Characterization and function of the bHLH-O protein XHes2: insight into the mechanisms controlling retinal cell fate decision

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Neurons and glial cells differentiate from common multipotent precursors in the vertebrate retina. We have identified a novel member of the hairy/Enhancer of split [E(spl)] gene family in Xenopus, XHes2, as a regulator to bias retinal precursor cells towards a glial fate. XHes2 expression is predominantly restricted to sensory organ territories, including the retina. Using in vivo lipofection in the optic vesicle, we found that XHes2 overexpression dramatically increases gliogenesis at the expense of neurogenesis. This increase in glial cells correlates with a delayed cell cycle withdrawal of some retinal progenitors. In addition, birthdating experiments suggest that XHes2 deviates some early born cell types towards a glial fate that would normally have given rise to neurons. Conversely, a significant inhibition of glial differentiation is observed upon XHes2 loss of function. The gliogenic activity of XHes2 relies on its ability to inhibit neuronal differentiation by at least two distinct mechanisms: it not only negatively regulates XNgnr1 and NeuroD transcription, but also physically interacts with a subset of proneural bHLH proteins.

KEY WORDS: bHLH, Hes2, Gliogenesis, Neurogenesis, Retina, Cell cycle, Xenopus

INTRODUCTION

The retina has been extensively used as a model system to investigate the molecular mechanisms governing cell fate decisions, as it contains only six types of neurons and one type of glia (Müller glia), properly organized in three nuclear layers. From numerous studies in a variety of organisms, a common model has emerged, where bHLH activators, in combination with homeodomain factors, are responsible for neuronal subtype specification (Wang and Harris, 2005) (reviewed by Hatakeyama and Kageyama, 2004). However, mechanisms allowing the generation of glial versus neuronal cells remain to be defined in detail.

Retinal Müller glial cells and neurons differentiate from a common multipotent precursor (Turner and Cepko, 1987; Wets and Fraser, 1988). Gliogenic activities of Notch signalling have been reported in this context (for reviews, see Perron and Harris, 2000; Pujic and Malikii, 2004). Activation of Notch signalling indeed promotes Müller glia differentiation (Bao and Cepko, 1997; Furukawa et al., 2000; Hojo et al., 2000; Scheer et al., 2001). Conversely, blocking Notch activity forces progenitors to differentiate prematurely, leading to a decrease in late cell types, including Müller glial cells (Austin et al., 1995; Dorsky et al., 1997; Silva et al., 2003). More recently, an instructive role of the Notch pathway in gliogenesis has been proposed, as the inhibition of both Notch and Delta function in the zebrafish retina prevents Müller glia cells from differentiating (Bernardos et al., 2005).

In mammals, it has also been shown that Notch effectors, such as Hes1 and Hes5, promote gliogenesis at the expense of the neuronal fate in the retina (Furukawa et al., 2000; Hojo et al., 2000; Kageyama and Nakanishi, 1997). Conversely, interfering with Hes1 function reduces the number of Müller glial cells (Furukawa et al., 2000; Takatsuka et al., 2004). Hes genes are vertebrate homologues of Drosophila hairy and Enhancer of split [E(spl)] genes, which encode basic helix-loop-helix (bHLH) transcriptional repressors that negatively regulate neuronal bHLH genes (Akazawa et al., 1992; Chen et al., 1997; Ishibashi et al., 1995; Sasai et al., 1992; Tomita et al., 1996). Gliogenic properties of Hes proteins could result from this anti-neuronal activity. Interestingly, however, some Hes-related proteins behave differently in the retina. For example, although Hes1, Hes5 and Hes2 bias retinal precursors towards gliogenesis, Hes1 or Hes3 do not (Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001). The Hes6 protein even promotes neurogenesis (Bae et al., 2000). The reason for these differences and the functional relevance for each Hes-related protein in gliogenesis are yet to be unravelled.

Recent work has provided evidence that cell fate determination is also linked to cell proliferation (reviewed by Ohnma and Harris, 2003). The timing of cell cycle exit may influence cell fate choice during retinogenesis, as, according to the ‘competence model’, precursors change their competence over time (Livesey and Cepko, 2001). A gliogenic phenotype could thus be related to defects in cell cycle exit. However, molecular pathways involved remain to be investigated.

To gain further insight into the mechanisms that control neuronal versus glial fate choice, we aimed to identify novel gliogenic genes in the Xenopus retina. We report herein the characterization of one of these, encoding the bHLH-O type protein XHes2. XHes2 is mainly expressed in groups of cells giving rise to sensory organs, including the retina. We show that blocking XHes2 function reduces gliogenesis. Conversely, XHes2 overexpression dramatically increases glial cell production. Analysis of the underlying mechanisms shows that XHes2 forces gliogenesis by repressing...
neurogenesis and by affecting the timing of cell cycle exit of a subset of precursors. XHes2 may function by inhibition of proneural gene transcription and through its direct interaction with a subset of bHLH proteins, including NeuroD.

MATERIALS AND METHODS

XHes2 cDNA isolation

Based on five XHair1-related, partially overlapping EST sequences from *Xenopus laevis* (GenBank Accession Numbers AW639943, AW642120, AW643378, AW644752 and BI350304), primers were designed to amplify the complete open reading frame (ORF) of XHes2: 5′-ACC ATG CCT CCC AAT GTA GCG CTC G-3′, forward primer; 5′-TCA CCA CGG CCT CCA GAT GGA GCT G-3′, reverse primer. XHes2-positive fractions of an oocyte λZap cDNA library (Claussens and Pieler, 2004) were selected for further screening performed with the ECL Labelling and detection system (Amersham), following the manufacturer’s protocol. The sequence of the longest obtained XHes2 cDNA consists of 1075 nucleotides including 5′- and 3′-untranslated regions, as well as a poly(A) tail (GenBank Accession Number DQ156231).

Expression constructs

pCS2+XHes2, pCS2+XHes2-ΔW and pCS2+XHes2-ΔC, respectively, encode the wild-type XHes2 protein, a shortened XHes2 protein devoid of the WRPW motif, and a C-terminal truncated XHes2 variant missing the WRPW motif and 51 additional amino acids, but still containing the Orange motif. These constructs were generated by cloning the PCR products of pBK-CMV XHes2 into the pCS2+ vector (Turner and Weintraub, 1994), pCS2+XHes2-ΔW-VP16 and pCS2+XHes2-ΔC-VP16 encode XHes2 variants where the WRPW motif or the C-terminal part was replaced by the VP16 transactivating domain (Sadowski et al., 1988). pCS2+XHes2-DBM encodes a DNA-binding mutant of XHes2, in which the basic amino acids 3EKRRRAR15 within the basic domain were replaced by the acidic amino acids 3KKEEEAID15, as was done to obtain the DNA-binding mutant of ESR6 (Deblay et al., 1999), and was generated by mutagenesis of pCS2+XHes2 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). To ensure proper nuclear localization of XHes2-DBM, pCS2+-NLS-XHes2-DBM was constructed by inserting XHes2-DBM into pCS2+NL5. To generate pCS2+XHes2(5′)-GFP the 5′-untranslated and N-terminal-coding region of XHes2 were amplified by PCR from pBK-CMV XHes2 and cloned in frame in front of GFP. Finally, pCS2+Flag-XHes2 and pCS2+MT-XHes2, encoding N-terminal Flag and myc tagged versions of XHes2, were engineered by subcloning XHes2 ORF from pCS2+XHes2 into pCS2+Flag and pCS2+MT (Turner and Weintraub, 1994). Cloning and mutagenesis primer sequences are available upon request. All other expression constructs used in this study have been previously described: nLab2 (Chitnis et al., 1995); MT-GFP (Rubenstein et al., 1997); NogginΔ5 (Smith and Harland, 1992); Notch ICD (Chitnis et al., 1995); MT-Ngnr1 (Ma et al., 1996); MT-NeuroD (Lee et al., 1995); MT-Xath3, Flag-Xhairy1, Flag-Xhairy2b, Flag-XHes6r, nlacZ RNA (50 pg) co-injection was used to visualize injected cells after histochemical staining for β-galactosidase activity. For the in vivo translation experiments, 10 pg of each Mo together with 400 pg XHes2(5′)-GFP RNA were co-injected into two animal blastomeres of the eight cell stage, GFP fluorescence was judged at stage 11. For the Mo injections, 10 to 20 pg of Mo were micro-injected into one ventral-animal blastomere at the eight-cell stage. Animal caps were dissected from stage 9 embryos and cultured until control siblings had reached the appropriate stages.

Real-time RT-PCR analysis

Total RNA from animal caps or embryos was isolated using the RNeasy Mini kit (Qiagen) and treated with DNase I (Qiagen). cDNA synthesis was carried out with the Gene Amp RNA PCR core kit (Perkin-Elmer) followed by real-time PCR analysis using gene specific primer pairs (available upon request), the iQ SYBR Green Supermix (BioRad) and the iCycler iQ real-time detection system (BioRad). Measurements of each experiment were done in duplicates. The copy number of transcripts in the samples was determined for each analyzed gene using the absolute standard curve method and then normalized to expression levels of ornithine decarboxylase (ODC). Relative values for each gene were obtained by relating the absolute copy number of each sample to the highest absolute copy number which was set to 100.

In vivo lipofection

DNA were transfected into the presumptive region of the retina of stage 17-18 embryos as reported (Holt et al., 1990; Ohnuma et al., 2002). Mo (0.5 mM) in vivo lipofection was performed as previously described (Boy et al., 2004). For retinal cell types distribution analysis, GFP-positive cells were counted on cryostat sections and identified based upon their laminar position and morphology, as previously described (Dorsky et al., 1995).

Brdu injection, immunohistochemistry and TUNEL assay

BrDU was injected intra-abdominally and was detected after a 45 minute treatment in 2 N HCl. Immunohistochemistry was performed on 4% paraformaldehyde fixed tissues. Cryostat sections (12 μm) were incubated with primary antibodies (monoclonal anti-BrDU, Roche; polyclonal anti-GFP, Molecular Probes; polyclonal anti-CRALB, a gift from Jack Saari), and specific binding sites were visualized using anti-mouse or anti-rabbit fluorescent secondary antibodies (Alexa, Molecular Probes). Detection of cell apoptosis was carried out with the DeadEnd fluorometric TUNEL system (Promega), following the manufacturer’s instructions.

Immunoprecipitation and western-blot analysis

Immunoprecipitation experiments and western analysis were performed on animal cap homogenates, as previously reported (Taelman et al., 2004).
RESULTS
Identification and structural analysis of Xenopus Hes2

A database search for Hairy-related proteins resulted in the identification of partially overlapping EST sequences encoding a novel bHLH protein in Xenopus laevis. A full-length cDNA clone was subsequently isolated from a total oocyte cDNA library and sequenced (see Materials and methods). Comparative sequence analysis (Fig. 1) reveals a high degree of sequence similarity to the previously known murine and human Hes2 proteins (Ishibashi et al., 1993; Ledent et al., 2002). Further database analysis identified two additional, closely related sequences from zebrafish and Xenopus tropicalis. The phylogenetic relationship of the Xenopus Hes2 proteins with mammalian and zebrafish Hes2 proteins is underscored by a significant conservation of amino acids within and flanking the bHLH and Orange (O) domains, which are crucial for DNA-binding and dimerization (Davis and Turner, 2001; Leimeister et al., 2000; Taelman et al., 2004), and by the presence of a C-terminal WRPW motif, which is known to bind the corepressor groucho (Fisher et al., 1996; Grbavec and Stifani, 1996; Paroush et al., 1994). Taken together, these vertebrate Hes2 proteins exhibit typical features of both the Hairy and E(spl) subfamilies of bHLH-O proteins (Davis and Turner, 2001) (Fig. 1A,B).

XHes2 is mainly expressed in developing sensory organs during Xenopus embryogenesis

The spatial and temporal distribution of XHes2 transcripts during embryogenesis of Xenopus laevis was analyzed using whole-mount in situ hybridization. Maternal XHes2 transcripts are present in the animal half of early cleavage stage embryos until the beginning of gastrulation (stage 10, data not shown). At stage 11, isolated XHes2-positive cells can be detected in a scattered pattern in the animal sphere of the embryo (Fig. 2A).

During neurulation and organogenesis, developing otic vesicles and retina emerge as the two main expression domains for XHes2. As early as stage 13.5 (early neural plate stage), two groups of cells, found at the lateroanterior borders of the neural plate and corresponding to the prospective otic placodes, are XHes2 positive (Fig. 2B); these become even more prominent as embryogenesis proceeds (Fig. 2C,H), and eventually persist in the dorsal region of the otic vesicles at stage 30 and beyond (Fig. 2J-M). XHes2 transcripts are also transiently found in the olfactory placodes (Fig. 2I). In addition, a weak expression of XHes2 can be detected in discrete groups of cells, first within the neuroectoderm during neurula stages and later in the brain (see Fig. 2 legend for details). In order to investigate whether XHes2 is expressed in primary neurons, double in situ hybridization against XHes2 and N-tubulin were performed (Fig. 2D). The stripes where XHes2 is expressed at stage 14.5 do indeed seem to overlap with the domains where primary neurons are present. We further prepared cross-sections from embryos stained for XHes2 or N-tubulin, respectively, in order to compare precisely their expression patterns (Fig. 2E,F). XHes2 appears to be expressed in the superficial layer where neuronal precursors continue to proliferate (Chalmers et al., 2002), whereas the N-tubulin-positive cells are present in the deeper layer containing primary neurons. This suggests that XHes2 expression is restricted to cycling neuronal precursor cells.

At stage 21, XHes2 expression is first observed in the developing retina (Fig. 2H). At stage 30, when most cells in the optic vesicle are dividing, XHes2 transcripts are found throughout the neural retina (Fig. 2J-N). From stage 35 onwards, XHes2 expression progressively declines in the central retina while remaining high in the margins, suggesting that XHes2 expression is turned off in post-mitotic cells while being maintained in proliferating progenitors (Fig. 2K,O). At stage 41, XHes2 expression becomes restricted to the ciliary marginal zone (CMZ), the only retinal region where retinogenesis is
Fig. 2. **XHes2** is mainly expressed in presumptive and developing sensory organs. (A-O) Whole-mount in situ hybridization analysis of **XHes2** expression during embryogenesis. (A) **XHes2** positive cells are transiently detected in the midgastrula animal ectoderm. (B-M) During neurulation and organogenesis, **XHes2** transcripts are predominantly expressed in dorsal parts of the otic placodes (B-D,G-I) and vesicles (J-M) (black arrowheads in H,J,M), as well as in the presumptive (H,I) and developing (J-M) retina (black arrows in H,I,K). A weaker expression is detected in the olfactory placodes (grey arrowhead in I), and later in the lateral line system (white arrowheads in L,M). **XHes2** transcripts are also found in lateral (grey arrow in C,D) and medial (light grey arrow in C,D) filamentous stripes within the posterior neuroectoderm, and in longitudinal domains between midline and otic placode in the anterior neuroectoderm (light grey arrow in C,D). **XHes2** and N-tubulin expression domains seem to overlap (double in situ hybridization in D), but **XHes2** expression is restricted to the superficial layer (E), while N-tubulin probes stain the deeper layer (F), as observed on vibratome cross sections. Further expression of **XHes2** is evident in some dorsal cells of the forming brain (H,I), and later in the forebrain and hindbrain (L,M; grey arrows in H,I,M). (N-Q) During retinogenesis, **XHes2** expression progressively moves from the central retina (NR) as differentiation proceeds (black arrowhead in O), and is finally restricted to the dividing precursors of the CMZ (N-P black arrows) and to the lens (LE; black arrowhead in P). In the CMZ, **XHes2** expression is excluded from the stem cell containing region (black arrowhead in Q). Embryos are orientated as follows: (A) lateral view, animal up; (B-D) dorsal view, anterior left; (M) dorsal view, anterior left. (N-Q) Vibratome cross-sections. Scale bar: 50 µm. (Q) Magnification of the CMZ in P.

still occurring (Fig. 2P). However, **XHes2**-positive cells are not detected in the stem cells containing region (Fig. 2Q) (Dorsky et al., 1995; Perron et al., 1998).

**XHes2** is atypically regulated by **XNgnr1** and **Notch**

Studies in *Xenopus* and other vertebrates have shown that the transcription of several bHLH-O genes is positively regulated by factors that are involved in key steps of neurogenesis, such as **XNgnr1** and **Notch** (Davis and Turner, 2001; Iso et al., 2003; Koyano-Nakagawa et al., 1999; Koyano-Nakagawa et al., 2000; Lamar and Kintner, 2005; Wettstein et al., 1997). Similarly, overexpression of **XNgnr1** resulted in a significant induction of **XHes2** expression in the animal cap assay (Fig. 3A). In whole embryos, misexpression of **XNgnr1** abrogated the endogenous domains of **XHes2** expression, whereas isolated scattered **XHes2** positive cells appeared ectopically, mainly in the non-neural ectoderm (Fig. 3B,C). Such differential regulation of **XHes2** in ectoderm and neuroectoderm differs from what has been observed for some other bHLH-O genes, such as **ESR1** and **ESR7**, which are uniformly activated in neuroectodermal and ectodermal cells of whole embryos upon XNgnr1 misexpression (Koyano-Nakagawa et al., 2000; Lamar and Kintner, 2005). Altogether, these data suggest that the **XHes2** response to **XNgnr1** depends on tissue/cell type-specific regulation.

In **Notch** ICD-injected embryos, ectopic **XHes2**-positive cells were only detected along the lateral border of the neural plate (Fig. 3D,E); similar effects were observed following injection of the constitutively active **Notch** mediator **XSu(H)-Ank** (data not shown). In the animal cap explant system, **Notch** ICD was unable to induce **XHes2** to any significant degree, both in naïve and neuralized animal caps (Fig. 3A). By contrast, the bHLH-O genes **ESR1** and **ESR7** were markedly induced by **Notch** ICD both in neuralized caps (Fig. 3A) and in neuroectoderm and ectoderm of neurula stage embryos (Koyano-Nakagawa et al., 2000) (data not shown). Thus, the transcriptional regulation of **XHes2** is clearly distinct from the one of other bHLH-O genes, such as **ESR1** and **ESR7**.

**Perturbation of **XHes2** activity in retinal progenitor cells affects gliogenesis and neuronal cell fate**

As **XHes2** is strongly expressed in the developing eye, we analysed whether it might function in the context of retinogenesis. For this purpose, **XHes2** was overexpressed in the developing retina by in vivo lipofection. Retinal cell clones overexpressing **XHes2** contained a dramatic increase of Müller-like cells (ranging from 45 to 75%), based on their morphology and position in the retina, compared with less than 5% in control retinas (Fig. 4A). Müller cell identity could be confirmed by immunostaining using an anti-CRALBP antibody, a marker of mature Müller glial cells (Bunt-Milam and Saari, 1983); in **XHes2** lipofected retinas, 93.8% of GFP-positive cells exhibiting a Müller cell morphology were indeed co-stained with the anti-CRALBP antibody, a marker of mature Müller glial cells (Bunt-Milam and Saari, 1983); in **XHes2** lipofected retinas, 93.8% of GFP-positive cells exhibiting a Müller cell morphology were indeed co-stained with the anti-CRALBP antibody (n=210 cells, Fig. 4B-F). Thus, overexpression of **XHes2** in the retina strongly affects precursor cell fate decision, leading to a dramatic increase of glial cells at the expense of neurons. Of note, retinas overexpressing a mutant version of **XHes2** [impaired in its DNA-binding activity (**XHes2-DBM**) did not exhibit any change in retinal cell distribution when compared with the controls (n=1648 analysed cells for the control and 923 for **XHes2-DBM**, data not shown), suggesting that the DNA binding domain is necessary for **XHes2** to promote the glial fate.
To confirm the gliogenic function of XHes2 during retinogenesis, we performed loss-of-function analysis using two antisense morpholino oligonucleotides against XHes2 (Mo1 and Mo2; Fig. 4G-L). In vivo lipofection of both morpholinos led to a significant decrease in Müller cells (Fig. 4M). This result, consistent with the above overexpression data, reinforces the view of XHes2 being critically involved in the control of gliogenesis. Of note, none of the other neuronal cell types showed any significant and reproducible change in its distribution (data not shown). Co-injection of Mo1 and a XHes2 construct devoid of the Mo1 target sequence rescued the loss-of-function phenotype and led to an increase in Müller cells similar to that observed with XHes2 alone.

To complete this loss-of-function analysis, we also performed in vivo lipofection experiments with two XHes2 constructs designed to block the endogenous XHes2 activity (a putative dominant negative and an antimorphic construct, named XHes2-W and XHes2-W-VP16, respectively, see Fig. 4N). As observed with morpholinos, both mutants induced a significant decrease of Müller cells upon expression in the developing retina (Fig. 4O). Notably, XHes2-W also generated a significant decrease of ganglion cells and amacrine cells, associated with a significant increase of photoreceptor cells (Fig. 4O). Thus, disrupting XHes2 function by XHes2-W not only affects gliogenesis, but also alters cell fate decisions of retinal neurons. One could expect an even more severe phenotype if XHes2-W-VP16 is employed, considering its transcriptional activation capability. Unexpectedly though, it produces the opposite effect on the distribution of neurons, i.e. a decrease of photoreceptor cells, as well as an increase of ganglion and amacrine cells (Fig. 4O). Thus, beside its role in gliogenesis, XHes2 could also be involved in neuronal cell type specification.

**XHes2 overexpression affects the proliferation of retinal precursors and the timing of Müller cell generation**

To gain more insight into the mechanisms underlying XHes2 gliogenic activity, we decided to investigate whether XHes2 affects precursor cell proliferation. We therefore performed BrdU injection at various stages of retinogenesis after prior lipofection with XHes2. At stage 30 and 33/34, the proportion of BrdU-positive cells among GFP clones in XHes2-overexpressing retinas did not differ from that observed in control retinas (Fig. 5A,B-G). However, it was found to be significantly increased at both stage 35/36 and stage 37/38 (Fig. 5A,H-M). This excessive proliferation was not due to enhanced cell survival, as assessed by the similar percentage of apoptotic cells found in GFP or XHes2-overexpressing clones at stage 37 (1.58±0.59, n=442 cells and 1.69±0.63, n=415 cells, respectively). Therefore, these data strongly suggests that, from stage 34 onwards, XHes2 maintains a subset of precursors that should have exit the cell cycle in a proliferative state. As Müller cells are normally the last cell type to be born, such an increase in the proportion of late progenitor cells surely contributes to the extra glial cell production. However this may not be sufficient to explain the huge predominance of Müller cells among retinal cell types. We thus investigated whether some Müller cells could have been born before stage 34, despite the absence of proliferation defects. For this purpose, we performed birthdating experiments by injecting BrdU into stage 34 to stage 41 embryos lipofected with XHes2. Such experiments allow to evaluate the timing of cell cycle exit of each differentiated cell type. We observed that the percentage of BrdU-positive Müller glial cells was significantly decreased in XHes2 lipofected retinas compared with control retinas (Fig. 5N-Q). Thus, a fraction of Müller cells observed in XHes2 transfected retinas at stage 41 were born prematurely.

As a whole, the above data reveal that XHes2-dependent excessive gliogenesis occurs during the whole course of retinogenesis. Before stage 34, XHes2 seems to drive some early born cells, that would normally have given rise to neurons, to adopt a glial fate, apparently without affecting their proliferation. Thereafter, XHes2 is also able to prolong proliferation of a subset of precursors resulting in a second wave of extra Müller cell production.

**XHes2 inhibits neuronal differentiation by repressing proneural gene transcription**

XHes2 could act either as an instructive gliogenic factor or as a repressor of neurogenesis. To test whether XHes2 has the ability to inhibit proneural gene transcription, we analyzed specific gene expression during the process of primary neurogenesis, following XHes2 overexpression or XHes2
knock-down. Ectopic XHes2 inhibited expression of both XNgnr1 and N-tubulin at the open neural plate stage (Fig. 6A,B). Repression of proneural and neuronal gene transcription persisted to late neurula stage (Fig. 6K,M,O). Conversely, inhibition of XHes2 expression with antisense morpholino oligonucleotides resulted in ectopic expression of XNgnr1 in the presumptive otic placode (Fig. 6C-E), strongly suggesting that XHes2 acts physiologically as a repressor of neurogenesis in its endogenous domain of expression.

As a first step to unravel potential mechanisms sustaining XHes2-dependent neurogenic inhibiting activity, we next investigated the effects of the different mutant XHes2 constructs. Tagged versions

Fig. 4. XHes2 is involved in retinal cell fate decisions. (A-F) XHes2 overexpression biases retinal precursors towards gliogenesis. (A) Percentage of retinal cell types observed in stage 41 retinas upon lipofection with GFP alone (control; 1244 cells, 11 retinas) or GFP plus XHes2 (939 cells, 13 retinas). (B-F) Typical sections of retinas transfected with GFP alone (B) or GFP plus XHes2 (C-F) showing the dramatic increase in Müller cell number (white arrows in B and C), as confirmed by anti-CRALBP immunostaining (E,F). White arrows in D-F point to cells with a Müller morphology that are indeed stained by CRALBP. (G-L) Two morpholinos were designed (G) that specifically affect in a dose-dependent manner the expression of a chimaeric XHes2-GFP construct, as assessed by in vitro translation (H) and in vivo GFP fluorescence (I-L) experiments. (M) XHes2 knockdown decreases the percentage of Müller cells (control Mo: 1144 cells, 10 retinas; Mo1: 2028 cells, 12 retinas; Mo2: 1534 cells, 10 retinas; XHes2: 432 cells, four retinas; XHes2+Mo1: 84 cells, three retinas). (N,O) XHes2 affects neuronal cell type specification. (N) Schematic representation of XHes2 variants employed. (O) Percentage of retinal cell types observed in stage 41 retinas co-lipofected with GFP plus XHes2-ΔW/1200 cells, 12 retinas) or GFP plus XHes2-ΔW-VP16 (1072 cells, 11 retinas). Values are given as mean±s.e.m. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

Abbreviations: AM, amacrine cells; BP, bipolar cells; GC, ganglion cells; GCL, ganglion cell layer; HC, horizontal cells; INL, inner nuclear layer; PR, photoreceptor cells; MU, Müller glial cells; ONL, outer nuclear layer.
injected in eight cells stage embryos drove the expression of equivalent levels of proteins as assessed by western-blotting experiments (data not shown). XHes2-DBM was not able to repress XNgnr1 nor N-tubulin (data not shown). XHes2-ΔW failed to inhibit XNgnr1 expression but still repressed N-tubulin (Fig. 6F,H). This suggests that, at least during primary neurogenesis, recruitment of transcriptional co-repressors through the WRPW motif (Fisher et al., 1996; Grbavec and Stifani, 1996; Paroush et al., 1994) is not an obligatory mechanism for XHes2 repressor activity. As XHes2 represses proneural gene transcription, we tested whether antimorphic variants could activate it. In an animal cap assay (Fig. 6J), overexpression of XHes2-ΔC-VP16 induced XNgnr1 and NeuroD expression in explants cultivated until the equivalent of early neurula stages; prolonged cultivation was found to be necessary to observe the induction of late neuronal markers such as N-tubulin and Xath3. We thus propose that XHes2-ΔC-VP16 is effective in promoting neuronal differentiation, albeit in a delayed manner. Accordingly, XHes2-ΔW-VP16 misexpression in whole embryos also promoted ectopic expression of XNgnr1 in the non-neural ectoderm as early as the open neural plate stage (Fig. 6G1-G3, 6L), whereas ectopic expression of N-tubulin could only be observed in later embryos (compare Fig. 6P with 6I). In order to determine the identity of these ectopic neurons, we performed in situ analysis using probes against sensory- (XHox11L2), motor- (Xlim3) or interneuron (Pax2) markers. Expression of these genes was repressed by XHes2. By contrast, XHes2-ΔW-VP16 inhibited Xlim3 and Pax2, but induced a strong ectopic expression of XHox11L2. Consequently, the ectopic neurons forming in response to XHes2-ΔW-VP16 can be classified as sensory neurons (Fig. 6Q-V).

**XHes2 physically interacts with a subset of bHLH proteins including NeuroD**

The observation, in early animal cap explants, of the simultaneous induction of XNgnr1 and NeuroD upon XHes2-ΔC-VP16 misexpression (Fig. 6J) suggests that both genes constitute potential transcriptional targets of XHes2. To investigate whether other

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**Fig. 5. XHes2 promotes extra Müller cell production by affecting both glial cell birthdate and retinal precursor proliferation.** (A-M) Analysis of BrdU incorporation (3 hour pulse) in retinal clones, following GFP or GFP plus XHes2 lipofection. (A) Percentage of BrdU-positive cells among GFP-transfected retinal cells (76, 509, 707 and 544 cells for the control; 124, 347, 1058 and 322 for XHes2 at stages 30, 33/34, 35/36 and 37/38, respectively). (B-M) Typical sections of stage 33/34 (B-G) or 37/38 (H-M) retinas immunostained for both GFP and BrdU, following lipofection of GFP alone (B-D,H-I) or GFP plus XHes2 (E-G,K-M). At stage 37/38, BrdU-positive cells in the control are mostly restricted to the CMZ, while many XHes2-overexpressing cells are still proliferating in the central retina (compare arrows in I and L). (N-Q) Birthdating experiments. Embryos lipofected with either GFP alone or GFP plus XHes2 were injected with BrdU every 8 hours from stage 34 to stage 41. (N) Percentage of BrdU-positive cells among GFP-transfected Müller cells (22 Müller cells for the control and 41 for XHes2). (O-Q) An example of GFP and BrdU double staining following XHes2 overexpression. Arrows indicate GFP-positive Müller cells that are BrdU negative. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; LE, lens; ONL, outer nuclear layer. Values are given as mean±s.e.m. *P<0.05, **P<0.001 (Student's t-test).
Fig. 6. XHes2 can regulate proneural gene transcription in both neural and non-neural ectoderm. (A-I,K-V) Whole-mount in situ hybridization analysis of neuro-specific gene expression in stage 14 (A-D,F-I) or 20 (E) or 24 (K-V) Xenopus embryos upon injection of XHes2 variants or morpholinos. Black arrowheads, posterior expression domains; black arrows, anterior expression domains; white arrowheads, ectopic expression. (J) Real-time RT-PCR analysis of neuro-specific gene expression in animal cap assays following injection with XHes2-ΔC-VP16. (A,B) XHes2 represses neurogenesis, as assessed by the downregulation of XNgnr1 (89%, n=9) (A) and N-tubulin (88%, n=16) (B). (C-E) Conversely, inhibition of endogenous XHes2 with XHes2-Mo2 extends XNgnr1 expression in the presumptive otic placodal region (D, injection into one of eight cells, 45%, n=42; one of two cells, 62%, n=13). XHes2-Mo1 shows the same phenotype (not shown, one of eight cells, 56%, n=63; one of two cells, 100%, n=14) in contrast to the control Mo (C, one of eight cells, 5%, n=60; one of two cells, 0%, n=37). (F) At some later neurula stage embryos, ectopic patches of XNgnr1 appear close to the otic placode region (arrow). Arrows in C,D point to the presumptive otic placodal region. (F-I) Effects of XHes2 variants on proneural gene expression at stage 14. (F,H) XHes2-ΔW does not significantly alter XNgnr1 expression (slightly reduced in 39%, n=13) (F), but still inhibits N-tubulin expression (91%, n=11) (H). (G,I) In stage 14 embryos, XHes2-ΔW-VP16 leads to ectopic XNgnr1 expression (G1, 100%, n=19; G2 and G3 show the non-injected and the injected lateral sides, respectively) but fails to activate N-tubulin expression (I, 100%, n=15). Moreover, transcription of N-tubulin is repressed in its endogenous domains (I). (J) XHes2-ΔC-VP16 induces neuronal differentiation of animal caps. In early caps (stage 13), XHes2-ΔC-VP16 induces the expression of the proneural genes XNgnr1 and NeuroD, while expression of late neuronal markers is low (Xath3) or even undetectable (N-tubulin). From stage 22 onwards, Xath3 and N-tubulin are significantly upregulated. Relative values are given as mean±s.e.m of two independent experiments. (K-P) Effects of XHes2 variants on proneural gene expression at stage 24. In XHes2-ΔW-VP16 overexpressing embryos, an ectopic activation of XNgnr1 (L, 29%, n=14), NeuroD (N, 100%, n=25) and N-tubulin (P, 85%, n=13) is observed. Conversely, expression of XNgnr1 (K, 93%, n=14), NeuroD (M, 100%, n=22) and N-tubulin (O, 85%, n=13) is strongly reduced at this stage following XHes2 injection. (Q-V) XHes2-ΔW-VP16 induces ectopic sensory neurons. The sensory neuron marker Xhox11L2 is ectopically activated by XHes2-ΔW-VP16 (R, 100%, n=13) while being repressed by XHes2 (Q, 100%, n=13). By contrast, transcription of the interneuron and motoneuron markers Pax2 (S,T) and Xlim3 (U,V) is downregulated by both XHes2 (S, 85%, n=26; U, 92%, n=12) and XHes2-ΔW-VP16 (T, 92%, n=24; V, 64%, n=14). Embryo orientation: (A-I) dorsal views, anterior downwards; (K-V) anterior views, dorsal upwards. is, injected side; nis, non-injected side.
mechanisms could also account for XHes2 anti-neuronal activity, we performed co-injection experiments of either XNgnr1 or NeuroD together with XHes2. XHes2 repressed the XNgnr1-induced ectopic activation of N-tubulin (Fig. 7A,B) and NeuroD (Fig. 7C,D) suggesting that XHes2 has the ability to repress NeuroD transcription downstream of XNgnr1. Furthermore, we observed that NeuroD RNA injection could not rescue the XHes2-dependent inhibition of neurogenesis (Fig. 7E,F). These observations clearly indicate that XHes2 serves a function downstream of NeuroD gene regulation.

bHLH-O proteins have been reported to inhibit neuronal differentiation not only as homodimers, but also by formation of heterodimers with other bHLH proteins, thereby blocking their ability to activate the transcription of downstream target genes (for reviews, see Davis and Turner, 2001; Iso et al., 2003). To investigate whether the inhibitory effect of XHes2 on neurogenesis could result from physical interactions with some bHLH proteins, co-immunoprecipitation experiments were carried out on animal cap explants misexpressing selected epitope tagged proteins. As shown in Fig. 7G, XHes2 has the ability to form homomeric complexes. In addition, it was also found to interact with NeuroD, Xath3, Xhairy1 and XHes6r, but not with other bHLH proteins tested, including XNgnr1 (Fig. 7G,H).

Taken together, these results suggest that XHes2 inhibits neuronal differentiation both by inhibition of XNgnr1 and NeuroD gene transcription, and by other mechanisms, such as physical interaction with proneural bHLH proteins, including NeuroD.

**DISCUSSION**

XHes2 is a bHLH-O protein-encoding gene that is primarily expressed in the developing sensory organs of *Xenopus* embryos. In the retina, it seems to be a crucial actor of the complex network governing glial versus neuronal differentiation. Our data suggest that XHes2 can inhibit neurogenesis at two levels, first by repressing the transcription of proneural bHLH genes, and second, via its ability to form inhibitory complexes with proneural bHLH proteins, such as NeuroD.

**Embryonic XHes2 expression profile is mainly restricted to developing sensory organs**

The main expression characteristics of XHes2 are clearly distinct from those of other known bHLH-O proteins in *Xenopus*. The most prominent expression domains of XHes2 define groups of cells giving rise to sensory organs. Some other bHLH-O genes, such as *XHRT1*, are also specifically expressed in a subset of cells of the nervous system (Pichon et al., 2002). By contrast, many others, such as Hes5-like genes, are expressed in general territories of primary neurogenesis and somitogenesis in *Xenopus* (Koyano-Nakagawa et al., 2000; Li et al., 2003; Schneider et al., 2001). Furthermore, XHes2 responsiveness to Notch signalling or proneural bHLH proteins differs from that of E(spl) related genes that are thought to be involved in lateral inhibition during primary neurogenesis in *Xenopus* (Koyano-Nakagawa et al., 1999; Koyano-Nakagawa et al., 2000; Lamar and Kintner, 2005; Wettstein et al., 1997). Indeed, injection of Notch ICD failed to induce XHes2 expression in naive or neuralized animal cap explants, as well as in ectodermal and most neuroectodermal cells of intact embryos. Moreover, XHes2 is activated only in a subset of ectodermal cells in whole embryos by ectopic XNgnr1 and is even inhibited within the neural plate. These findings are not compatible with the idea of XHes2 being a primary target for Notch and being inducible by XNgnr1, at least in the early phases of neurogenesis. A few other bHLH-O genes also display differences in the way they are regulated by Notch signalling and XNgnr1, such as Xhairy2 or XHRT1 (Lamar and Kintner, 2005; Lopez et al., 2005; Pichon et al., 2002), indicating a specific context-dependent control of bHLH-O gene expression. Promoter analysis may be required to bring more insights into the transcriptional regulation of XHes2.
Expression profiles of several *Hes* genes have been characterized during murine retinogenesis. *Hesr1*, *Hesr2*, *Hesr3*, *Hes1*, *Hes5* and *Hes6* are all first expressed in the ventricular zone of the retina, which contains common, multipotent precursors for neurons and glial cells (Bae et al., 2000; Hojo et al., 2000; Satow et al., 2001; Tomita et al., 1996). As development proceeds, some of them are maintained in differentiating or differentiated retinal cell types. *Hesr1* is expressed in both the inner and outer region of the inner nuclear layer (INL), that contain amacrine and horizontal cells, respectively (Satow et al., 2001). *Hesr2* expression is restricted to the middle region of the INL containing bipolar and Müller cells (Satow et al., 2001). *Hes5* is found in differentiating, but not mature, Müller cells (Hojo et al., 2000). *Hes6* is expressed in ganglion cells and in the INL (Bae et al., 2000). Thus, these Hes genes are likely to act at several steps of retinogenesis. By contrast, but similar to murine *Hes1* and *Hesr3* (Satow et al., 2001; Tomita et al., 1996), *XHes2* is restricted to precursor cells, suggesting that it could be involved in retinal cell fate decisions.

**XHes2 acts as a gliogenic factor in the retina**

In the rodent retina, *Hes1*, *Hes5* and *Hes2* have been shown to promote gliogenesis (Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001), while *Hes6* has an opposite inhibitory effect upon overexpression (Bae et al., 2000). By contrast, *Hesr1* and *Hesr3* do not seem to play any role in retinoblast fate decisions (Satow et al., 2001). These observations demonstrate that Hairy/Enhancer of Split family members expressed in the retina have distinct functions during retinogenesis.

In the present paper, we show that *XHes2* overexpression during retinogenesis in *Xenopus* leads to a dramatic increase of glial cells at the expense of neurons. Furthermore, *XHes2* loss of function results in the opposite effect, i.e. a decrease of Müller cells. Two different hypotheses can be considered. As Müller cells are the latest cells to be born during retinogenesis, *XHes2* overexpression could simply delay differentiation of retinal precursors. Alternatively, *XHes2* could specifically promote gliogenesis and/or inhibit neurogenesis. In the first case, *XHes2* overexpression should lead to an increase of all late born cells, including bipolar cells, combined with a decrease of early-born cells, such as ganglion cells. However, as we did not observe such a phenotype, we do not favour this hypothesis. Consequently, we propose that *XHes2* is involved in the choice of glial versus neuronal cell fate. Some Hes genes expressed broadly in the nervous system may be part of a common genetic cascade controlling gliogenesis. By contrast, the restricted expression profile of *XHes2* suggests that this repressor could control specific aspects of gliogenesis in sensory organs.

**XHes2 gliogenic activity relies on its ability to repress neurogenesis through different mechanisms**

Accumulating evidence suggests that components of the cell cycle molecular machinery influence cell fate decision in the nervous system (for reviews, see Ohnuma et al., 2001; Ohnuma and Harris, 2003). In line with this idea, we observed that *XHes2*-dependent excessive generation of Müller cells correlates with a delayed timing of cell cycle arrest for some retinal precursor cells. This is consistent with the recent finding that the Hes-related gene *Hes1* directly controls proliferation through transcriptional repression of the cyclin-dependent kinase inhibitor, p27(Kip1) (Murata et al., 2005). However, whether *XHes2* directly affects cell cycle exit and whether this is the underlying mechanism for excessive Müller cell generation remains to be elucidated. Alternatively, prolonged proliferation for *XHes2*-overexpressing progenitors could be a consequence of instructive Müller cell fate induction.

Besides, our results suggest that these changes in cell cycle exit timing are restricted to a subpopulation of retinal progenitors. Indeed, wild-type and *XHes2* overexpressing retina exhibited a similar proportion of cell cycle withdrawal between stage 30 and 33/34. This suggests that *XHes2* does not affect cell cycle exit timing of early-born cell types. Nevertheless, these early progenitors are sensitive to *XHes2* gliogenic activity because, as inferred by our birthdating experiment, they also give rise to Müller cells following cell cycle arrest. We therefore propose that in addition to maintaining some precursors longer in the cell cycle, *XHes2* is also able to redirect early born cell types, that would otherwise have given rise to neurons, towards a glial fate. This most probably results from the ability of *XHes2* to inhibit proneural gene activity. In the wild-type retina, cells that are born first express proneural genes, which lead them along the neurogenic pathway and inhibit gliogenesis. In view of our genetic interaction data, we propose that *XHes2* overexpression prevents neurogenesis of early-born cells by inhibiting the activity of proneural genes, such as *XNgnr1* or *NeuroD*. Consequently, the alternative fate is the Müller glial one.

Further investigations would be necessary to know whether, in addition to inhibiting neurogenesis, *XHes2* also possesses an instructive gliogenic activity. In addition, the putative coupling between the effects of *XHes2* on the cell cycle and its neurogenesis inhibiting activity remains to be investigated.

Our data highlight different possible mechanisms through which *XHes2* could exert its neuronal inhibiting activity. As the DNA-binding domain appeared necessary for *XHes2* to inhibit neurogenesis, *XHes2* is likely to operate through transcriptional control of target genes. We observed that endogenous expression of *XNgnr1* and *NeuroD* is repressed upon ectopic expression of *XHes2* in whole embryos. In addition, both genes are simultaneously induced in early animal cap explants upon *XHes2*-ΔC-VP16 misexpression. We cannot exclude the possibility that such effects may arise from non-specific action of the overexpressed *XHes2* constructs on any Hes target genes sharing the same binding sites. However, the observation that inhibition of endogenous *XHes2* with morpholino oligonucleotides results in ectopic *XNgnr1* expression in presumptive otic placodal cells strongly suggests that some proneural genes may indeed constitute potential transcriptional targets of *XHes2*.

The observation that neither *XNgnr1* nor *NeuroD* RNA injection could rescue the *XHes2*-dependent inhibition of neurogenesis suggested that *XHes2* may also act through alternative mechanism(s), in addition to its transcriptional repressing activity. Our data support the idea that *XHes2* forms multi-protein inhibitory complexes with proneural transcription factors. *XHes2* was found to co-immunoprecipitate in vivo with *NeuroD* and *Xath3*, but not with *XNgnr1* and *Xath5* proneural bHLH proteins (Kanekar et al., 1997; Lee et al., 1995; Ma et al., 1996; Perron et al., 1999; Takebayashi et al., 1997), implying that *XHes2* may antagonize *NeuroD* and *Xath3* function via protein-protein interactions. Furthermore, *XHes2* interacts with the bHLH-O proteins *Xhairy1* and *Xhes6r* (an allelic version of *Xhes6*; M.S., unpublished). Previous studies suggested that *Hes6* promotes neuronal differentiation by blocking the function of *Hes1* proteins (Bae et al., 2000; Gratton et al., 2003; Koyano-Nakagawa et al., 2000). The formation of *XHes2*-*XHes6r* heterodimers implies that *XHes6r* may also antagonize *XHes2* activity. Further investigations will be necessary to confirm that *XHes2* indeed inhibits proneural genes and
proteins in the retina. Nevertheless, the above data strongly suggest that XHes2 acts as a gliogenic factor by taking part in the complex bHLH-O network of interactions that finely control neurogenesis through mutual cross-regulations.

**XHes2 is involved in neural cell fate decision during retinogenesis**

Overexpression of the XHes2 dominant-negative constructs, XHes2-ΔW and XHes2-ΔW-VP16, decreases the number of Müller cells but also alters the proper ratio of neuronal cell types. This result suggests that XHes2, in addition to controlling the neuron-glia ratio, may also be involved in neuronal cell fate decisions. Surprisingly, lipoexpression of XHes2-ΔW and XHes2-ΔW-VP16 resulted in opposite effects on neuron distribution. XHes2-ΔW significantly increased photoreceptors at the expense of ganglion and amacrine neurons, whereas XHes2-ΔW-VP16 increased ganglion and amacrine neurons at the expense of photoreceptors. The different modes of action of XHes2-ΔW and XHes2-ΔW-VP16 could explain such a discrepancy. As XHes2 acts as a repressor, XHes2-ΔW, which cannot bind the corepressor groucho but still binds DNA, may act in a dominant-negative manner, leading to a release of XHes2-mediated gene inhibition. However, XHes2-ΔW-VP16 possesses a transcriptional activation domain and thus probably stimulates the transcription of XHes2 target genes over a basal level. Consequently, it is probable that the expression level of XHes2 target genes is different in retinas lipofected with XHes2-ΔW or with XHes2-ΔW-VP16. The balance in gene expression could thus differentially influence neuronal cell fate decisions. The Hes1-knock out phenotype indirectly suggested a role of Hes1 in controlling neuronal cell fate. Hes1-null mice develop severe developmental eye defects, resulting from impaired fate decisions. The Hes1-knock out phenotype indirectly suggested that XHes2 acts as a gliogenic factor by taking part in the complex bHLH-O network of interactions that finely control neurogenesis through mutual cross-regulations.


