Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions

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Lysine acetylation is a reversible posttranslational modification of proteins and plays a key role in regulating gene expression. Technological limitations have so far prevented a global analysis of lysine acetylation's cellular roles. We used high-resolution mass spectrometry to identify 3600 lysine acetylation sites on 1750 proteins and quantified acetylation changes in response to the deacetylase inhibitors suberoylanilide hydroxamic acid and MS-275. Lysine acetylation preferentially targets large macromolecular complexes involved in diverse cellular processes, such as chromatin remodeling, cell cycle, splicing, nuclear transport, and actin nucleation. Acetylation impaired phosphorylation-dependent interactions of 14-3-3 and regulated the yeast cyclin-dependent kinase Cdc28. Our data demonstrate that the regulatory scope of lysine acetylation is broad and comparable with that of other major posttranslational modifications.

Acetylation of lysine is a reversible posttranslational modification (PTM), which neutralizes the positive charge of this amino acid, changing protein function in diverse ways (*1*, *2*). It has a key role in the regulation of gene expression through the modification of core histone tails by histone acetyltransferases (HATs) or histone deacetylases (HDACs) (*3*, *4*). Some modified lysines specifically bind bromodomain-containing proteins, which are part of large complexes that modulate chromatin architecture. Lysine acetylation is also important for p53 functions and interactions and for microtubule stabilization (*5*). There are many individual reports of acetylation sites on proteins involved in diverse biological processes, suggesting that lysine acetylation has broad regulatory functions in addition to the few that are actively studied.

HDACs [or lysine deacetylases (KDACs)] are important drug targets in cancer and neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases, and there are more than 80 clinical trials currently underway (*6*–*9*). Two KDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and valproic acid, are in clinical use. Furthermore, KDAC inhibitors are potent reprogramming agents for the generation of induced pluripotent stem cells (*10*). However, the specific mode of action of KDAC inhibitors has remained enigmatic and may involve acetylation on histones and other proteins.

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Despite great biological and clinical interest in lysine acetylation, our knowledge of in vivo acetylation sites is limited. However, advances in quantitative mass spectrometry (MS)–based proteomics (*11*–*13*) allow PTMs to be studied at a proteomic scale (*14*, *15*). In the case of phosphorylation, affinity purification of modified peptides makes it possible to quantify phosphorylation at thousands of sites (*16*, *17*). Peptides containing acetylated lysines can also be partially enriched by an antibody directed against this modification. This strategy was used to identify 388 lysine acetylation sites, mainly in mitochondria, a cellular compartment where this modification had hardly been described before (*18*). We reasoned that high-resolution high-accuracy MS, when coupled to an efficient quantitation strategy, should enable a global view of the "lysine acetylome" and its changes upon KDAC inhibition.

Sequencing and quantifying the acetylome. We enriched acetylated peptides from trypsin-digested whole-cell lysates of MV4-11 cells, a human acute myeloid leukemia cell line with an antibody against acetyl-lysine (*19*). We identified approximately 1000 acetylation sites by means of high-resolution high-accuracy MS (*20*). However, biologically important acetylation sites of low abundance remained undetected because of a large background of nonacetylated peptides. We therefore further separated peptides from immunoaffinity purifications by means of isoelectric focusing into 12 fractions (*21*). We used stable-isotope labeling with amino acids in cell culture (SILAC) (*22*, *23*) so as to determine global acetylation changes in response to two KDAC inhibitors, SAHA and MS-275. Lastly, to assess reproducibility and depth of acetylome coverage, we performed similar analyses in two additional human cell lines of epithelial (A549) and lymphoid origin (Jurkat) (fig. S1A) (*24*). The resulting raw data were processed with the MaxQuant suite of algorithms (*25*).

Overall mass accuracy was in the parts-per-billion range; high resolution was achieved for all peptides (fig. S1B), which facilitated accurate quantitation. We detected more than 3600 acetylation sites on 1750 proteins (table S1) at an overall false discovery rate (FDR) for peptides of less than 1%. The FDR for acetylated lysine-containing peptides, as opposed to all peptides, was even lower (between 0.1 and 0.3%) because SILAC determines the number of arginines and lysines directly, greatly aiding specific identification. We precisely pinpointed the site of modification for more than 95% of the peptides (table S1).

In a separate experiment without affinity enrichment, the number of acetylation sites was 60-fold lower (0.058% as compared with 3.44%). Percentage of acetylation sites C-terminal to the peptide—an in vitro artifact—was 72% as compared with 2.5% in the enriched samples. We used D_3C_1-labeled acetic acid in the sample preparation so as to independently show that C-terminal lysine acetylation was mainly caused by this chemical. We selected nine proteins for which acetylation was not previously described and one known acetylated protein and independently confirmed their acetylation by means of immunostaining (Fig. 1A). Proteins from a human cervical cancer cell line (Hela) or a human osteosarcoma cell line (U2OS) that were expressed as fusions with green fluorescent protein (GFP) under the endogenous promoter (*26*) were immunoprecipitated, and all showed clear lysine-acetylation signals (Fig. 1A). CBX3 was immunoprecipitated from transiently transfected human embryonic kidney 293 cells and also showed clear acetylation. Together, this demonstrates that our large-scale acetylome is of in vivo origin.

The in vivo acetylome. We first assessed the coverage of our data set for histone acetylation sites and found that we detected all sites that we were able to extract from the literature (table S1). The known in vivo acetylation sites of proteins of low abundance, such as the tumor suppressor p53, Ku70, nuclear factor κB subunit RelA, and protooncogene protein c-Myc, were also present. A large number of acetylation sites on proteins exclusively annotated as mitochondrial (381 different sites) (*18*), but these did not constitute the largest category. We compared the acetylomes of the three different cell types (Fig. 1B). For any one cell line, 60 to 80% of the acetylated proteins and 60 to 75% of the acetylated sites were also found in the other two. This indicates that our experimental methodology is accurate and reproducible and that acetylation patterns are similar in cells derived from different tissue types. Our experiment covers the majority of known in vivo sites and expands by at least sixfold the number of such sites reported in UniProt (*27*). The large overlap (fig. S2), even on proteins of low abundance, also implies sub-

stantial coverage of the core acetylome. However, it is likely that many more sites are still to be discovered because they may only be acetylated in specific cell types or at particular developmental or cell-cycle stages.

Lysine acetylation is an ancient PTM that is conserved from prokaryotes to humans. We evaluated phylogenetic conservation of lysine-acetylated proteins and compared it with that of the entire proteome (*28*). Lysine-acetylated proteins are significantly more conserved across the evolutionary tree (fig. S3), indicating additional selective pressure. Acetylated proteins were as conserved as phosphorylated proteins across vertebrates. For more distantly related species such as yeast (fig. S3A) and especially in prokaryotes, the acetylome was much more conserved than the phosphoproteome ($P < 10^{-20}$) (fig. S3B).

Lysine acetyltransferases (KATs) and KDACs are thought to be predominantly nuclear or mitochondrial, and indeed we did find a large number of acetylated proteins in these compartments. However, proteins with exclusive cytoplasmic annotation were also highly represented in the acetylome (Fig. 2A). HDAC6 is the only known cytoplasmic KDAC, but our results make it unlikely that it is the sole deacetylase with cytoplasmic activity.

Phosphorylation mainly occurs in unstructured regions of proteins, such as hinges and loops (*28*, *29*). In contrast, and in concordance with an earlier report (*18*), acetylation sites were frequently located in regions with ordered secondary structure. Furthermore, compared with all lysines, acetylated lysines were significantly enriched in structured regions and depleted in unstructured regions (Fig. 2B).

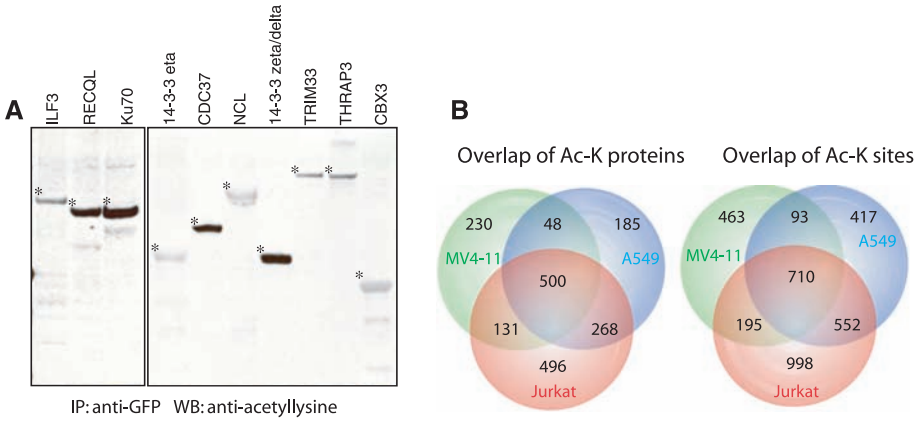

Fig. 1. Overview of in vivo acetylome analysis. (**A**) Independent validation of lysine acetylation of proteins. (**B**) Overlap of acetylated proteins and sites in three different cell lines. Ten different proteins from the acetylome data set were immunoprecipitated from GFP-tagged BAC transgenic cell lines and stained with antibody to acetyl-lysine. The bands marked with an asterisk indicate acetylated proteins.

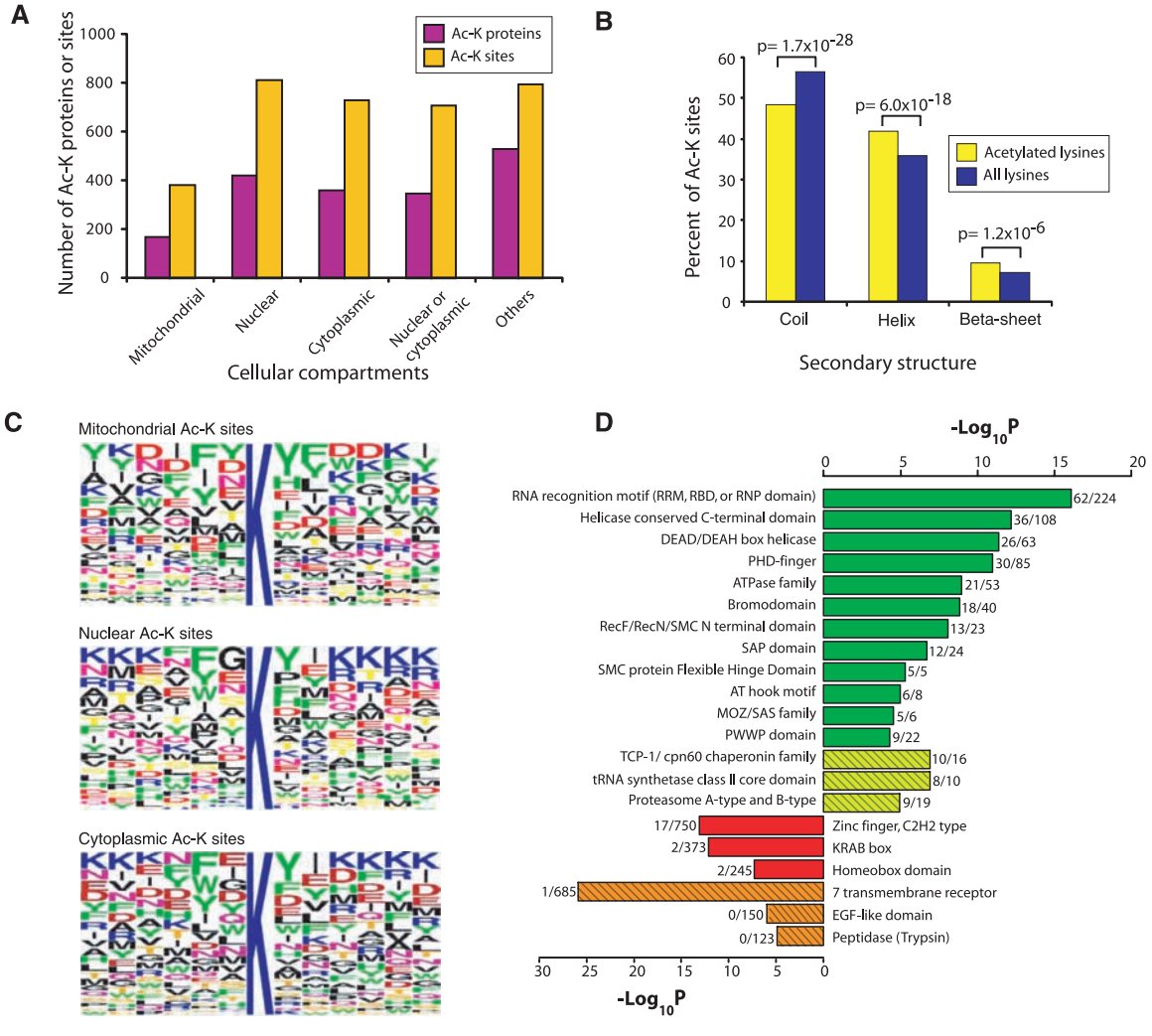

Fig. 2. Properties of acetylated proteins and sites. (**A**) Cellular distribution of acetylated proteins and sites. Proteins were assigned based on exclusive Gene Ontology (GO) annotations. (**B**) Distribution of all lysines and acetylated lysines in structured and unstructured regions of the proteins. (**C**) Sequence logo plots represent normalized amino acid frequencies for ±6 amino acids from the lysine acetylation site. (**D**) Domain architecture of acetylated proteins. The green bars indicate Pfam protein families and domains that are significantly overrepresented, and the red bars indicate underrepresented domains in the acetylome as compared with those in the entire proteome. The light green and orange striped bars represent cytoplasmic domains.

We analyzed local sequence context around the acetylation sites. Amino acids with a bulky side chain—mainly tyrosine and phenylalanine—were enriched in the –2 and +1 positions. This observation agrees with the frequent occurrence of acetylated lysines in ordered regions of proteins. Positively charged amino acids were almost completely excluded from the –1 position. Nuclear and cytoplasmic acetylation motifs are similar but different from mitochondrial motifs (Fig. 2C). For example, lysines were the preferred amino acids beyond the +2 and –3 positions for both nuclear and cytosolic proteins. In mitochondrial proteins, there was a preference for tyrosine (and histidine to a lesser degree) in the +1 position, and hydrophobic amino acids were enriched. Glycine at –1 was observed in vivo here and has been demonstrated on specific proteins (the GK motif) (*1*, *2*).

Cellular localization and function of proteins is often dictated by their domains. Pfam domains (*30*) associated with nuclear functions were most overrepresented in our acetylome, including RNA-binding motifs, various helicases, the PWWP domain, the PHD finger, and bromodomains ($P < 10^{-5}$) (Fig. 2D). Among the most underrepresented domains were those associated with membranes or extracellular space and peptidases.

Acetylation targets macromolecular complexes. We were intrigued by the presence of a large number of acetylated proteins in large macromolecular complexes and classified acetylated proteins according to known chromatin-associated processes (fig. S4). Known nuclear lysine acetyltransferase complexes (*3*) were themselves highly acetylated on multiple subunits (fig. S4A). Three subunits were known to be acetylated in these nine KAT complexes; our analysis detected more than 40. Autoacetylation of p300 is a well-described example of KAT acetylation that regulates its own enzymatic activity (*31*). Our data demonstrate that extensive acetylation of KAT complex subunits is a general property. ATAC is a newly described KAT complex in *Drosophila melanogaster* described after our data were acquired (*32*). Of the human homologs of ATAC subunits, seven were acetylated. SIN3A, the main deacetylase complex, contains at least eight subunits modified on 18 distinct sites (fig. S4B).

Almost no acetylation sites are currently known on chromatin-remodeling complexes, but we found that SWI/SNF, NURD, INO80, and NURF are heavily acetylated, usually on multiple lysines (fig. S4C). Two complexes involved in regulating transcription, FACT (facilitates chromatin transcription) and TAF [TATA-box binder protein (TBP)–associated factor], were also targets of acetylation (fig. S4D).

Histones and p53 are regulated by several different PTMs, suggesting extensive crosstalk between different PTMs. Major methyltransferase complexes that contain catalytic subunits encoded by the mixed lineage leukemia (MLL) genes were extensively modified by acetylation, suggesting crosstalk between KATs and methyltransferases directly at the level of the modifying enzymes (fig. S4E). Histone demethylases such as JARIDs (Jumonji/ARID domain–containing proteins) were also acetylated, further substantiating the regulatory link. Lastly, a large number of nuclear ubiquitin-modifying enzymes were also subject to this modification (fig. S4F). These data suggest a direct and intricate crosstalk between different enzymes involved in modulating chromatin-associated functions via PTMs.

To investigate acetylation-targeting of many macromolecular complexes in an unbiased way, we analyzed the complete acetylome data set using Comprehensive Resource of Mammalian protein complexes (CORUM), a database of manually curated and validated mammalian protein complexes (*33*). A total of 39 nuclear and five cytosolic complexes were enriched for acetylated proteins at $P < 0.05$ (Fisher's exact test) (table S2), including all of the complexes described above.

To quantify protein-interaction properties of the acetylome, we used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database of physical and functional interactions (*34*). Compared with similarly sized randomly selected protein data sets, the acetylome has significantly higher network connectivity ($P < 10^{-6}$) with six interactions per node as compared with less than three for random data sets (fig. S5). This further corroborates the notion of high connectivity among acetylated proteins. In the global STRING-generated protein-protein network, several complexes and cellular functions formed prominent, tightly connected clusters as assessed by means of molecular complex detection (fig. S6A) (*35*). In addition to the physical complexes found manually and by CORUM, cellular processes such as RNA splicing, nuclear transport, protein folding, and cytoskeletal regulation were highlighted (fig. S6B).

Cellular processes regulated by acetylation. With the exception of histone modification and DNA repair, in which acetylation has well-established roles, there is little evidence for a broad role of acetylation in other nuclear processes. Our data reveal that a large number of acetylation sites are present on proteins involved in all major nuclear processes, such as splicing, cell cycle, chromatin remodeling, DNA replication, transcription, and nuclear transport, which strongly suggests that these processes may be influenced by this modification (Table 1). Previously, only a small number of well-studied proteins were known to be acetylated in these processes. For example, there are only three known acetylation sites for proteins implicated in splicing, whereas we found 130 acetylation sites on proteins involved in this process. These acetylated proteins are connected in a dense protein-protein interaction network (Fig. 3A).

Acetylation of a few proteins such as p53, Ku70, FEN1, and WRN is known to be important in the repair of damaged DNA. Acetylation of p53 regulates its stability through crosstalk with the ubiquitination machinery, modulates interactions with TAF1, and regulates its transcriptional activity (*5*). We confirmed acetylation of these proteins and found further sites, which may also be functional. For example, Ku70 and Ku80, which form heterodimers and regulate the activity of DNA-dependent protein kinase (DNAPK), a key kinase involved in DNA damage repair, are both acetylated. Acetylation of Ku70 is already

Table 1. Acetylation regulates diverse protein classes and biological categories. Acetylated proteins were grouped into major cellular functional categories or into protein classes on the basis of their known functions or function predicted by functional domains.

Cellular functional categories and protein classes	Number of acetylated proteins	Number of acetylation sites
DNA replication	52	98
DNA damage and repair	72	167
Chromatin remodeling	26	46
Cell cycle	132	243
RNA transcription	31	71
RNA splicing	109	206
Nuclear hormone signaling	9	22
Nuclear transport	17	41
Cytoskeleton reorganization	50	137
Nucleotide exchange factors	55	92
Endocytosis and vesicular trafficking	39	62
DNA/RNA helicases	46	105
Ubiquitin ligases and deubiquitylases	46	70
Protein kinases	47	71
Acetyltransferases and deacetylases	21	61
Methyltransferases and demethylases	12	34
Transcription factors	29	40
Histones	15	61
Adaptor proteins	14	40
Chaperones	40	127
Ribosomal proteins	75	136

known to be important in this process. Many other proteins that are essential for DNA damage repair, such as MDC1, DDB1/2, RAD50, PCNA, WRNIP1, MSH2, BLM, and RAD54L, are acetylated (Fig. 3B). The human genome encodes six phosphoinositide-3-kinase–related protein kinases (PIKKs) that contain a conserved C-terminal FAT [FKBP12-rapamycin–associated protein (FRAP), ataxia telangiectasia mutated (ATM), transformation/transcription domain-associated protein (TRRAP)], a kinase domain, and FATC (FAT C terminus) domain structure: ATM, ATR, SGK1, DNAPK, TRRAP, and FRAP1. PIKKs play key roles in DNA damage repair, and we found acetylation sites in SGK1, DNAPK, TRRAP, and FRAP1. Acetylation of ATM has been reported (*36*). The FATC domains of these kinases mediate an interaction with Tip60, an acetyltransferase that has a critical role in DNA damage repair and, in the case of ATM and DNAPK, regulates their kinase activity (*36*, *37*). Thus, acetylation may represent a common regulatory mechanism of activation for FATC domain–containing PIKKs.

Acetylation is a widespread modification for nuclear ubiquitin ligases and deubiquitylases including UHRF1, HUWE, BRE1A, and BRE1B, which have roles in the modification of histones and chromatin-bound proteins (table S1). Similarly, HAUSP, USP22, and USP11 deubiquitylases, which have roles in chromatin biology, and Ubch37 and USP14, the two major proteasome-bound deubiquitylases, are acetylated.

The p53 interaction network controls its functions with several mechanisms. We discovered that not only p53 but several proteins that are critical for

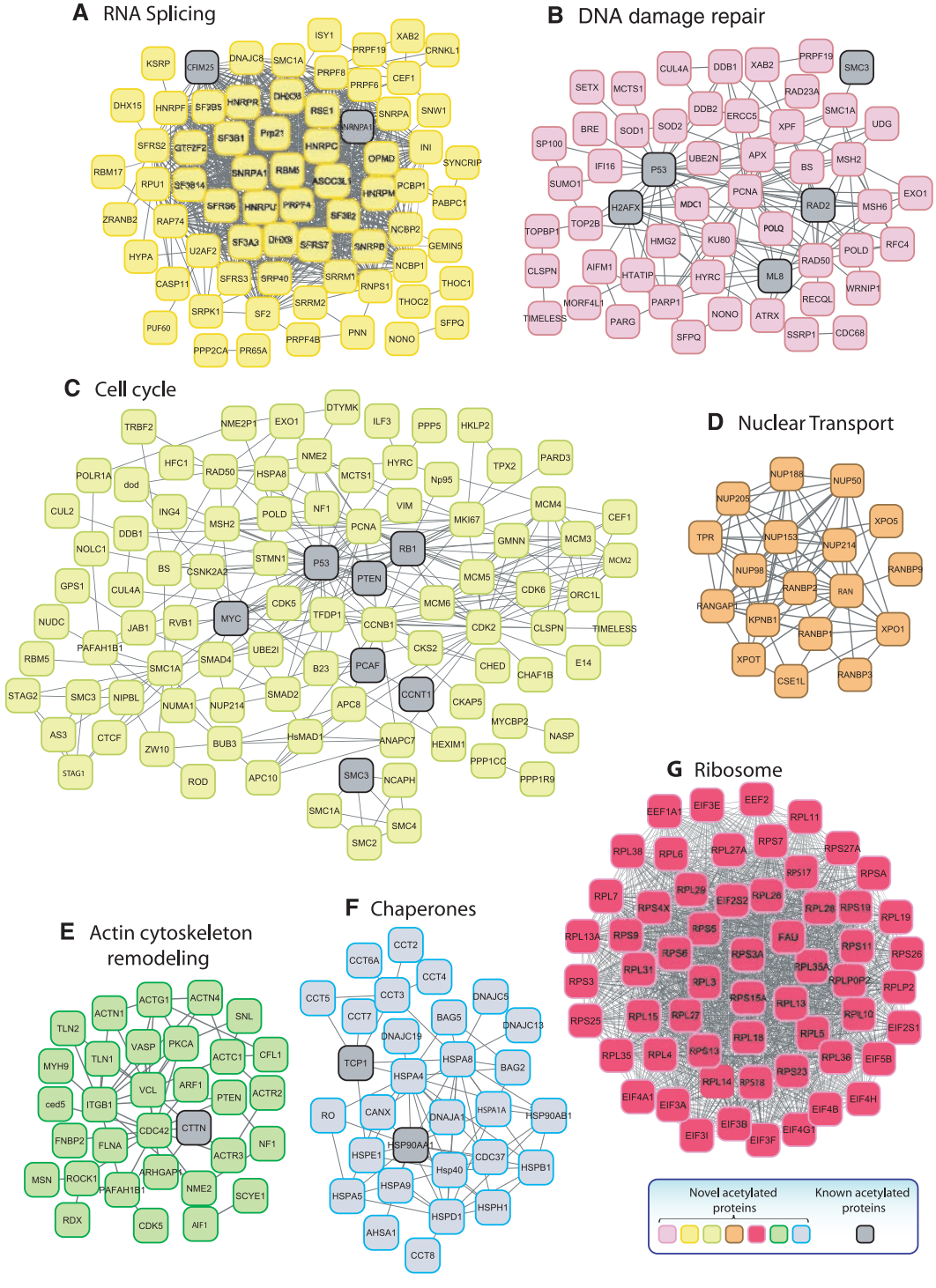

Fig. 3. Acetylation-modulated functional networks. (**A** to **G**) Interaction networks of acetylated proteins in different cellular functions from STRING analysis of the acetylome. Individual networks were generated for each specific functional category (table S1). Gray nodes indicate proteins previously reported to be acetylated.

its functions, such as DAXX, PML, PTEN, and HAUSP (*38*), are acetylated (table S1). HAUSP regulates the stability of p53 and MDM2, but less is known about its own regulation. The five HAUSP acetylation sites reported here represent a possible mechanism with which to modulate its activity. Collectively, these results indicate that not only p53 but many of the core proteins of the p53 circuitry also probably are regulated by acetylation.

A large number of proteins involved in chromatin remodeling and cell cycle are acetylated (table S1). For example, CDC2, a major cyclin-dependent kinase and regulator of S-phase progression and mitosis, was acetylated at two different lysines: K6 and K33. K6 is conserved between CDC2 and CDK2 and was acetylated on both kinases. The K33 acetylation site is located within the kinase domain and is important for the coordination of adenosine 5′-triphosphate (ATP) binding. This lysine is conserved even in the budding yeast homolog. Indeed, acetylation of yeast Cdc28 was confirmed by means of protein immunoblotting (fig. S7A). MS data furthermore confirmed that Cdc28 is acetylated on K40 (fig. S7B), a residue analogous to K33 of CDC2. To address functional consequences of altering this residue, we mutated K40 in yeast Cdc28 to arginine, which is charge-conserving, or to glutamine, which is noncharged and may mimic acetylated lysine. Both point mutants failed to rescue *cdc28*Δ yeast strains (Fig. 4A). This lysine therefore appears to be critical to the proper function of Cdc28 in yeast. CDK9, a non–cell-cycle kinase, is acetylated on the analogous site, and acetylation inhibits its kinase activity (*39*). CDK6, another cell-cycle kinase, and PRPF4B (PRP4 pre-mRNA–processing factor 4 homolog B), a kinase involved in mRNA splicing, were also acetylated on the ATP-binding lysine. Thus, lysine acetylation may have a wider role in the regulation of kinase activity. Acetylation of SMC3 (structural maintenance of chromosomes 3) in yeast and human is important for the separation of sister chromatids (*40*). We found 22 sites, including two recently described ones (*39*), on all major SMC proteins that are part of the cohesion complex (SMC1A, -2, -3, -4, and -5). These data point to a larger role of acetylation in the regulation of the cohesion complex and thus separation of sister chromatids. We also found acetylation of many other cell-cycle regulators, such as TPX2, NUMA1, cyclin T1 and B1, BUB3, CDK6, and STMN1. As shown in Fig. 3C, acetylated cell-cycle proteins are highly connected by functional and physical protein-protein interactions.

Interactions of nuclear receptor–binding proteins to nuclear receptors via LXXLL motifs (where X represents any amino acid) regulate transcriptional activation. Acetylation of nuclear receptor coactivator 3 (NcoA3) near LXXLL motifs regulates these interactions (*41*). We found that both NcoA2 and NcoA3 are acetylated within their LXXLL motifs, which is likely to influence binding properties and therefore the function of these important nuclear receptor coactivators.

Nuclear pores are the gatekeepers of nucleocytoplasmic transport and, together with the RAN guanidine triphosphate cycle, control protein traffic. To our knowledge, acetylation has not been implicated in the regulation of nuclear transport. We discovered that several constituents of this machinery are acetylated (Fig. 3D). RANGAP1 [RAN–guanosine triphosphatase (GTPase)–activating protein 1] is acetylated on K524, the same lysine residue that has an important role in the regulation of nuclear transport when modified by sumoylation (*42*). Acetylation of this lysine abolishes sumoylation and thus may regulate nuclear transport.

Cytoplasmic complexes regulated by acetylation. Acetylation of tubulin regulates microtubule function (*43*). We found several additional acet-

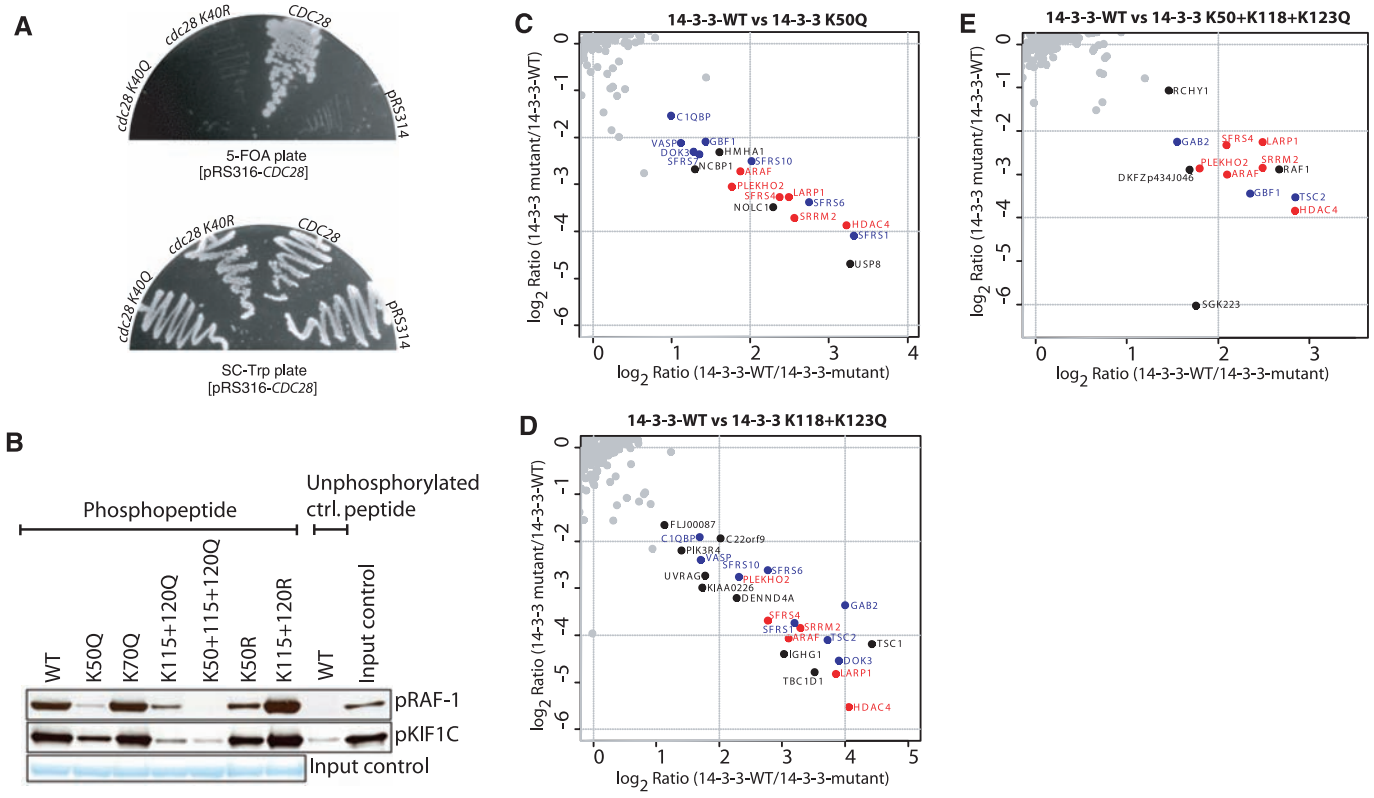

Fig. 4. Acetylation of Cdc28 and 14-3-3 impair their functions. (**A**) Mutation of acetylation site K40 on Cdc28 impairs its function. Growth of haploid yeast strains harboring empty vector or plasmids expressing *CDC28*, *cdc28-K40R*, or *cdc28-K40Q* in a *cdc28*Δ strain were tested for growth on 5-FOA plates, which select against the wild-type copy of *CDC28* on a separate URA3-marked plasmid. SC-Trp plates are shown as control. (**B**) Mutation of acetylated lysine on 14-3-3-ε abolishes binding to RAF1 and KIF1c phosphopeptide ligands. Binding of recombinant GST–14-3-3 proteins to phosphopeptides was analyzed by means of peptide pull-down assays. (**C** to **E**) Mutation of K50Q and K118+K123Q impairs binding of 14-3-3 to full-length proteins. Proteins interacting with 14-3-3 wild type (WT) or mutants were identified and quantified by use of SILAC-based MS as described in fig. S9. Proteins in red are quantified in all six experiments, and proteins that were quantified in four or more experiments are blue. The *x* axis shows the relative binding of 14-3-3–WT compared with the indicated 14-3-3 mutants (log2 SILAC ratio for WT/mutant). The *y* axis shows the relative binding of the indicated 14-3-3 mutants compared with 14-3-3–WT (log2 SILAC ratio for mutant/WT).

ylation sites on various tubulin isoforms. The machinery controlling actin-based cell motility is also highly acetylated (Fig. 3E). All but one of the subunits of the ARP2/3 complex, the major actin nucleation complex, are acetylated, as are cortactin, coffilin, and coronin, which interact with the ARP2/3 complex (table S1). Of the nine acetylation sites identified on purified cortactin (*44*), we confirmed five and identified two others. Thus, regulation of cytoskeleton reorganization and cell motility by lysine acetylation may involve many more proteins than tubulin and cortactin.

The function of chaperone protein HSP90 is inhibited by acetylation (*45*). Hyperacetylation of HSP90 is thought to be a major effector through which KDAC inhibitors interfere with protein folding, and one acetylation site on HSP90-α is known (*45*). We could not confirm this site, but we found HSP90-α to be acetylated on at least 14 lysines and HSP90-β on a minimum of four. Likewise, all the eight subunits of TCP1 or TriC chaperone complex (CCT1 to -8) were also heavily acetylated (Fig. 3F).

14-3-3 proteins are widely expressed proteins that specifically bind to phosphoserine or phosphothreonine and regulate a diverse set of cellular processes, such as signal transduction, cell cycle, and DNA damage repair (*46*). 14-3-3 proteins also interact with HDAC1, HDAC4, and HDAC5. Multiple isoforms of 14-3-3 were acetylated in different cell types (table S1 and fig. S8A). Moreover, analogous sites were acetylated in multiple isoforms (fig. S8B).

To understand the role of 14-3-3 acetylation and its impact on the interaction of 14-3-3 with phosphorylated peptides, we mutated four lysines (K50, K69, and K118+K123) that are acetylated in vivo and are highly conserved among 14-3-3 proteins (*47*). Acetylated lysines in 14-3-3-ε were mutated either to glutamine (Q), so as to mimic acetylated lysine, or arginine (R), so as to prevent acetylation but conserve a positive charge. Mutation of K50 to glutamine reduced binding to two model phosphopeptides, and mutation of both K118 and K123 severely impaired its binding (Fig. 4B). In contrast, K118+K123R showed no such binding deficiency. A triple acetylation mimetic mutant (K50+K118+K123Q) did not bind phosphopeptides. To further confirm the importance of these sites, we performed pull-downs using glutathione *S*-transferase (GST)–13-3-3-ε fusion protein or its mutants and quantified proteins that specifically bound to 14-3-3 (fig. S9). The acetylation mimetic mutants of 14-3-3 (K50Q, K118+K123Q, and K50+K118+K123Q) showed impaired binding to synthetic peptides as well as to full-length proteins from whole-cell lysates (Fig. 4, C to E). Among the proteins that showed decreased binding to acetylation, mimetic mutants of 14-3-3 in these five are known to bind (RAF1, HDAC4, TSC22, GAB2, and ARAF), which independently validates the results obtained with peptide associations. The crystal structure of 14-3-3 bound to its phosphopeptide ligand confirms the importance of K50 and K123 in binding to its ligand (*48*). Thus, our results uncover a mechanism that modulates phosphorylation-dependent interactions on the side of the phosphopeptide-binding domain and suggest crosstalk between phosphorylation and acetylation.

Our data also contain many further interesting leads for functional studies in a wide variety of biological areas. For example, an unexpected role of acetylation was recently reported for the activation of signaling from the interferon-α receptor (*49*). We identified acetylation sites in mediators of interferon actions such as DDX58, IRAK4, OAS2, and TRIM25. These data further strengthen the notion that acetylation may regulate innate immune responses.

Quantitative acetylation analysis upon KDAC inhibition. The clinical success of KDAC inhibitors for specific cancers as well as their promise for treating other diseases make it important to identify their targets at a site-specific level. We used SILAC to quantify downstream targets of SAHA and MS-275 in three different cell lines (fig. S1A). SAHA is reported to have broad inhibitory activity and to target many KDACs, with the exception of sirtuins (*6*). In contrast, MS-275 is thought to inhibit only three class I KDACs. Both inhibitors increased acetylation of specific histone sites, but neither changed acetylation of mitochondrial proteins, which are thought to be deacetylated by sirtuins. The inhibitors only upregulated about 10% of all acetylation sites by at least a factor of two (table S4). Thus, both inhibitors were unexpectedly specific in vivo. Acetylation sites that were regulated more than twofold by both of these inhibitors in the triple-SILAC experiment in MV4-11 cells are listed in table S5. SAHA is a more potent inducer of histone acetylation than MS-275 (fig. S6). For example, a number of sites on the core histones H3 and H4 are several times more highly regulated in response to SAHA than by MS-275 (table S5). Acetylation of the variant histone H2AZ—a mark for DNA damage sites—was upregulated almost 20-fold by SAHA. The fact that acetylation of all histone sites is not equally increased suggests that these drugs target the histone deacetylases differently. Important cytoplasmic targets, such as HSP90α and -β, were highly acetylated in the presence of SAHA but not in cells treated with MS-275 (fig. S1B). Conversely, the acetylation levels of four out of five sites on p53 were increased by MS-275 but not at all by SAHA.

Discussion. We describe a streamlined methodology for the proteome-wide identification and quantitation of acetylation sites and provide an in-depth view of the in vivo acetylome. We identified 3600 acetylation sites and implicated acetylation in the regulation of a diverse set of cellular functions. The data are available to the scientific community via Phosida (www.phosida.com) (*28*), a public database that is a member of ProteinExchange and provides detailed information about each acetylation site, such as evolutionary conservation and local secondary structure prediction of the protein sequence around the acetylation site.

The overall picture of lysine acetylation resulting from our experiments is that it contributes to regulation of almost all nuclear functions and to the control of a surprisingly large array of cytoplasmic functions as well. The relatively high overlap of the measured acetylome in three cell lines and the fact that we covered most of the in vivo sites described in the literature indicate that we sampled a substantial part of all acetylation sites. The several thousand sites detected here place the size of the measured acetylome between that of the phosphoproteome, in which tens of thousands of sites are now quantifiable, and that of the spectrum of ubiquitinated proteins, for which only a few hundred sites are currently known. In common with these major PTMs, acetylation seems to have been adopted in the regulation of a wide variety of dissimilar cellular functions. A striking feature of acetylation is that it tends to occur in large macromolecular complexes.

The application of quantitative proteomics enabled us to assess the effects of perturbing the acetylation network in a global and unbiased manner. KDAC inhibitors were much more specific than generally thought, potentially shedding new light on their unexpected clinical specificity.

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Materials and Methods
Figs S1 to S9
Tables S1 to S5
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Detection of 16 Gamma-Ray Pulsars Through Blind Frequency Searches Using the Fermi LAT

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Pulsars are rapidly rotating, highly magnetized neutron stars emitting radiation across the electromagnetic spectrum. Although there are more than 1800 known radio pulsars, until recently only seven were observed to pulse in gamma rays, and these were all discovered at other wavelengths. The Fermi Large Area Telescope (LAT) makes it possible to pinpoint neutron stars through their gamma-ray pulsations. We report the detection of 16 gamma-ray pulsars in blind frequency searches using the LAT. Most of these pulsars are coincident with previously unidentified gamma-ray sources, and many are associated with supernova remnants. Direct detection of gamma-ray pulsars enables studies of emission mechanisms, population statistics, and the energetics of pulsar wind nebulae and supernova remnants.

A wide variety of astrophysical phenomena, such as black holes, active galactic nuclei, gamma-ray bursts, and pulsars, are known to produce photons with energies exceeding tens of megaelectron volts. Detection and accurate localization of sources at these energies is challenging because of the low fluxes involved and the limitations of the detection techniques. The sky above 100 MeV was surveyed more than 30 years ago by the COS-B satellite (*1*) and more recently by the Energetic Gamma Ray Experiment Telescope (EGRET) (*2*) onboard the Compton Gamma Ray Observatory. One of the main legacies of EGRET was the detection of ~300 gamma-ray sources, many of which have remained unidentified despite searches at a wide variety of wavelengths (*3*). Many EGRET (and COS-B) unidentified sources are thought to be of galactic origin because of their lack of variability and concentration along the galactic plane. A large fraction of these have been suspected to be pulsars [e.g., (*4*–*6*)] despite deep radio and x-ray searches often failing to uncover pulsed emission, even when the gamma-ray sources were coincident with supernova remnants (SNRs) or pulsar wind nebulae (PWNs). The lack of radio pulsations has usually been explained as the narrow radio beams missing the line of sight toward Earth (*7*). We refer to such pulsars as "radio-quiet"; even though they may emit radio waves, these cannot be detected at Earth. Before the launch of the Fermi Gamma-ray Space Telescope, Geminga (*8*) was the only known radio-quiet gamma-ray pulsar. Current models of pulsar gamma-ray emission predict that gamma-ray beams are much wider than radio beams (*9*), thus suggesting that there may be a large population of radio-quiet gamma-ray pulsars.

Soon after launch on 11 June 2008, the Large Area Telescope (LAT) onboard Fermi began surveying the sky at energies above 20 MeV. A companion paper describes the LAT detection of a population of gamma-ray millisecond pulsars (MSPs) (*10*). Here we report the detection of 16 pulsars found in blind frequency searches using the LAT. Previously, gamma-ray pulsars had been detected only with the use of a radio (or, in the case of Geminga, an x-ray) ephemeris.

Observations and data analysis. The LAT is a high-energy gamma-ray telescope sensitive to photon energies from 20 MeV to >300 GeV, featuring a solid-state silicon tracker, a cesium-iodide calorimeter, and an anticoincidence detector (*11*). Gamma-ray events recorded in the LAT have time stamps derived from a GPS clock on the Fermi satellite with an accuracy of <1 μs (*12*). The LAT operates in continuous sky survey mode, covering the entire sky every 3 hours. Relative to EGRET, the LAT has a larger effective area (9500 cm^2 at normal incidence), larger field of view (2.4 sr), more efficient use of time on orbit for photon collection, and a finer point spread function (5° at 100 MeV, 0.8° at 1 GeV). The first three factors result in more rapid photon accumulation, and the fourth increases the signal-to-noise ratio by improving the background rejection.

Editor's Summary

Lysine Acetylation Catalog

Covalent posttranslational modification is an essential cellular regulatory mechanism by which the activity of proteins can be controlled. Advances in mass spectrometry made it possible for **Choudhary *et al.*** (p. 834, published online 16 July) to assess the prevalence of lysine acetylation throughout the whole proteome. Acetylation is much more widespread than previously appreciated and occurs on proteins participating in all sorts of biological functions. Acetylation can influence susceptibility of proteins to phosphorylation and occurs frequently on enzymes that control the modification of other proteins by covalent ubiquitination and on proteins that form large macromolecular complexes. The findings also help to characterize the actions of lysine deacetylase inhibitors, which have shown clinical promise in treatments for cancer.