentiation while protein levels are maintained in postmitotic cells. We will return to this issue later.

Knockdown of PRC2 function disrupts eye development

The expression pattern of PRC2 components indicates a possible role in proliferation and/or differentiation of retinal progenitor cells. To determine whether PRC2 is involved in eye development we chose a loss of function approach. We designed an antisense oligonucleotide morpholino that blocks the translation of *Xez* mRNA (termed Xez ATG MO hereafter) and injected it in one dorsal cell of 8 cell stage embryos along with GFP mRNA as a tracer, and observed the overall effect on eye development at the tadpole stage (stage 41). We found that Xez ATG MO caused a dose-dependent reduction in the brain and eye size on the injected side (Fig. 10A,B). Injection of Xez control MO (a scrambled sequence of Xez ATG MO) had a minimal effect (Fig. 10B).

A possible explanation for the observed tissue reduction on the injected side is that Xez MO may promote cell death. To investigate this possibility we first performed TUNEL analysis on Xez ATG MO injected embryos at stage 24 and found that these embryos do not show increased levels of apoptosis in the developing eye when compared to embryos injected with control MO (fold change in the number of TUNEL positive
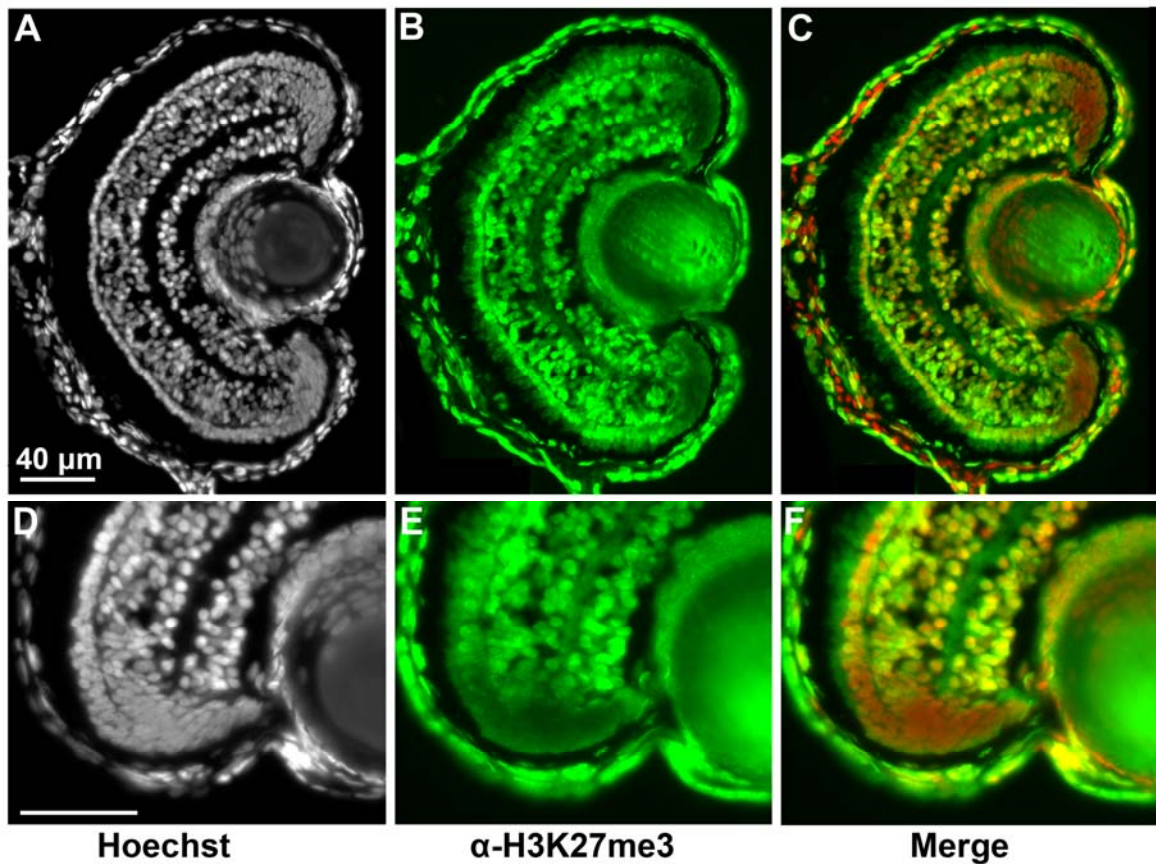
Figure 9. H3K27 me3 levels are elevated in differentiated retinal cells.
(A-C) Immunostaining of a retinal section with antibody against H3K27me3. (D-F) A
magnification of the peripheral part of the retina showing a sharp increase in H3K27me3
staining (green) as cells becomes fully differentiated. Retinal cells were counter stained with
Hoechst (red) to reveal nuclei. Scale bar 40 μm.
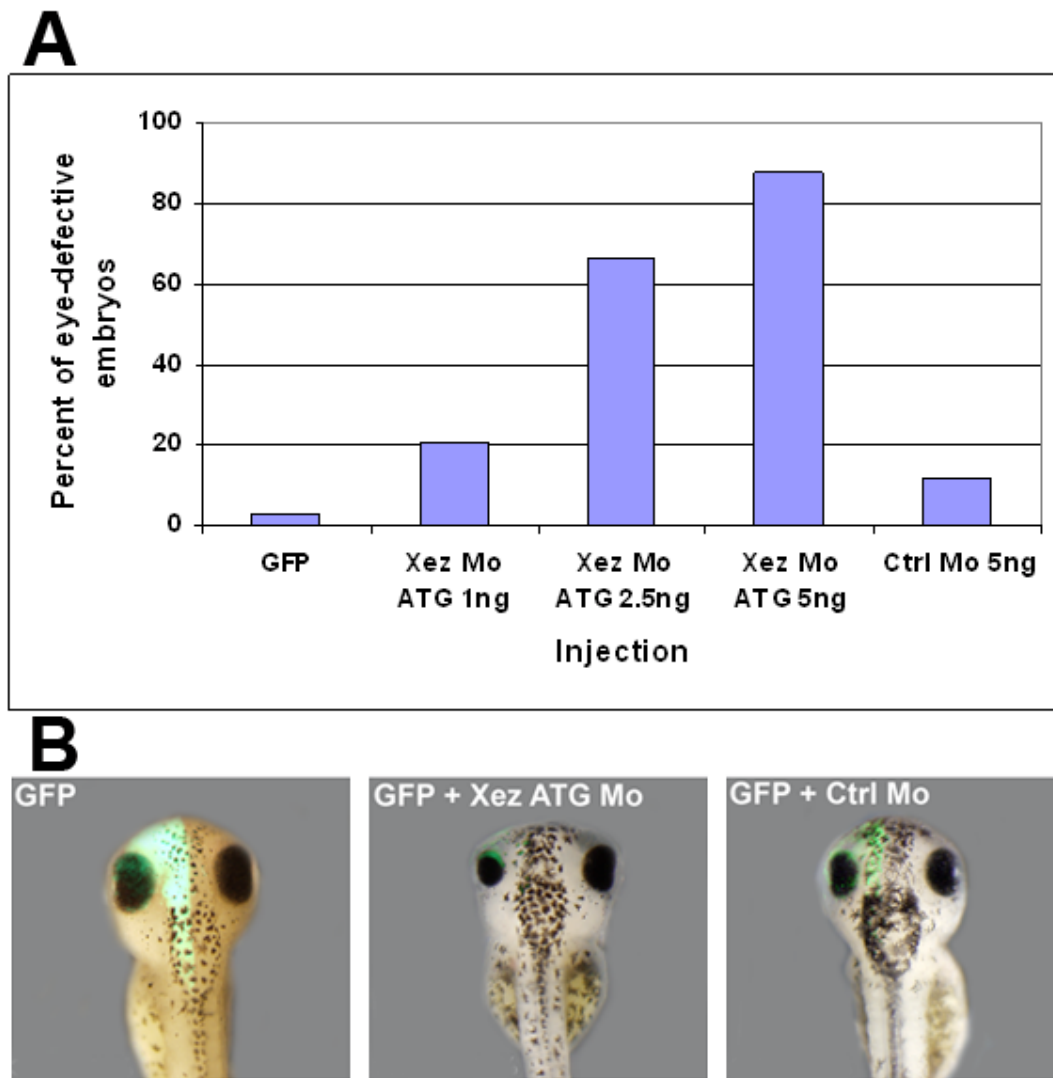
Figure 10. Xez knockdown causes reduction in the eye size in a dose dependent manner.
(A) Quantification of number of embryos with a small eye at the injected side when increasing doses of Xez morpholino was injected. (B) Representative embryos injected with GFP only, Xez MO or Control MO along with GFP mRNA.

cells in the injected eye: 1.5 ± 2.5 SEM, n= 43 for Xez ATG MO injected; 2.2 ± 0.8 SEM, n=21 for control MO and data not shown). Second, we reasoned that if this phenotype was primarily due to apoptosis then we should be able to rescue it by co-injecting the mRNA encoding the *Xenopus* homologue of the anti apoptotic factor *Bcl-xl* along with Xez ATG MO (Tribulo et al., 2004). In agreement with the TUNEL data, injection of *Bcl-xl* mRNA was unable to rescue the Xez MO-induced microphthalmia (embryos with microphthalmia: 76% n= 45 for Xez ATG MO injected embryos; 79% n= 84 for control MO + Bcl-xl mRNA injected embryos; Fig. 11). Taken together, we rule out apoptosis as a primary factor in Xez MO inducing eye malformation.

We were able to reproduce the effect on eye size by injecting either a morpholino that targets the 5' UTR region of *Xez* (termed Xez UTR MO hereafter), or a previously characterized Suz12 MO that inhibits the translation of the PRC2 core subunit *Suz12* (Peng et al., 2009). However, the effect of either morpholino was less penetrant than that of Xez ATG MO (embryos with reduced eye size: UTR MO 17% n = 36; SUZ12 MO 41% n= 34; Fig. 12). In summary, PRC2 function is required for the formation of normal eye size in apoptosis-independent manner. We speculate that this effect is due to reduced proliferation, since it has been shown that PRC2 is required for cell proliferation in a variety of tissues (Ezhkova et al., 2009; Margueron and Reinberg, 2011). This hypothesis will be tested in future studies.
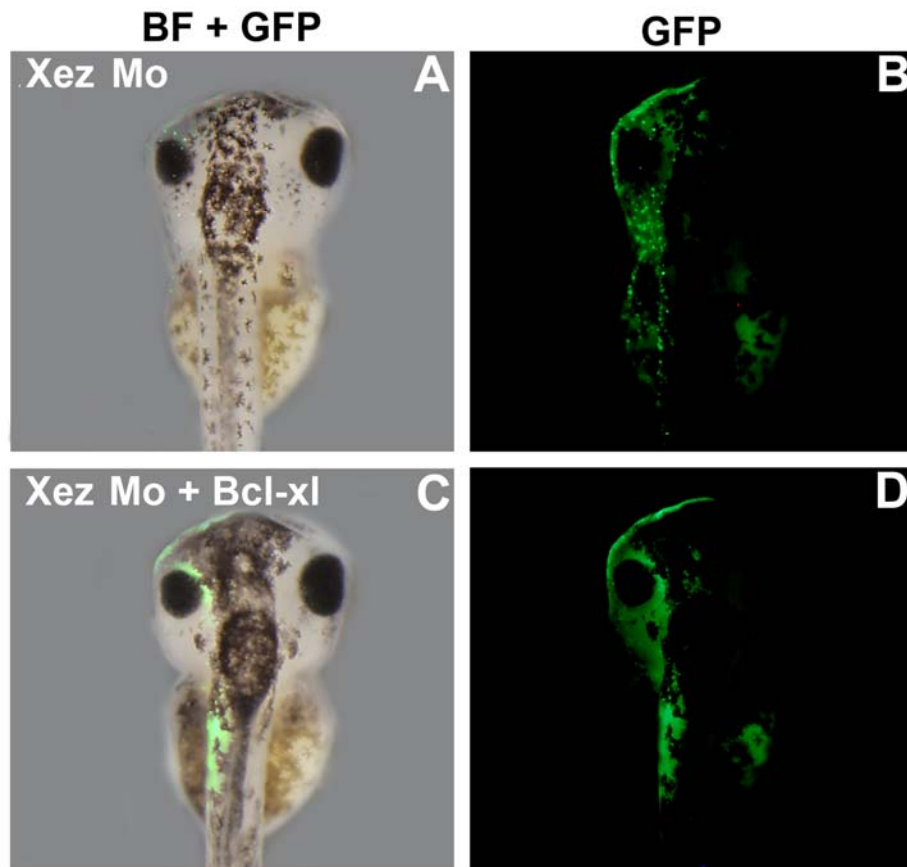
Figure 11. Apoptosis is unlikely to be responsible for microphthalmia. Coinjection of Bcl-xl mRNA with Xez MO cannot rescue the effect on eye size. B and D are the embryos shown in A and C, respectively, under green channel to highlight injected side.
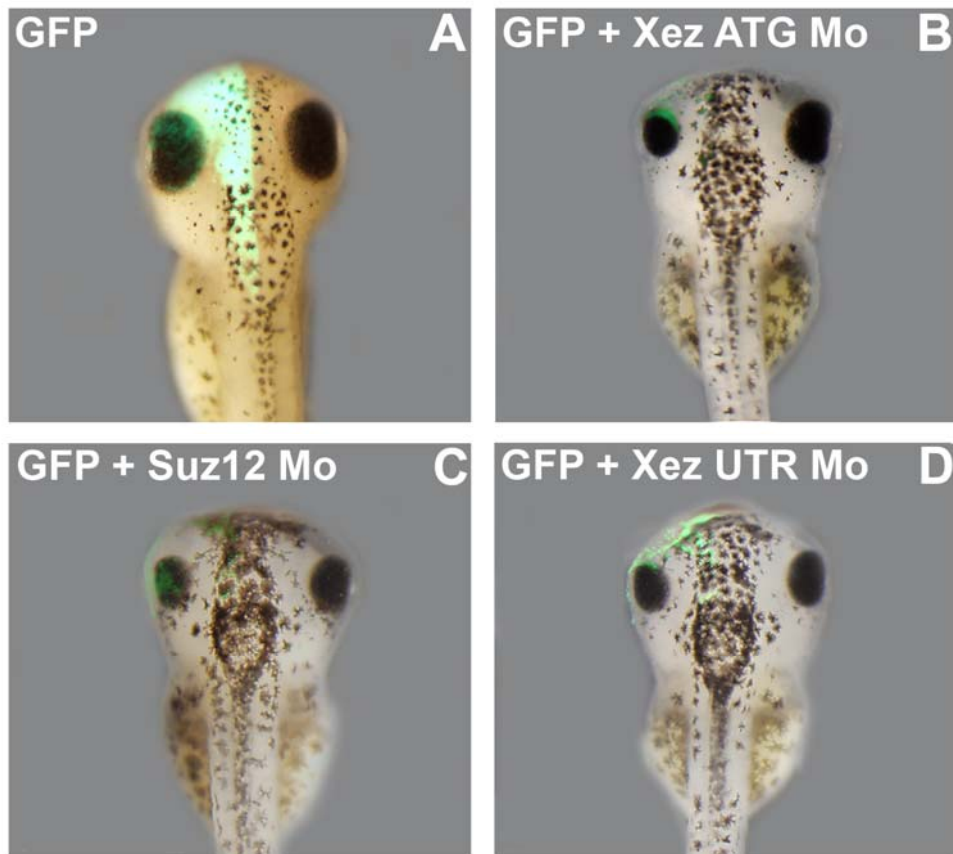
Figure 12. Knockdown of Xsuz12 phenocopies inhibition of Xez.
Injection of a translation blocking MO against Suz12 produces a small eye phenotype, mimicking the effect of Xez knockdown. An independent MO against the UTR region of Xez also affects eye size.

Knockdown of PRC2 does not affect retinal progenitor specification or
cell cycle genes

Next we characterized the molecular effect of inhibiting *Xez* on eye development.
We first tested whether Xez MO disrupts retinal progenitor specification by injecting one
dorsal blastomere of 8-cell stage embryos with Xez ATG MO or control MO plus β-gal
mRNA as a tracer and investigating the expression of *Rx, Pax6, Six3* and *Vsx1* by in situ
hybridization analysis at stage 20. There was no apparent change in the expression
intensity of all of the tested genes though there was a clear reduction in the size of the
expression domain on the injected side, in agreement with our previous observation of a
reduced eye size (Fig. 13A-D; embryos with a reduced expression domain 70% n= 90 for
Rx, 85% n= 47 for Pax6, 81% n= 43 for Six3, 82% n = 74 for Vsx1). Similarly, we saw
no effect on the expression intensity of *Frz5, Sox2* and *CyclinD1*, while the expression
domain was smaller (82% n=76 for Frz5, 81% n=62 for Sox2 and 81% n=16 for
CyclinD1; Fig. 13E-G). Embryos injected with control MO showed no or minimal effect
on the expression of the tested genes (embryos with normal expression: 100% n= 13 for
Rx, 100% n= 19 for Pax6, 91% n= 11 for Six3, 93% n = 14 for Vsx1, 94% n=16 for Frz5,
100% n=15 for Sox2 and 93% n=15 for CyclinD1; Fig. 14). Taken together, we conclude
that blocking *Xez* does not inhibit the expression of factors required for the specification
of the retinal progenitors or the expression of proliferation genes.

Knockdown of PRC2 blocks expression of retinal differentiation genes

The bHLH genes are major regulators of retinogenesis and shown to be heavily
involved in retinal cell type specification (Hatakeyama and Kageyama, 2004).
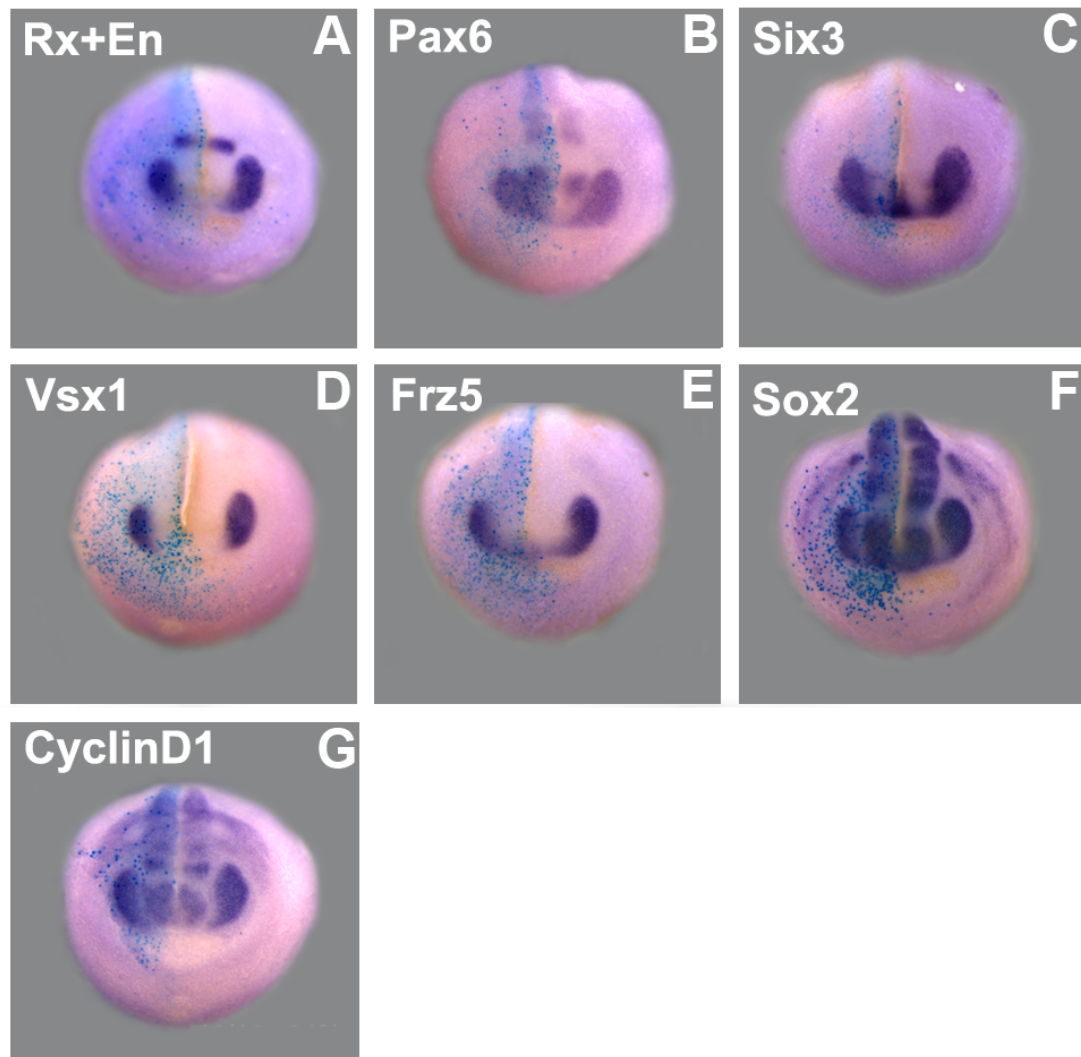
Figure 13. Inhibition of Xez does not affect the expression levels of retinal progenitor genes. Anterior view of stage 20 embryos after injection of Xez MO and β-gal to label injected side. The expression of Rx as well as all other markers revealed a relatively normal levels of expression, even though the eye is smaller. Dorsal side is up.
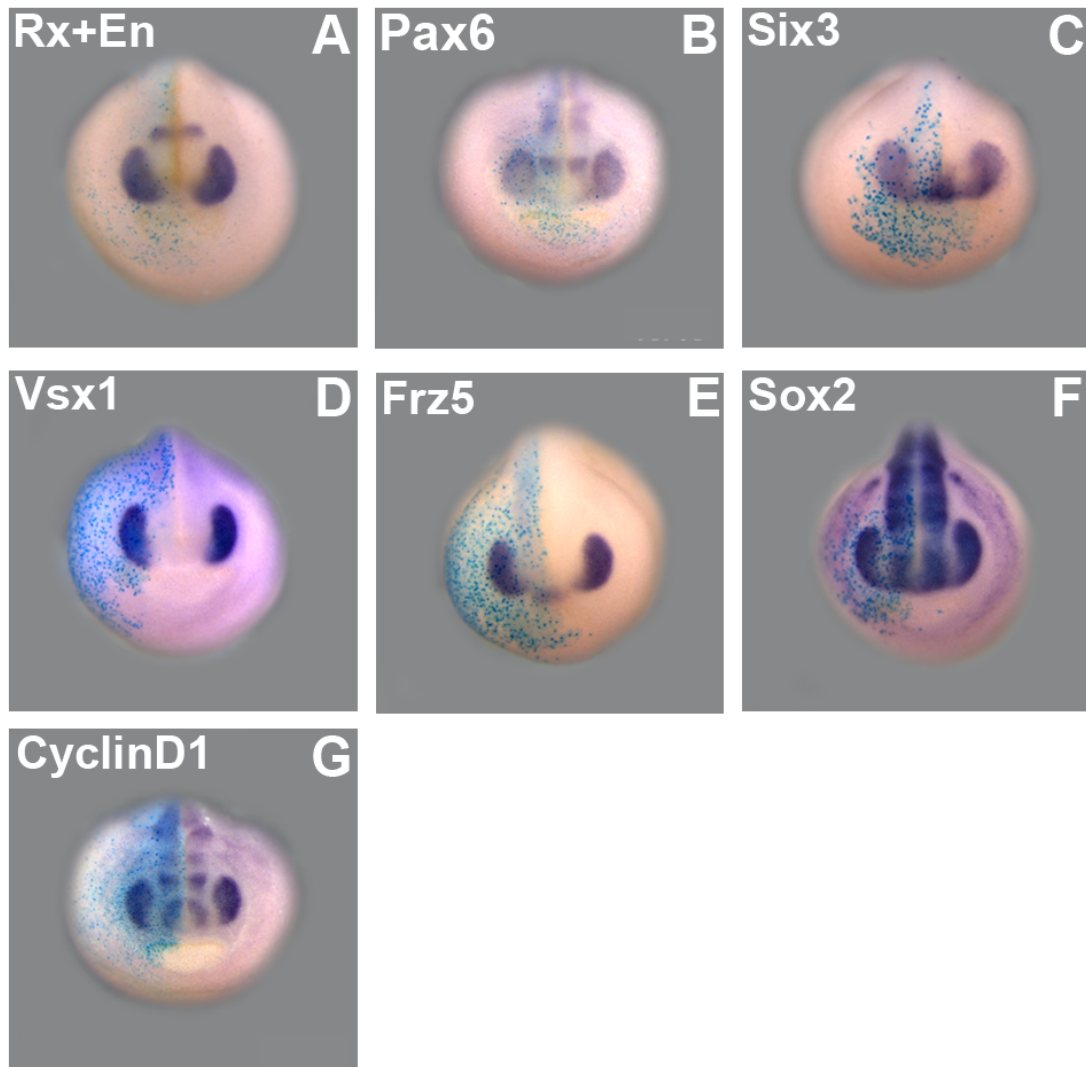
Figure 14. The expression domain of retinal progenitor markers is not affected in control MO injected embryos. Frontal view of stage 20 embryos injected with Xez control MO. Injected side is highlighted in blue (β-gal staining). Dorsal side is up.

Interestingly, previous work demonstrated that PRC2 can mediate repression of neural differentiation genes, including proneural bHLH factors (Boyer et al., 2006; Hirabayashi et al., 2009). The expression of the core subunits of PRC2 is observed before and during retinal neurogenesis in the optic cup stage, when proneural bHLH factors are active, suggesting that PRC2 may be involved in regulating retinal neural differentiation (Aldiri and Vetter, 2009). We therefore tested the effect of blocking the function of *Xez* on retinal neurogenesis by injecting XEZ ATG MO along with β-gal or GFP mRNA as a tracer in one dorsal blastomere at 8-cell stage embryos and determine the expression of several retinal differentiation factors by in situ hybridization analysis. We found that the expression of the proneural bHLH genes, *Xath5, Xash1, Xash3, NgnR1* and *NeuroD* was lost or dramatically reduced on the Xez ATG MO injected side (55% n= 53 for Xath5, 57% n=52 for Xash1, 47% n = 19 for NeuroD, 40% n=16 for Xash3, 43% n=30 for NgnR1; Fig. 15A-N). Further, the expression of *Sbt1*, a downstream target of *Xath5* and *NeuroD*, as well as *Hermes*, a ganglion cell marker was suppressed (67% n=14 for Sbt1, 84% n=31 for Hermes; Fig. 15G-L) (Patterson et al., 2000; Logan et al., 2005). The expression levels of *Rx, Vsx1* and *Sox2* at this stage were not affected (Fig. 15O-P and data not shown).

Additionally, injection of Xsuz12 MO or Xez UTR MO mirrored the effect of Xez ATG MO, thought the effect was weaker (Fig. 16A-N). Thus, while PRC2 does not disrupt the specification of retinal progenitors it is essential for the upregulation of retinal differentiation genes, suggesting that PRC2 ensures the proper execution of the transcription program that governs progression from proliferation to differentiation.

Figure 15. The initiation of the expression of retinal differentiation genes is blocked by Xez inhibition. (A-P) Lateral view of embryos injected with Xez MO along with β-gal mRNA to mark the injected side. The levels of bHLH gene expression and their target Sbt1 is markedly reduced on the injected side, while the expression of Rx remains unaffected. At stage 34, the expression of the ganglion cell marker Hermes is reduced as well. (Q-W) The expression of proneural bHLH genes in embryos injected with control MO is largely unaffected.

Injected side   Uninjected side   Injected side   Uninjected side

Xez MO

Xath 5 (A)   (B)   NgnR1 (C)   (D)

Xash1 (E)   (F)   Sbt1 (G)   (H)

NeuroD (I)   (J)   Hermes (K)   (L)

Xash3 (M)   (N)   Rx+En (O)   (P)

Control MO

Xash3 (Q)   (R)   Xash1 (S)   (T)

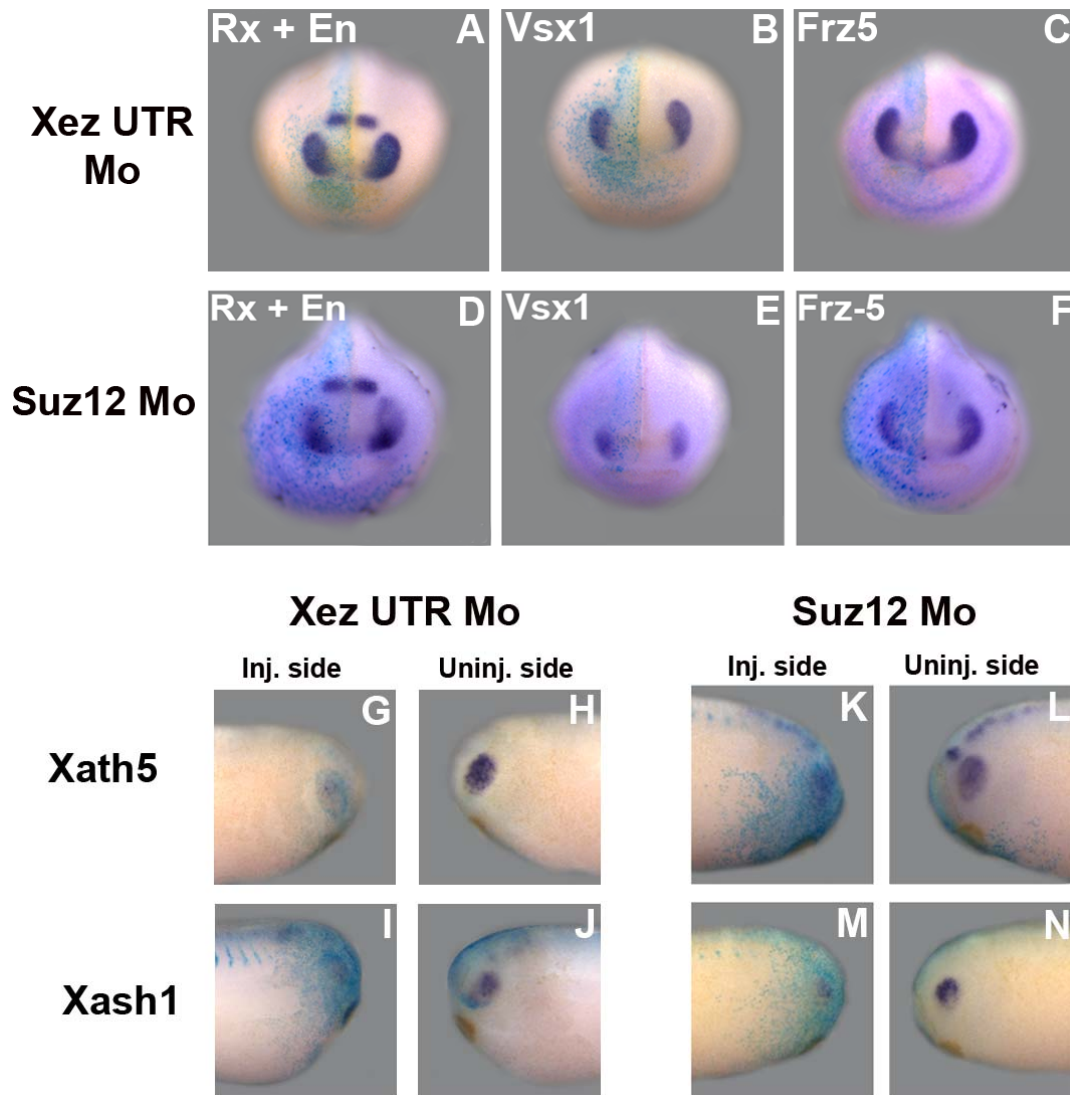Sbt1 (U)   (V)   Xath5 (W)   (X)
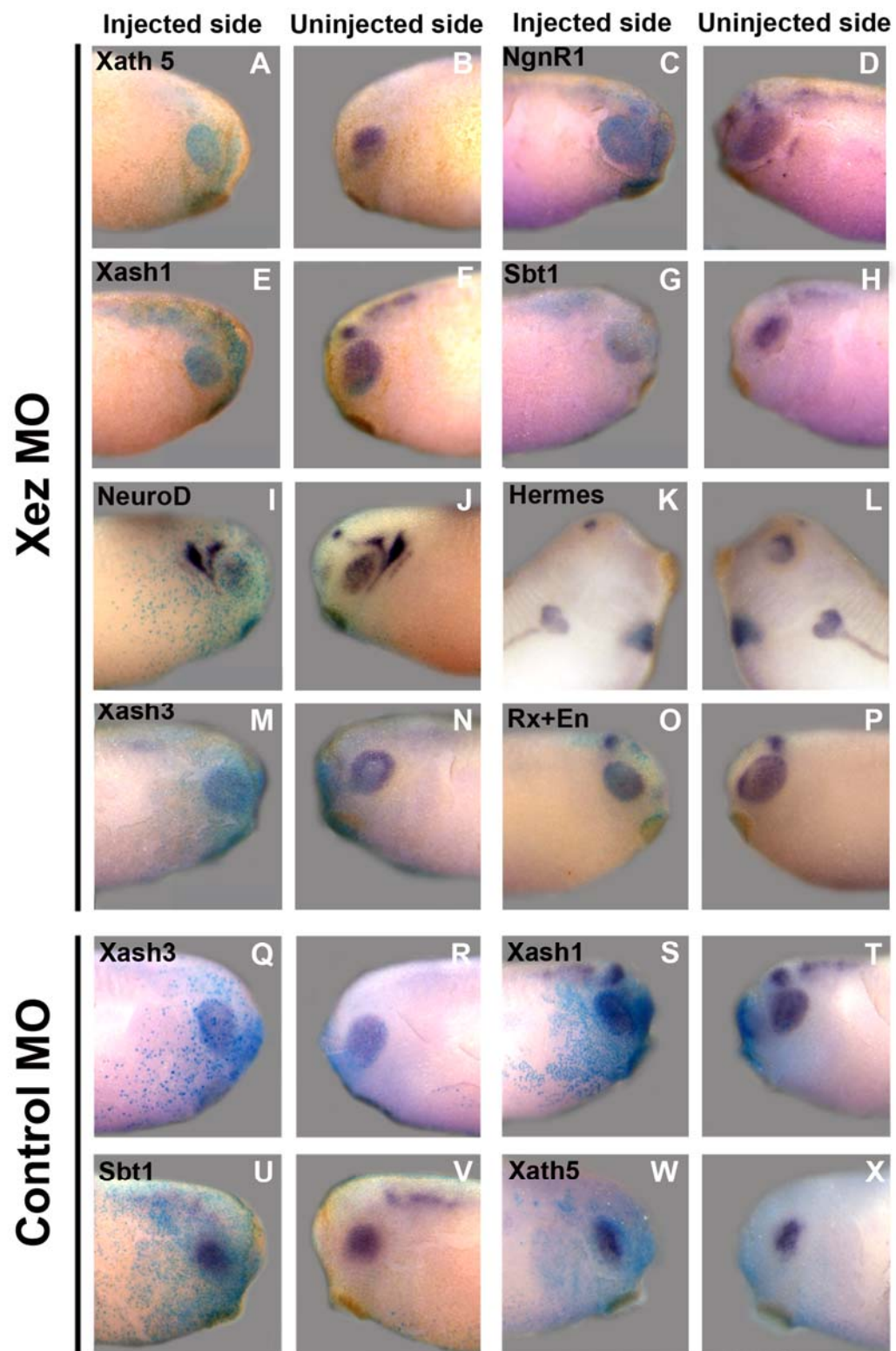
Figure 16. Inhibition of Suz12 mimics the effect of Xez knockdown during the transition from proliferation to differentiation. (A-F) Front view of stage 20 embryos injected with the indicated MOs after performing in situ hybridization for Rx+En, Vsx1 and Frz5. (G-N) Lateral view of stage 26 embryos.

Knockdown of PRC2 biases cell fate toward late born cell types

Since *Xez* is required for the initiation of the proneural bHLH genes, a major determinant of cell fate decisions, we reasoned that blocking PRC2 may affect subsequent cell fate decision as well. In principle, blocking of *Xez* function in retinoblasts might lead to inhibition or delay in the genesis of neuronal cell types and/or promotion of nonneural cell fate. To address this we injected one dorsal blastomere at the 16 cell stage with Xez UTR MO or Suz12 MO along with GFP mRNA and counted the number of each retinal cell type that are positive for GFP in stage 41 retinal sections. Retinal cells were identified based on morphology and laminar position (Moore et al., 2002). When compared to control, inhibition of *Xez* or *Suz12* caused a significant decrease in the percentage of early born cell types (e.g., ganglion cells), and an increase in the late born retinal cells, including nonneural cell types (Fig. 17). A preliminary analysis suggests that these non-neural retinal cells are positive for CRALBP, a Müller glia marker (data not shown).

PRC2 is required for H3K27me3 in *Xenopus* retina

The presence of high levels of H3K27me3 in retinal postmitotic cells contradicts the mRNA expression pattern of PRC2 subunits, raising the possibility that XEZ does not mediate the addition of H3K27me3 in *Xenopus*. Ezh2 has two mammalian paralogues; Ezh1 and Ezh2, both of which can catalyze the addition of H3K27me3 (Shen et al., 2008). Although not identified in *Xenopus,* it is still possible that EZH1 but not EZH2 is active in postmitotic retinal cells. Thus we wanted to know whether *Xenopus* Ezh2 (*Xez)* is a factor in trimethylating H3K27 in the retina. To address this question, we injected
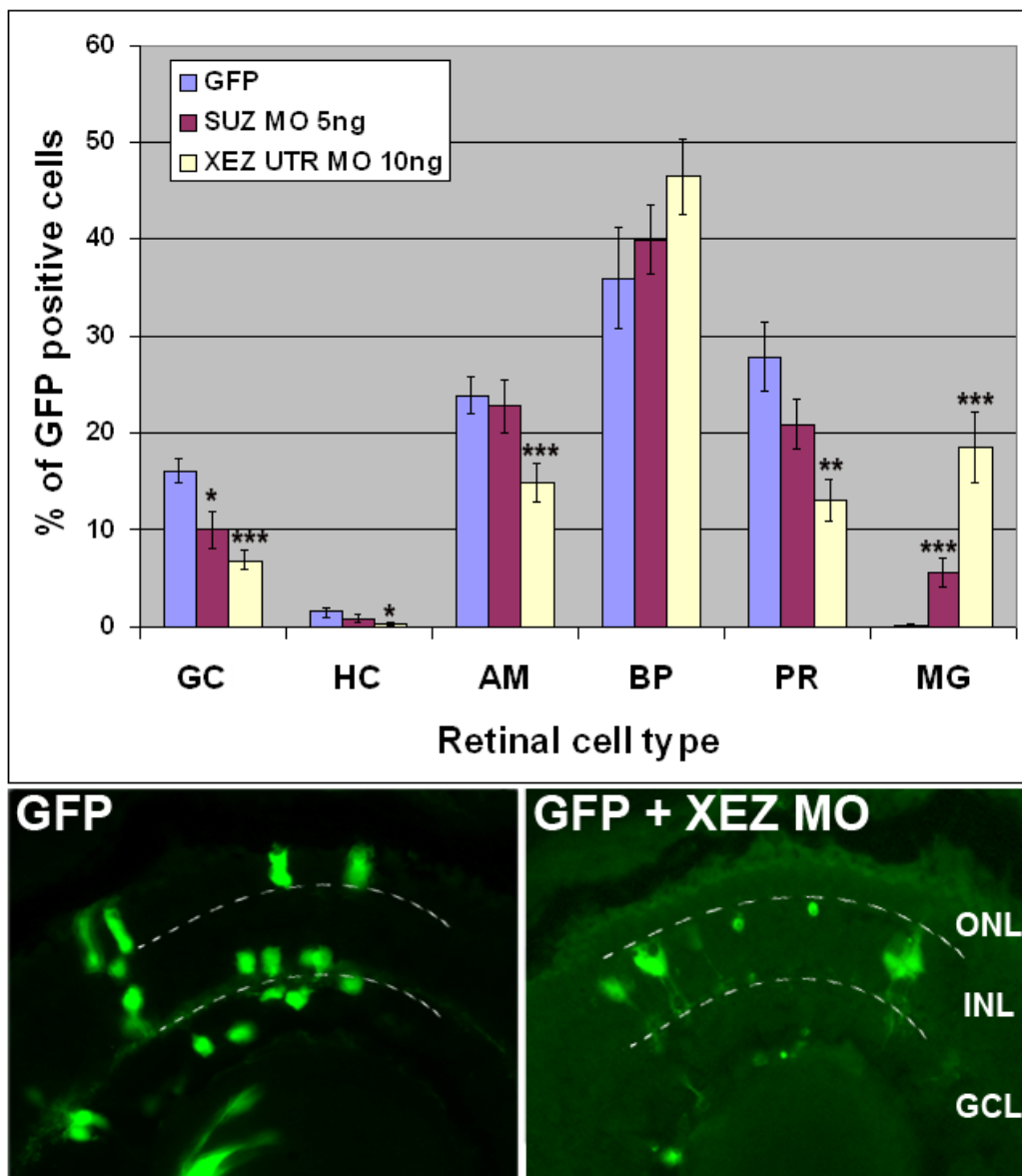
Figure 17. Blocking Xez or Xsuz12 inhibits early born retinal cell types.
Upper panel: retinal analysis after injection of Xez or Xsuz12 MOs. In both case there is an increase in later born cell types (i.e., Muller glia) at the expense of early born cell types (e.g., ganglion cells). Lower panel: representative images of retinal sections after injection of GFP mRNA only or with Xez UTR MO. Asterisks indicate degree of statistical significance when compared to injection of GFP mRNA only. Abbreviations: GC, ganglion cells; HC, horizontal cells; AM, amacrine cells; BP, bipolar cells; PR, photoreceptors; MG, Müller glia; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

XEZ ATG MO or SUZ12 MO with GFP mRNA in one cell of 32 cell stage embryos and immunostained retinal sections at stage 41 with an antibody that specifically recognizes H3K27 trimethylation. We found that GFP positive cells exhibit a marked reduction in the levels of H3K27me3, indicating that PRC2 function during development is required for H3K27me3 (Fig. 18A-D). Control MO does not affect H3K27me3 in *Xenopus* retina (Fig. 18E-F). Similarly, we found that Suz12 MO also causes a reduction in HEK27me3 levels (Fig. 19A-D). We conclude that XEZ and XSUZ12 contribute to trimethylating H3K27 in *Xenopus* retina. However, these data could not rule out the chance that a *Xenopus* homologue of Ezh1 is present and has residual activity in postmitotic retinal cells.

H3K27me3 is dynamic and selectively decorates a subset of *Xenopus* genes

Given that PRC2 is essential for retinal neurogenesis, we sought to understand the molecular mechanism underlying its function by defining retinal genes that are targeted for PRC2-mediated H3K27me3 deposition. For this purpose we collaborated with Dr Gert Veenstra's group who has previously analyzed the dynamics of H3K27me3, RNA Polymerase II (RNAPII) and H3K4me3 occupancy during development of *Xenopus tropicalis* using chromatin immunoprecipitation (ChIP-seq) and RNA-seq technology (Akkers et al., 2009). Here, they analyzed the dynamics of H3K27me3 deposition on 1841 genes that are known to be expressed in *Xenopus* eye based on Xenbase expression database of X. *tropicalis*. He compared the signal intensities of DNA methylation (DNAme; assessed by methyl-cap-sequencing; indicative of silencing), H3K4me3 (activation mark), RNAP II and RNA levels (indicating active transcription) to that of

Figure 18. Xez is required for H3K27me3 deposition in Xenopus retina.
Immunostaining of H3K27me3 (red) after co-injection of GFP mRNA with
Xez ATG MO (A-D) or Control MO (E-F). Hoechst labels nuclei (blue).
Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL,
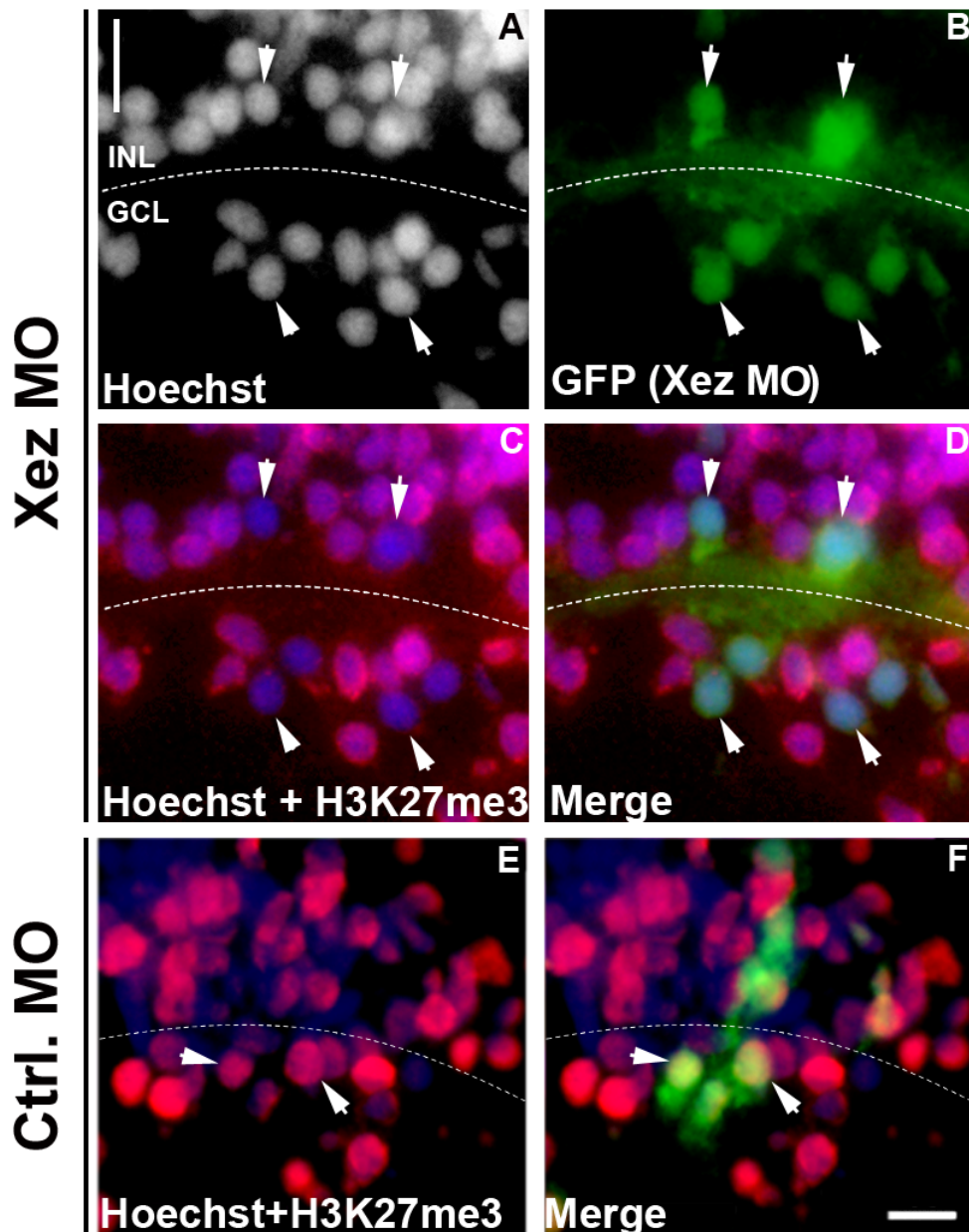ganglion cell layer. Scale bar 10 μm.
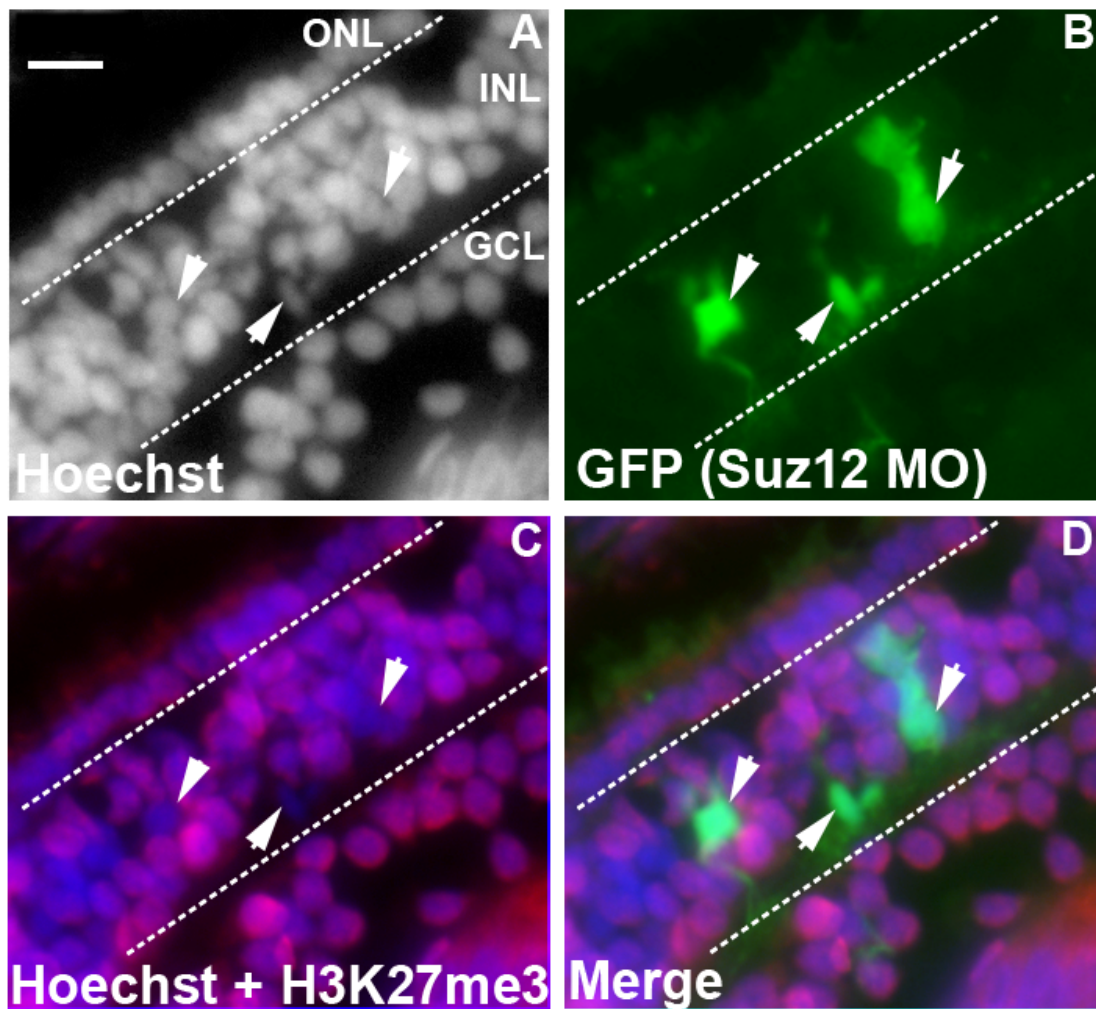
Figure 19. Suz12 is required for H3K27me3 deposition in Xenopus retina.
Immunostaining of H3K27me3 (red) after coinjection of GFP mRNA with
Xsuz12 ATG MO. Hoechst labels nuclei (blue). Abbreviations: ONL, outer nuclear
layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 10 μm.

H3K27me3 across blastula (stage 9), early neurulation (stage 12), open neural plate (stage 16) and optic cup stages (stage 30). Occupancy was examined across ±10 kb from the transcription start site (TSS), and the data were hierarchy clustered and visualized in a heat map.

Remarkably, his analysis revealed that only 43 of the examined genes showed a considerable enrichment with H3K27me3 (see supplementary data; Table 1, and data not shown). Many genes that are expressed in the eye might have been excluded from this analysis in part because the Xenbase database is not complete, and many genes that are expressed in the retina are not represented. Therefore, we prepared a list of specific candidate genes that are expressed in the retina and known to have important functions during retinogenesis, such as components of Wnt and Notch signaling pathways, EFTFs and Sox2 (Dorsky et al., 1995; Van Raay et al., 2005; Zuber et al., 2003). The analysis was then repeated for the genes on this list. Among those, only a group of 11 genes are highly decorated with H3K27me3, further suggesting target selectivity of PRC2 (see supplementary data; Table 2).

Overall, the analysis has revealed several aspects regarding the mode of action of PRC2 and the dynamics of its mark H3K27me3 (see supplementary data). First, we found that H3K27me3 abundantly marks only a fraction of the examined genes, suggesting that PRC2 recruitment to retinal gene promoters during development is selective. Interestingly, some promoters (i.e., *Vsx1*) seem to be repressed even when they are not highlighted with appreciable levels of H3K27me3 (compare RNA and H3K27me3 levels at stage 9 in supplementary data), indicating that H3K27me3-mediated repression is not the only mechanism for negative regulation of gene expression in *Xenopus*. Second, the

levels of H3K27me3 deposition on promoters fluctuate during development, suggesting that they

are temporally regulated (Akkers et al., 2009). For instance, the bHLH factor NeuroD2 is enriched with H3K27me3 only during neurulation stages (stage 12-16) then methylation is drastically decreased during later stages, indicating that H3K27me3 deposition is exquisitely dynamic (see supplementary data). Third, many of the H3K27me3-labled loci belong to the homeodomain gene family, suggesting a possible evolutionarily conserved role of the polycomb proteins in the regulation of homeodomain gene transcription (see supplementary data). Fourth, some known negative regulators of neurogenesis are targets for H3K27me3 methylation. For instance, the bHLH gene *Hes2* is strongly labeled with H3K27me3, and thus is a possible target for PRC2-mediated repression (see supplementary data). This may provide an explanation for why loss of *Xez* inhibits retinal differentiation since HES2 negatively

regulates retinal neurogenesis and has been shown to promote glial cell fate when overexpressed in *Xenopus* (Solter et al., 2006). Finally, we noticed that H3K27me3 does not label the PRC2 core components *Xez, Xeed, Suz12* or *Rbbp4*, suggesting that the catalytic function of PRC2 does not mediate repression of its own subunit expression during development (see supplementary data).

Table 1. List of genes that are expressed in Xenopus eye and are targets for H3K27me3

	H3K27me3-marked genes		H3K27me3-marked genes
1	lhx8|scaffold_187:638982-658982	**23**	tfap2b|scaffold_63:1588694-1608694
2	tal1|scaffold_1:2814226-2834226	**24**	six2|scaffold_25:1417565-1437565
3	slc32a1|scaffold_38:1173751-1193751	**25**	tbx5|scaffold_455:561233-581233
4	gsx2|scaffold_107:798886-818886	**26**	pitx2|scaffold_89:421117-441117
5	pax6|scaffold_399:634691-654691	**27**	isl2|scaffold_103:2341623-2361623
6	neurod1|scaffold_15:4573788-4593788	**28**	fst|scaffold_700:467993-487993
7	cldn5|scaffold_12:3162643-3182643	**29**	mab21l1|scaffold_80:320869-340869
8	neurod2|scaffold_610:667037-687037	**30**	lhx1|scaffold_516:532853-552853
9	dio3|scaffold_222:331385-351385	**31**	tbx2|scaffold_72:2271992-2291992
10	tfap2e|scaffold_411:848848-868848	**32**	foxc1|scaffold_95:2476171-2496171
11	fezf1|scaffold_121:2284147-2304147	**33**	foxd1|scaffold_113:1104930-1124930
12	grem1|scaffold_37:3633915-3653915	**34**	otx2|scaffold_68:1506061-1526061
13	nr2f1|scaffold_58:509696-529696	**35**	sox2|scaffold_245:1116801-1136801
14	dll1|scaffold_2:6640524-6660524	**36**	meis2|scaffold_37:3122165-3142165
15	nrp1|scaffold_503:281227-301227	**37**	pcdhl8|scaffold_16:2610731-2630731
16	cpeb1|scaffold_417:460548..496578	**38**	rbpms2|scaffold_417:601773-621773
17	tfap2a|scaffold_33:1101278..1120447	**39**	irx3|scaffold_146:2180665-2200665
18	ppm1l|scaffold_50:1000058-1020058	**40**	neurog2|scaffold_89:985047-986929
19	cyp26a1|scaffold_444:52089-72089	**41**	mrps30|scaffold_43:2621484..2629876
20	fam43a|scaffold_319:1098866-1118866	**42**	h1f0|scaffold_88:869330-889330
21	crx|scaffold_481:937053-957053	**43**	hes4|scaffold_207:1135429-1155429
22	fnta|scaffold_79:998022-1018022		

Table 2. List of genes that are expressed in Xenopus eye and are targets for H3K27me3.

	H3K27me3-marked genes
1	fzd5|scaffold_264:931001-951001
2	msx1|scaffold_441:684861-704861
3	hes1|scaffold_245:61668-81668
4	dll1|scaffold_2:6640524-6660524
5	id3|scaffold_106:2568946-2588946
6	sox2|scaffold_245:1116801-1136801
7	six3|scaffold_25:1488110-1508110
8	hes2|scaffold_73:304903-324903
9	lhx2|scaffold_405:715299-735299
10	six6|scaffold_68:3076029-3096029
11	vsx2|scaffold_389:612128-632128

Discussion

Expression of PRC2 core subunits and H3K27me3 in *Xenopus* retina

The roles of the PRC2-mediated repression in the maintenance and differentiation of ESCs, and during the development of several organs have been reported but its role underlying eye development remains unclear (Margueron and Reinberg, 2011). We reasoned that if PRC2 regulates some aspects of progression from retinal proliferation to differentiation then its components should be expressed in retinal progenitors during development. Indeed, in the retina the transcripts of PRC2 core subunits are transiently enriched in retinal progenitors in the CMZ region and downregulated in differentiated cells, in line with previously published data on skin and cortex development (Ezhkova et al., 2009; Pereira et al., 2010). This suggests the presence of a precise mechanism that tightly controls the maintenance of PRC2 transcription in retinal progenitors and shuts it off concomitant with differentiation. One possibility is that early transcription factors that drive eye specification control the expression of PRC2 and regulate its activities. Alternatively, PRC2 expression might be regulated by active signaling pathways in the retina such as Wnt signaling. Interestingly, loss of the Wnt component Frz5 partially mimics the effect of PRC2 inhibition, raising the possibility that retinal PRC2 expression is maintained by Wnt signaling (Van Raay et al., 2005).

The global level of the H3K27me3 mark is clearly elevated in postmitotic cells, which, though surprising, is still in agreement with the overall pattern of H3K27me3 in several tissues, including mouse retina and chick spinal cord (Akizu et al., 2010; Rao et al., 2010). The inverse correlation between PRC2 transcription levels and H3K27me3 depositions in *Xenopus* retina is puzzling, but may suggest that PRC2 proteins persist

during neural differentiation to modify chromatin in postmitotic cells. In support of this hypothesis, blocking translation of *Xez* in retinal progenitors negatively affects H3K27me3 levels in postmitotic cells. Yet, it remains necessary to directly investigate the presence of PRC2 proteins in mature retina by western blot or immunostaining. PRC2 protein functions might also be modulated in postmitotic cells by association with binding partners or by posttranslational modifications such as phosphorylation. Indeed EZH2 contains several phosphorylation sites that inhibit or enhance EZH2 catalytic function (Margueron and Reinberg, 2011).

Why H3K27me3 is enriched in retinal differentiated cells remains unclear but it is possible that it is used to stabilize terminal cell fate decisions by permanently suppressing the expression of all genes that are not related to the homeostasis of the fully differentiated cells. We speculate that H3K27me3 deposition in postmitotic cells is very stable and persists throughout adult life, unlike the case during development as will be discussed later.

Possible involvement of PRC2 in retinal proliferation

It has been shown that PRC2 is involved in controlling the proliferation capacity of cells during development and in cancer. For instance, skin and pancreas cells harboring *Ezh2* mutants are less proliferative due to derepression of tumor suppressor loci, which are known polycomb group targets (Bracken et al., 2007; Chen et al., 2009; Ezhkova et al., 2009). In cancer, altered levels of *Ezh2* have been associated with the progression and aggressiveness of many cancer types, underscoring the importance of PRC2 in proliferation (Bryant et al., 2007; Margueron and Reinberg, 2011; Varambally et

al., 2002). We found that there is a marked reduction in eye size upon inhibition of PRC2

function, an indicator of a possible effect on proliferation. Initial assessment suggests that

this microphthalmia is not due to increased levels of apoptosis or to a major alteration of

eye patterning but it still remains unclear whether progenitors retain their ability to divide

or are proliferating more slowly. Further analysis of BrdU uptake and proliferation

markers is required.

PRC2 regulates retinal differentiation in *Xenopus*

Given that PRC2 mediates the repression of differentiation genes in ESCs it was

initially predicted that the removal of PRC2 should lead to premature cellular

differentiation. However, it has become increasingly clear that PRC2 is essential for the

differentiation of ESCs in culture, as knockout of PRC2 function negatively affects the

ability of ESCs to differentiate (Boyer et al., 2006; Chamberlain et al., 2008; Pasini et al.,

2007). During organogenesis, the effect of *Ezh2* conditional knockouts is context-

dependent, but in certain cases it leads to accelerated differentiation and premature

upregulation of late terminal differentiation genes such as during epidermal development

(Ezhkova et al., 2009). In our model system, we found no evidence for premature

expression of retinal differentiation genes upon inhibition of PRC2 function. Rather, loss

of PRC2 inhibited the expression of the proneural bHLH genes, while retinal progenitor

specification genes were largely unaffected. Additionally, knocking down of PRC2 core

subunits altered subsequent cell fate choices by promoting a nonneural cell fate at the

expense of early born cell types, consistent with loss of proneural gene expression. Thus

our data support a model in which PRC2 is required for the proper activation of the

retinal differentiation program during the transition from proliferation to differentiation

and do not support a role of PRC2 in preventing premature differentiation in retinal progenitors.

Given that H3K27me3 levels increase concomitant with retinal differentiation, it is tempting to speculate that PRC2 may orchestrate several steps during neurogenesis. For instance, PRC2 might participate in coordinating the retinal differentiation programs executed by the proneural bHLH proteins. It will be interesting to examine whether proneural bHLH factors retain their ability to promote cell fate specification when PRC2 function is blocked.

H3K27me3 deposition during development

Understanding the mechanism by which PRC2 is regulating progression from proliferation to differentiation relies in part on identifying the retinal target genes of H3K27me3 during development. We found that the pattern of H3K27me3 deposition on retinal gene promoters is selective, where only a subgroup of the investigated genes are decorated with H3K27me3, suggesting that PRC2 is selectively recruited to the promoters of certain genes during retinal development. Interestingly, many of the H3K27me3 targets in our list belong to the homeodomain-containing group, a major regulator of eye development. However, whole mount in situ data upon knockdown of *Xez* does not indicate a major effect on homeodomain gene expression during the optic vesicle stage, although we saw a modest expansion in the expression of *Rx* domain during neurulation (data not shown). We do not know whether inhibition of PRC2 function affects homeodomain gene expression during the optic cup stage and/or later stages. A

quantitative PCR analysis might be necessary to accurately assess changes in the gene expression of H3K27me3-occupied genes after inhibition of PRC2 function.

Some of the identified H3K27me3 targets include negative regulators of neurogenesis such as the transcription factor *Hes2* (Fig. 24). This is interesting because it provides a possible mechanism by which PRC2 functions during eye development since overexpression of Hes2 blocks proneural gene expression, and promotes Müller glia formation in *Xenopus* (Solter et al., 2006). In this model PRC2 functions to prevent the premature expression of negative regulators of retinogenesis, thus promoting the progression of neural differentiation by preserving the neural potential of retinal progenitors. Consequently, blocking PRC2 function could lead to misexpression of negative regulators of retinogenesis, which would disrupt the progression from proliferation to differentiation.

Alternatively, since H3K27me3 also decorates non-neural genes (Akkers et al., 2009), blocking PRC2 may lead to a global misexpression of a large group of genes that ultimately disrupts the progression of neurogenesis. We do not favor this model because previous studies have shown that while PRC2 occupies the promoters of large number of genes in ESCs, its inhibition led to derepression of only a minority of those (Boyer et al., 2006; Ezhkova et al., 2009). Additionally, the overall effect of PRC2 conditional mutants on organ development is relatively mild, further supporting the hypothesis that PRC2 function is essential for the transcriptional regulation of a selective group of genes (Ezhkova et al., 2009; Hirabayashi et al., 2009). Eventually, determination of the H3K27me3-occupied genes that show significant changes in the expression level after

blocking PRC2 function may provide insights into the mechanism by which PRC2 operates during retinal development.

How PRC2 is recruited to certain retinal gene promoters remains unknown but this might occur because of the presence of unique DNA elements in the targeted promoters that facilitate this recruitment. Such unique sequences, termed Polycomb Response Elements (PRE), have been previously identified in mouse and Drosophila and shown to bind PRC2 via association with the polycomb protein YY1 (Bracken and Helin, 2009; Sing et al., 2009). Interestingly, YY1 expression in the nervous system during development of *Xenopus* mimics that of PRC2 subunits (Kwon and Chung, 2003). Whether PRE like elements exist in the promoters of retinal genes has not been determined.

Alternatively, PRC2 selective recruitment to retinal promoters may require the binding of factors that direct retinal development. This is a plausible hypothesis given that PRC2 is involved in the development of diverse organs including skin, muscle and cortex, and in every case it mediates the repression of a subset of organ-specific genes (Caretti et al., 2004; Ezhkova et al., 2009; Hirabayashi et al., 2009). A possible candidate is the canonical Wnt signaling effector β-catenin which has been shown to bind EZH2 in mammary epithelial cells and in MCF-7 cells (Li et al., 2009; Shi et al., 2007) . Canonical Wnt signaling is essential for retinal neural differentiation during *Xenopus* eye development (Agathocleous et al., 2009; Van Raay et al., 2005). Candidate transcription factors might themselves be targets for PRC2 (i.e., EFTFs), which could provide a potential feedback loop mechanism to regulate organ development (Bracken and Helin, 2009; Wang et al., 2010). Thus, to better understand the mechanism by which PRC2

operates during eye development it remains necessary to test whether PRC2 binds to retinal-specific factors by doing tissue-specific pull-down experiments or by performing screens using cDNA libraries extracted from retinal tissue.

Interestingly, we noticed that H3K27me3 deposition on retinal promoters changes during development, indicating that H3K27me3 addition is dynamic and thus may be used transiently to regulate retinal gene expression. This suggests the involvement of a H3K27me3 demethylase that is active during retinal development and catalyzes the removal of HEK27me3 in a temporal step-wise manner. Again this also may imply the involvement of retinal-specific signaling pathways or transcription factors that regulate demethylase expression and target selectivity. H3K27me3 demethylases have been identified in mammals and zebrafish, and have been implicated in neural differentiation but their characterization in *Xenopus* has not yet been reported (Burgold et al., 2008; Lan et al., 2007).

Finally, it is worth considering some limitations to the ChIP-seq analysis performed here. First we assumed that the pattern and targets for H3K27me3 depositions in *X. tropicalis* will be similar to those of *X.laevis* but this will need to be confirmed. Second, the ChIP was done using whole embryos and is not limited to retinal tissues, so we do not have a detailed picture of H3K27me3 changes specifically during eye development. Third, the analysis missed several developmental stages that would have revealed a more comprehensive view of H3K27me3 deposition during development.

The model

Based on current experimental data we propose a working model for PRC2 function during retinal development in *Xenopus*. PRC2 core subunits are provided maternally but their transcription is maintained in retinal progenitors by retinal-specific transcription factors and/or signaling pathways that are active in retinal tissue. During this period of development PRC2 catalyzes the addition of H3K27me3 to a select group of retinal genes, including negative regulators of neurogenesis, and thus mediates their repression. This allows retinal neurogenesis to proceed, and when the time is proper expression of the repressors is initiated to stop neural differentiation and/or promote Müller glia formation. Then, PRC2 transcription is shut down in postmitotic cells but protein levels are maintained to promote a permanent repression of genes irrelevant to the homeostasis of terminally differentiated cells.

References

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

Agathocleous, M., Iordanova, I., Willardsen, M. I., Xue, X. Y., Vetter, M. L., Harris, W. A., Moore, K. B., 2009. A directional Wnt/beta-catenin-Sox2-proneural pathway regulates the transition from proliferation to differentiation in the Xenopus retina. Development. 136**,** 3289-99.

Akizu, N., Estaras, C., Guerrero, L., Marti, E., Martinez-Balbas, M. A., 2010. H3K27me3 regulates BMP activity in developing spinal cord. Development. 137**,** 2915-25.

Akkers, R. C., van Heeringen, S. J., Jacobi, U. G., Janssen-Megens, E. M., Francoijs, K. J., Stunnenberg, H. G., Veenstra, G. J., 2009. A hierarchy of H3K4me3 and H3K27me3 acquisition in spatial gene regulation in Xenopus embryos. Dev Cell. 17**,** 425-34.

Aldiri, I., Vetter, M. L., 2009. Characterization of the expression pattern of the PRC2 core subunit Suz12 during embryonic development of Xenopus laevis. Dev Dyn. 238**,** 3185-92.

Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K., Bell, G. W., Otte, A. P., Vidal, M., Gifford, D. K., Young, R. A., Jaenisch, R., 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature. 441**,** 349-53.

Bracken, A. P., Helin, K., 2009. Polycomb group proteins: navigators of lineage pathways led astray in cancer. Nat Rev Cancer. 9**,** 773-84.

Bracken, A. P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., Theilgaard-Monch, K., Minucci, S., Porse, B. T., Marine, J. C., Hansen, K. H., Helin, K., 2007. The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev. 21**,** 525-30.

Bryant, R. J., Cross, N. A., Eaton, C. L., Hamdy, F. C., Cunliffe, V. T., 2007. EZH2 promotes proliferation and invasiveness of prostate cancer cells. Prostate. 67**,** 547-56.

Burgold, T., Spreafico, F., De Santa, F., Totaro, M. G., Prosperini, E., Natoli, G., Testa, G., 2008. The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. PLoS One. 3**,** e3034.

Caretti, G., Di Padova, M., Micales, B., Lyons, G. E., Sartorelli, V., 2004. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. Genes Dev. 18**,** 2627-38.

Chamberlain, S. J., Yee, D., Magnuson, T., 2008. Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells. 26**,** 1496-505.

Chen, H., Gu, X., Su, I. H., Bottino, R., Contreras, J. L., Tarakhovsky, A., Kim, S. K., 2009. Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. Genes Dev. 23**,** 975-85.

D'Autilia, S., Decembrini, S., Casarosa, S., He, R. Q., Barsacchi, G., Cremisi, F., Andreazzoli, M., 2006. Cloning and developmental expression of the Xenopus homeobox gene Xvsx1. Dev Genes Evol. 216**,** 829-34.

Dorsky, R. I., Rapaport, D. H., Harris, W. A., 1995. Xotch inhibits cell differentiation in the Xenopus retina. Neuron. 14**,** 487-96.

Ezhkova, E., Pasolli, H. A., Parker, J. S., Stokes, N., Su, I. H., Hannon, G., Tarakhovsky, A., Fuchs, E., 2009. Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell. 136**,** 1122-35.

Faust, C., Lawson, K. A., Schork, N. J., Thiel, B., Magnuson, T., 1998. The Polycomb-group gene eed is required for normal morphogenetic movements during gastrulation in the mouse embryo. Development. 125**,** 4495-506.

Ferreiro, B., Skoglund, P., Bailey, A., Dorsky, R., Harris, W. A., 1993. XASH1, a Xenopus homolog of achaete-scute: a proneural gene in anterior regions of the vertebrate CNS. Mech Dev. 40**,** 25-36.

Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D., Khorasanizadeh, S., 2003. Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev. 17**,** 1870-81.

Hatakeyama, J., Kageyama, R., 2004. Retinal cell fate determination and bHLH factors. Semin Cell Dev Biol. 15**,** 83-9.

Hemmati-Brivanlou, A., de la Torre, J. R., Holt, C., Harland, R. M., 1991. Cephalic expression and molecular characterization of Xenopus En-2. Development. 111**,** 715-24.

Hensey, C., Gautier, J., 1998. Programmed cell death during Xenopus development: a spatio-temporal analysis. Dev Biol. 203**,** 36-48.

Hirabayashi, Y., Suzki, N., Tsuboi, M., Endo, T. A., Toyoda, T., Shinga, J., Koseki, H., Vidal, M., Gotoh, Y., 2009. Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. Neuron. 63**,** 600-13.

Hirsch, N., Harris, W. A., 1997. Xenopus Pax-6 and retinal development. J Neurobiol. 32, 45-61.

Hsieh, J., Gage, F. H., 2004. Epigenetic control of neural stem cell fate. Curr Opin Genet Dev. 14, 461-9.

Huang, S., Moody, S. A., 1993. The retinal fate of Xenopus cleavage stage progenitors is dependent upon blastomere position and competence: studies of normal and regulated clones. J Neurosci. 13, 3193-210.

Hutcheson, D. A., Vetter, M. L., 2001. The bHLH factors Xath5 and XNeuroD can upregulate the expression of XBrn3d, a POU-homeodomain transcription factor. Dev Biol. 232, 327-38.

Kanekar, S., Perron, M., Dorsky, R., Harris, W. A., Jan, L. Y., Jan, Y. N., Vetter, M. L., 1997. Xath5 participates in a network of bHLH genes in the developing Xenopus retina. Neuron. 19, 981-94.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., Reinberg, D., 2002. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. 16, 2893-905.

Kwon, H. J., Chung, H. M., 2003. Yin Yang 1, a vertebrate polycomb group gene, regulates antero-posterior neural patterning. Biochem Biophys Res Commun. 306, 1008-13.

Lan, F., Bayliss, P. E., Rinn, J. L., Whetstine, J. R., Wang, J. K., Chen, S., Iwase, S., Alpatov, R., Issaeva, I., Canaani, E., Roberts, T. M., Chang, H. Y., Shi, Y., 2007. A histone H3 lysine 27 demethylase regulates animal posterior development. Nature. 449, 689-94.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., Weintraub, H., 1995. Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science. 268, 836-44.

Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., Chevalier, B., Johnstone, S. E., Cole, M. F., Isono, K., Koseki, H., Fuchikami, T., Abe, K., Murray, H. L., Zucker, J. P., Yuan, B., Bell, G. W., Herbolsheimer, E., Hannett, N. M., Sun, K., Odom, D. T., Otte, A. P., Volkert, T. L., Bartel, D. P., Melton, D. A., Gifford, D. K., Jaenisch, R., Young, R. A., 2006. Control of developmental regulators by Polycomb in human embryonic stem cells. Cell. 125, 301-13.

Lessard, J. A., Crabtree, G. R., 2010. Chromatin regulatory mechanisms in pluripotency. Annu Rev Cell Dev Biol. 26, 503-32.

Li, X., Gonzalez, M. E., Toy, K., Filzen, T., Merajver, S. D., Kleer, C. G., 2009. Targeted overexpression of EZH2 in the mammary gland disrupts ductal morphogenesis and causes epithelial hyperplasia. Am J Pathol. 175**,** 1246-54.

Livesey, F. J., Cepko, C. L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci. 2**,** 109-18.

Logan, M. A., Steele, M. R., Van Raay, T. J., Vetter, M. L., 2005. Identification of shared transcriptional targets for the proneural bHLH factors Xath5 and XNeuroD. Dev Biol. 285**,** 570-83.

Ma, Q., Kintner, C., Anderson, D. J., 1996. Identification of neurogenin, a vertebrate neuronal determination gene. Cell. 87**,** 43-52.

Margueron, R., Reinberg, D., 2011. The Polycomb complex PRC2 and its mark in life. Nature. 469**,** 343-9.

Mathers, P. H., Grinberg, A., Mahon, K. A., Jamrich, M., 1997. The Rx homeobox gene is essential for vertebrate eye development. Nature. 387**,** 603-7.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S., Sasai, Y., 1998. Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. Development. 125**,** 579-87.

Nekrasov, M., Wild, B., Muller, J., 2005. Nucleosome binding and histone methyltransferase activity of Drosophila PRC2. EMBO Rep. 6**,** 348-53.

O'Carroll, D., Erhardt, S., Pagani, M., Barton, S. C., Surani, M. A., Jenuwein, T., 2001. The polycomb-group gene Ezh2 is required for early mouse development. Mol Cell Biol. 21**,** 4330-6.

Ohsawa, R., Kageyama, R., 2008. Regulation of retinal cell fate specification by multiple transcription factors. Brain Res. 1192**,** 90-8.

Pasini, D., Bracken, A. P., Hansen, J. B., Capillo, M., Helin, K., 2007. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol. 27**,** 3769-79.

Pasini, D., Bracken, A. P., Jensen, M. R., Lazzerini Denchi, E., Helin, K., 2004. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J. 23**,** 4061-71.

Patterson, K. D., Cleaver, O., Gerber, W. V., White, F. G., Krieg, P. A., 2000. Distinct expression patterns for two Xenopus Bar homeobox genes. Dev Genes Evol. 210**,** 140-4.

Peng, J. C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A., Wysocka, J., 2009. Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell. 139**,** 1290-302.

Pereira, J. D., Sansom, S. N., Smith, J., Dobenecker, M. W., Tarakhovsky, A., Livesey, F. J., 2010. Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. Proc Natl Acad Sci U S A. 107**,** 15957-62.

Perron, M., Kanekar, S., Vetter, M. L., Harris, W. A., 1998. The genetic sequence of retinal development in the ciliary margin of the Xenopus eye. Dev Biol. 199**,** 185-200.

Pietersen, A. M., van Lohuizen, M., 2008. Stem cell regulation by polycomb repressors: postponing commitment. Curr Opin Cell Biol. 20**,** 201-7.

Rajasekhar, V. K., Begemann, M., 2007. Concise review: roles of polycomb group proteins in development and disease: a stem cell perspective. Stem Cells. 25**,** 2498-510.

Rao, R. C., Tchedre, K. T., Malik, M. T., Coleman, N., Fang, Y., Marquez, V. E., Chen, D. F., 2010. Dynamic patterns of histone lysine methylation in the developing retina. Invest Ophthalmol Vis Sci. 51**,** 6784-92.

Reijnen, M. J., Hamer, K. M., den Blaauwen, J. L., Lambrechts, C., Schoneveld, I., van Driel, R., Otte, A. P., 1995. Polycomb and bmi-1 homologs are expressed in overlapping patterns in Xenopus embryos and are able to interact with each other. Mech Dev. 53**,** 35-46.

Shen, X., Liu, Y., Hsu, Y. J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G. C., Orkin, S. H., 2008. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell. 32**,** 491-502.

Shi, B., Liang, J., Yang, X., Wang, Y., Zhao, Y., Wu, H., Sun, L., Zhang, Y., Chen, Y., Li, R., Zhang, Y., Hong, M., Shang, Y., 2007. Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells. Mol Cell Biol. 27**,** 5105-19.

Showell, C., Cunliffe, V. T., 2002. Identification of putative interaction partners for the Xenopus Polycomb-group protein Xeed. Gene. 291**,** 95-104.

Sing, A., Pannell, D., Karaiskakis, A., Sturgeon, K., Djabali, M., Ellis, J., Lipshitz, H. D., Cordes, S. P., 2009. A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. Cell. 138**,** 885-97.

Solter, M., Locker, M., Boy, S., Taelman, V., Bellefroid, E. J., Perron, M., Pieler, T., 2006. Characterization and function of the bHLH-O protein XHes2: insight into the mechanisms controlling retinal cell fate decision. Development. 133**,** 4097-108.

Sumanas, S., Ekker, S. C., 2001. Xenopus frizzled-5: a frizzled family member expressed exclusively in the neural retina of the developing eye. Mech Dev. 103**,** 133-6.

Turner, B. M., 2010. Environmental sensing by chromatin: An epigenetic contribution to evolutionary change. FEBS Lett.

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

Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., Rubin, M. A., Chinnaiyan, A. M., 2002. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 419**,** 624-9.

Vernon, A. E., Philpott, A., 2003. The developmental expression of cell cycle regulators in Xenopus laevis. Gene Expr Patterns. 3**,** 179-92.

Wang, L., Jin, Q., Lee, J. E., Su, I. H., Ge, K., 2010. Histone H3K27 methyltransferase Ezh2 represses Wnt genes to facilitate adipogenesis. Proc Natl Acad Sci U S A. 107**,** 7317-22.

Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Maeda, R., Nojima, Y., Li, H., Okamoto, H., Masai, I., 2005. Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. Development. 132**,** 3027-43.

Yoshitake, Y., Howard, T. L., Christian, J. L., Hollenberg, S. M., 1999. Misexpression of Polycomb-group proteins in Xenopus alters anterior neural development and represses neural target genes. Dev Biol. 215**,** 375-87.

Yu, J., Yu, J., Rhodes, D. R., Tomlins, S. A., Cao, X., Chen, G., Mehra, R., Wang, X., Ghosh, D., Shah, R. B., Varambally, S., Pienta, K. J., Chinnaiyan, A. M., 2007. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res. 67**,** 10657-63.

Zhou, X., Hollemann, T., Pieler, T., Gruss, P., 2000. Cloning and expression of xSix3, the Xenopus homologue of murine Six3. Mech Dev. 91**,** 327-30.

Zimmerman, K., Shih, J., Bars, J., Collazo, A., Anderson, D. J., 1993. XASH-3, a novel Xenopus achaete-scute homolog, provides an early marker of planar neural

induction and position along the mediolateral axis of the neural plate. Development. 119**,** 221-32.

Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G., Harris, W. A., 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development. 130**,** 5155-67.

CHAPTER 4

ANLYSIS OF *Jarid2* EXPRESSION AND FUNCTION IN

THE DEVELOPING CNS OF *XENOPUS*

Abstract

The Polycomb Repressive Complex PRC2 is essential for the regulation of key developmental events during organogenesis, including the proper progression from proliferation to differentiation in the developing *Xenopus* retina. However, the molecular mechanism underlying PRC2 function during eye development remains poorly understood. Recently, several studies found that JARID2, a member of the Jumonji C domain protein family, associates with PRC2 and modulates its DNA binding and catalytic activity. The expression pattern of the PRC2 core subunits has been characterized but *Jarid2* expression and its contribution to eye development in *Xenopus* is unknown. Here we show that *Jarid2* is maternally supplied and continues to be expressed throughout *Xenopus* development, including in the developing central nervous system. Surprisingly, *Jarid2* is highly transcribed in retinal and brain postmitotic neurons, in direct contrast to the expression pattern of the PRC2 core subunits. Given that JARID2 is associated with H3K9me2 deposition, we also characterized the levels of this modification in the developing central nervous system and found that H3K9me2 enrichment positively correlates with the expression of *Jarid2* in postmitotic cells. Knockdown of *Jarid2* function by a translation blocking morpholino suppresses retinal differentiation but does not affect retinoblast specification or H3K27me3 levels in retinal differentiated cells. Taken together, our data indicate that the expression pattern of *Jarid2* is distinct from that of the PRC2 core subunits and suggests a unique role for *Jarid2* in retinal differentiation.

Introduction

      Jarid2 (also known as Jumonji) is the founding member of the Jumonji C (Jmj C) domain containing protein family, and was initially identified in a mouse gene trap screen to define factors important for murine development (Motoyama and Takeuchi, 1995; Takeuchi et al., 2006). JmjC domain has been shown to bear a histone demethylase activity, however JARID2 is catalytically dead because its Jmj C domain lacks key residues necessary for the enzymatic activity (Klose et al., 2006; Takeuchi et al., 2006; Herz and Shilatifard, 2010). JARID2 has been associated with transcription repression due to the presence of distinct DNA binding, protein interaction and nuclear localization motifs (Fig. 20A) (Jung et al., 2005). Interestingly, the repression activity of JARID2 has been mapped to a small motif near its N-terminus, away from its characteristic domains (Li et al., 2010; Pasini et al., 2010; Fig. 20A).

      How JARID2 represses genes is not fully understood, but data suggest that it may do so by association with chromatin remodeling factors that control histone posttranslational modifications. As mentioned, JARID2 does not bear histone demethylase activity, yet it can interact with the G9A-GLP complex and enhances its ability to deposit the repressive mark H3K9me2 on selected promoters (Kato et al., 2008; Shirato et al., 2009). More recently, JARID2 has been identified as a binding partner for the PRC2 complex that mediates its recruitment to DNA (Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). As previously discussed, PRC2 complex is a histone methyltransferase that catalyzes the addition of the repressive mark H3K27me3 (Kuzmichev et al., 2002). Interestingly, JARID2 and PRC2 co-occupy a large set of promoters in embryonic stem cells (ESCs), suggesting that JARID2 association with
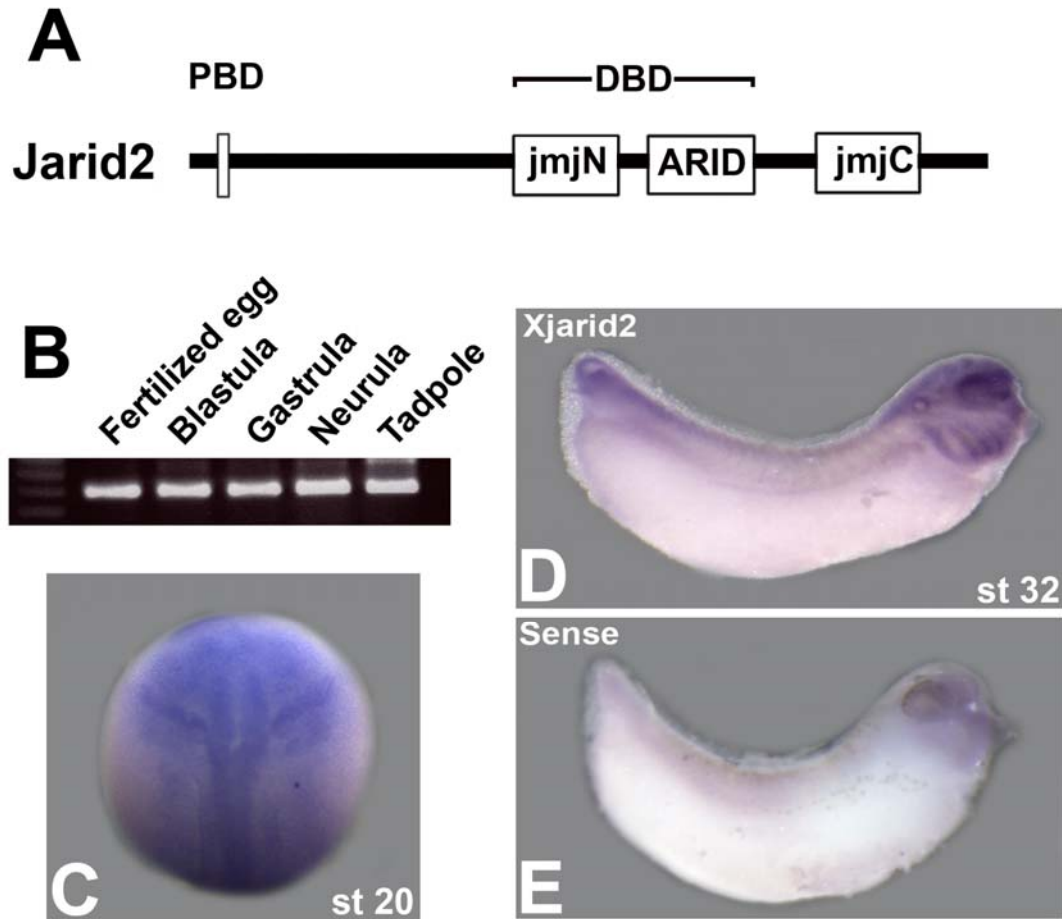
Figure 20. Expression of *Xjarid2* during *Xenopus* development. A) schematic representation of different domains in JARID2 (modified from Jung el al., 2005). B) RT-PCR analysis of *Xjarid2* expression during development. C-D) In situ analysis using *Xjarid2* anti-sense probe during stage 20 (C) and stage 32 (D). E) *Xjarid2* sense probe shows minimal signal. Abbreviations; PBD, PRC2 binding domain (also the repression domain); DBD, DNA binding domain; jmjN, jumonji N; ARID, AT-rich interacting domain; jmjC, jumonjiC.

PRC2 is important for the PRC2-mediated repression (Li et al., 2010; Shen et al., 2009). However, the exact function of JARID2 remains controversial as JARID2 does not seem to be required for global H3K27me3 deposition, and there are conflicting reports regarding its effect on PRC2 catalytic activity in vitro (Li et al., 2010; Peng et al., 2009). JARID2 can also interact with the tumor suppressor protein RB and enhance its ability to suppress E2F transcription in vitro (Jung et al., 2005).

Jarid2 is expressed during embryogenesis where it plays critical roles in the organogenesis of several tissues such as liver, brain and heart (Motoyama and Takeuchi, 1995; Motoyama et al., 1997; Takahashi et al., 2004; Takeuchi et al., 2006). Homozygous mutants for *Jarid2* are lethal mainly due to developmental defects in heart formation (Takahashi et al., 2004). Interestingly, the expression of *Jarid2* in multiple tissues during mouse development tends to increase concomitant with the onset of tissue differentiation, suggesting a possible conserved role in the transition from proliferation to differentiation (Takeuchi et al., 2006). For example, during heart development, *Jarid2* is expressed in differentiating cardiac myocytes where it negatively regulates proliferation by repressing the expression of the cell cycle gene *CyclinD1* (Shirato et al., 2009; Toyoda et al., 2003). In the brain, *Jarid2* is detected in neurons after final mitosis in the cerebrum and cerebellum, and is required to suppress *CyclinD1* expression in the hindbrain (Takahashi et al., 2007). *Jarid2* is also involved in the differentiation of the liver where it is critical for the proper maturation of fetal hepatocytes (Anzai et al., 2003). *Jarid2* may also have unique functions that are context-dependent. For instance, ESCs depleted of *Jarid2* have no proliferation defects, but their ability to differentiate into neural or mesodermal lineages is compromised (Landeira et al., 2010; Pasini et al., 2010). Finally, *Jarid2* is

required for the proper gastrulation in *Xenopus* embryos and the activin-dependent induction of mesoderm markers (Peng et al., 2009).

The above data underscore the importance of *Jarid2* in the progression from proliferation to differentiation during development of several tissues, but its possible roles in eye organogenesis have not been investigated. Given that PRC2 is expressed during *Xenopus* development and is involved in the proper differentiation of retinal progenitors (Chapter 3), we reasoned that X*jarid2* may also contribute to this process. This study has the potential to uncover unexplored roles of *Jarid2* during eye development and shed light on the mechanism by which PRC2 may regulate retinal differentiation in *Xenopus*.

Methods and Materials

Microinjections of morpholinos and mRNAs

Capped mRNAs were synthesized in vitro using Message Machine kit (Ambion). mRNAs were injected alone or in combination with antisense morpholino (MO) in one animal dorsal blastomere of 8 or 32 cell stage Xenopus embryos. The following mRNAs were used in injections: eGFP (500 pg), β-galactosidase (β-gal; 200 pg). Embryos were collected at the appropriate developmental stages and fixed with 4% PFA, and embryos marked with expression of eGFP or X-gal staining in the anterior part of the CNS were isolated for whole mount in situ hybridization analysis. X-gal staining was performed on β-gal injected embryos as previously described (Turner and Weintraub, 1994). *Xjarid2* MO was previously described (Peng et al., 2009).

In situ hybridization analysis

In situ hybridization was performed using whole embryos and brain sections as previously described (Hutcheson et al., 2005). The following DIG-labeled riboprobes were used for the analysis: Xash1 (Ferreiro et al., 1993), Xath5 (Kanekar et al., 1997), Xfz5 (Sumanas and Ekker, 2001), CyclinD1 (Vernon and Philpott, 2003), Six3 (Zhou et al., 2000), Xrx1 (Mathers et al., 1997), En-2 (Hemmati-Brivanlou et al., 1991), Hermes (Patterson et al., 2000).

To make *Xjarid2* probe, a cDNA fragment that spans the first 699 bp was amplified from a cDNA library prepared from whole embryos at stage 17-18 using the following primers: Forward: 5'-CCCCGAATTCATGAGCAAGGAAAGGCC-3'; Reverse: 5'-CCCCCTCGAGTCACCCATTGAAAAC-3'
The cDNA fragment was cloned into a BleuScript (BS) plasmid, and after linearization, DIG-labeled sense- and anti-sense probes were made using T7 and T3 RNA polymerase, respectively.

RT-PCR analysis

Total RNA was isolated from different stages of *Xenopus* embryos using Trizol (Inivtrogen) and was further purified by RNeasy Mini kit (Qiagen). First strand cDNA was synthesized as was previously described (Logan et al., 2005b). RT-PCR analysis for *Xjarid2* was performed using the following primers:
Forward: 5'-ATGGTCGGAAGAAAGGGTGG-3'
Reverse: 5'-ACTGTTTGGCTGGGATTGGG.

Immunohistochemistry

Immunostaining was performed using previously described methods. In brief, embryos were grown to stage 41 in 0.1X MMR, fixed in 4% PFA for 45 minutes, embedded in OCT and 14 micron sections were made using a cryostat. After blocking (5% goat serum and 0.1% Triton in PBS) for 30 minutes, sections were incubated with primary rabbit anti-H3K27me3 (Millipore, 1:100) or rabbit anti-H3K27me3 (Active Motif, 1:100) antibodies overnight at 4 °C. Then, slides were washed and a secondary Alexa Fluor 568-conjugated goat anti-rabbit antibody (Molecular Probes, 1:2000) was added for 2 hours at room temperature. Sections were counter stained with Hoechst (1:15000) to visualize nuclei.

Results

Jarid2 is expressed during *Xenopus* development

Given that PRC2 core subunits are expressed in the developing CNS of *Xenopus*, we reasoned that its binding partner, *Xjarid2*, might also have a similar expression pattern (Aldiri and Vetter, 2009). Thus, we first examined the temporal expression of *Xjarid2* by performing RT-PCR using RNA extracted from different developmental stages up to stage 41 (tadpole stage). *Xjarid2* expression was detected in the fertilized egg as well as in all developmental stages examined, suggesting that *Xjarid2* may have multiple roles during early and late stages of development (Fig. 20B and data not shown). To determine the spatial distribution of *Xjarid2* in postneurulation stages during development we performed whole-mount in situ hybridization analysis on selected stages using a probe that spans the first 699 bp of *Xjarid2* cDNA. At stage 20 (shortly after neural plate

closure), *Xjarid2* expression was weakly detectable in the presumptive spinal cord and head region (Fig. 20C). By stage 32 (tailbud stage), *Xjarid2* expression was observed in the developing central nervous system along the dorsoventral axis and in the head region, including the developing eye, branchial arches, otic vesicles and in the forebrain (Fig. 20D). These results are in agreement with the expression data of the PRC2 core subunits, *Xsuz12*, *Xeed*, *Rbbp4* and *Xez*, suggesting that *Xjarid2* and the core subunits of PRC2 may be coincidently expressed in neural tissues (Aldiri and Vetter, 2009).

To better define the expression domains of *Xjarid2* in the central nervous system, we performed in situ hybridization analysis of *Xjarid2* on retina, hindbrain and spinal cord sections at tadpole stage. We found that *Xjarid2* transcript in the nervous system is highly enriched in postmitotic cells and less detectable in proliferative zones (Fig. 21 and 22). For instance, in the retina, *Xjarid2* levels seem to be ubiquitously expressed in the ganglion cell layer (GCL), inner nuclear layer (INL) and, to a lesser extent, in the outer nuclear later (ONL) while expression in the Ciliary Marginal Zone (CMZ; the retinal proliferative zone) is hardly detectable (Fig. 21A-C). Similar results were found in the hindbrain and spinal cord: *Xjarid2* transcripts are downregulated in the ventricular wall region (where progenitors reside) and are highly expressed in postmitotic cells (Fig. 22A and C). These results are in stark contrast to the expression pattern of the core PRC2 subunits (i.e., *Xsuz12*) in the nervous system, which are primarily located in proliferative zones and downregulated upon neural differentiation (Fig. 22B, D; Chapter 3 and data not shown). Taken together, these data indicate that the expression of *Xjarid2* is distinct from that of the PRC2 core subunits, and suggest that *Xjarid2* function in the nervous system might be unique.
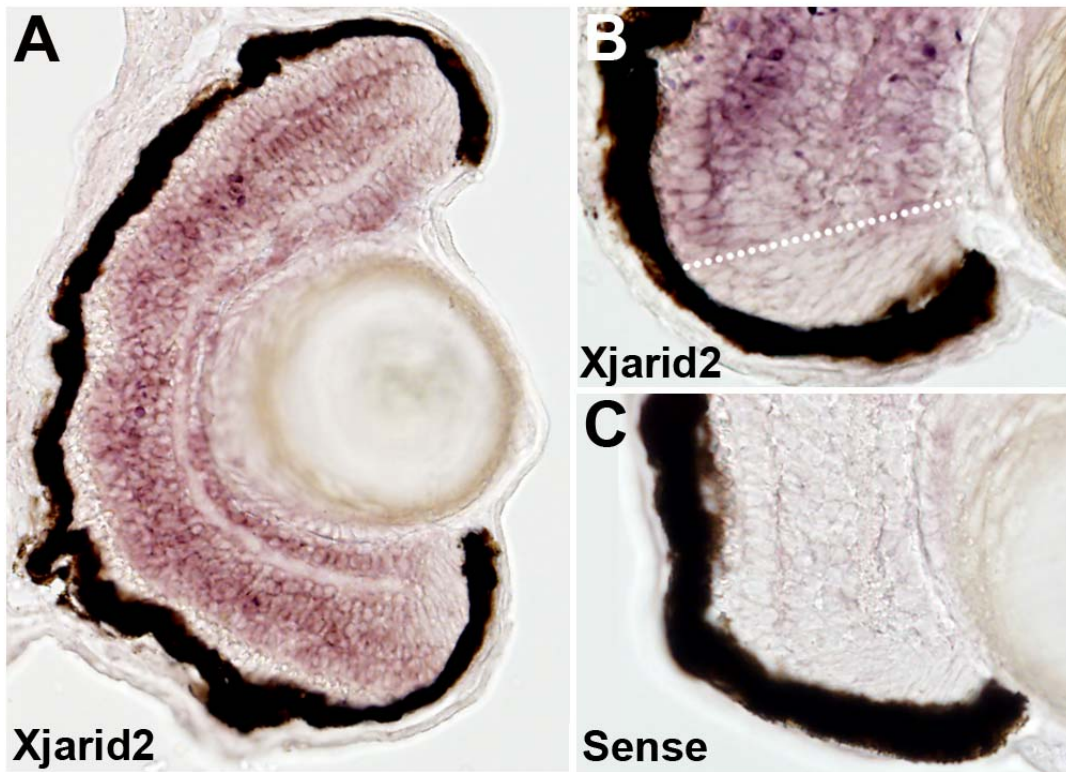
Figure 21. Expression of *Xjarid2* in mature retina at stage 41. A) *Xjarid2* is expressed in retinal postmitotic cells. B) The CMZ region of the retina shows reduced *Xjarid2* expression (compare the two areas flanking the dashed line). C) No staining is observed when retina is stained with *Xjarid2* sense probe.
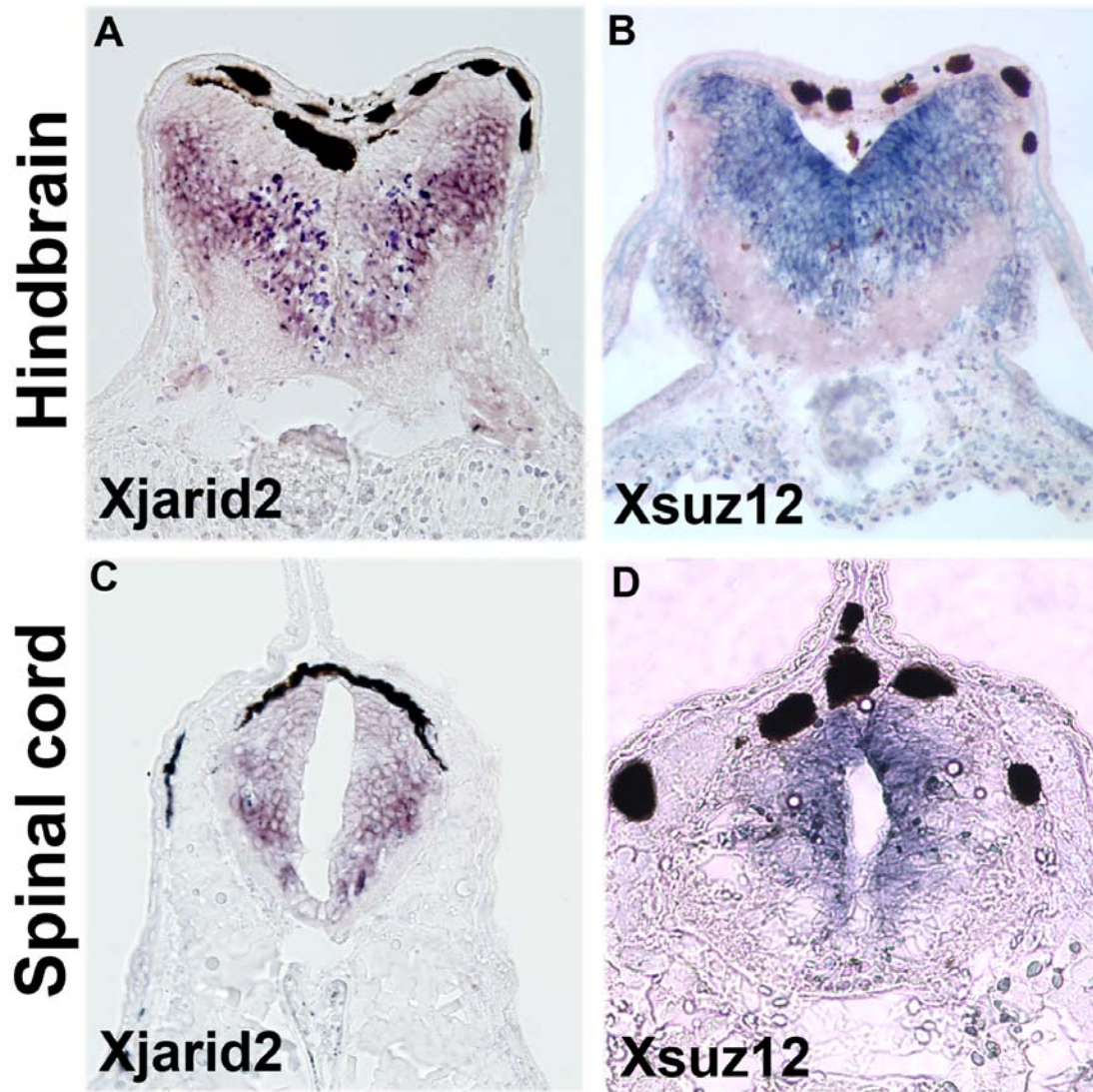
Figure 22. Comparative analysis of the expression of *Xjarid2* and *Xsuz12* in the brain and spinal cord. A and C) expression of *Xjarid2* is increased as cells differentiate. Note that in the spinal cord *Xjarid2* expression seems to be more apparent in the ventral side. B and D) Unlike *Xjarid2*, the expression of the PRC2 core subunit *Xsuz12* is enriched in the ventricular zone.

Xjarid2 is required for retinal differentiation

To determine the importance of *Xjarid2* during retinal development, we injected a previously characterized Xjarid2 MO in one dorsal blastomere of 8 cell stage embryos along with β-gal mRNA (to mark the injected side) and assayed the effect on eye development by performing in situ hybridization analysis (Peng et al., 2009). First, we analyzed the expression of the retinal progenitor specification and proliferation markers *Rx, Frz5, Six3* and *CyclinD1* at stage 20 and found that Xjarid2 MO does not alter the expression intensity of these genes (embryos with normal intensity: 100% n=48 for Rx, 100% n=19 for Six3, 100% n=14 for Frz5 and 100% n=14 for cycline D1; Fig. 23). However, we noticed that the expression domains of these markers tended to be a slightly larger on the injected side (embryos with larger expression domain: 42% n=48 for Rx, 37% n=19 for Six3, 50% n=14 for Frz5 and 57% n=14 for cyclinD1; Fig. 23). These data suggest that inhibition of *Xjarid2* function does not disrupt retinal progenitor specification.

Next we tested the effect of Xjarid2 MO on retinal differentiation by examining the expression of the proneural differentiation genes *Xath5* and *Xash,1* and the ganglion cell marker *Hermes*. We noticed a marked reduced in the expression of these markers on the injected side (embryos with reduced expression: 67% n=12 for Xath5, 41% n=22 for Xash1, 79% n=19 for Hermes; Fig. 24). Collectively, while retinal progenitor specification seems to proceed normally in *Jarid2* morphant embryos, expression of retinal differentiation genes is inhibited or delayed, suggesting that *Jarid2* is essential for retinal differentiation.
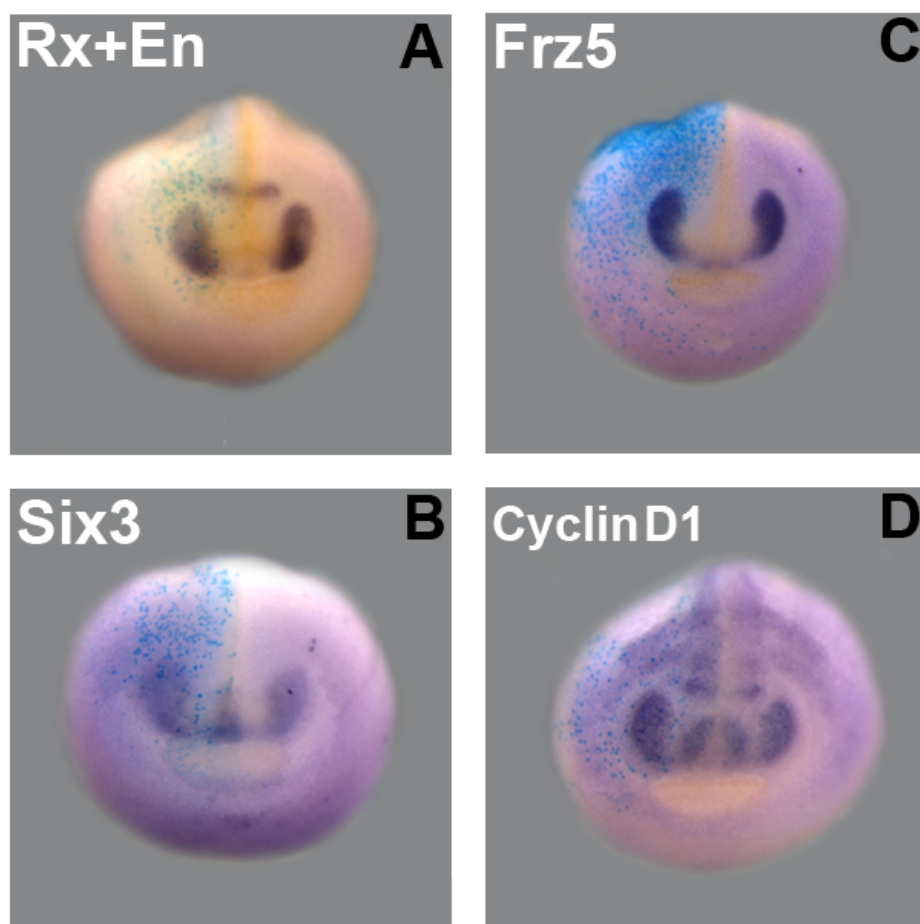
Figure 23. *Xjarid2* **is not required for retinal progenitor specification.**
The relative levels of expression of the indicated markers are unchanged.
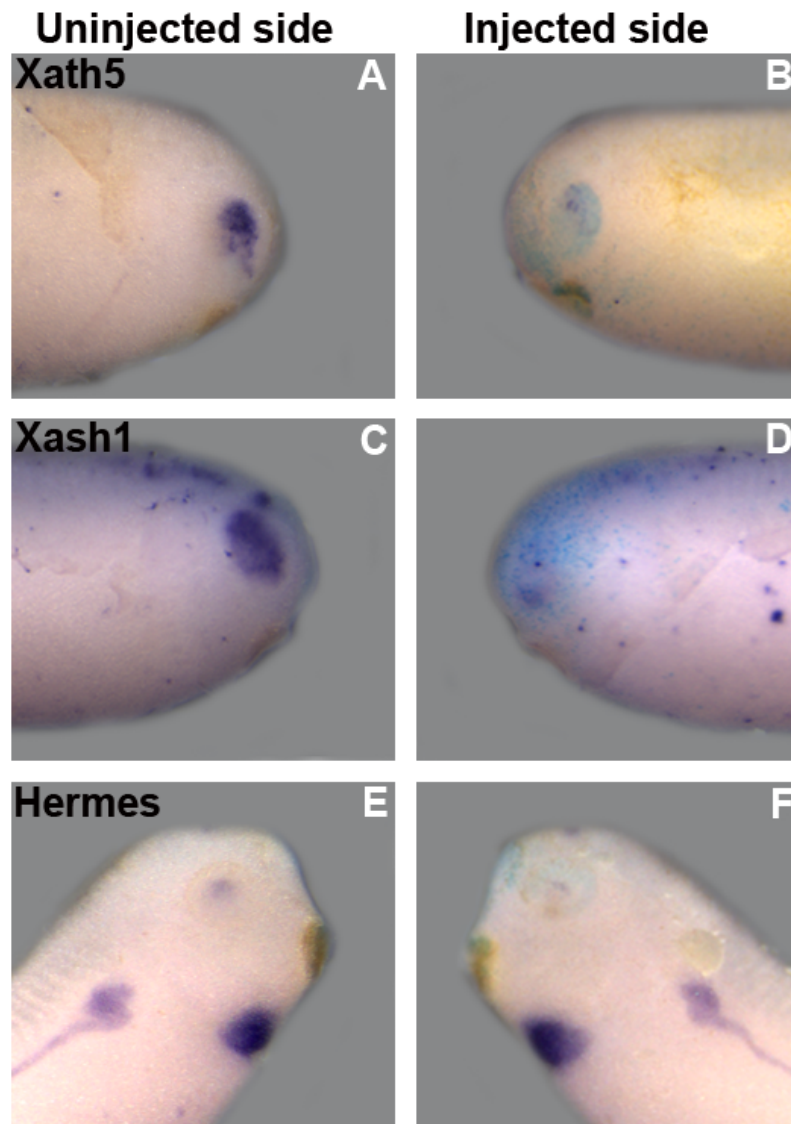Marker domains on the injected side tend to be a little larger.

Figure 24. Inhibition of *Xjarid2* blocks retinal neural differentiation.
The expression of proneural bHLH genes (A-D) and the ganglion cell
marker Hermes (E-F) is lost or reduced upon injection of Xjarid2 MO.

The expression of H3K9me2

Jarid2 function has been linked to chromatin regulation primarily through association with the methyltransferases responsible for H3K9me2 and H3K27me3 deposition (Pasini et al., 2010; Peng et al., 2009; Shirato et al., 2009). We have previously determined the global H3K27me3 levels in the mature retina and found that they are increased in retinal postmitotic cells (Chapter 3). However, the expression of H3K9me2 in *Xenopus* retina has not been characterized. We examined the distribution pattern of H3K9me2 by immunostaining using an antibody that specifically recognizes this mark, and similar to the expression of *Xjarid2* and HEK27me3, H3K29me2 levels were highly enriched in postmitotic cells in the nervous system (Fig. 25 and 26). In the retina, high levels of H3K9me2 were observed in the GCL and ONL and were weakly detectable in the ONL and the CMZ (Fig. 25A-F). Similar pattern was observed by examining sections from the brain and spinal cord (Fig. 26A-F). Collectively, we conclude that the global levels of H3K9me2 and H3K27me3 correlate positively with the expression of *Xjarid2* in the nervous system, suggesting that *Xjarid2* may be involved in the regulation of these marks.

Xjarid2 is not required for global H3K27me3 levels in the retina

How JARID2 contributes to the PRC2-mediated addition of H3K27me3 remains inconclusive. In vitro data from different groups suggest that the addition of JARID2 to PRC2 can inhibit or enhance its catalytic function (Li et al., 2010; Peng et al., 2009).
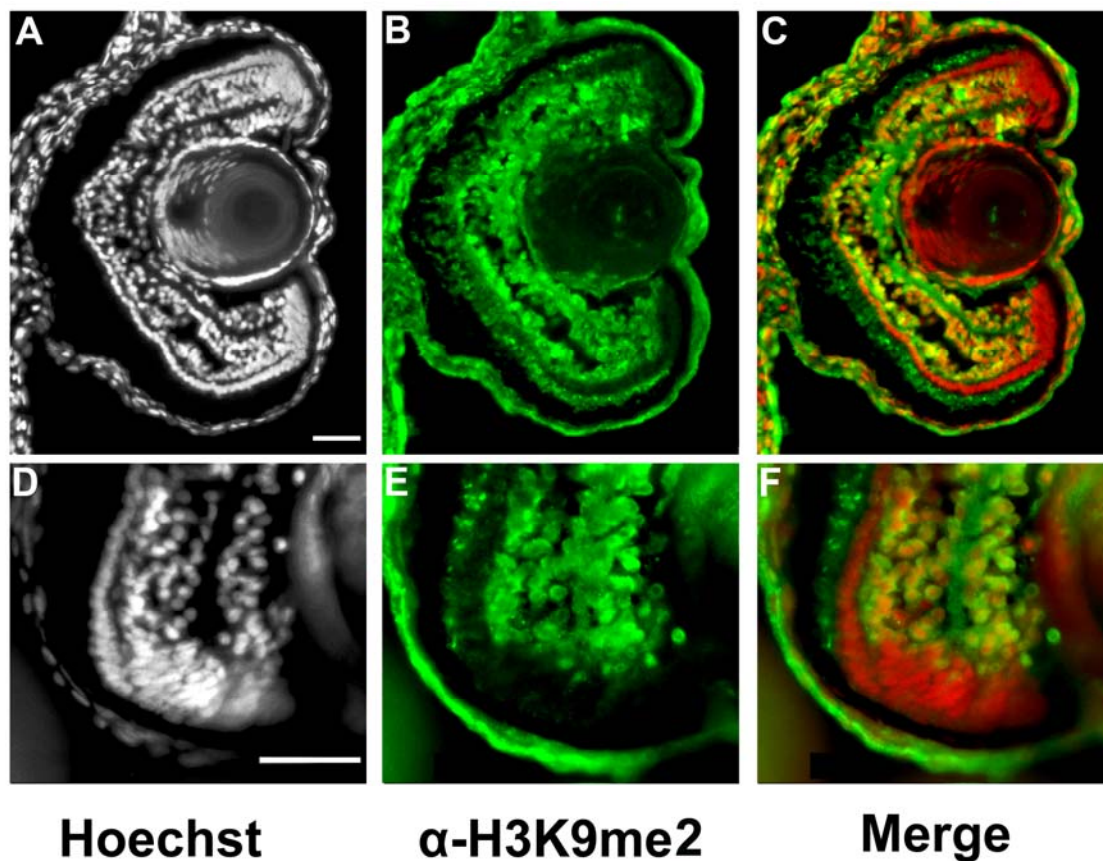
Figure 25. H3K9me2 levels are elevated in differentiated retinal cells.
(A-C) Immunostaining of a retinal section with antibody against H3K9me2. (D-F) A magnification of the peripheral part of the retina showing a sharp increase in H3K27me3 staining (green) as cells differentiate. Notice that the signal in the photoreceptor layer is not as strong as in other layers. Retinal cells were counter stained with Hoechst (red) to reveal nuclei. Scale bar 40 μm.
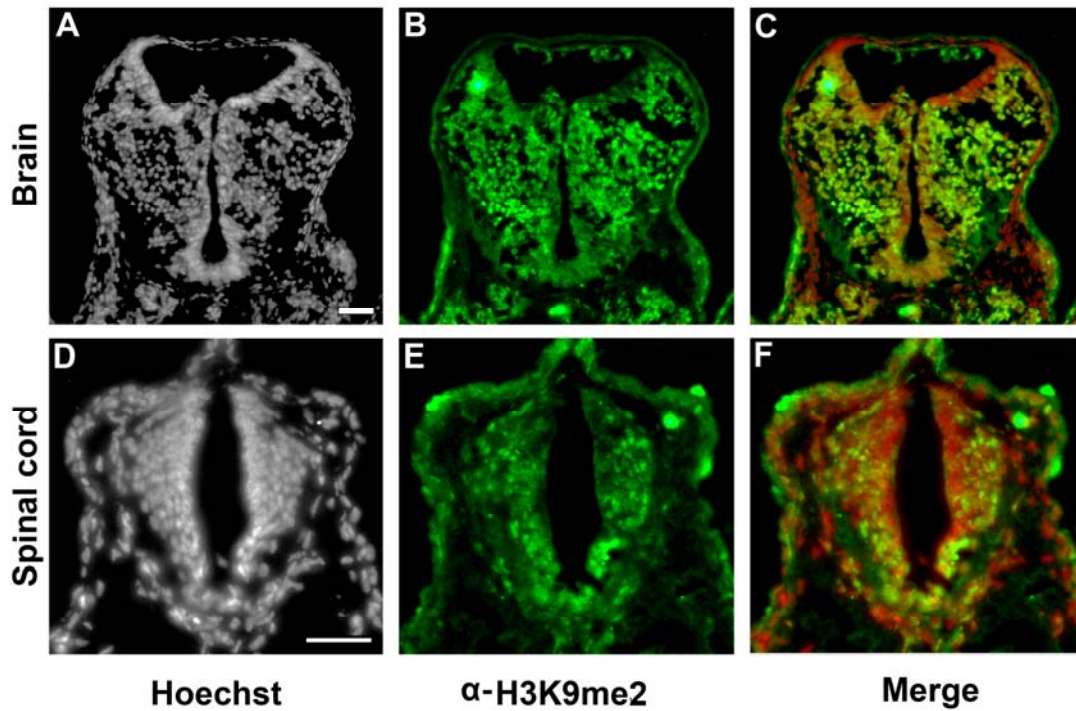
Figure 26. H3K9me2 levels are elevated in postmitotic cells in the brain and spinal cord. (A-C) Immunostaining of a retinal section with antibody against H3K9me2 (green) in the forebrain. (D-F) H3K9me2 staining in the spinal cord. Notice that signal is decreased in the areas adjacent to the ventricles. Sections were counter stained with Hoechst (red) to reveals nuclei. Scale bar 40 μm.

Similarly, levels of H3K27me3 on promoters are decreased (Li et al., 2010; Pasini et al., 2010) or increased (Peng et al., 2009; Shen et al., 2009) upon knockdown of *Jarid2* in ESCs. A common agreement among those studies is that JARID2 knockdown does not affect bulk H3K27me3 levels. To test whether this hypothesis is correct in *Xenopus* retina we injected one dorsal blastomere of 32 cell embryos with Xjarid2 MO and mRNA for GFP (to trace MO injected cells) and immunoassayed GFP-labeled retina with the H3K27me3 antibody. We did not observe any reduction in H3K27me3 levels in GFP positive cells, supporting the evidence that JARID2 is not required for global H3K27me3 in *Xenopus* (Fig. 27A-D). It remains to be determined whether it regulates H3K9me2 levels.

Discussion

Although the roles of the PRC2 core components during development have been under attention, the biological functions of its auxiliary subunits have been poorly characterized. Here we show that the PRC2 binding partner *Jarid2* is expressed during *Xenopus* development including in the CNS. Given that PRC2 core factors are largely transcribed in progenitor domains of the CNS (Chapter 3), we predicted that *Jarid2* expression would have a similar pattern. It was surprising to discover that *Jarid2* expression correlates positively with neural differentiation, suggesting that the mechanisms that control the transcription of *Jarid2* and the PRC2 core subunits might be distinct. We hypothesize that *Jarid2* expression in differentiated cells might be upregulated by factors that direct retinal neural differentiation programs such as the
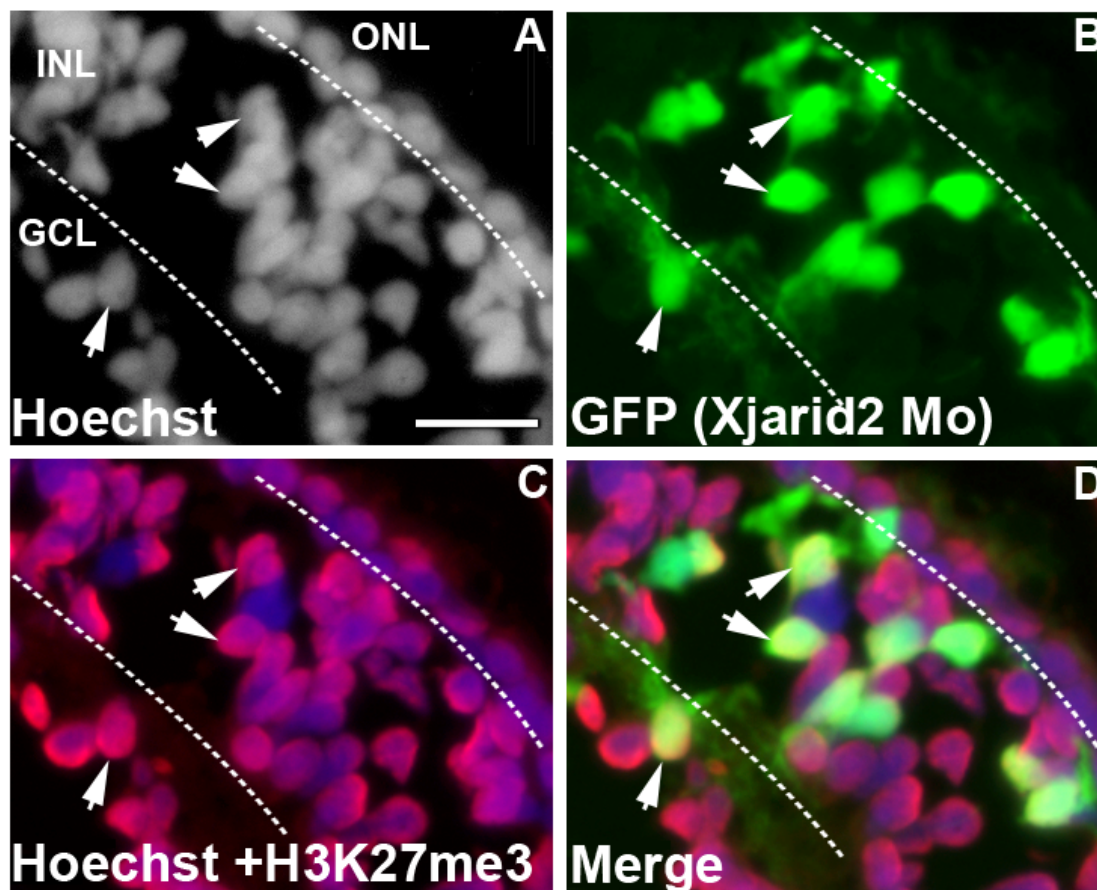
Figure 27. *Xjarid2* **is not required for global H3K27me3 deposition in Xenopus retina.**
(A-D) Immunostaining of H3K27me3 (red) after co-injection of GFP mRNA
with Xjarid2 MO. Hoechst labels nuclei (blue). Abbreviations: ONL, outer nuclear
layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 10 μm.

proneural bHLH proteins. Whether *Jarid2* is a downstream target of the proneural bHLH

factors remains to be addressed.

Why *Jarid2* is expressed in differentiated cells remains unclear but we speculate

that one of its primary roles is to repress proliferation by downregulating the expression

of positive cell cycle regulators such as *CyclinD1*.This is a plausible hypothesis given

that this function is conserved during hindbrain and heart development, and JARID2

directly binds and represses CyclinD1 promoter (Toyoda et al., 2003; Takahashi et al.,

2007). Our initial analysis suggests that blocking Jarid2 function in the open neural plate

and in the optic vesicle causes a mild expansion in the expression domain of *CyclinD1*,

but the expression intensity does not seem to change (Fig. 22 and data not shown).

Further analysis of the effect of blocking *Jarid2* on retinal proliferation and the

expression of cell cycle genes is required to test this hypothesis.

Alternatively, JARID2 may be cooperating with PRC2 in repressing a large group

of genes irrelevant to the homeostasis of fully differentiated cells. We do not favor this

model in part because Jarid2 is not required for bulk H3K27me3 in the retina. It is still

possible that JARID2 associates with PRC2 proteins only on a limited number of

promoters (will be discussed later). Interestingly, JARID2 might also be involved in the

transcriptional repression of the PRC2 core subunits themselves given the inverse

correlation between the expression of *Jarid2* and PRC2 core subunits in the mature

retina. Mechanistically, a possible regulatory mechanism for the JARID2-mediated

repression of PRC2 might be the RB-E2F pathway (Hallstrom and Nevins, 2009). It has

been shown that E2F induces the expression of the PRC2 core subunits *Eed* and *Ezh2* in

tumor cells and in fibroblasts (Muller et al., 2001; Bracken et al., 2003). E2F activities

are repressed by RB, a process that is potentiated by the interaction between JARID2 and RB (Jung et al., 2005; Swiss and Casaccia, 2010). Thus JARID2 might negatively control the expression of core PRC2 subunits in postmitotic cells by enhancing the repressive activity of RB on E2F which acts upstream of PRC2 core subunits.

Finally, there is a likelihood that JARID2 might be participating in coordinating the neural differentiation programs directed by the proneural bHLH factors. Evidence from the open neural plate stage suggests that Jarid2 is required for NeuroD's ability to promote primary neurogenesis (data not shown). Whether this is true during retinal development remains to be tested.

Based on RT-PCR analysis and in situ hybridization data we cannot rule out that low levels of *Jarid2* are expressed in retinal progenitors where it is actively cooperating with PRC2 core subunits to promote retinal differentiation. In support of this model, our preliminary loss of function data show that *Jarid2*, like PRC2, is not required for eye specification but is essential for the initiation of retinal differentiation genes, suggesting that Jarid2 is important for the proper progression from proliferation to differentiation. Interestingly, *Jarid2* function in promoting neurogenesis seems to be conserved in other parts of the CNS as we observed that blocking of *Jarid2* function inhibited primary neurogenesis at the open neural plate stage and blocked the expression of the proneural bHLH genes *NeuroD* and *NgnR1* (data not shown). It remains to be determined whether JARID2 interacts with PRC2 in retinal progenitors and whether both co-occupy similar targets during retinal development.

The relationship between JARID2 and H3K27me3 deposition is not well understood. Paradoxically, JARID2 is bona a fide binding partner of PRC2 but it does not

seem to be required for bulk H3K27me3 in either *Xenopus* or in ESCs (Peng et al., 2009; Shen et al., 2009). It has been proposed that PRC2 might exist in two modules: a JARID2-independent PRC2 that is responsible for the global deposition of H3K27me3 and a JARID2-containing PRC2 that works on selected promoters to fine tune H3K27me3 (Herz and Shilatifard, 2010). Therefore although *Jarid2* is not required for global H3K27me3 in the retina it might still function by modulating PRC2 activities on selected targets. It will be interesting to define target promoters that are occupied by PRC2 and JARID2 in retinal progenitors and in postmitotic cells.

Alternatively, JARID2 function in retinal development could be PRC2-independent. It was reported that JARID2 functions in promoting H3K9 methylation through binding the GLP-G9A complex, raising the possibility that JARID2 mediates retinal gene repression through H3K9me2. In support of this hypothesis, H3K9me2 deposition increases concomitant with retinal differentiation (Fig. 30). A third possibility is that JARID2 repressive ability requires the involvement of both H3K27me3 and H3K9me2 (Herz and Shilatifard, 2010). Distinguishing between these different possibilities can be addressed in part by comparing H3K9me2 targets to those occupied by H3K27me3 in wild type versus *Jarid2* morphant embryos.

References

Aldiri, I., Vetter, M. L., 2009. Characterization of the expression pattern of the PRC2 core subunit Suz12 during embryonic development of Xenopus laevis. Dev Dyn. 238**,** 3185-92.

Anzai, H., Kamiya, A., Shirato, H., Takeuchi, T., Miyajima, A., 2003. Impaired differentiation of fetal hepatocytes in homozygous jumonji mice. Mech Dev. 120**,** 791-800.

Ferreiro, B., Skoglund, P., Bailey, A., Dorsky, R., Harris, W. A., 1993. XASH1, a Xenopus homolog of achaete-scute: a proneural gene in anterior regions of the vertebrate CNS. Mech Dev. 40**,** 25-36.

Hallstrom, T. C., Nevins, J. R., 2009. Balancing the decision of cell proliferation and cell fate. Cell Cycle. 8**,** 532-5.

Herz, H. M., Shilatifard, A., 2010. The JARID2-PRC2 duality. Genes Dev. 24**,** 857-61.

Jung, J., Kim, T. G., Lyons, G. E., Kim, H. R., Lee, Y., 2005. Jumonji regulates cardiomyocyte proliferation via interaction with retinoblastoma protein. J Biol Chem. 280**,** 30916-23.

Landeira, D., Sauer, S., Poot, R., Dvorkina, M., Mazzarella, L., Jorgensen, H. F., Pereira, C. F., Leleu, M., Piccolo, F. M., Spivakov, M., Brookes, E., Pombo, A., Fisher, C., Skarnes, W. C., Snoek, T., Bezstarosti, K., Demmers, J., Klose, R. J., Casanova, M., Tavares, L., Brockdorff, N., Merkenschlager, M., Fisher, A. G., 2010. Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. Nat Cell Biol. 12**,** 618-24.

Li, G., Margueron, R., Ku, M., Chambon, P., Bernstein, B. E., Reinberg, D., 2010. Jarid2 and PRC2, partners in regulating gene expression. Genes Dev. 24**,** 368-80.

Logan, M. A., Steele, M. R., Vetter, M. L., 2005. Expression of synaptic vesicle two-related protein SVOP in the developing nervous system of Xenopus laevis. Dev Dyn. 234**,** 802-7.

Mathers, P. H., Grinberg, A., Mahon, K. A., Jamrich, M., 1997. The Rx homeobox gene is essential for vertebrate eye development. Nature. 387**,** 603-7.

Pasini, D., Cloos, P. A., Walfridsson, J., Olsson, L., Bukowski, J. P., Johansen, J. V., Bak, M., Tommerup, N., Rappsilber, J., Helin, K., 2010. JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. Nature. 464**,** 306-10.

Patterson, K. D., Cleaver, O., Gerber, W. V., White, F. G., Krieg, P. A., 2000. Distinct expression patterns for two Xenopus Bar homeobox genes. Dev Genes Evol. 210, 140-4.

Peng, J. C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A., Wysocka, J., 2009. Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell. 139, 1290-302.

Shen, X., Kim, W., Fujiwara, Y., Simon, M. D., Liu, Y., Mysliwiec, M. R., Yuan, G. C., Lee, Y., Orkin, S. H., 2009. Jumonji modulates polycomb activity and self-renewal versus differentiation of stem cells. Cell. 139, 1303-14.

Shirato, H., Ogawa, S., Nakajima, K., Inagawa, M., Kojima, M., Tachibana, M., Shinkai, Y., Takeuchi, T., 2009. A jumonji (Jarid2) protein complex represses cyclin D1 expression by methylation of histone H3-K9. J Biol Chem. 284, 733-9.

Sumanas, S., Ekker, S. C., 2001. Xenopus frizzled-5: a frizzled family member expressed exclusively in the neural retina of the developing eye. Mech Dev. 103, 133-6.

Takahashi, M., Kojima, M., Nakajima, K., Suzuki-Migishima, R., Motegi, Y., Yokoyama, M., Takeuchi, T., 2004. Cardiac abnormalities cause early lethality of jumonji mutant mice. Biochem Biophys Res Commun. 324, 1319-23.

Takahashi, M., Kojima, M., Nakajima, K., Suzuki-Migishima, R., Takeuchi, T., 2007. Functions of a jumonji-cyclin D1 pathway in the coordination of cell cycle exit and migration during neurogenesis in the mouse hindbrain. Dev Biol. 303, 549-60.

Takeuchi, T., Watanabe, Y., Takano-Shimizu, T., Kondo, S., 2006. Roles of jumonji and jumonji family genes in chromatin regulation and development. Dev Dyn. 235, 2449-59.

Toyoda, M., Shirato, H., Nakajima, K., Kojima, M., Takahashi, M., Kubota, M., Suzuki-Migishima, R., Motegi, Y., Yokoyama, M., Takeuchi, T., 2003. jumonji downregulates cardiac cell proliferation by repressing cyclin D1 expression. Dev Cell. 5, 85-97.

Turner, D. L., Weintraub, H., 1994. Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev. 8, 1434-47.

Vernon, A. E., Philpott, A., 2003. The developmental expression of cell cycle regulators in Xenopus laevis. Gene Expr Patterns. 3, 179-92.

Zhou, X., Hollemann, T., Pieler, T., Gruss, P., 2000. Cloning and expression of xSix3, the Xenopus homologue of murine Six3. Mech Dev. 91, 327-30.

CHAPTER 5

SUMMARY AND PERSPECTIVES

The role of chromatin remodeling factors during tissue-specific development has not been fully explored in part because loss of many of these factors is embryonic lethal at early stages of development. Inspired by data from ESCs, we focused on one of the complexes involved in mediating global gene repression, the PRC2 complex. In ESCs, PRC2 contributes to promoter repression of many developmental regulators, including genes that are essential for retinal differentiation (Lee et al., 2006). Hypothesizing that PRC2 might function in eye development we initially determined the expression of its components during *Xenopus* development.

PRC2 Expression During *Xenopus* Development

In total the expression pattern of five components of PRC2 has been characterized by in situ hybridization analysis, four of which are considered essential for its function. We found that the PRC2 core components, *Xez*, *Xeed*, *Xsuz12* and *Xrbbp4* are coincidently expressed in the developing CNS (Aldiri and Vetter, 2009). Developing organs that show high levels of PRC2 transcripts include eye, brain, spinal cord and branchial arches. During eye development, PRC2 genes are expressed in retinal progenitors and downregulated upon neural differentiation. One caveat to this analysis is that it was limited to detection of PRC2 transcripts and did not incorporate analysis of the protein levels. Future experiments will include using immunostaining and western blot to detect different components of PRC2 proteins during development.

Possible Regulatory Mechanisms of PRC2 Expression

The mechanism that controls the maintenance of PRC2 transcription in retinal progenitors has not been investigated but a possible candidate is the canonical Wnt signaling pathway which is active in retinal progenitors and is required for the progression from proliferation to differentiation (Agathocleous et al., 2009; Van Raay et al., 2005). In support of this hypothesis, loss of the canonical Wnt signaling partially mimics the effect of *Xez* inhibition on retinal differentiation, as it reduces retinal proliferation, blocks the expression of the proneural bHLH genes and biases cells toward late born cell fates (Van Raay et al., 2005). However, unlike the case in the canonical Wnt signaling, *Xez* is not required for the expression of *Sox2* (Chapter 3).

Alternatively, retinal expression of PRC2 components might be maintained by EFTFs, which control eye specification and proliferation (Zuber et al., 2003). A third candidate is the transcription factor E2F, which has been shown to control the expression of PRC2 subunits *Eed* and *Ezh2* in tumor cells and in fibroblasts (Bracken et al., 2003; Muller et al., 2001). It will be interesting to determine whether any of these candidates is necessary for the expression of PRC2 components in retinal progenitors.

PRC2 Is Required for Retinal Differentiation

Previously published studies on PRC2 during development indicated that PRC2 is involved in the regulation of cellular proliferation and/or differentiation in a context-dependent manner (Margueron and Reinberg, 2011; Surface et al., 2010). One model derived from studies on skin development suggests that PRC2 prevents premature activation of a late differentiation program, while another from ESCs postulates that

PRC2 is required for differentiation (Pasini et al., 2008; Pirrotta, 2009). Based on these models, we predicted that loss of PRC2 function could lead to either 1) premature differentiation of retinal progenitors, which can be manifested in the overproduction of early born retinal cell types, or 2) inhibition of retinal differentiation, which would be characterized by loss or delay in the expression of proneural bHLH factors and a bias toward generation of late born retinal cell types. We found that PRC2 is required for the expression of retinal differentiation genes, which is in agreement with the published data on roles of PRC2 in ESC differentiation (Pasini et al., 2008). Additionally, we found that loss of PRC2 function biases retinal cells toward late born cell fates. These data further demonstrate that PRC2 is utilized in a tissue-specific manner to perform distinct functions.

The molecular mechanism underlying PRC2 function during retinal development is not clear, but we hypothesize that PRC2 cooperates with known regulators of eye development to orchestrate the progression from proliferation to differentiation by promoting the neural potential of retinal progenitors. One of the candidate molecules that PRC2 function might be associated with is SOX2 which is an important transcription factor for the proper differentiation of the retina (Taranova et al., 2006; Van Raay et al., 2005). *Sox2* is expressed in retinal progenitors under the control of the canonical Wnt signaling (in *Xenopus*), and is required for the expression of the proneural bHLH genes. SOX2 blocks the ability of proneural bHLH factors to induce the expression of retinal downstream targets, suggesting that SOX2 promotes the neural identity of progenitors but prevents further differentiation (Agathocleous et al., 2009; Van Raay et al., 2005). Interestingly, a genome wide analysis of promoter occupancy in ESCs revealed that

SOX2 and SUZ12 co-occupy the promoters of many genes that are important for specification and differentiation of retinal cell types such as *Vsx1, Pax6, Ngn, NeuroD, Nrl, Isl-1, Prox-1*and E*bf3* (Lee et al., 2006). Thus PRC2 might function in retinal progenitors to promote neural differentiation by regulating the transcription of target genes in association with SOX2. Whether SOX2 and PRC2 share some of the retinal targets genes during *Xenopus* development, and are functionally linked remains to be addressed.

It is also possible that PRC2 is essential for orchestrating the proneural bHLH retinal differentiation programs. Future experiments will test whether overexpression of the proneural bHLH factors can promote cell fate specification when PRC2 function is blocked.

Possible Involvement of PRC2 in Retinal Proliferation

Although preliminary, our data suggest that loss of PRC2 might also be affecting retinal proliferation. This effect comes to no surprise as the role of PRC2 core components in controlling cellular proliferation during development and cancer progression is well documented (Margueron and Reinberg, 2011). For example, during skin development *Ezh2* is required to repress the tumor suppressor locus Ink4A-Ink4B in basal progenitors, and thus maintain their proliferative potential (Ezhkova et al., 2009). Given that *Ezh2* is a downstream target for the RB-E2F pathway in certain cancer types, it will be interesting to test whether *Ezh2* gain or loss of function mutants are associated with human eye malignancies and microphthalmia, respectively (Bracken et al., 2003; Muller et al., 2001).

Developmental H3K27me3 Deposition Is Selective and Dynamic

In an effort to understand how PRC2 functions during retinal differentiation, we characterized the levels of its mark H3K27me3 using immunohistochemistry. The analysis revealed that global H3K27me3 is enriched in retinal postmitotic cells, which was surprising given that the PRC2 core components are transcribed in retinal progenitors. It is possible that polycomb proteins are maintained during differentiation while their transcription is inhibited. In the future, experiments will be performed to test whether PRC2 proteins are expressed in postmitotic cells using immunostaining and/or western blot.

The presence of high levels of H3K27me3 in differentiated cells is intriguing and raises questions about its roles during adult life. One possible function is that H3K27me3 is involved in permanently repressing the expression of cell cycle genes in neurons, which prevents the cell from re-entering the cell cycle and allows stabilization of terminal cell fate. A second possibility is that deposition of H3K27me3 plays a role in regulating the expression of genes involved in visual processing and other physiological roles of adult retina. Interestingly, a previous study has shown that eliminating the repressive mark H3K9me2 from the mouse adult brain by conditionally deleting the GLP-G9A complex leads to complex behavioral defects and cognitive impairment (Schaefer et al., 2009).

To determine why PRC2 is required for retinal differentiation, a genome wide analysis to define H3K27me3 targets during development was performed. These data indicate that H3K27me3 decorates a small group of retinal genes, suggesting that the deposition of H3K27me3 is selective. The specific decoration of a limited number of

genes raises questions about the mechanism that mediates selective recruitment of PRC2 to its target genes. This process might involve association between PRC2 and eye-specific factors that recruit PRC2 to its intended targets and/or the presence of a DNA motif in the targeted promoters that facilitate recruitment.

Our data indicate H3K27me3 deposition changes during development, suggesting that H3K27me3 addition is dynamic and thus might be used transiently to regulate retinal gene expression. The loss of H3K27me3 during *Xenopus* development might indicate the presence of a demethylase that specifically catalyzes H3K27me3 removal, but such an enzyme has not been cloned yet in *Xenopus*. The presence of H3K27me3 demethylases in *Xenopus* is almost certain given that H3K27me3-specific demethylases have been identified in other vertebrates (Lan et al., 2007; Burgold et al., 2008).

**H3K27me3 targets Negative Regulators of Retinal Differentiation**

It is interesting to find that the list of H3K27me3 targets include factors that inhibit neural differentiation. This might suggest that one of the primary roles of PRC2 during retinal development is to facilitate the onset of neural differentiation by shutting off the expression of negative regulators of retinogenesis to allow differentiation to proceed (Fig. 33). If true, then blocking the expression of these genes should rescue (at least partially) loss of PRC2 function. It will be interesting to test whether the expression of candidate negative regulators of neurogenesis is unregulated upon knocking down of PRC2 function. Further, investigating the global consequences of loss of PRC2 function on retinal gene expression using ChIP-seq and microarray profiling could help in identifying such genes.

PRC2 Roles in Eye Development Might Be Conserved

Given that polycomb genes are evolutionarily conserved, we speculate that some of its biological functions might also be conserved. In support of this view, a mutation in the Drosophila E(z) (*Ezh2* homologue) causes a delay in photoreceptor differentiation during eye development (Janody et al., 2004). In mammals, *Ezh2* is expressed during eye development but further analysis of the expression pattern of the other PRC2 components has not been performed (Rao et al., 2010). Future studies will focus on characterizing the retinal expression of PRC2 genes and exploring the effect of loss of PRC2 function on murine retinal neurogenesis by generating eye-specific *Ezh2* conditional knockouts.

It has been shown that PRC1 is recruited to the H3K27me3-occupied territories, and is required for the PRC2-mediated repression, suggesting that PRC1 may also be involved in eye development. However, the expression of its components *Polycomb*, *Bmi1*, *Ph* and *Ring1* is not fully addressed in vertebrate retina. Characterization of the expression and the function of PRC1 may provide some insights into the functional link between PRC2 and PRC1 and how they might contribute to the proper development of vertebrate retina.

Figure. 28. A possible model for PRC2 function during retinal development. PRC2 might be required to suppress the expression of negative regulators of retinal neural differentiation to allow the initiation of proneural bHLH gene expression and subsequent cell fate acquisition to proceed.
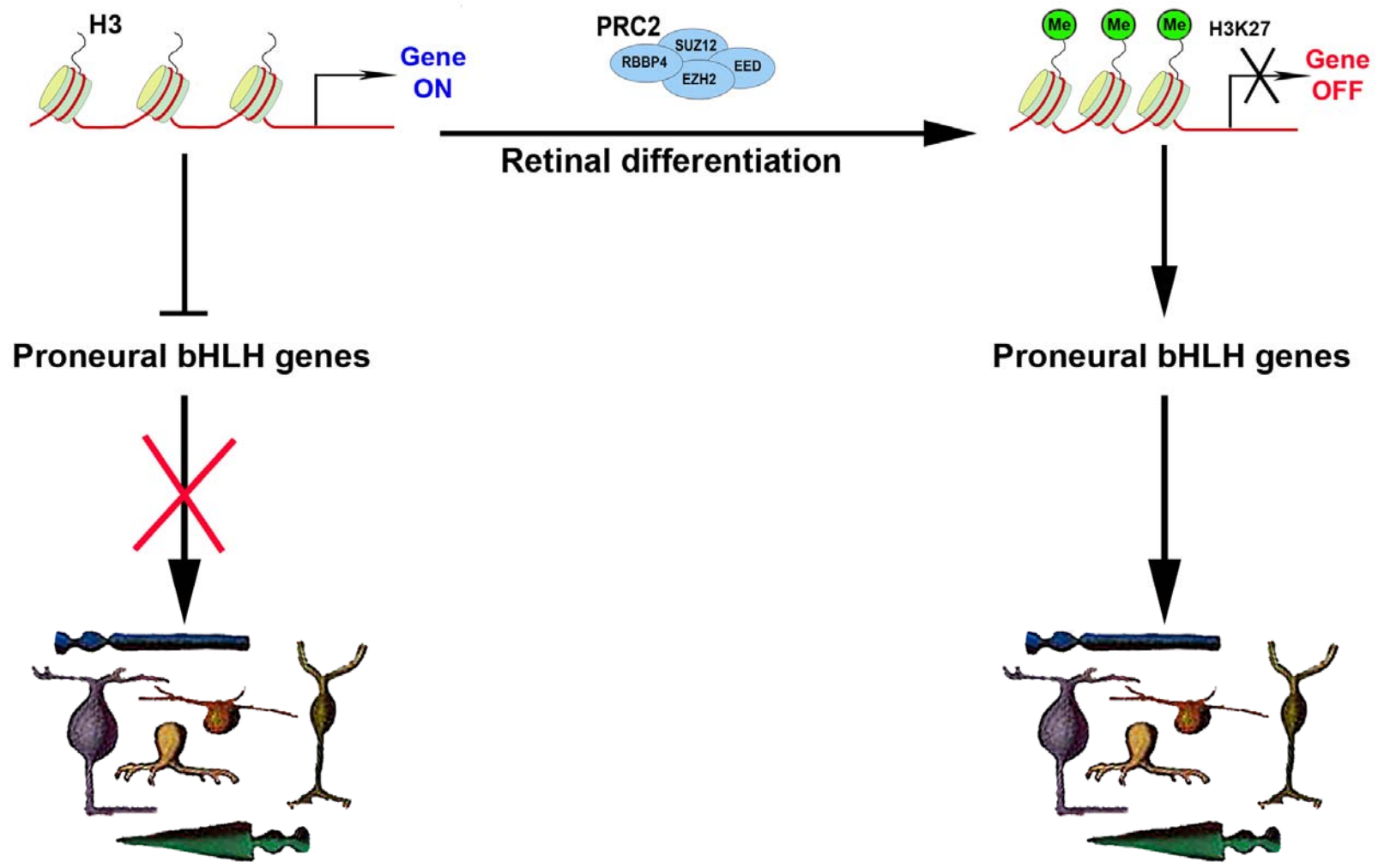
Possible Nonhistone Modifying Roles of PRC2 Components

Most studies on the roles of PRC2 during tissue organogenesis have assumed that its loss of function effect is mediated by the lack of its repressive mark H3K27me3. However, evidence suggests that PRC2 components can mediate non-histone modifying functions as well. For instance, PRC2 can localize to the cytoplasm in T-cells, where it is essential for the T-cell antigen receptor (TCR)-mediated actin polymerization through its methyltransferase activity (Su et al., 2005). Further, individual PRC2 subunits have been reported to have PRC2-independent functions, and thus might mediate distinct biological roles. EZH2, for example, can bind the Wnt effector beta-catenin and enhance the transactivation of beta-catenin targets independent of its SET domain (Shi et al., 2007). Interestingly, this function does not require the expression of the other PRC2 core subunits (Shi et al., 2007). These studies highlight the need for an examination of the cellular localization of different PRC2 components during development and whether they have roles that can be uncoupled from the methyltransferase activities.

Jarid2 Is Uniquely Expressed During _Xenopus_ Development

Given that PRC2 plays important biological roles, we wondered how its auxiliary subunits might contribute to its function. We have characterized the expression pattern of a newly identified partner of PRC2, JARID2 (Herz and Shilatifard, 2010). We found that _Jarid2_ is expressed in all stages of _Xenopus_ development but is highly enriched in postmitotic cells of the CNS, suggesting that _Jarid2_ may have distinct functions during neural differentiation. It has been shown that JARID2 contributes to the regulation of H3K27me3 and H3K9me2, and in agreement with these data, we found that both marks

are elevated in differentiated cells concomitant with the increase in the expression of *Jarid2* (Herz and Shilatifard, 2010). The biological functions of *Jarid2* in the nervous system remain largely unknown but previous data suggest it might be important in suppressing proliferation in differentiated cells. Our preliminary data indicate that *Jarid2* is essential for neural differentiation but is not required for global H3K27me3 in postmitotic cells. However, whether it is required to recruit PRC2 to positive regulators of cell cycle in retinal postmitotic cells remains unknown. Similarly, it is not clear whether JARID2 coordinates deposition of both H3K27me3 and H3K9me2 on the same targets or whether it regulates the addition of these marks on independent sets of promoters.

Concluding Remark

Although the biochemical functions of the polycomb group proteins have been extensively studied, much remains to be learned about how they are utilized in vertebrate development. We have revealed an unexplored role of PRC2 during eye development in *Xenopus*. PRC2 components are expressed during retinal development and are essential for the expression of the proneural bHLH factors during retinogenesis and for subsequent cell fate decisions. This study adds a new component to the network that controls the transcriptional reprogramming that occurs during cell transition from proliferation to differentiation in the retina.

References

Agathocleous, M., Iordanova, I., Willardsen, M. I., Xue, X. Y., Vetter, M. L., Harris, W. A., Moore, K. B., 2009. A directional Wnt/beta-catenin-Sox2-proneural pathway regulates the transition from proliferation to differentiation in the Xenopus retina. Development. 136**,** 3289-99.

Aldiri, I., Vetter, M. L., 2009. Characterization of the expression pattern of the PRC2 core subunit Suz12 during embryonic development of Xenopus laevis. Dev Dyn. 238**,** 3185-92.

Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E., Helin, K., 2003. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. Embo J. 22**,** 5323-35.

Ezhkova, E., Pasolli, H. A., Parker, J. S., Stokes, N., Su, I. H., Hannon, G., Tarakhovsky, A., Fuchs, E., 2009. Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell. 136**,** 1122-35.

Herz, H. M., Shilatifard, A., 2010. The JARID2-PRC2 duality. Genes Dev. 24**,** 857-61.

Janody, F., Lee, J. D., Jahren, N., Hazelett, D. J., Benlali, A., Miura, G. I., Draskovic, I., Treisman, J. E., 2004. A mosaic genetic screen reveals distinct roles for trithorax and polycomb group genes in Drosophila eye development. Genetics. 166**,** 187-200.

Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., Chevalier, B., Johnstone, S. E., Cole, M. F., Isono, K., Koseki, H., Fuchikami, T., Abe, K., Murray, H. L., Zucker, J. P., Yuan, B., Bell, G. W., Herbolsheimer, E., Hannett, N. M., Sun, K., Odom, D. T., Otte, A. P., Volkert, T. L., Bartel, D. P., Melton, D. A., Gifford, D. K., Jaenisch, R., Young, R. A., 2006. Control of developmental regulators by Polycomb in human embryonic stem cells. Cell. 125**,** 301-13.

Margueron, R., Reinberg, D., 2011. The Polycomb complex PRC2 and its mark in life. Nature. 469**,** 343-9.

Muller, H., Bracken, A. P., Vernell, R., Moroni, M. C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J. D., Helin, K., 2001. E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev. 15**,** 267-85.

Pasini, D., Bracken, A. P., Agger, K., Christensen, J., Hansen, K., Cloos, P. A., Helin, K., 2008. Regulation of stem cell differentiation by histone methyltransferases and demethylases. Cold Spring Harb Symp Quant Biol. 73**,** 253-63.

Pirrotta, V., 2009. Polycomb repression under the skin. Cell. 136**,** 992-4.

Rao, R. C., Tchedre, K. T., Malik, M. T., Coleman, N., Fang, Y., Marquez, V. E., Chen, D. F., 2010. Dynamic patterns of histone lysine methylation in the developing retina. Invest Ophthalmol Vis Sci. 51**,** 6784-92.

Schaefer, A., Sampath, S. C., Intrator, A., Min, A., Gertler, T. S., Surmeier, D. J., Tarakhovsky, A., Greengard, P., 2009. Control of cognition and adaptive behavior by the GLP/G9a epigenetic suppressor complex. Neuron. 64**,** 678-91.

Shi, B., Liang, J., Yang, X., Wang, Y., Zhao, Y., Wu, H., Sun, L., Zhang, Y., Chen, Y., Li, R., Zhang, Y., Hong, M., Shang, Y., 2007. Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells. Mol Cell Biol. 27**,** 5105-19.

Su, I. H., Dobenecker, M. W., Dickinson, E., Oser, M., Basavaraj, A., Marqueron, R., Viale, A., Reinberg, D., Wulfing, C., Tarakhovsky, A., 2005. Polycomb group protein ezh2 controls actin polymerization and cell signaling. Cell. 121**,** 425-36.

Surface, L. E., Thornton, S. R., Boyer, L. A., 2010. Polycomb group proteins set the stage for early lineage commitment. Cell Stem Cell. 7**,** 288-98.
Taranova, O. V., Magness, S. T., Fagan, B. M., Wu, Y., Surzenko, N., Hutton, S. R., Pevny, L. H., 2006. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev. 20**,** 1187-202.

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

Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G., Harris, W. A., 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development. 130**,** 5155-67.