INVESTIGATING TERMINAL DIVISIONS DURING RETINAL DEVELOPMENT IN ZEBRAFISH

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This thesis is dedicated to my uncle who passed away from Covid-19.

The brightest and the most innately curious person I will ever know.

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Abstract

The vertebrate central nervous system (CNS) consists of a vast array of neuronal and glial cell-classes. How this cellular diversity is generated during development is not fully understood. With its well-characterized diverse complement of cell-classes, the retina is an accessible part of the CNS that is well suited to investigate this question (Dowling 2012). During my doctoral work, I have strived to understand the establishment of the retinal tissue and the assignment of cell fates during retinogenesis (**Result section 2.1**. "Cellular and molecular determinants of retinal cell fate" Petridou and Godinho; *accepted manuscript, Annual Review of Vision Science*). I have been using the retina of zebrafish, a vertebrate model organism with attributes that make it particularly suitable to probe the mechanisms underlying how cell diversity is established. As in other CNS regions, distinct retinal cell-classes arise following progenitor mitotic divisions. Towards the end of retinogenesis progenitors divide terminally, generating post-mitotic cells that either acquire distinct fates (asymmetric division) or the same fate (symmetric division). Using genetic tools to label specific progenitors and *in vivo* time-lapse imaging to follow their fates, I have been examining the generation of distinct cell classes. In recently published work (**Results section 2.2**: "Notch-mediated re-specification of neuronal identity during central nervous system development", Engerer*, Petridou* et al., 2021), we showed that progenitors that express the *visual system homeobox 1 transcription factor* (*vsx1*) and were thought to exclusively generate bipolar cell (BC) interneurons (excitatory) via symmetric divisions can also divide asymmetrically to produce a BC and an amacrine cell (AC), a molecularly and functionally distinct inhibitory retinal interneuron. In these events, Notch signaling plays a major role, conferring nascent BCs with a degree of 'plasticity' in order to respecify and acquire the amacrine cell fate under physiological conditions. Notch acts synergistically with Pancreatic transcription factor 1a (Ptf1a), which is known to directly instruct the AC fate. By manipulating the *vsx1* lineage and exploiting the plasticity window conferred by overexpressing a Notch signaling component we managed to heterochronically direct the *vsx1* lineage to produce a cell class (ganglion cells (GCs)) that it normally does not. In a second study, I have described, for the first time *in vivo*, terminal asymmetric mitotic events that generate a Müller cell (MC) and a distinct subclass of BCs (**Results section 2.3**.: "Neuron-glia pairs arise from terminal progenitor divisions in the vertebrate retina", Petridou et al. *in preparation*). MCs, the principal glia cell class of the vertebrate retina, are concurrently generated along with other retinal interneurons like BCs during retinogenesis across species. In the zebrafish retina, I was able to trace individual mitotic events and directly illustrate that

MCs derive from asymmetric terminal events with their siblings being a distinct subtype of excitatory BCs expressing the *visual system homeobox 2 transcription factor* (*vsx2*). Notch signaling plays a major role here too, driving the fate specification of the MCs. Loss of function experiments illustrated the loss of MC and an increase in the *vsx2* BC population, reflecting the role of this pathway in the establishment of the *vsx2* lineage. Such terminal asymmetric events at the end of retinogenesis, could support a mechanism of cell production "on demand" that allows for the fine tuning of the retinal circuitry.

Table of contents

Acknowledgements....4

Abstract....6

List of abbreviations....3

1 Introduction....7

1.1 Overview of the vertebrate retina....9

1.1.1 Cone and Rod photoreceptors....10

1.1.2 Horizontal Cells....10

1.1.3 Bipolar Cells....11

1.1.4 Amacrine cells....12

1.1.5 Ganglion cells....12

1.1.6 Müller cells....13

1.2 Retinal development....14

1.2.1 Modes of divisions during retinal development....14

1.2.2 Birth of retinal cell classes....15

1.2.3 Progenitor multipotency....17

1.2.4 Heterogeneity of progenitors....17

1.2.5 Intrinsic determinants influencing progenitors....18

1.2.6 Extrinsic determinants influencing progenitors....19

1.2.7 Stochastic model versus deterministic model....19

1.3 Notch signaling in development....20

1.3.1 Overview of the Notch signaling cascade (Canonical pathway)....21

1.3.2 Overview of Notch receptors and ligands expressed in CNS....24

1.3.3 Notch downstream effectors....26

1.3.4 Notch signaling in the development of the CNS....27

1.4 Aims of the thesis....35

2 Results....37

2.1 Cellular and molecular determinants of retinal cell fate (*accepted for publication in Annual Review of Vision Science*).....39

2.2 Notch-mediated re-specification of neuronal identity during central nervous system development (*published in Current Biology*) 63

2.3 Neuron-glia pairs arise from terminal progenitor divisions in the vertebrate retina (*in preparation*) 91

3 Discussion.....115

3.1 *Vsx1* and *vsx2* terminal events 117

3.2 Notch signaling in establishing asymmetric fates.....118

3.3 The role of Notch signaling in establishing asymmetric fates in the *vsx1* lineage 119

3.4 The role of Notch signaling in establishing asymmetric fates in the *vsx2* lineage 120

3.5 Investigating specific Notch signaling components 121

3.6 Convergence of Notch signaling and fate specification factors.....121

3.7 Could cellular diversity simply arise from distinct progenitor populations?.....122

3.8 Synaptic connections between siblings and functional implications.....123

3.9 Concluding remarks.....126

4 References.....129

5 List of publications 155

6 Eidesstattliche Versicherung/Affidavit.....157

7 Declaration of author contributions 159

List of abbreviations

ACs	Amacrine cells
ADAM10	A Disintegrin and Metalloproteinase domain-containing protein 10
AF	Arborisation field
ANK	Ankrin
APEX	Ascorbate peroxidase
Ascl1	Achaete-Scute Complex Homolog-Like 1, also known as Mash1
Atoh7	Atonal homolog 7
BCs	Bipolar cells
bHLH	Basic helix–loop–helix
BLBP	Brain lipid binding protein
BMP	Bone Morphogenetic Protein
CAH	Carbonic anhydrase
CLEM	Correlative Light Electron Microscopy
CNS	Central nervous system
CTBP2	C-Terminal Binding Protein 2
Cx	Connexin
Dll	Delta-like
DOS	Delta and OSM-11-like proteins domain
Dscam	Down syndrome cell adhesion molecule
DSL	Delta/Serrate/Lag-2 family
E	Embryonic day
EGF	Epidermal growth factor
EM	Electron microscopy
ER	Endoplasmic Reticulum
ERB-B2	Erb-b2 receptor tyrosine kinase 2
Foxn4	Forkhead Box N4
GABA	Gamma-Aminobutyric acid
GCL	Ganglion cell layer
GCs	Ganglion cells
GFAP	Glial fibrillary acidic protein
GluMI	Glutamatergic monopolar interneuron
GMC	Ganglion mother cell
GCP	Granule cell progenitor
Grg	Groucho-related gene
GS	Glutamine synthetase
HCs	Horizontal cells
HD	Heterodimerization domain
Hes	Hairy and Enhancer of split (E(spl)) homologs
Hey	Hairy/Enhancer-of-split related with YRPW
hpf	Hours post fertilization
Ids	Inhibitors of DNA binding and cell differentiation
INL	Inner nuclear layer
INM	Interkinetic nuclear migration

IPC	Intermediate progenitor cell
IPL	Inner plexiform layer
ipRGCs	Intrinsically photosensitive melanopsin-containing retinal ganglion cells
Jag	Jagged
KA	Kolmer-Agduhr
L	Long
Lin12	Abnormal cell lineage protein 12
M	Medium
MAML	Mastermind-like protein
Math3	Neuronal Differentiation 4, also known as NeuroD4
MC	Müller cell
mib	Mindbomb mutant
MNNL	N'-terminal domain
MPC	Multipotent pancreatic progenitor cell
NECD	Notch Extracellular Domain
Ngn2	Neurogenin 2, also known as Neurog2
NFI	Nuclear factor I
NICD	Notch Intracellular Domain
NIRB	Near infrared branding
NLSs	Nuclear localization signals
NRR	Negative regulatory region
OL	Oligodendrocyte
Olig2	Oligodendrocyte transcription factor 2
ONL	Outer nuclear layer
OPC	Oligodendrocyte precursor cells
OPL	Outer plexiform layer
Otx2	Orthodenticle homeobox 2
P	Postnatal day
Par3	Partition defective protein 3 (Par3)
PEST	Proline-glutamic acid-serine and threonine rich motifs
PhR	Photoreceptors
PMN	Primary motor neuron
Prdm1	PR-SET domain zinc-finger
Ptf1a	Pancreatic transcription factor 1a
PTU	N-Phenylthiourea
RAM	RBP-J association module
RBP-J	Recombining binding protein suppressor of hairless
RGCs	Radial glia cells
RORβ1	Retinoid-related orphan receptor β
RPC	Retinal progenitor cell
S	Short
SAC	Starburst amacrine cell
Sara	Smad Anchor Receptor Activation
Shh	Sonic hedgehog

SOP	Sensory organ precursor
Sox9	SRY-Box Transcription Factor 9
TLE1–4	Transducin-like E(spl)
TMD	transmembrane domain
Trβ2	Thyroid hormone receptor β2
UV	Ultraviolet
Vsx1	Vsx1 homeodomain transcription factor
Vsx2	Vsx2 homeodomain transcription factor
vWF	von Willebrand Factor type C domain
WRPW	Trp-Arg-Pro-Trp motif
Xotch	Xenopus homolog of Notch
YRPW	Tyr-Arg-Pro-Trp motif

1 Introduction

1.1 Overview of the vertebrate retina

The vertebrate retina is an isolated, approachable part of the CNS (Dowling 2012) responsible for incorporating visual information from the surrounding visual scene. Stereotypically organized, the retina consists of six neuronal cell classes and one glial cell class (Müller cell, MC), all of which are derived from a common progenitor pool in the retina (**Figure 1**). This laminated structure has three distinct nuclear layers – from the apical to basal surface, these are the outer and inner nuclear layer (ONL, INL) and ganglion cell layer (GCL). The different retinal cell classes form intricate synaptic connections in the outer and inner plexiform layers (OPL, IPL) allowing the transmission and early processing of visual information before reaching the GCL. At the GCL, the visual information converges and thereafter is transmitted to higher-order brain areas of the visual processing pathways (Wässle & Boycott 1991; Wässle et al. 2004; Masland 2012). The individual cellular classes play distinct roles in the extraction of visual features in an achromatic and chromatic manner (Baden 2021).

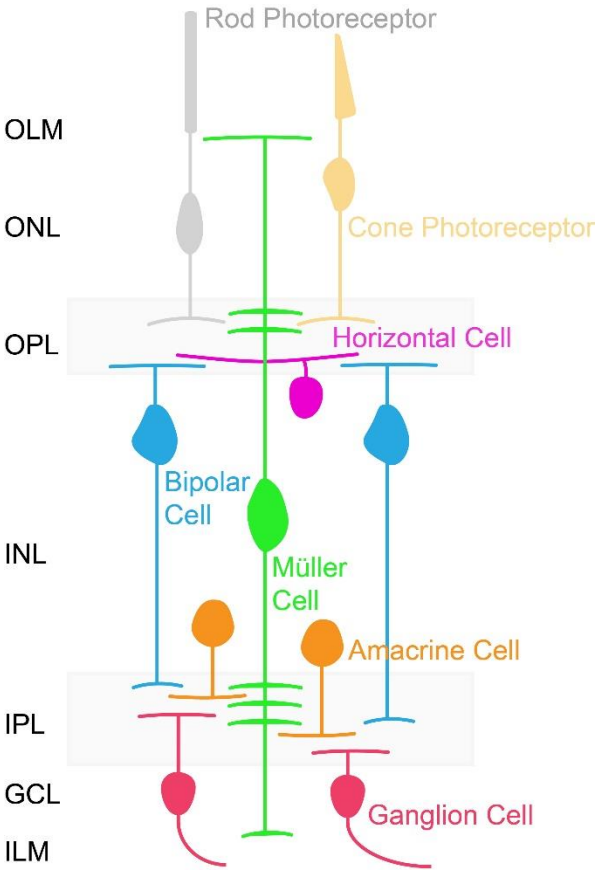

Figure 1: Overview of the vertebrate retina. Abbreviations: Outer limiting membrane (OLM), Outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), inner limiting membrane (ILM).

In the following section, I will introduce each retinal cell class, highlighting the intricacies of the zebrafish retina since my work was exclusively conducted using this model organism.

1.1.1 Cone and Rod photoreceptors

The photoreceptors (PhRs) are the first order neurons that receive photons and convert them to chemical messages in the OPL (Stryer 1991). PhRs are glutamatergic neurons that do not fire action potentials but rather display graded responses e.g. amplitude of response is proportional to the strength of the stimulus. In the presence of light, PhRs hyperpolarize leading to decreased glutamate release (Yau 1994). These cells contact both horizontal cells (HCs) and BCs via ribbon synapses in the OPL to pass this information for further processing (tripartite synapses) (Dieck & Brandstätter 2006). There are two types of PhRs: cones and rods. The cone PhRs are tuned to different wavelengths of light, mediating color vision and acuity in different species based on ecological adaptations (Baden & Osorio 2019; Baden et al. 2020). For example, in the mouse retina, there are two types of cone PhRs (medium and short wavelength). On the other hand, zebrafish are tetrachromats displaying four types of cones with distinct spectral opsin characteristics (Vihtelic et al. 1999). Zebrafish have long wavelength-sensitive (L or Red), medium wavelength-sensitive (M or Green), short wavelength-sensitive (S or Blue), and ultraviolet-sensitive (UV) cones. In addition, cones in zebrafish are classified based on their morphology as short single cones (UV cones), long single cones (S cones), and a double cone pair in which L and M cones intertwine their outer segments (Engström 1960; Meier et al. 2018). The cone PhRs form row mosaics presumably allowing a more efficient information flow and extraction of visual characteristics in a 2:2:1:1 density ratio (L, M, S, and UV cones) (Allison et al. 2010; Engström 1960; Raymond et al. 1993). On the other hand, rod PhRs are high–fidelity light detectors operating in low-light conditions (Kawamura & Tachibanaki 2012; Pugh 2018). Vertebrates have mainly one type of rod (expresses rhodopsin) apart from some amphibians that display two spectrally distinct rod types (Yovanovich et al. 2017).

1.1.2 Horizontal Cells

HCs lie at the interface between the INL and OPL and are part of a complex synaptic scheme (Wässle & Boycott 1991). HCs are OFF (hyperpolarize in the presence of light) Gamma-Aminobutyric acid (GABA)-ergic neurons, which fire graded potentials (Cervetto & Piccolino, 1974; Dowling & Ripps, 1973; Kaneko & Shimazaki, 1975). HCs integrate glutamatergic input from multiple PhRs and provide negative feedback to these cells (Liu et al. 2013; Vroman et al. 2013). This configuration allows HCs to contribute to the synaptic gain and integration time

of PhRs and the formation of the center-surround input to be processed by the BCs (Thoreson & Mangel 2012). HCs provide feedforward inhibition to BCs (Yang & Wu 1991), thus affecting them not only indirectly via the PhRs but also directly. In contrast to the mouse retina which has a single type of HC, in the zebrafish retina, four types of HCs have been distinguished based on morphology, connectivity, and molecular expression of connexins (Klaassen et al. 2016; Li et al. 2009; Yoshimatsu et al. 2014); the proteins that enable HCs to homotypically and laterally connect via gap junctions with one another. Thus, for example, H1, H2 and H3 types bear both dendritic and axonal terminals, whereas H4 is axon-less (Klaassen et al. 2011; Li et al. 2009). H1 cells are contacted by L, M, S, and UV cones and express connexin 52.9 (Cx52.9), H2 cells are innervated by M, S, and UV cones. H3 cells are innervated by S and UV cones while H4 cells contact rods and UV cones and exclusively express Cx52.7 (Klaassen et al. 2016; Li et al. 2009).

1.1.3 Bipolar Cells

BCs are glutamatergic neurons responsible for the processing of visual information in the INL integrating visual information from the PhRs and HCs and transmitting it to ACs and GCs, employing ribbon synapses at their axon terminals in the IPL (Euler et al., 2014). Based on their responses to light, BCs can broadly be characterized as ON (depolarization in response to light, metabotropic glutamate receptors and chloride channel forming glutamate transporter; (Grant & Dowling 1995) and OFF (hyperpolarization, AMPA/kainate glutamate receptors; (Connaughton & Nelson 2000; DeVries 2000). The axon terminals of OFF and ON BCs stratify in the upper and lower halves of the IPL respectively. In addition, based on PhR input (cone vs rod BCs), axonal stratification patterns (single- vs multi- stratified), morphology (dendritic and axonal arbors), and molecular marker expression up to fifteen subclasses of BCs have been described in rodents (Euler et al. 2014; Shekhar et al. 2016; Wässle et al. 2009). In the zebrafish retina, seventeen subclasses of BCs have been characterized to date (based on stratification and morphology) (Connaughton et al. 2004; Vitorino et al. 2009), which increase to thirty-three, if PhR connectivity is taken into account (Li et al. 2012). Functionally distinct BCs have been reported but not compared against existing subclasses (Bartel et al. 2021; Zimmermann et al. 2018). In contrast to the mouse retina (Shekhar et al. 2016), a comprehensive transcriptomic analysis of zebrafish BCs is yet to be published, leaving open the possibility of unidentified subclasses.

1.1.4 Amacrine cells

ACs are axonless interneurons located in the INL but also in the GCL (displaced ACs) (Wässle & Boycott 1991). ACs are part of a complex synaptic network. They receive glutamatergic input from BCs and in turn provide inhibitory feedback to BCs mediated by either GABA or glycine neurotransmitter release (Masland 2012). Additionally, ACs provide lateral inhibition to other ACs and feedforward inhibition to downstream GCs. ACs are dual-transmitter neurons and in addition to an inhibitory neurotransmitter (e.g. GABA or Glycine) they can additionally release glutamate, dopamine, acetylcholine, neuropeptides etc (Karten & Brecha 1983). Collectively, ACs are classified based on their dendritic arborisation field (narrow, medium and large), stratification (single or bi-stratified) in the IPL, and neurotransmitter release (Diamond 2016). In the zebrafish retina, twenty-eight different subclasses have been described (Connaughton et al. 2004; Jusuf & Harris 2009) whereas in the mouse retina sixty-three subclasses have been reported, based on recent transcriptome analysis (Yan et al. 2020, *bioRxiv doi: https://doi.org/10.1101/2020.03.10.985770*). Despite the plethora of AC subclasses, their involvement in the processing of visual information is not well understood. There are however some AC subclasses that have been extensively studied (mainly in rodents) such as the cholinergic Starburst ACs (SACs) that mediate, along with GCs, direction selectivity and the detection of centrifugal/centripetal motion (Diamond 2016; Euler et al. 2002; Hausselt et al. 2007) or A17 ACs that only contact rod BCs as part of the classical rod pathway (Chavez et al. 2010; Diamond 2016).

1.1.5 Ganglion cells

GCs are the output neurons of the vertebrate retina (Dhande & Huberman 2014). GC somata are located in the basal-most nuclear layer, the GCL where they receive input from retinal interneurons (BCs and ACs) (Wässle & Boycott 1991). GC axons converge to form the optic nerve, which in turn transfers information to higher brain areas. In the zebrafish retina, GC axons project to the midline and terminate in nine arborisation fields (AF1-9) before reaching the optic tectum, referred to as AF10 (Burrill & Easter 1995; Robles et al. 2014). Importantly, GCs do not exhibit graded responses but rather action potentials (Enroth-Cugell & Robson 1966; Kuffler 1953). These glutamatergic spiking neurons have been classified in approximately fourty-two categories based on dendritic morphology, expression of molecular markers, and light responses in the mouse retina (Baden et al. 2016; Goetz et al. 2022, *bioRxiv doi: https://doi.org/10.1101/2021.06.10.447922*; Sanes & Masland 2015; Shekhar et al. 2022). In the adult zebrafish, eleven to fourteen subclasses of RGCs have been identified based on

stratification within the IPL (monostratified, bistratified, diffused) or the OPL/IPL (biplexiform), their dendritic arborisation patterns (wide- or narrow field), and their response to light (ON-, OFF- or ON-OFF) (Mangrum et al. 2002; Robles et al. 2014; Zhang et al. 2010). Additionally, in zebrafish, GCs are further categorized based on their axonal projections in the tectum and pretectum, rounding up the number of different subclasses to 50 (Robles et al. 2014). Recently thirty subclasses of GCs have been recognized based on transcription profiles correlating molecular profiles with morphologically and physiologically known GC subclasses along with the underlying behaviors of the whole animal (Kölsch et al. 2020). Some GC subclasses stand out due to their direct link to a visual function and visual driven behavior. For example, the direction and orientation-selective GCs (Antinucci et al. 2016; Lowe et al., 2013; Wei 2018) or the intrinsically photosensitive melanopsin-containing RGCs (ipRGCs) which in mammals are implicated in non-image forming functions such as the regulation of the circadian rhythm and the pupillary reflex (Berson et al. 2002; Chen et al. 2011; Gamlin et al. 2007). In zebrafish, GCs have been shown to directly underlie specific behaviors such as prey-capture, looming-evoked escape, light preference, phototaxis (Kölsch et al. 2020; Kramer et al. 2019; Robles et al. 2014; Semmelhack et al. 2014; Temizer et al. 2015; Zhang et al. 2017).

1.1.6 Müller cells

MCs are the principal glial cell type of the vertebrate retina; other glial cells include microglia and astrocytes (Newman, 2009). MCs are derived from progenitors that are resident within the retina (Cepko et al. 1996). By contrast, astrocytes migrate into the retina via the optic nerve (Ling et al. 1989) and microglia derive from yolk sac progenitors (Alliot et al. 1999). MCs display polygonal-like shaped somata which are located in the INL and span the entire width of the retina (Magalhães & Coimbra 1972; Reichenbach & Reichelt 1986; Uga & Smelser 1973). Their apical and basal processes contribute to the formation of the outer and inner limiting membranes of the retina respectively (OLM and ILM, **Figure 1**). Apically they form adherens and tight junctions with the outer segments of the PhRs (Bunt-Milam et al. 1985; Omri et al. 2010) and basally MC endfeet delineate the end of the GCL. These cells form a pan-retinal network of homogeneous density albeit not a mosaic, contacting retinal interneurons in a stereotypic manner (Wang et al. 2017) and forming columnar micro units. MCs provide homeostatic support via water regulation, metabolic support, neurotransmitter exchange (Bringmann et al. 2006) and along with the microglia, play a role in immune support such as auto-phagocytosis (Bejarano-Escobar et al. 2017). Among newly assigned roles (Reichenbach & Bringmann 2013), MCs act as light guides due to their biophysical properties,

targeting light to the photoreceptor layer (Agte et al. 2011; Franze et al. 2007; Labin et al. 2014). Implications of this function are a reduction of light scattering and an increase in the signal to noise ratio (mainly affecting cone vision).

In the zebrafish retina, it has been suggested that MCs are essential for the overall tensile strength of the retinal tissue (MacDonald et al. 2015) and furthermore act as the resident stem cells in the mature retina, expressing molecular markers associated with retinal progenitors such as the *vsx2* (Vitorino et al. 2009) or the *glial fibrillary acidic protein* (*gfap*) (Bernardos & Raymond 2006; Marcus & Easter 1995) and providing the tissue with regenerative capacity (Bernardos et al. 2007). Thus, attempts have been made in the last years to elucidate the mechanisms underlying the MCs response to injury and apply the principles to higher vertebrates (Hoang et al. 2020; Jorstad et al. 2017; Lahne et al. 2020). MCs have thus far not been classified into subclasses although local differences in their regenerative response upon injury have been observed (Wan & Goldman 2017).

1.2 Retinal development

The retinal cell classes described above are derived from a common pool of multipotent progenitors (Fekete et al. 1994; Holt et al. 1988; Turner & Cepko 1987; Turner et al. 1990; Wetts & Fraser 1988). An influential model of retinogenesis, known as the temporal competence model, proposed that progenitors are capable of generating specific cell classes only during limited time windows. Moreover, as time progresses their potential, i.e. capacity to produce a particular cell class decreases (Cepko et al. 1996). Thus, distinct cell classes are generated in a sequential yet overlapping manner; with their 'birthdates' having been revealed by now classical studies (Sidman 1961; Young 1985). Almost 30 years later, there is still much that we do not comprehend about retinal development and fate specification, and as new experimental evidence is discovered the complexity of retinogenesis increases. In the following paragraphs, I will briefly describe certain aspects of retinal development that are relevant to this thesis (focusing mainly on studies of rodents and zebrafish). For a more extended version of this refer to the first manuscript of the current dissertation "Cellular and molecular determinants of retinal cell fate" (**Result section 2.1**).

1.2.1 Modes of divisions during retinal development

Three modes of divisions have been described to take place during CNS development (Götz and Huttner, 2005, Livesey and Cepko, 2001). First, progenitors can divide in a proliferative mode, generating two daughters that re-enter the cell cycle to expand the stem cell pool.

Secondly, a progenitor can divide differentiatively so that at least one daughter cell leaves the cell-cycle. Such a division mode, if asymmetric in its output, leads to the production of one progenitor and one post-mitotic cell which eventually differentiates. Finally, a progenitor can divide terminally. Such divisions are differentiative; both daughter cells exit the cell-cycle and acquire either the same (terminal symmetric) or different (terminal asymmetric) fates. Events that give rise to neurons are additionally referred to as neurogenic whereas the ones that give rise to glia cells as gliogenic. In zebrafish, it was illustrated that the division mode probability changes over time with proliferative events being more likely to occur early during retinal development whilst terminal events at the end of retinogenesis (He et al., 2012).

1.2.2 Birth of retinal cell classes

Retinal neuro- and gliogenesis take place in rodents from embryonic day 11 (E11) until post-natal day 7 (P7) (Rapaport et al. 2004; Young 1985) and in zebrafish from the first-day post fertilization until the third (Hu & Easter 1999). Remarkably, even though the period of cytogenesis is different across species, the sequence in which the different cell classes appear is conserved to a great degree (**Figure 2**) with GCs being consistently first born. This observation is not limited to rodents and fish but can be extended to Xenopus and Chick, as classical birth-dating studies have illustrated (Holt et al. 1988; Prada et al. 1991; Wong & Rapaport 2009). These studies were based on the use of tritiated thymidine or thymidine analogs and reported the time point at which a progenitor undergoes its last division and leaves the cell-cycle i.e., its "birth". Thus, as rodent studies indicated, in an overlapping manner, GCs are amongst the first to be generated followed by HCs and cone PhRs. Thereafter, ACs and rod PhRs start to differentiate, followed by BCs and MCs.

Nonetheless, species-specific differences that alter this sequence were detected as technical innovations became available. For example, *in vivo* imaging of HCs during retinal development in zebrafish, revealed that these cells are generated "late" during retinogenesis, in the time frame during which BCs are produced (Godinho et al. 2007) in contrast to the rodent retina, in which HCs along with GCs are among the first retinal neurons to be born. Further, a combination of the MAZe strategy (Collins et al. 2010)–a genetically encoded branding of clones -and *in vivo* imaging revealed that there is a sequence inversion among ACs and HCs in the zebrafish retina compared to other vertebrates (He et al. 2012). In recent days, classical birth-dating approaches are revisited in combination with specific molecular markers that allow for a more accurate identification of not only distinct cell classes but also cell subtypes within a cell class. For example, it was beautifully illustrated in rodent retina that certain BC subtypes

are generated before others within the same developmental window defined by older birth-dating studies combining up to sixteen different RNA markers differentially expressed in BC subtypes along with two different thymidine analogues (West et al. 2022).

However, in both mouse and zebrafish, the timing of MCs differentiation is still elusive. In rodents, MCs are thought to be among the last cells born and the same was thought to hold true for the fish retina as well. As previously mentioned, MCs share morphological features (retina-spanning) and display great molecular overlap with retinal progenitor cells (RPCs) (Blackshaw et al. 2004), thus rendering the development of tools that target exclusively differentiated MCs (and not RPCs) challenging. In addition, in lower vertebrates such as zebrafish, MCs retain their regenerative potential in order to respond upon injury (Goldman 2014) but importantly it was shown that these cells can re-enter the cell cycle in order to produce rod PhRs under physiological conditions (Bernardos et al. 2007). This led to the conclusion that a birth-dating approach might not be suitable to address when these cells appear during retinogenesis (Lenkowski & Raymond 2014). In addition, there is a body of work in the zebrafish retina which supports that MCs might not be the last cell type generated (Hu & Easter 1999; Peterson et al. 2000; Schmitt & Dowling 1999). For example, ultrastructural analysis via electron microscopy (EM) revealed that MCs display maturation characteristics as early as 50 hours post fertilization (hpf) (mid retinogenesis in fish and not late as expected). At this stage MCs start to form adherens junctions with the PhR inner segments in order to shape the outer limiting membrane (Schmitt & Dowling 1999). Another study used a battery of markers including Glutamine synthetase (GS) and Carbonic anhydrase (CAH) which are expressed in mature MCs and found that these proteins are present as early as 60hpf (Peterson et al. 2000). Whether the conflicting data reflect a species-specific difference, remains to be addressed.

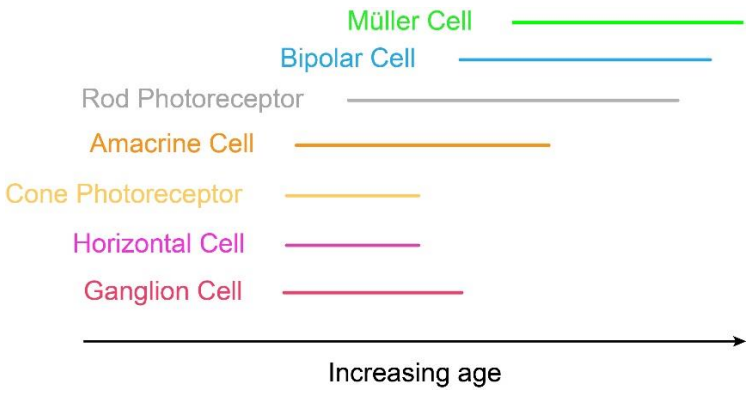

Figure 2: Birth of retinal cell classes in rodents.

1.2.3 Progenitor multipotency

Along with the birth-dating studies which address the temporal aspect of cell differentiation in the retina, there is a contemporary body of literature investigating the multipotency or lack thereof of RPCs (Fekete et al. 1994; Holt et al. 1988; Turner & Cepko 1987; Turner et al. 1990; Wetts & Fraser 1988). In these studies, progenitor cells were labelled via retroviral vectors or intracellular tracers at both embryonic and post-natal stages. Titration of the labelling agents was proposed to target individual progenitor cells, although direct evidence for this was not provided. The progeny derived from individually marked progenitors were traced at retinal maturity. The derived clones were analyzed and the cell types were identified based on their stereotypic location along with their particular morphological characteristics. These studies found a great variety of clones in terms of size and composition, which contained both neurons and MCs leading to the conclusion that RPCs are multipotent. In addition, the study of two-cell clones revealed two very interesting aspects of retinogenesis. Firstly, two-cell clones consisting of a single cell class e.g., rod PhRs alluded to the presence of biased/dedicated progenitors. Secondly, the scarcity of clones consisting of two MCs supported the idea that gliogenic progenitors do not exist. Time-lapse imaging of isolated rat retinal progenitors *in vitro* also revealed clones that varied in size and cellular composition with glial cells and neurons deriving from a common lineage (Gomes et al. 2011). Although these lineage experiments illustrated the broad developmental potential of RPCs, they did not specify whether these progenitors are intrinsically or extrinsically specified and which mechanisms underlie this variability.

1.2.4 Heterogeneity of progenitors

Major advances in the technologies utilized in single-cell transcriptomics studies have allowed scientists to address the heterogeneity of retinal progenitors (Shiau et al. 2021) and consequently the intrinsic differences that potentially allow for their diverse fate specification. Recent RNAseq data from the mouse retina allowed for a broad categorization of progenitors into primary vs. neurogenic progenitors and early vs. late ones. Primary progenitors were found to be enriched for cell cycle-related genes and neurogenic progenitors for proneural genes that are related to a differentiative fate. Despite the single-cell resolution and this gross classification along the temporal axis, the researchers did not identify different competence states of RPCs within the same developmental stage (Clark et al. 2019). RNAseq of the zebrafish retina at distinct days post-fertilization did not allow for the segregation of progenitors into “primary” and “neurogenic” categories, potentially due to the crude temporal

resolution (Xu et al. 2020). Nonetheless, *in vivo* imaging clearly illustrated a shift from proliferative divisions (both daughter cells re-enter the cell cycle) presumably representing primary progenitors to differentiative and terminal divisions (production of a progenitor cell and a post-mitotic cell or two post-mitotic cells) corresponding to neurogenic progenitors as retinogenesis progresses (He et al. 2012). Subtle intrinsic differences might be overlooked in the volume of the data and the manner of analysis (Shiau et al. 2021). Nonetheless, additional experimental evidence supports the idea of intrinsic fate determination at least to some extent.

1.2.5 Intrinsic determinants influencing progenitors

Manipulation of transcription factors has illustrated how the intrinsic properties of RPCs affect the fate output and what regulatory networks are at play. For example, Orthodenticle homeobox 2 (Otx2), Vsx2 and PR-SET domain zinc-finger (Prdm1 or Blimp1) are transcription factors integral to the decision of BC versus rod PhR fate. *Otx2* is expressed in the post-natal murine retina in the retinal pigmented epithelium, the cytoplasm of rod PhRs and in the upper part of INL, where it is expressed by some BCs (Baas et al. 2000). In *otx2* mutants both BCs and PhRs are reduced illustrating the importance of Otx2 for both fates (Koike et al. 2007). Prdm1 (Hsiau et al. 2007) is a transcription factor which represses BC-related genes in *otx2* cells (Brzezinski et al. 2010, 2013; Hsiau et al. 2007; Katoh et al. 2010; Park et al. 2017). Vsx2 is instructive in BC generation (Burmeister et al. 1996; Green et al. 2003; Horsford et al. 2004) and suppresses PhR related genes via direct binding at relevant regulatory sequences, thus inhibiting the acquisition of the PhR fate (Dorval et al. 2005; Livne-bar et al. 2006). Otx2 seems to be upstream of both Prdm1 and Vsx2 and regulates their expression via enhancer sequences. In turn Prdm1 and Vsx2 suppress the expression of one another to solidify the rod PhR over the bipolar cell fate (Brzezinski et al. 2010, 2013; Goodson et al. 2020a,b; Katoh et al. 2010; Kim et al. 2008; Mills et al. 2017; Wang et al. 2014). Another example is the specification of ACs. *Ptf1a* is expressed in AC committed progenitors (Fujitani et al. 2006; Jusuf et al. 2011; Nakhai et al. 2007) and has been shown to be activated upon interaction of Forkhead Box N4 (Foxn4) with Retinoid-related orphan receptor β (RORβ1) (Liu et al. 2013, 2020). Beyond individual transcription factors, signaling pathways also play an important role in fate specification. Notch signaling is instrumental for gliogenesis, namely the generation of MCs (Bao & Cepko 1997; Furukawa et al. 2000; Jadhav et al. 2006a, 2009; Perron et al. 1998; Scheer et al. 2001; Vetter & Moore 2001). Manipulations of the Notch1 receptor along with its downstream targets such as the hairy and Enhancer of split (E(spl)) homologs transcription factors (Hes) and Hairy/Enhancer-of-split related with YRPW motif (Hey also known as Hers) e.g.

Hes1(Furukawa et al. 2000), Hers2 (Ohtsuka et al. 1999; Satow et al. 2001), and Hes5 (Hojo et al. 2000) have illustrated that sustained Notch signaling is not only instructive of the glial fate but in parallel suppresses other neuronal fates of late born retinal cell types such as rod PhRs. Additionally, it has been shown that ablation of *notch1* in postnatal RPCs affected not only MCs but also BCs whilst increasing the number of rod PhRs (Jadhav et al. 2006b) reflecting a potential need of Notch signaling in the BC fate.

1.2.6 Extrinsic determinants influencing progenitors

Few studies provide evidence that extrinsic factors play a role not so much regarding the specification of individual cells but rather the control of an entire cell class. For example, it was shown *in vitro* in chick that the production of new GCs is negatively affected by pre-existing differentiated GCs (Waid & McLoon 1998) and it was later shown that the Sonic hedgehog (Shh) pathway is responsible for this inhibition in chick and mouse (Wang et al. 2005; Zhang & Yang 2001). A similar approach was used in the rat retina. Embryonic rat RPCs (E16) were reaggregated with postnatal retinal cultures that were depleted or enriched for differentiated ACs. The production of newly generated ACs was enhanced in the postnatal cultures depleted of mature ACs leading to the conclusion that differentiated cells affect the fate of newly generated ones albeit within the same lineage (Belliveau & Cepko 1999). Additionally, it has been suggested that the embryonic cellular milieu could respecify postnatal RPCs fated to give rise to rod PhRs into acquiring the BC fate (Belliveau et al. 2000).

1.2.7 Stochastic model versus deterministic model

In order to appreciate how retinal circuit elements are generated two broad models have been proposed: the stochastic and the deterministic model. The stochastic model predicts that RPCs are in principle equipotent and that they can divide and/or lead to different fates with a fixed probability within a window of time (Gomes et al., 2011; He et al., 2012: Boije et al., 2015). In the deterministic model the lineages are hardwired and either multiple specified progenitors exist in parallel or a single population of RPCs progresses from one competence state to the other irreversibly (competence model) and produces the different retinal lineages (Cepko 2014).

The variability in the size and clone composition observed in classical lineage studies (Holt et al. 1988; Turner & Cepko 1987; Turner et al. 1990) cannot be explained exclusively based on the deterministic model. Independent studies have illustrated a degree of stochasticity during retinal development (Boije et al. 2015; Gomes et al. 2011; He et al. 2012). Indeed,

investigations of isolated postnatal rat retinal progenitors *in vitro* (Gomes et al. 2011) and progenitors in the zebrafish retina *in vivo* (He et al. 2012), revealed that individual progenitors choose among distinct division patterns (proliferative, differentiative and, terminal) and cell fates with fixed and predictable probabilities. A subsequent study in the zebrafish retina, suggested that the cellular variability of the derived clones could be explained to a certain degree by the combination of key transcription factors/fate determinants (Atoh7, Ptf1a and Vsx1) and their probabilistic and independent action (Boije et al., 2015). In this latter study, also the effect of extrinsic factors was accounted for in the determination of the RPCs. Interestingly, morphant background (either for *ptf1a* and or *atoh7*) seemed to delay the start of neurogenesis by half cell cycle of transplanted *wild-type* clones. Adjustment of the author's model for this delay predicted the correct outcome of these lineages.

Nonetheless, stochastic mechanisms cannot explain the presence of biased progenitors that terminally divide and generate a specific cell lineage (Engerer et al. 2017; Godinho et al. 2007; Hafler et al. 2012; Suzuki et al. 2013; Weber et al. 2014b) at later stages of retinal development. For example, *oligodendrocyte transcription factor 2* (*olig2)* expressing progenitors in the mouse retina terminally divide to generate either two cone PhRs or two HCs (Hafler et al. 2012), *thyroid hormone receptor β2* (*trβ2)* progenitors in the zebrafish retina are biased towards the production of red cone PhRs (Suzuki et al. 2013) and *vsx1* expressing progenitors towards the production of BCs (Engerer et al. 2017; Weber et al. 2014b). Thus, biased progenitors imply that stochasticity does not universally apply and might be more relevant for the early neurogenic phase. Additionally, a recent study in zebrafish revealed a deterministic component in the generation of MCs from the beginning of retinogenesis (Rulands et al. 2018).

Collectively, these studies underline that in order to have an invariable tissue to a certain degree both stochastic and deterministic mechanisms need to be at play. Within a temporal window in which a selected number of fates are permitted (deterministic aspect) a progenitor can stochastically "choose" among them to satisfy the overall ratio of the individual cell classes by the independent activation of key transcription factors (Boije et al. 2015).

1.3 Notch signaling in development

If a developmental biologist was asked to single out a conserved signaling pathway crucial for all organisms from the moment of establishment of planar polarity to adulthood, the answer would have to be the Notch signaling pathway. Notch was first identified in Drosophila melanogaster as a genetic locus that affects the decision between neuronal and epidermal fate

(Artavanis-Tsakonas et al. 1999; Poulson 1940). Since then more than 80 years of extensive research in invertebrates and vertebrates has revealed a pleiotropic role of Notch signaling which has yet to be completely understood.

For the purpose of this thesis, I will focus on the main roles of Notch signaling in shaping CNS development after a brief overview of the mechanistic details of this pathway.

1.3.1 Overview of the Notch signaling cascade (Canonical pathway)

For the initiation of Notch signaling two adjacent cells need to interact i.e., the signal-sending cell and signal-receiving cell. Although the complete biophysical processes are not yet fully clear, the current proposed model is as follows (Henrique & Schweisguth 2019; Sprinzak & Blacklow 2021) (**Figure 3**):

1. On the membrane of the signal-sending cell, the extracellular domain of the Delta/Serrate/Lag-2 family (DSL) ligand interacts with the extracellular domain of the Notch receptor on the signal–receiving cell triggering a cascade of events known as the canonical Notch pathway (**Figure 3, step 1**).

2. On the signal-sending cell side, ubiquitylation of the ligand via Mindbomb induces the formation of clathrin-coated vesicles and the subsequent endocytosis of the ligand (**Figure 3, step 2 & 3**).

3. Endocytosis of the bound ligand allows a negative regulatory domain of the Notch receptor in the extracellular space to be exposed.

4. Consequently, A Disintegrin and Metalloproteinase domain-containing protein 10 (ADAM10) cleaves the Notch Extracellular Domain (NECD) allowing for *trans*-endocytosis to the signal sending cell (**Figure 3, step 3**).

5. In parallel, γ-Secretase leads to the release of the Notch Intracellular Domain (NICD) in the cytoplasm of the signal-receiving cell (**Figure 3, step 4**).

6. NICD is translocated to the nucleus of the cell (**Figure 3, step 5**).

7. There, NICD forms a complex with the recombining binding protein suppressor of hairless (RBP-J), the mastermind-like protein (MAMLs) and other co-activators (**Figure 3, step 5**).

8. Expression of the downstream Notch effectors depends on a balance between the NICD transcription activation complex and the transcription repression complex consisting of RBP-J and either co-activators or co-repressors respectively.

This mechanism refers to an interaction of *trans*-activation. Interestingly, it has been proposed that the contact area between the two cells correlates with Notch signaling strength, so that the smaller the contact area the smaller the signal (Shaya et al. 2017). Moreover, the effect of Notch signaling on the signal-sending cell depends on the combination of ligand/receptors. In the case of lateral inhibition for example during which the signal-receiving cell inhibits the sigmal-sending cell to acquire the same fate, the expression of Notch effectors (see **section 1.3.3**) will decrease the levels of the Notch receptor in the signal-receiving cell leading to a negative feedback loop to the signal-sending cell. Hence, lateral inhibition can amplify differences in Notch signaling amongst neighboring cells leading to different fate outcomes. Conversely, Notch induction leads to a positive feedback loop amongst the two interacting cells which can lead to the acquision of the same fate (Sjöqvist and Andersson, 2019).

In addition, it has been observed that ligands and receptors located in the same cell can either *cis*-inhibit (Baek et al. 2018) and *in vitro* even *cis*-activate (Sprinzak et al. 2010, 2011) creating very complex signaling patterns. For example, in Droshophila, during the dorso ventral patterning of the wings, Fringe proteins glycosylate the Notch1 receptor. When Fringe is not present, Jagged ligand cis-inhibits Notch receptor and becomes unavailable for trans-interactions. Conversely, in the presence of Fringe, glycosylated Notch receptors cannot cis and trans interact with Jagged 1(JAG1) resulting to a cell that can only trans-interact with Delta and send JAG1 signals to the surrounding cells (Bray 2016). Additionally, it is worth mentioning that there is also a non-canonical Notch signaling cascade although its role *in vivo* is not well understood yet (Andersen et al. 2012). As its name might imply the non-canonical Notch signaling does not necessarily require the ligand receptor interaction or other components like the γ-Secretase or RBP-J. Instead, there is a crossover with other signaling pathways such as the Wnt/β –catenin signaling cascade.

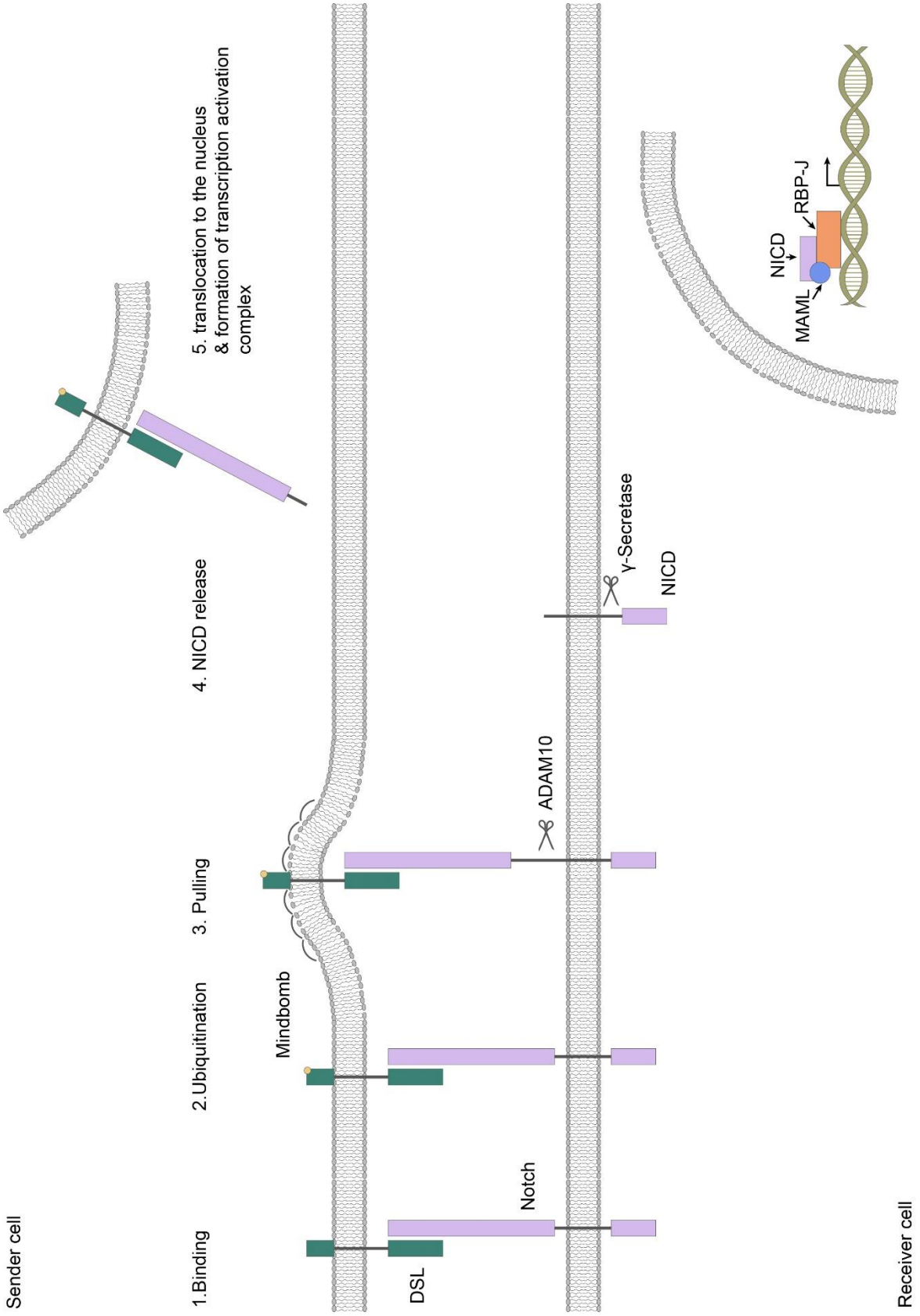

Figure 3: *Trans*-activation of Notch signaling. Figure adapted with permission from Sprinzak and Blacklow, 2021; copyrights Annual Reviews, Inc. and Bray, 2006; copyright Springer Nature BV.

1.3.2 Overview of Notch receptors and ligands expressed in CNS

Notch signaling requires two components: a Notch ligand in the membrane of the signal-sending cell and a Notch receptor in the membrane of the signal-receiving cell (at least in the case of *trans*-activation). Both Notch ligands and receptors are highly conserved among species, although the number of homologs varies. In the CNS of rodents four Notch receptors (Notch1-4), two Delta-like ligands (Dll1 and 3), and two Jagged ligands (Jag1-2), all belong to the DSL family (based on Uniprot, an open access database of protein sequences). On the other hand, the zebrafish CNS is characterized by the presence of four Notch receptors (Notch1a, 1b, 2b and 3), four Delta ligands (Delta A-D) and three Jagged ligands (Jag1a, 1b and 2b), based on the zfin database. Both Notch receptors and DSL ligands are single transmembrane proteins with extracellularly long chains of Epidermal growth factor (EGF)-like repeats (Kopan & Ilagan 2009; Kovall & Blacklow 2010). The Notch extracellular domain contains (from N' to C' terminal) twenty-nine to thirty-six EGF-like repeats, some of which are important for the ligand interaction (eleventh-twelfth & twenty-fourth to twenty-ninth). Subsequently, there is a negative regulatory region (NRR) consisting of three Abnormal cell lineage protein 12 (Lin12) Notch repeats and the heterodimerization domain (HD). After the transmembrane domain (TMD) which is the location of several cleavage positions, there are several regulatory intracellular regions of the receptor, which consist of the NICD. Firstly, The RBP-J association module (RAM) allows the interaction with RBP-J and the formation of the transcription activation complex. Downstream of RAM there are seven ankyrin repeats (ANK). At the end is the Transactivation domain, which includes the nuclear localization signals (NLSs) that directs NICD to the nucleus and a peptidic sequence that allows for the fast degradation of the receptor rich in proline-glutamic acid-serine and threonine motifs (PEST). On the other hand the DSL ligands consist of (from N' to C' terminal) the N'-terminal domain (MNLL), a Delta/Serrate/LAG-2 (DSL) domain, Delta and OSM-11-like proteins domain (DOS) which has two specialised EGF repeats, followed by EGF-like repeats (six to eight for Delta-like and fifteen to sixteen for Jagged ligands) and finally a TMD. In contrast to Delta, Jagged has between the EGF-like repeats and the TMD a von Willebrand Factor type C domain (vWF) and six Jagged domains. Both receptors and ligands display a signal peptide at the N- terminal end (Andersson & Lendahl 2014; Chillakuri et al. 2012; Kopan & Ilagan 2009) (**Figure 4**).

Notch receptors and ligands can be subjected to post-translational modifications in the Endoplasmic Reticulum (ER) such as N-linked glycans and O-linked glycan found on EGF repeats affecting various aspects such as the receptor proper conformation but also trafficking

to the membrane (Pandey et al. 2020). In addition, these modifications affect the interaction between the Notch receptors and DSL ligands and their differential binding strength. In the case of *cis*-inhibition, post-translational modifications affect the cascade's directionality (Kakuda & Haltiwanger 2017; Kakuda et al. 2020; Sprinzak & Blacklow 2021). Additionally, even in the case of *trans*-activation the exact combination of ligand and receptor expressed can affect the fate of the cell. For example, interaction of Dll/Notch leads to a salt and pepper pattern in which one cell can differentiate (signal sending) and laterally inhibit the differentiation of the other (signal-receiving cell). On the other hand, expression of Jag1 in the signal-sending cell will lead to induction of the same fate in both the signal-sending and signal-receiving cell driving the propagation of fate (Boareto 2020).

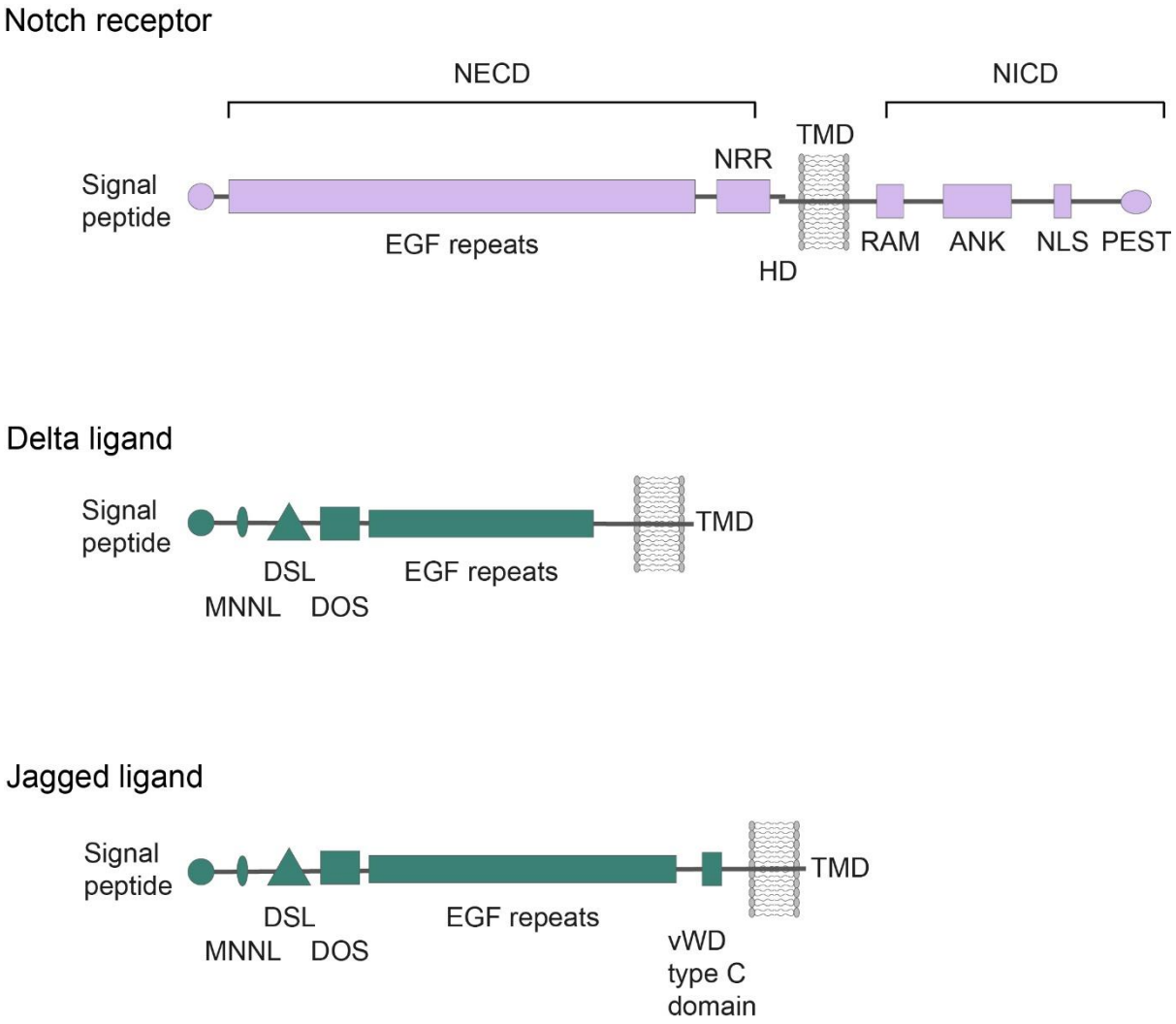

Figure 4: Overview of Notch receptor and ligand structural domains. Notch receptor: NECD (Notch extracellular domain), EGF (epithelial growth factor), NRR (negative regulatory region), HD (homodimerization domain), TMD (transmembrane domain), NICD (Notch intracellular domain), RAM (RBP-J association module), ANK (ankyrin repeats), NLS (Nuclear localization signal), PEST (proline-glutamic acid-serine and threonine motifs). Delta

ligand: MNNL (N'-terminal domain), DSL(Delta/Serrate/LAG-2), DOS (Delta and OSM-11-like proteins domain). Jagged ligand: vWD type C domain (von Willebrand Factor type C domain).

1.3.3 Notch downstream effectors

The Hes family (Hes1, 5, 7) represents the main downstream targets of Notch signaling (Boareto 2020; Nan & Zou 2021). Hes family members– are basic helix–loop–helix (bHLH) transcription factors that can act both as activators or repressors of gene expression. Hes factors have a bHLH domain (DNA binding domain and homo-/heterodimer formation), an Orange domain (protein interaction specificity), and the WRPW domain (Trp-Arg-Pro-Trp motif) which recruits the repressors Transducin-like E(spl) (TLE1–4)/Groucho-related gene (Grg). Homodimers of Hes inhibit gene expression by targeting N-box and class-C elements of the promoter of proneural genes like *ascl1 (achaete-Scute Complex Homolog-Like 1also known as mash1)*, *neurogenin 2(Ngn2)* (Heng & Guillemot 2013; Lee 1997) which in turn are responsible for the activation of cell cycle progression and differentiation genes (mediated by a sequence in the N terminal of these genes). Additionally, Hes1 has been shown to autoregulate its expression levels via N-box inhibition of its own promoter. This self-inhibition along with the active degradation of the protein (half-life ~20mins in mice) creates an oscillatory mode of expression with a period of 2 hours (Hirata et al. 2002; Shimojo et al. 2008). This oscillatory pattern of action is reflected in downstream targets of Hes1, for example, Dll-1 in mice and Delta C in zebrafish. Several models have been proposed as to how these oscillations can contribute to cell fate decisions with downstream Notch factors, activators, repressors, and target genes being expressed out of phase or in phase and affecting the outcome accordingly (Shimojo et al. 2008, 2016).

Moreover, another two groups of downstream targets of Notch signaling have been identified. The first one is the Hey protein family (Kokubo et al. 1999; Leimeister et al. 1999). These transcription factors are related to the *hes* genes and three Hey factors have been identified in mammals: Hey1, Hey2, and HeyL. They have a similar structure as the Hes transcription factors apart from the C-terminal sequence. The Hey family expresses the YRPW sequence domain but cannot strongly interact with the TLE factors as Hes do. Nonetheless, Hey family members act as transcriptional repressors and form dimers with both Hey and Hes factors (Iso et al. 2003; Kobayashi & Kageyama 2014; Weber et al. 2014a). Another group of downstream Notch factors consist of the Inhibitors of DNA binding and cell differentiation (Ids 1-4). Ids have a HLH domain (absence of the basic domain that permits DNA binding) that allows them to target proneural bHLH transcription factors (Benezra et al. 1990; Ruzinova & Benezra 2003)

forming non-functional heterodimers and thus inhibiting cell cycle progression and differentiation. Additionally, Ids can form heterodimers with Hes factors, inhibiting their ability to bind to the N-box regions and lowering their efficiency of binding to the class-C ones, relieving their auto-repression (Bai et al. 2007).

1.3.4 Notch signaling in the development of the CNS

One of the first experiments testing a role for Notch in the vertebrate nervous system employed the use of a mutated Notch receptor homologue in Xenopus (*xotch ΔE*). *Xotch ΔE* was unable to dimerize (lack of the extracellular dimerization properties of the receptor) and could lead to embryos displaying dorso-anterior defects along with a spatially restricted increase in neuronal, muscle or epidermal tissue (Coffman et al. 1993). Along the same lines, when the Notch receptor ligand was overexpressed (*x-delta-1*), primary neurogenesis was inhibited in these embryos (Chitnis & Kintner 1996; Chitnis et al. 1995), strengthening the concept that Notch signaling and lateral inhibition were at play in order to regulate neuronal differentiation. *In vitro* work in which P19 cells (murine carcinoma cells) were transfected with a dominant gain-of-function mutant of murine-*notch* showed that Notch could suppress neuronal differentiation albeit leaving gliogenesis unaffected (Nye et al. 1994). Meanwhile, the identification of the *delta* and *notch* homologs in chick (Henrique et al. 1995; Myat et al. 1996) led to the conclusion that Notch signaling bears a conserved role in neurogenesis, among vertebrates.

Over the years, a collective body of work has identified three roles of Notch during development:

1) Maintenance of neural stem cells.

2) Induction of gliogenesis.

3) Effect on binary fate decisions.

I will focus on the role of Notch in the developing CNS, including the retina.

1.3.4.1 Maintenance of neural stem cells

During cortical neurogenesis, there are several sources of progenitors that are apically or basally located, display different morphological characteristics (mono-, bi-vs non-polar) and give rise to neurons directly or in-directly (Arai & Taverna 2017; Fernández et al. 2016). Amongst these, there is a progenitor population named radial glia cells (RGCs). RGCs divide asymmetrically generating a radial glia cell and another cell type that is either a neuron or an intermediate progenitor cell (IPC) (Anthony et al. 2004; Malatesta et al. 2000, 2003; Miyata et

al. 2001; Noctor et al. 2001). The latter cell type can either self-amplify or self-deplete to generate two neurons (Arai & Taverna 2017; Fernández et al. 2016). In addition to being progenitors, RGCs act as a scaffold for radial migration (Rakic 1972) of neurons to the proper cortical layer. At the end of neurogenesis, RGCs acquire morphological and molecular characteristics of astrocytes (E16)-the nascent glia type of the cortex (Noctor et al. 2004; Schmechel & Rakic 1979; Voigt 1989).

Notch has been shown to play a major role in neural stem cell maintenance in the telencephalon. *Notch1* over-expression in neuroepithelial progenitors in the mouse cortex, via *in utero* electroporation, illustrated that Notch signaling could promote the formation of RGCs (Chambers et al. 2001; Gaiano et al. 2000). Activation of Notch in RGCs requires both IPCs and existing post-mitotic neurons that act as Notch ligand presenting cells (Kawaguchi et al. 2008; Nelson et al. 2013; Yoon et al. 2008). This interaction results in the expression of glia related genes like *brain lipid binding protein* (*blbp*) a direct target of Notch signaling (Anthony et al. 2005) and *erb-b2 receptor tyrosine kinase 2* (*erb-b2*) via both Notch canonical (Rbp-j mediated) and non-canonical pathways (Patten et al. 2003, 2006). Loss of function experiments confirmed the importance of Notch in the maintenance of this progenitor pool. For example, *rbp-j* conditional knock out mice led to the depletion of RGCs (Imayoshi et al. 2008, 2010) whilst inactivation of *hes1, 3* and *5* (null mice, lethal phenotype) accelerated cell differentiation at the expense of later-born neurons, depleted RGCs and led to a range of deficits in many regions of the CNS (Hatakeyama et al. 2004). Double knock-out mice for *hes1* and *hes5* led to a similar phenotype in the telencephalon (Ohtsuka et al. 2001). It was suggested that Hes1 and Hes5 might not act on their own but along with other downstream Notch effectors such as Hey1 and 2 and inhibit Ascl1 (Mash1) and Math3 (a pro-differentiation bHLH factor also known as Neuronal Differentiation 4, NeuroD4) in order to maintain RGCs during cortical development (Sakamoto et al. 2003). The oscillatory mode of expression of the *hes* genes which is reflected in the proneural bHLH factors is important for the maintenance of the RGC population whereas the sustained expression of proneuronal genes such as *asclI* (*mash1*) leads to neuronal differentiation (Baek et al. 2006; Imayoshi & Kageyama 2014; Imayoshi et al. 2013; Kageyama et al. 2009). Following the self-renewing asymmetric division of an RGC, the decision of whether the sibling cell will become an IPC, or a neuron lies in the epigenetic silencing of *hes1* and consequently the presence of proneuronal factors such as Ngn2 and Ascl1 which regulate Dll1 levels directly (Castro et al. 2006). Induction of Notch signaling in the remaining sibling cell will prohibit its differentiation (and presumably return to the RGC fate) (Agirman et al.

2017). This mechanism allows for the temporal control of neurogenesis (Kawaguchi et al. 2008). Recently, it was illustrated *in vitro* that Bcl6 – a transcriptional repressor -which among others represses *hes5* – acts in concert with Sirt1 to suppress Notch signaling in order to promote neurogenesis (Bonnefont et al. 2019). In the developing zebrafish brain, neurons are derived from the asymmetric divisions of RGCs in a stereotypic fashion. Basally located sibling cells expresses high Notch levels that will permit the re-entrance to the cell cycle whereas the apically located sibling cell will differentiate (Dong et al. 2012).

Notch signaling has also been implicated in the cerebellum. Conditional knock out of *notch1* in neuroepithelial progenitor cells (Lütolf et al. 2002) led to the initiation of their differentiation. However rather than acquiring a neuronal fate, these differentiating cells were eliminated via apoptosis leading to a Purkinje cell reduction in the adult cerebellum. In addition, it was illustrated that the Notch2 receptor is expressed by granule cell progenitors (GCPs) during proliferation but not in mature granule cells. Overexpression of *notch2* or the Notch activated gene *hes1* both *in vitro* and *ex vivo* stalled the differentiation of GNPs (Solecki et al. 2001). Recently, it was shown that the differentiation vs proliferation switch is mediated via intercellular interaction between two populations of GCPs. GCPs that express *jag1* (Notch-OFF) will give rise to mature granule cells whilst hindering the differentiation of their neighbouring 'Notch-ON' GCPs via Notch2 -Hes1 components (Adachi et al. 2021).

Implications of Delta-Notch signaling in primary neurogenesis have been explored both in rodents and fish (Dornseifer et al. 1997; Haddon et al. 1998; Pompa et al. 1997). Targeted mutagenesis of the downstream Notch gene *hes1 in vivo* resulted in defects of the neural tube and a lethal phenotype in null mice around birth. The proneural factor Ascl1 (Mash1) was upregulated and precursor cells in the telencephalon differentiated earlier than in wild-type embryos (Ishibashi et al. 1995). Dll1 and Dll4 are reported to be transiently expressed in the rodent spinal cord by differentiating neurons regulating neurogenesis in the pV2 domain (Rocha et al. 2009). In that context, Dll1 was predicted to maintain progenitors in a proliferating state while Dll4 was suggested to control the differentiation and diversity of the p2 domain. Furthermore, conditional knock-out of *mindbomb*, a gene encoding for a protein responsible for the endocytosis of Notch ligand, led to a depletion of progenitors in the spinal cord and premature differentiation (Kang et al. 2013). In the developing zebrafish spinal cord both precursor cells and neurons express *delta* and *notch* genes. *DeltaA* mutant embryos displayed fewer neural precursor cells along with excess numbers of early specified neurons and fewer late-specified neurons and glia (Appel et al. 2001). Later on, it was shown that Notch signaling

affects the maintenance of *olig2* precursors (Shin et al. 2007) whilst different combinations of Notch (Notch1a,b and Notch3) and Delta ligands (Delta A and D) regulate the maintenance of the p2 progenitors (Okigawa et al. 2014) in the ventral spinal cord. Further, *in vivo* imaging revealed that expression of high levels of DeltaD ligand in transient basal protrusions of differentiating neurons provides lateral inhibition to progenitor cells at different distances regulating their differentiation (temporally and spatially) (Hadjivasiliou et al. 2019).

In Drosophila, it was shown that the expression of the activated Notch receptor in photoreceptor precursor cells transiently inhibited their differentiation and led to the acquisition of an incomplete neuronal identity (Fortini et al. 1993). Furthermore, later pivotal work in Xenopus and Chick retina revealed the conserved role of Notch in maintaining a pool of neuroepithelial progenitors throughout retinogenesis, which sequentially adopt different fates via additional mechanisms (Ahmad et al. 1997; Austin et al. 1995; Dorsky et al. 1995, 1997). For example, injection of an activated form of Xotch in retinal cells inhibited cell differentiation (Dorsky et al. 1995) whilst it was shown *in vitro* and *ex vivo* chick tissue, that GCs were derived from a pool of progenitors via Notch action (Austin et al. 1995). Extension of these observations illustrated that downregulation of Delta 1 ligand results in an increase of GCs and conversely, exogenous supply of Delta leads to a reduction of differentiated GCs (Ahmad et al. 1997). Furthermore, when *delta* mRNA was misexpressed early during Xenopus retinogenesis, cells adopted GC and cone PhR fates whereas, at later stages transfection of *delta* led additionally to the generation of rod PhRs at the expense of later-born neurons, underlying the role of Notch in temporal control of the switch to neurogenesis (Dorsky et al. 1997). Further studies, in the rodent retina, shed light on the involvement of Notch in suppressing specific cell fates (Furukawa et al. 2000; Hojo et al. 2000; Jadhav et al. 2006b; Ohtsuka et al. 1999; Satow et al. 2001). For example, Notch1 receptor not only keeps retinal progenitors in a proliferative state but seems to specifically inhibit PhR fate, since progenitors in which *notch1* was deleted predominantly produced rod and cone PhRs (Jadhav et al. 2006b). In the zebrafish retina, a model that implicates Notch in neuronal differentiation was developed. During retinal development, the nuclei of neuroepithelial progenitors move apically and basally during the cell cycle in a phenomenon named interkinetic nuclear migration (INM). Studies suggested that asymmetric distribution of Notch components along the apico-basal axis influences the differentiation of the retinal neurons, with Notch signaling predominantly being active on the apical side and promoting proliferation (Bene et al. 2008; Murciano et al. 2002) . Nonetheless, a more recent study argued against this model, supporting a role for Notch signaling in

influencing neurogenic specification via the asymmetric distribution of Sara-positive endosomes (Smad Anchor Receptor Activation) implicated in the trafficking of internalized Delta and Notch (Nerli et al. 2020).

1.3.4.2 Notch signaling in gliogenesis

The role of Notch in glial fate specification and differentiation is apparent throughout the CNS. Viral-based over-expression of the Notch1 receptor in rodents at either embryonic or postnatal stages led to an increase of glial cells in cortical regions (Chambers et al. 2001; Gaiano et al. 2000). Conditional ablation of *rbp-j* during development in CNS progenitors led to decreased number of astrocytes both in the diencephalon and in the spinal cord of these mice along with decreased expression of SRY-Box Transcription Factor 9 (Sox9) - a glia-specification regulator (Stolt & Wegner 2010; Stolt et al. 2003; Taylor et al. 2007). Expression of the *notch1* and *notch3 intracellular domains* in multipotent progenitors derived from adult rat hippocampus induced the formation of astrocytes (Tanigaki et al. 2001) as did incubation of neurospheres *in vitro* with different soluble Notch ligands (Grandbarbe et al. 2003). In 2009, Namihira et al. extended these observations and provided a mechanistic insight into the Notch induced astrocytic differentiation, revealing that the activation of Nuclear factor I (NFI) and the demethylation of astrocyte-specific promoters were key mediators (Namihira et al. 2009). In the cerebellum, in addition to astrocytes, Bergmann glia are also affected by Notch signaling. Bergmann glia are thought to derive, like cortical astrocytes, from the direct transdifferentiation of RGCs (Buffo & Rossi 2013). Ablation of *notch1* in the cerebellum resulted, amongst other phenotypes, in the reduction of glial cells, including cerebellar astrocytes (Lütolf et al. 2002), whilst Cre-mediated deletion of *notch1*, *notch2*, and *rbp-j* genes specifically in Bergmann glia resulted in morphological irregularities and disorder of the cerebellar molecular layer already present from postnatal day 3 (Komine et al. 2007). These findings confirm the interaction of Bergman glia with Purkinje cells, during development, via Notch signaling in order to differentiate (Buffo & Rossi 2013). Of note is that not only canonical Notch signaling but also the non-canonical pathway plays a role during the monolayer formation of Bergman glia (Eiraku et al. 2005).

The contribution of Notch signaling in oligodendrocyte (OL) generation is more complex. OLs are generated by divisions of oligodendrocyte precursor cells (OPCs) (Hill et al. 2014; Marisca et al. 2020; Zhu et al. 2011), which in turn are derived from the ganglionic eminences and a specialized domain of the ventral ventricular zone in the spinal cord (Barry & McDermott 2005; Kessaris et al. 2006; Liu et al. 2002). Notch signaling seems to primarily suppress the

differentiation of OLs. This was illustrated in *in vitro* cultures of isolated and purified OPCs from the rat optic nerve upon exposure to a battery of Notch ligands (Wang et al. 1998) and confirmed *in vivo* (Gaiano et al. 2000). Specifically, inhibition of the Notch1 receptor in OPCs of the mouse spinal cord led to positional and temporal disruptions in their differentiation into OLs (Genoud et al. 2002). Additionally, *notch1* heterozygous mouse mutants were characterized by increased and ectopic myelination in the brain presumably by the prematurely differentiated OLs (Givogri et al. 2002). Studies in the zebrafish spinal cord support a model in which Notch signaling plays a dual role (data from the ventral spinal cord). According to this, Notch signaling induces OPC fate in line with a glial specification role. Nonetheless, as in rodents, Notch inhibits OPC differentiation into immature /pre-myelinating OLs (Park & Appel 2003; Park et al. 2005).

During retinogenesis, Notch signaling maintains RPCs in a proliferative state. Nonetheless, as retinal development progresses Notch signaling is downregulated in post mitotic cells that acquire a neuronal identity but not in cells that will acquire a Müller cell fate (Bao & Cepko 1997; Furukawa et al. 2000; Jadhav et al. 2006a; Vetter & Moore 2001). Multiple components of the Notch pathway have been shown to be instrumental in glial specification. In rats, retroviral delivery of a constitutively active form of Notch1 interfered with the differentiation of multiple retinal neurons. The derived clones displayed aberrant morphology and consisted of Müller cells and progenitors (Bao & Cepko 1997). Viral expression of *hes1* (and *notch1*) led to an increase in cells expressing glial markers while targeted expression of dominant negative Hes1 led to a decreased number of glia, accompanied by a decrease of bipolar cells (Furukawa et al. 2000). Additionally, overexpression of *hes5* increased the number of Müller cells at the expense of rod photoreceptors, presumably by direct conversion while examination of *hes5* deficient retinae illustrated that Hes5 directs glia fate but is not necessary for the survival of these cells (Hojo et al. 2000). Apart from Hes1 and Hes5, a member of the Hey family seems to contribute directly to gliogenesis and can substitute the action of the aforementioned genes (Ohtsuka et al. 1999). Thus, Hey2 (but not Hey1 or HeyL) promotes glia versus neuronal cell fate in the mouse retina and misexpression of it resulted in Müller cells at the expense of rod photoreceptors (Satow et al. 2001). The role of Notch in zebrafish retinal gliogenesis was initially reported by (Scheer et al. 2001) where the Gal4-UAS system, developed for Drosophila (Fischer et al. 1988), was exploited to express a constitutively active Notch receptor (Takke et al. 1999) under the control of either a *deltaD* or heat shock promoter (Dornseifer et al. 1997; Haddon et al. 1998; Halloran et al. 2000). Depending on the effector and the

developmental stage at which the *notch1a intracellular domain* expression was induced, the phenotype varied from anophthalmia, gross tissue abnormalities to neuronal deficits and gliosis. In addition, Notch over-activation led to undifferentiated cells that lacked a glial or neuronal marker and an increase in apoptotic cells (Scheer et al. 2001). On the other hand, inhibition of Notch signaling via a pharmacological reagent (γ-Secretase inhibitors) or the use of a mutant fish (*mib, mutant of mindbomb*) hindered Müller cells from acquiring their cellular identity and morphology (Bernardos et al. 2005; MacDonald et al. 2015). In addition, a genome-wide analysis of isolated Müller cells and progenitors from the mouse retina revealed that Notch not only instructs the glial fate but also is essential for the maintenance of this fate and cell maturation post-mitotically (Nelson et al. 2011). Collectively, these results indicate that Notch signaling in the developing retina inhibits the acquisition of neuronal fate, causing cells to remain either in an undifferentiated state or enter gliogenesis.

1.3.4.3 Notch signaling in binary fate decisions

Across species, Notch signaling has been implicated in binary fate decisions where one cell type is chosen from two alternative fates (Jukam & Desplan 2010; Pierfelice et al. 2011). Notch was firstly identified as a key player in the decision of neural versus epidermal fate in Drosophila (Poulson 1940). In recent years, binary fates have been described in different contexts in vertebrates. In both rodents and zebrafish, along the dorsal-ventral axis of the spinal cord, differentially expressed transcription factors in locally restricted domains give rise to distinct neuronal cell types. Binary decisions in which Notch is of major importance take place in two of these domains namely the pMN and p2 domains. In the pMN domain, terminal divisions generate a primary motor neuron (PMN) and a Kolmer-Agduhr (KA) cell (Park et al. 2004) whereas, in the p2 domain V2a and V2b -excitatory and inhibitory interneurons respectively- are generated (Kimura et al. 2008). Inactivation of Notch signaling produces an excess of PMNs at the expense of KA interneurons and conversely constitutive activation of Notch leads to an excess of KA interneurons at the expense of PMNs (Shin et al. 2007). In the p2 domain, activation of the Notch1 receptor triggers a genetic cascade of the V2b fate over the V2a (express high levels of Delta ligand) in mouse, chick and zebrafish (Barrio et al. 2007; Batista et al. 2008; Kimura et al. 2008; Okigawa et al. 2014; Peng et al. 2007). Other examples of binary fate decisions in which Notch is implicated include the rodent cerebellum and the epiphysis of zebrafish. (Zhang et al. 2021) showed that in the rodent cerebellum, the “Notch On” state permits the generation of Purkinje cells whereas the “Notch OFF” state promotes the generation of the excitatory granule cells, presumably via lateral inhibition. In the zebrafish

epiphysis, Notch acts synergistically with the Bone Morphogenetic Protein (BMP) signaling pathway to promote PhR over the projection neuron fate during mitosis (Cau et al. 2008; Quillien et al. 2011). Binary cell fate decisions have also been shown to occur in the vertebrate retina. Terminal asymmetric divisions consisting of PhRs/ interneurons and PhRs/ MCs take place in the rodent retina (Cayouette & Raff 2003). It was shown in retinal mouse explants, that asymmetric inhibition of Notch via selective segregation of Numb- a well-known Notch antagonist (McGill & McGlade 2003; McGill et al. 2009)-during mitosis lead to pairs of PhRs/ non-PhRs cells (Kechad et al. 2012).

1.4 Aims of the thesis

The goal of the current dissertation was to evaluate whether and how terminal asymmetric mitotic divisions contribute to generating cellular diversity in the retina. To this extent, I used the zebrafish retina as a model and investigated:

1) A Notch mediated mechanism that allows for the (re)specification of one retinal interneuron fate to another.
2) An asymmetric terminal event that generates an MC (the principal retinal glial cell-class) and a distinct type of excitatory interneuron.

2 Results

2.1 Cellular and molecular determinants of retinal cell fate (*accepted for publication in Annual Review of Vision Science*)

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Abstract

The vertebrate retina is regarded as a simple part of the central nervous system (CNS) and thus amenable to investigations of the determinants of cell fate. Its five neuronal cell classes and one glial cell class all derive from a common pool of progenitors. Here we review how each cell class is generated. Retinal progenitors progress through different competence states, in each of which they generate only a small repertoire of cell classes. The intrinsic state of the progenitor is determined by the complement of transcription factors it expresses. Thus, although progenitors are multipotent, there is a bias in the types of fates they generate during any particular time window. Overlying these competence states are stochastic mechanisms that influence fate decisions. These mechanisms are determined by a weighted set of probabilities based on the abundance of a cell class in the retina. Deterministic mechanisms also operate, especially late in development, when preprogrammed progenitors solely generate specific fates.

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Cellular and Molecular Determinants of Retinal Cell Fate

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Keywords

retinal development, cell fate, central nervous system, CNS, progenitor cells, neurogenesis, gliogenesis

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1. INTRODUCTION

The retina has long been used as a model to investigate the determinants of cell fate in the vertebrate central nervous system (CNS). Its peripheral location in the eye, compact structure, and relatively small number of cell classes make the retina a simple and "approachable part of the brain" (Dowling 2012). More recently, however, the impetus for studying retinal cell fate has expanded beyond it being a model for the CNS, focusing instead on the retina itself. The elucidation of molecular pathways that instruct distinct retinal cell fates has greatly aided work aimed at cell replacement therapies with the potential to restore compromised vision (Jorstad et al. 2017, Todd et al. 2021, Yao et al. 2018). Here we review what is known about cell fate acquisition in the vertebrate retina, focusing on the mouse retina, findings from which have been the biggest contributor to this field, but also drawing on work from other vertebrates, including zebrafish, chick, and *Xenopus laevis*.

1.1. Cell Composition of the Vertebrate Retina

Each of the six retinal cell classes occupies stereotypic positions within one of the three cellular layers (**Figure 1*a***). Photoreceptors (PhRs) (the rods and cones) are located in the outer nuclear layer. Three interneuron classes, horizontal cells (HCs), bipolar cells (BCs), and amacrine cells (ACs), reside in the inner nuclear layer (INL), and ganglion cells (GCs) can be found in the

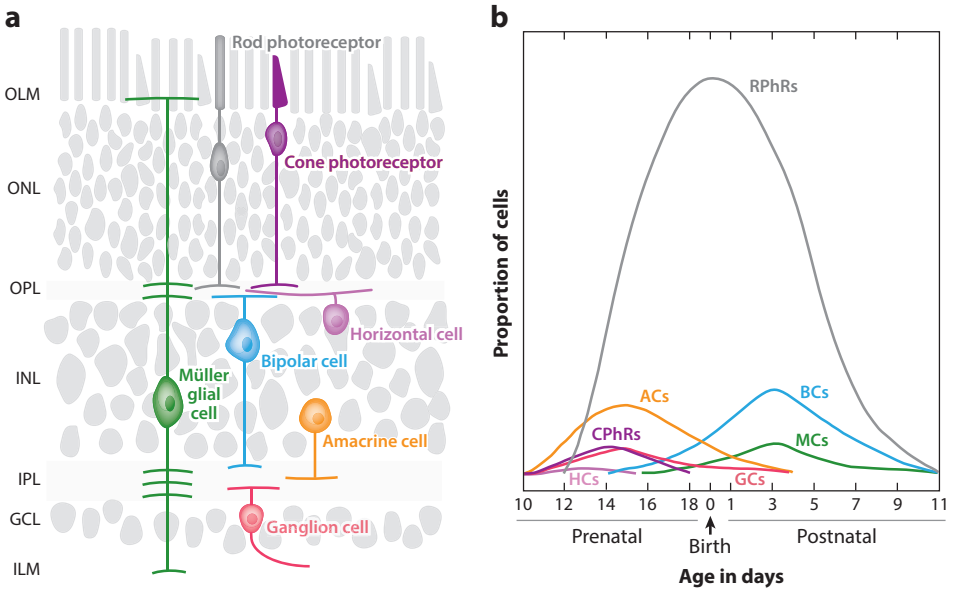


Figure 1

Schematic of the vertebrate retina. (*a*) Five neuronal cell classes and one glial cell class are stereotypically localized in the three nuclear layers: ONL, INL, and GCL. Retinal cells form synaptic connections in the OPL and IPL. MCs contribute to the formation of the OLM and ILM at the apical and basal part of the tissue, respectively. (*b*) The proportion of retinal cell classes that are born (i.e., undergo their last mitosis) from embryonic day 10 until postnatal day 11 in the mouse retina. Panel *b* adapted with permission from Young (1985); copyright John Wiley and Sons. Abbreviations: AC, amacrine cell; BC, bipolar cell; CPhR, cone photoreceptor; GC, ganglion cell; GCL, ganglion cell layer; HC, horizontal cell; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; MC, Müller glial cell; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; RPhR, rod photoreceptor.

ganglion cell layer. Müller glial cells (MCs), the major glial cell class, span the apico-basal extent of the retina, with their somata localized to the INL. The number of subtypes within each neuronal cell class varies across vertebrates, reflecting the specializations required in distinct visual environments. For instance, whereas the mouse retina contains two subtypes of cone PhRs, sensitive to short (S) and medium (M) wavelengths of light, the zebrafish retina is tetrachromatic with cones sensitive to long (L), M, S, and UV wavelengths (Baden & Osorio 2019). Similarly, whereas the mouse retina has a single type of HC (Peichl & González-Soriano 1994), the zebrafish retina has four (Song et al. 2008).

1.2. Cell Genesis in the Developing Retina

The period of cell genesis varies across species. Thus, whereas retinogenesis is protracted in the mouse, extending from embryonic day 11 until postnatal day 7, a period of almost 2 weeks (Young 1985), it is complete within 2 days in zebrafish (Hu & Easter 1999). Nevertheless, cells are generated in a conserved order. GCs are the first to be generated in all vertebrates studied thus far. In mice, in which the most thorough birth-dating studies have been conducted, the next cell classes generated are cones, ACs, and HCs, followed by rods, BCs, and MCs. Importantly, the time windows during which each of these cell classes is generated overlap considerably. Thus, at any given time point, cells with distinct fates are generated concurrently (**Figure 1*b***). How retinal progenitor cells (RPCs) generate the diverse cell classes that populate the retina has been the subject of intense study over the last three decades.

2. RETINAL PROGENITOR CELLS

RPCs are regarded to be multipotent, capable of generating more than one cell class. Evidence for this comes from lineage studies that marked single RPCs using retroviral infections or fluorescent tracers and analyzed their ensuing progeny at mature time points. Daughter cell clones arising from these RPCs were variable in size and composition and comprised both neuronal cell classes and Müller glia (Holt et al. 1988, Turner & Cepko 1987, Turner et al. 1990, Wetts & Fraser 1988).What remained unclear from these studies were the patterns of mitosis individual RPCs underwent and which specific cell classes were generated at each division. Cycling RPCs can undergo one of three modes of division: (*a*) Symmetric proliferative divisions are characterized by RPCs that divide to generate daughter cells that return to the cell cycle (**Figure 2*a***). Such divisions occur early during retinal development to increase the RPC pool. (*b*) Asymmetric differentiative divisions that generate an RPC and a postmitotic daughter allow for the generation of distinct retinal cell classes while maintaining the RPC pool (**Figure 2*b***). (*c*) Terminal divisions in which two postmitotic daughters are generated effectively deplete the RPC pool and largely occur toward the end of cell genesis (**Figure 2*c***). If both postmitotic daughters acquire the same fate, these terminal divisions are considered symmetric, whereas the acquisition of distinct fates would render the divisions asymmetric (**Figure 2*d***). Indeed, such asymmetric fate outcomes (e.g., a rod and an AC; Hafler et al. 2012, Turner & Cepko 1987) provide support for the multipotency of RPCs even in terminal divisions. There is, however, also evidence for RPCs committed to a single fate, for example, rods (Turner & Cepko 1987).

2.1. Cellular and Molecular Heterogeneity Among Retinal Progenitors

RPCs are present throughout the period of retinal histogenesis, coexisting alongside newly generated postmitotic cells. Cycling RPCs span the extent of the retinal epithelium and undergo characteristic nuclear translocations along their cytoplasmic processes that are tightly linked to the phase

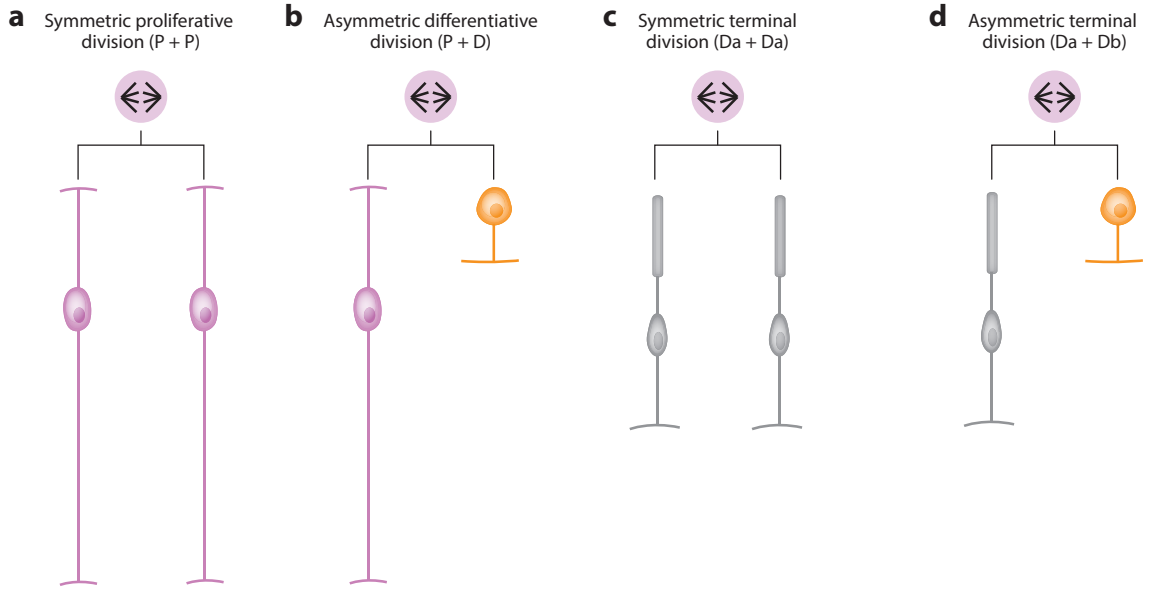


Figure 2

Modes of division of retinal progenitors. (*a*) Symmetric proliferative division produces two progenitor cells (P + P). (*b*) Asymmetric differentiative division generates a progenitor cell and a postmitotic cell that will undergo differentiation (P + D). (*c*) Symmetric terminal division produces two postmitotic cells that will acquire the same fate (Da + Da). (*d*) Asymmetric terminal division generates two postmitotic cells of different fates (Da + Db).

of the cell cycle they are in. Thus, for example, RPC nuclei are located closer to the basal surface during S-phase and at the apical surface at mitosis (Baye & Link 2007, Sauer 1935). This cellular behavior, termed interkinetic nuclear migration, is conserved across vertebrate species and CNS regions. However, not all RPCs resemble these neuroepithelium-spanning cells or exhibit their behavior. In the zebrafish retina, delaminated progenitors exclusively committed to the HC fate undergo terminal mitosis in the forming INL (Godinho et al. 2007, Weber et al. 2014), whereas HC-committed progenitors in the chick retina undergo mitosis at the basal surface (Boije et al. 2009). Terminally dividing BC progenitors in the zebrafish retina also undergo mitotic divisions in the INL (Engerer et al. 2017, Weber et al. 2014).

Although most RPCs are multipotent, they are not a homogeneous population. Gene expression studies of the developing mouse retina (Blackshaw et al. 2004, Trimarchi et al. 2008), including a large-scale single-cell RNA-sequencing (scRNA-seq) effort (Clark et al. 2019), revealed RPC subpopulations with distinct molecular signatures. Broadly, murine retinal RPCs were classified as primary or neurogenic. Although both RPC categories comprise cycling cells, only the neurogenic subpopulation expresses proneural transcription factors (TFs), indicative of an ensuing differentiative mitotic division in which at least one daughter exits the cell cycle. Primary RPCs at a given developmental stage were enriched for cell cycle–related genes and largely molecularly homogenous (Clark et al. 2019). However, primary RPCs from embryonic versus postnatal time windows exhibited distinct molecular signatures. Among the transcriptional regulators that embryonic primary RPCs expressed were *Fibroblast growth factor 15* (*Fgf15*), *Forkhead Box P1* (*Foxp1*), and *Foxp4*, whereas *Retinaldehyde Binding Protein 1* (*Rlbp1*), *SRY-Box Transcription Factor 8* (*Sox8*), *Argininosuccinate Synthase 1* (*Ass1*), and *Nuclear Factor I* (*Nfi*) TFs were

expressed by postnatal primary RPCs. Neurogenic RPCs from the embryonic and postnatal mouse retina could also be distinguished by the specific proneural TFs they express. For instance, the TF atonal homolog 7 (Atoh7, also known as Math5), which is necessary for specifying the GC fate (see Section 4.5), is expressed by neurogenic RPCs only in the embryonic retina, not in the postnatal mouse retina (Clark et al. 2019). This expression is in line with when GCs are generated and the fact that Atoh7 is pivotal in GC fate specification.

Heterogeneity in the RPC population was also reported in an RNA-seq study of the developing zebrafish retina (Xu et al. 2020). Given the speed of retinogenesis in the zebrafish retina—lasting only 2 days from 24 h postfertilization (hpf) until 72 hpf—RPCs were isolated at relatively short intervals and subdivided into clusters based on gene expression patterns. Three of the clusters were common to RPCs originating from distinct developmental time points, 24, 36, and 48 hpf. The gene expression profiles of Clusters 1 and 2 suggested they are akin to the primary RPCs described for the mouse retina, and Cluster 3 was classified as comprising neurogenic RPCs that give rise to the earliest-born cell classes in the zebrafish retina, GCs and ACs. RPCs isolated at 48 hpf were subdivided into four additional clusters: Cluster 4 expressed genes linked to the generation of BCs and PhRs, Cluster 5 represented precursors committed to the HC fate, and Clusters 6 and 7 represented precursors committed to the PhR and MC fates, respectively.

2.2. Temporal Patterning of Progenitors

The differential gene expression patterns between temporally distinct RPC cohorts reflect the prevailing model of cell fate determination. Originally proposed more than two decades ago (Cepko et al. 1996), the competence model suggested that RPCs transition through distinct states in which they can generate a limited repertoire of cell classes. Moreover, transitions between competence states are unidirectional so that once the time window for the generation of a specific cell class has passed, it can no longer be generated (Cepko et al. 1996, Livesey & Cepko 2001).

The competence state an RPC is in is determined by so-called temporal TFs. At distinct developmental time windows, RPCs express specific temporal TFs that are necessary and sufficient to generate early and late cell classes that are born in the embryonic and postnatal retina, respectively. Although temporal TFs do not directly instruct cell fate, they are upstream of transcriptional networks that do (see Section 4). Several temporal factors in the mouse retina have been identified, all of which have counterparts in the *Drosophila* CNS. *Ikaros Family Zinc Finger 1* (*Ikfz1*), a temporal TF, is expressed in embryonic mouse RPCs and confers the competence to generate GCs, ACs, and HCs, three of the four cell classes born prenatally (Elliott et al. 2008). Thus, when *Ikfz1* is experimentally misexpressed in the postnatal mouse retina, RPCs that normally generate PhRs and BCs acquire the competence to generate GCs, ACs, and HCs. Conversely, in the absence of *Ikfz1*, cell classes generated embryonically are reduced in number, whereas those generated in the postnatal retina are unaffected. The temporal TF *Castor Zinc Finger 1* (*Casz1*) is expressed by mouse RPCs at mid-to-late stages of retinogenesis, permitting the generation of rods and BCs (Mattar et al. 2015). Indeed, it has been proposed that through its interactions with the nucleosome remodeling and deacetylase (NuRD) complex and the Polycomb repressor complex, *Casz1* promotes the rod fate while suppressing MC glial fate (Mattar et al. 2021). A recently discovered temporal TF, POU Class 2 Homeobox 1 (Pou2f1), regulates the generation of cones (Javed et al. 2020), all of which are born prenatally in the mouse retina. Notably, Pou2f1 represses Casz1, thus preventing early RPCs from acquiring a late RPC competence state. Thus, temporal TFs not only confer specific competence states to the RPCs in which they are expressed but also regulate the transitions between states.

2.3. Biased Progenitors

In addition to the multipotent RPCs described in Section 2.2, some RPCs are biased, even stereotypic, in the cell classes they generate. This is particularly apparent in RPCs undergoing terminal divisions. For example, in the zebrafish retina, terminal divisions in the late stages of neurogenesis generate pairs of PhRs (He et al. 2012, Suzuki et al. 2013), BCs (Engerer et al. 2017, Weber et al. 2014), or HCs (Godinho et al. 2007, Weber et al. 2014). RPCs in the mouse retina, expressing the basic helix-loop-helix (bHLH) oligodendrocyte transcription factor 2 (Olig2), divide terminally, generating daughters with the same fate, either two cones or two HCs (Hafler et al. 2012). Pairs of HCs of the same subtype in the chick retina have been reported (Rompani & Cepko 2008). Moreover, Cadherin 6 (Cdh6)-expressing RPCs in the mouse retina give rise to multiple cell classes, but the GCs they generate are almost exclusively of a specific subtype: direction-selective GCs that also express Cdh6 (De la Huerta et al. 2012).

3. INTRINSIC VERSUS EXTRINSIC DETERMINANTS OF CELL FATE

Several lines of evidence suggest that intrinsic mechanisms rather than extrinsic cues are key players in determining cell fate in the retina. RPCs isolated from the rat retina and cultured at clonal density divided in predictable modes and gave rise to retinal cell classes in the same order they would have in vivo (Cayouette et al. 2003, Gomes et al. 2011). Moreover, RPCs from the embryonic retina generated cell classes with early fates even when cultured with (Belliveau & Cepko 1999) or transplanted into (Rapaport et al. 2001) a postnatal environment. Similarly, postnatal RPCs did not alter their output when placed in an embryonic retinal environment (Belliveau et al. 2000). Thus, being placed in a heterochronic milieu did not alter the innate capacity of RPCs. Nevertheless, cues from the environment might provide some feedback to RPCs, for example, by inhibiting the generation of more cells from a specific cell class when sufficient numbers have been generated (Waid & McLoon 1998).

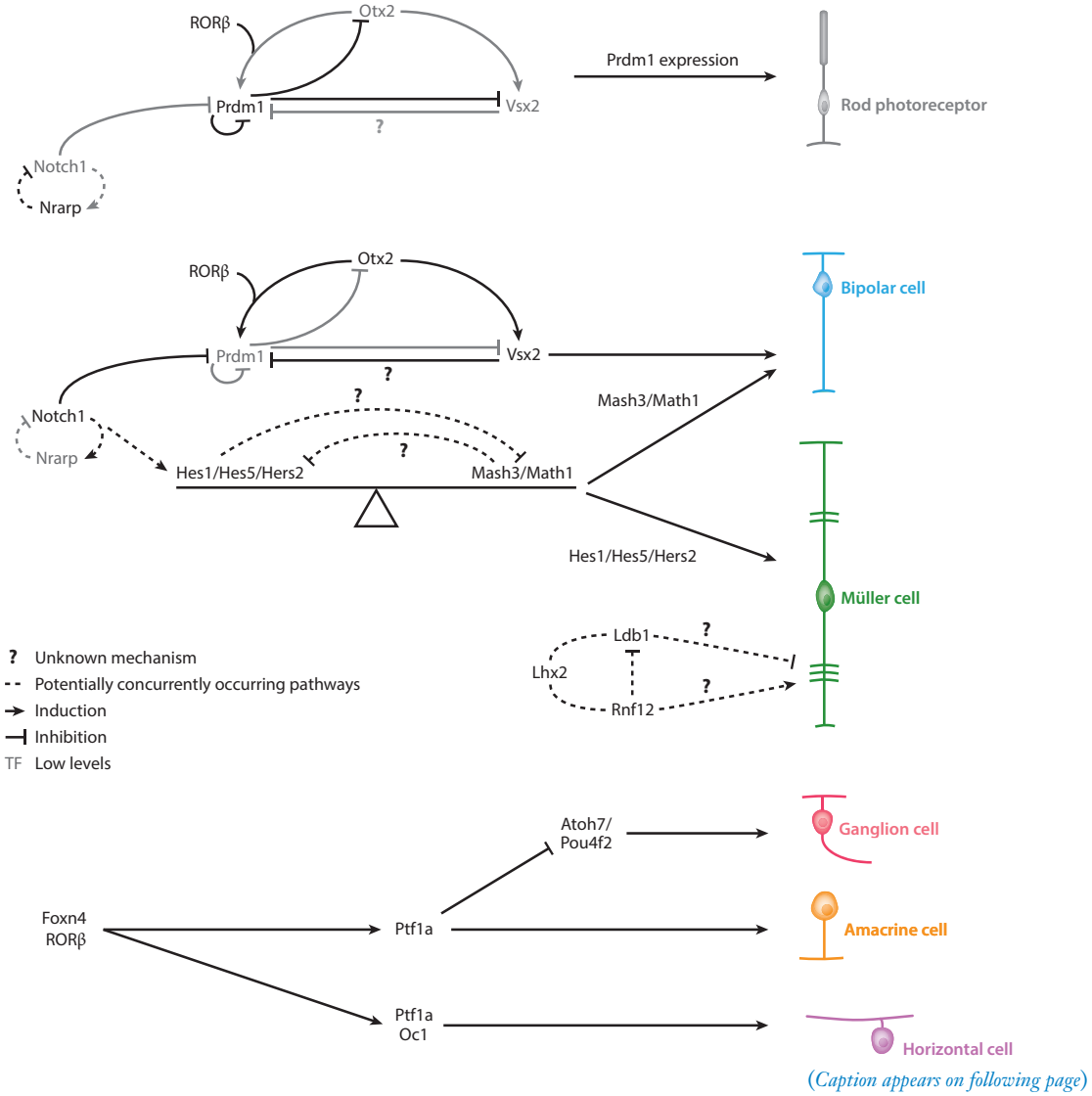
Intrinsic mechanisms that instruct cell fate involve specific TF cascades that are downstream of the competence factors described in Section 2.2. In Section 4, we describe the gene regulatory networks involved in the specification of each retinal cell class (**Figure 3**). The role that specific TFs play has been investigated largely through gain- and loss-of-function approaches. Cre recombinase–based fate mapping and sophisticated retroviral tools have enabled specific lineages to be targeted for labeling or manipulation (Hafler et al. 2012). Time-lapse imaging in vivo in the zebrafish retina (Engerer et al. 2021, Godinho et al. 2007, He et al. 2012, Jusuf et al. 2011, Poggi et al. 2005) and in vitro in postnatal rat RPCs (Gomes et al. 2011) has provided direct observations of dividing RPCs and their progeny in physiological conditions and following manipulation. Further, scRNA-seq studies of the developing mouse (Clark et al. 2019), human (Lu et al. 2020), and zebrafish retina (Wang et al. 2020, Xu et al. 2020) are providing detailed insights into the developmental programs that generate the diversity of retinal cell fates.

4. GENE REGULATORY NETWORKS INVOLVED IN FATE DETERMINATION

4.1. Photoreceptors

The subtypes and proportions of PhRs vary in different species. In zebrafish, rods make up a small proportion of the entire PhR population, with cone subtypes dominating (Baden & Osorio 2019). By contrast, in nocturnal mammals such as mice, rod PhRs dominate (Young 1985). In mice, rods are generated throughout the period of retinogenesis, peaking around birth. Given their abundance, almost every clone contained rods in lineage-tracing studies of mice and rats (Turner &

Cepko 1987, Turner et al. 1990), with some clones exclusively comprising rods. Cones are generated only prenatally in mice, and in many vertebrate species cone genesis is initiated prior to the commencement of rod genesis (Carter-Dawson & Lavail 1979, Sidman 1961, Young 1985). Both PhR types can be generated by terminal divisions in the mouse (Hafler et al. 2012) and zebrafish (He et al. 2012) retina. However, whereas these terminal divisions can generate heterotypic progeny in mice, homotypic PhR pairs are generated in zebrafish (Suzuki et al. 2013). Retrovirus-based clonal analysis of RPCs in mice expressing Olig2 suggested that they divide terminally, generating different combinations of two-cell clones comprising a PhR and an interneuron (Hafler et al. 2012). At embryonic time points, these two-cell clones comprise two cones, one cone and one HC, or two HCs. At postnatal ages, Olig2^{+} RPCs generated two rods or one rod and one AC.



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Molecular mediators of cell fate in the vertebrate retina. Gene regulatory networks involved in the fate determination of different cell classes are depicted. In the mouse retina, the acquisition of rod PhR and BC fates is linked, controlled by a TF network involving Otx2, Vsx2, and Prdm1. Otx2 is expressed by a subset of postmitotic precursors and activates Vsx2 and Prdm1, which are instructive for BC and rod PhR fates, respectively. Vsx2 and Prdm1 cross-repress each other to establish which TF will prevail and thus impact which cell fate is specified. Acquiring a definitive rod PhR fate requires the activation of Nrl (not shown). Otx2 and RORβ bind to Nrl in postmitotic PhR precursors to solidify the rod PhR fate. Notch signaling, in concert with its negative regulator Nrarp, also plays an important role in the rod PhR versus BC fate decision. High levels of Notch activation lead to the inhibition of Prdm1 and thus the BC fate. Conversely, low levels of Notch signaling favor the rod PhR fate at the expense of BCs. Notch signaling is also key to regulating the balance between neurogenesis (rod PhR and BC fates) and gliogenesis (MC fate). The Notch effector genes *Hes1*, *Hes5*, and *Hesr2*, which are important for MC fate specification, repress TFs such as Math3 and Mash1 that act in concert with Vsx2 to instruct the BC fate. A gene regulatory network involving Lhx2, its co-activator Ldb1, and Rnf12 has also been implicated in MC fate. The temporal TF Foxn4, together with RORβ, activates Ptf1a, which is essential for AC fate determination. Ptf1a, in combination with OC1, also a Foxn4 downstream target, is important for HC fate determination. The bHLH TF Atoh7, acting together with its downstream target Pou4f2, is key to GC fate specification and differentiation. Abbreviations: AC, amacrine cell; Atoh7, atonal homolog 7; BC, bipolar cell; bHLH, basic helix-loop-helix; Foxn4, winged helix/forkhead; GC, ganglion cell; HC, horizontal cell; Hesr2, Hes-Related Repressor Protein 2; Hes1,5, Hes Family BHLH Transcription Factor 1,5; Ldb1, LIM Domain Binding 1; MC, Müller glial cell; Nrarp, Notch-regulated ankyrin repeat protein; Nrl, neural retina leucine zipper; OC1, onecut 1; Otx2, orthodenticle homeobox 2; PhR, photoreceptor; Pou4f2, POU Class 2 Homeobox 1; Prdm1, PR domain zinc finger protein 1; Ptf1a, pancreas transcription factor 1a; Rnf12, RING Finger Protein 12; RORβ, retinoid related orphan nuclear receptor β; TF, transcription factor; Vsx2, visual system homeobox 2.

Notably, rods and cones were never immediate siblings. Moreover, not all rods are generated by the Olig2$^+$ RPCs; two-cell clones comprising one rod and one BC or one rod and one MC are generated by Olig2$^-$ RPCs at postnatal time points. The TF orthodenticle homeobox 2 (Otx2), which is upregulated in RPCs as they exit the cell cycle, lies at the heart of rod and cone fate determination (Nishida et al. 2003). In the absence of Otx2, both types of PhRs fail to form.

4.1.1. Cone photoreceptors. Cone fate in mice relies on Otx2 and onecut 1 (OC1), an atypical homeodomain TF. In combination with Otx2, OC1 binds enhancer elements of thyroid hormone receptor β2 (Thrβ2), which is active in RPCs that generate cones (as well as HCs). The timing of OC1 expression correlates with when cones, not rods, are generated. Indeed, OC1 is a key element in deciding whether a cone or a rod PhR is fated. Repression of OC1 allows rod PhR generation. Conversely, induced expression of OC1 in Otx2$^+$ cells in the postnatal mouse retina, when cone generation is normally long completed, results in the generation of cones (Emerson et al. 2013).

In the zebrafish retina, cones are generated in terminal divisions (He et al. 2012), with distinct cone subtypes sensitive to L, M, S, and UV wavelengths of light generated as homotypic pairs by dedicated progenitors (Suzuki et al. 2013). For example, Thrβ2$^+$ L cones are generated by Thrβ2$^+$ progenitors. Knockdown of Thrβ2 reduced the number of L cones and increased the number of UV cones. Similarly, in mice, the absence of Thrβ2 led to a loss of M cones and an increase in S cones, which are phylogenetically similar to zebrafish UV cones (Ng et al. 2001). UV cones in zebrafish are specified by the T-box TF Tbx2b; in its absence, UV cones are reduced significantly in number with a concomitant increase in rod number (Alvarez-Delfin et al. 2009). Thus, Tbx2b specifies UV cone fate and represses the rod fate.

4.1.2. Rod photoreceptors. At postnatal stages of mouse retinal development, Otx2$^+$ postmitotic precursors are bipotential, capable of adopting rod or BC fates. Otx2 directly activates the transcriptional repressor PR domain zinc finger protein 1 (Prdm1), also called B lymphocyte-induced maturation protein-1 (Blimp1), and visual system homeobox 2 (Vsx2), also called Ceh-10 homeodomain-containing homolog (Chx10). Prdm1 is associated with rod fate specification, and Vsx2 is linked to the BC fate. Prdm1 and Vsx2 cross-repress each other to establish which TF will prevail. The levels of each of these TFs thus critically determine which cell fate will be chosen. Prdm1 also inhibits Otx2. Once the rod or BC fate is specified, a transient period occurs during

which a switch can be induced by the experimental overexpression of the appropriate TF. However, once mature they are no longer amenable to fate changes. Acquiring a definitive rod fate requires activation of the TF neural retina leucine zipper (Nrl) (Mears et al. 2001) or its target, nuclear receptor subfamily 2 group E member 3 (Nr2e3) (Chen 2005). Otx2 and the protein encoding the retinoid related orphan nuclear receptor β (Rorβ) gene directly bind to Nrl in postmitotic PhR precursors to instruct the rod fate. If Nrl is knocked out, cones are generated at the expense of rods (Mears et al. 2001). Experimental induction of Nrl or Nr2e3 can transform cone precursors into rods (McIlvain & Knox 2007, Oh et al. 2007). Thus, rod fate specification requires repression of cone-specific genes.

4.2. Bipolar Cells

Acquisition of the BC fate is closely linked with the rod fate (described in Section 4.1.2) and is controlled by a network of TFs in which Otx2, Prdm1, and Vsx2 play key roles. In $Otx2^{-/-}$ mutant mice in which PhRs fail to form, BCs are also significantly decreased, implicating a role for Otx2 in PhR and BC fates (Koike et al. 2007). Vsx2 is instructive for the BC fate (Burmeister et al. 1996, Green et al. 2003, Horsford et al. 2004) and inhibits the acquisition of the PhR fate by suppressing the expression of PhR-related genes (Dorval et al. 2005, Livne-Bar et al. 2006). Conversely, Prdm1 represses BC-related genes in $Otx2^{+}$ cells (Brzezinski et al. 2010, 2013; Katoh et al. 2010; Park et al. 2017). In the absence of Prdm1, increased numbers of BCs are observed at the expense of rods. Thus, Prdm1 and Vsx2 suppress each other to solidify the rod PhR versus BC fate (Brzezinski et al. 2010, 2013; Goodson et al. 2020a,b; Katoh et al. 2010; Kim et al. 2008a; Mills et al. 2017; Wang et al. 2014). Recent studies reported newly found upstream regulatory sequences in the *otx2* and *vsx2* loci, specific to BCs (Chan et al. 2020, Norrie et al. 2019), displaying another level of specificity in the activation of these factors.

Notch signaling is an additional important component of the rod versus BC fate decision, affecting cell specification postmitotically (Mizeracka et al. 2013a). Inactivation of the Notch1 receptor in late retinal progenitors in the mouse retina increased the number of rod PhRs at the expense of BCs, whereas Notch1 ablation in early progenitors favored the production of cone PhRs (Jadhav et al. 2006b, Yaron et al. 2006). Microarray analysis of Notch1 conditional knockout retinae revealed the downregulation of Notch target genes and effectors along with an upregulation of rod precursor–specific genes, including *Prdm1* (Mizeracka et al. 2013b). Collectively, these data suggest a model in which BCs require the action of Notch1 in order to directly or indirectly reduce the levels of Prdm1. Low Prdm1 levels alleviate the inhibition of *Otx2* and *Vsx2* genes and permit their expression and action. When the effect of Notch signaling subsides, the balance favors the expression of proneuronal genes, such as *Math3* [additionally known as *atonal BHLH Transcription Factor 3* (*Atoh3*) or *Neuronal Differentiation 4* (*NeuroD4*)] and *Mash1* [also known as achaete-Scute Family BHLH Transcription Factor 1 (*Ascl1*)], that act along with *visual system homeobox 2* (*Vsx2*) to specify the BC fate (Hatakeyama et al. 2001, Tomita et al. 2000). In $Otx2^{+}$ cells in which Notch signaling is not active, *Prdm1* levels are high. As a result, the expression of *Otx2* and *Vsx2* are low or eliminated, leading the cell toward the rod PhR fate.

Additional TFs induce subtype specification of BCs in the rodent retina. Among these are *Vsx1* (Chow et al. 2001, 2004; Ohtoshi et al. 2001, 2004; Shi et al. 2011), basic helix-loop-helix domain containing class B 4 protein (*Bhlhb4*) (Bramblett et al. 2004, Kim et al. 2008b), *Bhlhb5* (Feng et al. 2006, Huang et al. 2014), iroquois Homeobox 5 (*Irx5*) (Cheng et al. 2005), insulin gene enhancer protein 1 (*Isl1*) (Elshatory et al. 2007a,b), PR/SET Domain 8 (*Prdm8*) (Jung et al. 2015), FEZ Family Zinc Finger 2 (*Fezf2*) (Suzuki-Kerr et al. 2018), and jumonji domain-containing protein-3 (*Jmjd3*) (Iida et al. 2014), which are differentially expressed in ON- and OFF-cone BCs as well as rod BCs and affect different aspects of cellular specification.

BC fate specification in the zebrafish retina has not been thoroughly investigated. Nonetheless, Vsx1 and Vsx2, which play important roles in BC fate specification in the mouse retina, are also key players in the zebrafish retina. Most zebrafish BCs express *Vsx1* (Engerer et al. 2017, 2021; Passini et al. 1997; Vitorino et al. 2009; Weber et al. 2014) and a smaller population (types S4 and S5) expresses *Vsx2* (Barabino et al. 1997, Passini et al. 1997, Vitorino et al. 2009). In zebrafish, *Vsx2* signals the BC fate (Vitorino et al. 2009) and acts as transcriptional repressor of *Vsx1* as in the mouse retina (Clark et al. 2008, Dorval et al. 2005, Passini et al. 1997, Vitorino et al. 2009).

4.3. Müller Glial Cells

Notch signaling is key for MC fate specification and differentiation across vertebrate species (Bao & Cepko 1997, Furukawa et al. 2000, Jadhav et al. 2006a, Perron et al. 1998, Scheer et al. 2001). Overexpression of the constitutively active form of the Notch1 receptor induced the expression of MC markers (Bao & Cepko 1997, Furukawa et al. 2000, Jadhav et al. 2006a, Scheer et al. 2001). Conversely, inhibition of Notch signaling led to a failure of MCs to differentiate (Bernardos et al. 2005, Scheer et al. 2001). Furthermore, sustained Notch signaling is essential for maintaining the MC identity (Nelson et al. 2011). Three Notch effectors, Hes Family BHLH Transcription Factor 1 (*Hes1*), *Hes5*, and HES-Related Repressor Protein 2 (*Hesr2*), all of which are bHLH transcriptional regulators, play a vital role in MC specification. Retrovirus-mediated overexpression of Hes1 in the mouse retina led to an increase in cells expressing glial markers; expression of a dominant negative form of *Hes1* led to a decreased number of MCs accompanied by a decrease in BCs (Furukawa et al. 2000). Overexpression of *Hes5* or *Hesr2* also increased the number of MCs, but at the expense of rods (Hojo et al. 2000, Satow et al. 2001), without inducing cell proliferation or death. Thus, *Hes5* and *Hesr2* promote glial fate in precursor cells while inhibiting neuronal fate. Manipulations that target MC fate affect both rods and BCs, retinal classes generated late during development (Young 1985). Thus, inactivation of the Notch1 receptor in late retinal progenitors led to an increased number of not only rod PhRs at the expense of BCs (as described in Section 4.2) but also MCs (Jadhav et al. 2006b). Notch-regulated ankyrin repeat protein (*Nrarp*), a downstream Notch target gene, is a negative regulator of Notch signaling (Krebs et al. 2001, Lamar et al. 2001, Pirot et al. 2004). When *Nrarp* is overexpressed in vivo, increased numbers of rod PhRs were generated at the expense of MCs, while BCs remained unaffected (Mizeracka et al. 2013a). Conversely, in the absence of *Prdm1*, increased numbers of MCs and BCs were seen at the expense of rods (Brzezinski et al. 2010, 2013). Collectively, Notch signaling appears key to achieving a balance between neurogenesis and gliogenesis via downstream effectors that repress proneurogenic bHLH TFs. Accordingly, retinae lacking bHLH neuronal specification factors displayed increased MC genesis (Akagi et al. 2004; Inoue et al. 2002; Tomita et al. 1996, 2000).

In addition to Notch signaling, a gene regulatory network centered on the LIM homeodomain TF *Lhx2* plays an important role in balancing neurogenesis and gliogenesis. *Lhx2*-deficient animals display a significant reduction of the variable Notch components, including Notch1 receptor (de Melo et al. 2016b), and of proneuronal bHLH factors such as NeuroD1 (de Melo et al. 2018). Recent work revealed that Lhx2 and LIM Domain Binding 1 (Ldb1), the transcriptional (co)activator of Lhx2, inhibit MC production, whereas in combination with another factor, RING Finger Protein 12 (Rnf12), they induce gliogenesis. Rnf12 is a negative regulator of Ldb1 and both are expressed in tandem in retinal progenitors (de Melo et al. 2016b,a, 2018), controlling Lhx2 action, which in turn coordinates chromatin accessibility (Zibetti et al. 2019). Downstream targets of Lhx2 act to specify different gliogenic properties of MCs (de Melo et al. 2016a). Coelectroporation of the Lhx2–Ldb1 complex increased PhRs at the expense of BCs and MCs (de Melo et al.

2018), implying that multiple mechanisms affecting fate decisions are in place and might occur concurrently.

Vsx2 (*Chx10*), described in Section 4.1.2 in the context of BC fate specification, also plays a role in MC fate determination (Burmeister et al. 1996, Green et al. 2003, Horsford et al. 2004). In the rodent retina, *Vsx2* is expressed in progenitors and at maturity in BCs and a subset of MCs (Liu et al. 1994, Rowan & Cepko 2004). In the zebrafish retina, the reverse pattern is observed: *vsx2* is expressed in MCs and a subset of BCs (Passini et al. 1997, Vitorino et al. 2009). Although it is not clear how *Vsx2* expression in progenitors and MCs is regulated, there are regulatory sequences upstream of the *vsx2* locus that are specific to BCs and potentially to MCs as well (Norrie et al. 2019). Current evidence suggests that *Vsx2* is permissive for but not necessarily instructive of the glial fate (Hatakeyama et al. 2001, Livne-Bar et al. 2006). Thus, further studies are required to elucidate the importance of *vsx2* in MC fate determination.

4.4. Horizontal Cells and Amacrine Cells

HCs and ACs are inhibitory interneurons that occupy distinct positions in the INL. Across vertebrate species examined thus far, both cell classes share common gene regulatory networks and arise from an RPC subpopulation that expresses the winged helix/forkhead TF Foxn4 (Li et al. 2004), which has been proposed to be a temporal TF. *Foxn4* confers progenitors with the competence to generate not only HCs and ACs but also cones and rods (Liu et al. 2020). Foxn4 together with retinoid-related orphan nuclear receptor β1 (RORβ1) acts to activate the expression of the bHLH TF pancreas transcription factor 1a (*Ptf1a*) (Liu et al. 2013), which is required for AC fate determination (Dullin et al. 2007, Fujitani et al. 2006, Jusuf et al. 2011, Nakhai et al. 2007). *OC1* is a downstream target of *Foxn4* and together with *Ptf1a* is required for the acquisition of HC fate (Wu et al. 2013). Postmitotic precursors that express *Ptf1a* and *OC1* are thus committed to the HC fate and begin to express markers of differentiating HCs, including the TFs LIM homeobox 1 (Lim1) (Poché et al. 2007) and Prospero Homeobox 1 (Prox1) (Dyer et al. 2003), while precursors solely expressing Ptf1a are committed to the AC fate. In the absence of *Ptf1a*, both ACs and HCs fail to form and an increase in the number of GCs is observed (Fujitani et al. 2006). This finding suggests an additional role for Ptf1a in repressing the GC fate.

4.5. Ganglion Cells

Atoh7 has long been identified as a key molecular player in GC fate specification (Brzezinski et al. 2012, Mu et al. 2005, Yang et al. 2003). Across vertebrate species a subset of neurogenic RPCs expressing Atoh7 was competent to generate GCs. In the absence of Atoh7, an almost complete loss of GCs was observed (Brown et al. 2001, Kay et al. 2001, Wang et al. 2001). Recent work, however, has called into question the role that Atoh7 plays in GC fate specification, suggesting instead that it promotes GC survival and axon pathfinding within the retina (Brodie-Kommit et al. 2021). When apoptosis was blocked [BCL2 Associated X, Apoptosis Regulator ($Bax^{-/-}$)] in Atoh7-deficient mice, GCs were largely specified, with only a 20% reduction in numbers compared with controls. TFs such as Isl1 and Pou domain class 4 transcription factor 2 (Pou4f2), normally regarded to be downstream of Atoh7 and to stabilize GC fate and differentiation (Gan et al. 1999, Pan et al. 2008, Wu et al. 2015), were expressed even in the absence of Atoh7. Thus, in addition to Atoh7, other unknown molecular regulators must work upstream of Isl1 and Pou4f2. A new model for GC fate has emerged in which Atoh7 specifies a small cohort of early-born retinal GCs that might be the source of prosurvival factors and pathfinding cues for later-born GCs. In parallel, TFs such as NeuroD1 (Mao et al. 2013), Sox4 (Jiang et al. 2013), and OC1 and OC2 (Sapkota et al. 2014) specify most GCs.

5. STOCHASTIC MECHANISMS IN CELL FATE DETERMINATION

As described in Section 2.2, the competence state of RPCs and the activation of specific downstream TF cascades contribute to cell fate specification. However, about a decade ago, two studies (Gomes et al. 2011, He et al. 2012) brought to the fore the concept that stochastic mechanisms also played a major role during retinogenesis. This notion did not imply randomness but rather that different modes of division or fate choices occurred with a fixed range of probabilities over the course of development and could not always be predicted. Both studies involved long-term time-lapse imaging of individual RPCs either in vitro in postnatal rat RPCs or in vivo in the zebrafish retina, quantitatively analyzing the modes of RPC mitotic divisions over multiple rounds and ascertaining the fate of the progeny at each division. In line with previous, now classical, lineage studies, the researchers found variability in the size and composition of RPC-derived clones. Was this variability a result of endogenously distinct RPC subpopulations, each generating specific cell classes in a deterministic manner? Or were there stochastic mechanisms operating on equivalent progenitors to yield different fate outcomes? Both mechanisms seem to operate during retinogenesis. The mode in which RPCs divided (symmetric proliferative, asymmetric differentiative, or symmetric differentiative; see **Figure 2**) was stochastic, that is, determined by a fixed set of probabilities. For example, in the developing zebrafish retina, at early stages all RPC divisions were symmetric and proliferative. At the next phase, all three division modes had an equal probability of occurring, and in the last phase symmetric differentiative divisions dominated. Similarly, stochastic mechanisms also dictated cell fate outcomes, with the probability for the acquisition of a specific fate being determined by the abundance of a particular cell class in the mature retina. Nevertheless, some cell fates are clearly generated by deterministic mechanisms. This is particularly true for late stages of retinogenesis, at least in the zebrafish retina. For example, rods, cones, BCs, and HCs in the zebrafish retina were almost entirely generated by terminal symmetric differentiative divisions (e.g., pairs of HCs or BCs). The frequency of such outcomes was much higher than would be expected if stochastic mechanisms were at play.

What could account for the stochastic nature of cell fate decisions in the retina? Several possibilities include variability at the level of gene expression and translational, posttranslational (Kærn et al. 2005), and epigenetic mechanisms (Hu et al. 2012, Raeisossadati et al. 2021). Indeed, the independent activation of specific core TFs, Atoh7, Ptf1a, and Vsx1 in RPCs early during retinogenesis in zebrafish was sufficient to explain the variability in clone composition. The TF or TF combinations individual RPCs expressed constrained their potential and thus the cell classes they produced (Boije et al. 2015).

6. WHEN IS CELL FATE DETERMINED?

Pinpointing exactly when a specific cell fate is determined is difficult. For cell classes that are exclusively generated by terminal symmetric mitotic divisions, one might assume that fate assignments must have already occurred at the level of the RPC. For other cell classes, the consensus is that fate determination occurs immediately prior to or soon after cell cycle exit. There appears to be a brief time window in nascent postmitotic cells during which they are malleable and amenable to fate switches, as shown for rod and BC fates in the murine retina (Goodson et al. 2020b). We recently showed in the zebrafish retina that at least some nascent BCs of the *vsx1* lineage switch to an AC fate during normal development and that this transdifferentiation is mediated by Notch signaling (Engerer et al. 2021). Indeed, Notch signaling has been implicated in binary fate decisions extensively across species and CNS regions, including in asymmetric terminal divisions in the mouse retina (Kechad et al. 2012). How differential Notch signaling is achieved in the daughter cells of

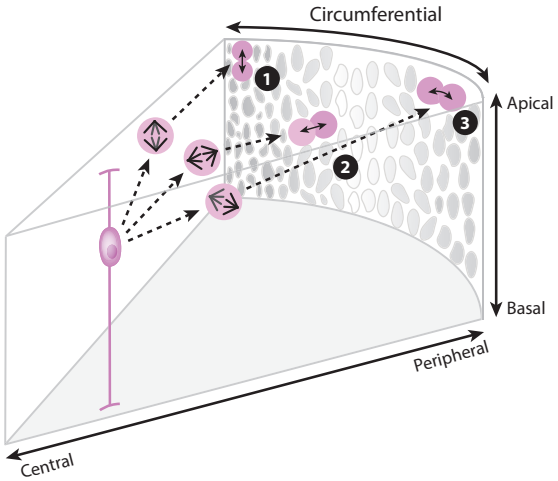


Figure 4

Angles of division. Mitotic divisions along the ① apical-basal axis, ② central-peripheral axis, and ③ circumferential axis. Figure adapted with permission from Cayouette et al. (2006); copyright Elsevier.

terminally dividing RPCs remains to be fully understood. In the mouse retina, the asymmetric inheritance of the Notch signaling antagonizer Numb was proposed to mediate distinct fates following terminal divisions (Kechad et al. 2012), but this mechanism does not seem to operate in the zebrafish *vsx1* lineage. Other, still unknown, mechanisms must therefore operate to underlie the asymmetric Notch activity. The unequal partitioning of a fate determinant in an RPC to its daughters relies both on the distribution of the determinant within the RPC and on the subsequent angle of cleavage at mitosis. In the mouse retina, Numb has a polarized distribution within RPCs and a cleavage plane that distributes it unequally to the emerging apically and basally located daughter cells, permitting diverging fates to emerge (Cayouette & Raff 2003, Kechad et al. 2012). In the zebrafish retina, mitotic divisions occur along multiple axes (**Figure 4**), but at least to date, no evidence has emerged to correlate asymmetric inheritance of a fate determinant and distinct fates.

7. CONCLUDING REMARKS

In the last few decades much progress has been made in our understanding of how different cell classes in the vertebrate retina are generated. The tools employed have expanded beyond classical gain- and loss-of-function experiments and have become increasingly sophisticated so that more intricate details of gene regulatory networks and epigenetic regulatory mechanisms are being revealed. Further, different model systems have provided insights into the evolutionarily conserved mechanisms of development across vertebrates. More recently, there have even been forays into directly understanding human retinal development, through scRNA-seq of fetal tissue and organoids. The insights gained from such investigations of cell fate regulators will undoubtedly contribute to regenerative medicine. Indeed the targeted generation of specific retinal cell types through either induced pluripotent cells or the coaxing of endogenous sources of cell replacement (e.g., MCs) is no longer a distant reality (Jorstad et al. 2017, Todd et al. 2021, Yao et al. 2018).

SUMMARY POINTS

1. The retina is an accessible part of the CNS. Its peripheral location, stereotypic cytoarchitecture, and readily identifiable cell classes make it particularly suitable to the investigation of mechanisms underlying cell fate acquisition.
2. Although the length of retinogenesis varies across vertebrates, retinal cell classes are generated in a conserved, albeit overlapping, order. Cells with distinct fates that are destined for different layers can be generated concurrently.
3. Retinal progenitor cells are largely multipotent but not molecularly homogeneous, suggesting that distinct lineages coexist. At any given time, individual retinal progenitors are competent to generate only a small repertoire of cell classes based on the specific transcription factor(s) that they express.
4. In addition to multipotent progenitors, progenitors that are biased to generate specific cell classes and those committed to the exclusive generation of particular cell classes exist.
5. Retinal cell classes can be generated via asymmetric mitotic divisions, in which their immediate sibling is a progenitor, or via terminal divisions, in which another postmitotic cell is their sibling. Terminal divisions can be further divided into symmetric or asymmetric modes depending on whether the daughter cells adopt the same or distinct fates, respectively.
6. Cell intrinsic mechanisms involving temporal transcription factors and downstream gene regulatory networks play major roles in instructing cell fate. By comparison, extrinsic cues play minor roles.
7. The gene regulatory networks instructing distinct retinal cell fates are beginning to be revealed in increasing detail. The use of scRNA-seq to analyze retinal development has expanded our knowledge of the molecular diversity of progenitors and the gene regulatory networks that mediate cell fates.

FUTURE ISSUES

1. The ways in which subtypes within retinal cell classes are generated should be investigated.
2. scRNA-seq analysis should be expanded to allow for the detection of mRNA isoforms of genes that likely play distinct roles in different contexts.
3. Newly emerging technologies in spatial genomics should be used to examine the expression of multiple transcripts at the single-cell level in developing retinal tissue.
4. Protein expression at the single-cell level during retinal development should be investigated.
5. New genetic sensors of key signaling pathways (e.g., the Notch pathway) should be generated, or existing sensors should be improved, to allow for more temporally resolved analysis of the activity of these pathways in in vivo or in vitro imaging contexts.

6. The link between lineage and connectivity should be explored to answer the question of whether cells that are born together or are clonally related preferentially form synaptic connections.

DISCLOSURE STATEMENT

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2.2 Notch-mediated re-specification of neuronal identity during central nervous system development (*published in Current Biology*)

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Summary

Neuronal identity has long been thought of as immutable, so that once a cell acquires a specific fate it is maintained for life.[1] Studies using over-expression of potent transcription factors to experimentally reprogram neuronal fate in the mouse neocortex[2, 3] and retina[4, 5] have challenged this notion by revealing that post-mitotic neurons can switch their identity. Whether fate reprogramming is part of normal development in the central nervous system (CNS) is unclear. While there are some reports of physiological cell fate reprogramming in invertebrates,[6, 7] and in the vertebrate peripheral nervous system,[8] endogenous fate reprogramming in the vertebrate CNS has not been documented. Here we demonstrate spontaneous fate re-specification in an interneuron lineage in the zebrafish retina. We show that the *visual system homeobox 1* (*vsx1*)-expressing lineage, that has been associated exclusively with excitatory bipolar cell (BC) interneurons [9, 10, 11, 12] also generates inhibitory amacrine cells (ACs). We identify a role for Notch signaling in conferring plasticity to nascent *vsx1* BCs, allowing suitable transcription factor programs to re-specify them to an AC fate. Over-stimulating Notch signaling enhances this physiological phenotype so that both daughters of a *vsx1* progenitor differentiate into ACs and partially differentiated *vsx1* BCs can be

converted into ACs. Furthermore this physiological re-specification can be mimicked to allow experimental induction of an entirely distinct fate, that of retinal projection neurons, from the *vsx1* lineage. Our observations reveal unanticipated plasticity of cell fate during normal retinal development.

Author contributions

Project design and experimental design: P.E., E.P., P.R.W., T.M., and L.G.

Project supervision: L.G. and T.M.

Experiments performed by P.E. and E.P.

Construct generation and transgenic lines: P.E., S.C.S., T.Y., and L.G.

Advice on the data analysis: R.P.

Manuscript writing with input from all of the authors: L.G.

Specifically, I contributed to the following figure panels in full (experiments and analysis):

Figure 1(F), Figure 3(B, glutamine synthetase marker), Figure 4(F), Figure S1(C-E), Figure S2(D), Figure S3(A&B), Figure S4(B).

I contributed data (equal or partial contribution of the dataset) and/or analysis (of data derived both from me and P.E.) to the following panels:

Figure 2(B&C), Figure 3(B&C), Figure 4(B,C,E), Figure S1(A&B), Figure S2(C), Figure S3(C).

Report

Current Biology

Notch-mediated re-specification of neuronal identity during central nervous system development

Highlights

- Spontaneous fate reprogramming in the *vsx1* lineage of the zebrafish retina
- Nascent *vsx1* bipolar cells are re-specified to an amacrine cell fate
- Notch signaling plays a major role in *vsx1* bipolar cell re-specification
- Over-stimulating Notch signaling enhances reprogramming in the *vsx1* lineage

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In brief

Engerer, Petridou et al. find evidence for spontaneous cell fate reprogramming during retinal development. Newly post-mitotic *vsx1* bipolar cells switch to an amacrine cell fate. Notch signaling plays a major role, conferring plasticity to nascent bipolar cells, allowing suitable transcription factors to instruct the amacrine cell fate.

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Report

Notch-mediated re-specification of neuronal identity during central nervous system development

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SUMMARY

Neuronal identity has long been thought of as immutable, so that once a cell acquires a specific fate, it is maintained for life.[1] Studies using the overexpression of potent transcription factors to experimentally reprogram neuronal fate in the mouse neocortex[2,3] and retina[4,5] have challenged this notion by revealing that post-mitotic neurons can switch their identity. Whether fate reprogramming is part of normal development in the central nervous system (CNS) is unclear. While there are some reports of physiological cell fate reprogramming in invertebrates,[6,7] and in the vertebrate peripheral nervous system,[8] endogenous fate reprogramming in the vertebrate CNS has not been documented. Here, we demonstrate spontaneous fate re-specification in an interneuron lineage in the zebrafish retina. We show that the *visual system homeobox 1* (*vsx1*)-expressing lineage, which has been associated exclusively with excitatory bipolar cell (BC) interneurons,[9–12] also generates inhibitory amacrine cells (ACs). We identify a role for Notch signaling in conferring plasticity to nascent *vsx1* BCs, allowing suitable transcription factor programs to re-specify them to an AC fate. Overstimulating Notch signaling enhances this physiological phenotype so that both daughters of a *vsx1* progenitor differentiate into ACs and partially differentiated *vsx1* BCs can be converted into ACs. Furthermore, this physiological re-specification can be mimicked to allow experimental induction of an entirely distinct fate, that of retinal projection neurons, from the *vsx1* lineage. Our observations reveal unanticipated plasticity of cell fate during retinal development.

RESULTS AND DISCUSSION

Vsx1 progenitors generate bipolar cells (BCs) and amacrine cells (ACs)

Vsx1 is a key determinant of BC fate across vertebrate species.[9–11,13] In the zebrafish retina, *vsx1* BCs are generated by *vsx1* progenitors via terminal, symmetric mitotic divisions,[14,15] suggesting they are hard-wired to the BC fate. However, in a *vsx1*:GFP transgenic line that faithfully reports *vsx1* expression,[12,16] small numbers of ACs are labeled[12] (Figure 1A). We investigated the origin of these ACs by *in vivo* time-lapse recordings in a Gal4-driver line (*Q32*) that provides a lineage label for a subset of *vsx1* cells.[14] ACs were generated by terminally dividing *Q32* progenitors, with BCs as their siblings. These AC-BC divisions represented almost half of all *Q32* divisions (41.2% ± 6%, mean ± SEM, 141 divisions, 16 fish; Figure 1B; see also lineage tracing studies[17,18]). *Q32* daughters that became ACs maintained somal contact with their BC sibling for several hours (5.3 ± 0.7 h, mean ± SEM, 13 pairs, 6 fish), before acquiring AC features. By contrast, BC-BC siblings did not lose somal contact after mitosis (at least over the recording, 14.5 ± 0.7 h, mean ± SEM, 10 pairs, 7 fish). Loss of somal contact with their BC sibling thus represents the earliest sign of morphological differentiation toward an AC fate (Figure S1A). *Q32* ACs express a bona fide pan AC marker, *ptf1a*[19]

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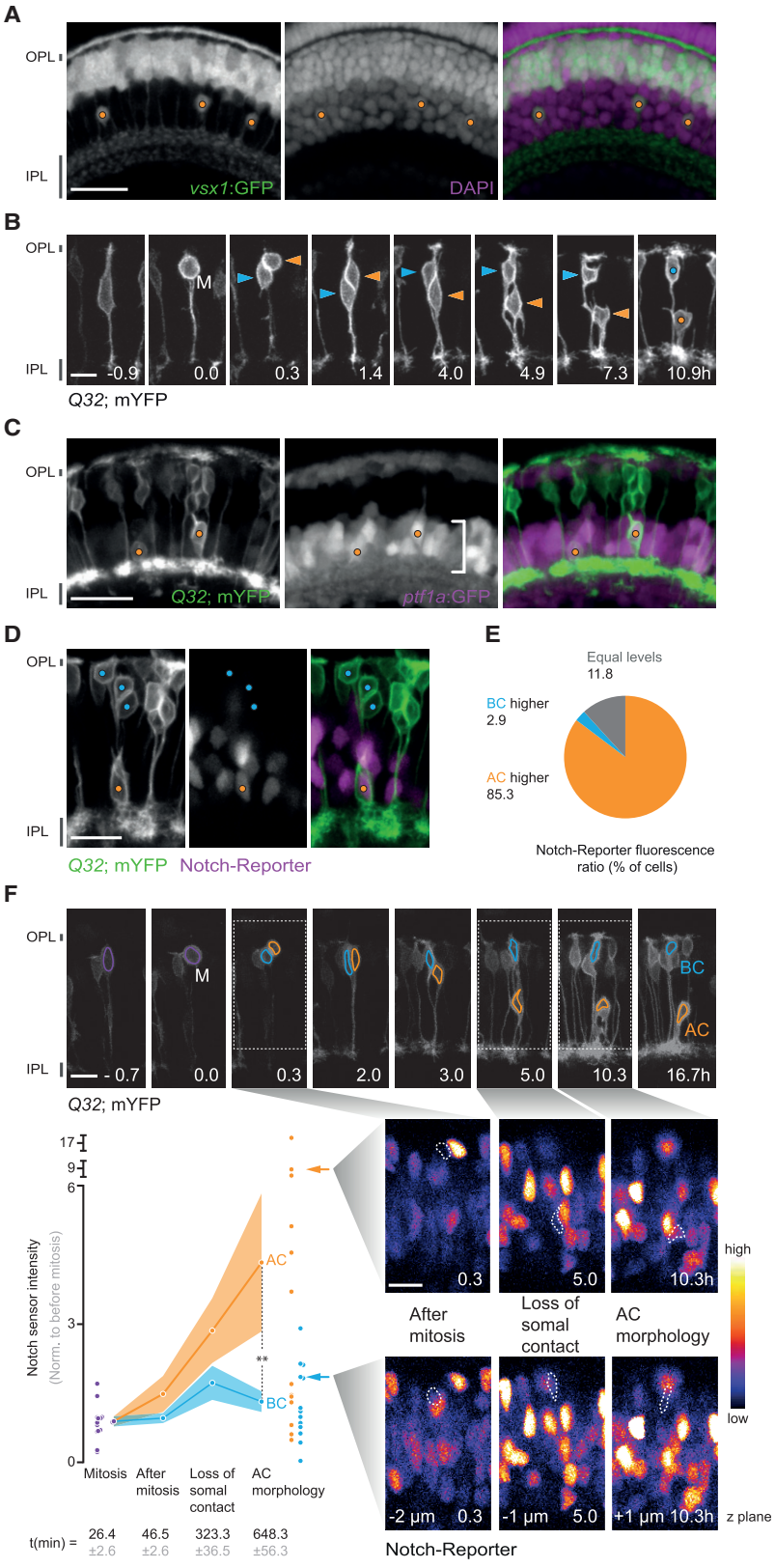

Figure 1. *Vsx1* progenitors generate ACs

(A) 3 dpf *vsx1*:GFP retina with *vsx1*$^+$ BCs and ACs (orange circles).

(B) 2 dpf *Q32* progenitor undergoing mitosis (M), generates a BC (cyan arrowhead; circle, final time point) and an AC (orange arrow-head, circle final time-point).

(C) 3 dpf WT retina showing *ptfa*:GFP$^+$ *Q32* ACs (orange circles). *Ptf1a*:GFP labels all ACs (INL, bracket, center panel). *Ptf1a*-GFP signal bleeds through *Q32*-YFP channel.

(D) 3 dpf retina showing Notch-reporter (*tp1*:hmgb1mCherry) expression in a *Q32* AC (orange circle), but not in surrounding *Q32* s BCs (cyan circles).

(E) Notch-reporter (*tp1*:hmgb1mCherry) fluorescence intensity in *Q32* ACs and BCs. AC and BC categories were tested against an expected frequency of 50% using a binomial test, $p < 0.0001$; 30 cells, 10 fish. Four ACs (of 34) displayed equal levels of fluorescence as neighboring BCs.

(F) 2 dpf transgenic retina (*Q32*; mYFP; *tp1*:H2BmCherry; top grayscale images, gamma adjusted) showing a WT *Q32* progenitor (purple outline) generating a BC (cyan) and an AC (orange). Notch-reporter levels in the AC and BC depicted using a Fire LUT (below, right). Notch-reporter intensity levels (means ± SEMs) for 13 *Q32* progenitors (8 fish) and their BC (cyan) and AC (orange) daughters (below, left). Times in relation to the time point before mitosis. Significant differences between BC-AC pairs were found when the AC acquired its characteristic morphology (Wilcoxon matched-pairs signed-rank test, $p = 0.0061$).

Scale bars, 20 µm (A and C); 10 µm (B, D, and F). IPL, inner plexiform layer; OPL, outer plexiform layer.

See also Figures S1A, S2, and S3A–S3C.

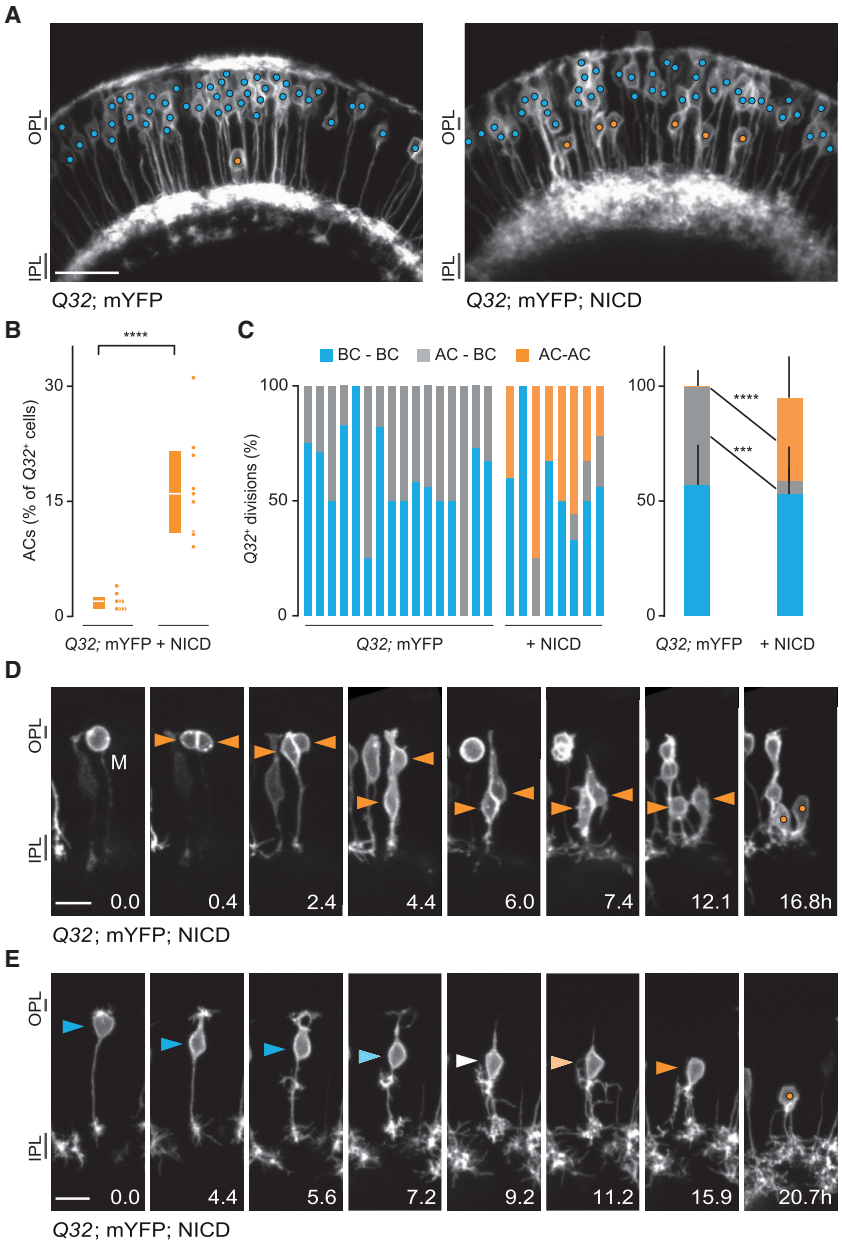

Figure 2. Notch signaling promotes AC fate in the *vsx1* lineage

(A) 3 dpf retinae from *Q32* WT and *Q32* NICD. BCs (cyan circles), ACs (orange circles).

(B) Proportion of ACs in WT and *Q32* NICD. Median and interquartile range (IQR) shown. *Q32* WT (21 ACs, 9 fish); *Q32* NICD (140 ACs, 9 fish), $p < 0.0001$, Mann-Whitney U test.

(C) Proportion of *Q32* divisions in WT and following NICD OE for each fish (left). Collective representation of *Q32* divisions, representing median and IQR per group (right). BC-BC: *Q32* WT versus NICD, $p = 0.5134$. AC-BC: *Q32* WT versus NICD, $p = 0.0004$. AC-AC: *Q32* WT versus NICD, $p < 0.0001$. WT: 141 *Q32* divisions, 16 fish; *Q32* NICD: 44 divisions, 8 fish. Mann-Whitney U test.

(D) 2 dpf *Q32* NICD retina, showing a mitotic progenitor (M) generating 2 ACs (orange arrowheads; circles, last time point).

(E) 2 dpf *Q32* NICD retina, showing a BC (cyan arrowhead), transdifferentiating into an AC (orange arrowhead; circle, last time point).

Scale bars, 20 μm (A); 10 μm (D and E).

See also Figures S1, S3D, and S3E.

(99.2% ± 0.8% of *Q32* ACs were *ptf1a*:GFP$^+$; mean ± SEM, 60 cells, 13 fish; Figure 1C), and only a small number maintain *vsx1* expression (1 of 26 *Q32* ACs was *vsx1*:GFP$^+$ at 3 days post-fertilization [dpf], 15 fish; Figures S2A–S2C), accounting for the low AC number in *vsx1*:GFP retinae (0.8% ± 0.2% of 2,295 *vsx1*:GFP$^+$ cells, mean ± SEM, 3 dpf, 8 fish). We also detected cells with an AC morphology in BC transgenic lines (*crx*:mCFP and *ctbp2*:mEGFP;[14,20,21] Figure S2D), hinting at their BC lineage origin.

Notch signaling promotes the AC fate in the *vsx1* lineage

To investigate whether Notch signaling underlies the asymmetric fates,[16,22–26] we crossed the *Q32* line to Notch-reporter lines[27,28] (Figure 1D). The majority of *Q32* ACs displayed higher Notch-reporter levels than *Q32* BCs in their vicinity (Figure 1E). To determine when the divergence of Notch signaling emerged, we monitored Notch-reporter expression in time-lapse recordings (Figure 1F). Notch-reporter levels were low in *Q32* progenitors and increased gradually post-mitosis. Significant increases were only detectable in the AC sibling of AC-BC pairs when it acquired its typical morphology (average 10 h post-mitosis), implicating the post-mitotic nature of this signaling event. We investigated whether mechanisms at the progenitor level[29–31] accounted for the signaling asymmetry in the ensuing daughters. However, we found neither asymmetric distribution of the Notch regulator Numb (10 cells, 4 fish) nor mitotic cleavage along a stereotypic axis (45 cells, 25 fish; Figures S3A and S3B), suggesting these key players are not involved.

We used DAPT, a γ-secretase inhibitor, to abrogate Notch signaling[32] and found an ~50% decrease in *vsx1*:GFP$^+$ ACs (DAPT-treated 2.0 ± 1.4 ACs per region, 23 regions, 12 fish versus DMSO controls 5.0 ± 1.6 ACs per region, 18 regions, 9 fish, median ± SD, $p < 0.0001$, Mann-Whitney U test; Figure S3B). Conversely, overexpression (OE) of the Notch intracellular domain (NICD)[33] in the *vsx1* lineage (*Q32* NICD) led to a 9-fold increase in *Q32* ACs (Figures 2A and 2B), that persisted at least until 5 dpf (Figure S3C). The increased AC number did not arise from increased *Q32* progenitor proliferation (0.21 ± 0.03 wild type [WT] versus 0.19 ± 0.05 NICD, means ± SEMs, divisions per hour, monitored 18 h from 2 to 3 dpf; $p = 0.75$, t test), but rather from the emergence of AC-AC pairs, a division mode not seen in *Q32* WT (Figures 2C and 2D), and by BC-to-AC

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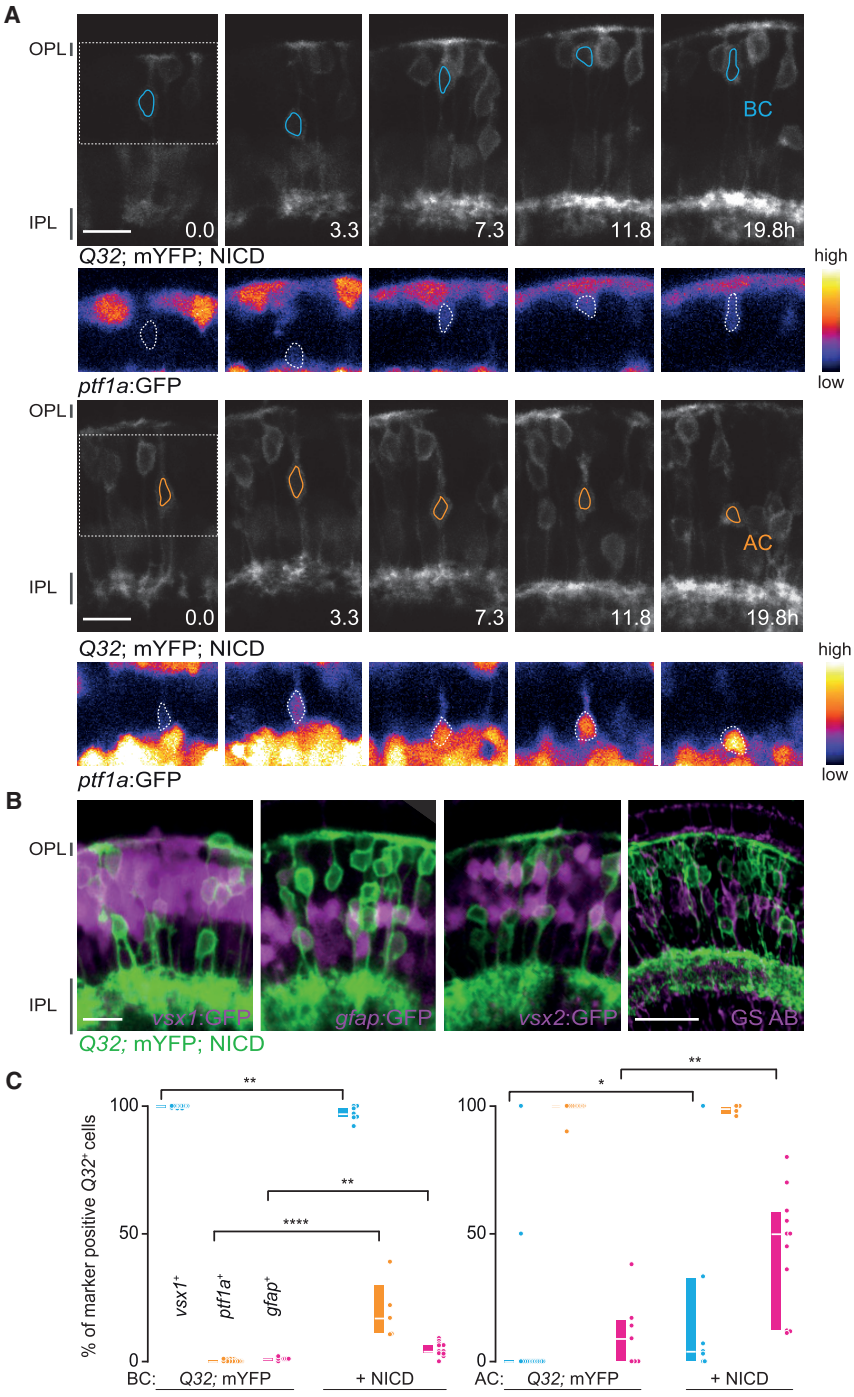

Figure 3. Marker expression in re-specified ACs

(A) In *Q32* NICD, BCs (outlined cyan, upper grayscale panels), express *ptf1a*:GFP (Fire LUT), albeit at lower levels than in ACs (outlined orange, lower grayscale panels). *Ptf1a*-GFP signal bleeds through *Q32*-YFP channel (grayscale panels).

(B) Following NICD OE, transdifferentiated *Q32* ACs do not express *vsx1*:GFP but *gfap*:GFP. *Q32* ACs are negative for *vsx2*:GFP and glutamine synthetase (GS antibody, AB).

(C) Percentage of *Q32* BCs (left) and ACs (right) expressing *vsx1*, *ptf1a* and *gfap* in WT or *Q32* NICD. Median and IQR represented. BCs: *vsx1*:GFP$^+$: *Q32* WT, (1227 BCs, 14 fish) versus NICD, (416 BCs, 7 fish); p = 0.0034. *Ptf1a*:GFP$^+$: *Q32* WT (1,614 BCs, 14 fish) versus NICD (486 BCs, 5 fish); p < 0.0001. *Gfap*:GFP$^+$: *Q32* WT (718 BCs, 7 fish) versus NICD (984 BCs, 11 fish); p = 0.0022. ACs: *vsx1*:GFP$^+$: *Q32* WT (28 ACs, 15 fish) versus *Q32* NICD (100 ACs, 7 fish); p = 0.0129. *Ptf1a*:GFP$^+$: *Q32* WT (60 ACs, 13 fish) versus *Q32* NICD (124 ACs, 4 fish); p = 0.1206. *Gfap*:GFP$^+$: *Q32* WT (43 ACs, 7 fish) versus *Q32* NICD (335 ACs, 11 fish); p = 0.0078. Mann-Whitney U test for pairwise comparisons.

Scale bars, 10 μm (A and B).

transdifferentiation (17 observations, 9 fish; Figure 2E). Transdifferentiating BCs exhibited process exuberance and took longer to acquire AC features compared to WT (Figure S1B), highlighting the fate switch that occurred along their original differentiation trajectory. Time-lapse of the Notch-reporter in *Q32* NICD during transdifferentiation showed that Notch signaling increases when the soma translocated toward the inner part of the inner nuclear layer (INL) and AC morphology emerged. Following AC-BC divisions, Notch-reporter levels increased significantly in the AC sibling several hours (average 6 h) post-mitosis (Figures S1C and S1D). Thus, also after NICD OE, Notch signaling appears to operate post-mitotically to re-specify fate.

Transdifferentiated ACs lose BC markers and express markers of immaturity

ACs in *Q32* NICD retinae were *ptf1a*:GFP$^+$ and most lost expression of *vsx1*:GFP (Figures 3A and 3C). The NICD-induced ACs also lacked other BC markers (0% *crx*:mCFP$^+$, 65 ACs, 3 fish; 1.9% ± 1.1% *ctbp2*:mEGFP$^+$, means ± SEMs, 95 ACs, 4 fish), resembling WT *Q32* ACs. Notably, 20% of BCs in *Q32* NICD expressed *ptf1a*:GFP, a phenotype that is absent in WT fish (Figures 3A and 3C). This suggests that after NICD OE, some BCs expressed AC markers, but did not adopt an AC morphology. Moreover, >40% of *Q32* NICD ACs were *gfap*:GFP$^+$, which is expressed in neural progenitors[34,35] (Figures 3B and 3C). Importantly, ~11% of WT *Q32* ACs also expressed this marker. Notch signaling is associated with Müller cell specification[36–39] and GFAP is expressed by Müller glia.[34] To exclude that NICD-induced *Q32* ACs were driven toward a Müller cell fate, we examined their expression of glutamine synthetase (GS)[40] and *vsx2*,[12] but found no evidence for this (GS: 0 of 68 ACs, 7 *Q32* NICD fish; 0 of 28 ACs, 9 *Q32* WT fish; *vsx2*:GFP: 0 of 274 ACs, 6 *Q32* NICD fish; 0 of 26 ACs, 6 *Q32* WT fish). Thus, *gfap*

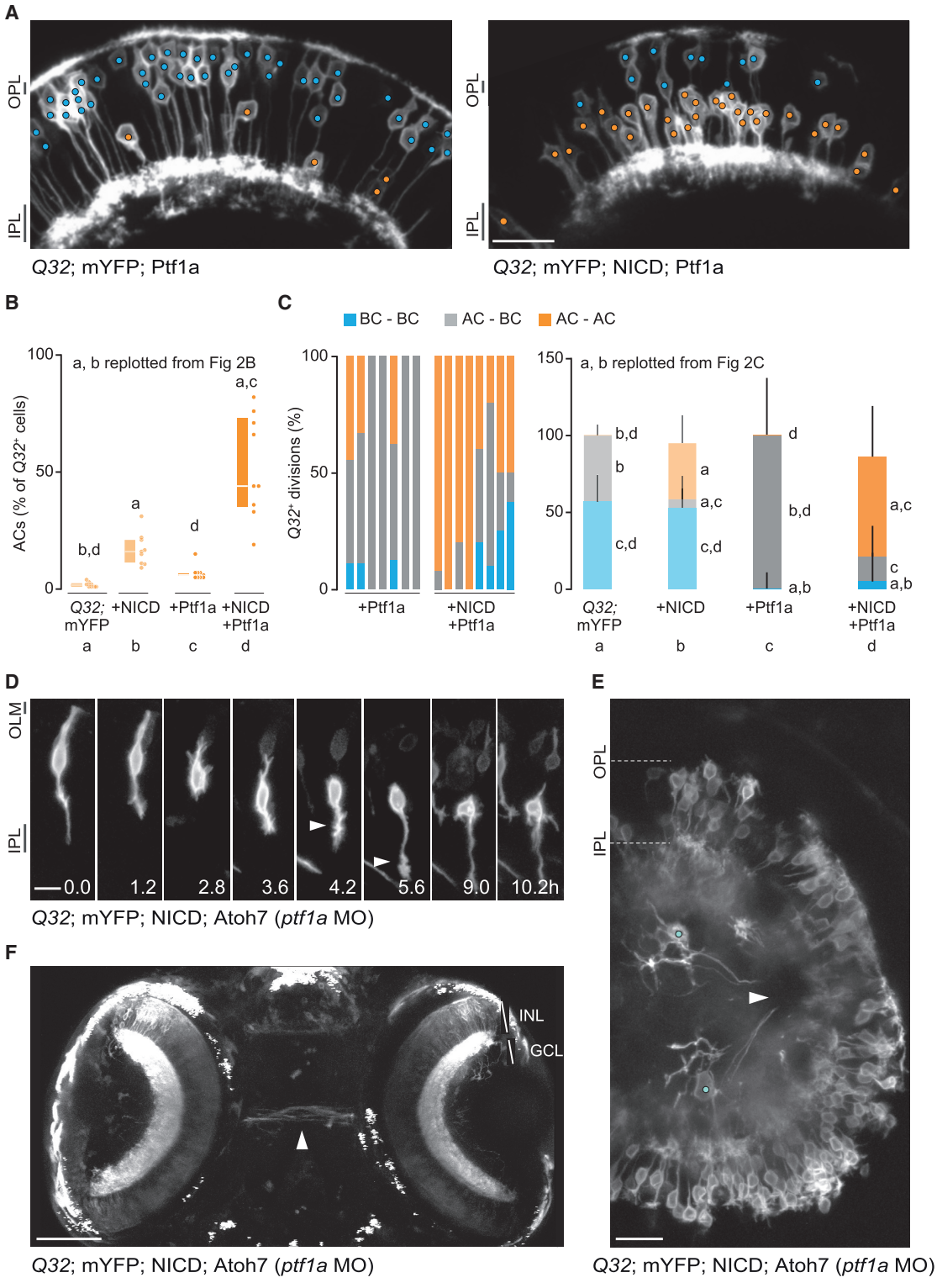

Figure 4. Notch signaling imparts plasticity to nascent post-mitotic cells

(A) 3 dpf retinae from *Q32* Ptf1a and *Q32* NICD Ptf1a fish. *Q32* BCs (cyan circles), ACs (orange circles).

(B) Proportion of ACs in *Q32* Ptf1a and *Q32* NICD Ptf1a represented as median and IQR. *Q32* Ptf1a (66 ACs, 9 fish), *Q32* NICD Ptf1a (372 ACs, 9 fish). *Q32* WT and *Q32* NICD originally plotted in Figure 2B. Significant differences found between WT and NICD ($p = 0.0003$), WT and NICD Ptf1a ($p < 0.0001$), and Ptf1a and NICD

(legend continued on next page)

in *Q32* ACs does not indicate Müller cell identity, but rather an immature state, permissive of a fate switch.

Ptf1a can induce the AC fate in the *vsx1* lineage

We next asked whether Ptf1a, a determinant of AC fate,[41–45] also governs the fate of *vsx1* ACs. Ptf1a OE in the *vsx1* lineage (*Q32* Ptf1a) resulted in a 3.6-fold increase in *Q32* ACs compared to WT (Figures 4A and 4B). Divisions generating at least 1 AC were higher in *Q32* Ptf1a (100% ± 6.19%, median ± SD) than in *Q32* WT (43% ± 23.8%, median ± SD, Mann-Whitney U test, p = 0.0001; Figure 4C) and in *Q32* NICD (47% ± 28.5%, median ± SD, Mann-Whitney U test, p = 0.0048). Surprisingly, we found a lower number of excess ACs in *Q32* Ptf1a compared to *Q32* NICD (Figure 4B), likely due to the lack of transdifferentiation events. Thus, Ptf1a appears to influence fate around mitosis or shortly thereafter. To examine whether NICD OE could extend this plasticity window, we generated *Q32* NICD Ptf1a fish and found a 28-fold increase in *Q32* ACs compared to WT (Figures 4A and 4B). AC-AC pairs accounted for the majority of divisions in these fish (66.5%; Figure 4C). Thus, physiological Notch signaling confers plasticity to nascent *vsx1* BCs, making them receptive to Ptf1a, which instructs the switch to an AC fate, a phenomenon that can be enhanced by combined NICD and Ptf1a OE. We next probed whether NICD OE combined with another transcription factor could instruct a cell fate beyond ACs.

Ganglion cell (GC)-like cells can be induced in the *vsx1* lineage

We tested whether GCs could be generated by knocking down *ptf1a*[43,46] to suppress AC fate and overexpressing NICD with atonal homolog 7 (Atoh7), a critical GC fate determinant.[47–51] We observed bipolar-shaped *Q32* cells, transforming morphologically while translocating to the GC layer (GCL) and initiating axon outgrowth (14 cells, 3 fish; Figure 4D). These cells expressed *isl2b*:GFP, a GC marker[52] (Figure S4). Of 68 *Q32* GCL-localized cells (24 fish), 30 bore morphologies reminiscent of GCs (Figure 4E), while the rest lacked a detectable axon or displayed few or no discernible neurites. At 5 dpf (4 fish), we observed *Q32* GC axons at the optic chiasm, suggesting successful retinal exit and path finding en route to the optic tectum (Figures 4F and S4).

Implications of developmental plasticity

Here, we report physiological Notch-dependent fate reprogramming in the *vsx1* lineage, allowing nascent BCs to re-specify to an AC fate. That Notch signaling confers plasticity to nascent post-mitotic cells has been suggested to occur in the postnatal mouse retina.[53] Notch signaling has also been implicated in transdifferentiation events in the damaged mammalian cochlea.[54] In the latter case, however, in contrast to our findings, attenuation of Notch signaling is involved.[55]

Vsx1-lineage-derived ACs represent a small fraction of the total AC population, the majority of which arise from a different lineage.[43,56] Could *vsx1* ACs have a specific functional significance? One can speculate that *vsx1* ACs are generated "on demand," with the BC-to-AC re-specification allowing local fine-tuning of excitation and inhibition. Reducing the AC number, using a *ptf1a* morpholino, led to an almost 2-fold increase in *vsx1* cells expressing *ptf1a*:GFP, thus commencing differentiation toward an inhibitory fate.

Finally, understanding the mechanisms of physiological fate re-specification could guide cell replacement strategies such as direct *in vivo* reprogramming.[57] While progress has been made converting glia to neurons,[58,59] re-specifying neurons from one subtype to another is limited to a short developmental time window, perhaps due to epigenetic changes that fixate cell type identities.[2,3,5] A pulse of Notch signaling may rejuvenate post-mitotic cells and re-open the window for transcription factor-induced re-specification of neuronal subtypes and thus should be explored in the ongoing search for efficient replacement strategies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Generation of constructs
  - Capped RNA synthesis and injections
  - Mounting zebrafish larvae for *in vivo* imaging
  - *In vivo* imaging
  - Photoconversion

Ptf1a (p = 0.0005), denoted by a (significantly different from *Q32* WT), b (from NICD), c (from Ptf1a), or d (from NICD Ptf1a). Kruskal-Wallis and post hoc Dunn's multiple comparisons test, further adjusted by the Benjamini-Hochberg false discovery rate (FDR) method.

(C) *Q32* divisions in *Q32* Ptf1a and *Q32* Ptf1a NICD for each fish (left). Divisions representing median and IQR per group (right). *Q32* Ptf1a: 42 divisions, 7 fish; *Q32* NICD Ptf1a: 52 divisions, 8 fish. *Q32* WT and *Q32* NICD originally plotted in Figure 2C. Significant differences were found in BC-BC divisions: WT versus Ptf1a (p = 0.0001), WT versus NICD Ptf1a (p = 0.0004), NICD versus Ptf1a (p = 0.0029), NICD versus NICD Ptf1a (p = 0.0075); AC-BC divisions: WT versus NICD (p = 0.0127), NICD versus Ptf1a (p = 0.0001), Ptf1a versus NICD Ptf1a (p = 0.0034); AC-AC divisions: WT versus NICD (p = 0.0007), WT versus NICD Ptf1a (p < 0.0001), Ptf1a versus NICD Ptf1a (p = 0.0035), denoted as in (B). Kruskal-Wallis and post hoc Dunn's multiple comparisons test, further adjusted by the Benjamini-Hochberg FDR method.

(D) An emerging GC-like cell in a 2-dpf *Q32* mYFP NICD Atoh7 retina with *ptf1a* morpholino (MO). The cell migrates basally, loses its bipolar morphology, and grows out an axon (arrowhead) and apically directed processes.

(E) Two *Q32* GC-like cells in a 3-dpf transgenic embryo (*Q32*; mYFP; NICD; Atoh7 and *ptf1a* MO). Putative axons exit the retina (arrowhead). Gamma adjusted.

(F) Dorsal view of a 3-dpf embryo (rostral top, eyes lateral) in which GC-like cells were induced (*Q32*; mYFP; NICD; Atoh7 and *ptf1a* MO), showing axons at the chiasm (arrowhead). Gamma adjusted.

Scale bars, 20 μm (A); 10 μm (D and E); 100 μm (F). GCL, ganglion cell layer; INL, inner nuclear layer; OLM, outer limiting membrane.

See also Figure S4.

- Morpholino injections
- DAPT treatment
- Immunostaining
- Genotyping of Q32 crosses

- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Cell type classification of Q32 cells
  - Quantification of Kaede recovery in photoconverted Q32 cells
  - Analysis of DAPT treated animals
  - Notch-Reporter levels in differentiated Q32 cells
  - Monitoring Notch-Reporter levels in time-lapse recordings of the Q32 lineage
  - Division rate analysis
  - Cleavage plane analysis
  - Statistics

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.08.049.

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AUTHOR CONTRIBUTIONS

P.E., E.P., P.R.W., T.M., and L.G. conceived of the project and designed the experiments. L.G. and T.M. supervised the project. P.E. and E.P. performed the experiments. P.E., S.C.S., T.Y., and L.G. generated new constructs and transgenic lines. R.P. advised on the data analysis. L.G. wrote the paper, with input from all of the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-c-myc, clone 9E10, 1:100	Sigma-Aldrich	Cat# M5546; RRID: AB_260581
Chicken polyclonal anti-GFP, 1:1000	Abcam	Cat# ab13970; RRID: AB_300798
Mouse monoclonal anti-glutamine synthetase, clone GS-6, 1:50	Sigma-Aldrich	Cat# MAB302; RRID: AB_2110656
Goat anti-mouse Alexa 647, IgG1, 1:250	Invitrogen	Cat# A-21240; RRID: AB_141658
Goat anti-chicken Alexa 488, IgG (H+L), 1:250	Invitrogen	Cat# A-11039; RRID: AB_142924
Goat anti-mouse Alexa 568, IgG2a, 1:250	Invitrogen	Cat# A-21134; RRID: AB_2535773
Chemicals, peptides, and recombinant proteins		
N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT)	Enzo Life Science	Cat# 50-200-8542
Critical commercial assays		
MyTaq Extract-PCR kit	Bioline	Cat# BIO-21126
Omniscript RT kit	QIAGEN	Cat# 205111
Ambion mMessage mMachine kit	Thermo Fisher Scientific	Cat# AM1340
Experimental models: Organisms/strains		
Zebrafish: Tg(vsx1:GFP)nns5: nns5Tg	[12,16]	ZFIN: ZDB-ALT-090116-1
Zebrafish: Tg(vsx2:GFP)nns1: nns1Tg	[12,60]	ZFIN: ZDB-ALT-061204-2
Zebrafish: Tg(UAS:gap43-YFP)q16b: q16Tg	[61]	ZFIN: ZDB-ALT-071129-3
Zebrafish: Tg(crx:MA-CFP)q20: q20Tg	[21]	ZFIN: ZDB-ALT-131118-1
Zebrafish: Tg(vsx1:Gal4)q32: q32Tg	[14]	ZFIN: ZDB-ALT-170831-2
Zebrafish: Tg(14xUAS:memTagRFP-T)	[14]	ZFIN: ZDB-ALT-170831-1
Zebrafish: Tg(ptf1a:eGFP)jh1: jh1Tg	[19]	ZFIN: ZDB-ALT-070531-2
Zebrafish: Tg(UAS:myc-notch-intra): kca3Tg	[33]	ZFIN: ZDB-ALT-020918-8
Zebrafish: Tg(T2KTp1bglob:hmgb1mCherry) jh11: jh11Tg	[28]	ZFIN: ZDB-ALT-101006-1
Zebrafish: Tg(*gfap*:GFP)mi2001: mi2001Tg	[34]	ZFIN: ZDB-ALT-060623-4
Zebrafish: Tg(UAS:Kaede)s1999t: s1999tTg	[62]	ZFIN: ZDB-ALT-070314-1
Zebrafish: Tg(−17.6isl2b:GFP): zc7Tg	[52]	ZFIN: ZDB-ALT-100322-2
Zebrafish: Tg(−1.8ctbp2:gap43-EGFP) lmb1: lmb1Tg	[20]	ZFIN: ZDB-ALT-120320-3
Zebrafish: Tg(EPV.TP1-Mmu.Hbb: hist2h2l-mCherry)s939: s939Tg	[27]	ZFIN: ZDB-ALT-110503-3
Zebrafish: Tg(14xUAS:mCherry)s1984t: s1984tTg	[63]	ZFIN: ZDB-ALT-130702-1
Zebrafish: Tg(5xUAS:Atoh7)	This paper	N/A
Zebrafish: Tg(5xUAS:Ptf1a)	This paper	N/A
Oligonucleotides		
For all primers used in cloning, see Table S1	Metabion	N/A
For all primers used in genotyping, see Table S2	Metabion	N/A
Morpholino p53: 5′- GCGCCATT GCTTTGCAAGAATTG-3′	Gene tools,[64]	ZFIN: ZDB-MRPHLNO-070126-7
Morpholino:MO1-ptf1a: 5′-CCAACA CAGTGTCCATTTTTTGTGC-3′	Gene tools,[43,46]	ZFIN: ZDB-MRPHLNO-070531-6
Recombinant DNA		
pCH-5xUAS:Ptf1a	This paper	N/A
pCH-5xUAS:Atoh7	This paper	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
atoh7 cDNA, I.M.A.G.E. clone	Source Bioscience	Cat# IRBVp5006D093D
pCS2 Numb-GFP	[65]	N/A
Software and algorithms		
ImageJ/Fiji	[66]	RRID: SCR_002285 ; https://fiji.sc/
Imaris	Bitplane	RRID: SCR_007370; https://imaris.oxinst.com/
Adobe Illustrator	Adobe	RRID: SCR_010279; https://www.adobe.com/
Adobe Photoshop	Adobe	RRID: SCR_014199; https://www.adobe.com/
GraphPad Prism	Graphpad Software	RRID: SCR_002798; https://www.graphpad.com

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Leanne Godinho (leanne.godinho@tum.de).

Materials Availability

Plasmids are available upon request to the lead contact.

Data and Code Availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experiments were performed according to local regulations (Regierung von Oberbayern). Zebrafish were maintained and bred as previously described.[67] Embryos were kept in 0.3x Danieau's solution at 28.5°C and staged as previously described.[68] Fish were either in an AB wild-type, Tuebingen Long Fin (TLN) or roy orbison[69] background. All experiments were performed on animals between 2 and 5 days post fertilization (dpf). During this period zebrafish are not sexually differentiated.[70] The transgenic lines used are listed in the key resources table. Note that line Tg(*vsx1*:Gal4)q32 (*Q32*) was referred to as Tg(*vsx1*:Gal4)q26 (*Q26*).[14] To generate the line, a 3.2 kb fragment upstream of the *vsx1* coding sequence was used to drive the expression of Gal4-VP16. Crossing the *Q32*:Gal4 line, in conjunction with a *uas*:memTag-RFP-T line, to *vsx1*:GFP revealed that the *Q32* driver faithfully labels a subset of *vsx1*:GFP+ cells.[14] We generated Tg(*5xuas*:Ptf1a) and Tg(*5xuas*:Atoh7) by Tol2 mediated insertion.[71]

METHOD DETAILS

Generation of constructs

To generate *pCH-5xuas*:Atoh7, the coding sequence of zebrafish *atoh7* (*atonal bHLH transcription factor 7*) was PCR amplified using primers containing EcoRI and NotI restriction sites (sequences of primers Atoh7 Forward and Atoh7 Reverse are available in Table S1) from a plasmid containing full-length *atoh7* cDNA (I.M.A.G.E Clone IRBVp5006D093D, Source Bioscience). The amplified *atoh7* sequence was cloned into a *5xuas* backbone using EcoRI and NotI. The *5xuas* backbone was generated by excising TagRFP-T out of *5xuas*:TagRFP-T (gift from Dr. M. Meyer, King's College, London, UK) using EcoRI and NotI. Subsequently, *5xuas*:Atoh7 was excised with AseI and AflII and ligated into the pColdHeart Tol2 vector (gift from Dr. M. Nonet, Washington University, St. Louis, USA) using blunted NheI and ClaI restriction sites.

To generate *pCH-5xuas*:Ptf1a, the coding sequence of zebrafish *ptf1a* was amplified using primers containing XmaI and NotI restriction sites (sequences of primers Ptf1a Forward and Pf1a Reverse in Table S1) from zebrafish cDNA (obtained by reverse transcription, Omniscript RT kit, QIAGEN) and cloned into the *5xuas* backbone using XmaI/NotI. *5xuas*:Ptf1a was released with AseI and AflII and ligated into the pColdHeart Tol2 vector using blunted NheI and ClaI restriction sites.

Capped RNA synthesis and injections

The PCS2 Numb-GFP plasmid was linearized using NotI. Capped mRNA was synthesized using the Ambion mMessage mMachine kit (Applied Biosystems) according to the manufacturer's instructions. Numb RNA was injected in one- to two-cell stage fertilized eggs at a concentration of 20ng/μl.

Mounting zebrafish larvae for *in vivo* imaging

Embryos were prepared for imaging as described previously.[14,72] Between 10 and 24 h post-fertilization (hpf), embryos were transferred to 0.3 × Danieau's solution containing 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to inhibit melanin formation. Embryos were manually dechorionated (when necessary), anesthetized using 0.02% tricaine (PharmaQ) in medium containing PTU and embedded laying on their side in low-melting agarose (0.7%–0.8%, Sigma) in glass covered 35mm dishes (MatTek, P35G-0-14-C).

In vivo imaging

Fish were imaged on an Olympus FV1000 confocal/2-photon, Olympus FVMPE-RS 2-photon, Olympus FV3000 or a Leica TCS SP8 microscope using water-immersion objectives (Olympus 20x/NA 0.95, Olympus 25x/NA 1.05, Zeiss 40x/NA 1.0, Nikon 25x/NA 1.1 and Nikon 40x/NA 0.8, Leica 25x/NA 0.95) or a silicon-immersion objective (Olympus 30x/NA 1.05). Embryos were maintained at 28.5°C during *in vivo* time-lapse recordings.

Photoconversion

The 'tornado scan' function on an Olympus FV1000 confocal was used to photoconvert Kaede in a local patch of *Q32* cells within the INL of *Q32*; *uas*:Kaede double transgenic fish with a 405 nm laser. Retinae were imaged immediately following photoconversion (Day 0). Fish were then unmounted from agarose and maintained in the dark at 28.5°C until subsequent imaging time-points at 24h (Day 1) and 48h (Day 2).

Morpholino injections

An antisense *ptf1a* translation-blocking morpholino (0.5mM, MO1, Gene tools, for sequence see key resources table), was injected using a picospritzer into the yolk of one or two-cell stage compound transgenic embryos (*Q32*; mYFP; NICD; Atoh7). A *p53* morpholino (0.02 to 1.0 mM, Gene tools, for sequence see key resources table) was injected into embryos from the *vsx1*:GFP line that were subsequently treated at 2 dpf with DAPT (see below).

DAPT treatment

N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was used at a final concentration of 50 μM in 0.3x Danieau's containing 1% DMSO. Embryos were injected with a *p53* morpholino to ameliorate DAPT induced toxicit.[64] At 2 dpf, embryos were transferred into DAPT containing medium (or DMSO containing medium as a control) and incubated for approximately 20 h before analysis.

Immunostaining

Immunostaining to detect glutamine synthetase, c-*myc* and YFP was performed on whole-mount compound transgenic embryos. Zebrafish embryos were fixed in 4% (wt/vol) paraformaldehyde (PFA) in 1x PBS at 4°C overnight. Following fixation, embryos were washed (3x5min) in 1x PBS, pH 7.4, and then incubated in 0.25% Trypsin in 1x PBS on ice for 3-4 min. Embryos were then washed 3-4 times in 1x PBS and incubated in 0.4% Blocking Reagent (Roche) in PBS for 1.5h. Incubation in primary antibodies, diluted in 1x PBS containing 1% Triton (PBST) and 0.4% Blocking reagent, proceeded for two days at 4°C. Following several washes in PBST for 4-5h, embryos were incubated in appropriate Alexa dye-coupled secondary antibodies overnight at 4°C in 0.4% Blocking reagent in PBST. Following several washes in 1x PBS, embryos were mounted in low-melting agarose (0.7%–0.8%, Sigma) and imaged. All incubation and washing steps were done on a rotating shaker.

Genotyping of Q32 crosses

The presence of the *uas*:NICD transgene was initially determined by immunostaining for the c-*myc* tag (99.1 ± 0.6% of YFP^+ cells were c-myc^+; 428 cells, 3 *Q32* NICD fish). The presence of c-*myc* staining correlated perfectly with exuberant neuritic arbors and the loss of lamination in the IPL as well as supernumerary ACs in retinae from putative *Q32* NICD fish (12 out of 12 fish). Thus, the presence of the NICD transgene could be readily determined by retinal morphology. The presence of the *uas*:Ptf1a or *uas*:Atoh7 transgene in embryos could be confirmed by cyan fluorescence in the heart as both constructs were generated using the pCold-Heart Tol2 vector in which *cmcl2* promoter elements drive the expression of CFP.

For some experiments we genotyped individual embryos after confocal imaging to definitively identify carriers of the *uas*:NICD or *uas*:Atoh7 transgenes. Between 6-11dpf, fish were anesthetized in 0.2% tricaine (PharmaQ) and processed for genotyping. DNA extraction was performed using a MyTaq Extract-PCR kit (Bioline, BIO-21126) according to the Manufacturer's guidelines. In brief, each reaction required 20μl Buffer A, 10μl Buffer B and 70μl Nuclease-Free Water (Ambion). Each larva was placed into a clean 1.5ml tube and the extraction mix was added. The reaction was incubated for 75°C for 5-6min and vortexed at least twice. Enzymatic deactivation was achieved by heating to 95°C for 10min followed by centrifugation at 14000rpm for 5min. The genomic DNA of each individual larva was transferred to a clean 1.5ml tube and used as a template for the genotyping. PCR to detect the transgenes used OneTaq hot Start DNA Polymerase (NEB, M0481) along with primers to detect Actin as a proxy to determine the integrity of the genomic DNA. Primers used for genotyping are listed in Table S2.

QUANTIFICATION AND STATISTICAL ANALYSIS

Cell type classification of Q32 cells

Q32 cells were classified as BC or AC based on neurite morphology (presence or absence respectively of an apical process) and position of the soma (lower or upper half of the INL). To obtain the AC-BC ratio of different *Q32* genotypes, a maximum intensity projection (MIP) of a few imaging planes (typically 5) was obtained, and all *Q32* cells (at least 70 per retina) in the MIP were classified. *Q32* divisions were classified as BC-BC, AC-BC or AC-AC based on the criteria described above.

Quantification of Kaede recovery in photoconverted Q32 cells

The recovery of green fluorescence intensity of Kaede protein (I_{CV}) was measured in a region of interest (ROI) encompassing the soma of individual BCs and ACs in a single image plane of a confocal stack and the background fluorescence (I_{BG}) averaged from six regions (*Q32* negative) was subtracted to correct for auto-fluorescence and potential out-of-focus contributions. In order to compare across different samples, fluorescence intensity values were normalized to the average fluorescence intensity of 6 unconverted BCs (I_{UC}) from the same section. The recovery of green fluorescent Kaede protein in *Q32* BCs and ACs was expressed as a percentage:

$$\textit{Recovery of green fluorescence in Q32 BC} = \frac{I_{CV\_BC} - I_{BG}}{I_{UC\_BCs} - I_{BG}}\%$$

$$\textit{Recovery of green fluorescence in Q32 AC} = \frac{I_{CV\_AC} - I_{BG}}{I_{UC\_BCs} - I_{BG}}\%$$

The unconverted BCs were chosen in the vicinity of the photoconverted area and ± 5 sections from the depth in which the photoconverted area was in focus. The same procedure was followed to obtain background values.

Analysis of DAPT treated animals

The effect of DAPT treatment was quantified in two independent quadrants of the peripheral retina of *vsx1*:GFP fish. In a given quadrant, the total number of *vsx1*:GFP$^+$ ACs were counted in 10 z-planes.

Notch-Reporter levels in differentiated Q32 cells

Tp1:hmgb1mCherry fluorescence intensity levels were determined in *Q32* ACs and their potential sibling BCs. If at least one of the *Q32* cells had a fluorescence intensity level twice above background, the cell group was used for analysis (34/77 cell groups could be used). To classify a cell group as 'AC high', 'BC high' or 'equal', Notch-Reporter levels in the *Q32* AC were compared to the surrounding *Q32* BCs with the highest Notch-Reporter fluorescence. If the *Q32* AC had twice the brightness of the *Q32* BC, the group was classified as 'AC high'. Similarly, in cases in which the *Q32* BC had twice the brightness of the *Q32* AC, the group was classified as 'BC high'. When no cell had at least twice the fluorescence intensity of the other, the group was classified as 'equal'.

Monitoring Notch-Reporter levels in time-lapse recordings of the Q32 lineage

Tp1:H2BmCherry fluorescence intensity levels were monitored in the *Q32* lineage by *in vivo* imaging. *Q32* BC-AC pairs were identified and their somata were manually demarcated based on their mYFP expression in a single image plane of a confocal stack using the ImageJ freehand tool. Notch-Reporter levels were measured based on the fluorescence intensity of the mCherry channel within the demarcated areas. The same approach was used for *Q32* transdifferentiation events in the NICD OE condition. Background values (mCherry channel) from three areas at the same time point were averaged and subtracted from the fluorescence intensity values of the cells being measured. Contributions of fluorescence from cells outside of the *Q32* lineage were identified as moving "objects" and used to correct the demarcation of each cell of interest accordingly. Finally, the fluorescence values were normalized to the time point before mitosis for the *Q32* BC-AC siblings or to the first time point seen for the transdifferentiation events after background subtraction.

Division rate analysis

The number of mitotic divisions in the *Q32* lineage in WT and NICD OE, irrespective of their outcome (BC-AC or BC-BC), were counted over a time span of ~18h between 52 hpf and 70 hpf. The division rate was expressed as the total number of divisions per hour.

Cleavage plane analysis

The cleavage planes of dividing progenitors that ultimately generated BC-AC daughters were analyzed. Cell divisions were analyzed in imaging volumes with a reference to the apical surface. Divisions were classified as apico-basal if the cells divided perpendicular to the apical surface and both siblings were present in the same imaging plane. Circumferential divisions occurred parallel to the apical surface and when both siblings were present in the same imaging plane. Central-peripheral divisions also occurred parallel to the

apical surface but the two cells were at different z-depths (larger than 2.4 μm) representing the central-peripheral axis of the retina. When dividing cells could not be strictly categorized in these three categories we ascribed them to an intermediate class.

Statistics

The Shapiro-Wilk test was used to determine whether parametric or non-parametric tests were appropriate for determining statistical significance with GraphPad Prism 8. The specific statistical tests used to compare data-sets are indicated in the respective part of the text or figure legend. $P \leq 0.05$ denoted with '*', ≤ 0.01 with '**', ≤ 0.001 with '***', ≤ 0.0001 ****.

Current Biology, Volume *31*

Supplemental Information

Notch-mediated re-specification of neuronal identity during central nervous system development

Peter Engerer, Eleni Petridou, Philip R. Williams, Sachihiro C. Suzuki, Takeshi Yoshimatsu, Ruben Portugues, Thomas Misgeld, and Leanne Godinho

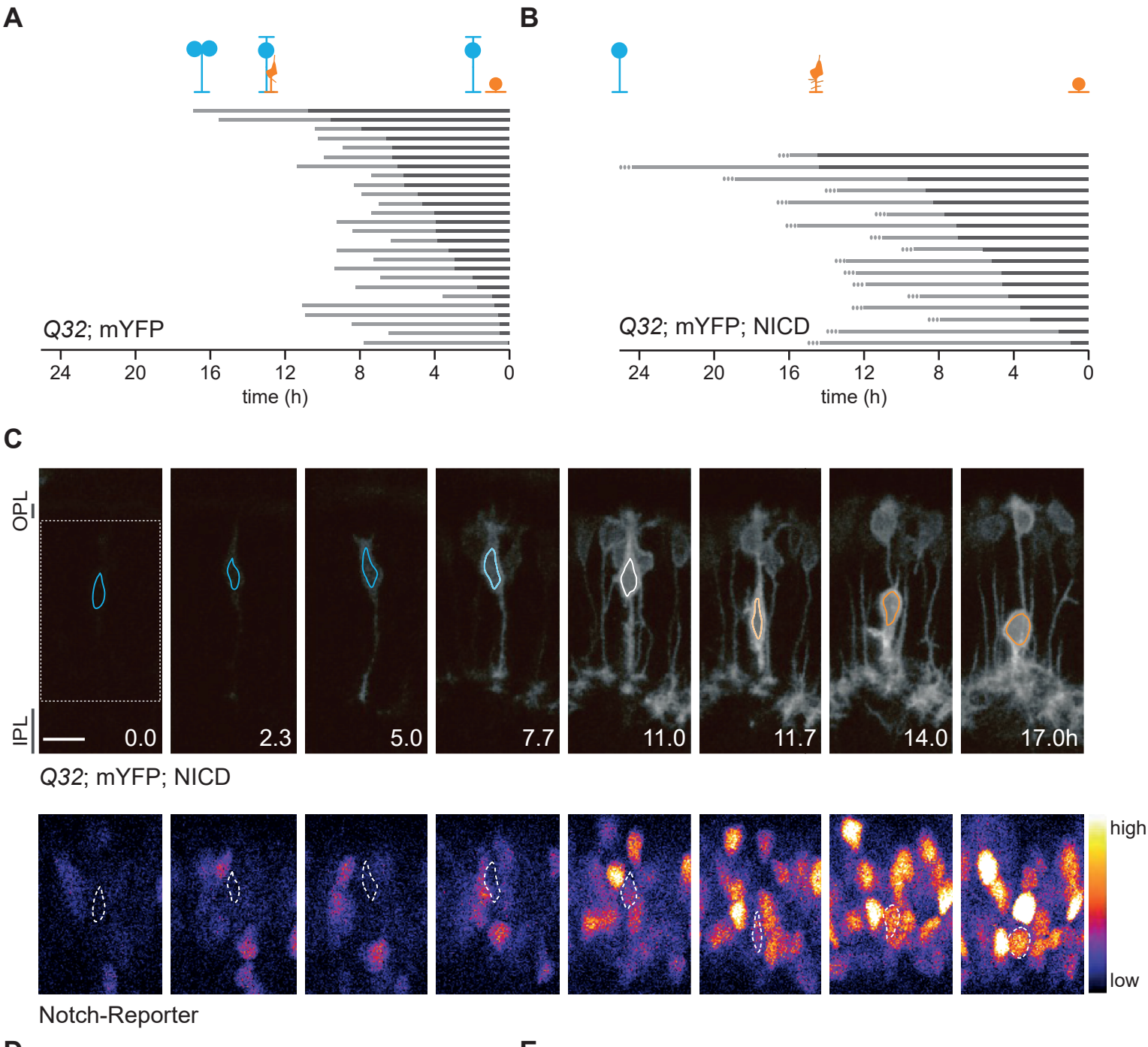

A
B
Q32; mYFP
Q32; mYFP; NICD
24
20
16
12
8
4
0
time (h)
C
OPL
IPL
0.0
2.3
5.0
7.7
11.0
11.7
14.0
17.0h
Q32; mYFP; NICD
high
low
Notch-Reporter
D
Notch sensor intensity
(Norm. to first time seen)
****
***
80
60
40
30
20
10
0
Imme-
diately
after
Exuberant
process
outgrowth
AC
morphology
t(min) =
20.3
±0.1
507.8
±53
894.5
±79.9
E
Notch sensor intensity
(Norm. to before mitosis)
15
10
5
4
2
0
AC
BC
Mitosis
After
mitosis
Loss of
somal
contact
AC
morphology
t(min) =
23.5
±3.3
43.5
±3.3
386.8
±63.2
518
±34.1

Figure S1. Dynamics of AC fate acquisition. Related to Figure 1 and Figure 2.

(A) In WT (*Q32*; mYFP), ACs (orange) are generated through asymmetric divisions in which BCs (cyan) are their immediate siblings. Each horizontal bar represents time-lapse tracks for individual cells one time-point after progenitor division until the acquisition of an AC fate, which was defined when the soma was localized to the inner half of the INL and the cell acquired a monopolar morphology with all neurites confined to the IPL. The average time-interval between the loss of somal contact between Q32 daughters and acquisition of AC morphology (dark gray shading in each track) was 4.2±0.6 h (mean ± SEM; 26 cells, 14 fish).

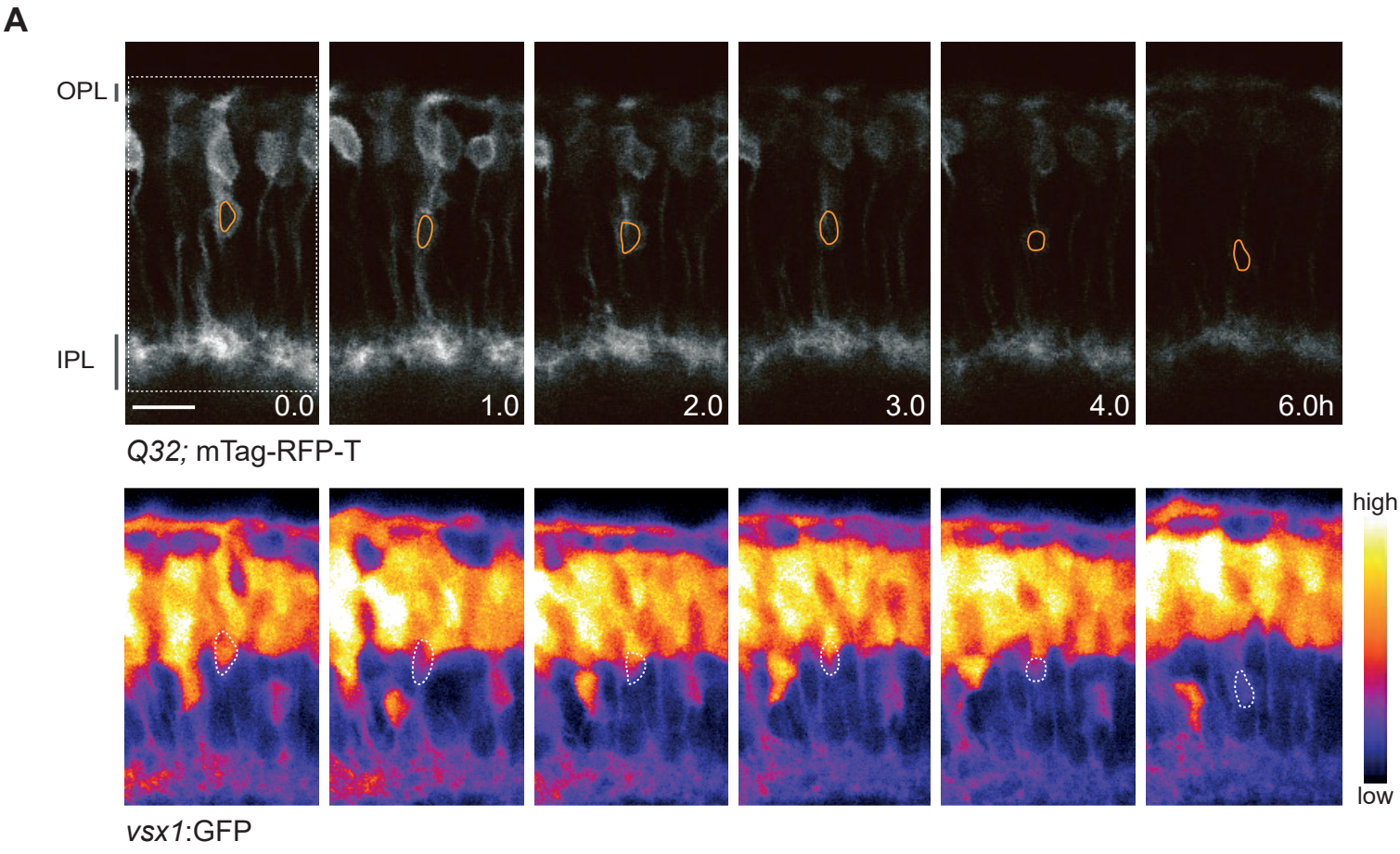
(B) Following NICD OE (*Q32*; mYFP; NICD), back-tracking ACs (horizontal bars as in A) for several hours beyond that for *Q32* WT did not reveal mitotic events (represented as three circles for each cell track), suggesting divisions must have occurred earlier. The starting point for analysis was thus cells bearing a BC morphology that appeared in the course of the time-lapse recording. These cells subsequently underwent a phase of exuberant process remodeling before acquiring an AC morphology (as described for A). The interval between the initiation of process exuberance and the adoption of AC fate (dark gray shading in each track) was 6.59±0.92h (mean ± SEM; 17 cells, 9 fish).

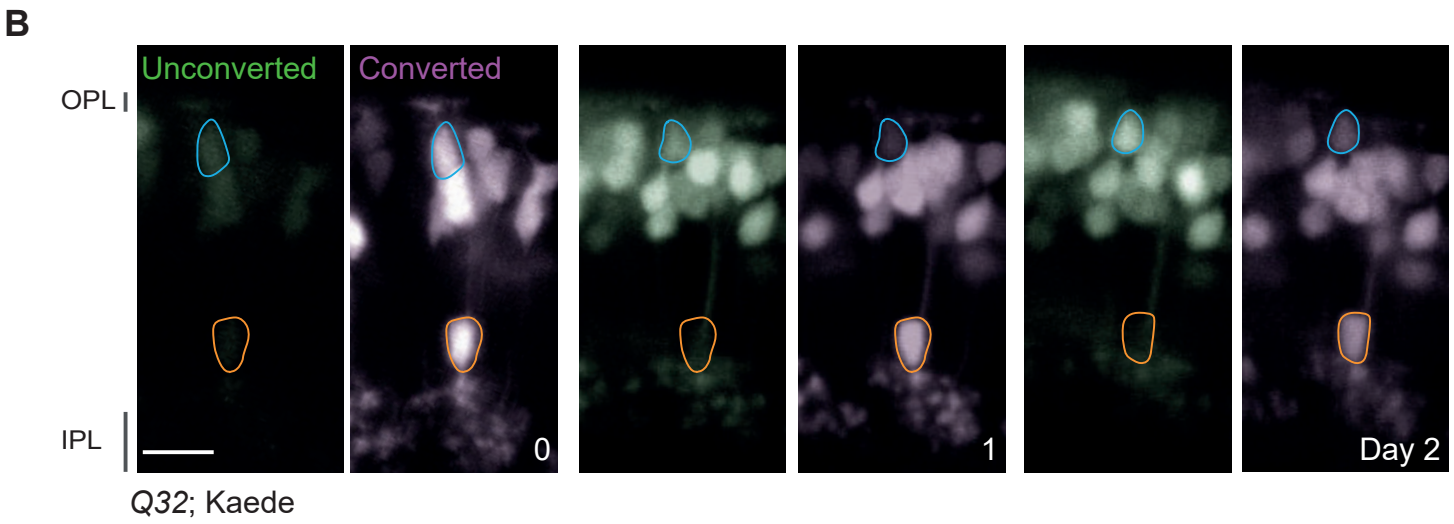
(C) *In vivo* confocal time-lapse recording of a 2 dpf retina from a quadruple transgenic line (Q32; mYFP; NICD; *tp1*:H2Bmcherry) showing a *Q32* BC transdifferentiating into an AC (soma outlined, upper gray-scale panels) that gradually increases Notch-Reporter expression (Fire LUT, lower panels). Scale bar: 10 µm.

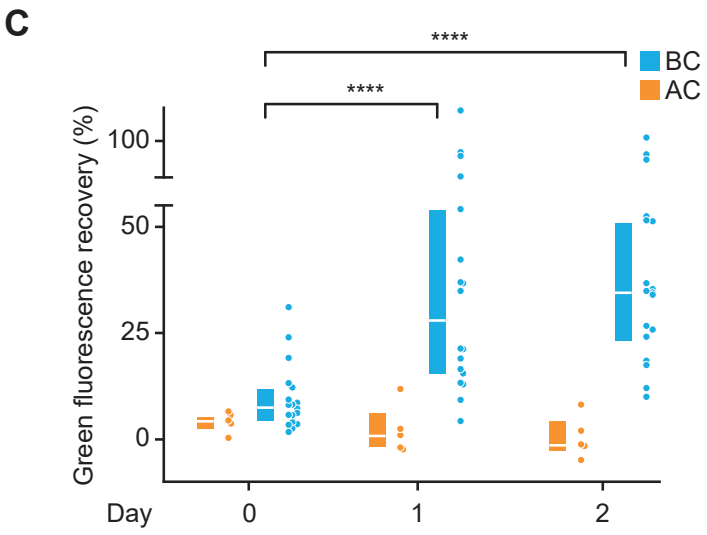
(D) Fluorescence intensity levels of the Notch sensor (*tp1*:H2Bmcherry) in *Q32* NICD measured for BCs that transdifferentiated into ACs (13 cells, 6 fish) during time-lapse recordings. Mean and SEM are depicted. Times indicated below each of the events were calculated in relation to the time-point the cell was first seen. Comparisons of fluorescence levels between all categories were done using the Friedman test and further adjusted by the Benjamini, Krieger and Yekutieli method. Statistically significant differences were found between “Immediately after” vs. “Exuberant processes outgrowth” ($p=0.0014$) and between “Immediately after” vs. “AC morphology” ($p<0.0001$). Arrow indicates Notch-Reporter fluorescence levels of the cell shown in (C).

(E) Fluorescence intensity levels of the Notch-Reporter (*tp1*:H2Bmcherry) following NICD OE in the *Q32* lineage measured in progenitors and their BC (cyan) and AC (orange) daughters during time-lapse recordings (6 AC-BC pairs, 5 fish). Mean and SEM are depicted. Statistically significant differences in fluorescence levels between BC and AC pairs were found at the last two time-points (Wilcoxon matched pairs signed-rank test, $p = 0.0313$). Times indicated below each of the events were calculated in relation to the time-point before mitosis.

OPL, outer plexiform layer; IPL inner plexiform layer.


A
OPL
IPL
0.0
1.0
2.0
3.0
4.0
6.0h
Q32; mTag-RFP-T
high
low
vsx1:GFP


B
Unconverted
Converted
OPL
IPL
0
1
Day 2
Q32; Kaede


C
****
****
BC
AC
Green fluorescence recovery (%)
100
50
25
0
Day
0
1
2

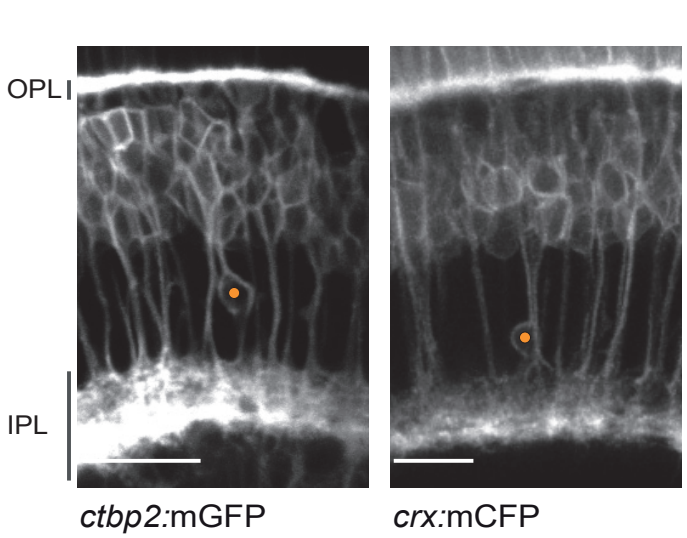

D
OPL
IPL
ctbp2:mGFP
crx:mCFP

Figure S2. BC-lineage derived ACs. Related to Figure 1

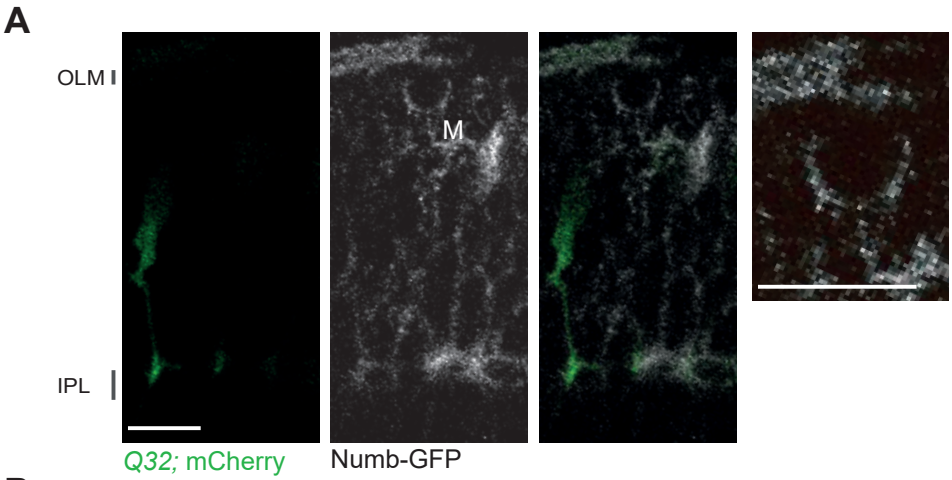
(A) *In vivo* confocal time-lapse recording of a 2 dpf retina from a triple transgenic line (*Q32*; mTag-RFP-T; *vsx1*:GFP) showing a *Q32* AC (orange outline, gray-scale, top panels) that is *vsx1*:GFP^{+} initially but gradually loses GFP expression (white-dashed outline, Fire LUT, lower panels). Scale bar: 10 µm.

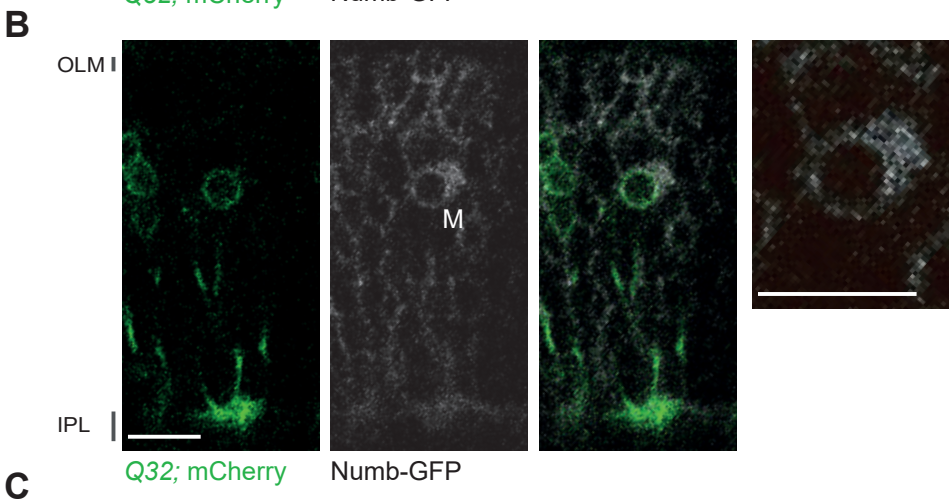
(B) To assess the extent to which *vsx1* promoter elements are active in *Q32* labeled ACs we photoconverted *Q32*-driven Kaede to the red fluorescent state in a BC (cyan outline) and an AC (orange outline, Day 0) in vivo. Recovery of the green fluorescence of Kaede only occurred in the BC (Day 2, left panel). For each day, the left panel depicts green fluorescence and the right panel depicts red fluorescence. Scale bar: 10 µm.

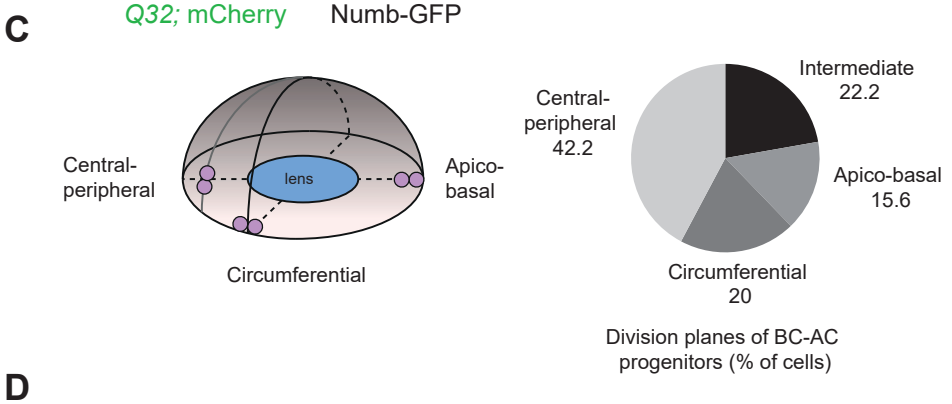
(C) Quantification of the recovery of *Q32*-driven green Kaede fluorescence in BCs and ACs, tracked *in vivo* over two days as shown in (B). Green fluorescence values (median and IQR) are plotted for 18 BCs and 5 ACs derived from 3 Q32 Kaede fish. Comparisons of fluorescence levels for BCs or ACs over time were done using the Friedman test and further adjusted by the Benjamini, Krieger and Yekutieli test. Statistically significant differences were found for BCs between Day 0 and Day 1 ($p = 0.0001$) and Day 0 and Day 2 ($p < 0.0001$). The lack of recovery of green fluorescence in *Q32* ACs suggests that vsx1 promoter elements are no longer active in these cells. *Q32*-driven fluorescence in ACs thus largely results from the amplification and prolongation of expression mediated by the Gal4-UAS system.

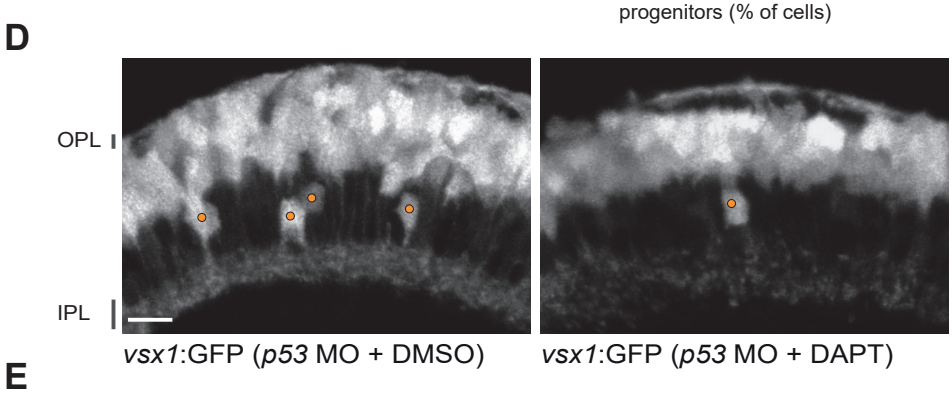
(D) Sparse labeling of ACs in BC transgenic lines. *In vivo* confocal images of 3 dpf retinae from the *ctbp2*:mEGFP and *crx*:mCFP transgenic lines showing cells with an AC morphology (orange circles). Scale bar 10 µm.

OPL, outer plexiform layer; IPL, inner plexiform layer.


A
OLM
IPL
M
Q32; mCherry
Numb-GFP


B
OLM
IPL
M
Q32; mCherry
Numb-GFP


C
Central-peripheral
lens
Apico-basal
Circumferential
Central-peripheral 42.2
Intermediate 22.2
Apico-basal 15.6
Circumferential 20
Division planes of BC-AC progenitors (% of cells)


D
OPL
IPL
vsx1:GFP (p53 MO + DMSO)
vsx1:GFP (p53 MO + DAPT)

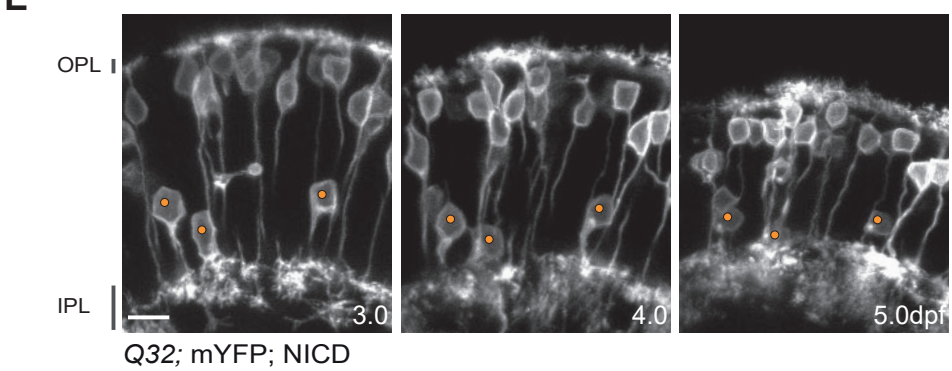

E
OPL
IPL
3.0
4.0
5.0dpf
Q32; mYFP; NICD

Figure S3. Characterization of Q32 progenitor divisions and the role of Notch signaling in mediating asymmetric fates. Related to Figure 1 and Figure 2.

(A) *In vivo* confocal image showing asymmetric (basolateral) distribution of Numb-GFP (gray scale) in a non-*Q32* mitotic (M) progenitor at the apical surface. Scale bar: 10μm.

(B) Symmetric Numb-GFP distribution in a non-apically dividing *Q32* mitotic cell (green) that eventually generated an AC-BC pair. Scale bar: 10μm.

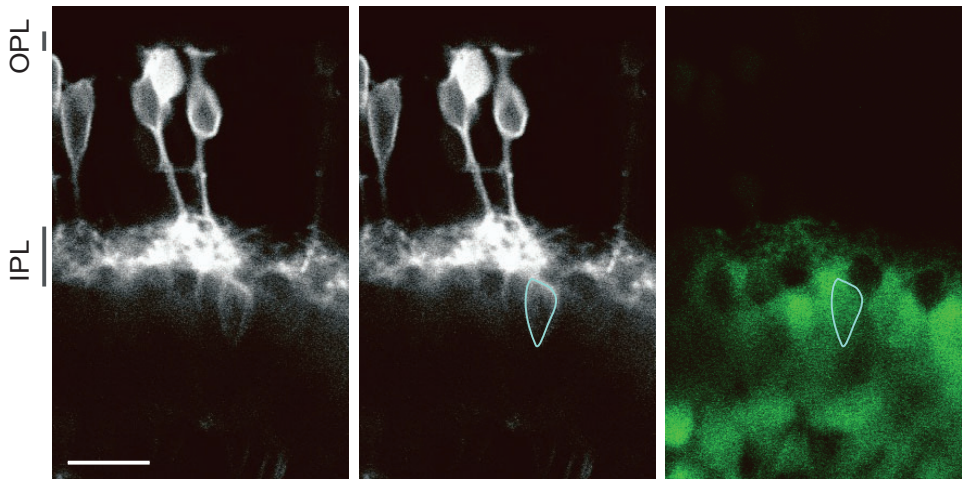
(C) Left: Graphical representation of progenitor division planes in relation to the retinal cup. Right: Percentage of *Q32* progenitor divisions resulting in AC-BC pairs with distinct cleavage plane orientations. Each category was tested against a frequency of 25% using a Chi-square test, $p = 0.057$, 45 cells, 24 fish.

(D) Pharmacological treatment using DAPT to abrogate Notch signaling in the *vsx1*:GFP line results in fewer *vsx1* ACs (orange circles). The p53 morpholino was used to ameliorate DAPT-induced toxicity. Scale bar: 10 μm.

(E) *In vivo* confocal time-lapse images of a retina in which *vsx1*-lineage restricted NICD OE (*Q32* mYFP NICD) results in supernumerary ACs (orange circles) that were maintained at least until 5 dpf. Scale bar: 10 μm.

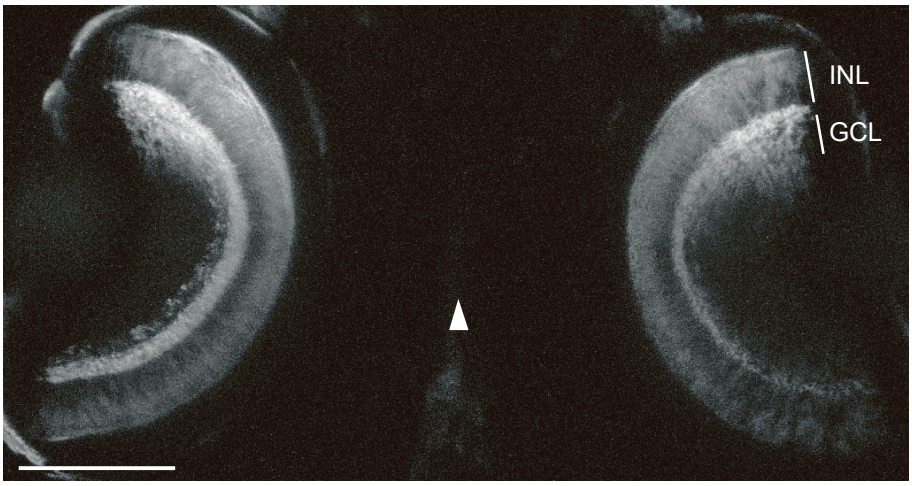
OLM, outer limiting membrane; OPL, outer plexiform layer; IPL, inner plexiform layer.

A



Q32; mYFP; NICD; Atoh7 (*ptf1a* MO) *isl2b:*GFP

B



Q32; mYFP

*isl2b:*GFP

Figure S4. Induction of GC-like cells in the vsx1 lineage. Related to Figure 4.

(A) Confocal image of a 3 dpf retina from a quintuple transgenic embryo (*Q32*; mYFP; NICD; Atoh7, isl2b:GFP) that was injected with a ptf1a MO showing a *Q32* GC-like cell (outlined) that is *isl2b*:GFP^{+}. Scale bar: 10 µm.

(B) *In vivo* two-photon images of the dorsal view of a 5 dpf *Q32* WT embryo (rostral up) in which no labeling can be seen at the optic chiasm (arrowhead, top panel). A tilt in the orientation of the embryo leads to a semi 'en face' view of the left eye. For reference, axons are clearly detectable at the chiasm (arrowhead) of a WT *isl2b*:GFP embryo (bottom panel, 3 dpf) in which GCs are labeled. Gamma was adjusted non-linearly for better visualization. Scale bar: 100 µm.

INL, inner nuclear layer; GCL, ganglion cell layer; OPL, outer plexiform layer; IPL, inner plexiform layer.

Primer name	Source	Sequence
atoh7 Forward	This paper	5'- GGAAAAGAATTCATGAAGCCCCGCAGGCC GAG -3'
atoh7 Reverse	This paper	5'- GGGCCCGCGGCCGCTCAGAGGCTTTCGTA GTGGT -3'
ptf1a Forward	This paper	5'- GGAAAAGCGGCCGCCCCGGGATGGACA CTGTGTTGGATCCATTCA-3'
ptf1a Reverse	This paper	5'- GGAAAAGCGGCCGCCCCGGGTTAGGAA ATGAAATTAAAGGG -3'

Table S1. Primers for cloning. Related to Star Methods.

Primer name	Source	Sequence
uas Forward	S1	5'-CATCGCGTCTCAGCCTCAC-3'
notch1a:intra Reverse	S1	5'-CGGAATCGTTTATTGGTGTCG-3'
5xuas Forward	This paper	5'-AACTCTGAATAGGGAATTGGC-3'
atoh7 Reverse	This paper	5'-CGTAGTGCATAAAATATCCCT-3'
ptf1a Reverse	This paper	5'GCCAATTGCTAGGCGTAAAG-3'
actin Forward	This paper	5'-CGGTAGCAGTTCCAGCTTTC-3'
actin Reverse	This paper	5'-GAAATCCCGGTGCTCACTAA-3'

Table S2. Primers for genotyping. Related to Star Methods.

Supplemental Reference:

S1. Scheer, N., Riedl, I., Warren, J.T., Kuwada, J.Y., and Campos-Ortega, J.A. (2002). A quantitative analysis of the kinetics of Gal4 activator and effector gene expression in the zebrafish. Mechanisms of development *112*, 9-14.

2.3 Neuron-glia pairs arise from terminal progenitor divisions in the vertebrate retina (*in preparation*)

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Abstract

Müller cells (MCs) are the principal glial cell-type in the vertebrate retina, providing structural and homeostatic support to retinal neurons. While MCs are derived from multipotent retinal progenitors, it remains unclear what types of progenitor divisions give rise to them, who their siblings are, and whether they are the last-born cells in the retina, as is the case for glia in other brain regions. We used *in vivo* time-lapse based lineage tracing in the zebrafish retina to establish the origins of MCs and their relationship to retinal neurons. Our observations reveal that MCs arise from terminal asymmetric divisions in which their siblings are *visual system homeobox gene 2 (vsx2)* expressing bipolar cell (BC) interneurons. Furthermore, Notch signaling plays a role in establishing these binary fates.

Author contributions

Project design and experimental design: E.P., T.M., and L.G.

Project supervision: L.G. and T.M.

Experiments performed by E.P.

Construct generation: E.P.

Manuscript writing: E.P. and L.G.

Introduction

Neurons and glia in the vertebrate central nervous system (CNS) are generated from a pool of multipotent progenitors that gradually lose their potential to produce different cell classes as development progresses (Kohwi & Doe 2013). In most parts of the CNS such as the neocortex and spinal cord, gliogenesis commences only after neurogenesis is complete (Götz et al. 2015; Guérout et al. 2014). For instance, at early time-points during cortical development in rodents and primates, radial glial progenitors divide in a self–renewing manner giving rise to different classes of neurons either directly or indirectly via intermediate progenitors. At later time-points, radial glia transdifferentiate into astrocytes, acquiring the morphological and molecular characteristics of these cells (Culican et al. 1990; Noctor et al. 2004, 2008; Schmechel & Rakic 1979; Voigt 1989). Similarly, in the spinal cord, after neurogenesis is completed, distinct progenitor populations generate astrocytes and oligodendrocytes (Barry & McDermott 2005; Liu et al. 2002). By contrast, in the vertebrate retina, the period of neurogenesis and gliogenesis overlap.

An isolated part of the CNS, the vertebrate retina consists of five neuronal classes and a single glial cell class, the Müller cells (MCs) (Dowling 2012). Birth-dating studies across species indicate that while there is a conserved order in which distinct cell classes are generated, MCs are generated concurrently with at least some neuronal cell classes. For example, in the rodent retina MCs are generated late in the period of cell genesis at a time when Rod photoreceptors (PhRs) and BCs are also generated (Prada et al. 1991; Rapaport et al. 2004; Wong & Rapaport 2009; Young 1985). Clonal analysis studies, in which single progenitors were labeled during development and the entirety of their progeny was traced at maturity, found no evidence for dedicated progenitors that exclusively generate MCs. Instead, clones were found to vary in size and composition, consisting of both neurons and glia (Holt et al. 1988; Turner & Cepko 1987; Turner et al. 1990). Thus, neurons and MCs in the retina share a common lineage. Moreover, in the rodent retina two-cell clones composed of a neuron and a glial cell were identified, implicating their origin from a single terminal mitotic event. Indeed, time-lapse monitoring of individual rat embryonic retinal progenitors and their progeny *in vitro* revealed single mitotic events that generated an MC and either a BC or PhR (Gomes et al. 2011). However, whether such mitotic divisions occur *in vivo* under physiological conditions remained unclear.

We set out to systemically investigate the occurrence of neuron-glia daughter cell pairs, in a vertebrate retina that allows *in vivo* monitoring of lineages. To do so, we turned to the zebrafish

retina where optical accessibility and rapid *ex utero* development allows lineage relationships and cell differentiation to be followed (Godinho et al. 2007; He et al. 2012; Poggi et al. 2005). Here we directly show that neuron-glia pairs arise during retinal development. Moreover, our experiments illustrate that the neuronal sibling of MCs is a distinct type of excitatory interneuron, the *vsx2* BC, and that Notch signaling plays a role in this binary fate decision.

Results

1. Asymmetric terminal divisions generate MCs

To investigate the origins of MCs by *in vivo* time-lapse imaging we sought transgenic lines that label these cells and the progenitors that generate them. Established lines such as Tg(*gfap*:GFP) label MCs at mature time-points (Bernardos & Raymond 2006), and not only the progenitors that generate them, but rather all retinal progenitors. Moreover, the density of labeling in this line precludes following individual mitotic divisions and their ensuing progeny. We thus turned to injections of genetic constructs into one-cell staged zygotes to sparsely label cells. Among the constructs we tried, a Notch reporter previously utilized to follow MC cell differentiation (MacDonald et al. 2015), proved the most useful. The construct is based on Notch-responsive *tp1* elements that drive the expression of a destabilized fluorescent protein (FP) to serve as a dynamic Notch sensor (Ninov et al. 2012). Injection of this construct allowed us to monitor mitotic events that generated MCs (**Figure 1A**). We identified MCs when laminar organization of the retina is well-established at 3 days post-fertilization (dpf) (Easter & Nicola 1996), based on their characteristic morphology, spanning the entire width of the retina and the location of their polygonal-shaped somata in the lower half of the inner nuclear layer (INL) (Magalhães & Coimbra 1972; Reichenbach & Reichelt 1986; Uga & Smelser 1973). We independently confirmed the MC identity of the *tp1*-FP-expressing cells, based on their expression of glutamine synthetase (GS), an established MC marker (Peterson et al. 2000; Riepe & Norenburg 1977) (**Supplementary Figure 1A**).

In all analyzed mitotic events (31 cases, 21 fish), MCs derived from asymmetric mitotic divisions, in which the sibling was another post-mitotic cell. These divisions occurred at the apical surface over the course of the second dpf (median and interquartile range (IQR), median: 50 hours post-fertilization (hpf); 25th percentile: 52 hpf; 75th percentile: 55.7 hpf, 31 cases, 21 animals) (**Supplementary Figure 1B**), across the gradient of retinal development (Burrill & Easter 1995; Hu & Easter 1999) (**Supplementary Figure 1C**).

To verify that the asymmetric mitotic divisions, described above, represented terminal events in which the sibling of the MC was a post-mitotic cell, we measured Notch sensor fluorescence levels of the two daughter cells over several hours. Notch sensor levels were upregulated in the future MC within ~3 hours post mitosis (Friedman test, multiple comparisons with FDR, p value 0.0001, 9 cases, 8 animals), in line with the known role of Notch signaling in instructing glial fate acquisition. By comparison, Notch sensor fluorescence levels in the sibling cell were downregulated for as long as it could be traced (**Figure 1B&C**). Thus, the sibling cell is most likely post-mitotic and not a progenitor that would be expected to exhibit increased Notch signaling as it re-enters the cell cycle (Henrique et al. 1997; Jadhav et al. 2006).

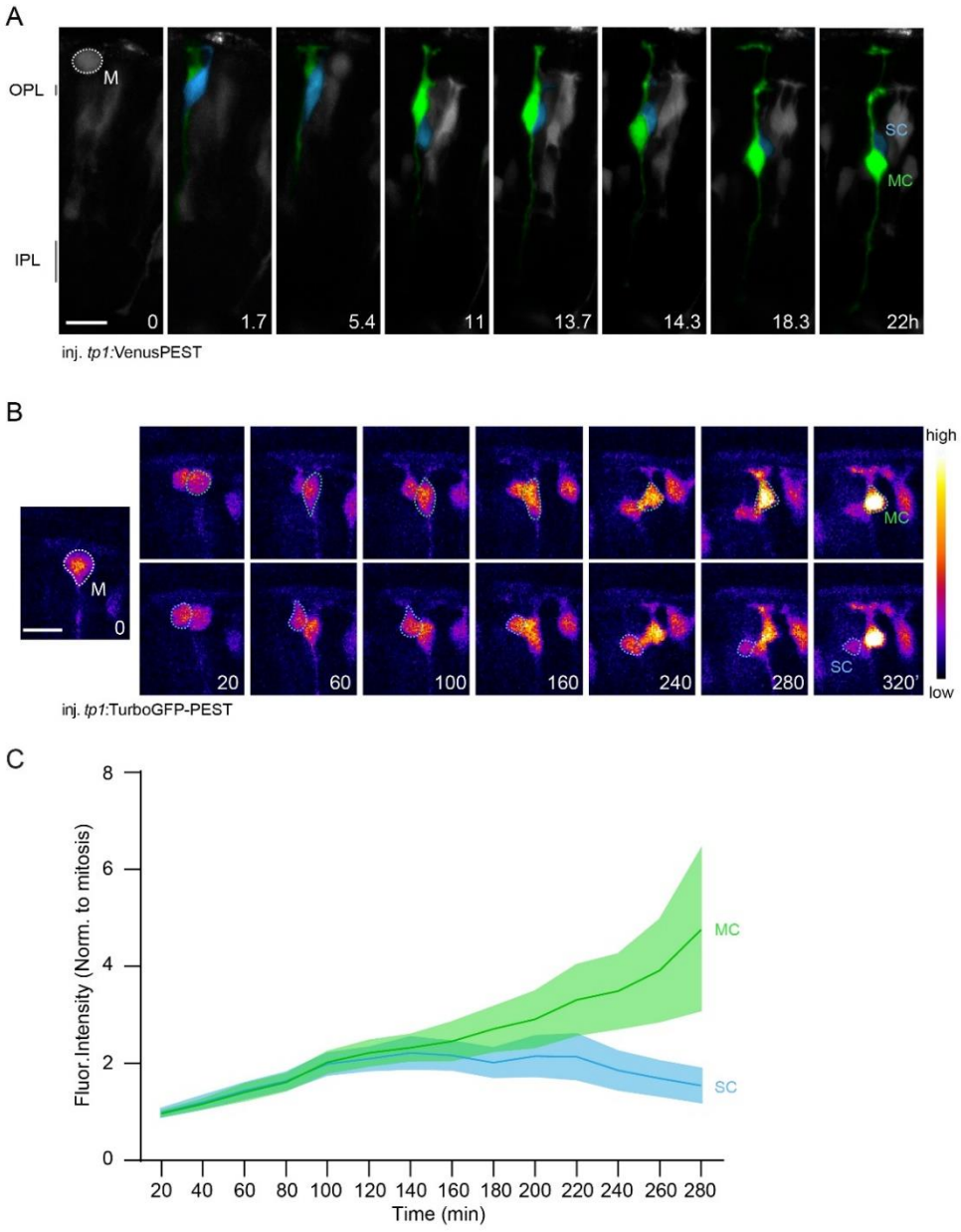


Figure 1: Terminal mitotic divisions generating a MC and a non-glial Sibling occur *in vivo*. A) *In vivo* time-lapse images of a retina from a *tp1*:VenusPEST injected embryo, showing a mitotic division that generates a MC and a Sibling cell (SC) that remains in the INL. B) *In vivo* time-lapse recording of a mitotic event labeled using a Notch sensor (*tp1*:TurboGFP-PEST) reveal differential Notch signaling levels between the MC and its SC. C) Quantification of Notch sensor (*tp1*:TurboGFP-PEST) levels, normalized to mitosis, following terminal divisions that generated a MC and another cell class in the first 4.7h post-mitosis (9 pairs, 8 fish). Line represents mean and opaque areas ±SEM (green for MCs and blue for SCs). hpf: hours post fertilization, MC: Müller cells, SC: Sibling cell. Scale bar: 10 µm

2. Vsx2 BCs are the siblings of MCs

The post-mitotic sibling of MCs remained within the INL. Their somata were smaller than that of MCs and occupied the outer half of the INL, where BCs reside (mean area ± SEM, MCs: 27.15±0.52µm2, 71 cells; *Vsx2* BCs: 18.66 ±0.26 µm2, 93 cells; 3 animals, 10dpf, unpaired t-test, $p < 0.0001$). In cases where it was possible to monitor both siblings until the end of the imaging session at 3 dpf, MCs and their prospective BC sibling remained closely associated, and occupied stereotypic apico-basal positions relative to each other. Two molecularly distinct BC sub-populations exist in the zebrafish retina. Those expressing *visual system homeobox gene 1* (*vsx1*) represent the overwhelming majority of BC interneurons (Vitorino et al. 2009) and are derived from *vsx1* progenitors via terminal divisions (Engerer et al. 2017, 2021; Weber et al. 2014). A smaller group of BCs express *vsx2* (Vitorino et al. 2009), but whose origin, while distinct from the *vsx1* population, has not been fully investigated. MCs in the zebrafish retina also express *vsx2*, opening up the tantalizing possibility that *vsx2* BCs are their immediate siblings. This expression of *vsx2* in BCs and MCs has also been reported in the rodent retina, albeit only in a subset of these cell classes. The potential common origin of these two cell classes has not been investigated (Rowan & Cepko 2004; Shekhar et al. 2016).

To investigate the possibility of a shared ancestry between *vsx2* MCs and *vsx2* BCs, we used the *vsx2*:GFP transgenic line that faithfully represents the endogenous expression of this transcription factor in the retina (Vitorino et al. 2009). Early in retinogenesis, *vsx2* is expressed in all retinal progenitor cells while being confined to MCs and a subset of BCs (that are *vsx1* negative) at later time points. *In vivo* time lapse imaging experiments in embryos from Tg(*vsx2*:GFP), to monitor *tp1* mitotic events that generated MCs, revealed that their sibling indeed acquired the *vsx2* BC fate (6 events, 5 animals; **Figure 2B**). Whether all MCs in the zebrafish retina are generated by terminal divisions, with *vsx2* BCs as their immediate sibling proved challenging to address. Attempts to answer this question using time-lapse imaging in

the *vsx2*:GFP transgenic line proved practically impossible as all retinal progenitors, rather than only those that exclusively generate MCs, are labeled. To circumvent this problem we asked if every MC was 'paired' with a *vsx2* BC, potentially representing sibling cells. We identified such *vsx2* MC - BC pairs (**Figure 2A**) at 10dpf. However the percentage of MCs that could be paired with *vsx2*:GFP^{+} BCs, as defined by apparent physical contact, was only approximately 15% of the population (31 out of 211 MCs, 7 fish, Wilcoxon test, p-value 0.0156). Similarly only 11% of *vsx2* BCs were found paired with MCs (31 out of 286 *vsx2* BCs, 7 fish). These results suggest that not all MCs and *vsx2* BCs are each other's immediate sibling. However, ascribing a sibling relationship through proximity several days after their generation may be misleading as other intervening retinal cells may have pushed the two daughters apart (ratio of MC/ *vsx2* BC per area of interest: 0.75, total number of MCs: 211 vs total number of *vsx2* BCs: 286, 10dpf, 7 fish).

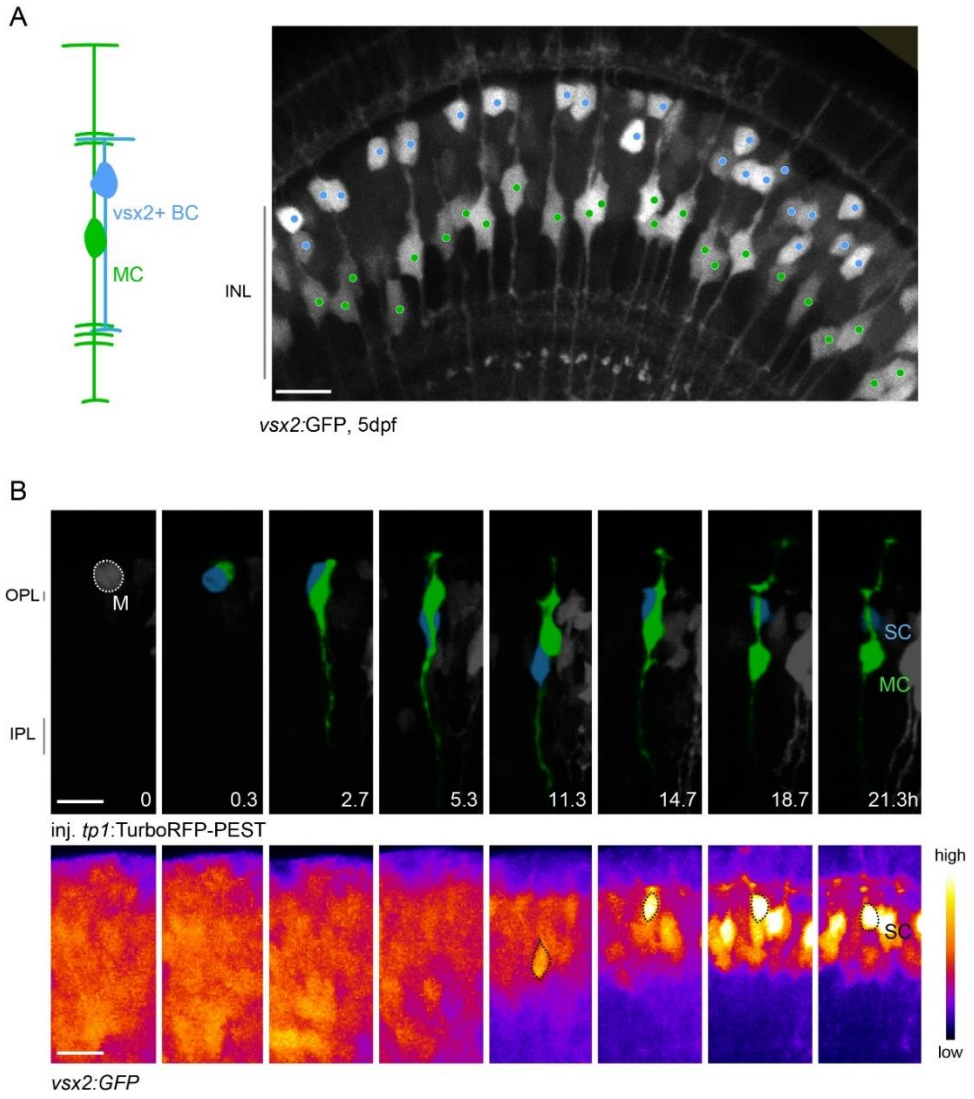


Figure 2: *Vsx2*-expressing BCs are the siblings of MCs. A) MCs and *vsx2* BCs display a particular configuration. *In vivo* 2-photon maximum z-plane projection image of MCs and *vsx2* BCs at a 5 dpf Tg(*vsx2*:GFP) animal. B) Confocal *in vivo* time-lapse images of a retina from a *vsx2*:GFP transgenic embryo injected with the Notch reporter *tp1*:TurboRFP-PEST at the one-cell stage. The sibling of the MC is a BC expressing higher levels of the *vsx2*:GFP transgene (outlined, lower panels-FIRE LUT). Both panels represent maximum z plane projection images. Scale bar: 10 μm.

3. Notch signaling influences the outcome of the vsx2 lineage

Notch signaling has been implicated in binary fate decisions (Jukam & Desplan 2010; Pierfelice et al. 2011). In order to elucidate the role of Notch in the MC-BC asymmetric events, we used N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl (DAPT) -a γ-Secretase inhibitor- (Geling et al. 2002) to abrogate Notch signaling. This treatment led to the abolishment of MCs when investigated in the background of transgenic animals positive for *vsx2*:dsRed and *gfap*:GFP (DMSO group, median and IQR: median: 6.65; 25th percentile: 4.225; 75th percentile 8.020 vs DAPT group, median and IQR: median 0, 25th percentile: 0, 75th percentile 0.21 cells per area), as was expected based on the instructive role of Notch for the glial cell fate (MacDonald et al. 2015; Randlett et al. 2013; Scheer et al. 2001). Notably, the number of *vsx2* BCs was increased (MC DMSO vs DAPT p-value < 0.001, BC DMSO vs DAPT p-value 0.0001. DAPT treated group 16 animals, DMSO group 17 animals, 3 independent experiments. Statistics: Mann-Whitney test) (**Figure 3B**). The BC identity of the DAPT induced cells was independently confirmed with a Tg(*ctbp2*:mEGFP) line, an established pan-BC marker (Odermatt et al. 2012) (**Supplementary Figure 3**). Thus, Notch signaling is implicated in these asymmetric events, in the absence of which a likely shift from terminal asymmetric to terminal symmetric events occurs, generating two *vsx2* BCs, and thus an increase in BC number.

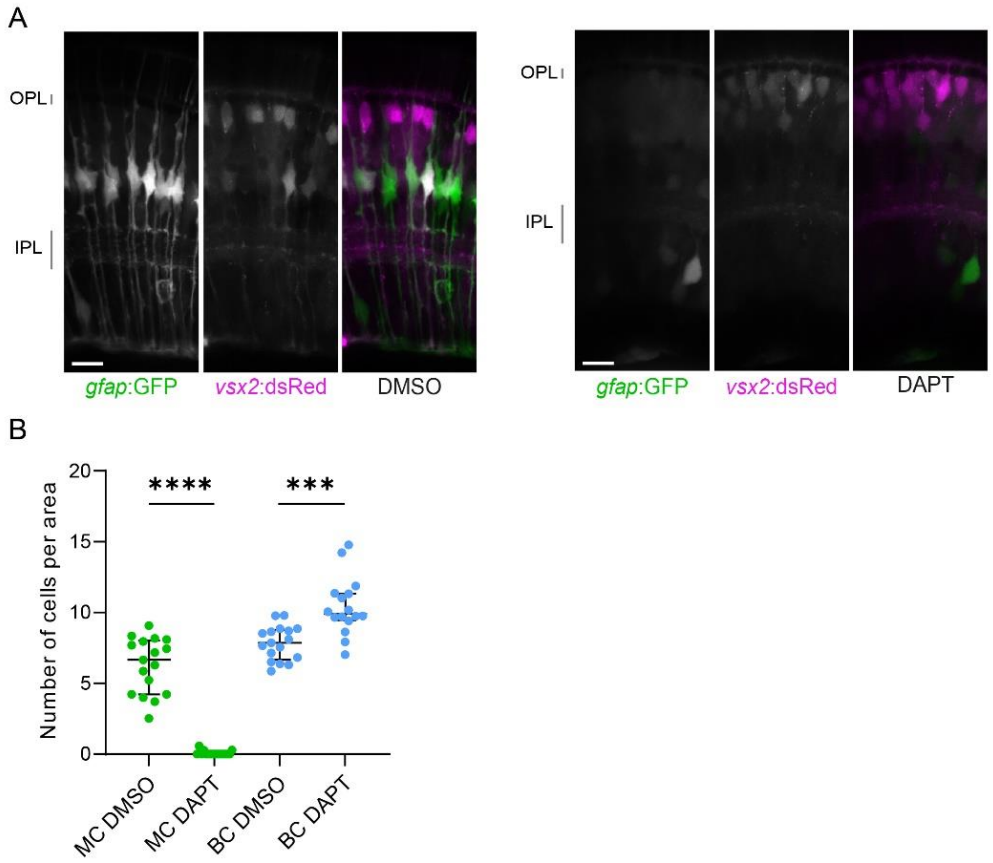


Figure 3: Notch manipulation of the *vsx2* lineage **A)** Double transgenic animals for *gfap*:GFP; *vsx2*:dsRed were incubated for 24hrs starting from 49hpf until 73hpf in 25µM DAPT/ 0.5% DMSO (DAPT group) or 0.5% DMSO (DMSO group). **B)** *vsx2*:GFP^{+} MCs and BCs are considered. Median and IQR are shown. Every dot represents the number of cells per 1000µm^{2}, green MCs and blue BCs. DAPT treated group 16 animals, DMSO group 17 animals, 3 independent experiments. Statistics: Kruskal-Wallis test with FDR for multiple comparisons. MC DMSO vs DAPT p-value 0.001, BC DMSO vs DAPT p-value 0.0174. Scale bar: 10 µm.

Discussion

Our time-lapse experiments reveal for the first time, *in vivo*, in a vertebrate retina, that MCs can be derived from asymmetric terminal divisions in which BC interneurons are their immediate siblings. A large-scale study of lineages in the zebrafish retina, that concentrated on the generation of distinct neuronal cell classes, also reported two instances of MC generation (He et al. 2012). In one case, a terminal symmetric division generating two MCs was reported. In a second, a terminal division was reported to generate an MC and a Rod PhR, an observation we did not make among the 31 MC-generating divisions we tracked. Nonetheless, MC – Rod PhR generating terminal divisions were reported in time-lapse recordings of cultured rat retinal progenitors (Gomes et al. 2011).

Notch signaling has been implicated in the context of non-terminal asymmetric divisions, maintaining one daughter in a proliferative state, while its sibling exits the cell-cycle and becomes post-mitotic (Alexandre et al. 2010; Bultje et al. 2009; Dong et al. 2012; Knoblich 2008; Skeath & Doe 1998). However, at least to our knowledge, a role for Notch signaling in the context of terminal asymmetric divisions that generate an MC and a *vsx2* BC, has not been described. The use of specific Notch sensor genetic constructs allowed us to visualize the dynamics of Notch signaling and predict, courtesy of disparate fluorescence levels, which of the two daughters would eventually acquire the MC fate. Ascribing a direct role for Notch signaling in the acquisition of the MC fate requires gain- and loss-of-function approaches. We were able to abrogate Notch signaling using a pharmacological inhibitor and observed a loss of MCs and concomitant increase in *vsx2* BCs. However, attempts at genetic manipulation proved challenging. In the absence of an MC specific promoter or promoter elements that specifically and exclusively target progenitors that generate them, genetically based gain- and loss-of-function experiments were not feasible. A pharmacological Notch gain-of-function approach-to our knowledge- has not yet been demonstrated in zebrafish. Nonetheless, in the field of cancer and immunology, agonist antibodies of the Notch 2 receptor have been used *in vivo* in rodents enhancing their antitumor response (Sugimoto et al. 2010). In addition, N-methylhemeanthidine chloride (NMHC) – a plant derived small molecule- was identified with the ability to act as a Notch agonist *in vitro* and inhibit acute myeloid leukemia (Ye et al. 2016). Whether these approaches could be used to address the issue at hand is unknown.

A common origin for MCs and *vsx2* BCs in the zebrafish retina was previously implied but not directly shown (Rulands et al. 2018). In that study, clones derived from progenitors marked early during cytogenesis comprised multiple cell-classes. However, single MCs and single *vsx2* BCs were consistently found within these clones, implicating a lineage relationship between these two cell fates. Although our *in vivo* time-lapse data clearly show terminal events giving rise to MC - *vsx2* BC siblings, pair analysis in the mature retina (10dpf) suggests that not all MCs may be generated this way. Several other possibilities exist. For example, other cell-classes, besides *vsx2* BCs, may be the siblings of MCs (Gomes et al. 2011; He et al. 2012). Alternatively, they may arise from non-terminal divisions in which a progenitor is their sibling. Yet another possibility, which has been alluded to in the literature (MacDonald et al. 2017) although with no direct evidence, is whether MCs can arise from transdifferentiating progenitors at the end of the period of cytogenesis. This would be akin to neocortical radial

glial progenitors transdifferentiating into astrocytes (Noctor et al. 2004; Schmechel & Rakic 1979; Voigt 1989).

To extend our *in vivo* imaging based observations, we performed birth-dating experiments to probe the extent to which the population of MCs and *vsx2* BCs are generated concurrently. We administered EdU systemically (injection into the heart) in *vsx2* transgenic embryos at specific developmental time-points and analyzed retinae at 4dpf (**Supplementary Figure 4A&4B**). Unexpectedly, we detected EdU positive *vsx2+* MCs and BCs as early as 36 hpf (**Supplementary Figure 4C**), at the very beginning of neurogenesis in the zebrafish retina, and in contrast to what has been reported for these cell classes in other vertebrates (Rapaport et al. 2004; Wong & Rapaport 2009; Young 1985). Independent experiments to verify our birth-dating approach by monitoring horizontal cell generation suggested that the EdU was bioavailable for more than 4 hours (**Supplementary Figure 4D&E)**, and thus did not exclusively label exiting cells immediately after exposure. Given the speed with which retinogenesis occurs in the zebrafish, this classical approach to birthdating may not be suitable to determine specific times at which distinct cell classes are generated in this species. Thus, our original aim to address how many of the "paired" MC-BCs are generated concurrently remains unanswered.

In the cortex, neurons that share ancestry establish preferential synaptic connections with each other than with their near-by non-siblings (Yu et al. 2009, 2012). Whether MCs exhibit a special connectivity pattern with their *vsx2* BC siblings compared to other neurons in their vicinity remains an open question. The impact of such connectivity patterns at a functional level remains open. One possibility would be to form electrical synapses via gap junctions, which are abundant in the retina and mediate fast inter-neuronal communication (Bloomfield & Völgyi 2009). In the mouse retina, MCs were reported to form gap junctions with a specific type of amacrine cell rendering this a unique connection compared to other surrounding neurons (Grimes et al. 2021). A preferential connectivity pattern between MCs and their *vsx2* BC siblings might represent a novel path through which visual information is processed in the retina (Euler et al., 2014).

Methods

1. Animals

All experiments were performed according to regulations as approved by the local regulatory bodies. Zebrafish were maintained, mated, and raised as described in (Mullins et al. 1994).

Embryos were kept in 0.3× Danieau's solution at 28.5°C and staged as previously described (Kimmel 1994). Fish were either in an AB wild-type or roy orbison (Ren et al. 2002) background. The transgenic lines used are listed in Appendix Table S1.

2. mRNA synthesis and DNA microinjection for sparse labeling

pCS2FA-Transposase was linearized using NotI. The linearized plasmid served as template for the production of capped mRNA of Tol2 Transposase (Kwan et al. 2007) using the Ambion mMESSAGE mMACHINE kit (FisherScientific, AM1340) according to the manufacturer's instructions. One- or two-cell stage fertilized eggs were injected with a mix of DNA and Transposase mRNA at 1:2 ratio (~5ng/µl/~10ng/µl). Injected constructs were: *tp1*:VenusPest, *tp1*:TurboGFP, *tp1*:TurboGFP-PEST and *tp1*:TurboRFP-PEST.

3. *In vivo* imaging

Embryos were prepared for imaging as described previously (Engerer et al. 2021). Between 20 to 24 hours post-fertilization (hpf) embryos were transferred to 0.3× Danieau's solution containing 0.003% 1-phenyl-2-thiourea (PTU, Sigma, P7629) to inhibit melanin formation. At 2 days post-fertilization (dpf), manually dechorionated embryos were anesthetized using 0.02% tricaine (PharmaQ) in medium containing PTU and embedded laying on their side in low-melting agarose (0.7-1%, Sigma, A4018). Fish were imaged on an upright Olympus FV1000 confocal/ and an inverted Olympus FV3000 confocal microscope using water-immersion objectives (Olympus 25×/NA 1.05) or a silicon-immersion objective (Olympus 30×/NA 1.05) respectively. Embryos were maintained at 28.5°C during all *in vivo* recordings. At a temporal resolution of 20min z-stacks of the peripheral retina were acquired until 3-3.5dpf, encompassing its entire circumference. Older fish (10dpf) were imaged on an upright Olympus FV1000 2-Photon system.

4. Generation of constructs

4.1. tp1:TurboGFP

The sequence of TurboGFP was PCR amplified with primers that contained EcoRI and ClaI restriction sites (f-primer: 5'-GGA AAA GAA TTC ATG GAG AGC GAC GAG AGC GG-3', r-primer: GGG CCC ATC GAT CTA TTC TTC ACC GGC ATC TGC AT) from the peTurboGFP-PRL-dest1 vector (gift from Prof. J. Ninkovic, Evrogen cat.# FP523). The amplified fragment was cloned into the *tp1*:VenusPEST backbone (Ninov et al. 2012), after excising VenusPEST with EcoRI and ClaI.

4.2. tp1:TurboGFP-PEST

The sequence of TurboGFPDest1(a.k.a TurboGFP-PEST) was PCR amplified with primers that contained EcoRI and ClaI restriction sites (f-primer: 5'-GGA AAA GAA TTC ATG GAG AGC GAC GAG AGC GG-3', r-primer: GGG CCC ATC GAT CTA CAC ATT GAT CCT AGC AGA AGC AC) from the peTurboGFP-PRL-dest1 vector (gift from Prof. J. Ninkovic, Evrogen cat.# FP523). The amplified fragment was cloned into the *tp1*:VenusPEST backbone (Ninov et al. 2012), after excising VenusPEST with EcoRI and ClaI.

4.3. tp1:TurboRFP-PEST

The sequence of TurboRFP-N was PCR amplified with primers that contained EcoRI and EcoRV restriction sites (f-primer: 5'- GGA AAA GAA TTC ATG AGC GAG CTG ATC AAG GA -3', r-primer: GGG CCC GAT ATC TCT GTG CCC CAG TTT GCT AG) from the pTurboRFP-N vector (Evrogen cat.# FP232). The amplified fragment was cloned downstream of *tp1* in the *tp1*:TurboGFP-PEST backbone after excising out TurboGFP-PEST vector with EcoRI and EcoRV.

5. EdU birth-dating

At 4-hour intervals, starting at 36hpf until 72hpf, groups of at least 10 fish were administered 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen, supplemented in Click-It reaction kit, C10340; e.g. 36hpf or 40hpf or 44hpf, etc). Each embryo was manually dechorionated, anesthetized using 0.02% tricaine (PharmaQ) and embedded laying on their side in single drops of low-melting agarose (0.7-1%, Sigma, A4018) with their yolk slightly tilled to allow access to the heart. After removing agarose from the heart area using a micro-blade, each embryo received two pulses (0.5nl per pulse) of EdU (400µM, 4% DMSO in 1x Danieau's) into the heart. Fish were subsequently recovered completely from the agarose and allowed to develop until 96-97hpf.

6. Immunohistochemistry

6.1. Detection of EdU and GFP

Larvae exposed to thymidine analogue were fixed for 15min in 1xPBS (4% PFA diluted from 16% EM Grade PFA, EMS, 15710) and incubated in 30% sucrose in 1xPBS overnight at 4°C. Animals were embedded in OCT (Tissue Tek) and cryo-sectioned (20 µM). Sections were mounted on SuperFrost slides (Fisher Scientific, 10149870), air-dried for 1h and washed

(3x5min) in 1x PBS, pH 7.4. Following a short permeabilization step (30min, 0.5%Triton-X in 1xPBS) and an additional washing step (3x5min in 1x PBS) a Click-It reaction was performed to develop the EdU signal (Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 dye, Invitrogen, C10340) according to manufacturer's instructions for 1.5h. The sections were washed (3x5min) in 1x PBS and incubated for 1h in blocking solution (20% Normal Goat Serum/ 0.5% PBST, Triton-X100). In order to enhance the endogenous GFP fluorescence driven by *vsx2* promoter elements, the blocking solution was replaced by GFP antibody (chicken 1:250-1:500, Abcam, ab13970) in blocking solution, overnight at 4°C. Following washes (3x5min in 1xPBS) the sections were incubated for 1h in goat anti-chicken Alexa 488, IgG (1:250, Invitrogen, A-11039), in blocking solution. After a final washing session (3x5min in 1xPBS), sections were cover-slipped using a mounting medium containing DAPI (Vectashield; Vector laboratories, H-1200).

6.2. GS immunostaining

Tp1:VenusPEST fish were fixed for 30min at room temperature and processed for cryosectioning as described above. Following air-drying for 1h and washing (3x5min) in 1x PBS, pH 7.4, sections were incubated for 1h in blocking solution (1% Bovine Serum Albumin, 10% Normal Goat Serum in PBST (0.3% Tween-20)) and then with mouse anti-GS IgG2a (1:50, clone GS-6, Sigma, MAB302) and chick anti-GFP antibody (1:1000, Abcam, ab13970) overnight at 4°C. Following washes (3x5min in 1xPBS) the sections were incubated for 1h with goat anti-mouse Alexa 568, IgG2a (1:250, Invitrogen A-21134) and goat anti-chick Alexa 488, IgG (1:250, Invitrogen, A-11039), in blocking solution. After a final washing session (3x5min in 1x PBS), sections were cover-slipped in Vectashield containing DAPI (Vector laboratories, H-1200).

7. DAPT treatment

Animals were incubated for 24h from 49hpf until 73hpf in 25μM DAPT (Sigma, D5942), 0.5% DMSO or 0.5% DMSO in 0.3X Danieau's supplemented with PTU. Animals were anesthetized, embedded in agarose and imaged immediately after treatment using confocal microscopy.

8. Image processing

Images were viewed and processed using open-source ImageJ/Fiji software (http://fiji.sc). Image panels were assembled in Photoshop CS6 (Adobe) and combined into figures using

Illustrator CS6 (Adobe). Gamma was not adjusted. Pseudo-coloring of cells in example panels was performed manually in Photoshop.

9. Data Analysis

9.1. Tracing of MC lineage

At the end of time-lapse imaging experiments (3 dpf), MCs were identified based on marker expression (Notch reporter/sensor), their spanning apical and basal processes and their somal shape (polygonal) and positioning (lower INL). For each MC, an attempt was made to back-trace its history until a mitotic event was reached. Cases for which we were not able to identify the mitotic event, either due to the starting point of the experiment or due to dim transgenic expression, were excluded from the data set.

9.2. Notch sensor analysis in MC–sibling cell pairs

Tp1:TurboGFP-PEST fluorescence intensity levels were monitored by *in vivo* imaging. MC - SC pairs were identified and their somata were manually demarcated based on their TurboGFP expression in a single image plane of a confocal stack using the ImageJ freehand tool. Notch-reporter levels were measured based on the fluorescence intensity of the TurboGFP channel within the demarcated areas. Background values (TurboGFP channel) from three areas at the same time point were averaged and subtracted from the fluorescence intensity values of the cells being measured.

9.3. Assessing paired vs single MCs and vsx2 BCs.

At 10dpf, a region of interest encompassing 10 planes (10 µm) in the ventro-nasal retina of *vsx2*:GFP transgenic fish was selected to assess potential MC-BC sibling pairs. Cells were considered to be siblings when their somata were aligned along the apico-basal axis and the BC soma lay at or along the MC apical process. In the absence of such a configuration, MCs were deemed to be 'single'. The reported ratio of MC/ *vsx2* BC was calculated based on the numbers of the respective cell classes in the areas of interest in which the sibling pair analysis took place.

9.4. DAPT analysis

Analysis was performed in the ventral retina. Maximum intensity projections of 21 optical sections (z step 1µm) were used to outline the analyzed area in the INL using ImageJ. The area was measured using the measure function after calibrating for pixel size. Cells were assigned

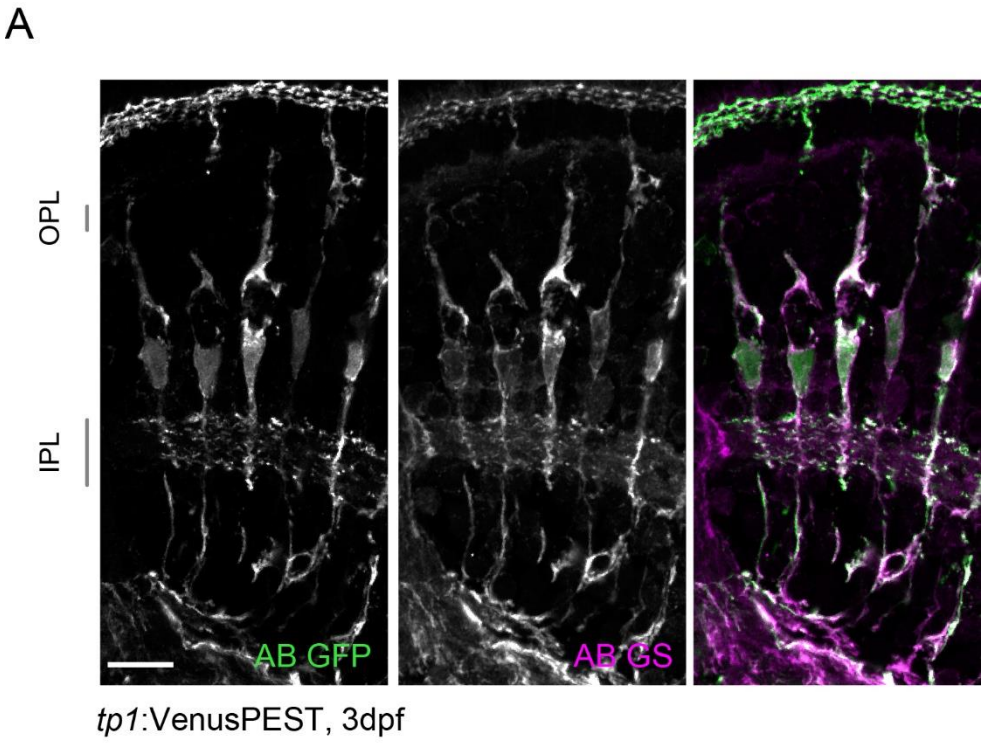
one of two categories, MCs or BCs using the cell counter function in a substack of 21 optical planes (the same ones that were maximum projected) within the outlined area. BCs and MCs were distinguished based on the *vsx2*-driven GFP signal (lower in MCs), location in the INL and overall morphology. In the case of dim *vsx2* signal, *gfap*:GFP was used to confirm MC identity.

10. Statistics

The Shapiro-Wilk test was used to determine whether parametric or non-parametric tests were appropriate for determining statistical significance with GraphPad Prism 8. The specific statistical tests used to compare data sets are indicated in the respective part of the text or figure legend. P-values 0.0332 are denoted with '*', 0.0021 with '**' , 0.0002 with '***', <0.0001 (****).

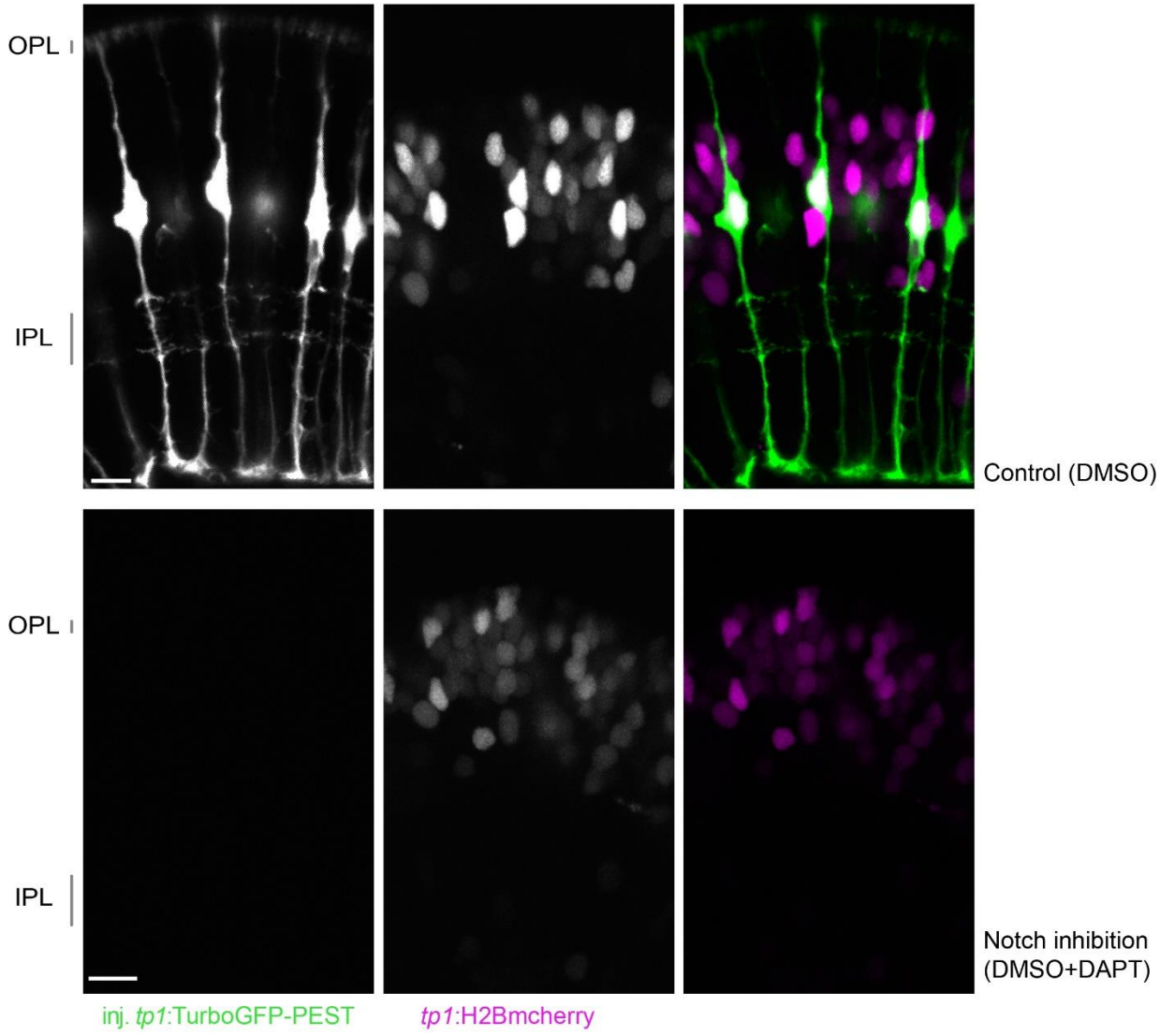
Table S1-Transgenic lines

Transgenic line	**Referred as**	**Reference**
Tg(*vsx2*:GFP)nns1	*vsx2*:GFP	(Kimura et al. 2006; Vitorino et al. 2009)
Tg(vsx2:LOXP-DsRed-LOXP-GFP)	*vsx2:*dsRed	(Kimura et al. 2006)
Tg(EPV.Tp1-Mmu.Hbb:Venus-Mmu.Odc1)	*tp1*:VenusPEST	(Ninov et al. 2012)
Tg(EPV.Tp1-Mmu.Hbb:hist2h2l-mCherry)	*tp1:*H2Bmcherry	(Ninov et al. 2012)
Tg(-1.8ctbp2a:GAP-EGFP)	*ctbp2*:mEGFP	Odermatt et al. 2012
Tg(gfap:GFP)mi2001	*gfap:*GFP	Bernardos & Raymond 2006

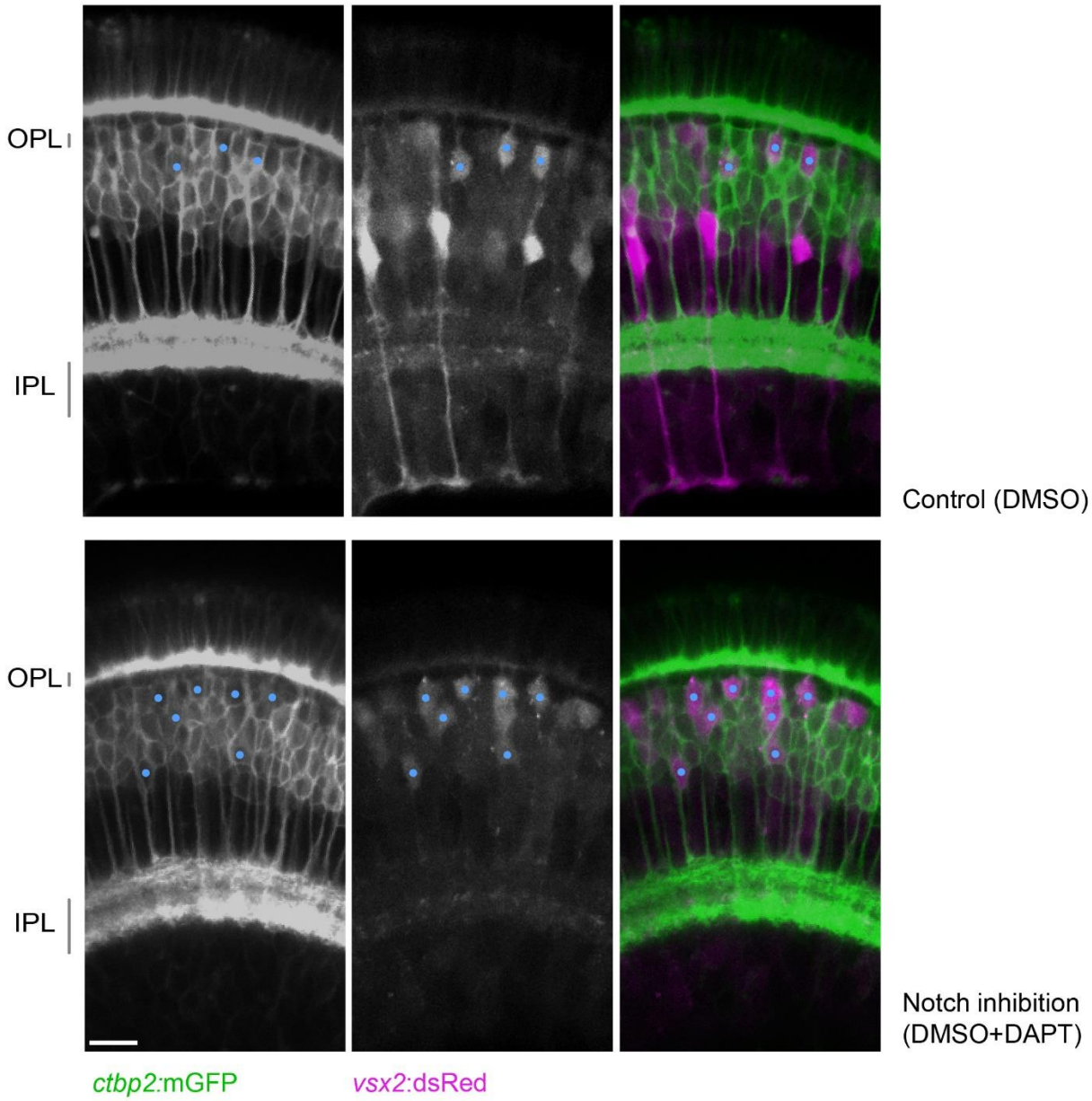


Supplementary figure 1: Characteristics of glia neuron-divisions. A) Immuno-staining against Müller cell marker Glutamine Synthetase (GS) and endogenous GFP in retina cross section of a 3dpf *tp1*:VenusPEST animal. B) Distribution of mitotic events from 2nd to 3rd day post

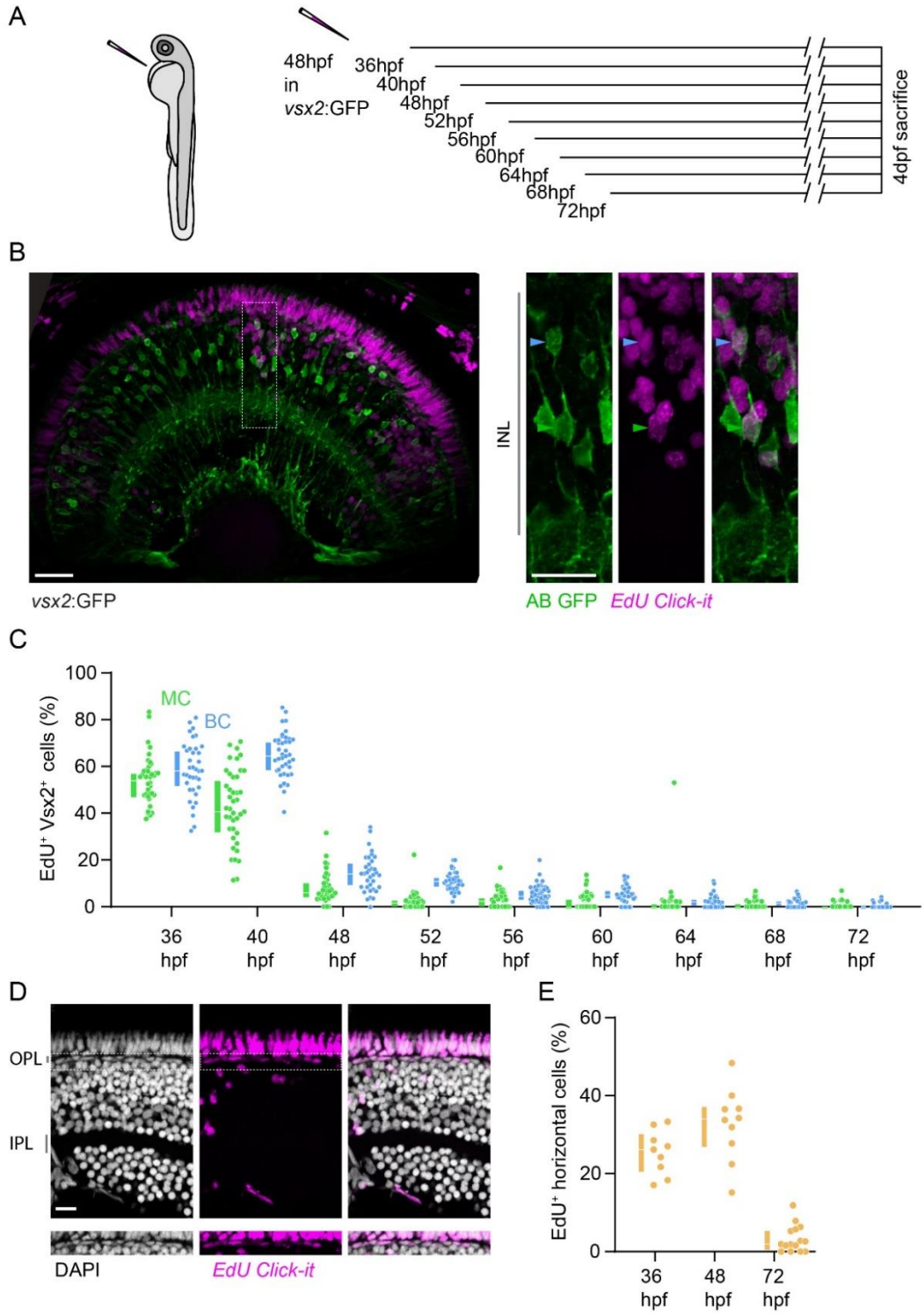
fertilization (Median and IQR, 31 cases, 22 animals). C) Mitotic events occur in all retina quadrants (Dorsal-Temporal 32.26%, Dorsal-Nasal 29.03%, Ventral-Nasal 12.9%, Ventral-Temporal 25.81%, 31 cases, 21 animals, Chi-square test p value = 0.4441). Scale bar: 10 µm.



Supplementary figure 2: Validation of Notch sensor. Double positive animals for *tp1*:H2Bmhcerry injected with *tp1*:TurboGFP-PEST were incubated for 24hrs starting from 49hpf until 73hpf in 25µM DAPT/ 0.5% DMSO (DAPT group) or 0.5% DMSO (DMSO group. Scale bar: 10 microns.



Supplementary figure 3: Notch inhibition promotes the production of vsx2 Bipolar cells. Double transgenic animals for ctbp2:GFP; vsx2:dsRed were incubated for 24hrs starting from 49hpf until 73hpf in 25µM DAPT/ 0.5% DMSO (DAPT group) or 0.5% DMSO (DMSO group). Scale bar: 10microns.



Supplementary figure 4: Birth-dating the *vsx2* lineage. **A)** Experimental model. Animals received an EdU-pulse in the heart at different time points (36, 40, 48, 52, 56, 60, 64, 68, 72hpf) and sacrificed at 4dpf. **B)** Left: Retinal cross-section of a 48hpf *vsx2*:GFP injected animal. Right: Area as indicated in the insert. Endogenous GFP represented by green and EdU developed via Click-It reaction represented by magenta. **C)** Quantification of EdU+ *vsx2* BCs (cyan color) and MCs (green). Boxes represent median and IQR. Individual points represent individual retinal sections analyzed. Scale bar: 20 µm.

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3 Discussion

How cellular diversity is established in the developing CNS and the underlying mechanisms involved has long been a topic of extensive study (Cepko 2014; Lodato & Arlotta 2014). While much progress has been made, many questions remain unanswered. Further, the majority of studies are based on single time points of fixed tissue or *in vitro* preparations, which may not always reflect the *in vivo* situation. In the current thesis, I used the retina as a model to understand how cellular diversity is established during development. The vertebrate retina, a compact, accessible and well-defined part of the CNS (Dowling 2012) is particularly suited for such a quest and studying it in zebrafish, a genetically and optically accessible model organism, allowed me to probe the mechanisms of cell fate acquisition *in vivo*. For my thesis, I concentrated on cell classes generated by terminal mitotic divisions. In the first published research manuscript (**Result section 2.2**), I investigated terminally dividing progenitors in the *vsx1* lineage. Together with my colleagues, I could show that *vsx1* progenitors are not restricted to generating BCs but can also generate ACs. We proposed that a Notch-mediated mechanism operating post-mitotically allows for a fate switch of newly born *vsx1* BCs (excitatory interneurons) to ACs (inhibitory interneurons). This suggests a degree of spontaneous plasticity in the CNS that has not been previously anticipated. In the second research manuscript (**Result section 2.3**), I observed terminal mitotic events that generated neuron-glial cell pairs, a mode of division not previously directly observed *in vivo*. Specifically, pairs comprised of a distinct type of excitatory interneuron (*vsx2* BCs) and a MC, the principal glial cell type in the vertebrate retina.

3.1 *Vsx1* and *vsx2* terminal events

Despite a degree of stochasticity that has been reported to govern fate acquisition (Boije et al. 2014, 2015; Gomes et al. 2011; He et al. 2012) there are terminal divisions with biased outputs. For instance, *olig2* embryonic progenitors in the rodent retina are biased towards the production of cone PhRs and HCs (Hafler et al. 2012). In the zebrafish retina, terminal symmetric divisions have been described that exclusively generate pairs of PhRs (He et al. 2012; Suzuki et al. 2013), HCs (Godinho et al. 2007; Weber et al. 2014b) or *vsx1* BCs (Engerer et al. 2017; Weber et al. 2014b), suggesting the existence of dedicated progenitors. *Vsx1*-expressing BCs constitute the majority of the BC population in the zebrafish retina, comprising multiple subclasses based on their axonal stratification within the IPL (Vitorino et al. 2009). While pairs of *vsx1* BCs are generated via terminal symmetric divisions, we discovered that a substantial proportion of nascent *vsx1* BCs undergo spontaneous respecification to acquire the AC fate (Engerer*, Petridou* et al. 2021). It should be noted that the vast majority of ACs are not generated via

vsx1 progenitors, but rather a completely distinct lineage (Dullin et al. 2007; Fujitani et al. 2006; Jusuf et al. 2011; Nakhai et al. 2007). Although we did not systematically address the specific neurotransmitter identity of ACs derived from the *vsx1* lineage, a recent study reported that the majority of these ACs are glycinergic with a lower number being GABAergic (Wang et al. 2020). Interestingly, in the mouse retina a study revealed the existence of a *vsx1* monopolar glutamatergic cell type (GluMI) that shares elements of both AC and BC identities (Della Santina et al. 2016; Shekhar et al. 2016). These *vsx1* expressing cells differentiate along with the *vsx1* BCs in the mouse retina, outside of the normal AC differentiation window. Initially, their morphology resembles nascent bipolar cells but later on, they seem to retract their apical processes beyond the OPL in order to acquire a monopolar amacrine-like morphology. Hence, it is tempting to hypothesize that GluMIs might derive from the same lineage as the *vsx1* BCs and derive from the same terminal events resembling what we observed *in vivo* in the zebrafish retina.

By comparison with the *vsx1* BC-AC pairs, less is known regarding the *vsx2* BC-MC siblings. These *vsx2* expressing BCs represent a smaller population of BCs that stratify in the S4 and S5 sublaminae of the IPL (Barabino et al. 1997; Passini et al. 1997; Vitorino et al. 2009). Here we provide evidence, for the first time, that at least a percentage of *vsx2* BCs are generated via terminal asymmetric divisions in which MCs are their sibling. Future studies can elucidate whether *vsx2* BCs are also produced via terminal symmetric divisions. The difficulty in addressing this question lies in the expression pattern of *vsx2*. In contrast to *vsx1* which is expressed mainly in postmitotic BCs and the progenitors that generate them (data from P. Engerer, not shown), *vsx2* appears first in all retinal progenitors and then limits its expression to a small subset of BCs and MCs (Vitorino et al. 2009). A recent study in mice illustrated that there are upstream regulatory sequences in the *vsx2* locus specific to BCs (Norrie et al. 2019) and different ones specific to the progenitors (Honnell et al. 2022). Thus, potentially, different upstream sequences could regulate the differential expression of *vsx2* in the zebrafish retina. Identification of such sequences could allow the genetic branding of divisions that are biased to produce a BC/MC pair or potentially a BC/BC pair.

3.2 Notch signaling in establishing asymmetric fates

Notch signaling has long been implicated in the acquisition of distinct fates in invertebrates and vertebrates. One of the most often cited examples comes from Drosophila neuroblasts. These cells divide to generate an apically located neuroblast and a basally located ganglion mother cell (GMC), which subsequently divides to generate two neurons or glial cells. This

correlation between the apico-basal position and subsequent acquisition of a neuroblast or GMC fate is not random. As the neuroblast enters the cell cycle, different polarization factors become asymmetrically segregated to opposite sides of the mitotic cell along the apico-basal axis. These polarity factors include, for example, proteins like Bazooka and Inscuteable/ Partner of Inscuteable (Insc/Pins) on the apical side whereas Prospero, Miranda, and Numb are segregated to the basal side (Jan & Jan 2001). The cell that inherits Numb, a Notch inhibitor (McGill & McGlade 2003; McGill et al. 2009), becomes a GMC, with a limited capacity for proliferation. Its apically located sibling, with comparatively higher levels of Notch signaling, returns to the cycle, acquiring a neuroblast fate. Similarly, in the Drosophila PNS, upon the division of the sensory organ precursor (SOP/pI) the fate of the two daughters Plla and Pllb depends on the asymmetric distribution of Numb and consequently Notch. Thus, the posteriorly located Plla (Notch +) will consequently divide once more to produce the shaft and socket cells of the bristle whereas the anteriorly located Pllb (Notch -) will give rise to the neuron and the sheath cell of the sensory organ (Couturier et al. 2012; Rhyu et al. 1994; Schweisguth 2015; Trylinski et al. 2017). In vertebrates, during cortical development, radial glial cells can divide asymmetrically to self-renew and generate a neuron or an intermediate progenitor (Anthony et al. 2004; Malatesta et al. 2000, 2003; Miyata et al. 2001; Noctor et al. 2001). During these asymmetric events, two key players have been proposed to be essential, namely the apical polarity determinant Partition defective protein 3 (Par3) and Notch signaling (Bultje et al. 2009). The asymmetric segregation of Par3 was suggested to ensure an imbalance of Notch signaling, consequently impacting the fate of the daughter cells. The authors proposed that high Par3 levels promoted high Notch signaling and thus the radial glial fate whilst low Par3 levels/Notch signaling the alternative fate e.g. neuron or intermediate progenitor. The ability of Par3 action to enhance Notch signaling was believed to be mediated via Numb and Numb-like protein.

3.3 The role of Notch signaling in establishing asymmetric fates in the *vsx1* lineage

Since Notch has been implicated in binary fate decisions across systems, we aimed to determine its role in the *vsx1* mitotic divisions that generated asymmetric outcomes, namely BC and AC daughters. It has been illustrated in several systems that the mitotic spindle orientation, i.e. plane of division, can be linked to the asymmetric distribution of Notch signaling components and/or inhibitors influencing the fate of the resulting sibling cells (Chenn & McConnell 1995; Das & Storey 2012; Dong et al. 2012; Kechad et al. 2012; Reugels et al. 2006). Although, a

clear correlation between the plane of division and the resulting fates of daughter cells has been shown in the mouse retina (Cayouette & Raff 2003; Kechad et al. 2012), this has not been reported in the zebrafish retina. Indeed, we were unable to find a correlation between mitotic plane orientation and a specific BC or AC fate in the *vsx1* lineage (n= 45 cells) (Figure S3 C of Engerer*, Petridou* et al. 2021). Additionally, in *Q32* mitotic events that generated BC-AC siblings, Numb (Figure S3 A&B of Engerer*, Petridou* et al. 2021) and Par3 (data not shown) were uniformly distributed, in line with observations of asymmetric terminal *vsx1* mitotic divisions in the zebrafish spinal cord (Kimura et al. 2008). The absence of an asymmetric distribution of Numb or Par3 together with a non-stereotypic division plane suggested that other, potentially post-mitotic, mechanisms might be at play, as previously suggested in the mouse retina (Mizeracka et al. 2013). *In vivo* imaging using a genetically based reporter to monitor Notch signaling levels in *Q32* divisions that generated BC-AC pairs revealed an increase only post-mitotically in the cell that ultimately acquired AC morphological and molecular characteristics. It remains unclear how differential Notch signaling is established post mitotically. Stochastic changes in Delta/Notch levels could lead to differences in Notch signaling strength between nascent BCs, allowing the cell with higher levels to re-specify to an AC fate, as was suggested for the V2a/ V2b specification of spinal cord neurons (Kimura et al. 2008). Interestingly, high Notch signaling is required for the specification of the inhibitory V2b fate in the zebrafish spinal cord, mirroring the scenario we describe for the acquisition of ACs, that are also inhibitory neurons. Another unanswered question remains as to whether the asymmetry in Notch signaling in these siblings arises due to their interaction with each other or their surrounding neighboring cells. Classical transplantation experiments could address this issue. Transplantation of wild-type *Q32* progenitors into embryos with abrogated Notch signaling (e.g. mib mutants), and time-lapse imaging to monitor whether BC-AC daughter pairs are generated could reveal whether relevant signaling comes exclusively from interactions between siblings. Similarly, the transplantation of *Q32* progenitors from a Notch mutant background into a wild type environment could reveal the influence of the surrounding cells.

3.4 The role of Notch signaling in establishing asymmetric fates in the *vsx2* lineage

The underlying mechanism by which Notch signaling mediates the *vsx2* BC/MC divisions is unknown. *In vivo* time lapse imaging illustrated that Notch signaling is upregulated in the future MC in line with the instructive role of Notch in gliogenesis (**Introduction section 1.3.4.2**). This imbalance between the siblings could be the result of the asymmetric segregation of Numb

for example (Alexandre et al. 2010) but further investigation is needed. Nonetheless, Notch signaling might be important for both BCs and MCs albeit at different levels. For example, it was shown in the rodent retina that the conditional knockout of the Notch1 receptor in postmitotic retinal cells led to the production of rod PhRs at the expense of both MCs and BCs (Mizeracka et al. 2013).

3.5 Investigating specific Notch signaling components

In order to better appreciate the role of Notch signaling in the *vsx1* and *vsx2* lineages, it will be important to understand the differential expression of Delta ligands and Notch receptors. In the zebrafish retina different Delta ligands and Notch receptors have been reported e.g., Notch1a, Notch 1b, DeltaA, Delta C e.t.c (Bene et al. 2008; Haddon et al. 1998; Nerli et al. 2020) either at the protein or RNA level. Nonetheless, investigating the expression pattern of these molecules in the specific lineages I have been studying has proven to be quite challenging. My attempts to address this for the *vsx1* population with available antibodies (Notch1, Delta C) did not yield conclusive results as the puncta-like expression pattern was distributed uniformly across the retinal tissue and not confined to the BCs or ACs in the *vsx1* lineage. An additional limitation is the poor temporal resolution; tissue fixation restricts an observation to a single time-point when the relevant dynamic event may or may not take place. Moreover, in this single snap-shot it is impossible to predict whether a *vsx1* bi-polar shaped cell is a progenitor or nascent BC that may subsequently undergo a fate re-specification or simply differentiate as a BC. Transcriptomic profiling of *vsx1* and *vsx2* populations in the established retina might allow the identification of differentially expressed Notch signaling components that could be used subsequently to develop genetic tools to monitor their expression *in vivo*. Of course, as technology progresses and spatial transcriptomics in intact tissues becomes increasingly accessible (Alon et al. 2021), basic developmental questions such as this can be answered with higher precision (Ratz et al. 2022).

3.6 Convergence of Notch signaling and fate specification factors

Notch signaling is involved in both types of cell diversity-generating terminal divisions described here. However, while Notch signaling is instructive for the MC fate, this is not the case for the *vsx1* lineage-derived ACs. Our hypothesis is that increased Notch signaling simply opens a window of plasticity in nascent *vsx1* BCs, allowing Ptf1a to directly instruct AC fate. Ptf1a has been extensively studied in the context of pancreatic cell fate determination and in instructing the fate of inhibitory interneurons throughout the CNS (Jin & Xiang 2019). In the

vertebrate retina, Ptf1a is fundamental to the production of ACs and HCs, both inhibitory interneuron cell classes (Dullin et al. 2007; Fujitani et al. 2006; Jusuf et al. 2011; Li et al. 2004; Nakhai et al. 2007). Whether Ptf1a operates similarly in the *vsx1* lineage as it does in the *vsx1* independent population to instruct AC fate remains elusive. Nonetheless, the convergence of Ptf1a and Notch signaling has been described in the literature mainly stemming from pancreatic development research (Bastidas-Ponce et al. 2017; Jin & Xiang 2019). For example, it has been suggested that Ptf1a directly induces the expression of Dll1 in the signal sending cell, a step crucial for the activation of Notch signaling in surrounding cells and the maintenance of the multipotent pancreatic progenitor cells (MPCs) pool (Ahnfelt-Rønne et al. 2011). Later during pancreatic development but also in adulthood, it has been shown that Ptf1a forms a tripartite complex with a bHLH protein potentially Hes1 (hypothesized to stabilize Ptf1a /(Ghosh & Leach 2006)) and RBP-J1 antagonizing NICD to form a transcription activation complex. Hence, the Ptf1a tripartite complex promotes pancreatic cell differentiation and maturity (Masui et al. 2007, 2010). How could this relate to the respecification mechanism we described in the retina? During retinal development, Foxn4 induces expression of *dll4* in order to stall PhR fate along with inducing both HC and AC fates (Luo et al. 2012). In addition, Foxn4 along with RORb activates the expression of *ptf1a* (Liu et al. 2013) which in turn autoregulates its expression and induces the AC fate (Dullin et al. 2007; Fujitani et al. 2006; Jusuf et al. 2011; Nakhai et al. 2007). We do not know whether, during the fate switch from *vsx1* BC to an AC, Foxn4 and RORb are responsible for the activation of *ptf1a*, but one could hypothesize that Ptf1a might act in a dual manner. It could promote the AC fate and concurrently act as the stop signal of the Notch signaling based on a simple balance between the tripartite complex Ptf1a/ RBP-J1/bHLH/ and NICD/ RBP-J1/MAML. This potentially could regulate the duration of the plasticity window.

3.7 Could cellular diversity simply arise from distinct progenitor populations?

One could argue that instead of a postmitotic mechanism that allows the respecification of a BC to an AC, two different pools of *vsx1* progenitors exist, one of which is committed exclusively to generating *vsx1* BCs and the other a bipotential progenitor that generates BC and ACs. This idea was proposed in a recent study (Wang et al. 2020). The authors generated a new transgenic construct targeting *vsx1* expressing cells and combined it with a lineage tracing technique (photo-conversion of Kaede) to allow for irreversible labeling of single *vsx1* progenitors. Subsequently, they analyzed the derived progeny at a later time point. The finding

of two cell clones containing *vsx1* BC-BC and *vsx1* BC-AC pairs led the authors to suggest that there are actually two distinct neurogenic lineages. Single cell RNAseq data from unspecified progenitors (whole retinae) during the same developmental window organized these cells in two *vsx1* clusters, *atoh7*$^{+}$ and *atoh7*$^{-}$ (atonal homolog 7), a transcription factor that is important for retinal ganglion cell determination early on (Brzezinski et al. 2012; Mu et al. 2005; Yang et al. 2003). Hence, it was suggested that *vsx1*$^{+}$*/atoh7*$^{-}$ progenitors might generate pairs of *vsx1* BC-BC and *vsx1*$^{+}$*/atoh7*$^{+}$ progenitors respectively *vsx1* BC-AC pairs. Nonetheless, overexpression of *atoh7* in *vsx1* progenitors did not increase the number of *vsx1* BC-AC pairs as one would expect if different neurogenic lineages were present. Stemming from different datasets of our work we do not believe that distinct vsx1 progenitor populations exist. The combinatorial overexpression of *NICD* and *ptf1a* in the *vsx1* lineage led to a shift of both BC-BC and BC-AC divisions to the AC-AC mode (Figure 4 B&C of Engerer*, Petridou* et al., 2021). This suggests that potentially all immature *vsx1* BCs can be respecified to ACs. In addition, at least at the mitotic level, we were unable to distinguish *vsx1* progenitors based on their Notch reporter expression (9 BC-BC generating progenitors, from 7 fish and 13 BC-AC generating progenitors from 8 fish; Mann-Whitney test) (Engerer*, Petridou* et al., 2021) irrespective of the outcome of these events. Of course, there could be intrinsic differences between these progenitors which are beyond the detection limit of our approaches. With the advance of transcriptomic methods, FACS-sorting *Q32*-labeled progenitors and proceeding with RNAseq could reveal whether distinct progenitor pools exist. Nonetheless, this might be challenging. Even with the use of the "full" stable transgenic line, Tg(*vsx1*:GFP), which faithfully reflects the endogenous expression pattern of *vsx1*, we might not reach the sensitivity needed to detect a small population of cells undergoing divisions within a defined window of time.

In the case of the *vsx2* lineage even less is known. A transcriptomic approach to determine whether distinct terminally dividing progenitor populations exist might not be feasible. *vsx2* is expressed in all retinal progenitors. Presently no genetic tools exist that would only target terminally dividing *vsx2* progenitors that generate either *vsx2* MC-BC, *vsx2* BC-BC or *vsx2* MC-MC pairs.

3.8 Synaptic connections between siblings and functional implications

In the rodent cortex, it was beautifully shown that siblings that are generated together tend to "wire" together forming functional connections (Yu et al. 2009, 2012). These excitatory sibling cells formed columnar microcircuits. Initially, the sister cells formed gap-junctions/electrical

synapses that allowed for their synchronous activity and later transitioned to form chemical synapses of higher synaptic strength among them than with other neighboring cells, illustrated via paired and also quadruple whole-cell recordings in acute brain slices.

It is thus conceivable that in both described cases of asymmetric fates (*vsx1* BC-AC and *vsx2* BC-MC) a special structural and functional connection might exist amongst siblings that differ from their connectivity with other surrounding cells in their vicinity. With the temporal resolution of our *in vivo* recordings (15-20 min), neither BCs nor MCs appeared to migrate tangentially post mitosis but rather find their position after some radial "wiggling" of their somata. The same applies for the *vsx1* derived ACs which migrated radially to the lower part of the INL devoid of any tangential movement. Hence, it is possible that *vsx1* BC-AC and *vsx2* BC-MC remain in close proximity with each other forming structural and functional synapses *in situ*. Nonetheless, addressing this experimentally would require a series of challenging experiments.

One approach to assessing the existence of synapses, either electrical or chemical, at the ultrastructural level would be to perform Correlative Light Electron Microscopy (CLEM) (Begemann & Galic 2016). Monitoring genetically labeled asymmetric events at the light microscopic level and identifying preferential synaptic connectivity between siblings derived from these events, using EM, would be the core idea of this approach. A combination of near infrared branding (NIRB) to delineate the area of interest (Bishop et al. 2011) and genetic tags that are specific for the *vsx1* or *vsx2* lineage and are identifiable by EM, could revealsuch connectivity patterns. One such available tag is a modified ascorbate peroxidase (APEX) which enables EM contrast in subcellular structures, organelles or molecules in which it is expressed and has been adapted for use in zebrafish (Ariotti et al. 2015; Zhang et al. 2019). Nonetheless, the APEX tag might actually obscure the identification of synaptic connections if it is, for example, targeted to the membranes or may not provide enough contrast if it is cytoplasmically expressed. Electrical synapses that are composed of gap junctions are notoriously difficult to identify via EM and these aspects should be taken into consideration. An alternative approach would be to perform Volumetric EM in the absence of an EM tag. This would require the segmentation and reconstruction of a whole volume, as its name implies, in the absence of genetic fiduciaries. Although it is feasible, such an approach demands an extensive amount of time, and the small number of samples that one can analyse can outweigh the benefits of the method. We explored the idea of EM (with the help of Dr. Schifferer, EM Hub, SyNergy, and Dr. Snaidero, HIH, University of Tubingen) using APEX2 (Böhm et al. 2022; Djenoune et al.

2017; Lam et al. 2015) expressed under the *vsx2* promoter elements in larvae at 9 dpf, at which time retinal circuitry is well established. Nonetheless, soon after, we realized that the chemical we routinely use to stall melanin formation, N-Phenylthiourea (PTU) (Karlsson et al., 2001), enabling us to visualize and perform *in vivo* imaging, seems to have detrimental effects on tissue integrity at the ultrastructural level (data not shown). Thus, we did not continue with CLEM.

There are two additional methods that could permit the identification of established synaptic connections. The first one is to combine immunostaining against known pre- and post-synaptic markers like Synaptophysin or PSD-95 and expansion microscopy which allows resolving fine cellular structures (Freifeld et al. 2017) or even the use of the respective transgenic lines such as Sypb-EGFP and Psd95-DsRedEx (Du et al. 2018). In the case of BCs, which form ribbon synapses one could combine specific markers (e.g. C-Terminal Binding Protein 2 (CTBP2) /Ribeye) that are incorporated in these structures along with super resolution imaging (Lv et al. 2012). The second approach, although not readily available, is the adaptation of a trans-synaptic method developed for Drosophila called *trans*-tango (Talay et al. 2017). This method has been adapted for zebrafish by the laboratory of Dr. Halpern (Geisel School of Medicine at Dartmouth, unpublished data, Zebrafish neurobiology & behaviour seminar series, 7th of April, 2022). Tango is a synthetic (foreign to the organism) signaling pathway inspired by Notch signaling. As such, it takes advantage of a ligand-receptor interaction between neighboring cells upon which a proteolytic event allows the release of a transcription factor that translocates to the nucleus and permits the expression of a reporter (Barnea et al. 2008). The expression of the ligand is limited to the presynaptic site whilst the expression of the remaining pathway components in all potential postsynaptic partners. The proper interaction allows the expression of the reporter gene in this postsynaptic cell (Talay et al. 2017). Such a mechanism could allow the identification of synaptic connections between the *vsx1* BC-ACs and *vsx2* BC-MCs.

Alternatively, one could explore the synaptic preference/ exclusivity of these siblings via a different route. Adhesion molecules such as members of the Down syndrome cell adhesion molecule (Dscam) and Sidekick family mediate homophilic interactions and have been shown to play an important role in the assembly of the inner plexiform layer where retinal interneurons synapse at specific sub-laminae with their retinal ganglion cell counterparts (Sanes & Yamagata 2009; Yamagata & Sanes 2008). For example, Dscam-like 1 protein is exclusively expressed by rod BCs, AII ACs and the GCs that these interact with (Fuerst et al. 2008; Yamagata & Sanes 2008). Potentially *vsx1* BC-AC and *vsx2* BC-MC pairs respectively express

specific combinations of adhesion molecules distinct from their cellular milieu. In addition, apart from the synaptic preference amongst the sibling cells one could explore the synaptic avoidance of the surrounding cells. Dscam proteins not only facilitate homophilic attraction but also repulsion as has been illustrated in the mouse retina (Fuerst et al. 2008, 2012). These molecules -at least in Drosophila- exhibit thousands of different isoforms via alternative splicing. As a result, different isoforms may not only not interact with one another but also repulse one another (Schmucker et al. 2000; Wojtowicz et al. 2004). Nonetheless, it is not entirely clear whether the same mechanisms apply in vertebrates.

In order to probe potential connectivity in these pairs using electrophysiological methods is extremely challenging. Experiments routinely performed in the neocortex or explanted retina of rodents such as paired electrophysiological recordings, are yet to be a standard procedure in the zebrafish retina, potentially due to the small scale of the animal, especially at larval stages. A combination of sensors like calcium indicators as a proxy for neuronal activity e.g., GCamp (Nakai et al. 2001) or neurotransmitter indicators e.g. glutamate (Marvin et al. 2013) and optogenetic stimulation could be an option. Nonetheless, optogenetic stimulations in the retina are troublesome since endogenously expressed opsin are affected by the illumination needed to activate the individual channels usually in the blue or red part of the spectrum (Antinucci et al. 2020). Advances made in modifying channelopsins in such a manner that could be excited via 2 photon illumination (Adesnik & Abdeladim 2021) allow for the manipulation of the retinal circuit without exciting endogenous opsins, and thus the assessment of functional connectivity of the sibling pairs of interest.

Finally and although viral tracing of synaptic connections is a state-of-the-art technique in higher vertebrates, it is not readily available in zebrafish. There are efforts to optimize viral infections and neuronal tracing (anterograde & retrograde) in adult fish (Dohaku et al. 2019; Ma et al. 2020) and quite recently in larvae (Satou et al. 2022) with the goal to enrich the toolkit of synaptic tracing methodologies in zebrafish research.

3.9 Concluding remarks

Terminal asymmetric events, such as those described in the current thesis (**Figure 5**), generate cellular diversity in a single mitotic division, a mode of cell genesis that is likely highly prevalent in rapidly developing organisms such as zebrafish. Specifically, in the first published research manuscript (**Result section 2.2**), together with my colleagues, we identified a mechanism of spontaneous fate specification which permits for an additional source of cellular

diversity and reflects the plasticity of nascent post mitotic cells. In the second research manuscript (**Result section 2.3**), I observed terminal mitotic events that generated neuron-glial cell pairs *in vivo* expanding the repertoire of possible terminal divisions in the developing CNS.

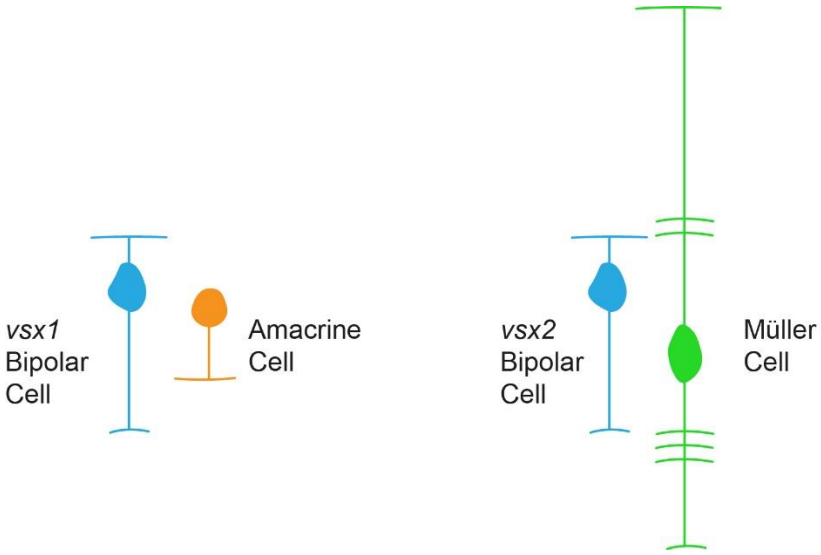

Figure 5: Newly identified sources of cellular diversity in the developing zebrafish retina.

What could be the role of these divisions and their impact on the retinal circuitry? These strategies could reflect the generation of specific cells "on demand", allowing for the fine tuning of emerging circuitry. Indeed, in the *vsx1* lineage, when the AC-instructive transcription factor Ptf1a was knocked down more *Q32* BCs started expressing *ptf1a*:GFP (data not shown, Dr. Engerer). However, the presence of these asymmetric pairs might not only affect the computational processes within the retinal circuit but also the encoded information that leaves the retina and impacts the visually-driven behavior of the animals. The genetic manipulations of the *vsx1* lineage and in the future of *vsx2* might allow addressing this issue. Overexpression of *ptf1a* and *NICD* in the *vsx1* lineage resulted in a dramatic increase in the number of *vsx1* ACs and an apparently corresponding decrease in *vsx1* bipolar cells, likely impacting the excitatory-inhibitory cell ratio (Figure 4 of Engerer*, Petridou* et al., 2021). The behavioral assessment of these animals might reveal the consequences of such manipulations and thus shed light on the physiological role these asymmetric pairs serve. Indeed, there are a variety of visually-driven behaviors like phototaxis, optomotor response (OMR), optokinetic response (OKR), prey capture etc that the animals can be assayed for (Bollmann 2019).

Collectively, the better we understand these asymmetric events and the functional implications of their presence, the better we will understand their impact on shaping an “invariably variable” tissue (phrase quoted from He et al. 2012).

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5 List of publications

Eleni Petridou and Leanne Godinho. "Cellular and molecular determinants of retinal cell fate." Annual Review of Vision Science (accepted, not published)

Engerer, Peter*, **Eleni Petridou***, Philip R. Williams, Sachihiro C. Suzuki, Takeshi Yoshimatsu, Ruben Portugues, Thomas Misgeld, and Leanne Godinho. "Notch-mediated re-specification of neuronal identity during central nervous system development." Current Biology 31, no. 21 (2021): 4870-4878. (*co-first)

Samardzija, Marijana, Andrea Corna, Raquel Gomez-Sintes, Mohamed Ali Jarboui, Angela Armento, Jerome E. Roger, **Eleni Petridou** et al. "HDAC inhibition ameliorates cone survival in retinitis pigmentosa mice." Cell Death & Differentiation 28, no. 4 (2021): 1317-1332.

Eldem Sadikoglou, Elena Daoutsali, **Eleni Petridou**, Maria Grigoriou, and George Skavdis. "Comparative analysis of internal ribosomal entry sites as molecular tools for bicistronic expression." Journal of Biotechnology 181 (2014): 31-34.

6 Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation “Investigating terminal divisions during retinal development in zebrafish” selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation “Investigating terminal divisions during retinal development in zebrafish” is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den 14.06.2022

Unterschrift Petridou, Eleni _____

7 Declaration of author contributions

1st Manuscript: “Cellular and Molecular Determinants of Retinal Cell Fate”, Petridou and Godinho, 2022, Annual Review of Vision Science.

Manuscript writing: E.P. and L.G.

2nd Manuscript: “Notch-mediated re-specification of neuronal identity during central nervous system development”, 2021, Current biology.

Project design and experimental design: P.E., E.P., P.R.W., T.M., and L.G.

Project supervision: L.G. and T.M.

Experiments performed by P.E. and E.P.

Construct generation and transgenic lines: P.E., S.C.S., T.Y., and L.G

Advice on the data analysis: R.P.

Manuscript writing with input from all of the authors: L.G.

3rd Manuscript: “Neuron-glia pairs arise from terminal progenitor divisions in the vertebrate retina”, under preparation.

Project design and experimental design: E.P., T.M., and L.G.

Project supervision: L.G. and T.M.

Experiments performed by E.P.

Construct generation: E.P.

Manuscript writing: E.P. and L.G.

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