Dosage Suppressors of *pds1* Implicate Ubiquitin-Associated Domains in Checkpoint Control

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In budding yeast, anaphase initiation is controlled by ubiquitin-dependent degradation of Pds1p. Analysis of *pds1* mutants implicated Pds1p in the DNA damage, spindle assembly, and S-phase checkpoints. Though some components of these pathways are known, others remain to be identified. Moreover, the essential function of Pds1p, independent of its role in checkpoint control, has not been elucidated. To identify loci that genetically interact with *PDS1*, we screened for dosage suppressors of a temperature-sensitive *pds1* allele, *pds1-128*, defective for checkpoint control at the permissive temperature and essential for viability at 37°C. Genetic and functional interactions of two suppressors are described. *RAD23* and *DDI1* suppress the temperature and hydroxyurea, but not radiation or nocodazole, sensitivity of *pds1-128*. *rad23* and *ddi1* mutants are partially defective in S-phase checkpoint control but are proficient in DNA damage and spindle assembly checkpoints. Therefore, Rad23p and Ddi1p participate in a subset of Pds1p-dependent cell cycle controls. Both Rad23p and Ddi1p contain ubiquitin-associated (UBA) domains which are required for dosage suppression of *pds1-128*. UBA domains are found in several proteins involved in ubiquitin-dependent proteolysis, though no function has been assigned to them. Deletion of the UBA domains of Rad23p and Ddi1p renders cells defective in S-phase checkpoint control, implicating UBA domains in checkpoint signaling. Since Pds1p destruction, and thus checkpoint regulation of mitosis, depends on ubiquitin-dependent proteolysis, we propose that the UBA domains functionally interact with the ubiquitin system to control Pds1p degradation in response to checkpoint activation.

When DNA is damaged or chromosomes are incompletely replicated, cells become checkpoint arrested. These checkpoints avoid replication of damaged template DNA and prevent aberrant segregation of damaged or partly replicated chromosomes. In budding yeast, proteolysis of anaphase inhibitors is regulated by these checkpoint systems. Progression from metaphase to anaphase is inhibited by Pds1p in *Saccharomyces cerevisiae* (6, 7, 29, 30). Before anaphase, Pds1p binds to Esp1p, inhibiting its anaphase-promoting activity (3). During an unperturbed cell cycle, Pds1p becomes polyubiquitinated at the metaphase-to-anaphase transition by multienzyme anaphase-promoting complex (APC)–cyclosome complexes. The modified forms are then recognized and degraded by 26S proteasomes (7). Once released from Pds1p, Esp1p activity induces the onset of anaphase.

pds1 mutants fail to execute checkpoint control in response to DNA damage, spindle poisons, or replication inhibition (4, 29, 30). Pds1p is required for replication checkpoint control only late in S phase, not in the context of an early S-phase replication block enforced by hydroxyurea (HU) (4, 29, 30). In

the presence of 0.1 M HU, replication proceeds more slowly. Under these conditions, cells perform other aspects of cell cycle progression, budding, and spindle assembly as rapidly as in the absence of HU; cell cycle arrest in G₂ is then necessary to delay anaphase while replication is completed. Growth in the presence of a non-replication-arresting concentration of HU therefore challenges the ability of cells to accurately couple S phase with mitosis. Although *pds1* mutants can prevent mitotic progression when replication is blocked, defective S-phase checkpoint control can be observed when replication is partially inhibited. Such experiments revealed a novel checkpoint pathway that is essential late in S phase. Components of this pathway, other than Pds1p and Mec1p (4; D. J. Clarke, M. Segal, S. Jensen, and S. I. Reed, submitted for publication), have not been identified. Moreover, the essential function of Pds1p at high temperature is not known. To gain insight into this function, we screened for dosage suppressors of *pds1-128* and have characterized two, encoded by *RAD23* and *DDI1*. Ubiquitin-associated (UBA) domains located at the C termini of both Rad23p and Ddi1p are required for suppression of *pds1-128*. Interestingly, these domains are found in several proteins involved in ubiquitin-dependent proteolysis, though no function has been assigned to UBA domains. Further experiments implicate these UBA domains in checkpoint signaling: strains with mutant versions of the proteins lacking the UBA domains are S-phase checkpoint defective.

MATERIALS AND METHODS

Yeast strain construction and growth conditions. All strains are derivatives of BF264-15DU *MATa ade1 his2 leu2-3,112 trp1-1^a ura3 Dns* (20). Cultures were grown at 30°C, unless otherwise stated, in yeast extract-peptone (YEP) medium

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containing 2% dextrose, raffinose, or galactose. Strains were constructed according to standard procedures (22) except for gene disruptions (27). *pds1*Δ cells were temperature sensitive at 28°C on YEP-dextrose plates in the BF264-15DU genetic background. Rad23p and Ddi1p were epitope tagged at the C-terminal extremities of their genomic coding regions with either six Myc or six His epitopes. The C-terminal region of each coding sequence was amplified by PCR using oligonucleotides that included *NotI* or *SalI* sites and then cloned into tagging vectors in frame with the epitope. Such constructs were linearized by restriction enzyme digestion within the *RAD23* or *DDI1* open reading frame (ORF) and then integrated at the endogenous *RAD23* and *DDI1* loci. *GAL1:RAD23* and *GAL1:DDI1* strains were constructed by amplifying the *RAD23* and *DDI1* coding sequences, cloning into a yeast integration vector behind a *GAL1* promoter and then integrating at the *LEU2* locus. Epitope tags could then be integrated behind the *GAL1:RAD23* and *GAL1:DDI1* sequences. *rad23^{subba2}* and *ddi1^{Δsuba}* mutant alleles, expressed endogenously or under *GAL1* promoter control, were constructed by integrating epitope tags directly 3' of the UBA domain sequences, thus truncating the corresponding coding sequence. Temperature-sensitive *esp1* strains were generated by PCR-based mutagenesis (13, 18).

Yeast strain genotypes. Genotypes used are shown in Table 1. Oligonucleotides used are shown in Table 2.

Isolation of *pds1-128* dosage suppressors. *pds1-128* suppressors were isolated by transformation with an *S. cerevisiae* genomic library contained in a vector which is maintained at high copy number in yeast cells (YE24 library [2]). Transformants were replicated onto YEP-galactose plates, and suppressors were selected at 37°C. About 160,000 transformants were analyzed to identify 110 colonies rescued in a plasmid-dependent manner; 80 of these colonies appeared wild type (WT) at 37°C on YEP-galactose medium. Of the 13 clones examined further, all contained *PDS1*; 30 of the 110 colonies grew more poorly than WT at 37°C on YEP-galactose medium. Plasmids isolated from each of these clones contained either *ESPI*, *RAD23*, or *DDI1*.

Cell biology protocols. Samples were prepared for FACS analysis as described elsewhere (14). Spindles were visualized by fluorescence microscopy by using strains expressing a *TUB1-GFP* construct (23). Cells were gamma irradiated in liquid medium (3.5 Gy per min). For G₁ arrest, strains were grown overnight to saturation and then diluted to an optical density of 0.15 to 0.2; then α-factor (200 ng/ml) was added; 90 to 100% arrest was complete within 2 to 2.5 h at 26 to 30°C. For experiments in which spindle morphology was analyzed, it was necessary to grow cells overnight in synthetic medium to allow good visualization of the spindles. When induction of genes from the *GAL1* promoter was required, overnight cultures were grown in YEP-raffinose. Synchrony was carried out in rich medium as described in the text.

RESULTS

Dosage suppressors of *pds1-128*. A genetic strategy was used to find proteins that functionally interact with Pds1p. We screened for genes whose increased dosage suppressed the temperature sensitivity of *pds1-128*. From a genomic *S. cerevisiae* library (maintained at high copy number in a yeast episomal vector), three dosage suppressors were identified: (i) *ESPI* (13 independent isolates, representing six plasmid types), (ii) *RAD23* (4 isolates, one plasmid type), and (iii) *DDI1* (8 isolates, two plasmid types). Since a genetic interaction between *ESPI* and *pds1* was previously reported (7), two novel suppressors of *pds1-128*, *RAD23* and *DDI1*, were identified (Fig. 1a).

pds1-128 cells become aneuploid when grown at the restrictive temperature, as a result of unequal nuclear division. Dosage suppression by *RAD23* and *DDI1* could result from a specific effect at the time of nuclear division or could improve cell viability in a less specific manner, not directly relevant to the function of Pds1p. To address this, cycle progression of *pds1-128* cells, with or without *RAD23* overexpression, was monitored by FACS analysis following release from G₁ (data not shown). While most *pds1-128* cells became aneuploid following nuclear division, the majority of cells overexpressing *RAD23* maintained 1C or 2C DNA contents. Similar results were obtained when *DDI1* was overexpressed. Therefore, dos-

age suppression of *pds1-128* at least partially corrected the primary functional defect in nuclear division at 37°C.

Specificity of *pds1-128* dosage suppression. Additional criteria were used to assess the specificity of the genetic interactions with *pds1-128*: first, whether the suppressors could rescue a *pds1* null mutant (*pds1*Δ); and second, whether genetic interactions with *esp1* (encoding a protein that physically interacts with Pds1p) could be detected (Fig. 1a and b).

Neither *DDI1* nor *RAD23* could rescue the inviability of *pds1*Δ cells at 28°C, the restrictive temperature of *pds1*Δ (strains harboring 2 μm plasmids or integrated versions of *RAD23* and *DDI1* induced from the *GAL1* promoter). Thus, high dosage of *DDI1* or *RAD23* cannot bypass the *pds1*Δ temperature sensitivity.

If Rad23p and Ddi1p are specific regulators of Pds1p, a genetic interaction with *esp1* mutants should be detectable. To test this, for this purpose, we constructed WT or *esp1* mutant strains which express *RAD23* or *DDI1* from the inducible *GAL1* promoter. Expression of either gene in WT cells had little effect on cell growth (not shown), but the lethality of strains with temperature-sensitive *esp1* alleles (*esp1-N5* and *esp1-B7* [13]) was enhanced by overexpression of *RAD23* or *DDI1* (Fig. 1b). This result can be rationalized in the context of the model described above; Pds1p inhibits the anaphase-promoting function of Esp1p, and Rad 23p and Ddi1p are putative positive regulators of Pds1p function. Therefore, high-dosage *RAD23* and *DDI1* would be expected to further compromise mutant *esp1* function, resulting in increased temperature sensitivity.

The genetic interactions of *RAD23* and *DDI1* with *pds1-128* were also examined in the context of Pds1p-dependent checkpoint pathways. The ability of *RAD23* or *DDI1* to rescue *pds1-128* temperature sensitivity in *mec1-1*, *rad53-1* or *chk1*Δ mutant backgrounds was examined (Fig. 1c). Experiments with the YE24-based plasmids, maintained at high copy number in yeast cells, or with *RAD23* and *DDI1* expressed from the *GAL1* promoter gave essentially the same results. *pds1-128 rad53-1* and *pds1-128 chk1*Δ cells were temperature sensitive at 37°C, as are *pds1-128* cells, and viability was restored by high dosage of the suppressors (not shown). Using the same analysis, *pds1-128 mec1-1* cells were only partly rescued by *RAD23* and *DDI1* overexpression (up to 35°C). *pds1-128 mec1-1* cells were temperature sensitive at 35°C, though *mec1-1* single mutants were not temperature sensitive. The inviability of *pds1-128 mec1-1* cells overexpressing *RAD23* or *DDI1* at 36°C suggests that rescue is partly dependent on Mec1p. Rad23p, Ddi1p, and Mec1p may function in a common pathway that targets Pds1p.

Dosage suppression of the *pds1-128* S-phase checkpoint defect. The genetic interactions of *RAD23* and *DDI1* with *pds1-128* were examined in the context of documented *pds1* checkpoint defects. We tested whether induction of *GAL1:RAD23* or *GAL1:DDI1* was able to suppress the sensitivity of *pds1-128* to nocodazole, gamma irradiation, or HU to examine whether Rad23p and Ddi1p could function in the spindle assembly, DNA damage, or S-phase checkpoint pathways. There was no suppression of the gamma irradiation sensitivity of *pds1-128* (data not shown). In contrast, a high-copy-number plasmid containing the *pds1-128* allele (YE-pds1-128) did rescue the gamma irradiation sensitivity. Thus, increasing the amount of the mutant Pds1-128p corrected the DNA damage checkpoint

TABLE 1. Yeast strain genotypes

Strain	Genotype
DCY1180.....	<i>MATa bar1Δ</i>
DCY1167.....	<i>MATα pds1-128</i>
DCY1283.....	<i>MATa bar1Δ rad23::KAN^R ddi1::KAN^R</i>
DCY1270.....	<i>MATa bar1Δ GAL1:RAD23(LEU2)</i>
DCY1278.....	<i>MATa bar1Δ GAL1:DDI1(LEU2)</i>
DCY1228.....	<i>MATa bar1Δ pds1::KAN^R</i>
DCY1234.....	<i>MATa bar1Δ pds1::KAN^R YEp-ESP1(URA3)</i>
DCY1235.....	<i>MATa bar1Δ pds1::KAN^R YEp-RAD23(URA3)</i>
DCY1236.....	<i>MATa bar1Δ pds1::KAN^R YEp-PDS1(URA3)</i>
DCY1237.....	<i>MATa bar1Δ pds1::KAN^R YEp-DDI1(URA3)</i>
DCY1233.....	<i>MATa bar1Δ pds1::KAN^R YEp24(URA3)</i>
DCY1195.....	<i>MATa pds1-128 YEp-DDI1(URA3)</i>
DCY1196.....	<i>MATa pds1-128 YEp-ESP1(URA3)</i>
DCY1199.....	<i>MATa pds1-128 YEp-RAD23(URA3)</i>
DCY1210.....	<i>MATa pds1-128 YEp-PDS1(URA3)</i>
DCY1790.....	<i>MATα pds1-128 YEp-pds1-128(URA3)</i>
DCY1213.....	<i>MATa pds1-128 YEp24(URA3)</i>
DCY1384.....	<i>MATa esp1::KAN^R YCp-esp1-N5(TRP1) GAL1:RAD23(LEU2)</i>
DCY1386.....	<i>MATa esp1::KAN^R YCp-esp1-B7(TRP1) GAL1:RAD23(LEU2)</i>
DCY1786.....	<i>MATa esp1-N5(TRP) GAL1:RAD23(LEU2)</i>
DCY1787.....	<i>MATa esp1-N5(TRP) GAL1:rad23^{Δuba2}(LEU2)</i>
DCY1788.....	<i>MATa bar1Δ esp1-N5(TRP) GAL1:DDI1-His₆(TRP1)::LEU2</i>
DCY1789.....	<i>MATa esp1-N5(TRP) bar1Δ GAL1:ddi1^{Δuba}-His₆(URA3)::LEU2</i>
DCY1668.....	<i>MATa esp1::KAN^R YCp-esp1-B7(TRP1) GAL1:DDI1(LEU2)</i>
DCY1264.....	<i>MATα bar1Δ rad23::KAN^R</i>
DCY1258.....	<i>MATα bar1Δ ddi1::KAN^R</i>
DCY1359.....	<i>MATα rad9::TRP1</i>
DCY1407.....	<i>MATa pds1-128 GAL1:DDI1(LEU2)</i>
DCY1272.....	<i>MATa bar1Δ pds1-128 GAL1:RAD23(LEU2)</i>
DCY1277.....	<i>MATa bar1Δ pds1::KAN^R GAL1:DDI1(LEU2)</i>
DCY1636.....	<i>MATa pds1::KAN^R GAL1:RAD23(LEU2)</i>
DCY1810.....	<i>MATa pds1-128 chk1::KAN^R GAL1:RAD23(LEU2)</i>
DCY1812.....	<i>MATa pds1-128 chk1::KAN^R GAL1:DDI1(LEU2)</i>
DCY1819.....	<i>MATα pds1-128 mec1-1 sml1Δ</i>
DCY1826.....	<i>MATa pds1-128 mec1-1 sml1Δ GAL1:DDI1(LEU2)</i>
DCY1824.....	<i>MATa pds1-128 mec1-1 sml1Δ GAL1:RAD23(LEU2)</i>
DCY1838.....	<i>MATa rad53-1 GAL1:RAD23(LEU2)</i>
DCY1836.....	<i>MATa pds1-128 rad53-1 GAL1:RAD23(LEU2)</i>
GY1492.....	<i>MATα pds1-128 GAL1:rad23^{Δuba2}-Myc₆(URA3, LEU2)</i>
GY1493.....	<i>MATα pds1-128 GAL1:DDI1-His₆(LEU2, TRP1)</i>
GY1494.....	<i>MATα pds1-128 GAL1:ddi1^{Δuba}-His₆(LEU2, URA3)</i>
DCY1671.....	<i>MATa bar1Δ GFP-TUB1::URA3</i>
DCY1672.....	<i>MATa bar1Δ pds1-128 GFP-TUB1::URA3</i>
DCY1679.....	<i>MATa bar1Δ rad23^{Δuba2}-myc₆::TRP1 ddi1^{Δuba}-His₆::LEU2 GFP-TUB1::URA3</i>
DCY1674.....	<i>MATa bar1Δ rad23::KAN^R ddi1::KAN^R GFP-TUB1::URA3</i>
DCY1859.....	<i>MATa pds1-128 rad53-1 YEp-DDI1(URA3)</i>
DCY1860.....	<i>MATa pds1-128 rad53-1 YEp-RAD23(URA3)</i>
DCY1858.....	<i>MATa pds1-128 rad53-1 YEp-PDS1(URA3)</i>
DCY1861.....	<i>MATa pds1-128 rad53-1 YCp50</i>
DCY1865.....	<i>MATa mec1-1 sml1 ΔYC50</i>
DCY1863.....	<i>MATa mec1-1 sml1Δ YEp-DDI1(URA3)</i>
DCY1864.....	<i>MATa mec1-1 sml1Δ YEp-RAD23(URA3)</i>
DCY1862.....	<i>MATa mec1-1 sml1Δ YEp-PDS1(URA3)</i>
DCY1869.....	<i>MATa pds1-128 mec1-1 sml1Δ YEp-DDI1(URA3)</i>
DCY1870.....	<i>MATa pds1-128 mec1-1 sml1Δ YEp-RAD23(URA3)</i>
DCY2078.....	<i>MATa bar1Δ rad23^{Δuba2}-Myc₆::TRP1 ddi1^{Δuba}-His₆::LEU2 CUP1:tetR-GFP(KAN^R) trp1::tetO(TRP1)</i>
DCY1662.....	<i>MATa bar1Δ CUP1:tetR-GFP(KAN^R) trp1::tetO(TRP1)</i>
DCY1866.....	<i>MATa pds1-128 mec1-1 sml1Δ YEp-PDS1(URA3)</i>
DCY1867.....	<i>MATa pds1-128 mec1-1 sml1Δ YEp24</i>

defect of these cells. Similarly, YEp-pds1-128 rescued the temperature sensitivity of *pds1-128* cells. By Western blot analysis, *pds1-128* cells have substantially less Pds1 than do WT cells (13). Therefore, in the context of the DNA damage checkpoint, *RAD23* or *DDI1* overexpression presumably cannot increase the amount of Pds1-128p. Overexpression of *RAD23* or

DDI1 was also unable to rescue the nocodazole sensitivity of *pds1-128* cells (data not shown). In this case, however, YEp-pds1-128 was similarly unable to rescue lethality caused by nocodazole treatment. Therefore, it was not possible to infer whether or not *RAD23* and *DDI1* overexpression affects Pds1-128p levels in the context of the spindle assembly checkpoint.

TABLE 2. Oligonucleotide used in plasmid or strain construction

PCR product	Oligonucleotide sequence (5'→3')
<i>RAD23</i> ORF for <i>GAL1</i> expression (5', <i>Bam</i> HI site)	CGGGATCCAAAAATGGTTAGCTTAACCTTT
<i>RAD23</i> ORF for <i>GAL1</i> expression (3', <i>Bam</i> HI site)	CGGGATCCAATCTCAGTCGGCATGATCGCT
<i>DDI1</i> ORF for <i>GAL1</i> expression (5', <i>Bam</i> HI site)	CGGGATCCAAAGATGGATTAAACAATTTCA
<i>DDI1</i> ORF for <i>GAL1</i> expression (3', <i>Bam</i> HI site)	CGGGATCCTTGATCATTGGAAAAGGAGGGA
Rad23p C terminus for tagging (5', <i>Sal</i> I site)	GTCGACCATCCGGTGCCTTGGCACAACCTG
Rad23p C terminus for tagging (3', <i>Not</i> I site)	GCGGCCGCCGTCGGCATGATCGCTGAATAG
Ddi1p C terminus for tagging (5', <i>Sal</i> I site)	GTCGACAATTATTGGGAGAATTCACCAAGC
Ddi1p C terminus for tagging (3', <i>Not</i> I site)	GCGGCCGCCCTTGGAAAAGGAGGGATGCAGC
Radp23p Δ UBA2 C terminus for tagging (3', <i>Not</i> I site)	GCGGCCCTCAATAGTCAACTTGGAAAAGAACCTTC
Ddi1p Δ UBA C terminus for tagging (3', <i>Not</i> I site)	GCGGCCCTCACGTTGTAGCACCGGCTGTACTTCT

In contrast, induction of *GAL1:RAD23* or *GAL1:DDI1* did suppress the sensitivity of *pds1-128* cells to HU (Fig. 2a). Growth on HU plates was also restored by YEp-*pds1-128* (not shown). Crucially, *GAL1:RAD23* and *GAL1:DDI1* could not rescue the HU sensitivity of *pds1* Δ strains, revealing that rescue was dependent on the presence of Pds1-128p. High-copy-number *RAD23* did increase Pds1-128p levels, and this effect was enhanced in the context of the S-phase checkpoint response (Fig. 2b). Moreover, overexpression of *RAD23* was not able to rescue the HU sensitivity of a *rad53-1* strain (another S-phase checkpoint-defective mutant), indicating that not all HU-sensitive checkpoint mutants are suppressed. The above experiments revealed that *RAD23* and *DDI1* are not dosage

suppressors of the DNA damage checkpoint defect of *pds1-128* mutant cells but can suppress the S-phase checkpoint defect. The data suggest that Rad23p and Ddi1p function specifically in a Pds1p-dependent S-phase checkpoint control.

Role of Rad23p and Ddi1p UBA domains in dosage suppression of *pds1-128*. One striking similarity between the C termini of Rad23p and Ddi1p is that both contain a UBA domain (Rad23p has a second, internally located UBA domain [Fig. 3a]). These are small domains (each about 10% of the full-length protein) of unknown function. Six UBA domain-containing proteins have been identified in budding yeast.

We tested whether the UBA domains of Rad23p and Ddi1p are required for suppression of *pds1-128*. Mutant versions of

FIG. 1. Dosage suppressors of *pds1-128*. (a) Suppression of *pds1-128* but not *pds1* Δ temperature sensitivity by episomal plasmids maintained at high copy number in yeast cells carrying YEp24(URA3), YEp-PDS1, YEp-RAD23, YEp-DDI1, and YEp-ESP1. Patches were grown at 25°C on synthetic medium containing dextrose but without uracil to prevent loss of the YEp24-based plasmids. These patches were replicated on to YEP-dextrose plates and grown at 25 to 37°C for 2 days. (b) Genetic interactions between *GAL1:RAD23* or *GAL1:DDI1* and temperature-sensitive *esp1* strains. Strains carrying *esp1* alleles (*esp1-N5* and *esp1-B7* [13]) with or without *RAD23* and *DDI1* alleles under control of the inducible *GAL1* promoter were grown at 30°C on YEP-dextrose plates, then replicated to YEP-galactose plates, and grown for 2 days at 30 to 35°C. (c) Suppression of *pds1-128 mec1-1* temperature sensitivity by episomal plasmids maintained at high copy number in yeast cells carrying YEp-PDS1, YEp-RAD23, YEp-DDI1, YEp24, and YEp50, grown as for panel a.

FIG. 2. Suppression of *pds1-128* S-phase checkpoint defects. (a) Suppression of *pds1-128* HU sensitivity by *RAD23* and *DDI1* overexpression. Strains were grown to midlog phase, and then serial dilutions were spotted on to YEP-dextrose or YEP-galactose plates (to repress or induce *GAL1:RAD23* and *GAL1:DDI1*) containing HU and grown for 2 to 3 days. (b) Overexpression of *RAD23* increases Pds1-128p levels in HU-treated cells. Cells containing a *GAL1:RAD23* gene and a hemagglutinin (HA) epitope-tagged *pds1-128* allele were grown to midlog phase (minus HU) or accumulated in S-phase with 100 mM HU for 3 h (plus HU) in dextrose (–) or galactose (+) medium to repress or induce *GAL1:RAD23*. Cellular protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Pds1-128-p-HA was detected after Western blotting. Cdc28p levels served as a loading control. A light exposure and a darker exposure of the same blot are shown.

RAD23 and *DDI1* that lack the C-terminal UBA domains (about 10% of each protein deleted) were created. To ensure that the mutant versions were stable in yeast cells, we added epitope tags at the C termini and confirmed endogenous expression to be at WT levels (or to be sufficiently elevated when under control of the *GAL1* promoter) by Western blotting (not shown). When expressed from the *GAL1* promoter, the mutant versions of Rad23, and Ddi1p (encoded by *GAL1:rad23^{Δuba2}* and *GAL1:ddi1^{Δuba}*) were unable to rescue the temperature sensitivity of *pds1-128*, though rescue was achieved by full-length versions of either protein (Fig. 3b). Suppression was also achieved by a Rad23p construct that lacked the N-terminal ubiquitin-like (UBL) domain and also by a Rad23p mutant lacking the internal UBA domain. Thus, the C-terminal UBA domains of Rad23p and Ddi1p seem to be the critical domains for *pds1-128* suppression. In the case of Ddi1p, we also constructed point mutants of the UBA domain. One such mutant,

L426A, could be expected to disrupt UBA integrity, based on structure prediction. In yeast two-hybrid experiments, this mutant was able to form Ddi1p-Ddi1p homodimers (B. L. Bertolaet and S. L. Reed, unpublished data), demonstrating that other than the UBA domain, the protein must be structurally intact. L426A did not, however, have the ability to suppress *pds1-128* temperature sensitivity (Fig. 3b). Thus, L426A specifically abolished the ability of Ddi1p to suppress *pds1-128*. The C-terminal UBA domains of Rad23p and Ddi1p were also required for enhancement of *esp1* temperature sensitivity (Fig. 3d and data not shown).

The ability of the mutant forms to rescue the HU sensitivity of *pds1-128* was also examined. Induction of *GAL1:ddi1^{Δuba}* was unable to rescue *pds1-128* HU sensitivity, whereas deletion of UBA2 from Rad23p produced a mutant that could only partially rescue the HU sensitivity (Fig. 3c). Therefore, the UBA domain of Ddi1p was essential for suppression of the *pds1-128* HU sensitivity, and deletion of the C-terminal UBA domain of Rad23p reduced the ability of Rad23p to rescue the HU sensitivity of *pds1-128* cells.

***rad23Δ ddi1Δ* null mutants are sensitive to HU and defective in cell cycle progression in the presence of HU.** The genetic and functional interactions of *RAD23* and *DDI1* with *pds1-128* suggested that Rad23p and Ddi1p may function in the S-phase checkpoint pathway and that the putative checkpoint function of Rad23p and Ddi1p depends on conserved UBA domains. It could therefore be expected that loss-of-function alleles of *RAD23* or *DDI1* would result in loss of S-phase checkpoint control. The common UBA domains of Rad23p and Ddi1p suggested that the two proteins may have some functional redundancy. We therefore constructed *RAD23* and *DDI1* deletion strains and tested their HU sensitivity. (Fig. 4a). *rad23Δ ddi1Δ* cells were as sensitive to HU as are *pds1-128* cells, though *rad23Δ* and *ddi1Δ* single mutants were either much less sensitive (*rad23Δ*) or not sensitive at all (*ddi1Δ*). *rad23Δ ddi1Δ* cells were not sensitive to gamma irradiation (Fig. 4b) or nocodazole (not shown).

To test whether *rad23Δ ddi1Δ* cells are S-phase checkpoint defective, a rebudding assay was performed (Fig. 5a). Cells released from G₁ following α -factor arrest were grown on solid medium containing 100 mM HU, and cell bodies per microcolony were counted. For WT *pds1-128* and *rad23Δ ddi1Δ* cells, budding occurred with very similar timing (not shown). The premature appearance of colonies containing three or more cell bodies (rebudding) would indicate an uncoupling of the normal timing of cell cycle progression from S phase. In these experiments, *pds1-128* cells rebudded before WT cells. However, if anything, *rad23Δ ddi1Δ* cells took longer to rebud than WT cells, suggesting a slight delay before or during mitosis (Fig. 5a). In a similar experiment, in which the strains were gamma irradiated before plating onto rich medium, WT and *rad23Δ ddi1Δ* cells rebudded with indistinguishable timing (Fig. 5b), whereas *pds1-128* cells rebudded before WT and *rad23Δ ddi1Δ* cells, due to the DNA damage checkpoint defect of this strain (Fig. 5b and reference 29).

To further examine the phenotype of *rad23Δ ddi1Δ* cells in the presence of HU, we directly compared S-phase progression with the onset of anaphase following release from G₁ (scoring budding, mitotic spindle formation using a *TUB1-GFP* construct, and DNA replication by FACScan analysis [Fig. 5c]).

FIG. 3. Suppression of *pds1-128* temperature and HU sensitivity depends on Rad23p and Ddi1p UBA domains. (a) Structural similarity between *RAD23* and *DDII*. Box diagrams show relative sizes of each ORF and the size and position of UBA, XPC, and UBL domains. C-terminal UBA domains (right) share 38% identity. The position of Ddi1p L426 is indicated in the alignment below. A hypothetical fission yeast protein (gb|Z69728 and gi|1204230) has an overall 32% identity to budding yeast Ddi1p. Probable Ddi1p homologues giving high identity scores are present in *Homo sapiens* (gb|AA406136 and gi|2064117; dbest database), *Caenorhabditis elegans* (gb|U50068 and gi|1208859) and *Leishmania major* (gb|AC002305 and gi|2429118). (b and c) Temperature sensitivity (b) and HU sensitivity (c) of *pds1-128* strains overexpressing *RAD23* and *DDII* or truncated versions of these genes expressing proteins lacking UBA domains or the *RAD23* UBL. Similar results were obtained when the WT or truncated proteins were tested as (Rad23p-MYC₆ and Ddi1p-His₆) fusions. Strains were handled as described in the legends to Fig. 1b and 2a. (d) Lack of genetic interaction between *GAL1:rad23^{Δuba2}* and temperature-sensitive *esp1*. Strains were handled as described in the legend to Fig. 1b.

Budding, spindle formation, and DNA replication (FACScan data not shown) occurred with similar timing in each strain. Short G₂ spindles were defined as those between 1 and 2 μ m long in these experiments. Strikingly, spindle elongation began in the *rad23 Δ ddi1 Δ* cells at the same time as in *pds1-128* cells, when about two-thirds of the DNA had been replicated. At this time, while spindles of WT cells remained 1 to 2 μ m in length, spindles of *rad23 Δ ddi1 Δ* cells elongated to 4 to 6 μ m. However, while spindles fully elongated (to 8 to 12 μ m) and then disassembled in a timely manner in *pds1-128* cells, *rad23 Δ ddi1 Δ* spindles remained about 4 to 6 μ m in length (mid-sized spindles), consistent with a midanaphase delay. *rad23 Δ ddi1 Δ* cells eventually elongated their spindles fully and exited mitosis (not shown), but later than WT cells did, in agreement with the rebudding experiments. These data suggest that *rad23 Δ ddi1 Δ* cells may be defective for restraining anaphase onset in HU but are unable to complete spindle elongation and exit mitosis rapidly.

Role of Rad23p and Ddi1p UBA domains in S-phase checkpoint control. The potential dual roles of Rad23p in nucleotide excision repair (NER) and checkpoint control might result in a somewhat ambiguous checkpoint-defective phenotype in the *rad23 Δ ddi1 Δ* mutant. Although these cells appeared to initiate anaphase before S phase was complete, they also appeared to be delayed for progression through the latter parts of mitosis. To resolve this issue, we attempted to create strains with mutant versions of Rad23p and Ddi1p that would be defective for checkpoint control but not compromised in NER or in the ability to progress through mitosis. We constructed strains in which endogenous *RAD23* and *DDII* were replaced with mutant versions lacking the C-terminal UBA domains (*rad23^{Δuba2}* and *ddi1^{Δuba}*), based on the genetic evidence that these mutant versions cannot suppress *pds1-128*. The UBA domain deletion alleles were epitope tagged to confirm that expression levels of the resulting products were similar to those of the WT proteins (not shown). We reasoned that since these domains are at least

FIG. 4. *rad23Δ ddi1Δ* cells are HU sensitive but not radiation sensitive. (a) Sensitivity of *pds1* and *rad23Δ ddi1Δ* mutants to HU. Strains were grown to midlog phase, then serial dilutions were spotted on to YEP-dextrose or YEP-dextrose plates containing HU, and growth continued for 2 to 3 days at 30°C. (b) Sensitivity of *pds1*, *rad23Δ*, *ddi1Δ*, and *rad9Δ* mutants to gamma irradiation. Strains were grown to midlog phase at 30°C in YEP-dextrose medium, irradiated, and then plated on YEP dextrose. Colonies were counted after 2 to 4 days.

partly required for suppression of *pds1-128* HU sensitivity, it may be possible to detect a checkpoint defect in strains with UBA domain deletions. Either a *rad23^{Δuba2}* or a *ddi1^{Δuba}* mutation showed only a marginal sensitivity to HU (not shown). In contrast, strains with both mutated alleles were HU sensitive (Fig. 6a), though not as sensitive as *pds1-128* strains. This suggested that *rad23^{Δuba2} ddi1^{Δuba}* cells might be partially defective in S-phase checkpoint regulation. *rad23^{Δuba2} ddi1^{Δuba}* cells were not sensitive to nocodazole, gamma irradiation, or UV irradiation (not shown), correlating with the failure of high dosage *RAD23* and *DDI1* to rescue the sensitivity of *pds1-128* to these agents and confirming that the *rad23^{Δuba2} ddi1^{Δuba}* strain is NER competent.

To determine whether *rad23^{Δuba2} ddi1^{Δuba}* cells are S-phase checkpoint defective, the rebudding assay was performed on medium containing 100 mM HU (Fig. 6b). WT, *pds1-128*, and *rad23^{Δuba2} ddi1^{Δuba}* cells budded with similar timing, but *pds1-128* and *rad23^{Δuba2} ddi1^{Δuba}* cells clearly rebudded prematurely, well before WT cells did. As a control, the rebudding assay was performed following gamma irradiation of *G₁* cells (as described for Fig. 5). In this case, the behavior of *rad23^{Δuba2} ddi1^{Δuba}* cells was indistinguishable from that of WT cells, demonstrating that the DNA damage checkpoint is intact in this mutant strain.

Next we compared S-phase progression with the onset of

FIG. 5. S-phase checkpoint defect of *rad23Δ ddi1Δ* mutants (a) Rebudding assay on HU plates. WT, *pds1-128*, and *rad23Δ ddi1Δ* cells were arrested in *G₁* at 30°C (α -factor), washed, and plated on YEP-dextrose plates containing 100 mM HU. Each strain budded with similar timing (not shown). Rebudding (the appearance of microcolonies containing three or more cell bodies) was scored at various time intervals (see also Fig. 6). (b) Rebudding assay following gamma irradiation. The procedure was as described for Fig. 5a. The ability of cells to rebud on YEP-dextrose plates following gamma irradiation in *G₁* was scored. (c) Timing of budding, *G₂* spindle formation, and spindle elongation in WT and mutant cells grown in liquid YEP-dextrose medium containing 100 mM HU following release from *G₁* (α -factor).

a **b**

Gamma irradiation

Hydroxyurea

c **d**

anaphase (measuring spindle elongation or loss of sister chromatid cohesion) following release from G₁ (Fig. 6c and d). Spindle formation and progression through S-phase occurred with similar timing in WT and *rad23^{Δuba2} ddi1^{Δuba}* cells. WT cells reached G₂ about 5 h following release from G₁ and then initiated anaphase ~30 to 40 min later. In contrast, spindle elongation in the *rad23^{Δuba2} ddi1^{Δuba}* mutant began earlier than in WT cells and before the cells reached a 2C DNA content. The *rad23^{Δuba2} ddi1^{Δuba}* mutant cells did not become delayed partway through spindle elongation, with 4- to 6-μm spindles, as was the case for the *rad23Δ ddi1Δ* mutant. Compared directly to *pds1-128* cells (not shown), anaphase began at an intermediate time in *rad23^{Δuba2} ddi1^{Δuba}* cells, later than in *pds1-128* cells but consistently earlier than in WT cells. When loss of sister chromatid cohesion was used as an indicator of anaphase onset, similar results were consistently obtained (Fig. 6d). These kinetics correlate well with the relative sensitivities of *rad23^{Δuba2} ddi1^{Δuba}* and *pds1-128* cells to HU and suggest that *rad23^{Δuba2} ddi1^{Δuba}* cells are partially defective in S-phase checkpoint control. The advancement of anaphase onset and of rebudding in the *rad23^{Δuba2} ddi1^{Δuba}* mutant clearly implicates the UBA domains of Rad23p and Ddi1p in cell cycle control.

DISCUSSION

Genetic interactions of *pds1-128* with *RAD23* and *DDI1*. The key role of Pds1p in M-phase cell cycle control has been well documented, but less is known about factors regulating Pds1p in response to checkpoint signals. We have used a genetic screen to identify proteins that interact with Pds1p. High dosage of *RAD23* or *DDI1* suppressed the temperature sensitivity of *pds1-128* and rescued the HU sensitivity of *pds1-128* at the permissive temperature. Neither the temperature sensitivity nor the HU sensitivity was rescued by *RAD23* or *DDI1* overexpression in *pds1Δ* strains; there was no bypass of the cellular requirement for Pds1p. In agreement, high-dosage *RAD23* and *DDI1* enhanced the temperature sensitivity of *esp1* mutants. This would be expected of proteins that positively regulate Pds1p, since Esp1p separin activity, required for progression through mitosis, is negatively regulated by Pds1p. These interactions implicate Rad23p and Ddi1p in promoting Pds1p-dependent functions. Moreover, *RAD23* and *DDI1* specifically corrected the S-phase checkpoint defect of *pds1-128* cells. Neither the gamma irradiation sensitivity nor the nocodazole sensitivity of *pds1-128* was rescued by overexpressing *RAD23* and *DDI1*. Thus, these data implicate Rad23p and Ddi1p in subset of the Pds1p-dependent checkpoint pathways.

UBA domains of Rad23p and Ddi1p are required for S-phase checkpoint control. Rad23p is a highly conserved protein with a NER function. The role of Rad23 in NER depends on binding to the NER complex via its XPC-binding domain and on an interaction with the proteasome cap via its UBL domain (9–11, 16, 17, 24, 28). Almost nothing is known about the function of Ddi1p. It has an upstream activation domain identical to that of *RAD23* and is induced by DNA damage and HU, but unlike *rad23Δ* mutants, *ddi1Δ* strains are not sensitive to UV light (data not shown) and are therefore not required for NER.

Since both Rad23p and Ddi1p have UBA domains at their C termini, we tested whether these domains are required for suppression of *pds1-128* defects. *rad23^{Δuba2} ddi1^{Δuba}* cells were not sensitive to gamma irradiation or nocodazole but were HU sensitive and underwent an advanced loss of sister chromatid cohesion and spindle elongation compared to WT cells when grown in the presence of 100 mM HU. *rad23^{Δuba2} ddi1^{Δuba}* mutant cells also exited mitosis and rebudded prematurely in the presence of 100 mM HU. It is not clear whether this rapid exit from mitosis could contribute to the HU sensitivity of these cells. Still, rapid exit from mitosis could be accounted for by deregulation of Pds1p proteolysis during anaphase in the *rad23^{Δuba2} ddi1^{Δuba}* mutant. As well as regulating anaphase onset, Pds1p plays a role in controlling exit from mitosis (1, 8, 15, 25). Although some Pds1p is degraded at the onset of anaphase (7), a subfraction of Pds1p remains and is localized on anaphase spindles (13). After anaphase onset, Pds1p inhibits exit from mitosis (5, 26). Thus, *rad23^{Δuba2} ddi1^{Δuba}* cells may be unable to efficiently regulate Pds1p proteolysis to control the onset of anaphase and to ensure a timely disassembly of anaphase spindles and subsequent exit from mitosis.

Regulation of Pds1p by Rad23p and Ddi1p? DNA damage, spindle perturbations, and replication inhibition increase Pds1p stability, which in turn inhibits the onset of anaphase. We have shown that *RAD23* overexpression increases the level of Pds1-128p in a manner that is enhanced by S-phase checkpoint activation. The proposal that Rad23p and Ddi1p regulate Pds1p stability is an attractive one given the dependence of rescue on UBA domains. The function of UBA domains is not known, though they are present in different classes of enzyme involved in ubiquitin-dependent proteolysis (12), an intriguing coincidence given the dependence of Pds1p proteolysis on the ubiquitin system. Rad23p also has a UBL domain at its N terminus; this domain was recently shown to be required for a physical interaction with 26S proteasomes, the particles that degrade ubiquitinated Pds1p to initiate the onset of anaphase. However, deletion of this domain did not abrogate the ability

FIG. 6. S-phase checkpoint defect of *rad23^{Δuba2} ddi1^{Δuba}* mutant cells. (a) Sensitivity of *rad23^{Δuba2} ddi1^{Δuba}* mutant cells to HU. Strains were grown to midlog phase, then serial dilutions were spotted onto YEP-dextrose or YEP-dextrose plates containing HU, and growth continued for 2 to 3 days at 30°C. (b) Rebudding assay on HU plates or following gamma irradiation (see legend to Fig. 5 for details). Microcolonies were scored based on the number of cell bodies per microcolony. (c) Timing of short spindle formation, S-phase progression, and spindle elongation in WT and mutant cells grown in liquid YEP-dextrose medium containing 100 mM HU following release from G₁ (α-factor). S-phase progression was monitored by FACScan analysis; histogram plots show DNA content throughout the experiment (in grey), and overlaid solid lines indicate positions of 1C and 2C DNA peaks taken from the asynchronous cultures prior to G₁ arrest. The percentage of the total cells that had elongated spindles above each histogram. (d) Timing of loss of sister chromatid cohesion in WT and *rad23^{Δuba2} ddi1^{Δuba}* mutant cells grown in liquid YEP-dextrose medium containing 100 mM HU following release from G₁ (α-factor). Both strains budded and replicated DNA with similar timing (FACScan analysis not shown), but loss of cohesion occurred prematurely in the mutant cells. Cohesion was monitored at the *TRP1* locus using the *tetR-GFP/tetO* system as previously described (4).

FIG. 7. Model for control of Pds1p stability. Pds1p can become polyubiquitinated by APC^{Cdc20}, a multienzyme E3 ubiquitin ligase. The ubiquitin chain enables a 26S proteasome to recognize and degrade Pds1p. These events must occur prior to the onset of anaphase, since Pds1p inhibits the activity of separin Esp1p, needed for anaphase spindle elongation and loss of sister chromatid cohesion. To block anaphase onset, checkpoint controls prevent Pds1p degradation. The spindle assembly checkpoint directly inhibits APC^{Cdc20}, and the DNA damage checkpoint is thought to promote Pds1p stability by a Chk1p-dependent phosphorylation of Pds1p. We propose a novel mechanism for anaphase control, in which Rad23p and Ddi1p are able to recognize ubiquitinated Pds1p, or the APC (not shown), and thereby inhibit the ubiquitin chain elongation that is required for targeting Pds1p to the 26S proteasome.

of Rad23p to rescue *pds1-128* when overproduced, and other data implicate the UBL domain in the NER function of Rad23p (21, 28). An alternative model for suppression of *pds1-128* by *RAD23* and *DDI1* was recently suggested by the finding that both Rad23p and Ddi1p could bind to ubiquitin and that this interaction was dependent on the UBA domains (Bertolaet and Reed, unpublished). These data make it tempting to propose an intriguing mechanism for Rad23p- and Ddi1p-dependent checkpoint regulation. Rad23p and Ddi1p could regulate Pds1p stability by binding to ubiquitinated Pds1p or to the ubiquitin ligase complex that ubiquitinates Pds1p via the UBA domains (Fig. 7). Indeed, a recent report described a novel activity of Rad23p as an inhibitor of ubiquitin chain elongation (19). Rad23p was shown to bind to a mono- or a diubiquitinated substrate and prevent extension of the ubiquitin chain. A similar interaction with ubiquitinated Pds1p, or the ubiquitin ligase complex, stimulated by ongoing DNA replication, may provide one mechanism through which the S-phase checkpoint is enforced. How UBA domains participate in such control systems is currently being addressed.

In summary, we propose that Rad23p and Ddi1p function in Pds1p-dependent S-phase checkpoint control in budding yeast. Rad23p and Ddi1p are likely to be universal checkpoint proteins. Their homologues in mammals and other eukaryotes are highly conserved, particularly within the UBA domains. Our data provide a first clue as to the cellular function of UBA domains in eukaryotes.

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