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Rad4 Regulates Protein Turnover at a Postubiquitylation Step

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The ubiquitin (Ub)-binding protein Rad23 plays an important role in facilitating the transfer of substrates to the proteasome. However, the mechanism underlying Rad23's function in proteolysis remains unknown. Here, we demonstrate that Rad4, a Rad23-binding protein, also regulates ubiquitylated substrate turnover. Rad4 was known previously only as a key repair factor that directly recognizes DNA damage and initiates DNA repair. Our results, however, reveal a novel function of Rad4. We found that Rad4 and Rad23 share several common substrates. Substrates in *rad4*Δ cells are ubiquitylated, indicating that Rad4 regulates a postubiquitylation event. Moreover, we found that Rad4 participates in the Rad23–Ufd2 pathway, but not the Rad23-Png1 pathway, consistent with previous findings that Png1 and Rad4 or Ufd2 form separate Rad23 complexes. The Rad4-binding domain is crucial for the functioning of Rad23 in degradation, suggesting that Rad4 and Rad23 work together in proteolysis. It is interesting to note that upon DNA damage, Rad4 becomes concentrated in the nucleus and degradation of the nonnuclear protein Pex29 is compromised, further suggesting that Rad4 may influence the coordination of various cellular processes. Our findings will help to unravel the detailed mechanisms underlying the roles of Rad23 and Rad4 in proteolysis and also the interplay between DNA repair and proteolysis.

INTRODUCTION

The ubiquitin (Ub)-proteasome system consists of two phases (Demartino and Gillette, 2007; Hanna and Finley, 2007; Ravid and Hochstrasser, 2008). In the Ub conjugative phase, Ub is activated and transferred to the substrate through several enzymes, including a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub-protein ligase (E3). For substrates targeted for degradation, successive Ub molecules are added to form a Ub chain on the substrates by E3 or E4 enzymes that are specifically involved in Ub chain elongation. In the second phase, the ubiquitylated substrate is delivered to and degraded by the 26S proteasome. Earlier studies in the Ub/proteasome field focused mostly on the proteasome, and the mechanisms of substrate recognition and Ub conjugation by E2s and E3s. The principles underlying substrate delivery to the proteasome remain elusive (Madura, 2004; Elsasser and Finley, 2005; Kim and Rao, 2006).

Recent evidence indicated that Ub-binding proteins (e.g., Rad23, Rpn10, and Cdc48) are key regulators of the substrate transfer process (Madura, 2004; Elsasser and Finley, 2005; Kim and Rao, 2006). How these Ub receptors work in vivo remains to be elucidated. Rad23 belongs to a family of proteins that contains both a Ub-associated (UBA) domain and a Ub-like (UBL) motif (Madura, 2004; Kim and Rao, 2006). It is important to note that the UBA motif binds to Ub chains in vivo and in vitro (Bertolaet *et al*., 2001; Wilkinson *et al*., 2001; Chen and Madura, 2002; Rao and Sastry, 2002; Kang *et al*., 2006). The UBL motif at the N terminus of these proteins shares ~20% identity with Ub and directly binds the proteasome (Schauber *et al*., 1998; Elsasser *et al*., 2002). Through the UBL- and UBA-mediated interactions, Rad23 serves as an adaptor in bridging the ubiquitylated proteins to the proteasome.

Rad23 is required for the efficient degradation of a subset of cellular proteins in vivo, including a UFD substrate (Lambertson *et al*., 1999; Rao and Sastry, 2002), Far1 (Verma *et al*., 2004), misfolded ricin A chain (Kim *et al*., 2006), and Hmg2 (Richly *et al*., 2005). In addition, Rad23 homologues are involved in the degradation of the CDK inhibitor Rum1 (Wilkinson *et al*., 2001), the myogenic transcription factor Pax3 (Boutet *et al*., 2007), and the tumor suppressor p53 (Glockzin *et al*., 2003). Stabilized substrates in *rad23* mutant cells are fully ubiquitylated, suggesting that Rad23 functions at a postubiquitylation step but at a preproteasome step (Lambertson *et al*., 1999; Rao and Sastry, 2002). These results indicate that Rad23 has a positive role in a subset of proteolytic pathways.

As indicated by its name, Rad23 was first known as a DNA repair protein because its mutant was sensitive to UV irradiation. In response to DNA damage, Rad23 is known to work with Rad4 (Dantuma *et al*., 2009). The Rad4–Rad23 complex is involved in nucleotide excision repair (NER), a major pathway for fixing a wide spectrum of DNA lesions that lead to large local distortions of the DNA structure, including UV-induced cyclobutane pyrimidine dimers and

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Abbreviations used: MMS, methyl methanesulfonate; NER, nucleotide excision repair; RTA, ricin A chain; His6, hexahistidine; TAP, tandem affinity purification; Ub, ubiquitin; UBA, Ub-associated domain; UBL, Ub-like element.

(6-4) photoproducts, cisplatin intrastrand cross-links, and other bulky DNA adducts (Hanawalt, 2003; Friedberg *et al*., 2006). Impaired NER activity leads to genomic instability and carcinogenesis as typified by the hereditary disease Xeroderma pigmentosum (XP) that exhibits extreme sunlight sensitivity and a strong propensity for skin cancer (Hanawalt, 2003; Friedberg *et al*., 2006).

Rad4 (termed XPC for its human homologue), an 87-kDa protein, plays an essential role in the detection of UV-induced lesions, a rate-limiting step in the DNA repair process (Sugasawa, 2008). Structural and functional analysis suggests that Rad4 can bind to DNA nonspecifically and probe for helical distortion along the DNA. On encountering a destabilized DNA conformation, Rad4 binds tightly to the DNA and in turn makes the lesion accessible for other DNA repair enzymes. Rad23, via binding to Rad4, was shown to have two accessory roles in NER: a stimulatory effect on damage recognition by Rad4, and protection of Rad4 from proteolysis (Lommel *et al*., 2002; Xie *et al*., 2004; Gillette *et al*., 2006). Subsequent to damage recognition, the Rad23–Rad4 complex recruits other proteins, including transcription factor II H (TFIIH), the single-stranded DNA binding protein RPA, and two nucleases XPG and XPF, to carry out repair synthesis and ligation, which in turn restore genome integrity (Friedberg *et al*., 2006; Sugasawa, 2008).

The cellular function of Rad4 previously seemed to be limited to DNA repair (Dantuma *et al*., 2009). Here, we demonstrate the involvement of Rad4 in Ub/proteasome-mediated protein turnover. Substrates which accumulate in *rad4*Δ mutant cells are ubiquitylated, suggesting that Rad4 regulates a post-ubiquitylation step. Rad4 binds the proteasome and Ub conjugates and shares common substrates with Rad23. The function of Rad23 in substrate degradation requires an intact XPCB domain, which is essential for Rad4 binding, further suggesting that Rad4 and Rad23 work together in proteolysis. Rad4 is not a regulator of global proteolysis because several proteasomal substrates maintain their normal degradation in *rad4*Δ cells. Furthermore, Rad4 is specifically involved in the Ufd2 branch of the Rad23 degradation pathway. It is interesting to note that upon

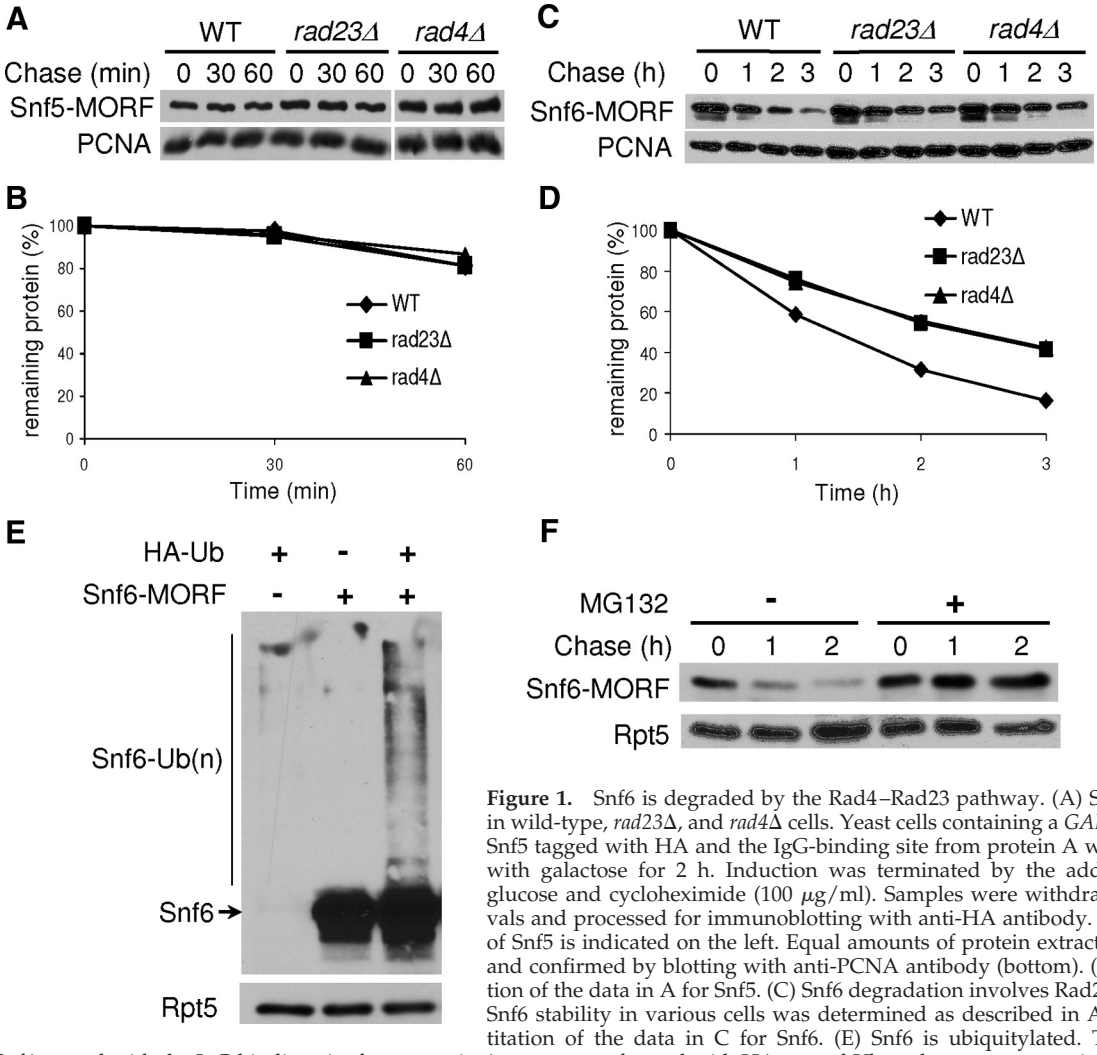

Figure 1. Snf6 is degraded by the Rad4–Rad23 pathway. (A) Snf5 is stable in wild-type, *rad23*Δ, and *rad4*Δ cells. Yeast cells containing a *GAL1*-regulated Snf5 tagged with HA and the IgG-binding site from protein A were induced with galactose for 2 h. Induction was terminated by the addition of 2% glucose and cycloheximide (100 μg/ml). Samples were withdrawn at intervals and processed for immunoblotting with anti-HA antibody. The identity of Snf5 is indicated on the left. Equal amounts of protein extracts were used and confirmed by blotting with anti-PCNA antibody (bottom). (B) Quantitation of the data in A for Snf5. (C) Snf6 degradation involves Rad23 and Rad4. Snf6 stability in various cells was determined as described in A. (D) Quantitation of the data in C for Snf6. (E) Snf6 is ubiquitylated. The plasmid expressing Snf6 tagged with the IgG-binding site from protein A was cotransformed with HA-tagged Ub or the empty vector into wild-type cells. Snf6 was precipitated with IgG beads and analyzed by immunoblotting with anti-HA antibody. Ubiquitylated and nonubiquitylated Snf6 proteins are indicated on the left. To ensure the equal loading, the amounts of the proteasome subunit Rpt5 in the extracts were determined by Western blotting in all of the subsequent expression shut-off experiments (bottom). (F) Snf6 is degraded by the proteasome. Wild-type yeast cells expressing Snf6 were treated with or without the proteasome inhibitor MG132 (75 μM). Snf6 degradation was monitored as described in C. Rpt5 levels served as the loading control (bottom).

exposure of cells to DNA damage, Rad4 became concentrated in the nucleus and the degradation of its nonnuclear substrate was impaired. Moreover, XPC also binds human Ufd2 and regulates UFD substrate degradation, suggesting the proteolytic regulation is a conserved feature of Rad4/XPC. Our results reveal a novel function of Rad4/XPC in proteolysis, and set the stage to further delineate the functions of Rad23 and Rad4 in proteolysis as well as the interplay between protein degradation and DNA repair.

MATERIALS AND METHODS

Yeast Strains and Plasmids

Cultures were grown in rich (YPD) or synthetic media containing standard ingredients and 2% glucose (SD medium), or 2% raffinose (SR medium), or 2% galactose (SG medium), or 2% raffinose + 2% galactose (SRG medium). Wild-type strain BY4741 (*MATa his3 leu2 met15 ura3*), isogenic mutant strains *rad4*Δ, *rad23*Δ, Rad23-TAP and Rad4-GFP strains, and the strains bearing Rad4 or Rad23 tagged with two epitopes V5 and hexahistidine (His6) at their chromosomal loci were obtained from Open Biosystems (Huntsville, AL). Yeast strain YHR242 (*RAD4-GFP*$^{\Delta}$NLS) was generated by replacing the nuclear localization signal (amino acids 28-46) with *URA3*-Myc cassette in *RAD4-GFP* strain, and then *URA3* was popped out by homologous recombination to leave the myc tag in place of deleted *RAD4* sequences. The deletions were confirmed by polymerase chain reaction (PCR) and DNA sequencing.

The plasmids pMORF-Snf5 and pMORF-Snf6 bearing the *GAL1* promoter-regulated Snf5 or Snf6 tagged at their C terminus with hemagglutinin (HA) and the immunoglobulin (Ig)G-binding site from protein A were obtained from Open Biosystems as well. The MORF tag does not significantly affect the function of Snf6 (Supplemental Figure 1). The plasmids expressing FLAG-tagged wild-type and mutant Rad23 from their endogenous promoters were generous gifts from Dr. Kiran Madura (Robert Wood Johnson Medical School, Piscataway, NJ).

Expression Shut-off Assay and Proteasome Inhibition Treatment in Yeast Cells

Yeast cells carrying plasmids expressing epitope-tagged proteins from the *GAL1* promoter were grown at 30°C to an OD_{600} of ~1 in SR-Ura medium with auxotrophic supplements and 2% raffinose as the carbon source. Protein expression was induced with 2% galactose for 2 h and then repressed by the addition of 2% glucose. Cycloheximide (100 μg/ml) was also added to stop translation. Samples were withdrawn at the indicated time points and harvested by centrifugation. Protein extracts were used for either Western blotting as indicated or immunoprecipitation with beads coated with various indicated antibodies, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblots were probed with various antibodies indicated followed by detection with goat anti-mouse horseradish peroxidase conjugate using enhanced chemiluminescence (ECL) reagents (GE Healthcare, Little

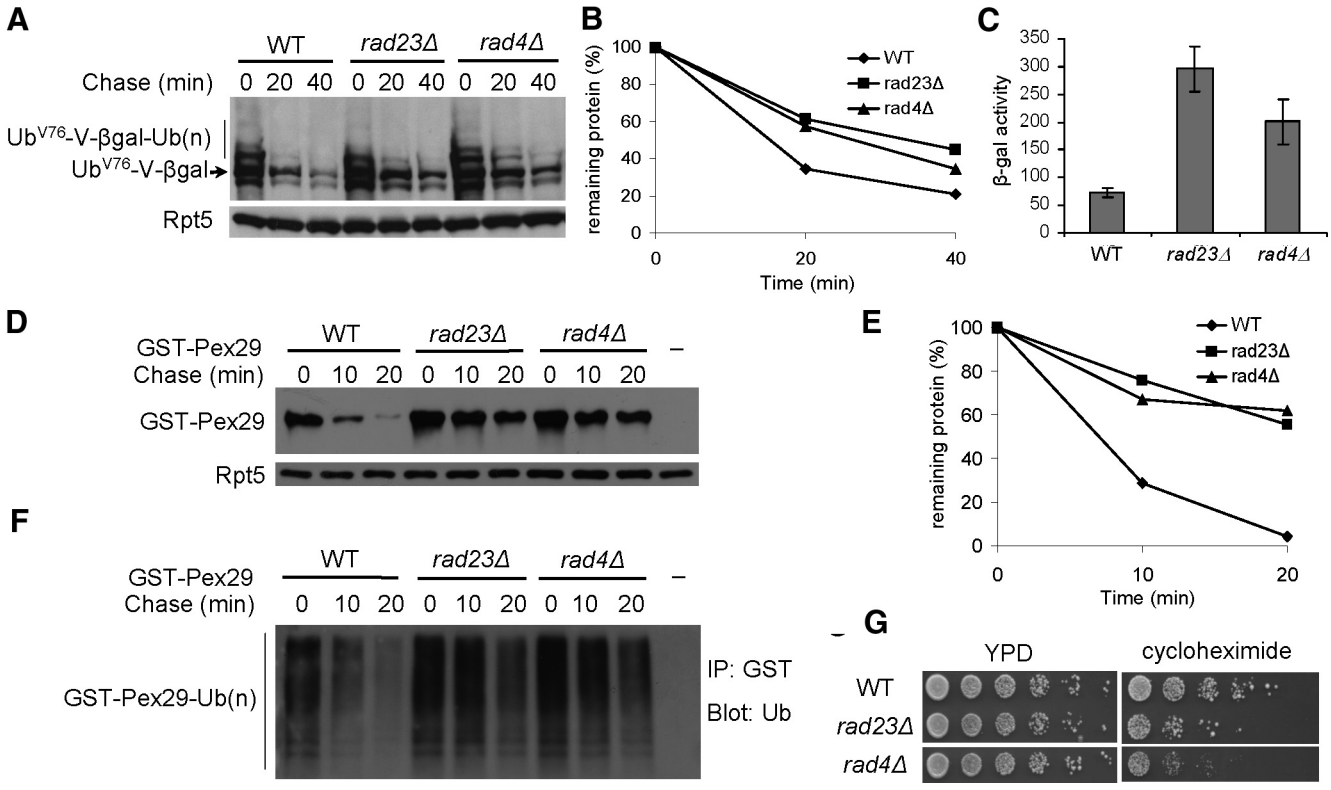

Figure 2. Rad4 and Rad23 share common substrates. (A) UFD substrate Ub^{V76}-V-βgal degradation is compromised in *rad23*Δ and *rad4*Δ cells. Wild-type (BY4741) and mutant cells containing a *GAL1* promoter-regulated Ub^{V76}-V-βgal were first grown in raffinose-containing medium. Expression of Ub^{V76}-V-βgal was induced by the addition of galactose for 1 h. Samples were taken after promoter shutoff at the time points indicated and analyzed by anti-βgal Western blot. An arrow indicates Ub^{V76}-V-βgal. A line indicates the "ladder" ubiquitylated Ub^{V76}-V-βgal. (B) Quantitation of the data in (A) for Ub^{V76}-V-βgal. (C) Levels of βgal activity in wild-type, *rad23*Δ, and *rad4*Δ cells carrying a plasmid bearing Ub^{V76}-V-βgal. Experiments were done three times, and the average values with SD are shown. (D) Efficient degradation of Pex29 requires Rad23 and Rad4. Wild-type and mutant cells containing a *GAL1* promoter-driven GST-His6-Pex29 were first grown in raffinose-containing medium. Expression of Pex29 was induced by the addition of galactose. Samples were taken after promoter shutoff at the time points indicated, immunoprecipitated with glutathione transferase (GST) beads, and later analyzed by anti-His6 Western blots. As a negative control, the extract containing no GST-Pex29 was processed similarly (last lane). The identities of proteins are indicated on the left. Equal amounts of protein extracts were used and confirmed by blotting with anti-Rpt5 antibody. (E) Quantitation of the data in D for Pex29. (F) Pex29 remains ubiquitylated in *rad23*Δ and *rad4*Δ cells. The immunoblot of Pex29 stability in D was probed with anti-Ub antibody. (G) Cells lacking Rad23 or Rad4 are sensitive to the translation inhibitor cycloheximide. Isogenic yeast strains were grown to an optical density A_{600} of ~1, and fivefold dilutions were spotted onto YPD plates with or without cycloheximide (0.5 μg/ml). The plates were incubated at 30°C for 2–4 d. The strain backgrounds are labeled on the left.

Chalfont, Buckinghamshire, United Kingdom). After the protein bands were detected by the ECL system and scanned, the densities of these bands were determined by ImageQuant 5.2 software (GE Healthcare) (Huang *et al*., 2006; Jutras *et al*., 2008). An example of using this quantification with known amounts of proteins is included as Supplemental Figure 2. The stable protein Rpt5 or proliferating cell nuclear antigen (PCNA) was used as a loading control in the expression shut-off experiments.

Proteasome inhibition was performed as described previously (Liu *et al*., 2007). In brief, yeast cells were grown in synthetic media using proline as the only nitrogen source. SDS (0.003%) was added to the media 3 h before galactose induction. MG132 (75 μM; BIOMOL Research Laboratories, Plymouth Meeting, PA) was added 30 min before the addition of glucose. Samples were collected at indicated time points and processed as described above.

Mammalian Cell Lines and Plasmids

The plasmids expressing HA-tagged Ufd2, FLAG-XPC, or the UFD substrate Ub^{G76V}-green fluorescent protein (GFP) are kind gifts from Drs. Keiichi I. Nakayama (Kyushu University, Fukuoka, Japan), Kaoru Sugasawa (Kobe University, Kobe, Japan), and Yong Tae Kwon, respectively. XPC wild-type cell line WI38 VA13 and XPC-deficient cell line XP4PASV are obtained from Dr. Kaoru Sugasawa.

Mammalian cells were cultured using DMEM supplemented with 10% fetal bovine serum. All media reagents were purchased from Cell-Gro Mediatech (Herndon, VA). Cells were kept in 37°C incubation with 5% CO_2.

RESULTS

Snf6, but Not Snf5, Requires Rad4 and Rad23 for Efficient Degradation

Rad4 and Rad23 were shown to interact with the chromatin remodeling complex containing Snf5 and Snf6 (Gong *et al*., 2006). Because Rad23 regulates proteolysis, Snf5 and Snf6 may be targeted by Rad23 for degradation. To test this possibility, we examined the stability of Snf5 or Snf6 in wild-type and *rad23* mutant cells. Although Snf5 was fairly stable (Figure 1, A and B), Snf6 was degraded in wild-type but stabilized in *rad23* mutant cells (Figure 1, C and D), suggesting that Snf6 is regulated by Rad23. It is interesting to note that Snf6 degradation was similarly impaired in a *rad4* mutant (Figure 1, C and D). Currently, it remains to be determined whether the Rad4/Rad23-mediated Snf6 degradation is physiologically significant for Snf6 function, which would require more careful analysis of the Snf6 degradation pathway. Nevertheless, this is the first evidence implicating a role of Rad4 in promoting substrate turnover.

Because Snf6 was not previously known to be degraded in a Ub/proteasome-dependent manner, we then examined whether Snf6 is ubiquitylated. We cotransformed the plasmid expressing Snf6 with the plasmid bearing HA-tagged Ub or the empty vector into wild-type cells. Snf6 was efficiently multi-ubiquitylated in wild-type cells (Figure 1E). We then evaluated whether Snf6 turnover is mediated by the proteasome. We found that Snf6 was markedly stabilized in the presence of the proteasome inhibitor MG132 (Figure 1F), suggesting that Snf6 is a substrate of the Ub/proteasome system.

Rad4 Regulates the Degradation of Ub^{V76}-V-βgal and Pex29

To confirm the role of Rad4 in Ub/proteasome-dependent proteolysis, we assessed the requirement of Rad4 for the degradation of a well-established Rad23 substrate, Ub^{V76}-V-βgal (Lambertson *et al*., 1999; Rao and Sastry, 2002). The

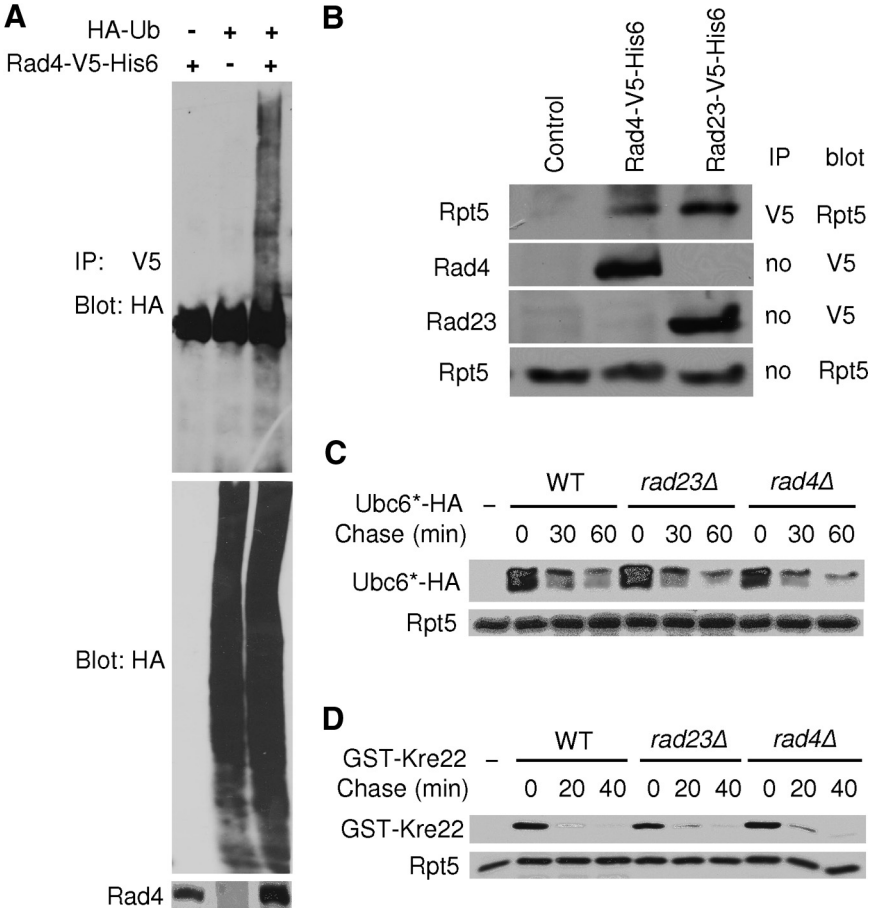

Figure 3. Specific roles of Rad4 in the Ub/proteasome system. (A) Rad4 interacts with Ub conjugates. The plasmid bearing HA-tagged Ub or the vector was transformed into a yeast strain expressing endogenous Rad4 fused to V5 and His6 tags. Rad4-binding proteins were enriched by immunoprecipitation with Dyna beads (Invitrogen, Carlsbad, CA) coated with V5 antibody and analyzed by immunoblotting with anti-HA antibody. Ubiquitylated bands associated with Rad4 are seen on the top panel. The bottom panels show the levels of Ub conjugates and Rad4 in these cells. (B) Rad4 binds the proteasome. The binding experiments were performed as described in A except the Rad4-immunoprecipitates were probed with the antibody against Rpt5, a proteasomal subunit, to determine the amount of proteasome. The identity of the bands is indicated on the left. The antibodies used for immunoprecipitation (IP) and Western blot (blot) are indicated to the right of the panels. (C) Degradation of Ubc6* is independent of Rad4 and Rad23. HA-tagged Ubc6* was expressed in wild-type and mutant cells. Expression shut-off experiments were performed as described previously (Ravid *et al*., 2006). (D) Kre22 degradation does not require Rad4 and Rad23. Expression shut-off analysis of a *GAL1* promoter-regulated Kre22 was performed similarly as described in Figure 2D.

genetic dissection of the proteolytic pathway that degrades the model substrate Ub^{V76}-V-βgal fusion led to the discovery of Ub fusion degradation (UFD) pathway (Johnson *et al.*, 1995). Using an expression shut-off assay, we found that Ub^{V76}-V-βgal degradation was compromised in cells lacking Rad23 or Rad4 (Figure 2, A and B). Consistent with this finding, our results derived from the LacZ assay also showed higher levels of β-gal activity in *rad4*Δ and *rad23*Δ than in wild-type cells (Figure 2C). It is interesting to note that Ub^{V76}-V-βgal ubiquitylation remained in *rad4*Δ cells (Figure 2A), suggesting that Rad4 functions after the substrate ubiquitylation step.

We recently identified another Rad23 substrate as the peroxisomal membrane protein Pex29 (Liu, unpublished

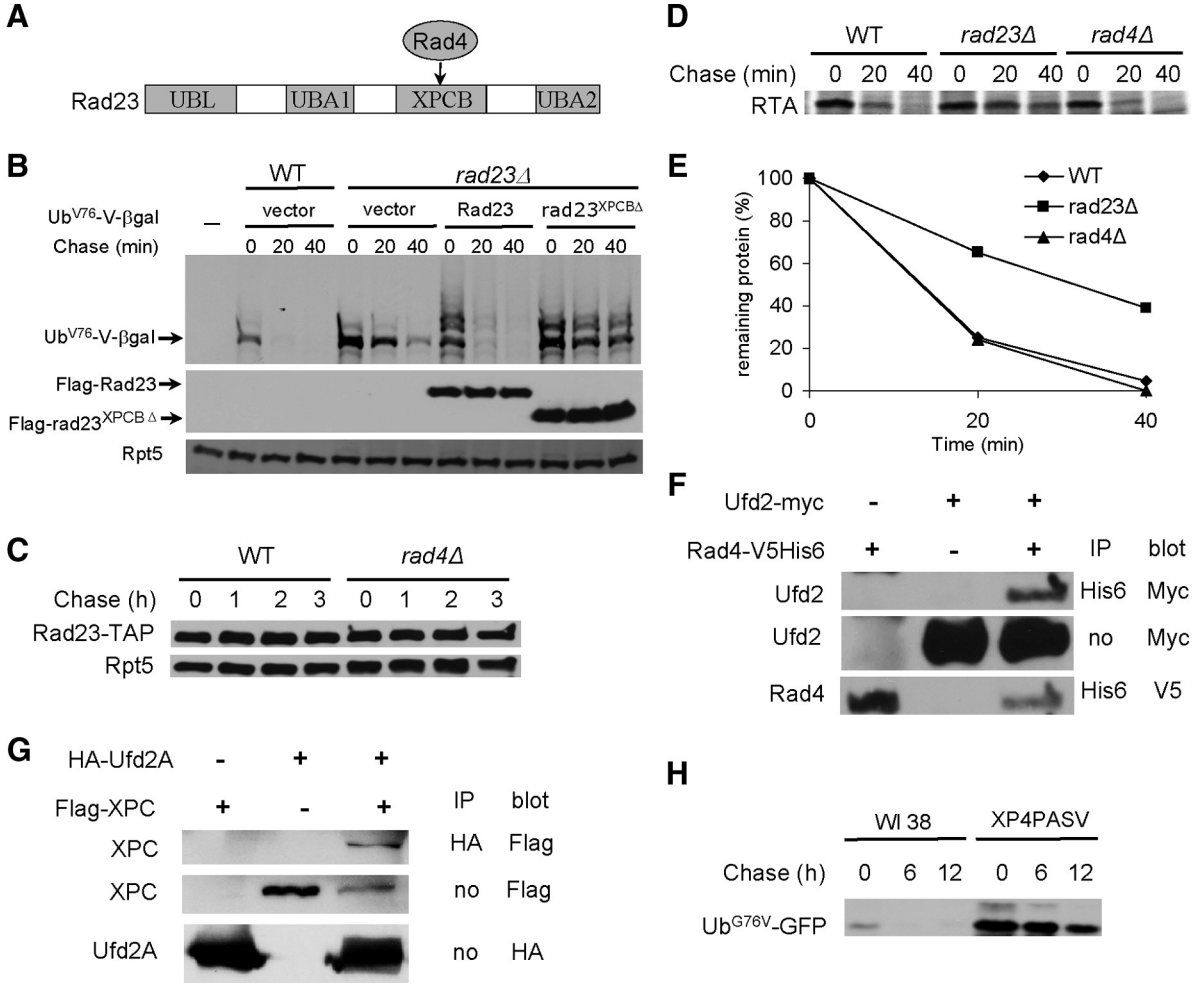

Figure 4. Rad4 is linked to the Ufd2 but not Png1 branch of the Rad23 pathway. (A) Domain organization of Rad23. Rad4 binds to the XPCB domain. (B) The intact XPCB domain is required for the degradation of the UFD substrate Ub^{V76}-V-βgal. The empty vector or the plasmid bearing indicated FLAG-tagged Rad23 derivatives were cotransformed with the plasmid expressing Ub^{V76}-V-βgal into wild-type or *rad23*Δ cells. Ub^{V76}-V-βgal degradation (top) was determined as described in Figure 2A. The amounts of Rad23 and the loading control Rpt5 are shown in the bottom panels. (C) Rad23 levels are not altered in *rad4*Δ cells. Wild-type or *rad4*Δ cells containing endogenous Rad23 appended with a TAP tag were grown to mid-log phase in YPD medium, and then cycloheximide was added to stop protein synthesis. The samples collected at various time points were analyzed by anti-TAP Western blot. (D) RTA degradation requires Rad23 but not Rad4. Pulse-chase analysis with yeast *rad23*Δ or *rad4*Δ cells expressing glycosylated RTA was done as described previously (Kim *et al.*, 2006). In brief, cells expressing FLAG-tagged RTA were pulse labeled with ^{35}S for 10 min, followed by a chase with cold methionine and cysteine. Samples taken at various time points were processed for immunoprecipitation with FLAG beads, followed by SDS-PAGE and autoradiography. (E) Quantitation of the data in D for glycosylated RTA. (F) Ufd2 interacts with Rad4. Coimmunoprecipitation analysis of the interaction between Rad4 and myc-Ufd2 was done as described previously (Kim *et al.*, 2004). In brief, proteins were extracted from cells expressing endogenous Rad4-V5His6 and galactose-inducible myc-tagged Ufd2 and immunoprecipitated with beads coupled to various antibodies as indicated. Immunoprecipitates were resolved by SDS-PAGE and probed with antibodies. The identity of the bands is indicated on the left. The antibodies for immunoprecipitation (IP), and blot are shown to the right of the panels. (G) XPC binds Ufd2A by coimmunoprecipitation. Human embryonic kidney 293 cells were transiently transfected with HA-Ufd2A and/or FLAG-XPC as indicated. After 48-h transfection, cell extracts were prepared with the lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, and protease inhibitors). Cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody, followed by the immunoblotting with anti-FLAG antibody. In the bottom panels, 10% input lysates were analyzed by immunoblotting to ascertain the expression of HA-Ufd2A and FLAG-XPC. (H) Ub^{V76}-GFP degradation is impaired in XPC deficient cells. XPC wild-type cell WI38 VA13 and XPC-deficient XP4PASV cells were transiently transfected with Ub^{V76}-GFP by using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were split into a six-well plate. After 48-h transfection, cells were treated with 100 μg/ml cycloheximide to start the chase. Indicated time points were taken after protein synthesis shut-off and analyzed by Western blotting with anti-GFP antibody.

data), which requires Doa10 and Ufd2 for ubiquitylation. We found Pex29 degradation was impaired to a similar extent in *rad4*Δ and *rad23*Δ cells (Figure 2, D and E). Stabilized Pex29 proteins were found to be ubiquitylated (Figure 2F). Together, our results demonstrate that Rad23 and Rad4 share common substrates and both regulate postubiquitylation events.

One of the major functions of the Ub system is to deal with various stresses (Demartino and Gillette, 2007; Hanna and Finley, 2007). Perturbations of the Ub/proteasome system affect cell survival in the presence of cycloheximide, which inhibits protein synthesis and promotes the formation of misfolded protein fragments. Loss of *RAD23* or *RAD4* rendered cells more sensitive to cycloheximide (Figure 2G).

Rad4 Binds the Proteasome and Ub Conjugates but Does Not Regulate Global Protein Turnover

If Rad4 is involved in escorting ubiquitylated proteins to the proteasome, it may thereby associate with the proteasome and ubiquitylated species in vivo. To this end, we carried out coimmunoprecipitation assays using a strain in which endogenous Rad4 is appended with V5 and hexahistidine (His6) epitopes. Like Rad23, we found that Rad4 also binds the proteasome and Ub conjugates (Figure 3, A and B).

Does Rad4 regulate specific substrate degradation or global proteasome-mediated proteolysis? We first evaluated the role of Rad4 in the degradation of a frequently used proteasomal substrate Ubc6*. Ubc6* is recognized as a misfolded protein by Doa10 E3 (Ravid *et al*., 2006; Ravid and Hochstrasser, 2008). We found that Ubc6* degradation was unaltered in cells lacking *RAD23* or *RAD4* (Figure 3C), indicating that Rad4 regulates a subset of substrate degradation.

All three yeast *Saccharomyces cerevisiae* UBL/UBA-containing proteins, Rad23, Dsk2, and Ddi1, promote substrate proteolysis (Elsasser and Finley, 2005; Dantuma *et al*., 2009). Rad23 and Dsk2 are often found to have overlapping functions because the degradation of several substrates was more severely compromised in *rad23*Δ *dsk2*Δ double mutants than in either single mutant (Madura, 2004; Elsasser and Finley, 2005; Richly *et al*., 2005). Recently, we identified Kre22 as a substrate that requires only Dsk2 but not Rad23 or Ddi1 for its degradation (Liu, unpublished data). We assessed whether Rad4 is involved in Kre22 turnover. It is interesting to note that deletion of *RAD4* did not affect Kre22 degradation (Figure 3D), suggesting that the proteolytic functions of Rad4 and Rad23 are tightly linked.

The XPCB Domain of Rad23 Is Critical for the Degradation of Its Substrates

To establish the significance of the Rad4–Rad23 complex in proteolysis, we investigated whether Rad23 mutants defective in Rad4 binding could maintain efficient proteolysis. Besides the UBL and UBA motifs described above, Rad23 contains a conserved XPCB domain for Rad4 binding (Figure 4A) (Dantuma *et al*., 2009). The plasmids bearing *RAD23* variants were cotransformed with a plasmid expressing the UFD substrate Ub^{V76}-V-βgal into *rad23*Δ cells. Consistent with previous studies, Ub^{V76}-V-βgal was partially stabilized in *rad23*Δ cells. The expression of wild-type but not the *rad23*$^{XPCB\Delta}$ mutant in the *rad23*Δ cells restored rapid degradation of Ub^{V76}-V-βgal (Figure 4B), suggesting that the in-

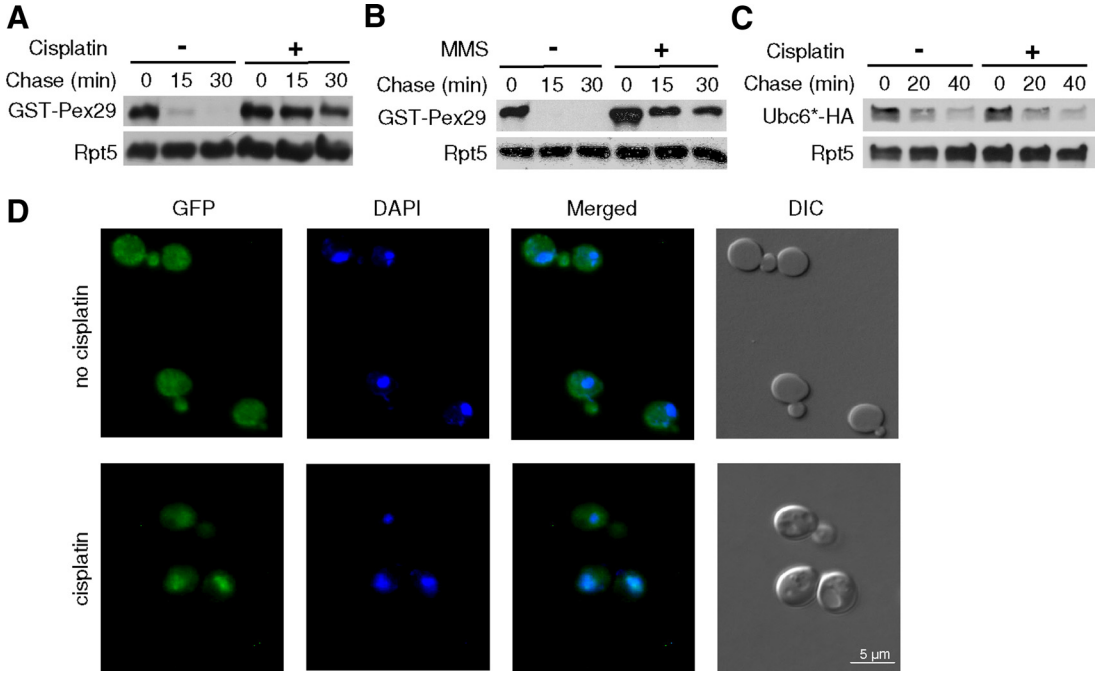

Figure 5. Functional alteration and cellular relocalization of Rad4 in response to DNA-damaging agents. (A and B) Pex29 degradation is impaired upon cisplatin- or MMS-induced DNA damage. Pex29 stability in wild-type cells was performed similarly as in Figure 2*D* except 0.8 mM cisplatin or 0.04% MMS were added for 1.5 or 4 h, respectively, before expression shut-off. (C) Cisplatin treatment does not affect the degradation of Ubc6*, a nonRad4 substrate. Ubc6* turnover was performed as described (Figure 3C) in the presence or absence of cisplatin. (D) Rad4-GFP localization with or without cisplatin treatment. Endogenous Rad4 is appended with GFP at its C terminus. Yeast cells were grown to mid-log phase and then treated with or without 1.6 mM cisplatin for 1.5 h. Cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), followed by 4′,6′-diamidino-2-phenylindole (DAPI) staining. Cells were then spotted on slides and mounted. Images were captured using an Axiocam digital camera (Carl Zeiss, Thornwood, NY) and processed with Axiovision 4.5 software.

tact XPCB domain is required for the proteolytic function of Rad23 in the degradation of the Ub^{V76}-V-βgal substrate.

Deletion of RAD4 Does Not Affect Rad23 Stability and Its Function in Glycoprotein Turnover

Because it was shown that the Rad23 binding protects Rad4 from degradation (Lommel *et al*., 2002; Gillette *et al*., 2006), we considered the possibility that interaction with Rad4 may shield Rad23 from proteolysis. We compared the half-life of endogenous Rad23 attached to a tandem affinity purification (TAP) tag in wild-type versus *rad4*Δ cells. We found that Rad23 was stable in both wild-type and *rad4*Δ cells (Figure 4C), suggesting that Rad4 is not needed to stabilize Rad23.

Rad4 and Png1 are known to compete for Rad23 to form separate Rad23-containing complexes (Suzuki *et al*., 2001). Png1 is a deglycosylation enzyme and the Rad23–Png1 complex recognizes and targets misfolded glycoproteins to the proteasome (Kim *et al*., 2006). We demonstrated previously that the Rad23–Png1 complex facilitates the degradation of the ricin A chain (RTA) (Kim *et al*., 2006). We determined RTA degradation in cells lacking *RAD4* and found that RTA degradation proceeded normally in *rad4*Δ cells (Figure 4, D and E), indicating that not all Rad23-dependent functions are impaired by the deletion of *RAD4*. It is important to note that the data suggest that Rad23 maintains its Rad4-independent functions.

The Ufd2–RAD4 Association Is Conserved from Yeast to Human

We have shown that Ufd2, a Ub chain elongation factor, binds the UBL domain of Rad23 and that the resulting complex is important for the function of Rad23 in the UFD substrate degradation (Kim *et al*., 2004), which also involves Rad4 (Figures 2A and 4B) but not Png1 (Kim *et al*., 2006). Ufd2 did not interact with Png1 in several binding experiments (Kim *et al*., 2006). Because Ufd2 and Png1 have distinct substrates, we proposed that Ufd2 and Png1 define different branches of the Rad23-dependent proteolytic pathway (Kim *et al*., 2006). We investigated whether Rad4 interacts with Ufd2 by coimmunoprecipitation. It is interesting that we found that Rad4 also binds Ufd2 (Figure 4F), consistent with the in vivo requirements of Ufd2 (Lambertson *et al*., 1999; Rao and Sastry, 2002) and Rad4 in the degradation of UFD substrates (Figure 2A) and Pex29 (Figure 2D; Liu, unpublished data), further suggesting that Ufd2 and Rad4 probably function in the same pathway.

Thus far, the known functions of Rad23 and Rad4 are conserved from yeast to human (Dantuma *et al*., 2009). We wondered whether XPC, the human counterpart of Rad4, is also involved in the UFD pathway (Kaneko *et al*., 2003; Park *et al*., 2009). There are two human homologues of Ufd2, which have been linked to Ub-mediated proteolysis (Kaneko *et al*., 2003; Park *et al*., 2009). We found that XPC interacts with Ufd2A by coimmunoprecipitation (Figure 4G). To assess the role of XPC in proteolysis, we used the XPC wild-type cell line WI38 and XPC-deficient cell line XP4PASV (Nishi *et al*., 2005). It is important to note that the degradation of UFD substrate Ub^{G76V}-GFP (Park *et al*., 2009) is impaired in XPC-deficient cell (Figure 4H). Combined, these results suggest the proteolytic function of Rad4/XPC is highly conserved.

Effects of DNA Damage on Rad4-mediated Proteolysis and Its Localization

Rad4 is localized to both the nucleus and the cytosol (Huh *et al*., 2003). Whereas the degradation of Pex29 and UFD substrates requires the action of Rad4 in the cytosol, NER occurs in the nucleus. We therefore set out to determine whether the cytosolic and NER activities of Rad4 are coordinated.

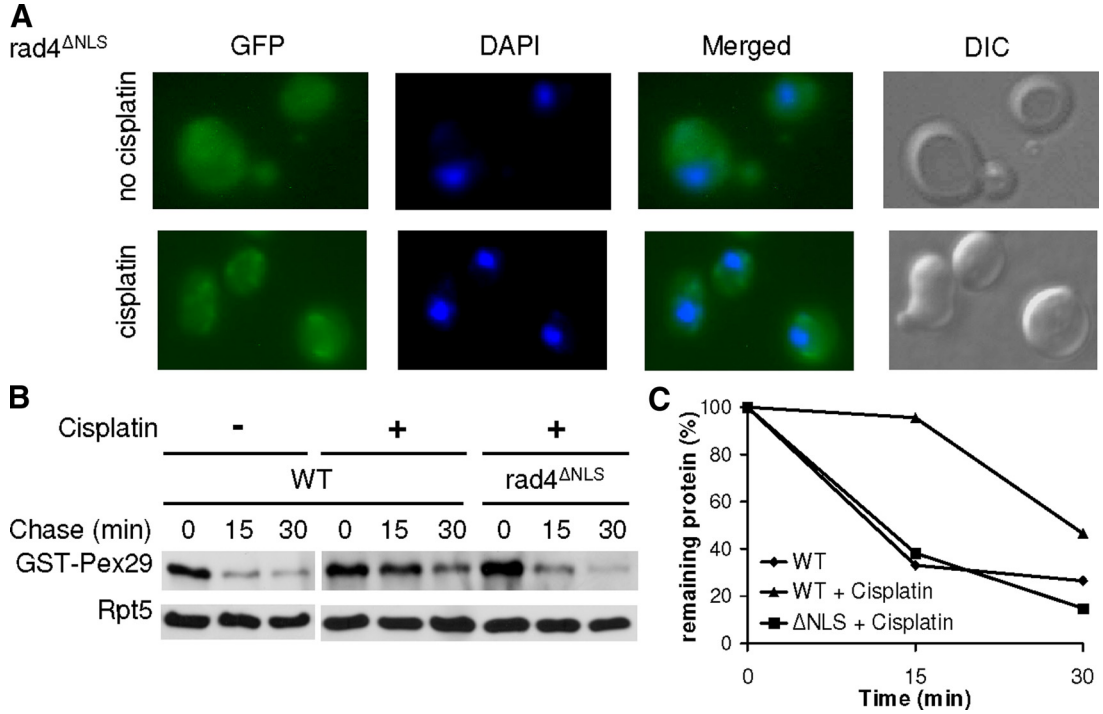

Figure 6. Effects NLS deletion on Rad4 localization and Pex29 degradation upon DNA damage. (A) $Rad4^{\Delta NLS}$-GFP localization with or without cisplatin treatment. The localization of $Rad4^{\Delta NLS}$-GFP was determined as described in Figure 5D. (B) Pex29 is efficiently degraded in $Rad4^{\Delta NLS}$-GFP mutant. Pex29 turnover was performed similarly as above (Figure 5A). (C) Quantitation of the data in B for Pex29.

First, we treated wild-type cells with cisplatin, which induces intrastrand DNA cross-links that require NER for their repair (Friedberg *et al*., 2006). It is interesting to note that Pex29 degradation was markedly compromised upon cisplatin treatment (Figure 5A). Furthermore, Rad4 has also been shown to be required for the repair of methyl methanesulfonate (MMS)-induced DNA damage (D'Errico *et al*., 2006). Similarly, Pex29 degradation was impaired upon treatment with MMS (Figure 5B).

It is possible that global proteolysis may be impaired upon DNA damage. We found that Ubc6* degradation, which is independent of *RAD23* or *RAD4* (Figure 3C), remains unaltered upon cisplatin treatment (Figure 5C), suggesting that DNA damage specifically affects a subset of substrate degradation. Rad4 is known to be degraded upon DNA damage (Gillette *et al*., 2006; Dantuma *et al*., 2009). If Rad4 level is reduced under DNA damage condition, this alteration could also account for compromised degradation of Pex29. We measured the Rad4 level with or without DNA damage. It is interesting to note that the amount of Rad4 remained the same under our conditions (Supplemental Figure 3). It was shown that RAD4 transcription is induced by DNA damage, which could maintain Rad4 level to facilitate efficient DNA repair (Jelinsky *et al*., 2000).

One possible explanation is that upon DNA damage, Rad4 is mobilized and relocalized to the nucleus. To detect Rad4 localization, we used a wild-type yeast strain expressing endogenous Rad4 fused to GFP at its C terminus (Huh *et al*., 2003). Without DNA damage, Rad4 was distributed throughout the cells. On cisplatin induction, Rad4 localized more in the nucleus (Figure 5D). To establish the significance of Rad4 mobility, we generated a *RAD4-GFP*$^{\Delta}$NLS mutant lacking its nuclear localization signal (amino acids 28-46). It is interesting to note that unlike wild-type Rad4, Rad4$^{\Delta NLS}$ mutant is not concentrated in the nucleus upon DNA damage (Figure 6A); Pex29 is efficiently degraded upon cisplatin treatment in *RAD4-GFP*$^{\Delta}$NLS mutant (Figure 6, B and C). Together, our results suggest that DNA damage triggers Rad4 redistribution in vivo and impairs its ability to fulfill its proteolytic functions in the cytosol.

DISCUSSION

Given its direct interactions with the proteasome and Ub conjugates, the function of Rad23 in proteolysis is easy to envision, although the mechanistic details remain sketchy (Madura, 2004; Kim and Rao, 2006; Dantuma *et al*., 2009). Here, we demonstrate that Rad4 also participates in the Rad23-mediated degradation pathway because the function of Rad23 requires an intact Rad4-binding XPCB motif and because these two proteins share common substrates, including the model substrate Ub^{V76}-V-βgal; the peroxisomal membrane protein Pex29; and Snf6, a subunit of the chromatin-remodeling complex.

What is the specific role of Rad4 in proteolysis? In response to DNA damage, Rad4 seems to play a major role in directly recognizing DNA lesions (Sugasawa, 2008). Rad23 plays accessory roles in shielding Rad4 from degradation and in stimulating Rad4 activity in damage recognition in an undefined manner (Dantuma *et al*., 2009). The significance of these proteins' contributions to proteolysis may be reversed. Given the biochemical properties of UBL and UBA domains, Rad23 has a major role in bridging ubiquitylated substrates to the proteasome (Madura, 2004; Dantuma *et al*., 2009). In contrast, the sequence of Rad4 provides little indication of how it may assist Rad23 in proteolysis. We propose that Rad4 likely plays an accessory role in proteolysis. NMR studies indicate that Rad23 contains four structured regions: UBL, XPCB, and two UBA domains (Figure 4A) (Walters *et al*., 2003; Kang *et al*., 2006). Furthermore, the intramolecular interactions between the UBL element and the UBA domains maintain Rad23 in a closed conformation (Walters *et al*., 2003; Goh *et al*., 2008). Rad23 exists in a stable complex with Rad4, which is pivotal for Rad23-mediated proteolysis. One possible biochemical role of the Rad23–Rad4 interaction is to maintain Rad23 in an open conformation (i.e., active state) and to promote its association with substrates and/or the proteasome.

Many NER factors participate in other non-DNA repair events (Hanawalt, 2003; Sugasawa, 2008). For example, RPA is essential for DNA replication. TFIIH is also required for RNA polymerase II–dependent transcription. Previously, Rad4/XPC was deemed as a DNA repair factor only. Our results reveal a novel function of Rad4/XPC in Ub/proteasome-mediated proteolysis. The multifunctional nature of repair proteins may not be coincidental. Traffic on the DNA is rather heavy, with ongoing replication and transcription. On DNA damage, lesions would require immediate attention to avoid genomic instability, which may be better served by mobilizing existing proteins than through de novo protein synthesis to meet the demands of the repair reactions. Once the lesions are fixed, these proteins could resume their normal functions, which in turn would facilitate the functional coupling of DNA repair with various cellular events. Our results on the spatial redistribution and functional alteration of Rad4 in response to cisplatin or MMS (Figures 5 and 6) indicate that Rad4 may be a rate-limiting factor in facilitating different cellular processes, and further highlight that the modulation and coordination of Rad4 activity could be an important regulatory mechanism under various stress conditions.

Mutations in XPC, the human homologue of yeast Rad4, lead to XP and skin cancers (Friedberg *et al*., 2006; Bernardes de Jesus *et al*., 2008). With >30 pathogenic mutations isolated in the XPC gene, defects in XPC constitute one of the most common forms of XP in patients. The phenotypes associated with XPC mutations were attributed previously to their defects in DNA repair. The major features of NER and Ub-mediated proteolysis are conserved from yeast to mammals (Friedberg *et al*., 2006). It is possible that some XPC mutations may affect substrate turnover and could be partly responsible for some disease phenotypes, a topic that is currently being pursued in our laboratory.

Recently, Cdc31, an essential protein involved in cell cycle control, was shown to associate with Rad4 and Rad23 (Chen and Madura, 2008). Cdc31 also participates in both DNA repair and protein degradation albeit by undefined mechanisms (Chen and Madura, 2008). The substrate examined in *cdc31* mutant cells was the artificial UFD substrate Ub-Pro-βgal. It will be important to determine whether Cdc31 regulates all or a subset of physiological Rad4/Rad23 substrates. Further studies also are needed to determine how their interactions are regulated and to elucidate the specific contribution of each protein in proteolysis and DNA repair.

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