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Cdkn1c drives muscle differentiation through a positive feedback loop with Myod

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Abstract

Differentiation often requires conversion of analogue signals to a stable binary output through positive feedback. Hedgehog (Hh) signalling promotes myogenesis in the vertebrate somite, in part by raising the activity of muscle regulatory factors (MRFs) of the Myod family above a threshold. Hh is known to enhance MRF expression. Here we show that Hh is also essential at a second step that increases Myod protein activity, permitting it to promote Myogenin expression. Hh acts by inducing expression of *cdkn1c* (*p57^{Kip2}*) in slow muscle precursor cells, but neither Hh nor Cdkn1c is required for their cell cycle exit. Cdkn1c co-operates with Myod to drive differentiation of several early zebrafish muscle fibre types. Myod in turn up-regulates *cdkn1c*, thereby providing a positive feedback loop that switches myogenic cells to terminal differentiation.

Keywords

muscle; *Cdkn1c*; zebrafish; Hedgehog; myod; myog; p57kip2

INTRODUCTION

Positive feedback helps commit cells to a differentiation step. A classical example is suggested by Myod auto-regulation during myogenesis (Weintraub, 1993). Myod is a transcription factor required for timely differentiation of certain muscle fibre populations in mice and zebrafish (Hammond et al., 2007; Hinits et al., 2009; Kablar et al., 1997; Maves et al., 2007; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). *Myod* reporter genes are down-regulated in *myod* null mice (Chargé et al., 2008; Kablar et al., 2003; Kablar et al., 1997), suggesting that positive feedback by Myod acts at the level of *myod* gene transcription. Indeed, Myod expression peaks early in differentiation of cultured myoblasts (Halevy et al., 1995). However, knockdown of Myod protein in zebrafish does not appear to decrease *myod* mRNA (Hinits et al., 2009; Maves et al., 2007). Thus, although Myod is essential for myogenesis in animals lacking the related Myf5/Mrf4 proteins (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993), whether Myod auto-regulation is required for muscle cell terminal differentiation is unclear.

MRF activation and cell cycle exit are two key steps in terminal myoblast differentiation (Andres and Walsh, 1996; Halevy et al., 1995). Studies in cell culture reveal that Myod can help drive cell cycle exit in multiple ways. One route is through activation of the cyclin-dependent kinase inhibitor Cdkn1a/p21^{Cip1}, a protein that regulates cell cycle exit in G1

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through its action on several CDKs (Nagahama et al., 2001). However, in vivo evidence that Cdkn1a is important for myogenesis is weak because *cdkn1a* null mice are viable and fertile (Deng et al., 1995). *cdkn1a* is a member of the Cip/Kip family of CDK inhibitors, which include *cdkn1b/p27^{Kip1}* and *cdkn1c/p57^{Kip2}* (Nagahama et al., 2001). Although null mutations of *cdkn1b* or *cdkn1c* are also viable, double mutants for *cdkn1a;cdkn1c* show, among other defects, a severe reduction in muscle differentiation (Fero et al., 1996; Yan et al., 1997; Zhang et al., 1997; Zhang et al., 1999). Why myogenesis fails in these mutants is unknown. Beyond mice, the function of the Cdkn1 family in myogenesis has only been analysed in *Xenopus laevis*, where the Cdkn1 gene *p27^{Xic1}* is required for myogenesis and can cooperate with Myod (Vernon and Philpott, 2003). Whether and how Cdkn1 proteins cooperate with Myod in embryonic myogenesis is unknown.

To understand the common themes of vertebrate myogenesis, we and others have recently begun to analyse the function of Myod and other MRFs in the zebrafish (Hammond et al., 2007; Hinits et al., 2009; Maves et al., 2007). As in mice, either Myod or Myf5 is required for early myogenesis in the somite (Hammond et al., 2007; Rudnicki et al., 1993). Distinct populations of muscle fibres in the zebrafish somite require distinct extrinsic signals in order to express MRFs and undergo terminal differentiation (Barresi et al., 2000; Blagden et al., 1997; Chong et al., 2007; Coutelle et al., 2001; Groves et al., 2005; Hammond et al., 2007; Hirsinger et al., 2004; Lewis et al., 1999; Ochi et al., 2008). For example, Hedgehog (Hh) signals from the ventral midline are required for proper *myf5* and *myod* expression and slow muscle formation by adaxial cells (Barresi et al., 2000; Coutelle et al., 2001; Lewis et al., 1999; Ochi et al., 2008; Schauerte et al., 1998). Strikingly, however, MRF expression is initiated normally in the absence of Hh, suggesting that Hh is necessary for MRF maintenance, not initial induction (Ochi et al., 2008). Thus, expression of both *myod* and *myf5* mRNAs is not sufficient to initiate an auto-regulatory loop maintaining MRF expression in vivo. Why not?

Here we show that a second action of Hh on muscle precursor cells is to activate expression of *cdkn1c/p57^{kip2}*, initiating a positive feedback loop that stabilizes Myod protein and permits it to activate *myogenin* expression and drive muscle terminal differentiation. Cdkn1c does not act by promoting cell cycle exit. These findings reveal a role of Cdkn1c in terminal differentiation that goes beyond its known function in other cells during cell cycle exit.

Materials and methods

Zebrafish lines and maintenance

Null mutant lines *smo^{b641}* (Varga et al., 2001), *myf5^{hu2022}* (Hinits et al., 2009) and *shha^{tbx392}* (Schauerte et al., 1998) were maintained on King's wild type background. Staging and husbandry were as described previously (Westerfield, 1995).

In situ mRNA hybridization, immunohistochemistry and Western analysis

In situ mRNA hybridization for *myf5, myod, myog, mrf4, eng2a* and *prdm1* was as described previously (Hinits et al., 2009). Additional probes were *hsp90a, cdkn1a* (Lee et al., 2008), *cdkn1b* (IMAGE 7002450), *cdkn1bl* (IMAGE 6799784) and *cdkn1c* (IMAGE 6892669). Embryos for immunohistochemistry were fixed in 4% PFA for 30 min and stained as described (Blagden et al., 1997; Groves et al., 2005). Primary antibodies used were MyHC (A4.1025) (Blagden et al., 1997), slow MyHC (F59) (Devoto et al., 1996), zebrafish Myod (Hammond et al., 2007), Myogenin (Hinits et al., 2009) and ß-Tubulin (Amersham N357). Myod antibody was absorbed against 8-24 hpf methanol-fixed embryos before use. HRP- (Vector) or Alexa dye-conjugated (Invitrogen) secondary antibodies were

used with Citifluor mountant (Agar). Confocal images were collected on a Zeiss LSM510. Western analysis was as described (Hinits et al., 2009).

Embryo Manipulations

Embryos were injected with MOs described previously to *myod* (2-4 ng), *myf5* (2-4 ng), *myog* (1-2 ng) (Hinits et al., 2009) and *cdkn1c* ATG MO (1-2 ng) (Shkumatava and Neumann, 2005), Park MO (3-5 ng) (Park and Chung, 2001) and 5′ MO (1-2 ng 5′tcaatgccgtgagccgacgtttgtt3′). Controls were vehicle or, in Fig. 6D, irrelevant mismatch MO 5′tgcttgatcatcctgagacaggcag3′. Cyclopamine (200 μM in fish medium) or vehicle control was added at 30-50% epiboly to embryos whose chorions had been punctured with a 30G hypodermic needle. BrdU treatment was performed as described (Appel et al., 2001). RNA (100 pg) was made with Ambion Megascript kit from pSP64T-*shha* or pßUT3 containing full length zebrafish *myod* subcloned into SacI/SalI sites and injected into embryos at 1-2 cell stage.

Full length zebrafish *cdkn1c* from IMAGE:6892669 was subcloned into the XbaI site of hsp70-4-MCS-IRES-mGFP6 (Hinits et al., 2007), adding a 5′ myc-tag and XbaI linkers with primers: 5′TCTTCTAGAATGCAGAAGCTGATCTCAGAGGAGGACCTGATGGCAAACGTGGACGTATC3′ and 5′TCTTCTAGATCATCTAATAGTTTTACGT3′ and sequence verified. DNA encoding this or Xenopus p28^{Kix1}, the closest *cdkn1c* homologue (Habermann et al., 2004), under hsp70/4 heatshock control (Yamaguchi et al., 2005) was injected at 1 cell stage. Heat shock 39°C was applied at 5-12 ss for 1 hour and rescue analysed 4-6 somite stages later. For analysis of whether an embryo was rescued or not, embryos were sorted into those with and without detectable MyHC. All, or in larger experiments a sample, of MyHC positive embryos were flatmounted and individually scored as either ‘strong’ or ‘weak’ based on the number and intensity of MyHC positive adaxial fibres. The data shown are the fraction of ‘strong’ embryos out of the entire sample across all experiments. Controls shown are heat-shock without vector injection, but also included vector injection without heat-shock. *Smo*^{b641} mutants were identified by *myod* mRNA pattern.

RESULTS

Myod protein accumulation is regulated by Hedgehog

Myf5 and Myod act together in adaxial cells to drive slow myogenesis (Hammond et al., 2007; Hinits et al., 2009; Maves et al., 2007). Initiation of *myf5* and *myod* expression in adaxial cells is independent of Hh (Ochi et al., 2008). However, *smoothened* (*smo*^{b641}) mutant embryos, which lack an essential component of the Hh signaling pathway, fail to maintain *myod* or *myf5* expression in adaxial cells and do not form adaxial-derived slow muscle fibres (Barresi et al., 2000). The same result arises in embryos treated with cyclopamine (cyA), a drug that inhibits all Hh signalling and prevents slow myogenesis (Barresi et al., 2001). In contrast, *sonic hedgehog a* mutants (*shha*^{tbx392}, *sonic you*) contain significant, albeit reduced, quantities of slow muscle, probably because two other Hh genes, *shhb* and *ihha*, are expressed in ventral midline tissues prior to the 5 som stage (5 ss) (Currie and Ingham, 1996; Ekker et al., 1995; Lewis et al., 1999). Surprisingly, we noticed that *smo*^{b641} and *shha*^{tbx39} mutants have an almost indistinguishable failure of *myf5* and *myod* mRNA maintenance in slow cells (Fig. 1A; see Supplementary Table S1 for numbers of embryos analysed and outcome(s) of all experiments). The sole difference is that, after an indistinguishable decline in expression in anterior presomitic mesoderm (PSM), the differentiated slow muscle cells in *shha*^{tbx392} mutant somites retain low levels of re-accumulated *myod* transcripts.

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Adaxial cells are not lost (Coutelle et al., 2001; Hirsinger et al., 2004). In cyA-treated, *smo*^{b641} and *shha*^{tbx392} embryos, adaxial cells retain strong *hsp90a* expression throughout the adaxial cell region (Fig. 1A). Similarly, adaxial cells, which are unlabelled by S-phase marker BrdU in wild type, remain unlabelled in cyA or *smo*^{b641} embryos (Fig S1). In 31 cyA or 15 *smo*^{b641} embryos, none of the approximately 480 adaxial cells per 8 ss embryo were BrdU labelled. Similarly, no Brdu labelling was observed in adaxial cells at later stages (data not shown). Thus, differential adaxial cell survival or proliferation does not account for the absence and presence of slow muscle in cyA/*smo*^{b641} and *shha*^{tbx392} mutants, respectively.

The formation of slow fibres in *shha*^{tbx392} mutants correlates with *myogenin* (*myog*) expression. *shha*^{tbx392} mutants express *myog* mRNA, whereas *smo*^{b641} or cyA embryos do not (Fig. 1B and data not shown). Myod protein was undetectable in adaxial cells of *smo*^{b641} and cyA embryos, although expression in presumed fast muscle precursors in the lateral somite is normal. In contrast, *shha*^{tbx392} embryos have significantly more Myod immunoreactivity in adaxial cell nuclei in PSM, although still much less than their siblings (Fig. 1C). We conclude that weak residual Hh signalling in *shha*^{tbx392} mutants promotes accumulation of Myod protein and *myog* expression, which might account for the residual slow myogenesis.

Myod promotes myogenin expression

As slow muscle formation correlates with MRF accumulation in adaxial cells, we asked whether Myod and/or Myog proteins drive adaxial slow muscle differentiation. Injection of wild type embryos with an antisense morpholino oligonucleotide (MO) to *myod*, which we have previously shown to knockdown Myod protein (Hammond et al., 2007), prevents *myog* mRNA accumulation in adaxial cells at 5 ss (Fig. 2A). This delays, but does not prevent, slow fibre formation, because of the presence of Myf5 (Hinits et al., 2009). Thus, in nascent adaxial cells Myod is rate limiting for *myog* expression and muscle differentiation.

We next tested whether Myogenin is also required for slow fibre formation. Two distinct *myog* MOs knockdown Myogenin protein and delay slow muscle differentiation (Hinits et al., 2009). However, *myog* MOs do not ultimately prevent slow myogenesis (Fig. 2C). Therefore, Myod-driven *myog* expression hastens slow muscle formation, possibly by raising overall MRF activity.

Myod and Myogenin are essential for slow myogenesis in shha mutant

We tested the role of MRFs in *shha*^{tbx392} mutants, a situation in which MRF activity may be limiting after failure of *myf5* and *myod* mRNA maintenance (Fig. 1A). Injection of either *myod* or *myog* MOs into a *shha*$^{tbx392/+}$ incross ablates the residual slow muscle normally observed in mutants (Fig. 2C). *Myf5* MO has no such effect. *Mrf4* is not expressed early in *shha*^{tbx392} mutants, and therefore cannot drive slow fibre formation (Hinits et al., 2007). Thus, by driving *myog* expression, Myod accounts for the presence of slow fibres in *shha*^{tbx392} mutants. As Hh signalling is essential for adaxial slow myogenesis and yet the accumulation of *myod* and *myf5* mRNAs are indistinguishable between *smo*^{b641} and *shha*^{tbx392} mutants, some other action of weak Hh signalling must account for the activation of Myod and induction of *myog*.

Cdkn1c is a Hh-dependent myogenic regulator present in adaxial cells

We hypothesized that residual Hh signalling promotes Myod stability and/or activity. In the retina, Shha drives terminal differentiation of neurons through Cdkn1c, also known as p57^{Kip2} (Shkumatava and Neumann, 2005). Loss of Cdkn1 activity leads to failure of murine myogenesis (Zhang et al., 1999). Zebrafish *cdkn1c* is expressed in adaxial cells

throughout their differentiation, as well as in the central nervous system and the base of the notochord (Fig. S2; Park et al., 2005). *Cdkn1c* mRNA is essentially eliminated from adaxial cells, but not from the notochord, by Hh blockade (Fig. 3A). Conversely, Shha over-expression up-regulates Cdkn1c (Fig. 3A). In contrast, *shha*^{tbx392} mutants have reduced but significant levels of adaxial *cdkn1c* mRNA; expression is near normal as cells leave the tailbud, but is much below the control level in more anterior adaxial cells of nascent somites (Fig. 3A). The correlation of residual *cdkn1c* mRNA with residual Myod protein (compare Figs 1C and 3A) makes it an excellent candidate Myod regulator.

Cdkn1c promotes Myod accumulation in adaxial cells

We used three different *cdkn1c* MOs, two previously shown to knockdown Cdkn1c in brain and retina (Park et al., 2005; Shkumatava and Neumann, 2005), and were able to reduce *myog* mRNA in *shha*^{tbx392} mutant embryos and delay it in siblings (Fig. 3B). *Cdkn1c* MO greatly decreased slow muscle differentiation in *shha*^{tbx392} mutants, but siblings were not noticeably affected (Fig. 3C lower panel). We conclude that Cdkn1c promotes slow fibre terminal differentiation when MRF levels are limiting.

Cdkn1c acts downstream of *myod* mRNA accumulation. *Cdkn1c* MOs did not reduce *myod* or *myf5* mRNA levels in the adaxial region, although *myod* expression in differentiated slow fibres within somites of *shha*^{tbx392} mutants was diminished, paralleling the reduction in slow fibre differentiation (Figs 3C and S3A). Cdkn1c has been suggested both to stabilize Myod protein through its effect on CDK2 activity and to enhance Myod DNA binding through direct protein:protein interaction (Reynaud et al., 2000; Reynaud et al., 1999). *Cdkn1c* MO-injected wild type embryos have reduced Myod immunoreactivity in adaxial nuclei, which is not apparent in the more numerous lateral fast muscle precursors (Fig. 3C,D). Consistent with this, Western analysis failed to reveal a significant alteration in level or size of Myod protein in 24 hpf embryos (Fig. S3B). *Cdkn1c* MO-injected *shha*^{tbx392} mutant (*cdkn1c*$^{-}$;*shha*^{tbx392}) embryos had Myod in the tailbud region, but it was reduced and did not persist in numerous adaxial cells in more anterior PSM, as it did in control *shha*^{tbx392} mutants (Fig. 3C upper panel). In *smo*^{b641} mutants, however, even less residual Myod protein was detected than in *cdkn1c*$^{-}$;*shha*^{tbx392}, paralleling their lower formation of residual slow fibres (Fig. 1C). Thus, in adaxial cells, Cdkn1c promotes Myod protein accumulation in vivo.

MRFs maintain Cdkn1c expression in adaxial cells

In mice, Cdkn1c co-operates with Cdkn1a to drive myogenesis (Zhang et al., 1999). Among the three additional zebrafish cdkn1 genes, *cdkn1a* (p21^{Cip1}), *cdkn1b* and *cdkn1b-like* (both p27^{Kip1} homologues), none are significantly expressed in adaxial cells, although *cdkn1b* mRNA is observed in the lateral somite, where fast muscle forms (Fig. S4). Thus, Cdkn1c appears to be the major Cdkn1 involved in zebrafish adaxial myogenesis.

Mammalian Cdkn1a and Xenopus p27^{Xic1} are Myod targets that mediate cell cycle withdrawal (Halevy et al., 1995; Vernon and Philpott, 2003; Wang and Walsh, 1996). Knockdown of Cdkn1c did not lead to proliferation of adaxial cells (none of the ~480 adaxial cells in any of 62 morphant embryos, Fig. S1, Table S1). We therefore tested whether MRFs were required to maintain *cdkn1c* expression. When both Myf5 and Myod proteins were depleted in the same embryo, adaxial *cdkn1c* mRNA was reduced in PSM and essentially ablated in somites (Fig. 4A). Nevertheless, significant adaxial *cdkn1c* mRNA was present in PSM adaxial cells flanking the tailbud (Fig. 4A). Loss of *myf5* alone did not diminish *cdkn1c* mRNA, but *myod* MO reduced *cdkn1c* mRNA, particularly in rostral somites (Fig. 4B). Conversely, when Myod was over-expressed by mRNA injection, ectopic *cdkn1c* and *myog* mRNAs and slow myogenesis were observed in both somitic and head

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mesoderm (Fig. S5). Thus, MRFs drive *cdkn1c* expression, implicating Cdkn1c in a positive feedback loop whereby MRF activity induces Cdkn1c, which then enhances MRF protein activity leading to terminal differentiation.

Hh promotes Cdkn1c expression specifically in tailbud

To distinguish better between a direct effect of Hh on *cdkn1c* expression and an indirect effect via MRF activity, we next knocked down Myod activity in embryos expressing ectopic Shha. Shha could still induce ectopic *cdkn1c* and ectopic slow fibres, showing that Myod was not essential (Fig. 4D,E). This finding raised the possibility that Myf5, which is also induced by ectopic Shha (Coutelle et al., 2001), might help mediate *cdkn1c* up-regulation. Knockdown of both Myf5 and Myod does not prevent Shha-driven up-regulation of *cdkn1c* mRNA in the lateral tailbud (Fig. 4D), confirming that nascent adaxial cells in tailbud regions do not require MRF activity, but that Hh signalling acts independently to promote *cdkn1c* expression. Strikingly, however, this ectopic *cdkn1c* mRNA is not maintained as tailbud cells enter the anterior PSM, and ectopic slow fibres do not form at 24 hpf, demonstrating that MRF activity is essential for the maintenance of Cdkn1c and slow myogenesis in the lateral PSM region (Fig. 4D,E).

Cdkn1c rescues adaxial myogenesis in the absence of Hedgehog signalling

We next asked whether Cdkn1c can act in the absence of Hh signalling. A notable, but little emphasized, characteristic of both *smo*^{b641} mutant and cyA-treated embryos, is the presence of rare residual slow muscle fibres, that form prior to the generation of secondary slow fibres (Barresi et al., 2001; Hirsinger et al., 2004)(Fig. 5). We dub this process ‘escape’, and asked whether it depends on Cdkn1c. Knockdown of Cdkn1c in *smo*^{b641} prevents most escape, whether assayed at 15 ss or 24 hpf (Fig. 5A,C). This suggests that Cdkn1c enhances slow myogenesis in the absence of Hh signalling, presumably through MRF-driven positive feedback.

A stringent test of the ability of Cdkn1c to promote MRF activity is to rescue adaxial myogenesis in embryos that entirely lack Hh signalling and fail to maintain MRF mRNAs. We induced zebrafish or Xenopus Cdkn1c in either cyA embryos or a *smo*$^{b641/+}$ incross by heat shock at 8-15 ss and assayed myogenesis at 12-18 ss (Fig. 5B,C). Isolated adaxial cells in *smo*^{b641} or cyA embryos expressing Cdkn1c and eGFP frequently differentiated into muscle, whereas control injected or un-heat-shocked adaxial cells did not (Fig. 5B,C). Rescued cells were restricted to anterior somites but less than half accumulated detectable eGFP, perhaps due to the short period post-heat-shock (Fig. 5B). No such rescue was observed in cyA-treated heat-shocked embryos injected with *myod* MO in addition to the *cdkn1c* heat-shock construct (Fig. 5C). As adaxial cells in *smo*^{b641} or cyA embryos do not proliferate despite failing to differentiate (Fig. S1), Cdkn1c does not simply act by forcing cell cycle exit. We conclude that Cdkn1c activity is sufficient to rescue adaxial myogenesis in the absence of Hh, presumably by promoting MRF activity. Thus, by promoting Myod accumulation from the otherwise transiently induced *myod* mRNA, Cdkn1c triggers a positive feedback loop that rescues adaxial myogenesis.

Cdkn1c drives fast fibre differentiation

Cdkn1c is expressed in fast myogenic cells from mid-somitogenesis until at least 24 hpf (Figs 3A and S2). Reduction of MRF activity severely curtails *cdkn1c* expression throughout the somites, including the precursors of fast cells, but does not inhibit neural expression (Fig. 4C). Myf5 and Myod knockdown leaves small groups of cells in the medial region of each somite retaining *cdkn1c* mRNA (Fig. 4C). These are the Hh-dependent residual fast muscle fibres that escape MRF knockdown, as shown by their expression of *myhz1* mRNA (Hinits et al., 2009). Knockdown of Cdkn1c entirely ablates these residual

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fast fibres, suggesting that Cdkn1c activity enhances the low residual activity of Myod in *myf5;myod* morphants or *myf5* mutants injected with *myod* MO (Fig. 6A and data not shown). Moreover, loss of Cdkn1c enhances the loss of fast muscle caused by *myod* MO alone (Fig. 6A). Therefore, Cdkn1c can also contribute to differentiation of fast fibres.

To test for the function of Cdkn1c in otherwise un-manipulated fast muscle, we injected *cdkn1c* MOs into embryos and analysed expression of fast muscle markers. Little change in *myhz1* mRNA or MyHC immunoreactivity was apparent in *cdkn1c* morphants when viewed in wholemount, yet sectioning at 24 hpf revealed a reduction in fast muscle area (Fig. 6B). No obvious change in accumulation of any MRF mRNA was detected in *cdkn1c* morphants, but Myod and Myogenin proteins were reduced in specific cell groups (Figs 6C and data not shown). Thus, Cdkn1c is required for efficient fast muscle differentiation, and appears to act on MRFs downstream of their transcription.

Myod and Cdkn1c interact genetically

To test the hypothesis that Myod and Cdkn1c act in the same pathway to drive muscle differentiation we looked for genetic interactions. Loss of Myod function in zebrafish leads to ablation of head myogenesis (Hinits et al., 2009). *Cdkn1c* is expressed in head muscle anlagen at 48 hpf, just as differentiation commences (Fig. S2). *Cdkn1c* morphant embryos showed a similar reduction in head myogenesis, which was particularly marked in the ventral hyoid muscles (Fig. 6D). Co-injection of low doses of *cdkn1c* MO and *myod* MO, which alone had no significant effect on myogenesis, led to depletion of head muscle, particularly the hyohyoideus and interhyoideus (Fig. 6D). Thus, *cdkn1c* is epistatic to *myod* and the genetic enhancement of *myod* loss of function shows that Cdkn1c and Myod operate in the same pathway.

DISCUSSION

The current work makes three major points. First, that Cdkn1c functions to promote both slow and fast myogenesis in the zebrafish. Second, that muscle terminal differentiation is driven by a positive feedback loop, with Cdkn1c stabilizing and activating Myod which, in turn, maintains *cdkn1c* expression. Third, that in slow muscle Hh drives *cdkn1c* expression leading to active Myod, *myog* expression and differentiation. Our work shows how a cell cycle regulator functions beyond the cell cycle to regulate terminal differentiation in vivo.

Cdkn1c and myogenesis

Our data show that Cdkn1c synergizes genetically with Myod to promote myogenesis. Although a major role of Cdkn1c elsewhere is to inhibit the G1/S transition, we find that Cdkn1c is not essential for cell cycle exit in slow muscle precursors: *cdkn1c* mRNA is lost in Hh signalling blockade and yet adaxial cells do not enter S-phase. Moreover, Cdkn1c knockdown does not prevent adaxial cell cycle exit, consistent with the absence of mRNA encoding cyclin D or E homologues in adaxial cells (www.zfin.org). Cdkn1c not only inhibits CDKs, but can also bind to a variety of other cellular proteins including Myod, PCNA, Skp2, Nr4a2 and LIMK-1 (www.hprd.org). It is highly likely that Cdkn1c promotes myogenesis through Myod. Firstly, we show that loss of Cdkn1c function mildly phenocopies loss of Myod. Second, Cdkn1c promotes accumulation of Myod immunoreactivity in adaxial cells. Third, Cdkn1c can rescue slow myogenesis only if Myod is present. Fourth, as has previously been shown, in cultured cells Cdkn1c can directly bind and stabilize Myod and may additionally enhance Myod activity by preventing CDK-dependent phosphorylation that otherwise leads to Myod degradation (Reynaud et al., 2000; Reynaud et al., 1999). Although zebrafish are not amenable to similar biochemical analyses,

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taken together, these data argue strongly that Cdkn1c acts in zebrafish myogenesis by promoting Myod activity.

The role of Cdkn1c in myogenesis appears to be ancient. In both mice and zebrafish, *cdkn1c* is highly expressed in muscle tissues and, while not essential for all myogenesis, contributes to muscle terminal differentiation (Yan et al., 1997; Zhang et al., 1997). Surprisingly, a *cdkn1c* gene has not been identified in *Xenopus laevis*, reptiles or birds. The lack of slow adaxial muscle in *X. laevis* trunk parallels this loss of the *cdkn1c* present in ancestral amphibia (Grimaldi et al., 2004; Habermann et al., 2004). Nevertheless, the unique amphibian Cdkn1 family member $p27^{Xic1}$ drives myogenesis in *X. laevis*, implying functional conservation of Cdkn1 action in muscle (Vernon and Philpott, 2003). In chicken, Cdkn1c function may also have been taken by Cdkn1b ($p27^{Kip1}$), which can promote muscle differentiation in cell culture (Leshem et al., 2000; Messina et al., 2005). We observe zebrafish *cdkn1b* expression specifically in fast muscle precursors, followed by *cdkn1c*. This suggests that each has a role in successive phases of the terminal differentiation process, as recently observed in murine pituitary development (Bilodeau et al., 2009). Mouse Cdkn1c is reported to accumulate in a subset of muscle nuclei (Zhang et al., 1997), reminiscent of the differential expression and role of *cdkn1c* in subsets of zebrafish myogenic cells. We wonder whether differing requirement for Cdkn1 function may reflect a fundamental distinction within vertebrate muscle cell populations.

In zebrafish, Cdkn1c is required for a subset of Myod-dependent head and lateral somitic muscles. Myod acts together with Myog in these muscles, independent of a Myf5 requirement, which we have termed the lateral mode of myogenesis (Hinits et al., 2009). In mice, *Cdkn1c* and *Myod* are also required in subsets of lateral somite and head muscles. Only certain somite-derived and head myogenic cells require MyoD, so that defects in *Myod* nulls appear transient (Kablar et al., 1997). Similarly, *cdkn1c* null mice lack lateral somite-derived abdominal muscle, yet some recover and survive (Zhang et al., 1997). The transient nature of the murine defects have prevented thorough analysis to date, but the similarities between species suggest an ancient function for Myod and Cdkn1c in lateral somitic myogenic mode.

We find that Cdkn1c co-operates with Myod to drive *myog* expression. However, Myogenin itself has a relatively minor role in early zebrafish myogenesis, contrasting with its major role in mice (Hasty et al., 1993; Nabeshima et al., 1993). The importance of Myogenin in mouse may relate to the greater myogenic role of Mrf4 (Kassar-Duchossoy et al., 2004; Rawls et al., 1998), and the more complex involvement of Cdkn1 family members. Murine *Cdkn1a* is a Myod target gene that is also expressed in early myogenesis and during terminal differentiation of myoblasts. *Cdkn1a* is redundant with *cdkn1c* for cell cycle exit. The phenotype of loss of function of both Cdkn1s is strikingly similar to a *myogenin* null (Halevy et al., 1995; Parker et al., 1995; Zhang et al., 1999). Although mouse *myogenin* expression is not entirely Cdkn1-dependent, these correlations demand further study.

Like *cdkn1c, cdkn1a* is also an ancient vertebrate gene, but is not detectably expressed in embryonic zebrafish muscle, suggesting it lacks a function in myogenesis, perhaps because of the limited proliferation in this species. Alternatively, murine *Cdkn1a* may have been recruited into myogenesis during the evolution in mammals of imprinting at the syntenically-conserved *Cdkn1c* region (Dunzinger et al., 2007). Imprinting represses paternal *Cdkn1c*, which might, without Cdkn1a, have prevented proper muscle differentiation in the fetus. As mammalian Cdkn1a now drives myoblast cell cycle exit, Cdkn1c may primarily regulate differentiation in mice, as in zebrafish adaxial cells. On the other hand, our data do not eliminate the possibility that Cdkn1c may regulate both cell cycle exit and differentiation in other zebrafish muscle cell types. Strikingly, repression of

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human *CDKN1C* in region 11p15 is implicated in Beckwith-Weidemann Syndrome (BWS), and polymorphisms in *CDKN1C* are associated with increased risk of atherosclerosis and myocardial infarction (Rodriguez et al., 2007; Zhang et al., 1997). The finding that loss of Cdkn1c disrupts cranial and lateral somitic myogenesis suggests that *CDKN1C* may contribute to the overgrowth of tongue muscle in BWS. Our data raise the question of whether Cdkn1c dysfunction contributes to other muscle disease, which often targets specific cranial or limb muscle groups.

Cdkn1c and Myod positive feedback loop

How does Cdkn1c promote Myod activity? It has been proposed that CDKs phosphorylate and inactivate Myod in proliferating myoblasts (Reynaud et al., 1999). In post-mitotic cells, similarly, CDK activity may decrease Myod activity and/or stability. Our data show that adaxial Cdkn1c promotes Myod accumulation, but is not required for cell cycle exit. So Cdkn1c may both confirm cell cycle exit, through inhibition of CDK activity, and also drive cells to terminal differentiation through the combined effects of decreased Myod phosphorylation and direct Myod binding by Cdkn1c, both of which stabilize Myod (Reynaud et al., 2000; Reynaud et al., 1999). Our data do not, however, rule out a contribution of other mechanisms by which Cdkn1c might enhance Myod activity. Indeed, we have been unable to quantify any stabilization or altered phosphorylation of Myod in vivo after Cdkn1c manipulation, although this may be due to the presence of Myod in multiple cell populations.

We find that Myod activity is also required to maintain adaxial *cdkn1c* expression. In cultured murine cell lines kept in differentiation conditions, Myod has been shown to regulate Cdkn1c indirectly when Cdkn1a is absent (Bean et al., 2005; Figliola et al., 2008; Vaccarello et al., 2006). Our finding that Cdkn1c promotes early zebrafish muscle differentiation in the absence of *cdkn1a* expression suggests that similar mechanisms operate in vivo. We propose a model in which positive feedback between Cdkn1c and Myod drives terminal differentiation (Fig. 7). The model contains a striking symmetry: two extracellular signals (Fgf and Hh) each initiate one half of a positive feedback system. Each initial response (MRF and *cdkn1c* expression, respectively) then acts to maintain and amplify the other. Ultimately, this leads to an irreversible commitment to terminal differentiation. Such a mechanism likely explains the correlations observed between *myod* and *cdkn1c* expression in various mammalian systems (Abou-Khalil et al., 2009; Bigot et al., 2008).

Cdkn1c and the role of Hedgehog in myogenesis

Positive feedback is a well-known mechanism for converting an analogue signal to a digital output; in this case, terminal differentiation. In zebrafish adaxial myogenesis, the level of Hh signaling regulates such a binary switch. We observed that, in *smo*^{b641} or cyA-treated embryos, which lack *cdkn1c* mRNA, *myod* mRNA is present in the tailbud but Myod protein generally does not accumulate. However, very rare cells do succeed in progressing to terminal differentiation. Such 'escape' is Myod-dependent and presumably, therefore, occurs in cells that accumulate enough Myod without the aid of normal Cdkn1c activity. We could ablate these residual slow fibres with *cdkn1c* MO. At an intermediate level of Hh signaling (in *shha*^{tbx392} mutants), a low level of *cdkn1c* mRNA accumulates. This Cdkn1c drives increase in Myod protein, initially without altering *myod* mRNA. The consequence is that about half the usual number of superficial slow muscle fibers form but, consistent with a binary switch model, those that do form express slow markers and undergo normal differentiation and migration. When Cdkn1c is experimentally reduced in *shha* mutants a further parallel reduction in adaxial Myod and residual slow muscle occurs. In the wild type condition, it seems that Cdkn1c is part of a system of genetic redundancy in terminal differentiation that establishes the correct number of slow muscle fibers. Both Hh and

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Cdkn1c promote fast muscle differentiation in *myf5+myod* morphant zebrafish (Hinits et al., 2009), suggesting that these proteins also cooperate in fast myogenesis. Hh-driven Cdkn1c expression appears to control the binary decision to cell cycle exit in the retina (Shkumatava and Neumann, 2005). It remains to be determined how widespread is the role of Hh and Cdkn1 in such analogue-to-digital conversion.

Hh signaling is a potent regulator of myogenesis, but has been described to both promote myoblast proliferation and drive terminal differentiation (Elia et al., 2007; Li et al., 2004). Susceptibility of Cdkn1 genes to Hh signaling may regulate a cell's response to Hh. Indeed, whereas *cdkn1c* expression is Hh-dependent in the muscle and retina, it is Hh-independent in notochord. Within muscle, situations in which Cdkn1 is not activated by Hh may favour proliferation. We have no evidence that Hh ever promotes proliferation of myogenic cells in zebrafish; its induction of Cdkn1c correlates with terminal differentiation. This suggests that, if Hh can drive Cdkn1 expression, promotion of terminal differentiation results. Our findings suggest that Cdkn1 function in myogenesis may form a node at which other signals can regulate Hh response.

That Cdkn1c over-expression alone can rescue slow myogenesis in *smo*^{b641} mutant or cyA-treated embryos is a dramatic demonstration of its central role in slow fibre formation. On the other hand, we did not observe ectopic slow fibres when Cdkn1c was over-expressed in lateral somitic cells, indicating that Cdkn1c is not responsible for determining the slow fate. At present, it is unclear if the action of Cdkn1c on Myod is sufficient to rescue slow fibres, or if other molecular targets are also involved. For example, adaxial expression of *prdm1*, which encodes a chromatin remodelling factor required for proper slow myogenesis, is Hh-dependent but MRF-independent (Baxendale et al., 2004; Hinits et al., 2009). It will be interesting to know if *prdm1* expression is also rescued by Cdkn1c. We note that Cdkn1c is insufficient to rescue all adaxial cells in which it is expressed. It is possible that Cdkn1c is required in a specific time window. Alternatively, only the adaxial cells with the highest level of residual *myod* mRNA may be rescued by Cdkn1c.

Our data show that *cdkn1c* sensitivity to Hh diminishes as the PSM matures. When MRF activity is blocked, to prevent the positive feedback loop we describe, *cdkn1c* is still Hh-responsive in tailbud, but not in anterior PSM. The molecular basis of this change in Hh sensitivity is unclear. It is known that, later in development, Hh signaling acts directly on lateral somitic cells to regulate dermomyotome behaviour (Feng et al., 2006). Although Fig. 4C shows that much *cdkn1c* expression in the lateral somite is MRF dependent, it will be important to determine how directly and independently Hh regulates MRFs and *cdkn1c* in dermomyotome.

Cdkn1c is one of the most highly up-regulated genes when murine myoblasts or muscle stem cells enter quiescence (Fukada et al., 2007). It seems likely that, in this context, Cdkn1c helps block the cell cycle but can not drive terminal differentiation, possibly due to a lack of MyoD. In human myoblasts, the parallel failure of MRF and Cdkn1c accumulation has been suggested to underlie replicative ageing (Bigot et al., 2008). It will be important to determine how cellular context controls the functioning of the positive feedback loop we have described.

Conclusion

Understanding the molecular mechanisms driving a committed cell to undergo terminal differentiation remains a fundamental problem in developmental and cell biology, with implications for stem cell science, regenerative medicine and cancer. Since the Weintraub lab's classic series of studies on *MyoD* auto-regulation, controlled positive feedback by

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MyoD has provided a paradigm in developmental biology. Subsequent work, however, has failed to prove that direct positive feedback of MyoD protein on the *Myod* gene has a significant role in either commitment to myogenesis or terminal differentiation. Here, we resurrect Weintraub's idea in new molecular clothes. We show how a major cell cycle regulator, Cdkn1c (p57^{Kip2}), helps drive terminal differentiation in vertebrate skeletal muscle in vivo by establishing a positive feedback loop with MyoD at the level of Myod protein activity, not *myod* transcription (Fig. 7). The work therefore fills a gap in the understanding of MyoD function in muscle differentiation; it shows that indirect positive feedback by Myod drives myogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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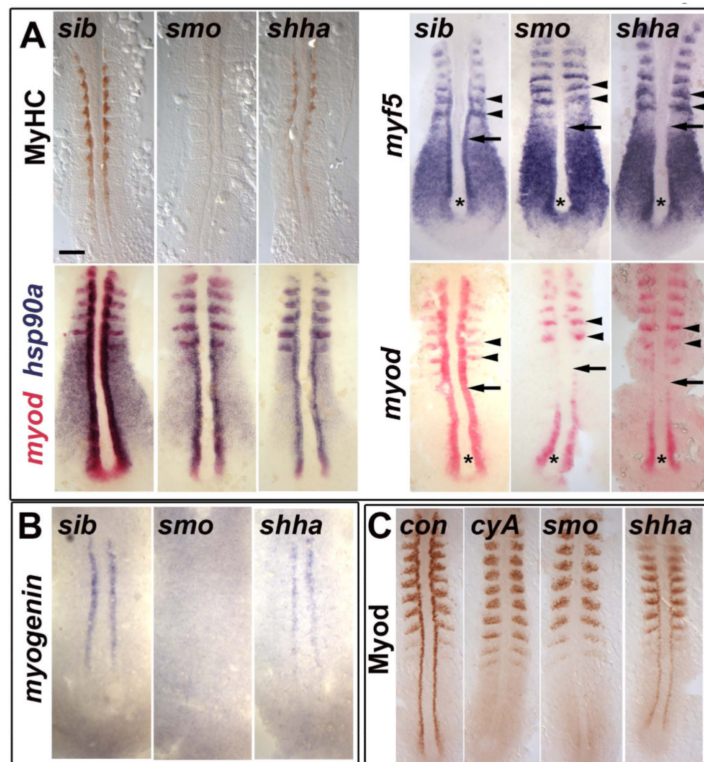

Figure 1. Myod protein level correlates with slow myogenesis in Hh signalling mutants

In situ mRNA hybridization for *myf5, myod, myog* and *hsp90a* and immunodetection of Myod and Myosin Heavy Chain (MyHC) in dorsal flatmounts of 5 ss stage control, cyclopamine-treated (cyA), smo^{b641} and $shha^{tbx392}$ mutant embryos. Mutants were identified by significant reduction in one or both markers in ~25% of lay ($shha^{tbx392}$: 9/41; smo^{b641} 14/50). Anterior to top. Bar: 100 μm. **(A).** Absence of MyHC in smo^{b641} but only reduction in $shha^{tbx392}$ mutants, despite indistinguishable loss of *myod* and *myf5* mRNAs in adaxial cells in anterior pre-somitic mesoderm (arrows). Note that *myf5* and *myod* expression in tailbud (asterisk) and presumptive fast muscle precursors (black arrowheads) is little affected by Hh manipulation. $Shha^{tbx392}$ differs from smo^{b641} only in expression of *myod* in differentiating adaxial muscle in somites (white arrowheads). **(B).** *Myog* mRNA is present in adaxial cells of $shha^{tbx392}$ but not smo^{b641} mutants. **(C).** Myod protein is absent in adaxial cells of cyA and smo^{b641} embryos but weakly present in $shha^{tbx392}$ mutant. Control embryos were either $shha^{tbx392}$ siblings or untreated wild type.

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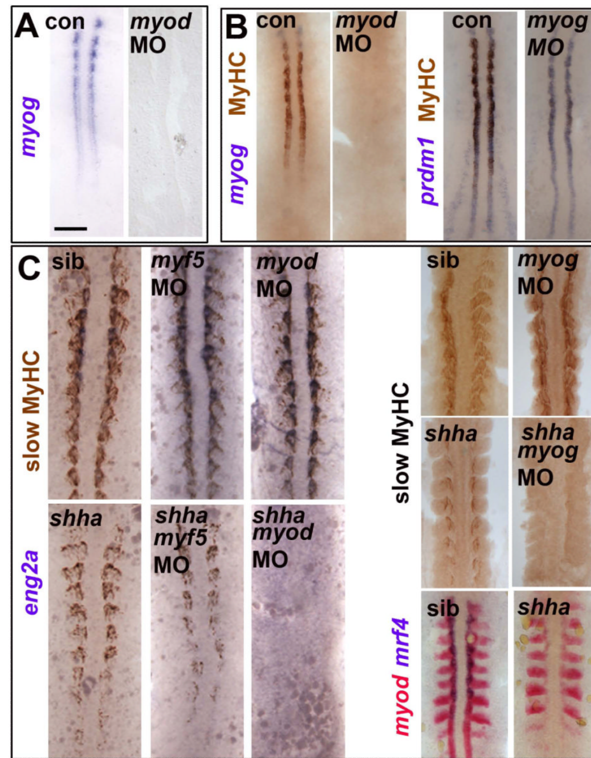

Figure 2. Myod-driven *myogenin* expression permits slow fibre formation in *shha* mutant

Myog mRNA or MyHC accumulation in uninjected, *myod* MO- or *myog* MO-injected embryos from wild type or $shha^{tbx/+}$ in-cross. Dorsal flatmounts of 5 ss (A,B) or 10-15 ss (C) embryos. Bar: 100 μm. **(A).** *Myog* mRNA is ablated by *myod* MO injection into wild type. **(B)** *Myod* and *myog* MOs each delay adaxial myogenesis. **(C)** *Myod* and *myog*, but not *myf5*, MOs ablate adaxial myogenesis in $shha^{tbx392}$ mutants (21/79), but not from siblings.

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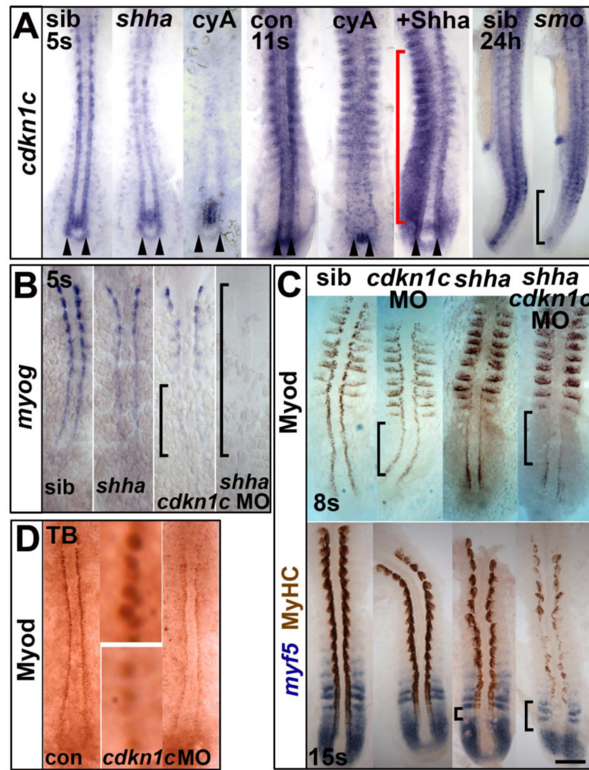

Figure 3. Hh causes Cdkn1c to stabilize Myod

(A). *Cdkn1c* mRNA is Hh-dependent in adaxial cells. *Shha*^{tbx392} or *smo*^{b641} mutation or cyA treatment reduces *cdkn1c* mRNA. Note that loss was proportional to reduction in Hh signal (arrowheads and black bracket). *Shha* mRNA injection caused unilateral somitic *cdkn1c* up-regulation (red bracket). **(B,C).** *Cdkn1c* 5′ MO injection into embryos from a *shha*$^{tbx392/+}$ incross reduced adaxial *myog* mRNA (B), Myod or MyHC proteins (C). Note delay in *myog* mRNA and reduction in Myod protein (brackets). **(D).** *Cdkn1c* ATG MO injection reduces adaxial Myod at tailbud stage. Bar: 100 μm.

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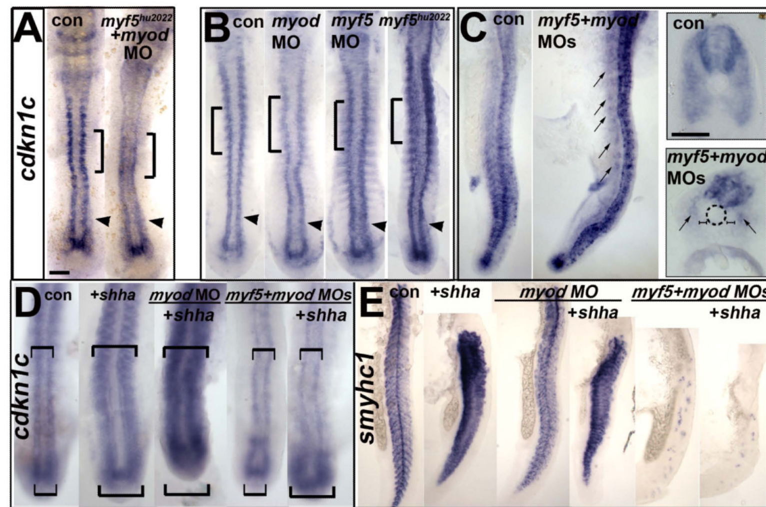

Figure 4. *Cdkn1c* expression depends on Hh in tailbud and on MRFs anteriorly

MO-injected wild type (B-D) or *myf5*^{hu2022} incross embryos (A,B) co-injected with *shha* mRNA (D,E) analysed for *cdkn1c* mRNA in 12-15 ss dorsal (A,B,D) or 24 hpf lateral flatmount, dorsal to right (C,E). Anterior is to top. Bars: flatmounts 100 μm, sections 50 μm. **(A-C)**. Adaxial *cdkn1c* mRNA accumulation initiates independent of MRF activity (arrowheads), but fails to be maintained (bracket). *Myod* MO reduces *cdkn1c* expression in older somites (brackets) but tailbud is unaffected (arrowheads). *Myf5* and *myod* knockdown ablates somite *cdkn1c* mRNA except in small groups of cells lateral to the un-migrated adaxial cells (arrows). Neural, cloacal and transient notochordal expression is unaffected. **(D).** Over-expression of Shha promotes *cdkn1c* up-regulation in anterior PSM and somite in contorls and after Myod knockdown, but not in *myf5+myod* double morphants (upper brackets). Note that Shha up-regulates *cdkn1c* in the lateral tailbud of *myf5+myod* double morphants (lower brackets), but this is not maintained in anterior PSM. **(E).** Over-expression of Shha promotes ectopic *smyhc1* expression at 24 hpf in control or *myod* morphants, but not in *myf5+myod* double morphant embryos.

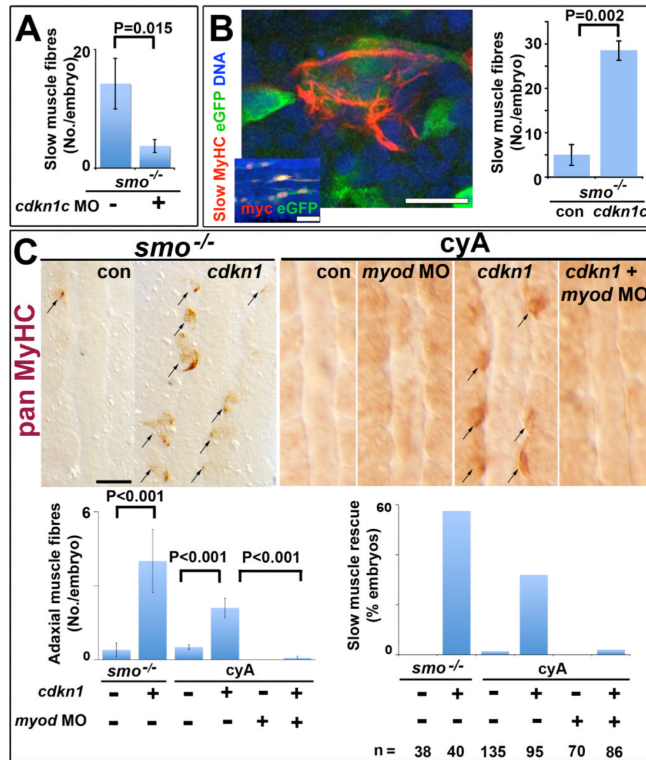

Figure 5. Cdkn1c rescue of slow myogenesis is Myod-dependent

Slow MyHC (A,B confocal sections) or pan MyHC (C, dorsal flatmount) in embryos lacking Hh signalling. (**A**). *Cdkn1c* MO injection reduces residual slow muscle fibres in 24 hpf *smo*^{b641} mutants. (**B**). Mosaic over-expression of zebrafish *cdkn1c* induced by heat-shock of *hs70/4:cdkn1cIRESeGFP* at 12 ss rescues slow MyHC at 18 ss. Inset shows coexpression of myc-Cdkn1c and eGFP. eGFP mosaicism was   5%, which corresponds to ~36 somitic adaxial cells at 18 ss. Bar: 10 μm; inset 50 μm. (**C**). Mosaic Xenopus *cdkn1* expression rescues adaxial muscle in *smo*^{b641} mutant or cyA-treated embryos at 10-12 ss. Cdkn1-driven rescue is prevented by *myod* knockdown. Charts show rescue of slow fibres (left, mean ±sem; Wilcoxon significance test) and fraction of embryos showing rescue (right; n = total embryos). Note that mosaic expression in ~10% of adaxial cells would mark ~33 somitic slow fibres per embryo at this stage. Bar: 50 μm.

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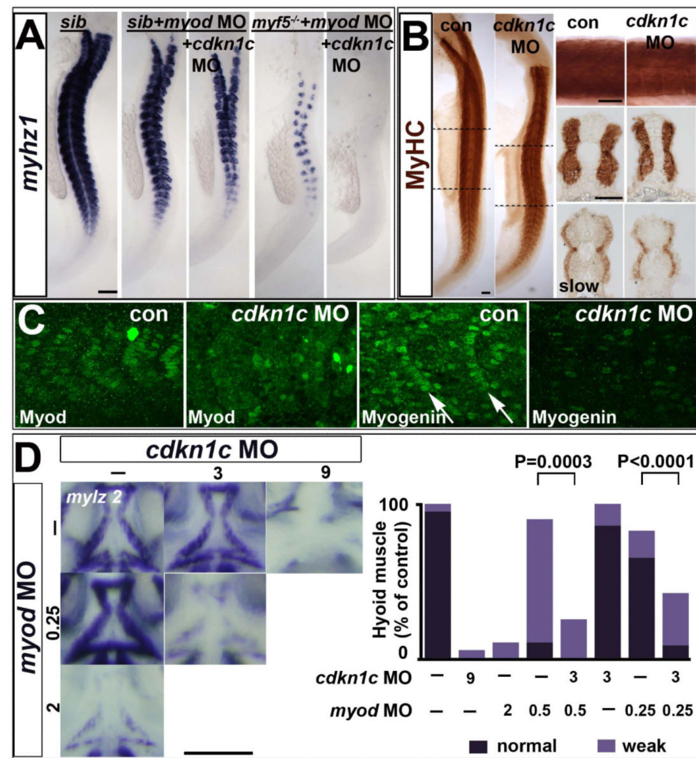

Figure 6. Cdkn1c drives fast myogenesis through Myod

In situ mRNA hybridization (A,D) or protein immunodetection (B,C) at 24 hpf (A-C; lateral flatmount) or 72 hpf (D, dorsal flatmount) of embryos injected with *cdkn1c* MO. **(A).** *Cdkn1c* MO exacerbated somitic fast muscle loss induced by *myod* MO, and ablated residual fast muscle in mutant *myf5*^{hu2022};*myod* knockdown. Bar: 100 μm **(B).** *Cdkn1c* MO alone diminishes fast muscle differentiation, without reducing slow muscle. Dashed lines indicate positions of upper and lower transverse sections. Bars: flatmounts 100 μm, somite lateral zoom and sections 50 μm **(C)**. *Cdkn1c* MO diminished Myod immunoreactivity in nuclei of nascent fast muscle and Myogenin immunoreactivity in more mature somites, particularly the expression at the posterior somite border (arrows). Bar: 50 μm. **(D).** *Cdkn1c* MO (9 ng) reduced ventral head myogenesis in a similar manner to *myod* MO (2 ng). Low doses of *cdkn1c* MO (3 ng) or *myod* MO (0.25 ng) that alone had little effect, synergised to reduce myogenesis in intermandibular, interhyoideus and hyohoideus muscles. Bar: 100 μm. Chart quantifies the extent of ventral hyoid muscle defects after injection of the indicated ng of each MO/embryo, with differences tested by X^2 test. All embryos were classified as having either ‘normal’, ‘weak’ or ‘no’ *mylz2* mRNA in the three stated muscles. ‘Normal’ embryos (dark bars) had strong *mylz2* expression, as shown in control panel. ‘Weak’ embryos (light bars) had *mylz2* expression visibly below the control range, as shown in the 2 ng *myod* MO panel. ‘No’ embryos entirely lacked *mylz2* expression, as in the 9 ng *cdkn1c* MO panel, are shown as white space above the bars. Data shown has n = 20 for each condition from within a single experiment.

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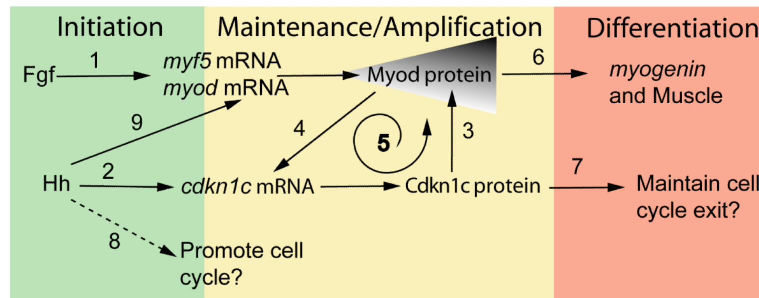

Figure 7. Model of Myod:Cdkn1c Positive Feedback

Adaxial MRF expression initiates independent of Hedgehog (Hh) signals, through Fgf (1). Hh initiates *cdkn1c* expression (2) leading to gradual accumulation of Myod protein (3) that maintains *cdkn1c* expression (4), creating a positive feedback loop (5) that triggers muscle differentiation (6). A possible additional role of Cdkn1 is maintenance of cell cycle exit (7). A proliferative effect of Hh in myogenesis has been reported in mouse myoblasts and chick somite cells, as indicated by the dashed line (8). However, all available evidence argues against a proliferative effect of Hh in zebrafish myogenesis. Instead, Hh apparently independently maintains MRF mRNA in anterior PSM (9).