Yeast mating switch Ho endonuclease is rapidly degraded by the ubiquitin system and this depends on the DNA damage response functions, MEC1, RAD9, and CHK1. A PEST sequence marks Ho for degradation. Here we show that the novel F-box receptor, Ufo1, recruits phosphorylated Ho for degradation. Mutation of PEST residue threonine 225 stabilizes Ho, yet HoT225A still binds Ufo1 in vitro. Stable HoT225A accumulates within the nucleus, whereas HoT225E is degraded. Deletion of the nuclear exportin Msn5 traps native Ho in the nucleus and extends its half-life. These experiments suggest that Ho is degraded in the cytoplasm. In mec1 mutants stable Ho accumulates within the nucleus; Ho produced in mec1 cells does not bind Ufo1. Thus the MEC1 pathway has functions both in phosphorylation of Thr-225 for nuclear export and in additional phosphorylations for binding Ufo1. Cells with HO under its genomic promoter, but stabilized by deletion of the Msn5 exportin, proliferate, but are multibudded. These experiments elucidate some of the links between the DNA damage response and degradation of Ho by the ubiquitin system.

Ho endonuclease introduces a site-specific double strand break (DSB) in the mating type (MAT) gene of Saccharomyces cerevisiae. HO expression is tightly regulated and occurs at the end of G1 in haploid mother cells, i.e. cells that have budded at least once (1). There are three copies of mating type information on chromosome III: two silent cassettes, MATα at HMLα and MATα at HMRα, and either mating type cassette at the transcribed MAT locus. The Ho DSB is repaired by gene conversion using one of the silent HM cassettes as a template and this leads to a mating type switch (2). Switching occurs before the onset of S phase and both progeny cells have the new MAT allele. At the four-cell stage a germinating spore gives rise to two switched cells derived from the mother cell and two progeny of the original mating type derived from the bud; these mate to form diploids that no longer express HO (3).

Ho belongs to the LAGLIDADG family of homing endonucleases that cleave long cognate sites. The recombination event by which homing endonuclease DSBs are repaired leads to insertion of the coding sequence of the endonuclease within the cognate site (homing) (4, 5). This destroys the site and protects the chromosome from further endonucleolytic cleavage. In contrast, repair of the Ho DSB by gene conversion leads to insertion of a new MAT allele and resurrects the Ho cognate site. We have shown that Ho endonuclease activity is confined to a narrow time window and that in addition to tightly regulated transcription of HO, the protein is degraded via the ubiquitin-26 S proteasome system with a short half-life of about 8 min (6).

Ubiquitylation of proteins involves a cascade of enzymes: ubiquitin is activated by an ubiquitin-activating enzyme (E1) that transfers the activated ubiquitin to an ubiquitin-conjugating enzyme (E2/UBC). Specificity of substrate ubiquitylation is mediated by an ubiquitin ligase (E3) that binds both the E2 and the substrate and mediates formation of the isopeptide bond between the terminal residue of ubiquitin and the substrate (7). Ho degradation involves two E2s, UBC9Rads6 and UBC3Cdc34 (6). UBC3Cdc34 ubiquitylates substrates as part of the Skp1-Cdc53-F-box receptor (SCF) E3 ubiquitin ligase complex (8, 9) and Ho is stabilized in mutants of Skp1 and Cdc53 and also in a deletion of the putative F-box coding gene ORF YML088w (6). The SCF mediates the ubiquitylation of substrates whose degradation is necessary for cell cycle progression at the G1/S transition. The SCF consists of a scaffold subunit Cdc53 that binds the RING finger protein Rbx1 at one end and a Skp1-F-box receptor complex at the other. Rbx1 and Cdc53 form a catalytic core complex that binds UBC3Cdc34 (10). F-box proteins recruit substrates for ubiquitylation and confer the specificity on the SCF (11). They bind the SCF by forming a complex that involves their N-terminal F-box domain and a similar domain in the adapter protein Skp1 (9). The F-box domain is followed by a leucine-rich or WD40 protein interacting domain (11); in many cases these have been shown directly to mediate the interaction with the ubiquitylation substrate (12–14).

Functions of the DNA damage response (DDR) MEC1, RAD9, and CHK1 are essential for degradation of Ho (6). The DDR is a network of interacting pathways for genome surveillance that leads to cell cycle arrest in response to DNA damage or to stalled replication and ensures that replication and chromosome segregation are completed with high fidelity (15, 16). MEC1 serves as the master signal transducer of all checkpoints of the DDR by activating a signaling network for cell cycle arrest, DNA repair, and cell recovery (15). The major DNA damage checkpoint is at the G2/M stage of the cell cycle and this is effected by phosphorylation of Rad9 by Mec1 and activation of downstream effector kinases Chk1 and Rad53 (17–19). Transient cell cycle arrest at the G2/M checkpoint is brought about by stabilization of Pds1/securin through Chk1

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The abbreviations used are: DSB, double strand break; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; SCF, Skp1-Cdc53-F-box receptor; DDR, DNA damage response; GFP, green fluorescent protein; MMS, methyl methanesulfonate; UIM, ubiquitin-interacting motif, CIAP, calf alkaline intestinal phosphatase; ORF, open reading frame.
and Rad53 (17). The involvement of functions of the DDR in the degradation of Ho via the ubiquitin-26 S proteasome protein degradation system has enabled us to probe for links between these two systems.

We originally identified ORF YML088w (now named Ufo1, UV-F-box-Ho) as a function required for degradation of Ho and postulated that it is the F-box receptor that recruits Ho for ubiquitylation by the SCF (6). Here we demonstrate a direct interaction between Ufo1 and its substrate, Ho, and with the SCF subunits, Skp1 and Cdc53, and map these interactions to specific domains of the Ufo1 protein. Substrates degraded by the SCF are usually phosphorylated (8) and we identified a PEST sequence in Ho that when deleted led to stabilization of the protein (6). We show that phosphorylation of Ho is essential for its interaction with Ufo1 and that, in a Δmec1 mutant of the DDR, Ho is not phosphorylated and cannot bind Ufo1.

We identified a critical threonine residue of the PEST sequence of Ho that when mutated leads to stabilization of the protein. However, this mutant form of Ho still binds Ufo1 in cell lysates. Subcellular localization experiments using GFP-fused wild type and mutant HoT225A show that the stabilized cell lysates. In complementtagged wild type and mutant HoT225A show that the stabilized cell lysates. GFP-HO protein also becomes stabilized. In a spores were chosen for deletion of the PEST sequence in Ho that when deleted led to stabilization of the SCF are usually phosphorylated (8) and we identified a specific domains of the Ufo1 protein. Substrates degraded by SCF subunits, Skp1 and Cdc53, and map these interactions to interaction between Ufo1 and its substrate, Ho, and with the DDR-mediated Ho Degradation Involves Nuclear Export

Microscopy—Cells expressing GFP-tagged proteins were observed with a Nikon fluorescence microscope fitted with 48–540-nm filters (dichromatic 505 nm, excitation 490– 480 nm, emission 515 nm). Images were captured with a Micromax 512 BFT camera (Roper Scientific) using WinView32 imaging software.

RESULTS

Degradation of Ho Proceeds via SCF\textsuperscript{T225A}.—Ho fused to the Gal4 DNA binding domain and Ufo1 fused to the activating domain gave a direct interaction in the two-hybrid protein interaction trap. This interaction between Ufo1 and Ho was confirmed by co-immunoprecipitation of Ho-LacZ and GFP-Ufo1 using anti-LacZ to precipitate the complex and anti-GFP antiserum to probe the Western blots for GFP-Ufo1 and -LacZ.
Ho-LacZ, respectively. Co-immunoprecipitation of control cells that expressed Ho-LacZ and the GFP epitope, or GFP-Ufo1 with LacZ, did not show this complex (Fig. 1B).

The amino acid sequence of Ufo1 shows three distinct domains: a N-terminal F-box motif between residues 4 and 49; a degenerate WD40 domain (36) between about 155 and 420, and a C-terminal domain that has 3 copies of a putative ubiquitin-interacting motif (UIM) between residues 547–566, 583–602, and 651–668 (37) (Fig. 1A). We subcloned each of these domains separately for the two-hybrid system and tested them for their interaction with Ho. A subclone centered around the putative WD40 domain (residues 156–512) interacted with Ho; no interaction was observed between the F-box subclone (residues 1–165) or the UIM domain subclone (residues 521–668) of Ufo1 and Ho (Fig. 1A).

To verify the F-box function of Ufo1 we tested for an interaction with components of the SCF. We found an interaction between Ufo1 and both Skp1 and Cdc53 in the two-hybrid system. The F-box domain subclone of Ufo1 interacts with both Skp1 and Cdc53, whereas the Ufo1 WD40 domain subclone was found to interact with Cdc53 (Fig. 1A). These interactions between Ufo1 and the SCF subunits were confirmed by transforming pGFP-UFO1 into cells expressing myc-tagged genomic SKP1 (24) or myc-CDC53 expressed from the GAL promoter (29) and co-immunoprecipitation. Cell lysates were immunoprecipitated with an anti-Myc antibody and the Western blots...
were probed with anti-GFP to detect GFP-Ufo1 and with anti-Myc to show the tagged SCF subunits. In cells expressing myc-Skp1 or myc-Cdc53, GFP-Ufo1 was precipitated with the anti-Myc antibody; this complex was not observed in the control cells in which Skp1 was not tagged or that expressed the GAL-myc vector alone (Fig. 1, B and C). These results confirm our hypothesis that Ufo1 acts as a F-box receptor and recruits Ho for ubiquitylation by the SCF.

The Interaction of Ufo1 and Ho Depends on Phosphorylation of Ho—Substrates recruited by the F-box receptors Cdc4, Met30, and Grr1 are phosphorylated (8) and we previously identified a PEST sequence in Ho between residues 216 and 236 that is necessary for its degradation. Indeed the N-terminal half of Ho truncated at residue 236 has the same half-life as full-length Ho, whereas the C-terminal fragment is stable (6).

To test directly whether phosphorylation of Ho determines its recruitment for ubiquitylation by Ufo1 we tested whether Ufo1-Ho complex formation is dependent on phosphorylation of Ho. Ho-LacZ was immunoprecipitated with anti-LacZ and Protein A-Sepharose and then treated with 0, 0.1, and 1.0 units of CIAP (calf alkaline intestinal phosphatase) for 15 min at 37 °C (29). Equal amounts of a lysate from cells expressing pGFP-UFO1 were added to each tube and the reactions were incubated at 4 °C overnight and then washed stringently and separated by SDS-PAGE for Western blot analysis. We found that dephosphorylation of Ho abrogated Ufo1-Ho complex formation (Fig. 2A). Next we attempted to map the phosphorylated residues responsible for complex formation between Ufo1 and Ho by examining the interaction of Ufo1 with the N- and C-terminal fragments of Ho described above. Ufo1 co-immunoprecipitated with full-length Ho and with the unstable N-terminal fragment, it did not form a complex with the C-terminal fragment of Ho. When the PEST residues were deleted from full-length Ho or from the N-terminal fragment, interaction with Ufo1 was only slightly reduced (Fig. 2B). This suggests that there are residues outside of the PEST sequence that interact with Ufo1. We mutated Thr-225 of the Ho PEST sequence to alanine; this residue is exposed on the protein surface according to our homology model of the Ho structure (56). In a pulse-chase experiment we found that the HoT225A mutant was stable with a half-life of about 20 min (Fig. 3A). Mutant HoT225A bound Ufo1 to the same extent as wild type Ho (Fig. 3B), as do the PEST deletions of Ho and its N-terminal fragment (above).

We previously identified a role for functions of the DDR, MEC1, RAD9, and CHK1, in degradation of Ho and postulated that these functions are part of a protein kinase cascade that marks Ho for degradation (6). We therefore looked at the phosphorylation status of Ho endonuclease isolated from wild type or mec1KD, rad9KD, and chk1KD mutants in which the protein is stabilized (6) ran from a single band that corresponds to the dephosphorylated form irrespective of phosphorylation treatment (Fig. 4A). Furthermore, Ho-LacZ isolated from Δmec1 (22) cells no longer bound Ufo1. In contrast, when Ufo1 was isolated from Δmec1 cells, it could still bind Ho-LacZ isolated from wild type cells (Fig. 4B). These experiments indicate that the MEC1 pathway is involved in marking Ho for degradation by phosphorylation leading to its recruitment by Ufo1 for ubiquitylation.

Subcellular Localization of GFP-Ho, GFP-HoT225A, GFP-HoT225E, and GFP-Ufo1—Given the anomaly between the stability of mutant HoT225A and its ability to bind Ufo1 in cell lysates, we asked whether perhaps in vivo these two proteins are segregated from one another in different cell compartments. We therefore compared the subcellular localization of GFP-Ho and GFP-HoT225A. As an additional control we constructed HoT225E fused to GFP; this mutant is anticipated to behave similarly to the phosphorylated form of wild type Ho. The cells were induced overnight with galactose, diluted to 0.4-0.6 of OD600 the next morning, grown for a further 3 h, and then observed. At this point 10 μm cycloheximide was added to the
The lanes on the separated by SDS-PAGE and then blotted with anti-GFP to detect Ufo1.

wild type or protein A and then incubated overnight with GFP-Ufo1 produced in either /H9004 mec1 and without treatment when extracted from /H9004 mec1KD mucase. Separation was by SDS-PAGE and the Western blots were not treated with CIAP. The lower arrow indicates phosphorylated Ho extracted from wild type cells that were treated with CIAP. The lower arrow indicates GFP-Ho after phosphatase treatment. GFP-Ho runs as the dephosphorylated form with and without treatment when extracted from mec1KD mutants. B, phosphorylated Ho interacts with Ufo1 irrespective of whether Ufo1 is made in wild type or ∆mec1 mutants. Ho-LacZ produced in wild type (w) and ∆mec1 mutants (m) was immunoprecipitated with anti-LacZ and Protein A and then incubated overnight with GFP-Ufo1 produced in either wild type or ∆mec1 mutants. The washed immunoprecipitate was separated by SDS-PAGE and then blotted with anti-GFP to detect Ufo1. The lanes on the left of the marker (M) show the GFP-Ufo1 in the total cell lysate by Western blotting. Subsequently the blot was treated with anti-LacZ to show Ho produced in each cell type (lower panel). w, wild type; m, ∆mec1 mutants. Only Ho produced in wild type cells binds Ufo1.

cell cultures to inhibit further translation of the GFP-HO mRNA. This treatment has no effect on the half-life of Ho measured in pulse-chase immunoprecipitation experiments (not shown). At the 0- and 10-min time points GFP-Ho, GFP-HoT225A, and GFP-HoT225E were observed in the nucleus. Native GFP-Ho is no longer nuclear after 20 min and by 30 min there is no longer any signal indicating that the protein has been degraded. In contrast GFP-HoT225A remained visible in the nucleus at all time points (Fig. 5A). GFP-HoT225E was no longer visible at 20 min. These observations imply that in vivo Ho exits the nucleus prior to binding Ufo1. Using a construct in which Ufo1 is expressed as a GFP fusion protein we determined that GFP-Ufo1 is distributed throughout the cell except for the vacuole (Fig. 5B).

Inhibition of Ho Export Leads to Stabilization of the Protein—Proteins traverse the nuclear pore complex of the nuclear envelope bound to importin and exportin receptors (reviewed in Ref. 39). The Msn5 nuclear exportin has been shown to recruit phosphorylated substrates (40). In a complementary experiment we asked whether trapping the protein inside the nucleus in a ∆msn5 mutant would extend the half-life of Ho. HO-LACZ was induced for 40 min in a pulse-chase experiment in isogenic control and ∆msn5 mutant cells and aliquots were immunoprecipitated with anti-LacZ at 0, 15, 30, and 45 min. We found that Ho is stabilized in the ∆msn5 mutant compared with the wild type and accumulates as a doublet indicative of its being phosphorylated (Fig. 6A). The same cells were transformed with pGFP-HO for microscopic observation. In wild type cells the GFP-Ho signal decayed by 30 min whereas in ∆msn5 mutants
GFP-Ho gave a very bright signal for at least 30 min; all of the signal was concentrated in the nucleus (Fig. 6B). Heterokaryon experiments in which Δmsn5 cells transformed with pGFP-HO were mated with kar1 MSN5 or kar1Δmsn5 cells showed that when Msn5 was supplied in trans, the GFP-Ho signal disappeared. In heterokaryons in which both parents were Δmsn5 the signal remained bright and Ho was not degraded during 40 min (Fig. 6C).

Subcellular Localization of Ho and Ufo1 in Mutants of the DDR—In light of our finding that HoT225A remains in the nucleus and that inhibition of its nuclear export in Δmsn5 mutants leads to stabilization of the protein we considered that perhaps one of the functions of the DDR may be to facilitate nuclear export of Ho. We therefore examined the subcellular localization of GFP-Ho in kinase-deficient mec1KD mutants. We found that Ho accumulated in a stable form within the nucleus up to 40 min after cycloheximide addition (Fig. 7). There was no difference in the distribution of GFP-Ufo1 and it was observed throughout the cell in mec1KD mutants and also in wild type cells treated with 0.1% MMS or UV to induce DNA damage, or after a Ho-induced DSB (not shown).

Effect of Stabilization of Ho on Cell Growth—The cleaved MATα intermediate formed by Ho endonucleolytic activity is very stable and the first switched alleles are visible only after 1.5 h (41, 42). By this time under normal conditions Ho would be degraded. We therefore asked what the biological effect of stabilizing the protein, so that it is present at an inappropriate stage of the cell cycle, would be. Expression of Ho from its native promoter occurs only in haploid cells and we therefore used a strain that has the a sequence at all three mating type cassettes. This enables the cells to go through the process of mating type interconversion without actually changing their mating type and diploidizing. We chose to stabilize Ho by deleting the Msn5 nuclear exportin so as to assess the effect of native rather than HoT225A mutant protein on cell cycle progression. Ho, HMLα, MATα, and HMRα cells transformed with a PCR product to delete the MSN5 nuclear exportin gene. In Ho, HMLα, MATα, and HMRα cells, wild type for the Msn5 exportin, the nucleus migrates to the neck between the mother cell and the bud. In Ho, HMLα, MATα, and HMRα cells that are Δmsn5, DNA replication is no longer synchronized with bud emergence; the cells are multibudded and mother cells are visible with divided nuclei. As the buds grow they acquire nuclei. Subsequent deletion of Ho in HoΔmsn5 cells restores the normal phenotype.

In this paper we provide direct evidence that Ho is a ubiquitylation substrate of the F-box protein Ufo1 and that the interaction is through the putative WD40 domain of Ufo1. Ufo1 (as ORF YML088w) appears among the Skp1-interacting pro-
teins in two-hybrid experiments (31) and as an in vivo component of the SCF by affinity purification of Cdc53 and mass spectrometry (24). Here we show that it is the F-box domain of Ufo1 that interacts with Skp1. Subclones expressing either the F-box or the WD40 domain of Ufo1 showed an interaction with Cdc53. This is compatible with the SCF structure (10) in which an interaction between Cdc53 and the F-box domain of the Skp2 F-box protein is observed in addition to that between Skp2 and the Skp1 adaptor protein. Given also that it is the WD40 domain that interacts with Ho, the interaction between the Ufo1 WD40 domain and Cdc53 places the Ufo1-Ho complex inside the SCF complex with the N-terminal F-box domain of Ufo1 bound also to Skp1. This would allow positioning of Ho close to its E2.

Like other substrates of the SCF, we show that Ho is phosphorylated when it binds to Ufo1 as treatment with alkaline phosphatase disrupts this interaction. The most obvious candidate position for phosphorylation of Ho is its PEST sequence. However, despite the fact that deletion of its PEST sequence stabilizes Ho (6), we found that this does not abolish binding to Ufo1. This suggests that there are other phosphorylated residues on Ho that participate in binding to Ufo1 and is consistent with the finding that PEST sequences alone, when fused to a heterologous protein, are not enough to destabilize it (12, 43). Indeed our data show that Ho with a single mutation in its PEST sequence, threonine 225 to alanine, although resistant to degradation, still binds Ufo1 with an affinity not visibly different from that shown by wild type Ho.

Stabilized mutant HoT225A accumulates within the nucleus. Furthermore, trapping wild type Ho in the nucleus in a msn5 exportin mutant leads to stabilization of the protein. These data indicate that Ho must exit the nucleus to be degraded. In mec1 mutants, in which Ho is stabilized, we find it within the nucleus. We propose that one of the functions of the MEC1 signal transcription cascade is to phosphorylate Ho on threonine 225 to facilitate its nuclear export and subsequent ubiquitylation by Ufo1 in the cytoplasm. Ho produced in mec1 mutants does not bind Ufo1. This indicates that whereas a single phosphorylation on T225A is sufficient for export of Ho from the nucleus, additional phosphorylations are necessary for recruitment of Ho by Ufo1 for ubiquitylation.

It is perhaps paradoxical that Ho needs to be exported to the cytoplasm to be degraded as localization studies of both 19 S and 20 S complexes in S. cerevisiae and Schizosaccharomyces pombe indicate that the majority of the proteasomes are nuclear (44–46). However, a number of reports show that ubiquitylation and degradation can take place in both the nucleus and cytoplasm. For example, in mammalian cells a single threonine-in mutation in cyclin D1(T286A) confers resistance to polyubiquitylation and an extended half-life (47). Normally during S phase cyclin D1 is exported to the cytoplasm; the single point mutation of Thr-286 by glycogen synthase kinase 3β is critical for binding of cyclin D1 to the CRM1 nuclear exportin (49). A further example of degradation being linked to nuclear export is the p53 tumor suppressor. P53 undergoes ubiquitylation by Mdm2 within the nucleus but must be exported to the cytoplasm to be efficiently degraded. Mutations in the RING finger of Mdm2 or in the E1 ubiquitin-activating enzyme that prevent p53 ubiquitylation lead to its accumulation in the nucleus (50, 51).

The conclusion from our experiments is that in vivo Ho is phosphorylated by the MEC1 pathway within the nucleus, but is ubiquitylated by Ufo1 in the cytoplasm. GFP-Ufo1 is distributed throughout the cell and DNA damage by a variety of agents including a DSB induced by Ho does not cause it to accumulate in the nucleus. Ufo1 may shuttle between the cytoplasm and the nucleus as its primary sequence shows a basic nuclear localization sequence, between residues 133 and 141. However, the finding that HoT225A is able to bind Ufo1 in a co-immunoprecipitation experiment, but is, nevertheless, stabilized, strongly suggests that ubiquitylation of Ho by Ufo1 occurs in the cytoplasm. The experiments showing that deletion of the Msn5 nuclear exportin causes Ho to accumulate in the nucleus in a stable form support this conclusion.

We conclude by following the fate of cells in which Ho is retained in the nucleus beyond its normal time window of activity. In budding yeast a single DSB can lead to cell cycle arrest at the G2/M stage through activation of both the Rad53 and Chk1 checkpoint kinases. During normal mating type interconversion, despite the prolonged persistence of the MAT cleaved intermediate, there is no checkpoint kinase activation for at least 1.5 h (52). In the presence of a single unrepaired DSB cells can resume normal cell cycle progression after a checkpoint-mediated delay, a process known as adaptation, during which Rad53 and Chk1 kinases are inactivated (52, 53). We have found that when HO expressed from its native promoter is retained in the nucleus by deletion of the Msn5 exportin, multibudded cells appear. This indicates that they have gone through several cell cycles. However, the fact that the buds do not separate from the mother cell indicates a deficiency in the cell cycle. We suggest that persistence of Ho activity leads to loss of synchrony between the independent DNA replication and bud emergence pathways of the cell cycle (54). This is particularly well illustrated in those cells in which nuclear division (on the DNA replication pathway) is no longer coordinated with nuclear migration (on the bud emergence pathway) and we see that the dividing nucleus has not migrated into its normal position in the neck between mother and daughter cells. Cytokinesis, where the two independent pathways merge, is delayed until the nucleus has found its way into the bud, however, meanwhile a new round of bud emergence commences giving the multibudded phenotype. This experiment emphasizes the importance of rapid Ho degradation in the life cycle of budding yeast and illustrates how the apparently fortuitous recruitment of functions of the DDR for targeting Ho to the ubiquitin system enabled establishment of the homothallic mating type interconversion in the evolution of this species.

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