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# The cdk inhibitor p27$^{Xic1}$ is required for differentiation of primary neurones in *Xenopus*

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## SUMMARY

**We have investigated the role of the cyclin-dependent kinase inhibitor, p27$^{Xic1}$, in the coordination of cell cycle exit and differentiation during early neurogenesis. We demonstrate that p27$^{Xic1}$ is highly expressed in cells destined to become primary neurones and is essential for an early stage of neurogenesis. Ablation of p27$^{Xic1}$ protein prevents differentiation of primary neurones, while overexpressing p27$^{Xic1}$ promotes their formation. p27$^{Xic1}$ may enhance neurogenesis by stabilising the bHLH protein, neurogenin. Moreover, the ability of p27$^{Xic1}$ to stabilise neurogenin and enhance neurogenesis localises to an N-terminal domain of the molecule and is separable from its ability to inhibit the cell cycle.**

Key words: Cell cycle, Cdk inhibitor, Neurone, Differentiation, p27$^{Xic1}$, *Xenopus*

## INTRODUCTION

Neural development is a complex process requiring precise coordination between proliferation and differentiation. During gastrulation in *Xenopus* embryos, a selection of initially undifferentiated, mitotically active cells located within the neural plate withdraw from the cell cycle and generate a defined pattern of primary neurones (Hartenstein, 1989). Cyclin-dependent kinase inhibitors (cdkis) are expressed in many cell types, including cells of the central nervous system (Yan et al., 1997; Durand and Raff, 2000), and may function in their differentiation as well as in cell cycle withdrawal (Dyer and Cepko, 2000; Zezula et al., 2001). Recent experiments in the *Xenopus* retina have shown that the cdki, p27$^{Xic1}$, biases determined neuroblast precursors toward a glial cell fate and this function is distinct from its regulation of the cell cycle (Ohnuma et al., 1999). We wished to determine the role of p27$^{Xic1}$ in cell fate determination and differentiation of primary neurones, the first neural cell type to differentiate from the neural plate.

Mammals express three members of the Cip/Kip family of cdkis, p21$^{Cip1}$, p27$^{Kip1}$ and p57$^{Kip2}$ (Sherr and Roberts, 1999). Although highly expressed in many neural tissues, redundancy and inaccessibility has complicated analysis of cdki function in nervous system development in null mouse models (Deng et al., 1995; Nakayama et al., 1996; Yan et al., 1997). As a result, a clear role for cdkis in neural differentiation has not been demonstrated. One major advantage of the *Xenopus* system is that there is only one described cdki, p27$^{Xic1}$, which shows structural and functional characteristics of p21$^{Cip1}$, p27$^{Kip1}$ and p57$^{Kip2}$ (Su et al., 1995; Shou and Dunphy, 1996).

Primary neurone differentiation requires the sequential activation of proneural basic helix-loop-helix (bHLH) transcription factors such as neurogenin (X-NGNR-1) (Ma et al., 1996), Xash3 (Zimmerman et al., 1993) and NeuroD (Lee et al., 1995), the roles of which have been likened to those of MyoD, Myf5 and myogenin during muscle differentiation. Although bHLH genes coordinate cell cycle exit and differentiation in muscle by upregulating cdkis (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995), no such relationship has been demonstrated in neural tissue.

We present the first clear in vivo evidence that a cdki has an essential role in the decision to adopt a neural fate. We show that p27$^{Xic1}$ is highly expressed in cells destined to become primary neurones, and that it is required for primary neurogenesis at a crucial step between X-NGNR-1 and NeuroD. Moreover, p27$^{Xic1}$ overexpression promotes ectopic neurone formation, while stabilising the proneural gene, X-NGNR-1.

## MATERIALS AND METHODS

### *Xenopus* embryos, fixation and β-galactosidase staining

*Xenopus laevis* embryos were obtained by standard methods and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Embryo fixation and β-galactosidase staining (200-300 pg injected per embryo) was performed as described (Sive et al., 2000).

### mRNA injection and morpholino antisense oligonucleotides

Capped RNAs were synthesised in vitro from nuc-β-gal and p27$^{Xic1}$ (Su et al., 1995); p27$^{Xic1}$ NT, p27$^{Xic1}$ CT, p27$^{Xic1}$ 35-96 and p21$^{Cip1}$

N50S (Ohnuma et al., 1999); X-NGNR-1 (Ma et al., 1996); NeuroD (Lee et al., 1995); $p21^{Cip1}$ (Harper et al., 1993); XMyT-1 (Bellefroid et al., 1996); Xath3 (Takebayashi et al., 1997); Xash3 (Zimmerman et al., 1993); and $p21^{Cip1}$ NT, $p21^{Cip1}$ CT, and $p21^{Cip1}$ 20-82, using the SP6 Message Machine kit (Ambion). Embryos are acutely sensitive to levels of $p27^{Xic1}$ RNA, so each new RNA batch must be carefully titrated for effect. The antisense morpholino oligodeoxynucleotide used was: 5′-GCAGGGCGATGTGGAAA-GCAGCCAT-3′ (Gene Tools LLC).

### Whole-mount in situ hybridisation, BrdU detection and antibody staining

Whole-mount in situ hybridisation was performed as described (Shimamura et al., 1994). Linearised Bluescript plasmid from X-NGNR-1 (BamH1/T7), NeuroD (Xba1/T7), Nβtub (BamH1/T3) or pCS2 XMyT-1 (BamH1/T7) and $p27^{Xic1}$ (BamH1/T7) was used to generate digoxigenin-11-UTP-labeled antisense RNA probes. BM Purple was used as a substrate. Double in situ hybridisation was performed as described (Sive et al., 2000) using BCIP (light blue) and NBT/BCIP (purple) as colour substrates. For 60-70 μm sections, specimens were post-fixed in MEMFA, embedded in 3% low melting point agarose and sectioned by Leica VT1000M vibratome, mounted in 100% glycerol and photographed with Nomarski optics. BrdU analysis was performed as described by Hardcastle and Papalopulu (Hardcastle and Papalopulu, 2000). Whole-mount antibody staining was performed as described previously (Sive et al., 2000) using anti-phospho-histone H3 (TCS Biologicals).

### Western blotting

$p27^{Xic1}$ was detected using a polyclonal antibody (Ohnuma et al., 1999). Blots were stripped (Chemicon International) and probed with anti-β-tubulin antibody (1:400) (Santa Cruz Biotechnology).

## RESULTS

### $p27^{Xic1}$ expression in the early embryo

$p27^{Xic1}$ expression has previously been shown in differentiating muscle and notochord at neurula stages and at lower levels in the neural plate (Ohnuma et al., 1999; Hardcastle and Papalopulu, 2000). To investigate the specific relationship between primary neurogenesis and $p27^{Xic1}$, we more carefully analysed $p27^{Xic1}$ expression during development using whole-mount in situ hybridisation.

$p27^{Xic1}$ is strongly expressed at stage 10.5-11, significantly before neural differentiation, in three stripes lateral to the midline in the prospective neural plate (data not shown), reminiscent of expression of genes involved in primary neurogenesis (Bellefroid et al., 1996; Ma et al., 1996). To examine this resemblance more closely, we have compared $p27^{Xic1}$ expression with that of XMyT-1 (Fig. 1) (Bellefroid et al., 1996).

XMyT-1, a zinc-finger transcription factor, is one of the earliest markers of primary neurogenesis. XMyT-1 staining is first detected at stage 11.5 in cells destined to differentiate into lateral, intermediate and medial primary neurones (Fig. 1D) (Bellefroid et al., 1996) and corresponds to the stripes of stronger $p27^{Xic1}$ expression (Fig. 1A). Overlapping $p27^{Xic1}$ and XMyT-1 staining can be seen at stage 13 by comparing single in situ hybridisations (compare Fig. 1B with 1E) and examining double in situ hybridisations (Fig. 1H). At this stage, $p27^{Xic1}$ placodal expression is clear (Fig. 1B, arrows), but the medial neural stripe of $p27^{Xic1}$ staining is obscured by stronger stain in the underlying myotome. $p27^{Xic1}$ expression is also apparent in the epidermis outside the neural plate between stages 10.5 and 15 (Fig. 1G). By stage 15, $p27^{Xic1}$ expression in the medial and intermediate stripes is completely obscured by staining in the underlying myotome. However, $p27^{Xic1}$ staining is still clear in the lateral primary neurone stripes (Fig. 1C) and placodal regions (Fig. 1C, arrows), and closely resembles the expression of XMyT-1, X-NGNR-1 and X-Delta-1 (Fig. 1F and data not shown) (Bellefroid et al., 1996; Ma et al., 1996). Moreover, $p27^{Xic1}$ expression is clearly seen in the primary neurone precursors in the sensorial layer of the neuroectoderm, both overlying and lateral to the staining in the myotome and notochord (Fig. 1I) (Hardcastle and Papalopulu, 2000). $p27^{Xic1}$ is not uniform in the neural plate at stage 15;

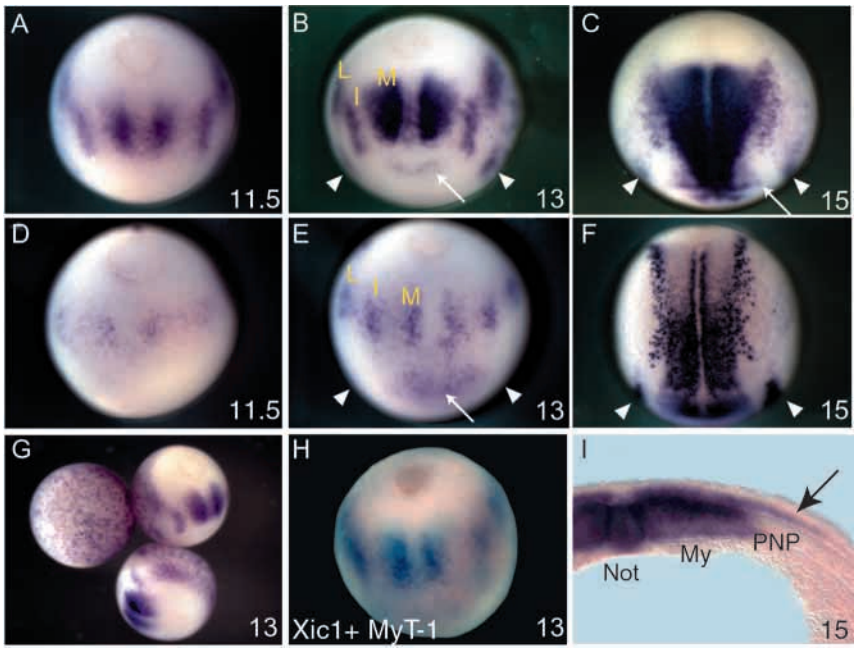


**Fig. 1.** $p27^{Xic1}$ is expressed in cells destined to become primary neurones. Whole-mount in situ hybridisation at indicated stages show expression of $p27^{Xic1}$ (A-C,G,I) or XMyT-1 (D-F,H). Except for G, figures show a dorsal view with anterior towards the bottom. Both $p27^{Xic1}$ and XMyT-1 are found in lateral, intermediate and medial stripes of primary neurones, placodes (arrows) and the trigeminal ganglia (arrowheads). (G) Stage 13 embryos show $p27^{Xic1}$ staining in the epidermis. (H) Double in situ hybridisation demonstrates overlap between $p27^{Xic1}$ (purple) and XMyT-1 (light blue). (I) Vibratome section of a stage 15 embryo shows $p27^{Xic1}$ expression in the myotome (My), notochord (Not) and primary neurone precursors (PNP) in the sensorial layer of the neural plate (arrow).

rather, its scattered distribution is similar to XMyT-1 and is reminiscent of proneural genes whose expression is restricted by Notch-mediated lateral inhibition (Fig. 1C).

In cultured myoblasts, the bHLH factor MyoD can transcriptionally upregulate expression of both muscle structural genes and the cdki, p21Cip1, thus coordinating cell cycle exit and differentiation (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). Analogous bHLH factors such as X-NGNR-1 and Xash3 function during neurogenesis. However, X-NGNR-1, Xash3 and XMyT-1 alone or in combination are unable to upregulate p27Xic1 expression either in whole embryos or in ectodermal explants (data not shown). Nonetheless, p27Xic1 is probably regulated in vivo by an unknown bHLH transcription factor or combination of factors, as p27Xic1 transcription increases on injection of XE12, a bHLH co-factor, and is inhibited in the presence of Id, a bHLH repressor (data not shown) (Lassar et al., 1991; Jen et al., 1992).

Thus, p27Xic1 is properly spatially and temporally expressed for involvement in primary neurogenesis. Interestingly, p27Xic1 expression precedes substantial proneural gene expression (Zimmerman et al., 1993; Lee et al., 1995; Ma et al., 1996) but coincides with the time that Rohon-Beard cells (primary sensory neurones) begin to exit the cell cycle (Lamborghini, 1980).

### p27Xic1 is necessary for neural differentiation

To investigate whether p27Xic1 is necessary for the differentiation of primary neurones, we used antisense morpholino oligonucleotides (Mo) to prevent translation of p27Xic1 message (Heasman et al., 2000). Injection of p27Xic1 Mo at the two-cell stage prevents the accumulation of p27Xic1 protein that normally occurs after the MBT (Shou and Dunphy, 1996), while injection of a control morpholino (Con Mo) has no effect (Fig. 2A).

As predicted, ablation of p27Xic1 can increase cell proliferation. Embryos injected with 10 ng p27Xic1 Mo have a 1.27-fold increase in mitotic cells expressing phosphorylated histone H3 (ph3), compared with the uninjected side ($n$=35, $P$=0.02, Student's $t$-test), as well as a small increase in BrdU incorporation (data not shown). The Con Mo had no effect on ph3 or BrdU (data not shown).

Embryos were assayed for primary neurone formation at stage 15 by staining for X-NGNR-1. X-NGNR-1 is the first proneural gene to be expressed in the neural plate, and specifies the formation of primary neurones (Ma et al., 1996). The Con Mo had no effect on any of the neural markers tested (Fig. 2B; data not shown). p27Xic1 Mo injection had very little effect on expression of X-NGNR-1 at stage 15 (no change in 91% of embryos, $n$=64), demonstrating that p27Xic1 is not required for this initial specification event (Fig. 2C). However, p27Xic1 Mo, but not Con Mo, reduced expression of XMyT-1, which is downstream of, and expressed shortly after X-NGNR-1 (Fig. 2D). NeuroD, a direct downstream target of X-NGNR-1, is also reduced in the absence of p27Xic1 (Fig. 2E) (Huang et al., 2000), demonstrating a role for p27Xic1 during early differentiation, but after specification. Interestingly, at stage 18, p27Xic1 Mo somewhat reduces expression of X-NGNR-1 in 77% of embryos ($n$=57), indicating that p27Xic1 may be required for X-NGNR-1 maintenance (data not shown). Terminal differentiation of primary neurones was

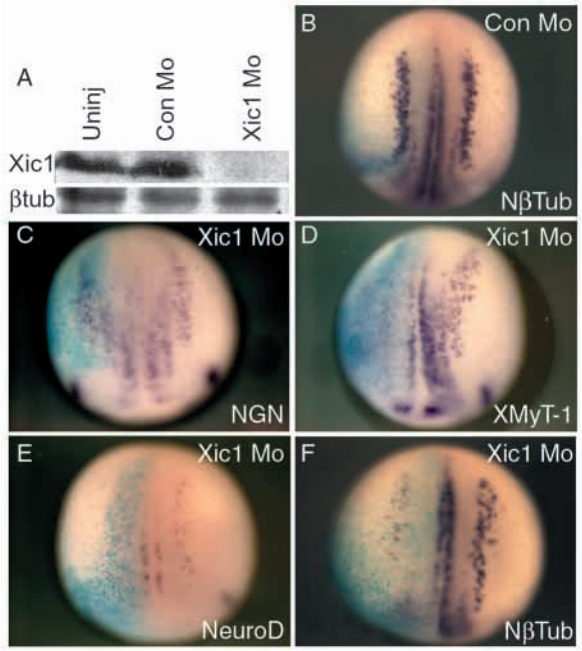


**Fig. 2.** Embryos depleted of p27Xic1 protein fail to produce differentiated primary neurones. (A) Western blot for endogenous p27Xic1 protein levels on stage 18 embryos that were injected with 20 ng Con Mo or p27Xic1 Mo. Cytoskeletal-β-tubulin demonstrates equal loading. Con Mo injection has no effect on any of the neural markers examined (B, data not shown). (C-F) p27Xic1 Mo-injected embryos were analysed for expression of X-NGNR-1, XMyT-1, NeuroD and Nβtub (purple) by whole-mount in situ hybridisation, injected side towards the left (β-gal, light blue). Injection of 20 ng p27Xic1 Mo has no effect on X-NGNR-1 (C) but ablates XMyT-1 (D), NeuroD (E) and Nβtub (F) expression.

examined by staining for Nβtub, a marker of differentiated neurones. 96% ($n$=136) of p27Xic1 Mo-injected embryos showed significant downregulation or absence of Nβtub on the injected side (Fig. 2F) that persisted until tailbud stages (data not shown).

p27Xic1 and a mammalian homologue, p21Cip1, share the ability to induce Müller glial cells at the expense of neural derivatives in the *Xenopus* retina, indicating that they may play similar roles both in cell cycle regulation and cell fate determination (Ohnuma et al., 1999). Using this functional homology, we investigated whether p21Cip1 could rescue the loss of primary neurones observed upon p27Xic1 Mo injection. In one typical experiment, after injection of p27Xic1 Mo, 55% of embryos ($n$=29) had no Nβtub-expressing primary neurones on the injected side (Fig. 3A). The remaining 45% of embryos had substantially reduced primary neurone numbers. Neurones in the lateral stripe were counted in the embryos with reduced Nβtub expression, and they displayed an average of 22 neurones on the injected side compared with an average of 45 on the uninjected side ($P$<0.001). After co-injection with p21Cip1, 100% of embryos showed some Nβtub-expressing primary neurones ($n$=29) with an average number of 44 neurones on the injected side compared with 45 on the uninjected side (Fig. 3B) ($P$<0.001). Thus, depletion of p27Xic1 protein by Mo injection prevents primary neurone formation. This effect is specific to the loss of cdki function because

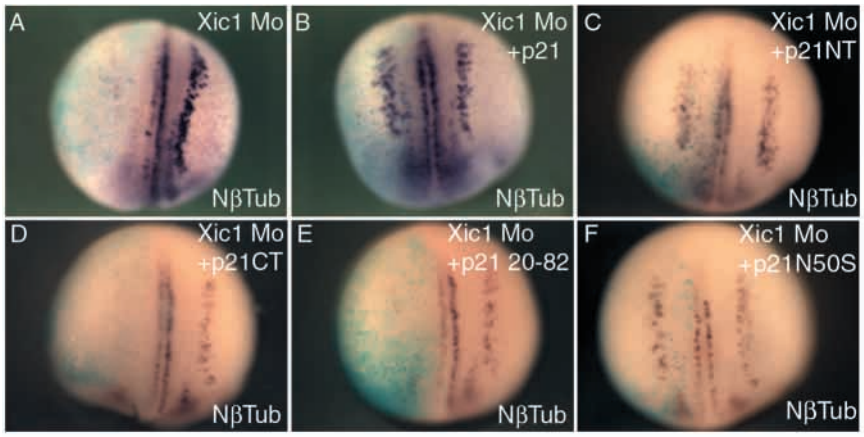


**Fig. 3.** $p27^{Xic1}$ promotes primary neurone differentiation independently of its cell cycle role. Nβtub expression abolished by $p27^{Xic1}$ Mo (A) is rescued by co-injection of $p21^{Cip1}$ (B). Embryos were co-injected with $p27^{Xic1}$ Mo and $p21^{Cip1}$ NT (C), $p21^{Cip1}$ CT (D), $p21^{Cip1}$ 20-82 (E) or $p21^{Cip1}$ N50S (F). Co-injection with $p21^{Cip1}$ NT (C) and or $p21^{Cip1}$ N50S (F) rescued Nβtub, while $p21^{Cip1}$ CT (D) and $p21^{Cip1}$ 20-82 (E) had no effect.

neurone formation can be rescued by co-injection of the mammalian homologue $p21^{Cip1}$.

We wished to determine whether the ability of $p21^{Cip1}$ to rescue primary neurogenesis in the absence of $p27^{Xic1}$ was solely related to its ability to arrest the cell cycle, or whether $p21^{Cip1}$ plays an additional role in determining primary neural cell fate. To address this question we used N- and C-terminal constructs of $p21^{Cip1}$ [amino acids 1-89 ($p21^{Cip1}$ NT) and 87-164 ($p21^{Cip1}$ CT)], which arrest the cell cycle in distinct ways. $p21^{Cip1}$ NT blocks cyclin/cdk kinase activity while $p21^{Cip1}$ CT binds and inactivates PCNA (Chen et al., 1995). We assayed the ability of these constructs to downregulate proliferation and compared this with their ability to rescue $p27^{Xic1}$ Mo-induced primary neurone loss. Injection of 20 pg full-length $p21^{Cip1}$ RNA substantially downregulates BrdU incorporation (data not shown) and rescues primary neurones (Fig. 3B). Although similar amounts of NT and CT construct RNAs (10 and 50 pg, respectively) greatly reduce or eliminate BrdU incorporation at this stage (data not shown), only expression of $p21^{Cip1}$ NT rescues primary neurogenesis (68% of embryos, *n*=38) (compare Fig. 3C with 3D). These data demonstrate that cell cycle arrest alone is insufficient to promote primary neurone formation, but rather some further function of the N terminus of $p21^{Cip1}$ is required.

Ohnuma et al. (Ohnuma et al., 1999) have shown that a mutant of $p27^{Xic1}$ (amino acids 35-96) blocks overall cdk2 kinase activity but cannot induce Müller glial cells, indicating a distinct, non-cell-cycle requirement for the $p27^{Xic1}$ NT region upstream of amino acid 35. To investigate whether a homologous deletion of $p21^{Cip1}$ can rescue primary neurones, we co-injected the mutant $p21^{Cip1}$ 20-82 with $p27^{Xic1}$ Mo. Although 50 pg of $p21^{Cip1}$ 20-82 was able to arrest the cell cycle by inhibiting overall cdk2 kinase (data not shown), it did not rescue primary neurogenesis (Fig. 3E). Thus, a separable differentiation function of $p21^{Cip1}$ is required to induce primary neurones.

To determine whether inhibition of cdk kinase activity is necessary for rescue, we injected the $p21^{Cip1}$ N50S point mutant, previously shown to have a reduced capacity for cdk2 inhibition (Ohnuma et al., 1999). $p21^{Cip1}$ N50S rescued the loss of primary neurones caused by injection of $p27^{Xic1}$ Mo in 66% of embryos (*n*=45, Fig. 3F). These data again show that the ability to promote primary neurogenesis is separable from the ability to inhibit overall cdk2 kinase activity.

## $p27^{Xic1}$ is required between X-NGNR-1 and NeuroD to promote neural fate determination

When overexpressed, X-NGNR-1 induces extensive ectopic neurogenesis on the flank of the embryo (Ma et al., 1996). Our in situ studies show that $p27^{Xic1}$ is expressed in the epidermis outside the neural plate at late gastrula and early neural plate stages (Fig. 1G) and is therefore available to cooperate with ectopic X-NGNR-1 to promote neurogenesis both inside and outside the neural plate. Thus, we investigated whether $p27^{Xic1}$ is required for X-NGNR-1-mediated primary neurone formation in the neural plate and embryonic skin. Embryos were injected with X-NGNR-1 and either Con Mo or $p27^{Xic1}$ Mo. While embryos co-injected with X-NGNR-1 and Con Mo show extensive ectopic neurogenesis in the epidermis (95%, *n*=37) (Fig. 4A), both endogenous and ectopic primary neurogenesis is substantially reduced or eliminated in the presence of $p27^{Xic1}$ Mo (100%, *n*=36) (Fig. 4B). The downregulation of Nβtub by $p27^{Xic1}$ Mo is rescued by co-injection of $p21^{Cip1}$ (97%, *n*=40) (Fig. 4C), again indicating that the effect of $p27^{Xic1}$ Mo on neurogenesis is specific to loss of cdki activity.

NeuroD, a bHLH factor that promotes terminal neural differentiation, lies downstream of X-NGNR-1 (Lee et al., 1995). To determine whether $p27^{Xic1}$ acts up- or downstream of NeuroD, we investigated Nβtub expression in response to NeuroD in the presence of Con Mo or $p27^{Xic1}$ Mo. As expected, NeuroD induced extensive ectopic neurogenesis in the presence of Con Mo (Fig. 4D). Strikingly, however, although $p27^{Xic1}$ Mo almost completely blocked Nβtub expression in 94% (*n*=70) of X-NGNR-1-injected embryos, NeuroD induced ectopic Nβtub in 82% (*n*=120) of embryos even in the presence of $p27^{Xic1}$ Mo (Fig. 4E). Therefore, $p27^{Xic1}$ is required at a crucial early step in neurogenesis and acts between X-NGNR-1 and NeuroD.

## $p27^{Xic1}$ promotes primary neurogenesis

We have shown that $p27^{Xic1}$ is necessary for primary neural differentiation. Next, we wished to determine whether $p27^{Xic1}$ expression is instructive for primary neurone cell fate determination and differentiation. Injection of high doses of $p27^{Xic1}$ message (250 pg and above) results in rapid cell cycle arrest and embryonic death, triggered by extensive apoptosis at early gastrula stages (Hardcastle and Papalopulu, 2000; Finkielstein et al., 2001). However, if lower doses are injected,

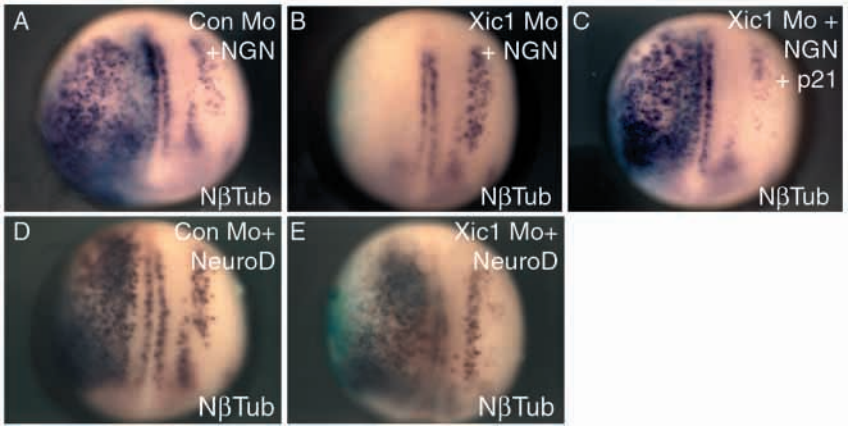


**Fig. 4.** p27Xic1 acts at an early stage of neurogenesis. Embryos co-injected with 50 pg X-NGNR-1 and 20 ng Con Mo (A) or p27Xic1 Mo (B) were analysed for Nβtub expression (purple) by whole-mount in situ hybridisation, injected side to the left (β-gal, light blue). Co-injection of X-NGNR-1 with Con Mo produces extensive ectopic neurones (A), while p27Xic1 Mo prevents both endogenous and ectopic primary neurogenesis (B). (C) Co-injection of p21Cip1 with X-NGNR-1 rescues downregulation of Nβtub expression by p27Xic1 Mo. Embryos were co-injected with 400 pg NeuroD and 20 ng Con Mo (D) or p27Xic1 Mo (E). NeuroD is able to induce extensive ectopic Nβtub even in the absence of p27Xic1 protein (E).

cells divide at a slower rate but apoptosis is greatly reduced or eliminated. After careful titration experiments, we chose to inject 30-60 pg p27Xic1 RNA, a dose that gives modest overexpression compared with endogenous levels (Fig. 5G). Injecting 30-60 pg of p27Xic1 into one cell of two-cell stage embryos causes, on average, 7% embryonic death. The surviving embryos appear healthy, although slowed cell division is evidenced by a 61% decrease in ph3 expression ($n$=10, $P$<0.001, Student's $t$-test) and a reduction of BrdU incorporation (81% of embryos, $n$=16) on the injected as compared with the uninjected side (Fig. 5A).

p27Xic1 overexpression upregulates the number of primary neurones on the injected side in the majority of embryos (67%, $n$=147) (Fig. 5B,C), but only within the primary neurone stripes in the neural plate. p27Xic1 does not lead to an appreciably earlier expression of Nβtub and, therefore, is unlikely to cause early birth of primary neurones (data not shown). Interestingly, ectopic p27Xic1 appears to induce neurones in the superficial layer of the neuroectoderm (Fig. 5C), which is usually refractory to primary neurogenesis (Chalmers et al., 2002). Expansion of primary neurogenesis in the neural plate is clear even in embryos where p27Xic1 RNA is overexpressed only in the ectoderm, as determined by β-gal tracer staining (Fig. 5C). Therefore, the ectopic induction of primary neurones by p27Xic1 is cell autonomous and not a secondary result of effects on the underlying myotome (Vernon and Philpott, 2003). To exclude the possibility that an increase in primary neurone cell size was responsible for the apparent increase in Nβtub expression, we used magnified images to count the number of Nβtub-positive cells in the lateral stripe on the injected versus the uninjected side. Of the 67% of embryos that showed an increase in the number of primary neurones, the injected side had, on average, 1.9 times as many neurones as the uninjected side ($P$=0.02, Student's $t$-test). This effect is dose dependent; at 30 pg, 39% of embryos had increased Nβtub expression, while 65% of embryos upregulated Nβtub at 60 pg.

To determine which region of p27Xic1 is required to promote ectopic primary neurones, we injected p27Xic1 NT (1-96), p27Xic1 CT (97-210) or p27Xic1 35-96. While the NT induced ectopic primary neurones (Fig. 4D) ($n$=123, average 1.7 fold increase, $P$<0.001), neither the CT nor 35-96 had any effect (Fig. 5E,F), again indicating that p27Xic1 has a separable N-terminal function required to promote primary neurogenesis that is distinct from its ability to inhibit overall cdk2 kinase activity or block the cell cycle.

In cultured muscle cell systems, the half-life of MyoD protein is extended by co-expression of p57Kip2 (Reynaud et

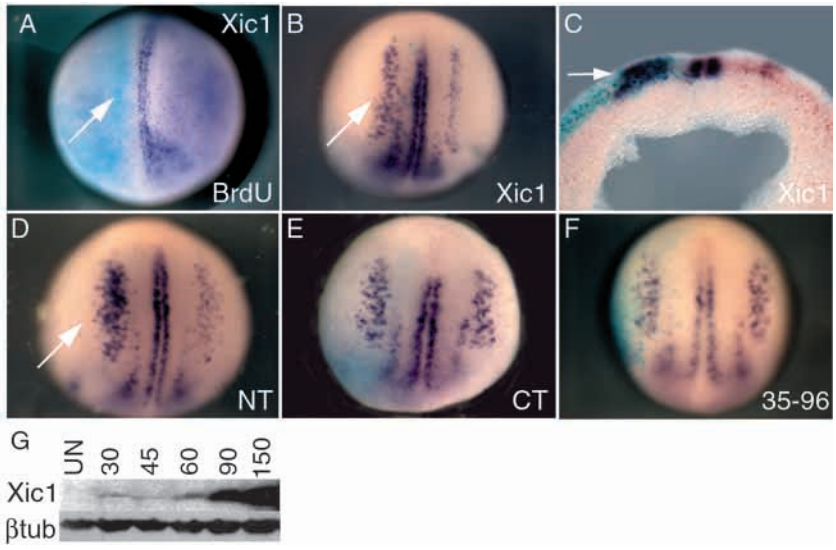


**Fig. 5.** Ectopic p27Xic1 promotes primary neurogenesis. Embryos were injected with (A) 45 pg p27Xic1 and βgal (light blue, injected side towards the left) and analysed at stage 15 for BrdU incorporation. Embryos were injected with (B) 60 pg p27Xic1, (D) 30 pg p27Xic1 NT, (E) 50 pg p27Xic1 CT or (F) 50 pg p27Xic1 35-96 with βgal and analysed at stage 15 for Nβtub expression (purple) by in situ hybridisation. Dorsal views show that p27Xic1 (B) and p27Xic1 NT (D) induce ectopic neurones within the neural plate (arrows), whereas p27Xic1 CT (E) and p27Xic1 35-96 (F) have no effect. (C) A section of a stage 15 embryo injected with 45 pg p27Xic1 indicating cell-autonomous Nβtub upregulation by p27Xic1. (G) Western blot for p27Xic1 levels on stage 10.5 embryos injected with increasing doses (30-150 pg) of p27Xic1 RNA.

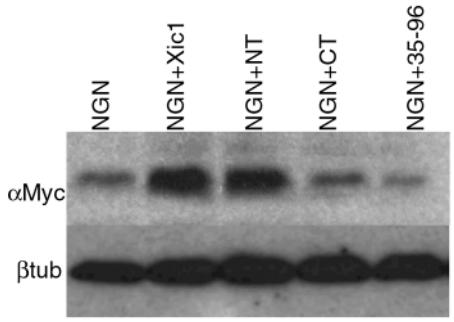


**Fig. 6.** $p27^{Xic1}$ stabilises X-NGNR-1 protein. Western blot for X-NGNR-1 levels on stage 20 embryos injected with X-NGNR-1 MT alone or in combination with $p27^{Xic1}$, $p27^{Xic1}$ NT, $p27^{Xic1}$ CT or $p27^{Xic1}$ 35-96. Cytoskeletal-β-tubulin demonstrates equal loading.

al., 1999). Originally, this stabilising property of $p57^{Kip2}$ was attributed to its inhibition of cdk-dependent phosphorylation of MyoD (Reynaud et al., 1999). However, a non-phosphorylatable form of MyoD ($MyoD^{Ala200}$) is also stabilised by co-expression of $p57^{Kip2}$ (Reynaud et al., 2000) and an N-terminal α-helix domain of $p57^{Kip2}$ is implicated in direct binding with the basic domain of MyoD and masking of potential degradation signals (Abu Hatoum et al., 1998). Because the processes of myogenesis and neurogenesis are highly analogous, we investigated the possibility that $p27^{Xic1}$ was promoting neurogenesis by stabilising X-NGNR-1 protein. We injected 50 pg of Myc-tagged X-NGNR-1 alone or in combination with 45 pg $p27^{Xic1}$, 30 pg NT, 50 pg CT or 50 pg 35-96 and performed western blots on stage 20 embryos. Co-injection of X-NGNR-1 with $p27^{Xic1}$ and $p27^{Xic1}$ NT significantly increased the amount of X-NGNR-1 protein persisting at this stage, while the CT and 35-96 had no effect (Fig. 6). However, neither native nor overexpressed $p27^{Xic1}$ immunoprecipitates with Myc-tagged X-NGNR-1 from embryo extracts (data not shown), indicating that $p27^{Xic1}$ may not stabilise X-NGNR-1 by direct binding. Therefore, $p27^{Xic1}$ both stabilises X-NGNR-1 protein and promotes neurogenesis in a manner that is distinct from its ability to inhibit cdk2 activity or arrest the cell cycle, but that may not involve direct binding. By analogy with MyoD, such mechanisms could involve regulation of DNA binding or nuclear localisation (Abu Hatoum et al., 1998; Floyd et al., 2001), but these possibilities remain to be investigated.

## DISCUSSION

$p27^{Xic1}$ is highly expressed in cells of the neural plate destined to form primary neurones (Fig. 1). Overexpression of $p27^{Xic1}$ promotes the formation of primary neurones (Fig. 5), while depletion of $p27^{Xic1}$ protein prevents neural differentiation (Fig. 2). Thus, $p27^{Xic1}$ is crucial for the specification and differentiation of neurones during early embryogenesis, and has a role distinct from its ability to inhibit overall cdk2 kinase activity or arrest the cell cycle. The discovery that $p27^{Xic1}$ is required downstream of X-NGNR-1 but upstream of NeuroD, suggests that this function occurs at the time of neural fate commitment, rather than during the final stages of neural differentiation. Indeed, terminal differentiation of primary neurones driven by NeuroD can occur in the absence of $p27^{Xic1}$ (Fig. 4). Furthermore, $p27^{Xic1}$ may promote primary neurogenesis via its ability to stabilise X-NGNR-1 protein (Fig. 6).

In the retina, $p27^{Xic1}$ regulates neural versus glial cell fate choice in addition to its role in cell cycle inhibition (Ohnuma et al., 1999). Cdkis have been previously implicated in the differentiation of neural derivatives in cultured cell systems such as oligodendrocytes and PC12 cells (Durand and Raff, 2000; Erhardt and Pittman, 1998). However, although highly expressed in many neural tissues, redundancy has complicated analysis of the roles of cdkis during development of the nervous system in null mouse models (Deng et al., 1995; Nakayama et al., 1996; Yan et al., 1997). Nevertheless, several lines of evidence indicate that mammalian cdkis may have analogous roles to $p27^{Xic1}$ during neurogenesis and gliogenesis. First, in the PC12 neural cell line, $p21^{Cip1}$ overexpression enhances differentiation in addition to arresting the cell cycle (Erhardt and Pittman, 1998). Secondly, mice null for $p27^{Kip1}$ have disrupted retinal cell differentiation and organisation, processes regulated by proneural gene function (Nakayama et al., 1996). Moreover, loss of $p57^{Kip2}$ causes inappropriate S-phase entry in the retina. Importantly, $p57^{Kip2}$ levels drop on cell cycle exit but it is re-expressed post-mitotically in a subset of amacrine neural precursor, and loss of $p57^{Kip2}$ leads to a significant increase in the calbindin amacrine cell subtype (Dyer and Cepko, 2000). These data indicate that $p57^{Kip2}$ is required for proper amacrine cell subpopulation distribution in vivo a process also regulated by proneural genes. Furthermore, Zezula et al. (Zezula et al., 2000) have demonstrated that $p21^{Cip1-/-}$ oligodendrocytes undergo prompt cell cycle withdrawal, but fail to differentiate. These results indicate that, in some contexts, cdkis may play a more general role in neural cell fate determination and differentiation, in addition to their ability to arrest the cell cycle. However, in contrast to the role of $p27^{Xic1}$ during primary neurone differentiation, Ohnuma et al. (Ohnuma et al., 2002) show that cdk kinase inhibition is essential to the ability of $p27^{Xic1}$ to synergise with proneural genes and promote early neural cell types in the retina.

The data presented here indicate that cell cycle exit is essential but insufficient for primary neurone differentiation. The NT and CT of $p27^{Xic1}$ both arrest the cell cycle (data not shown), but only the NT induces ectopic primary neurones (Fig. 5). Indeed, previous studies have shown that premature cell cycle arrest does not affect primary neurogenesis (Harris and Hartenstein, 1991; Kroll et al., 1998). Instead, our results suggest that a differentiation function of $p27^{Xic1}$, which is complementary to, but distinct from, its ability to arrest the cell cycle is required for primary neurone formation. An N-terminal mutant of $p27^{Xic1}$ that retains its ability to inhibit cdk2 kinase activity but is missing amino acids 31 to 35 is unable to induce extra primary neurones (Fig. 5F). Additionally, only the full NT of $p21^{Cip1}$ can rescue the $p27^{Xic1}$ Mo-induced loss of primary neurones (Fig. 3). Moreover, the $p21^{Cip1}$ N50S mutant, which has an impaired ability to inhibit cdk2 but contains an otherwise intact N-terminal region, is able to rescue the $p27^{Xic1}$ Mo phenotype (Fig. 3F).

Overexpression of $p27^{Xic1}$ results in the formation of ectopic neurones, but only within the neural plate (Fig. 5). $p27^{Xic1}$ does

not lead to an appreciable earlier expression of Nβtub and therefore is unlikely to cause early birth (data not shown). p27Xic1 doses higher than 75 pg did not enhance, and sometimes inhibited, primary neurone formation (data not shown), indicating acute sensitivity to p27Xic1 levels. This sensitivity may account for the difference between results presented here and those of Hardcastle and Papalopulu (Hardcastle and Papalopulu, 2000) who failed to detect ectopic neurones inside or outside of the neural plate on injecting 250pg of p27Xic1 message, a dose that induced 70% embryonic death. High doses of p27Xic1 may arrest neural plate cells before instructive proneural genes have accumulated or may result in selective death of the overexpressing cells. Indeed, work in keratinocytes has indicated that sustained overexpression of p21Cip1 at high levels may, in fact, inhibit differentiation (Di Cunto et al., 1998). However, our data clearly indicate that modest p27Xic1 overexpression promotes primary neurone formation within expanded proneural domains.

Full-length p27Xic1 and p27Xic1 NT stabilise X-NGNR-1 protein (Fig. 6) and promote primary neurogenesis (Fig. 5), although the molecular mechanism of this stabilisation is unknown. Initial co-immunoprecipitation studies indicate that p27Xic1 does not bind directly to overexpressed X-NGNR-1 (data not shown). Future investigations into the effects of p27Xic1 on X-NGNR-1 DNA binding, nuclear localisation and degradation may reveal alternative mechanisms for the observed upregulation of primary neurogenesis upon p27Xic1 overexpression.

In summary, the model presented in Fig. 7 proposes that p27Xic1 acts in parallel with X-NGNR-1 at an early stage of neurogenesis, and requires functions independent of its ability to arrest the cell cycle or inhibit overall cdk2 kinase activity. p27Xic1 may function in part by regulating X-NGNR-1 stability, and this is also independent of its ability to inhibit cdk2. By combining distinct cell cycle and differentiation functions, a single molecule, p27Xic1, may provide a powerful way to coordinate the processes of division and differentiation during primary neurogenesis.

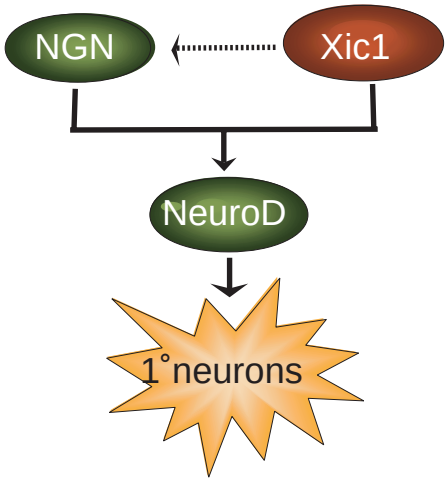


**Fig. 7.** Model demonstrating that p27Xic1 functions upstream of NeuroD, in parallel with X-NGNR-1, to stabilise X-NGNR-1 protein and promote primary neurone differentiation.

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