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Xiro homeoproteins coordinate cell cycle exit and primary neuron formation by upregulating neuronal-fate repressors and downregulating the cell-cycle inhibitor *XGadd45-γ*

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Abstract

The *iroquois* (*iro*) homeobox genes participate in many developmental processes both in vertebrates and invertebrates, among them are neural plate formation and neural patterning. In this work, we study in detail *Xenopus* Iro (Xiro) function in primary neurogenesis. We show that misexpression of *Xiro* genes promotes the activation of the proneural gene *Xngnr1* but suppresses neuronal differentiation. This is probably due to upregulation of at least two neuronal-fate repressors: *XHairy2A* and *XZic2*. Accordingly, primary neurons arise at the border of the *Xiro* expression domains. In addition, we identify *XGadd45-γ* as a new gene repressed by Xiro. *XGadd45-γ* encodes a cell-cycle inhibitor and is expressed in territories where cells will exit mitosis, such as those where primary neurons arise. Indeed, *XGadd45-γ* misexpression causes cell cycle arrest. We conclude that, during *Xenopus* primary neuron formation, in *Xiro* expressing territories neuronal differentiation is impaired, while in adjacent cells, *XGadd45-γ* may help cells stop dividing and differentiate as neurons.

Keywords: *Xenopus*; *Iroquois*; *Gadd45-γ*; Cell cycle; Neural patterning

1. Introduction

Proneural genes that encode transcriptional regulators of the basic helix–loop–helix (bHLH) family play pivotal roles in neurogenesis. It is thought that activation of the earliest proneural genes promotes a genetic cascade that causes the transition from competent neural precursors to terminally differentiated neurons. Members of this cascade are late-expressed bHLH genes, other transcription factors and the Notch pathway (reviewed in Chitnis, 1999). In *Xenopus*, the early proneural gene *Xngnr1* is expressed within the neural plate in three broad domains at each side of the midline (Ma et al., 1996). *Xngnr1* promotes activation of the lateral inhibition pathway mediated by Notch through the upregulation of its ligand *XDelta1* (*XDl1*) (Chitnis et al., 1995; Ma et al., 1996). As a consequence, only a subset of the cells expressing *Xngnr1* differentiate as primary neurons (Ma et al., 1996). Their differentiation requires the bHLH and zinc-finger factors XMyT1, XCoe2/Xebf2, Xath3, XHes6, NKL, NeuroD and Xebf3 (Bellefroid et al., 1996; Dubois et al., 1998; Koyano-Nakagawa et al., 2000; Lamar et al., 2001; Lee et al., 1995; Pozzoli et al., 2001; Takebayashi et al., 1997). Additional factors such as Gli proteins and XBF-1, which promote neuronal differentiation, and XZic2, XHairy1 and XHairy2A, which antagonize it, probably help define the position in which neurons differentiate (Bourguignon et al., 1998; Brewter et al., 1998; Dawson et al., 1995; Koyano-Nakagawa et al., 2000). The end result of this genetic cascade is the generation of primary neurons in bilateral stripes located at medial, intermediate and lateral positions within the neural plate (Bellefroid et al., 1996; Chitnis et al., 1995; Koyano-Nakagawa et al., 2000; Ma et al., 1996; Takebayashi et al., 1997).

Cell-cycle arrest is required for neuronal differentiation (Farah et al., 2000; Hollyday, 2001), although the details of how these two processes are interrelated are largely unknown. Some insights have been provided by *Xenopus XBF-1* (Hardcastle and Papalopulu, 2000). Mild overexpression of this gene stops the cell cycle and promotes generation of ectopic neurons. In contrast, strong overexpression promotes cell division and suppresses neuronal

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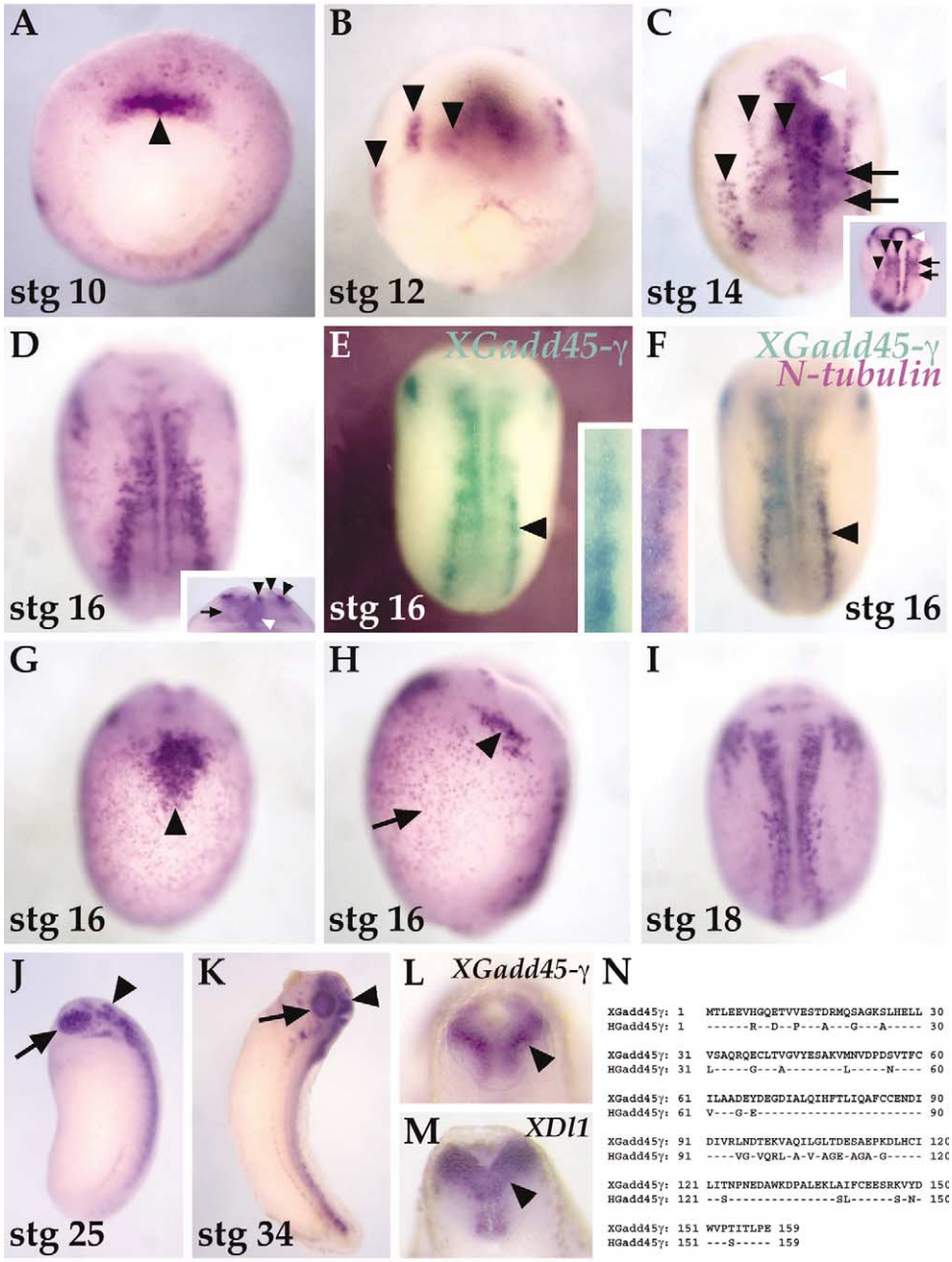

Fig. 1. Expression and protein sequence of *XGadd45-γ*. (A) Vegetal view; (B–F,I) dorsal views; (G) ventral view; (H,J,K) lateral views. (A) *XGadd45-γ* is expressed in the dorsal mesoderm (arrowhead) at early gastrula stages (stg). Throughout gastrulation, *XGadd45-γ* is also expressed in scattered cells of the animal region (not shown). (B) At mid-late gastrula, three rows of cells at each side of the midline start to accumulate *XGadd45-γ* mRNA (arrowheads). (C) These rows of *XGadd45-γ* expressing cells are clearly visible in the early neurula (black arrowheads). *XGadd45-γ* is also expressed in forming somites (arrows) (Jen et al., 1999) and in a horseshoe-shaped domain within the anterior neural plate (white arrowhead). This pattern is similar to that of *XDl1* (inset). (D) A similar expression profile is observed at mid neurula stages. (D, inset) Transverse section of a mid neurula revealing *XGadd45-γ* expression in three neuroectodermal rows of cells (black arrowheads), in the notocord (white arrowhead) and in somites (arrow). (E) Mid neurula doubly hybridized for *XGadd45-γ* and *N-tubulin* and shown after the first chromogenic reaction to detect *XGadd45-γ* mRNA (cian). (F) After second chromogenic reaction to detect *N-tubulin* (purple). Insets in (E) and (F): high magnifications views of the lateral neural stripe pointed at with an arrowhead in their corresponding panels. During neurula stages, *XGadd45-γ* is also expressed in the prospective cement gland (G, arrowhead), trigeminal placode (H, arrowhead) and in scattered cells in the non-neural ectoderm (H, arrow). *XGadd45-γ* expression is detected in the same sites at later stages (I). In late neurula (J) and tailbud stages (K), *XGadd45-γ* is expressed in territories in which secondary neurogenesis takes place, such as the eye (arrows) and brain (arrowheads). (L–M) Hindbrain transverse sections of tailbud embryos showing *XGadd45-γ* (L) and *XDl1* (M) domains of expression (arrowheads). (N) Amino acid sequence alignment of *Xenopus* and human Gadd45-γ proteins (accession numbers AJ414384 and AF078078, respectively). Identical residues are indicated by dashes.

differentiation. The effect of *XBF-1* on the cell cycle is mediated, at least in part, by its regulation of $p27^{XIC1}$, which encodes a cell-cycle inhibitor that is expressed in non-dividing cells (Hardcastle and Papalopulu, 2000).

The *iroquois* (*iro*) genes, which encode evolutionary conserved homeoproteins, participate in neurogenesis and in many other developmental processes (reviewed in Cavodeassi et al., 2001). In *Xenopus*, five members of the *iro* family have so far been identified (Bellefroid et al., 1998; Garriock et al., 2001; Gómez-Skarmeta et al., 1998). *Xiro1*, *2* and *3* are expressed in the neural plate in territories that partially overlap with those that express proneural genes (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). Moreover, overexpression of either one of these *Xiro* genes ectopically activate proneural genes (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998). However, in *Xenopus* and other vertebrates, this activation may be indirect, since Xiro1 functions as a repressor that downregulates *Bmp-4* (Gómez-Skarmeta et al., 2001). Moreover, *Ziro3*, the zebrafish ortholog of *Xiro3*, also encodes a repressor that is expressed in the organizer and also downregulates *Bmp-4* (Kudoh and Dawid, 2001).

Here, we further examine the role of *Xiro* genes in neurogenesis. We find that these genes, despite their ability to promote *Xngnr1* expression, suppress neuronal differentiation, probably through upregulation of neuronal repressors such as *XZic2* and *XHairy2A*. In addition, we identify another gene regulated by Xiro1, the *Xenopus* ortholog of the *growth-arrest-and-DNA-damage-induced gene gamma* (*XGadd45-γ*), which provides a link between neuronal differentiation and cell cycle exit. *XGadd45-γ* encodes a small protein whose homologs in humans and mice belong to three-member families (Abdollahi et al., 1991; Beadling et al., 1993; Fan et al., 1999; Fornace, 1992; Fornace et al., 1992; Takekawa and Saito, 1998; Zhang et al., 1999). These proteins interact with cell-cycle regulators and their overexpression promotes cell-cycle arrest and apoptosis (Azam et al., 2001; Hall et al., 1995; Kearsey et al., 1995; Sheikh et al., 2000; Smith et al., 1994; Takekawa and Saito, 1998; Vairapandi et al., 1996; Zhan et al., 1999; Zhang et al., 2001; Zhao et al., 2000). However, the details of their interference with the cell cycle are unknown. Here we find that, during *Xenopus* development, *XGadd45-γ* is expressed in territories in which cells will stop dividing. Accordingly, misexpression of *XGadd45-γ* reduces the number of dividing cells in *Xenopus* embryos. *XGadd45-γ* is activated by Xngnr1 and repressed by Xiro. As a consequence, it is expressed in primary neuron territories, where it may help cells exit the cell cycle and differentiate as neurons.

2. Results

2.1. Isolation and expression of XGadd45-γ

A *Xenopus* subtraction library enriched in cDNA fragments of genes putatively repressed by Xiro1 was prepared by subtracting the population of cDNAs from animal caps injected with *Noggin* and *Xiro1-VP16* mRNAs from those obtained from caps injected with only *Noggin* mRNA (Section 4). The rationale for this subtraction was that overexpression of *Noggin* neuralizes competent ectoderm (Lamb et al., 1993) and Xiro1 fused to the VP16 activator domain (*Xiro1-VP16*) activates genes that are normally repressed by wild-type Xiro1 (Gómez-Skarmeta et al., 2001). One cDNA (1290 bp) from this enriched library encoded a *Xenopus* homolog of the human (and mouse) Gadd45-γ protein (Fig. 1N). The corresponding gene was named *XGadd45-γ*.

The pattern of expression of *XGadd45-γ* during early embryonic development is described in Fig. 1. Interestingly, *XGadd45-γ* mRNA is detected in sites in which cells will exit the cell cycle (Saka and Smith, 2001), namely, in dorsal

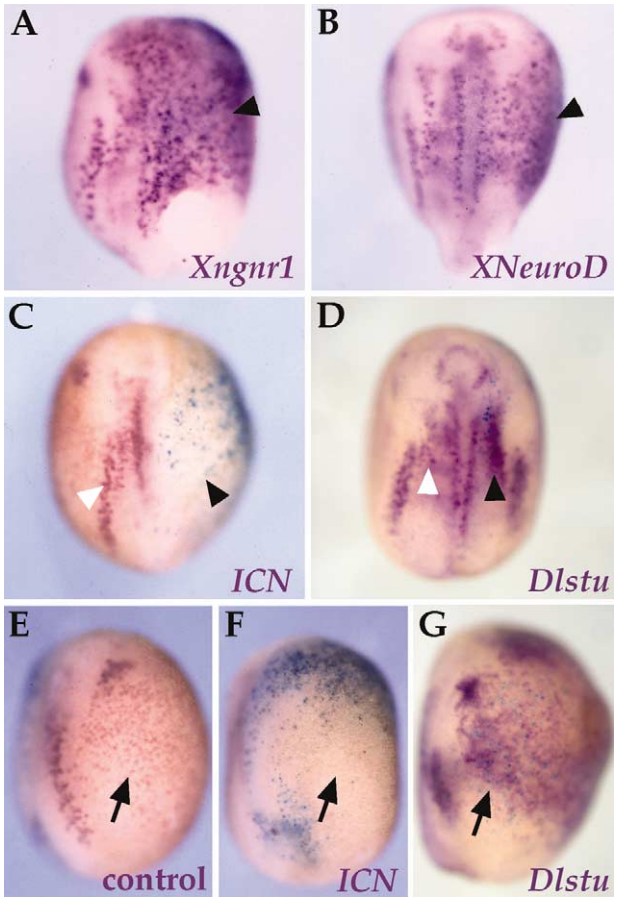

Fig. 2. Proneural genes activate and Notch signaling represses *XGadd45-γ*. Dorsal (A–D) and lateral (E–G) views of midneurula embryos. Embryos were injected with 1 ng of the indicated mRNAs in one blastomere at the two-cell stage together with 0.3 ng of *lacZ* mRNA. The injection side was determined by X-Gal staining. *Xngnr1* (A) and *XNeuroD* (B) mRNA promote ectopic *XGadd45-γ* expression (arrowheads). Increased (C) or decreased (D) Notch signaling, respectively, represses or activates *XGadd45-γ* expression (compare uninjected and injected sides, pointed at with white and black arrowheads, respectively). *XGadd45-γ* expression in scattered cells of the non-neural ectoderm (E) is also repressed (F) or activated (G) when Notch signaling is increased or decreased, respectively (arrowheads).

mesoderm cells during gastrulation (Fig. 1A), and in primary neurons (Fig. 1C–F), prospective cement gland (Fig. 1G) and putative ciliated cells (Fig. 1H) during neurulation. In general during neurulation, *XGadd45-γ* mRNA distribution is very similar to that of *XDl1* (Chitnis et al., 1995) (Fig. 1C, L, M).

2.2. XGadd45-γ is activated by proneural genes and repressed by notch signaling

The three bilateral stripes of *XGadd45-γ* expression in the neural plate (Fig. 1C) coincide with territories in which primary neurons will form (Fig 1E, F). Moreover, *XGadd45-γ* expression is preceded by that of the proneural gene *Xngnr1*(Ma et al., 1996). This suggested that proneural genes might activate *XGadd45-γ*. This was indeed the case. Injection of *Xngnr1* (1 ng) or *XNeuroD* (1 ng) mRNAs promoted ectopic expression of *XGadd45-γ* (Fig. 2A, B; 71 or 67% of embryos, $n = 21$ or 18, respectively). It is known that *Xngnr1* also activates *XDl1*, which promotes Notch signaling and lateral inhibition (Chitnis et al., 1995;Ma et al., 1996). Hence, we examined whether this signaling regulates *XGadd45-γ*. We increased or decreased Notch signaling by overexpressing either the intracellular domain of XNotch1 (ICD; Chitnis et al., 1995) or the dominant negative form of its ligand *XDl1* (Dlstu; Chitnis et al., 1995). These treatments respectively reduced (Fig. 2C; 75%, $n = 44$) or increased (Fig. 2D; 71%, $n = 56$) the number of cells expressing *XGadd45-γ* in the three-stripe domain. In addition, we observed that Notch signaling also negatively regulates *XGadd45-γ* in the non-neuronal ectoderm (Fig. 2E–G; 63–71%, $n = 38$–45).

Both, the coexpression of *XGadd45-γ* with *N-tubulin* (Fig. 1E, F) and its response to Notch signaling indicate that, in the neural plate, *XGadd45-γ* is expressed in differentiating neuronal precursors. In the non-neuronal ectoderm the scattered pattern of *XGadd45-γ* may correspond to ciliated cells, since *a-tubulin*, a marker of these cells, is also repressed by Notch signaling (Deblandre et al., 1999).

2.3. XGadd45-γ antagonizes cell division

Since *XGadd45-γ* expression occurs in sites where cells stop dividing (Saka and Smith, 2001) and the Gadd45 proteins are known to inhibit the cell cycle (Azam et al., 2001; Smith et al., 1994; Takekawa and Saito, 1998; Vairapandi et al., 1996; Zhang et al., 1999; Zhang et al., 2001; Zhao et al., 2000), we examined whether *XGadd45-γ* overexpression affects cell division. To this end we prepared *XGadd45-γ* constructs bearing a Myc epitope either at the amino (MT-XGadd45) or at the carboxy terminus (XGadd45-MT) of the protein. Injection of 1 ng of mRNA of either of these constructs caused lethality. Cells lost adherence, were extruded into the vitelline space, and the embryos failed to gastrulate. This phenotype is similar to that of other cell cycle inhibitors (Grammer et al., 2000; Hardcastle and Papalopulu, 2000). Milder overexpressions (500 pg) allowed approximately 50% of the embryos to develop until neurula stages. We monitored the number of dividing cells by staining with antibody against phosporylated histone H3 (PH3), which recognizes mitotic cells. Embryos were coinjected with *lacZ* mRNA to localize the injection site (blue in Fig. 3). Animal poles of embryos

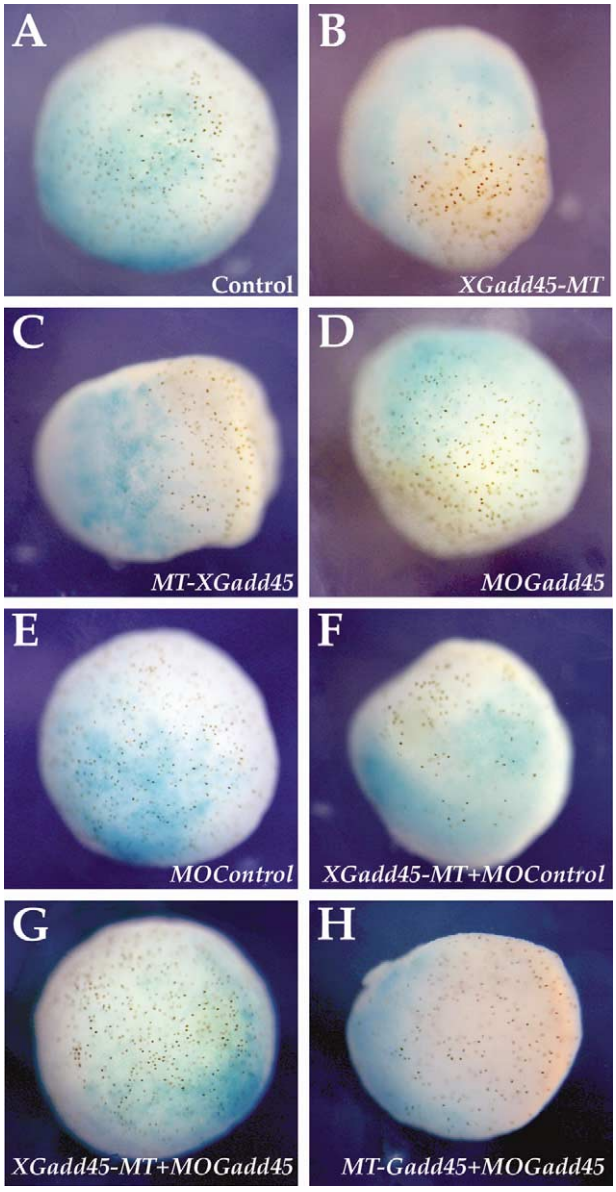

Fig. 3. *XGadd45-γ* overexpression antagonizes cell division. Animal views of stage 11 embryos injected with 0.5 ng of the indicated mRNAs and/or 10 ng of the indicated morpholino oligonucleotide and 0.3 ng of *lacZ* mRNA. Embryos were in situ hybridized for *lacZ* mRNA (blue) and stained for anti PH3 as a mitotic marker. (A) Distribution of mitotic cells in stage 11 control embryos injected with *lacZ* mRNA alone. (B,C) Expression of *XGadd45-MT* (B) or *MT-XGadd45* (C) reduces the number of dividing cells. (D,E) Injection of MOGadd45 (D) or a control morpholino oligonucleotide (E) did not affect the number of dividing cells. (F,G) Reduction of mitotic cells promoted by *XGadd45-MT* mRNA is not affected by coinjection of a control morpholino (F) but is antagonized by coinjection of MOGadd45 (G). (H) MOGadd45 cannot interfere with *MT-XGadd45* promoted cell cycle arrest.

injected with *lacZ* alone (Fig. 3A) showed a dense pattern of dividing cells at stage 11 (Saka and Smith, 2001). However, embryos with *XGadd45-MT* or *MT-Gadd45* mRNAs in this region showed a strong reduction in the number of dividing cells in the injected area (Fig. 3B, C; 68–82%, $n = 73$–85). This effect was also observed at neurula stages (not shown). We attempted to interfere with *XGadd45-γ* function by injecting 10 ng of a *XGadd45-γ* antisense morpholino oligonucleotide (MOGadd45) (Heasman et al., 2000). MOGadd45 or a control morpholino oligonucleotide did not significantly modify the number of mitotic cells (Fig. 3D, E; 100%, $n = 45$–25). However, MOGadd45, but not the control morpholino (Fig. 3F; 70%, $n = 27$), did block the inhibition of cell division by *XGadd45-MT* (Fig. 3G; 84%, $n = 45$). Importantly, MOGadd45 did not antagonize the interference of *MT-XGadd45* with cell division (Fig. 3H; 68%; $n = 31$). This indicated that the effect of MOGadd45 was specific, since these modified antisense oligonucleotides act by blocking translation and they are effective only if their target sequence is within 30 bases from the translation start site. This was the case for *XGadd45-MT* mRNA but not for the *MT-XGadd45* transcript.

In cell culture assays, overexpression of *Gadd45* genes can promote cell cycle arrest and apoptosis (Azam et al., 2001; Hall et al., 1995; Kearsey et al., 1995; Sheikh et al., 2000; Smith et al., 1994; Takekawa and Saito, 1998; Vairapandi et al., 1996; Zhan et al., 1999; Zhang et al., 2001; Zhao et al., 2000). Conceivably, the strong reduction of the number of mitotic cells observed in embryos injected with *XGadd45-γ* constructs could be a consequence of an increase in cell death. As determined by tunel staining, we did observe a slightly increase (10%, $n = 40$) in the number of apoptotic cells in stage 11 embryos injected with *XGadd45-MT* mRNA, as compared with control embryos (Fig. 4A, B). This effect was completely rescued by coinjection of MOGadd45 (100%, $n = 35$), but not by coinjection of the control morpholino (Fig. 4C, D). However, note that the number of dying cells observed upon *XGadd45-MT* overexpression is very reduced compared with that of cells exiting the cell cycle (compare Figs. 3B and 4B). Moreover, this increased apoptosis was only observed in about 10% of the injected embryos, while the reduction of mitotic cells was found in approximately 75% of the *XGadd45-MT* injected embryos. We conclude that, although the overexpression of *XGadd45-γ* causes some apoptosis, its main effect is to promote cell cycle arrest, similarly to the *Xenopus* cell cycle inhibitor $p27^{XIC1}$ (Hardcastle and Papalopulu, 2000).

As a further demonstration of the specificity of MOGadd45, we examined the presence of the Myc epitope, both by inmunostaining and western blots, in embryos injected with *XGadd45-MT* mRNA, without or with coinjection of MOGadd45. As expected, this epitope was present in the first case (Fig. 4E), but it was totally absent in the second (Fig. 4F, 100%; $n = 56$). This further indicated that MOGadd45 prevented translation of *XGadd45-MT* mRNA and most likely that of endogenous *XGadd45-γ* mRNA.

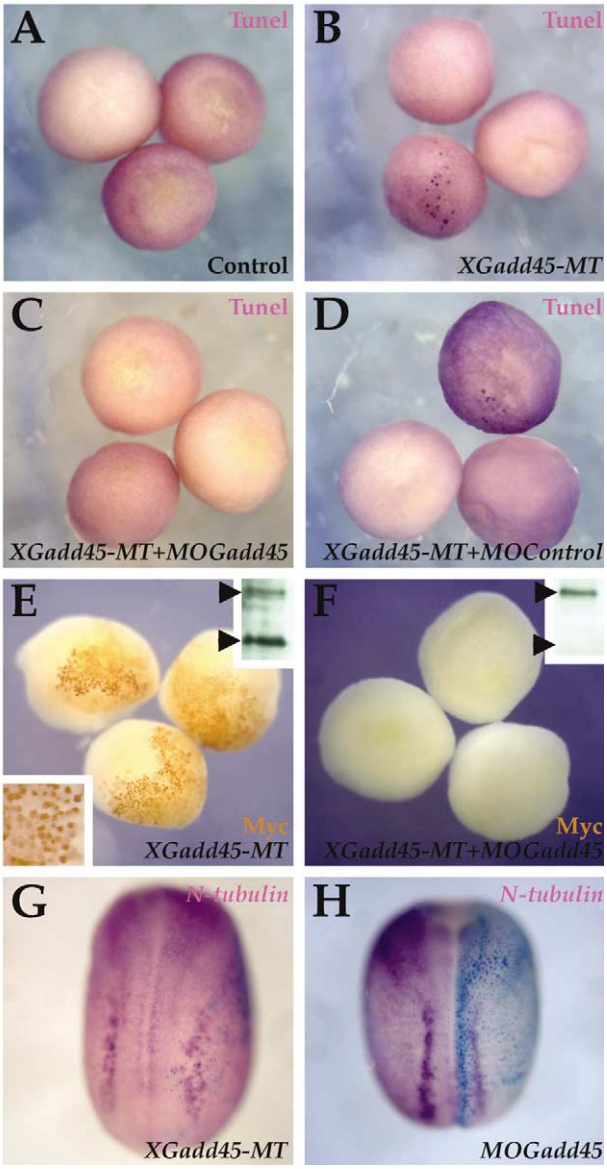

Fig. 4. *XGadd45-γ* overexpression causes a mild increase of cell death. Animal views of stage 11 (A–F) and dorsal views of stage 15 (G,H) embryos injected with 0.5 ng of the indicated mRNAs and/or 10 ng of the indicated morpholino oligonucleotide. (A–D) Tunel staining to label apoptotic cells. (A) Uninjected embryos showed no apoptotic cells. (B) In a few embryos injected with *XGadd45-MT* mRNA several dying cells were detected. (C,D) This effect was antagonized by coinjection of MOGadd45 (C), but not by a control morpholino (D). (E) The Myc epitope was detected in *XGadd45-MT* injected embryos. The XGadd45-MT protein was present both in the nucleous and in the cytoplasm (lower inset). Upper inset shows a western blot to detect Myc in *XGadd45-MT* injected embryos. Note the presence of both a control cross-reacting protein (upper band) and the XGadd45-MT protein (lower band). (F) In embryos coinjected with *XGadd45-MT* mRNA and MOGadd45 the Myc epitope was undetectable both in whole mount and in western blot (inset). However, the control cross-reacting protein (upper band, inset) is present.(G,H) N-tubulin expression is not affected in embryos injected with *XGadd45-MT* mRNA (G) or MOGadd45 (H).

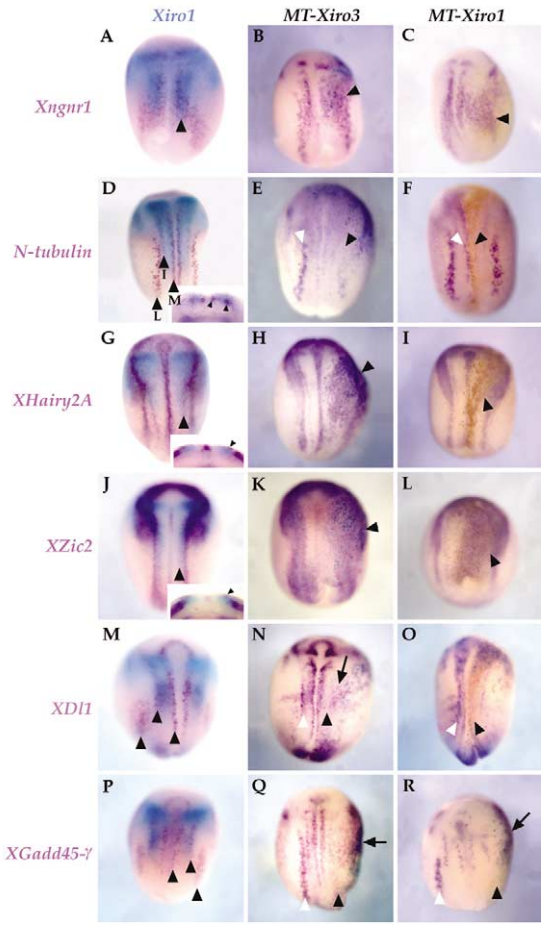

Fig. 5. *Xiro1* or *-3* mRNAs similarly affect neural markers. All panels show dorsal views of midneurula embryos. (A,D,G,J,M,P) Wild type embryos. (B,E,H,K,N,Q) *MT-Xiro3* mRNA (100 pg) injected embryos. This mRNA was coinjected with *lacZ* mRNA (300 pg) and the injection side was determined by X-Gal staining. (C,F,I,L,O,R) *MT-Xiro1* mRNA (0.5–1 ng) injected embryos. In these embryos, the injected site was determined by anti-myc staining. (A) *Xiro1* (blue) and *Xngnr1* (purple) expression domains overlap (arrowhead). (B,C) Misexpression of *MT-Xiro3* (B) or *MT-Xiro1* (C) mRNAs promoted ectopic *Xngnr1* expression (arrowheads). (D) The medial (M) and intermediate (I) *N-tubulin* stripes (purple) are located at the border of the *Xiro1* domain (blue). Inset: transverse section (arrowheads point at the M and I rows of neurons). (E,F) Ectopic expression of *MT-Xiro3* or *MT-Xiro1* mRNAs repressed *N-tubulin* (uninjected side, white arrowhead; injected side, black arrowhead). (G) The intermediate stripe of *XHairy2A* (purple, arrowhead) is within the *Xiro1* domain (blue). The overlapping domains of these genes are verified in transverse sections (inset, arrowhead). (H,I) *XHairy2A* is overexpressed in embryos injected with *MT-Xiro3* or *MT-Xiro1* mRNAs (arrowheads). (J) The intermediate stripe of *XZic2* (purple) is also within the *Xiro1* territory (blue) (arrowheads in whole mount and section, inset). (K,L) *MT-Xiro3* or *MT-Xiro1* mRNA promote ectopic *XZic2* (arrowheads). (M) The medial and intermediate *XDl1* stripes (purple) are located at the border of the *Xiro1* domain (blue). (N,O) *XDl1* is repressed by *MT-Xiro3* or *MT-Xiro1* misexpression (the stripes pointed at with a white arrowhead in the uninjected side are absent in the injected side, black arrowhead). In these embryos, *XDl1* is also activated in other regions (arrow in N). (P) Similarly to *XDl1*, *XGadd45-γ* (purple) medial and intermediate stripes are located at the border of *Xiro1* territories (blue). (Q,R) *MT-Xiro3* or *MT-Xiro1* mRNAs repress *XGadd45-γ* in the stripes territory (black arrowheads, compare with the stripes in the non-injected side, white arrowheads) and activate it in other regions (arrows).

We also examined the effect of overexpressing *XGadd45-MT* mRNA and MOGadd45 on primary neurogenesis. Neither the increase nor the reduction of *XGadd45-γ* function affected primary neurogenesis (Fig. 4G, H). Overexpression of other cell cycle inhibitors such as $p27^{XIC1}$ also do not affect primary neurogenesis (Hardcastle and Papalopulu, 2000). In addition, the absence of observable effects in embryos injected with MOGadd45 could be due to the rescue of *XGadd45-γ* function by other members of the Gadd45 family. Thus, the expression pattern of *XGadd45-γ* and the effects caused by its overexpression suggests a possible requirement of *XGadd45-γ* in cell cycle arrest necessary for primary neuron differentiation. Confirmation of this requirement may need functional interference with all Gadd45 family members.

2.4. Different Xiro genes similarly affect neural markers

Before further characterizing the interaction between *XGadd45-γ* and the *Xiro* genes, it was advisable to reexamine the function of each *Xiro* member in neurogenesis. Apparently conflicting results have been reported (Bellefroid et al., 1998; Gómez-Skarmeta et al., 1998), since at neurula stages Xiro1 and -2 promoted *Xngnr1* expression, while Xiro3 inhibited neuronal differentiation. To this end, we prepared similar myc-tagged constructs for each *Xiro* gene (*MT-Xiro1-3*) and assayed their effects on several neural markers. We found that the three genes behaved similarly, but *Xiro3* was five to ten times more effective that *Xiro1* or *Xiro2*. Consistently with the overlapping domains of *Xiro1, 3* and *Xngnr1* (Fig. 5A), injection of each *MT-Xiro* mRNA ectopically activated *Xngnr1* (Fig. 5B, C; 68–76%, $n = 40$–45). Primary neurons, as determined by *N-tubulin* expression, appear at the borders of *Xiro* territories (Fig. 5D and Bellefroid et al., 1998). Again, consistently with this localization, each *MT-Xiro* mRNA suppressed *N-tubulin* (Fig. 5E, F; 90–94%, $n = 20$–32). Finally, the *Xiro* domains overlap with the medial stripes of two neuronal repressors, *XHairy2A* and *XZic2* (Brewter et al., 1998; Dawson et al., 1995) (Fig. 5G, J). We found that each *MT-Xiro* mRNAs strongly upregulated these repressors (Fig. 5H, I, K, L; 75–86%, $n = 20$–35). We conclude that *Xiro* genes upregulate *Xngnr1* and also repressors that prevent neuronal differentiation.

2.5. Dual effects of Xiro overexpression on XDl1 and XGadd45-γ

XGadd45-γ and *XDl1* are similarly expressed, both temporally and spatially. In the neural plate, these genes are expressed in stripes that demarcate territories in which primary neurons arise. The medial and intermediate stripes are located at the borders of the *Xiro* expressing domains (Fig. 5M, P). These locations are consistent with Xiro being a repressor of *XDl1* and *XGadd45-γ*. Indeed, misexpression of each *MT-Xiro* mRNA eliminated the stripes of *XDl1* and

XGadd45-γ (Fig. 5N, O, Q, R; 55–64%, $n = 25$–38). However, these mRNAs also promoted ectopic expression of these genes in other regions (arrows in Fig. 5N, Q, R; 50–68%, $n = 25$–38). This non-autonomous effect of Xiro on *XDl1* and *XGadd45-γ* will be described elsewhere.

Xiro1, and probably Xiro 2 and 3, act as repressors (Gómez-Skarmeta et al., 2001). Hence, converting them to activators might cause the upregulation of normally repressed genes. We used an inducible dominant negative form of Xiro1 in which its homeodomain was fused to an activation domain (*HD-GR-E1A*; Gómez-Skarmeta et al., 2001). Embryos were injected with *HD-GR-E1A* mRNA (0.5 ng) and activity was induced at stage 12.5 by addition of dexamethasone. The panneural marker *Sox2* was not affected (Fig. 6A; 85%, $n = 20$), indicating that neural plate formation was not impared. However, *Xngnr1* and, probably as a consequence, *XGadd45-γ* and *N-tubulin* expressions were suppressed (Fig. 6B–D; 75–79%, $n = 28$–32). These effects were dexamethasone-dependent. Coinjection of *HD-GR-E1A* and *Xngnr1* mRNAs rescued *N-tubulin* and *XGadd45-γ* expression (Fig. 6E and not shown; 68%, $n = 25$). In contrast, *HD-GR-E1A* mRNA strongly upregulated *XDl1* (Fig. 6F; 61%, $n = 52$). We determined whether *HD-GR-E1A* could upregulate *XDl1* in the absence of protein synthesis. Indeed, this was the case since ectopic *XDl1* was observed in *HD-GR-E1A* injected embryos treated with cycloheximide (CHX) for 30 min, and then for 3 h with CHX and Dex (Fig. 6G, see Section 4 for detailed treatment). No ectopic *XDl1* was observed in injected embryos in the presence of CHX and in the absence of Dex (Fig. 6H) or just in the absence of Dex (Fig. 6I). Thus, Xiro proteins probably directly repress *XDl1*. In contrast, repression of *XGadd45-γ* and N-tubulin by Xiro proteins could be an indirect consequence of Xiro-promoted upregulation of neuronal repressors.

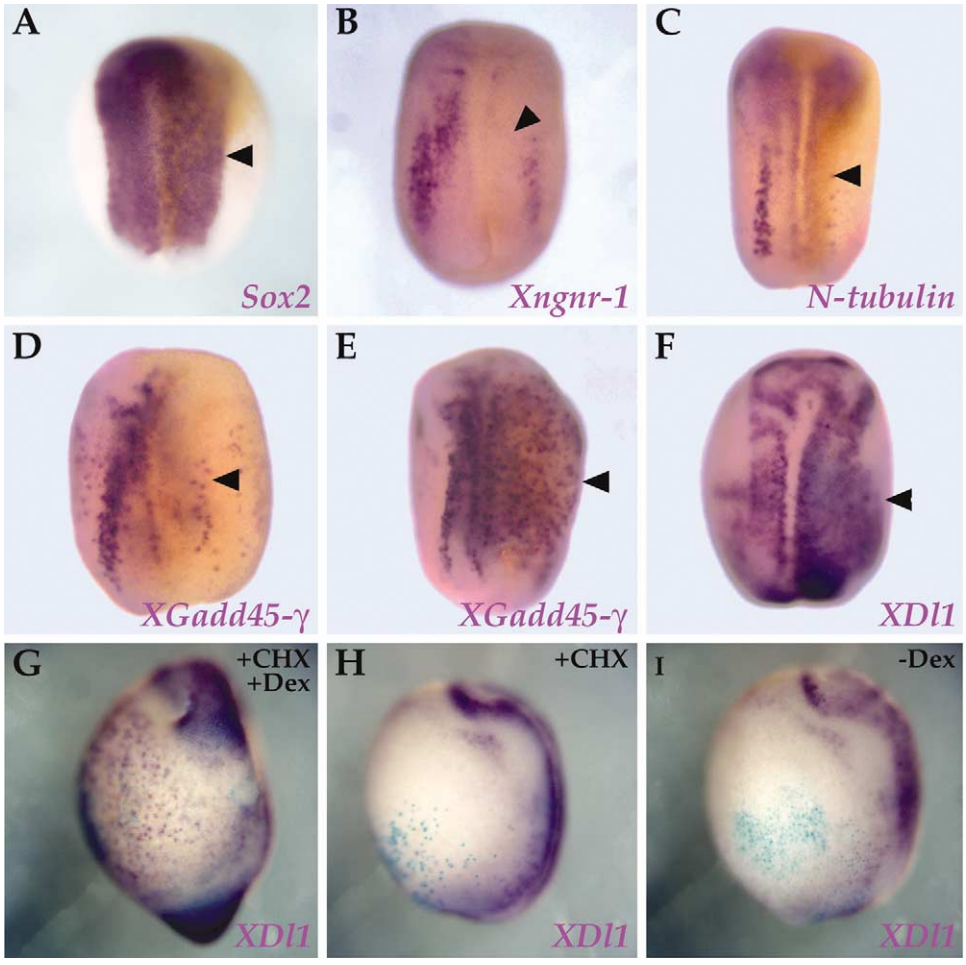

Fig. 6. Overexpression of *HD-GR-E1A* mRNA affects neural markers. Dorsal views (A–F) or lateral views (G–I) of neurula stage embryos injected with 0.5 ng of *HD-GR-E1A* mRNA with (G–I) or without (A–F) 0.3 ng of *LacZ* mRNA. Dexamethasone was added at stage 12.5 (A–F) or at stage 13.5 (G) to avoid suppression of neural plate formation caused by interference with *Xiro* function at gastrula stages (Gómez-Skarmeta et al., 2001). The injected side was determined by anti-Myc staining (A–F) or XGal staining (G–I). (A) *Sox2* is not affected. However, *Xngnr1* (B), *N-tubulin* (C) and *XGadd45-γ* (D) expressions are suppressed (arrowheads). Coinjection with *Xngnr1* mRNA (1 ng) rescued *N-tubulin* (not shown) and *XGadd45-γ* (E, arrowhead) expression. In embryos injected with 0.5 ng of *HD-GR-E1A* mRNA, *XDl1* (F) is overexpressed. (G) This activation was also observed when protein synthesis was blocked. (H,I) No ectopic *XDl1* was observed in injected embryos in the presence of CHX and in the absence of the hormone (H), or just in the absence of the hormone (I).

3. Discussion

3.1. XGadd45-γ is expressed in cells that will stop dividing and may provide a link between cell differentiation and cell-cycle arrest

Xiro1, and probably Xiro2 and -3, act as transcriptional repressors (Gómez-Skarmeta et al., 2001). In a subtraction screen designed to isolate genes repressed by Xiro1, we have identified the *Xenopus* ortholog of mammalian *Gadd45-γ*. This gene belongs to a three-member family that encodes the small (18 kDa), related proteins Gadd45-α, Gadd45-β/MyD118 and Gadd45-γ/CR6 (Abdollahi et al., 1991; Beadling et al., 1993; Fan et al., 1999; Fornace, 1992; Fornace et al., 1992; Takekawa and Saito, 1998; Zhang et al., 1999). Apparently, these proteins have similar functions in the arrest of cell growth and in apoptosis, although the details of their mechanism(s) of action are unknown. They all interact with the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$, the proliferating cell nuclear antigen (PCNA, a component of a complex which includes cyclin-dependent kinases and $p21^{WAF1/CIP1}$), and Cdc2/Cyclin B1 (Azam et al., 2001; Hall et al., 1995; Kearsey et al., 1995; Sheikh et al., 2000; Smith et al., 1994; Takekawa and Saito, 1998; Vairapandi et al., 1996; Zhan et al., 1999; Zhang et al., 2001; Zhao et al., 2000). Overexpression of each protein in cell culture promotes cell-cycle arrest and apoptosis (Azam et al., 2001; Smith et al., 1994; Takekawa and Saito, 1998; Vairapandi et al., 1996; Zhang et al., 1999; Zhang et al., 2001; Zhao et al., 2000). Some evidence supports that *Gadd45-α* inhibits Cdc2/Cyclin B1 kinase activity (Zhan et al., 1999). In addition, these proteins bind to and activate the MTK1/MEKK4 kinase, which itself activates the p38/JNK kinase pathway that promotes apoptosis in response to environmental stress (Takekawa and Saito, 1998). However, the Gadd45 requirement for p38/JNK kinase activation remains controversial (Shaulian and Karin, 1999; Wang et al., 1999; Zhang et al., 2001). In contrast, *Gadd45-α*, *Gadd45-β/MyD118* and *Gadd45-γ/CR6* are differentially activated in response to several signaling pathways and a variety of genetic and envionmental stresses, and show distinct patterns of expression in mice tissues (Guillouf et al., 1995; Kastan et al., 1992; Selvakumaran et al., 1994; Takekawa and Saito, 1998; Wang et al., 1999). These data suggest that, despite their very similar composition, each protein may have some functional specificity.

During *Xenopus* early development, *XGadd45-γ* is expressed in a dynamic pattern that prefigures many territories where cells will stop dividing (Saka and Smith, 2001). Examples are, during gastrulation, the dorsal mesoderm, and, in the neurula stages, the neuronal precursors, the prospective ciliated cells, the somites and the prospective cement gland. In all these territories, *XGadd45-γ* mRNA transcription precedes mitotic quiescence (Hardcastle and Papalopulu, 2000; Saka and Smith, 2001). As expected for a cell-cycle inhibitor, overexpression of *XGadd45-γ* at moderate levels reduces the number of dividing cells and this effect is not a consequence of an increase of cell death. Injection of high amounts of *XGadd45-γ* mRNA promotes lethality. Gastrulation is impeded, and cells lose adherence and are extruded into the vitelline space. These *XGadd45-γ* effects are similar to those observed with other cell-cycle inhibitors (Grammer et al., 2000; Hardcastle and Papalopulu, 2000). Sites of *XGadd45-γ* expression coincide, in some cases, with those of $p27^{XIC1}$, a gene that encodes a cyclin-dependent kinase inhibitor of the $p21^{WAF1/CIP1}$ family (Hardcastle and Papalopulu, 2000). Moreover, overexpression of either *XGadd45-γ* or $p27^{XIC1}$ arrests the cell-cycle in vivo (this work and Hardcastle and Papalopulu, 2000). Thus, it is possible that *Gadd45-γ* not only interacts with $p21^{WAF1/CIP1}$, but also with $p27^{XIC1}$. Interference with *XGadd45-γ* function with a specific morpholino oligonucleotide did not affect the cell cycle.

This may be due to redundancy between different Gadd45 proteins.The spatial and temporal patterns of expression of *Gadd45-γ* and the Notch ligand *XDl1* largely coincide. Moreover, both *XGadd45-γ* and *XDl1* (Chitnis et al., 1995; Ma et al., 1996; Wettstein et al., 1997) are positively regulated by proneural genes and negatively controlled by Notch signaling. According to the lateral inhibition model, activation of the Notch pathway within a cell, by signaling from neighbouring cells, maintains its mitotic potential and prevents its differentiation. In contrast, a cell that expresses high levels of Notch ligands and signals strongly, escapes lateral inhibition, exits the cell cycle and differentiates (reviewed in Artavanis-Tsakonas and Lake, 1999). *XGadd45-γ* may provide a link between Notch signaling, cell-cycle arrest and differentiation. Thus, in the neural plate, cells with high levels of proneural genes have also high levels of *XDl1* and *XGadd45-γ*. The first allows them to escape lateral inhibition, and the second to exit the cell cycle. These cells can then differentiate. Mitotic arrest mediated by *XGadd45-γ* probably occurs through interaction with cyclin and inhibitors of cyclin-dependent kinases. In neighbouring cells, the Notch pathway is activated, proneural genes and *XGadd45-γ* are downregulated, and cell-cycle arrest and differentiation cannot occur. It is of interest that induction of *Gadd45* genes in cell culture stops the cell cycle in G1 phase (Zhang et al., 2001). This phase is compatible with exiting the cell cycle, a requirement for terminal neuronal differentiation (Hollyday, 2001). Cells that differentiate outside the neural plate may resort to genes different from the proneural ones to accumulate Notch ligands and *XGadd45-γ*.

3.2. Distinct Xiro proteins have similar effects on neural development

We have compared the effects of overexpressing either *Xiro1*, *-2* or *-3* in neural development. To make comparisons more meaningful, we prepared equivalent constructs in the

pCS2MT plasmid. The overexpression of each *Xiro* gene caused similar effects, although *Xiro3* was approximately five to ten times more potent. Paradoxically, the overexpressions activated *Xngnr1* and repressed neuronal differentiation. This may be explained at least in part by our finding that *Xiro* upregulates the neuronal repressors *XHairy2A* and *XZic2* (Brewter et al., 1998; Dawson et al., 1995). Indeed, it has been shown that *XZic2* antagonizes development of *Xngnr1*-promoted ectopic neurons (Brewter et al., 1998). We have also observed that *XZic2* antagonizes *Xngnr1*-promoted *XGadd45-γ* and *XDl1* expression (not shown). Consistently with our findings, in wild type embryos, the intermediate stripes of expression of *XHairy2A* and *XZic2* are within the *Xiro1* domains. Also in accordance with our results, in the prospective spinal chord, the *Xiro1* domain is contained within the broader *Xngnr1* domain and neurons arise at the border of the *Xiro1* domain. Taken together, these observations suggest that Xiro proteins simultaneously participate in the activation of *Xngnr1* and of genes that antagonize primary neuron formation.

Overexpressions of *Xiro* genes repressed both *XGadd45-γ* and *XDl1* in territories where primary neurons arise. Consistently, in wild type embryos, *XGadd45-γ* and *XDl1* are expressed at the borders of *Xiro* domains. Moreover, *XDl1* is activated in embryos expressing a Xiro1 chimera that converts the Xiro1 repressor into an activator (HD-GR-E1A). This activation occurs even in the absence of protein synthesis. Thus, *XDl1* is probably directly repressed by Xiro. However, *XGadd45-γ* is repressed by HD-GR-E1A, probably because *Xngnr1* is also downregulated. Indeed, coinjection of *HD-GR-E1A* and *Xngnr1* mRNAs rescues the expression of *XGadd45-γ*. Thus, Xiro-mediated repression of *XGadd45-γ* is probably indirect and may take place, at least in part, by Xiro-upregulated neuronal repressors. In this case, interference with Xiro function would suppress neuronal repressors, but would also downregulate *Xngnr1*, which is needed for *XGadd45-γ* expression.

3.3. Model of Xiro function in neural patterning

We wish to propose a model for the function of Xiro in neural patterning that integrates the above data (Fig. 7). Xiro proteins, as well as other factors, participate in the activation of *Xngnr1*. Within the Xiro domains (blue area in Fig. 7), Xngnr1 does not activate *XDl1*, *XGadd45-γ* and cannot promote differentiation of primary neurons due to the upregulation by Xiro of neuronal repressors, such as *XHairy2A* and *XZic2*. In addition, Xiro probably represses *XDl1* directly. Outside the Xiro domains (purple area in Fig. 7), other factors, such as the Gli proteins (Brewter et al., 1998), activate *Xngnr1*, which in turn promotes the expression of *XDl1* and*XGadd45-γ* in those cells that will become primary neurons (red circles in Fig. 7). XDl1 switches on the lateral inhibition mechanism by which the Notch signaling pathway is activated in neighbouring cells (orange circles in Fig. 7). This pathway downregulates proneural genes, *XDl1* and *XGadd45-γ*. As a consequence, these cells keep dividing and do not differentiate. In contrast, cells with high levels of *Xngnr1*, *XDl1* and *XGadd45-γ* escape lateral inhibition, exit the cell cycle (in part due to the presence of *XGadd45-γ*) and differentiate as primary neurons. This differentiation is triggered by a genetic programme activated by Xngnr1. Thus, Xiro proteins may help coordinate cell cycle and differentiation, similarly to XBF1 (Hardcastle and Papalopulu, 2000).

4. Materials and methods

4.1. Isolation of XGadd45-γ

Twenty animal caps from embryos injected with either 100 pg of *noggin* or with 100 pg of *noggin* and 4 ng of *Xiro1-VP16* mRNAs were excised at stage ten and cultured until stage 18. Total RNA was extracted and cDNA was synthesized using the SMART PCR cDNA Synthesis Kit (Clontech). The resulting two populations of cDNAs were used to prepare a subtraction library using the Clontech PCR-Select cDNA Subtraction Kit. The library was ligated to the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli*. One of the clones contained a 1290 bp cDNA fragment with a full length open reading frame (ORF) which encode XGadd45-γ.

4.2. Plasmid constructions

The primers 5′-CCCG*GAATTC*CATGACTCTGGAAG-3′ and 5′-AC*CTCGAG*GAAAGATGTGAGCTCGGGCAGGG-3′ were used to amplify the *XGadd45-γ* coding region. The PCR fragment was subcloned in PGEM-T easy, sequenced, excised with EcoRI and XhoI (in bold in the primer sequences), and cloned in PCS2-MT plasmid (Turner and Weintraub, 1994) to obtain the MT-Gadd45 construct. In this construct, the region from the first methionine to the stop codon of the *XGadd45-γ* gene is fused in frame to and downstream from the Myc epitopes of the plasmid. The Gadd45-MT construct was prepared in a similar way but using primers 5′-GA*GGATCC*TTTTGTGGCTTACCCGG-3′ and 5′-GA*GGATCC*GGGCAGGGTGATAGTG-3′ and subcloning the PCR fragment in BamHI site in PCS2-MT plasmid. This construct contains the region of the *XGadd45-γ* gene from 23 base pairs upstream of the first methionine up to the last codon preceding and fused in frame to the Myc epitopes of the plasmid.

A set of equivalent tagged Xiro1, 2 and 3 constructs were prepared. A short DNA fragment, from the 5′ region of a cDNA of each gene, which includes unique sites within the ORF (AvaI site in *Xiro1*, BamHI site in *Xiro2* and PstI site in *Xiro3* cDNAs), were PCR-amplified. These unique sites allowed fusing the PCR fragments to the rest of each cDNA. In all cases, the 5′ primer contained an EcoRI site replacing the first methionine codon. This site allowed cloning these PCRs fragments in frame within the pCS2-MT plasmid. The

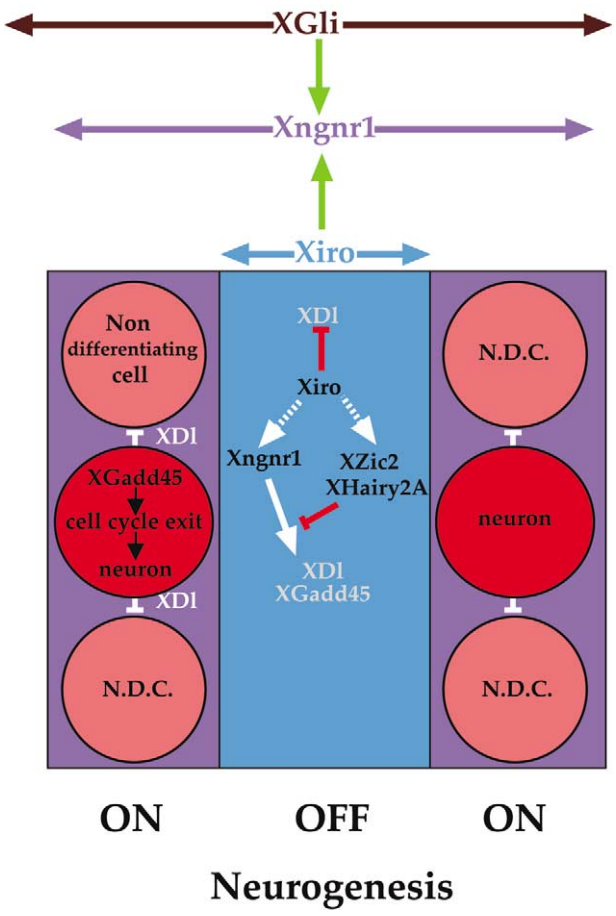

Fig. 7. Model of *Xiro* function in primary neurogenesis. See text for details.

PCR fragments were cloned in pGEM-T Easy vector and sequenced prior to fusion with their corresponding cDNA in the pCS2-MT plasmid.The primers used were: for *Xiro1*, 5′-GGAGAGA*GAATTC*CTTCCCG-3′ and 5′-GGGGGCGCTGTACGGGC-3′; for *Xiro2*, 5′-GAGCTAC-*GAATTC*CTATCC-3′ and 5′-TGGGCGCTGAAGGCAG-3′; and for *Xiro3*, 5′-CAC*GAATTC*CTTCCCACAG-CTGGGCTACC-3′ and 5′-CCAGCTGGGGGAAGATGG-GCA-3′.

4.3. DNA sequencing

DNA sequencing was performed with ABI chemistry in an automatic DNA sequencer. Custom synthesized oligonucleotides were from ISOGEN (Bioscience BV, Maarsesen, The Netherlands).

4.4. Whole-mount in situ hybridization, X-Gal, antibody and Tunel staining

Antisense RNA probes were prepared from cDNAs using digoxigenin or fluorescein (Boehringer Mannheim) as labels. Specimens were prepared, hybridized and stained as described (Harland, 1991). Double in situ hybridizations were performed as described (Gómez-Skarmeta et al., 1998). X-Gal staining was carried out according to Coffman et al. (1993). Antibody staining was performed (Turner and Weintraub, 1994) after in situ hybridization and bleaching of the embryos using mouse monoclonal anti-Myc from BabCo and rabbit anti-Phophohistone H3 from Upstate Biotechnology. For the Zic2 probe an IMAGE *Xenopus* clone IMAGp998E128490Q2 obtained from the German Resource Center and Primary Database (RZPD) in Berlin, was used. Tunel staining was performed according to Hensey and Gautier (1997).

4.5. In vitro RNA synthesis

All DNAs were linearized and transcribed as described (Harland and Weintraub, 1985) with GTP cap analog (New England Biolabs). After DNAse treatment, RNA was extracted with phenol–chloroform, column purified and precipitated with ethanol. mRNAs for injection were resuspended in water.

4.6. Embryos, explants, microinjection of mRNA, and morpholino and cycloheximide treatment

Xenopus embryos were obtained and animal caps were prepared as described (Gómez-Skarmeta et al., 1998) and staged according to Niewkoop and Faber (1967). Synthetic mRNAs were injected into embryos at the one- or two-cell stage using 8–12 nl. Ten nanograms of a *XGadd45-γ* antisense morpholino (5′-GTCCGTGAACTTCTTCCAGAG-TAGTCAT-3′) was injected into embryos at the one- or two-cell stage using 8–12 nl. To analyze whether HD-GR-E1A activates *XDl1* in the absence of protein synthesis, embryos injected with 0.5 ng of *HD-GR-E1A* mRNA were incubated with cycloheximide (10 μg/ml) during 30 min. This treatment was followed by another 3 h incubation with dexamethasone (10 μM) plus cycloheximide. After this, the embryos were fixed and processed for in situ hybridization (Casio and Gurdon, 1987).

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