Assembly of non-contractile dorsal stress fibers requires α-actinin-1 and Rac1 in migrating and spreading cells

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Summary

Cell migration and spreading is driven by actin polymerization and actin stress fibers. Actin stress fibers are considered to contain α-actinin crosslinkers and nonmuscle myosin II motors. Although several actin stress fiber subtypes have been identified in migrating and spreading cells, the degree of molecular diversity of their composition and the signaling pathways regulating fiber subtypes remain largely uncharacterized. In the present study we identify that dorsal stress fiber assembly requires α-actinin-1. Loss of dorsal stress fibers in α-actinin-1-depleted cells results in defective maturation of leading edge focal adhesions. This is accompanied by a delay in early cell spreading and slower cell migration without noticeable alterations in myosin light chain phosphorylation. In agreement with the unaltered myosin II activity, dorsal stress fiber trunks lack myosin II and are resistant to myosin II ATPase inhibition. Furthermore, the non-contractility of dorsal stress fibers is supported by the finding that Rac1 induces dorsal stress fiber assembly whereas contractile ventral stress fibers are induced by RhoA. Loss of dorsal stress fibers either by depleting α-actinin-1 or Rac1 results in a β-actin accumulation at the leading edge in migrating and spreading cells. These findings molecularly specify dorsal stress fibers from other actin stress fiber subtypes. Furthermore, we propose that non-contractile dorsal stress fibers promote cell migration and early cell spreading through Rac1-induced actin polymerization.

Key words: Actin stress fibers, Alpha-actinin, Migration, Myosin, Spreading

Introduction

The defining features of mesenchymal migration mode are polarized, fan-shaped cells having a prominent leading protrusion and a contractile rear (Friedl and Wolf, 2003; Friedl and Wolf, 2010). The migration and spreading of these cells need dynamic actin polymerization at the leading edge (Pollard and Borisy, 2003; Ponti et al., 2004) as well as actin stress fibers, which are considered to contain α-actinin crosslinkers and nonmuscle myosin II motors providing contractility (Friedl and Wolf, 2003; Pellegrin and Mellor, 2007). Noteworthy is that migrating cells may also contain other types of actin–myosin assemblies (e.g. Cramer et al., 1997; Svitkina et al., 1997). Studies of mesenchymal migrating or spreading cells describe distinct subtypes of actin stress fibers on the basis of their subcellular localization and termination sites (Hotulainen and Lappalainen, 2006; Small et al., 1998; Tojkander et al., 2011). The fiber subtypes are dubbed dorsal stress fibers, transverse arcs and ventral stress fibers. More recent studies show additional differences between actin stress fiber subtypes including their assembly mechanisms, dynamics, myosin II abundance and function (Ang et al., 2010; Burnette et al., 2011; Hotulainen and Lappalainen, 2006; Small et al., 1998; Tojkander et al., 2012; Tojkander et al., 2011). The fiber subtypes appear to assemble via annealing of Arp2/3 nucleated actin filaments at the leading edge focal adhesions and move constantly towards nucleus where they disassemble (Heath, 1983; Small et al., 1998). Transverse arcs appear to assemble through annealing of Arp2/3 nucleated actin fibers (Hotulainen and Lappalainen, 2006), and are abundant in myosin IIA (Burnette et al., 2011; Cai et al., 2010; Vicente-Manzanares et al., 2008).

Curve-shaped transverse arcs assemble on the lamellar region of migrating cells in parallel orientation with the leading edge (Hotulainen and Lappalainen, 2006; Small et al., 1998) (Fig. 1A open arrowhead). Transverse arcs do not directly associate with focal adhesions, and move constantly towards nucleus where they disassemble (Heath, 1983; Small et al., 1998). Transverse arcs appear to assemble through annealing of Arp2/3 nucleated actin fibers (Hotulainen and Lappalainen, 2006), and are abundant in myosin IIA (Burnette et al., 2011; Cai et al., 2010; Vicente-Manzanares et al., 2008).

In addition to transverse arcs, the lamellar region contains dorsal stress fibers (Fig. 1A filled arrowhead), which perpendicularly elongate from the leading edge focal adhesions and often attach with transverse arcs (Hotulainen and Lappalainen, 2006; Small et al., 1998). The assembly of dorsal stress fibers has been shown to be partially dependent on formin-family member mDia1 (Hotulainen and Lappalainen, 2006; Oakes et al., 2012). Recently, myosin II was reported to be incorporated into dorsal
stress fibers after these fibers associate with transverse arcs (Hotulainen and Lappalainen, 2006). The myosin II incorporation to dorsal stress fibers was shown to occur through tropomyosin 4 recruitment (Tojkander et al., 2011). In addition, a recent study focusing on focal adhesion maturation reported that partial α-actinin-1 downregulation inhibits radial (dorsal) stress fiber assembly at focal adhesions but retain a contractile lamella (Oakes et al., 2012).

Interestingly, despite the increasing number of studies demonstrating stress fiber subtype specific properties, largely uncharacterized is the possible degree of molecular specificity between fiber subtypes as well as signaling pathways regulating their dynamics. With this motivation we initially addressed a question whether either of the two major nonmuscle actin stress fiber crosslinkers α-actinin-1 or α-actinin-4 could show specificity in crosslinking actin stress fiber subtypes. Both of these α-actinin isoforms are ubiquitously expressed and have shown to be associated with cancer cell migration (Otey and Carpen, 2004). The finding that α-actinin-1 is specifically required to assemble dorsal stress fibers provided us an unique tool to investigate the role of this actin stress fiber subtype in cell migration and spreading.

**Results**

**α-Actinin-1 and α-actinin-4 localization on dorsal stress fibers**

In order to investigate whether α-actinin-1 or α-actinin-4 possess any specificity in crosslinking actin stress fiber subtypes, human osteosarcoma U2OS cells were selected due to their well-defined dorsal stress fibers, transverse arcs and ventral stress fibers (Hotulainen and Lappalainen, 2006; Vallenius et al., 2000) demonstrated by staining of focal adhesions and actin stress fibers (Fig. 1A). Prior to initiate subcellular localization studies we made an attempt to generate α-actinin-1 specific antibody (dubbed A1-341, see Materials and Methods), as the lack of it has been a major drawback in determining endogenous α-actinin-1 localization by immunofluorescence. Subsequent western blotting analysis from U2OS cells transiently expressing control, GFP-tagged α-actinin-4 or α-actinin-1 indicates that the generated A1-341 antibody only reacts with α-actinin-1 (Fig. 1B; A1-341), whereas a commercial BM-75.2 antibody reported to be specific for α-actinin-1 (Araki et al., 2000; Celli et al., 2006; Chen et al., 2006; Honda et al., 1998) recognizes both isoforms (Fig. 1B; BM-75.2), and ALX-210 is specific for α-actinin-4 (Chen et al., 2006) (Fig. 1B; ALX-210). The specificity of A1-341 and ALX-410 enabled to assess distributions of α-actinin-1 and α-actinin-4 on actin stress fibers by immunofluorescence analysis. Although α-actinin-1 and α-actinin-4 staining is noted on all actin stress fiber subtypes (Fig. 1C,D) an interesting differential staining pattern is observed on dorsal stress fibers. On dorsal stress fibers α-actinin-1

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**Fig. 1. Distinct distribution of α-actinin-1 and α-actinin-4 on dorsal stress fibers.** (A) Immunofluorescence analysis of a U2OS cell with anti-vinculin (white; focal adhesions) and phalloidin (green; F-actin) to distinguish ventral stress fibers (sf) (arrow), dorsal stress fibers (filled arrowhead) and transverse arcs (open arrowhead). (B) Western blotting analysis of U2OS cells expressing control, GFP-tagged α-actinin-4 (A4-GFP) or α-actinin-1 (A1-GFP) using a new polyclonal antibody raised against α-actinin-1 (A1-341), a commercial antibody (BM75.2) or α-actinin-4-specific ALX-210 antibody (Chen et al., 2006). Endogenous α-actinin is indicated with an asterisk (*). (C,D) Immunofluorescence analysis using α-actinin-1 (A1-341) or α-actinin-4 (ALX-210) antibodies co-stained with vinculin and F-actin as indicated. Arrows and arrowheads indicate actin stress fiber subtypes as in A. (E,F) Distribution of α-actinin-1 and α-actinin-4 staining on dorsal stress fibers (lower bracket, dorsal sf) and focal adhesions (upper bracket, FA) using indicated antibodies and F-actin staining. Arrows in F demonstrate prominent α-actinin-4 staining at the base of the fiber where it emerges from the focal adhesion, whereas α-actinin-1 stains the entire dorsal stress fiber. (G,H) Box-plot analyses demonstrate the ratio of α-actinin-1 (G) or α-actinin-4 (H) intensities against F-actin, separately from the base or trunk of dorsal stress fibers as visualized (zoom-in images from E and F, respectively). α-Actinin/ F-actin ratio values are normalized to the base of dorsal stress fibers. A total of 117 (G) or 81 (H) fibers were analyzed from three independent experiments. Outliers are marked with grey asterisks. **P<0.01 by Student’s t-test. Scale bars: 10 μm. ns, not significant.
is abundant throughout the fibers (Fig. 1E), whereas α-actinin-4 staining is prominent only at the base of the fibers where it emerges from the focal adhesion (Fig. 1F; arrow). Separation of a dorsal stress fiber into a base and a trunk area as depicted in Fig. 1G,H [also referred to distal and proximal parts, respectively (Hotulainen and Lappalainen, 2006; Tojkander et al., 2011)] demonstrates a significant base selectivity of α-actinin-4 compared to α-actinin-1 (Fig. 1G,H). This difference is also noted between focal adhesions and dorsal stress fiber trunks (supplementary material Fig. S1A,B). Similar subcellular localization pattern of α-actinin-1 on actin stress fiber subtypes can be detected upon ectopic expression of GFP-tagged α-actinin-1 (supplementary material Fig. S1C). Although GFP-tagged α-actinin-4 overexpression results in notable changes in actin stress fiber morphology, it also mimics endogenous α-actinin-4 localization (supplementary material Fig. S1D). Moreover, endogenous α-actinin-1 localization identified with A1-341 antibody is undetectable in α-actinin-1-depleted cells (supplementary material Fig. S1E). These results indicate that the newly generated α-actinin-1 antibody specifically recognizes subcellular localization of α-actinin-1, which is abundant throughout dorsal stress fibers.

**Assembly of dorsal stress fibers requires α-actinin-1**

In order to investigate whether the observed localization differences correlate with α-actinin-1 or α-actinin-4 functions on dorsal stress fibers we performed small interfering RNA (siRNA) mediated downregulation of α-actinin-1, α-actinin-4 or both, which after optimization showed undetectable protein levels in U2OS cells (Fig. 2A; supplementary material Fig. S2B,C,E). The F-actin staining of control and α-actinin downregulated cells reveals that while α-actinin-4 depletion does not result in appreciable changes in stress fiber subtypes (Fig. 2B; si-A4), α-actinin-1-silenced cells show an almost complete loss of dorsal stress fibers at the leading edge without affecting other stress fiber

![Fig. 2. α-Actinin-1 silencing results in loss of dorsal stress fibers, which associates with an increase of small adhesions at the leading edge.](image-url)
subtypes (Fig. 2B; si-A1; see also Fig. 2E; supplementary material Fig. S3A). As expected simultaneous downregulation of α-actinin-1 and α-actinin-4 causes nearly undetectable actin stress fibers indicating that these two α-actinins are major actin stress fiber crosslinkers in U2OS cells (supplementary material Fig. S2D,E). The loss of dorsal stress fibers in α-actinin-1-depleted cells is accompanied by significantly smaller (50%, P<0.001) vinculin-positive adhesion sites at the leading edge, not noted at the trailing edge (Fig. 2C; compare red and yellow circles and the adjacent box-plot for quantification). Furthermore the smaller area of adhesion sites associates with an increase in adhesion numbers (63%, P<0.001; Fig. 2D). The observed change in adhesion sites and numbers at the leading edge but not at trailing edge suggests that α-actinin-1-crosslinked dorsal stress fibers regulate adhesion maturation at the leading edge.

In order to gain further insight into the mechanism resulting in loss of dorsal stress fibers in α-actinin-1-silenced cells, we followed actin stress fiber dynamics using time-lapse imaging of the available EGF-CLP-36-expressing U2OS cells (Vallénius et al., 2004) (supplementary material Movies 1, 2). While in control cells prominent straight dorsal stress fibers form (Fig. 2E; filled arrowheads; supplementary material Movie 1) and attach to transverse arcs (open arrowhead), α-actinin-1-silenced cells occasionally display only a few weak nonlinear fibers that disappear abruptly (Fig. 2E; supplementary material Movie 2). Consistent with this, a focused analysis of dorsal stress fiber bases in α-actinin-1-silenced cells (not stably expressing EGF-CLP-36) stained with F-actin reveals short fibers exhibiting significantly reduced amount of filamentous actin (57%, P<0.001, Fig. 2F; supplementary material Fig. S3A). The loss of dorsal stress fibers following α-actinin-1 silencing cannot be rescued by ectopic expression of α-actinin-4 (data not shown), although in murine lung fibroblasts α-actinin-1 was reported to rescue the studied α-actinin-4 functions (Shao et al., 2010a). This strongly proposes the specific requirement of α-actinin-1 in the dorsal stress fiber assembly. Furthermore, in agreement with U2OS cells, α-actinin-1 downregulation causes a loss of α-actinin-1 rich dorsal stress fibers in mouse embryonic fibroblasts (MEFs), which exhibit clearly identifiable actin stress fiber subtypes (supplementary material Fig. S3B,D). This can be also detected in spreading MEFs (supplementary material Fig. S3C). Collectively, these results demonstrate that the assembly of dorsal stress fibers requires α-actinin-1 in U2OS cells and in MEFs.

α-Actinin-1 depletion causes decreased cell migration without altering myosin II activity

The established function of actin stress fibers is to regulate cell migration (Ridley, 2001). Growing number of studies suggest that actin stress fiber subtypes regulate cell migration differently. For example ventral stress fibers promote rear contraction and formation of front-to-rear polarity axis (Ang et al., 2010; Vicente-Manzanares et al., 2008). To assess a possible role of dorsal stress fibers in cell migration, we selected to use a scratch wound-healing assay where cells at wound edge show directional migration. F-actin and vinculin co-staining indicates that cells at the wound edge exhibit evident dorsal stress fibers, transverse arcs and ventral stress fibers containing α-actinin-1 and are often fan-shaped (supplementary material Fig. S4A–C). Comparison of wound closure areas at 24-hour time point when the control cells are about to close their wounds, α-actinin-1-silenced cells show minor but significant delay, an average of 27% (n=15; P<0.01) (Fig. 3A,B). Subsequent 24-hour time-lapse imaging of wound closure hourly (supplementary material Movie 3) revealed that control and α-actinin-1-silenced cells display similar migration pattern, but α-actinin-1-silenced cells migrate overall more
slowly (in control cells wound closure rate on average 33.4 μm/h (24.4–39.9 μm) and in α-actinin-1-depleted cells 26.0 μm/h (20.4–32.7 μm). The observed wound closure rate in control cells is consistent with previous reports analyzing mesenchymal migration mode (Clarke et al., 2004; Friedl and Wolf, 2010). In agreement with the wound-healing assay, α-actinin-1 depletion decreases U2OS cell migration through Matrigel-coated transwells on average 28% (Fig. 3D,E). Compared to control cells, the cell number and total cell area is not significantly altered in α-actinin-1-silenced cells (supplementary material Fig. S4D,E). Collectively, these results implicate that the decreased migration reflects a loss of dorsal stress fibers following α-actinin-1 downregulation.

Interestingly, the decreased migration in α-actinin-1-silenced cells is opposite to what has been noted following silencing or inhibition of myosin IIA (Even-Ram et al., 2007; Sandquist et al., 2006), which is the major myosin isoform in transverse arcs in lamella region. Therefore, we next probed myosin II activity following α-actinin-1 silencing by western blotting analysis using phospho-myosin light chain (pMLC) antibody. While inhibition of myosin IIA with a ROCK kinase inhibitor (Y27632) leads to a dramatic drop in pMLC, α-actinin-1 downregulation does not significantly change pMLC levels (Fig. 3F). These results together with a loss of dorsal stress fibers in α-actinin-1-silenced cells suggest that dorsal stress fibers promote cell migration in myosin-II-independent manner.

Dorsal stress fiber trunks are non-contractile

The results above prompted us to investigate the status of myosin II on dorsal stress fibers. Interestingly, although pMLC staining of migrating U2OS cells clearly decorated ventral stress fibers and transverse arcs (Fig. 4A; open arrowhead), dorsal stress fibers are not evident in pMLC staining (Fig. 4A; filled arrowhead) except in regions close to transverse arcs. Consistently, there is a significantly lower pMLC/F-actin ratio in dorsal stress fibers compared to transverse arcs (Fig. 4B). To further investigate the unexpected lack of pMLC on dorsal stress fibers (Hotulainen and Lappalainen, 2006; Tojkander et al., 2011) we analyzed these fibers in cells overexpressing GFP-tagged myosin IIA. Vinculin and F-actin staining of these cells indicates that whereas GFP-myosin IIA localizes in a dotted-like pattern on transverse arcs (Fig. 4C; open arrowhead) and on ventral stress fibers (Fig. 4C; arrow), myosin IIA signal on dorsal stress fiber trunks is undetectable (Fig. 4C; filled arrowhead). Similar to myosin IIA, myosin IIB localizes to transverse arcs but is not detectable on dorsal stress fiber trunks (supplementary material Fig. S4F). These results strongly suggest that dorsal stress fiber trunks are non-contractile.

To further assay the role of myosin II on dorsal stress fibers, we treated U2OS cells with the ROCK kinase inhibitor (Y27632) or with the myosin II ATPase inhibitor blebbistatin for 30 minutes. As expected, a 30 minute inhibition with Y27632 (data not shown) or blebbistatin (Fig. 4D) leads to a dramatic decrease of ventral stress fibers and transverse arcs, but interestingly dorsal stress fibers are significantly more resistant to blebbistatin (Fig. 4D). It is also interesting to note that α-actinin-1 remains on dorsal stress fibers (Fig. 4D; zoom-in, lower bracket: dorsal sf) indicating that myosin II driven tension is not needed for α-actinin-1 localization on these fibers. In contrast blebbistatin treatment removes both α-actinin-1 and vinculin from focal adhesions as reported previously (Pasapera et al., 2010; Schiller et al., 2011). This is noted as a 5–10 μm gap from the leading edge (Fig. 4D; zoom-in, upper bracket, FA and Fig. 4D; vinculin). To summarize, the lack of myosin II on dorsal stress fiber trunks and the finding that these fibers are resistant to myosin II inhibitors strongly propose that dorsal stress fibers are non-contractile.

Delayed early cell spreading and β-actin accumulation at cell periphery following α-actinin-1 depletion

The apparent minor role of myosin II on dorsal stress fibers prompted us to assess whether functions of these fibers would be

![Fig. 4. Dorsal stress fiber trunks lack myosin II.](image-url)

(A) A merged image of an untransfected migrating U2OS cell stained with phospho-MLC (pMLC) and F-actin. The filled arrowhead points to a dorsal stress fiber and the open arrowhead to the corresponding transverse arc. (B) Box-plot analysis of pMLC/F-actin signal ratio on transverse arcs (n=37) or dorsal stress fibers (n=43) from three independent experiments. Values are normalized to transverse arcs and outliers indicated with grey asterisks. (C) Immunofluorescence images of a U2OS cell expressing GFP-tagged myosin IIA (GFP-myosin IIA) stained with F-actin and vinculin to visualize actin stress fiber subtypes and focal adhesions. The filled arrowheads mark a dorsal stress fiber, open arrowheads a transverse arc and arrows a ventral stress fiber. Myosin IIA signal is also detectable in some focal adhesions (asterisks) as reported (Even-Ram et al., 2007). (D) Control or 30-minute blebbistatin-treated U2OS cells co-stained with α-actinin-1 and vinculin antibodies and F-actin as indicated. Right-hand panel: F-actin/α-actinin-1 merged zoom-in images of the indicated box areas to demonstrate α-actinin-1 staining along dorsal stress fibers (lower bracket, dorsal sf) but not at adhesion sites after blebbistatin treatment (blebbistatin; upper bracket, FA). **p<0.001 by a Student’s t-test. Scale bars: 10 μm.
mediated through actin polymerization. Actin polymerization at cell periphery has a major role during early cell spreading, where myosin II activity is low (Cai et al., 2006; Johnston et al., 2008; Price et al., 1998). During early spreading phase radial actin fibers resembling dorsal stress fibers initiate to assemble from peripheral adhesions in fibroblasts (Cai et al., 2010). Therefore, we first analyzed actin stress fibers and α-actinin-1 localization from spreading control and α-actinin-1-depleted cells seeded on fibronectin-coated coverslips. The majority of control cells initiate to assemble radial fibers at the cell periphery within 30 minutes (Fig. 5A; si-NT) consistent with studies in fibroblasts (Cai et al., 2010). These fibers also associate with transverse arcs and contain abundant α-actinin-1 (Fig. 5A; si-NT) whereas α-actinin-4 staining is detectable only at bases of emerging fibers (arrowhead in supplementary material Fig. S5A). Interestingly, the α-actinin-1-depleted cells fail to form these radial fibers (Fig. 5A; si-A1). Occasional small radial fiber buds are detected similarly to the migrating cells lacking α-actinin-1 (supplementary material Fig. S3A). These results significantly extend the observed similarities between dorsal stress fibers in migrating cell and radial fibers in spreading cells, which we therefore suggest to be redefined as dorsal stress fibers.

To investigate the consequences of dorsal stress fiber loss in early cell spreading, we analyzed control and α-actinin-1-depleted cells using time-lapse imaging of EGFP-CLP-36 U2OS cells (Vallenius et al., 2004) enabling to follow spreading in concomitantly with actin fiber formation. In control cells protrusions emerge from cell edge after plating and cells start spreading roughly 5 minutes afterwards. Dorsal stress fibers are noted at around 20 minutes following plating (supplementary material Movie 4). By contrast, in α-actinin-1-silenced cells spreading is delayed by ~30 minutes and prominent dorsal stress fibers are not noted during the 2-hour follow-up. Consistent with the cell spreading delay, cell circumference at 20 minutes after plating is significantly smaller in α-actinin-1-silenced cells (Fig. 5B,C). Analysis of time-lapse images taken every 2 minutes after plating demonstrates constant spreading delay between 4 to 20 minutes time points (supplementary material Fig. S5B). Despite this, the α-actinin-1-silenced cells do gradually spread, and acquire a shape and size comparable to control cells (Fig. 5B; 120 minutes; supplementary material Movie 4). At the 120 minutes point they have a 1.3-fold larger circumference (three independent experiments; n=20; P<0.001). This change is however transient based on the lack of detectable difference in cell area in continuously cultured cells (supplementary material Fig. S4E). During a two-hour follow-up significantly higher fraction of control cells (29%; 16/56) initiate migration compared to α-actinin-1-depleted cells (15%; 13/88), which is in agreement with wound-healing delay results (Fig. 3). Noteworthy is that early cell spreading delay is also observed in fixed U2OS cells not stably expressing EGFP-CLP-36 (data not shown). To summarize, the delay in early cell spreading together with the non-contractile nature of dorsal stress fibers imply that these fibers contribute to actin polymerization at the leading or spreading cell edge.

To investigate possible changes in actin isoforms associated with dorsal stress fiber assembly we analyzed subcellular localization of β-actin following α-actinin-1 silencing. Interestingly, both spreading and migrating α-actinin-1-silenced cells show an accumulation of β-actin at cell periphery (Fig. 5D,E). In agreement with the noted endogenous β-actin accumulation, GFP-tagged β-actin expression in α-actinin-1-silenced cells also accumulates at the leading edge of migrating cells (data not shown). This is specific for β-actin, as it is not observed in migrating cells stained for γ-actin (supplementary material Fig. S5C).

![Fig. 5. Impaired early cell spreading and β-actin accumulation at cell edge upon α-actinin-1 depletion.](image-url)
Rac1 induces assembly of dorsal stress fibers crosslinked by α-actinin-1

When considering the possible upstream signals regulating non-contractile dorsal stress fiber mediated functions, an interesting candidate is Rac1 as it is a critical regulator of actin polymerization at the leading edge (Ridley, 2011; Wu et al., 2009). To assess whether Rac1 promotes dorsal stress fiber assembly we expressed a GFP-tagged constitutively active Rac1 (Rac1 V12) or as a control constitutively active RhoA (RhoA V14) involved in contractility mediated cell migration (Parsons et al., 2010). Analysis of GFP signals indicate that Rac1 V12 localizes on focal adhesions (Fig. 6A,E) as reported previously (Nethe et al., 2010; ten Klooster et al., 2006), whereas RhoA exhibits only diffuse cytoplasmic pattern also consistent with previous studies (Ridley and Hall, 1992; Yonemura et al., 2004).

Strikingly, F-actin staining revealed that Rac1 V12-expressing cells exhibit pronounced dorsal stress fibers (Fig. 6B; arrowhead) and only few ventral fibers (Fig. 6B; arrow) whereas in RhoA V14-expressing cells the reverse is noted regarding actin fiber types (supplementary material Fig. S6A). Quantification demonstrates a twofold increase in dorsal stress fiber numbers in Rac1 V12-expressing cells (Fig. 6C) and the fibers are also significantly longer (Fig. 6D). In contrast, RhoA V14 expression results in a significant increase in ventral stress fiber numbers and decrease in dorsal stress fiber numbers (Fig. 6C). These results suggest that Rac1 is a critical inducer of dorsal stress fibers. To investigate a possible interdependency of Rac1 and α-actinin-1 in dorsal stress fibers, we subsequently analyzed whether Rac1 could induce these fibers in the absence of α-actinin-1. Concomitant depletion of α-actinin-1 and ectopic expression of Rac1 V12 results in severe defects in dorsal stress fiber formation (Fig. 6E). Only few short fibers emerge from focal adhesions at the leading edge similarly as noted in α-actinin-1-depleted migrating cells (Fig. 6E; supplementary material Fig. S3A).

Furthermore, subsequent analysis of dorsal stress fibers by F-actin staining following Rac1 downregulation indicates that Rac1 is required to assemble these fibers (Fig. 6F). Importantly, Rac1 silencing results in β-actin accumulation at the leading edge similarly as in α-actinin-1-depleted cells (supplementary material Fig. S6B). Overall, these results provide evidence that α-actinin-1 and Rac1 are critical molecules required for the assembly of dorsal stress fibers in migrating and spreading cells.

**Fig. 6. Rac1 induces dorsal stress fibers crosslinked by α-actinin-1.** (A) GFP-signals from the leading edge of migrating U2OS cells expressing constitutively active GFP-tagged Rac1 (Rac1 V12) or RhoA (RhoA V14). Rac1 V12 signal is noted as elongated structures (asterisk, *) identified as focal adhesions based on vinculin staining (data not shown). (B) Staining of F-actin and vinculin from a Rac1 V12-expressing cell; dorsal (arrowhead) and ventral (arrow) stress fibers as indicated. (C) Quantification of the numbers of dorsal and ventral stress fibers (sf) in control (n=12), Rac1 V12 (n=15) or RhoA V14 (n=10) expressing cells from three independent experiments. (D) Quantification of focal adhesion and dorsal stress fiber (sf) length in control (n=12) or Rac1 V12 (n=15) expressing cells from three independent experiments. Error bars in C and D indicate s.d. (E) Staining of actin fibers (F-actin) and GFP-Rac1 V12 (GFP signal) from cells expressing GFP-Rac1 V12 and also transfected with non-targeting siRNA (si-NT) or α-actinin-1-targeting siRNA (si-A1). Arrowheads demonstrate dorsal stress fibers, which are rudimentary in the α-actinin-1-depleted Rac1 V12-expressing cells. (F) F-actin-stained non-targeting siRNA (si-NT) or Rac1 (si-Rac1)-downregulated U2OS cells. **P<0.01, ***P<0.001 by a Student’s t-test. Scale bars: 10 μm.
Discussion

Our results demonstrate remarkable specificity of dorsal stress fibers both in molecular composition and in their regulation compared to ventral stress fibers and transverse arcs. α-Actinin-1 is required to crosslink linear dorsal stress fibers, as in its absence only rudiments of α-actinin-4 containing dorsal stress fibers emerge. α-Actinin-1 and α-actinin-4 are very similar in primary structure (87% identity), with less related regions in the third spectrin-like repeat and at the very N-terminus. Both of these regions interact with various proteins but their α-actinin isoform specificity has not been determined (Otey and Carpen, 2004). Interestingly, the N-terminal region of α-actinin-1, which associates with filamentous actin, has a focal adhesion kinase (FAK) site (Tyr12) (Craig et al., 2007; Izaguirre et al., 2001), which does not appear to be a major phosphorylation site in α-actinin-4 (Shao et al., 2010b). Moreover, this phosphorylation site was reported to promote tumor cell adhesion under pressure in an α-actinin-1-dependent manner suggesting a possible dorsal stress fiber-associated function. However, another in vitro study demonstrated that Tyr12 phosphorylation on α-actinin-1 decreased α-actinin-1 binding with filamentous actin (Izaguirre et al., 2001), and thereby future studies are needed to determine the role of Tyr12 phosphorylation.

The lack of dorsal stress fibers in α-actinin-1-silenced cells is accompanied with impaired adhesion maturation at the leading edge. This finding is consistent with observations made following α-actinin-1 downregulation in CHO.K1 cells, where α-actinin-1 is needed for the maturation of early stage adhesions in a myosin II-independent manner (Choi et al., 2008). In CHO.K1 cells myosin II independency was correlated with long-lived nascent adhesions whereas in U2OS cells or MEFs having short-lived nascent adhesions myosin II was reported to be needed for adhesion maturation (Choi et al., 2008). Another study on U2OS cells also utilizing α-actinin-1 downregulation showed that initial compositional maturation of focal adhesions is not tension dependent, but instead requires a stress fiber template (Oakes et al., 2012). The defective maturation of focal adhesions in α-actinin-1-downregulated cells was determined by the decreased phosphorylation of FAK (pY397) and paxillin (pY31) as well as smaller adhesion sites (Oakes et al., 2012), and these defects were not noted following the ROCK kinase inhibition. Here, we observe that the inhibition of myosin II ATPase activity results in compositional changes in focal adhesions determined by vinculin and α-actinin-1 disappearance from adhesion sites, which occurs at the presence of prominent dorsal stress fibers. This is in agreement with several recent studies including two large-scale proteomic purifications of focal adhesions showing a large set of proteins recruited to focal adhesions in myosin-II-dependent manner (Kuo et al., 2011; Pasapera et al., 2010; Schiller et al., 2011). In aggregate these studies suggest that dorsal stress fiber-mediated tension is required for focal adhesion maturation at the leading edge whereas initial adhesion formation apparently does not require tension. Presumably, the dorsal stress fiber mediated tension at the leading edge derives from lamellar retrograde flow including transverse arcs (Hotulainen and Lappalainen, 2006), which is coupled to focal adhesions through dorsal stress fibers.

The lack of myosin II on dorsal stress fiber trunks signifies another compositional specialization of stress fiber subtypes. This striking result challenges the widely presented view (Parsons et al., 2010; Pellegrin and Mellor, 2007; Vicente-Manzanares et al., 2009) that actin stress fibers including dorsal stress fibers (Hotulainen and Lappalainen, 2006; Tojkander et al., 2011) represent sarcomere-like structures containing myosin II. Interestingly, previous polarity studies demonstrate important differences between dorsal stress fibers and other stress fiber subtypes (Pellegrin and Mellor, 2007). While the actin filament polarity on ventral stress fibers and transverse arcs is alternating resembling that of muscle cell sarcomeres, dorsal stress fibers orient unipolarly, i.e. barbed ends at focal adhesions point to the leading edge and pointed ends towards the nucleus. Thereby, the generation of sarcomere-like fibers would not only require incorporation of myosin II molecules into dorsal stress fibers but also a drastic reorientation of actin filaments. Considering that myosin II appears to be incorporated following the association of dorsal stress fibers with transverse arcs (Hotulainen and Lappalainen, 2006) this association may trigger actin rearrangements and incorporation of myosin II at the tips of dorsal stress fibers following transverse arcs attachment.

This study additionally identifies that dorsal stress fibers are specifically induced by active Rac1. A wealth of literature highlights the importance of Rac1 as an inducer of Arp2/3 nucleated branched actin filaments pushing leading edge forward (Ridley, 2011). However, Rac1 also localizes to focal adhesions (Nethe et al., 2010; ten Klooster et al., 2006) and associates with mDia2 nucleating unbranched actin filaments (Ji et al., 2008; Lammers et al., 2008). Recently it was shown that mDia2 depletion abrogated formation of fibers termed lamellipodial filopodia in the presence of constitutively active Rac1 (Yang et al., 2007). Interestingly, these linear lamellipodial filopodia fibers oriented perpendicularly to the leading edge share characteristics with dorsal stress fibers. In U2OS cells mDia2 has been proposed to nucleate dorsal stress fibers (Oakes et al., 2012) (also our unpublished observation), although mDia1 is clearly additionally involved (Hotulainen and Lappalainen, 2006; Oakes et al., 2012), and associates with Rac1 (Lammers et al., 2008). In U2OS cells mDia2 also contributes to the nucleation of transverse arcs (Tojkander et al., 2011). Future experiments are needed to resolve links between Rac1 and mDia1, mDia2 as well as potential other fiber-specific nucleators (Ang et al., 2010).

The established role of Rac1 as a stimulator of cell migration and spreading through actin polymerization (Ridley et al., 2003) together with myosin II free dorsal stress fiber trunks propose that actin polymerization is important in dorsal stress fiber dependent cell migration and early cell spreading. The observed increase in β-actin at the leading edge of migrating and early spreading cells in the absence of dorsal stress fibers is consistent with this. Interestingly, β-actin deficient fibroblasts display a migration defect apparently associating with increased ventral stress fibers (Bunnell et al., 2011; Tondeleir et al., 2012) suggesting that β-actin is involved in dorsal stress fiber assembly. A possibility is also that dorsal stress fiber driven actin polymerization promotes cell migration and spreading by acting as ‘mother filaments’ for other lamellar actin network structures as suggested for lamellipodial filopodia as a way to increase nucleation points for the Arp2/3 complex (Yang et al., 2007).

Regarding cell migration the noted reciprocal assembly of dorsal stress fibers by Rac1 and ventral stress fibers by RhoA is interesting and demonstrates specificity in upstream regulators of stress fiber subtypes. The reciprocal activation of Rac1 and RhoA is often associated with temporal regulation such as when cell migration modes interconvert from amoeboid (RhoA) to mesenchymal (Rac1) (Sanz-Moreno et al., 2008), during cell...
protrusions (Machacek et al., 2009) and during neurite growth cone extension (Kozma et al., 1997). Our results suggest that spatial and temporal activation of Rac1 and RhoA results in assembly of distinct actin stress fiber subtypes, which coordinate leading edge protrusions and rear detachment, respectively.

Materials and Methods

Cell culture, reagents and antibodies

Human U2OS osteosarcoma cell line and immortal mouse embryonic fibroblasts (MEFs) were propagated in DMEM media (Invitrogen) supplemented with 10% fetal calf serum, penicillin and streptomycin and glutamine at 37°C in 5% CO2. To generate immortalized MEFS, cells from passage 3 were infected with a retrovirus encoding residues 302 to 390 of p53 (Klefstrom et al., 1997) and grown as pools following selection with hygromycin (Invitrogen) at 0.2 mg/ml. To inhibit myosin II ATPase activity U2OS cells were treated for 30 minutes with 100 µM blebbistatin (Sigma) or with 10 µM ROCK kinase inhibitor (Y27632, Tocris Bioscience). The rabbit polyclonal anti-α-actinin-1 antibody was raised using the N-terminal α-actinin-1 sequence (DHYDYSQQTNDN) as an immunogen, and subsequently affinity purified (Eurogentec). Other primary antibodies were: the rabbit polyclonal anti-α-actinin-4 (catalog no. ALX-210-356, Alex Biochemicals), the mouse monoclonal anti-vinculin antibody (catalog no. V9131, Sigma), the rabbit polyclonal anti-phospho-MLC (Ser19) antibody (catalog no. 3671, Cell Signalling), the mouse monoclonal total anti-MLC antibody (catalog no. M4401, Sigma), the hybridoma antibody myosin IB (CMI23) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa Department of Biology, Iowa City, IA 52242, mouse monoclonal anti-α-actin antibody (catalog no. A1978, Sigma), mouse monoclonal anti-γ-actin antibody was kindly provided by Dr Christine Chapronnier (Geneva Faculty of Medicine, Switzerland) (Dugina et al., 2009), the rabbit polyclonal anti-CDK2 antibody (catalog no. sc-163, Santa Cruz), and rabbit polyclonal anti-GAPDH antibody (catalog no. 14C10, Cell Signaling). Filamentous actin was stained by using Alexa Fluor® 488, 546 or 647 phallodin (Invitrogen). For secondary antibodies in western blotting anti-rabbit-HRP and anti-mouse-HRP (Chemicon International) were used and for immunofluorescence Alexa Fluor® anti-rabbit 594, Alexa Fluor® anti-mouse 647 or Alexa Fluor® anti-mouse 488 (Invitrogen). Other reagents used: 10 µg/ml fibronectin (catalog no. 374-0301, from VWR International) for coating coverslips.

Plasmid constructs and transfections

Myosin IIα (MYH9) was obtained from the human ORFeome library (Open Biosystems) as a Gateway pENTR223 entry clone, from where it was further transferred into the pcDNA6.2/N-EmGFP-DEST destination vector (Invitrogen) to generate GFP-tagged-myosin IIα using LR reaction kit (catalog no. 12538-013, Invitrogen). Human GFP-α-actinin-1 and GFP-α-actinin-4 were kindly provided by Prof. Carol Otey (University of North Carolina School of Medicine, USA) (Edlund et al., 2001), and GFP-RacV12 and GFP-RhoV14 by Dr Anne Debant (CRBM-Macromolecular Biochemistry Research Center, France) (Bellanger et al., 2000). For overexpression studies Fugene6 transfection reagent (Roche) was used and for siRNA-mediated gene silencing Lipofectamine2000 transfection reagent (Invitrogen). Human p3 (actinin-1 and Rac1 271 m was drawn to

Immunofluorescence and western bloting analyses

For immunofluorescence analysis U2OS cells seeded on glass or fibronectin (10 µg/ml) coated coverslips were fixed with 3.5% (w/v) paraformaldehyde (PFA) for 15 minutes and washed three times with TBS prior to permeabilization using 0.1% Triton X-100 for 5 minutes. Then washed three times with PBS, blocked with 5% goat serum in TBS for 30 minutes, labeled with primary antibody for 30 minutes, washed three times with TBS, labeled with secondary antibody for 30 minutes, washed three times with TBS, labeled with Hoechst, and mounted with Immu-mount (Thermo Scientific). Cells analyzed by β-actin antibody stainings were fixed in 1% PFA for 10 minutes and for 7 minutes with MeOH at −20°C followed by the staining protocol described above. For γ-actin antibody stainings antibody stainings were fixed in 1% PFA for 10 minutes (Dugina et al., 2009).

Stained coverslips were analyzed and imaged using upright Zeiss AxioImager M2 epifluorescence microscope equipped with 63x/Plan-Apochromat/1.40 Oil/ M27 and AxioCam HRm camera, inverted Zeiss LSM 510 Meta and upright Leica TCS SP5 laser scanning confocal microscopes equipped with 63x/Plan-Apochromat/1.40/DC and 63x/HCX APo/1.38/Corr (glycerol)/CS21 objectives, respectively. Fluorescence images were generated using Adobe Photoshop CS5, version 12.0 and Adobe Illustrator CS5, version 15.0.

For western blotting cells were lysed using SDS boiling buffer (2.5% SDS, 0.25 M Trizma base including 50 mM NaF, 10 mM β-glycerophosphate, 0.5 mM DTT, 0.5 mM PMSF, 2.5 µg/ml aprotinin and 1 µg/ml leupeptin), needle (25-gauge) and centrifuged at 20,800 g for 10 minutes at 4°C prior to protein concentration measurements using Bio-Rad DC protein assay (Bio-Rad Laboratories). 10–30 µg of lysates were run on SDS-PAGE gel, and for western blotting analysis TBS including 0.05% Tween 20 and 5% BSA was used for blocking and antibody incubations. Primary antibodies were incubated overnight and secondary antibodies for 30 minutes prior to detection with ECL reagents (SuperSignal West Femto, PIERCE).

Wound healing, transwell migration, cell spreading and live imaging

For the wound-healing assay 70,000 cells were initially seeded 24 hours before transfections and cell number was counted again following 24 hours of transfection. Wounds were generated to confluent monolayers of U2OS cells with a sterile tip 72 hours after transfection of pooled non-targeting or ACTN1 targeting siRNAs. Wound areas were marked to the bottom of each plate to enable image acquisition of the same area using Olympus CKX4 microscope equipped with UplanFLx4/0.13xPh objective and Canon D6004i EOS 300D digital camera. Images were taken 24 hours from the wound start when control cells almost reached confluence. Similar transfection set-up as above was used to perform time-lapse imaging during wound closure, except cells were seeded on 6-well plates. Using Cell-IQ (CM Technologies) platform, phase-contrast images were taken hourly using 10x objective from cells cultured at 37°C in 5% CO2 for 24 hours. Data derives from five independent experiments. Subsequently all images were maximum intensity projection (MIP) generated using Adobe Photoshop CS5, version 12.0, and the ImageJ software 1.46 calculating open areas in pixels. Transwell migration assay was performed using Matrigel-coated (catalog no. E1270, Sigma) 24 mm Transwell invasion chambers with 8 µm pore polycarbonate membrane inserts (catalog no. 3428, Corning) according to manufacturer’s instructed CSS5, version 15.0.

Time-lapse imaging of EGFP-CLP-36 expressing U2OS cells (Vallenius et al., 2004) was utilized in spreading assay and actin stress fiber dynamic analysis. For spreading assay 72 hours after transfection of pooled non-targeting or ACTN1 siRNA oligos cells were re-plated on fibronectin coated coverslips and allowed spreading for 30 minutes prior to 3.5% PFA fixation or were re-plated on fibronectin coated Lab-Tek chambers (catalog no. 155379, Nunc) for time-lapse imaging. For time-lapse imaging of actin stress fibers non-targeting or ACTN1 targeting siRNA oligos were conducted as mentioned above but cells were allowed to spread on fibronectin coated Lab-Tek chambers for 3 h prior live imaging. For all time-lapse imaging Zeiss Stellion HSI microscope equipped with Zeiss AxioCam MRm camera and EC Plan-Neofluar 63x/1.30 Oil Ph3 objective was used for spreading assay and actin stress fiber dynamic analysis. For F-actin stress fiber dynamic analysis images were acquired every 2 minutes for the period of 2 hours at 37°C and 5% CO2 by using phase contrast and GFP fields. SlideBook 4.2 software and QuickTime Player Pro 7.6 were used for generating movies.

Image analysis and quantifications

Dorsal stress fiber base and trunk areas were quantified by measuring the ratio of the integrated density of α-actinin-1 or α-actinin-4 antibodies and F-actin (phallolin) by using ImageJ software 1.46. A base part of dorsal stress fiber was determined to be 1:4 and a trunk 3:4 of dorsal stress fiber length attaching with a transverse arc (Fig. 1G,H; supplementary material Fig. S1A,B). To measure F-actin intensity of nonlinear rudimental dorsal stress fibers following α-actinin-1 silencing a 1 µm area from adhesion was determined as the base of the dorsal stress fiber (Fig. 2E; supplementary material Fig. S1A).

Using ImageJ software 1.46 the vinculin positive leading edge adhesion sites were quantified from an area of 10 µm×10 µm, which was drawn by first determining the midpoint of nucleus, from where the vertical axis was drawn towards the highest adhesion amount of vinculin positive adhesions at the leading edge. For the tragiing edge analysis a horizontal axis was determined perpendicular to the vertical leading edge axis and the boxed area of 10 µm×10 µm was drawn to width of most of adhesions at the edge. For the trailing edge analysis the area and amount of adhesions were quantified.

Circumference of spreading cells was measured from phase-contrast time-lapse images (Fig. 5B,C) or fixed cells (supplementary material Fig. S4E). For analysis cells reaching fan-shaped morphology during 120 minutes follow-up time without attaching neighboring cells were chosen, and cell circumference was analyzed by...


Dorsal fibers need α-actinin-1 and Rac1


**Fig. S1. α-actinin-1 localizes throughout dorsal stress fibers.** (A, B) Box-plot analyses show ratio of α-actinin-1 (A; fiber n=117) or α-actinin-4 (B; fiber n=81) intensities against F-actin, separately from focal adhesions (FA), base of dorsal stress fibers (base) or trunk of dorsal stress fibers (trunk). Values are normalized to ratios in focal adhesions. Grey asterisks stand for outliers. (C, D) Immunofluorescence analysis of U2OS cells transiently expressing GFP-tagged α-actinin-1 (A1-GFP) or α-actinin-4 (A4-GFP) co-stained with F-actin and vinculin antibody as indicated. Filled arrowheads mark dorsal stress fibers, open arrowheads transverse arcs and arrows ventral stress fibers. (E) Immunofluorescence analysis of α-actinin-1 stained non-targeting siRNA (si-NT) and α-actinin-1 silenced (si-A1) cells. Endogenous α-actinin-1 is localized on all stress fiber subtypes (as indicated above) whereas following α-actinin-1 depletion endogenous α-actinin-1 is undetectable. *p<0.05 and ***p<0.001 by Student’s t-test. Scale bar 10 µm.
Fig. S2. Requirement of α-actinin-1 for dorsal stress fiber assembly and double knockdown of α-actinin-1 and α-actinin-4. (A) F-actin staining of migrating U2OS cells transfected with non-targeting (si-NT) and two independent α-actinin-1 targeting siRNAs as indicated. Filled arrowheads mark dorsal stress fibers not noted in the two α-actinin-1-siRNA transfected cells (si-A1-oligo1 and si-A1-oligo2) whereas ventral stress fibers (arrow) and transverse arcs (open arrowhead) are unaltered. (B) Western blotting analysis of lysates from cells shown in (A) using α-actinin-1 (A1-341) or CDK2 (loading control) antibodies as indicated. (C) A longer exposure of an immunoblot following siRNA mediated downregulation of α-actinin-1, α-actinin-4 or both as indicated. (D) A merged image of F-actin and Hoechst staining of a U2OS cell transfected with α-actinin-1 and α-actinin-4 targeting oligos simultaneously. (E) Western blotting analysis of lysates from control cells and cells shown in (D) using indicated antibodies. Images are from the same blots and white lines indicate removal of intervening lanes. Scale bar 10 µm.
Fig. S3. Dorsal stress fiber base remnants following α-actinin-1 silencing and loss of dorsal stress fibers in α-actinin-1 silenced MEFs. (A) Merged immunofluorescence images of F-actin and vinculin stained non-targeting (si-NT) or α-actinin-1 (si-A1) downregulated U2OS cells. Zoom-in images of the boxed areas to demonstrate occasionally detected bases of dorsal stress fibers following α-actinin-1 silencing (compare filled arrowhead and asterisk). (B, C) Immunofluorescence analysis of fan-shaped (B) and spreading (C) immortal mouse embryonic fibroblasts (MEFs) following non-targeting (si-nt) or α-actinin-1 (si-a1) downregulation. Cells are stained using α-actinin-1 (A1-341), vinculin antibodies and F-actin staining as indicated. Dorsal stress fibers are marked with filled arrowheads, transverse arcs with open arrowheads and ventral stress fibers with arrows. (D) Western blotting analysis of MEFs shown in B. GAPDH is used as loading control. Scale bar 10 μm.
Fig. S4. Dorsal stress fibers at wound edge cells and a lack of myosin IIB on dorsal stress fibers (A) F-actin and vinculin co-staining of U2OS cells after a scratch wound. Arrowheads point examples of dorsal stress fibers. (B) Zoom-in of the boxed area in (A) to demonstrate prominent α-actinin-1 (A1-341) staining on dorsal stress fibers. Filled arrowheads mark dorsal stress fibers, open arrowheads transverse arcs and arrows ventral stress fibers. (C) Time-lapse frames of fan-shaped migrating U2OS cells. (D) Cell number of U2OS cells transfected with non-targeting (si-NT) or α-actinin-1 (si-A1) targeting siRNA 72 hours following first transfection demonstrate no significant difference. 70 000 cells were seeded 24 hours prior to first transfection. Cells are counted from five independent experiments. (E) Quantification of cell circumference (see material and methods) from α-actinin-1 (si-A1) targeted U2OS cells relative to non-targeted (si-NT) cells does not demonstrate significant difference from 20 analyzed cells form three independent experiments. (F) Immunofluorescence images of U2OS stained with myosin IIB antibody and F-actin. The filled arrowheads mark a dorsal stress fiber, which lacks myosin IIB staining whereas transverse arcs show myosin IIB staining (open arrowheads). Scale bar 10 µm.
Fig. S5. Decreased early cell spreading rate in α-actinin-1 downregulated cells and unaltered gamma-actin staining in α-actinin-1 silenced cells. (A) A merge immunofluorescence image of α-actinin-4 and F-actin or α-actinin-4 and vinculin stained spreading U2OS cell seeded on fibronectin coated coverslips for 30 minutes. Arrowhead mark dorsal stress fibers. (B) Arbitrary cell circumference of non-targeting (si-NT) and α-actinin-1 silenced (si-A1) cells from time-lapse images taken every two minutes after plating on fibronectin coated coverslips as indicated. (C) Immunofluorescence images of gamma-actin stained migrating non-targeting siRNA (si-NT) or α-actinin-1 silenced (si-A1) U2OS cells. Dorsal stress fibers (filled arrowhead), transverse arcs (open arrowhead) and ventral stress fibers (arrow) as indicated. Scale bar 10 µm.
Fig. S6. Active RhoA induces ventral stress fibers and loss of Rac1 leads to beta-actin accumulation at the leading edge. (A) Immunofluorescence analysis of cells transiently expressing GFP (control) or GFP-RhoA V14 (RhoA V14) co-stained with F-actin and vinculin antibody as indicated. Arrowheads point to dorsal stress fibers and arrows to ventral stress fibers used for quantification in Fig. 6C,D. (B) Immunofluorescence images of beta-actin stained migrating non-targeting siRNA (si-NT) or Rac1 silenced (si-Rac1) U2OS cells. Zoom-in images of the boxed areas are shown as insets. Asterisk indicates a broad beta-actin band at the leading edge. Scale bar 10 µm.
**Movie 1. Dorsal stress fiber dynamics in U2OS cells.** Time-lapse imaging of U2OS control cells stably expressing GFP-tagged CLP-36 (Vallenius et al., 2004) over a period of 82 min. In control cells prominent dorsal stress fibers (filled arrowhead) are noted and new dorsal stress fibers following 14 min of imaging (new filled arrowheads). Dorsal stress fiber connection to transverse arcs (open arrowhead) is evident starting from 34 min timepoint. N stands for nucleus.

**Movie 2. Loss of dorsal stress fibers upon α-actinin-1 silencing.** Time-lapse imaging of α-actinin-1 silenced cells stably expressing GFP-tagged CLP-36 (Vallenius et al., 2004) over a period of 82 min. Few dorsal stress fiber or its remnants (filled arrowhead) appear, which are weak, nonlinear and disappear abruptly. N stands for nucleus.
Movie 3. $\alpha$-actinin-1 depleted cells migrate more slowly. Series of phase-contrast images taken every hour for 24 hours during wound healing of control (si-NT) or $\alpha$-actinin-1 silenced (si-A1) cells.

Movie 4. $\alpha$-actinin-1 silencing leads to a loss of dorsal stress fibers and delayed early cell spreading. Series of fluorescent and phase-contrast images taken every 2 minutes for 120 minutes of control (si-NT) and $\alpha$-actinin-1 (si-A1) silenced cells both stably expressing EGFP-tagged CLP-36 (Vallenius et al., 2004) plated at time = 0 on fibronectin. The 20 minutes time point is marked. Control cells spread during 20 minutes, and start migrating at approximately 30 minutes, whereas $\alpha$-actinin-1 depleted cells spread significantly slower.