Proteomic Approaches in Circadian Biology

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Abstract Circadian clocks are endogenous oscillators that drive the rhythmic expression of a broad array of genes that orchestrate metabolism and physiology. Recent evidence indicates that posttranscriptional and posttranslational mechanisms play essential roles in modulating circadian gene expression, particularly for the molecular mechanism of the clock. In contrast to genetic technologies that have long been used to study circadian biology, proteomic approaches have so far been limited and, if applied at all, have used two-dimensional gel electrophoresis (2-DE). Here, we review the proteomics approaches applied to date in the circadian field, and we also discuss the exciting potential of using cutting-edge proteomics technology in circadian biology. Large-scale, quantitative protein abundance measurements will help to understand to what extent the circadian clock drives system wide rhythms of protein abundance downstream of transcription regulation.

Keywords Circadian rhythm • Proteomics • Mass spectrometry • Protein quantification • Posttranslation modifications

Abbreviations

CE-MS	Capillarity electrophoresis mass spectrometry
GC-MS	Gas chromatography mass spectrometry
LC-FT MS/MS	Liquid chromatography Fourier transformation tandem mass spectrometry
MALDI TOF MS	Matrix-assisted laser desorption/ionization time of flying mass spectrometry

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MS	Mass spectrometry
SELDI	Surface-enhanced laser desorption/ionization
SPE	Solid-phase extraction

1 Introduction

Genetic technologies have long been used to study circadian biology, as reviewed in the previous chapter (Reddy 2013). In contrast, proteomic approaches have so far been limited and if applied at all, have used two-dimensional gel electrophoresis (2-DE). Due to its technical limitations and to recent advances in high-resolution mass spectrometry (MS), this technology is becoming obsolete. Currently, the most powerful proteomics method is high-accuracy, quantitative MS shotgun proteomics. In that approach, the proteome is digested into peptides, and the resulting, very complex mixtures are separated by liquid chromatography (LC), which is coupled to high-resolution tandem mass spectrometric identification and quantification (MS/MS). MS-based proteomics is increasingly used to determine global protein abundance, protein–protein interactions, as well as posttranslational modifications (PTMs) (Aebersold and Mann 2003; Cox and Mann 2011; Mallick and Kuster 2010; Yates et al. 2005).

Here we review the proteomics approaches applied to date in the circadian field; these can be classified into three general methods: expression proteomics, interaction proteomics, and proteomics of PTMs. Moreover, we also discuss the exciting prospect and potential of using cutting-edge proteomics technology in circadian biology.

2 Expression Proteomics

Expression proteomics is defined as the measurement of the absolute or relative quantity of the proteins in a sample. Therefore, the notion of expression proteomics is similar to the widely applied transcriptomics approaches such as microarray measurements or, more recently, "deep sequencing." However, the goal of proteomics is to ideally measure the total protein complement of a biological system. In principle, this has the advantage over transcriptomics that the proteins are generally the functional units in the cell rather than intermediates.

MS-based expression proteomics relies on quantifying the mass spectrometric signals of the peptides to determine protein abundance in a complex mixture. The two most common methods are isotope labeling and label-free quantification (Bantscheff et al. 2007). The isotope-based methods make use of the fact that MS

can readily quantify the ratios between versions of the same peptide that have different mass. The stable isotopes can be introduced by chemical methods or by metabolic labeling. Among the latter method, stable isotope labeling by amino acids in cell culture (SILAC) is the most accurate and widely used; it relies on the incorporation of a "heavy" or "light" nonradioactive isotope of an essential amino acid into all proteins of the proteomes to be compared (Ong et al. 2002; Ong and Mann 2006). These proteomes are mixed, processed, and analyzed together. The heavy SILAC-labeled proteome can easily be distinguished from the light-labeled proteome by modern high-resolution MS techniques on the basis of the known peptide mass differences. The relative intensity between the peaks of the SILAC peptide pairs directly reflects the relative abundance of the protein in the original samples. This method can be very accurate because it eliminates quantitative differences due to sample processing. Although SILAC was originally developed for cell culture experiments, it has recently been applied to whole organisms and human tissues (Baker et al. 2009; Geiger et al. 2010; Kruger et al. 2008). Alternatively, protein quantification can also be achieved with label-free methods based on alignment of separate LC-MS/MS runs of peptide mixtures and comparison of the signal intensities of the same peptides between the runs. Label-free quantification is less accurate than isotope labeling, especially if several fractionation steps are involved; however, it is simpler and can be applied to any system.

For many years circadian regulation of metabolism and physiology has been investigated through the analysis of gene expression. This has been made possible by DNA array technology, which has facilitated large-scale circadian gene expression studies and provided essential information about circadian transcriptional control in mouse brain and peripheral tissues (Duffield 2003; Hughes et al. 2009; McCarthy et al. 2007; Panda et al. 2002; Storch et al. 2002).

By comparison, global proteomic analyses in the circadian field have been very limited in number and in the depth of coverage (Table 1). The majority of these analyses employed comparative two-dimensional gel electrophoresis (2-DE) and, specifically, difference gel electrophoresis (2-DIGE). Below we review proteomic reports characterizing daily variation of protein levels in several rodent organs mainly using that technology, which is now out of date and not recommended anymore. We also discuss the identification of circadian and electrical stimulation-dependent released peptides in the suprachiasmatic nucleus (SCN).

2.1 Proteomes of Brain and Eye

The circadian timekeeping system in mammals is organized in a hierarchical manner (Buhr and Takahashi 2013). Virtually, all tissues contain internal, self-autonomous clocks that regulate local physiology and metabolism (Stratmann and Schibler 2006). A master clock located in the brain, the suprachiasmatic nucleus (SCN), synchronizes and phase-dependently coordinates the peripheral clocks (Ko and Takahashi 2006). The SCN in turn receives light cues from the retina

Table 1 Summary of expression proteomics approaches applied to circadian biology

Tissue	Technique	Identified/rhythmic proteins	References
SCN	2-DIGE/MS	871/34	Deery et al. (2009)
SCN light stimulated	LC MS/MS	2,131/387	Tian et al. (2011)
SCN releasate RTH-stimulated	SPE-MALDI TOF MS	14	Hatcher et al. (2008)
SCN endogenous peptides	LC-FT MS	102	Lee et al. (2010)
Retina	LC-MALDI TOF MS LC-MS/MS	415/11	Tsuji et al. (2007)
Rat pineal gland	2-DE MALDI TOF	1,747/60	Moller et al. (2007)
Liver	2-DIGE MALDI TOF MS LC-MS/MS	642/39	Reddy et al. (2006)
Blood	SELDI MS LC-MS/MS	6	Martino et al. (2007)

through the retinohypothalamic tract (RHT), entraining it in phase to the external environment. This information is then transmitted by the SCN via humoral and neuronal signals to peripheral tissues, thus synchronizing behavior and physiology in the whole organism (Davidson et al. 2003). Expression profiling by microarrays in the SCN has identified hundreds of transcript that appear to be under circadian control (Panda et al. 2002; Ueda et al. 2002; for a review see Reddy 2013). Only recently has the SCN been the target of proteomic analyses to uncover rhythmic oscillations of intracellular as well as extracellular, secreted molecules.

Hastings and colleagues performed 2D-DIGE analyses with protein extracts from mouse SCN collected every 4 h across the circadian day (Deery et al. 2009). On the resulting gels, 871 protein spots were detected; among them, 115 showed circadian variation. Of these, 53 spots were analyzed using protein digestion followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Since some of the analyzed spots corresponded to isoforms of the same protein, only 34 unique proteins showing robust circadian changes were finally identified. The authors estimated that between 6 % and 13 % of SCN soluble proteins show daily oscillation, a proportion notably higher than the previously reported SCN cycling transcriptome (5 %) (Ueda et al. 2002). Moreover, only 11–38 % of the cycling proteins also showed rhythmicity at the transcript level, which was interpreted to imply a key role of posttranscriptional regulation in the circadian clock (Deery et al. 2009). Oscillating SCN soluble proteins covered diverse functional categories with considerable overrepresentation of molecules involved in synaptic vesicle recycling. Further experiments described in the study demonstrated the importance of vesicle recycling factors in the maintenance of electrical rhythmicity as well as neuronal circuitry, both known and central properties of the SCN (Weaver 1998).

This analysis complemented previous work describing MS-characterized rat SCN releasates across circadian time and after electrophysiological stimulation of the RHT (Hatcher et al. 2008). Secreted neuropeptides collected and concentrated on solid-phase extraction (SPE) materials were analyzed by off-line MALDI TOF MS. SCN releasates were found to contain several established circadian neuropeptides as well as some peptides that could not be identified because their masses did not match known compounds. Intriguingly, this work revealed that the releasate content is stimulation specific, characterized by a robust secretion of proSAAS-derived peptides after RHT stimulation. The role of one of these peptides in light-mediated cues was demonstrated by inducing an SCN phase shift response after exogenous application. A more recent study applied liquid chromatography in combination with high-resolution MS (LC-FTMS/MS) to detect neuropeptides in the rat SCN (Lee et al. 2010). The authors identified 102 SCN endogenous peptides in addition to 12 peptides bearing different posttranslation modifications (PTMs).

It is known that photic input induces transcriptional activation in the SCN with de novo transcription of immediate early and clock genes as well as other light-induced genes that ultimately mediate the phase reset of the clock (Albrecht et al. 2001; Araki et al. 2006; Castel et al. 1997; Porterfield et al. 2007). To investigate the effects of light stimulation on proteome-wide expression in the mouse SCN, Figeys and coworkers recently developed a much more sophisticated proteomic approach termed AutoProteome (Tian et al. 2011). It consisted of an automatic sample processing step followed by LC-MS/MS with two stages of peptide separation. This study demonstrated for the first time that light stimulation induces significant changes in the SCN proteome, as 387 proteins of a total of 2,131 quantified ones showed light-induced changes in their expression levels. Bioinformatic analysis indicated that light-inducible proteins are widely distributed into diverse canonical pathways. Among the light-responsive proteome, the authors selected several for confirmation. Two of these proteins were already previously associated with clock timing processes, vasopressin-neurophysin 2-copeptin, and casein kinase 1 delta, and three of them (Ras-specific guanine nucleotide-releasing factor, the deubiquitinating enzyme USP9X, and the ubiquitin-protein ligase UBE3A) had no previously recognized connection to the circadian clock. Moreover, the analysis showed enrichment of proteins from the ubiquitin and proteasome pathways indicating their potential role in controlling protein expression in the SCN connected to the light-resetting response.

Before reaching the SCN, light information is received and processed in the retina by photoreceptors and retinal ganglion cells. Besides being the essential organ for photic entrainment in mammals, the retina was the first peripheral organ where an intrinsic clock was identified (Tosini and Menaker 1996). Gene expression studies have indicated that circadian rhythms in the retina regulate many aspect of its physiology (Kamphuis et al. 2005; Storch et al. 2007). Furthermore, a proteomic analysis of the retina using 2-DE combined with MALDI TOF MS and LC-MS/MS identified 11 proteins with circadian oscillations (Tsuji et al. 2007). Despite the limited number of identification, rhythmic proteins covered different

biological functions suggesting that a broad range of physiological processes may be controlled at the protein level in the retina by the circadian clock.

Characterization of protein oscillations in the rat pineal gland has been the focus of another proteomic report (Moller et al. 2007). In mammals the pineal gland controls the circadian synthesis and secretion of the hormone melatonin. Production of melatonin peaks at night and its elevated nocturnal plasma level is used as an indicator of the photoperiodic time (Goldman 2001). Employing 2-DE followed by MALDI TOF MS/MS identification, the study identified 60 proteins with differential expression between day and night in the rat pineal gland (Moller et al. 2007). A total of 25 proteins were found to be up-regulated at night, which is the peak of the synthesis of the hormone. Bioinformatic classification showed that proteins highly expressed at night are involved in morphogenesis and local metabolism. Additionally and previously unreported, several proteins showed high expression during the day, suggesting a distinct rhythmic metabolism in anti-phase to the melatonin production.

2.2 Proteomes of Peripheral Organs

Proteomics has also been applied to understand circadian regulation of local metabolism in peripheral organs. Gene expression profiling has long illustrated the role of circadian transcription in the control of physiology in different mammalian peripheral organs. Early reports have identified hundreds (Akhtar et al. 2002; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002) and a more recent one thousands (Hughes et al. 2009) of rhythmic transcripts in the mouse liver (Reddy 2013; Brown and Azzi 2013). To complement gene expression analysis, Reddy et al. performed the first circadian proteomic study to identify protein oscillations in the mouse liver (Reddy et al. 2006). Proteins collected every 4 h across the circadian cycle were analyzed by 2D-DIGE and identified by MALDI TOF-MS or LC-MS/MS. The authors detected 642 proteins, 60 of which oscillated with high statistical significance, whereas 20 % of the identified proteins showed overall significant daily changes, a rate that differed notably from the reported circadian transcriptome (5–10 %) (Akhtar et al. 2002; Hughes et al. 2009; Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002). Additional validation revealed that almost half of the proteins found to be rhythmic did not have a cycling transcript, which would be consistent with a key role of posttranscriptional regulation in the circadian clock. Similar divergences between transcriptome and proteome have been previously reported in cancer (Hanash 2003) and many other systems, but more recent studies using high-precision instrumentation have generally shown higher mRNA–protein correlation (Cox and Mann 2011).

Interestingly, different protein isoforms encoded by individual genes were found among rhythmic liver proteins in the study. In addition, rate-limiting enzymes involved in central liver metabolic pathways like carbohydrate metabolism and the urea cycle showed daily oscillations at the protein level. This highlights the fundamental role of circadian regulation of protein expression in liver physiology. Further knowledge of hepatic metabolism regulation by circadian clocks at the level of proteins could contribute greatly to the understanding of pathologies of this organ. Furthermore, it would aid in the development of chronotherapeutic strategies aimed at minimizing drug toxicity (Akhtar et al. 2002; Sewlall et al. 2010).

SELDI and LC-MS/MS were used in an attempt to detect daily changes in peptides in mouse blood (Martino et al. 2007). This study attempted to find markers that could define body time of day, as indicative of changes in the organism's metabolism. While few peptides were identified, the potential of following daily changes in blood protein abundance in humans to monitor health and diseases is indisputable. In the future, using more modern proteomics technology, it may be possible to find such markers, which could then be applied in molecular diagnosis of aberrant clock function or to chronotherapy applications.

2.3 Current Capabilities of Expression Proteomics

Until now, daily dynamic expression of proteins has been mostly investigated with 2-DE followed by MS. This technique has several shortcomings, first of all limited resolution and throughput, leading to quantification of only a small subset of the proteome. Specifically, only soluble proteins can be assayed; analyzed proteins have to be detected as spots in the gels; and finally, these spots need to be identified one by one using MS. In addition to these general restrictions, 2-DE faces additional challenges when characterizing temporal changes of protein abundance: the spots need to be detected in all or the majority of the assayed gels, spot localization needs to match between gels (but PTMs alter the electrophoresis mobility of the proteins), and relative changes in their intensity have to be estimated by gel image analysis. In contrast, high-resolution MS-based quantitative proteomics enables drastically deeper proteome coverage as well as much more accurate comparison of protein abundance between samples. For example, feasibility of high-resolution, quantitative MS-based proteome analysis has been demonstrated by the quantification of more than 5,000 proteins in embryonic stem cells (Graumann et al. 2008). This method can also quantify tissue proteomes to considerable depths, by using either the SILAC mouse (Kruger et al. 2008) or SILAC "spike-in" strategies (Geiger et al. 2011). Proteome coverage of tissues is somewhat reduced compared to cell lines, mainly due to the large differences in protein abundance in the sample (dynamic range). Nevertheless, measurement of global changes in more than 4,000 proteins between young and old mice has already been

described (Walther and Mann 2011). These numbers still compare favorably to results from early gene arrays, and it is clear that this technology could be uniquely useful for comparative analysis of circadian transcriptome and proteome.

3 Interaction Proteomics

Novel mammalian clock components and modifiers have often been discovered by genetic screens (Takahashi et al. 2008; Buhr and Takahashi 2013). In particular, essential clock proteins were identified by using forward and reverse genetics, and more recently, chemical and functional genomics have revealed novel regulators; see review by Baggs and Hogenesch (2010). Proteomics can, in principle, complement these approaches through the determination of physical interactions of proteins involved in circadian function. However, so far there are only few examples of finding novel clock components in this way. "Interaction proteomics" is an entire field dedicated to mapping protein–protein interactions and protein complexes. The method involves affinity purification of a "bait" protein followed by MS-dependent identification of interaction partners. The lack of good quality, specific antibodies against many proteins often requires the use of tagged versions of the bait. A key concept in interaction proteomics is the necessity of quantification to distinguish specific from background interactions, reviewed in (Gingras et al. 2007; ten Have et al. 2011; Vermeulen et al. 2008). This is becoming increasingly important because high-sensitivity, high-resolution MS can identify hundreds of proteins in single pulldowns, almost all of them being nonspecific binders. Conversely, the ability to filter out unspecific binders allows mild elution conditions that retain weak and transient interactions. This in turn enables following time-dependent interactions. SILAC, chemical labeling, or label-free algorithms can all quantify the proteins (Gingras et al. 2007; Hubner et al. 2010; Sardiu and Washburn 2011; Wepf et al. 2009). In all cases the procedure requires parallel bait and control purifications (from tagged cell lines or specific antibody beads and untagged cell lines or control beads, respectively), because of the possibility of dynamic exchange of complex components in the combined samples. Peptides from unspecific binders will have similar intensities in MS spectra from both pulldowns (one-to-one ratios), while specific protein binders will be enriched relative to the control precipitation; see Fig. 1. This very powerful technology remains to be exploited for mammalian circadian biology; the examples below generally did not use MS-based quantification and instead filtered out probable unspecific binders and contaminants by comparison to control gels.

Some years ago Schibler and colleagues described the first interaction proteomics experiment using rat cell lines stably expressing exogenous tagged PER1 protein (Brown et al. 2005). Immunoprecipitation of tagged PER1 complexes from nuclear

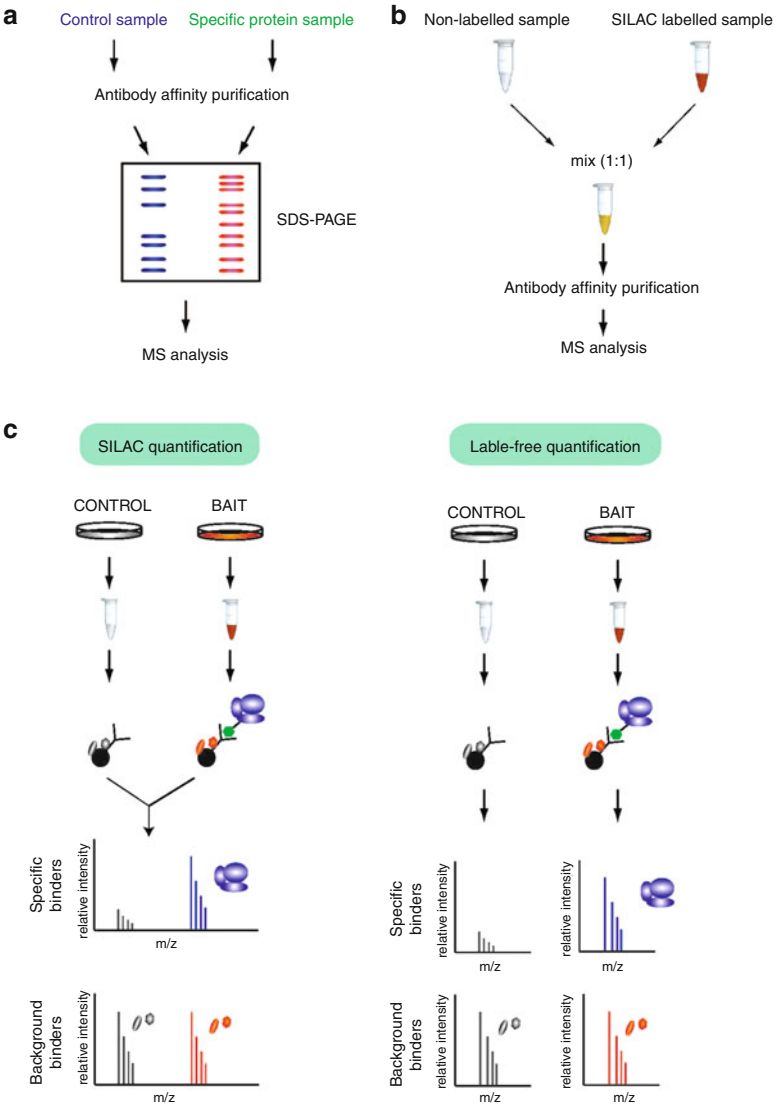

Fig. 1 Schematic representations of the interaction proteomics approaches employed in circadian biology and generally used quantitative affinity purification methods. (**a**) Workflow of the studies performed in mammals with control purification as a reference for unspecific protein pulldowns (Brown et al. 2005; Duong et al. 2011; Robles et al. 2010). (**b**) The method applied to *Neurospora*; reference sample for quantification was obtained with SILAC-labeled protein extracts (Baker et al. 2009). (**c**) Workflows of the two approaches used for quantitative interaction proteomics. *Left panel* shows the strategy followed for SILAC quantification: two cell populations, labeled with light or heavy SILAC and expressing control or bait protein, respectively, are lysed followed by affinity purification. After purification extracts are mixed and analyzed by LC-MS/MS, both light and heavy peptides will appear in the MS spectra, allowing direct comparison of intensities and therefore precise quantification. Strategy for label-free quantification is shown in the right panel.

cell extracts followed by MS analysis identified two novel interactors: NONO and WDR5. Knock-down validation studies showed that the RNA-binding protein NONO is essential for circadian rhythmicity in mammalian cells and in flies. In addition, WDR5, a member of the histone methyltransferase complex, associated to PER complexes and seemed to assist in their function (Brown et al. 2005).

More recently, two studies from the Weitz group described a more refined interaction proteomics approach in which the tagged proteins were endogenously expressed—substituting the untagged endogenous forms while preserving their function. This method ensures that the affinity-purified protein complex is the only complex present in the cell and that it is functional. In this way, novel clock regulators important for circadian function were discovered in protein complexes isolated from mouse cells and tissues (Duong et al. 2011; Robles et al. 2010). Affinity-purified nuclear protein complexes from mouse fibroblast containing tagged BMAL1 were analyzed by MS to identify RACK1 (receptor for activated C kinase 1) as a new BMAL1 interaction partner. Validation experiments showed that RACK1 binds to BMAL1 in a time-dependent manner, recruiting PKCα to the complex, and that it inhibits BMAL1-CLOCK activity. This effect could be mediated by PKCα-dependent phosphorylation of BMAL1, which was demonstrated in an in vitro assay (Robles et al. 2010). More recently, a similar interaction proteomics approach in mouse tissues identified novel components of the endogenous PER protein complexes. Importantly, this uncovered the first molecular mechanism for negative feedback in the mammalian circadian clock (Duong et al. 2011). Briefly, MS analysis identified the RNA-binding protein PSF (polypirimidine tract-binding protein-associated splicing factor) as a novel component of the PER nuclear protein complexes. PSF binds to the PER complex and functions as a transcription corepressor by recruiting SIN3A-HDAC. Consequently, PERs mediate the binding of PSF-SIN3A-HDAC in a time-dependent manner to the *per1* promoter inducing histone deacetylation and therefore transcriptional repression.

Another elegant application of proteomics was directed at the *Neurospora* circadian clock. For the first time in circadian biology, Dunlap and coworkers used SILAC-based interaction proteomics (Baker et al. 2009). To determine the dynamic interactome of the *Neurospora* clock gene FREQUENCY (FRQ), heavy SILAC-labeled *Neurospora* was used as a reference sample to assess relative protein abundance among the experimental time points. The heavy control consisted of a pooled mixture of protein lysates from six cultures collected every 4 h so as to contain all time-dependent FRQ isoforms and complexes. This pool was

Fig. 1 (continued) In this case control and bait expressing cell lysates are immunoprecipitated and analyzed by MS separately. Peptides from specific binders have different intensity between control and specific pulldown, while background proteins have similar intensities in both immunoprecipitations

mixed 1:1 to light protein lysates collected in the same time-dependent manner. The heavy-to-light (H/L) ratio of any given peptide identified by MS therefore represented the relative change in their abundance. In this way the authors defined the temporal interactome of FRQ. For instance, the FRQ-interacting RNA helicase (FRH) interacts with FRQ throughout the day, the heterodimeric transcription factor WHITE COLLAR-1 and WHITE COLLAR-2 complex (WCC) preferentially binds FRQ in the early part of the day.

4 Posttranslational Modifications in Circadian Biology

Daily rhythms are generated by a molecular mechanism consisting of negative and positive transcriptional feedback loops. Proper function of this molecular clock is regulated at multiple levels, transcription, posttranscription, translation, and posttranslation. In recent years, multiple studies have highlighted the role of PTMs in core clock components for general clock function as well as for fine-tuning (Mehra et al. 2009). An increasing number of PTMs have been described in clock proteins in different species, and furthermore, recent data in cyanobacteria indicate a clock based entirely on PTMs (Johnson et al. 2008). One of the most common PTMs of clock proteins is phosphorylation, regulation of which is temporal and phase-specific (Vanselow et al. 2006). In addition, acetylation, ubiquitination, and SUMOylation have been reported to regulate clock protein function or stability in mammals (Asher et al. 2008; Cardone et al. 2005; Lee et al. 2008; Mehra et al. 2009; Nakahata et al. 2008; Sahar et al. 2010). A common characteristic of most of the reported PTMs is their rhythmicity; for that reason measurement of PTM temporal changes would be extremely desirable in the circadian field. To date there is only one such study and it describes circadian dynamic changes of phosphorylation of the FRQ protein in *Neurospora crassa*. The data was obtained in the paper that characterized dynamic interactions of FRQ already mentioned above Baker et al. 2009. Using SILAC in combination with high-resolution MS, the authors identified and quantified 75 phosphorylation sites in the affinity-purified protein. Quantification of these sites across different circadian times allowed depicting phase-specific phosphorylation changes and demonstrated how this temporal regulation affects FRQ stability. Thus, this study showed for the first time the quantitative extent of rhythmic phosphorylation, similarly to what has been qualitatively reported for other clock proteins in different species (Chiu et al. 2008; Kivimae et al. 2008).

Technological advances in MS now allow to characterize modified peptides with high quantitative accuracy and to localize the PTM with single amino acid resolution in the peptide. Since one of the main challenges of PTM analysis is the low abundance of many modified peptides, enrichment strategies have been developed (Bantscheff et al. 2007; Choudhary and Mann 2010). Modification-specific enrichments can be done at the protein or peptide level and for the entire proteome or for specific, purified proteins of interest. In many biological fields and particularly in circadian biology, it is not only interesting to identify PTMs but even more

to determine their changes among different states of the proteome. This can be achieved by MS-based quantitative PTM analysis. Several studies have recently reported extensive dataset of different PTMs: phosphorylation (Beausoleil et al. 2004; Bodenmiller et al. 2007; Dephoure et al. 2008; Ficarro et al. 2002; Olsen et al. 2010), acetylation (Choudhary et al. 2009; Kim et al. 2006), N-glycosylation (Kaji et al. 2007; Zielinska et al. 2010), methylation (Ong et al. 2004), ubiquitination (Argenzio et al. 2011; Kim et al. 2011; Wagner et al. 2011), and SUMOylation (Andersen et al. 2009; Tatham et al. 2011). The first study of global dynamic changes in phosphorylation upon stimulation in cell lines reported more than 6,000 phosphorylation sites (Olsen et al. 2006), and a recent study quantified in vivo changes in more than 10,000 phosphosites in mouse liver upon insulin injection (Monetti et al. 2011). Additionally, this technology has been applied to the study of proteome and phosphoproteome dynamics during different stages of cell division, a system that experimentally resembles the circadian one. Remarkably, the study revealed that most of the detected phosphosites and approximately 20 % of the quantified proteins show changes during the cell cycle (Olsen et al. 2010). Additionally, phosphorylation site occupancy (or "stoichiometry") was determined for thousands of the detected phosphosites during different cell cycle stages. Estimation of phosphorylation site stoichiometry will be highly desirable also in circadian biology. This would be very interesting in particular for clock proteins, given that their progressive phosphorylation is a feature of timekeeping and that this phosphorylation kinetics determines proper clock function. Furthermore, phosphorylation deregulation in some clock proteins has been associated with disorders in humans (Reischl and Kramer 2011; Vanselow and Kramer 2007). Because several kinases play an essential role in the function of the clock (Lee et al. 2011; Reischl and Kramer 2011), determination of phosphorylation occupancy more generally in the whole circadian phosphoproteome could result in significant insights into the role of additional kinases in circadian rhythmicity.

5 Mass Spectrometry Applied to Metabolomic Studies

In addition to proteins, mass spectrometry can also be applied to the study of metabolites. Metabolomics technology, in particular gas chromatography–mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and capillarity electrophoresis-mass spectrometry (CE-MS), has been used to the study of circadian metabolomics. Daily oscillations in mouse blood and plasma as well as human plasma and urine have been reported in recent studies (Dallmann et al. 2012; Eckel-Mahan et al. 2012; Minami et al. 2009). A recent paper provides an overview of integrating other circadian datasets with metabolomics (Patel et al. 2012).

6 Perspectives for Proteomics in Circadian Biology

Proteomics is essential in the functional annotation of the genome and future attempts to build a quantitative, "systems-based" description of cell biology in general (Cox and Mann 2011) and of circadian biology in particular (Baggs and Hogenesch 2010). Circadian biology is an ideal field for the application of quantitative proteomics since circadian clocks control daily oscillations of gene expression and thereby protein abundance, modification, activity, and localization in the cell.

Circadian regulation of gene expression at the level of the transcriptome has been the focus of many studies in the circadian field. In contrast, very little is known about the circadian regulation of global protein abundance and PTMs, mainly because of the technical challenges described above. Given that proteins and not nucleic acids are the main executors of cellular functions, circadian biology would hugely benefit from the development and deployment of functional proteomics methods. In addition to the initial steps of the gene expression program, protein abundance in the cell is regulated by translation, stability, and degradation mechanisms. Interestingly, a recent comprehensive study showed that protein abundance is not only determined by message abundance but that translation control is at least as important (Schwanhausser et al. 2011). The above-reviewed proteomics studies, though relatively limited, are consistent with a fundamental contribution of posttranscriptional regulation in the generation of daily rhythms. Therefore, comprehensive and quantitative analysis of the circadian behavior of the proteome will be a pre-requisite for a systematic and complete understanding of the function of circadian clocks in metabolism and physiology as well as for the effective application of this knowledge to pathologies associated with circadian rhythms such as sleep and metabolic disorders, cancer, etc (Barnard and Nolan 2008; Bass and Takahashi 2010; Huang et al. 2011; Takahashi et al. 2008).

As discuss above, recent advances in high-resolution MS quantitative proteomics allow quantification of the proteome and PTMs at a dramatically larger scale and depth. Therefore, we foresee that application of this technology to the circadian field will lead to promising outcomes (Fig. 2). First of all, the comprehensive analysis of global circadian changes of the proteome (protein levels and PTMs) in tissues and its comparison and complementation to the circadian variation of the transcriptome will result in a better understanding of the role of transcriptional and posttranscriptional regulation in the circadian clock and its relation to daily changes of behavior and physiology. Secondly, dynamic interaction proteomics of the molecular clock can delineate the temporal behavior of the complexes, uncovering the presence of novel protein interactions as well as the dynamics of functionally important PTMs in core clock proteins. Finally, spatial–temporal proteomics can lead to crucial information about the subcellular dynamics of clock protein complexes and its correlation to protein composition.

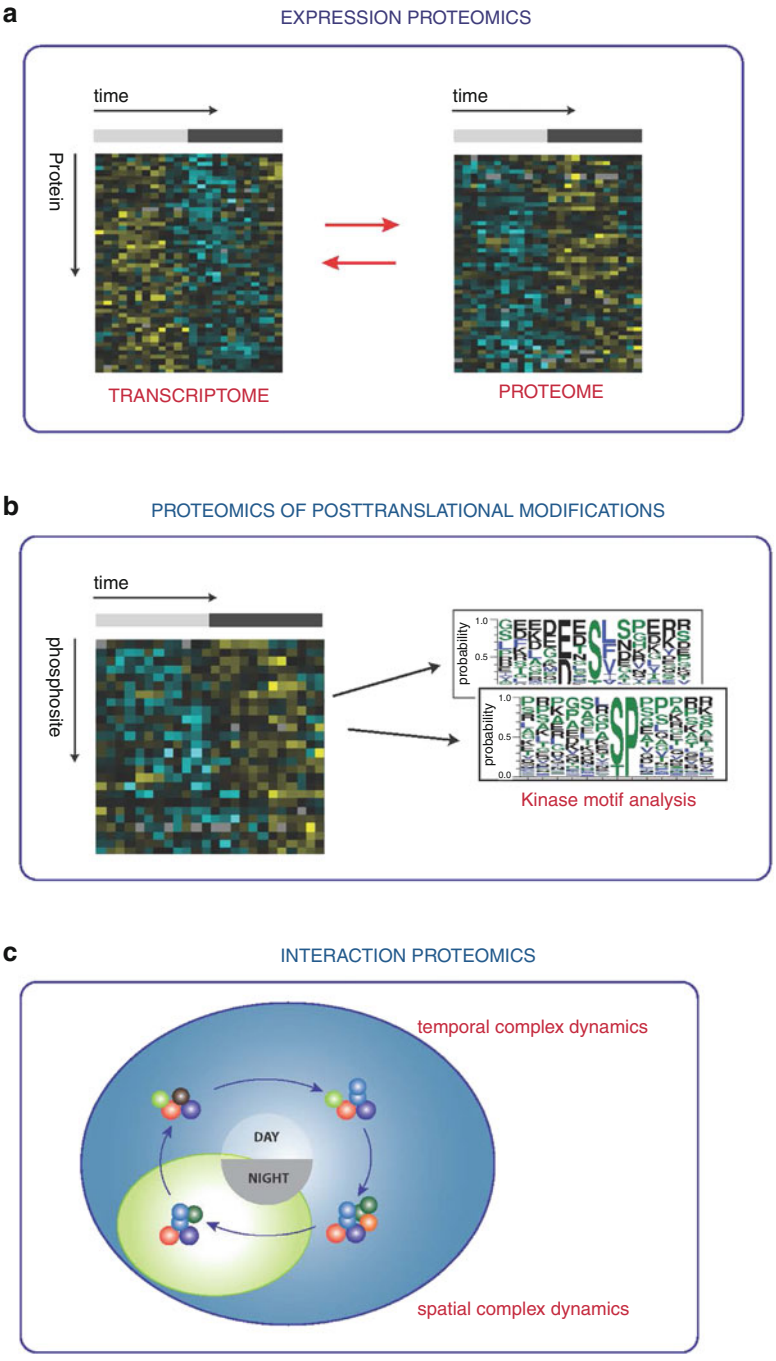

Fig. 2 Potential applications for high-resolution mass spectrometry based quantitative proteomics in the circadian field. (**a**) Expression proteomics application to the circadian clock could lead to

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Fig. 2 (continued) global temporal proteome datasets comparable in scale to previously reported transcriptomes. (**b**) Temporal quantification of PTMs, for example, phosphorylation, could generate large-scale data from which information about kinase activity could be retrieved. (**c**) Spatio-temporal interaction proteomics can also be applied to circadian biology to dissect dynamics and cellular localization of clock core complexes, both essential for proper clock function

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