LIM Proteins in Actin Cytoskeleton Mechanoresponse

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Abstract

The actin cytoskeleton assembles into branched networks or bundles to generate mechanical force for critical cellular processes such as establishment of polarity, adhesion, and migration. Stress fibers are contractile, actomyosin structures that physically couple to the extracellular matrix through integrin-based focal adhesions, thereby transmitting force into and across the cell. Recently, LIM domain proteins have been implicated in mediating this cytoskeletal mechanotransduction. Among the more well studied LIM domain adapter proteins is zyxin, a dynamic component of both focal adhesions and stress fibers. Here, we discuss recent research detailing the mechanisms by which stress fibers adjust their structure and composition to balance mechanical forces, and suggest ways zyxin and other LIM domain proteins mediate mechanoresponse.

Overview of mechanotransduction

Mechanical forces such as stretching and contraction direct a variety of cellular processes: epithelial sheets are stretched and deformed during embryonic development [1], muscle contraction contributes to the remodeling of connective tissue [2], and the vascular endothelia adjusts to changes in blood pressure [3]. To minimize mechanical damage to cells and to maintain tissue homeostasis, mechanical forces generated outside the cell must be balanced with forces inside the cell **(Figure 1A)** [4]. To maintain balance during cell migration, the cell responds through timely, appropriately graded adjustments in mechanical properties such as stiffness, contractility and tensile strength.

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While it is known that the cell can sense these changes in force through attachment points such as focal adhesions (FA) (cell-matrix) or adherens junctions (cell-cell), the cell must also transmit these forces over long distances via their actin cytoskeleton. Force is transmitted through attachment points, which act as bridges to connect intracellular structural elements [5], such as actin stress fibers (SF), to the extracellular matrix. Changes in mechanical stress (force per unit area) or strain (deformation due to stress) results in either a structural or a chemical signaling change that the cell must sense and compensate for by changing structural components, activating signaling pathways, and adjusting contractility [6].

The actin cytoskeleton adopts a variety of configurations and performs a number of essential cell functions. In concert with microtubules and intermediate filaments, actin networks confer shape, enable cell polarization, support cell-cell junctions, and promote cell adhesion and migration. Among its many configurations, actin can form SFs, first described as tension induced linear structures in the cytoplasm [7]. SFs are composed of 10-30 bundled, unbranched actin polymers [8] with periodic concentrations of the actin crosslinker α-actinin interleaved with non-muscle myosin II [9]. The actomyosin structure of SFs is similar to the sarcomeric patterning in muscle, with the concentrations of α-actinin possibly serving an actin tethering function analogous to its role in the Z-line in muscle [10]. However, unlike muscle, the actin polymers in SFs are overlaid with alternating actin polarity, span multiple sarcomeric units and are far less ordered [10]. As such, SFs may be better suited, functionally, to the

development of steady, isometric contraction, as opposed to the rapid contraction/relaxation cycles of skeletal and smooth muscle. Indeed, SFs arise in tissue where force generation is required, such as during dermal wound closure [11, 12], or in glands that require contraction to expel, for example, milk [13] or saliva [14]. In vascular endothelial cells, SFs are observed to be induced by fluid shear stresses resulting from blood flow [15, 16]. Three types of SF have been described: dorsal SFs, ventral SFs and transverse arcs.[9, 17] (Text Box 1). Among these SF types, ventral SFs, which we focus on in this review, span two FAs and are myosin II mediated force generating machines for the cell [17].

Actin SFs are the principal mediators of force dynamics as they are both mechanically sensitive and mechanically responsive. Additionally, SFs exhibit continuous adjustment of their configuration and composition through constant remodeling and repair [18-20]. While the response of SFs to both chemical and mechanical stress has been studied extensively, little is known about how this response is mediated. The LIM domain family of proteins has emerged as potential arbiters of the response to force in the actin cytoskeleton [20, 21]. Recent proteomic studies identified 26 LIM domain proteins in FAs (Table 1) [22]. Of these 26 proteins, the FA concentrations of 21 are sensitive to contractility inhibition [22, 23]. A subset of these proteins, zyxin, Hic-5, and CRP are recruited to SFs in response to stretch [21, 24], while zyxin and the adapter protein paxillin mediate strain induced SF repair [20, 25, 26]. These recent discoveries support the hypothesis that LIM proteins are mechanoresponders.

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The continuous adaptation of actin SFs and FAs to changing force is an exciting area of investigation at the interface of cell biology and mechanobiology. It is increasingly evident that mechanical force influences integrin-based adhesions, the actin cytoskeleton, and the connections between these two structures [18, 20, 21, 24, 25]. Here, we address the progress and challenges in understanding how SFs sense and respond to force, especially with regard to the emerging role of LIM-domain proteins, in particular zyxin, in mediating this response.

Regulation of force by stress fibers

Our understanding of actin SFs has evolved from a static cable of actin to a flexible, dynamic structure that functions as a tension sensor [27]. Actin SFs anchor at sites of integrin-based FAs forming a complex interface between SFs and FAs. In addition to providing a physical linkage for force transduction and the machinery for *de novo* SF generation, it is also a site of force sensing and signaling. Super resolution fluorescence microscopy of the FA to SF architecture has detailed a stratified hierarchy of protein distributions with distinct functional roles. At the layer proximal to the plasma membrane there is a concentration of integrin signaling molecules, including focal adhesion kinase and the LIM domain protein paxillin [28]. At the intermediary, force transducing layer, actin is linked to integrins through the tension sensitive protein talin [28], which binds vinculin when deformed by tension [29]. More closely associated with the actin SF is a concentration of actin regulatory proteins including zyxin, α-actinin and VASP [28].

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Among the various types of SFs, ventral SFs are essential for maintaining the balance between extracellular and intracellular forces. Besides varying levels of myosin II dependent contractility, other mechanisms can control the gradation of this force. For example, the interface between actin and integrins in FAs is not a rigid connection. Rather, this interface functions as a slip clutch, thereby creating a tension sensitive connection, which becomes more rigid as the FA undergoes force dependent maturation [30-35]. Initially, the relatively unstable connection between actin and integrins is mediated by talin [36]. However, force induced integrin clustering, focal adhesion kinase recruitment and activation and subsequent accumulation of vinculin, zyxin and α-actinin presumably reinforce the connection between actin and integrins. Additionally, SFs undergo continuous adjustment of their configuration and composition [18, 19], resulting in adaptive changes in tensile strength and force distribution **(Figure 1B and Movie S1).**

Execution of an adaptive response to force changes requires both a force sensing and response mechanism. Some proteins recruited to actin structures through their LIM domains in a force dependent manner also contain effector domains that serve to recruit and regulate the signaling of actin regulatory proteins. Therefore, LIM domain proteins appear well suited to mediate the SF's adaptive response to force. Throughout the review, we discuss the role of LIM domain proteins in regulating SF assembly, repair, and remodeling, which allow SFs to adapt to changing forces.

LIM domain proteins: mediators of stress fiber assembly and repair

LIM domains (LIN-11, Isl1 and MEC-3) are dual zinc finger protein-protein or protein-DNA binding interfaces [37]. LIM domain proteins contain multiple LIM domains that coordinately interact with a wide array of binding partners. While the structural core of cysteine and histidine amino acids that coordinates zinc is highly conserved, LIM domains also contain stretches of variable sequence that impart specific high affinity binding [37]. Although most LIM domain proteins contain other functional domains that result in their participation in a wide array of biological processes, these functions are primarily dependent on the interactions mediated by the LIM domains [37].

Proteomic studies have indicated that LIM domain proteins such as Hic-5, paxillin, CRP2, and zyxin are sensitive to mechanical stress in the actin cytoskeleton [22, 23]. Additionally, these proteins may play a variety of roles in regulating actin cytoskeleton dynamics. Among these proteins, zyxin has received extensive study regarding its role in force-regulated management of the actin cytoskeleton, and may provide a paradigm for understanding the roles of other LIM domain proteins in the regulation of cytoskeleton dynamics. Indeed, early studies of zyxin identified it as a cytoskeleton associated LIM domain protein [38], and further work detailed its role as an actin regulatory protein [39] participating in cell motility [40, 41].

Zyxin has three LIM domains at its C-terminus, which are essential for its localization to FAs [42, 43]. The N-terminus of zyxin, which interacts with the

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actin crosslinker α-actinin [40, 44, 45], also contains four proline-rich ActA repeats that mediate zyxin's interaction with actin regulators Mena and VASP [46-48]. Zyxin also contains two leucine-rich nuclear export sequences in its central region [49] **(Figure 2).**

Zyxin exhibits dynamic response to force changes and regulates SF remodeling and repair through recruitment of multiple actin regulatory binding partners. Zyxin dynamics in response to actin SF thinning are visible by live cell imaging of fluorescent labeled proteins **(Figure 1B and Movie S1)** [20]. Recently, the LIM domain adapter protein paxillin has also been shown to be mechanoresponsive, and like zyxin, is involved in SF repair [25, 26]. Below, we discuss the emerging roles of LIM domain proteins in SF remodeling and repair, with an emphasis on zyxin.

LIM proteins regulate SF assembly and remodeling

Ventral SFs frequently span the length of the ventral surface of cells plated on two-dimensional substrates, though length and morphology vary greatly with cell and substrate type. Ventral SFs have FAs at both its ends, which are capable of attaching to substrate, thereby allowing ventral SFs to be directly stretched when the substrate is stretched **(Figure 1A)**. The assembly of ventral SFs requires the involvement of several additional SFs as well as various LIM associated proteins. Three LIM domain proteins have been shown to be recruited to SFs in response to stretch: Hic-5 [24], CRP2 [24], and zyxin [21]. While it remains possible that Hic-5 and CRP2 regulate mechanically induced SF assembly and remodeling, to

date, only zyxin has clearly been shown to do so [21].

Ventral SF assembly initiates through the merging of transverse arcs and dorsal SF fragments **(Text box 1)** [17] and later forms complex, highly dynamic, linked networks within the cell **(Figure 1B and Movie S1)**. Additionally, ventral SF elongate through force dependent *de novo* actin polymerization occurring at FAs [17]. Similar to FA assembly, activation of Rho GTPase induces the assembly of SFs [50]. Rho activates the formin family of actin nucleators to stimulate actin polymerization [18, 51], while GTP-bound Rho activates Rho-associated protein kinase (ROCK), consequently triggering contractility through myosin light chain phosphorylation [52]. Without some minimal level of myosin-based contractility, SFs disperse [52] suggesting myosin is needed for the aggregation of actin filaments into SFs. Indeed, in cell free systems, a mixture of actin filaments and myosin II is sufficient, upon ATP activation, for self-assembly of contractile bundles [53]. Myosin activity is also essential for the formation and maintenance of FAs and consequently for the maintenance of stable substrate connections [52]. However, the requirement for myosin activity may not be strictly due to the tension generated by myosin contractility, as roles for myosin dependent retrograde flow and bundling have also been proposed [54, 55].

During SF assembly, zyxin flows away from FAs in synchrony with newly assembled actin bundles, while FA proteins vinculin and paxillin remain at FAs [56]. This retrograde flux of zyxin, which is force dependent and requires both Rho kinase activity and myosin contractility [56], indicates a role for zyxin in *de novo* SF generation. A variety of data supports this hypothesis. Microscopy of

fluorescently labelled actin in detergent-permeabilized cells showed that actin incorporation was high at zyxin-rich FAs and actin incorporation declined when zyxin localization was displaced by overexpression of the zyxin LIM domain [57]. Moreover, mild shear stress in cells, similar to interstitial fluid flow, induced a perinuclear actin cap structure that failed to form in the absence of zyxin [58]. Lastly, atomic force microscopy, which applied and measured force at cell adhesions [59], revealed a reduction in the pulling force at cell-fibronectin bead contact sites when zyxin expression was knocked down with RNAi [60].

The role of zyxin in the force dependent actin regulation on SFs is not limited to the FA/SF interface. Fibroblast cells respond to uniaxial cyclic stretch through cell wide remodeling, which includes actin SF reinforcement and SF reorientation perpendicular to the stretch axis [21]. Along with actin SF thickening, cyclic stretch stimulates zyxin mobilization from FAs to SFs [21], suggesting the localization of zyxin is important for the actin remodeling process. How does this relocalization occur? In preliminary experiments, when zyxin tagged with photoactivatable GFP was photoactivated in single FAs, Zyxin-GFP spread without preference to SFs attached to the photoactivated FA and to SFs and FAs unattached to photoactivated FA (unpublished results); however, further experiments need to be done to validate this finding. Nonetheless, these results suggest that the primary mode of redistribution is through rapid exchange and cytoplasmic diffusion. In the absence of zyxin, SF reinforcement in response to uniaxial stretch is abrogated, but reorientation persists [21]. Through a different experimental approach, tension-sensitive zyxin dynamics were observed on

SFs, where zyxin dissociated from SFs with relief of tension through laser severing, and was reversibly recruited to SFs in response to AFM stylus-driven tension induction [61].

The localization of zyxin in FAs is also force dependent. Fluorescence recovery after photobleaching was used to track changes in zyxin dissociation following abrogation of traction forces. Force was reduced through either treatment with the Rho-associated kinase inhibitor, Y27632, femtosecond pulsed laser ablation of the proximal attached SF, or plating on soft substrates, and demonstrated an increased rate of dissociation for zyxin when tension was released [62].

Targeting the many actin regulatory proteins to specific cytoskeletal locations at appropriate times is critical to SF response and function. Ena/VASP proteins are actin regulators that bind actin barbed ends and promote filament elongation [63]. Zyxin is required for Ena/VASP recruitment to focal adhesions and to mechanically stimulated SFs [21, 41] as disruption of the zyxin-VASP interaction causes VASP mislocalization and impaired actin remodeling [64]. This finding indicates that zyxin functions as a cytoskeletal adapter protein recruiting VASP to sites of actin remodeling.

Like zyxin, the LIM domain proteins Hic-5 and CRP2 are recruited to SFs in response to cyclic stretch and negatively influence cell contractility [24]. However, unlike zyxin, a scaffolding protein whose role in cytoskeletal regulation appears limited to the recruitment of actin regulators, Hic-5 and CRP2 may regulate contractility through G-protein signals, though this hypothesis remains

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untested. Loss of Hic-5 function in a Hic-5 knockout mouse model results in increased apoptosis both in wire injured femoral arteries and in stretched, cultured vascular smooth muscle cells [65]. Furthermore, cultured Hic-5 null cells exhibited reduced actin SFs following stretch when compared to wild type cells. Reduction of SFs in Hic-5 null cells was accompanied by dispersal of the FA protein vinculin, suggesting the stabilization of vinculin at FAs by Hic-5 confers resistance to stretch induced apoptosis [65]. CRP2 interacts with the LIM domains of Hic-5 [24], but it is not known whether this interaction is responsible for the stretch dependent SF recruitment of either protein. While both Hic-5 and CRP2 concentrate on SFs in response to stretch and regulate cytoskeletal function, further investigation is required to define their roles in SF remodeling and assembly.

LIM proteins mediate SF repair

Actin in SFs undergoes stochastic cycles of thinning and repair (strain elongation followed by restoration of actin and mechanical stabilization) [20] (**Figure 3A**). Traction force microscopy, to measure substrate deformation under FAs, showed attached SFs that underwent localized strain events were under increasing tension prior to initiation of the strain event. Once the strain event was initiated, tension was relieved, followed by a gradual return to baseline tension accompanied by restoration of the actin structure. In this case, the mechanoresponse appeared to be triggered by disruption of the actin bundle [20]. Indeed, if a strain site fails to repair, SF segments retract to the attached FAs.

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SF strain sites recruit at least four different proteins found at FAs: zyxin, paxillin, α-actinin and VASP [20, 25, 26]. However, SF strain sites do not contain vinculin, a hallmark FA protein that links integrins to the actin cytoskeleton [20], nor do they show detectable levels of focal adhesion kinase, or enriched phospho-tyrosine activation [25]. Additionally, SF strain sites do not mature into FA, or show any evidence of substrate attachment. Instead, they are restored to mature striated SF, often with the addition of new sarcomeric units [20, 66]. Therefore, SF strain sites do not appear to be FAs. These data suggest that a subset of actin regulatory components in FAs may resolve SF strain sites in the absence of integrin mediated transmembrane adhesion. Given the parallels between FAs and this 'focal repair complex', other LIM proteins may be targeted to these sites, where they may perform functions similar to those in FAs.

LIM proteins have been implicated in mediating this SF repair. For example, LIM proteins zyxin and paxillin are rapidly recruited to SF strain sites; however, neither is dependent on the other for recruitment, which is detailed below [25], and unlike zyxin, paxillin is not recruited to SFs in a cyclically stretched cell [24]. In cells lacking either zyxin or paxillin, SF strain sites fail to repair, resulting in a significant increase in the frequency of catastrophic SF breakage [20]. Cells lacking zyxin are unable to generate normal levels of traction force, suggesting that failure to stabilize SF ruptures reduces the load bearing capacity of SFs [20].

The repair functions of zyxin are executed by the actin crosslinker α-actinin and the actin regulator VASP, which are recruited to SF strain sites in a zyxin dependent manner [20]. α-actinin binds the N-terminal region of zyxin. Truncation

of the 42 N-terminal amino acids of zyxin disrupts α-actinin binding to zyxin, which significantly reduced α-actinin accumulation at SF strain sites, resulting in a near complete loss of actin repair [20]. This mutation in zyxin does not disrupt zyxin recruitment to SF strain sites, indicating that even though α-actinin binds both actin and zyxin, α-actinin appears to have no role in zyxin recruitment. In cells with non-mutated zyxin, α-actinin recruitment to strain sites slightly lags zyxin, suggesting that zyxin is recruited first, and then zyxin recruits α-actinin [20]. Zyxin binding to VASP is required for VASP recruitment to either cyclically stretched SFs [21] or to SF strain sites [20]. Mutation of the proline-rich ActA repeats in the N-terminal region of zyxin eliminates VASP binding to zyxin, thereby preventing VASP recruitment to SF strain sites and resulting in a failure to stabilize elongation [20]. Failure to stabilize elongation, in this case, may be from the slower accumulation observed with this zyxin mutant or from the loss of VASP's role in facilitating actin polymer elongation [46]. In cells with non-mutated zyxin, VASP is recruited in synchrony with zyxin [20]. Consistent with its role as an adapter, these data suggest that the primary function of zyxin in SF strain site repair is to recruit the actin regulators α-actinin and VASP [20] **(Figure 3B)**.

LIM domain proteins: recruitment to stress fibers

How are LIM proteins recruited in a force dependent manner to the actin cytoskeleton? It was found that a truncated form of zyxin expressing only the LIM domains localized normally to SFs and FAs and, like wild-type, was recruited strongly to SFs with stretch [64] and to SF strain sites [25]. A zyxin mutant lacking its C-terminal LIM domains retained weak localization to FAs, but showed

no SF recruitment response upon stretch [64] or to strain sites [25]. Since many of zyxin's N-terminal binding partners, including α-actinin and VASP also bind directly to actin, and zyxin has never been shown to directly bind actin, zyxin's N-terminal binding partners might recruit zyxin to SFs. However, based on evidence that LIM domains are both necessary and sufficient for recruitment, this hypothesis is not supported.

Similar to zyxin, the LIM domains of paxillin are necessary and sufficient for recruitment to SF strain sites [25] and to FAs [67], while the LIM domains of Hic-5 are required for its recruitment to stretched SFs [24]. For all LIM domain proteins studied in detail thus far, recruitment to SFs is dependent on the LIM domains. However, the mechanism of force driven LIM domain recruitment to cytoskeletal structures remains an open and actively studied question. Clues to answering this question may come from the localization of zyxin and paxillin to SF strain sites as described above. These events appear to be incomplete disruptions of the actin bundle in response to increasing tension [20]. Zyxin recruitment, presumably from a robust cytoplasmic pool, follows immediately **(Figure 3A)**. Recruitment of zyxin is specifically restricted to the ruptured region indicating that the signal for recruitment is resident within that region, and does not occur through a more distal signaling system such as the attached FA or local stress sensing membrane channels. Furthermore, SF strain sites are rich in actin free barbed ends [20], and are sites of rapid actin polymerization, which is required to promote repair. As noted earlier, zyxin is also enriched at FA and in retrograde fluxes [56], which are both sites of actin polymerization. Although not formally

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observed, zyxin could bind directly to strained actin, or bind via its association by an unidentified linker protein that serves as the 'first responder', bridging the gap between the stressed cytoskeleton and zyxin LIM domains. The molecular mark on the actin SF that activates zyxin accumulation in response to strain may be associated with the actin polymerization machinery. Zyxin's recruitment to cytoskeletal structures under tension could be driven by newly revealed actin barbed ends, conformational changes or post-translational modifications in actin or in zyxin binding partners **(Figure 3B)**. Determining which mechanisms regulate zyxin mechanoresponse provide exciting opportunities for future investigations.

Concluding remarks

While this review focused on the role of LIM proteins in the assembly and regulation of ventral SF response to force, similar mechanisms may also exist for other types of SFs. Recent discoveries detailing zyxin's tension sensitive response and roles in the regulation of actin dynamics have opened up new ways of thinking about mechanoresponse and mechanotransduction. First, current data indicate that the mechanically induced accumulation of zyxin on the cytoskeleton is dependent on SF resident feature(s). Although molecular details remain elusive, the signal that recruits zyxin from the cytoplasm appears to be the strained actin structure **(Figure 1B)**. As such, the zyxin mediated repair system resembles DNA break repair wherein a lesion is identified and the stabilization and repair system accumulates at the site of the break. Characterizing how the stress signal is communicated to the repair system will be

helpful in understanding how zyxin and the many other mechanoresponsive LIM domain proteins function. Second, the characterization of the bipartite structure and function of zyxin, with a LIM region dedicated to targeting and a separate region dedicated to actin regulation, may be relevant for understanding how other LIM proteins function **(Figure 2)**. Third, it is clear that the cytoarchitecture is highly dynamic, and as such, requires active management to maintain its structural and organizational functions. While this involves cell wide stress responses such as up or down regulation of cytoskeleton components, targeted repair of strain sites [20] and addition of new sarcomeres in the middle of stress fibers [66] indicate there is a tightly targeted response to spatially restricted sites of strain **(Figure 3)**. Investigating how these mechanically stressed sites are sensed and repaired will provide further insights into how cell structure is fine-tuned to maintain homeostasis. Finally, as noted previously, the LIM domains of both zyxin and paxillin are recruited independently to stress fiber strain sites [25, 26]. However, unlike zyxin and Hic-5, paxillin is not recruited to cyclically stretched SFs [24]. Thus, although LIM proteins as a group appear to share the capacity to accumulate on actin structures in response to increased contractility or mechanical stress, they do so with a degree of specificity that suggests the involvement of additional regulatory controls that serve to enhance or limit the participation of specific proteins to specific physiological circumstances. Understanding the regulation of mechanoresponse specificity provides a compelling challenge for future research.

Text Box1

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Text box 1; Stress fibers types and formation

Stress fibers are formed through a combination of *de novo* polymerization that occurs at FAs, and the merging of previously formed fragments. SFs form complex, highly dynamic linked networks within the cell. They have been classified as dorsal, ventral or transverse arcs. The formation of these three types of SF was described in a study performed in human osteosarcoma cells [17].

Dorsal Stress Fibers Dorsal SFs typically are associated with a single FA, where they are formed through formin mDia1 mediated actin polymerization. They contain α-actinin that does not take on a periodic appearance until the free end of the SF attaches to a transverse arc or ventral SF and myosin II displaces and interdigitates α-actinin rich nodes [17].

Transverse Arcs Transverse arcs are not associated with FA, but generated through the myosin II dependent merging of short Arp2/3 dependent actin filament fragments that are formed in the lamellipodia [17].

Ventral Stress Fibers Ventral SFs are connected to FAs at both ends and as such are the SF type responsible for force generation. Ventral SFs form when a region of transverse arc spanning connections to two dorsal SFs contracts and sheds regions not between the dorsal SFs [17].

Muscle While SFs do not display the crystalline orderliness of mature muscle, particularly in terms of the strict organization of actin polarity, SF are strikingly similar to developing myofibrils. Like SFs, premyofibrils and nascent myofibrils

contain alternately polarized actin polymers, periodically α-actinin rich z-bodies, and interdigitated non-muscle myosin. As myofibrils mature, their sarcomeric structure becomes more defined, and nonmuscle myosin is replaced by muscle myosin [68]. While a number of LIM proteins are found in muscle, little is known of their roles in muscle development, remodeling and maintenance. Zyxin is present in skeletal muscle, but is more enriched in smooth muscle, especially in the lung [69]. Future work may show key roles for LIM proteins in force bearing tissues like smooth and skeletal muscle.

Table 1

LIM Family	Presence in Focal Adhesions [22, 23, 70]	Sensitive to changes in myosin II contractility (assessed by Blebbistatin treatment) [22, 23]	Localize to stress fibers with cyclic stretch	Localize to stress fiber strain sites
ZYXIN				
Ajuba	Unknown	Unknown	Unknown	Unknown
LIMD1	YES	YES	Unknown	Unknown
LPP	YES	YES	Unknown	Unknown
Migfilin/FBLIM1	YES	YES	Unknown	Unknown
TRIP6	YES	YES	Unknown	Unknown
WTIP	Unknown	Unknown	Unknown	Unknown
Zyxin	YES	YES	YES[21]	YES[20]
PAXILLIN				
HIC5	YES	YES	YES[24]	Unknown
Leupaxin	Unknown	Unknown	Unknown	Unknown
Paxillin	YES	YES	NO[24]	YES[25]
LHX				

ISL1	Unknown	Unknown	Unknown	Unknown
ISL2	Unknown	Unknown	Unknown	Unknown
LHX1	Unknown	Unknown	Unknown	Unknown
LHX2	Unknown	Unknown	Unknown	Unknown
LHX3	Unknown	Unknown	Unknown	Unknown
LHX4	Unknown	Unknown	Unknown	Unknown
LHX5	Unknown	Unknown	Unknown	Unknown
LHX6	Unknown	Unknown	Unknown	Unknown
LHX9	Unknown	Unknown	Unknown	Unknown
LMX1a	Unknown	Unknown	Unknown	Unknown
LMX1b	Unknown	Unknown	Unknown	Unknown
LMO				
LMO1	Unknown	Unknown	Unknown	Unknown
LMO2	Unknown	Unknown	Unknown	Unknown
LMO4	Unknown	Unknown	Unknown	Unknown
LMOX	Unknown	Unknown	Unknown	Unknown
CRP				
CRP1	YES	YES	Unknown	Unknown
CRP2	YES	YES	YES[24]	Unknown
CRP3	Unknown	Unknown	Unknown	Unknown
FHL				
ACT	Unknown	Unknown	Unknown	Unknown
FHL1	YES	Unknown	Unknown	Unknown
FHL2	YES	YES	Unknown	Unknown
FHL3	YES	YES	Unknown	Unknown
PINCH				
PINCH/LIMS1	YES	Yes	Unknown	Unknown
PINCH2/LIMS2	YES	Unknown	Unknown	Unknown
Testin				
Dyxin	Unknown	Unknown	Unknown	Unknown
LMO6	Unknown	Unknown	Unknown	Unknown
RILP	Unknown	Unknown	Unknown	Unknown
Testin	YES	YES	Unknown	Unknown
Enigma				
Cypher/PDLIM6	Unknown	Unknown	Unknown	Unknown
ENH/PDLIM5	YES	YES	Unknown	Unknown
Enigma/PDLIM7	YES	YES	Unknown	Unknown
PDLIM1	YES	YES	Unknown	Unknown
ALP				
ALP/PDLIM3	Unknown	Unknown	Unknown	Unknown
CLP-36	Unknown	Unknown	Unknown	Unknown
Mystique/PDLIM2	YES	YES	Unknown	Unknown
RIL/PDLIM4	YES	YES	Unknown	Unknown
LASP				
LASP	YES	YES	Unknown	Unknown
LIM-nebulette	Unknown	Unknown	Unknown	Unknown

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NRAP	Unknown	Unknown	Unknown	Unknown
MICAL				
MICAL	YES	Unknown	Unknown	Unknown
MICAL-like	YES	Unknown	Unknown	Unknown
MIRAB	Unknown	Unknown	Unknown	Unknown
LIMK				
LIMK1	Unknown	Unknown	Unknown	Unknown
LIMK2	Unknown	Unknown	Unknown	Unknown
Other				
ABLIM	YES	YES	Unknown	Unknown
EPLIN/LIMA1	YES	YES	Unknown	Unknown
LMO7	YES	Unknown	Unknown	Unknown
Scielin	Unknown	Unknown	Unknown	Unknown
ZNF185	Unknown	Unknown	Unknown	Unknown

Text Box 2

Local response to global stress

Zyxin responds rapidly to a variety of signals through highly localized accumulation on cytoskeletal structures. These signals include internally driven contractility modulation or externally derived forces. The zyxin response varies according to the signal.

Focal adhesion maturation requires either myosin II contractility or application of external force [71]. As adhesions mature, they become larger and accumulate higher levels of zyxin. These mature adhesions exhibit lower levels of traction force transfer to the substrate [72], suggesting more of an anchoring than a mobilizing function. Several studies, wherein attached SF were either severed or tugged upon, showed zyxin binding kinetics decreased with a drop in tension [62] or increased with higher tension [61].

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Retrograde fluxes are displacements of actin and focal adhesion proteins along FA proximal SFs. As such, they have the appearance of comet tails. Proteins identified in fluxes include zyxin and VASP, as well as FAK. Zyxin movement in fluxes tracks with the flow of actin [56]. Fluxes can be enhanced through immobilization on micropatterned islands, applied stretching forces or plating on stiff substrates. Relief of SF contractility through blebbistatin treatment eliminates fluxes. Also of note, fluxes are dependent on tyrosine phosphorylation at FA [56].

Stress fiber strain sites are localized regions of SFs that undergo rapid extension accompanied by thinning and subsequent recovery of actin **(Figure 1B)**. These sites are initiated by increasing tension in the SF, which the strain event serves to relieve. Zyxin recruits α-actinin and VASP to the strain site where they aid repair. Failure to repair strain sites by the zyxin-dependent repair mechanism results in catastrophic rupture and retraction of the SF [20]. Recent work has shown that paxillin has a role in strain site repair independent of zyxin [25, 26].

Global stress fiber thickening in response to cyclic stretch also occurs through a zyxin dependent mechanism. Uniaxial cyclic stretch results in thickened SFs aligned perpendicular to the axis of stretch, suggesting an orientation that attempts to minimize mechanical stress to the SF. Thickening is accompanied by extensive localization of zyxin on the SF [21]. Global thickening and zyxin recruitment are also induced by jasplakinolide induces actin stabilization [21].

Acknowledgements

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Figure Legends

Figure 1 Force and stress fiber dynamics. (A) Cellular and tissue integrity require that intracellular and extracellular forces be balanced. Integrin-based focal adhesions anchor intracellular actin stress fibers to the extracellular matrix. Stress fibers have a characteristic 'sarcomeric' structure with α-actinin rich puncta (green dots on stress fibers) interdigitated with regions enriched in myosinII. (Inset) Transmembrane integrins (green) connect to the extracellular matrix (orange filaments) and to focal adhesions (magenta dots), which anchor actin filaments (magenta lines). Force is transferred from the extracellular matrix to the stress fiber via focal adhesions. (B) Mouse fibroblast cell labeled with zyxin-GFP and actin-mApple shows the dynamic nature of the actin structure and

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compensatory zyxin redistribution, including the development and repair of stress fiber strain sites. The magnified views show the development of a stress fiber strain site exhibiting rapid zyxin accumulation (bracket) (see also Movie S1).

Figure 2 Zyxin domain structure. (A) The cytoskeletal protein zyxin has proline rich act-A repeats, leucine-rich nuclear export sequences, serine phosphorylation sites and cysteine/histidinezinc coordinating LIM domains. Zyxin N-terminus has binding sites for α-actinin and VASP and mediates actin regulatory functions. Zyxin C-terminus has three LIM domains for protein interactions and mediates force induced targeting.

Figure 3 Development of a stress fiber strain site. Stress fibers have a periodic, muscle sarcomere-like structure with non-muscle myosin interleaved between α-actinin and zyxin rich densities. Increasing tension on the stress fiber, possibly because of myosin contractility, induces spontaneous ruptures, resulting in rapid elongation of the site and thinning of the actin structure. This generates actin free barbed ends, and triggers recruitment of a zyxin dependent repair system consisting of α-actinin and VASP. Resolution of the strain site is characterized by a reinforced actin bundle and insertion of new sarcomeric units. B) Hypothetical model of zyxin mechanoresponse.

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Supplemental information

Movie S1. Time lapse micrograph showing an unperturbed mouse fibroblast labeled with zyxin-GFP and actin-mApple.

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Figure 1 Force and stress fiber dynamics

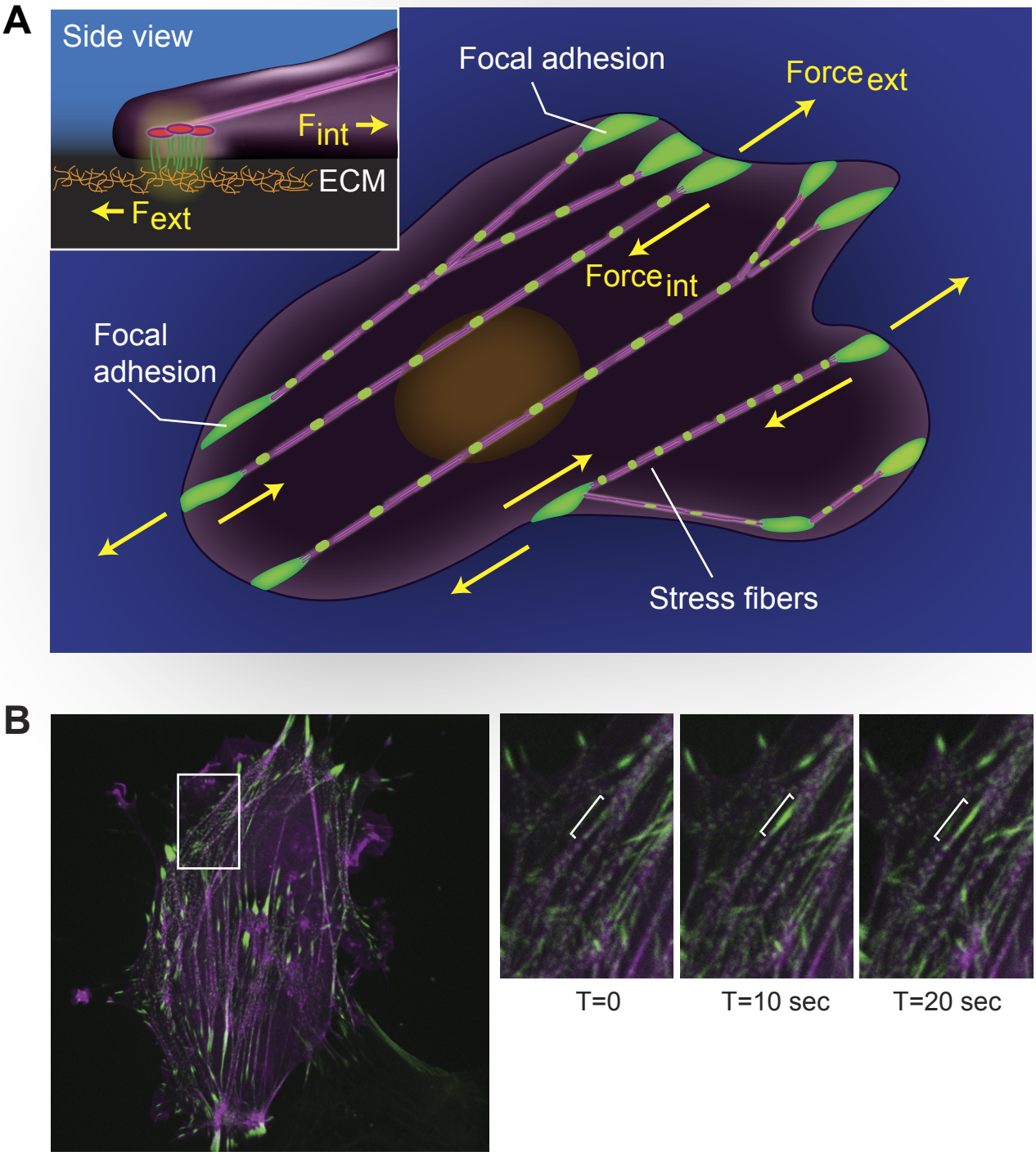


Figure 2 Zyxin domain structure and hypothetical mechanism of mechanoresponse

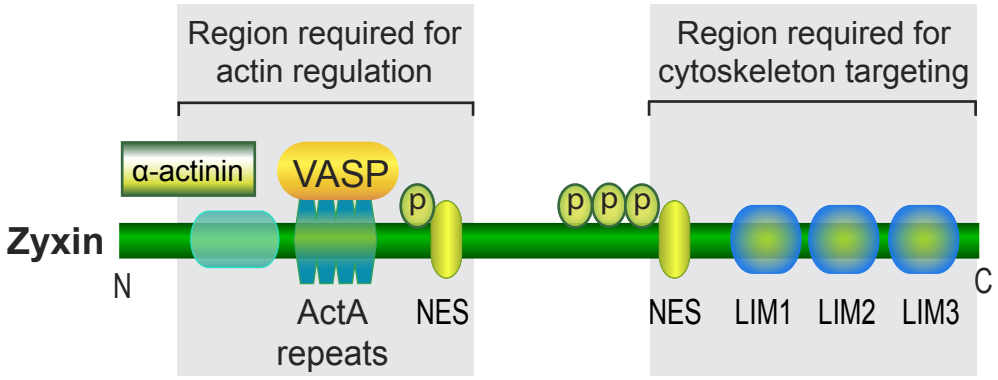
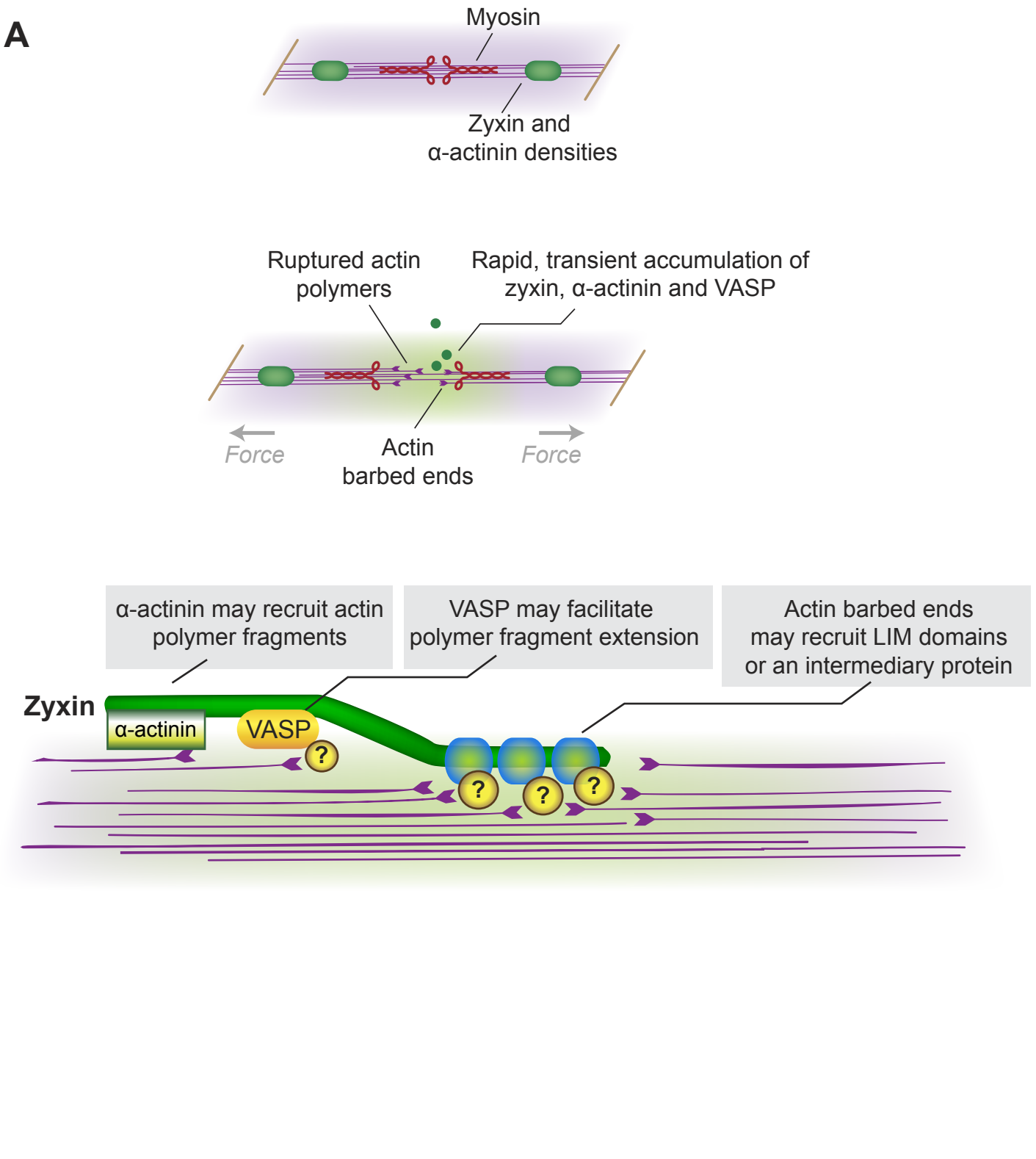


Figure 3 Development of a stress fiber strain site



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