Temporal Phosphoproteome Dynamics Induced by an ATP Synthase Inhibitor Citreoviridin

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Citreoviridin, one of toxic mycotoxins derived from fungal species, can suppress lung cancer cell growth by inhibiting the activity of ectopic ATP synthase, but has limited effect on normal cells. However, the mechanism of citreoviridin triggering dynamic molecular responses in cancer cells remains unclear. Here, we performed temporal phosphoproteomics to elucidate the dynamic changes after citreoviridin treatment in cells and xenograft model. We identified a total of 829 phosphoproteins and demonstrated that citreoviridin treatment affects protein folding, cell cycle, and cytoskeleton function. Furthermore, response network constructed by mathematical modeling shows the relationship between the phosphorylated heat shock protein 90 β and mitogen-activated protein kinase signaling pathway. This work describes that citreoviridin suppresses cancer cell growth and mitogen-activated protein kinase/extracellular signal-regulated kinase signaling by site-specific dephosphorylation of HSP90AB1 on Serine 255 and provides perspectives in cancer therapeutic strategies. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.051383, 3284–3298, 2015.

Citreoviridin is the pathogenic factor for several diseases, including acute cardiac beriberi (3) and Keshan disease (4). Many studies have elucidated the underlying mechanism of citreoviridin cytotoxicity in different cell types. Citreoviridin interferes with the metabolism of nerve and muscle tissues, competing with the absorption of vitamin B1, thus causing beriberi (5). In addition, citreoviridin initiates Keshan disease through oxidative stress mechanism (4). Citreoviridin elevates the activation of nuclear factor-κB (NF-κB) to enhance tumor necrosis factor (TNF)-α-induced endothelial adhesion in human umbilical vein endothelial cells (6) and atherogenesis in hypercholesterolemic ApoE-deficient mice (7). In hepatic cells, citreoviridin induces autophagic cell death through reactive oxygen species (ROS) (8) and lysosomal-mitochondrial axis (9).

In previous studies, we reported that citreoviridin could specifically kill cancer cells but not normal cells because of ectopically expressed ATP synthase (ecto-ATP synthase) on plasma membrane of cancer cells (10, 11). In general, ATP synthase is located in the inner membrane of the mitochondria. However, recent studies have revealed that ATP synthase is also expressed on the cell surface of keratinocyte, hepatocyte, and adipocytes, as well as endothelial, neuronal, and cancer cells (10–19). After citreoviridin treatment on cells with ectopically expressed ATP synthase, there was no change in the mitochondrial membrane potential, suggesting that citreoviridin inhibited the activity of ectopic, but not mitochondrial ATP synthase (10). Furthermore, the inhibition of ecto-ATP synthase by citreoviridin stimulates the unfolded-protein response and elevates levels of ROS in lung cancer cells (10). Quantitative proteomic analysis of human lung tumor xenografts showed that citreoviridin induces alternations in the expression of glucose metabolism-related enzymes and suggested that citreoviridin may reduce the glycolytic intermediates for macromolecule synthesis and inhibit cell proliferation (20). Furthermore, combined treatment of citreoviridin and bortezomib, a 26S proteasome inhibitor on breast cancer cells triggered lethality through unusual nonapoptotic caspase- and autophagy-independent cell death with a cytolytic vacuolization phenotype (11). However, the prompt and dynamic molecular responses triggered by citreoviridin...
and their underlying mechanisms in cancer cells remain unknown.

Protein phosphorylation is one of most widespread modes of post-transcriptional modification in cell signaling (21, 22). Many protein kinases such as mitogen-activated protein kinases (MAPKs) and serine/threonine protein kinase (Akt) are major enzymes that drive tumor growth by signal transduction, leading to transcriptional changes of oncoproteins (23, 24). Therefore, characterization of protein phosphorylation status following stimuli-induced signaling changes may provide important insight into the regulation of physiological events in cells. Recent advances in quantitative phosphoproteomic profiling not only allows researchers to study the aberrant regulation of signaling pathways but also assists in the discovery of appropriate therapeutic targets for various diseases (23, 25–27).

In the present study, we combined MS-based phosphoproteomics with functional analysis and mathematical modeling to comprehensively elucidate the global phosphorylation changes of citreoviridin treatment in lung cancer cells. Our findings uncover dynamic molecular responses underlying citreoviridin treatment in cancer cell growth and reveal new insight for cancer therapy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human lung adenocarcinoma cells CL1–0 and A549 were grown as previously described (10). Briefly, cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, New York, NY) containing 10% fetal bovine serum (Gibco) at 37 °C and 5% carbon dioxide.

**Drug Treatment of Cultured Cells—Citreoviridin** (Santa Cruz Biotechnology, Santa Cruz, CA) solution was solubilized in DMSO (AppliChem, Darmstadt, Germany). CL1–0 and A549 cells were treated with citreoviridin (4.6 μM for CL1–0 and 1.5 μM for A549) or with the same volume of DMSO, as previously described (10). Time points for treatment were 10 s; 1, 5, and 10 min; and 1, 2, 12, 24, 36, and 48 h. Two biological replicates for each time point experiment were performed. After treatments, cells were washed with phosphate-buffered saline and then centrifuged at 1200 × g. The collected cells were stored at −80 °C until use.

**Sample Preparation for Cell Phosphoproteome**—Proteins were extracted with 12 mM sodium deoxycholate (Sigma-Aldrich, St Louis, MO), 12 mM sodium lauroyl sarcosine, 50 mM triethylammonium bicarbonate (Sigma-Aldrich), protease mixture (Sigma-Aldrich), and phosphatase inhibitor mixture (Sigma-Aldrich), as described previously (28). Protein concentration was determined by using the bicinchoninic acid Protein Assay Reagent kit (Pierce, Rockford, IL). Cell lysates were reduced with 10 mM dithiothreitol (BioShop, Burlington, Canada) at room temperature for 30 min, and carbodiimethylated with 55 mM iodoacetamide (Sigma-Aldrich) in the dark at room temperature for 30 min. Alkylated proteins were digested with Lys-C (1:100 w/w; WAKO, Osaka, Japan) for 2 h and then digested with sequencing-grade modified trypsin (1:100 w/w; Promega, Mannheim, Germany) overnight. For detergent removal, the peptide mixture was combined with an equal volume of ethyl acetate (Sigma-Aldrich) and then acidified with trifluoroacetic acid (Sigma-Aldrich) to a pH of <3. The acidified sample was shaken for 1 min and then centrifuged at 15,700 × g for 2 min to separate the aqueous and organic phases. The collected aqueous sample without detergents was desalted with a 12 M sodium cyanoborohydride (Sigma-Aldrich) in the dark at room temperature (30). Ammonium hydroxide (1%, 24 μl) was added to stop the reaction by reacting with the excess formaldehyde. Formic acid (10%, 30 μl) was further added with functions of ending the labeling reaction and acidifying the samples. Finally, the H- and D-labeled samples were combined at 1:1 ratio and then desalted by using SDB-XC StageTips.

**Dimethyl Labeling of Peptides**—Tryptic peptide samples (150 μg) were dried under vacuum and then redissolved in 0.1 M TEAB solution. The control and drug-treated peptide samples were first mixed with 6 μl of 4% formaldehyde–H2O (Sigma-Aldrich) and 4% formaldehyde–H2O (Sigma-Aldrich), respectively, and then immediately 6 μl of freshly prepared 0.6 M sodium cyanoborohydride (Sigma-Aldrich) was added to each mixture. Each mixture was vigorously mixed and then reaction was allowed to proceed for 60 min at room temperature (30). Ammonium hydroxide (1%, 24 μl) was added to stop the reaction by reacting with the excess formaldehyde. Formic acid (10%, 30 μl) was further added with functions of ending the labeling reaction and acidifying the samples. Finally, the H- and D-labeled samples were combined at 1:1 ratio and then desalted by using SDB-XC StageTips.

**Phosphopeptide Enrichment**—The phosphopeptide was enriched by hydroxy acid-modified metal oxide chromatography (HAMMOC), as previously described (31, 32). Briefly, custom-made MOC tips were prepared by packing 0.5 mg of TiO2 beads (GL Sciences, Tokyo, Japan) into 10 μl C8 StageTips (29). Prior to sample loading, MOC tips were equilibrated with solution A (0.1% TFA, 80% acetonitrile, and 300 mg/ml lactic acid). About 200 μg of freshly digested peptide mixture was mixed with an equal volume of solution A. The resulting mixture was loaded onto the MOC tips (100 μg mixed peptides per tip) and then washed with solution A and with solution B (0.1% TFA and 80% acetonitrile). Phosphopeptides were eluted with 0.5 and 5% solutions of piperidine (Sigma-Aldrich). The eluate was acidified with TFA, desalted with SDB-XC StageTips, and then vacuum-dried. The phosphopeptides were then re-suspended in 0.5% TFA and analyzed by nLC-MS/MS. For each time point and xenograft group, two independent batches of biological samples were prepared.

**NanoLC-MS/MS Analysis**—Samples for cell phosphoproteomes were analyzed on an LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) equipped with a nanoACQUITY UPLC system (Waters Corp., Milford, MA). Peptide samples were loaded onto a 2 cm × 180 μm capillary trap column and then separated in a 75 μm × 25 cm nanoACQUITY 1.7 μm BEH C18 column at a flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid, and B consisted of 0.1% formic acid and 80% acetonitrile. A linear gradient of 10–40% B in 90 min and 40–85% B in 10 min was employed throughout this study. Mass spectra from survey full scans were acquired on the Orbitrap (m/z 350–1500). The resolution of the instrument was set to 60000 at m/z 400 with an automated gain control (AGC) value of 106. The top ten most-intense precursor ions were selected from the MS.
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scan for subsequent collision-induced dissociation MS/MS scan by ion trap (AGC target at 7000). For each biological sample, duplicate nanoLC-MS/MS analyses were performed.

Data Analysis for Cell Phosphoproteome—Raw MS spectra were processed for peak detection and quantitation by using MaxQuant software version 1.3.0.5 (33). Peptide identification was performed by using the Andromeda search engine (34) and the Swiss-Prot database (release 87, subset human, 20265 protein entries). Search criteria used in this study were trypsin specificity, fixed modification of carbamidomethyl, variable modifications of oxidation and phosphorylation, and allowed for up to two missed cleavages. A minimum of six amino acids in the peptide length was required. The precursor mass tolerance was 3 ppm and the fragment ion tolerance was 0.5 Da. By using a decoy database strategy (35), peptide identification was accepted based on the posterior error probability with a false discovery rate of 1%. Precursor intensities of already identified peptides were further searched and recalculated by using the “match between runs” option in MaxQuant. For multiple phosphopeptides, the localization probability of all putative phosphosites was determined by using the MaxQuant PTM score algorithm, which was calculated based on the peptide spectral match and the potential phosphosites in each peptide (33, 36). The spectra of peptides with more than three phosphosites were manually checked and provided in supplemental Fig. S1. For single peptide with multiple hits, all matched proteins were counted separately. The ratio of phosphosite was normalized by MaxQuant. Phosphosites that displayed a minimum of threefold change in abundance for at least three time points in the temporal phosphoproteomic study were considered as regulated phosphosites.

Phosphoproteomic Analysis of Xenograft Tissues—The tumorigenic assays in athymic mice was successfully performed to examine the effect of citreoviridin on tumor growth. Mice were first subcutaneously injected with lung cancer cell CL1–0 and intraperitoneally administered with control vehicle DMSO or ATP synthase inhibitor citreoviridin. The analyses results and detailed information were described in our previous study (20). Xenograft tissues excised and collected from this xenograft model were snap-frozen in liquid nitrogen and stored at –80 °C for phosphoproteomic analyses. The protein extract of each sample was denatured in 8 M urea solution (BioShop). Proteins were then reduced, carbamidomethylated, and then digested five times with 50 μm TEAB for trypsin digestion. Tryptic peptides were then processed by dimethyl labeling and phosphopeptide enrichment, and then analyzed by LC-MS/MS. Peptide identification and quantification were performed by using Mascot version 2.3 (Matrix Science, London, UK) and Mass Navigator version 1.3 (Mitsui Science, Tokyo, Japan). Details of the experimental procedure can be found in the supplemental Methods.

Phosphorylation Motif Analysis—Phosphopeptide sequences at any one time point were submitted to Motif-X algorithm (37) for identification of over-represented motifs. Only phosphopeptides with the phosphosite localization probability > 0.75 were selected for analysis. Sequences were centered on each phosphosite and extended to 15 amino acids (±7 residues). Where the phosphosite was close to the N- or C-terminal of the protein, the sequence was filled with the required number of “X”, which denotes any amino acid. The Human International Protein Index database in Motif-X was used as a background. The significance threshold was set at r < 10−6, and the occurrence threshold of input data was set at 20.

In order to associate motifs with specific kinases, over-represented motifs were matched to known kinase motifs. The position weight matrix (PWM) for each over-represented motif was generated based on motif-matching sequences from the results of Motif-X. PWM was constructed by counting the occurrence of each amino acid at each position and then normalizing at each position by the number of sequences. PWMs for 300 kinase recognition motifs, including 247 Ser/Thr and 53 Tyr kinases, were obtained from the PhosphoNetwork database (38). We calculated the similarity between PWMs of over-represented and kinase recognition motifs by determining the Pearson’s correlation coefficient and generated a similarity matrix. Because the phosphosites are noninformative, we excluded the central S/T/Y residue (column 8 in PWM) from similarity measurements to focus on flanking sequences. The similarity matrix was analyzed by hierarchical clustering to group similar motifs into motif clusters. Hierarchical clustering using average linkage with correlation distance was performed by constructing a generalized association plot (GAP) (39).

Fisher’s exact test was performed to assess whether phosphosites with regulation changes of a given time point are significantly enriched at a specific motif cluster. This test is commonly used to assess the relative between two variables, i.e. phosphorylation motif and phosphorylation intensity, and formulated as follows:

\[
P(X \geq k) = \sum_{j=x}^{n} \binom{n}{i} \binom{N-n}{K-i} \binom{N}{K}
\]

where \(N\) is the total number of phosphosites for which expressions may be quantified at a given time point, and \(n\) is the number of phosphosites that are significantly regulated (log2(fold-change) ≥ 1) at a given time point. \(K\) represents the number of phosphosites containing the motif of a given motif cluster, and \(k\) stands for the number of overlapping phosphosites.

Fuzzy c-Means Clustering—To examine relative temporal phosphorylation patterns, soft clustering was applied to phosphosites and the fuzzy c-means algorithm implemented in the “Mfuzz” package (40) for R was used. For clustering analysis, we used 898 phosphosites with < 2 missing values in the temporal expression profile of 10 s to 2 h. In the first step, temporal phosphorylation data for the changing phosphosites were transformed by base 2 logarithm. Second, missing values without quantitative data were imputed by k-nearest neighbors method (where k is 10) implemented in the “impute” package. Third, because clustering is performed in Euclidian space, change values of phosphosites were standardized to have a mean value of zero and a standard deviation of one. This step ensures that vectors of genes with similar changes in phosphorylation are close in Euclidean space. Finally, the fuzzy c-means algorithm was performed. The clustering parameter \(m\), which affects the “softness” of the cluster, was estimated through the Mfuzz internal function “mestimate” in the fuzzy c-means algorithm. The appropriate cluster number \(c\) was determined by manual observation of the real data.

Mathematical Modeling and Network Construction—We aimed to identify the mechanism of modulatory relationships of the selected phosphoproteins involving protein folding related proteins and kinases significantly regulated within short response time and to construct a phosphoprotein modulatory network. For each selected phosphoprotein, its log2 expression ratio was first determined. Because the selected phosphoproteins with multiple phosphosites were primarily quantified from the same phosphopeptides (supplemental Table S12), we used the expression ratios from the corresponding dominant phosphopeptides to model the dynamic change of theses phosphoproteins, ignoring their rare phosphorylated variants. The temporal expression of the selected phosphoprotein \(i\) was described with the following discrete dynamic model:

\[
x[t + 1] = x[t] + \sum_{j=1}^{4} \alpha_j x[t] - \lambda x[t] + k_i + e_i[t]
\]
where \( x_i(t) \) represents the log2 ratio of the phosphoprotein expression at time \( t \) for phosphoprotein \( i \), and \( a_i \) denotes the modulatory ability of the \( j \)-th phosphoprotein for the target phosphoprotein \( i \). \( \lambda_i \) represents the log2 ratio of the phosphoprotein expression for the \( j \)-th phosphoprotein that potentially regulates target phosphoprotein \( i \). \( \lambda_i \) denotes the number of phosphoproteins potentially regulating phosphoprotein \( i \). \( \delta_i \) indicates the degradation effect of the target phosphoprotein \( i \). \( k_i \) represents the basal expression ratio, and \( \epsilon_i \) represents the stochastic noise. In the mathematical model, \( x_i(t) \), \( \lambda_i \), and \( \delta_i \) could be obtained from the quantitative phosphoproteomics data, whereas \( a_i, \lambda_i, \) and \( k_i \) were the parameters to be identified. Among those model parameters, the modulatory abilities \( a_i \)'s were of special importance. The modulatory abilities implied the modulatory relationships between phosphoprotein \( j \) and phosphoprotein \( i \) with a positive value indicating activation effect and a negative value indicating inhibitory effect, respectively.

After mathematical models for the selected phosphoproteins were built, parameters in the models were then identified through the maximum likelihood estimation method (41, 42) with the aid of phosphoprotein expression data (details can be found in the Supplemental Methods). When parameters for all the selected phosphoproteins were identified, the significant modulatory relationships which constitute the phosphoprotein network were determined based on the estimated modulatory abilities \( a_i \)'s. Akaike information criterion (41, 43) and Student’s \( t \) test were employed for model order selection and to determine the significance of the modulatory relationships (see Supplemental Methods). In this manner, significant modulations of phosphorylation between proteins of protein folding and kinases were identified and the phosphoprotein network was constructed.

Western Blot Analysis—Cell lysates were separated according to molecular weight through polyacrylamide gel electrophoresis by using Bio-Rad Mini-PROTEIN 3 Electrophoresis Cell and then transferred onto polyvinyl difluoride membranes (Millipore, Bedford, MA, USA). After it was blocked with 5% nonfat milk or 1% bovine serum albumin, the membrane was probed with primary antibodies against the proteins of interest, and the primary antibodies were linked to secondary antibodies conjugated to horseradish peroxidase. Protein bands were visualized by using an enhanced chemiluminescence detection kit (Pierce Biotechnology Inc., Rockford, IL, USA). Antibodies, including rabbit anti-HSP90AB1, rabbit anti-MAPK1, rabbit anti-Raf-1, rabbit anti-MEK1/2, rabbit anti-PI3K, rabbit anti-P-HSP90AB1 (pSer255), rabbit anti-P-MAPK1/3 (pThr185/202 + pTyr187/204), rabbit anti-P-Raf-1 (pSer338), and rabbit anti-P-MEK1/2 (pSer217/221), were purchased from GenTex (San Antonio, TX, USA). Mouse anti-actin was obtained from Sigma.

RNA Extraction and Complementary DNA (cDNA) Synthesis—Total RNA was isolated from CL-1–0 cells by using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Quality and quantity of RNA were determined on a NanoDrop ND-1000 ultraviolet spectrophotometer (NanoDrop Technologies, Wilmington, DE). First-strand cDNA synthesis was prepared by using Oligo (dT) and the RevertAid H minus First Strand cDNA synthesis kit (Wilmington, DE). First-strand cDNA synthesis was prepared by using ND-1000 ultraviolet spectrophotometer (NanoDrop Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Mouse anti-actin was obtained from GenTex (San Antonio, TX). Mouse anti-actin was obtained from GenTex (San Antonio, TX). Mouse anti-actin was obtained from GenTex (San Antonio, TX).

RESULTS

Quantitative Analysis of Dynamic Phosphorylation Profiles of Lung Cancer Cells with Citreoviridin Treatment—To characterize the temporal phosphorylation dynamics in response to citreoviridin treatment in lung cancer cells, a quantitative phosphoproteomic approach was applied in this study. We previously detected ecto-ATP synthase and electron transport chain on the cell surface of the lung cancer cell line CL-1–0 and found that citreoviridin, an ATP synthase inhibitor, could effectively inhibit the growth of CL-1–0 cells (10). Most importantly, citreoviridin-mediated inhibition showed no obvious impact on the normal function of mitochondrial ATP synthase, suggesting citreoviridin mainly inhibits the activity of ectopic, but not mitochondrial ATP synthase in cancer cells (10). Accordingly, CL-1–0 cells in this study were exposed to citreoviridin (treatment group) or to dimethyl sulfoxide (DMSO, control group) for periods spanning 10 s to 48 h (Fig. 1). To profile the phosphorylation changes of citreoviridin treatment on a global scale, proteins extracted from different samples...
were digested and then processed by dimethyl labeling. Phosphopeptides were enriched using hydroxy acid-modified metal oxide chromatography (HAMMOC) (31, 32). Analyses of enriched samples were performed by nanoscale liquid chromatography-tandem MS (nanoLC-MS/MS) using two biological replicates. In the time-dependent phosphoproteome data, 2195 phosphopeptides corresponding to 707 phosphoproteins were identified and 2467 phosphorylation sites (phosphosites) were quantified using MaxQuant (33) (Fig. 2A, supplemental Table S1, and S2). Most peptides were singly or doubly phosphorylated (Fig. 2B). The phosphosites were divided into three categories based on the localization probability (P): class I (P > 0.75), class II (0.75 ≥ P > 0.5), and class III (P < 0.5) (45, 46). Among the 2467 phosphosites, 2038 (82.6%) phosphosites were assigned with a probability of at least 0.75 (class I; supplemental Table S2 and Supplemental Table S3). Class I phosphosites revealed a distribution of 82% phosphoserine (pSer), 13% phosphothreonine (pThr), and 5% phosphotyrosine residues (pTyr) (Fig. 2C).

**Phosphoproteomic profiling of xenograft tissues with citreoviridin treatment**—In parallel with the temporal phosphoproteome, we also performed a quantitative phosphoproteomics study of xenograft tissues. Tissue samples were obtained from a CL1–0 xenograft model, which was used to confirm the inhibitory effect of citreoviridin on tumor growth in vivo (20). Immunodeficient mice carrying subcutaneous CL1–0 tumors were abnormally injected with citreoviridin or DMSO and then sacrificed when control tumors reached a size of 1000 mm³. Proteins from the dissected tissues were digested and processed for phosphopeptide enrichment. Phosphopeptides were analyzed by LC-MS/MS using two biological replicates. We acquired 862 unique phosphopeptides corresponding to 679 quantified phosphosites on 423 phosphoproteins (Fig. 2D, supplemental Table S4 and S5). The identified peptides were predominantly monophosphorylated (Fig. 2E). Among all phosphosites, 497 (73.2%) were classified as confident assignments (class I; supplemental Table S3 and Table S5). The phosphosites are predominantly pSer (92%) relative to pThr (7%) and pTyr (1%) (Fig. 2F). Comparison between cell and xenograft profiles showed an overlap of 301 phosphoproteins (supplemental Fig. S2). By combining xenograft data with time-dependent cell phosphoproteome analysis, a total of 829 phosphoproteins were identified (supplemental Fig. S2).

**Global Profiles of Cell and Xenograft Phosphorylation Changes Reveal Specific Functions Associated with Citreoviridin Treatment**—In our studies utilizing citreoviridin in cultured cell and xenograft systems, we quantified a total of 829 phosphorylated proteins. To identify significantly regulated phosphoproteins in the temporal phosphoproteome, we considered phosphosites that displayed a minimum of threefold change in abundance (log₂ (fold - change) ≥ 1.58) for at least
three time points to be differently expressed. Hence, up to 96 phosphosites matching 61 proteins were regulated (supplemental Fig. S3A and supplemental Table S6). Using the same cutoff of threefold change in the xenograft data set, we identified 18 positively and 22 negatively regulated phosphosites on 14 and 18 proteins, respectively (supplemental Fig. S3A and supplemental Table S7). To assess the role of citreoviridin inhibition in lung cancer, functional processes relevant to the regulated phosphoproteins of cells and xenografts were generated by using MetaCore Analytical Suite (47) (supplemental Fig. S3B). Although only seven common proteins were significantly regulated in phosphorylation level, we observed considerable overlap of enriched functions between cell and xenograft in response to citreoviridin treatment. For example, proteins involved in protein folding, immune responses, and regulation of telomere length, and cytoskeleton proteins were overrepresented in both data sets. As previously reported, these biological processes are associated with cancer progression and are influenced by the regulation of signaling pathways such as MAPKs and Akt cascades (48–51). The results thus emphasize the importance of protein phosphorylation on cell physiology in response to citreoviridin treatment.

Motif Analyses From Global Phosphoproteomic Data Revealed Possible Kinase Families Affected by Citreoviridin—To investigate possible kinases involved in citreoviridin treatment, we performed a motif analysis to determine kinase-substrate relations. All identified phosphosites with seven flanking residues were submitted to the Motif-X software tool (37). Forty-one motifs containing 33 serine (Ser) and 8 threonine (Thr) phosphosites were overrepresented ($p < 10^{-6}$). To associate the enriched motifs with specific kinases, we examined similarities between enriched motifs and kinase recognition motifs. We obtained 300 kinase recognition motifs from the PhosphoNetworks database (38). All Tyr motifs were ignored because none of them were enriched in our dataset, and the remaining 247 human kinases were used for further analysis. A similarity matrix between 41 over-represented motifs and 247 human kinases was generated and hierarchical clustering was performed (Fig. 3A and supplemental Table S8). On the basis of motif-kinase analysis, 41 motifs were grouped into 20 motif clusters matched to specific kinases (Fig. 3B and supplemental Table S8). Furthermore, we used the Fisher’s exact test to assess if the phosphosites regulated at a given time point were significantly enriched within a specific motif cluster. As shown in Fig. 3C, phosphosites regulated at 10 s and enriched on clusters 13 and 16 were mostly associated with the members of casein kinase 2 (CK2) family, including casein kinase 2 α (CSNK2A1) and casein kinase 2 α isoform (CSNK2A2) (supplemental Table S8), whereas the late-response phosphosites enriched on cluster 3 were recognized by cyclin-dependent kinase (CDK) and MAPK families (supplemental Table S8). These analyses revealed that responsive signaling of citreoviridin might be under regulation by the CK2 family and might affect MAPK or CDK cascades.
Fig. 3. Enriched motifs of phosphopeptides indicating that CK2 and CDK/MAPK are regulators in the citreoviridin-treated cells. A, Similarity matrix enriched motifs and kinase recognition motifs were clustered based on the average linkage with Pearson’s correlation coefficient. B, The magnified clustering result of enriched motifs shows 20 motif clusters obtained by manual manipulation (blue line). C, Enrichment of each cluster motif of significantly regulated phosphoproteins at each time point is represented as a matrix. Cells filled in red denote cluster motifs that significantly overlap with regulated phosphoproteins (p < 0.05).
Temporal Clustering and Mathematical Modeling Reveal a Response Network in Lung Cancer Cells With Citreoviridin Treatment—To investigate the dynamics of phosphorylation responding to citreoviridin, phosphosites were classified based on their expression changes. In the global analyses, distinguished patterns of protein phosphorylation were observed between early (10 s–2 h)- and late-phase (12–24 h) responses (supplemental Fig. S4). To survey the molecules immediately responding to citreoviridin, we next classified the temporal patterns of early-phase phosphorylation. By utilizing the unsupervised clustering (fuzzy c-means) technique, phosphorylation events were further categorized into six different patterns (Fig. 4A and supplemental Table S9). Upon the stimulation with citreoviridin, ~49.6% of phosphorylation events (clusters 1, 2, and 3) displayed rapid and sustained downregulation within 10 min. Most proteins involved in these groups are related to protein folding or belonged to cytoskeletal regulation within 10 min. Most proteins involved in these (clusters 1, 2, and 3) displayed rapid and sustained down-regulation in citreoviridin-treated lung cancer cells (Fig. 4A) and supplemental Table S9). Upon the stimulation with citreoviridin, ~49.6% of phosphorylation events (clusters 1, 2, and 3) displayed rapid and sustained down-regulation within 10 min. Most proteins involved in these groups are related to protein folding or belonged to cytoskeleton (Fig. 4A and supplemental Table S9). For example, heat shock protein 90 β (HSP90AB1; clusters 1 and 3), heat shock protein 105 kDa (HSPH1; cluster 1), and histone acetyltransferase p300 (EP300; cluster 3) are required for the chaperon complex function (52–54). On the other hand, lamin-B1 (LMNB1; cluster 1 and 2), inner nuclear membrane protein Man1 (LEMD3; cluster 1 and 3), and lamina-associated polypeptide 2 (TMPO; cluster 1 and 3) are important regulators during the development of intermediate filaments (55–57). These findings are consistent with our results of functional processes (supplemental Fig. S3B). In contrast, most phosphoproteins of clusters 4, 5, and 6 were transiently regulated. Numerous phosphosites categorized in these groups are associated with mRNA processing and translation initiation, implying a regulatory effect on gene transcription and translation in citreoviridin-treated lung cancer cells (Fig. 4A and supplemental Table S9).

Given that physiological changes upon stimulation depend on interrelated processes of molecular signaling, we performed mathematical modeling to construct the response network with citreoviridin treatment (Fig. 4B, supplemental Table S10 and Table S11). The phosphorylation levels of some of these proteins were validated by Western blotting (Fig. 5C and supplemental Fig. S5). From our inferred response network, 15 proteins of interest for signaling connectivity were examined. Among them, phosphorylation of the tumor suppressor nucleoplasm (NPM1) was found to either stimulate or inhibit phosphorylation of other proteins responsible for unfolded protein responses and protein stability, such as histone deacetylase 1 (HDAC1), EP300, and ubiquitin carboxyl-terminal hydrolase 2 (USP2) (58–60). Furthermore, the phosphorylation of transcriptional mediators including RNA polymerase-associated protein (LEO1) and EP300, might transcriptionally regulate kinases CDK2 and MAPK1. We also noticed that one adhesion molecule expressed on the cell surface, Down syndrome cell adhesion molecule (DSCAM), behaved as an upstream regulator for other molecules associated with protein folding, including hsc70-interacting protein (ST13), EP300, and HSP90AB1. Moreover, the phosphorylation of HSP90AB1, a controller for chaperone complexes and cell signaling (52, 61), could activate a downstream component of the MAPK/extracellular signal-regulated kinase (ERK) pathway, MAPK1 (62). MAPK/ERK signaling is known for its role in cell growth by regulating cell cycle, cell death and protein folding responses (62). Therefore, MAPK/ERK might participate in the response to citreoviridin to suppress tumor growth through the upstream phosphorylation regulation of other proteins, such as HSP90AB1 and ST13.

Citreoviridin Suppresses HSP90 Phosphorylation and MAPK/ERK Signaling—Our results from clustering and constructed response network displayed the temporal relation of phosphorylated HSP90 and MAPK1 in citreoviridin treatment. The time-dependent profiling data showed that Ser255 of HSP90AB1 had decreasing levels of phosphorylation under a short response time (Fig. 5A, 5C and supplemental Table S6). This site and its surrounding residues were also predicted to be a substrate for the CK2 family, which was overrepresented at 10 s in the motif-kinase analysis (Fig. 3C and supplemental Table S8). Interestingly, data on not only cultured cells, but also on xenografts show markedly decreased levels of phosphorylated Ser255 (supplemental Table S7), all suggesting the importance of site-specific regulation in HSP90 upon citreoviridin stimulation.

As previously reported, inhibition of HSP90 expression can lead to depression of some signaling pathways involved in cancer progression and cell growth, including the MAPK/ERK cascade (61, 63, 64). Notably, our temporal profile of phosphorylation revealed that phosphorylated MAPK1 Thr185/Yr187, a conserved TEY activation motif (62), was downregulated under slower response times (Fig. 5A, 5C and supplemental Table S6). To confirm the effect of citreoviridin on phosphorylated HSP90AB1 and MAPK/ERK signaling, changes in protein phosphorylation were validated. We showed that quantitation of both HSP90AB1 Ser255 and MAPK1 Thr185/Yr187 by Western blotting produced results similar to those obtained by MS-based phosphoproteomic approaches (Fig. 5B–5D). Moreover, upstream kinases of MAPK/ERK signaling such as RAF proto-oncogene serine/threonine-protein kinase (Raf-1) and dual specificity mitogen-activated protein kinase (MEK1/2) were all under decreased phosphorylation levels (Fig. 5E and 5F). To examine whether the observed phosphorylation regulation came from the changes of protein translation, we also measured and quantified the protein abundance by Western blotting. As shown in Fig. 5, the decrease of protein expression was found in MAPK1, Raf1, and MEK1/2 during the late responses (12–48 h) of citreoviridin treatment, though the changes in protein abundance was not as significant as in protein phosphorylation. The results indicate that the regulated MAPKs phosphorylation in the late response time might be partially affected by the changes of protein translation. We further detected the
expression of HSP90AB1 Ser255 and MAPK in xenograft tissues and in another lung cancer cell line (A549) (Fig. 6). Unsurprisingly, phosphorylation of both HSP90AB1 Ser255 and MAPK1 Thr185/Tyr187 were evidently suppressed in xenograft tissues (Fig. 6A and 6C). Similar to the results for the temporal phosphoproteome of CL1–0 cells (Fig. 5B and 5C), HSP90AB1 Ser255 and MAPK1 Thr185/Tyr187 showed their decreasing phosphorylation in A549 cells on treatment with citreoviridin in rapid (1 min–1 h) and slow (12 h–48 h) responses times (Fig. 6B and 6D). All of these results confirm the inhibition of HSP90AB1 phosphorylation and MAPK/ERK
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A

B

C

D

E

F

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signaling under citreoviridin treatment in both lung cancer cells and xenograft model.

The Phosphosite Ser\textsuperscript{255} of HSP90AB1 is Crucial for MAPK/ERK1 Signaling—In this study, we found that citreoviridin treatment affected phosphorylation instead of protein levels in HSP90AB1 (Fig. 5B and 5C), implying the regulatory importance of site-specific phosphorylation of HSP90AB1. Although phosphosite Ser\textsuperscript{255} has been identified in a previous study (36), the influence of HSP90 on signal transductions was only investigated at the total protein level (61, 63). No evidence regarding the effects of HSP90 phosphorylation on MAPK activation has been reported (65). To elucidate the relationship between HSP90 phosphorylation and MAPK/ERK signaling, wild-type HSP90AB1 and mutant HSP90AB1 with an alanine substitution of residue Ser\textsuperscript{255} were independently transfected into the lung cancer cell line CL1–0. Dephosphorylation of the S255A (serine changed to alanine) mutant and the expression level of the wild-type and control S255E (serine changed to glutamate) were confirmed by Western blotting (Fig. 7A and 7B). We found that overexpression of wild-type HSP90 enhanced phosphorylation levels of Ser\textsuperscript{255} and up-regulated MAPK/ERK cascade in comparison with levels in vector-only cells. In contrast, the S255A mutant of HSP90 produced much less effect on MAPK/ERK signaling proteins, highlighting the necessity of HSP90 Ser\textsuperscript{255} phosphorylation in regulating MAPK/ERK signaling. Because the important role of MAPK/ERK signaling cascade in cell growth, we further examined the impact of S255A mutant of HSP90AB1 on cell viability. As expected, cancer cells carrying the S255A mutant displayed lower viability compared with wild-type and S255E control CL1–0 and A549 cells (Fig. 7C). Together, our results suggest that phosphorylation of HSP90AB1 Ser\textsuperscript{255} might affect cancer cell growth through its modulation of MAPK/ERK signaling.
The influence of citreoviridin on cancer cell physiology has been increasingly examined. Here, we applied a temporal phosphoproteomic approach to profile the global changes of protein phosphorylation in cultured cells and xenograft tumor tissues for a comprehensive understanding of the influence of citreoviridin on molecular responses. Our study revealed that citreoviridin treatment resulted in significant changes of phosphorylation levels in numerous proteins. Furthermore, we observed that the cell cycle, protein folding, and mRNA processing might be regulated by citreoviridin-stimulated responses, consistent with previous reports (10, 18, 66). We also identified other processes that have not been reported to be related to citreoviridin treatment, such as cytoskeleton-associated function, development regulation, and ubiquitin-proteasomal proteolysis. These findings might expand our knowledge of biological processes in response to citreoviridin, but they require further validation.

In this study, we identified groups of molecules with similar temporal responses in citreoviridin treatment by analyzing the time-dependent phosphoproteome of cells. Motif analyses of the temporal profile further revealed that various kinase families such as CK2 and MAPK were individually over-represented at different time points. This study uncovered the differential regulation of kinases from early to late responses during citreoviridin treatment. The results were consistent with our network and functional analyses showing that MAPK1 was involved in citreoviridin treatment. The strategy using clustering of predicted motifs and enrichment analysis allowed us to clearly observe the changes in important kinase-substrate relationships on a temporal scale.

In our time-course data, the number of regulated phosphosites displaying decreasing phosphorylation levels was much higher than the number of elevated phosphorylated sites (Supplemental Table S6). This implies that citreoviridin treatment might globally suppress phosphorylation, eventually leading to changes in protein activities. Our analysis showed that protein folding might play an important role in the early-response stage of citreoviridin-treated cells. Moreover, a significant decrease in HSP90AB1 phosphorylation was found not only in cultured cells but also in the xenograft phosphoproteome, indicating its crucial role in citreoviridin treatment. It has been reported that cells treated with HSP90 inhibitor showed reduced activity in signaling pathways, including Akt and MAPK/ERK cascades (63, 64, 67). Our study showed that the phosphorylation level of one key component involved in MAPK/ERK signaling, MAPK1, was markedly diminished at late stages in citreoviridin-stimulated responses. However, other upstream kinases in the MAPK/ERK cascade were not identified in our phosphoproteome data. This difficulty was probably because of the much lower abundance of kinases compared with that of their substrates (68). We used Western...
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 blotting to overcome this problem and successfully detected down-regulation of other kinases involved in MAPK/ERK signaling. Furthermore, many biological processes enriched in both cell and xenograft phosphoproteomes (supplemental Fig. S3B), including protein folding and the cell cycle, are known to be regulated by MAPK/ERK signaling (62, 69, 70), highlighting the connection between citreoviridin treatment and MAPK/ERK cascade in cancer cells.

To understand whether the regulatory effects of HSP90AB1-Ser255 dephosphorylation and MAPK signaling inactivation were specific to citreoviridin, we also examined the HSP90AB1 and MAPK1 phosphorylation in lung cancer cells with two independent treatments. One was the chemotherapeutic agent sorafenib, a multiple kinase inhibitor (71). The other one was Tanshinone IIA, an anti-tumor agent that can induce reactive oxygen species (ROS) accumulation in lung cancer cells (72, 73). Both reagents could induce the inactivation of MAPKs/ERKs at early or late time points (supplemental Fig. S6). On the other side, the dephosphorylation of HSP90AB1 was only observed at the early time-points in sorafenib treatment, but not in Tanshinone IIA-treated cells. In consistence with our observation of citreoviridin phosphoproteome, the regulation of MAPK phosphorylation under both conditions was accompanied by the changes in protein abundance during late-response time, indicating the involvement of translational regulation. All these results suggest that the regulation of HSP90AB1-Ser255 and MAPK phosphorylation could be induced by other stresses and stimuli as well.

Although the effects of HSP90 on cell signaling and cellular functions such as cell proliferation and apoptosis have been revealed by many studies (63–65, 74), much less is known about the influence of site-specific phosphorylation of HSP90. We previously found that citreoviridin could prohibit cell proliferation in both cancer cell (10) and xenograft tissues (20). However, the lower level of phosphorylated HSP90 Ser255 and MAPK/ERK cascade attributable to citreoviridin had no significant effect on apoptosis in xenograft model (supplemental Fig. S7) and lung cancer cells (10). In this study, we uncovered the regulatory effects of the identified phosphosite Ser255 on cell signaling. Our results revealed that alanine substitution of Ser255 had less effect on MAPK/ERK signaling compared with wild-type HSP90AB1 (Fig. 7A and 7B). Moreover, cells over-expressing mutant HSP90AB1 S255A also displayed lower viability than that of wild-type HSP90AB1 (Fig. 7C). All of these results indicate a crucial role for HSP90AB1 Ser255 phosphorylation in the regulation of MAPK/ERK signaling.

There is increasing interest in the capability of HSP90 as a therapeutic target because of its housekeeping function, and HSP90 inhibitors have been regarded as potential drug candidates in cancer therapy (65). Here, we found that the citreoviridin treatment could suppress cell growth and MAPK/ERK signaling by HSP90AB1 regulation at the phosphorylation level. This result implies that ATP synthase inhibitors might warrant further investigation with their major role in targeting therapy in comparison with HSP90 inhibitors. However, to compressively understand the role of site-specific phosphorylation to the housekeeping functions and other signaling transductions of HSP90 in cells, additional analyses such as protein activity are required to define the impact of Ser255 phosphorylation on HSP90.

Although citreoviridin can inhibit the activity of various molecules, according to our previous work, it might suppress the cancer growth by mainly interacting with ecto-ATP synthase of cancer cells (10). Because of the enzyme’s F₆ catalytic domain facing the extracellular space, ecto-ATP synthase affects ATP levels outside the cell (75). Furthermore, localization on the cell surface enables ecto-ATP synthase to function as a receptor for cell signaling; thus, the enzyme has widespread effects on cellular processes, including cell proliferation, cholesterol metabolism, and tumor progression (12, 76–78). Therefore, understanding the molecular mechanisms underlying the physiological effects of ecto-ATP synthase is important in cancer research, and we speculated that MAPK/ERK signaling by HSP90AB1 regulation might be one of the downstream of ecto-ATP synthase induced pathway.

Together, our data provide a global profile of protein phosphorylation changes stimulated by citreoviridin in both cancer cells and xenograft systems. We found that phosphorylation of Ser255 in HSP90AB1 might be an important regulatory factor in response to citreoviridin treatment. Site-specific phosphorylation of HSP90AB1 might affect cell viability through regulation of MAPK/ERK signaling. This investigation not only expands our understanding of the influence of citreoviridin on cancer cell growth at the post-translational level, but also provides new information for the development of targeting therapy for cancer.

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[5] This article contains supplemental Figs. S1 to S7, Tables S1 to S12, and Methods.

REFERENCES

1. Linnett, P. E., Mitchell, A. D., Osselton, M. D., Mulheirn, L. J., and Beechey, R. B. (1978) Citreoviridin, a specific inhibitor of the mitochondrial adenine-nucleotide triphosphatase. Biochem. J. 170, 503–510
2. Gause, E. M., Buck, M. A., and Douglas, M. G. (1981) Binding of citreoviridin to the beta subunit of the yeast F1-ATPase. J. Biol. Chem. 256,
21. Hunter, T. (1995) Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. Cell 80, 225–236

22. Macek, B., Mann, M., and Olsen, J. V. (2009) Global and site-specific quantitative phosphoproteomics: principles and applications. Annu. Rev. Pharmacol. Toxicol. 49, 199–221

23. Karisch, R., Fernandez, M., Taylor, P., Virtanen, C., St-Germain, Jonathan R., Jin, Lily L., Harris, Isaac S., Mori, J., Mak, Tak W., Senis, Yotis A., Ostman, A., Moran, Michael F., and Neel, Benjamin G. (2011) Global proteomic assessment of the classical protein-tyrosine phosphatase and redoxome Cell 146, 826–840

24. Harsha, H. C., and Pandey, A. (2010) Phosphoproteomics in cancer. Mol. Oncol. 4, 482–495

25. Palumbo, A. M., Smith, S. A., Kalcic, C. L., Dantus, M., Stemmer, P. M., and Reid, G. E. (2011) Tandem mass spectrometry strategies for phospho-protein quantification. Nat. Biotechnol. 29, 1367–1372

26. Lim, Y. P. (2005) Mining the tumor phosphoproteome for cancer markers. Clin. Cancer Res. 11, 3163–3169

27. Masuda, T., Tomita, M., and Ishihama, Y. (2008) Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. J. Proteome Res. 7, 731–740

28. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for multiparameter enrichment, purification and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906

29. Boersema, P. J., Rajmakers, R., Lemeer, S., Mohammed, S., and Heck, A. J. (2009) Multiplex peptide stable isotope dimethyl labeling for quantitive proteomics. Nat. Protoc. 4, 484–494

30. Kyono, Y., Sugiyama, N., Imari, K., Tomita, M., and Ishihama, Y. (2008) Successive and selective release of phosphorylated peptides captured by hydroxy acid-modified metal oxide chromatography. J. Proteome Res. 7, 4585–4593

31. Kyono, N., Masuda, T., Shinoda, K., Nakamura, A., Tomita, M., and Ishihama, Y. (2007) Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. Mol. Cell. Proteomics 6, 1103–1109

32. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide dynamic range. Nat. Biotechnol. 26, 396–400

33. Cox, J., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648

34. Schwartz, D., and Gygi, S. P. (2005) An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. Nat. Biotechnol. 23, 1391–1398

35. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for multiparameter enrichment, purification and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906

36. Reid, G. E. (2011) Tandem mass spectrometry strategies for phospho-protein quantification. Nat. Biotechnol. 29, 1367–1372

37. Olsen, J. V., Blagoev, B., Gnädig, F., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648

38. Schwarz, D., and Gygi, S. P. (2005) An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. Nat. Biotechnol. 23, 1391–1398

39. Hu, J., Rho, H. S., Newman, R. H., Zhang, J., Zhu, H., and Qian, J. (2014) PhosphoNetworks: a database for human phosphorylation networks. Bioinformatics 30, 141–142

40. Hu, H.-M., Tien, Y.-J., and Chen, C.-H. (2010) GAP: A graphical environment for matrix visualization and cluster analysis. Comput. Stat. Data Anal. 54, 767–776

41. Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M. L., Jensen, L. J., Gnad, F., Cox, J., Jensen, T. S., Nigg, E. A., Brunak, S., and Mann, M. (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. Sci. Signal 3, ra3

42. Johansson, R. (1993) System modeling and identification. Prentice Hall, Englewood Cliffs, NJ

43. Wang, Y. C., and Chen, B. S. (2010) Integrated cellular network of transcription regulation and protein–protein interactions. BMC Syst Biol. 4, 20

44. Akaike, H. (1974) A new look at the statistical model identification. IEEE Transactions on Automatic Control 19, 716–723

45. Vizcaíno, J. A., Cote, R. G., Csfardas, A., Dianes, J. A., Fabregat, A., Foster, J. M., Griss, J., Alpi, E., Birim, M., Contelli, J., K’Oelly, G., Schoenegger,
Dynamic Phosphoproteomics of Citreoviridin Inhibition

A. Ovelleiro, D. Perez-Riverol, Y. Reisinger, F. Rios, D. Wang, R., and Hermjakob, H. (2013) The PRoteomics IDENTifications (PRIDE) database and associated tools: status in 2013. *Nucleic Acids Res.* 41, D1063–1069

45. Nakagami, H., Sugiyama, N., Mochida, K., Daidi, A., Yoshida, Y., Toyoda, T., Tomita, M., Ishihama, Y., and Shirasu, K. (2010) Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants. *Plant Physiol.* 153, 1161–1174

46. Hu, C. W., Lin, M. H., Huang, H. C., Ku, W. C., Yi, T. H., Tsai, C. F., Chen, Y. J., Sugiyama, N., Ishihama, Y., Juan, H. F., and Wu, S. H. (2012) Phosphoproteomic analysis of *Rhodopseudomonas palustris* reveals the role of pyruvate phosphate dikinase phosphorylation in lipid production. *J. Proteome Res.* 11, 5362–5375

47. Nikolsky, Y., Nikolskaya, T., and Bugrim, A. (2005) Biological networks and analysis of experimental data in drug discovery. *Drug Discov. Today* 10, 653–663

48. Wang, Y., Kelber, J. A., Cao, H. S. T., Cantin, G. T., Chen, Y. J., Sugiyama, N., Ishihama, Y., Juan, H. F., and Wu, S. H. (2012) Pseudopodium-enriched atypical kinase 1 regulates the cytoskeleton and cancer progression. *Proc. Natl. Acad. Sci. U.S.A.* 107, 10920–10925

49. Cabodi, S., del Pilar Camacho-Leal, M., Di Stefano, P., and Dellafi, P. (2010) Integrin signalling adaptors: not only figurants in the cancer story. *Nat. Rev. Cancer* 10, 858–870

50. Ikushima, H., and Miyazono, K. (2010) TGFβ signalling: a complex web in cancer progression. *Nat. Rev. Cancer* 10, 415–424

51. Radpour, R., Barekati, Z., Hightower, L. E., and Lee, J. (2010) HSPB1, actin filament dynamics, and aging cells. *Ann. N.Y. Acad. Sci.* 1197, 76–84

52. Jakob, U., and Buchner, J. (1994) Assisting spontaneity: the role of Hsp90 small Hsps as molecular chaperones. *Trends Biochem. Sci.* 19, 205–211

53. Doshi, B. M., Hightower, L. E., and Lee, J. (2010) HSPB1, actin filament dynamics, and aging cells. *Ann. N.Y. Acad. Sci.* 1197, 76–84

54. Ito, T., Ikehara, T., Nakagawa, T., Kraus, W. L., and Muramatsu, M. (2000) p300-mediated acetylation facilitates the transfer of histone H2A–H2B dimers from nucleosomes to a histone chaperone. *Genes Dev.* 14, 1899–1907

55. Wilson, K. L., and Foisner, R. (2010) Lamin-binding Proteins. *Cold Spring Harb Perspect Biol.* 2, a000554

56. Warner, H. J., and Bonne, G. (2007) “Laminopathies”: a wide spectrum of human diseases. *Exp. Cell Res.* 313, 2121–2133

57. Dechat, T., Vlicek, S., and Foisner, R. (2000) Review: Lamina-associated polypeptide 2 isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. *J. Structural Biol.* 129, 335–345

58. Wang, F. M., Chen, Y. J., and Ouyang, H. J. (2011) Regulation of unfolded protein response modulator XBPs1 by acetylation and deacetylation. *Biochem. J.* 433, 245–250

59. Khan, O., and La Thangue, N. B. (2012) HDAC inhibitors in cancer biology: emerging mechanisms and clinical applications. *Immunol. Cell Biol.* 90, 85–94

60. Graner, E., Tang, D., Rossi, S., Baron, A., Migita, T., Weinstein, L. J., Lechpammer, M., Huesken, D., Zimmermann, J., Signoretti, S., and Loda, M. (2004) The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. *Cancer Cell* 5, 253–261

61. Scaltriti, M., Dawood, S., and Cortes, J. (2012) Molecular pathways: targeting hsp90—who benefits and who does not. *Clin. Cancer Res.* 18, 4508–4513

62. Morrison, D. K. (2012) MAP kinase pathways. *Cold Spring Harb Perspect Biol.* 4, a011254

63. Travers, J., Sharp, S., and Workman, P. (2012) HSP90 inhibition: two-pronged exploration of cancer dependencies. *Drug Discov. Today* 17, 242–252