Mechanical signals activate p38 MAPK pathway-dependent reinforcement of actin via mechanosensitive HspB1

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ABSTRACT Despite the importance of a cell’s ability to sense and respond to mechanical force, the molecular mechanisms by which physical cues are converted to cell-instructive chemical information to influence cell behaviors remain to be elucidated. Exposure of cultured fibroblasts to uniaxial cyclic stretch results in an actin stress fiber reinforcement response that stabilizes the actin cytoskeleton. p38 MAPK signaling is activated in response to stretch, and inhibition of p38 MAPK abrogates stretch-induced cytoskeletal reorganization. Here we show that the small heat shock protein HspB1 (hsp25/27) is phosphorylated in stretch-stimulated mouse fibroblasts via a p38 MAPK-dependent mechanism. Phosphorylated HspB1 is recruited to the actin cytoskeleton, displaying prominent accumulation on actin “comet tails” that emanate from focal adhesions in stretch-stimulated cells. Site-directed mutagenesis to block HspB1 phosphorylation inhibits the protein’s cytoskeletal recruitment in response to mechanical stimulation. HspB1-null cells, generated by CRISPR/Cas9 nuclease genome editing, display an abrogated stretch-stimulated actin reinforcement response and increased cell migration. HspB1 is recruited to sites of increased traction force in cells geometrically constrained on micropatterned substrates. Our findings elucidate a molecular pathway by which a mechanical signal is transduced via activation of p38 MAPK to influence actin remodeling and cell migration via a zyxin-independent process.

INTRODUCTION Mechanical forces are sensed by cells and transduced into biochemical signals that drive changes in gene expression and influence cell fate (Discher et al., 2009; Wozniak and Chen, 2009; Mammoto et al., 2012). This ability to sense and respond to mechanical cues is essential during development and throughout an organism’s life. Moreover, disturbance of mechanical homeostasis is associated with myriad disease phenotypes (Deanfield et al., 2007; Chen, 2008; Fournier et al., 2010; Ateshian and Humphrey, 2012). For a mechanical signal to influence biology, it must be converted to a chemical signal that is capable of regulating cell behavior and function; this is the process of mechanotransduction. A major challenge in cell biology is to ascertain the mechanistic details that underlie mechanotransduction.

A central element of a mechanotransduction pathway from the cell surface to the cell interior involves the coupling of cell-surface adhesion receptors, such as integrins, to mechanosensors that are themselves conformationally modulated in response to mechanical cues or adapted to recognize such force-induced changes (McGough et al., 1997; Sawada et al., 2006; del Rio et al., 2009; Shimozawa and Ishiwata, 2009; Ehrlicher et al., 2011; Hayakawa et al., 2011; Uyeda et al., 2011; Galkin et al., 2012). Integrins are transmembrane heterodimers that bind extracellular matrix and anchor actin-rich stress fibers (SFs) at prominent membrane-substratum adhesion sites called focal adhesions (FAs) (Burridge et al., 1988). Essential for maintaining tensional homeostasis, SFs are...
myosin-associated, contractile bundles of filamental actin (F-actin). SFs are prominent in cultured cells, particularly fibroblasts, and also assemble in vivo when cells experience mechanical stress (Wong et al., 1983; Byers et al., 1984). FAs enable internal SF-generated contractile forces to be transmitted to the cell exterior and external mechanical signals to be communicated to the cell interior (Harris et al., 1980; Wang et al., 1993; Lauffenburger and Horwitz, 1996). As such, FAs are recognized as a major site for the transmission of physical cues from the cell exterior to interior (Geiger et al., 2009; Yan et al., 2015; Horton et al., 2016).

The exposure of cells to mechanical stimulation results in remodeling of the actin cytoskeleton, providing an attractive system for the study of the mechanotransduction response. When fibroblasts are plated on a flexible substrate and subjected to uniaxial cyclic stretch, they reorient perpendicular to the stretch vector and undergo a SF thickening or “reinforcement” response (Yoshigi et al., 2005; Hoffman et al., 2012). The SF reinforcement response depends on the recruitment of LIM domain proteins, such as zyxin and paxillin, both of which contribute to the actin remodeling that occurs in response to mechanical stress (Yoshigi et al., 2005; Smith et al., 2010, 2013). Abrogation of the SF reinforcement response is evident in zyxin-null and paxillin-null cells, and this results in an increase in spontaneous SF breaks within the cells, illustrating that the mechanotransduction response that leads to SF remodeling and reinforcement is key to cytoskeletal homeostasis (Smith et al., 2013; Smith et al., 2014). Although several LIM proteins have now been implicated in the response of cells to mechanical force (Kim-Kaneyama et al., 2005; Hirata et al., 2008; Colombelli et al., 2009; Schiller et al., 2011), little is understood about the upstream signaling cascades that are activated as a prelude to the actin reinforcement response.

Mitogen-activated protein kinase (MAPK) signaling cascades are activated in mechanically stimulated fibroblasts (Nguyen et al., 2000; Sawada et al., 2001) as well as in organs such as the heart and lung when mechanical stress is elevated (Zhang et al., 2000; Dolinay et al., 2008). Activation of p38 MAPK signaling occurs in response to fluid shear stress (Li et al., 1996), in response to pulling on magnetic beads attached to cells (Wang et al., 2005), and in response to cyclic deformational strain (Chaudhuri and Smith, 2008). The p3 pathway is activated by multiple environmental stimuli that are transmitted via cell-surface proteins, including transmembrane growth factor receptors, receptor tyrosine kinases, and G-protein coupled receptors (Cuadrado and Nebreda, 2010; Hotamisligil and Davis, 2016; Rodriguez-Carballo et al., 2016). Mechanical stimulation has been shown to activate the small G-protein Rap1 and MKK3/6 kinases that phosphorylate p38 (Sawada et al., 2001). P38 MAPK signaling is also activated downstream of transmembrane integrin engagement via focal adhesion kinase and is antagonized by the protein tyrosine phosphatase PTEN (Aikawa et al., 2002). Integrin engagement has also been shown to be required for the response of fibroblasts to uniaxial cyclic stretch (Yoshigi et al., 2005), thus providing a link between FAs and mechanically sensitive MAPK signaling. Here we have interrogated the role of p38 MAPK signaling in the response of cells to uniaxial cyclic stretch and have uncovered a role for this pathway in the stretch-dependent actin-reinforcement response. We have explored the molecular mechanism by which stretch-activated p38 MAPK signaling influences the mechanotransduction response, defining the role of p38 MAPK-dependent phosphorylation of the small heat shock protein HspB1 (also known as mouse hsp25 and human hsp27) in the response of cells to mechanical stress.

RESULTS
Uniaxial cyclic stretch activates p38 MAPK and induces actin cytoskeletal remodeling

Despite the broad functional significance of a cell’s ability to sense and respond appropriately to mechanical cues, the molecular mechanisms by which physical cues are converted to cell-instructive chemical information remain to be elucidated. The response of fibroblasts to mechanical stress can be studied by exposing the cells to a regimen of controlled uniaxial cyclic stretch. This type of stimulation induces alignment of fibroblasts perpendicular to the stretch vector and also promotes an actin remodeling response. We have explored the molecular mechanism by which such mechanical signal influences cellular biochemistry is postulated to involve MAPK

Figure 1: Uniaxial cyclic stretch promotes actin remodeling and activation of p38 MAPK signaling. (A) Mouse fibroblasts were stimulated by uniaxial cyclic stretch (15%, 0.5 Hz, 1 h) followed by detection of F-actin with fluorescently tagged phalloidin; the stretch direction is on the horizontal plane, indicated by a double-headed arrow scaled to 50 μm. (B) Comparison of the SFTI of unstretched and stretched-stimulted cells revealed a statistically significant increase in SFTI. Graph is mean with SD, ***p < 0.0001 was calculated using unpaired Student’s t test that assumed Gaussian distribution and equal SD between populations, n >100 SFTI measurements in >13 microscopic fields per group. (C) Schematic representation of p38 MAPK pathway. (D) Western immunoblot analysis (20 μg protein per lane) revealed the activation of the p38 MAPK pathway in response to uniaxial stretch cyclic, as illustrated by phosphorylation of p38, MK2, and HspB1. Immunoblot detection of vinculin confirms equal protein loading across lanes and detection of ERK1/2 activation confirms effective delivery of the stretch signal. (E) Quantification of phosphorylation signals relative to unstretched samples (set at onefold baseline) illustrates the sustained activation of p38 signaling during the 60 min period of the stretch regimen, mean with SD as pooled from more than three independent stretch experiments.
Inhibition of the p38 pathway abrogates stretch-induced reinforcement of actin SFs

To test whether activation of the p38 pathway is required for the actin reinforcement response downstream of mechanical stress, we evaluated the actin stretch response in the presence of the p38 inhibitor SB203580 (Cuenda et al., 1995; Guay et al., 1997; Young et al., 1997). Cells were preincubated with dimethyl sulfoxide (DMSO) (control) or with SB203580 for 1 h and then stimulated with uniaxial cyclic stretch in the presence of DMSO or SB203580. Phosphorylation of both MK2 and HspB1 occurs in DMSO-treated cells exposed to uniaxial cyclic stretch, whereas phosphorylation of both proteins is abrogated in cells exposed to the p38 inhibitor (Figure 2A). These findings are consistent with the hypothesis that the stretch-dependent phosphorylation of MK2 and HspB1 is largely attributable to activation of the p38 pathway. The p38 inhibitor-sensitive phosphorylation of HspB1 is observed within 5 min of stretch application under conditions where no corresponding increase in HspB1 protein is observed (Figure 2B). Although exposure to SB203580 does not result in dissolution of the actin cytoskeleton and drug-treated cells properly align SFs perpendicular to the stretch vector (Figure 2C), cells stretched in the presence of the p38 inhibitor display a statistically significant deficiency in the SF reinforcement response (Figure 2, C and D). To determine whether the kinase responsible for elevated phosphorylation of MK2 and HspB1 is MK2, we stimulated MK2-null and rescue cells (generously provided by Matthias Gaestel) (Ronkina et al., 2007; Sousa et al., 2007). The levels of phospho-HspB1 are increased in the MK2 rescue cells compared with MK2-null cells (Figure 2E), suggesting that MK2 is likely the kinase responsible for the phospho-HspB1 signal we are detecting. Although our findings clearly illustrate that elimination of MK2 abrogates the stretch-induced phosphorylation of HspB1, the MK2-null cells are transformed with SV40 large T antigen (Ronkina et al., 2007) and thus cannot be directly compared with the other cell types used in this study. Moreover, we detect two MK2 isoforms in our WT fibroblasts consistent with previous reports while the rescue cell line expresses only one MK2 isoform (Ronkina et al., 2007; Sousa et al., 2007). Collectively, our findings illustrate that p38 MAPK signaling contributes to the mechanotransduction response and caused us to focus our research on understanding the role of MK2 in the mechanical reinforcement of actin SFs.
HspB1, a downstream target of the p38 pathway, is recruited to the actin cytoskeleton in response to mechanical cues

HspB1 was previously identified as a regulator of actin filament assembly that copurifies with vinculin (Miron et al., 1988, 1991). The p38-dependent phosphorylation of HspB1 in response to stretch raised the intriguing possibility that HspB1 might play a role in the actin remodeling that is induced by mechanical stimulation. If HspB1 contributes to the reinforcement of actin SFs downstream of mechanical stress, then we might expect to detect it in association with the actin cytoskeleton. As reported by others (Collier and Schlesinger, 1986; Lavoie et al., 1995; Clarke and Mearow, 2013), we find that HspB1 displays a diffuse cytoplasmic distribution in unstretched (0 min of stretch) fibroblasts (Figure 3A). However, when cells are stimulated by exposure to uniaxial cyclic stretch for 15 or 60 min, HspB1 is frequently detected on linear elements within the cytoplasm that we postulated might correspond to subdomains of actin SFs (Figure 3A). The colocalization of HspB1 with actin SFs in stretched cells was confirmed by coincidence of the HspB1 immunofluorescence signal (arrowheads) with that of F-actin as detected by Phalloidin staining (Figure 3B). These findings illustrate that HspB1 is recruited to actin SFs in response to cell stretch.

Since HspB1 is phosphorylated in a p38-dependent manner in response to stretch, we investigated whether phospho-HspB1 is the isoform that is recruited to the actin cytoskeleton in response to mechanical stimulation. Immunostaining of unstretched or stretched cells with an antibody that recognizes phosphorylated HspB1 (Ser86) revealed that phospho-HspB1 becomes concentrated on linear cytoplasmic elements in response to uniaxial cyclic stretch (Figure 3C). The localization of phospho-HspB1 is prominent at the cell periphery and is also observed overlaying the nucleus in structures reminiscent of transmembrane actin-associated nuclear (TAN) lines (Luxton et al., 2011) in some cells. Quantitative analysis of the percentage of cells that exhibited a discrete, SF-associated subcellular localization of phospho-HspB1 in response to stretch is shown in Figure 3D. Phospho-HspB1 is barely detectable in unstretched cells (<10% of cell population). In contrast, within 5 min (Figure 3C) we observe recruitment of phospho-HspB1 to subdomains of actin SFs in response to stretch that peaks with ~50% of cells displaying some cytoskeletal accumulation of phospho-HspB1 after exposure to a 60-min stretch regimen (Figure 3D). To evaluate whether there is a correlation between the detection of cytoskeletal-associated phospho-HspB1 and the reinforcement of actin SFs as evidenced by SFTI analysis, we compared the population-wide SFTI

FIGURE 3: Phospho-HspB1 is recruited to discrete SF domains in response to uniaxial cyclic stretch. (A) Indirect immunofluorescence localization of total HspB1 in stretch-stimulated mouse fibroblasts shows diffuse cytoplasmic HspB1 distribution in unstretched cells and HspB1 recruitment to short linear elements within the cytoplasm of stretch-stimulated cells. Two examples of labeling are shown for the 60 min timepoint to illustrate that we observe HspB1 can be found at both SF termini (arrowheads) and full SFs (arrows) in stretched cells. (B) Double labelling of HspB1 and F-actin reveals that HspB1 displays a subcellular distribution that overlaps with actin SFs (arrowheads) but does not generally populate the entire expanse of transcellular SFs. (C) Indirect immunofluorescence localization of phosphorylated HspB1 shows a rapid accumulation of phospho-HspB1 in association with SF termini in stretched cells. Phospho-HspB1 is also detected on linear nuclear lines in some stretched cells. (D) Quantitation of the percentage of cells displaying discrete phospho-HspB1 localization under unstretched or stretched conditions compared across four independent experiments. (E) SFTI analysis of stretch-stimulated F-actin is presented as total population (4.3 SFTI, 251 roi) and then stratified for phospho-HspB1-positive cells (5.0 SFTI, 187 roi) and phospho-HspB1-negative cells (4.0 SFTI, 140 roi). Cells with phospho-HspB1 on the cytoskeleton generally have higher SFTI. (F) Double labeling of mouse fibroblast on coverslip with antibodies directed against vinculin (magenta) and zyxin (green). Scale bar is 25 μm. (G) Double labeling of stretch-stimulated fibroblast with antibodies directed against vinculin (magenta) and phospho-HspB1 (green). (F, G) The region of zyxin and phospho-HspB1 at comet tails (white arrowheads) that do not colocalize with vinculin (yellow arrowheads) is indicated. Double-headed arrows indicate the stretch vector and scale of 25 or 50 μm as designated. Graphs are shown as mean with SD. P values were determined by unpaired Student’s t test assuming Gaussian distribution and equal SD between populations. **p < 0.001, ***p < 0.0001, n.s. = not statistically significant.
section with vinculin as detected by indirect immunofluorescence. Although the most striking and consistent distribution of phospho-HspB1 in stretched cells corresponds to actin comet tails, in the 50% of cells that display cytoskeletal association of HspB1, we observe diversity in the phospho-HspB1 staining patterns, having noted both variable staining intensity, as well as heterogeneity in extent of comet tail labeling and occasional labeling of transverse SFs (Supplemental Figure S1).

The elaboration of actin comet tails can be promoted by plating cells on square, micropatterned substrates (Parker et al., 2002). Under these conditions, the accumulation of zyxin and the formation of zyxin-rich comet tails are concentrated at the four corners of the cells where ruffling and the strongest traction forces are observed (Wang et al., 2002; Guo and Wang, 2007; Rape et al., 2011). Therefore, to test whether the accumulation of phospho-HspB1 is enhanced at areas of strong traction forces, we plated green fluorescent protein (GFP)-zyxin expressing cells on micropatterned, fibronectin surfaces of various dimensions and examined the localization of both zyxin and phospho-HspB1 (Figure 4). Zyxin and phospho-HspB1 both accumulated at the corners of the square-shaped cells, indicating that the recruitment of both proteins is force sensitive. However, the two proteins did not display complete overlapping distributions. In the first image of a cell spread on the large (10,000 μm²) micropatterned area (example 1), zyxin comet tails are readily apparent at the corners (arrowheads) but phospho-HspB1 appears in a diffuse cytoplasmic distribution. In the second image showing cells plated on the large (10,000 μm²) micropatterned area (example 2), one can observe overlapping distributions of zyxin and phospho-HspB1 at comet tails (Figure 4, arrows).

**Robust recruitment of HspB1 to comet tails depends on sustained mechanical stimulation and is inhibited by cytochalasin D**

To determine whether sustained mechanical stimulation is required for maintenance of HspB1 phosphorylation, cells were stretch stimulated for 1 h and then removed from stretch stimulation for another hour prior to fixation. Phospho-HspB1 increases on stretch stimulation and declines on removal of the stretch stimulation (Figure 5A). Immunolocalization studies show that phospho-HspB1's cytoskeletal accumulation declines on cessation of cell stretch (Figure 5B). The SFTI increases with stretch stimulation and returns to basal levels on removal of stretch stimulation (Figure 5C). Under conditions that do not result in complete dissolution of SFs, abrogates the accumulation of phospho-HspB1 on SF termini (Figure 5D). These results are consistent with the view that the cytoskeletal localization of phospho-HspB1 is.
depends on the availability of free F-actin barbed ends. The stretch-induced SF reinforcement response is also compromised in response to treatment with cytochalasin D (250 nM, preincubation 1 h, 7 μg protein) during stretch stimulation (15% uniaxial, 0.5 Hz, 15 min and 60 min) compromises the recruitment of phospho-HspB1 (green) to actin SFs (phalloidin, magenta) and abrogates the SF reinforcement response (E, SFTI analysis; n = 259, 149, 283, 147 roi, respectively). (F) Western immunoblot shows stretch-induced phosphorylation of HspB1 continues in the presence of cytochalasin D. Vinculin immunodetection confirms equal protein loading. Graphs are presented as mean with SD, values were derived from unpaired t tests that assumed Gaussian distribution and equal SDs between populations. ***p < 0.0001, n.s. = not statistically significant.

**Phosphorylation of HspB1 is required for its mechanically sensitive cytoskeletal accumulation**

Since the stretch-induced recruitment of HspB1 to cytoskeletal structures occurs concomitant with increased phosphorylation of HspB1, we tested the possibility that stretch-induced phosphorylation directs the cytoskeletal recruitment of HspB1. Murine HspB1 is phosphorylated by MK2 on two serine residues in response to p38 MAPK activation (Stokoe et al., 1992) (Figure 6A). We generated site-specific mutations in a GFP-tagged variant of HspB1 to enable direct visualization of the phospho variants in unstretched and stretched cells. The HspB1-GFP proteins were expressed in wild-type (WT) fibroblasts using a lentiviral delivery system and subsequent fluorescence-activated cell sorting (FACS) to isolate a population enriched in GFP-expressing cells. Importantly, C-terminally tagged HspB1 faithfully recapitulates the subcellular distribution of endogenous phospho-HspB1 in stretched cells (Figure 6B). Western immunoblot analysis shows the expression of wild-type HspB1-GFP as well as two mutant variants: the unphosphorylatable S2A variant in which Ser15 and Ser86 are mutated to alanine and the S2E variant in which Ser15 and Ser86 are mutated to the phosphomimetic residue glutamic acid (Figure 6C). WT, S2A, and S2E-HspB1-GFP do not display cytoskeletal localization in unstretched cells (Figure 6, D–F). Thus the phosphomimetic variant S2E is not sufficient to drive actin SF accumulation in the absence of a bona fide mechanical signal. Compared to WT HspB1 (Figure 6D), the S2A variant fails to associate with the actin cytoskeleton in response to stretch stimulation (Figure 6E), consistent with the view that phosphorylation of HspB1 on Ser15 and/or Ser86 is necessary for cytoskeletal recruitment. The phosphomimetic S2E variant displays visible recruitment to the actin cytoskeleton in response to stretch (Figure 6F). This recruitment typically appeared less robust compared with WT protein, possibly reflecting preferential cytoskeletal association of the endogenous HspB1 that is present in the GFP-expressing cells.

**HspB1-null cells generated by CRISPR/Cas9 genome editing are deficient in stress fiber remodeling**

A CRISPR/Cas9 nuclease strategy was employed to disrupt the murine HSPB1 gene to define the phenotypic consequences of loss of HspB1 function in cells (Figure 7A). Two regions in exon 1 were targeted (target sequences 23 and 27), and multiple cell lines were isolated and screened for HspB1 status. Immunoblot analysis of the parental WT cells and two independently derived CRISPR lines (lanes A and B) illustrate that HspB1 protein is not detected in the CRISPR lines, whereas upstream control proteins p38 and MK2 remain at WT levels (Figure 7B). The HspB1-null cells are readily propagated in cell culture and adhere to plastic dishes and glass coverslips. However, HspB1-null cells display abrogated spreading compared with WT cells on coverslips coated with increasing concentrations of the extracellular matrix fibronectin, as assessed by cell area measurements 3 h after plating the cells (Figure 7C). Cell morphology and FA status were assessed by staining cells plated for 3 h on coverslips coated with 10 μg/ml fibronectin (Figure 7D). Vinculin-rich FAs are detected in the HspB1-null cells; however, the FAs often appear smaller and less robust than in wild-type controls under these short-term culture conditions. Comparison of the actin cytoskeletons of unstretched parental WT cells to the HspB1-null cells does not reveal a consistent alteration in F-actin content by visual inspection (Figure 7E) or by SFTI analysis (Figure 7F), suggesting that HspB1 is not essential for baseline maintenance of the actin cytoskeleton in cultured fibroblasts. In response to uniaxial cyclic stretch, HspB1-null cells align perpendicular to the stretch vector comparable to wild-type cells; however, the actin reinforcement response is abrogated in HspB1-null cells compared with wild-type cells (Figure 7F).
HspB1-null cells display enhanced cell migration

The establishment of zyxin-rich actin comet tails is promoted by mechanical cues and is inversely correlated with cell migration (Guo and Wang, 2007). Zyxin-null cells display increased migration relative to their wild-type counterparts, consistent with the view that zyxin acts as a negative regulator of cell migration (Hoffman et al., 2006). Given the localization of phospho-HspB1 on focal adhesion-associated actin comet tails, we postulated that elimination of HspB1 expression might affect cell migration. We used time-lapse microscopy to compare the migratory behaviors of the parental WT cells and the 2 HspB1-null cell lines. Cells were seeded to confluent into small chambers that were removed prior to microscopy, giving an even starting line to follow cell migration. In this directional migration assay, the HspB1-null cells consistently migrated farther than the parental WT cells in the same amount of time (Figure 8, A and B). During the 12-h observation period, WT cells migrated an average distance of 239 μm, whereas HspB1-null cells A and B migrated an average distance of 332 and 360 μm, respectively, demonstrating a 45% increase in net migration when HspB1 protein was eliminated. The average velocity for each cell type was determined by analysis of multiple independent experiments (Figure 8C). WT cells migrated consistently slower than either HspB1-null cell, suggesting that HspB1 acts as a regulatory brake during the process of cell migration. To demonstrate that the enhanced migratory behavior of HspB1-null cells was specifically due to the loss of HspB1, we compared the HspB1-null cells with rescue cells programmed to reexpress HspB1. To reconstitute HspB1 expression in the CRISPR/Cas9 edited HspB1-null cells, we generated a CRISPR/Cas9 nuclease-resistant HspB1 construct by mutating the protospacer adjacent motif (PAM). The HspB1-null cells engineered to reexpress HspB1 displayed HspB1 expression comparable to the parental WT cells (Figure 8D). Cell migration approached WT levels by reintroduction of HspB1 (Figure 8E). These data illustrate that HspB1 acts as a negative regulator of cell migration.

HspB1 and Zyxin contribute independently to the actin SF remodeling response to uniaxial cyclic stretch

Since both zyxin (Yoshigi et al., 2005; Hoffman et al., 2012) and HspB1 associate with the actin cytoskeleton in response to mechanical cues and both contribute to the SF reinforcement response downstream of uniaxial cyclic stretch, we explored whether these two proteins act in the same or parallel pathways. Unlike zyxin, HspB1 is not associated with actin SFs in unstimulated cells (Supplemental Figure S2), but both proteins accumulate on actin SFs in response to mechanical cues. Elimination of HspB1 expression fails to perturb either zyxin expression levels (Figure 9A) or zyxin recruitment to actin SFs in response to uniaxial cyclic stretch (Figure 9B). We observed that the accumulation of zyxin on SFs of stretched HspB1-null cells appears diminished relative to what occurs in WT cells and postulate that this is due to the abrogated actin remodeling response in cells lacking HspB1 (Figure 7, E and F). Reciprocally, elimination of zyxin does not have deleterious effects on the HspB1 response to stretch. The phosphorylation of HspB1 in response to stretch is evident in zyxin-null cells (Figure 9A) and HspB1’s accumulation on actin comet tails in stretched cells occurs independent of zyxin (Figure 9C). These results suggest that the HspB1 and zyxin responses to stretch occur by independent, mechanically sensitive pathways. If this were the case, then we would expect the SF remodeling and reinforcement response to be more seriously compromised in double-mutant cells than in either of the single-mutant cells. To explore this possibility, we used the CRISPR/Cas9 genome strategy presented in Figure 7 to mutagenize the HSPB1 gene in our previously described zyxin-null cells (Yoshigi et al., 2005; Hoffman et al., 2006). As can be seen in Figure 9D, we have generated cells that lack detectable zyxin or HspB1 protein by Western immunoblot analysis. The double-mutant cells display vinculin-rich FAs and F-actin SFs when plated on glass coverslips (Figure 9E), although we noticed some subtle qualitative differences in the cytoskeletal organization of the double mutant cells, these were difficult to quantify in unchallenged cell populations. When HspB1/zyxin double-null cells are challenged by exposure to uniaxial cyclic stretch, we observe alignment of actin SFs perpendicular to the stretch vector both by visual inspection of phalloidin-labeled cells (Figure 9F) and...
by quantitative analysis of the SF Alignment Index (Figure 9G). In contrast, the HspB1/zyxin double-null cells display a striking deficit in the SF remodeling and reinforcement response downstream of uniaxial cyclic stretch as quantified by SFTI (Figure 9H). This actin reinforcement deficit was greater than that observed in either the HspB1-null or zyxin-null cells (Figure 9H). In addition to the lack of SF reinforcement in HspB1/zyxin double-null cells, we observe the presence of readily visible phalloidin-labeled aggregates (Figure 9, F and I; open arrowheads). These phalloidin-stained structures are not labeled by DAPI, indicating that they do not colocalize with cell nuclei and thus likely represent cell fragments or intracellular aggregates of filamentous actin. These findings illustrate that the capacity of cells to respond to mechanical stress depends on parallel, complementary pathways involving HspB1 and zyxin. Moreover, the elimination of both proteins results in a synthetic deterioration of the integrity of the actin cytoskeleton in cells exposed to cyclic stretch.

**DISCUSSION**

Mechanical forces are generated and transmitted by cells and these forces are critical for the regulation of cell shape, motility, and growth control (Discher et al., 2009; Wozniak and Chen, 2009; Mammino et al., 2012; Iskratsch et al., 2014). The role of mechanical force in shaping biological behavior is now a focal point for research in cell biology, developmental biology, biophysics, and bioengineering. Here we have shown that exposure of mouse fibroblasts to uniaxial cyclic stretch induces phosphorylation of p38 MAPK, activating a canonical kinase cascade (Stokoe et al., 1992; Landry and Huot, 1995; Guay et al., 1997; Kayyali et al., 2002). This results in MK2-dependent phosphorylation of the small heat shock protein, HspB1, its localization to discrete SF subdomains, the reinforcement of actin SFs, and reduced cell migration (Figure 10). Our findings illustrate that the capacity of cells to respond to mechanical stress depends on parallel, complementary pathways involving HspB1 and zyxin. Moreover, the elimination of both proteins results in a synthetic deterioration of the integrity of the actin cytoskeleton in cells exposed to cyclic stretch.
change in actin that is detected by HspB1. In addition to its generally accepted role as a protein chaperone, HspB1 was identified as a contaminant that copurifies with vinculin and acts as a potent inhibitor of actin polymerization (Miron et al., 1988, 1991). Subsequent biochemical studies of purified HspB1 revealed that unphosphorylated HspB1 forms large oligomers, binds stoichiometrically to monomeric actin (G-actin), and inhibits actin polymerization in vitro (Benndorf et al., 1994; Mounier and Arrigo, 2002; Salinthone et al., 2008). Thus HspB1 is reported to act as an actin monomer sequestering protein that prevents precocious actin polymerization.

Several studies have suggested that HspB1's function as an actin regulator is influenced by posttranslational modification. Phosphorylation of HspB1 by MK2 in vitro induces dissociation of oligomeric complexes and release of HspB1-bound actin monomer to promote actin assembly (Huot et al., 1997; An et al., 2004; During et al., 2007; Damarla et al., 2009). Models that incorporate these established biochemical properties of HspB1 suggest an attractive mechanism for how mechanical signals might be converted into a biochemical output that could modulate the actin response in a phosphorylation-dependent manner (Gerthoffer and Gunst, 2001; Mounier and Arrigo, 2002; Salinthone et al., 2008). However, historical efforts to localize HspB1 in cells has revealed only a diffuse cytoplasmic distribution (Collier and Schlesinger, 1986; Lavoie et al., 1995; Clarke and Mearow, 2013), a finding that called into question the physiological relevance of HspB1's ability to interact with actin in vitro. We reasoned that if HspB1 were involved in the actin remodeling response downstream of mechanical stress, a cytoskeletal localization might be evident only in cells exposed to a mechanical stimulus. Indeed, we show here that HspB1 is associated with discrete regions of the

FIGURE 8: HspB1-null cells display enhanced cell migration. (A) Time-lapse microscopy was used to evaluate cell migration and compare the migratory behavior of cells from the parental WT line, as well as two independently derived HspB1-null lines, CRISPR A and CRISPR B. Cells were cultured to confluence within a confined area (Ibidi chambers) and then the barrier was removed, enabling directed cell migration from a well-defined edge. Migration of cells outward from the confluent cell island was monitored for 12 h. Fields of cells are shown at the beginning and at 12 h completion. Example time-lapse movies for WT, HspB1-null A, HspB1-null B cells are included as Supplemental Data. (B) Graph of 12 h migration distances for each cell type are shown from a single time-lapse experiment (9 measurements for each cell type) and the migration difference was reproduced in at least three independent experiments. WT cells migrated an average of 239 μm, HspB1-null A cells migrated an average of 332 μm, and HspB1-null B cells migrated an average of 360 μm over the 12 h period. (C) Graph of average velocity for each cell type showed a persistent migration difference in HspB1-null cells (four independent migration experiments). (D) Western immunoblots (10 μg protein per lane) of WT parental cells, HspB1-null cells, and rescue HspB1 cells show the rescue HspB1 expression level approaches WT expression levels. Vinculin loading control confirms unchanged expression regardless of HspB1 status. (E) Quantitation of directed cell migration from a defined edge (Ibidi chambers) over a 12 h period. WT parental cells migrated an average of 230 μm (18 measurements), HspB1-null cells migrated an average of 372 μm (18 measurements), and HspB1 rescue cells migrated an average of 265 μm (18 measurements), suggesting that reexpression of HspB1 returned the cells to almost WT migration. Graphs are mean with SD; p values (*p < 0.05, **p < 0.001, ***p < 0.0001, n.s. = not significant) were determined using an unpaired t test that assumed Gaussian distribution and equal SDs between populations.
actin cytoskeleton in cells subjected to uniaxial cyclic stretch (Figure 3, A–C). Moreover, we have uncovered a relationship between our ability to detect HspB1 in association with the actin cytoskeleton and the cell’s SFTI, providing a correlation between cytoskeletal accumulation of HspB1 and SF remodeling and reinforcement (Figure 3E). Future analysis of what attracts HspB1 differentially to actin comet tails and dissection of the detailed molecular mechanism by which HspB1 contributes to actin SF remodeling and reinforcement will further enhance our understanding of the response of cells to mechanical stress.

Given the correlation between HspB1 cytoskeletal accumulation and SF reinforcement, it is of interest to understand what physiological conditions influence the association of HspB1 with the actin cytoskeleton. Our studies have revealed that retention of HspB1 in association with the actin cytoskeleton depends on sustained delivery of mechanical cues (Figure 5, A–C). Consistent with our findings that HspB1’s association with the actin cytoskeleton is promoted by mechanical stimulation, HspB1 was identified as a contractility-dependent component of FAs in unbiased proteomic studies (Kuo et al., 2011; Schiller et al., 2011).

Although the application of uniaxial cyclic stretch induces a pronounced enhancement of both cell types, and it was not different between WT and HspB1/zyxin double-null cells. The alignment index is graphed as mean with SEM, and p values were determined by unpaired t tests assuming Gaussian distributions with equal SD between populations. (H) SFTI analysis of unstretched (gray bars) and stretch-stimulated (black bars) cells (WT, HspB1-null, zyxin-null, HspB1/zyxin double-null). Unstretched/Stretched SFTI: WT cells 3.0/4.2, HspB1-null cells 3.0/3.6, zyxin-null 3.1/3.4, HspB1/zyxin double-null cells 3.0/3.0. Only HspB1/zyxin double-null cells were not significantly different (n.s.) by SFTI analysis. SFTI is graphed as mean with SD, greater than 100 measurements per condition, and unpaired t tests assumed Gaussian distributions with equal SD between populations. (I) Stretch-stimulated cells (uniaxial cyclic stretch, 15%, 0.5 Hz, 60 min) stained for F-actin (phalloidin) display alignment perpendicular to the stretch vector and variable stress fiber thickening (see H) depending on the presence or absence of HspB1, zyxin, or both proteins. Aggregates of F-actin in the HspB1/zyxin double-null cells are indicated by arrowheads. The stretch vector is represented by double-headed arrow of 50 μm scale. **p < 0.001, ***p < 0.0001, n.s. = not significantly different.
HspB1 response to mechanical stress

of HspB1 association with cytoskeletal elements, it is important to note that SF association of HspB1 is not uniformly observed on all SFs within a cell nor is cytoskeletal association observed in all cells. The underlying reason for the heterogeneous accumulation of HspB1 on SF subdomains of individual cells is not understood. It is possible that some threshold level of mechanical stress is required for recruitment of HspB1 to the cytoskeleton and that depends on cell orientation relative to the stretch vector or other factors; it is possible that cytoskeletal accumulation of HspB1 is short lived and the protein is thus not captured uniformly in large cell populations visualized at a single arbitrary timepoint, or it is possible that HspB1 association with the cytoskeleton is regulated by secondary physiological cues that vary from cell to cell and remain to be elucidated. Analysis of the subcellular localization of fluorescently labeled HspB1 in living cells subjected to controlled mechanical stimulation will help to distinguish among these and other possibilities.

It is notable that phospho-HspB1 accumulates predominantly at FA-proximal actin comet tails that emanate from vinculin-rich FAs. These SF domains represent sites of elevated tension and actin assembly that are promoted by mechanical signals such as those engendered by substrate stiffness, geometrical constraints, or stretching forces (Wang et al., 2002; Guo and Wang, 2007; Rape et al., 2011). We previously reported that zyxin is present both in FAs and in actin comet tails (Beckerle, 1986; Crawford and Beckerle, 1991). Others have found that zyxin-rich comet tails are induced when cells are plated on stiff substrates and geometrically constrained micropatterned substrates, and they represent sites of dynamic retrograde actin and zyxin flux that is correlated with actin assembly (Guo and Wang, 2007). Our discovery of phospho-HspB1 at actin comet tails along with zyxin supports the notion that both proteins contribute to the mechanical stress response. Confirmation of that hypothesis was provided by the demonstration that mutant cells lacking both HspB1 and zyxin fail to launch an actin SF reinforcement response when exposed to uniaxial cyclic stretch (Figure 9H). In addition, when the double-mutant cells are challenged by exposure to mechanical stress, they accumulate aggregates of phalloidin-stained material (Figure 9, F and I). These unusual actin structures may result when strained SFs break and retract in a myosin-dependent manner (Smith et al., 2010; Chapin et al., 2012) or could occur via some other mechanism that remains to be identified.

An important contribution of our work is the demonstration that the phosphorylation of HspB1 is necessary but not sufficient to promote association with the actin cytoskeleton in living cells. Use of site-directed mutagenesis to create a mutant variant of HspB1 that lacks the two best characterized MK2-dependent phosphorylation sites (S2A-HspB1) enabled us to demonstrate that phosphorylation of HspB1 is critical for its stretch-stimulated recruitment to the actin cytoskeleton (Figure 6, D–F). Generation of an HspB1 variant (S2E-HspB1) designed to mimic a constitutively phosphorylated HspB1 in cells revealed that the presence of a constitutively “active” HspB1 is not sufficient to promote cytoskeletal association, as evidenced by the fact that no actin localization of S2E-HspB1 is observed in unstretched cells (Figure 6F). The ability of the S2E-HspB1 variant to accumulate in association with actin SFs exclusively in stretched cells (Figure 6F) illustrates the fidelity of the protein construct and that accumulation of HspB1 on the actin cytoskeleton requires both the posttranslational modification of HspB1, as well as the creation of cytoskeletal docking sites in response to mechanical stimulation. The accumulation of HspB1 at actin comet tails, which are established sites of actin polymerization and retrograde flow, raised the possibility that HspB1 was attracted to free-barbed ends of actin filaments, which are concentrated at these regions (Symons and Mitchison, 1991; Gupoton et al., 2007). Our demonstration that low-dose cytochalasin D inhibits the accumulation of phospho-HspB1 at actin comet tails is consistent with the view that free F-actin barbed ends are required for the targeted recruitment of HspB1 to SFs (Figure 5D). Thus we postulate that the application of mechanical force promotes both the availability of free barbed ends and the posttranslational modification of HspB1, enabling cytoskeletal recruitment of HspB1 and SF reinforcement.

Disturbance of HspB1 function causes distal hereditary motor neuropathy and Charcot-Marie-Tooth disease type 2 (CMT2), the most common inherited neuromuscular disease in humans (Evgenio et al., 2004; Houlden et al., 2008). CMT2 is a disorder characterized by axonal peripheral neuropathy (Evgenio et al., 2004). Five autosomal dominant mutations in human HspB1 that cause CMT2 or distal hereditary motor neuropathy have been identified (Evgenio et al., 2004; Houlden et al., 2008), and each of these residues is conserved in murine HspB1. The mechanism by which HspB1 mutations contribute to peripheral neuropathy is not understood. Given the critical role of the actin cytoskeleton for axon guidance, growth cone dynamics, and neuronal function (Dent and Gertler, 2003), it is intriguing to consider the possibility that these disorders

FIGURE 10: Model illustrating a proposed mechanism by which p38 MAPK signaling influences the response of cells to mechanical stress. Stretch activation of p38 results in phosphorylation and recruitment of HspB1 to the actin cytoskeleton, especially actin comet tails. Elimination of HspB1 function abrogates SF reinforcement and promotes cell migration. The HspB1 pathway provides a previously uncharacterized mechanism for mechanotransduction that is independent of the role of zyxin in stretch-induced actin remodeling.
result from impairment of the neuronal cytoskeletal response to mechanical stress.

Collectively our findings highlight the role of p38 MAPK signaling in the mechanotransduction response and elucidate the role of a ubiquitous small heat shock protein in cytoskeletal remodeling and reinforcement in the face of mechanical challenge. We have provided a new framework for understanding the response of cells to mechanical cues by implicating a p38 MAPK signaling cascade and the downstream target, HspB1, as part of the mechanotransduction response (Figure 10). We show that phosphorylated HspB1 is recruited to actin comet tails that are established sites of mechanical tension and actin polymerization. Perturbation of the p38-MK2-HspB1 pathway with chemical inhibitors or genetic loss of function mutants results in a decreased actin SF remodelling and reinforcement response and increased cell migration. Prior studies have illustrated that HspB1 expression increases dramatically in cells exposed to high temperature and the protein accumulates on actin SFs in heat-challenged cells (Miron et al., 1991; Bryantsev et al., 2002), consistent with the idea that accumulation of HspB1 on the cytoskeleton is protective. Enhanced cell motility of HspB1-null fibroblasts is consistent with the inverse relationship of cell migration to comet tail formation (Guo and Wang, 2007) and with a report that HspB1-null mice display increased neutrophil migration in a wound closure model (Crowe et al., 2013). The novel, p38-regulated roles of HspB1 in the response to mechanical stress raise questions about how p38 is activated by mechanical cues and whether other effectors downstream of p38 play central roles in mechanotransduction. Further analysis of these questions is warranted given both the physiological significance of the mechanotransduction response and current knowledge of the complexity of the p38 MAPK signaling network.

MATERIALS AND METHODS

Reagents

Antibodies for vinculin (Sigma V-9131), phospho-p38 and total p38 (Cell Signaling Technology #4511 & #9212), phospho-MK2 and total MK2 (Cell Signaling Technology #3007 & #3042), phospho-HspB1 (Cell Signaling Technology #9709 for human hsp27 phosphorylated on Ser82 and for mouseSer86) and total hsp25 (Cell Signaling Technology #2442 and Enzo Life Sciences ADI-SPA-801), phospho-ERK1/2 (Cell Signaling Technology #4370), phospho-JNK (Cell Signaling #4668), SUN2 (Abcam #124916), GFP (Living Colors #632381), phospho-zyxin (Cell Signaling Technology #8467), and zyxin (Beck- erle Lab B71 and B72) were used as previously described (Hoffman et al., 2003, 2006); zyxinB72 antibody is now commercially available (EMD Millipore ABC1387). AlexaFluor conjugated secondary antibodies and Phallolidin, along with 4′,6-diamidino-2-phenylindole (DAPI) were used as recommended (Molecular Probes/Invitrogen). Horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) were used for Western immunoblots. P38 inhibitor SB203580 (Cell Signaling Technology #5633) and actin inhibitor cytochalasin D (Sigma #c8273) were resuspended in DMSO and used as directed by manufacturer.

Cells

Wild-type (WT) mouse fibroblast and GFP-zyxin cells were isolated and cultured as described previously (Hoffman et al., 2006) in high-glucose DMEM complete (DMEMc) supplemented with pyruvate, glutamine, penicillin/streptomycin (Invitrogen), and 10% fetal bovine serum (Hyclone Labs, Logan, UT). MK2-null and rescue cells were provided by Matthias Gaestel and cultured as described (Ronkina et al., 2007; Sousa et al., 2007).

Mutagenesis

To generate the HspB1 S2A phospho-mutant, Ser15 AGC was mutated to Ala GCT and Ser86 AGC was mutated to Ala GGT, using a Quik Change site-directed mutagenesis kit (Agilent Technologies). To generate the HspB1 S2E phospho-mutant, Ser15 AGC was mutated to Glu GAA and Ser86 AGC was mutated to Glu GGT. The S2A and S2E mutants were generated in a pDONR221 Gateway recombination plasmid (Invitrogen). After DNA sequencing to confirm mutations, the clones were subsequently Gateway cloned into a pLenti6.3/V5 plasmid with a GFP tag (Invitrogen) for transfection into 293FT cells. Packaged virus from the 293FT cells was used to transduce wild-type mouse fibroblasts previously described (Hoffman et al., 2006). The virally transduced cells were selected for resistance in 2.5 μg/ml blasticidin (Invitrogen).

CRISPR/Cas9 genome editing

HspB1-targeting sequences for CRISPR/Cas9 genome editing (Ran et al., 2013) were designed and constructed by Timothy Dahlem, (University of Utah Mutation Generation and Detection Core; http://cores.utah.edu). To generate HspB1-null cells, Cas9 HspB1 fusion plasmids that encoded 5′ CGTGGCCCTTCTGCTGTCG 3′ [CRISPR plasmid 23] and 5′ GGTCCTGGGATCCGGACAGA 3′ [CRISPR plasmid 27], both in exon 1 of the HspB1 gene, were transfected into 293 FT cells to generate CRISPR plasmid-containing lentivirus. The parental WT mouse fibroblasts previously described (Hoffman et al., 2006) were virally transduced, and cells were selected for resistance in 2.5 μg/ml Puromycin (Invitrogen). Two independently targeted and isolated cell lines (CRISPR 23 “A” and CRISPR 23/27 “B”) were used in experiments presented here. The HspB1 gene in CRISPR 23 A cells was sequenced and found to have a 253-base-pair deletion following the ATG start, which would result in a frame shift and multiple stop codons. HspB1 protein status was assessed by Western immunoblot analysis and by immunofluorescence localization using HspB1-specific antibodies and both CRISPR 23 A cells and CRISPR 23/27 B cells were determined to be null for HspB1 protein.

To generate an untagged HspB1 construct that was CRISPR/Cas9 nuclease resistant and could be reintroduced into HspB1-null cells as a “rescue” construct, the PAM site sequence 5′ to the start of the guide sequence in the HspB1 gene was conservatively mutated from CGG (Arg) to ACG (Arg), creating a caspase 9-resistant expression construct. This rescue construct was virally transduced into the HspB1-null cells using the same viral production and expression protocol used to generate the original HspB1-null CRISPR 23 A cells (described above).

To generate the HspB1/zyxin double-null cells, the HspB1-targeting CRISPR plasmid 23 was used as described above on fibroblasts originally isolated from zyxin-null mice (Hoffman et al., 2006). HspB1 protein status was assessed by Western immunoblot analysis to confirm the cells were null for both HspB1 and zyxin.

Uniaxial cyclic stretch, stress fiber thickening, and alignment analysis

Cells were stretch stimulated using a custom-designed system previously described (Yoshigi et al., 2005). Briefly, cells were seeded onto precoated (25 μg/ml collagen I and 2 μg/ml fibronectin) silicone membranes (2.2 million cells onto three 26 × 33 mm membranes in a 10-cm plate) and grown to confluence overnight. Cells were either subjected to uniaxial cyclic stretch (15%, 0.5 Hz, up to 1 h) and then lysed and proteins harvested for immunoblot analysis or fixed (3.7% formaldehyde 15 min) for cell staining and microscopy (Yoshigi et al., 2005; Hoffman et al., 2012). For stretch experiments with
inhibitors (SB203580 at 10 μM; cytochalasin D at 250 nM), cells on membranes were preincubated for 1 h prior to stretch, and the stretch was performed in media with fresh inhibitor added. SFTI analysis of phalloidin-stained cells utilized a custom erosion/brightness decay software written in LabView (National Instruments) and previously described (Yoshigi et al., 2005). Multiple stress fibers in multiple cells in >10 microscopic fields were measured (regions of interest, roi) for relative SFTI within single experiments in at least three independent stretch experiments. Stress fiber alignment analysis was performed as previously described (Yoshigi et al., 2003; Yoshigi et al., 2005). (For source code, contact M. Yoshigi.) Briefly, local stress fiber orientation was calculated by Sobel filter algorithm, and orientation histograms were obtained from ~400 cells. Distribution kurtosis of orientation histogram was designated as the alignment index. Higher alignment values indicate more aligned stress fibers (perpendicular to stretch vector), zero being mesokurtic (Gaussian distribution).

Immunofluorescence microscopy
Cells seeded onto glass coverslips or onto stretch-silicone membrane substrates were fixed (15 min in 3.7% formaldehyde/phosphate-buffered saline) and then permeabilized (5 min in 0.5% Triton X-100), blocked (1 h in 5% normal goat serum), and incubated with antibodies as recommended by suppliers. Cell images were captured with a Zeiss Axiophot fluorescence microscope (Zeiss 40X Neofluor 0.75 NA dry objective and Zeiss 63X AposChromat 1.4 NA oil objective), Zeiss AxiocamMRm camera, Zeiss AxioVision version 4.8.1 software. Quantitation of phospho-HspB1 (Cell Signaling Technology Antibody #9709) localization patterns utilized images from at least 10 microscopic fields (<50 cells total for each stretch condition). Image sets were collected with identical camera settings throughout each experiment, and images were evaluated for cytoskeletal distribution on screen. The phospho-HspB1 localization signals were quantitated for four independent stretch and stain experiments.

For the fibronectin dose–response experiment, 30,000–60,000 WT cells and HspB1-null cells were seeded into a 12-well dish with glass coverslips that were uncoated or precoated with 0.1 μg/ml, 1 μg/ml, or 10 μg/ml fibrobrinectin. The cells were incubated in DMEMc+10% serum for 3 h and then fixed and stained as described above. The spreading experiment was performed three times with similar results. For cell area quantitation, cells were outlined and measured (National Institutes of Health [NIH] FIJI ImageJ, v.2.0.0), with graph presentations and analysis using Graph Pad Prism software.

Western immunoblot analysis
Protein concentration of cell lysates was measured (Pierce Coomassie Protein Assay) and proteins (7.5–30 μg/lane, described in the figure legends) were electrophoresed through denaturing 15% polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose filters. Antibodies were diluted as recommended by the manufacturers and detected by HRP-conjugated antibodies and enhanced chemiluminescence (GE Healthcare). Quantitation of Western immunoblots to compare x-fold increase utilized the Analyze/Gels module of FIJI (NIH ImageJ) with the signals in unstretched lanes considered as onefold. Western immunoblot results were confirmed in at least three independent experiments.

Micropatterned substrates
A custom panel (20 mm × 20 mm) of square micropatterned adhesive areas (20 μg/ml Fibronectin) of increasing sizes of small 1024 μm² (32 × 32 μm), medium 2025 μm² (45 × 45 μm), and large 10,000 μm² (100 × 100 μm) (Cytochip, Grenoble France) were placed in a six-well plate. Cells were seeded (60,000 cells/well) and allowed 4 h to adhere and spread. Formaldehyde was added directly to the media to 3.7% concentration and cells were fixed for 15 min, followed by permeabilization, staining, and microscopy as described under Immunofluorescence microscopy.

Migration
Glass-bottom 12-well cell culture plates (Invitro Scientific PR-1.5HN) containing two-well culture insert chambers (Ibidi #80209) were seeded at 23,000 cells per insert chamber and grown for 24 h to confluent density. To begin the cell migration assay, the insert chambers were removed and cells were given fresh DMEMc + 10% fetal bovine serum media, and cells migrated out from the edge in a directed manner. Using a Nikon Ti Eclipse microscope and 10× objective with DIC, Nikon Elements software, and Pathology Devices environmental chamber (37°C, 5% CO₂, 70% humidity), multiple microscopic fields were imaged for 12–18 h at 10-min intervals. Image analysis and migration distances were measured with FIJI (NIH ImageJ). For each cell type, three microscopic fields were measured across the top, middle, and bottom of the field for a total of nine measurements per cell type. For the migration rescue experiments, six microscopic fields were measured as above for a total of 18 measurements per cell type. Cell migrations were compared between multiple cell types run within the same experiment, and migration experiments were performed at least three independent times.

Statistical analysis
Graphs are presented as mean with SD, and p values are noted in the figure legends with p < 0.05 considered statistically significant. The horizontal lines above the graphs identifies the two groups compared using unpaired Student’s t tests that assumed a Gaussian distribution and equal SDs between populations and were calculated using Prism 6 software (GraphPad). *p < 0.05, **p value < 0.001, ***p value less than 0.0001, comparisons that were not statistically significant (n.s.) are designated or not shown.

Figure preparation
Cell images were processed with Photoshop CC and assembled as figures in Illustrator CC (Adobe). Graphs were constructed in Prism 6 (GraphPad, San Diego CA).

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