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

Suchitra Devi Gopinath[1], Shuh Narumiya[2] and Jyotsna Dhawan[1,*]

[1]Center for Cellular and Molecular Biology, Uppal Road, Hyderabad, 500 007, India
[2]Department of Pharmacology, Kyoto University, Kyoto, Japan
*Author for correspondence (e-mail: jdhawan@ccmb.res.in)

Accepted 25 June 2007
Journal of Cell Science 120, 3086-3098 Published by The Company of Biologists 2007
doi:10.1242/jcs.006619

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 mDia1 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.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/17/3086/DC1

Key words: mDia1, MyoD, SRF, RhoA, Adhesion-dependent signaling, Quiescence, Synchronized myoblasts, β-catenin, TCF, APC

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 postnatal 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 Assoian, 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; Sotiropoulous 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).

mDiaphanous (mDia), 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, mDiaphanous (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 mDia 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 Assoian, 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) indicating that mDia is required for G1-S progression. Taken together, these results indicate that mDia function is required for both MyoD expression and cell cycle progression.

In fibroblasts, the other major RhoA effector – ROCK – modulates both acto-myosin contractility and SRF activity via its effects on focal adhesions and stress fibers (Sahai et al., 1998). However, inhibition of ROCK in myoblasts using the pharmacological inhibitor Y27632 led to altered morphology but did not affect MyoD expression (Dhawan and Helfman, 2004) or SRF activity (see 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*Hin*dIII 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*Hin*dIII 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

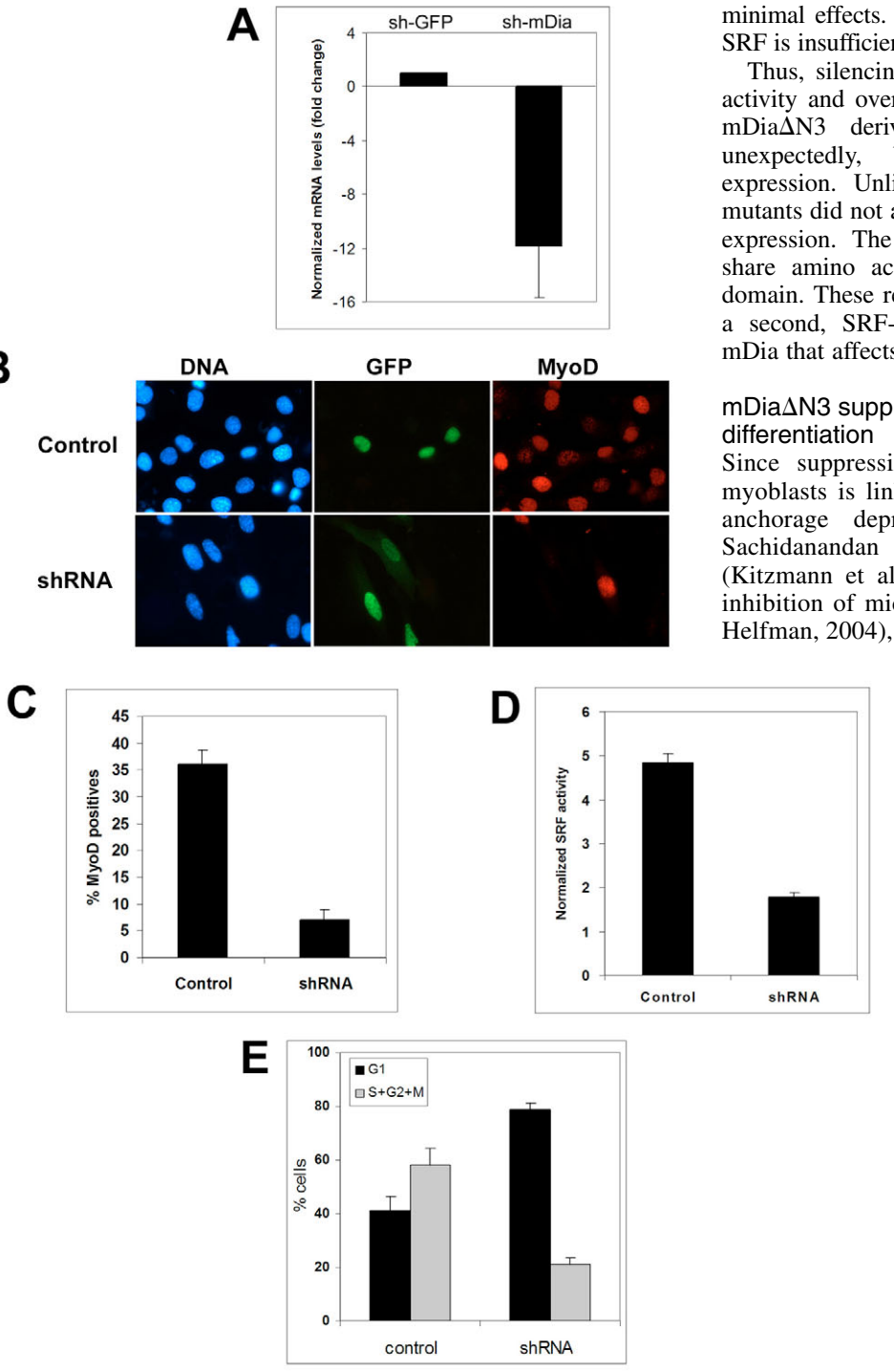

Fig. 1. Knockdown of mDia1 suppresses SRF activity, MyoD expression and cell cycle progression. (A) Quantitative real-time RT-PCR analysis of mDia1 mRNA in cells transfected with control GFP shRNA (sh-GFP) and mDia1 shRNA (sh-mDia). Values represent normalized fold differences between mDia1 and GAPDH 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 or empty vector, the SRF reporter 3D.Aluc and β-gal (mean ± s.d., n=4, P<0.0001). (E) FACS analysis of mDia1-knockdown cells shows an increased G1 population compared with control cells.

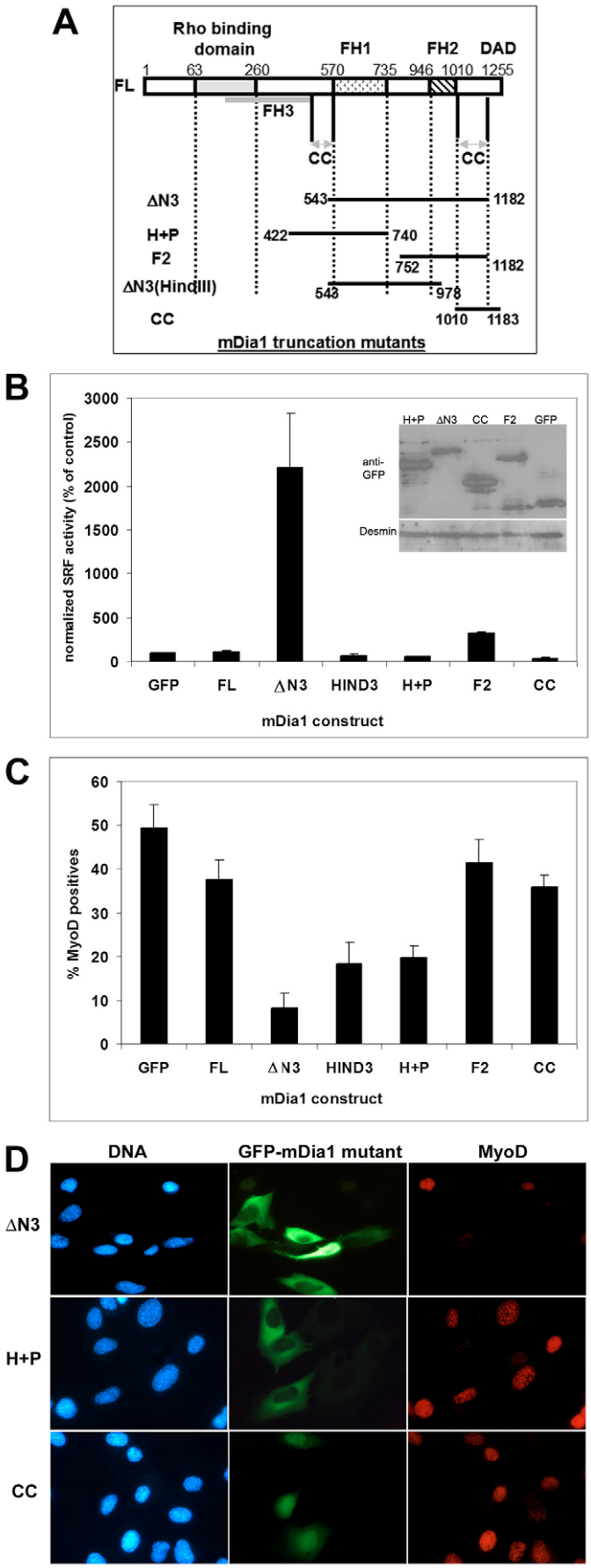

Fig. 2. mDia1 regulates MyoD by an SRF-independent pathway. (A) Schematic of full-length mDia1 (FL) and five truncation derivatives. (B,C) mDia1ΔN3 activates SRF. C2C12 myoblasts were co-transfected with full-length mDia1 (FL), mDia1 mutants or a GFP control, the SRF reporter and a β-gal plasmid. To minimize effects of serum on SRF activity, transfected cells were incubated in 0.5% serum for 24 hours before assay. mDiaΔN3 increased SRF activity >25 fold (mean ± s.e.m., n=4, P<0.0041). Western blotting with anti-GFP (panel B, inset) showed that all mutants were expressed at relatively equal levels. (C) Quantification of MyoD expression detected by immunofluorescence assay in cells overexpressing GFP (control), full-length mDia1 (FL) or mDia1 mutants (ΔN3, *Hin*d3, F2, H+P, CC). Despite strongly activating SRF, mDia1ΔN3 suppresses MyoD expression maximally (mean ± s.e.m., n=7, P<0.0001). (D) Immunodetection of MyoD expression in C2C12 myoblasts transiently transfected with GFP-tagged mDia1 truncation mutants (ΔN3, H+P, CC).

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 ΔN3*Hin*dIII 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, ΔN3*Hin*dIII, 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 ΔN3*Hin*dIII 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, ΔN3*Hin*dIII 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

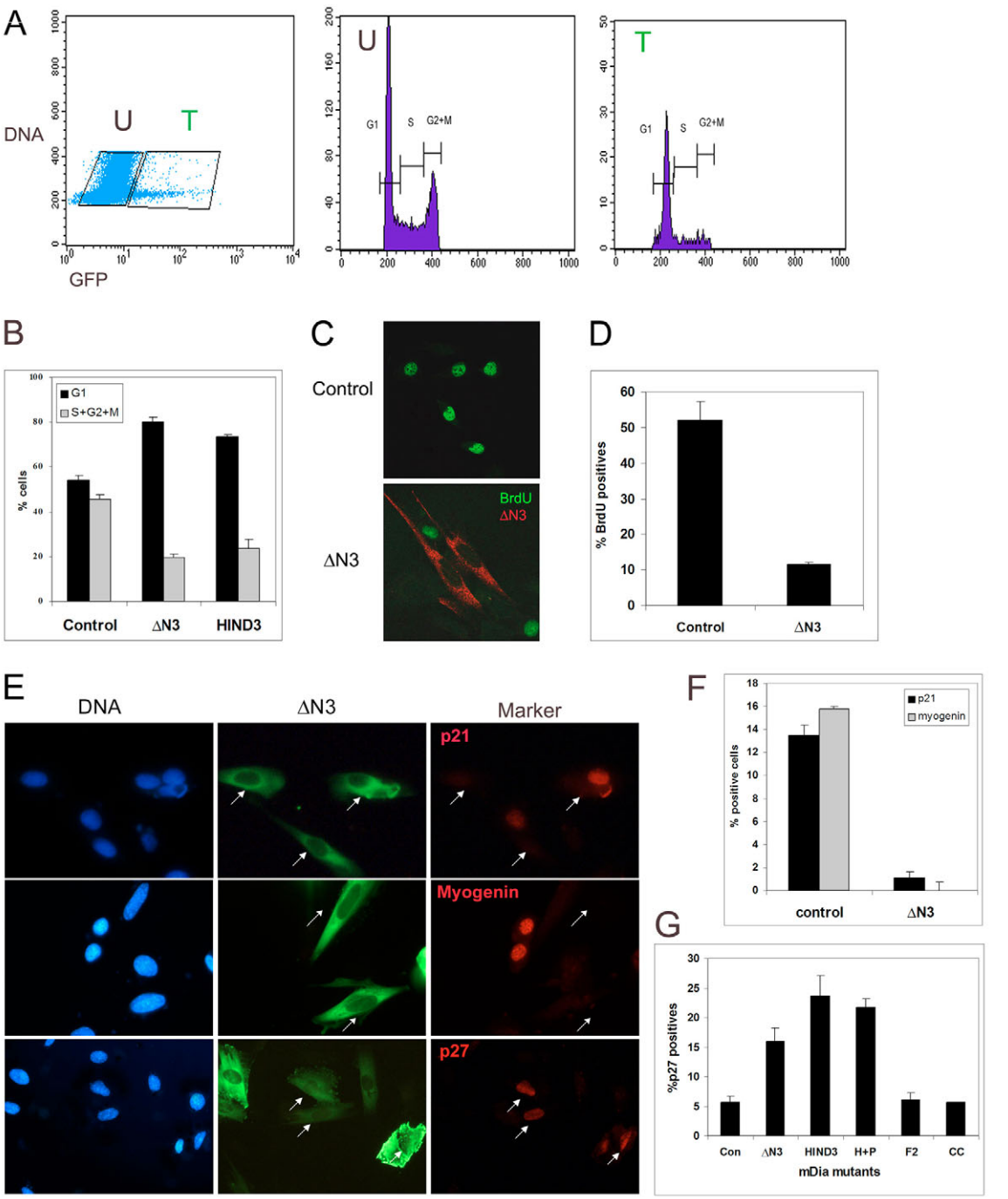

Fig. 3. mDia1 mutants that affect MyoD expression cause G1 arrest without differentiation. (A) Cell cycle analysis of C2C12 myoblasts transfected with GFP-tagged ΔN3 or N3*Hin*dIII 24 hours after transfection. Transfected (T) and untransfected (U) cells were distinguished by gating on GFP. (B) A greater proportion of ΔN3 and ΔN3*Hin*dIII transfected cells showed a G1 DNA content compared with untransfected cells (mean ± s.e.m., n=4, P<0.0002). (C,D) ΔN3 transfected cells show reduced BrdU incorporation. Immunodetection of BrdU (green) in cells transfected with Flag-tagged mDiaΔN3 (red) (mean ± s.d., n=3). (E,F) ΔN3 transfected cells do not differentiate. ΔN3 transfected cells were stained for Myogenin and p21, after 24 hours in differentiation medium (mean ± s.e.m., n=2). Arrows indicate transfected cells that are negative for two markers of differentiation: p21 (top) or Myogenin (middle), but positive for p27, a marker of reversible arrest (bottom). (G) Quantification of p27 induction: mDia mutants that affect MyoD expression (ΔN3, ΔN3*Hin*dIII, H+P) induce expression of p27 (mean ± s.e.m., n=4).

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*Hin*dIII 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.

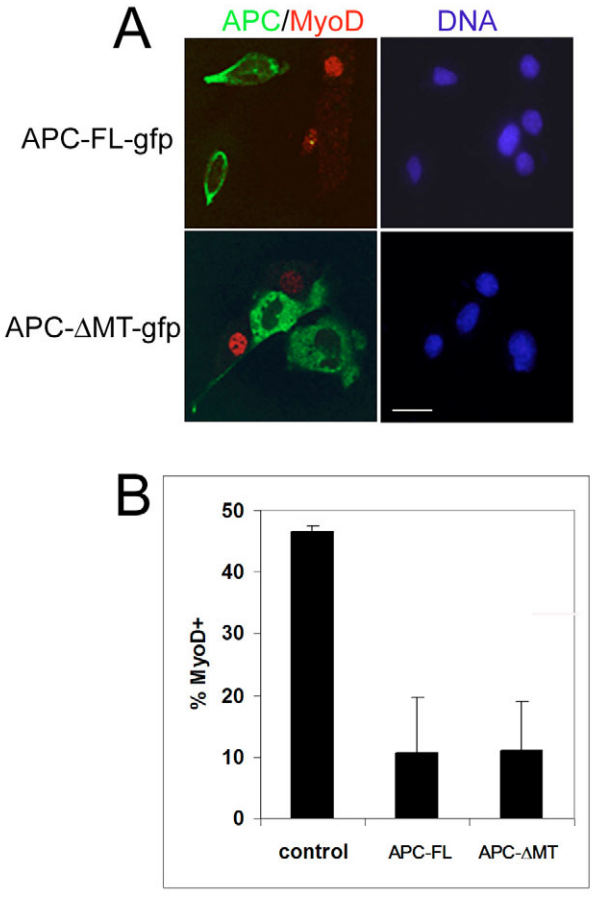

Fig. 4. APC inhibits MyoD expression: Microtubule-association of APC is not essential. (A) APC inhibits MyoD expression: Confocal analysis of cells transfected with full-length APC (APC-FL-GFP) or APC lacking the microtubule-binding domain (APCΔMT-GFP). Full-length APC is microtubule associated whereas APCΔMT-GFP localizes to the cytoplasm; both forms effectively inhibit MyoD expression (red). Bar, 20 μm. (B) Quantification of MyoD expression in cells transfected with GFP alone (control) APC-FL-GFP or APCΔMT-GFP (mean ± s.e.m., n=2).

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 role 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*Hin*dIII 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*Hin*dIII 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*Hin*dIII 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.

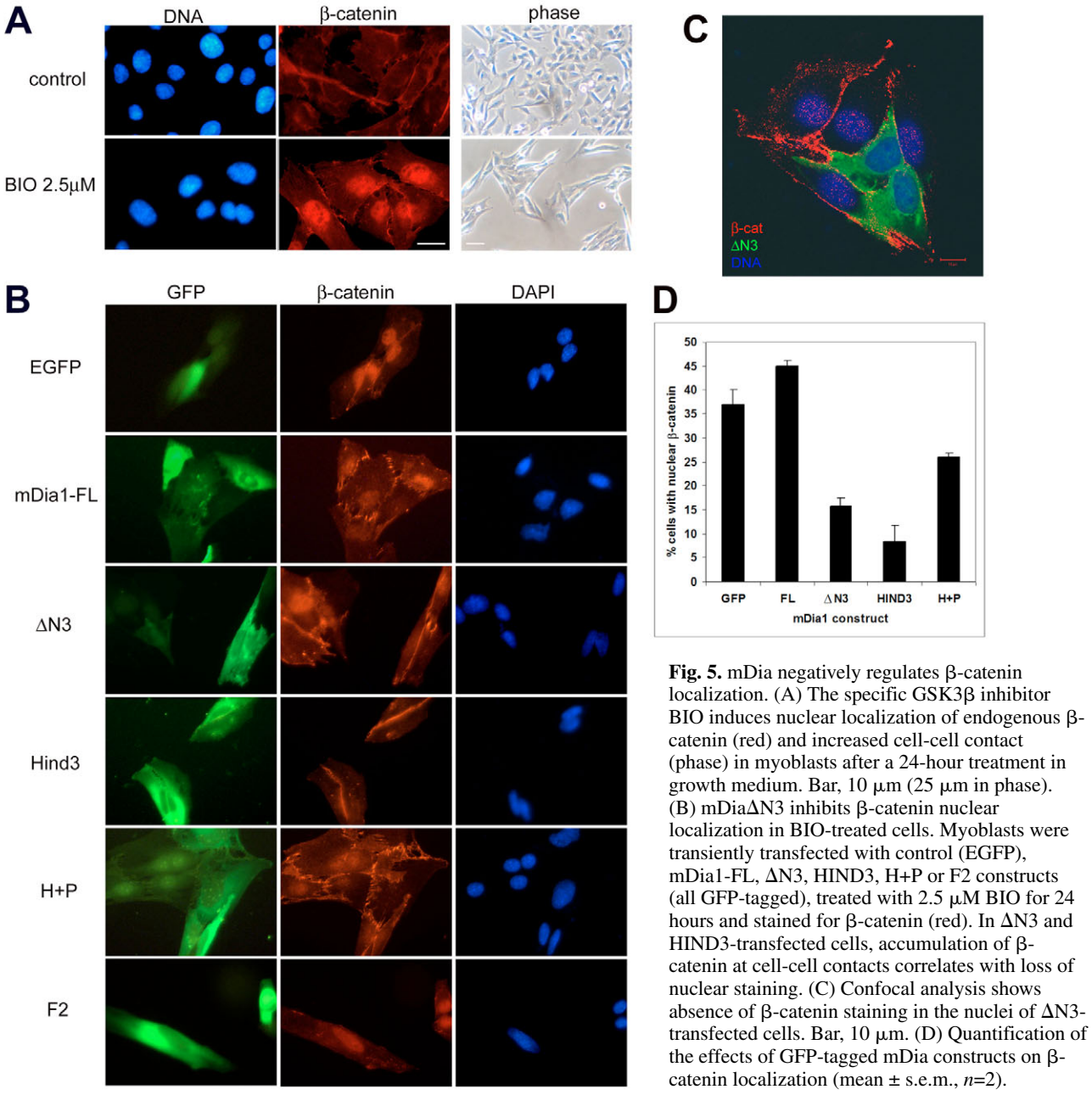

Fig. 5. mDia negatively regulates β-catenin localization. (A) The specific GSK3β inhibitor BIO induces nuclear localization of endogenous β-catenin (red) and increased cell-cell contact (phase) in myoblasts after a 24-hour treatment in growth medium. Bar, 10 μm (25 μm in phase). (B) mDiaΔN3 inhibits 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).

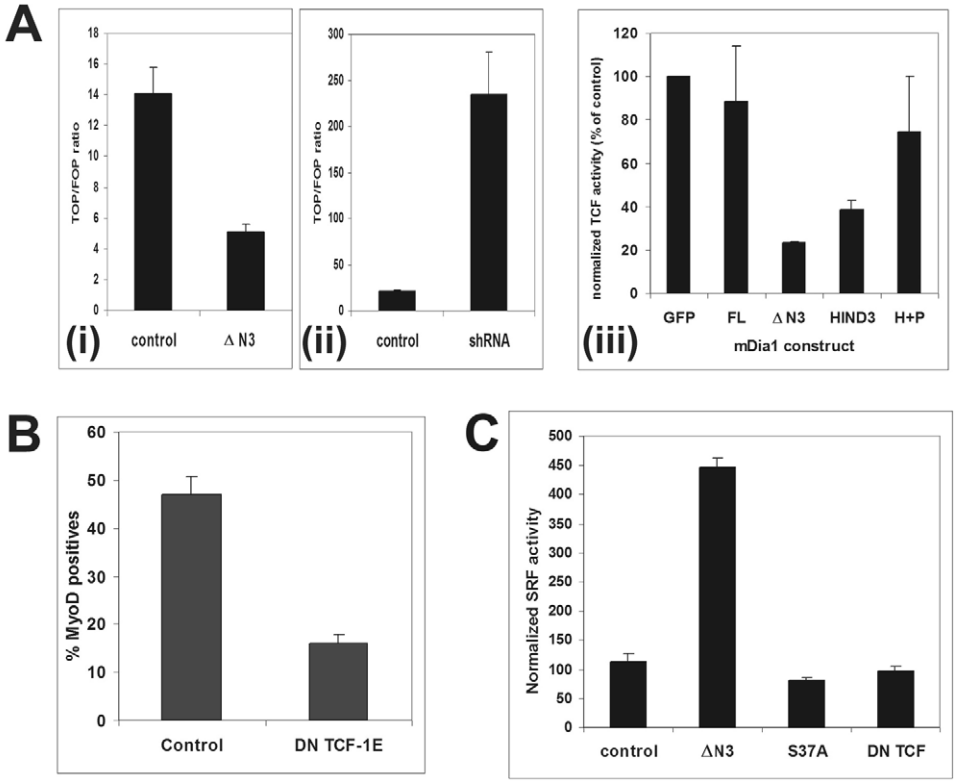

Fig. 6. mDia negatively regulates TCF. (A) TCF activity is suppressed by mDia1ΔN3 and activated by mDia knockdown. (i) Myoblasts were co-transfected with the TCF reporter plasmid TOP-flash + GFP (control) or mDia1ΔN3 (ΔN3) and TCF-dependent luciferase activity measured. Values represent normalized ratios of TOP-flash activity to the respective FOP-flash control (mean ± s.e.m., n=11, P<0.0001). (ii) Myoblasts were co-transfected with TOP-flash + mU6 vector (control) or mDia1 shRNA (shRNA) and luciferase activity measured as in (i) (mean ± s.e.m., n=5, P<0.0021). (iii) Comparison of effects of full-length (FL) and different mDia mutants on TCF activity (values represent normalized TCF activity, mean ± s.e.m., n=2). ΔN3 is the most effective at suppressing TCF activity. (B) Inhibition of TCF suppresses MyoD expression. Cells were transfected with GFP alone (control) or along with dominant negative TCF-1E (DN TCF-lacking the β-catenin-binding domain) and MyoD expression quantified (mean ± s.e.m., n=3, P<0.0021). (C) SRF activity is not affected by β-catenin S37A and dnTCF. Myoblasts were co-transfected with the SRF reporter with control (pBS), ΔN3, S37A or DN TCF constructs and luciferase activity measured. (mean ± s.e.m., n=4).

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, mDiaΔN3 strongly inhibited the MyoD DRR activity to ~9% of control, ΔN3*Hin*dIII 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 ΔN3 and ΔN3*Hin*dIII 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 mDiaΔN3 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 ΔN3*Hin*dIII 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 mDiaΔN3 inhibition and rescue MyoD expression. β-catenin S37A robustly activates TCF reporter activity in mDiaΔN3-transfected cells (Fig. 8A). Co-transfection of β-catenin S37A with mDiaΔN3 led to a twofold increase in MyoD expression compared with mDia-ΔN3 co-transfected with a control plasmid, suggesting a partial reversal of the effects of mDiaΔN3 (Fig. 8B,C). The milder suppressive effect of ΔN3*Hin*dIII 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 ΔN3*Hin*dIII 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	gggaaatCAAAgggcca	Site A
Wnt signaling	685-701	1.000	0.86	agcaaagCAAAgcaagc	Site B
pathway	695-711	1.000	0.87	gagcaaaCAAAgcaaag	

*Capital letters indicate the core sequence used by the MatInspector program; underlined letters indicate nucleotides conserved in vertebrates.

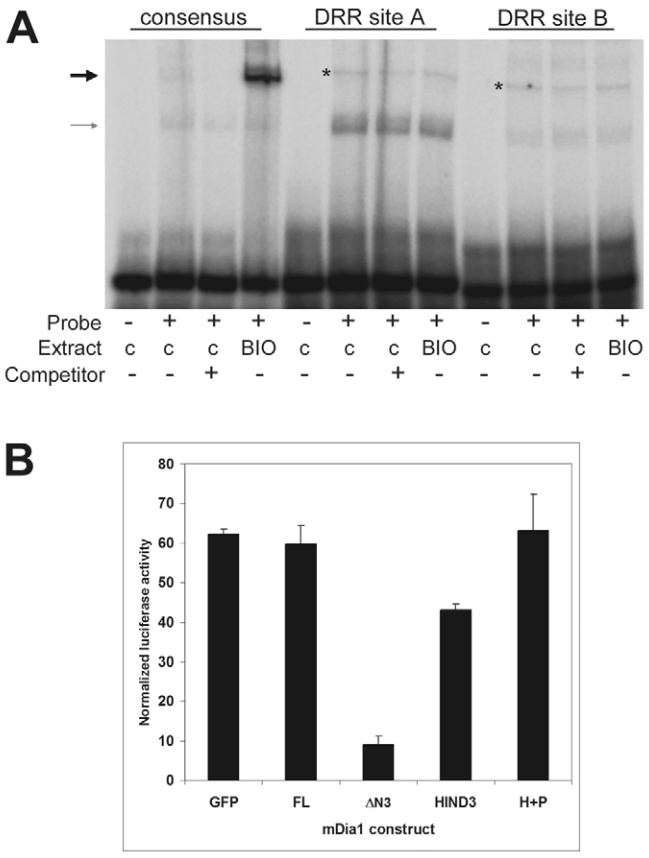

Fig. 7. MyoD DRR activity is inhibited by mDia. (A) Gel-shift assays of the putative TCF/LEF sites in the MyoD DRR. ^{32}P-end-labeled oligo probes representing a TCF consensus site or two of the three sites from the MyoD DRR (denoted DRR site A or DRR site B) were incubated with extracts prepared from control C2C12 myoblasts (indicated as 'c') or myoblasts treated with 2.5 μM BIO for 24 hours. Assays were performed in the presence or absence of 100-fold molar excess of the respective cold competitor oligo. The consensus TCF site participated in the formation of complexes that were competed by excess cold probe, and induced by BIO (black arrow) consistent with the binding of β-catenin–TCF. The gray arrow indicates a nonspecific complex. However, DRR sites A and B bound a nonspecific complex (indicated by *) that was neither competed nor BIO-inducible. Thus, the TCF consensus sites in the MyoD DRR do not appear to function as targets of specific nuclear factor binding. (B) MyoD DRR activity is inhibited by mDia. Myoblasts were transiently transfected in growth medium with a mouse MyoD DRR-pGL3 promoter construct along with pBS (control) or mDia constructs, and a βgal plasmid. Luciferase activity was quantified after 24 hours and normalized for transfection efficiency (mean ± s.e.m., n=3). ΔN3 is the most effective at suppressing DRR activity, and overall the DRR suppressive activity of the different forms of mDia1 correlated with suppression of TCF activity.

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

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.	–

*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.

(Geneste et al., 2002). SRF activity depends on its co-activator MAL that is sequestered by monomeric G-actin in the cytoplasm when actin is depolymerized (Miralles et al., 2003). When actin polymerizes, MAL is released, translocates to the nucleus and facilitates SRF-dependent transcription.

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 – ΔN3*Hin*dIII 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, ΔN3*Hin*dIII, H+P), ΔN3 suppresses MyoD expression maximally, whereas ΔN3*Hin*dIII 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

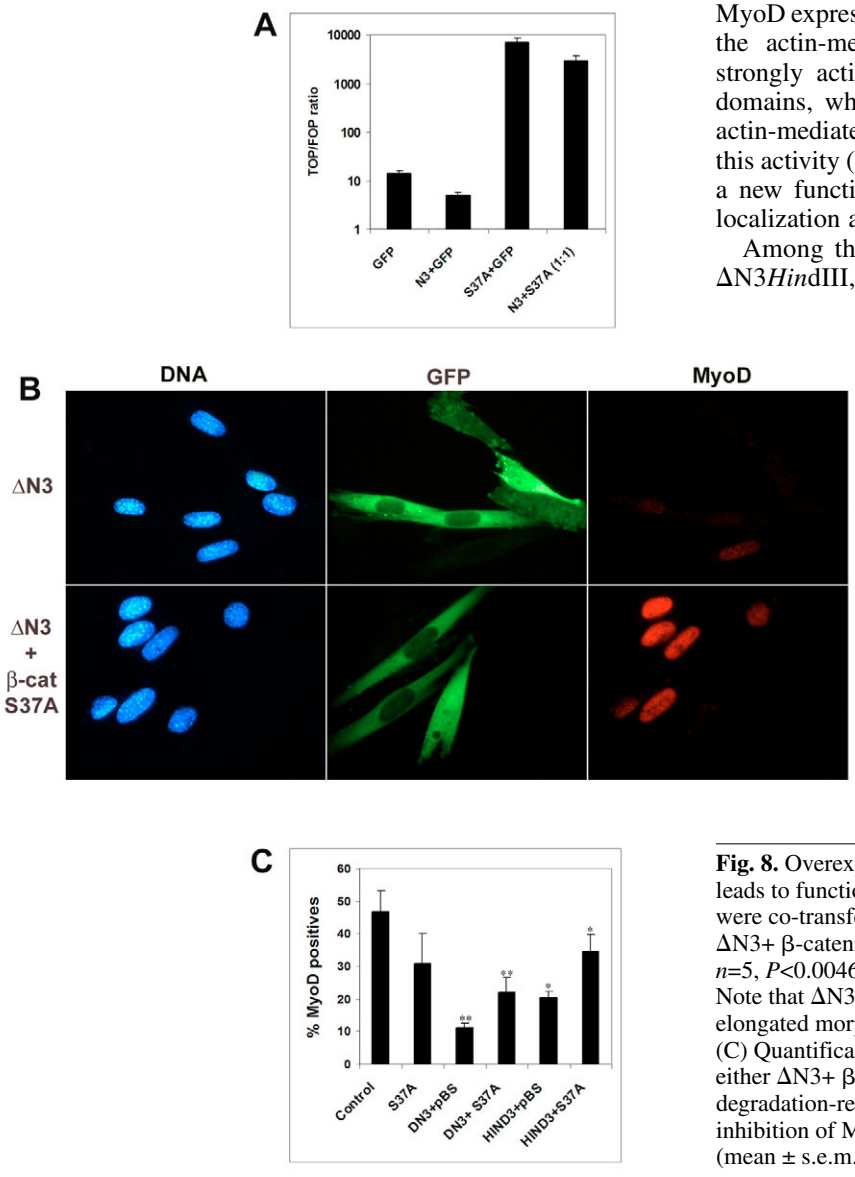

Fig. 8. Overexpression of an APC-independent form of β-catenin leads to functional bypass of mDiaΔN3 inhibition. (A) Myoblasts were co-transfected with a control plasmid (GFP), ΔN3+GFP or ΔN3+ β-catenin S37A and TCF activity determined (mean ± s.e.m., n=5, P<0.0046). (B) MyoD expression in cells transfected 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 ΔN3*Hin*dIII+ 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).

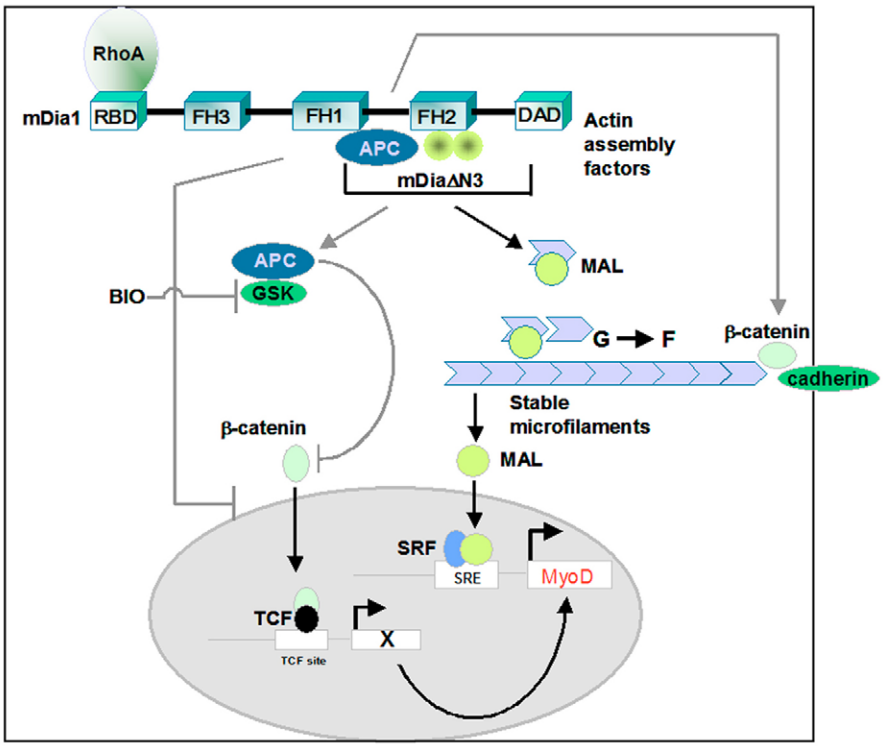

Fig. 9. mDiaΔN3 controls MyoD by reciprocal regulation of two transcription factors. A model for dual signaling to the MyoD gene by mDia via positive regulation of a directly acting Rho-actin-SRF pathway and negative regulation of an indirect APC–β-catenin–TCF pathway. Actin assembly factors bind to the FH1 and FH2 domains, drive polymerization of microfilaments to activate SRF via MAL release and thereby induce MyoD expression. The interaction of mDia with APC may increase cytoplasmic degradation of β-catenin or reduce nuclear shuttling of APC and thereby promote cytoplasmic retention of β-catenin. As dnTCF inhibits MyoD, TCF may play an activating role by inducing positive upstream factors (X). Taken together, the data suggest that MyoD expression is suppressed by any perturbation of mDia, because SRF and TCF are reciprocally regulated by this key signaling adaptor protein.

effects of mDia on APC's target, β-catenin (see below). At present, it is unclear whether other FH1-binding proteins are involved.

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 mDia1 interferes with endogenous β-catenin localization and function. Although mDiaΔN3 (a.a. 543-1182) blocks BIO-induced β-catenin nuclear localization, ΔN3*Hin*dIII (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*Hin*dIII 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*Hin*dIII 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*Hin*dIII 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 activitor of MyoD. Known upstream activators of MyoD expression such as Pax3 are induced in response to Wnts (Petropoulos 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 mechano-chemical signals activated by damage to this contractile tissue.

Materials and Methods

Cell culture

A subclone of C2C12 myoblasts (Yaffe and Saxel, 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, dnTCF1E, Src or mU6-shRNA (1.25 μg) using Lipofectamine 2000 (Invitrogen). For co-transfections, ΔN3 or mU6-shRNA plasmids and EGFP-C1 were used at a 4:1 ratio. For the rescue experiments, Dia 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 3DA.Luc or Super8XTOPFLASH or FOPFLASH + 6 ng of pRSV-βGal per well, 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-N3*Hin*dIII, GFP-H+P, GFP-F2 and GFP-CC are described in Watanabe et al. (Watanabe et al., 1999). mU6 vector was a gift from D. Turner (Yu et al., 2002). dnTCF-1E (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 β-catenin binding domain into the EVR vector (M. Waterman, personal communication). Super8X-TOP-flash (TCF site) and FOP-flash (mutated TCF site) (Veeman et al., 2003); full length APC-gfp and APCΔMT-gfp (Penman et al., 2005). The 714 bp MyoD DRR was amplified from mouse genomic DNA using the primers (F, 5′-CTTAAGAGCTC-GATCTACACTTGGTGGCAGGTAG-3′ and R, 5′-ATATTCTCGAGCGAGCAAA-CAAAGCAAAGC-3′) and cloned into the pGL3 promoter vector.

SRF, TCF and DRR reporter activity assays

Luciferase activity in lysates prepared from 3DA.Luc transfectants (SRF), TOP- or FOP-flash transfectants (TCF), or DRR transfectants (MyoD) was assayed using chemiluminescence (Promega) and expressed as relative light units (RLU), normalized to co-transfected β-gal and total protein. TCF activity was finally expressed as a ratio of TOP-FOP activity. BrdU incorporation was measured 24 hours post transfection, by pulsing with 100 μM BrdU for 15 minutes and detection as described (Dhawan and Helfman, 2004).

Fluorescence microscopy

24 hours after transfection, myoblasts were fixed and permeabilized in 2% paraformaldyhyde, 0.2% Triton X-100 in CSK buffer (10 mM PIPES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM $MgCl_2$, 100 μg/ml streptomycin, pH 7.0) (Herzog et al., 1994). All washes were in CSK buffer. Primary antibodies were: anti-β-catenin (BD Biosciences) 1:250; anti-MyoD (Novocastra) 1:80; anti-p21, 1:500; anti p27, 1:250; anti BrdU-FITC, 1:10 (BD Biosciences). Secondary antibodies (Molecular Probes) were goat anti-mouse Alexa Fluor 488, 1:500 and goat anti mouse Alexa Fluor 594, 1:500; Oregon green phalloidin (1:50). Samples were mounted in 50% glycerol and imaged at room temperature. ~250 transfected cells were counted per coverslip and all samples analyzed in duplicate in at least three independent experiments. Staining was recorded on a CCD camera using an Olympus microscope (40× UPlanFL Olympus objective, 0.75 NA; ImagePro Plus software) or on a Zeiss 510 Meta laser-scanning confocal microscope (63×, Plan Apochromat Zeiss objective, 1.4 NA; LSM5 software). Images were minimally adjusted for brightness and contrast using Adobe Photoshop 6.0.

Western blot analysis

Cells were solubilized in 2× Laemmli sample buffer, and 100 μg of total protein was analyzed as described (Sachidanandan et al., 2002). Antibodies were diluted in blocking buffer: MyoD polyclonal (Santa Cruz) 1:400, desmin polyclonal (Sigma) 1:500. GFP monoclonal, 1:500; HRP-conjugated secondary antibody (anti-rabbit, Bangalore Genei) (1:10,000) was detected using ECL (Amersham).

Cell cycle analysis

C2C12 cells transfected with mDiaΔN3-GFP or ΔN3*Hin*DIII-GFP were analyzed as described (Sachidanandan et al., 2002) on a FACS Calibur (BD Biosciences). Transfected cells were detected by gating for GFP.

RNA interference

Short hairpin RNAs (shRNAs) were designed against full-length mDia1 using OligoRetriever (http://www.cshl.org/)public/SCIENCE/hannon.html). The sequences for mDia1shRNA were 5′-TTTGTGGTGGTGGTATACCTGTACCGA-AGCGGTACAGCTATACCATCACCATTTTT-3′ and 5′-CTAGAAAAATGGTGA-TGGTATAGCTGTACCGCTTCGGTACAGGTATACCACCACCA-3′.

The sequences for GFP shRNA were 5′-TTTGAACTTCAAGGTCCGCCAC-AACGAAGCGTTTTGGCGGACCTTGAAATTTTTTT-3′ and 5′-CTAGAAAAA-AATTTCAAGGTCCGCCAAAACGCTTCGTTGTGGCGGACCTTGAAGTT-3′. Oligos were annealed and cloned into the mU6 vector (Yu et al., 2002). C2C12 cells were co-transfected with mU6-mDia shRNA or mU6-GFP shRNA or empty vector and pSV2Neo, and stable pools selected in G418 500 μg/ml.

Quantitative real-time RT-PCR

1 μg total RNA isolated from Dia-shRNA, control GFP-shRNA or mU6 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 cycler (Applied Biosystems) and the SDS2.1 ABI Prism software. Dissociation curves were used to verify the amplicons and normalized fold differences of cycle thresholds [$2^{-(-\Delta\Delta Ct)}$] calculated relative to a control GAPDH amplicon.

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′-AGGGGGAGATCAAAGGGCCACCT-3′ and 3′-AGGTGGCCCTTTGA-TCTCCCCCT-5′; DRR site A, 5′-AGGGGGAAATCAAAGGGCCACCT-3′ and 3′-AGGTGGCCCTTTGATTTCCCCCT-5′; DRR site B, 5′-GCTTGCTTTGTT-TGCTCGGG-3′ and 3′-CCCGAGCAAACAAAGCAAAGC-5′. Oligos were annealed and end-labeled with T4-polynucleotide kinase (New England Biolabs). Binding reactions (Prieve and Waterman, 1999) used 15,000 cpm purified probe incubated with 5 μg nuclear extract on ice for 30 seconds in the presence or absence of 100-fold molar excess of cold competitor oligo, followed by electrophoresis through 6% native gels and detection by phosphorimaging (Fuji).

We thank N. Rangaraj for expert assistance with confocal microscopy; S. Byers, R. Moon, I. Nathke, P. Salinas, A. Sarin, R. Treisman, D. Turner and M. Waterman for generous gifts of reagents; T. Rando, M. Waterman, P. Salinas, I. Siddiqi, G. Swarup and D. Kasbekar for critically reading the manuscript; L. Shashidhara and T. Vaidya for helpful discussions; and members of the lab for help with the analysis of β-catenin in mDia transfectants. This work was supported in part by grants from the Govt. of India Dept. of Biotechnology and the Council of Scientific and Industrial Research (CSIR) Network Program on Cell and Tissue Engineering (J.D.). S.D.G. was supported by a CSIR pre-doctoral research fellowship. J.D. is a Senior Research Fellow of the Wellcome Trust (UK).

References

Alberts, A. S. (2001). Identification of carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain. *J. Biol. Chem.* **276**, 2824-2830.

Alfarano, C., Andrade, C. E., Anthony, K., Bahroos, N., Bajec, M., Bantoft, K., Betel, D., Bobechko, B., Boutilier, K., Burgess, E. et al. (2005). The Biomolecular Interaction Network Database and related tools 2005 update. *Nucleic Acids Res.* **33**, D418-D424.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Natsura. Y.

and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase. *J. Biol. Chem.* **271**, 20246-20249.
Andrews, N. C. and Faller, D. V. (1991). A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res*. **19**, 2499.
Blau, H. M., Chiu, C. P. and Webster, C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell* **32**, 1171-1180.
Carnac, G., Primig, M., Kitzman, M., Chafey, P., Tui, L. D., Lamb, N. and Fernandez, A. (1998). Rho GTPase and serum response factor control selectively the expression of MyoD without affecting Myf-5 in mouse myoblasts. *Mol. Biol. Cell* **9**, 1891-1902.
Chen, J. C. J., Ramachander, R. and Goldhamer, D. J. (2002). Essential and redundant function of the MyoD DRR revealed by targeted mutagenesis. *Dev. Biol*. **245**, 213-223.
Chrzanowska-Wodnicka, M. and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol*. **133**, 1403-1415.
Clevers, H. (2000). Armadillo takes the APC shuttle. *Nat. Cell Biol*. **10**, E177-E178.
Collins, C. A. (2006). Satellite cell self-renewal. *Curr. Opin Pharmacol*. **6**, 301-306.
Copeland, J. W. and Treisman, R. (2002). The Diaphanous-related formin mDia1 controls serum response factor activity through its effects on actin polymerisation. *Mol. Biol. Cell* **13**, 4088-4099.
Dhawan, J. and Helfman, D. M. (2004). Modulation of acto-myosin contractility in skeletal muscle myoblasts uncouples growth arrest from differentiation. *J. Cell Sci*. **117**, 3735-3748.
Dhawan, J. and Rando, T. A. (2005). Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell arrest, activation and replenishment. *Trends Cell Biol*. **15**, 666-676.
Gauthier-Rouviere, C., Vandromme, M., Tuil, D., Lautredou, N., Morris, M., Soulez, M., Kahn, A., Fernandez, A. and Lamb, N. (1996). Expression and activity of serum response factor is required for expression of the muscle-determining factor MyoD in both dividing and differentiating mouse C2C12 myoblasts. *Mol. Biol. Cell* **7**, 719-729.
Geneste, O., Copeland, J. W. and Treisman, R. (2002). LIM kinase and Diaphanous cooperate to regulate serum response factor and actin dynamics. *J. Cell Biol*. **157**, 831-838.
Gineitis, D. and Treisman, R. (2001). Differential usage of signal transduction pathways defines two types of serum response factor target gene. *J. Biol. Chem*. **276**, 24531-24539.
Goldhamer, D. J., Faerman, A., Shani, M. and Emerson, C. P., Jr (1992). Regulatory elements that control the lineage-specific expression of myoD. *Science* **256**, 538-542.
Grounds, M. D., Garrett, K. L., Lai, M. C., Wright, W. E. and Beilharz, M. W. (1992). Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell Tissue Res.* **267**, 99-104.
Habas, R., Kato, Y. and He, X. (2001). Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel formin homology protein Daam1. *Cell* **107**, 843-854.
Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D. and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**, 1018-1021.
Herzog, M., Draeger, A., Ehler, E. and Small, J. V. (1994). Immunofluorescence microscopy of the cytoskeleton. In *Cell Biology Laboratory Handbook* (ed. J. Celis), pp. 355-360. San Diego, CA: Academic Press.
Kitzmann, M., Carnac, G., Vandromme, M., Primig, M., Lamb, N. and Fernandez, A. (1998). The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *J. Cell Biol.* **142**, 1447-1459.
L'honore, A., Lamb, N., Vandromme, M., Turowski, P., Carnac, G. and Fernandez, A. (2003). MyoD distal regulatory region contains an SRF binding CArG element required for MyoD expression in skeletal myoblasts and during muscle regeneration. *Mol. Biol. Cell* **14**, 2151-2162.
Mammoto, A., Huang, S., Moore, K., Philmo, O. and Ingber, D. E. (2004). Role of RhoA, mDia and ROCK in cell-shape dependent control of the Skp2-p27^{kip1} pathway and the G1/S transition. *J. Biol. Chem*. **279**, 26323-26330.
Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E. and Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev*. **10**, 1173-1183.
Meijer, L., Skaltsounis, A. L., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X. P., Vonica, C. A., Brivanlou, A. et al. (2003). GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol.* **10**, 1255-1266.
Milasincic, D. J., Dhawan, J. and Farmer, S. R. (1996). Anchorage dependent control of muscle specific gene expression in C2C12 myoblasts. *In Vitro Cell Dev. Biol. Anim*. **32**, 90-99.
Miralles, F., Posern, G., Zaromytidou, A. I. and Treisman, R. (2003). Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113**, 329-342.
Munsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P. and Lassar, A. B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **23**, 2911-2922.
Nelson, W. J. and Nusse, R. (2004). Convergence of Wnt, β-catenin, and cadherin pathways. *Science* **303**, 1483-1487.
Penman, G. A., Leung, L. and Nathke, I. S. (2005). The adenomatous polyposis coli (APC) protein exists in two distinct soluble complexes with different functions. *J. Cell Sci.* **118**, 4741-4750.
Petropoulos, H. and Skerjanc, I. S. (2002). Beta-catenin is essential and sufficient for skeletal myogenesis in P19 cells. *J. Biol. Chem*. **277**, 15393-15399.
Prieve, M. G. and Waterman, M. L. (1999). Nuclear localization and formation of beta-catenin-lymphoid enhancer factor 1 complexes are not sufficient for activation of gene expression. *Mol. Cell. Biol.* **19**, 4503-4515.
Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein Rho regulates the assembly of focal adhesions and stress fibers in response to growth factors. *Cell* **70**, 389-399.
Rochat, A., Fernandez, A., Vandromme, M., Moles, J. P., Bouschet, T., Carnac, G. and Lamb, N. J. (2004). Insulin and wnt1 pathways cooperate to induce reserve cell activation in differentiation and myotube hypertrophy. *Mol. Biol. Cell* **15**, 4544-4555.
Sachidanandan, C., Sambasivan, R. and Dhawan, J. (2002). Tristetraprolin and LPS-inducible CXC chemokine are rapidly induced in presumptive satellite cells in response to skeletal muscle injury. *J. Cell Sci.* **115**, 2701-2712.
Sahai, E., Alberts, A. S. and Treisman, R. (1998). RhoA effector mutants reveal distinct effector pathways for cytoskeletal organization, SRF activation and transformation. *EMBO J.* **17**, 1350-1361.
Seale, P. and Rudnicki, M. A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev. Biol.* **218**, 115-124.
Sotiropoulos, A., Ginetis, D., Copeland, J. W. and Treisman, R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* **98**, 159-169.
Soulez, M., Rouviere, C. G., Chafey, P., Hentzen, D., Vandromme, M., Lautredou, N., Lamb, N., Kahn, A. and Tuil, D. (1996). Growth and differentiation of C2 myogenic cells are dependent on SRF. *Mol. Cell. Biol.* **16**, 6065-6074.
Tajbakhsh, S. (2005). Skeletal muscle stem and progenitor cells: reconciling genetics and lineage. *Exp. Cell Res*. **306**, 364-372.
Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M. and Cossu, G. (1998). Differential activation of Myf-5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf-5. *Dev. Suppl.* **125**, 4155-4162.
Takano, H., Komuro, I., Oka, T., Shiojima, I., Hiroi, Y., Mizuno, T. and Yazaki, Y. (1998). The Rho family G proteins play a critical role in muscle differentiation. *Mol. Cell. Biol.* **18**, 1580-1589.
Tapscott, S. J. (2005). The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development* **132**, 2685-2695.
Tapscott, S. J., Lassar, A. B. and Weintraub, H. (1992). A novel myoblast enhancer element mediates MyoD transcription. *Mol. Cell Biol*. **11**, 4994-5003.
Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S. A. and Alberts, A. S. (2000). Diaphanous-related formins bridge Rho GTPase and Src Tyrosine kinase signaling. *Mol. Cell* **5**, 13-25.
Van Aelst, L. and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev.* **11**, 2295-2322.
van de Wetering, M., Sancho, E., Verweij, C,, de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P. et al. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**, 241-250.
Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H. and Moon, R. T. (2003). Zebrafish prickle a modulator of noncanonical Wnt/Frizzled signaling regulates gastrulation movements. *Curr. Biol.* **13**, 680-685.
Wallar, B. J. and Alberts, A. S. (2003). The formins: active scaffolds that remodel the cytoskeleton. *Trends Cell Biol*. **13**, 435-446.
Wasserman, S. (1998). FH proteins as cytoskeletal organizers. *Trends Cell Biol.* **8**, 111-115.
Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M. and Narumiya, S. (1997). p140mDia, a mammalian homolog of Drosophila Diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.* **16**, 3044-3056.
Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. and Narumiya, S. (1999). Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nat. Cell Biol.* **1**, 136-143.
Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75**, 1241-1244.
Wei, L., Zhou, W., Croissant, J. D., Johansen, F. E., Prywes, R., Balasubramanyam, A. and Schwartz, R. J. (1998). RhoA signaling via serum response factor plays an obligatory role in myogenic differentiation. *J. Biol. Chem.* **273**, 30287-30294.
Wei, Q. and Paterson, B. M. (2001). Regulation of MyoD function in the dividing myoblast. *FEBS Lett.* **490**, 171-178.
Welsh, C. F. and Assoian, R. K. (2000). A growing role for Rho family GTPases as intermediaries in growth factor- and adhesion-dependent cell cycle progression. *Biochim. Biophys. Acta* **1471**, M21-M29.
Wen, Y., Eng, C. H., Schmoranzer, J., Cabrera-Poch, N., Moris, E. J. S., Chen, M., Wallar, B. J., Alberts, A. S. and Gundersen, G. G. (2004). EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. *Nat. Cell Biol.* **6**, 820-830.
Yaffe, D. and Saxel, O. (1977). A myogenic cell line with altered serum requirements for differentiation. *Differentiation* **7**, 159-166.
Yoshida, N., Yoshida, S., Koishi, K., Masuda, K. and Nabeshima, Y. (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J. Cell Sci.* **111**, 769-779.
Young, C. S., Kitamura, M., Hardy, S. and Kitajewsky, J. (1998). Wnt-1 induces growth, cytosolic beta-catenin and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. *Mol. Cell. Biol.* **18**, 2474-2485.
Yu, J. Y., DeRuiter, S. L. and Turner, D. L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 6047-6052.
Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A. and Beauchamp, J. R. (2004). Mucle satellite cells adopt divergent fates: a mechanism for self renewal? *J. Cell Biol.* **166**, 347-357.