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 increased adrenergic and neurohormonal stimulation and perturbation of the spatial distribution of organelles) also imply an orchestrated action involving multiple organelles.5–7 Yet it is unclear exactly how the various organelles coordinate their responses under both physiologi-
Organelar interplay can occur via 2 routes: second messengers or structural connections (Figure 1). The concentration of second messengers (eg, 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 posttranslational 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 sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA)2 in the SR and troponin (Tn)C in the sarcromeres.\(^9\)\(^,\)\(^10\) Another example is cAMP, which can activate protein kinase (PK)A, resulting in phosphorylation of proteins throughout the cardiomyocyte.\(^11\) 

Direct physical connection is the other mechanism for organelle interplay, occurring through the 3 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 because they likely mediate, and therefore regulate, organellar crosstalk. To test this hypothesis, organelles can be analyzed after subcellular 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 the ability of proteomics to scrutinize biological processes and reveal the new levels of complexity introduced by PTMs. We have also included novel analytic 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 2D 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, or 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. The Table gives an overview of the most common technologies, with their advantages, limitations, and possible biological applications.

A few key concepts have emerged from the proteomic studies performed over the last 16 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, more than 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; Table I in the Online Data Supplement, available at [http://circres.ahajournals.org]). 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 majority of the potential PTMs have not been fully investigated, in part because of 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, plays major roles 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). These studies demonstrate that at least two-thirds of mitochondrial proteome are conserved across different tissues. Because the levels of these shared proteins greatly vary from organ to organ, 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. In addition, various combinations of the isoforms (slow and fast) differentiate cardiac and skeletal myocytes.

Third, technological development is fundamental for scientific advancement. Improvements in each step of the proteomic pipeline have expanded the number and types of proteins (eg, hydrophobic, basic, etc) that can be observed. Considerable contributions have arisen from methods that improved protein separation (eg, 2DE) or enrichment of PTM peptides (eg, O-linked N-acetylglucosamine and phosphorylation). Developments in mass spectrometers have also led to enhanced speed, sensitivity, and mass accuracy. When coupled with advanced

Figure 1. A proteomic strategy to study organellar communication. After organelle fractionation, proteomic methods including protein separation, quantification, and identification are used to study the different subproteomes. Integrated analysis of the data generated from several organelles can help highlight common molecular components involved in the coordination of different cell compartments. D indicates desmosome; G, gap junction; MT, mitochondrion; MYO, myofilament; nDLC, mono- or multidimensional LC. (Illustration elements provided by Cosmocyte/Ben Smith).

Figure 2. Current knowledge of PTMs in heart proteins. A, Occurrence of different types of PTMs in proteins annotated with “heart expression” in the human protein reference database (left, only site-specific PTMs that appeared in >15 entries are shown; for a full list, see Online Table I) with emphasis on phosphorylated proteins grouped based on the total number of phosphorylation sites (right). B, Distribution of proteins annotated with “heart” and subcellular localization in either SR, sarcomeres, or mitochondria; the total number (left) and the number of those that are phosphorylated (right) are provided, as reported in the Uniprot Protein Knowledgebase (UniprotKB).
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 (see selected reviews52–57). In the next section, we describe the different techniques commonly used in organellar proteomics, beginning with subcellular fractionation and following each step of the proteomic workflow through MS-based protein characterization.

Subcellular 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 (eg, by precipitation). Sensitivity and resolving power can instead be increased by focusing on a particular subproteome.35,37 This requires balancing 2 aspects: sample purity and preservation of the PTM status of each protein. Importantly, regardless of the methods used, 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 organelar 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 subcellular localization.25

When compared with 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 (eg, phosphatases, proteases inhibitors) or harsh denaturing conditions can be used.35,36 On the other hand, mild conditions are required when enzyme activity or protein–protein interactions (eg, 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. Although this method is appropriate for proteomics, it leaves the myofilament subproteome denatured, inactive, and not amenable to functional studies. Additional precautions may be required for some oxidative PTMs, where the procedures should be performed in the dark (eg, S-nitrosylation41,42). For PTMs that are extremely labile (because of 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 “Nonclassical” Two-Dimensional Gel Electrophoresis

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-1970s by O’Farrell. Since then, 2 notable improvements that greatly reduce technical variability have been developed: (1) the introduction of immobilized pH gradient strips for isoelectrofocusing (IEF) and (2) fluorescent protein labeling. In fact, researchers can now reproducibly separate thousands of protein forms and reliably quantify protein changes across different samples by combining these methods (Table).

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 the pI of one protein, inducing a shift along the IEF axis. In the second dimension, proteins are separated according to their size using SDS-PAGE. Other PTMs can modify protein size and, thus, electrophoretic mobility in the second dimension; these include proteolytic cleavage, glycosylation, and ubiquitination. Phosphorylation can also slow down electrophoretic mobility, inducing an apparent alteration in protein size, most likely because of the interference with protein–SDS interactions.

Recently, several “nonclassical” 2DE techniques have been rediscovered and optimized, combining different separation methods. One example is blue-native gel electrophoresis (BNE), where proteins are separated under nondenaturing 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 with additional information regarding protein–protein interactions and the composition of protein complexes.

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 in-gel electrophoresis approach offer a very reliable platform for quantifying changes in protein levels between different experimental conditions (e.g., disease versus healthy). Moreover, the image analysis that used to be extremely laborious can now be contracted to companies that perform this routinely. Despite the advantages of 2DE, 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. In addition, a single protein may be presented as multiple spots, each representing different forms. Therefore, a 2DE gel containing 1000 spots may only represent 300 to 400 different proteins. 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). Although we and others have shown previously that it 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: One- and Two-Dimensional LC

Different LC techniques such as ion exchange, size exclusion, affinity, and reversed-phase, can be used for the separation of complex protein and peptide mixtures based on their intrinsic properties (e.g., size, charge/pI, hydrophobicity, and protein–binding properties). 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 reversed-phase, either off- or on-line with MS. A wide selection of chromatographic materials for reversed-phase LC 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 analytic LC strategies (multidimensional LC) increases resolution. 2D LC can be used to separate peptides after protein digestion (referred to as shotgun approach), or intact proteins. The latter potentially provides additional information about protein isoforms and PTM status. Similar to 2DE, LC methods do not allow for unambiguous assignment of quantity changes based on elution profile alone, and require protein labeling before 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. The most common technical challenges with affinity chromatography are: determining which proteins are contaminants because of 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. This is particularly important when studying species that have poor or underrepresented annotation, such as rabbit or dog. 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, because of its speed and level of detail (e.g., PTM characterization; for historical account of MS development, see Zhou et al).
MS instruments consist of 3 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 and electrospray ionization (MALDI and ESI, respectively), 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 MS²) so that peptides can be further broken down using collision-induced dissociation. An advantage of MS² is that it provides detailed chemical information about the amino acid sequence.77 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, nondigested proteins, with or without prior separation (also known as a “top-down” approach).78,79 Because of 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 versus 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 tags83 via enzymatic (eg, stable isotope labeling with amino acid in cell culture [SILAC] and18O labeling52,84,85) or chemical derivatization (eg, 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 proteins 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 4 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) (Online Figure 1).87,88 MRM is a MS² technique that allows relative and absolute quantification of proteotypic peptides (ie, peptides that have a unique amino acid sequence specific to a particular protein or isoform) and, hence, of proteins. In short, triple quadrupole (Q3) MS can be used to select a specific peptide of interest (including modifications or polymorphism89,90) based on its m/z, in Q1. This parent ion is then fragmented in the Q2, and the daughter fragments (also called transition ions) are monitored and quantified in the Q3. The choice of the transition ions is critical because it allows monitoring a specific peptide, based on MS² identification. Normally, few transitions need to be monitored to achieve reliable quantification (n≥5). In many cases, heavy isotope-labeled peptides are used as internal standards for absolute quantification, but relative quantification (sample A versus 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 straightforward 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 the function of one protein (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 attributable 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 (See reviews on phospho-proteomics.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 phosphopeptides. There are a few protocols available, based either on the affinity of phosphopeptides for positively charged metal ions94,95 or on other LC methods: reversed-phase LC96 and hydrophilic interaction chromatography.97 Another consideration of PTM analyses is related to the stability of the modification. For example, the detection of phosphopeptides by MS is challenged by the lability of the phosphate bond during collision-induced dissociation. Phosphoesters 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 phosphopeptides in the MS has been developed (electron transfer dissociation).98 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 MS data handling.99 Bioinformatic tools are also used for the prediction of protein characteristics (such as pl and MW values, PTM consensus sequence, etc.), and several algorithms are publicly available (eg, www.expasy.net). Also freely accessible are a number of curated protein databases (see Online Table III). Although 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 consensus sequence of the modifying enzyme or if the modification has been observed in an isoform.

Nowadays, these protein databases are routinely mined by algorithms that can match experimental MS spectra to protein sequences fragmented in silico, in a semiautomated fashion.100 Algorithms such as OMSA, Mascot, X!tandem are able to extract the numeric 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” (eg, 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 (eg, 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 laboratory-to-laboratory 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 provide examples of how proteomics has been successfully used 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 Ca2+ in cardiomyocytes. It has been the dogma that release of Ca2+ from SR, in a Ca2+-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 Ca2+ cycling proteins. Particular focus has been targeted on 2 SR proteins: SERCA and its regulator, phospholamban (PLB). SERCA is the main regulator of Ca2+ uptake during cardiomyocyte relaxation, and its activity is tightly modulated through interaction with PLB.9

When studying SR proteins, the initial challenges are the extensive membrane network of the organelle 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 using shotgun methods in the presence of MS-friendly surfactants that are compatible with in-solution enzyme digestion and MS identification (eg, Rapigest105). 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 existing challenge separating SR from other membrane organelles. Most preparations contain other membrane proteins (in particular from mitochondria103,105,106), and this complicates the goal of a precise identification of the SR subproteome. Because of the ambiguous definition of the SR subproteome, leading to a poor implementation of the term “sarcoplasmic reticulum,” the proteins from this organelle are underrepresented in different databases (eg, 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 last year in a study by Andersson et al.\textsuperscript{107} This study showed that a cardiac-specific, inducible SERCA2 knockout 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\textsuperscript{2+} to the cardiomyocyte contractions. This result implies either that the heart uses an alternative mechanism for cycling Ca\textsuperscript{2+} into the SR or that a small, nonimmunoreactive fraction of SERCA2 is present. As mentioned, because of the problems in obtaining pure SR, it remains debatable which other SR proteins might be upregulated or modified as a consequence of severe SERCA2 depletion. This emphasizes the need for an accurate description of the SR subproteome and precisely how it changes with heart disease. An alternative strategy is to specifically target SERCA2 using affinity chromatography combined with targeted MS measurements (eg, MRM).

Several genetically modified mouse models targeting expression of SERCA and PLB have been constructed to study the overall biological effects on Ca\textsuperscript{2+} -cycling disruption (SERCA conditional knockout\textsuperscript{107}; PLB knockout and overexpression\textsuperscript{108}). As expected, significant effects on cardiac contractility attributable to altered Ca\textsuperscript{2+} homeostasis are commonly observed in these models. Interestingly, by applying different proteomic approaches (eg, 2DE, gel-free, MS\textsuperscript{2}), it is clear that alterations in Ca\textsuperscript{2+} handling induces phenotypic effects beyond the contractile apparatus. These include changes in cytoskeleton protein composition, membrane receptors and intracellular signaling pathways, and energy metabolism.\textsuperscript{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\textsuperscript{2+} released from SR binds to TnC, which alters the interaction between the thin (actin, troponin, and troponin: consisting of TnI, TnT, and TnC) and thick (myosin comprising myosin heavy chain and 2 light chains and myosin-binding protein C) filaments, leading to muscle contraction.\textsuperscript{10} Recently, it has been proposed that sarcomeres should be regarded as a cellular organelle because of its close involvement in structural, electric, and metabolic aspects of cardiomyocyte dynamics.\textsuperscript{111} In HF, the reduction in cardiac pump function can be related to changes in the SR but also to sarcomeric function.\textsuperscript{10} Although numerous mutations in the sarcomeric proteins causing familial hypertrophic and dilated cardiomyopathies have been described,\textsuperscript{112} the importance of PTMs for sarcomeric proteins is also well established.\textsuperscript{113}

In the Tn complex, TnI is the inhibitory subunit\textsuperscript{111} and its function is highly regulated by phosphorylation. The importance of Ser23 and Ser24 in controlling cardiac contractility and myofilaments responsiveness to Ca\textsuperscript{2+} has been extensively studied.\textsuperscript{114} Recently, several other phosphorylation residues were identified, and currently there are 7 known sites in humans (Ser23, Ser24, Ser31, Ser42, Ser44, Thr141, and Ser150)\textsuperscript{115}; some of these are also important in the control of cardiac dynamics.\textsuperscript{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.\textsuperscript{116} The biological importance of these TnI phospho-sites is under debate.\textsuperscript{115} Our group has developed MRM assays to specifically quantify and monitor the phosphorylation status of 6 of the identified TnI phospho-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 acetylgalactosamine at Ser and Thr residues (O-linked βN-acetylgalactosamine [O-GlcNAc]). When first discovered, identification of O-GlcNAcylation was challenging and time-consuming.\textsuperscript{117} However, enrichment methods of today, in combination with sensitive MS, have established widespread occurrence of this PTM.\textsuperscript{21} Recently, O-GlcNAcylation was implicated in myofilament contractile alterations.\textsuperscript{118} Ramirez-Correa et al discovered myofilament Ca\textsuperscript{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 identified 32 O-GlcNAcylation sites in the myofilament subproteome, one being Ser150 of TnI. Knowing that there is extensive crosstalk between O-GlcNAcylation and phosphorylation,\textsuperscript{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.\textsuperscript{119} During the development of HF, both the energy demands and metabolism change, showing a remarkable decrease in oxidative phosphorylation and a shift toward glucose over fatty acid utilization.\textsuperscript{120} Interestingly, during HF therapy such as cardiac resynchronization, 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.\textsuperscript{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 subfractionation.\textsuperscript{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.\textsuperscript{122} Now, curated databases for mitochondrial proteins are also available,\textsuperscript{123} including one recently compiled by Mootha and colleagues, who assigned 1098 mitochondrial proteins (MitoCharta) by
combining extensive MS characterization with microscopy and computation to confirm mitochondrial protein localization. Unfortunately, there is a lack of equivalent databases for the less annotated species that are used in cardiac research (eg, rat, canine, porcine). Moreover, according to Forner et al, 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.

Because of 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 and overrepresented in the mitochondria, are normally poorly separated by 2DE. We reported previously that ~1000 mitochondrial protein forms, purified from the heart tissue in an animal model of HF, can be separated using 2DE. These include basic membrane proteins such as Prohibitin 2 (pI 10.04) and voltage-dependent anion channels (pI 7.48), which could be nicely resolved through improved sample loading using paper bridge. Other groups used LC-MS in combination with 1DE or iTRAQ 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 5 protein complexes (I to V); the first 4 generate the proton gradient that is used by complex V to synthesize ATP. At least 3 of the 5 complexes in the chain have been investigated using proteomics. This is not surprising, because some of them could be considered a proteome on their own. For instance, complex I is constituted by 45 subunits. Moreover, this complexity is further increased by PTMs. Recently, Palmisano and colleagues were able to identify phosphorylation sites in at least 5 subunits of complex I by combining BNE and phosphopeptide enrichment through titanium dioxide. We demonstrated complex V (ATP-synthase) phosphorylation and its effects on ATP synthesis using, respectively, phosphopeptide enrichment and mutation analysis combined with functional assays. Recently, Ping et al identified 61 phosphorylation sites in all major mitochondrial pathways, including 10 for the electron transport chain and 4 for complex V, in the murine heart.

Other mitochondrial compartments can be enriched to peer deeper and deeper into this proteome and its modifications (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.

Subproteomics 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, 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, which cycles among sarclemma, SR, and sarcomeres. Ca enters the cell through ion channels in the sarclemma, triggering further release of Ca from the SR. This occurs where Ca 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 release and results in weakened cardiac contractions. It has also become evident that mitochondria can provide a significant storage of Ca and that mitochondrial Ca uptake increases ATP production. Thus, on a beat-to-beat basis, Ca both induces muscle contractions and increases ATP production to meet the needs of heightened energy demand. This supports the idea that Ca is an important factor in organellar interplay (Figure 4). Ca can also be stored intracellularly in microdomains, where, unlike in the cytosol, levels do not fluctuate during the action potential. This pool of Ca can bind to and activate cystolic enzymes (eg, calmodulin and calcineurin), thereby inducing Ca-dependent signaling cascades. One important aspect involved in cardiac disease is the prohypertrophic response mediated by the Ca/calcineurin-dependent signaling pathway.

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

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. Interestingly, both PKG and PKA signaling cascades con-
verge in the phosphorylation of several proteins (e.g., both enzymes specifically induce phosphorylation of Ser 23/24 in TnI). 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). 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 phosphopeptides where quantified in a single study. 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, methylation, ubiquitylation, and glycosylation, 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. In the heart, IFs are constituted by desmin and have been found to regulate the function of myofilaments, mitochondria, and gap junctions. The central role of IFs in HF is corroborated by recent proteomic studies that investigated all major subproteomes of the cardiac cell and found that modified desmin forms were altered as a common denominator. The increase in neurohormonal stimulation and mechanical stress affects cellular contractility, energy metabolism, conduction, intracellular signaling, and cell structure mislocalization. We propose that dysregulation 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 second messengers, resulting in dynamic regulation of PTMs, and by the disruption of cytoskeletal structures such as IFs. D indicates desmosome; G, gap junction; MT, mitochondrion; MYO, myofilament.
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 nonspecific.\textsuperscript{58,153–162}

Desmin coenriches with the myofilament fraction of the proteome because IFs are “wrapped” around the sarcomeres and keep them aligned.\textsuperscript{150} Desmin mutations cause contractile failure, and it was recently postulated that the misalignment of the sarcomeres that arises when desmin cytoskeleton is disrupted may represent a cause of contractile failure itself (Figure 4).\textsuperscript{150,162} 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 with both shams and animals rescued by cardiac resynchronization therapy.\textsuperscript{58} This observation opens an interesting line of investigation, because desmin is known to regulate mitochondrial positioning and behavior through, albeit, an unknown mechanism.\textsuperscript{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). In addition, the integrity of IFs cytoskeleton plays a major role in cardiac conduction because gap junction localization at the intercalated discs is ensured by desmin through the interaction with the desmosomes (Figures 1 and 4).\textsuperscript{152} Lateralization of gap junctions, which is observed in the failing heart, may be a cause of arrhythmias, and can be induced by mutation of desmin or desmosomal proteins, at the sites of interaction with the IFs.\textsuperscript{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,\textsuperscript{16} of autophagy,\textsuperscript{17} and, more recently, of translation.\textsuperscript{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 because different groups reported either an increase or the absence of desmin in the cardiomyocytes of HF patients compared to healthy subjects.\textsuperscript{163,164} On the other hand, desmin quantification by 2DE has shown that multiple PTM forms of the protein are increased during human HF.\textsuperscript{160} We reported the increase of modified forms of the protein in cultured cardiomyocytes that become hypertrophied with endothelin-1 treatment.\textsuperscript{155} Therefore, we postulate that the discrepancy generated by immunostaining studies may be attributable 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 not only would help elucidate the functions of the IFs in the heart but could also highlight novel candidate biomarkers for cardiac disease.\textsuperscript{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.\textsuperscript{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.\textsuperscript{157} Desmin has also been found phosphorylated or acetylated in noncardiac cells and tissue\textsuperscript{109,148,165}; this was unexpected, because 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 3 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,\textsuperscript{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 IF 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 subproteomes 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.

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Disclosures

None.

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Divide and Conquer: The Application of Organelle Proteomics to Heart Failure
Giulio Agnetti, Cathrine Husberg and Jennifer E. Van Eyk

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Online Figure 1. MRM quantification of modified and unmodified peptides by triple QMS. (A) Initial digestion of proteins into peptides. (B) The peptide mixture is injected in the triple quadrupole MS, where the quadrupole Q1 is used to select a specific peptide (parent). The parent ion is fragmented in the Q2 and transition (daughter) ions are monitored in the Q3. Relative quantification is obtained by comparing the spectral count from different transitions. Normally, five to six pairs (parent and daughter) for each peptide are monitored in order to achieve a reliable quantification. D: detector.
### Online Table I: See separate excel spreadsheet

### Online Table II: See separate excel spreadsheet

### Online Table III: Selected protein databases

<table>
<thead>
<tr>
<th>Database</th>
<th>Features</th>
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| NCBI                              | • Merging annotations from GenBank, RefSeq, TPA, SwissProt, PIR, PRF and PDB  
• The most comprehensive resource of protein sequences (ref Martens L. Methods Mol Biol. 2009)  
• Heterogenic in sequence reliability, annotation, and peptide sequence redundancy (ref Martens L. Methods Mol Biol. 2009)  
• Limited additional biological information  
• Curated by the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH)                                                                 | ncbi.nlm.nih.gov/protein    |
| Uniprot Protein knowledgebase (UniprotKB) | • Including both manually (Swiss-Prot) and automatically annotated (TrEMBL) entries  
• Currently containing more than 519 300 curated entries  
• No particular focus regarding species or PTMs  
• Extensive additional biological information  
• Curated by UniProt comprising the European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics (SIB) and the Protein Information Resource (PIR) | www.uniprot.org             |
| The human protein reference database (Hprd) | • Manually curated  
• Currently comprising more than 93 700 PTMs  
• Restricted to the human proteome  
• Curated by The Pandey lab and the Institute of Bioinformatics at Johns Hopkins University.                                                                 | www.hprd.org                |
| PhosphositePlus®                 | • Manually curated  
• Currently containing more than 71 400 curated entries on phosphorylation sites  
• Major focus on phosphorylation sites in mouse and human  
• Including non published PTMs from Cell Signaling Technology (CTS)  
• Curated by CTS                                                                 | www.phosphosite.org         |