Xenopus Bsx links daily cell cycle rhythms and pineal photoreceptor fate

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In the developing central nervous system, the cell cycle clock plays a crucial role in determining cell fate specification. A second clock, the circadian oscillator, generates daily rhythms of cell cycle progression. Although these two clocks interact, the mechanisms linking circadian cell cycle progression and cell fate determination are still poorly understood. A convenient system to address this issue is the pineal organ of lower vertebrates, which contains only two neuronal types, photoreceptors and projection neurons. In particular, photoreceptors constitute the core of the pineal circadian system, being able to transduce daily light inputs into the rhythmical production of melatonin. However, the genetic program leading to photoreceptor fate largely remains to be deciphered. Here, we report a previously undescribed function for the homeobox gene *Bsx* in controlling pineal proliferation and photoreceptor fate in *Xenopus*. We show that *Xenopus Bsx* (*Xbsx*) is expressed rhythmically in postmitotic photoreceptor precursors, reaching a peak during the night, with a cycle that is complementary to the daily rhythms of S-phase entry displayed by pineal cells. *Xbsx* knockdown results in increased night levels of pineal proliferation, whereas activation of a GR-Xbsx protein flattens the daily rhythms of S-phase entry to the lowest level. Furthermore, evidence is presented that *Xbsx* is necessary and sufficient to promote a photoreceptor fate. Altogether, these data indicate that *Xbsx* plays a dual role in contributing to shape the profile of the circadian cell cycle progression and in the specification of pineal photoreceptors, thus acting as a unique link between these two events.

homeobox | pineal organ | proliferation | differentiation | circadian

The correct balance between proliferation and differentiation is crucial to ensure the appropriate size of the different areas of the central nervous system and the proportionate generation of a remarkable variety of neuronal and glial cell types. In particular, in the retina and cerebral cortex, cell cycle exit of progenitors is strictly coordinated with cell fate specification following a temporal order that involves different competence stages (1, 2). An additional level of complexity in the control of cell proliferation was recently discovered with the observation that the circadian clock, which regulates metabolic and physiological rhythms, also generates daily rhythms of cell cycle progression. Indeed, several cell cycle regulators, including c-myc, cyclin D1, and Wee-1, are regulated in a circadian manner (3, 4). This results in S-phase entry of many cells at the end of the day or during the night, an evolutionarily conserved phenomenon that has been proposed to represent an adaptation of ancestral unicellular animals to reduce the risk of UV-induced DNA damage (5, 6).

Although significant progress has been made in understanding how the circadian clock and the cell cycle interact, it is still unclear what the molecular links are between the circadian control of cell proliferation and the generation of specific cell types.

A suitable system in which this issue can be addressed is the pineal organ, a dorsal diencephalic structure that plays a central role in the regulation of circadian rhythms. It is now clear, especially from studies on nonmammalian vertebrates, that the pineal organ shares many similarities, and apparently a common evolutionary origin, with the retina (7, 8). Compared with the retina, the pineal organ displays a simpler structure containing only two neuronal types, photoreceptors and projection neurons, which are generated from the same precursor and represent the functional homologues of retinal photoreceptors and ganglion cells, respectively (7, 9, 10). In particular, the photoreceptors constitute the core of the pineal circadian system, being able to transduce daily light inputs into the rhythmical production of melatonin. At the same time, the photoreceptors make contact with the projection neurons that innervate different areas of the brain. Despite the central role played by photoreceptors in pineal physiology, the mechanisms specifying this neuronal type are largely unknown. Studies in zebrafish have shown that the homeodomain transcription factor floating head (flh) and the proneural genes *achaete/scute* homolog 1a (*ascl1a*) and *neurogenin* (*ngn*) are required for pineal neurogenesis but are not involved in cell fate decision between photoreceptors and projection neurons (11). On the other hand, Notch plays a dual role in determining the pineal cell number and inhibiting the projection neuron fate (10). The lack of an instructive role for Notch in specifying the photoreceptor identity suggests the existence of positive signals that remain to be identified.

Here, we show that the homeobox gene *Xenopus Bsx* (*Xbsx*) (12) is expressed in photoreceptor precursors in a cyclical manner with a phase that is complementary to the daily rhythms of S-phase entry displayed by pineal cells. Our functional data indicate that *Xbsx* acts as a unique link between the rhythmical control of cell cycle progression and the specification of photoreceptors.

Results

***Xbsx* Demarcates the Early Pineal Territory and Is Expressed in Postmitotic Photoreceptor Precursors.** The expression pattern of *Xbsx* is closely related to the expression of its mouse homologue (12). In situ hybridization analysis shows that *Xbsx* transcripts are first detected at midneurula stage (stage 16) in a few cells located bilaterally in the anterior neural plate (Fig. 1*A*). With the closure of the neural tube, these expression domains converge medially in the dorsal diencephalon at the level of the epiphysial anlage (Fig. 1 *B–D*). *Xbsx* expression in the pineal complex persists throughout the analyzed stages. At tailbud stage, *Xbsx* is also expressed in the hypothalamus and in the ventral telencephalon in an area possibly corresponding to the septum (Fig. 1*E*). The onset of *Xbsx* expression in the pineal territory occurs later than that of other homeobox genes such as *Xrx1*, *Xotx5*, and *Xnot2*, thus suggesting that *Xbsx* is not involved in the earliest events of pineal development.

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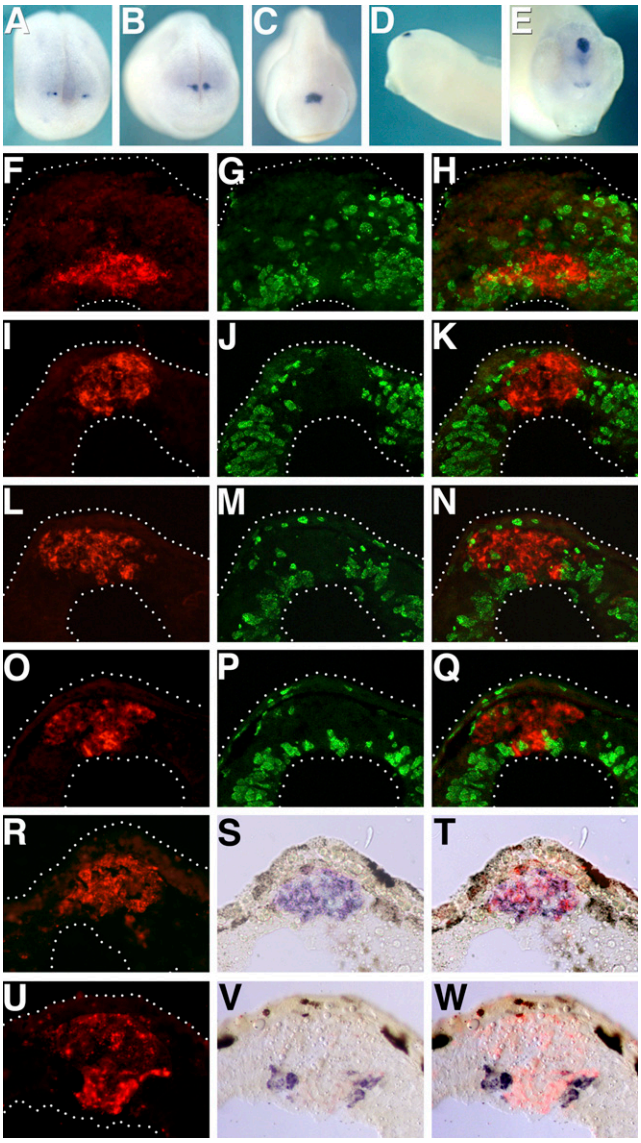

Fig. 1. *Xbsx* expression during pineal organ development. *Xbsx* whole-mount in situ hybridization was performed at stage 16 (*A*), stage 19 (*B*), stage 22 (*C*), stage 32 (*D*), and stage 38 (*E*). Immunostaining for BrdU incorporation (*G*, *J*, *M*, and *P*; green staining), in situ hybridization for *Xbsx* (*F*, *I*, *L*, and *O*; red staining), and merge (*H*, *K*, *N*, and *Q*) were performed on cryostat sections at stage 24 (*F–H*), stage 32 (*I–K*), stage 37 (*L–N*), and stage 40 (*O–Q*). In situ hybridization for *Xbsx* (*R*, red staining), *Xotx5* (*S*, blue staining), and merge (*T*) was performed on stage 37 cryostat sections. In situ hybridization for *Xbsx* (*U*, red staining), *Hermes* (*V*, blue staining), and merge (*W*) was performed on stage 37 cryostat sections.

To identify the pineal cells expressing *Xbsx*, we first tested whether this gene is expressed in proliferating or postmitotic cells. Analysis of BrdU incorporation as a marker of S-phase (Fig. 1 *F–Q*) as well as that of cyclin D1 expression to highlight cells in different phases of mitosis (Fig. S1) shows that *Xbsx* is expressed in postmitotic cells throughout pineal development. Because photoreceptors and projection neurons are the only two neuronal components of the pineal organ, we compared the expression of *Xbsx* with that of markers for these two cell types. *Xbsx* was found to colocalize with photoreceptor markers such as *Xotx5* (Fig. 1 *R–T*) and to be complementary to *Hermes*, which demarcates projection neurons (Fig. 1 *U–W*). In particular, *Xbsx* is already expressed at stage 24 in postmitotic photoreceptor precursors that express *Xotx5* (Fig. 1 *F–H*) but do not yet express the differentiation markers Interphotoreceptor retinoid-binding protein (IRBP) and Recoverin.

***Xbsx* Is Expressed in a Cyclical Manner in Light/Dark Conditions.** Because pineal photoreceptors represent a complete circadian system in lower vertebrates (8), we asked whether *Xbsx* expression might cycle over the 24 h. To this end, we performed in situ hybridization on brains dissected from tadpoles (stage 46) grown in a 12-h light/12-h dark (LD) cycle from the time of fertilization. Brains were collected at four time points, and the expression of *Tph*, which is known to undergo circadian variations (13), was used as a positive control. *Xbsx* expression was found to cycle similarly to *Tph* (Fig. 2 *A* and *B*), with the highest levels at zeitgeber time (ZT) 18 (ZT indicates hours after "lights on": ZT0, lights on; ZT12, lights off), the lowest at ZT6, and intermediate levels at the other time points. To understand whether *Xbsx* expression is controlled by light or by the circadian oscillator, we tested the persistence of *Xbsx* rhythmicity in constant darkness (DD). Under these conditions, only genes regulated by the circadian clock, such as *Tph* and *AANAT*, maintain cyclical expression (13, 14). Because an initial exposure to LD cycles is required in zebrafish to establish clock-controlled rhythms of gene expression, we raised embryos for several days in LD conditions and subsequently for 2 days in the DD cycle. Embryos were collected during the second day of the DD cycle at 6-h intervals. Based on *Tph* expression, we found that, although 3 and 5 days of LD cycles are not sufficient, 7 days represents an adequate period for the entrainment of the circadian machinery. In fact, following this protocol, *Tph* expression in DD appears to be rhythmical, although its level is significantly lower than that observed in the LD cycle (Fig. 2 *C* and *D*). On the contrary, under the same conditions, *Xbsx* expression remains low and arrhythmical (Fig. 2 *C* and *D*). Altogether these data suggest a circadian clock-independent regulation for *Xbsx*.

Daily Rhythmicity in Pineal Cell Cycle Progression. Daily rhythms of cell cycle progression have been documented in a wide variety of organisms (4, 15, 16). To assess whether entry in S-phase is regulated by light during *Xenopus* pineal development, embryos kept in the LD cycle were treated with BrdU at 6-h intervals for 2 days starting from stage 26. At this initial stage the pineal organ evaginates from the roof of the diencephalon and becomes functional, beginning to produce melatonin rhythmically (17).

To normalize for variations in proliferation occurring at different stages of development, we calculated the percentage of BrdU-positive cells over the total number of cells for each time point. In particular, we analyzed BrdU incorporation in the *Xotx5*-positive area, thus focusing the analysis on photoreceptor precursors. Our data show that pineal cell proliferation levels are characterized by a cyclical profile during a period of 24 h (Fig. 2 *E* and *F*). In particular, the highest percentage of BrdU-positive nuclei was observed at ZT6, whereas the lowest percentage was detected at ZT0. Embryos analyzed at ZT12 and ZT18 show decreasing intermediate percentages of S-phase nuclei. In accordance with data from other biological systems (16, 15), S-phase entry in the pineal organ displays a peak in the second half of the day and in the first part of the night. These effects are independent of the embryonic stage and appear to be linked to the daily progression of time.

***Xbsx* Is Necessary to Block S-Phase Entry During the Night.** The cyclical expression of *Xbsx* and its exclusion from proliferating pineal cells raised the hypothesis that this gene might play a role in controlling S-phase rhythmicity of pineal photoreceptor precursors. To address this issue, we performed BrdU incorporation in embryos injected with an antisense morpholino oligonucleotide that specifically targets *Xbsx* (*MoXbsx*) (Fig. S2*A*). We found that compared with control embryos, *MoXbsx*-injected embryos dis-

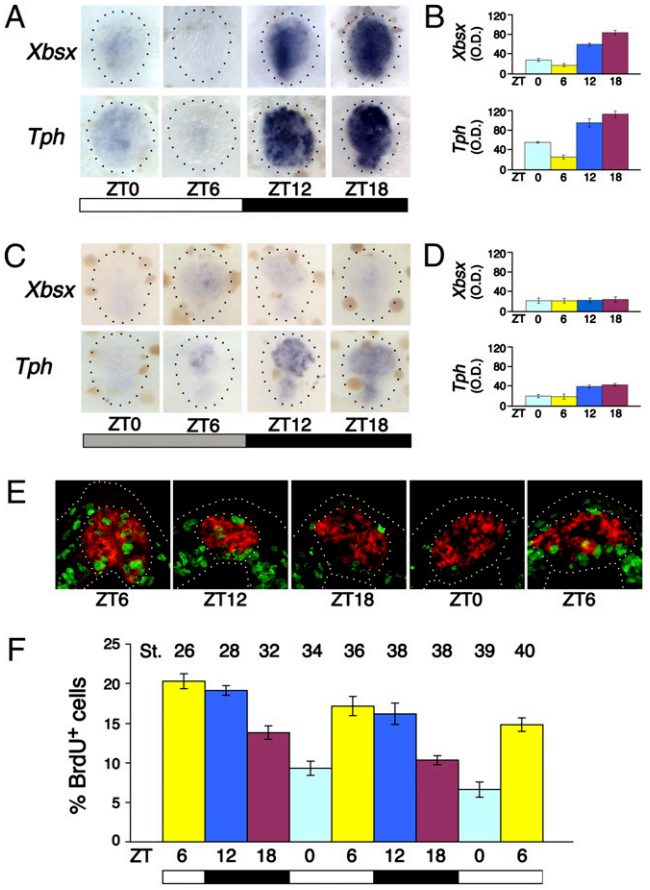

Fig. 2. Daily rhythms of *Xbsx* expression and S-phase entry of pineal cells. (*A* and *C*) Whole-mount in situ hybridization for *Xbsx* and *Tph* was performed on dissected stage 46 brains. Embryos were kept in an LD (*A*) or DD (*C*) cycle and collected at the indicated time points (ZT0, lights on; ZT12, lights off). Quantification was determined by optical density (O.D.) of *Xbsx* and *Tph* in situ hybridization signal for embryos kept in LD (*B*) or DD (*D*) condition. The peak-to-trough statistical difference was determined by the Student's *t* test: *Xbsx*, $P = 0.006$; *Tph*, $P = 0.002$ (*B*) and *Xbsx*, $P = 0.21$; *Tph*, $P = 0.008$ (*D*). (*E*) Representative cryostat sections showing BrdU incorporation (green staining) in the pineal organ at five time points between stage 26 and stage 36. The *Xotx5*-positive area (red staining) is circled. (*F*) Quantification of BrdU-labeled cells present in the *Xotx5*-positive area. At each time point, the mean percentage per pineal organ of BrdU-positive nuclei over the total number of nuclei was plotted against ZT time. The embryonic stage (St.) corresponding to each time point is indicated on the top. Data represent pooled results from three independent experiments. The peak-to-trough statistical difference was determined by the Student's *t* test: $P = 0.001$. The number of cells counted is shown in Table S1. In this and the following figures, the white and black bars indicate the light and dark phases, respectively. The gray bar in DD experiments indicates a dark phase at the time when control embryos are in the light phase.

play an increase in pineal BrdU-positive cells exclusively at ZT18 and ZT0, whereas no significant change is observed for ZT6 and ZT12 (Fig. 3 *A* and *B*). These results are consistent with a specific interference of the cyclical *Xbsx* expression that reaches the highest level during the night. The attenuation of *MoXbsx* effects on the second analyzed day (corresponding to the fifth day after the injection) is likely attributable to a decrease in stability and/or availability of the injected morpholino. Thus, *Xbsx* appears to contribute to the rhythmicity of pineal cell proliferation, specifically inhibiting S-phase entry during the night.

***Xbsx* Knockdown Results in Reduction of Pineal Photoreceptors.** Many physiological processes, including proper tissue development and homeostasis, require a balance between apoptosis and cell proliferation. We therefore decided to evaluate the level of pineal apoptosis during development of *MoXbsx*-injected embryos. TUNEL assays performed at ZT6 and ZT18 show that *Xbsx* knockdown causes an increase of pineal apoptotic cells in the *Xotx5*-positive area with respect to control embryos at both time points (Fig. 3*C*). Thus, inhibition of *Xbsx* induces an increase in both S-phase entry and cell death. To assess the consequence of these opposite actions, we analyzed the effects of *MoXbsx* injection on the generation of differentiated pineal cell types. To this aim, we used Recoverin and *Hermes* as specific markers to identify differentiated pineal photoreceptors and projection neurons, respectively (Fig. 3 *D* and *E*). Embryos injected with *MoXbsx* are characterized by a 25% reduction of photoreceptor cells compared with control injected embryos. Conversely, *Xbsx* knockdown does not significantly affect the genesis of pineal projection neurons. The specificity of this effect is supported by the efficient rescue of photoreceptors observed in embryos coinjected with *MoXbsx* and synthetic RNA encoding a hormone-inducible GR-Xbsx protein (Fig. S2*B*). Taken together, the loss of function data suggests that *Xbsx* is required for the proper cell cycle exit and differentiation of pineal photoreceptor precursors and the generation of mature photoreceptors.

***Xbsx* Gain of Function Inhibits Entry in S-Phase of Pineal Photoreceptor Precursors and Promotes Their Differentiation.** To analyze the role of *Xbsx* in pineal development further, we performed gain-of-function experiments. The effects observed on ectopic expression of a hormone-inducible GR-Xbsx protein are essentially opposite to the ones described for *MoXbsx* injection. In fact, comparison between dexamethasone-treated (i.e., activated) and untreated (control) GR-Xbsx-injected embryos shows that *Xbsx* overexpression leads to a decrease in BrdU incorporation at ZT6, ZT12, and ZT18, thus sensibly flattening the daily rhythms of cell cycle progression (Fig. 4 *A* and *B*). TUNEL assays show that activation of GR-Xbsx does not affect apoptosis in the *Xotx5*-positive area (Fig. 4*C*). Moreover, analysis of pineal cell types in GR-Xbsx-injected embryos shows that *Xbsx* induces a 29% increase in pineal photoreceptors, whereas it does not affect the generation of projection neurons (Fig. 4 *D* and *E*). Altogether, these results show that *Xbsx* is sufficient to prevent entry in S-phase of pineal photoreceptor precursors and to promote photoreceptor specification.

Cell Proliferation Inhibits *Xbsx* Expression. Because gain- and loss-of-function experiments indicate that *Xbsx* is necessary and sufficient to repress pineal cell proliferation, we decided to analyze how *Xbsx* expression is affected by high levels of proliferation. This was achieved by overexpressing cyclin A2 together with cdk2. Under these conditions, *Xbsx* expression is repressed at all the analyzed time points, thus indicating a mutual antagonism between cell proliferation and *Xbsx* activity (Fig. 4*F*).

Discussion

***Xbsx* Is Expressed in a Cyclical Manner in Pineal Photoreceptor Precursors.** The photosensitive pineal organ of lower vertebrates has been proposed to reflect an ancient form of the vertebrate retina (8). In particular, the retina and the pineal organ share genetic pathways involved in the control of photoreception, phototransduction, and melatonin production. Moreover, the developing pineal organ expresses a set of transcription factors that is known to direct retina development (18) and includes Pax6, Rx1, Six3, Otx2, Otx5, Lhx3, and ET. Although the signals that provide specific instructions leading to a pineal rather than retinal fate have not been clearly dissected yet, there are also rare examples of genes displaying selective expression in the pineal organ but not in the retina. These include pinopsin and exorhodopsin, two pineal-specific opsins (8), and the transcription

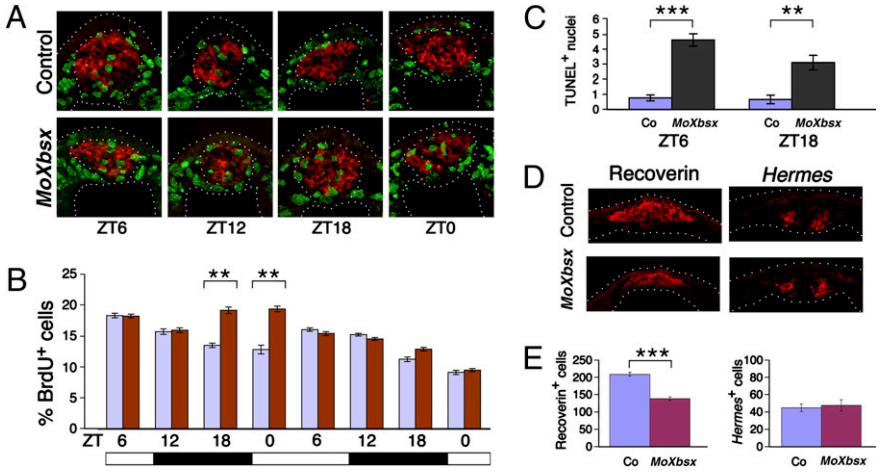

Fig. 3. *Xbsx* knockdown leads to increased S-phase entry during the night and to a reduction of pineal photoreceptors. (*A*) Representative cryostat sections showing BrdU incorporation (green staining) in control- and *MoXbsx*-injected embryos during 24 h between stage 26 and stage 34. The *Xotx5*-positive area (red staining) is circled. (*B*) Quantification of BrdU-labeled cells present in the *Xotx5*-positive area of control-injected (blue bars) and *MoXbsx*-injected (red bars) embryos at eight time points. At each time point, the mean percentage per pineal organ of BrdU-positive nuclei over the total number of nuclei is plotted against ZT time. Data represent pooled results from three independent experiments. The number of cells counted is shown in Table S1. (*C*) Average number of TUNEL-positive nuclei in the *Xotx5*-positive area per pineal organ for embryos collected at ZT6 and ZT18, corresponding to stage 36 and stage 38, respectively. Data represent pooled results from three independent experiments. Six control (Co)- and six *MoXbsx*-injected embryos were analyzed per time point in each experiment. (*D* and *E*) Analysis of pineal cell types in control- and *MoXbsx*-injected stage 42 tadpoles. (*D*) Representative cryostat sections showing expression of markers for photoreceptors (Recoverin) and projection neurons (*Hermes*). (*E*) Average numbers of cells positive for Recoverin and *Hermes* per pineal organ. Data represent pooled results from three independent experiments. For each marker, four control (Co)-injected and four *MoXbsx*-injected embryos were analyzed per time point in each experiment. Asterisks indicate statistical differences as determined by the Student's *t* test: $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars indicate SEM.

factor Not2 [denominated *floating head* (*flh*) in zebrafish], which is expressed in precursors of both photoreceptors and projection neurons (9). The homeodomain-containing transcription factor Xbsx is another example of this small subset of pineal-specific proteins. Compared with *Not2*, *Xbsx* shows the peculiarity of being expressed exclusively in photoreceptor precursors and is characterized by cyclical expression. What does control *Xbsx* pineal expression? *Xbsx* is activated in the pineal territory later than *Xotx5*, *Xnot2*, and *Xrx1*. Moreover, a survey of a 5-kb region located upstream of the mouse *Bsx* gene highlights the presence of

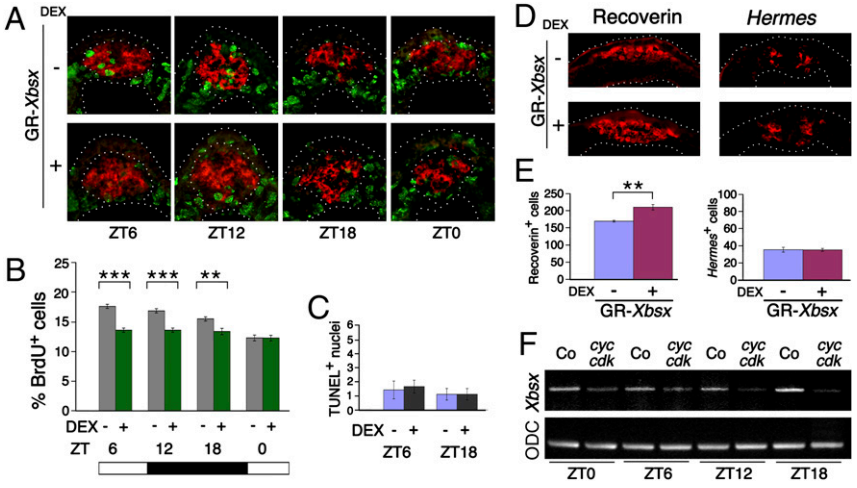

Fig. 4. Overexpression of *Xbsx* reduces S-phase entry and increases the number of pineal photoreceptors. (*A*) Representative cryostat sections showing BrdU incorporation (green staining) at four time points in embryos injected with GR-*Xbsx* and treated or untreated with dexamethasone (DEX). The *Xotx5*-positive area (red staining) is circled. (*B*) Quantification of BrdU-labeled cells present in the *Xotx5*-positive area of embryos injected with GR-*Xbsx* and untreated (gray bars) or treated (green bars) with DEX. At each time point, the mean percentage per pineal organ of BrdU-positive nuclei over the total number of nuclei is plotted against ZT time. Data represent pooled results from three independent experiments. The number of cells counted is shown in Table S1. $^{**}P < 0.01$; $^{***}P < 0.001$. (*C*) Average number of TUNEL-positive nuclei in the *Xotx5*-positive area per pineal organ for embryos collected at ZT6 and ZT18, corresponding to stage 36 and stage 38, respectively. Data represent pooled results from three independent experiments. Six DEX-treated and six untreated GR-*Xbsx*-injected embryos were analyzed per time point in each experiment. (*D* and *E*) Analysis of pineal cell types in stage 42 tadpoles. Embryos were injected with GR-*Xbsx* and allowed to develop in the presence or absence of DEX. (*D*) Representative cryostat sections showing expression of markers for photoreceptors (Recoverin) and projection neurons (*Hermes*). (*E*) Average numbers of cells positive for Recoverin and *Hermes* per pineal organ. Data represent pooled results from three independent experiments. For each marker, four DEX-treated and four untreated GR-*Xbsx*-injected embryos were analyzed per time point in each experiment. Asterisks indicate statistical differences as determined by the Student's *t* test: $^{**}P < 0.01$. Error bars indicate SEM. (*F*) RT-PCR analysis to evaluate the effects of cyclin A2/cdk2 overexpression on *Xbsx* expression. Control and injected embryos were collected at the indicated time points. ODC, ornithine decarboxylase.

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three Crx and two Otx2 binding sites, two transcription factors involved in mammalian pinealocyte specification and differentiation (19–21). Interestingly, *Crx* belongs to the *Otx* gene family and is considered to be the homologue of *Xenopus Otx5*. Altogether, these observations suggest that Bsx might function downstream of Otx5/Crx, Otx2, Not2, and Rx1 in events that follow the initial pineal territory specification. However, because none of the above-mentioned genes is expressed rhythmically, other factors should be responsible for *Xbsx* cyclical expression. *Xbsx* rhythmicity is lost in embryos kept in DD conditions, suggesting that this gene is controlled by the alternation of light and dark rather than by the circadian oscillator. A similar behavior, although with opposite rhythmicity, was described for *Xper2* expression in the *Xenopus* retina (22). *Xbsx* cyclical expression is consistent with a model in which light promotes the accumulation of an activator that reaches a threshold, or becomes otherwise active, at the end of the day, thus being able to stimulate the transcription of *Xbsx* during the night. Alternatively, *Xbsx* could be continuously transcribed, but inhibitory factors present during the daytime might degrade or destabilize its mRNA. Putative inhibitory factors could be represented by rhythmically expressed deadenilases (similar to Nocturnin but with an opposite phase of expression) (23) and micro-RNAs (24).

***Xbsx* Couples the Daily Rhythmicity of Pineal Cell Cycle Progression and the Specification of Pineal Photoreceptors.** Recent studies have implicated components of the circadian clock in the transcriptional control of cell cycle regulatory genes (4). In this work, we investigated the control of cell proliferation and cell fate specification in the pineal organ, which offers the advantage of being composed of only two neuronal types. Focusing on photoreceptor precursors, we found that they display daily rhythms of S-phase entry throughout pineal development, reaching a peak during the latter part of the light phase and the first part of the dark period. Interestingly, *Xbsx* is expressed in BrdU-negative cells, and the phase of its rhythmical expression is complementary to the daily phase of proliferation (Fig. 5*A*). Moreover, functional experiments indicate that *Xbsx* expression inversely correlates to cell proliferation. In fact, *Xbsx* knockdown raises the level of BrdU-positive cells specifically during the night, whereas *Xbsx* overexpression leads to a flattening of cell proliferation to the lowest level. Conversely, when cell proliferation is maintained at high levels by overexpressing cyclin A2/cdk2, *Xbsx* expression is drastically repressed. Accordingly, studies performed in cell cultures show that *Bsx* transcription is activated during retinoic acid-induced neuronal differentiation (25). Although the daily rhythms of cell proliferation are controlled by the circadian clock, light plays a central role in entraining and maintaining significant amplitude of S-phase rhythmicity (15). Moreover, light may exert independent effects on pacemaker rhythmical outputs, as was recently shown for control of the nocturnal release of melatonin (26). Bsx, whose expression does not appear to be under the control of circadian rhythms, could play an essential role in synchronizing photoreceptor differentiation with the end of the daily proliferative phase.

Cell fate determination during pineal development was recently addressed in zebrafish (10). In particular, it was shown that photoreceptors and projection neurons are born simultaneously and that although reduction of Notch activity promotes the projection neuron fate, constitutive activation of Notch is not sufficient to induce photoreceptors. This led to postulation of the existence of a still unidentified photoreceptor-inducing signal. Our data add significant information to this model, indicating that *Bsx* could be an effector of such a signal. Indeed, when *Xbsx* is knocked down, we observe both stimulation of cell proliferation and increased apoptosis, which results in a decrease of differentiated photoreceptors. Apoptosis may be attributable to the activation of excessive proliferation (27) or to the lack of an appropriate differentiation signal. These data are consistent with the hypothesis that *Xbsx* is required for cell cycle exit and/or differentiation of photoreceptor precursors (Fig. 5*B*). According to this hypothesis, in the absence of *Xbsx*, photoreceptor progenitors do not exit the cell cycle, as suggested by the increased

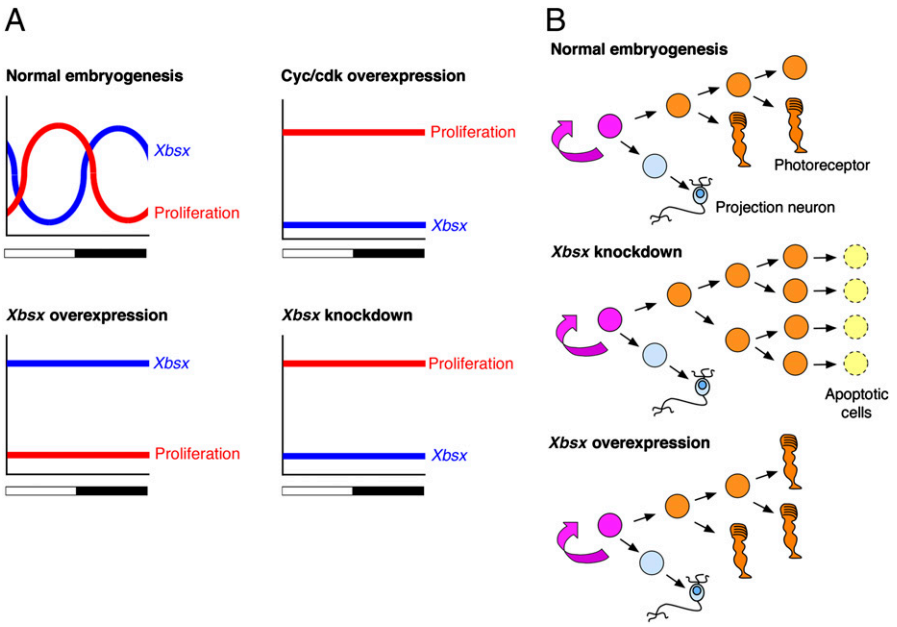

Fig. 5. Working model for *Xbsx* function in cell proliferation and differentiation of photoreceptor precursors. (*A*) Schematic summary of the relation between *Xbsx* expression and pineal cell proliferation in WT and manipulated embryos. (*B*) Model of action for *Xbsx*. The pink cell represents the common progenitor of projection neurons and photoreceptors. It is assumed that photoreceptor precursors undergo a limited number of asymmetrical cell divisions. *Xbsx* knockdown prevents cell cycle exit of photoreceptor precursors that eventually undergo apoptosis. *Xbsx* overexpression increases cell cycle exit of photoreceptor precursors and promotes their differentiation.

BrdU incorporation in *MoXbsx*-injected embryos, but, lacking appropriate specification cues, would eventually die. This model is also supported by *Xbsx* gain-of-function experiments that show a reduction of BrdU incorporation but no effect on apoptosis and an increase of pineal photoreceptors. In both gain-of-function and loss-of-function experiments, the number of projection neurons remains unchanged compared with control embryos, indicating that *Xbsx* functions exclusively in the lineage of photoreceptor precursors.

The remarkable conservation of the *Bsx* expression pattern between the mouse model and *Xenopus* suggests a conservation of function. Thus far, studies on *Bsx* knockout mice have focused on the hypothalamic role played by this gene in controlling the homeostasis of energy balance (28, 29). Nonetheless, it was noticed that these mice display pineal gland hypoplasticity (28). Because the mammalian pinealocyte and the nonmammalian pineal photoreceptor appear to have evolved from a common ancestral photoreceptor cell (8), it is tempting to speculate that the role in pineal photoreceptor specification we observe in *Xenopus* might be conserved among vertebrates. To be tested, this hypothesis awaits a cell type-specific analysis of the pineal gland in Bsx knockout mice as well as comparative studies of Bsx function in other vertebrate model systems.

In conclusion, this work provides insights into the molecular mechanisms coordinating the daily rhythms of cell proliferation and the generation of specific cell types. Our results highlight a previously undescribed essential role for *Bsx* in orchestrating these two events during the specification of pineal photoreceptors. Because endogenous circadian oscillators regulate cell proliferation in most cells, further studies are expected to identify additional factors linking circadian cell cycle exit to specific cell fates.

Materials and Methods

In situ hybridization, immunostaining, embryo microinjection, and BrdU incorporation were performed as previously described (30–32). TUNEL staining was performed on tissue cryosections using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon) and following the manufacturer's protocol. The *Xbsx* antisense morpholino and control morpholino were obtained from Gene Tools, LLC. Injections and embryo manipulations during the dark phase were performed under red safe lights. More details are described in *SI Text*.

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