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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.

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, 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 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, 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,

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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 30 G 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 signalling 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.

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