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Article

A Molecular Pathway for Myosin II Recruitment to Stress Fibers

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Summary

Background: Cell migration and morphogenesis are driven by both protrusive and contractile actin filament structures. The assembly mechanisms of lamellipodial and filopodial actin filament arrays, which provide the force for plasma membrane protrusions through actin filament treadmilling, are relatively well understood. In contrast, the mechanisms by which contractile actomyosin arrays such as stress fibers are generated in cells, and how myosin II is specifically recruited to these structures, are not known.
Results: We demonstrate that four functionally distinct tropomyosins are required for assembly of stress fibers in cultured osteosarcoma cells. Tm1, Tm2/3, and Tm5NM1/2 stabilize actin filaments at distinct stress fiber regions. In contrast, Tm4 promotes stress fiber assembly by recruiting myosin II to stress fiber precursors. Elimination of any one of the tropomyosins fatally compromises stress fiber formation. Importantly, Dia2 formin is critical to stress fiber assembly by nucleating Tm4-decorated actin filaments at the cell cortex. Myosin II is specifically recruited through a Tm4-dependent mechanism to the Dia2-nucleated filaments, which subsequently assemble endwise with Arp2/3-nucleated actin filament structures to yield contractile stress fibers.
Conclusions: These experiments identified a pathway, involving Dia2- and Arp2/3-promoted actin filament nucleation and several functionally distinct tropomyosins, that is required for generation of contractile actomyosin arrays in cells.

Introduction

Cell migration is driven by lamellipodial and filopodial actin filament arrays, which provide the force for plasma membrane protrusions through actin filament treadmilling, and by stress fibers that are contractile actomyosin bundles involved in cell adhesion, morphogenesis, and tail retraction [1–4]. Stress fibers also contribute to the integrity of endothelial barriers by providing the endothelial cell's support against tension [5, 6]. Stress fibers display a periodic α-actinin-myosin II pattern and alternating actin filament polarity, resembling muscle sarcomer structure [7]. However, actin filament organization in stress fibers is less regular compared to myofibrils of mature muscles, and stress fibers appear to contract constantly with occasional relaxation periods instead of continuous contraction/relaxation cycles [8].

Stress fibers can be divided into three subcategories based on their orientation in cells and interactions with focal adhesions [9]. Transverse arcs are curved actomyosin structures that flow from the leading edge toward the cell center. Arcs are not directly attached to the substrate via focal adhesions but transmit their contractile force through dorsal stress fibers. Recent studies provided evidence that arcs assemble through endwise association of myosin II bundles and relatively small α-actinin crosslinked F-actin bundles derived from the Arp2/3-nucleated lamellipodial actin filament network [10, 11]. Dorsal stress fibers, and related graded polarity filaments, attach to focal adhesions at their distal end and elongate toward the cell center through actin polymerization at focal adhesions [10, 12]. Dia1 formin plays an important role in stress fiber assembly, and, at least in U2OS cells, it promotes elongation of dorsal stress fibers at focal adhesions [10, 13]. The third class, ventral stress fibers, anchors to focal adhesions at each end. In cultured osteosarcoma cells, ventral stress fibers are derived from preexisting transverse arcs, which are connected to focal adhesion anchored dorsal stress fibers located at the opposite sides of the cell [10]. At least in fibroblasts, ventral stress fibers can also assemble through fusion of filopodia-derived dorsal stress fibers [14]. In addition, certain cell types also contain a fourth class of stress fiber structure called the perinuclear actin cap, which regulates nuclear shape [15]. Despite the wealth of information concerning the assembly of stress fibers, the mechanism by which myosin II is specifically incorporated into these structures is poorly understood.

Tropomyosins (Tms) are actin-binding proteins affecting actin dynamics and myosin II function in cells. They prevent actin filament depolymerization at pointed ends and can inhibit ADF/cofilin-promoted actin filament disassembly in vitro and in cells [16, 17]. Tms also regulate muscle contraction by controlling myosin binding to actin filaments [18]. Mammals have over 40 Tm isoforms, which can be further classified into high molecular weight (HMW) and low molecular weight (LMW) Tms. These isoforms are generated through alternative splicing of four *tropomyosin* genes. In nonmuscle cells, Tms have been linked to many contractile actin-based structures, and they participate in cell motility, adhesion, cytokinesis, and apoptosis [19]. Several Tm isoforms were shown to associate with stress fibers in various cell types. Overexpression of certain Tms induces more pronounced stress fibers in many cell types, whereas simultaneous inhibition of all Tms by small interfering RNA (siRNA) or by antibody injection resulted in a disappearance of stress fibers [20–24]. However, the exact role or roles of Tms in stress fiber assembly are poorly understood, and the possible functional differences between various Tm isoforms in stress fibers have not been reported to date.

Here we found that different tropomyosin isoforms have distinct roles in stabilization of actin filaments at specific stress fiber regions and in myosin recruitment. We also revealed that cooperation of Dia1 formin-induced actin assembly at focal adhesions, together with Arp2/3 and Dia2

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formin-promoted assembly at lamellipodia, is required for generation of stress fibers. Formin-nucleated filaments are subsequently decorated by tropomyosins to regulate their stability and to recruit myosin into these structures.

Results

Localization and Dynamics of Nonmuscle Tropomyosins in Stress Fibers

Simultaneous elimination of Tm isoforms results in a disappearance of stress fibers [22–24]. However, the exact mechanism by which Tms contribute to stress fiber assembly and stability has not been reported. Furthermore, whether individual Tm isoforms play redundant or distinct roles in stress fibers was not known. To explore the exact function of Tm isoforms in stress fibers, we chose a human osteosarcoma cell line (U2OS) as a model. These cells display a dynamic stress fiber network, and the three distinct stress fiber categories can be readily identified [10]. Western blot analysis of U2OS lysates using specific antibodies (see Experimental Procedures) revealed six Tm isoforms: HMW tropomyosins Tm1, Tm2, and Tm3 and LMW tropomyosins Tm4, Tm5NM1, and Tm5NM2 (Figure 1A).

To examine the subcellular localizations of the above-mentioned Tm isoforms, we expressed GFP or YFP fusions of each isoform in U2OS cells. Expression of fluorescently tagged tropomyosins itself did not markedly affect stress fiber morphology (see Figure S1A available online), although with higher expression levels more prominent ventral stress fibers were sometimes detected. The localization of endogenous proteins in U2OS cells was also verified by specific antibodies against Tm4 and Tm5 (Figure S1B).

Each isoform (Tm1, Tm2, Tm3, Tm4, Tm5NM1, and Tm5NM2) associated with transverse arcs and ventral stress fibers (Figure 1B and Figure S1A). In these structures, they displayed a periodic distribution by colocalizing with myosin II and by displaying a complementary localization pattern to α-actinin (Figure S1D). Fluorescence recovery after photobleaching analysis on ventral stress fibers revealed that Tm2, Tm3, Tm4, and Tm5NM2 display dynamic association with stress fibers. However, compared to other isoforms, the recovery of Tm4 at stress fibers was more rapid, suggesting that Tm4 may be functionally different from the other isoforms expressed in U2OS cells (Figure 1C).

Interestingly, Tms displayed isoform-specific localization patterns along dorsal stress fibers. Tm2 colocalized with F-actin along the entire dorsal stress fibers, whereas Tm1, Tm5NM1, and Tm5NM2 concentrated at the distal ends of dorsal stress fibers (Figure 1B and Figures S1A and S1C) corresponding to focal adhesions marked by vinculin (Figure 2). Tm3 and Tm4 localized proximally to focal adhesions, either as short segments or as a dotted pattern (Figure 1B, Figure 2, and Figures S1A and S1C). Live-cell imaging focusing on Tm2, Tm4, and Tm5NM2 (which represent the three different localization categories of Tms in dorsal stress fibers) revealed that Tm5NM2 is a relatively early marker for the focal adhesions. Tm2, which typically localized along the entire dorsal stress fiber, was also present in mature focal adhesions together with actin but associated with the newly forming adhesion sites after Tm5NM2 (Figure S2B; Movie S1).

Tm4 typically localized to short segments beneath the adhesion site in dorsal stress fibers and was found to incorporate into dorsal stress fibers both spatially and temporally immediately after late focal adhesion marker zyxin [25] (Figures S2A and S2C). Comparison of α-actinin and Tm4 localizations in elongating dorsal stress fibers revealed that the two proteins display a nonoverlapping localization pattern, with α-actinin being absent from the sites occupied by Tm4. Because a previous study demonstrated that myosin II bundles occasionally incorporate into dorsal stress fibers and simultaneously displace α-actinin from the site of association [10], we compared the subcellular localizations of endogenous myosin II and Tm4 to each other and found that the two proteins colocalize along dorsal stress fibers (Figure S3).

Collectively, these results demonstrate that Tm isoforms are sequentially recruited to focal adhesions (Tm1 and Tm5NM1/2) and to elongating dorsal stress fibers (Tm2). Importantly, Tm4 localizes to dorsal stress fibers later, and its localization to these structures coincides with myosin II incorporation to dorsal stress fibers.

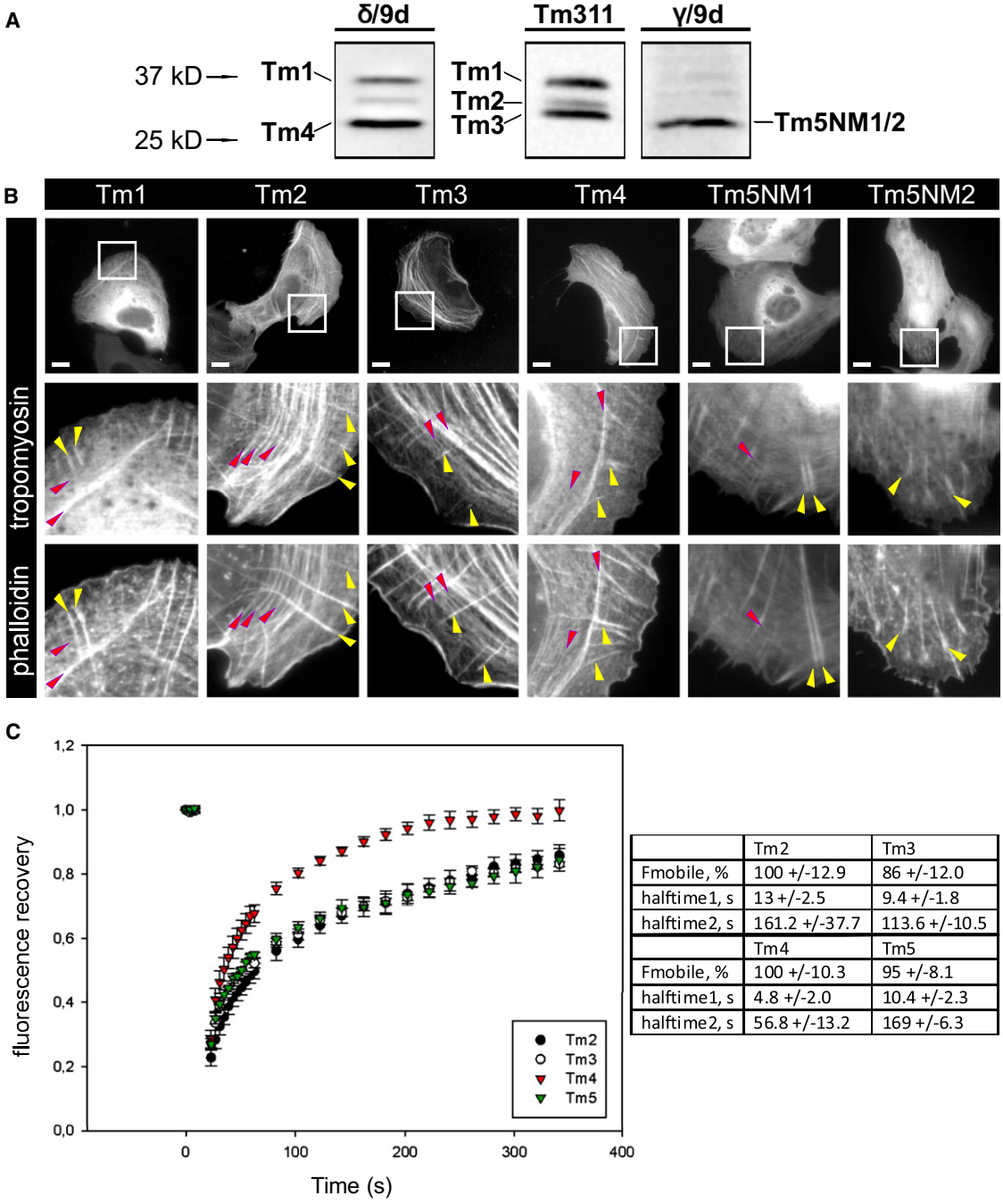
Distinct Tropomyosin Isoforms Regulate the Stability of Stress Fibers

RNA interference (RNAi) was applied to elucidate the cellular functions of Tm isoforms and to reveal whether different isoforms have redundant or distinct functions. With appropriate siRNA oligonucleotides, we succeeded in efficiently depleting Tm1, Tm2/3 (because of very similar exon compositions, these two isoforms were depleted simultaneously), Tm4, and Tm5NM1/2 (depleted simultaneously) from U2OS cells (Figure 3A). Strikingly, incubation of cells for 3 days with siRNAs against Tm1, Tm2/3, or Tm5NM1/2 led to changes in cell morphology and resulted in a dramatic decrease in the number of stress fibers, whereas the knockdown cells still contained similar amounts of cortical F-actin compared to control cells (Figure 3B and Figure S4). These data suggest that these isoforms have nonredundant roles in stress fiber assembly. Cells with milder knockdown phenotypes, observed 1–2 days after transfection, displayed thinner stress fibers compared to cells transfected with the control oligonucleotides, and sometimes the remaining stress fibers formed abnormal network-like structures (Figure S5).

Tm4 Is Required for Myosin II Incorporation to Stress Fibers

Whereas depletion of Tm1, Tm2/3, and Tm5NM1/2 resulted in a loss of stress fibers in U2OS cells, the phenotype of the Tm4 knockdown cells was different. Even after 3 days of incubation with RNAi oligonucleotides (and after efficient loss of Tm4), the majority of cells still contained visible stress fibers, which displayed comparable F-actin intensity to control cells (Figure S4). However, the stress fibers were abnormal, displaying a curly appearance (Figures 4A and 4B). This phenotype was often accompanied by an increase in the amount of filopodia and long dorsal stress fibers.

The phenotype of Tm4 knockdown cells, together with the faithful colocalization of myosin II with Tm4 in stress fibers, suggests that Tm4 may regulate myosin II association with stress fibers. Therefore, the amount of myosin II incorporated into the stress fibers of Tm4 knockdown cells was examined. Immunostaining of wild-type and Tm4 knockdown cells with myosin II antibody revealed severely diminished amounts of myosin II in stress fibers of Tm4-depleted cells, and this was accompanied by an increase in the amount of diffuse myosin II staining (Figures 4B and 4C). Rescue experiments revealed that cells expressing a Tm4 construct that is refractory to the RNAi oligonucleotide display normal stress fiber architecture and myosin incorporation to stress fibers, demonstrating



	Tm2	Tm3
Fmobile, %	100 +/-12.9	86 +/-12.0
halftime1, s	13 +/-2.5	9.4 +/-1.8
halftime2, s	161.2 +/-37.7	113.6 +/-10.5
	Tm4	Tm5
Fmobile, %	100 +/-10.3	95 +/-8.1
halftime1, s	4.8 +/-2.0	10.4 +/-2.3
halftime2, s	56.8 +/-13.2	169 +/-6.3

Figure 1. Expression, Localization, and Dynamics of Tropomyosin Isoforms in U2OS Cells

(A) Expression pattern of tropomyosins (Tms) in U2OS cells was examined from lysates with specific Tm antibodies. Tropomyosin high molecular weight (HMW) isoforms 1, 2, and 3, as well as the low molecular weight (LMW) isoforms 4 and 5NM1/2, were detected from this particular cell type. The following antibodies were used for detection: γ/9d (detecting Tm5NM1 and Tm5NM2), δ/9d (specific for Tm4, with some cross-reactivity to Tm1), and TM311 (specific for HMW Tms, Tm1–3, and 6).

(B) Distinct localization patterns of Tm isoforms expressed in U2OS cells. All fluorescently tagged Tm isoforms localized to contractile stress fibers (ventral stress fibers and arcs), whereas their localization in dorsal stress fibers varied in an isoform-dependent manner. Magnifications of the areas containing dorsal stress fibers are shown. Yellow arrowheads indicate Tm localization in dorsal stress fibers compared to F-actin (as visualized by phalloidin staining). Red arrowheads indicate arcs. Scale bar represents 10 μm.

(C) Dynamics of tropomyosin isoforms in ventral stress fibers. The dynamics of different GFP- or YFP-tagged Tm isoforms in ventral stress fibers of U2OS cells were examined by fluorescence recovery after photobleaching (FRAP). Average recovery curves of the raw data (mean of 10–20 separate FRAP experiments) ± standard error of the mean (SEM) are shown on the left. Data of the average curves were fitted with double exponential (see Experimental Procedures), and mobile fraction and $t_{1/2}$ values (± standard deviation) for each isoform were calculated from the fitted data of the individual recovery curves.

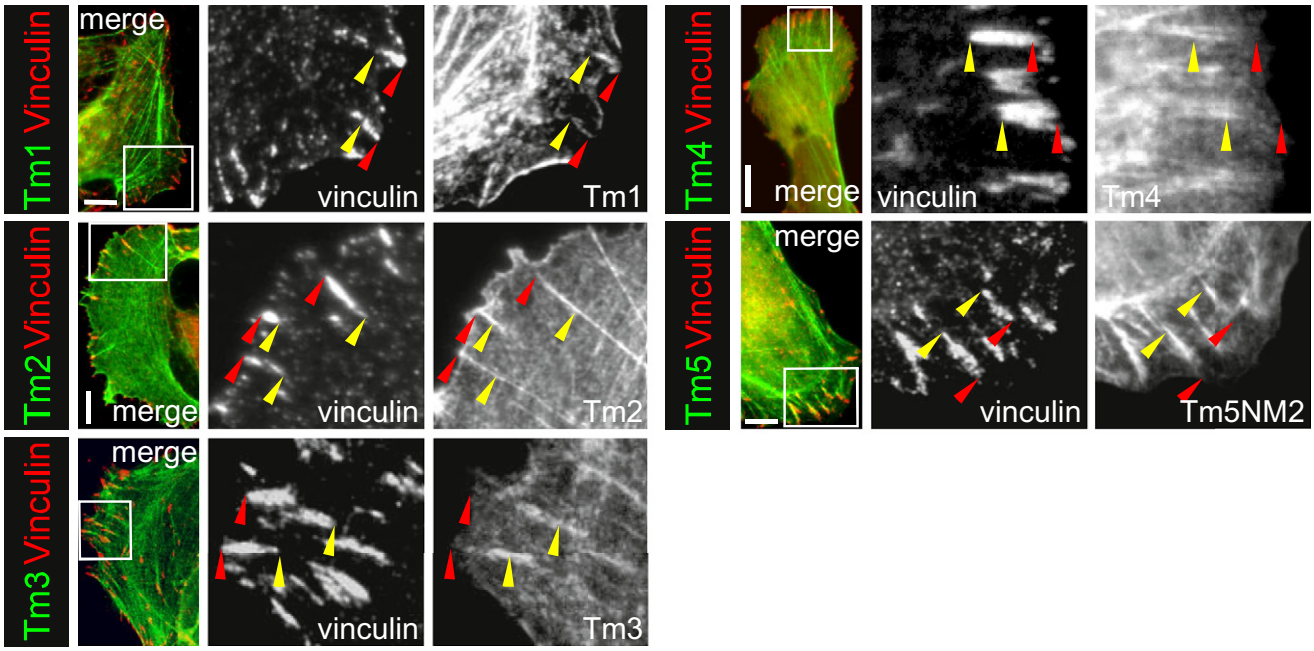


Figure 2. Distinct Localizations of Tm Isoforms in Focal Adhesion Attached Dorsal Stress Fibers

Comparison of localization patterns of Tm isoforms with vinculin. Yellow and red arrowheads indicate the proximal and distal tips of the focal adhesions, respectively. Vinculin is in green and Tms are in red. Scale bar represents 10 μm.

that the phenotypes observed in Tm4-RNAi cells result from specific depletion of this tropomyosin isoform (Figure 4D).

To further assess the function of Tm4 in myosin-dependent stress fiber contractility, we examined the ability of wild-type, Tm4, and Tm5NM1/2 knockdown cells to contract in a 3D collagen matrix. As a control for noncontracting cells, blebbistatin-treated cells were examined in a similar assay (Figure S6). As expected, Tm5NM1/2 knockdown cells, which do not contain visible stress fibers (Figures S6B and S6C), displayed severely diminished contraction in the collagen matrix. Importantly, Tm4 knockdown cells, which still contained visible stress fibers, also displayed severe contractility defects as compared to wild-type cells (Figures S6B and S6C). Taken together, these data suggest that Tm4 regulates the incorporation of myosin II into stress fibers. Because Tm1, Tm2, Tm3, and Tm5NM1/2 appear to regulate the stability of actin filaments in focal adhesions and stress fibers, and because their depletion leads to a loss of stress fiber network in U2OS cells, their possible contribution to myosin II recruitment could not be examined in this system.

Myosin II Is Recruited into Tropomyosin-Decorated Actin Filament Structures during Transverse Arc Formation

A previous study revealed that transverse arcs are generated through endwise assembly of myosin II bundles and α-actinin crosslinked actin bundles behind the lamellipodia of motile cells [10]. However, the origin of myosin II bundles, as well as the exact contribution of lamellipodial actin filament arrays in this process, is unknown. To study the incorporation of Tms into transverse arcs, we transfected U2OS cells with fluorescently tagged LMW Tms together with either α-actinin or myosin light chain. In fully formed transverse arcs, Tms displayed a periodic localization pattern that overlapped with myosin II and was complementary to the one of α-actinin (Figure S1D). Interestingly, live-cell imaging revealed diffuse localization of Tm4 and α-actinin at the edges of the cell. Relatively small Tm4 and α-actinin patches subsequently condensed from the diffuse Tm4 and α-actinin meshworks close to the cell edge and further assembled endwise to form a transverse arc (Figure 5A). Additionally, Tm5NM2 behaved in a similar way when expressed in U2OS cells together with α-actinin (Movie S2).

We next compared myosin II and Tm4 localization during transverse arc formation. Discrete Tm4 spots were detected at first, and myosin II concentrated to these Tm4-containing structures later upon emergence of transverse arcs (Figures 5B and 5C; Movie S3). Importantly, myosin intensity increased gradually on Tm spots, suggesting that the assembly of myosin II bundles may occur on these tropomyosin-decorated actin filaments (Figure 5C). Collectively, these data suggest that Tm4 recruits myosin II to specific actin filament structures during transverse arc formation and, in this way, confers contractility to the stress fiber.

Transverse Arcs Are Generated from Dia2- and Arp2/3-Nucleated Actin Filament Structures at the Leading Edge of the Cell

A previous study suggested that transverse arcs are generated through endwise assembly of Arp2/3-nucleated (α-actinin-crosslinked) actin filaments and myosin II bundles [10]. However, our observations demonstrating the presence of lamellipodial Tm structures that recruit myosin II and are subsequently crosslinked with α-actinin-containing actin structures to generate arcs suggested that arcs may arise from two distinct F-actin populations located at the lamellipodia. A recent study provided evidence that the lamellipodial actin filament network indeed consists of Arp2/3-nucleated branched filament structures and linear mDia2/DRF3 formin-nucleated actin filaments [26]. Furthermore, at least in yeasts, the tropomyosin-decorated actin filaments are typically

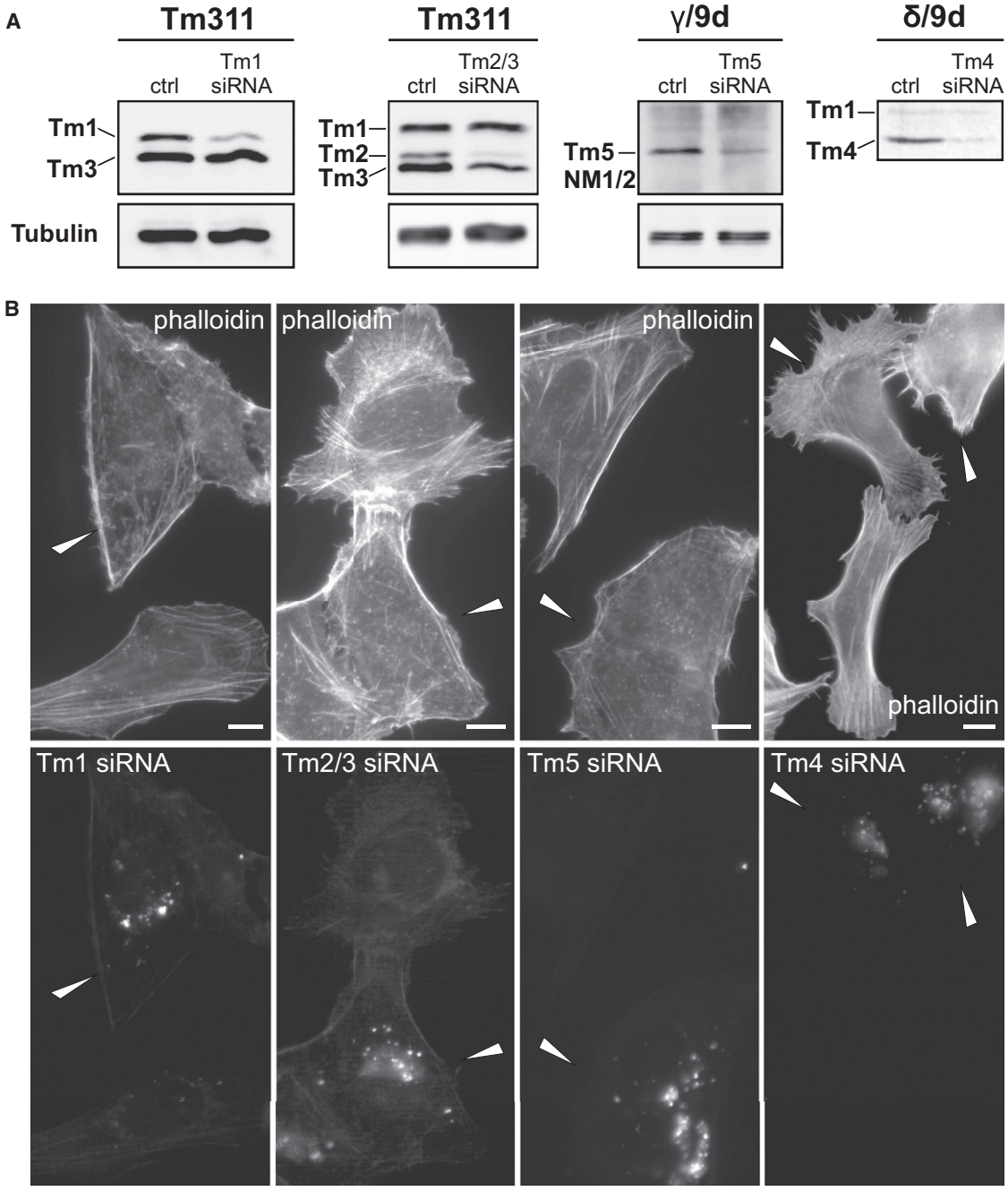


Figure 3. Depletion of Tropomyosins Disrupts the Stress Fiber Network

(A) Verification of the efficiency of different tropomyosin siRNAs. Cells were incubated with specific Tm siRNAs, and the specificity of depletion was determined by western blotting with isoform-specific antibodies.

(B) Phenotypes of tropomyosin knockdown cells. Cells treated for 3 days with specific Tm siRNAs against Tm1, Tm2/3, Tm4, and Tm5NM1/2 were fixed and stained with phalloidin. Arrowheads indicate the Tm knockdown cells containing 5′-Alexa Fluor 488-labeled siRNAs. Scale bar represents 10 μm.

nucleated by the formin family proteins [27, 28], and tropomyosin enhances formin-mediated actin assembly in vitro [29, 30]. Thus, we tested the possible role of mDia2/DRF3 formin in the formation of transverse arcs.

Rif GTPase is a regulator of lamellipodial and filopodial actin filament structures, and its main downstream effector is mDia2/DRF3 [26, 31–33]. Expression of dominant inactive Rif (Rif-TN) resulted in dramatic effects on transverse arc assembly. Depending on the severity of the phenotype (expression level of Rif-TN), α-actinin-containing arcs were either moderately affected or completely lost (Figure 6A). Abnormal transverse arc formation in Rif-TN-expressing cells was also detected by live-cell microscopy (Movie S4). In contrast to the negative effects on transverse arc formation, many Rif-TN-expressing cells displayed an increase in the density of focal adhesion-associated dorsal stress fibers. Importantly, in cells expressing Rif-TN, myosin II dots in arcs became less prominent or completely disappeared, and

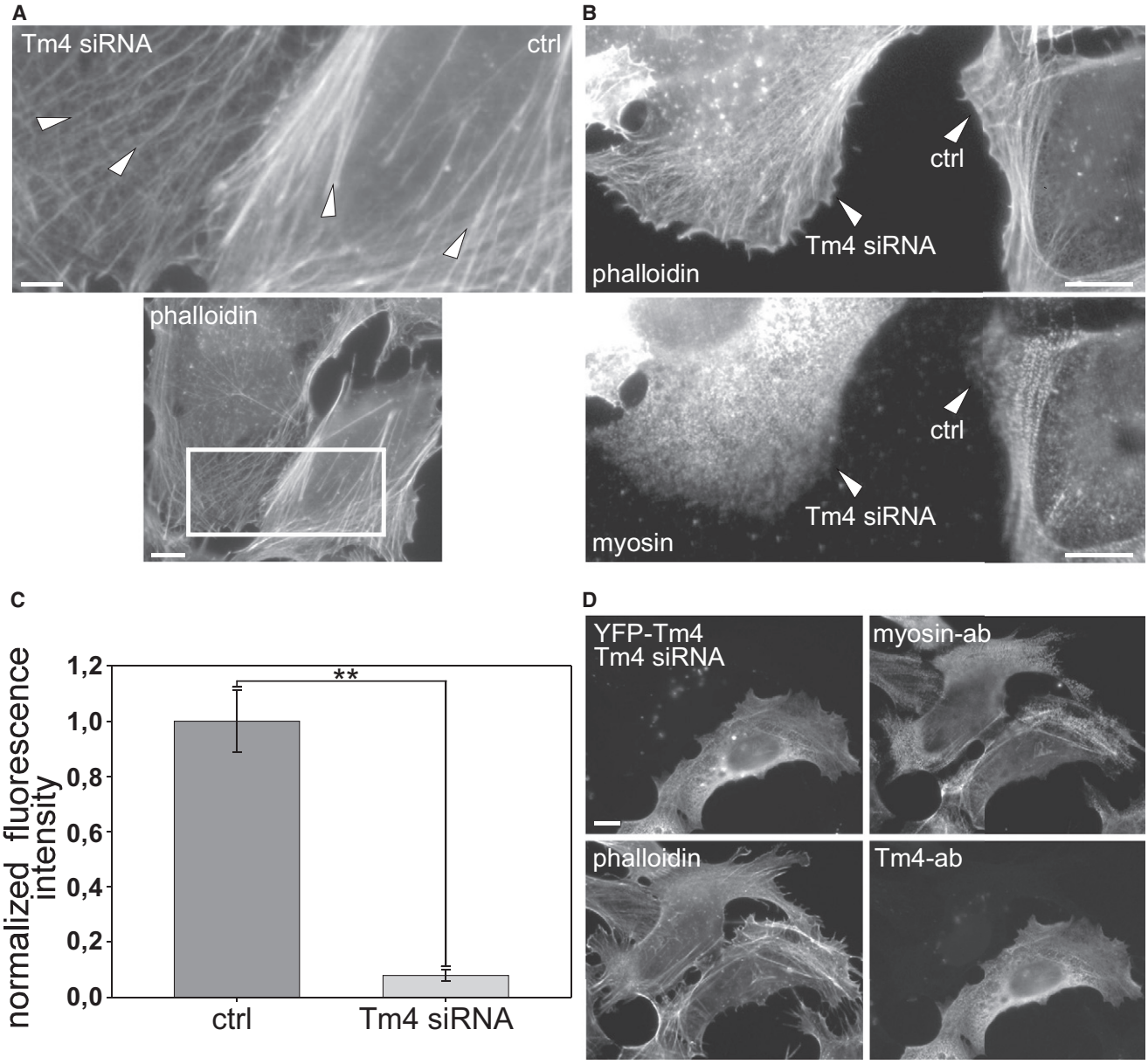


Figure 4. Tm4 Is Required for Myosin II Recruitment to Stress Fibers

(A) U2OS cells were incubated for 3 days with Tm4 siRNA, fixed, and stained with phalloidin. The stress fibers in Tm4 knockdown cells typically displayed a "curly" appearance compared to control (ctrl) cells with straight stress fibers. Scale bar represents 10 μm.

(B) Staining of Tm4 knockdown cells with myosin II antibody and phalloidin revealed that myosin II displays predominantly diffuse cytoplasmic localization in the knockdown cells compared to neighboring control cells. Scale bar represents 10 μm.

(C) Myosin incorporation into stress fibers was quantified from control and Tm4 knockdown cells stained with myosin II antibody and phalloidin. Myosin intensity in stress fibers was measured (TINA software) and normalized against corresponding phalloidin intensity of the fiber. Data are represented as mean ± SEM, n = 22. **$p < 0.001$, as determined by a Mann-Whitney test.

(D) Expression of YFP-Tm4 refractory to RNAi rescues Tm4 knockdown phenotype, demonstrating that the phenotypes observed in Tm4 RNAi cells result from specific depletion of this tropomyosin isoform. Alexa 488-conjugated siRNAs and YFP-Tm4 are shown on the green channel, whereas Alexa 647-conjugated phalloidin, and antibody stainings of myosin II and Tm4 with Alexa 555- and Alexa 405-conjugated secondary antibodies, respectively, are displayed on other channels.

myosin II displayed mostly diffuse cytoplasmic localization (Figure 6A). Furthermore, Tm4 failed to accumulate to the remaining stress fibers in Rif-TN-expressing cells and instead displayed mostly diffuse cytoplasmic localization (Figures S7B and S7C). Dominant active Rif (Rif-QL) induced the formation of filopodia in U2OS cells without dramatic effects on stress fiber density (Figure S7D).

Because Rif also has other downstream targets in addition to Dia2 [34], we directly examined the role of Dia2 in arc formation by RNAi. Similarly to Rif-TN, depletion of mDia2/DRF3 by RNAi from U2OS cells affected arc formation. α-actinin staining in arcs remained occasionally still visible, but myosin II became mostly diffuse and only occasionally localized as weaker spots along transverse arcs (Figures 6B and

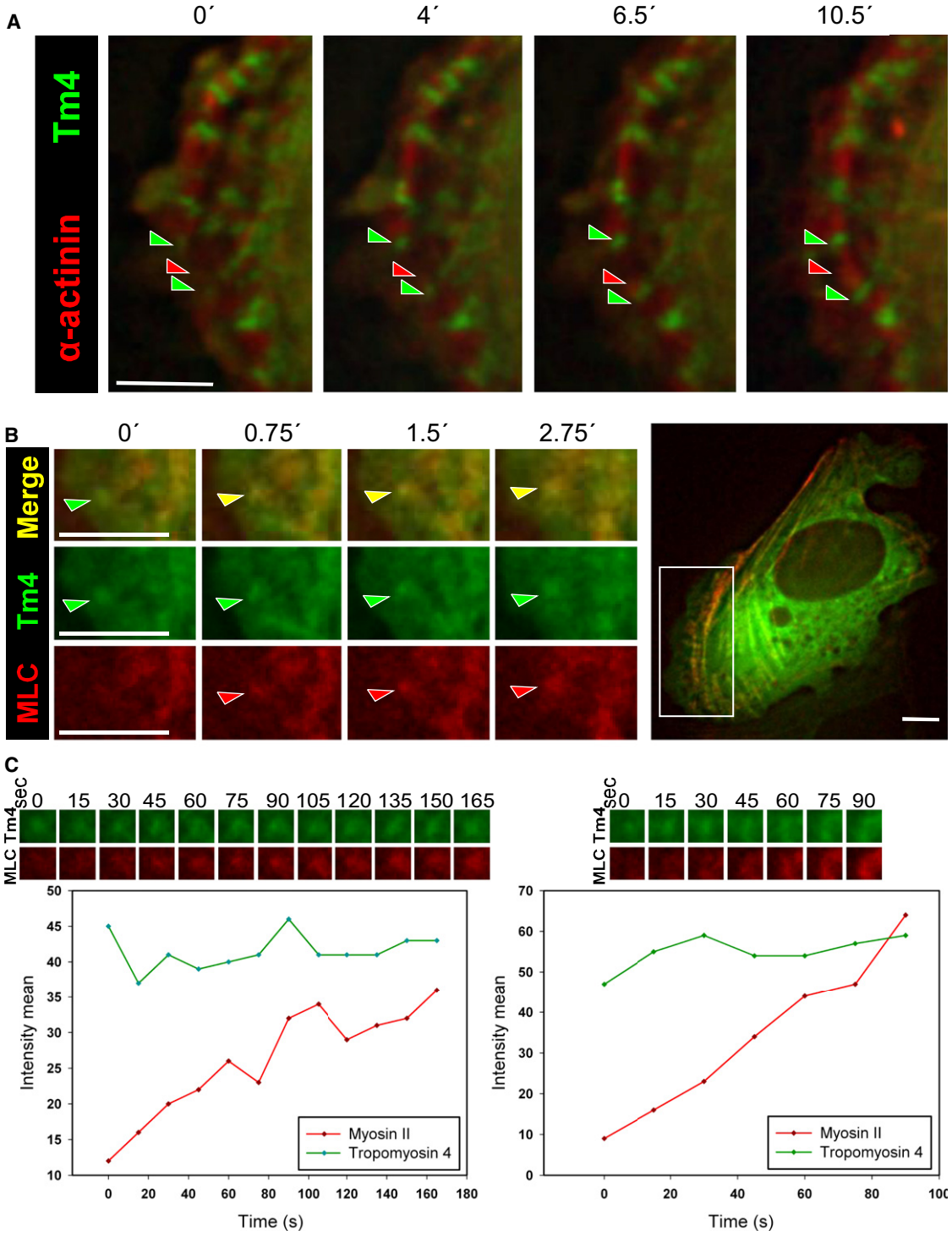
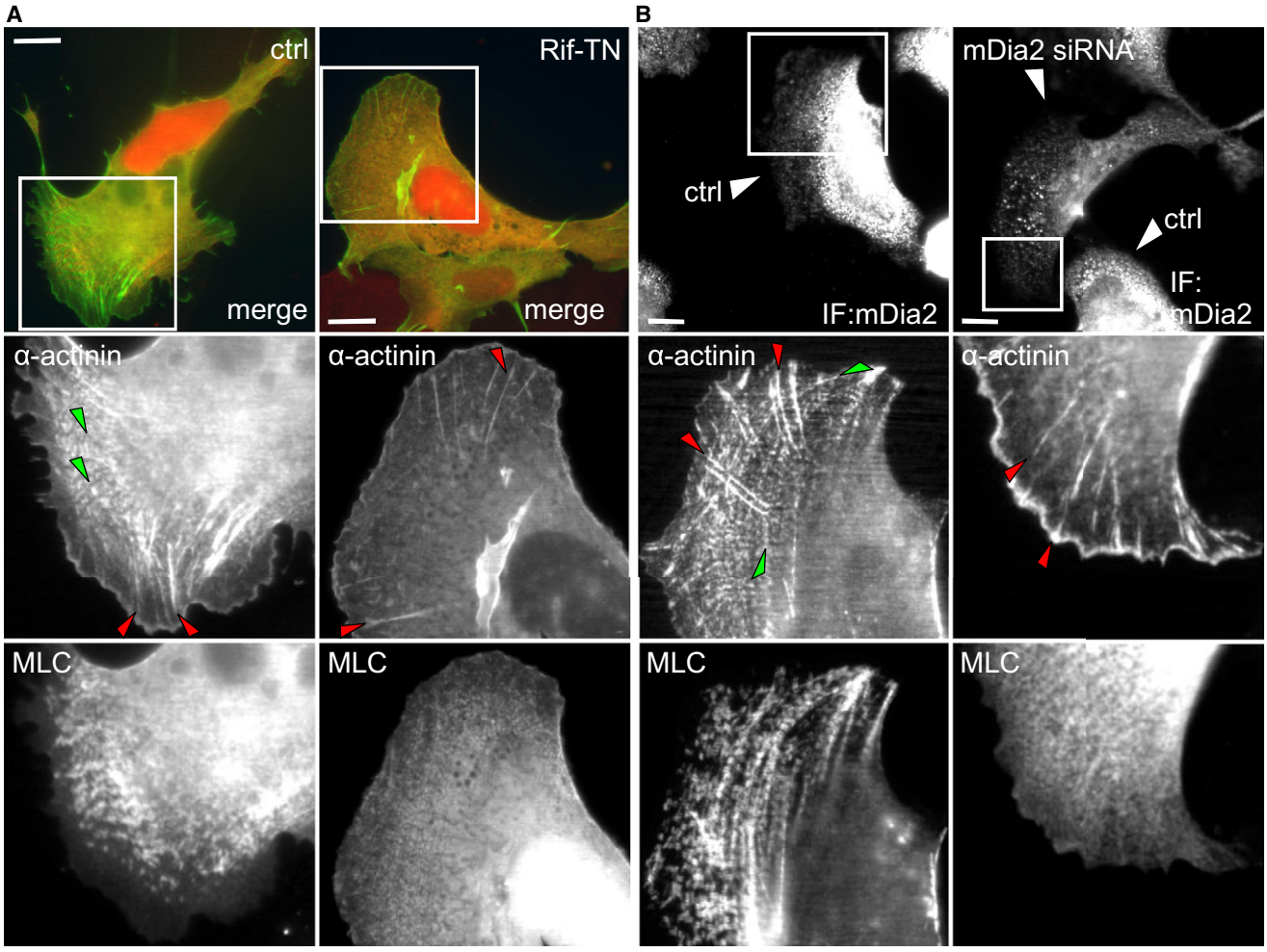


Figure 5. Incorporation of Tropomyosins and Myosin II into Transverse Arcs

(A) Time-lapse imaging of U2OS cells expressing YFP-Tm4 and CFP-α-actinin revealed that Tm4 and α-actinin display diffuse localizations at the cell edges. However, discrete Tm4 and α-actinin spots formed farther away from the cell edge, and these spots assembled endwise to form intact transverse arcs. Examples of Tm4 and α-actinin spots derived from the diffuse lamellipodial localization are indicated by green and red arrowheads, respectively. Scale bar represents 5 μm.

(B) Time-lapse imaging of U2OS cells transfected with Cherry-MLC and YFP-Tm4 revealed that tropomyosin (Tm4) precedes myosin during the assembly of transverse arcs. Four time frames from the edge of the same cell are shown in panels 1, 2, 3, and 4. Tm4 is in green, MLC is in red, and merge is in yellow. Arrows indicate distinct Tm4 spots where myosin is recruited after Tm4. The white box in the right panel corresponds to the cell region shown in Movie S3. Scale bar represents 5 μm.

(C) Quantification of the Cherry-MLC recruitment to defined YFP-Tm4 foci during transverse arc assembly.



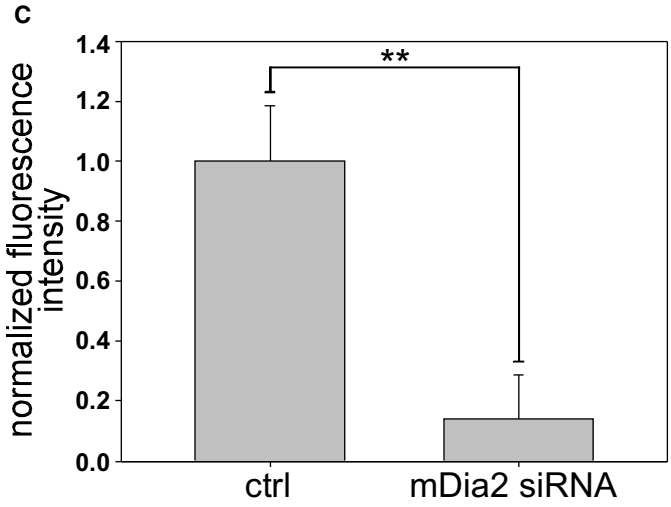


Figure 6. Rif-mDia2/DRF3 Pathway Is Essential for Myosin II Recruitment to Transverse Arcs

(A) Inactive Rif (Rif-TN) inhibits arc formation. Cells were transfected with plasmids expressing Rif-TN, YFP-α-actinin, and Cherry-myosin or YFP-α-actinin and Cherry-myosin alone (control cells). Cells were stained with myc antibody for verification of Rif-TN expression (data not shown). Red arrowheads indicate dorsal stress fibers, and green arrowheads in control cells indicate transverse arcs. Scale bar represents 10 μm.

(B) Depletion of mDia2/DRF3 leads to disruption of transverse arcs. Cells were treated with siRNA against mDia2/DRF3 and transfected with YFP-α-actinin and Cherry-myosin. Cells with low mDia2/DRF3 levels (an example shown in the top panel) did not contain transverse arcs. As in the case of Rif-TN expression, myosin II displayed mostly diffuse cytoplasmic staining in mDia2/DRF3 knockdown cells. Scale bar represents 10 μm.

(C) Quantification of myosin II levels in actin bundles of control and mDia2/DRF3 knockdown cells. Myosin intensity in stress fibers was measured (TINA software) and normalized against corresponding phalloidin intensity of the fiber. Data are represented as mean ± SEM, n = 16. **$p < 0.001$, as determined by a Mann-Whitney test.

6C and Figure S7A). Together, these data suggest that two distinct F-actin pools, (1) Arp2/3-nucleated, α-actinin-crosslinked filaments and (2) Dia2-nucleated, Tm-decorated actin filaments, are required for generation of transverse arcs. Recruitment of myosin II to the latter, Tm-decorated actin filaments, subsequently triggers the crosslinking of these actin filament structures to yield contractile transverse arcs.

Discussion

Actin filaments form functionally diverse protrusive and contractile structures in cells. The assembly mechanisms of protrusive lamellipodial and filopodial actin filament arrays are relatively well understood, whereas the pathways leading to the formation of myosin II-containing contractile actin filament structures, such as stress fibers and muscle myofibrils, are not known. By examining the cellular roles of the six Tm isoforms expressed in U2OS cells, we revealed several important new aspects concerning the mechanisms of assembly and turnover of stress fibers. We show three things. (1) Each Tm isoform has at least partly nonredundant function in stress fibers. Tm1, Tm5NM1, and Tm5NM2 appear to control the stability of actin filaments in focal adhesions, whereas Tm2 and/or Tm3 regulate the stability of actin filaments along the dorsal stress fibers. Importantly, Tm4 has a critical role in stress fiber contractility by recruiting myosin II to stress fibers. (2) Transverse arcs are generated from two distinct populations of lamellipodial actin filaments that are nucleated by the Arp2/3 complex and mDia2/DRF3 formin. (3) The Dia2-nucleated actin filaments are decorated by Tms, which subsequently recruit myosin II. The Tm/myosin II-containing actin filament arrays assemble with the Arp2/3-nucleated (and α-actinin-crosslinked) filaments to generate a contractile actomyosin bundle. Together, these findings reveal that, in comparison to the filopodial and lamellipodial actin filament arrays, contractile stress fibers are generated through a fundamentally different pathway that requires both Arp2/3- and Dia2-promoted actin nucleation and the activities of several functionally distinct tropomyosin isoforms. A working model for the assembly of stress fibers is presented in Figure 7.

Transverse arcs are gently curved actomyosin bundles oriented parallel to the leading edge of motile cells. Previous studies proposed that they form by endwise assembly of myosin II bundles and Arp2/3-nucleated, α-actinin-crosslinked actin structures [10]. Importantly, our new data show that Dia2-nucleated lamellipodial actin filaments, which are decorated by Tms, critically contribute to transverse arc formation. mDia2/DRF3 knockdown cells and cells expressing inactive Rif displayed a lack of transverse arcs, and this was accompanied by a diffuse cytoplasmic myosin II and Tm4 localization. Because wild-type U2OS cells display only a very small density of filopodia and microspikes, it is unlikely that the defects in Tm4 and myosin II localization in Dia2 knockdown cells would result from the lack of filopodia. Live-imaging analyses revealed that myosin II was gradually recruited to "arc precursors" after Tms. Thus, our data propose that the Dia2-nucleated, Tm-decorated actin filaments close to the plasma membrane form a platform that promotes the assembly of myosin II bundles during the formation of transverse arcs. In this respect, it is important to note that active Dia2 can associate with the plasma membrane [35, 36]. mDia2/DRF3 was recently also reported to contribute to focal adhesion assembly and dynamics [37]. Our data propose that, instead of directly regulating actin assembly at focal adhesions, mDia2/DRF3 may alternatively contribute to focal adhesion dynamics indirectly through its essential role in the generation of contractile transverse arcs.

Previous studies suggested that myosin II can be incorporated directly to focal adhesion attached dorsal stress fibers to generate contractile actomyosin bundles [10, 14]. However, our new data provide evidence that myosin II is recruited to stress fibers in transverse arcs. This is because disruption of arcs by depleting Tm4 or Dia2 resulted in a lack of myosin II in stress fibers. Furthermore, myosin II spots located along the dorsal stress fibers correspond to connection points between arc precursors and dorsal stress fibers. The exact mechanism by which myosin II-containing arcs connect to dorsal stress fibers is still elusive, but Tms may play an important role in this process because they are concentrated at the connection point of arcs and dorsals of stress fibers.

Earlier studies showed that simultaneous inactivation or depletion of Tms from mammalian cells leads to the loss of stress fibers, but they did not address the possible specific roles of various Tm isoforms [23, 24]. We now provide evidence that each Tm isoform has a specific function that is critical to the formation of a proper contractile actin stress fiber network in U2OS cells. All Tm isoforms displayed similar localizations in arcs and ventral stress fibers but had specific localization patterns in dorsal stress fibers. Tm1 and Tm5NM1/2 localization was restricted to focal adhesions. Thus, we propose that these isoforms stabilize specific actin filament populations at focal adhesions and at distal ends of dorsal stress fibers. This hypothesis is supported by a recent study demonstrating that overexpression of TM5NM1 stabilizes actin filaments in focal adhesions, increases focal adhesion size, and promotes the formation of fibrillar adhesions [38]. In addition, other regulators of actin dynamics and organization, such as coronin 2A, cofilin, and α-actinin, are important for focal adhesion dynamics and maturation [39, 40]. Tm2, on the other hand, localizes along the entire length of dorsal stress fibers, and its depletion, together with Tm3, leads to increased actin dynamics in stress fibers and ultimately to a complete loss of stress fibers. Because Tm3 was shown to confer increased turnover of actin filaments in B35 cells [41], the destabilization of stress fibers in Tm2/3 knockdown cells most likely results from the lack of Tm2. Thus, we propose that Tm2 stabilizes actin filaments along the stress fibers and that through its specific role in dorsal stress fibers, this isoform is essential for the maintenance and/or assembly of a contractile stress fiber network.

Importantly, our data provide evidence that Tm4 recruits, either directly or indirectly, myosin II to stress fibers during the formation of transverse arcs. This is because Tm4 displays faithful colocalization with myosin II in all three types of stress fibers, Tm4 knockdown cells display diminished association of myosin II with stress fibers, and the knockdown cells have severe defects in contractility despite still containing stress fibers. The stress fibers in Tm4 knockdown cells also display abnormal "curly" morphology that is similar to the one reported after inhibition of myosin II-dependent contractility by caldesmon overexpression [42, 43]. Interestingly, Tm4 knockdown cells have more filopodia, suggesting that in the absence of Tm4, the Dia2-nucleated actin filaments may be used for the formation of filopodia instead of becoming precursors of transverse arcs. It is important to note that Tm4 is also involved in the assembly and repair of myofibrils in skeletal muscles via the formation of premyofibrils [44]. Thus, we

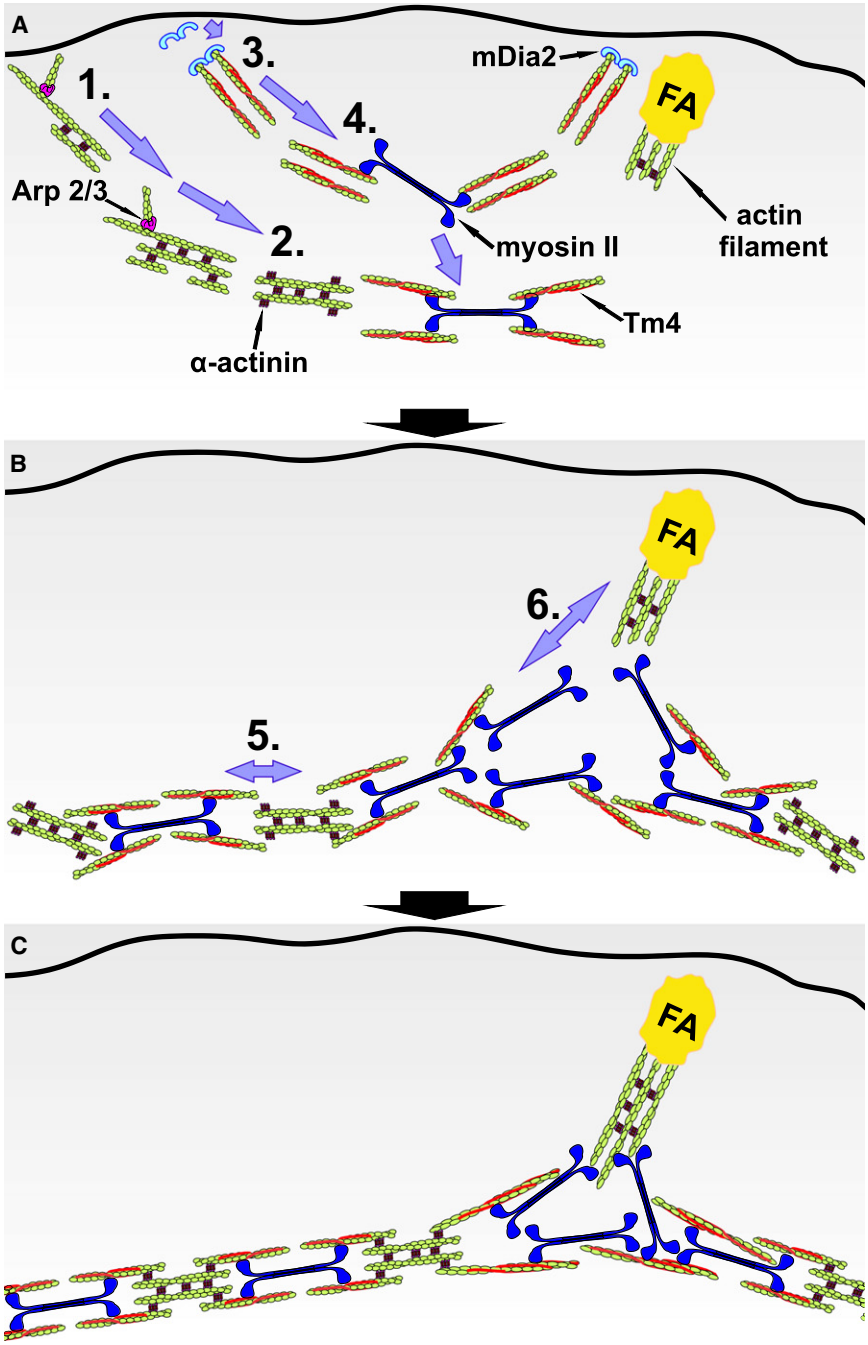


Figure 7. A Working Model for the Generation of Myosin II-Containing Contractile Stress Fibers in Motile Cells

(A) The Arp2/3-nucleated actin filament network is required for the formation of α-actinin-crosslinked actin filaments (1 and 2), whereas the mDia2/DRF3 formin is required for the assembly of tropomyosin (Tm4)-decorated actin filaments at the lamellipodium (3). Myosin II is specifically recruited to Tm4-containing (mDia2/DRF3-nucleated) actin filament bundles (4).

(B) Myosin II-containing actin filament bundles assemble endwise with α-actinin-crosslinked actin filaments to yield transverse arcs (5). The transverse arcs interact with the proximal tips of focal adhesion attached dorsal stress fibers (6).

(C) Contractile stress fiber network is formed. The dorsal stress fiber-attached transverse arc condenses (contracts) as it flows toward the cell center. Please note that Tm1 and Tm5NM1/2 localize to focal adhesions, whereas Tm2 localizes along the entire length of dorsal stress fibers, where it regulates the stability of actin filaments.

propose that Tm4 may also recruit myosin II to stress fiber-like precursors of myofibrils in muscles. However, the exact mechanism by which Tm4 recruits myosin II to stress fibers, as well as its biochemical and structural differences to other Tms expressed in U2OS cells, remains to be elucidated. In this context, it is important to note that recent studies demonstrated that fission yeast tropomyosin can induce the formation of functionally specified actin structures by increasing the affinity of class II and unconventional class V myosins for actin filaments and by competing for actin binding with the actin crosslinking protein fimbrin [45–48]. Thus, Tm4 may directly recruit myosin II to stress fiber precursors, or this may depend on the interplay with Tm4 and other proteins. In this context, it is important to note that whereas the other Tm isoforms colocalize with α-actinin in focal adhesions and/or dorsal stress fibers, Tm4 and α-actinin display mutually exclusive localization patterns in all stress fibers.

Recent studies provided evidence that myofibrils are derived from transverse arc-like precursors during myofibrillogenesis [49]. Our work demonstrating that both Dia2- and Arp2/3-nucleated cortical actin filament pools are critical to transverse arc assembly proposes that these two actin nucleation mechanisms also contribute to premyofibril formation in developing muscle cells. However, it is possible that instead of mDia2/DRF3, some other formin or formins contribute to the formation of Tm-decorated actin filaments during myofibrillogenesis. Furthermore, stress fiber-like actomyosin bundles contribute to endothelial cell branching in 3D environment [50], but the assembly mechanisms of these structures has not been reported. Thus, in the future it will be important to reveal the possible similarities and differences in the mechanisms regulating the assembly and turnover of actomyosin bundles in different cell types and in different environments.

Experimental Procedures

For information about the experimental procedures, please see the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes seven figures, Supplemental Experimental Procedures, and four movies and can be found with this article online at doi:10.1016/j.cub.2011.03.007.

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