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A proteomics approach to understanding protein ubiquitination

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There is a growing need for techniques that can identify and characterize protein modifications on a large or global scale. We report here a proteomics approach to enrich, recover, and identify ubiquitin conjugates from *Saccharomyces cerevisiae* lysate. Ubiquitin conjugates from a strain expressing 6xHis-tagged ubiquitin were isolated, proteolyzed with trypsin and analyzed by multidimensional liquid chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for amino acid sequence determination. We identified 1,075 proteins from the sample. In addition, we detected 110 precise ubiquitination sites present in 72 ubiquitin-protein conjugates. Finally, ubiquitin itself was found to be modified at seven lysine residues providing evidence for unexpected diversity in polyubiquitin chain topology *in vivo*. The methodology described here provides a general tool for the large-scale analysis and characterization of protein ubiquitination.

To characterize protein phosphorylation several strategies have been developed to capture modified peptides and determine their identity on a large scale[1–3]. However, no similar methods have been described for the analysis of ubiquitinated proteins. Although the biological significance of protein ubiquitination is appreciated[4–6], its study is inherently difficult because the modification is large (~8 kDa) and because the turnover of ubiquitinated proteins is very rapid, so that steady-state conjugate levels are characteristically low.

Trypsin proteolysis of a ubiquitin-conjugated protein produces a signature peptide at the ubiquitination site containing a two-residue remnant (glycine-glycine) that is derived from the C terminus of ubiquitin and that is still covalently attached to the target lysine residue via an isopeptide bond (**Fig. 1**). This signature peptide has a mass shift at the lysine residue of 114.1 Da as well as a missed proteolytic cleavage because trypsin proteolysis cannot occur at the modified lysines. The branched tryptic fragments are identifiable by database-searching algorithms, and the sequence of the signature peptide can be determined.

Isolation of yeast ubiquitin conjugates was accomplished as illustrated in **Figure 2**. Whole lysate was harvested from exponential cultures of cells expressing 6xHis-tagged ubiquitin; cells expressing wild-type ubiquitin served as a control[7]. It should be noted that 6xHis-ubiquitin in this strain is expressed at levels similar to that of ubiquitin in the wild-type strain[8]. We purified 6xHis-ubiquitin conjugates by affinity chromatography, using Ni-NTA resin under highly denaturing conditions, which minimizes copurification of proteins associated with ubiquitin conjugates. After the cell lysates were loaded, the columns were washed extensively with a denaturing buffer at pH 8.0 and again with a more stringent solution at pH 6.3. Finally, the bound proteins were eluted by adjusting the pH to 4.5, and an aliquot (0.5%) was examined by SDS-PAGE and silver staining. Only a few proteins were visibly purified from the control cell lysate, which included endogenous histidine-rich proteins. In contrast, many proteins were enriched in the 6xHis-ubiquitin lysate, including the monomeric form of 6xHis-ubiquitin, yielding approximately 0.2 mg protein per 100 mg cell lysate. The enrichment of ubiquitinated species was substantial, as shown by the high molecular weight smear on the gel (**Fig. 2**).

The purified proteins from both yeast strains were analyzed by LC/LC-MS/MS (**Fig. 2**). In the ubiquitin-conjugate sample, all proteins were directly proteolyzed with trypsin. Owing to the enormous complexity of the resulting peptide mixture, we separated it in two dimensions of chromatography to allow more than 120 h (5 d) of analysis time and permit many thousands of peptides to be sequenced. The first separation step was to fractionate the peptides by strong cation exchange (SCX) chromatography, which separates peptides based on ionic charge[9]. All peptide-containing fractions were collected and then subjected independently to nanoscale microcapillary reversed-phase chromatography. Sequence analysis of the eluting peptides was accomplished by online MS/MS. During the elution, peptide ions were constantly detected and sequenced (fragmented) in an automated fashion, with one peptide being sequenced on average every 2 s[10]. More than 96,000 sequencing attempts were acquired for the 6xHis-ubiquitin sample during the experiment. Each sequencing attempt (one MS/MS spectrum) was searched independently against a yeast protein database using the Sequest algorithm[11]. As a result, we identified 4,210 peptides corresponding to 1,075 candidate ubiquitin-conjugated proteins (see **Supplementary Table 1** online) from the 6xHis-ubiquitin sample after subtracting the 48 proteins found in the control experiment (**Supplementary Table 2** online). Performing the search a second time with a mass increase of 114.1 Da as a variable

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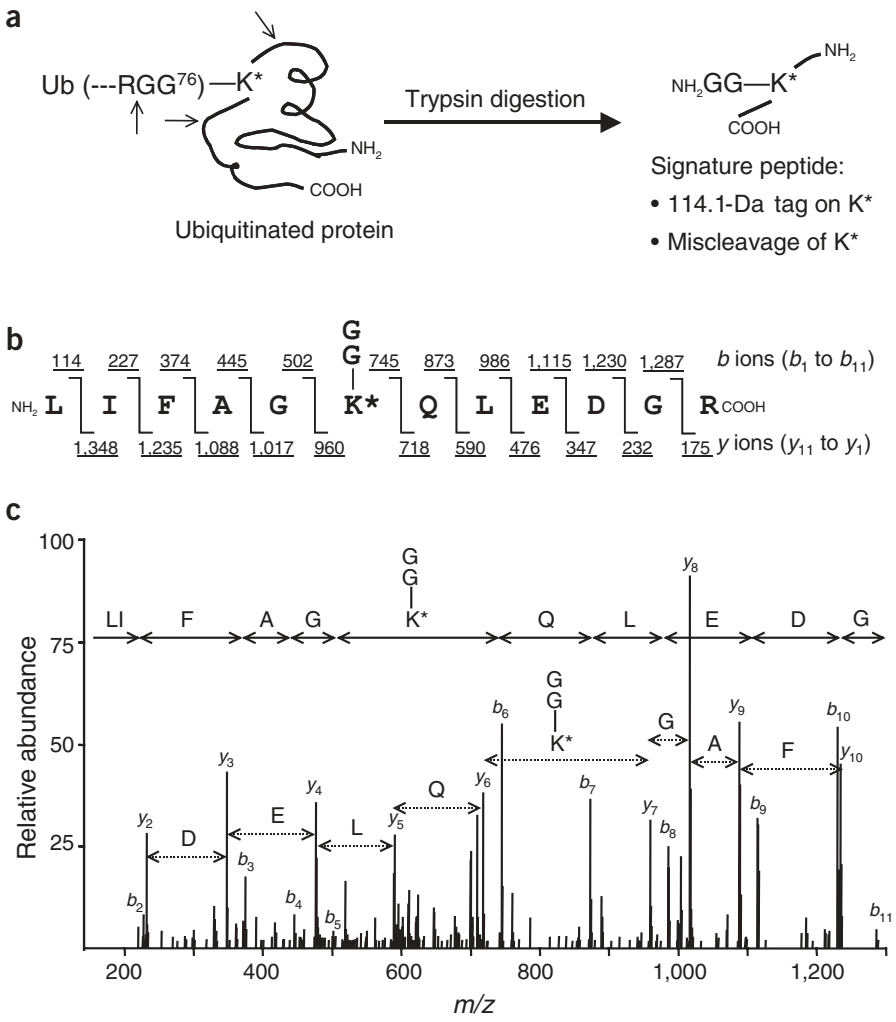

Figure 1 Strategy for identifying the precise site of ubiquitination by MS/MS. (**a**) After trypsin digestion, a ubiquitin-conjugated protein contains a diglycine remnant of ubiquitin (Ub) covalently attached to a lysine residue that is resistant to trypsin proteolysis. Amino acids are denoted by single-letter code. (**b**) Example showing the sequence of a signature peptide produced by trypsin proteolysis. Cleavage at the peptide backbone in the tandem mass spectrometer would result in the predicted fragment ion masses shown (*b*- and *y*-type ions) with the intact diglycine modification. (**c**) Fragmentation pattern (MS/MS spectrum) acquired for the peptide shown in **b**. Only the singly charged ions are shown for simplicity. Much of the primary amino acid sequence can be determined including the precise site of ubiquitination. The ubiquitin-conjugated protein used in this example was ubiquitin itself and the isopeptide bond linkage was through Lys48.

modification to lysine residues resulted in the identification of 110 ubiquitination sites from 72 different proteins (**Table 1**; **Supplementary Table 3** online).

To lend support to the identification of 72 putative ubiquitin conjugates, we used an independent method for assessing ubiquitination. Nineteen genes encoding proteins not previously known to be ubiquitinated were expressed in a hemagglutinin (HA)-tagged form in cells expressing myc-ubiquitin. Tagged proteins were immunoprecipitated with antibodies to the HA epitope, resolved by SDS-PAGE and immunoblotted. Ubiquitin conjugates were observed as high molecular weight species reacting with anti-myc antibodies. Such species were clearly detected for all 19 proteins tested (**Supplementary Fig. 1** online). In control samples from cells not expressing myc-ubiquitin, immunoreactive bands observed in the anti-myc panel are specific to myc-ubiquitin (data not shown). In addition, when the lysis was performed in the presence of 1% SDS, similar results were obtained, suggesting the ubiquitin conjugates are not derived from coprecipitated proteins (data not shown).

To compare proteins identified by this study with the entire yeast proteome, we examined their molecular environment (**Fig. 3**). Although proteins in all categories were identified, integral membrane proteins were preferentially detected. For example, we identified a large number of known or suspected substrates (amino acid permeases and hexose transporters) for the E3 ligase, Rsp5 (**Fig. 3b**)[12]. Of the 72 proteins with identified ubiquitination sites, about one-third were internal membrane proteins, which supports prior data indicating that ubiquitination plays multiple roles in the trafficking of membrane proteins, such as in their down-regulation via internalization and degradation in the lysosome and vacuole[12,13]. However, for most of the proteins detected, the subcellular localization was not found in the databases. Finally, the ubiquitination sites were found to be evenly distributed throughout the 72 proteins with no bias towards N or C termini (**Fig. 3c**).

An examination of the 110 sites of ubiquitination (**Table 1**) showed that, of the 72 proteins with defined sites, 24 (33%) were conjugated with ubiquitin on two sites or more, supporting a common theme of ubiquitination of multiple sites. In fact, for one protein (Ecm21), we detected six sites of ubiquitination, all but one within the middle third of the polypeptide (**Table 1**). In addition, in members of the same protein families, the same lysine residues were modified by ubiquitination. For example, the sites detected for Snc1 and Snc2 were identical, as were the sites for Hxt6 and Hxt7 (**Table 1**).

After ubiquitin is linked to the substrate through an isopeptide bond, additional ubiquitin molecules are frequently conjugated to the first one, forming a branched (polyubiquitin) chain also linked by isopeptide bonds, usually through the Lys48 residue[14]. However, alternative lysine residues can be used[15–17]. We found that ubiquitination in yeast can occur on seven sites within ubiquitin itself, with a relative abundance order of Lys48 > Lys63 and Lys11 >> Lys33, Lys27 and Lys6. The qualitative abundance was assessed based on the overall number of times each signature peptide was sequenced (**Supplementary Table 4** online). Lys63-linked chains are known to be present in yeast, and this site has previously been implicated in processes other than degradation (*e.g.*, DNA repair, translational control, endocytosis, protein kinase activation)[7,16,18,19]. The Lys11, Lys33, Lys27 and Lys6 sites are described here *in vivo* for the first time. The Lys29 ubiquitination site was identified only on a peptide modified at both Lys29 and Lys33. In particular, the Lys11 branched polyubiquitin chains were easily detectable

Table 1 Proteins with identified ubiquitination sites from *S. cerevisiae*

Protein	No. of sites	Peptide sequence
Ubiquitin	7	IQDK*EGIPPDQQR, SK*IQDK*EGIPPDQQR, LISEEDLGMQIFVK*TLTGK, TITLEVESSDTIDNVK*SK, TLTGK*TITLEVESSDTIDNVK, LIFAGK*QLEDGR, TLSDYNIQK*ESTLHLVLR
ECM21	6	FHQTIIK*SNSGLPVK, GYEYEYDQTDPVAK*DPYNPYYLDFASK, SHEENEK*PVYD, FNNLDK*LLSTPS#PVNR, EEIVENSFNDNLLSYSPFDDDSDSK*GNPK, TPVAVSTANK*PIYINR
GNP1	3	EK*QIGSIEPENEVEYFEK, NDLDDVSHYEMK*EIQPK, SSYITVDGIK*QSPQEQEQK
HXT7	3	GANYDAEEMTHDDK*PLYK, DEIK*AYGEGEEHEPVVEIPK, LAGNASWGELFSSK*TK
SAM2	3	DTIK*K*IGYDDSAK, K*IGYDDSAK*GFDYK,
URA3	3	DEGYDWLIMTPGVGLDDK*GDALGQQYR, K*AGWEAYLR, K*FADIGNTVK
YHR097C	3	K*STLDEVFGR, LPSYEEAAGTPK*QQAPYPK, NITQFDSK*MK
AKL1	2	DK*DS#NSS#ITISTSTPSEMR, VSPHASTAITENK*R
CDC48	2	K*TPLEPGLELT#AIAK, AAAPTVVFLDELDSIAK*AR
CIT2	2	LVSSIYEVAPGVLTEHGK*TK, YMAQRK*FAMDHFPDYELFK
CUE5	2	SSGIDEDEVVT#PAEDAK*EEEEEHPPLPAR K*NPDEDEFLINS#DDEM–
ERG3	2	EVEHFIK*EVEGDDNDR, RPDDSLFDPK*LR
ERG5	2	K*SLNGLFTK, LSK*ENNYEPQVFFHEMR
GDH1	2	STATGPSEAVWYGPPK*AANLGGVAVS GLEMAQNSQR, VDIALPCATQNEVSGEEAK*ALVAQGVK
JEN1	2	LILSDAVK*ANGGEPLPK, Ac–SSSITDEK*ISGEQQQPAGR
LSB1	2	LPEK*WDGNQR, TGDK*IQVLEK
PHO84	2	K*IHDTSDEDMAINGLER, SSVNK*DTIHVAER
SSA1	2	IASK*NQLESIAYSLK, MVAEAEK*FKEEDEKESQR,
UIP3	2	AAEIHK*EAEFEYWR –MQTPSENTDVK*LDTLDEPSAH
YGR268C	2	DTHDDELPSYEDVIK*EEER, SK*DTHDDELPSYEDVIK
YHL010C	2	K*DGELAANFLRLVQNEVDGK*LVEVGGSG DDDNNDIGNSDELQNVVYGNR
YIL041W	2	EFLSNSFAEEPEAK*PEVAEEEK*PQTAISM NDEDDA–
YMR295C	2	ASDVK*ISEDDK*AR
YOL109W	2	EQAEASIDNLK*NEATPEAEQVK, K*EEQNIADGVEQK
ACS2	1	MDTYLK*PYPGHYFTGDGAGR
ADE13	1	NDIGLRGVK*GTTGTQASFLALFHGNHDK
ALD6	1	TK*LHFDTAEPVK
ARO10	1	YTNSTLIQCPSK*LALK
BSD2	1	K*YLNQSQNQA–
CCT8	1	LPQNPNAGLFK*QGYNSYSNADGQIIK
CHD1	1	YLK*NLINSNYK
CHS3	1	ISDEGVAEDEFDK*DGDVDNFEESSTQPINK
COS4	1	LFNSEK*SWSPVGLEDAK
CSR2	1	IPQDK*NHNEVNDTNGNSNTSLQTSSN VPIQHYTR
CTR9	1	LLYQK*ENYMASLK
DDI1	1	LMANPDDPDNK*KR
DLD3	1	TAAHPVAQLTAEAYPK*VK
ELP3	1	K*DILIGLLR
ERG1	1	SLRPSFDEAVSQGK*FR
EXO84	1	QVQEEVK*LNINK
GAP1	1	VKPIEVDPNLSEAEK*VAIITAQTPLK
GLN1	1	SVAK*EGYGYFEDR
GLY1	1	SMGAPIGSVLVGNLK*FVK
GSC2	1	LLYHQVPSEIEGK*R
HXT5	1	PVSSYISHEGPPK*DELEELQK
HXT6	1	GANYDAEEMAHDDK*PLYK
ITR1	1	VHELK*YEPTQEIIEDI–
NNF2	1	K*VSHLQSLMNTK
PDR12	1	TLDIKPGYEDK*VPK
PDR5	1	GVLTEK*NANDPENVGER
PDX3	1	FTLNEK*QLTDDPIDLFTK
PHO87	1	GDSDEK*AIDGNNINEETIELDELSPQGK
PIN3	1	NASPASLEYVEALYQFDPQQDGDLGLKP GDK*VQLLEK
RPS20	1	EK*VEEQEQQQQQIIK
RPS3	1	ALPDAVTIIEPK*EEEPILAPSVK
RPS4B	1	IDLASGK*ITDFIK
RVS167	1	YNGQQGVFPGNYVQLNK*N–
SNA3	1	DLEAHPAEESQAQPPAYDEDDEAGADVPLM DNK*QQLSSGR
SNC1	1	LTSIEDK*ADNLAVSAQGFK
SNC2	1	LTSIEDK*ADNLAISAQGFK
SSA2	1	NTISEAGDK*LEQADKDAVTK
STE6	1	ILDEK*HNTLEVENAR
TNA1	1	NLEDSWFFNK*EEK
UBR2	1	K*AIS#PDNASTNENDSNK
URA7	1	K*DSHSAEFYPDIDEK
YDL203C	1	ILQEK*VYR
YER067W	1	LNYYPPFVLHESHEDPEK*ISDAANSHSK
YKL187C	1	FGDHNLGDDDDADFEK*QVNR
YKR041W	1	–MSDDDYMNSDDDNDAEK*R
YLR413W	1	IIEEHESPIDAEK*NFAR–
YPL207W	1	HGTNPVSK*NWR
YRO2	1	LGLIFDEEPAEHVGPVAEK*K

*, ubiquitination; #, phosphorylation; Ac, acetylation; –, protein terminus.

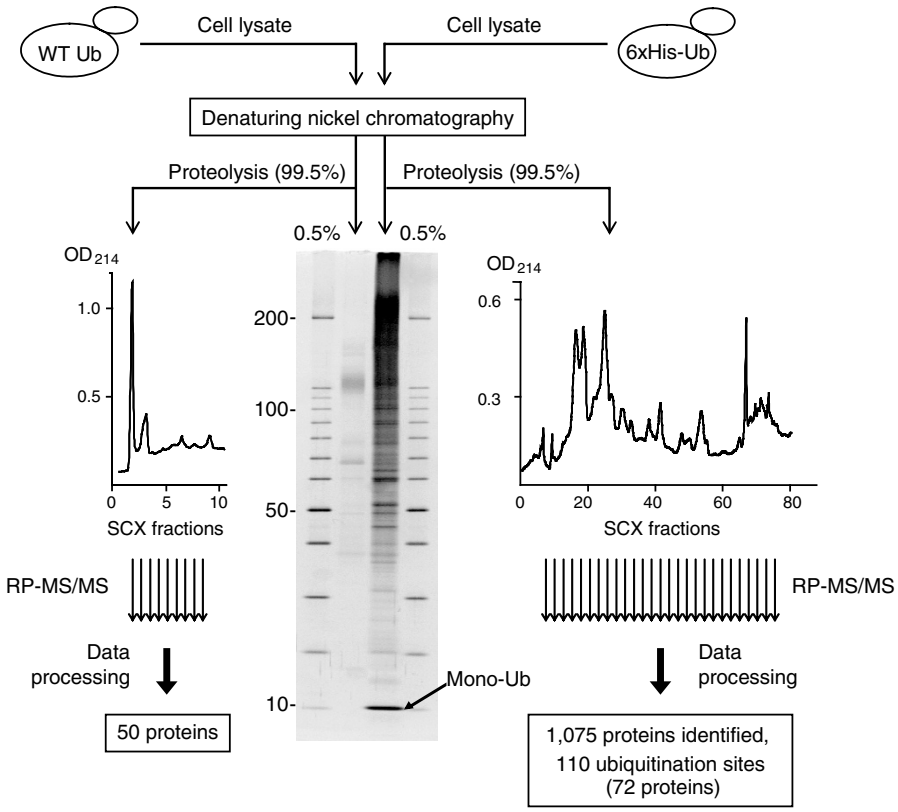

Figure 2 Isolation and sequence analysis of yeast ubiquitin conjugates. Ubiquitin conjugates were purified from 100 mg of whole cell lysate from a yeast strain expressing 6xHis-ubiquitin or a control strain by denaturing nickel-affinity chromatography. For visualization, 0.5% of the eluate was analyzed by 6•16% SDS-PAGE and silver staining. The remainder of the sample was directly trypsinized, and resulting peptides were separated by SCX chromatography with fraction collection. All fractions ($n = 80$) were sequentially analyzed by nanoscale microcapillary reversed-phase LC with online sequence analysis by MS/MS. We identified 1,075 proteins from the 6xHis-Ub sample and 110 precise ubiquitination sites. A molecular weight marker is shown on either side of the gel (10-kDa ladder of 10–120 kDa and 200 kDa).

at comparable levels to Lys63 chains. Collectively, these data may point to a broader role for ubiquitin in processes other than proteasome-dependent proteolysis than had been previously thought. These data provide evidence for unexpected diversity in polyubiquitin chain topology.

Phosphorylation of some proteins is known to be a prerequisite for ubiquitination and subsequent substrate degradation[20]. Examining the list of sequenced peptides, we found 125 phosphorylation sites from 97 phosphopeptides distributed among 60 proteins (**Supplementary Table 5** online). Only ten sites (7.9%) in our list were found in the previously published phosphoproteome analysis of *S. cerevisiae*[3]. For example, in addition to the six ubiquitination sites found for Ecm21, we found that this protein also contained twelve phosphorylation sites. Surprisingly, among the phosphorylated proteins detected was ubiquitin itself, at serine 57. To confirm this phosphorylation site, we synthesized the phosphopeptide and found its MS/MS spectrum to be the same as that detected in the *S. cerevisiae*–derived sample (**Supplementary Fig. 2** online). This serine has been described as nonessential for viability in an alanine-scanning mutation experiment[21]. However, from the crystal structure of tetraubiquitin[22], this residue is solvent exposed and could potentially interfere with proteasome recognition. The significance of this phosphorylation event merits further studies.

The phenomenon of protein ubiquitination has been known for more than 25 years[23]. The involvement of ubiquitination in processes as diverse as cell cycle regulation, DNA repair and receptor-mediated endocytosis[18,20] provides a measure of its biological significance. We have combined affinity isolation of ubiquitin conjugates with large-scale amino acid sequencing by MS/MS, with the goal of characterizing both ubiquitin conjugates and precise sites of ubiquitination (modified lysinyl residues).

The 110 ubiquitination sites presented here provide an initial framework for future genetic analysis whereby each modified lysine can be studied by site-directed mutagenesis. This is critical because it allows for the testing of the biological significance of each ubiquitination event. Only three proteins from the list (not including ubiquitin itself) were previously known to be ubiquitinated (Gap1, Pdr5 and Ste6). For five others, there was indirect evidence of ubiquitination (Hxt6, Hxt7, Itr1, Jen1 and Gnp1). All eight are membrane proteins[12]. We attempted to confirm the ubiquitination of 19 further proteins. All 19 proteins were found to be substrates for ubiquitin (**Supplementary Fig. 1** online), lending support to the basic method.

The ubiquitin conjugates detected here represent only a subset of all ubiquitin conjugates. The mass spectrometer is a concentration-sensitive detector and so the ubiquitin conjugates identified represented the more abundant conjugates in the cell. We failed to detect a number of known, short-lived regulators of the cell cycle (*e.g.*, Sic1, Cln1, Cln2, Clb1, Clb2, Clb3 and Clb4). Many of these proteins are so quickly degraded after ubiquitination that they can be measured only after stabilization either by chemical proteasome inhibition or after genetic deletion of required E2 or E3 ligases. We used no stabilization techniques in this first experiment. We are now repeating the experiment with the use of chemical proteasome inhibitors in an attempt to define the subset of ubiquitin conjugates that is rapidly degraded by the proteasome. In addition, we will focus on developing a methodology that will allow for the comparative analysis of ubiquitin conjugation in yeast strains with deletions of specific E2 and E3 ligases by combining the strategy described here with the isotope-coded affinity tags method[24].

A more general extension of the technique is in determining the targets of ubiquitin-like protein modifiers, nine of which have been described to date. With one exception, each ubiquitin-like protein modifier will leave its own signature remnant peptide bound to its target after trypsin digestion. Thus, the present mass spectrometry–based approach is capable of identifying proteins modified by ubiquitin and ubiquitin-like modifiers, mapping their sites and defining the specific modifier involved.

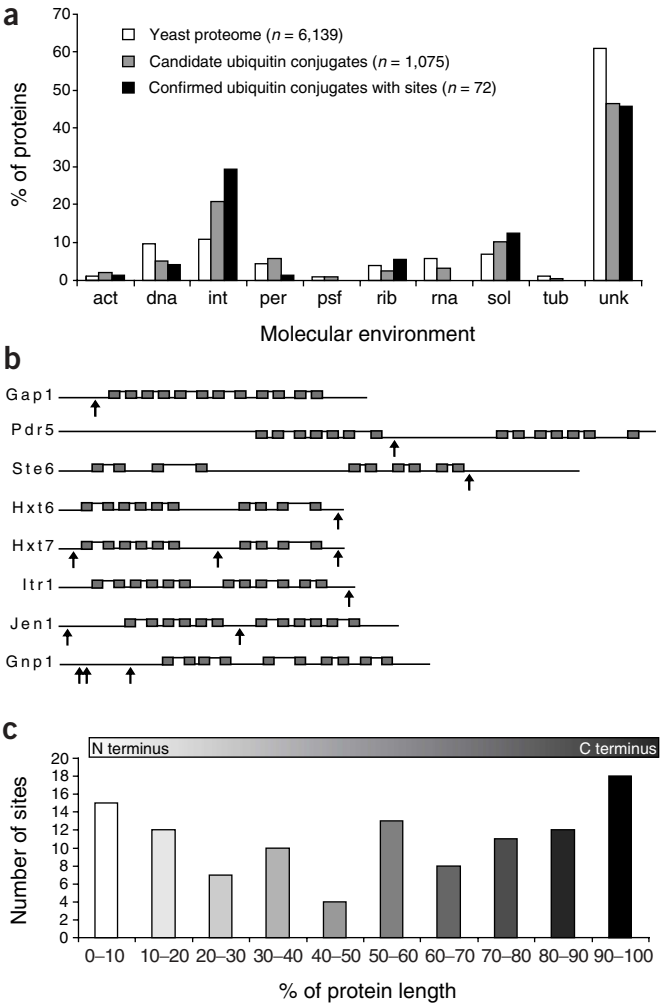

Figure 3 (**a**) Comparison among yeast proteome ($n = 6,139$), identified proteins in the candidate ubiquitin-conjugate fraction ($n = 1,075$) and proteins whose ubiquitination sites in the molecular environment is known ($n = 72$). act, actin-binding; dna, DNA-associated; int, integral membrane; per, peripheral membrane; psf, protein synthesis factor; rib, ribosome-associated; sol, soluble; tub, tubulin-associated; rna, RNA-associated; unk, unknown. (**b**) Location of ubiquitination sites in eight transmembrane proteins detected in this study. Ubiquitination sites are shown with an arrow, and lines are drawn either below or above the predicted transmembrane domains (rectangles) to show cytoplasmic or extracellular orientation, respectively. These proteins are all known or presumed ubiquitination targets of the same E3 ligase, Rsp5 (ref. 12). (**c**) Distribution of 110 ubiquitination sites within their protein sequences. The length of each protein is normalized to 100%.

METHODS

Preparation of ubiquitin conjugates from *S. cerevisiae*. Strain SUB592 (ref. 7) (also named JSY171, in which all ubiquitin genes were removed and a 6xHis-myc-ubiquitin–coding plasmid was introduced) and the control strain SUB280 (similar to SUB592 except for the introduction of a wild-type ubiquitin plasmid) were grown at 30 °C to log phase (OD_{600} 1–1.5) and lysed in buffer A (10 mM Tris-HCl, pH 8.0, 0.1 M NaH_2PO_4, 8 M urea, 10 mM β-mecaptoethanol) using glass beads. A 0.5 ml Ni^{2+}-NTA-agarose column (Qiagen) was loaded with the clarified lysates, sequentially washed with 30 bed volumes (V) of buffer A twice, 3 V of buffer B (10 mM Tris-HCl, pH 6.3, 0.1 M NaH_2PO_4, 8 M urea) and eluted with 3 V of buffer C (10 mM Tris, pH 4.5, 0.1 M NaH_2PO_4, 8 M urea). The eluents were adjusted to pH 8.5, reduced with 10 mM DTT at 37 °C for 1 h and alkylated with 50 mM iodoacetamide in the dark for 30 min. The protein mixtures were further dialyzed to reduce urea to 1 M, and digested with trypsin as described[9].

LC/LC-MS/MS. The tryptic peptides were separated in the first dimension by SCX chromatography using a 2.1 mm 20 cm Polysulfoethyl A column (Poly LC) and fraction collection every min (solvent A: 5 mM phosphate buffer, 25% acetonitrile, pH 3.0; solvent B: solvent A with 350 mM KCl; flow rate: 0.2 ml/min). Peptides from the control were eluted in a 10-min gradient of 0–100% solvent B. Peptides from the 6xHis-ubiquitin strain were fractionated in a 70-min gradient of 5–30% solvent B. All collected fractions ($n = 80$) were reduced in volume and then analyzed individually using 75 m inner diameter 12 cm self-packed fused-silica C18 capillary columns as described[9]. Peptides were eluted for each analysis during a 90-min gradient in which the ions were detected, isolated and fragmented in a completely automated fashion on an LCQ-DECA ion trap mass spectrometer (Thermo Finnigan). More than 96,000 sequencing attempts (MS/MS spectra) were acquired during the analysis of the 80 fractions containing ubiquitin conjugates.

Immunoprecipitations. Open reading frames (ORFs) were cloned into a YEplac195-derived plasmid using gateway cloning technology (Invitrogen). Final plasmids contained a *CUP1* promoter followed by a start methionine, a 10xHis-tag, a triple-HA tag, a gateway cloning linker, the respective ORF with its own stop codon and a second gateway cloning linker followed by a tobacco etch virus terminator. The control plasmid, pJR6, lacks an ORF as well as the gateway cloning linkers. Plasmids were transformed into a SUB280-derived strain[8] containing a plasmid with a *LYS2* marker that expresses a myc-tagged form of ubiquitin. Cells were grown to an OD_{600} of 0.6–1.2 in complete medium lacking uracil and lysine, supplemented with 100 M $CuSO_4$. Cells were collected, washed in H_2O, and lysed using glass beads in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 60 M PS-341 (Millenium), 15 mM iodoacetamide and Complete protease inhibitors (Roche). Lysates were cleared by centrifugation and anti-HA antibody was added (Y-11, Santa Cruz) to the supernatant. After a 2-h incubation at 4 °C, samples were centrifuged and the supernatants added to immobilized protein A. After 1 h incubation at 4 °C, protein A beads were washed four times in lysis buffer. Samples were resuspended in Laemmli sample buffer and boiled for 3 min. Proteins were then analyzed by SDS-PAGE and immunoblotting, using anti-HA antibody (12CA5, Roche) and anti-myc polyclonal antibody (Upstate). An additional experiment was performed under denaturing conditions where the immunoprecipitation buffer contained 2% SDS instead of 1% Triton. The samples were then diluted 30-fold in 1% Triton before immunoprecipitation.

Data processing. All MS/MS spectra were searched against the yeast ORFs database (from genome-www.stanford.edu/Saccharomyces/) supplemented with the sequence of the recombinant 6xHis-myc-ubiquitin using the Sequest algorithm[11]. Modifications were permitted to allow for the detection of the following (mass shift shown in daltons): oxidized methionine (+16), carboxyamidomethylated cysteine (+57), ubiquitinated lysine (+114), phosphorylated serine, threonine and tyrosine (+80), and N-terminal acetylation (+42). Sequest criteria were similar to those previously described[25], including (i) an *Xcorr* must be greater than 2.0, 2.2 and 3.75 for 1+, 2+ and 3+ charge-state peptides, respectively, (ii) a peptide must be fully or partially tryptic and (iii) a peptide must have a ΔCn score of >0.1 (ref. 11). We also manually verified peptides from each protein identified by two or fewer peptides based on previously reported standards[26–28]. For identification of phosphorylation and ubiquitination sites, every peptide was manually verified to identify the precise site. Raw MS/MS data for every modified peptide is also available from the author's website: http://deer.med.harvard.edu/pubs/ubiquitin. Transmembrane domains were predicted via the web at http://www.cbs.dtu.dk/services/TMHMM/ and http://www.ch.embnet.org/software/TMPRED_form.html.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Zhou, H., Watts, J.D. & Aebersold, R. A systematic approach to the analysis of protein phosphorylation. *Nat. Biotechnol.* **19**, 375–378 (2001).
2. Oda, Y., Nagasu, T. & Chait, B.T. Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat. Biotechnol.* **19**, 379–382 (2001).
3. Ficarro, S.B. *et al.* Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **20**, 301–305 (2002).
4. Weissman, A.M. Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell. Biol.* **2**, 169–178 (2001).
5. Glickman, M.H. & Ciechanover, A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* **82**, 373–428 (2002).
6. Layfield, R., Alban, A., Mayer, R.J. & Lowe, J. The ubiquitin protein catabolic disorders. *Neuropathol. Appl. Neurobiol.* **27**, 171–179 (2001).
7. Spence, J. *et al.* Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. *Cell* **102**, 67–76 (2000).
8. Finley, D. *et al.* Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol. Cell Biol.* **14**, 5501–5509 (1994).
9. Gygi, M.P., Licklider, L.J., Peng, J. & Gygi, S.P. Combining two-dimensional chromatography and mass spectrometry for the separation of complex peptide mixtures. in *Protein analysis: A Laboratory Manual* (ed. Simpson, R.) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2003), in the press.
10. Peng, J. & Gygi, S.P. Proteomics: the move to mixtures. *J. Mass Spectrom.* **36**, 1083–1091 (2001).
11. Eng, J., McCormack, A.L. & Yates, J.R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**, 976–989 (1994).
12. Rotin, D., Staub, O. & Haguenauer-Tsapis, R. Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J. Membr. Biol.* **176**, 1–17 (2000).
13. Hicke, L. Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* **9**, 107–112 (1999).
14. Pickart, C.M. Ubiquitin in chains. *Trends Biochem. Sci.* **25**, 544–548 (2000).
15. Mastrandrea, L.D., You, J., Niles, E.G. & Pickart, C.M. E2/E3-mediated assembly of lysine 29-linked polyubiquitin chains. *J. Biol. Chem.* **274**, 27299–27306 (1999).
16. Spence, J., Sadis, S., Haas, A.L. & Finley, D. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell Biol.* **15**, 1265–1273 (1995).
17. Baboshina, O.V. & Haas, A.L. Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5. *J. Biol. Chem.* **271**, 2823–2831 (1996).
18. Finley, D. Signal transduction. An alternative to destruction. *Nature* **412**, 283, 285–286 (2001).
19. Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G. & Jentsch, S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135–141 (2002).
20. Ciechanover, A., Orian, A. & Schwartz, A.L. Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* **22**, 442–451 (2000).
21. Sloper-Mould, K.E., Jemc, J.C., Pickart, C.M. & Hicke, L. Distinct functional surface regions on ubiquitin. *J. Biol. Chem.* **276**, 30483–30489 (2001).
22. Cook, W.J., Jeffrey, L.C., Kasperek, E. & Pickart, C.M. Structure of tetraubiquitin shows how multiubiquitin chains can be formed. *J. Mol. Biol.* **236**, 601–609 (1994).
23. Goldknopf, I.L. *et al.* Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein. *J. Biol. Chem.* **250**, 7182–7187 (1975).
24. Gygi, S.P. *et al.* Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17**, 994–999 (1999).
25. Washburn, M.P., Wolters, D. & Yates, J.R. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **19**, 242–247 (2001).
26. Link, A.J. *et al.* Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* **17**, 676–682 (1999).
27. Licklider, L.J., Thoreen, C.C., Peng, J. & Gygi, S.P. Automation of nanoscale microcapillary liquid chromatography–tandem mass spectrometry with a vented column. *Anal. Chem.* **74**, 3076–3083 (2002).
28. Gygi, S.P., Rist, B., Griffin, T.J., Eng, J. & Aebersold, R. Proteome analysis of low abundance proteins using multidimensional chromatography and isotope coded affinity tags. *J. Proteome Res.* **1**, 47–54 (2002).