Cop9/signalosome subunits and Pcu4 regulate ribonucleotide reductase by both checkpoint-dependent and -independent mechanisms

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The signalosome is implicated in regulating cullin-dependent ubiquitin ligases. We find that two signalosome subunits, Csn1 and Csn2, are required to regulate ribonucleotide reductase (RNR) through the degradation of a small protein, Spd1, that acts to anchor the small RNR subunit in the nucleus. Spd1 destruction correlates with the nuclear export of the small RNR subunit, which, in turn, correlates with a requirement for RNR in replication and repair. Spd1 degradation is promoted by two separate CSN-dependent mechanisms. During unperturbed S phase, Spd1 degradation is independent of checkpoint proteins. In irradiated G2 cells, Spd1 degradation requires the DNA damage checkpoint. The signalosome copurifies with Pcu4 (cullin 4). Pcu4, Csn1, and Csn2 promote the degradation of Spd1, identifying a new function for the signalosome as a regulator of Pcu4-containing E3 ubiquitin ligase.

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The COP9 signalosome (CSN) complex was originally identified as a negative regulator of photomorphogenesis in plants (for review, see Schwechheimer and Deng 2001). Subsequently, it was purified from human cell extracts during attempts to isolate the 19S regulatory lid complex of the proteasome (Seeger et al. 1998). The human signalosome consists of eight core subunits, each sharing significant homology with a corresponding subunit in the regulatory 19S lid complex of the proteasome (Deng et al. 2000). The purified CSN complex can cleave the ubiquitin-like Nedd8 protein from cullins (Lyapina et al. 2001; Wee et al. 2002). Csn5 contains a putative metalloprotease motif that is presumed to mediate deneddylation activity (Cope et al. 2002). Cullins are subunits of E3 ubiquitin ligases (Feldman et al. 1997; Skowyra et al. 1997), and deneddylation of cullins decreases SCF E3 ubiquitin ligase activity (Osaka et al. 2000). SCF E3 complexes typically consist of a cullin, the Rbx1 RING domain protein that binds an E2 enzyme [Kamura et al. 1999; Skowyra et al. 1999], and an adapter protein, Skp1, that binds an F-box protein that determines the substrate specificity [Skowyra et al. 1997].

In Arabidopsis, the signalosome is involved in the degradation of the two bZIP transcription factors [Hy5, HyH] that lie at the top of a transcriptional cascade required to induce ~30% of Arabidopsis genes during photomorphogenesis (Holm et al. 2002). An E2-like protein, Cop10, and an E3 RING protein, Cop1, are also required to degrade Hy5 and HyH, which occurs when seedlings are germinated in the dark (Osterlund et al. 2000; Holm et al. 2002; Suzuki et al. 2002). The biochemical role of the signalosome is unknown, although a correlation with Cop1 nuclear localization (von Arnim et al. 1997) and the associations between the signalosome and E3 ubiquitin ligases [Lyapina et al. 2001; Schwechheimer et al. 2002] suggest a regulatory role in ubiquitination that may be linked to subcellular localization [Chamovitz et al. 1996; Hellmann and Estelle 2002].

A highly conserved signalosome complex was identified in the fission yeast Schizosaccharomyces pombe (Mundt et al. 1999) and subsequently shown to be required to remove the Nedd8 ubiquitin-like protein from the cullins Pcu1 and Pcu3. Despite the fact that null mutants in csn1, csn2, csn3, csn4, and csn5 each cause constitutive Nedd8 modification of Pcu1 and Pcu3, only...
csn1-d and csn2-d mutants demonstrate obvious phenotypes (Zhou et al. 2001; Mundt et al. 2002). These include slow S-phase progression and a modest sensitivity to DNA-damaging agents. This suggested that some of the functions associated with the CSN complex could be independent of its role in removing Nedd8 from cullins. We have used both the slow S-phase phenotype and the DNA-damage sensitivity specific to csn1-d and csn2-d strains to investigate this novel biological role for the signalosome. Our results lead us to propose a regulatory function for the CSN complex in Pcu4-dependent ubiquitin ligase activity. We propose that the cullin Pcu4 is indirectly required for activation of ribonucleotide reductase through degradation of the protein Spd1, thereby supplying sufficient deoxyribonucleotides for DNA replication and repair.

Results

A link between the signalosome, ribonucleotide reductase, and Spd1

csn1-d- and csn2-d-null mutants are slow growing and spend an extended period of time in S phase (Mundt et al. 1999). Consistent with a defect in DNA replication, csn1-d and csn2-d are synthetically lethal with the loss of rad3 function. Rad3 is a PI3-like protein kinase homologous to Saccharomyces cerevisiae Mec1 and human ATR (Bentley et al. 1996). In Schizosaccharomyces pombe, Rad3 is required for all DNA-structure-dependent checkpoints. In response to S-phase perturbations, Rad3 activates the DNA replication checkpoint kinase Cds1, whereas upon DNA damage in late S/G2, Rad3 acts through the Chk1 kinase (Lindsay et al. 1998; Martinho et al. 1998). Cells harboring the rad3-ts allele are checkpoint-proficient at 27°C and checkpoint-deficient at 35.5°C. We previously reported that rad3-ts csn1-d double-mutant cells rapidly lose viability and accumulate evidence of mitotic catastrophe upon a temperature shift from 27°C to 35.5°C [Mundt et al. 1999].

To identify the cause of the slow S phase in signalosome mutants, we screened for multicopy suppressors of rad3-ts csn1-d lethality at 35.5°C. This analysis identified multiple independent clones of suc22 that efficiently rescued the growth defect of rad3-ts csn1-d cells. suc22 encodes the small subunit of S. pombe ribonucleotide reductase (RNR). Active RNR is a tetrameric enzyme composed of two small subunits (Suc22) and two large subunits (Cdc22) that generates deoxyribonucleotides. To confirm the suppression, we integrated a second suc22 gene at the ura4 locus, with its transcription driven by the constitutively active ADH promoter. Overexpression of suc22 (100-fold increase in Suc22 protein) allowed csn1-d cells to progress normally through S phase [Fig. 1A] without activating Cds1 kinase, an indication of checkpoint activation [Fig. 1B]. suc22 overexpression also complemented the UV- and IR-sensitivity of csn1-d cells (data not shown, cf. Fig. 4D, below). These data suggest a role for the signalosome in the regulation of RNR activity that is important for S-phase progression and DNA repair.

Figure 1. Suppression of Csn1 S-phase delay. (A) FACS analysis of csn1-d cells overexpressing suc22 from an integrated ADH promoter or deleted for spd1, an inhibitor of S phase (Woollard et al. 1996). Control nitrogen-starved cells with a G1 and G2 peak are shown at the top [Wt-N]. Logarithmically growing wild-type [Wt] cells show a G2 peak, csn1-d cells show a broad peak indicative of a delay in S phase (Mundt et al. 1999). Overexpression of suc22 or deletion of spd1 restores a normal FACS profile in csn1-d cells. Deletion of spd1 alone does not affect the FACS profile. (B) Cds1 kinase activity is suppressed by either suc22 overexpression or spd1 deletion. The same strains as used in A were tested for in vitro kinase activity using equal amounts of immunoprecipitated Cds1 against MBP as substrate. Cds1 derived from csn1-d cells is constitutively active because of the delay in S phase (Mundt et al. 1999). Either suc22 overexpression or spd1 deletion suppresses the constitutive activity as well as reducing the extent of the activation caused by treatment with 10 mM HU.

In S. cerevisiae, the essential function of MEC1 can be alleviated by the overexpression of a large RNR subunit or by the deletion of SML1, which encodes a 104-amino-acid inhibitor of RNR (Desany et al. 1998; Zhao et al. 1998; Chabes et al. 1999). rad3 is not an essential gene in S. pombe, but becomes essential in csn1-d cells. This essential function can be suppressed by overexpression of an RNR subunit [Fig. 1; data not shown]. This similarity between S. pombe csn1-d mutants and S. cerevisiae led us to explore the possibility that an Sml1-like inhibitor exists in S. pombe. We predicted that loss of such an inhibitor might rescue rad3-ts csn1-d cells at 35.5°C and its overexpression should delay cell cycle progression in S phase and be toxic to checkpoint-defective cells. Such a gene, spd1 (S-phase delayed), encoding a small protein (124 amino acids), has previously been characterized as a negative regulator of S phase in fission yeast (Woollard et al. 1996), and its overexpression is toxic to checkpoint-defective cells (Borgne and Nurse 2000). Although there is no significant sequence homology between Sml1 and Spd1, both proteins are small (104 and 124 amino acids, respectively) and predicted to contain similar α-helical regions. We therefore combined the deletion of spd1 with either csn1-d [Fig. 1] or csn2-d [data not shown]. In both cases, loss of spd1 phenocopied suc22 overexpression in csn1-d cells: a normal cell cycle

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profile was restored (Fig. 1A), endogenous activation of Cds1 was suppressed (Fig. 1B), and the lethality of csn1-d in an rad3-defective mutant background was alleviated (data not shown).

**Dynamic changes in RNR localization during S phase are Csn1- and Spd1-dependent**

Our data suggest that Spd1 inhibits RNR activity and that the CSN complex is required to release RNR from this inhibition during DNA replication or repair. To explore the mechanism by which Spd1 regulates RNR, we studied Suc22 by Western blot and indirect immunofluorescence. The suc22 gene expresses two transcripts (1.5 and 1.9 kb), both of which encode the same size protein. The 1.5-kb transcript is constitutively expressed, whereas the 1.9-kb transcript is only expressed at low levels during S phase and is undetectable in other cell cycle stages in unperturbed cells. However, in response to DNA damage or hydroxyurea (HU) treatment (which blocks cells in early S phase), the 1.9-kb transcript is induced (Harris et al. 1996). Surprisingly, no significant changes in Suc22 protein level were detected during S phase in synchronized cells or in cells blocked in S phase by HU treatment (Fig. 2A). However, Suc22 is predominantly nuclear throughout the cell cycle, but the nuclear localization of Suc22 decreases during unperturbed S phase (Fig. 2B,C) and is lost in cells exposed to HU (Fig. 3B). We next asked if loss of Suc22 nuclear staining during unperturbed S phase was dependent on Rad3. Consistent with the lack of an essential function for Rad3 in *S. pombe*, rad3-null mutants showed a similar Suc22 localization profile as wild-type cells. This indicates that Rad3 is not required to regulate Suc22 during the normal unperturbed cell cycle (Fig. 2D).

In contrast to rad3-d, deletion of csn1 (or csn2, data not shown) resulted in constitutive nuclear localization of Suc22 with no decrease during S phase [Fig. 3A], even when cells were arrested with HU for 2 h [Fig. 3B]. In spd1-d cells we did not observe any specific nuclear localization of Suc22 at any stage in the cell cycle [Fig. 3A]. A similar profile is seen in spd1-d csn1-d double-mutant cells [data not shown]. These data suggest an antagonistic role for Spd1 and the signalosome in RNR regulation: Spd1 maintains Suc22 in the nucleus, whereas the signalosome appears to assist in its nuclear export.

**Suc22 relocalization requires active nuclear export**

If crm1-dependent nuclear export is required for Suc22 loss from the nucleus, cells treated with Leptomycin B (LMB), an inhibitor of crm1-dependent nuclear export, should retain Suc22 nuclear staining after HU treatment. Indeed, this is the case [Fig. 3B,C]. However, although we see crm1-dependent loss of nuclear Suc22 in HU-treated cells, we do not observe an accompanying increase in the cytoplasmic signal. Our anti-Suc22 antibody was raised to a C-terminal peptide. This region of the small RNR...
subunit is tightly associated with the large subunit in active mammalian RNR complexes (Lycksell et al. 1994). Thus, the lack of cytoplasmic staining likely reflects epitope masking in the cytoplasm. To test this possibility, we examined Suc22 localization after HU treatment using an N-terminal TAP-tagged Suc22 (Werler et al. 2003). Loss of TAP-Suc22 from the nucleus was accompanied by the simultaneous appearance of TAP-Suc22 in the cytoplasm (Fig. 3D). If the Suc22 localized in the cytoplasm is not detectable with our α-Suc22 antibody because of epitope masking, this suggests that the large subunit of RNR (Cdc22) is resident in the cytoplasm. We have been unable to obtain a polyclonal antibody to Cdc22, but using a strain expressing GFP-Cdc22 (Fig. 3E) from the cdc22 locus and under the control of the native cdc22 promoter, we, indeed, observed that Cdc22 is constitutively localized throughout the cell, without obvious exclusion from, or concentration in, the nucleus.

RNR localization in response to the G2 DNA damage checkpoint

csn1-d and csn2-d cells are DNA-damage-sensitive. To ascertain the behavior of Suc22 following DNA damage, we first analyzed Suc22 localization after treatment of wild-type cells or chk1-d mutant cells with 100 J/m² of UV light (Fig. 4A) or with 250 Gy of IR (data not shown). Irradiation of cells synchronized in G2 resulted in a rapid chk1-dependent loss of nuclear Suc22 staining. Similar analysis of asynchronous cell populations (Fig. 4B) shows this effect is also dependent on rad3 and csn1, but not on csn4. Pretreating cells with LMB (Fig. 4B) before irradiation shows that Suc22 is exported from the nucleus in a signalosome- and checkpoint-dependent manner, presumably to achieve RNR activation in response to DNA damage.

If the relocalization of Suc22 from the nucleus to the cytoplasm reflects activation of RNR in G2 in response to DNA damage, failure to relocalize Suc22 should cause DNA damage sensitivity. Indeed, csn1-d cells (which do not relocalize Suc22 following damage) are UV- and IR-sensitive (Mundt et al. 1999). Because deletion of spd1 results in constitutive loss of Suc22 nuclear localization even in a csn1-d background, we tested the UV and IR sensitivity of csn1-d spd1-d double-mutant cells (Fig. 4D) and of csn1-d mutants overexpressing suc22 (data not shown). Loss of spd1 or overexpression of Suc22 in a csn1-d background restored UV and IR sensitivity to close to wild-type levels. These data suggest that the CSN- and checkpoint-dependent relocalization of Suc22 from the nucleus to the cytoplasm is required for an efficient DNA damage response.
Relocalization of Suc22 corresponds to degradation of Spd1

The data presented above indicate that Spd1 negatively controls nuclear export of Suc22 but Csn1 stimulates it. This predicts that Csn1 controls either a modification of Spd1 or its level in the cell. Using an antibody against Spd1 (Borgne and Nurse 2000) and epitope-tagged strains (see Materials and Methods), we characterized Spd1 levels throughout the unperturbed cell cycle, in cells treated with HU and in G2 cells following treatment with either UV or IR (Fig. 5A–C). Spd1 levels mirrored the nuclear export of Suc22: during a synchronized unperturbed cell cycle, Spd1 levels declined transiently in S phase in a manner that was dependent on csn1 but independent of rad3 and chk1 [Fig. 5A]. This is consistent with previously reported data [Borgne and Nurse 2000]. In response to ionizing irradiation of G2 cells, Spd1 levels decreased in a manner dependent on rad3, chk1, and csn1 [Fig. 5B]. In response to HU, Spd1 levels dropped in a rad3-dependent and csn1-dependent manner as cells accumulated in S phase [Fig. 5C]. Indirect immunofluorescence established that Spd1 is a nuclear protein, which disappeared in a signalosome-dependent manner upon ionizing radiation of G2 cells [Fig. 5D].

Proteosome and polyubiquitination-dependent Spd1 degradation

To confirm that Spd1 is degraded in a ubiquitin- and proteosome-dependent manner, we examined ubiquitin modification of an endogenous TAP-Spd1 protein (expressed from its own promoter at the spd1 locus) using an assay in which His6-Ubi is expressed in cells and ubiquitinated proteins are purified by IMAC chromatography. Probing the purified fractions derived from HU-treated csn1-d, mts2-1, and mts3-1 single-mutant cells (Gordon et al. 1997) and mts2-1 csn1-d double-mutant extracts for TAP-Spd1 [Fig. 6A,B] demonstrates that, when the proteosome function is attenuated, polyubiquitinated TAP-Spd1 can be detected. Mts2 and Mts3 are subunits of the regulatory particle of the proteosome. Whereas in mts+ cells polyubiquitinated proteins are rapidly degraded and are thus not detected, mts2 or mts3 mutants accumulate polyubiquitinated TAP-Spd1 at the restrictive temperature. Consistent with a role of the CSN complex in Spd1 degradation, polyubiquitinated TAP-Spd1 can be detected. Mts2 and Mts3 are subunits of the regulatory particle of the proteosome. Whereas in mts+ cells polyubiquitinated proteins are rapidly degraded and are thus not detected, mts2 or mts3 mutants accumulate polyubiquitinated TAP-Spd1 at the restrictive temperature. Consistent with a role of the CSN complex in Spd1 degradation, polyubiquitinated TAP-Spd1 is absent in two independent mts2-1 csn1-d double mutants at the restrictive temperature [Fig. 6B]. Interestingly, monoubiquitination of TAP-Spd1 is not significantly decreased in the csn1-d mutant background.

Spd1 degradation requires Pcu4, which copurifies with the signalosome

Identifying the mechanism of Spd1 degradation should provide insight into the function of the signalosome. The CSN complex is required for the deneddylation of the cullins, and the subunits have been identified as cullin-binding proteins [Lyapina et al. 2001]. Mutants in both pcu3 and pcu4, which encode two of the three S. pombe cullin homologs, have been reported to exhibit slow growth and DNA damage sensitivity, phenotypes that would be consistent with a role in Spd1 degradation [Kominami et al. 1998]. We therefore ascertained if Spd1 degradation was dependent on pcu3 or pcu4. First, we
tagged Spd1 with a $3 \times$ HA tag to simplify its identification (see Materials and Methods). In $pcu4$-d mutants (but not $pcu3$-d mutants, data not shown), loss of 3HA-Spd1 upon treatment of cells with HU is significantly attenuated (Fig. 7A), suggesting that Pcu4 plays a major role in Spd1 degradation.

Because the neddylation status of Pcu4 has not been reported, we used a strain in which the 3$^{\text{rd}}$ region of the $pcu4$ gene of $S. pombe$ was modified to encode the TAP-tag (see Materials and Methods). Although not fully functional, Pcu4-TAP separates on SDS-PAGE as two major bands, consistent with Nedd8 modification (Fig. 7B). Surprisingly, in $csn4$-d and $csn5$-d mutant backgrounds, these two bands were unaltered, whereas in a $csn1$-d background the upper band was overrepresented compared with the lower band (Fig. 7B). These data suggest that there may be a more direct relationship between Pcu4 and the Csn1/Csn2 subunits of the signalosome than exists between the signalosome and either Pcu1 or Pcu3.

To explore this further, we generated a TAP-tagged $csn2$ allele (integrated at the endogenous locus and expressed from its own promoter) and purified the signalosome to homogeneity (Fig. 7C; Tasto et al. 2001). Purified proteins were separated by SDS-PAGE and samples were stained with Coomassie blue (left) or silver (right). Individual Coomassie bands were excised and identified by trypsin cleavage and mass fingerprinting using mass spectrometry. Eleven bands generated fingerprinting data compatible with annotated ORFs in the $S. pombe$ genome database (Fig. 7D). In addition to the identification of Csn1, Csn2, Csn3, Csn4, Csn5, and Csn7, we also identified Ura1, Ddb1, Pcu4, an Hsp70-like protein, and an unknown ORF (SPBC651.07) encoding a protein of 30.48 kD that we have named Csa1 (for COP9/signalosome-associated protein 1). We did not identify the putative Csn6 homolog or the second ORF in $S. pombe$ with homology to Csn7 subunits (SPAC1751.03). This is consistent with the fact that, unlike the other csn genes, csn6 and SPAC1751.03 (annotated as csn7b) are both essential, suggesting functions distinct from the signalosome (C. Zhou and D.A. Wolf, pers. comm). Although deneddylation of Pcu1 and Pcu3 is signalosome-dependent, we did not find Pcu1 or Pcu3 association with the signalosome in these preparations, suggesting that these interactions [Lyapina et al. 2001; Zhou et al. 2001] are unstable. It is possible that the 11 proteins identified are present in a single complex. An alternative explanation would be that two or more independent complexes containing TAP-Csn2 are copurifying. To date, we have not distinguished between these two possibilities.

**Discussion**

Although mutations in $csn1$, $csn2$, $csn3$, $csn4$, and $csn5$ each cause constitutive Nedd8 modification of the cullins Pcu1 and Pcu3, only two subunits of the $S. pombe$ signalosome, Csn1 and Csn2, are important for proper progression through S phase and efficient survival of DNA damage [Mundt et al. 1999, 2002; Zhou et al. 2001].
Our data strongly suggest that the failure to degrade Spd1 in a Csn1/Csn2-dependent manner is the reason for both the S-phase delay and the repair defect. Spd1 acts to keep the small RNR subunit Suc22 in the nucleus, thereby regulating RNR activity. Because the large RNR subunit, Cdc22, is constitutively dispersed throughout the cell, we suggest: [1] There is a nuclear RNR pool that can be activated in the absence of signalosome function, because the complete loss of RNR would be a lethal event. [2] To increase the supply of deoxyribonucleotides during DNA replication and repair, Suc22 delocalizes from the nucleus to the cytoplasm in a signalosome-dependent manner to form additional active complexes with Cdc22. In cells deleted for spd1, Suc22 is constitutively cytoplasmic and most probably associated with Cdc22 (the C-terminal epitope is masked; cf. Figs. 3, 4 and Lycksell et al. 1994) and therefore constitutively active. Thus, our data suggest that the Csn1 and Csn2 subunits of the signalosome are required to degrade Spd1. This releases Suc22 from the nucleus so it can associate with Cdc22 and dNTPs can be produced.

This model is supported by the response of Spd1 and Suc22 after DNA damage: following irradiation of G2 cells, Csn1 and Csn2 are both required for degradation of Spd1 and Suc22 relocation to the cytoplasm. In csn1-d cells, Suc22 is always nuclear and relocation fails to occur after DNA damage. However, in cells deleted for both csn1 and spd1, Suc22 is constitutively cytoplasmic, which suppresses both the UV and ionizing radiation sensitivity, showing that DNA damage sensitivity is coincident with the inability to localize Suc22 to the cytoplasm after DNA damage. Thus, the sensitivity of csn1-d cells probably reflects inefficient repair caused by a lack of dNTPs. This hypothesis is supported by the observation that overexpression of suc22 in csn1-d cells rescues UV sensitivity as effectively as spd1 deletion.

The signalosome and Pcu4 cooperate to degrade Spd1

Csn1 and Csn2, but not Csn3, Csn4, or Csn5, are required in vivo for the degradation of Spd1. The cullin Pcu4 is also required for Spd1 degradation (Fig. 7; data not shown). Pcu4 is most homologous to human cullins CUL4A and CUL4B and like these human cullins lacks the N-terminal Skp1-binding motif found in Pcub1 and its human homolog CUL1. Skp1 is the adaptor protein that links CUL1 to F-box proteins that, in turn, target the SCF to its substrates. The other S. pombe cullin, Pcub3, is most homologous to human CUL3, which associates with an Skp1-like protein and has a N-terminal domain bearing homology to the Skp1-binding site of CUL1. It is not known if S. pombe Pcub3 associates with Skp1, but it is not thought that Pcu4 associates with the adapter protein Skp1, and therefore it is unlikely to bind F-box proteins.

Our data suggest a close relationship between Pcu4 and the signalosome that is distinct from the relationship between the signalosome and either Pcub1 or Pcub3: the genetic dependency of the Nedd8 modification status of Pcu4 is apparently distinct from that seen with Pcub1 or Pcub3 and appears to mirror the biological phenotypes of null mutations in signalosome subunits. Furthermore, the purified S. pombe signalosome contained Pcu4, but not Pcub1 or Pcub3, and no F-box proteins were found to copurify. There is a strong prediction in the literature that Csn5 encodes the isopeptidase that cleaves Nedd8 from cullins (Lyapina et al. 2001). We observe that Pcu4 migrates as two bands (usually diagnostic for Nedd8 modification) in the purified signalosome, although alternative explanations cannot be ruled out at this stage.

Figure 6. The dependency of Spd1 degradation on Pcu4 and copurification of Pcu4 with the signalosome. (A) Ubiquitinated TAP-Spd1 forms are evident in mts3 mutant cells at both the permissive and restrictive temperatures. Wild-type, csn1-d, and mts3-1 mutant cells were used to express His6-ubiquitin. Soluble extracts were prepared from each strain (mts3-1 cells were analyzed at both the permissive 25°C and restrictive 35°C temperatures) either with […] or without […] prior incubation in HU for 2.5 h. His6-ubiquitin was purified on Ni2+ beads; the beads were boiled and the eluate was resolved by 12% SDS-PAGE and probed for the TAP-tag. Ub indicates the multiple-ubiquitinated forms of TAP-Spd1; nUb indicates the monoubiquitinated form. (B) Ubiquitinated TAP-Spd1 forms are evident in mts2 mutant cells, and these are lost in mts2 csn1-d double mutants. Wild-type, csn1-d, mts2, and two csn1-d mts2 double-mutant isolates were used to express His6-ubiquitin. Soluble extracts were prepared and His6-ubiquitin was purified on Ni2+ beads. The resulting beads were boiled, and the eluate was resolved by 8% SDS-PAGE and probed for the TAP-tag. As before, nUb indicates the multiple-ubiquitinated forms of TAP-Spd1; Ub indicates the monoubiquitinated form. A control aliquot of TAP-Spd1 extract is also run to show the size of unmodified TAP-Spd1 and demonstrate that nonubiquitinated Spd1 is not purified by IMAC chromatography. The star indicates a nonspecific band.
Each signalosome subunit (Csn1–8) contains either an MPN or a PCI domain. In the human and Arabidopsis signalosomes, only the Csn5 and Csn6 subunits are MPN-domain proteins. Csn5 is a member of the MPN/JAMM motif subclass of MPN-domain proteins, whereas Csn6 contains a conventional MPN domain (Cope et al. 2002; Maytal-Kivity et al. 2002). The MPN+/JAMM motif contains five conserved polar residues embedded within the conventional MPN domain that may define a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, perhaps coordinating a metal ion. Although it has been predicted that the MPN+/JAMM motif relates to a novel isopeptidase activity, active recombinant protein has not been reported, and thus formal proof of this prediction is unavailable (Cope et al. 2002; Maytal-Kivity et al. 2002). The purified S. pombe signalosome did not contain the putative Csn6 homolog, which in any case would not be predicted to contain a catalytic domain, possibly in the regulation of transcription (Martinez et al. 2001; Schroeder et al. 2002). Interestingly, Ddb1 has been found in human cells to be associated with CUL4A (Shiyanov et al. 1999), and Ddb2 (the WD-repeat subunit of DDB) is targeted for degradation by CUL4A (Nag et al. 2001). This suggests that our finding Ddb1 in the complex is not coincidental and reflects a role for Ddb1 in Cul4-dependent polyubiquitination (Fig. 7E).

The S. pombe ddb1-d strain exhibits slow growth and modest DNA damage sensitivity (Zolezzi et al. 2002), and, in results to be presented elsewhere (A.M. Carr and C. Liu, unpubl.), we find that Ddb1 plays a role in Spd1 degradation. Whereas a Ddb1 homolog is present in S. cerevisiae, no clear homolog of p48 (Ddb2) can be identified. Intriguingly, S. cerevisiae lacks both Ddb2 and Ddb1 homologs, and also does not have a highly conserved signalosome or a cullin with clear homology to CUL4A/B. An attractive explanation for our observations would be that the signalosome provides a targeting function for Pcu4-dependent E3 ubiquitin ligases, recruiting this polyubiquitination apparatus to nuclear proteins such as Spd1 (and possibly a range of transcription factors) through interactions with Ddb1 [Fig. 7E]. In such a model, Csn1 and Csn2 could be required to target Pcu4-dependent E3 ligase to Spd1, whereas other subunits may target other (as yet unidentified) proteins. Alternatively, the regulation of Pcu4 by Csn1 and Csn2 through deetylation may be more strict than the regulation of Pcu1 by Csn-dependent deetylation [note that Pcu1 is an essential protein, but csn5-d mutants, which are defective in deetylation of Pcu1, display no discernable phenotype in log-phase cells].

The association of Ddb1 with histone deacetylases in human cells (Martinez et al. 2001) and of Arabidopsis Ddb1 with Det1 (Schroeder et al. 2002), an H2A-binding
protein required for photomorphogenic transcription [Benvenuto et al. 2002], suggests that there may be relationship between chromatin functions and Ddl1 that may involve Pcu4 and the signalosome in the regulation of transcriptional control.

**Two distinct signalosome-dependent mechanisms regulate Spd1 degradation and Suc22 relocalization**

We can clearly distinguish two separate inputs to the Spd1 degradation machinery: In unperturbed S phase, Spd1 degradation is csn1- and csn2-dependent but rad3-, chk1-, and cds1-independent. In G2 cells after DNA damage, Spd1 degradation requires both the signalosome subunits Csn1 and Csn2 plus the rad3- and chk1-dependent DNA damage checkpoint.

This suggests that Chk1 either directly or indirectly modifies Spd1 in response to DNA damage, or that it modifies an aspect of the protein degradation machinery to target Spd1. In *S. cerevisiae*, a similar small protein, Sm1, acts as a formal inhibitor of RNR activity [Chabes et al. 1999]. Although it is unknown if this acts in vivo by regulating the subcellular localization of RNR subunits, it is known that Sm1 is targeted by the Mccl–Dun1 kinase pathway for phosphorylation [Zhao and Rothstein 2002] and that this phosphorylation correlates with Sm1 degradation and RNR induction both during S phase and in response to DNA damage.

We have seen no evidence of Spd1 modification in response to cell cycle progression or DNA damage in *S. pombe* [Fig. 5A,B; data not shown]. We presently favor the probability that Csn1-dependent Spd1 degradation is controlled by modifications to the Pcu4 and signalosome-dependent protein-degradation machinery. These modifications could be directed by Chk1 in response to DNA damage and by alternative mechanisms during cell cycle progression (e.g., cyclin-dependent kinase activity).

**Materials and methods**

**Elutriation and FACS analysis**

Cell were synchronized using a JE-5.0 elutriating centrifuge [Beckman]. Small G2 cells were collected, harvested, and resuspended in fresh medium at 3 × 10⁶ cells/mL. Septation was determined by fixing cells in methanol and staining with DAPI and Calcofluor [Russell and Nurse 1986]. Samples for protein extract were washed in ice-cold water, frozen in LN₂, and stored at −80°C. Of this, 50 µg (total protein) was Western-blotted.

**Immunofluorescent microscopy**

Staining was as described [Hagan and Hyams 1988]. Cells were fixed in 3.7% paraformaldehyde for 10 min and stained with primary antibody (α-Suc22 at 1:50, α-GFP at 1:200) and secondary antibody (FITC-conjugated, DAKO, at 1:150). TAP-Tag was detected directly with FITC-conjugated rabbit anti-mouse (DAKO) at 1:150.

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