

Rad23 Interaction with the Proteasome Is Regulated by Phosphorylation of Its Ubiquitin-Like (UbL) Domain

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Abstract

Rad23 was identified as a DNA repair protein, although a role in protein degradation has been described. The protein degradation function of Rad23 contributes to cell cycle progression, stress response, endoplasmic reticulum proteolysis, and DNA repair. Rad23 binds the proteasome through a UbL (*ubiquitin-like*) domain and contains UBA (*ubiquitin-associated*) motifs that bind multiubiquitin chains. These domains allow Rad23 to function as a substrate shuttle-factor. This property is shared by structurally similar proteins (Dsk2 and Ddi1) and is conserved among the human and mouse counterparts of Rad23. Despite much effort, the regulation of Rad23 interactions with ubiquitinated substrates and the proteasome is unknown. We report here that Rad23 is extensively phosphorylated *in vivo* and *in vitro*. Serine residues in UbL are phosphorylated and influence Rad23 interaction with proteasomes. Replacement of these serine residues with acidic residues, to mimic phosphorylation, reduced proteasome binding. We reported that when UbL is overexpressed, it can compete with Rad23 for proteasome interaction and can inhibit substrate turnover. This effect is not observed with UbL containing acidic substitutions, consistent with results that phosphorylation inhibits interaction with the proteasome. Loss of both Rad23 and Rpn10 caused pleiotropic defects that were suppressed by overexpressing either Rad23 or Rpn10. Rad23 bearing a UbL domain with acidic substitutions failed to suppress *rad23Δ rpn10Δ*, confirming the importance of regulated Rad23/proteasome binding. Strikingly, threonine 75 in human HR23B also regulates interaction with the proteasome, suggesting that phosphorylation is a conserved mechanism for controlling Rad23/proteasome interaction.

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Introduction

Rad23 was first characterized as a DNA repair factor that is required for nucleotide excision repair (NER; reviewed in Refs. [1] and [2]). The NER mechanism is required for the removal of bulky DNA adducts, and mutations in multiple complementation groups can lead to *Xeroderma pigmentosum* in humans (reviewed in Ref. [1]). A complex consisting of Rad23 and Rad4 performs a key role in recognizing bulky lesions in DNA [2]. The loss of Rad4 (XPC in human) prevents DNA incision, which leads to a complete NER defect. In contrast, loss of yeast Rad23 causes a partial decrease in UV survival. However, DNA incision occurs in *rad23Δ*, suggesting that the

defect in this mutant occurs at a late step in the NER mechanism. We discovered that Rad23 could interact with the proteasome. This finding revealed a novel role for proteolysis in DNA repair [3]. In agreement, proteasome mutant's show reduced survival after UV light, although the specific requirement for proteolysis in NER has not been determined.

Distinct methods to assess the role of Rad23 and the proteasome in DNA repair have been utilized [3,4]. However, these studies have not yielded concordant results. We note that DNA incision is an early step in NER that does not reflect the efficiency of subsequent events including DNA patch filling, ligation, chromatin remodeling, and recovery from growth arrest. In contrast, yeast cell survival is

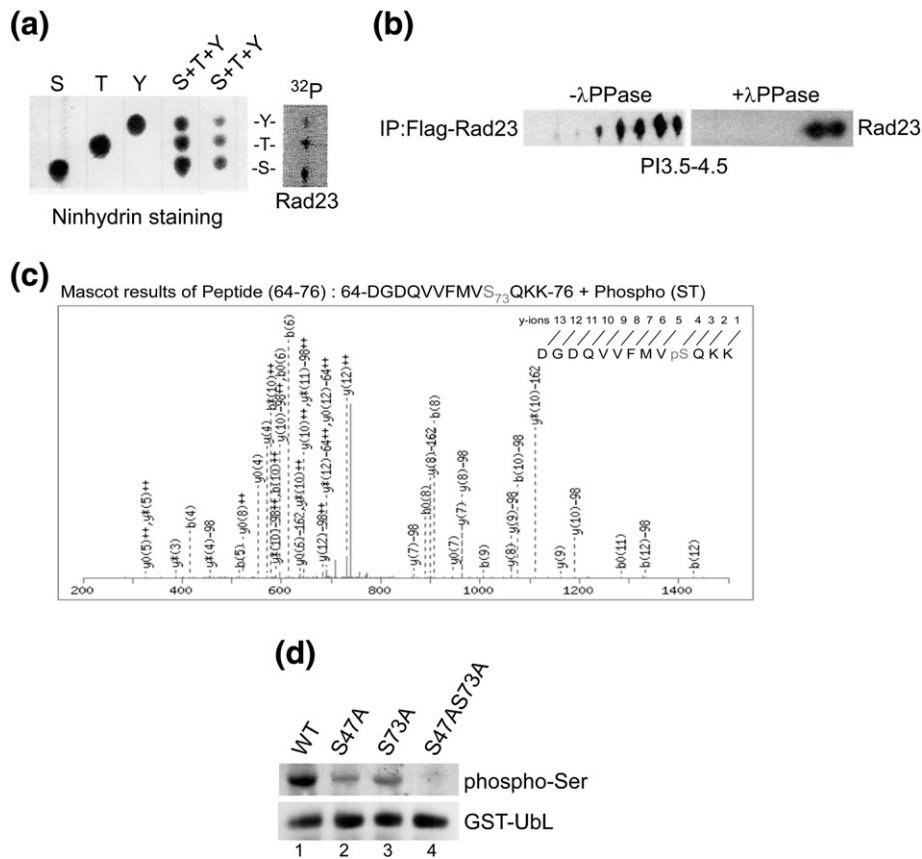


Fig. 1. Rad23 are phosphorylated at multiple residues *in vivo*. (a) Yeast cells expressing Flag-Rad23 were affinity purified from exponential-phase yeast cells that were incubated with [^{32}P]orthophosphate for 1 h. The *in vivo* phosphorylated Flag-Rad23 was separated by SDS-PAGE and transferred to PVDF membrane. ^{32}P -labeled Flag-Rad23 was identified by autoradiography, excised, and digested in acid. The hydrolysate was separated by one-dimensional TLC. The positions of non-radioactive phosphoamino acid standards were detected by staining with ninhydrin (left panel). The independent standards and two different amounts of a mixture of the three combined standards are shown. The pattern of radioactive spots generated from the hydrolyzed of ^{32}P -Flag-Rad23 is shown on the right (Flag-Rad23), and the positions of phosphorylated tyrosine (Y), threonine (T), and serine (S) residues are indicated. (b) Flag-Rad23 was immunoprecipitated from yeast cells and incubated with or without λ -phosphatase for 1 h. Proteins were released from the affinity beads and resolved by isoelectric focusing. The separated proteins were resolved in the second dimension in SDS-PAGE, transferred to nitrocellulose, and incubated with anti-Flag antibody. (c) Recombinant GST-Rad23 was purified from *E. coli* BL21 (DE3) cells, extensively washed by lysis buffer, and subjected to *in vitro* kinase assay followed by liquid chromatography–tandem mass spectrometry analysis. (d) GST-UbL and mutants with changes in candidate phosphorylation sites were isolated from *rad23* Δ strain and *in vivo* phosphorylation was investigated by immunoblotting, using antibodies that recognize phosphoserine residues.

an endpoint assay that is determined several days after DNA injury, and it does not take into account defects in earlier biochemical steps.

Rad4 protein is constitutively degraded by the ubiquitin/proteasome pathway but is stabilized through its interaction with Rad23 [5]. The intermediate UV sensitivity of *rad23* Δ is partly due to accelerated turnover of Rad4. However, the UV sensitivity of *rad23* Δ is not overcome by stabilizing Rad4, suggesting that Rad23 has additional functions that cannot be bypassed by solely providing more Rad4. Because DNA incision occurs efficiently in *rad23* Δ , Rad23 appears to function later in the NER pathway. The Rad23 requirement for efficient cell cycle

progression [6], cellular response to environmental toxins [6], and endoplasmic-reticulum-associated degradation [7] is all linked to its role as a shuttle-factor. The UbL (*ubiquitin-like*) domain in yeast Rad23 binds the Rpn1 subunit in the proteasome [8] and a substrate ubiquitination factor, Ufd2. In contrast, the UbL domains in two human counterparts (hHR23A and hHR23B) bind the proteasome subunit S5a [9] and Ataxin-3 [10,11]. A distinct binding domain in Rad23 binds Rad4 [12], and similarly, human Rad23 proteins contain a unique motif that binds XPC [13]. The two UBA (*ubiquitin-associated*) domains in Rad23 bind multiubiquitin chains. The carboxy-terminal UBA2 domain can also bind Png1 [14]. The human UBA2

domain interacts with other regulatory factors including HIV-1 (human immunodeficiency virus-1)-encoded Vpr [15], p300/CREB [16], and MPG1 [17]. The significance of these interactions is not clear.

The proteolytic role of Rad23 explains its widespread involvement in stress response, transcription, endoplasmic-reticulum-associated protein degradation, and NER [18]. Removal of the UbL prevents interaction with the proteasome and causes UV sensitivity, underscoring a role for the proteasome in NER. An important property of a “shuttle-factor” function (such as Rad23) is to interact with the proteasome only when it is bound to cargo (multiubiquitin proteolytic substrates). In agreement, overexpression of the UbL domain caused unregulated interaction with the proteasome and stabilization of proteolytic substrates. Significantly, it is not known how Rad23/proteasome interaction is regulated to prevent such an unregulated interaction. Similarly, the regulation of Rad23 interactions with multiubiquitinated substrates has not been investigated. Regulation of Rad23/proteasome interaction provides a way to repeatedly deliver multiubiquitinated proteins to the proteasome [19]. Although there is some evidence that monomeric and dimeric states of Rad23 might regulate its activity [20,21], it is unclear how it oscillates between these two forms. Rad23 interaction with the proteasome is independent of its interactions with other factors including MPG [17], Png1 [14], Vpr [15], and p300 [16] because the isolated UbL domain can bind proteasomes efficiently. It is also unclear how Rad23 interaction with Rad4 is distinct from its interaction with multiubiquitinated proteolytic substrates.

We determined that Rad23 is phosphorylated *in vivo*. This post-translational modification provides a promising avenue for understanding its regulation and function. Mass spectrometry analysis showed that several residues in Rad23 are phosphorylated *in vivo* and *in vitro*. In this report, we examine the phosphorylation of serine residues in the UbL domain, and test their effect on proteasome binding and substrate turnover. We determined that UbL phosphorylation inhibited Rad23/proteasome binding and reduced protein degradation by the proteasome. We speculate that Rad23 phosphorylation controls its association with the proteasome, thereby accomplishing the repeated delivery of ubiquitinated proteolytic substrates.

Results

Multiple residues in Rad23 are phosphorylated *in vivo*

Yeast cells were grown in medium containing [³²P] orthophosphate and protein extracts were prepared.

Flag-Rad23 was immunoprecipitated and separated in a denaturing polyacrylamide gel. ³²P-Flag-Rad23 was excised and subjected to acid hydrolysis, and the hydrolysate was resolved by thin-layer chromatography (TLC) (Fig. 1a). Unlabeled serine, threonine, and tyrosine were combined and two different loadings were separated (lanes 4 and 5). The positions of these unlabeled amino acid residues were determined by staining with ninhydrin. The TLC plate was subsequently exposed to X-ray film and ³²P-serine (S), ³²P-threonine (T), and ³²P-tyrosine (Y) were detected. To confirm these results, we immunoprecipitated Flag-Rad23 from unlabeled cells and treated one-half of the sample with lambda phosphatase (+λPPase; Fig. 1b). The proteins were separated by two-dimensional gel electrophoresis, and an antibody against Rad23 detected five spots. Significantly, most of these spots were lost following exposure to λPPase, consistent with their dephosphorylation. Two spots remained after dephosphorylation, representing the unphosphorylated form of Rad23 and one containing a single phosphate. We speculate that this could arise if this residue were inaccessible to λPPase.

We purified GST-Rad23 from *Escherichia coli* and incubated the immobilized protein with extract prepared from wild-type yeast. GST-Rad23 was then subjected to mass spectrometry analysis (liquid chromatography–tandem mass spectrometry), and a number of phosphorylated residues were identified. We were intrigued by the phosphorylation of residues in the UbL domain because this structure has a well-characterized role in binding the Rpn2 protein in yeast proteasomes. In contrast, the UBA domains in Rad23 have multiple binding partners that could confound the characterization of their phosphorylation. Because UbL/proteasome interaction is essential for all Rad23 activities, the regulation of this function is important. To strengthen our *in vitro* studies, we isolated GST-UbL from yeast cells and characterized the protein by mass spectrometry. These studies confirmed that Ser73 in the UbL domain is also phosphorylated *in vivo*. A representative mass spectrometry profile of peptides 64–76 shows the *in vitro* phosphorylation of Ser73 (Fig. 1c). However, we also identified residues that were differentially phosphorylated *in vitro* and *in vivo*. For instance, Ser47 was phosphorylated *in vitro*, whereas Ser57 and Ser59 were phosphorylated *in vivo*. Since there are seven Ser/Thr residues in this general region, there may be flexibility in which residues are targeted for phosphorylation. We also note that the phosphorylation of specific residues could be regulated *in vivo*, but not *in vitro*. Further study will be required to verify the physiological relevance of other phosphorylated residues. Full-length Rad23 that was characterized *in vivo* showed phosphorylation of Thr94 and Thr139. Both residues lie outside the UbL domain. Intriguingly, the polypeptide sequence flanking these residues are highly similar

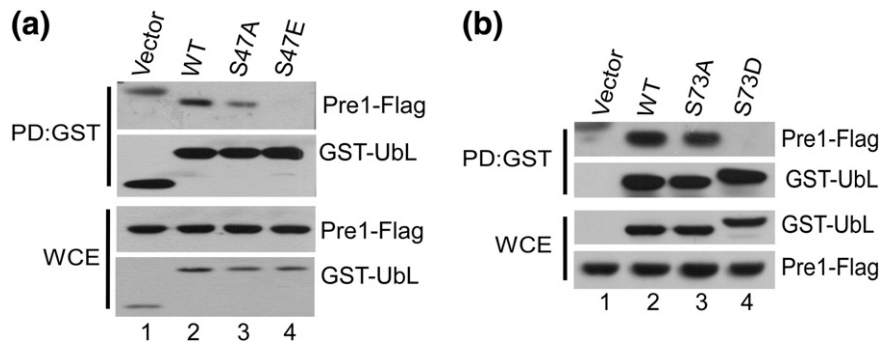


Fig. 2. Phosphomimetic mutations of Ser47 and Ser73 in UbL domain prevent Rad23/proteasome interaction *in vivo*. (a and b) GST-UbL and variants with mutations in this domain were expressed in *rad23Δ* strain that expressed Pre1-Flag, and the interaction between the proteasome and the UbL domain was examined.

(90-ESASTPG-96 and 135-ESATTPG-141, respectively), suggesting that they may be targeted by the same kinase. We note that ~70-amino-acid sequence between UbL and UBA1 is highly enriched in Ser/Thr residues (more than approximately one-third), and many conform to potential phosphorylation sites.

Serine 47 and serine 73 in the UbL domain are important sites for phosphorylation

The structure of the yeast UbL domain was determined at the atomic level, and strong similarity to ubiquitin was observed [22]. However, unlike ubiquitin and other UbL modifiers, the UbL domain in Rad23 protein is not excised [23] and conjugated to other proteins. The yeast UbL domain binds the proteasome subunit Rpn1 [8], whereas the human counterparts of Rad23 bind the S5a subunit in the proteasome [9]. The Rad23 UbL domain also interacts with Ufd2 [11,24] and Ataxin-3 [10], which are also associated with the protein degradation pathway. The absolute requirement for UbL in binding the proteasome [3] led us to focus on the effect of phosphorylation on its function. Human and mouse Rad23 counterparts contain a threonine residue at the position corresponding to Ser73 in yeast Rad23. Although serine and threonine residues are not necessarily interchangeable, as illustrated by the fact that only threonine can function as a nucleophile in the proteasome peptidases [25], both residues are structurally similar and can be phosphorylated. In addition to Ser73, mass spectrometry of UbL purified from yeast showed that three additional Ser/Thr residues were phosphorylated *in vivo*.

UbL was expressed as a fusion to glutathione S-transferase (GST) and Ser47 and Ser73 were converted to alanine. GST-UbL was affinity purified, separated by SDS-PAGE, and an immunoblot was incubated with antibody against phosphoserine (Fig. 1d). A strong cross-reaction was observed with the anti-phosphoserine antibody, whereas

the interaction with GST-UbL^{S47A} and GST-UbL^{S73A} was reduced significantly. We characterized GST-UbL to specifically focus on residues in this domain that might be phosphorylated. As expected, the phosphorylation of a double mutant (GST-UbL^{S47A S73A}) was reduced further.

We reasoned that conversion of a phosphorylated residue to an acidic amino acid might mimic the effect of a phosphorylated residue, as has been described by others [26,27]. Ser47 and Ser73 were converted to acidic residues (S47E and S73D). GST-UbL and mutant derivatives were expressed in yeast containing an epitope-tagged proteasome subunit (Pre1-Flag). GST proteins modified at Ser47 were affinity purified on glutathione Sepharose and immunoblots were reacted with antibodies against Flag and GST (Fig. 2a). Antibody reaction against the GST and GST-UbL proteins in whole cell extracts (WCE) showed that they were expressed at similar levels. The level of Pre1-Flag was also equivalent in all strains. After affinity purification, similar amounts of GST proteins were isolated. GST alone did not co-purify Pre1-Flag. (A higher-molecular-weight band represents a cross-reaction against GST.) Both GST-UbL (wild type) and GST-UbL^{S47A} co-precipitated Pre1-Flag (lanes 2 and 3). In contrast, GST-UbL^{S47E} showed significantly reduced interaction with the proteasome, as indicated by the lower co-purification of Pre1-Flag, despite high levels in the extract (lane 4). Similarly, the interaction between the proteasome and GST-UbL^{S73A} and GST-UbL^{S73D} was examined (Fig. 2b). In agreement with the findings in Fig. 2a, we observed reduced proteasome (Pre1-Flag) interaction with GST-UbL^{S73D} (lane 4). (The Flag antibody reaction showed non-specific reaction against GST, seen at the top of lane 1.) We also tested the co-purification of another proteasome subunit and similar findings were observed (Fig. S1).

We investigated if the reduced proteasome interaction displayed by specific UbL mutants also occurred in the context of the full-length protein. A

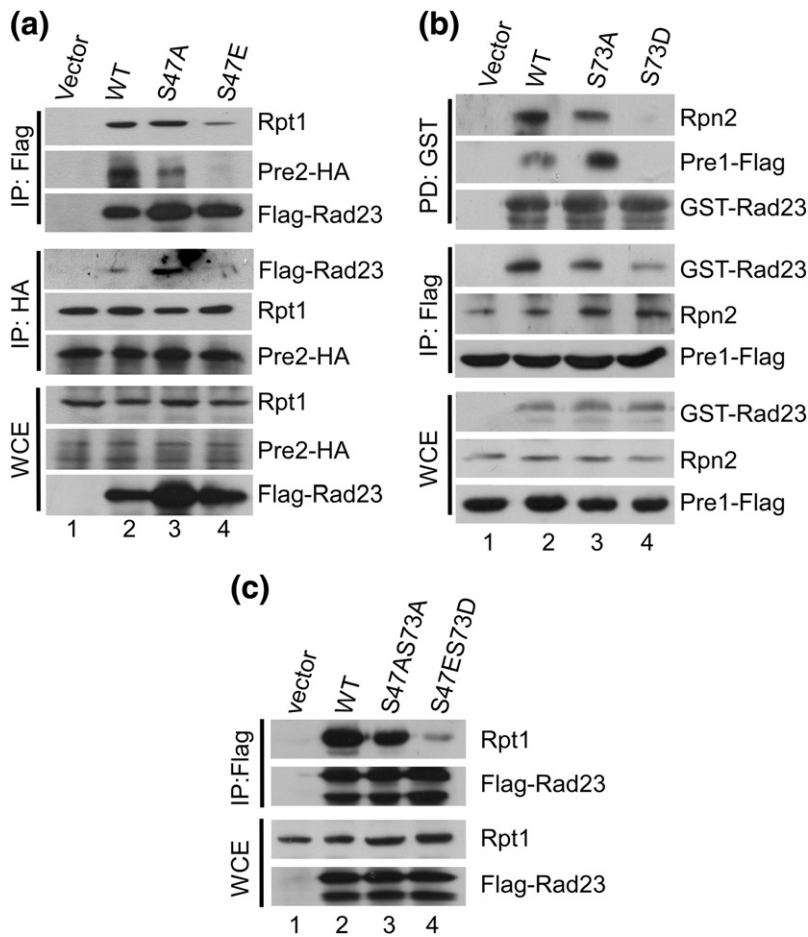


Fig. 3. Phosphomimetic mutations of Ser47 and Ser73 in Rad23 prevent Rad23/proteasome interaction *in vivo*. (a) Flag-Rad23, Flag-Rad23^{S47A}, and Flag-Rad23^{S47E} were expressed in *rad23Δ* that also expressed proteasome subunit Pre2-HA. WCE were prepared and equal amount of protein was incubated with Flag-agarose. The co-purification of the proteasome, with Rad23 and mutant derivatives, was determined by immunoblotting. (b) GST-Rad23, GST-Rad23^{S73A}, and GST-Rad23^{S73D} were expressed in *rad23Δ* expressing a different epitope-tagged proteasome subunit, Pre1-Flag. As described in (a), the interaction between 26S proteasome and Rad23 was investigated by purifying either the GST-tagged Rad23 proteins or the proteasome subunit Pre1-Flag. Rad23/proteasome interaction was gauged by immunoblotting. (c) The consequence of a UbL mutant bearing both Rad23^{S47AS73A} and Rad23^{S47ES73D} mutations was examined by isolating the full-length proteins from yeast cells and examining interaction with the proteasome. A vector control (lane 1) showed that non-specific precipitation of the proteasome and Rad23 was negligible.

yeast strain expressing the 20S proteasome subunit Pre2-HA was transformed with an empty vector or plasmids expressing wild-type Flag-Rad23, Flag-rad23^{S47A}, and Flag-rad23^{S47E}. Protein extracts were prepared and applied to Flag-agarose, and immunoblots were incubated with antibodies against hemagglutinin (HA) (Fig. 3a). The Flag-tagged Rad23/rad23 proteins were recovered efficiently on the affinity beads. However, the co-purification of Pre2-HA was reduced with Flag-rad23^{S47E} (lane 4), but not with Flag-rad23^{S47A}. The filter was incubated next with antibody against Rpt1 and reduced binding to this 19S proteasome subunit was observed. In contrast, the co-purification of Rpt1 with Flag-rad23^{S47A} was not affected. There were no detectable non-specific interactions associated with extracts containing vector and the Flag-agarose matrix (lane 1).

In a reciprocal study, Pre2-HA was immunoprecipitated and the co-purification of Flag-Rad23 was investigated. Immunoblotting showed that a lower amount of Flag-rad23^{S47E} was co-purified with Pre2-HA (lane 4). In contrast, Pre2-HA interaction with the 19S subunit Rpt1 was not affected, indicating that the interaction between the 19S and 20S protea-

some particles was unaffected. The Flag-tagged Rad23 proteins were expressed efficiently in all strains (WCE, lower panels). We also tested the effect of mutations of Ser73 (Fig. 3b). These Rad23 derivatives were expressed as fusions to GST. GST, GST-Rad23, GST-rad23^{S73A} and GST-rad23^{S73D} were expressed in a yeast strain containing Pre1-Flag. Protein extracts were applied to glutathione Sepharose and immunoblotting showed significantly reduced interaction between GST-rad23^{S73D} and the proteasome (lane 4). Neither Rpn2 nor Pre1-Flag was isolated with GST-rad23^{S73D}, whereas GST-Rad23 and GST-rad23^{S73A} showed similar interactions with the proteasome. In agreement, Pre1-Flag was purified with reduced levels of GST-rad23^{S73D}, but GST-Rad23 and GST-rad23^{S73A} were efficiently co-purified.

We investigated if a double mutant would have a more severe defect in binding the proteasome. Flag-Rad23, Flag-rad23^{S47AS73A}, and Flag-rad23^{S47ES73D} were expressed in wild-type cells. Protein extracts were applied to Flag-agarose and the bound proteins were characterized by immunoblotting (Fig. 3c). The interaction between Flag-rad23^{S47ES73D} and the

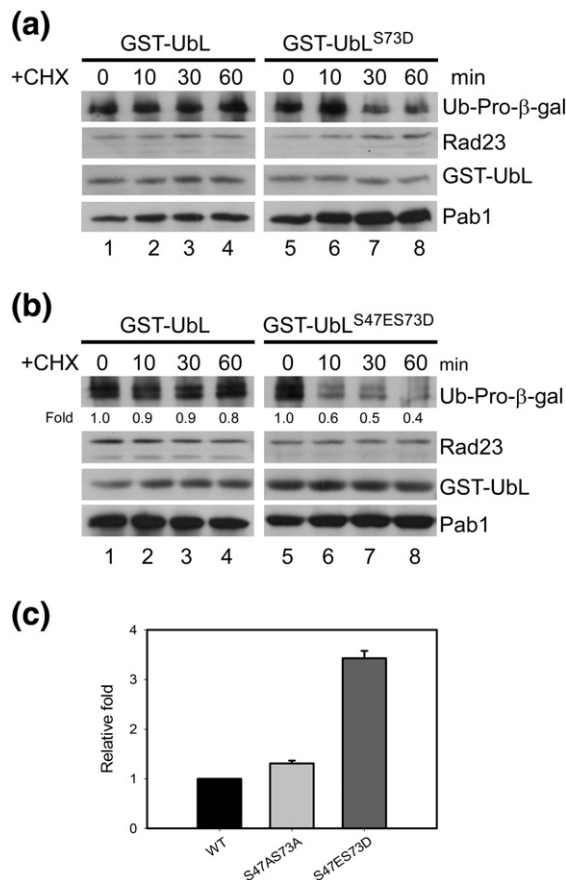


Fig. 4. Stabilization of proteolytic substrates by Rad23 phosphomimetic mutants. (a) Overexpression of the Rad23 UbL domain can interfere with protein degradation. Ub-Pro-β-gal was co-expressed with either GST-UbL or GST-UbL^{S73D}. Ub-Pro-β-gal was detected in total extract during a cycloheximide chase, by immunoblotting, and moderate stabilization was observed. (b) Ub-Pro-β-gal was co-expressed with GST-UbL or the double mutant GST-UbL^{S47E S73D}, in wild-type yeast. Pab1 levels were determined as a loading control. These results are representative of three independent experiments. (c) The reporter substrate Ub-Pro-β-gal was co-expressed with Flag-Rad23, Flag-Rad23^{S47A S73A}, or Flag-Rad23^{S47E S73D} in *rad23Δ*. β-Galactosidase activity was measured in WCE to estimate Ub-Pro-β-gal stability. β-Gal activity representing the average of four independent experiments was normalized to the value detected in the wild-type strain.

proteasome was significantly reduced, whereas Flag-rad23^{S47A, S73A} interaction with the proteasome was similar to that observed with Flag-Rad23.

Unregulated UbL/proteasome binding can inhibit protein turnover

We showed previously that overexpression of the UbL domain can stabilize proteolytic substrates [19]. This effect is caused by a non-productive interaction between the yeast UbL domain and the proteasome.

We therefore surmised that, if UbL phosphorylation reduced proteasome binding, then a mutation such as S73D should not affect the turnover of proteasome substrates. We expressed GST-UbL and GST-UbL^{S73D} in yeast cells that contained Ub-Pro-β-gal, which is a well-characterized test substrate of the proteasome [28]. High levels of GST-UbL caused stabilization of Ub-Pro-β-gal (Fig. 4a), which is degraded rapidly by the proteasome. The expression levels of native Rad23, GST-UbL, and a stable protein (Pab1) were unaffected during the cycloheximide chase. In contrast, expression of GST-UbL^{S73D} failed to affect the rapid turnover of Ub-Pro-β-gal, consistent with our findings reported here that phosphorylation of the UbL domain reduces interaction with the proteasome. Expression of a double mutant GST-UbL^{S47E S73D} also failed to inhibit the rapid degradation of Ub-Pro-β-gal, whereas the expression of GST-UbL caused strong stabilization (Fig. 4b). The double mutant exerted a stronger effect than the Ser73 → Asp73 substitution mutation, indicating that phosphorylation of both Ser47 and Ser73 affects proteasome binding. Next, we examined the effect of the Rad23^{S47E, S73D} mutation on proteolysis by the ubiquitin-proteasome pathway. Flag-Rad23 and its mutant variants were co-expressed in *rad23Δ* cells with the test substrate Ub-Pro-β-galactosidase (Ub-Pro-β-gal). An expression shut-off assay was used to measure steady-state β-gal enzymatic activity. We detected ~3-fold higher β-gal activity in *rad23Δ* cells expressing the double mutant Flag-Rad23^{S47E, S73D} (Fig. 4c). In contrast, similar β-gal activity was measured in extracts containing Flag-Rad23 and Flag-Rad23^{S47A, S73A}. We conclude that the lower β-gal activity was the result of successful delivery of Ub-Pro-β-gal to the proteasome by Flag-Rad23 and Flag-Rad23^{S47A, S73A}. These results were confirmed by immunoblotting analysis (Fig. S2).

Phosphorylation of the UbL domain is required for efficient growth and response to stress

We reported previously that the Rad23 and Rpn10 proteins have overlapping functions [6]. Loss of both proteins (*rad23Δ rpn10Δ*) causes pleiotropic defects that are not observed in either single mutant (*rad23Δ*; *rpn10Δ*). As expected, expression of either Rad23 or Rpn10 fully rescued the pleiotropic defects of *rad23Δ rpn10Δ*. This finding offered an opportunity to test the importance of UbL/proteasome interaction using specific phosphorylation mutants. Removal of the UbL domain from yeast Rad23 causes intermediate UV sensitivity [5,6]. However, the removal of this domain (77 amino acid residues) could cause unforeseen structural changes. The ability to regulate Rad23/proteasome binding through single amino acid substitutions (*rad23^{S47A, S73A}*) offered a unique way to characterize the significance of this interaction without inducing major steric and structural

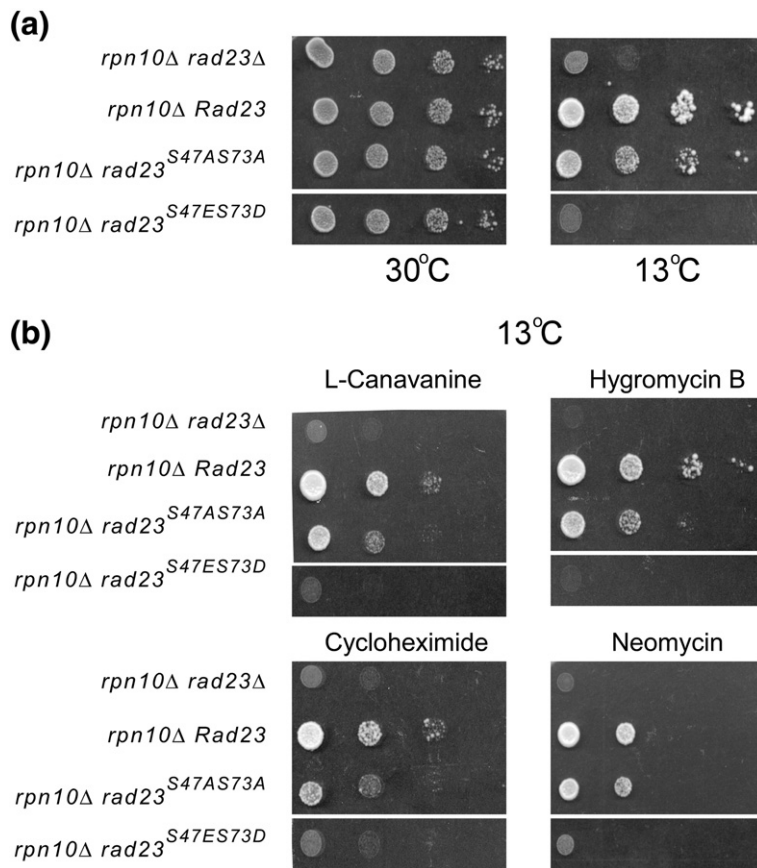


Fig. 5. Altered stress response by yeast cells expressing Rad23 phosphomimetic mutations. Wild-type Rad23 or derivatives with mutations in Ser47 and Ser73 were expressed in *rad23Δ rpn10Δ*. Mid-log-phase cultures were normalized to a density of $A_{600} \sim 1.0$, and 10-fold dilutions were spotted on selective synthetic medium plates. (a) The plates were incubated at 30 °C or at 13 °C until growth was observed. (b) Mid-log-phase cultures were spotted on medium containing 0.5 $\mu\text{g/ml}$ L-canavanine, 0.1 $\mu\text{g/ml}$ cycloheximide, 2 μM neomycin, or 0.2 μM hygromycin B. These results are representative of three independent experiments.

perturbations. We expressed Rad23, *rad23^{S47A,S73A}*, and *rad23^{S47E,S73D}* in *rad23Δ rpn10Δ* and spotted 10-fold dilutions of actively growing cultures on synthetic medium (Fig. 5a). The agar plates were incubated at the permissive (30 °C; left panel) and non-permissive temperatures (13 °C; right panel). The poor growth of *rad23Δ rpn10Δ* at 13 °C was suppressed by expression of Rad23 and *rad23^{S47A,S73A}*. In contrast, *rad23^{S47E,S73D}* was unable to confer growth to *rad23Δ rpn10Δ* at 13 °C.

We also examined growth in the presence of drugs that severely impede the growth of *rad23Δ rpn10Δ*. We confirmed that expression of Rad23 suppressed all of the drug-specific effects in *rad23Δ rpn10Δ* (Fig. 5b). Expression of *rad23^{S47A,S73A}* partially suppressed the poor growth of in *rad23Δ rpn10Δ*, caused by L-canavanine, hygromycin B, cycloheximide, and neomycin. The proteasome is required for the elimination of damaged proteins that are induced by exposure to these drugs [29].

Regulated proteasome interaction by Rad23 is required for efficient cell cycle progression

The *rad23Δ rpn10Δ* mutant displays a strong G2-specific delay during cell cycle progression [6]. This growth defect is likely to be caused by a failure to degrade cell-cycle-specific regulatory factors.

Regulated Rad23/proteasome interaction might contribute directly to the turnover of these key proteins. We therefore investigated if the absence of Rad23/proteasome interaction contributed to the G2 phase delay in *rad23Δ rpn10Δ*. We used a cell sorter to monitor cell cycle progression in *rad23Δ rpn10Δ* expressing Rad23 or phosphorylation defective mutants (Fig. 6). At 30 °C, the distribution of G1, G2, and M phase cells was similar in all four strains (a), although a higher proportion of G2 cells was evident in *rad23Δ rpn10Δ*. After transfer to 13 °C, cells expressing Rad23 had an equivalent proportion of G1 and G2 phase cells (38.4% and 34.8%, respectively). However, the level of G2 phase cells increased dramatically in *rad23Δ rpn10Δ* (G1 = 13.4%; G2 = 62.7%). Expression of either Rad23 or *rad23^{S47A,S73A}* in the double mutant restored normal distribution of cells at 13 °C. In contrast, *rad23^{S47E,S73D}* failed to alleviate the G2 phase growth delay in *rad23Δ rpn10Δ*, demonstrating that Rad23/proteasome interaction is required for efficient cell cycle progression.

Phosphorylation of the UbL domain in human hHR23B inhibits interaction with the proteasome

To determine if the mechanism for regulating yeast Rad23/proteasome interaction is conserved, we

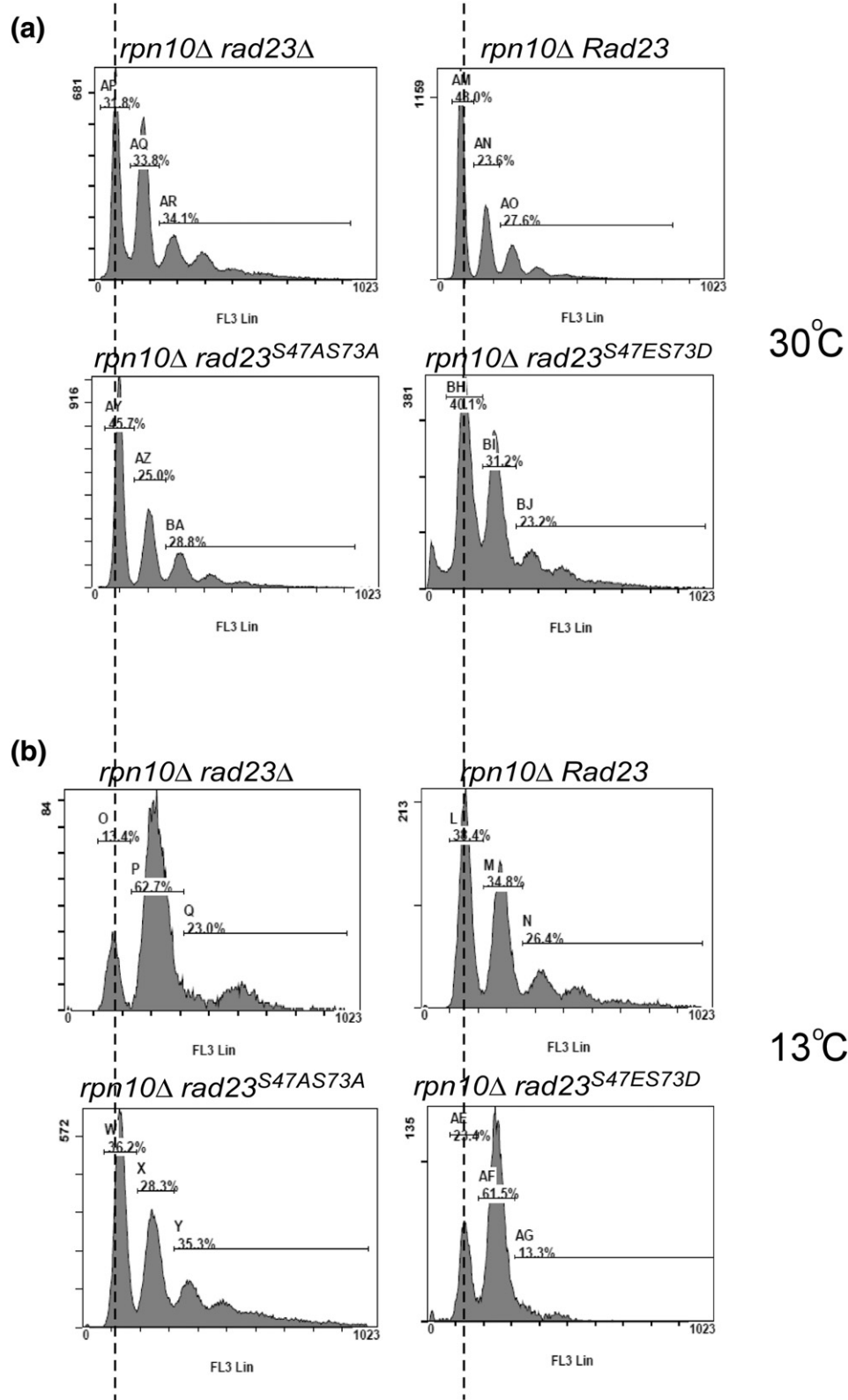


Fig. 6. The G2/M phase delay in *rad23Δ rpn10Δ* is not suppressed by Rad23^{S47ES73D}. Wild-type Rad23 or mutations in various phosphorylation sites were expressed in *rad23Δ rpn10Δ*. Yeast cells were grown to the mid-exponential phase at (a) 30 °C or (b) 13 °C, fixed in 70% ethanol at –20 °C, and examined by flow cytometry.

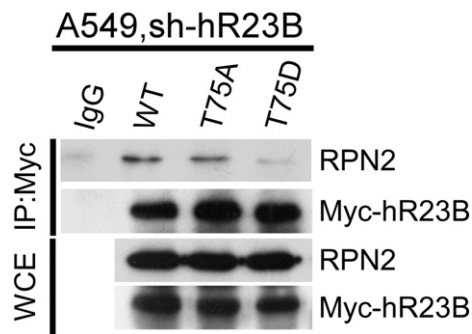


Fig. 7. Phosphorylation of the UbL domain in human hHR23B inhibits interaction with the proteasome. Thr75 in hHR23B was converted to either alanine (T75A) or aspartic acid (T75D), and the protein derivatives were expressed with a Myc epitope in human lung carcinoma cell line A549. A549 contained a hHR23B-specific lentiviral expressed knock-down construct. Protein extracts were incubated with anti-Myc antibody to immunoprecipitate Myc-hHR23B and mutant derivatives. The co-precipitated proteasome was determined by immunoblot analysis using antibody against Rpn2.

examined the human Rad23 counterpart hHR23B. Sequence analysis showed that Thr75 in hHR23B corresponds to the Ser73 residue in Rad23 that we examined here. Thr75 was converted to either alanine (T75A) or aspartate (T75D), and the protein derivatives were expressed with a Myc epitope in human lung carcinoma cell line A549. A549 contained an hHR23B-specific lentiviral expressed knock-down construct. Protein extracts were incubated with anti-Myc antibody to immunoprecipitate Myc-hHR23B and mutant derivatives. We found that both wild-type and T75A mutants were able to co-precipitate the Rpn2 proteasome subunit (Fig. 7). In contrast, the T75D mutant showed significantly reduced interaction with Rpn2. The expression of Rpn2 and the hHR23B proteins was similar in all strains (WCE). These findings demonstrate that our characterization of the Rad23 protein in yeast is applicable to the human Rad23 protein, implying a common regulatory mechanism.

Discussion

Three distinct structural motifs have been identified in the family of Rad23 proteins. The yeast Rad23 protein is the paradigm for this class of proteins and is generally believed to function as a shuttle-factor that can deliver proteolytic substrates to the proteasome [19]. This action requires two structures: an amino-terminal UbL domain that binds the proteasome [3], and two UBA domains that bind multiubiquitin chains [30,31]. In addition, Rad23 contains

a Rad4-binding sequence that can bind and control Rad4 stability [32]. The UbL domain has been reported to bind the Rpn1 subunit in the proteasome [8], the Ufd2 factor [11], and Ataxin-3 [10]. The UBA domains are believed to bind many multiubiquitinated cellular proteins, as well as numerous specific factors, including HIV-1-encoded Vpr protein [33], Png1 [14], and p300 [16]. The significance of these interactions is not understood.

Rad23/proteasome interaction has been examined extensively, and its link to the Rpn10 proteasome receptor and other factors has been investigated [6,34,35]. This interaction can be easily monitored, and its effect on protein turnover has been investigated. Previous studies that characterized a rad23 mutant protein lacking the entire UbL domain revealed proteolytic defects. Therefore, the discovery that phosphorylation of specific residues in the UbL domain affects Rad23/proteasome interaction allowed us to focus on investigating the significance of this association in protein degradation. Proteomic analysis of human Rad23 (hHR23B) that was purified in association with the proteasome showed phosphorylation of serine 160.

We show here that residues in the UbL domain are phosphorylated *in vivo* and *in vitro*. Conversion of these residues to alanine did not have an appreciable effect on growth or response to environmental stresses. However, conversion to an acidic residue caused strong inhibition of binding to the proteasome. This deficiency could be assessed directly by measuring the co-purification of proteasome subunits and also using recombinant protein that was incubated with yeast extracts. The biological relevance of blocking proteasome interaction using either the full-length protein (rad23^{S47E,S73D}) or just the UbL domain (UbL^{S47E,S73D}) was tested. Unregulated interaction between the UbL domain and the proteasome can strongly interfere with intracellular protein breakdown. However, a mutant version that is unable to bind the proteasome (UbL^{S47E,S73D}) did not interfere with the turnover of a test substrate of the proteasome. We suggest that regulated Rad23/proteasome interaction is physiologically important because rad23Δ rpn10Δ cells expressing rad23^{S47E,S73D} were sensitive to drugs that cause protein unfolding and showed a strong delay in the G2 phase of the cell cycle.

Our findings raise interesting questions about the nature of Rad23/proteasome binding. Based on the hypothesis that Rad23 can deliver multiubiquitinated proteins to the proteasome, it is important to determine if this interaction occurs only after Rad23 has bound cargo. Specifically, an unregulated interaction between UbL and the proteasome can inhibit protein degradation [19]. We speculate that unphosphorylated Rad23 binds multiubiquitinated proteins and traffics them to the proteasome. Following the delivery of a proteolytic substrate,

Rad23 might become phosphorylated to trigger its release from the proteasome. This mechanism would allow Rad23 to renew another cycle of substrate translocation to the proteasome. This mechanism might require a proteasome-associated kinase that specifically phosphorylated Rad23, as well as other shuttle-factors, after the delivery of substrates. Other scenarios are also possible, although we believe that this model provides a straightforward interpretation of the data. Many kinases are associated with the proteasome, although further study will be required to identify one that can target Rad23. It is intriguing in this regard that the Snf1 kinase has been linked to Rad23 function in DNA repair, although its site of action has not been described.

Materials and Methods

Yeast strains and plasmids

Yeast cultures were grown in rich (YPD) or synthetic media containing 2% glucose or galactose. The *Saccharomyces cerevisiae rad23Δ* and *rad23Δrpn10Δ* strains and strains expressing chromosomal HA-tagged Rad4 were as previously described [5]. Similarly, plasmids expressing Flag-tagged Rad23, *rad23^{ΔUbl}*, Pre2-HA, Pre1-Flag, Ub-Arg-β-galactosidase (Arg-β-gal), Ub-Pro-β-galactosidase (Ub-Pro-β-gal), Rpn8-V5, GST-tagged Rad23, and Ubl were as described previously [3,19,32,36]. Full-length human Rad23B (hHR23B) cDNA was amplified from A549 cells and cloned into pCMV-Myc with XhoI and EcoRI sites. The substitutions of the serine 47 and serine 73 residues to alanine or aspartic acid/glutamic acid were achieved using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following DNA oligonucleotides:

S47A forward: 5'-CAAATAAACTGATCTACGCGG
GTAAAGTGCTAC-3'
S47A reverse: 5'-GTAGCACTTTACCCGCGTAGTAC
AGTTTTATTTTG-3'
S47E forward: 5'-CAAATAAACTGATCTACGAGG
GTAAAGTGCTAC-3'
S47E reverse: 5'-GTAGCACTTTACCCGCGTAGTACAG
TTTTATTTTG-3'
S73A forward: 5'-GTCTTCATGGTTGCTCAAAAAAG-3'
S73A reverse: 5'-CTTTTTTTGAGCAACCATGAAGAC-3'
S73D forward: 5'-GTCTTCATGGTTGATCAAAAAAG-3'
S73D reverse: 5'-CTTTTTTTGATCAACCATGAAGAC-3'
T75A (hHR23B) forward: 5'-GTGGTTATGGTGGCCAA
ACCCAAAG-3'
T75A (hHR23B) reverse: 5'-CTTTGGGTTTGCCACCATA
ACCAC-3'
T75D (hHR23B) forward: 5'-GTGGTTATGGTGGACAAAC
CCAAAG-3'
T75D (hHR23B) reverse: 5'-CTTTGGGTTTGCCACCATA
ACCAC-3'.

In all cases, multiple isolates were confirmed by DNA sequencing.

Chemicals and antibodies

The antibody against Pab1 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The polyclonal antibodies against β-galactosidase, Rpn2, HA, and V5 were from Abcam, Inc. (Cambridge, MA, USA). Anti-Flag M2-agarose beads, iodoacetamide, hydroxyurea, L-canavanine, neomycin, hygromycin B, and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The polyclonal antibodies against Rpt1 and Rad23 were gifted from Dr. Madura (University of Medicine and Dentistry of New Jersey, Piscataway, NJ, USA). The glutathione Sepharose 4B beads were purchased from GE Healthcare (Piscataway, NJ, USA). *Ortho*-nitrophenyl-β-galactosidase was purchased from MP Biomedicals, Inc. (Solon, OH, USA). Dithiothreitol was purchased from Amresco, Inc. (Solon, OH, USA). λ-Phosphatase was purchased from Cell Signaling Technology (Beverly, MA, USA). 4-Nitroquinoline-1-oxide was from Lancaster (Morecambe, UK). All other chemicals were purchased from Sigma, unless otherwise specified.

Protein expression shut-off assay

Yeast cells expressing β-galactosidase from the *GAL1* promoter were grown at 30 °C to an OD₆₀₀ of ~1 in a synthetic 2% raffinose medium, lacking uracil. Protein expression was induced by the addition of 2% galactose for 2 h and then repressed by the addition of 2% glucose. Cycloheximide (0.5 mg/ml) was then added to stop protein synthesis. Samples were withdrawn at the indicated time points and cells harvested by centrifugation, and protein extracts were prepared for immunoblotting or immunoprecipitation, as indicated.

Immunoprecipitation and Western blotting

Yeast strains containing plasmids were grown in synthetic medium, pelleted, and frozen at -20 °C. For analysis, the cells were suspended in buffer A [50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM ethylenediamine-tetraacetic acid, 1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 10% glycerol, and protease inhibitors (Roche, Mannheim, Germany)] and lysed by disruption with glass beads. The extracts were centrifuged at 12,000 rpm for 5 min at 4 °C, and the protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of total protein were adjusted to 500 μl with buffer A containing 20 μl anti-Flag M2-agarose beads (for Flag-tagged proteins) or glutathione Sepharose 4B beads (for GST-fusion proteins). The samples were incubated at 4 °C for 2–3 h, and the beads were washed three times with 1 ml buffer A. The bound proteins were boiled for 5 min, separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the appropriate primary antibodies. For the detection of ubiquitin conjugates, the nitrocellulose membranes were boiled for 5 min prior to incubation with appropriate secondary antibodies. The signal was developed using an enhanced chemiluminescence kit (Perkin Elmer, Boston, MA, USA).

One-dimensional TLC

Yeast cells expressing Flag-Rad23 were grown to the logarithmic (Log) phase in the presence of [³²P] orthophosphate (GE Healthcare) for 1 h. *In vivo* phosphorylated Flag-Rad23 was recovered by immunoprecipitation, resolved by SDS-PAGE, and transferred to a PVDF membrane. ³²P-labeled Flag-Rad23 was excised and digested in acid, and the hydrolysate was separated by one-dimensional TLC. The positions of non-radioactive phosphoamino acid standards were detected by staining with ninhydrin.

Drug sensitivity assay

Yeast cells were grown to mid-log-phase in selective medium and normalized to a density of $A_{600\text{ nm}} \sim 1$. Serial dilutions (10-fold) were spotted on selective synthetic medium plates containing different chemicals. The plates were then wrapped in aluminum foil and incubated at 30 °C or 13 °C until colonies could be imaged.

Yeast cell cycle phase analysis

Yeast cells were grown to mid-log-phase in selective synthetic medium, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol at –20 °C. The cells were washed with 50 mM Tris (pH 7.5), suspended in PBS containing 1 mg/ml RNase A, and incubated at 37 °C for 2 h. The cells were centrifuged and washed with PBS, proteinase K (40 µg/ml) was added, and the cells were incubated at 55 °C for an additional hour. Propidium iodide was added at a final concentration of 20 µg/ml. For analysis, the cells were sonicated twice to disrupt aggregates and then immediately subjected to flow cytometry using a Beckman Coulter FC500 (Beckman, Brea, CA, USA). Cell cycle phases were identified and plotted.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmb.2014.10.004>.

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Conflict of Interest Statement: The authors have no competing financial interests in this work. KM is the Founding President and CEO of CellXplore, Inc., which is characterizing the ubiquitin-proteasome system to develop diagnostic assays for human breast cancer. KM is also an inventor on multiple patents. However, there is no conflict with this work.

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NER, nucleotide excision repair; TLC, thin-layer chromatography; GST, glutathione S-transferase; WCE, whole cell extracts; PBS, phosphate-buffered saline.

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