

Maintaining proteostasis under mechanical stress

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Abstract

Cell survival, tissue integrity and organismal health depend on the ability to maintain functional protein networks even under conditions that threaten protein integrity. Protection against such stress conditions involves the adaptation of folding and degradation machineries, which help to preserve the protein network by facilitating the refolding or disposal of damaged proteins. In multicellular organisms, cells are permanently exposed to stress resulting from mechanical forces. Yet, for long time mechanical stress was not recognized as a primary stressor that perturbs protein structure and threatens proteome integrity. The identification and characterization of protein folding and degradation systems, which handle force-unfolded proteins, marks a turning point in this regard. It has become apparent that mechanical stress protection operates during cell differentiation, adhesion and migration and is essential for maintaining tissues such as skeletal muscle, heart and kidney as well as the immune system. Here, we provide an overview of recent advances in our understanding of mechanical stress protection.

Keywords autophagy; chaperones; mechanobiology; proteostasis; signal transduction

Subject Categories Autophagy & Cell Death; Post-translational Modifications & Proteolysis; Translation & Protein Quality

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See the Glossary for abbreviations used in this article.

Introduction

In a living organism, the integrity of the cellular protein network is under constant threat. Increased temperature, the generation of reactive oxygen species or the accumulation of aberrant proteins lead to heat, oxidative and proteotoxic stress, which shifts the conformational equilibrium of proteins towards partially unfolded and misfolded states prone to aggregation (Richter *et al*, 2010; Dahl *et al*, 2015; Sala *et al*, 2017; Hipp *et al*, 2019). Protein aggregation can interfere with essential cellular processes causing severe pathology including neurodegeneration and dementia (Douglas & Dillin, 2010; Choe *et al*, 2016; Woerner *et al*, 2016; Hipp *et al*, 2019). To protect the proteome against stressful conditions, the cell employs protein folding and degradation systems. These highly regulated systems recognize partially unfolded and misfolded conformers and promote refolding or, in case of terminal damage, mediate disposal (Fig 1A) (Buchberger *et al*, 2010; Balchin *et al*, 2016; Cohen-Kaplan *et al*, 2016; Dikic, 2017; Klimek *et al*, 2017; Nillegoda *et al*, 2018). Key players in stress protection are molecular chaperones and their regulatory cochaperones. They sense protein unfolding based on the exposure of hydrophobic surfaces that are otherwise buried in the native structure (Kim *et al*, 2013b; Dahiya & Buchner, 2019; Rosenzweig *et al*, 2019). Chaperone binding prevents aggregation and directs the non-native client onto specific folding or degradation pathways. Protein degradation can be mediated by the ubiquitin–proteasome system (UPS) or selective autophagy (Cohen-Kaplan *et al*, 2016; Dikic, 2017; Höfeld & Hoppe, 2018). In both cases, degradation is usually initiated by the attachment of a ubiquitin-derived degradation signal onto the client, based on the activity of

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Glossary

ABD	actin-binding domain
AJ	adherens junction
CASA	chaperone-assisted selective autophagy
CIM	critical illness myopathy
CMA	chaperone-mediated autophagy
ECM	extracellular matrix
FA	focal adhesion
FAK	focal adhesion kinase
HSP	heat shock protein
ICU	intensive care unit

Ig	immunoglobulin
mTORC1	mechanistic target of rapamycin complex I
PDZ	PSD-95/Dlg1/ZO-1 domain
SD	slit diaphragm
sHSP	small heat shock protein
TPR	tetratricopeptide repeat
TSC	tuberous sclerosis complex
UCS	UNC-45/CRO1/She4p domain
UnDOx	unfolded domain oxidation
UPS	ubiquitin–proteasome system

client-specific ubiquitin conjugation systems (Fig 1A) (Khaminets *et al*, 2016; Gatica *et al*, 2018). Depending on the selected degradation pathway, the generated degradation signal is recognized either by ubiquitin receptors in the proteasome or by autophagic ubiquitin adaptors that facilitate an association of the ubiquitylated client with phagophore membranes leading to autophagosome formation and client degradation in autolysosomes. An extensive body of work has elucidated the adaptation of cellular folding and degradation machineries in response to heat, oxidative and proteotoxic stress (Richter *et al*, 2010; Vihervaara & Sistonen, 2014; Dahl *et al*, 2015; Sala *et al*, 2017; Nillegoda *et al*, 2018; Dahiya & Buchner, 2019; Hipp *et al*, 2019; Rosenzweig *et al*, 2019). In contrast, the impact of mechanical stress on these machineries became only recently apparent, when force-regulated proteostasis systems were identified and shown to be essential for cell and tissue homeostasis (Janiesch *et al*, 2007; Arndt *et al*, 2010; Gazda *et al*, 2013; Ulbricht *et al*, 2013, 2015; Kathage *et al*, 2017; Rinschen *et al*, 2017b; Donkervoort *et al*, 2020).

Mechanical stress is a highly relevant physiological stimulus

Cells in multicellular organisms are permanently exposed to mechanical forces, which are either passively applied or generated inside the cell (Discher *et al*, 2009; Irianto *et al*, 2016; Hu *et al*, 2017; Wolfenson *et al*, 2019) (Fig 1B). Indeed, the ability to generate and respond to mechanical forces is a basic requirement for the development, life and survival of organisms (Alon & Dustin, 2007; Johnson *et al*, 2007; Moore *et al*, 2010; DuFort *et al*, 2011; Hoffman *et al*, 2011; Mammoto *et al*, 2013; Yusko & Asbury, 2014). To stand and walk, to take a breath, or to pump blood through the body depends on force generation in skeletal and cardiac muscle. Cells in the skin, the cardio-vascular system and the kidney must withstand mechanical forces under touch and during blood circulation and filtration. Immune cells are subjected to mechanical forces when they attach to blood vessel walls upon exit from the vasculature and during migration to sites of infection and inflammation. Finally, the division, migration, functionality and differentiation of cells rely on their ability to sense the mechanical properties of their surrounding such as tissue elasticity and matrix stiffness (Engler *et al*, 2006; Gilbert *et al*, 2010; Hersch *et al*, 2013; Wolfenson *et al*, 2019). Impairment of these processes results in diverse diseases, including myopathies, heart and kidney failure, leukocyte adhesion deficiencies and cancer (Etzioni, 2007; DuFort *et al*, 2011; Hoffman *et al*,

2011; Yang *et al*, 2015; Ayad *et al*, 2019; Maurer & Lammerding, 2019).

The actin cytoskeleton is centrally involved in generating and responding to mechanical forces (Small & Resch, 2005; Johnson *et al*, 2007; Luo *et al*, 2013; Schiller & Fässler, 2013; Gautel & Djinić-Carugo, 2016; Schiffhauer *et al*, 2016). Comprised of globular actin molecules, which polymerize into a dynamic filament network, it provides shape and mechanical stability to cells, generates mechanical force and drives cell migration. Actin-anchoring sites are of particular importance for force generation and force transduction. At the plasma membrane, proteins of the integrin and cadherin families together with their interaction partners form cell adhesions that link the actin cytoskeleton to the extracellular matrix and neighbouring cells (Fig 1B and C) (Schiller & Fässler, 2013; Irianto *et al*, 2016; Schiffhauer *et al*, 2016). At the nucleocyto-skeletal interface, the actin cytoskeleton contacts the nuclear envelope to impact genome functions (Simon & Wilson, 2011; Isermann & Lammerding, 2013; Graham & Burridge, 2016; Cho *et al*, 2017; Nava *et al*, 2020). Furthermore, actin filaments are anchored to the constituents of the blood filtration barrier at the slit diaphragm in the kidney (Huber & Benzing, 2005; New *et al*, 2014), and in skeletal and cardiac muscle, actin anchoring is mediated by Z-discs, which limit the minimal contractile units of muscle, the sarcomeres (Fig 2) (Frank *et al*, 2006). Mechanical forces, externally applied through blood pressure and movement or internally generated through actomyosin contraction, accumulate at anchoring sites leading to a high stress concentration.

Force-induced protein unfolding

Many constituents of actin-anchoring sites undergo force-induced unfolding (Hu *et al*, 2017; Saini & Discher, 2019). One such protein is vinculin, which links the actin cytoskeleton to integrin complexes at cell–matrix adhesions (focal adhesions, FAs) and to cadherin complexes at cell–cell junctions (adherens junctions, AJs) (Atherton *et al*, 2016; Goldmann, 2016). Mechanical force disrupts auto-inhibitory interactions between the head and tail domains of vinculin and in this way stimulates binding to other anchoring components and actin filaments (Atherton *et al*, 2016). At FAs, vinculin interacts with talin, which is also a mechanosensitive protein (Fig 1C) (Austen *et al*, 2015; Ringer *et al*, 2017; Lemke *et al*, 2019; Fischer *et al*, 2021). Remarkably, mechanical forces do not just induce a domain rearrangement, but instead trigger the loss of tertiary and secondary structural elements within talin (del Rio *et al*,

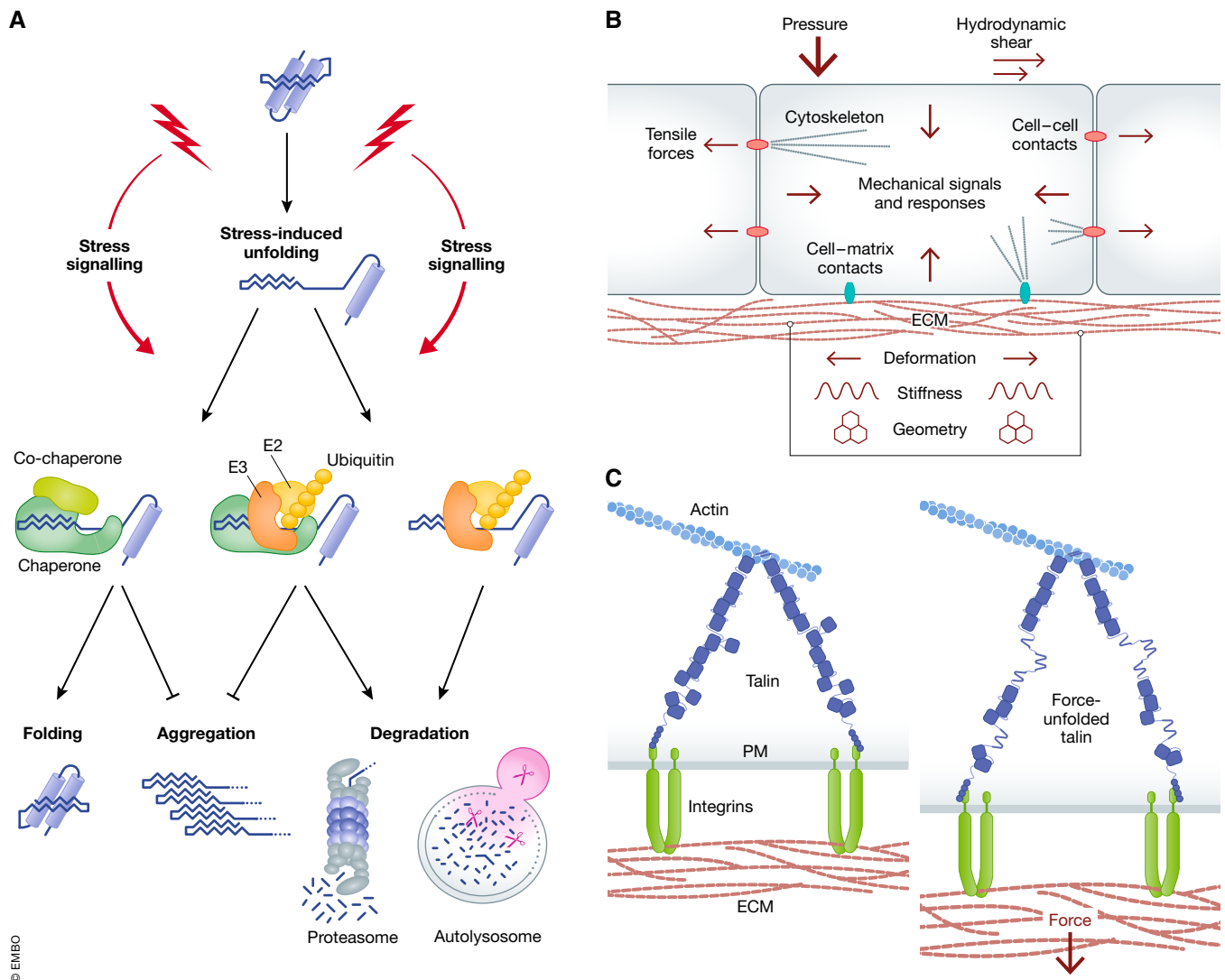


Figure 1. Protecting the proteome against mechanical stress relies on protein folding and degradation systems that recognize force-unfolded proteins.

(A) Stress protection systems comprise chaperone/cochaperone complexes and specialized E2/E3 ubiquitin conjugation systems, which recognize unfolded proteins under stress conditions to facilitate their refolding or their degradation by the proteasome and autophagic/lysosomal pathways. Ubiquitylation can be assisted by molecular chaperones or can proceed through a direct recognition of unfolded proteins by quality control E3 ubiquitin ligases. Stress-induced signalling pathways regulate the activity of the involved folding and degradation systems. (B) Cellular systems are permanently exposed to a wide variety of mechanical signals. After recognition and transmission through cell-cell and cell-matrix contacts as well as cytoskeletal systems, these signals induce a variety of specific cell responses. (C) Mechanical forces can trigger the unfolding of mechanosensory proteins such as talin, which links integrin-containing adhesion complexes in the plasma membrane (PM) to the actin cytoskeleton (ECM—extracellular matrix).

2009; Yao *et al*, 2014a, 2016). Talin possesses an amino-terminal integrin binding domain connected to a rod-like structure formed by 13 α -helical bundles, the last of which contacts actin. When talin is stretched in cells, tensile forces are exerted linearly along the molecule (Fig 1C) (Ringer *et al*, 2017). The mechanical properties of talin domains were analysed in biochemical experiments using magnetic tweezers, total internal reflection fluorescence and atomic force microscopy (del Rio *et al*, 2009; Yao *et al*, 2014a, 2016). These experiments demonstrated that 12 out of the 13 helical bundles undergo force-induced unfolding, leading to a disruption of helix-helix interactions and the complete unfolding of individual helices. In turn, buried vinculin binding sites become exposed. Talin

unfolding thus contributes to the transduction of mechanical signals. Importantly, the force required for the unfolding of different helical bundles varies between 5 and 20 pN (Yao *et al*, 2016). This suggests that the talin rod acts as a force buffer. Integrin-actin contacts are maintained by talin over a significant force range through the subsequent unfolding of single helical bundles (Fig 1C).

Another proposed mechanosensor and interactor of various FA proteins (i.e. vinculin and FAK) is the stretch-sensitive adaptor protein p130^{Cas} (Sawada *et al*, 2006). Following early FA maturation, p130^{Cas} is recruited to FAs, unfolds due to applied tension and exposes up to 15 formerly hidden phosphorylation sites (Sawada *et al*, 2006). Phosphorylated p130^{Cas} engages in different

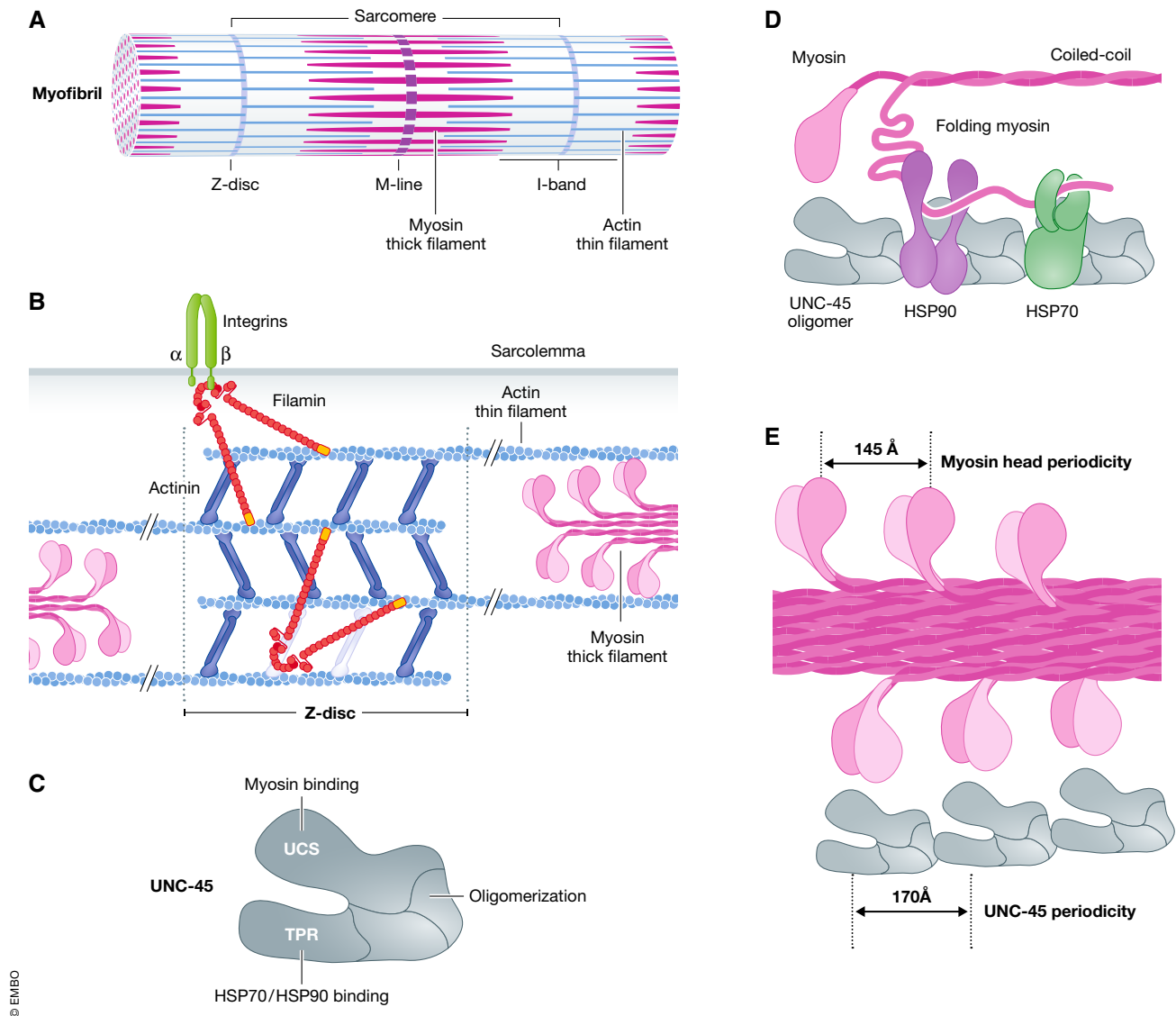


Figure 2. The UNC-45-containing chaperone system mediates the folding and assembly of myosin in muscle sarcomeres.

(A) The sarcomere represents the smallest contractile unit of striated muscles. It is repetitively arranged in tubular myofibrils, numerous bundles of which form the muscle fibre. Z-discs limit the sarcomere on both sides and mediate the anchoring of actin thin filaments. Myosin thick filaments are intercalated between the actin filaments and are connected at the M-line. The I-band is the region that contains exclusively actin filaments. (B) Actin and filamin crosslink actin filaments within the Z-disc. In addition, filamin interacts with integrin molecules in the sarcolemma. (C) Schematic representation of the domain structure of the cochaperone UNC-45. UCS, UNC-45/CRO1/She4p domain and TPR, tetratricopeptide repeat. (D) Oligomeric UNC-45 coordinates the activity of HSP70 and HSP90 chaperone proteins during the folding and assembly of myosin filaments. (E) The UNC-45 oligomer provides a molecular scaffold for enforcing the regular spacing of folded myosin head domains in the myosin thick filament.

mechanosensitive signalling cascades, in agreement with a role of the protein as a central hub for force transmission. Moreover, the interaction of p130^{Cas} with vinculin has been proposed to stabilize an open conformation of vinculin, thereby promoting talin binding and FA stability (Janošiak *et al*, 2014). Finally, p130^{Cas} has been shown to be important for actin reorientation in response to cyclic mechanical stretch (Niediek *et al*, 2012).

Importantly, mechanical forces are transduced not only at the cell–matrix interface but also at the cell–cell contacts through AJs. AJs contain α -catenin as a main mechanosensor (Huveneers & de

Rooij, 2013; Yao *et al*, 2014b; Ladoux *et al*, 2015). α -catenin physically couples the cadherin complex, the core component of AJs, to the actin cytoskeleton. Loss of α -catenin function disrupts epithelial integrity, the ability of cells to undergo coordinated cell movements and the ability to reorient actin fibres under cyclic strain (Noethel *et al*, 2018). α -catenin comprises three evolutionarily conserved regions: an N-terminal region that binds to β -catenin, a central M-region and a C-terminal actin-binding domain (ABD). Opening of the ABD under tension results in enforced actin binding and facilitates dimerization of the ABD to promote actin bundling (Buckley

et al, 2014; Ishiyama *et al*, 2018). In addition, also the M-region of α -catenin undergoes a conformational change when stretched to reveal a formerly hidden binding site for vinculin (Yonemura *et al*, 2010; Choi *et al*, 2012; Yao *et al*, 2014b). The recruitment of vinculin is thought to strengthen the AJ-actin link and seems to enhance the mechanosensitivity of α -catenin (Noethel *et al*, 2018).

A function as mechanotransducer and force buffer was also assigned to filamin, which binds actin along stress fibres, at focal adhesions and at the sarcomeric Z-disc (Fig 2B) (Frank *et al*, 2006; Ehrlicher *et al*, 2011; Nakamura *et al*, 2011, 2014). Filamin is a large homodimeric protein comprised of two rods with terminal actin and integrin binding sites, respectively, on opposing ends. This domain arrangement enables the protein to crosslink and anchor actin filaments. The filamin rod is formed by 24 immunoglobulin (Ig) domains, some of which engage in pairwise interactions (Nakamura *et al*, 2011). Single-molecule mechanical measurements demonstrated that the Ig domain pair 20–21 of filamin functions as an auto-inhibited force-activatable mechanosensor (Lad *et al*, 2007; Rognoni *et al*, 2012). In the inhibited state, the amino-terminal β -strand of domain 20 folds onto domain 21. Mechanical force disrupts this interaction, leading to rod extension and the exposure of binding sites in domain 21 for integrins and other partner proteins. Molecular dynamics simulation suggests that also other Ig domains of filamin are sensitive to mechanical force (Kesner *et al*, 2010). Thereby, unfolding of the amino-terminal β -strand seems to represent a common initial step, resulting in metastable states similar to those observed upon thermal unfolding (Kesner *et al*, 2010).

The gigantic myofilament protein titin (Mr: ~3,800 kDa) spans the muscle sarcomere from the Z-disc to the M-band and confers elasticity and passive force under stretch (Linke & Hamdani, 2014). Titin regions localized in the I-band adjacent to the Z-disc function as molecular springs that extend and condense as the muscle stretches and contracts. At low forces, regions containing tandemly arranged Ig domains elongate due to the straightening of interdomain linkers, whereas at higher forces, intrinsically disordered structures are extended (Linke & Hamdani, 2014; Mártonfalvi *et al*, 2014).

As already mentioned above, talin unfolding is observed at forces between 5 and 20 pN (Yao *et al*, 2016). Unfolding of the Ig domain 20–21 pair of filamin is triggered at forces between 2 and 4 pN (Rognoni *et al*, 2012), and conformational transitions of titin occur upon stretching with forces as low as 5 pN (Mártonfalvi *et al*, 2014). To put this into perspective, one should note that the contraction of a single actin–myosin unit generates a force of 3–4 pN (Finer *et al*, 1994). Thus, cytoskeleton components constantly undergo unfolding reactions under physiological conditions. Indeed, studies with cultured cells illustrate the prevalence and significance of force-induced protein unfolding in the cellular context. In one approach, the accessibility of cysteine residues was monitored (Johnson *et al*, 2007). Cysteines are often buried within tertiary and/or quaternary structure, and their force-induced exposure is therefore a readout for a significant loss of structural elements. Red blood cells were subjected to fluid shear stress to determine the force-induced unfolded proteome, and also adherent mesenchymal stem cells were analysed, in which traction forces are generated based on acto-myosin contraction (Johnson *et al*, 2007). In both cell types, mechanical stress caused unfolding of the actin-associated scaffolding protein spectrin, confirming previous biochemical studies on

the mechanosensitive properties of the cytoskeleton protein (Rief *et al*, 1999; Altmann *et al*, 2002; Johnson *et al*, 2007). Moreover, in adherent mesenchymal stem cells traction forces resulted in the unfolding of non-muscle myosin and filamin (Johnson *et al*, 2007). The latter was also at the focus of another study, in which a fluorescence resonance energy transfer (FRET)-based sensor was constructed to visualize the disruption of the Ig domain 20–21 pair of filamin in adherent mammalian cells (Nakamura *et al*, 2014). Maximal opening of filamin occurred predominantly at the cell edge and in protruding areas following pharmacological stimulation of cell spreading and migration.

Taken together, the described findings demonstrate that cells of a multicellular organism are challenged continuously by mechanical protein unfolding. Even in the absence of externally applied force, the tension generated inside adherent and migrating cells is sufficient to alter the conformation of abundant cytoskeleton proteins, leading to a significant loss of structural elements. This sets the stage for a critical involvement of protein folding and degradation machineries, which recognize force-unfolded proteins during mechanical stress protection.

Proteostasis machineries involved in mechanical stress protection

Molecular chaperones are defined by the ability to bind and stabilize unfolded proteins (see Fig 1A) (Hartl *et al*, 2011; Dahiya & Buchner, 2019; Rosenzweig *et al*, 2019). This enables them to assist in the folding, sorting and degradation of proteins, often in cooperation with regulating cochaperones. Small heat shock proteins (sHSPs), members of the HSP70 and HSP90 chaperone families as well as diverse cochaperones of HSP70 and HSP90, have been linked to mechanical stress protection (Arndt *et al*, 2010; Sarparanta *et al*, 2012; Gazda *et al*, 2013; Ulbricht *et al*, 2013; Adriaenssens *et al*, 2017; Jacko *et al*, 2020).

The HSP90 chaperone system is essential for muscle assembly in worms, fish, mice and men (Janiesch *et al*, 2007; Du *et al*, 2008; Gaiser *et al*, 2011; Donlin *et al*, 2012; Gazda *et al*, 2013; Echeverría *et al*, 2016). In zebrafish, for example, HSP90 α is abundantly expressed in skeletal muscle and its knockdown results in paralysed animals with massive perturbation of the myofibrillar organization of skeletal muscle (Etard *et al*, 2007; Du *et al*, 2008). The chaperone apparently exerts essential functions in the assembly of muscular structures during development. Yet, with respect to a possible involvement in mechanical stress protection, it is necessary to investigate the maintenance of muscle following, for example, a depletion of HSP90 isoforms in adult organisms. In fact, such an approach was performed in the nematode *C. elegans*. Adult worms were treated with silencing RNAs directed against the HSP90 homolog DAF-21, which shows strong expression in body wall muscle cells (Gaiser *et al*, 2011). Depletion severely reduced motility and caused a profound disruption of muscle structure. The findings demonstrate that HSP90 is required for both the initial assembly of skeletal muscles and their maintenance when force is generated. Furthermore, maintaining muscle structure and activity relies on the cooperation of HSP90 with several of its cochaperones, including the HSP70/HSP90 organizing protein HOP/STI1, AHA1 and p23, which regulate the ATP-regulated client binding

cycle of HSP90, as well as UNC-45 (Barral *et al*, 1998; Frumkin *et al*, 2014; Donkervoort *et al*, 2020).

What are clients of the HSP90 chaperone system under mechanical stress? Linke and coworkers showed that HSP90 binds to the N2-A region of cardiac and skeletal titin isoforms (Donlin *et al*, 2012; Voelkel *et al*, 2013). This region of titin is intrinsically unstructured and its extension under force contributes to the elasticity of the myofilament protein (Linke & Hamdani, 2014). Abrogating HSP90 recruitment to this region impaired skeletal and cardiac muscle activity in zebrafish and led to severe disorganization of the sarcomeric I-band, where the N2-A region is located (Donlin *et al*, 2012; Voelkel *et al*, 2013). Thus, HSP90 exerts an essential chaperone function on mechanosensitive domains of titin.

Myosin is another client of HSP90. The chaperone cooperates with HSP70 and its cochaperone UNC-45 to facilitate myosin folding and to organize sarcomere architecture (Etard *et al*, 2007; Wohlgemuth *et al*, 2007; Gazda *et al*, 2013). The sarcomeric repeat is a supra-molecular structure, in which thin (actin) and thick (myosin) filaments and their associated proteins are arranged in a precise order that is required to link the formation of actin–myosin cross-bridges with filament gliding and muscle contraction (Fig 2A and B) (Geeves & Holmes, 2005; Henderson *et al*, 2017). The *C. elegans* UNC-45 protein is the founding member of the conserved UCS (UNC-45/CRO1/She4p) domain family and orchestrates the activity and assembly of various myosin subtypes (Fig 2C) (Price *et al*, 2002; Etard *et al*, 2007; Wohlgemuth *et al*, 2007; Kim *et al*, 2008; Gazda *et al*, 2013). Mutations in UNC-45 were initially shown to induce myosin disorganization and severe motility defects (Venolia & Waterston, 1990; Barral *et al*, 1998, 2002; Price *et al*, 2002). In *Drosophila*, zebrafish and *Xenopus*, UNC-45 homologs are essential for muscle development and maintenance under stress (Etard *et al*, 2007, 2008; Wohlgemuth *et al*, 2007; Melkani *et al*, 2011). In zebrafish, for example, an UNC-45 homolog plays a role in the formation of myocyte attachment complexes that transmit forces from the contractile apparatus to the extracellular matrix, ensuring mechanical stability of muscles during contraction (Myhre *et al*, 2014). In mammals, two UNC-45 homologs exist, indicating an evolutionary conserved requirement for myosin-specific cochaperones (Price *et al*, 2002). UNC-45A represents a broadly expressed isoform, which is necessary for actomyosin bundling and plays a key role in cell adhesion and migration, promoting the proliferation and invasiveness of cancer cells (Bazzaro *et al*, 2007; Guo *et al*, 2011; Lee & Kumar, 2016). On the other hand, UNC-45B is predominantly expressed in striated muscles. Indeed, abnormal UNC-45B function is associated with severe muscle defects resulting in skeletal and cardiac myopathies (Janiesch *et al*, 2007; Donkervoort *et al*, 2020).

UNC-45 is able to interact with HSP70 and HSP90 simultaneously and to form multimeric linear assemblies (Fig 2D) (Gazda *et al*, 2013). It thus provides a multisite-docking platform to assemble myosin filaments in a precisely defined pattern into thick myofilaments and eventually myofibrils (Fig 2E). Importantly, this highly conserved chaperone system is required not only for sarcomere formation during development but also for its maintenance in the contracting muscle (Kim *et al*, 2008; Gaiser *et al*, 2011; Pokrzywa & Hoppe, 2013; Donkervoort *et al*, 2020). In response to stress or damage to the myofibre, HSP90 and UNC-45 dissociate from the Z-disc and transiently associate with myosin (Etard *et al*, 2008),

indicating their essential involvement in assembly processes under mechanical stress.

In single-molecule experiments using atomic force microscopy, UNC-45 was shown to bind to the myosin motor domain upon force-induced unfolding and to prevent aberrant protein interactions of the unfolded domain (Kaiser *et al*, 2012). Thus, stabilization of force-unfolded myosin appears to contribute to the observed essential activity of UNC-45 in contracting muscle.

Coping with mechanical stress does not only depend on the induction of chaperone-based folding and assembly machineries, but damaged proteins also need to be disposed to maintain the integrity of the proteome. Indeed, selective ubiquitin-dependent autophagy emerged in recent years as a degradation pathway of major importance in mechanical stress protection. First evidence was provided when a mechanically induced interaction of titin with the autophagic ubiquitin adaptor NBR1 was described (Lange *et al*, 2005). The adaptor protein participates in the selective autophagic removal of pathologic protein aggregates and peroxisomes in non-muscle cells (Kirkin *et al*, 2009; Odagiri *et al*, 2012; Deosaran *et al*, 2013). Accordingly, the force-induced recruitment of NBR1 to titin might localize the adaptor at the cytoskeleton for the autophagic disposal of mechanically damaged proteins. Many studies illustrate the importance of autophagy for muscle maintenance (Sandri, 2010). Deletion of key autophagy factors in transgenic animal models leads to a progressive deterioration of muscle structure and activity under mechanical stress, which is accompanied by the accumulation of protein aggregates (Tanaka *et al*, 2000; Raben *et al*, 2008; Masiero *et al*, 2009; He *et al*, 2012; Kim *et al*, 2013a). However, observed pathologies were usually attributed to impaired glucose and energy metabolism in the absence of autophagy (Sandri, 2010; He *et al*, 2012; Kim *et al*, 2013a). An essential involvement of autophagy in the removal of mechanically unfolded and damaged cytoskeleton proteins became apparent with the functional characterization of the cochaperone BAG3 (Arndt *et al*, 2010).

BAG3 can interact simultaneously with members of the HSP70 chaperone family and with small heat shock proteins, such as HSPB8 (also known as HSP22) (Carra *et al*, 2008; Gamerdinger *et al*, 2009, 2011; Arndt *et al*, 2010; Crippa *et al*, 2016; Ganassi *et al*, 2016; Judge *et al*, 2017; Rauch *et al*, 2017) (Fig 3A). Small heat shock proteins form oligomeric chaperone complexes that maintain non-native proteins in a state competent for processing by other proteostasis factors (Mainz *et al*, 2015; Ungelenk *et al*, 2016; Dahiya & Buchner, 2019). BAG3-mediated chaperone coupling allows to coordinate the holding function of small heat shock proteins with the chaperone function of HSP70 proteins (Rauch *et al*, 2017). In addition, the BAG3 chaperone complex can contain the HSP70-associated ubiquitin ligase CHIP, which mediates the ubiquitylation of chaperone-bound client proteins to initiate their disposal (Fig 3A) (Arndt *et al*, 2010). When ubiquitylation occurs in association with the BAG3 chaperone complex, CHIP generates a signal for autophagic degradation. It triggers the recruitment of the autophagic ubiquitin adaptor SQSTM1/p62 to stimulate the engulfment of the BAG3 chaperone complex by autophagosome precursor membranes, so-called phagophores, leading to client degradation in autolysosomes (Fig 3B) (Gamerdinger *et al*, 2009; Arndt *et al*, 2010). In muscle cells, autophagosome formation is further facilitated through cooperation of BAG3 with the actin-associated protein SYNPO2, which links the BAG3 chaperone complex to a

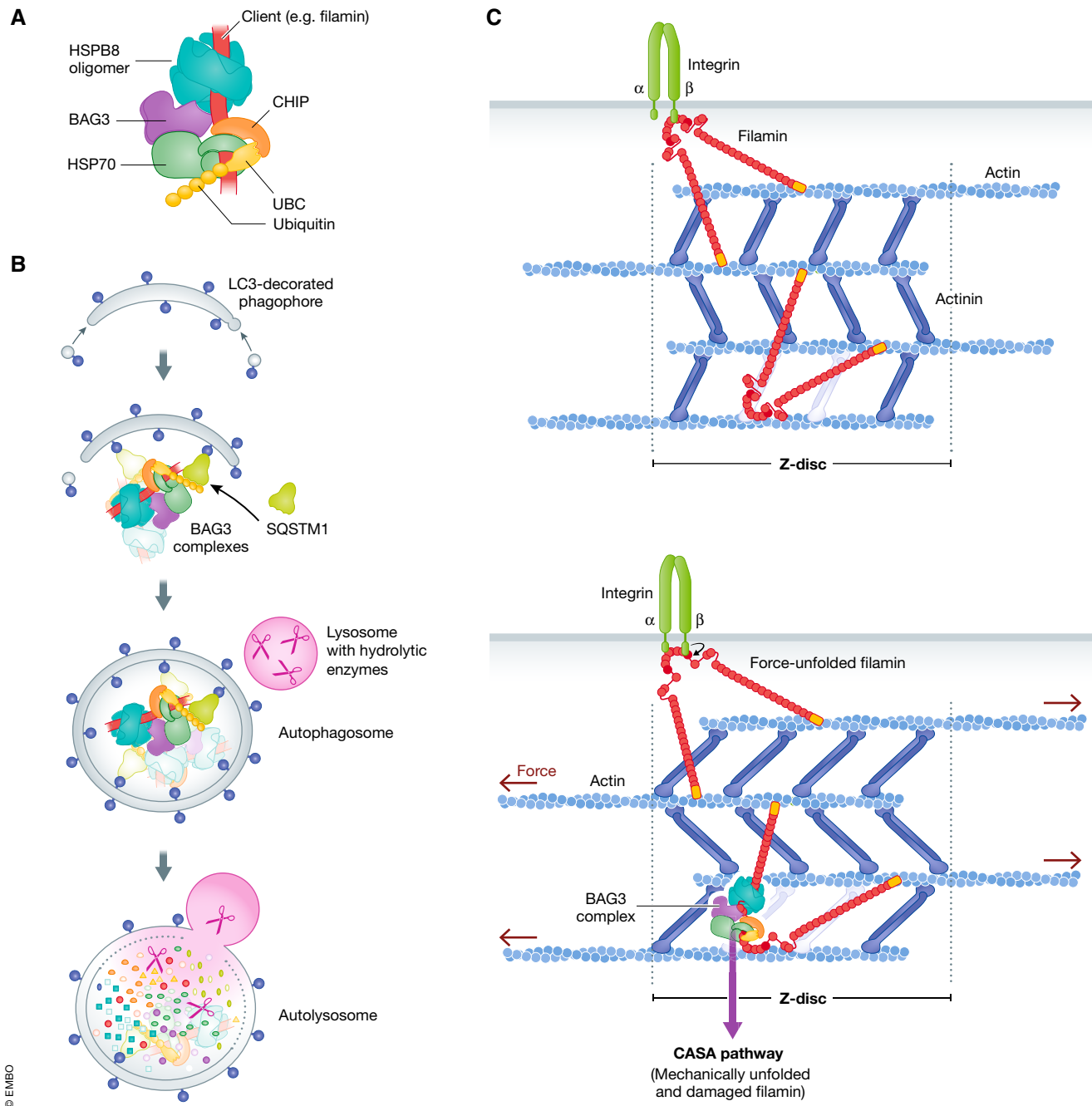


Figure 3. The BAG3 chaperone complex initiates a force-induced autophagy pathway in contracting muscle.

(A) BAG3 links chaperones of the HSP70 family to small heat shock proteins such as HSPB8. The HSP70-associated ubiquitin ligase CHIP can associate with the BAG3 chaperone complex to ubiquitinate a bound client protein in cooperation with a ubiquitin conjugation enzyme (UBC). (B) Enclosure of the BAG3 chaperone complex by phagophore membranes is facilitated by the autophagic ubiquitin adaptor SQSTM1, leading to autophagosome formation and ultimately to degradation in autolysosomes. (C) Actin–myosin contraction causes the force-induced unfolding of the actin-crosslinking protein filamin at the Z-disc of the muscle sarcomere. Mechanically unfolded and damaged forms of filamin are recognized by the BAG3 complex and directed onto the CASA pathway for disposal.

phagophore membrane fusion machinery (Ulbricht *et al*, 2013). The BAG3-mediated autophagy pathway was termed chaperone-assisted selective autophagy (CASA) to distinguish it from chaperone-mediated autophagy (CMA) (Arndt *et al*, 2010; Kaushik & Cuervo, 2012). In contrast to CASA, CMA does not involve autophagosome

formation but instead depends on a chaperone-mediated direct transfer of client proteins across the lysosomal membrane for degradation (Kaushik & Cuervo, 2012).

BAG3 was initially shown to participate in the autophagic degradation of aggregation-prone, pathologic proteins in neuronal cells

and later on also found to be involved in the turnover of the microtubule organizing protein tau in neuronal processes, thus providing an essential proteostasis function in the nervous system (Carra *et al*, 2008; Gamerdinger *et al*, 2009, 2011; Crippa *et al*, 2010; Lei *et al*, 2015). However, BAG3-deficient animals and patients carrying BAG3 mutant alleles display a profound pathology in skeletal and cardiac muscle, characterized by a progressive deterioration of sarcomeric structures under mechanical stress (Homma *et al*, 2006; Selcen *et al*, 2009; Arndt *et al*, 2010; Arimura *et al*, 2011; Claeys *et al*, 2013; Ruparelina *et al*, 2014; Konersman *et al*, 2015; Quintana *et al*, 2016; Fang *et al*, 2017; Meister-Broekema *et al*, 2018; Schänzer *et al*, 2018; Kimura *et al*, 2021). Moreover, a decline in BAG3-mediated sarcomere turnover contributes to the development of heart failure, the leading cause of mortality in the industrialized world (Martin *et al*, 2021). Ultimately, the mechanosensitive actin-crosslinking protein filamin was identified as a target of CASA (Arndt *et al*, 2010; Ulbricht *et al*, 2013). In an unbiased biochemical assay using isolated cytoskeleton from differentiated murine muscle cells and purified CASA components, BAG3 stimulated the release of filamin from the cytoskeleton, which can be considered as a requirement for subsequent sorting to the lysosome and autophagic degradation (Arndt *et al*, 2010). BAG3 and its chaperone partners HSP70 and HSPB8 bind to the mechanosensitive region of filamin comprising Ig domains 19–21 (see above) and direct mechanically unfolded and damaged forms onto the CASA pathway for disposal (Fig 3C) (Arndt *et al*, 2010; Klimek *et al*, 2017). Indeed, filamin forms aggregates in skeletal and cardiac muscle when the CASA pathway is compromised under pathological conditions (Arimura *et al*, 2011; Ruparelina *et al*, 2016; Fang *et al*, 2017; Kimura *et al*, 2021; Martin *et al*, 2021), illustrating the relevance of CASA for myofilament homeostasis in contracting muscle.

Recognition of mechanically unfolded filamin by the CASA machinery seems to be facilitated by LDB3 (Pathak *et al*, 2021). Like SYNPO2, LDB3 is a cytoskeleton-associated PDZ domain protein and acts as a multivalent interaction hub. It directly binds to the mechanosensitive region of filamin and also contacts HSP70. Similar to findings for BAG3, mutations in LDB3 cause skeletal and cardiac myopathy in animal models and patients (Cassandrini *et al*, 2021; Pathak *et al*, 2021). Pathology is accompanied by the formation of protein aggregates containing filamin, HSP70, HSPB8, BAG3 and ubiquitin, suggesting an impairment of CASA-mediated quality control upon expression of non-functional LDB3 (Pathak *et al*, 2021).

The HSP70 cochaperone DNAJB6 has been detected in association with BAG3, HSPB8 and CHIP in rat muscle (Sarparanta *et al*, 2012). DNAJB6 is a critical sensor of cellular stress, which contributes to the regulation of stress responses (Klaips *et al*, 2020). Mutations in DNAJB6 cause a form of limb-girdle muscular dystrophy, characterized by a progressive weakness and wasting of arm and leg muscles, consistent with a maintenance and stress protection function of DNAJB6 (Ruggieri *et al*, 2016). Strikingly, disease-associated mutations of DNAJB6 were shown to abrogate the ability of the cochaperone to bind to and stabilize unfolded proteins (Sarparanta *et al*, 2012). Because DNAJ-type cochaperones help to load clients onto HSP70 (Faust *et al*, 2020), it is conceivable that DNAJB6 may assist in the initial recognition of at least a subset of client proteins by the BAG3 machinery. However, physiological relevant clients of DNAJB6 in the context of mechanical stress protection remain to be identified.

An expansion of the CASA client repertoire may also be achieved through cooperation with different small heat shock proteins. In addition to HSPB8, BAG3 can bind HSPB1 (HSP27), HSPB5 (α B-crystallin) and HSPB6 (HSP20) (Rauch *et al*, 2017). Mutations in HSPB1 and HSPB5 have been linked to different forms of muscle weakness (Adriaenssens *et al*, 2017), and phosphorylation of HSPB5 at serine-59, which increases the chaperone activity of HSPB5 (Peschek *et al*, 2013), is induced in human leg muscle following resistance exercise (Jacko *et al*, 2020). Moreover, HSPB1 and HSPB5 were shown to engage in a force-induced association with mechanosensitive elements of sarcomeric titin, which prevents titin stiffening and aggregation in stretched sarcomeres (Kötter *et al*, 2014). Binding of BAG3 to HSPB1 and HSPB5 may thus provide access to specific client proteins during mechanical stress protection. Of note, HSPB1 interacts with SQSTM1/p62 and regulates autophagosome formation (Haidar *et al*, 2019), pointing to an active role of HSPB1 in the disposal of unfolded client proteins. Small heat shock proteins apparently emerge as key players in mechanical stress protection.

In a recent study, Martin *et al* (2021) monitored the BAG3-mediated release of proteins from the cytoskeleton of cardiomyocytes by mass spectrometry and identified several sarcomere proteins as potential new clients of the CASA pathway, including the mechanosensitive protein spectrin. Quality control functions of BAG3 under mechanical stress thus seem to extend beyond filamin homeostasis.

Protein triage under mechanical stress

What determines the fate of a force-unfolded mechanosensory protein? How is a decision reached between spontaneous or chaperone-assisted refolding and chaperone-assisted degradation? To answer these questions, it appears helpful to look at current concepts regarding protein triage in the proteostasis field. A prevalent view invokes a kinetic partitioning of proteins between free and chaperone-bound states to facilitate de novo folding and to ensure conformational flexibility required for function (Kim *et al*, 2013b). According to this concept, chaperones would bind to force-unfolded domains of mechanosensors engaged in the transduction of mechanical signals, when the rate of domain refolding is slower than the rate of chaperone binding. Transient interaction with chaperones would prevent aggregation and reduce the concentration of free folding intermediates at force-bearing and force-generating sites. Slow refolding kinetics may result in repeated recognition of unfolded mechanosensors by chaperone complexes and may ultimately lead to the recruitment of degradation factors. Dynamic low affinity interactions between mechanosensors, chaperones, degradation-inducing cochaperones, chaperone-associated ubiquitin ligases and ubiquitin receptors would shift the balance towards degradation. In such a scenario, a clear distinction between “productive” force-induced unfolding during mechanotransduction and “unproductive” mechanical damage would be difficult to draw. Instead, folding and refolding kinetics of mechanosensitive domains would determine functionality, chaperone recognition and initiation of degradation.

In case of the giant muscle protein titin, reversible oxidation was recently shown to reduce the protein’s elasticity through preferential

modification of mechanosensitive Ig domains in the I-band region of the muscle sarcomere (Loescher *et al*, 2020). This process, termed unfolded domain oxidation (UNDOx), increases cardiac stiffness under mechanical force and oxidative stress. By increasing the aggregation propensity of the unfolded and oxidized Ig domains, homotypic interactions between titin molecules are promoted to control myocardial stiffness. Of note, HSP90 and the autophagic ubiquitin adaptor NBR1 bind to the I-band region of titin during quality control (Lange *et al*, 2005; Donlin *et al*, 2012), and the small heat shock proteins HSPB1 and HSPB5 recognize unfolded Ig domains of titin to suppress titin stiffening (Kötter *et al*, 2014). UNDOx may thus facilitate recognition by proteostasis factors and may influence the kinetic partitioning of titin.

Reversible prolyl isomerization regulates the refolding kinetics of the mechanosensitive Ig 20–21 domain pair in the BAG3 client filamin (Rognoni *et al*, 2014). While disruption of the 20–21 interdomain interaction occurs at forces around 4 pN, higher forces lead to a cis-trans isomerization of a proline switch in domain 20 causing domain unfolding. The trans state persists up to minutes even when force is reduced, leaving the domain unstable for a prolonged time.

The studies by Loescher *et al* and Rognoni *et al* reveal molecular events that alter the folding and refolding kinetics of mechanosensitive domains, which in turn affect protein stability and chaperone recognition. Oxidation and force-induced isomerization could thus be decisive steps during protein triage. Yet, both studies emphasize the relevance of the observed processes for the regulation of the mechanical properties of the cytoskeleton. Transient population of unstable, aggregation-prone states of mechanosensors under surveillance of chaperones and other proteostasis factors appears to be an integral part of the adaptation of cells and tissues to mechanical forces.

Balancing transcription, translation and autophagy under mechanical stress

The BAG3 chaperone system mediates the degradation of filamin under mechanical stress. Yet, BAG3 also contributes to maintaining filamin homeostasis by stimulating the synthesis of new filamin molecules through interactions with components of the Hippo and mTOR signalling pathways, respectively (Ulbricht *et al*, 2013; Kathage *et al*, 2017). The Hippo pathway (named after the kinase Hippo involved in this pathway in *Drosophila*) is a mechanoresponsive signalling pathway that controls the expression of extracellular matrix components and cytoskeleton proteins including filamin (Dupont *et al*, 2011; Yu & Guan, 2013; Wackerhage *et al*, 2014). Hippo signalling proteins that are contacted by BAG3 include LATS1, LATS2, AMOT and AMOTL1 (Ulbricht *et al*, 2013; Taipale *et al*, 2014). In their activated state, these proteins interact with the transcriptional coactivators YAP and TAZ, which results in coactivator retention in the cytoplasm to attenuate target gene expression in the absence of a mechanical stimulus (Wackerhage *et al*, 2014; Yusko & Asbury, 2014). BAG3 interacts with the Hippo signalling proteins in a manner competitive with YAP/TAZ binding, causing YAP/TAZ release and translocation into the nucleus for the activation of target gene expression, including filamin (Ulbricht *et al*, 2013). BAG3-activated transcription of filamin allows to compensate the disposal of mechanically damaged filamin molecules and to maintain the cytoskeleton under mechanical stress.

Protein synthesis is stimulated by BAG3 through spatial regulation of the mTOR kinase complex I (mTORC1) (Kathage *et al*, 2017). mTORC1 represents a master regulator of anabolic and catabolic processes in eukaryotic cells, whose activity is controlled by the inhibitory tuberous sclerosis complex (TSC) (Laplanche & Sabatini, 2012; Betz & Hall, 2013; Menon *et al*, 2014). In adherent smooth muscle cells, BAG3 recruits the TSC complex to actin stress fibres by binding to the complex subunit TSC1 (Kathage *et al*, 2017). TSC recruitment results in an inhibition of mTORC1 along stress fibres, which is essential for the initiation of CASA at sites of mechanical protein unfolding and damage. At the same time, TSC recruitment and sequestration by BAG3 attenuates mTORC1 inhibition throughout the remaining cytoplasm, causing an upregulation of protein translation (Kathage *et al*, 2017). The BAG3-mediated spatial regulation of mTORC1 apparently allows for a simultaneous induction of autophagy and protein synthesis in response to mechanical stress.

Mechanical stress signalling

The expression of many proteostasis factors is elevated when non-native proteins accumulate to increase the folding and degradation capacity of the cell. A key component in this regulatory circuitry is the heat shock factor 1 (HSF1) (Vihervaara & Sistonen, 2014; Li *et al*, 2017; Naidu & Dinkova-Kostova, 2017; Barna *et al*, 2018; Klaips *et al*, 2020). Identified for the first time in heat shocked fruit flies (which explains the name), HSF1 has emerged as a general stress protective transcription factor that upregulates the expression of folding and degradation proteins in response to diverse stress stimuli (Vihervaara & Sistonen, 2014; Barna *et al*, 2018). In the repressed state, monomeric HSF1 is complexed with chaperone proteins, i.e. HSP70 and HSP90. When non-native proteins accumulate upon cell stress, chaperones dissociate from HSF1 to stabilize non-native clients. In turn, HSF1 trimerizes and activates target gene expression (Li *et al*, 2017). Importantly, also mechanical stress triggers HSF1 activation (Li *et al*, 2013; Ulbricht *et al*, 2013). This leads to an elevated expression of many proteins that were discussed above to contribute to mechanical stress protection, including core components of the CASA machinery such as HSP70, BAG3, SQSTM1 and ubiquitin (Franceschelli *et al*, 2008; Barna *et al*, 2018). Accordingly, an HSF1- and BAG3-dependent mechanotransduction pathway was proposed for the conversion of mechanical forces into biochemical and cell biological responses (Ulbricht *et al*, 2013). On this pathway, force-unfolded mechanosensory proteins are recognized by chaperone proteins, causing HSF1 derepression and activation. The resultant increase of the cellular concentration of BAG3 and its partner proteins facilitates degradation of mechanically damaged client proteins through CASA. At the same time, increased engagement of BAG3 with Hippo signalling proteins activates YAP/TAZ-dependent gene expression and stimulates protein synthesis through TSC complex sequestration and mTORC1 activation (Ulbricht *et al*, 2013). Balancing of transcription, translation and degradation is required to maintain the cytoskeleton under mechanical stress.

Recent work also reveals an unexpected link between HSF2 and mechanotransduction (Joutsen *et al*, 2020). Similar to HSF1, HSF2 is activated upon proteostasis imbalance, following proteasome inhibition for example. In this situation, HSF2-dependent gene expression

is required to maintain cadherin-mediated cell–cell adhesions and to ensure cell survival (Joutsen *et al*, 2020). The findings suggest that HSF2 could contribute to the transcriptional control of mechanical stress protection.

In addition to regulated gene expression, posttranslational modifications, in particular phosphorylation and dephosphorylation events, contribute to the induction and adjustment of mechanical stress protection systems. Mechanical forces activate diverse signalling pathways governed by multiple kinases and phosphatases to tightly control contractile functions and to maintain the cytoskeleton. For example, mechanical stress imposed by acute exercise was shown to activate the extracellular signal-regulated kinases ERK1 and ERK2, Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38, MAPK) (Kumar *et al*, 2002; Coffey *et al*, 2006; Hoffman *et al*, 2015; Nelson *et al*, 2019). Hypertrophy-stimulating resistance training activates the protein kinases Akt and mTOR (Léger *et al*, 2006), and the Akt pathway is involved in the adaptation of skeletal muscle fibres to changes in workload (Bodine *et al*, 2001), resulting in an increase in fibre size (Rommel *et al*, 2001). Further kinases implicated in controlling protective and adaptive processes to mechanical stress in muscle cells include protein kinase C (PKC), calmodulin-dependent protein kinase II (CaMKII), p21-activated kinase 1 (PAK1) and AMP-activated protein kinase (AMPK) (Egan & Zierath, 2013; Hoffman *et al*, 2015; Nelson *et al*, 2019). However, the lack of detailed knowledge about kinase–substrate and phosphatase–substrate relationships remains a major bottleneck in decoding signalling networks under mechanical stress. Only a few global phosphoproteomic studies of skeletal muscle exist to date. Phosphosites identified in proteins from non-exercised skeletal muscle and/or differentiated skeletal muscle cells without fully assembled sarcomeres were used to predict the involved kinases (Højlund *et al*, 2009; Hou *et al*, 2010; Drexler *et al*, 2012; Kettenbach *et al*, 2015). However, activities of many kinases drastically change in response to acute exercise as demonstrated by quantitative phosphoproteomics of human skeletal muscle (Hoffman *et al*, 2015; Nelson *et al*, 2019). Recently, a mouse myotube model was established to study force-regulated signalling processes (Reimann *et al*, 2017). Murine C2 or C2C12 skeletal muscle cells were differentiated to mature myotubes and their contraction was triggered by electrical pulse stimulation (EPS), followed by quantitative phosphoproteomics with a focus on Z-disc and Z-disc-associated proteins. Z-discs have been proposed as nodal points of myocyte signalling (Pyle & Solaro, 2004; Frank *et al*, 2006). Since Z-discs limit the force-generating sarcomeres, help to connect myofibrils to each other and further link the contractile apparatus to the sarcolemma and the extracellular matrix, they are in an ideal position to sense mechanical strain and orchestrate signalling processes (see Fig 2). Most importantly, the establishment of the Z-disc protein phosphorylation landscape identified clustered multisite phosphorylation in the CASA client filamin and core CASA components including BAG3, HSPB8 and SYNPO2 (Reimann *et al*, 2017). Moreover, force-induced and phosphorylation-regulated interactions of filamin with HSPB1 and the degradation factor FILIP1, respectively, were recently described (Collier *et al*, 2019; Reimann *et al*, 2020). The findings indicate that interactions, localization, dynamics and/or functions of mechanosensitive proteins and force-regulated proteostasis factors are modulated by reversible phosphorylation under mechanical stress.

In *Drosophila*, the serine/threonine kinase NUAK interacts with Starvin, the fly orthologue of BAG3 (Brooks *et al*, 2020). Starvin and NUAK mutant flies exhibit muscle degeneration and muscle contraction defects, and similar phenotypes are also observed following a depletion of *Drosophila* HSC70 and the autophagic membrane marker ATG8 (Arndt *et al*, 2010; Brooks *et al*, 2020). Moreover, muscle degeneration is accompanied by the accumulation of protein aggregates that contain filamin. NUAK thus seems to exert a central regulatory function in controlling CASA activity in fly muscles. It remains to be investigated how NUAK is regulated in response to muscle contraction and mechanical stress and whether NUAK orthologues exert a similar function in mammals.

Strikingly, phosphoproteomics of human exercised muscle and EPS-stimulated murine myoblasts revealed several conserved sites in BAG3 that are subjected to dephosphorylation in response to mechanical force (Hoffman *et al*, 2015; Reimann *et al*, 2017). This suggests an essential involvement of phosphatases in BAG3 regulation. However, involved enzymes and functional consequences of BAG3 dephosphorylation remain to be established. Notably, the protein phosphatase 5 (PP5) is a direct binding partner of HSP70 and HSP90 (Connarn *et al*, 2014; Oberoi *et al*, 2016) and acts as an HSP90-activated phosphatase at a mechanosensitive region of titin to modulate titin elasticity and myofilament-associated signalling pathways (Krysiak *et al*, 2018). The work provides a first conceptual framework to explain the involvement of phosphatases in mechanical stress protection.

Signalling pathways not only activate stress protection systems but can also contribute to the shutdown of these systems, when necessary. Indeed, the Hippo network kinase STK38 was recently identified as a CASA inhibitor, which binds BAG3 to abrogate the interplay of the cochaperone with HSPB8 and SYNPO2 during the initiation of autophagy (Klimek *et al*, 2019). STK38 is activated upon starvation or when mechanical signals cease during cell detachment (Joffre *et al*, 2015; Bettoun *et al*, 2016), and STK38 activation stimulates BAG3 binding (Klimek *et al*, 2019). The resultant CASA inhibition may increase the availability of commonly used autophagy factors for the execution of other autophagy pathways, i.e. starvation and detachment induced autophagy, which might be more important under these conditions for cell survival.

Mechanical stress protection in the kidney

The previous chapters highlighted the relevance of mechanical stress protection for muscle maintenance. It is important to note, however, that mechanical stress protection is not limited to skeletal and cardiac muscle but represents a universal process. To illustrate this, we will discuss stress protection systems in the kidney and in immune cells in more detail.

Ultrafiltration in the kidney is a necessary physiological function for maintaining organismal homeostasis. The human kidney generates 180 l of primary urine each day in the 1 million glomeruli of the renal filtration unit (Fig 4A). This three-layered filtration unit is formed by endothelia, the glomerular basement membrane and podocytes enwrapping the glomerular tuft from the outside (Fig 4B). The highly sophisticated ultrastructural architecture of podocytes consists of primary and secondary interdigitating processes covering the outer surface of glomerular capillaries (Brinkkoetter *et al*, 2013). Podocyte

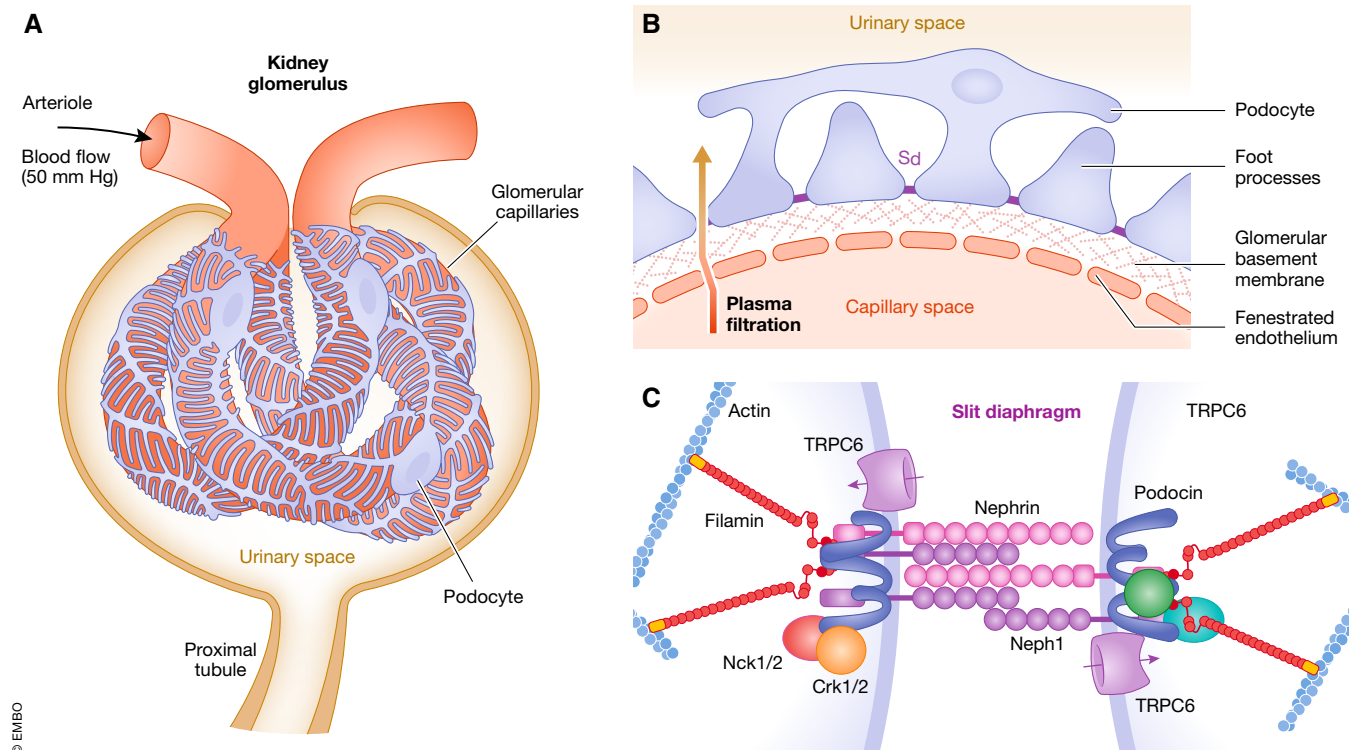


Figure 4. Podocytes withstand high mechanical forces at the kidney filtration barrier.

(A) Schematic representation of the kidney glomerulus. Blood is filtered across the walls of the glomerular capillaries lined with podocytes. The filtrate enters the urinary space and exits through the proximal tubule. (B) The glomerular filtration barrier is formed by a fenestrated endothelium, the glomerular basement membrane and podocytes. Podocytes possess long foot processes that wrap around the capillaries, leaving slit diaphragms (Sd) available for blood filtration. (C) At the slit diaphragm, foot processes are connected by a mechanosensitive protein complex containing nephrin, Neph1 and podocin. The complex is linked to TRPC6 ion channels, diverse kinases and the actin cytoskeleton. Filamin mediates actin anchoring and crosslinking at the slit diaphragm.

processes are connected to the underlying glomerular basement membrane by cell-specific focal adhesion/cytoskeleton interactions (Sachs & Sonnenberg, 2013; Lennon *et al*, 2014) and to neighbouring cells through a highly specialized membrane-like cell junction called the slit diaphragm (Benzing, 2004; Grahammer *et al*, 2013; Endlich *et al*, 2017). Failure of the filtration barrier becomes apparent with the key symptom of proteinuria—the loss of protein into the urine. Genetic causes for proteinuria affect genes almost exclusively coding for proteins of the focal adhesion/cytoskeleton interface or the slit diaphragm protein complex (Perico *et al*, 2016; Schell & Huber, 2017). The slit diaphragm contains an evolutionarily conserved mechanosensitive ion channel super-complex, comprised of the homodimeric podocin proteins and the ion channel TRPC6 (Fig 4C) (Benzing, 2004; Huber *et al*, 2006; Huber *et al*, 2007; Hagmann *et al*, 2014). In addition, the Ig superfamily members nephrin and Neph1, and signalling adapters such as PI3 kinase, Nck1/2 and Crk are part of the super-complex (Huber *et al*, 2003; Höhne *et al*, 2011; New *et al*, 2014; Grahammer *et al*, 2016). More recently identified members of this complex include the mechanosensitive cytoskeletal adapter and CASA client filamin (Venkatareddy *et al*, 2011), and the autophagy-regulating protein C1QBP (Rinschen *et al*, 2016). Mutations in virtually all of these proteins are a major cause of genetic forms of proteinuria indicating that integrity of this protein complex is essential for kidney function (Perico *et al*, 2016).

Due to their location, the podocytes are constantly subjected to mechanical stress resulting from the blood pressure (Endlich & Endlich, 2012; Brinkkoetter *et al*, 2013). The terminally differentiated podocytes have to withstand this mechanical stress to maintain a physiological kidney function, despite an only limited capacity for self-renewal (Assady *et al*, 2019). Podocytes thus rely on adaptive mechanisms that allow repair in response to mechanical damage to prevent their loss by detachment (Kriz & Lemley, 2015). Adaptation might be especially important in disease states with glomerular hypertension and hyperfiltration, when mechanical forces increase even further (Helal *et al*, 2012; Srivastava *et al*, 2018). Indeed, by combining quantitative analyses with advanced mathematical modelling, it could be demonstrated that the permeability of the filtration barrier is modulated through compression of the capillary wall. Disease-associated morphological alterations of podocytes lead to reduced compressive forces that counteract filtration pressure, thereby rendering the filtration barrier leaky (Butt *et al*, 2020). This work proves an active role of the podocyte in counteracting filtration pressure to sustain the filtration barrier.

Considering the presence of the CASA client filamin in the podocin super-complex (Fig 4C) and the key role of BAG3-dependent proteostasis mechanisms for filamin homeostasis in muscle cells, it appears likely that BAG3 also represents an important mechanical stress protection factor in podocytes. In support of this hypothesis,

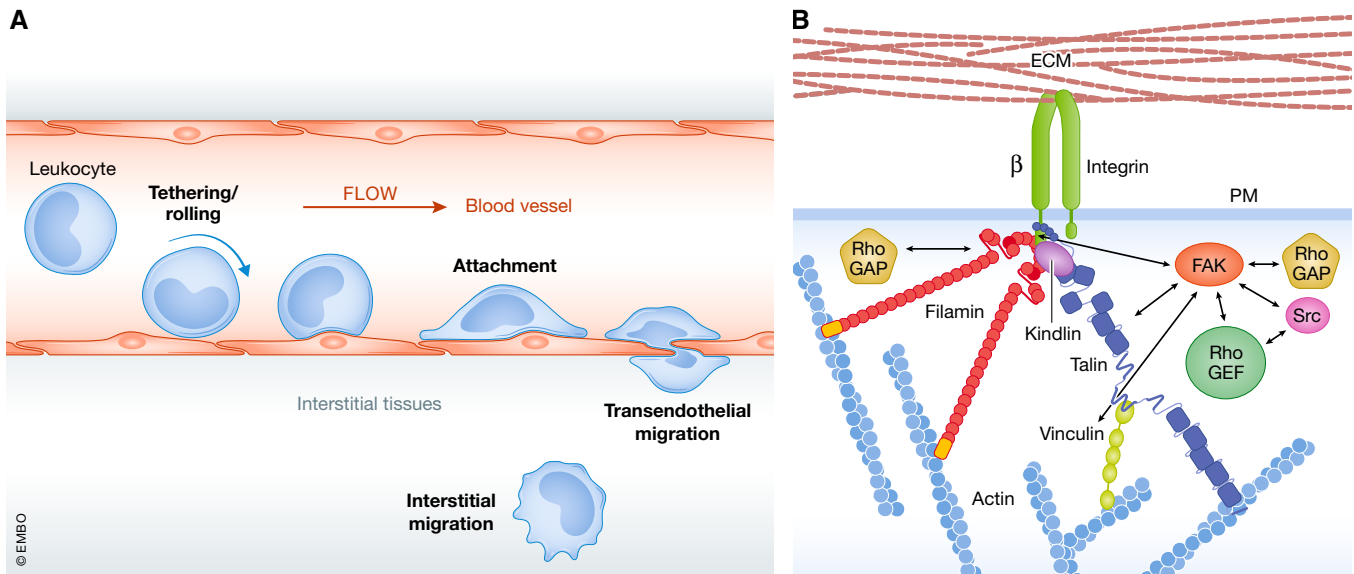


Figure 5. Mechanical forces govern the adhesion, migration and activity of immune cells.

(A) Immune cells, such as leukocytes, are subjected to variety of mechanical forces when they leave the blood stream and migrate through sites of infection and inflammation. (B) Schematic representation of the integrin machinery that operates in immune cells. The transduction of mechanical signals between intracellular processes and the extracellular matrix (ECM) via integrin heterodimers requires a two-way communication system that employs cytoskeletal adaptors (e.g. talin, kindlin, vinculin), kinases (e.g. FAK, Src) and mediators of Rho-GTPase signalling. Integrin engagement with extracellular ligands affects motility and cell fate decisions, including survival, proliferation and differentiation via downstream signalling cascades. PM, plasma membrane.

the entire CASA complex, including BAG3, HSPB8, HSP70, the E3 ubiquitin ligase CHIP and the ubiquitin adaptor SQSTM1, was detected in an unbiased proteomic screen for proteins enriched in podocytes (Rinschen *et al*, 2018). In addition, phosphoproteome studies revealed multiple phosphorylation sites in BAG3, CHIP and filamin in primary podocytes, which are altered during podocyte differentiation (Rinschen *et al*, 2014, 2016). Induction of filamin expression represents an early response upon filtration barrier failure and knockdown of filamin in *Drosophila* reduces filtration capacity, pointing to key role of the BAG3 client in determining the mechanical properties of the filtration barrier (Koehler *et al*, 2020). Of note, the central podocyte actin-cytoskeleton protein synaptopodin has been shown to play a role in BAG3-mediated autophagy in neurons (Ji *et al*, 2019). Recent evidence also suggests that a YAP-mediated mechanoresponse is required for maintaining podocyte integrity throughout lifetime (Keyvani Chahi *et al*, 2016; Schwartzman *et al*, 2016) and that YAP is an essential mediator of mechanical activation of podocytes (Rinschen *et al*, 2017a). Finally, mutations in the CASA component SYNPO2 were recently shown to contribute to monogenic nephrotic syndrome (Mao *et al*, 2020). Taken together, the data strongly suggest that mechanical stress protection systems are present and functional in podocytes and might be conserved, at least in part, between podocytes and muscle cells.

Mechanical stress protection in the immune system

The immune system may be viewed as a complex cell system, which is not embedded in “static” tissue architectures (Huse, 2017). This is even true for immune organs such as lymph nodes, which

have a highly dynamic content of motile cells (Miller *et al*, 2002). In mammals, most immune cells originate in the bone marrow (Schulz *et al*, 2012). Precursor cells, together with already mature immune cells, exit the bone marrow via the bloodstream and are transported to their destination, namely resting or inflamed tissues. In addition, T cells recirculate between blood and lymphatic organs, that is they are cycling between blood-borne and tissue-borne states. Thus, a universal property of immune cells is their capacity to exit the circulation and to enter tissues (Fig 5). The ability to sense and respond to mechanical cues is essential for the development, activation, differentiation and expansion of immune cells (Zhang *et al*, 2020). In the blood, immune cells are subjected to the flow forces generated by the heartbeat, which gives rise to blood pressure. When immune cells enter tissues, they need to adhere to the endothelia of the destination organ and to traverse the respective endothelial barriers. This process, also called extravasation, has been extensively studied and led to a widely accepted model, according to which immune cells utilize two major classes of adhesion molecules, termed integrins and selectins (Ley *et al*, 2007). Both play essential roles in the recruitment of such cells to endothelial membranes at so-called post-capillary venules and subsequently to the underlying tissues. Several laboratories have shown that haemodynamic, flow-mediated shear forces (or shear stresses) play an essential role in these processes (Shamri *et al*, 2005; Alon & Dustin, 2007; Alon & Ley, 2008). Flow forces alter the regulation and possibly the mechanism of extravasation, which involves two-dimensional crawling and transmigration of immune cells between endothelial cells (paracytosis) or through their cell bodies (transcytosis) (Fig 5A) (Carman *et al*, 2007). Haemodynamic forces were also shown to be relevant for the motile behaviour of non-classical monocytes, which move

on barrier membranes of capillaries, larger veins and arteries via intraluminal crawling, as such cells constantly patrol in these blood vessels (Auffray *et al*, 2007). Finally, also the intraluminal crawling of T cells has been shown to be affected by shear stress (Valignat *et al*, 2013).

Upon tissue entry, immune cells are further subjected to multiple forces or have to generate forces, respectively. Leukocytes migrate through a diverse array of three-dimensional environments, and their motility mechanisms highly depend on the cell type and on the surrounding matrix (Huse, 2017; Gauthier & Roca-Cusachs, 2018; Kechagia *et al*, 2019). Several classes of molecules have been shown to regulate functional force transduction in leukocytes. This includes several integrin adhesion receptors, which can bind either to cellular ligands or to extracellular matrix ligands such as collagen or fibronectin receptors (Fig 5B). Ligand binding of the integrin adhesome is dynamically regulated by conformational switching between active and inactive states (Horton *et al*, 2015; Li & Springer, 2017). Forces generated by the cell during crawling or extracellular forces applied by the surrounding tissues are transduced to the effector machinery of the actin cytoskeleton (Huse, 2017). Finally, an array of intracellular signalling molecules, including Rho, Rap and Arf-GTPases (Reiner & Lundquist, 2018), and the cytoskeletal adapters talin and kindlin (Goult *et al*, 2018; Rahikainen *et al*, 2019) regulate both adhesion and cytoskeletal dynamics (Fig 5B).

Of note, the stimulation of adhesion and spreading of human Jurkat T lymphoblasts induces the expression of BAG3 and filamin and results in increased filamin turnover (Ulbricht *et al*, 2013). Moreover, inhibition of autophagy strongly impairs the adhesion, spreading and migration of Jurkat cells and attenuates the resistance of lymphoblasts and human primary leukocytes to shear stress (Ulbricht *et al*, 2013). These findings point to an essential role of the CASA machinery and the autophagic disposal of force-unfolded proteins in mechanically stressed immune cells. Future studies will have to reveal the link between force-regulated signalling pathways and BAG3-dependent proteostasis mechanisms in these cells.

Always stressed

We use the term “mechanical stress” throughout this review. Per definition, the term “stress” refers to a state of increased strain or tension resulting from exceptionally adverse or demanding circumstances. Yet, in multicellular organisms, mechanical forces of a scale sufficient to disturb the structure of mechanosensitive proteins are not exceptional but ever-present and affect diverse types of cells and tissues, as outlined above. Recent results, however, revealed an even more unanticipated and fundamental role of mechanical stress protection systems in the determination of cell behaviour. The identification of BAG3, HSPB8 and SQSTM1 as essential factors for cell division points to a very general function of the CASA pathway in all higher eukaryotic cells (Fuchs *et al*, 2015; Varlet *et al*, 2017; Luthold *et al*, 2020). In dividing human HeLa and HEK 293T cells, BAG3 is hyperphosphorylated at mitotic entry and localizes to centrosomal regions. Depletion of BAG3 and HSPB8 severely affects spindle orientation in mitotic cells due to disorganized actin-rich retraction fibres, and similar phenotypic

aberrations are observed upon depletion of the autophagic ubiquitin adaptor SQSTM1, which cooperates with BAG3 and HSPB8 during CASA (Fuchs *et al*, 2015; Luthold *et al*, 2020). Furthermore, the BAG3/HSPB8 machinery was also found to be essential for the accurate disassembly of the actin-based contractile ring during cytokinesis, and alterations in this process triggered by the depletion of the CASA components could be alleviated by a pharmacological induction of autophagy (Varlet *et al*, 2017). Although the relevant CASA targets during mitosis remain to be identified, the CASA-mediated autophagic disposal of cytoskeleton proteins seems to be of critical importance during cell division at stages when profound changes in cell tension occur.

Relevance for human physiology and pathophysiology

The inactivation or deregulation of mechanical stress protection factors causes severe pathology in humans. For example, mutations in BAG3 give rise to skeletal muscle myopathies and cardiomyopathies (Claeys *et al*, 2013; Fang *et al*, 2017; Kimura *et al*, 2021), a decline of BAG3 activity contributes to heart failure (Martin *et al*, 2021), and mutation-induced functional impairment of UNC-45 leads to skeletal muscle myopathy and ovarian and breast cancer (Bazzaro *et al*, 2007; Janiesch *et al*, 2007; Donkervoort *et al*, 2020). Advancing our knowledge about mechanical stress protection systems in humans will thus be instrumental for the diagnosis of diverse diseases and may pave the road for therapeutic intervention using, for example, proteostasis modulators, such as the autophagy inducing compound rapamycin. In addition, such knowledge might provide means to sustain the fitness and increase the performance of healthy individuals.

It is generally accepted that performance and health in human beings is critically dependent on muscle mass and strength, especially in the elderly (McLeod *et al*, 2016). Mechanical strain induced by resistance exercise is a necessary cellular stimulus to trigger anabolic signalling (Gehlert *et al*, 2015) and to induce protein synthesis and muscle growth (Mayhew *et al*, 2009; Damas *et al*, 2015). However, muscle stimulation by resistance exercise in athletes or in patients during rehabilitation programmes needs to be repeated over several weeks to achieve significant muscle growth and strength gain (Bird *et al*, 2005). Yet, resistance exercise also causes the force-induced unfolding of sarcomeric proteins and muscle damage (Proske & Morgan, 2001; Ulbricht *et al*, 2015). Thus, increased muscle anabolism has to be accompanied by an induction of mechanoprotective systems to ensure a coordinated adaptation to mechanical stress in exercised muscle (Arndt *et al*, 2010; Ulbricht *et al*, 2015). In fact, strenuous resistance exercise leads to an immediate induction of the CASA machinery in human leg muscle, which will then continue at least for the next 24 h to degrade mechanically unfolded and damaged filamin and possibly other mechanosensory proteins (Ulbricht *et al*, 2015). Moreover, repeated resistance exercise during several weeks of training causes an elevated expression of CASA components, which limits muscular damage during a subsequent bout of exercise (Ulbricht *et al*, 2015). CASA apparently acts as a central adaptation mechanism in human muscle, responsive to acute physical exercise and repeated mechanical stimulation. The BAG3-mediated regulation of mTORC1, which activates protein synthesis in response to mechanical stress (Kathage *et al*, 2017),

might also contribute to the increase of muscle mass following exercise (Goodman, 2014; Jacobs *et al*, 2014).

The BAG3-interacting small heat shock protein HSPB5 is rapidly phosphorylated at serine-59 in human skeletal muscle after strenuous resistance exercise, and the extent of phosphorylation correlates with the intensity and number of muscle contractions (Jacko *et al*, 2019, 2020). Furthermore, HSPB5 phosphorylation declines during repeated exercise and re-increases upon exercise following training cessation (Jacko *et al*, 2020), suggesting rapid adaptation and de-adaptation in response to the timing of mechanical stimulation. Elucidating the relationship between exercise parameters, alterations in mechanical stress protection systems and muscle damage and growth will be important for establishing optimized training programmes for athletes and individuals who need to build up muscle during rehabilitation.

It is interesting to consider not only situations with increased mechanical stress such as resistance exercise, but also conditions when mechanical signals cease, for example in immobilized and ventilated patients in intensive care units (ICU), the number of which dramatically increased during the COVID pandemic. Life-saving treatment of ICU patients often involves periods of mechanical ventilation, sedation and complete mechanical silencing. Under these conditions, up to 30% of patients develop critical illness myopathy (CIM), characterized by a massive loss of muscular myosin (Friedrich *et al*, 2018). The resultant muscle weakness can be as severe as causing a complete quadriplegia and represents a major obstacle for the recovery of affected ICU patients. Elaborate animal models established the complete mechanical silencing as a predominant factor in the development of CIM, which causes aberrant autophagic flux, increased levels of stress protection factors including HSP70, SQSTM1 and HSPB5, and altered expression of components of the ubiquitin–proteasome system in affected muscle (Ochala *et al*, 2011; Llano-Diez *et al*, 2019). As outlined above, myosin is a critical client of the UNC-45/HSP90 chaperone machinery and, indeed, also this machinery is present at elevated levels in animal models of CIM (Llano-Diez *et al*, 2019). UNC-45 protein levels need to be precisely regulated to ensure the progression of myosin folding (Hoppe *et al*, 2004; Janiesch *et al*, 2007). Consequently, an increase of UNC-45 expression in CIM patients might directly contribute to the collapse of myosin homeostasis. In addition to myosin, also levels of the mechanosensitive protein titin are reduced in muscles of CIM patients and titin loss results in Z-disc disassembly and sarcomere disintegration (Swist *et al*, 2020).

Although more research is required to understand the molecular alterations that cause critical illness myopathy, available data already illustrate that mechanical silencing has a profound impact on the proteostasis network and causes drastic changes in mechanical stress protection systems. The altered homeostasis of mechanosensitive client proteins is apparently incompatible with cell and tissue maintenance. A tight interplay between mechanical stimulation and proteome maintenance is further supported by the findings that passive mechanical loading as well as a pharmacological induction of proteostasis factors, for example based on the administration of the chaperone co-inducer BGP-15, alleviates aspects of CIM pathology (Salah *et al*, 2016; Llano-Diez *et al*, 2019). It seems that our cells and tissues are not just mechanically stressed all the time, but that this stress is necessary for fitness and survival by establishing a functional proteostasis network.

In need of answers

- i What are the constituents of the force-unfolded proteome and mechanical stress protection systems?
 - a. State-of-the-art proteomic approaches of cells and tissues subjected to mechanical forces of defined intensity should be performed in future studies to identify the force-unfolded proteome and the proteostasis systems that are involved in maintaining this part of the proteome.
- ii What determines the recognition and processing of mechanosensitive domains by protein quality control systems?
 - a. Single-molecule studies have revealed force-induced unfolding events in mechanosensors. It will be necessary to include chaperones and other proteostasis factors in such studies to elucidate how the dynamic unfolding of mechanosensitive domains during mechanotransduction is linked to the surveillance by protein quality control systems. Analysis should also include posttranslational modifications of mechanosensors and proteostasis factors that alter folding and interaction kinetics.
- iii Which kinase/phosphatase networks control mechanical stress protection and what are their targets?
 - a. Our knowledge about the signalling pathways that operate under mechanical stress and their critical targets is still limited. The further development of techniques for elucidating phosphoproteomes coupled to force stimulation of cells, tissues and model organisms will be important to establish regulatory networks.
- iv Are there common and tissue-specific mechanical stress protection systems?
 - a. For obvious reasons, the analysis of mechanical stress protection initially focussed on muscle. A comparative analysis of different tissues will be required to elucidate common principles and more specific responses. This may pave the road for a tissue-specific pharmacological intervention to treat diverse diseases associated with a dysregulation of mechanical stress protection.

Concluding remarks

Mechanobiology and proteostasis research are booming areas in biochemistry, cell biology and biomedicine. However, until recently, there has been very little exchange between these areas. Mechanobiologists wanted to identify and understand force-generating, force-bearing and force-transducing structures, but usually overlooked that force-induced protein unfolding poses a problem in cells, coped with by providing specialized protein folding and degradation systems. Proteostasis researchers, on the other hand, focussed their attention on cellular and organismal responses to heat, oxidative and proteotoxic stress, but largely ignored mechanical forces as an ever-present major threat for protein integrity and proteome maintenance. The work described here illustrates that mechanobiology and proteostasis research need to be integrated to understand cell and tissue homeostasis in the presence of mechanical forces. Future studies should provide a comprehensive picture of the force-sensitive proteome and the protein folding and degradation systems necessary for its maintenance. Elucidating involved proteostasis systems and signalling networks may ultimately provide the means to treat

diseases caused by dysregulated mechanical stress protection ranging from myopathies to renal failure and immune deficiencies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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