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COMMENTARY

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Coordinating progenitor cell cycle exit and differentiation in the developing vertebrate retina

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ABSTRACT

The proper development of the vertebrate retina relies heavily on producing the correct number and type of differentiated retinal cell types. To achieve this, proliferating retinal progenitor cells (RPCs) must exit the cell cycle at an appropriate time and correctly express a subset of differentiation markers that help specify retinal cell fate. Homeobox genes, which encode a family of transcription factors, have been accredited to both these processes, implicated in the transcriptional regulation of important cell cycle components, such as cyclins and cyclin-dependent kinases, and proneural genes. This dual regulation of homeobox genes allows these factors to help co-ordinate the transition from the proliferating RPC to postmitotic, differentiated cell. However, understanding the exact molecular targets of these factors remains a challenging task. This commentary highlights the current knowledge we have about how these factors regulate cell cycle progression and differentiation, with particular emphasis on a recent discovery from our lab demonstrating an antagonistic relationship between Vsx2 and Dmbx1 to control RPC proliferation. Future studies should aim to further understand the direct transcriptional targets of these genes, additional co-factors/interacting proteins and the possible recruitment of epigenetic machinery by these homeobox genes.

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Introduction

The developing vertebrate neural retina emerges through a complex process of morphogenesis of the anterior neuroepithelium and initially consists of proliferating retinal progenitor cells (RPCs), which are multipotent cells capable of limited self-renewal. Differentiation begins in the center of the neural retina and expands toward the periphery as RPCs differentiate into one of the five main classes of retinal neurons (ganglion cells, amacrine cells, bipolar cells, horizontal cells, and photoreceptor cells), most of which are highly specialized into multiple subtypes, or Müller glial. In the mature retina, these cells are organized into 3 cellular layers [ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL)], which must be properly formed so that the cells are capable of relaying visual sensory information to the brain via the optic nerve.[8] Differentiation also occurs in a specific temporal order that is well conserved across vertebrate species, with ganglion cells appearing first while Müller glia are the last to appear.[57,76,83] Due to this birth order, the proportion of cells that exit the cell cycle and differentiate must be appropriately timed to ensure the correct number and type of cells are produced.

Therefore, proper neurogenesis of the retina relies on appropriately regulating the transition from a proliferating RPC to a post-mitotic differentiated retinal cell. At the heart of this process, two activities must be carefully coordinated: (1) cell cycle exit and (2) activation of an appropriate differentiation program. Failure to do so can result in proliferation defects resulting in microphthalmia or hyperplasia, differentiation defects and disruption to the lamination of the retina, and apoptosis (refs. 2,7,22,67,93). It is commonly believed that cell cycle exit and differentiation are intimately coupled, and that halting cell cycle progression is a prerequisite for differentiation. This is for the most part true,

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considering that they are temporally coupled: when cell cycle or proliferation-related genes are down regulated (i.e. *vsx2, cyclinD1*), differentiation specific genes are concurrently up-regulated.[14] Cell cycle kinetics is also observed to change during differentiation with a lengthening of the cell cycle as the retina matures.[4] Genetic manipulations that lengthen or reduce the G_1 phase are observed to result in premature terminal differentiation or increased progenitor proliferation, respectively.[52,78] In addition, genetic perturbations that prevent cell cycle exit in the retina often lead to a delay in retinal differentiation.[93] Although still controversial, evidence suggests that the two processes can be uncoupled and that cell cycle exit is not required for, or a consequence of, differentiation.[46,52,66] Supporting this idea, RPCs start to express differentiation markers before the onset of cell cycle exit and cell cycle proteins are still expressed well after terminal mitosis.[33,74,75] In addition, major perturbations on important cell cycle factors (i.e., Rb, CKIs) can cause ectopic division of differentiated neurons[28,67]). Therefore, the proliferation to differentiation transition is not as rigid as the classic perspective posits, although further research is required to fully explore this notion.

A flexible relationship likely represents the independence of the underlying molecular mechanisms governing cell cycle control and differentiated programs. As such, elucidating the molecular players is important to understand how each process is separately governed. Although these are separate processes, there may be unique opportunities for cross talk between these pathways. Advantageously, this can help ensure that cell cycle exit is properly coordinated with differentiation. For example, the proneural gene ATOH7/ATH5, which is required for the production of RGCs, also regulates cell cycle length via Notch signaling.[24] Many other transcription factors (TFs), including a broad class of homeobox genes, have also been associated with both cell cycle regulation and differentiation. Homeobox TFs are therefore properly positioned to activate/repress cell cycle genes while concurrently repressing/activating differentiation programs. This review will focus on understanding the molecular mechanisms controlling cell cycle exit and/or differentiation during retinal neurogenesis, and in particular the role of homeobox TFs including dmbx1 and vsx2, which may contribute to understanding the etiology of retinal developmental disorders.

Cell cycle control in the developing retina

Increasing evidence indicates that transcription factors that regulate retinal neurogenesis impinge on major cell cycle regulators. Terminal differentiation is characterized by stalling in the G_1 phase as cells arrest and exit the cell cycle into an irreversible G_0 phase.[19] Due to this, the G_1/S phase progression is an important regulatory point that determines whether a cell will transition from a proliferative to post-mitotic state. The key molecular event dictating whether a cell progresses from G_1 to S phase or exits the cell cycle culminates on the phosphorylation status of retinoblastoma (Rb) protein.[37] The main function of Rb is to inhibit division, promote cell cycle exit and suppress cell cycle re-entry of differentiated cells.[17] In agreement, loss of retinoblastoma 1 (*rb1*) function in zebrafish leads to a delay of cell cycle exit and delay of differentiation of early-born RGCs, which negatively affects proper retinotectal connectivity and phototactic behaviors.[51] In mice this phenotype is more severe, with *Rb1* knockouts exhibiting retinal hypoplasia, ectopic division, and considerable apoptosis of retinal cells.[67]

The phosphorylation status of Rb during G_1 depends on the activity of cyclin-dependent kinases (CDK), which are active when bound to a corresponding cyclin protein.[37] When active, the cyclin-CDK complex phosphorylates Rb, and dissociates Rb from the transcription factor, E2F, allowing E2F to transcribe genes important for the S and G_2 phases, leading to cell cycle progression (Fig. 1).[88] The major cyclin important in retinal development is CyclinD1 (Ccnd1), which bind to and activates Cdk4/6. Ccnd1 is highly expressed in RPCs but its expression is downregulated in differentiated retinal cells.[6,37,84] Consistent with a function in maintaining proliferation, *Ccnd1* loss in mice causes severe microphthalmia due to reduced RPC proliferation.[29,41] In addition, the cell cycle is prolonged, RPCs prematurely exit the cell cycle and retinas display differentiation defects showing a greater proportion of RGCs and photoreceptors at the expense of horizontal and amacrine cells.[28] In zebrafish, knockdown of *ccnd1* also results in microphthalmia although differentiation is not severely affected since all major cell types are produced, suggesting a role in cell cycle regulation independent of differentiation.[35] Conversely, ectopic *ccnd1* prevents normal cell cycle exit, causing excessive cell proliferation and apoptosis.[85] It is interesting to highlight that

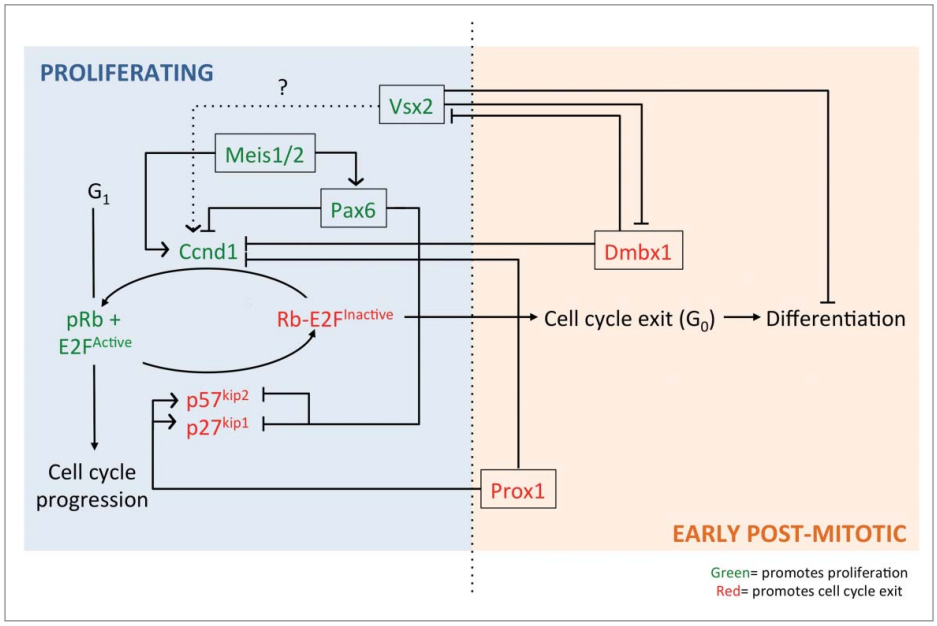

Figure 1. Schematic representing how the homeobox genes Pax6, Meis1/2, Prox1, Dmbx1 and Vsx2 control the cell cycle progression or exit of an RPC into an early post-mitotic neuron. Some of these genes (Pax6, vsx2 and Meis1/2) are expressed early in a proliferating RPC (seen on the 'blue' proliferating side) whereas others (Dmbx1 and Prox1) are expressed as a RPC exits the cell cycle (seen on the 'orange' early post-mitotic side). Each homeobox gene highlighted has been implicated in activating/inhibiting certain cell cycle factors/other homeobox genes via direct or indirect transcriptional regulation (see text for more details). Pointed arrows indicate an activating role whereas straight edge arrows indicate an inhibiting role.

recently Ccnd1 has also more broadly been associated with transcriptional regulation in mouse retinal development and therefore may have non-cell cycle related functions.[12] The role of other cyclins, including other D-type cyclins (D2 and D3) and E-type cyclins is not as pronounced in the retina, although cyclin E, but not D2/D3, is capable of rescuing RPC defects in *Ccnd1* knockouts.[20,29,30,47] Interestingly, *CyclinD3* levels are up regulated in *Ccnd1* knockouts although retinal proliferation is not rescued, indicating a compensating mechanism that cannot functionally substitute for Ccnd1.[48] As a result, the major cell cycle activator in the G_1 phase of the retina appears to be Ccnd1 and high expression in RPCs normally promotes cell cycle progression.

However, a mechanism must be in place to counter cyclin-CDK activity to promote cell cycle exit. One of the most prominent mechanisms involves the activity of cyclin kinase inhibitors (CKIs) (Fig. 1). Two families of CKIs have been identified including the Ink4 family and Cip/Kip family,[89] but only three CKIs have been implicated in retinal development including $p27^{kip1}$ and $p57^{kip2}$, and $p19^{ink4d}$. Consistent with their role in cell cycle exit, $p27^{kip1}$, $p57^{kip2}$ and $p19^{ink4d}$ are upregulated and highly expressed in a subset of RPCs undergoing cell cycle exit and in newly post-mitotic cells, although each CKI shows distinct spatial and temporal expression.[28,36,37] A loss of any of these CKIs results in an increase in proliferation of RPCs, whereas overexpression leads to premature cell cycle exit.[27,36,61] The main mechanism behind how CKIs exert their cell cycle inhibition appears to be by directly binding and inactivating cyclin-CDKs[89] For example, $p27^{kip1}$ can directly interact with Ccnd1-Cdk4/6 or cyclin E-Cdk2 and prevent their activity.[37] However, if there are sufficiently high levels of Ccnd1, excessive Ccnd1 can bind and sequester $p27^{kip1}$ and free other cyclin-cdk complexes (i.e. cyclin E-cdk2) to promote cell cycle progression. In agreement, simultaneous knockout of $p27^{kip1}$ in *Ccnd1* knockout mice can rescue retinal cellularity[48,87] Analysis indicates this is because cyclinE-cdk2 is freed from inhibition by $p27^{kip1}$, allowing partial restoration of pRb and promotion of the cell cycle.[87] It appears, therefore, that the balance between the levels of cell cycle activators (i.e., cyclin-CDK) and repressors (i.e. CKIs) is crucial in controlling the cell cycle of the RPCs. Moreover, the timing of expression of these positive and negative regulators is key to understanding this balance.

Homeobox genes control of cell cycle exit via regulation of cell cycle components

Homeobox genes encode a family of transcription factor proteins characterized by the presence of a DNA binding motif called the homeodomain and over the years, have been seen to have critical roles in retinal neurogenesis.[32,94] Traditionally, homeobox genes were predominantly linked to the transcription of genes responsible for maintaining a proliferative RPC identity, specifying cell fate of RPCs and/or dictating differentiation. However, given that the cell cycle needs to be dynamically regulated as a cell transitions from a RPC to a post-mitotic cell, the question of whether homeobox genes could also directly regulate the expression of cell cycle components gained interest. Now, multiple homeobox TFs have been observed to have direct or indirect roles in regulating cyclins and CKIs. This indicates that TFs are multifaceted factors capable of intervening and coordinating different, distinct molecular pathways. The importance of these roles is highlighted by the fact that homeobox genes are increasingly being implicated in human retinal disorders including microphthalmia and therefore understanding their multi-faceted function could help understand causes and possible treatments of these disorders.[94] The focus here will be on highlighting examples of homeobox genes controlling the cell cycle and differentiation in the retina with only minor consideration given to evidence for similar roles found in the central nervous system. However, it is important to note that a number of homeobox genes have also been implicated in other tissues and notably, carcinogenesis, given their intimate relationship with cell cycle components (reviewed in ref. 1).

One of the best recent examples of a retinal homeobox gene that regulates the cell cycle is the paired box (Pax) gene, Pax6. Pax6 is highly expressed in the developing eye, and is predominately viewed as an early retinal determination gene with homozygous mutations resulting in a complete absence of eye structures (congenital aniridia) in humans.[15,54] However, *Pax6* overexpression also causes microphthalmia and retinal dysplasia, consistent with a cell cycle effect.[98] Pax6 expression is found throughout the RPC population and subsequently in differentiated RGCs, amacrine and horizontal cells.[56] In controlling differentiation, Pax6 is required to maintain RPC multipotency with *Pax6*-deficient RPCs showing limited differentiation, producing exclusively amacrine cells.[68] Failure to create other neuron subtypes stems from the fact that Pax6 directly regulates the transcription of proneural genes, including RGC specification factor *Atoh7*, and neurogenesis factors *neurogenin-2* and *Ascl1*.[61,73,77,80] Pax6 expression in RPCs is also required to promote cell cycle progression. *Pax6* loss in the retina results in a significant reduction in the amount of cells in the S phase and an increase in the number of cells exiting the cell cycle.[42,77] Interestingly, *Pax6*-deficient mouse retinas show an increase in factors that both promote (cyclinD1-3) and inhibit ($p27^{kip1}$ and $p57^{kip2}$) the cell cycle, with *Pax6*-deficient cells typically co-expressing these opposing factors[42] This implies that Pax6s regulation of the cell cycle is complex, possessing the ability to either promote or inhibit the cell cycle. Evidence of its role in the retina versus the brain seems to support this idea. Although Pax6 appears to normally promote cell cycle progression/proliferation in RPCs, Pax6 in cortical progenitors actually inhibits proliferation.[9,40,42,68] Therefore, this may represent a context-specific difference that results from different extrinsic/intrinsic cues, or a presence of other TFs/proteins that interact with and affect Pax6s specific function. It is not clear from studies in the retina whether Pax6 can directly regulate the transcription of these cell cycle factors since other TFs, such as Vsx2, which are known to regulate cell cycle factors, are reduced in expression in *Pax6*-deficient RPCs.[42] Although this implies it is indirectly regulated, investigation in cortical progenitor cells indicate that Pax6 is capable of directly repressing the transcription of cell cycle factors, such as *Cdk6*, to attenuate proliferation.[69]

Other highly studied TFs are Meis1/2, which belong to the TALE-class of homeobox TFs and are the only Meis family members expressed in the eye;[16,53] Bumsted-O'Brien, 2007). Meis proteins are the vertebrate homolog of the *homothorax* (Hth) gene in *Drosophila* which is expressed in multipotent cells of the eye and is required to maintain proliferation and prevent premature differentiation.[10] A similar role in controlling RPC proliferation for Meis1/2 in the retina of chick, mouse and zebrafish has also been seen, suggesting a highly conserved role in retinal development.[6,53,55] In these 3 species, Meis1/2 is also expressed in proliferating RPCs, but down regulated when cells begin to differentiate.[6,53] Knockout or knockdown of *Meis1/2* in mice, chick and zebrafish all

result in microphthalmia due to impairment in the proliferation of RPCs.[6,53] Moreover, *meis1* and *meis2* work synergistically in zebrafish since the amount of mitotic cells are even more reduced when both genes are knocked down.[53] Evidence suggests that the mechanism by which Meis1 maintains proliferation is by promoting the G_1 to S transition of the cell cycle. This is because knockdown of *meis1* in zebrafish results in a significantly higher proportion of cells stalled in the G_1 phase.[11] Consistent with this cell cycle effect, *ccnd1* expression is reduced in *meis1/2* knockdowns. The proliferation defect in the retina can also be rescued by expressing *ccnd1*, implying that *meis1/2* regulates *ccnd1* expression.[6,53] If this regulation is direct or indirect is not known. This regulatory relationship is also observed in the retina where *Meis1* knockdown results in reduced *Pax6* transcript levels.[11,54] However, expression of *Pax6* in *Meis1* knockdown does not rescue the loss of *ccnd1* expression, suggesting that loss of *ccnd1* is independent of the loss of *Pax6*.[11] In contrast to Meis1's regulation of *Pax6*, biochemical and functional analysis of a Meis1 eye enhancer has identified *Pax6* binding sites, suggesting that *Pax6* may also regulate *Meis1* expression.[82] However, the importance of this regulation in the retina is uncertain, considering that *pax6* morphants do not exhibit alterations in *meis1* expression.[82] However, in light of this evidence and the fact that Meis and *Pax6* are expressed in similar domains within the eye, it suggests that homeobox TFs are capable of regulating each other's expression.[6]

Unlike the previous examples above, Prox1 homeobox TF negatively regulates RPC proliferation and is required to promote cell cycle exit. Prox1 is the vertebrate homolog of the *prospero* gene in *Drosophila*, which promotes cell cycle exit via repression of the transcription of cell cycle progression genes, *cdc25, cyclinE* and *cyclinA* during neurogenesis.[63] In the vertebrate retina, a similar role in promoting cell cycle exit also occurs. Prox1 expression is found in proliferating RPCs preceding the expression of $p27^{kip1}$ and $p57^{kip2}$.[39] Loss of *Prox1* results in expansion of *Ccnd1* expression, more proliferating RPCs, and fewer differentiated cells with a bias toward the production of later-born cell types.[39] Conversely, ectopic expression of *Prox1* results in premature cell cycle exit and bias toward early born cell types, and horizontal cells. This indicates that Prox1 may promote CKI expression to cause cell cycle exit, although whether Prox1 has a direct role in their transcription remains to be investigated. A similar role for Prox1-mediated cell cycle exit is also reported in the mouse lens, where $p27^{kip1}$ and $p57^{kip2}$ expression is decreased in *Prox1*-deficient lens, and in spinal interneurons.[70,91] Prox1 has also been observed to have a critical role in suppressing malignant neuroblastoma transformation by suppressing *Ccnd1, CyclinA, CyclinB1, Cdc25A* and inducing *p27kip1*.[45] These all indicate that Prox1 has important effects on the cell cycle factors in development and disease. Interestingly, Prox1 expression in RPCs is extinguished in cells exiting the cell cycle at the G_1/G_0 phase, and reinitiated in horizontal and amacrine cells, being necessary and sufficient for horizontal cell genesis.[39] This segregated expression patterns seems to suggest that regulation of the cell cycle and differentiation program by Prox1 are completely independent processes. Indeed, expression of Prox1 has also been reported in bipolar cells, rod precursors and Muller glia, although the requirement of Prox1 in these cells is not fully elucidated.[26,39] The role of Pax6, Meis1 and Prox1 in the cell cycle is summarized in the schematic in Figure 1.

Vsx2 and dmbx1: Coordinating cell cycle exit through antagonistic gene expression

The examples of Pax6, Meis1/2 and Prox1 highlight how homeobox TFs can have a profound effect on retinal development through the regulation of cell cycle machinery. In our lab, we have added to this understanding by investigating the role of visual system homeobox 2 (vsx2) and diencephalon/mesencephalon homeobox 1 (dmbx1) in zebrafish retinal development. We have shown how they function antagonistically to control the transition from a RPC to postmitotic cell by affecting each other's expression, and cell cycle factors to ultimately affect proliferation and cell cycle exit[92] (summarized in Fig. 1).

Vsx2 (also previously referred to as Chx10 in mice or alx in zebrafish) is a member of the paired-like homeobox TF family and contains both a homeodomain and an adjacent CVC domain. Both domains are required for high specificity DNA binding, with Vsx2 generally mediating transcriptional repression of its target genes.[34,65,97] Vsx2 is expressed in RPCs in the developing retina, but is downregulated as RPCs exit the cell cycle. In the mature retina, remaining RPCs

found in the ciliary or circumferential marginal zone (CMZ) and differentiated bipolar interneurons, and Muller glia maintain Vsx2 expression.[50,64,81] Null mutations in *VSX2*, are associated with severe microphthalmia and congenital blindness in humans and have also been previously identified as the cause of microphthalmia in the ocular retardation (or^J) mouse model.[18,43] Knockdown in zebrafish also results in microphthalmia highlighting a conserved role of *vsx2* in retinal development.[5,90,92] More specific analysis of Vsx2 loss of function experiments in the retina shows there are two major defects: (1) a reduction in the number of proliferating RPCs; and (2) an absence of bipolar cells due to defects in bipolar differentiation.[18] The early effect of Vsx2 on proliferation and later effect on bipolar development appear to be separable processes since Vsx2 is dispensable for cell proliferation but required to control bipolar cell genesis (by inhibition of rod differentiation) in the postnatal mouse retina.[65] However, transient ectopic *vsx2* expression in zebrafish is sufficient to prolong RPC proliferation in the central retina.[92] To maintain an RPC identity over a postmitotic cell state, Vsx2 partly acts to repress genes (i.e., *Vsx1* and *Ath5*) that would otherwise specify the fate of the cell.[26,90] Vsx2 maintains RPC proliferation via repression of the inhibitory cell cycle component, $p27^{kip1}$. Supporting this, *Vsx2*-null retinas express a higher portion of $p27^{kip1}$-positive cells, but when $p27^{kip1}$ is concurrently knocked out with *Vsx2*, there is a rescue in the proliferation defect, hypocellular retinal phenotype, and lamination defects.[50] It is proposed that Vsx2 regulates $p27^{kip1}$ at the post-transcriptional level through Ccnd1- mediated repression of $p27^{kip1}$.[50] This implies that Vsx2 may directly regulate *Ccnd1* levels, but this would require Vsx2 to act as a transcriptional activator, which has not yet been reported. Given these functions, Vsx2 must be appropriately down regulated to facilitate the transition from a RPC to postmitotic cell, although the factors responsible for this regulation remain unclear.

A promising candidate was Dmbx1 since contrary to Vsx2, Dmbx1 is required for cell cycle exit during retinal development. Dmbx1 also belongs to the paired-like homeobox gene family and is thought to act as a transcriptional repressor.[60] However, its transcriptional targets have not been well defined. Dmbx1 has been implicated in regulating neurogenesis in both the retina and midbrain.[71,93] Sequence analysis has indicated that this gene is conserved in mouse, humans and zebrafish although there is functional divergence in dmbx1 between species. In particular, mouse Dmbx1 is predominantly expressed and important in midbrain and hindbrain development, but its expression and function in the retina has not been thoroughly investigated.[49,71,72] In contrast, the zebrafish genome harbours two *dmbx1* paralogs, *dmbx1a* and *dmbx1b*, and both are expressed in the midbrain (pretectum/tectum), hindbrain and retina, with knockdown resulting in microphthalmia and defects in the retinotectal pathway.[21,59,93] Interestingly, a recently identified mutation in human *DMBX1* was associated with hyperopia indicating that this gene is relevant to human retinal disorders as well.[3] Zebrafish *dmbx1a* expression onset in the neural retina coincides with when RPCs start exiting the cell cycle and is found to coincide predominately in newly postmitotic cells in the INL.[21,93] Knockdown of *dmbx1a* results in significant reduction in retinal growth, and disruption to retinal differentiation and lamination,[93] Underlying this phenotype, more retinal cells remain proliferative, possess a prolonged cell cycle, and fail to differentiate.[93] These results suggest that dmbx1 promotes cell cycle exit, although the exact molecular mechanism responsible, remains to be fully elucidated.

Wong et al. (2015)[92] provide insight into this mechanism by establishing evidence for a signaling axis involving antagonistic functions of vsx2 and dmbx1. Since vsx2 is expressed in RPCs, but downregulated at the onset of dmbx1 expression, this suggested that these TFs might mutually repress each other. To investigate this, Wong et al. (2015)[92] analyzed changes in transcript levels in *dmbx1/vsx2* overexpression and knockdown embryos. Consistent with the hypothesis, *vsx2* expression is upregulated when *dmbx1* is knocked down, with *vsx2* expression expanding beyond RPCs of the CMZ and into the central retina. Conversely, knockdown of *vsx2* correspondingly results in an upregulation of *dmbx1*, while overexpression of *vsx2* significantly decreases *dmbx1* expression. This antagonistic regulation of expression supports the hypothesized mutual repression, with manipulation of gene expression either fostering or removing this repression.

Importantly, the changes in *vsx2* or *dmbx1* expression levels result in obvious changes to proliferation or timing of cell cycle exit, indicating that the proper temporal expression of these genes have important

implications on the state of RPCs. In particular, *vsx2* overexpression is sufficient to prolong proliferating RPC identity in the central retina, since central retinal cells continue to incorporate BrdU for longer when vsx2 is transiently induced. In contrast, overexpression of *dmbx1* results in premature cell cycle exit. A decrease in the number of mitotic cells (pHH3 and BrdU-positive cells) in an early retina (24hpf), normally dominated by mitotic cells, supports this conclusion. In addition, premature cell cycle exit results in an abundance of earlier born retinal cells type, RGCs, and decrease in later born cell types, including Müller glia and cone photoreceptors. This indicates that controlling the balance of *vsx2* and *dmbx1* expression down regulation and onset, respectively, is crucial in establishing proper retinal development and the mutual antagonism between these genes helps ensure that this balance is met.

The question now remained, what other factors fit into this molecular pathway, upstream and downstream of vsx2/dmbx1? Upstream of vsx2, we show that FGF signaling is necessary to maintain *vsx2* expression. Inhibition of FGF signaling causes *vsx2* levels to decrease, suggesting that FGF signaling is a positive regulator of *vsx2* in zebrafish, a relationship which is conserved with mammals.[27] Interestingly, we also found that this positive regulation by FGF must be sustained in order for *vsx2* levels to be up regulated in the absence of dmbx1, since the simultaneous inhibition of FGF signaling and *dmbx1a* knockdown did not result in an up regulation of *vsx2*.[92] This suggests that *vsx2* level is not directly regulated by *dmbx1* but requires continued FGF signaling to be upregulated. It is possible that *dmbx1* regulates the repression of FGF signaling components that lead to reduced *vsx2*, but future studies are required to address if there is direct binding of dmbx1 to the vsx2 promoter, or other promoters, in order to fully understand this regulation.

In addition, understanding the downstream factors affected, provide insight into why the cell cycle and proliferation ability of the cells are affected in *dmbx1* knockdown. We found that *ccnd1* was prominently affected in dmbx1 morphants,[92] showing a substantial increase in expression throughout the central retina. Conversely, overexpression of dmbx1 decreases the expression of *ccnd1*. Furthermore, combined knockdown of *dmbx1* and *ccnd1* is able to partially rescue the differentiation defect observed in dmbx1 morphants. This suggests that dmbx1 negatively regulates *ccnd1* to promote cell cycle exit, but does not seem to be necessary for controlling retinal differentiation *per se*. Therefore, dmbx1 represents a homeobox TF whose role is uniquely accredited to controlling cell cycle exit of proliferating RPCs by negatively regulating *ccnd1*.

It is important to note that due to the antagonistic nature of the vsx2-dmbx1 relationship, *vsx2* expression needs to be down regulated before *dmbx1* expression predominates and furthermore, *dmbx1* cannot reduce *vsx2* until it is more highly expressed. What is responsible for initially tipping the balance toward dmbx1 expression is unknown. Extrinsic factors implicated in retinal development (i.e. Wnt, FGF, Shh) may be responsible for prompting RPCs to start to exit the cell cycle, ultimately leading to changes in the transcription of factors including vsx2 and dmbx1. We know from our results that *vsx2* is influenced by FGF signaling, but it is unknown what other factors are capable of regulating *dmbx1*.

Further understanding cell cycle control: Transcriptional targets, protein interactions and epigenetic regulation

Most of the effects of homeobox genes, including vsx2 and dmbx1 on retinal development, are associated with the genes they transcriptionally regulate. As such, discovering the transcriptional targets is crucial to understanding their function. We conclude from our findings that vsx2 likely transcriptionally represses *dmbx1*, and dmbx1 likely transcriptionally represses *vsx2* and *ccnd1*. However direct evidence, using a biochemical technique such as chromatin immunoprecipitation (ChIP), is needed to show that these TFs are capable of binding to the promoters of these genes. Moreover, this influence is not necessarily an independent process and may require the activity of additional co-factors. In particular, TFs commonly hetero-dimerize with other TFs to affect their DNA binding specificity and targets. For example, Meis1 and Pbx, another homeobox gene implicated in controlling retinal RGC axon outgrowth, have been seen to interact and cooperatively bind to target genes.[13,45] In addition, Dmbx1 has been shown to form a heterodimer with Otx2.[60] Homeobox proteins can also physically interact with other types of proteins, most notably, including cell cycle factors. Prox1 has been shown to interact with proliferating cell nuclear antigen (PCNA) and this was seen to inhibit Prox1s ability to

transcriptionally activate the betaB1-crystallin promoter.[23] RPC proliferation promoting factor, Six3/6 has been shown physically interact with geminin, a DNA replication-inhibitor that promotes cell cycle arrest, to inhibit its activity and ultimately control the balance between proliferation and differentiation.[31,86] Whether other homeobox genes, including vsx2 and dmbx1, control RPC proliferation by physically interacting with cell cycle proteins remains an exciting question to address in the future. Discovering these new protein-protein interactions can add a complexity that aids in better understanding the function of these homeobox TFs, especially in different developmental contexts.

TFs can also recruit epigenetic machinery to affect retinal development. Given that the transition from a RPC to postmitotic, differentiated cell is accompanied by dramatic changes in gene expression, epigenetic regulation can promote long-term repression of cell cycle/progenitor related genes and represents an evolving field of study in retinal development. Highlighting the importance of epigenetics, studies support the idea that chromatin modifiers, including histone methyltransferases (HMT) have a substantial influence on the RPC proliferation and differentiation. For example, loss of Ezh2 activity, a subunit in polycomb repressive complex 2 (PRC2) responsible for di- and trimethylation of lysine 27 of histone H3, reduces RPC proliferation, disrupts retinal lamination and delays differentiation in the mouse retina.[95] A specific type of HMT, G9a, is also implicated in brain and retinal development, with *g9a* loss in zebrafish causing notable reductions in eye and brain size (Rai et al., 2010). In the mouse retina, G9a mediated dimethylation of lysine 9 of histone H3 in RPCs on progenitor cell-related genes, including cyclins and progenitor TFs (such as Vsx2) is essential to mediate repression and promote normal terminal differentiation and survival of retinal cells.[57] Since transcription factors are capable of recruiting/interacting with chromatin modifiers, it is possible that homeobox TFs interact with HMTs/other modifiers. In support of this motion, the TF PRDM4, recruits the HMT, PRMT5 to maintain neural stem cells in a proliferative state.[25] Whether dmbx1 interacts with epigenetic machinery, to stabilize repression of *ccnd1, vsx2* and other targets, and promote cell cycle exit is an exciting question to explore.

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