Epigenetic regulation of neural progenitor multipotency

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ABSTRACT

Time plays a key role in the histogenesis of all tissues. Still, developmental time is doubly important in the nervous system, where the large diversity of neuronal and glial cell types are produced by neural progenitor cells that undergo step-wise competence transitions in a time-dependent manner. This is particularly evident in the developing retina, where resident neural progenitors generate complex lineages comprising diverse neuronal and glial cell types in stereotyped sequences by altering their multipotency over developmental time. However, a major question that persists is how individual multipotent progenitors dynamically regulate their developmental potential to produce specific cell types at the right time and in the correct proportions and sequence. Landmark studies have suggested that competence transitions are cell-autonomously encoded by transcriptional regulators and epigenetic processes, but these mechanisms are not well understood in vertebrate lineages. Since nucleosome remodellers interact with both transcription factors and heterochromatic complexes, we sought to address the role of nucleosome remodeling complexes in developmental timing in the mouse retina. We generated conditional knockouts (cKOs) of *Chd4* – a key nucleosome remodelling enzyme in neural progenitors. *Chd4* cKOs exhibited a marked expansion in early-born retinal ganglion cells. Postnatally, later-born rod photoreceptors were drastically underproduced. This was partly due to progenitors failing to differentiate on schedule and continuing to proliferate beyond their normal developmental window. This ultimately led to a striking increase in Müller glia production. Histological marker analyses suggest that these effects were independent of alterations in cell death or proliferation at perinatal stages; however, as development progressed, *Chd4* cKO retinas exhibited elevated apoptosis, which might have

additionally contributed to the decreased rod generation. To determine whether Chd4 regulates retinal cell-type production by altering competence windows, we performed EdU birthdating. These experiments revealed that cell fates were altered without affecting the early RPC competence window. Next, we examined the effect of *Chd4* on the genome and transcriptome, focusing on the perinatal retinal progenitor pool. Multi-seq single-cell transcriptomics demonstrated that deletion of *Chd4* created divergent gene expression profiles and developmental trajectories. ATAC-seq experiments performed on sorted P1 retinal progenitors revealed that chromatin accessibility was significantly increased at ~10,000 genomic loci and ~4,000 genes in the *Chd4* cKO. The changes in accessibility in *Chd4* cKO RPCs correlated with increases in transcription, suggesting that Chd4 restricts the genome to repress progenitor identity and promote differentiation. Thus, despite a very strong shift in the production of early-born and late-born cell types, our data suggest that Chd4-dependent nucleosome remodelling plays a crucial role in the temporal transition that governs lineage termination but does not regulate earlier temporal transitions.

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List of Abbreviations

Adnp- Activity-dependent neuroprotective protein

ANOVA- Analysis of variance

ATAC-seq- Assay for transposase accessible chromatin with sequencing

bHLH- Basic helix-loop-helix

BrdU- Bromodeoxyuridine

Casz1- Castor transcription factor

ChAHP- Chd-Adnp-Hp1 complex

CHD- Chromodomain helicase DNA-binding

cHet- Conditional heterozygous

cKO- Conditional knock-out

CNS- Central nervous system

CTCF- CCCTC- binding factor

CTD- C-terminal domain

CUT&RUN- Cleavage under target and release under nuclease

DAR- Differentially accessible region

DEG- Differentially expressed gene

DUF- Domain of unknown function

E- Embryonic stage

EdU- 5-ethynyl-2’-deoxyuridine

EFTF- Eye field transcription factor

GATAD2A/B- GATA zinc finger domain containing 2(A/B)

GCL- Ganglion cell layer

Hd- Homeodomain

HDAC- Histone deacetylase

Hp1- Heterochromatin protein 1

IHC- Immunohistochemistry

INL- Inner nuclear layer

INBL- Inner neuroblastic layer

MBD- Methyl-CpG-binding protein

miRNA- MicroRNA

MTA- Metastasis associated protein

NuRD- Nucleosome remodelling and deacetylase

OCT- Optimal cutting temperature

ONL- Outer nuclear layer

ONBL- Outer neuroblastic layer

OS- Optic stalk

OV- Optic vesicles

P- Postnatal stage

PBS- Phosphate buffered saline

PFA- Paraformaldehyde

PHD- Plant homeodomain

PRC- Polycomb repressive complex

RBBP- Retinoblastoma binding proteins

RGC- Retinal ganglion cell

RIPA- Radioimmunoprecipitation assay

RPC- Retinal progenitor cell

RPE- Retinal pigmented epithelium

scRNA-seq- Single cell RNA sequencing

SWI/SNF- Switch/sucrose-non-fermenting

tTF- Temporal transcription factor

UMAP- Uniform manifold approximation and projection

Wt- Wild-type

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Chapter 1. Introduction

The human central nervous system (CNS) is composed of approximately 160 billion neuronal and glial cells (1). The proper functioning of the CNS requires an orderly generation of diversified cell types in correct proportions and numbers during development. Neural progenitors generate this cell type diversity on a tightly regulated developmental schedule. In virtually every lineage, neural progenitors progressively alter their output over developmental time, where they initially make neurons, but then irreversibly switch to producing glia (2). Moreover, intricate transitions in progenitor potential are observed in regions such as the developing retina and mammalian neocortex, where neural progenitors generate complex lineages consisting of many subtypes of neurons and glia in stereotyped sequences. While we know a great deal about the molecular determinants that control neural cell fate specification, we know much less about how the multipotency of neural progenitor cells is modified during development. In particular, neural progenitors progress through distinct phases of multipotency, which have been termed ‘competence’ states by the field. Dissecting the molecular mechanisms that regulate competence might provide a better understanding of how neural progenitors can alter their potential during development and help identify factors that drive certain cell fate choices and diversity.

The developing vertebrate retina provides a classic experimental model with which to understand the mechanisms that modify progenitor potential over time. It is composed of multipotent neural progenitor cells capable of generating complex lineages, with multiple neuronal and glial cell types produced simultaneously. The retina provides a tractable

system with a relatively small number of cell types, a stereotyped architecture, and a developmental trajectory that is well-conserved across vertebrates (3). These features make the retina an invaluable system to identify molecular mechanisms that regulate progenitor potential and fate specification (4,5).

1.1. Retinal development:

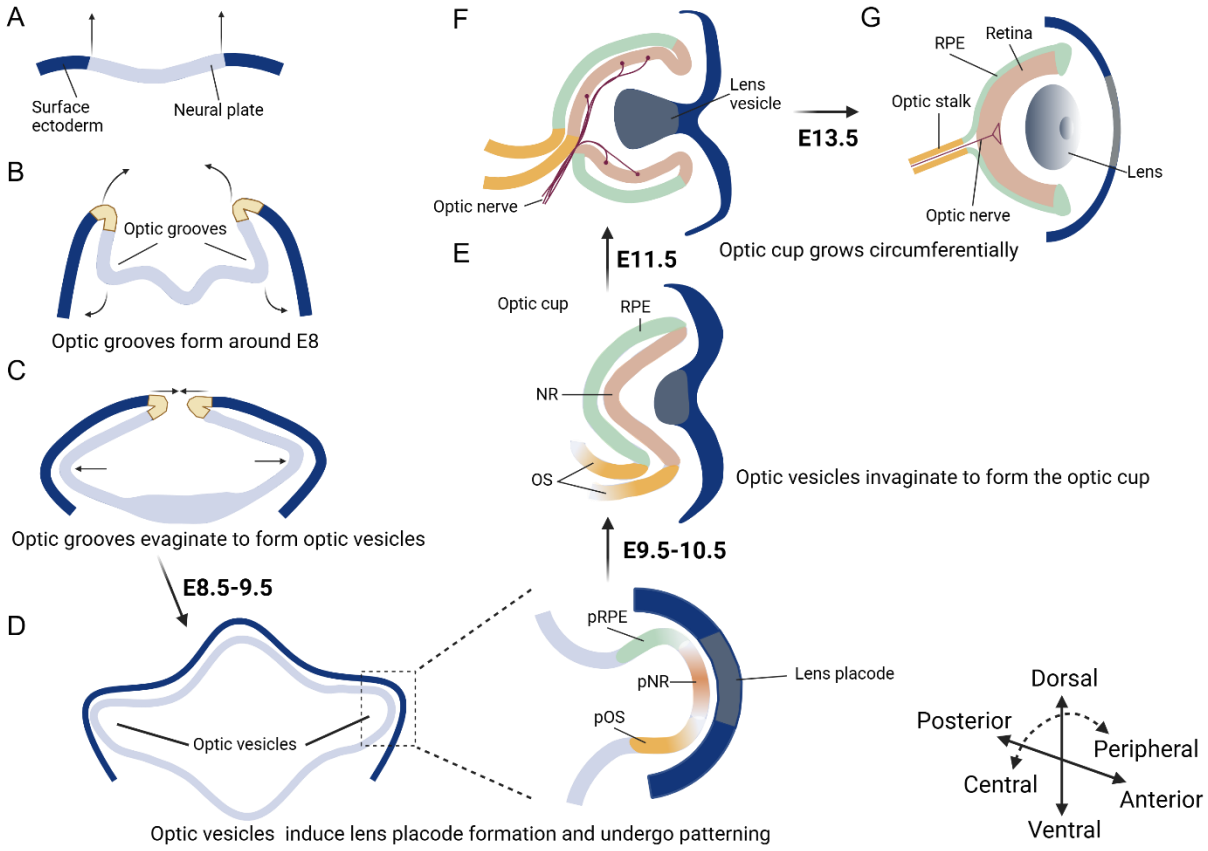

Fig. 1.1. Stages of early eye development in mouse. (A) The vertebrate eye develops from the diencephalic neural plate. (B) As the neural plate folds upwards and inwards, it forms the optic grooves. (C) The margins of the neural fold approach each other and the optic grooves expand outwards to initiate the formation of

the optic vesicle. (D) Subsequently, the neural fold is sealed and the neural tube is pinched off. The optic vesicles continue to grow outwards, coming in contact with the surface ectoderm, which is induced to form the lens placode. Simultaneously, the optic vesicle undergoes dorsal to ventral patterning where it is divided into presumptive retinal pigment epithelium (pRPE), presumptive neural retina (pNR) and presumptive optic stalk (pOS). (E) The optic vesicle undergoes invagination to form the bilayer optic cup, where the inner layer forms the NR and the outer layer forms the RPE. Additionally, the lens placode at the surface ectoderm begins to invaginate into the optic cup. (F,G) The optic stalk comes in contact with the ventral retina to generate the optic fissure that provides an exit for retinal axons. As the optic cup grows circumferentially, it closes the optic fissure enclosing the optic nerve. Additionally, the lens placode gets detached from the surface ectoderm to form the lens vesicle. Created with BioRender.com

Vertebrate eye development is initiated by the formation of the eye field within the anterior neural plate at a region where the expression of conserved eye field transcription factors (EFTFs) overlaps (6,7). In mice, the EFTFs include Pax6, Rax, Six3, and Lhx2, which form a regulatory network essential for eye formation, as mutations or knockdown of these genes can lead to severe developmental defects, such as anophthalmia (8–11). The induction of the eye-field by EFTFs causes morphogenetic changes in the anterior neural plate, where the optic grooves are formed. As the neural plate rises and infolds, the optic grooves expand outwards to become the optic vesicles (OV) (12) (Fig. 1.1 A-C). As development continues, the neural folds close and detach to form the neural tube, while the OV continues to evaginate or expand outward, coming into close contact with the overlying surface ectoderm and prompting the formation of the lens placode (8,9,12) (Fig. 1.1D).

In mice, this occurs around embryonic (E) day 8.5-9.5 (9). At this stage, the OV is composed of neuroepithelial progenitors that can generate RPE, OS, or neural retina.

Subsequently, the neuroepithelial progenitors undergo dorsal-ventral regional patterning, partitioning the cells into presumptive retinal pigment epithelium (dorsal), the neural retina (central), and the optic stalk (ventral) (8,9,13) (Fig. 1.1D). This process depends on a balance of signalling pathways — including FGF, BMP, Wnt/β-catenin, Shh and retinoic acid (8,13). These signals activate key transcription factors such as PAX6, RAX, SIX3, LHX2, OTX2, and VSX2 that establish retinal identity, guide tissue boundaries, and suppress alternative fates. For example, FGF1/2 secreted from the surface ectoderm activates VSX2 expression in the neural retina, which inhibits MITF to maintain neural retina identity over RPE identity (9). The dorsal OV then invaginates to form the bilayered optic cup, in which the inner layer forms the neural retinal and the outer layer forms the retinal pigment epithelium, lying apposed to one another (12,13) (Fig. 1.1 E). The ventral OV, which consists of the optic stalk, begins to elongate and as it comes into contact with the ventral retina it forms the optic fissure. By E11.5, the optic cup grows circumferentially, where the optic fissure closes to create the optic nerve, and the retinal pigment epithelium has fully encircled the neural retina (9,12,13) (Fig. 1.1F,G). Concomitantly, the lens placode invaginates to form the lens vesicle, which separates from the surface ectoderm and differentiates into the lens (7).

At this stage, the neural retina, is composed of proliferative neural progenitor cells called retinal progenitor cells (RPCs), that form a pseudostratified neuroepithelium known as the neuroblastic layer. At this time, RPCs continue to express EFTFs such as Pax6 and Lhx2 along with additional factors including Vsx2 and Sox2, which are required for maintaining RPC multipotency and supporting their continued proliferation (14,15). In mice, retinal neurogenesis begins around E11.5, where RPCs in the neuroblastic layer undergo a

complex sequence of events that alter their proliferation and output, alongside processes such as apoptosis that refine retinal cell numbers. Previous studies have shown that retinal development follows a central-to-peripheral gradient, with the earliest-born postmitotic cells arising near the optic nerve head, eventually generating the laminated retinal tissue, composed of all retinal neurons and glia (6,16).

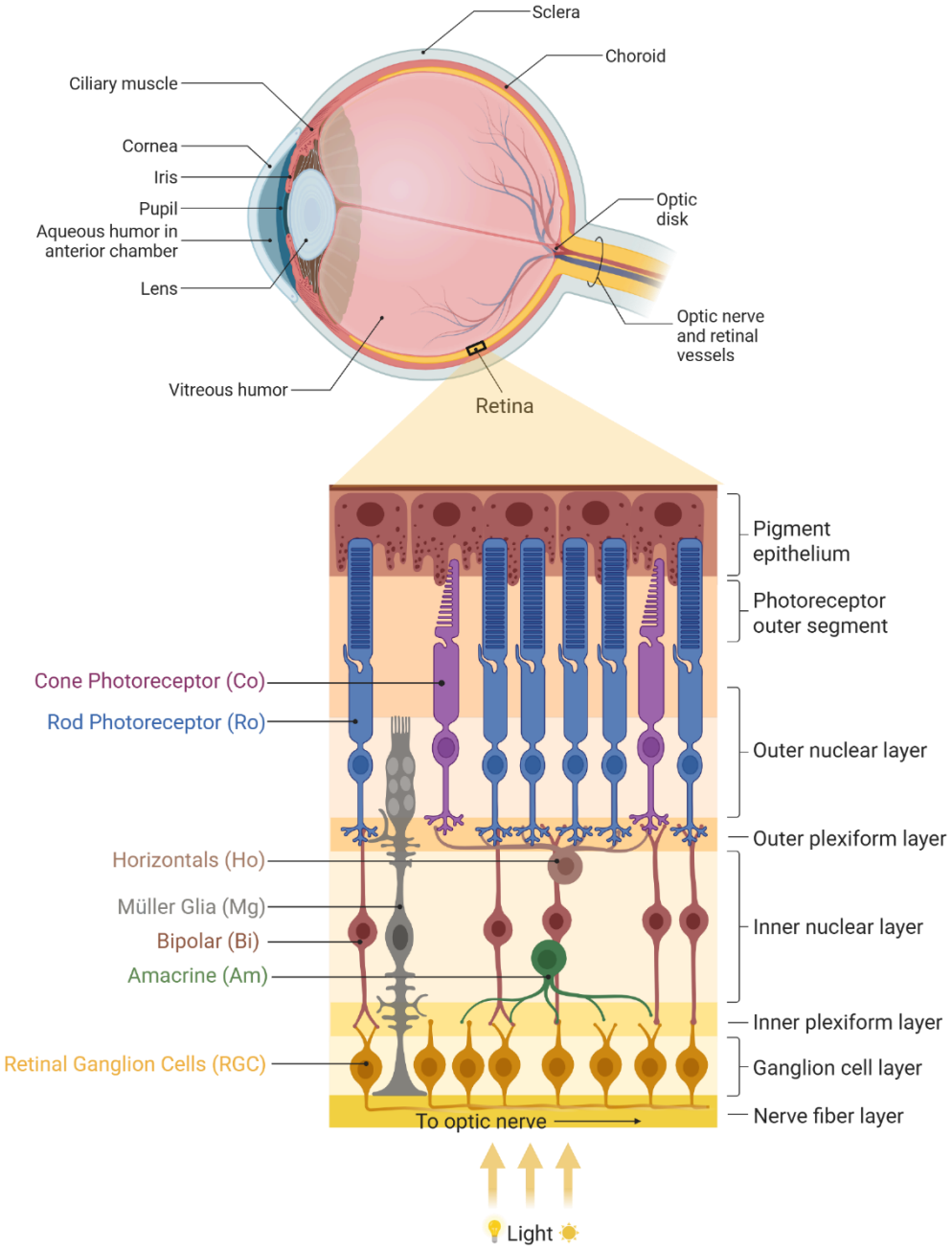

Fig 1.2. Schematic representation of the eye and the retina. (A) The eye is divided into an anterior segment, comprising the cornea, iris, and lens, and a posterior segment, consisting of vitreous humor, retina, and choroid. Light enters through the cornea, passes the anterior chamber and lens, and then travels through the vitreous humor before reaching the retina. The retina is the innermost neural tissue of the eye. (B) The retina is a laminated structure composed of three main layers, each containing cell bodies, with two plexiform layers. Below the retinal pigment epithelium lies the outer nuclear layer (ONL) that contains the

cell bodies of photoreceptor cells. Below this lies the outer plexiform layer, where the photoreceptors form synapses with bipolar cells and horizontal cells. The inner nuclear layer (INL) is composed of cell bodies of bipolar cells, horizontal cells, amacrine cells and Müller glia. The inner plexiform layer contains synaptic connections between bipolar cells and retinal ganglion cells (RGCs) along with amacrines. The last cell body-containing layer is the ganglion cell layer (GCL), which contains RGCs and displaced amacrine cells. The axons of RGCs traverse through the nerve fibre layer to the optic nerve. Created with BioRender.com

1.2.1 Retinal structure:

The retina is the innermost neural tissue of the eye (Fig. 1.2A) that plays a crucial role in phototransduction, which is the process of converting light energy from photons into neural signals, thereby enabling vision (17,18). Utilizing the Golgi staining method, Ramón y Cajal, in the 1890s, was able to provide a comprehensive description of the cellular organization of the vertebrate retina, along with its basic wiring, leading to the neuron doctrine (19,20). Subsequent advances in imaging technologies significantly improved the ability to acquire high-resolution images, enabling a deeper understanding of the retina's organization.

The mature vertebrate retina is a highly organized, laminated tissue composed of six major neuronal cell types, each with a distinct morphology and function and a single type of glial cell (Fig. 1.2B). These include primary sensory cells, which are the rod and cone photoreceptors, interneurons, namely, horizontal cells, bipolar cells and amacrine cells, and output neurons called retinal ganglion cells (RGCs), along with a single type of glial cell called Müller glia. These neurons are arranged into three discrete layers of cell bodies: the outer nuclear layer (ONL), which houses rod and cone photoreceptors; the inner nuclear layer (INL), which contains the somata of bipolar, horizontal, amacrine cells and

glia; and the ganglion cell layer (GCL), which includes RGCs and displaced amacrine cells. Interspersed between the cell body layers are two plexiform layers where retinal synaptic communication occurs (20). Together, this diverse ensemble of cell types forms morphologically and functionally distinct circuits that generate the retina's complex visual output.

The above-mentioned retinal structure is conserved across vertebrates, although species-specific differences are observed that adjust retinal structure or function to suit each species' visual requirements. For example, the human retina contains a high-acuity region in the central retina called the macula or fovea, which is mostly composed of cone photoreceptors responsible for colour vision. In contrast, the peripheral retina is thinner and is composed mostly of rods, which mediate scotopic vision(20). Another example is the composition of cone subtypes between human and mouse retinas. While the cones in mouse retinas express short wavelength (S)-opsin and middle wavelength (M)-opsin, in human retinas, there are three subtypes of cones which express S and M-opsin along with long wavelength (L)-opsin (18,21). In mice, rods are the most abundant retinal cells (≈70%), followed by bipolar and amacrine cells (≈10% and 8%, respectively). Cones and RGCs comprise 3-4% of retinal cells, while Müller glia make up around 5%. Lastly, horizontal cells represent less than 1% (22).

1.2.2 Birth order of retinal cell types:

The introduction of "birthdating" techniques has provided a powerful tool for accurately determining the developmental timing of the production of different retinal cell types. Nucleotide analogs such as tritiated thymidine or bromodeoxyuridine (BrdU), were used as birth-marking labels and were typically administered via intraperitoneal injections to

pulse-label the proliferating cells that were in S-phase, incorporating stably into any cell that is actively synthesizing DNA (23). The underlying concept is to introduce a permanent marker into a dividing cell and track the cell's development. As the marker remains stable, it is possible to infer retrospectively that a labelled cell underwent division at the time of marker administration. Thus, in cells that continue to divide, successive rounds of DNA replication would gradually dilute the label, whereas cells that exit the cell cycle at the subsequent mitosis would remain prominently marked, enabling precise determination of the timing of cell commitment.

Birthdating approaches have revealed that different retinal cell types are generated in a defined temporal sequence, largely conserved across vertebrates (24–27) (Fig. 1.3). More recently, single-cell RNA sequencing (scRNA-seq) has validated this temporal sequence of retinal cell production, as the emergence of distinct retinal cell types can be reconstructed from their transcriptomic signatures (28,29). In mice, early neurogenesis begins during embryonic stages, around E11.5, where the RPCs first generate RGCs, which are always the first-born retinal cell type. Subsequently, cone photoreceptors, horizontal cells and most amacrine cells are produced. These cell types differentiate during early embryonic stages and are considered early-born or early-fate cell types. As the generation of these cell types peaks, rod photoreceptors begin to be produced. Perinatally, RPCs undergo a competence transition in which they retain the ability to generate amacrine cells—with only a limited subset of amacrine subtypes produced postnatally—while completely losing the capacity to give rise to other early-born cell types. The late phase occurs postnatally. Late-born cell types include rods, generated in peak numbers during early postnatal stages, bipolar cells, and Müller glia. Studies have shown RPC

lineages can terminate through Müller glial differentiation, potentially via direct conversion of RPCs into Müller cells (30,31).

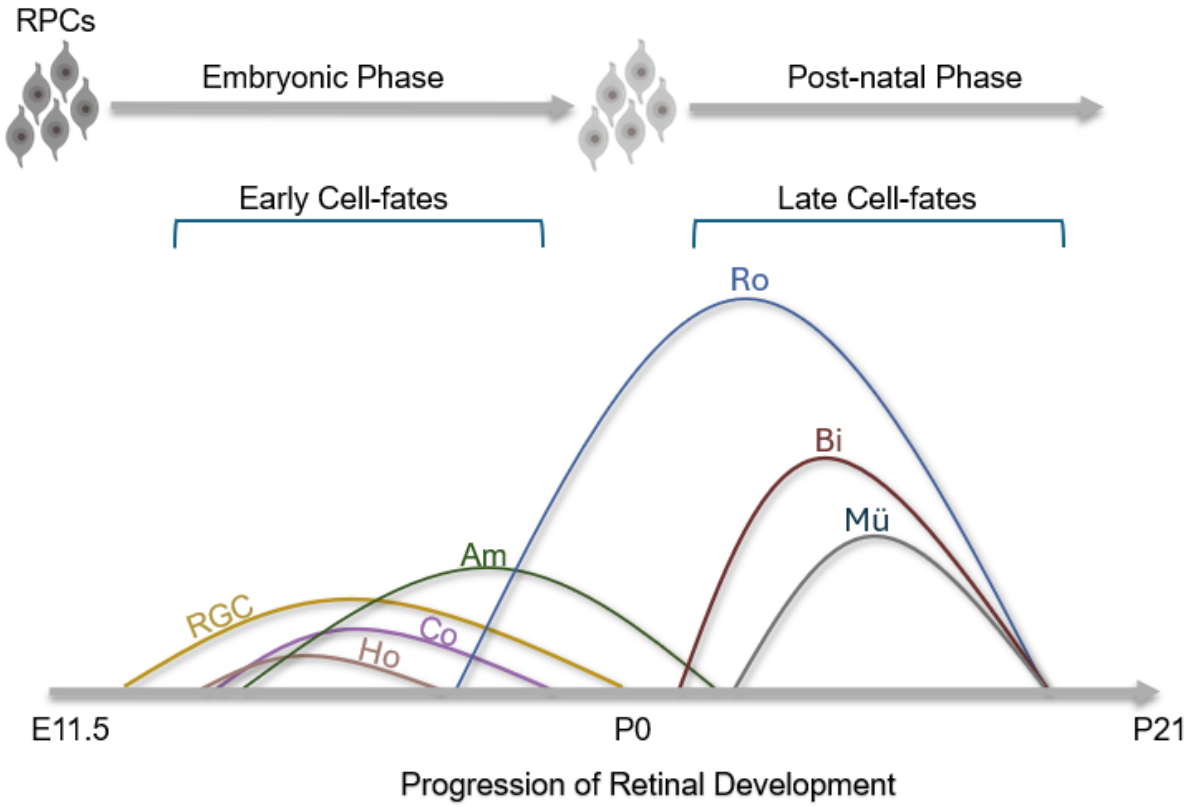

Fig 1.3. The birthdating curve of retinal cell types. The birth order of the different retinal cell types is based on previous birth-dating studies in mouse and rat retinas (24–27,32). The birthdating curve illustrates that RPCs exhibit distinct phases of multipotency, during which each phase confers competence to produce specific neuronal subtypes simultaneously. As development progresses, RPC potential becomes more restricted as they lose the competence to make early fate cells. The area under the curve is approximate and not to scale by abundance.

1.2.3 Multipotency of RPCs:

While birthdating methods reveal the population-level timing of retinal cell type generation, they cannot resolve lineage relationships or determine whether different cell

types arise from common or distinct progenitors. Lineage-tracing methodologies allow the retrospective reconstruction of individual progenitor cell behaviours as they mark progenitors at defined time points. However, a limitation to this approach is that these methods are often incapable of resolving lineage dynamics acutely, as some cell types require considerable time to express cell type markers and become morphologically differentiated, making it difficult to disentangle the order or timing of cell birth, as marked progenitors may continue to proliferate long after labelling.

Lineage-tracing experiments were first made possible by replication-incompetent gammaretroviruses that label only proliferative cells, as they require the mitotic breakdown of the nuclear envelope to integrate into the cell's genome. This cell-cycle dependence of retroviral transduction allows for largely unbiased labeling of individual proliferating RPCs without reliance on predefined gene-specific markers; however, residual biases persist, including preferential infection of specific proliferative cell populations and the possibility of transgene silencing, which can affect clone detection (33). Moreover, the size and composition of resultant lineages are constrained by the temporal competence state of the RPCs, such that the clonal output varies depending on the developmental stage at which infection occurs, reflecting a temporal-dependent bias in RPC output rather than solely methodological bias. Retroviruses are added at a low titre during development to facilitate sparse cell labelling, which helps identify the resulting clonal structure from individual RPCs. The clonal structure of individual RPCs can be reconstructed retrospectively, as the cells belonging to the same clonal tree are restricted to the radial axis of the retina, forming columnar units.

Retrovirus-based lineage tracing was performed in the Cepko lab, where retroviruses were administered during embryonic retinogenesis and at perinatal stages of development (34,35). These studies demonstrated that postnatally labelled RPCs were capable of producing all four postnatal cell types, with clones exhibiting diverse and overlapping combinations of these fates. Additionally, some clones contained both neurons and glia, indicating that a single progenitor can give rise to both lineages (34), suggesting that retinal glia do not arise from fate-restricted glioblasts. Embryonic retroviral labelling revealed that individual RPCs could generate large multicellular clones comprising various combinations of retinal cell types, with some clones containing six different cell types (35).

Subsequent studies used lipophilic dyes to mark cells in zebrafish and *Xenopus* (36,37). Other studies marked individual RPCs with reporters (typically GFP) through DNA transfections (38). This was further validated by generating retinal clones in chimeric mice using reporter transgenes, demonstrating that individual RPCs could generate clones of 100-200 cells in a radial column and included all seven cell types (39). These studies suggest that most RPCs are multipotent and can undergo both symmetric and asymmetric divisions, as evidenced by their large clonal sizes, which give rise to all retinal neuron and glial cell types. Time-lapse imaging has also advanced our understanding of retinal lineage dynamics by overcoming the limitations inherent to retroviral approaches. Time-lapse imaging allows direct visualization of cell production and thus enables the determination of the birth order of individual RPC lineages. Gomes et al. applied time-lapse microscopy to examine individual rat RPCs using long-term clonal cultures. They revealed that the birth order of neurons and glia was stereotyped, but not fixed within individual clones.

Additionally, individual RPCs underwent either asymmetric divisions, generating a progenitor and a post-mitotic cell, or symmetric divisions, producing either two post-mitotic cells or two progenitors. The terminal symmetric divisions that produce two post-mitotic cells could contain two different retinal cell types (40).

In the above study, live imaging was performed on clonal cultures of perinatal RPCs that had the potential to generate rods, amacrines, bipolars and Muller glia. The time-lapse revealed that the symmetric or asymmetric cell division patterns of individual RPCs varied unpredictably, fitting a stochastic model. This stochasticity was also observed in the cell-type composition of the clones, which generated a mix of all four cell types, not in a fixed sequence, and the probability of generating each cell type matched its overall abundance in the mature retina (40). Thus, at each division, there is a higher probability of generating a rod than other cell types, based on the cell-fate bias of the progenitor cell. Since these experiments were performed in culture, the stochasticity shown by RPCs is analogous to a "loaded dice," where the bias towards a certain fate is probabilistically determined by cell-intrinsic mechanisms, and the biases might change based on the developmental stage (40). This was further validated by a live-imaging study of zebrafish retinogenesis *in vivo* which showed that individual RPCs can generate all seven cell types and, like rat RPCs, exhibit stochasticity during development (41).

Recently, scRNA-seq studies have provided further insight into the multipotent nature of RPCs. Analysis of progenitor gene expression suggests that there is a single pool of multipotent RPCs (28,29). However, these studies have shown that RPCs also give rise to 'neurogenic' precursors that have more restricted proliferative and developmental potential. Retinal neurons are often thought to arise from specific neurogenic precursors,

whereas Müller glia are thought to differentiate directly from RPCs without passing through a neurogenic intermediate (28,29).

To identify mechanisms that control RPC bias, numerous studies have performed genetic fate mapping in mice, in which Cre-recombinase is expressed under the control of key developmental genes. Cre-based lineage tracing identified neurogenic precursor subsets that expressed bHLH transcription factors and revealed biases in progenitor fate specification. Under the control of the *Atoh7* promoter, Cre-based lineage analysis showed that Atoh7-expressing cells give rise to most neuronal cell types, including RGCs and cones, but bipolar and glial cells lie largely outside the Atoh7 lineage (42–44). Atoh7 acts upstream of RGC determinants and is thought to function as a permissive factor in establishing RGC competence, in which it is required for RGC development but not sufficient to specify RGC fate, and additionally, the Atoh7 lineage includes multiple non-RGC cell types (42,43,45–47). Similarly, Ascl1 is a proneural bHLH transcription factor that defines a subset of RPCs with restricted neurogenic potential. Brzezinski et al. performed inducible Cre-based lineage tracing studies showing that *Ascl1*-expressing progenitors generate most retinal neurons and glia but have limited competence to generate RGCs, whereas Neurog2-expressing progenitors produced few bipolars and Müller glia (48,49). Other factors such as Olig2 were also shown to define progenitor cell subpopulations where Cre-based mapping labelled early-born cell types including horizontals and cones but RGCs, bipolars and glia lied outside the Olig2 lineage (50).

However, in these cases, although the progenitors had the potential to generate multiple cell types, they had limited proliferative capacity relative to retroviral clones, suggesting that they might function within restricted RPC lineages and might represent different

neurogenic branches. Taken together, these data are consistent with the idea that the majority of the multipotent RPCs follow a stochastic model where their fate decisions are not preprogrammed. A notable exception is the Cdh6-expressing RPCs that are deterministic for a specific direction-sensitive RGC subtype (51). However, with respect to non-RGCs, Cdh6 -expressing progenitors generate other neuronal cell types in a stochastic manner (28).

1.2.4 Intrinsic regulation of RPC multipotency:

Multiple studies indicate that the temporal shifts in RPC competence are governed by intrinsic programs rather than by external environmental cues. In heterochronic transplantation experiments in *Xenopus laevis*, early-stage retinas were transplanted into older hosts, and the RGC axonal outgrowth roughly matched the original schedule of the grafted tissue, suggesting grafted RPCs preserved their temporal state despite the temporally inappropriate *in vivo* environment (52). Furthermore, culturing retinas *ex vivo* as explants had minimal effects on the timing of cell generation, and the initially undifferentiated neuroblastic retina subsequently generated retinal cell types that organized into layers similar to those formed *in vivo* (53–55).

Additionally, when RPCs were dissociated and cultured, they largely adopted temporally appropriate fates, and early heterochronic mixing experiments reported minimal shifts in their competence. When RPCs were mixed with early- or late-born cells at varying ratios, they did not convert from late-to-early or early-to-late competence in response to environmental cues (4). For instance, heterochronic combinations produced more early-born cones while reducing early-born amacrines (56), while other combinations likewise elevated late-born bipolar production while diminishing late-born rods (57). In some

paradigms, modest tendencies for early progenitors to generate late-born fates, such as rods, were observed; however, robust or systematic changes in competence were not evident. Nonetheless, the interpretation of these findings is limited by the technical constraints of the time, including restricted marker availability and limited resolution for defining cell states, thereby limiting the ability to fully assess the extent of shifts in competence. Re-examination of these paradigms using contemporary approaches, such as single-cell RNA sequencing, may provide a more comprehensive understanding of progenitor competence dynamics. Lastly, RPCs were cultured at clonal density to examine the output of individual cells under uniform environmental conditions. These experiments showed that isolated late-stage RPCs generated lineages with similar cell numbers and cell type composition to their *in vivo* counterparts, suggesting that intrinsic mechanisms regulate cell fate specification (40,58).

Collective findings from birthdating, lineage-tracing, and heterochronic clonal culture studies formed the basis for the competence model, which suggests that RPCs generate different cell types by undergoing transitions in their competence (59). According to this model, as individual RPCs progress through developmental stages, they transition through different competence states, such that at early stages of development, an RPC first acquires the ability to produce a particular set of cell types. As its competence shifts, it loses the potential to make those early cell types and gains the potential to generate later-born ones (60). These sequential changes in temporal competence establish developmental windows during which different cell types can be produced.

Although cell-intrinsic mechanisms regulate temporal competence, extrinsic cues have also been shown to influence RPC behavior (3). For instance, Notch signaling plays a role

in balancing RPC proliferation and differentiation (61). The Notch pathway consists of transmembrane receptors Notch1–4 and ligands of the Delta and Jagged family (62). Receptor–ligand interactions between neighbouring cells release the Notch intracellular domain (NICD), which activates downstream targets such as Rbpj, Hes1, and Hes5 that regulate RPC proliferation (61,63,64). In dividing RPCs, Notch signaling mediates lateral inhibition, which is a juxtacrine feedback mechanism between neighbouring cells. Lateral inhibition promotes asymmetric divisions in which a cell committing to a neuronal fate signals its neighbours to remain as progenitors (65). Overall, studies indicate that both the timing and levels of Notch signaling regulate multiple aspects of retinal development, including the balance between progenitor proliferation and differentiation. Active Notch signaling in early RPCs maintains them in a proliferative state by preventing cell-cycle exit, whereas downregulation of Notch promotes neuronal differentiation. Notch signaling also contributes to neuronal cell-type specification by influencing cell fate decisions in progenitors. In addition, Notch signaling plays a pivotal role in glial specification, with elevated activity observed in progenitors committing to gliogenesis (66–72). However, it remains unclear whether Notch signalling alters RPC competence to generate cell types outside their defined developmental window.

In addition to the growth factors required to sustain RPCs in culture, studies have identified additional extrinsic cues that can alter cell type production (73,74). However, the known extrinsic cues are believed to act as feedback regulators. Factors such as Gdf11, Tgf-beta, and Shh are secreted by early-born neurons. Once these particular cell types are generated in adequate numbers, they releases the aforementioned growth factors, leading to suppression of further production of the same cell type (75). Although extrinsic cues

influence RPC behaviour by modulating proliferation or restricting cell-type production, they are thought to have minimal effects on regulating temporal competence states. Rather, intrinsic mechanisms play a key role in regulating progressive changes in RPC competence over time, while extrinsic cues may fine-tune these intrinsic programs (76).

1.2. Mechanisms regulating RPC competence:

Overall, birthdating, lineage tracing, and time-lapse imaging have revealed distinct features that govern retinal development, namely, (i) individual RPCs are multipotent and can generate all the retinal cell types by transitioning through intrinsically determined competence states, and (ii) retinal cell type specification follows a chronological birth order that is conserved across vertebrates. Despite these advances, the mechanisms regulating the temporal transitions in RPC competence remain incompletely understood. A key question is how RPCs switch their competence during development to produce the correct cell types at the right time and in correct proportions. Numerous studies have identified potential cell-intrinsic mechanisms underlying these transitions and have proposed mechanisms that are not mutually exclusive but instead likely act cooperatively to orchestrate retinal development. These mechanisms include cascades of transcription factors, miRNAs, and epigenetic processes.

1.3.1 Combinatorial Coding

Transcription factors play a key role in retinal cell-fate specification (77). For example, Nrl is required for rod photoreceptor identity, while Ptf1a specifies horizontal and amacrine cells, with its loss reducing these cell types and increasing RGCs (78,79). As additional fate-determining transcription factors have been characterized, transcriptional hierarchies and regulatory networks have emerged in which these factors might cooperate

or negatively regulate one another to stabilize cell-type identity (3,60). For instance, Pou4f2 (Brn3b) is essential for RGC specification and represses the expression of factors involved in the differentiation of other early cell types (80).

The combinatorial coding model posits that the co-expression of specific transcription factor combinations determines neuronal or glial identity (81–83). This is observed during spinal cord development, where the spatial location of progenitor cells alters their exposure to morphogens secreted by signalling centers. Morphogen concentrations determine which combinations of transcription factors are induced or repressed, and these combinations are sufficient to confer competence for specific neuronal or glial type (84). Transcription factors of the basic helix-loop-helix (bHLH) and homeodomain (Hd) families play particularly prominent roles in this process (85) and in the developing mouse retina, several Hd and bHLH factors may act combinatorially to guide retinal cell specification (9).

However, Hd and bHLH factors by themselves were not sufficient to program retinal fates. In the retina, most of the important Hd transcription factors (including the EFTFs) are expressed throughout development. While bHLH factors exhibit more dynamic expression patterns, they were not found to be sufficient to generate their respective cell types. For example, in a study in *Xenopus,* six bHLH transcription factors, along with eight Hd factors, were misexpressed in RPCs, but this did not result in deterministic generation of any particular cell fates (85). Moreover, bHLH factors are mainly expressed in neurogenic precursors rather than in RPCs (28), and most fate determinants therefore cannot directly regulate competence. Competence factors should likely be expressed in dividing RPCs where they might act to suppress competing competence states while

minimally impacting proliferation and functioning upstream to bias the expression of fate-determinants (60). Nevertheless, a key limitation is that current single-cell RNA sequencing and lineage-tracing approaches lack sufficient resolution to discern whether multipotent competence is an intrinsic property of progenitors or instead represents transient intermediate states, where they regulate the generation of different retinal subtypes.

1.3.2 Temporal Transcription Factors

Temporal regulation of neural progenitor competence is well defined in *Drosophila* neuroblasts in the ventral nerve cord, where a sequential cascade of temporal transcription factors (tTFs), namely, *hunchback, Krüppel, pdm, castor, and grainyhead*, acts to impose stepwise changes in competence and ensure the correct timing and proportion of neuronal fates (86–89). Analogous mechanisms operate in the developing vertebrate retina, where dynamically expressed tTFs act in RPCs and are necessary and sufficient to specify distinct fate windows while suppressing inappropriate fates (90). The tTFs carry out these functions independently of changes in RPC proliferation and/or differentiation and also engage in cross-regulatory interactions that drive temporal competence transitions (86).

Being informed from the fly literature, our lab and others have shown that murine orthologs of *Drosophila hunchback* (*Ikaros/ Ikzf1*) and *castor* (*Casz1*), played a similar role in regulating temporal competence in the developing mouse retina. Expression studies in mice revealed that both Ikzf1 and Casz1 have a dynamic pattern, where Ikzf1 is expressed in RPCs during embryonic phases of retinogenesis, but not in post-natal stages, while Casz1 has a low expression level during embryonic phases, but steadily rises as retinal development progresses and peaks postnatally (91,92).

Functionally, the Cayouette lab showed that Ikzf1 loss reduces early-born cell types, while its overexpression in late retinal stages induced early fates at the expense of bipolar cells and Müller glia (91,92). In contrast, conditional knock-out of *Casz1* resulted in decreased rod photoreceptor production, accompanied by a small increase in early cell types, including horizontals, amacrines, and cones (92). Müller glia generation was also increased in the absence of *Casz1*, suggesting that Casz1 acts as a temporal competence factor in RPCs, promoting rod production and suppressing other early and late cell fates (93). Moreover, it was observed that the effects of Ikzf1 and Casz1 were independent of progenitor cell proliferation or death, and that Ikzf1 misexpression in late RPCs represses Casz1 expression (81).

Subsequent studies identified forkhead transcription factors as additional temporal regulators. Foxp1 exhibits a dynamic, early developmental expression pattern. It promotes early competence by extending the production of early-born cell types and repressing late fates through transcriptional activation of early RPC genes and repression of late temporal factors such as *Casz1* and *Nfib* (94,95). Foxn4 specifies early competence, being necessary and sufficient for amacrine and horizontal cell formation, acting upstream of key differentiation factors important for amacrine and horizontal cell differentiation, and engaging in cross-regulatory control by repressing Ikzf1 and activating Casz1 (96,97).

The Blackshaw lab additionally identified Nfia/b/x transcription factors as regulators of late temporal competence. Nfia/b/x transcription factors upregulate in RPCs at late stages and are necessary and sufficient for late bipolar/Müller competence and to terminate the RPC lineage (28,98,99). Overexpression of Nfia/b/x biases progenitors toward both bipolar cells and Müller glia at the expense of earlier neuronal types (28), while the

absence of Nfia/b/x severely impairs the gliogenic competence of RPCs and bipolar generation while prolonging the rod production beyond their normal neurogenic period due to impaired cell-cycle exit (28).

Although tTFs have been shown to regulate RPC competence transition during development, there are some research gaps that remain. While the genomic occupancy for select tTFs, such as Nfib and Ascl1 has been characterized, the mechanisms by which tTFs regulate the genome remains largely unknown. As a result, how these factors directly regulate target genes and modulate progenitor competence remains poorly understood, with current insights obtained primarily from phenotypic analyses. Furthermore, the extent to which alterations in tTFs expression influence neuronal birthdating remains unresolved. Although some studies report modest shifts within early temporal windows (91,94), definitive changes in neuronal birthdates have yet to be demonstrated. Addressing these gaps would delineate the molecular mechanisms by which tTFs regulate the genome to stably alter the gene expression patterns in RPCs to influence their temporal competence.

In addition to the tTFs, there are additional transcription factors that can influence RPC potential. For instance, Lhx2 which is an eye field transcription factor expressed continuously in RPCs from the onset of retinogenesis and later in Müller glia. Although it does not meet the criteria of a temporal transcription factor (72,100), it nonetheless, influences both early and late fate specification events and is essential for the proper temporal progression of RPC competence (72,98,101). Its loss prolongs retinal ganglion cell production beyond its normal window (101). Lhx2 is also required for RPCs to

respond to extrinsic cues, including Sonic hedgehog–mediated feedback, where Lhx2 was required for the expression of Shh co-receptors (102).

1.3.3 MicroRNAs

MicroRNAs (miRNAs) are 19–25 nucleotide non-coding RNAs that regulate gene expression by repressing translation or promoting degradation of target mRNAs (103). In the canonical pathway, RNA polymerase II transcribes miRNA genes into hairpin-containing primary transcripts (pri-miRNAs), which are processed by Drosha–DGCR8 into precursor miRNAs (pre-miRNAs) (103–105). Pre-miRNAs are further processed in the cytoplasm into mature miRNAs and loaded into RNA-induced silencing complex (RISC) to guide silencing of complementary mRNAs, by binding primarily to their 3' untranslated region (106). In the non-canonical pathway, miRNAs can also arise from introns or exons of protein-coding genes via splicing before entering the canonical processing pathway.

Multiple studies have used microarrays, quantitative real-time PCR, and in situ hybridization, among others, to profile miRNAs expression and temporal dynamics during retinal development (107–109). To assess miRNA function, the Reh lab conditionally knocked out the *Dicer* gene in RPCs. The Dicer enzyme plays a central role in miRNA biogenesis, being essential for both converting pre-miRNAs into their mature forms and functioning as an integral component of the RISC complex (107). In the absence of *Dicer*, the progression of RPC competence from early to late was severely affected, leading to a prolonged window of RGC production at the expense of late-born cell types such as rods and Müller glia (110). In the same study, the researchers performed miRNA profiling and

identified several miRNAs whose expression was dysregulated in the absence of *Dicer* (110).

In a subsequent study, three key miRNAs were identified as effectors of Dicer. La Torre et al. showed that miRNAs let-7, miRNA-125, and miRNA-9 were important in regulating the early-to-late competence transition in RPCs (111). The overexpression of these miRNAs rescued the *Dicer* mutant phenotype and promoted the transition to the late competence state, thereby inducing the production of late-born cell types, such as rod photoreceptors. Moreover, Lin28 and Prtg were identified as downstream targets, and miRNA inhibition of Lin28 and Prtg in RPCs is required for progression from the early to the late stages of competence, as overexpression of these targets maintained an early competence state in RPCs (111).

These studies suggest that miRNAs regulate RPC competence and, given their effects on mRNA stability or translation, might also regulate the expression of temporal factors and fate determinants, thereby influencing other aspects of retinal development, such as progenitor proliferation, cell fate determination, and maintenance (107,112). However, the mechanisms by which miRNAs orchestrate these processes remain poorly defined. Some gaps include defining how the temporal dynamics of miRNA expression are achieved during retinal development and distinguishing direct versus indirect targets of miRNA regulation. In addition, how miRNAs interact with transcription factors and epigenetic regulators to influence cell-fate decisions remains unresolved. Finally, many miRNAs are encoded by multiple paralogous genes, complicating the study of how different miRNA genes contribute to competence.

1.3.4 Epigenetic Mechanisms

Waddington first introduced the concept of epigenetics, which can be described as the study of heritable changes in gene expression that occur without altering the underlying DNA sequence (113). Epigenetic mechanisms such as DNA methylation, histone modification or nucleosome sliding are key in maintaining or suppressing gene expression plasticity during development. These cell-intrinsic mechanisms exert a profound effect on neural stem and progenitor cell potential through restructuring the chromatin and regulating gene expression, and hence, numerous studies have examined the roles of chromatin regulators during retinal development (114,115). Chromatin regulators are enzymes and protein complexes that can be grouped into three broad categories, namely, "writers", "readers" and "erasers", based on their ability to either modify or recognize epigenetic marks (116). Certain writers, such as the DNA methyltransferases Dnmt1, can covalently attach methyl groups to promoters of active genes, rendering them inactive, and their absence has been shown to affect RPC cell-cycle progression leading to reduced rod photoreceptor generation (117). DNA methylation is scarce in pluripotent cells, but this modification gradually spreads across the genome as development proceeds, helping to stabilize emerging cell identities. Conversely, erasers such as ten-eleven translocation (Tet) enzymes function to demethylate DNA. For example, a recent study by Hernández-Núñez et al. has shown that active DNA demethylation mediated by TET enzymes is essential for rod photoreceptor specification, where demethylation acts upstream of rod fate determinants such as Nrl and Nr2e3, and in its absence, photoreceptor precursors adopt the cone identity (118). Although DNA methylation and demethylation influence

cell type specification, they appear to have little impact on directing RPC lineage decisions (119).

On the other hand, epigenetic writers such as polycomb have been shown to function in neural progenitors to regulate the transition between neurogenesis and gliogenesis (93,120,121). Polycomb repressive complex 2 (PRC2) catalyzes the addition of the repressive mark H3K27me3, thereby establishing a heterochromatic nucleosome configuration (122). Conditional deletion of PRC2 components in the developing retina led to reduced RPC proliferation and enhanced Müller glial generation, along with alterations in other neuronal cell fates (120,121). More recently, the Vetter lab examined conditional knockouts of Jarid2. Jarid2 is an obligatory polycomb subunit in RPCs, and its loss alters temporal progression, leading to overproduction of early-born cell types at the expense of later-born neurons, but not glia (94). Zhang et al. showed that the birth window for generating early cell types was prolonged in these knockouts. Additionally, RPCs displayed an early-shifted transcriptional profile. Genes expressed in early RPCs failed to be downregulated and did not acquire the H3K27me3 mark, including the Ttf *Foxp1* (94).

The above studies indicate that heterochromatic modifications may explain how retinal temporal development is regulated by epigenetic marks, where they would function to reinforce temporal gene expression programs. Another mechanism that might regulate RPC competence is gene decommissioning, in which accessibility of gene regulatory elements are lost, leading to stable gene silencing. In this scenario, epigenetic erasers, such as the nucleosome remodelling and deacetylase (NuRD) complex (described further below in Section 1.4.1) could work in concert with polycomb. Reynolds et al. showed that in

embryonic stem cells, NuRD-mediated deacetylation of the active histone mark H3K27ac enabled PRC2 recruitment and subsequent H3K27 trimethylation at promoters, resulting in gene repression (123). The NuRD complex has also been linked to polycomb complexes and to temporal development in neural progenitors (93,124–127). The NuRD complex can decommission genes and regulatory elements, creating a barrier to future reactivation (128). Thus, gene decommissioning might represent a molecular mechanism driving the progressive restriction of neural progenitor potential and thereby regulating RPC competence (Fig. 1.4).

The mechanisms described above that govern temporal transitions in RPC competence are not mutually exclusive. Rather, proper retinal development likely relies on coordinated interactions among them. In many instances, Ttfs have been shown to interact with epigenetic regulators, such as the NuRD complex during retinal development (93). Additionally, the NuRD complex has also been shown to interact with Ikzf1, Foxp1 and Lhx2 in different contexts (129–131). miRNAs have also been shown to associate with epigenetic modulators, in which nuclear miRNAs form a complex with Argonaute proteins, which can then direct histone-modifying enzymes, such as Hdacs and the polycomb protein EZH2, to target gene promoters, thereby establishing repressive histone marks (e.g., H3K27me3) and inducing gene silencing (132–134). These observations suggest a model in which heterochromatic processes might act downstream of temporal transcription factors. However, while transcription factors have a well-studied role in retinal development, how they regulate and remodel the genome remains much more poorly understood. The NuRD complex is a strong candidate for integrating dynamically expressed temporal transcription factors with heterochromatic effectors. NuRD has both

histone deacetylation and nucleosome remodelling activities, and we sought to examine how chromatin remodelers regulate RPC potential.

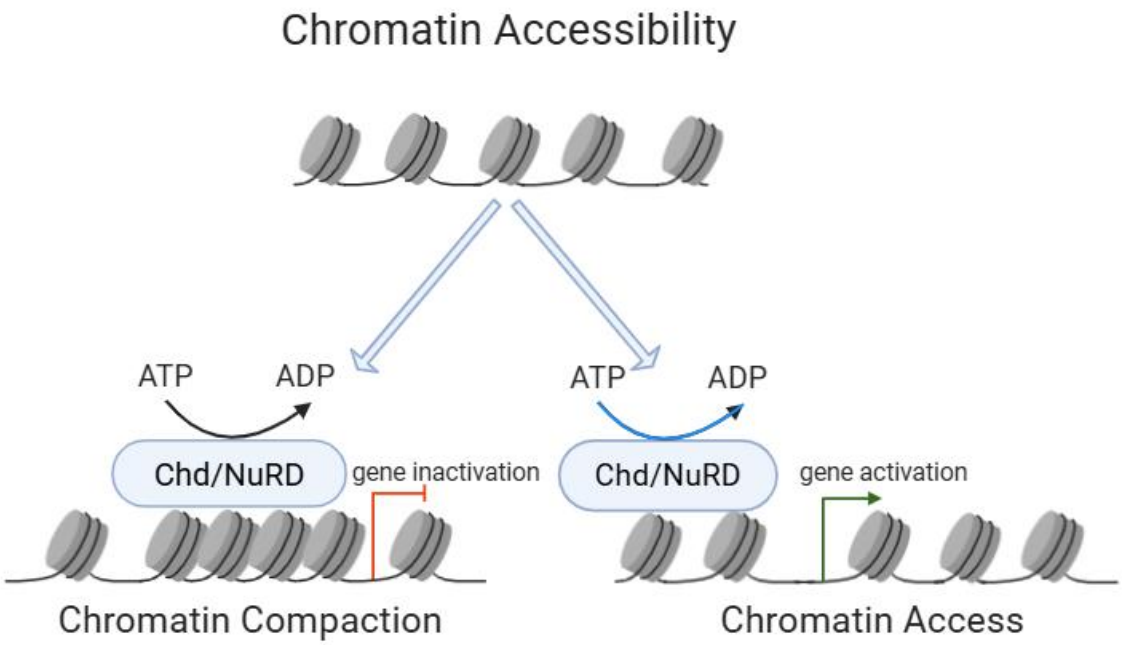

Fig 1.4. Nucleosome remodelling by Chd/NuRD. The ATPase/helicase domain of Chd proteins disrupts histone–DNA interactions via a twist-defect mechanism, driving ~5 bp nucleosome translocation along the DNA. This activity regulates nucleosome spacing and chromatin accessibility (135). Nucleosome sliding alters local chromatin accessibility, thereby exposing or closing regulatory elements and modulating gene expression. During cerebellar development, NuRD suppresses accessibility, thereby decommissioning a subset of promoters (128,136).

1.3. Nucleosome Remodellers:

The dynamic organization of chromatin within the cell plays a critical role in regulating gene activity, enabling cell-type–specific programs during development and differentiation. Central to this process is the nucleosome. Nucleosomes are the building blocks of chromatin, where DNA is wrapped around a disc of highly conserved proteins

called histones. Each nucleosome consists of a core histone octamer, composed of H2A, H2B, H3, and H4, wrapped by 146 base pairs (bp) of DNA (137). Between each nucleosome is a 10-80 bp linker DNA, to which H1 linker histones can be recruited. H1 linker histones function to stabilize and compact the nucleosome and the chromatin structure (138). The dynamic plasticity of the chromatin structure is partly driven by the action of specialized ATP-dependent nucleosome remodellers. Nucleosome remodelers include enzymes that bind to chromatin and alter interactions between histones and DNA, thereby regulating accessibility at regulatory elements and affecting gene activity (139). During development, nucleosome remodelling coordinates key processes, including progenitor proliferation, neural migration, and cell differentiation (122).

Nucleosome remodellers utilize the energy from ATP hydrolysis to reposition, eject, and replace histones within the nucleosome (135). This ability is provided by an SNF2 (sucrose nonfermenting 2)-like ATPase domain, which can be subdivided into two linked RecA-like lobes (also denoted as DExx and HELICc) that are linked by a variable insertion. Depending on the functional domains flanking the ATPase, the nucleosome remodellers can be classified into four distinct families. Namely, switch/sucrose-non-fermenting (SWI/SNF), imitation switch (ISWI), chromodomain-helicase-DNA-binding (CHD) and inositol requiring 80 (INO80) (137). Additionally, several orphan families of nucleosome remodellers have also been identified, such as the alpha-thalassemia/mental retardation (ATRX) family (140).

Nucleosome remodellers have been shown to regulate various aspects of retinal development, as loss-of-function studies have resulted in complex retinal phenotypes that (122). For instance, BRG1, a component of the SWI/SNF complex, is crucial for retinal

lamination and regulating cell-cycle length and exit in mice. Loss of Brg1 resulted in microphthalmia and impaired photoreceptor differentiation (141). Another SWI/SNF subunit, Brm plays a role in specifying the RGC fate by modulating Brn3b expression and function and promotes cell cycle exit during RGC differentiation (142). In another study, the ISWI subunit Snf2h was conditionally deleted, resulting in significantly smaller retinas lacking photoreceptors. Snf2h was shown to be expressed in RPCs and post-mitotic cells, and its deletion did not affect the generation of retinal cell types except rods and cones, indicating its requirement for photoreceptor maintenance (143). Although these studies indicate that nucleosome remodellers play an important role during neural differentiation, not much is known about how nucleosome remodelling might regulate RPC potential. Thus, in this study, we focused on one such nucleosome remodeler, Chd4, a member of the Chd family.

1.4. Chd4 nucleosome remodeller:

The chromodomain helicase (CHD) family encodes ATP-dependent chromatin remodelers that are highly conserved from yeast to humans and are associated with diverse biological functions, including transcriptional regulation, cell proliferation, and maintenance of genome stability (144). They are also linked to cancer, neurodevelopmental disorders, and other developmental diseases in humans (145). The CHD family consists of ten distinct members (CHD1L, CHD 1–9) that are classified into three subfamilies (I-III) based on their structural properties and presence of other conserved domains in addition to the centrally located SNF2-type helicase-ATPase domain and a pair of N-terminal chromodomains. While the ATPase domain confers ATP-dependent enzymatic activity

that enables CHD proteins to remodel the epigenetic state of target genes, the chromodomains facilitate chromatin interactions by binding to DNA, RNA, or methylated histones (145). Members of subfamily I (CHD1/CHD2) contain a DNA-binding domain with a preference for binding AT-rich sequences (146). In contrast, subfamily III members (CHD6, CHD7, CHD8, and CHD9) contain BRK domains, SANT-like regions, CR motifs, and an additional non-sequence-specific DNA-binding (145).

Chd4 is a ~219 kDa protein that belongs to sub-family II along with Chd3 and Chd5. The subfamily II members are characterized by the presence of two consecutive plant homeodomain (PHD) zinc finger motifs located upstream of their chromodomains (147). Additionally, two domains of unknown function have been identified at the C-terminus of these proteins (Fig. 1.5) (144). The different domains of Chd4 have unique functionalities that enhance its catalytic activities. The PHD fingers are readers of H3 histone tail modifications. Chd4 PHD fingers exhibit preference for methylated histone H3 lysine 4 (H3K4), and also to H3K9me3 and H3K9ac, which are found at both active and inactive gene regulatory elements (148,149). However, this latter preference was restricted in the presence of H3K4me3, which is a mark associated with active promoters (145,148–150). In Chd4, evidence suggests that both the PHD fingers can simultaneously bind to both the H3 tails within the same nucleosome and are required for the nucleosome remodelling and repressive functions of Chd4 (151,152). Similarly, the chromodomains were found to support the ATPase and transcriptional repression activities of Chd4 (151). The C-terminal domain (CTD) of Chd4 has been shown to be essential for interactions with Gatad2b and Adnp (153). The C-terminal part of the protein also contains two domains of unknown function (DUF), which are thought to mediate transcriptional modulation through

interactions with multiple co-repressors (154). Additionally, recent studies have indicated that the C-terminus of Chd4 harbours a SANT-SLIDE domain that reinforces its remodelling activity (155,156).

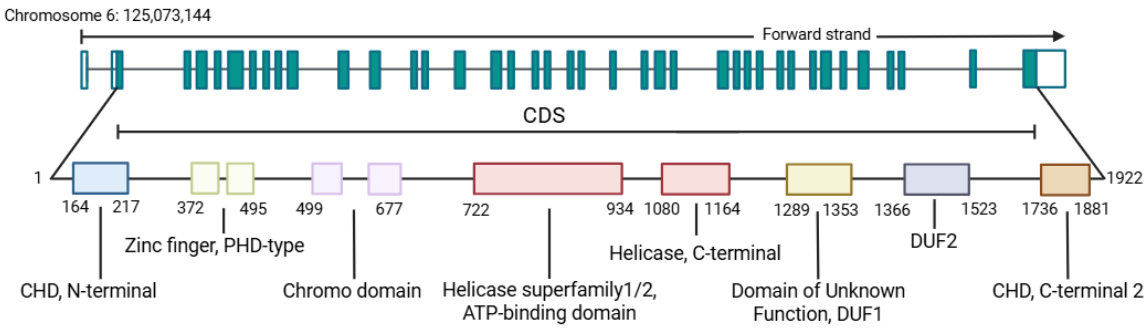

Fig 1.5 Domains of Chd4. The Chd4 gene is located on chromosome 6 in the mouse and 12p13 in humans. The gene is ~34kb and contains 39 exons, with the CDS start codon in the 2nd exon. The Chd4 protein is 1922 amino acids long and possesses N-terminal PHD fingers followed by chromodomains and the enzymatic ATPase-helicase domain. In the C-terminal region, it has two domains of unknown function.

1.4.1 Chd4 complexes

NuRD complex:

Chromatin remodellers commonly form multisubunit complexes to facilitate and enhance remodelling activities, and control recruitment to the genome. Chd4 is known to be a core component of the nucleosome remodelling and deacetylase (NuRD) complex. The NuRD complex is an epigenetic modulator with dual enzymatic activities that is composed of two subcomplexes, namely, the remodelling subcomplex and the deacetylase subcomplex (157). The NuRD complex contains several additional subunits, each encoded by multiple

paralogous genes. Recent studies indicate that the metastasis associated proteins (MTA1/2/3) form a dimer that associates with four copies of the retinoblastoma binding proteins (RBBP4 and/or RBBP7) and together with two histone deacetylases (HDAC1/2) subunits form the deacetylase submodule (158,159). The remodelling subcomplex is composed of Chd proteins that interact with the GATA zinc-finger domain proteins (Gatad2A/B). The sub-complexes are connected by methyl-CpG-binding protein (Mbd2/3), which binds to the MTA dimers and recruits the Gatad2 subunit to form the complete NuRD complex (158,160) (Fig. 1.6A). The enzymatic activities of the NuRD complex are provided by Chd3/4/5 and Hdac1/2, where Chd3/4/5 endows the NuRD complex with the ability to slide nucleosomes along the chromatin, modulating accessibility to gene regulatory elements and thereby affecting gene expression. Hdac1/2 provides the NuRD complex with the ability to deacetylate lysine residues on histone tails (161).

CHD, GATAD2, and MBD subunits are thought to be monomeric within NuRD, and their recruitment is therefore mutually exclusive with that of their respective paralogs (127,162,163). For instance, in the murine neocortex, CHD3, CHD4, and CHD5 homologs form distinct NuRD complexes that are proposed to regulate different aspects of cortical development (127). Similarly, MBD2/3 were also shown to be present in distinct NuRD complexes with different genomic occupancy. While MBD2 robustly binds methylated DNA leading to gene repression, MBD3 appears to prefer hydroxymethylated DNA (163,164). Moreover, the NuRD complex has been shown to interact with various transcription factors, such as Ikzf1, Casz1 and Lhx2, among others (93,129,130). Thus, the presence of distinct NuRD subunit associations enables numerous possible complex

configurations, which may help fine-tune NuRD activity. By altering its subunit composition and interacting partners, the NuRD complex can potentially adapt its function, supporting its broad roles in transcriptional regulation and genome integrity (165,166).

In the context of development, the NuRD complex was shown to be a vital regulator of stem and progenitor cell proliferation and the control of fate potential. Initially, the NuRD complex was believed to be involved in gene repression, however, gene expression analyses have revealed that the NuRD complex contributes to both repression and activation and is enriched at many transcriptional start sites and accessible regulatory elements (167). Comprehensive studies using embryonic stem cells and have shown that the NuRD complex fine-tunes transcription by changing chromatin architecture, suppressing inappropriate or noisy expression during cell-state transitions, and regulating lineage commitment by modulating expression of pluripotency genes (167–169).

ChAHP complex:

A recent study in mouse embryonic stem cells (mES) identified a new chromatin remodelling complex called the “ChAHP complex” that is composed of Chd4, Activity-dependent neuroprotective protein (Adnp) and Heterochromatin protein 1 (Hp1) (170) (Fig. 1.6B). Ostapcuk et al. showed that the ChAHP complex binds to specific DNA motifs and represses gene expression by establishing an inaccessible chromatin state around its binding sites. This study also showed that the loss of function of the ChAHP complex in mES cells led to impaired commitment to the neuronal lineage (170). A follow-up study showed that ChAHP counteracted CTCF binding in a competitive manner (171). ChAHP

was shown to prevent CTCF binding and chromatin loop formation, thereby affecting interactions between promoters and enhancers.

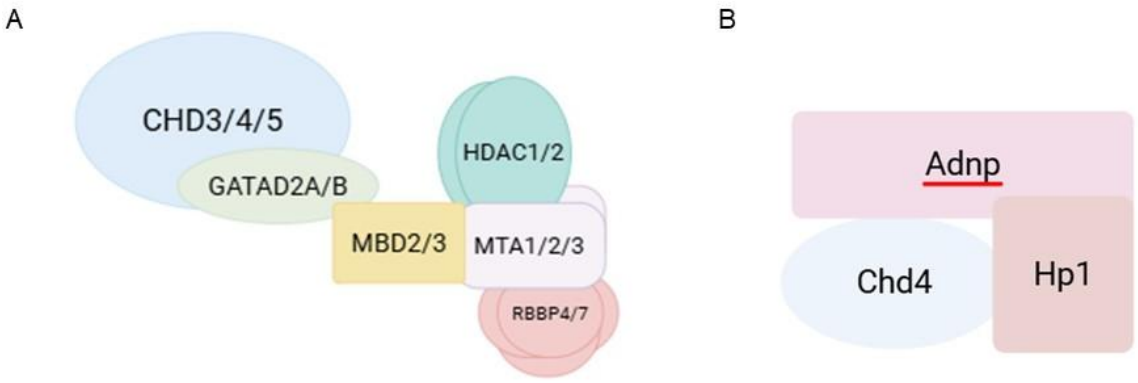

Fig 1.6. Chd4 complexes. (A) Chd4 is one of the core components of the NuRD complex and forms the remodelling subcomplex along with Gatad2 subunits. The deacetylase module is composed of Hdac, Mta and Rbbp subunits and Mbd domains bridges the two modules to form the complete NuRD complex. (B) Recent studies identified that Chd4 interacts with Adnp and Hp1 proteins to form the ChAHP complex.

1.5. Objectives and Hypothesis:

In the developing murine CNS, the Chd4/NuRD complex, along with the polycomb group of proteins, was observed to be required for normal transition from neurogenesis to gliogenesis through persistent repression of neuronal differentiation in late-stage neural progenitor cells (125,172). This is consistent with a previous study that observed the requirement of the Chd4/NuRD complex for suppressing neuronal differentiation (126). However, in the developing murine retina, the NuRD complex, along with Casz1 and

polycomb, were observed to suppress gliogenesis, as perturbations in the NuRD complex functionalities resulted in increased glial production (93). The reason for the disparities between different studies is not well understood; however, the above studies indicate the importance of epigenetic modulators in competence state transitions and cell fate specification of progenitor cells. Although we have previously identified the functional relevance of the NuRD complex during the transition from the production of rod photoreceptors to Müller glia, the Chd4-specific regulation of other neuronal fate decisions throughout retinal development and its effects on early-phase RPCs remain unclear.

Previous studies in the developing murine neocortex have shown that during early-stages of development, progenitor cells lacking *Chd4* exhibited increased cell-cycle exit and apoptosis, resulting in cortical thinning and altered cortical lamination (127). Conversely, Sparmann et al. showed that in late-stage neural progenitor cells Chd4 regulates the transition from neurogenesis to gliogenesis by restricting the early onset of glial production (124). Recent studies have shown that de novo mutations in *CHD4* lead to multisystemic neurodevelopmental disorder that includes several overlapping phenotypes such as developmental delay, intellectual disability and macrocephaly, as well as optic nerve abnormalities (173,174). However, mechanisms by which Chd4 regulates neural progenitor cell multipotency are not well understood, as germline *Chd4* knock-outs in mice are lethal (175).

Hence, the objective of this study is to utilize the developing mouse retina to dissect, *in-vivo*, the genetic requirement for *Chd4* during retinal development and identify mechanisms through which it might regulate the distinct states of RPC multipotency. We

hypothesize that Chd4 dynamically regulates the epigenetic landscape of RPCs to influence developmental timing and cell fate decisions. To accomplish the above objectives, we used a conditional genetics approach, in which *Chd4* was knocked out specifically in RPCs at the onset of retinogenesis.

We found that in the absence of *Chd4*, the cell-type composition of the retina was altered. Birthdating studies showed that Chd4 regulated competence transitions during retinal linage termination. In order to delineate the mechanism by which *Chd4* might regulate retinal development, we performed single-cell RNA-seq (scRNA-seq) on late-stage RPCs and examined the nucleosome remodelling activity of Chd4 using the Assay for Transposase-Accessible Chromatin (ATAC)- seq. We also performed Cleavage Under Target & Release Under Nuclease (CUT&RUN) to identify the occupancy of NuRD subunits such as Chd4 and Mbd3. Taken together, our data indicate that Chd4 regulates the competence transition that terminates the retinal lineage but not earlier competence transitions. These results show for the first time that the epigenetic mechanisms governing retinal competence transitions are context-dependent, wherein, nucleosome remodelling is required for the late-stage transition terminating the retinal lineage, whereas the early-to-late neurogenic switch is likely regulated by epigenetic factors and mechanisms distinct from Chd4.

Chapter 2. Materials and Methods

2.1. Animal Work:

Animal work was conducted in accordance with the guidelines established by the Canadian Council on Animal Care, under the supervision of the Animal Care and Veterinary Service (ACVS) at the University of Ottawa, in compliance with the ethical protocols OHRI-2856, OHRI-2867, OHRI-3949, and OHRI-4029. Mice with *Chd4* floxed alleles (*Chd4*$^{f/f}$) (176) were graciously donated by Katia Georgopoulos (Harvard, MA, USA), while the *Chx10-Cre* transgenic mice (177) were generously donated by the Catherine Tsilfidis laboratory (OHRI, ON, Canada). C57BL/6J mice were obtained from Jackson Laboratories (RRID: IMSR_JAX:000664) and used to backcross both *Chd4*$^{f/f}$ and *Chx10-Cre* alleles onto the C57BL/6J background. To generate retina-specific *Chd4* conditional knockout (cKO), *Chd4*$^{f/f}$ mice were crossed with the *Chx10-Cre* line. Both males and females were used for all experiments.

For genotyping, ear notches from adult mice or tail clippings from pre-natal/juvenile mice were collected. DNA was extracted using 50mM NaOH at 95°C for 30 minutes, followed by neutralization with 1M Tris at pH 7.4. The samples were mixed by vortexing and centrifuged (Legend Micro 21 Centrifuge, Sorvall, Thermo Scientific, Catalogue no. 75002437) for 5 minutes at max speed. Polymerase chain reaction (PCR) was performed using primers listed in Table 2.1, according to the manufacturer's protocol (Taq FroggaMix FBTAQM, Frogga). The PCR products were subsequently run on 1% agarose gel (1% w/v agarose, 0.003% Ethidium Bromide in 1X TAE buffer (24.2% w/v Tris base, 5.7% glacial acetic acid, 50mM EDTA pH≈8.0 in ddH_20), and visualized using UV detection (Gel Doc XR+ Molecular Imager, Bio-Rad). The amplicon sizes were verified

using 1kb DNA ladder (GeneRuler 1kb Plus DNA Ladder, FERSM1331, Thermo Scientific).

Name	Sequence
Mi-2ß Fwd strand	5′-CTCCAAGAAGAAGACGGCAGATCT-3′
Mi-2ß Rev strand	5′-GTCCTTCCAAGAAGAGCAAG-3′
Cre Fwd	5′-AGGTGTAGAGAAGGCACTTAGC-3′
Cre Rev	5′-CTAATCGCCATCTTCCAGCAGG-3′

Table 2.1. Primer sets used for genotyping.

2.2. Tissue Preparation:

For embryonic stages, whole heads were collected and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Subsequently, the whole heads underwent three washes with 1X PBS (0.137M NaCl, 2.7mM KCl, 0.01M Na_2HPO_4, 1.8mM KH_2PO_4, pH≈7.4 in ddH_20) for 3 minutes each on a rocker at room temperature, followed by immersion in 20% sucrose in 1X PBS at 4°C overnight. The next day, they were subjected to 3 washes in 1X PBS for 5 minutes each and submerged in a 1:1 solution of OCT:20% sucrose in 1X PBS overnight at 4°C. Finally, the whole heads were embedded in the OCT compound and were stored at -80°C.

For P0 and P2, the pups were euthanized via decapitation and the whole eyes were dissected out from the head using curved forceps under the dissecting microscope. Subsequently, a small slit was introduced between the lens and choroid to allow the fixative to penetrate the eye. Eyes were then fixed in 4% PFA for 10 minutes followed by

3 washes in 1X PBS for 2 minutes each at room temperature and incubated in 20% sucrose at 4°C overnight. Finally, the eyes were transferred into the O.C.T. compound (Fisher 23-730-571) and frozen at -80°C.

P15 mice were euthanized via CO2 inhalation followed by cervical dislocation, while P5 to P8 pups were euthanized via decapitation. At adult stages, from P5 onwards, eye-cups were generated as described previously (178). This was followed by 4% PFA fixation for 2-3 minutes. After fixation, the retinal tissues underwent 3 washes in 1X PBS for 2 minutes each at room temperature and were transferred to 20% sucrose in PBS at 4°C for 2-3 hours. They were finally immersed in the OCT compound for storage at -80°C.

2.3. Immunohistochemistry:

The retinal tissues were parallel-sectioned using a cryostat (Thermo Scientific). 14µm thick coronal cryosections were collected onto Superfrost Plus slides (Fisher) and processed for immunofluorescence as described previously (92,93). Briefly, the tissue sections were washed three times in 1X PBS, followed by incubation with the primary antibody overnight at 4 °C. Primary antibodies (Table 2.2) were diluted in blocking buffer (1X PBS supplemented with 0.4% Triton X-100, 3% w/v BSA, and 1:5000 Hoechst 33342). Subsequently, the slides were washed 3 times with 1X PBS. Alexa 555, or 647 – conjugated secondary antibodies (Table 2.2) were added at a dilution of 1:1000 in blocking buffer and incubated for 2-3 hours at room temperature. After 3 washes in 1X PBS, the coverslips were mounted using Mowiol mounting media (12% w/v Mowiol 4-88, 30% w/v glycerol, 120mM Tris-Cl pH 8.5, 2.5% DABCO) and stored at 4°C until imaged.

Antigen	Species	Dilution	Supplier	Catalog no.	Application
Brn3a	Mouse	1:100	Millipore Sigma	MAB1585 MI	IHC
Brn3b	Goat	1:200	Santa Cruz	sc-6026	IHC
Chd3	Rabbit	1:200	Fortis	A301-220A-T	IHC
Chd4	Rat	1:200	Biolegend	942302	IHC
Chd4	Rabbit	1:1000 1:100	Abcam	Ab72418	Western, CUT&RUN
Cleaved Caspase- 3 (Asp175)	Rabbit	1:500	Cell Signaling	9579	IHC
Cone Arrestin	Rabbit	1:200	Millipore Sigma	AB15282	IHC
Gfp	Chick	1:1000	Abcam	AB13970	IHC
Hoechst 33342		1:5000	Tocris	NB5117	IHC
Ki67	Rabbit	1:100	Millipore Sigma	SAB5500134	IHC
Lhx2	Rabbit	1:200	Millipore Sigma	ABE1402	IHC
Mbd3	Rabbit	1:100	Abcam	EPR18258	CUT&RUN
Neurofilament	Mouse	1:100	DSHB	2H3	IHC
Nr2e3	Mouse	1:100	R&D Systems	PP-H7223-00	IHC
Otx2	Goat	1:200	R&D Systems	AF1979	IHC
Pax6	Rabbit	1:100	Novus	NBP2-19711	IHC
pHH3	Rabbit	1:200	Cell Signaling	9701S	IHC
Rbpms	Guinea pig	1:100	Millipore Sigma	ABN1376	IHC
Rlbp1	Mouse	1:100	Invitrogen	MA1-813	IHC
Rxrγ	Mouse	1:100	Santa Cruz	Sc-365252	IHC
Sox2	Goat	1:100	R&D systems	AF2018-SP	IHC
Sox9	Rabbit	1:100	Novus		IHC
Tfap2a	Mouse	1:50	DSHB	3B5	IHC
Anti-guinea pig Alexa Fluor™ 555	Goat	1:1000	Rockland	606-142-129	2° (IHC)
anti-goat Alexa Fluor™ 488	Donkey	1:1000	Jackson	705-547-003	2° (IHC)
Anti-goat Alexa Fluor™ 555	Donkey	1:1000	Jackson	705-565-147	2° (IHC)
Anti- goat DyLight™ 650	Donkey	1:1000	Novus	NBP1-75604	2° (IHC)
Anti-mouse Alexa Fluor™ 555	Donkey	1:1000	Invitrogen	A-31570	2° (IHC)

Anti-mouse Alexa Fluor™ 647	Donkey	1:1000	Invitrogen	A-31571	2° (IHC)
Anti-rabbit Alexa Fluor™ 555	Donkey	1:1000	Invitrogen	A-21428	2° (IHC)
Anti-rabbit Alexa Fluor™ 647	Donkey	1:1000	Jackson	711-607-003	2° (IHC)
Anti-rat DyLight™ 550	Donkey	1:1000	Invitrogen	SA510027	2° (IHC)
Anti- Rabbit HRP	Donkey	1:10000	GE Healthcare	NA934	2° (Western)

Table 2.2. List of antibodies and dilutions along with its application.

2.4. Imaging and Cell Counting:

Images were acquired on an LSM900 confocal microscope (Zeiss), using a 63X objective (Plan-Apochromat 63x/1.40 Oil DIC f/ELYRA) with a 0.5X digital zoom. Where required, a 20X objective (Plan-Apochromat 20x/0.8 Ph2) with 1.0X digital zoom was also used to acquire whole eye images. The images were tiled using Zen software (Zeiss) where required. For each biological replicate, four optical sections (single Z planes) of the peripheral retina were used to generate the cell counts. Manual cell counting was performed using Fiji (ImageJ), wherein each optical section analyzed was cropped to a constant axial/tangential plane of 100µm. Images were further processed using Adobe Photoshop CS3 (Adobe) software.

2.5. EdU Incorporation:

P0, P1 and P8 pups were injected intraperitoneally with 10mM of EdU (Invitrogen #C10640; 50 mg/kg) and eyes were harvested at P2, P8 or P15, as indicated in the figure legends. EdU staining was performed on retinal sections using the Click-iT™ Plus EdU Cell Proliferation Kit for Imaging (Invitrogen #C10640) according to the manufacturer's protocol. In instances where EdU was co-stained with other antibodies, the tissues were

first processed with immunostaining with primary and secondary antibodies before the Click-iT staining reaction.

2.6. TUNEL Assay:

The P0 retinas were processed for TUNEL assay as described above. After obtaining 14μm coronal cryosections, the tissues were subjected to IHC staining with primary and secondary antibodies. Subsequently, the sections were washed three times with 1X PBS before being subjected to the TUNEL assay protocol as per the manufacturer's protocol (ThermoFisher #C10618).

2.7. Explant Transduction:

Retroviral preparation, *ex vivo* retroviral transduction, and clone reconstruction were performed as described previously (92), with minor modifications. pMSCV-IRES-Cre was obtained from Addgene and used for Cre transduction into *Chd4 f/+* and *Chd4 f/f* retinal explants at P0. The media was changed daily for 14 days, after which the explants were fixed in 4% PFA and frozen in OCT. Subsequently, the tissues were sectioned and analyzed by IHC. Cell types were annotated using morphology and laminar distribution as described previously (93).

2.8. Statistical Analysis:

Statistical analysis for image count data was performed using Microsoft Excel and GraphPad Prism version 8 (GraphPad) software. n-values refer to biological replicates, where each biological replicate is an independent animal, corresponding to an individual data point. For cell-counting, a minimum of 3 biological replicates were quantified, and statistical analysis was performed via one-way ANOVA with Tukey’s multiple comparison

test or two-tailed unpaired t-test. We did not perform statistical analyses to predetermine sample sizes. The count data is presented by mean ± standard error of mean (SEM). ∗p < 0.05; ∗∗p < 0.05; ∗∗∗p < 0.005; ∗∗∗∗p < 0.0005. ns = not significant.

2.9. Western Blot:

Western blotting was performed as previously described (93) with some modifications. P0 dissected retinas were homogenized in ice-cold RIPA lysis buffer with protease inhibitors (cOmplete, Mini, EDTA-free; 11836170001; Millipore Sigma) and 1mM PMSF. The samples were then incubated on ice for 15 minutes. Thereafter, they were sonicated on ice using a Cole-Parmer Ultrasonic Homogenizer (RK-04711-45) at 20% amplitude with an 8-sec pulse followed by a 30-sec interval for a total of 3 pulses and centrifuged at 21,000xg at 4°C for 15 mins. The supernatant was collected, and the protein concentration was quantified using BCA assay. Approximately 40µg of protein lysate was separated on a 6-10% gradient SDS-PAGE gel and semi-dry transferred onto PVDF membranes (Millipore).

The membranes were then washed 3 times in 1X TBST buffer and blocked for 1 hour at room temperature in the blocking buffer. Subsequently, the primary antibody (Table 2.2) was added at a dilution of 1:1000 in blocking buffer and incubated overnight at 4°C on a shaker. After 3 washes with 1X TBST, the membranes were incubated with secondary HRP-conjugated antibody at a dilution of 1:10,000 in blocking buffer at room temperature for 2 hours on a shaker. Subsequently, the membranes were washed 3 times with 1X TBST and 1ml of ECL solution was added. The membranes were then digitally imaged using BioRad chemiluminescence gel doc.

2.10. Retinal Single-cell Suspension:

To obtain a neonatal retinal dissociated cell suspension, retinas from P1 pups were dissected out using aseptic conditions into the Papain buffer (0.003N NaOH, 100U papain solution (Worthington), 0.4% DNaseI (Millipore Sigma 04716728001), 2mg L-cysteine crystal in 1X PBS). The dissociation steps were also performed under sterile conditions. The dissected retinas once transferred into the papain buffer were incubated at 37°C for 8 minutes. Subsequently, papain buffer was aspirated and replaced with LO-OVO solution (1X LO-OVO (Bio Basic), 0.4% DNaseI in 1X PBS). The retinal tissue was washed once more with gentle aspiration and the addition of fresh LO-OVO solution. Thereafter, the retinal tissue was triturated and centrifuged for 11 minutes at 200g at room temperature. The retinal cells were then resuspended in 1X PBS. Consequently, the cells were counted and viability assessed using trypan blue with a Countess II instrument (Invitrogen, AMQAX1000).

2.11. ATAC-seq:

Two biological replicates were used for each genotype, namely, control (wt, *Chd4*+/+$^{Cre+ve}$) and mutants (cKO, *Chd4* f/f^{Cre+ve}). P1 retinas from each biological replicate were dissected to make a dissociated suspension as described above. Subsequently, the dissociated cells were subjected to fluorescence-activated cell sorting (FACS) at the OHRI Flow Cytometry and Cell Sorting Facility using a Beckman Coulter MoFlo XDP, and 75,000 Gfp+ve cells were flow-sorted from each replicate and used for the ATAC-seq assay as described previously (179,180) using the manufacturer's protocol (Nextera library kit, Illumina), with assistance from the Stemcore Molecular Biology Core Facility at OHRI.

Briefly, 75,000 flow-sorted cells were lysed in cold lysis buffer (10mM Tris-Cl, pH 7.4, 10mM NaCl, 3mM $MgCl_2$ and 0.1% IGEPAL CA-6390). The lysed nuclei were then tagmented with 6.5µl of TDE1 transposase. Samples were subsequently purified using Zymo-Spin IC columns (Zymo), and the sequencing libraries were made according to the Nextera workflow. Libraries were purified using AMPure XP kit (Beckman Coulter) and sequenced by paired-end sequencing (150bp) using the NextSeq 500 platform to a read-depth of 25-35 million reads per sample.

The analysis of the ATAC-seq dataset was performed using the Galaxy interface (181). After the initial quality control, the NGS adapters were trimmed using Trimmomatic, and the reads were mapped to the mm9 reference genome using Bowtie2. The alignment files were generated in duplicates for each replicate. The duplicated alignment data for each replicate was then merged using Samtools 'merge'. Subsequently, peak calling was performed with MACS2. Differential peak detection and analysis were performed using DiffBind and Limma. DiffBind peaks were sorted via k-means clustering with Seqplots and annotated to nearby genes using GREAT. Footprinting analysis was performed using TOBIAS. The ATAC-seq and CUT&RUN-seq data is available on the GEO database, under accession GSE266039.

2.12. CUT&RUN-seq:

CUT&RUN was performed using the CUTANA™ ChIC / CUT&RUN Kit (EpiCypher #14-1048) according to the manufacturer's protocol. Briefly, P1 pups were euthanized via decapitation, and the retinal tissues were harvested. The dissected tissues were then submerged in 500µl of media (DMEM) supplemented with 10% DMSO and underwent slow freeze at -80°C. The retinal tissues were stored at -80°C until the genotypes of the

littermates were confirmed. For this experiment, we utilized individual pups for each antibody reaction and subjected the CUT&RUN protocol on both control and conditional knock-out littermates. The frozen retinal tissues were quickly thawed at 37°C and homogenized using mechanical disruption techniques. Approximately 500,000 cells were used for each antibody reaction. The cells were pelleted by spinning for 3 mins at 600 x g at room temperature and resuspended in wash buffer. The cells were washed twice before immobilizing them onto activated Concavanalin A beads. Subsequently, 2µl of primary antibodies, namely, anti-Chd4 (Abcam #Ab72418) and anti-Mbd3 (Abcam #Ab157464), were added. For negative control, we utilized the manufacturer-provided anti-rabbit IgG antibody. The antibody-bound beads were incubated overnight at 4°C on a rotator followed by permeabilization with 200µl of Digitonin buffer. The antibody-bound chromatin was then subjected to the manufacturer's protocol for pAG-MNase cleavage. Subsequently, a stop buffer containing E. coli spike-in DNA for sequencing normalization was added to stop the enzymatic reaction. The resulting cleaved chromatin was then isolated using the DNA purification kit and the purified DNA was eluted in 12µl of elution buffer, which was submitted to the Stemcore Molecular Biology Core Facility at OHRI for library preparation and sequencing.

The analysis for the CUT&RUN-seq datasets was performed using the Galaxy interface. The fastq raw sequence reads were first subjected to FastqGroomer for conversion between various FASTQ quality formats and then subjected to quality control steps to eliminate reads with phred scores below 20. Subsequently, Trimmomatic was used to trim the adapters and other Illumina-specific sequences from the reads. The reads were mapped to the mm9 reference genome using Bowtie2. Subsequently, the alignment files were

generated in duplicates for each replicate were merged was for each replicate via Samtools 'merge'. Peak calling was performed using MACS2 and lastly, the called peaks were clustered via k-means clustering.

2.13. Multi-seq:

Multi-seq was performed as described previously (179,182) with some modifications. For this assay, we used three biological replicates each for the control (Chd4 f/+Cre+ve) and mutant (Chd4 f/f Cre+ve) samples, totalling six replicates. P1 retinas from each biological replicate were dissociated to obtain a single cell suspension as described above. Approximately 250,000 dissociated cells per replicate were then barcoded by incubating with 'anchor' and 'co-anchor' lipid-modified oligonucleotides graciously provided by the Gartner lab (182). Barcode oligonucleotides were purchased from Integrated DNA Technologies. Multiseq barcodes 1,3 and 4 were used to tag individual control replicates while barcodes 2,5 and 6 tagged individual mutant replicates (Table 2.3). Individual replicates were co-incubated with barcode oligonucleotides for 10 minutes. The barcoded cells were then pelleted and washed thrice with PBS and pooled into a single tube at a 1:1 ratio. Approximately 20,000 pooled cells were used in a single Chromium™ run (3' Library & Gel Bead Kit v2, PN-120237, 10X Genomics).

Barcode no.	**Replicate Genotype**	**Sequence**
Multiseq# 1	Control-Chd4 F/+$^{Cre-}$	5′-CCTTGGCACCCGAGAATTCCAGGAGAAGAAAAAAAAAAAAAAAAAAAAAAAAAA-3′
Multiseq# 2	Mutant-Chd4 F/F^{Cre+}	5′-CCTTGGCACCCGAGAATTCCACCACAATGAAAAAAAAAAAAAAAAAAAAAAAAAA-3′
Multiseq# 3	Control-Chd4 F/+$^{Cre+}$	5′-CCTTGGCACCCGAGAATTCCATGAGACCTAAAAAAAAAAAAAAAAAAAAAAAAAA-3′

Multiseq# 4	Control-Chd4 F/+$^{Cre+}$	5′-CCTTGGCACCCGAGAATTCCAGCACACGCAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3′
Multiseq# 5	Mutant-Chd4 F/F^{Cre+}	5′-CCTTGGCACCCGAGAATTCCAAGAGAGAGAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3′
Multiseq# 6	Mutant-Chd4 F/F^{Cre+}	5′-CCTTGGCACCCGAGAATTCCATCACAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3′

Table 2.3. Sequence of the multiplex barcodes used for tagging individual replicates. The highlighted nucleotides are the unique replicate-specific barcode sequence.

The resulting expression library FASTQs were processed using CellRanger (10X Genomics). The deMULTIplex workflow was used to perform quality control, removing doublets and cells that lacked barcodes. Output files were filtered and analyzed using Scanpy version 1.9.190 in Python (Python Core Team n.d.). Genes detected in less than 3 cells were removed from the analysis. Low-quality cells (less than 5,000 genes detected, less than 20,000 reads/counts detected, or more than 0.05% of mitochondrial genes detected) were excluded. The cell types were annotated through an unbiased deep-learning model based on previously published retinal single-cell expression data (28) using scDeepSort version 1.0. Differential gene expression analysis was performed using Scanpy (Wilcoxon signed-rank test) or MAST version 1.24.042. The scRNA-seq data is available on the GEO database, under accession GSE300175.

Chapter 3. Results

3.1 Functional requirement of Chd4 during retinal development

3.1.1. Chd4 expression during retinal development:

We first utilized immunohistochemistry to examine the spatiotemporal expression profile of Chd4 during mouse retinal development. We found that Chd4 was ubiquitously expressed from embryonic day (E) 11.5 through to adult stages (Fig. 3.1). Expression levels were relatively constant within the nuclei of Ki67+ RPCs from E13.5 through to postnatal day (P) 2 (Fig. 3.1D-K). However, Chd4 levels became somewhat elevated in postmitotic neurons within the ganglion cell layer (GCL). At P0 and P2, elevated expression was also apparent within postmitotic Ki67-negative cells within the outer neuroblastic layer (ONBL; Fig. 3.1G-K). In the adult retina, Chd4 expression levels remained particularly elevated within inner nuclear layer (INL) and GCL neurons. Weak expression was also apparent in rod photoreceptors (Fig. 3.1L-O). To address other (group II subfamily) Chd4 paralogs, we examined previously published retinal scRNA-seq data (28). While Chd4 was expressed at high levels in RPCs, both Chd3 and Chd5 were mainly expressed in postmitotic neurons (Fig. 3.2). These results indicate that Chd4 is the main group II paralog in RPCs, but that its expression is not temporally dynamic.

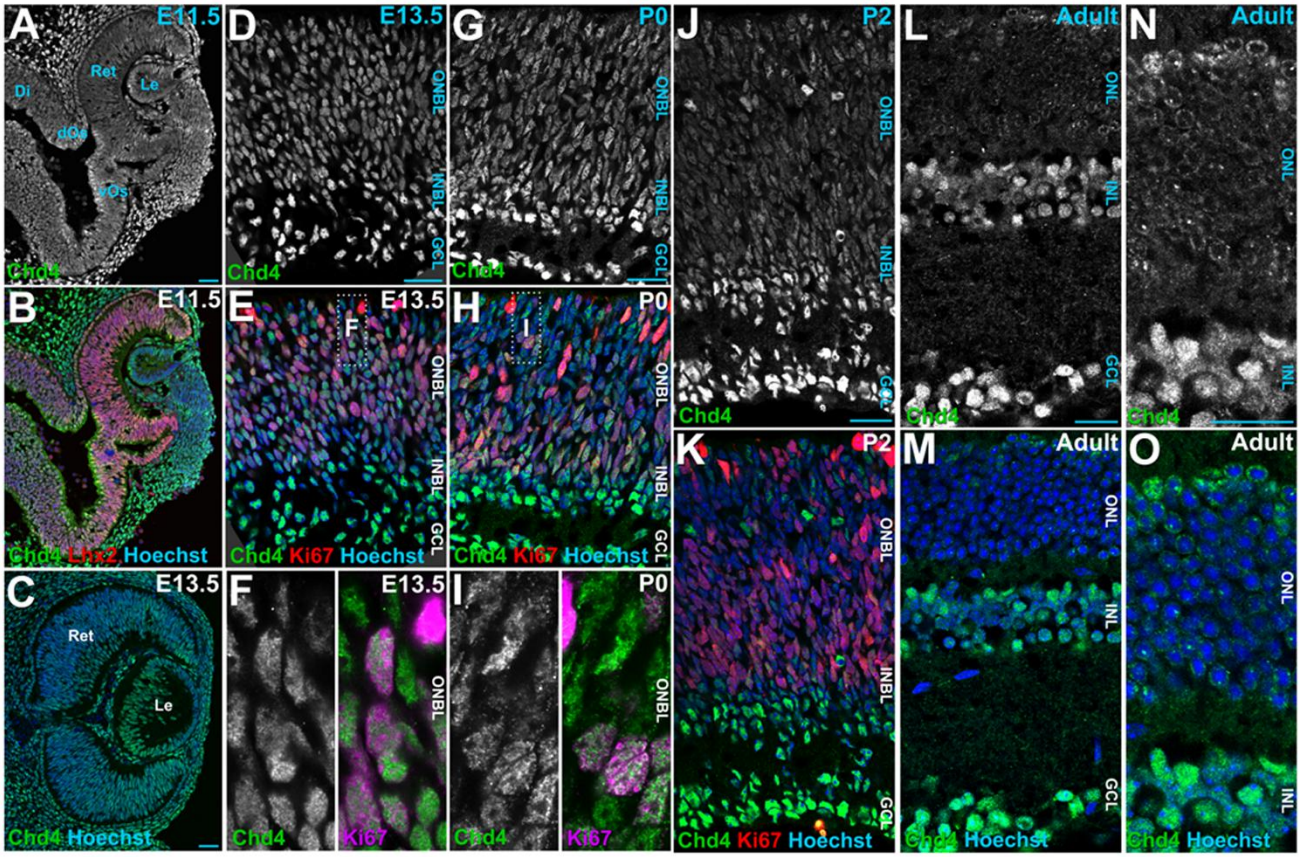

Fig. 3.1. Chd4 expression dynamics during retinal development. (A,B) E11.5 retinas stained for Chd4 and Lhx2. (C-F) Co-staining of Chd4 and Ki67 at E13.5 (D-F), P0 (G-I) and P2 (J,K). (L-O) Adult retinas stained for Chd4 and with Hoechst. Ret, retina; Le, lens; Di, diencephalon; dOs, dorsal optic stalk; vOs, ventral optic stalk; ONBL, outer neuroblastic layer; INBL, inner neuroblastic layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars: 20 μm.

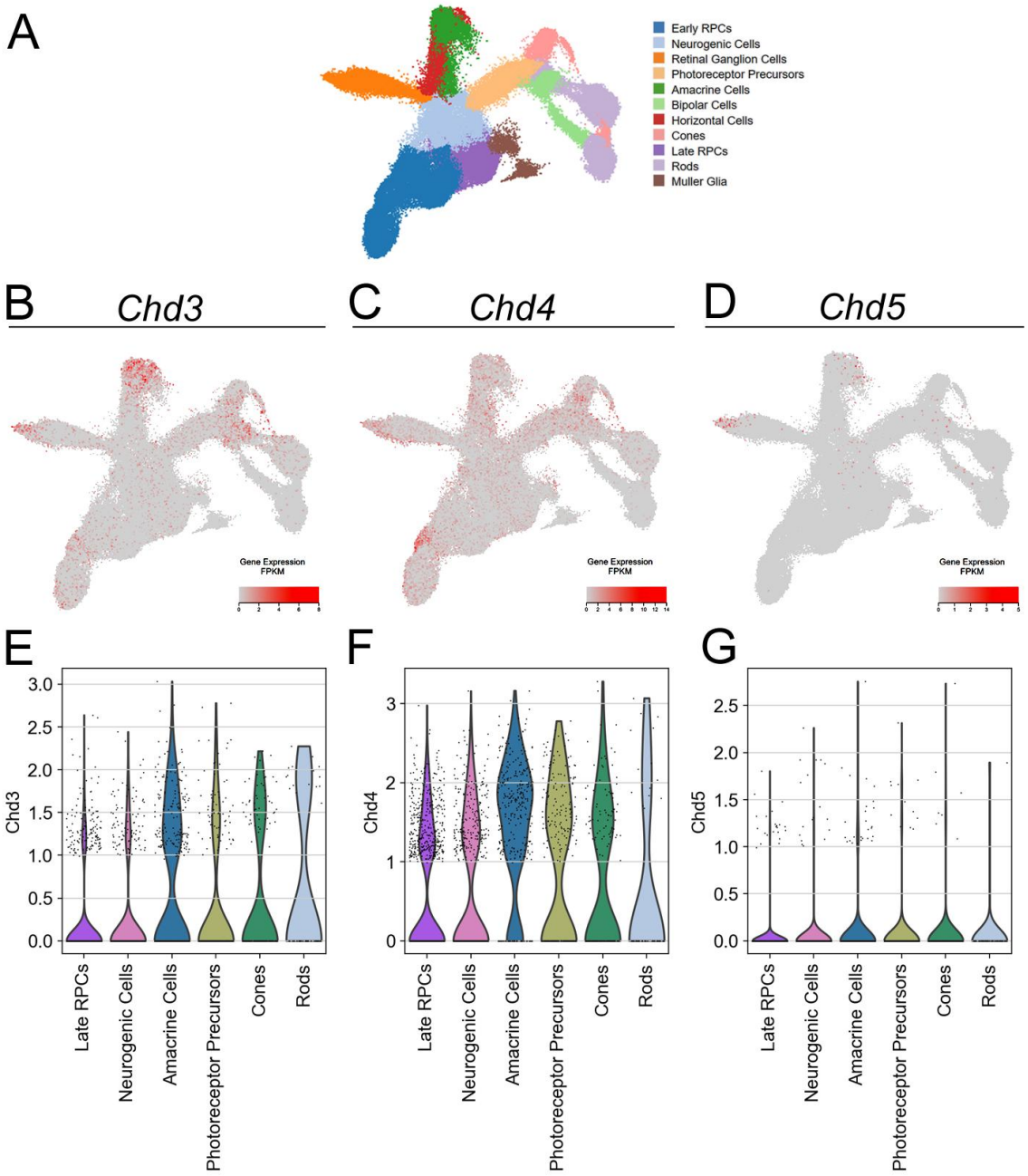

Fig. 3.2. Expression dynamics of *Chd3/4/5* during retinal development. (A) UMAP projection of retinal developmental trajectories from a previously published scRNA-seq dataset (28). (B-D) The expression of individual Chd paralogs during retinal development from the same atlas as indicated. (E-G) Violin plots displaying *Chd3* (E), *Chd4* (F), and *Chd5* (G) expression in retinal cell types as determined by scRNA-seq data from P1 control samples.

3.1.2. *Chd4* cKO affects postnatal retinal histogenesis:

Since *Chd4* is an essential gene (175), we next generated *Chd4* conditional knockouts (cKOs) using an allele with loxP sites flanking exons 12-21, which together encode the ATPase/helicase domain (176). This cassette was deleted using the Chx10-Cre-GFP driver (177), which expresses a Cre-GFP fusion protein in RPCs, beginning at ~E10.5. Towards the end of development, Cre-GFP expression is maintained in bipolars and weakly in some Müller glia. Full-length Chd4 protein (expected size 219 kDa) was efficiently abrogated in cKO retinas (Fig. 3.3A-C). Although the Chx10-Cre-GFP driver is prone to mosaicism, in our study we observed that an average of ~70% of cells within the ONBL expressed GFP in perinatal Cre+ animals (Fig. 3.4), which is similar to the overall proportions of RPCs within the layer (e.g. see Fig. 3.9). Next, the effect of Chd4 ablation was assessed at various stages between E16.5 and P15 (Fig. 3.3D-F, Fig. 3.5). *Chd4* cKOs exhibited a markedly expanded GCL along with a poorly formed inner plexiform layer. The distinct neuropil dividing the ONBL and GCL in the wild type and conditional heterozygote (chet) was missing in the cKOs (Fig. 3.3D-F, Fig. 3.5). However, when the total number of cells was quantified between the three genotypes, no significant changes were observed at P0 or P2 (Fig. 3.3G, H; Fig. 3.5). This indicates that the expansion of the GCL does not arise as a consequence of significant retinal overgrowth, nor from premature differentiation of the progenitor pool.

At P8, *Chd4* cKOs exhibited disorganized retinal lamination. Along with the expanded GCL, mutant retinas exhibited a thinned ONL as compared to wild type or chets, and additionally exhibited ectopic GFP+ nuclei within the ONL (Fig. 3.3F). Again, when cells were quantified, no significant difference was observed between wild-type, chet, and cKO

retinas (Fig. 3.3I). At P15 the loss of Chd4 resulted in a hyperplastic ONL containing ectopic GFP+ cells (Fig. 3.3F, Fig. 3.6). cKO retinas exhibited an approximately 1.5-fold decrease in cell numbers when compared to wild type or chet (Fig. 3.3J). These data suggest that, in the cKO, cell death significantly reduces cell numbers between P8 and P15 (Fig. 3.3K), likely corresponding to the wave of apoptosis previously shown to prune supernumerary bipolars and amacrines at ~P10 (183–185).

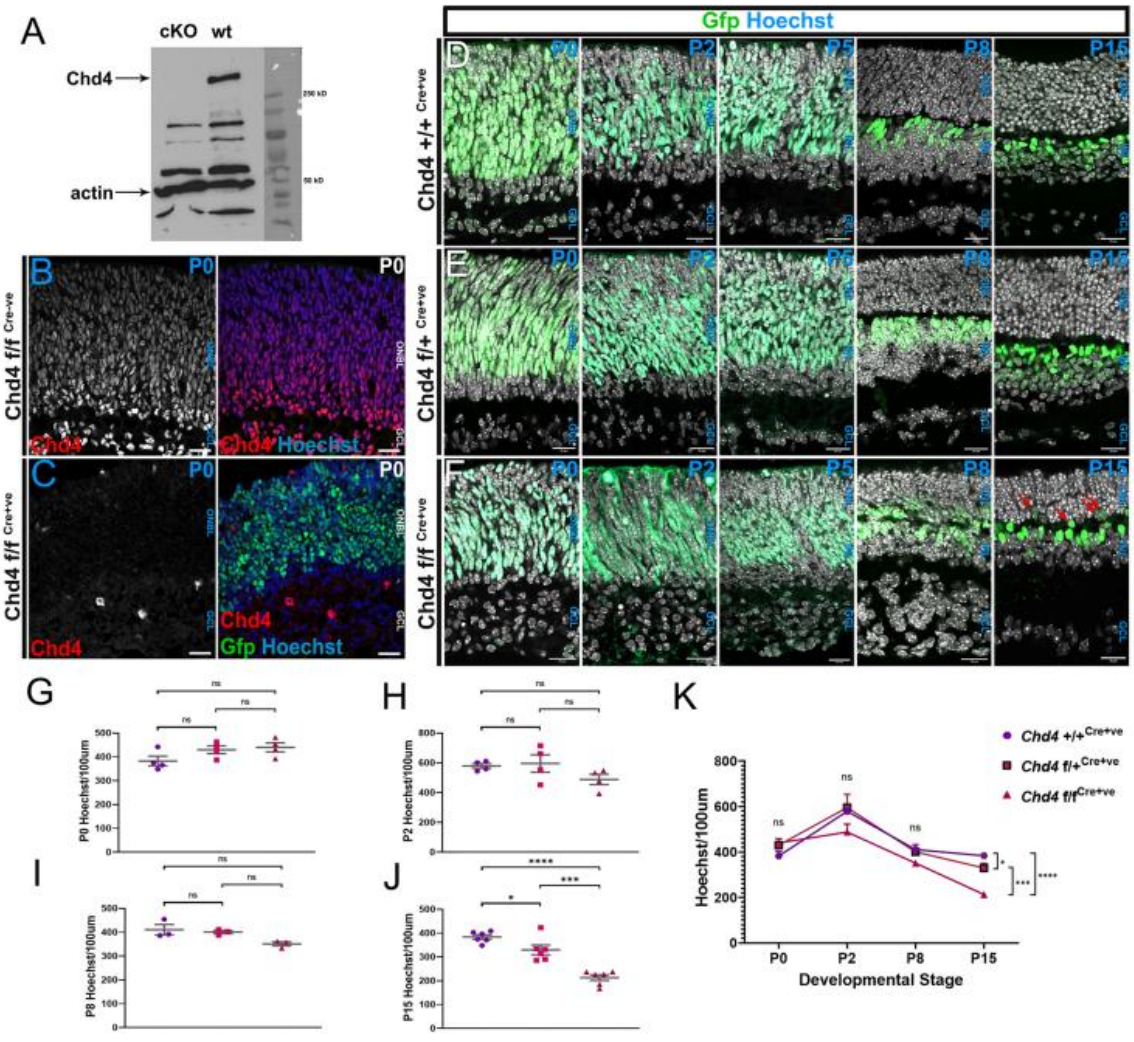

Fig. 3.3. Chd4 is required for retinal histogenesis. (A) Western blot analysis of Chd4 protein expression. (B-C) Chd4 immunostaining at P1 on wild-type (wt, *Chd4 f/f Cre-*; B) or mutant (mut, *Chd4 f/f Cre+*; C) retinal sections. (D-F) Wild-type (wt, *Chd4* $+/+^{Cre+}$), heterozygous (het, *Chd4* $f/+^{Cre+}$) and mutant retinas

from P0, 2, 5, 8 and 15 were stained with Hoechst and YFP. Red arrows indicate ectopic YFP+ cells in the ONL. (G-K) Quantification of total Hoechst counts at P0, P2, P8 and P15 as indicated. All data are presented as mean ±SEM. *p<0.05, ***p=0.0001, ****p<0.0001, ns=not significant by one-way ANOVA with Tukey's multiple comparisons test. Scale bar = 10 microns.

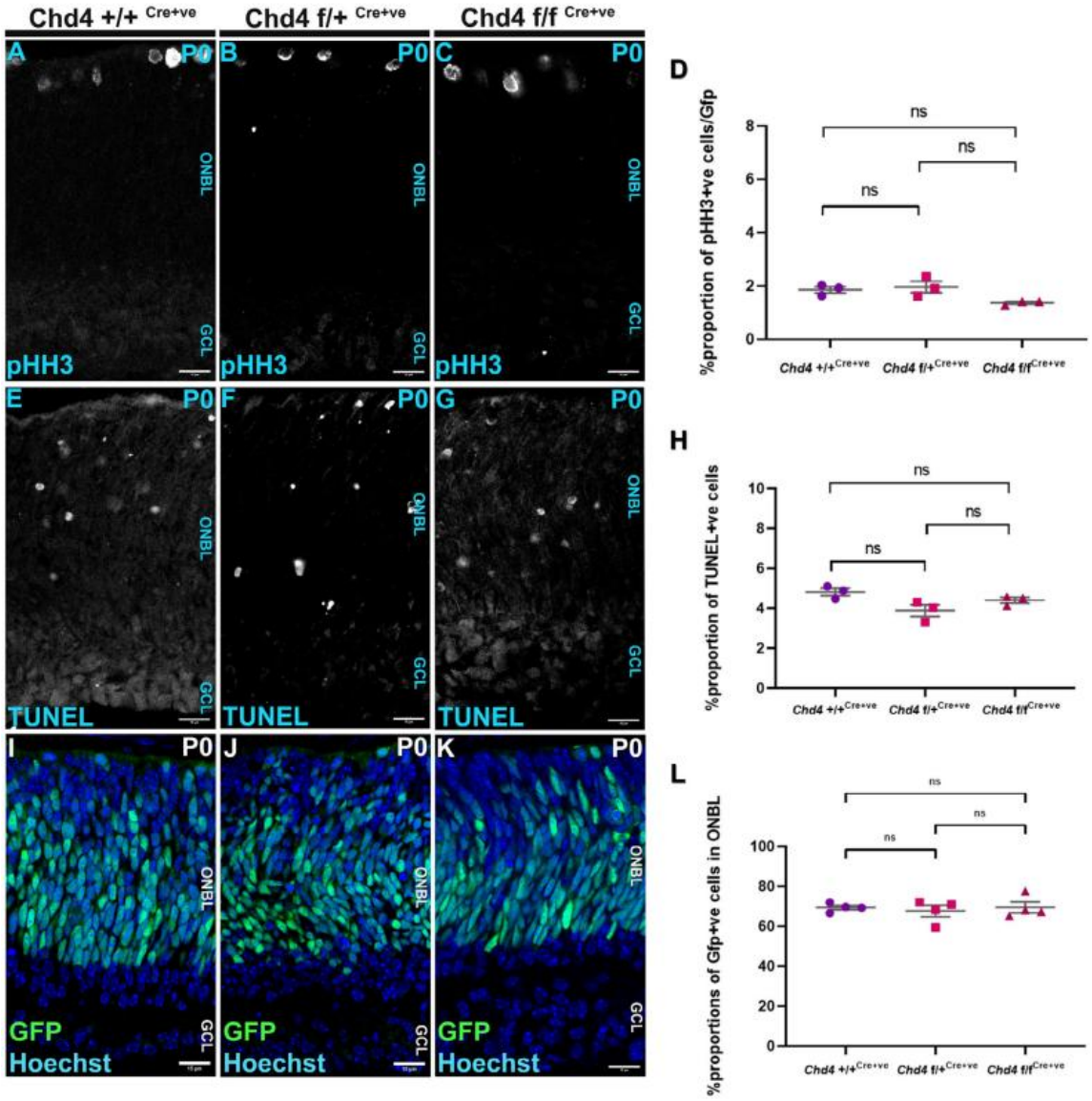

Fig. 3.4. *Chd4* cKO does not affect proliferation or viability at P0. (A-C) Wt, chet, and cKO retinal sections were stained with the mitotic marker phospho-histone H3. (D) Percentage of pHH3+ cells at P0. (E-H) TUNEL assay to quantify apoptotic cells. (I-K) GFP staining to mark Chx10+ progenitors. (L) Using GFP as a proxy for RPCs, the progenitor pool as quantified between the different genotypes. All data are

presented as mean ±SEM. ns: not significant by one-way ANOVA with Tukey's multiple comparison test. ONBL: outer neuroblastic layer; GCL: ganglion cell layer. Scale bar = 10 microns.

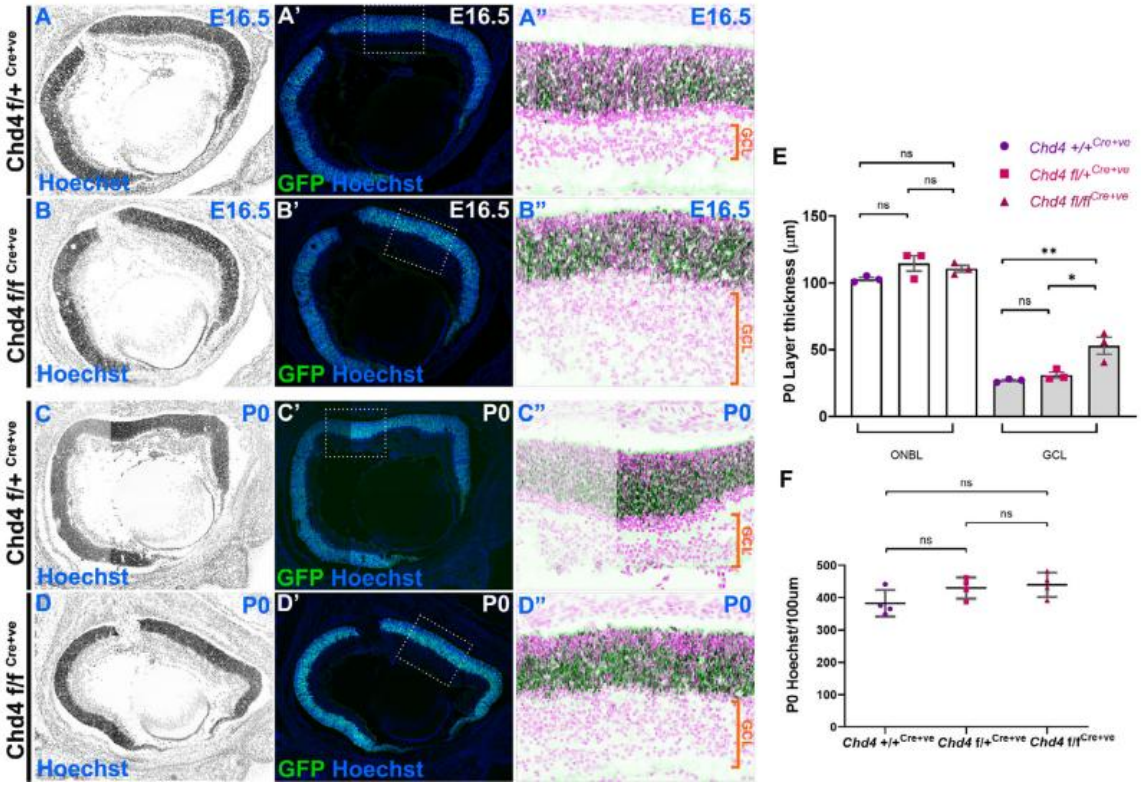

Fig. 3.5. *Chd4* cKO leads to embryonic expansion of the ganglion cell layer and lamination defects. (A-B″) E16.5 chet and cKO whole eye sections were stained with Hoechst and visualized for GFP epifluorescence. (C-D″) P0 chet and cKO whole eye sections were stained with Hoechst and visualized for GFP epifluorescence. (E) Quantification of individual layer thickness between the different genotypes. (F) Total Hoechst was quantified and compared across the three genotypes. All data are presented as mean ±SEM. p-value *p<0.05, **p<0.005, ns: not significant by one-way ANOVA with Tukey's multiple comparisons test.

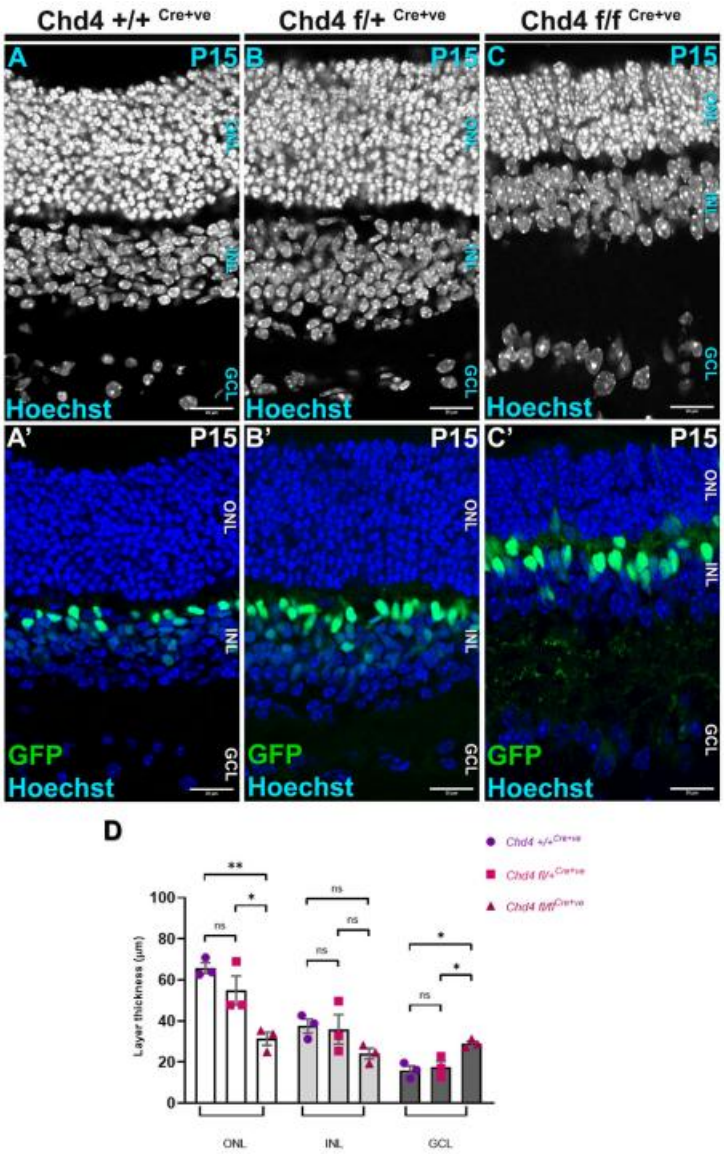

Fig. 3.6. Effect of *Chd4* cKO on P15 retinal histology. (A-C') Wt, chet and cKO retinal sections were stained with Hoechst to mark the cell bodies in the different retinal layers. (D) Quantification of individual layer thickness between the different genotypes. All data are presented as mean ±SEM. P-value *p<0.05, **p<0.005, ns=not significant by one-way ANOVA with Tukey's multiple comparisons test. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Scale bar = 10 microns.

3.1.3. Chd4 regulates retinal cell-type production:

We next examined cell-type markers at P15, when development is complete. We found that cKO retinas had an almost twofold increase in the proportion of Rbpms+ RGCs as compared to controls (Fig. 3.7A, B). As a partial measure of amacrines, we counted Pax6+ cells within the INL. cKO retinas displayed a slight increase in INL amacrines when compared to wild type (Fig. 3.7C, D). These changes were observed in proportional counts, although not in absolute numbers (Fig. 3.8). For cones, we found that cKOs exhibited an approximately twofold decrease in the percentage of cone arrestin+ cells when compared to wild-type and chet retinas (Fig. 3.7E, F).

During postnatal stages, RPCs generate amacrines, rods, bipolars and Müller glia. In the ONL, we counted marker-negative rods by excluding cone arrestin+ cones and GFP+ bipolars/Müllers. As expected from the thinning of the ONL (Fig. 3.6), rods were significantly reduced in *Chd4* cKO retinas as compared to wild type or chet (Fig. 3.7 E, F). The proportion of strongly Otx2+ bipolar cells did not differ among the three genotypes at P15. However, cKOs exhibited ectopic Otx2+ /GFP+ bipolar cells within the ONL (Fig. 3.7G, H). During retinal development, Müller glia are the latest-born cell type. Radially polarized Sox2+ cells were increased by almost twofold in the cKO, with many located within the ONL (Fig. 3.7 I, J).

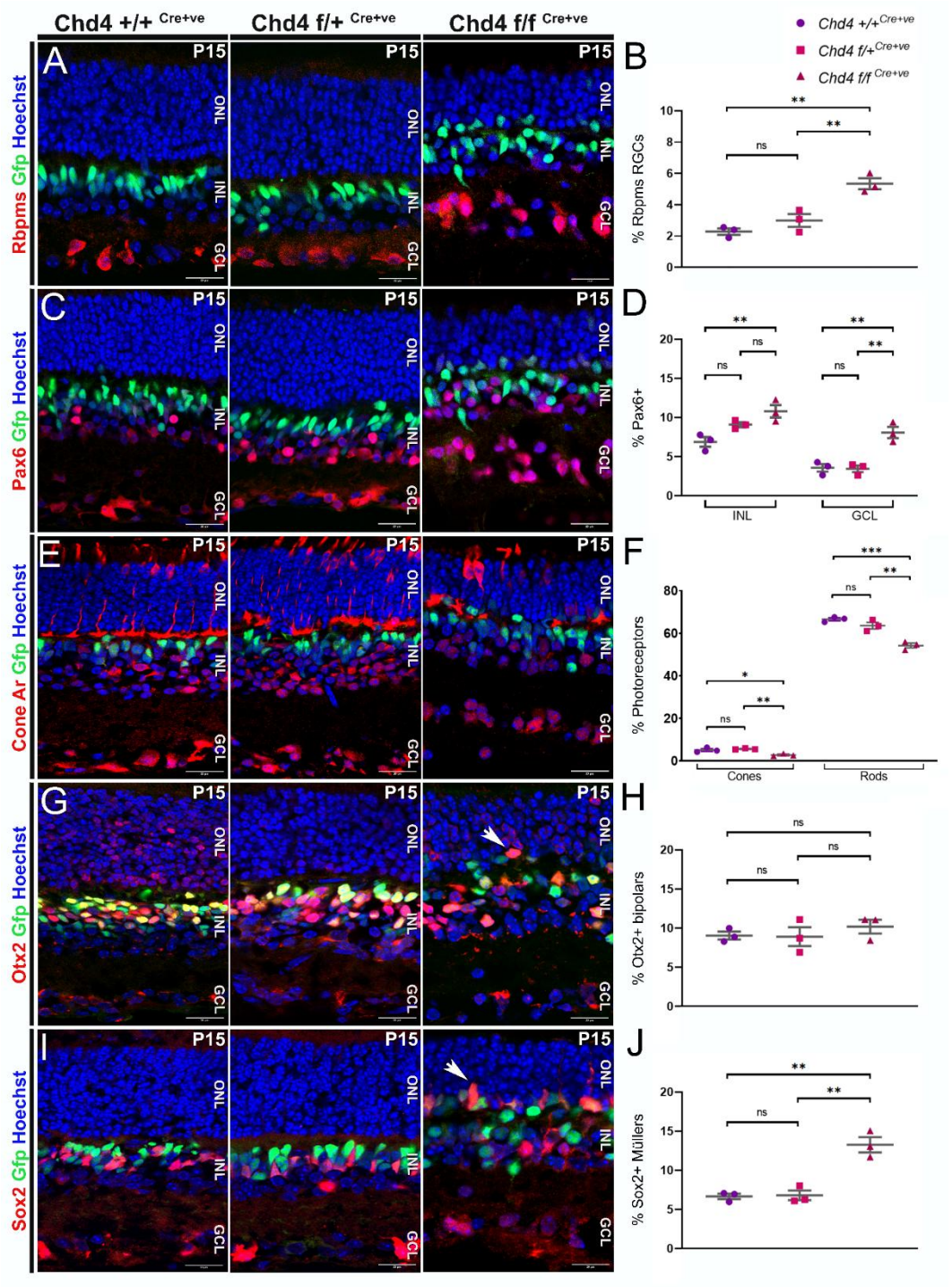

Fig. 3.7. Shifts in retinal cell type composition in the *Chd4* cKO. Marker staining was used to quantitate cell type proportions among the three genotypes at P15. (A, B) Rbpms+ RGCs. (C, D) Pax6+ amacrines (INL) along with RGCs (GCL). (E, F) Cone arrestin+ cones. GFP-negative and cone-arrestin negative ONL cells were counted as rods. (G, H) Brightly positive Otx2 cells were counted as bipolars. Arrow indicates

ectopic bipolars in the ONL. (I, J) Radially polarized Sox2+ cells were counted as Müller glia. Data from A-J are also shown in Fig. 3.8. Arrow indicates ectopic glia in the ONL. All data are presented as mean ±SEM. *p<0.05, **p<0.005, ***p<0.0005; ns=not significant by one-way ANOVA with Tukey's multiple comparison test. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars: 20 μm.

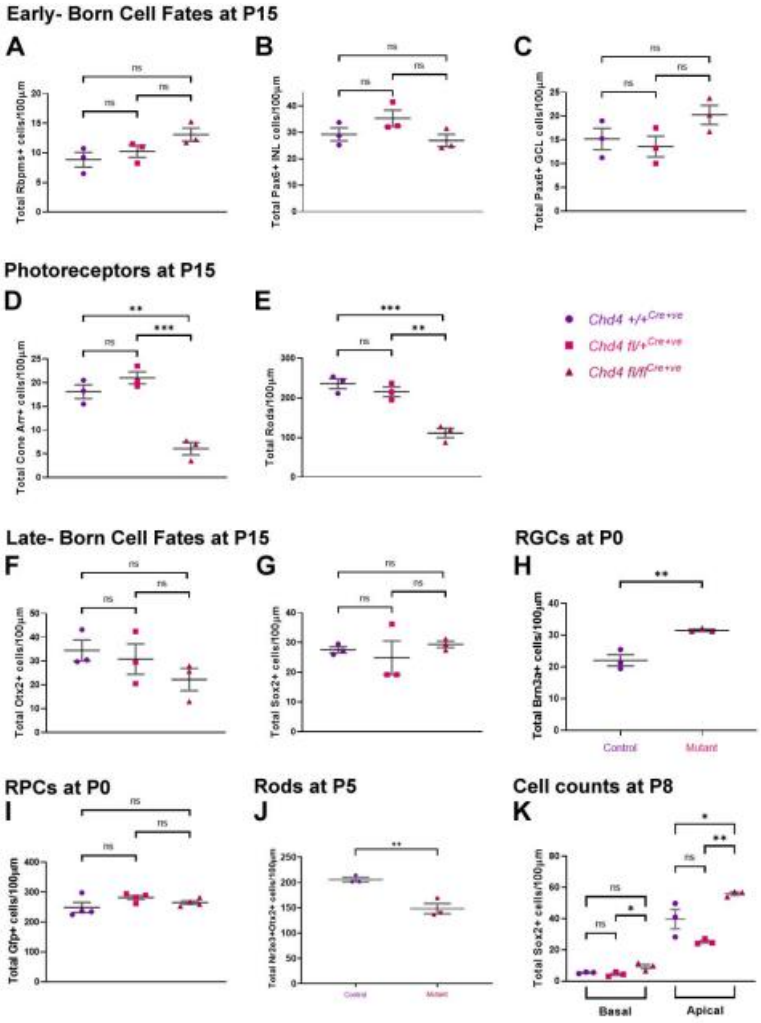

Fig. 3.8. Absolute cell numbers counted in hundred micron-wide bins. (A-G) Data from Fig. 3.7 re-expressed in absolute cell counts. (A) Rbpms+ RGCs. (B, C) Pax6+ amacrines (B; INL) along with RGCs (C; GCL). (D) Cone arrestin+ cones. (E) GFP-negative/cone-arrestin negative ONL cells were counted as rods. (F) Brightly positive Otx2 cells were counted as bipolars. (G) Radially polarized Sox2+ cells were

counted as Müller glia. (H, I) Data from Fig. 3.9 re-expressed in absolute counts. (H) Brn3a+ RGCs. (I) GFP+ RPCs. (J, K) Data from Fig. 3.12 re-expressed as absolute counts. (J) Nr2e3+/Otx2+ rods. (K) Sox2+ cells. All data are presented as mean ±SEM. *p<0.05, **p<0.005, ***p<0.0005, ns=not significant by one-way ANOVA with Tukey's multiple comparison test.

3.1.4. Chd4-dependent chromatin remodelling does not regulate progenitor proliferation:

The observed shifts in cell type composition at P15 could potentially be explained by alterations in proliferation or cell death. For example, if self-renewing divisions were undermined, this might prematurely exhaust progenitors, leading to overproduction of early-born cells such as RGCs, and underproduction of late fates such as rods. We therefore examined retinas at earlier stages. At P0, when RPCs lose the competence to generate early-born cell types and rod production peaks (25,27), Brn3a staining confirmed that RGCs were increased approximately twofold in the *Chd4* cKO (Fig. 3.9A-D) – similar to the increase observed at P15. The expansion in early-born neurons was also illustrated via the RGC marker Rbpms (Fig. 3.10). Horizontal cells are the rarest early-born cell type, representing ∼0.5% of the total retinal cell count (22). We visualized horizontals by performing Lhx1 and calbindin co-staining, but did not observe a significant difference in horizontal cell numbers (Fig. 3.10). Finally, we noted that the size of the progenitor pool – as reflected by the expression of the Chx10- Cre-GFP transgene – was not altered (Fig. 3.9A-E).

We next hypothesized that RGCs might be produced beyond their normal birth window. Postnatally, only trace levels of RGC production are observed in wild-type mice (25). We therefore injected EdU at P0. After 2 days, we observed small numbers of newly born

EdU+/Brn3b+ RGCs that were often localized apically, suggesting that they were migrating towards the GCL. However, very few of these cells were observed, and there was no marked difference between controls and cKOs (Fig. 3.11A-D). Similarly, EdU+ RGCs were not observed when EdU was injected at P1 and retinas were harvested at P8 (Fig. 3.11E). These results agree with the marked expansion of the GCL already observed by E16.5 and P0 in the *Chd4* cKO (Figs 3.3, 3.5 and 3.9), as well as scRNAseq data (see below), which together indicate that supernumerary RGCs are produced during embryogenesis within their normal birth window.

Next, we hypothesized that RGCs might increase at the expense of the rods and cones that are generated during embryonic stages. We therefore counted Otx2+ photoreceptor precursors at P0, as well as Rxrg+ cones. Surprisingly, we found that proportions of early-born photoreceptors in *Chd4* cKO retinas were comparable to controls (Fig. 3.9F-H), suggesting that the decrease in cone arrestin+ cells observed at P15 might reflect subsequent defects in cone differentiation or survival. Thus, while RGCs expand and photoreceptors contract in number, these changes appeared not to be linked to a common fate decision.

Since rod photoreceptor production normally peaks between P0 and P2 (25,27), we reasoned that RPCs might exhibit defects in proliferation that could undermine rod production at perinatal stages. We first examined phospho-histone H3+ mitotic cells, but found no difference between the genotypes (Fig. 3.4). Next, we injected EdU to mark S-phase cells at P0. At P2, RPCs were co-stained for EdU and Ki67, which marks proliferating cells. Both EdU and Ki67 were comparable between the three genotypes (Fig. 3.9I-M). Double-labelled EdU+ /Ki67+ RPCs that had undergone self-renewal were

also not significantly different (Fig. 3.9I-N). These data indicate that Chd4 does not affect proliferation dynamics at perinatal stages, in agreement with lack of significant differences in overall cell numbers observed up to P8. However, previous work in the developing neocortex had shown that loss of Chd4 led to elevated cell death (127). We therefore performed the TUNEL assay at P0, but changes in apoptotic frequency were not yet observed in the *Chd4* cKO versus controls (Fig. 3.4). Thus, while *Chd4* cKOs exhibited distorted cell-type proportions that could have arisen as a byproduct of premature RPC exhaustion or cell death, such effects were not yet evident perinatally during the normal peak of rod production.

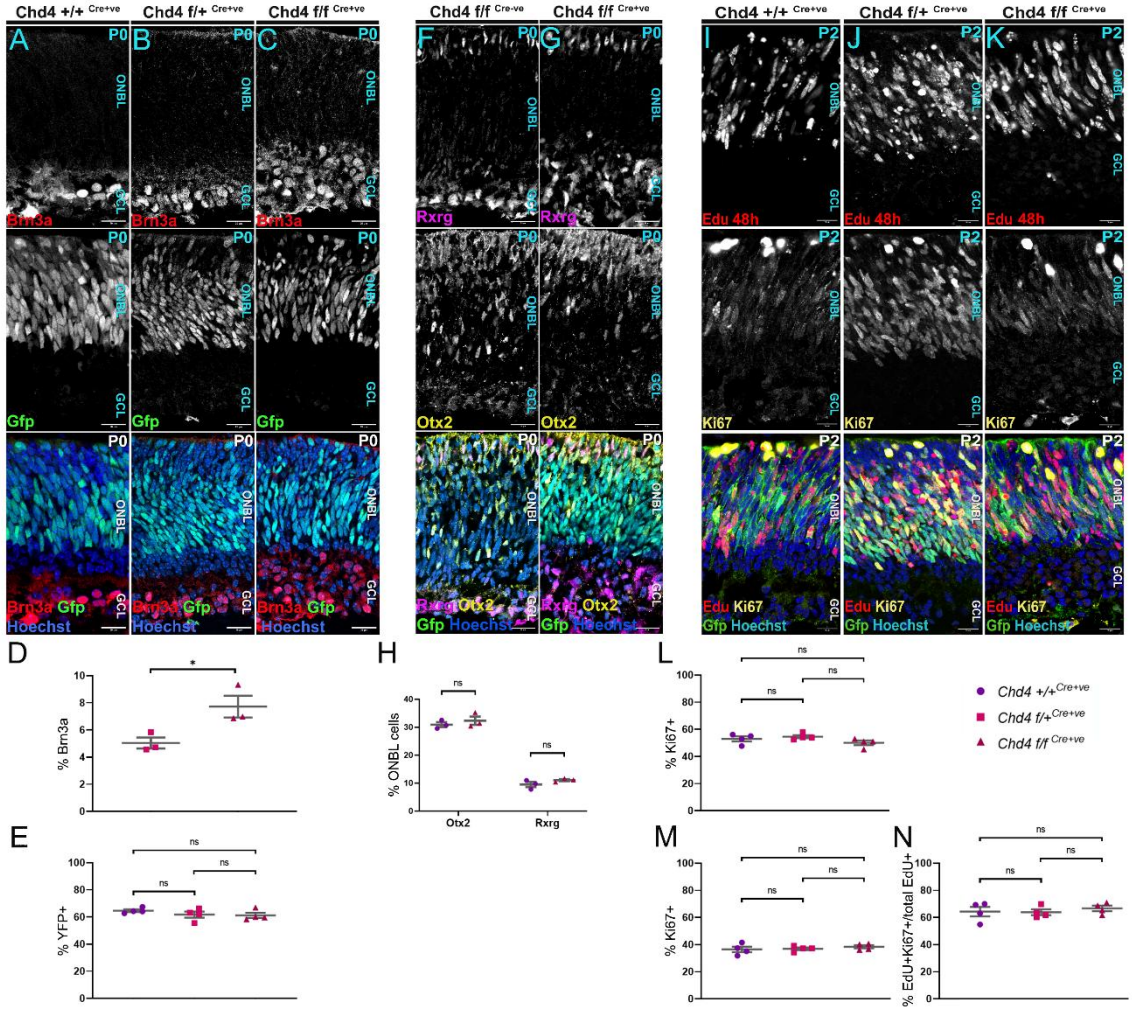

Fig. 3.9. Fate shifts are independent of perinatal alterations in RPC proliferation. (A-C) P0 retinal sections co-stained for the RGC marker Brn3a and imaged for GFP, which marks RPCs. (D, E) Percentage of Brn3a+ cells (D) or GFP+ cells (E). (F, G) P0 retinal sections were co-stained for the cone marker Rxrg and the photoreceptor precursor marker Otx2. (H) Percentage of Otx2+ and Rxrg+ cells between control and mutant retinas. (I-K) EdU was injected at P0. At P2, retinas were harvested and stained for EdU and Ki67. (L-N) Percentage of cells that were EdU+ (L), Ki67+ (M) or EdU+Ki67+ (N) as a percentage of total EdU+ cells. Data from D, E are also shown in Fig. 3.8. All data are presented as mean ±SEM. *p<0.05; ns= not significant by one-way ANOVA with Tukey's multiple comparisons test. ONBL, outer neuroblastic layer; GCL, ganglion cell layer. Scale bars: 20 µm.

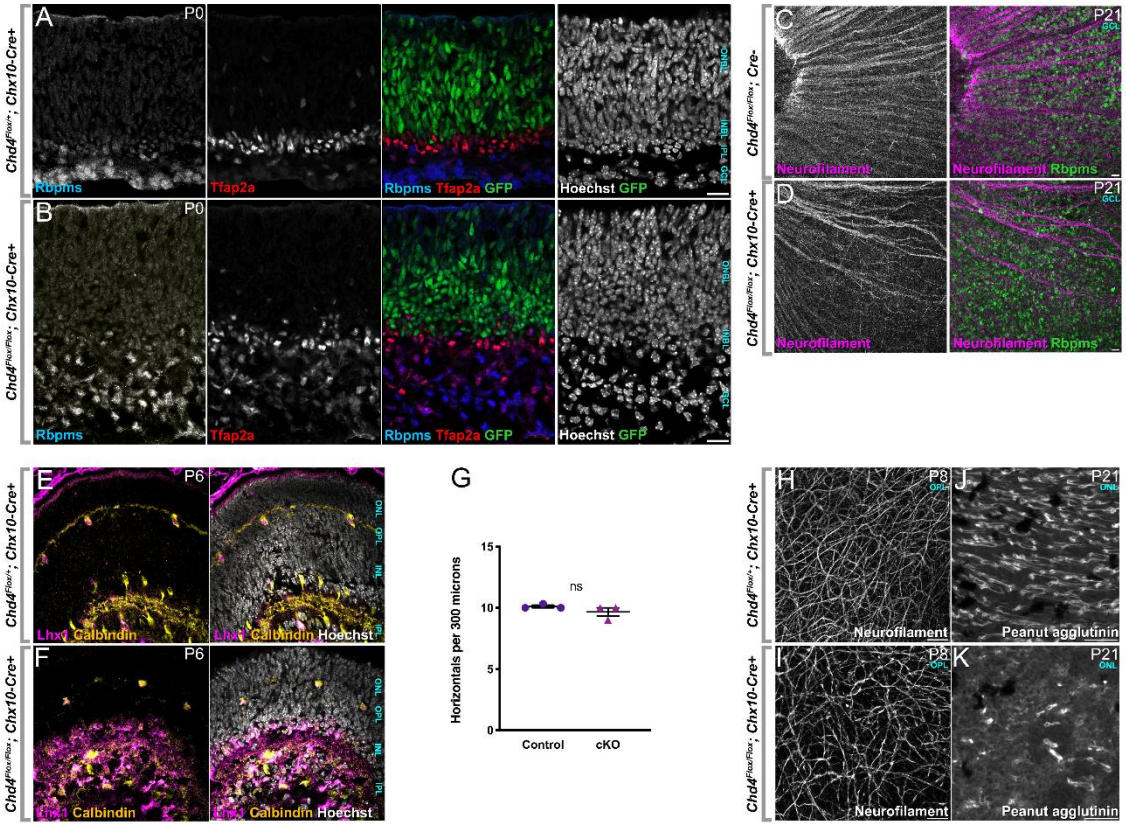

Fig. 3.10. Early-born cell type markers in the *Chd4* cKO. (A, B) P0 chet (A) and cKO (B) retinas co-stained with the ganglion cell marker Rbpms and the amacrine marker Tfap2a. Scale bar = 20 microns. (C, D) P21 wild-type (C) or cKO (D) whole-mount stainings for the RGC markers Rbpms and neurofilament. At the level of the GCL and nerve fiber layer, neurofilament specifically marks RGC axons. (E, F) P6 chet (C) and cKO (D) retinas co-stained with the horizontal marker Lhx1 and the horizontal/amacrine marker

calbindin. (G) Quantitation of Lhx1+ horizontal cells counted in 300 micron bins at P5. (H, I) P8 chet (E) and cKO (F) retinas stained in wholemount for neurofilament. Images depict single z-planes captured at the level of the outer plexiform layer, where neurofilament specifically marks horizontal cells. Scale bar = 30 microns. (J, K) P21 chet (J) and cKO (K) retinas stained in wholemount for peanut agglutinin. Images consist of extended focus composites at the level of the outer nuclear layer, where peanut agglutinin marks cone outer segments. Scale bar = 30 microns. ONBL: outer neuroblastic layer; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer; OPL: outer plexiform layer; IPL: inner plexiform layer.

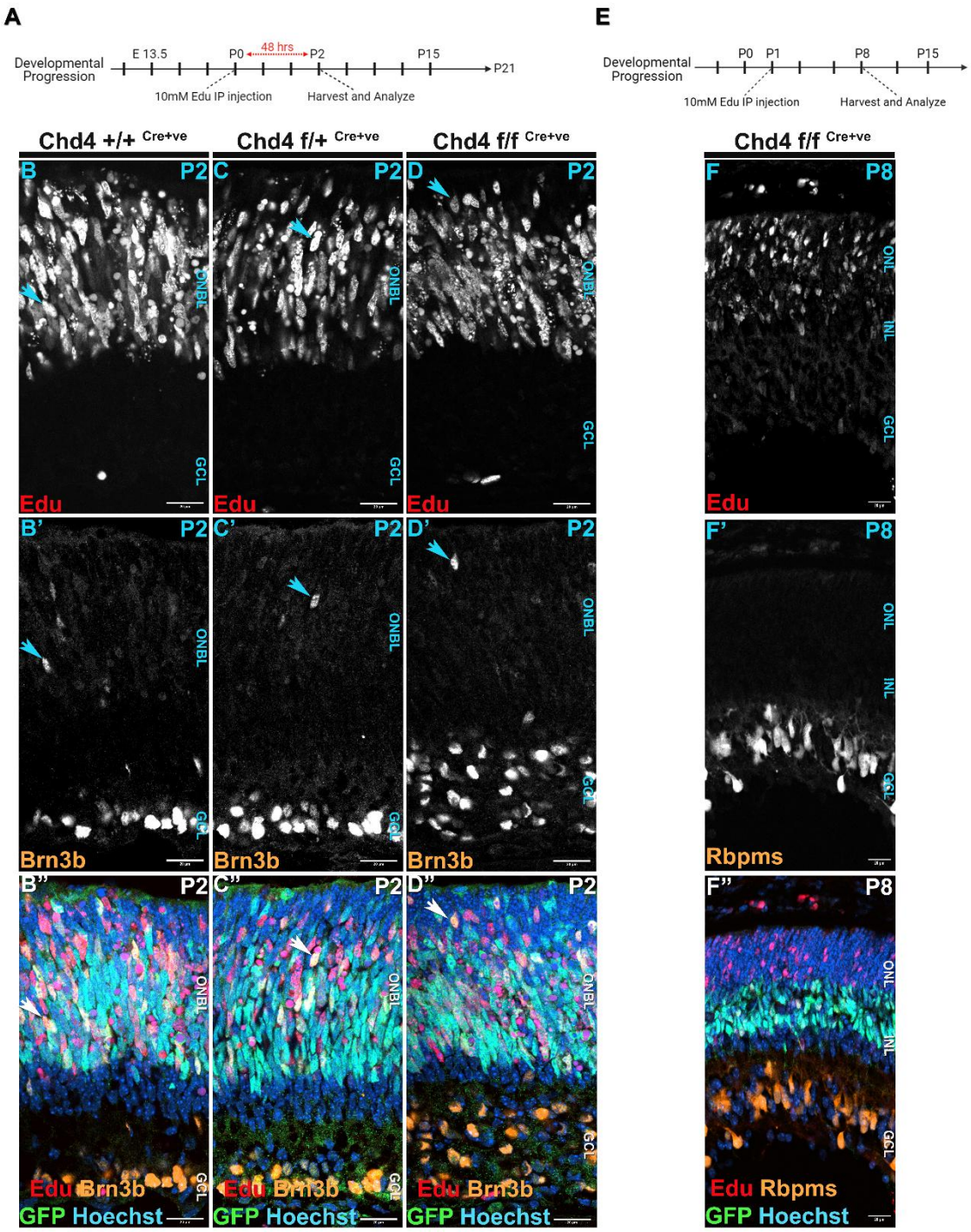

Fig. 3.11. RGCs are overproduced within their normal birth window in *Chd4* cKO retinas. (A) Schematic of 48-hours Edu birthdating assay. (B-D″) Wt, chet, and cKO retinas were stained with EdU and Brn3b. Arrows indicate cells that are double positive for EdU and Brn3b. (E) Schematic of 7-day Edu birthdating assay. (F-F″) cKO retina was stained with EdU and Rbpms. Scale bar = 10 microns.

3.1.5. Chd4 regulates neurogenic competence at late stages:

By P15, *Chd4* cKOs exhibit reductions in rods and a proportional expansion in Sox2+ glial cells, but also marked cell loss. To help determine whether these changes arose due to altered cell type production or through apoptosis, we next examined retinal development between P5 and P8. At P5, overall cell counts did not differ between controls (wild type/chet) and cKOs (Fig. 3.12A-D). However, rod photoreceptors expressing Otx2 and Nr2e3 were significantly reduced – both in absolute and proportional terms (Fig. 3.12A-E, Fig. 3.8). There was an overt expansion in Sox2+ /GFP+ RPCs in *Chd4* cKOs (Fig. 3.12F, G). However, when we stained P5 retinas for activated caspase 3, we observed a significant elevation in apoptotic cells (Fig. 3.12F-H). Thus, the reduction in rod photoreceptors was concomitant with increased cell death in the *Chd4* cKO.

At P8, the laminar distribution of cells was markedly altered. In control retinas, almost all GFP+ cells were basal to the forming plexiform layer that divides the ONL and INL. However, in the *Chd4* cKO, many GFP+ cells were located within the ONL – apical to the plexiform layer (Fig. 3.12I-Q), which was more discontinuous in comparison to controls. In the ONL, GFP-negative photoreceptor precursors were also visibly reduced in the cKO, again suggesting that late-stage rod production was reduced. To examine this more directly, we marked newly born cells via injection EdU at P1 and visualized their subsequent fates at P15. We found that the proportion of EdU+ cells was reduced in the ONL and increased in the INL (Fig. 3.13).

Next, we examined additional cell types. Tfap2a+ amacrines were not significantly altered, but Otx2+ bipolars were modestly but significantly increased (Fig. 3.12I-K). Next, we examined Sox2. In the basal INL and GCL, Sox2 marks early-born cholinergic

amacrine cells with large circular nuclei. These amacrines were significantly increased in the cKO (Fig. 3.12L-O; Fig. 3.14). More apically, radially polarized GFP+/Sox2+ cells were also significantly increased (Fig. 3.12L-O; Fig. 3.14). Since Sox2 marks both RPCs and Müllers, we next stained for markers that can distinguish between these cell types. Strikingly, while the proliferation marker Ki67 was restricted to the peripheral margins of the retina in controls, proliferating RPCs persisted throughout the retina in *Chd4* cKOs (Fig. 3.12P-R). Cell counts revealed a significant increase in RPC numbers (Fig. 3.12R). Next, we birthdated cell-type production from persisting RPCs in the *Chd4* cKO by injecting EdU at P8 (Fig. 3.15). At P15, control retinas exhibited EdU+ cells only at the peripheral margins of the retina, and EdU+ cells were often rod photoreceptors located within the ONL. By contrast, EdU+ cells were found throughout the central retina in the cKO, and while some ONL cells were labelled, the vast majority of EdU+ cells were Sox2+/GFP+ glia.

To definitively mark differentiated Müller glia, we next stained for Rlbp1. In controls, Rlbp1 expression stained brightly GFP+ cells in the basal INL. Strikingly, Rlbp1 was virtually absent from the Chd4 cKO retina (Fig. 3.12S, T). Rlbp1+ cells could be seen only in patches of Cre mosaicism (Fig. 3.16). In the cKO, Rlbp1 was little expressed at P6 and P8, but was expressed by P15 (Fig. 3.16). Taken together, these data demonstrate that RPCs fail to differentiate into quiescent glia on schedule and accumulate in the *Chd4* cKO but eventually differentiate.

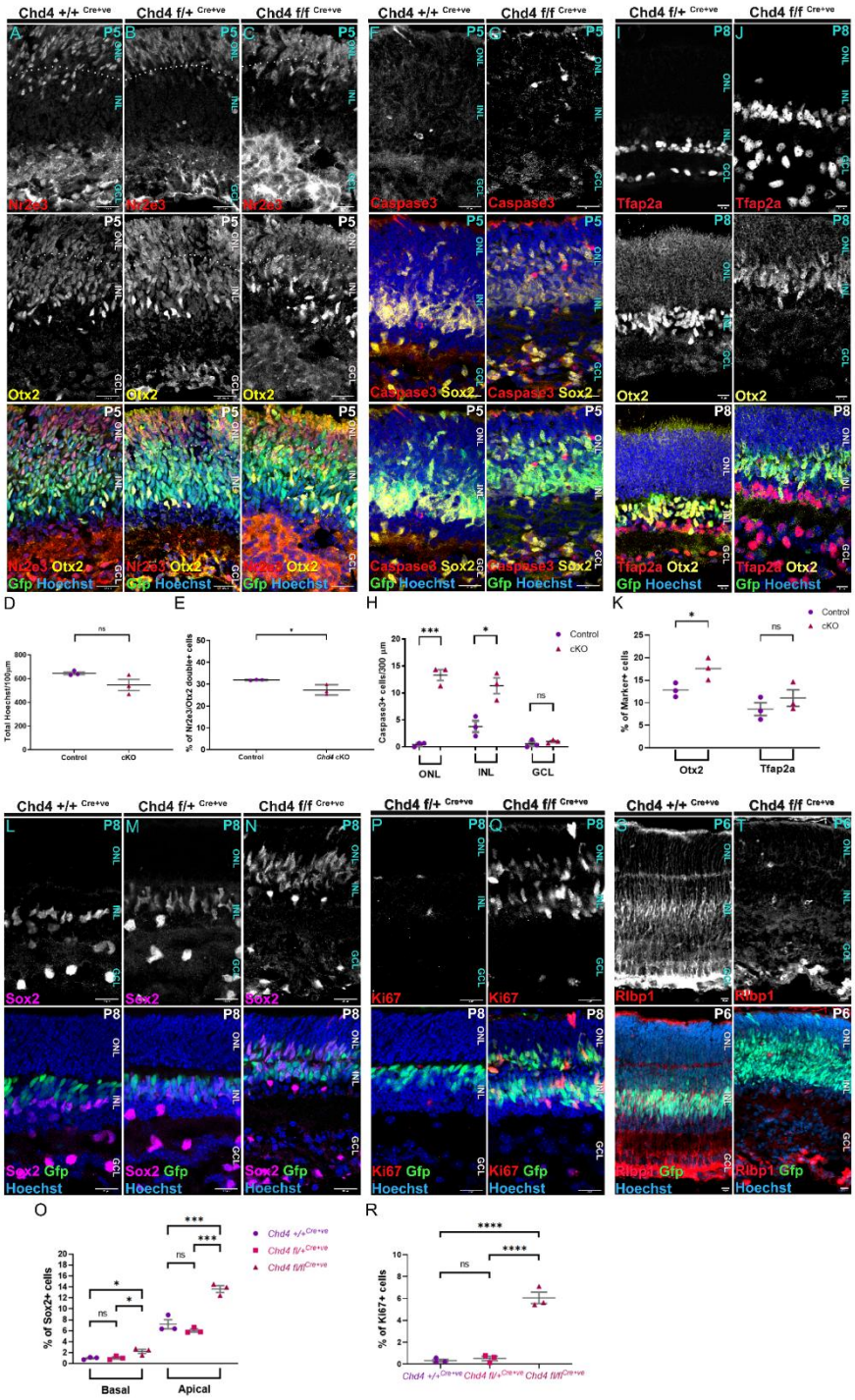

Fig. 3.12. Chd4 is required to terminate the retinal lineage. (A-H) Marker staining was used to quantitate cell type proportions at P5. (A-C) Nr2e3+ rods and Otx2+ photoreceptor precursors. (D) Hoechst counts per 100 µm. (E) Nr2e3+/Otx2+ rod precursors. (F, G) Sox2+ RPCs and apoptotic cells, marked by active caspase 3. (H) Active caspase 3+ cells per 300 µm. (I-R) Marker staining was used to quantitate cell type proportions

at P8. (I, J) Tfap2a+ amacrine cell Otx2+ bipolars. (K) Percentages of marker-positive cells, as indicated. (L-N) Cells brightly expressing Sox2 are cholinergic amacrines, whereas dimly positive, radially polarized cells are glia. (O) Quantitation of basal Sox2+ amacrines versus apical Sox2+ glia. (P, Q) Proliferative cells expressing Ki67 persist in the central retina in the cKO. Data from E and O are also shown in Fig. S5. (R) Quantitation of percentages of Ki67+ cells in the central retina. (S, T) Rlbp1 specifically marks Müller glia. All data are mean ±SEM. *p<0.05, ***p=0.0001, ****p<0.0001.ns=not significant by two-tailed unpaired Student's t-test or one-way ANOVA with Tukey's multiple comparison test. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars: 20 μm.

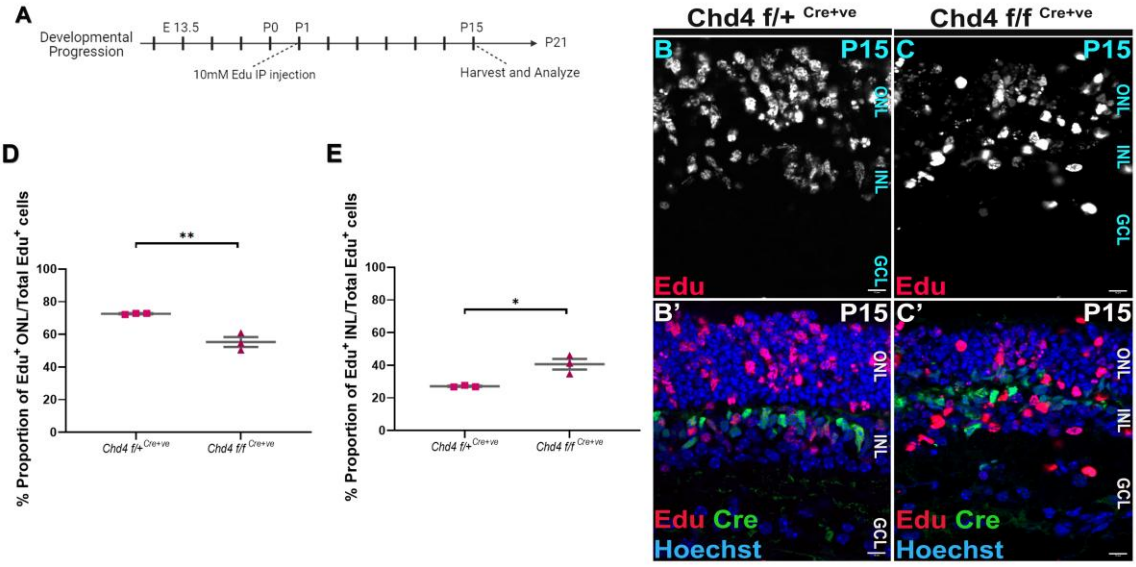

Fig. 3.13. Chd4 promotes the development of rod photoreceptors. (A) EdU was injected at P1 and retinas were harvested at P15. (B, C) EdU+ birthdated cells in cHet (B) versus cKO (C) retinas. (D, E) Quantification of the proportion EdU+ cells divided between the ONL (D) and INL (E). *p<0.05, **p<0.005, by Student's t test. Scale bar = 10 microns.

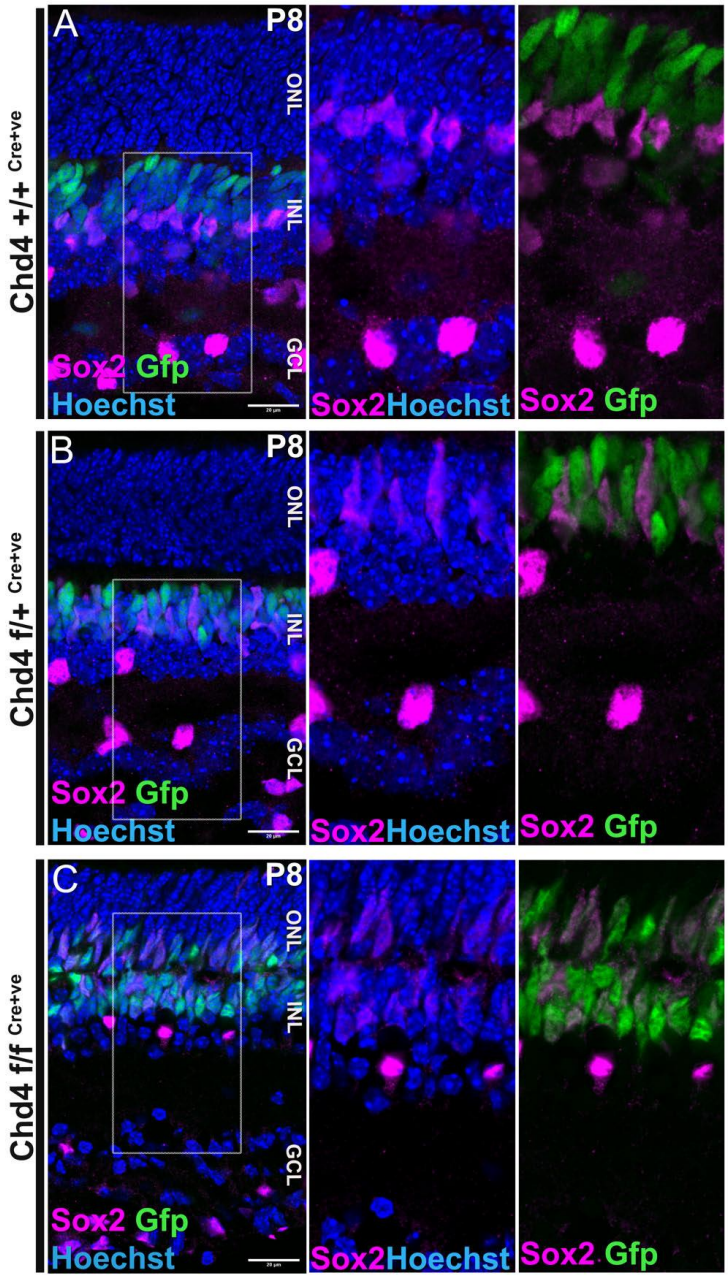

Fig. 3.14. Sox2 stainings at P8. In the basal INL and GCL, Sox2 marks early-born cholinergic amacrine cells with large circular nuclei. More apically, Sox2 marks radially polarized GFP+ Müllers. (A) Wild-type. (B) chet. (C) cKO. Data are from Fig. 5L-N. Scale bar = 20 microns.

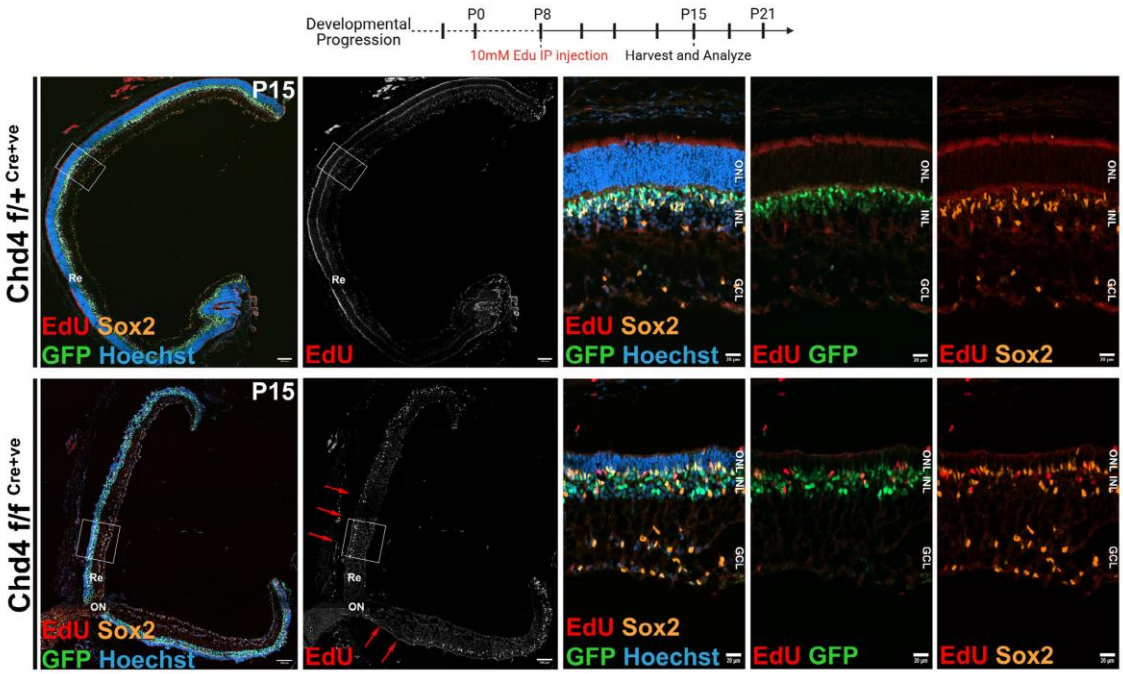

Fig. 3.15. Proliferating RPCs persist at late stages in the *Chd4* cKO and mainly generate Sox2+ glia. EdU was injected at P8 and retinas were harvested at P15. Birthdated EdU+ cells were absent in the central retina in cHets (top). By contrast, EdU+ cells were observed throughout central retinal regions in cKO retinas (bottom). Boxed regions indicate the magnified areas shown in the rightmost panels.

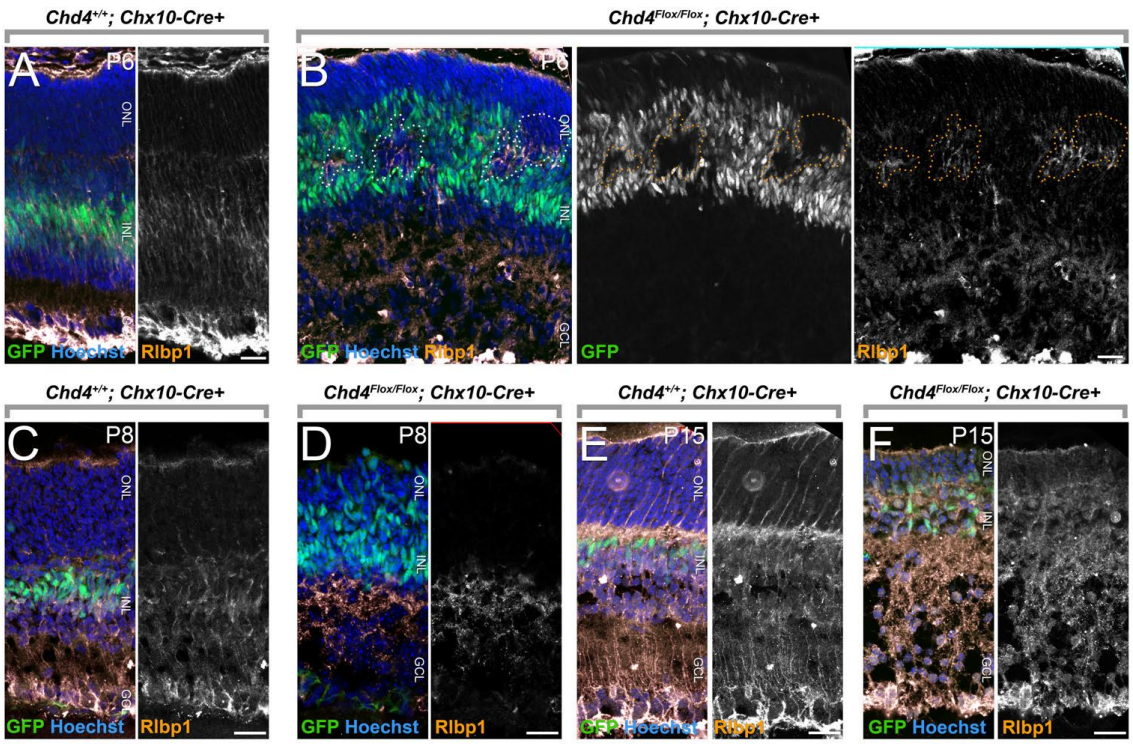

Fig. 3.16. Delayed glial differentiation in the *Chd4* cKO. (A-F) Rlbp1 staining on wild-type (A, C, E) versus cKO (B, D, F) retinas at P6 (A, B), P8 (C, D), and P15 (E, F). Retina in (B) includes patches of mosaicism (dotted outlines) as indicated by the lack of GFP expression. Scale bar = 20 microns.

3.2 Mechanistic insight into Chd4's role during retinal development

3.2.1. Loss of Chd4 results in divergent transcriptomic profiles:

To understand how *Chd4* mutation shifts the transcriptional state of RPCs, we performed scRNA-seq at P1, prior to the marked distortions in proliferation, cell death and differentiation observed at later stages. To avoid batch effects, we used the Multi-seq barcoding approach (182), allowing us to compare biological replicates for three *Chd4* cKOs versus three littermate control retinas processed together within the same 10X Genomics Chromium well. Cells were sequenced to a depth of 23,190 reads and 1980 genes per cell for an estimated sequencing saturation of 49.6%. After demultiplexing and removing low-quality cells and doublets, our dataset retained 9776 cells, with 2152 control cells and 7624 cKO cells. Next, cell types were annotated using scDeepSort (186) to perform unsupervised label transfer based on a previously published retinal scRNA-seq atlas (28).

Next, we visualized the data using uniform manifold approximation and projection (UMAP; Fig. 3.17A). We noted that cells annotated as 'late RPCs' formed a wheel-like structure, from which a neurogenic 'stem' emerged, followed by a bifurcation towards amacrine cells, or alternatively towards photoreceptor precursors. Marker gene expression confirmed the fidelity of the cell-type annotation (Fig. 3.18). Next, we examined each replicate (pup) individually (Fig. 3.17B). To confirm the genotype of each barcode, we examined how Chd4 and its paralogs were expressed in each replicate (Fig. 3.19). Focusing on RPCs, we observed that *Chd4* cKO cells exhibited a significant reduction in Chd4 transcription as compared to controls, but that Chd4 was not eliminated (Fig. 3.17C). However, this was expected, since the loxP-flanked cassette does not excise the 3′ end of

the gene (176). Since *Chd4* cKO has been shown to lead to a compensatory upregulation in its paralogs *Chd3* and *Chd5* (128,187), we additionally examined these transcripts and found that both were significantly upregulated in *Chd4* cKO samples, as expected (Fig. 3.17C). Immunohistochemistry confirmed that Chd3 protein was upregulated in *Chd4* cKO RPCs, further validating these observations (Fig. 3.20).

Next, we visualized *Chd4* cKOs versus controls. Control cells from each replicate clustered together in UMAP space (Fig. 3.17D). *Chd4* cKO cells overlapped with control cells but were additionally shifted into novel parallel clusters that did not contain control cells (Fig. 3.17E). We found that significantly upregulated genes – including *Chd5* (Fig. 3.17F) and *Tcfl5* (Fig. 3.17G) – were expressed only in the novel clusters that appeared in *Chd4* cKO samples, but not in clusters occupied by control cells. These data likely indicate that our *Chd4* cKO samples exhibit marked alterations in gene expression across the full developmental trajectory, and that these changes are observed despite some probable mosaicism in cKO replicates (Fig. 3.19), as well as compensation from *Chd3* and *Chd5* paralogs.

We next identified differentially expressed genes (DEGs). Focusing specifically on RPCs, *Chd4* cKOs exhibited both downregulated and upregulated DEGs (Fig. 3.17H). Downregulated DEGs included the transcription factor *Irx5*, which is involved in the specification of bipolar cell subtypes (188) and the proneural gene *Ascl1*, which is necessary for rod and bipolar cell production (189) (Fig. 3.17I). In accordance with the later expansion of the RPC pool, upregulated genes included *Apoe*, *Cdkn1a*, *Hes5*, *Mt1* and *Mt2*, which are all expressed in RPCs (Fig. 3.17H, I). Other upregulated genes included *Ifitm2*, *Tcfl5* and *Snhg11*, which were previously observed to be upregulated in

the *Chd4* cKO neocortex (187). We next called GO terms on these upregulated DEGs. Top terms included 'Growth', 'Cell population proliferation' and 'Cell death' (Fig. 3.17J), which are phenotypes that emerge in the cKO at later stages but were not yet evident in perinatal counts.

Despite the lack of obvious temporal shifts in cell-type production, we reasoned that a shift in the developmental stage of *Chd4* cKOs could be evaluated by comparing our dataset against other timepoints. We therefore integrated our dataset with an existing developmental scRNA-seq atlas (28). Using independent component analysis (ICA), we found that most of the timepoints in the published scRNA-seq atlas were arrayed in a logical continuum, with the earliest embryonic samples at the origin, perinatal samples differing most along the first component (ICA1) and later postnatal samples differing most along the second component (ICA2; Fig. 3.17K). In accordance with expectations, both P1 *Chd4* cKO and littermate control samples were localized near to P0 and P2 samples from the retinal atlas. However, we observed that the *Chd4* cKO samples were slightly shifted towards P5 samples, while littermate controls were located closer to P0 and P2 samples. To better visualize this potential shift, we plotted the integrated dataset in a comparison matrix (Fig. 3.17L). Both P1 control and cKO samples correlated most closely with P2 samples (control, 0.64; *Chd4* cKO, 0.52). Strikingly, *Chd4* cKOs correlated more strongly with P5 samples (0.47) than they did with E18.5 (0.27) or P0 (0.24) samples. By contrast, littermate control samples correlated more strongly with E18.5 (0.54) or P0 (0.48) samples, and much less well versus P5 (0.23), as would be expected. Thus, while a shift in temporal identity could not easily be discerned with respect to UMAP trajectories,

both global gene expression and DEG signatures suggest that *Chd4* cKOs may be slightly accelerated in their temporal state.

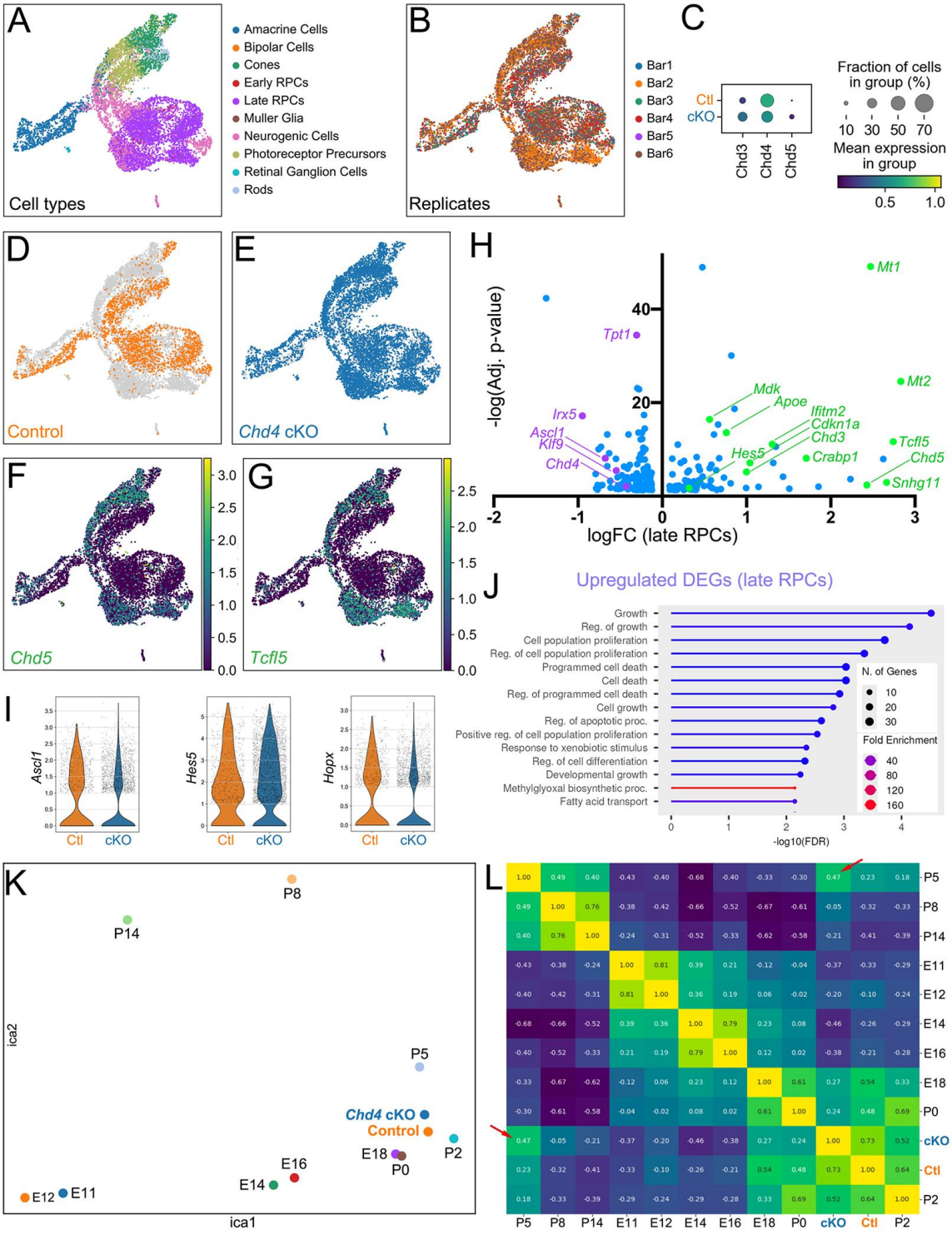

Fig. 3.17. Loss of Chd4 leads to global transcriptional dysregulation. (A) Leiden UMAP clustering of 9776 single cells from P1 control (n=3) or cKO (n=3) littermates. Cell types were annotated via unsupervised label transfer from a published atlas of retinal development (28). (B) UMAP projection of each demultiplexed sample. (C) Dotplot comparing the expression of Chd4 and its paralogs in late RPCs from control versus cKOs. (D, E) Comparison of control versus cKO cells. (F, G) UMAP projection of the expression of *Chd5* (F) and *Tcfl5* (G). (H) Volcano plot of differentially expressed genes (DEGs) from late RPCs in *Chd4* cKO samples versus control (adj. p-value<0.05; LogFC>0.4). (I) Violin plots comparing *Ascl1*, *Hes5* and *Hopx* expression in wild-type versus cKO late RPCs. (J) GO term analysis of significantly upregulated DEGs. (K) Independent component analysis (ICA) comparing wild-type and cKO scRNA-seq data with a published retinal RNA-seq atlas (28). (L) Pairwise comparison correlation matrix heatmap of the ICA analysis. Arrows indicate the elevated correlation between the cKO dataset and the P5 samples of the retinal atlas.

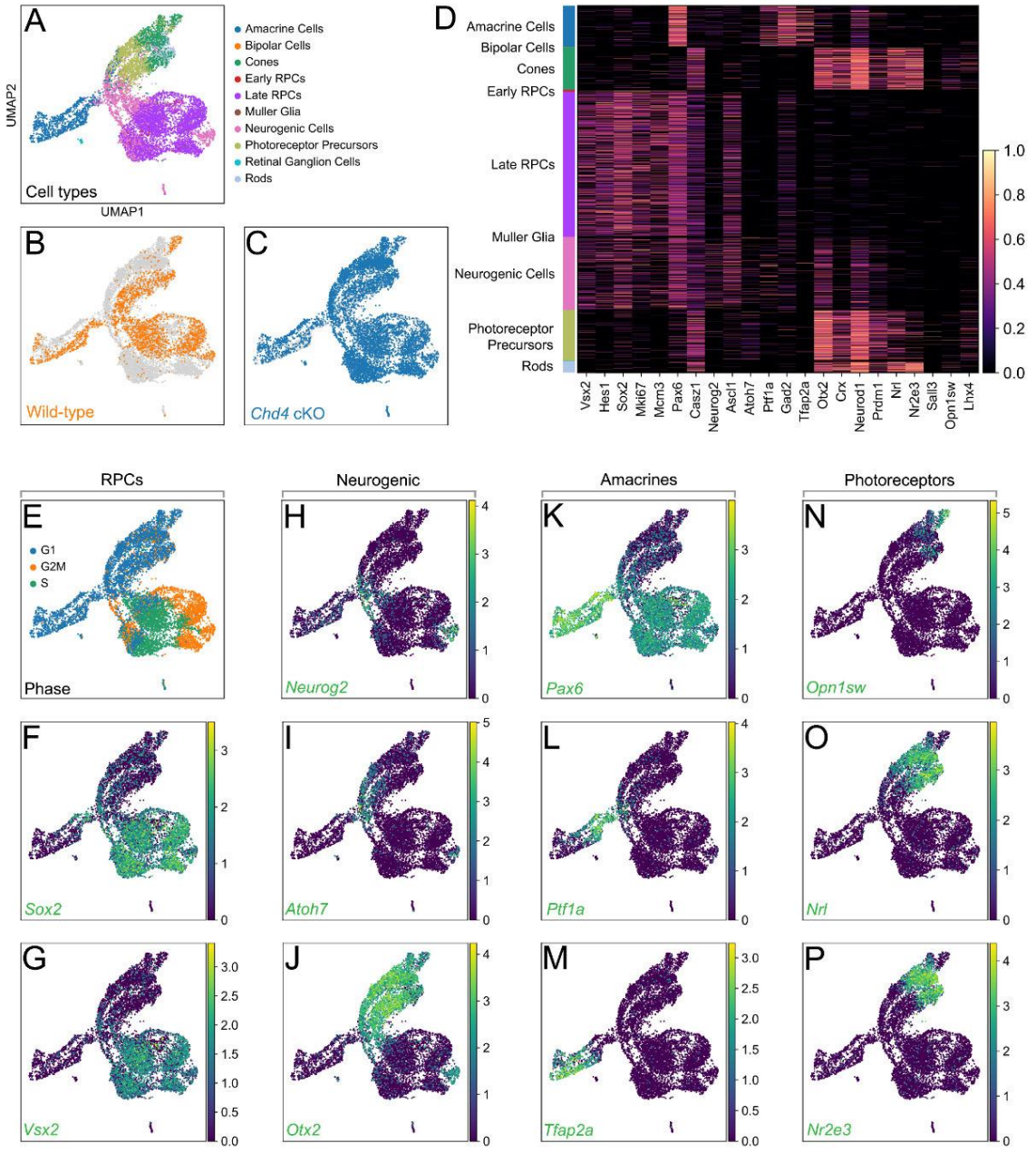

Fig. 3.18. Annotation of UMAP clusters using marker gene expression. (A) Cell-type annotation using label transfer from a published retinal scRNA-seq atlas (28). (B-C) Segregating the UMAP projection based on cell genotype. (D) Heat map of the expression of marker genes used to annotate the different cell type clusters present in the P1 scRNA-seq dataset. (E-P) UMAP projections of marker gene expression in control and *Chd4* cKO replicates. (E-G) RPC markers are based on the cell-cycle phase (E), along with the expression of progenitor-specific markers *Sox2* (F) and *Vsx2* (G). (H-J) Markers of neurogenic cells

determined by the expression of *Neurog2* (H), *Atoh7* (I), and *Otx2* (J). (K-M) Amacrine cell type markers *Pax6* (K), *Ptf1a* (L), and *Tfap2a* (M). (N-P) Photoreceptor markers *Opn1sw* (N), *Nrl* (O), and *Nr2e3 (*P).

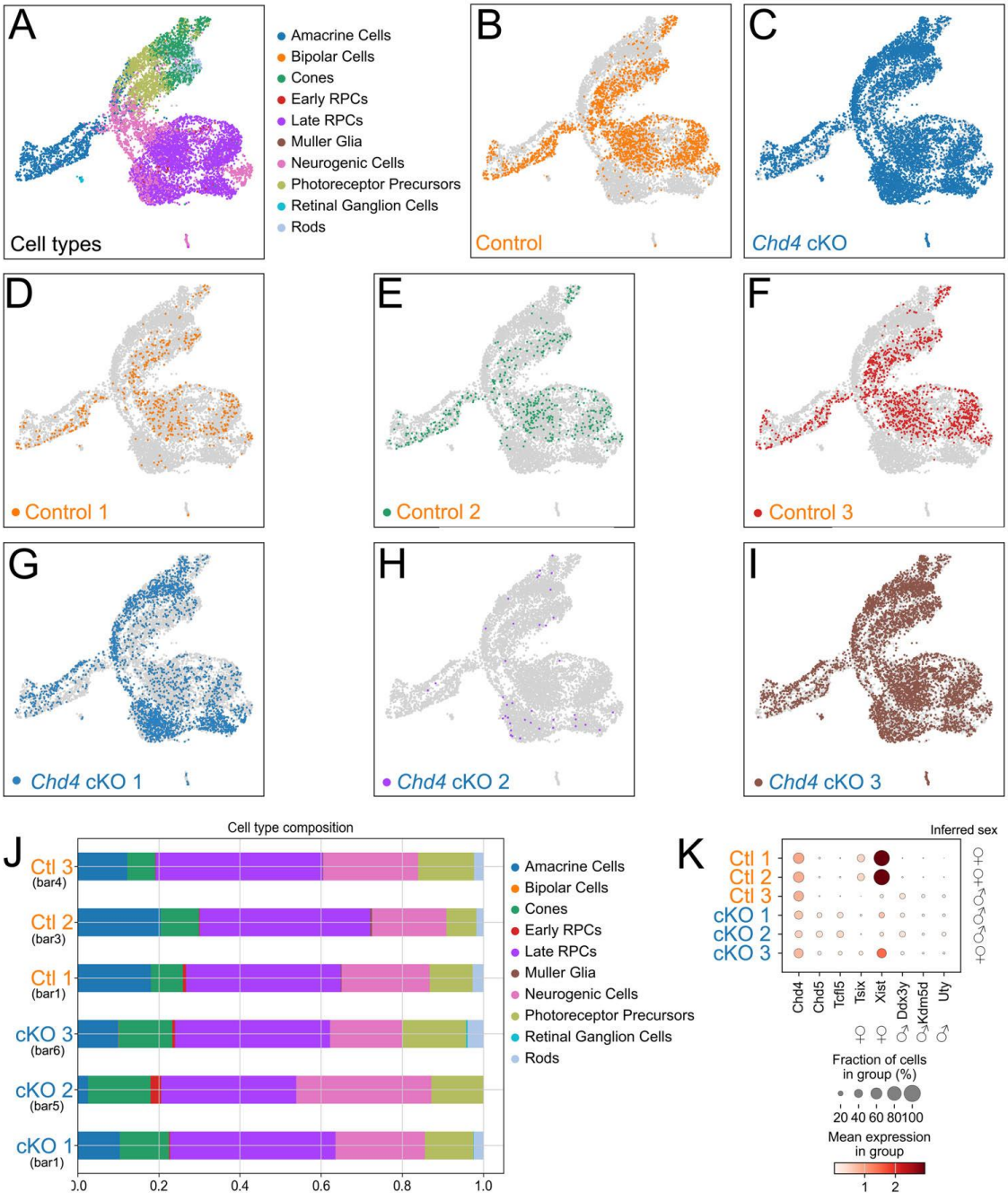

Fig. 3.19. Comparison of scRNA-seq replicates. (A) Cell-type annotation using label transfer from a published retinal scRNA-seq atlas (28). (B-C) UMAP projection of cells segregated by genotype. (D-I) UMAP projection of cells segregated by replicate. (J) Cell type proportions by replicate. (K) Dotplot of gene

expression by replicate. Note that *Chd4* cKO replicate 3 exhibits many cells that overlap with wild-type cells in UMAP space (I), suggesting that is likely mosaic. Accordingly, *Chd4* transcript is less reduced in this replicate, and the *Chd5* and *Tcfl5* transcripts that upregulate in the cKO are also less upregulated (K). Note also that the *Xist* and *Tsix* genes are transcribed from the inactivated X chromosome only in female cells, while *Ddx3y*, *Kdm5d*, and *Uty* genes are located only on the Y chromosome.

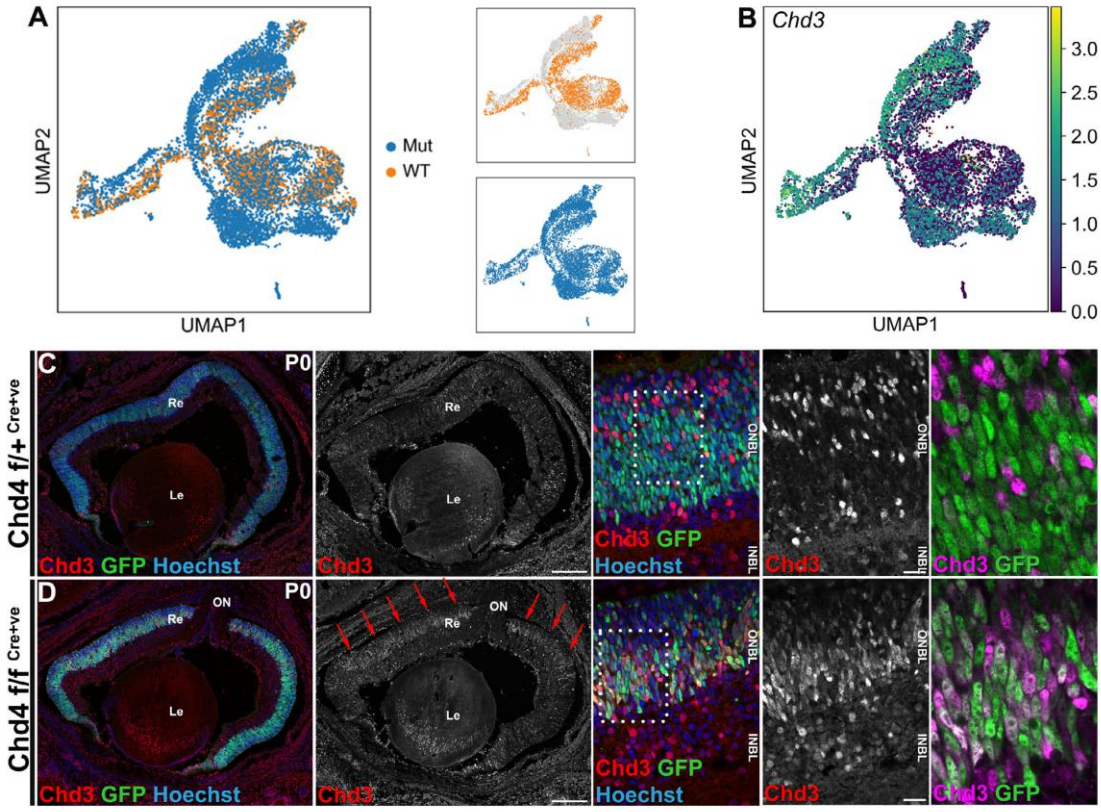

Fig. 3.20. Validation of the upregulation in Chd3 expression in the absence of Chd4. (A) UMAP representation of clusters segregated by genotype. (B) UMAP projection of *Chd3* expression in control and cKO samples. (C-D) IHC staining of Chd3 in chet and cKO P1 retinal sections.

3.2.2. Chd4 regulation of chromatin occupancy and accessibility in RPCs:

Next, we wished to determine how Chd4 regulates the genome. To examine the genome occupancy of Chd4, we performed CUT&RUN-seq on P1 wild-type and cKO retinas using a validated Chd4 antibody (128,187). We additionally examined Mbd3, which is specific to the NuRD complex. Visual comparison of these datasets revealed correspondence between Chd4 and Mbd3 (Fig. 3.23A). Across the genome, Chd4 occupied ~10,000 peaks in wild-type retinas, which was comparable to peak numbers observed in the neocortex and cerebellum (128,187). Mbd3 occupied ~3500 peaks, with most of these peaks co-occupied by Chd4 (Fig. 3.21A-C). In *Chd4* cKO retinas, Chd4 and Mbd3 peak numbers were drastically reduced to ~2500 and 1000, respectively (Fig. 3.21A). When compared to published retinal ChIP-seq data (190), we found that approximately two-thirds of the Chd4 peaks localized to gene promoters marked by H3K4me3 (Fig. 3.21B).

To directly visualize the nucleosome remodelling activity of Chd4 in RPCs, we performed ATAC-seq on two wild-type and two cKO littermates, by sorting RPCs marked by the *Chx10-Cre-GFP* transgene at P1. Inspection of the loxP-flanked cassette revealed almost complete excision in the cKO, validating the sorting strategy (Fig. 3.22). To identify differentially accessible regions (DARs) in *Chd4* cKO RPCs, we next performed diffbind analysis (Fig. 3.23B), yielding approximately 10,000 DARs between control and mutant RPCs. Most DARs exhibited increased accessibility (Fig. 3.23A-C). While the NuRD complex has previously been shown to decommission some regulatory elements, we found that most DARs were still nominally accessible in the control datasets (Fig. 3.23C). More surprisingly, most of these DARs exhibited little Chd4/NuRD complex occupancy (Fig.

3.23B), indicating a probable 'kiss-and-run' transient interaction or indirect regulation. By contrast, DARs that were reduced in accessibility in Chd4 cKOs appeared to be directly bound, suggesting that loss of the NuRD complex footprint might drive the effect.

To determine how differential accessibility might relate to gene expression, we performed peak-to-gene annotation. We selected only gene-proximal peaks (within 5 kb upstream and 1 kb downstream of the gene body inclusive) in order to filter the overall peak number. Peaks located more proximally to the gene can be annotated with higher confidence, whereas more distal peaks yield increasingly ambiguous associations; thus, closer proximity generally supports more reliable conclusions. Gene proximal DARs were associated with ~1600 genes. Gene ontology analysis showed that neuron fate commitment, neurogenesis and neuron differentiation were highly enriched (Fig. 3.23D), suggesting potential misregulation of these processes.

To determine how changes in accessibility affect gene expression, we examined genes associated with proximal DARs in our scRNAseq dataset. We found that DAR-associated genes overlapped with only ~10% of DEGs. However, more extensive overlap was observed with Chd4 CUT&RUN-seq peaks, with more than half of the DEGs and approximately one-third of DAR-associated genes directly occupied by Chd4. Additionally, 33 target genes were common between all three of the datasets (Fig. 3.23E), including genes such as *Cited2* (Fig. 3.23A), *Mbnl2*, *Jund* and *Plagl1* (Fig. 3.24). In accordance with these observations, when we generated a gene scoring module for genes associated with a proximal DAR, we found a slight but significant upregulation in the *Chd4* cKO (Fig. 3.23F), suggesting that changes in accessibility correlate with transcription. We next measured accessibility across gene bodies using LIMMA, which

allowed us to integrate accessibility data for each gene body rather than on an individual peak-by-peak basis. Comparing foldchanges in gene accessibility versus fold-changes in transcription, we observed a significant ($P<0.0001$) positive correlation between increased accessibility and transcriptional upregulation across the genome (Fig. 3.23G).

Lastly, we performed footprinting analysis on accessible peaks in order to identify differential transcription factor occupancy using the TOBIAS algorithm (Fig. 3.23H) (191). Using this approach, we found that Ctcf was one of the over-represented motifs in *Chd4* cKO RPCs. This suggests that the loss of Chd4 might lead to increased recruitment of Ctcf to sites that are typically inaccessible, resulting in disorganization of genome looping, as previously shown in cerebellar granule cells (136). Additionally, Tcfl5 motifs were also over-represented in mutant RPCs, corroborating increased *Tcfl5* transcript levels observed in the scRNA-seq data. Taken together, these data suggest that Chd4 may have a broad role in restricting nucleosome accessibility and consequent transcription across the genome, which might stabilize the RPC identity and drive self-renewal.

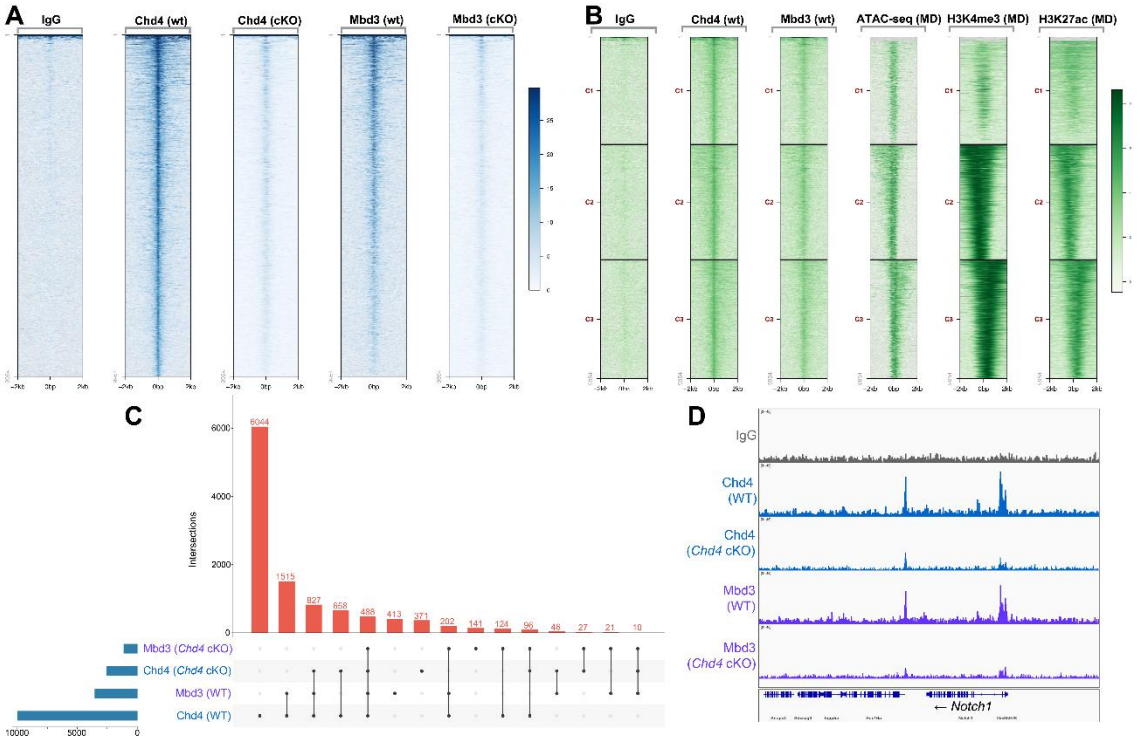

Fig. 3.21. Genomic occupancy of Chd4. (A) Heat map of CUT&RUN-seq peaks of IgG, Chd4 and Mbd3 from wild-type and *Chd4* cKO P1 retinas centered on Chd4 wild-type peaks. (B) Comparing the wildtype CUT&RUN-seq dataset with previously published ChIP-seq data (190) on age-matched retinas to determine the genomic occupancy of Chd4 and Mbd3. (C) Upset plot of CUT&RUN-seq peak intersections. (D) IGV track of CUT&RUN-seq peaks on the *Notch1* locus.

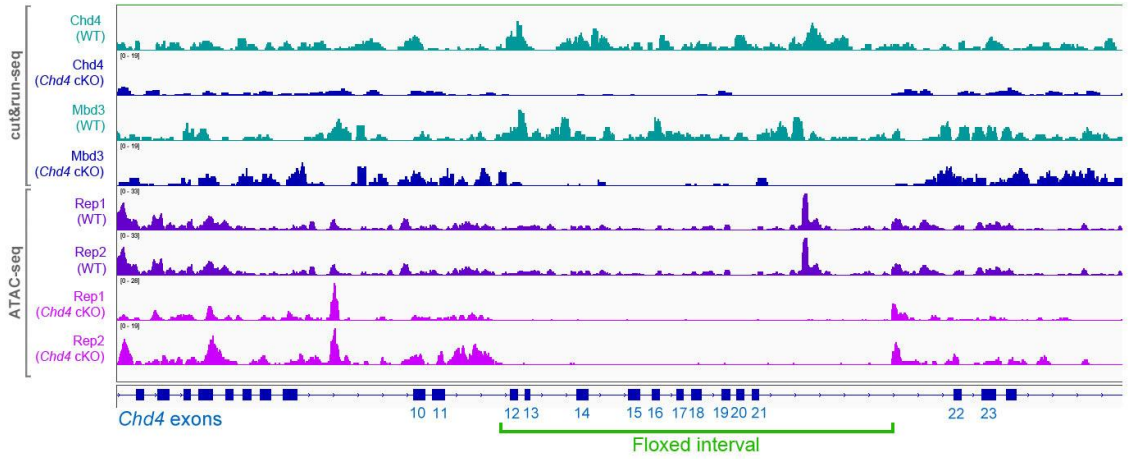

Fig. 3.22. Excision of the loxp flanked region in *Chd4* cKO genomic data.

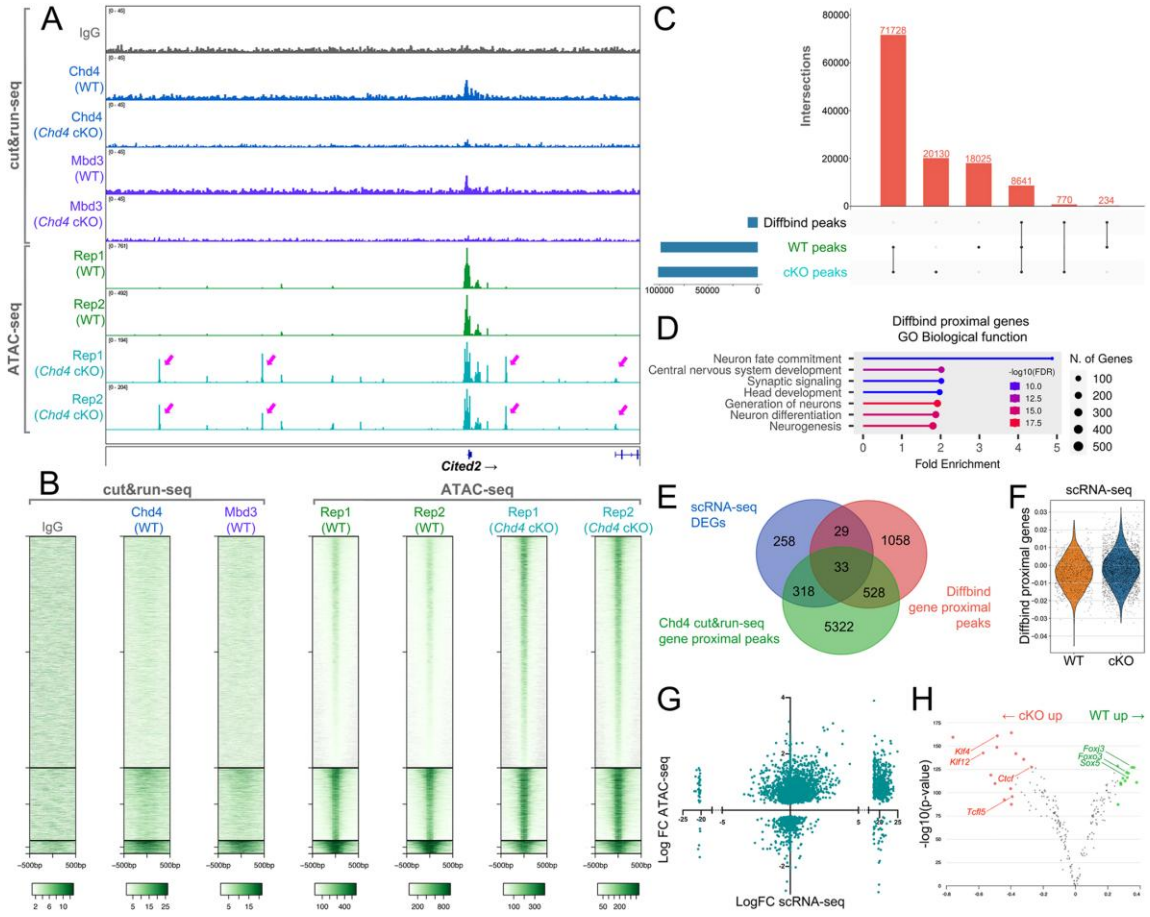

Fig. 3.23. Chd4 restricts chromatin accessibility in RPCs. (A) CUT&RUN-seq and ATAC-seq tracks showing called peaks at *Cited2* locus from P1 control and Chd4 cKO RPCs. Arrows indicate ectopic peaks present in the cKO samples but not in control. (B) CUT&RUN-seq and ATAC-seq datasets centred on differentially accessible regions (DARs) identified via Diffbind. (C) Upset plot comparing the overlap between ATAC-seq peaks comparing control and cKO datasets versus DARs. (D) GO terms analysis of Diffbind proximal genes. Diffbind proximal peaks were assigned to genes located within 5 kb upstream and 1 kb downstream of the peak. (E) Integration of DEGs from the scRNA-seq analysis with genes associated with gene-proximal DARs and Chd4 CUT&RUNseq gene-proximal peaks. (F) Violin plot of scRNA-seq gene scores for genes associated with a proximal DAR, comparing control versus *Chd4* cKO RPCs. (G) Integration of scRNA-seq gene expression with gene body accessibility measured via LIMMA. (H) Footprinting analysis on accessible peaks using the TOBIAS algorithm.

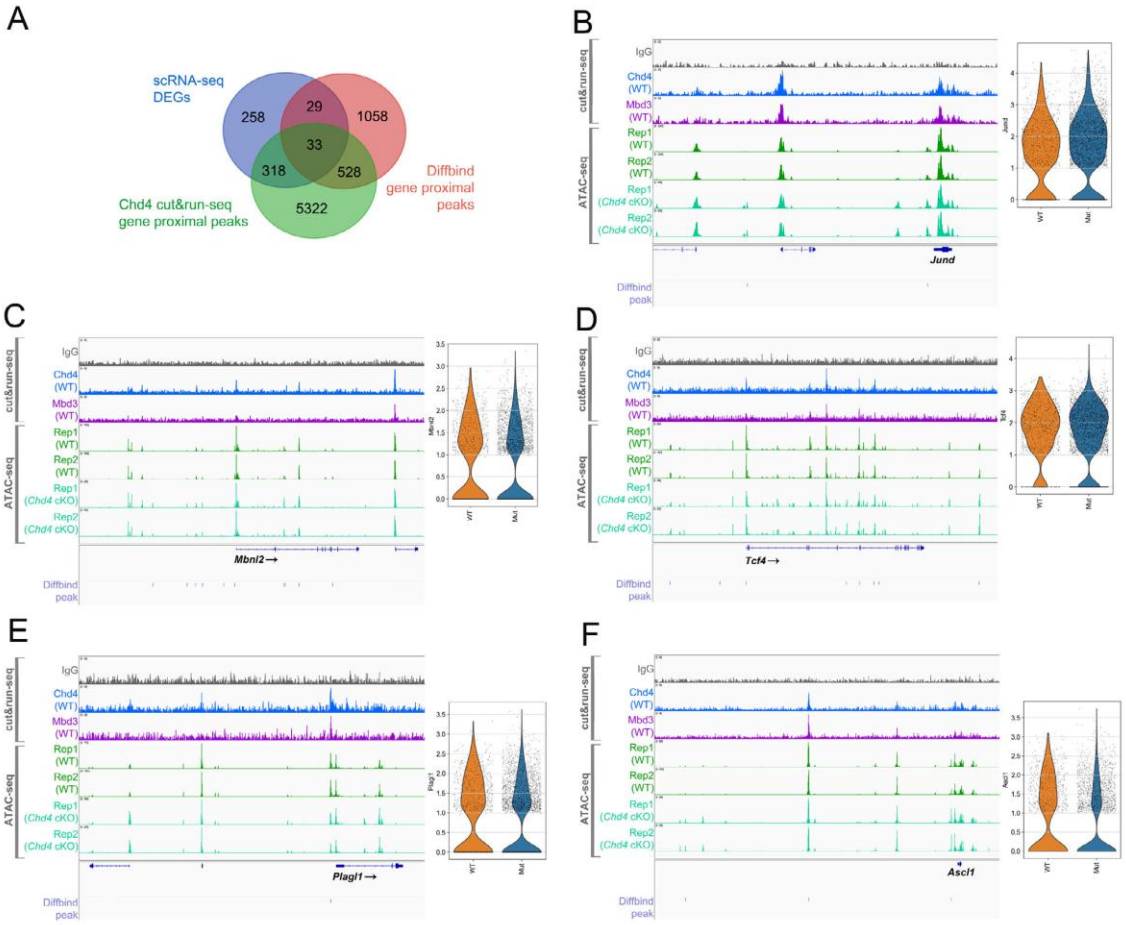

Fig. 3.24. Integration of ATAC-seq, CUT&RUN-seq, and scRNA-seq datasets. (A) Integration of DEGs from the scRNA-seq analysis with genes associated with gene proximal DARs and Chd4 CUT&RUN-seq gene-proximal peaks. (B-F) CUT&RUN-seq and ATAC-seq tracks for selected DEGs that are directly occupied by Chd4. Diffbind peaks indicate DARs from control vs. *Chd4* cKO ATAC-seq data. Violin plots display scRNA-seq expression data from late RPCs.

Chapter 4. Discussion

During CNS development, neural progenitors undergo temporal transitions in competence to generate a diversified population of neuronal and glial subtypes in correct proportions and order. Although progenitor competence plays a critical role in establishing cell type diversity in the nervous system, the underlying molecular mechanisms are poorly understood. The developing retina provides a tractable system with which to decipher the mechanisms that regulate the temporally dependent competence transitions in neural progenitors, as a single population of multipotent RPCs sequentially generates the six neuronal and one glial cell type seen in the mature retina. Here, we address the role of a specific epigenetic process, namely nucleosome remodelling, in regulating the neurogenic competence of RPCs. We demonstrate that the Chd4 chromatin remodeler is essential for balancing retinal cell type production and termination of the retinal lineage by regulating genome accessibility to prevent transcriptomic dysregulation and maintain normal developmental trajectories.

4.1 Focus on Chd4:

Previous research in our lab has suggested that the NuRD complex influences progenitor competence transitions during retinal development (93). The NuRD complex is a multi-subunit epigenetic modulator with dual enzymatic functions. Hdac1/2 provides the complex with deacetylase activity, while Chd subfamily II proteins give the complex remodelling abilities. While studies have highlighted the importance of Hdacs (161,192) during retinal development, less is known about the role of Chd subfamily II proteins or how they might impact progenitor competence.

We focused our study on Chd4, as previous research in the developing murine neocortex demonstrated that while Chd3/5 associated with the NuRD complex in post-mitotic neurons, Chd4 associated with the complex in neural progenitors (127). Another study utilizing neural progenitor cells (NPCs) showed that Chd4 knock-down led to precocious gliogenesis, indicating that it might regulate the transition from neurogenesis to gliogenesis (124). Moreover, *Chd4* cKOs exhibited a loss in late-born upper-layer cortical neurons, suggesting that Chd4 might also regulate earlier competence transitions (127,193). Previous work in the cerebellum has shown that Chd4 is required to decommission genes and regulate higher-order genome looping (128,136). However, the genetic requirement for *Chd4* during retinal development has not previously been examined.

We initially mapped the spatiotemporal expression profile of Chd4 in the developing retina, from E11.5 to P21. Our immunohistochemistry (IHC) revealed that Chd4 was ubiquitously expressed in retinal progenitor cells (RPCs) from E11.5 through to perinatal stages, suggesting it may regulate RPC output during development. Postnatally, Chd4 expression persisted in post-mitotic neurons, with high levels observed in the inner nuclear layer (INL) and ganglion cell layer (GCL). This matches a previously published single-cell transcriptomic atlas of the developing retina, covering from E11 to P14, which showed that *Chd4* transcripts were present in both RPCs and neurons, whereas *Chd3/5* transcripts were mainly confined to post-mitotic neurons with negligible expression in RPCs. Therefore, to understand the role of Chd4 in controlling RPC potential, we employed a conditional genetic approach.

4.2 Utilizing retina-specific knock-out:

Since *Chd4* is an essential gene (175), and previous studies in the developing neocortex showed changes in cortical histogenesis and neuronal generation in the absence of *Chd4* (127,193), we opted for a targeted approach to characterize its role in retinal development. We generated *Chd4* cKOs by crossing the $Chd4^{flox}$ allele with the Chx10-Cre-GFP driver. The expression of the Cre-GFP fusion protein is controlled by the promoter of *Chx10*, which is a retina-specific gene whose expression begins at around ~E10.5 and is present in almost all RPCs until later postnatal stages of development. At the end of development, Chx10 expression is maintained in bipolars and a few Müller glia. The Cre recombinase excises the exons that encode the ATPase domain of Chd4, thereby abolishing its enzymatic activity. Thus, the resulting cross results in a retina-specific knockout of *Chd4* right at the beginning of retinogenesis, with GFP acting as a reporter for progenitor identity. To assess the robustness of this approach, we analysed Chd4 protein levels in P0 control and *Chd4* cKO retinas via IHC as well as western blot and successfully demonstrated the efficient abrogation of the Chd4 protein in the mutant retinas. Although the Chx10-Cre-GFP driver is prone to mosaicism, in our study, we observed an average of ~70% of cells within the ONBL expressed GFP in perinatal Cre+ animals, which is similar to the overall proportions of RPCs within the layer.

4.3 Chd4 regulation of retinal temporal states

During retinal development, RPCs, like neural progenitors in the developing cortex, undergo temporally dependent competence transitions to generate the diverse neuronal and glial cell types. In rodents, RPCs initially undergo a competence transition perinatally where they lose competence to make early neuronal cell-fates and switch to generating

late-fates. As development progresses, RPCs undergo another competence transition, losing their proliferative and neurogenic potential and directly differentiating into Müller glia, thereby terminating the RPC lineage. However, the underlying molecular mechanisms are poorly understood. Utilizing the retina-specific *Chd4* cKO model, we addressed the role of nucleosome remodelling in regulating the chronology of cell type production from retinal progenitors. We hypothesized that Chd4-dependent chromatin remodelling might dynamically regulate the epigenetic landscape of RPCs to influence developmental timing, by regulating the competence transitions of RPCs and thereby controlling cell-fate decisions.

Chd4 cKOs accordingly exhibited increases in early-born RGCs, and later-born rods were drastically decreased (Fig. 4.1 A, B). While we predicted that this shift in cell-type composition might arise due to prolongation in the early competence window, this proved not to be the case. Instead, RGCs were overproduced during their normal developmental window, as evidenced by the expanded GCL in E16.5 mutant retinal sections. Our birth-dating studies also suggested that RGCs are not born beyond their temporal window. We injected EdU in perinatal control and mutant retinas that were harvested at later time points and did not detect any elevation in EdU+ RGCs.

Additional validation was provided through our scRNA-seq analysis. We utilised the scRNA-seq data to address whether there might be a prolongation of early competence during the perinatal stage of retinal development in the absence of *Chd4* and saw no evidence that supports it. For example, we did not observe the persistence of an RGC neurogenic trajectory in our UMAP. However, like other postnatal scRNA-seq datasets (194), we captured very few RGCs in our experiment and almost all of the recovered

RGCs came from the *Chd4* cKO samples. Since the few RGCs recovered were segregated from neurogenic cells, these data reinforce our birthdating data that indicate that P1 RPCs do not continue to produce significant numbers of RGCs in the *Chd4* cKO. Rather, our scRNA-seq profiling suggested that *Chd4* cKOs were instead slightly accelerated in their temporal state. Moreover, comparison of our differentially expressed genes (DEGs) with the pseudotime DEGs identified by Clark et al. along the RPC–Müller glia trajectory, visualized as heatmaps of transcript expression across the transition, revealed that many genes upregulated during the transition to the glial state are also upregulated in *Chd4* cKO retinas, while genes that decline along this trajectory are similarly downregulated in the cKO (28). Suggesting that the accelerated temporal state in the *Chd4* cKOs appears to mimic glial differentiation. However, we observed that Ki67+ RPCs persisted beyond their normal developmental window, which eventually differentiated into glia, albeit much later than controls. The observed discrepancies in temporal state—apparent acceleration in P1 scRNA-seq and delayed glial differentiation at P8—are challenging to reconcile but likely reflect asynchrony within the temporal program, wherein certain processes advance while others lag. This disconnect may further arise from Chd4 not functioning as a central regulator of the perinatal competence transition, whereas postnatally it appears essential for RPCs to exit the cell cycle and differentiate. Taken together, these data suggest that Chd4-dependent nucleosome remodelling regulates the temporal transition that terminates the retinal lineage but does not control earlier competence transitions. These data suggest that multiple molecular mechanisms regulate the competence transitions of RPCs, and that only the terminal transition between neurogenesis and gliogenesis requires Chd4-dependent chromatin remodelling.

4.4 Chd4 is required to balance retinal cell-type production

Chd4 cKOs exhibited an expansion of early-born RGCs and amacrines, while rods were significantly reduced (Table 4.1). Chd4 ablation had a marginal effect on other neuronal cell types but increased glial generation by P15. The observed changes in the cell-type composition of the mutant retinas could arise if RPC proliferation and cell-fate specification were affected. To that effect, we analyzed P0 *Chd4* cKO and control retinas when the RPCs are undergoing competence transitions. Surprisingly, the late RPC population in the mutants was comparable in cell numbers to that of the controls, indicating no exhaustion of the late RPC pool. Additionally, the proliferation of late RPCs, as well as changes in cell death, were also unaffected by the loss of *Chd4* (Table 4.1). Thus, the increases in early-born cell-fates such as RGCs in the mutant retinas, occurred without a complementary decrease in alternative fates could be explained by RPC hyperproliferation during embryonic stages of development. Another possibility is that RGC apoptosis might be reduced in the absence of *Chd4*. There is a wave of apoptosis during the perinatal stage of retinal development that fine-tunes the proportions of different cell types, where supernumerary RGCs die. However, there is also an earlier wave of embryonic RGC cell death that we did not investigate (195–197).

While distortions in cell type composition were observed prior to the significant cell loss that occurs between P8 and P15, alterations in cell death may nonetheless be responsible for the observed shifts in cell type composition. At P0, the number of rod and cone photoreceptors in the *Chd4* cKO retinas was comparable to that of controls; however, by P15, they were significantly reduced. However, the reduction in rod photoreceptors was also evident at P5 at the onset of the observed elevation in apoptosis, suggesting that they

are underproduced (Table 4.1). While rods could be underproduced at later stages, cone production is complete by P0, meaning that the subsequent reduction in cone numbers is likely driven by apoptosis. This suggests that the alterations in cell death in *Chd4* cKO retinas might partly be responsible for the reduced numbers of photoreceptors at postnatal stages of development. In addition to the elevated apoptosis, the mutant retinas also exhibited an increased number of RPCs cycling well beyond their normal temporal window. Together, these results suggest that the decrease in photoreceptors at P15 may be due to a combination of factors, including increased apoptosis and the failure of RPCs to undergo neurogenic differentiation.

Müller glia are the latest-born retinal cell types and have been shown to arise directly from the late RPC pool rather than from neurogenic precursors (28). Thus, the increase in glial generation in the mutant retinas can be attributed to the expansion of the cycling RPCs seen at P8 that fail to undergo neurogenic divisions and terminally differentiate into Müller glia. Further evidence was provided from birthdating studies where EdU was injected into P8 mutant and control retinas and analysed at P15. In the controls, Edu+ cells were mostly found in the peripheral edge, and labelling was observed in many rod photoreceptors. In the mutant retinas, EdU+ cells were found scattered across the entire tissue, with most EdU+ cells also being positive for the glial marker Sox2. Taken together, the above results indicate that Chd4 might be required during postnatal retinal development to promote neurogenic differentiation of RPCs, as well as regulating photoreceptor generation and survival.

Chd4 cKO versus Controls (wt and het)					
Development stage / Phenotype	P0	P2	P5	P8	P15
Total Cell Count (Hoechst)	≈	≈	≈	≈	↓
RGCs	↑	↑	nd	nd	↑
Horizontal cells	nd	nd	≈	nd	nd
Total Amacrine cells	nd	nd	nd	≈	vs wt ↑
Cholinergic Amacrine cells	nd	nd	nd	vs wt ↑	nd
Cone and Rod photoreceptors	≈	nd	Rods ↓	nd	Rods and Cones ↓
Bipolar cells	nd	nd	nd	↑	≈
Müller Glia	nd	nd	nd	delayed differentiation ↓	↑
RPCs	≈	≈	nd	↑	nd
Cell death	≈	nd	↑	nd	↑
Tissue Lamination	• Expanded GCL • Defective IPL	• Expanded GCL • Defective IPL	Expanded GCL	• Expanded GCL • Defective OPL • Ectopic glia and bipolars in ONL	• Thinned ONL • Expanded GCL • Ectopic glia and bipolars in ONL

Table 4.1. Summary of changes in *Chd4* cKO retinas versus controls. The phenotypic changes of mutant retinas versus controls at different stages of retinal development. Red arrows denote a significant decrease; green arrows denote a significant increase; ≈ indicates no significant difference between genotypes; nd= not determined, indicates that data were not quantified.

4.5 Molecular control of progenitor competence:

Molecular determinants of temporal competence states were first identified in *Drosophila* neuroblasts (198), where transcription factor cascades were shown to progressively modify progenitor potential, to generate chronological sequences of neurons. Analogous to *Drosophila* temporal factors, temporal transcription factor cascades that regulate RPC competence during retinal development have been identified more recently (91,92,96,101). Additionally, in both the retina and in other regions of the CNS, competence transitions have also been shown to be dependent on heterochromatic determinants, including DNA methylation and the polycomb repressor complex (94,172,199). This suggests a model where heterochromatic processes might act downstream of transcription factors to "decommission" genes that impart early competence and progressively restrict the developmental potential of RPCs. However, it remains unclear whether/how transcription factors and heterochromatic determinants converge. This is especially true for polycomb, as the mechanisms regulating its temporally dependent recruitment to the genome remain poorly understood, and transcription factors that directly recruit polycomb have yet to be fully elucidated in vertebrates.

Multiple temporal transcription factors, including Ikzf1, Casz1 and Foxp1, have been shown to interact with the NuRD complex in various contexts (93,200–202). Additionally, the NuRD complex has been shown to either regulate or interact with heterochromatic regulators such as the polycomb repressor complexes (123,125). Therefore, we hypothesized that the NuRD complex might represent a general pathway that links the

temporal transcription factors with the heterochromatic machinery to regulate the temporal transitions in RPC potential in a stage-dependent manner.

With respect to temporal transcription factors, the *Chd4* cKOs resemble *Casz1* cKOs, although they display a more pronounced phenotype. Postnatally, *Chd4* was crucial for promoting rod photoreceptor generation and restricting excess glial generation, phenocopying *Casz1* cKO retinas (92). This suggests that Casz1 and Chd4 may be part of a shared pathway that governs the late-RPC-to-Müller-glia transition, during which neurogenic competence is lost. This aligns with previous biochemical evidence showing that Casz1, Chd4, and/or NuRD interact physically and have been previously associated with late neurogenic competence (93,125). On the other hand, Ikzf1 was shown to be necessary and sufficient to endow RPCs with early competence, and in the absence of *Ikzf1*, the early-born cell types were shown to be underproduced (91,203). This contrasts with *Chd4* cKO retinas, where the absence of *Chd4* led to a significant overproduction of early-born RGCs, indicating that even though Chd4 and Ikzf1 are known to be cofactors, different mechanisms might regulate these temporal transitions. Rather, the increase in RGCs in the *Chd4* cKO retinas bears a resemblance to *Lhx2* cKOs (101), which would be consistent with biochemical and functional data linking Lhx2 to NuRD in the cortex (129). These observations suggest that distinct mechanisms may govern different aspects of competence transitions.

In addition to changes in cell-type composition, abrogation of *Chd4* also resulted in defects in retinal histology. *Chd4* cKO retinas displayed defects in the formation of the inner plexiform layer, which was evident in perinatal stages of development and in adult stages exhibited marked changes in retinal lamination. This indicates that Chd4 may have

additional functions during retinal development and may be required for additional processes such as neurite outgrowth, as shown previously in cerebellar granular neurons (128). *Chd4* cKO retinas also displayed ectopic presence of bipolars and glia in the ONL, which may arise due to defects in lamination. Compared to Casz1, Chd4 elicits a more severe phenotype, suggesting that additional factors may interact with the Chd4/NuRD complex in a stage-dependent manner (Fig. 4.1C). Therefore, further work will be required to better understand the mechanisms that control retinal histogenesis.

The above observation that different epigenetic mechanisms may govern each temporal transition has been previously described. For instance, in the retina, polycomb was shown to regulate the competence transition between the production of early versus late-born neurons (94), while in cortical development, it influences the switch between early-to-late neuronal production in addition to regulating the transition from neurogenesis to gliogenesis (172,204). In cortical lineages, NuRD and Chd4 were previously found to suppress precocious gliogenesis during the neurogenic phase of development, but did not regulate earlier temporal transitions (124,125). Our findings are consistent with the latter findings, as although Chd4 significantly affected the production of early- versus late-born neurons, our results indicate that the Chd4/NuRD complex is not necessary for the transition between early versus late neurogenic competence in the retina.

Once late competence is established, postnatal RPCs generate rods, bipolars, amacrines, and Müller glia (3,24,25). Our data suggest that while Chd4 does not regulate the perinatal early-to-late RPC competence transition, Chd4 (and likely Casz1) controls the late-RPC-to-Müller-glia transition, where neurogenic competence is lost (Fig. 4.1). This is in accordance with the notion that neurogenic cells are unable to adopt a Müller glia fate,

suggesting that the loss of neurogenic competence might coincide with the acquisition of gliogenic competence. In contrast, RPCs in the *Nfia/b/x* triple mutants were previously shown to underproduce bipolars and glia and the persisting cycling RPCs overproduced rods (28). This suggests that *Chd4* and *Nfia/b/x* have complementary roles in terminating retinal lineage and would indicate two alternative mechanisms that might act in parallel.

4.6 Role of nucleosome remodelling in gene regulation:

To understand how Chd4 regulates the genome, we focused on the perinatal stages of retinal development, where our birthdating analyses showed that the *Chd4* abrogation in RPCs resulted in an altered cell-type composition, but did not exhibit any changes in RPC proliferation or death. Moreover, at P1, *Chd4* cKO retinas exhibit some aspects of their phenotype, with the earliest-born ganglion cells being significantly increased. Still, the late RPC pool in the mutant retinas is not depleted. We therefore focused on this stage. Our scRNA-seq analysis revealed that the loss of *Chd4* resulted in widespread transcriptomic dysregulation. We applied a non-biased label-transfer approach utilizing a previously published transcriptomic atlas of the developing retina (28) and were able to annotate most of the cell types present in P1 retina. Our UMAP resembles the published scRNA-seq dataset by Clark et al. (28) that showed RPCs forming a niche of neurogenic cells, which branch further into clusters of amacrine cells and photoreceptor precursors. This is also in agreement to the scRNA-seq study performed by Zhang et al. on E18.5 retinas (94). Segregating the transcriptional signatures based on genotypes, our results showed that loss of *Chd4* exhibited shifted transcriptomic trajectories, implying a global transcriptomic dysregulation.

The DEG analysis revealed that loss of *Chd4* affected the expression of various genes ranging from cell-cycle regulators to long non-coding RNAs and transcription factors, indicating that *Chd4* might play a multi-faceted role during retinal development. Downregulated genes included transcription factors involved in cell fate specification, such as the proneural gene *Ascl1,* which is in agreement with the observation that *Chd4* is required for late neurogenic competence. By contrast, upregulated DEGs included genes linked to RPC identity. This likely reflects the requirement of *Chd4* to restrict RPC proliferation at later stages of retinal development. Similarly, upregulated DEGs were also linked to proliferation and apoptosis, which were shown to be increased in later stages of development. Furthermore, our scRNA-seq and IHC data showed that Chd3/5 are not normally expressed in progenitors, however, in the absence of *Chd4*, both *Chd3* and *Chd5* upregulated, suggesting a possible genetic compensation similar to that reported in the developing cortex (187). Nevertheless, since Chd3/4/5 are reported to have non-redundant functionalities (127), the compensation effect might be minimal, as the loss of Chd4 alone was sufficient to elicit a robust phenotypic response at the end of retinogenesis. Nonetheless, further studies will be required to dissect the individual contribution of Chd3 and Chd5 during postnatal retinal development.

Here, we also report that the changes in gene expression seen in *Chd4* cKO retinas directly correlate to changes in accessibility. As a direct readout of Chd4 functionalities, we performed ATAC-seq on sorted RPCs. We showed that in the absence of *Chd4*, modest increases in accessibility occurred at thousands of regulatory elements, which correlated with changes in transcription at associated genes. Similar findings were previously reported in the granule neurons of the murine cerebellum, where Chd4 depletion led to a

widespread increase in genomic accessibility (128,136). These genome-wide changes in accessibility have also been reported in embryonic stem cells, where the NuRD complex was required to silence inappropriate gene programs, such as those that promote pluripotency, and to facilitate the exit from self-renewal to activate genes necessary for lineage commitment (167,169). Taken together, our data suggest that without Chd4 remodelling functions, the suppression of RPC gene expression signatures is undermined, leading to the reinforcement of the RPC state and hindering neuronal differentiation (Fig. 4.1D).

In the developing retina, Chd4 occupied approximately 10,000 peaks, similar to what has been reported in the cerebellum and neocortex (128,187). Two-thirds of these peaks correlated with acetylated histone H3K27 and trimethylated histone H3K4, which mark active enhancers and promoters, respectively, confirming findings in post-mitotic cerebellar granule neurons, where Chd4 was shown to bind to active promoters and enhancers. However, Mbd3 CUT&RUN resulted in only 3,500 peaks, perhaps indicating the NuRD-independent functions of Chd4. Recent studies have shown that, apart from the NuRD complex, Chd4 can also form a distinct chromatin remodeling complex with Adnp, known as the ChAHP complex, which has been demonstrated to regulate neurogenesis in the developing cortex (170,187). The Chd4 phenotype might also arise due to the dysregulation of genome looping. Ctcf was one of the overrepresented footprints in *Chd4* cKO RPCs. Chd4 was previously shown to regulate Ctcf binding and thereby control genome architecture (136). Chd4 might also regulate cohesin through the ChAHP complex, as Adnp and Ctcf have been shown to bind the same DNA motifs (171). Thus, future studies will be required to determine whether Chd4 regulates neurogenic

competence through NuRD, ChAHP, and/or via interactions with additional cofactors, such as temporal transcription factors.

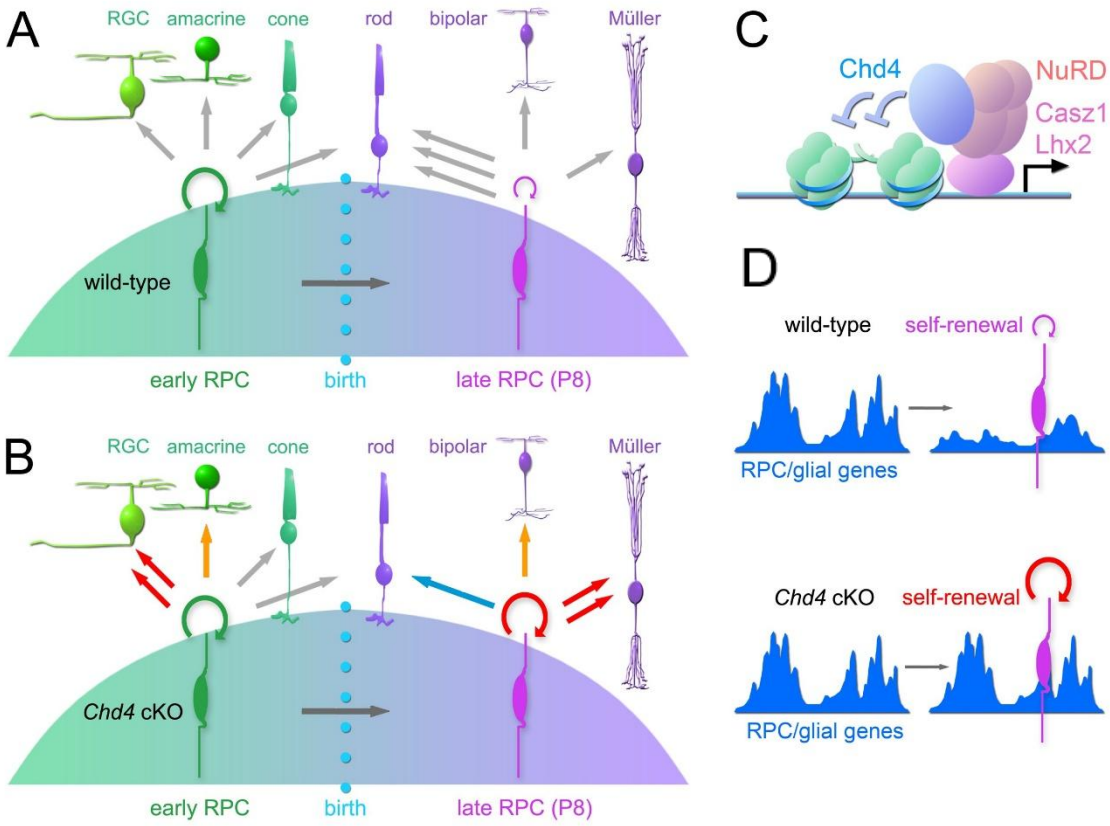

Fig. 4.1. Summary of the effects of *Chd4* cko during retinal development. (A, B) Alterations in cell-type production in wild-type versus *Chd4* cKO retinas. Red arrows: increased; orange arrows: slightly increased; blue arrows: decreased. (C) Chd4/NuRD may associate with different transcription factors at various developmental stages to control cell fate specification. (D) Model for the role of nucleosome remodelling in the temporal progression of RPCs. In the absence of Chd4, expressed genes remain more accessible, reinforcing the progenitor state.

4.7 Future directions:

In this study, we show that Chd4-dependent nucleosome remodeling regulates progenitor potential by balancing cell-type production and driving retinal lineage termination, without affecting earlier temporal transitions. Mechanistically, Chd4 limits genome accessibility to repress progenitor identity and promote differentiation. However, there are other fundamental questions that remain unanswered that can be tackled in future studies. For instance, it remains unclear as to how *Chd4* cKO led to increased production of RGCs. Our scRNA-seq dataset includes only 22 RGCs, all from cKO replicates, making it challenging to evaluate transcriptomic differences between RGCs from wild-type and mutant retinas. Future studies investigating Chd4's role during embryonic retinal development could reveal how it regulates early RPC potential. Integrating scRNA-seq of early RPCs from wild-type and *Chd4* cKO retinas with ATAC-seq and genomic occupancy analyses may elucidate mechanisms by which Chd4 limits the overproduction of early-born cell types, such as RGCs. Another unresolved aspect of the study is the limited occupancy of the Chd4/NuRD complex at regions of increased chromatin accessibility in mutant RPCs, suggesting that genome accessibility at many loci may be regulated indirectly. To explore this, we performed motif footprinting on differentially accessible regions and identified Ctcf as an overrepresented motif in *Chd4* cKO RPCs. Given Ctcf's role in genome looping and regulating chromatin structure, these findings implicate Chd4 in the regulation of genome architecture (205). Further studies will be required to determine how Chd4-dependent genome remodeling in RPCs influences their developmental potential.

The NuRD complex has a multi-faceted role in stem and progenitor cells and this is due to the variable composition of its subunits along with the myriad of co-factors and transcription factors that it can interact with (165). We have previously shown that Casz1 interacts with the NuRD complex in perinatal retinas, raising the question of whether other temporal factors also interact with the NuRD complex. Ikzf1 is known to be a NuRD interactor in B-cells; however, our phenotyping data suggests that during early phases of retinal development, the NuRD complex might interact with other factors instead of Ikzf1 to regulate RGC production. Identifying the proteomic interactome of the NuRD complex through BioID using Chd4 and Mbd3 as bait at different stages of retinal development might provide an insight into the different cofactors that associate with NuRD during retinal development. Additionally, Chd4 is also known to be a part of the ChAHP complex, but the role of ChAHP in retinal development remains unknown. Future studies exploring ChAHP and NuRD complex-specific regulation of retinal development might provide insight into how different epigenetic regulators with overlapping subunit members function during development. Finally, in the absence of *Chd4*, we observed increased transcription of *Chd3* and *Chd5,* indicating some functional compensation. However, the phenotype observed was still severe, indicating that Chd3 and Chd5 have non-redundant functionalities in the developing retina that need further exploration.

This study has provided insights into how an epigenetic process, namely nucleosome remodelling by Chd4, could regulate progenitor cell potential and impact cell-fate specification during retinal development. There are predominantly two proposed models of RPC cell-fate determination: the serial competence model, in which RPCs gain and lose competence in a stepwise, unidirectional manner to adopt specific cell fates in a

temporally dependent manner, and the progressive restriction model, in which RPC potential becomes more restricted as development progresses. Our results correspond well with the latter model. Early RPCs have the potential to generate all the cell types in the retina, however as development progresses, epigenetic mechanisms such as chromatin compaction by Chd4 limit the potential of RPCs by possibly decommissioning multipotency and proliferation genes along with constraining the expression of cell fate specification genes, thereby regulating cell type production in a temporal dependent manner. This might explain why late RPCs lose the ability to make early cell fates.

A link between Chd4 and progressive restriction also raises the question of whether RPC potential can be restored by reversing or removing these epigenetic barriers. That is important because once retinal neurons are lost, they cannot be replaced. For this reason, vision impairment in retinal degeneration is permanent. Therefore, it is of immense interest to develop regenerative cell therapies to utilize the properties of neural stem cells to replenish damaged neurons. However, regenerative therapies for the eye have proved to be difficult to bring to the clinic thus far, partly due to the poor understanding of the intricate molecular mechanisms involved in retinal development. Many of the cell types that are lost in retinal degenerative disease are produced only during early phases of development, and in relatively low numbers. This includes cone photoreceptors lost in age-related macular degeneration, as well as retinal ganglion cells lost in glaucoma. Together, these two diseases afflict approximately three million Canadians (206,207).

One strategy to replace the lost cell types is through transplantation, where the damaged cells are produced in the lab by reprogramming embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) and are then transferred into the retinas of patients with

retinal degenerative diseases (208). Previous studies have shown that the NuRD complex acts as a barrier to reprogramming (160,209). Our study provides some mechanistic insight into how Chd4/NuRD might restrict RGC production and enhance photoreceptor specification, and future studies could examine strategies to enhance cell type production from ESCs or iPSCs by blocking NuRD functionalities, along with complementing with temporal transcription factors or fate specifiers to generate specific retinal cell types for transplantation.

Although transplantation strategies are promising to replace damaged neurons, there are some drawbacks with one of them being material transfer, where previous studies have shown that the GFP from transplanted donor cells were transmitted into hosts cells (210,211). Thus, studies have investigated the possibility of performing in vivo reprogramming of glial cells in the retina into neurons. Lower vertebrates, such as zebrafish, have the ability to regenerate their retina upon injury by activating endogenous Müller glia that re-enter the cell cycle and are able to replace the lost neurons (212). However, mice lack the ability to naturally regenerate the retina through Müller glia–mediated mechanisms. The Reh lab attempted to recapitulate the glial regeneration observed in zebrafish in mouse retinas following injury and observed that epigenetic constraints, such as reduced chromatin accessibility, limited the regenerative capabilities of murine Müller glia (213). In their studies, they directly targeted murine Muller glia and overexpressed temporal factors and fate determinants in the presence of histone deacetylase inhibitor to unlock the neurogenic potential of mammalian glia (213,214). In future studies, it would be interesting to see if glial regeneration can be enhanced by conditionally knocking out Chd4 specifically in the mammalian Müller glia.

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Appendix:

List of Publications:

1. **Shah S**, Medisetti S, Fernandes JAL, Mattar P. Chd4 remodels chromatin to control retinal cell type specification and lineage termination. Development. 2025 Sept 18; 152(20):dev204697.
   Available at: https://doi.org/10.1242/dev.204697

2. **Shah S**, Medisetti S, Fernandes JAL, Mattar P. Competent to Stand Trial-The Case for Temporal Control of Retinal Development. In: Encyclopedia of the Eye. Elsevier; 2025. p. 577–96. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780443138201000530
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Competent to Stand Trial—The Case for Temporal Control of Retinal Development

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Key Points

- Review the literature that underpins our understanding of retinal lineage, focusing on the birth order of different cell types
- Provide a detailed overview of genes that have been shown to regulate retinal lineage
- Critically examine molecular mechanisms that have been proposed to underlie temporal changes in retinal progenitor competence

Glossary of Terms

Cell type Major categories of differentiated cells. Also called "classes" in the literature. The retina contains 7 such cell types. Cell types differ markedly in gene expression, physiology, and function. Morphological differences are generally distinctive enough that cell types can be distinguished by morphology alone.

Competence The potential that a progenitor has at a given time. Eg. a progenitor might have the potential to generate multiple cell types. However, within a given temporal window, the same progenitor may have the competence to produce only a restricted subset of those types. The progenitor would gain the competence to produce the other cell types at a later stage.

Competence factor A factor that is dynamically expressed in progenitors, and regulates their multipotency to help define a temporal window. This is a temporal factor, but one that operates similarly in multiple neural lineages.

Fate determinant A gene that promotes the differentiation of a specific cell type. In the developing retina, many fate determinants drive cell cycle exit, or are expressed postmitotically, whereas temporal factors are compatible with the progenitor state.

Genetic fate mapping A strategy for lineage tracing cells that express a given gene. Usually, this is performed by knocking Cre recombinase into the locus of the gene-of-interest.

Multipotent The potential to give rise to multiple cell types.

Neurogenic A progenitor that is committed to produce neurons. Note that "neurogenic" has a different meaning in *Drosophila* neurodevelopment.

Potential The cell types that a given progenitor can produce over the course of development (e.g. if genetically fate-mapped).

Precursor A postmitotic cell that is committed, but has not yet fully differentiated. Note that this definition is specific to the retina development field.

Progenitor A proliferating cell that can give rise to retinal cell types.

Subtype Further subdivisions within the "cell type" framework. Also called "subclasses" in the literature. Subtypes usually differ in gene expression, physiology, and function, but to a lesser degree. Subtypes are often morphologically similar, and typically cannot be distinguished definitively without using marker genes.
Temporal factor A factor that is dynamically expressed in retinal progenitors, and that is necessary to control their output within that expression window.
Temporal window The developmental period in which a given cell type is generated.

Abstract

The functions of the visual system depend on the precise wiring patterns of its constituent circuits. This in turn requires the ordered production of a constellation of different neuronal and glial cell types, each with unique morphologies, projection patterns, and physiology. During retinal development, this cellular diversity must be generated with chronological precision. To do this, retinal progenitor cells alter their behavior over developmental time—producing retinal cell types in a stereotyped sequence. However, the molecular mechanisms that temporally control progenitor competence are not fully understood. Here we review the evidence and concepts that underpin current thinking about retinal progenitor competence, and how this in turn relates to retinal lineage and cell fate determination.

Introduction—*Opening Arguments*

Individual retinal cells were first visualized by Treviranus (Finger, 1994), who proposed that the carpet-like photoreceptor outer segments were "papillae," that were contiguous with the fibers of the optic nerve. However, in defiance of this simple arrangement, subsequent investigators were confronted with a bewildering array of different cell types lying in between the photoreceptive outer segments and the optic nerve. Like other regions of the CNS, we now appreciate that the retina harbors a vast array of specialized cell types and subtypes. The diversity of cellular forms and functions establishes at least 30 different circuits—each tuned to a different visual feature (Baden et al., 2020; Demb and Singer, 2015). Since our visual system therefore depends directly on the production of a multitude of different cell types, there has been intense interest in deciphering the developmental mechanisms and genetic programs that control retinal cell type specification.

In developmental biology, *time* is the central framework upon which every theory and experiment is anchored. Over time, organs develop progressively from fields of relatively simple progenitor cells into complex tissues with differentiated cell types and specialized architecture. However, in the retina—unlike many other tissues, developmental time also plays a central role in changing the behavior of progenitor cells. This review will focus on how time alters the developmental competence of retinal progenitors during neurogenesis.

Establishing the Chronology of the Crime—Birthdates and Clones

Early histological characterizations of retinal development suggested that different retinal cell types were produced in a characteristic sequence, which was generally shared across different vertebrate species. At the gross histological level, the ganglion cell layer was always found to be produced first (Cameron, 1905; Coulombre, 1955; Weysse, 1906). The ontogeny of different cell types could also be guessed at in these early studies. For example, amacrine cells ("inner horizontal cells") appeared before cell types such as bipolars and Müller glia (Cajal, 1972; Cameron, 1905). Cones appeared before rods (Weysse, 1906). While these inferences were largely made using morphological criteria, the advent of "birthdating" techniques allowed cell generation to be precisely resolved. Nucleotide analogs such as tritiated thymidine or bromodeoxyuridine were used to pulse-label proliferating cells during DNA synthesis in S-phase. Additional rounds of DNA replication would progressively dilute the label from continuously proliferating cells, whereas cells that terminally exited the cell cycle at the subsequent mitosis would remain prominently and permanently marked, allowing cell commitment to be timed. In species that developed *in ovo*, labels could be applied chronically, leading to cumulative labeling of all cell types born during specific developmental windows.

Birthdating approaches have revealed that beneath the chaos of retinal neurogenesis lies an evolutionarily conserved and quantitatively robust birth order (Fujita and Horii, 1963; Hollyfield, 1972; Holt et al., 1988; Kahn, 1974; La Vail et al., 1991; Rapaport et al., 2004; Sidman, 1961; Wong and Rapaport, 2009; Young, 1985) (**Fig. 1A**). These data have recently been corroborated by single-cell RNA-sequencing (scRNA-seq) techniques, in which the ordered appearance of different cell types can be reconstructed using transcriptomic signatures (Clark et al., 2019; Cowan et al., 2020; Hoang et al., 2020; Lu et al., 2020). Retinal ganglion cells (RGCs) are always born first, followed by cones and horizontals. Amacrines begin to be generated very early during mouse development (Sidman, 1961; Voinescu et al., 2009), but appear to be born somewhat later during primate retinal development (La Vail et al., 1991; Lu et al., 2020). Cone production is also protracted in primates relative to rodents (La Vail et al., 1991; Rapaport et al., 2004; Young, 1985). The neurogenesis of these "early" cell types is followed by the production of rod photoreceptors, and then by bipolar cells. The differentiation of Müller glia terminates the sequence.

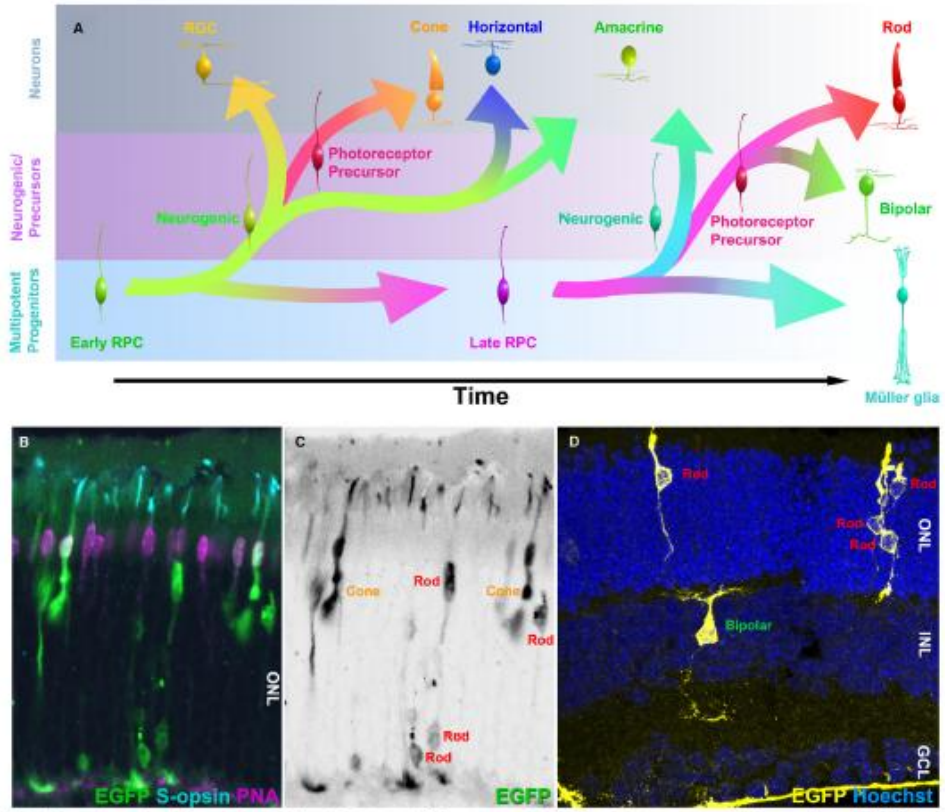

Fig. 1 Lineage dynamics during vertebrate retinal development. (A) In mouse, during embryonic stages, early retinal progenitor cells (RPCs) generate neurogenic progenitors or precursors that are restricted in potential—both in terms of proliferation and multipotency. These committed cells differentiate into retinal ganglion cells (RGCs), horizontal cells, cones, and amacrines. Early-born photoreceptor precursors also generate some rods, although the peak of rod genesis is postnatal. Early RPCs self-renew extensively, which increases the size of the progenitor pool. By perinatal stages, RPCs have undergone a competence transition to become late RPCs. At this point, RGCs, cones, and horizontal cells are no longer produced. RPCs continue to generate amacrine cells, and also generate photoreceptor precursors that can produce rods or bipolar cells. Late RPCs become depleted over time, but RPCs that persist at the end of development differentiate directly into Müller glia. (B, C) Since early RPCs convert into late RPCs, marking early RPCs with EGFP leads to the labeling of "early-born" cones and "late-born" rods. S-opsin and peanut agglutinin (PNA) are cone markers. Murine RPCs were transfected *in utero* at E14.5, and fully developed retinas were harvested at P21. (D) Transducing "late" RPCs using an EGFP-expressing retrovirus exclusively leads to the labeling of late-born cell types. Retroviral clones were generated using murine P0 retinal explant cultures, and harvested after 14 days *in vitro*.

Birthdating techniques constitute a powerful means with which to characterize the population dynamics of cell type development. However, these techniques do not establish lineage relationships, and therefore do not resolve whether cell types are born from common or distinct progenitors. Lineage relationships were elucidated using a variety of methodologies that permitted the behavior of individual progenitor cells to be reconstructed retrospectively. Since lineage tracing involves the marking of progenitors at specific timepoints, these assays share some characteristics with birthdating experiments. However, the exact order and timing of cell birth is usually unresolved since progenitors can continue to proliferate long after being marked (see Fig. 1B–D).

Lineage tracing experiments were initially made possible via the development of techniques that permitted the labeling of individual RPCs. Analogously to the nucleotide analogs used for birthdating, gammaretroviruses only integrate into proliferative cells, when the disassembly of the nuclear envelope permits viral access to the genome. The cell cycle -dependence of retroviral transduction was leveraged to label individual RPCs in an unbiased fashion. Administration of virus at low titre during development led to very sparse labeling of RPCs, which ensured that the resultant lineages would arise clonally from individual RPCs. Fortuitously, the migration of clonally-related cells is generally restricted to the radial axis of the retina, and so marked cells take up their final

positions in columnar units, which greatly facilitates analysis. In mice, administration of retroviruses during embryonic retinogenesis led to the production of highly variable clones that could contain any retinal cell type (Turner et al., 1990). Administration of retroviruses postnatally in rats led to the production of smaller clones that contained only late-born cell types—amacrines, rods, bipolars, and Müller glia (Turner and Cepko, 1987).

Retroviral studies were soon followed by reports that utilized lipophilic dyes to mark cells in zebrafish and *Xenopus* (Holt et al., 1988; Wetts and Fraser, 1988). Later, DNA transfections were also used to mark individual RPCs with reporters—typically GFP (Holt et al., 1990; Wong and Rapaport, 2009). Clones were also obtained through the generation of chimeric animals harboring reporter transgenes. These latter experiments demonstrated that over murine retinal development, the unitary output of each initial RPC is a radial column containing 100–200 cells that can include all 7 cell types (Williams and Goldowitz, 1992). Together, these studies demonstrated that retinal lineages shared common features in vertebrates. Rather than the existence of dedicated progenitors for each cell type as is observed in other regions of the CNS, RPCs were instead multipotent—with individual RPCs capable of giving rise to all of the retinal neuron and glial cell types.

Finally, time-lapse imaging has also made a key contribution to our understanding of retinal lineage dynamics. Both birthdating and clonal analyses are retrospective assays, in which RPC behavior is inferred *post hoc* after development is complete. By directly observing cell production, time-lapse imaging permits the birth order of the lineage to be fully elucidated. Time-lapse microscopy was first used to examine individual rat RPCs using long-term clonal cultures (Gomes et al., 2011). At the population level, individual RPCs in culture produced neurons and glia in similar numbers and proportions to their *in vivo* counterparts (Cayouette et al., 2003; Gomes et al., 2011). However, time-lapse reconstructions revealed that the birth order of neurons and glia was not fixed within individual clones. Individual RPCs could produce cell types out of the canonical order, and different cell types could be produced from a single mitotic division (Gomes et al., 2011). Time-lapse studies have also been performed *in vivo* using zebrafish (Almeida et al., 2014; He et al., 2012). Zebrafish are particularly amenable to long-term time-lapse imaging, given the transparent nature of zebrafish larvae in concert with the very rapid timecourse of retinal neurogenesis. Time-lapse imaging of zebrafish retinogenesis revealed that individual RPCs *in vivo* behaved similarly to rat RPCs *ex vivo*, exhibiting a great deal of stochasticity, and a lack of fixed birth order within clones (Almeida et al., 2014; He et al., 2012).

The DNA Evidence—Genetic Control of Retinal Lineage

Lineage tracing, birthdating, and time-lapse imaging have collectively revealed fundamental principles that govern retinal development. (i) As a population, RPCs generate cell types in a characteristic birth order. (ii) Individual RPCs are multipotent—capable of generating all of the retinal cell types. Taking stock of these landmark advances, Takashi Watanabe and Martin Raff wrote, "The challenge now is to determine how individual multipotential precursor cells decide what type of differentiated retinal cell to become" (Watanabe and Raff, 1992). Despite many breathtaking advances, in many ways, this question remains just as relevant now as it did 30 years ago.

And yet, perhaps this is the wrong question. The extensive stochasticity exhibited by RPCs suggests that they may not "decide." Yet, if RPC fate decisions are not preprogrammed, how then would cell types be produced in the correct times and ratios? It is important to point out that stochasticity does not imply that events are completely uncontrolled. The 7 canonical cell types produced by RPCs are not generated at random with equal probability. RPCs are biased in their rates of proliferation, self-renewal, differentiation, and cell type production, and these biases are regulated by developmental mechanisms (Gomes et al., 2011; He et al., 2012). Viewed this way, the important question is not "how does the RPC decide?," but rather, "what are the mechanisms that control RPC biases?."

An alternative idea is that the different clone structures represent the jumbled output of an ensemble of different RPC subtypes (Cepko, 2014). In such a scenario, stochasticity would be an illusion. Rather than one RPC with varied behavior, the retina would instead be generated by a large variety of different RPC types—each programmed to produce a specific type of clone. This idea could be verified by several types of data. First, transcriptional profiling could help to identify RPC subtypes, under the assumption that they would vary in gene expression signatures. However, while evidence for variability in RPC gene expression has been observed (Blackshaw et al., 2004; Jasoni and Reh, 1996; Trimarchi et al., 2008), scRNA-seq studies have thus far not revealed discrete RPC subtypes with specific marker genes and developmental potential (Clark et al., 2019; Cowan et al., 2020; Hoang et al., 2020; Lu et al., 2020).

Second, genetic fate mapping could allow RPC subtypes to be marked based on differences in their gene expression profiles. To date, one study has indeed revealed the existence of a highly proliferative multipotent RPC that is nonetheless determined to generate a specific RGC subtype. The Sanes laboratory performed genetic lineage tracing by knocking Cre recombinase into the *Cdh6* locus (De la Huerta et al., 2012). *Cdh6* is an adhesion protein that functions as a recognition molecule to help regulate synapse assembly. It is specifically expressed in a specific subtype of (ON-OFF) direction sensitive RGCs. However, genetic fate mapping revealed that *Cdh6* was additionally expressed in the upstream RPCs. Cdh6-expressing RPCs selectively generated direction sensitive RGCs, with little-to-no production of other RGC subtypes. However, with respect to non-RGCs, *Cdh6* -expressing progenitors produced other neuron subtypes seemingly at random, and clones were highly variable. *Cdh6* -expressing RPCs are therefore deterministic with respect to RGCs, but otherwise appear not to be preprogrammed. The identification of an RPC *subtype* that nonetheless exhibits extensive stochasticity strongly argues against the existence of an ensemble of different RPCs with fixed behavior.

In accordance with the idea that deterministic rules may govern particular fate decisions, genetic fate mapping experiments have hinted at the existence of additional deterministic lineage branches. In the simplest case, genes have been identified whose

expression determines cell type identity. For example, the transcription factor *Nrl* determines rod photoreceptor identity (Mears et al., 2001). Accordingly, *Nrl* expression is restricted to postmitotic cells fated to differentiate into rods (Adnani et al., 2018; Phillips et al., 2018). Another example is the Ptf1a transcription factor, which marks postmitotic cells that give rise to horizontals or amacrines (Fujitani et al., 2006; Nakhai et al., 2007). Ptf1a is not expressed in any other cell types. Moreover, *Ptf1a* mutation leads to the loss of amacrines and horizontals, and the concomitant gain of RGCs. While such postmitotic cell fate determinants are of critical importance for retinal development, we will here focus our discussion on determinants of lineage structure that operate in dividing cells. The reader is directed to several other excellent reviews that cover retinal cell fate determinants (Bassett and Wallace, 2012; Cepko, 2014; Zhang et al., 2023b) (but also see subtype-specification below).

While Nrl and Ptf1a are expressed exclusively in postmitotic cells, fate mapping has also suggested the existence of proliferative cells that appear to be governed by deterministic rules. For example, the Hattar lab knocked Cre recombinase into the *Opn4* locus in order to gain genetic access to intrinsically photosensitive (ip) RGC subtypes. When crossed to recombination reporter mice, ipRGCs were labeled as expected. However, rods and cones were additionally marked by the strategy (Ecker et al., 2010). Since rods and cones do not express *Opn4*, *Opn4* is likely expressed in progenitors/precursors—just prior to differentiation—perhaps analogously to *Cdh6* in the direction sensitive RGC lineage. If so, this would suggest the existence of an *Opn4* -expressing progenitor with the potential to make only rods, cones, and ipRGCs.

In another example, a combination of methods was used in order to lineage trace *Olig2* -expressing cells in the mouse retina (Hafler et al., 2012). During embryonic stages, *Olig2* -expressing cells divided terminally and mainly made cone photoreceptors and horizontals. Postnatally, *Olig2* -expressing progenitors mainly generated rod photoreceptors and some amacrines. Similarly, *Atoh7*+ expressing cells in zebrafish were recently shown to divide to generate a pair of progenitors that each divide terminally. One of these progenitors always produced two photoreceptors, although it remains unclear whether these photoreceptors were rods or cones (Nerli et al., 2023).

In the above cases, the relatively small clone size and/or number of cells marked by the lineage tracing strategies suggests that *Olig2*/*Opn4*/*Atoh7* -expressing cells may be fate-restricted (but nonetheless multipotent) progenitors that have very limited proliferative potential. These studies therefore illustrate deterministic rules that regulate specific modes of neuronal differentiation. However, it remains unclear whether all cells belonging to a given cell type are produced by these deterministic progenitors *or only a subset*. For example, Olig2+ or Atoh7+ progenitors can both produce cones (Brzezinski et al., 2012; Emerson et al., 2013; Ghinia Tegla et al., 2020; Hafler et al., 2012; Inoue et al., 2002; Yang et al., 2003), but it remains unclear whether Olig2 and Atoh7 are co-expressed or expressed sequentially, or alternatively, whether Olig2+, and Atoh7+ progenitors are on completely separate lineage branches—each of which can independently produce cones.

Genetic fate mapping has also pointed to the existence of neurogenic progenitors with more stochastic behavior. Genetic marking of cells expressing *Neurog2*, *Otx2*, or *Ascl1*, suggests that these cells exhibit reduced proliferative potential in comparison to retroviral clones that sample RPCs at random. Marked progenitors were capable of producing multiple cell types (Brzezinski et al., 2011; Emerson and Cepko, 2011; Emerson et al., 2013; Ghinia Tegla et al., 2020; Ma and Wang, 2006), although in each case, there was a bias in the cell types generated. For example, Ascl1 -expressing progenitors marked at early stages produced all cell types, except that they generated few RGCs. By contrast, Atoh7 or Neurog2 -expressing progenitors produced few bipolars and Müller glia (Brzezinski et al., 2011, 2012; Yang et al., 2003; Poggi et al., 2005). Again, the precise hierarchy of these determinants is not entirely clear. Ascl1 and Neurog2 have been shown to be cross-repressive in some contexts (Akagi et al., 2004; Fode et al., 2000), but these bHLH factors were found to be co-expressed or even interdependent in other experiments (Brzezinski et al., 2011; Han et al., 2021; Hufnagel et al., 2010; Nelson et al., 2009).

In addition to lineage tracing and fate mapping experiments, genetic loss-of-function experiments have also provided insight into RPC multipotency. In particular, the mutation of specific cell fate determinants showed repeatedly that the loss of one cell type could be counterbalanced by the gain of another. For example, mutations were identified in which RGCs were gained at the expense of amacrines (Inoue et al., 2002), RGCs and amacrines gained at the expense of rod photoreceptors (Ghinia Tegla et al., 2020), Müller glia gained at the expense of RGCs (Le et al., 2006), bipolars gained at the expense of rods (Brzezinski et al., 2010; Katoh et al., 2010), cones gained at the expense of rods (Haider et al., 2000; Mears et al., 2001; Milam et al., 2002), rods gained at the expense of cones (Emerson et al., 2013; Javed et al., 2020; Sapkota et al., 2014), and Müller glia gained at the expense of rods (Mattar et al., 2015, 2021) or bipolars (Livne-Bar et al., 2006; Tomita et al., 1996).

These phenotypes have often been interpreted to illustrate binary fate decisions that operate *naturally* in retinal lineages during development. While such interpretations are likely to be correct in many instances, it is important to emphasize that these outcomes do not necessarily mean that *all* cells of a given type are generated through a specific binary fate choice. For example, a mutation might affect a cone vs. rod fate choice but leave unaffected an alternative lineage branch harboring a binary fate choice between cones and horizontals. Again, there might be more than one way to make each cell type. Moreover, observed imbalances might represent *mutant* lineages that may not necessarily correspond with the wild-type situation. In lineage tracing experiments, the observance of two-cell clones reflects the binary output of terminal divisions. In the natural wild-type condition, cell type pairs such as rod/bipolar, rod/amacrine, rod/Müller glia are commonly observed (Turner and Cepko, 1987). By contrast, two cell clones containing both "early" and "late" cell types—such as cones with bipolars—are much rarer (Adnani et al., 2018; He et al., 2012; Kechad et al., 2012; Turner et al., 1990; Wong and Rapaport, 2009), suggesting that they likely do not normally arise from a bipotent precursor, or that such precursors produce only a small minority of each cell type.

Offsetting effects on cell fate may also occur as a result of alterations in multipotent RPCs rather than due to changes in bipotent precursor cells. For example, we now understand that Müller glia arise directly from RPCs rather than through neurogenic

progenitors that might simultaneously generate neurons in a terminal division (Clark et al., 2019; Lu et al., 2020; Zhang et al., 2023b). Thus, genetic mutations that disrupt neuronal determination might lead to overproduction of Müller glia. Indeed, this explanation was previously suggested to account for the striking observation that in *Atoh7* mutant mice, cells birthdated at early embryonic stages fail to produce RGCs and instead produce Müllers (Le et al., 2006)—which would not ordinarily be produced during early stages of development.

In summary, genetic fate mapping experiments have been crucial for revealing the existence of progenitor subtypes. Committed progenitors often appear to obey deterministic rules, which would govern the terminal branches of retinal lineages. However, the central structure of the lineage tree—which would tend arise through the more proliferative behavior of multipotent RPCs, appears to be largely stochastic. Although RPCs do not appear to exhibit preprogrammed fate decisions with respect to characteristics such as proliferation, self-renewal, and differentiation, these behaviors are nonetheless still regulated—but by what mechanisms?

The Suspect Acted Alone—Evidence for Intrinsic Controls

In the retinas of bird, fish, and frog species, the retina develops very rapidly. At the population-level, different cell types exhibit considerable temporal overlap in their production windows (Belecky-Adams et al., 1996; Fujita and Horii, 1963; Hollyfield, 1972; Holt et al., 1988; Jusuf et al., 2011; Kahn, 1974). For example, whereas cones are exclusively born during embryonic phases of neurogenesis in rodents, a considerable proportion of cones were born during later phases of retinal neurogenesis in *Xenopus* (Wong and Rapaport, 2009). Similarly, birthdating the middle phases of *Xenopus* neurogenesis (stages 32–33) led to the labeling of all cell types, although only a few RGCs were labeled in accordance with their earlier production window. However, within individual clones, RPCs generally produced cell types in the same sequences found in mammalian species (He et al., 2012; Wong and Rapaport, 2009). These data suggest that RPCs with early temporal identity might be found side-by-side with progenitors harboring later identity in these species. If so, the asynchrony in the temporal state of the progenitor pool might suggest a lack of environmental coordination.

In accordance with this postulate, a variety of lines of research have strongly suggested that the temporal transitions exhibited by RPCs are intrinsically programmed, rather than being controlled by extrinsic environmental signals. Heterochronic transplantation experiments were performed in *Xenopus*, in which early-stage (22–23) dorsal retinas were transplanted into later-staged (27–28) hosts (Holt, 1984). The observed delay in RGC axon outgrowth more-or-less matched the original schedule of the grafted tissue—suggesting that the temporal state of grafted RPCs was not altered by the temporally inappropriate *in vivo* environment. Moreover, culturing retinas *ex vivo* had little impact on the timing of cell production, although proliferation was reduced (Austin et al., 1995; Belecky-Adams et al., 1996; Caffe et al., 1989; Ezzeddine et al., 1997; Ha et al., 2017; Sparrow et al., 1990; Spence and Robson, 1989). Next, retinal RPCs were dissociated and cultured, or mixed with early- or late-born cells in various ratios (Adler and Hatlee, 1989; Altshuler and Cepko, 1992; Austin et al., 1995; Lillien and Cepko, 1992; Reh, 1992; Reh and Kljavin, 1989; Watanabe and Raff, 1990, 1992). When RPCs were exposed to different cell mixtures, they did not convert from late-to-early or early-to-late competence in response to environmental cues. For example, heterochronic mixtures led to the overproduction of early-born cones at the expense of early-born amacrines (Belliveau and Cepko, 1999), or increased late-born bipolars at the expense of late-born rods (Belliveau et al., 2000). Finally, RPCs were cultured individually in low-density conditions, allowing their unitary output to be studied under constant environmental conditions. Again, these cultures revealed that isolated RPCs behaved very similarly to their *in vivo* counterparts (Cayouette et al., 2003; Gomes et al., 2011), suggesting that the temporal competence states of RPCs are intrinsically controlled.

Taken together with the results of birthdating and lineage experiments, the results of heterochronic and clonal cultures led to the proposal of the competence model (Livesey and Cepko, 2001), in which RPCs undergo transitions in their developmental potential in a cell-intrinsic manner. At the beginning of development, an RPC would initially gain the competence to generate a set of cell types. A shift in competence would cause this developmental potential to be lost, while the ability to generate later cell types would be gained. Changes in temporal competence would thereby create developmental windows for the production of each cell type.

It is important to recognize that while the evidence strongly suggests that temporal competence is controlled via cell-intrinsic mechanisms, extrinsic signals nonetheless have profound effects on RPC behavior (Bassett and Wallace, 2012; Zhang et al., 2023b). Obviously, many growth factors are needed in order to sustain RPCs. Several experiments have additionally shown that the competence state of RPCs can be altered through extrinsic signals (Close et al., 2005; Kim et al., 2005; Wang et al., 2005). However, in each case, these signals are thought to function as feedback modifiers. In other words, the known examples are based on quorum sensing. When a given cell type is produced in sufficient numbers, it secretes a factor that limits further production of that cell type. Such mechanisms can restrict cell type production, but have not thus far been shown to be *necessary* to induce the production of later cell types.

Interestingly, in other regions of the CNS, extrinsic signals appear paramount. Initial experiments in the neocortex indicated that like RPCs, late-stage cortical progenitors were not temporally re-specified when engrafted into early environments (Desai and McConnell, 2000; McConnell and Kaznowski, 1991). However, these experiments marked proliferating cells indiscriminately with nucleotide analogs. The initial results therefore did not take into account the diversification of the progenitor pool. In the cortex, intermediate (or basal) progenitors with more limited proliferative potential increase in number between early and late stages. This leads to an "apples-to-oranges" comparison, where "late" progenitor preparations contained increased ratios of more committed progenitors. When this change in the progenitor pool was accounted for, it was observed that apical progenitors (radial glia) *could* alter their competence to match heterochronic environments—either early or late (Oberst et al., 2019).

A similar principle could conceivably have masked extrinsic RPC respecification in previous studies, since retinal neurogenic progenitors vary over developmental time—both in numbers and in molecular identity. In any case, it seems clear that extrinsic mechanisms—whether permissive or instructive, must ultimately rely on intrinsic factors for their effects to be transformed into stable changes in cellular behavior (Li et al., 2022).

Reconstructing the Crime Scene—Proposed Mechanisms for Temporal Competence

What is the molecular nature of competence, and how are temporal transitions achieved? A number of hypotheses and observations have informed our understanding of the potential mechanisms. Here we review various concepts and mechanisms that have been proposed to explain RPC competence. It should be emphasized that the mechanisms proposed below are not mutually exclusive. Retinal development likely depends on all of these mechanisms.

Combinatorial Coding

Throughout the CNS, progenitors are initially patterned based on their position within the developing neural tube under the influence of morphogen gradients. In the ventral spinal cord, different transcription factors were induced or repressed based on a given progenitor's distance from signaling centers. Combinations of transcription factors were found to be necessary and sufficient to impart competence for specific neuronal or glial types (Osseward and Pfaff, 2019; Shirasaki and Pfaff, 2002). These factors tended to be either homeodomain or basic helix-loop-helix (bHLH) transcription factors. Many of the same factors were found to be expressed during retinal development, including Ascl1, Pax6, Neurog2, Sox2, and others. This led to the hypothesis that homeodomain and bHLH transcription factors would be necessary and sufficient for retinal cell fate specification. The temporal control of retinal cell type production would arise from the ordered deployment of these factors over time (Fig. 2A).

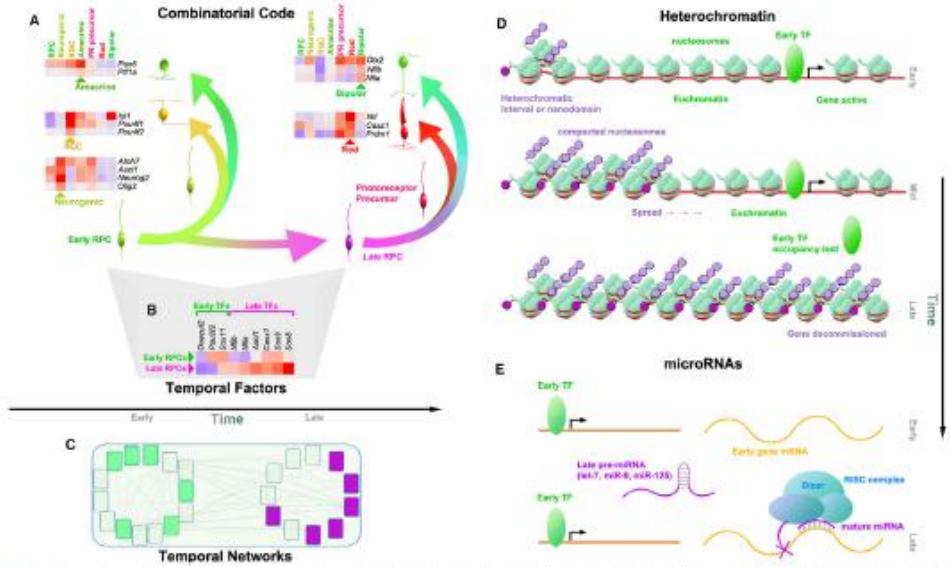

Fig. 2 Proposed mechanisms underlying competence. (A) Cell fates are determined by a transcription factor code. Temporal development can be achieved by the ordered deployment of these codes. However, most retinal cell fate determinants described thus far are expressed only in committed progenitors/precursors. This suggests that the temporal deployment of cell fate determinants must be regulated by upstream factors operating in the RPCs. (B) Temporal factors generate transcription factor codes in RPCs, which change dynamically over developmental time. Heatmaps in (A, B) were generated from murine scRNA-seq data using *Ptae* (Swamy et al., 2021). (C) Temporal development is controlled by networks of transcription factors—which include some or all of the temporal factors shown in (B). Network properties control the competence state of the RPC. (D) Heterochromatin spreading is stochastic, time-dependent, and heritable in daughter cells. Heterochromatin can spread across euchromatic intervals through the actions of Polycomb repressor complexes. In this way, genes can be progressively decommissioned. Progressive restriction of the genome could temporally gate the output of temporal factors or fate determinants, and thereby alter progenitor behavior. (E) MicroRNAs similarly gate the transcriptome of the cell by controlling which mRNAs can be expressed. Dynamic expression of microRNAs can regulate temporal development by controlling the expression of temporal factors or fate determinants (not depicted), or by interfering with the translation of their target effector genes (depicted).

However, this model proved inadequate to explain retinal development. In RPCs, homeodomain transcription factors were often expressed at constant levels throughout development. While bHLH transcription factors like Atoh7 and Ascl1 proved to be more dynamic, they tended not to be sufficient to produce their cognate cell types. For example, overexpression of Atoh7 was sufficient to increase RGC production during the normal (early) temporal window for RGC production in *Xenopus* (Kanekar et al., 1997). However, overexpression of Atoh7 at later stages of frog eye development had no effect on RGC production (Moore et al., 2002). In another example, the cell types induced by Neurod1 were found to shift depending on the stage of misexpression (Morrow et al., 1999). In another study, a variety of factors associated with Müller glia development were misexpressed. Despite the fact that the experiments were performed within the normal birth window for Müller glia, overexpression of the glial fate determinants Nfia, Plagl1, Rax, Sox2, Sox8, or Sox9 could not elevate Müller production (de Melo et al., 2016a). In the most dramatic example, 288 combinations of bHLH and homeodomain transcription factors were systematically misexpressed in RPCs in *Xenopus*, but with little additive effect (Wang and Harris, 2005). Collectively, these experiments illustrate that the "usual suspect" fate determinants were insufficient to specify fates outside of their natural temporal windows. Other factors have been identified that are sufficient to specify particular cell types, including Ptf1a and Prox1. However, these factors are in most cases restricted to precursor cells, and are not expressed in RPCs.

Temporal Factors

While transcription factors that are necessary for retinal cell fate specification were repeatedly observed not to be sufficient, advances in *Drosophila* pointed to another possible explanation. In parallel to "spatial" cell fate determinants, a second group of transcription factors diversified progenitor gene expression over developmental time. Cascades of transcription factors were identified that changed sequentially with each cell division, allowing competence to be altered in a stepwise fashion (Kohwi and Doe, 2013; Li et al., 2013). In fly neuroblasts, temporal factors are thought to act as a generic molecular clock, because the same sequences of transcription factors are utilized by different neuroblasts to produce markedly different cell types and lineages. Fly temporal factors therefore establish competence windows, rather than directly specifying particular cell fates.

In vertebrates, a number of genes have been shown to alter the production of cell types in a manner that is analogous to the fly temporal cascades (Fig. 2B). The criteria for a *bona fide* temporal factor is as follows. (i) The factor must be expressed in RPCs in a dynamic fashion. (ii) The factor must be necessary to promote one or more fates within its expression window, and/or sufficient to suppress fates from outside of its window. Phenotypes should be "early at the expense of late," or "late at the expense of early." Importantly, differences in proliferation/differentiation that expand or prematurely deplete RPCs should not explain these outcomes. For example, Prox1 is necessary for the production of early born cell types, including horizontal cells (Dyer et al., 2003). Late born cell types are overproduced in *Prox1* mutants. However, neuronal differentiation is reduced at early stages, potentially leading to a buildup of progenitors, which likely go on to overproduce late-born cell types at later stages (Dyer et al., 2003). Cross-regulatory interactions between temporal factors might additionally be expected based on the transcription factor cascades that have been described in *Drosophila* (Kohwi and Doe, 2013; Konstantinides et al., 2022; Li et al., 2013). Finally, as per *Drosophila* neuroblasts, one might expect temporal factors to function similarly outside of the retinal context to provide generic temporal identities in multiple regions of the CNS. Below, we review transcription factors that have been shown to meet some of these criteria. We apologize to authors whose work was not cited due to space constraints.

Early Factors

Atoh7 and Prox1. As described above, the bHLH transcription factor Atoh7 is mainly expressed in neurogenic progenitors with limited proliferative potential. Atoh7 also has a very dynamic expression pattern that is restricted to early developmental stages (Brown et al., 1998). Atoh7 expressing progenitors can give rise to most neuronal cell types, but glia and bipolars are largely outside of the Atoh7 lineage (Brzezinski et al., 2012; Nerli et al., 2023; Poggi et al., 2005; Yang et al., 2003). In general, the *Atoh7* mutant phenotype exhibits an "early-to-early" fate switch, with RGCs dramatically lost, and different early cell types concomitantly increased in different species. However, increases in late cell types are also observed in mutants (Brown et al., 2001; Brzezinski et al., 2012; Kay et al., 2001; Wang et al., 2001). Nonetheless, Atoh7 is not sufficient to induce RGCs outside of its normal developmental context (Moore et al., 2002; Prasov and Glaser, 2012).

As mentioned above, like Atoh7, Prox1 is likewise expressed at early stages in progenitor cells at the point of cycle exit (Dyer et al., 2003). Prox1 is required for early born cell types—particularly horizontals, which are completely lost in mutants. Late cell types are overproduced in mutants—possibly as a byproduct of progenitor amplification. However, Prox1 is also sufficient to induce horizontals when misexpressed during late stages of development (Dyer et al., 2003). Thus, Atoh7 and Prox1 are both important for early fates, but likely act within more committed branches of the RPC lineage.

Foxn4. Foxn4 is a winged-helix/forkhead transcription factor that is expressed in multipotent RPCs. The production of horizontal and amacrine cells is drastically reduced in *Foxn4* mutants (Li et al., 2004). While these early-born cell types are lost, some early-born cell types are increased, including both RGCs and cones. However, later-born rod photoreceptors are also overproduced, suggesting a possible temporal shift (Li et al., 2004). Accordingly, Foxn4 has been shown to lie upstream of multiple early fate determinants, including Neurod1, Neurod6, Prox1, Tfap2a, and Tfap2b—all of which are important for amacrine and horizontal cell specification and differentiation (Fujitani et al., 2006; Jin et al., 2015; Li et al., 2004). Foxn4 was also shown to cooperate with Rorβ1 to upregulate Ptf1a (Liu et al., 2013). Foxn4 was also shown to repress the early temporal factor Ikzf1, and to upregulate the late temporal factor Casz1 (Liu et al., 2020) (see below). Heterochronic misexpression of Foxn4 induces amacrines at the

expense of rods, bipolars and Müller glia (Li et al., 2004). Despite its clear connection to early RPC competence, *Foxn4* expression persists into later stages of development—perhaps matching the broad temporal window for amacrine production. Accordingly, its expression in RPCs downregulates prior to the latest stages of neurogenesis (Gouge et al., 2001; Li et al., 2004; Shiau et al., 2021).

Foxp1. A connection between Foxp1 and competence was first made in the developing neocortex, where Foxp1 was found to promote progenitor self-renewal and to prolong the production of early-fate cells (Pearson et al., 2020). In the retina, the Vetter and Watanabe laboratories showed that Foxp1 was initially expressed in RPCs, but that its levels rapidly declined over developmental time (Suzuki-Kerr et al., 2017; Zhang et al., 2023a). *Foxp1* mutants exhibited reduced numbers of early-fate cells, including RGCs, horizontals, and cones (Zhang et al., 2023a). Gain-of-function experiments expanded these early cell types, and reduced the numbers of late-born rods and Müller glia (Suzuki-Kerr et al., 2017; Zhang et al., 2023a). Foxp1 overexpression also promoted cell cycle exit, potentially explaining these effects. However, the RGC production window also appeared to be prolonged (Zhang et al., 2023a). Accordingly, RPC gene expression was shown to be temporally shifted by Foxp1 manipulations. In RPCs, *Foxp1* loss-of-function led to transcriptional upregulation of "late" temporal factor genes, including *Casz1*, *Nfib*, and *Sox9* (see below), whereas overexpression led to their downregulation. Because Foxp1 works similarly in two different contexts (cortex and retina), it likely meets the criteria for a *bona fide* early competence determinant.

Ikzf1. *Ikzf1* is a zinc finger transcription factor that is homologous to *Drosophila hunchback*. In fly neuroblasts, *hunchback* specifies the earliest temporal state (Isshiki et al., 2001; Kambadur et al., 1998). Hypothesizing that such activities might be conserved in vertebrates, the Cayouette lab initially focused on *Ikzf1* (Elliott et al., 2008). Ikzf1 was found to mark early RPCs, but was not detected in late-stage RPCs. Accordingly, genetic fate mapping marked all cell types found in the retina, suggesting that the gene is actively expressed at the onset of retinogenesis (Tarchini et al., 2012). Ikzf1 was also expressed in all early-born retinal neurons except for cone photoreceptors. Next, *Ikzf1* knockouts were examined (Elliott et al., 2008). Early-born cell types were found to be underproduced, although there was no effect on cone photoreceptors. Late-born cell types were unaffected. Gain-of-function experiments were performed by administering Ikzf1-expressing retroviruses during late stages of neurogenesis. Remarkably, Ikzf1 was sufficient to induce the production of early-born horizontals, and amacrine cell production was also increased. These changes came at the expense of late-born bipolars and Müller glia. RGCs were not observed *in vivo*, but were produced when gain-of-function was performed in culture, perhaps indicating that heterochronic RGC production was blocked *in vivo* by feedback mechanisms. In both loss- and gain-of-function experiments, cell cycle was not affected, suggesting that shifts in cell birthdates or proliferation kinetics cannot explain the observed phenotypic effects. Moreover, gain-of-function experiments yielded similar effects whether Ikzf1 was misexpressed via single-copy retrovirus, or via high-copy plasmid electroporation (Elliott et al., 2008; Mattar et al., 2015). Finally, Ikzf1 was found to yield analogous effects when misexpressed in the developing neocortex (Alsio et al., 2013). Like Foxp1, Ikzf1 thus meets the criteria for an early competence factor.

In search of an explanation for why rod and cone photoreceptors were unaffected by Ikzf1 manipulations, Javed et al. additionally examined a second *hunchback* ortholog, namely *Ikzf4*. Ikzf4 was found to be expressed in RPCs throughout retinal development, suggesting that it is not a temporal factor (Javed et al., 2023). Nonetheless, Ikzf4 appears to affect cell fate specification at both early and late phases of development. *Ikzf1*, *Ikzf4* double-mutants exhibited reductions in cone photoreceptors, suggesting that *Ikzf1* and *Ikzf4* might act redundantly. Perhaps accordingly, Ikzf4 was shown to bind and directly regulate several cone fate determination genes, including *Pou2f2* (see below). However, Ikzf4 was also found to be necessary and sufficient for Müller glia production. These activities perhaps illustrate that Ikzf paralogs may act combinatorially or redundantly to affect early and/or late fate decisions.

Onecut1/2, Pou2f1/2, Sox4/11. Onecut1, Onecut2, Pou2f1, and Pou2f2 are expressed in neural progenitors during early developmental stages throughout the CNS (Sagner et al., 2021). All four of these transcription factors are expressed in RPCs during early phases of development, but are downregulated during later phases (Emerson et al., 2013; Goetz et al., 2014; Javed et al., 2020; Shiau et al., 2021; Wu et al., 2012). Onecut and Pou factors are also expressed in postmitotic precursors and in early-born neurons. *Onecut1* and *Onecut2* single mutants both exhibit marked reductions in the numbers of horizontal cells generated during development. Onecut1/2 double mutants had defects in the production of all early-born cell types, including cones, RGCs, and early-born amacrines (Sapkota et al., 2014). Remarkably, late-born cell types were little affected. Like *Onecut* mutants, *Pou2f2* conditional mutants also exhibit reductions in cones and horizontals (Javed et al., 2020).

Interestingly, overexpression of *Onecut1* in the postnatal retina was sufficient to extend the developmental window for horizontal and cone genesis (Emerson et al., 2013; Wu et al., 2013), suggesting that it can act as an early competence determinant. Examination of single and double mutants suggests that Onecut factors act in parallel with Ptf1a and/or Prox1 to specify horizontal cell fates (Klimova et al., 2015; Sapkota et al., 2014). Heterochronic misexpression of Pou2f1 and Pou2f2 in late RPCs similarly induced cone production, and Pou2f2 additionally induced some horizontals (Javed et al., 2020). With respect to cone production, Onecut1 cooperated with Otx2 to induce the expression of the cone determinant *Thrb* (Emerson et al., 2013), whereas Pou2f2 acted by suppressing the rod determination gene *Nrl* (Javed et al., 2020). In all of the above cases, the data are consistent with these factors regulating cell fate in postmitotic precursors or committed neurogenic progenitors. However, since Onecut1/2 and Pou2f1/2 are also dynamically expressed in RPCs, their effects on competence might instead map to multipotent RPCs. Indeed, Javed et al. additionally showed that Pou2f1 was also induced by Ikzf1, but acted to suppress the late temporal factor Casz1.

Sox4/11 are SRY box transcription factors. Like Onecut and Pou factors, Sox4/11 have been shown to act as early temporal factors in other CNS contexts (Bergsland et al., 2006; Li et al., 2012; Shim et al., 2012; Sagner et al., 2021). In the retina, Sox4 and Sox11 were both expressed in early RPCs and early-born postmitotic cell types (Usui et al., 2013). Gain-of-function experiments in mouse retinas revealed a role for Sox11 in promoting cone photoreceptors at the expense of late cell types, including bipolars and Müllers. However, *Sox11* knockouts did not exhibit defects in late-born cell types. Moreover, cone production was delayed in *Sox11*

knockouts but not abolished, and Sox11 misexpression did not extend cone production into the postnatal period. These data leave open the possibility that Sox11 might again regulate cone specification in postmitotic precursors rather than in progenitors. In summary, Onecut1/2, Pou2f1/2, and Sox4/11 are candidate temporal factors, but they might also/instead regulate cell fate in committed precursors rather than in RPCs. Alternatively, they may act in RPCs, but their regulatory functions might be inherited and executed in precursors.

Late Factors

Ascl1. As discussed above, genetic fate mapping has suggested that *Ascl1* is expressed in multipotent progenitors that nevertheless exhibit somewhat restricted proliferative potential (Brzezinski et al., 2011). *Ascl1* -expressing progenitors exhibit a greatly diminished competence to generate RGCs—even during early stages of development. However, *Ascl1* mutants do not exhibit corresponding increases in RGC numbers, perhaps suggesting that Ascl1 marks a lineage branch point between RPCs and more committed progenitors that bypasses RGC production. Perhaps accordingly, RGCs are enriched within lineages that express the bHLH transcription factors Neurog2 and Atoh7. Although *Ascl1* is not required to restrict RGC production, *Ascl1* mutants overproduce Müller glia at the expense of bipolars (Tomita et al., 1996, 2000).

What happens when Ascl1 is heterochronically misexpressed during earlier phases of neurogenesis? Knocking *Ascl1* into the *Atoh7* locus did not alter the generation or timing of late cell types, including bipolars and Müllers (Hufnagel et al., 2013). Early-fate RGC production was both delayed and reduced. Knock-in retinas exhibited increased proliferation and apoptosis, which might partially explain the observed reductions in RGCs. However, other "early" cell types—like horizontals and cones, were actually increased in numbers when the knock-in allele was bred to homozygosity. Ascl1 therefore seems to oppose RGC production. What happens when Ascl1 is misexpressed during the latest temporal state—namely, in differentiated Müller glia? Indeed, this scenario actually occurs during retinal regeneration in species such as the chick and zebrafish (Fausett et al., 2008; Fischer and Reh, 2001; Yurco and Cameron, 2007), but not in mammals (Karl et al., 2008). In landmark work, the Reh laboratory generated transgenic mice that permitted Ascl1 to be induced in Müllers artificially. Remarkably, Müllers could be induced to re-enter neurogenesis when Ascl1 was induced at the end of development (Pollak et al., 2013; Ueki et al., 2015). In adult Müllers, Ascl1 did not promote neurogenesis in the absence of damage (Ueki et al., 2015), however, the inclusion of a histone deacetylase inhibitor allowed Ascl1 to function in mature Müllers (Jorstad et al., 2017), suggesting that adult glia have an additional epigenetic barrier that impedes neurogenic competence. Resultant neurons were often bipolar-like, perhaps suggesting that Ascl1 ties a late temporal identity to neurogenic competence.

Casz1. *Casz1* is homologous to *Drosophila castor*—a temporal competence factor in fly neuroblasts (Cui and Doe, 1992; Grosskortenhaus et al., 2006; Kambadur et al., 1998; Mellerick et al., 1992; Vacalla and Theil, 2002). *Casz1* and *castor* genes share unique zinc finger motifs that are not found in other transcription factors (Mattar et al., 2015). In RPCs, Casz1 is dynamically expressed during murine retinal development. Expression levels in RPCs are initially low, and then increase—peaking at birth, and then declining again at late stages of development (Blackshaw et al., 2004; Mattar et al., 2015, 2021). In the adult retina, Casz1 is also prominently expressed in rod and cone photoreceptors as well as GABAergic amacrines. *Casz1* conditional knockouts exhibited a reduction in rod photoreceptors, with early-born cell types proportionately increased. Müller glia were also increased. Heterochronic misexpression in early RPCs led to the opposite effect, with rod photoreceptors promoted at the expense of early cell fates and Müller glia. Similar phenotypes were obtained whether gain-of-function was performed via plasmid electroporations that drive high level expression, or via single-copy retroviral transduction, which has a much lower and more physiological expression level (Mattar et al., 2015, 2021). Loss- and gain-of-function experiments revealed no effect on RPC proliferation, indicating that Casz1 expression is compatible with progenitor identity and does not drive cell cycle exit or proliferation. These data are consistent with Casz1 acting to enhance rod photoreceptor production, and to suppress the earliest and latest temporal states.

Lhx2. Lhx2 is an eyefield transcription factor that is required for eye growth and development (Porter et al., 1997). Lhx2 is expressed continuously in RPCs throughout retinal development (de Melo et al., 2016b). Lhx2 is therefore not an obvious candidate for a typical temporal factor based on the criteria listed above. Accordingly, Lhx2 has been shown to affect both early and late fate decisions, and regulates different target genes at different developmental stages (de Melo et al., 2016b; Gordon et al., 2013; Zibetti et al., 2019). However, ablation of *Lhx2* or its cofactors *Ldb1/2* strikingly arrests the progression of RPC competence (Gordon et al., 2013; Gueta et al., 2016). In *Lhx2* conditional mutants, RGCs were overproduced continually through to the end of the normal production window. Lhx2 thus plays an important role in the temporal progression of competence. Lhx2 and its cofactors were also shown to be required for RPCs to receive extrinsic signals, including feedback signals from the sonic hedgehog pathway (Gueta et al., 2016; Li et al., 2022). It will be important to determine to what degree this mechanism can explain Lhx2's role in competence transitions.

Nfia/b/x. Histological and transcriptomic profiling revealed that Nfia/b/x transcription factors were strikingly upregulated during late stages of retinal development, and expressed in bipolars and Müller glia, as well as some amacrines in the mature retina (Clark et al., 2019; de Melo et al., 2016a; El-Hodiri et al., 2022; Zibetti et al., 2019). Conditional triple knockouts exhibited a dramatic phenotype during late stages of retinal development. The normal termination of the RPC lineage failed to occur during late stages of development. Müller glia were abolished and bipolar production was greatly reduced (Clark et al., 2019). Late-staged RPCs continued to proliferate and produced rods for several weeks beyond the normal neurogenetic period. Triple mutant RPCs therefore lost gliogenic potential. Misexpression experiments were also performed. Nfia overexpression alone did not increase the production of Müller glia when introduced during late stages of development (de Melo et al., 2016a), but Müller glia genes were directly bound and upregulated when Nfia/b/x were triply transfected (Lyu et al., 2021). Moreover, misexpression during early stages reduced the

production of RGCs and increased the production of photoreceptors (Lyu et al., 2021). Nfia/b/x are therefore necessary and sufficient for late bipolar/Müller competence, and to terminate the RPC lineage. *Nfi* genes may cooperate with Sox8/9 to specify glial identities throughout the CNS (see below).

Prdm1. Prdm1 was found to be highly expressed in postmitotic precursor cells. In conditional mutants, bipolar cell generation was strikingly increased at the expense of rod photoreceptors (Brzezinski et al., 2010; Katoh et al., 2010). These data suggest that Prdm1 is required in bipotent photoreceptor/bipolar precursors to diminish bipolar differentiation. This interpretation is supported by scRNA-seq data that reveal a postmitotic bifurcation in precursors that give rise to rods and bipolars (Clark et al., 2019; Lu et al., 2020). However, conditional knockouts also exhibited increases in dividing progenitors during late stages of neurogenesis, which likely differentiated into Müller glia at the completion of development (Brzezinski et al., 2010; Katoh et al., 2010). In early stages, *Prdm1* mutants were also observed to exhibit reductions in cone photoreceptors (Brzezinski et al., 2013; Katoh et al., 2010). Remarkably, bipolars and Müllers were produced precociously in *Prdm1* mutants (Brzezinski et al., 2013). These data indicate that *Prdm1* is required to restrict the competence to produce late-born bipolars and Müllers.

Prdm1 genetic fate mapping revealed striking features about its effects on lineage. Almost all rod and cone photoreceptors were present in the *Prdm1* lineage (Brzezinski et al., 2013). By contrast, earliest born RGCs and latest born Müller glia were completely excluded from the *Prdm1* lineage. All other neuron types were intermediate, with about one-third of the cells present within the *Prdm1* lineage. In addition to high-level expression in photoreceptor precursors, Prdm1 was found to be expressed at low levels in a subset of dividing progenitors. Next, genetic fate-mapping was performed on *Prdm1* conditional mutants. When rod photoreceptor precursors were marked using *Crx-Cre* or *Nrl-Cre* drivers, many rod precursors were found to convert into bipolars or glia (Brzezinski et al., 2013; Katoh et al., 2010). Prdm1 was subsequently shown to lie downstream of Otx2 (Wang et al., 2014), which tends to mark committed progenitors that are undergoing terminal divisions (Muranishi et al., 2011). Together with the *Crx-Cre* and *Nrl-Cre* lineage data, these data argue that *Prdm1* acts to restrict late competence at or even after cell cycle exit.

Sox8/9. Sox8 and Sox9 expression levels increase considerably after the initial wave of neurogenesis is complete (Muto et al., 2009; Poche et al., 2008). *Sox9* conditional mutants appear to have no defect in neuron numbers, but Müller glia are lost (Poche et al., 2008). Functional data suggest that both Sox8 and Sox9 are necessary for the latest competence state—somewhat analogous to *Nfia/b/x* conditional mutants. However, it is unclear whether Sox8/9 upregulation might participate in closing the early temporal window. Moreover, gain-of-function experiments suggest that Sox8/9 are not sufficient to promote Müller glia by themselves (de Melo et al., 2016a). However, Sox9 has been shown to cooperate with Nfi factors to promote gliogenesis in other regions of the CNS, suggesting that Sox9 and Nfi factors may both represent *bona fide* late competence determinants (Deneen et al., 2006; Kang et al., 2012; Sagner et al., 2021; Stolt et al., 2003).

Temporal Networks

One hypothesis is that competence is controlled by a network of transcription factors rather than by individual genes. As discussed above, scRNA-seq studies have helped to identify temporal transcription factors and fate determinants with dynamic, stage-specific expression profiles in RPCs (Clark et al., 2019; Lu et al., 2020). Taking this one step further, Lyu et al. used scATAC-seq to infer transcription factor occupancy via footprinting analysis. Integrating scRNA-seq expression data with scATAC-seq accessibility/occupancy data allowed RPC transcription factor networks to be collectively tracked over developmental time. Networks of early-expressed and late-expressed transcription factors were identified and shown to be cross-repressive, suggesting that the system has multiple stable states, with each network acting to reinforce its expression and repress the alternative temporal states (Lu et al., 2020) (Fig. 2C). In such a scenario, networks might be highly resistant to perturbation. Extensive compensation might thereby mask temporal phenotypes associated with the genetic ablation of individual network nodes. This might explain why temporal factors that single-handedly define temporal windows have not yet been found. Instead, the activities of multiple transcription factors might work collectively to determine the competence state of RPCs.

One potential criticism of this model is that self-reinforcing networks would appear to be highly stable. This would seem to be at odds with the highly stochastic outcomes produced by RPC fate decisions. Perhaps stochasticity can potentially be explained by the observation that the expression of many key neural fate determinants have been shown to be oscillatory over very short timescales. This is particularly true of Notch pathway components, as well as the associated proneural bHLH transcription factors (Imayoshi et al., 2013; Imayoshi and Kageyama, 2014). The Notch pathway and bHLH transcription factors are thought to participate in "lateral inhibition," which is a juxtracrine feedback loop between neighboring cells. In lateral inhibition, the Notch pathway helps to establish asymmetric divisions that coordinate progenitor self-renewal vs. neuronal differentiation, and also diversify terminal fate decisions. Stochasticity might therefore map mainly to the fate decisions made by neurogenic progenitors and precursors, while RPC fate decisions would be robustly governed by stable networks. Yet, as discussed above, terminal fate decisions appear to be more deterministically programmed versus the decisions made by multipotent RPCs. The existence of temporal transcription factor networks is clear (Lyu et al., 2021), but the properties of these networks will need to be further explored in the future.

In *Drosophila*, neuroblasts undergo competence transitions that are highly analogous to RPCs, although RPC lineages are much more deterministic (Kohwi and Doe, 2013; Li et al., 2013). Fly transcription factors also form complex networks, but importantly, each temporal transcription factor inhibits the expression of the preceding transcription factor, which is necessary to drive progressive competence transitions (Konstantinides et al., 2022; Li et al., 2013). Moreover, feed-forward network activities have been described (Baumgardt et al., 2009). Going forward, it will be important to define the feedback and feed-forward mechanisms that are critical for driving temporal progression of vertebrate temporal gene networks.

Heterochromatin

While experiments in *Drosophila* neuroblasts pointed to the role of transcription factor cascades in temporal competence, classic experiments in vertebrate neural progenitors implicated heterochromatin in competence transitions. Focusing on the competence transition from neurogenesis to gliogenesis, several investigations have revealed the importance of heterochromatic processes such as DNA methylation (Takizawa et al., 2001) and polycomb (Amberg et al., 2022; Hirabayashi et al., 2009; Pereira et al., 2010; Telley et al., 2019; Morimoto-Suzki et al., 2014) in temporal control of competence. DNA methylation is largely absent in pluripotent cells, and is progressively added to the genome over developmental time. This progressive methylation is clearly important for reinforcing cell identity. However, DNA methylation appears to be largely dispensable for neural cell fate decisions (Hahn et al., 2013; Nasonkin et al., 2013; Singh et al., 2017). Moreover, DNA methylation is virtually absent in *Drosophila*, suggesting circumstantially that it is not essential for temporal patterning mechanisms during neurodevelopment.

With respect to polycomb, investigations were performed by multiple groups without necessarily revealing a clear temporal phenotype—except on the neurogenesis vs. gliogenesis fate decision (Aldiri et al., 2013; Fujimura et al., 2018; Iida et al., 2015; Mattar et al., 2021; Zhang et al., 2015). More recently, the Vetter laboratory performed careful examination of *Jarid2* conditional knockouts. Jarid2 is an obligatory polycomb subunit in RPCs. *Jarid2* mutants exhibited a clear defect in temporal progression, with early-born cell types systematically overproduced at the expense of late-born neurons (but not glia) (Zhang et al., 2023a). Differentiation was elevated in *Jarid2* mutants, which might contribute to the observed overproduction of early-fate cells. However, Zhang et al. also demonstrated that the birth window for early cell types was extended in knockouts. Moreover, RPCs exhibited an early-shifted gene expression signature. Genes expressed in early RPCs failed to be downregulated, and did not accumulate the polycomb associated heterochromatic histone mark H3K27me3. Such misregulated genes notably included the temporal factor Foxp1, which was discussed above.

Collectively, these studies suggest mechanisms that can potentially explain how heterochromatin might regulate temporal development. First, heterochromatic processes might simply be required to suppress the expression of temporal factors like Foxp1. In this scenario, the requirement for heterochromatic "writers" such as polycomb would simply reflect a requirement for conventional transcriptional repression in temporal gene expression programs.

A second idea is that the *stability* of heterochromatic modifications over time is the more important feature. Instead of merely shutting off transcription, heterochromatic writers mark target genes with epigenetic modifications that can be stably inherited through multiple rounds of DNA replication. In this scenario, the importance for heterochromatic writers would be for gene decommissioning—the prevention of future gene expression/reactivation. Indeed, chromatin remodellers such as the NuRD complex have been physically and functionally linked to polycomb complexes, and to temporal development in neural progenitors (Kehle et al., 1998; Knock et al., 2015; Mattar et al., 2021; Muralidharan et al., 2017; Nitarska et al., 2016; Sparmann et al., 2013; Tsuboi et al., 2018; Zhang et al., 2013). The NuRD complex can decommission genes and regulatory elements (Yamada et al., 2014; Yang et al., 2016), which would constitute a formidable barrier to their subsequent reactivation. Gene decommissioning could thereby constitute the molecular mechanism that underlies progressive restriction of neural progenitors. If so, competence would be defined by the ability or inability for a given transcription factor to upregulate its target genes due to accessibility/inaccessibility of cognate cis regulatory elements.

A third idea, is that the *spreading* of heterochromatin could directly function as the molecular clock that regulates RPCs. Classic experiments in yeast and *Drosophila* revealed that heterochromatin can progressively spread across euchromatic regions of the genome (Grewal, 2023). This spreading heterochromatinization of genomic intervals is both time dependent and stochastic, which leads to variegation from cell-to-cell. Variegation is nonetheless heritable, such that heterochromatinization is generally not reversed in daughter cells. The spreading of heterochromatin through neighboring euchromatic regulatory elements might drive stochastic but permanent gene silencing, which could directly decommission genes without requiring additional transcriptional repressors (Fig. 2D). While heterochromatic marks have been comprehensively profiled during retinal development (Aldiri et al., 2017; Norrie et al., 2019), most experiments have been performed on retinal tissue as a whole, rather than on RPCs. In the future, single-cell genomic methods might be required in order to visualize heterochromatin spreading in RPCs. It will also be important to determine how temporal transcription factors and epigenetic writers and erasers interact to orchestrate genome regulation.

MicroRNAs

MicroRNAs are endogenously expressed transcripts encoded in genes or introns, which can suppress the translation of target genes. MicroRNAs possess stretches of self-complementary double-stranded RNA (pre-miRNAs). Upon formation of double-stranded hairpins, transcripts are processed into short single-stranded mature microRNA molecules of approximately 21–25 bases, and loaded into the RNA induced silencing complex (RISC). Using the mature microRNA as a guide, the RISC complex can surveil endogenous messenger RNAs. Complementarity between the mature microRNA "seed" with the mRNA leads to suppression of mRNA expression—either by blocking translation, or through enzymatic cleavage of the mRNA. The Dicer enzyme is critical—both for the enzymatic processing of pre-miRNAs into mature microRNAs, and as a component of the RISC.

In *Xenopus*, interfering with early-expressed microRNAs was found to accelerate the translation of bipolar fate determinants, including *Xotx2* and *Xsox3* (Decembrini et al., 2008). Subsequently, the role of microRNAs in retina development was investigated systematically by conditionally deleting the *Dicer* gene in RPCs. Like *Lhx2* conditional knockouts, *Dicer* ablation strikingly arrested the progression of competence, and led to prolonged RGC production (Georgi and Reh, 2010; Iida et al., 2011). Which microRNAs mediate these effects? Clarity was provided via microRNA profiling that identified several with strikingly dynamic expression

profiles (Decembrini et al., 2009; La Torre et al., 2013). In mouse, La Torre et al. identified microRNAs that upregulated in RPCs during later stages of retinal development, including *let-7*, *miR-125*, and *miR-9* (La Torre et al., 2013). Strikingly, misexpression of these three microRNAs was sufficient to rescue the *Dicer* mutant phenotype. Moreover, heterochronic misexpression of these microRNAs in early RPCs induced the production of late cell types, including rod photoreceptors. Additional downstream target genes were also identified, including *Lin28* and *Ptg*, which were accordingly shown to be sufficient to promote early fates and suppress late cell types when misexpressed in later-stage RPCs (La Torre et al., 2013).

Together, Decembrini et al. and La Torre et al. demonstrate that microRNAs can control competence by regulating temporal factors and fate determinants. Alternatively, like heterochromatic modifications, microRNAs could restrict gene expression by blocking the translation of target mRNAs. Even if a temporal factor or fate determinant is expressed in an RPC, the presence of microRNAs might ensure that it cannot upregulate key target genes (Fig. 2E), which might explain why many fate determinants have little activity when misexpressed heterochronically. While microRNAs might therefore play a dominant role in regulating retinal development, how the temporally dynamic expression patterns exhibited by various microRNAs are achieved remains poorly understood. In the future, it will be important to improve our understanding of microRNA regulation during retinal development.

Like several of the temporal factors described above, microRNAs have additionally been shown to play analogous roles in temporal development elsewhere in the CNS, including in neocortical progenitors (De Pietri Tonelli et al., 2008; Nishino et al., 2013; Saurat et al., 2013; Shimazaki and Okano, 2016). Indeed, a role for microRNAs in developmental timing is deeply conserved in evolution, with microRNAs such as *let-7* timing vertebrate development in a variety of contexts, but also during the development of *C. elegans*, where much microRNA biology was originally described (Faunes and Larrain, 2016; Moss, 2007). Additionally, given the highly pleiotropic roles of microRNAs, it is perhaps not surprising that they have been implicated in many other retinal functions, including progenitor proliferation, late-stage fate decisions, differentiation, and viability (Damiani et al., 2008; De Pietri Tonelli et al., 2008; Decembrini et al., 2009; Fairchild et al., 2019; Iida et al., 2011; Ohana et al., 2015; Aldunate et al., 2019). A detailed review of microRNA biology in retinal development is beyond the scope of this review, but the reader is directed to an excellent review on this subject (Reh and Hindges, 2018).

The Nuts and Bolts of the Case—Neuronal Subtype Development

Cellular differentiation does not cease with the upregulation of hallmark cell-type specific markers. It is now believed that there may be as many as 135 cell subtypes in the retina—most of which are specialized subtypes of amacrines, bipolars and RGCs (Shekhar and Sanes, 2021; Shiau et al., 2021). Only recently have molecular methods been developed with which to mark or manipulate individual subtypes. How do specific subtypes arise during development?

Subtype specification is best understood for RGCs. Shekhar et al. used high resolution scRNA-seq to track the specification of the ~45 RGC subtypes over time (Shekhar et al., 2022). Rather than observing 45 different RGC precursors, at the earliest timepoints, RGCs instead exhibited considerable overlap in their gene expression signatures. Newly born RGCs could be separated into ~10 distinctive clusters, but these clusters were not completely discrete. Subsequently, between embryonic day 13 and postnatal day 5, each of the 45 murine RGC subtypes progressively coalesced from the initial 10 precursor clusters. Reconstructing developmental trajectories over time led to the conclusion that RGC precursors are multipotent (although only at the level of subtypes), with each initial precursor cluster being capable of generating multiple RGC subtypes. Conversely, each of the 45 subtypes did not appear to arise from any one initial precursor cluster. Instead, the differentiation of each RGC subtype could be correlated back to multiple precursor clusters. These data suggest that RGCs are first generically specified at the cell type -level. The resultant undifferentiated RGCs are postmitotic, but capable of giving rise to multiple RGC subtypes during differentiation.

The development of amacrine and bipolar cell subtypes might follow analogous principles. Like RGCs, amacrine subtype differentiation appears to occur well after cell cycle exit. For example, in rodents, cholinergic amacrine cells are born several days earlier than the expression of characteristic subtype markers (Elshatory et al., 2007a; Galli-Resta et al., 1997). Genetic experiments also suggest that amacrine precursors may have the potential to generate multiple subtypes. When specific amacrine subtype determinants are mutated, an alternative subtype can be gained at the expense of the lost identity, suggesting that fate decisions continue to be actively made in committed precursors after cell cycle exit (Agreda, 2023; Elshatory et al., 2007b; Huang et al., 2014).

However, it is important to note that other data conflict with this model. As mentioned above, genetic lineage tracing data have revealed the existence of a multipotent RPC that appears to deterministically produce only on-off direction sensitive RGCs without exhibiting potential for other RGC subtypes (De La Huerta et al., 2012). It is perhaps also worth pointing out that the transcriptomic signatures of precursor states appear to be highly convergent in many scRNA-seq experiments. For example, amacrine and horizontal precursors are difficult to resolve transcriptomically, even though immunohistochemical methods can readily distinguish these precursors using cell-type markers that include Lhx1, Sall3, and Tfap2a among others (de Melo et al., 2011; Jin et al., 2015; Liu et al., 2000; Poche et al., 2007). This suggests the possibility that type and subtype differentiation could perhaps be driven by a very small number of fate determination genes without initially exhibiting broadly divergent transcriptomic signatures. Finally, a number of reports have shown that RGC, amacrine, and bipolar cell subtypes exhibit different birthdates (McNeill et al., 2011; Morrow et al., 2008; Osterhout et al., 2014; Voinescu et al., 2009; West et al., 2022). For bipolars, these differences are quite subtle (West et al., 2022), but they are much more protracted in the case of RGC and amacrine cell subtypes (McNeill et al., 2011; Osterhout et al., 2014; Voinescu et al., 2009). It seems unclear why subtype birthdates would differ systematically if their identities were specified postmitotically.

Perhaps one explanation might be that subtype differentiation is instead specified by neuronal activity, and that subtype birthdates arise due to temporal differences in activity patterns within specific circuits. Interestingly, despite the fact that both RGCs and amacrine subtypes appear to differentiate during a period that includes considerable circuit activity—through processes that include retinal waves and/or intrinsically photosensitive RGCs, light deprivation does not appear to disrupt the production of subtypes, although it impairs their full differentiation (Tiriac et al., 2022; Whitney et al., 2023). These data hint that retinal subtype differentiation might be intrinsically programmed, but much remains to be done to clarify the mechanisms that regulate subtype specification and differentiation.

Conclusions—*Closing Arguments*

Why temporal development? In many regions of the brain, neural progenitors are developmentally programmed to generate specific types of neurons based on their position in space. For example, in the spinal cord, secreted morphogens program neural progenitors according to their location along the dorsoventral axis (Jessell, 2000; Lai et al., 2016). In this way, spatial position within the neural tube is translated into differences in progenitor competence. While the tissue architecture of the mature spinal cord is far from simple, neuron subtypes tend to be sorted into distinct columns or pools—each containing clusters of functionally equivalent neurons (Osseward and Pfaff, 2019). In many other parts of the CNS, neurons are grouped together in "nuclei" that contain cells with similar molecular identities. In these regions, circuit assembly often depends on the ability to guide axons with precision over long distances between nuclei. However, the developmental migration of the neurons to their destinations is usually short-range.

In the retina, producing cell types/subtypes using spatial mechanisms would create an enormous challenge for circuit assembly. Unlike many other regions of the CNS, retinal circuits are arranged in columns that contain all 7 cell types sorted into three distinct layers. These columns must be repeatedly arrayed across an enormous volume of tissue—approximately 1000 mm^2 in humans. On average, each of the approximately 1.2 million RGCs present in the human retina subserves a columnar unit containing approximately 100–150 cells. Producing these circuit units from multiple spatially discrete progenitor pools would necessitate the long-range migration of huge numbers of cells. This would likely be error-prone and expensive. Via temporal development, the migratory distances faced by newborn neurons and glia are dramatically reduced. Another potential advantage is that the expansion or contraction of retinal tissue across evolution could be scaled in a straightforward fashion to produce eyes of different sizes.

Temporal development might also provide a mechanism to guide circuit assembly. Clonal columns might "self-wire" to establish light detecting circuits. Indeed, this principle is observed in the neocortex, where clonal units preferentially form functional units (Yu et al., 2009). However, the requirement for retinotopic circuits can clearly be overcome without lineage mechanisms. Somatotopy must be preserved across the vertebrate visual system, as well as for other sensory systems. In each case, the spatial relationships between sensory inputs must be maintained and coordinated across a variety of CNS divisions that develop from independent progenitor pools. Although axon guidance mechanisms appear to readily solve problems of circuit topography, temporal development might nonetheless constitute an advantageous process for guiding local circuit establishment.

Finally, one major advantage of temporal systems might be exemplified by vertebrate systems such as zebrafish and *Xenopus* that exhibit regenerative potential. In these systems, when the retina is damaged, the logic of temporal development appears to be reversible. Müller glia—which are the quiescent terminal state of the RPC temporal program (Zhang et al., 2023b), are rewound back towards multipotency. Each Müller thereby becomes a regenerative unit—capable of replacing all retinal cell types. This regenerative response would be much more complex to achieve if separate progenitor types were required to regenerate each cell type. If regeneration truly does rely on developmental competence mechanisms, unlocking retinal regeneration in mammals might simply be a matter of "reversing" the molecular clock that drives retinal histogenesis. Perhaps accordingly, several groups have shown that the deployment of temporal factors and fate determinants in mammalian Müller glia can drive neurogenesis (Boudreau-Pinsonneault et al., 2023; Hoang et al., 2020; Jorstad et al., 2017; Pollak et al., 2013; Todd et al., 2021, 2022; Ueki et al., 2015; Wohl et al., 2019). There remains a large gap in our ability to restore meaningful function to the degenerated retina. However, the important progress made in understanding temporal competence mechanisms provides hope that recapturing lost regenerative potential might soon be feasible.

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Other Publications:

1. Larrigan S, **Shah S**, Fernandes JAL, Mattar P. Chromatin Remodeling in the Brain-a *NuRD*evelopmental Odyssey. Int J Mol Sci. 2021 Apr 30;22(9):4768.

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List of scientific communications:

Oral Presentations:

1. **Shah S**, Fernandes A, Mattar P. Epigenetic Regulation of Neural Progenitor Multipotency. The Association for Research in Vision and Ophthalmology (ARVO 2023), New Orleans, USA; April 23-27 2023.

2. **Shah S**, Dang T, Mattar P. The chromatin re-modeller *Chd4* regulates neural progenitor multipotency. OHRI Research Day; Nov. 19th 2020.

3. **Shah S**, Mattar P. Dissecting the role of NuRD complex in retinal development. 33rd Annual Research Day. Department of Ophthalmology, University of Ottawa; May 22nd 2019.

Poster Presentations:

1. **Shah S**, Fernandes A, Medisetti S, Mattar P. Epigenetic regulation of neural progenitor multipotency.

- NFRF Team iNeuron Annual Symposium. King City, ON; Jan 21st- 23rd 2025.

2. **Shah S**, Clemont-Dupont S, Medisetti S, Fernandes A, Mattar P. The chromatin remodeller Chd4 progressively restricts the developmental potential of retinal progenitors.

- Gordon Research Conference on Visual System Development. Lucca, Italy; May 18th– 24th 2024.

3. **Shah S**, Fernandes A, Mattar P. Epigenetic regulation of neural progenitor multipotency.

- ARVO 2022 Annual Meeting. (virtual) Denver, USA; May 1st-4th 2022. (Travel award recipient)
- Till and McCulloch Meetings. Vancouver, BC; Oct. 3rd-5th 2022. (Travel award recipient)

4. **Shah S**, Dang N, Mattar P. The chromatin re-modeller Chd4 regulates neural progenitor multipotency.

- CMM/NSC Research Day. University of Ottawa; Oct. 24th 2019
- Till and McCulloch Meetings. Montreal, QC; Nov. 4th-6th 2019. (Travel award recipient)

- OHRI Research Day. Ottawa, ON; Nov. 7th 2019.

Scholarships:

1. University of Ottawa Admission Scholarship, 2018-2022
2. David Shillito Scholarship- University of Ottawa, 2019-2022
3. James M Inglis Fellowship- University of Ottawa, 2020-2021

Additional Results:

Background information:

RNA-binding proteins (RBPs) have been shown to be involved in post-transcriptional regulation, where they form functional interactions with RNA during transcription, splicing, polyadenylation, subcellular localization, translation, and decay, and thus play an essential role in mRNA processing, stability, and transport (1). RBPs typically recognize specific RNA sequences or structural motifs through specialized RNA-binding domains such as the RNA recognition motif, K-homology domain, double-strand RNA-binding domain, or zinc finger domains. Dysregulation of RBP functions has been implicated in various disorders, including neurodegenerative diseases (2).

Recent research has also uncovered interactions between RBPs and chromatin-associated proteins, revealing a complex regulatory network that integrates RNA biology with epigenetic control. A previous study surveying the RNA-binding activity of 24 chromatin-associated proteins showed the binding of these proteins to a broad spectrum of RNAs, including coding and noncoding transcripts (3). Chd4 was one of the proteins surveyed that showed a high affinity for binding mRNA and long-non-coding (lnc) RNA Xist. Chd4

was also shown to bind lncRNA PAPAs derived from rRNA promoters, and this interaction has been suggested to recruit Chd4/NuRD complex to rDNA promoter for transcriptional regulation (4). Recently, Chd4's role in RNA binding and its functional significance was identified where Chd4 was shown to associate with thousands of mRNAs and the consequence of RNA binding was to antagonize the chromatin binding and remodeling activity of Chd4 at actively transcribed genes to prevent the establishment of repressive chromatin structures (5). These studies implicate a link between RNA biology and chromatin dynamics that might potentially provide additional insights into epigenetic regulation of gene expression.

Objective:

While Chd4 is known for its role in chromatin remodeling and transcriptional regulation, recent findings suggest that CHD4 also binds RNA, highlighting a potential regulatory mechanism that has yet to be fully understood. Previous studies provided insight into the RNA binding activities of Chd4, but the biological significance of this association remains unclear.

Given Chd4's involvement in developmental processes, and neurodevelopmental disorders, elucidating its RNA interactions could reveal novel layers of epigenetic regulation. Thus, the objective is to gain a better understanding of Chd4/RNA interactions in vivo and identify its biological significance during development using the retina as a model system.

Aim: To delineate the association of Chd4/RNA interactions and identify its significance during development. For this aim, we will focus on P1 retinas as it would allow us to compare with the other datasets that we have generated.

Preliminary Results:

Chd4 associates with RNA in-vivo:

To assess Chd4/RNA interactions, we performed RIP-seq on wild-type P1 retinas. Briefly, P1 retinas were dissected and homogenized, in asepctic conditions before applying them to the RNA immunoprecipitation protocol (Fig. 1B). We performed native RIP, following the manufacturer's protocol, using anti-rabbit Chd4 and anti-rabbit IgG as a negative control. Chd4 associated RNA was purified and sequenced through paired-end sequencing with around 40 million reads per sample. The sequencing results were analyzed using the Galaxy web interface. Post-filtering we obtained approximately 1000 RNAs that were enriched more than 2-fold in Chd4 as compared to controls (Fig. 1C). These results hint at either direct or indirect binding to RNA by Chd4 *in vivo*.

Gene ontology analysis of Chd4 RIP-seq transcripts:

In order to identify the biological significance of the Chd4 bound RNA transcripts, we performed GO term analysis on transcripts that were enriched more than 2-fold. This analysis hinted that the bound transcripts played an important role in retinal development. The GO-term enrichment identified biological processes that are involved in cell-fate commitment, regulation of cell-cycle, neural precursor proliferation, optic nerve development among others (Fig. 2). These results hint that Chd4 association with RNA

may have a developmentally important role and provide an additional layer of epigenetic regulation that may modulate cell-fate specification.

Future Directions:

The preliminary results obtained thus far indicate that the Chd4 interaction with RNA might play a key role in regulating developmentally important processes. However, this study is still in its nascent stages, and there are significant gaps that need addressing. For example, it remains unclear whether the Chd4 association with RNA is direct or whether Chd4 is binding RNA through the RNA-binding proteins. Additionally, how the disruption of these associations in *Chd4* cKO leads to the observed phenotype remains unclear. To address some of these queries, we can perform IHC stainings on P1 control and mutant retinas for transcripts identified in the RIP-seq. Alternatively, we could perform RNA FISH to visualize the bound transcripts and observe their stability in the absence of Chd4.

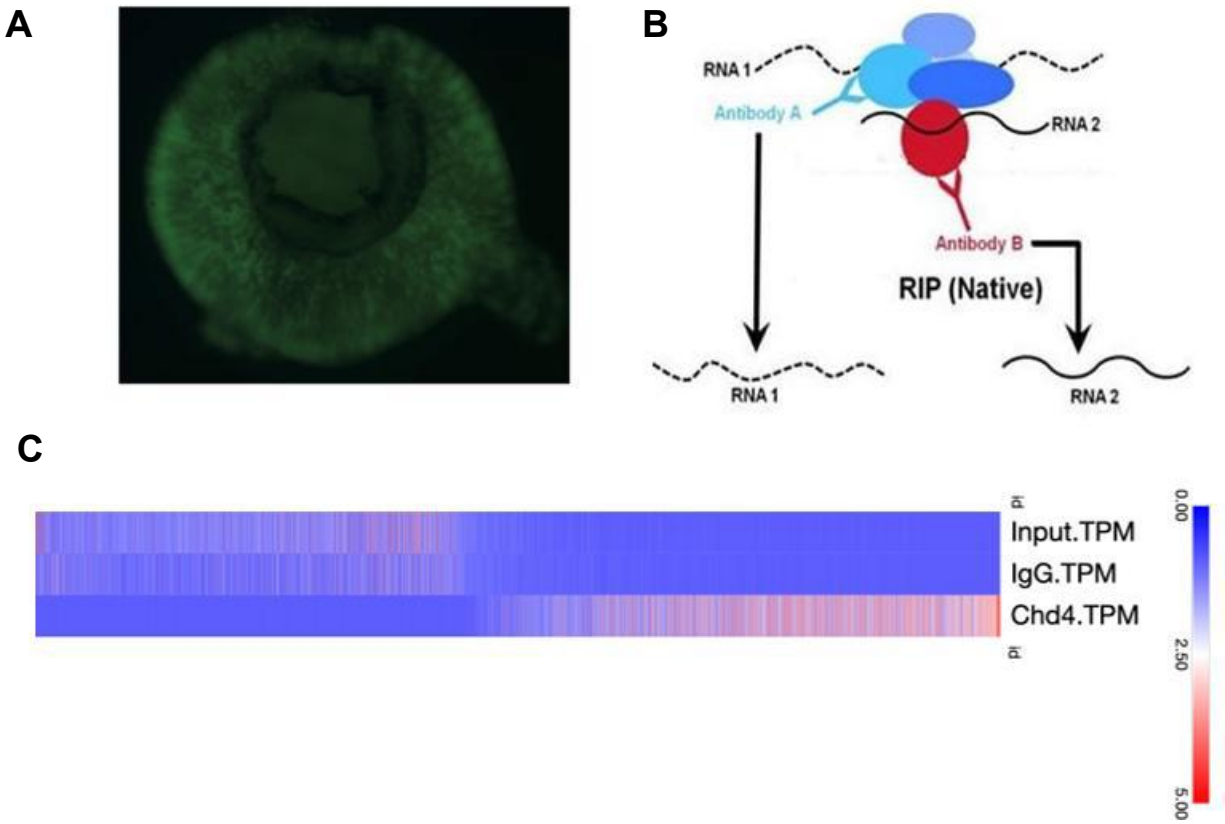

Fig. 1: Chd4 RIP-seq on P1 retinas. (A)Wild-type P1 retinas were used to perform the RIP-seq assay where the retinas were dissected and homogenized**.** (B) RNA immunoprecipitation was performed according to the manufacturer's protocol using rabbit anti-Chd4 and anti-rabbit IgG antibodies. (C) Sequencing result post quality-control on raw reads and filtering for 2-fold enrichment resulting in approximately 1000 RNAs associating with Chd4.

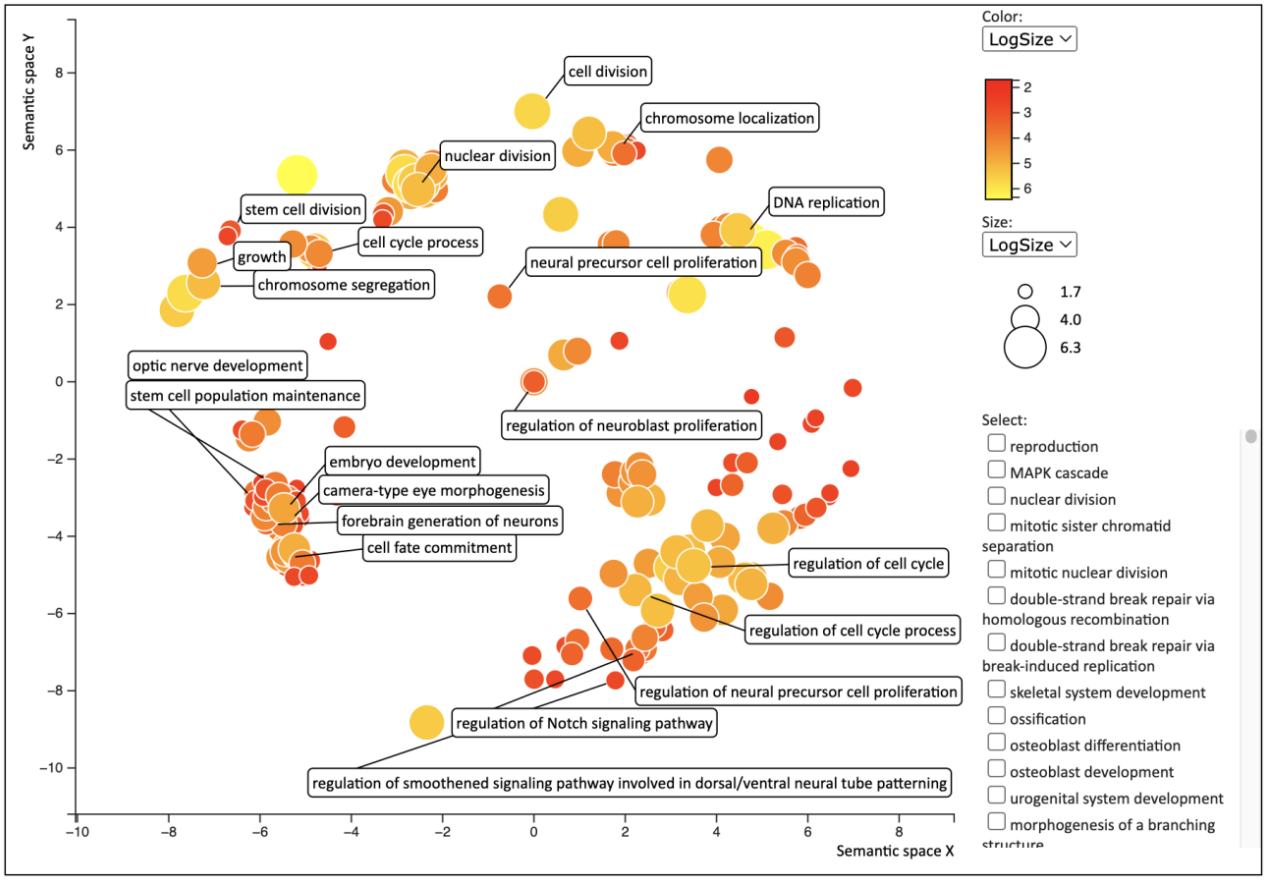

Fig. 2: GO term analysis of Chd4 RIP-seq transcripts.

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