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Global analysis of phosphorylation and ubiquitylation crosstalk in protein degradation

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Abstract

Crosstalk between different types of post-translational modifications (PTMs) on the same protein molecule adds specificity and combinatorial logic to signal processing, but has not been characterized on a large-scale basis. Here, we developed two methods to identify protein isoforms that are both phosphorylated and ubiquitylated in the yeast *Saccharomyces cerevisiae*, identifying 466 proteins with 2,100 phosphorylation sites co-occurring with 2,189 ubiquitylation sites. We applied these methods quantitatively to identify phosphorylation sites that regulate protein degradation via the ubiquitin-proteasome system. Our results demonstrate that distinct phosphorylation sites are often used in conjunction with ubiquitylation, and these sites are more highly conserved than the entire set of phosphorylation sites. Finally, we investigated how the phosphorylation machinery can be regulated by ubiquitylation. We found evidence for novel regulatory mechanisms of kinases and 14-3-3 scaffold proteins via proteasome-independent ubiquitylation.

Introduction

Protein post-translational modifications (PTMs) function as highly versatile switches that regulate protein activity, concentration and subcellular localization, and maintain homeostasis. One or more residues in a protein can be modified, either in an independent fashion or combinatorially, to confer specific protein regulation[1,2]. However, both the basis and prevalence of PTM crosstalk, in which the presence of one modification influences the appearance of others, remain unknown.

Two of the most prevalent PTMs in eukaryotic proteomes, phosphorylation and ubiquitylation, are integral to almost every cellular process. Phosphorylation is the primary

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mechanism for regulating cellular signaling, while ubiquitylation plays a prominent role in protein degradation. Cross-regulation between phosphorylation and ubiquitylation can take many forms[2]. PTMs can regulate the machinery of other modification type, such as phosphorylation activating E3 ubiquitin ligase activity[3-5]. The coordinated targeting of a substrate by multiple modification types provides another example of crosstalk. This is perhaps best exemplified by phosphodegrons, in which one or more phosphorylation site(s) function in a *cis*-regulatory manner to promote the subsequent ubiquitylation of a substrate. Phosphodegrons are critical to cell cycle progression, where they impart irreversibility and robustness. Given the evidence for co-regulation between phosphorylation and ubiquitylation, and the influence of these PTMs on a wide variety of cellular processes, we set out to characterize the scope of crosstalk between these two modifications.

Proteomics based on mass spectrometry has emerged as a powerful tool to characterize protein modifications and has demonstrated that thousands of proteins are post-translationally modified at any given time[5-9]. To date, most studies have focused on a single type of modification in isolation due to two limitations[10-12]. First, modifications often exist at sub-stoichiometric levels and require highly specific enrichment methods. Second, proteomics is most commonly performed on peptides, rather than intact proteins, which often severs the connection between multiple modifications present on a single protein isoform. With these limitations in mind, we developed two enrichment strategies that permit the global identification of co-modified proteins and applied these methods to investigate ubiquitylation-phosphorylation crosstalk in the context of protein degradation. Using these approaches we find that distinct phosphorylation sites are often found co-occurring with ubiquitylation and describe the identification of several new phosphodegrons.

Results

Development of methods to identify co-modified proteins

To probe combinatorial PTM cross-talk, we developed two methods to enrich for proteins containing two PTM types. The specific enrichment approaches developed here are targeted at the evaluation of ubiquitylation and phosphorylation cross-talk but should be generally applicable to many PTM types.

In the first enrichment approach, we applied cobalt-NTA (nitrilotriacetic acid) affinity purification to enrich for proteins modified with His-tagged ubiquitin during log-phase growth in *S. cerevisiae*[13] (Fig. 1a). This ubiquitin-enriched population we refer to as ubiquitylated proteins, whereas the ubiquitin-depleted flow-through we refer to as nonubiquitylated proteins (Supplementary Fig. 1). After carrying out tryptic digestion, we enriched for phosphopeptides in both the ubiquitin-enriched population and the flow-through. Additionally, to identify individual ubiquitylation sites in the ubiquitin-enriched population, we used an antibody to enrich for peptides with the characteristic glycine-glycine (diGly) remnant that remains on ubiquitylated lysine residues after tryptic digestion. We analyzed the three samples (nonubiquitylated phosphopeptides, ubiquitylated phosphopeptides and ubiquitylated nonphosphopeptides) with nano–reversed-phase liquid chromatography (nRPLC) coupled to tandem mass spectrometry (MS/MS). The quantities of PTM sites and protein identifications are listed in Table 1.

We used two enrichment strategies (illustrated here in the context of the proteasome inhibition experiment). (a) In the first enrichment method, samples were enriched for ubiquitylated (Ub) proteins with affinity purification of His-tagged ubiquitin. Nonubiquitylated (Non-Ub) proteins (dotted lines) were digested with LysC and enriched for phosphopeptides. Ub proteins (solid lines) were digested with trypsin and further enriched for diGly peptides and for phosphopeptides, which was followed by mass

spectrometry analysis. (b) In the second enrichment method, proteins were digested with trypsin into peptides and enriched for doubly charged peptides via strong-cation exchange (SCX). All SCX fractions were further enriched for diGly peptides before mass spectrometry analysis. (c) Four cases showing how the same protein can exist in different combinations of modification states: a phosphorylation site unique to the ubiquitylated protein isoform (i), phosphorylation sites found in both ubiquitylated (ii) and nonubiquitylated (iii) isoforms, and a phosphorylation site unique to nonubiquitylated protein isoforms (iv). (d) Overlap of non-Ub and Ub samples with respect to phosphorylation sites and phosphoproteins. z, charge state. nRPLC, nano–reversed-phase liquid chromatography. Though this first method permits identification of proteins modified by both ubiquitylation and phosphorylation regardless of the proximity of these modifications on the protein sequence, it cannot establish which two sites are present on the same isoform. Thus, we developed a second approach to directly identify peptides concurrently modified by phosphorylation and ubiquitylation.

The second approach relies on sequential peptide-based enrichment to identify peptides containing multiple PTM types. Here, one can establish that both PTMS are present on the same protein isoform, but this approach is limited to identifying PTMS sites found in close sequence proximity. We used strong-cation exchange (SCX) chromatography to first separate peptides by their solution charge (Fig. 1b). SCX has previously been applied to enrich for PTMs that alter solution charge state, and we show here that it can also be utilized to enrich for both ubiquitylated peptides and ubiquitylated phosphopeptides (Supplementary Fig. 2a)[14,15]. Ubiquitylated peptides represent a substoichiometric population; thus, each SCX fraction was further enriched for the diGly ubiquitin remnant with an antibody, and all fractions were analyzed by nRPLC coupled to MS/MS. This second method resulted in 1,008 unique identifications of ubiquitylated phosphopeptides as compared to only 56 with the first method (Supplementary Fig. 2b, Table 1 and Supplementary Data 1).

A total of 466 proteins co-modified with ubiquitylation and phosphorylation were identified in these experiments (Table 1, Fig. 1c, d). Nearly all ubiquitylated phosphoproteins were also identified in a non-ubiquitylated phosphoprotein isoform (Fig. 1e, g). However, a smaller degree of overlap was observed at the phosphorylation site level (Fig1. f, g). These results suggest that protein isoforms modified with ubiquitin carry unique phosphorylation sites that likely distinguish their specific function relative to that of their non-ubiquitylated isoforms. We also compared the Gene Ontology enrichment of ubiquitylated phosphoproteins *vs*. non-ubiquitylated phosphoproteins. Ubiquitylated phosphoproteins were enriched for localization in the ribosome, membranes, the cell bud, and processes such as protein translation and transmembrane transport (Fig. 2a, Supplementary Table 1). In agreement with these results, we found ubiquitylated phosphoproteins to be enriched for several transporter and arrestin domains (Supplementary Table 2).

Conservation and function of sites on co-modified proteins

In order to study the evolutionary conservation of phosphorylation and ubiquitylation sites, we aligned yeast proteins with their respective human orthologs. A PTM was defined as conserved when the yeast protein and the human protein were modified in the same region of the alignment. We found ubiquitylation sites in yeast to be more conserved than expected by random chance. However, the level of conservation was not specific to the absence or presence of phosphorylation. Ubiquitylation sites on phosphoproteins had a similar level of conservation over random expectation with respect to all ubiquitylation sites ($P = 0.26$, Kolmogorov-Smirnov test, Supplementary Fig. 3).

We next examined the conservation of phosphorylation sites. As displayed in Figure 2b, we compared the conservation over random of four groups of yeast phosphorylation sites: (1) all

phosphorylation sites; (2) phosphorylation sites on non-ubiquitylated protein isoforms (Fig. 1f); (3) phosphorylation sites on ubiquitylated proteins, but identified in non-ubiquitylated isoforms (Fig. 1e); and (4) phosphorylation sites found co-occurring with ubiquitylation (Fig. 1c, d). We observed that phosphorylation sites found co-occurring with ubiquitylation are more conserved (over random expectation) than all other phosphorylation site groups tested ($P = 0.0027$, Kolmogorov-Smirnov test). This result suggests that phosphorylation sites found co-occurring with protein ubiquitylation are more likely to be functionally important[16,17].

Characterization of the response to proteasome inhibition

After developing methodologies to identify proteins co-modified with phosphorylation and ubiquitylation, we sought to globally investigate the relationship between phosphorylation and ubiquitylation in the context of proteasome-mediated degradation. The proteasome is responsible for the degradation of ubiquitylated proteins. Globally it functions to modulate protein concentrations and remove misfolded or damaged proteins. It also serves to promote cell cycle progression by degrading specific proteins at precise phase transitions of the cell division cycle[18-21]. Certain proteasome substrates are known to require the presence of specific phosphorylation sites, known as phosphodegrons, prior to ubiquitylation and proteasome-mediated degradation.

We measured the quantitative changes in proteins, phosphorylation sites and ubiquitylation sites upon proteasome inhibition with bortezomib by performing a stable-isotope labeling by amino acids in cell culture (SILAC) experiment (Fig. 3a,b, Table 2 and Supplementary Data 1). We analyzed both ubiquitylated and nonubiquitylated protein samples (Fig. 1a,b) via mass spectrometry before phosphopeptide and diGly remnant enrichment to assess quantitative changes at the protein level; the distribution of protein abundances was mostly unaffected by proteasome inhibition (Fig. 3a). Protein changes were mostly observed in the ubiquitylated population, in which an abundance fold increase of >2 was observed for 11.1% of proteins, whereas <1% of nonubiquitylated protein increased by more than twofold (Fig. 3a and Table 2). Overall, increases in protein abundances among ubiquitylated isoforms did not cause depletions of these proteins from the nonubiquitylated pool. This result supports the hypothesis that ubiquitylated protein isoforms represent a small fraction of the total pool of a given protein[6].

Proteasome inhibition caused an increase in the median abundance of ubiquitylated peptides, with 12.9% of ubiquitylated peptide isoforms increasing in abundance by more than twofold, whereas phosphorylation sites on ubiquitylated proteins were affected to a lesser extent: 3.4% increased by more than twofold (Fig. 3b and Table 2). Increases in ubiquitylation-site abundance were not correlated with increases in protein abundance. Only 13.4% of proteins with an increase in ubiquitylation sites of more than twofold also displayed a similar increase in protein abundance. This suggests that proteasome inhibition increases the stoichiometry of ubiquitylation sites on already ubiquitylated proteins rather than promoting ubiquitin conjugation to previously nonubiquitylated protein molecules. As further evidence, we found that proteins containing a site of ubiquitylation doubling in abundance were half as likely to be represented by a single isoform as compared to all ubiquitylated proteins (20.2% *versus* 40.5%, $P = 4.4 \times 10^{-12}$). Thus, the majority of ubiquitylated proteins contained multiple isoforms that could be differentially regulated upon proteasome inhibition.

Properties of regulated phosphorylation sites

The expectation is that upon proteasome inhibition, phosphorylation sites within phosphodegrons will increase in abundance. We found that phosphorylation sites on

ubiquitylated proteins that increase in abundance are indeed more likely to match a degron motif (Fig. 3c). The increase in abundance of a phosphorylation site often corresponds to an increase in ubiquitylation site abundance (Supplementary Fig. 4a); however, the presence of a degron motif is not sufficient to predict ubiquitylation site abundance changes (Supplementary Fig. 4b). These results suggest that measuring the response to proteasome inhibition of both phosphorylation sites and ubiquitylation sites is important to identifying phosphodegrons.

Many canonical phosphodegrons are found in short-lived cell cycle proteins. Thus, we analyzed the distribution of half-lives for ubiquitylated proteins, ubiquitylated phosphoproteins, and non-ubiquitylated phosphoproteins[22] (Fig. 3d). We compared the distribution of the whole group, and the subset of proteins from each group that contained a site increasing in abundance by >75%. We found that the ubiquitylated phosphoproteins with a phosphorylation site increasing in abundance have significantly shorter half-lives ($P = 6.3 \times 10^{-3}$) whereas sites increasing in abundance in other protein groups were not significantly different. Functional enrichment analysis of this population revealed that 30% could be classified as either cell cycle or cell division proteins ($P = 0.02$). These results reinforce the role of phosphodegrons in the regulation of cell cycle proteins, but also show that the degradation of proteins with alternative functional roles are also regulated by phosphodegrons.

We hypothesized that crosstalk between phosphorylation and ubiquitylation would be found in pairs of modification sites in close proximity. To study this, we mapped modification sites onto protein structure homology models of yeast proteins. We calculated the spatial distance between either (a) phosphorylation sites co-occurring with lysine ubiquitylation sites (Fig. 1c, d), or (b) between the position of these same ubiquitylation sites to phosphorylation sites identified on non-ubiquitylated isoforms (Fig. 1e). We observed that phosphorylation sites co-occurring with ubiquitylation show a small but highly significant decrease in distance to ubiquitylation sites when compared to other phosphorylation sites (Fig. 3e, $P = 3.1 \times 10^{-9}$). Phosphorylation–ubiquitylation pairs that were co-regulated in abundance and increased together upon proteasome inhibition were significantly closer than other pairs ($P = 0.0043$). This result was not observed with regulated phosphorylation sites not co-occurring with ubiquitylation (Fig. 3e). Together, these results suggest that the molecular mechanisms that mediate the crosstalk between these two modifications impose spatial constraints and that the pairs of sites identified by our method have mechanistic implications.

Co-regulation of phosphorylation and ubiquitylation sites

The current literature contains a collection of phosphodegrons that have been identified through targeted biochemical assays. To identify novel potential phosphodegrons, we considered ubiquitylation and phosphorylation site pairs that displayed correlated accumulation upon proteasome inhibition. We identified a total of 180 ubiquitin-phosphorylation site pairs in which both modifications increased in abundance (Supplementary Data 2). These pairs included several proteins with known phosphodegron sites (e.g. Ash1, Far1, and Ho), but in many cases represented different phosphorylation sites than previously reported[20,23,24]. In some instances however, phosphodegrons are comprised of multiple phosphorylation sites and different combinations of sites on the same protein can stimulate protein degradation[18]. Therefore, these new sites on previously identified phosphodegron-containing proteins may contribute to the previously observed effects, or may represent alternative degradation signals.

To further validate our approach, we tested the impact of a specific phosphorylation site on protein degradation via a complementary methodology. In the present work, we identified the protein Swi5 containing one phosphorylation site (Thr323) and three ubiquitylation sites

in this region (Lys227, Lys270, and Lys315) all increasing in abundance upon proteasome inhibition. In yeast, Swi5 regulates the transcription of *SIC1*, and during early G_1 phase, Swi5 is targeted for degradation via the SCF^{Cdc4} complex[25]. In previous work, eight potential Cdk target sites (Ser225, Ser231, Ser246, Ser250, Ser261, Ser300, Thr320, Thr323) were simultaneously mutated to alanine, which increased protein stability and delayed entry into S-phase[25,26]. We tested the candidate phosphodegron site identified in our study, Swi5 Thr323, by generating a yeast strain carrying a T323A mutation. The protein degradation of the mutant was compared to that of the wild type protein. The T323A mutation stabilized the protein relative to the wild type protein (Fig. 4a, Supplementary Figure 5a-b). We also generated a strain containing a T323E mutation, to mimic a permanently phosphorylated state. Upon proteasome inhibition, this mutant accumulated at a faster rate and to a greater extent than Swi5 WT (Figure 4b). These results demonstrate that Swi5 Thr323 phosphorylation is a phosphodegron site and validate the ability of our approach to identify functional PTM crosstalk.

We also evaluated an additional phosphorylation site that represents the diversity in function of phosphodegrons. We observed a single site of ubiquitylation (Lys329) on the GTPase Gic2 and numerous phosphorylation sites all increasing in abundance upon proteasome inhibition. Previous work has demonstrated that phosphorylation of Gic2 Ser254 and/or Ser258 serves as a phosphodegron and mutations to alanine inhibited, but did not block Gic2 degradation[27]. Here we mutated an alternative potential phosphodegron site, Gic2 S360A. This inhibited, but did not block Gic2 degradation, while the phosphomimetic Gic2 S360E mutant caused more rapid degradation of Gic2 relative to that of wild type (Supplementary Fig. 5c and d). In agreement with previous work, these results suggest that several phosphorylation sites contribute to the total phosphodegron signal on Gic2, much like the well-studied case of Sic1.

Ubiquitylation regulates the phosphorylation machinery

We examined the potential for ubiquitylation to regulate the phosphorylation machinery by evaluating the enrichment (that is, the conservation) of ubiquitylation sites in protein kinases, phosphatases and phosphobinding domains, using previously described methods[12]. To obtain robust statistics, we included ubiquitylation sites found here along with sites from human and mouse studies[5–7]. We mapped these sites onto a representative structure of each domain family and performed statistical analysis to find regions that were commonly modified by ubiquitin. Within the protein kinase domain family, a total of 72 proteins showed enrichment of ubiquitylation sites either in the glycine-rich loop (30 of 388 sites) or in Region A, N-terminal to the activation loop (55 of 388 sites ($P < 0.005$); Fig. 4c).

Proteins of the 14-3-3 domain family bind phosphorylated targets. These proteins primarily function as dimers, with each monomer binding a different protein target, or a different region of the same protein target[28]. Ubiquitylation sites in the 14-3-3 domain from 19 proteins from either human, mouse, of the yeast *S. cerevisiae* were significantly enriched in two functionally important regions: the N-terminal helix (25 of 171 sites), required for dimerization, and a helix that forms the phosphopeptide binding pocket (60 of 171 sites, ($P < 0.005$), Fig. 4d). The function of this ubiquitylation is independent of degradation as ubiquitylation sites from the two 14-3-3 proteins in *S. cerevisiae*, Bmh1 and Bmh2, did not change upon proteasome inhibition (Fig. 4d).

No regions were significantly enriched for ubiquitylation within phosphatases, or significantly enriched for phosphorylation sites among different types of the ubiquitylation machinery (data not shown).

Discussion

Cross-talk between different protein PTMs is an emerging theme in biology, but thus far it has been observed only on histones and a few other proteins. The study of PTM cross-talk has been a major challenge because methods are lacking to determine whether two PTMs co-occur on the same protein. We have developed two methods to identify PTM cross-talk between any PTM pair on a proteome-wide basis. Our first approach relies on affinity-purification enrichment of proteins containing a specific PTM followed by subsequent enrichment of the other PTM type. This approach is best suited for applications of cross-talk in which one PTM type can be isolated at high purity. Our second approach, based on SCX fractionation, is applicable to the study of cross-talk between any PTMs in which one PTM changes the solution charge state, though the method can characterize only PTMs that are close in sequence proximity. When used in combination, however, these methods provide a more comprehensive view of PTM cross-talk, and we expect that these methods will find application in future studies of protein regulation by multiple PTM types.

A central question in PTM crosstalk is how one modification site regulates surrounding modification sites. In the case of phosphodegrons, phosphorylation promotes subsequent ubiquitylation. However, is this directionality globally true? We found greater conservation of phosphorylation on ubiquitylated proteins, a characteristic indicative of biological function[12,16,17]. The reverse analysis evaluating the conservation of ubiquitylation sites on phosphoproteins *vs*. non-phosphorylated proteins revealed no differences. These results suggest a global crosstalk directionality, in which phosphorylation more frequently precedes ubiquitylation.

In addition to investigating PTM crosstalk, this work revealed new insights into the regulatory roles of ubiquitylation. To our surprise, many instances of increased ubiquitylation were not accompanied by corresponding changes in protein abundance. Instead, already ubiquitylated proteins became ubiquitylated on other residues, increasing the stoichiometry. One interpretation of these results is that ubiquitylation of these other residues serves as a distress signal for the cell to rapidly degrade that protein molecule. Alternatively, more complex signaling may be at play, in which specific ubiquitylation sites are utilized as degradation markers, while others serve to modulate protein function in a reversible fashion.

Besides the combinatorial PTM crosstalk found in co-modified proteins, PTMs can alter the activity of proteins that regulate a different PTM. Kinases have a highly conserved kinase domain that can be regulated by phosphorylation. Phosphorylation of the activation loop causes activating structural changes, while phosphorylation of the glycine-rich region can regulate ATP binding[29]. The precise enrichment of ubiquitylation near the domain activation loop and in the glycine-rich region suggests a additional mode of kinase regulation by ubiquitylation: reversible inhibition. We hypothesize that the steric hindrance of a ubiquitin attachment in either of these regions serves as a rapid means to transiently inhibit kinase activity. Further experiments are required to validate this possible regulatory mechanism.

A second example of such regulation is the 14-3-3 phospho-binding domain family. The highly specific enrichment of ubiquitylation in the phospho-binding region suggests that ubiquitylation can be used to inhibit 14-3-3 interaction with its targets. Inhibition of dimerization is plausible, given the enrichment of ubiquitylation in the dimerization region. It is likely that ubiquitin functions to reversibly regulate 14-3-3 activity, however, the nature of this regulation remains unclear.

PTM crosstalk is a complex and multifaceted landscape that is largely unexplored. New proteomics technologies, such as we describe here, now offer a practical approach to characterize modification crosstalk and to elucidate its complexity and influence.

Methods

Yeast Culture

For proteomic experiments, the following strain was used: *S. cerevisiae* LSY207 *(MATa lys2-801, ura3-52, leu2-3,112, his3Δ200, trp1-1, ubi1ΔTRP1, ubi2Δura3, ubi3Δ, ubi4ΔLEU2, pdr5::KanMX* [pUB221] [pUB100]), pUB221 is a *URA3*-marked plasmid that expresses 6His-*myc*-ubiquitin under a *CUP1* promoter while pUB100 expresses the essential ribosomal protein encoded by the Ubi1 gene[3,4,30,31]. LSY207 was grown in lysine-free synthetic complete media supplemented with either 436 μM light lysine (K0) or heavy lysine (K8). For qualitative experiments 1 L of cells were grown in K0 media. For proteasome inhibition experiments two 1 L cultures were grown. The control culture was grown in K0 media to which 1.54 mL of DMSO were added 1 hour prior to harvesting[18,19,21,32,33]. The other culture was grown in K8 media and was exposed to 50 μM Bortezomib in DMSO (1.54 mL) 1 hour prior to harvesting (LC Laboratories). All cultures were harvested during mid-log phase (OD_{600} ~1.0).

Sample preparation

All steps were performed at 4°C unless otherwise noted. Harvested cells were washed with water and resuspended in 8-10 mL of lysis buffer containing 8 M urea, 300 mM NaCl, 50 mM Tris pH 8.2, 50 mM NaF, 50 mM Na-β-glycerophosphate, 10 mM Na-pyrophosphate, 1mM Na-orthovanadate, and 1 tablet mini protease inhibitor (Roche). For SILAC experiments cells were mixed 1:1. Cell suspensions were transferred to 2 mL screw-cap tubes and lysed by four repetitions of bead beating (1 min beating, 1.5 min rest). Tubes were spun first at 100 g to remove beads and subsequently at 10,000 g to pellet cellular debris. The supernatants were pooled. At this point 10% of the sample was removed for SCX-IP experiments (~9-20 mg protein). The remaining sample was transferred to a tube containing 2.5 mL of equilibrated Cobalt-NTA resin (Talon Superflow Resin Clontech Laboratories, Inc.) and incubated for 40 min at 4°C to affinity purify ubiquitylated proteins via the His tag. The flow through was further depleted of ubiquitin containing proteins by two subsequent incubations with fresh cobalt resin. The ubiquitin containing proteins bound to the resin were washed twice with 8 mL of equilibration buffer (8 M urea, 300 mM NaCl, 50 mM Tris pH 8.2), followed by two washes with equilibration buffer supplemented with 10 mM imidazole. Finally ubiquitin containing proteins were eluted with 7 mL of equilibration buffer containing 150 mM imidazole followed by 1.5 mL of equilibration buffer containing 300 mM imidazole.

Proteins were reduced in 5 mM DTT for 45 min at RT, alkylated with 15 mM iodoacetamide for 45 min at RT in the dark, and alkylation was capped by incubation with 15 mM DTT at RT for 15 min.

The qualitative experiment resulted in three aliquots: (1) for SCX-IP purification, (2) ubiquitin-enriched proteins eluted from the cobalt resin (“ubiquitylated proteins”), and (3) ubiquitin-depleted proteins that did not bind to the cobalt resin (“non-ubiquitylated proteins”). Each of these aliquots were diluted 4.5-fold with 50 mM Tris, pH 8.2 and digested overnight at 37°C with 100 μg of trypsin (Promega).

In the proteasome experiment, we used the same three aliquots as described above for the qualitative experiments, however the digestion schema was different to ensure that all

peptides contained a lysine residue required for peptide quantification. In this case, the ubiquitin-enriched and ubiquitin-depleted aliquots were diluted 2-fold with 50 mM Tris, pH 8.9 and 80 μg or 240 μg of lysyl endopeptidase (LysC) were added, respectively (Wako). After 4 hours incubation at RT half of the ubiquitin-enriched sample was removed, this was used for phosphopeptide enrichment. The remaining half of this aliquot and the sample for SCX-IP are both to be enriched for diGly containing peptides, thus they were diluted to 1.8 M urea with 50 mM Tris, pH 8.2, and digested with 100 μg trypsin overnight at 37°C (Promega). After enzymatic digestion, all samples were acidified to ~ 2 pH with TFA and desalted on tC18 SepPak cartridges (Waters).

SCX-based peptide fractionation

Peptides not subjected to His-tag purification (i.e. SCX-IP samples) were fractionated over a Polysulfoethyl A strong cation exchange cartridge (PolyLC)[2,34]. Two buffers were used to fractionate the peptides: (A) 5 mM KH_2PO_4, 30% acetonitrile, pH 2.65, and (B) 5 mM KH_2PO_4, 350 mM KCl, 30% acetonitrile, pH 2.65. Cartridges were first conditioned with a decreasing percentage of SCX buffer B, followed by equilibration in SCX buffer A. Samples dissolved in SCX buffer A were loaded onto the cartridge, and peptides were stepwise eluted with increasing concentration of salt (0%, 15%, 18%, 21%, 24%, 27%, 80% buffer B). Eluents were lyophilized and then desalted on tC18 SepPak cartridges (Waters) prior to enrichment of diGly containing peptides via immunoprecipitation. This method was used to enrich for peptides concurrently modified with phosphorylation and diGly.

Non-ubiquitylated peptide samples were separated into 12 fractions via column based strong cation exchange. For qualitative experiments 2 mg peptides were separated on a 4.6mm × 200 mm Polysulfoethyl A (5 μm, 300 Å) column, while for SILAC experiments 10 mg of peptides were separated on a 9.4 mm × 200 mm column. The gradient conditions were essentially as described elsewhere[8,9,34,35]. All fractions were dried and desalted on tC18 SepPak cartridges prior to phosphopeptide enrichment. A separate aliquot of 1mg of ubiquitin-depleted sample was also fractionated on a 30 mg waters Oasis cartridge by reverse-phase at basic pH. The cartridge was conditioned with 80% acetonitrile in 20 mM ammonium hydroxide and equilibrated with 20 mM ammonium hydroxide. The sample was loaded in 1 mL of water and step-wise elution of peptides was achieved by increasing acetonitrile concentration in 20mM ammonium hydroxide (0%, 6%, 9%, 12%, 15%, 18%, 30%, and 80% acetonitrile elutions). A 10 μg aliquot of the ubiquitin enriched LysC digest was also fractionated in this manner on an Empore C18 stage tip, except elution at 15% and 30% acetonitrile were omitted (3M)[5-7,36]. This sample was used to acquire protein quantitation on ubiquitylated proteins.

Immunoprecipitation of diGly containing peptides

Peptide immunoprecipitations were performed essentially as described elsewhere[10,11,37]. Samples were dissolved in 1.4 mL of IAP buffer (50 mM MOPS-NaOH, pH 7.2, 10 mM Na_2HPO_4, 50 mM NaCl) and spun at 4°C for 10 min at × 4000 g. For each sample, 80 μg of diGly antibody loaded on protein A agarose beads were used (Cell Signaling)[6,13]. The antibody was first washed twice with PBS, followed by 3 washes with IAP. The supernatant of the samples were then incubated with the antibody at 4°C for 40 min with gentle rotation. The antibody was then washed three times with IAP, followed by two washes with water. The antibody was incubated for 10 min in 100 μL of 0.15% TFA to elute diGly peptides. This elution was repeated two more times and all elutions were pooled and desalted on stage tips[14,15,36].

The flow-through of the diGly immunoprecipitations of the SCX-IP samples were each desalted on 100 mg tC18 SepPak cartridges and an aliquot of this was analyzed via MS.

Enrichment of phosphopeptides

Both the ubiquitin-enriched LysC digested sample (SILAC) and the flow-through of the qualitative ubiquitin-enriched trypsin diGly IP peptide mixtures were enriched for phosphopeptides using 150 μL of magnetic NTA-IMAC beads (Qiagen)[13,30,38]. Phosphopeptide enrichment was also performed on the peptides from the ubiquitin-depleted sample that were fractionated via SCX. Here 300 μL of magnetic NTA-IMAC beads were used in total. The beads were washed three times with water, then incubated with 40 mM EDTA, pH 8 for 30 min. Next, the beads were washed three times with water and then incubated with 100 mM $FeCl_3$ for 30 min. The beads were then washed with 80% acetonitrile, 0.1% TFA three times. Samples were dissolved in either 150 μL (fractions) or 300 μL (diGly IP flow-through) and incubated with the IMAC beads for 30 min with agitation. The beads were then washed three times with 80% acetonitrile, 0.1% TFA and the phosphopeptides eluted with 100 μL of 50% acetonitrile, 25% ammonium hydroxide. The eluent was quickly passed through a stage tip to filter out any beads and immediately acidified to pH ~ 3 with formic acid. The eluent was then dried and resuspended in 4% formic acid, 3% acetonitrile for MS analysis.

Mass spectrometry analysis

Peptides were resuspended in 4% formic acid, 3% acetonitrile and loaded onto a 100 μm ID × 3 cm pre-column packed with Maccel C18 3 μm, 200 Å particles (The Nest Group, Inc.). Peptides were eluted over a 75 μm ID × 26 cm analytical column packed with the same material. The exact gradient conditions were tailored to the complexity and chemical properties of each sample, however generally the gradient was from 8%-30% acetonitrile in 0.15% formic acid over the course of 90 or 120 min. All MS spectra were collected with orbitrap detection. Two types of MS/MS spectra were collected, either resonant-excitation collision-activated dissociation with ion trap detection (CAD), or beam-type collision-activation dissociation (HCD) with orbitrap detection. Non-modified peptides samples were analyzed by CAD, whereas modified peptides samples were independently analyzed by both HCD and CAD. When MS/MS spectra were collected in the linear ion trap the 20 most abundant ions were selected in a data dependent manner, while for MS/MS collected in the orbitrap the ten most abundant ions were selected. The exact mass spectrometer settings can be found in the RAW files provided at http://faculty.washington.edu/jvillen/lab/.

Identification and quantification of peptides, proteins, and modifications

MS/MS spectra were searched with Sequest[10,39] against the SGD yeast proteome (downloaded Jan 5th 2011). The precursor mass tolerance was set to 50 ppm and the fragment ion tolerance was set to 0.36 Da with a 0.11 Da offset. A static modification on cysteine residues (57.021464 Da) and a variable modification of methionine oxidation (15.994914 Da) were used for all searches. For non-SILAC experiments, additional variable modifications representing phosphorylation of serine, threonine, or tyrosine (79.966333 Da) or diGly on lysine (114.042928 Da) were used where appropriate. For SILAC experiments, an additional variable modification of heavy lysine (8.014198 Da) was considered. Search results were filtered to a 1% FDR at the peptide and protein level based on experimental group (i.e. ubiquitin enriched, depleted, or SCX-IP)[18-21,40]. Phosphorylation and ubiquitylation sites were localized using an in-house implementation of the Ascore algorithm[41]. Sites with an Ascore > 13 ($P < 0.05$) were considered confidently localized, and diGly modifications localized to the C-terminal peptide residue were discarded. For non-SILAC experiments only confidently localized modification sites are reported. For SILAC experiments, modification isoforms, rather than single sites are listed. Only those proteins that were identified containing a diGly modification were considered ubiquitylated. For SCX-IP experiments, phosphorylation sites on ubiquitylated proteins were considered to

be only those found on the same peptide as a diGly modification. From the His-tag elution experiments, phosphorylation sites reported were restricted to those identified on proteins, which we also identified a diGly modification on.

Peptides were quantified using in-house software measuring chromatographic peak maximum intensities. Only peptides with a summed SNR ratio of the light and heavy peptide > 7 were considered. The peak maxima for peptides with the same combination of modification sites were summed before calculating a ratio. When modification sites could not be localized, the region of amino acids the site could reside in is listed. Proteins were quantified by first summing maximum peak intensities of all unmodified peptides for a given protein. The ratio was calculated between the sum of all peptides for a protein from heavy labeled cells and the sum from light labeled cells. In instances where only the light or heavy form was identified, the noise signal level was used as the signal of the missing peptide. Abundance ratios for both modification sites and proteins were all normalized by the same magnitude, to adjust for mixing errors. This normalization adjusted the distribution of protein quantitative ratios such that the median was set to $\log_2 = 0$. Co-regulated phosphorylation and ubiquitylation was defined as pair of modification site isoforms that either (1) had a minimum abundance change of ∼60% and the magnitude of change were within 15% of each other, or (2) both modifications had a minimum abundance change of 100% (2-fold).

Site mutagenesis and Western blotting

Plasmids encoding HA-tagged proteins (Open Biosystems yeast ORF collection) were purified and subjected to site directed mutagenesis[6,42]. Wild type amino acids were either converted to alanine (to represent a non-phosphorylated state) or glutamic acid (to represent a phosphorylated state). The wild type and Swi5 T323A mutant plasmids were introduced into the Y258 strain (*MAT*a *his4-580, ura3-52, leu2-3,112, pep4-3)*, while the Swi5 WT and Swi5 T323E mutant plasmid was introduced into a Y258 strain carrying a *PDR5* deletion (*MAT*a *his4-580, ura3-52, leu2-3,112, pep4-3, pdr5Δ::KanMX)*. Over expression of the plasmid was induced with galactose incubation (4-6 h). For Swi5 T323A and Swi5 WT, cycloheximide was added to inhibit translation (50 μg/mL) and 2 mL of cell culture were harvested after 0, 20, and 40 min. For Swi5 WT and Swi5 T323E Bortezomib was added to inhibit the proteasome (100 μM) and 1.5 mL of cell culture were harvested after 0, 30, 60, 90 min. Additional strains containing mutants in the Gic2 protein were generated from GST-tagged proteins (Open Biosystems yeast GST collection) and cycloheximide degradation assays performed exactly as described above for Swi5. For Gic2, WT protein expression was observed to be higher than either mutant; therefore mutant strains were induced for 4 h while the WT strain was induced for 30 min. This produced similar protein expression for the mutant and WT forms. Harvested cells were lysed by bead beating in 50 mM Tris pH 8.2, 1 mM EDTA, 0.5% Triton X-100, 100 mM NaCl, 1 mM DTT, and 1 tablet mini protease inhibitor (Roche). LDS denaturing buffer was added (1×) and samples were incubated at 100 °C for 5 min. Clarified cell lysates were separated by SDS-PAGE followed by immunoblotting with the anti HA-tag antibody (0.33μg/mL, GenScript catalog# A01244), anti-GST antibody (0.25μg/mL, GenScript catalog# A000865), or anti-α-tubulin (loading control, 0.17μg/mL, GenScript catalog# A01410). Western blots images were processed and quantified using the freely available ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Bioinformatics: Conservation, functional enrichment, and structural analysis

Protein sequence alignments between yeast, human, and mouse were done with MUSCLE version 3.6 using standard alignments options[22,43]. Human and mouse PTMs were obtained from Phosphosite plus (http://www.phosphosite.org/)[44]. In order to avoid potential redundancies and issues with gene duplication we only used putative 1-to-1 orthologs as

predicted with 90% confidence by the inparanoid algorithm (orthology information available at http://inparanoid.sbc.su.se/)[45]. To account for errors in the PTM positional assignments we defined a PTM site to be conserved if the aligned peptide of the ortholog protein is also known to be modified within a window of +/−2 alignment positions. In order to estimate a random expectation (i.e. null model) for the conservation of PTM sites from species A to species B, we randomly shuffled the PTM sites of each protein in species A. The random shuffling was done only from possible acceptor residues (K for ubiquitylation and S/T for phosphorylation). We excluded tyrosine acceptor residues from the sampling, as these constitute a minority of phosphosites. The ratios of conserved over expected values for the different groups of PTMs were compared using a Mann-Whitney ranked test.

Gene ontology enrichment analysis was performed using Babelomics4 (http://babelomics.bioinfo.cipf.es/) and p-values were calculated using a two-tailed Fisher exact test and correcting for multiple testing[46]. For assessment of differences in protein half-lives a two-tailed Wilcoxon test was performed.

In order to search for significant enrichment of PTMs within specific regions of domain families we selected for each domain a representative structure and transferred the PTMs, occurring in all instances of this domain in different species (mouse, human, and the yeast *S. cerevisiae*), using protein sequence alignments[5-7]. We then used a sliding window of ten amino acids and random sampling to identify regions, within each domain family, that are enriched for PTMs. Any ten amino-acid peptide with a significant ($P < 0.005$) enrichment of PTMs when compared to random was defined a potential regulatory region[12].

Three known phosphodegron motifs (or protein sequence patterns) were obtained from the ELM database (http://elm.eu.org). The three patterns used were [LIVMP]X{0,2}[ST]PXX[ST] or [LIVMP]X{0,2}[ST]PXXE or [ST]GX{1,3}[ST]. X represents any amino acid, square brackets represent a position that accepts any of the amino acids within brackets and curly brackets describe the number of times that the preceding amino acid can be repeated. As an example X{0,2} means that 0 to 2 random amino acids can fit this position(s). For analysis of modification site protein structural proximity, yeast homology models were obtained from Modbase database (modbase.compbio.ucsf.edu) and calculated the spatial distance (i.e. ångströms between alpha carbons) between modification sites[47].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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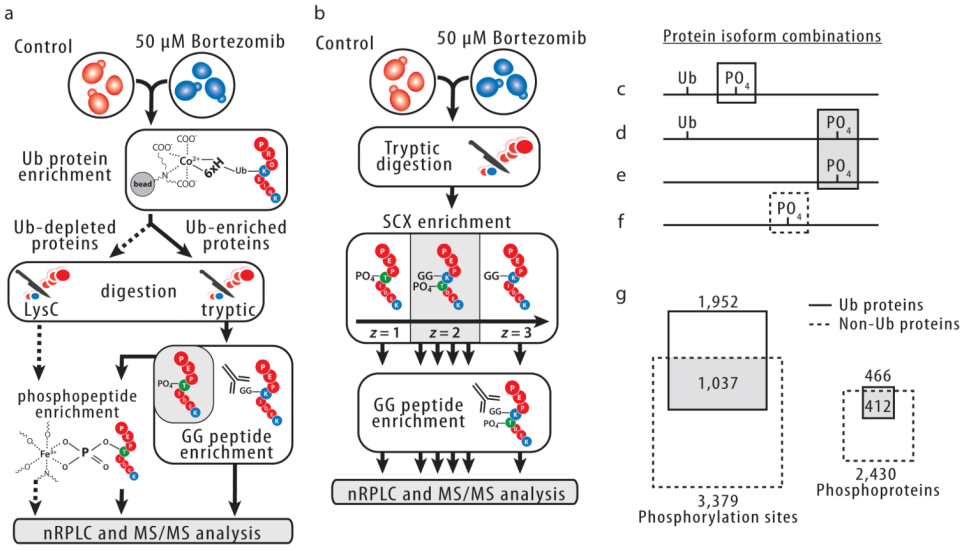


Figure 1. Overview of methodology and modifications identified

Two sets of experiments were performed. The first was a qualitative assessment (non-SILAC), and the second, as illustrated here, was a SILAC experiment, in which cells from control and proteasome-inhibited cultures were mixed. We utilized two enrichment strategies for both experiments (**a-b**). (**a**) First, samples were enriched for Ub proteins. Non-Ub proteins (dashed lines) were digested and enriched for phosphopeptides. Ub proteins (solid lines) were further enriched for diGly-peptides and for phosphopeptides. (**b**) Proteins were digested to peptides and enriched for doubly charged peptides via strong-cation exchange (SCX). SCX fractions were then enriched for diGly-peptides prior to mass spectrometry analysis. (**c-f**) Illustration of four cases of how the same protein can exist in different combinations of modification states. (**c**) displays a phosphorylation site unique to the ubiquitylated protein isoform, while (**d**) and (**e**) display that some phosphorylation sites are found in both ubiquitylated and non-ubiquitylated isoforms. Finally, (**f**) displays a phosphorylation site unique to non-ubiquitylated proteins. (**g**) Overlap of phosphorylation sites and phosphoproteins from the Non-Ub (dashed lines) and Ub samples (solid lines).

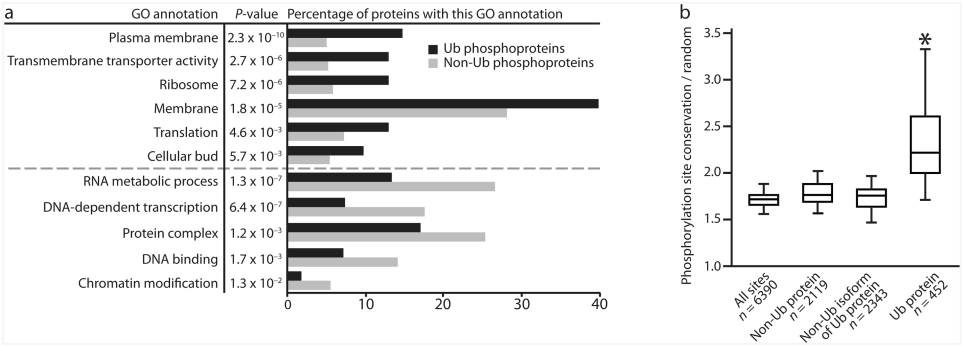


Figure 2. Evolution and functional enrichment of modification sites

(**a**) GO enrichment analysis of ubiquitylated phosphoproteins *vs*. non-ubiquitylated phosphoproteins. GO enrichment was performed using Babelomics4. (**b**) For different populations of proteins we calculated the ratio of conserved phosphorylation sites over conserved amino acids from random sampling of the same number of phosphor-acceptor residues (Ser, Thr). Whiskers represent the lowest/highest data point within 1.5 times the interquartile range (**P* = 0.0027, Kolmogorov-Smirnov test).

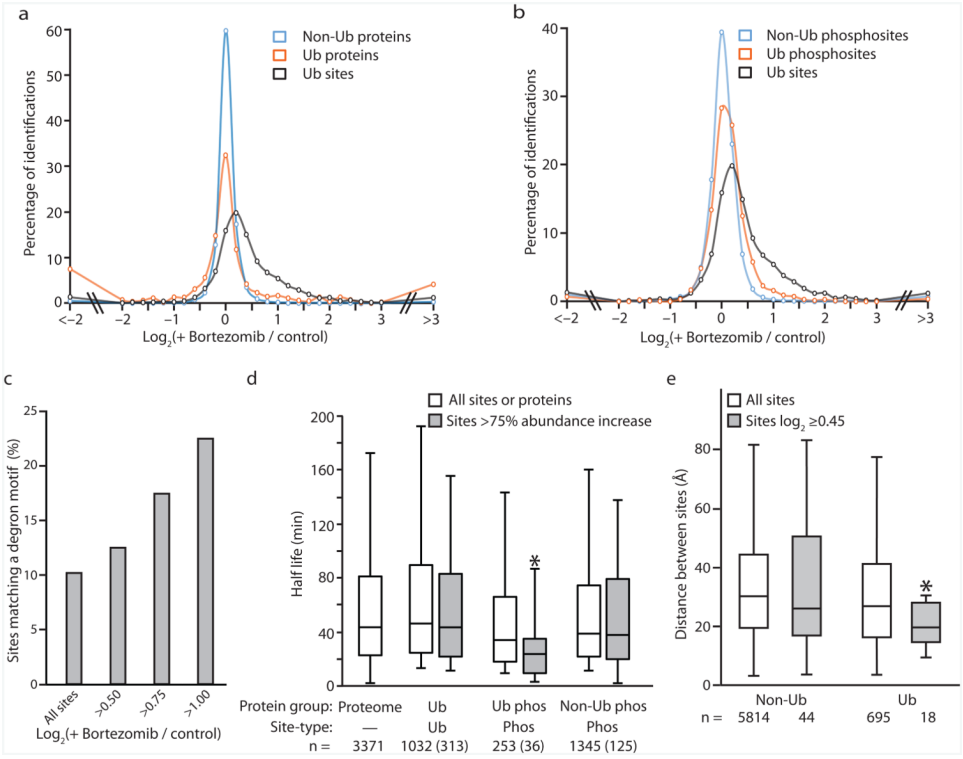
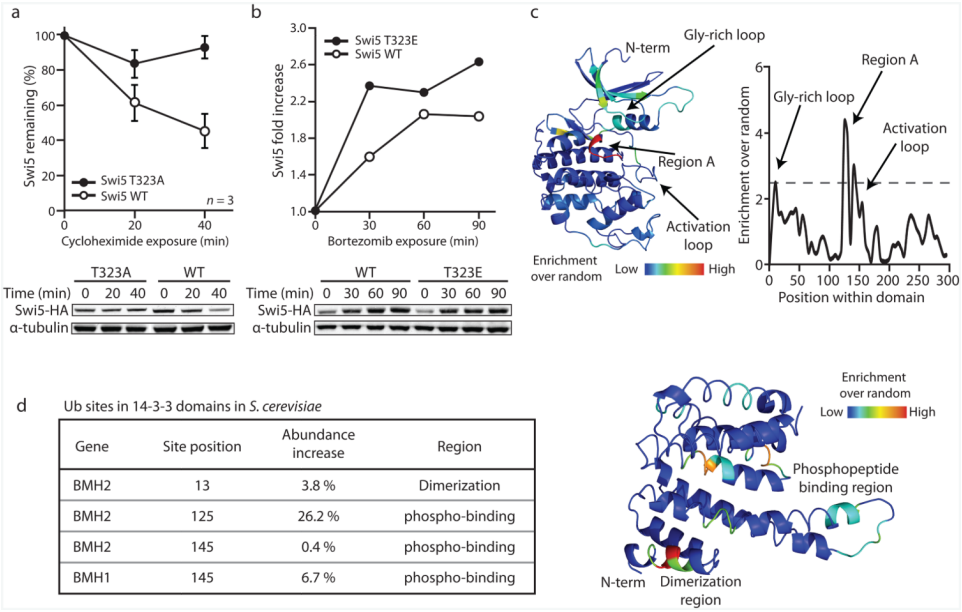


Figure 3. The effect of proteasome inhibition on protein and PTM site abundance, and properties of regulated phosphorylation sites

(**a**) Log_2 distributions of proteins and ubiquitylation sites abundance changes in response to proteasome inhibition (50 μm Bortezomib for 1 hour). (**b**) Log_2 distributions of phosphorylation and ubiquitylation sites abundance changes. (**c**) The distribution of sites matching a phosphodegron motif at various intervals of abundance changes after proteasome inhibition. (**d**) Half-life distributions for all protein identifications are compared to those proteins containing sites increasing in abundance by >75% (*$P = 6.3 \times 10^{-3}$), whiskers represent the 10th and 90th percentile. Protein half-life values were obtained from the literature[22]. (**e**) The pairwise spatial distance (i.e. ångströms between alpha carbons) between either phosphorylation sites co-occurring with lysine ubiquitylation sites (Ub), or between the position of these same ubiquitylation sites to phosphorylation sites identified on non-ubiquitylated isoforms (Non-Ub). Distances between all pairs (white boxes) are compared to the distances between pairs for which both sites increase in abundance upon proteasome inhibition (grey boxes). ($P < 0.0043$). Whiskers represent the lowest/highest data point within 1.5 times the interquartile range.



Gene	Site position	Abundance increase	Region
BMH2	13	3.8 %	Dimerization
BMH2	125	26.2 %	phospho-binding
BMH2	145	0.4 %	phospho-binding
BMH1	145	6.7 %	phospho-binding

Figure 4. Validation of a new phosphodegron and ubiquitin mediated regulation of phosphorylation machinery

(**a-b**) Yeast cells were treated with galactose to induce Swi5 expression and incubated with either cycloheximide to measure protein degradation (*n* = 3) (**a**) or Bortezomib to measure protein accumulation (**b**). Aliquots were taken at the indicated times and protein expression was monitored by immunoblotting with anti-HA to detect Swi5, or anti-α-tubulin for a loading control. Degradation levels measured from immunoblotting were quantified in triplicate, while Bortezomib induced accumulation was quantified from a single replicate. (**b**) Enrichment of ubiquitylation sites in the structure of the kinase domain. A representative structure pertaining to PDB ID: 1QMZ is shown. A total of 72 proteins showed enrichment of Ub sites within the Gly-rich loop or in Region A adjacent to the activation loop. Graph in the right represents the numeric values of enrichment over random for different positions within the kinase domain. Relevant regions within the domain are indicated. (**c**) Enrichment of ubiquitylation sites within the 14-3-3 phosphorylation-binding domain (PDB ID: 3MHR, 19 proteins from either human, mouse, or the yeast *S. cerevisiae*). The embedded table represents ubiquitylation sites identified in the phospho-binding and dimerization regions of the two 14-3-3 domain containing proteins in *S. cerevisiae* and the percent increase in abundance upon proteasome inhibition measured in this study.

Table 1

Protein and modification identifications from qualitative experiments.

Group	Sample	Ubiquitylated proteins	Ubiquitylated phosphoproteins	Ubiquitylation sites	Phosphorylation sites
1	Ub-protein enrichment	891	321	2,395	1,769
2	SCX-IP	1,817	245	4,659	437
	Total	1,920	466	5,629	2,100
			Phosphoproteins		Phosphorylation sites
3	Non-Ub proteins		2,376		11,704

Table 2

Quantifications from proteasome inhibition experiments (SILAC). Where applicable, values in parentheses indicate the number of proteins or isoforms increasing in abundance by > 2-fold.

Group	Sample	Ubiquitylated proteins	Ubiquitylated phosphoproteins	Ubiquitylated isoforms	Phosphorylated isoforms
1	Ub-protein enrichment	558 (62)	322	3,816 (628)	1,710 (54)
2	SCX-IP	1,176	108	2,787 (119)	303 (10)
	Total	1,307	378	5,465	2,048
		Proteins	Phosphoproteins		Phosphorylated isoforms
3	Non-Ub proteins	3,484 (28)	1,939	NA	10,656 (159)