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A directional Wnt/β-catenin-Sox2-proneural pathway regulates the transition from proliferation to differentiation in the *Xenopus* retina

Michalis Agathocleous[1], Ilina Iordanova[1], Minde I. Willardsen[2], Xiao Yan Xue[1], Monica L. Vetter[2,*,†], William A. Harris[1,*,†] and Kathryn B. Moore[2]

Progenitor cells in the central nervous system must leave the cell cycle to become neurons and glia, but the signals that coordinate this transition remain largely unknown. We previously found that Wnt signaling, acting through Sox2, promotes neural competence in the *Xenopus* retina by activating proneural gene expression. We now report that Wnt and Sox2 inhibit neural differentiation through Notch activation. Independently of Sox2, Wnt stimulates retinal progenitor proliferation and this, when combined with the block on differentiation, maintains retinal progenitor fates. Feedback inhibition by Sox2 on Wnt signaling and by the proneural transcription factors on Sox2 mean that each element of the core pathway activates the next element and inhibits the previous one, providing a directional network that ensures retinal cells make the transition from progenitors to neurons and glia.

KEY WORDS: Sox, Neuron, Progenitor, Proneural, Retina, Wnt, *Xenopus*

INTRODUCTION

Multipotent retinal progenitors must solve two fundamental problems. First, they must initially expand their numbers but later limit their proliferation so that the right number of differentiated cells is produced at the appropriate developmental time. Second, the distinct processes of division and differentiation must be coordinated so that differentiation can be initiated when cells stop dividing (Harris, 1997; Livesey and Cepko, 2001).

Limiting proliferation while promoting differentiation involves extensive cross-talk between molecular components of the cell cycle and the cell fate machinery (Ohnuma and Harris, 2003). Examples include intercellular signals, such as Delta-Notch, which affect cell proliferation while inhibiting neuronal differentiation (Dorsky et al., 1997; Henrique et al., 1997; Jadhav et al., 2006; Schneider et al., 2001), and intrinsic transcription factors, such as the proneural bHLH genes, which bias progenitors to exit the cell cycle and assume particular neuronal fates (Hatakeyama and Kageyama, 2004; Vetter and Brown, 2001). Factors such as these help to ensure that cells do one thing or the other, but do not account for how cells progress along this proliferation-to-differentiation axis.

In the canonical Wnt/β-catenin signaling pathway, Wnt ligands bind to Frizzled (Fz) receptors, and the binding initiates a cascade that results in the translocation of β-catenin to the nucleus, where it partners with TCF/LEF transcription factors to activate transcription (Logan and Nusse, 2004). Wnt promotes cell proliferation in multiple tissues (Michaelidis and Lie, 2008), in particular in the developing retina (Denayer et al., 2008; Kubo et al., 2003; Kubo et al., 2005; Yamaguchi et al., 2005). Wnt can also inhibit neural retina differentiation, in some cases promoting ciliary body and iris fates (Cho and Cepko, 2006; Liu et al., 2007; Ouchi et al., 2005). It is puzzling, then, that neurogenesis requires Wnt/β-catenin signaling in a number of systems (Otero et al., 2004; Zhou et al., 2006).

The SoxB1 family of genes (Sox1-3) may be key effectors of Wnt/β-catenin signaling in the developing nervous system (Lee et al., 2006; Van Raay et al., 2005). During neurogenesis, Sox2 antagonizes proneural genes and can maintain progenitors (Bylund et al., 2003; Graham et al., 2003). In the frog retina, Wnt/β-catenin signaling through Fz5 is necessary for Sox2 expression, which is required for proneural gene expression and the transition from progenitors to neurons (Van Raay et al., 2005).

The requirement for both Wnt/β-catenin signaling and Sox2 during neurogenesis appears at odds with the finding that Wnt and Sox2 favor proliferation and progenitor cell maintenance. We therefore investigated the effects of Wnt, Sox2 and proneural genes on each other and on retinal progenitor cells in a variety of misexpression and transgenesis studies in *Xenopus*. We discovered that these factors are core components of a conserved hierarchical cascade and propose that they form a powerful directional network that drives cells from a proliferative, undifferentiated state to a non-proliferative, differentiated neuronal or glial fate.

MATERIALS AND METHODS

Embryo collection

Xenopus laevis embryos were obtained by in vitro fertilization, dejellied in 2% cysteine (pH 8.0), grown in 0.1×MMR or 0.1×MBS (Falk et al., 2007) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

Constructs

Constructs used have been described previously: pCS2-Sox2, pCS2-Sox2BD(–), pCS2-Sox2EnR (Kishi et al., 2000; Mizuseki et al., 1998b), pCS2-TCF3-VP16, pCS2-dnTCF3 (Molenaar et al., 1996; Vonica et al., 2000), pCS2-dnLEF1 (Kengaku et al., 1998), pCS2-Act-β-catenin (Baker et al., 1999), TOP-dGFP (Dorsky et al., 2002), and pCS2-Xath5-hGR (Moore et al., 2002), which is induced with 4 μg/ml dexamethasone (Sigma).

[1]Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK. [2]Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132, USA.

*These authors contributed equally to this work
†Authors for correspondence (monica.vetter@neuro.utah.edu; harris@mole.bio.cam.ac.uk)

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Transgenesis procedure

The generation of transgenics was carried out as described (Kroll and Amaya, 1996) with modifications (Hutcheson and Vetter, 2002). Embryos were collected at stage 23 or 34 and analyzed for GFP expression by whole-mount anti-GFP staining.

In vivo transfection and injections

DNA was lipofected at stages 15-18 into the presumptive retina (Holt et al., 1990; Ohnuma et al., 2002b), or electroporated at stage 22-23 (Falk et al., 2007). pCS2-eGFP, pCS2-GAPGFP or pCS2-mRFP (monomeric RFP) was co-lipofected to mark transfected cells. Sox2 (300 pg), DeltaSTU or Su(H)DBM (200-400 pg) mRNA was injected into one dorsal animal blastomere at the 8-cell stage. Statistical significance was assessed using Student's *t*-test and the binomial test.

In situ hybridization

In situ hybridization with digoxigenin-labeled antisense mRNA probes was performed as described (Van Raay et al., 2005). Probes used were: *Xath5* (Kanekar et al., 1997); *Xbrn3d* (Hutcheson and Vetter, 2001); *Xdelta-1* (Dorsky et al., 1997); pBS-*Xebf3* (Pozzoli et al., 2001); pXelc-3 (*ElrC*) (Good, 1995); *En2* (Hemmati-Brivanlou et al., 1991); pBS-XETOR (Cao et al., 2002); pGEMT-*Xgadd45g* (de la Calle-Mustienes et al., 2002); Hermes (Patterson et al., 2000); pBSXNeuroD (Lee et al., 1995); pCMV-SPORT6-NKL (IMAGE clone 4058009) (Lennon et al., 1996); *Xnotch-1* (Coffman et al., 1990); *X-ngnr-1* (Ma et al., 1996; Sommer et al., 1996); *Xrx1* (Mathers et al., 1997); *Xpax6* (Hirsch and Harris, 1997); *Sbt1* (Logan et al., 2005); *Xsix6* (or *XOptx2*) (Zuber et al., 1999); *Xsox2* (Mizuseki et al., 1998a); *Vsx1* (D'Autilia et al., 2006).

BrdU/EdU labeling and immunostaining

BrdU or EdU (10 mM) (Salic and Mitchison, 2008) was injected intra-abdominally 2 hours before fixation to mark cycling cells, or repeatedly every 8-10 hours for birthdating (Ohnuma et al., 1999). Immunostaining was performed on 10-µm sections using primary antibodies: rabbit anti-CRALBP [1:1000; a gift from Dr J. Saari (Bunt-Milam and Saari, 1983)], mouse anti-Cyclin A2 [1:100, a gift from Tim Hunt and Anna Philpott (Howe et al., 1995)], mouse anti-PCNA (1:200, Santa Cruz Biotechnology), rabbit anti-phospho-histone H3 (1:500, Upstate), rabbit anti-GFP (1:1000, Molecular Probes or Torrey Pines), mouse IgM R5 (1:2) (Drager et al., 1984), mouse monoclonal anti-GFP (1:500, Roche), rabbit anti-Sox2 (1:500, Chemicon ab5603), mouse anti-BrdU (1:80, clone BMC 9318, Roche) and rat anti-BrdU (1:100, Abcam ab6326). Secondary antibodies were: Alexa Fluor 568 goat anti-rabbit (1:2500), Alexa Fluor 488 goat anti-rabbit, (1:2000), Alexa Fluor 488 goat anti-mouse (1:1000) (all Molecular Probes), anti-mouse FITC-adsorbed (1:200) and anti-rat biotin-adsorbed (1:200) (all Jackson) with Neutravidin-Alexa 350 at 1:200 (Molecular Probes). For Sox2 staining, sections were heated at 95°C for 10 minutes in 10 mM sodium citrate (pH6), and for BrdU they were bathed in 2M HCl for 20 minutes. EdU staining was performed according to the manufacturer's instructions (Invitrogen). Cell nuclei were counterstained with 0.1 µg/ml DAPI (Sigma).

Flow cytometry

Single-cell suspensions of retinal tissue were obtained as described (Locker et al., 2006), fixed in paraformaldehyde and stained with 0.2 µg/ml DAPI. Samples were analyzed on a CyanADP (Dako) flow cytometer. Data were acquired using Summit (Dako) and analyzed with FlowJo (Tree Star).

RESULTS

Canonical Wnt signaling activation blocks retinal neuron differentiation in *Xenopus*

Canonical Wnt/β-catenin signaling through the Fz5 receptor is required for neuronal differentiation in the *Xenopus* retina (Van Raay et al., 2005). We were therefore interested in activating this pathway to drive neurogenesis. We co-lipofected a GFP marker with TCF3-VP16, a modified version of the transcriptional effector of canonical Wnt signaling in which the β-catenin-binding domain has been replaced with the VP16 transcriptional activator (Vonica et al., 2000), into cells of the eye fields of stage 17 embryos (see Fig. S1 in the supplementary material). At stage 40/41, when all the cells in the central retina are fully differentiated, a significant fraction of the TCF3-VP16-transfected cells (identified by the co-lipofected GFP marker), retained neuroepithelial morphologies characteristic of retinal progenitor cells (RPCs) (Fig. 1A), with large cell bodies and

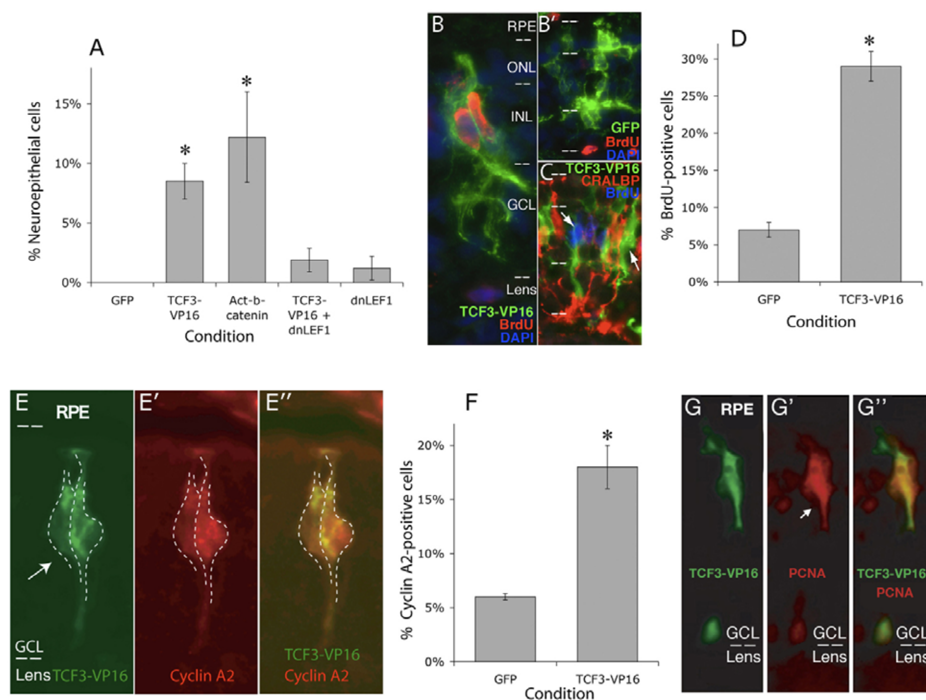

Fig. 1. Canonical Wnt signaling promotes progenitor cell maintenance. (**A**) Lipofection of TCF3-VP16 or activated β-catenin increases the proportion of neuroepithelial (NE) cells as compared with GFP controls. The effect was blocked by co-expression of dominant-negative (dn) LEF1, whereas dnLEF1 alone had no significant effect on the proportion of NE cells (GFP, *n*=6 retinas, 391 cells; TCF3-VP16, *n*=7, 519 cells; activated β-catenin, *n*=5, 211 cells; dnLEF1+TCF3-VP16, *n*=6, 297 cells; dnLEF1, *n*=3, 321 cells). (**B**,**B′**) Cells transfected with TCF3-VP16 show typical neuroepithelial morphology and incorporate BrdU (B), unlike control cells (B′). (**C-G″**) TCF3-VP16-expressing cells with long processes do not stain for the Müller glial cell marker CRALPB (C), they incorporate BrdU more frequently than controls (D), and are positive for Cyclin A2 (E-F) and PCNA (G-G″). Error bars indicate s.e.m. $^*P<0.001$ by Student's *t*-test. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

DEVELOPMENT

processes spanning the retina (Fig. 1B). Moreover, they incorporated BrdU, indicating that they were still actively cycling (Fig. 1B,D). By contrast, cells transfected with GFP alone showed typical neuronal morphologies, with cell bodies and processes restricted to one layer, and were BrdU negative (Fig. 1B′). Müller cells are the only retinal cell type somewhat similar in shape to neuroepithelial cells, but the TCF3-VP16-transfected cells were negative for the Müller cell marker CRALBP (Bunt-Milam and Saari, 1983) (Fig. 1C, arrows). TCF3-VP16 transfection resulted in a 4-fold increase in the proportion of cells expressing Cyclin A2 (Fig. 1E-E″,F) (Vernon and Philpott, 2003) and stained for the ubiquitous cell cycle marker PCNA (Fig. 1G-G″) (Maga and Hubscher, 2003). Thus, many of the retinal progenitors with activated canonical Wnt signaling failed to differentiate, retaining morphological and proliferative characteristics of RPCs.

A constitutively active β-catenin construct, from which the GSK3-β phosphorylation sites have been removed (Baker et al., 1999), also resulted in 12% of transfected cells retaining neuroepithelial morphology, comparable to the fraction obtained by TCF3-VP16 lipofection. When TCF3-VP16 was antagonized by co-transfection with dominant-negative LEF1 (Molenaar et al., 1996), less than 2% of the transfected cells showed RPC-like morphologies (Fig. 1A). These results strongly suggest that the promotion of neuroepithelial cells is a specific effect of the canonical Wnt/β-catenin pathway.

Canonical Wnt signaling blocks neuronal differentiation downstream of proneural genes

The progression of retinal progenitors to differentiated neurons depends upon the sequential expression of a number of genes (Perron et al., 1998). To determine where in this cascade Wnt activation blocks differentiation, we generated transgenic embryos (Kroll and Amaya, 1996) expressing constitutively active β-catenin under the control of the retinal-specific Rx1A promoter (Rx:Act-β-catenin). The promoter is activated in the early eye field (Zhang et al., 2003; Zuber et al., 2003) so that all retinal progenitors have enhanced Wnt signaling. The first retinal-specific genes expressed in RPCs are the eye field transcription factors (EFTFs), which specify retinal identity. Expression of the EFTFs Rx1, Vsx1 and Six6 at stage 23 was not affected by Wnt activation (Fig. 2A,B and see Table S1 in the supplementary material), suggesting that this early phase of establishing RPC identity proceeded normally. The expression of En2, which marks the midbrain/hindbrain boundary, was also normal (Fig. 2A), indicating that Wnt activation in retinal progenitors does not alter general anteroposterior patterning. Activation of Wnt signaling also did not diminish Sox2 expression (Fig. 2C).

We next asked whether retinal differentiation was blocked after Wnt activation owing to a failure to initiate proneural gene expression. In the chick retina, Wnt2b overexpression downregulates proneural gene expression (Kubo et al., 2005), so this might be a possibility. However, we found that expression of the proneural genes Xath5, NgnR and Xash3 was normal in the Rx:Act-β-catenin transgenic embryos, indicating that canonical Wnt activation does not inhibit their expression in the *Xenopus* retina (Fig. 2E,F and see Table S1 in the supplementary material).

If proneural genes are unaffected, perhaps Wnt activation affects the next step in the differentiation cascade, i.e. the proneural target genes and differentiation markers. Strikingly, we found that the expression of all the proneural target genes we tested, including Brn3d (Hutcheson and Vetter, 2001) (Fig. 2G,H), Ebf3 (Pozzoli et al., 2001) (Fig. 2I,J), ElrC (Logan et al., 2005) (Fig. 2K,L), Gadd45

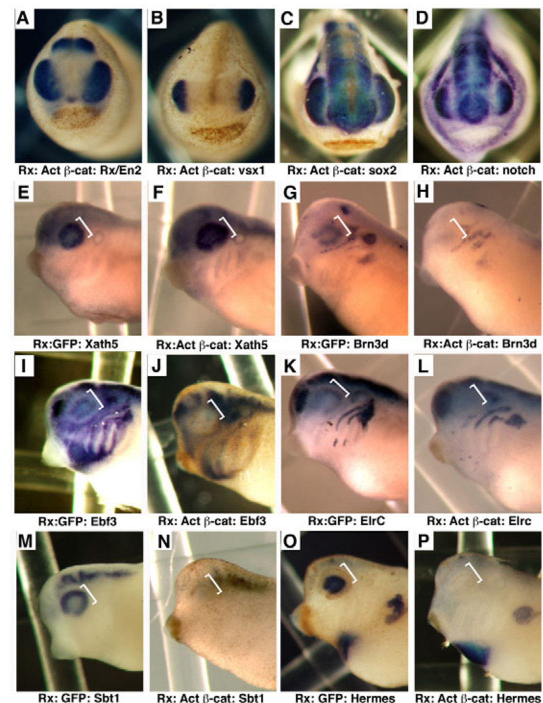

Fig. 2. Canonical Wnt signaling does not affect retinal identity but blocks neuronal differentiation downstream of the proneural genes. (**A**,**B**) Rx:Act-β-catenin (Act β-cat) transgenic embryos (stage 23) show normal expression of Rx and En (A) and of the retinal progenitor marker Vsx1 (B). (**C**,**D**) Act β-cat transgenic embryos show normal expression of Sox2 (C), a gene required for neural competence, and of the neurogenic gene Notch (D). (**E**,**F**) Act β-cat transgenic embryos (stage 34) show normal expression of the proneural gene Xath5. (**G-P**) Act β-cat transgenic embryos show loss of retinal expression of the proneural target genes Brn3D (G,H), Ebf3 (I,J), ElrC (K,L) and Sbt1 (M,N), as well as of Hermes (O,P), a marker of differentiated ganglion cells. Brackets indicate the retina.

(Logan et al., 2005) (see Table S1 in the supplementary material), Sbt1 (Logan et al., 2005) (Fig. 2M,N) and Xetor (Logan et al., 2005) (see Table S1 in the supplementary material), was absent or dramatically reduced in Rx:Act-β-catenin embryos as compared with Rx:GFP control embryos. Loss of expression was observed only in the areas where the Rx transgene is expressed. This suggests that canonical Wnt/β-catenin pathway activation prevents retinal neuron differentiation by blocking the expression of proneural target genes.

We also observed loss of expression of the retinal ganglion cell (RGC) marker Hermes, the bipolar markers Otx2 and Vsx1, and the bipolar/photoreceptor marker Otx5b in the Rx:Act-β-catenin transgenic embryos at stage 34 (Fig. 2O,P and see Table S1 in the supplementary material). Gadd45, Sbt1 and Xetor are expressed in late progenitors or early postmitotic neurons, whereas ElrC and Ebf3 are expressed in RGCs and in some inner nuclear layer cells (Logan et al., 2005), and Brn3d is expressed in differentiated RGCs, suggesting that the block in proneural gene action by Wnt is not specific to a particular neuronal cell fate, but is a global effect on neuronal differentiation. Together, these data suggest that Wnt pathway activation arrests development at the neural-competent progenitor state, in which EFTFs, Sox2 and the proneural genes are expressed but further differentiation is blocked because proneural gene function is compromised.

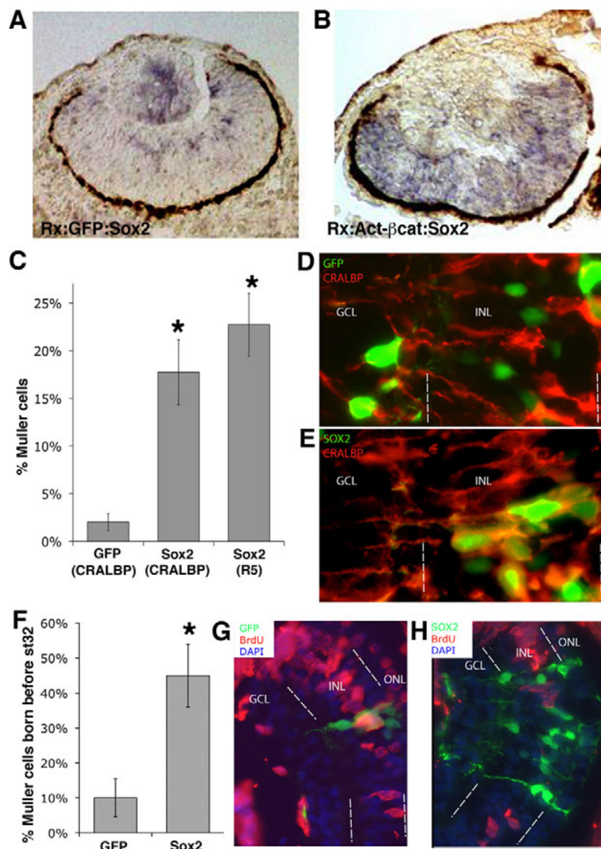

Fig. 3. Sustained Sox2 expression promotes Müller glial cell formation. (**A**,**B**) Sox2 expression is expanded in Act β-cat transgenic embryos (A) as compared with control (B; Rx:GFP) transgenic embryos. (**C-E**) Lipofection of Sox2 causes a large increase in the representation of CRALBP- or R5-positive Müller cells [D, GFP, *n*=8, 942 cells; E, Sox2 (CRALBP), *n*=7, 485 cells; Sox2 (R5), *n*=9, 616 cells]. **P*<0.001. (**F-H**) Sox2 causes an increase in the proportion of Müller cells born before stage 32, which are BrdU negative (G, GFP, *n*=5, 30 cells; H, Sox2, *n*=3, 31 cells). Error bars indicate s.e.m. **P*<0.001.

Sox2 expression also inhibits neuronal differentiation in the *Xenopus* retina

In the neural tube, Sox2 inhibits the activity of proneural genes and maintains the neural progenitor state (Bylund et al., 2003; Graham et al., 2003), and in the retina Sox2 is downstream of canonical Wnt (Van Raay et al., 2005). Sox2 is mostly downregulated by stage 39 in the central differentiated retina, but expression is maintained in the still growing ciliary marginal zone (Fig. 3A). We found that Wnt activation is sufficient to maintain Sox2 expression throughout the central retina at this late stage (Fig. 3B).

We next tested whether this sustained Sox2 expression in retinal progenitors is sufficient to prevent neuronal differentiation. Lipofection of Sox2 did indeed block neurogenesis; however, in contrast to TCF3-VP16-transfected cells, Sox2-transfected cells adopted a Müller glial morphology (Fig. 3C), were positive for the Müller cell markers CRALBP and R5 (Drager et al., 1984) and did not incorporate BrdU (Fig. 3D,E). We tested whether the promotion of Müller cells by Sox2 could also occur at Sox2 levels that could inhibit proneural activity, by injecting Sox2 mRNA into one dorsal animal blastomere at the 32-cell stage [targeting over 60% of the retina (Huang and Moody, 1993)]. Over an 80-fold range of dosages, we observed a significant increase in the proportion of CRALBP$^+$ Müller glia cells (400 pg Sox2 mRNA, 23±1.35%, *n*=12, 2514 cells versus control, 5.2±0.66%, *n*=9, 1880 cells; 100 pg, 22.0±1.4%, *n*=11, 2305 cells; 25 pg, 23.3±2.06%, *n*=7, 1914 cells; 5 pg, 16.7±1.51%, *n*=5, 862 cells). The results of these Sox2 lipofections and injections suggest that Sox2, over a wide range of levels, specifically promotes Müller glia.

We also wondered whether the increase in Müller cells caused by Sox2 overexpression could be due to a weak phenocopy of a constitutive Wnt/β-catenin signaling effect, in that extra Sox2 might maintain the progenitor fate for longer, so that when these Sox2-transfected cells eventually differentiate, they do so in a glial-promoting late environment. After continuous BrdU administration from stage 32 to 40, only 10% of control Müller cells were BrdU negative, i.e. born before stage 32, as compared with 45% of Sox2-transfected Müller cells (Fig. 3F-H). Therefore, Sox2 does not promote the glial fate by delaying cell cycle exit and differentiation.

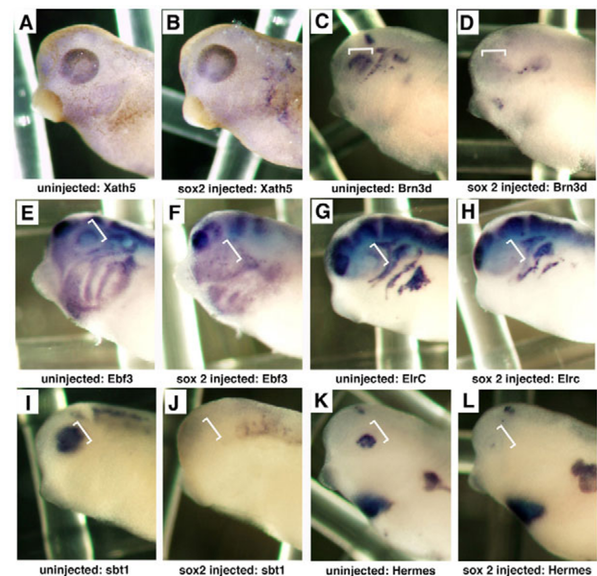

Fig. 4. Sox2 blocks neuronal differentiation downstream of the proneural genes. (**A**,**B**) Overexpression of Sox2 in retinal progenitors by mRNA injection at the 8-cell stage does not decrease the expression of the proneural gene Xath5 on the injected side (B), as compared with the control side (A), in stage 34 embryos. (**C-L**) Retinal expression of the proneural targets Brn3d (C,D), Ebf3 (E,F), ElrC (G,H) and Sbt1 (I, J) is lost, as compared with the control (uninjected) side of the embryos. Sox2-injected embryos also lose retinal expression of Hermes (K,L), a marker of differentiated ganglion cells. Brackets indicate the retina.

Sustained Sox2 expression blocks proneural function

Although Sox2 promotes gliogenesis and Wnt/β-catenin promotes progenitor maintenance, it is important to remember that neither of these cell types are neurons. Thus, it is a strong possibility that maintained Sox2 expression, although not an exclusive effector of the Wnt pathway in this system, might still be the primary reason why activation of Wnt/β-catenin signaling prevents neuronal differentiation. If so, Sox2 should block neurogenesis at the same step as activation of Wnt/β-catenin signaling. To address this, we overexpressed Sox2 by mRNA injection at the 8-cell stage and assayed the developing retina for changes in the expression of genes in the differentiation cascade by in situ hybridization at stage 23. No change was observed in the EFTF progenitor markers Vsx1, Rx or

Six6 (see Table S1 in the supplementary material). Similarly, the retinal expression of the proneural genes Xath5 (Fig. 4A,B; *n*=22) and Nngr1 (see Table S1 in the supplementary material) at stage 34 was unaffected by Sox2 overexpression. However, just as in Rx:Act-β-catenin embryos, the expression of proneural target genes and markers of differentiated neurons was consistently absent or dramatically reduced in almost all embryos: Brn3d (absent or dramatically reduced in 90% of embryos; Fig. 4C-D), Ebf3 (75%; Fig. 4E,F), ElrC (94%; Fig. 4G,H), Gadd45 (84%; see Table S1 in the supplementary material), Xetor (87%; see Table S1 in the supplementary material), Sbt1 (90%; Fig. 4I,J) and Hermes (96%; Fig. 4K,L) were lost or their expression delayed in stage 34 embryos. Thus, Sox2 expression appears to cause retinal progenitors to become blocked at the same step in the neurogenic program as does activated Wnt/β-catenin signaling.

Notch is required for Wnt/β-catenin signaling and Sox2 to block retinal progenitor differentiation

Notch is a good candidate for the anti-neuronal differentiation effects of both Wnt/β-catenin and Sox2 signaling, as it antagonizes proneural genes in the retina, in particular by suppressing their activity (Dorsky et al., 1995; Schneider et al., 2001). The expression of Notch and its ligand Delta in the *Xenopus* retina is dependent on canonical Wnt/β-catenin signaling and Sox2 expression (Van Raay et al., 2005), and in the mouse retina Notch is a direct target of Sox2 (Taranova et al., 2006).

To test the role of Notch signaling downstream of the Wnt-Sox2 pathway, we co-lipofected TCF3-VP16 with DeltaSTU, a truncated version of the Notch ligand Delta, which blocks Notch signaling (Chitnis et al., 1995). DeltaSTU almost completely abolished the effect of TCF3-VP16 in maintaining cells in an undifferentiated state, resulting in less than 1% neuroepithelial cells (Fig. 5A). Blocking Notch by injection of DeltaSTU mRNA in Rx:Act-β-catenin transgenic embryos was also sufficient to restore the expression of the proneural target genes Brn3d (70% of embryos showed retinal expression; Fig. 5B), ElrC (71% retinal expression; Fig. 5C), Sbt1 (75% retinal expression; Fig. 5D), as well as of the differentiation marker Hermes (82% retinal expression; Fig. 5E, see Fig. 2 for comparison). Similarly, concurrent overexpression of DeltaSTU with Sox2 by mRNA injection at the 8-cell stage restored expression of Brn3d (83% retinal expression; Fig. 5F), ElrC (100% retinal expression; Fig. 5G), Sbt1 (89% retinal expression; Fig. 5H) Ebf3, Gadd45, Xetor (see Table S1 in the supplementary material) and Hermes (78% retinal expression; Fig. 5I, see Fig. 4 for comparison).

To test whether Notch signaling is required cell-autonomously for Sox2 to inhibit neurogenesis, we co-lipofected Sox2 and a dominant-negative construct Su(H)DBM, which inhibits the Suppressor of Hairless intracellular effector of Notch (Wettstein et al., 1997). Su(H)DBM abolished the effect of Sox2 in promoting Müller glia (Fig. 5J-L). The same result was observed upon co-injection of Sox2 and Su(H)DBM mRNAs at the 32-cell stage [Sox2, 22±4.9%, *n*=2, 400 cells; Sox2+Su(H)DBM, 4.7±1.7%, *n*=3, 341 cells; Su(H)DBM, 5.0±2.1%, *n*=4, 337 cells; GFP, 4.2±2.7%, *n*=4, 347 cells; data not shown]. These findings suggest that Wnt/β-catenin signaling drives progenitors into a neural-competent state, in which Sox2 and then proneural genes are expressed, but the further differentiation of these cells is blocked through Notch signaling.

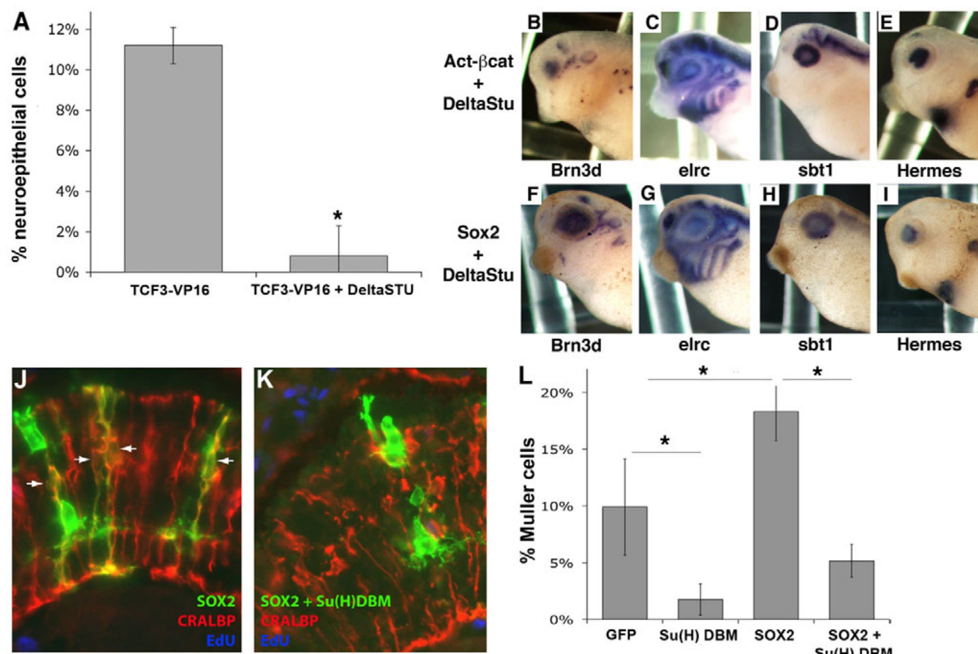

Fig. 5. Blocking Notch signaling suppresses the ability of Wnt/β-catenin signaling or Sox2 to inhibit neuronal differentiation. (**A**) Co-expression of TCF3-VP16 and a dominant-negative form of Delta (DeltaSTU) that blocks Notch signaling in retinal progenitors, inhibits the TCF3-VP16-mediated increase in neuroepithelial cells (TCF3-VP16, *n*=7, 405 cells; TCF3-VP16+DeltaSTU, *n*=5, 234 cells). Error bars indicate s.e.m. **P*<0.001. (**B-E**) Act β-cat transgenic embryos (stage 34) injected with DeltaSTU mRNA show restored retinal expression of the proneural target genes Brn3D (B), ElrC (C) and Sbt1 (D), and of Hermes (E), a marker of differentiated ganglion cells. (**F-I**) Expression of Sox2 and DeltaSTU in retinal progenitors by mRNA injection at the 8-cell stage also restored retinal expression of Brn3D (F), ElrC (G), Sbt1 (H) and Hermes (I). (**J-L**) Lipofection of Sox2 promotes CRALBP$^+$/EdU$^-$ Müller cells (J arrows, L), whereas co-transfection with Su(H)DBM produces CRALBP$^-$ cells (K,L) (GFP, *n*=4, 192 cells; Su(H)DBM, *n*=6, 342 cells; Sox2, *n*=5, 868 cells; Sox2+Su(H)DBM, *n*=9, 892 cells). Error bars indicate 95% confidence interval (C.I.). *P<0.001.

Wnt activation promotes retinal progenitor proliferation, whereas Sox2 activation does not

If both Wnt/β-catenin activation and Sox2 overexpression prevent neuronal differentiation through the same mechanism, why is it that Sox2 promotes gliogenesis whereas Wnt/β-catenin promotes progenitor maintenance? It seemed reasonable to hypothesize that at least some of the differences in the effects of Sox2 and Wnt activation might be due to differential stimulation of proliferation. TCF3-VP16 or Sox2 were electroporated at stage 23, which results in expression of the constructs around the start of neurogenesis (Falk et al., 2007). TCF3-VP16 caused a 5-fold increase in the proportion of BrdU-positive cells in the central retina at stage 37 (Fig. 6B,D) as compared with controls (Fig. 6A,D), whereas Sox2 had no effect (Fig. 6C,D). The DNA profiles of transfected retinal cells were assessed by flow cytometry at stage 37 (Fig. 6E). We found that TCF3-VP16, but not Sox2 or GFP, transfection doubled the number of cells in the S/G2/M phases, indicating a larger proliferating cell fraction (Fig. 6F and inset). Thus, a fundamental difference between Wnt/β-catenin pathway activation and Sox2 overexpression in retinal progenitors is the ability to stimulate proliferation. Activated caspase staining did not detect abnormally increased apoptosis after TCF3-VP16 or Sox2 lipofection or electroporation (see Fig. S2 in the supplementary material).

These results show that Sox2 alone is not sufficient for enhanced proliferation; however, stimulation of Sox2 might still be necessary for the increase in proliferation downstream of Wnt/β-catenin signaling. To test this, we activated Wnt/β-catenin signaling while blocking Sox2 activity using Sox2BD(–), a dominant-negative Sox2 construct lacking the DNA-binding domain. This construct specifically inhibits Sox2-mediated function in *Xenopus* (Kishi et al., 2000). Co-transfection of TCF3-VP16 with Sox2BD(–) (Fig. 6G) did not abrogate the TCF3-induced increase in proliferation [TCF3-VP16, 18.0±1.9% BrdU-positive cells; TCF3-VP16+Sox2BD(–), 19.2±2.2%], although not all cells had a neuroepithelial morphology (data not shown), suggesting that Sox2 might contribute to other aspects of the neuroepithelial phenotype. Co-transfection with another dominant-negative construct, Sox2EnR (Kishi et al., 2000), gave similar results (TCF3-VP16+Sox2EnR, 18.5±3.0% BrdU-positive cells; data not shown). These results suggest that the canonical Wnt pathway stimulates cell proliferation independently of Sox2.

Sox2 and cell cycle activation can recapitulate the effects of Wnt/β-catenin signaling on retinal progenitor cell maintenance

Our results suggest that there are two facets to the neuroepithelial fate: inhibition of neuronal differentiation and maintenance of an actively dividing state. Wnt/β-catenin signaling activation promotes both aspects, whereas Sox2 activation is only able to achieve the former. We therefore predicted that activation of the cell cycle and Sox2 together should replicate the effects of Wnt/β-catenin signaling alone. Indeed, co-lipofection of Sox2 with Cyclin E1 (Vernon and Philpott, 2003) significantly increased the proportion of BrdU-positive neuroepithelial cells in the central retina (Fig. 7B-D; 16.7±1.8%), as compared with Cyclin E1 (6.5±1.0%), Sox2 (0.2±0.2%) or GFP (0.9±0.4%) (Fig. 7A,D), to the levels caused by Wnt/β-catenin signaling activation (compare with Fig. 1). At the same time, co-transfection with Cyclin E1 inhibited the increase in Müller cells observed with Sox2, suggesting that cells that were destined to become Müller glia in response to Sox2 instead remain progenitors when their cell cycle is concurrently activated. Additionally, Sox2+Cyclin E1 significantly increased the proportion of mitotic cells marked with phospho-histone H3 (Fig. 7H,I; 4.7±1.0%), as compared with Sox2 (Fig. 7F,I; 0%), Cyclin E1 (Fig. 7G,I; 0.7±0.7%) or control GFP (Fig. 7E,I; 0.1±0.2%), suggesting that the co-transfected cells are, like normal neuroepithelial cells, both synthesizing DNA and dividing.

Negative-feedback loops may drive the progression of the Wnt-Sox2-Proneural pathway

If Wnt/β-catenin signaling drives cells into a proliferative, neural-competent progenitor state, how do these progenitors ever progress out of the cell cycle, and how do they escape Sox2 suppression of neuronal differentiation?

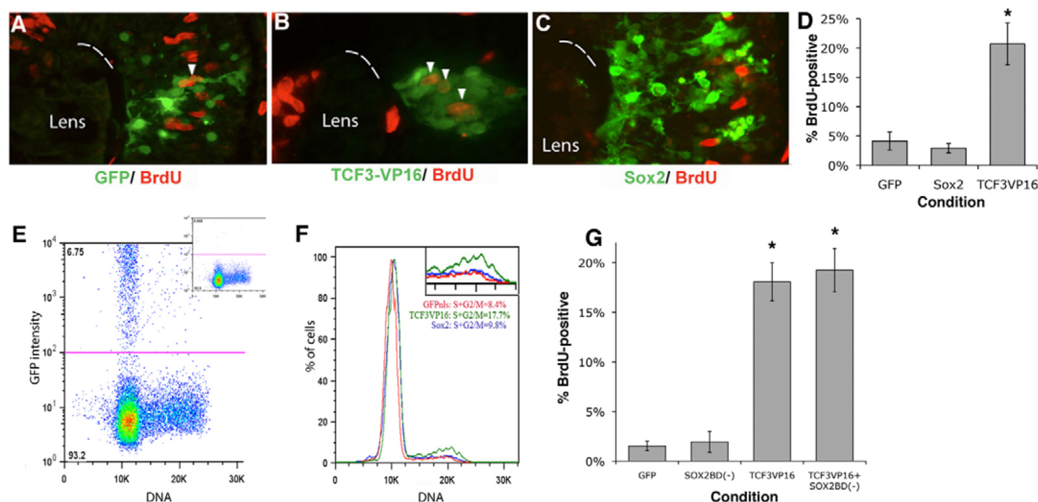

Fig. 6. Wnt/β-catenin activation, but not Sox2, promotes cell proliferation. (**A-D**) Electroporation with TCF3-VP16 (*n*=5, 493 cells), but not Sox2 (*n*=3, 1719 cells), significantly increases the proportion of BrdU$^+$ cells (arrowheads) at stage 37, as compared with control GFP (*n*=4, 653 cells). (**E**) GFP, Sox2 and TCF3-VP16 were electroporated and proliferation assessed by flow cytometry at stage 37. Transfected cells display a GFP intensity above background, whereas non-electroporated retinas do not have any GFP$^+$ cells (inset). (**F**) TCF3-VP16 transfection causes a 2-fold increase in the proportion of cells in the S/G2/M phases, as compared with Sox2 or GFP transfection, indicating a larger proliferating cell fraction (see inset). (**G**) Co-lipofection of the dominant-negative Sox2BD(–) does not inhibit BrdU incorporation caused by TCF3-VP16 at stage 41 (GFP, *n*=12, 2615 cells; Sox2BD(–), *n*=9, 670 cells; TCF3-VP16, *n*=11, 1539 cells; TCF3-VP16+Sox2BD(–), *n*=10, 1242 cells. Error bars indicate 95% C.I. **P*<0.001 compared with GFP control.

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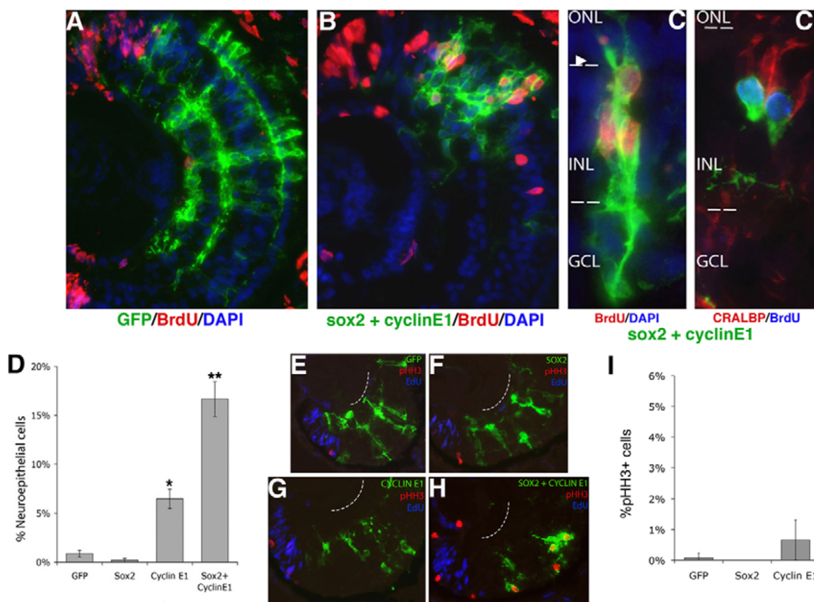

Fig. 7. Sox2 and Cyclin E1 can recapitulate the effects of Wnt/β-catenin signaling on the proliferation and maintenance of neuroepithelial morphology. (**A**) GFP-lipofected retinas show a typical cell type distribution at stage 41. (**B-C′**) Lipofection of Sox2+Cyclin E1 results in BrdU-positive cells that do not stain for the Müller cell marker CRALBP (C′). (**D**) The increase in BrdU-positive, neuroepithelial-like cells is significant in the double transfection versus the Cyclin E1-only control (***P*<0.001). Cyclin E1 lipofection causes a slight increase in BrdU-positive central retina cells (GFP, *n*=10, 2642 cells; Sox2, *n*=11, 2043 cells; Cyclin E1, *n*=13, 2388 cells; Sox2+Cyclin E1, *n*=10, 1688 cells. Error bars indicate 95% C.I. **P*<0.001. (**E-I**) Co-expression of Sox2 and Cyclin E1 promotes mitosis, as seen with anti-phospho-histone H3 staining (H,I), in contrast to overexpression of GFP (E), Sox2 (F) or Cyclin E1 (G) alone (GFP, *n*=10, 1308 cells; Sox2, *n*=6, 474 cells; Cyclin E1, *n*=6, 605 cells; Sox2+Cyclin E1, *n*=11, 1697 cells). Error bars indicate 95% C.I. ***P*<0.005.

It has been reported in several contexts that Sox family genes inhibit canonical Wnt signaling (Kan et al., 2004; Mansukhani et al., 2005; Zorn et al., 1999). We therefore assessed the effects of Sox2 expression on the levels of the canonical Wnt reporter TOP-FLASH (TOP-dGFP) in the retina by co-injecting plasmids for TOP-dGFP [which, in the frog retina, is widely expressed in cells with an active Wnt pathway (Van Raay et al., 2005)] and mRFP (to label all the injected cells), with or without Sox2 at early blastula stages. When just mRFP and TOP-dGFP were co-injected, 62% of mRFP-positive retinal cells at stage 33 were also GFP positive, but when Sox2 was also overexpressed, the number of mRFP-positive GFP-positive cells decreased to 33% (Fig. 8A-C). To confirm this by an alternative approach, Sox2 and mRFP were co-injected at blastula stages, and then later, at stage 17, TOP-dGFP DNA was lipofected into the eye field. Thus, in the same retina, some TOP-dGFP-lipofected cells had been injected with mRFP+Sox2 and some had not. In control retinas, 48% of the GFP-positive cells were mRFP-positive, but when mRFP-positive cells also expressed Sox2 this fell to 30% (Fig. 8D-H), as GFP-positive (active Wnt signaling) cells were excluded from Sox2-expressing domains. These results show that canonical Wnt signaling is antagonized by Sox2 expression.

SoxB1 members can directly bind to, and inhibit, β-catenin via their C-terminus, independently of transcription (Kan et al., 2004; Mansukhani et al., 2005; Zorn et al., 1999). Sox2BD(–), which lacks the DNA-binding domain but retains the C-terminus, had a similar activity to Sox2 itself in suppressing TOP-dGFP expression (Fig. 8H). This suggests that *Xenopus* Sox2 inhibits Wnt signaling independently of DNA binding, and probably transcription (Kelberman et al., 2008). Thus, Wnt/β-catenin is necessary for Sox2 expression (Van Raay et al., 2005) and Sox2 in turn is able to quench Wnt/β-catenin signaling.

Could a negative-feedback mechanism be responsible for turning off Sox2? Work in the neural tube suggested that Sox2 is inhibited by proneural gene overexpression (Bylund et al., 2003). We

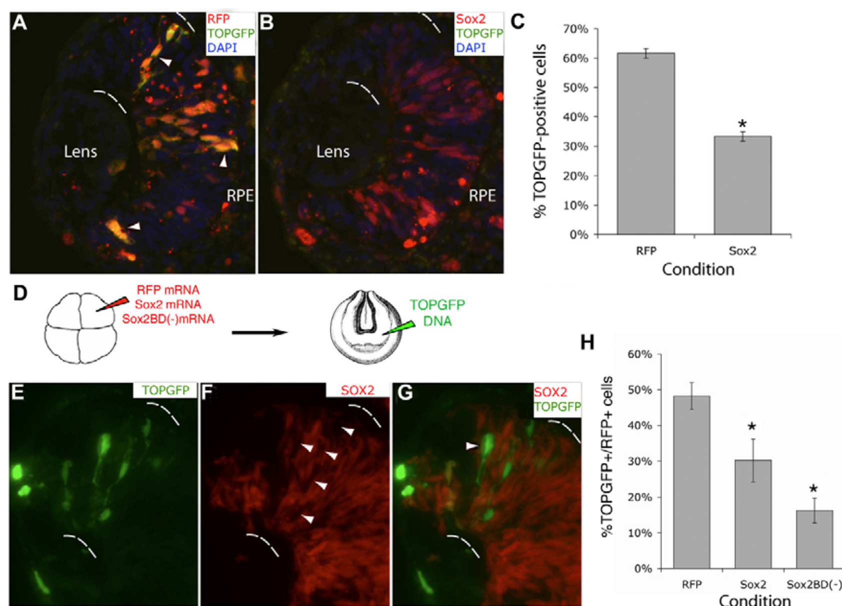

Fig. 8. Sox2 inhibits canonical Wnt/β-catenin signaling. (**A**,**B**) mRFP-only-injected cells co-express the TOP-dGFP reporter (A, arrowheads), unlike Sox2-injected cells (B). (**C**) The proportion of TOP-dGFP$^+$ cells out of the total injected (RFP$^+$) cells is reduced in the presence of Sox2 (mRFP, *n*=13, 3471 cells; Sox2, *n*=10, 3420 cells). (**D**) mRNAs for mRFP (tracer) alone, mRFP+Sox2 or mRFP+Sox2BD(–) (dominant-negative Sox2) were injected into dorsal animal cells of cleavage-stage embryos. TOP-dGFP DNA was subsequently lipofected into stage 17 optic vesicles. (**E-G**) In a Sox2-injected embryo, the cells that express GFP (E) are those that do not express Sox2 (gaps indicated by arrowheads in F). (**H**) In retinal cells expressing Sox2 or Sox2BD(–), there is a 2-fold reduction in the proportion of TOP-dGFP$^+$ cells that express mRFP (mRFP, 682 cells; Sox2, 228 cells; Sox2BD(–), 437 cells). Error bars indicate 95% C.I. **P*<0.01.

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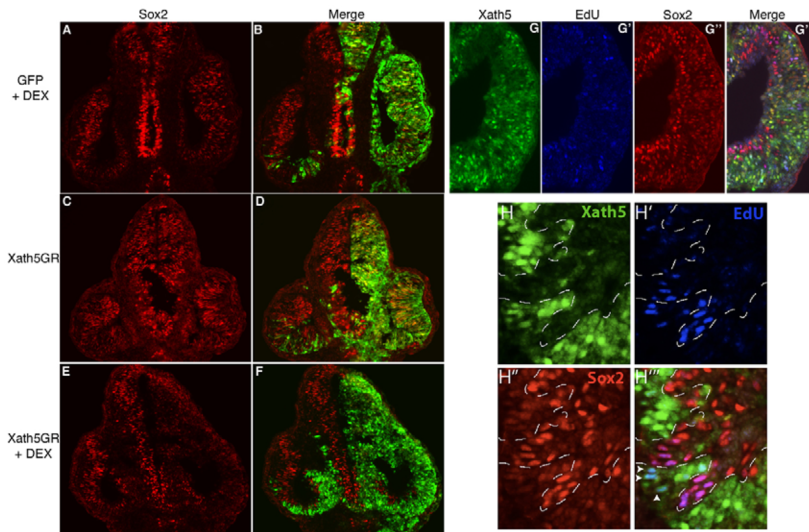

Fig. 9. Xath5 injection suppresses Sox2 protein levels. (**A-F**) Control embryos injected with GFP alone and dexamethasone (DEX) treated (A,B), or co-injected with Xath5GR and GFP without DEX (C,D), did not show downregulation of Sox2 protein in the injected areas. By contrast, embryos co-injected with Xath5GR and GFP and DEX treated (E,F) displayed a pronounced Sox2 downregulation in Xath5-expressing cells. (**G-H‴**) Staining 6 hours after the start of DEX treatment shows a downregulation of Sox2 (G″) and EdU (G′) in areas of the retina that overexpress Xath5GR (G). Areas overexpressing Xath5GR are outlined in a magnified view in H-H‴. Occasionally, a reduction in Sox2 is seen in cells that are still cycling (arrowheads, H‴).

therefore tested whether proneural genes in the retina could inhibit Sox2, using blastomere injections of mRNA encoding the proneural protein Xath5 fused to the glucocorticoid receptor (Xath5GR), followed by dexamethasone (DEX) treatment at stage 18 to activate Xath5GR when Sox2 is already present in the retina. Xath5GR+DEX embryos exhibited a dramatic disappearance of Sox2 protein at stage 26, whereas Sox2 remained high in controls (Fig. 9A-F). Levels of Sox2 were already reduced in Xath5GR embryos 6 hours after DEX addition, in parallel with a reduction in EdU incorporation (Fig. 9G-G‴,H′,H‴) but preceding neurogenesis, as at this time cells with reduced Sox2 signaling were still distributed throughout the neuroepithelium, did not display an RGC morphology (Fig. 9G-H‴) and did not stain for the RGC markers Isl-1 and Hermes (data not shown). Since maintaining Sox2 prevents neurogenesis, even though Xath5 is still present (Figs 3, 4), these results suggest that the downregulation of Sox2 by Xath5 is necessary for neuronal differentiation.

DISCUSSION

Wnt/β-catenin signaling acting through Sox2 activates proneural gene expression in the frog retina (Van Raay et al., 2005). We now show that Wnt and Sox2 inhibit proneural action through Notch, thereby blocking neuronal differentiation. In addition, Wnt signaling stimulates proliferation independently of Sox2, maintaining the progenitor fate, while Sox2 pushes retinal progenitors to Müller glial fates. Concurrent activation of Sox2 and the cell cycle can recapitulate the effects of Wnt in maintaining the RPC fate. Finally, inhibition of Wnt signaling by Sox2, and of Sox2 by the proneural transcription factors, facilitates a transition from proliferation to differentiation, thereby ensuring that progenitors progress forwards to a differentiated state (see Fig. 10 and Fig. S3 in the supplementary material).

The regulation of neurogenesis and gliogenesis by Wnt/β-catenin signaling and Sox2

These results tie together disparate strands in the function of Wnt/β-catenin and Sox2 signaling as investigated in various vertebrate models. Sox2 both sets up neural potential (Kishi et al., 2000; Taranova et al., 2006; Van Raay et al., 2005) and inhibits terminal neuronal differentiation (Bylund et al., 2003; Graham et al., 2003). The present study shows that Sox2 plays a central role in suppressing retinal neurogenesis downstream of Wnt/β-catenin signaling, but it enhances Müller glial differentiation and does not maintain progenitors. Similarly, Sox2 overexpression increases Müller cells in mouse retinal explants (Lin et al., 2009) and promotes the in vitro differentiation of neocortical progenitors into astroglial cells (Bani-Yaghoub et al., 2006). Notch activation by Sox2 may be involved in this gliogenic effect, as activated Notch signaling promotes gliogenesis (Furukawa et al., 2000; Ohnuma et al., 2002a; Scheer et al., 2001). Therefore, either the absence of proneural gene expression (Van Raay et al., 2005) or the suppression of proneural activity allows retinal progenitors to adopt the glial fate.

The Wnt pathway is activated in the peripheral retina near the ciliary marginal zone in other species besides *Xenopus* (Cho and Cepko, 2006; Denayer et al., 2008; Liu et al., 2007). Yet, Wnt activation in the chick causes cells to be blocked in a proneural-negative progenitor state (Kubo et al., 2005) and in the mouse they assume non-neuronal peripheral fates (Cho and Cepko, 2006; Liu et al., 2007). In chick and mouse, Wnt signaling does not appear to regulate Sox gene expression; however, the suppression of neurogenesis via activation of Wnt/β-catenin is common to the frog, chick and mammalian retina (Burns et al., 2008; Cho and Cepko, 2006; Fu et al., 2006; Liu et al., 2007).

There is strong evidence for connections between Wnt/β-catenin, SoxB1 and proneural genes in the regulation of neural differentiation in other tissues. In the zebrafish hypothalamus, canonical Wnt signaling, acting via Sox3, is necessary for the expression of proneural and neurogenic genes (Lee et al., 2006). LRP mutant mice exhibit dramatic hypoplasia of the developing neocortex owing to a reduction in neurogenesis as well as in proliferation (Zhou et al., 2006). Similarly, in the adult hippocampus, Wnt activation promotes both neurogenesis and stem cell proliferation in a dissociable manner (Lie et al., 2005), which fits with our explanation that Wnt/β-catenin signaling sets up neuronal potential but then suppresses differentiation and maintains progenitor cells.

Wnt couples the suppression of neuronal differentiation with the maintenance of proliferation in promoting the progenitor fate

Our results suggest two parallel aspects of the progenitor cell fate: the suppression of neuronal differentiation and the maintenance of proliferative ability, controlled by two branches of Wnt signaling, one of which is Sox2 dependent. This model fits with

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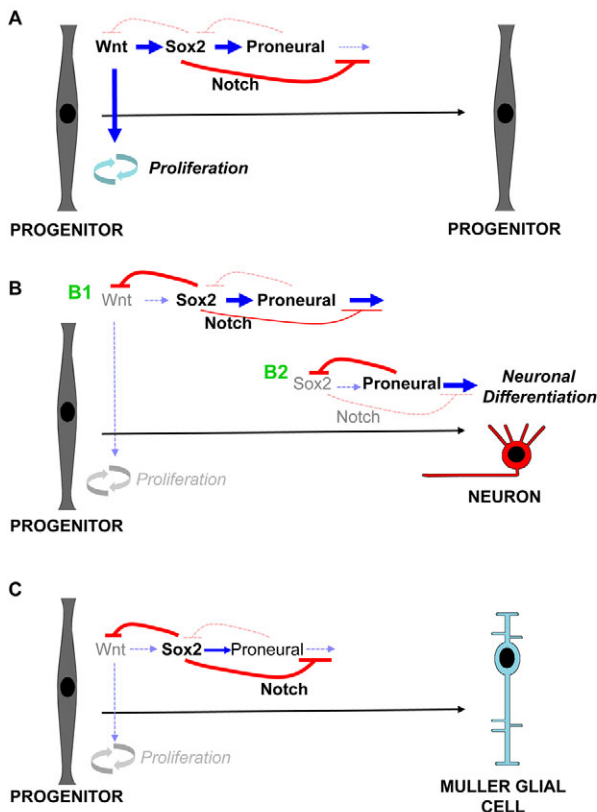

Fig. 10. A model for the role of the Wnt-Sox2 pathway in the transition from a progenitor to a differentiated cell. (**A**) Wnt signaling activation in a neuroepithelial cell activates Sox2 and the proneural genes, but through Sox2 and Notch it blocks proneural activity and neuronal differentiation. Wnt also independently maintains proliferation, and this leads to progenitor maintenance and expansion. (**B**) The build-up of Sox2 switches off Wnt, inhibiting proliferation (B1), and then the accumulation of proneural activity switches off Sox2, relieving the inhibition of neuronal differentiation and leading to neurogenesis (B2). (**C**) Alternatively, if Sox2 levels remain high it will limit proneural activity and neuronal differentiation will be blocked, but Wnt signaling will also be inhibited, leading to cell cycle exit and glial differentiation.

findings in the spinal cord that Wnt activates proliferation (Megason and McMahon, 2002), whereas Sox2 does not (Bylund et al., 2003). The parallel control of differentiation and proliferation might be a more general feature of Wnt signaling; for example, in the developing limb, Wnt/β-catenin signaling and Sox9 interact to couple proliferation and chondrocyte differentiation (ten Berge et al., 2008).

If Sox2 is not mediating the proliferative effects of Wnt/β-catenin signaling, other effectors must be involved. Although exogenous Cyclin E1 was able to cooperate with Sox2 in progenitor maintenance, we detected little or no change in Cyclin E1 retinal expression after Wnt signaling perturbations, nor in the expression of other cell cycle activators including Cyclin D1, Cyclin A2, n-Myc and c-Myc (Van Raay et al., 2005) (data not shown; see Table S1 in the supplementary material), suggesting that these genes might not be transcriptional targets in the frog retina. Perhaps other genes might function as Wnt-dependent effectors of proliferation here, or perhaps proliferation is regulated through post-transcriptional mechanisms or by changing the mode of progenitor division (Mizumoto and Sawa, 2007; Nakamura et al., 2005).

Müller cells are transcriptionally very similar to neuroepithelial progenitor cells (Blackshaw et al., 2004). They can divide after injury or provision of growth factors (Dyer and Cepko, 2000; Fischer et al., 2002), at which point they may return to a neuroepithelial-like state (Raymond et al., 2006), perhaps through a Wnt-dependent mechanism (Osakada et al., 2007). These and our results therefore suggest that a crucial distinction between RPCs and Müller cells is a Wnt-mediated capacity to proliferate.

Progression from a proliferative to a neuronal or glial fate is controlled by a cascade of Wnt, Sox2 and proneural genes

For the progression from a progenitor to a neuronal fate, both Wnt/β-catenin signaling and Sox2 must be switched off, relieving the inhibition of proneural activity and stopping proliferation. The inhibition of Wnt by Sox2 is likely to take place during retinogenesis, as Sox2 injections do not result in early defects in the specification of retinal progenitor identity. This therefore suggests a negative-feedback mechanism of Sox2 on Wnt signaling. Interestingly, mutations in human *SOX2* associated with anophthalmia have been mapped to the C-terminal domain, which normally interacts with β-catenin, resulting in an inability of Sox2 to inhibit canonical Wnt signaling in vitro (Kelberman et al., 2008).

For neuronal differentiation to proceed, Sox2 must also be switched off to relieve the inhibition of proneural activity. In the *Xenopus* retina, we found that the proneural bHLH transcription factor Xath5 induced a dramatic reduction of the Sox2 protein. In the cortex, a serine protease cleaves Sox2 specifically in neuronal but not glial precursors, thus relieving the block on neurogenesis (Bani-Yaghoub et al., 2006). It will be interesting to see whether in the retina, proneural genes feed back on Sox2 through this mechanism or through transcriptional repression.

Wnt, Sox2 and the proneural genes appear to form a modular circuit in which each step activates the subsequent step and is in turn inactivated by it, driving cells towards differentiation, while limiting the ability of an external proliferation signal, such as Wnt, to continue signaling indefinitely. The relative levels of Wnt, Sox2 and proneural genes determine where a cell lies along the pathway from proliferation to differentiation and whether it assumes a progenitor, glial or neuronal fate (see Fig. 10 and Fig. S3 in the supplementary material). Understanding fully the function of each interaction in the cascade must await a more quantitative analysis of the relationship between the participating factors. This mechanism of transition from one cell state to another by the integration of directional interactions and feedback loops resembles that reported in diverse systems; for example, during sporulation of *Bacillus subtilis*, where a circuit with five basic nodes displays successive hierarchical gene activations, coupled with negative-feedback loops that switch off the previous state (Eichenberger et al., 2004). Further investigations will reveal whether general aspects of the mechanism that we describe here are at work in other neural and non-neural tissues, and how this directional pathway integrates with other factors that help to coordinate neuronal proliferation and differentiation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/19/3289/DC1

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