**"Functional proteomics of cellular mechanosensing mechanisms"**

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**Abstract**

Mechanosensing enables cells to coordinate their phenotype with the mechanical properties of their tissue microenvironment. In this process, cells probe their surroundings by applying contractile forces, which produces different amounts of mechanical strain within the cells as a function of the stiffness of their extracellular substrates. Tension within cells can then affect the structure and composition of most cellular organelles, including cell adhesions, the cytoskeleton, the plasma membrane and the nucleus. On a molecular level, the conformations, modifications, interactions, and subcellular localizations of proteins have been shown to be altered by biomechanical forces. Functional proteomics aims at the analysis of these effects in a proteome wide, unbiased and high throughput manner. Emerging methods, such as crosslinking mass spectrometry and advanced protein correlation profiling, will enable future analysis of mechanosensing on the level of protein interactions *in situ*, and subcellular protein localization, which can now be determined with very high accuracy from whole cell analysis for thousands of proteins at once. Combined use of these mass spectrometry toolsets with the analysis of posttranslational modifications will ultimately move the field to a comprehensive list of molecular alterations in cellular mechanosensing. We will give an overview on current developments in functional proteomics and the latest applications on questions related to mechanobiology.

## Keywords

Proteomics; Mass spectrometry; Protein interactions; PTM; Mechanosensing; Systems biology

1. **Introduction**

Cells actively sense and respond to mechanical inputs from their environment [1]. In this process the mechanical properties of the extracellular matrix (ECM), such as stiffness, topology, and dimensionality, are transduced into biochemical signals that will induce changes of cellular behavior. In the last 10 years the field of mechanobiology rapidly evolved and a variety of data shows that biomechanics critically affects almost all aspects of multicellular life. How do cells sense and transduce mechanical cues? It is conceivable that biomechanics is likely to impinge on proteins with functions in cell-matrix and cell-cell adhesions, the cytoskeleton, and ultimately the nucleus, which is the biggest and most rigid organelle of the cell. Whilst cell-cell adhesion is mediated mainly by cadherins, cell adhesion to the ECM is predominantly orchestrated by integrins. Both integrin- and cadherin-mediated adhesions connect to the filamentous (F-) actin cytoskeleton by using a variety of adaptor and signaling proteins. These proteins assemble into a dense and highly dynamic network visible as a protein plaque at the plasma membrane, which we refer to as the adhesome [2-4]. Force on cell–matrix adhesions can directly change the conformation of proteins and expose cryptic-binding sites within mechanosensitive adhesome proteins leading to alterations in protein interactions and postranslational modifications, which affect both the local adhesion site and through signaling the cell in its entirety [3, 5-9].

Cells within tissues experience different degrees of ECM rigidity, ranging from very soft environments (as in brain and adipose tissue) to more rigid surroundings (such as those found within bones or muscle). By recapitulating these different ECM properties *in vitro*, it was found that mesenchymal stem cells (MSCs) differentiate towards different cell lineages in direct response to tissue mechanics [10]. Moreover, it is well appreciated that abnormal changes in ECM stiffness and mechanosensitive signaling pathways contribute to the development and progression of various diseases, such a cancer and fibrosis [11-14]. For instance, it has been shown that a physiological stiffness range (0.2 - 2 kPa) keeps human lung fibroblasts in a quiescent state, while substrates with higher stiffness (2-35 kPa), as observed in fibrotic lungs, induce a profibrogenic phenotype with high proliferation and matrix synthesis rates [15]. The fibrotic ECM of patients with idiopathic pulmonary fibrosis (IPF) instructs fibroblasts to further increase ECM production [16], which might be partially due to differences in ECM stiffness. Mechanosensing by the alpha6-integrin confers an invasive fibroblast phenotype and mediates lung fibrosis [17], and inhibition of αv-class integrins attenuated mouse models of lung and liver fibrosis [18, 19]. Tumor development is often accompanied by lysyl oxidase (LOX)-dependent collagen crosslinking, resulting in stiffening of the ECM and thereby enhancing tumor cell proliferation and metastatic colonization [12, 20]. Furthermore, ECM stifness directly upregulates the number and activity of invadopodia, thereby promoting cancer invasiveness and metastasis [21, 22]. Thus, it is well appreciated that mechanobiology is important in the pathophysiology of many diseases, which makes cellular mechanosensing a potential target for therapeutic intervention.

On encounter of their extracellular substrate cells reciprocate the stiffness of that substrate. This mechanoreciprocity is generated by feedback connections between cell-matrix adhesions and the cytoskeleton, which tune the strength of contractile forces to an equilibrium of applied force and tensile strength of the ECM substrate [5]. In this process, the structure and organization of the cytoskeleton is altered, which was shown to also induce long term gene expression changes [23]. Individual integrins in cell-matrix adhesions were shown to contribute to signaling and rigidity sensing in different ways. For instance, αVβ3 and α5β1 integrin differ in their generation of traction forces [24] or induction of actin cytoskeleton remodeling [25]. Furthermore, these different integrin classes were shown to cooperate in sensing ECM rigidity [26, 27]. While β1-class integrins (α5β1) regulate activation of the Rho–ROCK–myosin II pathway to induce actomyosin generated forces, αv-class integrins (αvβ3, αvβ5) are important for adaptation to external forces and regulate the reinforcement of cytoskeletal linkage and the enlargement of focal adhesions by activating the GEF-H1–Rho–mDia pathway [26]. The precise molecular nature of many elements in these feedback connections is currently unknown and it is also unclear to which extend an equilibrium of mechanosensing to mechanoresponse differs between cell types, and how such differences can be programmed.

Currently it is unclear at which molecular levels the mechanical inputs are transduced into biochemical signals and how these signals affect cellular responses. Despite numerous observations of mechanosensitive behavior of cellular structures, most studies looked at individual components and did not provide insight on network level information about cellular pathways, protein complexes and organelles under the influence of mechanical forces. Functional proteomics addresses the dynamic aspects of protein abundance, localization, structure, post-translational modification, and interactions with other proteins, typically using unbiased methods such as quantitative mass spectrometry (MS) driven proteomics. A key characteristic of functional proteomics studies is their proteome-wide approach to these questions, generally involving high-throughput methods rather than a more traditional “protein-by-protein” approach. For instance, MS analysis of adult mouse [28] and human [29] tissue proteomes revealed a linear scaling relationship over several orders of magnitude between tissue stiffness and protein abundance of nuclear lamins and collagen type I α 1 chain (Col1a1) [28]. Future functional proteomics studies in aggregation will ultimately deliver a complete list of molecular events involved in mechanosensing. In this review we will give an overview on MS driven functional proteomics and its application in systems biology studies of cellular (mechano-)biology.

1. **Mass spectrometry driven functional proteomics**
   1. The principles of mass spectrometry based proteomics

Due to the dynamic nature and complexity of biological systems it is impossible to predict their behavior from the properties of individual parts, but rather by considering the interplay of systems components. Thus, central to the field of systems biology is an understanding of the organization of individual components into functional networks and the identity and dynamics of molecular interactions spanning from genome to proteome to phenotype. Describing, predicting and solving challenging biological problems, such as cellular mechanosensing, require the development of new technologies and analytical tools. Proteomics has become an essential discipline for the accurate building of network models [30]. Recent and ongoing advances in MS-based quantitative proteomics enabled the generation of a growing number of datasets detailing the quantity, structure, function, activity and molecular interactions of proteins [31]. Different aspects of the proteome can be explored by choosing the right combination of sample preparation, MS instrumentation and data processing [32].

The principal components of a typical MS include: the ion source, the mass analyser and the detector. The analysis of complex biological systems is highly dependent on separation technologies such as 2-dimensional gel electrophoresis (2D-GE) [33] or, most importantly, high performance liquid chromatography (HPLC) [34]. A mass spectrometer generates a beam of gas phase ions from samples (analytes), sorts and detects the produced ions according to their mass-to-charge (*m/z*) ratios using electrical/magnetic fields, and provides output signals, indicating the *m/z* ratios and intensities (abundances) of all the detected ions. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are commonly used to ionize proteins or peptides for MS analysis. These techniques can be coupled to different mass analyzers, such as the quadrupole (Q) and time-of-flight (TOF), ion-trap (IT), Fourier transform ion cyclotron (FT-MS), and the Orbitrap mass analyzer. Each mass analyzer has inherent advantages/disadvantages with respect to accuracy, mass resolution, mass range and sensitivity, which has been reviewed elsewhere [35, 36].

The identification of proteins by MS can be achieved by two main approaches: the analysis of peptide mixtures (bottom-up proteomics) and the analysis of intact proteins (top-down proteomics). In the top-down approach (native MS) single proteins or simple mixtures of intact proteins are separated using LC or 2-D electrophoresis, followed by MS analysis. Detection of all existing proteoforms (different versions of a protein derived from the same gene) can be achieved simultaneously in one spectrum. Current high-throughput top-down workflows successfully identified large numbers of proteins present in human cells [37]. Nevertheless, as efficient separation of intact proteins is very challenging, proteome-wide analysis of intact proteins using top-down proteomics is not achieving similar success as the bottom-up approach in terms of proteome coverage, sensitivity, and throughput. Thus, native MS is currently mainly used on single proteins or for structural proteomics analysis of highly purified protein complexes. In bottom up proteomics, proteins are extracted from the source material, denatured and then proteolytically digested by sequence specific enzymes, such as trypsin. The resulting peptides are separated by liquid chromatography (LC) prior to MS analysis (LC-MS/MS or LC-MALDI MS/MS). MS analysis in bottom-up proteomics is done with three main approaches (reviewed in [31]): (1) Data dependent acquisition (DDA), (2) data independent acquisition (DIA), which are both used to achieve an unbiased analysis of complete proteomes; and (3) targeted proteomics to detect proteins of interest with higher sensitivity, quantitative accuracy and reproducibility. Targeted proteomics can simplify the analysis, as it focuses on a subset of known proteins (peptides) of interest.

In a typical DDA setup two types of spectra are collected. In a first scan (MS1) the masses of tryptic peptides over the full mass range of the instrument is determined, while a subsequent series of second scans (MS2) determines the mass of all possible peptide fragments - differing by one amino acid in length - that are generated in the mass spectrometer by collision induced dissociation of the peptide backbone. The combination of these two scan modes allows peptide sequence identification based on spectral matching against theoretical spectra (e.g., protein database search).

In DIA, predetermined *m/z* ranges are interrogated either by fragmenting all ions entering the mass spectrometer at a given time (broadband DIA, MSE or MSAll) or by fragmenting the full m/z range into smaller *m/z* isolation windows that are each independently and successively analyzed [38]. One prominent DIA method is known as SWATH (Sequential Windowed Acquisition of All Theoretical Fragment Ion) [39].SWATH performs data-independent fragmentation of all precursor ions entering the MS in 20-30 m/z isolation windows. The whole m/z range of interest is covered by continuous stepping of the isolation window. This results in a complete fragment ion map of the sample. However, the data analysis is generally challenging and requires prior knowledge on the sample of interest (in contrast to the methods name). Currently, DIA analysis is dependent on prior DDA experiments to adopt database-based search engines used in DDA to search the produced multiplexed spectra.

In case of targeted proteomics, the MS analyzes a preselected group of proteins. Using pre-existing information, characteristic (proteotypic) peptides (PTP) are analyzed with methods such as the selected reaction monitoring technique (SRM; also referred to as multiple reaction monitoring, MRM). The method usually uses the capability of triple quadrupole mass spectrometers (QQQ) [40]. In MRM the first quadrupole of a QQQ selects the previously defined PTPs based on their m/z ratio. The second quadrupole is activating the selected peptides in the collision cell through collision induced dissociation (CID) to generate specific product ions (fragments), which are further analyzed in the third quadrupole [41]. Recent advances in targeted techniques allow measuring several thousand peptides in a single analysis. Targeted approaches can be more sensitive and reproducible compared to discovery proteomics methods and it simplifies the complex bioinformatical downstream analysis required to interpret the MS data. Thus, targeted MS is increasingly applied in clinical biomarker studies [42].

* 1. Interaction proteomics

The majority of proteins form multiprotein complexes to exert their biological functions. Characterizing the dynamic interactions of proteoforms, ideally in their native environment in situ, and determine the functional implications of these interactions is the main aim of functional proteomics [43, 44]. A range of approaches are currently used to identify protein-protein interactions, including affinity purification-mass spectrometry (AP-MS), cross-linking mass spectrometry (XL-MS), MS-based protein correlation profiling (PCP), yeast two-hybrid (Y2H) screening, and proximity ligation strategies.

AP-MS has become the most popular method of choice to map and track the dynamic changes in protein-protein interactions, including the ones occurring during cellular signaling events [45]. AP-MS has already been the basis of large-scale interaction mapping in Saccharomyces cerevisiae [46, 47] as well as in human cells [48, 49]. In this technique endogenous or tagged bait proteins are depleted from cell lysates using an affinity resin. Proteins that are associated with the bait can then be identified by LC-MS/MS. In the context of novel high sensitivity MS instrumentation, new analysis methods take advantage of specific enrichment of interactors in the context of a large amount (thousands) of unspecific background binders that are used for normalization of the data [50]. Furthermore, using such methods and label free quantification of proteins in both the input cell lysates and affinity purifications enables the analysis of protein complex stoichiometries, which has recently been attempted at a proteome wide scale [43]. Future studies will likely use such methods to derive changes in the interactome in response to specific perturbations, such as mechanical stimuli.

AP-MS studies are limited by (1) the necessity of isolating and purifying complexes out of their native environment, which may lead to loss of weak affinity interactors, and (2) the fact that protein complex topology cannot be inferred directly from the AP-MS data. In other words, proteins that co-purify in AP-MS studies do not necessarily interact directly with the bait. The recent successful use of chemical crosslinking in combination with mass spectrometry (XL-MS) promises to overcome these limitations and enable true functional proteomics *in situ* [51-53]. The concept of XL-MS is to treat a native sample with bifunctional chemical crosslinkers in order to introduce covalent links between two amino acid side chains in very close proximity (<2 nm) to each other and thereby fix the spatial information. Upon denaturation and digestion of proteins with sequence specific enzymes, peptides that were close enough to the interaction interfaces of protein surfaces are still covalently linked to each other. A number of different strategies have been developed in recent years to identify these crosslinked peptide pairs in the mass spectrometer in order to build topological models of protein complexes [54-59]. XL-MS was used to identify interesting interaction partners or novel co-factors within specific complexes [60], or to provide topology of their interactions [59]. Most recent attempts to apply XL-MS to whole cells showed the potential of using this technique to analyze protein interaction networks *in situ* [54, 61]. Since conceptually XL-MS is able to address both the structural configurations and rearrangements within proteins and the interaction between proteins at the same time and *in situ,* this method is about to bring major insights into mechanisms of mechanosensing. Current difficulties with sensitivity and throughput of this method are likely to be overcome by improvements in MS-instrumentation and biochemical methods in the near future [53, 54].

Another interesting strategy to screen for protein interactions in the living cell in situ is based on proximity-dependent labeling methods. These methods are based on modified enzymes genetically fused to proteins of interest that are capable of attaching known reactive groups to potentially interacting proteins in close proximity to the protein of interest, in order to enable their isolation and identification by MS. Examples of such techniques include biotin ligase-based methods, e.g. proximity-dependent biotin identification (BioID) [62], or peroxidase-based techniques, e.g. ascorbate peroxidase (APEX) [63, 64]. The BioID approach makes use of the ability of a mutant form of the Escherichia coli biotin ligase (BirA\*) to biotinylate proteins in close proximity [65]. In the APEX method, mutated ascorbate peroxidase oxidizes biotin-phenol in the presence of hydrogen peroxide, generating radicals that that covalently tag proximal endogenous proteins [64, 66]. This technique is suitable for purification of both protein complexes and organelles. Hung *et al.* developed a new variant of APEX, called APEX2, showing enhanced catalytic efficiency [67]. Recently, APEX was used to analyze the mitochondrial proteome [63, 64, 68], the endoplasmic reticulum-plasma membrane junction [69], and mechanosensitive primary cilia [70].

* 1. Organellar proteomics

The eukaryotic cell is characterized by a high degree of subcellular compartmentalization. The composition of cellular compartments is dynamic and many proteins partition only temporarily into subcellular structures. Thus, proteins may engage different multiprotein complexes depending on where they are in the cell. The subcellular localization of specific proteins is routinely determined by microscopy-based methods, but can also be studied using biochemical fractionation methods. The combination of these enrichment methods for subcellular compartments with MS-driven discovery proteomics tremendously increased our understanding of proteome dynamics and organellar composition [71-75]. Several studies using organelle proteomics have led to a better understanding of the molecular mechanisms involved in the regulation of the function of various cellular organelles, such as the nucleus [76], mitochondria [72], Golgi [77, 78], autophagosome [79], phagosome [80], and focal adhesions [71, 81-83].

Due to the high sensitivity of mass spectrometers and the difficulties inherent in purifying organelles, it has been challenging to distinguish real organellar proteins from those that are contaminating. To overcome this problem, methods such as protein correlation proﬁling (PCP) [84, 85], have been developed. PCP looks at similar quantification profiles of proteins with known organellar markers across a biochemical isolation procedure, such as a density centrifugation gradient. Using this approach, Andersen *et al.* identiﬁed 41 likely candidates of the centrosome and validated 23 novel components, many of which were later described to cause genetic diseases when mutated [86]. Similarly, Foster *et al*. mapped 1,404 proteins to ten subcellular locations in mouse liver, which were validated using enzymatic assays, and confocal microscopy [84]. Several `flavours´of PCP approaches have been used. With a method called LOPIT more than 500 proteins were simultaneously localized in different organelles, resulting in the determination of steady-state protein distributions between these organelles [87]. Moreover, a group led by Kathryn S. Lilley reported the development of a method they called hyperLOPIT [88]. Using this approach they provided localization data for over 5,000 proteins to reveal the organization of organelles, suborganellar compartments, and protein complexes. Most recent developments in PCP approaches enabled the generation of `Dynamic Organellar Maps´, providing quantitative cellular localization data for around 9,000 proteins, resolving all major organelles with a prediction accuracy of >90% for individual proteins [74]. Borner *et al.* demonstrate the power of their `Dynamic Organellar Maps´ analysis pipeline by providing a quantitative mapping of EGF-triggered subcellular translocation events with accurate quantification of the dynamic protein copy number changes in different compartments upon EGF treatment [74]. Future combinations of such PCP approaches may be applied to the field of mechanobiology. For instance, the combined analysis of post-translational modifications (PTMs) of proteins with their subcellular localization, will reveal causative relationships of PTMs and changes in protein localization.

2.4. Analysis of post-translational modifictions by mass spectrometry

The human proteome is greatly more complex than the human genome. The complexity of proteoforms is a consequence of differential splicing and the dynamic occurrence of post-translational modifications (PTMs). PTMs refer to chemical modifications of specific amino acid residues of a protein and/or cleavage of the translated sequence. Currently, more than 300 different types of PTMs have been identified [89], including phosphorylation, glycosylation, acetylation, nitrosylation, ubiquitination, ribosylation or methylation. For many such PTMs, tens of thousands of sites can now be confidently identified and localized in the sequence of the protein by MS [90]. These modifications result in structural and functional diversity of proteins, including changes of activity, interaction with other cellular molecules, localization as well as stability. Thus, PTMs are essential elements in the coordination of signaling networks. Since for all PTMs the site occupancy (the ratio of unmodified protein to its modified counterpart) is usually rather low it can be difficult to identify these modified versions without prior enrichment of modified peptides [90]*.* Enrichment strategies can be divided into: 1. ionic interaction-based approaches (e.g. Serine/Threonine/Tyrosine phosphorylation); 2. antibody-based affinity enrichment (e.g. tyrosine phosphoryled, lysine acetylated and uniquitin-like modified peptides); 3. chemical labeling of the PTMs, including in vitro chemical reactions and in vivo metabolic labeling, which could be conjugated to an affinity linker (such a biotin or fluorous affinity tag) for the identification of O-GlcNAc-modifications N-glycosylated and N-protein acetylated peptides; 4. pull-down with a macrodomain-containing proteins, used for instance to investigate PARylation. For many other PTMs there is a need to develop enrichment methods, in order to enable their systematic analysis by MS.

In particular, phosphorylation of serine, threonine and tyrosine residues, is one of the most important and well-studied modifications in cellular signal transduction networks [91] and will thus be discussed in more detail here as an example of PTM proteomics. The most popular methodology for general phosphopeptide enrichment is metal ion chromatography, e.g. immobilized metal affinity chromatography (IMAC) with Fe3+ [92] and metal oxide affinity chromatography (MOAC) with TiO2 [93, 94]. Several complementary approaches have also been described, including IMAC with Ga3+ [95, 96] and MOAC with ZrO2 [97-99] or Nb2O5 [100]. Furthermore, calcium phosphate precipitation (CPP) has been proven to be a simple enrichment technique and exhibits a reasonable coverage of phosphorylation sites [101]. In addition, phosphoramidate chemistry (PAC) is another important approach in which phosphopeptides are coupled to a solid-phase support [102]. The methods, however, differed in their specificity of isolation and in the set of isolated phosphopeptides [102]. Optimized sample preparation methods together with continuous improvements in MS have allowed to accurately identify and quantify phosphorylation at the proteome scale, with the deepest dataset covering more than 50,000 distinct phosphorylated peptides in a single human cancer cell line [103-106]. Importantly, if done with the right experimental and computational workflow, also information about phosphosite occupancy can be obtained from the MS experiments [105, 106].

Currently, there are to our knowledge no published large scale phosphoproteomics studies on cellular mechanosensing. Future studies may also combine the analysis of PTMs, organellar proteomics and protein-protein interaction studies, to reveal molecular switches in network organization that control cellular responses to force.

1. **Quantitative proteomics of cellular mechanosensing**
   1. Cell-matrix adhesions and the cytoskeleton

As the major molecular link between cells and the ECM, integrins play a central role in determining how cells sense and respond to their mechanical environment. To accomplish these tasks, integrins cluster and assemble ancillary proteins in specialized adhesion structures that differ in their morphology, subcellular localization, lifespan and protein composition. Nascent adhesions (NA) are the smallest adhesive structures in membrane protrusions [107], which either disassemble after a short lifespan (< 1 min), or mature into larger and longer-lived (> 5 min) structures, termed focal adhesions (FA). The formation of FA is dependent on myosin-II mediated cell contractility, indicating that indeed mechanical forces play a pivotal role in determining FA structure and composition. The important role of individual integrin subunits and distinct FA molecules in shaping mechanosensitivity of FA has been appreciated [26, 108-110]. However, the molecular mechanisms driving the effects of specific FA components on cellular mechanosensitivity remain largely unknown.

The composition of cell-matrix adhesions is dynamic, so it is conceivable that many proteins partition into adhesions only temporarily as a function of cell perturbation or in a cell-type specific manner [3]. A pioneering study by the Sheetz lab, using two-dimensional gel electrophoresis to analyze protein binding onto Triton X-100–insoluble cytoskeletons as a result of mechanical stretch, revealed that transduction of matrix forces likely occurs through force-dependent conformational changes in the integrated cytoskeleton [111]. Subsequently, the structure and function of several prominent FA proteins, including talins [8], vinculin [108], and p130Cas [9], was shown to be affected directly by mechanical inputs. In those cases mechanical protein unfolding upon stretch was shown to either generate novel binding sites leading to protein recruitment and/or new PTMs on the stretched proteins. These new PTMs can then induce changes in protein complex formation. Thus, these examples show that transduction of mechanical forces implicates alterations in protein complex formation and localization at cell-matrix adhesions and/or the cytoskeleton. Recruited proteins can either reinforce the mechanical connections, or induce signaling events, or de-stabilize connections and induce FA turnover. For instance, the adhesome protein kank2 was recently shown to interfere with F-actin binding to the talin rod, leading to the suppression of mechanical force transmission across activated integrins [112].

Future functional proteomics studies will attempt to establish a global view on these molecular alterations. In order to analyze the dynamics of the full complement of proteins that can be recruited towards cell-matrix adhesions (adhesome) several groups developed protocols to deplete the cell body and analyze the remaining sample, which is enriched for cell-matrix adhesions, using MS [26, 71, 81, 82, 113-116] (reviewed in [117]). A meta-analysis of these first proteomic studies of the adhesome revealed a core set of 60 proteins that were robustly identified in the majority of datasets, likely representing key structural constituents of cell-matrix adhesions, including adaptor proteins such as Talin and Kindlin as well as actin-binding proteins and proteins containing LIM domains, such as the zyxin, Pdlim, Fhl, paxillin and Crp families [116]. The relative MS-quantification of adhesome proteins within FA preparations from cells subjected to myosin-II inhibition enabled a first unbiased analysis of myosin-II dependent recruitment to FA [71, 82], discovering the surprising prevalence of LIM domains in mechanosensitive proteins [5, 71]. The myosin-II dependent recruitment of LIM domain proteins was observed in both initial studies and Kuo *et al.* also showed a role of the Rac guanine nucleotide exchange factor β‑Pix in the negative regulation of focal adhesion maturation and the promotion of lamellipodial protrusion and FA turnover [82]. Moreover, the composition of cell-matrix adhesions was also studied using biotin proximity ligation in combination with MS. The authors identified 27 known adhesome proteins and 8 previously unknown components in close proximity to paxillin, including kank2, as well as new kindlin-2 interacting partners, liprin b1 and EFR3A [118]. Using the same technique Guo *et al.* characterized a network of proteins that putatively maintain and regulate cell-cell adhesions [119]. Overall 561 proteins were identified by proximity ligation to be associated with E-cadherin, and 419 of these interactions were completely novel. The majority of proteins were identified as adaptor proteins, proteins involved in cellular transport and protein synthesis [119].

A large number of kinases and phosphatases are also recruited to adhesion sites. In fact, the FA is the major site of protein tyrosine phosphorylation in the cell, resulting in prominent staining of FA when using a phosphotyrosine specific antibody [4, 120]. Using a combination of phosphoproteomic analyzes of integrin signaling and small interfering RNA-based functional screening, novel integrin-regulated kinases (DBF4, PAK2 and GRK6), with critical roles in cell adhesion and migration were discovered [121]. Both non-receptor tyrosine kinases such as FAK and SRC [122-127], and receptor tyrosine kinases (RTKs), such as AXL, and ROR2 [128], have been implicated in the mechanosensing response. Src kinase was shown to associate preferentially with β3- integrins [123, 126, 129, 130], and phosphoproteomics on cells expressing either β1- and β3- integrins alone or in combination, revealed that indeed integrin specific phosphorylation landscapes are generated upon adhesion to fibronectin [83]. Quantification of 7,529 phosphorylation sites showed that ~13% of the sites, including proteins in SRC target pathways, were regulated in an integrin subtype specific manner upon fibronectin engagement. A limitation of this study was that only the total phosphoproteome was compared between cells. It would be important to derive subcellular (`organellar´) phosphoproteomes and correlate the PTM site stoichiometry with protein recruitment and complex formation.

By applying a combination of the FA enrichment procedure used for adhesome proteomics with a phosphoproteomics workflow, Robertson *et al.* recently generated a first unbiased snapshot of the phosphoproteome in close proximity to FA (`phospho-adhesome´) [131]. Their analysis identified 499 phosphoproteins that were detected in fibronectin-induced adhesion complexes. Interestingly, FAK and paxillin, two of the most highly connected proteins in the PPI network, were also the most highly phosphorylated adhesome proteins. This highlights the fundamental role for phosphorylation in the regulation of PPI within the adhesome. However, this study did not address dynamic changes of PTMs during the mechanosensing response.

Cell-matrix adhesions form a continuum with the actin cytoskeleton, which assembles into branched networks or bundles in response to the cells mechanical environment and thereby generates both passive structural support and active mechanical forces. Thus, in concert with microtubules and intermediate filaments, actin networks confer shape, enable cell polarization, support cell-cell junctions, and promote cell adhesion and migration. Studying cytoskeletal functions in the cytoskeleton's native state is inherently difficult due to its rigid and insoluble nature. Klemke *et al*. developed a purification method that enriches for the cytoskeleton and its associated proteins in their native state that is compatible with current MS-based protein detection methods [132]. Proteomics studies have also shown that mechanical tension in cells induces abundance changes in cytoskeletal proteins [133]. For instance, vascular smooth muscle cells (VSMCs) from different regions along the aorta with opposing mechanical properties expressed different amounts of cytoskeletal and FA proteins [134]. Temporal phosphoproteomic profiles of VSMCs in response to cyclic stretch indicated that key regulators of the actin cytoskeleton, such as the protein kinase C (PKCs) family, Rho-associated coiled-coil containing protein kinase 1 (ROCK1) and Akt participate in the adaption of VSMCs to stretch [135].

* 1. Nucleus

Similar to the cytoskeleton, the nucleus is in dynamic reciprocity with the cells environment and stiffens with mechanical loading [136, 137]. A growing body of evidence suggests that the shape of the nucleus and associated regulation of chromatin architecture controls gene expression and cell fate determination [138]. Interestingly, it was observed that functionally related genes that are actively transcribed cluster into co-localized `transcription hotspots´ even if they reside on different chromosomes [139]. These hotspots are usually observed in the nuclear periphery, were chromosomes can be attached to the nuclear envelope (NE) [140, 141]. The structure and composition of the nuclear envelope can be altered by mechanical inputs [28, 136, 142], which triggered the idea that chromosome architecture and the localization and activity of gene expression hotspots could be targeted directly by mechanical forces. The NE is composed of the inner and outer nuclear membrane that is studded with nuclear pores. The NE’s main structural support is a fibrillar nuclear lamina (NL) at the inside of the inner nuclear membrane with the lamin A-type (lamins A and C) or B-type (lamins B1 and B2) proteins as its main components. A proteomic study on A-type lamins-depleted dermal fibroblasts revealed that the largest fraction of differentially expressed proteins is involved in actin cytoskeleton organization and FA dynamics, suggesting a feedback between the NL and the cell adhesion machinery [143].

The NL is attached to the cytoskeleton via the `linker of nucleoskeleton and cytoskeleton´ (LINC) protein complexes situated in the NE and containing Nesprins and Sun proteins amongst others [144]. Furthermore, the NL connects to DNA via a host of specialized adaptor proteins, such as emerin [145]. Recent MS studies revealed that cells contain hundreds of NE proteins, many of them specific to the nuclear membranes, and their expression varying between cell-types and tissues [146-151]. Each of those proteomic studies identified novel proteins previously not reported at the NE, with several having cytoskeletal associations that may help in keeping the nucleus in direct mechanical connection to the tissue microenvironment. Indeed, a direct physical and thus mechanical link from the desmosomes and hemidesmosomes to the nucleus, has recently been visualized in 3D cultures of mammary epithelial cells, using electron microscopy and super-resolution light microscopy [152]. Furthermore, deletion of the LINC complex protein Sun2 affected epidermal integrity in vivo, emphasizing the importance of the cross talk between the nucleus, cytoskeleton, and intercellular adhesions [153]. By applying the BioID method, Roux *et al*. identified over 120 proteins as being in close proximity to lamin-A, including a number of previously characterized NE components, known lamin interactors, proteins associated with nuclear‐cytoplasmic transport, as well as previously uncharacterized proteins, including FAM169A (later renamed SLAP75) [62].

Lamin-A and lamin-B confer different mechanical properties to the NL. In a landmark functional proteomics paper, Swift & Discher *et al.* developed MS strategies to measure protein abundance, stoichiometry, conformation, and interactions within the NL of tissues and cells in relation to stiffness of tissues and extracellular matrix [28]. They found that protein abundance of A-type lamins scaled with tissue stiffness so that they dominated in stiff environments while B-type lamins dominate in soft environments. Lamin-A confers mechanical resistance to the nucleus and using a method called cysteine-shotgun MS, which covalently labels cysteins that are involved in structurally labile disulfide bonds, they demonstrate conformation changes of lamin-A upon force application to the nucleus. Both, the incorporation of lamin-A to the NL and its turnover is regulated by phosphorylation. Swift & Discher *et al.* show with their MS approach that stiff environments induce lamin-A dephosphorylation at the important regulatory sites (e.g. S22), thereby keeping lamin-A in the NL and stabilizing it [28, 142]. Also emerin, another adaptor protein of the NE becomes tyrosine phosphorylated in response to force and was shown to be essential for the nuclear mechanical response to tension [137]. A proteomics approach, using AP-MS, showed that emerin scaffolds a variety of functionally distinct multiprotein complexes at the nuclear envelope in vivo [154]. Notably included were nuclear myosin I & II-containing complexes that might sense and regulate mechanical tension at the nuclear envelope [154]. He & Wickström *et al.* recently demonstrated an important role of an emerin/myosin-II mechanosensory complex at the outer NE in lineage commitment of epidermal stem cells. They show that force driven accumulation of emerin at the NE alters heterochromatin anchoring to the NL and leads to a switch in histone methylation landmarks at constitutive heterochromatin that control lineage commitment [155]. Another recent study demonstrates that tensile loading of mesenchymal stem cells alters chromatin organization, nuclear mechanics and histone methylation status [156]. Interestingly, they found that increasing strain levels and number of loading events led to a greater degree of chromatin condensation that persisted for longer periods of time after the cessation of loading [156]. This `mechanical memory´ effect reemphasizes the importance of epigenetic modifications in manifesting long term effects of mechanotransduction.

# In addition to the direct effects of mechanics on chromatin architecture, gene expression can also be regulated by nuclear translocation of transcription factors [157]. Nuclear translocation of these factors has been shown to be regulated by phosphorylation events [158], by cellular architecture and the physical state of the cytoskeleton [23, 159], and the composition and integrity of the NE [28]. Future functional proteomics studies will certainly analyze these changes of subcellular localization during mechanosensing in a proteome wide manner. The best characterized proteins whose nuclear translocation is clearly regulated by mechanical forces are the transcriptional effectors of the Hippo pathway YAP and TAZ [157]. The main target of YAP/TAZ is the TEAD family of transcription factors, which drive the expression of proliferative and anti-apoptotic genes [160]. Localization and activity of YAP and TAZ are regulated by several physical and biochemical milieus. In sparsely populated cells, YAP and TAZ localize to the nucleus. In the presence of cell to cell contact however, YAP and TAZ are inactivated and localize to the cytoplasm [161]. The group of Stefano Piccolo reported that a stiff matrix substrate activates YAP and TAZ, and promotes their nuclear localization, whereas a soft substrate inactivates them, and promotes their cytoplasmic retention [158, 162]. Subsequent studies provide evidence that YAP/TAZ activity in the nucleus critically depends on cytoskeletal integrity and is regulated by phosphorylation, which can be induced by actomyosin dependent contractility [163, 164]. When present in the nucleus, YAP and TAZ induce a wide range of downstream signaling cascades and also contribute to storage of mechanical “memory” of past ECM interactions [165]. By culturing human MSCs on stiff hydrogels for several days and re-locating them to soft substrates, it was found that YAP remained activated in the nucleus even 10 days after softening, indicating that this transcription factor is involved in translating the memory of past physical signals [165]. Using time-resolved proteomics on kidney podocytes shows that podocyte injury stimulates the expression of YAP target genes. Podocytes are constantly subjected to physiological filtration pressure and considerable mechanical strain. Interestingly, injury reduces YAP activity in cultured podocytes grown on stiff substrates. Culturing these cells on soft substrate or inhibiting stress fiber formation recapitulated the injury-induced YAP up-regulation observed *in vivo* [166]*.* Moreover,using label-free quantitative proteomic analysis, Kohli *et al.* identified novel TAZ/YAP interactors involved in regulation of gene expression by matrix stiffness [167].

Another example of a mechanosensitive transcription regulator is [serum response factor](http://www.sdbonline.org/sites/fly/newgene/serumrf1.htm) (SRF) and its cofactor myocardin-related transcription factor (MRTF), known also as MKL1 or MAL. The nuclear translocation and activity of MAL is regulated by the ratio of monomeric globular (G-) actin to filamentous (F-) actin content. Upon release from G-actin, MAL binds to SRF and activates the transcription of cytoskeletal and FA proteins [159]. Thus, the activity of SRF/MAL can be induced by tension, which is known to alter the G-/F-actin balance in an integrin dependent manner [168]. Accordingly, the expression of αV and β1-class integrins differentially affects the G to F-actin ratio and activity of SRF/MAL [169]. Interestingly, integrin signaling can also affect nuclear actin dynamics and reduce the fraction of G-actin bound inactive MAL in the nucleus [170]. The transmission of tension along stress fibers to the nucleus and subsequent formation of nuclear F-actin is dependent on the LINC complex, lamin A/C and emerin so that deletion of these proteins also affects SRF-MAL activity [170, 171].

Future functional proteomics studies of mechanosensing will likely reveal the spatiotemporal changes of proteins between cellular compartments and also improve our understanding of molecular alterations in the nucleus.

1. **Perspectives**

Proteomics has significantly contributed to our understanding of cell biology in the last decade. Recent developments in MS methods substantially increase quantitative accuracy, sensitivity of detection and sample throughput and will thus enable a more systematic characterization of cellular mechanosensing. Cell-matrix adhesions, the cytoskeleton, and the nucleus are remodeled by mechanical tension. The accompanying alterations in subcellular localization of proteins, and how these are regulated by protein interactions and modifications such as phosphorylation are currently not very well characterized. The use of MS-driven proteomics to study mechanobiology is in its early days. Future studies will use a combination of cellular fractionation protocols and PTM proteomics to correlate protein modification occupancy with subcellular localization and protein interactions. Proteome wide XL-MS is an exciting new field of research, which holds great promise in bringing an unbiased interaction proteomics approach to the molecular events *in situ* as they happen when tissues or cells are subjected to mechanical perturbations.

We propose that a functional proteomics approach to cellular mechanotransduction needs to employ a hybrid set of MS discovery methods together with medium to high throughput means of functional interrogation such as CRISPR/Cas mediated genome editing. In order to build hierarchical networks of functional relationships during cellular mechanosensing, it will be necessary to analyze the effects of mutants on a proteome wide scale. For instance, deleting one protein in a pathway or mutating a selected phosphosite can affect one distinct mechanosensitive signaling module, which can be observed on the level of the phosphoproteome, while other mechanosensitive modules may stay unaffected. With such observations we will learn a lot about the interdependence of signaling events during mechanosensing. Furthermore, such experiments will reveal novel signaling intermediates that serve as essential hubs and/or threshold modulators for mechanical inputs in cells. High content data analysis is necessary for the interpretation of such experiments. We envision that datasets with many perturbations (mutants), some of which may cause similar effects, while others cause unique effects, may enable to predict mechanosensitive `pathways´ de novo. Ultimately, the identification of signaling switches and their effects during cellular mechanosensing will enable a better understanding of cellular activities in diseases such as tissue fibrosis and cancer, and may reveal potential targets for therapy.

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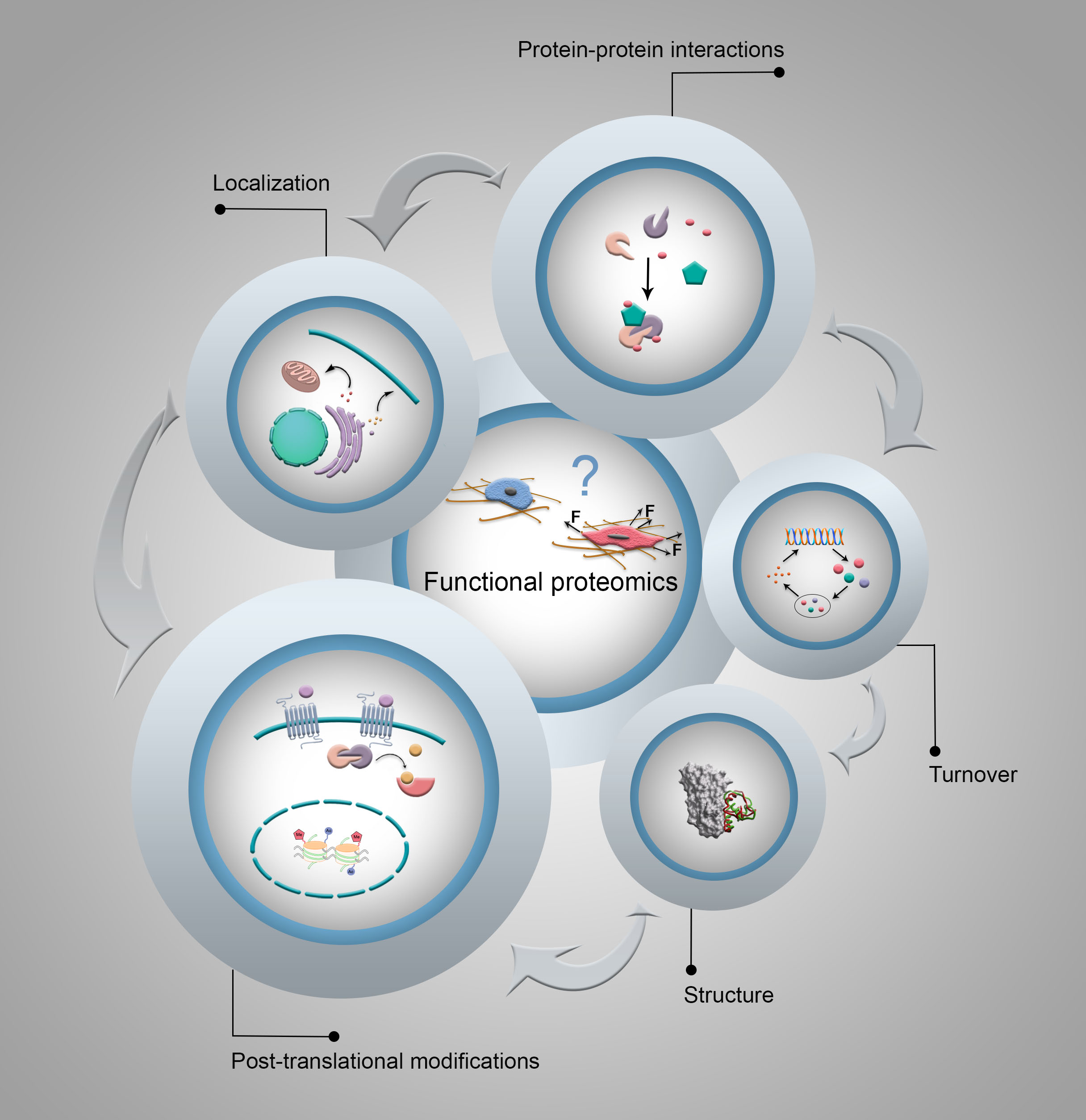
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**Figure 1.** **Application of mass spectrometry (MS)-driven functional proteomics in systems biology studies of cellular mechanosensing.** The translation of biomechanics (F, force) into biochemical signals likely involves a multitude of protein complexes within cell adhesions, the cytoskeleton and the nucleus that are directly or indirectly affected by mechanical strain. Functional proteomics typically aims at the analysis of molecular alterations on a proteome wide scale and is one of the power houses of modern life science. Recent developments in sample preparation methods, MS instrumentation and computational workflows, enables the accurate and sensitive analysis of protein abundance and stability, post-translational modifications (PTMs), structure, interactions, and subcellular localization. Future systems biology studies of cellular mechanotransduction will integrate these layers of information to identify critical signaling intermediates that serve as essential hubs and/or threshold modulators for mechanical inputs in cells.