Supporting Information

D'Autilia et al. 10.1073/pnas.1000854107

SI Materials and Methods

Animals. To obtain embryos, induction of ovulation of *Xenopus* females, in vitro fertilization, and embryo culture were carried out as described by Newport and Kirschner (1). Embryos were staged according to Nieuwkoop and Faber (2). Embryos were raised at 18 °C on a 12-h/12-h LD cycle with lights on at ZT0 and lights off at ZT12.

In Situ Hybridization. Whole-mount in situ hybridization and in situ hybridization on cryosections were performed as described previously (3). For in situ hybridization on dissected brains, stage 46 embryos were fixed in MEMFA [100 mM Mops (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% (vol/vol) formaldehyde] for 30 min at room temperature. Brains were manually dissected using Dumont number 5 tweezers, postfixed in MEMFA for 30 min at room temperature, and finally stored in 100% (vol/vol) ethyl alcohol at −20 °C.

The *Xbsx* probe was transcribed from the pBS-Xbsx plasmid, which contains the full *Xbsx* coding sequence. The templates for the production of in situ hybridization probes for *Tph* (4), *Xotx5b* (5), *Hermes* (6), and cyclin D1 (7) have been described previously.

Embryo Microinjections and BrdU Incorporation. To make the GR-Xbsx expression construct, the ORF of Xbsx was PCR-amplified using the primers gcAGATATCATGAATTTAAATTTTACTT CCCCTGGG (forward) and GTCCTCGAGCTATAGCAAAT GCTGCG (reverse) and was inserted into the EcoRV/Xho1 sites of $pCS2^+$/GR. Capped synthetic RNAs were generated in vitro by SP6 transcription from pCS2GRXbsx, pCS2mt*XcyclinA2*, pCS2mt*Xcdk2* (8, 9), and pCS2GFP. GR-Xbsx mRNA (50 pg), *MoXbsx* (1 pmol), and cyclin A2 (50 pg)/*cdk2* (50 pg) were coinjected with GFP mRNA (200 pg) into both dorsal blastomeres at the four-cell stage. GR-Xbsx protein was activated at the end of gastrulation (stage 11.5) by adding dexamethasone to the culture medium (final concentration 10^{-5} M). Addition of dexamethasone to control uninjected embryos did not affect pineal cell proliferation and photoreceptor generation.

BrdU was delivered by means of intraabdominal injection at the indicated stage, and embryos were fixed 2 h after the treatment. BrdU incorporation was detected as previously described (10).

Immunostaining. Immunohistochemistry were performed according to the method of Viczian et al. (5). To identify pineal photoreceptors, an anti-RECOVERIN antibody (code AB5585; Chemicon International) was used at a final dilution of 1:100. Cy2 goat anti-mouse (Oregon green, code 011038; Molecular Probes) secondary antibodies were used at a final concentration of 1:500. Hoechst 33258 (final concentration 0.12 g/mL; Sigma) was used to counterstain cell nuclei.

Antisense Oligonucleotide Morpholino. The *Xbsx* antisense morpholino used was: 5′- CTATAAACAAGATGAGACCTGTTAC -3′ (Gene Tools, LLC). A standard morpholino oligo (Gene Tools, LLC) was injected as a control. The specificity of XbsxMo was assayed by Western blot analysis, testing the effects on the translation of Myc-tagged constructs carrying either the WT Xbsx morpholino target sequence (5′-gtaacaggtctcatcttgtttatag-3′) or a five-mismatch mutated sequence (5′-gtaCcTggtGtcatAttgtAtatag-3′) (Fig. S1).

RT-PCR. For RT-PCR, total RNA was isolated from *Xenopus* embryos at the indicated time points starting at stage 26. The corresponding cDNAs were prepared using oligo deoxythymidine primers and superscript reverse transcriptase (Gibco BRL). To amplify Xbsx and ornithine decarboxylase (ODC) cDNA, the following primers were used: Xbsx, ATCAGGGT TGCCAGTACCAG (forward), CTGCGATGGACACAAAT CATC (reverse); and ODC, 5′-AATGGATTTCAGAGACCA-3′(forward), 5′-CCAAGGCTAAAGTTGCAG-3′(reverse).

1. Newport J, Kirschner M (1982) A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* 30:675–686.
2. Nieuwkoop PD, Faber J (1967) *Normal Table of Development of Xenopus laevis (Daudin). North-Holland* (Elsevier, Amsterdam).
3. D'Autilia S, et al. (2006) Cloning and developmental expression of the Xenopus homeobox gene Xvsx1. *Dev Genes Evol* 216:829–834.
4. Green CB, Cahill GM, Besharse JC (1995) Regulation of tryptophan hydroxylase expression by a retinal circadian oscillator in vitro. *Brain Res* 677:283–290.
5. Viczian AS, Vignali R, Zuber ME, Barsacchi G, Harris WA (2003) XOtx5b and XOtx2 regulate photoreceptor and bipolar fates in the Xenopus retina. *Development* 130:1281–1294.
6. Gerber WV, et al. (1999) The RNA-binding protein gene, *hermes*, is expressed at high levels in the developing heart. *Mech Dev* 80:77–86.
7. Vernon AE, Philpott A (2003) The developmental expression of cell cycle regulators in Xenopus laevis. *Gene Expression Patterns* 3:179–192.
8. Paris J, et al. (1991) Cloning by differential screening of a *Xenopus* cDNA coding for a protein highly homologous to cdc2. *Proc Natl Acad Sci USA* 88:1039–1043.
9. Howe JA, Howell M, Hunt T, Newport JW (1995) Identification of a developmental timer regulating the stability of embryonic cyclin A and a new somatic A-type cyclin at gastrulation. *Genes Dev* 9:1164–1176.
10. Casarosa S, et al. (2003) Xrx1 controls proliferation and multipotency of retinal progenitors. *Mol Cell Neurosci* 22:25–36.

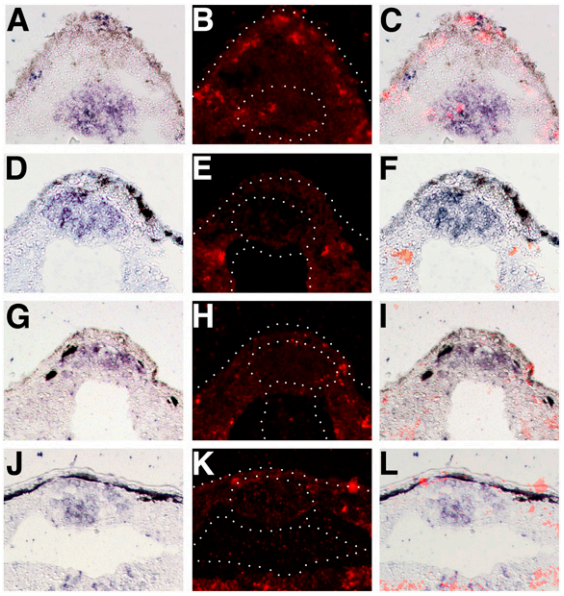

Fig. S1. *Xbsx* is not expressed in cyclin D1-positive cells. In situ hybridization for *Xbsx* (*A*, *D*, *G*, and *J*; blue staining), cyclin D1 (*B*, *E*, *H*, and *K*; red staining), and merge (*C*, *F*, *I*, and *L*) was performed on cryostat sections of stage 24 (*A*–*C*), stage 32 (*D*–*F*), stage 37 (*G*–*I*), and stage 40 (*J*–*L*) embryos.

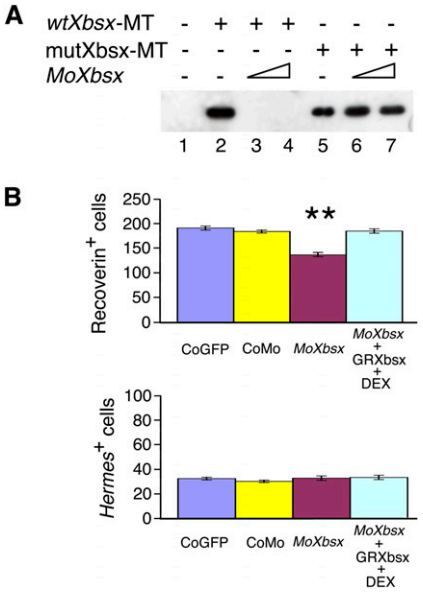

Fig. S2. Specificity of *Xbsx* morpholino. (*A*) *MoXbsx*, a morpholino antisense oligonucleotide complementary to the 5′ UTR of *Xbsx*, efficiently abrogates translation in *Xenopus* embryos of a myc-tag construct carrying the *Xbsx* target sequence (wt*Xbsx*-MT; lane 3, 1 pmol *MoXbsx*; lane 4, 2 pmol *MoXbsx*). *MoXbsx* does not affect the translation of a myc-tag construct carrying a mutated target sequence (mut*Xbsx*-MT; lane 6, 1 pmol *MoXbsx*; lane 7, 2 pmol *MoXbsx*). (*B*) GR-*Xbsx* injection rescues the inhibitory effects of *MoXbsx* on the generation of pineal photoreceptors (Recoverin-positive cells). No effect is observed on injection of control standard morpholino (CoMo). CoGFP indicates control embryos injected with GFP mRNA alone. DEX, dexamethasone. Asterisks indicate statistical differences as determined by the Student's *t* test: **, $P < 0.01$. Error bars indicate SEM.

Table S1. Total number of cells counted in BrdU incorporation experiments

WT									
Stage	26	28	32	34	36	38	38	39	40
ZT	6	12	18	0	6	12	18	0	6
$BrdU^+,Otx5^+$	164	174	129	76	164	179	85	75	201
$Otx5^+$	816	893	941	803	940	1,119	843	1,143	1,364
MoXbsx									
ZT	6	12	18	0	6	12	18	0	
Control									
$BrdU^+,Otx5^+$	98	97	111	101	149	147	54	80	
$Otx5^+$	584	684	889	933	961	1013	545	940	
MoXbsx									
$BrdU^+,Otx5^+$	151	109	117	127	123	116	100	85	
$Otx5^+$	908	774	625	668	843	832	817	902	
GR-*Xbsx*									
ZT	6	12	18	0					
GR-Xbsx − DEX									
$BrdU^+,Otx5^+$	252	174	206	183					
$Otx5^+$	1,473	1,055	1,415	1,591					
GR-Xbsx + DEX									
$BrdU^+,Otx5^+$	147	188	173	179					
$Otx5^+$	1,346	1,465	1,363	1,480					

The number of cells positive for both BrdU and Otx5 ($BrdU^+,Otx5^+$) and the total number of Otx5-positive cells ($Otx5^+$) are indicated. DEX, dexamethasone.