Uncovering the Proteome Response of the Master Circadian Clock to Light Using an AutoProteome System*§

Ruijun Tian‡§¶¶, Matias Alvarez-Saavedra‡§, Hai-Ying M. Cheng**‡§¶¶, and Daniel Figeys**‡§¶¶*

In mammals, the suprachiasmatic nucleus (SCN) is the central circadian pacemaker that governs rhythmic fluctuations in behavior and physiology in a 24-hr cycle and synchronizes them to the external environment by daily resetting in response to light. The bilateral SCN is comprised of a mere ~20,000 neurons serving as cellular oscillators, a fact that has, until now, hindered the systematic study of the SCN on a global proteome level. Here we developed a fully automated and integrated proteomics platform, termed AutoProteome system, for an in-depth analysis of the light-responsive proteome of the murine SCN. All requisite steps for a large-scale proteomic study, including preconcentration, buffer exchanging, reduction, alkylation, digestion and online two-dimensional liquid chromatography-tandem MS analysis, are performed automatically on a standard liquid chromatography-MS system. As low as 2 ng of model protein bovine serum albumin and up to 20 μg and 200 μg of SCN proteins can be readily processed and analyzed by this system. From the SCN tissue of a single mouse, we were able to confidently identify 2131 proteins, of which 387 were light-regulated based on a spectral counts quantification approach. Bioinformatics analysis of the light-inducible proteins reveals their diverse distribution in different canonical pathways and their heavy connection in 19 protein interaction networks. The AutoProteome system identified vasopressin-neurophysin 2-copetin and casein kinase 1 delta, both of which had been previously implicated in clock timing processes, as light-inducible proteins in the SCN. Ras-specific guanine nucleotide-releasing factor 1, ubiquitin protein ligase E3A, and X-linked ubiquitin specific protease 9, none of which had previously been implicated in SCN clock timing processes, were also identified in this study as light-inducible proteins. The AutoProteome system opens a new avenue to systematically explore the proteome-wide events that occur in the SCN, either in response to light or other stimuli, or as a consequence of its intrinsic pacemaker capacity. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.007252, 1–15, 2011.

Through evolution, the circadian timekeeping system has arisen to ensure that an organism can anticipate and adapt to regular environmental changes resulting from a 24-hr day/night cycle. Virtually all aspects of mammalian physiology and behavior are governed by the circadian clock and exhibit daily rhythms. Intense research and interest in understanding clock timing mechanisms is fueled by the recent discoveries that disruptions in circadian rhythms are linked to a host of pathophysiological disorders, including cancer, cardiovascular disease, metabolic syndrome, and various neurological syndromes (1–3). Genetic studies have established that circadian rhythms are driven by a network of core clock components that interact within a series of dynamically regulated transcription-translation feedback loops (4–6). As the master circadian pacemaker in mammals, the suprachiasmatic nucleus (SCN) can run autonomously with near 24-hr periodicity and coordinate the phase of peripheral oscillators throughout the body. Moreover, the SCN resets its phase in direct response to environment light, ensuring that clock-controlled processes remain tied to the rhythms of the environment. Large-scale gene expression analyses of the murine SCN have revealed hundreds of cyclic transcripts (7, 8). For a handful of these genes, rhythms in protein expression or post-translational modification have also been documented (9, 10). In comparison, proteome-wide analysis of the SCN in response to light has been limited (11, 12), and studies on light-induced protein expression or post-translational modifications are generally restricted to several of the core clock genes (e.g. Period1 and Period2), immediate early genes (e.g. c-Fos), kinases and histones (13–15). A systematic study of...
studied by both in situ (20). Endogenous peptides secreted from the SCN were also analyzed using difference gel electrophoresis two-dimensional-DIGE successfultly visualized 871 protein “spots” from the murine SCN and identified 34 circadian regulated proteins by preparative scale gel and liquid chromatography-tandem MS (LC-MS/MS) (20). Endogenous peptides secreted from the SCN were also studied by both in situ solid-phase extraction followed by off-line matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) MS as well as LC-Fourier transform (FT) MS using multiple tissues (21, 22).

One of the main technical challenges when dealing with minute amounts of starting material is sample loss and lower yield. In recent years, great success has been made to develop integrated fluidic systems for online LC-MS/MS analysis, which provided a clear improvement in sensitivity (23–25). Moreover, immobilized enzymes coupled with high performance liquid chromatography (HPLC) were reported to improve the processing of proteomic samples prior to mass spectrometry (26–30). However, these sample processing systems are not readily compatible with large-scale proteomics studies of minute sample amounts. The automated LC-MS/MS systems are often complex systems, and do not incorporate protein preconcentration and most of the biochemical and chemical processing for proteomic samples. Furthermore, two-dimensional liquid chromatography fractionation, which is necessary for large-scale proteomics study, is still performed with difficulty on current automated systems.

Here, we report a fully automated online proteomics sample processing and mass spectrometry identification system, termed AutoProteome system, for minute amounts of protein samples. All requisite steps for proteomics sample processing, including protein preconcentration, buffer exchange, reduction/alkylation, and digestion are integrated. Furthermore, the system includes online two-dimensional chromatography separation and mass spectrometry identification (two-dimensional LC-MS/MS) based on a standard nanoflow LC-MS/MS system. We used this automated system to study the effect of light stimulation on protein expression in the murine SCN. In total, 196,109 peptides corresponding to 4189 unique proteins were identified by the AutoProteome system with FPR of 1%, and 2131 proteins were quantified with spectral counts-based quantitation, a marked improvement over previous studies. Of these 4189 unique proteins, 387 proteins have statistically significant changes in expression levels (either up- or down-regulated) in response to light. Systematic function analysis revealed their tight connection and distribution in multiple pathways related to circadian rhythms. Subsequent validation by immunostaining and Western blot analysis demonstrated the light-inducible expression of two key proteins that have previously been implicated in clock timing processes, as well as three additional proteins that have not been linked to circadian rhythms.

**EXPERIMENTAL PROCEDURES**

Materials—Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was purchased from Pierce (Rockford, IL). Trypsin was purchased from Promega (Madison, WI). Protease inhibitors and phosphatase inhibitor mixture plates were obtained from Roche (Mannheim, Germany). PolySULFOTETHYL A was obtained from (PolyLC, Columbia, MD). POROS HS 20 µm with dynamic binding capacity of 75 mg/ml for lysozyme at pH 6.2 was obtained from Applied Biosystems (Foster City, CA), and Magic C18 AQ (5 µm, 200 Å pore) was obtained from Michrom BioResources (Auburn, CA). Fused-silica capillaries with ID of 200 µm and 700 µm were obtained from Polymicro (Phoenix, AZ). Acetonitrile with 0.1% formic acid and water with 0.1% formic acid were purchased from J.T. Baker (Phillipsburg, NJ). All other chemicals were obtained from Sigma-Aldrich (Saint Louis, MO).

**Light Treatment Paradigm and Tissue Processing—**Adult (8- to 10-week-old) C57Bl6/J male mice (The Jackson Laboratory, Bar Harbor, ME) were housed in individual cages with a 12 h light:12 h dark (LD) cycle (100 lux intensity) for a minimum of 2 weeks, and then transferred to total darkness for 2 consecutive days. Following dark adaptation, mice received a single light pulse (LP: 15 min, 100 lux) at circadian time (CT) 15, returned to darkness and killed 4 h later for tissue harvesting. Mice were killed by cervical dislocation and decapitated, and eyes were covered with black electrical tape under dim red light. For immunofluorescence (IF) labeling, brains were dissected and immersed in chilled oxygenated physiological saline, cut into 600 µm coronal sections with an oscillating tissue slicer, and fixed (6 h, room temperature) in a 4% (w/v) formaldehyde/phosphate-buffered saline (PBS) solution, pH 7.4. Tissue was cryoprotected in 30% sucrose (w/v) overnight, and then cut into thin (40 µm) sections using a freezing microtome. For Western blotting and MS, SCN tissue was dissected using a micropunch, frozen on dry ice, and stored at −80 °C until use. Dark control (DD) mice were killed at the same CT without exposure to a single light pulse. For these experiments, CT was estimated based on the prior LD cycle, where Zeitgeber time (ZT) 12 (i.e. lights off) was used to define CT 12. Mice were maintained at the animal facility of the University of Ottawa in accordance with institutional guidelines. All animal handling and experimental procedures were approved by the Animal Welfare Committee of the University of Ottawa.

**Immunofluorescent Labeling and Image Acquisition—**Thin (40 µm) brain sections were washed five times in PBST (PBS with 0.1% Triton X-100), blocked (1 h, room temperature) in 10% horse serum/PBST, and incubated (overnight, 4 °C) in primary antibodies. The following primary antibodies were used: mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:1000; Molecular Probes, Eugene, OR), guinea pig polyclonal anti-arginine vasopressin (AVP) (1:4000; Peninsula Laboratories, San Carlos, CA); rabbit polyclonal anti-RASGRF1 (1:500; Cell Signaling Technology, Danvers, MA); rabbit polyclonal anti-UBE3A (1:3000; LifeSpan Biosciences, Seattle, WA); rabbit polyclonal anti-USP9X (1:500; Novus Biologicals, Littleton, CO); and rabbit polyclonal anti-DesRED (1:200; Clontech, Mountain View, CA). The following day, sections were washed five times in PBST and incubated (2 h,
room temperature) with Alexa Fluor® 488- and/or Alexa Fluor® 594-conjugated secondary antibodies (1:1000; Molecular Probes) against the IgG domains of the primary antibodies. In some experiments, sections were counterstained with the nuclear marker DRAQ5™ (1:2000; Biostatus Ltd., Shepshed, Leicestershire, UK). Sections were mounted on slides with Dako Fluorescence Mounting Medium (Dako Canada, Inc., Mississauga, Ontario, Canada).

Images were captured using a Zeiss 510 laser scanning confocal microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with argon (488 nm), helium/neon (546 nm), and helium/neon (633 nm) lasers. Images were acquired with ZEN 2008 software (Carl Zeiss MicroImaging GmbH). All confocal parameters (pinhole, contrast, brightness, etc.) were held constant for all data sets from the same experiment. For quantitative analysis, central SCN images were inverted and the SCN was outlined using a polygon. The “measure” function in ImageJ (http://rsbweb.nih.gov/ij/) yielded a “mean gray” value for the SCN. Values were presented as mean relative abundance of the protein examined.

**Western Blot Analysis**—SCN tissue was pooled from three animals per condition, and homogenized in ice-cold radioimmunoprecipitation assay buffer supplemented with protease inhibitor mixture, incubated for 20 min on ice, and passed several times through a 27-gauge syringe needle. After preclearing by centrifugation (15 min at 17,000 × g), proteins were quantified by the Bradford method using the Coo massie Plus™ Protein Assay Reagent (Fisher Scientific Canada, Whitby, ON, Canada). Protein samples were resolved on sodium dodecyl sulfate polyacrylamide gels under denaturing conditions, and blotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) by wet transfer for 2 h at 80 V for low molecular weight proteins or overnight (~16 h) at 30 V for high molecular weight proteins. Membranes were blocked (45 min, room temperature) with 5% skim milk in TBST (Tris-buffered saline containing 0.05% Triton X-100), and incubated (4 °C, overnight) with the following antibodies: rabbit anti-casein kinase Iβ (CSNK1D) (1:2500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-Ras-GRF1 (1:2000, Cell Signaling Technology) and rabbit anti-actin (1:30,000, Sigma-Aldrich). Membranes were incubated (2 h, room temperature) with ImmunoPure® HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:250,000; Pierce, Rockford, IL). Membranes were washed 5 × 5 min in TBST after antibody incubations, and the signal was detected by chemiluminescence using the SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce). All western quantitation were performed with ImageJ software (http://rsbweb.nih.gov/ij/).

**Protein Extract Preparation**—For tissue lysis for MS, one SCN tissue was suspended in 100 μl lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM CaCl2, 2 mM MgCl2, and fresh protease inhibitor mixture), and sonicated four times for 3 s each with at least 1 min on ice between each pulse. After incubating for 30 min on ice, the extract was clarified by centrifugation at 13,000 × g for 10 min (4 °C). The supernatant was transferred to a
Light-responsive Proteome by a Novel AutoProteome System

Fresh tube and the protein concentration was determined by the Bradford method.

**Automated Online Proteomics System (AutoProteome System)**—As shown in Fig. 1, the AutoProteome system consists of an Agilent 1200 series capillary pump, an Agilent 1200 series micro-autosampler, and a LTQ linear ion trap mass spectrometer equipped with a nanospray source (Thermo, San Jose, CA). The spray voltage was set at 1.8 kV. All MS and MS/MS spectra were acquired in the data-dependent mode. The mass spectrometer was set such that one full MS scan was followed by ten MS/MS scans for LTQ linear ion trap mass spectrometer. Different dimensions of reactor columns (i.e., 200 μm ID × 2 cm, 200 μm ID × 20 cm and 700 μm ID × 10 cm) packed with POROS SCX beads in-house were connected in Agilent 1200 autosampler. Both C18 trap columns (200 μm ID × 5 cm) and C18 analytical columns (75 μm ID × 5 cm) were packed with Magic C18 AQ beads and connected in mass spectrometry part. All of the protein samples and reagents were kept on 96-well sample plates in autosampler at 4 °C in the dark. The flow rate was set at 10 μL/min with 0.1% formic acid for automated sample processing operation, and switched to 5 μL/min to reach a final flow rate of 200 nL/min after splitting for LC-MS/MS analysis.

All automated proteomic reactor operations were programmed and controlled by the HPLC system and mass spectrometry software in coordination (as shown in supplemental Table S1). The reactor column was first conditioned with 0.1% formic acid by capillary pump, and system pressure was kept below 20 bar. Protein samples were acidified to pH 3 with 100 mM citric acid and loaded onto the reactor column. Trypsin was dissolved into 10 mM citric acid, pH 3 and loaded onto the reactor column in a ratio of 1:5 to protein sample. After the sample and trypsin were loaded, the reactor column was washed with 20% acetonitrile, 10 mM citric acid, pH 3 to remove buffers and contaminants. For protein reduction, 20 mM TCEP on the proteomic reactor, allowing the protein reduction to be accomplished under acidic conditions, and some of the systems. Significant difference were evaluated by the Fisher’s exact test as reported previously,(32) and the identified proteins with p value lower than 0.05 were kept for further function analysis.

Protein function, pathway, and network of the identified proteins were mapped and summarized by Ingenuity Pathways Analysis (IPA), version 8.5 (Ingenuity Systems, Redwood City, CA). Canonical pathways analyses were performed with p value of 0.05 (Fisher’s exact test). Networks were displayed with minimum significant score of 16 (Fisher’s exact test).

**RESULTS AND DISCUSSION**

**Automated Online Sample Processing and Analysis**—We developed an automated system, termed the AutoProteome system, for the processing of minute levels of proteomic samples. The AutoProteome system fully integrates the extraction and concentration of proteins, the chemical and biochemical processing of proteins, and the separation of peptides by two-dimensional HPLC. The AutoProteome system consists of a modified proteomic reactor(33) incorporated in a standard microflow LC-MS setup using only one pump, one autosampler, and one mass spectrometer (Fig. 1), thereby greatly minimizing the complexity of the system. Briefly, in this arrangement the proteomic reactor is introduced right after the autosampler arm prior to the switching valve in the autosampler, thus allowing the whole protein processing procedure to be performed automatically and controlled by the HPLC system software. The autosampler in combination with the HPLC pump can automatically introduce the proteomic samples, the enzymes, and the chemicals necessary for the processing of the proteomic samples. Moreover, the proteomic reactor simultaneously serves as a preconcentration device for the proteins. The protocol for the proteomic reactor (33) was modified such that dithiotreitol was replaced with TCEP on the proteomic reactor, allowing the protein reduction to be accomplished under acidic conditions, and some of the buffer exchange procedures used in the manual proteomic reactor protocol were eliminated. As shown in supplemental Fig. S1, the reduction reaction efficiency is not affected by the replacement of dithiotreitol with TCEP, whereas the online sample processing procedure is greatly simplified. The processing of proteomic samples on the AutoProteome system, including the concentration, and biochemical and chemical processing of the samples, is completed within 120 to 170 min, depending on the sample volume.

The AutoProteome system also performs online two-dimensional LC-MS/MS analysis. SCX based peptide separation has been extensively used as the first separation mode in
two-dimensional LC-MS/MS analysis (34). Interestingly, the proteomic reactor is based on a strong cation exchange mechanism: therefore, once the processing of the samples has occurred on the proteomic reactor, it can then be used as the first dimension separation column in two-dimensional LC-MS/MS analysis. This is accomplished by sequential step-elution of the peptides from the proteomic reactor to a C18 precolumn (Fig. 1 and supplemental Table S1).

We first compared the performance of the AutoProteome system with the manual proteomic reactor. Different amounts of BSA were processed on the AutoProteome system with online one dimension LC-MS/MS and the manual proteomic reactor with offline one dimension LC-MS/MS. As shown in Fig. 2A, better performance was obtained on the AutoProteome system for the analysis performed using 20 ng to 2 μg of protein. The most probable reason for this improvement is the reduction in sample loss in the fully automated system.

We then tested the performance of the system for the analysis of the complex SCN proteome. In particular, we tested whether a single or a fraction of a mouse SCN would provide sufficient amounts of starting material for analysis on the AutoProteome system. We also tested whether different

---

**Fig. 2.** The performance of the AutoProteome system for BSA protein standard and SCN tissue from single mouse. A, Detection limit of the AutoProteome system and manual proteomic reactor for the BSA protein standard from 2 ng to 2 μg (n = 3); B, The distribution of identified proteins and identified peptides from different sample amounts and two-dimensional LC-MS/MS methods (n = 2). For the analysis of 20 μg of SCN tissue extract, a reactor column with dimensions of 200 μm ID × 20 cm was used, and NH4OAc with concentrations of 50, 100, 300, 700, and 1000 mM was used. For the analysis of 200 μg of SCN tissue extract, a reactor column with dimensions of 700 μm ID × 20 cm was used, and NH4OAc with concentrations of 50, 100, 150, 200, 250, 300, 350, 400, 700, and 1000 mM were used for 10 SCX fractions elution and 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 500, 600, 700, and 1000 mM were applied for 20 SCX fraction elution. C, The distribution of the unique peptides identified from 200 μg of SCN tissue extract in 10 SCX fractions, and (D) their overlap between different fractions.
numbers of step elution from the proteomic reactor would augment the number of identified proteins. We tested the performance of the system for 20 and 200 μg of protein extract of SCN tissue (corresponding to 1/10 and 1 mouse SCN, respectively) and for 5- to 20-step elutions during the two-dimensional peptide separation. The number of proteins identified from the SCN using the AutoProteome system depends on the amount of samples injected on the system and the depth of the two-dimensional-HPLC separation (Fig. 2B). Moreover, a doubling in the number of identified peptides was obtained when the step elution was increased from 10 to 20. However, the number of unique identified proteins did not significantly change, and therefore, we decided to utilize 200 μg of sample and perform 10-step elutions per analysis for subsequent analyses. The total time for the AutoProteome process including the sample processing and the two-dimensional LC-MS/MS was 28 h. Based on this strategy as shown in Fig. 2C, approximately on average each fraction is composed of 76% unique peptides and two subsequent fractions are composed of 90% unique peptides. Furthermore, the heat map analysis of the identified unique peptides across different fractions confirmed their tight distribution at single fraction (Fig. 2D). These results demonstrate the efficient fractionation of the digested peptides and the possibility of using the AutoProteome system for the analysis of the SCN proteome.

Characterization of the Proteomic Response of the SCN to Light—As the master circadian pacemaker in mammals, the SCN coordinates peripheral clocks throughout the body in order to maintain proper phase alignment in relation to the external environment. The SCN is innervated by glutamatergic and PACAPergic projections arising from the retina, and can therefore directly reset (or entrain) its clock phase in response to light, the most potent synchronizer or “Zeitgeber” (“Zeit” = time, “geber” = giver) of circadian rhythms. Photic stimulation during the circadian night activates a number of kinase pathways (e.g. p42/p44 MAPK) within the SCN that ultimately trigger de novo transcription of several immediate early genes (e.g. c-fos, fos-B, jun-B) (35), core clock genes (e.g. PER1 and PER2) (14, 36), and other light-inducible genes (12). Studies have shown that transcriptional activation within the SCN is essential for light-induced resetting of behavioral, physiological, and metabolic rhythms (37). In addition, various studies have demonstrated a requirement for de novo protein synthesis in light-evoked clock entrainment (38, 39). More recently, we have shown that light-induced microRNA expression in the SCN acts as a feedback modulator of clock entrainment via regulation of protein translation (40, 41). To better understand the molecular processes that underlie the ability of the SCN to synchronize to environmental light, we set forth to identify the proteins that are altered in expression in the murine SCN in response to photic stimulation.

To this end, C57Bl/6J mice that had been stably entrained to a fixed 12 h:12 h LD cycle were maintained in constant darkness for 2 consecutive days prior to receiving a 15-min light pulse (LP) at CT 15. Following light exposure mice were returned to dark conditions and killed 4 h later for harvesting of SCN tissue. Dark control (DD) mice were killed at the same CT without receiving the LP. SCN tissues from separate cohorts of animals were used for immunohistochemical analysis, Western blotting and proteomic analysis. The protein extracts from the SCN of LP- and DD-treated mice were sequentially analyzed on the AutoProteome system. In order to explore the light-responsive proteome of the SCN, a quantitative proteomic study based on spectral counts was performed following photic stimulation (42–44). For each treatment condition (i.e. LP and DD), four biological replicates of 200 μg proteins (i.e. ~1 bilateral SCN) were analyzed on the AutoProteome system. For each individual protein, relative quantitation of its abundance between the light- versus dark-exposed mice was obtained. As a control to confirm the efficiency of our light treatment paradigm, we verified by indirect immunofluorescence that the mPeriod 2 (mPER2) gene, a known light-induced clock gene in the SCN (14), was up-regulated in the SCN of LP-treated mice relative to DD subjects (Fig. 3A).

A total of 196,109 peptides corresponding to 4189 unique proteins were identified using the AutoProteome system with a FPR of 1%. In order to meet our very stringent filtering criteria established to decrease the false positive rate, each individual protein must be identified: (1) in at least three out of four biological replicates; and (2) with a minimum of two unique peptides. We identified 1958 proteins from the LP sample with FPR of 0.18%, and 1716 proteins from the DD sample with FPR of 0.11% (supplemental Table S2). Four hundred fifteen (19.5%) and 173 proteins (8.2%) were uniquely identified in the LP and DD samples, respectively (Fig. 3B).

We evaluated the reproducibility of the spectral count method and our filtering criteria for four biological replicates of the light and dark treated animals (as shown in supplemental Fig. S3A and B). The normalized spectral counts of each protein between any of two biological replicates were correlated and compared, and the correlation values were calculated. The average correlations between replicates for the LP and DD samples are 0.96 and 0.95, respectively. These results demonstrate that the AutoProteome system, in combination with the spectral count method and our filtering criteria, has good reproducibility for the semi-quantification of proteins.

We were particularly interested in the proteins that had statistically significant changes in their expression levels following light exposure. We established an MA plot based on the log2 transformed normalized spectral counts of each identified proteins where M (log2LP-log2DD) represents the differential protein abundance between DD and LP samples, and A ((log2LP+log2DD)/2) represents the average protein abundance. Proteins that do not vary in expression following a light pulse are expected to center at zero on the M axis. As
shown in Fig. 3C, the majority of the proteins quantified were tightly centered at zero. All of the proteins present on the MA plot were color-coded according to the \( p \) value calculated by Fisher’s exact test as previously described (32). Proteins with a \( p \) value \(< 0.05 \) were coded blue, whereas proteins with a \( p \) value \(\geq 0.05 \) were coded red or green if their expression levels were enhanced or decreased, respectively, following the light treatment. From our analysis, 218 proteins were significantly up-regulated (supplemental Table S3), and 169 proteins were significantly down-regulated (Table S4). Interestingly, the minimum spectral counts for up-regulated proteins and down-regulated proteins were 6 and 4, respectively, reinforcing the notion of our statistic filtering approach.

We next explored the possible overlap between rhythmically expressed genes and light-inducible genes in the SCN. To this end, we compiled a list of 416 cycling transcripts from two microarray gene expression profiling studies of the murine SCN (7, 8). This list of rhythmically expressed genes was compared with our list of proteins whose expression was affected by light treatment. Of the 416 previously reported rhythmically expressed genes, 120 were detected by the AutoProteome system in this study. Out of these 120 proteins, 23 exhibited a significant change at the protein level following photic stimulation (supplemental Table S5). This is a reasonable correlation between the circadian transcriptome and the light-responsive proteome considering that they are not fully overlapping processes and that the changes at the protein levels are often out-of-phase or not directly correlated with the changes at the transcript level.

Interestingly, of the 23 proteins that were significantly altered by light treatment and exhibited circadian rhythms in gene expression, five proteins that are implicated in the ubiquitin-proteosome pathway were found: four of these (\textit{i.e.} PSMD5, UBA3, UBQLN1, and USP9X) were significantly up-regulated, whereas one (\textit{i.e.} FBXO3) showed light-dependent down-regulation. Protein ubiquitination has been previously associated with the degradation of several core clock genes, subsequently influencing the accumulation of the protein and, hence, the pace of circadian transcription and circadian oscillations (45). For instance, PER1 and PER2 are targeted to the ubiquitin-proteasome degradation pathway by the actions of casein kinase 1 epsilon (CK1\(\varepsilon\)) and \(\beta\)TrCP, an F-box protein

\[ \text{Molecular & Cellular Proteomics 10.11} \]
that comprises the SCF (Skp1-Cul1-F-box protein) ubiquitin ligase (46, 47). Loss of FBXL3, another F-box protein that targets the ubiquitination and degradation of the core clock genes cryptochrome 1 (Cry1) and cryptochrome 2 (Cry2), slows the circadian clock (48, 49). With respect to our findings, PSMD5 belongs to the 26S proteasome complex and functions as one of the non-ATPases. UBA3 (ubiquitin-activating enzyme 3) was shown to mediate protein degradation through the 26S proteasome (50). UBQLN1 (ubiquilin 1) physically interacts with both proteasomes and ubiquitin ligases in large complexes, and is postulated to regulate protein degradation (51). FBXO3 (F box only protein 3) was recently identified as a new component of the SCF ubiquitin ligase complex (52). The clustering of these ubiquitination-related proteins demonstrates their potential involvement in regulating light-induced clock entrainment.

In addition to factors implicated in the ubiquitin-proteasome pathway, molecular functions of our light-responsive proteome data and the circadian transcriptome data both show enrichment in functions such as transporter, transcription regulator, kinase, and oxidoreductase, demonstrating potential overlap between circadian genes and light-inducible genes in these molecular function groups (supplemental Fig. S3). Moreover, we compared our results with the recent study of the light-responsive proteome of the SCN, analysis of canonical pathways was performed by fitting the identified proteins into the Ingenuity Pathways Analysis (IPA). Based on the p value cutoff of 0.05, the identified proteins in this study distribute widely into 155 different pathways with at least one light-inducible protein in each (Fig. 4A and supplemental Fig. S4). On average, ~28 proteins per pathway can be confidently identified by the stringent quantification criteria, ~15% of which are light-inducible. This result demonstrates that light-inducible proteins represent a relatively large portion of the SCN proteome as identified by the AutoProteome system from the SCN of a single mouse. Moreover, photic stimulation potentially influences a broad range of molecular pathways within cells of the SCN. Four canonical pathways with reported relevance to circadian rhythms and light-evoked clock entrainment were selected for further analysis: (1) the Ca2+/cAMP response element binding protein (CREB) transcriptional pathway; (2) the molecular clockwork; (3) the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway; and (4) the ubiquitin-proteasome degradation pathway (Fig. 4B and supplemental Figs. S4B and C). Because of their high connection to each other as well as their direct relevance to circadian rhythms, the CREB transcriptional pathway and the molecular clockwork were combined together for the analysis. The identified proteins with significant, light-induced changes (p value < 0.05) associated with the four canonical pathways are listed in Table I.

Of particular interest, 22 identified proteins were discovered to be connected with the CREB transcriptional pathway and/or the molecular clockwork (Fig. 4B). The latter, which governs circadian oscillations in nearly all cells, is comprised of interlocking positive and negative transcription/translation feedback loops that drive rhythmic expression of critical core clock components. The transcription factors CLOCK and BMAL1 bind to E-box promoter elements and activate the Period and Cryptochrome genes, whose protein products subsequently heterodimerize and translocate to the nucleus, where they repress CLOCK-BMAL1 activity and shut down their own transcription. Other rhythmic, or clock-controlled genes (ccg), are also regulated by CLOCK/BMAL1-mediated transcription. Photic regulation of this molecular loop requires, in part, on CREB, which mediates light-induced transcriptional activation of the Period genes, subsequent to its own activation by the light-triggered ERK/MAPK signaling cascade (13, 37, 53, 54). Within these pathways, seven proteins are significantly up-regulated and two proteins are significantly down-regulated following light exposure. One of the proteins is Vasopressin-neurophysin 2-copeptin, the precursor of the neuropeptide arginine vasopressin (AVP), which is expressed by SCN neurons and is both clocked-controlled (i.e. rhythmic) and regulated by the CREB transcriptional pathway (7, 21, 55). Consistent with previous observations at the mRNA level (56), our proteomics data demonstrate that nocturnal light exposure induces the expression of AVP in the SCN. To further confirm this light-induced up-regulation, we performed high-resolution confocal imaging of AVP expres-
FIG. 4. Canonical pathway analysis and validation of identified proteins with significant alterations in expression level in the SCN in response to light. A, The summary of centralized canonical pathways with more than five light-inducible proteins (also see supplemental Fig. S4A for canonical pathways with more than one light-inducible proteins); B, Schematic representation of the CREB transcriptional pathway and molecular clockwork. Identified proteins in this study are in blue color; identified proteins with significant up-regulation and down-regulation are in red and green color, respectively; C, immunofluorescent analysis of AVP peptide expression in the SCN of light-pulsed mice and untreated controls. Low magnification images (left): scale bar = 50 μm. High magnification images (right): scale bar = 5 μm. GFAP (Glial fibrillary acidic protein), glial marker; DRAQ5, nuclear marker. Arrows indicate cells in which AVP peptide is up-regulated in response to light, and arrowheads indicate cells expressing basal levels of AVP peptide in dark controls; The mean ± S.E. relative abundance of AVP in untreated versus light-pulsed SCN samples is presented at the bottom-left of panel C; D, Western blot analysis of CSNK1D expression in SCN extract from LP- and DD-treated mice. Actin expression served as the loading control. Values presented below the blot represent the mean relative abundance of CSNK1D, normalized to actin expression. n = 6 mice per treatment condition, grouped in pools of three.

Fig. 4. Canonical pathway analysis and validation of identified proteins with significant alterations in expression level in the SCN in response to light. A, The summary of centralized canonical pathways with more than five light-inducible proteins (also see supplemental Fig. S4A for canonical pathways with more than one light-inducible proteins); B, Schematic representation of the CREB transcriptional pathway and molecular clockwork. Identified proteins in this study are in blue color; identified proteins with significant up-regulation and down-regulation are in red and green color, respectively; C, immunofluorescent analysis of AVP peptide expression in the SCN of light-pulsed mice and untreated controls. Low magnification images (left): scale bar = 50 μm. High magnification images (right): scale bar = 5 μm. GFAP (Glial fibrillary acidic protein), glial marker; DRAQ5, nuclear marker. Arrows indicate cells in which AVP peptide is up-regulated in response to light, and arrowheads indicate cells expressing basal levels of AVP peptide in dark controls; The mean ± S.E. relative abundance of AVP in untreated versus light-pulsed SCN samples is presented at the bottom-left of panel C; D, Western blot analysis of CSNK1D expression in SCN extract from LP- and DD-treated mice. Actin expression served as the loading control. Values presented below the blot represent the mean relative abundance of CSNK1D, normalized to actin expression. n = 6 mice per treatment condition, grouped in pools of three.
TABLE I
Identified proteins with significant changes in canonical pathway analysis. Canonical pathways are: CREB/molecular clockwork pathway, ERK/MAPK signaling pathway and protein ubiquitination pathway

| Canonical pathway name | IPI no. | Gene symbol | Sequence name | N-SC±S.D. (LP/DD)\(^a\) | Expression/p value\(^b\) |
|------------------------|---------|-------------|---------------|--------------------------|-------------------------|
| AVP                    | IPI00118471.1 | AVP         | Vasopressin-neurophysin 2-copeptin | 3.35 ± 2.71/0 | ↑/6.2E-3 |
| CAMKII                 | IPI00124695.1 | CAMK2G      | Isoform 1 of Calcium/calmodulin-dependent kinase type II gamma chain | 2.95 ± 0.75/0 | ↑/1.13E-2 |
| CK1δ                   | IPI00138790.2 | CSNK1D      | Casein kinase 1, delta, isoform CRA_b | 2.41 ± 1.74/0 | ↑/3.86E-2 |
| Gα(Gai/o)              | IPI00230192.5 | GNAO1       | Isoform Alpha-1 of Guanine nucleotide-binding protein G(o) subunit alpha | 7.23 ± 8.05/0 | ↑/7.41E-05 |
| Gγ                     | IPI00230194.5 | GNG2        | Guanine nucleotide-binding protein G(i)/G(S)/G(O) subunit gamma-2 | 3.38 ± 1.83/0 | ↑/1.13E-2 |
| iGLUR                  | IPI00136967.2 | GRIA2       | Isoform 1 of Glutamate receptor 2 | 3.98 ± 2.39/0 | ↑/3.41E-3 |
| mGLUR                  | IPI00136716.1 | GRM3        | Metabotropic glutamate receptor 3 | 2.53 ± 1.72/0 | ↑/2.08E-2 |
| Integrin               | IPI00132474.3 | ITGB1       | Integrin beta-1 | 2.87 ± 1.76/0 | ↑/1.13E-2 |
| PP1/PP2A               | IPI00224697.1 | PPP2R5E     | Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform | 6.88 ± 0.97/0 | ↑/1.16E-05 |
| PP1/PP2A               | IPI00851027.1 | PPP1R11     | Protein phosphatase 1, regulatory (inhibitor) subunit 11 | 3.41 ± 1.93/0 | ↑/1.13E-2 |
| E3 ring                | IPI00124047.1 | CUL1        | Cullin-1 | 2.32 ± 1/0 | ↑/3.86E-2 |
| E3 ring                | IPI00131224.1 | TCEB2       | Transcription elongation factor B poly peptide 2 | 3.23 ± 0.99/0 | ↑/6.2E-3 |
| DUB                    | IPI00463367.2 | USP7        | Isoform 2 of Ubiquitin carboxyl-terminal hydrolase 7 | 2.09 ± 0.62/0 | ↑/3.86E-2 |
| DUB                    | IPI00798468.1 | USP9X       | Ubiquitin specific protease 9, X chromosome | 11.16 ± 1.7/0 | ↑/4.42E-08 |
| PSMD                   | IPI00457661.5 | PSMD5       | 26S proteosome non-ATPase regulatory subunit 5 | 13.19 ± 6.62/5.48 ± 1.64 | ↑/1.6E-2 |
| PSMD                   | IPI00457661.5 | PSMD9       | Putative uncharacterized protein | 2.41 ± 1.74/0 | ↑/3.86E-2 |
| RAS                    | IPI00131248.1 | KRAS        | Isoform 2A of GTPase Kras | 0/1.67 ± 0.42 | ↑/3.86E-2 |
| RAS                    | IPI00328322.1 | RRAS2       | Ras-related protein R-Ras2 | 0/2.16 ± 1.17 | ↑/3.61E-2 |
| 14-3-3                 | IPI00227392.5 | YWHAH       | 14-3-3 protein eta | 41.83 ± 12.22/53.73 ± 4.56 | ↑/3.12E-2 |
| PP1/PP2A               | IPI00123862.1 | PPP1C       | Isoform Gamma-1 of Serine/threonine-protein phosphatase PP1-gamma catalytic subunit | 2.2 ± 1.65/6.59 ± 1.98 | ↑/1.98E-3 |
| E3/E3 HECT             | IPI00399840.2 | UAE3A       | Ubiquitin protein ligase E3A | 0/2.45 ± 1.09 | ↑/3.09E-3 |
| PSME1                  | IPI00124223.3 | PSME1       | Proteasome activator complex subunit 1 | 0/3.35 ± 2.24 | ↑/5.94E-4 |

\(^a\) Normalized spectral counts (N-SC) with standard deviation (S.D.).

\(^b\) Identified proteins with significant up-regulation are labeled with up arrow; identified proteins with significant down-regulation are labeled with down arrow.
sion in the SCN of LP- and DD-treated mice by indirect immunofluorescence (Fig. 4C). Lack of colocalization with the glial cell-specific marker glial fibrillary acidic protein (GFAP) confirmed the neuronal distribution of AVP within the SCN. AVP-positive cells were restricted to the dorsomedial aspect of the SCN (considered to be the “rhythmic” compartment, in contrast with the retino-recipient ventrolateral aspect, of the SCN), and light stimulation markedly augmented AVP expression (Fig. 4C).

Another protein of interest from our canonical pathway analysis of the proteomics data set is Casein kinase 1 delta (CSNK1D or CK1δ). Its homolog, casein kinase 1 epsilon (CK1ε), is firmly established as a core clock component, phosphorylating the PER2 protein at a critical residue and targeting it for degradation; a mutation in CK1ε underlies the now-famous tau mutation in hamsters, which results in a dramatic shortening of the circadian period (57). However, the role of CK1δ in light-induced clock entrainment remains to be addressed. In our proteomics study, CK1δ was identified as significantly up-regulated following photic stimulation. This finding was subsequently confirmed by Western blotting (Fig. 4D), and is consistent with a previous study that noted a light-induced increase in CK1δ expression at the transcript level in the SCN following nocturnal light exposure (58).

Notably, the ubiquitin-proteasome pathway is highly enriched for light-inducible proteins (eight in total), suggesting a key role for this pathway in the regulation of photic entrainment of the circadian clock (9, 48). Interestingly, two deubiquitinating enzymes (i.e., USP7 and USP9X) were identified by our MS approach as being significantly up-regulated in the SCN following photic stimulation, whereas one ubiquitin-protein ligase (i.e., UBE3A) was significantly down-regulated (as

*Fig. 5. Light regulates the expression of the deubiquitinase USP9X and ubiquitin protein ligase UBE3A within the murine SCN.* Immunofluorescent analysis of (A) USP9X and (B) UBE3A expression in the SCN of light-pulsed mice and untreated controls. Low magnification images (left): scale bar = 100 μm. High magnification images (right): scale bar = 50 μm. DRAQ5, nuclear marker. The mean ± S.E. relative abundance of the protein examined in untreated versus light-pulsed SCN samples is presented below the micrographs.
shown in Table I). As shown in Fig. 5, the light-regulated expression of USP9X and UBE3A was subsequently validated by indirect immunofluorescent analysis. A recent study reported that USP9X-mediated deubiquitination of SMAD4 is essential for TGFβ signaling (59), which in turn has been implicated in the resetting of circadian clocks in peripheral tissues (60). Furthermore, components of the TGFβ signaling pathway, including TGFβ itself and SMAD3, are expressed in the murine SCN, leaving open the possibility that USP9X regulates TGFβ signaling within the master pacemaker (61). In another study, genetic disruption of dube3a, the Drosophila homolog of UBE3A, reduced or abolished the rest and activity rhythms in flies, suggesting that expression of core proteins of the molecular clock may be affected (62). We argue that a potential interplay between deubiquitinating enzymes (e.g. USP7 and USP9X) and ubiquitin ligases (e.g. UBE3A) is essential for regulating changes in protein expression and function following photic stimulation, and that these changes, in turn, may play a role in the resetting response.

To further explore potential connections of the identified light-inducible proteome and their up-stream and down-stream interactions, network analysis based on the known interactions of the identified proteins was performed. This approach can highlight clusters of interacting proteins that are affected by photic stimulation. Interestingly, of the 387 proteins affected by light treatment, 352 (91%) are grouped into 19 protein interaction subnetworks with more than 13 proteins affected by photic stimulation. Interestingly, of the 387 proteins affected by light treatment, 352 (91%) are grouped into 19 protein interaction subnetworks with more than 13 proteins affected by photic stimulation. Furthermore, 18 interaction networks were associated with at least one common protein as noted in Fig. 6A. These results demonstrate the significantly high biological correlation of the identified light-inducible proteins, predicting their possible functional correlation in response to light. For example, networks 3, 5, 7, 12, and 13 are associated with each other by at least two common proteins as highlighted in Fig. 6A. This connected network complex is highly centralized at three core nodes: huntingtin (HTT), tumor necrosis factor (TNF), and Glucose Transporter-4 (SLC2A4) (supplemental Fig. S6). The roles of these HTT-, TNF-, and SLC2A4-centered networks in circadian clock processes are currently unknown but warrant future investigation.

From the first network, Ras-specific guanine nucleotide-releasing factor 1 (RASGRF1), which was uniquely identified in LP samples with a normalized spectral count of 2.21, was selected for further validation (Fig. 6B). In network 1, RASGRF1 is functionally associated with the transcription factor nuclear factor kappaB (NF-kB), which has been shown to be expressed by glial cells within the SCN and modulates light-induced phase shifts of behavioral rhythms, and which may participate in cytokine-mediated modulation of the circadian system (63, 64). Biochemically, RASGRF1 is a guanine nucleotide exchange factor for specific Ras or Rho GTPases and itself is activated by various glutamate and G protein-coupled receptors, both of which are important for light-induced SCN clock entrainment (65). As shown in Fig. 6C, there is significant up-regulation of RASGRF1 in SCN neurons following photic stimulation. Western blotting revealed a ~2-fold increase in RASGRF1 expression in the SCN in response to light (Fig. 6D). Although RASGRF1 has not been previously implicated in the regulation of circadian rhythms, based on its biochemical profile, one may speculate that it plays a role in the intracellular signaling events that couple light-induced activation of cell surface receptors to changes in Ras/MAPK-dependent gene transcription or to possible Rho-mediated structural remodeling of SCN neurons. Moreover, genetic ablation of RASGRF1 in mice impairs hippocampal-dependent learning and memory consolidation (66), raising the possibility that RASGRF1 may serve a similar role in the SCN in controlling neuronal plasticity in response to light.

In summary, the AutoProteome system represents the first fully automated and integrated proteomics platform that incorporates all requisite sample processing steps and online two-dimensional LC-MS/MS analysis. It should be noted that this system is essentially based on a standard LC-MS system, which can easily be adapted and applied for different large-scale proteomics studies (i.e. phosphoproteomics) where the starting material is in limiting quantities. In addition, we report the first light-responsive proteome of the master circadian pacemaker in mammals, the SCN, using tissue harvested from a single mouse for each experiment. Broad distribution into different key canonical pathways and a tight connection of the entire light-responsive proteome demonstrate the potential biological significance of our findings to the regulation
of mammalian circadian rhythms. Lastly, from our proteomics results, we confirmed the light-inducible nature of two proteins that had previously been implicated in clock timing processes within the SCN, as well as three other proteins which are as yet uncharacterized with respect to their role in circadian rhythms.

Acknowledgments—We thank Kyle Jones from BIOINQUIRE, LLC for the help with ProteoIQ software.

* This work was supported by operating grants to D.F. from Genome Canada, the National Sciences and Engineering Research Council (NSERC) of Canada, the Canadian Foundation for Innovation (CFI) and the Ontario Research Fund and to H.-Y.M.C. from the Canadian Institute of Health Research (CIHR) #086549 and an infrastructure grant from the Canadian Foundation for Innovation (CFI). D.F. is a Canada Research Chair (CRC) Tier I in Proteomics and Systems Biology, and H.-Y.M.C. is a Canada Research Chair (CRC) Tier II in the Genetics of Biological Timing and both are recipients of the Early Researcher Award from the Ontario Ministry of Research and Innovation.

** To whom correspondence should be addressed: Ottawa Institute of Systems Biology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5. Tel.: (613) 562-5800, ext. 8674; Fax: (613) 562-5655; E-mail: dfigeys@uottawa.ca; Department of Biology, University of Toronto Mississauga, 3359 Mississauga Road North, Mississauga, Ontario, Canada L5L 1C6. Tel.: (905) 569-4299; Fax: (905) 569-4275; E-mail: hajing.cheng@utoronto.ca.

[This article contains supplemental Figs. S1 to S6 and Tables S1 to S6.]

Current address: The Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, ON, Canada MSG X5.

†† Department of Biology, University of Toronto Mississauga, 3359 Mississauga Road North, Mississauga, Ontario, Canada L5L 1C6.

REFERENCES

1. Fu, L., and Lee, C. C. (2003) The circadian clock: pacemaker and tumor suppressor. Nat. Rev. Cancer 3, 350–361
2. Rosbash, M., and Takahashi, J. S. (2002) Circadian rhythms: the cancer connection. Nature 420, 373–374
3. Staels, B. (2006) When the Clock stops ticking, metabolic syndrome emerges. Nat. Med. 12, 54–55; discussion 55
4. Roenneberg, T., and Merrow, M. (2005) Circadian clocks - the fall and rise of physiology. Nat. Rev. Mol. Cell Biol. 6, 965–971
5. Hastings, M. H., Reddy, A. B., and Maywood, E. S. (2003) A clockwork web: circadian timing in brain and periphery, in health and disease. Nat. Rev. Neurosci. 4, 649–661
6. Herzog, E. D. (2007) Neurons and networks in daily rhythms. Nat. Rev. Neurosci. 8, 790–802
7. Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., and Hogenesch, J. B. (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307–320
8. Ueda, H. R., Chen, W., Adachi, A., Nakamura, K., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K., Suzuki, Y., Sugano, S., Inou, M., Shigeyshi, Y., and Hashimoto, S. (2002) A transcription factor response element for gene expression during circadian night. Nature 418, 534–539
9. Godinho, S. I., Maywood, E. S., Shaw, L., Tucci, V., Barnard, A. R., Busino, L., Pagano, M., Kendall, R., Quwailid, M. M., Romero, M. R., O’Neill, J., Chesham, J. E., Brooker, D., Lalanne, Z., Hastings, M. H., and Nolan, P. M. (2007) The after-hours mutant reveals a role for Fbx3 in determining mammalian circadian period. Science 316, 897–900
10. Cardone, L., Hirayama, J., Giordano, F., Tamaru, T., Palvimo, J. J., and Sassone-Corsi, P. (2005) Circadian clock control by SUMOylation of BMAL1. Science 309, 1390–1394
11. Araki, R., Nakahara, M., Fukushima, R., Takahashi, H., Mori, K., Umeda, N., Sujino, M., Inouye, S. T., and Abe, M. (2006) Identification of genes that respond in expression to light exposure and express rhythmically in a circadian manner in the mouse suprachiasmatic nuclei. Brain Res. 1098, 9–16
12. Porterfield, V. M., Piontkivska, H., and Mintz, E. M. (2007) Identification of novel light-induced genes in the suprachiasmatic nucleus. BMC Neurosci. 8, 83
13. Obrietan, K., Impeny, S., and Storm, D. R. (1998) Light and circadian rhythm regulate MAP kinase activation in the suprachiasmatic nuclei. Nat. Neurosci. 1, 693–700
14. Albrecht, U., Zheng, B., Larkin, D., Sun, Z. S., and Lee, C. C. (2001) Mper1 and mper2 are essential for normal resetting of the circadian clock. J. Biol. Rhythms. 16, 100–104
15. Castens, B., Beilkeny, M., Cohen, S., Wagner, S., and Schwartz, W. J. (1997) Light-induced c-Fos expression in the mouse suprachiasmatic nucleus: immunoelectron microscopy reveals co-localization in multiple cell types. Eur. J. Neurosci. 9, 1950–1960
16. Abu-Farha, M., Elisma, F., Zhou, H., Tian, R., Zhou, H., Asmer, S. M., and Figyes, D. (2009) Proteomics: from technology developments to biological applications. Anat. Chem. 81, 4585–4599
17. Aebersold, R., and Mann, M. (2003) Mass spectrometry-based proteomics. Nature 422, 198–207
18. Köcher, T., and Superti-Furga, G. (2007) Mass spectrometry-based functional proteomics: from molecular machines to protein networks. Nat. Methods 4, 807–815
19. Motyama, A., and Yates, J. R., 3rd (2008) Multidimensional LC separations in shotgun proteomics. Anal. Chem. 80, 7167–7193
20. Deayy, J. R., lawmaker, E. S., Graham, J. E., Sladek, M., Karp, N. A., Green, E. W., Charles, P. D., Reddy, A. B., Kyriacou, C. P., Lilley, K. S., and Hastings, M. H. (2009) Proteomic analysis reveals the role of synaptic vesicle cycling in sustaining the suprachiasmatic circadian clock. Curr. Biol. 19, 2031–2036
21. Hatcher, N. G., Atkins, N., Jr., Annangudi, S. P., Forbes, A. J., Kelleher, N. L., Gillette, M. U., and Sweedler, J. V. (2008) Mass spectrometry-based discovery of circadian peptides. Proc. Natl. Acad. Sci. U.S.A. 105, 12967–12972
22. Lee, J. E., Atkins, N., Jr., Hatcher, N. G., Zamborg, L., Gillette, M. U., Sweedler, J. V., and Kelleher, N. L. (2010) Endogenous peptide discovery of the rat circadian clock: a focused study of the suprachromatic nucleus by ultrahigh performance tandem mass spectrometry. Mol. Cell Proteomics 9, 285–297
23. Waanders, L. F., Chwalek, K., Moretti, M., Kumar, C., Lammert, E., and Mann, M. (2009) Quantitative proteomic analysis of single pancreatic islets. Proc. Natl. Acad. Sci. U.S.A. 106, 18902–18907
24. Pinkse, M. W., Mohammed, S., Gouw, J. W., van Breukelen, B., Vos, H. R., and Heck, A. J. (2008) Highly robust, automated, and sensitive online TiO2-based phosphoproteomics applied to study endogenous phosphorylation in Drosophila melanogaster. J. Proteome Res. 7, 687–697
25. Link, A. J., Eng, J., Schiltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvick, B. M., and Yates, J. R., 3rd (1999) Direct analysis of protein complexes using mass spectrometry. Nat. Biotechnol. 17, 676–682
26. Hsieh, Y. L., Wang, H., Elcione, C., Mark, J., Martin, S. A., and Regnier, F. (1996) Automated analytical system for the examination of protein primary structure. Anal. Chem. 68, 456–462
27. Krenkova, J., Lacher, N. A., and Svec, F. (2009) Highly efficient enzyme reactors containing trypsin and endoproteinase LysC immobilized on porous polymer monolith coupled to MS suitable for analysis of antibiotics. Anal. Chem. 24, 2004–2012
28. Callier, E., Temporini, C., Perani, E., De Palma, A., Lubbda, D., Mellerio, G., Sala, A., Galliano, M., Caccianiga, G., and Massolini, G. (2005) Trypsin-based monolithic bioreactor coupled on-line with LC/MS/MS system for protein digestion and variant identification in standard solutions and serum samples. J. Proteome Res. 4, 481–490
29. Lopez-Ferrer, D., Petritis, K., Robinson, E. W., Hixson, K. T., Tian, Z., Lee, J. H., Lee, S. W., Toile, N., Weitz, K. K., Belov, M. E., Smith, R. D., and Pasa-Tolic, L. (2010) Pressurized pepsin digestion in proteomics: An autamatable alternative to trypsin for integrated top-down bottom-up proteomics. Mol. Cell Proteomics 10, M110.0007479, 1–11
30. Ma, J., Liu, J., Sun, L., Gao, L., Liang, Z., Zhang, L., and Zhang, Y. (2009) Online Integration of Multiple Sample Pretreatment Steps Involving De-
Light-responsive Proteome by a Novel AutoProteome System

nutation, Reduction, and Digestion with Microflow Reversed-Phase Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry for High-Throughput Proteome Profiling. Anal. Chem. 81, 6534–6540

31. Elias, J. E., and Gygi, S. P. (2007) Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat. Methods 4, 207–214

32. Li, R.-X., Chen, H.-B., Tu, K., Zhao, S.-L., Zhou, H., Li, S.-J., Dai, J., Li, Q.-R., Nie, S., Li, Y.-X., Jia, W.-P., Zeng, R., and Wu, J. R. (2008) Localized-statistical quantification of human serum proteome associated with type 2 diabetes. PLoS One 3, e3224

33. Ethier, M., Hou, W., Duwelis, H. S., and Figeys, D. (2006) The proteomic reactor: a microfluidic device for processing minute amounts of protein prior to mass spectrometry analysis. J. Proteome Res. 5, 2754–2759

34. Fournier, M. L., Gilmore, J. M., Martin-Brown, S. A., and Washburn, M. P. (2007) Multidimensional separations-based shotgun proteomics. Chem. Rev. 107, 3654–3686

35. Morris, M. E., Viswanathan, N., Kuhlman, S., Davis, F. C., and Weltz, C. J. (1998) A screen for genes induced in the suprachiasmatic nucleus by light. Science 279, 1544–1547

36. Shearman, L. P., Zykova, M. J., Weaver, D. R., Kolakowski, L. F., Jr., and Reppert, S. M. (1997) Two period homologs: circadian expression and photic regulation in the suprachiasmatic nucleus. Neuron 19, 1281–1289

37. Obrietan, K., Impey, S., Smith, D., Aths, J., and Storm, D. R. (1999) Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nucleus. J. Biol. Chem. 274, 17748–17756

38. Murakami, N., Nishi, R., Katayama, T., and Nasu, T. (1995) Inhibitor of protein synthesis phase-shifts the circadian oscillator and inhibits the light induced-phase shift of the melatonin rhythm in pigeon pineal cells. Brain Res. 693, 1–7

39. Zhang, Y., Takahashi, J. S., and Turek, F. W. (1996) Critical period for cycloheximide blockade of light-induced phase advances of the circadian locomotor activity rhythm in golden hamsters. Brain Res. 740, 285–290

40. Cheng, H.-Y., Papp, J. W., Varlamova, O., Dziema, H., Russell, B., Cuprman, J. P., Nakazawa, T., Simizu, K., Obrietan, I., Impey, S., and Obrietan, K. (2007) microRNA modulation of circadian-clock period and entrainment. Neuron 54, 813–829

41. Alvarez-Saavedra, M., Antoun, G., Yanagiya, A., Oliva-Hernandez, R., Cerjo-Palma, D., Perez-Iratxeta, C., Sonenberg, N., and Cheng, H. Y. (2010) miRNA-132 orchestrates chromatin remodeling and translational control of the circadian clock. Hum. Mol. Genet. 20, 731–751

42. Liu, H., Sadovyg, R. G., and Yates, J. R. (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal. Chem. 76, 4193–4201

43. Dix, M. M., Simon, G. M., and Gravatt, B. F. (2008) Global mapping of the topography and magnitude of proteolytic events in apoptosis. Cell 134, 679–691

44. Roth, A. F., Wan, J., Bailey, A. O., Sun, B., Kuchar, J. A., Green, W. N., Bailey, A. O., Sun, B., Kuchar, J. A., Green, W. N., and Leutz, A. (2007) Dual modification of BMAL1 by SUMO2/3 and ubiquitin selectively modulates transcription of clock-regulated genes in neurons and glia: possible divergent roles of mPeriod1 and mPeriod2 in the mouse suprachiasmatic nucleus. J. Neurosci. Res. 86, 612–616

45. Duport, S., Mamidi, A., Cordonens, M., Montagner, M., Zacchigna, L., Audom, M., Martello, G., Stinchfield, M., Soligo, S., Irni, M., Muro, S., Modena, N., Argenton, F., Newfield, S. J., and Piccolo, S. (2009) FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell 136, 123–135

46. Kondo, N., Hirota, T., Kawamoto, T., Kato, Y., Tsubota, T., and Fukada, Y. (2008) Activation of TGF-beta/activin signalling resets the circadian clock through rapid induction of Dec1 transcripts. Nat. Cell Biol. 10, 1463–1469

47. Yoshimura, T., Thorne, J., and Coogan, A. N. (2009) Age and time of day influences on the expression of transforming growth factor-beta and phosphorylated SMAD3 in the mouse suprachiasmatic and paraventricular nuclei. Neuroimmunomodulation 16, 392–399

48. Wu, Y., Bolduc, F. V., Bell, K., Tully, T., Fang, Y., Sehgal, A., and Fischer, J. A. (2008) A Drosophila model for Angelman syndrome. Proc. Natl. Acad. Sci. U.S.A. 105, 12399–12404

49. Leone, M. J., Marpegan, L., Bekinschtie, T. A., Costas, M. A., and Golombek, D. A. (2006) Suprachiasmatic astrocytes as an interface for immune-circadian signalling. J Neurosci. Res. 84, 1521–1527

50. Marpegan, L., Bekinschtie, T. A., Freudenthal, R., Rubio, M. F., Ferreyra, A. G., Romano, A., and Golombek, D. A. (2004) Participation of transcription factors from the Rel/NF-kappa B family in the circadian system in hamsters. Neurosci. Lett. 359, 9–12

51. Ding, J. M., Faiman, L. E., Hurst, W. J., Kuriyashina, L. R., and Gillette, M. U. (1998) Resetting the biological clock: mediation of nocturnal CREB phosphorylation via light, glutamate, and nitric oxide. J. Neurosci. 18, 667–675

52. Giese, K. P., Friedman, E., Tellez, J. B., Fedorov, N. B., Wines, M., Feig, L. A., and Silva, A. J. (2001) Hippocampus-dependent learning and memory is impaired in mice lacking the Ras-guanine-nucleotide releasing factor 1 (Ras-GRF1). Neuropharmacology 41, 791–800

53. Golombek, D. A., Alvarez-Saavedra, M., Dziema, H., Choi, Y. S., Li, A., and Obrietan, K. (2009) Segregation of expression of mPeriod gene homologs in neurons and glia: possible divergent roles of mPeriod1 and mPeriod2 in the brain. Hum. Mol. Genet. 18, 3110–3124
In order to cite this article properly, please include all of the following information: Tian, R., Alvarez-Saavedra, M., Cheng, H.-Y. M., and Figeys, D. (2011) Uncovering the Proteome Response of the Master Circadian Clock to Light Using an AutoProteome System. Mol. Cell. Proteomics 10(11):M110.007252. DOI: 10.1074/mcp.M110.007252.