The RhoA effector mDiaphanous regulates MyoD expression and cell cycle progression via SRF-dependent and SRF-independent pathways

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Summary

Expression of the key muscle transcription factor MyoD is regulated by RhoA GTPase, which is an important regulator of adhesion-dependent signaling. We show that mDiaphanous (mDia) – an adaptor protein that mediates the effects of RhoA on cell motility and the cytoskeleton – is an upstream regulator of MyoD in C2C12 mouse myoblasts. Knockdown of mDia1 reduced MyoD expression and proliferation via a serum-response factor (SRF)-dependent pathway. Surprisingly, overexpression of a Rho-independent form of mDia1 (mDiaΔN3), despite activating SRF, also suppressed MyoD and the cell cycle, suggesting the presence of a second pathway downstream of mDia1. We present evidence that the alternative pathway by which mDia regulates MyoD involves T-cell factor (TCF)/lymphoid enhancer factor (LEF) and its co-activator, β-catenin. TCF activity was suppressed by mDiaΔN3 and induced by silencing mDia. mDiaΔN3 disrupted the signal-dependent nuclear localization of β-catenin and suppressed MyoD expression. Co-expression of a degradation-resistant form of β-catenin with mDiaΔN3 restored MyoD expression, suggesting a mechanistic link between the two signaling proteins. We also implicate a region encompassing the FH1 domain of mDia1 in β-catenin-TCF regulation. Taken together, our results suggest that a balance between two pathways downstream of mDia regulates MyoD expression and cell cycle progression.

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Introduction

Skeletal muscle formation occurs through a series of regulated events involving the specification of myoblasts, their expansion, migration, and withdrawal from the cell cycle, culminating in their differentiation into muscle fibers (reviewed by Tajbakhsh, 2005). Of the four myogenic regulatory factors (MRFs) – MyoD, Myf5, Myogenin and MRF4 – that control muscle-specific gene expression, MyoD and Myf5 are expressed in proliferating myoblasts and play crucial and partially overlapping roles in lineage restriction, whereas Myogenin, a target of MyoD, is induced during cell cycle exit associated with differentiation. During the regeneration of adult skeletal muscle after injury, dormant postnatale myoblasts or satellite cells are activated to divide and differentiate, recapitulating many embryonic myogenic processes (Seale and Rudnicki, 2000; Collins, 2006). MyoD expression in satellite cells is essential for effective regeneration (Megeny et al., 1996). However, the mechanisms that regulate MyoD expression in quiescent and activated satellite cells are poorly understood.

MyoD has potent muscle-determining activity, first defined by its ability to convert nonmyogenic cells to the myogenic fate (reviewed by Weintraub, 1993). Multiple inhibitory mechanisms counter MyoD activity in proliferating myoblasts (Wei and Paterson, 2001), preventing precocious differentiation. Less is known about the upstream mechanisms that regulate MyoD expression, in particular during the entry and exit of satellite cells from G0. MyoD is not detected in quiescent satellite cells (Grounds et al., 1992), is rapidly induced during activation in response to muscle injury, expressed in proliferating satellite cell progeny, but is suppressed in those cells that return to quiescence and replenish the resting progenitor pool (Zammit et al., 2004).

The current understanding of the molecular mechanisms by which regulation of MyoD expression is coupled to the cell cycle has benefited from studies in cultured myoblast lines derived from muscle satellite cells (reviewed by Dhawan and Rando, 2005). Using different strategies to generate synchronized myoblasts (Milasincic et al., 1996; Sachidanandan et al., 2002; Dhawan and Helfman, 2004), we have demonstrated that suppression of MyoD in G0 and induction during G1 progression is regulated by adhesion-dependent mechanisms. Induction of MyoD during G1 is associated with competence for myogenesis, but additional events govern the actual transition to differentiation (Wei and Paterson, 2001).

The small GTPase RhoA, a key regulator of adhesion-
dependent signaling (Ridley and Hall, 1992) and G1 events (Welsh and Aassoian, 2000), has been implicated in the regulation of MyoD expression (Carnac et al., 1998; Gauthier-Rouviere et al., 1996; Dhawan and Helfman, 2004) and reversible cell cycle arrest (Dhawan and Helfman, 2004). Signaling through RhoA (but not Rac or Cdc42) is required for the expression of MyoD, but not Myf5, in growing myoblasts. RhoA-dependent transcription of MyoD is mediated through its effects on serum response factor (SRF) (Gauthier-Rouviere et al., 1996; Carnac et al., 1998; L’honore et al., 2003). In fibroblasts, alterations in actin dynamics are both necessary and sufficient for the Rho-dependent activation of SRF (Miralles et al., 2003; Sotiropoulos et al., 1999). In myoblasts, MyoD expression also responds to perturbation of microfilaments (Dhawan and Helfman, 2004), consistent with the involvement of SRF.

The immediate effectors of RhoA regulation of MyoD in myoblasts are unknown. ROCK (one of several downstream mediators of Rho action) is a serine-threonine kinase that activates microfilament contractility and facilitates the formation of stress fibers and focal adhesions (Amano et al., 1996). However, pharmacological inhibition of ROCK does not affect MyoD expression or myogenic differentiation in C2C12 cells (Dhawan and Helfman, 2004). mDia1, another immediate effector of RhoA, acts on the cytoskeleton and in cell migration. In mammalian cells, mDia regulates microfilament dynamics and SRF activity (Wasserman, 1998), and in Xenopus, diaphanous-related formin proteins mediate crosstalk between the Rho and Wnt pathways to regulate morphogenetic events (Habas et al., 2001). In this study, we show that mDia plays a complex role in controlling both MyoD expression and the cell cycle. We provide evidence for an SRF-alternative pathway downstream of mDia, and show that this signaling intermediary regulates localization of the multifunctional protein β-catenin and activity of its target transcription factor TCF (T-cell factor). Regulation of TCF–β-catenin by mDia involves a domain distinct from that required for SRF regulation. Thus, mDia regulates MyoD expression via two different transcription factors – SRF and TCF.

We propose that the reversible expression of MyoD during quiescence and activation of satellite cells may also respond to pathways that regulate cytoskeletal dynamics and cell adhesion.

Results

The Rho GTPases regulate adhesion-dependent signaling pathways through effects on the cytoskeleton (Ridley and Hall, 1992). In myogenic cells, RhoA specifically regulates the expression of the lineage determination factor MyoD (Carnac et al., 1998; Takano et al., 1998; Wei et al., 1998) via transcriptional activation of the MyoD gene by serum response factor (SRF) (L’honore et al., 2003). Previously, we showed that RhoA-dependent mechanisms couple MyoD expression to cell cycle exit: overexpression of active Rho led to sustained MyoD expression and differentiation, whereas dominant-negative Rho caused suppression of MyoD, and G0 arrest in an undifferentiated state (Dhawan and Helfman, 2004). In this study, we sought to identify the immediate effector responsible for transducing the RhoA signal and to delineate the pathway by which RhoA regulates MyoD.

The RhoA effector mDia1 is required for MyoD expression and cell cycle progression

Of the known RhoA effectors, mDia1 (mDia, an adaptor protein of the formin family) has been implicated in remodeling cytoskeletal networks and regulating SRF activity (Wasserman, 1998). To assess the involvement of mDia1 in MyoD regulation we used RNA interference to knockdown mDia1 expression. mDia1 mRNA levels in C2C12 myoblasts transfected with mDia1 short hairpin (sh)RNA were reduced to <10% of levels in control cells transfected with GFP-shRNA (Fig. 1A). To determine the effect of reduced mDia1 expression on MyoD, C2C12 myoblasts were co-transfected with the mDia1 shRNA-encoding plasmid and a GFP marker, and analyzed by antibody staining (Fig. 1B,C). The frequency of MyoD-positive cells among shRNA transfectants was reduced to 20% of that seen in control transfectants, indicating that mDia1 is required for MyoD expression.

To confirm that knockdown of mDia mRNA had consequences on a known target – SRF – we measured SRF activity using co-transfection of mDia1 shRNA with the SRF reporter 3DA.Luc. mDia1 shRNA-transfected cells showed ~40% of the SRF activity of control cells (Fig. 1D). Thus, reduced MyoD expression in mDia1-knockdown myoblasts correlated with decreased SRF activity. Conversely, induction of SRF activity during cell cycle activation is required for expression of MyoD (supplementary material Fig. S1).

RhoA also plays an important role in proliferation (Van Aelst and D’Souza-Schorey, 1997; Welsh and Aassoian, 2000; Dhawan and Helfman, 2004), in which mDia has also been implicated (Mammoto et al., 2004). To determine the effects of knockdown of mDia1 on the myoblast cell cycle, we used FACs analysis of cells co-transfected with GFP and mDia1 shRNA (Fig. 1E). A greater proportion of mDia1 shRNA cells (GFP+) showed a 2C DNA content (80% vs 40% in controls) correlated with decreased SRF activity. Conversely, induction of SRF activity during cell cycle activation is required for expression of MyoD (supplementary material Fig. S2). Thus, ROCK is not an immediate effector of Rho-mediated regulation of MyoD.

Rho-independent forms of mDia1 also suppress MyoD: a second pathway for MyoD regulation

mDia is an adaptor protein that contains three formin homology (FH) domains in addition to a RhoA-binding domain (RBD; schematic in Fig. 2A). Models of mDia-Rho interaction predict that the C-terminal diaphanous autoregulatory domain (DAD) binds the N-terminal RBD and is displaced by binding of activated Rho, resulting in a conformation that opens the centrally located FH domains to new interactions (Alberts, 2001). In fibroblasts, although a derivative of mDia1 that lacks the RBD and FH3 domains (mDiaΔN3, residues 543-1182) (Watanabe et al., 1999) is constitutively active in increasing F-actin content and SRF activity (Geneste et al., 2002), full-length mDia1 is not active,
consistent with the auto-inhibitory model (Copeland and Treisman, 2002). Similarly, in myoblasts, full-length mDia1 did not affect SRF activity, but the Rho-independent derivative mDia1ΔN3 upregulated SRF activity ~25-fold (Fig. 2B) and induced stress fibers (not shown). The mDia F2 mutant that comprises the FH2 domain was also mildly activating, but other forms lacking either part or all of the FH1 or FH2 domains did not activate SRF. All mutants were expressed at relatively similar levels to the EGFP control (Fig. 2B, inset). The effects of individual mDia1 derivatives on SRF in myoblasts recapitulated their activity in fibroblasts, where the FH2 domain has been shown to be essential for SRF activation and the FH1 domain for enhancement of FH2 function (Copeland and Treisman, 2002).

To determine the effect of mDia1 overexpression on MyoD, we transfected C2C12 myoblasts with individual GFP-tagged mDia1 truncation mutants. Surprisingly, the SRF-activating ΔN3 derivative of mDia1 strongly inhibited MyoD expression (Fig. 2C,D). Almost all mDiaΔN3-expressing cells were negative for MyoD expression. ΔN3/HindIII and H+P mutants of mDia1 also suppressed MyoD expression, albeit to a lesser extent than ΔN3, whereas full-length mDia1, F2 and CC mutants had minimal effects. These results suggest that activation of SRF is insufficient for MyoD expression.

Thus, silencing endogenous mDia1 suppressed SRF activity and overexpression of the constitutively active mDiaΔN3 derivative strongly activated SRF, yet unexpectedly, both perturbations inhibit MyoD expression. Unlike ΔN3, the ΔN3/HindIII and H+P mutants did not affect SRF activity, yet inhibited MyoD expression. The three suppressive mDia1 derivatives share amino acids 543-740, encompassing the FH1 domain. These results strongly suggest the existence of a second, SRF-independent pathway downstream of mDia that affects MyoD regulation.

mDiaΔN3 suppresses proliferation and differentiation

Since suppression of MyoD expression in C2C12 myoblasts is linked to G0 arrest, whether induced by anchorage deprivation (Milasincic et al., 1996; Sachidanandan et al., 2002), by serum deprivation (Kitzmann et al., 1998; Yoshida et al., 1998) or by inhibition of microfilament contractility (Dhawan and Helfman, 2004), we hypothesized that mDiaΔN3 might

Fig. 1. Knockdown of mDia1 suppresses SRF activity, MyoD expression and cell cycle progression. (A) Quantitative real-time RT-PCR analysis of mDia1 mRNA in cells transfected with control GFP shRNA (sh-GFP) and mDia1 shRNA (sh-mDia). Values represent normalized fold differences between mDia1 and GAPDH mRNA in each sample (n=3) ± s.d. (B,C) MyoD expression in myoblasts co-transfected with empty vector or mDia1 shRNA and a GFP reporter, 24 hours after transfection (mean ± s.d., n=4, P<0.0013). (D) Knockdown of mDia1 reduces SRF activity. Normalized SRF activity in C2C12 myoblasts co-transfected with mDia1 shRNA and a GFP reporter, 24 hours after transfection (mean ± s.d., n=4, P<0.0013). (E) FACS analysis of mDia1-knockdown cells shows an increased G1 population compared with control cells.
mDia1 regulates MyoD expression also cause G0 arrest. Indeed, FACS analysis of mDia1 mutant-transfected myoblasts (Fig. 3A,B) and pulse labeling with BrdU (Fig. 3C,D) confirmed that the ΔN3 and ΔN3HindIII derivatives that inhibit MyoD expression also reduce proliferation. Furthermore, mDiaΔN3-expressing cells were negative for Myogenin, an early marker of differentiation as well as for the cyclin-dependent kinase inhibitor (CDKI) p21, a marker of irreversible arrest (Halevy et al., 1995). Interestingly, all three forms that suppressed MyoD (ΔN3, ΔN3HindIII, H+P) also activated the CDKI p27, a marker of reversible arrest (Dhawan and Helfman, 2004) (Fig. 3E-G).

The experiments described thus far demonstrate that silencing of endogenous mDia1 suppresses SRF activity, MyoD expression and cell cycle progression. Overexpression of the Rho-independent mDia1ΔN3, despite increasing SRF activity, also arrests myoblasts in G0 and suppresses MyoD expression and differentiation. The ΔN3HindIII and H+P derivatives do not activate SRF, yet inhibit MyoD and the cell cycle. Together, these observations suggest a model in which two pathways stem from mDia to converge on MyoD. One pathway acts through SRF, and the other is mediated by unknown mechanisms involving the FH1 domain shared by the ΔN3, ΔN3HindIII and H+P mutants.

Investigating the SRF-independent pathway downstream of mDia

To identify candidate SRF-independent pathways by which mDia might act, we searched the BIND protein interaction database (Alfarano et al., 2005) for mDia-binding proteins, and identified the adenomatous polyposis coli (APC) protein, a known regulator of microtubule stability (Wen et al., 2004) and of β-catenin expression (reviewed by Nelson and Nusse, 2004). In fibroblasts, a tripartite complex of APC, end binding protein 1 (EB1) and mDia caps the plus ends of microtubules and leads to their stabilization (Wen et al., 2004), and in myoblasts mDiaΔN3 also stabilized microtubules against nocodazole-induced depolymerization (S.D.G. and J.D., unpublished). We therefore investigated a possible role for APC in MyoD regulation.

Over-expression of APC inhibits MyoD expression: microtubule association is not essential APC participates in a complex that activates GSK3β, a kinase...
that phosphorylates the multifunctional protein β-catenin, which is associated with cadherin cell adhesion complexes as well as nuclear transcription factors. Phosphorylation by GSK3β controls cytoplasmic degradation of β-catenin (Nelson and Nusse, 2004). Wnt signaling neutralizes APC function and inhibits the β-catenin destruction complex, permitting translocation of β-catenin to the nucleus, where it acts as a transcriptional co-activator (Young et al., 1998).
To assess the effects of APC on MyoD expression, we overexpressed GFP-tagged full-length APC, or mutant APC (APCΔMT), which lacks the microtubule-binding domain but retains the ability to induce β-catenin turnover (Penman et al., 2005). Both forms of APC strongly suppressed MyoD expression (Fig. 4). Thus, although mDia may bind APC and stabilize microtubules, as well as inhibit MyoD expression, these appear to be independent functions and interactions between APC and microtubules were not essential for MyoD downregulation. Taken together, these results suggest that the mechanism by which mDia suppresses MyoD may involve APC but not microtubules.

mDia affects β-catenin nuclear accumulation

APC plays a major role in regulation of β-catenin localization: inactivation of APC leads to nuclear accumulation of β-catenin and activation of gene expression in conjunction with TCF/LEF (Young et al., 1998). Although not directly implicated as a TCF target, MyoD expression during somitogenesis as well as myogenic differentiation in culture is induced by Wnt signaling (Munsterberg et al., 1995; Tajbakhsh et al., 1998; Rochat et al., 2004). We hypothesized that the mechanism by which overexpressed mDiaΔN3 suppresses MyoD expression may involve disruption of β-catenin localization. β-catenin is not detectable in the nuclei of untreated C2C12 cells, but cells exposed to the specific GSK3-β inhibitor 6-bromo-indirubin-3-oxime (BIO) (Meijer et al., 2003) showed clear β-catenin nuclear localization and enhanced cell-cell contact (Fig. 5A). mDiaΔN3 inhibited the BIO-induced translocation of β-catenin (Fig. 5B-D), while markedly increasing β-catenin at sites of cell-cell contact. As with SRF activity, full-length (FL) mDia1 did not affect this second function. Importantly, other derivatives that contain the FH1 domain also blocked β-catenin nuclear localization: ΔN3/HindIII was as active as ΔN3 in impeding β-catenin localization whereas H+P was less active. The F2 derivative that lacks the FH1 domain was inactive. Thus, overexpressed mDiaΔN3, despite activating SRF, may dominantly inhibit MyoD expression by altering localization of β-catenin, through a mechanism involving the FH1 domain.

TCF activity is modulated by mDia and required for MyoD expression

TCF/LEF factors are the target of the β-catenin nuclear co-activator function. To investigate the role of mDia in β-catenin regulation, we used the TOP-flash TCF reporter assay (Veeman et al., 2003). Consistent with its inhibition of MyoD expression, mDiaΔN3 suppressed TCF activity to ~30% of levels in the control (Fig. 6A panel i). Interestingly, mDia1 shRNA strongly induced TCF activity (~tenfold, Fig. 6A panel ii), suggesting that endogenous mDia negatively regulates TCF activity. The ΔN3/HindIII mutant also inhibited TCF activity, whereas FL mDia1 and the H+P mutant did not (Fig. 6A panel iii). These results are broadly consistent with the effects of each of these forms of mDia in regulating localization of the essential TCF co-activator β-catenin, a putative positive regulator of MyoD. Endogenous mDia1, as shown by the shRNA experiments, activates SRF and inhibits TCF, and the ΔN3 derivative shows the same effect. ΔN3/HindIII and H+P derivatives do not activate SRF and are less effective than ΔN3 in inhibiting TCF. Therefore, it is unlikely that ΔN3 and the other deletion derivatives act by a dominant negative mechanism.

The experiments described above show that mDia is an upstream regulator of both TCF activity and MyoD expression. To determine whether TCF activity is required for MyoD expression, we overexpressed a dominant negative TCF1E lacking the β-catenin binding domain (M. Waterman, personal communication) and found that it inhibited MyoD expression (Fig. 6B). Since mDiaΔN3 and ΔN3/HindIII suppressed β-catenin nuclear localization, TCF activity and MyoD expression, TCF may represent the SRF-independent pathway for MyoD regulation downstream of mDia. Thus, a potential resolution of the surprising finding that both overexpression and knockdown of mDia1 reduce MyoD expression may be that despite activating SRF, mDiaΔN3 suppresses MyoD expression by inhibiting TCF, whereas despite activating TCF, mDia1 shRNA suppresses MyoD expression by inhibiting SRF.

To assess whether modulation of TCF activity affects SRF,
we co-transfected the SRF reporter 3DA.luc with constitutively active β-catenin S37A or dnTCF (Fig. 6C). Whereas mDiaΔN3 induced SRF activity as expected, neither S37A nor dnTCF affected the basal activity, confirming that SRF and TCF act in different pathways.

TCF may regulate MyoD expression by an indirect mechanism

The Wnt pathway has been implicated in activation of MyoD expression during embryonic myogenesis (Munsterberg et al., 1995; Tajbakhsh et al., 1998). The region of the MyoD enhancer that is active during development is the –20 kb element known as the core enhancer (Goldhamer et al., 1992). However, in adult satellite cells, activation of MyoD is mediated by an element at –5 kb called the distal regulatory region (DRR) (Tapscott et al., 1992; Chen et al., 2002), which contains an active SRF-binding site [serum-response element (SRE) or CArG box] (L’honore et al., 2003). MyoD has not been implicated as a direct target of TCF/LEF. Interestingly, examination of the 714 bp DRR using MatInspector v7.4 (www.genomatix.de) revealed two consensus sites for TCF/LEF1 (Table 1).

To determine whether the putative TCF/LEF sites in the MyoD DRR are functional, we used gel-shift assays. Neither of the sites in the DRR showed specific binding activity under conditions where a canonical TCF site bound nuclear factors that were BIO inducible (Fig. 7A), nor were they able to compete out binding of the consensus sequence. These data suggest that MyoD is not a direct target of β-catenin or TCF.

Fig. 5. mDia negatively regulates β-catenin localization. (A) The specific GSK3β inhibitor BIO induces nuclear localization of endogenous β-catenin (red) and increased cell-cell contact (phase) in myoblasts after a 24-hour treatment in growth medium. Bar, 10 μm (25 μm in phase). (B) mDiaΔN3 inhibits β-catenin nuclear localization in BIO-treated cells. Myoblasts were transiently transfected with control (EGFP), mDia1-FL, ΔN3, Hind3, H+P or F2 constructs (all GFP-tagged), treated with 2.5 μM BIO for 24 hours and stained for β-catenin (red). In ΔN3 and Hind3-transfected cells, accumulation of β-catenin at cell-cell contacts correlates with loss of nuclear staining. (C) Confocal analysis shows absence of β-catenin staining in the nuclei of ΔN3-transfected cells. Bar, 10 μm. (D) Quantification of the effects of GFP-tagged mDia constructs on β-catenin localization (mean ± s.e.m., n=2).
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Transcriptional activity of the MyoD DRR is inhibited by mDia

To determine whether mDia affects the transcriptional activity of the MyoD DRR, we co-transfected a DRR-luciferase reporter construct (DRR-pGL3) along with either control or mDia constructs. In this transient assay, mDia/H9004N3 strongly inhibited the MyoD DRR activity to ~9% of control, H9004N3HindIII was mildly inhibitory and the H+P and FL forms were ineffective (Fig. 7B). Suppression of DRR activity by the different mDia1 derivatives correlated well with suppression of TCF activity, and in the case of the H9004N3 and H9004N3HindIII derivatives, also correlated with their effects on β-catenin localization. Despite the presence of the positive serum response element (SRE) in the DRR, the net effect of overexpressed active mDiaH9004N3 on DRR transcriptional activity is negative, consistent with the observed inhibition of MyoD expression. Taken together, the observations suggest a model wherein MyoD is an indirect target of β-catenin–TCF signaling.

Co-expression of β-catenin partially rescues the suppressive effect of mDia on MyoD

The inhibitory effect of the ΔN3 and ΔN3HindIII derivatives of mDia on MyoD correlated with exclusion of β-catenin from the nucleus, and accumulation at cell contacts (Fig. 5B). To determine whether β-catenin levels and/or localization play a role in its inhibitory effect, we examined whether a degradation-resistant active β-catenin mutant S37A could functionally bypass the mDiaH9004N3 inhibition and rescue MyoD expression. β-catenin S37A robustly activates TCF reporter activity in mDiaH9004N3-transfected cells (Fig. 8A). Co-transfection of β-catenin S37A with mDiaH9004N3 led to a twofold increase in MyoD expression compared with mDia-H9004N3 co-transfected with a control plasmid, suggesting a partial reversal of the effects of mDiaH9004N3 (Fig. 8B,C). The milder suppressive effect of ΔN3HindIII on MyoD expression could also be partially reversed by co-expression of β-catenin S37A (Fig. 8C). These results are consistent with the observations that the ΔN3 and ΔN3HindIII derivatives suppress MyoD expression, perturb β-

Table 1. TCF consensus sites in the 714 bp MyoD distal regulatory region

| Family/matrix   | Position | Core similarity | Matrix similarity | Sequence*   | Site designation |
|-----------------|----------|-----------------|-------------------|-------------|-----------------|
| TCF/LEF1        | 440-456  | 1.000           | 0.972             | gggaggtCAAAggtgcc | Site A          |
| Wnt signaling   | 685-701  | 1.000           | 0.86              | agcaagCAAAggaagc | Site B          |
| pathway         | 695-711  | 1.000           | 0.87              | pgagcaagCAAAggaag |                 |

*Capital letters indicate the core sequence used by the MatInspector program; underlined letters indicate nucleotides conserved in vertebrates.
catenin localization and inhibit TCF, and suggest a mechanistic link between these three activities. A comparison between the effects of the different mDia1 derivatives is shown in Table 2.

Considered in the context of earlier results that established its role in cytoskeletal signaling, the results obtained from our experiments using RNAi and deletion derivatives of mDia1 led us to propose a model for the pathways downstream of this Rho effector in myoblasts (Fig. 9). In summary, this study reveals a novel dual mechanism by which mDia1 regulates MyoD: via positive regulation of SRF and negative regulation of β-catenin-TCF.

Discussion

We have delineated a complex pathway by which MyoD expression is regulated in C2C12 myoblasts. Four new findings emerge from our study: first, we show that the RhoA effector mDia1 regulates MyoD expression and myoblast quiescence; second, we demonstrate that SRF-independent pathways downstream of mDia1 regulate MyoD; third, we provide evidence that mDia1 regulates β-catenin and TCF, and implicate a region encompassing the FH1 domain in this activity; finally, we demonstrate that MyoD is an indirect target of these nuclear effectors of canonical Wnt signaling.

MyoD expression is coupled to the cell cycle by Rho-SRF signaling

Regulation of MyoD during differentiation is well understood (Tapscott, 2005), but less is known about reversible regulation of this myogenic regulator during the cell cycle – an important feature of adult muscle stem cells (Dhawan and Rando, 2005). Adhesion-dependent signaling regulates MyoD expression, cell cycle progression (Sachidanandan et al., 2002; Dhawan and Helfman, 2004) and SRF activity (see supplementary material Fig. S1). Coupling of MyoD regulation to the cell cycle occurs via RhoA, a major regulator of G1 progression, cytoskeletal signaling and SRF. Activated RhoA drives monomer G-actin into filamentous F-actin (Chrzanowska-Wodnicka and Burridge, 1996), leading to activation of SRF

![Fig. 7. MyoD DRR activity is inhibited by mDia.](image)

| Table 2. Summary of effects of mDia1 and its derivatives in myoblasts |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| mDia1 construct | Region included (a.a.) | Inhibition of MyoD expression | Induction of SRF activity | Inhibition of TCF activity | Inhibition of β-catenin nuclear localization | Inhibition of DRR activity | Inhibition of cell cycle |
| FL mDia1       | 1-1255          | –               | –               | –               | –               | –               | n.d.            |
| ΔN3*           | 543-1182        | +++             | +++             | +++             | +++             | +++             | +               |
| HIND3*         | 543-978         | +              | –               | +/–             | +               | ++              | +++             |
| H+P*           | 422-740         | –              | –               | –               | +/–             | +              | +++             |
| F2             | 752-1182        | –              | –               | –               | n.d.            | –               | n.d.            |
| CC             | 1010-1183       | –              | –               | n.d.            | n.d.            | n.d.            | n.d.            |

*The three mDia1 derivatives that inhibit MyoD expression share a region spanning residues 543-740, containing the FH1 domain (aa 570-735). This ‘FH1+’ region may play a key role in the mechanism by which mDia controls the SRF-independent pathway. ΔN3 is the most active form in all assays, suggesting that although the FH2 domain may not play a role on its own, co-operation between the FH1 and FH2 domains may be required for full activity. DRR, distal regulatory region; FL, full length; n.d., not determined; SRF, serum-response factor; TCF, T-cell factor.
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SRF target genes differ in their sensitivity to Rho-Actin dynamics (Gineitis and Treisman, 2001), and although targets such as vinculin and SRF itself are sensitive to actin polymer status, egr1 and junB are unaffected. MyoD has a functional SRF-binding site (L’honore et al., 2003), is regulated by RhoA (Carnac et al., 1998; Dhawan and Helfman, 2004), and responds to actin dynamics (Dhawan and Helfman, 2004) (see supplementary material Fig. S1). Inclusion of MyoD as an actin-dependent target suggests a role for SRF in coupling lineage determination with cytoskeletal dynamics and cell cycle activation.

Evidence for SRF-dependent and SRF-independent control of MyoD expression

SRF is clearly involved in MyoD regulation because microinjected anti-SRF antibodies (Gauthier-Rouviere et al., 1996), dominant-negative SRF (Soulez et al., 1996; Carnac et al., 1998) and disruption of microfilaments by latrunculin B reduce both SRF activity and MyoD expression (Dhawan and Helfman, 2004) (see supplementary material Fig. S1). However, our data show that SRF activity is not sufficient for MyoD induction, since a Rho-independent derivative of the effector mDia1 (mDiaΔN3) activates SRF but unexpectedly, suppresses MyoD. Two other Dia truncation mutants – ΔN3HindIII and H+P – have no effect on SRF but also suppress MyoD expression. Thus, an antagonistic SRF-alternative pathway emanates from mDia1 to regulate MyoD. We have used truncation mutants and RNAi to delineate this new SRF-alternative pathway.

SRF-independent regulation of MyoD by mDia1 requires a region spanning the FH1 and FH2 domains

Silencing of endogenous mDia1 or overexpression of Rho-independent mDia1ΔN3 led to MyoD suppression and cell cycle arrest, suggesting a critical threshold of signaling through this Rho effector. Downregulation of both SRF activity and MyoD expression in mDia1-knockdown cells is consistent with the actin-mediated function of mDia. mDia1ΔN3, which strongly activates SRF, is comprised of the FH1 and FH2 domains, where the FH2 domain is the key determinant of actin-mediated SRF regulation and the FH1 domain enhances this activity (Copeland and Treisman, 2002). Our results reveal a new function for the FH1 domain in regulating β-catenin localization and TCF activity.

Among the mutants that possess the FH1 domain (ΔN3, ΔN3HindIII, H+P), ΔN3 suppresses MyoD expression maximally, whereas ΔN3HindIII and H+P, which lack part or all of FH2 respectively, are less active, suggesting that FH2 and the region between FH1 and FH2 may be required for full activity. The proline-rich FH1 domain interacts with the Src tyrosine kinase (Tominaga et al., 2000) profilin (an actin cross linker) (Watanabe et al., 1997) and WW domain proteins (Wallar and Alberts, 2003). Co-transfection of dominant negative Src does not relieve the ΔN3-mediated suppression of MyoD (S.D.G. and J.D., unpublished). However, APC whose binding has been mapped to a region encompassing the FH1 and FH2 domains (Wen et al., 2004), negatively regulates MyoD, consistent with the

![Fig. 8](https://example.com/fig8.png)

Fig. 8. Overexpression of an APC-independent form of β-catenin leads to functional bypass of mDiaΔN3 inhibition. (A) Myoblasts were co-transfected with a control plasmid (GFP), ΔN3+GFP or ΔN3+β-catenin S37A and TCF activity determined (mean ± s.e.m., n=5, P<0.0046). (B) MyoD expression in cells transfected as in A. Note that ΔN3+β-catenin S37A transfected cells retain the elongated morphology typical of ΔN3 transfectants but are MyoD+. (C) Quantification of MyoD expression in myoblasts transfected with either ΔN3+β-catenin S37A or ΔN3HindIII+β-catenin S37A. The degradation-resistant β-catenin S37A mutant partially reverses the inhibition of MyoD expression mediated by both mDia derivatives. (mean ± s.e.m., **P<0.0002, n=6; *P<0.046, n=3).
Regulation of MyoD by Wnt–β-catenin–TCF signaling

The APC-dependent β-catenin degradation complex is inhibited by Wnt signaling, enhancing β-catenin levels and nuclear translocation, leading to activation of TCF target genes (Clevers, 2000). Wnt signaling induces MyoD expression in the embryo (Munsterberg et al., 1995; Tajbakhsh et al., 1998) and during differentiation of cultured muscle cells (Rochat et al., 2004). MyoD in proliferating C2C12 myoblasts also appears to be positively regulated by Wnt signaling, because APC and dominant-negative TCF suppress MyoD expression. Other Wnt inhibitors such as Axin and sFRP also inhibit MyoD, but overexpression of full-length LEF (an activator) does not further increase MyoD expression (S.D.G. and J.D., unpublished), suggesting that Wnt pathway activity is necessary but not limiting.

mDiaΔN3 prevents nuclear accumulation of β-catenin and inhibits TCF activity, and despite the activation of a positive regulator (SRF), inhibits the expression of MyoD protein as well as the transcriptional activity of the MyoD DRR. Most importantly, co-expression of β-catenin functionally bypasses the negative effect of mDia on MyoD, suggesting that overexpression of the constitutively active form of mDia interferes with endogenous β-catenin localization and function. Although mDiaΔN3 (a.a. 543-1182) blocks BIO-induced β-catenin nuclear localization, ΔN3/HindIII (a.a. 543-978) is as effective as ΔN3, H+P (a.a. 422-740) is less effective and F2 (a.a. 752-1182) is ineffective, suggesting that a.a. 543-751 represent the minimal β-cat inhibitory domain. This corresponds closely to the FH1 domain (a.a. 570-735). The difference between the activities of H+P and ΔN3/HindIII can be attributed to residues 741-978, which is outside the FH1 domain, and suggests that an extended region is required for full activity. This extended region corresponds to the APC-binding region reported by Wen et al. (Wen et al., 2004). Thus, the mechanism by which mDia inhibits β-catenin localization and MyoD expression could be mediated by APC, but our results do not exclude APC-independent mechanisms such as the effects of mDia on cytoskeletal configuration.

The DRR-inhibitory activity of the different mDia derivatives correlates well with their TCF-inhibitory activity, but is not as strictly correlated with the regulation of β-catenin localization. Thus, H+P (like ΔN3/HindIII and ΔN3) does block β-catenin nuclear localization albeit less effectively, but this mDia derivative does not perturb TCF (or DRR) activity. These observations may suggest additional mechanisms by which the ΔN3/HindIII and ΔN3 derivatives affect TCF.

MyoD might be an indirect target of TCF

Reciprocal regulation of two transcription factors leads to inhibition of MyoD when mDia levels are perturbed. Silencing mDia reduces SRF activity but potentiates TCF activity. By contrast, mDiaΔN3 induces SRF activity and inhibits TCF activity. SRF directly activates MyoD transcription by binding to the SRE-CarG box in the MyoD DRR (L’honore et al., 2003). As the consensus TCF sites in the MyoD DRR do not specifically bind nuclear factors, TCF is likely to be an indirect activator of MyoD. Known upstream activators of MyoD expression such as Pax3 are induced in response to Wnts (Petroopoulos and Skerjanc, 2002). It is therefore likely that MyoD is indirectly regulated by TCF.

mDia as a regulator of myogenic gene expression

The role of mDia in regulating the cytoskeleton and cell motility is well established, but less is known of its
involvement in the control of gene expression. An intriguing cytoskeletal dimension in the regulation of MyoD emerges from this study, and shows that MyoD is not only a direct target of SRF, whose co-activator is regulated by actin dynamics, but also an indirect target of TCF whose co-activator, β-catenin, is itself involved in cell adhesion and microfilament-membrane interactions. mDia acts as a nodal modulator of two pathways, resulting in reciprocal regulation of SRF and TCF/LEF via reciprocal effects on the localization of their cytoplasmic co-activators, MAL and β-catenin, respectively. Collectively, our results demonstrate that signals emanating from mDia co-regulate MyoD and the cell cycle. Thus, in culture, expression of this lineage determinant is coupled to proliferation and responsive to cytoskeletal dynamics and adhesion-dependent signaling pathways. It is conceivable that MyoD regulation in quiescent satellite cells in muscle is triggered by mechno-chemical signals activated by damage to this contractile tissue.

**Materials and Methods**

**Cell culture**

A subclone of C2C12 myoblasts (Yaffe and Saxeal, 1977; Blau et al., 1983) designated C2C12A2 derived earlier (Sachidanandan et al., 2002) was used (referred to as C2C12 in this study). C2C12 myoblasts were cultured in growth medium (GM: DMEM + 20% FBS).

**Transient transfections**

C2C12 myoblasts were plated on coverslips 14 hours before transfection with plasmids encoding mDia1, APC, dl-ctD1F1E, Src or miR-16-shRNA (1.25 μg) using Lipofectamine 2000 (Invitrogen). For co-transfections, AN3 or mL6-shRNA plasmids and EGFP-C1 were used at a 4:1 ratio. For the rescue experiments, ΔN derivatives and β-catenin S37A (gift of S. Byers, Georgetown University, Washington, DC) were transfected in a ratio of 1:1. Cells were transfected for 6 hours in OptiMEM (Invitrogen) then incubated in GM for 24 hours. For luciferase assays, cells plated in 24-well dishes were transfected with 300 ng of the test plasmids or control (pBS) + 60 ng of DA.Luc or SuperX-TOPLFLASH or FOPFLASH + 6 ng of pRSV-ShGFP or empty vector and incubated in 0.5% serum for 24 hours. The efficiency of transfection was 30-50%.

**Plasmids**

Expression plasmids of mouse mDia1 (GFP-ΔN3, GFP-ΔN3HindIII, GFP-H+P, GFP-F2 and GFP-CC are described in Watanabe et al. (Watanabe et al., 1999). mL6 vector was a gift from D. Turner (Yu et al., 2002), dl-ctD1F1E (gift of T. J. Ting and M. L. Waterman, University of California, Irvine, CA) was constructed by sub-cloning of human TCF1E (Van de Wetering et al., 2002) missing the N-terminal domain into the EVR vector (M. Waterman, personal communication). SuperX-TOPLFLASH (TCF site) and FOP-flash (mutated TCF site) (Veeman et al., 2003); full length APC-β and APCβMT-gfp (Pennanen et al., 2005). The 714 bp MyoD DRR was amplified from mouse genomic DNA using the primers (F: 5’-CTTTAGAGGCTTCGATCTCAGATTTGGTGCCGGTTGTGAGTT-3’ and R: 5’-ATATCTTCTCGACGGCAAGGCTTTCCTGTGAGTT-3’). Cells were co-transfected with mL6-mDia shRNA or mL6-GFP shRNA or empty vector and pSV2Neo, and stable pools selected in G418 500 μg/ml.

**Quantitative real-time RT-PCR**

1 pg total RNA isolated from Dia-shRNA, control GFP-shRNA or mL6 vector-transfected pools was used to generate cDNA (Clontech). 2 μl cDNA (diluted 1:5) were mixed with 10 μl of SYBR Green PCR Master Mix (Applied Biosystems) and analyzed in triplicate using the 7900HT Sequence Detection Systems cycle (Applied Biosystems) and the SDS2.1 ABI Prism software. Dissociation curves were used to verify the amplicons and normalized fold differences of cycle thresholds [2−ΔΔCt] calculated relative to a control GAPDH amplex.

**Mobility shift assays**

Nuclear extracts (Andrews and Faller, 1991) were prepared from control C2C12 cells or cells treated for 24 hours with the GSK3β inhibitor BIO (Meijer et al., 2003). Double-stranded oligonucleotide probes were as follows: Consensus TCF site, 5’-AGGCGCGATCAAAGGCCACCTCT-3’; and 3’-AGTTGGCCCTTTGATTTCCCCCT-3’. Binding reactions (Prieve and Waterman, 1999) used 15,000 cpm purified probe in a volume of 20 μl. N2 and R, 5 μl of cDNA (diluted 1:5) were co-transfected with mL6-mDia shRNA or mL6-GFP shRNA or empty vector and pSV2Neo, and stable pools selected in G418 500 μg/ml.

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