Supporting Information

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SI Materials and Methods

Embryos and Injection. *Xenopus* embryos were obtained by in vitro fertilization and staged according to Nieuwkoop and Faber (1). cRNAs were prepared from pCS2 derivatives (Ambion) or pBSRN3. Except when otherwise specified, *MObarhl2* (60 ng) and *MOcasp3* (40 ng) or cRNA-encoding *bcl*-X_L (200 pg) or *hbcl2* cRNA (200 pg) were injected together with *β-gal* or *gfp* cRNA (100 pg) as tracers into one or two dorsal blastomere of four-cell stage embryos. β-catenin morpholino (MO; 10 ng) and cRNA encoding glycogen synthase kinase-3β (*gsk3β*; 100 pg), *gsk3βDN* (100 pg) (2), *barhl2* (50 pg), or *casp3* (100 pg) together with a tracer were injected into the dorsal blastomere, D1.1 or D1.2 (3), of 8- or 16-cell stage embryos. For all rescue experiments, we tested a range of cRNA (50–300 pg) and selected the minimal cRNA quantity that both induced the specific phenotype and displayed no toxicity. Three independent experiments were performed, and the results were pooled. The Caspase3 (Casp3) expression vector was a gift of K. Nakajima (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan) (4). *gsk3β* and *gsk3βDN* expression vectors were gifts of S. Y. Sokol (Mount Sinai School of Medicine, New York) (2).

Antisense MOs. Antisense oligonucleotides coupled to fluorescein were made by Gene-Tools: MOcasp3I: CAAG**ATG**GAAGAATCCCAGAATGGT; MOcasp3ct: CAAG**GTGT**AAG**C**ATC**A**CAGAAT**A**GT; MOcasp3II: GTTTGGATCAGATCGGGTTTTGGTA; and MOßcat: TTTCAACCGTTTCCAAAGAACCAGG. The underlined nucleotides are the mutated ones compared with MOcasp3I sequence. The initiating ATG is indicated in bold. Both MOs were used for each type of experiment. Because the effects of MOcasp3I and MOcasp3II were similar, data were pooled and referred to as MOcasp3.

RT-PCR. Total RNA from embryos was extracted using the Qiagen RNeasy Kit. RT-PCR analysis was carried out with the SuperScript One-Step Kit (Invitrogen) using specific primers for *ncam*, *chordin*, *otx2*, *foxg1*, *barhl2*, and *histone-H4* as previously described in ref. 5 and 5′GGGGATCCATGGAAGAATCCCAGAATGGTG3′ and 5′TCGAACCATCCTCCTCTCCATGGC3′ for *casp3*. Water was used as a negative control.

Whole-Mount in Situ Hybridization. Double and single in situ hybridization (ISH) were performed using digoxigenin- or fluorescein-labeled probes as previously described (6) with modifications (7). The ectoderm overlying the anterior neural tube together with the eyes was removed before ISH. The neural tubes of chosen specimens were dissected in PBS-Tween 0.1% and stored in 90% glycerol. For flat-mounted embryos, the neural tubes were bisected along the dorsal and ventral midlines with a tungsten needle and mounted in 90% glycerol. The same settings were used for image acquisition of both control and injected sides of whole-mount ISH of each dissected neural tube to allow for direct comparison. In this way, we have a complete view of both forebrain and midbrain territories, and we compare the extent of expression territory between the control and injected sides of the embryo. For transverse sections, the specimens were embedded in 3% agarose, and sections (50 μm) were cut on a Leica VT1000E vibratome.

X-Gal Staining. Embryos were fixed in paraformaldehyde (PFA) 4% for 30 min, washed in phosphate buffer, and transferred into Red-Gal (Research Organics) staining solution (5).

Protein Isolation and Western Blot Analysis. Total protein from embryos was extracted as previously described (5). Nuclear and cytoplasmic extracts were prepared with the Compartmental Protein Extraction Kit (K3013010; BioChain). In each case, 10 μg protein were used for Western blot analysis. Antibodies against activated Caspase-3 (1/1,000, #9661; Cell Signaling Technology), β-catenin (dilution 1/1,000, #610154; BD Transduction Laboratories), active β-catenin in which Ser37 and Thr41 are dephosphorylated (dilution 1/300, #05–665; Millipore), α-actin (dilution 1/500, #ab1801; ABCAM), and Sam 68 (dilution 1/500, sc-333; Santa Cruz) were used.

TUNEL Staining and Cell Death Detection by ELISA. Whole-mount TUNEL staining and detection of cytoplasmic nucleosomal DNA were performed as described (5). TUNEL staining was performed on stage 18–20 embryos or stage 26–27 and 33 dissected neural tubes, excluding the retinas and optic stalks.

BrdU Incorporation and Immunohistochemistry. The vitelline membranes of stage 26 *Xenopus* embryos were removed, and the embryos were immersed in a solution of 2.5 μg/μL BrdU (B-5002; Sigma) diluted in 9 g/L NaCl. Embryos were fixed (4% PFA) after various lengths of time as indicated. Embryos were vibratome-sectioned (50 μm), cellular nuclei were stained with bisbenzimide (BB), and BrdU-positive cells or phosphorylated histone ^{3}H (P-^{3}H)-positive cells were revealed by immunostaining. β-gal revelation or GFP immunolocalization were used to mark the injected side. Immunolocalization of BrdU (#347580; Beckson Dickinson), P-^{3}H (#06–570; Upstate Biotechnology), GFP (GFP-1020; Aveslab), and β-catenin (dilution 1/300, C 2206; Sigma) was performed as previously described in ref. 8. *n* indicates the total number of embryos counted. At least three sections were analyzed per embryo.

For BrdU cumulative analysis, we assessed the BrdU labeling index (LI; the proportion of BrdU-labeled cells over the total number of cells) in *MObarhl2*- or *MOcasp3*-injected sides and their respective control sides after different periods of BrdU incorporation starting at stage 26. Histograms indicate the ratio of BrdU-immunopositive cells among BB-positive cells in the injected (red) or control side (blue) after different lengths of time of BrdU incorporation; h indicates the length of time (in hours). A linear regression function was used to calculate the duration of the cell cycle according to Takahashi et al. (9); abscissae indicate time (in hours), ordinates show LI, and Tc and Ts estimate the duration in hours of the total cell cycle and S phase, respectively.

Firefly and Renilla Luciferase Activity. Eight-cell stage embryos were injected into the four animal blastomeres with either Tcf binding sites-cfos-promoter-firefly-luciferase (TOP-Flash) or Tcf mutated binding sites-cfos-promoter-firefly-luciferase (FOP-Flash) (20 pg/blastomere) and pRL (Renilla luciferase, 5 pg/blastomere) plasmids (10). We injected *noggin* cRNA (50 pg/blastomere) to anteriorize the animal caps (ACs) (11), GFP cRNA (20 pg/blastomere) as a tracer, *wnt8b* cRNA (50 pg/blastomere) as a positive control, and water as a negative control. *MObarhl2* (10 ng/blastomere) was injected alone or together with *casp3* cRNA (50 pg/blastomere). *MOcasp3* (10 ng/blastomere) was injected alone or together with *barhl2* cRNA (25 pg/blastomere). The embryos were injected and left to develop until stage 9. ACs were prepared and left to develop until their siblings had reached stage 20. Three ACs were pooled per activity mea-

surement point, and we assessed at least five measurement points for each experimental condition. Proteins were extracted, and a double luciferase assay was performed according to the manufacturer's instructions (Promega) using a bioluminometer TriStar LB 941 (Berthold). Firefly luciferase activity was normalized to Renilla luciferase activity. Three independent experiments were performed.

Confocal Microscopy—Images and Statistical Analysis. The specimens were embedded in 3% agarose, and sections (50 μm) were cut on a Leica VT1000E vibratome. Sections were photographed under a Leica SP2 Visible Laser Confocal Microscope (Leica). Bisbenzimide and BrdU- and P-^{3}H–positive cells were counted on the images using ImageJ software (http://rsb.info.nih.gov/ij/). Each embryo was individually analyzed, and *n* is the number of embryos analyzed. On average, two ImageJ-generated slices of 10 μm per section were counted. All statistical analyses were done using the SPSS 11 program (www.spss.com). Normality of the data distribution between sections of one embryo was assessed using the Shapiro Wilk statistic test (SPSS). Variances between two datasets were confirmed to be insignificant according to the Fisher test. The results are shown as mean ± SEM. The means between two different datasets were compared using the paired sample *t* test, and significant differences are indicated with asterisks.

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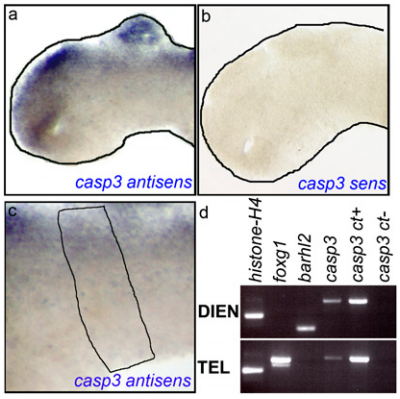

Fig. S1. casp3 and barhl2 are coexpressed in the stage (st) 26 prosomere P2. (*A*–*C*) ISH with *casp3* antisens (*A* and *C*) or *casp3* sens (*B*) probes on st 26 dissected neural tubes. (*A* and *B*) At st 26, *casp3* is mostly expressed in the telencephalic prospective pallium, in the diencephalic and mesencephalic alar plates of the neural tube, and in the pineal gland. (*C*) Enlargement of the prosomere P2 area of *A*. *barhl2* expression domain is indicated with a black line. (*D*) RT-PCR analysis on RNA purified from telencephalic (TEL) or diencephalic (DIEN) st 26 neural tube parts. *Foxg1* and *barhl2* are pallial TEL and DIEN markers, respectively. *Histone H4* and the plasmid encoding Casp3 were used as positive controls, and water was a negative control (ct).

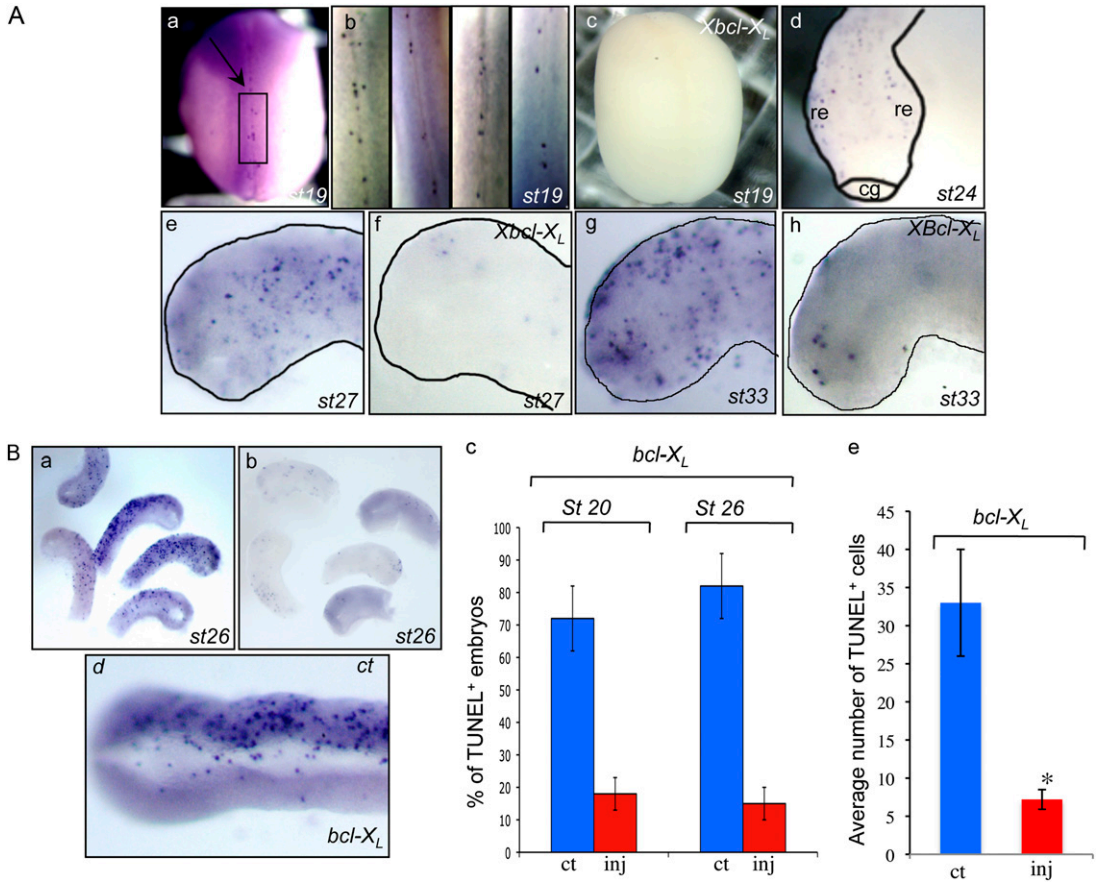

Fig. S2. From st 20 to 33, apoptotic nuclei are uniformly distributed in the anterior neural tube, and Bcl-X_L overexpression protects anterior neural tube cells from apoptosis. (*A*) Apoptosis patterns. (*a* and *b*) TUNEL-positive cells occurred mainly along the dorsal closure line of the neural tube during neurulation (st 18–20; 70%, $n = 122$). (*d*, *e*, and *g*) From st 20 to 33, cells died in the anterior part of the neural tube but were not confined to specific areas ($n \geq 150$ for each stage). (*d*) WT representative embryos at st 24. Representative dissected neural tubes at (*e*) st 26–27 and (*g*) st 33. *bcl-X_L* cRNA was injected into one dorsal blastomeres of four-cell embryos together with GFP as a tracer. TUNEL staining was performed on these embryos at (*c*) st 18–20, (*f*) 26 and 27, and (*h*) 33. (*a*–*c*) Dorsal view, anterior up; (*b*) an enlargement of WT st 19 embryos. (*d*) Anterior view, dorsal up. (*e*–*h*) Lateral view, anterior to the left. Re, retina; cg, cement gland. (*B*) *Bcl-X_L* overexpression prevents apoptosis. (*a*–*c*) *bcl-X_L* cRNA was injected into two dorsal blastomeres at the four-cell stage. TUNEL staining of (*a*) control or (*b*) *bcl-X_L*–injected representative dissected neural tubes is shown at st 26. (*c*) Comparison of the percentage of TUNEL-positive embryos (defined as having more than 30 apoptotic nuclei) at st 20 and 26 in control embryos (ct) and in embryos injected in the two dorsal blastomeres at the four-cell stage with *bcl-X_L* cRNA (inj). Each experimental batch ($n \geq 60$) was assessed independently. (*d* and *e*) *bcl-X_L* cRNA was injected into one dorsal blastomere at the four-cell stage. (*d*) TUNEL staining of a representative *bcl-X_L*–injected dissected neural tube is shown at st 26 (dorsal view, anterior to the left). (*e*) Average numbers of TUNEL-positive cells on the control or injected sides at st 26 of *bcl-X_L*–injected anterior neural tubes (forebrain to midbrain; $n = 14$, *bcl-X_L*–injected vs. ct, $P \leq 0.002$).

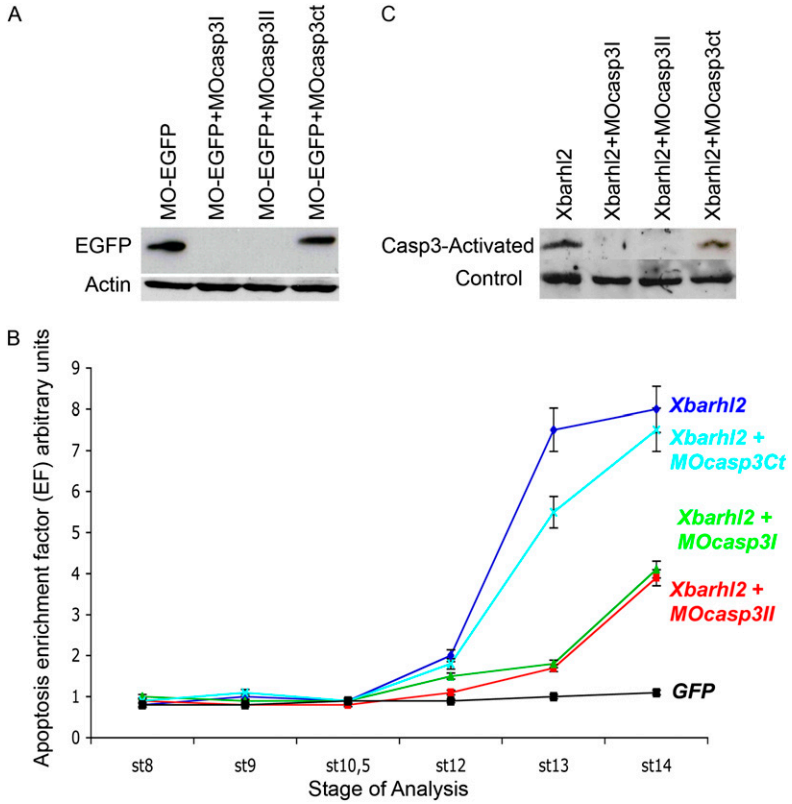

Fig. S3. MO against Casp3 protect *Xenopus* embryos from barhl2-induced apoptosis. Two MOs, *MOcasp3I* and *MOcasp3II*, were designed to inhibit endogenous Casp3 activity together with a control MO, *MOcasp3ct*. We used three different means to evaluate the capacity of the Casp3 MO to specifically block endogenous Casp3 activity. (*A*) MOcasp3I and MOcasp3II bind their complementary sequences and inhibit translation in embryos. The 5′ end of the *casp3* gene containing the *MOcasp3I* and *MOcasp3II* sequences was subcloned in-frame upstream of the *EGFP* cDNA. The 5′-gtttggatcagatcgggttttggtagccaagATGgaagaatcccagaatggt-3′ sequence was subcloned in the ClaI site of the vector pCS2-EGFP to generate the *MO-EGFP* expression vector. Western blotting analysis showed that translation of this construct containing the 5′ end of the *casp3* gene fused upstream of the *EGFP* gene was completely inhibited by coinjection with each specific MO, whereas coinjection with the control MO had no effect. (*A*) Western blot analysis against *EGFP* with 3-μg protein extracts from *Xenopus* st 13 embryos. Translation of the MO-EGFP fusion protein (lane 1) is inhibited by coinjection with *MOcasp3I* (lane 2) or *MOcasp3II* (lane 3), whereas *MOcasp3ct* has no effect (lane 4). α-actin (42 kDa) was used as a loading control. (*B* and *C*) MOcasp3I and MOcasp3II protect *Xenopus* embryos from apoptosis induced by Xbarhl2 overexpression. The protective effect of *MOcasp3I* and *MOcasp3II* against Barhl2-induced apoptosis was shown by ELISA to detect cytoplasmic histone-associated mono- and oligo-nucleosomes, which are specifically released during apoptosis (*B*) or by Western blotting (*C*). (*B*) Kinetics of Barhl2-induced apoptosis between st 8 and st 14 in the presence or absence of *MOcasp3*. Embryos were injected in one dorsal blastomere at the two-cell stage with *EGFP*, *barhl2* cRNA together with *EGFP*, *barhl2* cRNA together with *MOcasp3I*, *MOcasp3II*, or *MOcasp3ct* and collected at the indicated developmental stages. Cell death was measured by an ELISA for cytoplasmic histone-associated mono- and oligo-nucleosomes that are specifically released during apoptosis. The apoptosis enrichment factor (EF) was calculated using GFP-injected embryos as a control. From st 10.5 on, a steady increase in the apoptosis EF can be observed in *barhl2*-injected embryos (EF = 8 at st 14). Coinjection of *barhl2* cRNA with *MOcasp3I* or *MOcasp3II* induces a significant delay in the onset of apoptosis (EF = 4 at st 14), whereas embryos injected with *barhl2* cRNA together with *MOcasp3ct* behave like embryos injected with *barhl2* cRNA alone (EF = 7.8 at st 14). (*C*) Western blot analysis of 15 μg protein extracted from the same batches of embryos at st 13: lane 1, *barhl2* (50 pg) and *EGFP* (50 pg); lane 2, *barhl2* (50 pg) and *MOcasp3I*; lane 3, *barhl2* (50 pg) and *MOcasp3II*; lane 4, *barhl2* (50 pg) and *MOcasp3ct*. A nonspecific band was used as a loading control.

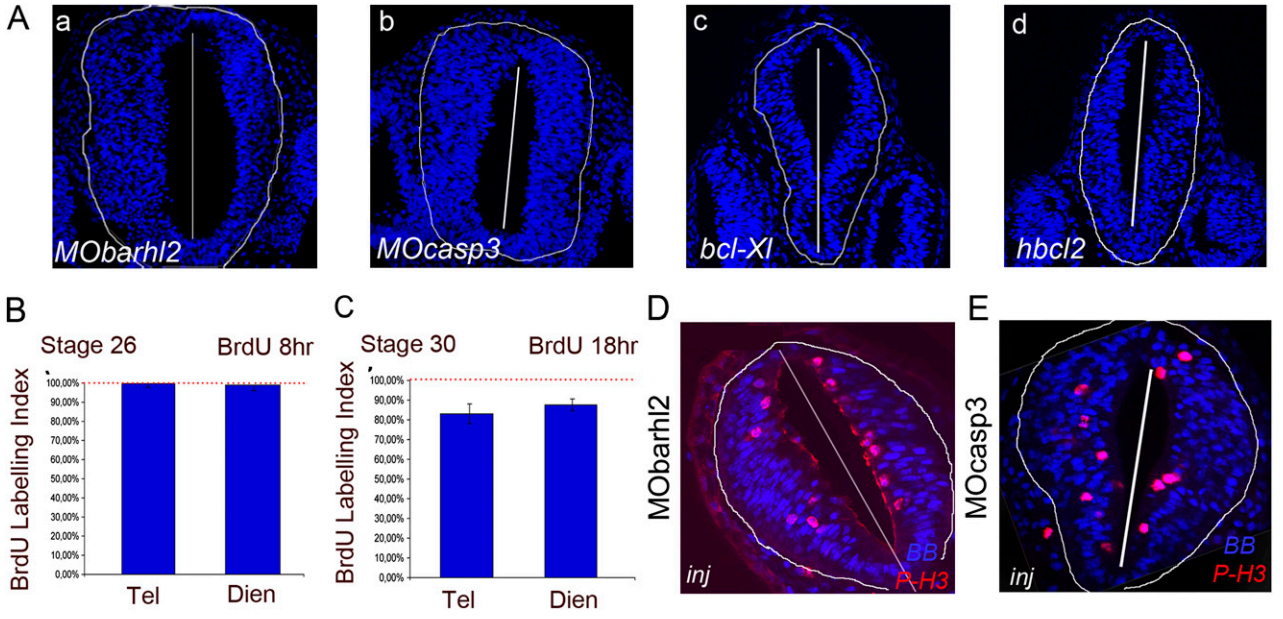

Fig. S4. Depletion of Barhl2 or Casp3 generates neural tube hyperplasia, an increase in the BrdU LI, and ectopic mitosis. (*A*) Depletion of Barhl2 or Casp3 generates neural tube hyperplasia. *Xenopus* embryos were injected with (*a*) MObarhl2-60 (60 ng), (*b*) MOcasp3-40 (40 ng), (*c*) bcl-XL cRNA, or (*d*) hbcl2 cRNA. Representative sections of st 26 embryos at the diencephalic level are shown dorsal side up. Cell nuclei are stained with BB. The white line indicates the midline of the neural tube and the limits of the neuroepithelium. The left side is the injected side. (*B* and *C*) Neuroepithelial cells all proliferate until st 26, and some neuroepithelial cells cease division after this point. The BrdU LI was measured after (*B*) 8 h starting at st 24 until st 26/27 or (*C*) 18 h starting at st 26/27 until st 30 of BrdU exposure in telencephalic and diencephalic sections. Each embryo was assessed independently. (*B*) Between st 24 and 27, 99.7% of telencephalic ($n = 3$) and 98.9% of diencephalic ($n = 3$) neural tube cells had incorporated BrdU and undergone at least one cell cycle. (*C*) Between st 26 and 30, only 84% of telencephalic ($n = 3$) and 88% of diencephalic ($n = 3$) neural tube cells had divided, arguing that some neuroepithelium cells had exited the cell cycle during this period. (*D* and *E*) Ectopic mitosis in Barhl2- and Casp3-depleted st 26 embryos. Representative sections of st 26 embryos at the diencephalic level, shown dorsal side up and immunostained for P-^{3}H (red), merged with BB (blue) images.

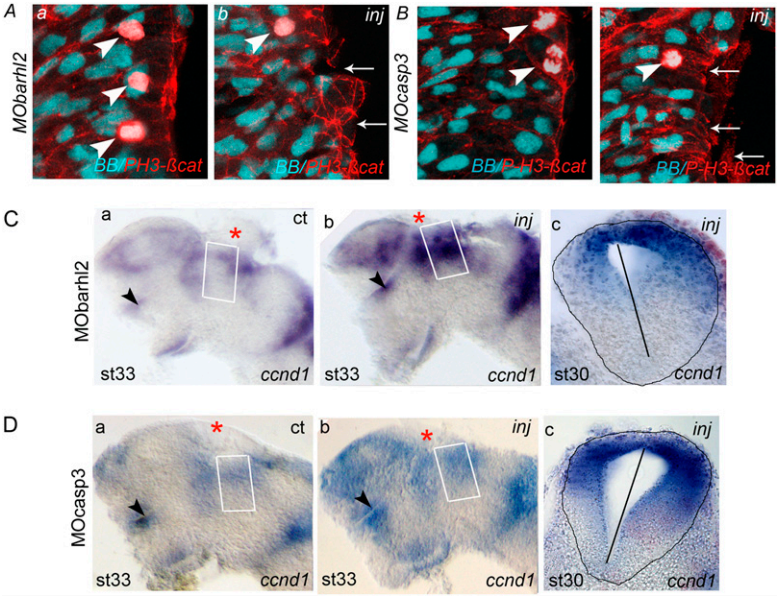

Fig. S5. Barhl2 or Casp3 depletion phenotypic defects. (*A* and *B*) Disorganization of the neuroepithelium. Representative IHC for β-catenin (red; membrane staining) together with P-^{3}H (red; nuclear staining) merged with BB (cyan) images on diencephalic sections of st 26/27 *Xenopus* embryos injected with *MO-barhl2* (*A*) or *MOcasp3* (*B*). The (*Aa*) control and (*Ab*) injected sides of representative sections are shown. Inj, injected side. Arrows indicate part of the apical surface where β-catenin staining is abnormal; arrow end indicates mitotic nuclei. (*C* and *D*) Increase in Wnt signaling in the prosomere P2 alar plate *ccnd1* expression profiles at st 33 or 30 analyzed by ISH. The ct (*Ca*) and injected (*Cb*) sides of one representative dissected neural tube flat-mounted and (*Cc*) a section at the diencephalic level are shown. The percent of embryos exhibiting the phenotype is indicated. (*Ca*) ct and (*Cb* and *Cc*) Barhl2-depleted (89%, $n = 19$); (*Da*) ct and (*Db* and *Dc*) Casp3-depleted neural tubes (82%, $n = 39$). The white rectangle delineates the prosomere P2 alar plate. The black arrow indicates localization of the optic stalk.

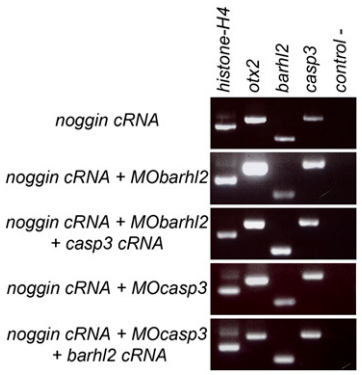

Fig. S6. *otx2*, *Barhl2*, and *casp3* transcripts are present in anteriorized ACs. RT-PCR analysis. *casp3* and *barhl2* transcripts are present in st 20 anteriorized ACs prepared in different conditions. *Otx2* is used as an anterior neuroectodermal marker, *Histone H4* is a positive control, and water is a negative control.

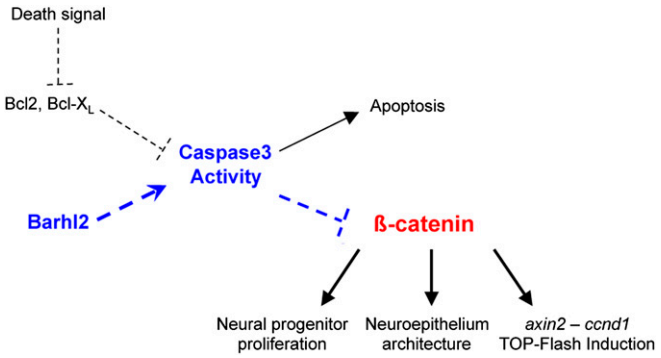

Fig. S7. A model for the genetic pathway for the regulation of β-catenin activity by Barhl2 and Casp3.