Cytoskeletal interactions of eukaryotic initiation factor 6 regulate mechanical responses in cells

Adam N. Keen\textsuperscript{1,2}, Luke A. Payne\textsuperscript{1,2}, Alistair Rice\textsuperscript{3}, Lisa J. Simpson\textsuperscript{1,2}, Kar Lai Pang\textsuperscript{1,2}, Armando del Rio Hernandez\textsuperscript{3}, John S. Reader\textsuperscript{1,2} and Ellie Tzima\textsuperscript{1,2*}

\textsuperscript{*}Authors jointly supervised this work

\textsuperscript{*}Corresponding author

\textsuperscript{1} Radcliffe Department of Medicine

\textsuperscript{2} Wellcome Centre for Human Genetics

\textsuperscript{3} Cellular and Molecular Biomechanics Laboratory, Department of Bioengineering, Imperial College London, London, UK
Abstract

There is an elaborate interaction between the force transducing cytoskeleton and the protein translation apparatus, yet our understanding of the functional significance of the link between these two major cellular machineries is rudimentary. Here, we demonstrate that the ribosomal eukaryotic initiation factor 6 (eIF6) associates with the cytoskeleton and regulates cellular mechanobiology. eIF6 co-localises with actin in vitro and in vivo, and cells deficient in eIF6 show cytoskeletal and focal adhesion defects accompanied by reduced cellular stiffness and traction force generation. Tensional force experiments reveal that eIF6 tunes cellular responses to external mechanical tension. Mechanistically, loss of eIF6 is not associated with defects in nascent protein synthesis or altered expression of cytoskeletal proteins; instead, eIF6 regulates the spatio-temporal activation of cellular signalling pathways critical for force transduction and focal adhesion growth. Furthermore, we identify a novel, eIF6-dependent mechano-complex that directs spatial activation of ERK1/2. These results reveal an extra-ribosomal function for eIF6 and a novel paradigm for how mechanotransduction, the cellular cytoskeleton and protein translation constituents are linked.
Introduction

Cells respond and adapt to a variety of mechanical stresses that regulate cellular signalling and function. Whether externally applied or internally generated, forces are transduced via the cytoskeleton machinery, an intricate fibrous network composed of filamentous actin (F-actin), intermediate filaments and microtubules, which provides the structural architecture and governs shape, size and mechanical properties of the cell. The cytoskeleton is anchored to the base of the cell by large macromolecular complexes with both mechanical and cell signalling components, called focal adhesions. Focal adhesions constitute well-described sites of mechanosensing; cytoskeletonally-generated forces lead to stresses in these adhesions because of the opposite forces that arise in the extracellular cell matrix (ECM). Focal adhesions are highly dynamic, which requires the correct spatio-temporal activation of signalling cascades including focal adhesion kinase (FAK) and extra-cellular signal-regulated 1/2 (ERK1/2). Externally-applied forces (through the ECM, ion channels or other mechano-receptors) also trigger active changes in cytoskeletal structures and cellular force generation. Force application on integrins or cell adhesion molecules such as platelet endothelial cell adhesion molecule-1 (PECAM-1) or cadherins leads to signalling cascades that ultimately lead to growth of adhesions and reinforcement of the cytoskeleton.

In addition to structural roles, the cytoskeleton modulates many cellular processes by providing a structural/physical platform which influences the activity and/or subcellular localisation of signalling proteins and their downstream targets. Elaborate and functionally important interactions between the cytoskeleton and the protein translation machinery suggest that the cytoskeleton plays a role in the spatial and temporal pattern of protein synthesis. Protein synthesis, or translation, is a complex process requiring choreographed interactions of numerous macromolecules. Pioneering electron microscopy studies first reported monosomes and polysomes entangled within the cytoskeletal network. Combined with in situ hybridisation, electron microscopy also allowed
visualisation of the association of cytoskeletal mRNAs with cytoskeletal filaments\textsuperscript{21}. Furthermore, cellular fractionation has confirmed that polysomes co-localise with the detergent-insoluble cytoskeleton\textsuperscript{22}. An increasing number of protein translational regulators have been shown to associate with the cytoskeleton\textsuperscript{23-28}, and proteomic analysis has revealed that a number of proteins involved in protein translation are present at the integrin adhesome\textsuperscript{29}. Thus, there is growing evidence that suggests co-regulation between the cytoskeleton and the protein translational machinery, however, our understanding of this is rudimentary at best.

Throughout evolution, ribosomal proteins have been co-opted to carry out auxiliary extra-ribosomal functions\textsuperscript{30}, often associated with their diverse subcellular localisations. A ribosome-associated factor that has been described in two distinct subcellular pools is eIF6. In the cytoplasm, eIF6 binds to the large ribosomal subunit (60S) and regulates the formation of an active 80S ribosome capable of protein translation\textsuperscript{31-35}. Although incompletely understood, the translational activity of eIF6 seems to be facilitated by the small ribosomal sub-unit (40S) binding and cytoskeleton regulating protein RACK1 (receptor for activated C kinase 1)\textsuperscript{33,36,37}. In addition to the cytoplasm, a pool of eIF6 is found in the nucleus where it is required for ribosome biogenesis\textsuperscript{38-40}. eIF6 haploinsufficiency does not affect basal protein synthesis\textsuperscript{34}, but eIF6 is required for efficient insulin-stimulated protein translation and global regulation of metabolism\textsuperscript{34,41,42}. eIF6 has been linked to a variety of processes, including tumour biology\textsuperscript{43,44}, regulation of metabolism\textsuperscript{41,42} and, more recently, non-canonical roles of eIF6 in wound healing have been reported\textsuperscript{45,46}. Here, we have utilised biophysical, biochemical and cell biological approaches to reveal a role for eIF6 in regulating cell mechanics through a novel mechanotransduction pathway.

Results

\textit{eIF6 interacts with the actin cytoskeleton}
Endogenous eIF6 has previously been shown to localise to the nucleus/nucleolus and cytoplasm\textsuperscript{38,47,48}. By using three commercially available anti-eIF6 antibodies in combination with confocal microscopy in two different cell types (bovine aortic endothelial cells and human epidermoid carcinoma A431 cells), we were able to visualise these subcellular localisations of eIF6 (Supplementary Figure 1a-b). To test specificity of these antibodies, we used scrambled (Scr) or eIF6 siRNAs (si eIF6) to knockdown endogenous eIF6; western blot analysis showed a reduction of \textasciitilde70\% to \textasciitilde90\% in band intensities (Supplementary Figure 1c-n).

In addition to its cytoplasmic and nucleolar localisation, endogenous eIF6 has been shown to associate with the cytoskeletal components spectrin, cytokeratin and tubulin\textsuperscript{49}, as well as intermediate filaments, β4-integrin and hemidesmosomes\textsuperscript{38,47}. Using an antibody that recognises both cytoplasmic and nuclear pools of eIF6, we examined the subcellular localisation of eIF6 in primary endothelial cells (ECs), as these cells have a highly dynamic cytoskeleton, are mechanically active and respond to exogenously applied mechanical forces. Analysis of eIF6 distribution by confocal laser scanning microscopy revealed that eIF6 is found throughout the cytoplasm and nucleus as well as in distinct network structures that were also stained with phallloidin (Figure 1a). Visual overlay of the red and green channels showed that eIF6 fluorescence was enriched in areas co-localising with actin stress fibres, signified by the appearance of yellow sections in the merged image (Figure 1a-b), which we confirmed was specific to eIF6 by immunofluorescent staining with a second commercial antibody (Supplementary Figure 2). Furthermore, fluorescent intensity profiles showed correlation in channel intensities, with spikes in eIF6 intensity coinciding with actin stress fibres (Figure 1c). To further explore the significance of this finding and its relevance in a physiologically-relevant setting \textit{in vivo}, we isolated the mouse descending aorta (a highly mechanically active tissue), prepared it \textit{en face} and applied the same immunofluorescent staining regimen to assess eIF6 localisation (Figure 1d). Similar to the \textit{in vitro} experiments, we found that eIF6 localised in areas co-stained with DAPI and phallolidin,
suggesting nuclear and cytoplasmic localisation (Figure 1d-f). To quantify eIF6 co-localisation with actin, we used Pearson’s co-localisation coefficient (ranging from 1, perfect correlation, to -1, perfect anti-correlation), which showed positive co-localisation between eIF6 and F-actin in ECs both in vitro and in vivo (Figure 1g).

To corroborate the subcellular distribution of eIF6 using an additional approach, we performed subcellular fractionation of cell lysates into cytoskeletal (actin-positive) and non-cytoskeletal (HSP90-positive) fractions. We found that eIF6 was present in both the cytoskeletal and non-cytoskeletal fractions (Figure 1h-i). To definitively confirm the co-localisation observed between eIF6 and actin occurs via a protein-protein association, we performed immunoprecipitation from cell lysates with either eIF6 or non-specific IgG antibodies. Robust co-immunoprecipitation of actin was observed in eIF6 immunoprecipitates, but not control IgG immunoprecipitates, thereby confirming their specific interaction (Figure 1j). In addition, co-immunoprecipitation of actin with eIF6 was lost in cell lysates where endogenous eIF6 had been depleted by siRNA (Figure 1j). To determine whether eIF6 binds directly to actin or via intermediary proteins we performed in vitro co-immunoprecipitation using purified proteins. Again, robust co-immunoprecipitation of actin was observed with eIF6 immunoprecipitates, which was lost when we used heat denatured actin (Figure 1k). Taken together, these experiments clearly demonstrate that eIF6 co-localises with the actin cytoskeleton in vitro and in vivo; endogenous eIF6 co-immunoprecipitates with actin in cell lysates; and purified eIF6 binds to actin in vitro.

Endogenous eIF6 regulates cytoskeletal organisation and cell-mediated forces

The cytoskeleton determines the size, shape and mechanical properties of the cell. The co-localisation of eIF6 with the actin cytoskeleton prompted us to ask if eIF6 is required for proper formation of the cytoskeleton. Examination of cells transfected with either Scr or eIF6 siRNAs revealed that loss of endogenous eIF6 altered overall cell morphology, increasing cell surface area (Figure 2a-b). Cells with reduced eIF6 displayed disorganised F-actin, as
assayed by a reduction in orientation coherency (ranging from 0 to 1; Figure 2a, c). To determine whether eIF6 is also involved in mechanosensitive focal adhesions, we quantified the number and size of vinculin-positive focal adhesions and showed that eIF6-depleted cells displayed significantly smaller and fewer vinculin-positive focal adhesion complexes relative to eIF6-expressing cells (Figure 2d-f). Similar results were obtained when we examined focal adhesions in A431 cells (Supplementary Figure 3). As additional controls, we also tested two additional single siRNAs for eIF6, which displayed the same phenotypes (Supplementary Figure 4). In addition to this loss-of-function approach, we examined the effects of gain-of-function by overexpression of eIF6; we found that overexpression of eIF6 gave a small, but statistically significant, increase in the number of vinculin positive focal adhesions (Supplementary Figure 5). Together, these results suggest that eIF6 plays a regulatory role in the organisation of the structural components of the cell.

The cytoskeleton is a fundamental and highly dynamic structure that regulates the mechanical properties of the cell via transmission of force. Given the effects of eIF6 silencing on both actin stress fibres and focal adhesions, we assessed endogenous force generation and stiffness of eIF6-depleted cells. To investigate if loss of eIF6 leads to changes in the forces cells exert on their substrate, ECs were grown on a substrate consisting of an array of elastic polydimethylsiloxane micropillars coated with fibronectin. The deflection of each pillar is proportional to cell traction force and was optically monitored (Figure 2g). Scr siRNA transfected cells generated traction stresses that were mostly concentrated around the periphery (Figure 2h). In contrast, eIF6-depleted cells showed a marked decrease in the overall traction force generation (Figure 2h-i).

The capacity for force application on substrates is intimately connected to cytoskeletal stiffness. Using atomic force microscopy, we measured the surface stiffness of individual ECs by nanoindentation following si Scr or si eIF6 transfection (Figure 2j). We ensured that our analysis would assess the contribution of the cytoskeleton to cell compliance by
indenting the cells at points between the nucleus and the cell edges. We observed that loss of eIF6 resulted in reduced Young’s modulus and, thus, reduced cellular stiffness (Figure 2k). Collectively, these results suggest that eIF6, via regulation of the actin cytoskeleton, determined endogenous force generation and stiffness of cells.

**Cellular phenotypes are not due to defects in canonical roles of eIF6**

Given the role of eIF6 in protein translation, we assessed whether the cytoskeleton and mechanical defects in eIF6-depleted cells could be attributed to reduced protein synthesis. To examine basal levels of protein translation in ECs we used O-propargyl-puromycin (OPP) to label nascent proteins in control and eIF6-depleted cells. OPP contains an alkyne group, which through Click chemistry can be covalently coupled to fluorescent tags for visualisation of nascent proteins. Despite almost complete knockdown of eIF6, unstimulated eIF6-depleted cells did not display defects in nascent protein synthesis, consistent with previous reports showing that loss of eIF6 does not affect basal protein synthesis in eIF6 haploinsufficient hepatocytes and skeletal muscle cells as well as in eIF6 siRNA-transfected fibroblasts and HeLa cells (Figure 3a-b). To corroborate these findings, we used sucrose gradient fractionation of cytoplasmic/ribonucleoprotein extracts of si Scr and si eIF6 cells to generate polysome profiles. With this technique, the macromolecules and structures involved in protein translation are physically separated, allowing efficiency of protein translation to be evaluated. In agreement with previous findings, we found no change in the general profile of the polysome peaks, indicative of no visible defects in protein translation efficiency (Figure 3c). This finding is consistent with our results showing no defects in nascent protein synthesis in eIF6-depleted cells. Although loss of eIF6 does not cause basal defects in protein translation, eIF6 has been reported to be required for efficient protein translation in response to insulin stimulation. To test if this is also true in our system, we assessed nascent protein synthesis and associated signalling in response to insulin in control and eIF6-depleted cells. In agreement with previous studies, we found that insulin-induced nascent protein synthesis was abrogated in eIF6-depleted cells.
(Supplementary Figure 6a-b), with corresponding reductions in activation of p70S6K
(Supplementary Figure 6c). However, activation of extracellular signal-related kinase
(ERK1/2), Akt and mammalian target of rapamycin (mTOR) were unaffected with loss of
eIF6 (Supplementary Figure 6d-f), again consistent with previous reports\textsuperscript{34,41}. Finally, given
the role of eIF6 in regulating the cytoskeletal machinery and cell mechanics (Figure 2) and to
explore whether eIF6 specifically regulates protein levels of cytoskeletal proteins, we
measured expression of a subset of key cytoskeleton proteins in our cell lysates. In
agreement with the Click-iT assay and polysome profiles, total expression levels of
cytoskeletal proteins were unaffected by knockdown of eIF6 (Figure 3d-j). Taken together,
these results show that regulation of cell mechanics by eIF6 is not a by-product of reduced
nascent protein synthesis or altered cytoskeletal protein levels.

Another previously described role of eIF6 is in ribosomal biogenesis\textsuperscript{40,41}, which raised the
question of whether the cytoskeleton and mechanical phenotypes of eIF6-depleted cells
could be due to defects in these processes. To assess for possible ribosomal biogenesis
defects in our system, we used a multi-pronged approach. First, we assayed nucleolar stress
by quantifying nucleolar size and number in control and eIF6 siRNA transfected cells.
Immunofluorescent staining of nucleolin revealed a small decrease in nucleolar number and
a decrease in nucleolar size in eIF6-depleted cells (Supplementary Figure 7a-c). We then
sought to see if this apparent nucleolar stress manifested as a defect in precursor ribosomal
RNA (pre-rRNA) levels by quantitative PCR (qPCR). We found no differences in any of the
transcripts we measured (45S, 28S, 18S and 5.8S) despite a ~90% knockdown efficiency
(Supplementary Figure 7d-j). Finally, we examined protein expression levels of four key 60S
ribosomal proteins; RPL7a, RPL10a, RPL26 and RPL23, which revealed small or no
detectable differences following loss of eIF6 (Supplementary Figure 7k-o). Given that the
half-life of ribosomes is ~5 days\textsuperscript{57,58} and all our experiments are carried out within a 48-72
hour window of siRNA-mediated knockdown, it is perhaps unsurprising that levels of
ribosomal proteins are relatively unaffected with loss of eIF6. Taken together, these results
show that although eIF6 depletion causes mild nucleolar stress, there are no eIF6-dependent changes in pre-rRNA, ribosomal or cytoskeletal protein expression, or nascent protein synthesis (as assayed by ClickIT and polysome profiles) in our system. These results are consistent with previous studies showing eIF6 siRNA-mediated knockdown in mammalian cells maintains ribosomal biogenesis. Based on this comprehensive characterisation, it is our conjecture that eIF6 regulates cell mechanics separately from its known roles in protein synthesis and/or ribosome biogenesis.

Mechanoresponses of ECs are dependent on eIF6

In addition to endogenous forces generated when a cell pulls on the ECM via the cytoskeleton, cells also remodel their cytoskeleton and focal adhesions in response to externally applied forces. There is a dynamic feedback system that allows coupling of externally applied forces to internal forces via mechanosignalling. In ECs specifically, application of localised tensional forces on PECAM-1, a transmembrane mechanosensor, initiates mechanosignalling cascades that ultimately result in cell-wide growth of focal adhesions and changes in cytoskeletal architecture. Having identified a role for eIF6 in endogenous force generation that results in focal adhesion homeostasis, we next determined if eIF6 is also required for exogenous force transduction. For this, we utilised a well-established approach to directly apply force to PECAM-1. Magnetic beads were coated with an antibody against the extracellular domain of PECAM-1 (or CD44 as a control (Supplementary Figure 8) and allowed to bind to ECs before a constant force (~10 pN) was applied for the indicated times using a permanent magnet (Figure 4a). We first tested whether the subcellular localisation of eIF6 was force-regulated. Confocal analysis revealed that force application increased the co-localisation of eIF6 with actin (Supplementary Figure 9), presumably congruent with the increase in stress fibers in response to force. Next, we tested whether eIF6 regulates the dynamic activation of mechanically-induced signalling pathways important for focal adhesion formation. When phosphorylated at Tyrosine (Y)397, FAK localises to cell-matrix contact sites and leads to the assembly of focal adhesions.
Using the same magnetic bead assay, we found that force on PECAM-1 induced phosphorylation of FAK at Y397 in control cells, however, loss of eIF6 resulted in reduced pFAK<sup>Y397</sup> in response to force (Figure 4b-c). This response was also observed in another primary endothelial cell type (Supplementary Figure 10). Crk-associated substrate, p130<sup>Cas</sup> is a focal adhesion protein that directly associates with FAK<sup>63-65</sup> and which links mechanical forces to tyrosine phosphorylation signalling<sup>66</sup>. Phosphorylation of Cas at Y165 (pCas<sup>Y165</sup>) in focal adhesions has been linked to several fundamental cell processes including regulation of F-actin and focal adhesion turnover<sup>67-71</sup>. Crk-like protein (CrkL) is an adaptor protein and binds to Cas and forms part of the mechanical scaffold of the adhesion site; CrkL has been shown to be phosphorylated at Y207 in response to force on E-cadherin<sup>72</sup>. Similar to what we found for FAK, force increased phosphorylation of both pCas<sup>Y165</sup> and pCrkL<sup>Y207</sup> in control cells (Figure 4b, d-e); in contrast, depletion of eIF6 abolished the force-induced activation of all these signals (Figure 4b, d-e). Importantly, the lack of mechanically-induced signalling was evident as early as 5 mins after force application in eIF6-depleted cells, indicative of a role for eIF6 in early mechanotransduction events.

We then asked if eIF6 is involved in force-induced focal adhesion growth, by assaying the focal adhesion profile of cells by immunostaining for vinculin (Figure 4f). Cells expressing eIF6 exhibited robust focal adhesion growth in both size and number in response to force on PECAM-1 (Figure 4f-h). In contrast, eIF6-depleted cells not only showed basal defects in focal adhesion number and size, consistent with Figure 2, but also failed to respond to force as they did not increase focal adhesion size or number (Figure 4f-h). Taken together, these results show that eIF6 triggers dynamic activation of force-driven mechanical pathways and downstream cell-wide focal adhesion growth.

The force-induced increase in vinculin-positive focal adhesions could be a result of increased protein synthesis of pre-existing mRNAs, assembly of existing vinculin molecules into focal adhesions, or both. To address this, we first determined if force application affected the expression levels of key cytoskeletal proteins in whole cell lysates of si Scr and
si eIF6 cells. Quantification showed no changes in total levels of vinculin, FAK or paxillin following force application in either group and no differences in the levels of cytoskeletal proteins following depletion of eIF6 (Supplementary Figure 11a-d), suggesting that neither force application nor depletion of eIF6 affects expression levels of focal adhesion proteins. To investigate this further, we examined the force response in the presence of the protein synthesis inhibitor cycloheximide; protein levels of vinculin, FAK, paxillin or the signalling mediator ERK1/2 were unaffected by force or in response to cycloheximide (Supplementary Figure 12). To ensure validity of this result, we tested the efficacy of cycloheximide treatment over a longer time-period (Supplementary Figure 13). We then examined the effect of cycloheximide in force-induced focal adhesion growth: our results showed that cells increased vinculin-positive focal adhesion size and number despite the presence of cycloheximide (Supplementary Figure 12), further demonstrating that new protein synthesis is not required for this force response.

To definitively address the requirement for eIF6 in protein synthesis, we examined nascent protein synthesis and activation of the p70S6K pathway in response to force application. As shown in Figure 4i-j, mechanical force application increases nascent protein synthesis (as assayed by puromycin) as well as phosphorylation of p70S6K in both control and eIF6 siRNA transfected cells, thus providing solid evidence that loss of eIF6 does not disrupt global protein synthesis in response to force (Figure 4i-j). Taken together, these results allow for the first time, uncoupling of the mechanical and translational functions of eIF6 and show that eIF6 is required for the dynamic activation of mechanical pathways and downstream cell-wide focal adhesion growth in response to force, independent of effects on protein expression levels.

*eIF6-mediated tension regulates spatial activation of the ERK1/2 pathway*

Having shown that eIF6 regulates mechanical responses independent of effects on protein synthesis, we sought to determine the molecular mechanisms of this mechano-regulation. A
prominent mechanosensitive pathway known to be regulated by both intracellular and extracellular mechanical cues is the ERK signalling cascade. Importantly, published work has demonstrated force-dependent activation of the ERK1/2 pathway downstream of mechanical tension on PECAM-1\textsuperscript{12,73-75}. Given that eIF6 has a role in dynamic mechanosignalling (Figure 4), we asked if eIF6 also regulates the mechano-activation of ERK1/2. Similar to previous reports, application of force on PECAM-1 induced activation of ERK1/2 in si Scr cells, as assayed by increased phosphorylation at threonine (T)\textsuperscript{202} and Y204 (ERK\textsuperscript{T202/Y204}) (Figure 5a-b). Surprisingly, despite defective force-induced focal adhesion growth, eIF6-depleted cells showed normal levels of pERK\textsuperscript{T202/Y204} in response to force, similar to those seen in control cells (Figure 5a-b). Total ERK1/2 expression levels were also unchanged with loss of eIF6 (Supplementary Figure 11a,e). On first look, these results suggested that the defect does not lie with ERK1/2. However, we also considered possible differences in the spatial activation of the ERK1/2 pathway, as active ERK1/2 has been reported to localise to the actin cytoskeleton and focal adhesions\textsuperscript{10,76-80} and the correct spatial localisation of active ERK1/2 is important for downstream cytoskeletal remodelling. We, therefore, hypothesised that eIF6 is also important for localised activation of ERK1/2 in response to force. Super-resolution confocal microscopy showed increased pERK\textsuperscript{T202/Y204} in both control and eIF6 siRNA transfected cells subjected to force (Figure 5c-d), consistent with the western blotting results presented above (Figure 5a-b). However, when we examined the spatial activation of ERK1/2, by assaying co-localisation of pERK\textsuperscript{T202/Y204} with vinculin, we found that while force promoted localisation of pERK\textsuperscript{T202/Y204} at focal adhesions (Figure 5c,e) in control cells, this force-induced co-localisation was lost in eIF6-depleted cells (Figure 5c,e). Similar defects in the spatial localisation of pERK\textsuperscript{T202/Y204} were also observed in migrating (and thus mechanically active cells) eIF6-depleted cells in the absence of external force (Supplementary Figure 14). Conversely, eIF6 overexpression induced a small increase in the localisation of pERK\textsuperscript{T202/Y204} to focal adhesions (Supplementary Figure 15). Taken together, these data demonstrate that eIF6 regulates the correct spatial mechano-activation of ERK1/2 and is essential for the cellular response to force.
A possible candidate for providing spatial cues for polarised activation of ERK1/2 is the scaffold protein RACK1 which interacts with actin and regulates focal adhesions. Importantly, in addition to binding ERK1/2, RACK1 is also an interactor of eIF6. Indeed, we observed an association between eIF6 and RACK1 in our system (Figure 6a). Based on this rationale, we hypothesised that eIF6 supports the association of ERK1/2 with RACK1 and consequent spatial activation of ERK1/2. To test this possibility, we immunoprecipitated GFP from ECs transfected with GFP or GFP-RACK1 plasmids using GFP capture technology (Supplementary Figure 16). To assess if loss of eIF6 altered RACK1 complexes, we examined RACK1 immunoprecipitates for presence of ERK1/2. We found a significant reduction (~50%) in the RACK1-ERK1/2 complex with loss of eIF6 (Figure 6a-b), suggesting that eIF6 supports RACK1 mediated localisation of ERK1/2 to the cytoskeleton and focal adhesions. Given that FAK also associates with RACK1 and regulates pERK localisation to focal adhesions, we also tested the effect of eIF6 depletion on this association. Similar to the RACK1-ERK1/2 complex, loss of eIF6 disrupted the RACK1-FAK association (reduced by ~50%) (Figure 6c-d). Overall, our results point towards a role for eIF6 in stabilisation of a RACK1-ERK1/2-FAK complex which is necessary for focal adhesion dynamics and force-induced structural remodelling.

To further evaluate the relevance of this complex, we assessed the subcellular localisation of the mechano-complex components in response to force. Confocal co-localisation analyses revealed that force application induced an increase in the colocalization of pERK1/2 and RACK1 as well as pERK1/2 and FAK (Figure 6d-g) which occurred at actin filaments and focal adhesions (Supplementary Figure 17). These increases were blocked by siRNA against eIF6, suggesting that the mechanosensitive subcellular localisation of eIF6 (Supplementary Figure 9) provides a spatial cue for the recruitment of other proteins that direct proper localization of activated ERK1/2 and subsequent cytoskeletal remodelling. Taken together, these results suggest that eIF6 facilitates and regulates the force-induced interactions of a mechanosensitive RACK1-ERK1/2-FAK protein complex, which localises
with the actin cytoskeleton and focal adhesions. This complex regulates force-dependent focal adhesion and cytoskeletal dynamics through regulation of pFAK$^\text{Y379}$, pCas$^\text{Y165}$ and pCrkL$^\text{Y207}$, correct spatial activation of ERK1/2 (Figure 6h) and downstream mechanical responses.

**Discussion**

Since the discovery that specific mRNAs associate with the cytoskeleton, there has been increasing interest in the complex cross-talk between the protein translation machinery and the cellular cytoskeleton. The eukaryotic initiation factor eIF6 is a component of the protein translation apparatus that binds to the large 60S ribosomal subunit, but it has also been reported to co-localise with the cytoskeleton$^{38,47,49}$. We provide evidence that eIF6 associates with actin and regulates cellular mechanics through a mechanosignalling cascade that requires synergistic mechano-complex formation and distinct spatial activation of ERK1/2. Our results demonstrate that eIF6 is required for the cellular response to both cell-generated and externally applied forces via regulation of mechanosensitive pathways. We show that loss of eIF6 lowers the magnitude of traction forces cells exert on their substrate, lowers their stiffness and suppresses the ability of cells to respond to external tensional forces.

An intriguing aspect of our work is the observation that the mechanical defects not attributed to canonical roles of eIF6 in protein translation as nascent protein synthesis, polysome profiles and cytoskeleton protein expression were all unchanged with loss of eIF6. This result is consistent with previous reports showing normal polysome profiles in cells with ~50-80% knockdown of eIF6 as well as eIF6 heterozygous mice$^{34,41,54,55}$, although there is some discrepancy as to the effects of eIF6 loss on 60S and 80S profiles, perhaps due to cell-dependent variability and differences in knockdown efficiency$^{55,86}$. In ECs, even ~90% knockdown of eIF6 showed no visible defects in basal protein synthesis and, importantly, no
changes in key cytoskeleton components. An advantage of our force application protocol was the ability to examine early events, including activation of key cytoskeleton signalling mediators and focal adhesion growth. We showed that these early mechanotransduction responses do not require new proteins to be made, yet they do require eIF6, thus allowing decoupling of the translational vs signalling functions of eIF6. That is of course not to say that cytoskeleton-dependent functions over a longer timeframe (such as cell migration, endothelial cell alignment, etc) that require proteolytic cleavage and degradation of proteins over multiple cycles of focal adhesion assembly and disassembly will not be affected by inhibition of translation and eIF6 depletion. Indeed, our results, consistent with previous reports\textsuperscript{34,41,42}, show that eIF6 is required for insulin-induced nascent protein synthesis but not nascent synthesis in response to force. These data raise the intriguing possibility that the requirement for eIF6 in regulation of protein synthesis is context- and stimulus-dependent. Overall, these critical observations uncovered a novel, non-canonical role for eIF6 in the regulation of cell mechanics, independent of translation and expand the ever-increasing repertoire of the extra-ribosomal functions of ribosomal proteins.

A ribosomal protein that controls both mRNA-specific protein synthesis and signalling is RACK1. RACK1 is a scaffold protein that has been shown to be critical for shuttling or anchoring several proteins to specific subcellular locations. In addition to regulating focal adhesions, RACK1 also has a key role to play in the translation machinery, as it binds to the 40S ribosomal subunit\textsuperscript{87,88}. RACK1 has been described to exist in a complex with eIF6 across diverse species\textsuperscript{33,89}, however, the functional significance of this interaction is poorly understood. We now establish an eIF6-dependent RACK1-ERK1/2 complex that is responsible for the distinct, spatial localisation of activated ERK1/2 at focal adhesions. We show that while eIF6 is not required for the global mechanical activation of ERK1/2 in response to tensional force, it is essential for the localised activation of ERK1/2 at focal adhesions both under basal conditions and in response to mechanical force. Tension developed in focal adhesions and actin stress fibres has been proposed to be a critical
mechanism driving ERK signalling, as spatially distinct pERK localisation on these structures has been described⁹⁰. Furthermore, both RACK1 and FAK have been shown to regulate ERK activation at focal adhesions and downstream focal adhesion remodelling⁹¹. The relationship of this mechano-complex with the ribosome is currently unknown; unlike most ribosomal proteins, RACK1 and eIF6 are not members of actively translating ribosomes and can thus play analogous roles not only at the nexus between the cell signalling and translational machineries, but also as signalling hubs away from the ribosome.

In summary, our results show formation of an eIF6 dependent mechano-complex is required to regulate the structural components of the cell and dynamic remodelling in response to mechanical stimulation by mediating phosphorylation of FAK, Cas and CrkL and localisation of pERK⁴⁴⁸T²⁰²Y²⁰⁴ to focal adhesions. Hence, our findings provide a novel physical and signalling link between the cytoskeletal and translational machinery that is required for dynamic remodelling of the cytoskeleton in response to mechanical stimulation by coordinating correct localisation of cellular signalling cascades.

Methods

Cell culture and transfections

Bovine aortic endothelial cells (BAECs), human embryonic kidney 293A cells (HEK293) and human epidermoid carcinoma A431 cells were cultured in DMEM (Corning) supplemented with 10% FBS and 1% Penicillin and Streptomycin (Gibco). Human umbilical vein endothelial cells (HUVECs) were cultured in EGM2 growth medium (Lonza). Cells were cultured at 37 °C in a humid atmosphere of 5% CO₂. siRNA reverse transfections for scrambled (siGENOME, Dhamacon), eIF6 (SMARTpool, Dhamacon) eIF6 1 (ON TARGETplus, Dhamacon), or eIF6 2 (ON TARGETplus, Dhamacon) were performed using the Lipofectamine RNAmax reagent (Invitrogen).
Plasmid overexpression for pEGFP-N1-Empty vector and p-EGFP-N1-RACK1 were performed using Lipofectamine 2000 reagent as per manufacturer's instructions. pEGFP-N1-RACK1 was purchased from Addgene plasmid # 41088; http://n2t.net/addgene:41088; RRID:Addgene_41088.

**Cloning and adenoviral generation**

Wild-type eIF6 was cloned into the pENTR/TOPO entry vector of the Gateway System (Invitrogen) using the KOD Hot Start High Fidelity polymerase. After confirmation of successful cloning by Sanger sequencing, the constructs were sub-cloned into the pAd/CMV/V5-Dest destination vector by LR Clonase II reaction. All steps were performed according to the manufacturer's instructions. The destination vector encoding eIF6 or LACZ was linearized by PacI digestion and transfected into HEK293A cells for adenoviral generation and subsequent amplification according to the manufacturer's instructions.

**Antibodies, inhibitors and other Reagents**

The antibodies included total(t)-eIF6 (611120, BD Biosciences), t-paxillin (610568, BD Biosciences), t-actin (Ab179467, Abcam), α-actinin (ab50599, Abcam), t-eIF6 (D16E9, Cell Signaling Technology), t-ERK1/2 (9102, Cell Signaling Technology), t-FAK (3285, Cell Signaling Technology), t-β-actin (4970, Cell Signaling Technology), PECAM-1 (gift from Newman lab), Phospho(p)-p130CasY165 (4015, Cell Signaling Technology), t-p130Cas (4011, Cell Signaling Technology), t-CrkL (3182, Cell Signaling Technology), p-CrkLY207 (3181, Cell Signaling Technology), p-ERK1/2T202:Y204 (9106, Cell Signaling Technology), ribosomal protein L7a (2415, Cell Signaling Technology), ribosomal protein L26 (2065, Cell Signaling Technology), p-AktS473 (4060, Cell Signaling Technology), t-Akt (9272, Cell Signaling Technology), p-p70s6kT389 (9205, Cell Signaling Technologies), p70s6k (9202, Cell Signaling Technology), p-mTORS2448 (5536, Cell Signaling Technologies), mTOR (4517, Cell Signaling Technology), t-actin (Ab179467, Abcam), α-actinin (ab50599, Abcam), t-cofilin (ab54532, Abcam), ribosomal protein L10a (WH0004736M1, Sigma), ribosomal
protein L23 (SAB4503628, Sigma), t-Vinculin (V9131, Sigma Aldrich), pFAKY397 (44-624, ThermoFisher Scientific), t-eIF6 (PA5-31066, Invitrogen), Alexafluor 488 goat anti-mouse (A11001, Invitrogen), Alexafluor 488 goat anti-rabbit (A11034, Invitrogen), Alexafluor 568 goat anti-mouse (A11061, Invitrogen), Alexafluor 568 goat anti-rabbit (A11011, Invitrogen), Alexafluor 647 goat anti-mouse (A21235, Invitrogen), Alexafluor 647 goat anti-rabbit (A21244, Invitrogen), AlexaFluor 647 goat ant0-rat (A21247, Invitrogen), Alexafluor 790 goat anti-rabbit (A11367, Invitrogen), Alexafluor 680 goat anti-rabbit (A21076, Invitrogen), Alexafluor 790 goat anti-mouse (A11375, Invitrogen), Alexafluor 680 goat anti-mouse (A21058, Invitrogen).

Inhibitors included cycloheximide (UltraPure, VWR) and RNase block Ribonuclease inhibitor (Agilent).

Ex vivo en face descending aorta preparations

All mouse experiments were approved and authorized by both the University of Oxford Local Animals Ethics and Welfare Committee and by the UK Home Office, and carried out under the project licence P0C27F69A. Mice used in this study were < 8 weeks old housed in individually ventilated cages at 22 °C, with 56% relative humidity and a light–dark cycle of 12 h–12 h, and were fed a standard chow diet (B&K).

C57BL/6J mice were placed under a terminal general anaesthesia with isoflurane, followed by exsanguination and perfusion fixation with 4% paraformaldehyde. The descending aorta was dissected out and the surrounding connective tissue and adventitial fat were removed. The aorta was fixed in 4% paraformaldehyde and stored at 4 °C in PBS until staining.

Immunofluorescence staining

Cells and fixed aortas were permeabilised in 0.2% TritonX-100 (Sigma-Aldrich) and blocked with 10% normal goat serum/1% BSA. Cells were incubated with primary antibodies (1:100)
before incubation with Alexa Fluor 488– and/or Alexa Fluor 568– and/or Alexa Fluor 647–
conjugated secondary antibodies (1:150; Invitrogen), followed by Alexa Fluor 488/568/647–
conjugated phallodin (Invitrogen) and DAPI (Invitrogen) and mounted with SlowFade Gold
(Invitrogen).

Confocal and Super-resolution microscopy
Images were acquired on a Zeiss LSM880 upright confocal microscope using a Zeiss air
plan-apochromat 20x 1 NA or a Zeiss oil plan-apochromat 63x 1.4 NA objective, equipped
with 405nm solid state, 488nm argon and 561 diode lasers. For confocal images a Quasar
detector was used. For super-resolution images, airyscan mode was used with an airyscan
detector. Images were acquired using Zen 2.3 software and airy processing also performed
on this software with a correction of 6.

G-actin/F-actin Assay in Live cells
Amounts of G-actin and F-actin were assessed from lysates of BAECs transfected with
non-targeting or elf6 siRNA using G-actin/F-actin in vivo assay biochem kit according to
the manufacturer’s recommendations (Cytoskeleton, BK037). Briefly, BAECs were
transfected for 48hr and lysed with F-actin stabilization buffer. Lysates were
ultracentrifuged (100,000Xg for 1hr), cytoplasmic fractions were separated and insoluble
fraction resuspended in F-actin depolymerisation buffer. Samples were run out on an
SDS-PAGE gel. Antibodies specific for actin were then used to visualize amounts of G-
actin and F-actin.

Co-immunoprecipitation and Western blotting
Cells were harvested in Cell Lysis Buffer (Cell Signaling, #9803) and supplemented with
protease and phosphatase inhibitor cocktail tablets. Lysates were pre-cleared with 20μl
protein A agarose beads (Cell Signaling, #9863) for 1hr at 4°C. The pre-cleared lysates were
then incubated with appropriate primary antibody for overnight at 4°C on an orbital shaker. The antibody incubated lysates were then incubated with 20 μl of protein A agarose beads for 2hr at 4°C on an orbital shaker. The beads were washed three times with the lysis buffer supplemented with protease and phosphatase inhibitors. The immunoprecipitation complexes were eluted from the beads by boiling in 2X SDS buffer for 5 minutes.

For western blotting analyses, protein lysates/co-immunoprecipitation complexes were resolved on a 4-12% gradient gel (Invitrogen) with the appropriate primary antibodies and IRDye-conjugated anti-mouse, anti-goat or anti-rabbit secondary antibodies, as appropriate. Images were acquired on a LICOR Odyssey infra-red scanner. Densitometric quantification of bands was performed using the ImageStudio software (LICOR Biosciences).

**In vitro binding assay**

Purified proteins for eIF6 and actin were purchased from commercial sources (Origene and Cytoskeleton, Inc, AKL99, respectively). Actin (5 μM) and eIF6 (0.5 μM) were added to binding buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM β-mercaptoethanol, 2.5 mM EDTA, 2.5 mM EGTA, 0.05% BSA, 10% glycerol, pH7.5). For heat-denatures actin controls, actin was heated to 95 °C for 5 mins. Immuno-precipitation buffer (150, M NaCl, 50mM HEPES, 1mM EDTA, 2.5mM EGTA, 1% Triton X-100, 10% Glycerol, 1% Sodium deoxycholate, cOmplete EDTA-free protease inhibitor, pH 7.5) was added to protein reaction. Reaction mixtures were then incubated with appropriate primary antibody for 2hrs at 4°C on an orbital shaker. The antibody incubated lysates were then incubated with 20 μl of protein A agarose beads for 1hr at 4°C on an orbital shaker. The beads were washed three times with the lysis buffer supplemented with protease and phosphatase inhibitors. The immunoprecipitation complexes were eluted from the beads by boiling in 2X SDS buffer for 5 minutes.

**GFP-Trap® Immunoprecipitation**
GFP-fusion pulldowns with GFP-Trap® (Chromotek) were performed as to the manufacturer's recommendations. Briefly, HEK293A cells were lysed in 10 mM Tris/HCl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40 and supplemented with protease and phosphatase inhibitor cocktail tablets. Cell lysates were cleared by centrifugation at 4°C for 10 min and supernatants were incubated with GFP-trap beads for 1 hr at 4°C with gentle rotation. The beads were washed three times with wash buffer (10 mM Tris/HCl pH 7.5; 150 mM NaCl; 0.5 mM EDTA, supplemented with protease and phosphatase inhibitor cocktail tablets) and resuspended in 2X SDS-PAGE sample buffer. The immunoprecipitation complexes were eluted from the beads by boiling for 10 min.

Atomic force microscopy (AFM)
AFM indentation was conducted on a JPK nanowizard-1 (JPK Instruments) operating in force spectroscopy mode, mounted on an inverted optical microscope (IX-81; Olympus). AFM pyramidal cantilevers (MLCT; Bruker) with a spring constant of 0.07 N m⁻¹ were used with a 35 μm glass bead attached to cantilever tip. Before measurements with the adapted cantilevers, their sensitivity was calculated by measuring the slope of force-distance curve in the AFM software on an empty region of the Petri dish. For cell indentation tests, the cantilever was aligned over regions in the middle of the cells using an inverted optical microscope (IX-81; Olympus). For each group, 30 individual cells were tested. Force-curve acquisition was carried out with an approach speed of 5 μm s⁻¹ and a maximum set force of 1.5 nN. Elastic moduli were calculated from the force-distance curves by fitting the contact region of the approach curve with the Hertz contact model using the AFM software (JPK).

Micropillar video microscopy and traction force measurements
Elastic micropillars were fabricated in PDMS following a previous protocol. Pillar arrays were coated with human plasma FN (10 μg ml⁻¹; Sigma) and incubated at 37 °C for 1 h before measurements. Cells were plated onto the pillar substrates, left for one hour, and then imaged for a maximum of 30 mins. Time-lapse imaging of the pillars was conducted.
with an inverted microscope (Eclipse Ti; Nikon) operating in bright-field mode at an ambient temperature of 37 °C. Image sequences were recorded with a sCMOS camera (Neo sCMOS Andor) at 0.5 Hz using a × 40 (0.6 NA, air; Nikon) objective over the early spreading phase \((t<60\ \text{min})\) and late spreading phase \((90\ \text{min}<t<120\ \text{min})\). The position of each pillar in the time-lapse videos was tracked using a custom MATLAB program to track the centre of a point spread function of the intensity of the pillars across all frames. By selecting a location free of cells, tracking of a small set of pillars allowed a measurement of the stage drift to be obtained and corrected for in the data set. The time-dependent displacement of a given pillar was obtained by subtracting the initial position of the pillar (zero force) from the position in a given frame. Traction forces were obtained by multiplying the pillar displacements by the pillar stiffness, the maxima for each pillar were found to obtain the peak forces across the cell.

Nascent protein synthesis assay

Nascent protein synthesis was analysed using non-radioactive metabolic labelling, Click-iT® OPP (O-propargyl-puromycin) Alexa Fluor 568 Protein Synthesis Assay Kit (Thermo Fisher Scientific, C10457) as per the manufacturer’s instruction. Briefly, cells were grown in media containing OPP reagent for 30 mins. Cells were fixed (3.7% formaldehyde in PBS) and permeabilised (0.5% Triton® X-100) before OPP detection. The signal intensity of incorporated OPP-Alexa Fluor 568 was measured by microscopy.

Polysome Profiling

Cells were grown in 15cm dishes until 80% confluence, as described above. Growth media was supplemented with cycloheximide (100 μg/ml) for 5 minutes and cells were scraped in ice-cold PBS containing cycloheximide (100 μg/ml). Cells were collected by centrifugation and lysed by vortexing for 10 seconds in 200 μl of polysome extraction buffer (5 mM Tris-HCl, 2.5 mM MgCl₂, 1.5 mM KCl, 1 mM DTT, 100 μg/ml cycloheximide, 100 units/ml RNase
block, 1% TritonX-100, 1% Sodium deoxycholate, Complete EDTA-free protease inhibitor).

Lysates were centrifuged at 16,000 g and supernatants collected.

Supernatants were layered on 13 ml of 5%-45% (wt/wt) sucrose density gradients (containing 20 mM HEPES at pH 7.6, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 100 μg/ml cycloheximide, 10 units/ml RNase block, cOmplete EDTA-free protease inhibitor), then centrifuged at 40,000 rpm for 2 hrs at 4 °C in a Beckman SW41Ti rotor. The samples were fractionated into 10 samples (each ~1300 μl) using density gradient fractionation (Brandel) with continuous measurement of absorbance at 254 nm.

**RNA extraction and qPCR**

Total RNA extraction was performed on HUVECs using the RNeasy Plus mini kit (Qiagen), with an additional genomic DNA wipeout treatment. First strand cDNA synthesis was performed using the Superscript III cDNA synthesis kit. Quantitative real-time PCR was performed in triplicate with SYBR green and CFX96TM real-time system. Thermocycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 30s, 60°C for 1min.

Gene expression was normalised to the constitutively expressed housekeeping gene GAPDH, and relative expression was calculated and plotted employing the ΔΔCt method.

Primer sequences used were as follows: human 28S rRNA- 5’-AGTCGGGTTGCTTGGGAA-3’, 5’-ATGC-3’; human 5.8S rRNA- 5’-ACTCTTAGCGGTGGATC ACTC-3’, 5’-AGAAGCGACGCTCAGACAG G-3’; human 18s rRNA – 5’-AGGAATTGACGGAAGGGCACCA-3, 5’-GTGCAGCCCCGGACATCTAAG-3’; human 45S rRNA- 5’-GTTCGAGGCGGTTTGAG TGA-3’, 5’-CTCCGAAGTCAACCCAC ACA-3’; human eIF6- 5’-TTGGTGCATCCCAAGACT TCAAT-3’, 5’-TCACAGTCCCGGCACACA-3’.

**Bead pulling**

Tosyl activated paramagnetic beads (4.5 μm) were washed with PBS and coated with an antibody to the extracellular domain of PECAM-1 (a gift from Peter Newman) or fibronectin.
Prior to use, beads were quenched in 0.2 M Tris, pH 7.4 to eliminate any remaining tosyl groups. ECs were seeded on fibronectin coated coverslips and then incubated in reduced serum media (0.5%) overnight. ECs were incubated with the beads (and cycloheximide, if appropriate) for 30 minutes prior to force application and then for 5 or 30 minutes of force at 37°C.

**Image analysis**

Image analyses performed on ImageJ. Quantification of the co-localisation was performed using the coloc2 plugin and cell area, focal adhesion number and focal adhesion area were measured using an in-house macro. For mechanically stimulated cells, only cells with 1-3 beads bound were used for analysis.

**Statistics**

Statistical analyses were performed using GraphPad Prizm 8 (La Jolla, CA, USA). Comparisons between groups were assessed by t test or ANOVA with a Tukey multiple comparisons post hoc test, where appropriate. Differences were considered significant when $p < 0.05$.

**Acknowledgements**

This work was supported in part by grants from the Wellcome Trust (Senior Research Fellowship to E.T.), BHF (PG/18/18/33574 to E.T.), John Fell Fund (to E.T.), BBSRC (BB/T003553/1 to E.T. and J.S.R.), the BHF Centre of Excellence, Oxford (RE/13/1/30181), and Wellcome Trust grant 203141/Z/16/Z supporting the Wellcome Centre for Human Genetics. Thanks also to Drs Carina Monico, Nadia Halidi and James Bancroft and the Micron imaging facility (supported by Wellcome Strategic Awards 091911/B/10/Z and 107457/Z/15/Z) for technical advice and access to equipment. We thank Peter and Debra Newman for providing the antibody to PECAM1.3.
Author Contributions

A.N.K. performed or was involved in most of the experiments and analyses. L.A.P. performed the G-F actin fractionation and co-immunoprecipitation and analysed the data. A.R. performed the traction force and atomic force microscopy experiment and analysed the data. K. L. P. performed RT-qPCR reactions and analysed the data. L.J.S. quantified expression of proteins following eIF6 siRNA transfection. A.E.d.R.H. led and supervised the traction force and atomic force microscopy experiments. J.S.R. co-supervised, conceived and developed the idea of the project, interpreted data and generated research funds. E.T. initiated the project, conceived and developed the idea of the project, generated research funds, directed and coordinated the project. A.N.K., J.S.R. and E.T. designed experiments, interpreted data and wrote the manuscript, with inputs from other authors.

Figure Legends

Figure 1. eIF6 associates with the actin cytoskeleton. (a) Representative immunofluorescent confocal micrographs of ECs, showing eIF6 (green), F-actin (red), DAPI (blue), scale bar = 20 μm. (b) Higher magnification image of selected region in a. (c) Representative fluorescent intensities along white line indicated in the merged image shown in b were quantified using the line scan mode. Line scans are plotted in the graph shown. (d) Representative immunofluorescent confocal micrographs from en face descending aorta preparations showing eIF6 (green), F-actin (red), DAPI (blue), scale bar = 20 μm. (e) Higher magnification image of selected region in d. (f) Representative fluorescent intensities along white line indicated in the merged image shown in e were quantified using the line scan mode. Line scans are plotted in the graph shown. (g) Co-localisation of eIF6 to F-actin was quantified using Pearson’s coefficient for in vitro and in vivo cells (n > 30 cells, across 3 separate experiments). (h-i) ECs were fractionated to separate non-cytoskeletal (HSP90-positive) and cytoskeletal compartments (actin positive). Localisation of eIF6 is shown in h.
by western blot and quantified in i. (j) Western blot showing eIF6 knockdown (on the left).

Immunoprecipitation using an IgG or eIF6 specific antibody from control siRNA (si Scr) or eIF6 siRNA (si eIF6) treated EC lysates showed that actin co-immunoprecipitates with eIF6. (k) Immunoprecipitation using an IgG or eIF6 specific antibody using purified proteins showed that actin, but not heat-denatured actin, co-immunoprecipitates with eIF6. Values in g and i are represented as mean ± SEM (n > 3 biological replicates).

**Figure 2.** eIF6 regulates cell-generated forces. (a) Representative immunofluorescent micrographs showing F-actin (phalloidin; red) and DAPI (blue) in ECs transfected with scrambled siRNA (si Scr) or eIF6 siRNA (si eIF6) imaged at 20x (upper) and 63x (lower). Scale bars = 50 μm and 20 μm, respectively. (b-c) Quantification of b mean cell area and c coherency in alignment of F-actin fibres in si Scr and si eIF6 cells. (d) Representative immunofluorescent micrographs showing vinculin-positive focal adhesions in si Scr and si eIF6 ECs, scale bar = 20 μm. (e-f) Quantification of e mean number and f mean area of vinculin-positive focal adhesions. (g-k) Traction force microscopy and atomic force microscopy measurements in si Scr and si eIF6 ECs (schematic representation shown in g and j, respectively). h force vector maps indicating the magnitude of traction forces calculated from maximum pillar displacement. Quantification of mean i micro-pillar displacement and k young’s modulus. Values in b, c, e, f, i and k are mean ± SEM (n > 30 cells, across 3 separate experiments) and significance was determined by t-test; **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 3. Depletion of endogenous eIF6 does not affect basal levels of protein synthesis.** (a) Representative fluorescent micrographs of scrambled (si Scr) or eIF6 (si eIF6) siRNA-transfected ECs, or cycloheximide (CHX) ECs, following incorporation of OPP to label nascent proteins (red) using a Click-iT assay and co-staining of cell nuclei (DAPI; blue). Scale = 20μm. (b) Quantification of cell fluorescence following OPP incorporation ClickIT assay. (c) Representative polysome profiles from A431 cells following sucrose
gradient fractionation, showing the small ribosomal subunit (40S), the large ribosomal subunit (60S) and the monoribosome (80S). (d) Representative western blots for cytoskeletal proteins from EC lysates. (e-j) Quantification of band intensity for western blot analysis for; e paxillin, f focal adhesion kinase (FAK), g cofilin, h vinculin, i extracellular signal-related kinase (ERK1/2) and j Crk-associated substrate p130\textsuperscript{cas} (Cas). Values in b, e, f, g, h, i and j are mean ± SEM (n > 3) and significance was determined by t-test; ***p<0.001, ****p<0.0001.

**Figure 4. Decoupling of eIF6 cellular functions.** (a) Schematic representation of permanent magnet system used to apply direct force on cellular mechanosensors. Using a permanent magnet, ECs transfected with scrambled (si Scr) or eIF6 siRNA (si eIF6) were exposed to mechanical force for 0, 5 or 30 minutes. (b) Representative western blots of phosphorylated and total protein levels from EC lysates. (c-e) Western blot band quantification of b phosphorylated FAK (pFAK\textsuperscript{Y397}) relative to FAK, c phosphorylated CrkL (pCrkL\textsuperscript{Y207}) relative to CrkL and d phosphorylated Cas (pCas\textsuperscript{Y165}) relative to Cas following mechanical force. (f) Representative immunofluorescent micrographs showing focal adhesions (vinculin; white) in ECs following force. Magnetic beads are highlighted by red circles scale = 20 μm. (g-h) Quantification of g mean number per cell and h mean area of vinculin-positive focal adhesions. (i-j) Mechanical force was applied using a permanent magnet for 0 (no force) or 30 minutes (force) in si Scr or si eIF6 A431 cells incubated with puromycin. Quantification of mean fluorescence of i puromycin and j phosphorylated p70S6k (pp70S6k\textsuperscript{T389}). Values in c, e, g, h, l and j are as mean ± SEM (n > 3 for c-e and n > 30 cells for g-i, across 3 separate experiments) and significance was determined by ANOVA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 5. eIF6 regulates the spatial mechano-activation of ERK1/2.** ECs transfected with scrambled (si Scr) or eIF6 siRNA (si eIF6) were exposed to force for 0, 5 or 30 minutes. (a-b) Representative western blots of phosphorylated ERK1/2 (pERK\textsuperscript{T202/Y204}) and total ERK1/2.
and quantification. (c) Representative super-resolution immunofluorescent micrographs showing co-localisation of pERK\textsuperscript{T202/Y204} (green) with focal adhesions (vinculin; red) following application of force. Larger images are higher magnification images of indicated region of whole cells shown in smaller images. Magnetic beads are highlighted by white circles. Scale = 20 μm. (d) Quantification of mean fluorescence intensities of pERK\textsuperscript{T202/Y204} following application of force. (e) Image analysis quantification of co-localisation of pERK\textsuperscript{T202/Y204} with focal adhesions, using Pearson’s coefficient, following force. Values in b, d and e are mean ± SEM (n > 30 cells, across 3 separate experiments) and significance determined by ANOVA; *p<0.05, **p<0.01, ****p<0.0001.

**Figure 6. elf6 stabilises mechano-complexes.** (a) Immunoprecipitation using an IgG or RACK1 specific antibody from A431 cell lysates showed that elf6 co-immunoprecipitates with RACK1. (b-c) HEK293 cells were transfected with scrambled (si Scr) or elf6 siRNA (si elf6) and transiently transfected with plasmids expressing GFP alone or GFP-RACK1, lysed and incubated with GFP-trap beads to immunoprecipitate GFP. Immunoprecipitates were tested for association of b ERK1/2 and c FAK; representative blots show elf6-dependent associations. (d-g) ECs transfected with scrambled (si Scr) or elf6 siRNA (si elf6) were exposed to force on PECAM-1 for 0 (no force) or 30 minutes (force). (d, f) Representative immunofluorescent micrographs showing co-localisation of d pERK\textsuperscript{T202/Y204} (green) and RACK1 (red), and f pERK\textsuperscript{T202/Y204} (green) and FAK (red) following application of force. PECAM-1 coated beads are highlighted by white circles. Scale = 20 μm. (e, g) Image analysis quantification of co-localisation of e pERK\textsuperscript{T202/Y204} with RACK1 and g pERK\textsuperscript{T202/Y204} with FAK, using Pearson’s coefficient, following force. Values in e and g are mean ± SEM (n > 30 cells, across 3 separate experiments) and significance determined by ANOVA; ***p<0.001, ****p<0.0001. (h) Schematic representation of the elf6-dependent mechano-complex. elf6 facilitates correct spatiotemporal control of the mechano-complex, regulating cytoskeletal and focal adhesions dynamics.
References

1. Wang, N., Butler, J. & Ingber, D. Mechanotransduction across the cell surface and through the cytoskeleton. Science 260, 1124-1127, doi:10.1126/science.7684161 (1993).

2. Chang, L. & Goldman, R. D. Intermediate filaments mediate cytoskeletal crosstalk. Nature Reviews Molecular Cell Biology 5, 601-613, doi:10.1038/nrm1438 (2004).

3. Harris, A. R., Jreij, P. & Fletcher, D. A. Mechanotransduction by the Actin Cytoskeleton: Converting Mechanical Stimuli into Biochemical Signals. Annual Review of Biophysics 47, 617-631, doi:10.1146/annurev-biophys-070816-033547 (2016).

4. Pegoraro, A. F., Janmey, P. & Weitz, D. A. Mechanical Properties of the Cytoskeleton and Cells. Cold Spring Harbor Perspectives in Biology 9, doi:10.1101/cshperspect.a022038 (2017).

5. Fletcher, D. A. & Mullins, R. D. Cell mechanics and the cytoskeleton. Nature 463, 485-492, doi:10.1038/nature08908 (2010).

6. Mitra, S. K., Hanson, D. A. & Schlaepfer, D. D. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol 6, 56-68 (2005).

7. DeMali, K. A., Wennerberg, K. & Burridge, K. Integrin signaling to the actin cytoskeleton. Current Opinion in Cell Biology 15, 572-582, doi:https://doi.org/10.1016/S0955-0674(03)00109-1 (2003).

8. Parsons, J. T. Focal adhesion kinase: the first ten years. Journal of Cell Science 116, 1409-1416, doi:10.1242/jcs.00373 (2003).

9. Mitra, S. K. & Schlaepfer, D. D. Integrin-regulated FAK–Src signaling in normal and cancer cells. Current Opinion in Cell Biology 18, 516-523, doi:https://doi.org/10.1016/j.jceb.2006.08.011 (2006).

10. Fincham, V. J., James, M., Frame, M. C. & Winder, S. J. Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. The EMBO Journal 19, 2911-2923, doi:10.1093/emboj/19.12.2911 (2000).

11. Choquet, D., Felsenfeld, D. P. & Sheetz, M. P. Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. Cell 88, 39-48 (1997).

12. Collins, C. et al. Localized Tensional Forces on PECAM-1 Elicit a Global Mechanotransduction Response via the Integrin-RhoA Pathway. Current Biology 22, 2087-2094, doi:10.1016/j.cub.2012.08.051 (2012).

13. Collins, C. et al. Haemodynamic and extracellular matrix cues regulate the mechanical phenotype and stiffness of aortic endothelial cells. Nature communications 5, doi:10.1038/ncomms4984 (2014).

14. Barry, A. K., Wang, N. & Leckband, D. E. Local VE-cadherin mechanotransduction triggers long-ranged remodeling of endothelial monolayers. Journal of cell science 128, 1341-1351, doi:10.1242/jcs.159954 (2015).

15. Bays, J. L., Campbell, H. K., Heidema, C., Sebbagh, M. & DeMali, K. A. Linking E-cadherin mechanotransduction to cell metabolism through force-mediated activation of AMPK. Nat Cell Biol 19, 724-731, doi:10.1038/ncb3537

http://www.nature.com/ncb/journal/v19/n6/abs/ncb3537.html#supplementary-information (2017).

16. Muhamed, I. et al. E-cadherin-mediated force transduction signals regulate global cell mechanics. Journal of Cell Science 129, 1843-1854, doi:10.1242/jcs.185447 (2016).

17. Bezanilla, M., Gladfelter, A. S., Kovar, D. R. & Lee, W.-L. Cytoskeletal dynamics: A view from the membrane. The Journal of Cell Biology 209, 329-337, doi:10.1083/jcb.201502062 (2015).

18. JANMEY, P. A. The Cytoskeleton and Cell Signaling: Component Localization and Mechanical Coupling. Physiological Reviews 78, 763-781, doi:10.1152/physrev.1998.78.3.763 (1998).

19. Wolosewick, J. J. & Porter, K. R. Stereo high-voltage electron microscopy of whole cells of the human diploid line, WI-38. The American journal of anatomy 147, 303-323, doi:10.1002/aja.1001470305 (1976).
Wolosewick, J. J. & Porter, K. R. Observation on the morphological heterogeneity of WI-38 cells. *The American journal of anatomy* **149**, 197-225, doi:10.1002/aja.1001490206 (1977).

Singer, R. H., Langevin, G. L. & Lawrence, J. B. Ultrastructural visualization of cytoskeletal mRNAs and their associated proteins using double-label in situ hybridization. *The Journal of Cell Biology* **108**, 2343-2353, doi:10.1083/jcb.108.6.2343 (1989).

Lenk, R., Ransom, L., Kaufmann, Y. & Penman, S. A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell* **10**, 67-78, doi:https://doi.org/10.1016/0092-8674(77)90141-6 (1977).

Kim, S. & Coulombe, P. A. Emerging role for the cytoskeleton as an organizer and regulator of translation. *Nat Rev Mol Cell Biol* **11**, 75-81, doi:http://www.nature.com/nrm/journal/v11/n1/suppinfo/nrm2818_S1.html (2010).

Smart, F. M., Edelman, G. M. & Vanderklish, P. W. BDNF induces translocation of initiation factor 4E to mRNA granules: Evidence for a role of synaptic microfilaments and integrins. *Proceedings of the National Academy of Sciences* **100**, 14403-14408, doi:10.1073/pnas.2436349100 (2003).

Gross, S. R. & Kinzy, T. G. Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. *Nature Structural & Molecular Biology* **12**, 772-778, doi:10.1038/nsmb979 (2005).

Fujimura, K. et al. Eukaryotic Translation Initiation Factor 5A (EIF5A) Regulates Pancreatic Cancer Metastasis by Modulating RhoA and Rho-associated Kinase (ROCK) Protein Expression Levels. *Journal of Biological Chemistry* **290**, 29907-29919, doi:10.1074/jbc.M115.687418 (2015).

Willett, M., Pollard, H. J., Vласак, M. & Morley, S. J. Localization of ribosomes and translation initiation factors to talin/integrin-enriched adhesion complexes in spreading and migrating mammalian cells. *Biology of the Cell* **102**, 265-276, doi:10.1042/bc20090141 (2010).

Liu, G. et al. Interactions of elongation factor 1alpha with F-actin and beta-actin mRNA: implications for anchoring mRNA in cell protrusions. *Molecular biology of the cell* **13**, 579-592, doi:10.1091/mbc.01-03-0140 (2002).

Horton, E. R. et al. Definition of a consensus integrin adhesome and its dynamics during adhesion complex assembly and disassembly. *Nature cell biology* **17**, 1577-1587, doi:10.1038/ncb3257 (2015).

Warner, J. R. & McIntosh, K. B. How Common Are Extraribosomal Functions of Ribosomal Proteins? *Molecular Cell* **34**, 3-11, doi:https://doi.org/10.1016/j.molcel.2009.03.006 (2009).

Warren, A. J. Molecular basis of the human ribosomopathy Shwachman-Diamond syndrome. *Adv Biol Regul* **67**, 109-127, doi:10.1016/j.jbior.2017.09.002 (2018).

Valenzuela, D. M., Chaudhuri, A. & Maitra, U. Eukaryotic ribosomal subunit anti-association activity of calf liver is contained in a single polypeptide chain protein of Mr = 25,500 (eukaryotic initiation factor 6). *J Biol Chem* **257**, 7712-7719 (1982).

Ceci, M. et al. Release of elf6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature* **426**, doi:10.1038/nature02160 (2003).

Gandin, V. et al. Eukaryotic initiation factor 6 is rate-limiting in translation, growth and transformation. *Nature* **455**, doi:10.1038/nature07267 (2008).

Russell, D. W. & Spremulli, L. L. Purification and characterization of a ribosome dissociation factor (eukaryotic initiation factor 6) from wheat germ. *Journal of Biological Chemistry* **254**, 8796-8800 (1979).

Gallo, S. & Manfrini, N. Working hard at the nexus between cell signaling and the ribosomal machinery: An insight into the roles of RACK1 in translational regulation. *Translation* **3**, e1120382, doi:10.1080/21690731.2015.1120382 (2015).

Grosso, S. et al. PKCβII modulates translation independently from mTOR and through RACK1. *Biochemical Journal* **415**, 77-85, doi:10.1042/bj20080463 (2008).
Sanvito, F. et al. The β4 Integrin Interactor p27BBP/eIF6 Is an Essential Nuclear Matrix Protein Involved in 60S Ribosomal Subunit Assembly. *The Journal of Cell Biology* **144**, 823-838, doi:10.1083/jcb.144.5.823 (1999).

Brina, D., Miluzio, A., Ricciardi, S. & Biffo, S. eIF6, anti-association activity is required for ribosome biogenesis, translational control and tumor progression. *Biochim Biophys Acta* **1849**, 830-835, doi:10.1016/j.bbagrm.2014.09.010 (2015).

Basu, U., Si, K., Warner, J. R. & Maitra, U. The Saccharomyces cerevisiae TIF6 gene encoding translation initiation factor 6 is required for 60S ribosomal subunit biogenesis. *Mol Cell Biol* **21**, 1453-1462, doi:10.1128/MCB.21.5.1453-1462.2001 (2001).

Brina, D. et al. eIF6 coordinates insulin sensitivity and lipid metabolism by coupling translation to transcription. *Nature communications* **6**, 8261, doi:10.1038/ncomms9261 (2015).

http://www.nature.com/articles/ncomms9261#supplementary-information (2015).

Miluzio, A. et al. Translational control by mTOR-independent routes: how eIF6 organizes metabolism. *Biochemical Society Transactions* **44**, 1667-1673, doi:10.1042/bst20160179 (2016).

Miluzio, A. et al. Expression and activity of eIF6 trigger malignant pleural mesothelioma growth in vivo. *Oncotarget* **6**, 37471-37485, doi:10.18632/oncotarget.5462 (2015).

Sanvito, F. et al. Expression of a highly conserved protein, p27BBP, during the progression of human colorectal cancer. *Cancer Res* **60**, 510-516 (2000).

Yang, Q.-Q. et al. Process of Hypertrophic Scar Formation: Expression of Eukaryotic Initiation Factor 6. *Chinese Medical Journal* **128**, 2787-2791, doi:10.4103/0366-6999.167359 (2015).

Shu, Q. et al. Involvement of eIF6 in external mechanical stretch–mediated murine dermal fibroblast function via TGF–β1 pathway. *Scientific Reports* **6**, 36075, doi:10.1038/srep36075 (2016).

Biffo, S. et al. Isolation of a Novel β4 Integrin-binding Protein (p27BBP) Highly Expressed in Epithelial Cells. *Journal of Biological Chemistry* **272**, 30314-30321, doi:10.1074/jbc.272.48.30314 (1997).

Ji, Y. et al. Eukaryotic initiation factor 6 selectively regulates Wnt signaling and beta-catenin protein synthesis. *Oncogene* **27**, 755-762, doi:10.1088/sj.occ.12100667 (2008).

Carotenuto, R. et al. Phosphorylation of p27BBP/eIF6 and its association with the cytoskeleton are developmentally regulated in Xenopus oogenesis. *Cellular and Molecular Life Sciences* **CMSL* **62**, 1641-1652, doi:10.1007/s00018-005-5153-9 (2005).

Chronopoulos, A. et al. ATRA mechanically reprograms pancreatic stellate cells to suppress matrix remodelling and inhibit cancer cell invasion. 7, 12630, doi:10.1038/ncomms12630 (2016).

https://www.nature.com/articles/ncomms12630#supplementary-information (2016).

Kraning-Rush, C. M., Carey, S. P., Califano, J. P., Smith, B. N. & Reinhart-King, C. A. The role of the cytoskeleton in cellular force generation in 2D and 3D environments. *Phys Biol* **8**, 015009-015009, doi:10.1088/1478-3975/8/1/015009 (2011).

Stamenović, D. Effects of cytoskeletal prestress on cell rheological behavior. *Acta Biomaterialia* **1**, 255-262, doi:https://doi.org/10.1016/j.actbio.2005.01.004 (2005).

Signer, R. A., Magee, J. A., Salic, A. & Morrison, S. J. Haematopoietic stem cells require a highly regulated protein synthesis rate. *Nature* **509**, 49-54, doi:10.1038/nature13035 (2014).

Clarke, K. et al. The Role of Eif6 in Skeletal Muscle Homeostasis Revealed by Endurance Training Co-expression Networks. *Cell reports* **21**, 1507-1520, doi:10.1016/j.celrep.2017.10.040 (2017).

Chendrimada, T. P. et al. MicroRNA silencing through RISC recruitment of eIF6. *Nature* **447**, 823-828, doi:http://www.nature.com/nature/journal/v447/n7146/suppinfo/nature05841_S1.html (2007).
Roux, P. P. & Topisirovic, I. Signaling Pathways Involved in the Regulation of mRNA Translation. *Mol Cell Biol* **38**, do:10.1128/MCB.00070-18 (2018).

Nikolov, E. N., Dineva, B. B., Dareva, M. D. & Nikolov, T. K. Turnover of ribosomal proteins in regenerating rat liver after partial hepatectomy. *Int J Biochem* **19**, 159-163, doi:10.1016/0020-711x(87)90326-0 (1987).

Hirsch, C. A. & Hiatt, H. H. Turnover of liver ribosomes in fed and in fasted rats. *J Biol Chem* **241**, 5936-5940 (1966).

Chen, C. S. Mechanotransduction – a field pulling together? *Journal of Cell Science* **121**, 3285-3292, doi:10.1242/jcs.023507 (2008).

Collins, C. et al. Localized tensional forces on PECAM-1 elicit a global mechanotransduction response via the integrin-RhoA pathway. *Current biology : CB* **22**, 2087-2094, doi:10.1016/j.cub.2012.08.051 (2012).

Geiger, B. & Bershadsky, A. Exploring the Neighborhood: Adhesion-Coupled Cell Mechanosensors. *Cell* **110**, 139-142, doi:https://doi.org/10.1016/S0092-8674(02)00831-0 (2002).

Humphries, J. D., Paul, N. R., Humphries, M. J. & Morgan, M. R. Emerging properties of adhesion complexes: what are they and what do they do? *Trends in Cell Biology* **25**, 388-397, doi:https://doi.org/10.1016/j.tcb.2015.02.008 (2015).

Polte, T. R. & Hanks, S. K. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proc Natl Acad Sci U S A* **92**, 10678-10682 (1995).

Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H. & Parsons, J. T. p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *J Biol Chem* **271**, 13649-13655 (1996).

Astier, A. et al. The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after beta1-integrin stimulation in B cells and binds to p130cas. *J Biol Chem* **272**, 228-232 (1997).

Sawada, Y. et al. Force Sensing by Mechanical Extension of the Src Family Kinase Substrate p130Cas. *Cell* **127**, 1015-1026, doi:https://doi.org/10.1016/j.cell.2006.09.044 (2006).

Donato, D. M., Ryzhova, L. M., Meenderink, L. M., Kaverina, I. & Hanks, S. K. Dynamics and Mechanism of p130Cas Localization to Focal Adhesions. *Journal of Biological Chemistry* **285**, 20769-20779, doi:10.1074/jbc.M109.091207 (2010).

Honda, H. et al. Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. *Nature Genetics* **19**, 361-365, doi:10.1038/1246 (1998).

Petch, L. A., Bockholt, S. M., Bouton, A., Parsons, J. T. & Burridge, K. Adhesion-induced tyrosine phosphorylation of the p130 src substrate. *J Cell Sci* **108**, 1371-1379 (1995).

Vuori, K. & Ruoslahti, E. Tyrosine Phosphorylation of p130Cas and Correlation of Colocalization with Integrin-Mediated Cell Adhesion to Extracellular Matrix. *Journal of Biological Chemistry* **270**, 22259-22262, doi:10.1074/jbc.270.38.22259 (1995).

Sharma, A. & Mayer, B. J. Phosphorylation of p130Cas initiates Rac activation and membrane ruffling. *BMC Cell Biology* **9**, 50, doi:10.1186/1471-2121-9-50 (2008).

Sehgal, P. et al. Epidermal growth factor receptor and integrins control force-dependent vinculin recruitment to E-cadherin junctions. *Journal of Cell Science* **131**, jcs206656, doi:10.1242/jcs.206656 (2018).

Osawa, M., Masuda, M., Kusano, K.-i. & Fujiwara, K. Evidence for a role of platelet endothelial cell adhesion molecule-1 in endothelial cell mechanosignal transduction: is it a mechanoresponsive molecule? *The Journal of Cell Biology* **158**, 773-785, doi:10.1083/jcb.200205049 (2002).

Chiu, Y.-J., McBeath, E. & Fujiwara, K. Mechanotransduction in an extracted cell model: Fyn drives stretch- and flow-elicited PECAM-1 phosphorylation. *The Journal of Cell Biology* **182**, 753-763, doi:10.1083/jcb.200801062 (2008).
Chrétién, M. L., Zhang, M., Jackson, M. R., Kapus, A. & Langille, B. L. Mechanotransduction by endothelial cells is locally generated, direction-dependent, and ligand-specific. *Journal of cellular biology* **224**, 352-361, doi:10.1002/jcp.22125 (2010).

Zuckerbraun, B. S., Shapiro, R. A., Billiar, T. R. & Tzeng, E. RhoA Influences the Nuclear Localization of Extracellular Signal-Regulated Kinases to Modulate p21Waf1/Cip1 Expression. *Circulation* **108**, 876-881, doi:10.1161/01.CIR.0000081947.00070.07 (2003).

Pritchard, C. A. et al. B-Raf Acts via the ROCKII/LIMK/Cofilin Pathway To Maintain Actin Stress Fibers in Fibroblasts. *Molecular and Cellular Biology* **24**, 5937-5952, doi:10.1128/mcb.24.13.5937-5952.2004 (2004).

Vetterkind, S., Poythress, R. H., Lin, Q. Q. & Morgan, K. G. Hierarchical scaffolding of an ERK1/2 activation pathway. *Cell Commun Signal* **11**, 65-65, doi:10.1186/1478-811X-11-65 (2013).

Vetterkind, S., Saphirstein, R. J. & Morgan, K. G. Stimulus-Specific Activation and Actin Dependency of Distinct, Spatially Separated ERK1/2 Fractions in A7r5 Smooth Muscle Cells. *PloS one* **7**, e30409, doi:10.1371/journal.pone.0030409 (2012).

Appel, S., Allen, P. G., Vetterkind, S., Jin, J.-P. & Morgan, K. G. h3/Acidic calponin: an actin-binding protein that controls extracellular signal-regulated kinase 1/2 activity in nonmuscle cells. *Molecular biology of the cell* **21**, 1409-1422, doi:10.1091/mbc.e09-06-0451 (2010).

Vomastek, T. et al. RACK1 targets the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway to link integrin engagement with focal adhesion disassembly and cell motility. *Molecular and cellular biology* **27**, 8296-8305, doi:10.1128/MCB.00598-07 (2007).

Neasta, J. et al. Activation of the cAMP Pathway Induces RACK1-Dependent Binding of beta-Actin to BDNF Promoter. *PloS one* **11**, e0160948, doi:10.1371/journal.pone.0160948 (2016).

Cox, E. A., Bennin, D., Doan, A. T., O'Toole, T. & Huttenlocher, A. RACK1 regulates integrin-mediated adhesion, protrusion, and chemotactic cell migration via its Src-binding site. *Molecular biology of the cell* **14**, 658-669, doi:10.1091/mbc.e02-03-0142 (2003).

Miluzio, A., Beugnet, A., Volta, V. & Biffo, S. Eukaryotic initiation factor 6 mediates a continuum between 60S ribosome biogenesis and translation. *EMBO reports* **10**, 459-465, doi:10.1038/jrpc.2010.301 (2009).

Kiely, P. A. et al. Phosphorylation of RACK1 on Tyrosine 52 by c-Abl Is Required for Insulin-like Growth Factor I-mediated Regulation of Focal Adhesion Kinase. *Journal of Biological Chemistry* **284**, 20263-20274, doi:10.1074/jbc.M109.017640 (2009).

Brina, D., Grosso, S., Miluzio, A. & Biffo, S. Translational control by 80S formation and 60S availability: the central role of elf6, a rate limiting factor in cell cycle progression and tumorigenesis. *Cell Cycle* **10**, 3441-3446, doi:10.4161/cc.10.20.17796 (2011).

Nilsson, J., Sengupta, J., Frank, J. & Nissen, P. Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. *EMBO reports* **5**, 1137-1141, doi:10.1038/sj.embor.7400291 (2004).

Adams, D. R., Ron, D. & Kiely, P. A. RACK1, A multifaceted scaffolding protein: Structure and function. *Cell Commun Signal* **9**, 22-22, doi:10.1186/1478-811X-9-22 (2011).

Guo, J. et al. Involvement of Arabidopsis RACK1 in Protein Translation and Its Regulation by Abscisic Acid. *Plant Physiology* **155**, 370-383, doi:10.1104/pp.110.160663 (2011).

Hirata, H. et al. Actomyosin bundles serve as a tension sensor and a platform for ERK activation. *EMBO reports* **16**, 250-257, doi:10.15252/embr.201439140 (2015).

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**, 671-675, doi:10.1038/nmeth.2089 (2012).