Crosstalk among the proteome, lysine phosphorylation, and acetylation in romidepsin-treated colon cancer cells

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ABSTRACT

Romidepsin (FK228) is one of the most promising histone-deacetylase inhibitors due to its potent antitumor activity, and has been used as a practical option for cancer therapy. However, FK228-induced changes in protein modifications and the crosstalk between different modifications has not been reported. To better understand the underlying mechanisms of FK228-related cancer therapy, we investigated the acetylome, phosphorylation, and crosstalk between modification datasets in colon cancer cells treated with FK228 by using stable-isotope labeling with amino acids in cell culture and affinity enrichment, followed by high-resolution liquid chromatography tandem mass spectrometry analysis. In total, 2728 protein groups, 1175 lysine-acetylation sites, and 4119 lysine-phosphorylation sites were quantified. When the quantification ratio thresholds were set to > 2.0 and < 0.5, respectively, a total of 115 and 38 lysine-acetylation sites in 85 and 32 proteins were quantified as increased and decreased targets, respectively, and 889 and 370 lysine-phosphorylation sites in 599 and 289 proteins were quantified as increased and decreased targets, respectively. Furthermore, we identified 274 proteins exhibiting both acetylation and phosphorylation modifications. These findings indicated possible involvement of these proteins in FK228-related treatment of colon cancer, and provided insight for further analysis of their biological function.

INTRODUCTION

Aberrant transcriptional repression of genes regulating cell growth and differentiation is a hallmark of cancer [1]. Altered activation of histone deacetylases (HDACs) is a key mechanism underlying transcriptional repression in malignancies [2], and in solid tumors, including colon cancer and malignant melanoma, HDAC overexpression is believed to similarly contribute to oncogenesis [3]. Accordingly, the use of small compounds that inhibit HDAC activity has become a novel strategy for cancer treatment [4, 5]. HDAC inhibitors (HDACis) are able to restore the expression of aberrantly suppressed genes capable of inhibiting tumor cell growth and/

or survival [6, 7]. Previously investigated HDACis include suberoylanilide hydroxamic acid (SAHA) [8, 9], depsipeptide (romidepsin), sodium phenylbutyrate [10, 11], and MS-275 [12, 13].

Romidepsin (FK228) is a novel HDACi that was approved by the United States Food and Drug Administration for treatment of cutaneous T cell lymphoma in 2009 and peripheral T cell lymphoma in 2011 [14, 15]. Its activities were also confirmed against other solid-tumor cancers, such as non-Hodgkin's lymphoma [16, 17], lung cancer [18, 19], breast cancer [20, 21], and colon cancer [22, 23]. FK228 exerts an antitumor effect through histone hyperacetylation and the subsequent transcription of genes that inhibit tumor growth [24, 25].

Although FK228 is already used in clinical trials, its mechanism of action remains largely undefined. Protein post-translational modifications (PTMs) function as highly versatile switches that regulate protein activity, concentration, subcellular localization, and maintain homeostasis. Chen et al. [26] performed a differential proteomic analysis to identify proteins associated with FK228-induced cytotoxicity in human lung cancer cells, identifying 27 proteins involved in signal transduction, transcription regulation, metabolism, and cytoskeletal organization. Wu et al. [27, 28] found that acetylation and ubiquitination sites changed in response to SAHA treatment, and that 55 common sites were capable of being both acetylated and ubiquitinated, with ubiquitination levels in 43 sites (78.2%) correlated with acetylation levels. Additionally, FK228-induced histone hyperacetylation in lung carcinoma cells was associated with increases in p21 and hypophosphorylated retinoblastoma protein expression [29].

To expand on these findings and reveal relationships between phosphorylation and the acetylome in response to FK228 treatment, we performed stable isotope labeling by amino acids (SILAC), affinity enrichment by antibodies, and high-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) to quantitatively compare the proteome, global phosphorylation levels, and the acetylome of HCT-8 and HCT-116 colon cancer cells before and after FK228 treatment. Additionally, the crosstalk between phosphorylation events and the acetylome was also analyzed in order to strengthen our understanding of FK228-dependent cancer therapy.
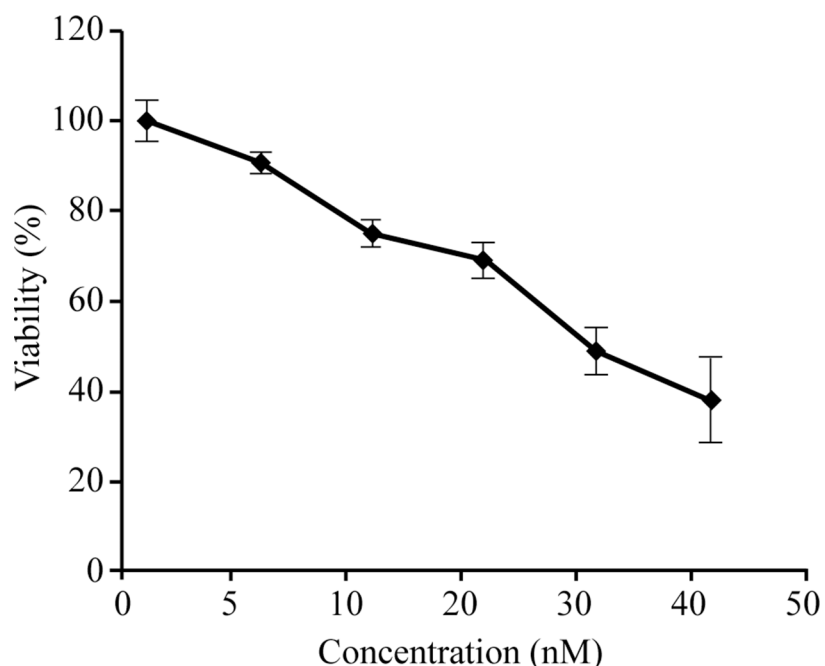
RESULTS

Cell cytotoxicity assay

Previous reports indicated that 50% of the HCT-8 cells were viable (IC_{50}) at FK228 concentrations of 29.46 nM [30]; therefore, this dosage was used for the 18-h treatment period in all subsequent experiments. In this study, we performed cytotoxicity assays on HCT-116 cells using varying concentrations of FK228. Our results demonstrated a dose-dependent response, whereby the viability of cultured HCT-116 cells decreased in response to increasing FK228 concentrations (Figure 1). Notably, ~50% of the cells were viable (IC_{50}) at a FK228 concentration of 26.86 nM, which was similar to the previous study [31]. Therefore, this dosage was used for the 18-h treatment period for HCT-116 cells in all of the subsequent experiments.

Proteome profile changes following FK228 treatment

Quantitative lysine-acetylome and global-phosphorylation analyses were performed in triplicate on HCT-8 and HCT-116 cell lines using SILAC and affinity enrichment, followed by high-resolution LC-MS/MS analysis. In total, 4910 protein groups were identified, with 4860 of them exhibiting differential regulation patterns between FK288-treated and -untreated cells, including 3940 and 3698 protein groups and 3986 and 3551 differential

Figure 1: Establishment of the appropriate FK228 concentration in HCT-116 cells. HCT-116 cells were cultured, and cell-proliferation and cytotoxicity assays were performed. The FK228 concentration resulting in 50% cell viability (IC_{50}) was used as the working concentration.

regulation patterns in HCT-8 and HCT-116 cells, respectively. We identified 2728 common protein groups and found that 2677 of them exhibited differential regulation patterns between FK288-treated and -untreated cells in the two cell lines (Figure 2A and 2B). After setting quantification ratios to > 2.0 and < 0.5 representing the increased- and decreased-regulation thresholds, respectively, among the 2677 common quantifiable proteins, 684 were classified as targets of increased regulation and 321 as targets of decreased regulation. Within these targets, 889 phosphorylation sites in 599 proteins were identified as exhibiting increased phosphorylation and 370 sites in 289 proteins were identified as exhibiting decreased phosphorylation, while 115 lysine-acetylation sites in 85 proteins were identified exhibiting increased acetylation and 38 sites in 32 proteins were identified as exhibiting decreased acetylation. These data are listed in Supplementary Table S1.

Alterations of phosphorylation profiles in FK228-treated cells

Phosphorylation involves the addition of a phosphate (PO_4^{3-}) group to a protein or other organic molecule and is among the most important PTMs. Phosphorylation stimulates the active state of many enzymes, thereby altering their function. Additionally, phosphorylation is involved in a wide range of cellular processes, including cell signaling, immune-system modulation, and tumor suppression [32–34].

In this study, we combined SILAC and affinity enrichment, followed by high-resolution LC-MS/MS analysis, for quantitative phosphoproteomics analysis in HCT-8 and HCT-116 cells with or without FK228 treatment.

Altogether, 7601 phosphorylation sites in 3441 proteins were identified as differing between treated

and untreated cells, resulting in quantification of 6178 sites in 2316 proteins. We found that 4119 common phosphorylation sites in 1930 proteins differed between treated and untreated HCT-8 and HCT-116 cell lines (Figure 3A and 3B). After setting quantification ratios to > 2.0 and < 0.5 as representing thresholds of increased and decreased phosphorylation events, respectively, 889 phosphorylation sites in 599 proteins were identified as targets exhibiting increased phosphorylation and 370 sites in 289 proteins were identified as targets exhibiting decreased phosphorylation. All data are listed in Supplementary Table S2.

GO analysis and protein domains involved in phosphorylation

According to the functional differences observed between increased and decreased phosphorylation events, we analyzed the proteins with quantified phosphorylation sites using GO enrichment-based clustering (Figure 4A−4C). Using the same threshold significance ratios, we performed these clustering analyses by dividing all proteins exhibiting statistically significant alterations into four quantiles (Q1−Q4) according to the L/H ratios (Q1: < 0.50, Q2: 0.50−0.67, Q3: 1.50−2.0, Q4: > 2.0) in order to determine the biological functions of the proteins exhibiting large (> 2.0 or < 0.50) or relatively small changes in ratios (1.5−2.0 or 0.50−0.67) in response to FK228 treatment.

The results of our analyses of the biological processes associated with phosphorylation are shown in Figure 4A. These results indicated that the proteins exhibiting decreased lysine phosphorylation in the Q1 quantile were highly enriched for cell division and cell cycle functions and regulation of transmembrane transport. Proteins with functions involving antigen processing and presentation, response to toxic substances, lipid
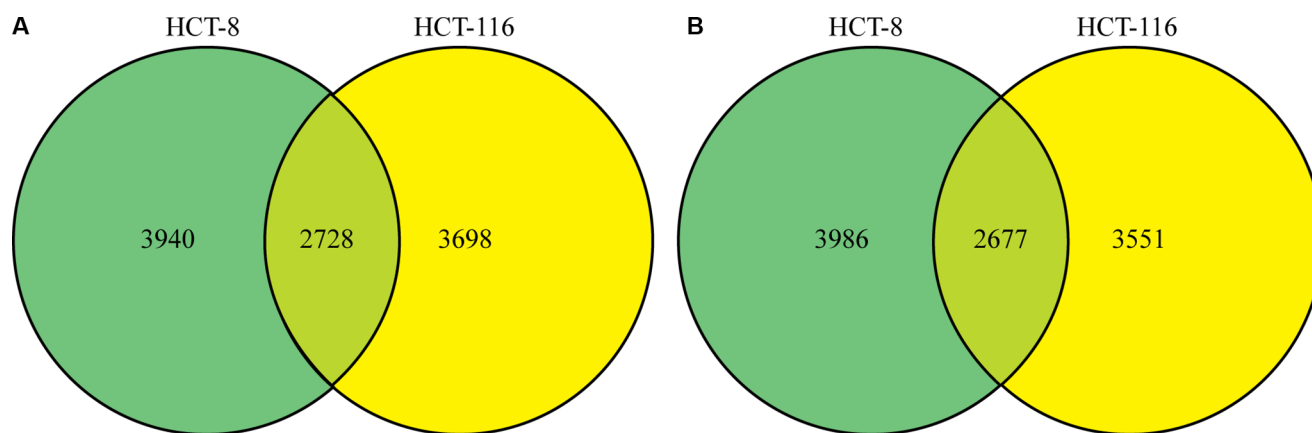
Figure 2: Changes in the proteome profile following FK228 treatment between HCT-8 and HCT-116 cells. Quantitative lysine-acetylome and global-phosphorylation analyses were performed in HCT-8 and HCT-116 cells using SILAC and affinity enrichment, followed by high-resolution LC-MS/MS analysis. Total and differentially modified proteins and regulation patterns were identified. (**A**) Differentially regulated protein groups between the two cell lines. (**B**) Differential regulation patterns between the two cell lines.

localization, lipid transport, blood circulation, circulatory system processes, macromolecular methylation, and microtubule-based movement were segregated into the Q2 quantile. The proteins exhibiting increased lysine phosphorylation were found to be involved in many biological process, such as female gamete generation, protein localization to the adherens junction, and some response proteins, including those involved in responses to alcohol, drugs, hepatocyte growth-factor stimulus, and indole-3-methanol. There were some proteins involved in regulatory functions, such as regulation of epithelial cell differentiation, cellular-component size, and actin filament-based movement.

The cellular-component analysis revealed that proteins in Q1 and Q2 exhibiting decreased phosphorylation events were preferentially located at chromosomal regions, such as the contractile ring and kinetochore, as well as other areas, including the myelin sheath and postsynaptic regions. These proteins were classified as brush border and nuclear inner-membrane proteins (Figure 4B). In contrast, the proteins exhibiting increased phosphorylation events were constituents of nuclear and cell organelles, such as the nuclear inner membrane, nuclear euchromatin and heterochromatin, rDNA heterochromatin, and the mitochondrial membrane. Some proteins were involved in kinase complexes, such as the AMP-activated protein kinase complex and histone deacetylase complex.

We also analyzed the molecular functions associated with phosphorylation events (Figure 4C). The proteins exhibiting decreased phosphorylation were highly enriched in phosphorylation-related processes, including binding proteins, such as ribosomal small-subunit binding, ankyrin binding, Y-form DNA binding, damaged-DNA binding, and 1-phosphatidylinositol binding. Methyltransferase activity was also enriched in phosphorylation-related processes, such as lysine *N*-methyltransferase activity and histone methyltransferase activity. Moreover, other proteins were involved as structural constituents of muscle and channel-regulator activity. Furthermore, proteins exhibiting decreased phosphorylation levels were also involved in RNA transcription and translation, such as DNA-directed RNA polymerase activity, RNA polymerase activity, DNA helicase activity, DNA-directed DNA polymerase activity, Rab guanyl-nucleotide-exchange factor activity, and ATPase capacity. Proteins exhibiting increased phosphorylation levels were enriched in nucleic acid binding, receptor-regulator activity, NAD-dependent enzyme activity, hydrolase activity, and ligase activity.

Following protein-domain analysis, we observed that Q1 was enriched with proteins having ribosomal protein domains, domains containing a sterile alpha motif, homeodomain-like structures, LEM-like domains, a rabaptin-like structure, a SET domain, a post-SET domain, NOP domains, and thioredoxin domains. Furthermore, some disease-associated domains were also observed, including retinoblastoma-associated domains and LisH-dimerization motifs, whereas Q2 was enriched with proteins containing lambda repressor-like domains, ABC-transporter domains, and guanylate-kinase domains. In contrast, Q3 was enriched with proteins exhibiting increased phosphorylation and having Kelch-type beta-propeller domains and dehydrogenase domains, while Q4 was enriched with cadherin domains, SAND domain-like structures, MAD homology-1 domains, CTF transcription factor/nuclear factor domains, and NET domains (Figure 4D).

Protein-complex and KEGG-pathway analysis of quantitatively altered phosphorylated proteins

Protein-complex and KEGG-pathway analyses of quantitatively altered phosphorylated proteins were also performed. Protein-complex analysis of the quantitatively altered phosphorylated proteins revealed that Q1 was enriched with proteins involved in the DNA-synthesis
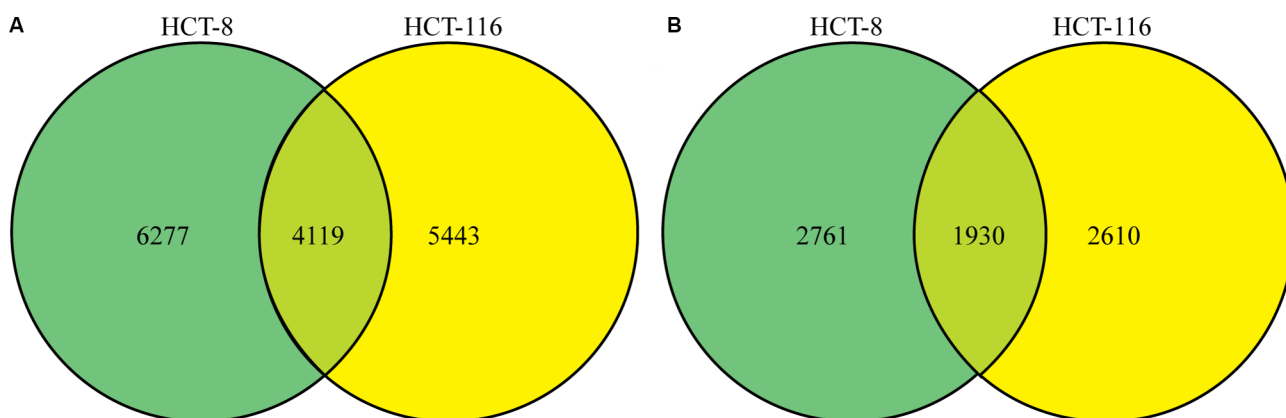
Figure 3: Phosphorylation profile changes following FK228 treatment of HCT-8 and HCT-116 cells. SILAC, affinity enrichment, and high-resolution LC-MS/MS analysis was used for quantitative phosphoproteomics analysis of HCT-8 and HCT-116 cells following FK228 treatment. Total and differentially phosphorylated sites and phosphorylated proteins were assessed. (**A**) Sites exhibiting differential phosphorylation patterns in the two cell lines. (**B**) Differentially phosphorylated proteins between the two cell lines.

A Biological process

Decreased fold change		Increased fold chang	
(>2)	(1.5~2)	(1.5~2)	(>2)

Cellular respiration
Antigen processing and presentation of exogenous peptide antigen
Antigen processing and presentation of peptide or polysaccharide antigen
Antigen processing and presentation of peptide antigen via MHC class
Response to toxic substance
Lipid localization
Lipid transport
Circulatory system process
Antigen processing and presentation of exogenous peptide antigen
Macromolecule methy lation
Microtubule-based movement
Methy lation
Chromosome localization
Sister chromatid segregation
Ribosome biogenesis
Cell cycle checkpoint
Regeneration
Nuclear division
Cytokinesis
Organelle fission
Regulation of transmembrane transport
Response to osmotic stress
Chromosome segregation
Regulation of cell cycle process
Ribonucleoprotein complex biogenesis
Hemophilic cell adhesion
Negative regulation of cellular response to growth factor stimulus
Epithelium migration
Regulation of epidermis development
Cellular carbohydrate metabolic process
Female gamete generation
Regulation of response to alcohol
Protein localization to cell junction
Protein localization to adherens junction
Response to hepatocyte growth factor stimulus
Regulation of cellular component size
Response to alcohol
Regulation of glucose transport
Positive chemotaxis
Desmosome organization
Response to tumor necrosis factor
Regulation of kidney development
Regulation of anatomical structure size
Positive regulation of reproductive process
Regulation of chromosome organization
Regulation of carbohydrate metabolic process
Negative regulation of homeostatic process
Regulation of vasculature development
Positive regulation of cell adhesion
Regulation of peptide transport
Positive regulation of membrane potential
Carbohydrate homeostasis
Response to lipid
Regulation of morphogenesis of a branching structure
Regulation of homeostatic process
Regulation of membrane depolarization
Regulation of ion homeostasis
Regulation of stem cell proliferation
Cellular response to drug
Regulation of dense core granule biogenesis
Positive regulation of organelle organization
Response to indole-3-methanol
Regulation of epithelial cell differentiation
Regulation of cellular ketone metabolic process
Mesenchymal cell proliferation
Positive regulation of cellular component movement
Regulation of muscle system process
Adherens junction organization
Response to steroid hormoe stimulus
Regulation of epithelial cell proliferation
Positive regulation of locomotion
Actin filament-based movement

B Cellular component

Decreased fold change — Increased fold chang

(>2)	(1.5~2)	(1.5~2)	(>2)	
				Nuclear heterochromatin
				Postsynaptic density
				Neuron projection terminus
				PRC1 complex
				Sin3-type complex
				Sin3 complex
				Terminal bouton
				AMP-activated protein kinase complex
				PcG protein complex
				NuRD complex
				CHD-type complex
				Axon terminus
				Histone deacetylase complex
				Mitochondrial membrane part
				Contractile ring
				Chromosome, centromeric region
				Microtubule associated complex
				Kinetochore
				Condensed chromosome
				Condensed chromosome, centromeric region
				Condensed chromosome kinetochore
				Myelin sheath
				Condensed nuclear chromosome
				Main axon
				Centriolar satellite
				Basement membrane
				Replication fork
				Sarcolemma
				Sarcoplasm
				Synaptonemal combrane
				Cell cortex part
				Postsynaptic membrane
				Paraspeckles
				Brush border
				Intermediate filament
				Heterochromatin
				Telomeric heterochromatin
				Centralspindlin complex
				Mitotic spindle
				Lamin filament
				Nuclear lamina
				Intrinsic to golgi membrane
				Integral to golgi membrane
				Gap junction
				Nuclear inner membrane
				Perikaryon
				Chromatin silencing complex
				RSF complex
				rDNA heterochromatin
				Desmosome
				Intercalated disc
				Nuclear cyclin-dependent protein kinase holoenzyme complex
				Nuclear pore nuclear basket
				Scrib-APC-beta-catenin complex
				Ribonucleoprotein granule
				Nuclear euchromatin
				Catenin-TCF7L2 complex
				Fascia adherens
				Nucleal cell body
				Cell-cell contact zone
				Dendrite

c
Molecular function

Decreased fold change   Increased fold chang

(>2)   (1.5~2)   (1.5~2)   (>2)

Microtubule motor activity
Ribosomal small subunit binding
Ankyrin binding
Lysine n-methyltransferase activity
Protein methyltransferase activity
Histong-lysine n-methyltransferase activity
Protein-lysine n-methyltransferase activity
S-adenosylmethionine-dependent methyltransferase activity
Demaged dna binding
N-methyltransferase activity
1-phosphatidylinositol binding
Structural constituent of muscle
Channel regulator activity
Y-form dna binding
DNA helicase activity
DNA-directed dna polymerase activity
Cyclic-nucleotide phosphodiesterase activity
Potassium: chloride symporter activity
Solute: cation symporter activity
Rab guany1-nucleotide exchange factor activity
Tubulin binding
Alpha-tubulin binding
DNA-directed RNA polymerase activity
RNA polymerase activity
Cholesterol binding
Serine hydrolase activity
ATPase activity, coupled to transmembrane movement of substances
P-P-bond-hydrolysis-driven transmembrane transporter activity
Hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
ATPase activity, coupled to movement of substances
Microtubule binding
Serine-type peptidase activity
Primary activity transmembrane transporter activity
Sterol binding
Nucleocytoplasmic transporter activity
Double-stranded RNA bingding
Ribonucleoprotein complex bingding
Ras GTPase bingding
Protein phosphatase 2A bingding
Ligand-dependent nuclear receptor bingding
Oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor
Lamin binding
Receptor regulator activity
Receptor activator activity
Ligand-dependent nuclear receptor transcription coactivator activity
NAD-dependent protein deacetylase activity
Histone deacetylase activity (H3-K9 specific)
NAD-dependent histone deacetylase activity
NAD-dependent histone deacetylase activity (H3-K9 specific)
Ran guanyl-nucleotide exchange factor activity
Alkali metal ion binding
Histone deacetylase binding
Potassium ion binding
Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
Core promoter proximal region DNA binding
Aminoacy1-tRNA ligase activity
Core promoter proximal region sequence-specific DNA binding
Ligase activity, forming carbon-oxygen bonds
Chromatin DNA binding
Ligase activity, forming aminoacy1-tRNA and related compounds

Figure 4: Functional enrichment-based clustering analysis for the quantified phosphorylated proteins. According to the functional differences observed between increased and decreased proteins, GO-term association and enrichment analysis using the DAVID program (https://david.ncifcrf.gov/) were performed. (**A**) Biological process analysis. (**B**) Cellular component analysis. (**C**) Molecular function analysis. (**D**) Protein domain analysis.

and -repair core complex, while Q2 was enriched with proteins involved in the nucleolar-remodeling and MDC1 complexes. The proteins exhibiting increased phosphorylation events were mainly included in the large Drosha complex, MLL-HCF complex, the MecP1 complex in the Q3 quantile, while proteins involved in the ctBP complex, SAP complex, and ING2 complex were found to be enriched in Q4 quantile (Figure 5A).

We also performed KEGG-pathway analysis of the quantitatively altered proteins, with the results showing that pathways involved in ribosome biogenesis in eukaryotes, cell cycle, DNA replication, drug metabolism, nucleotide-excision repair, and RNA polymerase activity were highly represented in Q1- and Q2-quantile proteins exhibiting decreased lysine-phosphorylation events, while proteins exhibiting increased lysine-ubiquitination events were involved in cancer misregulation and digestive-system functions, such as gastric acid and salivary secretion (Figure 5B). These results suggested that proteins exhibiting increased phosphorylation levels following FK228 stimulation were highly associated with cell signaling activity and disease progression.

Protein-protein interaction networks associate with differentially phosphorylated proteins

Protein-interaction networks can serve as an alternative strategy for analysis of physical and functional interactions [35]. The protein-protein interaction networks associated with the differentially phosphorylated proteins were analyzed, with the results showing that proteins exhibiting increased lysine phosphorylation following FK228 treatment were highly enriched in the spliceosome. In contrast, proteins exhibiting decreases in lysine phosphorylation in response to FK228 treatment were involved in the mitotic cell cycle, transcription, DNA-template activity, Poly(A) RNA binding, protein polyubiquitination, and the MLL1 complex (Figure 6A). In the spliceosome-related phosphorylated-protein-interaction network, there were 16 sites exhibiting increases in modifications in response to FK228 treatment, two sites exhibiting decreased modification, and two sites exhibiting both increased and decreased modifications (Figure 6B). In contrast, dysregulated modification sites associated with the mitotic cell cycle-related phosphorylated-protein-interaction network included 16 sites exhibiting decreased modifications and 16 sites exhibiting increased modifications, with only two sites exhibiting both increased and decreased modifications (Figure 6C). The phosphorylated-protein-interaction networks associated with DNA-template-related transcription involved 20 sites exhibiting increased modifications and five sites exhibiting decreased modifications, five sites exhibiting both increased and decreased modifications (Figure 6D). The phosphorylated-protein-interaction networks associated

with poly(A) RNA binding contained seven sites exhibiting increased modifications, two sites exhibiting decreased modifications, and one site exhibiting both increased and decreased modifications (Figure 6E). The phosphorylated-protein-interaction networks associated with polyubiquitination- and MLL1-complexes included 11 sites exhibiting increased modifications, eight sites exhibiting decreased modifications, and one site exhibiting both increased and decreased modifications (Figure 6F). All data are listed in Supplementary Table S3.

Acetylome profile alterations following FK228 treatment

We investigated the acetylation levels of non-histone proteins in response to FK228 treatment using SILAC, Kac-antibody enrichment, and LC-MS/MS analysis. A total of 1289 Kac sites corresponding to 756 proteins were identified from HCT-8 and HCT-116 cells, and 1175 sites in 747 proteins were quantified (Supplementary Table S4).To the best of our knowledge, this is the first comprehensive profiling of Kac data following FK228 treatment in HCT-8 and HCT-116 cells.

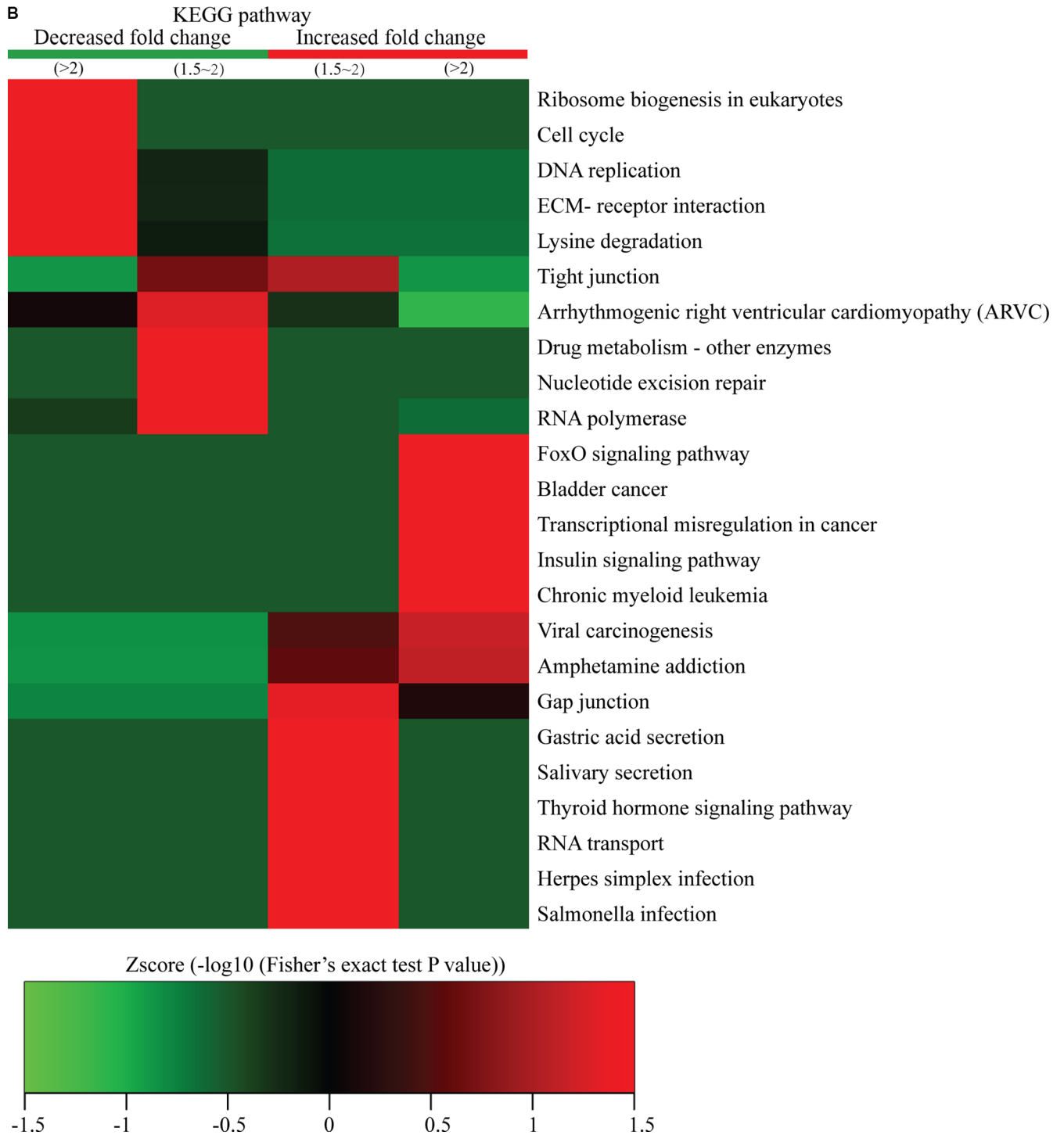## GO analysis and protein domains associated with quantitatively altered acetylated proteins

We performed enrichment-based clustering analyses to compare the functions of identified proteins exhibiting increased and decreased acetylation levels (Figure 5A–5F). For clustering analysis, all the quantified acetylation sites were also divided into four quantiles (Q1–Q4) according to L/H ratios as previously described.

The results of our analyses regarding the biological processes associated with acetylation are shown in Figure 7A. Proteins exhibiting decreased lysine acetylation were involved in the regulation of metabolic processes, including positive regulation of homeostatic processes, cholesterol regulation, ossification regulation, and response to organic substances, indicating that FK228 treatment can influence protein metabolism. Additionally, the functions of proteins exhibiting decreased Kac were also focused on cell signaling-transduction mechanisms, such as signaling, single-organism signaling, programmed cell death, extracellular matrix organization, and extracellular structure organization. Conversely, the proteins exhibiting increased Kac sites were involved in response to stress and regulation of organismal processes, including carbohydrate catabolic processes, antigen processing, presentation of peptide antigens, reproductive processes, and mitotic cell cycle regulation.

Cellular-component analysis showed that some proteins located on the membrane, such as Acyl-CoA *N*-acyltransferase, histidine kinase, and DNA topoisomerase, were highly enriched in Q1, while homeodomain-like domain-containing proteins, helicases,
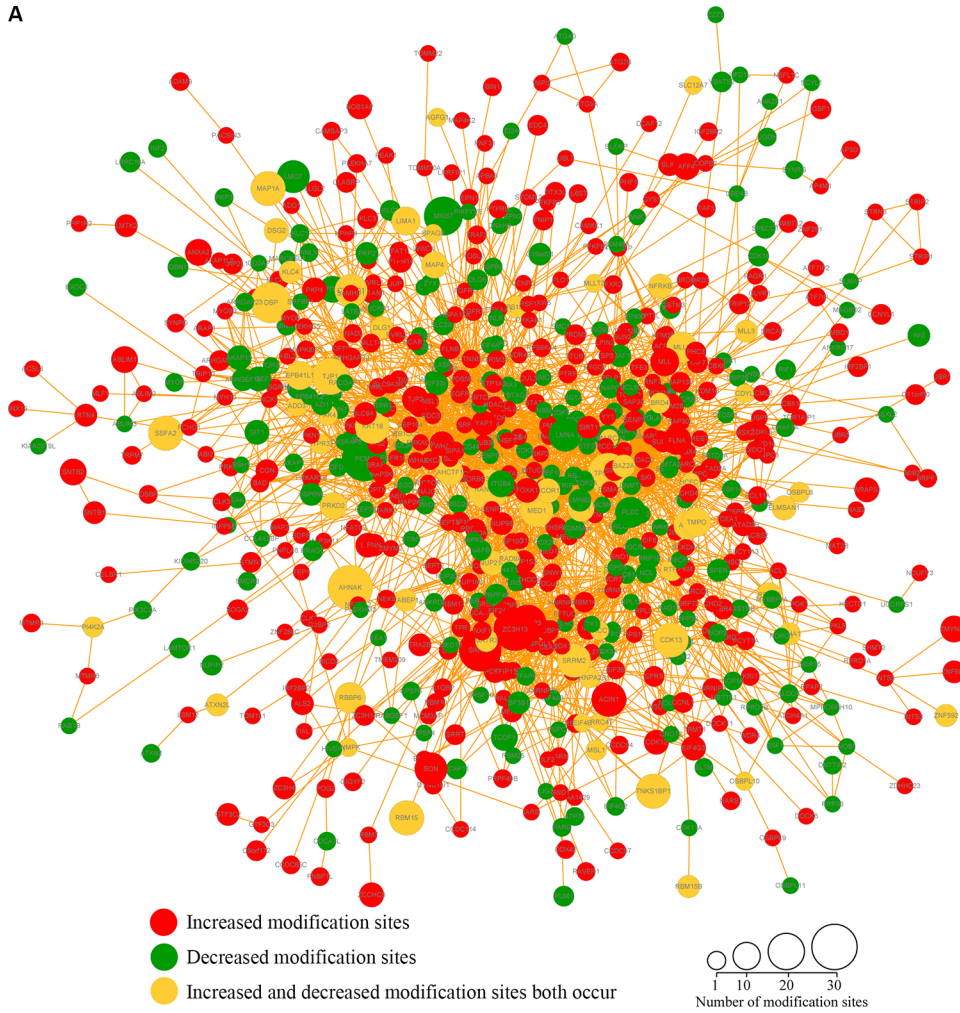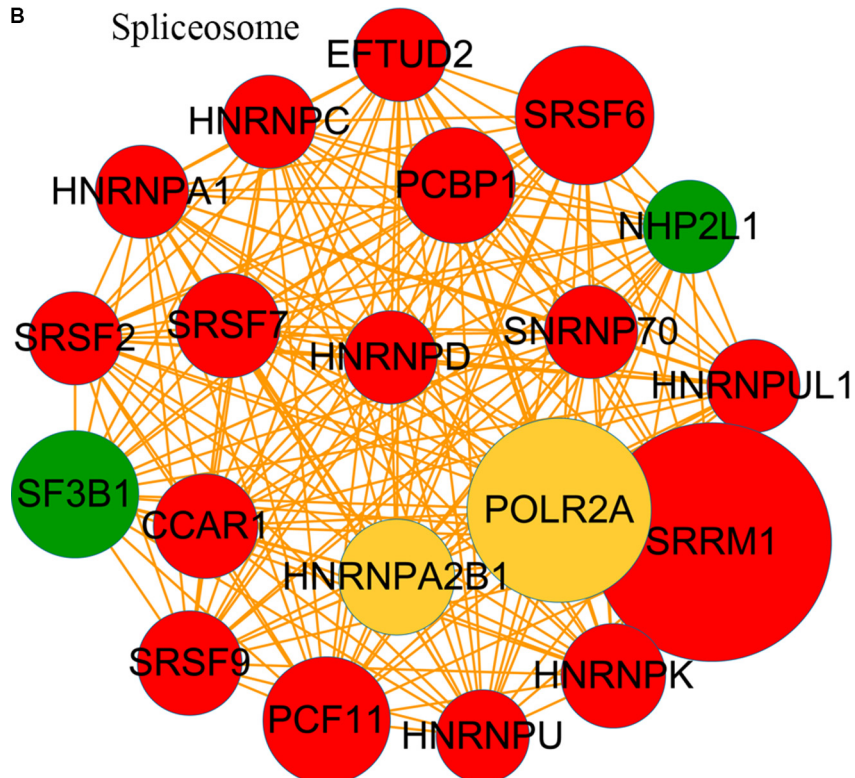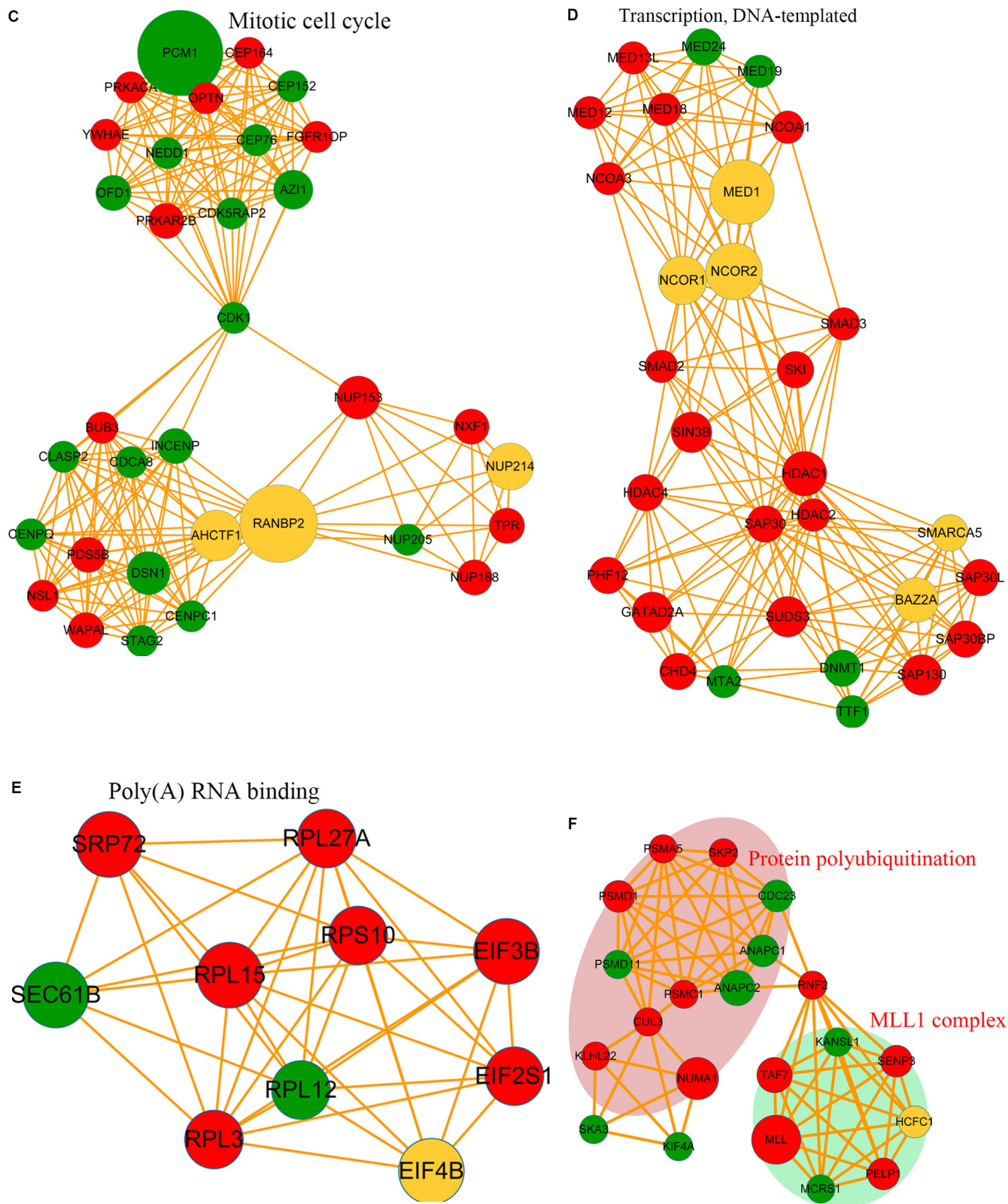
A

Protein domain

Decreased fold change   Increased fold change

(>2)	(1.5~2)	(1.5~2)	(>2)	
				BRCT domain
				Sterile alpha motif, type 2
				Homeodomain-like
				LEM-like domain
				Rabaptin, GTPase-Rab5 binding domain
				SET domain
				Post-SET domain
				NOPS
				Thioredoxin domain
				Retinoblastoma-associated protein, B-box
				Retinoblastoma-associated protein, A-box
				LisH dimerisation motif
				Ribosomal protein L7Ae/L30e/S12e/Gadd45
				Retinoblastoma-associated protein, N-terminal
				Kelch-type beta propeller
				Dehydrogenase, E1 component
				G-patch domain
				EGF-like calcium-binding domain
				HTH CenpB-type DNA-binding domain
				RAVE complex protein Rav1 C-terminal
				B-box, C-terminal
				Immunoglobulin-like fold
				Concanavalin A-like lectin/glucanases superfamily
				Six-bladed bête-propeller, TolB-like
				Zinc finger, B-box
				SPla/RYanodine receptor subgroup
				SPla/RYanodine receptor SPRY
				B30.2/SPRY domain
				Cadherin
				SAND domain-like
				MAD homology 1, Dwarfin-type
				CTF transcription factor/nuclear factor 1, N-terminal
				CTF transcription factor/nuclear factor 1, DNA-binding domain
				NET domain
				Spen paralogue and orthologur SPOC, C-terminal
				Lambda repressor-like, DNA-binding domain domain
				ABC transporter, transmembrane domain
				ABC transporter type 1, transmembrane domain
				Vacuolar sorting protein 9, subgroup
				Guanylate kinase-like
				Guanylate kinase/L-type calcium channel beta subunit
				Spen paralogue/orthologur C-terminal, metazoan
				Zinc finger, C2H2-type matrin
				Lamin tail domain
				Ataxin 2, SM domain
				Ataxin 2, C-terminal
				LsmAD domain
				SAB domain
				Band 4.1, C-terminal
				Vacuolar sorting protein 9
				CBS domain

Figure 5: Protein-complex and KEGG-pathway analysis of quantitatively altered phosphorylated proteins. The CORUM protein complex database for humans (http://mips.helmholtz-muenchen.de/genre/proj/corum/) was used for protein-complex analysis. The KEGG (http://www.genome.jp/kegg/) database was used to investigate enriched pathways using the DAVID Functional Annotation Tool against the background of *Homo sapiens*. (**A**) Protein complex analysis. (**B**) KEGG pathway analysis.

A

- 🔴 Increased modification sites
- 🟢 Decreased modification sites
- 🟡 Increased and decreased modification sites both occur

○ ○ ○ ○
1 10 20 30
Number of modification sites

B

Spliceosome

EFTUD2, HNRNPC, SRSF6, HNRNPA1, PCBP1, NHP2L1, SRSF2, SRSF7, HNRNPD, SNRNP70, HNRNPUL1, SF3B1, CCAR1, POLR2A, SRRM1, HNRNPA2B1, SRSF9, PCF11, HNRNPU, HNRNPK

Figure 6: Phosphorylated protein-protein interaction network analysis. The STRING database system (http://string-db.org/) was used to construct a protein-protein interaction network, and functional protein-interaction networks were visualized using Cytoscape (http://www.cytoscape.org/). (**A**) All identified phosphorylated protein-interaction network. (**B**) Spliceosome-related phosphorylated-protein-interaction network. (**C**) Mitotic cell cycle-related phosphorylated-protein-interaction network. (**D**) Transcription DNA-template-related phosphorylated-protein-interaction network. (**E**) Poly(A) RNA-binding-related phosphorylated-protein-interaction network. (**F**) Protein polyubiquitination and MLL1-complex-related phosphorylated-protein-interaction network.

RanBP2-type zinc-finger domain-containing proteins, and DEAD/DEAH box-type DNA/RNA helicases were abundant in Q2 (Figure 7B). In contrast, increased Kac sites were found in ubiquitin-like domain-containing proteins, linker histone H1/H5, glutathione S-transferase, SET domain-containing proteins, post-SET domain-containing proteins, and MOZ/SAS-like proteins.

From molecular-function analysis (Figure 7C), we observed that proteins classified as members of the melanosome, pigment-granule proteins, and histone acetyltransferase complex-related proteins were enriched in the Q1 quantile. Catalytic step 2 spliceosome proteins, nucleolus proteins, and some complex-associated proteins were enriched in the Q2 quantile. In contrast, proteins exhibiting increased Kac sites in Q3 were classified as DNA-bending complexes, protein-DNA complexes, cortical cytoskeleton, spindle, chromatin, action cytoskeleton, cell projections, cytoskeleton, cell-cell adherens junction, and cell-junction proteins.

Following protein-domain analysis, we observed that the domains involved in ligand-activated sequence-specific DNA binding, oxidase activity, synthase activity, transporter activity, phospholipase C activity, and tyrosine kinase activity were enriched in Q1 proteins exhibiting decreased sites involving Kac modifications. The domains involved in oxidoreductase activity, core-promoter binding, isomerases, nucleoside-triphosphatases, helicases, hydrolases, and phosphatase activity were enriched in Q2 proteins. The proteins exhibiting increased Kac-modification sites in Q3 contained domains involving transferase activity, DNA binding, lyase activity, methyltransferase activity, and transferase activity. Domains involved in isomerase activity, cis-trans isomerase activity, peptide binding, amide binding, DNA binding, protein-complex binding, actin-dependent ATPase activity, phosphatase-regulator activity, protein phosphatase-inhibitor activity, and protein phosphatase-regulator activity were abundant in Q4 proteins (Figure 7D).

Protein-complex and KEGG-pathway analysis of quantitatively altered acetylated proteins

Protein-complex analysis of the quantitatively altered acetylated proteins (Figure 8A) showed that proteins involved in the HBO1 complex and emerin complex were enriched in proteins exhibiting decreased Kac-modification sites. The 53 BP1-containing complexes, RNA poly II-containing coactivator complexes, Tat-SF complexes, G2/M-phase of the cell cycle, histone H3.1 complexes, and DNA-PK-Ku involved proteins exhibiting increased Kac-modification sites.

KEGG-pathway analysis of the quantitatively altered proteins showed that pathways involving antigen processing and presentation, legionellosis, alpha-linolenic acid metabolism, amyotrophic lateral sclerosis, SNARE

correlates in vesicular transport, the MAPK-signaling pathway, the spliceosome, and RNA transport were enriched in Q1 and Q2 proteins, while proteins exhibiting increased Kac-modification sites were involved in carbon metabolism, base-excision repair, mismatch repair, alcoholism, arrhythmogenic right-ventricular cardiomyopathy, adherens junction activity, viral carcinogenesis, endometrial cancer, tight-junction activity, and systemic lupus erythematosus (Figure 8B). These results showed that acetylation was highly associated with cell signaling and diseases.

Protein-protein interaction network analysis of the differentially acetylated proteins

We next constructed a protein-protein interaction network for the proteins containing Kac sites. Our results revealed that proteins with Kac sites were involved in the spliceosome, ribosome, and RNA transport, while dysregulation of protein export, the proteasome, unfolded-protein binding, poly(A) RNA binding, and the histone core was observed in response to FK228 treatment was observed (Figure 9A). In the acetylated-protein-interaction network associated with the spliceosome, there were 10 modification sites exhibiting increased acetylation, seven modification sites exhibiting decreased acetylation, and one site exhibiting both increased and decreased acetylation (Figure 9B). The proteasome- and spliceosome-related networks associated with proteins exhibiting dysregulated acetylation included six modification sites exhibiting increased acetylation and three modification sites exhibiting decreased acetylation, respectively (Figure 9C). The acetylated-protein networks related to unfolded-protein binding, poly (A) RNA binding, and histone core included nine modification sites exhibiting increased acetylation and four modification sites exhibiting decreased modification, with two sites exhibiting both increased and decreased modifications (Figure 9D). These data are listed in Supplementary Table S5.

Crosstalk between quantitated phosphorylation and the acetylome

To study the crosstalk between acetylation and phosphorylation, we compared the data from the acetylome with global phosphorylation data obtained from FK228-treated HCT-8 and HCT-116 cells and identified 274 proteins that underwent both acetylation and phosphorylation (Figure 10A). These proteins exhibited consistent changes in modification, suggesting positive correlations between acetylation and phosphorylation in these proteins.

To further investigate potential crosstalk between the acetylome and phosphorylation states, a protein-protein interaction network based on Kac and
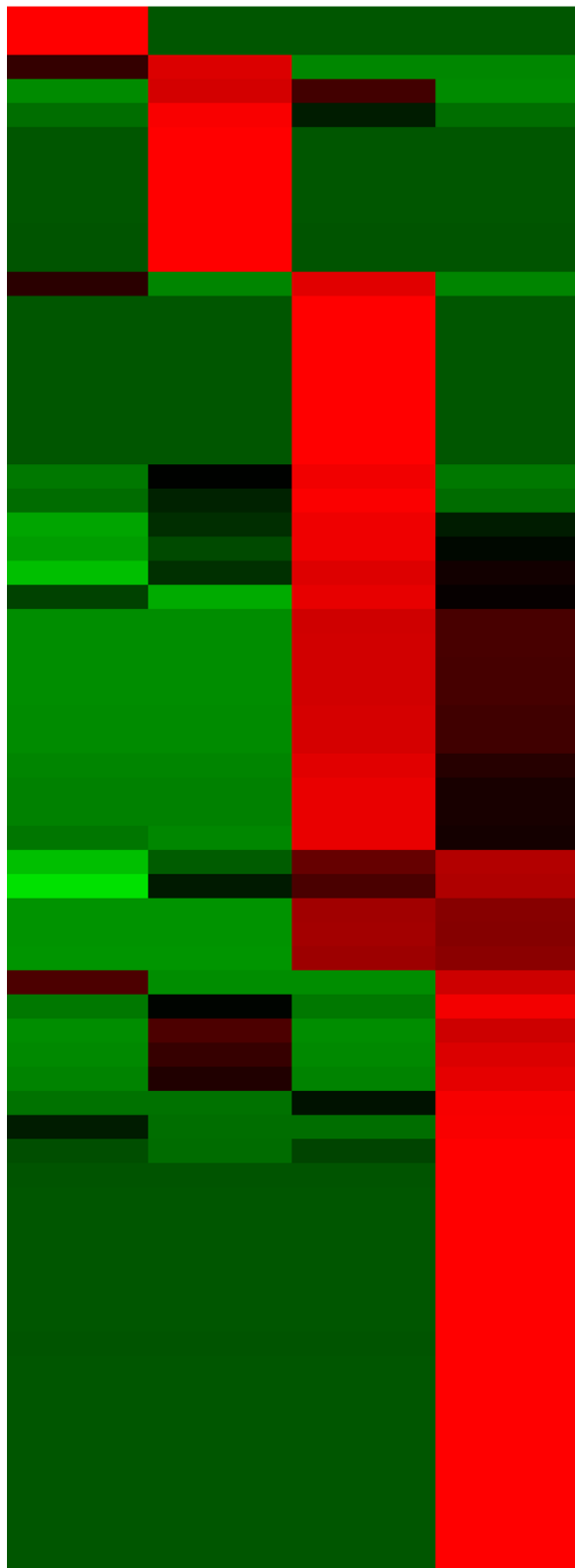
A

Biological process

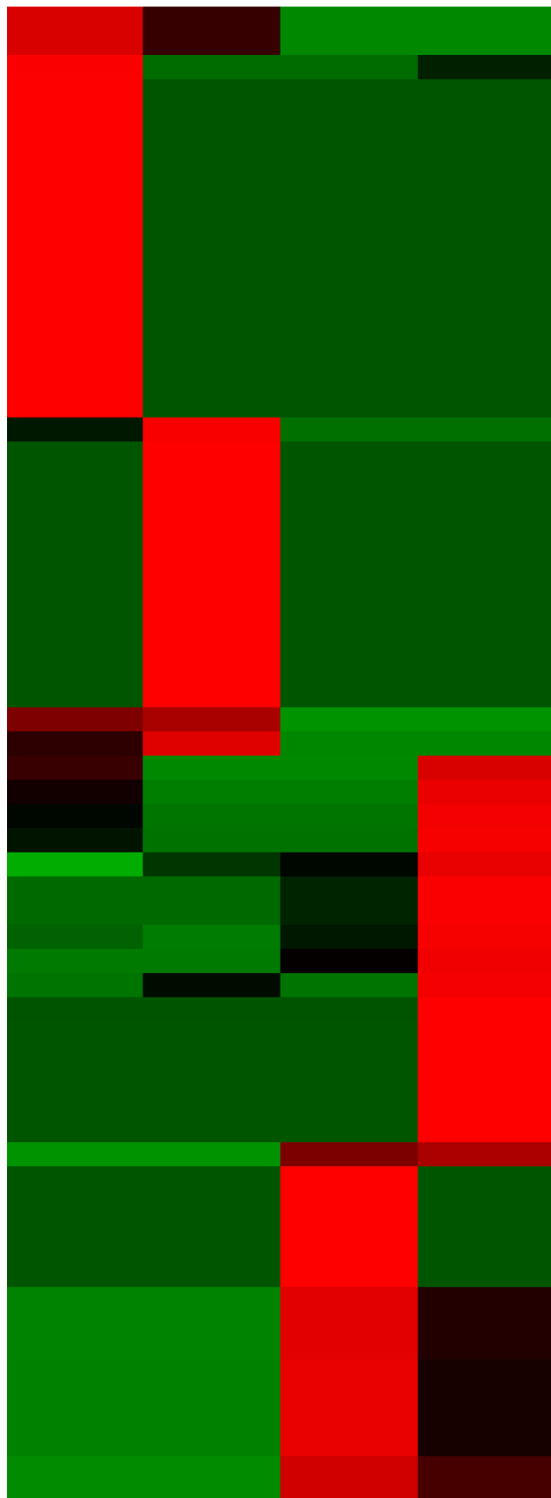Decreased fold change | Increased fold change

(>2) (1.5~2) (1.5~2) (>2)

regulation of cholesterol homeostasis
positive regulation of homeostatic process
programmed cell death
response to cytokine stimulus
regulation of ossification
response to organic substance
extracellular matrix organization
extracellular structure organization
signal transduction
signaling
single organism signaling
cellular response to stress
defense response
inflammatory response
carbohydrate catabolic process
antigen processing and presentation of peptide antigen
antigen processing and presentation of exogenous peptide antigen
response to lipid
response to external stimulus
regulation of muscle tissue development
negative regulation of multicellular organismal process
multicellular organism reproduction
sex differentiation
reproductive process
cellular process involved in reproduction
cellular component movement
cellular process involved in reproduction in multicellular organism
taxis
chemotaxis
locomotion
response to oxygen−containing compound
immune system process
antigen processing and presentation of exogenous antigen
antigen processing and presentation
reproduction
response to ionizing radiation
mitotic cell cycle
developmental process involved in reproduction
cell differentiation
anatomical structure homeostasis
chromosome organization
regulation of multicellular organismal development
cell cycle
regulation of cell differentiation
negative regulation of developmental process
response to radiation
organelle organization
protein−DNA complex subunit organization
macromolecular complex assembly
response to antibiotic
cell cycle process
tissue development
regulation of gene silencing
anatomical structure morphogenesis
establishment or maintenance of cell polarity
response to wounding
regulation of developmental process
positive regulation of developmental process
protein complex biogenesis
regulation of anatomical structure morphogenesis
macromolecular complex subunit organization
protein complex subunit organization
cellular component assembly
cellular developmental process
cellular component biogenesis

B Cellular component

Decreased fold change | Increased fold change

(>2) (1.5~2) (1.5~2) (>2)

Ubiquitin−like
SPOC like C−terminal domain
Ku70/Ku80 beta−barrel domain
Ku70/Ku80 C−terminal arm
Ku70/Ku80, N−terminal alpha/beta
High mobility group box domain
Linker histone H1/H5, domain H15
von Willebrand factor, type A
Glutathione S−transferase, C−terminal−like
SET domain
FY−rich, N−terminal
HIT−like domain
Zinc finger, double−stranded RNA binding
Glutathione S−transferase, N−terminal
FY−rich, C−terminal
Post−SET domain
MOZ/SAS−like protein
Winged helix−turn−helix DNA−binding domain
Cyclophilin−type peptidyl−prolyl cis−trans isomerase domain
Histone−fold
Histone core
Protein kinase−like domain
Sterile alpha motif domain
Myosin−like IQ motif−containing domain
Sterile alpha motif/pointed domain
Homeodomain−like
Helicase, C−terminal
Helicase, superfamily 1/2, ATP−binding domain
SANT/Myb domain
Zinc finger, RanBP2−type
DNA/RNA helicase, DEAD/DEAH box type, N−terminal
SAP domain
Acyl−CoA N−acyltransferase
Histidine kinase−like ATPase, ATP−binding domain
GNAT domain
Zinc finger, PHD−finger
Zinc finger, FYVE/PHD−type
Dbl homology (DH) domain
DNA topoisomerase, type IIA, subunit B, domain 2
DNA topoisomerase, type IIA, subunit A/C−terminal
Mab−21 domain
Major facilitator superfamily domain, general substrate transporter
Synaptobrevin
CRAL−TRIO domain
DNA topoisomerase, type IIA−like domain
Inhibitor of growth protein, N−terminal
Ribosomal protein L6, alpha−beta domain
DNA topoisomerase, type IIA, central domain
Major facilitator superfamily domain
DTHCT
Type IIA DNA topoisomerase subunit A, alpha−helical domain
Acyl−CoA oxidase, C−terminal
Longin domain
DNA topoisomerase, type IIA, subunit A/ C−terminal, alpha−beta
Toprim domain

c

Molecular function

Decreased fold change — Increased fold change

(>2)	(1.5~2)	(1.5~2)	(>2)	

melanosome
pigment granule
chromosome, centromeric region
nuclear part
nuclear lumen
DNA topoisomerase complex (ATP−hydrolyzing)
clathrin−sculpted vesicle
clathrin−sculpted gamma−aminobutyric acid transport vesicle
caveola
Ada2/Gcn5/Ada3 transcription activator complex
clathrin−sculpted gamma−aminobutyric acid transport vesicle membrane
female pronucleus
epsilon DNA polymerase complex
intermediate filament cytoskeleton
pronucleus
condensed chromosome
histone acetyltransferase complex
catalytic step 2 spliceosome
nuclear pore
integral to endoplasmic reticulum membrane
endoplasmic reticulum part
nuclear pore nuclear basket
nuclear inclusion body
pore complex
endoplasmic reticulum lumen
intrinsic to endoplasmic reticulum membrane
neuron spine
nuclear membrane
dendritic spine
nucleolus
Prp19 complex
chromosome
PML body
chromosomal part
cell−cell junction
transcription factor complex
nucleosome
DNA bending complex
protein−DNA complex
cortical cytoskeleton
spindle
chromatin
actin cytoskeleton
cell projection
cytoskeleton
cell−cell adherens junction
cell junction
tRNA−splicing ligase complex
A band
eukaryotic translation elongation factor 1 complex
mitochondrial alpha−ketoglutarate dehydrogenase complex
M band
mitochondrial tricarboxylic acid cycle enzyme complex
extracellular organelle
extracellular membrane−bounded organelle
extracellular vesicular exosome
nuclear telomere cap complex
Ku70:Ku80 complex
trans−Golgi network membrane
telomere cap complex
DNA−dependent protein kinase−DNA ligase 4 complex
nonhomologous end joining complex
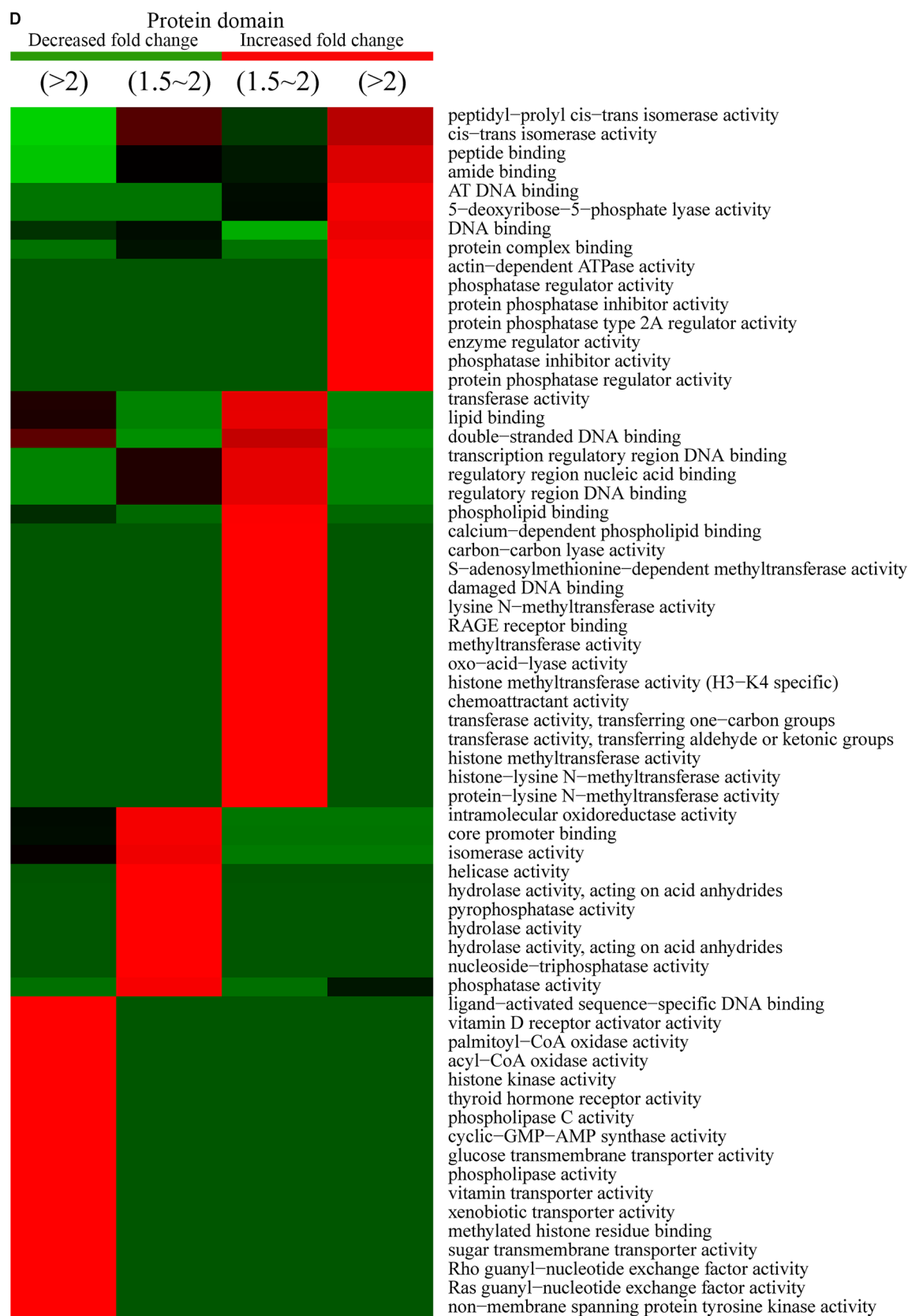
Figure 7: Functional enrichment-based clustering analysis for the quantified acetylated proteins. According to the functional differences observed between proteins exhibiting increased or decreased modification sites, GO-term association and enrichment analysis using DAVID (https://david.ncifcrf.gov/) was performed. (**A**) Proteins involved in biological processes, (**B**) Cellular component processes, (**C**) molecular functions, and (**D**) protein domain processes.

A

Protein comple

Decreased fold change | Increased fold change

(>2) (1.5~2) (1.5~2) (>2)

HMGB1−HMGB2−HSC70−ERP60−GAPDH complex
HBO1 complex
Emerin complex 24
Emerin complex 52
SNW1 complex
CDC5L core complex
CDC5L complex
DNMT3B complex
C complex spliceosome
SNF2h−cohesin−NuRD complex
53BP1−containing complex
RNA pol II containing coactivator complex Tat−SF
RC complex during S−phase of cell cycle
Histone H3.1 complex
H2AX complex II
RC complex during G2/M−phase of cell cycle
Rap1 complex
TERF2−RAP1 complex
DNA double−strand break end−joining complex
TRF2−Ku complex
Ku antigen−NARG1 complex
WRN−Ku70−Ku80−PARP1 complex
Ku70/Ku86/Werner complex
Ku−ORC complex
Ku antigen−YY1−alphaMyHC promoter complex
Ku70/Ku86 complex
Ku antigen complex
PCNA−KU antigen complex
DHX9−ADAR−vigilin−DNA−PK−Ku antigen complex
DNA−PK−Ku−eIF2−NF90−NF45 complex
Vigilin−DNA−PK−Ku antigen complex
NCOA6−DNA−PK−Ku−PARP1 complex
MGC1−DNA−PKcs−Ku complex
DNA−PK−Ku antigen complex
DNA−PK−Ku complex

Figure 8: Protein-complex and KEGG-pathway analysis of quantitatively altered acetylated proteins. Methods used for analysis were similar to those described for the proteins exhibiting altered phosphorylation levels. (**A**) Protein complex analysis. (**B**) KEGG pathway analysis.
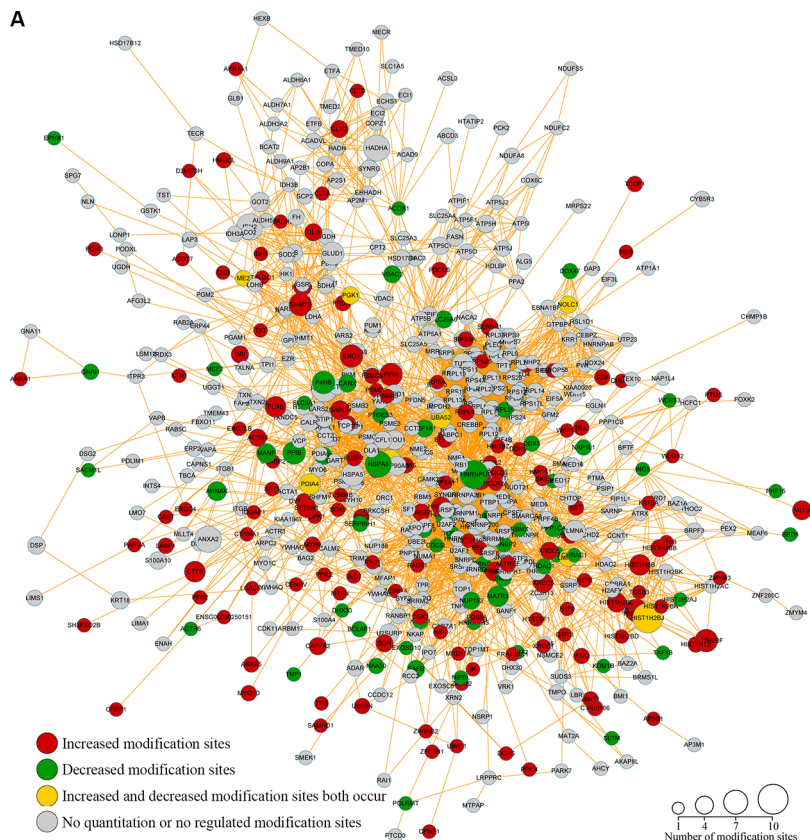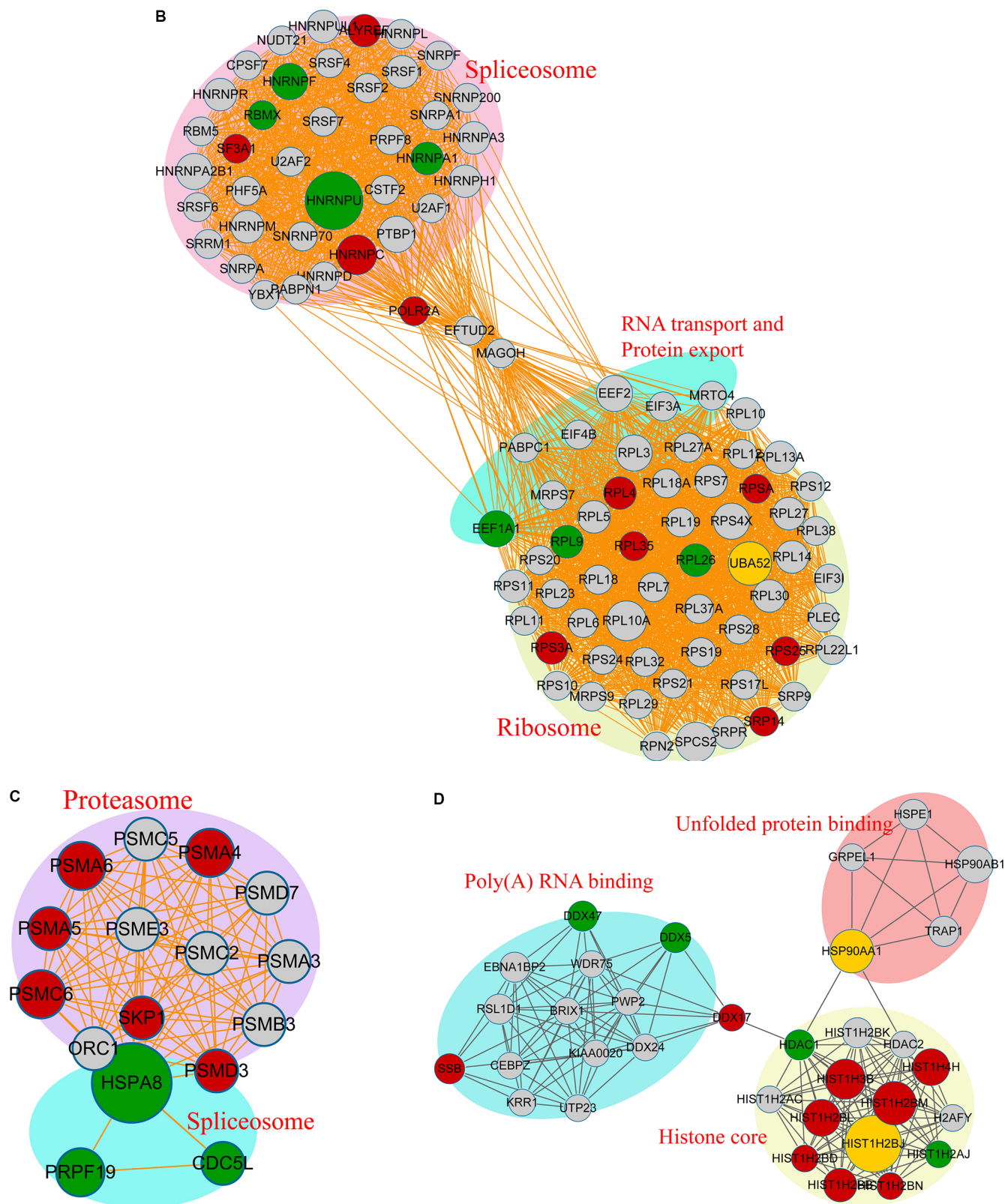
Figure 9: Acetylated protein-protein interaction analysis. The STRING database system (http://string-db.org/) was used to construct a protein-protein interaction network, and functional protein-interaction networks were visualized using Cytoscape (http://www.cytoscape.org/). (**A**) All identified acetylated protein-interaction networks. (**B**) The acetylated-protein-interaction network associated with the spliceosome, ribosome, RNA transport, and protein export. (**C**) The acetylated-protein-interaction network associated with the proteasome and spliceosome. (**D**) The acetylated-protein-interaction network associated with unfolded-protein binding, Poly(A) RNA binding, and the histone core.

lysine-phosphorylated proteins was constructed (Figure 10B). The global overview of the protein-protein interaction network between Kac and lysine-phosphorylated proteins was obtained (Figure 10B), followed by clustering these proteins according to multiple biological processes (Figure 10C). We observed that Kac and lysine-phosphorylated proteins both participated in the spliceosome, nucleoplasm activity, mitotic prometaphase, G2/M transition of the mitotic cell cycle, nuclear-transcribed mRNA catabolic processes, histone core interactions, RNA-biosynthetic processes, and MLL1-WDR5 complexes (Figure 10D–10H; Supplementary Table S6). Some important proteins involved in these networks were both acetylated and phosphorylated, including HNRNP A1, HNRNP U, and the CHD4 complex. These proteins might be important in mediating the effects of FK228 treatment of HCT-8 and HCT-116 cells, and, therefore, their biological functions in cancer should be further investigated. The representative spectra of P08238, Q8NEZ4, Q13547, Q01105, O14617, Q9Y3Z3, Q6ZU65, P18583, Q8WUA2, P20810 proteins that underwent both acetylation and phosphorylation are presented in Figure 11 and Supplementary Figure S1.

DISCUSSION

Protein function is regulated by diverse PTMs, which represent a key means of regulating cellular processes and diversifying the proteome, resulting in regulation of protein activity, subcellular localization, and maintenance of homeostasis. Modification of specific protein residues can confer different regulatory outcomes individually or in a combinatorial manner [32]. Phosphorylation and acetylation are the two most prevalent PTMs observed in the eukaryotic proteome. Phosphorylation is the primary mechanism involved in cellular signaling, while acetylation plays a prominent role in protein expression, and can inhibit protein degradation. Phosphorylation and acetylation of lysine residues represent prominent and ubiquitous regulatory mechanisms that are conserved throughout the human proteome [33–38]. For example, van Noort et al. [39] reported that phosphorylation and lysine acetylation could be simultaneously measured in the small bacterium *Mycoplasma pneumoniae*. Cheung et al. [40] found that histone H3 phosphorylation and acetylation are synergistic in response to epidermal growth factor stimulation.

Here, we determined both the quantitative proteome and the acetylome in HCT-8 and HCT-116 cells following FK228 treatment. We found that FK228 treatment altered protein acetylation levels, resulting in both increases and decreases in acetylation events. The quantitative acetylome from HCT-8 and HCT-116 cells following FK228 treatment was also obtained, with > 1000 Kac sites quantified. From these sites, modifications to 115 lysine-acetylation sites in 85 proteins were quantified as being increased and modifications to 38 sites in 32 proteins were decreased in response to FK228 treatment. Interestingly, 47 histone lysine-acetylation sites were identified in core histone proteins. These significantly altered proteins are involved in multiple biological functions, as well as a myriad of metabolic and enzyme-regulated pathways. For these interesting events, further study should be performed to elucidate the associated mechanisms.

Quantitative phosphoproteomics analysis was also performed in HCT-8 and HCT-116 cells following FK228 treatment. We identified 4982 phosphorylation sites in 2062 protein groups in total, among which, 4166 sites in 1937 proteins were quantified. When setting a quantification ratio of > 2.0 as the threshold for sites exhibiting increased modifications and < 0.5 as the threshold for sites exhibiting decreased modifications, 901 phosphorylation sites in 603 proteins were quantified as exhibiting increased modifications and 375 sites in 292 proteins were quantified as exhibiting decreased modifications. Finally, the crosstalk between Kac and phosphorylation was also analyzed. According to our results, Kac and lysine-phosphorylated proteins both participated in the spliceosome, nucleoplasm activity, mitotic prometaphase, G2/M transition of the mitotic cell cycle, histone core interactions, RNA-biosynthetic processes, and MLL1-WDR5 complexes. Some important proteins involved in these networks were both acetylated and phosphorylated, including coexistence of phosphorylation and Kac in hnRNPA1, hnRNPU, MKI67, sAFB, lMNB1, SET, MAP4, SKP1, PARVA, AP3D1, SAMHD1, RFC1, UBN2, TOP2A, SON, CTNNA1, PPIL4, CAST, NOLC1, and CHD4. These proteins might be important in mediating the effects of FK228 treatment of cancer cells, and, therefore, their biological functions in cancer should be further investigated.

Wu et al. [27, 28] investigated the quantitative proteome, acetylome, and ubiquitylome, as well as crosstalk between the three datasets in A549 cells following SAHA treatment. Their results identified 55 common sites exhibiting both acetylation and ubiquitination. Sakaguchi et al. [41] reported that p53 is acetylated *in vitro* at separate sites by two different histone acetyltransferases, suggesting that DNA damage enhances p53 activity as a transcription factor in part through carboxy-terminal acetylation that is directed by N-terminal phosphorylation. Wang et al. [42] showed that an ATM- and Rad3-related signaling pathway and a phosphorylation-acetylation cascade are involved in activation of p53/p21Waf1/Cip1 in response to 5-aza-2′-deoxycytidine treatment. Chen et al. [43] revealed that the acetylation of RelA at Lys310 is regulated by prior phosphorylation of Ser276 and Ser536. Such phosphorylated and acetylated forms of RelA display enhanced transcriptional activity [43].

According to the results presented here, FK228 treatment directly altered lysine acetylation and phosphorylation levels in HCT-8 and HCT-116
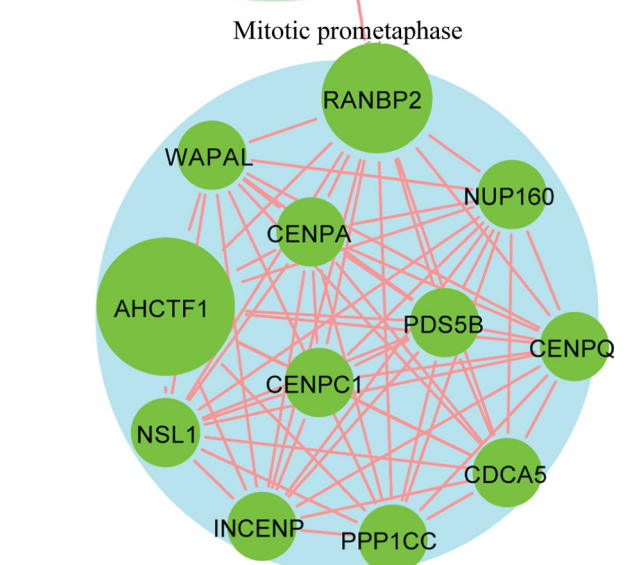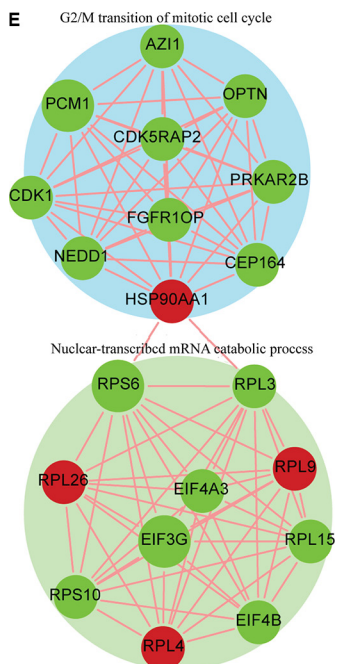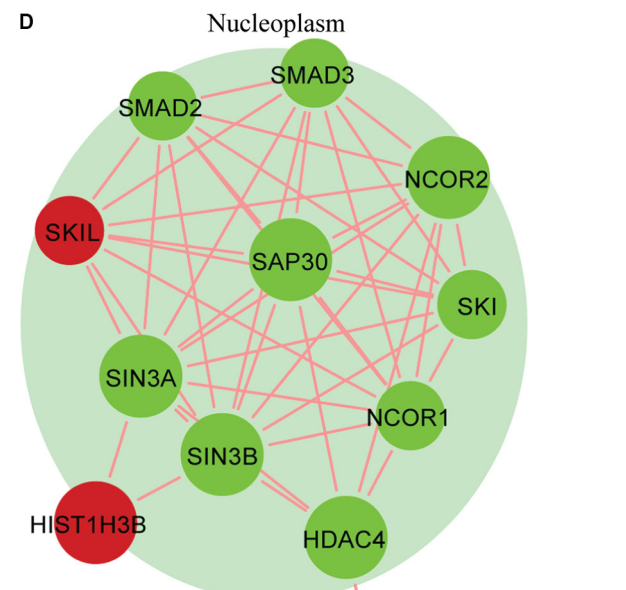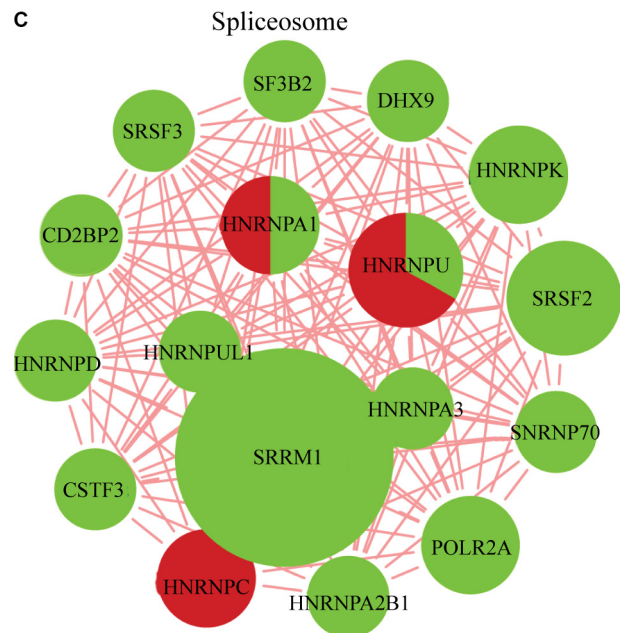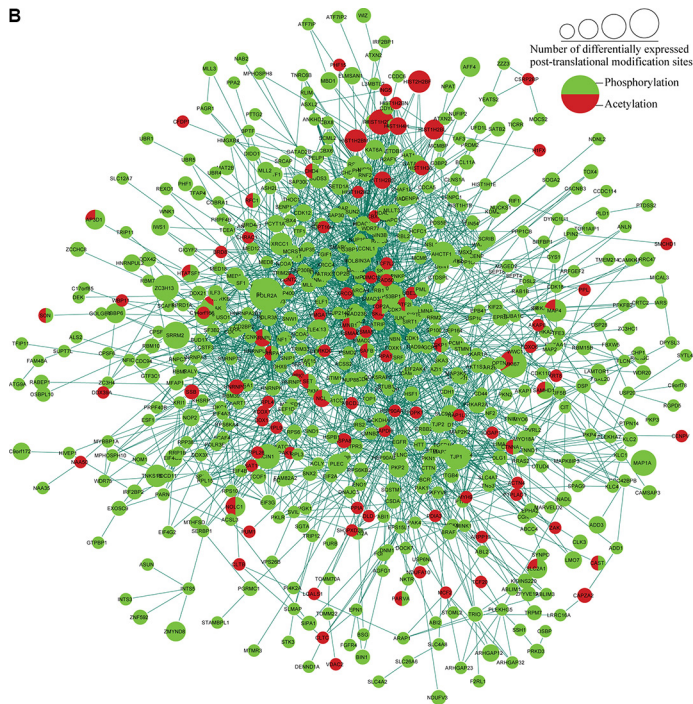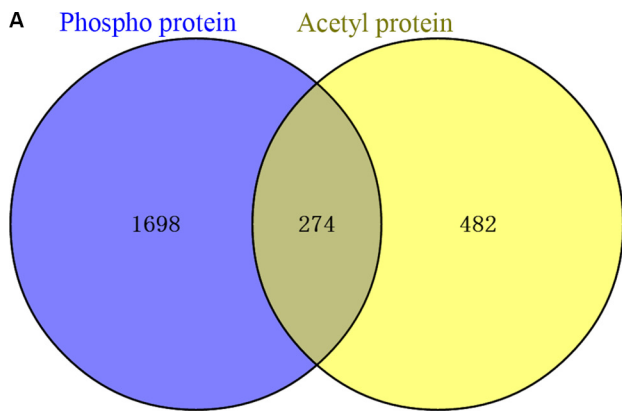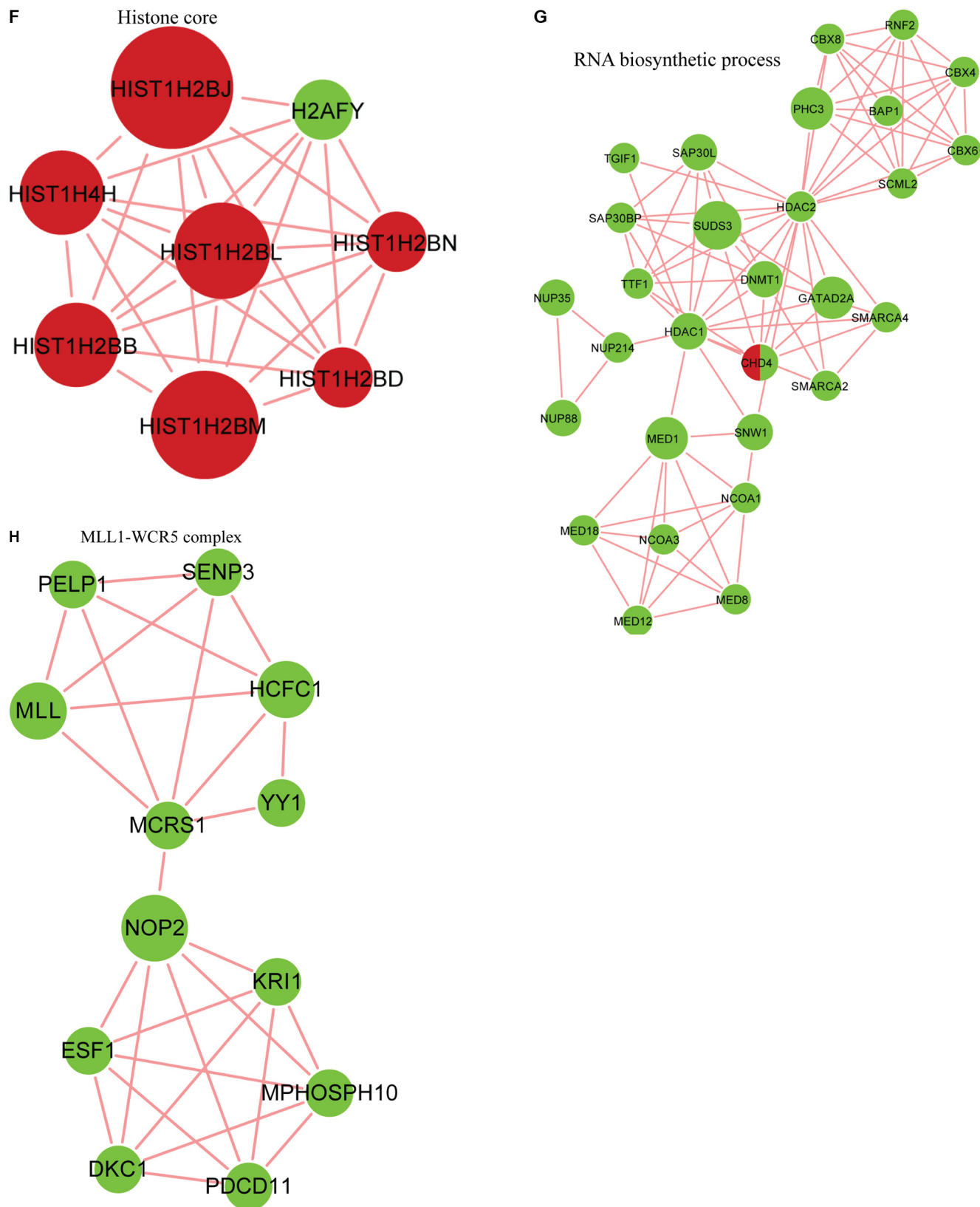
Figure 10: Analysis of the crosstalk between phosphorylation levels and the acetylome. (**A**) Overlap between phosphorylated proteins and acetylated proteins. Protein-protein interaction network between phosphorylated proteins and acetylated proteins obtained from Cytoscape (**B**), in the spliceosome (**C**), the nucleoplasm and mitotic prometaphase (**D**), in G2/M transition of the mitotic cell cycle and nuclear-transcribed mRNA catabolic processes (**E**), in the histone core(F),in RNA-biosynthetic processes (**G**) and clustered in the MLL1-WDR5 complex (**H**).

A

Raw file	scan	Method	Score	Mass	Gene names
1	4757	FTMS;HCD	72.43	1910.04	HSP90AB1
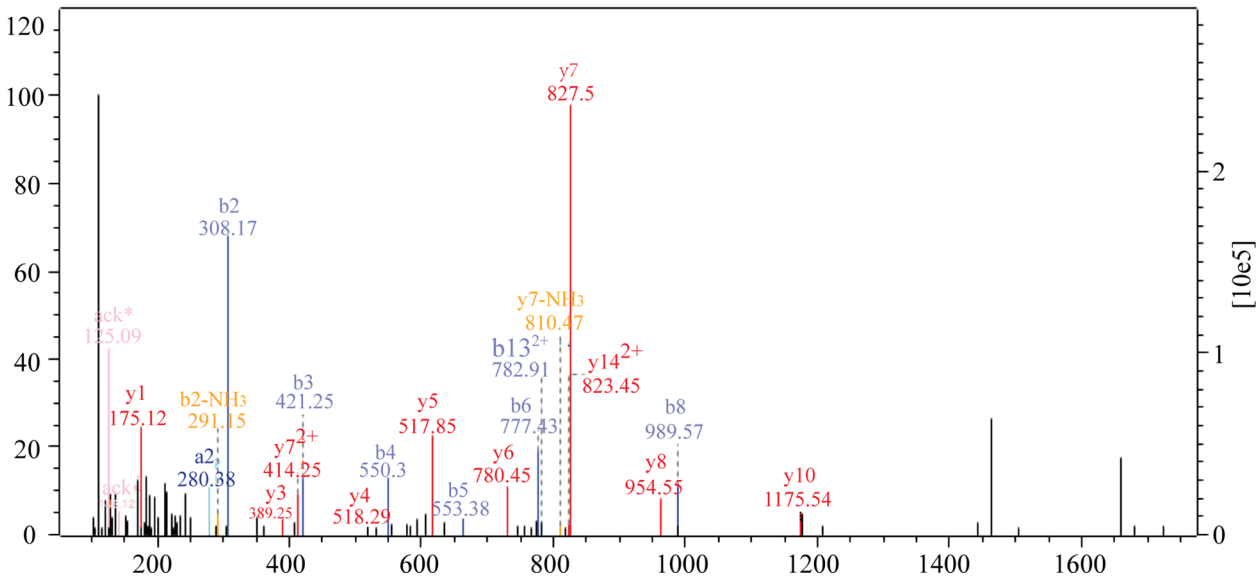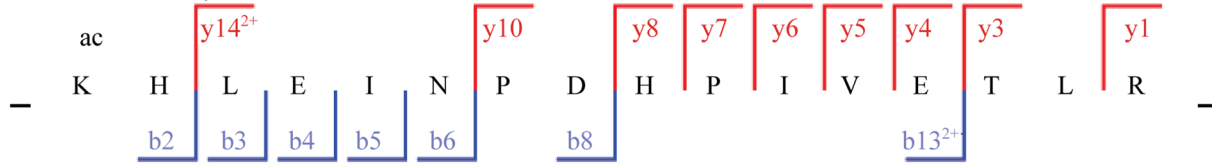
B

Raw file	scan	Method	Score	Mass	Gene names
21	500	FTMS;HCD	160.64	1493.6	HSP90AB1; HSP90AB2P
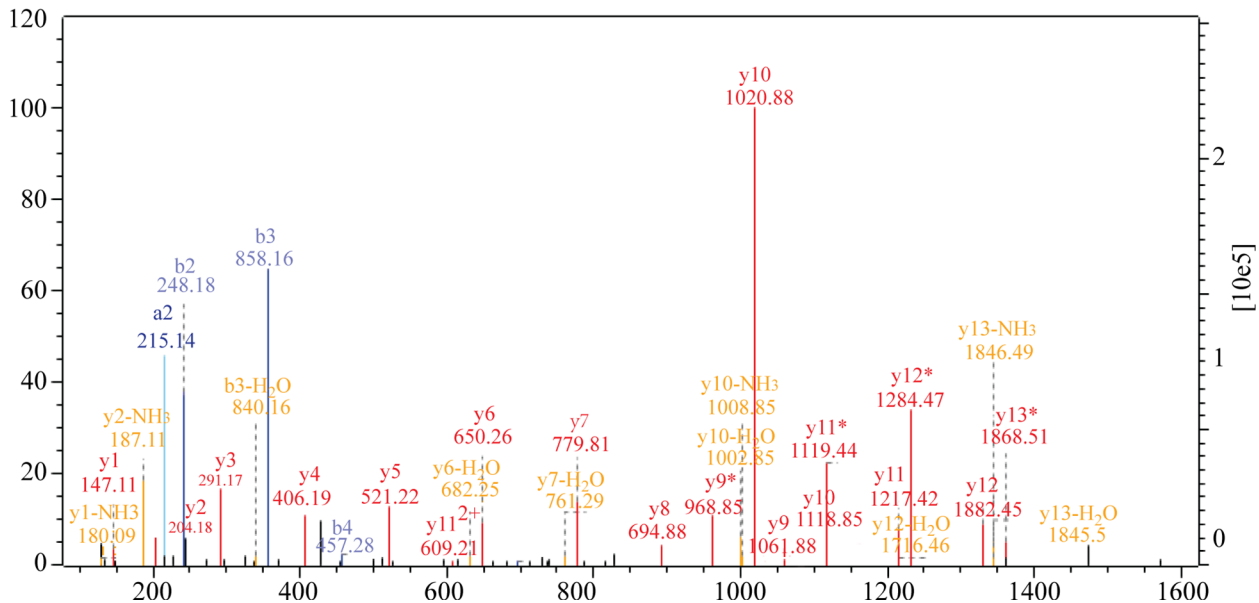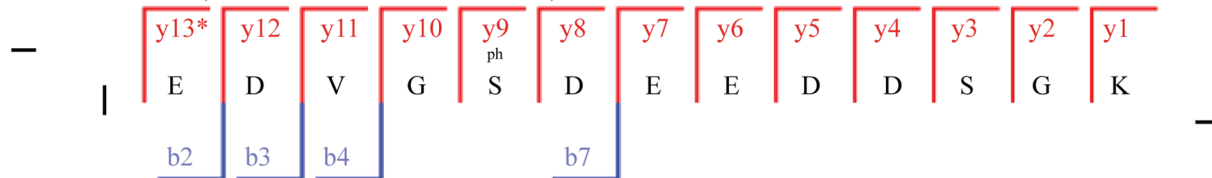
y10 y9 y8 y7 y6 ac y5 y4 y3 y1

T L V L S D K H S P Q K

b2 b3 b4

a2 187.14 b2 275.14
ack 143.12
y1-NH3 180.09
y2-NH3 258.14
ack+ 125.09
y1 147.11
b3 314.21
y3 372.22
b4+ 427.29
y4 459.26
y4-H2O 441.25
y8²⁺ 484.74
y9²⁺ 541.29
y10²⁺ 590.82
y5 595.82
y5-H2O 578.3
y6 755.42
y7 661.45
y8 968.48
y9 1091.55
y9-H2O 1053.5
y10 1190.63
y10-NH3 1163.6

y15* y13* y12* y11 y10 y9 y8* y7 y6 pn y5 y4 y3 y2 y1

P L S E E Q L D G I L S P E L D K

b2 b3 b4 b5 b6 b7 b8 b9 b10

y1 147.11
a2 188.15
b2 211.14
y2 262.14
b3 298.18
y3 875.22
b3-H2O 280.17
b7²⁺ 899.21
b4-H2O 409.21
y2-NH3 245.11
b5-H2O 588.25
b5 556.26
y5-H2O 588.81
y4 504.27
y4-H2O 486.26
b4 427.22
y6* 670.84
y6-H2O 666.81
y6-H2O 642.25
b6 684.82
y6 768.2
y7* 788.42
y7-NH3 765.4
y7 881.4
b7 797.4
y7-H2O 766.41
b8-H2O 894.42
y8* 896.51
b8 912.48
b9 969.45
y9* 958.58
b9-NH3 951.48
y10* 1068.96
b10-H2O 1004.58
y9 1051.51
b8-NH3 895.4
y10 1166.58
b10 1082.54
y11* 1181.64
y11 1279.82
b10-NH6 1065.5
y12-NH3 1292.67
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y12* 1809.7
y13* 1488.74
y15* 1654.82
y15-NH3 1687.79
y5 601.82
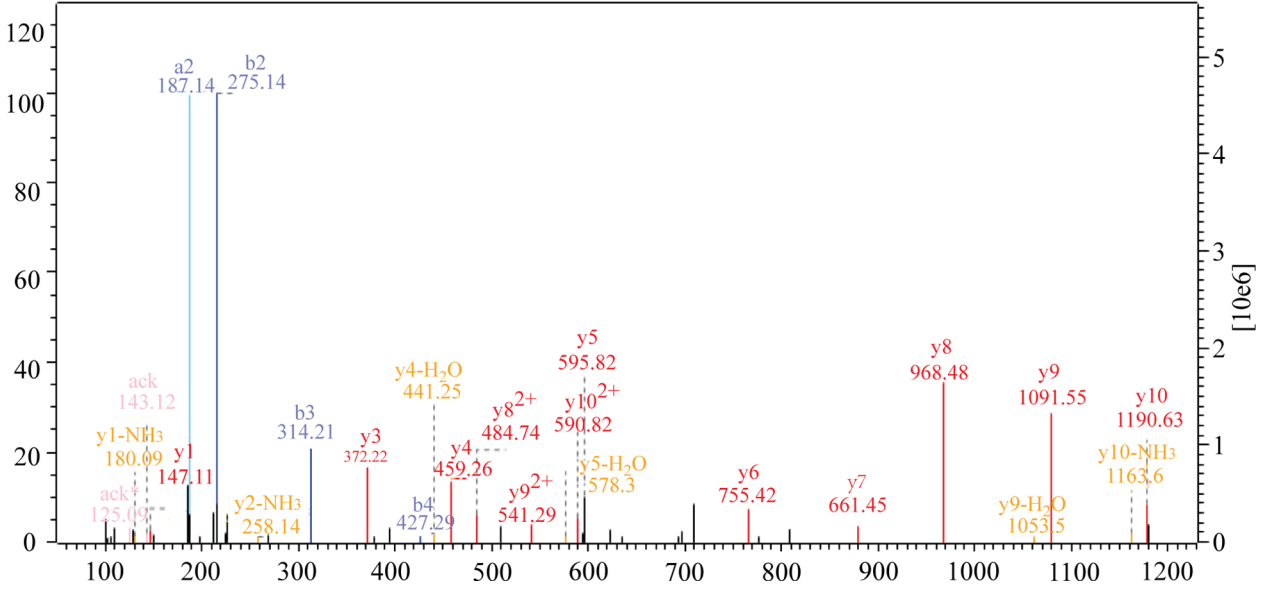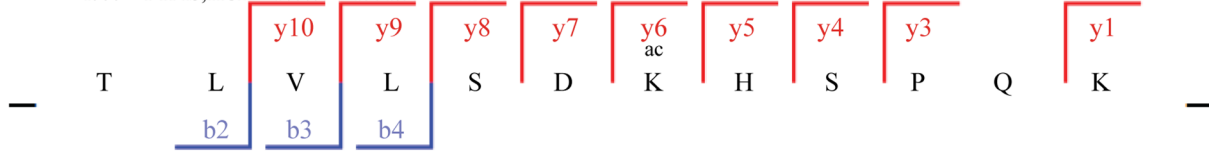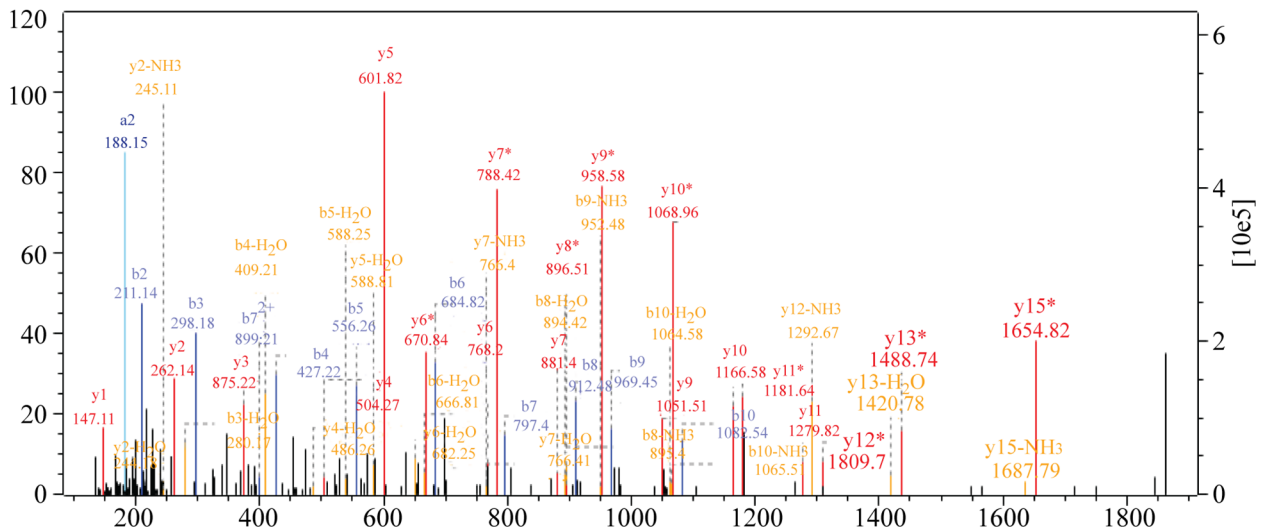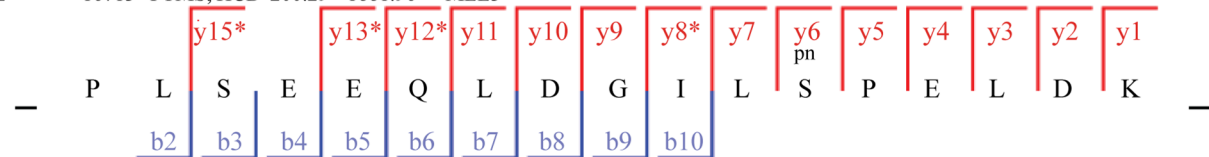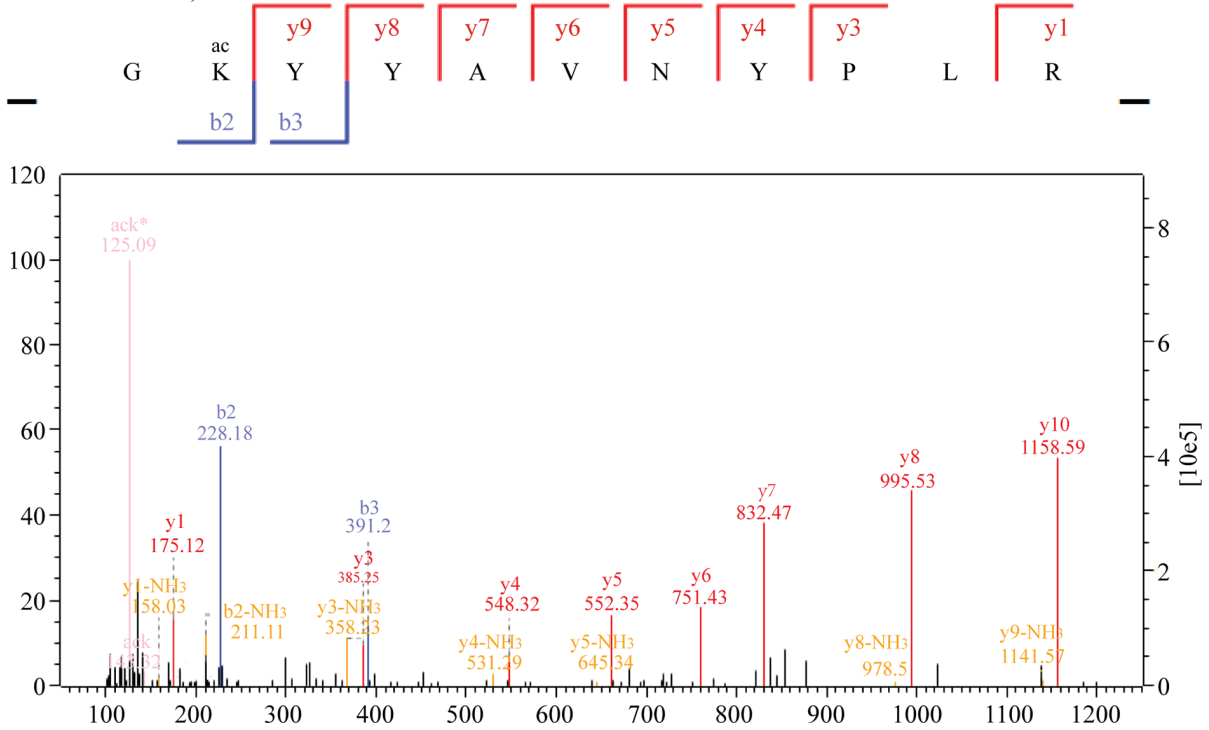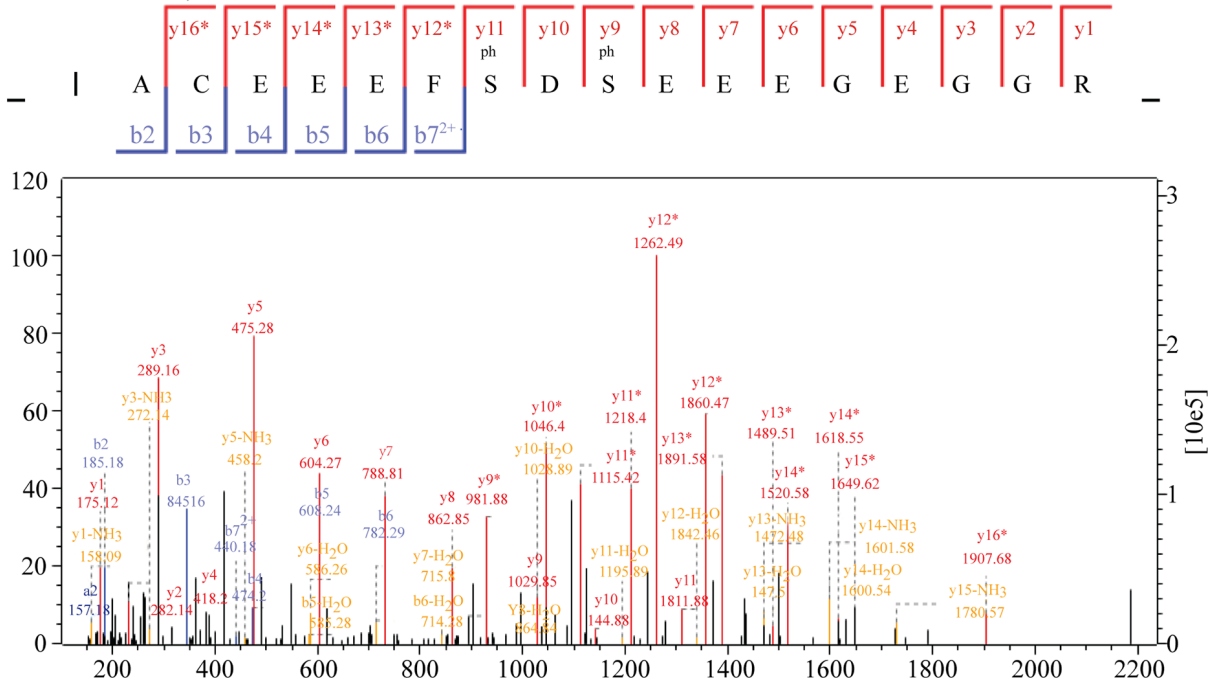
Figure 11: MS/MS spectra of P08238, Q8NEZ4, and Q13547 acetylation and phosphorylation. (**A**) P08238 acetylation, (**B**) P08238 phosphorylation, (**C**) Q8NEZ4 acetylation, (**D**) Q8NEZ4 phosphorylation, (**E**) Q13547 acetylation, (**F**) Q13547 phosphorylation. These proteins were underwent both acetylation and phosphorylation.

cells. Moreover, the alteration of acetylation and phosphorylation levels also ultimately regulate each other due to the existing crosstalk between acetylation and phosphorylation events.

Additionally, the changing levels of lysine acetylation could induce alterations in the global proteome. Moreover, transcription factors could also undergo phosphorylation and induce alterations to the global proteome via the transcriptome. However, these constitute hypotheses and require confirmation through additional experimentation.

In conclusion, using SILAC labeling, antibody-based affinity enrichment, and high-resolution LC-MS/MS, we obtained results suggested that FK228 treatment broadly altered the proteome, phosphorylation levels, and the acetylome in HCT-8 and HCT-116 cells. Additionally, potential crosstalk between phosphorylation states and the acetylome associated with FK228 treatment were observed through bioinformatics analyses, which expanded our current understanding of FK228-related cancer therapy. We noted potential interactions between the acetylome, phosphorylation levels, and the proteome based on positive regulation between the acetylome and phosphorylation events. However, further experiments are necessary to enhance validation and interpretation of the predicted mechanisms.

MATERIALS AND METHODS

Cell culture

HCT-8 and HCT-116 colon cancer cells were purchased from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in glucose (4.5 g/L) and Dulbecco's Modified Eagle Medium (DMEM) supplemented with glutamine and sodium pyruvate and containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with 95% air and 5% CO_2. The cells were subcultured every 2 to 3 days after digestion with 0.02% ethylenediaminetetraacetic acid (EDTA) and 0.1% trypsin.

Cell cytotoxicity assay

Previous reports indicated that 50% of HCT-8 cells were viable (IC_{50}) following administration of FK228 at concentrations of 29.46 nM [30]. We performed cytotoxicity assays on HCT-116 cells using varying concentrations of FK228 according to previously described methods [30]. When HCT-116 cells reached 75% confluence, a cell proliferation and cytotoxicity assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to manufacturer instructions. Briefly, a 96-well plate was pre-incubated for 24 h, followed by addition of different

concentrations of FK228 (Sigma-Aldrich, St. Louis, MO, USA) to each well. CCK-8 solution (10 μL) was added and incubated for an additional 1 to 4 h. Cell viability was determined by measuring the absorbance at 450 nm. Cell cytotoxicity was calculated using the HTC-116 cell-viability measurements following treatment with FK228 The FK228 concentration resulting in 50% cell viability (IC_{50}) was used as the fixed working concentration for later experiments.

SILAC labeling and FK228 treatment

Cells were grown to 80% confluence and labeled with either "heavy isotopic lysine" (13C-Lysine) or "light isotopic lysine" (12C-Lysine) using a SILAC protein quantitation kit (Pierce; Thermo Scientific, Rockford, IL, USA) according to manufacturer instructions. Briefly, cells were grown in DMEM supplemented with 10% FBS and either the "heavy" form of [U-13C6]-l-lysine or "light" [U-12C6]-l-lysine for more than six generations in order to achieve > 97% labeling efficiency before being harvested, following by expanding in SILAC media to achieve the ~5 × 10^8 cell number in 150-cm^2 flasks.

The "light"-labeled cells were treated with 29.46 nM FK228, and the "heavy"-labeled cells were treated with same concentration of dimethyl sulfoxide. After treatment, the cells were maintained in SILAC media for another 48 h before being harvested and washed twice with ice-cold phosphate-buffered saline (PBS) containg 2 μM trichostatin A and 30 mM nicotinamide. After freezing in liquid nitrogen, cell pellets were stored at −80°C until further use.

Protein extraction

The harvested "heavy"- and "light"-labeled cells were lysed with 2× NETN buffer [200 mM NaCl, 100 mM Tris-Cl, 2 mM EDTA, and 1.0% NP-40 (pH 7.2)] supplemented with 0.5% Triton X-100 on ice for 30 min. The supernatant was saved following centrifugation at 20,000 g for 10 min at 4°C. After concentration measurement, equal amounts of crude proteins from the "heavy"- or "light"-labeled supernatant were mixed and precipitated by adding trifluoroacetic acid (TFA) to a 15% (v/v) final concentration (soluble fraction). After washing twice with −20°C acetone, the protein pellets were dissolved in 100 mM NH_4HCO_3 (pH 8.0) for trypsin digestion. The remaining cell pellets were dissolved in 8 M urea to extract the chromatin-binding proteins. After measurement of protein concentration, equal amounts of chromatin-binding proteins in the urea solution were mixed, and the proteins were precipitated by adding TFA to a 15% (v/v) final concentration (nuclear-pellet fraction). After washing twice with −20°C acetone, the protein pellets were dissolved in 100 mM NH_4HCO_3 for trypsin digestion.

Trypsin digestion

Protein solutions containing 1:50 (w/w) ratio of trypsin (Promega, Madison, WI, USA) to protein was prepared and digested at 37°C for 16 h. Dithiothreitol was added to a final concentration of 5 mM, incubated at 50°C for 30 min. In order to alkylate proteins, indole-3-acetic acid was added to a final concentration of 15 mM, and incubated in the dark for 30 min. The alkylation reaction was quenched by 30 mM cysteine for another 30 min. Trypsin was then added at a trypsin-to-protein ratio of 1:100 (w/w) for digestion at 37°C for 4 h to complete the digestion cycle.

High-performance liquid chromatography (HPLC) fractionation

The sample was then fractionated by high pH, reversed-phase HPLC using an Agilent 300 Extend C18 column (5-μm particles, 4.6-mm internal diameter, 250-mm length; Agilent Technologies, Santa Clara, CA, USA). Peptides were initially separated into 80 fractions using a gradient of 2% to 60% acetonitrile in 10 mM ammonium bicarbonate (pH 10) over 80 min, followed by combining the peptides into six fractions and drying by vacuum centrifugation.

Affinity enrichment

Before affinity enrichment, anti-lysine-acetylation (anti-Kac) and anti-lysine-phosphorylation antibody beads (PTM Biolabs, Inc., Hangzhou, China) were washed twice with ice-cold PBS. To enrich Kac and lysine-phosphorylated peptides, tryptic peptides bound to Kac and lysine-phosphorylation antibody beads dissolved in NETN buffer were incubated with pre-washed anti-Kac and anti-lysine-phosphorylated antibody beads at a ratio of 15 mL beads/mg protein at 4°C overnight with gentle shaking. The beads were washed four times with NETN buffer and twice with ddH$_2$O. The bound peptides were eluted from the beads with 0.1% TFA, and the eluted fractions were combined, vacuum-dried, and analyzed by LC-MS/MS.

LC-MS/MS analysis

Peptides were dissolved in 0.1% formic acid (FA) and directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100; Thermo Fisher Scientific, Danvers, MA, USA). Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC; Thermo Fisher Scientific). The gradient comprised of an increase from 6% to 22% solvent B (0.1% FA in 98% acetonitrile) for 24 min, 22% to 35% for 10 min, a climb to 80% in 5 min, then holding at 80% for the last 3 min, all at a constant flow rate of 300 mL/min on an EASY-nLC

1000 UPLC system (Thermo Fisher Scientific). The resulting peptides were analyzed using a Q ExactiveTM hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

The fractionated peptides were subjected to a nanospray ionization source, followed by MS/MS using the Q ExactiveTM hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) connected online to the UPLC system. Intact peptides were detected at a resolution of 70,000. Peptides were selected for MS/MS using a normalized collision energy setting of 28, and ion fragments were detected at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan, followed by 20 MS/MS scans, was applied for the top 20 precursor ions above a threshold ion count of 2E4 in the MS survey scan, with a 15.0-s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control was used to prevent overfilling of the ion trap, and 5E4 ions were accumulated for generation of the MS/MS spectra. For MS scans, the m/z scan range was 350 to 1800.

Database search

The resulting MS/MS data were processed using MaxQuant with an integrated Andromeda search engine (v.1.4.1.2; http://www.biochem.mpg.de/5111795/maxquant). Tandem mass spectra were searched against the SwissProt database (20,274 sequences) concatenated with a reverse-decoy database. Trypsin/P was specified as the cleavage enzyme, allowing up to three missing cleavages, five modifications per peptide, and five charges. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethylation on Cys was specified as a fixed modification, and oxidation on Met, acetylation on Lys, and acetylation on the protein N-terminus were specified as variable modifications. False discovery rate thresholds for protein, peptide, and modification sites were specified at 1%, and the minimum peptide length was set at 7 residues. All other parameters in MaxQuant were set to their default values. The site-localization probability was set at > 0.75.

Bioinformatics analysis

We performed Gene Ontology (GO) term association and enrichment analysis using the Database for Annotation and Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/). The Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) database was used to investigate enriched pathways using the DAVID Functional Annotation Tool against the background of *Homo sapiens*. Additionally, the InterPro database (http://www.ebi.ac.uk/interpro/) was searched using the DAVID Functional Annotation Tool against the background of *Homo sapiens*. The manually curated

CORUM protein complex database for humans (http://mips.helmholtz-muenchen.de/genre/proj/corum/) was used for protein-complex analysis. The STRING database system (http://string-db.org/) was used to construct a protein-protein interaction network, and functional protein-protein interaction networks were visualized using Cytoscape (http://www.cytoscape.org/). When performing the bioinformatics analysis, a corrected $p = 0.05$ was considered significant.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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