The UBA2 Domain Functions as an Intrinsic Stabilization Signal that Protects Rad23 from Proteasomal Degradation

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Summary

The proteasome-interacting protein Rad23 is a long-lived protein. Interaction between Rad23 and the proteasome is required for Rad23’s functions in nucleotide excision repair and ubiquitin-dependent degradation. Here, we show that the ubiquitin-associated (UBA)-2 domain of yeast Rad23 is a cis-acting, transferable stabilization signal that protects Rad23 from proteasomal degradation. Disruption of the UBA2 domain converts Rad23 into a short-lived protein that is targeted for degradation through its N-terminal ubiquitin-like domain. UBA2-dependent stabilization is required for Rad23 function because a yeast strain expressing a mutant Rad23 that lacks a functional UBA2 domain shows increased sensitivity to UV light and, in the absence of Rpn10, severe growth defects. The C-terminal UBA domains of Dsk2, Ddi1, Ede1, and the human Rad23 homolog hHR23A have similar protective activities. Thus, the UBA2 domain of Rad23 is an evolutionarily conserved stabilization signal that allows Rad23 to interact with the proteasome without facing destruction.

Introduction

The Saccharomyces cerevisiae Rad23 protein is involved in regulation of nucleotide excision repair, proteolysis and cell cycle progression (Clarke et al., 2001; Lamberton et al., 1999; Watkins et al., 1993). While the precise mode of action of Rad23 is unknown, increasing evidence suggests that a close interplay between Rad23 and the ubiquitin/proteasome system is essential for its diverse functions (Chen and Madura, 2002; Russell et al., 1999; Schauer et al., 1998). The ubiquitin/proteasome system was originally identified as the primary degradation machinery in the cytosol and nucleus of eukaryotic cells (Baumeister et al., 1998; Hershko and Ciechanover, 1998), but recent studies have revealed additional nonproteolytic functions in DNA repair and transcription (Ferdous et al., 2001; Gonzalez et al., 2002; Russell et al., 1999).

The presence of several characteristic structural domains highlights the close relationship of Rad23 with the ubiquitin/proteasome system. Rad23 contains an N-terminal ubiquitin-like (UbL) domain and two ubiquitin-associated (UBA) domains: an internal UBA1 domain and a C-terminal UBA2 domain (Buchberger, 2002). The UbL domain, which can be functionally replaced by ubiquitin (Watkins et al., 1993), mediates the interaction between Rad23 and the proteasome (Schauer et al., 1998). The UBA domain was originally identified as a sequence motif present in proteins linked to the ubiquitination system (Hofmann and Bucher, 1996). It was later reported that, notwithstanding their low sequence homology, all UBA domains share the ability to bind ubiquitin (Bertolaet et al., 2001b; Chen et al., 2001; Wilkinson et al., 2001). Based on its ability to simultaneously bind ubiquitinated proteins and the proteasome, it has been proposed that Rad23 may function as a scaffold that facilitates interactions between substrates and the proteasome (Chen and Madura, 2002; Kim et al., 2004). Recently, direct biochemical evidence has been provided for a role of Rad23 in targeting ubiquitinated substrates to the proteasome (Elsasser et al., 2004; Saeki et al., 2002a; Verma et al., 2004). Several studies revealed that a concerted action of the ubiquitin/proteasome system and Rad23 is important for nucleotide excision repair (NER) although the precise mode of action of the proteasome in this event remains obscure (Russell et al., 1999; Schauer et al., 1998). Interestingly, this DNA repair activity requires only the 19S regulatory particle of the proteasome and is independent of the proteolytically active 20S core particle (Gillette et al., 2001).

Substrates of the ubiquitin/proteasome system are targeted by degradation signals, which are small motifs or domains that accommodate interaction with the proteasome in a ubiquitin-dependent (Hershko and Ciechanover, 1998) or, less common, a ubiquitin-independent manner (Chen et al., 2004; Murakami et al., 1992). As a general rule, recruitment of proteins to the proteasome results in their rapid inactivation by processive degradation. However, the interaction between Rad23 and the proteasome does not lead to Rad23 destruction (Schauer et al., 1998; Watkins et al., 1993). We have previously described a similar phenomenon with a viral repetitive sequence that can be used for cis-stabilization of proteasome substrates (Heessen et al., 2002; Levitskaya et al., 1995; Sharipo et al., 1998). Based on these findings, we postulated that cellular proteins might contain similar protective “stabilization signals” that spare them from proteolysis (Dantuma and Masucci, 2002). Here, we show that the C-terminal UBA domains of Rad23, Dsk2, Ddi1, and Ede1 function as stabilization signals that can protect proteins from proteasomal degradation.
Figure 1. Cis Stabilization of Proteasome Reporter Substrates by the UBA2 Domain

(A) Schematic drawing of the fusion of the N-end rule reporter substrate Ub-R-GFP and Rad23 fragments. The locations of the UbL, UBA1, and UBA2 domains in Rad23 are indicated.

(B) Lysates of 10 OD_600 units of log-phase yeast coexpressing Ub-R-GFP-Rad23^{UBL} and 3HA ubiquitin were subjected to immunoprecipitation with an anti-GFP antibody, followed by Western blotting with an anti-HA antibody. Yeast cells transformed with an empty vector and 3HA ubiquitin were used as negative control. The molecular weight markers, ubiquitinated species, and the immunoglobulin heavy chain (HC) are indicated.

(C) Representative histograms of flow cytometric analysis of yeast expressing the Ub-M-GFP, Ub-R-GFP, or the Ub-R-GFP fusions shown in (A). The mean fluorescence intensity of each sample is indicated in the upper-right corner (corrected for background fluorescence of vector transformed yeast).

(D) Quantification of mean fluorescence intensities of yeast cells expressing Ub-M-GFP, Ub-R-GFP, or the Ub-R-GFP fusion proteins measured by flow cytometry. Ub-M-GFP was standardized as 100%. Values are means and standard deviations of three independent experiments. Asterisks indicate values that are significantly different from Ub-R-GFP (t test, p < 0.05).

(E) Representative histograms of flow cytometric analysis of yeast cells expressing Ub^{G76V}-GFP or Ub^{G76V}-GFP-Rad23^{UBL}.
UBA2 Is an Intrinsic Stabilization Signal

Figure 2. An Intact UBA2 Domain Protects from Proteasomal Degradation and Does Not Cause General Impairment of Proteasomal Degradation

(A) Western blot analysis of turnover of Ub-R-GFP, Ub-R-GFP-UBA1, and Ub-R-GFP-UBA2 levels in wild-type yeast and Ub-R-GFP in the ubr1Δ strain at 0, 10, 20, and 40 min after promoter shutoff.

(B) Densitometric quantification of the Ub-R-GFP (closed circles) and Ub-R-GFP-UBA2 (open circles) signal in (A). The signal at t = 0 is standardized as 100%.

(C) Mean fluorescence intensities of yeast expressing Ub-R-GFP-UBA1, Ub-R-GFP-UBA2, and Ub-R-GFP-UBA2L392A. Mean and standard deviations of three independent experiments are shown. Asterisk indicates values that are significantly different from Ub-R-GFP-UBA1 (t test, p < 0.01).

(D) Western blot analysis with specific GFP antibody of Ub-R-GFP and Ub-R-GFP-Rad23ΔUbL levels at 0, 10, 20, and 40 min after switching off the GAL1 promoter in yeast expressing Ub-R-GFP alone or together with Ub-R-GFP-Rad23ΔUbL. Putative ubiquitinated species (asterisks) and molecular weight markers are indicated.

Results

Cis Stabilization of Proteasome Reporter Substrates by the UBA2 Domain
The remarkable stability of the proteasome-interacting protein Rad23 prompted us to investigate whether this protein may harbor domains that protect it from proteasomal degradation. For this purpose, we analyzed whether insertion of Rad23 fragments in green fluorescent protein (GFP)-based substrates of the ubiquitin/proteasome system could inhibit their degradation (Figure 1A). These GFP-based substrates are targeted to the proteasome through the presence of well-defined degradation signals that deem the GFP for ubiquitin-
the inhibitory activity (Figure 2C).

while the UBA1 domain had no effect on degradation (Schauber et al., 1998). Deletion of the UbL domain re-
ncreased steady-state levels of UBA2-containing sub- domain could fulfill this role as it has been shown to
from Proteasomal Degradation
An Intact UBA2 Domain Is Required for Protection

Rad23 mediates interaction with the proteasome, we general inhibitors of ubiquitination (Ortolan et al., 2000)
cause it is well established that the UbL domain of It has been reported that UBA domains can act as
dependent proteolysis (Dantuma et al., 2000b). Be-
ubr1 signal, UbG76V-GFP. Of note, N-end rule and UFD sub-
strate carrying an ubiquitin fusion degradation (UFD) protective role in the context of native Rad23, we took
alyzed the effect of the UBA2 domain on a reporter sub-
In order to explore whether the UBA2 domain plays a
protective role in the context of native Rad23, we took
ubiquitin ligase (Table 1), confirming that the ubiquitin/
proteasome system is responsible for degradation of
this substrate in yeast (Bartel et al., 1990). Thus, the
UBA2 domain increases the steady-state levels of
Ub-R-GFP by delaying its proteosomal degradation.

Structural analysis of the UBA2 domains of the hu-
man homolog of Rad23 A (hHR23A) has revealed that a
conserved leucine corresponding with leucine 392 in
yeast Rad23 is important for the structural integrity of
the characteristic UBA fold (Mueller and Feigon, 2002).
Substitution of this single amino acid with an alanine
was sufficient to completely abrogate the protective ef-
fect, suggesting that a proper UBA fold is important for
the inhibitory activity (Figure 2C).

The UBA2 Domain Does Not Cause General
Impairment of the Ubiquitin/Proteasome System
It has been reported that UBA domains can act as
general inhibitors of ubiquitination (Ortolan et al., 2000)
d and deubiquitination (Hartmann-Petersen et al., 2003).
Thus, stabilization of the GFP reporter could be the
consequence of a general impairment of the ubiquitin/
proteasome system caused by the overexpressed UBA2 domain. To probe into this possibility, we exam-
ined whether overexpression of the stable Ub-R-GFP-
Rad23L392A fusion protein led to a general accumula-
tion of proteasome substrates. Ub-R-GFP-Rad23L392A
and the Ub-R-GFP or UbG76V-GFP substrates were coex-
pressed in yeast and their degradation was followed
after simultaneously switching off expression of both
proteins. Overexpression of Ub-R-GFP-Rad23L392A did
not affect the turnover of Ub-R-GFP (Figure 2D) or
UbG76V-GFP (data not shown). Furthermore, while gen-
el impairment of the ubiquitin/proteasome system re-
results in accumulation of ubiquitinated substrates and
induction of cell cycle arrest and apoptosis (Dantuma
et al., 2000b), overexpression of Ub-R-GFP-Rad23L392A
did not increase the total pool of ubiquitin-conjugates	nor did it affect proliferation (data not shown). We con-
clude that insertion of the Rad23 UBA2 domain can se-
lectively protect proteasome substrates carrying the
UBA2 domain from degradation without disturbing
overall proteasomal degradation.

The UBA2 Domain Functions as an Intrinsic
Stabilization Signal in Rad23
In order to explore whether the UBA2 domain plays a
protective role in the context of native Rad23, we took
substrates were targeted through distinct ubiquitination
paths for degradation by the proteasome (Bartel et al.,
1990; Johnson et al., 1995; Koegl et al., 1999). We
found a very similar protective effect on the UFD
reporter substrate, suggesting that the effect is largely
independent of the type of degradation signal (Figure 1E).

Table 1. Yeast Strains Used in this Study

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<td>DFS FLAG-RAD23</td>
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<td>SHY015</td>
<td>DFS FLAG-rad23L392A rnp10L::HIS3</td>
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dependent proteolysis (Dantuma et al., 2000b). Be-
cause it is well established that the UbL domain of
Rad23 mediates interaction with the proteasome, we
omitted the UbL domain to assure that only the degra-
dation signal of the reporter substrate would target the
fusion to the proteasome.

Introduction of the Rad23L392L did not interfere with
cleavage of the N-terminal ubiquitin of ubiquitin-R-GFP
(Ub-R-GFP; data not shown), which is required for ac-
quisition of the N-end rule degradation signal in this re-
porter substrate (Varshavsky, 1996), nor did it affect
targeting of the Ub-R-GFP for ubiquitination because
we could readily detect ubiquitinated reporter sub-
strates (Figure 1B). Insertion of the Rad23L392L in the
Ub-R-GFP reporter resulted in a 14-fold increase in the
mean fluorescent intensity of the reporter-expressing
yeast to approximately 50% of the level observed with
the stable Ub-M-GFP that lacks a degradation signal
(Figures 1C and 1D). To identify the region in Rad23 that
was responsible for this effect, we produced a set of
C-terminal deletions of the Ub-R-GFP-Rad23L392L fu-
sion protein (Figure 1A). Removal of the C-terminal
UBA2 domain brought the fluorescence intensity al-
most back to the Ub-R-GFP level (Figures 1C and 1D).
Insertion of the UBA2 domain was sufficient to recapit-
ulate the effect observed with Rad23L392L, whereas the
related UBA1 domain alone had no appreciable effect
on the mean fluorescent intensity (Figures 1C and 1D).

To test whether the stabilizing effect of UBA2 was
dependent on the type of degradation signal, we an-
alyzed the effect of the UBA2 domain on a reporter sub-
strate carrying an ubiquitin fusion degradation (UFD)
signal, UbG76V-GFP. Of note, N-end rule and UFD sub-
strates are targeted through distinct ubiquitination
paths for degradation by the proteasome (Bartel et al.,
1990; Johnson et al., 1995; Koegl et al., 1999). We
found a very similar protective effect on the UFD re-
porter substrate, suggesting that the effect is largely
independent of the type of degradation signal (Figure 1E).

Table 1. Yeast Strains Used in this Study

An Intact UBA2 Domain Is Required for Protection
from Proteasomal Degradation
Promoter shutoff experiments demonstrated that the
increased steady-state levels of UBA2-containing sub-
strates were due to delayed turnover of the reporter
while the UBA1 domain had no effect on degradation
(Figures 2A and 2B). The Ub-R-GFP reporter was stable in
the ubr1Δ yeast strain, which lacks the N-end rule
ubiquitin ligase (Table 1), confirming that the ubiquitin/
**UBA2 Is an Intrinsic Stabilization Signal**

Figure 3. The UBA2 Domain Functions as an Intrinsic Stabilization Signal in Rad23

(A) Turnover of FLAGRad23, FLAGRad23L392A, and FLAGRad23L392A after promoter shutoff. Samples were collected at the indicated time points after shutting off the GAL1 promoter and analyzed in anti-FLAG Western blot analysis.

(B) Densitometric quantification of Western blot shown in (A). FLAGRad23 (closed circles), FLAGRad23L392A (open circles), and FLAGRad23L392A (squares). The signal at t = 0 for each of the constructs was standardized as 100%.

(C) Steady-state levels of FLAG-tagged Rad23 in the wild-type, rad23D, FLAGRad23, and FLAGRad23L392A yeast strains determined in an anti-FLAG Western blot on 0.25 OD600 units. FLAGRad23 and FLAGRad23L392A are expressed from the endogenous Rad23 promoter.

(D) Turnover of FLAGRad23 and FLAGRad23L392A was determined by metabolic labeling with 35S-methionine/cysteine followed by chasing within the presence of an excess cold methionine and cysteine for the indicated times. FLAGRad23 and FLAGRad23L392A were immunoprecipitated with anti-FLAG antibodies. Asterisks indicate a nonspecific band.

Figure 3C shows that UbL domain is important for targeting Rad23 to the proteasome (Schauber et al., 1998) and, in the absence of the protective UBA2 domain, for induction of Rad23 proteolysis.

Three yeast strains were generated to gain insight into the role and functional significance of the UBA2 domain under physiological conditions: (1) a rad23D strain, which lacks the Rad23 gene, (2) a FLAGRad23 strain, and (3) a FLAGRad23L392A strain, which express FLAG-tagged wild-type Rad23 and FLAG-tagged mutant Rad23L392A, respectively, from the endogenous Rad23 promoter (Table 1). The FLAGRad23 could easily be detected in Western blot analysis, whereas the level of the FLAGRad23L392A mutant was below the detection limit (Figure 3C). Pulse-chase analysis revealed that this striking difference in steady-state levels was due to accelerated degradation of the Rad23L392A mutant (Figure 3D). We conclude that the UBA2 domain is important for physiological expression levels of Rad23 from its endogenous promoter.

**Rad23 Lacking a Protective UBA2 Domain Is Functionally Compromised**

It has been reported that the UBA1 and UBA2 domains of Rad23 are dispensable for functional NER (Bertola et al., 2001b). Consistent with this earlier report, we
Figure 4. Rad23 Lacking a Protective UBA2 Domain Is Functionally Compromised

(A) UV sensitivity of the wild-type, rad23Δ, FLAGRad23, and FLAGRad23L392A yeast strains. Serial dilutions of yeast cultures were spotted on YPD agar and were left untreated or exposed to 300 J/m² UV light.

(B) UV sensitivity of the wild-type, rad23Δ, FLAGRad23, and FLAGRad23L392A yeast strains. Serial dilutions of yeast cultures were spotted on YPD agar and were left untreated or exposed to one, two, or three exposures of 100 J/m² UV light with 20 min intervals between each exposure.

(C) Quantification of UV sensitivity by colony survival assay. Yeast was exposed to 3 × 100 J/m² or left untreated. The numbers were standardized to untreated controls. Mean and standards deviation of triplicate measurements are shown. Asterisks indicate that values are significantly different from wild-type strain (t test, p < 0.02).

(D) Serial dilutions of the indicated yeast cultures were spotted on YPD agar and were grown at 30°C or 25°C.

found that yeast cells expressing the destabilized FLAGRad23L392A were only slightly affected in their ability to survive a 300 J/m² UV light exposure, whereas rad23Δ cells were highly sensitive to this UV dose (Figure 4A). This suggests that the reduced Rad23 levels in the rad23Δ strain, whereas the FLAGRad23 and FLAGRad23L392A strains coped equally well (Figure 4B).
UBA2 Is an Intrinsic Stabilization Signal

Rad23 as multiubiquitin chain receptor engaged in the targeting of substrates to the proteasome, a notion that was recently supported by detailed biochemical studies (Verma et al., 2004). We investigated the significance of the protective UBA2 domain in relation to this function of Rad23. Deletion of Rad23 and Rpn10 resulted indeed in slow growth, in particular at 25°C (Figure 4D). Whereas introduction of the FLAGRad23 restored the growth to wild-type levels, the destabilized FLAGRad23L392A did not rescue the growth phenotype of the rad23/rpn10 double mutant. Thus, the presence of a protective UBA2 domain is of critical significance for Rad23 function.

The C-Terminal UBA Domains of Dsk2, Ddi1, and Ede1 Act as Stabilization Signals

Dsk2 and Ddi1 share with Rad23 the presence of an N-terminal UbL domain and a C-terminal UBA domain (Figure 5A). In addition, some proteins such as Ede1, a protein engaged in membrane trafficking (Aguilar et al., 2003), carry a C-terminal UBA domain but lack an UbL domain (Figure 5A). In order to examine whether the UBA domains of these proteins share the protective capacity of Rad23’s UBA2 domain, they were fused to the C terminus of the Ub-R-GFP reporter. Flow cytometric analysis clearly demonstrated a significant increase in the steady-state fluorescence levels of the chimeric reporters (Figure 5B). Most striking was the effect of the UBA domain of Dsk2, which even exceeded the effect of the Rad23 UBA2 domain, while the Ddi1 and Ede1 had weaker effects. Analysis of the turnover of these reporter constructs confirmed that the increase in the steady-state levels was due to a delay in degradation (Figure 5C). We conclude that the stabilizing effect of the C-terminal UBA domain is not unique for Rad23 but is a more general phenomenon shared with at least three other UBA domains.

The Protective Effect of the UBA2 Domain Is Evolutionarily Conserved

To examine whether the protective effect of the UBA2 domain has been conserved during evolution, we next tested the activity of the UBA1 and UBA2 domains of the human homolog hHR23A. We found that the UBA2 domains of Rad23 and hHR23A but not their UBA1 counterparts are equally capable of stabilizing the Ub-R-GFP reporter in yeast (Figure 6A). In order to assess whether the protective effect also operates in mammalian cells, we used a previously described assay in which human cervix carcinoma HeLa cells are transiently transfected with these fusions and the percentage of fluorescent cells is determined in the absence or presence of a specific proteasome inhibitor (Dantuma et al., 2000a). Introduction of the UBA1 domain of Rad23 or hHR23A in Ub-R-GFP did not affect the stability of this reporter in HeLa cells, whereas the UBA2 domains inhibited degradation (Figures 6B and 6C). We conclude that the stabilizing effect of the UBA2 domain in Rad23 is conserved during evolution.

Discussion

We have shown that the UBA2 domain of Rad23 act as an intrinsic, cis-acting, transferable stabilization signal.
Thus, Rad23 harbors counteracting degradation and stabilization signals, which enable the protein to interact with the proteasome without being degraded. UBA2-dependent stabilization is required for Rad23 function in DNA repair and protein degradation.

Our data show that protein stabilization is a characteristic shared by at least four C-terminally positioned UBA domains present in Rad23, Dsk2, Ddi1, and Ede1. Rad23, Dsk2, and Ddi1 each contain an N-terminal proteasome-interacting UbL domain and a C-terminal UBA domain (Jentsch and Pyrowolakis, 2000), whereas Ede1 lacks the UbL domain. Whether Ede1, which is the yeast homolog of eps15 and involved in ubiquitin-dependent membrane trafficking (Aguilar et al., 2003), interacts with the proteasome awaits clarification. Rad23, Dsk2, Ddi1, and Ede1 are to our knowledge the first examples of cellular proteins that harbor a specific domain that enables proteins to resist degradation. It remains to be seen how widespread this phenomenon is, but there are several findings in the literature suggesting that other proteins may contain related and unrelated stabilization signals (Dantuma and Masucci, 2002). For instance, a truncated variant of the ataxin 1-interacting ubiquitin-like protein lacking the UBA domain was also found to be degraded by the ubiquitin/proteasome system (Riley et al., 2004).

How do C-terminal UBA domains protect from degradation? There are indications that UBA domains do not only bind ubiquitin but also UbL domains (Raasi and Pickart, 2003; Wang et al., 2003). Based on these findings, it has been proposed that intramolecular interactions between the UbL and UBA domains may give Rad23 a closed conformation that cannot bind to the proteasome (Madura, 2002; Wang et al., 2003). Others have proposed that UBA domains can inhibit elongation of polyubiquitin chains by capping conjugated ubiquitin (Chen et al., 2001; Ortolan et al., 2000). Although either UbL binding or inhibition of polyubiquitination provides possible explanations for the stabilizing effect of the C-terminal UBA domain, there are several observations that do not support these models. First, the ubiquitin binding activity of the UBA domains is not sufficient for the protective effect since the very similar UBA1 domains of Rad23 and hHR23A, that bind ubiquitin and block ubiquitination equally well as UBA2 domains (Bertolaet et al., 2001b; Chen et al., 2001; Rao and Sastry, 2002), lacked this protective activity. Second, the presence of the UBA domain did not have any striking effects on the handling of ubiquitin on the substrate: the N-terminal ubiquitin was efficiently cleaved (Schauber et al., 1998). We have obtained preliminary data suggesting that a C-terminal extension of the native protein in the context of the native protein (Bertolaet et al., 2001b). Fourth, the interaction between Rad23 and the protea-
some has been studied in much detail (Elsasser et al., 2002; Kim et al., 2004; Saeki et al., 2002b; Schauber et al., 1998), leaving little doubt about the fact that Rad23 does interact with the proteasome in vivo.

A possibility that cannot be excluded is masking of polyubiquitin chains by the UBA2 domain. Thus the UBA2 domain could bind to polyubiquitin chains conjugated to Rad23, thereby modifying the interaction between Rad23 to the proteasome in such a way that it does not promote degradation. It remains unclear whether Rad23 itself is a target for ubiquitination, but the fission yeast homolog Rph23 was shown to be polyubiquitinated (Elder et al., 2002). In disfavor of this model, it was recently shown that the UBA1 and UBA2 domains bind synthetic Lys48 tetraubiquitin chains equally well (Raasi and Pickart, 2003), but more detailed analyses on the in vivo preferences of UBA domains for different polyubiquitin chains is required to conclusively address this issue.

Our study shows that the structural integrity of the UBA2 domain is an essential constraint for the protective effect. An attractive hypothesis that could explain these results is that the C-terminal UBA2 domain hinders unfolding of proteasome-bound Rad23. Prakash and coworkers have recently shown that degradation signals require an unstructured initiation site for efficient unfolding and proteasomal degradation (Prakash et al., 2004). Unfolding is a rate-limiting and crucial step in the sequence of events that leads to degradation of ubiquitinated substrates (Thrower et al., 2000), and in vitro studies have shown that tightly folded C-terminal domains can delay or block proteasomal degradation (Navon and Goldberg, 2001). Disturbing the UBA2 fold may enable the proteasome to unfold and degrade Rad23. This would also explain the intriguing observation that simple C-terminal tagging can destabilize Rad23 (Schauber et al., 1998) because extending the UBA2 domain with unstructured epitope tags may provide the proteasome with a “handle” to unwind the UBA domain. Notably, it has been hypothesized that processing domains, i.e., domains that interrupt proteasomal degradation of a precursor protein at a specific point (Rape et al., 2001), may mediate protein-protein interactions that terminate degradation by forming stable structures (Rape and Jentsch, 2002). UBA domains can interact with each other (Bertolaet et al., 2001a), and a similar mechanism may therefore apply for stabilization by UBA domains.

We have shown that yeast expressing destabilized Rad23 can resist a high, single dose of UV exposure, despite extremely low Rad23 levels. This observation is consistent with earlier reports showing that deletion of the UBA2 domain does not affect UV sensitivity after a single UV exposure (Bertolaet et al., 2001b; Chen and Madura, 2002). Apparently cells contain excess amounts of Rad23 because extending the UBA2 domain with unstructured epitope tags may provide the proteasome with a “handle” to unwind the UBA domain. Notably, it has been hypothesized that processing domains, i.e., domains that interrupt proteasomal degradation of a precursor protein at a specific point (Rape et al., 2001), may mediate protein-protein interactions that terminate degradation by forming stable structures (Rape and Jentsch, 2002). UBA domains can interact with each other (Bertolaet et al., 2001a), and a similar mechanism may therefore apply for stabilization by UBA domains.

Turnover Analysis
For GAL1 promoter shutoff experiments, transcription and translation were arrested by adding glucose and cycloheximide (Sigma) to final concentrations of 2% and 0.5 mg/ml, respectively. For endogenously expressed proteins, only cycloheximide was added to the growth media (final concentration 100 μg/ml). Aliquots were taken at the indicated time points, and total protein extracts were prepared by NaOH lysis and TCA precipitation. Samples were heated

UV Survival Assays

For drop assays, yeast cells were resuspended in sterile water and Mol. Cell. Biol.


onine and 1 mM cold cysteine for the indicated time. Yeast was Roberts, J.M. (2004). N-acetylation and ubiquitin-independent pro-

spread out over YPD plates and the number of colonies was deter-

mined after growth at 30°C for 2 days in the dark.

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