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The zebrafish *flotte lotte* mutant reveals that the local retinal environment promotes the differentiation of proliferating precursors emerging from their stem cell niche

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SUMMARY

It is currently unclear how intrinsic and extrinsic mechanisms cooperate to control the progression from self-renewing to neurogenic divisions in retinal precursor cells. Here, we use the zebrafish *flotte lotte* (*flo*) mutant, which carries a mutation in the *elys* (*ahctf1*) gene, to study the relationship between cell cycle progression and neuronal differentiation by investigating how proliferating progenitor cells transition towards differentiation in a retinal stem cell niche termed the ciliary marginal zone (CMZ). In zebrafish embryos without Elys, CMZ cells retain the capacity to proliferate but lose the ability to enter their final neurogenic divisions to differentiate as neurons. However, mosaic retinae composed of wild-type and *flo* cells show that despite inherent cell cycle defects, *flo* mutant cells progress from proliferation to differentiation when in the vicinity of wild-type retinal neurons. We propose that the differentiated retinal environment limits the proliferation of precursors emerging from the CMZ in a manner that explains the spatial organisation of cells in the CMZ and ensures that proliferative retinal progenitors are driven towards differentiation.

KEY WORDS: Ciliary marginal zone, Cell cycle progression, Differentiation, Neurogenesis, Retinal stem cells, Zebrafish

INTRODUCTION

The precise coordination of cell proliferation, differentiation and death ensures that organs of the correct size, morphology and composition are formed; the orchestration of these programmes is particularly evident during neurogenesis in the vertebrate retina. Neuronal differentiation in the eye commences when multipotent retinal progenitor cells (RPCs) begin to exit the cell cycle and acquire postmitotic fates in a temporally and spatially coordinated manner, with retinal ganglion cells (RGCs) born first and rod photoreceptors and Müller glia last (Agathocleous and Harris, 2009; Livesey and Cepko, 2001). As in other systems, RPC proliferation is intrinsically controlled by the ordered synthesis and destruction of regulatory proteins that propel cells through phases of growth, DNA replication and chromosome segregation. As cells pass from one cell cycle phase to the next, they assess their internal and external environments to verify that previous events have been accurately completed and to confirm that their choice to continue cycling or to exit is appropriate. A variety of inhibitory mechanisms arrest cells at key checkpoints to enable repair of replication mistakes, DNA damage or chromosome alignment errors, or, if the mistakes are irreparable, to allow induction of apoptosis (reviewed by Malumbres and Barbacid, 2009).

Retinal neurogenesis continues throughout life in fish and amphibians as their eyes continue to grow. In these animals, after the onset of neurogenesis in the central retina, neurons are added to the eye from a population of proliferating progenitors located at the retinal periphery in a stem cell niche termed the ciliary marginal zone (CMZ). The developmental history of embryonic retinal neurons is spatially recapitulated in the CMZ, such that the youngest and least determined cells are found nearest the periphery, the proliferative retinoblasts in the middle, and the differentiating cells closest to the central retina (reviewed by Amato et al., 2004; Perron et al., 1998).

We understand very little about what triggers retinoblasts to transition from a programme of proliferative self-renewal to one of terminal cell cycle exit and differentiation (Agathocleous and Harris, 2009; Cayouette et al., 2006). To date, it has proven difficult to address this issue because directly modifying cell cycle progression often leads to pleiotropic phenotypes involving apoptosis (e.g. Ma et al., 1998; Ryu and Driever, 2006). However, evidence from a variety of species and developmental paradigms implicates both intrinsic and extrinsic signals in the regulation of the cell cycle behaviour and fate of proliferative RPCs (Agathocleous and Harris, 2009).

In this study, we use the zebrafish *flotte lotte* (*flo*) mutant to explore the mechanisms that regulate terminal cell cycle exit of RPCs in the CMZ. The *flo* locus encodes Elys (also known as Ahctf1) (Davuluri et al., 2008; de Jong-Curtain et al., 2009), a component of the Nup107-160 complex that localises to kinetochores during mitosis and participates in the reformation of functional nuclear pores immediately after mitosis (Rasala et al., 2006). Elys is required for cell cycle progression, playing key roles in kinetochore function (Mishra et al., 2010) and in the regulation of mitotic entrance and exit (Davuluri et al., 2008; Fernandez and Piano, 2006; Franz et al., 2007; Galy et al., 2006; Gillespie et al., 2007).

Our results reveal that extrinsic environmental signals, most likely from differentiating neurons, influence when and how RPCs stop proliferating and start differentiating as they emerge from the

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CMZ. When Elys function is compromised, RPCs cycle more slowly, taking longer than normal to progress from S to M phase, and often undergo apoptosis as a result of failed cell cycle progression. When programmed cell death is blocked, aberrantly cycling RPCs accumulate, primarily in the CMZ, but fail to make the transition to differentiation. Surprisingly, this failure to differentiate is independent of functional Elys protein as *flo* mutant cells survive and differentiate into neurons when provided with a wild-type retinal environment. Therefore, the wild-type retinal environment promotes the differentiation, rather than apoptosis, of aberrantly cycling neural progenitors. This phenomenon, which we term environmentally driven differentiation, is likely to act alongside apoptosis as a mechanism to prevent the overproliferation of aberrantly cycling cells. In addition, our data suggest a simple feedback mechanism to explain the spatial organisation of cycling progenitors, committed precursors and differentiating neurons in the CMZ.

MATERIALS AND METHODS

Zebrafish lines and genotyping

AB and *tupl* wild-type and *flotte lotte* (*flo*^{ti262c}) zebrafish (*Danio rerio*) strains were bred and maintained according to standard procedures (Westerfield, 2000). Embryos were genotyped as described (de Jong-Curtain et al., 2009), except that the digestion products were resolved on a 2.5% Metasieve agarose (Flowgen) gel in Tris-borate-EDTA buffer (Sigma).

Histology

Whole-mount immunolabelling and in situ hybridisation procedures were performed as previously described (Xu et al., 1994). For antibody staining of cryosections, embryos were first protected by sequential incubation in 15% then 30% sucrose in phosphate-buffered saline supplemented with 0.5% Triton X-100 (PBST) for 12-16 hours at 4°C, then embedded in OCT, stored at –80°C, and sectioned at 16-20 μm using a Leica cryostat. The following antisera were used: β-catenin (Sigma; 1:500); γ-tubulin (Sigma; 1:200); zn5 [Zebrafish International Resource Center (ZIRC); 1:250]; GFP (Abcam or AMS Biotechnology; 1:1000); glutamine synthetase (Chemicon International; 1:500); zpr1 (ZIRC; 1:250); BrdU (Roche; 1:300); PH3 (Upstate Biochemical; 1:500); mab414 (Abcam; 1:100); calretinin (Abcam; 1:100).

To prepare in situ hybridisation probes, linear DNA templates were prepared by restriction digestion of plasmids for *ath5*, *ccnd1* and *cdkn1c* or PCR for *elys* and *mz98*. The first 876 nucleotides of *elys* cDNA were PCR amplified from 24 hpf whole-embryo cDNA with oligonucleotides #87 (5′-GGATTTGAAGTTTCTCTTCGCTACTGC-3′) and #88 (5′-GGATCCATTAACCCTCACTAAAGGGAAGGTTGGTGCGCTGGATG-TACTGA-3′) and used as a template for T3 RNA polymerase (site encoded in #88). A fragment corresponding to the last 528 bp of the *mz98* (sb:cb491 – Zebrafish Information Network) gene was PCR amplified from plasmid 913 as described (Pujic et al., 2006). Antisense RNAs were synthesised using the appropriate polymerase (Promega) and digoxigenin-labelled nucleotides (Roche) following the manufacturer's instructions. To detect in situ hybridisation, embryos were incubated with NBT/BCIP or FastRed (both from Roche) according to the manufacturer's instructions.

TUNEL labelling to detect apoptosis was performed using the ApopTag Kit (Chemicon International). Manufacturer's instructions were followed for the labelling reaction, then embryos were washed in PBST, blocked and developed as described in the whole-mount in situ hybridisation protocol (Xu et al., 1994).

For histology sections, embryos were embedded in JB-4 polymer (Polysciences) following the manufacturer's instructions and sectioned with a Leica microtome. Sections were stained with Methylene Blue alone or combined with Fuchsin (Schmitt and Dowling, 1999).

Microinjections

To inhibit p53 function, one-cell stage embryos resulting from *flo*$^{+/–}$ incrosses were injected with 1 nl of 1 mM p53MO (5′-GCGCCAT-TGCTTTGCAAGAATTG-3′; GeneTools). For each individual p53MO experiment, embryos from the same clutch were used as experimental subjects and controls. Capped GFP mRNAs for injection were prepared using the mMessage mMachine RNA Synthesis Kit (Ambion) according to the manufacturer's instructions and injected into one-cell stage embryos.

Cell proliferation assays

BrdU incorporation was performed on ~52-54 hpf embryos embedded in 1% low-melting-point agarose. One nanolitre of 10 mM BrdU (Sigma; diluted in fish water from 50 mM stock made with DMSO) was injected into the heart of zebrafish embryos, some of which had been injected with p53MO at the one-cell stage. Embryos were then removed from the agarose and incubated at 28.5°C until 3 dpf, sorted into *flo* and wild-type populations and fixed. To document cell cycle progression, 1 nl of 10 mM BrdU was injected twice into the heart of each embryo: once at ~52 hpf and then 2 hours later. Embryos were fixed ~1.5 or 24 hours after the second injection. Tails were genotyped, and then *flo* and wild-type heads embedded for cryosectioning.

Cell transplantation experiments

Embryos resulting from *flo*$^{+/–}$ incrosses were injected with GFP mRNA (40-50 pg per embryo) at the one-cell stage. Thirty to 40 GFP^{+} cells were transplanted from the apical region of mid-blastula donor embryos into early-gastrula-staged hosts in the region fated to become the eye (Cavodeassi et al., 2005; Woo and Fraser, 1995). Donor embryos were either genotyped or allowed to grow until 3 dpf to distinguish mutants from siblings. Host embryos were fixed at the stages indicated in the figures, genotyped if necessary, then prepared for cryosectioning and antibody staining.

Imaging and data processing

Embryos subjected to whole-mount in situ hybridisation were cleared in serial incubations of glycerol (25, 50, 75 and 95%), the eyes dissected and placed in a drop of glycerol, cover-slipped, and imaged with a 40× (0.8 NA) water-immersion lens using a Nikon E1000 microscope connected to a digital camera (Jenoptik) operated by Openlab (Improvision) software. JB-4 sections were covered with DPX mounting medium (BDH), cover-slipped and imaged as above.

Cryosections were examined by confocal fluorescence microscopy (Leica Systems) using a 40× (1.2 NA) or 63× (1.4 NA) oil-immersion lens. Whole-mount immunostained embryos were imaged using a 40× (0.8 NA) water-immersion lens. Counting of PH3^{+} and BrdU^{+} PH3^{+} cells for Figs 4 and 5 was performed blind. Numbers and simple arithmetic were managed on an Excel (Microsoft) spreadsheet, statistical calculations performed and the output graphed using Prism4 (GraphPad). All confocal images were processed using Volocity (Improvision) software; single slices were exported as tiffs; all figures were composed with Photoshop and Illustrator (CS3, Adobe).

RESULTS

Apoptosis at the interface between the CMZ and central retina is responsible for decreased eye growth in the *flo* mutant

flo$^{ti262c/ti262c}$ embryos are initially indistinguishable from wild-type siblings, but their eyes exhibit growth defects that are evident by 2-3 days post-fertilisation (dpf) (Fig. 1A,B) (Wallace et al., 2005). Other than small eyes, mutants show no overt phenotypes until later stages, when defects in intestinal development lead to larval death (Davuluri et al., 2008; de Jong-Curtain et al., 2009; Wallace et al., 2005). In histological sections, the phenotype of *flo* mutants could first be identified at 2 dpf by the presence of occasional acellular holes in the retina (Fig. 1C,D). At later stages, retinal layers were evident in the *flo* eye (Fig. 1E,F), but γ-tubulin labelling of apically positioned centrosomes at 3 dpf showed that lamination is irregular, with some misaligned cells and incompletely formed outer and inner plexiform layers (Fig. 1G,H). Consistent with the identity of Elys as a component of the Nup107-

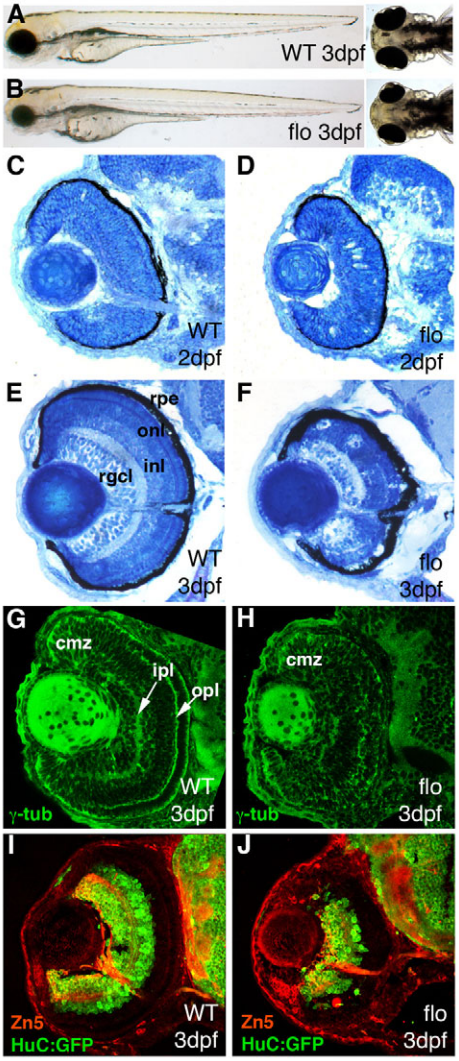

Fig. 1. *flo* mutants have small eyes. (**A**,**B**) Lateral (left) and dorsal (right) views of live wild-type (A) and *flo* (B) zebrafish embryos at 3 dpf. (**C-F**) Frontal transverse sections through wild-type (C,E) and *flo* (D,F) embryos at 2 dpf (C,D) and 3 dpf (E,F). (**G-J**) Frontal cryosections of wild-type (G,I) and *flo* (H,J) eyes immunostained to detect markers for polarity (G,H, γ-tubulin, green) and differentiation [I,J; zn5 (Alcama), RGCs, red; HuC::GFP (Elavl3), RGCs and amacrine cells, green]. rgcl, retinal ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigmented epithelium; cmz, ciliary marginal zone; ipl, inner plexiform layer; opl, outer plexiform layer.

160 nuclear pore complex, which is also required for kinetochore function, nuclear pores are disrupted and their components aggregated in retinal cells (Davuluri et al., 2008; de Jong-Curtain et al., 2009) (see Fig. S1 in the supplementary material).

Differentiated neurons and glia of all major classes were found in the central retina of *flo* mutants, albeit in reduced numbers (Fig. 1I,J; see Fig. S2 in the supplementary material; data not shown), with the numbers of rod and cone photoreceptors severely reduced. Only a few scattered rod photoreceptors differentiated and, with the exception of the earliest born cluster of cells in the ventronasal retina, cone photoreceptors were absent (see Fig. S2 in the supplementary material). The reduction of neurons in the central retina of *flo* mutants is presaged by slight defects in the early waves of expression of genes such as *ath5* (*atoh7* – Zebrafish Information Network) and

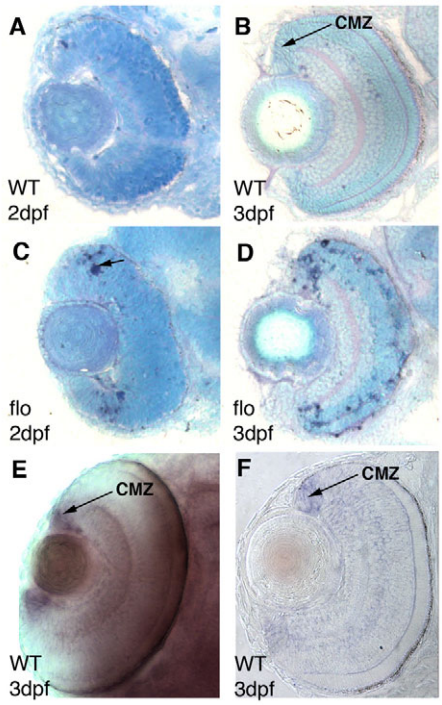

Fig. 2. *elys* is expressed in the CMZ and central CMZ cells apoptose in *flo* mutants. (**A-D**) Frontal plastic sections of wild-type (A,B) and *flo* (C,D) zebrafish eyes at 2 dpf (A,C) and 3 dpf (B,D). TUNEL$^+$ apoptotic cells are present at the central limit of the CMZ in the *flo* retina (dark blue nuclei, arrow in C). (**E**,**F**) Whole-mount eye (E) and frontal plastic section (F) showing that at 3 dpf, *elys* expression is restricted to the CMZ.

shh that sweep across the central retina accompanying neurogenesis (Masai et al., 2000; Neumann and Nuesslein-Volhard, 2000). For example, both *ath5* and *shh*:GFP expression was initiated normally in the ventronasal retina of *flo* mutants, but subsequently spread more slowly and less widely across the central retina (see Fig. S3 in the supplementary material).

Irrespective of these early defects, *flo* eye size was only noticeably reduced from ~2.5 dpf, a stage by which central retinal neurogenesis is largely complete and new neurons are being added from the CMZ; therefore, the small eye phenotype of *flo* mutants is consistent with an absence of retinal growth from the CMZ. Although a CMZ was evident in *flo* eyes (Fig. 1I,J; see Fig. 3A,B), we suspected that cells emerging from this stem cell niche could be defective in proliferation and/or survival. In support of this, by 2 dpf, apoptotic cells were localised to a region adjacent to, or overlapping with, the CMZ of *flo* mutants (Fig. 2C). By 3 dpf, the number of dying cells had increased from a few cells near the retinal periphery to many cells located mainly at the central edge of the CMZ and apical surface of the retina (Fig. 2D; data not shown). At these stages, cell death was rarely detected in the wild-type retina (Fig. 2A,B). These results suggest that the small eyes in *flo* mutants are primarily due to a failure of viable neurons to emerge from the CMZ.

Elys is required for the transition from retinal stem cell to differentiating neuron

The apoptosis of cells at the interface between domains of proliferation and differentiation in *flo* retinae suggested that defects in cell cycling or timely cell cycle exit might underlie the *flo* retinal

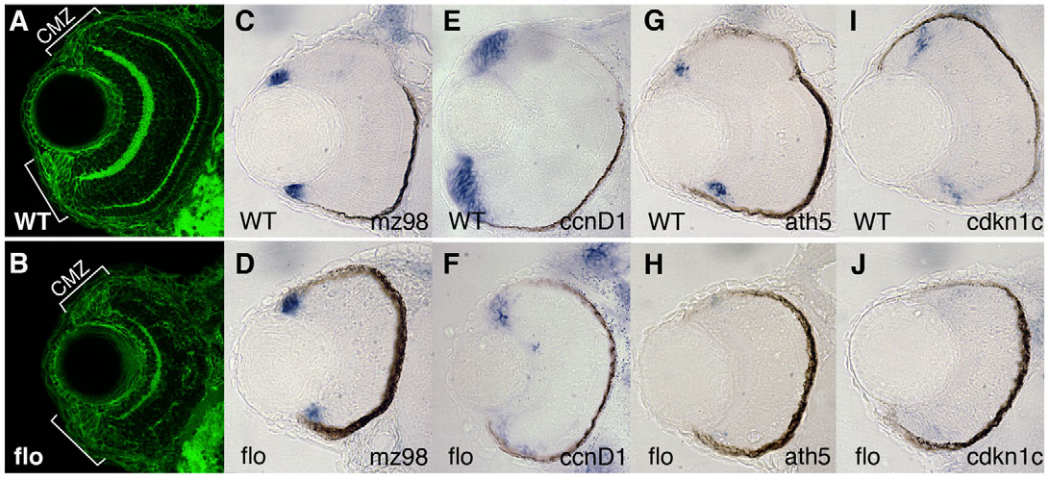

Fig. 3. CMZ cells in *flo* embryos show reduced expression of cycling and differentiation markers. (**A**,**B**) CMZ cells are present in *flo* retinas. Frontal transverse cryosections of zebrafish retinae stained for β-catenin (green). Brackets denote CMZ boundaries. (**C-J**) Gene expression in subpopulations of cells within the CMZ. *mz98*, a marker of the peripheral, putative stem cell compartment, is expressed in wild-type siblings (C) and *flo* (D) embryos. *ccnd1* is highly expressed in the wild-type proliferating progenitors of the CMZ (E) but is reduced in *flo* eyes (F). *ath5* (G,H) and *cdkn1c* (I,J) are expressed in cells in central CMZ concomitant with the final division cycle in the wild-type CMZ (G,I), but are absent in the *flo* retina (H,J).

phenotype. *flo* mutants carry a null mutation in *elys* (Davuluri et al., 2008; de Jong-Curtain et al., 2009), which encodes a component of the Nup107-160 complex that functions prior to, and during, mitosis (Davuluri et al., 2008; Gillespie et al., 2007; Rasala et al., 2006). Confirming previous observations, we found that *elys* is maternally inherited and initially expressed ubiquitously, before becoming progressively restricted to domains that contain highly proliferative cells, particularly the intestinal epithelium and CMZ of the eyes (Fig. 2E,F; see Fig. S4 in the supplementary material) (Davuluri et al., 2008; de Jong-Curtain et al., 2009).

What is the consequence of the lack of Elys in the CMZ cells? To determine whether *elys* is required in specific regions of this germinal zone, we examined the expression of markers for subdomains of the CMZ in *flo* mutants. The most peripheral *mz98*-expressing region of the CMZ (Pujic et al., 2006) appeared to be properly specified and of approximately the same size and shape in wild-type and mutant eyes (Fig. 3C,D). This is consistent with the observation that apoptosis is absent from this peripheral, presumptive stem cell-like domain of the CMZ.

In contrast to the peripheral-most compartment of the CMZ, cycling progenitors and committed neuronal precursors in the central regions of the CMZ showed striking differences in gene expression between *flo* and wild-type eyes. Expression of *ccnd1*, which encodes a G1 cyclin, was considerably reduced in *flo* eyes (Fig. 3E,F), and markers for cells transitioning from proliferation to differentiation were absent from the *flo* CMZ. Specifically, *ath5*, which encodes a bHLH transcription factor that is expressed in a subset of RPCs prior to their final division (Masai et al., 2000; Poggi et al., 2005), was undetectable in *flo* CMZs (Fig. 3G,H). Likewise, *cdkn1c*, which encodes the $p57^{cip/kip}$ cyclin-dependent kinase inhibitor (CKI) that is required for cell cycle exit and differentiation of many retinal neurons (Ohnuma et al., 1999; Shkumatava and Neumann, 2005), was absent from the central-most limit of the *flo* CMZ (Fig. 3I,J). Together, these data illustrate that Elys function is important for the normal behaviour of cycling progenitors and/or committed neuronal precursors prior to the final divisions that generate neurons.

flo RPCs fail to enter their terminal neurogenic divisions

Is the *elys* mutant phenotype made manifest in self-renewing progenitors or in committed neuronal precursors? Because cell death obscures the final phenotype of cells in the *flo* CMZ, we inhibited apoptosis and assessed markers for cycling progenitors (*ccnd1*) and committed neuronal precursors (*ath5* and *cdkn1c*). To block apoptosis of the CMZ cells, we injected a morpholino (MO) against the tumour suppressor *p53* (*tp53* – Zebrafish Information Network), which is activated by a variety of cell-stress situations, including disruptions in DNA replication and cell cycle progression (Bill et al., 2009). Injection of p53MO prevents all detectable apoptosis in *flo* retinae (data not shown) (see also Davuluri et al., 2008).

Blocking p53-dependent apoptosis robustly restored *ccnd1* expression in the CMZ (Fig. 4A-C; see Fig. S5 in the supplementary material), but failed to rescue differentiating cells. Neither *ath5* (Fig. 4D-F) nor *cdkn1c* (Fig. 4G-I) was expressed at 3 dpf in the *flo* retina, irrespective of the presence of p53MO. Thus, *flo* mutant cells fail to progress from cycling progenitor to differentiating neuronal precursor, and it is possible that the apoptotic cells at the central limit of the CMZ (Fig. 2C,D) die because they are unable to make this transition. These data suggest that the critical defect in *flo* mutant cells is an inability to advance from a proliferating to a differentiating state.

To determine why *flo* mutant cells are compromised in this transition, we tested whether Elys is required for cell cycle progression. Markers for the S and M phases [BrdU and phosphorylated histone H3 (PH3), respectively] showed that *flo* cells proliferate aberrantly, with mutants containing elevated levels of proliferating and dividing cells, some of which were positioned outside of the CMZ (Fig. 5D-F). Likewise, when p53-mediated apoptosis was blocked, the number of $PH3^+$ $BrdU^+$ cells remained elevated, with nearly twice as many $PH3^+$ cells in *flo* eyes as compared with their sibling counterparts (Fig. 4J-L; see Fig. S5 in the supplementary material). The majority of $PH3^+$ cells in such retinae were confined to the CMZ (Fig. 4M,N; see Fig. S5 in the supplementary material).

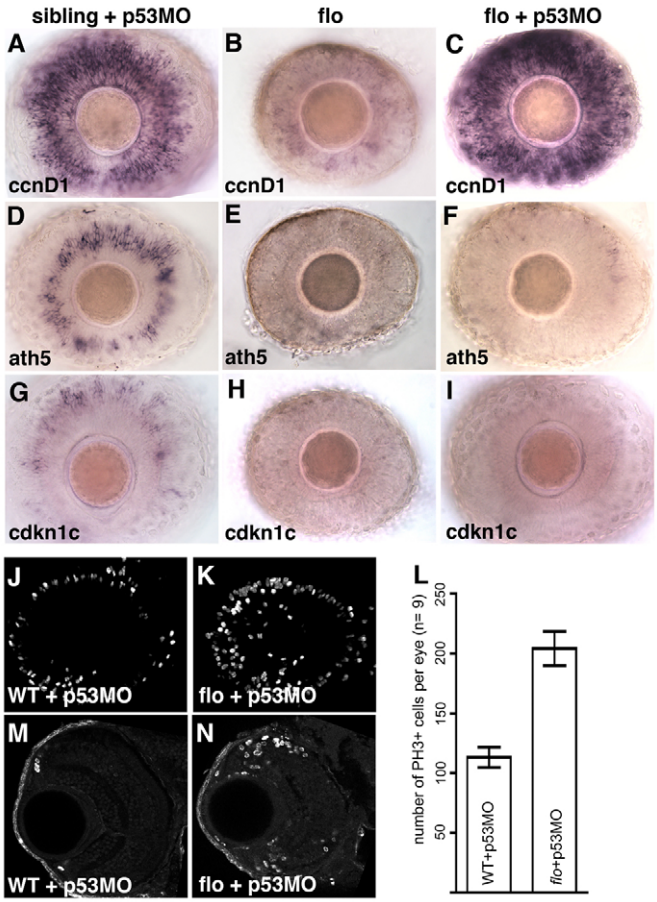

Fig. 4. *flo* CMZ cells fail to transition from proliferation to differentiation, even in the absence of apoptosis. (**A-I**) Intact retinae from 3-dpf wild-type (A,D,G) and *flo* (B,E,H) zebrafish embryos and *flo* embryos injected with p53MO (C,F,I), labelled to show expression of the genes indicated bottom left. *flo* CMZ cells robustly express the proliferative marker *ccnd1* (C) but not markers of cell cycle exit (F,I) when p53-mediated apoptosis is inhibited. (**J**,**K**,**M**,**N**) Confocal projections of laterally viewed wild-type + p53MO (J) and *flo* + p53MO (K) whole retinae and coronal sections of wild-type + p53MO (M) and *flo* + p53MO (N) retinae, all stained with anti-phosphohistone H3 (PH3). *flo* + p53MO eyes exhibit increased proliferation in/near the CMZ. (**L**) *flo* + p53MO embryos contain significantly more mitotic cells than wild-type + p53MO siblings (P<0.0001, Student's t-test). The average number of mitotic retinal cells per eye (n=9 eyes from 9 different fish, examples shown in J,K) was calculated and graphed with error bars (95% confidence limits).

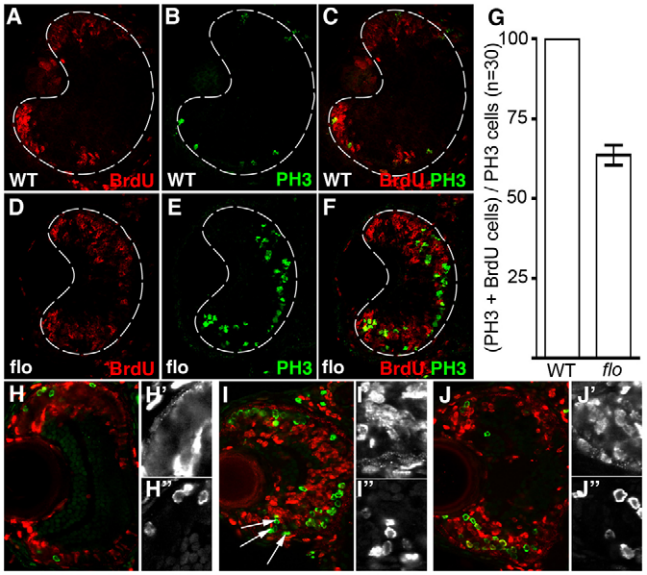

Fig. 5. *flo* retinal cells fail to progress efficiently through the cell cycle. (**A-F**,**H-J″**) Frontal transverse cryosections of zebrafish retina stained for markers of DNA replication (BrdU, red) and M phase (PH3, green). Wild-type (A-C) and *flo* + p53MO (D-F) embryos were injected with BrdU at 53 hpf and again at 55 hpf, then fixed ~75 minutes later. Single-channel magnifications of BrdU (H′,I′,J′) and PH3 (H″,I″,J″) in the CMZ are shown. (**G**) The average number of PH3$^+$ cells that are also BrdU$^+$ per section (n=30 eyes) was calculated and graphed with error bars (99% confidence limits). All of the PH3$^+$ cells in wild-type siblings were also BrdU$^+$. Significantly fewer cells (27.5%) were double labelled in *flo* embryos (P<0.0001, Student's t-test). As above, wild-type (H), *flo* (I) and *flo* + p53MO (J) embryos were injected with BrdU but then fixed 24 hours later.

flo retinal cells cycle more slowly than wild-type cells

Increased numbers of cells positive for S-phase and M-phase markers suggests that cycling progenitors in *flo* retinae either hyperproliferate or cycle more slowly and consequently do not exit the cell cycle at the developmentally appropriate time. To distinguish between these two possibilities, we measured the time required for cycling progenitors in *flo* retinae to advance from S to M phase. We carried out the majority of these experiments in *flo* + p53MO embryos to avoid the difficulties associated with quantitation in the presence of dead or dying cells. First, all S-phase cells were labelled with BrdU for a period of 3.5 hours prior to fixation (see Materials and methods), and then the BrdU labelling of all M-phase (PH3$^+$) cells was assessed. At ~52 hours post-fertilisation (hpf), wild-type cycling progenitors in retinae require no more than 2.5 hours to progress from S phase to M phase (data not shown), and so this labelling regime results in 100% of PH3$^+$ cells in wild-type retinae being BrdU$^+$ (Fig. 5A-C,G). In striking contrast, nearly 30% of the PH3$^+$ cycling progenitors in the *flo* + p53MO eyes were not labelled by BrdU (Fig. 5D-G). These cells either spent the entire time course of the experiment at the beginning of M phase or they took over 3.5 hours to progress through G2. By extending the period following BrdU injection to 24 hours, we found that the population of *flo* PH3$^+$ BrdU$^-$ cells (Fig. 5D-F) eventually progressed to a PH3$^+$ BrdU$^+$ state (Fig. 5H-J). Consistent with the idea that many *flo* RPCs eventually arrest at the G2/M checkpoint in a p53-dependent manner, significant numbers of PH3$^+$ BrdU$^-$ cells were still observed in *flo* retinae (without p53MO) even when the period following BrdU injection was extended to 24 hours (Fig. 5I).

A wild-type environment drives the aberrantly cycling *flo* cells to differentiation

Thus far, the data presented support the idea that Elys is required for RPCs to transition from cycling progenitor to committed neuronal precursor. To examine whether *flo* cells are intrinsically compromised in their ability to transition from proliferating progenitor to differentiating neuron because of their cell cycle defects, we created mosaic retinae composed of wild-type and *flo* cells. As a member of the Nup107-160 complex, Elys is required to form nuclear pores in retinal cells (Davuluri et al., 2008; de

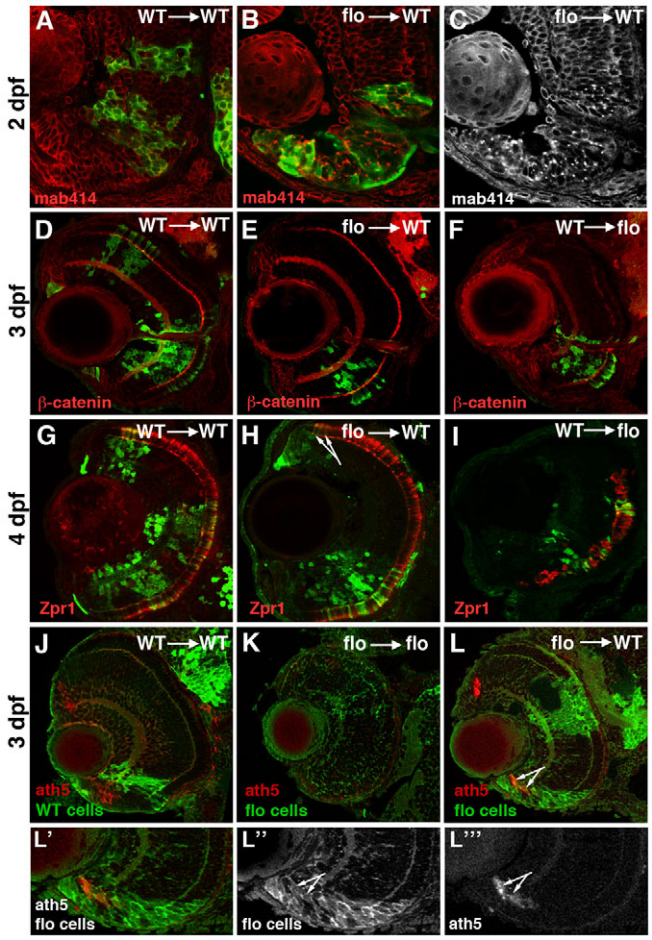

Fig. 6. *flo* cells survive and differentiate in a wild-type environment. (**A-L‴**) Frontal sections of zebrafish eyes at various ages labelled with antibodies as indicated bottom left; mab414 recognises the Nup107-160 subcomplex of nuclear pores, β-catenin highlights cell membranes and plexiform layers; zpr1 recognises cone photoreceptors. GFP-labelled cells from either wild-type (A,D,F,G,I,J) or *flo* (B,C,E,H,K,L) donor embryos were transplanted into wild-type (A-E,G,H,J,L) or *flo* (F,I,K) hosts. The arrows in H and L point to *flo* mutant photoreceptors and *ath5*-expressing cells emerging from the CMZ in wild-type eyes, respectively. See text for the specific numbers of mosaic eyes examined for each condition. *ath5* expression (or lack thereof) in wild-type (J) and *flo* (K) retinae is shown for reference. (L′,L″,L‴) Single-channel magnifications from L illustrate *flo* CMZ cells in a wild-type environment.

Jong-Curtain et al., 2009) (see Fig. S2 in the supplementary material). Not surprisingly, nuclear pore labelling remained disrupted in *flo* mutant cells in a wild-type environment and the presence of mutant cells had no effect on nuclear pore distribution in adjacent wild-type cells (Fig. 6A-C). Thus, the environment has no effect on the role of Elys in constructing nuclear pores.

In striking contrast to the lack of a non-cell-autonomous influence of the environment on nuclear pore formation, wild-type cells had a substantial effect upon the ability of *flo* mutant cells to differentiate as retinal neurons. *flo* cells always integrated completely into the differentiated neuronal layers of wild-type retina (n=18 eyes) (Fig. 6E), and were able to differentiate into all neuronal types, including cone photoreceptors (n=12 eyes) (Fig. 6H), which were almost completely absent in *flo* mutants. These results are consistent with two possibilities: either the *flo* retinal environment abnormally promotes proliferation or the wild-type retina promotes differentiation. To distinguish between these alternative scenarios, we transplanted wild-type cells into *flo* retinae. Consistent with the wild-type environment promoting RPC differentiation, small clusters of wild-type cells transplanted into *flo* retinae restored lamination and differentiation to the surrounding mutant cells (n=18 eyes, Fig. 6F; n=13 eyes, Fig. 6I; compare Fig. 6I with Fig. S2 in the supplementary material to see the comprehensive rescue of cone photoreceptors and Fig. 6F with Fig. 3B for lamination). Together, these results indicate that a wild-type neuronal environment provides signals that promote the differentiation and organisation of *flo* cells in the central retina. However, as shown above (Fig. 1; see Figs S1 and S2 in the supplementary material), some *flo* central retinal cells undergo differentiation in their mutant environment, albeit at a reduced rate. Therefore, the rescue of *flo* cell differentiation in the central retina could be a consequence of a prevention of apoptosis of postmitotic cells by wild-type signals and/or a restoration of the ability of *flo* cells to transition from proliferation to differentiation.

To determine whether the environment can indeed drive the differentiation of aberrantly cycling RPCs, we asked if *flo* progenitors in the CMZ are able to transition from cycling progenitor to committed precursor in the presence of wild-type retinal neurons. We transplanted *flo* cells into wild-type eyes and selected the rare cases in which they were incorporated into the CMZ. Under mutant conditions, *flo* CMZ cells did not express *ath5*, a gene that is associated with progression from cycling progenitor to differentiating neuron, even when apoptosis was blocked (Fig. 3G,H, Fig. 4E,F, Fig. 6K). However, when *flo* cells were integrated into the CMZ of a wild-type retina, they behaved like their wild-type counterparts and expressed *ath5* as they progressed from the peripheral to the central retina (n=4 eyes) (example in Fig. 6L). *flo* cells emerging from the CMZ subsequently differentiated as neurons (Fig. 6H,L, arrows). Further supporting the idea that the wild-type environment drives *flo* cells out of the cell cycle so that they can differentiate, we found that by 2.5 dpf, *flo* cells located in a wild-type retina rarely display PH3 staining (n=12 eyes) (see Fig. S6 in the supplementary material). By 3 dpf, none of the *flo* cells in wild-type eyes that we examined exhibited PH3 staining (n=12 eyes) (see Fig. S6 in the supplementary material), whereas *flo* retinae contained many $PH3^+$ cells at this stage (n>30 eyes) (see Fig. S5 in the supplementary material).

Taken together, all of our transplantation studies indicate that a wild-type environment can promote cell cycle exit and differentiation of *flo* mutant cells, most notably the abnormally cycling *flo* RPCs that emerge from the CMZ stem cell niche. These extrinsic signals override the apoptotic programme and force aberrantly cycling neuronal precursors to differentiate without restoring nuclear pores.

DISCUSSION

In this study, we examined the proliferation and differentiation defects of the zebrafish *flo* retina and gained new insight into the transition from proliferating progenitor to differentiating neuron, a process that regulates eye size and growth by controlling the ratio of dividing cells to neurons. Our results suggest that the *flo* gene product, Elys, is dispensable for maintaining a stem cell-like state, but is important for the transition from proliferation to differentiation. *flo* retinal cells cycle more slowly and eventually undergo apoptosis (this study) (Davuluri et al., 2008). When p53-

mediated cell death is inhibited, *flo* cells continue to cycle but are unable to progress from cycling progenitor to differentiating neuron. Unexpectedly, a wild-type retinal environment rescues the ability of *flo* cells to transition from proliferation to differentiation. Notably, this rescue is most pronounced in the CMZ, highlighting the importance of extrinsic signals in regulating the progression from proliferating progenitor to differentiating neuron, and indicating that the local environment can regulate the proliferation of aberrantly cycling RPCs by promoting neuronal differentiation. Together, our data indicate that the transition from proliferation to differentiation is tightly controlled by a combination of cell-intrinsic and cell-extrinsic mechanisms.

Extrinsic signals promote cell cycle exit and differentiation of proliferating retinal progenitors

We propose that the local environment of the differentiating/differentiated neural retina promotes terminal cell cycle exit and differentiation of the rapidly cycling progenitors that emerge from the CMZ (Fig. 7). Our data show that when cell cycle progression is disrupted in RPCs (as in the case in *flo* mutants), the wild-type environment can override proliferation defects that would otherwise lead to apoptosis and instead pushes cells towards cell cycle exit and differentiation.

Perhaps the simplest model to explain how RPCs could be stimulated to exit the cell cycle and differentiate is one in which the distal-most CMZ provides environmental cues that maintain stem and progenitor cells by promoting proliferation. As cells in the niche divide, their daughters are displaced towards the central retina, away from the source of the proliferation signal, and consequently undergo differentiation. Similar models have been proposed to explain certain cell behaviours in various stem cell niches (e.g. Li and Clevers, 2010; Morrison and Spradling, 2008). However, our data, as well as observations from other neuronal stem cell niches (reviewed by Kaslin et al., 2008), suggest an alternative model. We propose the presence of at least two sets of environmental signals: one that promotes the proliferation of stem/progenitor cells and one that limits the proliferation of rapidly cycling precursors by promoting differentiation. In the retina, these pro-differentiation signals are most likely provided by the differentiating (*ath5*- and *cdkn1*-expressing) cells and/or newly differentiated neurons and glia adjacent to the central edge of the CMZ (most of which are missing in *flo* mutant eyes). In support of this more complex model for retinal stem cell behaviour, we find that *flo* CMZ cells retain the ability to proliferate, but fail to progress from proliferation to differentiation, especially when checkpoint-induced p53-mediated apoptosis is blocked. These data suggest that proliferation signals are intact in the *flo* CMZ, but that cell cycle exit/differentiation signals are compromised such that *flo* cells in the CMZ are unable to undergo terminal cell cycle exit and differentiation. This is further supported by our transplantation studies in which *flo* cell differentiation is rescued in a non-cell-autonomous manner, such that the wild-type environment promotes survival and differentiation.

Our model also provides an explanation for the spatial organisation and limited size of the CMZ stem cell zone (Fig. 7). Relatively quiescent stem cells are believed to reside in the peripheral-most CMZ, and as cells emerge centrally from this zone their proliferation rate increases (Agathocleous and Harris, 2009; Ohnuma and Harris, 2003). This has the inevitable consequence that the more centrally positioned of the proliferative cells will come into proximity with the differentiating retina. We suggest that this environment promotes cell cycle exit and differentiation of cycling RPCs located in the CMZ. Consequently, whatever the intrinsic proliferative capacity of the RPC might be, the movement of the cell out of the stem cell niche will inevitably lead to its differentiation. Thus, in our model, the feedback from the mature retinal environment ensures that the proliferative zone of the CMZ is self-limiting. This process, which we term 'environmentally driven differentiation', also ensures that any cell that escapes normal intrinsic cell cycle control is driven towards differentiation. We suggest that this mechanism acts redundantly with apoptosis as a means of halting the proliferation of cells with cell cycle defects.

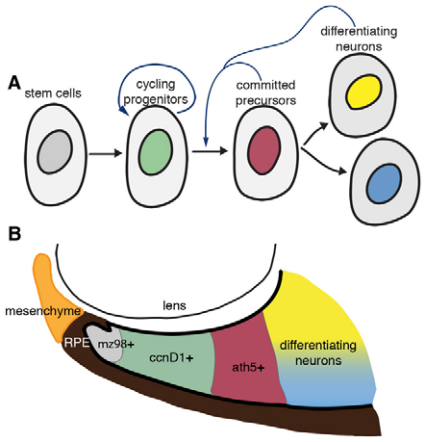

Fig. 7. A model suggesting that the mature zebrafish retina drives cell cycle exit and differentiation of CMZ cells. (**A**) Cycling progenitors receive extrinsic feedback from surrounding cells to transition from proliferation to differentiation. Retinal stem cells at the periphery of the eye give rise to proliferating progenitors, the behaviour of which is regulated by intrinsic (cell cycle) and extrinsic (environmental) cues that determine the probability that these cells will stop cycling and instead differentiate. Although *flo* cells (not shown) have intrinsically compromised cell cycle kinetics, they are able to respond to wild-type extrinsic signals that promote their transition from proliferation to differentiation. (**B**) The spatial organisation of the CMZ. Putative stem cells (*mz98*-expressing), cycling progenitors (*ccnd1*-expressing), committed precursors (*ath5*-expressing) and differentiating neurons are sequentially arranged, such that dividing cells inevitably come into contact with differentiating neurons as they move centrally from the CMZ. We suggest these differentiating cells encourage the dividing cells to enter their final cell cycle and differentiate (see A).

We show that environmentally driven differentiation occurs when *flo* RPCs are exposed to a wild-type environment, but does a similar mechanism occur during normal development and growth? Limited investigations of chromosomal abnormalities of differentiated neurons in vivo reveal that a small, but significant, population of mature neurons has a history of cell cycle defects. In zebrafish, nearly 2.5% of all neurons in the adult brain show deficits in chromosomal number that are consistent with the survival and differentiation of cells with DNA damage and/or mitotic defects (Zupanc et al., 2009). Strikingly, similar numbers of aneuploid neurons are also found in adult mouse and human brains (Kingsbury et al., 2005; Rehen et al., 2001; Rehen et al., 2005). Together with these data, our results raise the possibility that environmentally driven differentiation can operate in the central nervous system to limit proliferation and promote differentiation of chromosomally compromised cells under normal circumstances.

How might the environment drive differentiation?

Recent mathematical modelling suggests that the environment around stem cells/neural progenitors can feedback onto cycling cells to control their behaviour and fate by adjusting cell cycle kinetics and modifying the probability that the progenitor chooses cell cycle exit over proliferation (Lander et al., 2009). A tenet of this and related models is that differentiating neurons produce signals that limit the production of neurons of the same class – a classical negative-feedback loop. We suggest that wild-type extrinsic signals promote *flo* cell differentiation by reducing the probability that they will continue to divide through induction of CKIs and other cell cycle exit genes. Thus, our data are consistent with models employing environmental feedback to control progenitor cell behaviour in cell lineages and stem cell niches (e.g. Lander et al., 2009; Lo et al., 2009).

Multiple extrinsic feedback loops provide stability in the regulation of proliferating progenitors (Lander et al., 2009; Lo et al., 2009). We suspect that the intrinsic properties of *flo* cells – DNA replication errors (Davuluri et al., 2008), slowed cell cycle progression and inefficient neurogenesis (this study) – perturb the *flo* retina such that the normal feedback signals governing proliferation and differentiation are imbalanced. The complete absence of differentiating neurons emerging from the CMZ of *flo* mutants is likely to alter many local environmental cues and, in consequence, generate an unstable environment that cannot robustly control cell behaviour. Although we have yet to identify the signals responsible for environmentally driven differentiation, there are a few candidates that might work together in the processes that we have described.

The TGFβ family member Gdf11 promotes differentiation by inhibiting cell cycle progression through the induction of Cip/Kip CKIs in mouse olfactory epithelium (Kawauchi et al., 2004; Wu et al., 2003). In the mouse retina, Gdf11 may promote differentiation by a different mechanism: controlling the competence window of progenitors to produce RGCs by inducing the proneural gene *Ath5* (Kim et al., 2005). However, these two processes might actually be similar because Atonal proteins can act as tumour suppressors, promoting cell cycle exit and differentiation (Bossuyt et al., 2009a; Bossuyt et al., 2009b), with Ath5 also cooperating with Cip/Kip CKIs during retinal neurogenesis (Ohnuma et al., 2002).

Similar to TGFβ family members, Shh can influence cell fate choice by regulating proliferation (Agathocleous et al., 2007; Cayuso et al., 2006; Sakagami et al., 2009). In the vertebrate retina, Shh regulates the length of the G1 and G2 growth phases of RPCs (Locker et al., 2006; Sakagami et al., 2009), and these data raise the possibility that Hh does not actively promote cell cycle exit (Shkumatava and Neumann, 2005) but instead moderates the length of time RPCs spend cycling, thereby influencing the timing of progenitor cell cycle exit. *shh* is expressed by differentiating neurons in the retina (Pujic and Malicki, 2004; Shkumatava et al., 2004), and given that *flo* retinae contain fewer differentiating/differentiated neurons, they are likely to contain lower levels of Shh. This might contribute to the slowed cell cycling phenotype of *flo* RPCs. The spatial and temporal dynamics of Hh signalling in the CMZ have proved difficult to document and therefore it is unclear whether reduced Hh signalling contributes to the failure of *flo* CMZ cells to exit the cell cycle and whether Hh signalling normally contributes to the differentiation of such cells in wild-type retinae.

In a variety of stem cell niches, Hh signalling appears to be complemented by a combination of other signals to ensure the appropriate progression from proliferation to differentiation (reviewed by Brabletz et al., 2009; Crosnier et al., 2006). It seems likely, therefore, that the rescue of *flo* cells by a wild-type environment is due to the provision of a cocktail of signals secreted by the surrounding wild-type cells. In the normal CMZ, this combination of extrinsic signals modulates retinal stem cell behaviour to limit CMZ size and produce the appropriate numbers and types of neurons as the eye grows throughout life.

Elys and differences between central and peripheral retinal neurogenesis

Retinal neurogenesis occurs in two distinct phases in the fish eye: RPCs in the central retina differentiate to form the first neurons and glia, whereas RPCs in the CMZ generate neurons and glia during subsequent eye growth (Agathocleous and Harris, 2009). In the central retinae of *flo* embryos, some proliferating progenitors are able to generate differentiated neurons of all major classes, whereas in the CMZ markers for differentiating cells are completely absent. Why do *flo* central retina cells retain the ability to transition from proliferation to differentiation, albeit with reduced frequency? The most likely explanation is that maternally inherited Elys is sufficient to support the terminal mitosis of early-born neurons in *flo* retinae, but that all such protein is depleted by the stage when the CMZ is generating neurons. Alternatively, Elys might be differentially required depending on cell type. For example, in human cells, Elys and one of its binding partners, Nup96, are phosphorylated and expressed similarly to cell cycle checkpoint proteins only when cells are actively cycling (Chakraborty et al., 2008; Nousiainen et al., 2006; Olsen et al., 2006). In addition, Elys function appears to be essential in rapidly cycling cells (Davuluri et al., 2008).

Another possibility is that central retinal neurogenesis is subject to different mechanisms of regulation than the CMZ. For instance, signals from intact midline tissue and optic stalk initiate neurogenesis in the central retina (Kay et al., 2005; Martinez-Morales et al., 2005; Masai et al., 2000; Stenkamp et al., 2000), whereas such signals are likely to be irrelevant for CMZ neurogenesis. Moreover, the timescale of regulation of neurogenesis is very different between central retina and CMZ. In the central retina, a pool of RPCs rapidly undergoes a limited number of divisions in a short period of time, generating neurons and depleting the generative pool of RPCs (Hu and Easter, 1999; Li et al., 2000). In marked contrast, the peripheral retina must employ robust self-contained regulatory mechanisms that ensure that RPCs and eye growth are tightly controlled throughout life. We suggest that feedback mechanisms, such as environmentally driven differentiation, are, consequently, likely to be more important for the peripheral than central retina.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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