Non-muscle myosin II induces disassembly of actin stress fibres independently of myosin light chain dephosphorylation

Tsubasa S. Matsui^{1,†}, Roland Kaunas³, Makoto Kanzaki¹,
Masaaki Sato^{1,2} and Shinji Deguchi^{1,*}

¹*Department of Biomedical Engineering, and* ²*Department of Bioengineering and Robotics, Tohoku University, Sendai 980-8579, Japan*

³*Department of Biomedical Engineering, Texas A&M University, College Station, TX 77843-3120, USA*

Dynamic remodelling of actin stress fibres (SFs) allows non-muscle cells to adapt to applied forces such as uniaxial cell shortening. However, the mechanism underlying rapid and selective disassembly of SFs oriented in the direction of shortening remains to be elucidated. Here, we investigated how myosin crossbridge cycling induced by MgATP is associated with SF disassembly. Moderate concentrations of MgATP, or [MgATP], induced SF contraction. Meanwhile, at [MgATP] slightly higher than the physiological level, periodic actin patterns emerged along the length of SFs and dispersed within seconds. The actin fragments were diverse in length, but comparable to those in characteristic sarcomeric units of SFs. These results suggest that MgATP-bound non-muscle myosin II dissociates from the individual actin filaments that constitute the sarcomeric units, resulting in unbundling-induced disassembly rather than end-to-end actin depolymerization. This rapid SF disassembly occurred independent of dephosphorylation of myosin light chain. In terms of effects on actin–myosin interactions, a rise in [MgATP] is functionally equivalent to a temporal decrease in the total number of actin–myosin crossbridges. Actin–myosin crossbridges are known to be reduced by an assisting load on myosin. Thus, the present study suggests that reducing the number of actin–myosin crossbridges promotes rapid and orientation-dependent disassembly of SFs after cell shortening.

Keywords: non-muscle myosin II; actin; stress fibres; cell shortening; mechanobiology

1. INTRODUCTION

Actin stress fibres (SFs) are contractile structures in non-muscle cells [1]. SFs are primarily composed of actin, non-muscle myosin II (NMII), α -actinin and tropomyosin [2]. The structure of mature SFs has periodic arrays of these proteins similar to the organization of myofibrils. The periodicity measured in non-muscle PtK2 cells is approximately 1.2 μm [3]. Contractile forces generated by NMII–actin interactions can lead to shortening of existing SFs, as well as contribute to the assembly of the extracellular matrix [4,5], focal adhesions and SFs themselves [2,6–10]. SF reorganization is

also induced by externally applied forces. For example, cyclic matrix stretch causes cells to reorient their SFs away from the direction of stretch [11–17].

Molecular mechanisms of SF assembly and disassembly have been extensively studied [18]. Increased phosphorylation of myosin regulatory light chain (MLC) promotes the assembly of new SFs in a gradual process that takes place over several minutes. The conventional mechanism for SF disassembly involves MLC dephosphorylation, via MLC phosphatase (MLCP), and the subsequent decreases in actomyosin MgATPase activity and contractile force required for maintaining SF structural organization. This relatively slow disassembly process occurs through kinetic competition between MLCP and the Rho kinases (Rho-kinase, myosin light chain kinase (MLCK), and zipper-interacting protein kinase) over several minutes, similar in timescale to the phosphorylation of MLC and the resulting assembly of SFs. In contrast, orientation-dependent disassembly of SFs can occur in a matter of seconds in response to sudden large-magnitude shortening of the matrix to which cells adhere [19] or SF detachment via localized

*Author and address for correspondence: Department of Bioengineering and Robotics, Tohoku University, Sendai, Japan (deguchi@bml.mech.tohoku.ac.jp).

[†]Present address: Department of Biomolecular Sciences, Tohoku University, Sendai, Japan.

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rsfs.2011.0031> or via <http://rsfs.royalsocietypublishing.org>.

One contribution of 9 to a Theme Issue ‘Nanoengineering life: from cell to tissue’.

application of trypsin at focal adhesions [20,21]. These studies suggest that MLC dephosphorylation alone is insufficient to account for such rapid and selective disassembly of SFs oriented in the direction of cell shortening.

Studies using extracted SFs, which allow control over the chemical environment interacting with the SFs, have provided important insight into the mechanisms of SF disassembly. MgATP is ubiquitous in living cells, and its intracellular concentrations (i.e. approx. 2–8 mM) are maintained very precisely under various physiological conditions [22]. Using extracted SFs, we showed that SFs contracted and remained relatively stable at [MgATP] below 2 mM [23]. At high physiological levels ([MgATP] > 5 mM), addition of MgATP induced rapid SF disassembly at rates similar to that observed for shortening- and trypsin-induced SF disassembly. These results suggest that rapid turnover of actin–myosin bonds via the powerstroke cycle contributes to SF disassembly independent of MLCP activity. This mechanism may be important for rapid SF disassembly caused by cell shortening.

Our previous experiments observing MgATP-induced SF disassembly were performed using phase-contrast microscopy that limited our ability to analyse disassembled actin at dimensions below optical resolution. Consequently, we were uncertain how SFs, having a width of approximately 200–500 nm [24], disperse into smaller constituent molecules when placed in MgATP-rich environments. Here, we found that the process of MgATP-induced SF disassembly occurs via shedding of actin filaments with lengths similar in size to those of the sarcomeric units.

2. MATERIAL AND METHODS

2.1. Cell lines

A7r5 vascular smooth muscle cell lines (ATCC, CRL-1444) were cultured in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10 per cent foetal bovine serum (JRH Biosciences) and 1 per cent penicillin–streptomycin (Invitrogen). We confirmed by immunostaining that the cells expressed non-muscle β -actin (anti- β -actin, Applied Biological Materials) and myosins IIA (anti-non-muscle myosin heavy chain IIA, Covance) and IIB (anti-non-muscle heavy chain myosin, Abcam) along the length of the SFs.

2.2. Solutions

Low ionic strength (μ) solution contained 2.5 mM triethanolamine [25], 1 mM dithiothreitol (DTT), $1 \mu\text{g ml}^{-1}$ leupeptin and $1 \mu\text{g ml}^{-1}$ pepstatin. Wash solution contained 2 mM free Mg^{2+} , 20 mM imidazole, 2 mM EGTA, 10 mM DTT, $1 \mu\text{g ml}^{-1}$ leupeptin and $1 \mu\text{g ml}^{-1}$ pepstatin. Activating solution contained 2 mM free Mg^{2+} , 20 mM imidazole, 2.1 mM CaCl_2 , 2 mM EGTA (to adjust pCa to 4.5 at pH 7.2), 10 mM MgATP and oxygen-removal reagents (2.3 mg ml^{-1} glucose, 0.018 mg ml^{-1} catalase and 0.1 mg ml^{-1} glucose oxidase; [26]). The pH was adjusted to 8.2 (low μ solution) or 7.2 (wash solution and activating solution) with KOH at room temperature. The μ -value of both

wash solution and activating solution was adjusted to 170 mM with potassium chloride. The compositions were calculated based on the equilibrium constants for all the above metals and ligands described by Fabiato & Fabiato [27].

2.3. Exposure of stress fibres

Cells cultured on a cover glass (18 mm square) were washed twice with cold PBS. Cells were rinsed once with ice-cold low μ solution, and then quickly de-roofed (approx. 1 min) by exposing to repeated applications of fresh low μ solution from a pipette [28–30]. After observing under a phase-contrast microscope that the apical plasma membrane and most cellular organelles, including the nucleus, were removed (figure 1), low μ solution was replaced with ice-cold wash solution. Ventral SFs and the cell cortex lying along the basal plasma membrane remained on the cover glass. Since most, if not all, cytoplasmic MgATP and free actin and myosin molecules were removed, all remaining myosin heads were expected to bind to actin in the rigour state.

2.4. Observation of dynamic behaviour of stress fibres

The cover glass supporting the exposed SFs was placed upside down over a glass plate to make a perfusion chamber with 10 mm width, 18 mm length and approximately 100 μm gap height using two parallel strips of scotch tape and a nail polish adhesive. The channel between the two glass surfaces was filled with wash solution containing 200 μM cytochalasin D (CytoD, Santa Cruz) with or without 300 nM Alexa 488-phalloidin (Molecular Probes) for 3 min, and then perfused by wash solution containing 200 μM CytoD via capillary action. The chamber was mounted on the stage of a microscope (IX-71, Olympus), activating solution containing MgATP was perfused from one side of the chamber and time-lapse epifluorescence images of SF behaviour were captured with a UPlanFL N objective (100X, NA 1.3, oil, Olympus) using a charge-coupled device camera (ORCA-R2, Hamamatsu). After treatment with activating solution for 3 min, the solution was exchanged with wash solution containing 200 μM CytoD. Actin-associated structures that dispersed from the SFs during MgATP treatment and later adhered to the opposite coverglass in the chamber were imaged to detect the sizes of actin-containing fragments. All experiments were performed at room temperature (22–24°C). For preparation of solutions, water-insoluble CytoD was dissolved in dimethyl sulphoxide to a concentration of 20 mM and stored at -80°C . These stocks were diluted with either wash solution or activating solution to the working concentration.

2.5. Characterization of actin-containing fragments

We characterized the composition of the individual dispersed actin fragments by assessing the effect of including CytoD in the wash solution. CytoD caps the barbed ends of actin filaments to block polymerization. Thus, 200 μM CytoD was included in the wash solution to prevent elongation (polymerization and end-to-end

Figure 1. SFs exposed to high [MgATP] disassemble, while the cell cortex remains. (a) Phase contrast and (b) fluorescence images of a cell stained with Alexa-conjugated phalloidin. (c,d) Phase contrast image of extracted ventral SFs (c) before and approximately 1 min (d) after the addition of 10 mM MgATP. (e) Fluorescence image of the same cell stained with Alexa-phalloidin showed that only the basal cell cortex and no SFs remained after MgATP treatment. (f) Magnified view of a part in (e), showing a wavy actin network, but no long and straight SFs. Fluorescence image of (b) was acquired with a standard exposure time of the camera of <1 s, whereas that in (e) required a longer exposure time of >5 s because of much lower fluorescence intensity. See also electronic supplementary material, movie S1. Scale bars, (a–e) 20 μm ; (f) 4 μm .

annealing) of actin filaments in the activating solution after their dispersal from intact SFs. To evaluate the efficiency with which CytoD inhibited fragment growth, we performed the following experiment. Two coverslips were prepared containing SFs labelled with two different dyes conjugated to phalloidin. Specifically, SFs on one coverslip were labelled with Alexa 488-phalloidin and the other with Alexa 546-phalloidin. The two coverslips were then adhered to each other with the SFs on the inner surfaces to form a single perfusion chamber. The chamber was then treated with wash solution either lacking CytoD or containing CytoD and then treated with the activating solution containing 10 mM MgATP. Epifluorescence images of shed actin fragments were captured on IX-71 microscope with a UPlanSApo objective ($100\times$, NA 1.4, oil, Olympus) using the ORCA-R2 camera.

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting

Cells and extracted SFs were solubilized in sodium dodecylsulphate (SDS) sample buffer. Samples were

boiled for 5 min and fractionated on 10 per cent polyacrylamide gels. Gels were stained with CBB (Coomassie Brilliant Blue) G-250 (Bio-rad Laboratories). For immunoblotting, samples were resolved on polyacrylamide gels (7.5% or 12.5%) and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were incubated with antibody against NMIIA (1 : 1000, Covance), NMIIIB (1 : 1000, Abcam), ROCK-2 (1 : 1000, Abcam), MYPT1 (myosin phosphatase1, 1 : 1000, Cell Signaling Technology), MLCK (1 : 1000, Abcam), α -actinin (1 : 1000, Santa Cruz Biotechnology), caldesmon (1 : 1000, Abcam), α -SMA (1 : 1000, Abcam), β -actin (1 : 1000, Applied Biological Materials), tropomyosin (1 : 1000, Sigma-Aldrich), MLC (1 : 1000, Cell Signaling Technology), 1P-MLC (1 : 1000, Cell Signaling Technology), 2P-MLC (1 : 1000, Cell Signaling Technology) and calmodulin (1 : 1000, Abcam). The primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Bio-Rad Laboratories) and enhanced chemiluminescence (Immobilon Western, Millipore).

Figure 2. SDS-PAGE and immunoblotting of extracted SFs. (a) CBB-stained gel and (b) immunoblotting analyses of a crude extract from intact cells and an extracted SF preparation.

2.7. Immunostaining

Cells were fixed with 4 per cent paraformaldehyde for 10 min, washed with PBS containing 30 mM glycine, permeabilized with 0.1 per cent Triton X-100 (TX) for 3 min, treated with 10 per cent normal goat serum in blocking solution (Block Ace, AbD Serotec) for 1 h at room temperature. The cells were then incubated with anti- α -actinin mouse monoclonal antibody (Sigma) at 4°C overnight, incubated with Alexa 647-conjugated secondary antibody (Molecular Probes) and then double-stained with Alexa 488-conjugated phalloidin. Epifluorescence images were captured using the ORCA-R2 camera.

2.8. Image analysis

Fluorescence intensity along a particular line in acquired images was obtained by IMAGEJ (NIH). Fast-Fourier transform analysis on the intensity profile was performed using a custom-made program written in LabVIEW (National Instruments) to obtain the distance between peaks. To quantify the length of fluorescent actin filaments, individual filaments in acquired images were detected semi-automatically based on the difference in fluorescence intensity between the background and filaments using another custom-made program written in Vision (National Instruments), thus yielding the contour length of each filament. In cases where filaments cross, we measured the length by careful manual tracing. We did not count lengths of $\leq 0.5 \mu\text{m}$ because of the limit of optical resolution. Unpaired Student's *t*-tests (for variables with a Gaussian distribution) or Mann-Whitney *U*-tests (for variables with a non-Gaussian distribution) were used as appropriate to compare independent variables between groups.

3. RESULTS

3.1. Contraction of extracted stress fibres

SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed to examine the composition of the SF preparation extracted from A7r5 cells (figure 2). Compared with the whole-cell preparation, the SFs from de-roofed cells contained a limited number of polypeptide bands. A total of 12 proteins (NMIIA, NMIIIB, ROCK-2, MYPT1, MLCK, α -actinin, caldesmon, α -SMA, β -actin, tropomyosin, MLC and calmodulin) were identified by immunoblotting (figures 2*b* and 3). The relative amounts of 1P-MLC (i.e. monophosphorylation of MLC at Ser19) and 2P-MLC (i.e. diphosphorylation of MLC at Thr18/Ser19) to total MLC showed that MLC in the extracted SFs is not dephosphorylated (figure 3). Using this SF preparation with actin filaments labelled with Alexa-phalloidin, we performed time-lapse fluorescence microscopy to confirm that the SFs maintained MgATP-induced contractile function in the presence of phalloidin. Our previous time-lapse imaging by phase-contrast microscopy showed that unlabelled extracted SFs promptly disassembled at [MgATP] above 4 mM, whereas the SFs shortened in axial length at [MgATP] below this critical value [23]. In the present study, we used an [MgATP] of 2.7 mM ($\mu = 157 \text{ mM}$, $\text{pMg} = 2$), which caused phalloidin-bound SFs to shorten at a constant rate of approximately $0.8 \mu\text{m s}^{-1}$ before collapsing into a tight mass without inducing disassembly (figure 4). This rate is smaller than but comparable to the rates we previously measured for unlabelled extracted SFs (approx. $1.8 \mu\text{m s}^{-1}$ at 3 mM [MgATP]; [23]).

3.2. Sarcomeric pattern along stress fibres

We next used α -actinin immunostaining to characterize the sarcomeric pattern within SFs. Periodic striations

Figure 3. MLC of extracted SFs is not dephosphorylated. Extracted SFs were processed for immunoblotting to detect total, 1P- and 2P-MLC. The relative amounts of 1P- and 2P-MLC to total MLC are shown.

were detected along the length of individual SFs within intact cells (figure 5*a*). Striation patterns with similar periodicity were detected in extracted SFs (figure 5*b*). These striations were not as distinct as those typically observed in myofibrils, which have a highly ordered periodic structure composed of thin filaments attached to the Z-line (consisting of α -actinin) and thick filaments located in the middle of the sarcomere [31,32]. Interestingly, thinner SFs tended to have the most consistent striations (figure 5*c,e(i)*). Meanwhile, thicker SFs, which were more common than thin SFs, exhibited apparently irregular striations characterized by non-uniform α -actinin staining not only in the axial direction but also across the width of the SF (figure 5*d,e(ii)*). We quantified the periods of the relatively regular striations of thin SFs by spectral analysis as a representative of the sarcomeric unit length (figure 5*f,g*). Statistical analysis using an unpaired Student's *t*-test showed that there was not a significant difference in the length distributions of SFs from intact cells and extracted SFs.

3.3. MgATP-induced disassembly of stress fibres

Next, we characterized the disassembly of extracted SFs upon treatment with high [MgATP]. To accomplish this, the wash solution was exchanged with activating solution containing 10 mM MgATP, resulting in complete SF disassembly in approximately 10 s (figure 6*a*). Prior to treatment with 10 mM MgATP, phalloidin staining appeared relatively uniform along the length of the SFs (figure 6*a*, left). After exposure

to 10 mM MgATP, periodic patterns emerged along the length of the SFs after approximately 3 s, soon followed by the dispersal of short fragments of the SFs into the solution. Intensity measurement and its spectral analysis indicated a periodicity in phalloidin staining of approximately 2 μ m (figure 6*b,c*).

Following MgATP exposure, the reaction was quenched by washing away the activating solution. Actin fragments that dispersed from the SFs and passively adhered to the opposing coverslip at the edges of the sample chamber were imaged and analysed. If the chamber was washed only once, some of the actin fragments maintained the capability to exhibit mobility when incubated with wash solution (electronic supplementary material, figure S1). By comparing images taken over a time interval of 2 s, it is apparent that this mobility was predominantly oriented along the fragment major axes. This mobility was lost after a second wash, suggesting that the mobility of these fragments was caused by myosin motor activity driven by residual MgATP remaining after the first wash. This mobility was also inhibited if the extracted SFs were treated with 0.1 per cent TX for 30 min at 4°C prior to MgATP administration. TX inactivates RhoA/Rho-kinase without dissociating actin, myosin and α -actinin from extracted SFs [33]. These results suggest that the mobility of these actin fragments was caused by both MgATP- and Rho-dependent actomyosin interactions. Further, TX pretreatment rendered the periodic patterns created after 10 mM MgATP exposure (figure 6*d-f*) to be more distinct than the patterns observed when TX was not applied (electronic supplementary material, figure S2). Statistical analysis using a Mann-Whitney *U*-test showed that there was not a significant difference in the intensity peak-to-peak distance between SFs with and without TX pretreatment.

3.4. Characterization of shed actin fragments

Quantification revealed that these shed actin fragments have very uniform fluorescence intensity (figure 7*a*). Specifically, intensities quantified from various positions along the length of individual filaments and between different filaments differed by only approximately 2 per cent, resulting in a very small variance (figure 7*b*, first bar). As phalloidin attaches to actin subunits at a 1:1 molar ratio [34], fluorescence intensity is expected to be proportional with the amount of actin. Indeed, the average intensity increased in a step-wise manner at points where two or three actin fragments crossed (figure 7*a*, arrows and arrowheads; figure 7*b*, second and third bars, respectively). If these fragments consisted of filament bundles, some bundles would be expected to fluoresce more brightly than others owing to differences in the number of filaments bundled together. Taken together, these results indicated that these fragments consist of individual actin filaments.

Next, we characterized the composition of the individual shed actin fragments by assessing the effect of including CytoD in the wash solution. Dual-labelled actin fragments were observed when CytoD was

Figure 4. The extracted SFs stained with Alexa-phalloidin are contractile at a moderate $[MgATP]$ of 2.7 mM. The graph depicts the gradual decrease in length of a representative SF (arrowheads in micrographs) after the initiation of contraction. See also electronic supplementary material, movie S2. Scale bar, 20 μ m.

absent (figure 8a). The presence of CytoD almost completely inhibited such dual-labelling of the fragments (figure 8b). We quantified the distribution in actin fragment lengths when CytoD was not included in the wash solution as compared to when the wash solution contained CytoD (figure 9). Substantially long ($>4 \mu$ m) fragments were observed in the absence of CytoD regardless of phalloidin labelling and/or TX pretreatment. In contrast, fragments in the presence of CytoD were substantially shorter, with virtually no filaments longer than 2.5 μ m. Here, in the case of unlabelled SFs (Phl-), the shed actin fragments were labelled with Alexa 488-phalloidin after MgATP treatment, washing and capture.

4. DISCUSSION

It is well accepted that NMII plays a critical role in SF assembly. By studying the behaviour of extracted SFs exposed to various concentrations of MgATP, we have obtained new insight into the additional role of NMII in SF disassembly. At 2.7 mM MgATP, SFs were stable and contracted steadily over tens of seconds. Phalloidin binding slows but did not impair SF contractility in our system. This is consistent with previous observations that phalloidin did not affect interaction of purified single actin with skeletal myosins [35]. Raising MgATP concentration to 10 mM, while maintaining the μ -value of the wash and activating solutions, caused the SFs to completely disassemble within 10 s. SF disassembly resulted in the dispersal of individual actin filaments with a length of approximately 1 μ m (figure 9). Note that the length of F-actin that constitutes the muscle sarcomere is approximately half the size of the sarcomeric unit [36]. The shed actin filaments are just half the length of an SF sarcomeric unit (figure 5), which is also comparable to that of peak distances that emerged along the length of the SFs (figure 6). These results suggest that the disassembly of SFs is not mediated by end-to-end depolymerization of constituent actin filaments, but rather by dissociation of the actomyosin

complex and unbundling of intact actin filaments that originally constituted the sarcomeric units.

To our knowledge, nebulin that is believed to determine F-actin length in myofibrils is not expressed in non-muscle cells. Thus, the length of SFs in non-muscle cells may inherently have a variety of lengths. Electron microscopy has been the sole method for analysing the microstructure of SFs such as the length of constituent actin filaments [3,37,38]; however, F-actin is present in a cross-linked actin architecture, which makes it difficult to identify individual filaments to determine their lengths [39]. The present method greatly simplifies the characterization of actin filament lengths. The extracted SFs in the rigour state were treated with CytoD and phalloidin at high concentrations to inhibit polymerization, end-to-end annealing [40] and depolymerization of the actin filaments. Subsequent MgATP treatment followed by dissociation of the actomyosin complex thus allows easy detection of the native lengths of actin filaments in SFs. It should be pointed out that the increase in filament length observed in the absence of CytoD pretreatment (cf. figure 8a) was owing to filament annealing after shedding, i.e. CytoD was not artificially shortening the filaments while they were still part of intact SFs.

MgATPase activity of NMII allowing actomyosin contraction, responsible for the maintenance of SF structures, is regulated by phosphorylation of MLC [6,41]. MLC is dephosphorylated by MLCP, and MLCP activity is regulated by Rho-kinase, an effector of RhoA. It has been reported that intracellular cyclic adenosine monophosphate (cAMP) levels are sharply elevated within 10 min following a release of cellular tension [42] or new integrin ligation with matrix [43]. Elevated cAMP mediates deactivations of RhoA and MLCK through protein kinase A (PKA), resulting in the dephosphorylation of MLC and subsequent SF disassembly [18]. However, it is unlikely the cAMP/PKA pathway is able to disassemble entire SFs within seconds through kinetic competition among MLCP, Rho-kinase and MLCK since that usually occurs over several minutes. In addition, there is no obvious

Figure 5. Characterization of sarcomere-like structures in SFs. (*a, b*) SFs in cells (*a*) and de-roofed cells (*b*) were dual-labelled with actin-binding phalloidin (green) and anti- α -actinin (red). (*c, d*) Some SFs displayed relatively regular striations along the long axis (*c*, magnified view of (i) in (*b*) containing apparently thin fibres), while in the others patches assessed by anti- α -actinin were non-uniform across their width ((*d*) magnified view of (ii) in (*b*) containing relatively thick fibres). (*e*) Intensity profiles of anti- α -actinin staining along the lengths of two representative SFs indicate that peak-to-peak distance was of the order of 1 μm in (i) of (*b*), whereas irregular distances were observed even along the long axis in (ii). (*f, g*) Histograms of peak-to-peak distances of anti- α -actinin intensity along the lengths of periodic thin SFs in cells (*f*) and extracted SFs (*g*). Scale bars, (*a, b*) 20 μm ; (*c–e*) 4 μm .

mechanism by which quick cell shortening is able to cause selective disassembly of only the SFs oriented in the direction of shortening [19–21] if this disassembly is caused only by diffusively transported biochemical signals [18].

Our results provide additional lines of evidence for the putative mechanism underlying selective disassembly of SFs (figure 10), which we previously discussed

[23] and here delve more deeply into the underlying mechanism. In addition to MLC-regulating intracellular signals, SF remodelling is controlled by mechanical cues from their environment. Such external loads transmit force to the SFs through focal adhesions, which are ultimately transmitted to NMII-actin bonds. Recent kinetic and single-molecule biophysical studies of skeletal muscle myosin II, smooth muscle myosin II and

Figure 6. Periodic actin patterns emerge along the length of SFs during MgATP-induced rapid disassembly. (a) Representative images from $n = 7$ experiments illustrating SFs before (left) and 5.3 s after exposure to 10 mM MgATP. (b) Intensity profile and magnified views of randomly selected regions (i)–(iii) from (a) show that SFs disassemble into short fragments of approximately $2 \mu\text{m}$ in length before completely dispersing. (c) Histograms of peak-to-peak distances measured by spectral analysis on the right image in (a). (d) Representative images from $n = 3$ experiments illustrating TX-treated SFs before (left) and 10 s after exposure to 10 mM MgATP. (e) As in (b), intensity measurements along regions (i)–(iii) in (d) indicate that exposure of TX-treated SFs to 10 mM MgATP induces SF breakdown into actin fragments of approximately $2 \mu\text{m}$ in length. (f) Histograms of peak-to-peak distances measured by spectral analysis on the right image in (d). The images in (b) and (e) are widened in the lateral direction for visual clarity. See also electronic supplementary material, movies S3 and S4. Scale bars, (a,d) $20 \mu\text{m}$; (b,e) $4 \mu\text{m}$.

Figure 7. Actin fragments shed from disassembling SFs consist of individual actin filaments. (a) In the absence of CytoD, polymerization and/or annealing of shed actin fragments into longer fragments were observed. (b) Fluorescence intensity on the actin filaments was nearly constant (1, mean + s.e.m., $n = 22$), except at locations where two (2, $n = 21$; e.g. arrowheads in (a)) or three (3, $n = 6$; e.g. arrow in (a)) different actin filaments cross. The intensity in those points increased in a stepwise manner, indicating that the filaments represent single actin filaments. Scale bar, 5 μm .

Figure 8. The effect of including CytoD in wash solution on the shed actin filaments. (a,b) Interactions of Alexa 488-labelled (green) and Alexa 546-labelled (red) actin fragments mixed in the same activating solutions without (a) or with (b) CytoD. In (a), where end-to-end annealing was permitted, actin filaments labelled with different dyes were observed. In (b), where CytoD blocked polymerization, very few actin filaments were observed to be labelled with both dyes. Scale bar, 5 μm .

NMII have revealed that the fraction of the MgATPase cycle time that the myosin head is attached to actin (i.e. the duty ratio) is significantly lowered by an assistive forward load on the myosin molecule [44–47]. Sudden SF shortening could provide a forward load on the constituent NMII. The expected decrease in the duty ratio (whose original value in unstrained condition was estimated to be 0.05 in NMIIA isoform and 0.23–0.4 in NMIIIB isoform; [48,49]) caused by the forward load could promote the detachment of entire myosin minifilaments. We submit that the binding of cytoplasmic MgATP to SFs being shortened quickly (enough to prevent new formation of actin–myosin crossbridges) may be sufficient to cause their prompt disassembly in a similar manner. This model is consistent with experimental observations that rapid SF disassembly is observed only when SFs are shortened faster than actomyosin sliding velocity [50,51].

Different from the selective SF disassembly we have discussed, Costa *et al.* [50] reported that all SFs within a cell disassemble after quick cell shortening. In their paper, three of the four images (cf. fig. 3c,d, 4a,b of their paper) used to demonstrate cell shortening-induced disassembly still contained actin bundles oriented preferentially away from the direction of cell shortening. SFs within an individual cell tend to be co-aligned along the major axis of the cell; hence, it is possible that Costa *et al.* observed the disassembly of all SFs in their fig. 3d because most SFs were originally aligned in the direction

of shortening. A subsequent study by Sato *et al.* [19] carefully quantified the proportion of cells that still contained SFs 15 min after cell shortening and reported that there were significantly fewer SFs in cells oriented in the direction of unloading than in cells oriented away from the direction of stretch. Additional experiments are underway in our group to definitively characterize the effects of SF shortening on disassembly.

While there are several similarities between SFs and myofibrils [36], these structures are clearly different in terms of stability. SFs are relatively unstable structures prone to fast turnover compared with myofibrils [52,53]. SFs have approximately 8–10 NMIIIs in each half of a myosin minifilament [45,54]. Myofibrils, in comparison, have approximately 150 skeletal myosin II per half a myosin thick filament [55]. The small number of myosin molecules in non-muscle myosin minifilaments may be responsible for the instability of SFs. In addition, SFs are expected to be particularly sensitive to assistive loads because of the markedly high load-dependent MgADP release rates of NMIIIB, the major NMII isoform present along the length of SFs [49]. Given that myofibrils are used for repeated shortening and lengthening, it would be highly disadvantageous to have such structural instability. SF turnover in non-muscle cells, on the other hand, is critical to cell shape changes and migration. Thus, non-muscle cells need their SFs to be inherently unstable in order to allow rapid SF remodelling.

Figure 9. Characterization of actin fragments shed from disassembling SFs. (a) Actin filaments obtained with (+) and/or without (–) CytoD, TX and phalloidin (Phl). Images are representative field-of-views from four or five independent experiments. (b) Length distributions of actin fragments obtained in each case. SF-derived actin filaments, whose polymerization and annealing are inhibited by CytoD, have lengths of the order of 1 μm and less than 4 μm . In contrast, the actin filaments in the absence of CytoD have longer lengths of more than 4 μm . Scale bar, 5 μm .

To our knowledge, there are no data regarding the dependence of NMII MgATPase activity and the duty ratio on [MgATP] measured in a physiological range of >2.5 mM. The only kinetic data we have noticed were reported by Wang *et al.* [49] in which MgATP-induced dissociation of purified actin and NMII was examined at ≤ 2.5 mM [MgATP]. They showed that [MgATP] dependence of the dissociation rates is still gradually increasing but reaching a saturated state at a concentration of 2.5 mM. The transition of SF condition from shortening (figure 4) to disassembly (figure 6) was induced only by increasing [MgATP] with a fixed μ -value of the solutions. Thus, actin–myosin crossbridges with a limited number of NMII in a sarcomeric unit may be close to the limit for complete dissociation around the physiological [MgATP] [23].

Our results are consistent with previous reports that NMII has a role in driving actin network disassembly [46,56–59]. Direct *in vitro* studies showed that NMII was involved in unbundling actin bundles composed of fascin, a globular actin filament cross-linker. When myosin II attached to the surface of actin–fascin bundles at high concentrations, the bundles disintegrated into single actin filaments. It was hypothesized that the forces produced by NMII motors, which may

penetrate into the bundles, break the bonds between F-actin and fascin [58]. Unlike actin–fascin bundles that form filopodia independent of NMII [60,61], SFs are crosslinked predominately by NMII, whose motor activity is necessary for maintaining the structural integrity of SFs as well as focal adhesions [18].

Non-muscle α -actinin is another major SF crosslinker whose role in SF stability should be considered. α -Actinin has a lower affinity for F-actin than NMII does [52,62]. We speculate that after actin–myosin crossbridges fail, α -actinin crossbridges are not sufficiently strong to resist SF disassembly. While NMII is necessary for SF stability, perhaps α -actinin serves the purpose of modulating the stability of SFs. Binding of α -actinin to actin is virtually inhibited in the presence of Ca^{2+} for pCa less than approximately 6 [62], which is much lower in concentration than the present experimental condition, pCa 4.5. This Ca^{2+} -dependency of non-muscle α -actinin is different from fascin, which is insensitive to Ca^{2+} [63,64]. Therefore, calcium influx after stress imposition [17,65–67] may also promote disassembly of SFs (but not of filopodia) by decreasing the affinity of α -actinin. Our extracted SFs contain other crossbridge-regulating proteins including caldesmon and tropomyosin (figure 2). While these proteins inhibit

Figure 10. A model of cell shortening-induced SF disassembly. (a) Actin–myosin crossbridges are known to be required for SF assembly and maintenance. (b) If MLC is dephosphorylated, NMII MgATPase activity and bipolar filament integrity are lost, which leads to the loss of actin–myosin crossbridges and full dissociation of NMII from actin. α -Actinin crossbridges are not sufficiently strong to resist the resulting disassembly particularly at high $[Ca^{2+}]$ (see §4). (c) All NMII heads may be unbound from actin at high $[MgATP]$, resulting in crossbridge loss-induced disassembly similar to that depicted in (b). (d) When cell shortening in the direction of SFs is much slower than the rate of NMII sliding velocity, NMII can form new crossbridges through MgATPase. Thus, SFs can shorten without inducing disassembly. (e) When cell shortening is much faster than the rate of NMII sliding velocity, the NMII heads attached to actin filaments are more likely to experience a forward push (i.e. an assisting load in the direction of the actin barbed ends), which is reported to accelerate MgADP release. Thus, all NMIIs with a decreased duty ratio can dissociate from actin, leading to SF disassembly regardless of MLC phosphorylation.

actin–myosin interactions, this inhibition is removed under high $[Ca^{2+}]$ such as that found in the activation buffer [68]. Thus, it seems to be reasonable to attribute MgATP-induced dissociation of NMII from actin to the rapid disassembly of SFs we observed.

5. CONCLUSIONS

We observed that periodic actin patterns emerged along the length of SFs, and the pattern disappeared during rapid SF disassembly caused by high $[MgATP]$. This disassembly occurred independent of MLC dephosphorylation. Fluorescence microscopy shows that the shed actin fragments had lengths comparable to those in the sarcomeric units of SFs, suggesting that unbundling of SFs is responsible for the disassembly. In terms of qualitative behaviour of actin–myosin interaction, a rise in $[MgATP]$ is expected to be functionally equivalent to: (i) a reduction in MgATPase activity owing to decreased MLC phosphorylation levels, and (ii) a temporal decrease in the total number of actin–myosin crossbridges. Importantly, considering recent kinetic data on NMII load-dependent MgATPase cycle, the latter mechanism involving a decreased number of

actin–myosin crossbridges is more likely to occur during quick cell shortening. Therefore, disappearance of particular SFs after cell shortening, which would reduce actin–myosin crossbridges in SFs oriented in the same direction with the shortening, may be caused by the same unbundling process induced by high $[MgATP]$ observed in this study.

The authors thank Hitomi Onodera for technical assistance. This work was supported by Special Coordination Funds for Promoting Science and Technology (to S.D.), and also in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (nos 21680039 and 22650098 to S.D. and no. 20001007 to M.S.).

REFERENCES

- 1 Pellegrin, S. & Mellor, H. 2007 Actin stress fibres. *J. Cell Sci.* **120**, 3491–3499. (doi:10.1242/jcs.018473)
- 2 Tojkander, S., Gateva, G., Schevzov, G., Hotulainen, P., Naumanen, P., Martin, C., Gunning, P. W. & Lappalainen, P. 2011 A molecular pathway for myosin II recruitment to stress fibers. *Curr. Biol.* **21**, 539–550. (doi:10.1016/j.cub.2011.03.007)
- 3 Cramer, L. P., Siebert, M. & Mitchison, T. J. 1997 Identification of novel graded polarity actin filament bundles in

- locomoting heart fibroblasts: implications for the generation of motile force. *J. Cell Biol.* **136**, 1287–1305. (doi:10.1083/jcb.136.6.1287)
- 4 Krammer, A., Lu, H., Isralewitz, B., Schulten, K. & Vogel, V. 1999 Forced unfolding of the fibronectin type III module reveals a tensile molecular recognition switch. *Proc. Natl Acad. Sci. USA* **96**, 1351–1356. (doi:10.1073/pnas.96.4.1351)
 - 5 Zhong, C., Chrzanowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A. & Burridge, K. 1998 Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. *J. Cell Biol.* **141**, 539–551. (doi:10.1083/jcb.141.2.539)
 - 6 Chrzanowska-Wodnicka, M. & Burridge, K. 1996 Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* **133**, 1403–1415. (doi:10.1083/jcb.133.6.1403)
 - 7 Colombelli, J. *et al.* 2009 Mechanosensing in actin stress fibers revealed by a close correlation between force and protein localization. *J. Cell Sci.* **122**, 1665–1679. (doi:10.1242/jcs.042986)
 - 8 Goffin, J. M., Pittet, P., Csucs, G., Lussi, J. W., Meister, J. J. & Hinz, B. 2006 Focal adhesion size controls tension-dependent recruitment of α -smooth muscle actin to stress fibers. *J. Cell Biol.* **172**, 259–268. (doi:10.1083/jcb.200506179)
 - 9 Hirata, H., Tatsumi, H. & Sokabe, M. 2008 Mechanical forces facilitate actin polymerization at focal adhesions in a zyxin-dependent manner. *J. Cell Sci.* **121**, 2795–2804. (doi:10.1242/jcs.030320)
 - 10 Rivelino, D., Zamir, E., Balaban, N. Q., Schwarz, U. S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B. & Bershadsky, A. D. 2001 Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDial1-dependent and ROCK-independent mechanism. *J. Cell Biol.* **153**, 1175–1186. (doi:10.1083/jcb.153.6.1175)
 - 11 De, R., Zemel, A. & Safran, S. A. 2007 Dynamics of cell orientation. *Nat. Phys.* **3**, 655–659. (doi:10.1038/nphys680)
 - 12 Hayakawa, K., Sato, N. & Obinata, T. 2001 Dynamic reorientation of cultured cells and stress fibers under mechanical stress from periodic stretching. *Exp. Cell Res.* **268**, 104–114. (doi:10.1006/excr.2001.5270)
 - 13 Kaunas, R., Nguyen, P., Usami, S. & Chien, S. 2005 Cooperative effects of Rho and mechanical stretch on stress fiber organization. *Proc. Natl Acad. Sci. USA* **102**, 15 895–15 900. (doi:10.1073/pnas.0506041102)
 - 14 Kaunas, R. & Hsu, H. J. 2009 A kinematic model of stretch-induced stress fiber turnover and reorientation. *J. Theor. Biol.* **257**, 320–330. (doi:10.1016/j.jtbi.2008.11.024)
 - 15 Kaunas, R., Hsu, H. J. & Deguchi, S. 2011 Sarcomeric model of stretch-induced stress fiber reorganization. *Cell Health Cytoskeleton* **3**, 13–22. (doi:10.2147/CHC.S14984)
 - 16 Lee, C. F., Haase, C., Deguchi, S. & Kaunas, R. 2010 Cyclic stretch-induced stress fiber dynamics: dependence on strain rate, Rho-kinase and MLCK. *Biochem. Biophys. Res. Commun.* **401**, 344–349. (doi:10.1016/j.bbrc.2010.09.046)
 - 17 Naruse, K., Yamada, T. & Sokabe, M. 1998 Involvement of SA channels in orienting response of cultured endothelial cells to cyclic stretch. *Am. J. Physiol.* **274**, H1532–H1538.
 - 18 Schoenwaelder, S. M. & Burridge, K. 1999 Bidirectional signaling between the cytoskeleton and integrins. *Curr. Opin. Cell Biol.* **11**, 274–286. (doi:10.1016/S0955-0674(99)80037-4)
 - 19 Sato, K., Adachi, T., Matsuo, M. & Tomita, Y. 2005 Quantitative evaluation of threshold fiber strain that induces reorganization of cytoskeletal actin fiber structure in osteoblastic cells. *J. Biomech.* **38**, 1895–1901. (doi:10.1016/j.jbiomech.2004.08.012)
 - 20 Deguchi, S., Ohashi, T. & Sato, M. 2005 Intracellular stress transmission through actin stress fiber network in adherent vascular cells. *Mol. Cell Biomech.* **2**, 205–216.
 - 21 Sato, K., Adachi, T., Shirai, Y., Saito, N. & Tomita, Y. 2006 Local disassembly of actin stress fibers induced by selected release of intracellular tension in osteoblastic cell. *J. Biomech. Sci. Eng.* **1**, 204–214. (doi:10.1299/jbse.1.204)
 - 22 Harris, D. A. 1996 Cellular ATP. In *Principles of medical biology*, vol. 4 part 2 (eds E. E. Bittar & N. Bittar), ch. 1, pp. 1–47, Cell chemistry and physiology. New York, NY: Elsevier Science.
 - 23 Matsui, T. S., Ito, K., Kaunas, R., Sato, M. & Deguchi, S. 2010 Actin stress fibers are at a tipping point between conventional shortening and rapid disassembly at physiological levels of MgATP. *Biochem. Biophys. Res. Commun.* **395**, 301–306. (doi:10.1016/j.bbrc.2010.03.150)
 - 24 Deguchi, S., Ohashi, T. & Sato, M. 2006 Tensile properties of single stress fibers isolated from cultured vascular smooth muscle cells. *J. Biomech.* **39**, 2603–2610. (doi:10.1016/j.jbiomech.2005.08.026)
 - 25 Katoh, K., Kano, Y., Masuda, M., Onishi, H. & Fujiwara, K. 1998 Isolation and contraction of the stress fiber. *Mol. Biol. Cell* **9**, 1919–1938.
 - 26 Kishino, A. & Yanagida, T. 1988 Force measurements by micromanipulation of a single actin filament by glass needles. *Nature* **334**, 74–76. (doi:10.1038/334074a0)
 - 27 Fabiato, A. & Fabiato, F. 1979 Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. Paris* **75**, 463–505.
 - 28 Hirata, H., Ohki, K. & Miyata, H. 2004 Dynamic change in the distribution of integrin on isolated ventral membrane: effect of divalent cation species. *Cell Motil. Cytoskeleton* **59**, 131–140. (doi:10.1002/cm.20029)
 - 29 Hirata, H., Ohki, K. & Miyata, H. 2005 Mobility of integrin $\alpha 5 \beta 1$ measured on the isolated ventral membranes of human skin fibroblasts. *Biochim. Biophys. Acta* **1723**, 100–105.
 - 30 Matsui, T. S., Deguchi, S., Sakamoto, N., Ohashi, T. & Sato, M. 2009 A versatile micro-mechanical tester for actin stress fibers isolated from cells. *Biorheology* **46**, 401–415. (doi:10.3233/BIR-2009-0551)
 - 31 Shimamoto, Y., Kono, F., Suzuki, M. & Ishiwata, S. 2007 Nonlinear force-length relationship in the ADP-induced contraction of skeletal myofibrils. *Biophys. J.* **93**, 4330–4341. (doi:10.1529/biophysj.107.110650)
 - 32 Telley, I. A., Denoth, J., Stüssi, E., Pfister, G. & Stehle, R. 2006 Half-sarcomere dynamics in myofibrils during activation and relaxation studied by tracking fluorescent markers. *Biophys. J.* **90**, 514–530. (doi:10.1529/biophysj.105.070334)
 - 33 Katoh, K., Kano, Y., Amano, M., Onishi, H., Kaibuchi, K. & Fujiwara, K. 2001 Rho-kinase-mediated contraction of isolated stress fibers. *J. Cell Biol.* **153**, 569–584. (doi:10.1083/jcb.153.3.569)
 - 34 Harada, Y., Sakurada, K., Aoki, T., Thomas, D. D. & Yanagida, T. 1990 Mechanochemical coupling in actomyosin energy transduction studied by *in vitro* movement assay. *J. Mol. Biol.* **216**, 49–68. (doi:10.1016/S0022-2836(05)80060-9)
 - 35 Yanagida, T., Nakase, M., Nishiyama, K. & Oosawa, F. 1984 Direct observation of motion of single F-actin filaments in the presence of myosin. *Nature* **307**, 58–60. (doi:10.1038/307058a0)

- 36 Dlugosz, A. A., Antin, P. B., Nachmias, V. T. & Holtzer, H. 1984 The relationship between stress fiber-like structures and nascent myofibrils in cultured cardiac myocytes. *J. Cell Biol.* **99**, 2268–2278. (doi:10.1083/jcb.99.6.2268)
- 37 Verkhovskiy, A. B. & Borisy, G. G. 1993 Non-sarcomeric mode of myosin II organization in the fibroblast lamellum. *J. Cell Biol.* **123**, 637–652. (doi:10.1083/jcb.123.3.637)
- 38 Verkhovskiy, A. B., Svitkina, T. M. & Borisy, G. G. 1995 Myosin II filament assemblies in the active lamella of fibroblasts: their morphogenesis and role in the formation of actin filament bundles. *J. Cell Biol.* **131**, 989–1002. (doi:10.1083/jcb.131.4.989)
- 39 Cano, M. L., Lauffenburger, D. A. & Zigmond, S. H. 1991 Kinetic analysis of F-actin depolymerization in polymorphonuclear leukocyte lysates indicates that chemoattractant stimulation increases actin filament number without altering the filament length distribution. *J. Cell Biol.* **115**, 3677–3687. (doi:10.1083/jcb.115.3.677)
- 40 Andrianantoandro, E., Blanchoin, L., Sept, D., McCammon, J. A. & Pollard, T. D. 2001 Kinetic mechanism of end-to-end annealing of actin filaments. *J. Mol. Biol.* **312**, 721–730. (doi:10.1006/jmbi.2001.5005)
- 41 Watanabe, T., Hosoya, H. & Yonemura, S. 2007 Regulation of myosin II dynamics by phosphorylation and dephosphorylation of its light chain in epithelial cells. *Mol. Biol. Cell.* **18**, 605–616. (doi:10.1091/mbc.E06-07-0590)
- 42 He, Y. & Grinnell, F. 1994 Stress relaxation of fibroblasts activates a cyclic AMP signaling pathway. *J. Cell Biol.* **126**, 457–464. (doi:10.1083/jcb.126.2.457)
- 43 DeMali, K. A., Wennerberg, K. & Burridge, K. 2003 Integrin signaling to the actin cytoskeleton. *Curr. Opin. Cell. Biol.* **15**, 572–582. (doi:10.1016/S0955-0674(03) 00109-1)
- 44 Kee, Y. S. & Robinson, D. N. 2008 Motor proteins: myosin mechanosensors. *Curr. Biol.* **18**, R860–R862. (doi:10.1016/j.cub.2008.07.071)
- 45 Kovacs, M., Thirumurugan, K., Knight, P. J. & Sellers, J. R. 2007 Load-dependent mechanism of nonmuscle myosin 2. *Proc. Natl Acad. Sci. USA* **104**, 9994–9999. (doi:10.1073/pnas.0701181104)
- 46 Norstrom, M. F., Smithback, P. A. & Rock, R. S. 2010 Unconventional processive mechanics of non-muscle myosin IIB. *J. Biol. Chem.* **285**, 26326–26334. (doi:10.1074/jbc.M110.123851)
- 47 Nyitrai, M. & Geeves, M. A. 2004 Adenosine diphosphate and strain sensitivity in myosin motors. *Phil. Trans. R. Soc. Lond. B* **359**, 1867–1877. (doi:10.1098/rstb.2004.1560)
- 48 Kovacs, M., Wang, F., Hu, A., Zhang, Y. & Sellers, J. R. 2003 Functional divergence of human cytoplasmic myosin II: kinetic characterization of the non-muscle IIA isoform. *J. Biol. Chem.* **278**, 38 132–38 140. (doi:10.1074/jbc.M305453200)
- 49 Wang, F., Kovacs, M., Hu, A., Limouze, J., Harvey, E. V. & Sellers, J. R. 2003 Kinetic mechanism of non-muscle myosin IIB: functional adaptations for tension generation and maintenance. *J. Biol. Chem.* **278**, 27 439–27 448. (doi:10.1074/jbc.M302510200)
- 50 Costa, K., Hucker, W. J. & Yin, F. 2002 Buckling of actin stress fibers: a new wrinkle in the cytoskeletal tapestry. *Cell Motil. Cytoskeleton* **52**, 266–274. (doi:10.1002/cm.10056)
- 51 Kumar, S., Maxwell, I. Z., Heisterkamp, A., Polte, T. R., Lele, T. P., Salanga, M., Mazur, E. & Ingber, D. E. 2006 Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization, and extracellular matrix mechanics. *Biophys. J.* **90**, 3762–3773. (doi:10.1529/biophysj.105.071506)
- 52 Hotulainen, P. & Lappalainen, P. 2006 Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J. Cell Biol.* **173**, 383–394. (doi:10.1083/jcb.200511093)
- 53 Peterson, L. J., Rajfur, Z., Maddox, A. S., Freel, C. D., Chen, Y., Edlund, M., Otey, C. & Burridge, K. 2004 Simultaneous stretching and contraction of stress fibers *in vivo*. *Mol. Biol. Cell* **15**, 3497–3508. (doi:10.1091/mbc.E03-09-0696)
- 54 Vicente-Manzanares, M., Ma, X., Adelstein, R. S. & Horwitz, A. R. 2009 Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nat. Rev. Mol. Cell Biol.* **10**, 778–790. (doi:10.1038/nrm2786)
- 55 Howard, J. 1997 Molecular motors: structural adaptations to cellular functions. *Nature* **389**, 561–567. (doi:10.1038/39247)
- 56 Ishikawa, R., Sakamoto, T., Ando, T., Fujime, S. & Kohama, K. 2003 Polarized actin bundles formed by human fascin-1: their sliding and disassembly on myosin II and myosin V *in vitro*. *J. Neurochem.* **87**, 676–685. (doi:10.1046/j.1471-4159.2003.02058.x)
- 57 Medeiros, N. A., Burnette, D. T. & Forscher, P. 2006 Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nat. Cell Biol.* **8**, 216–226. (doi:10.1038/ncb1367)
- 58 Haviv, L., Gillo, D., Backouche, F. & Bernheim-Groswasser, A. 2008 A cytoskeletal demolition worker: myosin II acts as an actin depolymerization agent. *J. Mol. Biol.* **375**, 325–330. (doi:10.1016/j.jmb.2007.09.066)
- 59 Wilson, C. A. *et al.* 2010 Myosin II contributes to cell-scale actin network treadmilling through network disassembly. *Nature* **465**, 373–377. (doi:10.1038/nature08994)
- 60 Nemethova, M., Auinger, S. & Small, J. V. 2008 Building the actin cytoskeleton: filopodia contribute to the construction of contractile bundles in the lamella. *J. Cell Biol.* **180**, 1233–1244. (doi:10.1083/jcb.200709134)
- 61 Totsukawa, G., Yamakita, Y., Yamashiro, S., Hartshorne, D. J., Sasaki, Y. & Matsumura, F. 2000 Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* **150**, 797–806. (doi:10.1083/jcb.150.4.797)
- 62 Burridge, K. & Feramisco, J. R. 1981 Non-muscle α -actinins are calcium-sensitive actin-binding proteins. *Nature* **294**, 565–567. (doi:10.1038/294565a0)
- 63 Craig, S. W. & Pollard, T. D. 1982 Actin-binding proteins. *Trends Biochem. Sci.* **7**, 88–92. (doi:10.1016/0968-0004(82)90153-0)
- 64 Furukawa, R. & Fechheimer, M. 1997 The structure, function, and assembly of actin filament bundle. *Int. Rev. Cytol.* **175**, 29–90. (doi:10.1016/S0074-7696(08) 62125-7)
- 65 Ando, J., Komatsuda, T. & Kamiya, A. 1988 Cytoplasmic calcium response to fluid shear stress in cultured vascular endothelial cells. *In vitro Cell Dev. Biol.* **24**, 871–877. (doi:10.1007/BF02623896)
- 66 Geiger, R. V., Berk, B. C., Alexander, R. W. & Nerem, R. M. 1992 Flow-induced calcium transients in single endothelial cells: spatial and temporal analysis. *Am. J. Physiol.* **262**, C1411–C1417.
- 67 Shen, J., Lusinskas, F. W., Connolly, A., Dewey Jr, C. F. & Gimbrone Jr, M. A. 1992 Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells. *Am. J. Physiol.* **262**, C384–C390.
- 68 Tanaka, J., Watanabe, T., Nakamura, N. & Sobue, K. 1993 Morphological and biochemical analyses of contractile proteins (actin, myosin, caldesmon and tropomyosin) in normal and transformed cells. *J. Cell Sci.* **104**, 595–606.