Development 130, 71-83

doi:10.1242/dev.00180

A single cdk inhibitor, p27^{Xic1}, functions beyond cell cycle regulation to promote muscle differentiation in *Xenopus*

Ann E. Vernon and Anna Philpott*

Department of Oncology, University of Cambridge, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK
*Author for correspondence (e-mail: ap113@hermes.cam.ac.uk)

Accepted 28 September 2002

SUMMARY

The molecular basis of the antagonism between cellular proliferation and differentiation is poorly understood. We have investigated the role of the cyclin-dependent kinase inhibitor p27^{Xic1} in the co-ordination of cell cycle exit and differentiation during early myogenesis in vivo using *Xenopus* embryos. In this report, we demonstrate that p27^{Xic1} is highly expressed in the developing myotome, that ablation of p27^{Xic1} protein prevents muscle differentiation and that p27^{Xic1} synergizes with the transcription factor MyoD to promote muscle differentiation. Furthermore, the ability of p27^{Xic1} to promote myogenesis resides in an N-terminal domain and is separable from its cell cycle regulation function. This data demonstrates that a single cyclin-dependent kinase inhibitor, p27^{Xic1}, controls in vivo muscle differentiation in *Xenopus* and that regulation of this process by p27^{Xic1} requires activities beyond cell cycle inhibition.

Key words: Cell cycle, Cdk inhibitor, Muscle, Xenopus

INTRODUCTION

The decision to divide or differentiate is determined by a fine balance of opposing developmental signals. During differentiation, multi-potential cells initiate genetic programs that commit them to progressively restricted lineages. In muscle, the cascade of myogenic helix-loop-helix (mHLH) proteins, which comprises MyoD, Myf5, myogenin and MRF4, signals cells to undergo myogenic commitment, to express muscle-structural genes and, finally, to become functional myofibers. To terminally differentiate, cells must not only receive the appropriate differentiation cues, but must also exit the cell cycle. While the molecules and mechanisms involved in early differentiation of muscle are relatively well understood, the link between promoters of terminal differentiation and regulators of cell cycle arrest has been extensively studied primarily in tissue culture systems.

Cell cycle arrest can be mediated through the inactivation of cyclin-dependent kinases (cdks) by cdk inhibitors (cdkis), of which there are two families. The p16^{Ink4} family (p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c} and p19^{Ink4d}) specifically inhibits cdk4 and cdk6, while the p21^{Cip1} family (p21^{Cip1}, p57^{Kip2} and p27^{Kip1}) inhibits all cdks involved in the G1/S transition (reviewed by Sherr and Roberts, 1999). In myogenic tissue culture systems, the link between proliferation and differentiation has been proposed to be via MyoD and p21^{Cip1}. Upon serum withdrawal, 10T1/2 cells transfected with MyoD can transcriptionally upregulate p21^{Cip1} expression, causing cell cycle arrest and subsequent myotube fusion (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995).

This straightforward in vitro model has not been supported by the creation of mice with homozygous deletions for p21^{Cip1} (Deng et al., 1995). Mice that lack p21^{Cip1} develop normally, with no signs of defective muscle differentiation (Deng et al., 1995). Additionally, p21^{Cip1} expression in myogenic cells of mice lacking the genes encoding MyoD and myogenin is normal, indicating that p21^{Cip1} expression does not require these mHLH factors (Parker et al., 1995; Sabourin et al., 1999). Nevertheless, mice lacking both p21^{Cip1} and a second cdki, p57^{Kip2}, fail to form myotubes and display increased proliferation and apoptosis of myoblasts, demonstrating that p21^{Cip1} and p57^{Kip2} redundantly control differentiation of mouse skeletal muscle (Zhang et al., 1999). Therefore, while confirming their importance and providing useful insight into their in vivo roles during muscle differentiation, redundancy makes information about the mode of action of cdkis from knockout mouse models difficult to interpret. In contrast to the redundancy observed in cdkis in mice, there is only one known cdki in *Xenopus*, p27^{Xic1}, and it exhibits structural and functional characteristics of all three p21^{Cip1} family members (Su et al., 1995; Shou and Dunphy, 1996).

Recent reports have shown that p27^{Xic1}, p21^{Cip1} and p57^{Kip2} have a role in neural/glial cell fate determination that is distinct and separable from their regulation of the cell cycle (Ohnuma et al., 1999; Dyer and Cepko, 2000; Zezula et al., 2001). Previous work has demonstrated that the capacity of p27^{Xic1} to bias committed neuroblasts towards a glial fate lies in a distinct region of the N terminus of the molecule but is independent of cell cycle exit and overall cdk2 kinase inhibition (Ohnuma et al., 1999). These findings indicate the possibility of a

differentiation function for $p27^{Xic1}$ in myogenesis, which is distinct from its regulation of the cell cycle.

We show that $p27^{Xic1}$ is first expressed in the embryonic myotome at stage 11, after MyoD expression, but prior to muscle structural gene expression. Its early myotomal expression makes $p27^{Xic1}$ a prime candidate molecule for coordinating cell cycle exit and myogenic differentiation. Consequently, we have used the many advantages of the *Xenopus* system to study the role of cdkis during in vivo muscle differentiation and cell cycle exit during the early stages of embryogenesis.

In this report we demonstrate that, whereas initial expression of MyoD is uniform, cell cycle exit in the myotome occurs in a wave from the front to the back of the embryo. Although MyoD does not cause cell cycle arrest by upregulating $p27^{Xic1}$ in vivo, $p27^{Xic1}$ does synergize with MyoD to promote muscle differentiation. Furthermore, the ability of $p27^{Xic1}$ to promote myogenesis is separable from its role in regulating the cell cycle. Finally, we provide evidence that $p27^{Xic1}$ is not needed for myogenic commitment, but is required for differentiation of muscle in *Xenopus*.

MATERIALS AND METHODS

Xenopus embryos, fixation and β-galactosidase staining

Xenopus laevis embryos obtained by hormone induced laying were in vitro fertilized, dejellied in 2% cysteine pH 7.8-8.0, washed and incubated in 0.1× MBS. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994), fixed and stained for β-galactosidase (200-300 pg injected per embryo) as described (Sive et al., 2000).

mRNA injection and morpholino antisense oligo procedures

Capped RNAs were synthesized in vitro from nuc-βgal (Chitnis et al., 1995), MyoDb, MyoD-enR (Wittenberger et al., 1999), $p27^{Xic1}$ (Su et al., 1995), $p27^{Xic1}$ NT, $p27^{Xic1}$ CT, $p27^{Xic1}$ NT 35-96 (Ohnuma et al., 1999), $p21^{Cip1}$ (Harper et al., 1993), using the SP6 Message Machine kit (Ambion). Embryos were injected in 0.2× MBS supplemented with 6% Ficoll.

The base composition of the $p27^{Xic1}$ antisense morpholino oligodeoxynucleotide is 5′-GCAGGGCGATGTGGAAAGCAGCCA-T-3′ (Gene Tools LLC). The control morpholino is a random sequence of the same length.

Explants and RT/PCR

Animal caps were dissected at stage 8 and ventral marginal zones were dissected at stage 10. Explants were incubated in 0.7×MBS until collection. RNA was isolated using RNAzolB (Tel-Test), oligo-dT primed and reverse transcribed into cDNA using standard methods. RT-PCR primers were calibrated to yield linear results that directly correlate template abundance and PCR amounts (Steinbach et al., 1998). The PCR primers used were: $p27^{Xic1}$, 5′-GTGGCACCCC-TCTTAAGGGC-3′ (forward) and 5′-TTCCAGTGGGCACAATAG-GT-3′ (reverse); MA (Stutz and Spohr, 1986), MHC, 5′-TTCAGCTGGAGTCTAAAC-3′ (forward) and 5′-TCTGTGGCATG-CTTCTCC-3′ (reverse) and ODC (Agius et al., 2000).

Immunohistochemistry

Monoclonal anti-sarcomeric actin (5C5) (Sigma) applied at a dilution of 1:500 for 2 hours at room temperature was recognized with either a goat anti-mouse IgM cy3 (1:800) (Jackson Immunoresearch) or a goat anti-mouse IgM-AP, using NBT/BCIP as color substrates. Tissue culture supernatant from the hybridoma D7F2 (anti-MyoD) was applied at a dilution of 1:4, recognized with a sheep anti-mouse Ig-AP secondary (1:150) (Jackson Immunoresearch) and amplified using an anti-AP antibody (APAAP complex, 1:50, Serotec). NBT/BCIP were used as color substrates. BrdU incorporation and detection was performed essentially as described in the Boehringer Mannheim instructions for the 5-bromo-2′-deoxy-uridine Labeling and Detection kit 1 (1296 736). Embryos were injected with 1 nmol of BrdU 2 hours before MEMFA fixation. Anti-BrdU (1:10) was applied to 10 μM paraffin wax embedded sections for 30 minutes at 37°C, washed and detected with an anti-mouse Ig-fluorescein (1:100) for 30 minutes at 37°C.

Whole mount in situ hybridization, antibody staining and TUNEL assay

Whole-mount in situ hybridization was performed as described (Shimamura et al., 1994). Linearized plasmid from MyoD (*Hin*dIII/T7), MA (*Hin*dIII/T7), MHC (*Nco*I/SP6) and $p27^{Xic1}$ (*Bam*H1/T7) was used to generate digoxigenin-11-UTP-labeled (Boehringer Mannheim) antisense RNA probes from the polymerases indicated. Double in situ hybridization was performed as described (Sive et al., 2000). BM Purple, NBT/BCIP and BCIP (Roche) were used as substrates.

Whole-mount antibody staining was performed as described (Sive et al., 2000) using an anti-phospho-histone H3 (TCS Biologicals) at 1:500 or an anti-muscle ATP-ase 12/101 (DSHB) at 1:400, and detected with an alkaline phosphatase-conjugated secondary using NBT/BCIP as substrates.

TUNEL staining was performed as described (Hensey and Gautier, 1998).

Western blotting

Protein extracts were prepared as described (Philpott and Friend, 1994). Total protein was separated by SDS-PAGE and western blotted to nitrocellulose by standard methods. $p27^{Xic1}$ was detected using a polyclonal antibody (Ohnuma et al., 1999). Antibody binding was detected using the Pierce SuperSignal chemiluminescence detection system. Blots were stripped (Chemicon International) and probed with anti-β-tubulin antibody (1:400) (Santa Cruz Biotechnology).

RESULTS

Pattern of cell cycle exit is directly related to myogenic differentiation

The highly ordered events in the process of skeletal myogenesis can be temporally separated in cultured cells (Andres and Walsh, 1996). The onset of C2C12 myoblast differentiation is signified by myogenin expression, followed by $p21^{Cip1}$ induction, cell cycle arrest, and, finally, cell fusion (Andres and Walsh, 1996). In *Xenopus*, a temporal cascade of the mHLH transcription factors MyoD, Myf5, and later, MRF4 regulate the progression of uncommitted, proliferating cells to determined myoblasts that exit the cell cycle and terminally differentiate into myotubes (Hopwood et al., 1989; Hopwood et al., 1991; Jennings, 1992). Myogenin is expressed only in the adult skeletal muscle of *Xenopus* (Jennings, 1992; Nicolas et al., 1998; Charbonnier et al., 2002). The initial broad and ubiquitous expression of MyoD is restricted to committed muscle cells (Harvey, 1990; Scales et al., 1990; Harvey, 1991; Rupp and Weintraub, 1991) shortly after the mid-blastula transition (MBT), when zygotic transcription begins (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). MyoD protein synthesis is synchronous across the future somite (Fig.

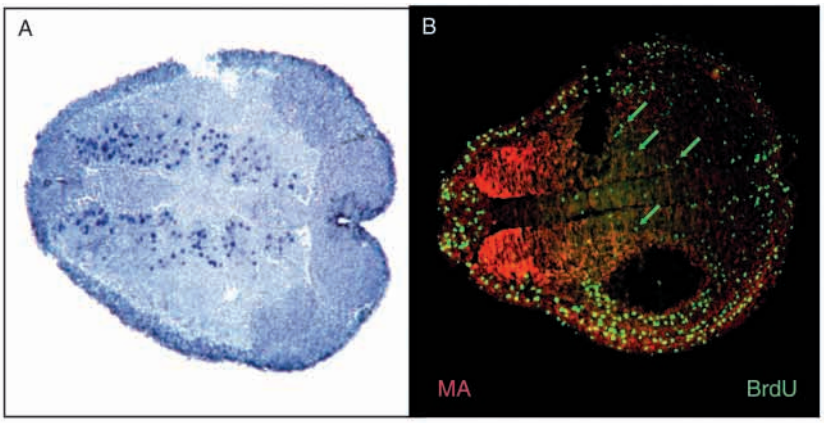

Fig. 1. Cell cycle exit is directly related to myogenic differentiation. (A) Longitudinal section of a stage 15 embryo stained with an antibody against MyoD (dark purple, anterior to left). (B) Longitudinal section of a stage 15 embryo was analyzed for the expression of BrdU (green, arrows) and muscle actin (MA) (red).

1A) (Harvey, 1992) whereas myogenic differentiation occurs in an anterior-posterior wave (Fig. 1B) (Harvey, 1992). MyoD protein levels cannot, therefore, be the only factor driving myogenic differentiation.

In order for cells to differentiate fully, they must exit the cell cycle. Accordingly, one explanation for the anteroposterior wave of muscle maturation is that, despite equal levels of MyoD protein, cells exit the cell cycle first in the front of the embryo and thus differentiate earlier than those in the rear of the embryo. We investigated this possibility by injecting BrdU into the blastocoel of stage 12-12.5 embryos and allowing them to develop for 2 hours until they reached mid-neural plate stage (stage 15), the stage at which muscle structural genes are first expressed (Gurdon et al., 1997). Longitudinal sections of these embryos were stained for the early myogenic marker, muscle actin (MA), and for BrdU, a marker of S-phase cells. As expected at this stage, MA is intensely expressed in the front of the embryo, but is virtually absent from the rear, reflecting the anteroposterior wave of differentiation (Fig. 1B) (Harvey, 1992). We observed that while cells in the differentiated anterior region do not stain for BrdU, many BrdU-positive cells are located in the rear of the myotome (Fig. 1B, arrows). Thus, anterior muscle cells exit the cell cycle before cells in the posterior of the embryo, confirming that proliferation and differentiation are mutually exclusive events and that temporospatially regulated cell cycle exit is likely to contribute to the front-to-back wave of muscle differentiation.

p27Xic1 and MyoD expression in the early embryo

Data presented in Fig. 1 indicates that MyoD expression alone is insufficient to cause cell cycle exit and differentiation in the myotome. However, evidence from tissue culture cells suggests that, in these systems, MyoD can drive cell cycle exit and transcriptionally upregulate p21Cip1 (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). Because of these data, we wished to determine whether MyoD expression temporally and spatially overlaps with that of p27Xic1 during early *Xenopus* development.

Using whole-mount in situ hybridization, we found that p27Xic1 is first detected throughout the animal pole at stage 10 (Fig. 2A). Importantly, its expression is specifically excluded from the area above the blastopore, which corresponds to the band of presumptive mesoderm (Keller, 2000). By stage 11, p27Xic1 localizes to the presomitic mesoderm (Fig. 2B, arrow), and is also found in the developing notochord and forming primary neurons. Additionally, p27Xic1 is transiently expressed in the epidermis between stages 10.5 and 15, demonstrated at stage 13 in Fig. 2L. At mid-neural plate stage (stage 15), p27Xic1 expression in the myotome is greatly intensified (Fig. 2C), obscuring staining in the medial and intermediate neural stripes. However, p27Xic1 is still visible in the lateral stripe, placodal regions (Fig. 2C, arrows) and notochord. By early tailbud stages (stage 22), p27Xic1 is expressed most strongly in the muscle, in a gradient with the most intense staining in the posterior myotome (Fig. 2D) (Ohnuma et al., 1999). By stage 26, p27Xic1 is diminished in the differentiated anterior myotome, but is still prominent in the posterior muscle, as well as being expressed in the eye and brain (Fig. 2I) (Ohnuma et al., 1999).

We then compared expression of p27Xic1 with that of MyoD. Early gastrula stage embryos (stage 10-11) show substantial differences between MyoD and p27Xic1 expression (compare Fig. 2A,B with 2E,F). While MyoD localizes to mesodermal precursors above the invaginating blastopore lip (Fig. 2E) (Hopwood et al., 1992), p27Xic1 expression is strikingly excluded from this region, with strong staining only in the animal pole ectoderm (Fig. 2A). At later gastrula stages, in contrast to p27Xic1, MyoD is found in both the lateral and ventral marginal zones in a horseshoe-like pattern around the blastopore (Fig. 2F), and is excluded from the dorsal region corresponding to the Spemann organizer and forming notochord (Fig. 2F, asterisk) (Frank and Harland, 1991). However, expression of MyoD in the ventral marginal zone, containing cells fated for non-somitic lineages, such as lateral plate mesoderm and blood (Keller, 1975; Keller, 1976; Dale and Slack, 1987a; Keller, 1991), is transient and is insufficient to convert these cells to muscle (Frank and Harland, 1991). During late gastrulation (stage 13), p27Xic1 is only detected in the anterior subset of MyoD-expressing cells in the lateral marginal zone that are destined to a somitic fate (Fig. 2K), but at stage 15, p27Xic1 and MyoD are both expressed throughout the entire myotome (Fig. 2C,G). Although MyoD expression is fairly uniform throughout the myotome at stage 22 (Fig. 2H), p27Xic1 is expressed in a gradient with the most intense staining in the rear of the embryo (Fig. 2D). By stage 26, MyoD is also reduced anteriorly and is expressed in an anterior to posterior gradient (Fig. 2J) similar to that of p27Xic1 (Fig. 2I).

p27Xic1 protein is also observed in the developing myotome at stage 15, using a p27Xic1-specific antibody (Fig. 2M). This staining appears to be more intense in the anterior than the

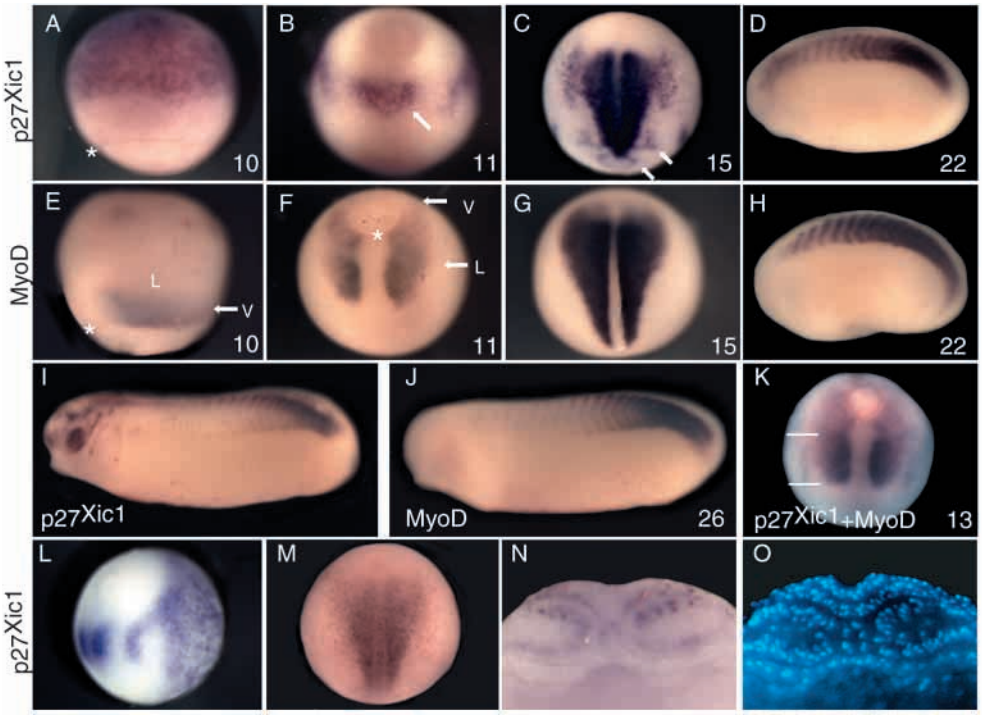

Fig. 2. p27^{Xic1} is expressed in cells destined to a somitic fate. Embryos were analyzed by whole-mount in situ hybridization at the indicated stages for the expression of p27^{Xic1} (A-D,I,L) and MyoD (E-H,J). (A) p27^{Xic1} expression in the animal pole at stage 10. Lateral view with dorsal towards the left, asterisk marks the involuting dorsal lip. (B) p27^{Xic1} localizes to the presomitic mesoderm at stage 11 (arrow). Dorsal view, anterior is downwards. (C) Stage 15 embryo with p27^{Xic1} in the myotome, notochord, primary neurons and anterior placodes (arrows). Dorsal view with anterior downwards. (D) By stage 22, p27^{Xic1} is downregulated in the more mature, anterior somites. Lateral view, anterior left. (E) Lateral view of an embryo stained for MyoD at stage 10 with dorsal leftwards and animal pole upwards. V, ventral marginal zone; L, lateral marginal zone; asterisk, involuting dorsal lip. (F) MyoD is expressed in a horseshoe around the blastopore at stage 11. Dorsal view, asterisk indicates the notochord. (G) MyoD expression at stage 15. Dorsal view with anterior downwards. (H) MyoD expression at stage 22. (I) At stage 26, p27^{Xic1} is virtually absent from the anterior myotome. (J) MyoD is still highly expressed posteriorly at stage 26. (K) Double in situ hybridization demonstrating that p27^{Xic1} (light blue) is only expressed in the subset of MyoD (purple)-expressing cells that are destined to become skeletal muscle. Parallel horizontal lines indicate the extent of the region of overlap (purple/black). (L) Lateral view of a stage 13 embryo showing epidermal p27^{Xic1} expression. Dorsal left, anterior down. (M) p27^{Xic1} protein expression at stage 15. (N) Bisected embryo showing nuclear p27^{Xic1} expression at stage 15. (O) Hoechst staining of DNA in bisected embryo shown in N.

posterior of the myotome, mirroring the wave of cell cycle exit and differentiation in this tissue (Fig. 1B). Co-staining bisected embryos with DNA-specific Hoechst and p27^{Xic1} demonstrates that p27^{Xic1} protein is nuclear (Fig. 2N,O), similar to MyoD protein at this stage (Rupp et al., 1994).

Thus, the early expression of p27^{Xic1} and MyoD is markedly different, suggesting that MyoD may not transcriptionally regulate the onset of p27^{Xic1} expression (compare Fig. 2A with 2E). However, MyoD and p27^{Xic1} do share regions of overlap in cells committed to a somitic fate beginning at stage 11 (Fig. 2B,F), and p27^{Xic1} is highly expressed in the myotome at stage 15 (Fig. 2C,M) when overt signs of muscle differentiation are first detected (Gurdon et al., 1997). Therefore, p27^{Xic1} is appropriately temporally and spatially expressed to function during myogenesis and could be regulated by MyoD later in development. Moreover, the subsequent downregulation of MyoD and p27^{Xic1} in terminally differentiated muscle indicates that although both may be important in the initiation of muscle differentiation, they are not required for its maintenance.

MyoD does not upregulate p27^{Xic1}

Data from tissue culture systems suggests that MyoD can transcriptionally upregulate the cdki, p21^{Cip1}, causing cell cycle arrest and differentiation upon serum withdrawal (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). The conspicuous differences in the initial expression patterns of MyoD and p27^{Xic1} revealed by our in situ hybridization experiments indicate that MyoD may not be regulating p27^{Xic1} at the earliest stages. Later, however, both MyoD and p27^{Xic1} are very highly expressed in the embryonic myotome during muscle differentiation. By analogy with cell culture, we wanted to determine whether MyoD could upregulate transcription of the *Xenopus* cdki, p27^{Xic1}, during in vivo development.

To investigate this question, first we used ectodermal explants (animal caps) isolated from late blastula stage embryos, which normally differentiate into ciliated epidermis (Chang and Hemmati-Brivanlou, 1998). However, overexpression of MyoD can induce MA expression in this tissue (Hopwood and Gurdon, 1990). To determine whether MyoD can transcriptionally upregulate p27^{Xic1}, we injected MyoD into both cells of two-cell stage embryos, dissected animal caps at stage 8, allowed them to develop until early gastrula and mid-neural plate stages and performed quantitative RT/PCR. Using primers for the early muscle structural gene, MA, p27^{Xic1}, and ornithine decarboxylase (ODC) as a loading control, we found that while MyoD can efficiently upregulate MA expression as early as stage 10, it has no effect on p27^{Xic1} levels at any of the stages examined (Fig. 3A). Moreover, overexpression of Myf5 and co-overexpression of MyoD and Myf5 were also unable to upregulate p27^{Xic1} in animal caps (data not shown). Although MyoD and Myf5 overexpression had no effect on p27^{Xic1} levels, we noted that p27^{Xic1} was expressed at a moderate level

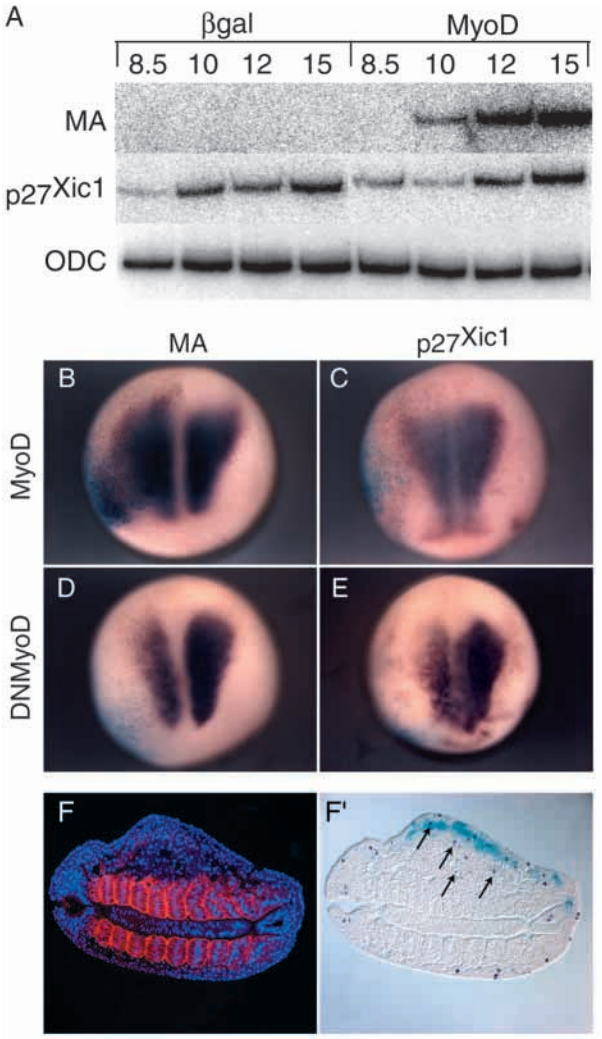

Fig. 3. MyoD is unable to upregulate expression of p27^{Xic1} in vivo. (A) Quantitative RT-PCR for MA, p27^{Xic1} and ODC on cDNA from animal caps of embryos injected with 100 pg MyoD. (B-F′) Embryos were injected with 100 pg MyoD (B,C,F,F′) or 50 pg DNMyoD (D,E) into one cell of two-cell stage embryos, along with β-gal as a tracer (light blue, injected side towards the left in B-E or upwards in F,F′). Dorsal views of embryos analyzed for (B,D) MA or (C,E) p27^{Xic1} expression by whole-mount in situ hybridization at stage 15 (F,F′). (F,F′) MyoD-injected embryos allowed to develop to stage 21 were stained for phospho-histone-H3 then longitudinally sectioned and stained for MA (red) and Hoechst (blue, DNA specific). MyoD overexpression enlarges the area staining for MA (F) and also causes extra proliferation (F′, arrows).

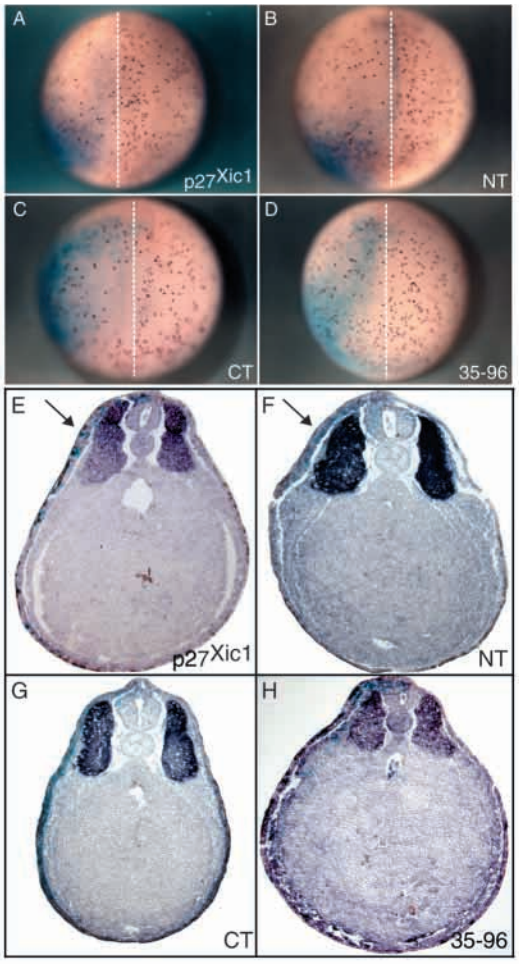

Fig. 4. p27^{Xic1} can enlarge the myotome independently of its ability to arrest the cell cycle. One cell of two-cell stage embryos were injected with (A,E) 45 pg p27^{Xic1}, (B,F) 15 pg p27^{Xic1} NT, (C,G) 50 pg p27^{Xic1} CT or (D,H) 50 pg p27^{Xic1} 35-96 and β-gal as a tracer (light blue, injected side to left) and allowed to develop until stage 15 (A-D) or 22 (E-H). Dorsal views with anterior downwards (midline indicated by broken white line) demonstrate that phospho-histone-H3 staining (purple) is reduced after injection of all four p27^{Xic1} constructs (A-D). Transverse sections stained with an antibody against muscle actin (dark blue) indicate that full-length p27^{Xic1} (A) and p27^{Xic1} NT (B) can increase the size of the myotome (arrows), while p27^{Xic1} CT (C) and p27^{Xic1} 35-96 (D) have no effect.

in both injected and uninjected caps, and so may still be required for MyoD-induced myogenesis.

To confirm that MyoD alone cannot upregulate p27^{Xic1} in vivo, we injected 100pg of MyoD into one cell of two-cell stage embryos, along with β-gal as a tracer, and performed whole-mount in situ hybridization for MA and p27^{Xic1} at stage 15. Again, although injection of synthetic MyoD message caused considerable ectopic MA expression (100% of embryos, *n*=34) (Fig. 3B), no appreciable difference in p27^{Xic1} staining was observed at this stage (Fig. 3C) (*n*=35). At doses of 500 pg, we sometimes observed a slight expansion in the region staining for p27^{Xic1} (data not shown, 33% of embryos, *n*=39). However, this upregulated expression is contiguous with the domain where p27^{Xic1} is normally expressed and does not correspond with the ventral areas of ectopic MA expression that can be induced by high-level MyoD overexpression (data not shown).

Although MyoD alone does not upregulate p27^{Xic1}, it may be responsible for its maintenance. To investigate this possibility, we injected a construct of MyoD fused to the repressor domain of the *Drosophila* Engrailed protein (DNMyoD) (Wittenberger et al., 1999), along with β-gal, into one cell of two-cell stage embryos and assayed for MA and p27^{Xic1} expression by whole-mount in situ hybridization (Fig. 3D,E). Seventy-eight percent of embryos exhibited

downregulated MA (*n*=131), while 62% of embryos had decreased p27^{Xic1} (*n*=85). Thus, loss of MyoD activity leads to a decrease in p27^{Xic1} expression and a concomitant loss in muscle differentiation, indicating that MyoD may be required for p27^{Xic1} maintenance.

Knockout mouse studies demonstrate that multiple cdkis can redundantly control myogenic differentiation. Mice homozygous for a deletion in p21^{Cip1} have no obvious defect in myogenesis (Deng et al., 1995; Brugarolas et al., 1998). p57^{Kip2} knockout mice have altered cell proliferation in several tissues, again with no gross myogenic defect (Yan et al., 1997; Zhang et al., 1997). However, mice lacking both p21^{Cip1} and p57^{Kip2} experience complete failure in muscle differentiation (Zhang et al., 1999). Therefore, MyoD could perhaps be acting through an unidentified cdki to bring about cell cycle arrest in *Xenopus*. To examine this possibility, we tested the ability of injected MyoD to induce cell cycle arrest. We injected MyoD into one cell of two-cell stage embryos and allowed them to develop until stage 21. These embryos were then stained for phospho-histone-H3 (ph3), a marker of mitosis (Saka and Smith, 2001), longitudinally sectioned and stained for MA. The MyoD-injected side of the embryo displayed a dorsoanterior bulge, comprising an enlarged myotome and, in addition, a large area between the differentiated myotome and the epidermis that does not stain for muscle or neural markers (Fig. 3F, data not shown) (Ludolph et al., 1994). Strikingly, there were more ph3-positive cells on the injected side than the uninjected side of the embryo, indicating that MyoD overexpression can promote proliferation under some circumstances (100% of embryos, *n*=9) (Fig. 3F′, arrows), and making it unlikely that it directly upregulates an unknown cdki. However, note that no ph3-positive cells are found in the expanded muscle (Fig. 3F′), indicating that MyoD does not substantially extend the proliferative period of this tissue.

p27^{Xic1} overexpression enlarges the myotome

Does p27^{Xic1} have a role in myogenesis? MyoD can apparently induce ectopic MA expression without upregulating p27^{Xic1} in animal caps and in whole embryos (Fig. 3A-C). To investigate directly whether p27^{Xic1} expression can promote myogenesis, we overexpressed p27^{Xic1}, along with β-gal, in one cell of two cell-stage embryos, allowed them to develop until stage 22 and stained transverse sections for MA (Fig. 4E). Injecting between 30-45 pg of p27^{Xic1} RNA is sufficient to slow the cell cycle without causing the extensive apoptosis and death, which occurs at higher concentrations (70% decrease of ph3, *n*=10) (Fig. 4A). p27^{Xic1} overexpression at this level causes a modest, but consistent and statistically significant expansion of the myotome area (injected side 1.40±0.001 times larger than uninjected side of embryo), indicating that levels of p27^{Xic1} are limiting for myogenesis (Fig. 4E).

p27^{Xic1} has homology to p21^{Cip1}, p27^{Kip1} and p57^{Kip2} (Su et al., 1995; Shou and Dunphy, 1996). In its N terminus, p27^{Xic1} has a cdk/cyclin binding motif similar to that found in p21^{Cip1}, p27^{Kip1} and p57^{Kip2}. The C terminus contains both a proliferating cell nuclear antigen (PCNA) binding region found in p21^{Cip1} (Waga et al., 1994; Chen et al., 1995) and a potential cdc2 phosphorylation site, the QT domain, shared with p27^{Kip1} and p57^{Kip2} (Polyak et al., 1994; Toyoshima and Hunter, 1994). Therefore, p27^{Xic1} has the ability to stop the cell cycle either by inhibition of cdks via its N terminus or through DNA replication inhibition via its C terminus (Su et al., 1995).

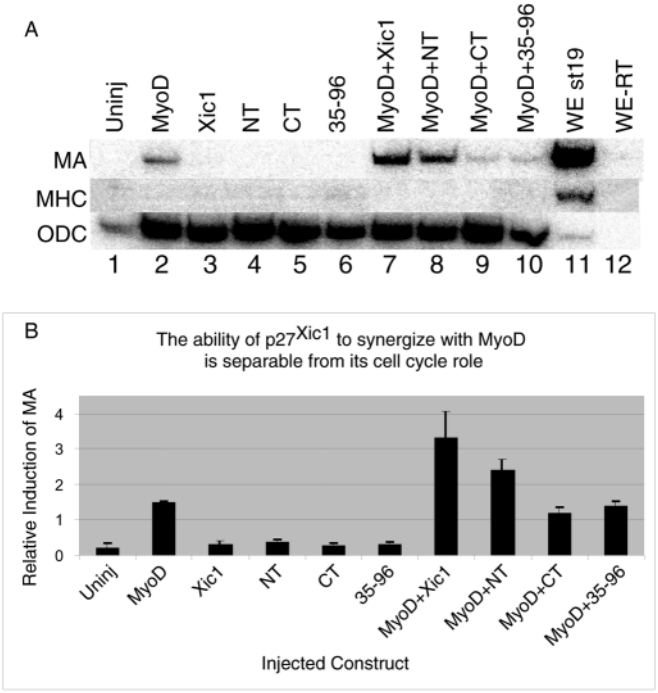

Fig. 5. p27^{Xic1} synergizes with MyoD to promote muscle differentiation. (A) Embryos were injected in both cells of two-cell stage embryos with MyoD (100 pg), p27^{Xic1} (45 pg), p27^{Xic1} NT (15 pg), p27^{Xic1} CT (50 pg), p27^{Xic1} 35-96 (50 pg) or a combination thereof. Ventral marginal zones dissected from injected embryos at stage10 were allowed to develop until parallel embryos reached stage 19 and analyzed by RT-PCR for expression of muscle actin (MA), myosin heavy chain (MHC) and ornithine decarboxylase (ODC) as an internal control. (B) Quantification of the synergistic upregulation of MA by MyoD and p27^{Xic1} mutant overexpression in ventral marginal zones. The relative induction of MA was determined by measuring the incorporated [^{32}P]dATP and normalizing to ODC. The standard error of the mean was obtained by performing this typical experiment in triplicate.

Previous work has indicated that cdkis have roles in neural differentiation that are distinct and separable from their role in regulating the cell cycle (Ohnuma et al., 1999; Dyer and Cepko, 2000; Zezula et al., 2001). In particular, Ohnuma et al. (Ohnuma et al., 1999) showed that p27^{Xic1} can induce preferential differentiation of Müller glia from retinoblasts in addition to its known function in inhibiting cell division. The ability to divert cell fate from neuroblast to glia is mediated by the N terminus of the molecule in a region that is overlapping with, but distinct from the cdk-binding domain (Ohnuma et al., 1999). Thus, p27^{Xic1} has a role in influencing neural cell fate, which goes beyond its role in inhibiting the cell cycle.

We wanted to determine which part of p27^{Xic1} was responsible for myotome expansion and whether this ability of p27^{Xic1} was separable from its role in regulating the cell cycle. To do this, we injected one cell of two-cell stage embryos with 15 pg p27^{Xic1} NT (1-96), 50 pg p27^{Xic1} CT (97-210) or 50 pg p27^{Xic1} 35-96, an N-terminal mutant that retains the ability to inhibit overall cdk2 kinase activity (Ohnuma et al., 1999) but is unable to induce Müller glia cells. ph3 staining demonstrates

that overexpressing similar doses of these three p27Xic1 mutants downregulates proliferation in the myotomal region, but does not cause excessive apoptosis or death (NT 62% decrease; CT 60% decrease; 35-96 49% decrease of ph3-expressing cells, *n*=10) (Fig. 4B-D). After developing to stage 22, injected embryos were transversely sectioned and stained for MA (Fig. 4F-H). While p27Xic1 NT was able to expand significantly the region staining for MA (injected side 1.46±0.016 times larger than uninjected side), neither p27Xic1 CT nor p27Xic1 35-96 had any significant effect (injected sides were 1.02±0.0 and 1.07±0.024 times larger than uninjected sides, respectively) (Fig. 4F-H). Therefore, cell cycle inhibition by the CT and 35-96 is insufficient for myotomal expansion, demonstrating that the N terminus differentiation domain of p27Xic1 is required for this function.

p27Xic1 and MyoD act together to promote myogenic differentiation

The increase in myotome size caused by overexpression of p27Xic1 and p27Xic1 NT was only observed within and adjacent to the region where embryonic muscle normally forms (i.e. ectopic myogenesis in lateral or ventral regions was not observed). Several reports indicate that muscle creatine kinase transcription is enhanced by co-expression of the cdki, p21Cip1 and MyoD in 10T1/2 cells (Skapek et al., 1995; Guo and Walsh, 1997; Reynaud et al., 1999). These findings, combined with our overexpression results, led us to hypothesize that p27Xic1 must be working to promote muscle differentiation within the population of cells that already express a threshold level of myogenic factors such as MyoD. To test this hypothesis more quantitatively, we injected both cells of two-cell stage embryos with MyoD alone or in conjunction with full-length p27Xic1, p27Xic1 NT, p27Xic1 CT or p27Xic1 35-96. At stage 10, ventral marginal zone explants (VMZs) were dissected and allowed to develop until parallel embryos reached stage 19. Quantitative RT-PCR was performed using primers for MA with ODC as a loading control (Fig. 5). Ventral marginal zone tissue is not specified at stage 10 and develops into non-somitic ventral structures in isolation (Dale and Slack, 1987b), but we find that MyoD overexpression will induce MA expression in this tissue.

When overexpressed alone, neither full-length p27Xic1 nor any of the p27Xic1 mutants induces MA expression in VMZs (Fig. 5A, lanes 3-6). MyoD alone induces minimal MA expression, and this is unchanged by co-expression of p27Xic1 CT or p27Xic1 35-96 (Fig. 5A, lanes 2, 9 and 10). However, when MyoD+full-length p27Xic1 or MyoD+p27Xic1 NT are injected together, a much greater upregulation of MA expression is observed (Fig. 5A, lanes 7 and 8) (3.32 times and 2.39 times more, respectively). This synergy between MyoD and full-length p27Xic1 or p27Xic1 NT (but not between MyoD and p27Xic1 CT or p27Xic1 35-96) was also seen in animal caps and dorsal marginal zone explants tested at various stages (data not shown). Thus, MyoD and p27Xic1 synergize to promote muscle differentiation. Furthermore, the synergistic property of p27Xic1 resides in the N-terminal differentiation domain and, therefore, this property can be distinguished from the ability of p27Xic1 to inhibit overall cdk2 kinase activity and arrest the cell cycle.

Although able to induce the early muscle differentiation marker MA, MyoD is unable to promote terminal differentiation of muscle both in animal caps and in whole embryos (data not shown) (Hopwood and Gurdon, 1990), suggesting a requirement for complementary regulatory factors. As cells committed to the myogenic lineage must withdraw from the cell cycle to differentiate, and mHLH factors alone do not induce cell cycle withdrawal at physiological concentrations in vivo (Davis et al., 1987; Tapscott et al., 1988; Braun et al., 1989; Olson, 1992), we hypothesized that co-expression of MyoD and p27Xic1 might promote expression of terminal muscle markers. Therefore, we used primers for the terminally differentiated muscle marker, myosin heavy chain (MHC), and performed RT-PCR on the VMZs co-injected with MyoD, p27Xic1 and the p27Xic1 mutants described above. However, none of the combinations of MyoD, p27Xic1 or p27Xic1 mutants was able to induce expression of MHC in the VMZs (Fig. 5) or in whole embryos, as assayed by in situ hybridization (data not shown). MyoD, p27Xic1 and the p27Xic1 mutants were also unable to upregulate expression of the late muscle marker 12/101 in VMZs at stage 19 (data not shown). Thus, although MyoD and p27Xic1 promote the early stages of muscle differentiation, additional factors must be present or active for these explants to terminally differentiate.

p27Xic1 is required for myogenic differentiation

Overexpression of p27Xic1 causes a modest increase in the size of the embryonic myotome. The ability of p27Xic1 to enlarge the myotome and to synergize with MyoD is separable from its ability to arrest the cell cycle. However, we wanted to know whether p27Xic1 was required for myogenesis or whether it simply promoted differentiation of cells already committed to a myogenic fate when overexpressed. To address this question, we used antisense morpholino oligonucleotides which bind over the translation initiation site of RNA and prevent accumulation of the targeted protein (Heasman et al., 2000). The production of p27Xic1 protein was completely inhibited by injection of p27Xic1 morpholino (p27Xic1 Mo), while injection of a control morpholino (Con Mo) had no effect (Fig. 6A).

To determine if p27Xic1 is required for myogenesis and, if so, where in the myogenic pathway it acts, we injected 20ng of either Con Mo or p27Xic1 Mo, along with β-gal as a tracer, into one cell of two-cell stage embryos and performed in situ hybridization for MyoD, Myf5, MA and MHC at stage 15 (Fig. 6). Interestingly, loss of p27Xic1 had no effect on the expression of MyoD (93% of embryos, *n*=136) (Fig. 6B) or Myf5 (86% of embryos, *n*=66) (Fig. 6C). However, MA expression was greatly reduced (69% of embryos, *n*=111) (Fig. 6D) and the terminal differentiation marker, MHC, was almost entirely absent (96% of embryos, *n*=100) (Fig. 6F). Furthermore, whole-mount antibody staining revealed significantly reduced expression of the muscle ATPase, 12/101 (75% of embryos, *n*=24) (Fig. 6H,I). Injection of the Con Mo had no effect on any of the markers tested (Fig. 6E and data not shown). Ohnuma et al. (Ohnuma et al., 1999) have previously demonstrated that p21Cip1 can substitute for p27Xic1 in Müller glial cell induction in the retina. The loss of myogenic differentiation caused by injection of p27Xic1 Mo is specific to loss of cdki function because it can be rescued by co-injection with p21Cip1 (Fig. 6G). While 96% of embryos injected with p27Xic1 Mo significantly downregulated MHC staining (Fig. 6F), 84% of embryos co-injected with p27Xic1 Mo and p21Cip1

had nearly normal MHC expression (*n*=149) (Fig. 6G). This data indicates that although p27^{Xic1} is not required for myogenic determination (i.e. MyoD and Myf5 expression), it is absolutely required for muscle differentiation.

One predicted phenotype resulting from preventing translation of a cdki required for muscle differentiation is increased myotomal proliferation. To test this hypothesis, we injected 20 ng of either Con Mo or p27^{Xic1} Mo into one cell of two-cell stage embryos, allowed them to develop until stage 21 and stained for ph3. These embryos were then longitudinally sectioned and stained with an antibody against MA (Fig. 7). Injection of the Con Mo had no effect on proliferation or MA expression (100% of embryos, *n*=7) (Fig. 7A,A′). However, in accordance with the data from our whole-mount in situ hybridization, loss of p27^{Xic1} led to a dramatic decrease in MA expression (100% of embryos, *n*=11) (Fig. 7B). These embryos also had ph3-positive cells within the area that would normally stain for MA and an increase in the number of dividing cells throughout the mesenchyme and epidermis (82% of embryos, *n*=11) (Fig. 7B′, arrows).

Cell cycle exit is a prerequisite for muscle differentiation. Therefore, we wished to determine whether the reduced muscle phenotype observed upon loss of p27^{Xic1} protein is the result of a failure to undergo cell cycle arrest, or whether a separate p27^{Xic1} function is required for muscle differentiation. To address this question, we treated p27^{Xic1} Mo- or Con Mo-injected embryos with hydroxyurea and aphidicolin (HUA), reagents that arrest cells at the G1/S phase transition of the cell cycle. Even after HUA treatment, p27^{Xic1} Mo-injected embryos failed to develop normal, differentiated muscle (Fig. 6H) when compared with both the uninjected side of the embryo (Fig. 6I) and the Con Mo-injected controls (data not shown). Therefore, in agreement with our overexpression data (Fig. 4), p27^{Xic1} is required for myogenesis independent of its ability to arrest the cell cycle.

What is the fate of the myocytes that fail to differentiate in the absence of p27^{Xic1}? When deprived of mitogenic stimulation, a large proportion of differentiating myocytes undergo programmed cell death (Wang and Walsh, 1996). However, differentiated myotubes remain viable in low-serum culture for more than 2 weeks (Wang and Walsh, 1996). The acquisition of this apoptosis-resistant phenotype correlates with the induction of p21^{Cip1}, and ectopic expression of p21^{Cip1} confers apoptotic protection to myocytes (Wang and Walsh, 1996). To determine whether loss of the cdki p27^{Xic1} resulted in excessive programmed cell death in vivo, embryos were injected in one of two cells with 20 ng Con Mo or p27^{Xic1} Mo and allowed to develop until stage 15. Injected embryos were analyzed for apoptotic cells by whole-mount TUNEL assay staining (Hensey and Gautier, 1998). Overall, Con Mo-injected embryos demonstrated very few apoptotic cells and there was no observable difference between the injected and uninjected sides of the embryo (no change in 100% of embryos, *n*=31) (Fig. 6J). However, the number of apoptotic cells increased upon injection of p27^{Xic1} Mo (47% of embryos, *n*=30) (Fig. 6K, representative embryo). Despite the increase in apoptotic figures observed upon injection of p27^{Xic1} Mo, the decrease in myogenic differentiation cannot be attributed entirely to extensive programmed cell death of the prospective muscle cells because MyoD and Myf5 appear unaffected by the absence of p27^{Xic1} (Fig. 6B,C). These data demonstrate that although loss of p27^{Xic1} increases the incidence of apoptosis, a significant proportion of the affected cells must either remain undifferentiated myotomal cells or adopt alternative differentiation fates.

Embryos injected with 20 ng of p27^{Xic1} Mo were allowed

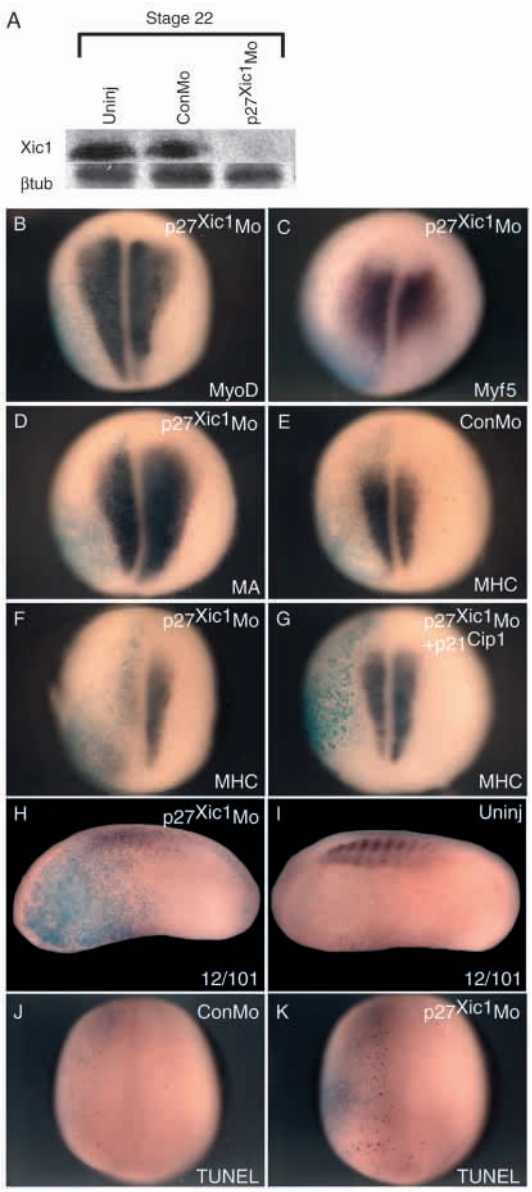

Fig. 6. p27^{Xic1} is required for muscle differentiation. Western blot for endogenous p27^{Xic1} protein in uninjected embryos and embryos injected with 20 ng Con Mo or 20 ng p27^{Xic1} Mo harvested at stage 22. Cytoskeletal β-tubulin is used as a loading control. Embryos were injected with 20 ng p27^{Xic1} Mo (B-D,F,H,K), 20 ng Con Mo (E,J) or 20 ng p27^{Xic1} Mo + 20 pg p21^{Cip1} (G) along with β-gal (light blue, injected side towards the left) and analyzed at stage 15 for expression of MyoD (B), Myf5 (C), MA (E) and MHC (E-G) by whole-mount in situ hybridization. Embryos injected with 20 ng p27^{Xic1} Mo were incubated in HUA from gastrulation until stage 22 and analyzed by whole-mount antibody staining for 12/101 expression (H,I). Embryos injected with 20 ng Con Mo (J) or p27^{Xic1} Mo (K) were analyzed for apoptotic cells at stage 15 by whole-mount TUNEL staining.

to develop until stage 26 and longitudinally sectioned to determine whether the downregulation of MA expression persisted and what effect this had on embryonic development. At this stage, p27Xic1 translation is still inhibited by injection of the p27Xic1 Mo (data not shown). The p27Xic1 Mo-injected embryos are bent, possibly because of the failure to develop muscle (Kopan et al., 1994). The injected side of these embryos completely fails to form somitic structures, has little or no MA expression and, indeed, appears to have more cells (Fig. 7C,C′). Thus, loss of p27Xic1 leads to a total failure in muscle formation, somitogenesis and differentiation that persists at least until late tailbud stages.

DISCUSSION

The coordinate regulation of the cell cycle with myogenic differentiation has most extensively been studied using cell culture techniques (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). These systems are somewhat artificial, though, as an exceptionally high level of MyoD expression is needed to inhibit the cell cycle and serum withdrawal is required to induce differentiation (Crescenzi et al., 1990; Sorrentino et al., 1990; Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). Mice with homozygous deletions for a number of cdkis have also been employed to investigate the importance of these molecules in the process of myogenesis in vivo (Deng et al., 1995; Kiyokawa et al., 1996; Nakayama et al., 1996; Yan et al., 1997; Zhang et al., 1997; Brugarolas et al., 1998; Fero et al., 1998; Zhang et al., 1998; Zhang et al., 1999). However, despite all three mammalian p21Cip1 family members being highly expressed in differentiating skeletal muscle (Polyak et al., 1994; Matsuoka et al., 1995; Parker et al., 1995; Nakayama et al., 1996; Yan et al., 1997; Zabludoff et al., 1998), their individual importance in the process of myogenesis is difficult to determine because of a high level of redundancy. Therefore, we have examined the interactions between myogenic determination factors and the cell cycle in vivo using *Xenopus laevis* as a model organism. This highly accessible, readily manipulable system is attractive because several myogenic genes have been cloned, the timing of differentiation has been defined, and there is little apparent redundancy as *Xenopus* has only one known cdki, p27Xic1. We present here clear in vivo evidence demonstrating the importance of this single cdki in coordinating the processes of cell cycle exit and differentiation.

Our whole-mount in situ hybridization revealed that p27Xic1 is highly expressed in the developing myotome, making it a prime candidate molecule in the coordination of cell cycle exit and differentiation (Fig. 2). During early development, p27Xic1 and the mHLH transcription factor, MyoD, are expressed in substantially different embryonic regions (Fig. 2A,E). However, when muscle differentiation begins, the mesodermal staining patterns of p27Xic1 and MyoD are indistinguishable (Fig. 2C,G) and both p27Xic1 and MyoD protein localize in the nuclei of differentiating muscle cells (Fig. 2M-O) (Rupp et al., 1994). The dynamic temporal and spatial expression of p27Xic1 is consistent with a role in the maintenance and promotion of the myogenic differentiation program in synergy with MyoD.

Data from tissue culture systems have suggested that MyoD can transcriptionally upregulate expression of the cdki, p21Cip1 and, upon serum withdrawal, induce myogenic differentiation

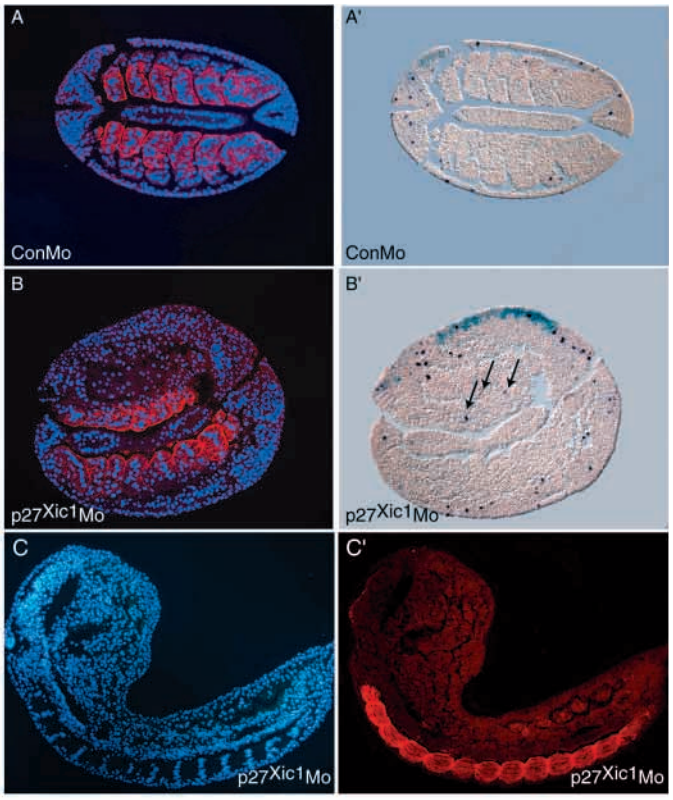

Fig. 7. Ablation of p27Xic1 causes excess proliferation and loss of differentiated muscle. Embryos were injected in one cell of two-cell stage embryos with (A,A′) 10 ng Con Mo or (B-C′) 10 ng p27Xic1 Mo and β-gal (light blue in A′,B′) (injected side upwards) and allowed to develop until stage 21 (A-B′) or stage 26 (C,C′). Embryos were stained for phospho-histone-H3 (purple in A′,B′), longitudinally sectioned and stained with an antibody against muscle actin (MA) (red in A,B,C′). DNA is stained with Hoechst (blue, A,B,C).

(Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995). However, the in vivo relevance of this finding is questionable, as cells cultured from MyoD-null mice do not show altered p21Cip1 expression (Parker et al., 1995; Sabourin et al., 1999). We have directly investigated whether MyoD can regulate expression of the *Xenopus* cdki, p27Xic1, both in vivo and in embryonic tissue explants (Fig. 3). When overexpressed at levels capable of upregulating MA expression, MyoD alone, or in combination with Myf5, is unable to upregulate p27Xic1 expression (Fig. 3 and data not shown). Thus, MyoD overexpression can induce myogenesis laterally and ventrally without inducing p27Xic1. However, p27Xic1 is expressed in the ectoderm outside the neural plate between stages 10.5-15 (Fig. 2L), and is therefore available to synergize with injected MyoD to promote muscle differentiation in these areas. Moreover, MyoD is unlikely to be acting through an unidentified cdki to arrest the cell cycle and initiate muscle differentiation, because when MyoD is overexpressed at a level capable of enlarging the myotome, we observed an upregulation of the mitotic marker ph3 (Fig. 3F′).

Interestingly, the increase in ph3 staining upon MyoD overexpression was not seen in the myotome, but rather in the epidermis and in the mesenchymal tissue between the skin and

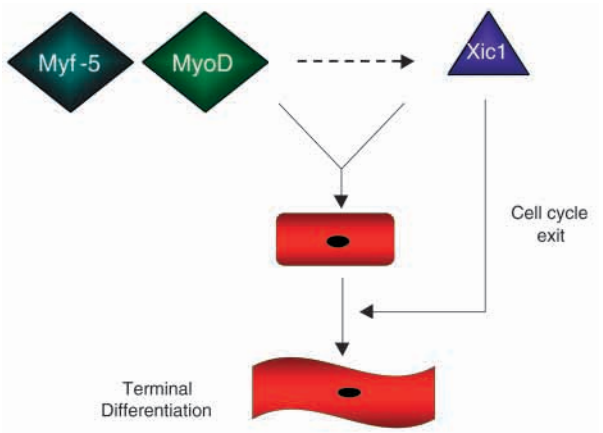

Fig. 8. p27^{Xic1} function during myogenic differentiation. During myogenesis, p27^{Xic1} is required in parallel with, or downstream of, the determination factors MyoD and Myf5. p27^{Xic1} both arrests the cell cycle and, independent of its cell cycle role, promotes differentiation.

the myotome (Fig. 3F′). Ludolph et al. (Ludolph et al., 1994) have previously reported that MyoD overexpression enlarged the myotome even in the absence of post-gastrulation cell division. However, the effect of blocking proliferation on the dorsoanterior mass, enlarged brain and epidermal thickening induced by MyoD overexpression was not discussed (Ludolph et al., 1994). Therefore, although the enlarged myotome may be due to increased myoblast recruitment, the mesenchymal mass may be attributable to enhanced proliferation. In any case, our results indicate that cell cycle exit in the myotome is unlikely to result from MyoD-mediated upregulation of p27^{Xic1} or another undiscovered cdki.

MyoD is thought to be involved in several regulatory feedback loops (Thayer et al., 1989; Steinbach et al., 1998). Therefore, we wanted to determine whether MyoD is necessary for p27^{Xic1} expression, even though it appears to be insufficient to initiate its transcription. Injection of a DNMyoD construct inhibited expression of both MA and p27^{Xic1}, indicating that MyoD may be necessary for maintenance of p27^{Xic1} (Fig. 3D,E). However, p27^{Xic1} may be upregulated by an unidentified bHLH factor that requires the activating partner, E12 (Rashbass et al., 1992). Therefore overexpression of DNMyoD could be inhibiting both MA and p27^{Xic1} by binding up this molecule. Additionally, as DNMyoD reduces the size of the myotome, the observed effect on p27^{Xic1}, which is highly expressed in the myotome, may be indirect.

p27^{Xic1} is highly expressed in the differentiating myotome, consistent with an essential role in myogenesis. Using an antisense morpholino oligonucleotide directed at p27^{Xic1}, we found that p27^{Xic1} is absolutely required for myogenic differentiation (Fig. 6). Inhibiting expression of the p27^{Xic1} protein had no effect on the expression of the determination markers MyoD and Myf5 (Fig. 6B,C), but significantly downregulated MA and 12/101 expression (Fig. 6D,H,I) and completely inhibited expression of the terminal muscle marker, MHC (Fig. 6F). These data indicate that p27^{Xic1} acts downstream of myogenic commitment, but prior to terminal differentiation. The block in myogenic differentiation caused by loss of p27^{Xic1} is specific as it can be rescued by injection of p21^{Cip1} (Fig. 6G). Embryos allowed to develop until stage 26 still exhibited near total loss of somitic muscle formation. In our experience, translation of p27^{Xic1} is inhibited by injection of p27^{Xic1} Mo to at least stage 32 (data not shown). An interesting question to investigate would be whether the ability to differentiate is recovered upon loss of the p27^{Xic1} Mo and re-accumulation of p27^{Xic1} protein or whether the window of competence for muscle differentiation has been lost.

In some instances, myogenic repression is a consequence of de-regulated growth control, while in others it is independent of cell proliferation (reviewed by Olson, 1992). Longitudinal sections of embryos injected with p27^{Xic1} Mo and stained for both ph3 and MA revealed an upregulation in mitotic cells and a downregulation of differentiation (Fig. 7B,B′). To investigate whether this failure to exit the cell cycle was responsible for the lack of differentiated muscle seen in the absence of p27^{Xic1} protein, we blocked cell division in p27^{Xic1} Mo-injected embryos by incubating them in HUA. Even when cells were arrested, they failed to differentiate into muscle without functional p27^{Xic1} (Fig. 6H,I). Therefore, we propose that, in vivo, a combination of cell cycle exit failure and lack of the N-terminal function of p27^{Xic1} contributes to the loss of myogenic differentiation observed upon loss of p27^{Xic1}. Although p27^{Xic1} is likely to be crucial for proper cell cycle regulation, it plays a separate distinct role in promoting differentiation, as described below.

We have shown that p27^{Xic1} overexpression blocks cell proliferation, actively promotes myogenesis and enlarges the size of the myotome, suggesting that p27^{Xic1} is a limiting factor during muscle differentiation (Fig. 4). *Xenopus* embryonic cells do not grow before independent feeding stages, but rather subdivide existing tissue. Therefore, the p27^{Xic1}-mediated increase in myotome size is probably due to enhanced recruitment into the muscle lineage rather than an increase in cell number or enhanced muscle cell growth. This hypothesis is supported by the finding that co-injection of MyoD and p27^{Xic1} enhances the expression of the early muscle structural gene, MA, in ventral marginal zones and in whole embryos (Fig. 5; data not shown). However, neither VMZs nor whole embryos co-injected with MyoD and p27^{Xic1} upregulated expression of the terminal differentiation markers MHC or 12/101 (Fig. 5; data not shown). These data also indicate that although p27^{Xic1} can promote early myogenic differentiation in synergy with MyoD, further regulatory molecules are required to promote the full differentiation program.

Most interestingly, the ability of p27^{Xic1} to promote myogenic differentiation is complementary to, but separable from, its ability to inhibit the cell cycle. Recent studies have revealed that cdkis can act as dual-function molecules that participate in the regulation of both the cell cycle and differentiation (Ohnuma et al., 1999; Dyer and Cepko, 2000; Zezula et al., 2001). However, these analyses have been performed in determined neural systems such as the PC12 neural cell line (Erhardt and Pittman, 1998), oligodendrocytes (Zezula et al., 2001) or the retina (Ohnuma et al., 1999; Dyer and Cepko, 2000). As the processes of myogenesis and neurogenesis are highly analogous (reviewed by Jan and Jan, 1993; Relaix and Buckingham, 1999), we wanted to extend these observations and investigate whether a similar differentiation role for cdkis existed during myogenesis in the

early embryo. We have shown that overexpression of full-length p27Xic1 and p27Xic1 NT can enlarge the size of the myotome, but p27Xic1 CT and p27Xic1 NT 35-96, although still capable of cell cycle inhibition, cannot (Fig. 4). As a more quantitative method of analysis, we chose to examine this phenomenon further in ventral marginal zone explants (VMZs). MyoD overexpression alone induces minimal MA expression that is unchanged by co-expression with p27Xic1 CT or p27Xic1 NT 35-96 (Fig. 5). However, co-expression of MyoD with p27Xic1 FL or p27Xic1 NT greatly enhances expression of MA over that of MyoD alone, demonstrating that the ability of p27Xic1 to synergize with MyoD is distinct from its ability to inhibit overall cdk2 kinase activity or to arrest the cell cycle and encompasses an N-terminal region upstream of amino acid 35 (Fig. 5).

p27Xic1 overexpression increases the size of the myotome and synergizes with MyoD to promote muscle differentiation. Moreover, p27Xic1 is required in parallel with, or downstream of, MyoD and Myf5 for muscle formation, and is, therefore, well placed to play a dual role in myogenesis. In Fig. 8, we propose that, to differentiate into myocytes, cells must express a threshold level of MyoD and p27Xic1, as well as exit the cell cycle. p27Xic1 clearly performs two functions: cell cycle arrest and promotion of differentiation via its N terminus. Both functions are essential for development to occur normally. The existence of a single molecule that is responsible for both of these crucial functions, allows elegant, coordinated control of division and differentiation.

Reynaud et al. (Reynaud et al., 1999) have demonstrated that p57Kip2 can stabilize MyoD protein and this ability is dependent upon the N terminus of the molecule. Although MyoD overexpression has been shown to induce growth arrest in a number of cell lines, myoblasts are able to proliferate despite its expression (Davis et al., 1987; Tapscott et al., 1988; Olson, 1992), indicating that its activity must be regulated in proliferating myoblasts. Several methods of inhibitory regulation of MyoD have been suggested, including modulation by binding partners such as Id, phosphorylation and either direct or indirect inhibition by the cyclin D-dependent kinases (Benezra et al., 1990; Jen et al., 1992; Rao et al., 1994; Skapek et al., 1995; Song et al., 1998; Kitzmann et al., 1999). The ability of p57Kip2 to extend the half-life of MyoD protein was originally postulated to be due to its inhibition of cdk-dependent phosphorylation of MyoD (Reynaud et al., 1999). However, the activity and stability of a non-phosphorylatable form of MyoD (MyoDAla200) is also enhanced by co-expression of p57Kip2 (Reynaud et al., 2000). Further data from this report implicates an N-terminal α-helix domain in p57Kip2 in direct binding with the basic domain of MyoD and masking potential degradation signals (Abu Hatoum et al., 1998). Although our initial experiments do not demonstrate direct binding between an overexpressed Myc-tagged MyoD and p27Xic1, nor do we see p27Xic1-mediated stabilization of overexpressed MyoD, how p27Xic1 is able to synergize with MyoD to promote myogenesis is an important issue. Future investigations into the ability of p27Xic1 to stabilize MyoD either by inhibition of phosphorylation by cyclins/cdks, regulation of nuclear localization, alteration of transcriptional activity through regulation of DNA binding or a combination of these mechanisms may prove interesting. Such data may reveal exciting paradigms for how cdkis might regulate cell fate determination and differentiation in several developmental contexts.

We thank Shinichi Ohnuma and Ralph Rupp for generously supplying plasmids, Michael Zuber for supplying the MHC primers, Phil Jones for support with statistical analysis and William Harris for helpful discussions. The anti-p27Xic1 antibody was a very generous gift from Dr Renee Yew. This work was funded by the Cancer Research Campaign (Grant SP2476/0101) and A. E. V. is supported by a Howard Hughes Medical Institute Predoctoral Fellowship.

REFERENCES

Abu Hatoum, O., Gross-Mesilaty, S., Breitschopf, K., Hoffman, A., Gonen, H., Ciechanover, A. and Bengal, E. (1998). Degradation of myogenic transcription factor MyoD by the ubiquitin pathway in vivo and in vitro: regulation by specific DNA binding. *Mol. Cell Biol.* **18**, 5670-5677.

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C. and de Robertis, E. M. (2000). Endodermal Nodal-related signals and mesoderm induction in Xenopus. *Development* **127**, 1173-1183.

Andres, V. and Walsh, K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *J. Cell Biol.* **132**, 657-666.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**, 49-59.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H. H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* **8**, 701-709.

Brugarolas, J., Bronson, R. T. and Jacks, T. (1998). p21 is a critical CDK2 regulator essential for proliferation control in Rb-deficient cells. *J. Cell Biol.* **141**, 503-514.

Chang, C. and Hemmati-Brivanlou, A. (1998). Cell fate determination in embryonic ectoderm. *J. Neurobiol.* **36**, 128-151.

Charbonnier, F., Gaspera, B. D., Armand, A. S., van der Laarse, W. J., Launay, T., Becker, C., Gallien, C. L. and Chanoine, C. (2002). Two myogenin-related genes are differentially expressed in Xenopus laevis myogenesis and differ in their ability to transactivate muscle structural genes. *J. Biol. Chem.* **277**, 1139-1147.

Chen, J., Jackson, P. K., Kirschner, M. W. and Dutta, A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* **374**, 386-388.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.

Crescenzi, M., Fleming, T. P., Lassar, A. B., Weintraub, H. and Aaronson, S. A. (1990). MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc. Natl. Acad. Sci. USA* **87**, 8442-8446.

Dale, L. and Slack, J. M. (1987a). Fate map for the 32-cell stage of Xenopus laevis. *Development* **99**, 527-551.

Dale, L. and Slack, J. M. (1987b). Regional specification within the mesoderm of early embryos of *Xenopus laevis*. *Development* **100**, 279-295.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**, 675-684.

Dyer, M. A. and Cepko, C. L. (2000). p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. *Development* **127**, 3593-3605.

Erhardt, J. A. and Pittman, R. N. (1998). Ectopic p21(WAF1) expression induces differentiation-specific cell cycle changes in PC12 cells characteristic of nerve growth factor treatment. *J. Biol. Chem.* **273**, 23517-23523.

Fero, M. L., Randel, E., Gurley, K. E., Roberts, J. M. and Kemp, C. J. (1998). The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* **396**, 177-180.

Frank, D. and Harland, R. M. (1991). Transient expression of XMyoD in non-somitic mesoderm of Xenopus gastrulae. *Development* **113**, 1387-1393.

Guo, K. and Walsh, K. (1997). Inhibition of myogenesis by multiple cyclin-

Cdk complexes. Coordinate regulation of myogenesis and cell cycle activity at the level of E2F. *J. Biol. Chem.* **272**, 791-797.

Guo, K., Wang, J., Andres, V., Smith, R. C. and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol. Cell. Biol.* **15**, 3823-3829.

Gurdon, J. B., Lemaire, P. and Mohun, T. J. (1997). Myogenesis in Xenopus embryos. *Methods Cell Biol.* **52**, 53-66.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D. and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**, 1018-1021.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805-816.

Harvey, R. P. (1990). The Xenopus MyoD gene: an unlocalised maternal mRNA predates lineage-restricted expression in the early embryo. *Development* **108**, 669-680.

Harvey, R. P. (1991). Widespread expression of MyoD genes in Xenopus embryos is amplified in presumptive muscle as a delayed response to mesoderm induction. *Proc. Natl. Acad. Sci. USA* **88**, 9198-9202.

Harvey, R. P. (1992). MyoD protein expression in Xenopus embryos closely follows a mesoderm induction-dependent amplification of MyoD transcription and is synchronous across the future somite axis. *Mech. Dev.* **37**, 141-149.

Heasman, J., Kofron, M. and Wylie, C. (2000). Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Hensey, C. and Gautier, J. (1998). Programmed cell death during Xenopus development: a spatio-temporal analysis. *Dev. Biol.* **203**, 36-48.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. *EMBO J.* **8**, 3409-3417.

Hopwood, N. D. and Gurdon, J. B. (1990). Activation of muscle genes without myogenesis by ectopic expression of MyoD in frog embryo cells. *Nature* **347**, 197-200.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1991). Xenopus Myf-5 marks early muscle cells and can activate muscle genes ectopically in early embryos. *Development* **111**, 551-560.

Hopwood, N. D., Pluck, A., Gurdon, J. B. and Dilworth, S. M. (1992). Expression of XMyoD protein in early *Xenopus laevis* embryos. *Development* **114**, 31-38.

Jan, Y. N. and Jan, L. Y. (1993). HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell* **75**, 827-830.

Jen, Y., Weintraub, H. and Benezra, R. (1992). Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. *Genes Dev.* **6**, 1466-1479.

Jennings, C. G. (1992). Expression of the myogenic gene MRF4 during Xenopus development. *Dev. Biol.* **151**, 319-332.

Keller, R. E. (1975). Vital dye mapping of the gastrula and neurula of Xenopus laevis. I. Prospective areas and morphogenetic movements of the superficial layer. *Dev. Biol.* **42**, 222-241.

Keller, R. E. (1976). Vital dye mapping of the gastrula and neurula of Xenopus laevis. II. Prospective areas and morphogenetic movements of the deep layer. *Dev. Biol.* **51**, 118-137.

Keller, R. (1991). Early embryonic development of Xenopus laevis. *Methods Cell Biol.* **36**, 61-113.

Keller, R. (2000). The origin and morphogenesis of amphibian somites. *Curr. Top. Dev. Biol.* **47**, 183-246.

Kitzmann, M., Vandromme, M., Schaeffer, V., Carnac, G., Labbe, J. C., Lamb, N. and Fernandez, A. (1999). cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. *Mol. Cell. Biol.* **19**, 3167-3176.

Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A. and Koff, A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* **85**, 721-732.

Kopan, R., Nye, J. S. and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120**, 2385-2396.

Ludolph, D. C., Neff, A. W., Mescher, A. L., Malacinski, G. M., Parker, M. A. and Smith, R. C. (1994). Overexpression of XMyoD or XMyf5 in Xenopus embryos induces the formation of enlarged myotomes through recruitment of cells of nonsomitic lineage. *Dev. Biol.* **166**, 18-33.

Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W. and Elledge, S. J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev.* **9**, 650-662.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I. and Loh, D. Y. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**, 707-720.

Newport, J. and Kirschner, M. (1982a). A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686.

Newport, J. and Kirschner, M. (1982b). A major developmental transition in early Xenopus embryos: II. Control of the onset of transcription. *Cell* **30**, 687-696.

Nicolas, N., Gallien, C. L. and Chanoine, C. (1998). Expression of myogenic regulatory factors during muscle development of Xenopus: myogenin mRNA accumulation is limited strictly to secondary myogenesis. *Dev. Dyn.* **213**, 309-321.

Nieuwkoop, P. D. and Faber, J. (1994). *Normal table of* Xenopus laevis. New York: Garland Publishing.

Ohnuma, S., Philpott, A., Wang, K., Holt, C. E. and Harris, W. A. (1999). p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. *Cell* **99**, 499-510.

Olson, E. N. (1992). Interplay between proliferation and differentiation within the myogenic lineage. *Dev. Biol.* **154**, 261-272.

Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W. and Elledge, S. J. (1995). p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. *Science* **267**, 1024-1027.

Philpott, A. and Friend, S. H. (1994). E2F and its developmental regulation in Xenopus laevis. *Mol. Cell. Biol.* **14**, 5000-5009.

Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P. and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**, 59-66.

Rao, S. S., Chu, C. and Kohtz, D. S. (1994). Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. *Mol. Cell. Biol.* **14**, 5259-5267.

Rashbass, J., Taylor, M. V. and Gurdon, J. B. (1992). The DNA-binding protein E12 co-operates with XMyoD in the activation of muscle-specific gene expression in Xenopus embryos. *EMBO J.* **11**, 2981-2990.

Relaix, F. and Buckingham, M. (1999). From insect eye to vertebrate muscle: redeployment of a regulatory network. *Genes Dev.* **13**, 3171-3178.

Reynaud, E. G., Pelpel, K., Guillier, M., Leibovitch, M. P. and Leibovitch, S. A. (1999). p57(Kip2) stabilizes the MyoD protein by inhibiting cyclin E-Cdk2 kinase activity in growing myoblasts. *Mol. Cell. Biol.* **19**, 7621-7629.

Reynaud, E. G., Leibovitch, M. P., Tintignac, L. A., Pelpel, K., Guillier, M. and Leibovitch, S. A. (2000). Stabilization of MyoD by direct binding to p57(Kip2). *J. Biol. Chem.* **275**, 18767-18776.

Rupp, R. A. and Weintraub, H. (1991). Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of X. laevis. *Cell* **65**, 927-937.

Rupp, R. A., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8**, 1311-1323.

Sabourin, L. A., Girgis-Gabardo, A., Seale, P., Asakura, A. and Rudnicki, M. A. (1999). Reduced differentiation potential of primary MyoD–/– myogenic cells derived from adult skeletal muscle. *J. Cell Biol.* **144**, 631-643.

Saka, Y. and Smith, J. C. (2001). Spatial and temporal patterns of cell division during early Xenopus embryogenesis. *Dev. Biol.* **229**, 307-318.

Scales, J. B., Olson, E. N. and Perry, M. (1990). Two distinct Xenopus genes with homology to MyoD1 are expressed before somite formation in early embryogenesis. *Mol. Cell. Biol.* **10**, 1516-1524.

Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501-1512.

Shimamura, K., Hirano, S., McMahon, A. P. and Takeichi, M. (1994). Wnt-1-dependent regulation of local E-cadherin and alpha N-catenin expression in the embryonic mouse brain. *Development* **120**, 2225-2234.

Shou, W. and Dunphy, W. G. (1996). Cell cycle control by Xenopus p28Kix1, a developmentally regulated inhibitor of cyclin-dependent kinases. *Mol. Biol. Cell.* **7**, 457-469.

Sive, H., Grainger, R. and Harland, R. (2000). *Early Development of* Xenopus laevis*: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Skapek, S. X., Rhee, J., Spicer, D. B. and Lassar, A. B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science* **267**, 1022-1024.

Song, A., Wang, Q., Goebl, M. G. and Harrington, M. A. (1998). Phosphorylation of nuclear MyoD is required for its rapid degradation. *Mol. Cell. Biol.* **18**, 4994-4999.

Sorrentino, V., Pepperkok, R., Davis, R. L., Ansorge, W. and Philipson, L. (1990). Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. *Nature* **345**, 813-815.

Steinbach, O. C., Ulshofer, A., Authaler, A. and Rupp, R. A. (1998). Temporal restriction of MyoD induction and autocatalysis during Xenopus mesoderm formation. *Dev. Biol.* **202**, 280-292.

Stutz, F. and Spohr, G. (1986). Isolation and characterization of sarcomeric actin genes expressed in Xenopus laevis embryos. *J. Mol. Biol.* **187**, 349-361.

Su, J. Y., Rempel, R. E., Erikson, E. and Maller, J. L. (1995). Cloning and characterization of the Xenopus cyclin-dependent kinase inhibitor p27XIC1. *Proc. Natl. Acad. Sci. USA* **92**, 10187-10191.

Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P. F., Weintraub, H. and Lassar, A. B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* **242**, 405-411.

Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B. and Weintraub, H. (1989). Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58**, 241-248.

Toyoshima, H. and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**, 67-74.

Waga, S., Hannon, G. J., Beach, D. and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**, 574-578.

Wang, J. and Walsh, K. (1996). Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. *Science* **273**, 359-361.

Wittenberger, T., Steinbach, O. C., Authaler, A., Kopan, R. and Rupp, R. A. (1999). MyoD stimulates delta-1 transcription and triggers notch signaling in the Xenopus gastrula. *EMBO J.* **18**, 1915-1922.

Yan, Y., Frisen, J., Lee, M. H., Massague, J. and Barbacid, M. (1997). Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11**, 973-983.

Zabludoff, S. D., Csete, M., Wagner, R., Yu, X. and Wold, B. J. (1998). p27Kip1 is expressed transiently in developing myotomes and enhances myogenesis. *Cell Growth Differ.* **9**, 1-11.

Zezula, J., Casaccia-Bonnefil, P., Ezhevsky, S. A., Osterhout, D. J., Levine, J. M., Dowdy, S. F., Chao, M. V. and Koff, A. (2001). p21cip1 is required for the differentiation of oligodendrocytes independently of cell cycle withdrawal. *EMBO Rep.* **2**, 27-34.

Zhang, P., Liegeois, N. J., Wong, C., Finegold, M., Hou, H., Thompson, J. C., Silverman, A., Harper, J. W., DePinho, R. A. and Elledge, S. J. (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* **387**, 151-158.

Zhang, P., Wong, C., DePinho, R. A., Harper, J. W. and Elledge, S. J. (1998). Cooperation between the Cdk inhibitors p27(KIP1) and p57(KIP2) in the control of tissue growth and development. *Genes Dev.* **12**, 3162-3167.

Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W. and Elledge, S. J. (1999). p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev.* **13**, 213-224.