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COMMENTARY

The inner workings of stress fibers – from contractile machinery to focal adhesions and back

Ariel Livne and Benjamin Geiger*

ABSTRACT

Ventral stress fibers and focal adhesions are physically coupled structures that play key roles in cellular mechanics and force sensing. The tight functional interdependence between the two is manifested not only by their apparent proximity but also by the fact that ventral stress fibers and focal adhesions are simultaneously diminished upon actomyosin relaxation, and grow when subjected to external stretching. However, whereas the apparent co-regulation of the two structures is well-documented, the underlying mechanisms remains poorly understood. In this Commentary, we discuss some of the fundamental, yet still open questions regarding ventral stress fiber structure, its force-dependent assembly, as well as its capacity to generate force. We also challenge the common approach – i.e. ventral stress fibers are variants of the well-studied striated or smooth muscle machinery – by presenting and critically discussing alternative venues. By highlighting some of the less-explored aspects of the interplay between stress fibers and focal adhesions, we hope that this Commentary will encourage further investigation in this field.

KEY WORDS: Cell mechanics, Focal adhesions, Mechanosensitivity, Sarcomeres, Stress fibers

Introduction – cellular mechanosensitivity as an emerging paradigm

In radical contrast to the common analogy of cells as 'biochemical machines' driven primarily by chemical interactions, most cells, throughout our body, constantly generate, transmit, sense and respond to mechanical inputs. We witness such biomechanical processes with every movement we make, every beat of our heart and every breath we take. Force perturbations are created by cells as they push and pull on their immediate surroundings, be it neighboring cells or the extracellular matrix (ECM), as they change their morphology or orientation in response to external mechanical constraints and, of course, as they migrate and divide. The underlying basis of these diverse examples is the ability of essentially all cells, not just muscle cells, to generate and apply force (Discher et al., 2005; Harris et al., 1980; Liang et al., 2010) and, no less important, their capacity to sense 'informative mechanical cues' and develop a specific physiological (often biochemical) response.

These forces may be divided, superficially, into two types: pushing and pulling. At the molecular level, pushing is typically attributed to the polymerization-based extension of actin bundles or individual fibers. As actin filaments elongate, they can push against any physical obstacle they encounter. Such a pushing mechanism can drive the leading edge of the cell forward during migration (Keren et al., 2008; Mogilner and Oster, 1996; Pollard et al., 2001), and extend thin protrusions to probe the surrounding ECM by using filopodia (Mattila and Lappalainen, 2008) or invade it with invadopodia (Buccione et al., 2004; Revach and Geiger, 2014). It is important to note, however, that cellular pushing does not necessarily take place through cytoskeletal polymerization, as in the cases of bleb (Fackler and Grosse, 2008) and lobopodium formation (Petrie et al., 2014), which are driven by the hydrostatic pressure in the cell. By contrast, pulling forces are generated, primarily, when bipolar bundles of myosin II motor proteins slide along actin fibers (Alberts et al., 2007; Vicente-Manzanares et al., 2009). Notably, myosin II moves along actin filaments in a directed, processive manner; however, when arranged in a bipolar configuration, the myosin motor heads can attach to different actin filaments, sliding one towards the other. This way, cells can apply contractile forces in a number of situations, ranging from classic muscle contraction (striated and smooth) (Gordon et al., 2000) and ECM remodeling (Larsen et al., 2006; Stopak and Harris, 1982) to wound closure (Brugués et al., 2014) and retraction of the trailing edge of migrating cells (Vicente-Manzanares et al., 2007). Nevertheless, contractile forces can also be effectively generated through alternative mechanisms. One such relevant example is the depolymerization of microtubule and actin filaments (see below) (Sun et al., 2010), both of which have been associated with chromosome movement (Kline-Smith and Walczak, 2004) and cytokinesis (Pinto et al., 2012).

In this Commentary, we discuss the mechanisms that underlie force generation by stress fibers, the transmission of the apparent contractile (and other) forces to the ECM through their anchorage sites to the matrix, i.e. focal adhesions, and the coordinated response of the two structures to externally applied forces. Stress fibers and focal adhesions are readily formed by many different cell systems in culture, e.g. fibroblasts, epithelial and endothelial cells (Tojkander et al., 2012), and analogous structures are found *in vivo* (Delon and Brown, 2009; North et al., 1993; Wong et al., 1983). With the growing interest in cellular mechanosensitivity, stress fibers and focal adhesions have drawn increasing attention over the last decades (see, e.g. Chrzanowska-Wodnicka and Burridge, 1996; Geiger et al., 2009; Pelham and Wang, 1997; Wang et al., 2001; Zemel et al., 2010). Here, we refrain from providing a comprehensive literature review of stress fibers and focal adhesions, information that can be readily found in several recent and comprehensive review articles (Burridge and Guilluy, 2015; Burridge and Wittchen, 2013; Geiger et al., 2009; Geiger and Yamada, 2011; Kassianidou and Kumar, 2015; Naumanen et al., 2008; Pellegrin and Mellor, 2007; Smith et al., 2014; Tojkander et al., 2012; Vallenius, 2013). Rather, we highlight and challenge fundamental, yet mostly open, questions regarding the mechanical crosstalk and interdependence between stress fibers and focal adhesions.

To that end, we present the main players, and provide a short overview of the different stress fiber types and their specific modes of association with focal adhesions, before exploring the similarities between stress fibers of non-muscle cells and the well-studied

Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 7610001, Israel.

*Author for correspondence (benny.geiger@weizmann.ac.il)

striated myofibrils. On the basis of theoretical and experimental considerations, we postulate that the commonly held comparison between stress fibers and myofibrils is problematic, and should be revised. We also present a set of fundamental open questions that deserve to be addressed in future research; these include: (i) where are the basic stress fiber building blocks (sarcomeres) assembled, and how are they maintained under the constant treadmilling of new actin from the focal adhesions? (ii) How are stress fibers and focal adhesions mechanically coupled and co-regulated, and which of the two is the 'primary driver'? (iii) Are all sarcomeres along the stress fiber identical, both structurally and dynamically? (iv) Stress fibers generate a stronger force than theoretically expected from simple considerations (e.g. number of motors, dimensions), whereas measurements of their mechanical properties obtained from different experiments appear to be inconsistent. How can these findings be reconciled?

Motivated by the above questions, we propose to extend the, currently, prevailing actomyosin model. We consider the possibility that, in addition to the sliding of actin and myosin II filaments against one another, a force-regulated association and dissociation of actin monomers has a key role in the generation of stress-fiber tension.

Stress fiber diversity and interdependence with focal adhesions

Stress fibers are crosslinked bundles of actin fibers. They are, typically, divided into three types – ventral stress fibers, dorsal stress fibers and transverse arcs – that differ in function, cellular location, structure and composition (Hotulainen and Lappalainen, 2006; Small et al., 1998) (Fig. 1A). Dorsal stress fibers are long, linear and non-contractile bundles of actin crosslinked by α-actinin, and composed of palladin and vasodilator-stimulated phosphoprotein (VASP) (Burnette et al., 2014; Gateva et al., 2014). They appear to be attached solely to the ventral membrane through focal adhesions at a single, discrete end, usually near the cell edges. From there, they extend towards the cell center through formin- and VASP-driven actin polymerization (Hotulainen and Lappalainen, 2006; Skau et al., 2015; Tojkander et al., 2015), apparently to serve as tracks on which transverse arcs can slide centripetally (Hotulainen and Lappalainen, 2006; Tee et al., 2015). Transverse arcs, however, are long, curved actin structures along which alternating and repetitive bands of α-actinin and myosin are arranged. After their formation at the lamellipodium from Arp2/3- and formin-nucleated actin fibers (Burnette et al., 2011; Hotulainen and Lappalainen, 2006; Tojkander et al., 2011; Zhang et al., 2003), the transverse arcs slide centripetally – not anchored to any focal adhesions – along the dorsal stress fibers in a myosin II-dependent fashion (Tee et al., 2015). They eventually fuse towards the cell center, forming thick and contractile actomyosin bundles. Finally, ventral stress fibers appear to combine the properties of transverse arcs and dorsal stress fibers. These long, linear actin structures typically extend from one side of the cell to the other, and attach at either end to focal adhesions (Fig. 1B). Towards their center, however, they are made up by alternating bands of α-actinin and myosin II of non-uniform lengths. Indeed, one of the mechanisms for ventral stress fiber formation involves the merger of a transverse arc bundle with two dorsal stress fibers at its sides in a tension-mediated manner (Hotulainen and Lappalainen, 2006; Tojkander et al., 2015). Ventral stress fibers also include two additional subtypes: peripheral stress fibers that run along the long stable edges of a cell (Prager-Khoutorsky et al., 2011) and actin cap stress fibers, which are draped over the nucleus (Kim et al., 2014). Interestingly, whereas myosin II motors generate contractile forces in both transverse arcs and ventral stress fibers, their overall effect is quite distinct. In transverse arcs, these forces act within the cell to centripetally drive the arcs (Hotulainen and Lappalainen, 2006). By contrast, ventral stress fibers transmit the myosin-II-generated forces externally through their anchorage to focal adhesions, to probe and interact with their microenvironment (Balaban et al., 2001; Grashoff et al., 2010; Sabass et al., 2008). Thus, although dorsal stress fibers and transverse arcs are prominent during cell spreading, it is the third type of stress fiber, the ventral stress fiber that are most commonly found in mature cells and believed to have a key role in cellular mechanosensing (Pellegrin and Mellor, 2007). We, therefore, focus in this Commentary on ventral stress fibers (hereafter referring to them as 'stress fibers') and discuss their mechanical coupling through focal adhesions to the ECM.

Located at the ventral membrane of adherent cells, the micrometer-long focal adhesions form a bridge between the terminal segment of stress fibers and the ECM. Their cytoplasmic components attach to stress fibers, whereas their extracellular parts are anchored to the ECM through transmembrane receptors of the integrin family. Focal adhesions are highly regulated multi-protein complexes that comprise over 200 distinct proteins, which are known collectively as the 'integrin adhesome' (Byron et al., 2011; Kuo et al., 2011; Schiller et al., 2011; Winograd-Katz et al., 2014; Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007; Zamir and Geiger, 2001). Focal adhesion proteins are typically sorted into two functional molecular classes: scaffolding proteins (e.g. vinculin, paxillin, talin, zyxin) and signaling proteins (e.g. focal adhesion kinases, specific phosphatases, Rho-family G-protein activators or inhibitors). The former are primarily involved in the formation and maintenance of a stable structural scaffold, thereby linking the stress fibers to the ECM through integrins. The latter, by contrast, are recruited to the adhesion sites, where they generate and mediate adhesion-dependent signals that act locally to control the development and sustainability of focal adhesions and, at the same time, also globally regulate key cellular processes, such as cell proliferation, differentiation, survival and migration (Wozniak et al., 2004).

Although stress fibers and focal adhesions are two distinct structures, they are clearly highly interdependent. Stress fiber disruption (e.g. inhibition of myosin II with blebbistatin or Y-27632) is accompanied by rapid disassembly of the attached focal adhesions. Similarly, stress fibers diminish when their anchorage sites disassemble during cell migration (Laukaitis et al., 2001), either owing to interactions with microtubules (Ezratty et al., 2005) or following the downregulation of specific focal adhesion constituents, such as talin (Humphries et al., 2007). In addition, stress fibers substantially contract when separated from their focal adhesions – as seen, for instance, when they are severed in the middle, leaving the two free ends unanchored (Colombelli et al., 2009; Kumar et al., 2006). Until new adhesion sites are formed, the focal adhesions that are still connected to the severed stress fiber display a rapid loss of the focal adhesion protein zyxin, whose localization is highly dependent on local mechanical stress (Colombelli et al., 2009; Zaidel-Bar et al., 2003). This reciprocal interdependence between stress fibers and focal adhesions is also manifested by the apparent correlation between key physical parameters of stress fibers and focal adhesions. Specifically, it was found that the size of focal adhesions changes according to the mechanical load applied to them: as the myosin II-generated contractile forces in a stress fiber rise, the surface area of the anchoring focal adhesion also increases (and vice versa). On an elastomeric substrate with a

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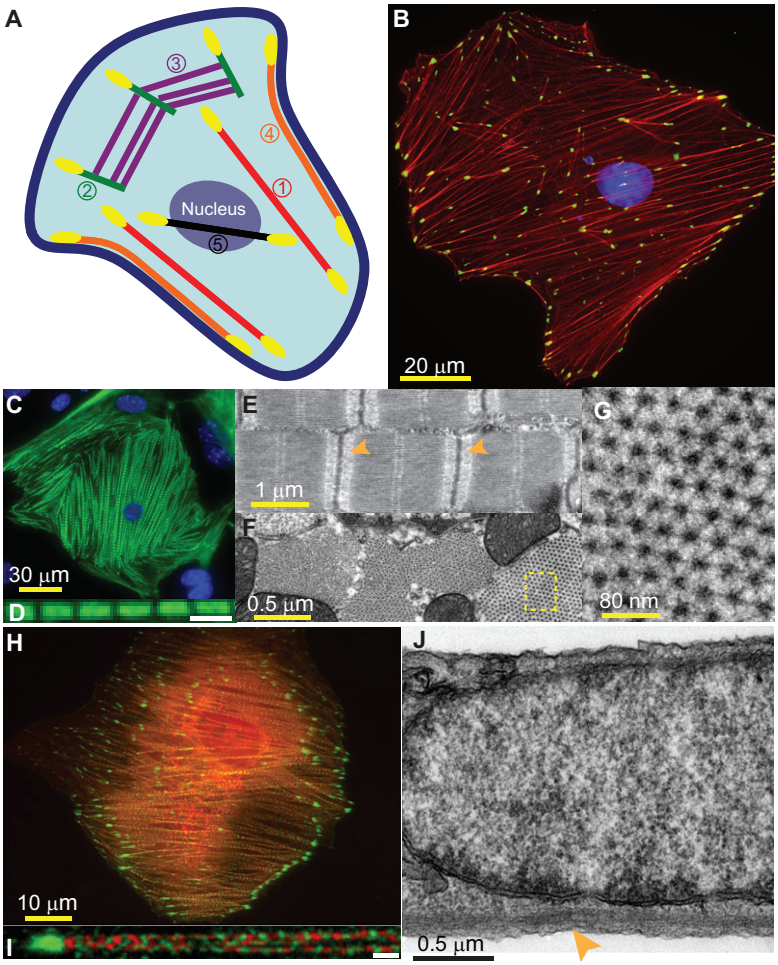

Fig. 1. Stress fibers, myofibrils and sarcomeres. (A) Different types of cellular stress fibers: ventral stress fibers (red, 1), dorsal stress fibers (green, 2), transverse arcs (purple, 3), peripheral stress fibers (orange, 4) and actin cap stress fibers (black, 5). All stress fibers, except transverse arcs, are anchored to the matrix by focal adhesions (yellow). (B) Ventral stress fibers (red) and their focal adhesion anchorage sites (yellow) are highly interdependent; disruption of one will lead to rapid disassembly of the other. Shown is a cell of the rat REF-52 cell line expressing YFP-tagged paxillin (a focal adhesion protein) and stained with TRITC–phalloidin (labeling stress fiber actin) and DAPI (nucleus). (C) Myofibrils (green) within a mouse cardiomyocyte; immunostaining of a 1-day-old newborn mouse for cardiac troponin T (green) and nucleus (DAPI staining, blue). These rod-like structures are the contractile elements of muscle cells. (D) Myofibrils are composed of sarcomeres, which tile them from one end to the other. Shown is a magnification of one of the myofibrils seen in C, illustrating the precise sarcomeric repeat. Scale bar: 2 µm. (E) Longitudinal section of striated myofibrils in mouse cardiac muscle displaying a high level of organization and alignment. This transmission electron microscopy (TEM) image clearly shows individual sarcomeres and their internal organization. Arrowheads mark z-lines, the α-actinin rich boundaries between adjacent sarcomeres. (F) Cross-sectional view (TEM) of a striated mouse cardiac muscle indicating the crystal-like organization of actin and myosin II filaments. The pattern differences between the three myofibrils are due to their lack of lateral alignment (i.e. each pattern corresponds to a myofibril cross-sectional view at a different location along the sarcomere). (G) Magnification of the dashed area shown in F shows the thick filament arrangement in a hexagonal array with ∼40 nm spacing. (H) Immunostaining of MLC (red) and α-actinin (green) in a REF-52 cell. Stress fibers display a repetitive sarcomeric substructure of myosin II and α-actinin, similar to that in striated muscle, although not as well-ordered. Since α-actinin is also found in focal adhesions, it is clearly visible at the ends of the stress fibers. (I) Magnification of one of the stress fibers in H, showing alternating bands of myosin II and α-actinin across it. Scale bar: 2 µm. (J) TEM image of a stress fiber (arrowhead) next to the nucleus in a fibroblast (longitudinal section). In contrast to striated muscle (e.g. E), no discernible substructure is observed at the sarcomeric scale.

∼10 kPa Young's modulus, this relationship between force and area was measured to be ∼5.5 nN/µm^2 (Balaban et al., 2001). This is equivalent to a constant shear stress of ∼5.5 kPa applied by focal adhesions on the underlying substrate, some three orders of magnitude higher than the maximal shear stress applied by blood flow on the arterial walls (Samijo et al., 1998).

Remarkably, although significant progress has been reached in recent years in classifying the different stress fiber structures and understanding their roles and modes of assembly, little is known about the differences between their corresponding adhesion sites. Thus, for example, because focal adhesion composition is differentially regulated by mechanical stress (Lavelin et al., 2013; Yoshigi et al., 2005), the lower tension in dorsal stress fibers relative to ventral stress fibers (Soiné et al., 2015; Tojkander et al., 2015) might result in substantial variations between their focal adhesions. Namely, we suggest that each type of stress fiber is associated with a characteristic focal adhesion type. Moreover, the forces transmitted by adhesions associated with the different types of stress fiber might significantly vary not only in magnitude but also in direction. Whereas, ventral stress fibers run parallel to the underlying substrate, thereby predominantly applying tangential forces (Balaban et al., 2001), dorsal stress fibers (Burnette et al., 2014) and actin cap stress fibers (Kim et al., 2014) can anchor to their adhesion sites at a significant pitch, indicative of considerable

out-of-plane contribution (Hur et al., 2009), for which most two-dimensional (2D) traction force microscopy setups are not sensitive enough. Hence, because current studies are prone to yield a mix of measurements from the potentially different adhesion types, an improved characterization (as in Kim et al., 2012) can influence the results of focal adhesion analysis. This, in turn, might explain, why in some cases a linear force–area relationship of focal adhesions is only partly observed (Oakes et al., 2014; Stricker et al., 2011; Tan et al., 2003), whereas in others it is discerned clearly (Balaban et al., 2001; Trichet et al., 2012).

Naturally, stress fibers interact mechanically not only with focal adhesions but also with additional cellular structures, including neighboring stress fibers and the actin cortex (see below); similarly, focal adhesions do not exclusively interact with stress fibers. Yet, for the sake of simplicity, we consider a scenario in the next sections, in which the interface between stress fiber and focal adhesion is the dominant – if not exclusive – mechanical connection between the two systems, and stress-fiber-generated forces are transmitted to the ECM solely through focal adhesions.

Stress fibers and myofibrils – some similarities, main differences

Stress fibers and striated muscle myofibrils are mechanistically distinct structures but, for the purpose of our discussion, their apparent similarities are highly appealing. Both are elongated linear structures that generate actomyosin-based contractile forces. Both are also organized in alternating bands of myosin II and α-actinin across a long actin cable and, in both systems, there are alternating segments in which actin displays an opposite polarity. Given that myofibrils are much better characterized than stress fibers (see Box 1), owing to their fundamental physiological role in muscle it is not surprising that they often serve as a primary reference system for stress fiber study.

Stress fibers display a repetitive segmented substructure that is reminiscent of the internal order found in striated muscle; both display ordered actin filaments, crosslinked at one end by α-actinin and interconnected at the other end by myosin motors. Just as in striated muscle, they are contractile – owing to sliding of myosin II on actin fibers – and play a key role in force generation. In view of this apparent similarity, the contractile units of stress fibers are often generally referred to also as sarcomeres (Kreis and Birchmeier, 1980; Sanger et al., 2006). Nevertheless, beyond these rather superficial similarities, fundamental differences exist between stress fiber and myofibril sarcomeres. First, their molecular composition is somewhat different; for example, some of the key components (such as α-actinin, myosin II, actin-binding proteins) have muscle-specific and stress-fiber-specific isoforms. Second, stress fiber contraction is regulated by phosphorylation of myosin light chain (MLC), as is seen in smooth muscle, rather than by troponin switching (Huxley, 1969). Third, the stress fiber sarcomere is not as precisely aligned as its counterpart in muscle (Fig. 1E). Fourth, the sarcomeres along a stress fiber are not uniform, displaying both temporal and spatial variations in length (Chapin et al., 2012; Peterson et al., 2004). Owing to these differences, key aspects of the organization and contractile function of stress fiber sarcomeres remain poorly characterized (e.g. the number of motors per sarcomere, actomyosin packing, etc.).

At the organizational and functional levels, stress fibers are typically one order of magnitude thinner than myofibrils, and their contraction is considerably less coordinated. In the same cell, stress fibers may align in different directions, span different lengths (typically, 10–100 µm) and show limited or no sarcomere registration (Fig. 1H–J). Moreover, along a single stress fiber, different sarcomere behaviors can be observed. Thus, although stress fibers generate contractile forces, they appear far less efficient in doing so than myofibrils. This, naturally, calls into question the similarities between the two systems.

One main distinction between stress fibers and myofibrils is the location and magnitude of the forces they produce through their contractile machinery. Stress fiber contractile forces are applied by individual cells to their immediate environment through focal adhesions. These forces, which may locally rearrange the ECM (Avnur and Geiger, 1981), do not result in significant matrix deformations – <1 µm over a typical stress fiber length of ∼100 µm (Trichet et al., 2012). Thus, stress fibers generate seemingly isometric forces, even when they undergo a high level of contraction (Deguchi et al., 2006). In contrast, striated myofibrils can undergo significant length changes (shortening by up to ∼30%), which is necessary for skeletal movement and heart contraction. The resulting non-isometric forces are transmitted over macroscopic lengths; skeletal myofibrils span between bones to form (together with joints) effective lever systems, and cardiac myofibrils of neighboring cells are mechanically coupled through adherens junctions along the intercalated discs to efficiently contract the heart. Finally, although myofibrils can also interact locally with the surrounding ECM (e.g. through costameres or tendons), this is principally a secondary effect whose magnitude still remains unclear (Hersch et al., 2013).

Another clear difference between striated myofibril and stress fiber contractility relates to their activation dynamics. Striated

Box 1. Myofibrils

Striated myofibrils are extended cylindrical structures, spanning the entire length of muscle cells (∼100 µm in cardiac muscle; a few millimeters to tens of centimeters in skeletal muscle). Their fundamental building block is the sarcomere, which, at rest, is typically ∼2 µm long but is able to shrink by ∼30% at the peak of muscle contraction (Lodish et al., 2000). Thus, 10^2–10^5 sarcomeres, precisely ordered one after the other, tile a striated myofibril from end to end (see Fig. 1C–E). The sarcomeres themselves are organized as thick complexes of bipolar myosin II filaments (thick filaments) that are flanked by individual actin fibers (thin filaments) (Alberts et al., 2007). In cross-section, electron microscopy reveals that the thick filaments are arranged in a hexagonal array (∼40 nm spacing), with actin fibers located at the center of each triangle of myosin filaments (Huxley and Faruqi, 1983) (see Fig. 1F,G). In addition, the actin filaments of a sarcomere are all oriented with their pointed (minus) ends pointing inwards, towards the thick filaments at the center. At their barbed (plus) end, the filaments of neighboring sarcomeres are crosslinked by α-actinin, thus giving rise to the repetitive sarcomeric structure (Alberts et al., 2007). Finally, although many different proteins are involved in the regulation of myofibril structure and function (Ono, 2010), titin (connectin) and nebulin (Horowits et al., 1986) stand out in their relevance to the mechanical properties of the individual sarcomere. These giant proteins (∼1 µm long) span from the ends of the sarcomere along the thin filaments (nebulin), up to the sarcomere center (titin). Thus, the mechanical behavior of the myofibril depends not only on its actomyosin machinery, but also on the elasticity of these proteins (Maruyama, 1997).

By shortening their sarcomeric units, myofibrils apply contractile forces to their anchorage sites. This process takes place through a controlled attachment-pulling-detachment cycle of myosin II motors on the actin fibers. In addition, the polarity of the actin fiber in each sarcomere ensures that the ratchet-like dynamics of myosin will always lead to sarcomere contraction (i.e. pulling the actin toward the sarcomere center). This entire process is regulated by a muscle-specific mechanism that is based on availability of Ca^{2+} and ATP (Goody, 2003; Huxley, 1969).

Journal of Cell Science (2016) 129, 1293-1304 doi:10.1242/jcs.180927

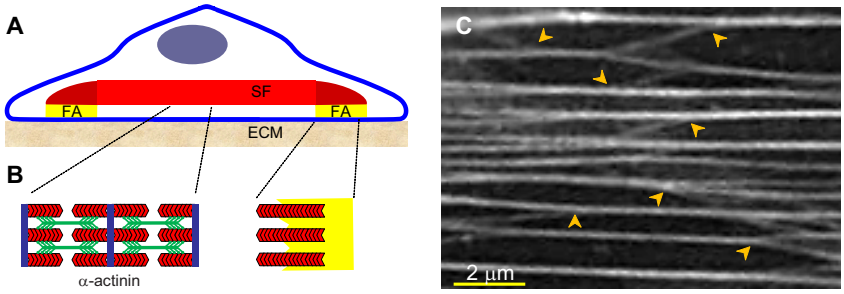

Fig. 2. Stress fiber organization: from 1D idealization to 2D reality. (A) Schematic of an isolated, single stress fiber (SF, red) running between two focal adhesions (FA, yellow). Stress fibers typically extend a few tens of microns, with a cross-section of less than 0.5 µm, giving rise to their rod-like morphology. (B) Simplified model of the stress fiber substructure illustrating the organization of actin (red), α-actinin (blue) and myosin II (green). The short actin filaments present in the stress fiber change from uniform polarity at and near the focal adhesions, to bi-directional polarity inside the stress fiber sarcomeres. Pointed ends of the red actin monomers depict their orientation. (C) In mature cells, multiple interconnections are observed between adjacent stress fibers (arrowheads), resulting in the formation of a 2D mechanical network from the 1D structures illustrated in A. This allows stress fibers to respond to external force input in a coordinated manner. Note that finer sub-resolution connections can also exist between neighboring stress fibers [e.g. actin fibers (Xu et al., 2012), microtubules, intermediate filaments] that are not observed in standard fluorescence microscopy.

muscles repeatedly shorten and relax, triggered by rapid action potentials and Ca^{2+} release (Alberts et al., 2007). Thus, skeletal muscles can switch from rest to full contraction within 10–100 ms (Wood, 2012) to drive quick body movement, and cardiac muscles may beat every 100 ms in small mammals. In contrast, smooth muscle cells and stress fibers respond much slower. Their contractility is regulated by phosphorylation of MLC (Katoh et al., 2001) and does not depend (solely) on electrical pulses. Consequently, hollow organs (e.g. blood vessels, epithelial tubes or glands) that are enveloped by smooth muscle, contract and expand at timescales that are up to ∼30× longer than those for striated muscle (Wood, 2012). In addition, stress fiber contractile forces take minutes to reach peak amplitude (Mbikou et al., 2006; Peterson et al., 2004), with hardly any changes in stress fiber length (Chapin et al., 2012). In effect, it was not until non-muscle cells were shown to be able to induce wrinkles in thin, deformable substrates (Harris, 1984) that the contractile nature of stress fibers was, indeed, confirmed. Since then, a number of different methods, ranging from explicit measurements (Sugita et al., 2011) to observations of direct consequences – e.g. deformations of the underlying substrate (Balaban et al., 2001; Trichet et al., 2012), or stress fiber retraction following laser cutting (Colombelli et al., 2009; Kumar et al., 2006; Russell et al., 2009) – have established that stress fibers are, in fact, constantly under tension. Therefore, whereas muscles can rapidly switch from rest to full power stroke, stress fibers remain at a quasi-constant operational level.

The distinct force characteristics of stress fibers, compared to those of myofibrils, suggest they have a different physiological role. It is conceivable that the relatively low and constant forces generated by stress fibers are sufficient for cells to monitor and explore the mechanical properties of their microenvironment, without damaging the surrounding tissue or the focal adhesions. In addition, just as forces are propagated from stress fibers to the ECM through focal adhesions, external forces applied to the focal adhesion-stress fiber complex can be sensed by this system, and modulate its organization and turnover (Livne et al., 2014). This is further facilitated by the slow temporal response of a stress fiber; whereas the myofibril actomyosin machinery needs to be fully assembled at all times to assure a quick response rate, stress fibers continuously assemble and disassemble. Thus, the limited co-alignment and registry that lowers the capacity of force generation, might improve the mechanosensing abilities of a cell with regard to probing and sensing its surroundings from different directions.

In light of the fundamental differences between stress fibers and myofibrils, we should find a better description of the mechanism underlying stress fiber mechanics. Moreover, given the interdependence between stress fibers and focal adhesions, the regulatory role of the adhesion sites also needs to be further investigated. The questions presented in the following section represent attempts to highlight and explore the inner workings of stress fibers.

Where are the stress fiber sarcomeric units assembled, and what is their fate?

We begin our discussion of this aspect with the observation that actin filaments are continuously polymerized in focal adhesions (Chorev et al., 2014; Skau et al., 2015; Tojkander et al., 2015). The newly formed actin filaments are then treadmilled away along the attached stress fibers towards the cell center, at a velocity of 0.02–0.4 µm/min (Endlich et al., 2007; Russell et al., 2011; Tojkander et al., 2015). As the pointed end of an actin filament always grows away from the nucleation site, these filaments all possess the same uniform polarity. Accordingly, the actin crosslinker fascin, which binds to unipolar, bundled actin filaments, is only found in focal adhesions and at stress fiber termini (Elkhatib et al., 2014). In contrast, towards the stress fiber center, actin polarity appears to be bi-directional, as is the case in myofibrils (Cramer et al., 1997) (Fig. 2A,B). Since sarcomeres are found throughout the length of the stress fiber, up to the adhesion sites, this suggests two possible scenarios; either the unipolar actin bundles that emanate from focal adhesions are quickly reorganized (partially or completely) to the alternating polarities observed in the central region (Cramer et al., 1997), or that actin organization along the stress fibers is non-uniform, resulting in variations in elastic and contractile behaviors (Peterson et al., 2004; Vogel et al., 2013).

Actin polymerization at focal adhesions appears to be a force-dependent process (Hirata et al., 2008; Kozlov and Bershadsky, 2004). Diminishing stress fiber-generated forces by using inhibitors of myosin or Rho kinases (Endlich et al., 2007) leads to the cessation of actin treadmilling at the stress fiber end. The fact that, despite the centripetal flow of actin, the length of stress fibers remains constant or even decreases, raises the question of what happens to all the actin incoming from the focal adhesions. One possibility is that the newly created actin pushes against and compresses the central part of the stress fiber. This, however, does not occur in practice, as the central stress fiber sarcomeres tend to

Journal of Cell Science (2016) 129, 1293-1304 doi:10.1242/jcs.180927

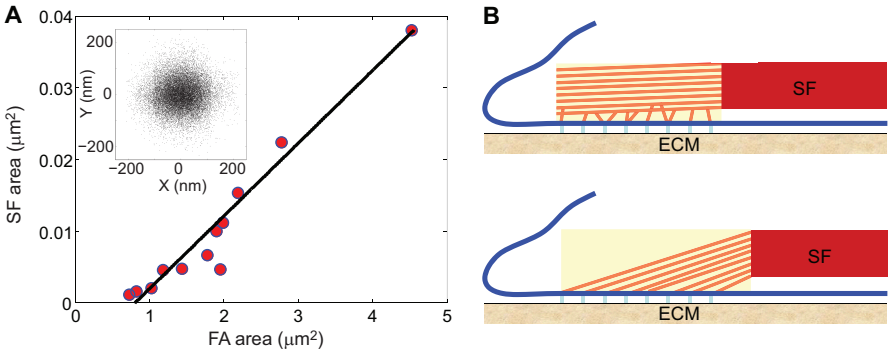

Fig. 3. Relationships between stress fibers and focal adhesions. (A) A linear relationship is observed between the stress fiber (SF) cross-sectional area and the focal adhesion (FA) area. The cross-sectional area of a stress fiber is close to the diffraction limit; here, it was measured by using PALM super-resolution microscopy in fixed REF-52 cells near the adhesion sites (our unpublished data). The inset shows a typical super-resolution plot of phalloidin-labeled actin in the stress fiber cross-section. (B) Possible organization of the interface between stress fibers and focal adhesions. Stress fiber actin fibers (long red lines) have been previously proposed to be decoupled from the underlying substrate through short 'suspension fibers' (short red lines, top panel) (Patla et al., 2010). Here, we suggest an alternative view, whereby the stress fiber actin fibers are attached to the underlying substrate through membrane-based actin nucleators (i.e. Arp2/3 or formins) followed by integrins (light blue lines) (bottom panel). The main difference between the two scenarios is that the latter suggests a direct correlation between focal adhesion size (light yellow rectangle) and the number of actin fibers across the stress fiber bundle (namely the stress fiber cross-sectional area). This exact correlation is demonstrated in A.

expand over time compared to those located closer to the focal adhesions (Elkhatib et al., 2014; Peterson et al., 2004). Another possibility is that, as well-spread cells have multiple interconnections between stress fibers that together form dynamic 2D branched networks (Fig. 2C) (Kumar et al., 2006), the excess actin might be transported away from the main cables into side branches (Russell et al., 2011). Moreover, these networks are likely to be mechanically coupled (Kumar et al., 2006) and can respond to forces in a coordinated manner (Livne et al., 2014). Another consequence of stress fiber branching is that individual stress fibers that extend between two discrete adhesion sites are rarely observed. Given that stress fiber stability and growth are mechanically regulated, it appears that local changes in a single stress fiber (for whatever reason) are likely to induce long-range effects on the entire stress fiber network.

Evidently, actin incorporation into stress fibers occurs not only at focal adhesions, but also along the entire length of the structure, as was directly observed by fluorescence recovery after photobleaching (FRAP) experiments, which demonstrated times for actin recovery of ∼10 min (Campbell and Knight, 2007; Hotulainen and Lappalainen, 2006). This recovery rate suggests that incorporation of new actin monomers occurs throughout the entire length of stress fibers, and not only at their focal-adhesion-associated ends, as it would take hours for actin polymerizing at focal adhesions to arrive at the stress fiber center. Thus, although stress fibers recruit or release actin throughout their entire length, actin is also continuously polymerized at focal adhesions, and flows centripetally. However, despite this exchange and new actin polymerization at adhesion sites, the overall length of stress fibers and the tension along them, remain largely unchanged. How these different features of the stress-fiber–focal-adhesion system are coordinated is unclear and deserves further consideration.

What mechanism underlies the coupling and co-regulation between stress fibers and focal adhesions through the terminal sarcomere?

Physical interactions between focal adhesions and stress fibers, whether related to actin flow or to force transmission from one to the other, take place at the so-called 'terminal sarcomere'. This is the final segment of the stress fiber that still contains myosin II, and links the sarcomeric chain on the one side and the adhesion site on the other. In recent years, significant progress has been made in our understanding of the molecular architecture of focal adhesions (Case et al., 2015; Kanchanawong et al., 2010; Patla et al., 2010). Nevertheless, the structural connection between the focal adhesion and the stress fiber terminus remains largely unclear (Gimona et al., 2003; Kaverina et al., 2003).

Intrigued by this coupling between the terminal sarcomere and focal adhesions, we took a rather naïve approach, and searched for structural (i.e. geometrical) features at the interface. By comparing, in fixed cells, the areas of focal adhesions and stress fiber cross-sections near the adhesion sites, we found that there is a linear correlation between the two (Fig. 3A; our unpublished data). Keeping in mind that focal adhesion area also linearly depends on the pulling force of the stress fiber (Balaban et al., 2001; Trichet et al., 2012), it appears that the two structures are not merely connected to one other and behave quasi-autonomously. Rather, they appear to grow and shrink in unison in response to mechanical stimulation. Explicitly, this suggests a mechanical link between individual stress fiber actin fibers and a specific region of the focal adhesion (Fig. 3B). This scenario is further supported by the recent finding, using super-resolution microscopy, that focal adhesion proteins are organized in elongated patches of similar width compared with the stress fibers connected to them (Hu et al., 2015). These results, in turn, naturally raise the question of the hierarchy between focal adhesions and stress fibers. Akin to the 'chicken and egg' dilemma, we might ask which of the two is the 'leader', whose force-dependent growth regulates the development of the other and determines the stress level transmitted to the ECM (Balaban et al., 2001; Trichet et al., 2012)?

Notably, the limited available EM data suggests that a considerably higher number of actin fibers might be found in the terminal sarcomere (Patla et al., 2010) compared with those in the central stress fiber sarcomeres (Cramer et al., 1997; Rigort et al., 2012). High-resolution cryo-EM tomography of focal adhesions reveals an actin inter-filament spacing of∼8 nm (Patla et al., 2010), which is probably due to bundling by fascin (Jansen et al., 2011). Towards the center of a stress fiber, similar measurements reveal that there are far fewer actin fibers per stress fiber thickness (Rigort et al., 2012). This sparser packing could be attributed to the larger

physical dimensions of either α-actinin (~35 nm) or myosin II [~30–50 nm (MacIntosh, 2003)], both of which crosslink the sarcomeric actin. However, as actin is constantly being polymerized at focal adhesions and treadmilled along the stress fibers, components of the initial terminal sarcomere necessarily form regular sarcomeres over time. This, therefore, raises the question of what happens to the excess of actin fibers in focal adhesions, and what other changes in its molecular architecture take place during this treadmilling process?

Are all sarcomeres along the stress fiber identical?

Variations in sarcomere behavior are observed along the stress fibers. Sarcomeres near the stress fiber center, for example, are slightly longer than their counterparts closer to the termini (Elkhatib et al., 2014; Peterson et al., 2004). However, when stress fiber contractility is stimulated by calyculin A or lipoprotein-a (LPA), this small difference becomes quite pronounced. Thus, a ~50% expansion of the (originally slightly longer) central sarcomeres is accompanied by a 30–40% shortening of sarcomeres at the periphery upon increased phosphorylation of MLC in fibroblasts (Peterson et al., 2004). Furthermore, the concentrations of myosin II and α-actinin, and their exchange timescales also vary significantly between central and peripheral sarcomeres (Peterson et al., 2004). At the single sarcomere level, spontaneous length variations may also take place without any apparent external perturbation (Chapin et al., 2012). As the stress fiber is a closed system over such short timescales (i.e. its overall length hardly changes), such spontaneous shortening (elongation) events are necessarily accompanied by an elongation (shortening) somewhere else along the structure. Interestingly, when an individual sarcomere suddenly changes its length, only a small region around it (~3 sarcomeres on either side) is affected (Chapin et al., 2012).

The variations in sarcomere dynamics, however, do not appear to arise directly from differences in the actin organization along the stress fibers. First, as actin bundles of mixed polarity are assumed to be more contractile than those of uniform polarity, in which some of the myosin heads are attached to the actin fibers in the wrong direction (Vogel et al., 2013), the bulk of stress fiber contraction should take place near its center. In reality, the exact opposite behavior is observed, with the sarcomeres next to focal adhesions being more contractile (Peterson et al., 2004). What, then, is the origin of the sarcomere variability? While this largely remains an open question, one interesting direction of research involves the combination of biochemical signaling processes with stress fiber mechanics (Besser and Schwarz, 2007), which showed that inhomogeneities in biochemical reaction–diffusion fields around the focal adhesions can account for the observed spatial variations in sarcomere contractility.

Owing to the linear structure of stress fibers, a uniform force magnitude is propagated across all their sarcomeres. Nevertheless, the observations of a differential contractile behavior suggest that the production of this force is not evenly distributed. Localized force generation, whether close to focal adhesions or further away, might explain the limited contraction of stress fibers compared to myofibrils. After all, whereas all the sarcomeres in a myofibril contract simultaneously and lead to its dramatic shortening (up to ~30%), the contraction of stress fibers is nearly isometric, with almost no significant length change. Moreover, in isolated form, stress fibers can shorten to less than 25% of their original length (Katoh et al., 1998). Thus, the limited stress fiber contraction might serve a physiological purpose. As non-muscle cells need to apply force to their environment to probe its mechanical properties, it can be advantageous not to cause any deformations (crucial for contracting the heart or generating large skeletal movements) that are too large, which could tear or damage the soft, surrounding ECM. In conclusion, the exact location where stress fiber contractility takes place remains unclear.

An enigma of force generation and transduction along stress fibers

An interesting correlation has been observed between the stress fiber-generated contractile forces and the focal adhesion area through which they are transmitted to the ECM (Balaban et al., 2001; Trichet et al., 2012). When focal adhesions increase (or decrease) in size, the traction forces they apply on the underlying substrate increase (or decrease) accordingly. Moreover, although focal adhesion dynamics are strongly related to the chemical and structural properties of the underlying matrix, different force-area behaviors are observed when cells are attached to pillar substrates of similar composition but varying effective rigidities (Trichet et al., 2012). Specifically, higher forces per adhesion size were measured on stiffer substrates, with only little change in chemistry and rigidity of the local surface, with which the focal adhesions directly interact. One interesting explanation for this force-area correlation stems from the molecular composition of focal adhesions. The load-dependent exchange rates of key adhesion components (Lavelin et al., 2013) suggest that under increasing tension, focal adhesions preferably recruit new proteins and, thus, grow. Inversely, when tension is removed, disassociation rates dominate and the molecular adhesion structure breaks down. However, this explanation, like other focal adhesion-based and force-oriented approaches, cannot account for the pillar measurements, which have shown that the force-area relationship is dependent on the effective substrate rigidity (i.e. pillar geometry) rather than on local surface stiffness (with which the focal adhesions come in to contact). Rigidity sensing, therefore, does not appear to take place solely at the focal adhesion level.

To understand how the stress-fiber–focal-adhesion system senses matrix stiffness and responds with different force–area dynamics, the mechanical behavior of the different elements at play need to be characterized first. Beginning with the relevant structural rigidities, no direct measurements of the elastic properties of focal adhesions are available to our knowledge. In contrast, stress fibers have been analyzed by using a wide range of experimental techniques, leading to values that differ by more than two orders of magnitude. *In vivo* estimates for the Young's modulus of stress fibers range from 1–10 kPa as determined by AFM nano-indentation (Lu et al., 2008) to ~100 kPa, a value that has been obtained by stretching adherent cells through their underlying substrate and measuring the resulting deformation fields (Nagayama et al., 2011; Nagayama and Matsumoto, 2010). For isolated stress fibers, the obtained values (≥1 MPa) differ even more (Deguchi et al., 2006). In addition, stress fiber rigidity can also be inferred indirectly: the Young's modulus of a rod-like stress fiber is simply the ratio between the stresses applied at its ends and the resulting strain. As the internal stress fiber forces are expected to be identical to those transmitted by focal adhesions, the stresses in both structures should differ according to their respective areas (stress is the force transmitted across a unit area). For a wide range of substrate rigidities, focal adhesion traction stresses are typically 5–20 kPa [(Balaban et al., 2001; Trichet et al., 2012) although the practical limit might even be higher (Ghassemi et al., 2012)] applied over a surface area that is ~100× larger than the corresponding area of the stress fiber cross-section (Fig. 3A). This implies that internal stresses in stress fibers are ~100× higher than

those seen in focal adhesions, i.e. 0.5–2 MPa [in comparison, estimates of the internal stress in striated muscle yield ∼0.3 MPa (Bloch and Gonzalez-Serratos, 2003)]. Moreover, the extensional strain in stress fibers (defined as the change in length relative to the original length) is estimated to be in the range of 2–20% (Deguchi et al., 2006). Taken together, these numbers yield a stress fiber Young's modulus of at least 3–10 MPa, which is significantly higher than that suggested by the experimental results (note that in this calculation, our unpublished data presented in Fig. 3A was used). In summary, there is a wide variability, both between the different stress fiber rigidity measurements (Fig. 4A), but also between their indirectly estimated values. However, because all these numbers are well below the ∼1 GPa rigidity of individual bare actin fibers (Kojima et al., 1994; Tsuda et al., 1996), it stands to reason that the elastic response of stress fibers is dictated rather by something else, possibly by the actin crosslinkers themselves.

As discussed above, the spacing of actin fibers across the stress fiber thickness can be estimated from high-resolution EM images (∼35–40 nm spacing, comparable to the lengths of the main stress fiber crosslinkers α-actinin and myosin II). Together with the internal stress fiber stress, these two numbers enable us to calculate the contractile force applied on individual actin fibers (the force being the product of the stress and area). Thus, for example, for highly rigid substrates (e.g. glass), this force is predicted (using our unpublished data presented in Fig. 3A) to be ∼2 nN (Fig. 4B). Assuming now that this force is generated solely by actomyosin contractility, we ask ourselves how many myosin II power strokes are needed to produce it. As in each cycle of attachment-pulling-detachment an individual motor generates up to ∼2 pN force (Norstrom et al., 2010), then 1000 such cycles would be needed to generate ∼2 nN. However, because in each cycle, a myosin motor advances along the actin fiber by one actin monomer, which spans ∼5.5 nm (Norstrom et al., 2010), these 1000 cycles would also amount to a sarcomere shortening of 5.5 µm. As this contraction is far greater than the sarcomere rest length of 1.5–2 µm (Peterson et al., 2004), it appears that the stress fiber contractile forces cannot be generated by regular sarcomeres. This puzzling and controversial result requires further elaboration, and, together with the wide variability of stress fiber rigidity measurements, highlights the need to better clarify our understanding of stress fiber mechanics.

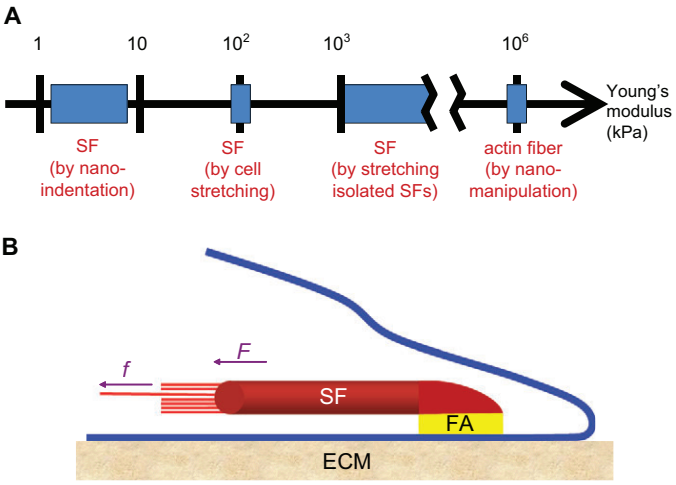

Fig. 4. Mechanical properties of stress fibers. (A) Stress fiber (SF) rigidity has been analyzed by using a wide range of experimental techniques (for details see main text). The resulting Young's modulus estimates range from ∼1 kPa to >1 MPa, suggesting that an improved understanding of stress fiber structure and mechanics is necessary for the correct interpretation of the existing measurements. Nevertheless, the fact that all measurements and estimates are well below the ∼1 GPa rigidity of individual bare actin filaments suggests that the elastic response of stress fibers is not dictated by the constituting actin fibers but, rather, by their crosslinkers. (B) The contractile tension (*F*) in a stress fiber is generated by (and distributed between) the individual comprising actin fibers. These single-filament forces (*f*) can be estimated from measurements of *F* and the number of filaments per stress fiber cross-section.

An alternative mechanism of stress fiber force generation based on regulated global actin turnover

The considerations discussed above suggest that the one-dimensional picture of a focal adhesion/stress fiber/focal adhesion system (Fig. 2A), which is mechanically driven purely by a uniform, actomyosin-based (muscle-like) contraction, might need to be reviewed. It is incompatible with the accumulating experimental data for the fine structure of the stress fiber network, the structural diversity of the stress fiber sarcomeres, and the isometric nature of the actomyosin-based contractile machinery. Filament sliding per se might, therefore, not be the sole generator of force. As indicated, stress fibers are dynamic structures whose building blocks (actin, myosin II, α-actinin and the like) are constantly exchanged with a soluble cytoplasmic pool (Fig. 5A). The exact mechanisms underlying this exchange processes are still poorly characterized, yet they are most likely to be regulated by a balance between the incorporation of new molecules into the structure, and the dissociation of molecules from it. Along these lines, O'Shaughnessy and colleagues took into account sarcomere remodeling to explain different stress fiber-related measurements (Stachowiak and O'Shaughnessy, 2008; Stachowiak et al., 2014). Essentially, the extent of actin fiber overlap originating from both ends of the stress fiber sarcomere was assumed to have a defined steady-state value and to be dynamically regulated by actin polymerization and disassembly rates. It is tempting, however, to take this assumption one step further and consider the possibility that the molecular association and dissociation rates of key stress fiber component(s) is affected by the tension along the structure. This is in line with differences in the dissociation rates of specific stress fiber and focal adhesion components observed following blocking of actomyosin contractility (Fig. 5B) (Lavelin et al., 2013). In addition, a recent study suggests that myosin II-derived forces inhibit vectorial actin polymerization at focal adhesions (Tojkander et al., 2015). Thus, it is conceivable that a reduction in stress (for example, due to actin polymerization at the terminal sarcomeres) increases dissociation events (but without rupturing the structure because only a small number of fibers are cut at a time), effectively leading to a shortening of the stress fiber. In this manner, the effective contractile stress of the stress fiber can increase again (Sun et al., 2010) until regaining its optimal tension level [akin to the constant stress in focal adhesions (Balaban et al., 2001)] (Fig. 5C). These events can take place in discrete regions along the stress fiber, such as its center, which would account for the observed inhomogeneity discussed above. Moreover, stress fiber branching or interactions with either cortical actin or other cytoskeletal systems, could locally affect stress fiber mechanics, which, in turn, might be balanced by the proposed force-induced regulation of actin assembly and disassembly.

The above scenario we propose suggests the dynamic regulation of stress fiber length and tension by a dual mechanism involving, on one hand, actin polymerization and its flow from focal adhesions and, on the other hand, exchange throughout the entire length of the stress fiber. Accordingly, sarcomere formation and their dissolution depend on the balance between actin dissociation and incorporation (Fig. 5D).

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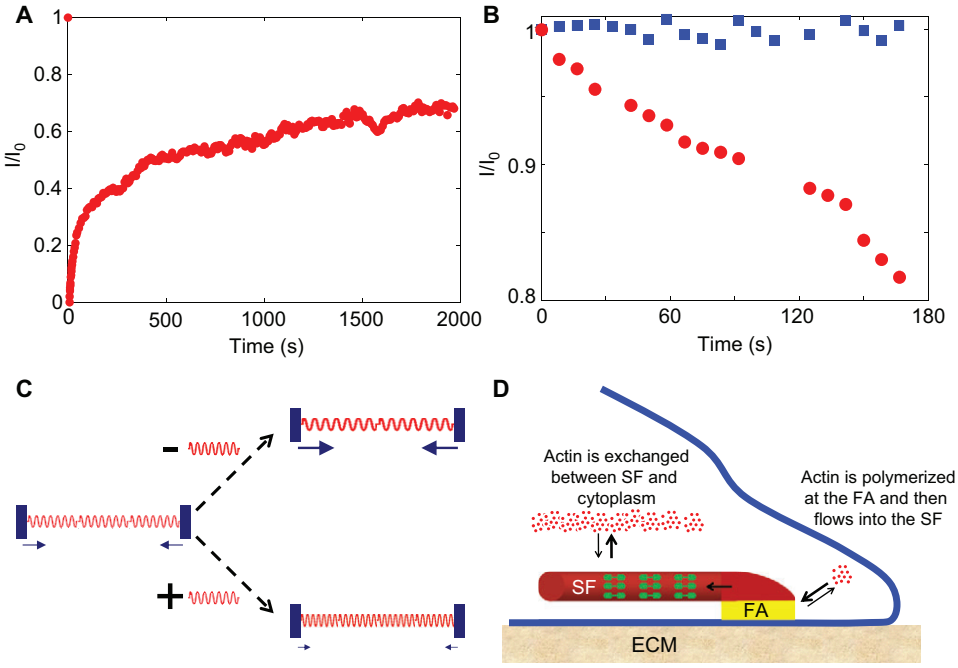

Fig. 5. Actin dynamics in stress fibers. (A) Stress fiber actin is constantly exchanged with that of the cytoplasmic pool, as illustrated by fluorescence recovery after photobleaching (FRAP) (at *t*=0s). To obtain the graph shown here, EGFP–actin-expressing REF-52 cells were used and FRAP data were fully normalized (our unpublished data). (B) Inhibition of Rho-kinase leads to the dissociation of actin from stress fibers. A rapid drop in actin intensity (I) after treatment of cells with 10 µM of Y-27632 (red circles) reflects a decrease in the amount of stress fiber actin. Untreated cells show no change in actin intensity (blue squares). For the graph shown here, EGFP–actin-expressing REF-52 cells were used and actin intensity measurements were taken 2 min after treatment and normalized relative to the first time point (our unpublished data). (C) Schematic of elastic springs in series, fixed between two immovable walls (left). The springs are stretched and apply an inward force (blue arrows) to the walls. If one of the springs is removed, the remaining springs need to be stretched further in order to remain attached to the walls and to each other (top right). This results in an increased tension (thicker arrows). By contrast, addition of another spring reduces the force along the chain of connected springs (bottom right). Analogously, incorporation of actin into and dissociation of actin from a stress fiber can affect its internal stress. (D) Proposed model of an alternative mechanism of force generation in stress fibers based on regulated global actin turnover. Here, the length and tension of a stress fiber (SF) can be dynamically regulated by a dual mechanism that involves actin polymerization and its flow from focal adhesions (FAs) as well as exchange of actin throughout the entire stress fiber. In this manner, internal stresses can be generated as described in C. This proposed mechanism extends the currently prevailing model of actomyosin contractility, suggesting that stress fiber tension is not exclusively generated and regulated by myosin motors (green).

Thus, for example, if more sarcomeres dissolve over time than new ones form, the stress fiber effectively shortens. This can result in an increase in pulling force applied to the adhesion sites, similar to that exerted by actomyosin contractility. In conclusion, instead of a single source of force generation in a stress fiber, there might well be a number of complementary mechanisms – myosin contraction, mechanically regulated polymerization at focal adhesions and actin exchange along the stress fiber – that work in synchrony.

Conclusions

Conceptually, stress fibers appear to be very simple objects – bundles of actin and myosin fibers that generate pulling forces through a mechanism that involves actomyosin contractility. Nevertheless, a closer examination of this system, together with our current understanding of its workings, lead us to conclude that many aspects of stress fibers and their coupling to focal adhesions remain unclear or, simply, do not fit the experimental results. To tackle this enigma, a multi-level and interdisciplinary approach needs to be developed. More high-resolution EM and super-resolution microscopy data are needed to shed light on the structure at play. Additional biophysical investigations are also required to better understand stress fibers and focal adhesions, both in their static and dynamic states. Finally, a comprehensive theoretical framework should be developed that can account for the wide range of experimental observations and measurements. We hope this Commentary will encourage further research within this field and towards answering the key questions raised here.

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Competing interests

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