Divide and Conquer: the Application of Organelle Proteomics to Heart Failure

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Abstract

Chronic heart failure is a worldwide cause of mortality and morbidity and is the final outcome of a number of different etiologies. This reflects both the complexity of the disease and our incomplete understanding of its underlying molecular mechanisms. One experimental approach to address this is to study subcellular organelles and how their functions are activated and synchronized under physiological and pathological conditions. In this review, we discuss the application of proteomic technologies to organelles and how this has deepened our perception of the cellular proteome and its alterations with heart failure. The use of proteomics to monitor protein quantity and post-translational modifications (PTMs) has revealed a highly intricate and sophisticated level of protein regulation. PTMs have the potential to regulate organelle function and interplay most likely by targeting both structural and signaling proteins throughout the cell, ultimately coordinating their responses. The potentials and limitations of current proteomic technologies are also discussed emphasizing that the development of novel methods will enhance our ability to further investigate organelles and decode intracellular communication.

Keywords

Proteomics; Heart Failure; Posttranslational Modifications; Organelle; Communication

INTRODUCTION

Chronic heart failure (HF) can be the outcome of several disease states such as hypertension, ischemic heart disease or valvular defects[1]. Despite these diverse etiologies, a number of features are frequently shared, including impaired Ca^{2+}-handling, depressed contractility and altered energy metabolism. Each feature reflects functional changes in specific subcellular organelles such as sarcoplasmic reticulum (SR), sarcomeres and mitochondria[2-4]. Other common features of HF (such as the activation of intracellular signaling in response to

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DISCLOSURES
None

increased adrenergic and neuro-hormonal stimulation, and perturbation of organelles' spatial distribution) also imply an orchestrated action involving multiple organelles[5-7]. Yet it is unclear exactly how the various organelles coordinate their responses under both physiological and pathological conditions. Indeed, their dys-coordination could be considered another feature of the failing heart[8].

Organellar interplay can occur via two routes: second messengers or structural connections (Figure 1). The concentration of second messengers (e.g. Ca^{2+}, NO• and cAMP) fluctuates throughout the cell. It is their local levels that regulate the activity of downstream protein targets, either directly or indirectly, via signaling cascades; and a common final outcome is the post-translational modification (PTM) of their protein targets. These proteins can be localized to different organelles providing a means for widespread cellular action. A good example is Ca^{2+}, which binds directly to both the sarco(endo) plasmic reticulum calcium-ATPase 2 (SERCA2) in the SR and troponin C (TnC) in the sarcomeres[9, 10]. Another example is cAMP which can activate protein kinase A (PKA), resulting in phosphorylation of proteins throughout the cardiomyocyte[11].

Direct physical connection is the other mechanism for organelle interplay, occurring through the three major components of the cytoskeleton: microfilaments (actin), intermediate filaments (desmin which is specific to cardiomyocytes) and microtubules (tubulin)[12]. The roles of microtubules and the actin cytoskeleton in cellular plasticity, motility and protein trafficking are well known[13, 14], as is the role of intermediate filaments in maintaining proper localization of different organelles[15]. However, novel functions for modified cytoskeletal proteins in diverse cellular functions are emerging[16-19].

We postulate that PTMs are important in the development of HF as they likely mediate, and therefore regulate, organellar cross-talk. To test this hypothesis, organelles can be analyzed after sub-cellular fractionation using a variety of proteomic technologies. An additional advantage of this approach is the enrichment of low abundant, organelle-specific proteins. The integrated analysis of the data generated from several organelles can help highlight common molecular components involved in the coordination among different cell compartments.

In the present review, we describe how our knowledge of HF has benefitted from proteomics' ability to scrutinize biological processes and reveal the new levels of complexity introduced by PTMs. We have also included novel analytical approaches, and discuss how these can be applied to specific biological questions regarding HF. These hands-on examples illustrate the contribution of proteomic approaches in moving the field forward.

Proteomics: When, Why and How

Historically, proteomics originated when separation technologies such as two-dimensional gel electrophoresis (2DE) and liquid chromatography (LC) were combined with mass spectrometry (MS). The need to increase our understanding of the "PROTEin complement of the genOME", the PROTEOME, has driven the continuous development of these technologies. With the implementation and integration of various technological platforms it has become possible to separate, identify, characterize and quantify complex mixtures comprising hundreds to thousands of proteins or peptides[20]. These are key features to gather a more thorough understanding of intricate and elusive diseases such as heart failure. Table 1 gives an overview of the most common technologies, with their advantages, limits and possible biological applications.

A few key concepts have emerged from the proteomic studies performed over the last sixteen years. First, even a single protein can be a complex mixture of its many modified forms. Through the specific measurement of a number of PTMs, and the determination of the actual modified amino acid residues, it has become clear that a protein can have multiple PTMs at multiple amino acid residues. Furthermore, different PTMs may compete with each other for a particular residue[21]. To date, over 400 different PTMs have been described, although far fewer have been documented in higher organisms such as mammals (see a detailed list at http://www.uniprot.org/docs/ptmlist). In the cardiac subproteome, phosphorylation is by far the best described PTM and most modified proteins have multiple phosphorylation sites (Figure 2A, Online Table I). To date, phosphorylation has been observed for 78%, 80% and 48% of cardiac proteins located in the SR, myofilaments and mitochondria, respectively (Figure 2B, Online Table II). However, the wide majority of the potential PTMs have not been fully investigated, in part due to a lack of technologies that specifically target them. Moreover, as a cautionary note, because the extent of PTMs occurrence is not clear and PTMs can modify the antigenic properties of proteins, quantification methods that rely solely on antibodies may lead to biased conclusions.

Second, in multicellular organisms, different cell types share many of the same proteins (proteome redundancy). These common proteins are part of essential cellular systems and may reflect the minimum number of functional proteins required for cell survival. This implies that the unique attributes of a specific cell type must be derived from variations in protein quantity, isoform expressions (from splice variants or different genes) or PTMs. Furthermore, this suggests that the regulation of proteins at the time of transcription and translation, as well as protein complex assembly, play a major role in determining the exact composition of the proteome and thus the cellular fate. Data to support this concept arise from a number of detailed MS-based analyses, including work on mitochondria isolated from different tissues (heart, kidney, liver and brain)[22, 23]. These studies demonstrate that, at least two third of mitochondrial proteome are conserved across different tissues. As the levels of these shared proteins greatly vary from organ to organ[23] it is possible that the *quantity*, rather than the type of protein within functional clusters discriminates among different tissues. However, protein isoforms are also important, as illustrated by the cell specificity of the Tn-complex (comprising TnT, TnC and TnI): the different cell-specific isoforms for each subunit constitute unique attributes of a cardiomyocyte. As well, various combinations of the subunits' isoforms (slow and fast) differentiate cardiac and skeletal myocytes[24].

Third, technological development is fundamental for scientific advancement. Improvements in each step of the proteomic pipeline have expanded the number and types of proteins (e.g. hydrophobic, basic, etc) that can be observed. Considerable contributions have arisen from methods that improved protein separation (e.g. 2DE[25, 26]), or enrichment of PTM peptides (e.g. O-linked beta-N-acetylglucosamine, O-GlcNAc[27, 28] and phosphorylation[29-31]). Developments in mass spectrometers have also lead to enhanced speed, sensitivity and mass accuracy. When coupled with advanced bioinformatic techniques, this has resulted in reduced false discovery rates and improved protein identification.

Hundreds of review articles on proteomics and human diseases have been published, but not as many address the application of proteomic technologies in the elucidation of cardiac disease (selected reviews[32-37]). In the next section we will describe the different techniques commonly utilized in organelle proteomics, beginning with sub-cellular fractionation and following each step of the proteomic workflow through MS-based protein characterization.

Sub-Cellular Fractionation for Proteomics

Each separation technique used in proteomics has a limited capacity regarding the number and types of proteins that can be resolved. This technical limitation prevents the detection of low abundant proteins and cannot always be overcome by simply increasing the total protein load as this may paradoxically result in protein loss (e.g. by precipitation). Sensitivity and resolving power can instead be increased by focusing on a particular sub-proteome[35, 37]. This requires balancing two aspects: sample purity, and preservation of one protein's PTM status. Importantly, regardless of the methods employed, it is difficult to isolate a pure organelle preparation. It is equally difficult to prove that a preparation is pure. This arises from the dilemma that a subproteome can comprise resident organellar proteins, those with multiple cellular localizations and contaminating proteins. Enrichment of a particular protein during the preparation can help, but validation through other techniques, such as microscopy, is ultimately required to confirm sub-cellular localization[23].

When compared to traditional biochemical organelle purification methods, a major concern for proteomic studies is keeping modifications intact during fractionation. To avoid introducing artificial PTMs during sample processing, either pharmacological inhibitors (e.g. phosphatases, proteases inhibitors) or harsh denaturing conditions can be used[35, 38]. On the other hand, mild conditions are required when enzyme activity or protein-protein interactions (e.g. complexes) are being addressed[39]. For example, our group has optimized a fast and reproducible method (called IN Sequence) to fractionate frozen heart tissue for proteomic applications: dividing the cardiac proteome into separate myofilament- and cytosolic-enriched fractions[40]. This method was developed to deplete the high abundant myofilament subproteome, allowing enhanced observation of the cytosolic proteins and preserving the original endogenous PTM status. While this method is appropriate for proteomics, it leaves the myofilament subproteome denatured, inactive and not amenable for functional studies. Additional precautions may be required for some oxidative PTMs, where the procedures should be performed in the dark (e.g. S-nitrosylation[41, 42]). For PTMs that are extremely labile (due to chemical instability or activation of regulatory enzymes), the speed of the procedure will be a factor in protocol optimization. The decision on which approach to use depends on the biological question and the assays that will be performed downstream of the proteomic pipeline (such as enzyme activity and other functional assays).

Protein and Peptide Separation Techniques

Electrophoretic Methods: Classical and Non-classical 2DE

Two-dimensional gel electrophoresis (2DE), has been used for protein separation since the first proteomic study[20]. The 2DE protocol most widely used today was developed in the mid '70s by O'Farrell[43]. Two notable improvements since then are: the introduction of immobilized pH gradient strips for isoelectrofocusing (IEF)[44] and fluorescent protein labeling[45], which greatly reduce technical variability. In fact, researchers can now reproducibly separate thousands of protein forms and reliably quantify protein changes across different samples by combining these methods (Table 1)[25, 36, 46].

In the first dimension of classical 2DE, proteins are separated according to their isoelectric points (pI) by IEF. Some PTMs, such as phosphorylation, acetylation, deamination, or alkylation can modify one protein's pI, inducing a shift along the IEF axis[47]. In the second dimension, proteins are separated according to their size using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). Other PTMs can modify protein size and thus, electrophoretic mobility in the second dimension; these include proteolytic cleavage, glycosylation and ubiquitination[48]. Phosphorylation can also

slow down electrophoretic mobility, inducing an apparent alteration in protein size, most likely because of the interference with protein-SDS interactions[49].

Recently, several "non-classical" 2DE techniques have been re-discovered and optimized combining different separation methods. One example is blue-native gel electrophoresis (BNE) where proteins are separated under non-denaturing conditions. Protein complexes can be separated by BNE followed by further separation under denaturing conditions, either by SDS-PAGE or classical 2DE. The initial BNE step contributes additional information regarding protein-protein interactions and the composition of protein complexes[50].

2DE has the advantage of providing a quantification method based on the area/volume of a protein spot after gel staining. Spot matching and differential display analysis are carried out through semi-automated software analysis. Fluorescent stains and, in particular, the difference gel electrophoresis approach (DIGE[45]) offer a very reliable platform for quantifying changes in protein levels between different experimental conditions (e.g. disease vs. healthy). Moreover, the image analysis that used to be extremely laborious can now be contracted to companies that perform this routinely[51]. Despite 2DE's advantages, a few considerations have to be mentioned. Each spot visualized can be a mixture of several proteins. In this case, another quantitative step is required to determine the specific protein responsible for the difference in spot intensity[52, 53]. As well, a single protein maybe presented as multiple spots, each representing different forms. Therefore, a 2DE gel containing 1,000 spots may only represent 300-400 different proteins[54]. These factors should be taken into account when protein identity and quantity changes are assigned based on 2DE.

One physical limitation of 2DE is the poor capacity for studying hydrophobic proteins, because anionic detergents cannot be used in the first dimension (IEF)[55, 56]. Although we[57, 58] and others have shown previously that is possible to separate highly hydrophobic proteins on 2DE, alternative techniques such as liquid chromatography (LC) or SDS-PAGE may be a preferable strategy. 2DE is, however, currently the only method that provides a direct indication of many different PTMs in a single analysis.

Chromatographic Methods: 1 and 2DLC

Different LC techniques such as ion exchange, size exclusion, affinity and reversed-phase (RP), can be employed for the separation of complex protein and peptide mixtures based on their intrinsic properties (i.e. size, charge/pI, hydrophobicity and protein-binding properties[40, 59, 60]). In these methods, analytes are separated in solution according to differences in their partitioning between the stationary and mobile phases. The most widely used LC in proteomics is RP, either off- or on-line with MS. A wide selection of chromatographic materials for RPLC is available: the choice of stationary phase (particle size, pore size, surface area, and chemistry of the substrate surface), and, to a lesser degree, flow rate and buffer composition, can be optimized to improve resolution.

As with gel-based methods, the combination of different analytical strategies such as in multi-dimensional LC (nDLC) increases resolution[59-62]. The separation of peptides by two-dimensional LC (2DLC) after protein digestion is referred to as shotgun approach[63, 64], as opposed to 2DLC systems that separate intact proteins, potentially providing additional information about protein isoforms and PTM status[65-67]. LC methods are advantageous due to minimal operator-dependence, although they require more complex MS capability and have a far greater MS workload than gel-based methods (Table 1). Similar to 2DE, LC methods do not allow for unambiguous assignment of quantity changes based on elution profile alone, and require protein labeling prior to MS (see below).

A specific LC technique that is worth mentioning is affinity chromatography. Affinity chromatography is based on reversible antigen-antibody interaction, and allows protein or protein complexes to be recovered with good purity and in some cases with retained biological activity[68-70]. The most common technical challenges with affinity chromatography are: determining which proteins are contaminants due to nonspecific binding; and distinguishing between the direct and indirect binding partners that form a protein complex. Different factors that can contribute to nonspecific binding are ionic or hydrophobic interactions. These can be limited by carefully optimizing both washing and binding conditions.

The use of multiple proteomic methods that combine different protein and/or peptide separation methods increases the overall coverage of the proteome[71, 72]. This is particularly important when studying species which have poor or underrepresented annotation, such as rabbit or dog[73, 74]. In these species, isolation of a higher number of peptides is required for exact protein identification and characterization. In fact, enhanced detection power increases the probability of matching a peptide sequence to a homolog in a more annotated species.

Protein Identification and Quantification Methods: Mass Spectrometry

Today, MS is the most common method for identification of proteins. MS has eclipsed other techniques, such as Edman's degradation, due to its speed and level of detail (e.g. PTM characterization; for historical account of MS development see Zhou et al.[75]).

MS instruments consist of three modules: an ion source, a mass analyzer and a detector. Once ionized, particles are accelerated and sorted according to their mass to charge ratio (m/z) in the mass analyzer, before they are detected. Thanks to the development of soft ionization methods, such as matrix assisted laser desorption (MALDI) and electrospray ionization (ESI), it is now possible to ionize large biological molecules including peptides or even entire proteins. The impact of these technologies on modern science is fundamental and their inventors were awarded the Nobel Prize for chemistry in 2002[76]. Common analyzers include time-of-flight (TOF), quadrupoles (Q) and ion traps. Each of these has been combined in tandem mass spectrometry (MS/MS or MS^2) so that peptides can be further broken down using collision induced dissociation (CID). An advantage of MS^2 is that it provides detailed chemical information about the amino acid sequence[77] (Online Figure I). Because mass accuracy is best over the low mass range, analysis of peptides rather than intact proteins allows for superior mass detection and, consequently, improved amino acid sequence assignment (called a "bottom-up" analysis). This fact, along with other benefits of peptide-centric proteomics (which include better separation, ionization, and database searching, see above), has determined the larger use of bottom-up approaches. However, MS can also be used to characterize intact, non-digested proteins, with or without prior separation (also known as a "top-down" approach)[78, 79]. Due to the continuous improvements of MS technologies, it is foreseeable that top-down approaches will become increasingly popular in the future.

Several approaches based on MS have been developed for quantification. These can be done with or without adding a mass tag to the peptide (tag- vs. label-free methods, respectively)[80]. Label-free methods base quantification on the signal intensity originated in the MS (peak area) or on the number of times a peptide is observed[81, 82]. Tag methods require peptide labeling with isotopic tags[83] via enzymatic (e.g. stable isotope labeling with amino acid in cell culture (SILAC) and ^{18}O-labeling[52, 84, 85]) or chemical derivatization (e.g. isobaric tag for relative and absolute quantification (iTRAQ))[86]. SILAC can only be used in proliferating cells, but chemical derivatization methods are more flexible and allow labeling of protein extracted from tissue and body fluids. In chemical methods, samples are

labeled using tags with the same molecular weight (isobaric). Once labeled peptides are introduced in the MS^2, each tag releases a different fragment with a specific mass that can be tracked in the lower portion of the spectra (~100 Da m/z). The intensity of the peaks generated by these reporter ions is proportional to the quantity of the protein in the original sample. Typically, at least four different tags are available, allowing multiple biological conditions to be analyzed simultaneously (multiplexing).

A newly adapted MS technology is multiple reaction monitoring (MRM) and its closely related method single/selective reactive monitoring (SRM)[87, 88] (Online Figure I). MRM is a tandem MS technique that allows relative and absolute quantification of proteotypic peptides (i.e. peptides that have a unique amino acid sequence specific to a particular protein or isoform) and, hence, of proteins. In short, triple quadrupoles (Q_3) MS can be used to select a specific peptide of interest (including modifications or polymorphism[89, 90]) based on its m/z, in quadrupole Q_1. This parent ion is then fragmented in the Q_2 and the daughter fragments (also called transition ions) are monitored and quantified in the Q_3. The choice of the transition ions is critical as it allows monitoring a specific peptide, based on MS^2 identification. Normally, few transitions need to be monitored in order to achieve reliable quantification (n 5). In many cases, heavy isotope-labeled standard peptides are spiked in for absolute quantification, but relative quantification (sample A vs. B) is relatively straightforward. The biggest advantages with MRM are the unbiased quantification and the ability to distinguish between PTMs and other protein forms. This capacity is often missing when using antibodies[88]. In addition, multiplexing is straight-forward compared to ELISA and a relative large number of peptides can be quantified during a single run.

PTM Characterization

As mentioned earlier "a protein" can be a complex and highly dynamic mixture of its modified forms. Important aspects of one protein's function (such as activity, localization, half-life and ability to form complexes) are all regulated by PTMs, and thus it is crucial that they be characterized. MS, alone or in combination with separation and/or enrichment techniques, currently represents the preferred method for PTM characterization. Most PTMs are recognizable in a MS spectrum through specific mass signatures. For instance, phosphorylation typically induces an increase in mass of 80 Da due to the weight of the phosphate group. A more common observation is the occurrence of neutral loss: the loss of a phosphate group alone (80 Da) or plus water (80+18=98 Da)[91] (for a review on phospho-proteomics see[92, 93]).

Two main factors limit the detection of peptides containing PTMs. One is the stoichiometric relation between the modified peptide and all other peptides in the samples. In other words, because peptide detection is a competitive process and the amount of a peptide with a single type of modification is relatively low, more abundant, variously unmodified and modified peptides are likely to be detected first. In some cases this limitation can be overcome by enriching for the specific modified peptides downstream of protein digestion (Figure 1). Such is the case for phospho-peptides. There are a few protocols available, based either on the affinity of phospho-peptides for positively charged metal ions[94, 95] or on other LC methods; RPLC[96] and hydrophilic interaction chromatography[97]. Another consideration of PTM analyses is related to the modification's stability. For example, the detection of phospho-peptides by MS is challenged by the lability of the phosphate bond during CID. Phospho-esters are weaker than peptide bonds; therefore they tend to break during fragmentation. For this reason a method that catalyzes the preferential fragmentation of peptide bonds with improved detection of phospho-peptides in the MS has been developed (electron transfer dissociation)[91]. Similar considerations can be made for other PTMs, although phosphorylation is currently the most studied[98]. Until recently, a technological

challenge was site-specific quantification of a given PTM. With MRM, it is now possible to measure the relative and absolute abundance of PTMs at each site within and across different proteins[89, 90].

Bioinformatics

Bioinformatics is integral to proteomics. In fact, bioinformatic tools are needed to assist almost every step of the proteomic workflow. This includes the quantitative analysis of proteins and peptides after separation, as well as the MS data handling[99]. Bioinformatic tools are also utilized for the prediction of protein characteristics (such as pI and MW values, PTM consensus sequence, etc.), and several algorithms are publicly available (e.g. www.expasy.net). Also freely accessible are a number of curated protein databases (see Online Table III). While these databases are essential for proteomic studies, it is important to mention that they are still under development and therefore far from being exhaustive. The number of proteins assigned to a specific tissue or organelle (Figure 2) depends on the way these are annotated in the database. Also the total number of residues modified by a given PTM in a protein (Figure 2A, right pie chart, Online Table I) will vary depending on the annotation criteria (Online Table I). For instance, modifications can be assigned to a given protein entry based on the consensuses sequence of the modifying enzyme or if the modification has been observed in an isoform.

It was the establishment of the first genomic databases, as a basis for the creation of protein databases that allowed proteomics to develop. Nowadays these protein databases are routinely mined by algorithms that can match experimental MS spectra to protein sequences fragmented *in silico*, in a semi-automated fashion[100]. Algorithms, like OMSA, Mascot, Sequest, X!tandem are able to extract the numerical values of peptide masses from MS spectra and then search against protein databases to assign peptides to a protein entry. In all cases, manual inspection of the spectra is warranted, especially when there is an indication of a potential modification. In fact, being programmed "to retrieve" (e.g. assign a peptide to a protein), these algorithms show an inherent bias for false positive identifications. Another issue associated with database-based identification is the fact that the databases can have multiple entries for a given protein (database redundancy). This can inflate the list of assigned entries. To overcome this issue and ensure single assignments, protein clustering algorithms can be helpful identifying sequence similarities based on a high degree of global (across the whole protein) or local (short protein domains) homology. Moreover, the correct assignment of a particular protein isoform or taxonomy (species) often requires extensive sequence coverage. This is because an amino acid sequence that is unique for a particular isoform/taxonomy must be assigned. On one hand, database redundancy and poor sequence quality can generate false positive identifications; on the other hand, the poor annotation of some species (e.g. dog or rabbit) can decrease the probability of identifying a protein correctly (false negative). These and other issues with database search were recently identified by the Human Proteome Organization as the major cause of lab-to-lab variability in reporting protein identification[101]. Even though databases are likely to improve in the future along with MS performance, awareness and education on these issues will be crucial for the generation of reliable data.

DIVIDE AND CONQUER: ONE SUBPROTEOME AT A TIME

The different proteomic techniques described above, from cellular fractionation to detailed protein characterization by MS, constitute a powerful tool in studying such a complex disease as HF. Even deeper insight on the disease can be gained by dividing the cardiomyocyte into separate fractions, and analyzing the resulting subproteomes, rather than studying the whole tissue. In this section we will provide examples of how proteomics has

been successfully employed to increase our understanding of the role of sarcoplasmic reticulum, myofilament and mitochondria in relation to cardiac disease.

The Sarcoplasmic Reticulum – Control of Calcium and Beyond

The SR organelle, appearing as an intracellular membrane network, serves as the main storage for intracellular Ca^{2+} in cardiomyocytes. It has been the dogma that release of Ca^{2+} from SR, in a Ca^{2+}-sensitive manner, is essential and necessary for normal cardiac function[102]. Thus, the observed reduction in cardiac force in HF patients has been partly explained by a reduction in the amounts and activity of Ca^{2+} cycling proteins. Particular focus has been targeted on two SR proteins: SERCA and its regulator, phospholamban (PLB). SERCA is the main regulator of Ca^{2+} up-take during cardiomyocyte relaxation, and its activity is tightly modulated through interaction with PLB[9].

When studying SR proteins, the initial challenges are the organelle's extensive membrane network and its connection to other cellular compartments, which complicate the isolation of a pure structure. Also the hydrophobic properties and relatively low abundance of the proteins comprising the SR can make them difficult to study. A number of general strategies have been developed for the enrichment of membrane proteins and these are applicable to SR proteins. These methods include fractionation by density gradients and/or differential centrifugation steps. To prevent precipitation of the hydrophobic proteins, they can be solubilized using high concentrations of detergents. This can be followed by electrophoretic separation and MS identification[103, 104]. Another approach is to avoid the gel separation step by employing shotgun methods in the presence of MS-friendly surfactants that are compatible with in solution enzyme digestion and MS identification (e.g. Rapigest[TM105]). The first study to identify SERCA using 2DE, successfully combined centrifugation enrichment with diheptanoyl-phosphatidylcholine solubilization[105]. Although the report showed that it is possible to achieve considerable enrichment of the SR, and that the SERCA protein can be separated by 2DE, it also illustrated the current challenge separating SR from other membrane organelles. Most preparations contain other membrane proteins (in particular from mitochondria[103, 105, 106]) and this complicates the goal of a precise identification of the SR sub-proteome. Due to the ambiguous definition of the SR sub-proteome, leading to a poor implementation of the term "sarcoplasmic reticulum", the proteins from this organelle are underrepresented in different databases (e.g. the ryanodine receptor is not annotated to the SR in the UniprotKB, Figure 2B, Online Table II).

A central role for SERCA2 in cardiac disease was questioned in last year's study by Andersson and colleagues[107]. This study showed that a cardiac-specific, inducible SERCA2 KO mouse survived for several weeks even with no detectible levels of SERCA2 protein in the cardiomyocytes (based on western blotting). Surprisingly, under these conditions the SR still contributed with Ca^{2+} to the cardiomyocyte contractions. This result implies either that the heart uses an alternative mechanism for cycling Ca^{2+} into the SR or that a small, non-immuno-reactive fraction of SERCA2 is present. As mentioned, due to the problems in obtaining pure SR, it remains debatable which other SR proteins might be up-regulated or modified as a consequence of severe SERCA2 depletion. This emphasizes the need for an accurate description of the SR sub-proteome and precisely how it changes with heart disease. An alternative strategy is to specifically target SERCA2 using affinity chromatography combined with targeted MS measurements (e.g. MRM).

Several genetically modified mouse models targeting expression of SERCA and PLB have been constructed to study the overall biological effects on Ca^{2+}-cycling disruption (SERCA conditional knockout (KO)[107]; PLB KO and over-expression[108]). As expected, significant effects on cardiac contractility due to altered Ca^{2+}-homeostasis are commonly observed in

these models. Interestingly, by applying different proteomic approaches (e.g. 2DE, gel-free, MS^2), it is clear that alterations in Ca^{2+}-handling induces phenotypical effects beyond the contractile apparatus. These include changes in cytoskeleton protein composition, membrane receptors and intracellular signaling pathways, and energy metabolism[109, 110].

The Sarcomeric Organelle – Power is Nothing without Control

Sarcomeric proteins are generally thought of as the molecular motors in the cardiomyocyte, generating force and conducting cellular shortening. Briefly, Ca^{2+} released from SR binds to TnC which alters the interaction between the thin (actin, tropomyosin, and troponin - consisting of TnI, TnT and TnC) and thick (myosin comprising myosin heavy chain and two light chains, and myosin binding protein C) filaments leading to muscle contraction[10]. Recently, it has been proposed that sarcomeres should be regarded as a cellular organelle because of its close involvement in structural, electrical, and metabolic aspects of cardiomyocyte dynamics[111]. In HF, this reduction in cardiac pump function can be related to changes in the SR but also to sarcomeric function[10]. While numerous mutations in the sarcomeric proteins causing familial hypertrophic and dilated cardiomyopathies have been described[112], the importance of PTMs for sarcomeric proteins is also well established[113].

In the Tn complex, TnI is the inhibitory subunit[111] and its function is highly regulated by phosphorylation. The importance of Ser23 and Ser24 in controlling cardiac contractility and myofilaments responsiveness to Ca^{2+} has been extensively studied[114]. Recently, several other phosphorylation residues were identified, and currently there are seven known sites in humans (Ser23, Ser24, Ser31, Ser42, Ser44, Thr141 and Ser150)[115]; some of these are also important in the control of cardiac dynamics[114]. In contrast, when exploring the basal *in vivo* phosphorylation of TnI in a murine model, only the Ser22/23 sites (equivalent to the human Ser23/24) were found to be modified[116]. The biological importance of these TnI phospho-sites is under debate[115]. Our group has developed MRM assays to specifically quantify and monitor the phosphorylation status of six of the identified TnI phosphor-sites to determine the stoichiometric ratios of each residue *in vivo*.

One contribution of proteomic technologies to basic science is the rapid discovery of new PTMs, including the modified residues. An example, which was recently identified in the sarcomeric proteins, is acetylglucoseamine at Ser and Thr residues (O-linked beta-N-acetylglucosamine, O-GlcNAc). When first discovered, identification of O-GlcNAcylation was challenging and time consuming[117]. However, today's enrichment methods, in combination with sensitive MS, have established widespread occurrence of this PTM[21]. Recently, O-GlcNAcylation was implicated in myofilament contractile alterations[118]. Ramirez-Correa and colleagues discovered myofilament Ca^{2+} desensitizing when exposing cardiac TX-100-skinned muscle fibers to GlcNAc (without disturbing the phosphorylation status of Ser23/24). Using MS-based methods, the group indentified 32 O-GlcNAcylation sites in the myofilament subproteome, one being Ser150 of TnI. Knowing that there is extensive crosstalk between O-GlcNAcylation and phosphorylation[21], it will be interesting to see how the alternate modification at Ser150 contributes to the biological function of TnI and the regulation of muscle contractile function.

Mitochondrial Proteomics: PTM Switches to Control Energy Production

Because the heart consumes more ATP than any organ of the body, it is particularly rich in mitochondria[119]. During the development of HF, both the energy demands and metabolism change, showing a remarkable decrease in oxidative phosphorylation and a shift towards glucose over fatty acid utilization[120]. Interestingly, during HF therapy like cardiac resynchronization (CRT), coordinated changes in the mitochondrial proteome suggest a

reversal to a healthier phenotype. These alterations include proteins controlling metabolism and ATP production, establishing the importance of mitochondria in modulating the development of HF[58].

The extensive application of proteomics to mitochondria also has historical and practical reasons. The study of mitochondria has exploited protein biochemistry for many decades, and proteomics itself can be considered an evolution of protein biochemistry. Furthermore, methods to enrich and purify the mitochondria are also several decades old, representing one of the most long-lived examples of proteome sub-fractionation[121]. Proteomic studies have certainly increased our understanding of mitochondria by providing evidence for the large and unexpected occurrence of PTMs.

The human mitochondrial proteome was originally characterized by Taylor et al., who initially described 651 mitochondrial and mitochondrial associated proteins[122]. Now, curated databases for mitochondrial proteins are also available[123], including one recently compiled by Mootha and colleagues, who assigned 1,098 mitochondrial proteins (MitoCharta) by combining extensive MS characterization with microscopy and computation to confirm mitochondrial protein localization[23]. Unfortunately there is a lack of equivalent databases for the less annotated species that are used in cardiac research (e.g. rat, canine, porcine). Moreover, according to Forner et al.,[22] who recently compared the proteome of mitochondria extracted from different rat organs (skeletal muscle, heart and liver), mitochondrial proteins can be annotated for other organelles (but not mitochondria) in widely used databases. The Authors also pointed out that mitochondrial proteomes from different organs are similar, at least qualitatively, with one third of the proteins identified being expressed exclusively in one organ. However, what seem to make a functional difference are the levels of expression of certain proteins.

Due to the high complexity of the mitochondrial proteome, and its inclusion of proteins with highly diverse properties, the proteomic approach for its study must be carefully selected. For instance, membrane proteins, which are more basic[124] and overrepresented in the mitochondria[122], are normally poorly separated by 2DE[56]. We reported previously that ~1,000 mitochondrial protein forms, purified from the heart tissue in an animal model of HF, can be separated using 2DE[58]. These include basic membrane proteins such as Prohibitin 2 (pI=10.04) and VDACs (pI 7,48), which could be nicely resolved through improved sample loading using paper-bridge[57]. Other groups used LC-MS[2] in combination with 1DE[22] or iTRAQ[125] to compare the proteins from cardiac mitochondria under different conditions.

Targeted strategies can be used to focus on a specific portion of the mitochondria, such as the respiratory chain. The respiratory chain consists of five protein complexes (I-V); the first four generate the proton gradient that is used by complex V to synthesize ATP[126]. At least three of the five complexes in the chain have been investigated using proteomics[127, 128]. This is not surprising, as some of them could be considered a proteome on their own. For instance, complex I is constituted by 45 subunits[129]. Moreover this complexity is further increased by PTMs[130-132]. Recently, Palmisano and colleagues were able to identify phosphorylation sites in at least five subunits of complex I by combining BNE and phospho-peptide enrichment through titanium dioxide[35, 130]. We demonstrated complex V (ATP-synthase) phosphorylation and its effects on ATP synthesis using, respectively, phospho-peptide enrichment and mutation analysis combined with functional assays[58, 133]. Recently, Ping et al. identified 61 phosphorylation sites in all major mitochondrial pathways including 10 for the electron transport chain and four for complex V, in the murine heart[134].

Other mitochondrial compartments can be enriched to peer deeper and deeper into this proteome and its modifications[135-137] (Figure 3). The very notion that mitochondrial proteins can be modified is a fairly recent advent and it opens the way to a more accurate description of the regulation of energy production in the normal and failing hearts. PTMs, among others, govern the way proteins interact to form supramolecular complexes, and their characterization is crucial.

Sub-proteomics and Cellular Interplay: Putting humpty dumpty Back Together

As described so far, isolating and studying single organelles is a good approach to uncover substantial information regarding their regulation and interplay. However, organelles can be tied together by either oscillations in the levels of second messengers or physically, through structural proteins. In the heart, perturbation of the complex and coordinated interplay between different cellular compartments might be an underlying sign of disease.

Second Messengers and Proteomics: Identifying the Targets

In this section we discuss the involvement of the second messengers Ca^{2+}, NO•, cGMP and cAMP in organelle communication in relation to HF. In healthy cardiomyocytes, contraction and relaxation are triggered by a transient rise and decline in the concentration of intracellular Ca^{2+}; which cycles between sarcolemma, SR and sarcomeres[10]. Ca^{2+} enters the cell through ion channels in the sarcolemma, triggering further release of Ca^{2+} from the SR. This occurs where Ca^{2+} channels in the plasma membrane and SR membrane are in close proximity. A common feature in HF is that this proximity becomes disorganized, which inhibits efficient SR-Ca^{2+} release and results in weakened cardiac contractions[7]. It has also become evident that mitochondria can provide a significant storage of Ca^{2+} and that mitochondrial Ca^{2+} uptake increases ATP production[138]. Thus, on a beat-to-beat basis, Ca^{2+} both induces muscle contractions and increases ATP production to meet the needs of heightened energy demand. This supports the idea that Ca^{2+} is an important factor in organellar interplay (Figure 4). Ca^{2+} can also be stored intra-cellularly in microdomains, where, unlike in the cytosol, levels do not fluctuate during the action potential[139]. This pool of Ca^{2+} can bind to and activate cytosolic enzymes (e.g calmodulin and calcineurin), thereby inducing Ca^{2+}-dependent signaling cascades. One important aspect involved in cardiac disease is the pro-hypertrophic response mediated by the Ca^{2+}-calcineurin dependent signaling pathway[140].

In contrast to Ca^{2+}, NO• diffuses across the sarcolemma. NO• can directly alter protein function by covalently modifying cysteine thiols to generate S-nitrosothiol (SNO). Recently, Lima and colleagues suggested that a majority of the NO• effects are mediated by this PTM[141]. Because of its labile nature, considerable work has been invested in the improvement of proteomic techniques to study SNO[42]. It is important to develop robust technology for the identification and quantification of SNO, as its presumed role as a regulator in cardiovascular disease is growing[141]. Recently, impaired S-nitrosylation of the ryanodine receptor was shown to induce Ca^{2+} leak from the SR and thus contribute to cardiac dysfunction[142].

Another important feature of NO• is the induction of another second messenger, cGMP, through activation of the soluble guanylyl cyclase. cGMP activates PKG, similar to cAMP activation of PKA; both induce signaling cascades, resulting in altered cardiomyocyte growth and function[11, 143]. Interestingly, both PKG and PKA signaling cascades converge in the phosphorylation of several proteins (e.g. both enzymes specifically induce phosphorylation of Ser 23/24 in TnI[115, 144]). With respect to organelle communication, PKA

and PKG phosphorylate proteins that are located in different cellular compartments: SR (PKA/PKG: Ryanodine receptor and PLB); myofilaments (PKA/PKG:TnI, PKA: myosin binding protein C); mitochondria (PKA/PKG: mitochondrial ATP regulated potassium channel); and sarcolemma (PKA/PKG: L-type Ca^{2+}-channel)[11, 145-147]. Phosphorylation via these kinase signaling cascades can be important in transmitting signals throughout the cell and coordinating the action of several organelles. Recently, an impressive 20,443 phospho peptides where quantified in a single study[148]. Although this was done in HeLa cells, similar levels will most likely occur in cardiomyocytes. Other PTMs that have been successfully enriched for are acetylation, metylation, ubiquitylation and glycosylation[149]; but such investigation in the cardiac system remains recent.

Organelles Physical Connection – One Ring to Rule Them All

Organelles can also communicate through the physical connection mediated by the cytoskeleton. For instance, the many interactions between intermediate filaments (IFs) and all major cell compartments make them strong candidates to mediate organelles communication[15]. In the heart, IFs are constituted by desmin and have been found to regulate the function of myofilaments[150, 151] mitochondria[15] and gap junctions[152]. The central role of IFs in HF is corroborated by recent proteomic studies that investigated all major sub-proteomes of the cardiac cell and found that modified desmin forms were altered as a common denominator. The increase of desmin PTM-forms with disease is a consistent observation from proteomic studies that investigated various human and animal models of HF, so it is highly unlikely that its alterations are non-specific[58, 153-162].

Desmin co-enriches with the myofilament fraction of the proteome as IFs are "wrapped" around the sarcomeres and keep them aligned[150]. Desmin mutations cause contractile failure and it was recently postulated that the mis-alignment of the sarcomeres that arises when desmin cytoskeleton is disrupted may represent a cause of contractile failure itself[150, 162] (Figure 4). Our group has recently reported that the levels of PTM-forms of desmin are altered in the mitochondrial fraction of a canine model of HF compared both to shams and animals rescued by CRT[58]. This observation opens an interesting line of investigation, as desmin is known to regulate mitochondrial positioning and behavior through, albeit, an unknown mechanism[5, 155]. One potential explanation is that desmin, maintaining the spatial organization between sarcomeres and mitochondria, ensures that energy production and consumption are properly coupled (Figure 4). As well, the integrity of IFs cytoskeleton plays a major role in cardiac conduction as gap-junction localization at the intercalated discs is ensured by desmin through the interaction with the desmosomes[152] (Figure 1 and 4). Lateralization of gap-junctions, which are observed in the failing heart, may be a cause of arrhythmias, and can be induced by mutation of desmin, or of desmosomal proteins, at the sites of interaction with the IFs[19]. Moreover, a number of novel functions have been attributed to the IFs in the heart including the regulation of the cardiac commitment of stem cells[16], of autophagy[17], and more recently of translation[18]. We believe that PTMs finely regulate these functions of the IFs.

Quantification of desmin in human HF, using immunostaining techniques, has generated controversial results as different groups reported either an increase or the absence of desmin in the cardiomyocytes of HF patients compared to healthy subjects[163, 164]. On the other hand, desmin quantification by 2DE has shown that multiple PTM-forms of the protein are increased during human HF[160]. We reported the increase of modified forms of the protein in cultured cardiomyocytes that become hypertrophied with endothelin-1 treatment[155]. Therefore we postulate that the discrepancy generated by immuno-detection studies may be due to PTMs. This exemplifies how proteomics could help in deciphering the molecular reasons for this controversy, and most importantly the biological value of these

modifications. A detailed characterization and quantification of desmin PTM species would not only help elucidate the functions of the IFs in the heart, but could also highlight novel candidate biomarkers for cardiac disease[157].

Several groups have reported changes in desmin modified forms with disease, *in vivo*, and Capetanaki et al. established a causal link between desmin modification and the formation of aggregates in a transgenic mouse model of HF[15]. The increase of an "acidic" form of desmin by 2DE was reported in the same study. However, despite the existence of several reported *in vitro* desmin modifications, desmin PTMs have been characterized in detail *in vivo* only recently. We identified the first phosphorylation sites of desmin in the heart, and linked them to HF in both a canine model and in humans[157]. Desmin has also been found phosphorylated or acetylated in non-cardiac cells and tissue[109, 148, 165]; this was unexpected, as desmin is thought to be specifically expressed in myocytes. The discoveries of desmin PTMs open new and exciting lines of investigation to address the functional roles of these modifications, particularly in HF.

In conclusion, the disruption of IF networks affects the function of at least three of the most important cell compartments in the heart, and possibly regulates many other aspects of cell function as well. Desmin phosphorylation and proteolysis have been long-known regulators of its assembly and function *in vitro*[166, 167], yet without site information, *in vivo*, progress has been hampered. Further study of the biological role played by these PTMs will be helpful in elucidating the mechanisms that lead to IFs lattice modification during HF and its effect on organellar interplay.

CONCLUSIONS AND FUTURE PERSPECTIVES

There is a need to develop novel, experimental approaches to study HF. We have reviewed how proteomic studies and technologies have enhanced our understanding and revealed new levels of complexity in HF at the cellular, organelle and protein levels. Integration of data obtained from different sub-proteomes and system-wide proteomic studies is important in understanding cellular phenotypes. We have emphasized the need to understand the complex and coordinated interplay between organelles. We hypothesize that the mechanisms involved in synchronizing different cellular compartments may be disrupted with HF. Furthermore, we propose that the regulation and end effectors of this communication reside, at least in part, with the induction of PTMs of target proteins. The quantification and characterization of proteome changes are a necessity, and will drive the development and adaption of new proteomic approaches. In this way, we will eventually understand the global communication that drives the cellular phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard Abbreviations and Acronyms

1/2/nDLC	One/two/multi-dimensional liquid chromatography
1/2DE	One/two dimensional electrophoresis

BNE	Blue native electrophoresis
CID	Collision induced dissociation
CRT	Cardiac resynchronization therapy
DIGE	Difference gel electrophoresis
ESI	Electrospray ionization
HF	Heart failure
IEF	Isoelectrofocusing
IFs	Intermediate filaments
MALDI	Matrix assisted laser desorption
MS^2	Tandem mass spectrometry
O-GlcNAc	O-linked beta-N-acetylglucosamine
Q	Quadrupole
pI	Isoelectric point
PLB	Phospholamban
PTMs	Posttranslational modifications
RP	Reversed-phase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA2	Sarco(endo) plasmic reticulum calcium-ATPase 2
SILAC	Stable isotope labeling with amino acid in cell culture
S/MRM	Single (selective)/ multiple reaction monitoring
SR	Sarcoplasmic reticulum
Tn	Troponin
TOF	Time-of-flight

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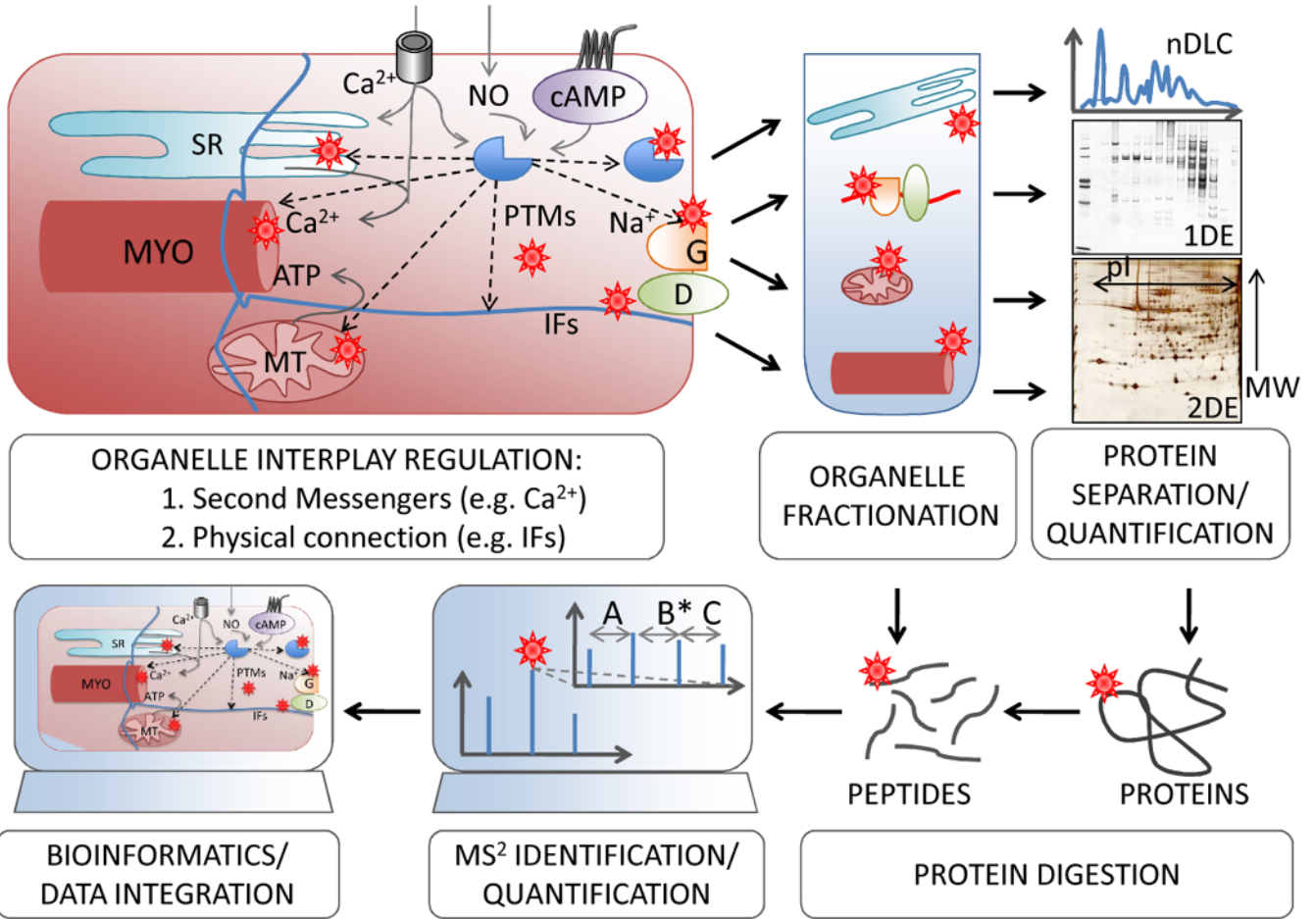
Figure 1. A proteomic strategy to study organellar communication
After organelle fractionation, proteomic methods including protein separation, quantification and identification are employed to study the different sub-proteomes. Integrated analysis of the data generated from several organelles can help highlighting common molecular components involved in the coordination of different cell compartments. MYO, myofilament; MT, mitochondrion; G, gap junction; D, desmosome.
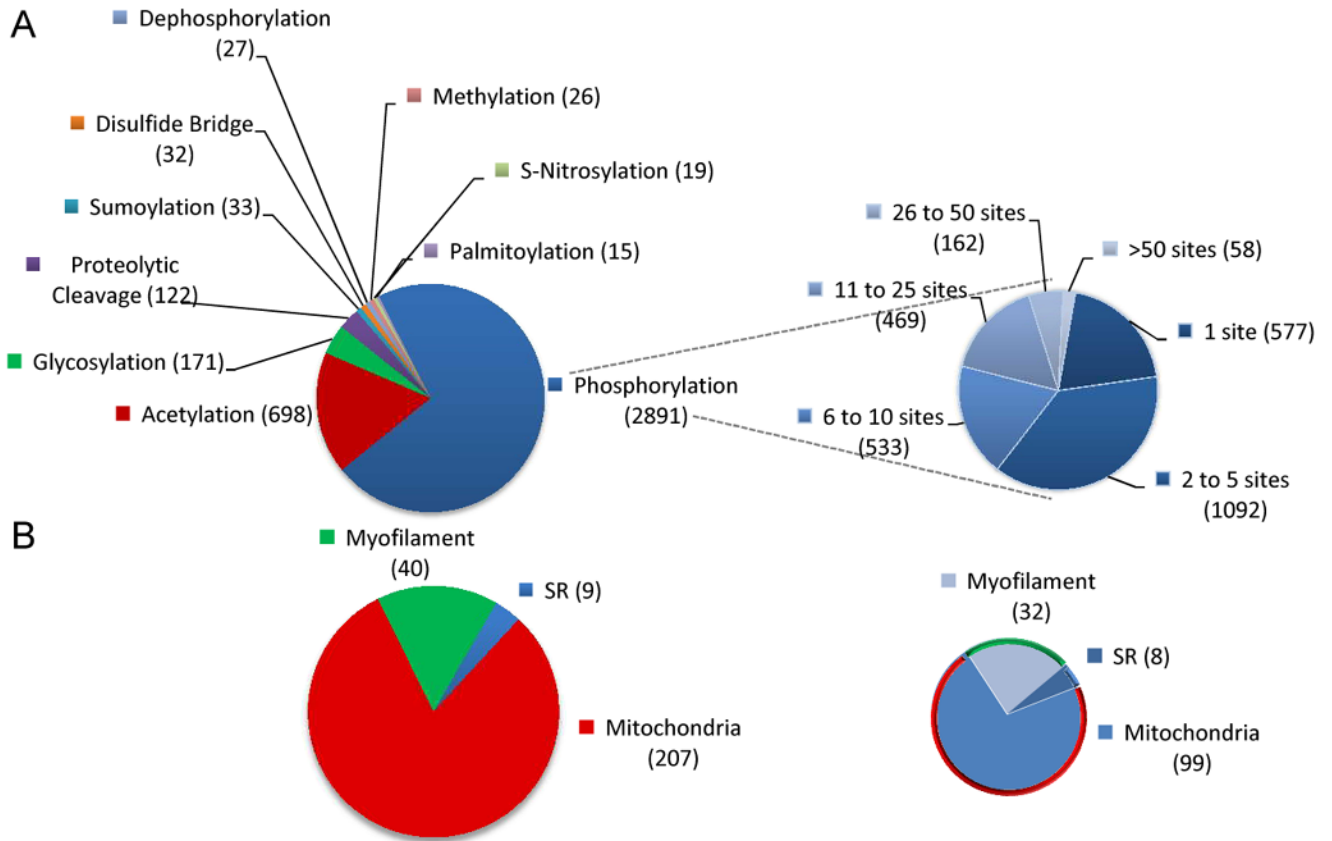
Figure 2. PTMs in heart proteins
A) Proteins annotated as "heart" with their site specific PTMs* (left) with emphasis on phosphorylated proteins grouped based on the total number of phosphorylation sites (right).
B) Proteins annotated as "heart" with their subcellular localization in either SR , sarcomeres or mitochondria; the total number (left) and the number of the ones that are phosphorylated (right) are provided, as reported in the Uniprot Protein Knowledgebase (UniprotKB). *Only PTMs that appeared in more than 15 entries in the Human protein reference database were considered.
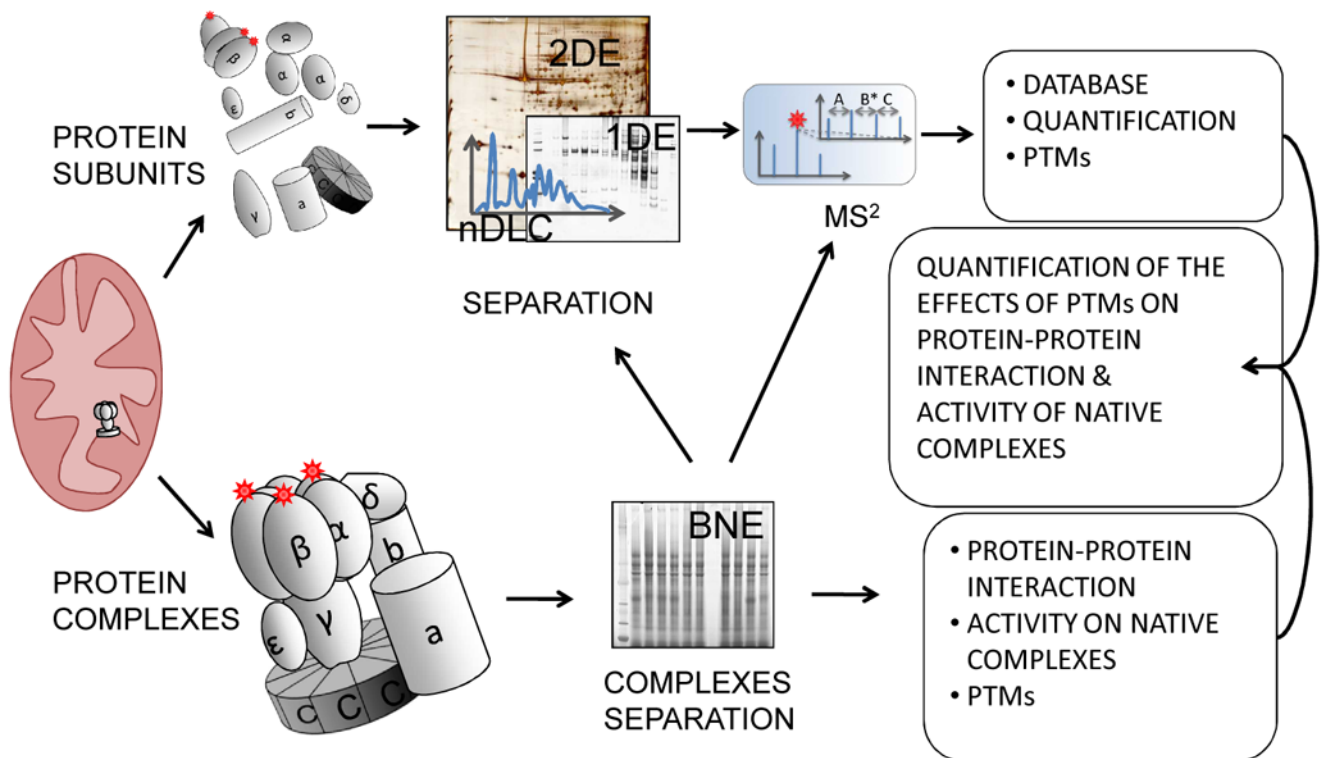
Figure 3. Proteomic to study PTM regulation of protein-protein interaction
Proteins and protein complexes can be alternatively separated from organelles, such as mitochondria. As an example, schematically presented, ATP-synthase (complex V, adapted from Elston T, et al., Nature. 1998, 29;391:510-3)can be denatured into its individual subunits or separated as a whole. The first approach is more suitable to monitor PTMs, the latter provides information on complex composition. The integrated analysis of the two strategies can indicate the role of PTMs in complex formation.
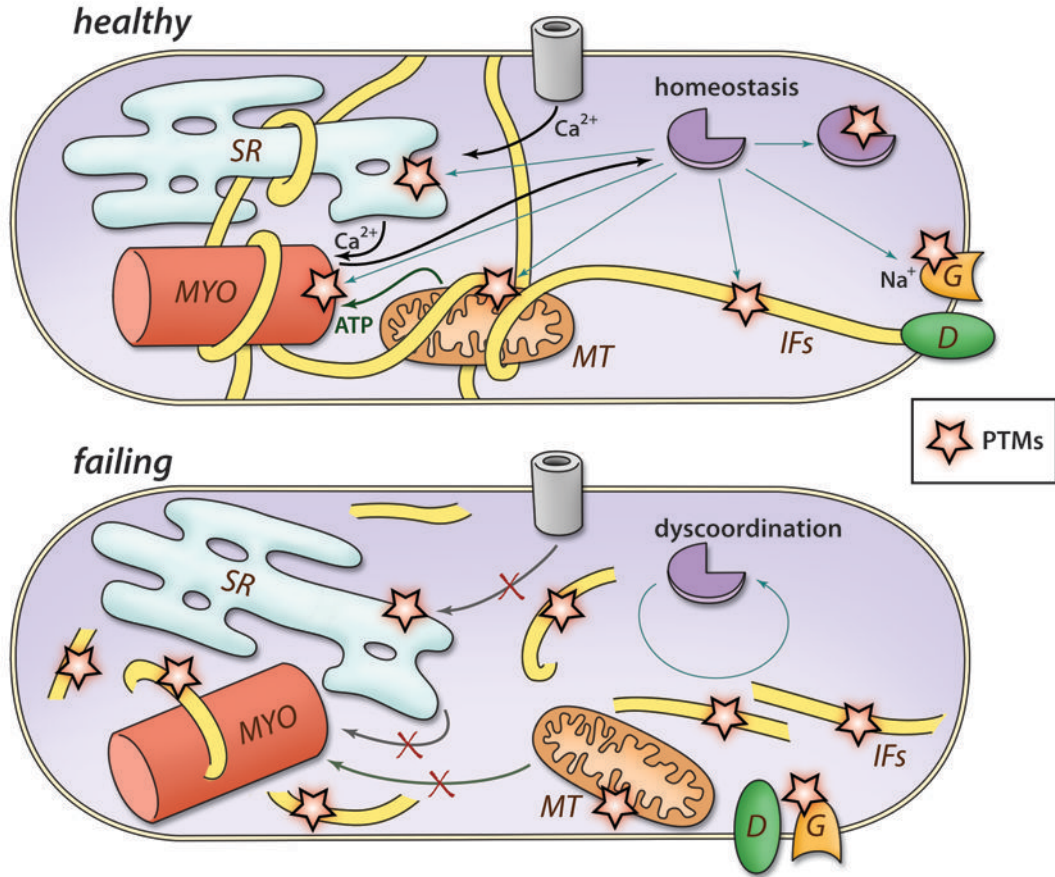
Figure 4. Organelle Dyscoordination in Heart Failure

Schematic presentation of features in a failing cardiomyocyte: increased neurohormonal stimulation and mechanical stress affects cellular contractility, energy metabolism, conduction, intracellular signaling and cell structure mislocalization. We propose that dys-regulation of various organelles could be considered another feature of the failing heart. We further hypothesize that this is regulated through changes in the homeostasis of secondmessengers, resulting in dynamic regulation of PTMs, and by the disruption of cytoskeletal structures such as IFs. MYO, myofilament; MT, mitochondrion; G, gap junction; D, desmosome.

Table 1

Proteomic techniques

Name	Advantages	Limits	Resolution	Quantification	Applications[*]
1DE[22,23,48,67,122,127,128,134,148,165]	• Fast and easy • Hydrophobic proteins • Inexpensive	• Low resolution • Quantification	good	poor	D, ID
2DE[20,43,58,72,103,110,127,135,155,157,160]	• Quantitative • PTM indication • Three-plex (DIGE, 3)	• Hydrophobic proteins • Operator dependent	very good	very good	D, Q, ID, PTM
1DLC[870,105,127,129,131,135,148]	• Automatized • Hydrophobic Proteins • Off/Online with MS	• Low resolution • Quantification	good	poor	D, ID
2DLC[65-67,106,135]	• Automatized • Off/Online with MS • Sensitivity	• MS workload • Quantification	good	N/A	D, ID, PTM
Tag-free[83]	• Fast • Good dynamic range	• Requires careful normalization	N/A	good	Q
MS-tag[52,53,72,86,148]	• Multiplex (8) • Reproducible	• Limited dynamic range	N/A	very good	Q
MRM[88,89]	• Absolute quantification • Customizable • Multiplex (40)	• Expensive • Time consuming to establish	N/A	very good	Q[‡], PTM

D: creating tissue, cellular or organelle database; Q: protein quantification ([‡]relative and absolute); ID: protein identification; PTM: protein discovery and/or validation; N/A: non applicable; [§]mainly reverse-phase. Original articles cited in the text are also listed in the table, in bold the ones that used cardiac samples.