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Multiple Functional Categories of Proteins Identified in an in Vitro Cellular Ubiquitin Affinity Extract Using Shotgun Peptide Sequencing

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To construct a high information content assay for examination of the function of the cellular ubiquitin system, we added his-tagged ubiquitin, ATP, and an ATP-regenerating system to endogenous human cellular ubiquitin system enzymes, and labeled cellular proteins with hexa-histidine tagged ubiquitin in vitro. Labeling depended on ATP, the ATP recycling system, the proteasome inhibitor MG132, and the ubiquitin protease inhibitor ubiquitin aldehyde, and was inhibited by iodoacetamide. Quadruplicate affinity extracted proteins were digested with trypsin, and the peptides were analyzed by 2D capillary LC−MS/MS, SEQUEST, MEDUSA, and support vector machine calculations. Identified proteins included 22 proteasome subunits or associated proteins, 18 E1, E2, or E3 ubiquitin system enzymes or related proteins, 4 ubiquitin domain proteins and 36 proteins in functional clusters associated with redox processes, endocytosis/vesicle trafficking, the cytoskeleton, DNA damage/repair, calcium binding, and mRNA splicing. This suggests a link between the ubiquitin system and these cellular processes. This map of cellular ubiquitin-associated proteins may be useful for further studies of ubiquitin system function.

Keywords: ubiquitin • proteasome • proteomics • tandem mass spectrometry • shotgun peptide sequencing

Introduction

Ubiquitination is a eukaryotic post-translational modification that targets substrate proteins for recognition and degradation by the 26S proteasome. The 76mer protein ubiquitin is covalently attached to substrate proteins by an E1 ubiquitin activating enzyme/ E2 ubiquitin conjugating enzyme/E3 ubiquitin ligase cascade.[1] Proteasome degradation of polyubiquitinated proteins regulates a variety of cellular processes[1] such as organ development, the cell cycle, proliferation, differentiation and apoptosis, antigen processing, release of membrane bound transcription factors, mRNA synthesis, TGF-beta signal transduction, endocytosis,[2] TNF-alpha related muscle wasting, and DNA repair.[3]

Ubiquitination is also used for processes other than direct proteasomal protein degradation. Monoubiquitination may serve a regulatory function,[4] and has been linked to ligand-induced receptor internalization and endosomal sorting,[5] the fanconi anemia signaling pathway, the response to DNA damaging agents and oxidative stress, histones and the control of chromatin dynamics during transcription,[6] and the release and maturation of infectious HIV particles.[7] Ubiquitin may be part of the retroviral budding machinery.[8] The ubiquitin−proteasome system is involved in a variety of diseases, including Parkinson's disease, Huntington's disease, Alzheimer's disease, Angelman syndrome, cystic fibrosis, muscle wasting, immune and inflammatory disorders, and cancer.[9]

A map of ubiquitinated cellular proteins may be useful for examining changes in ubiquitination in different cellular states, the effects of internal or external cellular perturbations such as the addition of inhibitors of enzymes of the ubiquitin-proteasome system or other proteins linked to this system, the effects of overexpression or deletion of macromolecules of functional interest, or disregulation of the ubiquitin-proteasome system in disease states. Affinity extraction of pig brain ubiquitinated proteins using a GST-proteasome subunit S5a fusion identified the rad23 protein.[10] Because S5a tightly binds chains with 4 or more ubiquitins,[11] such extracts should selectively isolate proteins with longer attached polyubiquitin chains. To isolate monoubiquitinated and polyubiquitinated proteins and their associated cellular proteins, we have added his-tagged ubiquitin[12] to lysed Hela cells. Utilizing endogenous ubiquitin system enzymes we have labeled cellular proteins in the presence of added ATP, an ATP regenerating system, and

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proteasome and ubiquitin protease inhibitors. Using quadruplicate affinity extractions, 2D strong cation exchange/reversed phase microcapillary hplc-quadrupole time-of-flight tandem mass spectrometry of peptides obtained from tryptic digests of the affinity extracts, SEQUEST[13] and MEDUSA[14] analyses, and support vector machine calculations[15] we have identified an array of ubiquitin-associated proteins. This set of proteins contains a variety of different functional clusters. This map may be useful for further examination of the function of these ubiquitinated proteins or their interacting partners in different cellular processes.

Experimental Section

$(His)_6$-Ubiquitin Conjugation Reactions and Affinity Chromatography. HeLa adenocarcinoma cells (ATCC, Manassas, VA) were cultured in modified DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (all from Mediatech, Herndon, VA) in a 37 °C incubator with 5% CO_2. Adherent asynchronously growing cells were harvested after 48 h by trypsinization followed by low speed centrifugation. Cells washed twice in isotonic phosphate buffered saline (Mediatech) were lysed in 50mM pH 7.5 HEPES buffer containing 100 mM KCl, 0.2% v/v NP-40, and 5 mM $MgCl_2$ (all from Sigma Chemical Co., St. Louis, MO), 0.5 mM tris[2-carboxyethyl] phosphine (Pierce Chemical Co., Rockford, IL), in a nitrogen cell disruption bomb (Parr Instrument Company, Moline IL). After low speed centrifugation, the supernatant was centrifuged at 100 000 × *g* for 1 h in a Sorvall RCM120EX mini-ultracentrifuge (Newton, CT). For the in vitro conjugation reaction, 2.15 mL of HeLa S-100 fraction (5 mg/mL protein) was used, with 1 mg purified his_6-ubiquitin, 5 μM ubiquitin aldehyde, and 10 μM MG132 (all from Boston Biochem, Boston MA). Labeling was initiated by addition of an ATP regeneration system containing 2.5 mM ATP, 2.5 mM magnesium chloride, 10 mM phosphocreatine and creatine kinase (Boston Biochem, Boston MA) at 20 C. Control experiments included a reaction mixture without the ATP regeneration system as well as pretreatment of cell-lysates with 10 mM iodoacetamide (Sigma Chemical Co., St. Louis MO) to block endogenous E1/E2 activity.[10] After the reaction, the mixture was desalted on a PD10 column (Amersham Biosciences, Piscataway, NJ) equilibrated in pH 7.5 PBS containing 0.3 M sodium chloride and 10 mM imidazole, and loaded onto a 1.5 mL column containing immobilized nickel-nitrilotriacetate resin (Sigma Chemical Co., St. Louis, MO). This column was washed with 20 column volumes of the same buffer, buffer at pH 6.0, buffer at pH 5.5, and finally PBS.

All affinity extractions and controls were done in quadruplicate and the washed beads containing protein complexes were stored at 4 °C until analyzed**.** Proteins eluted from the nickel beads were separated by SDS−PAGE using a 4−20% Tris-glycine PAGE system and Western blotted as per instructions (Novex, San Diego, CA). Proteins electroblotted onto PVDF membranes were probed with an anti-his tag antibody (Santa Cruz Biotechnology, Santa Cruz CA) or anti-ubiquitin antibody (Zymed, South San Francisco, CA) and developed using an ECL plus enhanced chemiluminescence reagent kit followed by detection on ECL hyperfilm (Amersham Biosciences, Piscataway NJ).

Proteolytic Digestion of IMAC Affinity Extracts. The his-tagged ubiquitin-protein conjugates bound to the Ni−NTA resin (50 μl bed volume) were incubated with 50 μl of 8 M urea containing methylamine (20 mM), LiCl (1 M), EDTA (2 mM) in Tris-HCl buffer (0.1 M, pH 8.5) for 1 h at room temperature with constant mixing. Proteins were reduced by dithiothreitol and carboxamidomethylated[14]. Lys-C endoproteinase (Boehringer Mannheim, Germany) was added to the protein solution (diluted to 4 M urea) to a final concentration of 5 μg/mL, and incubated at 37 °C for 15 h. Modified trypsin (Boehringer Mannheim, Germany) was added to the solution (diluted to 2 M urea) to a final concentration of 25 μg/mL and incubated overnight at 37 °C. Proteolysis was stopped by the addition of 1 μL glacial acetic acid and the samples stored at −80 C until analyzed.

Microcapillary 2D LC−MS/MS. Five μL of the trypsin digest was desalted on a C18 guard column (Vydac, Hesperia, CA) offline, and was injected onto a biphasic 16 cm × 75 μm i.d. microcapillary HPLC column with a fused silica PicoFrit 15μ spray tip (New Objective Inc., Woburn MA). The first phase of the column contained 4 cm of polysulfoethyl aspartamide strong-cation exchange media (PolyLC, Columbia, MA), and the second phase contained 12 cm of reversed-phase Aquasil C_{18} 3 μm resin (Thermo Hypersil, Keystone Scientific, Bellefonte, PA). Separation of the tryptic peptides used step elutions of 5, 10, 20, 50, 100, and 500 mM ammonium acetate containing 5% v/v acetonitrile and 0.5% pH 4 acetic acid in the first dimension. Second dimension reversed phase elution was from 100% buffer A (5% acetonitrile, 0.5% acetic acid) to 80% buffer B (80% acetonitrile, 0.5% acetic acid) for 2.5 h at a flow rate of ca. 200 nl/min. The capillary column was eluted by an LC Packings (San Francisco, CA) Ultimate microcapillary HPLC. Tandem mass spectrometry used a quadrupole time-of-flight tandem mass spectrometer (Micromass, Manchester, UK).

Database Searches and Bioinformatics. MS/MS data files from reversed phase hplc runs for all of the ion exchange steps were combined into one file for each affinity extraction. These data were digitally compared with concatenated files for all four controls using the program MS2filter and MS/MS spectra in any control run were removed. Remaining mass spectra were analyzed using TurboSequest (ThermoFinnigan, San Jose, CA), which uses the SEQUEST algorithm,[13] against a human protein sequence database derived from the NCBI nonredundant human database. Results were summarized and further analyzed using the Oracle 8.0 database MEDUSA.[14] Websites used to examine domains and motifs present in identified proteins include emotif (http://dna.stanford.edu/cgi-bin/emotif), the ISREC profile scan (http://hits.isb-sib.ch/cgi-bin/PFSCAN), blocks (http://blocks.fhcrc.org), pfam (http://pfam.wustl.edu), NCBI entrez (http://www.ncbi.nlm.nih.gov:80/entrez) and clustal W (http://clustalw.genome.ad.jp/).

Support Vector Machine Analysis. Proteins present in at least two of the four affinity extracts were subjected to further analysis. Peptide data used for the analysis included 13 parameters.[15] Three parameters are properties of the observed peptide: mass, charge, and ion current. SEQUEST-generated parameters included Xcorr, delta Cn, RSp, Sp, and percent y and b ion match. Four additional parameters used to evaluate the peptides included the sequence similarity of the top two SEQUEST-matched proteins for each peptide, the fraction of the peptide MS/MS ion current matched by each tryptic peptide, the fraction of the total number of MS/MS peaks matched by predicted peptide ions, and the total number of MS/MS peaks observed. A training set obtained on the same quadrupole time-of-flight mass spectrometer under similar conditions, containing positive and negative peptides, was used to analyze the peptides found in these experiments. Proteins

identified by fewer than three different peptides were selected, and peptides used for these identifications were analyzed for the correctness of the match between the 13 parameter data and the peptide sequence using support vector machine calculations.[15] Discriminant scores produced by the SVM were converted to probabilities by fitting a sigmoid function using a held-out portion of the training data.[16] Proteins identified using one or more peptides with an 80% or higher probability were examined using different bioinformatics tools. Identification of proteins by one or two peptides was checked for ambiguity by BLAST analysis[17]. Proteins identified using 13 parameter data predicted to be correctly matched to a peptide sequence with a probability of 50−79% are listed in the results section but are not discussed in detail except as noted.

Results

In Vitro Ubiquitin Labeling and Affinity Extraction. Preliminary experiments examined several methods for epitope-tagging ubiquitinated proteins before their affinity extraction[18]. Retroviral delivery of N-flag tag-labeled ubiquitin resulted in labeling of a number of spots on 2D gels; however, the extracted protein yield was low, perhaps due to dilution of tagged ubiquitin with endogenous cellular ubiquitin during labeling. S5a binds polyubiquitin containing at least four ubiquitin monomers;[11] thus, this approach may miss monoubiquitinated proteins. To include these proteins, we examined in vitro labeling using N-his$_6$-epitope tagged ubiquitin. This approach also allows direct addition to the cell lysate of inhibitors such as ubiquitin aldehyde that might not penetrate the membrane of live cells.

Figure 1A shows the in vitro ubiquitination of Hela cell lysate proteins using added N-his$_6$-ubiquitin as a label, as detected by anti-his tag Western blotting. Cells were lysed in phosphate buffered saline containing 0.2% NP-40, and 2.5 mM ATP and an ATP regenerating system containing 10 mM phosphocreatine and creatine kinase were added. In the presence of the full regeneration system (lanes 3 and 3′) multiple high molecular weight bands were generated in a time-dependent fashion. Bands corresponding in mass to the his$_6$-ubiquitin monomer (∼9 kDa), tetramer (∼36 kDa), and pentamer are present, as well as many other bands. Removal of ATP and the ATP regeneration system (lanes 1 and 1′) resulted in few if any detectable bands, suggesting incorporation of labeled his$_6$-ubiquitin was ATP-dependent. Addition of the E1 ubiquitin activating enyme and E2 ubiquitin conjugating enzyme inhibitor iodoacetamide[10] blocked the appearance of almost all bands (lanes 2 and 2′), consistent with the importance of these enzymes in the appearance of the bands. Addition of the ubiquitin protease inhibitor ubiquitin aldehyde and the proteasome inhibitor MG132 (lanes 4 and 4′) also resulted in the disappearance of most of the bands. These results are consistent with the incorporation of his$_6$-ubiquitin into protein conjugates, some of which may include tetramers and larger polyubiquitin chains. Figure 1B shows the cross-reactivity of proteins with the anti-his tag antibody and an anti-ubiquitin antibody in cell lysates and anti-his tag affinity extracts. The silver stained gel shows differential protein extraction by the anti-his tag antibody. Based on densitometry of the silver stained gel, ca. 10% of the added his$_6$-ubiquitin was incorporated into other proteins. These results are consistent with the ATP-dependent labeling and differential extraction of a number of proteins using this epitope tag after addition of his$_6$-ubiquitin, proteasome and ubiquitin protease inhibitors. Figure 1C shows the MS/MS spectra of two ubiquitin-like proteins identified by this procedure, rad23 homologue B and the ubiquitin-like 4 protein.

Affinity Extracted Proteins. Identified proteins could be grouped into different categories, summarized in Figure 2. One category includes proteasome subunits or proteasome-associated proteins (Table 1). Of the 32 or more 26S proteasome subunits,[19] 22 were identified. Sixteen were from the 19S regulatory particle. A lid complex subunit (subunit 3) was isolated, as were four alpha and beta subunits of the 20S core particle. Two deubiquitinating enzymes and two proteins that bind ubiquitin chains were isolated. A valosin containing protein was isolated which purifies with the 26S proteasome[20] and targets multi-ubiquitinated proteins to the proteasome. This could explain the presence in the affinity extracts of a number of known ubiquitinated proteins. An 805mer homologue of this protein that is 93% identical in sequence over the length of the shorter protein was also isolated.

Proteins in seven additional categories were also identified. A second category includes other ubiquitin-proteasome system proteins (Table 2). Seven E3 ligases or ring finger proteins were identified, as were 7 ubiquitin conjugating enzymes or homologues and 3 ubiquitin hydrolases or homologues (UCH 11 and 37, and elongation factor 1 alpha). The most prominent proteins, represented by the largest number of peptides, were N-recognin (responsible for recognition of N-end rule pathway substrates[21]), the E3 ligase hyd,[22] Rb associated protein p600, and the E2 ubiquitin conjugating enzyme IAP repeat-containing 6 protein. A progestin-induced protein E3 ligase similar to hyd was also isolated.

A third category of proteins includes ubiquitin and ubiquitin domain proteins (Table 2)**.** Two tryptic peptides identified ubiquitin. Three different peptides identified BAT3, a large proline-rich protein with a ubiquitin-like domain near its N-terminus.[23] UBL4/GDX was identified by two peptides. Rad23 homologue B, which has one ubiquitin-like and two UBA ubiquitin binding domains, was identified by one peptide. It binds the Machado−Joseph disease protein MJD1/josephin[24] which was also isolated. The ubiquitin domain protein elongin B was also isolated.[25]

A fourth category includes seventeen proteins known to be ubiquitinated, or proteins associated with ubiquitin−proteasome system proteins (Table 3). Insulin receptor substrate 2,[26] histone H2A,[27] and possibly vimentin are ubiquitinated in vivo. CENP E binding to kinetochores is regulated by its ubiquitination.[28] Epidermal growth factor receptor pathway substrate 15 (Eps15) is monoubiquitinated.[29] HnRNP-U is a pseudosubstrate for the SCF E3 ubiquitin ligase.[30] KIAA1578 has a WWE domain found in ubiquitin conjugating systems. Proteins which bind known ubiquitinated proteins, such as c-myc binding protein[31] or the thyroid hormone receptor binding protein[32] were also found.

A fifth category includes proteins not known to be ubiquitinated (Table 4). These can be grouped into several functional subsets. One includes five cytoskeletal proteins. A second includes redox proteins, four with thioredoxin domains and two related to protein disulfide isomerase. A third includes four proteins related to DNA damage or DNA repair. Two members of a putative E cadherin complex, p120 catenin and the ras GTPase activating protein IQGAP1, were identified. Four calcium binding proteins, including the ligand pair annexin II and

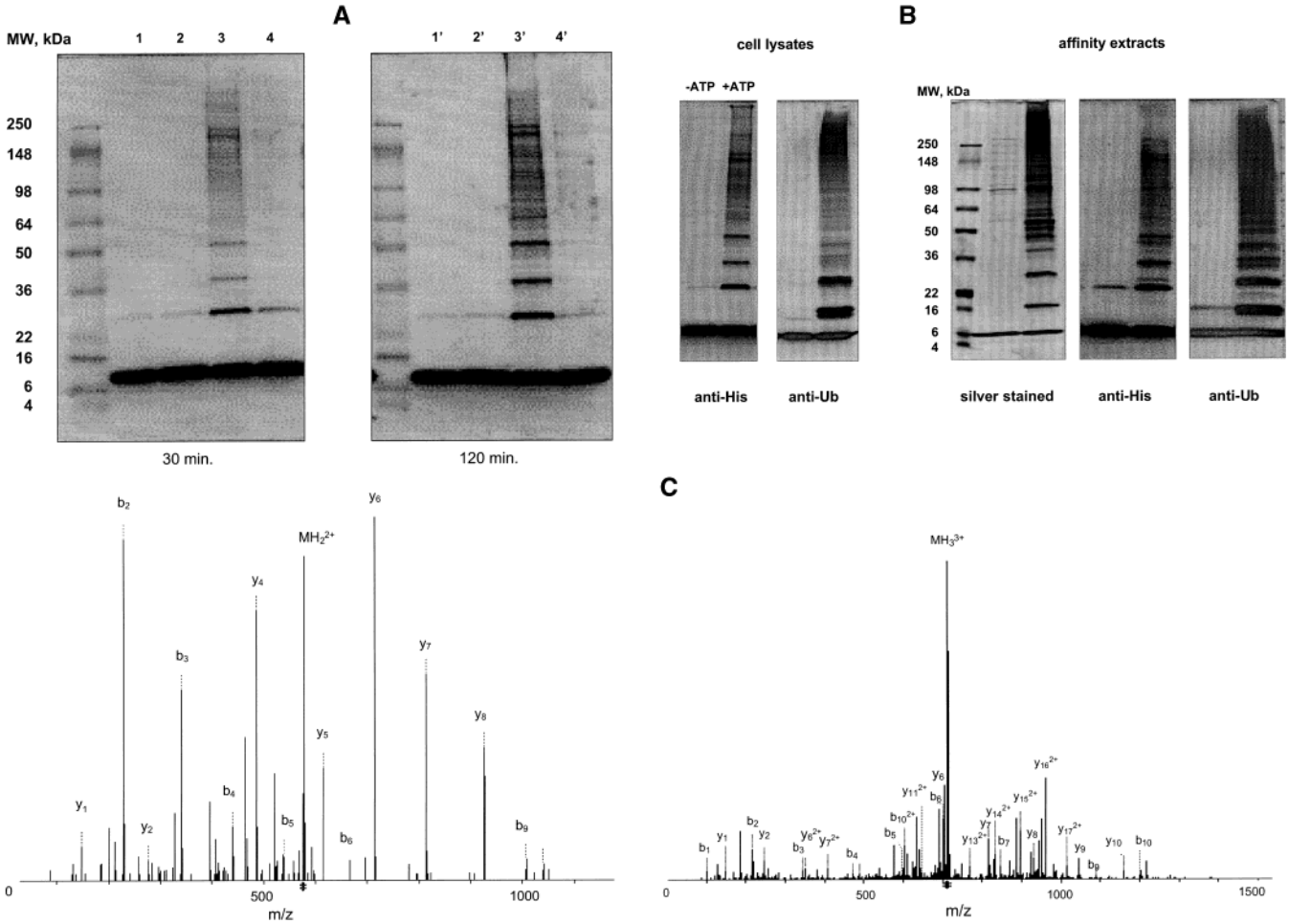

Figure 1. In vitro ubiquitination of cell lysate proteins. **A.** In vitro ubiquitination of cell lysate proteins using his_6-ubiquitin as a label, visualized as an anti-his tag Western blot of cell lysate incubated with different combinations of reagents. HeLa cells were lysed in 0.2% NP-40 and incubated with N-his_6-tagged ubiquitin, and when present an ATP regenerating system, the ubiquitin protease inhibitor ubiquitin aldehyde, the proteasome inhibitor MG132, and the E1 ubiquitin activating enzyme and E2 ubiquitin conjugating inhibitor iodoacetamide. **1**−**4**, 30 min. incubation of reagents in cell lysate followed by SDS-PAGE and Western blotting. **1**, incubation with his-ubiquitin, ubiquitin aldehyde, MG132, but no ATP recycling system. Few higher molecular weight his-ubiquitin bands are detected. The major band at ca. 9 kDa is his-ubiquitin. **2**, incubation with these reagents, the ATP recycling system, and the E1 and E2 inhibitor iodoacetamide. This is the full in vitro ubiquitin labeling system, but with the E1 and E2 inhibitor iodoacetamide added. This inhibitor abolishes the generation of higher molecular weight his-ubiquitin bands. **3**, incubation as in 2 without iodoacetamide; this is the complete in vitro ubiquitin labeling system. **4**, incubation with all reagents in 3 except ubiquitin aldehyde and MG132. In the absence of these inhibitors, most of the higher molecular weight bands disappear, suggesting that inhibition of this catabolism is necessary for significant accumulation of the his-tagged bands. **1′**−**4′.** These lanes show the 120 min. timepoint for the same reactions as in 1−4. The intensity of bands in lane 3′ has increased but the other lanes still have few bands; the creation of higher molecular weight bands is time-dependent. **B.** Detection of ubiquitinated proteins in Hela cell lysates and nickel-chelate affinity chromatography extracts of in vitro labeled cells. Left panels, blot of cell lysates 120 min. after addition of his_6-ubiquitin and the ATP regeneration system. Anti-his tag and anti-ubiquitin antibodies were used in the absence (lane 1) and presence (lane 2) of ATP and the ATP regenerating system. Ladders of proteins cross-reactive with both the anti-his tag and anti-ubiquitin antibodies are visible. Right panels, affinity extracts using nickel-chelate chromatography. A silver stained gel of in vitro labeled cellular proteins shows extraction of more proteins in the presence than in the absence of ATP and the ATP regeneration system. Anti-his tag and anti-ubiquitin Western blots show the same result. **C.** MS/MS spectra of two ubiquitin like proteins identified in the ubiquitin map. Left: spectrum of the ubiquitin-like 4 peptide LNLVVKPLEK; y and b ions are as indicated. Right: spectrum of the rad23 homologue B peptide TLQQQTFKIDIDPEETVK.

caplactin/S100, were observed. Five proteins related to splicing factors, and a WD repeat protein were identified. Twenty-one additional proteins not in these categories were also affinity extracted.

Thirty additional proteins were identified that were predicted by SVM calculations to be positives; they have a probability of a correct match between the peptide sequence and mass spectrometry parameters of between 50% and 80%. These include the ubiquitin conjugating enzyme transcription factor IID, t-box transcription factor Tbx22, the Ig superfamily receptor LNIR, translocase of inner mitochondrial membrane 8 homologue A, methenyltetrahydrofolate cyclohydrolase, connexin 37, the protein Zeb, a disintegrin and metalloproteinase domain 8, PRO2472, transmembrane similar to mucin 1, the zinc finger protein hrx, and IL-1 receptor related protein 2. The hypothetical proteins XP_107624, XP_104755, XP_101255, XP_094661, XP_065315, XP_065730, XP_059954, XP_059750, XP_060020, XP_062074, XP_087802, FLJ10044, FLJ10154, FLJ12543, KIAA0306, KIAA0786, 214K23.2.1 isoform 1, and AAH02675 were also isolated.

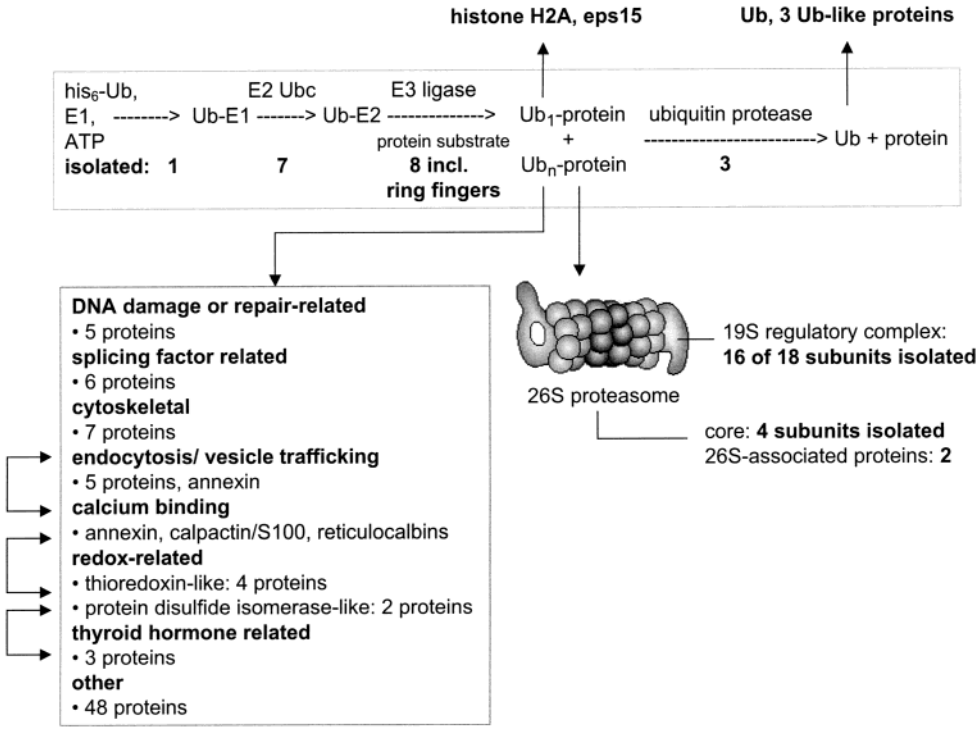

Figure 2. Summary and functional relationships of affinity extracted proteins. The number of isolated proteins in a variety of different categories are indicated in **bold.** Examples of all of these ubiquitin system proteins were isolated, as were the catabolic ubiquitin proteases and subunits of the 26S proteasome. Besides 3 ubiquitin-like proteins, multiple proteins in several functional categories were isolated, suggesting that labeling was not random but may have centered on protein complexes of defined function.

Discussion

As a first step in obtaining a set of ubiquitinated proteins or ubiquitin-interacting proteins, we have examined in vitro labeling of cellular proteins with epitope tagged ubiquitin. To maximize labeling and facilitate identification of affinity extracted proteins, catabolic pathways for ubiquitinated proteins involving the proteasome and deubiquitinating enzymes were blocked by added inhibitors. We have not added enzymes of the ubiquitin-proteasome system, thus labeling utilizes endogenous E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzymes, E3 ubiquitin ligases, their regulators, and other members of this system that may not be known. Ubiquitin levels in mammalian cells have been reported in the range of 7-300 pmol per 10^6 cells[33]; assuming a cell volume of 1 pl, this is roughly a concentration range of 10−400 μM. Our use of 50 μM ubiquitin for labeling is thus in the observed range for mammalian cells.

The extracted proteins in many cases are members of discrete functional categories, suggesting in vitro labeling with ubiquitin is not random. Identification of some of these clusters with processes known to be associated with the ubiquitin system suggests functional complexes may be associated with the labeling. A total of 244 proteins were present in at least two of the four affinity extracts, 144 were identified by peptides predicted by support vector machine calculations to be positives with over a 50% probability of being correctly sequenced), and 113 were identified by at least 3 peptides, or one or two peptides with at least an 80% chance of being correct. Twenty-two subunits of the 26S proteasome, or associated proteins, including 16 of 18 known 19S regulatory complex subunits, were isolated. Two alpha-type and two beta-type subunits from the 20S proteasome core were extracted; all were identified by 2 or more peptides. Because more peptides are present that identify 19S subunits than core subunits, higher levels of the 19S regulatory complex appear to be extracted than of the 20S core of the proteasome. The proteasome 19S regulatory complex S5a subunit binds polyubiquitin containing at least 4 ubiquitins[11] and was identified by 9 peptides. This may explain the relative abundance of the 19S complex in the affinity extract. The proteasome subunit S5a,[34] as well as the 26S proteasome subunits rpn1 and rpn2[35] bind rad23, which may explain its presence.

Several monoubiquitinated proteins were isolated, including histone H2A,[4] Sjogren's syndrome antigen,[36] and epidermal growth factor receptor pathway substrate 15 (eps15), which is important for clathrin-mediated endocytosis of transmembrane receptors.[29] One of the peptides used to identify histone H2A, VTIAQGGVLPNIQAVLLPK, contains lysine 119, which is thought to be the site of ubiquitination.[37] This peptide would not be identified by its MS/MS spectrum with an attached ubiquitin, thus it may be in a complex containing another ubiquitinated protein, or histone H2A may be ubiquitinated at another site. Endocytic vesicular sorting of monoubiquitinated proteins is facilitated by the UIM motif, which was present in KIAA1578, josephin, and eps15, all of which were isolated here. A ubiquitin map based on S5a affinity extractions might not detect these proteins. CENP-E, whose targeting to kinetochores is regulated by ubiquitination not involving proteasomal degradation,[28] was also isolated.

Another large set of isolated proteins contains enzymes and other proteins associated with the ubiquitin system. The most prominent E3 ligase was KIAA0312. Seven E2 ubiquitin conjugating enzymes or analogues were isolated, along with three ubiquitin proteases and the E1 ubiquitin activating enzyme. A

Table 1. Identification of Proteasome Subunits by in Vitro His_6-Ubiquitin Labeling and 2D LC–MS/MS

protein	function	different peptides	reproducibility
CGI-70	subunit of 19S regulatory complex; deubiquitinating enzyme UCH37; UCH-L5	14	4/4
subunit 6A	subunit of regulatory complex; HIV tat binding protein; ATPase; p27k protein	13	4/4
subunit S4	26S regulatory subunit, ATPase	13	4/4
subunit 11	26S regulatory subunit p44.5; non-ATPase; recognizes N-end rule substrates	10	4/4
subunit 2	26S regulatory subunit, ATPase, binds HIV TAT transactivator; MSS1	10	4/4
subunit 8	26S regulatory complex subunit, ATPase; p45, thyroid hormone receptor interacting protein 1	9	4/4
subunit 5a	26S regulatory subunit, binds polyubiquitin	9	4/4
subunit p44S10	KIAA0107; regulatory subunit	7	3/4
subunit 1	26S non-ATPase regulatory subunit 1, p112	7	4/4
subunit 2	non-ATPase regulatory subunit, p97, rpn1, binds p55 TNF receptor protein	6	4/4
valosin containing protein	305mer ATPase, targets multi-ubiquitinated proteins to the proteasome; purifies with 26S proteasome	6	3/4
subunit 3	26S regulatory subunit non-ATPase, PCI domain containing, p58	5	4/4
subunit 4	26S regulatory subunit 6B, ATPase, tat-binding protein 7	3	4/4
subunit 7	alpha type, isoform 4; PSMA7	3	3/4
pad1	26S regulatory subunit, homologue of yeast pad1; overexpression causes pleiotropic drug resistance	2	3/4
subunit 7	26S regulatory subunit, non-ATPase, mov34 homolog	2	4/4
similar to valosin containing protein	806mer found in small cell lung carcinomas	2	3/4
subunit 6	alpha type; similar to p27k protein	2	3/4
subunit 7	beta type	2	2/4
subunit 5b	26S non-ATPase regulatory subunit; has dileucine repeats; may be important in transmembrane protein trafficking	2	2/4
subunit 1	beta type, C5	2	2/4
similar to subunit 12	XP_093547; similar to 26S proteasome regulatory subunit 12; p55	1	2/4

number of E2 enzymes and a HECT domain E3 ligase have been reported to co-immunoprecipitate with the proteasome;[19] some of the proteins in Table 2 may have been isolated due to such an interaction. Because the E1 and E2 inhibitor and cysteine alkylator iodoacetamide was present, these enzymes may have been isolated in complexes not involving ubiquitination of their active site cysteines, but instead containing another ubiquitinated site or protein. These results suggest that E1, E,2 and E3 enzymes may exist in stable complexes containing at least one protein ubiquitin acceptor.

Other proteins may have been isolated due to their binding to the proteasome.[19] Subunit 8 of the 19S regulatory complex binds the thyroid hormone receptor, as does activating signal cointegrator 1[38] and a protein similar to thyroid hormone receptor binding protein. All three were isolated here, suggesting the extraction of a complex containing these proteins.

Two peptides from ubiquitin were isolated; they were predicted to be positives by SVM calculations, but ion currents in the range of $(0.9-9) \times 10^6$ indicate they are not present at high levels. Due to the high levels of added his_6-ubiquitin (48 μM), more peptides might have been expected. Ubiquitin is a very stable protein,[39] may have refolded after heat denaturation, and may thus not have been well digested by trypsin. Other identified ubiquitin domain proteins include BAT3, which may be involved in the control of apoptosis as well as HSP70 activity, and ubiquitin-like 4 protein, a ubiquitin-like 157mer protein. Rad23 homologue B has a ubiquitin like domain and two UBA domains. Both BAT3 and rad23 homologue B may bind the proteasome Rpn1 subunit,[35] which could explain their isolation here. These proteins may thus have been extracted along with the 19S regulatory complex of the proteasome. The ubiquitin domain protein elongin B is a part of the VHL E3 ligase complex.[25]

We have isolated seven proteins, including several novel proteins, that may be part of the response to DNA damage or DNA repair, which involves ubiquitination.[3] The HECT domain E3 ligase hyd ubiquitinates DNA topoisomerase II binding protein in the DNA damage response.[22] Levels of BRE mRNA are responsive to DNA damage.[40] Rad23 homologue B is important for nucleotide excision repair.[41] SMC6 is part of a 2 MDa complex required for both proliferation and DNA repair.[42] Eps15 has a DNA topoisomerase II motif. Expression of the KARP-1 protein is induced by DNA damage in a p53-dependent fashion and may be involved in double strand break repair.[43] Using pfam and emotif, KIAA0204 contains a 35 residue UVR domain, thought to be involved in DNA damage recognition and nucleotide excision repair. Further work will be necessary to explore the relationship of these proteins to each other, and functional significance of their relationship to the ubiquitin system.

Another class includes proteins involved in cellular redox control. One is thioredoxin, which is involved in processes such

Table 2. Ubiquitin System Proteins Identified Using in Vitro His_6-Ubiquitin Labeling and 2D LC–MS/MS

protein	function	peptides	reproducibility
KIAA0312	HECT domain protein	20	4/4
hyd	HECT domain E3 ligase impt. in DNA damage response	15	4/4
N-recognin	involved in N-end rule pathway recognition; KIAA0462; similar to Rb-associated factor 600	15	4/4
KIAA1578	has UBA and UIM ubiquitin interaction domains; has WWE domain found in ubiquitin conjugation systems	12	3/4
Rb associated protein p600	subunit of Rb-associated complex; contains zinc finger characteristic of N-recognin	9	3/4
baculoviral IAP repeat-containing 6	E2 ubiquitin conjugating BIR-domain enzyme	8	3/4
KIAA1734	E2 ubiquitin conjugating enzyme 230 kDa ortholog	5	4/4
ring finger protein 20	has zinc ring finger domain	5	3/4
UBCH7	ubiquitin conjugating enzyme	3	4/4
HSPC150	has ubiquitin conjugating enzyme region	3	3/4
FLJ20552	C3HC4 zinc ring finger protein	3	3/4
ubiquitin conjugating enzyme	E2−24 kDa ubiquitin conjugating enzyme EPF	3	4/4
Sjogren's syndrome antigen	RING finger protein that is both mono- and poly-ubiquitinated in cells[36]	2	2/4
progestin induced protein	E3 ubiquitin ligase similar to hyd protein	2	2/4
E2N	ubiquitin conjugating enzyme	1	2/4
elongation factor 1 alpha/ EF-tu	essential for ubiquitin-mediated degradation of *N*-acetylated proteins; may be a ubiquitin protease[63]	1	2/4
UCH-X	ubiquitin protease 11	1	2/4
E1 ubiquitin activating enzyme	activates ubiquitin for eventual transfer to substrates	1	2/4
ubiquitin domain proteins			
ubiquitin	present as mono- and polyubiquitin with and without a his-tag	2	3/4
BAT3	proline-rich protein with N-terminal ubiquitin-like domain[23]; may control apoptosis	3	4/4
UBL4	ubiquitin-like protein GDX or ubiquitin-like 4	2	2/4
rad23	homologue B; has 1 ubiquitin and 2 UBA domains; involved in DNA excision repair[41]; the proteasome subunit Rpn1 binds ubiquitin-like domains including rad23[24]	1	2/4
elongin B, polypeptide 2	elongation factor with a ubiquitin-like domain that is part of the VHL E3 ligase complex[25]	1	2/4

as redox control, oxidative stress response, proliferation, apoptosis and different signaling pathways.[39] The novel protein MGC14353 contains a 79 residue thioredoxin motif. The novel protein MGC3178 contains 3 thioredoxin regions and a C-terminal KDEL endoplasmic reticulum retention sequence. The novel protein BC008913 contains a 74 residue thioredoxin region. Vimentin is glutathionylated in oxidatively stressed cells[45] and binds ubiquitin related proteins such as IAP.[46] Protein disulfide isomerase ER60 precursor has a thiol-dependent reductase activity, may control extracellular and intracellular redox activities, and may be involved in oncogenic transformation.[47] The protein disulfide isomerase-related calcium binding protein ERp72[48] contains a calsequestrin domain, three thioredoxin domains and three protein disulfide isomerase active site sequences. It is a luminal endoplasmic reticulum protein with a C-terminal KEEL sequence. It is part of a large heterocomplex in the endoplasmic reticulum that folds and post-translationally modifies the thyroid hormone precursor thyroglobulin. Three other proteins related to the thyroid hormone receptor system were isolated (above); we may thus have isolated part of a complex important in thyroglobulin folding. Our results suggest a link between the ubiquitin system, this large complex, the thyroid receptor and thyroid hormone.

A set of four calcium binding proteins isolated here may be linked to the above redox proteins, to cellular proliferation, and to the ubiquitin-proteasome system. Annexin II is involved in cell proliferation,[49] intracellular calcium signaling,[50] and a variety of other functions. Annexins are important in regulated endo- and exocytosis[51] and oxidative stress-induced carcinogenesis.[52] The calcium binding protein calpactin/S100 forms a heterotetramer with annexin II.[53] Reticulocalbins 1 and 2 are endoplasmic reticulum-resident EF hand calcium binding proteins that are members of the CREC family of secretory pathway proteins.[54] Reticulocalbin is upregulated 6-fold along with other oxidation resistance enzymes in a hydrogen peroxide resistant epithelial cell line.[55] All four of these proteins were isolated from affinity extracts with anti-proliferative peptides that cause cell cycle arrest in A549 cells,[56] suggesting their participation in this phenotype. Their isolation here links their function to the ubiquitin system.

Seven proteins associated with mRNA splicing or interactions were isolated. KIAA0017 contains a 193 residue domain similar to the spliceosome associated protein splicing factor subunit 3B and a 79 residue domain similar to one in the spliceosomal protein SAP130. Splicing factor CC1.3 was isolated, as was splicing factor 3B subunit 3 (SAP130) and PTB-associated splicing factor. The protein AAH06474, which contains the RNA-recognition RRM domain and the SURP module found in splicing regulators, was also isolated. FLJ20294 contains WD repeat and WD40 domains, which are involved in RNA binding and modification reactions. The protein hnRNP−U binds the ubiquitin ligase SCF as a pseudosubstrate, binds RNA, and is thought to participate in RNA processing.[57] These results suggest an interaction between the ubiquitin system and proteins associated with RNA splicing and other RNA interactions.

Table 3. Affinity Extracted Ubiquitinated or Related Proteins Identified by in Vitro His_6-Ubiquitin Labeling

protein identified	function	peptides	reproducibility
vimentin	possibly ubiquitinated intermediate filament protein; ubiquitin like proteins regulate the interaction of vimentin with the plasma membrane	8	4/4
tubulin beta chain	in yeast, the ubiquitin conjugating enzyme CDC34 colocalizes with beta tubulin at the mitotic spindle[64]	4	4/4
hnRNP U	nuclear phosphoprotein that binds the SCF (beta-TrCP) ubiquitin E3 ligase as a pseudosubstrate[30]	5	4/4
eps 15	EGF receptor pathway substrate 15; monoubiquitinated by EGF receptor[29]; part of endocytic pathway	5	4/4
insulin receptor substrate 2	ubiquitinated and degraded by the 26S proteasome in response to insulin or IGF-1; this is blocked by PI3-kinase inhibitors	3	4/4
activating signal cointegrator 1	thyroid receptor interactor 4/TRIP-4; TRIPs bind the transcription factor thyroid hormone receptor; TRIP-4 acts as a transactivator; another TRIP may ubiquitinate specific target proteins[38]	3	4/4
histone H2A	ubiquitinated on lys 119[37]; this may have a role in control of DNA replication	2	3/4
josephin/MJD1	ataxin-3, interacts with ubiquitin-like domain of rad23B homolog[24] using the UIM motif	3	3/4
CENP E	mitotic motor protein whose binding to kinetochores is regulated by its ubiquitination[28]	3	2/4
ribosomal protein S17	expression is downregulated along with ubiquitin in leukemia cell differentiation	2	2/4
c-myc binding protein	c-myc levels are regulated by ubiquitination[31]	1	2/4
thyroid hormone receptor binding protein	thyroid hormone receptor is subject to degradation by the proteasome[32]	3	2/4
mannose 6-phosphate receptor binding protein	mannose 6-phosphate receptor levels are stabilized by a proteasome inhibitor	3	2/4
endocytosis/budding/vesicle trafficking			
cortactin/ems1	oncogene; interconnects the membrane cytoskeleton and vesicle budding machinery[60], a process involving ubiquitination[8]; nucleates branched actin filaments	9	4/4
rabaptin 4 or 5	direct effectors of the GTPases rab 4 and 5, which are important in endocytic membrane fusion; internalization/targeting of proteins to multivesicular endosomes involves ubiquitin[5]	1	3/4
similar to rab 11 binding protein	rab 11 is implicated in vesicle trafficking[61]	2	2/4
snare Vti1a-beta	found in small synaptic vesicles[62]	1	2/4

A number of cytoskeletal proteins were identified in the affinity extract. The intermediate filament proteins desmin, vimentin, and keratin 8 were present, as were myosin 1XB, the protein XP_036740 which is similar to tropomyosin, the hypothetical actin-binding protein FLJ22056, beta-tubulin, and cortactin. Two components of a cadherin complex that were isolated include p120 catenin, which binds the cytoplasmic domain of transmembrane cadherins such as E cadherin,[58] and the ras GTPase activating protein IQGAP1, which forms a complex with E cadherin and catenin, and negatively regulates cell−cell adhesion.[59] These results indicate a potential regulatory role for ubiquitin in cadherin-related cytoskeletal events.

A last category of isolated proteins includes those associated with vesicle budding, trafficking, or regulated endocytosis and exocytosis. Ubiquitin-mediated protein degradation has been implicated in endocytosis[2] and monoubiquitination has been linked to receptor internalization and endosomal sorting,[5] retroviral budding[8] and the release and maturation of HIV particles.[7] Proteins isolated here include rabaptin 4 or 5, both of which are direct effectors of the GTPases rab 4 and 5, respectively. These are important in endocytic membrane fusion, and the internalization/targeting of proteins to multivesicular endosomes. Eps 15, which is monoubiquitinated by the EGF receptor,[29] is part of the endocytic pathway. Cortactin interconnects the cytoskeleton and vesicle budding machinery[60] and nucleates branched actin filaments. A member of a small family of nearly identical proteins similar to Rab 11 was also identified. Rab 11 has been implicated in vesicle trafficking,[61] and a rab 11 interacting protein also interacts with myosin Vb. It is possible that the six cytoskeletal proteins identified interact with some of the proteins involved in endocytosis isolated here. SNARE Vti1a-beta, a component of a snare complex localized to synaptic vesicles[62] was identified by the SVM by a single positive peptide with a probability of 67%. These results suggest that proteins known to be involved in ubiquitin-regulated processes can be identified using our approach.

Overall, this ubiquitin map is weighted to discrete categories of proteins, many but not all of which are known to have links to the ubiquitin system. A number of the proteins may have been isolated by binding to the proteasome in the presence of the proteasome inhibitor MG132. Many identified proteins, such as 19S and 20S proteasome subunits, splicing factors, ribosomal protein S17, hnRNP−U, elongin B, cortactin, p120 catenin and IQGAP1, josephin and rad23B, annexin II and calpactin, CENP-E, SMC6, SNARE Vti1a-beta, and several proteins in the thyroid hormone receptor system, are part of defined complexes. Due to the addition of 0.3 M sodium chloride to one of the bead washes, weakly bound proteins may have been washed off the nickel beads, leaving a set of proteins with tightly bound core protein complex members. Some proteins may be directly ubiquitinated, others may be present in protein complexes without being directly ubiquitinated.

Table 4. Identification of Other Proteins Using 2D LC−MS/MS Analysis of in Vitro His_6-Ubiquitin Labeled Proteins

protein	function	peptides	reproducibility
cytoskeletal-related			
desmin	intermediate filament protein; accumulates along with ubiquitin and other proteins in protein surplus myopathies	4	4/4
myosin 1XB	has ras-association, large ATPase and calmodulin binding domains or motifs	3	3/4
XP_042658	intermediate filament protein; keratin 8	2	2/4
FLJ22056	hypothetical protein with WH2 Wiskott Aldrich homology region, which binds actin	2	2/4
XP_036740	similar to tropomyosin; peptide is present in 3 similar proteins	1	2/4
redox-related proteins			
MGC3178 protein	protein with 3 internal thioredoxin-like domains and C-terminal KDEL endoplasmic reticulum retention sequence	7	4/4
hypothetical protein BC008913	protein with 74 aa thioredoxin family region	6	4/4
protein disulfide isomerase-related	ERp60, has thiol reductase activity, may control cellular redox potential[47]	6	4/4
protein disulfide isomerase related	calcium-binding ERP-72, contains calsequestrin and thioredoxin domains	5	4/4
thioredoxin	multiple cellular redox roles[44]	4	2/4
MGC14353	unknown protein with 74 aa thioredoxin family sequence motif	3	3/4
DNA damage or repair-related			
KIAA0204	contains protein kinase and tyrosine kinase domains, and a UVR domain involved in nucleotide excision repair and DNA damage recognition in prokaryotes	4	3/4
KARP-1 binding protein	involved in double strand break repair[43]	2	3/4
SMC6	part of a complex required for DNA repair and proliferation[42]	1	2/4
BRE	brain and reproductive organ-expressed protein; responsive to DNA damage[40]	1	2/4
cadherin complex			
p120 catenin	binds cytoplasmic domain of transmembrane cadherins such as E cadherin; phosphorylated by src	4	3/4
ras GTPase activating protein IQGAP1	forms a complex with E cadherin/catenin and negatively regulates cell−cell adhesion	3	3/4
receptor-related			
receptor interacting S/T kinase 2	regulates CD95-mediated apoptosis	3	3/4
opioid growth factor receptor	not known to be ubiquitinated	1	3/4
calcium binding proteins			
annexin II	calcium binding protein; has multiple functions in cell	2	2/4
reticulocalbin 1	EF hand calcium binding protein	3	3/4
reticulocalbin 2	EF hand calcium binding protein	1	3/4
calpactin/S100	S100 EF hand calcium binding protein A11; annexin II ligand	3	3/4
splicing factor-related			
KIAA0017	has splicing factor 3b and DNA subunit binding factor domains	4	3/4
splicing factor CC1.3	has RNA recognition motif; estrogen receptor coactivator	4	2/4
PTB-associated splicing factor	pre-mRNA splicing factor that binds polypyrimidine tract binding protein	4	4/4
splicing factor 3b subunit 3	spliceosome-associated protein p130	2	2/4
AAH06474 protein	has RRM RNA recognition motif, SURP module found in splicing regulators, glu-, ser-, pro-rich motifs	1	2/4
FLJ20294	WD repeat protein, may be involved in mRNA interactions	1	2/4
other			
FLJ13614	no obvious motif	6	3/4
MGC19464	cysteine protease-like	5	4/4
carbamoyl phosphate synthetase	trifunctional enzyme	5	3/4
rho guanine nucleotide exchange factor 7	PAK-interacting exchange factor beta	3	4/4
cell membrane glycoprotein	110 kDa interferon-upregulated cell surface antigen in gastric carcinoma cells; ammonium transporter motif	3	3/4
eukaryotic translation initiation factor 2 subunit 3	XP_006922; contains GTP binding domain; elongation factor 1 alpha is a ubiquitin protease[63]	3	3/4
cystatin B	cysteine protease inhibitor	2	3/4
rhoA	ras homologue A	2	2/4
XP_107789	no known motif	2	2/4
XP_088406	no known motif	2	2/4
60S ribosomal protein L5	shuttling protein involved in nucleocytoplasmic transport of 5S RNA	2	2/4
serine protease inhibitor	heat shock protein 47; binds collagen	2	2/4
MGC14289	hypothetical protein with CCCH zinc finger motif	2	2/4
BC006227	unknown protein	2	2/4
KIAA1771	no known motifs	2	2/4
SB92	peptide identifies SB92 and 3 truncated versions containing a FtsJ methyltransferase domain	1	3/4
FLJ20602	no obvious motif	1	2/4
ATP binding cassette protein XM_086117	consistent with several proteins in this class; many are transmembrane pumps	1	2/4
p30 DBC protein	contains glutamic acid rich domain	1	2/4
DKFZp761A052	hypothetical protein	1	2/4
FLJ20093	ankyrin repeat protein	1	2/4

Apart from allowing a systems biology approach to more global questions about the function of the ubiquitin-proteasome system in cells, this set of ubiquitin-labeled proteins may be useful for testing the effects of some classes of ubiquitin-proteasome system enzyme inhibitors. Examining changes in the set of proteins as a function of added inhibitors of E1, E2, or E3 ubiquitin system enzymes may allow an assessment of the selectivity of these inhibitors. Changes as a function of overexpressed or dominant negative versions of proteins, after anti-sense or RNAi treatment targeting particular functional proteins, or after other cellular perturbations, may be useful for examining their role in the functioning of the cellular ubiquitin system. Future work will be directed toward developing in vivo labeled and subcellular versions of the ubiquitin sub-proteome.

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