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MOLECULAR CONTROL OF DIAPHANOUS-RELATED FORMINS IN CELL BIOLOGY AND DISEASE

By

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ABSTRACT

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Formins are a conserved family of proteins that govern cytoskeletal remodeling in the cell. Common among all formins is the Formin Homology-2 domain, which is responsible for nucleating and elongating non-branched actin filaments. One particular subset of the formin family, the mammalian Diaphanous-related (mDia) formins, are downstream effectors for small GTP-binding proteins whose activities are controlled by an intricate autoregulatory mechanism.

Actin remodeling plays a central role during vesicle trafficking. It was found that the small GTPase RhoB interacts with mDia2 on intracellular vesicles. In addition, interfering with RhoB or mDia2 activity diminished vesicle movement. These studies suggest that controlled actin dynamics are required for normal trafficking events, and mDia formins play a significant role in this process.

A mouse model of human myelodysplastic syndrome was previously generated by knocking out *Drf1*, the gene that encodes mDia1. These studies led to the generation of mice lacking both mDia1 and RhoB expression, because of the known interaction between these two proteins and because of the proposed tumor suppressor function of RhoB. It was found that the additional loss of RhoB expression enhances the myelodysplastic phenotype of mDia1 knockout mice. The mechanism by which mDia1 and RhoB loss contributes to the observed

phenotype may be centered on the actin sensing pathway mediated by serum response factor, an important transcription factor that is activated in response to mDia activity.

Formins are required during cell division to mediate the assembly of the contractile actin ring for the completion of cytokinesis. However, important questions remained about the specific role of mDia2 during cell division. It was discovered that mDia2 expression is cell cycle-dependent and is degraded by ubiquitin-mediated proteolysis. mDia2 proteolytic degradation is a regulatory mechanism to shut down formin activity during cell division and is likely to function in other contexts as well. Preliminary studies suggest that the anaphase-promoting complex serves as the ubiquitin ligase to promote mDia2 ubiquitination.

Despite the numerous cytoskeletal remodeling events controlled by formins, relatively few instances have directly implicated formins in disease processes. A more extensive characterization of formin function will be important to determine their contributions to normal cell biology and to disease.

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KEY TO ABBREVIATIONS

aCGH	Array comparative genomic hybridization
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
APC/C	Anaphase-promoting complex/cyclosome
CAR	Contractile actin ring
CDR	Commonly deleted region
CFP	Cyan fluorescent protein
CNS	Central nervous system
CytD	Cytochalasin D
DAAM	Dishevelled-associated activator of morphogenesis
DAD	Dia-autoregulatory domain
DBA	Diamond-Blackfan anemia
dDia2	*D. discoideum* Dia2
DID	Dia-inhibitory domain
DIP	Dia-interacting protein
DRF	Diaphanous-related formin
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Egr1	Early growth response-1
EMH	Extramedullary hematopoiesis
ENU	N-ethyl-nitrosourea

F-actin	Filamentous actin
FH1	Formin-homology-1
FH2	Formin-homology-2
FHOD	Formin-homology-2-domain-containing protein
FMN	Formin
FMN1-IV	Formin-1 isoform IV
FMNL	Formin-like
FRET	Fluorescence resonance energy transfer
FRL	Formin-related gene in leukocytes
FTI	Farnesyl-transferase inhibitors
GAP	GTPase-activating protein
GBD	GTPase-binding domain
GEO	Gene expression omnibus
GFP	Green fluorescent protein
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
Jasp	Jasplakinolide
LatA	Latrunculin A
Ld	Limb deformity
mDia	Mammalian Diaphanous-related formin
MDS	Myelodysplastic syndrome
MEF	Mouse embryonic fibroblast
MPN	Myeloproliferative neoplasm

MTOC	Microtubule organizing center
NPM	Nucleophosmin
PI3-K	Phosphoinositide 3-kinase
POF	Premature ovarian failure
PTEN	Phosphate and tensin homolog
RAGE	Receptor for advanced glycation end products
RNAi	Interfering RNA
SRE	Serum response element
SRF	Serum response factor
TCR	T cell receptor
TR	Texas red
WASp	Wiskott-Aldrich Syndrome protein
YFP	Yellow fluorescent protein
zDia2	Zebrafish Dia2

Chapter 1

The Role of Formins in Human Disease

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Abstract

Formins are a conserved family of proteins that play key roles in cytoskeletal remodeling. They nucleate and processively elongate non-branched actin filaments, and also modulate microtubule dynamics. Despite their significant contributions to cell biology and development, few studies have directly implicated formins in disease pathogenesis. This review highlights the roles of formins in cell division, migration, immunity, and microvesicle formation in the context of human disease. In addition, we discuss the importance of controlling formin activity and protein expression to maintain cell homeostasis.

Introduction

Formin family proteins – so-called because of conserved formin-homology-1 and -2 (FH1 and FH2) domains – have emerged as key regulators of actin and microtubule cytoskeletal dynamics during cell division and migration. The FH1 and FH2 domains were identified by Castrillon and Wasserman in the initial characterization of the *Diaphanous* gene in *Drosophila* [1], and both domains participate in the control of cytoskeletal remodeling. The proline-rich FH1 domains have been shown to bind to numerous WW- and SH3-domain containing proteins in addition to profilin-actin which contributes to the ability of formins to produce non-branched actin filaments. FH2 domains dimerize, then nucleate and processively elongate linear actin filaments by associating with their growing barbed (+) ends. These FH2 dimers create an environment that favors actin monomer addition to generate actin filaments.

While formins are important for actin remodeling events, formins can also modulate microtubule dynamics [2] in at least two different ways. Diaphanous-related formins (DRFs), the family of formins most closely related to the canonical formin Diaphanous, bind directly to microtubules to promote their stabilization [3]. In addition, mammalian DRF (mDia) proteins have been shown to associate with microtubule-end binding proteins EB1 and APC [4]. APC is the product of the adenomatous polyposis coli (*APC*) familial colon cancer tumor suppressor gene, and its role in disease progression may be mediated in part by mDia family proteins. Emerging evidence suggests that the formin mDia1

possesses tumor suppressor activity [5], again pointing to a role for formins in cancer formation.

Defects in cytoskeletal remodeling proteins have previously been implicated in malignancy [6] and the aforementioned association between mDia proteins and tumor suppression may point to specific roles for formins in cancer and other diseases. But despite the significant roles formins play in cell biology and development [7], relatively few studies have directly linked formins with disease pathogenesis. This review highlights the existing body of knowledge suggesting that defects in formin gene and gene product function contribute to disease.

Cytoskeletal remodeling and the cell cycle.

Inappropriate cell cycle regulation and cell division are often responsible for the cellular changes that lead to human disease. Changes in cell morphology, chromosome segregation, and vesicular trafficking are all fundamental events that occur during cell division. Each of these events are governed by cytoskeletal remodeling, and it is not surprising then that formins are centrally involved in many aspects of cell division. In fact, one of the best-characterized roles for formins is a necessary function during cytokinesis [1, 8, 9].

Cytokinesis occurs in the last stage of cell division to physically separate the mother cell into two daughter cells. This process requires the formation of an actin-rich contractile ring that constricts to induce plasma membrane invagination. Completion of cytokinesis is marked by abscission of the daughter

cells. Importantly, disruption of formin function by mutation or genetic deletion often results in cytokinesis failure (Fig. 1.1). This initial discovery was made in *Drosophila* after loss of the *Dia* allele led to aneuploidy in germ cells [1]. Subsequent work in other species has shown that numerous formins play a critical role in cytokinesis [7].

Failure to divide the daughter cells after karyokinesis results in a tetraploid cell. Surviving tetraploid cells are prone to genomic instability, widely thought to contribute to cancer initiation and progression [10]. Therefore, inappropriate control of formin function or expression in humans may be a critical event in cancer development. However, no evidence directly links cytokinesis failure with cancer as a consequence of defective formin function, despite the conserved role for formins in cytokinesis.

As mentioned previously, formins can also modulate microtubule dynamics. How formins stabilize microtubules is reviewed in detail by Gundersen and colleagues [2]. In the context of cell division, microtubule stabilization is required to facilitate chromosome segregation and midbody formation [11]. mDia1 has been shown to localize to spindle microtubules in dividing Hela cells [12]. However, the contribution of mDia function toward spindle assembly and dynamics remains unclear. mDia1 and mDia2 have also been shown to decorate the midbodies of dividing cells [13]. The midbody, a dense region of stable microtubules at the site of abscission, helps coordinate vesicle trafficking to promote the membrane remodeling required for cell separation. It is likely that formin-mediated microtubule stabilization contributes to the trafficking events

during cell division, especially considering that formins control vesicle trafficking in other cellular contexts as well [14-16].

In summary, formin activity is critical for proper cell division and thus for the maintenance of genomic integrity during cell division. Future studies are likely to link formins with cancer initiation or other diseases directly, given the fundamental role of formins during cytokinesis.

Formins in Cancer Cell Migration and Invasion

Mammalian cells display remarkable capacities for migration, invasion, and morphological plasticity, and these attributes make possible numerous biological processes of central interest in the understanding of development, homeostasis and disease. Cells of the immune system, in particular, are capable of precisely-targeted homing and invasion of tissues; cells in metastatic cancer are similarly capable of migration and invasion. These processes are known to depend on dynamic modulation of the cytoskeleton. A thorough understanding of these processes is essential for progress in diagnosis and therapy of malignancy and other disease states in which cell migration or invasion is centrally involved.

The reorganization of the actin cytoskeleton drives morphological changes during directed cell migration and invasion in normal and malignant cells. Much attention has focused upon the Rho subfamily of small GTPases, and their enhanced expression and/or activation in human cancers, proteins fundamental to actin remodeling; yet, historically, little is known about the role of key downstream effecter proteins propagating Rho signaling in migrating/invading

cancer cells. In the past several years, significant progress in the understanding of cytoskeletal dynamics has led to the construction of detailed and useful models of these pathways. In particular, an emerging understanding of the functions and roles of formins has generated a model of formin-based actin polymerization and microtubule stabilization, usually in the context of Rho GTPase signaling. Collectively, this emerging evidence (briefly discussed below) suggests a role for the formin family of proteins in modulating both actin- and microtubule-based cytoskeletal networks to promote cancer cell adhesion, migration and ultimately invasion (Fig. 1.2).

mDia1

mDia1 has been implicated in a variety of distinct processes driving normal and cancer cell polarity and migration. mDia1 has been demonstrated to bind, stabilize and polarize microtubules from the cell center to the periphery in migrating cells [4, 17-21]. mDia1-dependent microtubule polarization and reorientation of both the microtubule organizing center (MTOC) and the Golgi towards the direction of cellular migration is fundamental in driving migration and is dependent upon members of the Rho family of GTPases, including Cdc42 and RhoA (reviewed in [22, 23]).

Acting as a Rho GTPase effecter protein, mDia1 also has a critical role in promoting the formation of actin-rich protrusions by building F-actin filaments and directing signaling networks at the leading edge of migrating cells. For instance, in the context of N-WASp-depleted adenocarcinoma cells, mDia1 drives actin-

enriched cellular protrusions and stress fiber formation in a RhoA-dependent manner [24]. Furthermore, through interaction with the cytoskeletal scaffolding protein IQGAP, mDia1 was localized to the leading edge of migrating fibroblasts [25]. mDia1 has been shown to control the formation of stable actin filaments and promote the formation and turnover of focal adhesions [13, 17, 19, 20]. Recently, it was shown that mDia1 knockdown inhibited formation of focal contacts, decreased lamellipodial thickness and impeded leading edge dynamics in migrating cells [20], while another study showed that mDia1 depletion inhibited Src accumulation into focal adhesions in migrating glioma cells, impairing focal adhesion function and stability, as well as cellular migration [19]. Furthermore, through direct interaction with the cell-surface receptor for advanced glycation end products (RAGE), mDia1 was shown to promote RAGE-ligand stimulated cellular migration in a manner dependent upon activated Rac and Cdc42 [26]. Thus, the role of mDia1 in cellular migration appears to be complex, involving multiple platforms (actin and microtubule cytoskeletons) and affecting cell polarity and the assembly of cytoskeletal structures supporting leading edge dynamics.

mDia2

Like mDia1, the related formin mDia2 has also very recently been implicated in cellular migration in both normal and cancer cells. For instance, the zebrafish homologue of mDia2, zDia2 (sharing approximately 50 and 80% sequence similarity to human and mouse mDia2, respectively), was shown to be involved in cell motility observed during gastrulation [27]. A morpholino-based

zDia2 knockdown strategy uncovered a role for zDia2 in the formation of actin-rich protrusions observed during gastrulation, and revealed that profilin I was coordinately involved downstream of zDia2 signaling to control cellular migration during gastrulation. Interestingly, the authors showed that zDia2 expression directed the formation of membrane blebs in the front row of marginal deep cells in zebrafish at the germ-ring stage of gastrulation. As membrane blebbing is an initial indicator of cellular motility accompanying the transformation of non-motile blastomeres into motile blastula cells, these results suggest a role for mDia2 homologues in cellular migration *in vivo*. Moreover, these results confirm *in vitro* evidence that mDia formins control cortical actin contractility and that their disruption promotes the plasma membrane blebbing that is a hallmark of the amoeboid-type of cellular motility demonstrated in cervical and prostate cancer cells [28, 29]. In a recent study by Di Vizio and colleagues (discussed further below), mDia2 depletion in DU145 and LNCaP prostate cancer cells enhanced blebbing upon EGF addition and a concomitant increase in motility and invasion was observed. Furthermore, invasion of MDA-MB-231 breast cancer cells in Matrigel was dependent upon mDia formins, and mDia2 was localized with Src to invadopodia; mDia2-depleted MDA-MB-231 cells had few invadopodia, pointing towards mDia2 as an important component in breast cancer cell invasion [30].

Finally, in addition to affecting the actin cytoskeleton during cellular migration and invasion, it is very likely that, like mDia1, mDia2 also influences the microtubule cytoskeleton in migrating cells. Indeed, it was recently shown that mDia2 stabilized microtubules independent of its ability to modulate actin filament

assembly [3]; this study illustrated the importance of understanding whether the microtubule stabilizing activity of mDia2 formin is also fundamental to cellular processes in which this formin previously had been implicated, including cellular adhesion and focal adhesion stability [31].

Other formin family members (FHOD/FHOS, Formin-1, dDia2, FRL)

In addition to the mDia family, other formins have been shown to play roles in generating actin-rich protrusions that promote cellular migration in a variety of cells and organisms. The Formin-homology-2-domain-containing protein (FHOD1) was demonstrated to enhance cellular migration upon overexpression in human melanoma cells, as well as in NIH 3T3 cells, without significantly affecting integrin expression/activation or cellular adhesion as a whole [32].

Other formins with only moderate homology to mDia formins were also shown to function in cellular migration, including Formin-1 isoform IV (Fmn1-IV), *D. discoideum* Dia2 (dDia2), and formin-related gene in leukocytes (FRL). For instance, Fmn1-IV is a formin protein with some sequence similarity to DAAM1 (Dishevelled-associated activator of morphogenesis-1). Dettenhoffer and colleagues generated Fmn1-IV knock-in mice and demonstrated in primary kidney epithelial cells a role for Fmn1-IV in cell spreading and focal adhesion formation [33]. Conversely, mouse embryonic fibroblasts (MEFs) derived from Fmn1-IV knock-out mice, which show weakly penetrent kidney aplasia [34], had altered protrusive behavior at the leading edge of the cells and had defective cell

spreading and focal adhesion formation [33]. Overexpression in *D. discoideum* of dDia2, which has some sequence similarity to mammalian DAAM1 (~42%) and to a lesser extent hDia2, led to the formation of more persistent, larger adhesive contacts between filopodia and substrate, suggesting a role for dDia2 in controlling filopodia dynamics and the formation of adhesive structures important for cell motility [35]. Finally, FRL (Formin-related gene in leukocytes) was shown to influence cell adhesion, cell spreading, lamellipodia formation and chemotactic migration [36]. In those studies, overexpression in macrophages of the FH3 domain (referred to as the DID domain in recent studies [37]), containing the Rac-binding domain, was sufficient to inhibit cell spreading as well as lamellipodia formation upon stimulation with the chemokine SDF1α (CXCL12); expression of the FH3 domain was also sufficient to ablate cell adhesion to fibronectin, and inhibit chemotactic migration towards SDF1α, indicating a role for this understudied formin in cell motility.

Formins in microvesicles

In addition to soluble proteins and other hormones, cells shed membrane vesicles into the extracellular environment. These extruded vesicles have been shown to contain various signaling proteins and microRNAs, and their contents can be transferred to neighboring cells, resulting in changes in proliferative and or migratory capacity [29, 38]. These structures range in size from ~50 nm to several microns in diameter and have been characterized in various guises as

exosomes, ectosomes, argosomes, microparticles, and oncosomes [39]. We will refer to them as microvesicles here.

Microvesicles are believed to arise by budding directly from the plasma membrane, but they appear to originate from multiple sources, which include organelles from the endocytotic and endosome processing/trafficking apparatus. Several lines of evidence suggest that formins are involved in both the formation and the emission of microvesicles (Fig. 1.2). Early work on mDia1 and mDia2 found both proteins on endosomal structures along with Src non-receptor tyrosine kinases [13]. Subsequent studies demonstrated that all mammalian Diaphanous-related formins (mDia1-3) have roles in endosome trafficking [15, 40]. Moreover, formin inhibition leads to an induction of non-apoptotic membrane blebbing in numerous cell types [28].

Whether any of the formin-associated vesicles gives rise to microvesicles has yet to be demonstrated. But recent work by Di Vizio and colleagues [29] has provided solid evidence that formins directly participate in microvesicle production and thereby function to encourage tumor growth and metastasis. Their studies focused on EGF-induced blebbing of prostate tumor cells, which results in the shedding of microvesicles termed 'oncosomes.' These microvesicles contain various signaling proteins, and alter signaling in other cells. Notably, Akt phosphorylation is induced in cells exposed to microvesicles shed by prostate cancer cells. Similar results obtained in glioblastoma cells [38] suggest that such phenomena are occasionally associated with metastatic cancer cells and/or amoeboid cell migration. Di Vizio and colleagues also tested

whether mDia2 knockdown in cells produced microvesicles. They incubated recipient cells with vesicles shed from mDia2 knockdown cells and saw measurable increases in proliferation and migration in *in vitro* assays.

These intriguing findings do not specifically implicate mDia2 or other formins in cancer progression. But they raise interesting questions about such roles. Expression of dominant interfering mDia1 (truncated mDia1 FH2 domain), the same variant known to induce blebbing, inhibits both mDia2 and mDia1 signaling to SRF [41]. Moreover, expression of the interfering mDia1 significantly enhances the tumor-forming capacity of Ras-transformed mouse embryo fibroblasts [42]. Whether the boost in tumor-forming ability is due to increased microvesicle production (perhaps secondary to enhanced non-apoptotic membrane blebbing), or is due to diminished signaling to the apoptotic machinery through SRF signaling to Egr1 [43], or to other effects is unknown. Clearly, the role of mDia2 in such signaling systems needs to be addressed in more detail.

Formins in Immunity

Cytoskeletal remodeling is required to generate an effective immune response. While formins are critical for cellular migration and invasion (discussed in the previous section), they also mediate polarity, adhesion, and activation of immune cells. Because of their critical roles in T cells, neutrophils, and other hematopoietic cells, formins may contribute to disease when their activity is disrupted in immune cells.

T cell responses

Recent work by our group, and independently confirmed by others, discovered a central role for the formin mDia1 for *in vivo* dynamic cytoskeletal remodeling driving T cell responses [28, 44]. Focusing upon mice with targeted deletion of the gene encoding mDia1, these studies collectively revealed the involvement of mDia1 in T cell development, proliferation and emigration into peripheral lymphoid organs, including spleen and lymph node. These data are consistent with considerable genetic evidence that Rho GTPases (*e.g.*, RhoA), their regulatory exchange factors (*e.g.*, Vav), and their effecters (*e.g.*, WASp) participate in normal T cell function. For example, while both $Vav^{-/-}$ and $WASp^{-/-}$ animals are defective in dynamic remodeling of actin following T cell receptor (TCR) ligation [45, 46], it is possible that the RhoGEF activity of Vav (specifically towards RhoA) directly affects cytoskeletal remodeling upon TCR stimulation. Indeed the small GTPase RhoA regulates integrin-mediated T cell adhesion and migration [47, 48]; while mDia1 is a known RhoA effector, it is plausible that the defects in adhesion and chemotactic migration may be due to disruption of RhoA-mediated mDia1 activation and dysregulation of formin-mediated actin dynamics. Indeed, constitutively-activated mDia1 (lacking the GTPase binding and a portion of the Dia-inhibitory domain) dramatically enhanced actin accumulation in Jurkat T cells *in vitro* while decreasing chemotactic migration upon TCR engagement [49]; these results suggest that arresting the dynamic actin and microtubule cytoskeletons controlled by mDia1 [4, 50, 51] inhibits normal T cell function, consistent with our results utilizing T cells derived from the

Drf1 knockout mouse. However, a recent study suggested that upon depletion of mDia1, actin dynamics at the immune synapse were not affected upon TCR stimulation [52]. mDia1 depletion was incomplete in those studies (90% depletion as estimated by Chabbra *et al* [53]), and, along with the modest effects observed in F-actin accumulation and cellular adhesion/migration, these results suggest that a lower level of mDia1 protein is sufficient to mediate actin dynamics in stimulated T cells.

Neutrophil responses

Following the discovery of a role for mDia1 in regulating actin dynamics critical for T cell migration, two recent studies also demonstrated a role for mDia1 in neutrophil migration and activation [54, 55]. Using neutrophils isolated from mDia1- [56], WASp- [46] or mDia1/WASp double-knockouts, these studies revealed an association between mDia1 and WASp that was important for mediating neutrophil polarization and chemotactic responses towards the chemokine MIP-2 or the fMLP formyl-peptide. These studies further demonstrated that both Src-family kinases and LARG/RhoA/ROCK signaling contributed to the mDia1-mediated signaling axis promoting neutrophil chemotaxis.

Other hematopoietic cells

The assembly of F-actin filaments into a contractile actin ring (CAR) was previously shown to be fundamental to enucleation [57, 58], a process

fundamental to erythyrocyte maturation in which the pycnotic nucleus is extruded from the mature erythroid cell. Until recently, the identity of actin-assembly factors mediating CAR formation and enucleation remained elusive. However, Lodish and colleagues recently demonstrated a specific requirement for the formin mDia2, through an association with Rac1 and Rac2, for CAR formation and subsequent enucleation [59]. In those studies, Rac inhibition ablated enucleation, while constitutively activated mDia2 was shown to rescue the defect. As it has been postulated that enucleation is a specialized form of cytokinesis, these results are consistent with previous findings revealing a role for mDia2 in contractile ring formation and in the completion of cytokinesis [13, 60, 61].

Finally, platelets exposed to thrombin are rapidly activated and undergo dramatic RhoA-dependent reorganization of their actin cytoskeleton [62, 63]; yet until recently, it was unclear what Rho effecter proteins are required for propagating Rho-dependent signaling in platelets. Two studies demonstrated a role for the formins mDia1 and DAAM1 in promoting actin assembly/remodeling and cell spreading in activated platelets in response to RhoA signaling [64, 65]. Collectively these findings, and those obtained in the experiments on hematopoietic cells described above, suggest an essential role for formin-mediated actin assembly in maintaining proper homeostasis during immune function. Additional *in vivo* analysis of mice deficient in formin protein expression and validation in human samples will be an important avenue for future studies.

Mouse models linking formins with disease

Multiple formin family members have been genetically manipulated in mice to determine their *in vivo* functions. These studies have revealed important roles for formins in development, immunity, and disease. In some instances, the subtle phenotypes suggest that there is functional redundancy between formins. Based on the current mouse models, several diseases have been associated with impaired formin function.

Models of Fmn gene function

Leder and colleagues identified the initial *Limb Deformity* (*Ld*) loci genes as having key roles in development programs that guide limb formation [66, 67]. Different transcripts with potential truncations were identified at those loci and hybridization experiments pointed to their expression in limb buds. It was then hypothesized that defects in their gene function affected limb formation [7]. Continued analysis of the *Ld* locus showed that while the *Formin* (*Fmn)* gene was near the *Ld* locus, the protein encoded by *Fmn* was not affected in the original knockout mice. Instead, defects in the *Gremlin* gene, which has important functions in limb formation, were shown to account for defects in *Ld* mice [7].

Recently, *Fmn1* or Formin 1 knockout mice were shown to have a limb deformity phenotype by reduction of digit number to four [68]. Importantly, there were no effects on Gremlin expression or function as a result of *Fmn1* knockout. These data suggest that Formin 1 function is indeed required for normal limb

development in certain contexts. Nevertheless, a role for Formin 1 in human development or disease remains to be described.

Fmn2 knockout mice identified a role for Fmn2 function in the meiotic cell divisions of mouse oocytes [69]. Knockout mice had reduced fertility as a result of defects in spindle positioning and polyploidy in oocytes. Since defects in oocyte maturation often result in birth defects and pregnancy loss in humans, it is interesting to speculate that problems with formin activity or expression may contribute to these errors [69]. However, neither Fmn2 nor any other formin family member has been specifically implicated in human fertility defects to date.

Models of Drf gene function

Human myelodysplastic syndrome (MDS) is a hematopoietic disorder due to defects in the control or differentiation of hematopoietic stem or progenitor cells [70]. Clinically, MDS patients present with various cytopenias, hypercellular marrow, dysplastic erythrocytes, and an increased risk to develop AML [71]. One subset of MDS is 5q- syndrome, which involves deletion of all or part of chromosome 5. Interestingly, *DIAPH1*, which encodes mDia1, is located at or near commonly deleted sites in 5q- patients. In fact, gene expression analysis of patients with 5q- MDS shows decreased *DIAPH1* expression [43]. This led to the hypothesis that mDia1 may have suppressor functions in the maintenance of hematopoietic stem or progenitor cell proliferation. This idea was recently supported through genetic deletion of the *Drf1* gene in mice. Knockout mice developed an age-dependent myeloproliferative disorder, including

splenomegaly, hypercellular bone marrow, and expansion of the myeloid and erythroid compartments in the spleen and bone marrow [5]. The specific mechanism by which loss of mDia1 contributes to this phenotype remains to be determined, but it is suggested that inappropriate signaling through the transcription factor SRF plays a critical role [7, 43, 72].

Moreover, additional loss of RhoB enhanced the myeloproliferative phenotype observed in mDia1 knockout mice alone. Compared to mDia1 knockout mice, mice lacking both RhoB and mDia1 developed a more severe myelodysplasia [73]. This is intriguing, given that RhoB has been proposed to have tumor suppressor activity [74], and low expression often correlates with late stage malignancy [75, 76]. It will be important to examine the role of formins and RhoB in the context of stem or progenitor cell function, since myelodysplasia is considered a disease of the stem cell compartment.

Alterations in formin expression or function associated with disease

Aberrant formin function and expression have been directly implicated in maladies as diverse as deafness [77], fertility defects [78], and cancer [79] (Table 1.1). However, the mechanisms demonstrating a causal role for formins in the formation of these diseases have not been rigorously tested. Nevertheless, these studies point to the importance of controlling formin activity for proper cell function.

DFNA1 – Autosomal Dominant non-syndromic Deafness

A progressive deafness disorder was discovered among a kindred in Costa Rica that had descended from the Conquistadore invasions. This disorder was named *DFNA1* and was subsequently mapped to the *DIAPH1* gene [77]. The mutation in *DIAPH1* is a four basepair (TTAA) insertion that generates a frameshift mutation in the coding sequence that is predicted to generate an inappropriate stop codon, truncating 32 amino acids (Fig. 1.3). However, the impact of this mutation on formin function has never been tested. Given the location of the truncation, it is possible that the mutation may induce a gain-of-function, by disrupting the autoinhibitory mechanism of mDia1.

Premature ovarian failure - POF

Previous work has shown that a critical region on the X chromosome is interrupted by a breakpoint associated with a familial case of POF [78]. Mapping of the gene responsible for the ovarian defect revealed that *DIAPH2* (mDia3) may be the gene responsible due to a breakpoint in the last intron. These findings suggest a possible role for mDia3 in ovarian development. The cellular consequences of the breakpoint for mDia3 function are unknown. But a role for formins in ovarian development is consistent with the spermatogenesis and oogenesis defects observed in mutant versions of the *Drosophila Dia* gene [1].

Expression data associated with disease

Formin-like 2 (FMNL2) expression was shown to be elevated in colorectal metastatic cancer cell lines compared to normal colorectal cancer cell lines [79].

In addition, FMNL2 expression was higher in primary colorectal cancer and lymph node metastases, with the highest expression in the metastatic derived cell lines. However, a correlation between FMNL2 expression and clinical diagnosis was not performed. Also unknown is whether increased FMNL2 expression contributes to colorectal cancer formation, or if it is simply a passenger effect due to other required genetic abnormalities.

The human leukocyte formin (FMNL1) is highly expressed in the thymus, spleen, and peripheral blood leukocytes. It was reported that high expression of FMNL1 is present in several lymphoid cancer cell lines [80]. However, whether FMNL1 expression correlates with lymphoid cancers from patient samples has not been tested.

Experiments that implicate mDia2 in the negative regulation of non-apoptotic blebbing and microvesicle formation suggest that mDia2 (and perhaps other formins) exerts control over amoeboid cell migration and/or other aspects of cancer progression [28, 29]. For instance, genomic analysis of prostate cancer samples, comparing primary tumors to metastases, suggests that deletions at the *DIAPH3* locus are significantly more common in metastatic disease [29]. *DIAPH3* is located in a region of the q arm of chromosome 13 (13q21.2), and while this region has been long thought to harbor tumor suppressor genes [81], no tumor suppressor genes have been specifically identified there. Further work will clarify the extent to which mDia2 is functioning in that role, but the findings to date indicate that such hypotheses are worthy of careful examination.

Concluding remarks

Formins govern both actin and microtubule cytoskeletal dynamics, and play important roles in cell division, cell migration, immunity, and development. While much of the knowledge about formin function has been established using model organisms and *in vitro* systems, relatively few studies have directly linked formins with disease pathogenesis. The generation of new mouse models and a more extensive characterization of the current models will be useful to provide insights regarding the function of formin family members *in vivo*. Future studies should address specific hypotheses related to aberrant formin expression and activity in disease. Are there disease-causing mutations in formins that affect their activity? How does increased or decreased formin expression facilitate disease, if at all? At this point, there are many more questions than answers regarding the role of formins in disease. Given the numerous cellular functions mediated by formins, determining answers to these questions should prove to be a rewarding endeavor.

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Table 1.1 Formin chromosome locations and human disease association.

Formin Subfamily	Name	GeneID	Chromosome	Disease Relevancy
Dia Diaphanous	mDia1/*DIAPH1*	1729	5q31	5q- Myelodysplastic Syndrome [5, 43] DFNA1 non-syndromic deafness [77]
	mDia2/*DIAPH3*	1730	13q21.2	Chromosome deletion in metastatic prostate cancer [29]
	mDia3/*DIAPH2*	81624	Xq21.33	Premature Ovarian Failure [78]
FRL Formin-Related gene in Leukocytes	FMNL1	752	17q21	Increased expression in lymphoid malignancies and peripheral blood leukocytes from CLL patients [80]
	FMNL2	114793	2q23.3	Increased expression in colorectal cancer [79]
	FMNL3	91010	12q13.12	
DAAM Dishevelled-Associated Activator of Morphogenesis	DAAM1	23002	14q23.1	
	DAAM2	23500	6p21.2	
Delphilin	GRID2IP	392862	7p22.1	
INF Inverted Formin	FHDC1	85462	4q31.3	
	INF2	64423	14q32.33	
FHOD Formin Homology Domain-containing protein	FHOD1/FHOS	29109	16q22	
	FHOD3/FHOS2	80206	18q12	
FMN Formin	FMN1	342184	15q13.3	
	FMN2	56776	1q43	

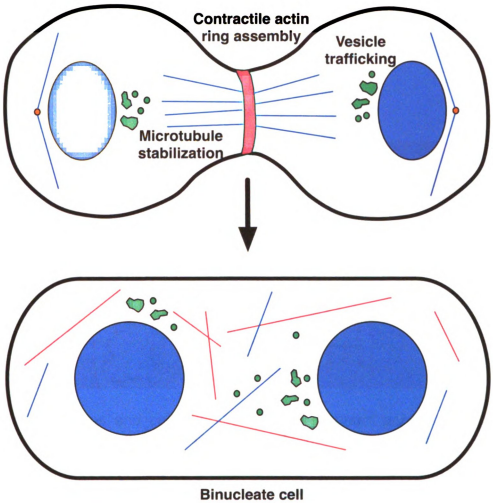


Figure 1.1 Formins in cytokinesis. Formins are required for cytokinesis through the assembly of the contractile actin ring. The ability of formins to stabilize microtubules and control vesicle trafficking may also be a required formin function during cytokinesis. Loss of formin activity or deregulation of formin activity has been shown to interfere with cytokinesis and lead to binucleate cells, which can result in chromosome instability.

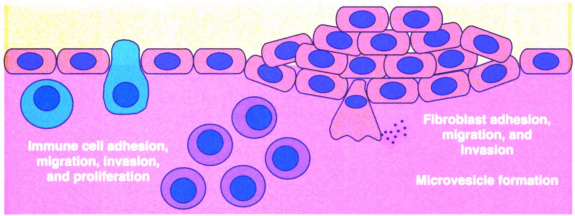
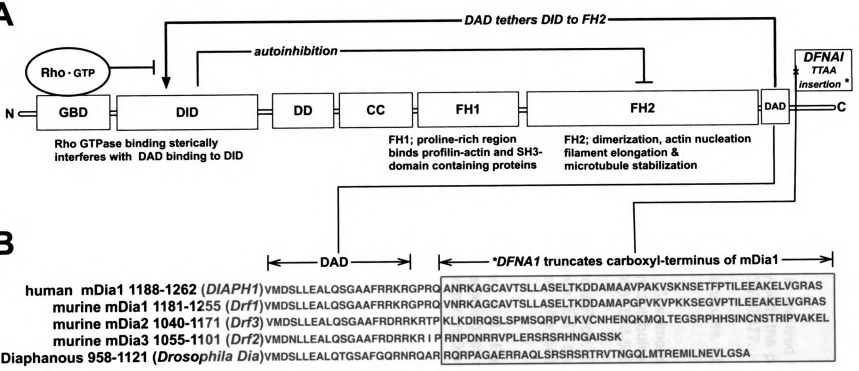


Figure 1.2. Formins and cell migration. Formins control adherent cancer cell migration and invasion by assembling actin-rich protrusive structures and stabilizing microtubules. Formins perform similar functions in immune cells, and studies from mDia1 knockout mice show that they also play a role in immune cell proliferation. In prostate cancer cells, the formin mDia2 was shown to control microvesicle formation, which could lead to oncogenic signal transmission to nearby cells.

Figure 1.3. Mutation in *DIAPH1* leads to an autosomal dominant non-syndromic deafness disorder. A. Schematic diagram of the *DIAPH1* encoded mDia1 protein. GTP-bound Rho binds the GTPase binding domain of autoregulated formins (e.g. mDia1) to sterically interfere with Dia inhibitory domain interaction with the Dia autoregulatory domain (DAD). Release of autoregulation promotes Formin Homology-2 domain mediated actin polymerization. The DFNA1 insertion is located at the C-terminal end of the DAD domain. B. ClustalW amino acid sequence alignment of human mDia1, murine mDia1-3, and *Drosophila* diaphanous. The DFNA1 insertion generates a truncated version of mDia1 that may disrupt autoregulation given the proximity of the truncation to the DAD domain. However, the truncation is on the C-terminal end of the most conserved DAD region, so the DFNA1 insertion may result in defective protein function not related to autoregulation.


A
DAD tethers DID to FH2
autoinhibition
Rho · GTP
DFNA1
TTAA
insertion *
N
GBD
DID
DD
CC
FH1
FH2
DAD
C
Rho GTPase binding sterically
interferes with DAD binding to DID
FH1; proline-rich region
binds profilin-actin and SH3-
domain containing proteins
FH2; dimerization, actin nucleation
filament elongation &
microtubule stabilization
B
DAD
*DFNA1 truncates carboxyl-terminus of mDia1
human mDia1 1188-1262 (DIAPH1) VMDSLLEALQSGAAFRRKRGPRQ ANRKAGCAVTSLLASELTKDDAMAAVPAKVSKNSETFPTILEEAKELVGRAS
murine mDia1 1181-1255 (Drf1) VMDSLLEALQSGAAFRRKRGPRQ VNRKAGCAVTSLLASELTKDDAMAPGPVKVPKKSEGVPTILEEAKELVGRAS
murine mDia2 1040-1171 (Drf3) VMDSLLEALQSGAAFRDRRKRTP KLKDIRQSLSPMSQRPVLKVCNHENQKMQLTEGSRPHHSINCNSTRIPVAKEL
murine mDia3 1055-1101 (Drf2) VMDNLLEALQSGAAFRDRRKR I P RNPDNRRVPLERSRSRHNGAISSK
Diaphanous 958-1121 (Drosophila Dia) VMDSLLEALQTGSAFGQRNRQAR RQRPAGAERRAQLSRSRSRTRVTNGQLMTREMILNEVLGSA

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Chapter 2

RhoB and the Mammalian Diaphanous-related Formin mDia2 in Endosome Trafficking

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Abstract

Rho GTPases and the dynamic assembly and disassembly of actin filaments have been shown to have critical roles in both the internalization and trafficking of growth factor receptors. While all three mammalian Diaphanous-related (mDia1/2/3) formin GTPase effector proteins have been localized on endosomes, a role for their actin nucleation, filament elongation, and/or bundling remains poorly understood in the context of intracellular trafficking. In a study of a functional relationship between RhoB, a GTPase known to associate with both early- and late-endosomes, and the formin mDia2, we show that 1) RhoB and mDia2 interact on endosomes; 2) GTPase activity—the ability to hydrolyze GTP to GDP—is required for the ability of RhoB to govern endosome dynamics; and 3) the actin dynamics controlled by RhoB and mDia2 are necessary for vesicle trafficking. These studies further suggest that Rho GTPases significantly influence the activity of mDia family formins in driving cellular membrane remodeling through the regulation of actin dynamics.

Introduction

Controlled remodeling of the actin cytoskeleton contributes to membrane dynamics during phagocytosis, filopodial extension, and organelle trafficking [1-4]. During endocytosis, actin filaments contribute to membrane deformation, vesicle scission, and force generation sufficient to move vesicles within the cell [5]. Furthermore, organelle- and membrane-bound actin assembly has also been shown to have a role in membrane fusion or in the mixing of lipid bilayers [6]. For example, drugs that decrease actin dynamics by promoting filamentous (F)-actin stabilization [7] or by causing net depolymerization can enhance membrane fusion. Similar effects have been observed for the intracellular trafficking of endocytic vesicles [8]. These and similar studies suggest that the coupled assembly and disassembly of F-actin ("dynamic actin") drive intracellular trafficking and membrane remodeling.

Many aspects of membrane dynamics are controlled by Rho family GTPases [9]. Rho proteins act as binary nucleotide-dependent switches and modulate the activity of effector proteins that serve to carry out various cellular responses. One such effector protein family, the mammalian Diaphanous (mDia)-related formins, has the ability to be autoregulated, that is they can maintain themselves in an inhibited form until activated by the Rho GTPases [10]. Once activated by the GTPase, the Diaphanous-related formin can directly nucleate G-actin, cause the processive elongation of F-actin, and in some cases, bundle nonbranched actin filaments [11-13]. Actin nucleation promoting factors [14], such as the Wiskott-Aldrich Syndrome protein (WASp)/Scar family of proteins,

also trigger the generation of new branched actin filaments. However, these proteins cause an increase in actin polymerization by activating the Arp2/3 complex [15]. While activated Arp2/3 has been implicated in membrane fusion and trafficking events in numerous systems [6], a role for formins in the assembly of organelle-bound F-actin has not been well characterized.

First shown for mDia1 and mDia2 [16], all three mammalian mDia proteins have now been localized on endosomes [17]. mDia3/hDia2C is thought to function as an effector for RhoD in the trafficking of Rab5-positive vesicles [17], and mDia1 was recently shown to be directed to early endosomes by RhoB through an unknown mechanism [18]. In each of these studies, expression of activated or "deregulated" versions of either mDia1 or mDia3 have been shown to block endosome movement [17, 18]. These reports proposed that activated GTPases, as well as activated mDia proteins, stabilize the interactions of endosomes with the actin stress fibers and together act as conduits for trafficking, and diminished vesicle movement [19].

In this study, we have used fluorescence resonance energy transfer (FRET) to demonstrate that RhoB and mDia2 specifically interact on endosomes. In addition, we have examined the role of RhoB and formin-mediated actin assembly on endosome motility by observing the effects of mDia2 activation or inhibition and comparing the effects caused by compounds that are well known to disrupt actin dynamics. Our results show that actin dynamics controlled by mDia2 contributes to the process of vesicle movement.

Experimental Procedures

Cell culture, microinjection, and time-lapse image acquisition – NIH 3T3, RhoB mouse embryo fibroblasts [20], and HeLa cells were maintained in DMEM containing 10% (v/v) fetal bovine serum (FBS). For microinjection experiments, cell lines were changed to medium containing 0.1% (v/v) FBS 24 h prior to all microinjections. Plasmids were injected at 0.1 mg/ml, unless indicated otherwise using a semi-automated injection system as previously described [16]. HeLa cells were plated onto glass-bottom dishes (MatTek #P35GC) and incubated for 14 h in DMEM lacking phenol red for live-cell time-lapse imaging. Cell microinjection and immunofluorescence were performed as previously described [16]. Antibody solutions were co-injected with Oregon Green Dextran (1 mg/ml) to identify successfully injected cells.

Time-lapse imaging was acquired using either a 100X or 63X objective with a Zeiss Axiovert 100M fitted with an environmental chamber to maintain cells at 37 °C and in a 10% CO_2–containing atmosphere. Images were acquired at 3-s intervals using a Zeiss Axiocam controlled by the Improvision software. Vesicle velocity was determined by measuring changes in the centroid along a single axis of 8–10 individual vesicles from four different cells in two separate experiments; velocities are expressed as the mean +/– sample standard deviation.

Recombinant proteins, antibodies, immunofluorescence, drugs, and pyrene actin nucleation assay – Latrunculin A, jasplakinolide, and cytochalasin D (Sigma) were dissolved in DMSO and used at concentrations of 1 μM or 10 μM.

Texas Red–labeled epidermal growth factor (TR-EGF; Molecular Probes, OR) was used at 50 ng/ml. Rabbit anti-mDia2 (1358) polyclonal antisera was generated against purified recombinant protein composed of amino acids 520–1040 from murine mDia2 [21]. Immunofluorescent detection of mDia2 was obtained by using rabbit anti-mDia2 (158)[16] followed by staining with anti-rabbit-AMCA secondary antibody (Jackson Immunoresearch). Indirect immunofluorescent localization of early endosomes was performed by using mouse anti-EEA1 (Abcam), followed by staining with anti-mouse-FITC secondary antibody (Jackson Immunoresearch).

Pyrene actin nucleation assays were performed as described by Li and Higgs [22] using recombinant mDia1 (amino acids 748–1203) and mDia2 (521–1171) proteins. Antibodies were added at the indicated concentrations prior to the start of the reactions.

Plasmids and FRET acquisition – C3 transferase was expressed from pEF-C3 [23]. For fluorescence resonance energy transfer (FRET) experiments, pEYFP, pEYFP-mDia2, pEYFP-ΔGBD-mDia2, and all pECFP-RhoA/B/C fusion constructs were based on the pEYFP-C1 and pECFP-C1 expression vectors (Clontech)[24]. The EYFP and ECFP plasmids were originally altered (A206K) to eliminate artifactual dimerization of fluorescent proteins [25]. The accession number for mDia2 [21] is AF094519. Details of plasmid construction are available upon request.

For FRET experiments, the indicated plasmids (100 ng/μl in 0.5X PBS) were microinjected into NIH 3T3 cells and fixed 4 h later with 3.7% formaldehyde.

For acquisition of FRET data on the Zeiss Axiovert 100M, a Chroma 86002BS dichroic mirror was used, and the corresponding filter sets (Chroma 86002) were CFP (excitation, 436/10 nm; emission, 470/30 nm), YFP (excitation, 500/20 nm; emission, 535/30 nm), and FRET channel (excitation, 436/10 nm; emission, 535/30 nm). For all of the data acquired, 2 x 2 binning mode with a 50- to 600-ms integration time was used. Background images were subtracted from the raw data before carrying out any FRET calculations. For valid FRET images, it was important to determine the degree of bleedthrough of the CFP/CFP and YFP/YFP channels into the FRET channel (CFP/YFP). To determine bleedthrough factors, ECFP-RhoB and EYFP-mDia2 were independently expressed, and for many different cells (n > 15), the ratio of the CFP/YFP intensity was calculated on a pixel-by-pixel basis relative to the respective CFP/CFP (RhoB) and YFP/YFP (mDia2) channels. This factor for each fluorophore indicates the degree of bleedthrough that occurs from CFP (0.55) and YFP (0.16) into the FRET channel; therefore, the bleedthrough factor represents the portion of the FRET signal that is not deriving from real energy transfer. In the case of pECFP-RhoB + pEYFP-mDia2 plasmids, images were acquired sequentially through the YFP, CFP, and then FRET channels. After background subtraction from each image, the corrected FRET was calculated on a pixel-by-pixel basis for the entire image using the equation: FRET = [CFP/YFP – (0.55 x CFP/CFP) – (0.16 x YFP/YFP)], incorporating the bleedthrough factors (0.55 and 0.16) as calculated above. The data were renormalized to their minimum and maximum values for a 14-bit gray scale (0–16,384 arbitrary

fluorescence intensity units) with a pseudocolor spectrum based on temperature being applied to the image, stretching from blue (low FRET) to red (high FRET). All calculations were performed using Openlab software package (Improvision, MA, USA).

Results

Inhibition of RhoB Interferes With Vesicle Movement – In order to examine the specific contribution of different Rho family members in endosome motility, we employed a rescue strategy (shown schematically in Figure 2.1A) that had been initially used to examine RhoA signaling to the transcription factor SRF [23, 26]. RhoA, RhoB, and RhoC were first inhibited by expressing *Clostridium botulinum* C3 transferase (C3), a toxin that ADP-ribosylates all three Rho family members and interferes with their function [27], along with CFP in cells for 4 h. Cells were then stimulated for 10 min with TR-EGF prior to the initiation of imaging. Whereas C3 expression had no effect on the overall uptake of TR-EGF, the large difference in vesicle motility in CFP - and CFP/C3 - expressing HeLa cells demonstrated that inhibition of RhoA-C blocked vesicle movement as expected (Fig 2.1B). Next, it was determined if either RhoA, RhoB, or RhoC activity could be individually rescued by co-expressing ribosylation-resistant versions (N41I) along with the toxin. We discovered that expression of CFP-RhoB-N41I, but not the other family members, rescued normal EGF-containing vesicle movement (Fig. 2.1B). Interestingly, activated (GTPase-deficient) CFP-RhoB-G14V/N41I failed to rescue the effects of C3 transferase. This observation

was not unexpected as expression of RhoB has been shown to interfere with EGFR motility [28]. These data also suggested that RhoB and its GTPase activity (ability to hydrolyze GTP) are necessary for the normal movement of vesicles bearing internalized TR-EGF.

mDia2 Interacts with RhoB on Endosomes – Given that RhoB was shown to be involved in the movement of EGF-containing vesicles, we hypothesized that RhoB may be working through the effector protein, the Diaphanous-related formin mDia2 that was previously shown to be on transferrin receptor-positive endosomes [16]. Therefore, it was important to test whether RhoB and mDia2 specifically interacted on endosomes. Initially, we used FRET to compare the interactions between the Rho family members RhoA, RhoB, RhoC and mDia2. The FRET approach was previously used to identify the interaction of mDia2 with Cdc42 [24]. The results for the potential interactions between RhoA-C and mDia2 are shown schematically in Fig. 2.2A-C. For the FRET experiments, activated RhoA-C (G14V) was fused to cyan fluorescent protein (CFP) and was used as the FRET donor, while mDia2 fused to yellow fluorescent protein (YFP) was the FRET acceptor [24]. For both of the YFP and CFP proteins, a mutation had been inserted (A206K) to eliminate artifactual dimerization of the fluorescent proteins [25]. The FRET signal in these experiments should only occur where the Rho GTPase would interact with the mDia protein's GTPase-binding domain (GBD [29, 30]). The binding of the GTPase to the GBD would cause the adjacent Dia-autoinhibitory domain (DID [22]) to release the carboxy-terminal Dia-autoregulatory domain (DAD [31]). GTPase-induced release of the DID-DAD

interaction then activates the full length formin protein and allows the formin homology-2 (FH2) domain to nucleate and elongate actin filaments [11, 29, 30, 32] .

The FRET expression constructs were microinjected into NIH 3T3 fibroblasts previously maintained in low serum (0.1% FCS) and were allowed to express for 4 h prior to fixation as described previously [24]. As expected, CFP-RhoB-G14V was targeted (Fig. 2.2A) to puncta that co-localized with markers of both early and late endosomes (data not shown) [33-37]. In a negative control experiment, FRET was not detected in cells expressing activated RhoB and a version of mDia2 lacking a region of the protein that contains the GTPase-binding domain [29, 30] and a portion of DID (Fig. 2.2B). The lack of an interaction between RhoB and the ΔGBD-mDia2 also indicates a requirement for that missing region (mDia2, residues 1–255) in the binding to RhoB [16, 24]. Despite the lack of a FRET signal, we found that activated RhoB was sufficient to localize the deregulated YFP-ΔGBD-mDia2 protein on endosomes. The results are similar to those previously described for a GBD-truncated version of mDia1 [18].

FRET analysis detected no significant interaction between activated RhoC and mDia2 despite significant co-localization (Fig. 2.2C). The lack of mDia2 FRET with RhoC was surprising given the homology between RhoA, RhoB, and RhoC. Within the experiment, we could demonstrate that CFP-RhoC-G14V fusion was active, as cells clearly displayed organized stress fibers upon staining with fluorescently labeled phalloidin (Fig 2.2C, right panel). Furthermore, the

CFP-RhoC fusion protein has been observed to act as a FRET donor with mDia1 (K. Eisenmann, unpublished observation). Consistent with the original identification of mDia2 as a RhoA-binding protein [21], CFP-RhoA and YFP-mDia2 were observed to interact within membrane ruffles of migrating cells (K. Eisenmann & B.J.W., unpublished observation). The result was similar to recent observations made using RhoA and an mDia1-derived FRET reporter [38]. Given the differences observed between RhoA, RhoB, RhoC, and Cdc42 [24] and their respective interactions with mDia2, the specific interaction between RhoB and mDia2 on endosomes appears to be unique for this GTPase-formin pair. While the interaction between RhoB and mDia2 on endosomes has been established here, it became important to demonstrate that this interaction was involved in the movement of cargo-containing vesicles.

mDia2 Interacts with RhoB on EGF-Containing Vesicles – Like RhoB [35-37, 39, 40], mDia proteins have been shown to localize on endosomes [16-19, 41]. CFP-RhoB was expressed along with YFP-mDia2 in HeLa cells for 4 h before stimulation with TR-EGF for 10 min as before (Fig. 2.3). A FRET signal was observed between mDia2 and RhoB on TR-EGF–containing vesicles. FRET between RhoB and mDia2 was also observed on vesicles bearing internalized transferrin (data not shown), which was consistent with our previous observation of mDia2 colocalization with transferrin receptor [16]. These data showed that the GTPase RhoB and mDia2 physically interact at sites where they could function to direct actin assembly on endosomes. We then examined if RhoB was necessary to recruit the mDia2 protein to endosomes.

RhoB is Required for mDia2 Vesicular Targeting – mDia2 localization was first found in mouse embryonic fibroblasts (MEFs) lacking RhoB (*RhoB –/–*) [20] by indirect immunofluorescence using previously characterized mDia2-specific antibodies [16]. In the control *RhoB* +/+ MEFS, mDia2 was typically observed in punctate regions (Fig. 2.4A) and co-localized with markers of both early and late endosomes (data not shown). On the other hand, in the *RhoB –/–* MEFS, mDia2 was diffusely localized throughout the cytoplasm and at the cell edge (see Fig 2.4B). In both cell lines, mDia2 had a similar localization at the cell periphery. These observations suggested that mDia2 localization to endosomes, and perhaps to other vesicles, was influenced by the presence of RhoB. To test this hypothesis, we expressed CFP-RhoB or CFP-RhoA in *RhoB –/–* MEFs to ascertain if they could "rescue" endogenous mDia2 localization. Shown in Fig. 2.4C, CFP-RhoB expression in the *RhoB –/–* MEFs caused mDia2 to become located in more punctate regions and condensed around the nucleus; endogenous mDia2 also co-localized with the expressed CFP-RhoB fusion protein. In contrast, CFP-RhoA expression (Fig. 2.4D) caused mDia2 to be diffusely distributed throughout the cell, with some puncta appearing only at the cell edge. In studies still underway, it is at these sites where RhoA interacts with both mDia1 and mDia2 (K. Eisenmann, B.J.W., & A.S.A., unpublished observation). Combined with the FRET results shown in Fig. 2.2, these observations indicate that RhoB is required for the subcellular targeting of mDia2 to endosomal compartments. However, because activated RhoB also drives ΔGBD-mDia2 to vesicles (Fig. 2.4E-H), the influence of RhoB appeared to be

indirect and not mediated by direct protein-protein interactions. Instead, activated RhoB must be involved in the mDia2 targeting to endosomes via a distinct mechanism. We then examined the functional consequences of RhoB signaling to mDia2 on endosomes.

Activation of RhoB-mDia2 Signaling Blocks Vesicle Trafficking – Since the RhoB-mDia2 (GTPase-effector) pair has been demonstrated to interact on endosomes, the role of mDia2 in endosome dynamics was investigated. First, it was hypothesized that mDia activation would have the same effect on vesicle motility as activated RhoB, which has been shown to interfere with EGF-receptor dynamics [42]. The results are shown in Figure 2.5. To activate mDia proteins in cells, we expressed EGFP-DAD, which serves to disrupt the intramolecular autoinhibition of endogenous mDia proteins, thus activating them in cells [22, 31, 43]. A version of DAD that is deficient in binding to DID, EGFP-DAD-M1041A [30, 32], was included as an inactive negative control. The activation of endogenous mDia by EGFP-DAD was compared with the effects of expression of CFP-RhoB or activated CFP-RhoB-G14V. Figure 2.5 shows the mean velocities of vesicles imaged from time-lapse wide-field epifluorescence microscopy of HeLa cells expressing the indicated proteins for 4 h before stimulation with 50 ng/ml TR-EGF. Consistent with the hypothesis that mDia proteins were effectors for activated RhoB, expression of EGFP-DAD (but not the inactive variant DAD-M1041A) significantly diminished trafficking of TR-EGF positive vesicles (monitored 10 min after stimulation; corresponding to late endosome-multivesicular body transitions). In agreement with the findings from other labs for

mDia1 [18] and mDia3 [17], expression of either GBD-truncated/deregulated mDia1 or mDia2 also significantly impaired vesicle trafficking (data not shown). Together with the effects of EGFP-DAD expression, these data suggest that deregulation (activation) of cellular mDia proteins by DAD or expression of the constitutively activated mDia proteins can mimic the effects of activated RhoB (G14V).

Inactivation of mDia2 Blocks Vesicle Movement – Expression of constitutively activated RhoB (G14V) caused a decrease in vesicle motility (Fig. 2.5), and it also failed to significantly rescue endosome dynamics in C3-treated cells (Fig. 2.1B). However, with the rescue of vesicle movement in C3-treated cells by a version of RhoB that has intact GTPase activity, it appears that wild type RhoB can play an important role in vesicle motility. Since RhoB recruits and activates the effector protein mDia2 on endosomes, it was hypothesized that the same cycling of activated/inactivated forms of mDia2 is required for efficient movement of vesicles. Indeed, we also found that a constitutively activated mDia (mDia2-M1041A [32]), with a mutation in DAD that abolishes binding to DID, causes a decrease in endosome dynamics (data now shown). However, the absolute requirement for mDia2 in vesicle movement had not yet been established.

In order to assess the requirement for mDia2, we used two approaches to interfere with mDia activity. The first method was to microinject inhibitory polyclonal antibodies that specifically block mDia2-, but not mDia1-, mediated actin polymerization *in vitro* (Fig. 2.6A). After microinjection and stimulation with

TR-EGF, vesicle movement was inhibited (Fig. 2.6B). The inhibition was similar to the results obtained upon expression of EGFP-DAD or activated RhoB (Fig. 2.5). On the other hand, microinjection of anti-mDia1 antibodies, previously shown to block both cytokinesis and activation of SRF-mediated gene expression [16], had no effect (data not shown). A requirement for mDia proteins in vesicle movement was also supported by the effects of a version of mDia1 that has been shown to act as a dominant interfering mDia protein capable of blocking both mDia1 and mDia2 signaling (YFP-mDia1-FH2ΔN) (Fig. 2.6B [44, 45]). The results show not only that deregulation or hyperactivation of RhoB and mDia2 interferes with vesicle trafficking, but that their normal "regulated" activities must remain intact for vesicle trafficking to occur.

Blocking Actin Dynamics Mimics the Effects of RhoB-mDia2 Activation/Inhibition – Combined with the observations that activation (deregulation) of both RhoB and mDia signaling blocked vesicle movement, the RhoB/mDia2 inhibition experiments showed that *dynamic* regulation of GTPase signaling through the formin effector is necessary for trafficking. That is, both the GTPase and the autoregulated formin must cycle between "on" and "off" states in order to function correctly.

The results are similar to what has been observed following the treatment of cells with drugs that affect the dynamic assembly and disassembly of F-actin, including: latrunculin A (latA [46]), which binds to actin monomers and induces a net depolymerization of F-actin; jasplakinolide (jasp [47]), which stabilizes F-actin while having the potential to be a potent inducer of actin polymerization; and

cytochalasin D (cytD [48]), which binds to and caps the barbed (fast-growing) end of F-actin. Each drug has been shown to block the trafficking of endosomes [6, 8].

As expected, all three drugs interfered with TR-EGF–labeled vesicle movement, which indicates that actin dynamics are required for efficient vesicle motility (Fig. 2.6B). A past study has shown that treatment with cytD reverses the effects of expression of constitutively activated ΔGBD-mDia3 [17], which interferes with vesicle trafficking. In our hands, however, cytD failed to relieve the blockade caused by expression of the activated mDia proteins (mDia1 and mDia2) or following expression of EGFP-DAD (data not shown). Furthermore, since treatment of cells with cytD alone strongly interfered with trafficking (Fig. 2.6B), the result was not surprising. Taken together, our comparison of the effects of drugs that affect actin dynamics, mDia regulation, and the ability of the formin mDia2 to assemble actin filaments, shows that discrete control of actin assembly and disassembly is required for normal trafficking of endosomes.

Discussion

Our studies of intracellular trafficking of endosomes bearing internalized EGF suggest a pathway in which dynamic actin assembled by mDia proteins is tightly controlled by the cycling RhoB GTPase. The effects of manipulation of actin dynamics, as well as GTPase and mDia function are summarized in Figure 2.7 along with a model for their contribution to endosome motility. While mDia2 and other family members interact with numerous GTPases *in vitro* [10], we show

here that RhoB interacts with mDia2 on endosomes within cells. The prevailing model is that RhoB binding to the GTPase binding domain of an autoinhibited mDia2 (Fig. 2.7A) will interfere with intramolecular interactions between the DID and DAD autoregulatory domains; the release of DAD from the Armadillo-like repeats in the GBD-DID region would relieve the negative effect of DID on FH2-mediated nucleation and processive elongation [11]. Activation allows mDia2 to generate non-branched actin filaments at the site of activation, thus generating force to propel vesicles in short-range movements (Fig. 2.7B) [49].

We also propose that the GTP-GDP hydrolysis cycle (Fig. 2.7C) is required to coordinate the cytoskeletal dynamics necessary for trafficking. This idea is supported by the observation that inhibition of RhoB by C3 impedes trafficking and that this inhibitory effect can be rescued by a ribosylation-resistant version of RhoB, but not by a constitutively active version of RhoB (G14V) in which GTP-hydrolysis is impaired. This is not the first observation that has demonstrated that hydrolysis is important for GTPase-regulated biological processes. The function of tubulin [50], dynamin [51], and peptide elongation factors [52] are all dependent upon hydrolysis cycles in order to function correctly. Critical roles for GTP-hydrolysis has also been shown previously for Rho function in bud site selection [53], exocytosis [54], and in signal transduction [26, 55].

GTP-hydrolysis may potentially have a role in the mechanism(s) that allow mDia proteins to respond to GTPase binding; addition of constitutively active GTP-bound RhoA, for example, only partially relieves autoinhibited mDia1 *in vitro*

[22, 56]. In cells, expression of constitutively active mDia proteins and DAD peptide block vesicle trafficking; DAD binds to the GBD-DID region and disrupts intramolecular autoregulation [11, 22, 29, 30, 32, 57]. One possible explanation is that mDia proteins locked in their 'on' states form a non-productive complex at the barbed-ends of filaments that participate in trafficking and impede the function of other actin assembly/disassembly proteins such as capping protein [58].

Taken together, both RhoB and mDia proteins appear to require the ability to autoregulate within the intracellular trafficking machinery. This raises the question of how GTPases and exchange factors and/or GTPase-activating proteins (GAPs) participate in the activation of the mDia proteins in cells. Already, we know that Src non-receptor kinases, known to regulate RhoGAPs [59], are capable of binding to formins [16, 17, 60]. While Src can phosphorylate mDia2, it has no effect on the ability of mDia2 to nucleate actin (H. Higgs, K.M. Eisenmann, A.S.A *unpublished observation*). Therefore, we speculate that mDia2-bound Src has a separate target, perhaps a RhoGAP, that may be involved in the indirect control of the formin in cells. Already, p190 RhoGAP has been implicated in mDia function as a binding partner for the mDia2-interacting protein DIP [61]. We are similarly interested in not only how GAPs, but exchange factors that activate GTPases by triggering release of GDP and GTP uptake, participate in the local control of mDia activity at specific sites in cells.

The signals emanating from RhoB that direct mDia2 to endosomes appear to be complex. Targeting occurs independently of direct protein-protein

interactions. Similar observations have been made for mDia1 in cells expressing RhoB [18]. While the mechanism for indirect targeting of mDia proteins to endosomes by RhoB is not clear, we speculate that RhoB, like its budding yeast counterpart Bni1p, is capable of integrating additional signals downstream of the Rho GTPases [43, 62]. Interactions with membrane lipids or accessory proteins may drive targeting. One candidate is again Src, which has already been shown to colocalize with mDia1, mDia2, and mDia3 on endosomes [16, 17]. There indeed appears to be a link between RhoB and Src; recent experiments have shown that RhoB traffics Src to the plasma membrane [40]. Future studies will address if Src has a role in RhoB-directed vesicle targeting.

Dynamic actin has been shown to be critical for other steps in the endocytic machinery. For example, Yarar *et al* discovered that disruption of the F-actin assembly and disassembly cycle with latA or jasp resulted in a nearly complete cessation of all aspects of clathrin-coated vesicle dynamics [8]. While we find no role for mDia proteins in endocytosis, we show that inhibition of actin assembly by latrunculin A or cytochalasin D interferes with the movement of vesicles carrying internalized fluorescent EGF following endocytosis. Paradoxically, treatment of cells with jasplakinolide, which stabilizes actin filaments, also blocked vesicle motility. These data have led us to conclude that the dynamic assembly and disassembly of F-actin are both required for the movement of EGF-bearing vesicles in cells (Fig. 2.7B). And, as discussed above, disruption of the normal regulatory mechanisms that control the activity of mDia proteins leads to a similar effect; expression of activated mDia1/2 [16] or

expression of DAD, which activates cellular mDia proteins [31], diminishes vesicle movement (Fig. 2.7C).

Several possible mechanisms for how constitutive activation of mDia proteins impedes movement have been suggested. First, formins assemble F-actin structures on vesicles that impede movement through the cytoplasm [18]. Secondly, mDia-assembled actin affects the ability of the vesicle to associate with the machinery that facilitates long-distance trafficking [17]. We suggest an alternative possibility that activated mDia proteins associated with the barbed-ends of F-actin interfere with treadmilling processes that have been suggested to occur in systems dependent upon Arp2/3-mediated branched filament assembly [63]. A second, less likely, explanation for the effects of constitutive mDia activation is that the formins generate force on vesicle that arrest movement. Formin-mediated actin polymerization has been shown to generate force *in vitro* [49, 64]. Under physiological conditions with cells, these "processive motors" could propel vesicles when applied asymmetrically (Fig. 2.7D). However, upon forced activation by expression of DAD or by expression of deregulated GBD/DID-truncated mDia1-3, the formins would push on vesicles in a fashion that approximates symmetrical opposing forces that would arrest movement (Fig. 2.7E). Conversely, disruption of formin processivity by inhibitory mDia2 antibodies (Fig. 2.6A) would negate force generation and hence block movement as well.

In summary, we have shown here that the other mDia family member, mDia2, has a role in vesicle trafficking. In addition to mDia1, mDia2 is also an

effector for RhoB. While we demonstrate a direct interaction between mDia2 and RhoB on endosomes, their functional relationship remains unclear. Future studies will address the role of mDia-mediated actin assembly on endosomes and address the mechanisms by which RhoB influences mDia targeting within cells.

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Figure 2.1. Rescue of RhoB activity restores endosome motility in cells expressing *Clostridium botulinum* toxin C3 transferase.
A. The strategy of the rescue of specific Rho GTPase activity using ribosylation-resistant (N41I) versions of RhoA, RhoB, or RhoC. C3 transferase, which ribosylates and inactivates Rho proteins on Asp-41, was co-expressed from an injected plasmid along with the indicated CFP-Rho fusion constructs. In all cases, cells were microinjected with plasmids expressing C3 transferase and a resistant version (N41I) of one of the Rho GTPases (A-C), allowed to express for 4 h, then stimulated with TR-EGF (50 ng/ml) for 5 min prior to the image acquisition.
B. Summary of the effects of the Rho rescue experiments on the mean velocities of TR-EGF–bearing vesicles. HeLa cells were microinjected with the indicated expression plasmids and allowed to express for 4 h, followed by a 5-min stimulation with TR-EGF (50 ng/ml). Monitoring the fluorescent imaging of TR-EGF–bearing vesicles over time allowed for the determination of the average velocity of the center of each vesicle; error bars depict the standard deviation. p-values were determined by Student's *t*-test of the indicated paired means.

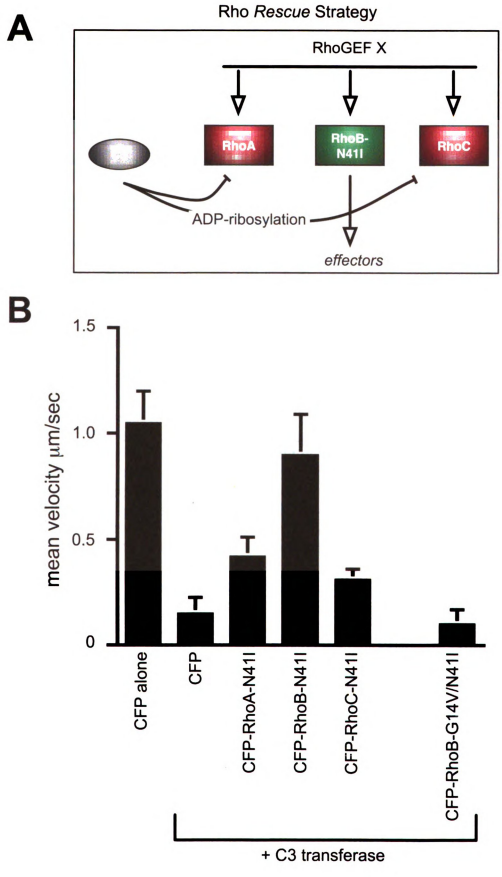
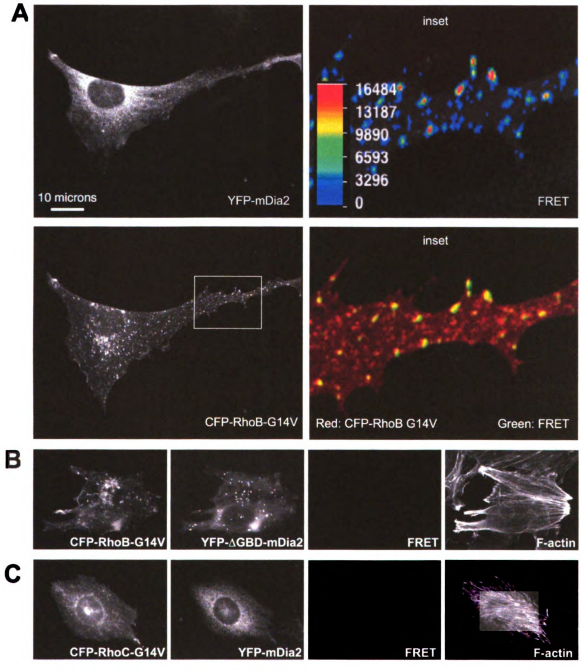

A
Rho Rescue Strategy
RhoGEF X
RhoA
RhoB-N41I
RhoC
ADP-ribosylation
effectors
B
mean velocity µm/sec
1.5
1.0
0.5
0
CFP alone
CFP
CFP-RhoA-N41I
CFP-RhoB-N41I
CFP-RhoC-N41I
CFP-RhoB-G14V/N41I
+ C3 transferase

Figure 2.2. RhoB and mDia2 interact on endosomes.
A. FRET interaction between YFP-mDia2 and activated CFP-RhoB-G14V. NIH 3T3 cells maintained in 0.1% FCS/DMEM were microinjected with the appropriate expression vectors before cell fixation and FRET acquisition. FRET is displayed in the inset (top right) using an artificial temperature scale in which red indicates the highest detected FRET signal. The lower right panel shows the FRET signal (green) in an overlay of CFP-RhoB-G14V (red), indicating that not all puncta bearing the GTPase interact with the formin mDia2.
B. FRET analysis in cells expressing CFP-RhoB-G14V and a version of mDia2 that lacks its GTPase-binding domain (YFP-ΔGBD-mDia2). Cells were microinjected with the respective expression vectors, fixed 4 h later, and stained with fluorescent phalloidin in order to monitor F-actin architecture. TRITC-phalloidin staining (far right panel) shows F-actin assembled into stress fiber–like bundles, indicative of expression of constitutively activated RhoB and mDia2.
C. FRET analysis in cells expressing CFP-RhoC-G14V expressed with YFP-mDia2. TRITC-phalloidin staining (far right panel) shows F-actin assembled into stress fiber–like bundles, indicative of expression of constitutively activated RhoC.


A
inset
16484
13187
9890
6593
3296
0
10 microns
YFP-mDia2
FRET
inset
CFP-RhoB-G14V
Red: CFP-RhoB G14V
Green: FRET
B
CFP-RhoB-G14V
YFP-ΔGBD-mDia2
FRET
F-actin
C
CFP-RhoC-G14V
YFP-mDia2
FRET
F-actin

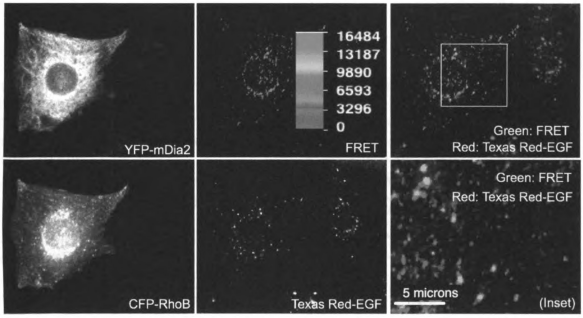
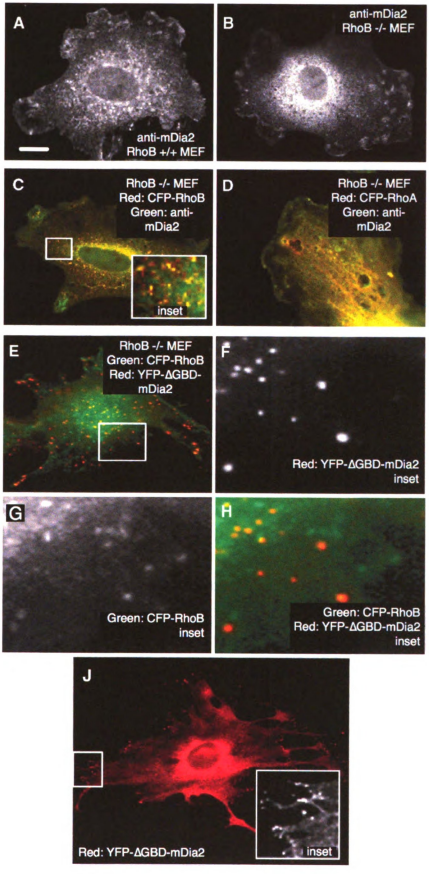


Figure 2.3. RhoB and mDia2 interact on a subset of vesicles bearing internalized EGF.

CFP-RhoB and YFP-mDia2 interact on vesicles bearing internalized Texas Red–labeled EGF. Cells expressing the two respective FRET probes (4 h after injection) were incubated with fluorescent EGF for 5 min prior to fixation. Consistent with previous results showing endogenous mDia2 on endosomes associated with transferrin receptor [16], RhoB-mDia2 FRET occurs on a subset of vesicles (FRET is false-colored green, with Texas Red-EGF indicated in red).

Figure 2.4. RhoB is required for mDia2 targeting to endosomes.
A. Wild type mouse embryo fibroblasts (generously provided by Dr. G. Prendergast) were fixed and incubated with rabbit anti-mDia2 (P158 [16]) followed by FITC-conjugated donkey anti-rabbit IgG. Cells were imaged at 100X; bar equals 10 mm.
B. Mouse embryo fibroblast from *RhoB*$^{-/-}$ mouse stained with anti-mDia2 as in **A**.
C. Expression of CFP-RhoB in a *RhoB*$^{-/-}$ MEF. In both **C** and **D**, cells were microinjected with the indicated expression plasmid, returned to the incubator and stained with anti-mDia2 as in **A**. Inset shows colocalization of RhoB with endogenous mDia2.
D. Expression of CFP-RhoA in a *RhoB*$^{-/-}$ MEF.
E-H. *RhoB*$^{-/-}$ mouse embryo fibroblasts expressing YFP-ΔGBD-mDia2 along with CFP-RhoB. YFP and CFP have been false colored red and green, respectively.
J. YFP-ΔGBD-mDia2 expressed in a *RhoB*$^{-/-}$ MEF.


A
anti-mDia2
RhoB +/+ MEF
B
anti-mDia2
RhoB -/- MEF
C
RhoB -/- MEF
Red: CFP-RhoB
Green: anti-
mDia2
inset
D
RhoB -/- MEF
Red: CFP-RhoA
Green: anti-
mDia2
E
RhoB -/- MEF
Green: CFP-RhoB
Red: YFP-ΔGBD-
mDia2
F
Red: YFP-ΔGBD-mDia2
inset
G
Green: CFP-RhoB
inset
H
Green: CFP-RhoB
Red: YFP-ΔGBD-mDia2
inset
J
Red: YFP-ΔGBD-mDia2
inset

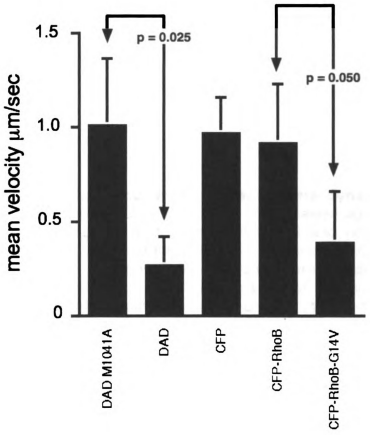


Figure 2.5. Deregulation of mDia2 function impairs endosomal dynamics. Fluorescent imaging of TR-EGF–bearing vesicles in HeLa cells were measured after expression of either EGFP-DAD, an inactive variant (DAD-M1041A, control), CFP (control), CFP-RhoB, or CFP-RhoB-G14V. Cells were microinjected with the indicated expression plasmids and allowed to express for 4 h, followed by a 5-min stimulation with TR-EGF (50 ng/ml). Mean velocities of TR-EGF–bearing vesicles were determined by averaging the change in distance between the center of each vesicle over time; error bars depict the standard deviation. p-values were determined by Student's *t*-test of the indicated paired means.

Figure 2.6. Deregulation of mDia2 function impairs endosomal dynamics.
A. In order to confirm that the anti-mDia2 antibodies (1358, raised against the FH1-FH2 domain of mDia2) specifically interfere with mDia2 and not mDia1, pyrene actin assays (left panel) were performed in the presence of increasing concentrations (0–10 mM) of antibody that had been preincubated with recombinant mDia1 (blue) or mDia2 (red). The presence of the anti-mDia2 antibody inhibited mDia2-mediated actin nucleation but not mDia1-mediated nucleation.
B. Summary of mean vesicle velocities following treatment with drugs that either stabilize F-actin (jasplakinolide), block assembly at the barbed end (cytochalasin D), or by binding to G-actin (latrunculin A), relative to the expression of interfering mDia1 (YFP-mDia1-FH2ΔN) or microinjection of anti-mDia2 antibody protein.

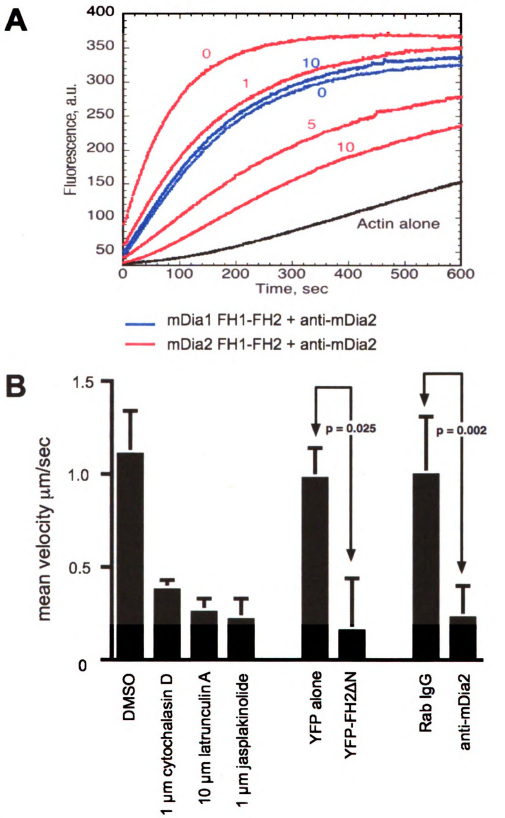
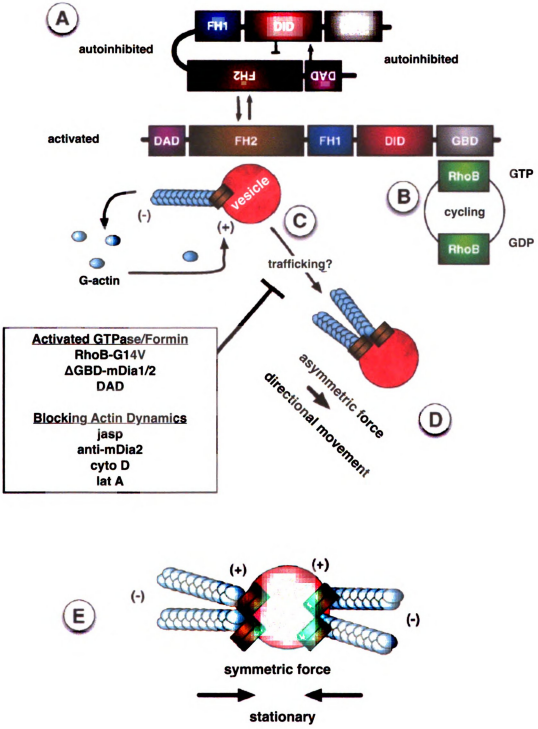

A
400
350
300
250
200
150
100
50
Fluorescence, a.u.
0
100
200
300
400
500
600
Time, sec
0
1
10
0
5
10
Actin alone
mDia1 FH1-FH2 + anti-mDia2
mDia2 FH1-FH2 + anti-mDia2
B
1.5
1.0
0.5
0
mean velocity µm/sec
p = 0.025
p = 0.002
DMSO
1 µm cytochalasin D
10 µm latrunculin A
1 µm jasplakinolide
YFP alone
YFP-FH2ΔN
Rab IgG
anti-mDia2

Figure 2.7. Role for RhoB and mDia2 in actin dynamics on endosomes.
A. Autoinhibited (inactive) mDia2 where the Dia-inhibitory domain (DID) is anchored to the formin homology-2 [FH2] domain by the adjacent Dia-autoregulatory domain (DAD).
B. Binding to the GTPase-binding domain disrupts DAD [31] binding to DID [22] and its ability to inhibit FH2-mediated nucleation/elongation of actin. In this study, constitutively active RhoB did not rescue vesicle motility; therefore, it seems that GTP/GDP turnover is required for this process. The activation/inhibition ("cycling") of mDia proteins also appears to be required, since the constitutive activation of mDia proteins (either by GBD/DID-truncation or expression of DAD) arrests vesicle movement.
C. Actin treadmilling ("dynamic" actin) is also critical for endosome movement, since both jasplakinolide (which stabilizes F-actin) and cytochalasin D (which inhibits F-actin polymerization) block the movement of vesicles.
D. Formin mediated actin assembly could drive short-range vesicle motility by applying assymetric force.
E. Hyperactivation of mDia proteins by expression of DAD or 'activated' mDia proteins would exert force on vesicles from multiple directions that would interfere with directed movement. Alternatively (not shown), activated mDia proteins bind constitutively to barbed ends of filaments on endosomes thereby blocking interactions with other end-binding proteins whose activities are necessary for endosome movement.


A
autoinhibited
FH1
DID
DAD
FH2
autoinhibited
activated
DAD
FH2
FH1
DID
GBD
B
RhoB
GTP
cycling
RhoB
GDP
vesicle
(-)
(+)
C
G-actin
trafficking?
Activated GTPase/Formin
RhoB-G14V
ΔGBD-mDia1/2
DAD
Blocking Actin Dynamics
jasp
anti-mDia2
cyto D
lat A
asymmetric force
directional movement
D
E
(+)
(+)
(-)
(-)
symmetric force
stationary

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Chapter 3.1

5q– Myelodysplastic Syndromes: Chromosome 5q Genes Direct a Tumor Suppression Network Sensing Actin Dynamics

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Abstract

Complete loss or interstitial deletions of chromosome 5 are the most common karyotypic abnormalities in myelodysplastic syndromes (MDS). MDS patients with isolated del(5q)/5q– have a more favorable prognosis than those with additional karyotypic defects who tend to develop myeloproliferative neoplasms (MPN) and acute myeloid leukemia (AML). The frequency of unbalanced chromosome 5 deletions has led to the idea that 5q harbors one or more tumor suppressor genes that have fundamental roles in the growth control of hematopoietic stem/progenitor cells (HSC/HPCs). Cytogenetic mapping of two distinct *commonly deleted regions* (CDRs) centered upon 5q31 and 5q32 respectively, identified candidate tumor suppressor genes, including the ribosomal subunit RPS14, the transcription factor Egr1/Krox24, and the cytoskeletal remodeling protein α-catenin. While each acts as a tumor suppressor, alone or in combination, no molecular mechanism accounts for how defects in individual 5q candidates may act as a lesion driving MDS or contributing to malignant progression in MPN. One candidate gene that resides between the conventional del(5q)/5q– MDS-associated CDRs is *DIAPH1* (5q31.3). *DIAPH1* encodes the mammalian Diaphanous-related formin mDia1. mDia1 has critical roles in actin remodeling in cell division and in response to adhesive and migratory stimuli. This review examines evidence, with a focus on mouse gene-targeting experiments, that mDia1 acts as a *node* in a tumor suppressor network that involves multiple 5q gene products. The network has the potential to sense dynamic changes in actin assembly. At the root of the network

is a transcriptional response mechanism mediated by the MADS-box transcription factor Serum Response Factor (SRF), its actin-binding myocardin family coactivator MAL, and the SRF-target 5q gene *EGR1*, which regulate the expression of PTEN and p53-family tumor suppressor proteins. We hypothesize that the network provides a homeostatic mechanism balancing HPC/HSC growth control and differentiation decisions in response to microenvironment and other external stimuli.

Introduction

Myelodysplastic syndromes are a heterogeneous collection of clonal hematopoietic disorders that arise due to defects in the control and differentiation of hematopoietic stem cells and/or hematopoietic progenitor cells (HSC/HPCs) (Malcovati and Nimer, 2008). Myelodysplastic syndromes are characterized by ineffective formation of hematopoietic cell lineages with dysplastic features (Nimer 2008). The clinical picture in MDS ranges from a spectrum of anemias, leuko- or thrombocytopenias to severe transfusion-dependent peripheral pancytopenias. Thrombocytosis and leukocytosis occur in certain MDS subtypes (Nimer, 2008b). Often the bone marrow is normo- to hypercellular with paradoxically increased apoptosis (Nimer, 2008a). Patients with MDS have an increased risk of progression to AML (Malcovati and Nimer, 2008). Median onset of MDS is $\geq$ 65 years with a male predominance (Nimer, 2008b).

The most common karyotypic defects in MDS are loss of all or part of chromosome 5 [(del)5 or 5q–], chromosome 7, the Y chromosome, and trisomy of chromosomes 20 and 8 (Nolte and Hofmann, 2008). Most patients have large interstitial deletions of 5q, and when these deletions occur in the context of more complex karyotypes, the prognosis is poor (Nimer, 2008b). The 5q deletions can be either more focused, such as in MDS with isolated del(5q) (Nolte and Hofmann, 2008), or quite large, including loss of the entire long arm of chromosome 5 or monosomy of chromosome 5 (5q-) (Olney and Le Beau, 2007). Aberrations in chromosome 5 (either [(del)5 or 5q–]) are frequently found in AML as well, however often these harbor different break points and deletion sizes

(Nolte and Hofman, 2008), are associated with additional cytogenetic changes and portend a worse prognosis (Nimer, 2008b; Vardiman *et al.*, 2009). A separate MDS entity involving *only* chromosome 5q, and the focus of the actin-sensing mechanism discussed below, is the trait formerly designated 5q-syndrome and now called MDS with isolated del(5q) (herein referred to as [(del)5 or 5q–]) (Vardiman *et al.*, 2009). This syndrome is characterized by interstitial deletions in chromosome 5q (different than those found in AML) and commonly presents as anemia, mild leukopenia and thrombocytosis with a female predominance, and has a more benign clinical course and good response to lenalidomide (List *et al.*, 2005).

Myeloproliferative neoplasms belong to the group of clonal myeloid disorders with mainly proliferative changes in one or more hematopoietic lineages such as thrombo-, leuko- or erythrocytes. Many oncogenic events were shown to drive proliferation of these myeloid cells (Tefferi and Gilliland, 2007). An overlapping group of MDS-MPN exists which shows both dysplastic and proliferative features (Neuwirtova *et al.*, 1996; Vardiman *et al.*, 2009). MPNs can transform into aggressive phenotypes such as AML and this process is frequently associated with additional 5q-/del(5q) chromosomal aberrations found in certain MPN subtypes such as primary myelofibrosis (Santana-Davila *et al.*, 2008).

Chromosome 5 abnormalities are amongst the most common and frequent in MDS and in AML, respectively, as well as at progression of MPN to AML; therefore, 5q has been postulated to harbor one or more tumor suppressor genes whose loss of function triggers the progression to malignancy in a multi-

step carcinogenesis program (Giagounidis *et al.*, 2006; Van den Berghe *et al.*, 1985).

Cytogenetic studies have attempted to classify *commonly deleted regions* (CDRs) in order to pinpoint candidate genes and/or to identify a common chromosome 5 breakpoint (Boultwood *et al.*, 2007; Le Beau *et al.*, 1993). Thus far, in (del)5 or 5q– MDS, no biallelic deletions or point mutations have been identified in genes associated with the 5q CDRs, leading to the idea that genes residing at 5q are behaving as *haploinsufficient tumor suppressors* (Shannon and Le Beau, 2008). The connection between changes in expression of (del)5 or 5q– MDS tumor suppressor candidate genes and defects in growth control *in vivo* is largely correlative and limited to associations in gene expression in hematopoietic progenitor cells (HPCs) from (del)5 or 5q– patients. In this review, we will discuss the molecular evidence and mouse models supporting specific 5q candidates as tumor suppressors of (del)5 or 5q– MDS and their roles in growth control.

Assessing the 5q tumor suppressor candidates

Commonly deleted regions in 5q

Despite study-to-study and patient-to-patient variability, different groups have identified a number of putative tumor suppressor genes within the two CDRs indicated in **Figure 3.1** (Boultwood *et al.*, 2002; Liu *et al.*, 2007). The working list of genes residing within the CDRs is relatively limited and includes *EGR1* (5q31.2), *CTNNA1* (5q31.2), and *RPS14* (5q33.1). Conventional

cytogenetic and array-based comparative genomic hybridization (aCGH) analyses point to CDRs in aggressive MDS and AML centered upon 5q31, as well as a CDR associated with the 5q– subset of MDS localized to 5q32 (Boultwood and Fidler, 1995; Boultwood *et al.*, 2002; Crescenzi *et al.*, 2004; Evers *et al.*, 2007; Giagounidis *et al.*, 2004; Herry *et al.*, 2007; Horrigan *et al.*, 2000; Le Beau *et al.*, 1993; Van den Berghe *et al.*, 1985).

The emergence of array-based, high-resolution, DNA copy number analysis has allowed the chromosome 5q region to be examined in more detail (Evers *et al.*, 2007). In addition, array-based approaches allow the determination of gene expression changes that accompany the progression to acute leukemia. Interestingly, aCGH studies reinforce previous cytogenetic mapping studies and highlight the deletion of a fairly large region of chromosome 5 (5q31-5q33; **Figure 3.2A**). Coupled with the lack of evidence for a recurrent chromosome 5q breakpoint, these studies suggest that deregulation of one or more candidate genes that map within the region of frequent 5q deletion contributes to (del)5 or 5q– MDS development.

However, the identification of specific candidate genes that lie within the del(5q) region is complicated by the global effects that chromosomal abnormalities have upon gene transcription. Gene expression profiling studies of MDS and other tumors have revealed that chromosome losses lead to dramatic gene expression defects within the deleted region (Greer *et al.*, 2000). For example, based on transcriptional profiling, when cells isolated from normal individuals were compared with cells from (del)5 or 5q– MDS patients, 146 of the

644 genes (23%) mapping to chromosome 5q were significantly downregulated in the (del)5 or 5q– MDS cells (Pellagatti *et al.*, 2006). In contrast, no genes within this same region were significantly downregulated in MDS samples that contain a balanced chromosome 5q. aCGH and other microarray approaches have the potential to reveal other genes that possess tumor suppressor properties. In each case, further functional studies are warranted to determine which gene or network of genes are "drivers" of MDS or simply "passengers" of the chromosome 5q deletion. Data emerging from multiple labs, using diverse approaches including targeted knockdown by interfering RNA (RNAi) and gene knockout mice, are beginning to suggest that multiple 5q gene candidates harbor tumor suppressor function.

The two CDRs mapped to 5q by conventional cytogenetics flank the *DRF1/DIAPH1* (5q31.3) gene **(Figure 3.1)**, which encodes the mammalian Diaphanous-related formin mDia1, a canonical member of the formin family of filamentous (F-) actin assembly proteins (discussed in more detail in following sections). Furthermore, detailed microarray gene expression profiling of samples from MDS patients with detectable 5q loss have revealed that *DIAPH1* expression is diminished as significantly as other notable candidate 5q– tumor suppressors, including *RPS14*, *EGR1*, and *CTNNA1* (**Figure 3.2B**). These data suggested to us a potential role for loss of mDia1 function in the etiology of (del)5 or 5q– MDS. This led us to target the murine *Drf1* gene for knockout (Peng *et al.*, 2007); as previously published and described in detail below, $Drf1^{-/-}$ mice developed an age-dependent myelodysplasia, similar in phenotype to other

knockout mice targeting genes residing in the 5q CDRs, including *EGR1* and *RPS14*. First, let us consider the 5q candidates historically associated with the (del)5 or 5q– subset of MDS.

RPS14: A role for defective translation in MDS?

Ribosomal proteins play a critical function in protein translation, and their dysregulation can promote tumorigenesis. RPS14 is an essential component of the 40S ribosome. Consistent with tumor suppressor function, *RPS14* gene (5q33.1) expression is diminished in (del)5 or 5q– MDS patients (Boultwood *et al.*, 2007; Ebert *et al.*, 2008; Lehmann *et al.*, 2007; Pellagatti *et al.*, 2008; Valencia *et al.*, 2008), and RPS14 re-expression in $CD34^+$ hematopoietic stem cells from affected patients slows proliferation and rescues protein synthesis defects (Ebert *et al.*, 2008). Notably, Diamond-Blackfan anemia (DBA), which shares several clinical features with MDS, is characterized by loss-of-function mutations or deletion of the ribosomal components RPS19, RPS24, RPS17, and RPL35A (Cmejla *et al.*, 2007; Farrar *et al.*, 2008; Gazda *et al.*, 2006; Shannon and Le Beau, 2008).

Translational control is a critical target of common oncogenes and tumor suppressors (reviewed in detail by Bilanges and Stokoe (Bilanges and Stokoe, 2007)). Interestingly, ribosomal proteins associated with DBA and (del)5 or 5q– MDS have been proposed to function in p53 activation in response to nucleolar stress. Indeed, RPS proteins have been demonstrated to inhibit p53 degradation by suppressing its ubiquitination; conversely, haploinsufficiency of RPS proteins

could adversely affect ribosomal biogenesis itself, consequently affecting the expression of tumor suppressors such as p53 (Dai and Lu, 2008). Furthermore, RPS14 is thought to lie downstream of the phosphoinositide 3-kinase (PI3-kinase) signaling pathway, which has a central role in governing translation. PI3K is a *bona fide* oncogene, as one of its catalytic subunits is either amplified or mutated in various solid tumors (Samuels *et al.*, 2004; Shayesteh *et al.*, 1999). The tumor suppressor PTEN (phosphatase and tensin homolog) is a phospholipid phosphatase that antagonizes PI3K by dephosphorylating phosphatidylinositol triphosphate (Tamguney and Stokoe, 2007).

PTEN is mutated in many late-stage tumors, including those arising in brain, prostate, and endometrium (Li *et al.*, 1997; Steck *et al.*, 1997). The PI3K pathway appears to be activated in AML, but thus far no mutations of *PTEN* or other components such as *AKT* have been found in AML (Tibes *et al.*, 2008). Upregulation of PI3K activity contributes to malignant alterations in proliferation, survival, metabolism, migration, and membrane trafficking (Tamguney and Stokoe, 2007). In addition to *RPS14*, other 5q genes are functionally connected to PI3K, p53, and PTEN, including *EGR1* and *CTNNA1*.

Early Growth Response–1 (Egr1) as a tumor suppressor in (del)5 or 5q– MDS

Residing within the 5q CDR is the gene encoding Egr1, a zinc finger protein belonging to the WT1 family of transcriptional regulators. *EGR1* is an early response gene and can mediate cellular responses to mitogens and growth

factors. Such activity is typically associated with oncogenes, but interestingly, Egr1 possesses significant tumor suppressor properties through its ability to directly regulate key target genes including *TGFb1*, *PTEN,* and *P53(TPR53)* (Baron *et al.*, 2006). *Egr1*$^{+/-}$ or *Egr1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) bypass senescence and, therefore, Egr1 was suggested to be an upstream regulator or "gatekeeper" of p53-dependent growth regulation (Krones-Herzig *et al.*, 2003). Egr1 is known to regulate the promoters of both the *PTEN* and *p53/TP53* tumor suppressor genes (Yu *et al.*, 2007) and was shown to physically interact with the p53 protein itself (Liu *et al.*, 2001). Egr1 upregulates PTEN expression in response to both radiation and calyculin A (Virolle *et al.*, 2001; Virolle *et al.*, 2003). Additionally, Egr1-mediated control of p53 and PTEN was demonstrated to have a role in DNA damage-induced apoptosis in both prostate and breast cancer cells (Adamson *et al.*, 2003; Adamson and Mercola, 2002; Yu *et al.*, 2007). Collectively, these data suggest a critical role for Egr1 in tumor suppression.

Beyond adhesion: A role for α-catenin in hyperproliferation in myeloid progenitors.

CTNNA1, the gene encoding α-catenin, resides at 5q31.2, within a CDR linked to (del)5 or 5q– MDS. Through an association with its well-known counterparts β-catenin and E-cadherin, the canonical roles for α-catenin were to promote the assembly of cell-cell junctions and to stabilize the actin cytoskeleton by directly binding actin (reviewed extensively in (Benjamin and Nelson, 2008)).

The quaternary complex then stabilized cell-cell linkages to promote cell adhesion.

However, recent data have prompted a modification of this classic model. In the updated model, α-catenin is maintained at a low concentration proximal to the plasma membrane and binds directly (albeit weakly) to β-catenin, and subsequently complexes with E-cadherin. Cell-cell adhesions drive E-cadherin clustering, and α-catenin dissociates from β-catenin; the juxtamembrane α-catenin concentration is increased sufficiently to promote its homodimerization. The α-catenin homodimer undergoes a conformational change, preferentially binds to F-actin (as opposed to β-catenin/E-cadherin), and promotes the bundling of actin filaments (Rimm *et al.*, 1995). Furthermore, the homodimer negatively regulates the Arp2/3 complex, impeding actin filament nucleation and elongation as well as the formation of dynamic Arp2/3-dependent lamellae (Gates and Peifer, 2005). Cell-cell adhesions would be predicted to strengthen, potentially inhibiting cell migration and invasion.

This model strongly suggests that changes in the expression or subcellular localization of α-catenin may influence disease progression. Diminished (or complete loss of) α-catenin expression is observed in a host of primary cancers (*e.g.*, breast, colorectal, prostate), as well as in cancer cell lines derived from primary tumors (*e.g.,* leukemia, leukocyte, colon, prostate) (Benjamin and Nelson, 2008). Furthermore, human samples from MDS patients revealed a loss of α-catenin protein expression within myeloid progenitor cells (Desmond *et al.*, 2007; Liu *et al.*, 2007), and, in some cancers, diminished α-

catenin is a strong predictor of invasive and metastatic behaviors, increased survival, and proliferation.

Recent studies suggest that α-catenin also functions in disease progression via the regulation of cell survival and apoptotic signaling pathways, independent of its traditional association with E-cadherin. Conditional knockout of α-catenin in mouse epidermis revealed not only adhesion and migration defects in the skin, but marked increases in Ras/MAPK signaling, hyperproliferation, and a significant presence of multinucleated cells (Vasioukhin *et al.*, 2001). Conditional deletion of α-catenin in the mouse central nervous system (CNS) led to severe hyperproliferation and dysplasia in the brain at E13.5 that was attributed to decreased cellular apoptosis and an accelerated cell cycle (Lien *et al.*, 2006; Vasioukhin *et al.*, 2001). Re-introduction of α-catenin into HL-60 myeloid leukemic cells (which harbor a 5q31 deletion encompassing *CTNNA1*) suppressed cellular proliferation while enhancing apoptotic death (Liu *et al.*, 2007). Collectively, these data implicate α-catenin in the control of cellular proliferation and cell death pathways and imply that loss of α-catenin protein may also adversely affect the ability of HSCs to migrate correctly from the bone marrow.

The exact mechanism(s) of action that α-catenin utilizes to specifically maintain proper hematopoiesis is unclear. Is perturbation of α-catenin-dependent HSC migration to and from the bone marrow sufficient to promote disease? This mechanism would presumably involve disruption of the actin binding/bundling activities of α-catenin. This proposed role for actin bundling proteins such as α-

catenin in hematopoiesis is consistent with a recent study from Qian *et al.* demonstrating a role for APC in hematopoietic stem and progenitor cell survival (Qian *et al.*, 2008). In that study, conditional knockout of APC in the hematopoietic compartment led to rapid and dramatic hematopoietic failure; specifically, Apc-depleted mice experienced exhaustion of the myeloid progenitor pool. Like α-catenin, Apc directly binds to and bundles actin filaments (Moseley *et al.*, 2007), suggesting a role for actin bundling proteins in the maintenance of the hematopoietic stem and progenitor compartments.

An expanding role for mDia1 in cytoskeletal regulation

The *RPS14*, *EGR1* and *CTNNA1* genes reside within CDRs historically associated with (del)5 or 5q– MDS, yet recent evidence suggests that *DIAPH1/DRF1* influences the etiology of the disease. The *DIAPH1* gene resides at 5q31.3, between the two most oft-cited CDRs, and encodes the formin mDia1. Mining of pre-existing expression data shows that *DIAPH1* expression is diminished to a similar degree as the other 5q– candidates **(Figure 3.2B).** We hypothesize that, in conjunction with other proteins possessing tumor-suppressor activity, mDia1 and its flanking neighbors operate together as a functional node sensing cytoskeletal dynamics and whose deletion or dysfunction promotes the development of (del)5 or 5q– MDS. First, we provide a brief primer of the multiple functions of mDia proteins.

Structure and function of mDia proteins

Structurally, all formin proteins share a Formin homology-2 (FH2) domain (Higgs, 2005) (**Figure 3.3A**). The FH2 domain nucleates and processively elongates actin filaments by associating with growing barbed ends and creating a biochemical environment favoring actin monomer addition (Kovar, 2006) (**Figure 3.3B**). mDia family formins are tightly regulated (Goode and Eck, 2007) by a Rho-controlled autoregulatory mechanism mediated by the interaction between their N-terminal (Dia-inhibitory, or DID) and C-terminal (Dia-autoregulatory, or DAD) domains (Alberts, 2001; Li and Higgs, 2005). Activated GTP-bound Rho proteins bind to the GTPase-binding domains (GBDs) and occlude DAD binding to DID, thus alleviating its inhibitory influence over the FH2 domain (Goode and Eck, 2007). Newly-generated actin filaments provide the underlying structures that drive changes in cell morphology to facilitate events as divergent as cell division, intracellular trafficking and chemotaxis (Chhabra and Higgs, 2007).

One mechanism of actin remodeling in response to external stimuli includes Rho GTPase signaling through their mDia formin effectors (Wallar and Alberts, 2003). mDia formins remodel the actin cytoskeleton through binding a variety of different Rho GTPases (**Fig 3.3**), and the specificity of the actin-rich structure is dictated by the association of distinct mDia:Rho GTPase pairs. For instance, the interaction between either mDia1 or mDia2 and activated RhoB is integral to early endosomal trafficking (Wallar *et al.*, 2007), while the interaction between mDia2 and activated Cdc42 promotes filopodia formation (Peng *et al.*, 2003). mDia formins participate in changes in cell morphology previously thought to depend largely on activated Arp2/3. These processes include the dynamic actin

remodeling underlying filopodia/microspike (Peng *et al.*, 2003; Tominaga *et al.*, 2002) and neurite formation (Dent *et al.*, 2007), phagocytosis (Colucci-Guyon *et al.*, 2005), vesicle trafficking (Fernandez-Borja *et al.*, 2005; Wallar *et al.*, 2007), and lamella/lamellipodial dynamics (Eisenmann *et al.*, 2007; Gupton *et al.*, 2007; Yang *et al.*, 2007).

Evidence for mDia1 in tumor suppression

Both mDia1 and the related mDia2 directly bind to RhoB and act as effectors for RhoB signaling (Fernandez-Borja *et al.*, 2005; Wallar *et al.*, 2007). RhoB has a critical role in apoptotic responses to DNA damage, and the GTPase was shown to be a target of farnesyl-transferase inhibitors (FTIs) (Adini *et al.*, 2003; Lebowitz *et al.*, 1997; Prendergast, 2001a; Prendergast, 2001b). RhoB, like other Ras family members, is post-translationally modified on a C-terminal motif (CAAX) by farnesylation. In cells treated with FTIs, RhoB shifts to become geranyl-geranylated. This change in post-translational modification leads to enhanced activation of serum-response factor (SRF)-mediated gene expression and elevated sensitivity to DNA-damaging agents such as doxorubicin (Du *et al.*, 1999; Lebowitz *et al.*, 1997; Lebowitz and Prendergast, 1998). While FTIs are in active clinical development, no mechanism accounting for how they trigger programmed cell death has yet been identified (Basso *et al.*, 2006). Interestingly, a recent study found that expression of a dominant-negative version of mDia1 not only enhanced tumorigenesis of Ras-transformed mouse embryonic fibroblasts, but it also impeded the ability of FTIs to suppress tumor growth

(Kamasani *et al.*, 2007). This result indicates an important role for mDia1 in the FTI response and suggests a functional relationship between RhoB and mDia1 in tumor suppression. These observations also point to a potential mechanism for how FTIs control tumor cell growth and supports exploration into the clinical use of FTIs in the control of MPS and/or MDS, an idea that has received some attention (Cortes *et al.*, 2002; Huang *et al.*, 2003; Kotsianidis *et al.*, 2008; Kurzrock, 2002; Kurzrock *et al.*, 2002).

Potential mouse models of (del)5 or 5q– MDS

The effects of knocking out various murine equivalents of 5q genes within the hematopoietic compartment have been extensively documented; those genes targeted for knockout include *EGR1* and other 5q genes lying outside of the conventional 5q– CDRs, such as *APC* and nucleophosmin-1 (*NPM1*). The resulting phenotypes are compared within **Table 3.1** and described briefly below.

Egr1-targeted mice

Egr1 has central roles in the proliferation and localization of hematopoietic stem cells (Min *et al.*, 2008). In the context of myeloid function, $EGR1^{-/-}$ mice have enhanced mobilization of HSCs into the bloodstream (Min *et al.*, 2008). It was not clear if the phenotype was due to hyperproliferation that would potentially cause the excess progenitors (stem cells) to outstrip the capacity of the bone marrow to house them. A second study, indicating Egr1 as a tumor suppressor, determined that a subset of mice haploinsufficient for *EGR1* develop

myelodysplastic features. To test the hypothesis that murine *Egr1* had a role in tumor suppression, Joslin *et al.* exposed both $Egr1^{+/-}$ and $Egr1^{-/-}$ mice to *N*-ethyl-nitrosourea (ENU). While neither $Egr1^{+/-}$ nor $Egr1^{-/-}$ mice had any outright myeloproliferative defects without ENU treatment, both types of mice developed myeloproliferative defects at an increased rate with a shorter latency period than wild-type mice treated with ENU (Joslin *et al.*, 2007). The effects (summarized in **Table 3.1**) included an elevated level of white blood cells, anemia, and thrombocytopenia. While this points to a tumor suppressor role for this transcription factor, *Egr1* haploinsufficiency alone does not appear to be sufficient to trigger myeloproliferative disorders in mice.

Mx1-cre/Flox-Apc mice

The gene encoding the actin-bundling protein APC (5q22.2) lies outside of the conventional CDR associated with (del)5 or 5q–subsets of MDS (Moseley *et al.*, 2007). Ablating APC in developing myeloid progenitor cells in mice results in a failure of normal hematopoiesis due to an increase of apoptosis of HSC and HPC cells (Qian *et al.*, 2008). Exhaustion of HSC and HPC cells leads to immune collapse and bone marrow failure. These mice show decreased levels of white blood cells and hemoglobin, are anemic and thrombocytopenic, and display defective erythrocytic and myeloid differentiation. Ineffective erythropoiesis is a result of differentiation arrest of late erythroblasts. Collectively, the severe hematopoietic defects observed in *Mx1*-cre/Flox-*Apc* mice suggest an important

role for APC in the maintenance of the hematopoietic stem and progenitor compartments.

Npm1-targeted mice

Nucleophosmin is a nucleolar phospho-protein that plays a role in centrosomal duplication and genomic stability in normal cells. Like APC, the gene encoding nucleophosmin-1 lies outside of conventional (del)5 or 5q– CDRs. Mice heterozygous for NPM1 develop many features similar to human MDS (Sportoletti *et al.*, 2008); 75% of such mice develop myeloid malignancies, while others develop B and T cell malignancies. The peripheral blood shows elevated levels of white blood cells and leukemic blasts, myeloid expansion and proliferation, and anemia and thrombocytopenia. Myeloid expansion and increased levels of leukemic blasts are seen in the bone marrow, spleen and liver. Splenomegaly is observed, with atypical lymphoid cells replacing normal spleen pulp.

Drf1-targeted/mDia1 knockout mice develop age-dependent myelodysplasia

Based upon the potential functional role between RhoB and mDia1 in tumor suppression and its location in the 5q region associated with MDS, we targeted the murine *Drf1* gene for knockout (Watanabe *et al.*, 1997; Peng *et al.,* 2003). Our working hypothesis was that mice would develop myeloproliferative defects or would progress to myelodysplasia. Upon birth *Drf1*$^{-/-}$ mice were

developmentally and morphologically indistinguishable from their wild-type littermates, yet both *Drf1*$^{+/-}$ and *Drf1*$^{-/-}$ mice developed age-dependent myeloproliferative defects. The resulting phenotype (detailed in **Table 3.1**) included marked splenomegaly (attributed to hyperproliferation within the spleen) and hypercellular bone marrow (due to expansion of activated monocytes and macrophages). Analysis of the erythroid compartment showed both a significant increase in the percentage of splenic cells in S phase and the expansion of erythroid precursors. Collectively, the overall phenotype of the *Drf1* knockout mouse, specifically the marked increased proliferation of hematopoietic progenitors *in vivo*, supports a role for mDia1 as a tumor suppressor for (del)5 or 5q– MDS. The exact mechanism by which mDia1-mediated tumor suppression is mediated remains unclear. An answer may lie in the interrelated contribution(s) of neighboring genes within the 5q CDRs and the proteins they encode, namely Egr1, α-catenin, and RPS14, as described below.

Networking the 5q tumor suppressor candidates: Serum response factor as a sensor for actin dynamics

Upon loss of each candidate 5q gene, including *EGR1*, *RPS14, CTNNA1* and *DIAPH1/DRF1*, similar defects in hematopoiesis are observed; data from these studies suggest that defects in expression of one or more candidate genes mapping within the region of frequent deletion contributes to MDS development. We hypothesize that there is a common functional mechanism driving the progression towards MDS upon loss of 5q. We suggest that the link binding the

5q candidates is SRF, which acts as an actin sensor to control the expression of other 5q genes, including *EGR1*. And we propose that the SRF sensor is controlled by two more 5q candidates: mDia1 and α-catenin.

In addition to directly affecting the actin architecture through the nucleation, elongation, and (in some cases) bundling of actin filaments (Chhabra and Higgs, 2007), mDia proteins regulate the expression of cytoskeletal proteins by activating the transcriptional regulator SRF. SRF was initially characterized as a regulator of immediate-early gene expression caused by stimulation of cells with growth factors (Hill and Treisman, 1995; Treisman, 1986; Treisman, 1996). Alone or in combination with other transcription factors, SRF plays a central role in controlling transcriptional responses to growth factors and other external stimuli (Posern and Treisman, 2006). Over the last two decades, studies have demonstrated that SRF regulates the expression of numerous genes associated with cytoskeletal remodeling, including the β-actin gene (Posern and Treisman, 2006; Sotiropoulos *et al.*, 1999). Rho family members and their actin-nucleating effectors, including Rho-activated mDia1, are strong activators of SRF-mediated gene expression (Copeland and Treisman, 2002; Hill *et al.*, 1995; Sahai *et al.*, 1998; Sotiropoulos *et al.*, 1999; Tominaga *et al.*, 2000).

Furthermore, the 5q candidate α-catenin also modifies gene transcription through SRF. α-catenin was shown to activate a serum-response element (SRE) reporter (a readout for SRF activity) (Merdek *et al.*, 2008), potentially by acting downstream of Rho/ROCK or through a parallel, Rho-independent pathway. These findings were consistent with a role for the downstream anti-proliferation

effects of α-catenin, as SRF is known to induce various genes associated with cell differentiation. The loss of α-catenin expression and subsequent defective growth control observed in many cancers may therefore be mediated through dysfunction of this novel α-catenin-SRF pathway.

Our proposed model is centered upon the activity of SRF, and incorporates an actin sensor controlling the expression of other candidate 5q tumor suppressor genes, including *EGR1* (**Figure 3.4**). How might this actin sensor function? The SRF co-activator MAL bears multiple *RPEL* motifs—so-called because of the amino acid sequence on the protein that mediates actin binding—that bind directly to monomeric actin; as concentrations of G-actin decrease in the cytoplasm, occupancy of the RPEL motifs diminishes (Guettler *et al.*, 2008; Posern *et al.*, 2004; Posern and Treisman, 2006). Non-actin-bound MAL, which normally shuttles between the nucleus and the cytoplasm, occupies the nucleus and is free to activate SRF (Guettler *et al.*, 2008).

Within the nucleus, SRF binds to conserved serum response elements harbored in the promoters of numerous genes, including that of *EGR1*. SRF can, in fact, regulate the *EGR1* promoter at no less than five consensus SRE/SRF binding sites (**Figure 3.5A**) (Mora-Garcia and Sakamoto, 2000). Hence, via its dual activities of diminishing the cellular pools of G-actin in the process of nucleating and elongating F-actin filaments and of activating SRF, mDia1 potentially acts as part of a node for regulating the expression of *Egr1*. This mDia1-driven mechanism may act in parallel with α-catenin-mediated SRF activation. Because Egr1 regulates the promoters of both *PTEN* and *p53/TP53*

tumor suppressor genes (Yu *et al.*, 2007) (**Figure 3.5A**), we postulate a role for the mDia1/α-catenin-SRF-Egr1 node in promoting malignancy through the disruption of *PTEN* and *p53/TP53* expression (**Figure 3.5B**). To date, there are no data to suggest that inactivating mutations of SRF or loss of SRF expression coincides with malignancies. However, expression of a constitutively active form of SRF fused to a transcriptional activation domain from the viral VP16 protein suppressed the ability of activated/oncogenic Ras to transform cultured fibroblasts (Kim *et al.*, 1994). While this result was published roughly 15 years ago and was confirmed by several groups, the explanation for the result has never been adequate. Our model, which assigns tumor-suppressor roles to SRF and many of its 5q neighbors, deftly accounts for this important observation.

Concluding remarks

No clear molecular mechanism accounts for how, individually or together, 5q tumor suppressor candidates either trigger myeloproliferative neoplasms or the myelodysplastic phenotype, or support the progression to malignancy. Candidate 5q tumor suppressor genes mapped to conventional CDRs in the 5q31-33 regions include *RPS14, EGR1,* and *CTNNA1*, and disruption of their expression leads to defects in hematopoiesis in mice. By driving actin filament assembly and bundling, respectively, mDia1 and α-catenin diminish cellular pools of G-actin to activate SRF, which in turn induces the expression of Egr1. Coupled with α-catenin-mediated SRF activation, multiple 5q candidates feed into this actin-sensing node, potentially affecting p53/PTEN signaling through

EGR1. Furthermore, through its association with the PI3K pathway, the 5q candidate RPS14 can also amplify the p53/PTEN signal. Hence, multiple interdependent mechanisms exist within this node that, upon disruption of one or more candidates, may lead to the progression towards malignancy in the (del)5 or 5q– subset of MDS.

An actin-dynamics sensing mechanism has the potential to control, or at least modulate, both the proliferation and proper migration of hematopoietic progenitors and stem cells. The mechanism could facilitate cells' ability to make 'go' or 'grow' decisions as hematopoietic cells differentiate and migrate to new compartments while they progress through their differentiation program.

Intriguingly, APC (a 5q gene product) binds directly and bundles F-actin and thus has the potential to modulate SRF and Egr1-regulated gene expression. Likewise, defective function and/or expression of Wiskott-Aldrich Syndrome protein (WASp), a canonical regulator of actin nucleation via the Arp2/3 complex and an activator of SRF, could release the growth-control machinery by affecting PTEN and p53 expression in the myeloid malignancies that arise in affected WAS patients. Thus, defects in an SRF-directed actin-dynamics sensing tumor suppression mechanism may have an additional role in carcinogenesis in non-MDS tumors.

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TABLE 3.1. The effects of knocking out murine equivalents of 5q genes.

Mouse Phenotype	*Drf1*	*Egr1*	*Apc*	*Npm1*
Elevated WBC	+	+	–	+
Lymphopenia	+	–	+	–
Monocytosis	+	+	–	+
Granulocytosis	+	+	–	+
Anemia	+	+	+	+
Thrombocytosis	+	–	–	–
Thrombocytopenia	–	+	+	+
Splenomegaly	+	+	–	+
Hepatomegaly	–	–	–	+
Extramedullary hematopoiesis	+	+	–	+
Loss of splenic organization	+	+	–	+
Myeloproliferative defects	+	+	–	+
Ineffective erythropoiesis in:				
bone marrow	+	+	+	+
spleen	+	+	+	+

Unique Features:

Drf1	Myeloproliferative defects (MPD) are age dependent.
Egr1	Increased rate of MPD with shorter latency. Absence of blasts.
Apc	Depletion of HSC and HPC pools lead to immune failure.
Npm1	Numerical and structural chromosomal abnormalities in genomic DNA; 75% myeloid malignancy, 25% B and T cell malignancy.

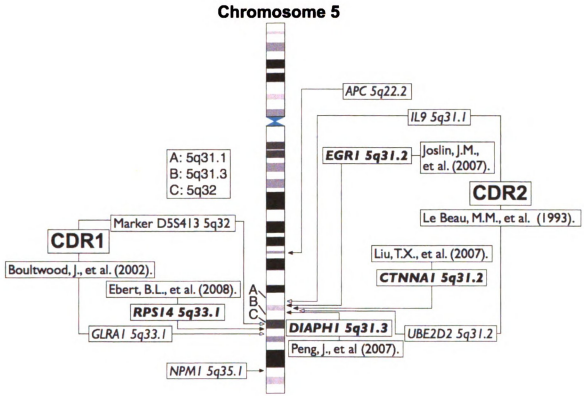
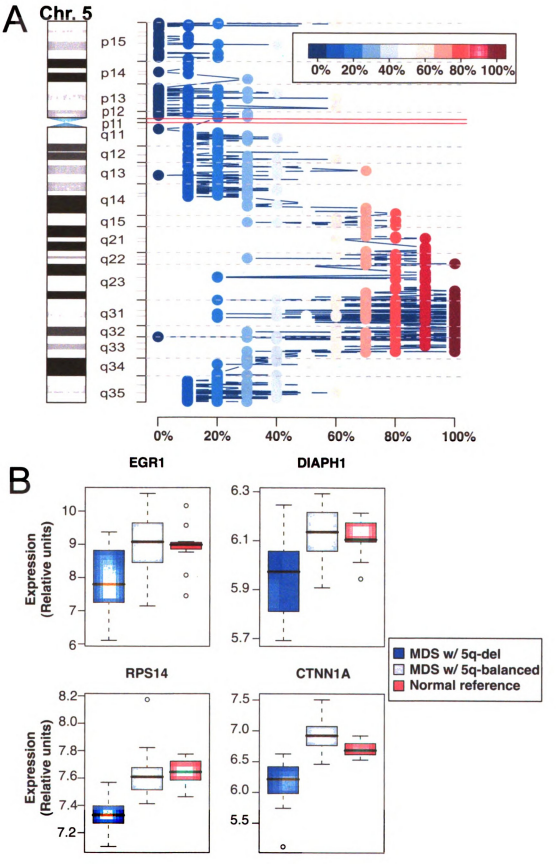


Figure 3.1. Commonly deleted regions (CDRs) and marker genes defined by conventional cytogenetics.

The two CDRs (Boultwood *et al.*, 2002; Le Beau *et al.*, 1993) mapped by G-banding flank the human gene *DIAPH1* (*Drf1*) encoding human mDia1 and located at 5q31.3. The CDR boundaries are indicated by the open arrowheads; the *RPS14* gene is located in CDR1 at 5q33.1, and both *EGR1* (5.31.2) and *CTNNA1* (5q31.2) genes are in CDR2.

Figure 3.2. DNA copy number and gene expression analysis of chromosome 5q in (del)5 or 5q– MDS: Comparison of *DIAPH1/Drf1* expression *versus* other candidate 5q tumor suppressors.
A. The percentage of patients having a copy number loss, computed for 374 genomic locations on chromosome 5 (red lines indicate the centromere). MDS cells with chromosome 5q deletions were examined by high-resolution array comparative genomic hybridization (n = 10) as described in (Evers *et al.*, 2007). The region of most frequent deletion maps between 5q23.3 and 5q33.3. To construct this plot, the Evers et al. DNA copy number data was obtained from the Gene Expression Omnibus database (GEO, GSE8804), replicate data were averaged, and genomic regions that had DNA copy number ratios less than 0.8 were considered regions of deletion.
B. Relative gene expression as determined by microarray analysis for several candidate genes that map within the del(5q) region. Gene expression values were obtained from CD34+ cells isolated from normal individuals (n = 11), from MDS patients who do not have a detectable 5q loss (MDS, n = 25), and from balanced and unbalanced del(5q) MDS patients; (n = 20). Significant decreases in expression are found in all of these genes in the 5q– versus normal individuals ($P < 0.05$). To construct this plot, gene expression data generated by Pellagatti et al. (Pellagatti *et al.*, 2006; Pellagatti *et al.*, 2004; Pellagatti *et al.*, 2008) were obtained from GEO (GSE4619), replicate gene expression measurements were averaged, and data plotted as log2-transformed intensity values.


A
Chr. 5
p15
p14
p13
p12
p11
q11
q12
q13
q14
q15
q21
q22
q23
q31
q32
q33
q34
q35
0% 20% 40% 60% 80% 100%
0% 20% 40% 60% 80% 100%
B
EGR1
DIAPH1
RPS14
CTNN1A
Expression (Relative units)
MDS w/ 5q-del
MDS w/ 5q-balanced
Normal reference

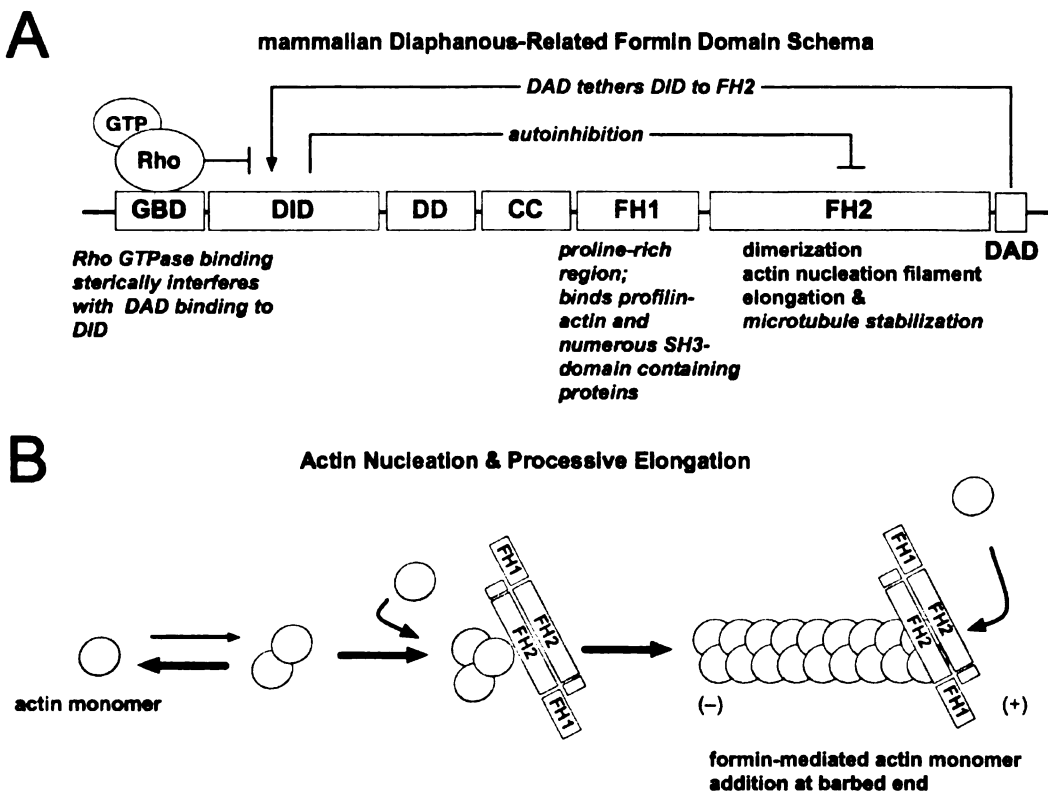
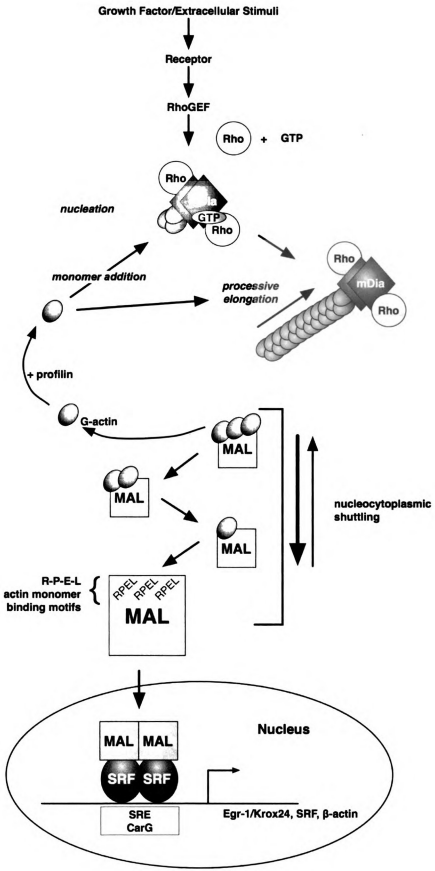


Figure 3.3. Schematics of mDia1 domain structure and mDia1-mediated actin filament assembly.

A. Like all Diaphanous-related formins, mDia1 is autoregulated (Higgs, 2005). DID weakly binds to and inhibits the actin-nucleating FH2 domain (Li and Higgs, 2003). DAD acts as a high-affinity anchor or catch that is released upon Rho binding to the GBD (Alberts, 2001; Wallar *et al.*, 2006). Bound GTP-Rho sterically interferes with DAD binding, thus releasing the inhibitory effects of DID over actin assembly. This leads to activation of F-actin assembly and microtubule stabilization (Alberts, 2001; Palazzo *et al.*, 2001; Wallar *et al.*, 2006).

B. Spontaneous actin assembly progresses from monomers to actin dimers and trimers; in the absence of assembly factors, these quickly dissociate. Formin FH2 domains, comprised of dimers linked by flexible tethers, bind to and stabilize actin dimer intermediates. Formins processively elongate filaments by creating an environment at the barbed (+) end that favors monomer addition (Otomo *et al.*, 2005). The crystal structures (Otomo *et al.*, 2005; Xu *et al.*, 2004) of yeast and mammalian formins revealed unique "lasso and post"-like dimers between FH2 domains. This so-called "tethered dimer" allows for a dynamic association with the barbed end of growing filaments.

Figure 3.4. mDia-directed SRF activation by changes in F-actin assembly. Growth factors or chemotactic stimuli propagate intracellular signals activating mDia1, in part, through binding to activated Rho GTPases. This subsequently allows for nucleation and processive elongation of non-branched actin filaments from a G-actin monomer pool. Diminishing concentration of actin monomer in the cytoplasm decreases the occupancy of RPEL motifs on the transcriptional co-activator MAL. In the absence of RPEL occupancy by G-actin, MAL translocates to the nucleus to bind and stimulate SRF (Posern *et al.*, 2004; Posern and Treisman, 2006). In addition to other enhancer elements, the *EGR1* promoter contains five SRF-binding sites called SREs. Upon docking to the SRE, MAL-bound SRF can promote the transcription of numerous genes including those for EGR1 as well as β-actin. mDia1 has the unique capacity to both diminish the cellular pools of G-actin in the process of nucleating and elongating F-actin filaments and activate SRF, thus amplifying the downstream readout of gene transcription.


Growth Factor/Extracellular Stimuli
Receptor
RhoGEF
Rho + GTP
Rho
GTP
Rho
nucleation
monomer addition
processive elongation
Rho
mDia
Rho
+ profilin
G-actin
MAL
MAL
MAL
nucleocytoplasmic shuttling
R-P-E-L actin monomer binding motifs
RPEL
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SRF
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SRE
CarG
Egr-1/Krox24, SRF, β-actin

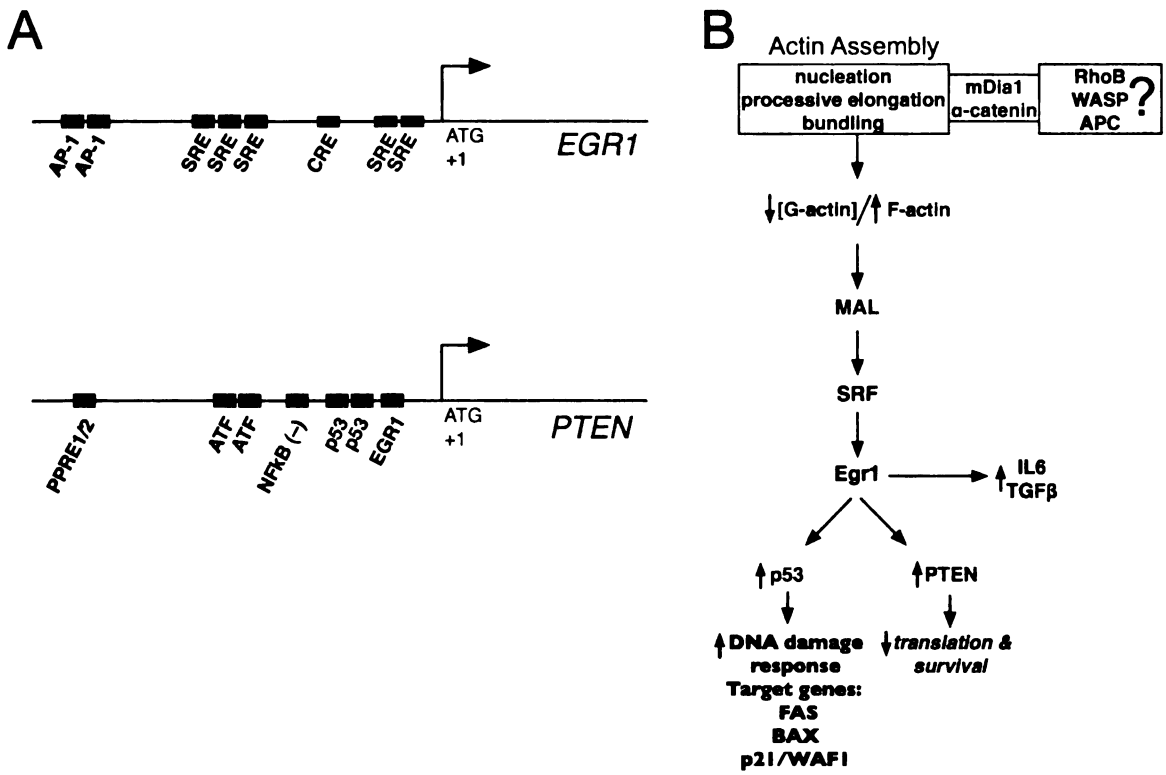

Figure 3.5. A model for an EGR1-dependent actin-sensing node in (del)5 or 5q– MDS that propagates p53/PTEN.

A. Schematic of the *EGR1* promoter, whose activity is regulated by at least 5 SRE consensus sites. In turn, Egr1 protein can act as a transcriptional activator to enhance the promoter activity of the tumor suppressor PTEN.

B. By driving actin filament assembly and diminishing the cellular pools of G-actin, mDia1 is a potent activator of SRF, which, in turn, induces the expression of Egr1. Egr1 can act as a transcriptional regulator, with target genes including TGFβ, p53, and PTEN, thereby regulating cell proliferation and survival signaling in response to stress. Coupled with α-catenin-mediated SRF activation, multiple 5q candidates feed into this actin-sensing node, potentially propagating p53/PTEN signaling through Egr1. Furthermore, through its association with the PI3K pathway, the 5q candidate RPS14 can also amplify the p53/PTEN signal. Exquisite control of this actin-sensing mechanism centered upon 5q candidate tumor suppressor genes would be critical to controlling the proliferation and proper migration of hematopoietic progenitors and stem cells.

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Chapter 3.2

Loss of RhoB Expression Enhances the Myelodysplastic Phenotype of mammalian Diaphanous-related Formin mDia1 Knockout Mice

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A.D. DeWard wrote entire manuscript and designed and performed all experiments. Flow cytometry analysis was performed by K. Leali and R. West.

Abstract

Myelodysplastic syndrome (MDS) is characterized by ineffective hematopoiesis and hyperplastic bone marrow. Complete loss or interstitial deletions of the long arm of chromosome 5 occur frequently in MDS. One candidate tumor suppressor on 5q is the mammalian Diaphanous (mDia)-related formin mDia1, encoded by *DIAPH1* (5q31.3). mDia-family formins act as effectors for Rho-family small GTP-binding proteins including RhoB, which has also been shown to possess tumor suppressor activity. Mice lacking the *Drf1* gene that encodes mDia1 develop age-dependent myelodysplastic features. We crossed mDia1 and RhoB knockout mice to test whether the additional loss of RhoB expression would compound the myelodysplastic phenotype. $Drf1^{-/-}$ $RhoB^{-/-}$ mice are fertile and develop normally. Relative to age-matched $Drf1^{-/-}$ $RhoB^{+/-}$ mice, the age of myelodysplasia onset was earlier in $Drf1^{-/-}RhoB^{-/-}$ animals including abnormally shaped erythrocytes, splenomegaly, and extramedullary hematopoiesis. In addition, we observed a statistically significant increase in the number of activated monocytes/macrophages in both the spleen and bone marrow of $Drf1^{-/-}RhoB^{-/-}$ mice relative to $Drf1^{-/-}RhoB^{+/-}$ mice. These data suggest a role for RhoB-regulated mDia1 in the regulation of hematopoietic progenitor cells.

Introduction

mDia-family formins assemble linear actin filaments and modulate microtubule dynamics in response to adhesive and proliferative stimuli [1]. They are regulated by Rho-family small GTP-binding proteins such as RhoB [2,3]. Rho GTPases activate formins through direct binding and disruption of an autoinhibitory mechanism mediated by regulatory domains that flank the actin/microtubule-binding formin homology-2 (FH2) domain [1]. While the roles of mDia formins in directed cell migration, cell division, and development are well established [4], only recently have gene-targeting experiments in mice revealed roles for mDia family formins in immune and myeloid cell proliferation [5,6,7].

The *DIAPH1* gene encoding human mDia1 (located at 5q31.3) lies between the two commonly deleted regions mapped by conventional cytogenetics in myelodysplastic syndrome (MDS) patient samples [8]. 5q- MDS is characterized by peripheral cytopenias and ineffective hematopoiesis [9]. How defects in one or more 5q genes trigger MDS or contribute to malignant progression in conjunction with additional chromosomal abnormalities remains unknown [10]. Gene targeting experiments on the murine *DIAPH1* homolog *Drf1* show that loss of mDia1 expression impairs the growth control of hematopoietic progenitors [6]. However, the specific mechanism by which loss of mDia1 expression triggers the MDS-like phenotype is currently under investigation.

The small GTPase RhoB binds to both mDia1 and mDia2 on endosomes and has a role in endosome trafficking [2,3]. RhoB also plays an important role in the apoptotic response to DNA damage [11], and loss of RhoB expression has

been shown to correlate with late-stage malignancy [12,13]. Mice lacking RhoB alone do not show any signs of myelodysplasia or any developmental or fertility defects, but Ras-transformed mouse embryonic fibroblasts (MEFs) from these mice are resistant to apoptosis in the presence of farnesyltransferase inhibitors (FTIs), doxorubicin, or taxol [14,15,16]. Together, these studies suggest RhoB possesses tumor suppressor activity.

In this study, we hypothesized that the myelodysplasia observed in *Drf1*-null mice would be enhanced by the additional loss of one of its regulators, RhoB. By examining the peripheral blood, bone marrow, and spleen hematopoietic progenitor cells, we found that $Drf1^{-/-}RhoB^{-/-}$ mice develop age-dependent myelodysplasia at and earlier age than $Drf1^{-/-}RhoB^{+/-}$ mice. These results are consistent with a model for disease progression in MDS that includes the alteration of multiple tumor suppressors in hematopoietic stem or progenitor cells.

Experimental Procedures

Gene targeting. The gene targeting and genotyping of *Drf1*-null mice were performed as previously described [17]; targeting and genotyping of *RhoB*-null mice were performed as described in [15]. The mice used in this study were a mixed 129/B6 genetic background. For all experiments, data were acquired from six $Drf1^{-/-}RhoB^{+/-}$ and six $Drf1^{-/-}RhoB^{-/-}$ mice at 100 days of age, and from eight $Drf1^{-/-}RhoB^{+/-}$ and nine $Drf1^{-/-}RhoB^{-/-}$ mice at 400 days of age.

Ethics Statement. All experiments performed were approved in advance by the Van Andel Research Institute Institutional Animal Care and Use Committee.

Flow cytometry analysis. Peripheral blood, bone marrow, and splenic single-cell suspensions were characterized by flow cytometric analysis. Peripheral blood was extracted from the heart using a syringe equipped with a fine gauge needle. Bone marrow was flushed from femurs using a syringe with a fine gauge needle and 3 mL of PBS. Single-cell suspensions of the spleen were obtained by mincing tissue with glass slides and subsequent passage and scraping of tissue in a ThermoShandon biopsy bag (Thermo Fisher Scientific).

Cells were incubated for 15 min at 20 °C in the dark. Incubation was followed by addition of 1× FACSLyse reagent (Becton Dickinson) for 15 min at 20 °C in the dark. After RBC lysis, the remaining cells were washed in 2 mL PBS with 0.1% sodium azide. Cells were fixed in 1.0% methanol-free formaldehyde (Polysciences, Inc.) in PBS containing 0.1% bovine serum albumin and refrigerated at 4.0 °C until acquisition. Appropriate subclass and negative controls were used to detect nonspecific binding of antibody and autofluorescence. A minimum of 10,000 events for fresh mononuclear cells and 5,000 events for splenic cells were acquired. Flow cytometric analyses were conducted using either a FACSCalibur 4-color or a FACSAria 12-color flow cytometer (Becton Dickinson). Data were analyzed using Becton Dickinson CellQuest Pro and FACSDiVa software.

Monoclonal antibodies. The following monoclonal antibodies were used: anti-CD29APC from BioLegend; anti-CD45PerCP (30-F11), anti-CD41FITC

(MWReg30), anti-CD71FITC (C2), anti-CD74FITC (In-1), anti-TER-119PE (Ly-76, Ter-119), anti-CD13PE (R3-242), anti-CD19PE (1D3), and anti-CD11bAPC (D12) from BD PharMingen; and anti-F4/80 (BM8), anti-CD8aPE (5H10), anti-CD4APC (RM4-5), anti-CD34APC (MEC14.7), and anti-CD3FITC (500A2) from Invitrogen/Caltag Laboratories.

Cell cycle analysis, complete blood count (CBC), and statistics. Cell cycle analyses used propidium iodide (Sigma) in a modified Vindelov's preparation. A minimum of 10,000 events were collected by flow cytometry. Data were analyzed using Becton Dickinson CellQUEST Pro and Verity House ModFIT LT software. CBC analysis was performed using a VetScan HM2 Hematology System (Abaxis). All statistical analysis was performed using the one-tailed Mann Whitney test for significance. Each point on scatter plots represents a single mouse, and each plot includes a horizontal line to indicate the median of the data. Scatter plots and statistics were performed using GraphPad Prism 5.0 software.

Results

We previously reported that *Drf1*$^{-/-}$ and *Drf1*$^{+/-}$ mice develop age-dependent myelodysplasia, typically around 450 days of age [6]. We asked whether the additional homozygous loss of *RhoB* would enhance disease progression relative to *Drf1*-null mice that still contain a functional allele of *RhoB*. Previous reports have shown that *RhoB*$^{+/-}$ cells are indistinguishable from *RhoB*$^{+/+}$ cells in their apoptotic response to DNA damage [16]. We characterized

multiple mice of each genotype at 100 and 400 days of age to assess the presence of myelodysplastic features.

Upon necropsy, we found that several 400-day-old *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice had splenomegaly, as determined by whole spleen weight compared to that of *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice (Fig. 3.6*A*). Pathological examination of H&E-stained splenic sections showed significant dysplasia, and there was often atrophy of the white pulp with poorly formed germinal centers (Fig. 3.6*B*). Splenic sections from *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice also revealed abnormal ratios of myeloid and erythroid composition (frequently a myeloid:erythroid ratio greater than 2.0) and an increased presence of extramedullary hematopoiesis (EMH) (Fig. 3.6*C*).

Based on our previous work in *Drf1*-null mice [6] and the recent finding that the mDia2 formin contributes to erythroblast enucleation [18], we examined the peripheral blood to determine if there was morphological evidence of erythrocyte dysplasia. Peripheral blood smears from *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice showed a marked elevation in abnormally shaped erythrocytes compared with blood from *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice. The erythrocytes were often spiked (echinocyte) or teardrop in appearance (Fig. 3.7*A*), consistent with dysplastic features observed in patients with MDS. *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice did show signs of dysplasia at 400 days, with several teardrop-shaped erythrocytes, but to a lesser extent than *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice. CBC analysis of peripheral blood revealed a significant increase in the WBC count of *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice relative to that of *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice at 400 days of age (Fig. 3.7*B*). Abnormal platelet counts are sometimes observed in

certain subsets of MDS, but we did not observe any significant differences in these mice (Fig. 3.7*C*).

We then focused our analysis specifically on the bone marrow and spleen to examine myelopoiesis in these compartments. Flow cytometry was used to determine the percentage of lymphocytes, monocytes, and granulocytes based on the pan-leukocyte marker CD45. Using the gating strategy outlined previously [6], we found that 400-day-old *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice had an increased percentage of granulocytes and a concomitant decrease in lymphocytes in the bone marrow compartment (Fig. 3.8*A*). Analysis of splenic single-cell suspensions isolated from 400-day-old mice showed a similar increase of granulocytes in *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice (Fig. 3.8*B*).

To further examine potential differences in myelopoiesis between *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice and *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice, we performed flow cytometry on cells from the bone marrow and spleen to detect levels of F4/80 (a pan macrophage marker) and CD11b (integrin αM; monocyte development marker). By 400 days of age, *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice had an increased percentage of F4/80^{+} cells in the spleen, but we observed no significant difference in the bone marrow (Fig. 3.8*C*). On the other hand, CD11b^{+} cells were significantly elevated in both the marrow and spleen by 400 days of age. We also examined cellular expression of the marker CD29 (β1 integrin; important for homing and retention in lymphoid organs). We found an increased percentage of CD29^{+} cells in the marrow, with an even more pronounced increase in the spleen of *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice relative

to *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice (Fig. 3.8*C*). These results are consistent with the observed increase in myeloid cell proportion found by histopathology (Fig. 3.6).

Finally, we analyzed the levels of TER-119 (erythroid-specific marker) and CD71 (transferrin receptor; a marker of proliferating erythroid precursors) in the marrow and spleen of *Drf1*$^{-/-}$*RhoB*$^{-/-}$ and *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice. While there were no differences in the bone marrow, the levels of CD71^{+} and TER-119^{+} cells were elevated in the spleens of 400-day-old *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice (Fig. 3.9, *A* and *B*). An increase in erythroid precursors was also evident in H&E-stained splenic sections (data not shown). Consistent with this observation and the presence of splenic EMH, we also found a substantial increase in splenic cells undergoing S phase in 400-day-old *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice relative to the number in *Drf1*$^{-/-}$ *RhoB*$^{+/-}$ mice (Fig. 3.9*C*).

Discussion

MDS is thought to arise because of multiple alterations in a hematopoietic stem cell [10]. We previously found that knocking out mDia1 expression in mice leads to the age-dependent development of myelodysplasia [6]. Here, we show that the additional knockout of RhoB expression in *Drf1*-null mice accelerates the progression to myelodysplasia. Several candidate tumor suppressor genes in humans reside on the long arm of chromosome 5 [8]. One of these genes is *DIAPH1*, which encodes the actin assembly protein mDia1. While genetic ablation of mDia1 expression alone in mice resembles 5q- MDS, it is likely that

mDia1 acts in concert with multiple other genes on the same commonly deleted region to suppress malignancy [8].

MDS can progress to a more advanced stage and ultimately develop into leukemia. The transformation events involved in disease progression are thought to include genes that contribute to cell-cycle control, apoptosis, and differentiation [10]. The small GTPase RhoB is required for apoptosis in response to DNA-damaging agents and farnesyltransferase inhibitors [14,16]. Previous reports have shown that RhoB negatively regulates Akt survival signaling and mediates apoptosis in a p53-dependent manner [19,20]. Given its role in apoptosis and its known interactions with the mDia formins, we hypothesized that deletion of *RhoB* would enhance the progression of MDS in mice lacking mDia1 expression. Our results support this hypothesis and further substantiate our mouse model of age-associated myelodysplasia. It is also interesting to speculate that loss of RhoB expression may interfere with hematopoietic stem cell maintenance, since recent work has highlighted a role for *RhoB* in stem cell self-renewal [21]. Together, these findings suggest that multiple mechanisms may contribute to the myelodysplastic phenotype in our mice.

Our mouse model uniquely positions us to test some important questions related to the *in vivo* role of RhoB in the anti-neoplastic affects of FTIs. FTIs have been tested clinically to treat various myelodysplasias, with varying degrees of efficacy [22]. But whether FTIs can alleviate the disease phenotype in *Drf1*-null or heterozygous mice and whether RhoB is required *in vivo* to mediate this response remains to be determined.

Our results point to the down-regulation of RhoB as a potential marker for late-stage MDS, similar to its diminished expression in other late-stage cancers [12,13]. Examination of the gene expression profile of RhoB in human patient samples that have a more advanced disease signature could help determine better treatment options for patients diagnosed with MDS.

In summary, we show that mice lacking mDia1 and RhoB expression progress to MDS faster than mice lacking mDia1 alone. These data parallel observations of MDS in humans, in which multiple alterations in hematopoietic stem cells contribute to disease pathogenesis. Our mouse model will be useful in characterizing the mechanism of disease progression further and in testing potential therapeutics to treat this chronic disease.

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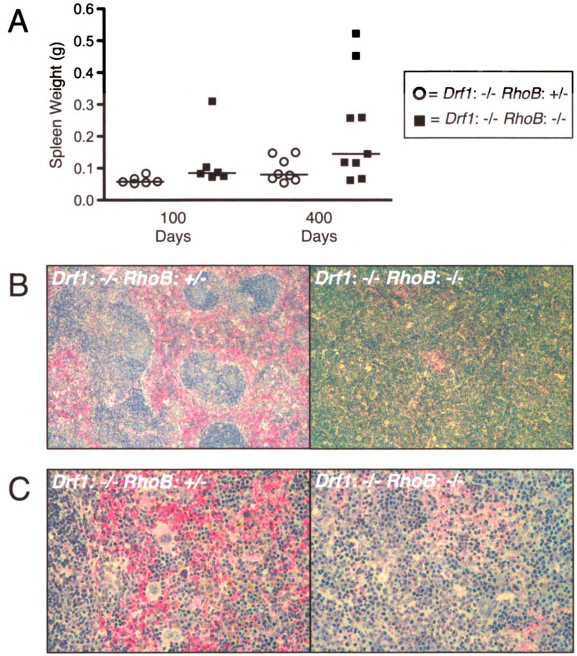


Figure 3.6. Mice lacking *Drf1* and *RhoB* develop splenomegaly and splenic disorganization.

Drf1$^{-/-}$*RhoB*$^{-/-}$ and *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice were necropsied at 100 and 400 days of age.

A. Spleens were removed from mice and weighed immediately. Each point on scatter plot represents data from a single mouse.

B. Formalin-fixed spleens from 400-day-old mice were paraffin-embedded and stained with H&E. Sections shown are at 10x magnification.

C. Splenic sections from **B**, but at 40x magnification.

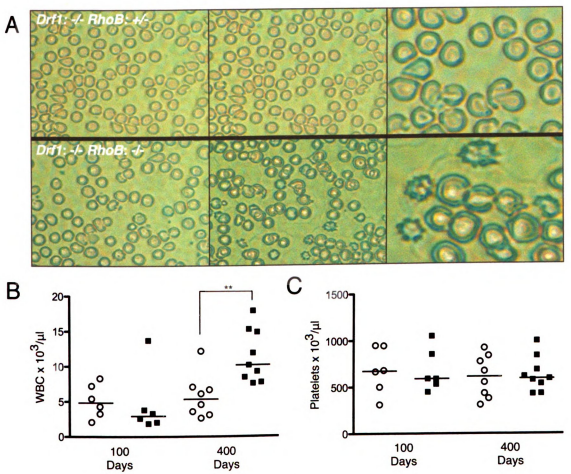


Figure 3.7. Peripheral blood from *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice show age-dependent abnormalities.

A. Peripheral blood smears stained with Wright-Giemsa from 400-day-old mice. Left and center panels are images at 40x; right panels are 60x.

B. Total WBC count from peripheral blood.

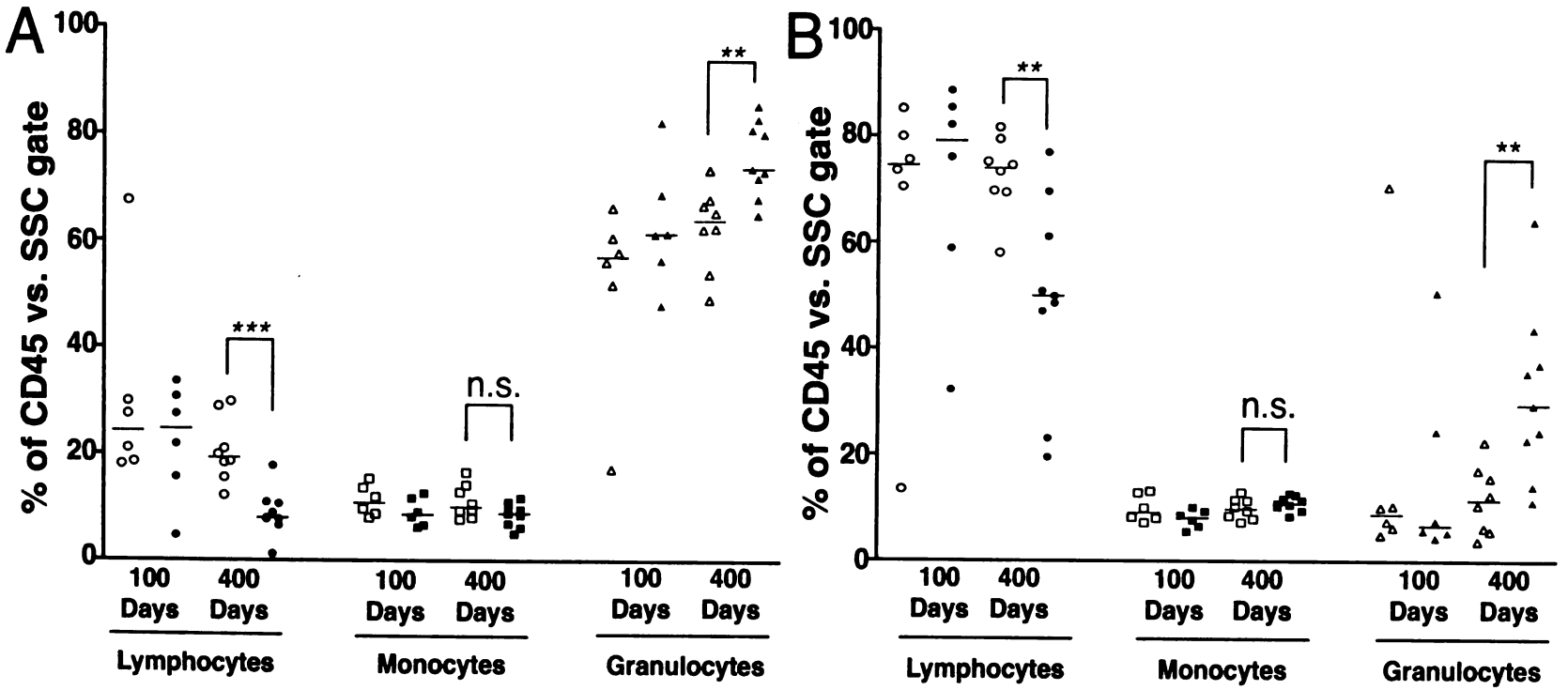
C. Platelet numbers from peripheral blood CBC analysis. In both **B** and **C**, each point on scatter plot represents data from a single mouse (o = *Drf1*$^{-/-}$*RhoB*$^{+/-}$; ■ = *Drf1*$^{-/-}$*RhoB*$^{-/-}$) (** denotes $P \leq 0.01$).

Figure 3.8. Flow cytometry analysis of mouse bone marrow and splenic cells.

A. Scatter plot showing the percentage of lymphocytes, monocytes, and granulocytes from bone morrow. Open shapes represent *Drf1*$^{-/-}$*RhoB*$^{+/-}$ mice and filled shapes represent *Drf1*$^{-/-}$*RhoB*$^{-/-}$ mice (** denotes $P \leq 0.01$; *** denotes $P \leq 0.001$).

B. Scatter plot showing the percentage of lymphocytes, monocytes, and granulocytes from mice splenic single-cell suspensions. Legend is the same as in **A** (** denotes $P \leq 0.01$).

C. Percentage of F4/80^{+}, CD11b^{+}, and CD29^{+} cells from the bone marrow and spleen of mice (* denotes $P \leq 0.05$; ** denotes $P \leq 0.01$) (o = *Drf1*$^{-/-}$*RhoB*$^{+/-}$; ■ = *Drf1*$^{-/-}$*RhoB*$^{-/-}$).


A
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% of CD45 vs. SSC gate
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100 Days
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Lymphocytes
Monocytes
Granulocytes

Figure 3.8 continued

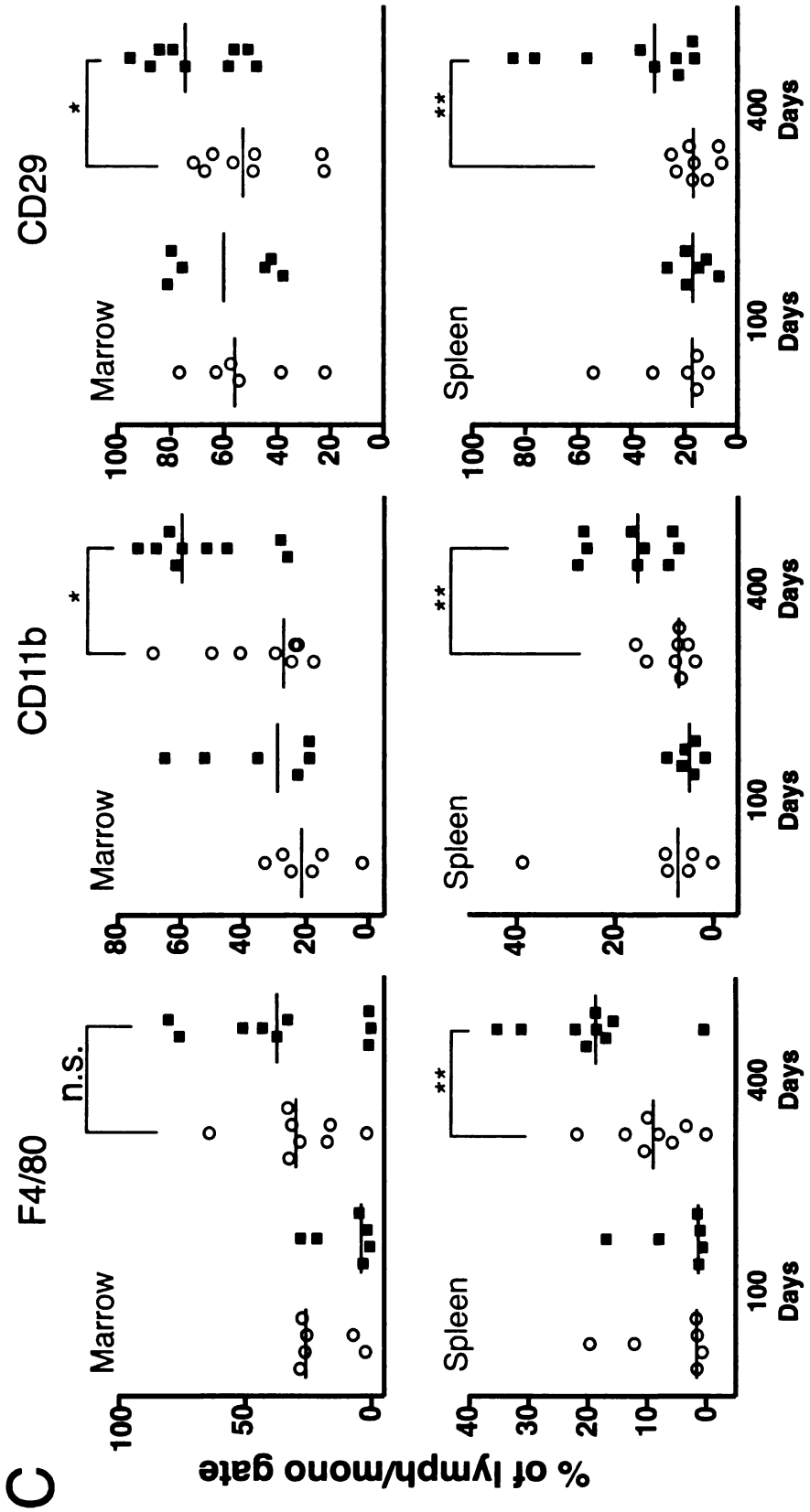


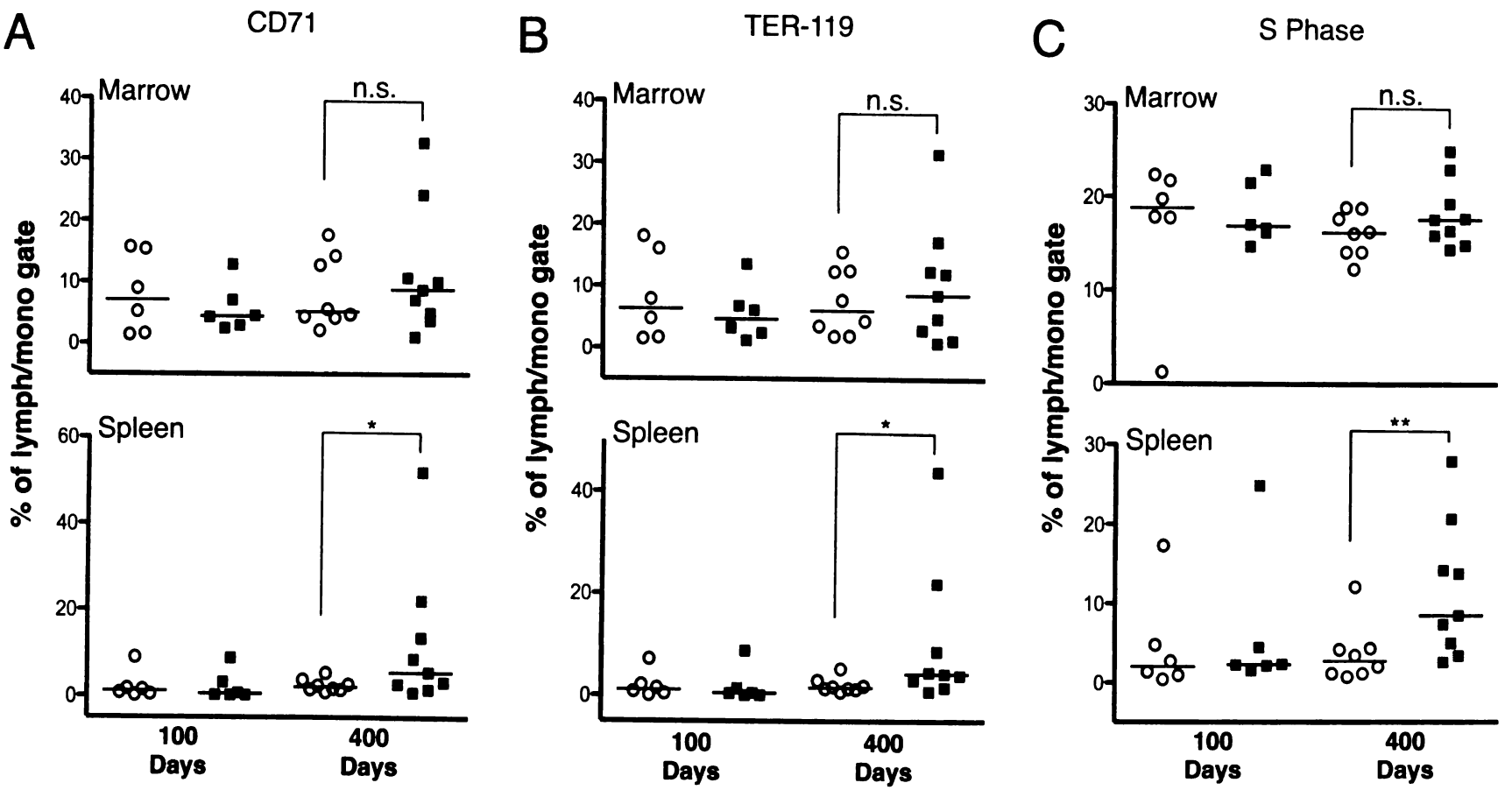
Figure 3.9. Flow cytometry analysis of erythroid precursors in mouse bone marrow and spleen.

Each point on the scatter plots represents data from a single mouse (o = *Drf1*$^{-/-}$ *RhoB*$^{+/-}$; ■ = *Drf1*$^{-/-}$*RhoB*$^{-/-}$)

A. Scatter plot showing the percentage of CD71^{+} cells from the bone marrow and spleen of mice (* denotes $P \leq 0.05$).

B. Percentage of TER-119^{+} cells from the bone marrow and spleen of mice (* denotes $P \leq 0.05$).

C. Percentage of cells undergoing S phase from the bone marrow and spleen of mice (** denotes $P \leq 0.01$).


A
CD71
Marrow
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Spleen
*
% of lymph/mono gate
100 Days
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B
TER-119
Marrow
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% of lymph/mono gate
100 Days
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Chapter 4.1

Ubiquitin-Mediated Degradation of the Formin mDia2 upon Completion of Cell Division

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Abstract

Formins assemble non-branched actin filaments and modulate microtubule dynamics during cell migration and cell division. At the end of mitosis, formins contribute to the generation of actin filaments that form the contractile ring. Rho small GTP-binding proteins activate mammalian Diaphanous-related (mDia) formins by directly binding and disrupting an intramolecular autoinhibitory mechanism. While the Rho-regulated activation mechanism is well-characterized, little is known about how formins are switched off. Here, we reveal a novel mechanism of formin regulation during cytokinesis based on the following observations: 1) mDia2 is degraded at the end of mitosis; 2) mDia2 is targeted for disposal by post-translational ubiquitin modification; 3) forced expression of activated mDia2 yields binucleate cells due to failed cytokinesis; and 4) the cytokinesis block is dependent upon mDia2-mediated actin assembly, because versions of mDia2 incapable of nucleating actin, but that still stabilize microtubules, have no effect on cytokinesis. We propose that the tight control of mDia2 expression and ubiquitin-mediated degradation is essential for the completion of cell division. Because of the many roles for formins in cell morphology, we discuss the relevance of mDia protein turnover in other processes where ubiquitin-mediated proteolysis is an essential component.

Introduction

Formin proteins play a role in diverse processes such as cell migration (1,2), vesicle trafficking (3,4), tumor suppression (5,6), and microtubule stabilization (7,8). Formins also play an essential and conserved role in cytokinesis (9-11). Proper cell division is essential in all animals to maintain the integrity of their genome. Failure to complete cytokinesis can result in genomic instability and ultimately lead to disease such as cancer (12).

The members of the mDia family of formins are autoregulated Rho effectors that remodel the cytoskeleton by nucleating and elongating non-branched actin filaments (13). The amino terminus of mDia contains a GTPase binding domain (GBD) that directs interaction with specific Rho small GTP-binding proteins. The adjacent Dia-inhibitory domain (DID) mediates mDia autoregulation through its interaction with the carboxyl terminal Diaphanous autoregulatory domain (DAD) (14,15). Between the DID and DAD domains lie the conserved Formin Homology 1 (FH1) and FH2 domains. The FH1 domain is a proline-rich region that mediates binding to other proteins such as profilin, Src, and Dia interacting protein (DIP) (16-19). In contrast, the FH2 domain binds monomeric actin to generate filamentous actin (F-actin) and can also bind microtubules directly to induce their stabilization (8,20).

While the mechanism of mDia activation is well-characterized, little is known about its inactivation. Previous reports have suggested that formins can cycle between active, partially active, and inactive states (21,22), due to GTP hydrolysis upon Rho binding to GTPase activating proteins (GAPs). Another

formin inactivation mechanism is through mDia interactions with DIP (23). In the context of cortical actin assembly, DIP negatively regulates mDia2 actin polymerization but has no effect on mDia1 actin polymerization, despite its ability to interact with both proteins directly (17). Because of the fundamental role for formins in cell division, we sought to identify how mDia2 is inactivated in mitosis.

During cell division, the expression level and activity of many proteins (*e.g.*, cyclins, Aurora and Polo kinases) are tightly regulated (24). A unifying regulatory mechanism among these proteins is ubiquitin-mediated proteolysis. In this study, we find that mDia2 protein levels are constant from S phase into mitosis and dramatically decrease at the end of mitosis due to ubiquitin-mediated degradation. Failure to inhibit mDia2 actin assembly results in multinucleation, which supports an essential role for the tight regulation of mDia2 during cell division.

Experimental Procedures

Cells and plasmids– HEK293T and HeLa cells were grown in DMEM containing 10% (v/v) FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Plasmids encoding Myc-mDia2, Myc-EGFP-mDia2-GBD, Myc-mDia2-ΔDAD, Myc-EGFP-mDia2-ΔFH1, Myc-EGFP-mDia2-DAD, and Myc-EGFP-mDia2-DAD-M1041A were previously described (15,16). EGFP-mDia2-ΔGBD/ΔDAD (AA 521-1040) and EGFP-mDia2-ΔGBD/ΔDAD-I704A were a gift from Gregg Gundersen. HA-Ubiquitin was a gift from Richard Cerione. Plasmids encoding Myc-mDia2 K118R, K118/119R, and

K493/494R mutants were constructed using site-directed mutagenesis (Stratagene) following the manufacturer's protocol.

Antibodies and reagents– The following antibodies were used in this study: anti-HA (clone 12CA5) and anti-Myc (clone 9E10) were generated at the Van Andel Research Institute Monoclonal Antibody core facility; anti-cyclin E, anti-Myc, and anti-GFP were from Santa Cruz Biotechnology; anti-GFP, as well as Hoechst 33342 and Texas Red phalloidin, were from Molecular Probes. Anti-β-catenin was from BD Transduction Laboratories; anti-ubiquitin (clone P4G7) was from Covance; anti-ubiquitin (clone FK1) was from BioMol; anti-β-tubulin (clone E7) was from the Developmental Studies Hybridoma Bank; anti-EF1α was from Upstate Biotechnologies; rabbit anti-mDia2 (1358) was raised against the mDia2 FH2 domain (generated as recombinant protein in *E. coli*) as described previously (3,17); and anti-rabbit conjugated to FITC, anti-mouse conjugated to TRITC, and anti-rabbit and anti-mouse conjugated to horseradish peroxidase were from Jackson Immunoresearch. MG132 proteasome inhibitor was from Calbiochem. Cell lysis and immunoprecipitation were performed as previously described (17). Immunoblots were performed using 4-20% Tris-glycine gels (Invitrogen) transferred to a 0.45-μm PVDF membrane (Invitrogen).

Cell cycle arrest and release– G1/S phase cell cycle arrest was performed by incubating HeLa and HEK293T cells in growth medium containing 2 mM thymidine (Sigma) for 16 h. Cells were briefly washed in 1X PBS and incubated in growth medium for 10 h followed by another thymidine incubation for 16 h. Cells were released into growth medium after washing with PBS. Cells were

collected for flow cytometry analysis or lysed at specific timepoints for immunoblotting. Mitotic cell cycle arrest was performed by incubating HEK293T cells in growth medium containing 100 ng/ml nocodazole (Sigma) for 18 h. Cells were briefly washed and allowed to progress through the cell cycle by incubating in growth medium.

Flow cytometry– HeLa and HEK293T cells were briefly washed in 1X PBS. Cells were stained with a propidium iodide (Sigma) solution containing Nonidet P-40 and RNAse. DNA content was acquired using a FACSCalibur flow cytometer (BD Biosciences). The percentages of cells in G0/G1, S, or G2/M phase were determined using ModFit LT software.

Microscopy and microinjection– HeLa cells were plated onto glass coverslips as previously described (25). Cells were microinjected with plasmid DNA at a concentration of 50 μg/ml as previously described (15). Cells were fixed with 3.7 % formaldehyde and permeabilized with 0.3% Triton-X 100.

Results

mDia2 localization and expression is cell cycle–dependent. Previously, mDia2 was shown to localize to the cleavage furrow and midbody in dividing NIH 3T3 cells (26). We show a similar cell cycle–dependent localization of mDia2 in human cells. HeLa cells were grown on coverslips and stained for endogenous mDia2 as well as for F-actin or β-tubulin (Fig. 4.1*A*). We observed that mDia2 co-localized with the actin-rich cleavage furrow and with the microtubule-rich central spindle during cytokinesis. Because mDia2 localization is tightly linked to the cell

cycle, we then asked whether mDia2 protein expression is also cell cycle–dependent. HeLa cells were arrested at the G1/S phase transition using a double thymidine block and allowed to progress through the remainder of the cell cycle. Immunoblots revealed that mDia2 protein expression was increased in S phase and mitotic cells compared to cells predominantly in G0/G1 phase (Fig. 4.1*B*). As cells completed mitosis and re-entered the G0/G1 phase, mDia2 protein expression decreased to levels similar to those found in asynchronously growing cells. Consistent with these results, we observed increased endogenous mDia2 fluorescence in cells undergoing mitosis (Fig. 4.1*A*, lower panel). Flow cytometry was performed in parallel to measure DNA content and quantify the percentage of cells in each cell cycle phase (Fig. 4.1*C*).

Proteasome inhibition blocks mDia2 degradation at the end of mitosis. The ordered proteolysis of numerous cell cycle proteins is required for cell division to occur properly (24). We asked if the decrease in mDia2 levels was the result of proteasomal degradation during the cell cycle. HeLa cells were arrested using a double thymidine block and released into growth medium with and without the proteasome inhibitor MG132. Cells treated with MG132 maintained elevated mDia2 levels relative to untreated cells that completed mitosis (Fig. 4.2*A*). The corresponding flow cytometry analysis confirmed the G1/S phase arrest, with subsequent completion of cell division upon thymidine washout (Fig. 4.2*B*).

We then wanted to address whether proteolytic degradation of mDia2 primarily occurs in late mitosis, when formins contribute to contractile ring formation, or if mDia2 is degraded earlier in the cell cycle. HeLa cells were

arrested in G1/S phase and released into growth medium containing nocodazole. This procedure allows cells to progress through S phase but causes arrest in early mitosis as a result of blocked microtubule assembly. Cells were then washed in normal growth medium to remove the nocodazole for the completion of cell division. Immunoblotting revealed that mDia2 protein levels remain constant from G1/S phase into mitosis (t = 0 to t = 6 hrs) (Fig. 4.2*C*). After nocodazole washout, mDia2 expression diminished to levels observed in asynchronously growing cells. However, cells treated with MG132 after nocodazole washout did not show diminished mDia2 expression, suggesting that mDia2 was proteolytically degraded in late mitosis. Flow cytometry confirmed the mitotic arrest in nocodazole-treated cells, with subsequent completion of mitosis upon nocodazole washout (Fig. 4.2*D*).

mDia2 is post-translationally modified by ubiquitin. Cell cycle–dependent protein degradation is often a result of polyubiquitination, which targets proteins for degradation in the proteasome (27). We hypothesized that polyubiquitination of mDia2 may signal for its degradation during mitosis. HEK293T cells were transiently transfected with epitope-tagged ubiquitin and mDia2. After mDia2 immunoprecipitation and immunoblotting against ubiquitin, we found that ubiquitin covalently modified mDia2 (Fig. 4.3*A*). This was also confirmed by performing the reverse immunoprecipitation to pull down ubiquitin and probing for mDia2 (Fig. 4.3*A*). We often observed a "laddering" of mDia2 at increased molecular sizes on immunoblots, characteristic of polyubiquitination. While mDia2 ubiquitination was shown to occur in cells, we wanted to verify that it was

not simply an artifact of overexpressed proteins. To test this, endogenous mDia2 was immunoprecipitated in HeLa cells and blotted for endogenous ubiquitin. β-Catenin, well known for its modification by ubiquitin, was immunoblotted as a positive control. Our results confirmed that endogenous mDia2 was ubiquitinated, which supports our data using transiently expressed mDia2 (Fig. 4.3*B*).

Different types of ubiquitin modification often dictate different cellular outcomes (28). Monoubiquitination—the attachment of a single ubiquitin to a lysine of a substrate protein—is usually associated with endocytosis and intracellular trafficking. On the other hand, polyubiquitination—attaching several linked ubiquitin proteins to a lysine of the substrate—usually results in proteasomal targeting and degradation. We tested the hypothesis that mDia2 is polyubiquitinated, consistent with our finding that mDia2 is proteolytically degraded at the end of mitosis. We took advantage of an antibody that will recognize only polyubiquitin chains, and not mono- or free ubiquitin. Myc-mDia2 was transfected in HEK293T cells and immunoprecipitated. Subsequent immunoblotting using the anti-polyubiquitin antibody revealed that mDia2 is modified by a polyubiquitin chain (Fig. 4.3*C*). Endogenous β-catenin was immunoprecipitated from HeLa cells and blotted with mDia2 as a positive control.

mDia2 ubiquitination is cell cycle–dependent. It is possible that mDia2 ubiquitination may be required for multiple cellular functions. Therefore, we asked whether mDia2 ubiquitination was regulated differently during cell division, as opposed to a constitutive process not dependent on the cell cycle. HEK293T cells expressing Myc-mDia2 and HA-ubiquitin were arrested in G1/S phase using

a double thymidine block and allowed to progress through the cell cycle. Cells were collected at various timepoints and subjected to immunoprecipitation and immunoblotting to examine the extent of mDia2 ubiquitination. Flow cytometry was also performed to confirm progression through the cell cycle. We found that ubiquitination of mDia2 increased as cells progressed from S phase (t = 0 h, 93% of cells in S) into mitosis (t = 4 to 10 h) (Fig. 4.4*A* and *B*). To further examine the timing of ubiquitination, HEK293T cells expressing epitope-tagged mDia2 and ubiquitin were arrested in mitosis after nocodazole treatment. Cells were collected at various times after nocodazole washout and subjected to immunoprecipitation and immunoblotting. In support of our hypothesis, the levels of ubiquitinated mDia2 increased at the end of mitosis (Fig. 4.4, *C* and *D*), which is consistent with the timing of mDia2 degradation observed earlier (see Fig. 4.2). These data do not rule out the possibility that mDia2 ubiquitination occurs at other times, but it demonstrates that mDia2 modification by ubiquitin is regulated at the end of mitosis.

mDia2 is ubiquitinated on multiple lysine residues. Next, we wanted to identify the region(s) of mDia2 attached to ubiquitin, which may reveal additional functions of ubiquitination beyond proteasomal degradation and help determine the functional consequence of stabilized mDia2. Mass spectrometry has been used to examine ubiquitinated substrates in MCF-7 human breast cancer cells, and that study revealed numerous proteins that were covalently modified by ubiquitin, one of which was mDia2 (29). This data not only substantiates our finding that mDia2 is ubiquitinated in cells at endogenous levels, but it also

identified a specific lysine residue attached to ubiquitin. That lysine (K118) is located in the mDia2 GBD. Interestingly, mDia1 was also found to contain a ubiquitinated lysine adjacent to the FH1 domain (corresponding to K493 of mDia2). ClustalW protein sequence alignment showed conservation of the corresponding lysines across the mouse and human sequences of mDia1 and mDia2 (Fig. 4.5*A*). We then asked if replacing the lysines with arginines would be sufficient to prevent ubiquitination. mDia2 K118, K118/K119, and K118/119/493/494 were replaced with arginines to generate a single, double, and quadruple mutant, respectively, because E3 ligases are known to be promiscuous in their attachment of ubiquitin to substrate proteins. The altered versions of mDia2 were expressed in HEK293T cells with HA-ubiquitin to examine the levels of ubiquitination. Interestingly, mDia2 ubiquitination was not abolished in any of the Arg-substituted variants, suggesting that there are additional lysine residues that can act as acceptors for ubiquitin attachment (Fig. 4.5*B* and *C*).

To better define where ubiquitin is attached, we expressed versions of mDia2 lacking certain domains, or expressed specific mDia2 domains individually, in HEK293T cells (Fig. 4.5*B*). Immunoblots showed that the mDia2 GBD was sufficient but not required for ubiquitination, since the ΔGBD/ΔDAD version of mDia2 could still be ubiquitinated. In addition, we found that the FH1 domain was not required for ubiquitination (Fig. 4.5*D*). These data confirm that ubiquitin can be attached to many different residues on mDia2 and is not specific to one domain or region of the protein.

Deregulated mDia2 expression induces cytokinesis failure due to excessive actin accumulation. No definitive mechanism of inactivating the mDia formins has been revealed. It is interesting to speculate that ubiquitination and proteolytic degradation of the mDias may be a general regulatory mechanism to ensure their inactivation, particularly during cell division. In this case, the failure to degrade mDia efficiently would result in excessive actin accumulation.

To test the biological significance of such a scenario, we microinjected HeLa cells with a ΔGBD/ΔDAD version of mDia2 (AA 521-1040), which lacks key autoregulatory domains. Plasmid DNA was microinjected into synchronized cells during late S phase. Cells were fixed 16 hours later and stained with phalloidin and Hoechst. We found that 40% of the cells expressing deregulated mDia2 were predominantly binucleate or to a lesser extent, multinucleate, which are hallmarks of failed cytokinesis (Fig. 4.6*A*). We also showed that a version of mDia2 that can stabilize microtubules but is deficient in actin nucleation (EGFP-mDia2-ΔGBD/ΔDAD-I704A (8)), does not result in multinucleation (Fig. 4.6*A*). This important distinction shows that failed cytokinesis is due to deregulated actin polymerization and not decreased microtubule dynamics, since mDia2 was recently shown to control both properties independently (8).

To further address whether mDia2 hyperactivation would impede the completion of cytokinesis, plasmid DNA encoding the DAD domain was microinjected into synchronized HeLa cells. Expression of this domain disrupts the formin autoregulatory mechanism in cells and results in increased actin polymerization (15,30). DAD expression promoted multinucleation in

approximately 40% of cells (Fig. 4.6*A* and *B*). An altered version of DAD (M1041A) that prevents binding to DID (31,32) and does not affect formin autoregulation (15) resulted in significantly fewer multinucleate cells (Fig. 4.6*A* and *C*).

Discussion

The later stages of cell division are part of a highly regulated process requiring the contribution of many proteins at specific times (33). Inappropriate regulation of cytokinesis results in genetic defects (aneuploidy) that can support the progression to malignancy. Here, we report the cell cycle–dependent localization and expression of mDia2: we found that mDia2 was ubiquitinated and degraded at the end of mitosis. We propose that mDia2 degradation is a potential mechanism to inactivate the formin, thus ensuring its appropriate activity within the cell cycle. A consequence of deregulated actin polymerization during mitosis is cytokinesis failure, since activated mDia2 results in the multinucleation of cells.

Contractile ring formation during the latter stages of mitosis requires highly regulated and localized actin assembly, while its constriction depends upon the action of myosin (34). In both budding and fission yeast, formin FH2 domains nucleate and processively elongate new actin filaments that form the contractile ring (35,36). FH2-mediated actin polymerization is increased in the presence of the FH1 domain because of its ability to interact with profilin-bound actin monomers. Once the dense, bundled actin network of the contractile ring is finally formed (37), myosin-II pulls on the actin fibers to invaginate the plasma

membrane. Soon after, components of the contractile ring are removed or degraded to allow disassembly of the ring (35).

It was recently shown that mDia2 is essential for cytokinesis of NIH 3T3 fibroblasts, presumably by acting as a scaffold for the contractile ring and maintaining furrow position (26). mDia2 is normally autoregulated in the cytosol, and activated to promote actin assembly at desired locations, such as the contractile ring. But it was interesting to find coordinated mDia2 protein expression during cell division. Why are the protein levels not sustained once cell division is complete if the formin could simply become autoregulated again? Interestingly, the yeast formin cdc12p contains conserved DID and DAD domains, but its activity does not appear to be mediated by autoregulation during cytokinesis (10). This suggests that additional regulatory mechanisms mediate the specific timing of actin assembly for this formin.

Traditionally, it has been assumed that formins are activated by certain Rho GTPases and eventually become inactivated upon release of Rho or other proteins that might keep formins in an activated conformation. One way to guarantee that mDia2 (or similar formins, such as cdc12p) would no longer be activated would be to dispose of the protein entirely. Ubiquitin-mediated proteolysis is a well-characterized mechanism for degrading cellular proteins via the proteasome. Protein ubiquitination has also been shown to play a crucial role in the completion of cytokinesis (38). Our discovery that mDia2 is ubiquitinated and degraded at the end of mitosis suggests a new layer of formin regulation.

In addition, we found that mDia2-mediated actin assembly will result in failed cytokinesis if not properly controlled. Deregulated versions of mDia2 also stabilize microtubules, thus making it difficult to determine whether failed cytokinesis is due to deregulated actin assembly or due to effects on microtubule stabilization. Stable microtubules play an important role in central spindle assembly (39), and the localization of mDia2 to the midbody during cytokinesis suggests that it could contribute to this process. Since mDia2 mediated actin assembly is independent of its microtubule stabilization properties, we took advantage of this distinction and used a mutant version of mDia2 (I704A) that is unable to nucleate actin but can still stabilize microtubules. We determined that failed cytokinesis is specifically the result of deregulated actin assembly.

Our findings are consistent with a similar model showing that deregulated actin polymerization by WASp (Wiskott-Aldrich syndrome protein) can lead to defective cytokinesis (40). This study showed that activating mutations in WASp, which are known to cause X-linked neutropenia, can lead to the hyperactivation and mislocalization of actin polymerization. The authors of this study concluded that failed cytokinesis was likely due to disruption of contractile ring formation and potential physical inhibition of mitosis by interfering with required signaling proteins.

It is well-known that intracellular trafficking of membrane to the central spindle is crucial for cleavage furrow invagination and subsequent separation (41,42). Our lab has demonstrated that constitutively active RhoB and deregulated mDia2, an effector for RhoB, slows vesicle trafficking (3). mDia2 localizes to

endosomes and contributes to their motility by mediating actin dynamics. Therefore, deregulated mDia2 may also hinder the normal trafficking of membrane and other components required for cell abscission. Defective abscission results in multinucleate cells, which is what we observed in the context of deregulated mDia2. Thus, mDia2 degradation may be required for both normal actin dynamics related to contractile ring assembly and for intracellular trafficking dynamics. This is an interesting possibility considering that protein ubiquitination contributes to the normal turnover of many proteins involved in endosome trafficking (43).

The anaphase-promoting complex (APC) is an E3 ligase important for the inactivation of cytokinesis machinery by triggering its degradation (44). In budding yeast, genetic deletion of the APC activator Cdh1 results in cells that do not disassemble the contractile actin ring properly because specific proteins remain stabilized after cell contraction (45). Furthermore, a stable version of the protein IQGAP, which crosslinks F-actin, can partially induce the phenotype observed in Cdh1-deficient cells. It was concluded that protein degradation of multiple contractile ring components must be responsible for efficient ring disassembly. Our findings suggest that mDia2 ubiquitination and degradation may contribute to the disassembly of the contractile ring, especially considering that mDia2 is a required component for proper actin ring assembly (26), and mDia2 was previously shown to bundle actin filaments directly (46).

A logical question is whether mDia2 can act as a substrate for APC-mediated ubiquitination. mDia2 sequence analysis shows that it contains over 10 putative

destruction box motifs (RXXL or KEN) (47). We show that mDia2 deletion mutants and lysine point mutants did not prevent ubiquitination, suggesting that there are multiple residues that can attach to ubiquitin. The large number of putative APC binding sites that span mDia2 is consistent with these results. We are currently testing the hypothesis that APC is responsible for promoting mDia2 ubiquitination and degradation during cell division. A stabilized mutant version of mDia2 will be important to fully characterize the functional consequence of mDia2 ubiquitination.

Taken together, our data provide new insights for mDia2 function during cell division. Given the importance of proper cell division for genomic stability, it will be important to study the role of mDia2 ubiquitination and degradation further, as well as examine other formins that might share similar properties.

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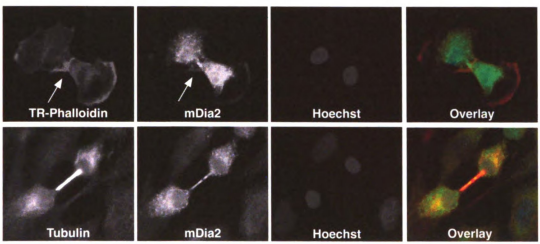
Figure 4.1. mDia2 localization and expression is cell cycle–dependent.
A. HeLa cells were stained with rabbit anti-mDia2 (FITC) and Phalloidin (Texas Red) or mDia2 (FITC) and tubulin (TRITC) along with DNA (Hoechst, blue). Overlay shows the merged image of all three channels. Arrows in top panels denote the actin-rich cleavage furrow containing mDia2.
B. HeLa cell lysates were collected at timepoints indicated after double thymidine G1/S arrest and release. "A" represents lysate from asynchronous population of cells. Immunoblots were probed with anti-mDia2 (1358) and anti-EF1α as a loading control.
C. Cells from **B** were labeled as described in "Experimental Procedures" to determine DNA content and analyzed on a flow cytometer. Plots show cell numbers relative to DNA content. The percentages of cells in G1, S, or M phase are shown for each timepoint.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mDia2
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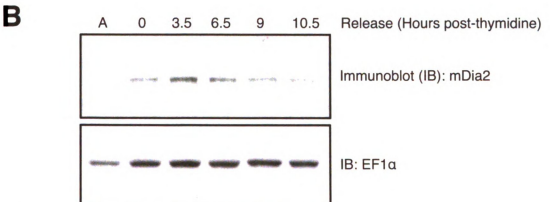
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3.5
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9
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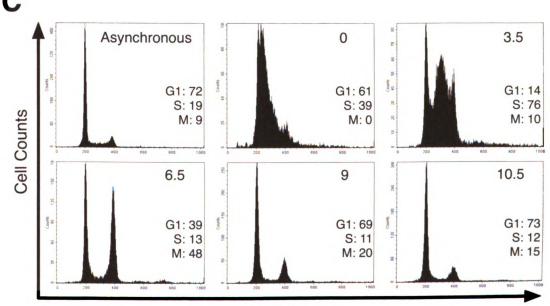
Release (Hours post-thymidine)
Immunoblot (IB): mDia2
IB: EF1α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Asynchronous
G1: 72
S: 19
M: 9
0
G1: 61
S: 39
M: 0
3.5
G1: 14
S: 76
M: 10
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G1: 39
S: 13
M: 48
9
G1: 69
S: 11
M: 20
10.5
G1: 73
S: 12
M: 15
Cell Counts
DNA Content

Figure 4.2. Proteasome inhibition prevents mDia2 degradation.
A. HeLa cells were arrested with a double thymidine block and released into growth media. MG132 proteasome inhibitor (20 μM) was added to a population of cells upon thymidine release and incubated for 10 h (lane 6). Lysates were collected at the timepoints indicated and immunoblotted for mDia2. EF1α was probed as a loading control. "A" represents cell lysate from an asynchronous population.
B. Cells from **A** were labeled as described in "Experimental Procedures" to determine DNA content and analyzed on a flow cytometer. Plots show cell numbers relative to DNA content. The percentages of cells in G1, S, or M phase are shown for each timepoint.
C. HeLa cells were thymidine arrested as in **A** (t = 0 h), but released into growth media containing 100 ng/mL nocodazole for 6 h (t = 0 to t = 6 h). Cells were then rinsed and released into normal growth media. MG132 (20 μM) was added to a population of cells 1 h after the nocodazole release and incubated for 5 h (lane 10). Immunoblotting of lysates were performed as in **A**.
D. Flow cytometry profiles showing DNA content of cells examined in **C**.

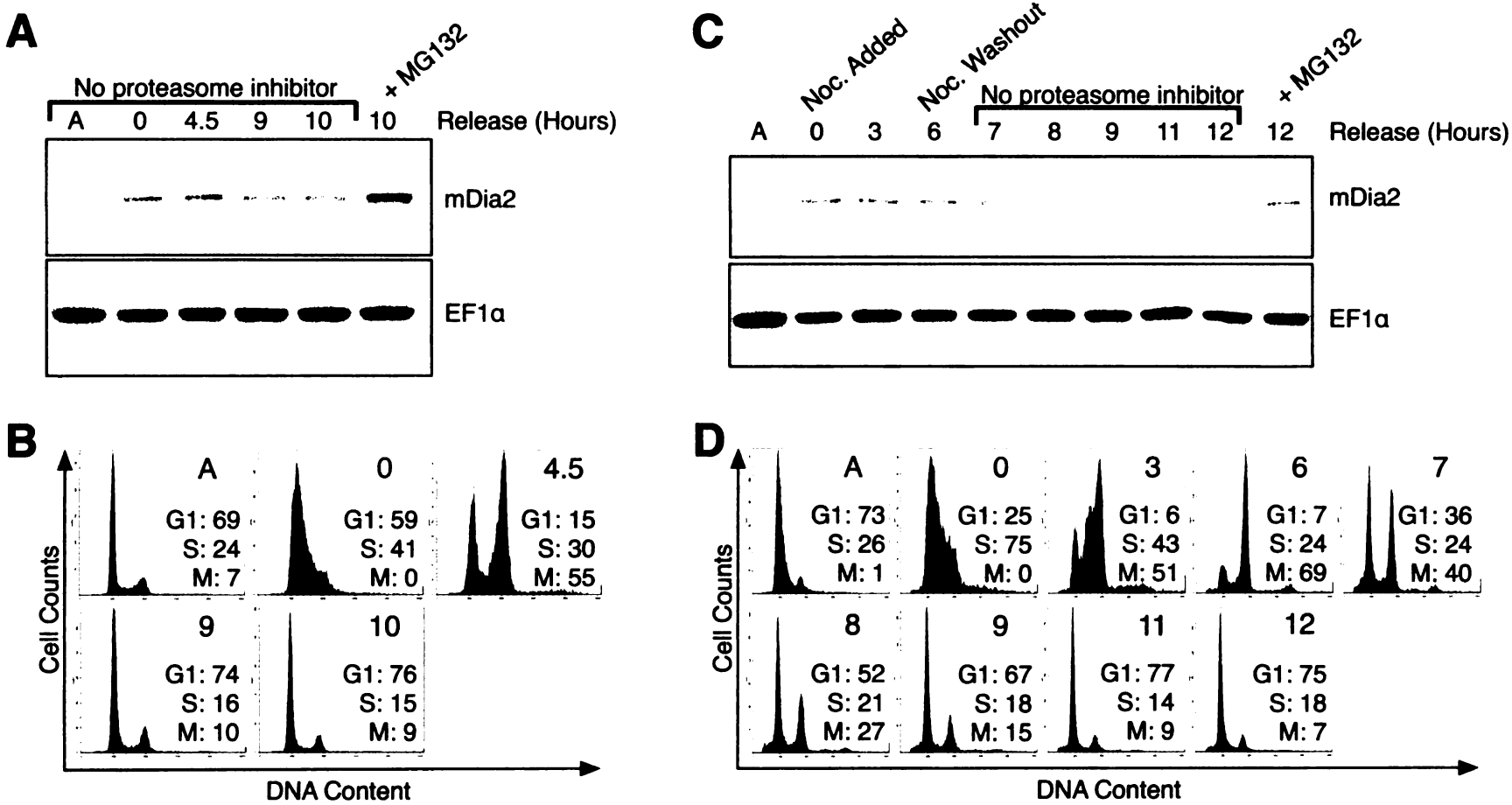

A
No proteasome inhibitor
+ MG132
A 0 4.5 9 10 10 Release (Hours)
mDia2
EF1α
B
A
G1: 69
S: 24
M: 7
0
G1: 59
S: 41
M: 0
4.5
G1: 15
S: 30
M: 55
9
G1: 74
S: 16
M: 10
10
G1: 76
S: 15
M: 9
Cell Counts
DNA Content
C
Noc. Added
Noc. Washout
No proteasome inhibitor
+ MG132
A 0 3 6 7 8 9 11 12 12 Release (Hours)
mDia2
EF1α
D
A
G1: 73
S: 26
M: 1
0
G1: 25
S: 75
M: 0
3
G1: 6
S: 43
M: 51
6
G1: 7
S: 24
M: 69
7
G1: 36
S: 24
M: 40
8
G1: 52
S: 21
M: 27
9
G1: 67
S: 18
M: 15
11
G1: 77
S: 14
M: 9
12
G1: 75
S: 18
M: 7
Cell Counts
DNA Content

Figure 4.3. mDia2 is polyubiquitinated.
A. HEK293T cells were transfected with Myc-mDia2, HA-ubiquitin, or co-transfected with both plasmids. Lysates were immunoprecipitated for either Myc or HA. Immunoblots of lysates and immunoprecipitations were probed with anti-Myc or anti-HA to examine the extent of ubiquitination.
B. HeLa cells were incubated with 10 μM MG132 for 18 h. Lysates were immunoprecipitated using anti-β-catenin or anti-mDia2. Immunoblots were probed with anti-ubiquitin, anti-β-catenin, or anti-mDia2 (1358) to examine endogenous ubiquitination.
C. HEK293T cells were co-transfected with Myc-mDia2 and HA-ubiquitin. Lysates were immunoprecipitated with anti-Myc. HeLa cells were incubated with 20 μM MG132 for 4 h. Lysates were immunoprecipitated with anti-β-catenin. Immunoprecipitations were immunoblotted with anti-Myc and an antibody specific to polyubiquitin chains.

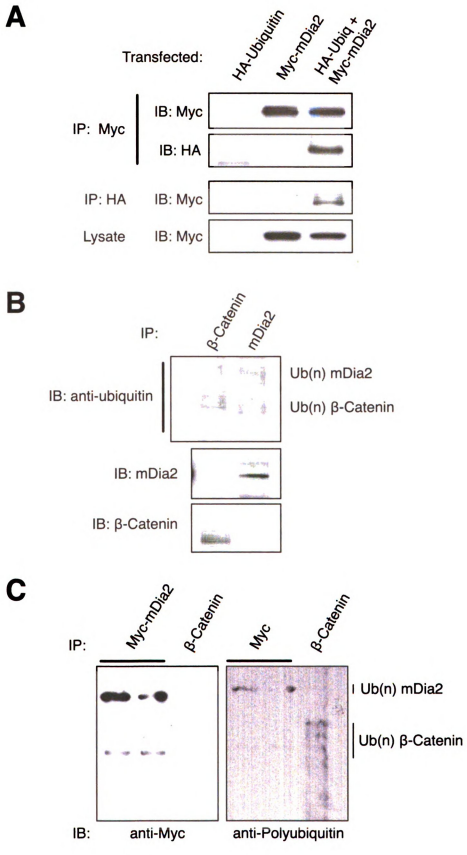

A
Transfected:
HA-Ubiquitin
Myc-mDia2
HA-Ubiq + Myc-mDia2
IP: Myc
IB: Myc
IB: HA
IP: HA
IB: Myc
Lysate
IB: Myc
B
IP:
β-Catenin
mDia2
IB: anti-ubiquitin
Ub(n) mDia2
Ub(n) β-Catenin
IB: mDia2
IB: β-Catenin
C
IP:
Myc-mDia2
β-Catenin
Myc
β-Catenin
Ub(n) mDia2
Ub(n) β-Catenin
IB:
anti-Myc
anti-Polyubiquitin

Figure 4.4. mDia2 ubiquitination increases at the end of mitosis.
A. HEK293T cells were co-transfected with Myc-mDia2 and HA-ubiquitin. Cells were arrested at G1/S phase with a double thymidine block. Cells were released into growth media and lysates were collected at timepoints indicated and subjected to Myc immunoprecipitation. Immunoblots were probed with Myc and HA to examine the extent of mDia2 ubiquitination. EF1α was blotted as a loading control and cyclin E was blotted to verify progression through the cell cycle.
B. Cells from **A** were labeled as described in "Experimental Procedures" to determine DNA content and analyzed on a flow cytometer. Plots show cell numbers relative to DNA content. The percentages of cells in G1, S, or M phase are shown for each timepoint.
C. HEK293T cells were co-transfected with Myc-mDia2 and HA-ubiquitin (lanes 2-7) in addition to Myc-GFP and HA-ubiquitin as a negative control (lane 1). Cells were incubated with 100 ng/mL nocodazole for 24 h to arrest cells in mitosis. Cells were rinsed and lysates were collected at indicated times after nocodazole washout. Immunoblots were probed with anti-Myc and anti-HA.
D. Flow cytometry profiles showing DNA content of cells from **C**.

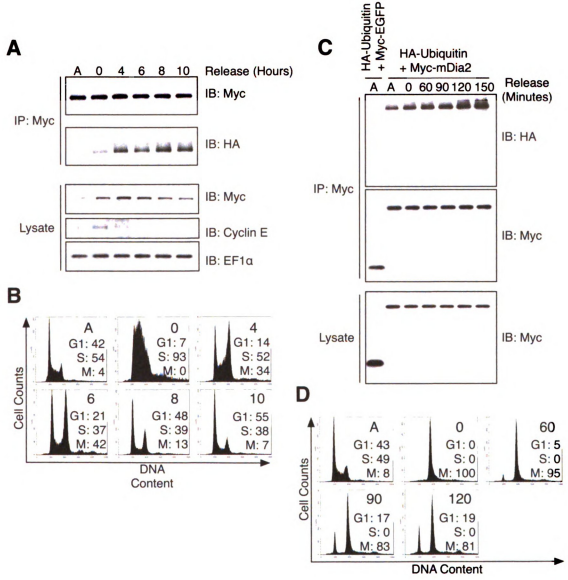

A
A 0 4 6 8 10
Release (Hours)
IP: Myc
IB: Myc
IB: HA
Lysate
IB: Myc
IB: Cyclin E
IB: EF1α
B
A
G1: 42
S: 54
M: 4
0
G1: 7
S: 93
M: 0
4
G1: 14
S: 52
M: 34
6
G1: 21
S: 37
M: 42
8
G1: 48
S: 39
M: 13
10
G1: 55
S: 38
M: 7
Cell Counts
DNA Content
C
HA-Ubiquitin + Myc-EGFP
HA-Ubiquitin + Myc-mDia2
A A 0 60 90 120 150
Release (Minutes)
IP: Myc
IB: HA
IB: Myc
Lysate
IB: Myc
D
A
G1: 43
S: 49
M: 8
0
G1: 0
S: 0
M: 100
60
G1: 5
S: 0
M: 95
90
G1: 17
S: 0
M: 83
120
G1: 19
S: 0
M: 81
Cell Counts
DNA Content

Figure 4.5. Ubiquitin is attached to multiple residues on mDia2.
A. ClustalW protein sequence alignment of mouse mDia1, human mDia1, mouse mDia2, and human mDia2 (Accession Numbers O08808, NP_005210, NP_062644, NP_001035982, respectively). Bold lysines represent ubiquitinated residues identified in (29).
B. Schematic profile of mDia2 mutants examined for ubiquitination.
C. Lysine-to-arginine mutant versions of Myc-mDia2 were co-transfected with HA-ubiquitin in HEK293T cells. Lysates were Myc-immunoprecipitated and immunoblots were probed with anti-Myc and anti-HA.
D. mDia2 mutants were co-transfected with HA-ubiquitin in HEK293T cells. Lysates were immunoprecipitated and immunoblots probed with anti-GFP, anti-Myc, or anti-HA to examine the extent of ubiquitination.

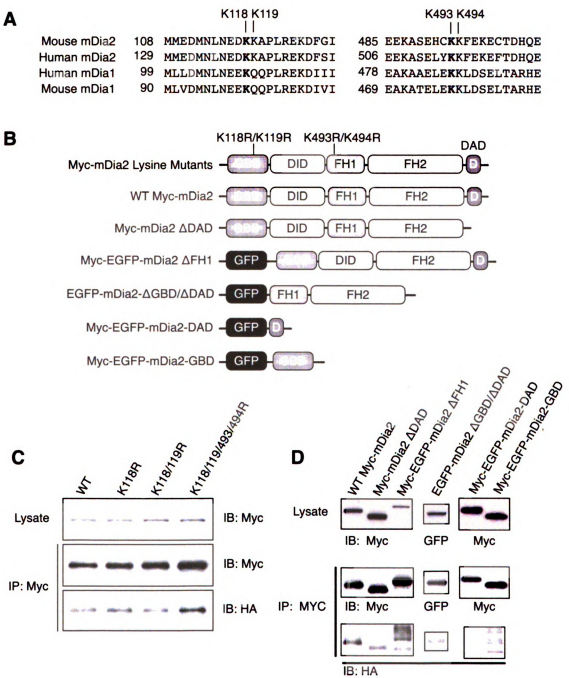
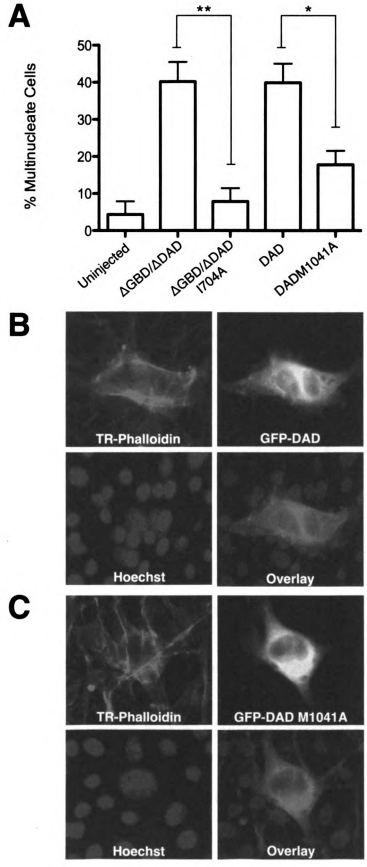

A
K118 K119
K493 K494
Mouse mDia2 108 MMEDMNLNEDKKAPLREKDFGI 485 EEKASEHCKKFEKECTDHQE
Human mDia2 129 MMEDMNLNEDKKAPLREKDFSI 506 EEKASELYKKFEKEFTDHQE
Human mDia1 99 MLLDMNLNEEKQQPLREKDIII 478 EAKAAELEKKLDSELTARHE
Mouse mDia1 90 MLVDMNLNEEKQQPLREKDIVI 469 EAKATELEKKLDSELTARHE
B
K118R/K119R
K493R/K494R
DAD
Myc-mDia2 Lysine Mutants
DID
FH1
FH2
WT Myc-mDia2
Myc-mDia2 ΔDAD
Myc-EGFP-mDia2 ΔFH1
GFP
EGFP-mDia2-ΔGBD/ΔDAD
Myc-EGFP-mDia2-DAD
Myc-EGFP-mDia2-GBD
C
WT
K118R
K118/119R
K118/119/493/494R
Lysate
IB: Myc
IP: Myc
IB: Myc
IB: HA
D
WT Myc-mDia2
Myc-mDia2 ΔDAD
Myc-EGFP-mDia2 ΔFH1
EGFP-mDia2 ΔGBD/ΔDAD
Myc-EGFP-mDia2-DAD
Myc-EGFP-mDia2-GBD
Lysate
IB: Myc
GFP
Myc
IP: MYC
IB: Myc
GFP
Myc
IB: HA

Figure 4.6. Deregulated mDia2 results in cytokinesis failure.
A. Bar graph showing the percent of multinucleate cells after injection with plasmids that encode EGFP-mDia2-ΔGBD/ΔDAD (AA 521-1040), EGFP-mDia2-ΔGBD/ΔDAD-I704A, EGFP-mDia2-DAD, or EGFP-mDia2-DAD-M1041A. Error bars represent the standard deviation from three independent experiments (** indicates $p < 0.01$, * indicates $p < 0.05$ using *t*-test for significance, $n = 30$).
B. HeLa cells were microinjected with plasmid DNA encoding EGFP-mDia2-DAD, which interferes with mDia autoregulation. Cells were stained for F-actin (Texas Red) and nuclei (Hoechst, blue).
C. HeLa cells were microinjected with a mutant version of the plasmid described in **B** that is unable to bind to DID and interfere with autoregulation (DAD-M1041A). Cells were stained as in **B**.


A
% Multinucleate Cells
50
40
30
20
10
0
**
*
Uninjected
ΔGBD/ΔDAD
ΔGBD/ΔDAD I704A
DAD
DADM1041A
B
TR-Phalloidin
GFP-DAD
Hoechst
Overlay
C
TR-Phalloidin
GFP-DAD M1041A
Hoechst
Overlay

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Chapter 4.2

The Anaphase-Promoting Complex Interacts with mDia2

Introduction

Progression through mitosis requires the timed proteolytic degradation of cell-cycle proteins. Targeting of substrate proteins for degradation during cell division is often mediated by the anaphase-promoting complex/cyclosome (APC/C) [1]. Mitotic cyclins are some of the best characterized targets of APC/C, and other well-characterized targets include the Polo and Aurora kinases.

The transfer of ubiquitin to a substrate protein begins with the covalent attachment of ubiquitin to an E1 ubiquitin-activating enzyme. The E1 enzyme interacts with an E2 ubiquitin-conjugating enzyme to allow transfer of the bound ubiquitin. Finally, an E3 ligase transfers ubiquitin to substrate proteins by interacting with the E2 enzyme. E3 ligases in the HECT domain-containing family of proteins form a covalent interaction with ubiquitin before its transfer to the substrate protein. On the other hand, ligases in the RING finger family mediate transfer of ubiquitin from the E2 directly to the substrate protein (reviewed in [2]).

APC/C is a member of the RING family of E3 ligases. It is a large protein complex whose ligase activity and substrate specificity is mediated by two different proteins, Cdc20 and Cdh1 [3]. Either Cdc20 or Cdh1 can bind APC/C to promote ligase activity, which is often dictated by the cell cycle phase. Cdc20 protein levels vary during cell cycle progression and its expression is greatest

from S phase through mitosis, then drops as cells enter G_1. On the other hand, Cdh1 protein levels are highest during mitosis, but are lowered during the G_1 and S phases. Both Cdc20 and Cdh1 recognize and bind to D box motifs (RXXL), while Cdh1 has the additional capacity to bind KEN motifs of substrate proteins [3, 4]. Because mDia2 contains one KEN motif and over ten D box motifs, we hypothesized that Cdc20 or Cdh1 interacts with mDia2 to mediate its ubiquitination during cell division.

Experimental Procedures

Cells, Antibodies, and Reagents – HeLa and HEK293T cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum. The following antibodies were used in this study; anti-Cdh1 (clone DH01), anti-Cdc20 (clone BA8), and anti-Apc5 were from Abcam, anti-EF1α was from Upstate Biotechnologies, rabbit anti-mDia2 (1358) was raised against the mDia2 FH2 domain (generated as recombinant protein in *Escherichia coli*) as described previously [5, 6]. Cell lysis and immunoprecipitation were performed as previously described [6]. Immunoblots were performed using 4-20% Tris-glycine gels (Invitrogen) transferred to a 0.45-μm polyvinylidene difluoride membrane (Invitrogen). Myc-mDia2 KEN and D box mutants were constructed using site-directed mutagenesis (Stratagene) following the manufacturer's protocol.

Cell Cycle Arrest and Release – G_1/S phase cell cycle arrest was performed by incubating HeLa cells in growth medium containing 2 mM thymidine (Sigma) for 16 h. Cells were briefly washed in 1X phosphate-buffered

saline and incubated in growth medium for 10 h followed by another thymidine incubation for 16 h. Cells were released into growth medium after washing with phosphate-buffered saline. Cells were collected for flow cytometry analysis or lysed at specific time points for immunoblotting.

Flow cytometry – HeLa cells were briefly washed in 1X phosphate-buffered saline. Cells were stained with a propidium iodide (Sigma) solution containing Nonidet P-40 and RNase. DNA content was acquired using a FACSCalibur flow cytometer (BD Biosciences). The percentages of cells in G_0/G_1, S, or G_2/M phase were determined using ModFit LT software.

Results

Cdh1 interacts with mDia2.

We previously reported that mDia2 is degraded at the end of cell division [7]. Because of the timing of mDia2 degradation and the presence of multiple D box motifs in mDia2 that may be recognized by Cdh1, we reasoned that there may be an association between mDia2 and Cdh1 to promote mDia2 ubiquitination. To test this hypothesis, Hela cells were synchronized at the G_1/S phase transition using a double thymidine arrest. Cells were released in growth media to allow progression through the remainder of the cell cycle. Samples were taken at time points during the arrest and release for subsequent analysis. Cells were lysed and endogenous mDia2 was immunoprecipitated and immunoblotted to reveal an interaction between mDia2 and endogenous Cdh1 (Figure 4.7). Intriguingly, there was an interaction observed at each time point, including the asynchronously

growing population of cells, suggesting that the interaction occurs during multiple cell cycle phases.

Cdc20 and APC/C interact with mDia2.

The APC/C activator Cdc20 is also known to recognize D box motifs similar to Cdh1. We then asked whether mDia2 also interacts with Cdc20 in cells. To test this, we arrested Hela cells with a double thymidine block and released the cells into growth media. Samples were collected at time points during the arrest and release for subsequent analysis. Cells were lysed and endogenous mDia2 was immunoprecipitated and immunoblotted to reveal an interaction between endogenous mDia2 and Cdc20 (Figure 4.8). We also probed the immunoblots for Apc5, a protein subunit of the APC/C complex. Consistent with our hypothesis, we found an interaction between mDia2 and the APC/C (Figure 4.8).

Mutation of KEN box does not prevent mDia2 ubiquitination.

mDia2 contains one KEN box and over 10 D box motifs (13 D box motifs in murine mDia2, 11 in human mDia2). Because Cdc20 and Cdh1 can target substrates containing D box motifs, and Cdh1 has the additional capacity to recognize the KEN motif, we asked whether mutation of the KEN motif or several D box motifs in mDia2 might diminish or prevent its ubiquitination. To test this, we mutated the KEN motif to encode AAA, which is known to prevent Cdh1 from recognizing the motif. HEK293T cells were co-transfected with wild-type Myc-mDia2 or the mutated Myc-mDia2 along with HA epitope tagged ubiquitin. Cells

were lysed and mDia2 was immunoprecipitated against the Myc epitope. Immunoblotting revealed that mutation of the mDia2 KEN motif did not diminish its ubiquitination compared to wild-type mDia2 (Figure 4.9). The mutation of two additional D box motifs showed a similar result (data not shown), suggesting that multiple D box and KEN motifs are sufficient to interact with Cdh1 or Cdc20 and promote mDia2 ubiquitination.

Discussion

The APC/C plays a central role during cell division to target substrate proteins for degradation in the proteasome [3]. Because mDia2 is extensively ubiquitinated and degraded during cell division [7], we hypothesized that the APC/C may be the E3 ligase mediating mDia2 ubiquitination. We discovered that both of the APC/C activators, Cdc20 and Cdh1, interact with mDia2 at endogenous levels. This is consistent with the Cdc20 and Cdh1 binding motifs located in mDia2. However, mutation of the KEN motif and two D box motifs in mDia2 did not prevent its ubiquitination. Multiple motifs would likely need to be mutated to prevent association with Cdc20 or Cdh1. Intriguingly, the interaction occurs during multiple phases of the cell cycle, which suggests that mDia2 degradation may have important functions beyond its role during cell division.

Our results support the hypothesis that APC/C is the E3 ligase that mediates mDia2 ubiquitination. Future experiments should test the ability of the APC/C to ubiquitinate mDia2 *in vitro*. In addition, it would be interesting to test whether mDia2 directly interacts with Cdc20 or Cdh1 using purified proteins or

fluorescence resonance energy transfer. A more extensive analysis of mDia2 D box mutants may reveal the specific region(s) of interaction with Cdc20 or Cdh1.

Inhibition of mDia2 ubiquitination will be important to assess the fundamental roles of its ubiquitination for cell function. Our data suggests that mDia2 ubiquitination may be a mechanism to inactivate formins. However there might be additional roles yet to be discovered. Does ubiquitination localize formins to distinct sites within the cell? Are formins ubiquitinated during vesicle trafficking or during filopodia formation? What are the cellular consequences if formins are not ubiquitinated? Further elucidation of the mechanisms controlling mDia2 ubiquitination will help address these questions.

The data presented here provide a foundation that will guide future studies related to the mechanisms of mDia2 ubiquitination. Unpublished work suggests that budding yeast formins are also ubiquitinated and degraded during cell division (David Pellman, personal communication). Whether the APC/C is the E3 ligase mediating degradation in this system is unknown. But the conservation of formin ubiquitination across species points to the importance of this event. More research into the control of formin function and regulation during cell division will provide valuable insight for basic mechanisms of cell biology.

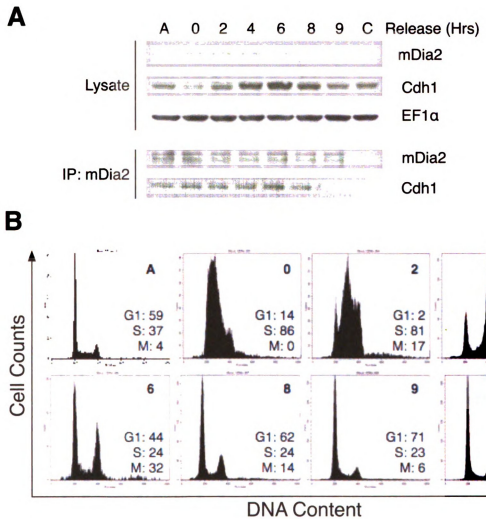

Figure 4.7. Cdh1 interacts with mDia2. A. Hela cells were arrested with a double thymidine block and released into growth media. Lysates were collected at the time points indicated and immunoprecipitated using mDia2 (1358) and immunoblotted for mDia2 and Cdh1. EF1α was probed as a lysate loading control. *A* represents cell lysate from an asynchronous population. *C* represents negative control immunoprecipitation. **B.** Cells from **A** were labeled as described under "Experimental Procedures" to determine DNA content and analyzed on a flow cytometer. Plots show cell numbers relative to DNA content. The percentages of cells in G_1, S, or M phase are shown for each time point.

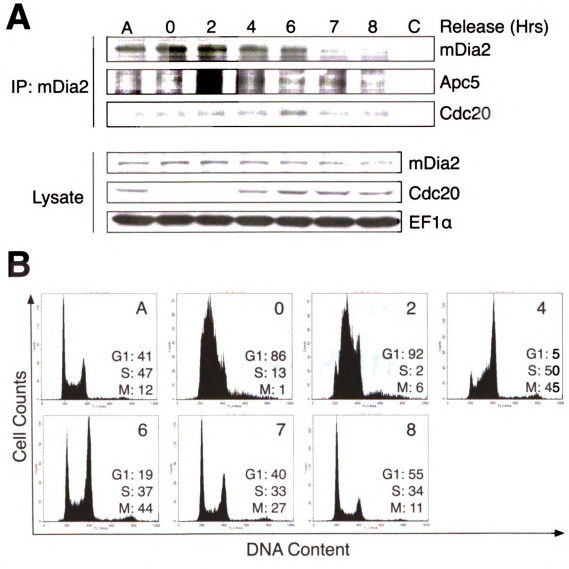

Figure 4.8. Cdc20 and APC/C interact with mDia2. A. Hela cells were arrested with a double thymidine block and released into growth media. Lysates were collected at the time points indicated and immunoprecipitated using mDia2 (1358) and immunoblotted for mDia2, Cdc20, and Apc5. EF1α was probed as a lysate loading control. *A* represents cell lysate from an asynchronous population. *C* represents negative control immunoprecipitation. **B.** Cells from **A** were labeled as described under "Experimental Procedures" to determine DNA content and analyzed on a flow cytometer. Plots show cell numbers relative to DNA content. The percentages of cells in G_1, S, or M phase are shown for each time point.

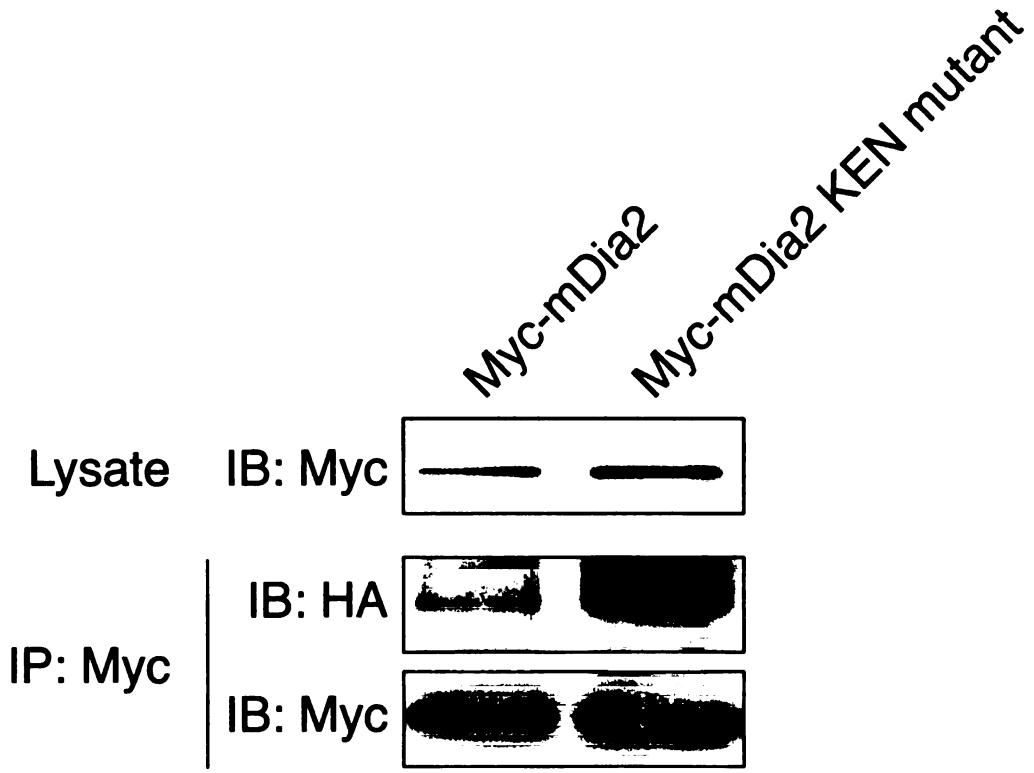

Figure 4.9. mDia2 KEN mutation does not prevent ubiquitination. HEK293T cells were transfected with Myc-mDia2 and HA-ubiquitin or the Myc-mDia2 KEN mutant (KEN motif mutated to AAA) and HA-ubiquitin. Lysates were immunoprecipitated for Myc. Immunoblots of lysates and immunoprecipitations were probed with anti-Myc or anti-HA to examine the extent of ubiquitination.

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Chapter 5

Summary and Conclusions

Formins mediate cytoskeletal remodeling events that are critical to maintain cell homeostasis. We have shown that the Diaphanous-related formin mDia2 is important for vesicle trafficking downstream of the small GTPase RhoB [1]. Versions of RhoB or mDia2 that promote constitutive actin assembly dramatically reduce the movement of vesicles bearing internalized EGF. Similar results have also been shown for mDia1 and mDia3 [2, 3]. These studies suggest that the dynamic assembly and disassembly of actin filaments is required for vesicle trafficking, and formin activity must be tightly regulated to effectively control this process.

Recently, the formin mDia1 was shown to possess tumor suppressor activity in hematopoietic stem or progenitor cells [4]. RhoB, which directly interacts and activates mDia proteins, has also been suggested to possess tumor suppressor activity [5]. Studies from our lab indicate that genetic deletion of RhoB enhances the myelodysplastic phenotype observed in mDia1 knockout mice [6]. The mechanism by which mDia1 controls hematopoietic cell proliferation is unclear, but one intriguing hypothesis is focused on SRF-mediated signaling to well-known tumor suppressors such as p53 or PTEN [7].

There are several ways in which *RhoB* deletion may enhance the myelodysplastic phenotype observed in *Drf1* null mice. First, it is important to

consider the multiple alterations that lead to MDS and eventually to AML [8]. Initial insults may occur to a normal stem cell due to exposure to chemicals, radiation, or age (Figure 5.1). In early stage MDS, these alterations lead to cells with impaired differentiation and a paradoxical increase in apoptosis. Additional mutations lead to late stage MDS or AML which is defined by the presence of cells with decreased apoptosis, impaired differentiation, and increased proliferation.

The MDS phenotype seen in *Drf1* null mice may reflect what is observed in early stage MDS, although apoptotic frequency has not been determined in these mice. Additional alterations then occur, possibly due to a lack of mDia1 protein expression, and could lead to late stage MDS or even AML. The loss of RhoB expression, however, may support a faster progression to late stage MDS because RhoB is required for apoptosis in transformed cells (Figure 5.2). An important distinction here is that loss of RhoB expression does not appear to affect apoptosis in normal cells [5]. This is consistent with the lack of phenotype in *RhoB* null mice. Since RhoB is required for apoptosis in transformed cells, the initial transforming event in hematopoietic cells may be induced by the lack of mDia1 expression. If these transformed cells then encounter further DNA damage, a lack of RhoB expression would prevent these cells from undergoing apoptosis, allowing them to expand and become mutated further.

Complicating the pathway to transformation is the fact that mDia function appears to be required for RhoB mediated apoptosis [9]. How then would loss of RhoB and mDia1 expression, in which both proteins interact in the same

cytoskeletal signaling pathway, lead to an enhanced phenotype from that observed in mDia1 null mice? If these proteins functioned exclusively in the same pathway as was proposed in Figure 3.5B, then an enhanced phenotype would not make sense in this instance. It has been shown that RhoB is required for DNA damage or FTI induced apoptosis in transformed cells [5]. However, RhoB interacts with additional formins other than mDia1 (Figure 5.3). These formins can also promote SRF activity, which could induce apoptosis downstream of p53 or through another mechanism. A dominant interfering mDia mutant was shown to block RhoB mediated apoptosis [9], but this particular mutant likely interferes with all of the mDia formins, thus blocking their downstream signaling. The lack of mDia1 expression alone does not prevent RhoB from interacting with mDia2 or mDia3, so lack of mDia1 alone would not prevent apoptosis. Therefore, loss of mDia1 expression leads to alterations in normal HSCs independent of RhoB, as evidenced by *Drf1* null mice.

Future studies should examine how mDia1 or RhoB may affect stem or progenitor cell function in greater detail. In particular, it would be interesting to test the contribution of formins for asymmetric cell division, which is an important feature of stem cell proliferation and maintenance. Rationale for these studies stem from the recent observation that RhoB may contribute to stem cell self-renewal [10]. Also, the link between formins and cell division has been characterized in several species [11]. Given the relationship between stem cells and cancer [12], these studies could provide significant insights into the mechanism of disease pathogenesis.

Based on the role of formins in vesicle trafficking and tumor suppression, it is apparent that formin activity must be tightly regulated for normal cell function. The majority of studies on formin mediated actin assembly has focused on the autoregulatory mechanism controlling their activation. However, little is known about the mechanisms that inactivate formins. We discovered that mDia2 is ubiquitinated and degraded upon completion of cell division [13]. We propose that this method of protein turnover for mDia2 may explain a general mechanism of formin inactivation. Expression of activated mDia2 in Hela cells leads to cytokinesis failure. Again, this points to the importance of controlling formin activity, since deregulated mDia protein expression disrupts cytoskeletal dynamics necessary for vesicle trafficking and cell division.

We also have evidence that mDia2 ubiquitination may be mediated by the E3 ligase APC/C. mDia2 interacts with the APC/C activators Cdc20 and Cdh1. While this interaction occurs during cell division, it may also be a general mechanism to promote mDia2 ubiquitination and degradation in non-dividing cells. Future studies should determine whether the interactions with mDia2 are direct, and if APC/C does, in fact, serve as the E3 ligase for mDia2 ubiquitination.

In conclusion, we provide compelling evidence that formin mediated actin assembly is critical for vesicle trafficking, cell division, and tumor suppression. To date, there have been relatively few studies that directly link formin function or expression with the initiation of disease. New mouse models should be developed to better define the *in vivo* role of formins. In addition, future studies should focus on the understudied formins that are not in the Diaphanous-related

family. There are still many unanswered questions regarding formin function, and further characterization will certainly expand our knowledge on the importance of this protein family for cell biology and disease.

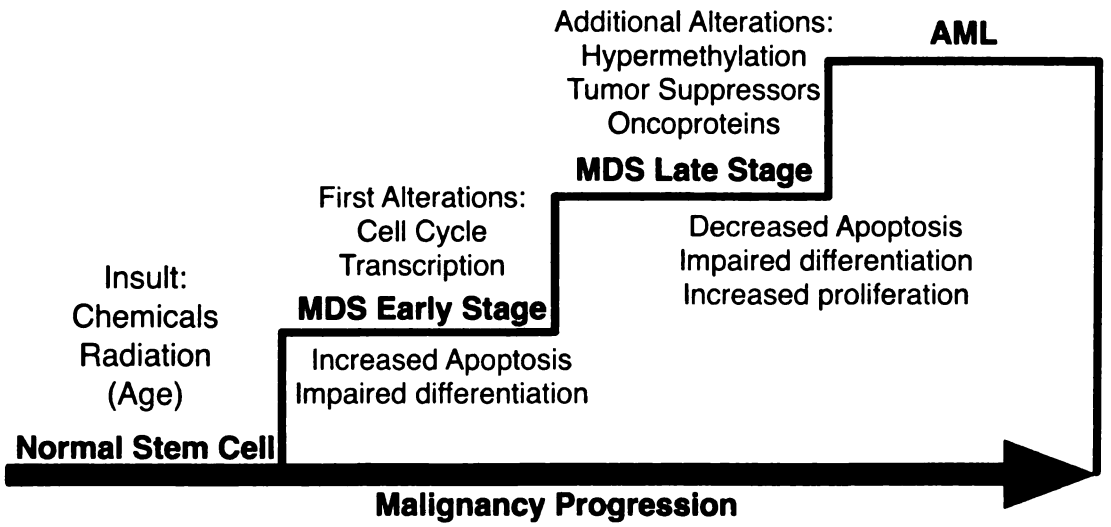

Figure 5.1. Multiple alterations are required for MDS progression. The initial insults that lead to MDS may be the result of chemicals, radiation, or age acting on a normal hematopoietic stem cell. Early stage MDS is defined by increased apoptosis and impaired differentiation. Additional mutations occur that lead to late stage MDS and AML. These cells show decreased apoptosis, impaired differentiation, and increased proliferation. This figure was adapted from [8].

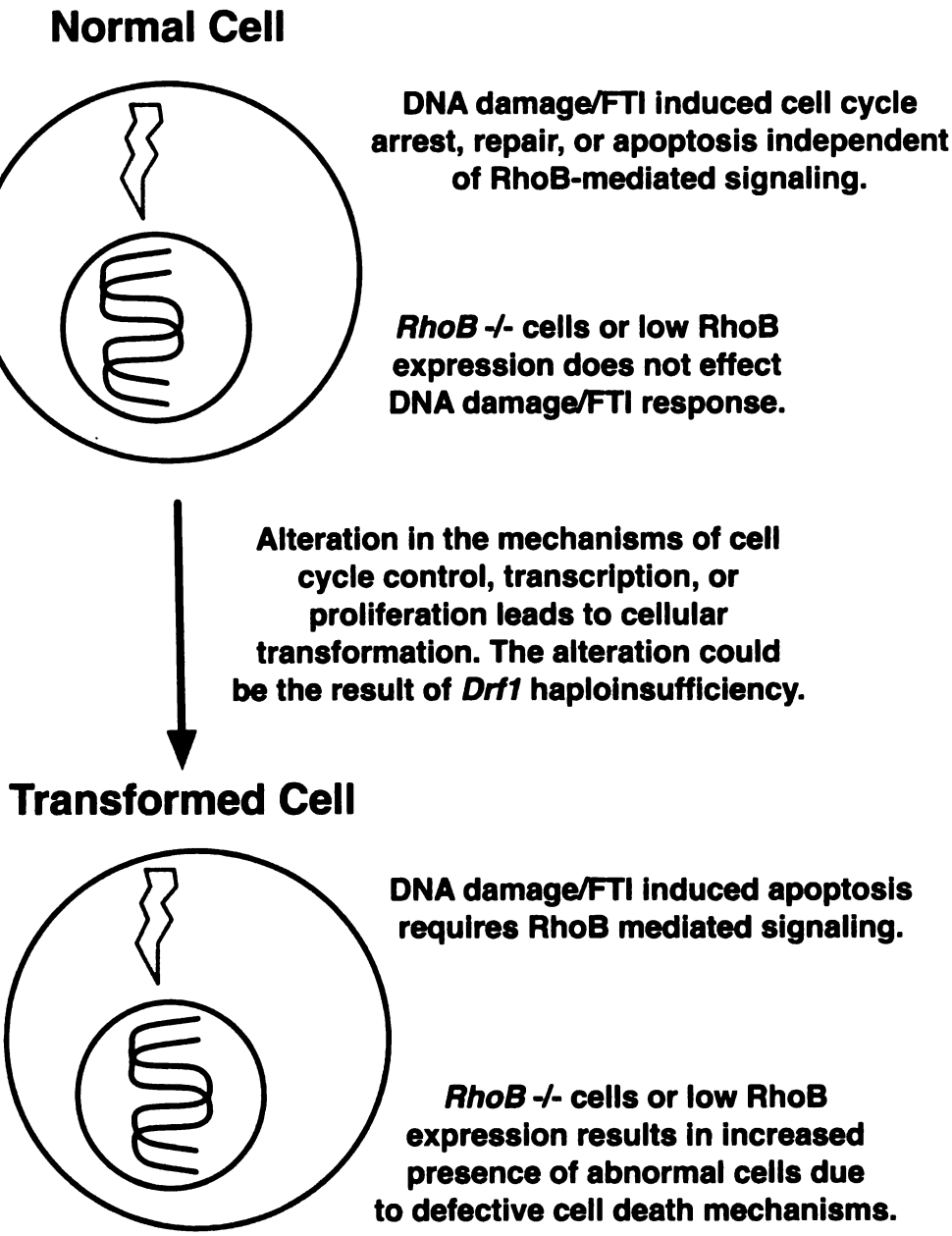

Figure 5.2. RhoB is required for DNA damage or FTI induced apoptosis in transformed cells. It was shown that RhoB is required for DNA damage or FTI induced apoptosis in transformed cells, but not normal cells [5]. Transformed hematopoietic stem or progenitor cells in *Drf1* null mice may fail to induce apoptosis after loss of RhoB expression. This could explain the enhanced myelodysplastic phenotype in mDia1 and RhoB double knockout mice.

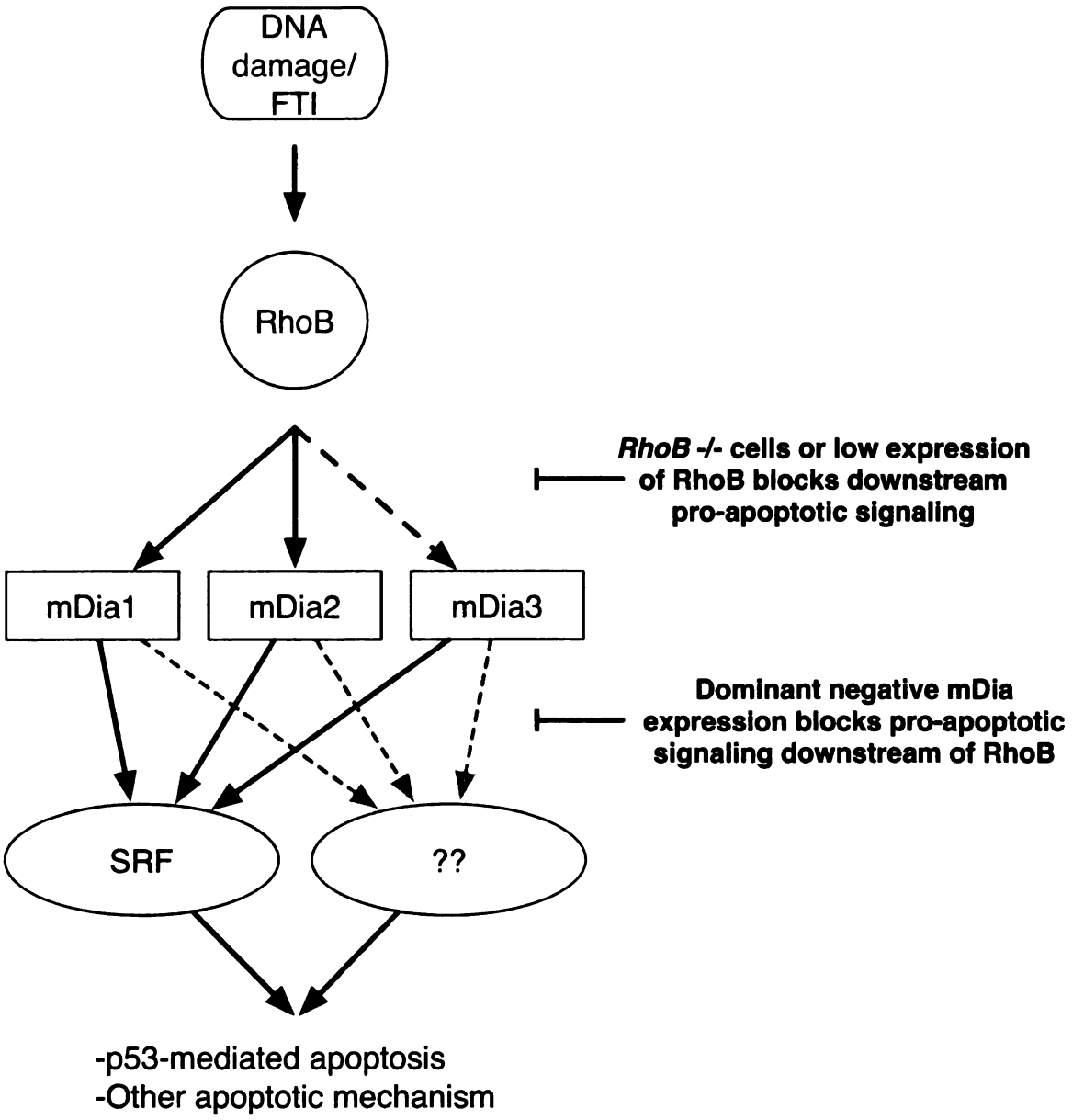

Figure 5.3. RhoB interacts with multiple mDia formins to mediate downstream signaling. RhoB is required for DNA damage or FTI induced apoptosis [5]. RhoB interacts with mDia1 and mDia2, and likely interacts with mDia3. These formins can activate SRF activity and may promote apoptotic mechanisms as proposed in Figure 3.5B.

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