The Rho effector mDia1 regulates MyoD expression and cell cycle progression via two antagonistic pathways.
Introduction

Formins are a family of highly conserved eukaryotic proteins implicated in a wide range of actin-based processes, including cell polarization, cytokinesis, sperm cell acrosome formation and limb and kidney morphogenesis (reviewed in Ridley, 1999; Wasserman, 1998; Zeller et al., 1999). The most conserved feature of all formins in vertebrates, Drosophila, Caenorhabditis and yeast is the presence of two juxtaposed formin homology domains, FH1 and FH2 (Castrillon and Wasserman, 1994; Wasserman 1998) (Figure 31). Some formins contain a conserved FH3 motif before the FH1 domain: this motif determines subcellular localization of the formin molecule (Petersen et al, 1998). The proline rich FH1 domain binds to the G-actin binding protein profilin (Watanabe et al, 1997) delivering ATP-bound actin monomers to the growing end of actin filaments. SH3 and WW proteins that participate in cytokinesis also associate with the FH1 domain of formins. The FH2 domain represents a unique and defining feature of formin proteins, sharing no obvious sequence similarity with any other domain or polypeptide. It controls actin nucleation in vitro (Pruyne et al, 2002) and actin assembly in vivo (Copeland and Treisman, 2002). Thus, formins function as a scaffold, recruiting proteins that regulate the cytoskeleton.

Different pathways and different mechanisms regulate several formins. Some of the known formins include Delphilin that links the glutamate receptor involved in postsynaptic transmission to the actin cytoskeleton (Miyagi et al, 2002) or the human formin, Daam1 that forms a complex with Dishevelled (DVL) of the Wnt pathway and Rho GTPase (Habas et al, 2001). Diaphanous-related formins (DRFs) constitute a sub-family of the formin homology (FH) proteins first defined after the discovery of the conserved FH1 and FH2 domains shared between Diaphanous (in Drosophila), Bni1p (in yeast) and Formins (limb deformity genes in mice). The DRFs have an amino terminal Rho GTPase-binding and carboxy-terminal Dia-autoregulatory domain (called RBD and DAD respectively) besides the conserved FH1 and FH2 domains (Evangelista et al, 1997; Imamura et al, 1997; Watanabe et al, 1997).
A) Schematic showing different domains of a DRF and their functions. RBD, RhoGTPase-Binding Domain; FH, Formin Homology; DAD, Diaphanous Autoregulatory Domain. (Figure modified from Boone et al., JCS, 2003).

B) Intramolecular repression of DRFs by binding of DAD and activation by Rho GTPase. (Figure modified from webpage of Christoff Ballestrem)

C) Transfection of active GFP-mDia (green) aligns actin bundles (stained with rhodamine phalloidin) parallel to the long axis of the cell compared to the meshed appearance in myoblasts transfected with GFP alone.

Figure 31: Molecular organization and regulation of the DRFs
Rho GTPase activates DRFs by binding to the RBD and disrupting the RBD-DAD interaction (Alberts, 2001; Watanabe, 1999). Activation of DRFs by Rho and repression by auto-inhibition can be experimentally mimicked by deleting the RBD (Figure 31) However, other formins also possess an intra-molecular mode of regulation with a regulatory N-terminal domain containing an RBD that does not bear a significant sequence similarity to the DRFs (for example the human formin Daam1 binds Rho but is not related to diaphanous (Habas et al, 2001). Thus, DRFs have been classified by a functional definition.

Earlier work to determine the functions of DRFs showed that Drosophila Dia null alleles were lethal while the alleles conferring a partial loss of gene function caused a failure to complete meiosis during germ cell proliferation resulting in sterile adult males and females (Wasserman et al, 1994). Further studies that localized mDia1, the mammalian orthologue of Diaphanous to the mitotic spindle in Hela cells (Kato et al, 2000), suggested that mDia1 is essential for proper completion of cell division in vertebrates. Consistent with the role of formins as cytoskeletal regulators in yeast and Drosophila, mDia was discovered in a screen for Rho effectors that could correct the aberrant orientation induced by over-expression of ROCK, another Rho effector (Watanabe et al, 1999). In this screen, mDia was found to align microtubules and actin parallel to the long axis of the cell (Figure 31C) terminating in mDia1-enriched foot-like processes that invariably contained focal adhesions. This cytoskeletal polarization is a characteristic feature of differentiated cells and migrating wound edge cells (Cook et al, 1998). Simultaneous with the discovery of mDia's role in regulating polarity of microtubules and microfilaments, several groups showed that a dominant negative form of mDia 1 comprising the Rho-binding domain (1-571 amino acids) and dominant negative ROCK could block the formation of Rho-induced actin stress fibers and focal adhesions in vertebrates (Nakano et al, 1999; Watanabe et al, 1999). Studies on the effects of individual functions of mDia and ROCK revealed that mDia promotes F-actin accumulation whereas ROCK controls actin filament bundling (Nakano et al, 1999; Tominaga et al, 2000). Since the main consequence of Rho-actin dynamics on gene expression is known to be mediated through the MADS box transcription
factor SRF (See introduction), perturbing mDia activity by antibodies against mDia1 blocked serum-induced activity of SRF in NIH3T3 cells (Sotiropoulos et al, 1999; Tominaga et al, 2000; Mack et al, 2001) with the same efficiency as the over-expression of non-polymerizable mutants of actin (Copeland and Treisman, 2002).

Thus, the unambiguous role of mDia in regulating the microfilament and microtubule cytoskeleton and SRF activity indicated that mDia is the dominant effector mediating Rho's effects on the cytoskeleton. However, the possibility that mDia could also be the mediator of other functions of Rho GTPase that involve cell cycle progression, differentiation and myogenic determination have not been explored.

Unlike earlier reports that established mDia's role in motility and the interaction of the mitotic spindle and cleavage furrow during cytokinesis, my studies show a role for active mDia in interphase, specifically in G1 progression and regulation of MyoD expression. However, the mechanism of regulation suggests the presence of two pathways emanating from mDia: one that is dependent on contractile, polymerized microfilaments acting through SRF and another unexplored pathway that is independent of SRF.

In this chapter I show that in addition to the canonical SRF dependent pathway, a TCF/β-catenin pathway operates downstream of mDia1. I present evidence that mDia1 regulates these two pathways antagonistically and propose that MyoD expression is regulated by the dynamic integration of these two pathways.

Results

Experiments described in Chapter 4 showed that over-expression of a Rho-independent form of mDia1, ΔN3 maximally suppressed MyoD expression without decreasing SRF activity. Additionally, the ability of active mDia1-ΔN3 to interact with src tyrosine kinase (Uetz et al, 1996) suggested
the possibility that MyoD expression could be modulated by mDia-mediated recruitment of src. As src is a prominent signalling molecule that is located on the cytoplasmic face of focal adhesions (Shalloway et al, 1992) and has been shown to play a role in suppressing MyoD's expression (Falcone et al, 1991), it was possible that mDia might suppress MyoD via src.

**ΔN3 active mutant of mDia1 exerts its effect on MyoD in Src-independent manner**

The mDia1 mutants that have a significant affect on the expression of MyoD (ΔN3, N3HindIII and H+P) share a common domain FH1 that is known to interact with Profilin and c-src tyrosine kinase. To assess the contribution of src to MyoD regulation in C2C12 myoblasts, cells were co-transfected with constitutively active mutant of src along with GFP as a marker of transfected cells and MyoD expression was analyzed by immunofluorescence. Myoblasts co-expressing the constitutively active form of src (Y529F) and GFP showed at least 50% reduction in MyoD positives (Figure 32, panels a,b,c) when compared to myoblasts expressing GFP alone. However, MyoD expression in myoblasts co-expressing mDiaΔN3 and c-srcY529F did not differ from MyoD expression in cells expressing mDia ΔN3 alone (panels g,h,i).

To address the possibility of a Src-mediated inhibition of MyoD through a Src-FH1 recruitment of mDia1, we co-transfected growing C2C12 myoblasts with GFPmDiaΔN3 or GFP alone and a dominant negative mutant of src (K296R/Y528F). While the control myoblasts co-expressing GFP and DNsrc were positive for MyoD expression (panels d,e,f) similar to GFP expressing cells, ΔN3 expressing cells continued to be negative for MyoD (panels j,k,l). Similar results were obtained when ΔN3 expressing cells were treated with 5 μM of a pharmacologic src tyrosine kinase inhibitor, PP2. MyoD positives were 47.5% in control and 4.1 % in ΔN3 expressing cells plated in the presence of the drug. Thus, suppression of MyoD in mDiaΔN3 expressing cells could not be rescued by suppression of endogenous src and the
Figure 32: \(\Delta N3\) suppresses MyoD expression in a Src-independent manner

A) C2C12 myoblasts were co-transfected with GFP-\(\Delta N3\) or control (EGFP-C1) along with a constitutively active form of src, Y529F or dominant negative src (DN-src). 24 hrs after transfection, cells were fixed and stained for MyoD protein. Panels a-c show cells transfected with control EGFP and Y529F, d-f show EGFP and dominant negative src, g-i show \(\Delta N3\) and Y529F, j-k show \(\Delta N3\) and dominant negative (DNsrc). As expected, cells transfected with active src are negative for MyoD expression while transfected cells in which Src activity has been abolished express MyoD. Notably, \(\Delta N3\) transfectants remain negative for MyoD despite the presence of DN-src.

B) Quantitation of transfection data. Values represent mean + SEM (n=2).
mechanism of inhibition of MyoD in mDiaΔN3 expressing cells does not correlate with its ability to recruit src.

*mDiaΔN3-expressing cells have a greater proportion of stable microtubules*

Analyses of the microtubule population in the migrating cells in wounded monolayers of serum starved NIH-3T3 fibroblasts revealed that the microtubule subunits at the progressive end of the cell were selectively stabilized due to the presence of LPA in serum that activated Rho and its effector mDia (Cook et al, 1998; Palazzo et al, 2004). Microtubules have been implicated in the intracellular organization during myogenesis, albeit at a much later stage, during the formation of myotubes (Gunderssen et al, 1989).

Since active mDia-ΔN3 is known to increase the pool of stable microtubules in fibroblasts, I determined whether ΔN3-expressing cells have altered microtubules. I assessed levels of acetylated tubulin, a marker of stable microtubules, as well as used a functional assay for stable microtubules by estimating their susceptibility to the microtubule depolymerizing drug, nocodazole. mDiaΔN3-expressing cells have higher levels of acetylated tubulin as well as a greater proportion of nocodazole-resistant microtubules than drug-treated control myoblasts. (Figure 33A). Thus, the suppression of MyoD expression by ΔN3 correlates with increase microtubule stability (Figure 33B).

Terminally differentiated multinucleated C2C12 muscle cells are irreversibly arrested and accumulate stable microtubules (Gunderssen et al, 1989). To examine whether microtubules are also stabilized during reversible arrest in untransfected myoblasts, I used the nocodazole resistance assay. While the majority of adherent asynchronous myoblasts displayed nocodazole-sensitive microtubules, adhesion-deprived G_0 myoblasts uniformly possessed a pool of intact microtubules after drug treatment (Figure 33C). Nocodazole-resistant microtubules persisted until at least 4 hrs after
mDia1-ΔN3 transfected cells display stable microtubules: A) C2C12 myoblasts were transfected with GFP-ΔN3 or a control plasmid (EGFPC1) and 24 hours later were treated with 2 μM nocodazole for 30 minutes, and immuno-stained for total tubulin. Microtubules in ΔN3 expressing cells display microtubules that are arranged parallel to the long axis of the cell and are resistant to nocodazole-induced depolymerization. B) Expression of MyoD in nocodazole-treated control cell but not in ΔN3 expressing cell with stable microtubules.
Suspension-arrested myoblasts show reciprocal alterations in microtubules (top and middle panels) and microfilaments (bottom panel). C2C12 myoblasts were suspended for 48 hours (G0) and replated in growth medium for 30 minutes, 4 hrs or 16 hrs (top panel). Parallel cultures were treated with 2 μM nocodazole or vehicle and total tubulin detected. Nocodazole resistant microtubules were detected in G0 and early G1 (R30 min, R4 hrs) but not in late G1/S phase (R16 hrs) (middle panel). Stress fibers (bottom panel) are lost in suspension and reform during replating at a time when microtubules become dynamic. Images show the same control cells as in top panel, co-stained with Oregon-green phalloidin to detect microfilaments.
reactivation (early G_1), a time when MyoD expression is induced. However, at 16 hrs after activation (late G_1/early S), most cells showed susceptibility to nocodazole. Thus, microtubule stability changes during the cell cycle with a timing that correlates with changes in MyoD expression.

Actin dynamics showed a reciprocal regulation to microtubules - the well defined stress fibers seen in adherent asynchronous cells were completely lost in G_0, and reformed during G_1 with kinetics that paralleled the appearance of nocodazole-sensitive microtubules (Figure 33C, bottom panels) and MyoD expression. Therefore, high SRF activity during adhesion-dependent cell cycle re-entry (Figure 21) correlated with stable microfilaments and dynamic microtubules.

To identify candidate pathways by which microtubules might act, I searched the BIND protein interaction database (Alfarano et al, 2005) for microtubule-associated mDia-binding proteins, and identified the Wnt pathway regulator APC (adenomatous polyposis coli) (Wen et al, 2004). A tripartite complex of APC, EB1 and mDia caps the plus ends of microtubules and leads to their stabilization. I hypothesized that mDia might modulate MyoD via effects on the Wnt pathway.

**The MyoD distal regulatory region contains consensus sites for the Wnt pathway target regulator TCF/LEF**

The Wnt pathway has been implicated in the Rho-dependent regulation of morphogenetic events involving formins (Habas et al, 2001), as well in the induction of embryonic myogenesis (Tajbakhsh et al, 1998). However, the role of Wnt signaling in post-natal myogenesis is less well established, and the MyoD gene has not been implicated as a direct target. The MyoD promoter contains a distal regulatory region (DRR; Tapscott et al, 1992; Chen et al, 2002) with an SRF binding site (CArG box) involved in activation of MyoD in satellite cells (L'Honore et al, 2003) (Schematic of DRR in Figure 34A and DRR sequence from Ensemble database in Figure 34B). DRR activity in transgenic mice is restricted to differentiated muscle and persists
A) Location of the Distal Regulatory Element (DRR) is 5 kb upstream of the MyoD transcription start site and the relative locations of the Serum response element (SRE) * and the TCF/LEF response elements (TCFRE)*.

B) Sequence of the DRR obtained from the Ensembl database. SRE of MyoD (red), and TCF response elements (blue). Below is the sequence of the consensus TCF binding site.

C) 1.5% agarose gel showing amplification of the DRR from C2C12 genomic DNA. Lane 1 is 100bp ladder, lane 2 and 3 show the 714 bp product that was sequenced, found to be mutation-free and cloned into pGL3 promoter.

D) Colony PCR of several clones of pGL3p+DRR. Extreme left lanes at the top and bottom of the gel (+) show positive controls from the initial genomic DNA amplification.

Figure 34: Organization and sequence of the mouse MyoD DRR

Consensus TCF site: 
\[
\text{ga(a/t)caaaag} \\
\text{ct(t/a)gtttcc}
\]
postnatally, suggesting an important role for the DRR in maintaining MyoD expression in myocytes and muscle fibers (Chen et al, 2002). Examination of the 714 bp DRR using MatInspector v7.4 (www.genomatix.de) revealed 3 consensus sites for the most downstream component of the Wnt pathway-T-Cell Factor1/Lymphocyte Enhancer Factor1 (TCF/LEF1), suggesting the possibility that MyoD is a direct transcriptional target. Figure 34A shows a schematic representation of the TCF response elements (TCFRE) and their relative position in the DRR. Site A differs from the canonical TCFRE by a single base in the non-conserved portion of the consensus site, while Site B is a combination of 2 overlapping TCFRE and represent the conserved portion of the TCFRE. The DRR was cloned into a reporter vector pGL3 Promoter for further analysis (Figure 34 C,D).

**A β-catenin insensitive form of TCF blocks MyoD expression**

Like SRF, TCF transcriptional activity is dependent on a co-activator that is normally cytoplasmic but translocated to the nucleus in a signal-dependent fashion. β-catenin, the TCF co-activator is found in cadherin complexes that mediate cell-cell adhesion and is regulated at the level of protein stability (reviewed in Nelson and Nusse, 2004. Figure 35). When Wnt signaling is active, turnover of β-catenin is suppressed, allowing it to translocate to the nucleus and stimulate TCF-dependent transcription (Young et al, 1998) from sites where TCF is already bound to DNA.

To test whether TCF is required for MyoD expression, I over-expressed a dominant negative TCF1 that lacks the β-catenin domain (Atcha et al, 2005 unpublished). Dominant negative TCF1 suppressed MyoD expression (Figure 36A,B). These results indicate that MyoD expression in adult myoblasts is controlled by TCF, a known effector of the Wnt pathway. However, these results do not allow a distinction between direct and indirect mechanisms of MyoD promoter regulation by TCF.
Figure 35: The canonical Wnt pathway

Wnts are secreted lipid modified signaling proteins that have multiple roles in animal development. Signaling is initiated by the binding of Wnt to its receptor Frizzled and the consequent activation of Dishevelled (Dvl), a scaffold protein. Wnt signaling is also negatively influenced by the occupation of a co-receptor Arrow/LRP5/6 with its ligand, Dickkopf (Dkk). Conventional Wnt signaling results in the nuclear accumulation of β-catenin with another transcription factor T-cell factor/Lymphoid enhancer factor (TCF/LEF). In the absence of a Wnt signal, the level of β-catenin is kept low by a degradation complex constituted by APC, Axin, GSK-3β and the F-box protein, β-TrCP. β-catenin also exists in a cadherin-bound form at the plasma membrane and regulates adhesion. The prevailing view in the field is that several growth factors/receptor pathways intersect with the Wnt pathway by regulating the availability of signaling β-catenin, either by disrupting the cadherin-catenin complex or repressing cadherin expression suggesting that the cadherin bound β-catenin possibly participates in nuclear events when required.

(Figure and text modified from Nelson and Nusse, 2004)
Figure 36: TCF regulates MyoD and is negatively regulated by mDia
A, B). Dominant negative TCF1E suppresses MyoD expression. C2C12
cells were co-transfected with GFP + dnTCF1E or GFP alone and MyoD
expression quantitated (mean + SEM, n=3, p< 0.0021).
C) TCF activity is suppressed by mDia1ΔN3. Myoblasts were
cotransfected with TOP-flash + GFP (control) or mDia1ΔN3, and
TCF-dependent luciferase activity measured. Values represent normalized
ratios of TOP-flash activity to the respective FOP-flash control
(mean + SEM, n=11, p< 0.0001).
D). Knocking down mDia1 induces TCF activity. Myoblasts were
cotransfected with TOP-flash + mU6 vector (control) or mDia1 shRNA and
TCF-dependent luciferase activity measured as in (C) (mean + SEM,
n=5, p< 0.0021).
TCF activity is modulated by mDia

To examine the effects of altering mDia levels on TCF activity I used the TOP-flash TCF reporter assay (Veeman et al, 2003). Over-expression of active mDia ΔN3 suppressed TCF activity to ~1/4th of control (Figure 36C). More interestingly, transfection of the mDia1 shRNA induced TCF activity ~10 fold (Figure 36D), suggesting that endogenous mDia1 keeps TCF activity suppressed. Taken together, these findings suggest that mDia negatively regulates TCF activity, thereby mediating cross talk between the Rho and Wnt pathways.

Activity of the MyoD DRR is modulated by mDia

To determine whether the effects of mDia ΔN3 on TCF activity and MyoD expression are mediated by direct effects on the MyoD promoter I cloned the DRR into the pGL3-promoter, a reporter vector and assessed the basal activity of the pGL3-DRR construct in transient transfection analysis. I found that DRR activity was at least 4 fold above the basal activity of the pGL3-promoter vector alone (Figure 37A) and displayed higher activity in DM when compared to GM consistent with increased MyoD transcription observed in DM (Figure 37B). Over-expression of mDia ΔN3 suppressed DRR activity to 1/8th of control (Figure 37C) confirming the observation that active mDia negatively regulates MyoD expression at the level of the promoter.

Thus, the following observations support the hypothesis that MyoD expression is antagonistically regulated by SRF and TCF in response to active mDia. Firstly, mDia-ΔN3 activates SRF-mediated transcription and inhibits TCF-mediated transcription from the respective canonical DNA-binding elements (3DAIuc and TOPflash). Secondly, the MyoD DRR bears binding sites for both SRF and TCF/LEF and the overall activity of the transfected DRR is suppressed by active mDia. Thirdly, the RNAi-mediated silencing of endogenous mDia leads to reduced SRF activity, increased TCF activity and suppression of MyoD. Fourthly, MyoD expression is suppressed by a
Figure 37: Analysis of the MyoD DRR by transient transfection

A) Basal activity of the 714 DRR in C2C12 myoblasts transfected with empty vector (pGL3Prom+pBS) or with DRR in pGL3 along with control plasmid (DRR+pBS) at a ratio of 1:4. Luciferase activity is normalized to a co-transfected βgal plasmid and total protein. The DRR shows at least 4-fold activation above control.

B) C2C12 myoblasts transfected with the 714 DRR or empty vector were plated in growth medium (GM) or differentiation medium (DM) after transfection. DRR activity in DM is 1.5 fold above that in GM.

C) C2C12 myoblasts transfected with the DRR +control plasmid (pBSKS-DRR) or the DRR +ΔN3 (N3-DRR) at a ratio of 1:4. ΔN3 inhibits DRR activity by at least 8-fold.
dominant negative TCF suggesting that normal expression is dependent on the presence of functional TCF. Therefore, it is tempting to speculate that the lack of MyoD expression in ΔN3 expressing cells may be due to interference in endogenous TCF activity at the MyoD promoter. However, the mechanism by which TCF activity could be modulated by mDia needs to be elucidated.

Is the microtubule stabilizing function of mDia ΔN3 linked to its ability to modulate TCF activity?

TCF/LEF is the transcriptional effector of the Wnt signaling pathway. In the canonical Wnt pathway, the binding of Wnts to the receptors of the Frizzled family leads to the activation of a scaffold protein Dishevelled (Dvl) and inhibition of the serine/threonine kinase, glycogen synthase kinase (GSK-3β). As a consequence, the GSK-3β target β-catenin accumulates and translocates to the nucleus where it activates TCF-mediated transcription. Independent reports have shown that in neurons, specific members of the Wnt pathway such as DVL and GSK-3β associate with the microtubule cytoskeleton (Krylova et al, 2000) forming a signaling hub that alters the activity of the β-catenin degradation complex and causing a dual impact on TCF-mediated transcription and the activity of microtubule binding proteins (Ciani et al, 2003). Thus, in the Wnt pathway, microtubules not only provide a scaffold permitting the local interaction of Wnt effectors (thereby influencing the final outcome of the pathway) but also are themselves regulated by microtubule binding proteins that are direct targets of the Wnt effectors.

Because of the correlation between altered microtubule dynamics and the Wnt pathway, I asked whether the mechanism by which mDia-ΔN3 modulates TCF activity is linked to its ability to induce stable microtubules. I used two drugs with opposing modes of action to disrupt microtubule function and study the impact of this disruption on TCF activity in C2C12 myoblasts stably expressing the TOPflash reporter, (C2TFC1). Asynchronous myoblasts (Mb), reversibly arrested myoblasts (G0) and synchronized myoblasts at 24 hours after reactivation (R24) were either treated with 10 µM Taxol, a
microtubule stabilizing agent or 2 μM Nocodazole, a microtubule depolymerizing agent or vehicle control for 24 hrs. Unlike changes in microtubule stability with the cell cycle, TCF activity between asynchronous, G0 synchronized and reactivated myoblasts did not differ significantly (Figure 38A, vehicle control bars). Further, TCF activity of nocodazole treated myoblasts was similar to control levels. Finally, stabilization of microtubules induced by Taxol, although inherently variable revealed a moderate but consistent increase in TCF activity above control levels. Thus, drug-induced changes in microtubule dynamics do not vastly perturb TCF activity.

The minor alterations in levels of active TCF in drug treated cells coupled with the observation that MyoD continues to be expressed in taxol and nocodazole treated myoblasts (Figure 38B) do not support a major role for microtubule stability in regulation of TCF activity in myoblasts (see Discussion). Further, preliminary experiments using transfection of an APC molecule lacking the microtubule binding domain shows as much inhibition of MyoD as the full length APC, suggesting that this Wnt inhibitor and mediator of microtubule stability does not require microtubule association for its inhibitory ability (J. Dhawan, unpublished). Thus, the correlation between mDia-mediated induction of stable microtubules and repression of TCF activity remains unresolved at present.

Co-expression of β-catenin partially rescues the suppressive effect of ΔN3 on TCF and MyoD

Since mDia is known to bind APC, a key negative modulator of the Wnt pathway (Wen et al, 2004), I hypothesized that active mDia might suppress TOPflash reporter activity by regulating β-catenin levels. Therefore, I examined whether over-expression of a non-degradable, APC-resistant form of β-catenin could functionally bypass the mDia-ΔN3 inhibition of TCF reporter activity and MyoD expression. Over-expression of the stable mutant β-catenin S37A alone inhibits MyoD expression slightly (Figure 39C), consistent with previous reports on inhibition of Myogenin expression and differentiation
Figure 38: Agents that disrupt microtubule stability do not affect TCF activity

A) Growing and arrested C2TCF myoblasts were treated with vehicle control (VC), 10 mM taxol or 2 mM Nocodazole for 24 hrs. Untreated growing and arrested myoblasts have similar levels of TCF activity. Nocodazole treated myoblasts do not differ in TCF activity from control, while taxol treated myoblasts display a slight increase (mean +/- SD, n=3) Note: Variability in taxol-treated myoblasts seemed inherent. B) 10 mM taxol treated myoblasts continue to express MyoD like the control untreated myoblasts. Note the change in microtubule organization and nuclear fragmentation compared to control.
Figure 39: Over-expression of an APC-independent form of β-catenin leads to functional bypass of mDiaΔN3 inhibition

A). C2C12 cells were co-transfected with ΔN3 alone or ΔN3 + β-catenin S37A and TCF activity (mean + SEM, n=5, p< 0.0046) expression determined.

B, C). MyoD expression in cells transfected with ΔN3 alone or + β-catenin (mean + SEM, n=4, p< 0.0009). Note that ΔN3+ β-catenin S37A transfected cells in panel E retain the elongated morphology typical of ΔN3 transfectants but are MyoD+ (panel F).
(Goichberg et al, 2001). However, co-transfection of the degradation-resistant active β-catenin mutant S37A with mDia-ΔN3 led to a robust increase of TCF reporter activity (Figure 39A) and a 2-fold increase in MyoD expression compared to mDia-ΔN3 alone (Fig 39B,C). These results suggest that overexpressed β-catenin can completely reverse the effect of mDia-ΔN3 on TCF-reporter activity but only partially reverse its effect on MyoD expression. However it is notable that ΔN3-mediated suppression of MyoD is alleviated in β-catenin over-expressing cells.

**Over-expression of the terminal Wnt effectors do not affect SRF activity**

To investigate the possibility of cross talk between the TCF/ β-catenin pathway and SRF activity, I co-transfected C2C12 myoblasts with the SRE reporter plasmid 3DAiuc and the β-catenin mutant S37A or dominant negative TCF (DNTCF). Compared to a control plasmid, neither S37A nor DNTCF affected SRF activity (Figure 40). Thus, SRF activity is unperturbed by modulating the activity of the most downstream components of the Wnt pathway and therefore does not contribute to the effects of over-expression of these terminal Wnt effectors.

**The peak of endogenous mDia1 transcript levels correlate with initiation of increase in SRF activity and the presence of stable microtubules**

To investigate the possibility that levels of endogenous mDia1 correlate with increased SRF activity, a real-time RTPCR analysis was used to assess mDia1 transcript levels in arrested and growing and differentiated myoblasts (protein levels could not be assessed since neither commercial antibodies nor gift antisera (Y. Takai, Osaka University) convincingly detected p140mDia in either western blots or immuno-fluorescence assays). Synchronized early reactivated myoblasts at 6 hrs (R6) showed the highest mDia1 transcript levels during the time course of arrest and reactivation suggesting that mDia is cell-cycle regulated and that endogenous induction of Dia expression are compatible with MyoD induction during G1 (Figure 41).
Figure 40: Over-expression of β-catenin and dominant negative TCF do not affect SRF activity

C2C12 myoblasts were co-transfected with control plasmid pBSKS or β-catenin S37A or dominant negative TCF (DNTCF) and the SRE reporter plasmid, 3DAIuc at 4:1 ratio. SRF activity of control myoblasts and those transfected with Wnt effectors is similar. Controls for the same experiment include a known activator of SRF activity, mDia-ΔN3 and known inhibitor of SRF activity shDia as well as a neutral GFP shRNA plasmid. Note that although unrelated, shGFP appears to reduce SRF activity. However this reduction is not significant when compared to SRF activity levels in cells transfected with shDia (mean +/- SD, n=3).
Figure 41: Levels of endogenous mDia transcript in growing and arrested myoblasts

Real time RT-PCR analysis to quantify endogenous mDia1 transcript. mDia1 mRNA levels are mildly induced during reversibly arrest (S48) but not during differentiation (MT). The peak of mDia1 expression is in activated myoblasts at 6 hrs (R6), returning to the levels seen in arrested cells by 18 hrs (R18). Synchronized reactivated myoblasts (R6) show a 3-fold increase above asynchronously growing myoblasts (MB) and a 2 fold induction over arrested cells (S48) (mean +/- SD, triplicate samples).
Conclusions and Discussion

In this chapter, I have analyzed possible mediators of mDia’s function and ascribed an additional role to mDia1 in modulating the Wnt pathway. I suggest that this novel function of mDia1 may impinge on MyoD expression.

I have shown that over-expression of mDiaΔN3 in myoblasts represses MyoD in a src-independent manner. Earlier studies have shown that in Rous Sarcoma Virus (RSV)-transformed quail myoblasts, expression of oncogenic pp60v-src repressed the transcription of MyoD transcripts (Falcone et al, 1991) in a mechanism independent of cell proliferation. Expectedly therefore, in my experiments, over-expression of the constitutively active src mutant Y529F represses MyoD expression (Figure 32 panels a,b,c). In addition, abolishing the activity of endogenous src using DNsrc or the general tyrosine kinase inhibitor PP2A at 2-10 µM resulted in sustained level of expression of MyoD. Thus, myogenic gene expression in C2C12 myoblasts is dependent on the presence of functional src. However the continued absence of MyoD protein in ΔN3 over-expressing cells in which src activity has been abolished (Figure 32, panels j,k,l) suggests that mDia-ΔN3 does not inhibit MyoD through its ability to interact with src.

The Wnt pathway, mDia and regulation of MyoD

The identity of the second mDia1-binding protein, APC, key inhibitor of the Wnt pathway led me to investigate the involvement of this important signaling pathway in the regulation of MyoD and mDia’s potential role therein. The conceptual and experimental links among MyoD, mDia and the Wnt pathway are discussed below.

Is MyoD a Wnt target?

The microtubule binding protein APC directly interacts with mDia through its basic domain (Wen et al, 2004). The consequence of this
interaction has been examined only at the level of cytoskeletal reorganization, i.e., in the stabilization of microtubules. However APC is a multifunctional protein that also inhibits the Wnt pathway by preventing β-catenin from translocating into the nucleus (reviewed by Arias et al, 1999; Huelsken and Birchmeir, 2001) suggesting that active mDia could be regulating MyoD by modulating the Wnt pathway. The only evidence in literature of cross talk between the Wnt pathway and formins is a report of the human formin Daam1, which interacts with DVL and induces Rho activity in gastrulating Xenopus embryos (Habas et al, 2001).

Although Wnt proteins were shown to be sufficient to initiate myogenesis in explants of mouse paraxial mesoderm by activating the expression of Myf-5 and MyoD (Tajbaksh et al, 1998) and in Xenopus mesoderm formation (Christian et al, 1991), less is known of the existence of the Wnt pathway in postnatal myogenesis. Adult haematopoietic stem cells isolated from regenerating muscle and identified by the presence of the surface marker CD45 adopted a myogenic fate when co-cultured with cell lines stably expressing Wnt isoforms 5a, 5b, 7a and 7b (Polesskaya et al, 2003). However, subsequent studies showed that none of the Wnt isoforms used in the co-culture experiment are expressed at the appropriate time in vivo in the adult (Zhao and Hoffman, 2004) raising doubts as to whether Wnt signaling is functional in adult skeletal muscle. An independent study (Bedada et al, 2005) used two separate techniques to show that adult murine bone marrow stem cells expressed MyoD when co-cultured with cell lines expressing Wnt1, 3, 4, 7a and 7b and by overexpressing a ROBO-related surface protein CDO. CDO is thought to mediate effects of cell-cell interactions between muscle precursor cells and participates in a positive feedback loop with MyoD, enhancing skeletal myogenesis. In quiescent C2 reserve cells, another culture model for satellite cells, insulin and LiCl, an inducer of the Wnt pathway cooperate to activate and induce the expression of MyoD and myogenin in a differentiation-promoting environment (Rochat et al, 2004). Neither in any of these studies, nor in those on embryonic myogenesis, has the level at which Wnts may affect myogenesis (by direct
transcriptional activation of muscle regulators or by indirect means) been addressed. Thus, there is a surprising scarcity of evidence available in the literature that addresses the mechanism(s) by which Wnts may be involved in adult myogenesis.

The lack of any reports on the TCF consensus sites in the MyoD DRR was also quite surprising given the extensive analysis to which this enhancer sequence has been subjected by multiple groups (Tapscott et al, 1992; Asakura et al, 1995; Chen et al, 2001). Thus, my observation of 3 potential TCF binding sites in the DRR although suggestive of a direct mechanism for Wnt action needs substantial experimental proof. Although the results of my study suggest that MyoD expression in C2C12 myoblasts is dependent on functional TCF (Figure 36), biochemical evidence of TCF binding to the TCFRE(s) in the DRR, as well as the effects of other Wnt effectors in regulating the myogenic program needs to be demonstrated. Preliminary experiments in the lab suggest that Axin, sFRP and APC, all inhibitors of the Wnt pathway, suppress MyoD expression, lending support to notion that MyoD is a Wnt target (J. Dhwaw, unpublished). Further, microarray experiments (Sindhu Subramaniam and J. Dhwaw, unpublished) reveal a large number of Wnt pathway genes, including direct Wnt targets to be induced during G0 arrest, when MyoD is suppressed. However, these experiments taken together do not distinguish between direct and indirect mechanisms for Wnt action.

*mDia regulates the Wnt pathway*

I have shown that knockdown of mDia elevates TOPFlash activity while over-expression of active mDia down-regulates TCF activity (Figure 36C,D). These observations raise the likelihood that one of the functions of endogenous mDia is to repress TCF activity, ascribing a novel role to mDia in modulating the canonical Wnt pathway. Although my experiments suggest that active mDia suppresses TCF reporter activity by enhancing the degradation of β-catenin, a direct assessment of the levels of nuclear β-
catenin protein in the active mDia1 over-expressing cell compared to untransfected cells has yet to be demonstrated. A predominant fraction of the β-catenin pool is found associated with cadherins at the adherens junctions while the transcriptionally active fraction in the nucleus of unstimulated cells is undetectable by routine immunostaining analysis. Studies to establish the link between the two pools have shown that over-expression of cadherins in Xenopus and Drosophila reduced the availability of β-catenin by sequestering it at the plasma membrane and making it unavailable for signaling to the nucleus. Conversely, a target of β-catenin/TCF signaling called Slug (belonging to the Slug/Snail family of zinc finger proteins), directly represses the expression of cadherins, resulting in increased β-catenin in the nucleus and consequently increased TCF/LEF activity (reviewed in Nelson and Nusse, 2004). Thus, the availability of β-catenin to act as a transcriptional regulator appears to be linked to its partitioning between adherens junctions and nuclear pools. However, this conclusion seems to be cell type specific as mutated cadherins in 15 breast cancer cell lines that are incapable of binding the cytosolic pool of β-catenin, do not result in constitutive activation of TCF activity, thus suggesting that the two fractions do not mingle (Van de Wetering et al, 2001) at least in breast cancer cell lines. Since my experiments address a downstream effect of β-catenin signaling, the challenge lies in detecting nuclear β-catenin protein, in particular the β-catenin in the active mDia transfected population alone. Enhancing the sensitivity of the detection systems may help in circumventing these problems.

*mDia might regulate MyoD expression through the Wnt pathway*

In this chapter I have shown that signaling through mDia represses MyoD at the level of the promoter. Although I have shown that active mDia can suppress the activity of a surrogate reporter bearing the canonical TCF sites, as well as the entire MyoD DRR (Figure 36C,D) it remains to be established if mDia affects the activity of the individual endogenous TCF sites in the DRR of the MyoD promoter.
To support my assumption of the repression of endogenous TCF activity in the MyoD promoter by active ΔN3, I co-transfected an active mutant of β-catenin that is known to activate TCF reporter activity. I observed an increase in the number of ΔN3 positive cells expressing MyoD (2-fold) suggesting that ΔN3 can potentially suppress endogenous TCF activity, and that S37A causes a functional bypass of this inhibition. The possibility that β-catenin might independently increase MyoD expression via stimulation of transcription through its interaction with histone acetyl transferase CBP (CREB binding protein) is unlikely, because over-expression of β-catenin alone at the same dose does not increase MyoD expression (Figure 39C). On the contrary, there is an inhibition of MyoD expression in myoblasts over-expressing β-catenin as has been observed with myogenin (Goichburg et al, 2001), who attributed the effect on myogenin to disruptions in the integrity of adherens junctions that are sensitive to the amounts of β-catenin available for binding to the cadherins. How then, does the overexpression of β-catenin rescue the effect of ΔN3 on MyoD? Since S37A is resistant to degradation, over-expression of β-catenin could have two consequences: first, a positive effect where sufficient amounts of β-catenin enter the nucleus and partially complement TCF activity which might activate MyoD, and second a negative effect mediated by disruption of adherens junctions which might suppress MyoD expression. Since the outcome is an increased level of MyoD expression, it can be assumed that the positive effect predominates. Thus, levels of MyoD expression might be the concerted output of these two opposing effects of β-catenin over-expression.

**Role of microtubules in the Wnt pathway and MyoD expression**

In fibroblasts, active mDia1 increases the proportion of stable microtubules by recruitment of APC and EB1 to the growing tips of microtubules in a Rho-stimulated manner, the APC-binding domain of mDia being located between the FH1 and FH2 domains (Wen et al, 2004). Our experiments show that over-expressed mDia1-ΔN3 induces stable microtubules in myoblasts, accompanied by the loss of MyoD expression and
G₀ arrest. We also observed a correlation between stable microtubules and loss of MyoD in G₀ myoblasts, whether induced by suspension culture (Figure 33C) or mDia over-expression (Figure 33A,B) leading us to speculate that over-expressed active mDia1 could co-operate with APC to stabilize microtubules, leading to enhanced negative regulation of β-catenin. Thus, TCF-dependent activation of MyoD would be prevented. However, whether the cytoskeletal role of APC is linked with its ability to function as a signal transducer is unclear. Using genetic means, APC translocation along microtubules was found to be critical for β-catenin turnover (Cui et al, 2002). Recent biochemical studies show that APC exists in two distinct protein pools in the cytoplasm, one of which was competent to bind microtubules. However both pools were functional, suggesting that association of APC with microtubules does not affect its ability to inhibit TCF-reporter activity (Penman et al, 2005).

Localization studies of mDia show that it is located in mitotic spindles in mitosis (Kato et al, 2000). In G₁, in addition to a diffuse cytoplasmic distribution, active GFP-mDia appears predominantly localized with APC near stable microtubule ends although mDia itself lacks a direct microtubule-binding site (Wen et al, 2004). In the absence of a detailed biochemical fractionation, it appears that mDia may itself exist in distinct pools in the cytoplasm like APC. In such a scenario, the consequences of drug-induced disruption of microtubule function could result in a perturbation of only a selective pool of mDia. In addition, the possibility that the mDia-APC-tubulin complex continues to exist after treating the cells with empirically determined doses of microtubule depolymerising drugs and therefore unaffected downstream signaling events, cannot be ruled out. Thus, whether changes in microtubule dynamics are the causal factor in affecting the activity of a transcription factor (TCF), in a manner analogous to the regulation of SRF activity by actin dynamics is presently unclear, but warrants a detailed investigation.
A model for mDia1-mediated regulation of MyoD expression

Taken together, my results demonstrate that two opposing signaling pathways emanate from mDia culminating in the reciprocal regulation of the activity of two transcription factors, SRF from the Rho pathway and TCF/LEF from the Wnt pathway. While SRF activity is dependent on Rho-Actin dynamics, TCF/LEF activity is possibly modulated by modulation of β-catenin stability. A model for the regulation of MyoD expression by mDia1 via SRF and TCF/LEF is proposed in Figure 42.

The significance of my studies is firstly, that they address the mechanism by which a lineage determining transcription factor MyoD is coupled to the cell cycle and show that these are inextricably linked; secondly, they identify a novel effector of adhesion-dependent signaling, mDia as the mediator of these controls. Given that skeletal muscle is a contractile tissue whose function is critically dependent on adhesive interactions and cytoskeletal dynamics, the notion that the stem cells associated with this tissue are also responsive to similar signals is intriguing and worthy of further analysis.
Figure 42: mDia-ΔN3 may control MyoD by two antagonistic pathways:

A model for dual signaling by mDia via SRF and TCF to MyoD. The level of MyoD expression is the integrated outcome of these opposing pathways. Actin assembly factors bind to the FH1 and FH2 domains driving polymerization of microfilaments and activating SRF via MAL release. mDia negatively regulates TCF activity and thereby may suppress MyoD expression. To decrease TCF activity mDia may employ mechanisms to prevent β-catenin from translocating into the nucleus. In this aspect, the link between mDia's ability to induce stable microtubules by recruiting the Wnt inhibitor APC is still unclear.