

## Co-ordinating retinal histogenesis: early cell cycle exit enhances early cell fate determination in the *Xenopus* retina

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### SUMMARY

The laminar arrays of distinct cell types in the vertebrate retina are built by a histogenic process in which cell fate is correlated with birth order. To explore this co-ordination mechanistically, we altered the relative timing of cell cycle exit in the developing *Xenopus* retina and asked whether this affected the activity of neural determinants. We found that *Xath5*, a bHLH proneural gene that promotes retinal ganglion cell (RGC) fate, (Kanekar, S., Perron, M., Dorsky, R., Harris, W. A., Jan, L. Y., Jan, Y. N. and Vetter, M. L. (1997) *Neuron* 19, 981-994), does not cause these cells to be born prematurely. To drive cells out of the cell cycle early, therefore, we misexpressed the cyclin kinase inhibitor, p27Xic1. We found that early cell cycle exit potentiates the ability of *Xath5* to promote RGC fate. Conversely, the cell

cycle activator, cyclin E1, which inhibits cell cycle exit, biases *Xath5*-expressing cells toward later neuronal fates. We found that Notch activation in this system caused cells to exit the cell cycle prematurely, and when it is misexpressed with *Xath5*, it also potentiates the induction of RGCs. The potentiation is counteracted by co-expression of cyclin E1. These results suggest a model of histogenesis in which the activity of factors that promote early cell cycle exit enhances the activity of factors that promote early cellular fates.

Key words: Neurogenesis, Gliogenesis, Cell cycle regulation, Notch, Proneural gene, *Xenopus laevis*, *Xath5*

### INTRODUCTION

In the central nervous system, neurons and glia often differentiate in a conserved histogenic order. In the *Xenopus* retina, for example, retinal ganglion cells (RGCs) are born and differentiate first, followed by horizontal neurons, amacrine neurons, photoreceptors and bipolar cells. Finally Müller glial cells differentiate (Cepko, 1999; Harris, 1997). DNA synthesis inhibitors have been used to show that birth order is not an essential determinant of cellular diversity in the retina (Harris and Hartenstein, 1991). However, in normal development, neurons that exit cell cycle early, differentiate early and assume early fates, while those that exit the cell cycle later, differentiate later and assume later fates. Molecular insights into this relationship are lacking. One possibility is that determination factors involved in early fates cause cells to exit the cell cycle early, while the converse possibility – that early cell cycle exit time influences cells towards early fates is also tenable. While several previous studies have shown that the determination factors can aid cell cycle exit, there have been few studies in the CNS showing how manipulating the cell cycle influences particular cell fates (Edlund and Jessell, 1999; Ohnuma et al., 2001). However, recent work in the vertebrate

retina suggests that cyclin-dependent kinase inhibitors p57Kip2 and p27Xic1 not only inhibit the cell cycle, but also influence cell fates (Dyer and Cepko, 2001; Ohnuma et al., 1999). The discovery of other such bifunctional molecules, like *geminin* (Kroll et al., 1998), suggests the existence of complex links between the cell cycle and cell fate determination, the understanding of which may shed light on the problem of histogenesis at a molecular level. Recent work in the *Drosophila* CNS (Isshiki et al., 2001) suggests that neuroblasts go through phases of expressing particular temporally co-ordinated transcription factors. When a neuroblast divides asymmetrically, the fate of its daughter cell is determined by the transcription factor expressed by the parent neuroblast at the time of the daughter cell's birth. If a similar system was involved in vertebrate histogenesis, then forcing a cell out of the cell cycle when it is expressing a particular transcription factor might well influence its fate.

The best molecular model for fate determination in the vertebrate nervous system involves the interaction of the proneural genes, which encode basic-helix-loop-helix (bHLH) transcription factors, and the Notch pathway. In the vertebrate retina, overexpression experiments have shown that proneural genes of the *atonal* class bias cells towards specific fates. For

example, *Xath5* influences progenitors towards early RGC fates (Acharya et al., 1997; Kanekar et al., 1997; Yan and Wang, 1998). Overexpression of active Notch pathway components in this system delays or blocks differentiation, leading to an increase in the percentages of cells that are determined at later stages, while inhibition of the Notch pathway leads to an increase in the percentages of cells types that differentiate early (Bao and Cepko, 1997; Dorsky et al., 1997; Dorsky et al., 1995; Henrique et al., 1997). As cells differentiate in the retina, they downregulate Notch and Delta, releasing themselves from inhibition. Successive release of different cells from Notch inhibitory activity is involved in orchestrating progenitor cells to differentiate at different times along different pathways (Cagan and Ready, 1989; Dorsky et al., 1997; Dorsky et al., 1995). The relationship between neurons and glia is particularly interesting in this regard. By suppressing the transcription of proneural bHLH transcription factors like neurogenin-1, overexpression of activated Notch inhibits neuronal determination and can promote the differentiation of glia from neural progenitors (Dorsky et al., 1995; Scheer et al., 2001; Wang and Barres, 2000). Conversely, neurogenin-1 has recently been found to block astrocyte differentiation by sequestering and inhibiting transcription complexes that are necessary for gliogenesis (Sun et al., 2001).

A key to understanding the orderly generation of neuronal diversity in a system like the vertebrate retina may, therefore, involve a deeper insight into relationships between the cell cycle on the one hand and the activities of Notch and the proneural genes on the other. A systematic study of how these determination pathways affect the cell cycle, and perhaps more importantly, how the cell cycle influences the function of the determination pathways has not yet been attempted. In this study, therefore, we took advantage of the developing *Xenopus* retinal system to examine the temporal and functional relationships between the genes that control cell cycle regulation and the genes that influence neural and glial cell fate in the retina. We show that the cell cycle feeds back on the determination functions of these factors so that early cell cycle exit favours the RGC fate while late cell cycle exit favours later fates. We also show that Notch may have an important novel role in histogenesis through its effects on the cell cycle.

## MATERIALS AND METHODS

### Materials

*Xenopus* embryos obtained by in vitro fertilisation were dejellied in 2% cysteine (pH 8.0) and allowed to develop in 0.1× MBS. Embryos were staged according to Nieuwkoop and Faber. All expression constructs of *Xath3* (Takebayashi et al., 1997), *Xath5* (Kanekar et al., 1997), *Xngnr-1* (Ma et al., 1996), *XNeuroD* (Lee et al., 1995), *Notch-ICD* (Chitnis and Kintner, 1996), *Delta-stu* (Chitnis et al., 1995), *XSu(H)* constructs (Wettstein et al., 1997), *p27Xic1* (Su et al., 1995), *cdc2* (Pickham et al., 1992), *cdk2* (Paris et al., 1991), *cyclin A2* (Howe et al., 1995), *cyclin E1*, *cdk4*, and *cyclin D1* (a gift from Dr Tim Hunt, ICRF, UK) were constructed using pCS2+ or pCS2+MT vector.

### In situ hybridisation and immunohistochemistry

Digoxigenin-labelled antisense RNA probes and the fluorescein-labelled probes were produced from the corresponding constructs and then in situ hybridisations were performed on 10 µm cryostat section at stage 41 as described previously (Harland, 1991). Immunostaining was performed on 10 µm cryostat section using the following primary

and secondary antibodies. Primary antibodies: anti-R5 (1:1) (Drager et al., 1984), anti-myc (9E10, 1:500; Sigma), anti-calbindin (1:200; Sigma), anti-rhodopsin (1:100; Dr Paul Hargrave), anti-phosphohistone H3 (1:500; Upstate), MPM-2 (1:500; Upstate), anti-cdc2 (1:100; Santa Cruz), anti-cyclin A2 (1:100). Secondary antibodies: Cy3-conjugated donkey anti-mouse IgG (1:1000), Cy3-conjugated donkey anti-rabbit IgG (1:1000), FITC-conjugated goat anti-rabbit IgG (1:500), FITC-conjugated goat anti-mouse IgG (1:500; Jackson Laboratories).

### Lipofection

pCS2+ or pCS2+MT constructs were lipofected into eye primordia of *Xenopus* embryos at stage 15, unless otherwise stated, according to the methods as described previously (Holt et al., 1990; Ohnuma et al., 1999). A GFP expression plasmid was colipofected to mark transfected cells. In double and triple lipofection, to normalise the amount of DNA introduced, pCS2+ was colipofected. Some colipofection efficiencies were checked using myc-tagged constructs and showed that more than 90% cells were colipofected.

### BrdU staining and TUNEL assay

After lipofection of the indicated constructs at stage 15, BrdU solution at 10 mM was injected intra-abdominally every 6 hours from the indicated stage. The embryos were fixed with 4% paraformaldehyde at stage 41 or 42, and then the BrdU incorporation was analysed as described previously (Ohnuma et al., 1999). For the experiments shown in Fig. 2A-C, BrdU was injected an hour before the fixation. After the in situ hybridisation, BrdU staining was performed. TUNEL assay was performed as described previously (Ohnuma et al., 1999).

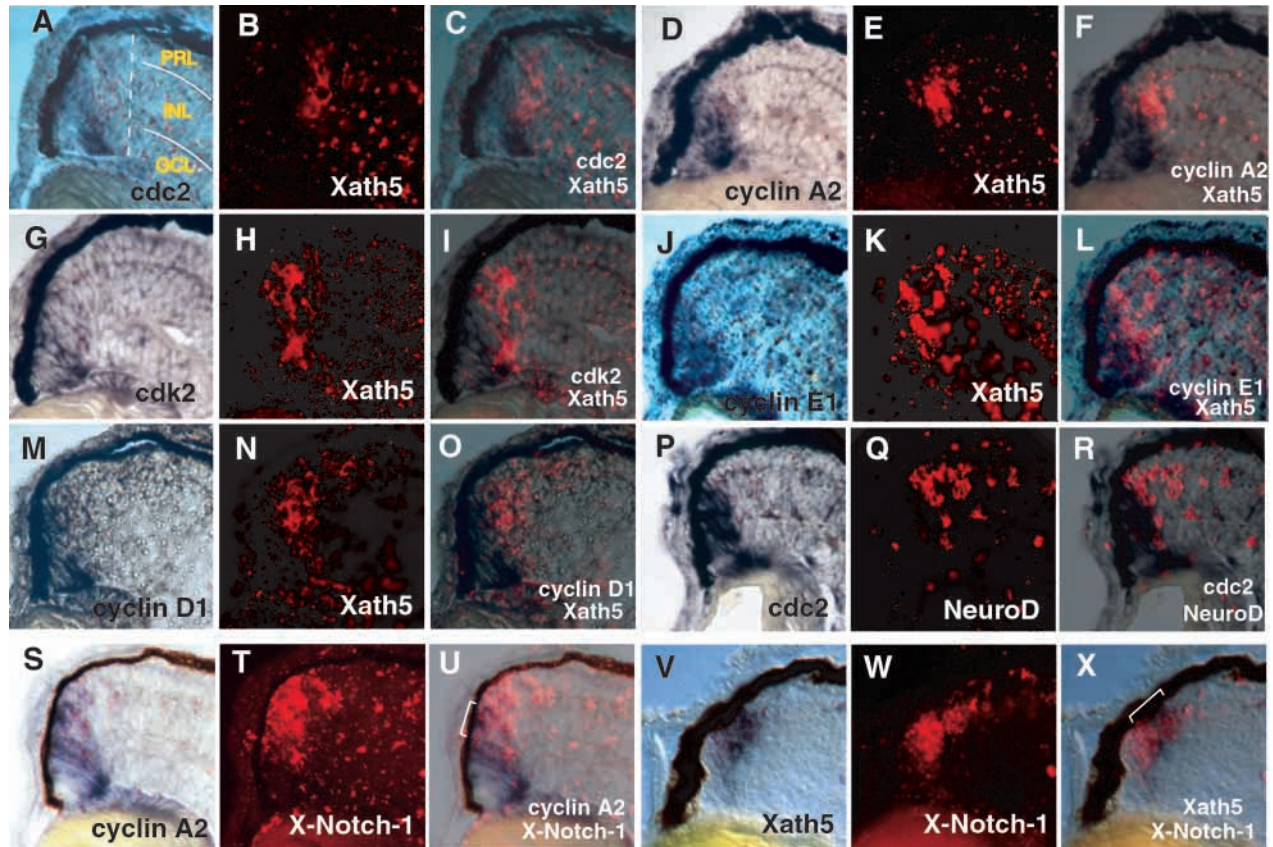
### Analysis

Cell types were identified using fluorescence microscopy of cryostat sections from stage 41 embryos. All transfected cells were identified by GFP labelling or myc staining of lipofected constructs. We used two criteria: morphology and layer for cell type identification. For example, a cell was scored as an RGC only if it had characteristic RGC morphology, such as an axon in the optic fibre layer, and a soma in the RGC layer. This means we would not have counted displaced RGCs and may have mistakenly counted some displaced amacrine cells as RGCs. Müller cells were distinguished from bipolar cells by their obvious vitreal process and this identification was sometimes confirmed with the Müller-specific antibody R5. We did not distinguish between rods and cones in this study. Because of variability between batches of embryos spawned on different days, experiments reported here were always done by comparing different experimental and control conditions from sibling embryos raised in identical conditions. For most experiments presented, we scored at least 500 cells from 8 sectioned retinas for each condition, (unless otherwise stated in the figure legend). The results from single experiments are presented in the graphs, but all key experiments were repeated at least twice with similar results. Error bars on all graphs indicate s.e.m.. Statistical significance was assessed using Student's *t*-test (in the figures, statistical significance is as follows: \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001).

## RESULTS

### Proneural genes and Notch pathway genes are upregulated in the ciliary marginal zone (CMZ) of the retina as cell cycle activators are downregulated

To analyse the expression of cell cycle genes during retinal development with respect to differentiation, we looked at the CMZ of stage 41 retinas. In previous studies, we have shown that genes that are expressed at the more peripheral regions in CMZ are usually expressed earlier during retinal development.

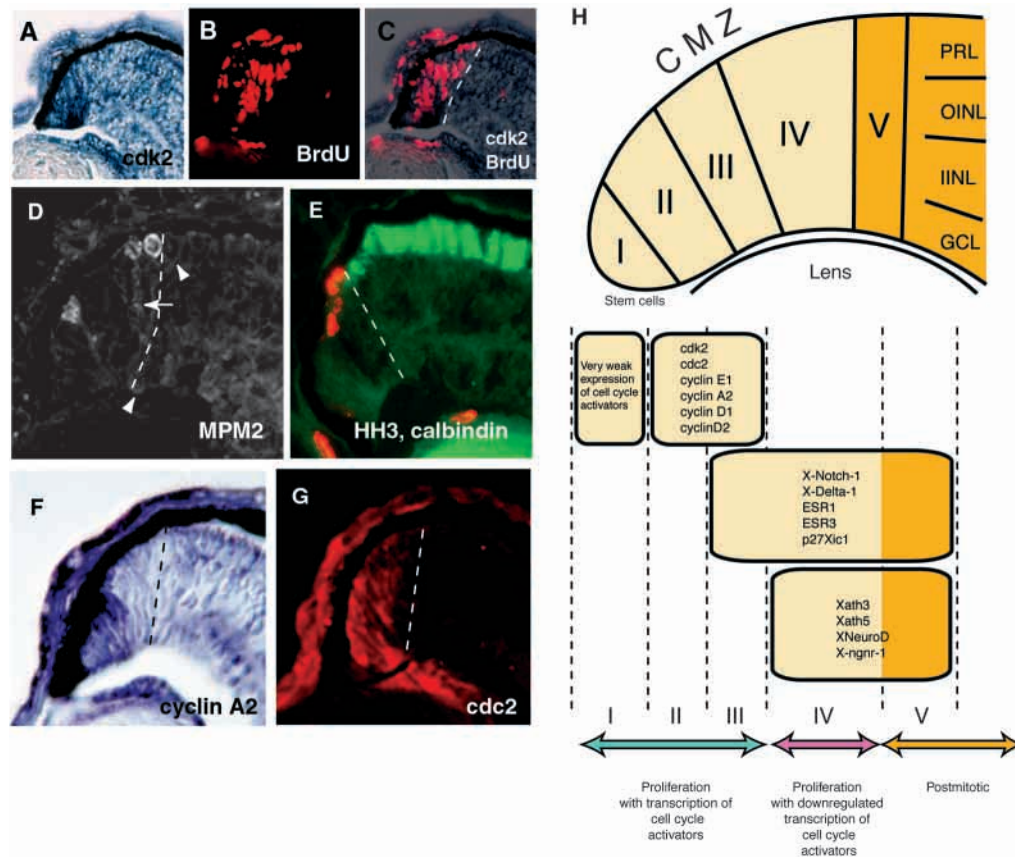


**Fig. 1.** Cell cycle genes are downregulated as neuronal determination genes are upregulated in the CMZ of stage 41 *Xenopus* embryos. In situ hybridisations to identify expression of the genes indicated on each panel. C,I,O,U,F,L,R and X are overlaid images of the single hybridisations. In A the dashed line indicates the end of the CMZ and the solid lines, the boundaries between differentiated layers. Brackets indicate regions of co-expression.

Thus, cellularly and molecularly, the CMZ spatially recapitulates the temporal differentiation of the embryonic retina (Perron et al., 1998). The CMZ continuously generates neurons and glial cells from the peripheral region of retina, during the entire life of the animal (Perron and Harris, 2000b). We can divide the CMZ roughly into five regions from peripheral to central, although in reality there may be finer gradations than we could distinguish here. Retinal stem cells are located at the most peripheral edge of the CMZ (region I, Fig. 2H) and differentiating cells are found at the central edge (region V). Between these, in regions II, III and IV, dividing retinoblasts become increasingly specified. In this system, therefore, double in situ hybridisations of the cell cycle genes in relation to proneural and Notch pathway genes reveal the relative timing of expression. As shown in Fig. 1A-U, all of major cell cycle activators, *cdc2*, *cdk2*, *cyclin D1*, *cyclin A2*, *cyclin E1*, and *cdk4* (data not shown) are expressed at their highest levels in an approximately identical region of the CMZ (within regions II and III) and their levels dramatically decrease just as the expression of the *atonal* related proneural genes such as *Xath5*, *XNeuroD*, *Xath3* and *Xenopus neurogenin-1* (*Xngnr-1*) increase (Fig. 1A-R and data not shown). These latter genes are all heavily expressed in region IV (Perron et al., 1998). In addition, the cell cycle activators are expressed more peripherally than the cdk inhibitor, p27Xic1 (Ohnuma et al., 1999). XNotch-1 expression starts in

region III suggesting that the Notch pathway might be involved in the transcriptional downregulation of cell cycle activators (Fig. 1S-U). The lower levels of cell cycle gene expression in region I, where retinal stem cells are located, suggest that these cells do not divide as rapidly as the retinoblasts that they generate. We present data (below) to show that Notch activity can help retinal cells exit the cell cycle, so it is also important to investigate the relationship between *XNotch-1* and *Xath5* expression. As expected, *XNotch-1* starts more peripherally than *Xath5*, yet there is a clear overlap in the expression of these two genes (Fig. 1V-X) (see also Perron et al., 1998).

The results of double in situ hybridisations (summarised in Fig. 2H) indicate that cell cycle activators are strongly downregulated as proneural genes become highly expressed. Previous studies have shown that *Xath5* and p27Xic1 are expressed in BrdU-positive cells, suggesting that these cells can still cycle where there are low levels of transcribed cyclins and rising levels of p27Xic1 (Ohnuma et al., 1999; Perron et al., 1998). To test this further, we used BrdU incorporation and markers of mitosis to monitor cell division. Fig. 2A-C shows that after downregulation of the cell cycle activator, *cdk2*, cells in region IV can still proceed through S phase. Also, mitotic cells are observed in this region as both cell bodies stained for the MPM-2 antibody (Fig. 2D) and nuclei for the phosphohistone H3 antibody (Fig. 2E). These results indicate that some cells are still able to proceed with at least one more



**Fig. 2.** Proliferation of precursor cells in the CMZ. (A-C) Double staining for cdk2 expression (A) and BrdU uptake (B). BrdU was injected at stage 41. The embryos were fixed one hour later and then in situ hybridisation and BrdU immunostaining were performed. (C) Overlaid image. (D) Immunostaining of stage 41 CMZ with MPM-2 antibody. Mitotic cells with endfeet are indicated by arrows, and differentiated photoreceptors and RGCs close to CMZ are indicated by arrowheads. (E), Double staining of stage 41 CMZ using phosphohistone H3 antibody (red) and calbindin antibody (green) to identify cone photoreceptors. (F) Immunostaining with cyclin A2 antibody. (G) Immunostaining with cdc2 antibody. The end of the CMZ is indicated by a dashed line in C, D, E, F and G. (H), Schematic model of the expression of cell cycle components and determination genes in the CMZ of the stage 41 embryo.

division after the transcription of cell cycle activators is downregulated. As the cell cycle is also regulated post-transcriptionally, we analysed protein levels by immunocytochemistry (Fig. 2F-G). Cyclin A2 and cdc2 proteins are highly expressed in the region II and III of the CMZ. The amount of cyclin A2 dramatically decreases in the peripheral end of region IV while that of cdc2 gradually decreases in region IV.

These results suggest that cell cycle activators are downregulated at the transcriptional and then the translational level as retinal cells begin to upregulate the Notch pathway and the proneural genes and go through their last divisions. These relationships seen in the CMZ are suggestive of what might be happening in retinal cells through developmental time. Indeed in all genes so far tested, the spatial arrangement of gene expression in the CMZ predicts the temporal order of expression in embryonic development of the central retina (Perron et al., 1998). We are therefore able to use these data as a model for developmental gene expression as we turn our attention to experiments on the central retina with the caveat that timing of expression of these genes in retinal cells during development remains speculative until analysed in detail.

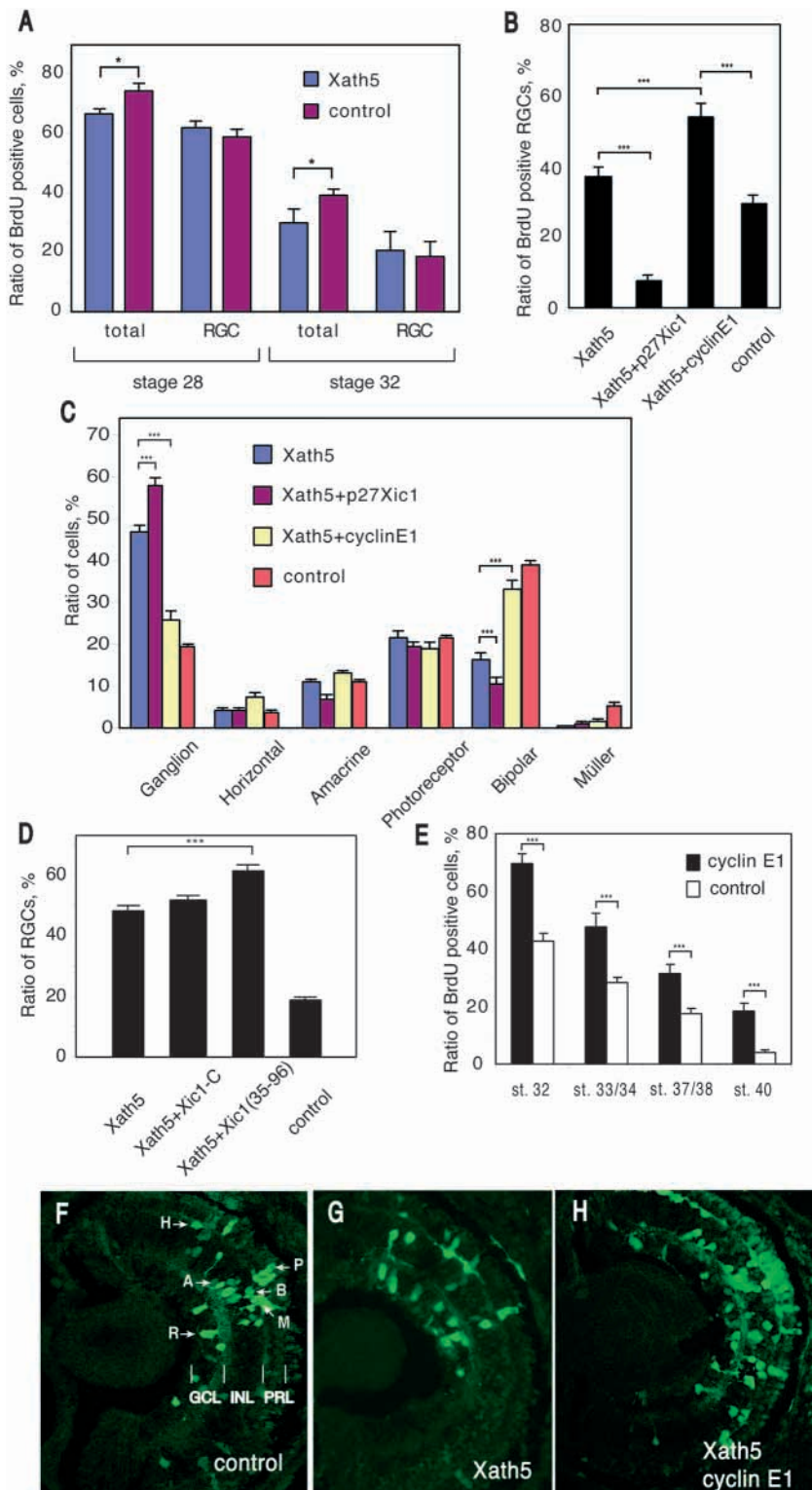
### Does *Xath5* have a role in cell cycle exit?

The correlation between the downregulation of cell cycle genes and the upregulation of proneural genes raises questions about the possible role of genes like *Xath5* in the cell cycle. Recent results with the *Ath5* zebrafish mutant, *lakritz*, suggest that *Ath5* does have a role. In this mutant, there is a lack of early born RGCs (Brown et al., 2001; Kay et al., 2001; Wang et al.,

2001). Instead the cells that would have given rise to RGCs appear to stay in the cell cycle longer and give rise to extra later born cell types. Thus *Ath5* appears to be necessary for RGCs to exit the cell cycle at the appropriate histogenetic time. But is it able to drive cell cycle exit? To test this possibility, we lipofected retinas with *Xath5* at stage 15 and injected BrdU into these embryos at either stage 28 or stage 32. The ratios of BrdU-positive cells were determined at stage 41 (Fig. 3A). The overall fraction of cells taking up BrdU slightly but significantly decreased when *Xath5* was transfected at either stage. When analysed by cell type, however, we found in both cases that *Xath5*-overexpressing RGCs exit the cell cycle at the same time as control RGCs. Thus, although more RGCs are born under the influence of *Xath5* overexpression, they do not exit the cell cycle earlier than normal RGCs. Although some *Xath5*-overexpressing cells become other cell types, these are also not born earlier than their control counterparts (data not shown). These results indicate that the proneural genes like *Xath5* may contribute to normal histogenesis by helping cell cycle withdrawal at the appropriate time but that they do not drive premature cell cycle exit.

### Early cell cycle exit enhances the ability of *Xath5* to induce RGCs

If *Xath5* does not drive cell cycle exit, the question remains as to why RGCs are born first in the sequence of retinal development. The expression sequence of proneural and cell cycle genes, shown above, suggests that proneural proteins act in a cellular environment of reduced cell cycle activation, so it is possible that cell cycle inhibition is influential to their

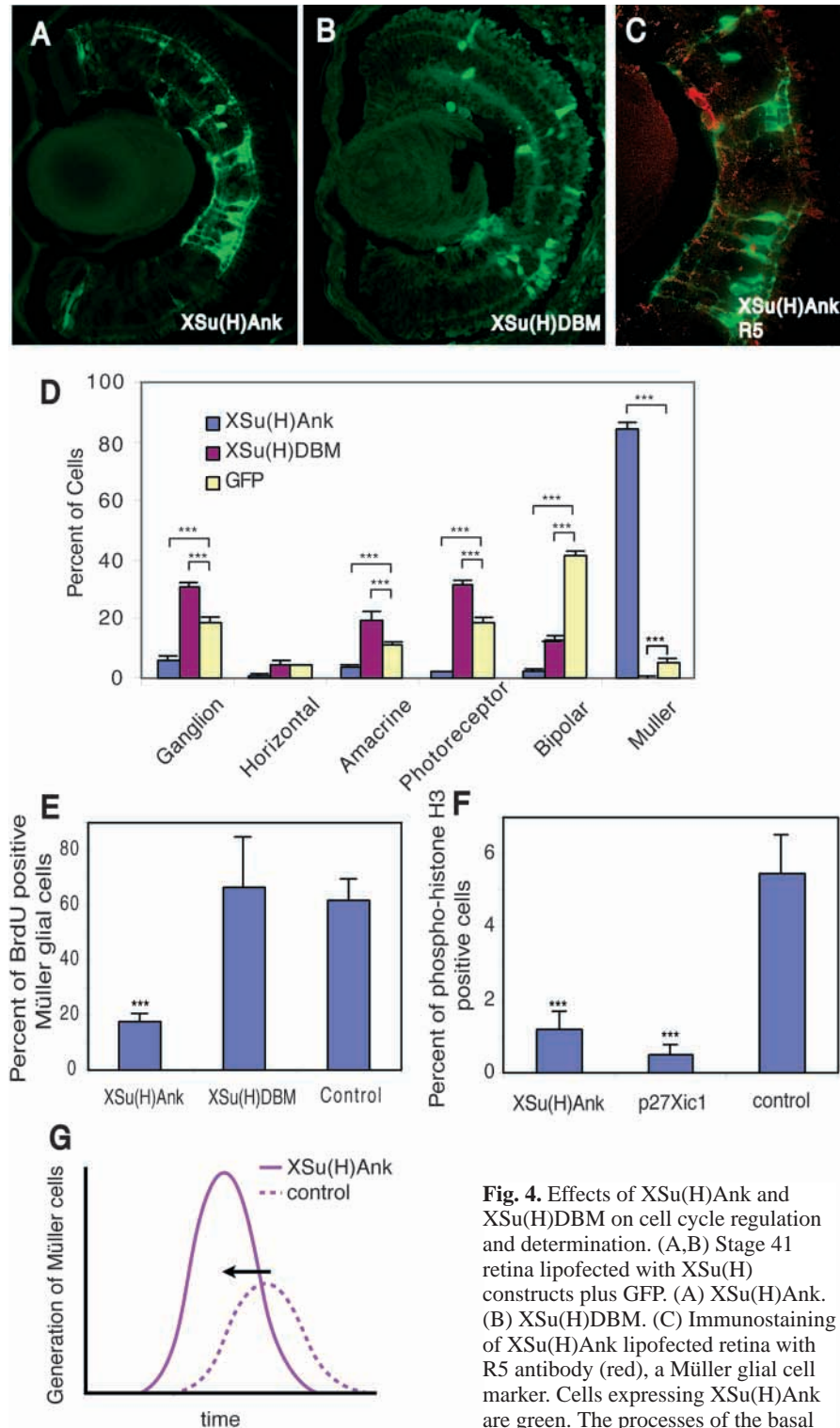


**Fig. 3.** Activity of *Xath5* as an RGC determinant is affected by modulation of cell cycle activity. (A) Cell cycle exit of RGC precursors is unaffected by *Xath5*. *Xath5* plus GFP or GFP only was lipofected at stage 15, and then BrdU injection started at stage 28 or late stage 32. At stage 41, the ratio of BrdU-positive cells in all lipofected cells or lipofected RGCs was analysed. (B) Cell cycle exit of RGC precursors is affected by p27Xic1 and cyclin E1. p27Xic1 or cyclin E1 was colipofected with *Xath5* at stage 15, and then BrdU injection started at early stage 32. At stage 41, the ratio of BrdU-positive lipofected RGCs to all lipofected RGCs was analysed. (C) Activity of *Xath5* as a RGC determinant is affected by modulation of cell cycle activity. *Xath5* was colipofected with p27Xic1 or cyclin E1 at stage 15, and then their effect on cell fate determination was analyzed. (D) Deleted constructs of p27Xic1 also increase the fraction of cells that become RGCs. (E) Overexpression of cyclin E1 in retinal precursor cells transiently activates the cell cycle. An expression construct of cyclin E1 was lipofected at stage 15 and BrdU injection started at the indicated stages. At stage 42, BrdU incorporation was analyzed. (F-H) Retina lipofected with *Xath5* by itself and in combination with cyclin E1. (F) Retina lipofected with GFP. R, RGC; P, photoreceptor cell; H, horizontal cell; A, amacrine cell; B, bipolar cell; M, Müller glial cell. (G) Retina lipofected with *Xath5* and GFP. The ratio of RGCs is 50% (12 RGCs out of 24 lipofected cells). (H) Retina lipofected with *Xath5*, cyclin E1 and GFP. The ratio of RGCs is 22% (11 RGCs out of 50 lipofected cells).

controls lipofected with GFP (see below), while co-lipofection with *p27Xic1* dramatically decreases the ratio of BrdU-positive RGCs to just 10%, implying *p27Xic1* drives the majority of these cells out of the cell cycle before stage 32. Cells transfected with *Xath5* have an increased probability of becoming RGCs and a decreased probability of becoming late cell types (Fig. 3C,F,G) (Kanekar et al., 1997). Co-transfection with *p27Xic1* strikingly augments this phenotype such that even more cells become RGCs than later cell types (Fig. 3C). *p27Xic1* also enhances the function of two other proneural genes that promote early neuronal fate in the *Xenopus* retina, *Xngnr-1* and *Xath3* (data not shown). These results suggest that early cell cycle exit potentiates the affects of proneural genes in promoting early cell fates.

In a previous study, we showed that p27Xic1 has determinative effects on its own (Ohnuma et al., 1999), through a domain of the molecule that promotes glial fate in the retina. In that study, we also found that p27Xic1 could be functionally dissected by generating N-terminal constructs that could no longer induce glial cells but still had kinase inhibitory activity (Ohnuma et al., 1999). Therefore, to assess whether the kinase-inhibitory activity or the Müller-inducing activity of

determinative activities. If so, we would predict that driving cell cycle exit early in cells that expressed these proneural genes might enhance their function. To test this, we co-expressed the cyclin kinase inhibitor p27Xic1 with *Xath5* and examined the retinas at stage 41 (Fig. 3B). BrdU incorporation from stage 32 indicates that when *Xath5* is overexpressed alone, 38% of RGCs are BrdU positive, which is similar to



**Fig. 4.** Effects of XSu(H)Ank and XSu(H)DBM on cell cycle regulation and determination. (A,B) Stage 41 retina lipofected with XSu(H) constructs plus GFP. (A) XSu(H)Ank. (B) XSu(H)DBM. (C) Immunostaining of XSu(H)Ank lipofected retina with R5 staining. The processes of the basal side of the lipofected cells show R5 staining. (D) Distribution of retinal

cell type in stage 41 retina lipofected with XSu(H) constructs at stage 15. (E) Effect of XSu(H)Ank and XSu(H)DBM on BrdU incorporation into Müller glial cells. The construct was lipofected at stage 15. After injection of BrdU at stage 34, the ratio of BrdU-positive cells in Müller glial cells was analysed at stage 41. XSu(H)Ank stops the cell cycle in the precursors earlier than the natural timing. (F) Effect of XSu(H)Ank on the ratio of phosphohistone H3-positive cells. After lipofection of the construct at stage 15, the ratio of phosphohistone H3-positive cells in the lipofected cell population was measured at stage 36. (G) Model of Müller glial cell generation in retina overexpressing XSu(H)Ank.

p27Xic1 was responsible for the enhancement of the Xath5 phenotype, we used the construct, Xic1(35-96), which retains kinase-inhibitory but has no Müller-inducing activity. When lipofected with proneural genes, this construct gives similar results to the full-length p27Xic1 (Fig. 3D). We also tested the p27Xic1 C-terminal construct, Xic1-C, which contains just the PCNA binding domain. Xic1-C inhibits the cell cycle in S phase instead of at the G<sub>1</sub>-S transition where the N-terminal construct, Xic1(35-96) acts (Ohnuma et al., 1999). Interestingly, Xic1-C shows an insignificant enhancement of proneural genes function (Fig. 3D), suggesting that it is not just cell cycle arrest, but the point of cell cycle arrest in G1 that is important for this function.

#### Late cell cycle exit inhibits the determinative effects of Xath5 on RGCs

If the activity of proneural genes is modulated by the timing of cell cycle exit, not only should it be enhanced by cell cycle inhibitors, but it should also be diminished by cell cycle activators. To see if this was the case, we assayed the effects of several cell cycle activators on cell cycle. *cdc2*, *cdk2*, *cdk4*, *cyclin A2*, *cyclin E1*, or *cyclin D1* were lipofected at stage 15 and BrdU incorporation starting at stage 32 was assayed at stage 41 when cell fates could be assayed. Only cyclin E1 was found to be effective on its own in stimulating BrdU incorporation in lipofected retinal cells. Cyclin E1 activates DNA synthesis at all stages tested compared to controls, but the activation declines as the animal ages parallel to the decline in DNA synthesis in control retinas, indicating that lipofection of cyclin E1 can only keep cells cycling a little longer than normal (Fig. 3E).

To test if this extra cell cycle activity inhibited proneural gene function as predicted, we colipofected cyclin E1 with Xath5 and GFP. Co-lipofection with cyclin E1 strikingly decreased the number of transfected cells that became RGCs, compared to cells transfected with Xath5 alone, while increasing later born cell types like bipolar cells (Fig. 3C,G,H). BrdU incorporation studies, showed as expected, that those few co-transfected cells that do become RGCs are born on average later than those that are transfected by Xath5 alone (Fig. 3B). This suggests that delaying cell cycle exit in Xath5 overexpressing cells interferes with

its ability to induce RGCs. There is, however, an alternative explanation for this result. It could be that a large fraction of the GFP-transfected cells in retinas that were co-lipofected with Xath5 and cyclin E1 did not actually express Xath5 (due to, for example, a possible low co-transfection frequency or interference between the two constructs), and that this accounts for the lower number of GFP-expressing RGCs. To test this possibility directly, a set of experiments was done using a myc-tagged Xath5. In this case, 25% of the myc-labelled cells co-lipofected with cyclin E1 differentiated into RGCs while 48% of the myc-labelled cells lipofected with myc-tagged Xath5 alone differentiated into RGCs. This result confirms the GFP tracer analysis (above), and supports the hypothesis that delayed cell cycle exit inhibits Xath5's RGC-inducing activity. Simultaneous overexpression of cyclin E1 with Xngnr-1 also inhibits the latter's RGC and photoreceptor cell inductive activity (data not shown). None of other cell cycle activator genes (*cdc2*, *cdk2*, *cdk4*, *cyclin D1*, and *cyclin A2*), which failed on their own to stimulate BrdU uptake, affected the function of Xath5 in this co-lipofection assay (data not shown), strongly suggesting that this fate-inhibition is linked to activation the cell cycle.

It has been reported that activation of the cell cycle in differentiating neurons by cyclin E1 may result in apoptosis (Neufeld et al., 1998; O'Hare et al., 2000). Thus, it is important to know whether overexpression of cyclin E1 also causes apoptosis here, as another potential explanation for the results is that Xath5-cyclin E1 co-transfected cells that would otherwise become RGCs, die instead. TUNEL staining of cells lipofected with cyclin E1 at stage 15 was therefore compared with GFP lipofected control cells at stages 29/30, 32, 35/36, and 40. We did not see any statistically significant difference at any of these stages (data not shown), suggesting that cyclin E1 overexpression does not cause significant apoptosis in these cells when introduced this way.

### Early cell cycle exit is induced by the Notch pathway

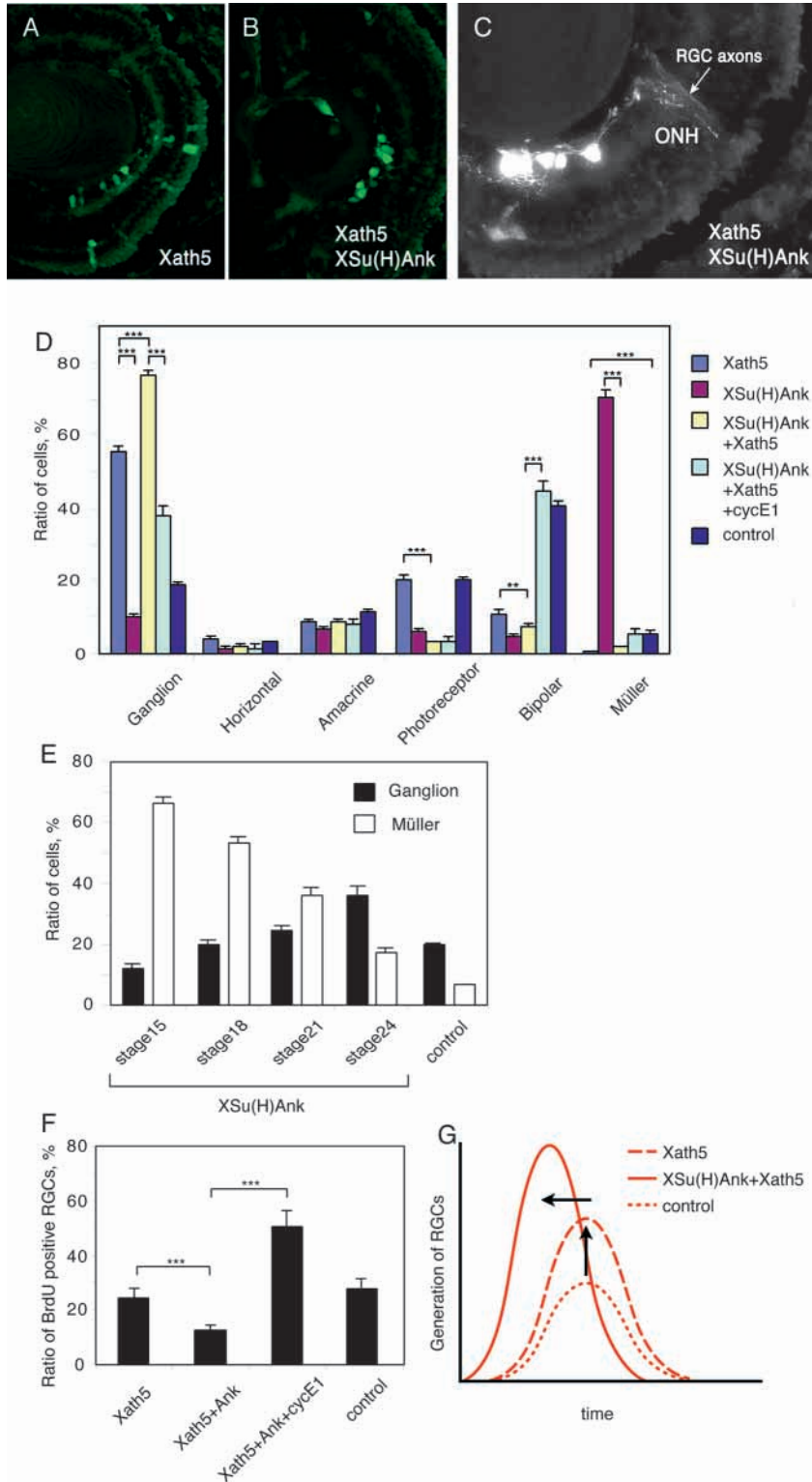
In the CMZ, proneural genes like *Xath5* are first expressed in cells that express low levels of *p27Xic1* (Fig. 2H) (Ohnuma et al., 1999) suggesting that in normal development, *Xath5*-positive cells may use another mechanism to exit the cell cycle. Notch is highly expressed in regions of *Xath5*-positive cells (Fig. 1V-X), and Notch activity has been linked in several studies to cell cycling, with effects that are highly context specific, enhancing proliferation in some systems and inhibiting it in others (Bao and Cepko, 1997; Dorsky et al., 1995; Scheer et al., 2001). Indeed, our previous work on activated Notch in the *Xenopus* retina suggested that Notch activation inhibits proliferation, as clone size was always small (one or two cells) in XNotch $\Delta$ E lipofections (Dorsky et al., 1995). We therefore wanted to know whether Notch activation promoted cell cycle exit in this system. To address this question, we used a constitutively active form of XSu(H), XSu(H)Ank (Wettstein et al., 1997). We first examined the role of the XSu(H)Ank on the cell cycle using BrdU incorporation and phosphohistone H3 labelling. XSu(H)Ank by itself induces Müller cells (Fig. 3F, Fig. 4A,C,D) possibly by interfering with the expression or activity of proneural bHLH genes, but it strongly decreases BrdU uptake by stage 34 in these overexpressing Müller cells compared to control Müller cells (Fig. 4E). It also decreases overall levels of

mitosis as seen by phosphohistone H3 labelling (Fig. 4F). These observations indicate that activation of Notch results in both the induction and accelerated cell cycle exit of Müller cell precursors (Fig. 4G). XSu(H)DBM is an inactive variant of XSu(H) which, when overexpressed, acts as a dominant negative possibly by competing with the native molecule. In contrast to XSu(H)Ank, XSu(H)DBM does not promote early cell cycle exit of Müller glia (Fig. 4E). It does, however, act in opposition to XSu(H)Ank in that cells transfected with XSu(H)DBM tend to differentiate into RGCs and photoreceptors but not glia, consistent with its effect on blocking the Notch regulated lateral inhibition (Fig. 4B,D). This effect is mimicked by other blockers of the Notch pathway, such as the modified ligand Delta<sup>STU</sup> (Dorsky et al., 1997). XSu(H)DBM does not significantly enhance or delay cell cycle exit of these overexpressing RGCs or photoreceptors (data not shown).

### Notch activation potentiates the induction of RGCs by Xath5

If early cell cycle exit enhances early neural fates, it might be expected that Su(H)Ank should enhance rather than inhibit Xath5 function in this system. However, Notch is a multifunctional protein that can also inhibit differentiation, and retinal cells overexpressing activated forms of Notch on their own often fail to differentiate or become glial cells as shown above (Dorsky et al., 1995; Scheer et al., 2001). Thus, a Notch plus proneural co-expression experiment raises the interesting question of whether proneural genes can produce early cell types in the presence of activated Notch, especially as this appears to be the normal context of expression of these genes in early histogenesis (Fig. 1V-X, Fig. 2H) (Perron et al., 1998). It should be noted in this regard that in the early *Drosophila* eye disc, Notch activity first induces and enhances the *atonal* expression in prospective R8 cells, while later it inhibits proneural activity (Baker et al., 1996; Baker and Yu, 1997; Cagan and Ready, 1989; Ligoxygakis et al., 1998). To test whether Notch activity enhances proneural function in the *Xenopus* retina, we co-expressed XSu(H)Ank with Xath5. As predicted, the gliogenic activity of XSu(H)Ank is completely inhibited by Xath5 (Fig. 4A, Fig. 5B,C). What is also clear, however, is that the RGC inductive activity of Xath5 was dramatically enhanced by XSu(H)Ank (Fig. 5A-D). In this experiment, overexpression of Xath5 alone yielded 56% of lipofected cells that differentiated into RGCs, while upon co-overexpression of Xath5 and XSu(H)Ank this rose to a stunning 78% (Fig. 5B,D). Co-expression of a constitutively active Notch receptor, Notch-ICD, with Xath5 also enhanced the RGC inductive activity of Xath5 (data not shown) at the expense of later born cell types. XSu(H)Ank also enhances the ability of Xath3, Xngnr-1, or XNeuroD to induce early cell types, particularly RGCs (data not shown), with attendant decreases of late cell types.

These results show that activation of Notch pathway in the context of misexpressed proneural genes in the developing retina does not inhibit neural differentiation, as might have been suspected from previous results where active Notch was transfected by itself, but rather enhances the function of co-expressed proneural genes. Does it have such an effect during normal histogenesis, for example in the cells that co-express



Xath5 and XNotch-1 (Fig. 1V-X)? If it did have such a role, then one might expect that later transfection of XSu(H)Ank by itself, at a stage that did not interfere with Xath5 expression, might promote RGC fate. We therefore, transfected retinas with GFP or GFP plus XSu(H)Ank at successively later stages and found that between stage 15 and 24, there was a gradual increase in the ratio of transfected cells that became RGCs, and

**Fig. 5.** XSu(H)Ank enhances co-expressed Xath5 activity through its effect on the cell cycle. (A-B) Stage 41 retina lipofected with Xath5 alone (A) and in combination with XSu(H)Ank (B). (C) Almost all RGCs induced by overexpression of Xath5 and XSu(H)Ank send axons that exit the eye through the optic nerve head (ONH). (D) Ratio of cells in the retina lipofected with Xath5 and/or XSu(H)Ank. Retina was lipofected with the indicated constructs at stage 15. The effect on cell type distribution was analysed at stage 41. (E) Effect of XSu(H)Ank on RGC versus Müller cell ratios as a function of stage of transfection. XSu(H)Ank plus GFP was lipofected at the indicated stages, and the effects on cell fate determination analyzed at stage 41. In control, GFP was lipofected at stage 15. Control at stage 18, 21, or 24 was similar to that of stage 15. (F) XSu(H)Ank induces earlier cell cycle exit of RGC precursors induced by Xath5. Xath5 was colipofected with XSu(H)Ank or with XSu(H)Ank plus cyclin E1 at stage 15. BrdU injection started at stage 32. At stage 41, the ratio of BrdU-positive cells in the lipofected RGCs was analyzed. (G) Model of RGC generation in retina overexpressing Xath5 alone or XSu(H)Ank plus Xath5.

a corresponding decrease in those that became Müller cells (Fig. 5E). These results are consistent with a role for Notch in the potentiation of RGC fate in normal development.

### Notch potentiates Xath5 function through its effect on cell cycle exit

Is this enhanced proneural function actually a result of Notch's effect on cell cycle exit as was shown above for p27Xic1? If so, then BrdU labelling should show that in the presence of Xath5, XSu(H)Ank enhances the early cell cycle exit of RGCs. Indeed, this is the case (Fig. 5F,G). There is, however, the possibility that the effect on the cell cycle and the effect on proneural enhancement could be a direct effect of Notch signalling on Xath5 function and have nothing to do with the former's effect on the cell cycle. To resolve this, it is necessary to test whether the Notch pathway is actually working through the cell cycle to enhance the effects of Xath5. Therefore, we attempted to override the cell cycle exit caused by XSu(H)Ank by adding cyclin E1 into the lipofection mixture. When we performed BrdU studies on these triple lipofected cells, we found that cyclin E1 reversed the early cell cycle exit caused by XSu(H)Ank (Fig. 5F). Importantly, when

Xath5 was co-expressed with both XSu(H)Ank and cyclin E1, the enhanced RGC induction due to XSu(H)Ank was also blocked (Fig. 5D). These results clearly implicate the cell cycle exit component of Notch function in proneural enhancement. Similarly, the potentiation by XSu(H)Ank of RGC induction by Xngnr-1 was also reversed by co-lipofection with cyclin E1 (data not shown).



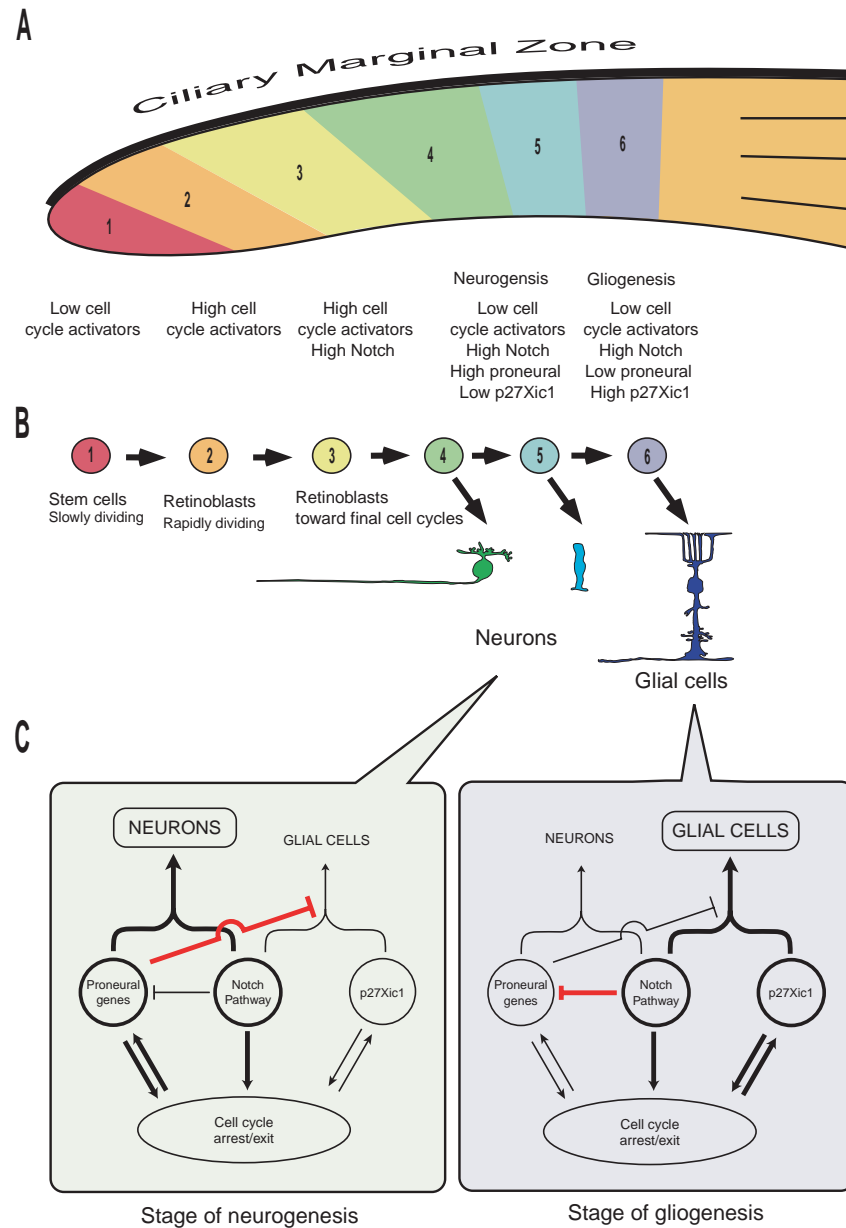
## DISCUSSION

In the *Xenopus* retina, cell cycle exit is co-ordinated with and influenced by the combined activity of proneural genes and the Notch pathway. Conversely, the state of the cell cycle appears

to influence the effectiveness of these determination factors. This interplay contributes significantly to retinal histogenesis. We found Xath5 by itself, although it promoted RGC fate, did not appear to do so by causing these cells to exit cell cycle prematurely. However both p27Xic1 and Su(H)Ank, by stimulating cell cycle exit, strongly enhanced the ability of Xath5 to induce RGCs, while cyclin E1, by delaying cell cycle exit, had the reverse effect on Xath5 activity. Both Xic1(35-96) by itself and cyclin E1 by itself, when transfected into the retina, had only weak effects on cell fate (data not shown) suggesting that cell cycle exit by itself is not a key determiner of cell fate, consistent with previous results (Harris and Hartenstein, 1991). Rather our results suggest that cell cycle exit time modulates the activity of transcription factors like Xath5, thereby helping to co-ordinate early cell cycle exit with the activity of transcription factors that promote early fate.

### The contribution of the Notch pathway and the proneural pathway to retinal histogenesis

Proneural gene products like Xath5 bias progenitor cells towards early neural fates (Perron and Harris, 2000a). In zebrafish, the optic stalk induces the first cells to express Ath5. These cells then leave the cell cycle and begin to differentiate as RGCs. Ath5 expression spreads across the retina like a wave driven by a preceding wave of Sonic hedgehog (Masai et al., 2000; Neumann and Nusslein-Volhard, 2000). In *Xenopus*, we do not know how Xath5 is turned on initially but we suggest in this paper that part of the function of these proneural genes is to help guide progenitors out of the cell cycle at the times normally associated with these early fates. In support of this suggestion, it has recently been reported that *lakritz*, a blind zebrafish mutant that lacks RGCs, is a loss-of-function mutant of the *ath5* proneural gene. Strikingly, in this mutant, cells tend to stay in the cycle at the normal birth date of RGC precursors. Instead more cells exit the cell cycle slightly later and differentiate as amacrine cells, the second born cell type (Kay et al., 2001). Other INL cells, including bipolars, are also increased, while photoreceptors (the last retinal cells to be born in zebrafish) are unchanged in number. In mice, cones are the second born cell type, and *Math5* mutants show a dramatic increase in cones at the expense of RGCs (Brown et al., 2001). These observations indicate that *ath5* homologues clearly participate in the normal timing of cell cycle exit for RGC precursors. Farah et al., reported that NeuroD2, Math5, and Mash1 all induce P19 embryonic



**Fig. 6.** Model of interaction among Notch pathway, proneural genes and cell cycle regulation in the *Xenopus* retinal development. (A,B) The spatial development of retinal cells in the CMZ (A) and the temporal development of retinal cells (B). (1) Stem cells divide slowly with low level of cell cycle activators; (2) retinoblasts divide rapidly as cell cycle activators rise; (3) as cell cycle activators are downregulated, retinoblasts enter their last cell cycles; (4) as expression of proneural genes of the *atonal* family rise in the presence of Notch, the first cells exit the cell cycle and acquire early neural fates; (5) later neural fates are the result of progressive cell cycle exit and the effect of the Notch pathway on specific proneural genes; (6) in the last phase of generating retinal cells, proneural gene expression has decreased while p27Xic1 expression has increased, favouring a glial fate. (C) Interaction models showing how the same network produces neurons when Notch and proneural levels are high (left), and glia when Notch and p27Xic1 levels are high (right).

carcinoma cells to exit the cell cycle and differentiate into neurons (Farah et al., 2000). The fraction of cells in the *Xenopus* retina taking up BrdU slightly, but significantly, decreases in Xath5-transfected cells indicating that Xath5 may indeed help get cells out of the cell cycle early. When analysed by cell type, however, we found no significant difference in the time of histogenesis between Xath5-overexpressing and control cells of the same type (Fig. 3A). Overexpression of Xath5 clearly causes misexpressing cells to become RGCs, the first cell type born, but these misexpressing cells are not born any earlier than normal RGCs. One possible explanation for these results is that a precursor specified to become an RGC, perhaps through the activity of Xath5, is particularly sensitive to other cell cycle exit signals, either extrinsic or intrinsic. Thus, although the influence of Xath5 on the cell cycle has yet to be clarified, our results suggest that it may be involved in the co-ordination of cell cycle exit without actively driving cells out of the cell cycle.

While it is well known that the Notch pathway influences cellular determination in the developing nervous system through its indirect transcriptional regulation of the proneural genes, it also has a variety of other effects, such as the one that it has on the cell cycle. Yet, its influence on cell fate through its effect on the cell cycle has not previously been addressed. Activation of at least part of the Notch pathway by XSu(H)Ank downregulates proliferative potential and results in early cell cycle arrest, as was previously shown by overexpression of Notch $\Delta$ E (Dorsky et al., 1995). These results are different from those in the rat, where Notch expression via retroviral infection induced abnormal growth and larger than normal clones (Bao and Cepko, 1997). Although the reason for this difference is not clear, it emphasises the fact that Notch has different effects on the cell cycle depending upon the context, as has been seen elsewhere (Baonza and Garcia-Bellido, 2000; Chambers et al., 2001; Johnston and Edgar, 1998). In recent work in the *Xenopus* neural plate, it appears that XSu(H)Ank activity can downregulate the expression of both cell cycle activators like cyclinA2 and cdk2 and cell cycle inhibitors like p27Xic1 (A. Vernon and A. P., unpublished data). In line with the observation reported here, it has recently been described in transgenic zebrafish retinas that cells overexpressing a constitutively active Notch also show early cell cycle exit (Scheer et al., 2001). These results indicate that, at least in *Xenopus* and zebrafish retina, Notch activity negatively regulates the cell cycle. Consistent with this finding, the normal expression of Notch in the CMZ of the *Xenopus* retina clearly overlaps the mitotic to postmitotic transition region (Fig. 2H).

A previous study, using Delta<sup>STU</sup>, a dominant negative Notch ligand, showed that misexpressing cells tend to differentiate earlier than control cells, as one might expect of cells in which a lateral inhibitory pathway that blocks cell differentiation is compromised (Dorsky et al., 1997). However, that study did not address the question of whether Delta<sup>STU</sup> affected the birthdate of misexpressing cells. In this study, we examined this question directly using XSu(H)DBM and found that blocking the Notch pathway appeared to have no significant effect on cell cycle exit times of any of the cell types investigated, although it slightly, but insignificantly, advanced the average cell cycle exit for all cells (data not shown). A probable explanation for this is that XSu(H)DBM biases retinal progenitors towards early fates such as RGCs and

photoreceptors by de-repressing proneural genes like *Xngnr-1*, *Xath5*, *Xath3* and *NeuroD*. These studies strongly suggest that in this system, activation but not inactivation of the Notch pathway using XSu(H) constructs, drives retinal precursors out of the cell cycle prematurely.

### Notch and proneural genes in gliogenesis and neurogenesis

In this paper, we show that Notch pathway activation, in the form of overexpressed XSu(H)Ank, strongly enhances the RGC inductive activity of Xath5. It also enhances the RGC inductive activities of Xath3, Xngnr-1, and XNeuroD (data not shown). In the *Xenopus* retina, expression of Notch pathway genes (*XNotch-1*, *XDelta-1*, *ESR1* and *ESR3*), starts before the expression of the atonal family of proneural genes (Perron et al., 1998). Thus, it does not appear that Notch expression can inhibit the expression of proneural genes in this region. This is in contrast to the effect of activating the Notch pathway in the neural plate, which clearly downregulates the expression of some proneural genes. By analogy with the role of Notch in the induction of *atonal* in the *Drosophila* eye disc (Li and Baker, 2001), high levels of Xath5 expression in the *Xenopus* CMZ even in the presence of active Notch are not surprising. It is interesting that blocking the Notch pathway by lipofection of XSu(H)DBM or Delta-Stu leads to the induction of early neural fates and the suppression of late glial fates. This is similar to the phenotype caused by activating the Notch pathway by XSu(H)Ank or Notch-ICD in the presence of proneural genes like *Xath5*. Our original lipofection technique targets retinal precursors very early, before they naturally begin to express proneural genes. Thus, it may be that in the former case, what is important is blocking the lateral inhibition pathway leading to de-repression of Xath5, whereas in the latter case, as Xath5 is artificially expressed, the lateral inhibition pathway is less important than the early cell cycle exit, which enhances Xath5 function. In an attempt to begin to address the question of whether the Notch pathway enhances the RGC inducing activity in cells that express Xath5 normally, we transfected XSu(H)Ank at later stages, under the assumption that doing so would be less likely to inhibit endogenous Xath5 expression and would therefore be more likely to result in coexpression of XSu(H)Ank and endogenous Xath5, thus driving cells out of the cell cycle while they express endogenous Xath5. We observed a relative increase in the ratio of RGCs to Müller cells with later transfections confirming the stage dependency of Notch driven phenotypes, consistent with this possibility. While, it is therefore reasonable to consider that activation of Notch signalling in the cells expressing Xath5 potentiates its cell fate determination activity by promoting cell cycle exit in vivo, we must point out that this experiment does not distinguish between the possibility that XSu(H)Ank has this temporal effect by inhibiting other proneural genes that drive other fates, or driving cells out of the cell cycle early. Indeed, it may do both.

The Müller cell inducing activities of both p27Xic1 and XSu(H)Ank are strongly suppressed by the proneural genes tested in the *Xenopus* retina (Fig. 3B, Fig. 5D) (Ohnuma et al., 1999). These proneural genes also inhibit endogenous glial determination in the *Xenopus* retina (Kanekar et al., 1997; Perron et al., 1999). Indeed, overexpression of any of several proneural bHLH genes, including *Mash1*, *Math2*, *Math3*,

*Ngn1*, *Ngn2*, *NeuroD*, or *NeuroD2*, inhibits differentiation into Müller glial cells in rat retina (Cai et al., 2000). Sun et al., reported that, in cortical cell culture, neurogenin-1 inhibits astrocyte differentiation by sequestering a CBP-Smad1 transcription complex away from astrocyte differentiation genes (Sun et al., 2001). While the proneural genes appear to be dominant when co-expressed with glial inducers such as p27Xic1 and XSu(H)Ank, it is also clear that these glial inducers when lipofected on their own prevent neurogenesis. Activation of Notch is known to inhibit primary neurogenesis induced by Xash3 in early *Xenopus* embryogenesis (Chitnis and Kintner, 1996). Similarly, when Xath5 is co-overexpressed with Notch-ICD from the cleavage stage by mRNA injection, Xath5 induction of RGCs in the retina is inhibited (Schneider et al., 2001). This result is contrary to our present findings using lipofection at later stages where Notch promotes the function of co-expressed proneural genes. This discrepancy might be explained in part by the zinc-finger gene transcription factor XMyT1 (Bellefroid et al., 1996), which is expressed in the differentiating retina but not in early stages of development (Perron et al., 1999). When XMyT1 is simultaneously overexpressed by RNA injection into blastomeres, activated Notch no longer inhibits the ability of Xath5 to induce RGCs (Schneider et al., 2001). Therefore the effect of Notch on neural versus glial development is highly dependent on the molecular context of its activation, e.g. the co-expression state of the proneural genes, of XMyT1 and of p27Xic1.

It is not clear how Notch function affects the cell cycle in this system, but it may seem surprising that Notch activity by itself tends to keep retinal cells undifferentiated, while when it is activated conjointly with either proneural or glial determinants, it enhances their respective functions. One potential explanation for this is it that Notch activation leads cells to pause at G<sub>1</sub> but does not promote full exit to G<sub>0</sub>, thus delaying differentiation. In the retina then, Notch alone might 'suspend' the cells from differentiating until they receive a cue to exit the cell cycle fully. The proneural genes or p27Xic1 could provide such exit cues, so that when functioning with Notch activity, they bias cells towards early neural or late glial fates, whereas Notch activation alone simply causes a delay or block of differentiation. We find that overexpression of cyclin E1 which promotes G<sub>1</sub> and S phase and prevents exit to G<sub>0</sub>, inhibits early differentiation, suggesting that full cell cycle arrest at this point may be critical for normal histogenesis.

### A model of retinal histogenesis

Based on the observations reported in this paper, we propose a hypothetical model of the interactions between cell cycle regulation and cell fate determination which contribute to conserved histogenic process of different cell type fates arising at different developmental stages (Fig. 6). In this model, the timing of cell cycle exit influences cell fate, and is thus important for the process of histogenesis. Taking our cue from the CMZ, we propose that at early stages, the cell cycle activators are expressed in the absence of any of the determination genes (Fig. 6A,B), and so cells cycle without differentiating. Next, Notch pathway genes begin to be expressed at high levels and as a result cell cycle activator expression decreases (A. Vernon and A. P., unpublished data). The *atonal* proneural genes come on next and reach high levels in the presence of Notch. These proneural genes act as

transcription factors that push cells down a neural pathway and they inhibit gliogenesis (Fig. 6C left). Notch activation causes some proneural-expressing cells to leave the cell cycle at this point and thus, it seems, enhances the determination activities of these proneural genes. It may be that high levels of proneural gene expression at the time of cell cycle exit help determine the fate of these cells, not unlike the case in *Drosophila* neuroblasts in which the transcription factor expressed at the time the ganglion mother cell is born is critical for its fate (Isshiki et al., 2001). The Notch pathway eventually suppresses the transcription of proneural genes, leading to a decrease in proneural expression at the same time that the bi-functional cell cycle inhibitor and glial inducer p27Xic1 is upregulated (Fig. 6A). This changes the balance from neural to glial and the combined activity of Notch and p27Xic1 drive these last dividing cells out of the cell cycle toward a glial fate (Ohnuma et al., 1999) and (Fig. 6C right). While this model may not be accurate in detail, the results presented here support a critical aspect of it, i.e., early cell cycle withdrawal enhances proneural function and thereby early neuronal fates. Conversely, late cell cycle withdrawal inhibits proneural function and pushes cells towards later fates. This interplay between the cell cycle machine and the determination machine helps to co-ordinate retinal histogenesis.

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