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The DNA Damage-Inducible UbL-UbA Protein Ddi1 Participates in Mec1-Mediated Degradation of Ho Endonuclease

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Ho endonuclease initiates a mating type switch by making a double-strand break at the mating type locus, *MAT*. Ho is marked by phosphorylation for rapid destruction by functions of the DNA damage response, *MEC1*, *RAD9*, and *CHK1*. Phosphorylated Ho is recruited for ubiquitylation via the SCF ubiquitin ligase complex by the F-box protein, Ufo1. Here we identify a further DNA damage-inducible protein, the UbL-UbA protein Ddi1, specifically required for Ho degradation. Ho interacts only with Ddi1; it does not interact with the other UbL-UbA proteins, Rad23 or Dsk2. Ho must be ubiquitylated to interact with Ddi1, and there is no interaction when Ho is produced in *mec1* or Δ*ufo1* mutants that do not support its degradation. Ddi1 binds the proteasome via its N-terminal ubiquitinlike domain (UbL) and interacts with ubiquitylated Ho via its ubiquitin-associated domain (UbA); both domains of Ddi1 are required for association of ubiquitylated Ho with the proteasome. Despite being a nuclear protein, Ho is exported to the cytoplasm for degradation. In the absence of Ddi1, ubiquitylated Ho is stabilized and accumulates in the cytoplasm. These results establish a role for Ddi1 in the degradation of a natural ubiquitylated substrate. The specific interaction between Ho and Ddi1 identifies an additional function associated with DNA damage involved in its degradation.

Ho endonuclease of *Saccharomyces cerevisiae* makes a site-specific double-strand break (DSB) at the mating type locus, *MAT*, in late G_1. The DSB is repaired by gene conversion using one of the silent mating type cassettes as a template, and this leads to a mating type switch (57). Repair of the DSB regenerates the Ho cognate site and in addition to tight transcriptional regulation of *HO* (10), the protein is rapidly degraded via the ubiquitin-26S proteasome system with a half-life of ca. 8 min (30).

Functions of the DNA damage response (DDR), *MEC1*, *RAD9*, and *CHK1*, are responsible for phosphorylation of Ho that targets it for degradation via the ubiquitin-26S proteasome system (30). The DDR is a cellular response that coordinates cell cycle progression with repair of lesions in DNA and with DNA replication (66). Ubiquitin conjugation involves an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase (22). In most cases substrate ubiquitylation occurs in multiprotein complexes (11). Ho is ubiquitylated by the SCF (Skp1-Cdc53-F-box protein) ubiquitin ligase (E3) complex. The SCF consists of a Cdc53/Cullin scaffold complexed at one end with the Skp1 adaptor protein and at the other with the RING protein, Rbx1. A series of F-box proteins recruit degradation substrates to the SCF by forming a complex with the Skp1 adaptor (54, 55). F-box proteins recruit substrate molecules that are marked by phosphorylation, usually at multiple sites (29, 41). The novel F-box protein Ufo1 recruits Ho to the SCF for ubiquitylation (29, 30). Transcription of *UFO1* is induced by the DDR in response to DNA damage, and this involves the Mec1 pathway (24). Deletion of *UFO1* affects maintenance of genome stability; however the mechanism underlying this observation has not been elucidated (56).

Despite being a nuclear protein, Ho degradation occurs in the cytoplasm, and if Ho is trapped within the nucleus either by a point mutation that eliminates a critical phosphorylation site, or by deletion of its nuclear exportin, Msn5, the protein is stabilized. The *MEC1* pathway leads to phosphorylation of residue HoT225, which facilitates its nuclear export, and of additional residues necessary for binding the F-box protein, Ufo1. Stabilization of Ho expressed from its native promoter, e.g., by deletion of its nuclear exportin, leads to genome instability (29).

The 26S proteasome consists of a 20S catalytic core complexed at one or both ends to a 19S regulatory particle (RP) that functions in substrate recognition, binding, deubiquitylation, unfolding, and in gating of the 20S (45). Substrates are marked for proteasomal degradation by covalent attachment of a chain comprising at least four ubiquitins linked via ubiquitinK48 (15). Initially the C-terminal glycine, G76, of ubiquitin is linked to a substrate lysine and a chain is formed by addition of successive ubiquitin molecules to the K48 residue of the preceding ubiquitin. Ubiquitin has seven lysine residues and besides K48-linked chains, K29- and K63-linked chains are formed in vivo. However, modification with these chains does not target a substrate for degradation (46).

There is growing evidence that additional functions subsequent to ubiquitylation determine whether a given substrate will indeed be degraded by the proteasome. These include the ubiquitin chain binding proteins. Originally it was proposed that the Rpn10 (mammalian S5a) subunit of the 19S RP binds polyubiquitylated substrates as its C-terminal ubiquitin inter-

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acting motif binds polyubiquitin chains (12, 52, 62). However, deletion of Rpn10 is not lethal (59). Cross-linking experiments indicate that Rpt5, an ATPase subunit of the base of the 19S, interacts with polyubiquitin chains (33). A further class of polyubiquitin-binding proteins is the UbL-UbA protein family exemplified by Rad23, Dsk2, and Ddi1 (7, 17, 65). The UbA domain (23) is a degenerate motif of 40 to 50 residues that folds into a compact three-helix bundle with a hydrophobic surface patch for protein-protein interactions (13, 40, 64). The UbA domain shows a high affinity for K48-linked polyubiquitin chains (48, 65). In a recent detailed study of the proximal UbA domain of human Rad23A (HHR23A) Raasi and colleagues have shown that the UbA domain binds ubiquitin chains with a 1:1 molar stoichiometry. Binding affinity increases with chain length and is optimal at 4 to 6 ubiquitin residues. Experiments employing chimeric K48-Ub_4 tetramers mutated in a critical residue (UbL8A) show that all the L8 side chains in Ub_4 contribute to the interaction with HHR23A-UbA (48).

UbL-UbA proteins have an N-terminal ubiquitinlike (UbL) domain of about 70 residues with primary sequence similarity to ubiquitin that adopts a ubiquitin fold (6, 23, 26, 31). The Dsk2 and Rad23 UbLs bind the 19S RP (14, 63). Degradation of certain artificial substrates is dependent on UbL-UbA proteins (17, 34, 50), and a number of natural substrates stabilized in the absence of Rad23 and Dsk2 and their *Schizosaccharomyces pombe*, frog, and human orthologs have been identified (see, e.g., references 3, 16, 32, 43, 50, and 62). These include misfolded endoplasmic reticulum (ER) substrates degraded via the ER-associated protein degradation pathway (39). These findings have led to the proposal that UbL-UbA proteins serve as adaptors that deliver ubiquitin-conjugated substrates to the proteasome (20, 65). However, in some instances the UbL-UbA proteins protect substrates from degradation in vivo (4, 36, 42) and in vitro (49). This has been suggested to be due to their preventing an interaction of the substrate ubiquitin chain with the proteasome perhaps by capping the chain and preventing access to both E3s and deubiquitylating enzymes (DUBs) (43, 49).

The least-studied UbL-UbA protein is Ddi1 (*D*NA *d*amage *i*nducible). Transcription of *DDI1* is induced in response to DNA damage (25, 68); however, no role for Ddi1 in the DDR has been defined. Δ*ddi1* and Δ*rad23* mutants are partially defective in a subset of Pds1/securin-mediated functions involved in S-phase checkpoint signaling (8). In addition Ddi1 (alias Vsm1) may have a role in intracellular membrane transport as it binds v- and t-SNARES that mediate docking and fusion of intracellular vesicles (37, 38). Ddi1 binds the proteasome and polyubiquitylated conjugates (52); however, these interactions have not been mapped to specific domains of the protein. Deletion of *DDI1* leads to accumulation of polyubiquitylated conjugates (52); however, Ddi1 has not been reported to be involved in the degradation of any physiological substrate. Recently an engineered version of Ddi1 was shown to mediate degradation of an artificial substrate (31). Here we show that Ddi1 is necessary for degradation of Ho endonuclease. Our results indicate a physiological role for Ddi1 as a proteasome receptor and identify ubiquitylated Ho endonuclease as its first natural substrate.

MATERIALS AND METHODS

Strains. W303 is *MAT***a** *his3 leu2 trp1 ura3-52*. The Δ*ddi1*, Δ*rad23*, and Δ*dsk2* strains and their isogenic wild type, BY4741, are from Euroscarf. The *ufo1* wild type and Δ*ufo1* mutants are Research Genetics BY4730 and #142, respectively. The Δ*mec1* mutant (*his3 leu2 ura3 sml1-1 mec1*::*TRP1*) was obtained from B. Garvick; *ufo1 rad6* mutants are in DF5 (58). NSY1 has *DDI1* with a C-terminal TAP tag (47) in DF5 integrated using primers (F-CAGACTAACGGAAATGC AGAATTTGCTGCATCCCTCCTTTTCCAATCCATGGAAAAGAGAAG and R-GGGCTACATACGTAGAGGCCGATCACAATATCAGTGGTTGCT CATACGACTCACTATAGGG) to make a PCR product consisting of the tag and the *TRP1* marker.

Plasmid construction and expression. p*TET-HO-LACZ* and p*TET-LACZ* are Ho^{LZ} and LacZ expressed from the TET promoter of plasmid pCM190, respectively (30). Construction of p*GFP-UFO1* and p*GFP-HO* is described in references 29 and 2, respectively. *DDI1* was expressed from the *ADH1* promoter in plasmid p*AD6* (37). Subclones of Ddi1 with the UbL or UbA domain deleted were LexA fusions expressed from the *GAL* promoter (4). pYES3 (Invitrogen) was used for expressing high levels of FLAG-tagged *RPN1* (^{F}*RPN1*), ^{F}*RAD23*, ^{F}*DSK2*, and ^{F}*DDI1* from the *GAL* promoter (52). Wild-type and (K48R,G76A) mutant ubiquitins (15) were expressed from the *CUP1* promoter. Transformation of yeast cells was performed by lithium acetate (1).

Metabolic labeling, immunoprecipitation (IP), and pulse-chase are based on reference 18 and described in reference 30. Coimmunoprecipitation (co-IP)-immunoblotting was based on the method described in reference 37. These experiments were done either by (i) coexpression of both the potential interacting partners in yeast or (ii) mixing two cell lysates, each with one of the potential interacting partners. In both cases the lysates were incubated with the appropriate antibody directed against one of the interacting partners; after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) the presence of the second protein was detected by Western analysis.

Yeast cells were grown overnight to late logarithmic phase (optical density at 600 nm = 0.8) in 50 ml of the appropriate inductive synthetic minimal medium with 2% galactose for expression of the *GAL*-regulated genes; for induction of the TET-regulated constructs, *HO-LACZ* and *LACZ*, cells were incubated for 40 min in medium without doxycycline. The chase was performed by addition of 10 mM cycloheximide and methionine in radioactive experiments or 3% glucose for galactose-induced genes. For experiments in which Ho protein was made in cells expressing native or mutant (K48R,G76A) ubiquitin, we cotransformed W303 cells with p*TET-HO-LACZ* and with the vector or native- or mutant-ubiquitin-expressing plasmids. The cotransformed yeast cells were grown overnight without doxycycline to induce *HO-LACZ*, and with 0.2 mM $CuSO_4$ to induce ubiquitin. In these experiments the specific activity of Ho^{LZ} in each cotransformant was determined by the o-nitrophenyl-β-D-galactopyranoside (ONPG) reaction and equal aliquots of ONPG units were taken for each co-IP.

The cells were harvested by centrifugation, washed in 50 ml of Tris-EDTA, and resuspended in 400 μl co-IP buffer (0.1% NP-40, 250 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl [pH 7.5], 1:25 of Boehringer protease inhibitor cocktail). Glass beads (0.5 to 0.6 g) were added, and cells were broken with a glass beater (Biospec Products) using five 1-min cycles at 4°C. The lysate was clarified by centrifugation at 13,000 rpm for 15 min at 4°C, and protein concentration was measured with the Bio-Rad protein reagent. Protein lysate (5 to 15 mg) was used for IP with the appropriate antibodies in co-IP buffer at 4°C for 1 to 2 h with mild shaking. Thirty microliters of 50% protein A-Sepharose (Amersham) was added to each sample, and incubation was continued under the same conditions for 0.5 to 1 h. The samples were washed 6 times with co-IP buffer with 1% of Triton and centrifuged. The pellet was resuspended in 30 μl 2× Laemmli sample buffer, boiled for 10 min, and electrophoresed on a 10% polyacrylamide SDS gel (PAGE) with protein size standards. The gel was transferred to nitrocellulose membrane (Protran BA 85; Schleicher & Schuell), and Western blotting was performed with the appropriate antisera. One milligram of crude protein was used to determine input by IP-Western blot, and 30 μg was taken for direct Western blotting. For Ddi1-TAP pulldowns, immunoglobulin G (IgG) was covalently coupled to M-280 tosyl-activated Dynabeads (DYNAL) according to the manufacturer's instructions. The beads were incubated with crude yeast lysate for 2 h at 4°C. The bead pellet was washed three times with co-IP buffer as above with increasing NaCl concentrations up to 250 mM. A Dynal magnetic particle concentrator (Dynal Biotech) was used to separate the beads from the lysate supernatant.

Anti-green fluorescent protein (anti-GFP) antibody purchased from Roche Molecular Biochemicals was used at dilutions of 1:200 for IP and 1:1,000 for Western blotting; anti-LacZ from Santa Cruz Biotechnology was used at 1:600 for IP and 1:1,000 for Western blotting; anti-Flag was purchased from Sigma and used at 1:250 for IP and 1:3,000 for Western blotting; anti-Ddi1 antiserum was a

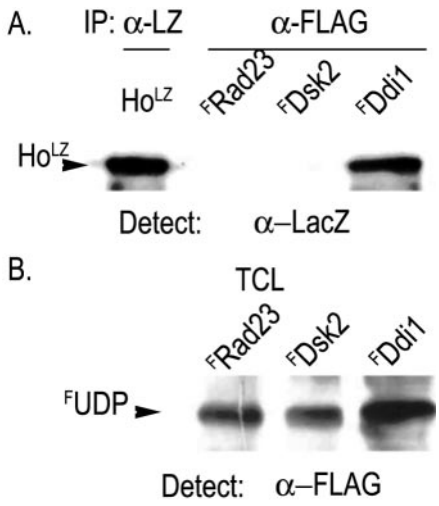

FIG. 1. Ho coimmunoprecipitates with Ddi1. Lysates from cells expressing *FRAD23*, *FDSK2*, or *FDDI1* were incubated with equal aliquots of lysate from cells with Ho-LacZ (Ho^{LZ}). (A) The FUbL-UbA proteins, *FRAD23*, *FDSK2*, or *FDDI1*, were immunoprecipitated with anti-FLAG (α-FLAG), blotted, and subjected to Western analysis using anti-LacZ antibodies to detect Ho^{LZ}. Left lane shows Ho^{LZ} by IP-Western. (B) Western blots of total cell lysates (TCL) from cells expressing *FRAD23*, *FDSK2*, or *FDDI1* were reacted with anti-FLAG antibodies. FUDP represents FLAGUbL-UbA proteins as indicated above each lane.

gift from Jeff Gerst and was used at 1:1,000 for IP and 1:5,000 for Western blotting; anti-Rpn12 antiserum was a gift from Dorota Skowyra and used at 1:10,000 for Western blotting; antiubiquitin antiserum from Affiniti Research Products was used at 1:5,000. Goat anti-rabbit antiserum, used at 1:1,000, and goat anti-mouse antiserum, used at 1:1,000, were purchased from Santa Cruz Biotechnology. Detection was by enhanced chemiluminescence (ECL) using an Amersham Pharmacia Biotech ECL Western blotting kit.

Microscopy. Cells expressing GFP-tagged proteins were observed with a Nikon fluorescence microscope as described previously (2).

RESULTS

Interaction of Ho with a UbL-UbA protein. To test the interaction of Ho with a UbL-UbA protein, lysates from cells expressing p*YES-FLAG*(F)-*RAD23*, *FDSK2*, or *FDDI1* (52) were incubated with lysate from cells that produced Ho^{LZ} (30). The FUbL-UbA proteins were immunoprecipitated with anti-FLAG antibodies and protein A. After washing the retained proteins were analyzed by PAGE and Western blotting with anti-LacZ antiserum to detect Ho^{LZ}. An aliquot of the Ho^{LZ} lysate was immunoprecipitated with anti-LacZ to assay the input of Ho^{LZ}. The FUbL-UbA proteins were detected in total cell lysates by Western blot with anti-FLAG antibodies as they could not be distinguished from the immunoglobulin chains after IP. We observed that Ho^{LZ} forms a complex only with FDdi1 and not with FRad23 or FDsk2 (Fig. 1).

The UbA domain of Ddi1 is necessary for interaction with Ho. Ddi1 has an N-terminal UbL of ca. 70 residues and a C-terminal UbA domain of ca. 40 residues. To map the interaction between Ho and Ddi1, we mixed Ho^{LZ} or control LacZ lysates with lysates from wild-type or Δ*ddi1* cells or from Δ*ddi1*cells that produced LexA fusions of Ddi1ΔUbL or Ddi1ΔUbA (4). Full-length and truncated Ddi1 proteins were immunoprecipitated with anti-Ddi1 antiserum and protein A, and the pellet was separated by PAGE and blotted. The Western blot was analyzed for the presence of Ho^{LZ} with anti-LacZ antiserum. We observed Ho^{LZ} in co-IPs with Ddi1 and Ddi1ΔUbL, but not with Ddi1ΔUbA indicating that Ho interacts with the UbA domain of Ddi1. The complex is between the Ho moiety and Ddi1 as the control LacZ protein did not interact with Ddi1 (Fig. 2A).

Ddi1 binds the 19S RP of the proteasome via its UbL domain. To test which domain of Ddi1 binds the proteasome, the above lysates were mixed with lysates from cells expressing p*YES-FRPN1*, a subunit of the 19S RP (53). Ddi1 proteins were subjected to IP with anti-Ddi1 and protein A, and the precipitated proteins separated by PAGE and blotted. Anti-FLAG antibodies were used to detect FRpn1 on the Western blots. The conditions we use in these experiments do not lead to disassociation of the 19S RP, and therefore interaction between Ddi1 and any subunit of the 19S RP complex would lead to coimmunoprecipitation of FRpn1. We found that full-length Ddi1 and Ddi1ΔUbA coimmunoprecipitate with FRpn1; however, FRpn1 did not coimmunoprecipitate with Ddi1ΔUbL (Fig. 2B). To exclude the possibility that Ddi1 could be interacting with Rpn1 that is not part of a 19S RP, we performed a pull-down experiment in which a lysate from cells producing TAP-tagged Ddi1 was incubated with magnetic beads coated with IgG. After stringent washing the proteins were separated by PAGE and blotted with anti-Rpn12 antiserum. A band corresponding to the size of Rpn12 was detected in the bead fraction of cells that produced Ddi1-TAP and was absent from the IgG beads incubated with a control DF5 lysate (Fig. 2C). Taken together these results indicate that Ddi1 binds the 19S RP via its UbL domain and interacts with ubiquitylated Ho via its UbA as reported for Rad23 and Dsk2 (14, 52).

We next tested whether Ddi1 is essential for formation of a complex between Ho^{LZ} and the 19S RP. Ho^{LZ} was produced in isogenic wild type and Δ*ddi1* mutants and immunoprecipitated with anti-LacZ antiserum, and the washed protein A-Ho^{LZ} beads were then incubated with equal aliquots of FRpn1 lysates for 4 h and washed thoroughly in co-IP buffer for separation by PAGE and Western blotting. Anti-FLAG was used to detect FRpn1 on the Western blots. We found that only Ho^{LZ} produced in wild-type cells in the presence of Ddi1 coimmunoprecipitated with FRpn1; when produced in Δ*ddi1* mutants it did not coimmunoprecipitate with FRpn1 (Fig. 3A). Similar experiments were next performed in which Ho^{LZ} was made in Δ*ddi1* mutants cotransformed with the LexA-DdiΔUbL and LexA-DdiΔUbA plasmids. In the absence of either the UbL or the UbA domain of Ddi1 no complex was formed that included Ho^{LZ} and FRpn1 (Fig. 3B).

Ho interacts with Ddi1 and the 19S RP only when ubiquitylated. To test whether Ho must be ubiquitylated to interact with Ddi1 and the 19S RP, we produced Ho^{LZ} in the presence of overexpressed native or double-mutant (K48R, G76A [Ub*]) ubiquitin. The double-mutant ubiquitin acts as a dominant negative leading to a prevalence of short ubiquitin conjugates and stabilization of substrates (15). In the presence of Ub* Ho degradation is retarded (28). The Ho^{LZ} lysates were mixed with equal aliquots of Ddi1 or of FRpn1 lysates. For assaying for interaction between Ho^{LZ} and Ddi1 we used anti-Ddi1 antibodies to IP Ddi1 and, after PAGE, anti-LacZ to detect Ho^{LZ} on the Western blots. To assay for interaction

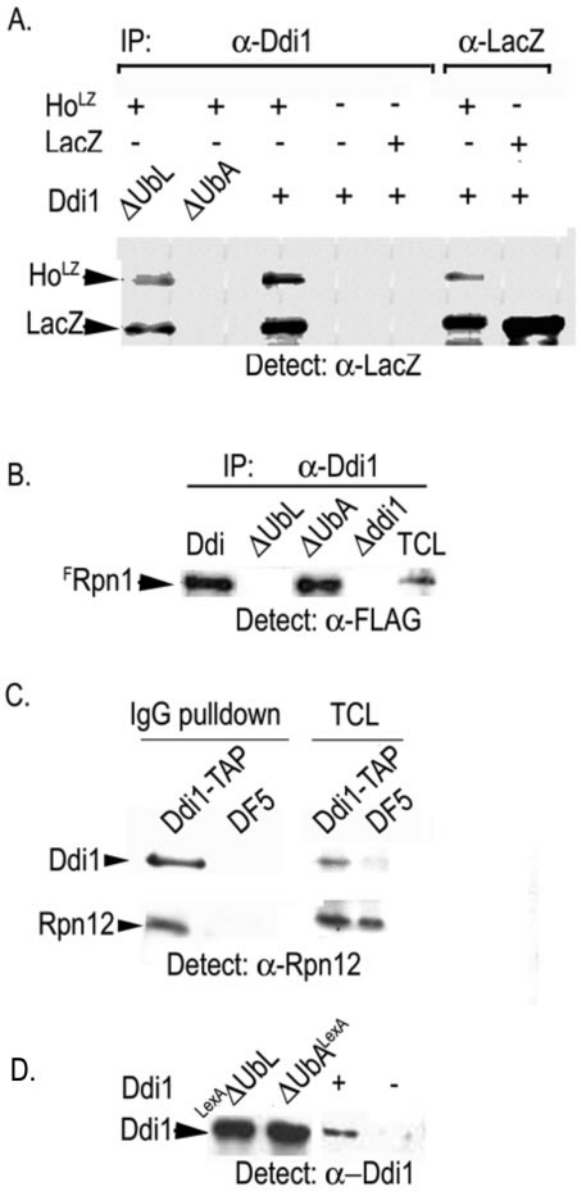

FIG. 2. (A) Ho interacts with Ddi1 via its UBA domain. Ho^{LZ} and control LacZ lysates were mixed with lysates from wild-type, Δ*ddi1*, or from Δ*ddi1* cells with LexA-DdiI1ΔUbL (ΔUbL), or LexA-Ddi1ΔUbA (ΔUbA). Ddi1 proteins were immunoprecipitated with anti-Ddi1 (α-Ddi1; left panel), and detection of Ho^{LZ} and LacZ was with anti-LacZ. Right panel shows Ho^{LZ} and LacZ in the lysates by IP-Western. (The LacZ band appears also in all Ho^{LZ} IPs in which degradation of Ho is observed.) Ho^{LZ} is observed in co-IPs with Ddi1 and Ddi1ΔUbL, but not with Ddi1ΔUbA. The LacZ control did not bind Ddi1. (B) Ddi1 binds the 19S RP via its UBL domain. The Ddi1 lysates were mixed with ^{F}Rpn1 lysates; Ddi1 proteins were immunoprecipitated with anti-Ddi1 and blotted, and anti-FLAG was used to detect ^{F}Rpn1 on the membrane. ^{F}Rpn1 coimmunoprecipitated only with full-length Ddi1 and Ddi1ΔUbA; it did not coimmunoprecipitate with Ddi1ΔUbL. (C) Ddi1-TAP on IgG beads pulls down the 19S RP. (Left panel) Cell extracts from DF5 yeast or NSY1 yeast that produce Ddi1-TAP were incubated with IgG magnetic beads. The bead fraction was gel separated and blotted with anti-Rpn12 antiserum. A band corresponding in size to Rpn12 is visible in the presence of Ddi1-TAP. Right panel shows total cell lysates (TCL). The anti-rabbit antiserum detects Ddi1-TAP by its TAP tag. (D) Western blot of TCL showing the presence of the different Ddi1 proteins used.

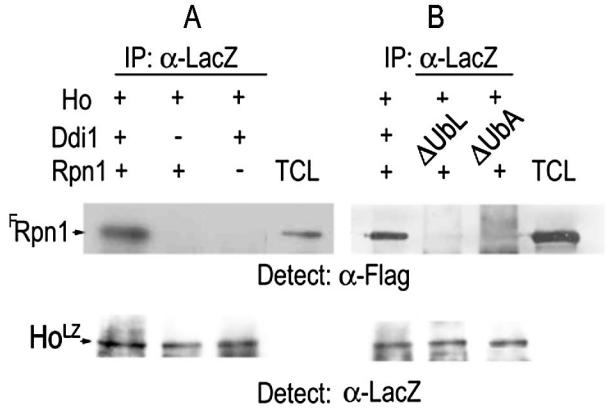

FIG. 3. Ho forms a complex with ^{F}Rpn1, and this requires both domains of Ddi1. (A) Protein A-Ho^{LZ} beads from IPs in which Ho^{LZ} was produced in the presence or the absence of Ddi1 were washed and then incubated with equal aliquots of ^{F}Rpn1 lysate. Ho^{LZ} coimmunoprecipitates with ^{F}Rpn1 only in the presence of Ddi1. (B) The above protein A-Ho^{LZ} beads were produced in extracts of cells producing Ddi1, LexA-Ddi1ΔUbL, or LexA-Ddi1ΔUbA as in Fig. 2D. They were incubated with equal aliquots of the ^{F}Rpn1 lysate. The coimmunoprecipitated pellets were gel separated and blotted and anti-FLAG (α-FLAG) was used to detect ^{F}Rpn1. No complex is formed between Ho and Rpn1 in the absence of either the UbL or the UbA domain of Ddi1. Total cell lysate (TCL) shows the ^{F}Rpn1 band. Lower panel shows a control aliquot of Ho^{LZ} from each cell type by anti-LacZ IP-Western blot.

between Ho^{LZ} and the 19S RP we used anti-LacZ to IP Ho^{LZ} and anti-FLAG antiserum for detection of ^{F}Rpn1 on the Western blots. Control lanes show an aliquot of each Ho^{LZ} lysate immunoprecipitated with anti-LacZ and analyzed by anti-LacZ Western blots. We found that whereas Ho^{LZ} produced in the presence of empty vector or overexpressed native ubiquitin interacts with both Ddi1 and the 19S RP, Ho^{LZ} made in the presence of overexpressed Ub∗ did not interact with either Ddi1 (Fig. 4A) or Rpn1(Fig. 4B).

The experiments with mutant ubiquitin suggest that ubiquitin chain formation is important for the interaction of Ho^{LZ} with Ddi1. To test this hypothesis, we therefore produced Ho^{LZ} in mutants where it does not undergo degradation and tested whether it could interact with Ddi1. Mutants used for Ho^{LZ} production are *mec1* mutants of the DDR in which Ho is not phosphorylated (29), and mutants of both the F-box protein that recruits Ho to the SCF, Δ*ufo1*, and in combination with the Rad6 E2 that has a role in Ho degradation (30), *rad6* Δ*ufo1* double mutants. Ddi1 was immunoprecipitated with anti-Ddi1 antibodies, the pellet was separated by PAGE, and detection of Ho^{LZ} on the Western blot was with anti-LacZ. To ensure that Ho was produced in all cell types, we performed control anti-LacZ IPs that were blotted with anti-LacZ antibodies. The results of these experiments show that only Ho^{LZ} produced in wild-type cells interacted with Ddi1; Ho^{LZ} made in mutants in which it could not be ubiquitylated did not coimmunoprecipitate with Ddi1 (Fig. 5A and B).

Ho is stabilized in the absence of Ddi1. To determine whether the association of ubiquitylated Ho with Ddi1 is a critical step in its degradation, we determined the half-life of Ho^{LZ} in wild-type and Δ*ddi1* cells by pulse-chase with radioactive methionine and anti-LacZ IP. In wild-type cells Ho was

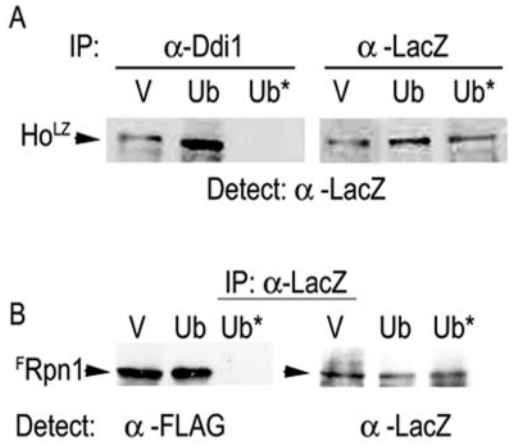

FIG. 4. Ho^{LZ} made in the presence of K48R, G76A ubiquitin does not interact with Ddi1 or the 19S RP. (A) Lysates with Ho^{LZ} from cells expressing vector, native, or K48R, G76A ubiquitin were mixed with equal aliquots of the wild type (Ddi1) (A) or with ^{F}Rpn1 (B) lysates. Anti-Ddi1 (α-Ddi1) was used to immunoprecipitate Ddi1, the pellet was gel separated and blotted, and Ho^{LZ} complexed to Ddi1 was detected with anti-LacZ. (B) Lysates were as in panel A. Anti-LacZ was used to immunoprecipitate Ho^{LZ}, the pellet was gel separated and blotted, and ^{F}Rpn1 was detected with anti-FLAG. Right panels of panels A and B indicate the presence of Ho^{LZ} by anti-LacZ IP and Western blot in each experiment. V represents the *CUP1* vector; Ub is the *CUP1* vector expressing native ubiquitin (Ub); Ub*, mutant ubiquitin. Ho^{LZ} produced in the presence of empty vector or native ubiquitin interacts with both Ddi1 and the 19S RP whereas Ho^{LZ} made in the presence of mutant ubiquitin did not interact with either protein.

rapidly degraded, whereas in Δ*ddi1* cells there was no observable degradation during the 30 min of the experiment (Fig. 6).

Ho accumulates in the cytoplasm of Δ*ddi1* mutants. Ho must exit the nucleus to be degraded and in mutants that affect early stages of the pathway, e.g., in *mec1* mutants, or in mutants of the nuclear exportin Msn5, stabilized Ho accumulates within the nucleus (29). The present results suggest that it is ubiquitylated Ho that interacts with Ddi1. This leads to the

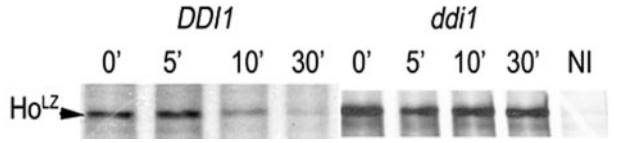

FIG. 6. Ho-LacZ is stabilized in the absence of Ddi1. The half-life of Ho^{LZ} in wild type and Δddi1 cells determined by pulse-chase and anti-LacZ IP. NI, noninduced.

prediction that Ho stabilized due to the absence of Ddi1 should accumulate in the cytoplasm. We therefore followed the degradation of ^{GFP}Ho in isogenic wild-type and Δ*ddi1* cells by inducing p*GFP-HO* and following the GFP signal by microscopy. Cycloheximide was added at the zero time point to inhibit protein synthesis. In wild-type cells a strong nuclear ^{GFP}Ho signal was seen at the zero time point and the GFP signal became less strong at 15 min and disappeared altogether by 30 min. In contrast, in Δ*ddi1* cells the ^{GFP}Ho signal disappeared from the nuclei of some cells by 15 min and was visible within the cytoplasm at both the 30- and 60-min time points (Fig. 7A). Stabilization of ^{GFP}Ho in Δ*ddi1* cells was confirmed in a parallel pulse-chase IP experiment. In contrast to Δ*ddi1* cells there was no stabilization of Ho^{LZ} in *rad23* or *dsk2* mutants (Fig. 7B).

Stabilization of Ho in Δ*ddi1* mutants could be the result of its being deubiquitylated in the absence of Ddi1. In this event it would no longer be recognized as a substrate for the proteasome. We therefore attempted to assay the ubiquitylation status of Ho directly. Using anti-GFP antiserum we immunoprecipitated ^{GFP}Ho from wild type and Δ*ddi1* mutants, and GFP from wild-type cells. The three immunoprecipitates were run in duplicate on the same gel followed by Western blotting with either anti-GFP or antiubiquitin antibodies. The lanes blotted with anti-GFP show the position of ^{GFP}Ho and a small number of faster migrating bands that may be degradation intermediates of ^{GFP}Ho; they do not appear in the GFP IP. The duplicate lanes blotted with antiubiquitin antibodies show high-

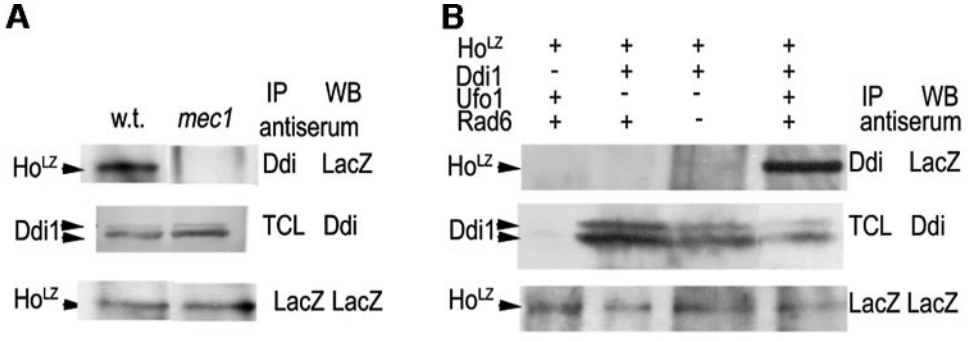

FIG. 5. (A) Ho^{LZ} made in *mec1* mutants does not interact with Ddi1. Ddi1 was immunoprecipitated with anti-Ddi1 (α-Ddi1), and the coimmunoprecipitated pellet was separated by PAGE and analyzed by Western blot with anti-LacZ antibodies to detect Ho^{LZ}. Middle panel shows the presence of Ddi1 in total cell lysates (TCLs) of wild-type and *mec1* cells. (Lower panel) Control anti-LacZ IP-Western blot indicates the presence of Ho^{LZ} in both cell lysates. Only Ho^{LZ} produced in wild-type cells interacts with Ddi1; Ho^{LZ} made in *mec1* mutants does not. (B) Ho^{LZ} made in Δ*ufo1* and in Δ*rad6* Δ*ufo1* double mutants does not interact with Ddi1. (Top panel) Ddi1 was immunoprecipitated with anti-Ddi1 and blotted, and Ho^{LZ} was detected with anti-LacZ; (middle panel) Western blot of TCL with anti-Ddi1 showing the presence of the Ddi1 doublet; (lower panel) anti-LacZ immunoprecipitates blotted with anti-LacZ indicate the presence of Ho^{LZ} in all cell types. Only Ho^{LZ} produced in wild-type cells interacts with Ddi1; Ho^{LZ} made in mutants in which it could not be ubiquitylated did not coimmunoprecipitate with Ddi1.

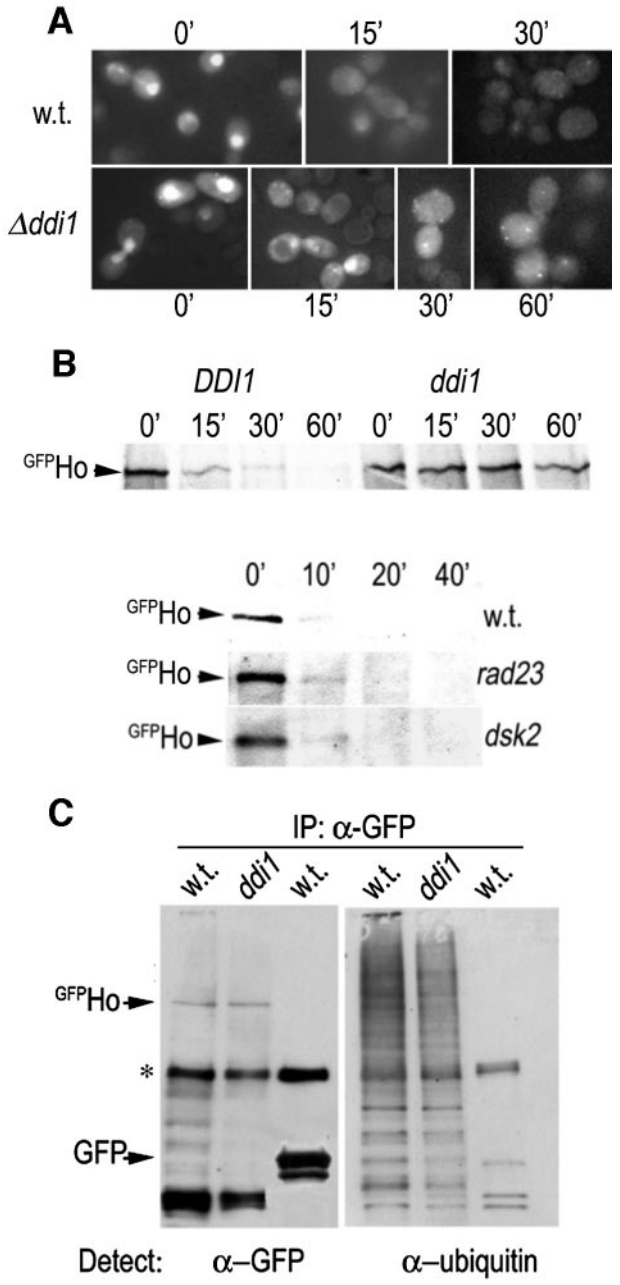

FIG. 7. (A) ^{GFP}Ho accumulates in the cytoplasm of Δ*ddi1* mutants. p*GFP-HO* was induced in wild-type and Δ*ddi1* cells, and the GFP signal was followed by microscopy. In wild-type cells the ^{GFP}Ho strong nuclear signal seen at the zero time point is no longer visible in the nucleus after 15 min and has completely disappeared by 30 min. In Δ*ddi1* cells the ^{GFP}Ho signal accumulates in the cytoplasm and is still visible at both the 30- and 60-min time points both as a dispersed cytoplasmic signal and as strong fluorescent spots that are probably aggresomes. (B) ^{GFP}Ho is stabilized in Δ*ddi1* cells, but not in Δ*rad23* or Δ*dsk2* mutants. The half-life of ^{GFP}Ho in wild-type and Δ*ddi1* cells was determined by pulse-chase and anti-GFP IP. (Lower panel) Half-life of Ho^{LZ} in wild-type and isogenic Δ*rad23* and Δ*dsk2* cells. (C) Ho is ubiquitylated in Δ*ddi1* mutants. ^{GFP}Ho was immunoprecipitated from wild-type and Δ*ddi1* mutants, and GFP was immunoprecipitated from wild-type (w.t.) cells. The IP pellets were run in duplicate and blotted with anti-GFP (α-GFP; left panel) and antiubiquitin antibodies (right panel). The anti-GFP blot shows the ^{GFP}Ho band and a small number of lower bands that may be degradation intermediates of ^{GFP}Ho; they do not appear in the GFP IP. * marks IgG chain. The antiubiquitin blot shows high-MW bands in both wild-type and Δ*ddi1* ^{GFP}Ho IPs; no ubiquitin conjugate bands are visible in the GFP IP. This is also the case after an extremely long exposure time (not shown).

molecular-weight (MW) bands in ^{GFP}Ho IPs from both wild type and Δ*ddi1* mutants; these antibodies do not detect any bands in the GFP IP. We interpret the high-MW bands to be ubiquitin conjugates of ^{GFP}Ho that are detectable with the antiubiquitin antiserum, but given the ratio of GFP/ubiquitin moiety of each ubiquitin-conjugated ^{GFP}Ho molecule would be below the level of detection by the anti-GFP antibodies (Fig. 7C). Thus Ho does not loose its ubiquitin tag in the absence of Ddi1.

DISCUSSION

There is growing evidence that additional functions subsequent to ubiquitylation determine whether a given substrate will indeed be degraded by the proteasome (39, 62). Our present results indicate that degradation of ubiquitylated Ho involves an additional DNA damage-associated protein, Ddi1. These results show that Ddi1 has a physiological role in the ubiquitin-proteasome system. However, they do not explain the specificity of Ho for this particular UbL-UbA protein. Stringent specificity of an ubiquitylated substrate and a single UbL-UbA protein is not always observed in vivo. For example, the mutant version of CPY* that is degraded via the ER-associated protein degradation pathway, shows a requirement for both Rad23 and Dsk2. Single mutants of either Rad23 or Dsk2 do not lead to CPY* stabilization (39, 62), and it is only in the double *rad23 dsk2* mutant that CPY* is stable. In these mutants control cytosolic substrates undergo normal degradation indicating that proteasome function is not impaired in the absence of Rad23 and Dsk2 (39). Similarly Rad23 and Rpn10 have redundant roles in degradation of the cyclin-dependent protein kinase inhibitor Sic1 (62).

In the case of Ho we observe a strong requirement for Ddi1 and not for either Rad23 or Dsk2. This is shown in co-IP experiments where Ho is found in a complex only with Ddi1 and not with either Rad23 or Dsk2. Furthermore it is supported by evidence showing stabilization of Ho in vivo in Δ*ddi1* mutants despite the presence in these cells of functional Rad23 and Dsk2 and additional polyubiquitin chain binding proteins (21). Given that all three UbL-UbA family proteins have a UbA domain and that this domain binds tetraubiquitin chains with high affinity, it is clear that the ubiquitin chain can be responsible for only part of the binding affinity and that residues of the substrate itself must be the predominant determinant of complex formation.

This raises the question of whether a substrate protein can interact with a UbL-UbA protein prior to its ubiquitylation. In this context it is interesting to note the presence of an integral UbL-UbA subunit in the recently discovered SCF complex responsible for degradation of p27 in G_1 cells in the cytoplasm (27). Here we used two experimental approaches to address this question: (i) mutant ubiquitin that leads to premature chain termination and (ii) production of Ho in mutants that do not support its degradation. In *mec1* mutants Ho is not phosphorylated and does not bind the F-box protein Ufo1 and is

therefore not recruited for ubiquitylation by the SCF. In fact lack of phosphorylation leads to trapping of Ho in the nucleus and to its total stabilization (29). In addition we produced Ho in mutants that act downstream of Mec1—an E3 mutant Δ*ufo1* and a Δ*ufo1* mutant in which we also deleted the E2, Rad6. (We used the double *ufo1 rad6* mutant as previously we found that Ho is stable in *rad6* mutants [30] and we do not know whether the Rad6 pathway acts upstream or in parallel with the SCF pathway of ubiquitylation). In all instances in which we produced Ho in cells that cannot support its degradation we did not observe any interaction with Ddi1. Thus the initial interaction must be between an ubiquitin chain and a UbL-UbA protein. This is probably a dynamic interaction that would be stabilized by complex formation between additional residues of both the substrate and the specific UbL-UbA protein.

We observe stabilized Ho that accumulates in the cytoplasm of Δ*ddi1* cells. One explanation for this stabilization could be deubiquitylation of Ho by cellular DUBs. Ho without its ubiquitin chains would be rescued from degradation. $Rad23^{Rhp23}$ and $Dsk2^{Dph1}$ of fission yeast bind tetraubiquitin-conjugated substrates, and this protects the chains from the activity of DUBs. Protection is conferred by isolated UbA domains of these proteins (20). Furthermore Rad23 inhibits proteasomal degradation of the nucleotide excision repair protein Rad4/XPC in both yeast and mammals (36, 42). In vitro Rad23 inhibits both K48-polyubiquitin chain extension and chain disassembly (49). However, in our experiments we found that Ho retains its ubiquitin chains in Δ*ddi1* mutants. This result indicates that ubiquitylated Ho is protected from the activity of cellular DUBs in the absence of Ddi1. We suggest a number of alternative hypotheses to explain this finding. (i) The DUBs are strictly compartmentalized. At least two DUBs are associated with the proteasome, the Rpn11 subunit of the 19S RP (60) and Ubp6 (19, 35). A facet of UbL-UbA protein activity may be to orient the substrate polyubiquitin chains so that they can be processed by the proteasomal DUBs. (ii) UbL-UbA protein activity may be necessary to extract the ubiquitylated substrate from the E3 complex and/or to terminate ubiquitin chain elongation. This could possibly involve the chaperone activity of the ATPase subunits of the 19S RP (5). Subunits of both the SCF and the UbL-UbA proteins, Rad23 and Dsk2, have been identified as components of complexes that copurify with affinity-purified 26S proteasomes (61). Therefore it is possible that the role of Ddi1 is to release Ho from the SCF^{Ufo1} and to make it available to the subunits of the 19S RP for deubiquitylation and unfolding. In the absence of Ddi1 ubiquitylated Ho may be retained within the SCF^{Ufo1} complex. We do indeed find Ho in a co-IP complex with Ufo1 in Δ*ddi1* cells (not shown). This result indicates that recruitment of Ho to SCF^{Ufo1} and its ubiquitylation do not require Ddi1. Experiments in which Ho ubiquitylation and degradation are reconstituted in vitro are necessary to determine the exact function performed by Ddi1.

In addition to the DDR functions Mec1, Rad9, and Chk1, which target Ho for degradation, we have now identified a further two functions associated with DNA damage that have a role in Ho degradation. These are the F-box protein Ufo1 described previously (29, 30) and the UbL-UbA protein, Ddi1. Both *UFO1* and *DDI1* are transcribed in response to DNA damage (25), and this is regulated by Mec1 although different effector kinases are involved (L. Kaplun, unpublished data; 67). Mating type switching is a very slow process, and the Ho-cleaved *MAT* allele is very stable (9, 51). However, the DDR checkpoint response is only evoked if DSB repair does not occur within 4 h (44). Ho degradation is very rapid, and the half-life of Ho does not exceed 10 min (30). We therefore conclude that it is the normal basal levels of the DDR functions, Ufo1 and Ddi1, that function in degradation of Ho.

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