Rad25 Protein Is Targeted for Degradation by the Ubc4-Ufd4 Pathway

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Proteasome-mediated proteolysis provides dynamic spatial and temporal modulation of protein concentration in response to various intrinsic and extrinsic challenges. To gain a better understanding of the role of the proteasome in DNA repair, we systematically monitored the stability of 26 proteins involved in nucleotide excision repair (NER) under normal growth conditions. Among six NER factors found to be regulated by the proteasome, we further delineated the specific pathway involved in the degradation of Rad25, a subunit of TFIIH. We demonstrate that Rad25 turnover requires the ubiquitin-conjugating enzyme Ubc4 and the ubiquitin ligase Ufd4. Interestingly, the deletion of UFD4 specifically suppresses the rad25 mutant defective in transcription. Our results reveal a novel function of the Ufd4 pathway and another tie between the proteasome and NER regulators.

Background: The proteolytic regulation of DNA repair factors remains poorly understood.

Results: Six proteins involved in nucleotide excision repair are found to be degraded by the proteasome.

Conclusion: Rad25 is selected for proteasomal degradation by the Ubc4-Ufd4 pathway.

Significance: Our results provide novel insights into the role of the proteasome in DNA repair.

Ubiquitin often serves as a molecular flag that marks proteins for proteasome-dependent destruction (1, 2). Ubiquitin-mediated proteolysis facilitates rapid adjustment of protein concentration, which is key to cell growth and development. Proteasomal targets are selected by a ubiquitin-protein ligase, E3, which works with several enzymes, including a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2) to covalently append ubiquitin molecules onto substrates (1, 2). Ubiquitin binding proteins (e.g. Rad23, Cdc48, and Rpn10) facilitate the docking of ubiquitylated substrates onto the proteasome for subsequent destruction (1, 3).

The ubiquitin-proteasome system (UPS)3 regulates a broad range of biological processes, including cell cycle progression, immune response, signal transduction, gene expression, and DNA synthesis and repair (2). Despite the fundamental importance of UPS in cell biology, our understanding of UPS function remains far from clear. One critical role of the UPS is to facilitate cellular responses to a myriad of internal or environmental challenges, including DNA damage (4, 5). A major challenge in UPS biology is to establish the network that links ubiquitin ligases to their cognate targets. Here we start to evaluate the involvement of ubiquitin-mediated proteolysis in nucleotide excision repair (NER), a cellular defense mechanism against a wide spectrum of DNA lesions that cause DNA structural distortion, such as cyclobutane pyrimidine dimers (6–4) and photoproducts as well as other bulky DNA adducts (6, 7).

Because these helix-distorting DNA lesions can be inflicted by many challenges imposed on cells, including exposure to sunlight that contains UV rays, NER is a pivotal cellular pathway for genome maintenance. Impaired NER activity can lead to diseases such as xeroderma pigmentosum (XP), Cockayne syndrome, and trichothiodystrophy (6, 7).

There are over 30 proteins involved in various NER steps, including DNA lesion detection, damage verification, lesion incision, and gap filling (6, 7). NER comprises two subpathways, global genome NER and transcription-coupled NER (TC-NER). TC-NER deals with lesions located in the transcribed strand. In TC-NER, upon encountering DNA damage, RNA polymerase II stalls and recruits proteins, including CSA and CSB to the damage site. In global genome NER, XPC works with the DDB1-DDB2 complex to detect DNA lesions. Global genome NER and TC-NER converge after lesion detection. A multisubunit TFIIH complex is recruited to unwind DNA around the lesion, which further attracts other repair proteins to carry out repair reaction (8). Emerging evidence suggests an intimate link between the UPS and NER (4, 5). Several NER factors (e.g. CSB, Rpb1, DDB2, and XPC) are degraded by the UPS, and some NER regulators also directly participate in substrate turnover as UPS components such as ubiquitin ligases (e.g. CSA, Ssl1, and Rad7) or ubiquitin-binding proteins (e.g. Rad23) (4, 5). However, the precise relationship between the UPS and NER is far from clear. As a first step to comprehensively assess the role of proteasome-dependent degradation in NER, we systematically examine the stability of 26 NER factors under normal growth conditions. Six proteins are found to be degraded by the proteasome. We further define the proteolytic...
pathway for Rad25 (also called Ssl2), which involves Ubc4 E2 and ubiquitin ligase Ufd4 (E3).

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids—**Strains MHY501 (MATa ura3-52 lys2-801 his2-Δ200 leu2-2 trp1-1), MHY498 (UBC4::HIS3 in MHY501), and a collection of yeast strains lacking non-essential E2s and E3s in the BY4741 background were obtained from Dr. Mark Hochstrasser (Yale University) (9). The **UFD4** gene was replaced by **KanMX4** using PCR-mediated homologous recombination to construct strain YHR260 (**UFD4::KanMX4**) with the parental strain J546 (MATa ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11, 15 can1-100 met2-Δ lys2-Δ RAD25::MET2/PRS315-rad25-ts24) (10). The replacement was confirmed by PCR and sequencing. To create the strains YHR269 (RAD25::MET2/pRS315-rad25-XP), YHR270 (RAD25::MET2/pRS315-rad25-ts24), YHR264 (RAD25::MET2/pRS315-rad25-XP **UFD4::KanMX4**), and YHR265 (RAD25::MET2/pRS315-rad25-ts24 **UFD4::KanMX4**), the yeast strains J546 (RAD25::MET2/Ycp50-RAD25) and YHR260 (RAD25::MET2/Ycp50-RAD25 **UFD4::KanMX4**) bearing the plasmid pRS315-rad25-XP or pRS315-rad25-ts24 were grown on 5-FOA plates to cure the URA3-bearing plasmid Ycp50-RAD25 (10). Haploid yeast strains expressing endogenously tagged Rad16-TAP, Rad24-TAP, Rad25-TAP, Rad30-TAP, Rad33-TAP, or Ufd4-GFP in the BY4741 background were obtained from Open Biosystems (Huntsville, AL).

The plasmids pRS425-TEF-FLAG-RAD25, pRS315-RAD25, pRS315-rad25-ts24 and pRS315-rad25-XP have been described previously (10). FLAG-tagged RAD25-XP or RAD25-ts24 alleles were constructed by site-directed mutagenesis using pRS425-TEF-FLAG-RAD25 as the template. The plasmids expressing wild-type or C86A mutant **UBC4** were obtained from Dr. Michael J. Ellison. The plasmids expressing the MORF-tagged genes RAD1, RAD2, RAD3, RAD7, RAD9, RAD10, RAD14, RAD28, RAD34, SSL1, TFB1, TFB2, TFB3, TFB4, RBP4, RBP9, ABF1, DEF1, SPT4, SPT5, and TOP1 were obtained from Open Biosystems. The plasmid pRS426-Rad25TAP was constructed by amplifying Rad25-TAP along with its endogenous promoter by PCR and then cloning it into the vector plasmid pRS426.

Yeast cells were grown in rich (YPD) or synthetic media containing standard ingredients and 2% galactose (the URA3-bearing plasmid **UFD4::KanMX4**), 3% raffinose (SR medium), 2% galactose (SG medium), or 2% raffinose + 2% galactose (SGM medium). 4-Nitroquinoline N-oxide (4-NQO) and Adriamycin were obtained from Sigma.

**Expression Shutoff Assay**—As described previously, (11), yeast cells expressing TAP- or MORF-tagged proteins were grown at 30 °C to an A_{600} of ~1 in YPD or synthetic medium, respectively. Although TAP-tagged proteins are regulated by their endogenous promoters, the expression of MORF-tagged proteins is controlled by a GAL1 promoter and induced by galactose for 3 h (11). Protein translation was shut off by the addition of cycloheximide (100 μg/ml). Samples were withdrawn at the indicated time points and harvested by centrifugation. Proteins were extracted by glass bead lysis of cells, processed for immunoblotting with anti-TAP (Open Biosystems) or anti-His6 for MORF-tagged proteins, followed by detection with goat anti-mouse HRP conjugate using ECL reagents (GE Healthcare). The stable protein Rpt5 was used as a loading control in the expression shutoff experiments.

**Immunoprecipitation Assay**—Yeast wild-type cells with or without **UFD4-GFP** carrying either vector pRS425 or pRS425-TEF-FLAG-RAD25 were grown in synthetic medium (SD-leu) to A_{600}~1.0 at 30 °C and treated with 75 μM MG132 for 1 h. Samples were harvested by centrifugation, and proteins were extracted by glass beads with lysis buffer (50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, and 1% TritonX-100 with protease inhibitors). Protein lysates were immunoprecipitated with protein G beads coated with FLAG or GFP antibody and resolved by 6% SDS-PAGE. Immunoblots were probed with anti-GFP (Covance, 1:1000) and anti-FLAG (Sigma, 1:5000) as indicated. The expression of **Ufd4-GFP** in the same samples was determined similarly.

**RESULTS**

To elucidate the involvement of the UPS in NER, we evaluated the protein stability of 26 known yeast NER factors (Fig. 1) that are appended with MORF or TAP tags (see “Experimental Procedures” for more detail) and available from Open Biosystems. Under normal growth conditions, we shut off protein expression by cycloheximide and monitored the change of protein level in wild-type yeast cells. Six proteins were found to be degraded in the absence of exogenous DNA damage (Fig. 1). Among them, only the DNA polymerase η Rad30 has been shown previously to be degraded (12). Rad30 is ubiquitylated by SCF-Tfi11E3 and degraded by the proteasome under normal conditions but stabilized upon DNA damage (12).

Both Rad25 and Tfb4 belong to a 10-subunit transcription initiator TFIH complex that is essential for transcription and NER (6, 8). Rad25/Ssl2 contains ATPase activity and can unwind DNA around the lesion in a NER reaction. Rad33 and Rad34 are two interacting proteins identified recently as important NER regulators, although their precise roles in DNA repair remain unclear (13). Rad28 participates in transcription-coupled NER, and its human counterpart CSA can target CSB (a homologue of yeast Rad26) for ubiquitylation and degradation upon DNA damage (4, 6).

We then assessed whether these proteins are destroyed by the proteasome (14). We found that the degradation of all six proteins was compromised upon treatment with the proteasome inhibitor MG132, suggesting that the proteasome is involved in their turnover (Fig. 2).

Because the physiological function of Rad25 is better characterized among these proteasomal substrates, we decided to delineate the mechanism involved in Rad25 turnover. Rad25 was tagged with TAP and expressed from its endogenous promoter. We wondered whether Rad25 turnover is regulated by DNA damage. We treated cells with 4NQO, which inflicts DNA damage. We found that Rad30 has been shown previously to be degraded (12). Rad30 is ubiquitylated by SCF-Tfi11E3 and degraded by the proteasome under normal conditions but stabilized upon DNA damage (12).

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Rad25 turnover was not significantly altered by Adriamycin (Fig. 3B). The substrate selection of proteasome-mediated proteolysis lies in ubiquitin ligases (1, 2). In yeast, more than 11 E2s and more than 60 E3s are involved in ubiquitin conjugation (1, 9). To uncover the specific pathway involved in Rad25 turnover, we first looked for the E2 enzyme critical for Rad25 degradation. We examined the stability of Rad25 in eight non-essential E2 mutants (Fig. 4A). We found that Rad25 turnover was compromised in yeast cells lacking UBC4 (Fig. 4, A and B). It has been shown previously that Rad25 overexpression rendered yeast cells more resistant to Adriamycin (18) (Fig. 4C). Interestingly, yeast cells lacking UBC4 were more tolerant of Adriamycin than wild-type cells (Fig. 4C), consistent with Rad25 accumulation in ubc4Δ (Fig. 4A). Adriamycin sensitivity could be restored in ubc4Δ cells by expression of wild-type UBC4 but not mutant UBC4 lacking its catalytic activity (Fig. 4C), suggesting that the E2 function of Ubc4 is required for Rad25 turnover.

FIGURE 1. Six NER factors are degraded under normal growth conditions. Wild-type yeast cells containing TAP- or MORF-tagged NER factors (see “Experimental Procedures”) were grown to an A600 of ~1. Samples were taken after expression shutoff by cycloheximide (CHX) at the indicated time points and analyzed by Western blotting using anti-TAP for TAP-tagged proteins or anti-His6 for MORF-tagged proteins. Equal amounts of samples were used and ascertained by blotting with Rpt5 antibody in all expression shutoff experiments (bottom panels). The identities of NER factors are indicated at the top.

FIGURE 2. The involvement of the proteasome in the degradation of NER factors. Wild-type yeast cells expressing NER factors were treated with or without the proteasome inhibitor MG132. The experiments were done as described above, except MG132 (75 μM) or dimethyl sulfoxide (DMSO) control was added 30 min prior to the addition of cycloheximide (CHX) for protein expression shutoff.
Given the apparent involvement of UBC4 in Rad25 turnover, we reasoned that an Ubc4-associated E3 ligase likely would regulate Rad25. Therefore, we evaluated the degradation of Rad25 in yeast mutants lacking one of 15 E3s known to work with Ubc4 (Fig. 5A). We found that Ufd4, a HECT (homologous to E6-AP terminus) domain containing E3, was required for efficient Rad25 turnover (Fig. 5, A and B).

Because an E3 can directly bind its substrate, we assessed whether Ufd4 interacts with Rad25 by coimmunoprecipitation experiment. We introduced the plasmid bearing FLAG-tagged Rad25 into the Ufd4-GFP strain, in which the chromosomal copy of Ufd4 is tagged by GFP. We found that Rad25 associated with Ufd4 (Fig. 5C), supporting the link between Rad25 and Ufd4.

Rad25 is an ATP-dependent DNA helicase composed of 884 amino acids. Distinct Rad25 mutants have been isolated previously with different defects (8, 10). Although some of them are sensitive to UV light because of impaired NER defects, some rad25 mutants are temperature-sensitive, likely because of transcription defects (8, 10). If Rad25 turnover is compromised in ufd4Δ cells, we wondered whether the UFD4 deletion could suppress the phenotypes of RAD25 mutants (Fig. 6A). One mutant employed was the rad25-XP allele, which contains the C-terminal deletion (missing amino acids 750–843), similar to one XPB patient (10). The rad25-XP mutant abolishes its NER activity and impairs transcription. The rad25-ts24 allele was sensitive to high temperature because the double mutations

**FIGURE 3.** Rad25 turnover is not altered significantly by DNA damage or Adriamycin. Immunoblot analysis of endogenously expressed, C-terminally TAP-tagged Rad25 in the absence or presence of 4NQO (15 μg/ml) or Adriamycin (30 μM) as indicated. The chemicals were added to YPD cultures 1 h before the addition of cycloheximide (CHX).

**FIGURE 4.** Ubc4 E2 is involved in Rad25 regulation. A, Rad25 turnover is compromised in cells lacking UBC4. Rad25 degradation in wild-type and eight mutant cells was determined as above. CHX, cycloheximide. B, quantitation of the data in A. C, ubc4Δ mutant cells are resistant to the chemotherapeutic drug Adriamycin. Isogenic wild-type or ubc4Δ strains bearing a vector, a multicopy plasmid expressing RAD25, or a plasmid expressing wild-type or mutant UBC4, as indicated, were grown to similar densities, and 5-fold serial dilutions were spotted onto SD plates with or without Adriamycin (30 μM).
V552I and E556K contained have been found to mainly disrupt transcription (10).

Consistent with previous studies, the rad25-ts24 allele was extremely sensitive to UV light (Fig. 6A). The deletion of UFD4 did not significantly alter the UV sensitivity associated with rad25-ts24 (Fig. 6A). Interestingly, the deletion of UFD4 restored the growth of rad25-ts24 cells at a higher temperature (Fig. 6A), providing a genetic link between rad25-ts24 and UFD4. Further

![Fig 5](image-url)  
**FIGURE 5. Ufd4 E3 is important for Rad25 turnover.** A, Rad25 turnover in Ubc4-associated E3 mutants. The plasmid bearing Rad25-TAP under its own promoter regulation was introduced into 15 E3 mutants. The ubc4Δ mutant was included as a control. CHX, cycloheximide. B, Rad25 turnover is impaired in ufd4Δ cells. Rad25 stability was determined in wild-type and ufd4Δ cells more than three times. Quantitation of the data with S.D. is shown on the left. C, Ufd4 interacts with Rad25. The plasmid bearing FLAG-Rad25 or a control vector was transformed into yeast cells expressing Ufd4-tagged with GFP at its chromosomal locus. Proteins were extracted from the cells indicated and incubated with the IgG beads coated with either FLAG or GFP antibody. Samples were resolved by SDS-6%PAGE and visualized by Western blotting using FLAG or GFP antibody as indicated. The identity of the bands is shown on the left. The antibodies for IP and Western blotting are indicated on the right. IP, immunoprecipitation.

![Fig 6](image-url)  
**FIGURE 6. The deletion of UFD4 suppresses the temperature sensitivity of rad25-ts24 but not the UV sensitivity of the rad25-XP allele.** A, Mid-log phase yeast cultures were spotted onto the plates in serial 4-fold dilution and split into three sets for 2 days of incubation. One set was placed at normal temperature (30 °C), the second set was kept at 37 °C, and the third set was exposed to 5 J/m² UV irradiation as indicated. The genotypes of the strains are shown on the left. B, degradation of Rad25 alleles in wild-type and ufd4Δ cells. The plasmids bearing FLAG-tagged Rad25 wild-type or mutant alleles (i.e. Rad25-ts24 and Rad25-XP) were transformed into wild-type and ufd4Δ cells. FLAG-Rad25 turnover was determined as described in Fig. 3. C, quantitation of the data in B.
thermore, we found that both the rad25-ts24 and rad25-XP alleles are regulated by Ufd4 (Fig. 6, B and C). The results suggest that the rad25-ts24 mutant likely retains partial activity because increased rad25-ts24 levels in ufd4Δ cells (Fig. 6B) restored temperature sensitivity (Fig. 6A). In contrast, the depletion of C-terminal Rad25/XPB abolishes its NER function (6, 8, 10), and increased rad25-XP levels in ufd4Δ cells (Fig. 6B) did not restore rad25-XP-conferring sensitivity to UV light (Fig. 6A).

**DISCUSSION**

DNA damage triggers a series of cellular responses to promptly repair DNA injury and maintain genome stability. A number of proteomic studies have sought to uncover posttranslational modifications (e.g. phosphorylation, sumoylation, and ubiquitylation) that are key to the activity of DNA repair factors (4, 5, 7, 19–21). Ubiquitin is emerging as a key player in NER because a number of NER factors (e.g. Rad7-Rad16, Ssl1, Rad4, Rad23, CSA, CSB, and DDB1) have been found to contain ubiquitin ligase activity and/or ubiquitin-binding ability (4, 5, 22, 23). Ubiquitin often serves as a molecular marker for proteolysis. Here we surveyed the stability of 26 NER factors and identified six of them that are subjected to proteasome-mediated destruction under normal conditions (Figs. 1 and 2), indicating that proteolysis provides one means to modulate the activity of some NER proteins. We further delineated the specific pathway responsible for Rad25 turnover.

Rad25 is an ATPase subunit of the general transcription factor TFIIH complex that is essential for in vivo transcription (8). In RNA polymerase II-mediated gene expression, Rad25 works as a helicase to open up a transcription bubble on promoter DNA that is essential for transcription initiation (6, 8). In NER, Rad25 facilitates the loading of TFIIH to the XPC complex docked onto a DNA lesion and is crucial for DNA duplex unwinding around the damage site, and, in turn, promotes the recruitment of other NER factors for subsequent damage removal and DNA ligation (6). How two distinct functions of Rad25 in transcription and NER are coordinated remains unclear. XPB is the human counterpart of Rad25 and shares conserved function with yeast Rad25. Importantly, mutations in XBP can lead to hereditary disorders, including xeroderma pigmentosum, trichothiodystrophy, and Cockayne syndrome (6, 8). It will be interesting to assess whether XBP is regulated similarly by the ubiquitin-proteasome pathway. If XBP is degraded normally, it will be important to evaluate whether pathogenic mutations interfere with XBP turnover. The suppression of some Rad25 phenotypes by Lfid4 deletion (Fig. 6) suggest that modulation of XBP levels may be a therapeutic strategy effective in some XBP patients.

The involvement of UPS in Rad25 regulation adds to a growing link between NER and the proteasome (4, 5). The proteasome regulates multiple NER proteins at distinct steps differently. Although some repair factors are kept at low levels under normal conditions, some NER regulators (e.g. Rpb1 and CSB) are destroyed upon UV irradiation (4, 5). For example, yeast Rad30 (DNA polymerase η) is degraded in the absence of exogenous damage (12), which likely prevents an elevated mutation rate and genome instability caused by Rad30 accumulation.

Following UV irradiation, Rad30 is spared from proteasome-mediated degradation to meet the demand of NER (12). Another well-documented case is the regulation of the p53 tumor suppressor, also known as the guardian of the genome (4, 5). p53 is normally degraded rapidly because dysregulated p53 expression is detrimental to cell growth and survival. Upon UV damage, p53 is stabilized and activates the DNA damage response program that is essential for genome maintenance. On the other hand, Rpb1, the largest subunit of RNA polymerase II, is normally a stable protein critical for transcription but degraded upon UV irradiation (15, 16). In TC-NER, RNA polymerase II stalls at lesion sites, and the degradation of Rpb1 likely dislocates RNA polymerase II from the damage and enables subsequent repair by other NER factors. Proteasome-mediated proteolysis clearly is key to the intricate dance of NER factors in response to DNA damage.

The identification of Ufd4-mediated Rad25 turnover also encourages us to further expand our strategy. The current screening is mainly limited to proteins that participate directly in the NER reaction. There are a number of proteins (e.g. Mccl and Tel1 kinase) that are involved in transmitting damage signals to activate the repair pathway and/or facilitate the spatial and temporal control of NER. The synthesis and turnover of these signaling molecules may be regulated tightly in response to DNA injury and will be included in future analyses. Moreover, to better understand the role of proteolysis in DNA repair, we will systematically monitor the stability of NER factors during and after DNA damage. Ultimately we hope to gain a comprehensive understanding of the precise role of ubiquitin-mediated proteolysis in DNA damage response and recovery, which have been tied to various human diseases.

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