Concurrent Quantification of Proteome and Phosphoproteome to Reveal System-wide Association of Protein Phosphorylation and Gene Expression*

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Reversible phosphorylation of proteins is an important process modulating cellular activities from upstream, which mainly involves sequential phosphorylation of signaling molecules, to downstream where phosphorylation of transcription factors regulates gene expression. In this study, we combined quantitative labeling with multidimensional liquid chromatography-mass spectrometry to monitor the proteome and phosphoproteome changes in the initial period of adipocyte differentiation. The phosphorylation level of a specific protein may be regulated by a kinase or phosphatase without involvement of gene expression or as a phenomenon that accompanies the alteration of its gene expression. Concurrent quantification of phosphopeptides and non-phosphorylated peptides makes it possible to differentiate cellular phosphorylation changes at these two levels. Furthermore, on the system level, certain proteins were predicted as the targeted gene products regulated by identified transcription factors. Among them, several proteins showed significant expression changes along with the phosphorylation alteration of their transcription factors. This is to date the first work to concurrently quantify proteome and phosphoproteome changes during the initial period of adipocyte differentiation, providing an approach to reveal the system-wide association of protein phosphorylation and gene expression. Molecular & Cellular Proteomics 8: 2809–2826, 2009.

Protein phosphorylation has been considered as a central role for cell regulation and signaling transduction. It is estimated that one-third of eukaryotic proteins are phosphorylated (1). In eukaryotes, the residues that undergo protein phosphorylation are mainly serine, threonine, and to a lesser extent tyrosine (2). Reversible phosphorylation of proteins is an important process for modulating cellular activities. At the upstream level, regulation of signaling transduction mainly relies on the appropriate phosphorylation events, such as sequential phosphorylation of various signaling molecules. At the downstream level, however, signaling transduction in the nucleus generally involves transcription of certain genes. Transcription factor activity, besides being regulated at the level of gene expression, is prominently regulated via post-translational events such as protein phosphorylation, processing, or localization (3). Phosphorylation or dephosphorylation of transcription-associated factors plays a crucial role in transcriptional regulation (see Fig. 1A).

Most of the previous studies have focused on the initial period of signaling transduction, which mainly refers to the dynamic changes of phosphorylation events only. For example, Krüger et al. (4) have applied immunoprecipitation of phosphoproteins to investigate the insulin pathway in the first 20 min. For the epidermal growth factor receptor signaling pathway, Blagoev et al. (5) have studied the period from 0 to 10 min, whereas Olsen et al. (6) have further studied phosphorylation dynamics for the first 20 min. However, there has been little investigation of the downstream gene expression regulated by phosphorylation.

Recent developments in mass spectrometry promise to provide novel insights into the dynamics of protein expression and activities regulated by post-translational modifications (7, 8). However, the isolation of phosphoproteins from complex mixtures and the determination of phosphorylation sites still remain a challenge. Different strategies have been applied for enrichment of phosphorylated peptides and proteins, including immunopurification and affinity chromatography (9–11). However, one of the vital limitations of all these methods is that, when phosphopeptides are successfully enriched, the nonphosphopeptides are excluded as flow-through. Thus, it is hard to detect both the proteome and phosphoproteome in one analysis. Because of the significance of concurrent identification and quantification of phosphopeptides and non-phosphopeptides, many recent attempts have been made.
For intertissue comparison, Trinidad et al. (12) applied strong cation exchange (SCX) and a titanium dioxide (TiO2) column combined with iTRAQ to compare relative protein expression and phosphorylation status of murine cortex, midbrain, cerebellum, and hippocampus. Using 15N-labeled rat brain as an internal standard, Yates and co-workers (13) have quantified phosphopeptides from p1 and p45 rat brain cortices and observed phosphorylation regulated distinctly on different sites compared with the protein level for a set of proteins. Whereas for the investigation of cells, Munton et al. (14) used isobaric peptide tags to determine the absolute quantity of both phosphorylated and unphosphorylated peptides of candidate proteins. Mann and co-workers (15) have applied the stable isotope labeling by amino acids in cell culture (SILAC) strategy to quantify kinome-wide protein expression and phosphorylation changes between S and M phase-arrested HeLa S3 cells. In their study, phosphopeptide identification was performed with a gel-based strategy as well as SCX and a TiO2 column, whereas identification of unphosphorylated peptides was obtained by additional analysis of total peptide fractions without phosphopeptide enrichment.

In our recent work, we have developed the yin-yang MDLC-MS/MS system, which combined SCX, strong anion exchange (SAX), and reverse-phase columns for comprehensive proteome and phosphoproteome identification (16). The SCX-RP-MS and SAX-RP-MS in the yin-yang MDLC-MS/MS system displayed complementary features for separation and identification of phospho- and nonphosphopeptides, providing an unbiased profiling of protein expression and phosphorylation without anyprefractionation or chemical derivation. On the other hand, for a single protein, it is also of great significance to identify and quantify phospho- and nonphosphopeptides concurrently, not only because the identification of nonphosphopeptides may confirm the characterization of their corresponding phosphorylated form but also because concurrent quantification helps to distinguish the effects of protein expression changes on the measurement of phosphorylation changes. For some proteins, their phosphorylation level may be regulated directly by a kinase or phosphatase without involvement of its gene expression (see Fig. 1B). A phosphoprotein may generally contain phosphopeptides and their non-phosphorylated counterparts and other nonphosphopeptides. Phosphorylation, as a post-translational protein modification, may change rapidly in response to extracellular stimuli without protein expression changes. If phosphorylation of a certain peptide increases upon stimulation, the peptide ratio of its corresponding nonphosphopeptide may decrease because phosphorylation of the nonphosphopeptides has reduced the percentage of the non-phosphorylated form when protein expression remains unchanged (see Fig. 1B). For some other proteins, phosphorylation sometimes is a phenomenon accompanied by the alteration of its gene expression. The phosphorylation of a protein changes with the same trend as that of protein expression. Consequently phosphorylation degree may change at the cellular level, but the relative ratio of phosphorylation versus non-phosphorylation of a specific protein remains unchanged (see Fig. 1C). Concurrent quantification of phosphopeptides and their non-phosphorylated counterparts as well as other non-phosphorylated peptides from the same protein makes it possible to differentiate cellular phosphorylation changes at these two levels.

Adipocyte differentiation is a complicated process involving sequential expression of multiple adipocyte-specific genes during which phosphorylation plays a crucial role for transcriptional activity regulation (17–19). For investigation of the effect of site-specific phosphorylation on transcriptional activity, the strong power of the on-line yin-yang MDLC-MS/MS system resulted in the large scale identification of both the proteome and phosphoproteome in one analysis, whereas combination with SILAC achieved the quantification of protein expression as well as site-specific phosphorylation at the same time (Fig. 2), thus giving direct insight into the effect of phosphorylation on transcriptional activity regulation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The stable isotope-containing amino acid (13C6,15N2)lysine was purchased from Cambridge Isotope Laboratories (Andover, MA). The D9785 deficient medium was a Dulbecco’s modified Eagle’s medium/F-12:1 mixture deficient in L-lysine and three other amino acids from Sigma-Aldrich. Complete protease inhibitor mixture tablets were purchased from Roche Applied Science, and sodium orthovanadate and sodium fluoride were from Sigma-Aldrich. Urea, DTT, ammonium bicarbonate, and iodoacetamide were all purchased from Bio-Rad. Trypsin was purchased from Promega (Madison, WI). All the water used in these experiments was prepared using a Milli-Q system (Millipore, Bedford, MA).

Cell Culture and Differentiation Induction—The SILAC strategy was applied to 3T3-L1 preadipocytes cultured in D9785 medium supplemented with 10% dialyzed bovine serum containing either light or heavy (15N2,15N2)lysine, respectively, in an atmosphere of 10% CO2 at 37 °C. Complete replacement was ensured prior to the experiment. Detailed instructions about this protocol are available. Two days postconfluence, 3T3-L1 fibroblasts cultured with light lysine were stimulated with 1 μg/ml insulin, 115 μg/ml 3-isobutyl-1-methyloxanthine, and 0.39 μg/ml dexamethasone for 1 h at 37 °C, whereas the 3T3-L1 fibroblasts cultured with heavy lysine were left untreated as a control. Two biological replicates using a different culture of cells were applied and subjected to further procedures.

Sample Preparation—After treatment, the “light” and “heavy” cells, respectively, were lysed in buffer containing 8 M urea, 4% CHAPS, 40
mm Tris, 65 mm DTT, protease inhibitor mixture, 1 mm NaF, and 1 mm Na$_3$VO$_4$, and the cell lysate was ultrasonicated and then centrifuged at 25,000 × g at 4 °C for 1 h to remove the insoluble material. After centrifugation, the protein concentration of the cell lysate was quantitated by Bradford assay, and then equal amounts of light and heavy cell lysates were combined for the subsequent processes.

**Protein Precipitation and Digestion**—Briefly each 250 μg of heavy and light lysates were mixed, and after 2 μl of 1 m DTT was added, the mixture was incubated at 37 °C for 2.5 h. Then 10 μl of 1 m iodoacetamide was added and incubated with the mixture for an additional 40 min at room temperature in darkness. After that, the protein mixtures were subjected to precipitation with 50% acetone, 50% ethanol, and 0.1% acetic acid. After precipitation for 20 h at −20 °C, the mixtures were centrifuged at 14,000 × g two times, and then the precipitates were dissolved in 50 mm NH$_4$HCO$_3$ and incubated with trypsin (25:1) at 37 °C for 20 h. The tryptic peptide mixtures were collected and lyophilized for further analysis.

**The On-line Yin-Yang MDLC-MS/MS System**—The peptide fractionation and MS identification were performed on the on-line yin-yang MDLC system coupled with a mass spectrometer, which was developed by our laboratory (16). Briefly two subsystems were built. One subsystem involves an SCX column (320 μm × 100 mm; Column Technology Inc.), two C$_{18}$ trap columns (300 μm × 5 mm; Agilent Technologies), and an analytical C$_{18}$ column (75 μm × 150 mm;
To obtain quantification of proteome and phosphoproteome changes during the adipocyte differentiation, 3T3-L1 fibroblasts were cultured in heavy (\[^{15}C_6{^{15}N_2}\] ) and light lysine. Two days postconfluence, light cell were stimulated with MDI for 1 h at 37 °C whereas the heavy cells were left untreated as a control. Equal protein amounts of the heavy and light cell lysates were combined and subjected to trypsin digestion. Then the peptide mixture was separated by the on-line yin-yang MDLC system followed by LTQ-Orbitrap identification. Further analysis included quantification of protein expression and phosphorylation and prediction of transcription factors and their targets.

Column Technology Inc.). For the other subsystem, an SAX column (320 μm × 100 mm; Column Technology Inc.) was utilized, replacing the SCX column in the first subsystem. The SCX column and SAX column subsystems were equilibrated by pH 2.5 and 8.5 buffers, respectively. First the peptide mixture dissolved in pH 2.5 buffer solution was loaded by the Surveyor autosampler (Thermo Electron, San Jose, CA) to the first subsystem with the SCX column mounted, and the flow-through peptide mixture was collected and lyophilized. Then the flow-through peptide mixture from the SCX column was redissolved in 80 μl of pH 8.5 buffer and loaded into the SAX column subsystem. The pH continuous gradient elution was performed from pH 2.5 to 8.5 for the SCX subsystem and from pH 8.5 to 2.0 for the SAX subsystem. The HPLC solvents for the reverse phase were 0.1% formic acid (v/v) aqueous (A) and 0.1% formic acid (v/v) in acetonitrile (B). The RP gradient was from 2 to 35% mobile phase B in 165 min at a 120 μl/min flow rate before the split and at 250 nl/min after the split. Finally, 10 fractions for each subsystem were used for peptide separation from the SCX or SAX column to the C18 trap column followed by further analysis by reverse-phase chromatography.

A linear ion trap (LTQ)-Orbitrap hybrid mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanospray ion source was operated in data-dependent mode in which acquisition was automatically switched between MS in the Orbitrap and MS/MS in the LTQ. The mass spectrometer was set so that full MS scan was followed by 10 MS/MS scans on the 10 most intense ions from the full MS spectrum with the following Dynamic Exclusion™ settings: repeat counts, 2; repeat duration, 30 s; and exclusion duration, 120 s. The resolving power of the LTQ-Orbitrap mass analyzer was set at 100,000 for the precursor ion scans (m/z 50% at m/z 400).

Database Searching, Phosphorylation Site Localization, and Quantitative Analysis Using Census—All .dta files were created using BioWorks 3.2 with precursor mass tolerance of 500 ppm, and all acquired MS/MS spectra were automatically searched against the mouse International Protein Index protein sequence database (version 3.35) containing 51,490 protein entries combined with real protein and reverse sequences of proteins by using the TurboSEQUEST searching program (University of Washington, licensed to Thermo Finnigan). Trypsin was designated as the protease with two missed cleavage sites allowed. Carbamidomethylation was searched as a fixed modification, whereas isocele-labeled lysine (+8.014199 Da), phosphorylation of serine/threonine/tyrosine residues (+79.96633 Da), and oxidized methionine (+15.99492 Da) were allowed as variable modifications.

First, for nonphosphopeptide identification and phosphorylation site localization, all accepted SEQUEST results must have a ΔCn score of at least 0.1 regardless of charge state as ΔCn ≥ 0.1 is significant for discriminating the first candidate peptide from the second candidate peptide. Second, all output results were combined using an in-house software called BuildSummary, and peptides and phosphopeptides were calculated separately and filtered to assure a false discovery rate of 0.05%. The false discovery rate was calculated based on the following formula: ΔCn = 1 − ln(1 − 0.05) where ΔCn is the number of peptide hits matched to “reverse” protein and ΔCn is the number of peptide hits matched to “real” protein (20). Third, every spectrum of the phosphopeptides was manually checked according to the following criteria: 1) its fragment ion peaks have a high signal-to-noise ratio, 2) it shows sequential members of the b- or y-ion series with phosphorylation site included, and 3) it shows intense proline-directed fragment ions. In addition, the phosphoric acid neutral loss peaks were checked to assist in phosphorylation site identification. When a scan was matched to phosphopeptides with a common sequence and the same number of phosphates but with different phosphorylation sites, the site was considered as ambiguous when ΔCn was < 0.08. Moreover, all identified phospho- and nonphosphopeptides were filtered with a precursor ion mass tolerance of 10 ppm and a fragment ion mass tolerance of 0.8 Da.

First, to eliminate redundancy, if the same peptide(s) was assigned to multiple proteins, then the multiple proteins were clustered to a “protein group.” If all of the peptides in protein group A were covered by protein group B, which has another peptide assigned, then protein group A was removed. Second, a single protein with the highest sequence coverage was selected from one protein group for further analysis. For quantitative analysis, only the lysine-containing peptides were subjected to the Census program (version 1.28) as quantification candidates to determine the 12C6-14N2-peptide/13C6-15N2-peptide ratio (21). The results of quantification were filtered so that peptides with determinant scores (R2) ≥ 0.5 were retained, and the correction factor (ln) was set at 0.0 when data were exported. Peptides with negative R2 were removed, and singleton peptides were discarded.

To determine the quantification of protein expression, we measured the SILAC ratio of all non-phosphorylated peptides except for those that have corresponding phosphopeptides with the common sequence (Fig. 1, B and C) as changes in phosphorylation state may alter the percentage of its non-phosphorylated form. Outliers were eliminated from all peptides that were assigned to the same protein using the biweight algorithm (22), whereas the weighted mean of the peptide ratio was determined as the protein expression ratio. For the phosphopeptide quantification, outlier elimination and weighted mean determination were also processed with the biweight algorithm for all peptide hits of a unique peptide (Fig. 1, B and C).
Phosphoprotein Purification and Western Blotting—To verify the quantification of protein expression and phosphorylation, 3T3-L1 preadipocytes were grown in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Two days postconfluence, the cells were stimulated with 1 μg/ml insulin, 115 μg/ml 3-isobutyl-1-methylxanthine, and 0.39 μg/ml dexamethasone for 1 h at 37 °C or left untreated as a control. For Western blotting verification of proteins expression, cells were lysed and centrifuged as described previously. For the phosphoprotein verification, a phosphopeptide purification kit from Qiagen (Valencia, CA) was applied (23, 24). Briefly cells were lysed in the Lysis Buffer and incubated 30 min at 4 °C, and then the cell lysate was centrifuged at 10,000 × g at 4 °C for 30 min to remove the insoluble materials. After centrifugation, the concentration of supernatant was quantified by Bradford assay. The lysate was then diluted to 0.1 mg/ml with Lysis Buffer, and 25 μl of the extracted proteins were applied to a Lysis Buffer-equilibrated phosphopeptide purification column at room temperature. After washing the column with 6 ml of Lysis Buffer, the phosphoproteins were eluted with 2 ml of the Phosphoprotein Elution Buffer.

For validation of protein expression, 10 μg of total protein of 115 μg/ml 3-isobutyl-1-methylxanthine, 0.39 μg/ml dexamethasone, and 1 μg/ml insulin (MDI)-stimulated and control samples were loaded. For verification of phosphorylation, the elution fractions were loaded at the same volume of 3.6 μl. After transfer, the PVDF membranes were blocked by 1X Net-gelatin (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.05% Triton X-100, and 0.25% gelatin) and then incubated with the corresponding primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) at 1:10,000 followed by detection with ECL (Pierce).

Prediction of Transcription Factors and Their Targets—To determine the effect of phosphorylation on transcriptional activity, the phosphorylation of transcription factors and expression of their targets were investigated. First a combination approach of support vector machine and error-correcting output coding was applied to identify transcription factors among quantified phosphoproteins (25). A database containing 3134 putative transcription factors for mouse was used. To detect downstream regulated genes of specific TFs, a reverse engineering algorithm named ARACNE (26, 27) was adopted that used gene expression profile data and TF terms as inputs and produced TF and target pairs as the outcome (28). The gene expression profile data were downloaded from the Gene Expression Omnibus (GEO) database and refined to build primary materials for the algorithm. For the mouse genome, gene expression profile data from Affymetrix mouse Genome 430 2.0 Array platform was collected. The refining process was carried out using the following steps. 1) Microarray data were selected from the raw data set, and a subset was built in which the microarray number of diverse tissues was kept in comparative proportion. 2) Microarray data in the subset were normalized to eliminate bias of different samples. Accordingly, a microarray subset containing 310 (34 tissues) samples was set up for the mouse genome. Then these subsets and TFs predicted in the previous work were utilized as inputs for the ARACNE method. At last, 37,040 TF-target pairs (related to 1340 TFs) in mouse were gathered with a middle threshold (p < 1e-4) and were used as a database for the TF-target prediction in our study.

RESULTS

Peptide and Phosphopeptide Identification by the On-line Yin-Yang MDLC-MS/MS System—As the SCX and SAX showed complementary features of binding and separation for peptides (16), the yin-yang MDLC system has proved to be a powerful strategy to identify the phosphopeptides and nonphosphopeptides in one analysis without a prefractionation procedure and has shown the most comprehensive identifications in the MDLC-MS/MS system. The basic peptides were retained and separated by the SCX, whereas the acidic peptides were collected as flow-through and further separated by the SAX. Both columns were then eluted by pH gradient and subjected to a reverse-phase column and then to LTQ-Orbitrap for identification. Generally, phosphopeptides are relatively more acidic than other peptides and thus are preferably detected in the SAX. Combination with the high resolution LTQ-Orbitrap assures accuracy of the consequent identification of both phospho- and nonphosphopeptides. In this work, two biological duplicates were conducted with independently cultured cells, and the identification depth of the proteome and phosphoproteome in each experiment is shown in Table I. The identification of the proteome and phosphoproteome in two replicates is quite similar with the shared part reaching ~70% for proteins and ~50% for phosphopeptides (Fig. 3A), indicating an acceptable reproducibility of the on-line yin-yang MDLC-MS/MS system. Obviously, the SCX column and the SAX column display complementary features for peptide identification. When ~70% of nonphosphopeptide hits were identified in the SCX-RP-MS, the SAX-RP-MS covered more than 97% of phosphopeptide hits of the total phosphopeptides identified by the on-line yin-yang MDLC-MS/MS system (Table I). Meanwhile, although the SAX column also identified thousands of unique nonphosphopeptides, only 4.8–16.0% of peptides are shared in the SCX and SAX columns. The theoretical pI distribution of the peptide backbones is shown in Fig. 4. Supplemental Tables 1 and 2 present the whole proteome and phosphoproteome identification in the on-line yin-yang MDLC system in two biological replicates. For the phosphoproteome, we identified 1639 distinct phosphosites and 241 ambiguous phosphosites on 1924 phosphopeptides of 765 phosphoproteins in Experiment I and 1897 distinct and 323 ambiguous phosphosites on 2233 phosphopeptides of 848 phosphoproteins in Experiment II. About half of these phosphopeptides were identified in both experiments (Fig. 3A). For these 765 and 848 phosphoproteins identified, 59.5 and 64.0% were also present in the peptide-based protein identification (Table I). Spectra of all phosphopeptides identified in each experiment are shown in supplemental Figs. 1 and 2.

Quantitative Analysis of Regulated Protein Expression upon MDI Stimulation—Combining SILAC for quantification and the on-line yin-yang MDLC-MS/MS for peptide fractionation, we quantified expression of a total of 2000 and 2123 proteins in Experiments I and II, respectively (supplemental Table 3) with 1342 proteins shared in the two biological replicates (Fig. 3B). As mentioned above, for quantification of protein expression, only the nonphosphopeptides without corresponding phosphopeptides were considered (Fig. 1, B and C). The distribution of the fold changes of protein expression for the 2000 and 2123 proteins is shown in Fig. 5A with the solid line representing the quantification in Experiment I and the dashed
**Quantitative Proteomics-revealed Transcriptional Regulation**

TABLE I

Proteome and phosphoproteome identification and quantification in each experiment

|                  | Exp. I | Exp. II |
|------------------|--------|---------|
| Total non-redundant protein group | 3,826  | 4,226   |
| Total labeled non-redundant protein group | 2,674  | 2,928   |
| Proteins identified without phosphopeptide | 3,060  | 3,376   |
| Proteins identified with phosphopeptide | 765    | 848     |
| Phosphoproteins identified with phosphopeptide | 455 (59.5%) | 543 (64.0%) |
| Nonphosphopeptide hits | 101,054 | 113,187 |
| Nonphosphopeptide hits in SCX | 68,844 (68.1%) | 84,316 (74.5%) |
| Nonphosphopeptide hits in SAX | 32,210 (31.9%) | 28,871 (25.5%) |
| Phosphopeptide hits | 11,180 | 10,642  |
| Phosphopeptide hits in SCX | 303 (2.7%) | 158 (1.5%) |
| Phosphopeptide hits in SAX | 10,877 (97.3%) | 10,484 (98.5%) |
| Unique nonphosphopeptides | 12,377 | 12,774  |
| Shared unique nonphosphopeptides in SCX and SAX | 1,949 (15.7%) | 625 (4.9%) |
| Unique phosphopeptides | 1,924  | 2,233   |
| Candidate phosphorylation sites | 1,880  | 2,220   |
| Positive phosphorylation sites | 1,639  | 1,897   |
| Ambiguous phosphorylation sites | 241    | 323     |
| Total proteins quantified | 2,154  | 2,338   |
| Proteins quantified without phosphopeptide | 1,873  | 1,946   |
| Proteins quantified with phosphopeptide | 281    | 392     |
| Phosphoproteins quantified with nonphosphopeptide | 134 (47.7%) | 187 (47.7%) |

* Phosphopeptides with a different sequence or a common sequence with distinct phosphosites or a different charge were considered as unique peptides, whereas the heavy/light peptides with the same sequence, phosphosites, and charge were considered as one unique peptide.

**Fig. 3. Reproducibility of the yin-yang MDLC-MS/MS systems.**

Two biological replicates were subjected to the yin-yang MDLC-MS/MS systems analysis. A, non-redundant proteins and phosphopeptides identified in Experiments I (gray) and II (white). B, proteins and phosphopeptides quantified in Experiments I (gray) and II (white).

line denoting Experiment II. Both display almost symmetrical patterns. To calculate the quantification error, the relative S.D. was determined by S.D./average. For our data, the relative S.D. of all proteins in each independent analysis revealed a quantification precision of better than ±5% (supplemental Fig. 3A), and the relative S.D. between the two biological replicates is 13.8%, indicating an acceptable reproducibility.

**Fig. 4. The distribution of peptide pl in SCX-RP-MS and SAX-RP-MS.**

As the typical quantification error arising from the SILAC quantification method was better than 10% relative S.D. for an individual peptide (29), a minimum of a 1.3-fold change was chosen as biologically significant. Under this criterion, 16 proteins changed significantly in expression in both replicates (Table II). This number is reasonable because expressions of most proteins are not expected to change appreciably within the time frame of our experiment. These proteins include many known differentiation-related molecules as well as proteins with no previous reference to adipocyte differentiation.
The Complementary Features of SCX and SAX Have Facilitated Peptide Isoform Identification and Quantification—One of the major limits for quantitative phosphoproteomics until now was the quantification of phosphopeptides and non-phosphopeptides of the same protein simultaneously. This is mainly due to the widely used phosphoprotein or phosphopeptide enrichment procedure in which most of the non-phosphoproteins or nonphosphopeptides are excluded as flow-through. In this study, we applied the on-line yin-yang MDLC system to fractionate phosphopeptides and nonphosphopeptides instead of preparations or chemical derivations; thus, all of the peptide mixtures were subjected to the high accuracy LTQ-Orbitrap for characterization. This made it possible to identify and quantify peptide isoforms with com-

**Table II**

| IPI number | Protein name | Exp. I | Exp. II | Average ratio | Relative S.D. | Quantified peptides (Exp. I/Exp. II) |
|------------|--------------|--------|---------|---------------|---------------|-------------------------------------|
| IPI00399449 | Nsf1lc       | 0.06   | 0.04    | 0.05          | 0.33          | 4/11                                |
| IPI00109221 | Sacm1l       | 0.59   | 0.29    | 0.44          | 0.48          | 4/4                                 |
| IPI00756198 | Kntc1        | 0.49   | 0.69    | 0.59          | 0.24          | 2/4                                 |
| IPI00114833 | Wfs1         | 0.66   | 0.62    | 0.64          | 0.04          | 1/2                                 |
| IPI00379876 | Ttll12       | 0.69   | 0.67    | 0.68          | 0.02          | 6/1                                 |
| IPI00123410 | Usp24        | 0.70   | 0.68    | 0.69          | 0.02          | 2/1                                 |
| IPI00137177 | Ctsa         | 1.32   | 1.30    | 1.31          | 0.01          | 25/10                               |
| IPI00133006 | Ndufab1      | 1.35   | 1.31    | 1.33          | 0.02          | 2/4                                 |
| IPI00313998 | Sqrdl        | 1.46   | 1.36    | 1.41          | 0.05          | 2/13                                |
| IPI00111960 | Gaa          | 1.43   | 1.44    | 1.44          | 0.01          | 18/14                               |
| IPI00229796 | Pygb         | 1.48   | 1.40    | 1.44          | 0.04          | 12/8                                |
| IPI00338178 | Fosl2        | 1.55   | 1.33    | 1.44          | 0.11          | 12/5                                |
| IPI00124640 | Grn          | 1.30   | 1.78    | 1.54          | 0.22          | 16/39                                |
| IPI00123635 | Islr         | 2.29   | 1.52    | 1.91          | 0.29          | 6/1                                 |
| IPI00118413 | Thbs1        | 1.51   | 4.04    | 2.77          | 0.65          | 48/9                                |
| IPI00785343 | H3f3b        | 4.27   | 4.92    | 4.60          | 0.10          | 1/1                                 |

\(^{a}\) International Protein Index.

\(^{b}\) The biweight of \(^{12}\)C\(_{6},^{14}\)N\(_{2}\)-peptide/\(^{13}\)C\(_{6},^{15}\)N\(_{2}\)-peptide (MDI/control) ratio of peptides assigned to the corresponding protein in Experiment I.

\(^{c}\) The biweight of \(^{12}\)C\(_{6},^{14}\)N\(_{2}\)-peptide/\(^{13}\)C\(_{6},^{15}\)N\(_{2}\)-peptide (MDI/control) ratio of peptides assigned to the corresponding protein in Experiment II.

Fig. 5. Quantification of protein expression and peptide phosphorylation in two biological replicates. A, the -fold change of protein expression was determined by the light-to-heavy ratio and transformed into the log scale with base e. Results of Experiments I and II are represented as solid and dashed lines, respectively. B, the -fold change of peptide phosphorylation was calculated and transformed as above. Results of Experiments I and II are represented as solid and dashed lines, respectively.

![Protein Expression](image1)

![Peptide Phosphorylation](image2)
common sequence, such as sequence-unique peptides in an unmodified state, singly phosphorylated state, and multiply phosphorylated state. Using this strategy, 980 isoforms of 323 sequence-unique peptides and 1125 isoforms of 374 sequence-unique peptides were identified in Experiments I and II, respectively (supplemental Table 4). For example, the sequence GSDAVSETSSVSHEIDLEK was identified in four distinct isoforms: non-, singly, doubly, and triply phosphorylated. For each phosphorylated state, however, the position of phosphorylation may also be different. Take EEEASEPEEAAS#PTTPK for instance; it has been identified with singly, doubly, and triply phosphorylated forms, whereas for the singly phosphorylated state, EEEAS#EPEEAASPTTPK and EEEASEPEEAAS#PTTPK (where # represents phosphorylation modification) were both identified to be positive. Combination with SILAC resulted in the quantification of 353 isoforms corresponding to 139 sequence-unique peptides and 677 isoforms corresponding to 242 sequence-unique peptides in each experiment as listed in supplemental Table 5. First, as MDI stimulation induces a series of phosphorylation events, each phosphorylation site may be distinctly regulated, and the ratios of some sequence-unique peptides with different phosphorylation sites are distinguished. Second, because phosphorylation alters the percentage of phospho- and non-phosphopeptides, the ratios of some of the peptides at the phosphorylated state and non-phosphorylated state are different from each other (Fig. 1A). As an example, for Zyxin (Fig. 6A) we identified and quantified the phosphopeptide VCS#IDLEIDSLSSLLDDMTK and its corresponding unphosphorylated form with the common sequence as well as other nonphosphopeptides, although the quantification ratios were different from each other. As the phosphorylated form increased to an average of 5.15-fold without protein expression alteration, the non-phosphorylated form showed a 0.55 ± 0.03-fold decrease. The ratio is quite reasonable because phosphorylation of the non-phosphorylated state reduced its percentage although the total amount of this protein remained unchanged.

Quantification of Site-specific Phosphorylation—Combining SILAC and the on-line yin-yang MDLC system, we achieved quantification of 606 unique phosphopeptides on 281 phosphoproteins and 900 unique phosphopeptides on 394 phosphoproteins, respectively, in two analysis (supplemental Table 6). About half of the phosphopeptides have been quantified in both experiments (Fig. 3B). For proteins quantified with phosphopeptides, 47.7% have peptide-based protein quantification in Experiments I and II (Table I). The -fold changes of phosphorylation of all these phosphopeptides are shown in Fig. 5B. The solid line indicates results in Experiment I, whereas the dashed line represents Experiment II. Both of them show almost symmetrical patterns. Similarly, quantification of peptide phosphorylation showed acceptable relative S.D. in each experiment (supplemental Fig. 3B). Quantification of the 362 phosphopeptides shared in the two replicates revealed a relative S.D. of 16.9%, indicating an acceptable reproducibility. Using a minimum of a 1.3-fold change for biological significance, 80 of the 362 shared phosphopeptides showed significant change in response to MDI stimulation in both experiments or showed significant changed in one experiment and showed a consistent trend in the other as shown in Table III. Two of the 80 phosphopeptides changed with significance similar to that of protein expression. In addition 221 phosphopeptides displayed significant changes in phosphorylation in one of the two replicates, among which nine phosphopeptides showed a similar ratio with corresponding protein expression (supplemental Table 7).

In particular, the complementary feature of SAX and SCX has made it possible to quantify the expression of most of the phosphoproteins, thus eliminating the effect of the expression ratio on the site-specific phosphorylation measurement of a certain protein. For some proteins, phosphorylation changed without obvious expression alteration, such as Zyxin mentioned above; in addition, some proteins still showed similar changes of phosphorylation and protein expression. For instance, Baz1b was identified as a phosphoprotein whose phosphorylation was quantified as 1.33-fold and expression was quantified as 1.67-fold (Fig. 6B). If only the phosphopeptides were quantified, the phosphorylation may be misesti-
| IPI number | Phosphopeptide sequences | Exp. I^a | Exp. II^b | Relative S.D. | No. of phosphates |
|------------|--------------------------|---------|-----------|-------------|------------------|
| IP00120691 | SNSSDAPGEESpSpSETEK | 0.35 | 0.76 | 0.52 | 1 |
| IP00120691 | SNSSDAPGEESpSpSETEK | 0.35 | 0.76 | 0.52 | 2 |
| IP00407425 | NKLGEDS#VDVS#ELEDR | 0.42 | 0.72 | 0.37 | 2 |
| IP00667973 | LGEAS#DSELADAK | 0.42 | 0.59 | 0.24 | 1 |
| IP00318048 | SpSpPKEEVAD#PEEAEASPTTPK | 0.44 | 0.97 | 0.53 | 2 |
| IP00118875 | GAT#PAEDDIDKDIDLFGS#DEEEDKEAR | 0.45 | 0.63 | 0.24 | 2 |
| IP00553784 | TLSVAAAFNEEDDE#PEEPEMPPEAK | 0.45 | 0.71 | 0.31 | 1 |
| IP00116498 | TAFDEAELTDLS#EESYK | 0.47 | 0.96 | 0.48 | 1 |
| IP00318048 | EEAEAS#PEEAEASPTTPK | 0.52 | 0.97 | 0.43 | 2 |
| IP00474783 | FIIQGSVSEDNS#EDISNLVK | 0.53 | 0.67 | 0.16 | 1 |
| IP00230597 | KDELS#DWS#LAGEDDDR | 0.54 | 0.47 | 0.10 | 2 |
| IP00120691 | EIITEPS#EEDAMMPFK | 0.57 | 0.79 | 0.23 | 1 |
| IP00120691 | EIITpEEPSpEEDAMMPFK | 0.57 | 0.79 | 0.23 | 1 |
| IP00153715 | NLETLPFS#S#DEEDEVK | 0.57 | 0.99 | 0.38 | 2 |
| IP00408909 | ALGLEES#EPEEKG | 0.57 | 0.67 | 0.11 | 1 |
| IP00226222 | EVDVEDAALLS#DLTELEGK | 0.58 | 0.81 | 0.23 | 1 |
| IP00115231 | LESLYS#DEEESVAGADK | 0.60 | 0.72 | 0.13 | 1 |
| IP00126317 | ELSE#ES#DEQLEEPMFLK | 0.60 | 0.83 | 0.23 | 2 |
| IP00396739 | GGPEGGAAPAACAGSPAGPDTEMEEVFDHGS#PGK | 0.61 | 0.85 | 0.23 | 1 |
| IP00126317 | KELS#EES#DEQLEEPMFLK | 0.63 | 0.87 | 0.22 | 2 |
| IP00622783 | TEDGWEWS#DDEMEDEK | 0.64 | 0.92 | 0.16 | 2 |
| IP00113576 | AQA#SES#EEEEOGQSSSPK | 0.64 | 0.83 | 0.18 | 1 |
| IP00420601 | SDLIEEEDLTEGKS#DEDEEVPQGPK | 0.64 | 0.94 | 0.27 | 1 |
| IP00751009 | AADLEDpEpEEETAK | 0.64 | 0.56 | 0.09 | 1 |
| IP00336503 | GSDAV#ETSpSpVSpHIEDLEK | 0.64 | 0.64 | 0.00 | 3 |
| IP00336503 | GSDAVpEpSpVSpHIEDLEK | 0.64 | 0.64 | 0.00 | 3 |
| IP00119320 | LLASPDASTLENS#WSPDEEK | 0.66 | 0.74 | 0.08 | 1 |
| IP00404725 | FSHYSLS#DS#DTEAK | 0.66 | 0.75 | 0.09 | 2 |
| IP00453851 | NTTKpETIPDMEDS#PPVSdSpESsEQCSESVR | 0.66 | 0.91 | 0.23 | 2 |
| IP00349560 | VLRKpsVPFGSpSpSddddTSVSELSQR | 0.68 | 0.91 | 0.20 | 2 |
| IP00108338 | DGESYD#FDFSEAETMPQV#H#PK | 0.68 | 0.63 | 0.05 | 1 |
| IP00674617 | VVCDADD#SpSpSVdVSOK | 0.69 | 0.88 | 0.17 | 1 |
| IP00336715 | MASVVPGOFDDADS#SpSpENK | 0.69 | 0.68 | 0.01 | 2 |
| IP00336715 | MASVVPGOFDDADS#SpSpENK | 0.69 | 0.68 | 0.01 | 2 |
| IP00671847 | STGVSFWtpQodSpDENEGEROspSpDPGSSK | 0.70 | 0.88 | 0.16 | 2 |
| IP00474974 | ADDDEADD#ssVSp#BP#PK | 0.70 | 0.68 | 0.02 | 1 |
| IP00319956 | TEEVEES#EEDPILEHPPENPVK | 0.73 | 0.66 | 0.08 | 1 |
| IP00458153 | VENDENETL#EPEGSSPK | 0.75 | 0.40 | 0.43 | 1 |
| IP00320208 | DDDEE#LS#DENSEE#AAK | 0.77 | 0.68 | 0.09 | 1 |
| IP00230429 | NVPSEQESLD#VDADF|K | 0.77 | 0.65 | 0.12 | 1 |
| IP00309481 | EVENE#T#PSV#EPEEEK | 0.78 | 0.65 0.24 | 0.13 |
| IP00222813 | AVE#SpDpEDH#TTLDK | 0.82 | 0.64 | 0.17 | 1 |
| IP00331612 | KPAQETEET#SpSpQES#AEED | 0.91 | 0.69 | 0.19 | 2 |
| IP00331817 | KGS#AE#S#S#DEEGKLVDEPAK | 0.92 | 0.31 | 0.25 | 3 |
| IP00331612 | KPAQETEET#SpSpQES#AEED | 0.96 | 0.66 | 0.26 | 2 |
| IP00229895 | A#PKAQA#SEETAP#SpSpPAAS#PTPQSAER | 0.97 | 1.39 | 0.25 | 2 |
| IP00131890 | LADLYGS#KTDFFDODS | 0.99 | 0.67 | 0.27 | 1 |
| IP00230668 | TpSpSpTNEDEDLNP#EQK | 1.04 | 1.41 | 0.21 | 1 |
| IP00131125 | DHIA#S#Q#S#EEVEVEG#EK | 1.05 | 4.10 | 0.84 | 1 |
| IP00808191 | SpKFSpSpDEDEDAENLEA#VESSGK | 1.13 | 1.53 | 0.21 | 1 |
| IP00340860 | TAE#GS#EA#EPEAK | 1.15 | 1.35 | 0.11 | 2 |
| IP00340860 | TpSpEsGspEAE#ETAPK | 1.15 | 1.35 | 0.11 | 2 |
| IP00122845 | GRS#T#PYpSpEADEDDEE#G#PSR | 1.30 | 1.32 | 0.01 | 3 |
| IP00605037 | KET#E#S#EADNLDLDER | 1.30 | 1.02 | 0.17 | 2 |
| IP00351206 | LLKPGEPSpSYpETpDEEDT | 1.31 | 1.02 | 0.18 | 1 |
| IP00173248 | MNVP#T#MNEVLSMD#DDEG#DAITG#GDTDK | 1.34 | 1.08 | 0.15 | 1 |
| IP00341869 | SGKNS#QEDS#S#E#EDEKDV | 1.34 | 1.13 | 0.12 | 3 |
| IP00341869 | SpGKNS#QEDS#S#EDS#E#EDEKDV | 1.34 | 1.13 | 0.12 | 3 |
| IP00317891 | QES#DPEDDDV#K | 1.35 | 1.04 | 0.18 | 1 |
mated as up-regulated to 1.33-fold. However, the quantification of the nonphosphopeptides of the same protein indicates that it is the expression change rather than the phosphorylation states that give rise to the increased ratio of the phosphopeptide. Representative extracted ion chromatograms of several quantified proteins and phosphopeptides are shown in supplemental Fig. 4.

Phosphorylation-regulated Transcriptional Activities—Protein phosphorylation is a widely diffuse and versatile post-translational modification that controls many cellular processes from signal transduction to gene transcription. Many critical events involved in cellular responses are mediated by changes in phosphorylation rather than transcriptional changes. For transcription factors, phosphorylation to a large extent regulates their transcriptional activities from Drosophila to higher eukaryotes (30–32). Using a combination approach of support vector machine and error-correcting output coding for transcription factor identification, 115 phosphoproteins quantified in either or both of the two experiments were identified as transcription factor. Among these 115 identified TFs, 73 have been reported to have transcriptional activities or to be transcription factor coactivators or corepressors. Several TFs and their predicted targets have been reported to be related in other analysis, such as co-expression or protein-protein interaction, indicating the reliability of the prediction strategy. Combined with an ARACNE-derived algorithm with a middle threshold ($p < 1e^{-4}$) (25), 683 proteins with expression quantification were predicted to be the targets of 90 transcription factors among the 115 TFs identified, giving rise to 1547 TF-target pairs.

First, to determine the effect of phosphorylation of the transcription factors on transcriptional activity, phosphorylation and expression of the TFs were investigated simultaneously. For the 115 phosphoproteins identified as transcription factors, 45 of them changed significantly in their phosphorylation states, 29 in 45 have quantified predicted targets, and 3 of the 29 TFs showed significant changes in their expression after MDI stimulation (Table IV). 381 proteins with their expression quantified were predicted to be targets of the 29 TFs, giving rise to 554 TF-target pairs. Additionally, expression of their targets was analyzed at the same time. Interestingly, 71 among the 381 targets showed increased or decreased expression in one or both experiments corresponding to the phosphorylation changes or, to a lesser extent, expression of their transcription factor (supplemental Fig. 5). In contrast, targets of TFs whose phosphorylation did not change significantly seldom showed protein expression alteration (data not shown).

Zyxin, a phosphoprotein discussed above, was identified as a transcription factor. Meanwhile, 16 proteins were predicted to be its targets, among which three proteins, Ctsa, Gm, and Capns1, have increased in expression. The expression of Zyxin remained almost unchanged, suggesting that it was the phosphorylation change that mostly changed the transcriptional activity (Fig. 9A). Rfc1, also named differentiation-specific element-binding protein, binds to a specific cis-acting

| IPI number | Phosphopeptide sequences | Exp. I | Exp. II | Relative S.D. | No. of phosphates |
|------------|--------------------------|-------|---------|---------------|-------------------|
| IP00856140 | IDEPNTPHPMNIGDDEDAYpSpDSEGNEVMTPDILAK | 1.36  | 1.22    | 0.08          | 1                 |
| IP00468994 | DYDEEEQYDSIEKEK           | 1.38  | 1.04    | 0.20          | 1                 |
| IP00319973 | EGEEPTYVPspDDEEPK         | 1.38  | 1.04    | 0.20          | 1                 |
| IP00228590 | SLAAALNITDNDENDEEEEAWK    | 1.42  | 1.02    | 0.23          | 1                 |
| IP00221566 | LFDDSDEKEDEEDTDGK         | 1.54  | 1.06    | 0.26          | 1                 |
| IP00605037 | KVELS#ESE#EDKGSK          | 1.61  | 1.03    | 0.31          | 2                 |
| IP00229828 | LAKS#VEDEMSD#$PGEPPFYTGQGR| 1.62  | 4.00    | 0.60          | 2                 |
| IP00222208 | AVEEQQDQDQS#EKS#KPGAS#DGER| 1.65  | 1.10    | 0.28          | 3                 |
| IP00222240 | LPEEPspSpDEEQQPEK         | 1.67  | 1.14    | 0.27          | 1                 |
| IP00222409 | LPEEPSS#EDEQQPEK          | 1.67  | 1.14    | 0.27          | 1                 |
| IP00399449 | RHS#GQDVHVVLK             | 1.81  | 2.18    | 0.13          | 1                 |
| IP00273491 | HTpPNTpSpDNEGSpDTpEVCGSNPSK| 2.24  | 3.84    | 0.37          | 2                 |
| IP00229069 | EIVPPPVEES#EEDDDGPLK      | 2.37  | 1.10    | 0.52          | 1                 |
| IP00338745 | QADVADQQTELPAENGETENOS#PAS#EERE | 2.50  | 1.05    | 0.58          | 2                 |
| IP00338745 | QADVADQQTELPAENGETENQSpPAS#EERE | 2.51  | 1.07    | 0.57          | 2                 |
| IP00311490 | VLS#DS#EEDADVPGTSTR       | 2.54  | 1.25    | 0.48          | 2                 |
| IP00387422 | VCS#DLEIDLSLSLLDDMTK      | 3.24  | 7.05    | 0.52          | 1                 |
| IP00608118 | PGVTSSTDSDSEDEDDEGEK      | 9.95  | 3.35    | 0.70          | 1                 |
| IP00608118 | PGVTSSTDSpSEDEDDEGEK      | 10.02 | 3.35    | 0.71          | 1                 |
| IP00131388 | APS#VANIGSHCDSLK          | 10.05 | 1.31    | 1.09          | 1                 |

a International Protein Index.
b Distinct phosphorylation sites are indicated as “#”, whereas “p” indicates a site with less confidence.
c The biweight of $^{12}C_6,^{14}N_2$-peptide/$^{13}C_6,^{15}N_2$-peptide (MDI/control) ratio of all peptide hits of the corresponding phosphopeptide in Experiment I.
d The biweight of $^{12}C_6,^{14}N_2$-peptide/$^{13}C_6,^{15}N_2$-peptide (MDI/control) ratio of all peptide hits of the corresponding phosphopeptide in Experiment II.
DNA element (differentiation-specific element) responsible for the irreversible continued expression of the angiotensinogen gene after differentiation of 3T3-L1 fibroblasts to adipocytes (33). It is also a coactivator for the transcription factor CCAAT/enhancer-binding protein α, which has a vital role in cell growth and differentiation (34). Recently, phosphorylation of Rfc1 was found to be associated with adipocyte differentiation, and calmodulin-dependent protein kinase II was respon-

### Table IV

**MDI-regulated transcription factor phosphosite**

| IPI^a number | Protein name | Phosphopeptide sequences^b | Exp. I^c | Exp. II^d | Relative S.D. |
|-------------|--------------|-----------------------------|---------|---------|---------------|
| IPI00230597 | Fxr1         | KDELS#DWS#LAGEDDR           | 0.54    | 0.47    | 0.10          |
| IPI00120691 | Ddx21^e      | SNSSDAPGEESpSpSETEK         | 0.35    | 0.76    | 0.52          |
| IPI00108338 | Mcm3^e       | DGEYDPYDFSEAEQTPMQVHT#PK   | 0.68    | 0.63    | 0.05          |
| IPI00230429 | Kpn3a^e      | NVQPEGSSLEDS#DVDADFK        | 0.77    | 0.65    | 0.12          |
| IPI00396739 | Smarca5      | GEGPAAAPCAAGSGPAdTEM EEFVDHG#PGK | 0.61 | 0.85 | 0.23 |
| IPI00113356 | Ddx24        | AQAVS#EVEEEEEEQSSSPK       | 0.64    | 0.83    | 0.18          |
| IPI00671847 | Ppp1r12a^e   | STGVSFWTPQDSpDENEGERQoSpDTP#EDGSSK | 0.70 | 0.88 | 0.16 |
| IPI00331612 | Hmg2a^e      | KPAQETEETspSpQoESe#AEED     | 0.96    | 0.66    | 0.26          |
| IPI00230668 | Tcf12        | TpSpStNTEDELDNPEQK          | 1.04    | 1.41    | 0.21          |
| IPI00340860 | Larp7^e      | TASS#EGSe#EATEpEKK          | 1.15    | 1.35    | 0.11          |
| IPI00221566 | Htatsf1^e    | LFDDSADKEDEDETDGK           | 1.54    | 1.06    | 0.26          |
| IPI00229069 | Antx1        | EVPPPPVEEs#EDEEEDGLPK       | 2.37    | 1.10    | 0.52          |
| IPI00311480 | Phip         | VLS#DES#EEEDAVTGTSTR        | 2.54    | 1.25    | 0.48          |
| IPI00397422 | Zyp          | VCS#IDLEDSLSSLLDMTK         | 3.24    | 7.05    | 0.52          |
| IPI00608118 | Psp1^e       | PGVTSSTspStspDEEDEOGEK      | 10.02   | 3.35    | 0.71          |
| IPI00553538 | Hist1h3a     | FQSSAVMALOEACEAY#VLGFEDTNLCAlAIK | 0.21 | NA | NA |
| IPI00848889 | Fnbp4        | ATGGCLLCGLAYAD#DDDESVEK     | NA      | 0.28    | NA            |
| IPI00421162 | Zc3h11a^e    | VESSENVPSP#PTTHPPVNAADDDEDEDQQDF#SEEGESK | NA | 0.38 | NA |
| IPI00395223 | Adar         | GEPLGSLDGDPA#S#IDLEGSEPLDMAEIK | 0.40 | NA | NA |
| IPI00353579 | Up18^e       | QGLHGS#S#IDIDSENEAKE       | 0.45    | NA      | NA            |
| IPI00453808 | Ddx55        | KDEQESD#DDDEDEEMLNNDTR     | 0.49    | NA      | NA            |
| IPI00470995 | Git1         | HGS#GA#DspDypEpEntQSDPGLLEGK | 0.50   | NA      | NA            |
| IPI00473314 | Lig1         | VAQVLSCEGDEDEAPG#PK         | 0.56    | NA      | NA            |
| IPI00750042 | Brwd1        | ILS#DS#EDCCEKE              | 0.62    | NA      | NA            |
| IPI00120886 | Ybx1^e       | NYQONYQONS#ESGEK            | 0.66    | NA      | NA            |
| IPI00227351 | Phf8         | DADEYIYPSLE#DDDDPALK        | 0.67    | NA      | NA            |
| IPI00338904 | Ctdp1^e      | EDLESMKEDVDEILGES#DDS#DIEK  | NA      | 0.68    | NA            |
| IPI00415329 | Phf14        | NISADDEEL#NDS#LTLSSQK       | NA      | 0.68    | NA            |
| IPI00515361 | Smarcc1^e    | NTEKEQDS#DVESEVKEE          | NA      | 0.70    | NA            |
| IPI00337844 | Ranbp2^e     | SHETDGS#AHQDEEDGGHFEPVPLPDK | NA      | 0.70    | NA            |
| IPI00125443 | Nr3c1        | SSTPAAGCAT#PTPEK            | 1.30    | NA      | NA            |
| IPI00221805 | Mta3         | MPTQSDEEKS#EPSP#TPtpAEPR    | NA      | 1.30    | NA            |
| IPI00130957 | Baz1b^e      | CDVoQEDS#E#GStEDIDNK        | NA      | 1.33    | NA            |
| IPI00674554 | Dmd1^e       | FS#PGDEFFAT#AGK             | 1.35    | NA      | NA            |
| IPI00556637 | Smarcd1^e    | RNSD#S#ELEDSLSELEDK         | NA      | 1.40    | NA            |
| IPI00468726 | Ddef1        | QDEMDES#DDDDLDKPSPIK        | 1.44    | NA      | NA            |
| IPI00121135 | Sfs2^e       | SpRSpKSpPPKS#PPEEGAVSpSp    | 1.56    | NA      | NA            |
| IPI00113300 | Dhx34        | LQEDGCS#S#SEEDERK           | NA      | 1.59    | NA            |
| IPI00120529 | Rfc1         | DPVTVYSmET#IDDDFVCK         | NA      | 1.60    | NA            |
| IPI00230653 | Prx1^e       | SLLES#PGLTpSpSdPT#POQDNQDNQNEEK | NA   | 1.61 | NA |
| IPI00130959 | Nsp          | EDMIS#EPEEK                 | NA      | 1.87    | NA            |
| IPI00461396 | Baz1a        | DLDPDVT#EDEDDPISK           | NA      | 2.80    | NA            |
| IPI00461396 | Baz1a        | DLDPDVT#EDEDDPISK           | NA      | 2.80    | NA            |
| IPI00153375 | Pdlim2       | FS#SpSpLDLSEDESVFK          | NA      | 2.95    | NA            |
| IPI00230677 | Acin1        | SpSpSpFSpEpIEGE#S#DEEKPR    | NA      | 5.26    | NA            |
| IPI00321739 | Akap8        | GEDPFVNETADLET#EGEVENVEEK   | 12.11   | NA      | NA            |

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^a International Protein Index.

^b Distinct phosphorylation sites are indicated as “#”, whereas “p” indicates a site with less confidence.

^c The biweight of ^12^C_6,^14^N_2-peptide/^13^C_6,^15^N_2-peptide (MDI/control) ratio of all peptide hits of the corresponding phosphopeptide in Experiment I. NA, not applicable.

^d The biweight of ^12^C_6,^14^N_2-peptide/^13^C_6,^15^N_2-peptide (MDI/control) ratio of all peptide hits of the corresponding phosphopeptide in Experiment II.

^e The corresponding protein has more than one phosphopeptide, but only the one with the most significant phosphorylation change is shown.
sible for phosphorylating Rfc1 in the process of adipocyte differentiation (35). An in vitro assay showed that Ser\(^{518}\) was one of the major targets of calmodulin-dependent protein kinase II phosphorylation. In our study, Ser\(^{107}\) and Thr\(^{109}\) were identified and quantified as increased to 1.60-fold in response to MDI stimulation. Here Rfc1 was identified as a transfection factor with 19 quantified proteins predicted to be its targets, four of which obviously changed in expression. Interestingly, one of them increased, whereas three decreased in expression (Fig. 9A).

DISCUSSION
The On-line Yin-Yang MDLC-MS/MS System Has Shown Strong Power for Concurrent Identification and Quantification of Both Proteome and Phosphoproteome—The different features of phospho- and nonphosphoproteins have made it challenging to identify them simultaneously in one analysis. Traditional strategies, such as antibody purification, chemical deviation, etc., enrich the phosphoproteins or phosphopeptides with the concomitant exclusion of the non-phosphorylated part. Previously in our laboratory, we have applied the yin-yang MDLC system for total peptide fractionation using a pH gradient and proved that yin-yang MDLC-MS/MS is a powerful strategy to identify both the proteome and phosphoproteome in one analysis (16). Here in our study, we further developed this strategy and accomplished automatic elution with an on-line pH gradient instead of the off-line gradient in our previous work. These improvements reduced the operative error and time consumption and, more importantly, increased identification efficiency and reproducibility and minimized the sample lost (36). A new shotgun proteomics approach, combing SILAC for quantification, the on-line yin-yang MDLC system for peptide fractionation, and a high accuracy LTQ-Orbitrap, was successfully developed and applied for characterization of both proteome and phosphoproteome changes during adipocyte differentiation. Identification and quantification of numerous phosphorylation sites demonstrate this strategy as a powerful approach to tackle the cellular phosphoproteome in considerable depth. For the phosphoproteins identified, an average of 61.7% of them were also present in the peptide-based protein identification (Table I), indicating the capability of the yin-yang MDLC system to identify the peptide and phosphopeptide for a certain protein concurrently. In addition peptide isoforms of multiple phosphorylation states, such as sequence-unique peptides in an unmodified state, singly phosphorylated state, and multiply phosphorylated states, have all been identified. Supplemental Fig. 6 shows the distribution of mono-, di-, and triphosphorylated sites in SAX and SCX. Not surprisingly, the percentages of di- and triphosphorylated sites are obviously higher in SAX than in SCX, whereas they showed no bias on identification of serine, threonine, and tyrosine phosphorylation. The results are reasonable because the yin-yang MDLC systems fractionate peptides based on their pi, not on the difference among serine, threonine, and tyrosine; and the more phosphate the peptide has, the more acidic it is and the more easily it will bind to the SAX column. Furthermore the percentages of phosphorylated serine, threonine, and tyrosine between Experiments I and II are quite similar, showing an ideal reproducibility of the yin-yang MDLC systems. This methodology can routinely investigate protein expression and phosphorylation of complex protein mixtures from various biological samples.

Several known differentiation-related molecules showed obvious alteration in protein expression. TSP1 is a multifunctional extracellular matrix glycoprotein that is induced by mitogens, acts to enhance mitogen-stimulated cell division, and promotes cell attachment and migration (37). It also acts as a tumor suppressor in vivo that can inhibit angiogenesis and may be important in cancer invasiveness (38). As an antiangiogenic factor and regulator of transforming growth factor-\(\beta\) activity, obesity, adipose inflammation, and insulin resistance, TSP1 is highly expressed in obese, insulin-resistant subjects; correlates with adipose inflammation; and was identified as one of the immediate early genes induced by differential hybridization in 3T3-L1 preadipocytes (39). In our study, TSP-1 showed an obvious increase in both replicates with an average of 2.78-fold change at 1 h of MDI stimulation (Table II), consistent with the microarray data (40).

Another protein, NADH-ubiquinone oxidoreductase (Ndufab1), also named acyl carrier protein (ACP), showed a significant increase in expression in two biological replicates. ACP is a universal and highly conserved carrier of acyl intermediates during fatty acid synthesis that is crucial for adipogenesis (41). It is an accessory and non-catalytic subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), which functions in the transfer of electrons from NADH to the respiratory chain. Studies in brown adipocytes have illustrated that the differentiation of brown adipocytes in cell culture is associated with increased gene expression for components of the respiratory chain/oxidative phosphorylation system. Here in our study, ACP, also as a key regulator of lipogenesis, was up-regulated to 1.33 ± 0.02-fold in two replicates (Table II), indicating it as an early response gene during the initial process of adipocyte differentiation.

In addition to known adipogenesis-related molecules, several possible candidates were quantified with significant expression changes after MDI stimulation. Cathepsins are usually characterized as members of the lysosomal cysteine protease family, and the cathepsin family name has been synonymous with lysosomal proteolytic enzymes. Furthermore, the cathepsin family also contains members of the serine protease (Cathepsins A and G) and aspartic protease (Cathepsins D and E) families as well. Several members of the cathepsin family are known to play important role in adipogenesis. For instance, in vitro differentiated human adipocytes show elevations of Cathepsin K and Cathepsin L mRNA compared with human preadipocytes, indicating their participation.
Cathepsin S was also reported to promote the early steps of human preadipocyte differentiation (43). Here we quantified another member of the cathepsin family, Ctsa, with an average increase of 1.31-fold and relative S.D. of only 1% among two replicates. As a serine carboxypeptidase, Ctsa can regulate peptide hormone activity at the cell surface and possibly intracellularly after receptor-mediated endocytosis and may also participate in peptide hormone processing (44). Hiraiwa (45) has demonstrated Ctsa as a lysosomal multifunctional protein, whereas our results indicated its potential role in white adipocyte differentiation.

More importantly, several interesting molecules were identified as phosphoproteins whose cellular phosphorylation level changed obviously in response to MDI stimulation. The endothelial actin-binding protein Flna has been shown to interact with the insulin receptor to exert an inhibitory tone along the mitogen-activated protein kinase (MAPK) activation pathway, which is crucial during the process of adipocyte differentiation (46). It is a scaffolding protein that recruits numerous proteins involved in a completely different set of functions, including signal transduction, gene transcription regulation, and receptor translocation. Flna has been found to undergo phosphorylation in vivo in response to serum, lysophosphatidic acid, or other stimuli (47). Quantification results in our study showed that expression of Flna remained with the phosphorylation on Ser^{2152} in the carboxyl-terminal part extremely increased with an average of 5.68-fold. A representative MS/MS spectrum of phosphorylation on Ser^{2152} of Flna.

Fig. 7. Representative MS/MS spectrum of site-specific phosphorylation identification. A, Ser^{2152} on APS#VANIGSHCDLSLK of Isoform 1 of Filamin A. B, Ser^{88} on VCS#IDLEIDSLSSLLDDMTK* of Zyxin. C, Ser^{20} on FIIGSVSEDNS#EDEISNLVK of acetyl-CoA carboxylase 1. K* and # represent [^{13}C_6,^{15}N_2]lysine and phosphorylation modification, respectively.
Validation of SILAC quantification of protein expression and phosphorylation. Western blotting was applied to the total cell lysate and the elution fraction of the phosphoprotein purification strategy for control (Ctrl) and 1-h MDI-stimulated samples. The SILAC ratio of protein expression and site-specific phosphorylation are indicated, respectively. Annexin I and activity-dependent neuroprotector homeobox protein (ADNP) were used as quantification control of protein expression and phosphorylation, respectively. Phos: phosphorylated.

Fig. 8. Validation of SILAC quantification of protein expression and phosphorylation. Western blotting was applied to the total cell lysate and the elution fraction of the phosphoprotein purification strategy for control (Ctrl) and 1-h MDI-stimulated samples. The SILAC ratio of protein expression and site-specific phosphorylation are indicated, respectively. Annexin I and activity-dependent neuroprotector homeobox protein (ADNP) were used as quantification control of protein expression and phosphorylation, respectively. Phos: phosphorylated.

is displayed in Fig. 7A. To further confirm the quantification, changes of expression and phosphorylation were detected by Western blotting (Fig. 8). To validate quantification of phosphorylation on Ser\(^{2152}\), a site-specific antibody to phospho-Ser\(^{2152}\) on Flna was applied. Obviously, expression of Flna showed no difference between MDI and control samples, whereas phosphorylation on Ser\(^{2152}\) increased. Meanwhile, an antibody to Flna itself was applied to detect the Flna amount in the total cell lysate and elution fraction of the phosphoprotein purification kit. Similarly, Flna increased after MDI stimulation in the elution fraction, whereas Flna remained the same in the total cell lysate. This illustrated that, when no site-specific antibody is available, this Western strategy still may confirm the quantification of site-specific phosphorylation. Ser\(^{2152}\) in the carboxyl-terminal tail of Flna has been reported to be the unique substrate in the carboxyl-terminal region for endogenously activated cAMP-dependent protein kinase (48) and is an important regulatory site that may be phosphorylated by ribosomal S6 kinase (49). Our result showed that functional alterations in Flna in relation to adipocyte differentiation might occur as a consequence of phosphorylation on Ser\(^{2152}\).

Zyxin, a low abundance phosphoprotein localized at the cellular sites of adhesion in fibroblasts, displays the architectural features of an intracellular signal transducer. It has three copies of the LIM motif, a double zinc finger domain found in many proteins that play central roles in regulation of cell differentiation, and was also reported to act as a tumor suppressor in tumor cells (50). Moreover, it has been reported to shuttle from the cytoplasm to the nucleus where it can affect cell fate, and the nuclear translocation is phosphorylation-dependent (51). In our results, phosphorylation on Ser\(^{88}\) of Zyxin increased an average of 5.15-fold after MDI stimulation, whereas its expression level remained unchanged. A representative MS/MS spectrum of phosphorylation on Ser\(^{88}\) of Zyxin is shown in Fig. 7B. The quantification results were also confirmed by Western blotting. As shown in Fig. 8, the amount of Zyxin significantly increased after MDI stimulation in the elution fraction (similar to the SILAC ratio) without an obvious change of the total amount. Because Zyxin has transcriptional activity and can shuttle to the nucleus in a phosphorylation-dependent manner and Ser\(^{88}\) is the only phosphorylation site identified and with high abundance, it is quite possible that phosphorylation on Ser\(^{88}\) contributes to the changes of transcriptional activity or localization of Zyxin, resulting in adipocyte fate determination of preadipocytes.

For some phosphoproteins that showed a significant change in phosphorylation level, their phosphorylation has previously been reported to be crucial to adipocyte differentiation. ACC is one of the key lipogenic enzymes. It carries out three functions as biotin carboxyl carrier protein, biotin carboxylase, and carboxytransferase and catalyzes the rate-limiting reaction in the biogenesis of long-chain fatty acids. Inhibition of acetyl-CoA carboxylase both inhibits fatty acid synthesis and stimulates fatty acid oxidation (52). In addition ACC is a substrate of adenosine monophosphate-activated protein kinase, and phosphorylation of both adenosine monophosphate-activated protein kinase and ACC was reported to be negative for lipogenesis. 5-amino-4-imidazolecarboxamide ribonucleoside, a cell-permeable activator of AMP-activated protein kinase, was also shown to profoundly inhibit lipogenesis through increased phosphorylation of ACC (53, 54). In our study, phosphorylation of ACC1 on Ser\(^{29}\) decreased to 0.60 \(\pm\) 0.07-fold with its expression almost unchanged. A representative MS/MS spectrum of phosphorylation on Ser\(^{29}\) of ACC1 is displayed in Fig. 7C. Protein expression and phosphorylation were also confirmed by Western blotting (Fig. 8). In total cell lysate, the amount of ACC1 remained almost the same, whereas in the elution fraction, the ACC1 level obviously decreased after MDI stimulation. This result indicated that Ser\(^{29}\) may be a key regulation site for the ACC activity and thus has a potential relationship with lipogenesis and adipocyte differentiation.

Concurrent Quantification of Phosphorylation and Protein Expression Has Distinguished Two Manners of Cellular Phosphorylation Alterations—When cells receive signals from their surroundings, they transmit signals mainly through sequential phosphorylation. A large portion of cellular proteins is phosphorylated; however, only a small portion of the total sites are regulated by a certain stimulus. As proteins generally serve as a signal integrating platform for various incoming stimuli, the phosphorylation on different sites of the same protein may
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either activate or deactivate the protein activity and consequent events; thus they are often distinctly regulated. This finding reflects that protein phosphorylation displays different functions on different sites of the same protein. This also suggests that, to obtain accurate and functional control of phosphorylation events, the degree of phosphorylation should be measured site-specifically rather than for the protein as a whole. Thus, signaling outcomes can be more directly connected to responsible upstream or downstream events. In our study, combination of SILAC and the yin-yang MDLC system has made it to possible to quantify protein expression as well as site-specific phosphorylation at the same time. In each of the replicates, expression of ~2000 proteins was quantified with relative S.D. less than 14% of the shared proteins between the two biological replicates. Meanwhile, hundreds of unique phosphopeptides were quantified site-specifically. More importantly, about half of the proteins quantified with phosphopeptides have peptide-based protein quantification, facilitating discrimination of phosphorylation alterations on the protein level or that are site-specific. For a system-wide investigation, concurrent quantification of protein phosphorylation and expression can give direct insights into the association of protein phosphorylation and gene expression for a specific protein. Many critical events involved in cellular responses are mediated by changes in post-translational protein modifications rather than gene expression changes (55). For a certain protein, phosphorylation may be altered without a significant change in protein expression (Fig. 1B), such as Zyxin (Fig. 6A) mentioned above, indicating a mechanism of phosphorylation changes regulated directly by protein kinases or phosphatases and without involvement of its gene expression. Subsequently, the counterpart unphosphorylated peptide VCSIDLEIDSLLDDLDTMK showed a reciprocal behavior, indicating a stoichiometric phosphorylation event at Ser\(^{38}\) of Zyxin. For some other proteins, however, phosphorylation changed to a degree similar to that of protein expression change (Fig. 1C) or the degrees of phosphorylation increased or decreased at the cellular level, but the relative degree of phosphorylation remained the same at protein level, such as for Baz1b (Fig. 6B). In this situation, phosphorylation is only a phenomenon that accompanied the alteration of its gene expression. Another example was for Band 4.1-like protein 2 whose phosphorylation on peptide EVENEQTPVS\(^{38}\)#EPEEEK was down-regulated to 0.67-fold, whereas its counterpart unphosphorylated peptide and other nonphosphopeptides showed no obvious changes (supplemental Tables 3 and 5). This indicated that Ser\(^{38}\) was only phosphorylated in a small portion of total Band 4.1-like protein 2. Thus, the yin-yang MDLC-MS/MS system combined with the SILAC approach provides the opportunity to discern between changes in protein dosage linked to phosphorylation and changes merely in the level of protein phosphorylation.

**Potential Effects of Site-specific Phosphorylation on Transcriptional Activity Regulation**—Generally, transcription factors and their targets show correlated expression among various tissues or different physiological status. Using co-expression data, reverse engineering has been widely applied to predict TF and their target genes or to explain regulation of TF on their targets (26, 56). Protein transcriptional modification, as another way of regulating target gene expression, may function before protein expression changes of TF itself. Previous studies have given some hints on phosphorylation-dependent regulation of certain TFs from plant to mammalian cells (57, 58). However, system-wide association of protein phosphorylation and gene expression was still poorly investigated because of technical limitations.

Fine tuning mechanisms of biological processes are not limited to the upstream level but may affect proteins at all levels of signaling transductions. A 1-h stimulation of 3T3-L1 preadipocyte differentiation was chosen to attempt to catch the transition from the upstream phosphorylation cascade to the downstream signaling transduction involving transcription regulation in response to MDI stimulation. Combining TF-target prediction and quantitative proteomics information, our results have illustrated the possible effects of phosphorylation on transcriptional activities. For the phosphorylation events quantified, some have a potential positive effect on transcriptional activities, such as Ser\(^{38}\) on Zyxin, mentioned above, for which three of its targets increased in expression. Similarly, phosphorylation of Psip1, a phosphoprotein known to be a transcriptional coactivator, obviously increased in response to MDI stimulation with five of the Psip1 targets showing increased expression. Whereas for some other TFs, phosphorylation on specific sites seemed to be negative for their transcriptional activities. One-fifth of the quantified targets of a transcriptional regulator, Prrx1, decreased as the phosphorylation of the protein increased. For some others such as Larp7 protein, phosphorylation of peptide TAS#EGS#EAEET-PEAPK increased in response to MDI stimulation, whereas two of its targets increased and another three decreased in expression. This indicated the possible existence of other regulators of these targets. Among the targets that were regulated by several TFs, however, protein expression of most of them seldom changed. For example, Sfrs1 was predicted to be the target of four TFs, Tcf12, Ddx21, Akap8, and Sfrs2, with which co-expression has been reported. The quantification result showed no significant expression alteration of Sfrs1, although several other targets of its four TFs increased or decreased, respectively. This raised the possibility that phosphorylation has a different effect on each of these four TFs; thus the retained expression level of Sfrs1 was the consequence of the counteraction of its four regulators. This also implied a mechanism of feedback control among transcriptional regulation networks.

On the other hand, we also detected the co-expression of TF and its targets, such as Baz1b whose phosphorylation on Ser\(^{706}\) and Ser\(^{709}\) increased with a ratio similar to that of
protein expression, implying that the phosphorylation level on these two sites was relatively unchanged. The increased expression of Baz1b indicates that it is one of the earliest molecules responsible for the gene transcription regulators during adipocyte differentiation. Interestingly, five downstream proteins showed significant expression alteration, whereas four of them increased with the up-regulated expression of Baz1b, indicating a positive correlation of TF expression with transcription of their target genes (Fig. 9B).

This high throughput quantitative analysis has illuminated the effects of phosphorylation changes from upstream of signaling pathways (such as interaction with the cell membrane receptors) to, more importantly, downstream of signal transduction (that is regulation of transcriptional activities).
Future studies may apply this strategy to a time course analysis of the adipocyte differentiation process. Furthermore, gene expression data measured by microarray may also be included. This may provide a comprehensive view for a system-wide modeling of signaling transduction and gene transcription regulation.

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