SEE COMMENTARY

Numerous proteins with unique characteristics are degraded by the 26S proteasome following monoubiquitination

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Contributed by Aaron Ciechanover, June 4, 2016 (sent for review March 21, 2016; reviewed by Yinon Ben-Neriah and Ivan Dikic)

The "canonical" proteasomal degradation signal is a substrate-anchored polyubiquitin chain. However, a handful of proteins were shown to be targeted following monoubiquitination. In this study, we established—in both human and yeast cells—a systematic approach for the identification of monoubiquitination-dependent proteasomal substrates. The cellular wild-type polymerizable ubiquitin was replaced with ubiquitin that cannot form chains. Using proteomic analysis, we screened for substrates that are nevertheless degraded under these conditions compared with those that are stabilized, and therefore require polyubiquitination for their degradation. For randomly sampled representative substrates, we confirmed that their cellular stability is in agreement with our screening prediction. Importantly, the two groups display unique features: monoubiquitinated substrates are smaller than the polyubiquitinated ones, are enriched in specific pathways, and, in humans, are structurally less disordered. We suggest that monoubiquitination-dependent degradation is more widespread than assumed previously, and plays key roles in various cellular processes.

monoubiquitination | 26S proteasome | protein degradation | ubiquitin replacement

Polymers of ubiquitin (Ub) are formed on proteasomal substrates in eukaryotic cells by the concerted action of three enzymes: the Ub-activating enzyme (E1), a Ub-carrier protein [E2; known also as Ub-conjugating enzyme (UBC)], and a Ub ligase (E3), which is the specific substrate-recognizing element of the system. The Ub chains typically consist of multiple moieties linked to one another via an isopeptide bond between the C-terminal Gly residue of the distal moiety and the ε-NH_2 group of Lys48 of the proximal one (1).

In addition, the system can also catalyze modification by a single Ub moiety (monoubiquitination) or multiple single Ub moieties (multiple monoubiquitinations), each modifying a distinct lysine residue (2). Generally, monoubiquitination has been conceived as a nondestructive signal. Furthermore, it has been suggested that efficient proteasomal targeting requires a chain with a minimal length of four Ub moieties (3). Monoubiquitination is known to be involved in multiple biological processes. For example, monoubiquitination of proteins containing a ubiquitin-binding domain (UBD) often mediates autoinhibition by a UBD–Ub interaction (4, 5). Signal transduction by membrane receptors, such as the EGFR, is attenuated by monoubiquitination-mediated receptor internalization (6). The subcellular localization of small GTPases is controlled, among other posttranslational modifications, by monoubiquitination (7, 8), and histone monoubiquitination regulates nucleasomal structure, thus affecting gene expression (9). Emerging reports indicate, however, that several substrates can be degraded following monoubiquitination (10–13). Partial degradation/processing of the p105 precursor of NF-κB, which results in release of the p50 active subunit of the transcription factor, is dependent on multiple monoubiquitinations (14). Importantly, these findings demonstrate that the proteasome can recognize a single Ub moiety (moieties) and imply the existence of monoubiquitination vs. polyubiquitination "decision" mechanisms. In this context, a previous study has suggested that the chain length required for proteasomal degradation is determined by the size of the substrate, and possibly other characteristics that affect the affinity of the modified substrate to the proteasome. Specifically, it was suggested that substrates smaller than 150 aa are degraded following monoubiquitination, whereas longer substrates require longer chains. Thus, a dynamic model was proposed, according to which the chain elongates to a point where the affinity to the proteasome is high enough to secure a stable binding

Significance

A substrate-conjugated polyubiquitin chain is accepted as the "canonical" proteasomal degradation signal. Using a cellular (human and yeast) proteomic screen in the exclusive presence of nonpolymerizable ubiquitin, we show that a large group of proteins is degraded by the proteasome following monoubiquitination. The screen also unraveled polyubiquitin-dependent substrates, as they are stabilized in the presence of this ubiquitin mutant. Notably, monoubiquitination- and polyubiquitination-dependent substrates display distinct important characteristics. Monoubiquitinated proteins are of lower molecular mass and of lesser structural disorder. The two groups can be assigned to defined cellular pathways. Furthermore, some of the characteristics are confined to either human or yeast cells, suggesting that the mechanism of action/recognition of the ubiquitin system in the two organisms are different somehow.

Author contributions: O.B., I.L., T.Z., A.A., H.G., B.B., T.S., Y.T.K., M.G., P.T., and A.C. designed research; O.B., I.L., T.Z., I.K., L.H.C., H.G., and B.B. performed research; O.B., I.L., T.Z., I.K., and L.H.C. contributed new reagents/analytic tools; O.B., I.L., T.Z., A.A., A.G., S.J., L.J., T.S., Y.T.K., M.G., P.T., and A.C. analyzed data; and O.B., I.L., T.Z., T.S., Y.T.K., M.G., P.T., and A.C. wrote the paper.

Reviewers: Y.B.-N., Hebrew University; and I.D., University of Frankfurt.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1608644113/-/DCSupplemental.

of the conjugated substrate, its concomitant detachment from the E3, and its subsequent degradation (15). Another study demonstrated that restricting the number of ubiquitinatable Lys residues can switch the mode of modification necessary for degradation from multiple monoubiquitinations to polyubiquitination, suggesting that, in the cell, the masking of Lys residues by protein–protein interactions or post-translational modifications can affect the mode of ubiquitination (11).

However, all of these studies have been carried out using specific substrates. Therefore, general conclusions regarding monoubiquitination-dependent degradation mechanisms, the population of substrates that are degraded following this modification, and importantly, whether they have common distinct characteristics, have remained limited.

In this study, we used a systematic proteomic approach for the identification and characterization of monoubiquitination-dependent proteasomal substrates. By silencing the endogenous WT Ub followed by expression of nonpolymerizable lysineless Ub, we identified numerous substrates in both mammalian and yeast cells that are targeted by the proteasome following monoubiquitination or multiple monoubiquitinations. Interestingly, we confirmed a previous hypothesis (15) that there is a correlation between the length of the substrate and its requirement for either monoubiquitination or polyubiquitination. Also, the monoubiquitinated substrates are enriched in specific pathways [e.g., oxidative stress, carbohydrate transport, and components of the ubiquitin–proteasome system (UPS) itself] and, in humans, are structurally less disordered.

Results

Establishing a System for Induction of Monoubiquitination in Cells. In general, to model monoubiquitination (or multiple monoubiquitinations), we silenced endogenous Ub expression and replaced it with a lysineless Ub (Ub^{K0}). This nonpolymerizable Ub species, in which all seven Lys residues were replaced with Arg, can modify each Lys in the target substrate only once and cannot be further ubiquitinated (16–19).

To study monoubiquitination in yeast, we used a modification of a previously described Ub replacement method (20). Briefly, all of the Ub-coding genes were deleted and replaced by galactose-inducible Ub (ΔUb strain), and either copper-inducible Ub^{WT} or Ub^{K0} (ΔUb^{UbWT} or ΔUb^{UbK0}, respectively). Thus, Ub expression can be silenced by adding glucose, and Ub reexpression can be induced by adding copper to the growth medium (Fig. 1*A*). To validate Ub silencing and reexpression, ΔUb, ΔUb^{UbWT}, and ΔUb^{UbK0} yeast strains were treated with glucose and copper. As shown in Fig. 1*B*, Ub expression was efficiently suppressed, and both Ub^{WT} and Ub^{K0} were markedly expressed.

To assess monoubiquitination in human cells, we used a modification of a previously described Ub replacement model in human cultured cells (21). Briefly, endogenous Ub is silenced in U2OS cells by a Ub-specific tetracycline-induced shRNA (shUb), and either HA-Ub^{WT} or HA-Ub^{K0} is expressed following infection with an adenoviral vector (Fig. 1*A*). To evaluate Ub silencing efficiency, we monitored Ub and Ub conjugates level in $U2OS^{shUb}$ cells following tetracycline treatment. As demonstrated in Fig. 1*Ci*, the level of both Ub and Ub–protein conjugates were significantly decreased. In the endogenous Ub-silenced cells, both HA-Ub^{WT} and HA-Ub^{K0} were efficiently expressed and assembled into high–molecular-mass conjugates following adenoviral expression (Fig. 1*Cii*). It should be noted that the pattern of conjugation appears similar for both Ub^{WT} and Ub^{K0} expression. This is probably due to the numerous substrates with a broad range of molecular mass that are conjugated, and from the possibility that many of them are modified by multiple monoubiquitinations.

To demonstrate Ub replacement using an additional method, we quantified Ub using mass spectrometry (MS). As shown in Fig. S1*A*, tryptic digestion of Ub^{WT} and Ub^{K0} yields both common and differential MS-detectable peptides. To assess Ub replacement in yeast, we treated ΔUb^{UbK0} cells with glucose and copper, and quantified Ub-derived peptides by MS. As illustrated in Fig. S1*B*, Ub^{K0} was markedly more abundant than endogenous Ub. To evaluate Ub^{K0} expression in human cells, we overexpressed HA-Ub^{K0} via adenoviral infection using increasing multiplicities of infection (MOIs). As displayed in Fig. S1*C*, Ub^{K0} expression level was MOI dependent and significantly exceeded the level of endogenous Ub.

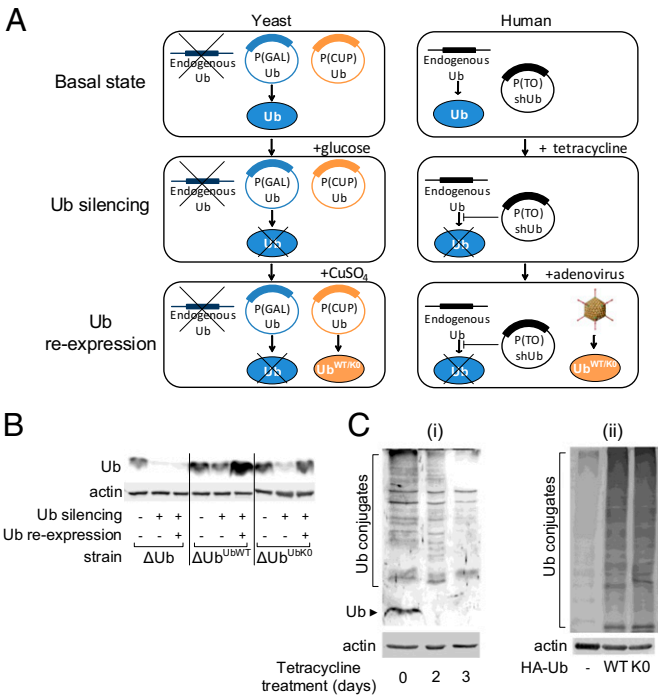

Fig. 1. Replacement of endogenous Ub by Ub^{K0} in yeast and mammalian cells. (*A*) Workflow describing Ub silencing and reexpression (see a detailed description under *Experimental Procedures*). (*B*) Ub replacement in yeast cells. ΔUb, ΔUb^{UbWT}, and ΔUb^{UbK0} yeast cells were treated for Ub silencing and Ub reexpression as indicated. Yeast cells were analyzed by trichloroacetic acid (TCA) lysis followed by SDS/PAGE and Western blotting (WB) using the indicated antibodies. (*C*) Ub replacement in human cells. (*i*) Ub silencing. To silence Ub, $U2OS^{shUb}$ cells were treated with tetracycline (1 μg/mL) for the indicated times. (*ii*) Ub reexpression. Following Ub silencing, cells were infected with viral vectors expressing Ub^{WT} or Ub^{K0}. In both panels, lysates were analyzed via SDS/PAGE followed by WB using the indicated antibodies.

Taken together, these data demonstrate the effectiveness of our Ub replacement strategies and suggest that our experimental systems are suitable for studying protein monoubiquitination.

Systematic Identification of Monoubiquitination-Dependent Proteasome Substrates. To identify substrates that are degraded following monoubiquitination, we replaced Ub with either Ub^{K0} or Ub^{WT} (as a control). We then used anti–K-ε-GG immunoprecipitation (Fig. S2) to enrich and quantify by MS GlyGly-modified peptides derived from tryptic digestion of ubiquitinated proteins (Fig. S2). This method enabled us also to identify ubiquitination sites (Ubsites). To verify that the ubiquitinated proteins serve indeed as proteolytic substrates, we monitored also the level of nonmodified peptides derived from them (Fig. 2*A*). To ascertain reproducibility, we performed several independent biological replicates for each model organism, using both stable isotope labeling by amino acids in cell culture (SILAC) and label-free quantification (Table S1). As shown in Fig. 2*B*, identification of the proteins was quite reproducible. Similar to previous data (22), identification of Ubsites within proteins was less reproducible.

This established experimental setup enabled us to discriminate between proteins degraded following modification by monoubiquitination (or multiple monoubiquitination), and those that are degraded

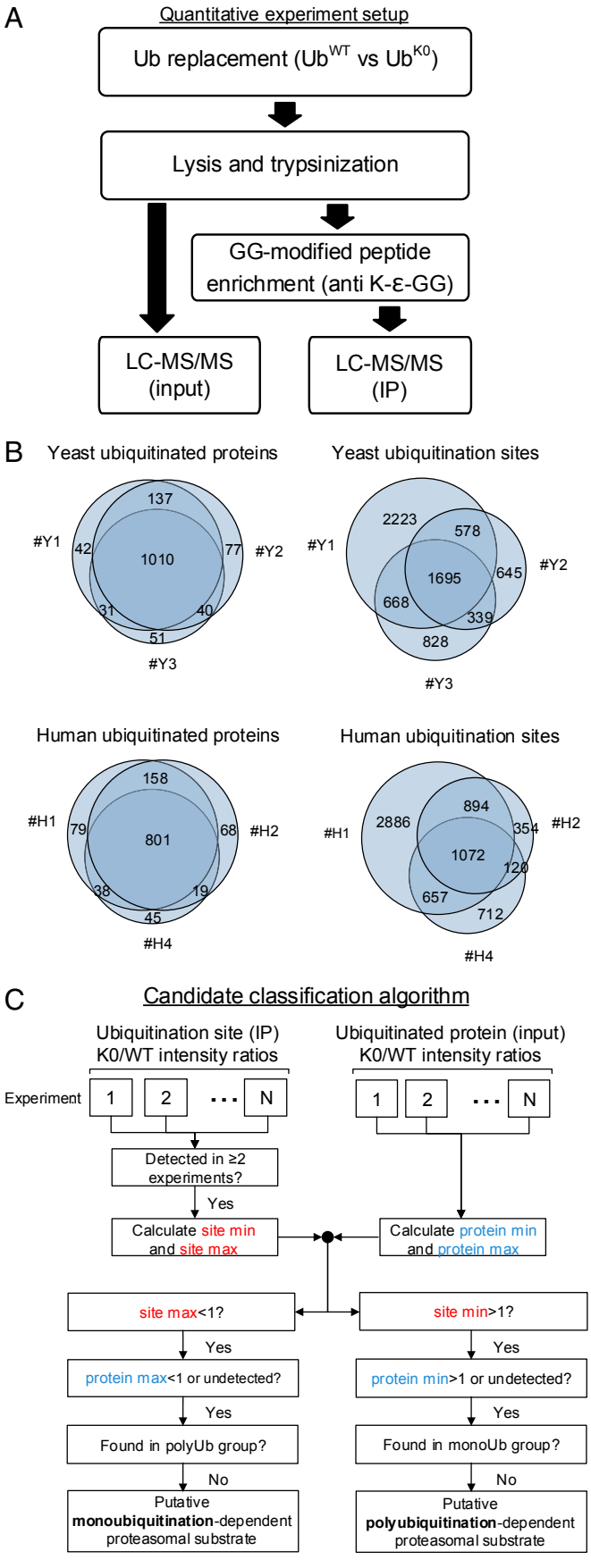

Fig. 2. Identification of monoubiquitination- and polyubiquitination-dependent proteasome substrates. (*A*) Experimental workflow. See a detailed description in *Results*. (*B*) Ubiquitination sites and ubiquitinated proteins identified in independent experiments. See Table S1 for experiment names. (*C*) The algorithm used for classification to monoubiquitination- and polyubiquitination-dependent substrates.

only following polyubiquitination. We calculated the MS signal intensity ratios following either Ub^{K0} or Ub^{WT} expression for both proteins and immunoprecipitated ubiquitinated sites (denoted "protein K0/WT intensity ratio" and "site K0/WT intensity ratio," respectively; see Dataset S1 for raw data). As illustrated in Fig. 2*C* and based on K0/WT intensity ratios, we classified ubiquitinated proteins to putative monoubiquitination- and polyubiquitination-dependent proteasome substrates as follows.

Monoubiquitination-dependent proteasomal substrates are expected to be unaffected by Ub^{K0} expression. Alternatively, as Ub^{K0} expression renders proteasomes less occupied by polyubiquitination-dependent substrates, increased proteasome availability may result in accelerated degradation of monoubiquitination-dependent substrates. Thus, we required these substrates to (*i*) have a site K0/WT ratio <1; (*ii*) have a detectable MS signal in at least two independent experiments; and (*iii*) have a protein K0/WT ratio <1 (if the protein is detectable).

The degradation of a polyubiquitination-dependent substrate is expected to be inhibited upon Ub^{K0} expression. Consequently, we expect their level to increase. Thus, we require these substrates to (*i*) have a site K0/WT ratio >1; (*ii*) have a detectable MS signal in at least two independent experiments; and (*iii*) have a protein K0/WT ratio >1 (if the protein is detectable).

A small fraction (<3%) of proteins were identified as belonging to the two groups. These proteins were excluded from the survey.

Applying these criteria in both yeast and human cells, we identified 82 and 220 monoubiquitination-dependent and 416 and 303 polyubiquitination-dependent putative proteasomal substrates, respectively (Dataset S2). Samples of each group are presented in Table 1, describing gene names and ubiquitinated Lys positions. As expected, the polyubiquitination-dependent substrate group included several previously suggested proteasomal substrates, e.g., Pdc1p, Ole1p, and Eno1p in yeast, and HIF1A, POLD2, and IER3 in human cells (23, 24).

Candidate Substrate Validation. To validate the results of our algorithm and experimental setup, we monitored the cellular stability (using cycloheximide chase) of randomly sampled representative candidate substrates following Ub replacement. As demonstrated in Fig. 3*A*, replacing Ub^{WT} with Ub^{K0} stabilized Ard1p in yeast and CDC20 in human cells (polyubiquitination-dependent substrates; Table 1). In contrast, the predicted monoubiquitination-dependent substrates, Gre1p in yeast and GOT1 in human cells, remained unstable. All four substrates were clearly degraded by the 26S proteasome, as they were stabilized following treatment with a proteasome inhibitor (Fig. 3*B*). Taken together, these results strongly suggest that our experimental setup is suitable for the systematic identification of monoubiquitination-dependent proteasomal substrates.

Physical Characteristics of Protein Substrates Play a Role in Their Mode of Ubiquitination: (*i*) Structural Disorder. Evidently, a significant number of proteins are degraded following monoubiquitination (and probably also multiple monoubiquitinations) in both yeast and human cells. This observation challenges the prevailing paradigm of polyubiquitination being the prerequisite for protein degradation. Intriguingly, bioinformatics analysis of the data reveals that yeast and human cells significantly differ in their preferences for using the two types of signals. In yeast, the ratio of proteins degraded by polyubiquitination vs. monoubiquitination is 5.07 (416/82), whereas in human cells it is only 1.37 (303/220). Because the identification method was unbiased, the significant difference in the ratios likely

Table 1. Putative ubiquitination-mediated proteasomal substrates

Entry	Protein name	Gene name	Ubiquitination positions	Ubiquitination mode/organism
Q00416	Helicase SEN1	SEN1	464	Mono/yeast
P22943	12-kDa heat shock protein	HSP12	12, 18, 23, 30, 36, 50, 64, 86, 88, 108	
Q08969	Protein GRE1	GRE1	7	
Q12734	Transcription factor CSR2	CSR2	670	
Q92325	Cullin-associated NEDD8-dissociated protein 1 homolog	LAG2	7	
Q04602	Vacuolar basic amino acid transporter 4	VBA4	368	
P36035	Carboxylic acid transporter protein homolog	JEN1	607	
P01094	Protease A inhibitor 3	PAI3	16, 18, 31, 32, 50	
Q12358	α-Ketoglutarate-dependent sulfonate dioxygenase	JLP1	114	Poly/yeast
P08679	Citrate synthase, peroxisomal	CIT2	96, 208, 454	
Q05637	Phosphate metabolism protein 6	PHM6	19	
P40483	Putative zinc metalloproteinase YIL108W	YIL108W	5, 348, 524	
P07347	N-terminal acetyltransferase A complex catalytic subunit ARD1	ARD1	191	
P00924	Enolase 1	ENO1	195, 409	
P06169	Pyruvate decarboxylase isozyme 1	PDC1	8	
P21147	Acyl-CoA desaturase 1	OLE1	457	
Q92890	Ubiquitin fusion degradation protein 1 homolog	UFD1L	279	Mono/human
Q9BT67	NEDD4 family-interacting protein 1	NDFIP1, N4WBP5, PSEC0192, PSEC0223	83	
Q9P2T1	GMP reductase 2	GMPR2	190	
Q92621	Nuclear pore complex protein Nup205	NUP205, C7orf14, KIAA0225	69	
Q9H9T3	Elongator complex protein 3	ELP3	338, 392, 517, 544	
P17174	Aspartate aminotransferase, cytoplasmic	GOT1	97, 99	
Q5JVF3	PCI domain-containing protein 2	PCID2, HT004	213	
O95302	Peptidyl-prolyl cis-trans isomerase FKBP9	FKBP9, FKBP60, FKBP63	527	
Q5JW28	Double-stranded RNA-binding protein Staufen homolog 1	STAU1	127	Poly/human
P36543	V-type proton ATPase subunit E 1	ATP6V1E1, ATP6E, ATP6E2	10	
Q9NWF9	E3 ubiquitin-protein ligase RNF216	RNF216, TRIAD3, UBCE7IP1, ZIN	487, 584, 773	
Q9NS91	E3 ubiquitin-protein ligase RAD18	RAD18, RNF73	115, 376	
Q12834	Cell division cycle protein 20 homolog	CDC20	136	
Q16665	Hypoxia-inducible factor 1α	HIF1A, BHLHE78, MOP1, PASD8	377, 389, 674, 709	
P46695	Radiation-inducible immediate-early gene IEX-1	IER3, DIF2, IEX1, PRG1	84	
P49005	DNA polymerase δ-subunit 2	POLD2	267	

points to important differences in the mode of recognition of the UPS in the two species. Yeast apparently operates much more by the traditional signal, polyubiquitin, whereas in humans monoubiquitination is used almost as frequently as polyubiquitination to mark proteins for degradation. The difference may arise from a combination of factors such as using different sets of conjugating enzymes (E2s and E3s), different preferences for local sequence and structural features of substrates, and the recognition elements of the proteasome. Some of these issues are addressed below.

In previous studies, it has been shown that protein ubiquitination and degradation are intimately linked with structural disorder. Intrinsically disordered protein regions lack a well-defined tertiary structure, yet they fulfill important functional roles linked with their highly flexible and adaptable structure (25–27). Structural disorder correlates with all three elements of degradation signals: location of the ubiquitin ligase recognition motif on substrates, the Lys residue(s) to which ubiquitin is attached, and a nearby long disordered region (LDR) (a region of at least 30 consecutive disordered residues) that initiates the unfolding of the substrate engaged with the proteasome (15, 28–30). Structural disorder may also be required for ubiquitin conjugation itself, in two different ways. It is repeatedly reported that the mutation of the Lys residue that is the site of modification does not usually abrogate sensitivity to UPS degradation (11, 31), because flexibility of the substrate enables multiple modifications on neighboring Lys resides. By a similar logic, the buildup of a polyubiquitin chain can also benefit from local structural disorder, because it enables the processive addition of subsequent ubiquitin moieties to the end of the growing polyubiquitin chain.

To test whether these disorder features may be related to the use of monoubiquitination vs. polyubiquitination, we tested whether predicted structural disorder of proteins differs in the different datasets (Fig. 4*A*). We found significant differences between yeast and human proteins: in yeast, the occurrence of structural disorder does not differ between monoubiquitinated and polyubiquitinated substrates, whereas in human cells, structural disorder prevails in polyubiquitinated substrates. Given that polyubiquitin is a stronger signal, we presume that

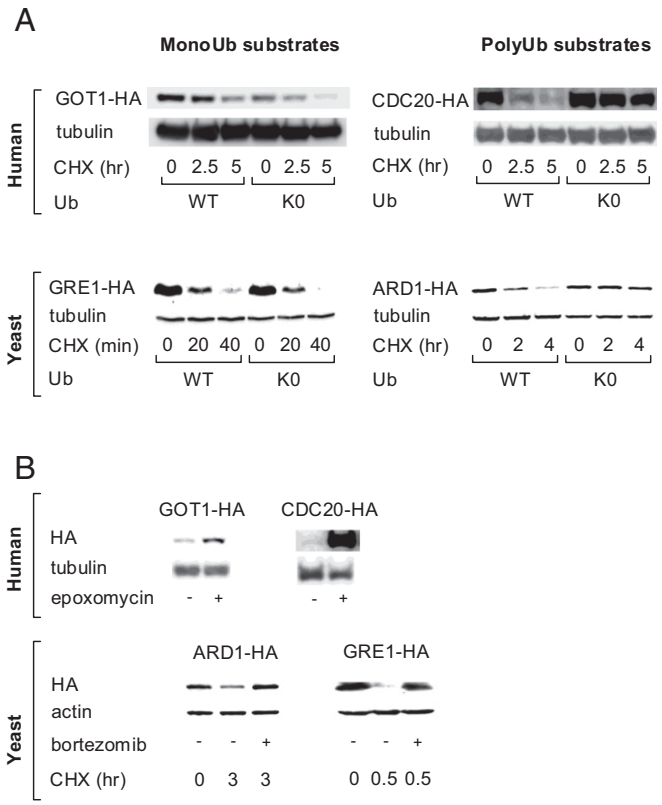

Fig. 3. Validation of representative candidate substrates degraded by the proteasome following monoubiquitination and polyubiquitination. (*A*, *Upper*) MCF7 cells were cotransfected with plasmids coding for GOT1-HA or CDC20-HA along with plasmids coding for Ub^{WT} or Ub^{K0} as indicated. (*Lower*) ΔUb^{UbWT} and ΔUb^{UbK0} yeast cells were transformed with plasmids coding for Gre1p-HA or Ard1p-HA, and Ub replacement was carried out as described in Fig. 1. In all experiments, substrate stability was monitored as described in *Experimental Procedures*. (*B*, *Upper*) MCF7 cells were transfected with either GOT1-HA or CDC20-HA, followed by the treatment with epoxomicin (2 μM, 24 h) as indicated. (*Lower*) ΔPDR5 yeast cells (strain Y12409 from the EUROSCARF collection) were transformed with plasmids coding for either Ard1p-HA or Gre1p-HA. Strains were treated with cycloheximide (CHX) and bortezomib (100 μM each) as indicated. Samples were collected at the indicated time points. Cell lysates were analyzed via SDS/PAGE followed by WB using the indicated antibodies.

structural disorder is primarily used for the buildup of the polyubiquitin chain. Because yeast relies more heavily on polyubiquitination, yet its polyubiquitinated substrates do not have more disorder, it is possible that their E3 ligases differ from humans in some critical features. These can be their number/redundancy, structural disorder, or binding heterogeneity, the structural disorder of which ensures processive addition of ubiquitin moieties in the ubiquitin chain.

These differences also have to manifest themselves in the local disorder of the protein chain around the ubiquitinated Lys residues. In general, the sites of posttranslational modifications (PTMs) in proteins tend to exhibit local disorder, as studied in detail for phosphorylation (32) and also ubiquitination (28). In our entire dataset, the lysines that are the sites of ubiquitination tend to be locally disordered, but even more importantly, they show a highly characteristic difference between the two species. In yeast, monoubiquitination sites, whereas in human cells polyubiquitination sites, are significantly more disordered (Fig. 4*B*). Our interpretation, again, is that these differences point to likely differences in the UPS in the two species. In yeast, polyubiquitination is robust, and it is the rare monoubiquitination sites that have to be supported by local disorder, probably more for initiation of degradation than modification (29). In human cells, the sites of polyubiquitination are significantly more disordered, probably as much for the processive buildup of the polyubiquitin chain as for initiation of degradation (as suggested above). These are genuine differences and do not result from the natural tendency of lysines to be locally disordered, which, as disorder-promoting amino acids, tend to be located in disordered regions of proteins (33) (Fig. S3*A*).

The distinction between the signaling strength and functionality of monoubiquitin and polyubiquitin chains in yeast and human cells is also reflected in characteristic differences in the proximity of LDRs to Ubsites, which might be the sites of initiation of proteasomal degradation (28–30). The need of such assistance for monoubiquitination sites in yeast is apparent from the larger proportion of such sites that are close to an LDR (Fig. 4*C*), and probably also by a larger proportion of such sites that are close to the termini of the proteins, which are generally flexible (Fig. S3*B*).

Conservation in evolution can be an important indicator of the functionality of PTM sites. For example, functional phosphorylation sites (i.e., were shown to have a functional role by direct or indirect evidence, e.g., mutagenesis and/or functional assays) evolve significantly slower than those without evidence for a functional role (34). Interestingly, Ubsites are significantly more conserved in human cells than in yeast (Fig. S3*C*) (these differences are significant because the phylogenetic coverage of the respective multiple alignments is comparable). These results infer that yeast sites are under a significantly lower evolutionary pressure, which may point to the fact that they are functionally more promiscuous.

Physical Characteristics of the Proteins Play a Role in Their Mode of Ubiquitination: (*ii*) Size of the Protein. In a previous study, we demonstrated that substrates degraded following conjugation of a single Ub moiety are usually shorter than 150 aa (15), which suggests that monoubiquitination is a weaker signal. Consistently, we found in this study a significant enrichment of shorter proteins among monoubiquitinated substrates in both yeast and human (Fig. S3*D*). This difference is not reflected in a preference of multiple modifications in longer proteins: there are no significant differences in the length of substrates that are degraded after the attachment of a single vs. multiple monoubiquitin or polyubiquitin chains (Fig. S3*E*).

Monoubiquitination- and Polyubiquitination-Dependent Substrates Are Differentially Enriched in Specific Biological Processes. To characterize the two different modes of ubiquitination in a cellular function perspective, we searched for enriched gene ontology (GO) terms, using the GOrilla tool (35, 36). As illustrated in Fig. 5*A*, it appears that monoubiquitination-dependent proteasomal substrates are enriched in oxidative stress response and carbohydrate transport pathways. As presented in Fig. 5*B*, molecular-function–specific GO terms were also identified. For example, monoubiquitination-dependent substrates were found enriched among ribosomal and proteasomal subunits. Similarities between the monoubiquitinated and polyubiquitinated substrates were also observed, as Ub system components were enriched in both groups. Interestingly, however, monoubiquitinated substrates consisted of mostly Ub ligases, E3s (e.g., HERC3, ITCH, XIAP), whereas polyubiquitinated substrates were enriched with Ub-conjugating enzymes, E2s (e.g., UBE2J1, UBE2T, and UBE2L6).

Ubiquitination Site Sequence Analysis Reveals Unique Patterns. Previous studies have demonstrated motifs and sequence patterns specific for protein posttranslational modifications such as phosphorylation, acetylation, and SUMOylation (29, 30, 37, 38). To identify Ubsite motifs, we analyzed our data using the Motif-X algorithm (39).

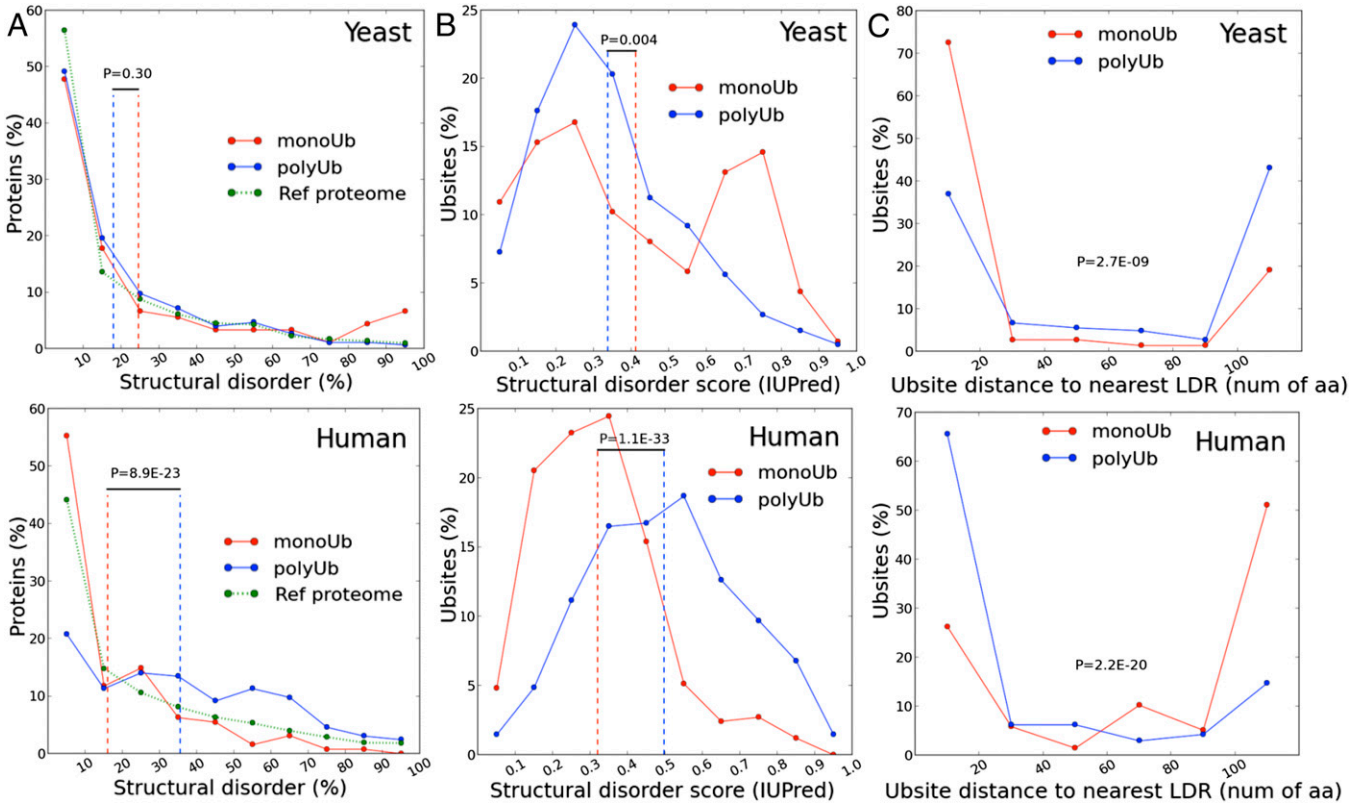

Fig. 4. Yeast and human substrates demonstrate distinct patterns of structural disorder. (*A*) Human polyubiquitinated substrates are more disordered than monoubiquitinated substrates. The distribution of overall disorder content in monoubiquitinated and polyubiquitinated substrates is plotted for yeast (*Upper*) and human (*Lower*). Overall disorder content is the fraction of predicted disordered residues in a given protein sequence. The distribution for the reference proteome corresponding to each species is shown for comparison. Dotted vertical lines correspond to the average values for the distribution. (*B*) Local disorder at the ubiquitination sites is more prominent in yeast monoubiquitination substrates and in human polyubiquitination substrates. The distribution of local disorder score is plotted for monoubiquitinated and polyubiquitinated sites from yeast (*Upper*) and human (*Lower*). The disorder profile for each sequence is first calculated using IUPred, and then the average disorder score of a 21-residue sequence window centered on each Ubsite is calculated. The distribution for the reference proteome corresponding to each species is shown for comparison. Dotted vertical lines correspond to the average values for the distribution. (*C*) The distance of Ubsites from their nearest LDR (see *Experimental Procedures* for definition) is plotted for yeast (*Upper*) and human (*Lower*) sites. The averages of the distributions are not shown here for the sake of clarity.

Consistent with previous findings (24), no specific motif was found when analyzing all identified Ubsites in either yeast or human cells. To analyze Ubsite amino acid composition, all identified Ubsites were examined. We constructed an alignment of peptides that are surrounding each modified Lys residue. Residue- and position-specific amino acid occurrences were calculated and were compared with the corresponding proteomic occurrence in a lysine-centered peptide. As depicted in Fig. S4*A*, ubiquitination sites demonstrated residues-specific enrichment (e.g., Ala, Gly, Gln) and underrepresentation (e.g., Cys, His, Lys, Met, Trp) for both yeast and human cells. Enrichment of Glu, and to a lesser extent Asp, was specific to yeast sites. Importantly, for several amino acids, the enrichment factor depended on the proximity to the modified Lys, suggesting that these residues can affect conjugation mechanisms.

To compare the ubiquitination site composition of monoubiquitination- vs. polyubiquitination-dependent substrates, we performed the above analysis for each group separately. As shown in Fig. S4 *B* and *C*, each group displays a unique pattern of enrichment factors, and several differences can be observed between the patterns of monoubiquitination- and polyubiquitination-dependent substrates.

Discussion

In this study, we identified 82 yeast and 220 mammalian proteins that are degraded by the proteasome following monoubiquitination. These significant numbers point to a much broader phenomenon to what was considered until recently as an exception. Therefore, it allows for analysis that sheds light on the mechanisms that underlie the different modes of ubiquitination. Because we did not use proteasome inhibitors, it is likely that some low level, rapidly degrading proteins were below the detection threshold, and therefore the numbers are probably larger.

Experimentally, we replaced Ub^{WT} with Ub^{K0} in cells to enforce monoubiquitination. This strategy has been used successfully to inhibit polyubiquitination-dependent proteasomal degradation (14, 17–19). Other methods to study monoubiquitination have been reported, such as inhibition of polyubiquitin chain formation by methylated Ub (40), or detection by Western blotting of specific substrates that appear to be monoubiquitinated (10). However, using methylated Ub is limited to cell-free systems, and using endogenous Ub can identify only individual substrates. Therefore, these methods limit the ability to identify and characterize the broad population of target substrates degraded by the proteasome following monoubiquitination and polyubiquitination. Thus, Ub^{WT}-to-Ub^{K0} replacement in cells seemed to be the most suitable strategy for our objective of carrying out a proteome-wide screen to identify these two distinct populations.

Notably, although efficient Ub^{K0} expression inhibits polyubiquitination, it may still support the conjugation of several Ub^{K0} molecules to a protein substrate, resulting in multiple monoubiquitinations. In our study, we included monoubiquitinated

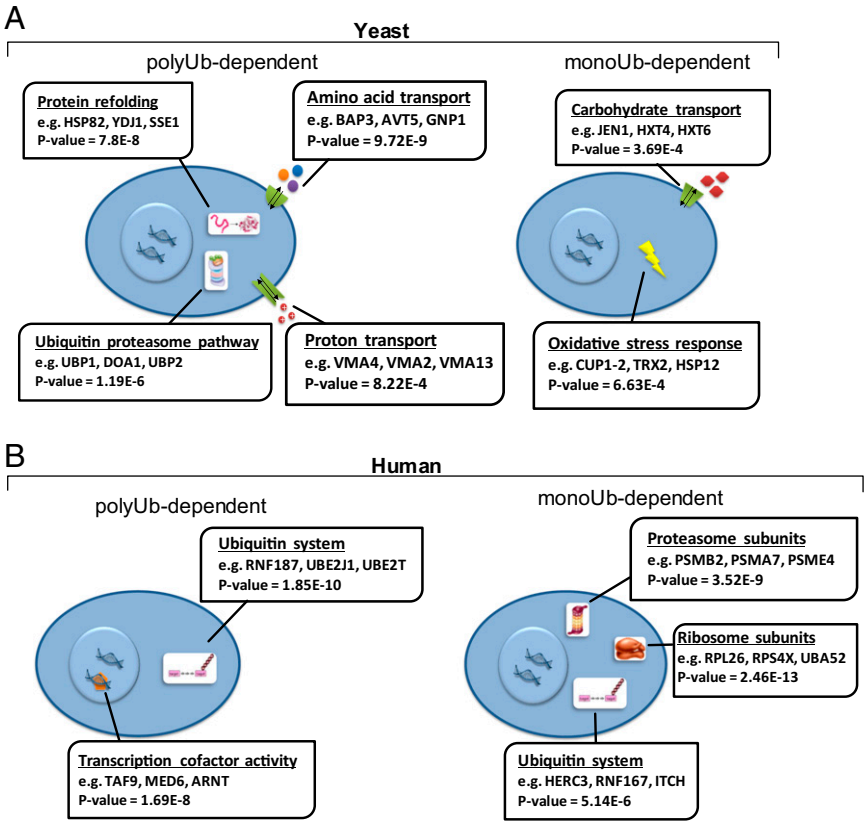

Fig. 5. Analysis of molecular functions and involvement in different cellular processes for monoubiquitination and polyubiquitination-dependent substrates. Monoubiquitination- and polyubiquitination-dependent substrate groups were analyzed for gene ontology (GO) term enrichment using the GOrilla tool, as described under *Experimental Procedures*. Representative proteins are shown for each enriched term, and the full list is shown in Table S2. (*A*) Biological-process GO term analysis for yeast substrates. (*B*) Molecular-function GO term analysis for human substrates.

and multiple-monoubiquitinated substrates in the same group, as discriminating between the two is complicated experimentally.

Importantly, in our screen, we decided not to use proteasome inhibitors as a tool to identify substrates that are nevertheless degraded following Ub replacement; the reason being that, in human cells infected with adenoviral HA-Ub^{WT}, we could not observe up-regulation of Ub conjugates following treatment with a proteasome inhibitor (Fig. S5*A*). It should be noted that the proteasome in the control cells was active (Fig. S5*B*), and its inhibition was efficient (Fig. S5*C*). This finding is consistent with partial proteasome inhibition by free Ub chains that are accumulated due to the high level of HA-Ub^{WT} (41). Also, the use of proteasome inhibitors is challenging in yeast cells, as low permeability results in low cellular concentration of the drugs (42). Notably, in human cells, it was reported that proteasome inhibitors impair protein synthesis in a selective manner, thus affecting the proteome in more than one mode. Therefore, interpretation of proteomic data of experiments carried out in their presence is not straightforward (43). Accordingly, we have adapted alternative classification criteria to identify monoubiquitinated vs. polyubiquitinated proteasomal substrates based on MS analyses of the proteome and ubiquitome under normal conditions and where only monoUb is available (*Results* and Fig. 2*B*). To confirm our results and to nevertheless relate them to proteasomal degradation, we integrated data from previous studies (23, 24, 44) and constructed a reference list of known ubiquitin–proteasome substrates (Dataset S3). Compared with this list, our polyubiquitination-dependent substrates were highly enriched with known proteasomal substrates (P value$_{yeast}$ = 6.90 × 10^{-23} and P value$_{human}$ = 5.06 × 10^{-5}, by hypergeometric test). Furthermore, our monoubiquitination-dependent candidates were also highly enriched with proteasomal substrates (P value$_{human}$ = 1.10 × 10^{-36}). These findings strongly suggest that our experimental model is faithful and offers a reliable method for the identification of UPS substrates. Furthermore, we validated biochemically that several randomly sampled monoubiquitination and polyubiquitination-dependent candidates that emerged from the screen do indeed belong to their respective predicted categories (Fig. 3).

Reportedly, membrane proteins such as receptor tyrosine kinases (RTKs) (45) are subjected to monoubiquitination-dependent lysosomal degradation. To specifically identify proteasomal substrates in the absence of proteasome inhibitors, plasma membrane proteins were excluded from our screen.

It is interesting to refer to specific proteins that were identified in previous studies as targeted by monoubiquitination. For example, Syndecan-4 (12) did exhibit site K0/WT ratio <1 (for Lys105) but was detected in one replicate only. Cks-2 (15) displayed protein K0/WT ratio <1 at the protein level but had conflicting ratios at site level. Other substrates including Pax-3 (13) and α-synuclein (10) could not be detected, probably due to low abundance in the bone-derived U2OS cells (46). Taken together, it seems that our results are in agreement with random previous data but should be further substantiated by experiments in cells from different tissues.

From the bioinformatics analyses of the substrates, several important and intriguing conclusions can be drawn. First, in agreement with previous studies in which it was shown that substrates of up to 150 residues can be degraded following monoubiquitination (15), we found that the distribution of monoubiquitination-dependent substrates is shifted toward shorter proteins (Fig. S3*D*).

Furthermore, a difference between yeast and human is also apparent when comparing the ratio of monoubiquitination- and polyubiquitination-dependent substrates. Yeast relies more heavily on polyubiquitination, whereas human cells use both monoubiquitination and polyubiquitination with a similar frequency. This difference can be interpreted if structural disorder is considered as shown by our own data (Fig. 4 and Fig. S3), and if we assume that a single ubiquitin moiety is a weaker signal for degradation than polyubiquitin. As suggested, local structural disorder is involved in various steps of the UPS cascade, from recognition motifs of E3 ligases through local disorder of ubiquitination sites to an LDR initiation site of substrate unfolding (28–30).

We have screened our monoubiquitination-dependent substrates for enriched biological-process–related GO terms and found a highly significant overrepresentation of genes associated with carbohydrate transport and oxidative stress response pathways (Fig. 5*A*). Because carbohydrate transporters are plasma membrane proteins, this finding is consistent with previous studies that demonstrated membrane receptors down-regulation via monoubiquitination-mediated endocytosis (47). Notably, as this pathway results in lysosomal/vacuolar rather than proteasomal degradation, this finding highlights the challenge in distinguishing between these two degradation modes using our experimental system. Oxidative stress was shown to activate cellular signal transduction cascades, and to result in gene expression modulation (48). The enrichment of oxidative stress response proteins in our monoubiquitination-dependent substrates may suggest that they are regulated by a common monoubiquitinating E3 ligase(s). Thus, oxidative stress-mediated down-regulation of this putative E3 may result in up-regulation of oxidative stress pathway components and activate the respective cellular response. Consistently, microarray experiments have shown that the expression of the E3s *UBR1* and *HUL4*, and the E2s *CDC43*, *RAD6*, and *UBC11*, is decreased following exposure to oxidative stress (49).

UPS components were enriched in both groups (Fig. 5*B*). This finding most likely represents the previously reported autoubiquitination of E2s (50–52) and E3s (53–55). Our findings show that E3s are preferably classified as monoubiquitination-dependent substrates.

In this study, we have determined the sequence positions of thousands of ubiquitinated lysines. The unsuccessful attempts to identify a ubiquitination-site motif in this study and in others (24, 56) reflect site-level promiscuity, which is supported by low ubiquitination sites conservation across eukaryotic species (56), and by the flexible selection of the ubiquitinated lysines within a given substrate (11, 31). Additionally, we used position-specific analysis of relative amino acid abundance to characterize ubiquitination sites (Fig. S4). This method yields a more thorough representation of the amino acid composition and reflects both enrichment and underrepresentation trends. According to the notion of promiscuity, a ubiquitination site should merely provide a sterically available ε-amino group of a Lys residue. Consistently, we found enrichment of small residues (e.g., Ala and Gly) and decrease in bulky (e.g., Trp) residues in the proximity of the ubiquitinated Lys. The depletion of Pro at position −1 also supports this concept, because Pro disrupts the protein's secondary structure and might impair the solvent accessibility of a following Lys residue.

Experimental Procedures

Adenovirus-Mediated Ub Replacement in Human Cells. For Ub silencing, U2OS^{shUb} (described in ref. 21) cells were treated with 1 μg/mL tetracycline for 24 h. Fresh tetracycline and adenoviruses encoding either HA-Ub^{WT} or HA-Ub^{K0} were added, and cells were incubated for additional 24 h.

Ub Replacement in Yeast. The construction of ΔUb strain was described previously [SUB328 (57)]. Briefly, endogenous Ub genes were deleted and replaced with a Ub gene expressed under a Gal promoter. To construct ΔUb^{UbWT} and ΔUb^{UbK0} strains, ΔUb yeast cells were transformed with pUb39 Ub^{WT} and pUb39 Ub^{K0}, respectively (both genes are under the Cup1 promoter). To replace the Gal-induced Ub, ΔUb^{UbWT} and ΔUb^{UbK0} yeast cells were grown to 1.0 OD_{600nm} in standard Hartwell's complete medium (HC) without glucose, supplemented with 2% (wt/vol) galactose and 2% (wt/vol) raffinose. Cells were then washed in double-distilled water (DDW) to remove galactose and raffinose, and resuspended in HC medium with glucose and 50 μM $CuSO_4$. Cells were incubated for 16 h at 30 °C, collected by centrifugation, washed in DDW, and frozen in liquid N_2.

Ubiquitination Sites Detection Using GG-Modified Peptide Enrichment. Dried peptides (*SI Experimental Procedures*, *Sample Preparation for MS*) were resuspended in immunoaffinity purification (IAP) buffer (50 mM Mops/NaOH, pH 7.2, 10 mM Na_2HPO_4, and 50 mM NaCl), and cleared by centrifugation. Supernatants were adjusted to pH 7.0 with NaOH and incubated with immobilized anti–K-ε-GG antibody (Cell Signaling Technology) at 4 °C for 3 h. Beads were washed with IAP buffer and then with a wash buffer (500 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% octyl glucoside, pH 7.4). GG-modified peptides were eluted with 0.2% TFA, desalted on C18 tips, and eluted in two fractions of 20% and 80% (vol/vol) acetonitrile. Peptides were analyzed as described in *SI Experimental Procedures*, *MS*.

For additional experimental procedures, see *SI Experimental Procedures*.

ACKNOWLEDGMENTS. We thank Drs. Zhijian James Chen (University of Texas Southwestern) and Daniel Finley (Harvard Medical School) for providing us with the mammalian and yeast systems for deleting endogenous Ub genes, respectively. Research in the laboratory of A.C. is supported by grants from the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, the Israel Science Foundation (ISF), the I-CORE Program of the Planning and Budgeting Committee and the ISF (Grant 1775/12), and the Deutsch–Israelische Projektkooperation. I.L. is supported by the Foulkes Fellowship. A.C. is an Israel Cancer Research Fund USA Professor. A.G. is supported by NIH Grant R01 GM101457. This work was supported by Odysseus Grant G.0029.12 from Research Foundation Flanders (to P.T.) and a VIB/Marie Curie COFUND Postdoctoral (omics@VIB) fellowship (to M.G.).

1. Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: Destruction for the sake of construction. *Physiol Rev* 82(2):373–428.
2. Komander D (2009) The emerging complexity of protein ubiquitination. *Biochem Soc Trans* 37(Pt 5):937–953.
3. Thrower JS, Hoffman L, Rechsteiner M, Pickart CM (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J* 19(1):94–102.
4. Hicke L, Schubert HL, Hill CP (2005) Ubiquitin-binding domains. *Nat Rev Mol Cell Biol* 6(8):610–621.
5. Hoeller D, et al. (2006) Regulation of ubiquitin-binding proteins by monoubiquitination. *Nat Cell Biol* 8(2):163–169.
6. Goh LK, Sorkin A (2013) Endocytosis of receptor tyrosine kinases. *Cold Spring Harb Perspect Biol* 5(5):a017459.
7. Ahearn IM, Haigis K, Bar-Sagi D, Philips MR (2011) Regulating the regulator: Post-translational modification of RAS. *Nat Rev Mol Cell Biol* 13(1):39–51.
8. Jura N, Scotto-Lavino E, Sobczyk A, Bar-Sagi D (2006) Differential modification of Ras proteins by ubiquitination. *Mol Cell* 21(5):679–687.
9. Chandrasekharan MB, Huang F, Sun ZW (2009) Ubiquitination of histone H2B regulates chromatin dynamics by enhancing nucleosome stability. *Proc Natl Acad Sci USA* 106(39):16686–16691.
10. Rott R, et al. (2011) α-Synuclein fate is determined by USP9X-regulated monoubiquitination. *Proc Natl Acad Sci USA* 108(46):18666–18671.
11. Dimova NV, et al. (2012) APC/C-mediated multiple monoubiquitylation provides an alternative degradation signal for cyclin B1. *Nat Cell Biol* 14(2):168–176.
12. Carvallo L, et al. (2010) Non-canonical Wnt signaling induces ubiquitination and degradation of Syndecan4. *J Biol Chem* 285(38):29546–29555.
13. Boutet SC, Disatnik MH, Chan LS, Iori K, Rando TA (2007) Regulation of Pax3 by proteasomal degradation of monoubiquitinated protein in skeletal muscle progenitors. *Cell* 130(2):349–362.
14. Kravtsova-Ivantsiv Y, Cohen S, Ciechanover A (2009) Modification by single ubiquitin moieties rather than polyubiquitination is sufficient for proteasomal processing of the p105 NF-kappaB precursor. *Mol Cell* 33(4):496–504.
15. Shabek N, et al. (2012) The size of the proteasomal substrate determines whether its degradation will be mediated by mono- or polyubiquitylation. *Mol Cell* 48(1):87–97.
16. Braten O, Shabek N, Kravtsova-Ivantsiv Y, Ciechanover A (2012) Generation of free ubiquitin chains is up-regulated in stress and facilitated by the HECT domain ubiquitin ligases UFD4 and HUL5. *Biochem J* 444(3):611–617.
17. Hospenthal MK, Freund SM, Komander D (2013) Assembly, analysis and architecture of atypical ubiquitin chains. *Nat Struct Mol Biol* 20(5):555–565.
18. Sun T, et al. (2011) The role of monoubiquitination in endocytic degradation of human ether-a-go-go-related gene (hERG) channels under low K^+ conditions. *J Biol Chem* 286(8):6751–6759.

19. Ward CL, Omura S, Kopito RR (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* 83(1):121–127.
20. Finley D, et al. (1994) Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol* 14(8):5501–5509.
21. Xu M, Skaug B, Zeng W, Chen ZJ (2009) A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta. *Mol Cell* 36(2):302–314.
22. Emanuele MJ, et al. (2011) Global identification of modular cullin-RING ligase substrates. *Cell* 147(2):459–474.
23. Mayor T, Graumann J, Bryan J, MacCoss MJ, Deshaies RJ (2007) Quantitative profiling of ubiquitylated proteins reveals proteasome substrates and the substrate repertoire influenced by the Rpn10 receptor pathway. *Mol Cell Proteomics* 6(11):1885–1895.
24. Kim W, et al. (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 44(2):325–340.
25. Tompa P (2011) Unstructural biology coming of age. *Curr Opin Struct Biol* 21(3): 419–425.
26. van der Lee R, et al. (2014) Classification of intrinsically disordered regions and proteins. *Chem Rev* 114(13):6589–6631.
27. Guharoy M, Bhowmick P, Tompa P (2016) Design principles involving protein disorder facilitate specific substrate selection and degradation by the ubiquitin-proteasome system. *J Biol Chem* 291(13):6723–6731.
28. Guharoy M, Bhowmick P, Sallam M, Tompa P (2016) Tripartite degrons confer diversity and specificity on regulated protein degradation in the ubiquitin-proteasome system. *Nat Commun* 7:10239.
29. Inobe T, Fishbain S, Prakash S, Matouschek A (2011) Defining the geometry of the two-component proteasome degron. *Nat Chem Biol* 7(3):161–167.
30. Ravid T, Hochstrasser M (2008) Diversity of degradation signals in the ubiquitin-proteasome system. *Nat Rev Mol Cell Biol* 9(9):679–690.
31. King RW, Glotzer M, Kirschner MW (1996) Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. *Mol Biol Cell* 7(9):1343–1357.
32. Iakoucheva LM, et al. (2004) The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res* 32(3):1037–1049.
33. Dunker AK, et al. (2001) Intrinsically disordered protein. *J Mol Graph Model* 19(1): 26–59.
34. Landry CR, Levy ED, Michnick SW (2009) Weak functional constraints on phosphoproteomes. *Trends Genet* 25(5):193–197.
35. Eden E, Lipson D, Yogev S, Yakhini Z (2007) Discovering motifs in ranked lists of DNA sequences. *PLoS Comput Biol* 3(3):e39.
36. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z (2009) GOrilla: A tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 10: 48.
37. Amanchy R, et al. (2007) A curated compendium of phosphorylation motifs. *Nat Biotechnol* 25(3):285–286.
38. Basu A, et al. (2009) Proteome-wide prediction of acetylation substrates. *Proc Natl Acad Sci USA* 106(33):13785–13790.
39. Chou MF, Schwartz D (2011) Biological sequence motif discovery using Motif-X. *Curr Protoc Bioinformatics* Chap 13:Unit 13.15–24.
40. Hershko A, Ganoth D, Pehrson J, Palazzo RE, Cohen LH (1991) Methylated ubiquitin inhibits cyclin degradation in clam embryo extracts. *J Biol Chem* 266(25):16376–16379.
41. Amerik AYu, Swaminathan S, Krantz BA, Wilkinson KD, Hochstrasser M (1997) In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. *EMBO J* 16(16):4826–4838.
42. Lee DH, Goldberg AL (1996) Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. *J Biol Chem* 271(44):27280–27284.
43. Milner E, et al. (2013) The effect of proteasome inhibition on the generation of the human leukocyte antigen (HLA) peptidome. *Mol Cell Proteomics* 12(7):1853–1864.
44. Udeshi ND, et al. (2013) Refined preparation and use of anti-diglycine remnant (K-ε-GG) antibody enables routine quantification of 10,000s of ubiquitination sites in single proteomics experiments. *Mol Cell Proteomics* 12(3):825–831.
45. Haglund K, et al. (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* 5(5):461–466.
46. Liu X, Yu X, Zack DJ, Zhu H, Qian J (2008) TiGER: A database for tissue-specific gene expression and regulation. *BMC Bioinformatics* 9:271.
47. Hicke L, Dunn R (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* 19:141–172.
48. Ikner A, Shiozaki K (2005) Yeast signaling pathways in the oxidative stress response. *Mutat Res* 569(1-2):13–27.
49. Gasch AP, et al. (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* 11(12):4241–4257.
50. David Y, Ziv T, Admon A, Navon A (2010) The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *J Biol Chem* 285(12):8595–8604.
51. Rape M, Kirschner MW (2004) Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry. *Nature* 432(7017):588–595.
52. Ravid T, Hochstrasser M (2007) Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. *Nat Cell Biol* 9(4):422–427.
53. Amemiya Y, Azmi P, Seth A (2008) Autoubiquitination of BCA2 RING E3 ligase regulates its own stability and affects cell migration. *Mol Cancer Res* 6(9):1385–1396.
54. Lorick KL, et al. (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci USA* 96(20):11364–11369.
55. Noels H, et al. (2009) Auto-ubiquitination-induced degradation of MALT1-API2 prevents BCL10 destabilization in t(11;18)(q21;q21)-positive MALT lymphoma. *PLoS One* 4(3):e4822.
56. Danielsen JM, et al. (2011) Mass spectrometric analysis of lysine ubiquitylation reveals promiscuity at site level. *Mol Cell Proteomics* 10(3):M110.003590.
57. Spence J, Sadis S, Haas AL, Finley D (1995) A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol Cell Biol* 15(3):1265–1273.
58. Hershko A, Eytan E, Ciechanover A, Haas AL (1982) Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. Relationship to the breakdown of abnormal proteins. *J Biol Chem* 257(23):13964–13970.
59. Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108(2):193–199.
60. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
61. Gietz RD, Woods RA (2006) Yeast transformation by the LiAc/SS carrier DNA/PEG method. *Methods Mol Biol* 313:107–120.
62. Dormond E, et al. (2010) An efficient process for the purification of helper-dependent adenoviral vector and removal of helper virus by iodixanol ultracentrifugation. *J Virol Methods* 165(1):83–89.
63. Dosztányi Z, Csizmok V, Tompa P, Simon I (2005) IUPred: Web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. *Bioinformatics* 21(16):3433–3434.