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Distinct effects of *XBF-1* in regulating the cell cycle inhibitor *p27^{XIC1}* and imparting a neural fate

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SUMMARY

XBF-1 is an anterior neural plate-specific, winged helix transcription factor that affects neural development in a concentration-dependent manner. A high concentration of XBF-1 results in suppression of endogenous neuronal differentiation and an expansion of undifferentiated neuroectoderm. Here we investigate the mechanism by which this expansion is achieved. Our findings suggest that *XBF-1* converts ectoderm to a neural fate and it does so independently of any effects on the mesoderm. In addition, we show that a high dose of *XBF-1* promotes the proliferation of neuroectodermal cells while a low dose inhibits ectodermal proliferation. Thus, the neural expansion observed after high dose *XBF-1* misexpression is due both to an increase in the number of ectodermal cells devoted to a neural fate and an increase in their proliferation. We show that the effect on cell proliferation is likely to be mediated by *p27^{XIC1}*, a cyclin-dependent kinase (cdk) inhibitor. We show that *p27^{XIC1}* is expressed in a spatially restricted pattern in the embryo, including the anterior neural plate, and when misexpressed it is sufficient to block the cell cycle in vivo. We find that *p27^{XIC1}* is transcriptionally regulated by *XBF-1* in a dose-dependent manner such that it is suppressed or ectopically induced by a high or low dose of *XBF-1*, respectively. However, while a low dose of *XBF-1* induces ectopic *p27^{XIC1}* and ectopic neurons, misexpression of *p27^{XIC1}* does not induce ectopic neurons, suggesting that the effects of *XBF-1* on cell fate and cell proliferation are distinct. Finally, we show that *p27^{XIC1}* is suppressed by *XBF-1* in the absence of protein synthesis, suggesting that at least one component of *p27^{XIC1}* regulation by *XBF-1* may be direct. Thus, XBF-1 is a neural-specific transcription factor that can independently affect both the cell fate choice and the proliferative status of the cells in which it is expressed.

Key words: XBF-1, Cell proliferation, Cell fate, *Xenopus*

INTRODUCTION

During gastrulation in vertebrates, signals from the dorsal mesoderm convert part of the ectoderm into a neural fate while the remaining ectoderm develops as epidermis (reviewed in Saxen, 1989). It is believed that the signals from the dorsal mesoderm induce neural tissue by antagonising BMP-4 signalling, which induces epidermis (reviewed in Tanabe and Jessell, 1996; Bier, 1997). The early neuroectoderm is an epithelial structure that contains the precursors of the central nervous system (Hartenstein, 1989). In fish and amphibians, a number of early differentiating neurons appear shortly after gastrulation within this neuroectodermal domain.

Neural induction, the conversion of ectodermal cells to neural precursors, results in the activation of regulatory genes that are characterised by early and broad expression domains in the neural plate (e.g. Ferreiro et al., 1994; Turner and Weintaub, 1994; Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998; Kroll et al., 1998; Mizuseki et al., 1998a,b; Zygar et al., 1998). Neuronal differentiation, or neurogenesis, takes place within the neural plate and is under the control of later acting genes, namely proneural and neurogenic genes. These genes show a more restricted pattern of expression and regulatory interactions between them allow a certain number of cells within the neural plate to switch from a neural to a neuronal fate (Chitnis et al., 1995; Lee et al., 1995; Bellefroid et al., 1996; Chitnis and Kintner, 1996; Ma et al., 1996; Takebayashi et al., 1997; Dubois et al., 1998).

The expression pattern of proneural and neurogenic genes broadly mark the sites of prospective neuronal differentiation where *N-tubulin*, a marker of differentiated neurons, will appear (e.g. Chitnis et al., 1995; Bellefroid et al., 1996; Ma et al., 1996; Dubois et al., 1998). This restricted expression shows that neuronal differentiation follows a stereotypical temporal and spatial order. Thus, in the posterior neural plate, neuronal differentiation takes place in three longitudinal domains on either side of the dorsal midline (Chitnis et al., 1995). Anteriorly, neuronal differentiation is delayed until the tadpole stage (Papalopulu and Kintner, 1996). At that point, neuronal differentiation appears in two clusters inside the forebrain, namely in the telencephalon and the ventral diencephalon, and two peripheral clusters that are connected with the brain, namely in the epiphysis and olfactory placodes (Papalopulu and Kintner, 1996; see also Hartenstein, 1993; Ross et al.,

1992). Neuronal cells are gradually added on to these initial clusters such that differentiation progressively spreads to the rest of the neuroectoderm. On the basis of the expression pattern of proneural and neurogenic genes, it has been suggested that these clusters in the forebrain are likely to be derived from two semicircular, horseshoe-shaped domains in the anterior neural plate (Bellefroid et al., 1996; Bourguignon et al., 1998).

How are the spatial and temporal controls of neurogenesis achieved? It has been suggested that members of the Gli and Zic families play a role in spatially controlling neurogenesis in the posterior neural plate since they induce and inhibit neurogenesis, respectively (Brewster et al., 1998). We have previously described the cloning of *XBF-1*, a transcription factor of the forkhead (winged helix) family (Papalopulu and Kintner, 1996; Bourguignon et al., 1998; independently cloned by Dirksen and Jamrich, 1995) and suggested that it may play a role in controlling the spatial pattern of neurogenesis in the anterior neural plate.

By misexpressing *XBF-1* in the posterior neural plate, we have shown that *XBF-1* causes an expansion of the neuroectoderm that coincides with the area that expresses high levels of *XBF-1* (Bourguignon et al., 1998). This expanded neuroectodermal domain does not itself express neuronal differentiation markers but is surrounded at its border by ectopic neuronal cells. We have also shown that *XBF-1* has a dose-dependent activity of suppressing or activating neuronal differentiation at high or low doses, respectively (Bourguignon et al., 1998). Thus, positioning neurogenesis at the border by high *XBF-1* expressing ectoderm may be explained by the dose-dependent activity of *XBF-1*, provided that cells at the border express lower levels of *XBF-1*. Although the levels and/or activity of endogenous *XBF-1* mRNA and protein are at present not known, this hypothesis is consistent with the normal expression pattern of *XBF-1* since proneural and neurogenic genes are expressed at the border of the *XBF-1* expression domain in the anterior neural plate (Bourguignon et al., 1998). Therefore, our previous findings suggested a general mechanism whereby a single molecule may be sufficient to position neurogenesis in vivo by virtue of its dual, inducing and repressing, activity on neurogenesis. Furthermore, the mouse homologue *BF-1* has been implicated in the temporal control of neurogenesis since a mouse knock-out shows premature neuronal differentiation in the forebrain (Xuan et al., 1995).

In this paper we investigate further the mechanism by which *XBF-1* exerts its effects. Specifically, we concentrate on the molecular mechanism whereby a high dose of *XBF-1* leads to neural plate expansion. We consider two possibilities: first, that *XBF-1* mediates a neural fate switch in the ectoderm such that additional neural ectoderm forms at the expense of epidermis; second, that *XBF-1* promotes the proliferation of neuroectodemal cells, resulting in an apparent expansion of the neural plate. Our findings support both possibilities. First, we present evidence that *XBF-1* switches ectodermal cells to a neural fate in isolated animal caps in the absence of inducing signals from the mesoderm. Second, we show that a high dose of *XBF-1* causes tissue outgrowths in the ectoderm, increases BrdU incorporation and inhibits the expression of the cyclin-dependent kinase (cdk) inhibitor $p27^{XIC1}$ (Su et al., 1995). We show that $p27^{XIC1}$ is normally expressed in non-dividing cells in the neurula embryo and is sufficient to block BrdU incorporation when misexpressed. The effect of *XBF-1* on cell division is dose-dependent such that at low concentrations *XBF-1* has the opposite effect, namely it decreases BrdU incorporation and induces ectopic $p27^{XIC1}$ expression. We conclude that a high dose of *XBF-1* expands the neural plate both by specifying additional ectodermal cells into a neural fate *and* by exerting a positive effect on the cell cycle of neuroectodermal cells mediated, at least in part, by suppression of a cell cycle inhibitor at the mRNA level. We show that *XBF-1* suppresses the expression of $p27^{XIC1}$ in the absence of protein synthesis, suggesting that $p27^{XIC1}$ may be a direct target of *XBF-1* and its vertebrate homologues. Finally, we present evidence in support of distinct effects of *XBF-1* on cell fate and proliferation. First, *XBF-1* mediates a neural cell-fate change under conditions in which cell division is blocked. Second, misexpression of $p27^{XIC1}$ stops the cell cycle but, unlike a low dose of *XBF-1*, does not induce ectopic or additional *N-tubulin*. We propose that *XBF-1* is a key regulatory gene that co-ordinates cell division and neural cell fate specification in the ectoderm.

MATERIALS AND METHODS

DNA constructs

Full-length *XBF-1* RNA, lacking the 5′ and 3′ non-coding sequences, was transcribed in vitro, as previously described (Bourguignon et al., 1998). Typically, 0.5 ng (high dose) or 90 pg (low dose) of *XBF-1* RNA were injected in a volume of 10 nl, as previously described (Bourguignon et al., 1998). However, the potency of in vitro transcribed RNA varied between different reactions, therefore each batch of RNA was biologically titrated before each experiment to identify the optimal concentration for a high- or low-dose phenotype. Generally, we found that a two- to fivefold difference in the concentration of the injected RNA was sufficient to significantly change the frequency of high- versus low-dose phenotype. $p27^{XIC1}$ RNA was transcribed from a *pCS2-Xic-1* construct (a gift of Dr S. Ohnuma). *XSox3* RNA was transcribed from a *T7TS-XSox3* construct (a gift of Dr R. Grainger). The full-length *XBF-1* cDNA without the untranslated regions was cloned in-frame into the p5′ HGR vector, which contains the ligand-binding domain of the Human Glucocorticoid Receptor (a gift of Dr K. Ryan) to produce a dexamethasone-inducible *XBF-1* construct (Kolm and Sive, 1995).

Embryo culture and injections

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg laying, in vitro fertilised using standard methods and chemically dejellied (3% cysteine in 0.1× Marc's Modified Ringer's solution (MMR), pH 7.9). Embryos were staged according to Nieuwkoop and Faber (1967). One blastomere of two-cell stage embryos was injected with capped, synthetic RNAs or plasmid DNA. 500-90 pg of *XBF-1* RNA or 100 pg of plasmid DNA were injected in a volume of 10 nl. Experimental RNAs were coinjected with lacZ RNA, which serves as a lineage label for cells that have inherited the injected RNA mixture. As a negative control, embryos were similarly injected with lacZ RNA alone. At neural plate stage, the injected embryos were fixed, stained with X-gal to reveal the distribution of the lacZ tracer, and then analysed by whole-mount in situ hybridisation. The lacZ RNA used in this study carries a nuclear localisation signal and therefore the blue staining is localised in the nucleus. In contrast, the in situ hybridisation signal is predominantly cytoplasmic.

In situ hybridisation

In situ hybridisation was performed essentially as described (Harland,

1991). The substrate for the chromogenic reaction was Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; purple colour). Antisense probes for *collagen type II* (Amaya et al., 1993), muscle actin (i.e. *cardiac actin*; Mohun et al., 1984), *XSox3* (Zygar et al., 1998), *N-tubulin* (Good et al., 1989); Chitnis et al., 1995), *p27^{XIC1}* (a gift of Dr S. Ohnuma) and *XBF-1* (Bourguignon et al., 1998), were transcribed with digoxigenin-11-UTP or fluorescein-12-UTP, as previously described (Harland, 1991).

Double in situ hybridisation was performed according to a protocol developed by Dr T. Doniach and described in Knecht et al. (1995). The substrate of the first chromogenic reaction was 5-bromo-6-chloro-3-indolyl phosphate (magenta phosp.; magenta colour) and of the second, 5-bromo-4-chloro-3-indolyl-phopshate (BCIP; light blue colour). Some specimens were sectioned after staining, and these were post-fixed overnight in MEMFA, and then embedded in gelatin/albumin mixture, solidified with glutaraldehyde. Sections (10 μm-30 μm) were cut on a Leica VT1000M vibratome, mounted in 90% glycerol, and photographed with Nomarski optics.

X-gal staining

Embryos were grown to the desired stage, devitellinised and fixed in MEMFA for 1 hour. Following a brief wash in phosphate buffer (pH 6.3) embryos were transferred into the X-gal staining solution (Coffman et al., 1990) until staining was apparent, typically a few hours. The reaction was terminated by rinsing in phosphate buffer and the embryos were dehydrated and stored in ethanol at −20°C until further processing.

BrdU incorporation

To examine levels of proliferation in the developing embryo, 10 nl of 5-bromo-deoxyuridine (BrdU) (Boehringer Mannheim, kit 1299964) were injected into each of three areas of stage 15.5 embryos: either side of the neural plate and once in the ventral midline. After 1 hour of incubation with BrdU, embryos were fixed in MEMFA for 1 hour and then dehydrated in a graded series of ethanols. BrdU was then detected with an anti-BrdU antibody, as described below.

To combine in situ hybridisation with immunohistochemistry, embryos were taken through the whole-mount in situ hybridisation procedure first, omitting the proteinase K digestion. After in situ hybridisation was complete, alkaline phosphatase activity was 'killed' by rinsing in methanol for 30 minutes. Embryos were rehydrated, washed in PBS and treated with 2 N HCl for 1 hour. Embryos were rinsed in PBS+0.3% Triton X-100 and blocked in 3% heat-treated lamb serum for 1 hour, followed by a wash in incubation buffer (66 mM Tris, 0.66 mM $MgCl_2$, 1 mM 2-mercaptoethanol: Boehringer Mannheim, kit 1299964). Incubation buffer was replaced with mouse monoclonal anti-BrdU solution (diluted 1:60 with incubation buffer: Boehringer Mannheim, kit 1299964) for 3 hours at 37°C, after which embryos were washed overnight in PBS. This was followed by rinsing in Tris-buffered saline+0.1% Tween 20 (TBST), then 20% heat-treated lamb serum in TBST and subsequently incubated for 5 hours at room temperature in a horseradish peroxidase-coupled secondary antibody, anti-mouse IgG (Jackson ImmunoResearch Lab. 115-035-006) (1:50 with 20% heat-treated lamb serum in TBST), after which embryos were washed overnight in TBST. Horseradish peroxidase activity was detected with diaminobenzidine (Sigma; brown colour).

Hydroxyurea and aphidicolin treatment

Embryos were dejellied with cysteine, grown to stage 10, devitellinised and added to a solution of 20 mM hydroxyurea (Sigma H8627) and 150 μM aphidicolin (Sigma A0781) in 0.1× MMR, as described in Harris and Hartenstein (1991). Aphidicolin was diluted from a 10 mg/ml frozen stock in dimethyl sulfoxide. Embryos were kept in this solution continuously until fixation.

Cycloheximide and dexamethasone treatment

Embryos were injected with 0.5 ng of *XBF-1-GR* RNA and treated with dexamethasone (Sigma) at various stages. A 20 mM stock of dexamethasone was stored frozen in 100% ethanol and before use was freshly diluted with 0.1× MMR to 10 μM final concentration. Protein synthesis was blocked by incubating embryos in cycloheximide (5 μg/ml) for 30 minutes; they were then transferred to dexamethasone plus cycloheximide for another 30 minutes and fixed a maximum of 4 hours later (Cascio and Gurdon, 1987).

Animal cap dissections

Animal caps were dissected from stage 9-9.5 embryos in 0.5× MMR, some were combined in pairs and were incubated until sibling controls reached neurula stage (stage 16-18). Animal caps were derived from embryos injected bilaterally with either *XBF-1* RNA or a lineage tracer, 10 mg/ml dextran tetramethylrhodamine (RLDx; D-1817, Molecular Probes) at the two-cell stage.

RESULTS

XBF-1 converts ectoderm to a neural fate

In embryos injected with a high dose of *XBF-1*, the expression of the neural marker *XSox3* expands to the lateral sides of the embryo (Bourguignon et al., 1998). Concomitantly, the expression of epidermal keratin is suppressed on the injected side (Fig. 1A,B) suggesting that *XBF-1* converts ectoderm to a neural fate. Since dorsal mesoderm induces neural tissue we tested whether *XBF-1* expanded the domain of dorsal mesoderm. Embryos injected with *XBF-1* did not show ectopic activation of the mesodermal markers, *muscle actin* (Fig. 1D), and *collagen type II* (data not shown), a marker for notochord and somites (Fig. 1F) while embryos from the same batch showed abundant ectopic *N-tubulin* formation (Fig. 1C).

To test whether *XBF-1* would promote a neural fate in isolated ectoderm, we combined *XBF-1* expressing animal caps with control animal caps that were lineage labelled with RLDx (Fig. 1E,G,H,J,K). Control uninjected animal caps that were cultured alone developed into atypical epidermis and showed no *XSox3* expression (Fig. 1H). In contrast, *XBF-1* injected animal caps expressed high levels of *XSox3* (Fig. 1G), but no *collagen type II* (Fig. 1E). Expression of *XSox3* was localised in the *XBF-1* injected, non RLDx-labelled part of the animal cap conjugates, demonstrating that *XBF-1* activates *XSox3* cell-autonomously (Fig. 1J,K). Since a few RLDx-positive cells were observed within the *XSox3* expressing domain (Fig. 1J,K) we cannot exclude the possibility that a short-range inducing signal operates as well. However, the majority of the induction appears cell-autonomous. Consistent with this finding, the expression of *XSox3* in the anterior neural plate is coincident with *XBF-1* (Fig. 5). These findings suggest that *XBF-1* can convert ectoderm to a neural fate autonomously, in the absence of neural inducing signals from dorsal mesoderm.

Blocking cell division with HUA does not prevent the ability of *XBF-1* to expand *XSox3*

To test the effect of *XBF-1* on neural specification further, we asked whether it induces expanded or ectopic *XSox3* expression when cell division is blocked (Fig. 1L-N). We treated *XBF-1* injected embryos with the DNA synthesis inhibitors hydroxyurea and aphidicolin (HUA) at stage 10, as described in Harris and Hartenstein (1991), and assayed for expanded or ectopic *XSox3* expression at stage 16. HUA-treated neurula-stage embryos had larger cells, demonstrating that cell division

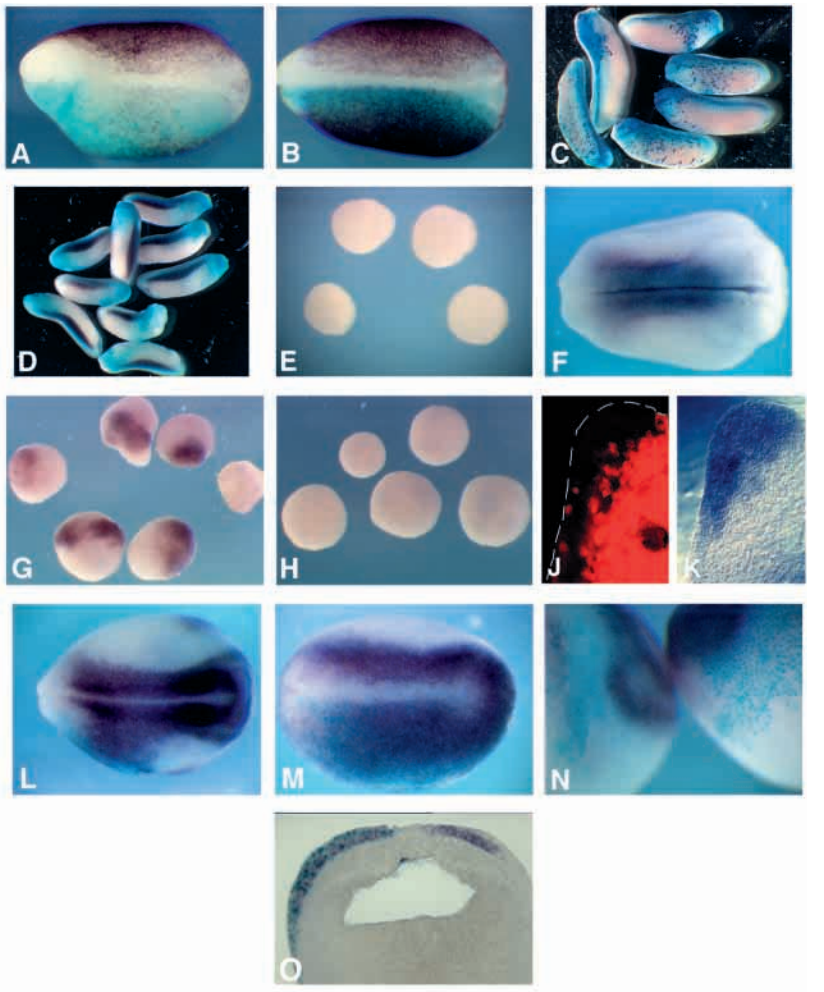

Fig. 1. *XBF-1* induces neural tissue autonomously, at the expense of epidermis and in the absence of mesodermal signals. *XBF-1* induces ectopic *XSox3* even when cell division is blocked. Embryos were injected with a high dose of *XBF-1* plus lacZ RNA or lacZ RNA alone (A or B respectively; light blue) and hybridised with a probe for epidermal keratin (dark magenta). Other embryos (C,D) were injected with *XBF-1* plus lacZ RNA and were analysed at the tadpole stage for lacZ (C,D; light blue), *N-tubulin* (C) and *muscle actin* (D) expression (magenta). Note that ectopic *N-tubulin* expression is not accompanied by a corresponding expansion or ectopic expression of *muscle actin*. *XBF-1* injected animal caps do not express *collagen type II* (E), which in the embryo is expressed in the somites and notochord (F). In contrast, *XBF-1* injected (G), but not control uninjected (H), animal caps express *XSox3*. In this experiment, *XBF-1* injected animal caps were combined with control RLDx labelled caps (E,G,H,J). Fluorescent (J) and bright-field (K) images of a sectioned animal cap demonstrate that *XSox3* expression is localised in the *XBF-1* injected part of the *XBF-1*/RLDx animal cap conjugate. Embryos (L,M) were injected with a high dose of *XBF-1* and lacZ RNA and some of these were treated with HUA at the early gastrula stage (M). All embryos shown (L-N) are hybridised with *XSox3*. Embryos injected with lacZ and treated with HUA have normal *XSox3* expression while both *XBF-1* injected (L) and *XBF-1* injected plus HUA (M) treated embryos show an expansion of the *XSox3* expression domain. The expansion of the *XSox-3* expressing domain in HUA-treated *XBF-1* injected embryos can be clearly seen in section (O). HUA treated embryos (N, embryo on the right) have larger nuclei than sibling controls (N, embryo on the left) illustrating that cell division has been effectively blocked by the HUA treatment.

had been blocked effectively, but *XSox3* expression was normal (Fig. 1N). Interestingly, in *XBF-1* injected/HUA treated embryos *XSox3* was expanded, just as in *XBF-1* injected controls (Fig. 1L,M,O), suggesting that *XBF-1* can convert ectoderm to a neural fate in the absence of cell division.

XBF-1 causes outgrowths of the ectoderm

Visual inspection of neurulae injected with a high dose of *XBF-1* revealed that they were 'swollen' on the injected side (12/18) and often developed externally visible convoluted outgrowths of the ectoderm (7/18, 9/12, Fig. 2A) that were visible as early as stage 12.5. Later stage embryos showed protrusions in the ectoderm on the injected side (Fig. 2D-G). Sectioning of affected neurula stage embryos showed that the ectoderm was thickened on the injected side and contained many more cells than were found in a comparable region of the ectoderm on the uninjected side. The ectodermal thickenings expressed *XSox3* (Fig. 2C) while the convoluted outgrowths contained *N-tubulin* positive cells (Fig. 2B,D,F). *XSox3* and *N-tubulin* were never coexpressed (not shown; Bourguignon et al., 1998). Dorsal mesoderm cell types were not detected in these outgrowths suggesting that they are purely ectodermal (Fig. 2G).

While formation of ectopic neural tissue can be explained if *XBF-1* mediated an epidermal-to-neural cell-fate switch, as described above, such a cell-fate switch cannot easily explain the hypertrophy of the ectopic neural tissue in comparison either to the normal neural or epidermal tissue (Fig. 2B,C). Therefore, we decided to investigate further whether *XBF-1* affects cell proliferation in addition to affecting cell fate specification. Although the *Xenopus* embryo does not feed at these early stages, increased cell divisions can result in enlarged organs despite the absence of net dry mass increase, by water uptake and/or utilisation of yolk reserves in the endoderm (see for example, Zuber et al., 1999).

High *XBF-1* causes increased BrdU incorporation in the ectoderm

We used BrdU incorporation as a measure of proliferation in normal (Fig. 3) and *XBF-1* injected embryos (Fig. 4). BrdU labelling of control embryos showed that at the neural plate stage most deep ectodermal cells, epidermal and neural, are dividing. At this stage, the superficial layer of the ectoderm is negative for BrdU all around the embryo with the exception of a small area of the neural plate close to the dorsal midline (Figs 3, 4). In addition, bilaterally symmetrical longitudinal domains that do not incorporate BrdU are visible in the deep layer of the posterior neural plate. These BrdU negative domains contain the *N-tubulin* expressing cells (Fig. 3). Interestingly, the domain of BrdU negative cells is wider that the domain of *N-tubulin* positive cells (Fig. 3, section) suggesting that not all cells that stop dividing express *N-tubulin* at this stage. It is possible that these cells adopt an alternative fate, are delayed

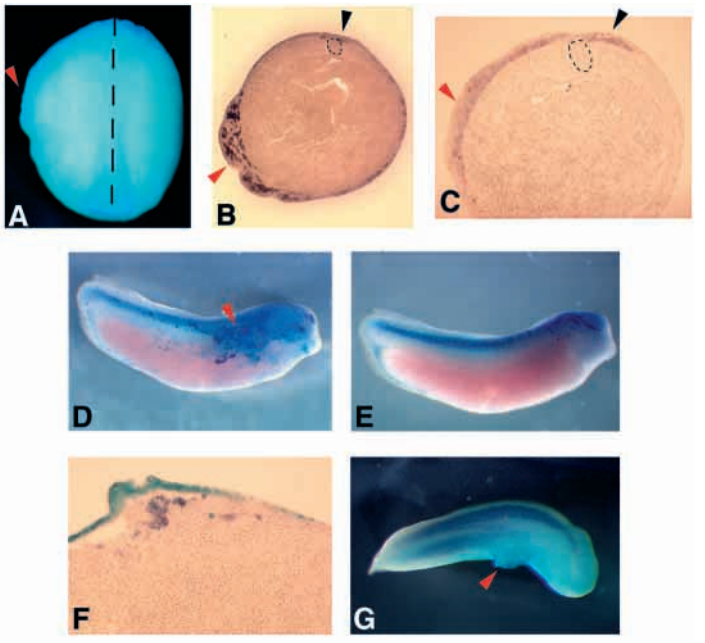

Fig. 2. *XBF-1* induces tissue outgrowths in the ectoderm. In all panels, a red arrowhead indicates convoluted and/or thickened ectoderm on the side that has been injected with *XBF-1* RNA (all panels except E and F) or DNA (F). Whole embryo (A) and transverse sections (B,C) of neurula embryos hybridised with *N-tubulin* (B) and *XSox3* (C; C is also lightly stained for BrdU incorporation). Note the dramatic thickening of the ectopic neural tissue (red arrowhead) compared with the normal neural tissue on the control side (black arrowhead). The dotted line demarcates the dorsal midline (A) or the notochord (B,C), separating the injected (left) from the uninjected side. Tadpole stage embryos were hybridised with *N-tubulin* (D, the injected side; E, the control side; F, high magnification of a section through D) and *muscle actin* (G) (magenta staining in all). In G, note that there is no ectopic *muscle actin* expression in the lateral protrusion. In embryos shown in D-G, *XBF-1* RNA or DNA was coinjected with lacZ RNA (light blue).

in expressing *N-tubulin* or are eliminated later by apoptosis. In contrast to *N-tubulin*, *XSox3* expression coincides with BrdU positive neural cells (Fig. 3).

In *XBF-1* injected embryos, the effect on the pattern of BrdU staining was twofold (Fig. 4A). When injected at a high dose, *XBF-1* expanded laterally the domain of dividing cells, in both the superficial and the deep layers, coincidentally with the expansion of *XSox3* expression. In addition, however, *XBF-1* increased proliferation *within* the *XSox3* expressing domain. For example, while the BrdU positive cells are mostly arranged as a monolayer in the deep layer of the control side, BrdU positive cells were stacked in a multilayer on the experimental side (arrows, Fig. 4A). By counting BrdU positive cells we have found a 50% increase of positive cells in areas that express ectopic *XSox3* compared to control neuroectoderm (an average of 24.5±4.3 cells in ectopic neural tissue versus 16.3±2.5 cells in control neural tissue, over an 100×10 μm^2 area, n=21 sections from three embryos). This moderate increase is expected at this stage where most ectodermal cells are normally dividing (Figs 3, 4), if *XBF-1* does not affect the rate of proliferation but prevents cells from becoming post-mitotic.

XBF-1 affects proliferation in a dose-dependent manner

A lower dose of injected *XBF-1* RNA had the opposite effect on BrdU incorporation in that it expanded the domain of BrdU negative cells. Triple *XSox3*, *N-tubulin* and BrdU staining (Fig. 4A) showed that this expansion was coincident with the expansion of *N-tubulin*. Interestingly, the spatial organisation of cell division along the medio-lateral axis of the neural plate was disrupted. As mentioned above, normally, the lateral stripe of *N-tubulin* expressing cells appear within a wider 'zone' of BrdU negative cells located next to the *XSox3* expression domain (Figs 3, 4). In experimental embryos, this well-defined BrdU negative zone was missing and was replaced by a domain of BrdU negative, *N-tubulin* positive,

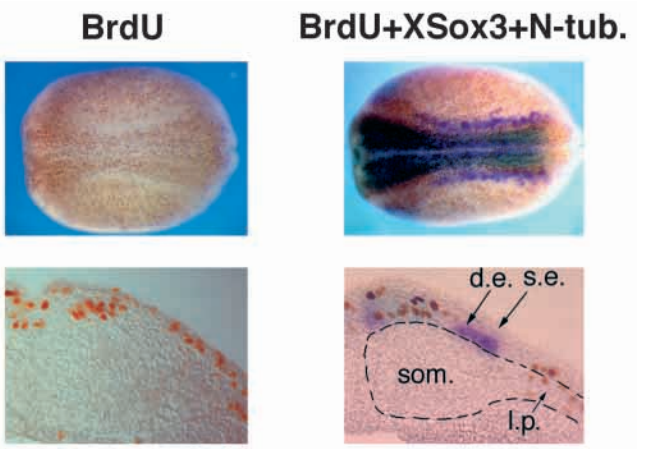

Fig. 3. *XSox3* and *N-tubulin* expression marks proliferating and post-mitotic neural cells, respectively. Embryos were processed either for BrdU incorporation alone (left panels) or simultaneously for BrdU incorporation, *XSox3* (light blue or green) and *N-tubulin* (magenta) expression (right panels), as described in Materials and Methods. Top panels show the whole mounted embryos and the bottom panels the respective transverse sections. *XSox3* expression overlaps with BrdU incorporating cells while *N-tubulin* is expressed in cells that do not incorporate BrdU. Note that the deep layer of the epidermal ectoderm is also heavily dividing at this stage. A dotted line demarcates the underlying somitic and lateral plate mesoderm. d.e., deep layer of the ectoderm; l.p., lateral plate mesoderm; s.e., superficial layer of the ectoderm; som., somitic mesoderm.

cells closely mingled with BrdU positive, *N-tubulin* negative, cells (Fig. 4). Taken together, these results suggest that *XBF-1* affects the proliferation of ectodermal cells in a dose dependent manner.

The effective dose of *XBF-1* can be determined by a synergistic interaction with *XSox3*

The phenotype of *XBF-1* injected embryos depends on the dose

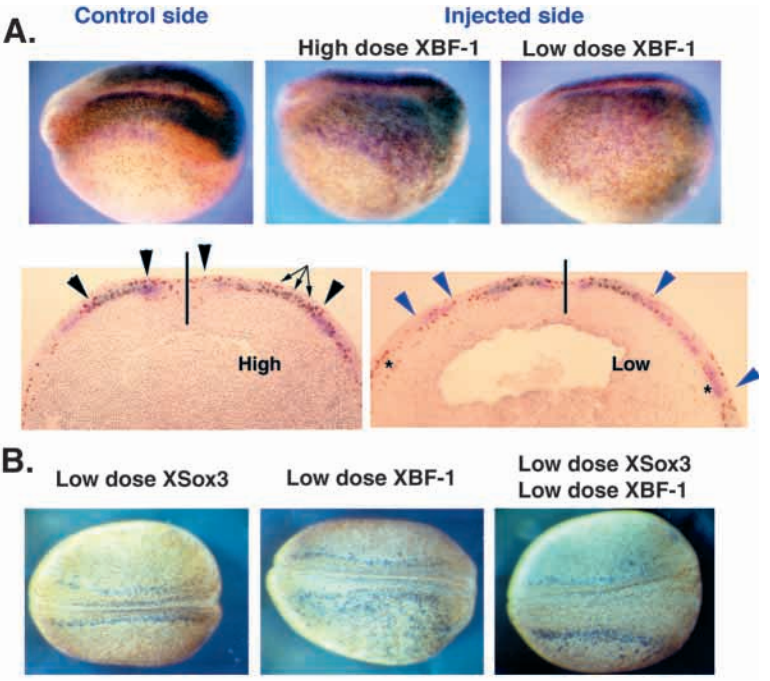

Fig. 4. *XBF-1* affects BrdU incorporation in the ectoderm and co-operates with *XSox3*. (A) Embryos were injected with either a high or a low dose of *XBF-1* RNA, as indicated, in one blastomere of the two-cell stage embryo and were processed for double whole-mount in situ hybridisation for *XSox3* (blue/green) and *N-tubulin* (magenta), followed by BrdU incorporation (brown). Whole mounts and transverse section are shown. In the sections a black line indicates the dorsal midline, separating the injected (right) from the uninjected side (left). Black and blue arrowheads delimit the *XSox3* and *N-tubulin* expression domains, respectively. In the high-dose sections, arrows show an area of increased BrdU incorporation on the injected side. In the low-dose sections, an asterisk on the injected side (right) shows an area of the ectoderm where dividing cells should have been located, when compared with the equivalent region of the control side (asterisk on control side, left). Also note that in the control side *N-tubulin* positive cells are located within a wider area of BrdU negative cells while in the experimental side *N-tubulin* positive and BrdU positive cells are intermingled. (B) Embryos were injected with either low-dose *XSox3*, low-dose *XBF-1*, or both, and processed for *N-tubulin* (purple) and BrdU incorporation (brown). A low dose of *XSox3* did not affect the expression of *N-tubulin*, whereas low-dose *XBF-1* induced additional ectopic *N-tubulin* positive cells. Coinjection of both low-dose *XSox3* and low-dose *XBF-1* suppressed endogenous and induced ectopic *N-tubulin*, in the same way that a high-dose *XBF-1* affects *N-tubulin* expression (see also Table 1).

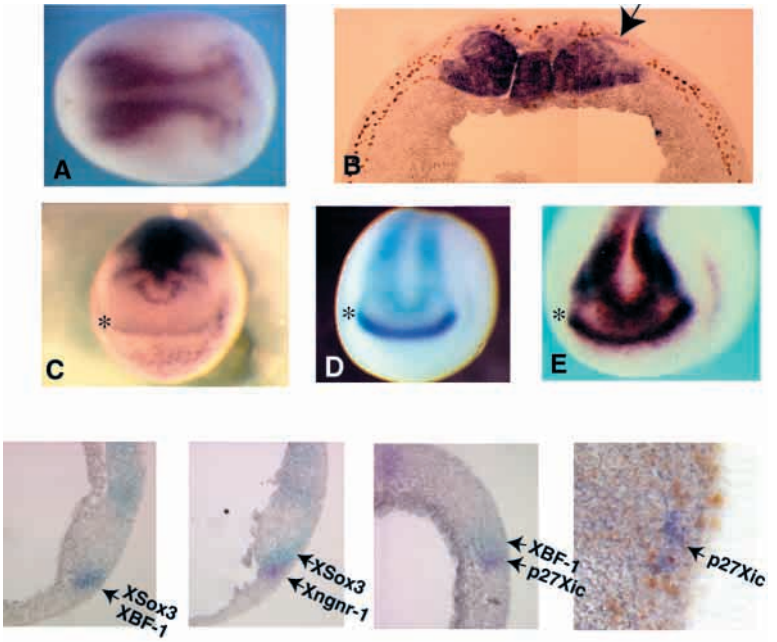

Fig. 5. $p27^{XIC1}$ is normally expressed in non-dividing cells. Expression of *XBF-1* in the anterior neural plate coincides with the expression of *XSox3*, but not that of $p27^{XIC1}$. Neural plate-stage embryos were analysed for the expression of $p27^{XIC1}$ (A-C). The sectioned embryo (B) was also labelled for BrdU incorporation (brown nuclear staining). $p27^{XIC1}$ is expressed in non-BrdU incorporating cells both in the mesoderm and in the neural ectoderm (B, arrow). In the anterior neural plate (C), $p27^{XIC1}$ was expressed in a band in a similar region to that of *XBF-1* (D) and *XSox3* (D,E) (marked by asterisks; D, *XBF-1* in purple and *XSox3* in light blue and E, *XSox3* only in purple/brown). (Lower panels) Sections of double in situ hybridisations in light blue and purple, showing that *XSox3* and *XBF-1* are largely coexpressed and that $p27^{XIC1}$ is expressed more rostrally than *XBF-1* in a more comparable region to that of neurogenin, *X-ngnr-1*. $p27^{XIC1}$ was expressed in BrdU negative cells in the anterior neural plate (bottom right panel).

of injected RNA (Bourguignon et al., 1998; Fig. 4). A high dose of *XBF-1* suppresses *N-tubulin* and at the same time induces ectopic *N-tubulin* at the border of the high *XBF-1* expressing ectoderm (high-dose phenotype) while a low dose expands endogenous *N-tubulin* and/or induces ectopic *N-tubulin* in the lateral and ventral ectoderm (low-dose phenotype). In order to test whether the effective *XBF-1* dose that the cells experience can be modulated by the presence of other factors, we coinjected *XSox3* and *XBF-1*. Embryos were injected with a low dose of *XSox3*, a low dose of *XBF-1* and with a combination of low dose *XSox3* plus a low dose of *XBF-1* and analysed with *N-tubulin* and BrdU staining. (Fig. 4B, lower panels; Table 1). In most experiments, embryos injected with a low dose of *XSox3* (250-100 pg) were normal. In some experiments, a percentage of *XSox3* injected embryos showed suppression of *N-tubulin* expression (see Table 1). As expected, a low dose of *XBF-1* gave a high frequency of expanded and/or ectopic *N-tubulin* (low dose phenotype; Fig. 4B; Table 1). Interestingly, when low doses of *XSox3* and *XBF-1* were coinjected, the frequency of the low-dose phenotype was

Table 1. Coinjection of *XSox3* potentiates the *XBF-1* phenotype

	Normal (%)	*N-tubulin* suppression (%)	*N-tubulin* expansion/ectopic (Low-dose phenotype) (%)	*N-tubulin* suppression + ectopic induction (High-dose phenotype) (%)	*n*
Exp. 1					
Low *XSox3*	100	0	0	0	13
Low *XBF-1*	0	0	48	52	25
Low *XSox3+XBF-1*	9	22	17	52	23
Exp. 2					
Low *XSox3*	100	0	0	0	36
Low *XBF-1*	35	0	65	0	26
Low *XSox3+XBF-1*	3	13	32	52	31
Exp. 3					
Low *XSox3*	50	36	14	0	14
Low *XBF-1*	22	0	78	0	18
Low *XSox3+XBF-1*	20	11	20	49	35

Embryos were injected with a low dose of *XSox3*, a low dose of *XBF-1* and with a combination of low dose *XSox3* plus a low dose of *XBF-1*. Results from three independent experiments are shown.

A 'high-dose phenotype' consists of suppression of endogenous *N-tubulin* and at the same time induction of ectopic *N-tubulin* at the border of the high *XBF-1* expressing ectoderm. A 'low-dose phenotype' consists of expansion of endogenous *N-tubulin* and/or induction of ectopic *N-tubulin* in the lateral and ventral ectoderm. Results show that there is a reproducible trend for the coinjection of *XSox3* and *XBF-1* to increase the frequency of the high-dose and decrease the frequency of the low-dose phenotype, when compared with low *XBF-1* alone. In some experiments, the frequency of the embryos showing complete suppression of *N-tubulin* also increased after coinjection. This may be due to the dose of *XBF-1* being too high to allow neuronal differentiation anywhere on the injected side.

reproducibly decreased while the frequency of the high-dose phenotype increased (Fig. 4; Table 1). These results suggest that a synergistic interaction between *XSox3* and *XBF-1* may be needed to increase the effective dose of *XBF-1*.

p27XIC1 expression is spatially restricted in the neural plate

To gain insight into the molecular mechanism by which *XBF-1* affects proliferation, we examined whether it can alter the expression of a regulator of the cell cycle. p27XIC1 is a cdk inhibitor that was isolated from *Xenopus* ovary and has been shown to inhibit cyclin/cdk activities and DNA synthesis in vitro (Su et al., 1995). First, we determined the expression pattern of *p27XIC1* (Fig. 5) and the effects of misexpression in whole embryos (Fig. 6). *p27XIC1* was heavily expressed in the developing somites and notochord, tissues that did not show BrdU incorporation. In contrast, lateral mesoderm incorporates BrdU and does not express *p27XIC1*. *p27XIC1* is also expressed in BrdU negative cells in the neural ectoderm, albeit at a lower level (Fig. 5B and lower panels). Specifically, in the posterior neural plate *p27XIC1* is expressed in bilaterally restricted domains of the deep layer (Fig. 5B) while in the anterior neural plate it is expressed in two horseshoe-shaped domains (Fig. 5C). The most anterior of these domains is positioned around the domain of expression of *XSox3* and *XBF-1*, with at most a partial overlap (Fig. 5C-E and lower panels).

p27XIC1 misexpression stops cell division

These findings suggest that *p27XIC1* is expressed in non-dividing, most likely post-mitotic, cells. To test whether *p27XIC1* has a role in cell-cycle inhibition in vivo we injected *p27XIC1* RNA in early embryos. Embryos injected with the usual dose of RNA (1-0.5 ng) died before gastrulation as preventing cell division before the Mid-Blastula-Transition activates apoptosis (Stack and Newport, 1997). However, around 30% of embryos injected with a lower dose of *p27XIC1*

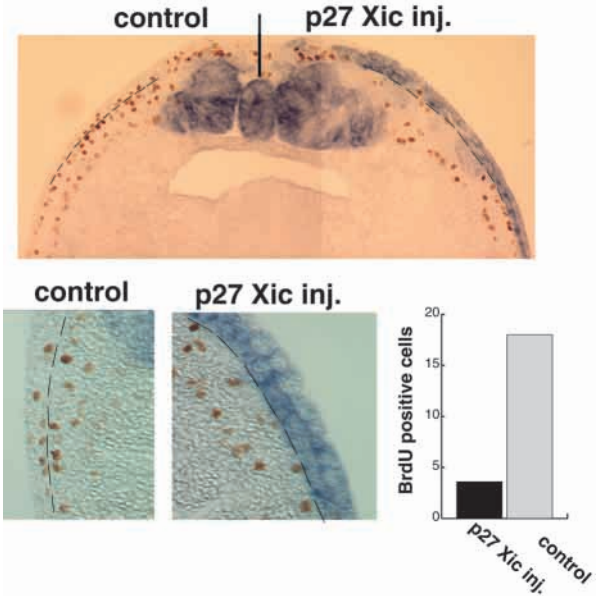

Fig. 6. *p27XIC1* blocks cell division when misexpressed. *p27XIC1* misexpression abolishes BrdU incorporation in the ectoderm. Embryos were injected with *p27XIC1* RNA at the two-cell stage and processed for *p27XIC1* expression by in situ hybridisation (blue/purple) and BrdU incorporation (brown nuclei) in transverse sections. In situ hybridisation with a *p27XIC1* probe revealed both the expression of the endogenous gene and the distribution of the injected *p27XIC1* RNA, both of which coincide with a complete absence of BrdU staining. The numbers of BrdU positive cells on the *p27XIC1* injected side in comparison with the uninjected side are also represented as a column chart. The BrdU count is represented as BrdU positive cells per section, over the neural fold area and was averaged from 15 sections over three embryos in two independent experiments. Sections scored were from the anterior spinal cord level. Error bars were too small to illustrate.

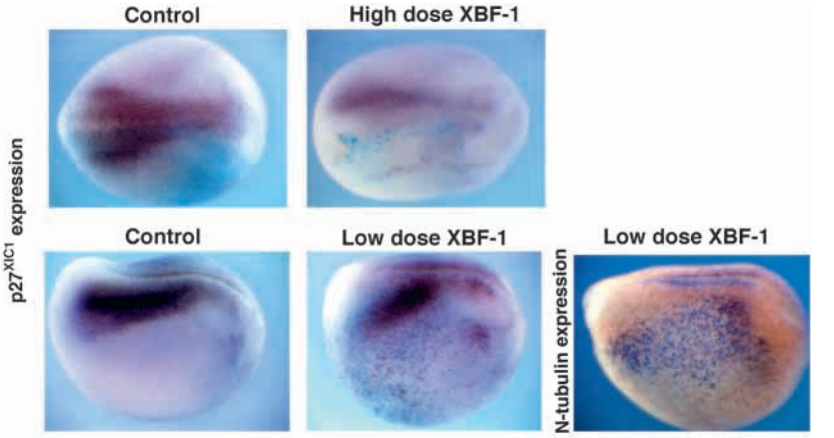

Fig. 7. *XBF-1* regulates *p27XIC1* in a dose-dependent manner. Embryos were injected with either a high or a low dose of *XBF-1* RNA and analysed for expression of *p27XIC1* or *N-tubulin.* A high dose of *XBF-1* suppressed *p27XIC1* and repositioned residual *p27XIC1* expression laterally (top right panel, dorsal view) while a low dose induced ectopic *p27XIC1* in the lateral ectoderm (bottom middle panel, side view), in the same way that a low dose of *XBF-1* induces ectopic *N-tubulin* (shown for comparison in bottom right panel). *XBF-1* RNA was coinjected with lacZ RNA as a lineage marker; embryos injected with lacZ alone were normal (left panels).

RNA (0.25 ng) survived to neurula stage. We found that such embryos showed a dramatic absence of BrdU staining in the expressing tissue (Fig. 6), suggesting that *p27XIC1* misexpression is sufficient to block the cell cycle in vivo. Injection of *p27XIC1* plasmid DNA (0.1 ng per embryo) allowed better survival of the embryos and gave otherwise identical results.

XBF-1 regulates the cell cycle inhibitor *p27XIC1*

To test whether *XBF-1* regulates *p27XIC1* we examined the expression of *p27XIC1* in *XBF-1* injected embryos. Embryos injected with a high dose of *XBF-1* showed a strong suppression of *p27XIC1* on the injected side (Fig. 7, upper panels). Often, there was residual *p27XIC1* expression displaced laterally. Interestingly, when injected at lower doses, *XBF-1* induced ectopic *p27XIC1* expression (Fig. 7, lower panels), suggesting that *XBF-1* can either suppress or activate *p27XIC1* in a concentration-dependent manner. This is consistent with the ability of *XBF-1* to induce either expanded/ectopic dividing neuroepithelial (marked by *XSox3*) or expanded/ectopic post-mitotic neural domains (marked by *N-tubulin*) in high or low concentrations, respectively (Fig. 4).

Since a low dose of *XBF-1* induced ectopic *p27XIC1 and* ectopic *N-tubulin* (Fig. 7, lower panels) we asked whether ectopic expression of *p27XIC1* would be sufficient to induce ectopic *N-tubulin*. In other words, whether the effect of a low dose *XBF-1* on *N-tubulin* are mediated by *p27XIC1*. We injected *p27XIC1* RNA or DNA and assayed for the pattern of expression of *XSox3* and *N-tubulin* (Fig. 8). In contrast to the effects of *XBF-1*, *p27XIC1* overexpression did not induce either ectopic neural (*XSox3*) or neuronal (*N-tubulin*) cells although it was sufficient to block the cell cycle (Fig. 8). Thus, only the effects of *XBF-1* on the proliferation of neuroectodermal cells are likely to be mediated by regulating the expression of *p27XIC1*. In turn, this finding suggests that the effects of *XBF-1* on cell proliferation and cell-fate specification are distinct.

p27XIC1 may be a direct target of *XBF-1*

Since *XBF-1* belongs to a family of winged helix transcription factors, we were interested in determining whether *p27XIC1* may be a direct target of *XBF-1*. We used a dexamethasone inducible fusion of *XBF-1* (*XBF-1-GR;* see Materials and Methods) in order to be able to block protein synthesis before activating *XBF-1* function. By injecting embryos with *XBF-1-GR* and adding dexamethasone at various stages, we determined that the ability of *XBF-1* to suppress *p27XIC1* is high at stage 11.5 but declines sharply at the end of gastrulation (Table 2). In the absence of dexamethasone no suppression was observed (Fig. 9; Table 2). When injected embryos were pretreated with CHX for 30 minutes before adding dexamethasone, *p27XIC1* was suppressed with similar frequency (Fig. 9; Table 3). CHX alone had no effect on *p27XIC1* expression in the neural ectoderm (Table 3). As a positive control for the efficacy of the CHX treatment, sibling embryos were treated with CHX at the early gastrula stage and these showed complete suppression of muscle actin gene expression, as previously reported (data not shown; Cascio and

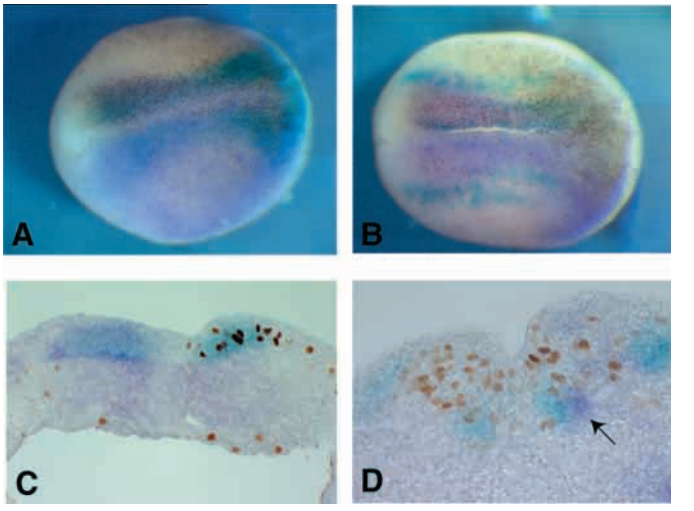

Fig. 8. *p27XIC1* misexpression does not alter the pattern of expression of *N-tubulin* or *XSox3*. *p27XIC1* misexpression has no effect in inducing ectopic *XSox3* (A,C) or *N-tubulin* (B,D) expression. Embryos were injected with *p27XIC1* RNA and were analysed for *p27XIC1* (magenta), *XSox3* (light blue, A,C) and *N-tubulin* expression (light blue, B,D) and for BrdU incorporation (brown nuclei). Magenta colour shows primarily the ectopic *p27XIC1* expression although some endogenous expression can be seen in B. Cells expressing *p27XIC1* did not incorporate BrdU; however cells in the neural plate that were expressing injected *p27XIC1* still expressed *XSox3*, excluding the possibility of non-specific toxic effects of *p27XIC1*. Arrow in D, shows a cell within the neural plate that has received *p27XIC1* RNA but does not express N-tubulin.

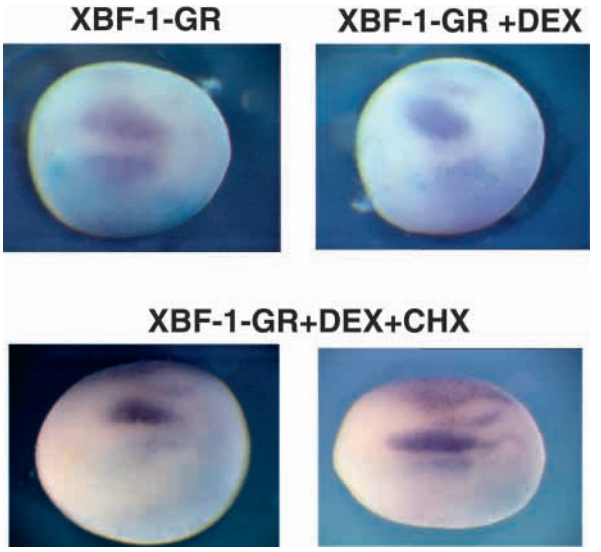

Fig. 9. *p27^{XIC1}* is a direct target of *XBF-1*. Dexamethasone-inducible *XBF-1* RNA suppresses *p27^{XIC1}* in the presence (bottom panels) and absence (top right) of the protein synthesis inhibitor, cycloheximide. In the absence of both dexamethasone and cycloheximide, *XBF-1-GR* did not suppress *p27^{XIC1}* expression (top left panel).

Table 2. The ability of *XBF-1* to suppress *p27^{XIC1}* expression declines towards the end of gastrulation

XBF-1-GR injected	*p27^{XIC1}* suppression (%)	*n*
+DEX, added at:		
stage 11.5	70	17
stage 12	37	27
stage 12.5	16	19
–DEX	0	17

Embryos were injected with a dexamethasone-inducible construct at the two-cell stage and were treated with dexamethasone (+DEX) at the stages indicated. Control embryos were similarly injected but not treated with dexamethasone (–DEX) and these were normal.

Gurdon, 1987). These findings suggest that the suppression of *p27^{XIC1}* by *XBF-1* may be a direct effect that does not require prior protein synthesis. We have been unable to determine whether the ectopic activation of *p27^{XIC1}* by a low dose of *XBF-1* is also a direct response because treatment with CHX alone resulted in increased expression of *p27^{XIC1}* in the epidermis (not shown but visible in Fig. 9, on uninjected side of CHX-treated embryos, bottom right panel), making it impossible to score for ectopic *p27^{XIC1}* activation in response to *XBF-1*.

DISCUSSION

During gastrulation, part of the ectoderm receives a neural inducing signal and is converted to a neural fate while the remaining ectoderm becomes epidermis. At the neural plate stage, most cells in the neural plate are dividing (Hartenstein, 1989; this work). Within this neuroectodermal domain, a limited number of cells exit the cell cycle at the early neurula stage (Hartenstein, 1989; this work). Thus, there is a number of distinct decisions that early ectodermal cells have to make, namely whether to acquire a neural or an epidermal fate and also whether to divide or differentiate. In addition, since differentiating cells are found in well-defined domains in the neural plate, a decision has to be made as to where differentiation will be positioned on the neural plate.

How are these processes controlled and co-ordinated? Previously, we showed that *XBF-1* either suppresses or activates neuronal differentiation and proposed that this dual, dose-dependent property may underlie the positioning of prospective neurogenesis around a high *XBF-1* expressing domain in the anterior neural plate. Here, we show that *XBF-1* also affects cell division and is therefore likely to play a central role in co-ordinating the decision to divide or differentiate. In addition, we show that *XBF-1* turns ectodermal cells into neural cells, thus is likely to play a role in neural cell fate specification.

The decisions to become neural or epidermal precursor and to divide or differentiate are co-ordinated during normal development, but how are they mechanistically linked? One can imagine for example, the existence of regulatory molecules that affect cell division but do not affect the cell's choice of cell type. Indeed, it has been recently shown that the homeobox gene *Optx2* promotes cell division without affecting cell fate in the *Xenopus* retina (Zuber et al., 1999). Conversely, one may expect the existence of regulatory molecules that mediate an epidermal to neural fate switch without affecting cell division. Indeed, the enlargement of the neural plate caused by several proneural genes has been attributed to a neural cell-fate switch (e.g. Turner and Weintaub, 1994; Takebayashi et al., 1997; Bellefroid et al., 1998; Kroll et al., 1998; see also Coffman et al., 1990). *XBF-1* stands out from both classes of molecules in that it affects both processes: (1) neural cell fate specification and (2) cell division/differentiation, thus providing a potential molecular link between the two processes.

Table 3. *XBF-1* suppresses *p27^{XIC1}* in the absence of preceding or ongoing protein synthesis

XBF-1-GR injected	*p27^{XIC1}* suppression (%)	*n*
+DEX, +CHX, added at:		
stage 11.5	75	16
stage 12	51	43
stage 12.5	26	19
Non-injected control		
+CHX, added at:		
stage 11	0	5
stage 12	0	14

Embryos were injected with a dexamethasone-inducible construct at the two-cell stage and were treated with cycloheximide (+CHX) and dexamethasone (+DEX) at the stages indicated (see Materials and Methods). CHX treatment alone had not effect in *p27^{XIC1}* suppression.

XBF-1 imparts a neural fate

In the embryo, *XBF-1* induces ectopic neural tissue in the ventral, prospective epidermal ectoderm, without the concomitant induction of ectopic dorsal mesoderm. In isolated animal caps, *XBF-1* can induce neural markers in the absence of mesoderm-derived neural inducing signals. These findings suggest that *XBF-1* can turn prospective epidermal cells into

neural precursors. Furthermore, the ability of *XBF-1* to enlarge the neural plate does not depend on cell division, as it takes place when cell division is blocked by HUA.

However, the morphology of embryos injected with *XBF-1* suggests that cell proliferation is affected. Embryos that are injected with a high dose of *XBF-1* show convolutions of the ectoderm that develop into pronounced neural outgrowths in the tadpole. When the RNA is directed to the dorsal side, the neural tube is hypertrophic. *XBF-1*'s neuralising activity could explain the hypertrophic neural tube as it would result in additional neural tissue being incorporated into the neural tube during neurulation. However, this neuralising activity does not explain the thickening of the ectopic neural tissue at the neural plate stage, when compared either to the corresponding area of the epidermis or to the endogenous neural plate on the uninjected side. These effects could be explained if *XBF-1* also affects the division of ectodermal cells, as discussed below.

XBF-1 affects cell proliferation

In this paper, by examining the pattern of BrdU incorporation in conjunction with *XSox3* and *N-tubulin* expression, we have established that *XSox3* is expressed in dividing neuroepithelial cells (similar to a related mouse gene *Sox1*; Pevny et al., 1998), while *N-tubulin* is expressed in post-mitotic cells. By the same method of triple labelling, we showed that embryos injected with a high *XBF-1* concentration show increased BrdU incorporation over an area of expanded or ectopic neuroectoderm, such that the normally bilayered neuroectoderm becomes multilayered. Furthermore, in high *XBF-1* injected embryos, we found BrdU-incorporating neural precursor cells in lateral domains where post-mitotic cells would normally be found. Thus, cells that express a high dose of *XBF-1* may be prevented from exiting the cell cycle. At present, it is not clear whether high *XBF-1* also affects the rate of division such that progenitor cells undergo more divisions within a given period of time. *XBF-1* also affects cell division when misexpressed at a low dose but in the opposite way: the domain where BrdU-negative cells are normally located, was expanded but was less well defined as positive and ectopic BrdU negative cells were intermingled.

The ability of *XBF-1* to affect the cell cycle is consistent with the observation that a dominant mutant of the chicken homologue *qin* is oncogenic (Chang et al., 1995; Li et al., 1997). It is also consistent with the phenotype of the BF-1 knock-out mouse, since telencephalic cells lacking BF-1 fail to continue dividing and differentiate prematurely (Xuan et al., 1995). However, these studies did not distinguish any effects that BF-1 may have in neural cell-fate specification and did not address the molecular mechanism by which BF-1 may affect the cell cycle.

XBF-1 regulates a cell-cycle inhibitor

In this report we have shown that *XBF-1* affects the expression of a cell cycle-regulating molecule, $p27^{XIC1}$. This gene was isolated from *Xenopus*, is most closely related to the mammalian $p27^{Kip1}$ and inhibits the cyclin E/cdk complex activity in vitro (Su et al., 1995). Since $p27^{XIC1}$ was isolated from an ovarian cDNA library it was not clear whether it is expressed later in development or whether it has a role in controlling the cell cycle in vivo (Su et al., 1995). We showed that at the neural plate stage $p27^{XIC1}$ is spatially restricted in the mesoderm and neural ectoderm and its expression coincides with BrdU-negative cells (see also Ohnuma et al., 1999). Misexpression of $p27^{XIC1}$ is sufficient to block the cell cycle in vivo, suggesting that it is a regulatory molecule rather than simply a marker of non-dividing cells. Subsequently, we showed that *XBF-1* affects the expression of $p27^{XIC1}$ in a dose-dependent manner; a high dose suppresses $p27^{XIC1}$ and a low dose induces it ectopically. The causal link between *XBF-1* and $p27^{XIC1}$ is further strengthened by two additional observations. First, $p27^{XIC1}$ is expressed in the anterior neural plate, close to the normal *XBF-1* expression domain. The two domains show only small partial overlap, consistent with the idea that *XBF-1* may have a repressing as well as an activating effect on $p27^{XIC1}$. Second, *XBF-1* can suppress $p27^{XIC1}$ in the absence of protein synthesis, suggesting that this interaction may be direct and likely to occur at the transcriptional level. Taken together, these findings suggest that high and low doses of *XBF-1* enhance or block cell division by suppressing or activating $p27^{XIC1}$, respectively. Our interpretations are based on misexpression experiments but are consistent with the pattern of expression of *XBF-1* in relation to other genes during normal development (e.g. *XSox3*, *X-ngnr-1*, *p27Xic*; see Results).

Our findings are also interesting from the point of view of $p27^{XIC1}$ regulation. Indeed, while there is a large body of evidence that the mammalian relative of $p27^{XIC1}$, $p27^{Kip1}$, is post-transcriptionally regulated (see, for example, Carrano et al., 1999; Sutterluty et al., 1999; Tomoda et al., 1999 and refs therein; reviewed in Amati and Valch, 1999), much less is known about the possible transcriptional regulation of $p27^{Kip1}$ (Kolluri et al., 1999). Our data show that, in a developmental context, at least part of the $p27^{XIC1}$ regulation occurs at the mRNA level, and suggest that *XBF-1* is one of the upstream transcription factors that can exert a direct effect on $p27^{XIC1}$. Interestingly, the promoter region of the human p27Kip1 gene (Ito et al., 1999) contains an exact match to the optimal binding site of the chicken *XBF-1* homologue, Qin, as defined by PCR selection (Li et al., 1997).

The effects of *XBF-1* on cell division and differentiation or cell-fate specification are distinct

Taken together with our previous work, our findings suggest that cells that express a high dose of *XBF-1* divide and do not differentiate while cells that express a low dose stop dividing and differentiate. An important question that arises from these findings is whether the effects of *XBF-1* on cell-fate specification, differentiation and cell division are distinct. Of particular interest is the potential link between cell differentiation and division because in normal development most cells stop dividing before they differentiate. Therefore, it was formally possible that the effects of *XBF-1* on differentiation were merely a consequence of its effect on the cell cycle. In other words, cells would differentiate or not, merely as a consequence of preventing progression through, or preventing exit from the cell cycle, respectively. In turn, this would imply that all of the effects of *XBF-1* are mediated by $p27^{XIC1}$. However, our findings argue against this scenario: blocking cell division by $p27^{XIC1}$ overexpression does not induce additional *N-tubulin* inside the neural plate, suggesting that stopping the division of neuroectodermal cells is not sufficient to turn them into *N-tubulin* positive cells. Blocking

cell division by *p27^{XIC1}* overexpression does not induce ectopic *N-tubulin* or *XSox3* outside the neural plate either, suggesting that stopping ectodermal cells dividing does not turn them into either neural or neuronal cells. Since *XBF-1* is very effective in inducing expanded/ectopic *XSox3* or *N-tubulin* (depending on the dose) these findings suggest that the effects of *XBF-1* on proliferation are distinct from effects on cell-fate specification or differentiation, the former only being mediated by *p27^{XIC1}*. One caveat of our proposal is that although we have shown that the injected *p27^{XIC1}*is active in stopping cells dividing, we cannot formally exclude the possibility that it is not fully functionally equivalent with the *p27^{XIC1}*that is induced by *XBF-1*. In other words, *XBF-1* may induce *p27^{XIC1} and* a gene X that may modify the activity of *p27^{XIC1}* such that it would acquire the property to effect cell fate. However, we note that this scenario would not invalidate our basic conclusion, which is that the effects of *XBF-1* include, but are not limited to, the regulation of *p27^{XIC1}* expression.

Synergism between *XSox3* and *XBF-1* may determine the effective dose of *XBF-1*

One striking feature of the activity of *XBF-1* is its dose-dependence. In our experimental set up, different doses of *XBF-1* are generated by varying the quantity of the injected in vitro transcribed RNA by two- to fivefold (see Materials and Methods). However, it is not clear whether the dose of *XBF-1* in vivo is regulated at the transcriptional or post-transcriptional level. An alternative possibility is that the dose of *XBF-1* is modulated in vivo by interactions with other factors. In support of this possibility we have shown that coinjection of *XBF-1* and *XSox3* potentiates the effect of *XBF-1* and converts a low-dose phenotype to a high-dose one. While the molecular basis of the synergism between *XSox3* and *XBF-1* warrants further investigation, this finding suggests that, in vivo, co-operative interactions rather than, or in addition to, RNA or protein distribution may be important for determining the effective dose of a transcription factor. According to this model, the high-dose effect of *XBF-1* is determined by the presence of *XSox3* while a low-dose effect of *XBF-1* could be determined either by the absence of *XSox3* or by interaction with other factors that lower the activity of *XBF-1*. An interesting twist to this model is that the expression of *XSox3* is upregulated by *XBF-1* itself, but only by a high, not a low, dose of *XBF-1* (Bourguignon et al., 1998; this work). Thus, it is equally important to identify factors that lower the effective dose of *XBF-1*. In relation to this, we note that AFX, another member of the forkhead family, is negatively regulated by post-transcriptional modification (Kops et al., 1999).

XBF-1 in the context of other genes

Several neural specification (i.e. proneural) genes and a related *Xenopus* winged-helix gene, *XBF-2*, have been shown to enlarge the neural plate (Turner and Weintaub, 1994; Nakata et al., 1997; Ravassard et al., 1997; Takebayashi et al., 1997; Bellefroid et al., 1998; Kroll et al., 1998; Mariani and Harland, 1998; Gomez-Skarmeta et al., 1999). In a few cases the ability of proneural genes to enlarge the neural plate in the absence of cell division (blocked with HUA) has been tested. In these cases, the results have been interpreted as suggesting that the neural plate enlargement is primarily the result of extra neuralisation (e.g. Turner and Weintaub, 1994; Takebayashi et al., 1997; Bellefroid et al., 1998; Kroll et al., 1998; see also Coffman et al., 1990). However, the effect on cell division or on genes that regulate the cell cycle has not been examined in the case of any of the above proneural genes. Therefore, we do not know how widespread is *XBF-1*'s dual property of regulating neural cell-fate specification and cell division among proneural genes. However, given the recent isolation of a patterning gene, *Optx2*, that can affect cell division without affecting cell fate (Zuber et al., 1999), we suspect that *XBF-1* may be part of only a small number of regulatory molecules that can affect both processes. A homeobox containing gene, *Xrx1*, is also thought to have both a proliferative and anterior patterning activity (Andreazzoli et al., 1999) but again the effect on the cell cycle has not been directly examined. *Geminin*, a recently isolated novel gene, is interesting since it is thought to be involved both in inhibiting DNA replication (McGarry and Kirschner, 1998) and in specifying a neural cell fate (Kroll et al., 1998), albeit in different systems.

Finally, it may appear surprising that a single gene can affect so many processes. However, the processes of cell-fate specification, cell division and differentiation must be co-ordinated in vivo, so one would expect the existence of regulatory molecules that lie in the cross-roads of these pathways. Our findings argue that *XBF-1* is such a key regulator; the challenge for the future is to understand how these diverse effects are integrated.

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