Ddb1 controls genome stability and meiosis in fission yeast

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The human UV-damaged DNA-binding protein Ddb1 associates with cullin 4 ubiquitin ligases implicated in nucleotide excision repair (NER). These complexes also contain the signalosome (CSN), but NER-relevant ubiquitination targets have not yet been identified. We report that fission yeast Ddb1, Cullin 4 (Pcu4), and CSN subunits Csn1 and Csn2 are required for degradation of the ribonucleotide reductase (RNR) inhibitor protein Spd1. Ddb1-deficient cells have >20-fold increased spontaneous mutation rate. This is partly dependent on the error-prone translesion DNA polymerases. Spd1 deletion substantially reduced the mutation rate, suggesting that insufficient RNR activity accounts for ~50% of observed mutations. Epistasis analysis indicated that Ddb1 contributed to mutation avoidance and tolerance to DNA damage in a pathway distinct from NER. Finally, we show that Ddb1/Csn1/Cullin 4-mediated Spd1 degradation becomes essential when cells differentiate into meiosis. These results suggest that Ddb1, along with Cullin 4 and the signalosome, constitute a major pathway controlling genome stability, repair, and differentiation via RNR regulation.

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Correct regulation of the availability of deoxynucleotide triphosphate (dNTP) is important for genome integrity. In budding yeast, each S phase requires the activation of the ribonucleotide reductase (RNR) enzyme that catalyses dNTP formation (Huang and Elledge 1997; Huang et al. 1998). This in turn requires activation of the DNA-integrity-checkpoint pathways, and checkpoint mutants are inviable because they fail to degrade the RNR inhibitor Sml1 when cells are in S phase (Zhao et al. 1998). In response to genotoxic stress, genes encoding RNR are transcriptionally induced in a checkpoint-dependent manner. However, elevated nucleotide pools cause increased mutation rates in vivo (Chabes et al. 2003), suggesting that fine tuning of dNTP levels is important for the fidelity of DNA synthesis.

In *Schizosaccharomyces pombe*, the COP9/signalosome (CSN) is required for RNR activation prior to DNA synthesis and in response to DNA damage (Liu et al. 2003). Active RNR is a heterotetramer of two large (Cdc22) and two small (Suc22) subunits (Fernandez-Sarabia et al. 1993). Outside of S phase, RNR assembly is inhibited by a small protein Spd1, which anchors Suc22 in the nucleus, away from Cdc22 in the cytoplasm. When cells enter S phase, Spd1 gets ubiquitinated and degraded, and Suc22 is transported to the cytoplasm to form active complexes with Cdc22. Mutants lacking the signalosome subunits Csn1 or Csn2, or the Cullin 4 homolog Pcu4, fail to degrade Spd1 upon S-phase entry, and so Suc22 remains in the nucleus, causing slow S-phase progression and sensitivity to DNA-damaging agents (Liu et al. 2003).

The CSN is a conserved multiprotein complex required for proper development in multicellular organisms (Cope and Deshaies 2003). Genetic and biochemical evidence suggest CSN functions as both a positive and a negative regulator of Cullin-based ubiquitin ligases. CSN mutants are defective in Cullin-based ubiquitination (Cope and Deshaies 2003), suggesting a role in correct modulation of these activities. Conversely, purified CSN can cleave the stimulatory Nedd8 modification (termed neddylation) from Cullins (Lyapina et al. 2001). Fission yeast cells lacking CSN subunits accumulate Cullins in their active, neddylated state (Zhou et al. 2001; Mundt et al. 2002). Furthermore, both human and fission yeast CSN copurify with deubiquitinating activi-

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ties (Groisman et al. 2003; Zhou et al. 2003), suggesting CSN may also prevent Cullin-based ubiquitination under certain circumstances.

In fission yeast, the signalosome can be isolated as a complex, which also contains Cullin 4 (Pcu4) and Ddb1 (Liu et al. 2003). Ddb1 is the fission yeast ortholog of human p127 DDB1, which together with the WD-repeat protein p48-DDB2 forms a UV-damaged DNA-binding heterodimer (UV-DDB) implicated in nucleotide excision repair (NER) (see below). A second Ddb1-like protein, Rik1, seems to be present only in fission yeast and is required for heterochromatin formation and meiotic telomere clustering (Ekwall et al. 1996; Nakayama et al. 2001; Tuzon et al. 2004). The Ddb1 family of proteins contain putative RNA-binding regions (Neuwald and Poleksic 2000; Dichtl et al. 2002), but the function of these has not been defined. Direct sequence orthologs of Ddb1 and Cullin 4 are not present in *Saccharomyces cerevisiae*, suggesting that this entire pathway may have been lost or changed during evolution.

Two distinct, yet related, DDB1 complexes containing CSN and Cullin 4 were recently purified from human cells (Groisman et al. 2003). One contained DDB2, while the other contained another WD40-repeat protein CSA. DDB2 is mutated in xeroderma pigmentosum group E (XP-E) patients, who are hypersensitive to UV-light, predisposed to a range of cancers, and suffer defective global genomic NER (GG-NER) (see Wittschieben and Wood 2003), and defective UV-induced apoptosis (Itoh et al. 2003, 2004). Mutation of CSA causes Cockayne syndrome, a transcription-coupled (TC-) NER defect (for review, see Svejstrup 2002).

The dynamic behavior of the human DDB1 complexes is consistent with the idea that the CSN modulates Cullin-based ubiquitination. Upon UV-irradiation, the CSN leaves the DDB2 complex, which then becomes tightly associated with chromatin. At the same time, Cullin 4 becomes activated by neddylation and presumably ubiquitinates substrates important for GG-NER. Subsequently, CSN reassociates and Cullin 4 becomes deneddylated. siRNA knockdown of CSN subunit 5 significantly decreases GG-NER, suggesting a positive role for CSN in this process. Conversely, CSN is only recruited to the CSA complex following UV-irradiation, presumably inactivating Cullin 4, and suggesting that suppression of ubiquitination is important for the execution of TC-NER (Groisman et al. 2003). Taken together, these observations suggest that DDB1 functions to regulate protein ubiquitination, although the genetic evidence for this is still circumstantial.

Deletion of *S. pombe ddb1* has been reported to confer chromosome segregation defects, mild UV-sensitivity, and slow S-phase progression (Zolezzi et al. 2002; Bondar et al. 2003). Here, we show that fission yeast Ddb1 is required together with the signalosome and Pcu4 for degradation of Spd1 following DNA checkpoint activation. We demonstrate that *ddb1* loss is synthetically lethal with loss of the DNA-damage checkpoint genes *cds1*, *chk1*, and *rad3*, and that these colethalities are readily suppressed by deletion of *spd1*. Importantly, we find that *ddb1* and *csn1* mutants have an increased spontaneous mutation rate, in part due to a deprived dNTP pool. Interestingly, the elevated mutation rates are also partially suppressed by inactivating translesion DNA polymerases. Finally, we demonstrate that mutants in *ddb1*, *csn1*, and *pcu4* are completely unable to undergo meiotic differentiation, since these cells fail to enter premeiotic S phase. This defect is suppressed by Spd1 loss, or alternatively, by Suc22 overexpression, consistent with the notion that these mutants suffer from insufficient levels of DNA building blocks.

Results

Δddb1 *cells have a mutator phenotype distinct from NER and MMR*

Since human DDB1 has been implicated in NER, we tested the sensitivity to UV and methyl methane sulphonate (MMS) of fission yeast cells deleted for *ddb1* (Fig. 1). Δ*ddb1* cells are mildly sensitive to UV and highly sensitive to MMS. In contrast, NER-deficient Δ*swi10* cells (lacking the ortholog of human ERCC1 excision nuclease (Rödel et al. 1992) are very sensitive to UV, but only modestly sensitive to MMS. Double-mutant Δ*ddb1* Δ*swi10* cells showed increased sensitivity to both UV and MMS when compared with either of the single mutants. These findings suggest that Ddb1 is important for the cellular responses to damaged DNA, but unlikely to have a significant function in NER.

We next tested whether Ddb1 functions in mutation avoidance by measuring the spontaneous reversion rates of the *ade6* alleles *485* and *(GTCC)$_2$-1399*. The *485* allele reverts to *ade*$^+$ via base substitution (Fleck et al. 1999), while *(GTCC)$_2$-1399* reverts by deletion or insertion of nucleotides (Marti et al. 2003b). The mutation rates of both *ade6* alleles were increased >20-fold in Δ*ddb1* mutants when compared with *ddb1*$^+$ (Table 1), suggesting that the function of Ddb1 is important for preventing mutations. NER and mismatch repair (MMR) are two of the important mutation-avoidance pathways in fission yeast (Rödel et al. 1992; Fleck et al. 1999; Rudolph et al. 1999). We thus examined the reversion rates of double mutants Δ*ddb1* Δ*swi10* (NER) and Δ*ddb1* Δ*msh2*

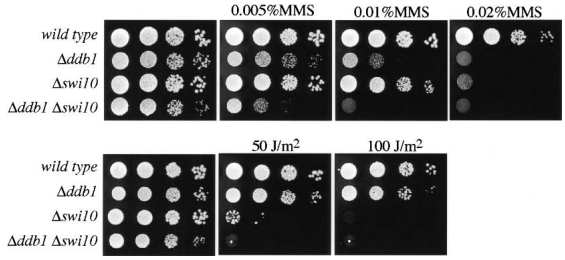


Figure 1. DNA-damage sensitivity of Δ*ddb1* is nonepistatic to nucleotide excision repair. Strains of indicated genotypes were spotted in 10-fold dilutions and either left untreated (*left*), grown on MMS containing plates as indicated (*top*), or exposed to UV-C irradiation (*bottom*).

Table 1. *Mutation rates*

Genotype	*485* Rate ($\times 10^{-9}$)	*485* Increase	*(GTCC)$_2$* Rate ($\times 10^{-10}$)	*(GTCC)$_2$* Increase
wild type	3.2 (0.8)	1	1.6 (0.9)	1
Δ*ddb1*	75 (13)	23	58 (15)	36
Δ*msh2*	58 (12)	18	16 (6.4)	10
Δ*swi10*	24 (5.7)	7.5	13 (9.9)	8.1
Δ*csn1*	18 (3.6)	5.6	ND	
Δ*spd1*	2.0 (1.2)	0.6	ND	
Δ*ddb1* Δ*msh2*	252 (54)	79	ND	
Δ*ddb1* Δ*swi10*	146 (40)	46	183 (28)	114
Δ*ddb1* Δ*csn1*	69 (35)	22	ND	
Δ*ddb1* Δ*spd1*	37 (15)	12	15 (4.0)	9.4
Δ*csn1* Δ*spd1*	7.6 (1.3)	2.4	ND	
Δ*swi10* Δ*spd1*	21 (4.8)	6.6	ND	
Δ*ddb1* Δ*rev3*	39 (3.1)	12	40 (9.1)	25
Δ*ddb1* Δ*dinB*	ND		33 (14)	21
Δ*ddb1* Δ*rev3* Δ*dinB*	ND		21 (11)	13
Δ*ddb1* Δ*rev3* Δ*spd1*	24 (6.3)	7.5	27 (4.1)	17

Reversion rates of the *ade6* alleles *485* and *(GTCC)$_2$* in the various backgrounds are mean values of at least three experiments with standard deviations in parenthesis (see Materials and Methods). Fold increases are relative to wild type. (ND) Not determined.

(MMR). Both exhibited higher mutation rates than either single mutant (Table 1), suggesting that Ddb1 does not prevent mutations by participating in MMR or NER.

We determined the mutation spectra of *ade6-485* reversions in the various backgrounds. In wild-type cells, *485* reverted at about the same frequencies by G-to-C, G-to-T, A-to-G, or A-to-C base substitutions (Supplementary Table S1). In contrast, the Δ*ddb1* mutant almost exclusively showed G-to-C and A-to-G events. This pattern is different from that of Δ*swi10* and Δ*msh2*, further supporting the idea that Ddb1 defines a novel mutation-avoidance pathway in fission yeast.

Ddb1 is required for Spd1 degradation

Ddb1 copurifies with the CSN and Pcu4, in a protein complex required for degradation of the RNR inhibitor Spd1 (Liu et al. 2003). This prompted us to test whether Ddb1 is required together with Csn1 and Pcu4 for degradation of Spd1 in response to S-phase arrest. Figure 2A shows that Δ*ddb1* cells—similar to Δ*csn1* and Δ*pcu4* cells—are defective for Spd1 degradation following treatment with 20 mM hydroxyurea (HU). Consistent with Ddb1 being required for Spd1 degradation, concomitant *spd1* deletion improved the growth rates of Δ*ddb1*, Δ*csn1*, and Δ*pcu4* cells, and partially suppressed their elongated cellular appearance (data not shown). Flow cytometry for DNA content revealed a significant sharpening of the broad G2-peaks observed in Δ*csn1* and Δ*ddb1* single mutants, suggesting that their slow S-phase progression was suppressed by deletion of *spd1* (Fig. 2B; Liu et al. 2003). In Δ*pcu4*, which shows the most severe phenotype of the three, concomitant *spd1* deletion only marginally improved the flow cytometry profile. Thus, Δ*pcu4* cells suffer additional problems in cell cycle progression.

Since Spd1 is thought to inhibit RNR, and hence, to keep the deoxunucleotide pool low, we directly tested whether Δ*ddb1* cells have reduced pools. We ^{32}P-labeled asynchronously growing cells and extracted nucleotides in formic acid and resolved the individual nucleotide triphosphates by thin-layer chromatography. As shown in Supplementary Figure S1 and summarized in Table 2, the deoxy-pools are approximately threefold decreased in Δ*ddb1*, an effect that is suppressed by deletion of *spd1*. This relatively moderate reduction in pool sizes was expected, since only ~10% of asynchronously growing cells are in S phase.

Δddb1 *is synthetically lethal with loss of the DNA-structure checkpoints in an* spd1$^+$*-dependent manner*

Cells deleted for *csn1* and *csn2* are synthetically lethal with loss of any of the DNA-structure checkpoint genes *rad3*, *cds1*, and *chk1*, and this colethality is suppressed by overexpression of the small RNR subunit Suc22 (Mundt et al. 1999; Liu et al. 2003). The DNA-structure checkpoints are not essential under normal conditions (Caspari and Carr 1999), but become required for progression through S phase when the availability of dNTP is limiting. To establish whether Δ*ddb1* cells require DNA structure checkpoints for viability, we analyzed tetrads from crosses of Δ*ddb1* with Δ*rad3*, Δ*cds1*, and Δ*chk1*. In all three crosses, we failed to recover double-mutant colonies (Fig. 3), and double-mutant cells survived only a few divisions (data not shown). Thus, like *csn1* and *csn2* mutants, Δ*ddb1* is colethal with loss of either the S phase or the G2 DNA-damage checkpoint. When we made the same crosses in *spd1*-deleted backgrounds, Δ*ddb1* checkpoint double mutants were readily recovered (Fig. 3). We conclude that the inability to degrade

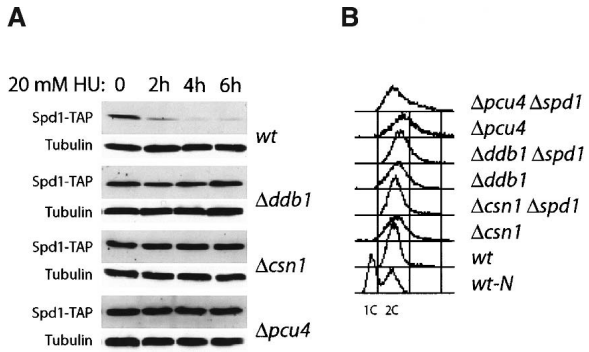


Figure 2. Δ*ddb1*, Δ*csn1*, and Δ*pcu4* are unable to degrade Spd1 in response to hydroxy-urea (HU). (*A*) TCA extracts of cells expressing Spd1-TAP tag and growing exponentially or treated with 20 mM HU for 2, 4, and 6 h were subjected to Western blotting. (*B*) DNA content flow cytometry profiles of exponentially growing cultures as indicated. As control for G1 and G2 peaks (1C and 2C DNA content) the profile of wild-type cells starved for nitrogen for 6 h was included.

Table 2. *Nucleotide pools*

	Wild type	Δ*ddb1*	Δ*ddb1* Δ*spd1*	Δ*spd1*
ATP	1	1	1	1
GTP	0.373	0.343	0.377	0.309
CTP	0.051	0.081	0.063	0.071
UTP	0.080	0.125	0.079	0.101
dATP	0.0156	0.0059	0.0180	0.0265
dGTP	0.0114	0.0037	0.0130	0.0169
dCTP	ND	ND	ND	ND
dTTP	0.0046	0.0016	0.0078	0.0135

Relative amounts of the nucleotide triphosphates as normalized to the values obtained for ATP. dCTP has not been determined (ND), since we failed to detect signals above background for this nucleotide. The results have been reproduced in an independent experiment.

Spd1 makes Δ*ddb1* cells strictly dependent on intact DNA structure checkpoints. Consistent with this, we found that cells without Ddb1 have elevated Cds1 kinase activity (data not shown), as also reported previously (Bondar et al. 2003).

spd1 *deletion partially suppresses sensitivity to DNA damage in* Δddb1 *and* Δcsn1

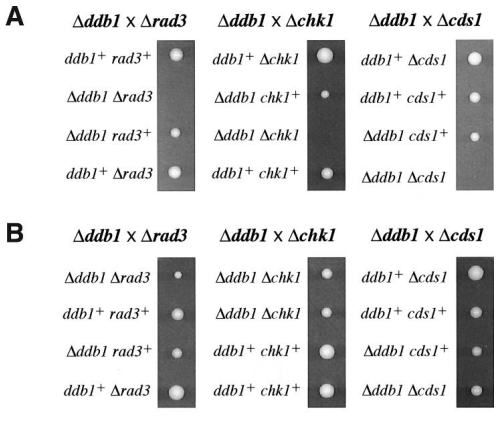
Since loss of *spd1* largely suppressed the slow S-phase profile and damage sensitivity of Δ*csn1* (Liu et al. 2003) and the DNA structure checkpoint dependency of the Δ*ddb1* mutant (this study), we speculated that *spd1* loss might also affect the DNA damage sensitivity of Δ*ddb1*. In Figure 4A we show that the mild UV sensitivity of Δ*ddb1* and Δ*csn1* cells at a relatively high dose of 160 J/m^2 was almost completely suppressed by deletion of *spd1*. In contrast, the UV sensitivity of NER-defective Δ*swi10* cells was not suppressed by *spd1* inactivation (Supplementary Fig. S3). Thus, the inability to activate RNR by Spd1 degradation upon severe UV damage causes lethal events at an elevated rate, at least partly due to lack of dNTP for DNA synthesis during repair and replication. In contrast, *pcu4*-deleted cells were more sensitive than Δ*ddb1* and Δ*csn1*, and their sensitivity was not suppressed by *spd1* loss, suggesting that Pcu4 controls additional aspects of the UV-damage response.

MMS treatment causes a variety of lesions, including DNA-strand breaks, and all three mutants were found to be highly sensitive to this drug (Fig. 4B); Δ*pcu4* cells showed the strongest sensitivity and, again, this was only marginally suppressed by lack of Spd1. Cells deleted for *ddb1* were slightly less sensitive than Δ*pcu4*, and elimination of Spd1 significantly suppressed this at the lower dose used (0.005% MMS), whereas less suppression was observed at 0.01% MMS. Finally, *csn1*-deleted cells were the least sensitive of the three, and here, we observed the best suppression by *spd1* deletion. Analysis of the double-mutant Δ*ddb1* Δ*csn1* indicated that *ddb1* and *csn1* belong to the same epistasis group with respect to MMS sensitivity (Fig. 4B). Taken together, these observations suggest that Ddb1/Pcu4/Csn1-mediated degradation of Spd1 is important in response to moderate levels of DNA damage. After more severe damage, other Ddb1/Pcu4-mediated reactions that do not require the signalosome become necessary.

Lack of Spd1 partially suppresses the mutator phenotype

Given these results, we next asked whether the mutator phenotype of Δ*ddb1* cells also involves inability to degrade Spd1. First, we tested whether Csn1 was part of this mutation-avoidance pathway. Indeed, Δ*csn1* cells showed a significantly increased reversion rate of *ade6-485*, albeit not as high as that observed in Δ*ddb1* cells (Table 1). The Δ*ddb1* Δ*csn1* mutant had about the same reversion rate of *ade6-485* as Δ*ddb1*, suggesting an epistatic relationship between Δ*ddb1* and Δ*csn1*, also in mutation avoidance. However, since mutation rates of Δ*ddb1* cells were significantly higher than that of Δ*csn1*, Ddb1 likely contributes to genomic stability, also through mechanisms that do not depend on the CSN. Due to their very poor plating efficiency, we have been unable to measure mutation rates in Δ*pcu4* cells.

We reasoned that the inability of Δ*ddb1* and Δ*csn1* cells to degrade Spd1 might create mutations via repli-



C

Cross	TT	NPD
Δ*ddb1* X Δ*rad3*	4	0
Δ*ddb1* X Δ*chk1*	7	0
Δ*ddb1* X Δ*cds1*	4	1
Δ*ddb1* Δ*spd1* X Δ*rad3* Δ*spd1*	6	2
Δ*ddb1* Δ*spd1* X Δ*chk1* Δ*spd1*	5	3
Δ*ddb1* Δ*spd1* X Δ*cds1* Δ*spd1*	4	4

Figure 3. Deletion of *spd1* suppresses synthetic lethality of Δ*ddb1* with loss of the DNA-damage checkpoint. (*A*) Examples of tetratype tetrads of the crosses indicated. Genotypes are indicated at *left*, and they demonstrate that coloss of *ddb1* and the DNA-damage checkpoint is lethal. (*B*) As in *A*, except all mutants used were also deleted for *spd1*. (*C*) Table summarizing the number of tetrads analyzed from the crosses indicated at *left*, all with the outcome that deletion of *ddb1* is colethal with deletion of *rad3*, *chk1*, and *cds1* in a manner suppressed by deletion of *spd1*. (TT) Meiotic segregation of the mutant alleles into tetra-type asci, i.e., all four spores have different genotypes; (NPD) nonparental ditypes, i.e., the mutant alleles cosegregate.

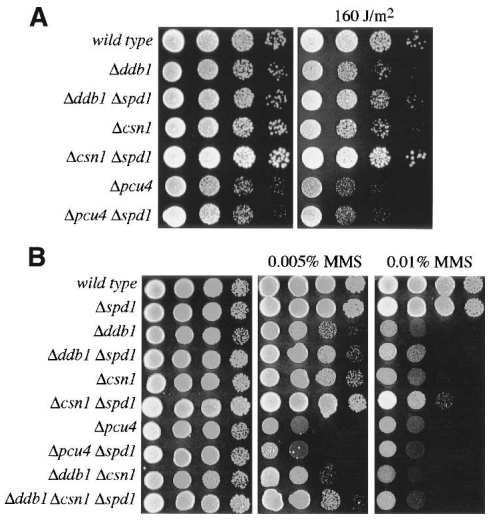


Figure 4. Deletion of *spd1* suppresses sensitivity of Δ*ddb1* and of Δ*csn1* to DNA damage. Survival tests of 10-fold dilution series of the indicated strains. (*A*) Right after spotting, plates were either left unirradiated or exposed to 160 J/m^2 of UV-C light. (*B*) The strains were spotted onto plates containing 0%, 0.005%, or 0.01% MMS as shown.

cation errors caused by insufficient dNTP pools. Consistent with this idea, we found that the *ade6-485* reversion rates of the Δ*ddb1* and Δ*csn1* mutants were reduced by ~50% by deletion of the *spd1* gene (Table 1). However, with significant mutator activity left in both double mutants, most notably in Δ*ddb1* Δ*spd1*, it is clear that Ddb1 and Csn1 are required for other aspects of mutation avoidance than for controlling RNR activation through Spd1 degradation.

Involvement of translesion DNA synthesis

We next tested whether translesion synthesis (TLS) was involved in the increased mutation rate of Δ*ddb1* cells. Unlike the replicative DNA polymerases, TLS polymerases can bypass certain types of lesions in DNA, but in doing so, they often incorporate erroneous nucleotides, thereby creating mutations (Friedberg et al. 2002; Hubscher et al. 2002). We tested the effects of mutating *rev3*, encoding the catalytic subunit of PolZ and *dinB*, coding for the Y-polymerase PolK (Wang 2001; Friedberg et al. 2002; Lehmann 2002; Kai and Wang 2003). Inactivation of one or both TLS polymerases partially suppressed the mutator phenotype of Δ*ddb1* by ~50% (Table 1). Thus, a considerable proportion of mutational events in Δ*ddb1* cells depend on active TLS. Interestingly, mutation rates in the triple-mutant Δ*ddb1* Δ*rev3* Δ*spd1* were not, or only slightly further reduced when compared with the double-mutants Δ*ddb1* Δ*spd1* and Δ*ddb1* Δ*rev3*, suggesting an epistatic relationship between the Δ*spd1* and Δ*rev3* mutants in this context. In contrast to Δ*ddb1* cells, the mutator phenotype of NER-defective Δ*swi10* cells was unaffected by *spd1* inactivation (Table 1), but is suppressed to wild-type level in the Δ*rev3* background (Marti et al. 2003a).

In the *(GTCC)$_2$* reversion assay, the Δ*ddb1* mutant exhibited a mutational hotspot of C_2 to C_1 1-bp deletions, as well as some complex events, both of which were not found in the wild type (Supplementary Fig. S2). The complex events represented deletion or insertion of nucleotides accompanied by one or more base substitutions in the vicinity. Such events were not observed in the double-mutants Δ*ddb1* Δ*rev3* and Δ*ddb1* Δ*dinB* or the triple mutant Δ*ddb1* Δ*dinB* Δ*rev3*, suggesting that they were due to error-prone TLS opposite spontaneous damage. However, the complex events were still observed in the Δ*ddb1* Δ*spd1* strain, again consistent with Ddb1 being required for other aspects of mutation avoidance than Spd1 degradation.

The C_2 to C_1 hotspot remained in the Δ*ddb1* double mutants with concomitant deletion of *rev3*, *dinB*, or *spd1* (Supplementary Fig. S2). However, when considering the mutation rates, deletion of *spd1* clearly reduced their occurrence, while no major effect could be seen by deleting Δ*rev3* and/or Δ*dinB* (Supplementary Table 2). Taken together, these results suggest that Ddb1 loss causes mutations both by Spd1-dependent and independent mechanisms; for both types translesion polymerases can be involved.

Spd1 degradation is required for entry into meiosis

So far in this report, we have focused on the damage/repair aspects of the Ddb1/Pcu4/Csn1 complex. However, CSN has been implicated in several developmental processes, and it has been proposed that its activity is crucial when cells must rapidly modify their physiology in response to signals received from the environment (Wei and Deng 2003). We therefore examined whether the Ddb1 pathway was required when *S. pombe* cells undergo sexual differentiation. In response to nitrogen starvation, fission yeast cells first conjugate to form diploid zygotes, which then undergo meiosis and sporulation (Nielsen 2004; Yamamoto 2004). While homothallic h^{90} strains deleted for *csn1*, *ddb1*, or *pcu4* did conjugate, we found that the resulting zygotes were completely unable to undergo meiosis (Fig. 5; Table 3). Genetic analysis revealed that only *csn1* and *csn2* were required for meiosis, while *csn3–csn7* were not (data not shown). Thus, the same two CSN subunits that control Spd1 degradation are also required for entry into meiosis.

We next investigated whether Spd1 loss could restore meiosis in these mutant cells. As shown in Figure 5 and Table 3, *ddb1*, *csn1*, and *pcu4* mutant strains readily underwent meiosis and sporulation when the *spd1* gene had been deleted. Suc22 overproduction also suppressed the meiotic defects of these mutants (Table 3). The restored meiosis was phenotypically normal with wild-type recombination frequencies (Supplementary Fig. S4). Taken together, these findings show that Csn1, Csn2, Ddb1, and Pcu4 all function in the same pathway to liberate Suc22 from Spd1 repression during meiotic differentiation.

To identify where meiosis was blocked, we developed a meiotic synchronization procedure based on the obser-

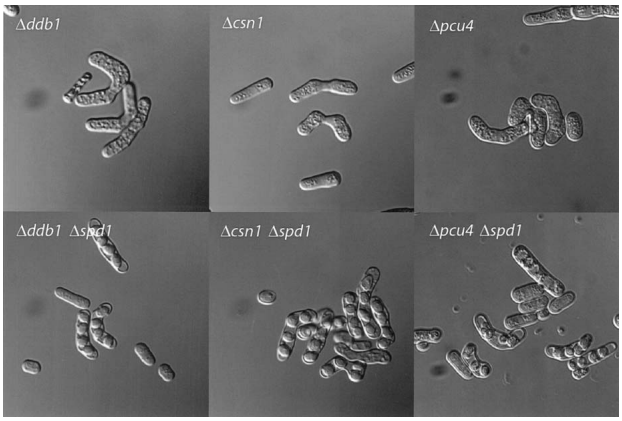


Figure 5. Deletion of *spd1* suppresses the complete meiotic failure of Δ*ddb1*, Δ*csn1*, and Δ*pcu4*. Microphotographs of h^{90} mutant strains as indicated after 4 d on plates inducing mating and meiotic differentiation.

vation that stable diploid h^+/h^- cells mutated in the *mat1-Mc* gene require M-factor pheromone in order to enter meiosis (Willer et al. 1995). When a growing culture of such cells was transferred to nitrogen-free medium containing M-factor, cells first rapidly accelerated into mitosis consistent with earlier reports (Fantes and Nurse 1977; Stern and Nurse 1997). This gave rise to a transient accumulation of G1 cells, which then directly entered meiosis. Following this procedure, >95% of wild-type cells had completed premeiotic S phase after 5 h (Fig. 6A, first row). If entry into meiosis was blocked by the *mat1-Mm* mutation (Willer et al. 1995), all of the cells arrested in G1 (Fig. 6B, second row), showing that they could no longer enter mitotic S phase.

Analysis of diploid Δ*ddb1*/Δ*ddb1* cells by this procedure was obscured by their slow S phase, reflected in very broad flow cytometry profiles of DNA content (Fig. 2B; data not shown). We therefore studied the meiotic block in a Δ*csn1* mutant background, where the DNA profile is less severely affected. Diploid Δ*csn1*/Δ*csn1* cells still responded to the meiotic conditions by accumulating in G1, but little or no entry into premeiotic DNA synthesis was apparent during the course of the experiment (Fig. 6A, third row). Mutating *spd1* or overproducing Suc22 restored entry into premeiotic S phase with almost wild-type kinetics in both Δ*csn1*Δ/*csn1* and Δ*ddb1*/Δ*ddb1* (Fig. 6A; data not shown).

Next, we looked at Spd1 protein levels in cells induced to undergo meiosis. Spd1 was barely detectable in growing wild-type cells, but its level increased during the first 2 h following the shift to meiotic conditions, only to decrease again as the cells entered premeiotic S phase (Fig. 6B). This pattern suggests that premeiotic S phase—similar to mitotic S phase (Liu et al. 2003)—is accompanied by degradation of the Spd1 protein. In the Δ*csn1*/Δ*csn1* cells, the level of Spd1 was not reduced; rather, it gradually increased during the meiotic conditions. In cells suppressed for the meiotic defect by Suc22 overexpression, the basal Spd1 content was relatively high and stayed high during meiotic progression, suggesting the existence of a positive feedback between high Suc22 and Spd1. In Δ*ddb1*/Δ*ddb1* diploids, the level of Spd1 was higher than in Δ*csn1*/Δ*csn1* and stayed high during meiotic conditions, in agreement with the meiotic block being caused by failure to degrade Spd1.

To compare directly the level of Spd1 in the different strains, we ran the 5-h sample from each experiment on the same gel (Fig. 6C). This confirmed that Δ*csn1*/Δ*csn1* and Δ*ddb1*/Δ*ddb1* diploid cells arrest before premeiotic S phase with elevated levels of Spd1 protein. We conclude that Δ*csn1*/Δ*csn1* and Δ*ddb1*/Δ*ddb1* mutant cells arrest before or in early premeiotic S phase, due to defects in down-regulation of Spd1. Hence, meiotic DNA replication is absolutely reliant on the ability to degrade the RNR inhibitor Spd1 by a Ddb1-dependent process.

Discussion

The human p127 DDB1 protein has attracted considerable interest because of its implication in DNA repair reactions, and recently, DDB1 was shown to be a central component of two related Cullin 4-based E3 ubiquitin ligases (Groisman et al. 2003). One complex contained the DDB2 protein, which is defective in patients suffering from the GG-NER disease XP-E, while the other contained CSA, a protein function lacking in patients with the TC-NER disease CS. Hence, Cullin 4-mediated ubiquitination reactions seem important for the execution of nucleotide excision repair, but no substrates for these reactions have yet been identified. Both E3 complexes also contain the COP9/signalosome, suggesting that the CSN may modulate the spatial and/or temporal activity of these ligases.

Spd1 as a major target for Ddb1

In the present study, we have examined the function of fission yeast Ddb1. We provide evidence that in this system, the protein also collaborates with the Cullin 4 homolog Pcu4 and the CSN in ubiquitin-mediated protein degradation, suggesting an evolutionary conservation of this pathway. We have identified the RNR inhibitor protein Spd1 as a major critical target. All of the phenotypes

Table 3. *Frequency of spore containing zygotes (%)*

h^{90} strains	2 d	4 d
wild type	74	97
Δ*ddb1*	0	0
Δ*ddb1* Δ*spd1*	62	84
Δ*ddb1 adh-suc22*$^+$	47	82
Δ*csn1*	0	0
Δ*csn1* Δ*spd1*	66	75
Δ*csn1 adh-suc22*$^+$	54	76
Δ*pcu4*	0	0
Δ*pcu4* Δ*spd1*	21	46
Δ*csn5*	72	81

Percentage of zygotes developed into asci after 2 or 4 d incubation on plates supporting mating and meiosis. Genotypes are indicated on the left side.

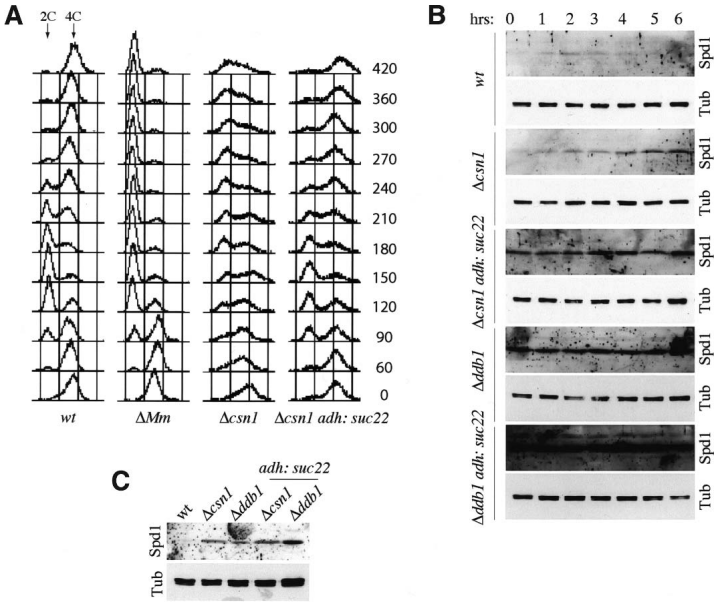


Figure 6. Pre-meiotic S-phase defect correlates with failure to down-regulate Spd1. (*A*) DNA content profiles of diploids as indicated shifted to meiotic conditions at time 0 with samples taken up to 420 min as shown at *right* of the profiles. (*B*) Western blots for Spd1 in the diploids induced to undergo meiosis with samples taken every hour up to 6 h. Anti-tubulin blots were included as loading control. Blots were run and developed in parallel to ensure direct comparability. (*C*) Western blot for Spd1 using the 6-h time-point samples from *B* to demonstrate on the same gel the higher levels of Spd1 in the Δ*ddb1*/Δ*ddb1* and Δ*csn1*/Δ*csn1* strains.

we have found associated with Ddb1 loss can, to a certain extent, be suppressed by deletion of *spd1*.

Our results demonstrate that Ddb1/Pcu4/Csn1-mediated degradation of Spd1 defines a new damage-response pathway important for genome integrity in fission yeast. Failure to degrade Spd1 causes slow S-phase progression, sensitivity to DNA-damaging agents, and increased mutation rates. In addition, survival of unperturbed cells becomes reliant on intact DNA structure checkpoints. Finally, the ability to degrade Spd1 is absolutely necessary when cells switch to the alternative developmental program of meiosis. This result suggests that meiotic S phase is more sensitive to Spd1-mediated RNR inhibition than mitotic S phase, which does proceed, albeit more slowly than normal. Consistent with this, it was previously reported that the *cdc22-45* mutant carrying a temperature-sensitive allele in the gene for the large RNR subunit prevented premeiotic S phase at intermediate temperatures that still allowed mitotic S phase (Grallert and Sipiczki 1991).

Our observations suggest that the phenotypes associated with defective Spd1 degradation during DNA replication and repair are due to insufficient dNTPs levels. Thus, meiosis could be completely restored in diploid *ddb1*/*ddb1* cells by overexpression of the small RNR subunit Suc22. Similarly, the radiation sensitivity and dependency on checkpoints of *csn1* cells could be rescued by Suc22 overexpression (Liu et al. 2003). Our measurements confirmed that Δ*ddb1* cells have reduced dNTP pools, and that deletion of *spd1* could restore the pools above wild-type levels.

The involvement of Ddb1 in Spd1 degradation was anticipated from the observation that Ddb1 copurifies with the Pcu4/Csn complex (Liu et al. 2003), and a recent study has reported the requirement for Ddb1 in this process (Bondar et al. 2004). However, the same group arrived at a conclusion entirely different from ours regarding the relationship between Ddb1 and the DNA structure checkpoint pathway. Whereas we found that Δ*ddb1* cells die in an Spd1-dependent manner in the absence of Rad3, Chk1, or Cds1, Bondar et al. (2003) reported that Cds1 loss suppressed the slow S-phase progression and sensitivity to DNA damage of Δ*ddb1* cells. We do not know the basis for this discrepancy.

Although Spd1 clearly is a key substrate for the Ddb1/Pcu4 pathway, it is evident from the incomplete suppression by *spd1* loss we observe—especially of the mutator phenotype and the MMS sensitivity—that other targets exist. Recently, it was shown that Cullin 4 prevents rereplication in *Caenorhabditis elegans* by ubiquitination of the licensing protein Cdt1 (Zhong et al. 2003). Furthermore, in both HeLa and *Drosophila* cells, Cullin 4, Ddb1, and the CSN are required for degradation of Cdt1 following irradiation (Higa et al. 2003; Hu et al. 2004). Thus, the picture emerging is that Cullin 4-mediated ubiquitination reactions play an important role in maintenance of genome integrity at the G1–S transition and during repair. In budding yeast, where direct sequence orthologs of Ddb1 and Cullin 4 are absent, the checkpoint protein Rad53 controls RNR activity. It is possible, though, that less-obvious orthologs exist in this yeast to maintain vestigial Ddb1/Cullin 4-like functions, e.g., mutation of a cullin-like encoding gene *rtt101* confers increased MMS sensitivity (Laplaza et al. 2004).

Human DDB1 was initially identified, together with the WD-40 repeat protein p48 DDB2, as an activity that associates tightly to UV-damaged DNA. Subsequently, NER-deficient XP-E patients were shown to be unable to form this complex, due to mutational inactivation of the *DDB2* gene (for review, see Tang and Chu 2002). The role of UV-DDB in NER is unclear. Albeit not directly required for reconstituted NER on naked DNA in vitro, UV-DDB does stimulate NER in such an assay (Aboussekhra et al. 1995; Mu et al. 1995). It has been

proposed that UV-DDB may function to assist the NER complex in gaining access to lesions in chromatin (Tang and Chu 2002), but other studies have shown that DDB1/DDB2 does not stimulate excision from nucleosomal DNA in vitro (Hara et al. 2000). The *S. pombe* genome contains no apparent DDB2 ortholog, and our genetic results suggest that Ddb1 functions in an epistasis group different from NER with respect to DNA-damage sensitivity and spontaneous mutation rates.

Fine tuning of dNTP levels is important for genome stability

We have provided evidence that Δ*ddb1* cells have a strong mutator phenotype. The reversions we observed include both base substitutions and small insertion/deletions at highly increased frequency. As *spd1* deletion suppressed the reversion frequencies to approximately half, our results suggest that a reduced nucleotide pool may be directly mutagenic. The fact that *spd1* deletion appeared largely epistatic with inactivation of the DNA translesion polymerase PolZ in the mutation analysis, indicates that insufficient availability of dNTPs may favor recruitment of error-prone TLS polymerases, perhaps to extend stalled forks of the normal replicative polymerases. Indeed, it has been shown previously that replicational stress causing checkpoint activation promotes mutagenic translesion synthesis (Kai and Wang 2003). The increased dNTP pools in the *spd1* background (Table 2) may be sufficient to circumvent checkpoint activation and the recruitment of translesion synthesis; hence, additional deletion of *rev3* would reduce the mutation rate only a little further. This model is in agreement with our observation that viability of Δ*ddb1* cells is dependent on the checkpoint kinases only in the presence of Spd1.

It will be interesting to learn whether the Ddb1 pathway also contributes to genome stability via activation of RNR in other systems like human cells. In budding yeast, it was—oppositely—demonstrated that an elevated dNTP pool could be mutagenic (Chabes et al. 2003), emphasizing the importance of proper RNR regulation for maintenance of genomic integrity. Our results may be important in cancer biology. Many cancer cells have constitutively high RNR activity due to inactivation of the pRB pathway (Angus et al. 2002), but we hypothesise that other cancer cells may adapt to a mutator strategy involving down-regulation of RNR and, hence, a slow, mutagenic S phase. Indeed, despite clear oncogenic effects of RNR overexpression, recent studies suggest that compromised RNR activity may also be common in human cancers, as the gene encoding the large RNR subunit R1 is located in the 11p15.5 region, which frequently displays one-allelic loss in solid tumors (for review, see Yen 2003).

Materials and methods

Strains construction

Δ*ddb1* was generated by transforming a wild-type strain with a linearized construct containing ~500 bp of 5′- and 3′-UTR on either side of the G418 resistance cassette for replacement of the entire *ddb1* open reading frame, and was used for all experiments except for the spontaneous mutation assays, where we used a *ddb1*-disrupted strain. Stable diploid *mat1-P/mat1-Mc* strains for synchronization of meiosis were generated by protoplast fusion essentially as described by Willer et al. (1995). In order to eliminate haploidization by chromosome loss caused by Δ*csn1* and Δ*ddb1* (Mundt et al. 2002; Zolezzi et al. 2002), complementing *ade6-M210/ade6-M216* allele pairs were included in all diploid strains.

Test systems to measure mutation rates

Mutation rates of the *ade6* alleles *485* and *(GTCC)$_2$-1399* were determined by fluctuation tests as described (Kunz and Fleck 2001; Marti et al. 2003b). Allele *485* is a C-to-G transversion that reverts to Ade$^+$ via base substitutions (Fleck et al. 1999); *(GTCC)$_2$-1399* is an insertion of a GTCC tetra nucleotide at an existing GTCC sequence at position 1399 of the *ade6* gene (the ATG start codon is at position 875). The insertion causes a frameshift and a premature stop codon in *ade6* and reverts to Ade$^+$ through 1-bp/4-bp net deletions or 2-bp/5-bp net insertions at or around the GTCC insertion (Marti et al. 2003b). Each fluctuation test included seven or nine cultures grown in 1.5 mL of YEL in the case of *485*, and 15 cultures grown in 5 mL of YEL in the case of *(GTCC)$_2$-1399*. Mutation rates were determined by the method of the median or from the proportion of cultures without Ade$^+$ (Luria and Delbrück 1943; Lea and Coulson 1949). For each genetic background, fluctuation tests were carried out at least three times. The nature of Ade$^+$ revertants was determined by sequencing of single-stranded DNA derived from PCR fragments as described (Kunz and Fleck 2001; Mansour et al. 2001).

Western blots

Protein extracts were made by the TCA precipitation method (Caspari et al. 2000). Spd1 was detected using anti-Spd1 polyclonal antibodies awo-1 (Woollard et al. 1996) diluted 1:200. Spd1-TAP was detected on Western blots using HRP-coupled antibodies from rabbit (DakoCytomation, cat. #P0161) diluted 1:3000. For loading control, we used anti-Tubulin monoclonal antibodies (Woods et al. 1989) diluted 1:3000. Primary antibodies were detected using HRP-coupled secondary antibodies diluted 1:5000. Blots were developed with ECL+ (for Spd1-TAP and Spd1) and with ECL (for Tubulin) from Amersham according to instructions.

Induction of meiosis

Synchronous meiosis was induced in the following way: Stable diploid *mat1-P/mat1-Mc* cells of the indicated genotype were grown in MSL medium (Egel et al. 1994) at 30°C to a density of 5×10^6 cells/mL, and then transferred at the same density to MSL without nitrogen and containing 1 μg/mL of synthetic M-factor (supplied by Schafer-N) (Wang et al. 1994). A detailed account for this synchronization procedure will be published elsewhere.

Flow cytometry

Samples for flow cytometry were fixed and stained with propidium iodine as described by Pereira and Jones (2001) with the following modification: Prior to RNase treatment, the cells were incubated with 1 mg/mL of pepsin in 0.1 N HCl for 1 h at

room temperature. The cells were analyzed on a Beckton Dickinson FACScan.

Nucleotide pool measurements

For labeling, 200 µCi of ^{32}P was added to a 0.5-mL culture containing 4×10^6 cells in MSL medium buffered with acetic acid and containing 50 µM cold phosphate. After 5 h, 400 µL was transferred to 50 µL of prechilled 26.5 M formic acid, followed by three freeze–thaw cycles. Cell debris was removed by centrifugation and 100 µL spotted onto a polyethyleneimine plate to separate nucleotides in two dimensions as described (Jensen et al. 1979). First-dimension thin-layer chromatography was developed in 0.85 M KH_2PO_4 adjusted to pH 3.4 with equimolar H_3PO_4. Following chromatography, the plates were washed twice in 10% (wt/vol) citrate and twice in water. The dried plates were developed in the second dimension in 0.75 M LiCl/7.5% H_3BO_3 adjusted to pH 6.8 by addition of solid LiOH. The plates were quantified using a Storm PhosphorImager, and the spots were indentified by addition of cold trinucleotides.

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