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Smt3/SUMO and Ubc9 are required for efficient APC/C-mediated proteolysis in budding yeast

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Summary

Ubiquitin-mediated proteolysis triggered by the anaphase-promoting complex/cyclosome (APC/C) is essential for sister chromatid separation and the mitotic exit. Like ubiquitylation, protein modification with the small ubiquitin-related modifier SUMO appears to be important during mitosis, because yeast cells impaired in the SUMO-conjugating enzyme Ubc9 were found to be blocked in mitosis and defective in cyclin degradation. Here, we analysed the role of SUMOylation in the metaphase/anaphase transition and in APC/C-mediated proteolysis in *Saccharomyces cerevisiae*. We show that cells depleted of Ubc9 or Smt3, the yeast SUMO protein, mostly arrested with undivided nuclei and with high levels of securin Pds1. This metaphase block was partially relieved by a deletion of *PDS1*. The absence of Ubc9 or Smt3 also resulted in defects in chromosome segregation. Temperature-sensitive *ubc9-2* mutants were delayed in proteolysis of Pds1 and of cyclin Clb2 during mitosis. The requirement of SUMOylation for APC/C-mediated degradation was tested more directly in G1-arrested cells. Both *ubc9-2* and *smt3-331* mutants were defective in efficient degradation of Pds1 and mitotic cyclins, whereas proteolysis of unstable proteins that are not APC/C substrates was unaffected. We conclude that SUMOylation is needed for efficient proteolysis mediated by APC/C in budding yeast.

Introduction

Important cell cycle transitions, such as the initiation of DNA replication, sister chromatid separation and the exit from mitosis, require the proteolytic destruction of specific regulatory proteins. Degradation is initiated by the attachment of chains of ubiquitin molecules to these target proteins (reviewed by Hershko and Ciechanover, 1998; Kornitzer and Ciechanover, 2000). Ubiquitin-mediated proteolysis ensures that proteins are degraded by the 26S proteasome only after they are tagged with ubiquitin.

Ubiquitylation requires the activity of three enzymes, termed E1, E2 and E3 (reviewed by Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002). Ubiquitin, a 76 kDa protein, is first bound to E1, a ubiquitin-activating enzyme. From E1, it is transferred to one of several E2 enzymes, called ubiquitin-conjugating enzymes. Subsequently, ubiquitin is attached to lysine residues of the substrate. This final step is catalysed by ubiquitin ligases, the E3 enzymes. Polyubiquitinated substrates are recognized and degraded by the 26S proteasome.

Two ubiquitin ligases are known to play fundamental roles during the cell cycle, the Skp1–cullin–F-box complex (SCF) and the anaphase-promoting complex/cyclosome (APC/C) (Jackson *et al*., 2000; Peters, 2002). In budding yeast, the major cell cycle role of SCF is the regulation of the G1/S transition, whereas APC/C is required for sister chromatid separation and the exit from mitosis. APC/C is a large complex consisting of at least 13 subunits and is highly conserved among eukaryotes (Harper *et al*., 2002; Peters, 2002). This ubiquitin ligase is activated at the metaphase/anaphase transition and is turned off at the end of the subsequent G1 phase. Important regulatory proteins are Cdc20 and Cdh1, which contain WD40 repeat motifs and are implicated in substrate recognition (Pfleger *et al*., 2001; Vodermaier, 2001). A key role of APC/C^{Cdc20} is the ubiquitylation of securins, known as Pds1 in budding yeast (Cohen-Fix *et al*., 1996). Securins are inhibitors of separases that trigger the separation of sister chromatids by cleavage of a cohesin subunit (Nasmyth, 2002). APC/C^{Cdh1} is activated in late anaphase and triggers the complete destruction of mitotic cyclins (Harper *et al*., 2002; Peters, 2002).

Previously, Seufert *et al*. (1995) found that degradation of the budding yeast B-type cyclins Clb2 and Clb5 requires the Ubc9 protein. *UBC9* encodes a protein homologous to ubiquitin-conjugating enzymes and was initially proposed to act as an E2 enzyme for the APC/C ubiquitin ligase. Later, it was shown that Ubc9 does not transfer ubiquitin to its target proteins, but instead modifies its targets with a protein related to ubiquitin, termed small ubiquitin-related modifier or SUMO (Johnson and Blobel, 1997; Schwarz *et al*., 1998).

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The sequence identity between SUMO and ubiquitin is only 18%, but these proteins share a similar three-dimensional structure (Bayer *et al*., 1998). Like ubiquitin, SUMO is highly conserved in eukaryotes. Budding yeast has a single SUMO gene, known as the essential *SMT3* gene, whereas three members of the SUMO family have been identified in vertebrates (reviewed by Melchior, 2000; Müller *et al*., 2001; Kim *et al*., 2002). The pathway of SUMO conjugation, termed SUMOylation, is similar to ubiquitylation, but requires different enzymes. SUMO is first bound to a heterodimeric E1 composed of Aos1 and Uba2. It is then transferred to Ubc9, the only known SUMO-conjugating enzyme, which in turn catalyses the formation of an isopeptide bond between the C-terminus of SUMO and an ∈-lysine residue of a target protein. Ubc9 shares structural similarities with ubiquitin-conjugating enzymes but, unlike these E2s, Ubc9 contains a positively charged surface. By analogy with ubiquitylation, E3 SUMO ligases were recently identified in yeast and mammals (Johnson and Gupta, 2001; Takahashi *et al*., 2001; Pichler *et al*., 2002).

In contrast to ubiquitylation, the modification of proteins with SUMO does not result in their proteolytic degradation. Instead, multiple other effects of SUMOylation have been described, for example the modulation of the subcellular localization of proteins, of protein–protein interactions or of the activity of transcription factors (Müller *et al*., 2001; Wilson and Rangasamy, 2001). Intensively studied SUMO substrates are, for example, mammalian RanGAP1, a factor required for nucleocytoplasmic transport, and the tumour suppressor p53. SUMO targets RanGAP1 to nuclear pores and stimulates the transcriptional and apoptotic activities of p53 (Gostissa *et al*., 1999; Rodriguez *et al*., 1999). Previous studies have also revealed a functional link between SUMOylation and ubiquitylation. In the case of IκBα, an inhibitor of the NF-κb transcription factor, SUMOylation was shown to antagonize ubiquitylation, thus preventing degradation of the protein by the ubiquitin pathway (Desterro *et al*., 1998).

The defects of yeast *ubc9-1* mutants in the degradation of B-type cyclins may also indicate a link between SUMOylation and ubiquitin-mediated proteolysis (Seufert *et al*., 1995). Furthermore, several reports have implicated important functions for SUMO during M-phase of the cell cycle. Yeast cells depleted of *UBC9* were impaired in mitosis, and temperature-sensitive *smt3* mutants, defective in the yeast SUMO gene, were identified in a screen for mutants defective in chromosome segregation (Biggins *et al*., 2001). *Schizosaccharomyces pombe* cells lacking the SUMO gene *pmt3* and the *UBC9* homologue *hus5* strains also displayed defects in chromosome segregation (al-Khodairy *et al*., 1995; Tanaka *et al*., 1999).

Here, we found that yeast cells lacking functional Smt3 and Ubc9 proteins fail to degrade Pds1 in mitosis and that the metaphase block of these cells can be partially bypassed by a *PDS1* deletion. Furthermore, we show that mutant strains defective in SUMOylation are impaired in proper proteolysis of securin and other APC/C target proteins in mitosis and in G1-arrested cells.

Results

Yeast cells depleted of Ubc9 and Smt3 arrest with short spindles and high levels of Pds1

It was reported previously that yeast *ubc9Δ* cells, which were kept alive by *UBC9* expressed from the galactose-inducible *GAL1-10* promoter, arrested in mitosis upon promoter shut-off in glucose medium (Seufert *et al*., 1995). Despite this cell cycle defect, we found that most cells of a *ubc9Δ GAL-UBC9* strain were viable and able to form colonies on plates containing glucose (data not shown). These findings prompted us to reinvestigate the requirement of *UBC9* for viability, by analysing cells completely depleted of Ubc9. Haploid segregants containing the *ubc9Δ* allele were obtained from a sporulating heterozygous diploid *UBC9*/*ubc9Δ* strain. The analysis of about 30 haploid *ubc9Δ* segregants revealed that cells lacking *UBC9* were able to produce microcolonies (Fig. 1A), mostly consisting of 30–50 cells, before cells ceased cell division and finally lysed. Thus, *ubc9Δ* segregants were able to divide on average four to six times, implying that Ubc9 protein inherited from the parental diploid strain is sufficient for *ubc9Δ* cells to undergo several cell divisions. A depletion of the SUMO protein Smt3 resulted in a similar phenotype. *smt3Δ* segregants obtained from a *SMT3/smt3Δ* diploid strain were able to undergo three to five cell divisions on average. Thus, low amounts of Ubc9 and Smt3 are sufficient for viability. The dissection of *ubc9Δ* and *smt3Δ* cell microcolonies showed that at least 80% of these cells were large budded, suggesting that they terminally arrested in G2/M phase. These findings underline the essential role of Ubc9 and Smt3 in G2/M phase.

To characterize further the mitotic arrest of cells defective in SUMOylation, we introduced a Myc-tagged version of Pds1 into a strain in which the *SMT3* gene was replaced by a *GAL-SMT3* construct (Biggins *et al*., 2001) and into a *ubc9Δ GAL-UBC9* strain. These strains were pregrown in galactose medium and then transferred to glucose medium. After a 15 h incubation period, most of the cells were large budded. Nuclei, spindles and Pds1-Myc were analysed by indirect immunofluorescence. About 70–80% of these large-budded cells had a single nucleus and a short mitotic spindle, and virtually all these cells contained Pds1-Myc (Fig. 1B and C). In contrast, 20–30% of large-budded cells had elongated anaphase spindles, and Pds1-Myc signals were only rarely detectable. Only a few cells had clearly separated masses of DNA. Instead, the

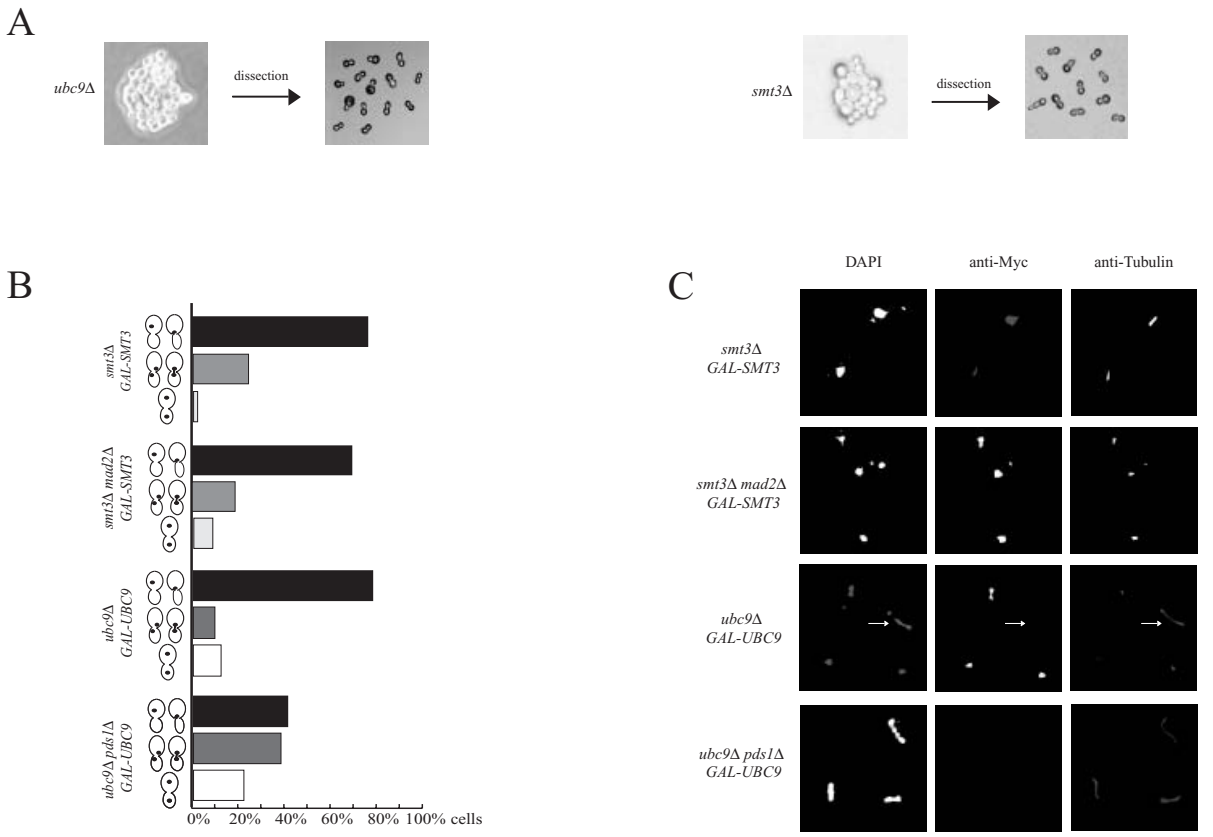

Fig. 1. Yeast cells depleted of Ubc9 and Smt3 mostly arrest in metaphase with high Pds1 levels, but this arrest can be bypassed by a deletion of *PDS1*.

A. Diploid *UBC9*/*ubc9Δ* and diploid *SMT3/smt3Δ* yeast strains were sporulated, and tetrads were dissected. Spores were grown on YEPD plates at 30°C. Representative segregants containing the *ubc9Δ* and *smt3Δ* alleles were photographed after 36 h. Microcolonies from segregants containing the deletion alleles were dissected with a micromanipulator to determine the phenotype of individual cells.

B and C. The following yeast strains were used for these experiments: (i) a strain containing *SMT3* expressed from the *GAL1-10* promoter instead of the wild-type *SMT3* gene (*smt3Δ GAL-SMT3*, S550); (ii) a strain containing in addition a deletion of the *MAD2* gene (*smt3Δ mad2Δ*, *GAL-SMT3*, S566); (iii) a strain with a deletion of the *UBC9* gene, but kept alive by the *UBC9* gene from the *GAL1-10* promoter (*ubc9Δ GAL-UBC9*, S552); and (iv) a strain containing in addition a deletion of the *PDS1* gene (*ubc9Δ GAL-UBC9 pds1Δ*, S483). Strains (i)–(iii) also contained a Myc18-tagged version of Pds1. These strains were pregrown in YEP medium containing 2% raffinose and 2% galactose. Cells were then transferred to YEP medium containing 2% glucose (YEPD) and incubated for 15 h. Subsequently, cells were collected for indirect immunofluorescence microscopy. Large-budded cells were analysed using DAPI, anti-tubulin antibodies and anti-Myc antibodies to visualize nuclei, spindles and Pds1-Myc respectively.

B. Analysis of large-budded cells. Black columns, percentage of cells with short spindles and a single nucleus; grey columns, percentage of cells with elongated spindles and dispersed or barely separated nuclei; white columns, percentage of cells with elongated spindles and distinctly separated nuclei. At least 200 cells from each strain were analysed.

C. DAPI, anti-tubulin and anti-Myc staining of representative cells The arrow marks a cell containing a dispersed nucleus and an elongated spindle.

DAPI signal was often dispersed, and chromosomes appeared to be lagging along the spindles (Fig. 1C, arrow), indicating that these cells failed to segregate chromosomes to opposite poles properly.

We then asked whether the metaphase arrest of Smt3-depleted cells and the failure to degrade Pds1 may be caused by the activation of the spindle checkpoint (Wassmann and Benezra, 2001). To test this, we constructed a *GAL-SMT3* strain lacking *MAD2*, a gene required for the checkpoint arrest (Li and Murray, 1991). Upon a shift to glucose medium, the *GAL-SMT3 mad2Δ* strain arrested similarly to the *GAL-SMT3* strain (Fig. 1B and C). The percentage of cells containing short spindles and the Pds1-Myc signal was not reduced, implying that the metaphase block of Smt3-depleted cells is independent of Mad2.

These results show that SUMOylation is needed for Pds1 degradation and the onset of anaphase, independently of the spindle checkpoint.

Deletion of PDS1 alleviates anaphase onset in Ubc9-depleted cells

We next addressed the question whether the metaphase

arrest of cells defective in SUMOylation may be caused by a failure to degrade Pds1. We argued that a deletion of the *PDS1* gene would then allow these cells to elongate their spindles and segregate their chromosomes, as shown previously for other mutants defective in metaphase, such as *apc* or *cdc20* mutants (Yamamoto *et al*., 1996; Lim *et al*., 1998). To test this, we crossed *pds1Δ* mutants with a *ubc9Δ GAL-UBC9* strain and received a *ubc9Δ GAL-UBC9 pds1Δ* strain that was reasonably viable at 25°C. This strain and a *ubc9Δ GAL-UBC9* control strain were pregrown at 25°C in galactose medium and then transferred to glucose medium. A large fraction of *ubc9Δ GAL-UBC9 pds1Δ* cells were large budded after incubation in glucose medium, and these were analysed by immunofluorescence microscopy (Fig. 1B and C). In contrast to *ubc9Δ GAL-UBC9* cells containing the *PDS1* gene, about 50% of *pds1Δ* cells had elongated spindles and separated or dispersed DNA masses. DNA masses were only poorly segregating in many cells containing elongated spindles, indicating defects in chromosome segregation.

Thus, a deletion of *PDS1* partially suppresses the metaphase arrest of Ubc9-depleted cells, implying that the metaphase block of cells defective in SUMOylation is caused, at least in part, by a failure to degrade Pds1.

Temperature-sensitive ubc9-2 mutants are delayed in degradation of securin Pds1 and cyclin Clb2 during mitosis

To characterize further the role of Ubc9 in mitosis, we used a temperature-sensitive *ubc9-2* mutant. The cell cycle arrest of this mutant at the restrictive temperature was less distinct than for Ubc9-depleted cells. After a shift for to 37°C 4 h, both large-budded and unbudded cells accumulated, but the number of small-budded cells was reduced compared with wild-type cultures (data not shown). A similar phenotype was described previously for a *smt3-331* temperature-sensitive mutant, which only moderately accumulated G2/M cells at the non-permissive temperature (Biggins *et al*., 2001). By analysing large-budded cells of *ubc9-2* mutants containing a Myc-tagged version of Pds1 by immunofluorescence microscopy, we found that about 70% of these cells contained short spindles, whereas about 30% of cells had elongated spindles (Fig. 2A). Virtually every cell with a short spindle contained a Pds1-Myc signal (data not shown). *ubc9-2* mutants containing long spindles frequently failed to segregate chromosomes to opposite poles properly. In a *ubc9-2 mad2Δ* mutant strain, cells with short spindles and Pds1-Myc accumulated similarly, as in *ubc9-2* mutants, implying that the observed mitotic delay occurs independently of the spindle checkpoint. This phenotype of *ubc9-2* mutants was similar to that described previously for *smt3-331* mutants (Biggins *et al*., 2001). Thus, both *ubc9-2* and *smt3-331* mutants are characterized by the accumulation of cells with undivided nuclei and by obstructions in chromosome segregation.

We next monitored the levels of Pds1 and cyclin Clb2 in synchronized wild-type and *ubc9-2* cultures. For this purpose, cultures were pregrown at 25°C and then arrested in G1 phase with α-factor. Subsequently, cells were shifted to 36°C and released from the pheromone arrest. Both wild-type and *ubc9-2* strains synchronously entered the cell cycle, as monitored by the appearance of small buds. The *ubc9-2* mutation affected neither budding nor the initiation of DNA replication (data not shown). Immunoblot analysis revealed that haemagglutinin (HA)-tagged Pds1 and Clb2 accumulated with similar kinetics in wild-type cells and *ubc9-2* mutants (Fig. 2B and C). However, *ubc9-2* cells were delayed in the degradation of Pds1 and Clb2. At the 75 min time point, Pds1 levels were decreased in wild-type cells, but remained at high levels in the mutant strain, before they dropped with a 15 min delay (Fig. 2B and C). The Clb2 protein levels decreased in the wild-type culture after 90 min, whereas only a slow decrease occurred in *ubc9-2* mutants at later time points.

These results show that proteolysis of the two APC/C substrates Pds1 and Clb2 is delayed in *ubc9-2* mutants, suggesting that proper proteolysis of these substrates during mitosis requires a functional Ubc9 protein.

Proteolysis of securin Pds1 depends on UBC9 and SMT3

To test more directly whether Ubc9 and Smt3 are required for Pds1 proteolysis, we tested its stability in α-factor-arrested G1 cells. In these G1 cells, APC/C is fully active, and Pds1 is highly unstable (Amon *et al*., 1994; Cohen-Fix *et al*., 1996). The stability of Pds1 in cells arrested in G1 phase was determined by promoter shut-off experiments. Wild-type cells, *ubc9-2* and *smt3-331* mutants were arrested with α-factor at 25°C, and then *PDS1-HA* was transiently expressed by galactose addition. After a temperature shift to 36°C, *PDS1-HA* expression was turned off by transferring cells to glucose medium. In wild-type cells, Pds1 was rapidly degraded under these conditions (Fig. 3). In *ubc9-2* and *smt3-331* mutants, Pds1 proteolysis was inefficient, and its half-life increased to more than 10 min, compared with less than 5 min in wild-type cells. The analysis of the DNA content by fluorescence-activated cell sorting (FACS) analysis confirmed that cultures remained arrested in G1 phase during the course of the experiment. Thus, proteolysis of Pds1 is impaired in the absence of functional Ubc9 and Smt3 proteins, suggesting that SUMOylation is required for efficient securin degradation during G1 phase.

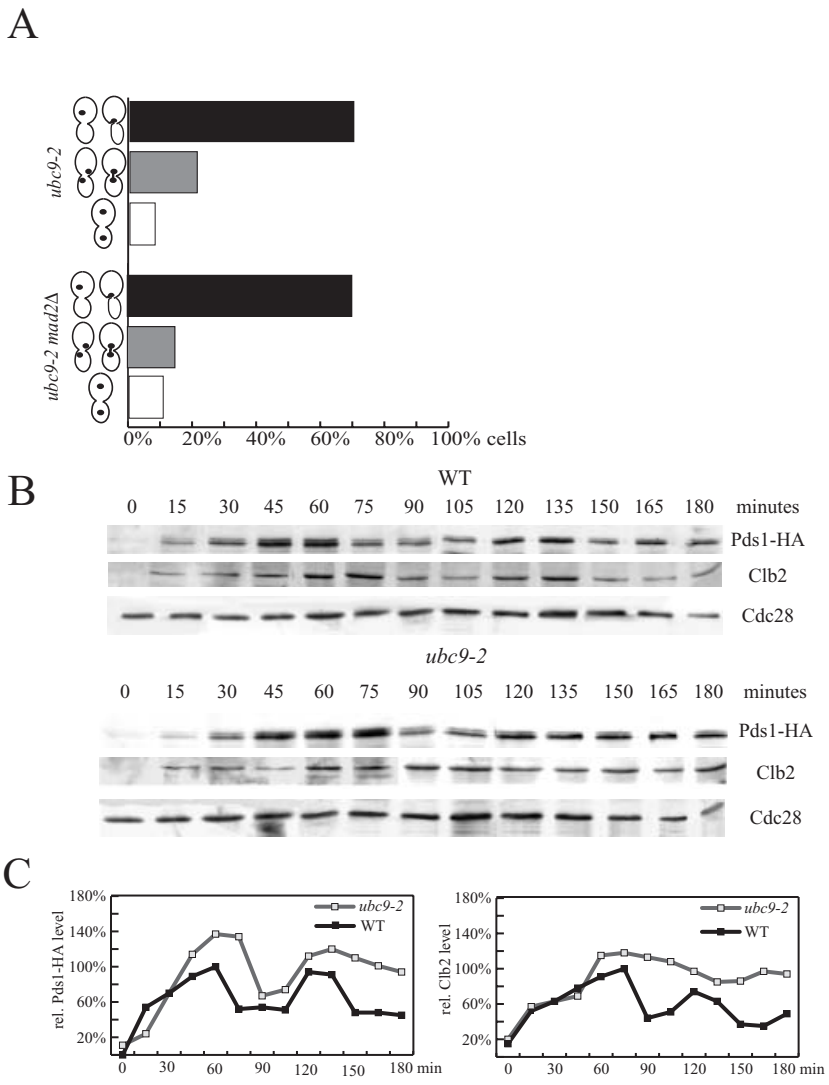

Fig. 2. Temperature-sensitive *ubc9-2* mutants are delayed in degradation of Pds1 and Clb2.
A. *ubc9-2* (S546) and *ubc9-2 mad2Δ* (S568) mutants, both containing an Myc18-tagged version of Pds1, were pregrown at 25°C and then shifted to 37°C for 4 h. Then, cells were collected for indirect immunofluorescence microscopy. Large-budded cells were analysed by DAPI, anti-tubulin antibodies and anti-Myc antibodies to visualize nuclei, spindles and Pds1-Myc respectively. Black columns, percentage of cells with short spindles and a single nucleus; grey columns, percentage of cells with elongated spindles and dispersed or barely separated nuclei; white columns, percentage of cells with elongated spindles and distinctly separated nuclei. At least 200 cells from each strain were analysed.
B and C. Log-phase cultures of a wild-type strain (S185) and a *ubc9-2* mutant strain (S406), both containing *PDS1-HA*, were pregrown in YEPD medium at 25°C and then treated with the pheromone α-factor for 2.5 h. Then, cultures were shifted to a restrictive temperature, 36°C, and incubated for an additional 30 min in the presence of α-factor. To remove the pheromone, cells were filtered, washed and transferred to fresh YEPD medium. Synchronized cultures were incubated further at 36°C. At the indicated time points after the release, samples were collected and analysed by immunoblotting, using the HA antibody to detect Pds1-HA and Clb2 antibodies to visualize Clb2 (B). Cdc28 was used as a loading control. Pds1-HA and Clb2 protein levels were quantified using a densitometer (C).

Proteolysis of cyclin and non-cyclin APC/C substrates is impaired in ubc9-2 *and* smt3-331 *mutants*

Previous results described a role for Ubc9 in the degradation of cyclins Clb2 and Clb5 (Seufert *et al*., 1995). These and our results indicate that SUMOylation may generally be required for proteolysis mediated by APC/C. To address these assumptions, we tested whether *ubc9-2* and *smt3-331* mutants are defective in degradation of cyclin and non-cyclin substrates. Consistent with the defects observed previously with *ubc9-1* mutants (Seufert *et al*., 1995), Clb2 was partially stabilized in *smt3-331* mutants (Fig. 4A). To test whether a further mitotic cyclin, Clb3, is stabilized in *ubc9-2* and *smt3-331* mutants, we performed promoter shut-off experiments. We found that proteolysis of Clb3 was delayed in *smt3-331* and *ubc9-2* mutants (Fig. 4B and C). The stabilization of Clb2 and Clb3 in G1-arrested cells suggests that Ubc9 and Smt3 are required for efficient proteolysis of mitotic cyclins.

To test the requirement of Ubc9 for degradation of another non-cyclin APC/C substrate, we analysed the accumulation of the polo-like kinase Cdc5 in G1-arrested cells. Upon expression of *CDC5-HA* from the *GAL1-10* promoter, Cdc5 accumulates only to low levels in wild-type G1 cells, because of its instability (Shirayama *et al*., 1998). We found that Cdc5 accumulates to higher levels in *ubc9-2* mutants than in wild-type cells at the restrictive temperature, indicating that its rapid degradation is impaired (Fig. 4D).

In summary, these results indicate that Ubc9 and Smt3 are required for efficient proteolysis of various APC/C substrates, implying that SUMOylation is generally important for the proper function of this ubiquitin ligase.

To elucidate further the involvement of Ubc9 in APC/C-mediated proteolysis, we tested whether the *ubc9-2* mutation displays genetic interactions with mutations in the APC/C subunit genes *CDC16* and *APC10*. Double mutants containing the *ubc9-2* mutation in combination

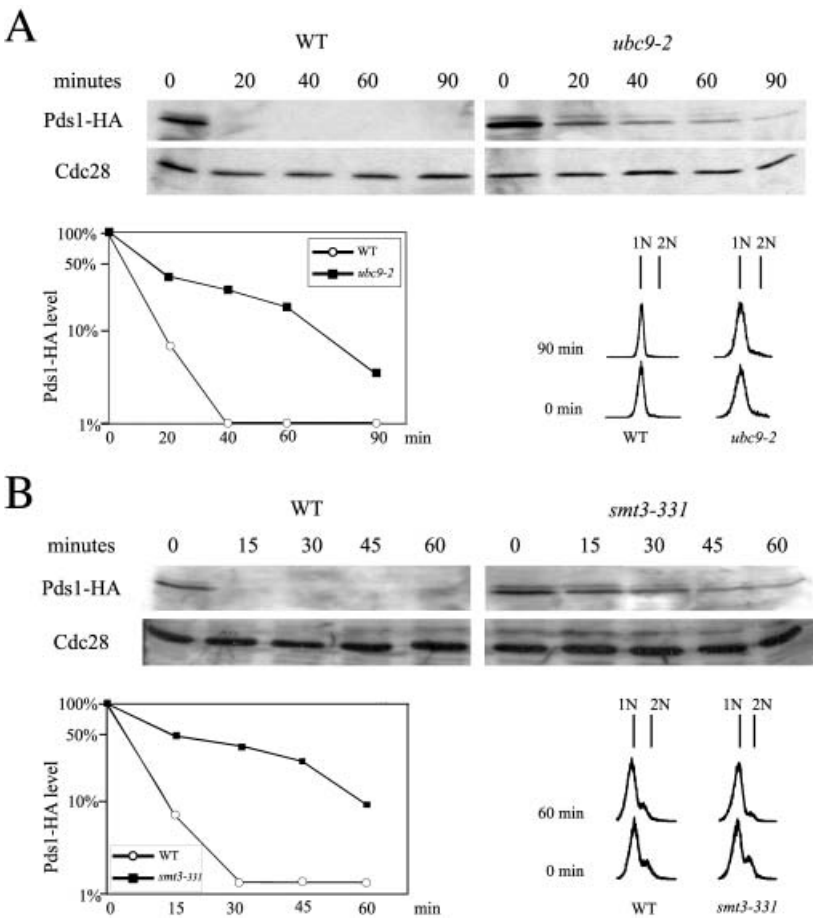

Fig. 3. Proteolysis of securin Pds1 is impaired in yeast *ubc9-2* and *smt3-331* mutants.
A. A wild-type strain (S206) and a *ubc9-2* (S365) mutant strain, both containing *bar1* deletions and *GAL-PDS1-HA* constructs, were pregrown in YEP + raffinose medium at 25°C to log phase. α-factor was added to arrest cells in G1 phase. After 2.5 h incubation with α-factor, 2% galactose was added to induce *PDS1-HA* expression. After 30 min, cultures were shifted to the restrictive temperature, 36°C, and incubated for another 30 min. To turn off the *GAL1-10* promoter, the cultures were filtered, transferred to YEPD medium containing α-factor and incubated at 36°C. Samples were collected at the indicated time points and analysed by immunoblotting. The HA antibody was used to detect Pds1-HA. Cdc28 served as a loading control. Pds1-HA protein levels were quantified using a densitometer. Samples at 0 and 90 min time points were collected for determining the DNA content by FACS analysis to confirm the G1 arrest (right). Microscopic analysis showed that more than 90% of cells were unbudded and displayed a shmoo-like phenotype.
B. A wild-type strain (S206) and a *smt3-331* (S535) mutant strain, both containing *bar1* deletions and *GAL-PDS1-HA* constructs, were treated as described in (A). Samples at the 0 and 60 min time points were analysed by FACS (right). Microscopic analysis confirmed that more than 90% of cells were unbudded.

with either the *cdc16-123* or *apc10-22* mutations were constructed. Both *ubc9-2 apc10-22* and *ubc9-2 cdc16-123* were non-viable at 30°C, whereas each of the single mutants was viable (data not shown). These synthetic phenotypes reveal at least moderate genetic interactions between *UBC9* and genes encoding APC/C subunits.

Other unstable proteins are normally degraded in ubc9-2 *and* smt3-331 *mutant strains*

It may be possible that SUMOylation does not specifically affect APC/C-mediated proteolysis, but may instead be needed for proteolysis in general, for example for the proper function of the 26S proteasome. We therefore tested whether *ubc9-2* and *smt3-331* mutants are generally impaired in the rapid degradation of unstable proteins. To test this possibility, we compared the stability of the transcription activator Gcn4 in wild-type cells and in *ubc9-2* and *smt3-331* mutants. Gcn4 proteolysis is not dependent on APC/C, but is instead a substrate of the SCF^{Cdc4} ubiquitin ligase (Meimoun *et al*., 2000). Wild-type and mutant strains were transformed with a centromeric plasmid containing a *GAL-GCN4-MYC* construct. *GCN4-MYC* was transiently expressed by galactose addition. The temperature was shifted to 36°C, and Gcn4 stability was determined by transferring cells to glucose medium. We found that Gcn4 degradation occurred with a similar efficiency in wild-type cells and in *ubc9-2* and *smt3-331* mutants (Fig. 5A). Ime2 is a further unstable protein, the proteolysis of which appears to be independent of the SCF and APC/C ubiquitin ligases (Bolte *et al*., 2002). To test whether Ime2 is stabilized in *ubc9-2* mutants, *IME2-HA* was transiently expressed in wild-type and mutant cells. We found that Ime2 is similarly degraded in both strains at the restrictive temperature (Fig. 5B).

The undisturbed degradation of Gcn4 and Ime2 in these mutants suggests that Ubc9 and Smt3 are not generally required for proteolysis of unstable proteins.

APC/C and Pds1 are localized to the nucleus in ubc9-2 *and* smt3-331 *mutant strains*

Previous findings have implicated SUMO modification in the subcellular localization of proteins (Wilson and Rangasamy, 2001; Kim *et al*., 2002). APC/C is mainly localized to the nucleus (Tugendreich *et al*., 1995) and, most

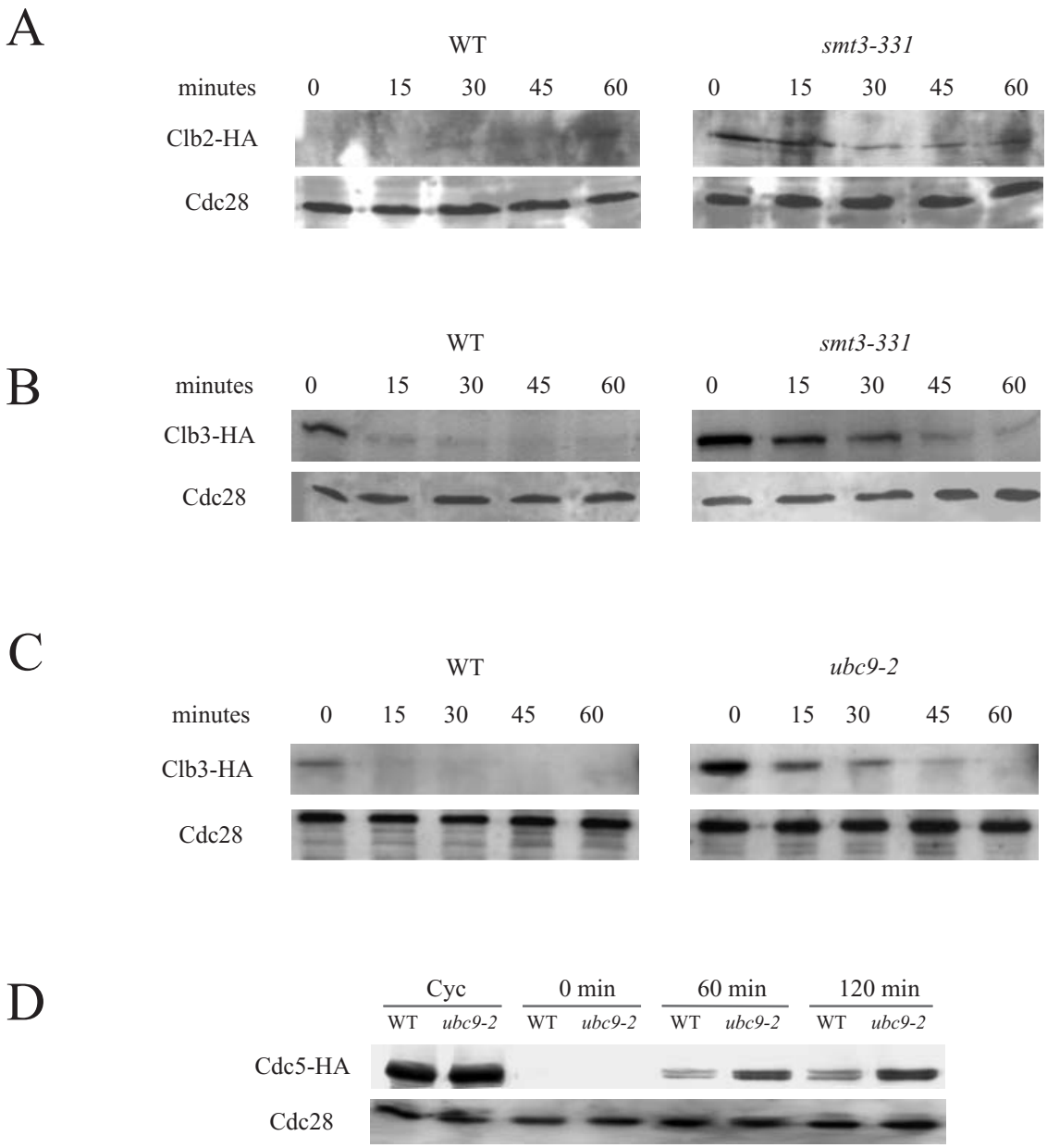

Fig. 4. Efficient proteolysis of APC/C substrates is impaired in *ubc9-2* and *smt3-331* mutants.
A. A wild-type strain (S057) and a *smt3-331* mutant strain (S543), both containing *bar1* deletions and the *GAL-CLB2-HA* construct, were pregrown in YEP medium containing 2% raffinose at 25°C. α-factor was added to arrest cells in G1 phase. After 3 h incubation with α-factor, 2% galactose was added to induce *CLB2-HA* expression, and then cultures were incubated for 30 min. Then, they were shifted to the restrictive temperature, 36°C, for another 30 min. To turn off the *GAL1-10* promoter, the cultures were filtered, transferred to YEPD medium containing α-factor and incubated at 36°C. Microscopic analysis confirmed that at least 90% of cells were unbudded and displayed a shmoo-like phenotype, implying that these cells were arrested in G1 phase (not shown). Samples were collected at the indicated time points and analysed by immunoblotting. The HA antibody was used to detect Clb2-HA. Cdc28 served as a loading control.
B. A wild-type strain (S056) and a *smt3-331* mutant strain (S544), both containing *bar1* deletions and *GAL-CLB3-HA* constructs, were treated as described in (A). The HA antibody was used to detect Clb3-HA.
C. A wild-type strain (S056) and a *ubc9-2* mutant strain (S390), both containing *bar1* deletions and *GAL-CLB3-HA* constructs, were treated as described in (A). The HA antibody was used to detect Clb3-HA.
D. A wild-type strain (S088) and a *ubc9-2* mutant strain (S487), both containing *bar1* deletions and *GAL-CDC5-HA* constructs, were pregrown at 25°C overnight in YEP + Raf medium. α-factor was added to arrest cells in G1 phase. After 2.5 h, galactose (2%) was added to induce *CDC5-HA* expression. At the same time, cells were shifted to 36°C and incubated for 120 min in the presence of α-factor. Microscopic analysis confirmed that at least 90% of cells were unbudded. Samples were collected at 0, 60 and 120 min and analysed by immunoblotting. Cyc, sample of cycling cultures at the 120 min time point. Cdc5-HA levels were analysed by immunoblotting using the HA antibody.

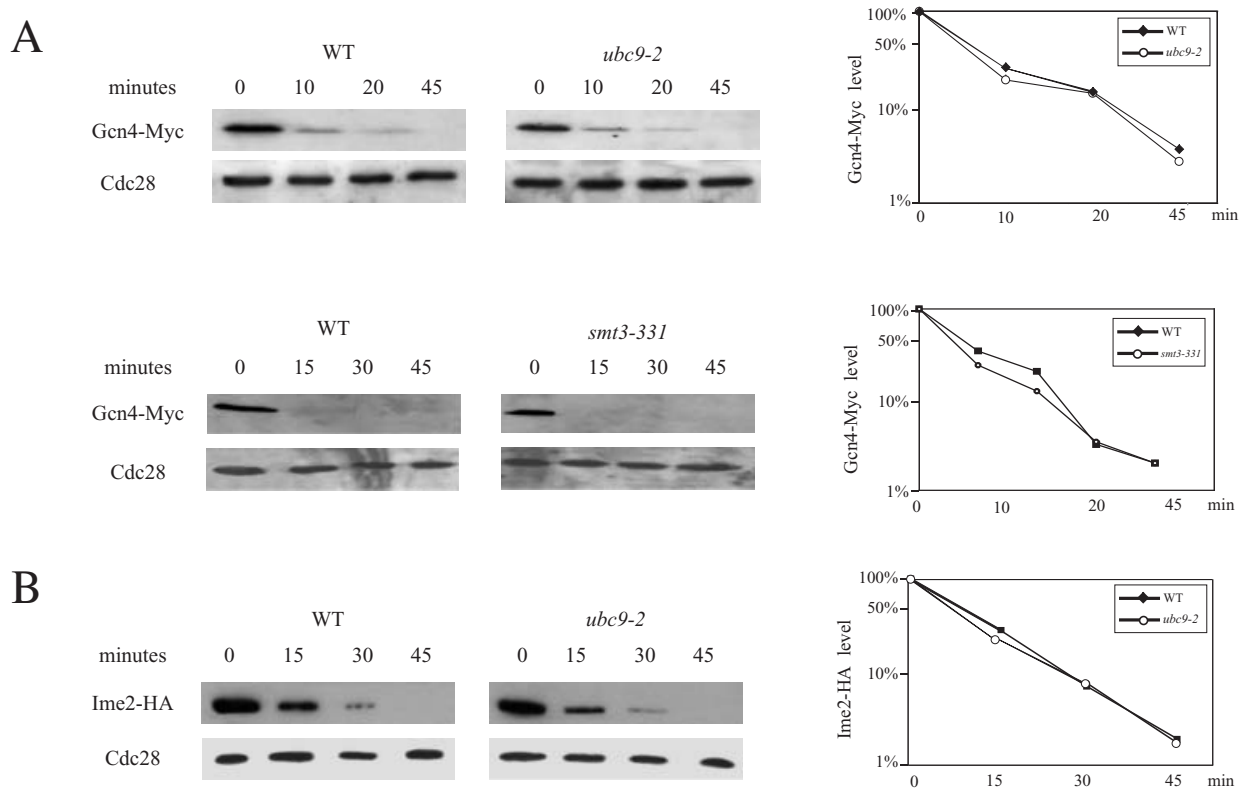

Fig. 5. *ubc9-2* and *smt3-331* mutants are not generally defective in proteolysis of unstable proteins.
A. A wild-type yeast strain (S001) and *ubc9-2* (S099) and *smt3-331* (S542) mutant strains were transformed with a centromeric plasmid containing *GAL-GCN4-MYC*. Transformants were pregrown at 25°C in minimal medium lacking uracil and containing 2% raffinose. Galactose was added to induce *GCN4-MYC* expression, and cells were incubated for 30 min. Then, cultures were shifted to a restrictive temperature, 36°C, and incubated for another 30 min. To turn off the *GAL1-10* promoter, cultures were filtered, transferred to minimal medium containing 2% glucose and incubated at 36°C. Samples were collected at the indicated time points and analysed by immunoblotting, using the MYC antibody to detect Gcn4-Myc. Cdc28 served as a loading control. Protein levels were quantified using a densitometer.
B. A wild-type yeast strain (S396) and a *ubc9-2* mutant strain (S534), both containing a *GAL-IME2-HA* construct, were pregrown in YEP medium containing 2% raffinose at 25°C. Galactose (2%) was added to induce *IME2-HA* expression. After 30 min, cultures were shifted to 36°C and incubated for another 30 min. To turn off the *GAL1-10* promoter, the cultures were filtered, transferred to YEPD medium and incubated at 36°C. Samples were collected at the indicated time points and analysed by immunoblotting. The HA antibody was used to detect Ime2-HA. Cdc28 served as a loading control. Protein levels were quantified using a densitometer.

probably, this ubiquitin ligase is highly active predominantly in this cellular compartment. We asked whether impaired APC/C-mediated proteolysis in mutants defective in SUMOylation may primarily be caused by the mislocalization of the APC/C core complex, regulatory proteins or substrates.

To test this, we analysed the subcellular localization of the core subunit Cdc16, the regulatory protein Cdc20 and of Pds1 in *ubc9-2* and *smt3-331* mutants. Cells were shifted to the restrictive temperature and, after 4 h, samples were collected for indirect immunofluorescence. We found that tagged versions of Cdc16, Cdc20 and Pds1 were localized mainly in the nucleus in *ubc9-2* and *smt3-331* mutants, as found for wild-type cells (Fig. 6). Similar data were obtained with Clb2 (data not shown). Thus, *ubc9-2* and *smt3-331* mutants apparently do not affect the nuclear localization of either of these proteins.

Discussion

A role for SUMO in the onset of anaphase and in chromosome segregation

In this report, we have analysed the roles of the budding yeast SUMO protein Smt3 and of the SUMO-conjugating enzyme Ubc9 in cell cycle progression and in proteolysis mediated by the anaphase-promoting complex/cyclosome (APC/C). Consistent with previous reports (al-Khodairy *et al*., 1995; Seufert *et al*., 1995; Tanaka *et al*., 1999; Biggins *et al*., 2001), our data showed that Smt3 and Ubc9 have pivotal functions during mitosis. A large proportion of budding yeast cells depleted of either Ubc9 or Smt3 accumulated as large-budded cells that contained short mitotic spindles, undivided nuclei and high levels of securin Pds1. These findings imply that SUMO modification is an essential process for the metaphase/anaphase transition.

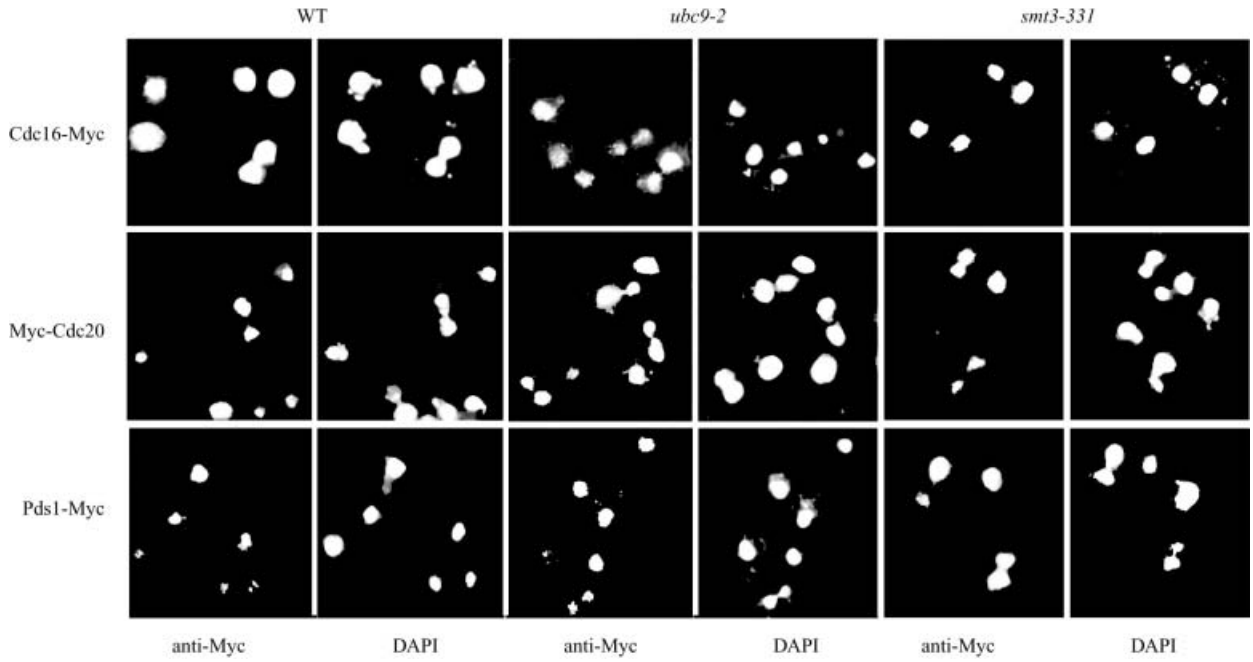

Fig. 6. The localization of APC/C and substrates is similar in wild-type cells and *ubc9-2* and *smt3-331* mutants. A wild-type strain and *ubc9-2* and *smt3-331* mutant strains containing MYC-tagged versions of the APC/C subunit Cdc16 (Cdc16-Myc), the activator protein Cdc20 (Myc-Cdc20) or securin Pds1 (Pds1-Myc) were pregrown in YEPD medium at 25°C. Cultures were shifted to 36°C for 4 h. Then, cells were fixed, spheroplasted and analysed by indirect immunofluorescence using the MYC antibody. DAPI was used to visualize nuclei.

The onset of anaphase requires proteolytic destruction of securins, which act as inhibitors of separases (Nasmyth, 2002). As Ubc9- or Smt3-depleted cells arrest with stable Pds1 protein, we addressed the question whether the metaphase arrest of cells impaired in SUMOylation is caused by a failure to degrade Pds1. We found that a deletion of *PDS1* allowed a significant fraction of *ubc9Δ* cells to elongate their spindles and to start to segregate their chromosomes, suggesting that Pds1 proteolysis is indeed an important function of SUMO at the metaphase/anaphase transition. As a fraction of *ubc9Δ pds1Δ* cells still contained short spindles and a single mass of DNA, it is likely that SUMO has important roles other than securin proteolysis for the onset of anaphase. In contrast to the *PDS1* deletion, cells impaired in SUMOylation were not affected by a deletion of the spindle checkpoint gene *MAD2*, suggesting that the mitotic delay is not a consequence of the checkpoint activation.

We have presented evidence that, in addition to the onset of anaphase, SUMOylation is also required for proper chromosome segregation. A fraction of Ubc9- or Smt3-depleted cells had elongated spindles, and these mostly contained DNA masses dispersed along the elongating spindle. Temperature-sensitive *ubc9-2* and *smt3-331* mutants displayed similar defects. Interestingly, the temperature-sensitive *smt3-331* mutant was initially identified in a screen for mutants defective in chromosome segregation (Biggins *et al*., 2001). In *Schizosaccharomyces pombe*, cells lacking either the SUMO gene *pmt3* or the *UBC9* homologue *hus5* were found to have abortive mitosis, and it was suggested that this resulted from defects in chromosome segregation (al-Khodairy *et al*., 1995; Tanaka *et al*., 1999). In conclusion, these and our data imply that SUMOylation plays an important role in sister chromatid separation and in chromosome segregation.

A role for SUMO in APC/C-mediated proteolysis

We have demonstrated that Ubc9 and Smt3 are needed for efficient securin proteolysis in cells arrested in G1 phase, a period of the cell cycle in which APC/C is highly active (Amon *et al*., 1994). Similar to securin degradation, proteolysis of mitotic cyclins was also delayed in *ubc9-2* and *smt3-331* mutants. These data are consistent with the previously demonstrated defect of *ubc9-1* mutants in the degradation of Clb2 and Clb5 (Seufert *et al*., 1995). The findings that APC/C-mediated proteolysis is perturbed in these mutants implies that SUMOylation is required for efficient function of the APC/C.

In contrast to mutants in APC/C subunit genes, we found that *ubc9-2* and *smt3-331* mutants display only partial defects in Pds1 degradation during mitosis of synchronous cultures and in G1-arrested cells. It is possible that SUMO modification plays only a minor role in proper APC/C function, for example for the fine tuning of its

activity. Alternatively, a residual activity of SUMOylation in *ubc9-2* and *smt3-331* mutants at restrictive temperatures may be sufficient for degradation of APC/C substrates, at least with reduced kinetics. Consistent with this hypothesis, we found that low levels of Ubc9 and Smt3 are sufficient for the viability of yeast cells (Fig. 1A).

How may SUMO affect APC/C-mediated proteolysis? We have provided evidence that Ubc9 and Smt3 are not needed for proteolysis in general. Unstable proteins that are not APC/C substrates, such as the SCF^{Cdc4} substrate Gcn4, were normally degraded in *ubc9-2* and *smt3-331* mutants, suggesting that the 26S proteasome is functional. These data favour the model in which specifically APC/C function may be affected in these mutants.

At present, it is unknown how SUMO modification influences this ubiquitin ligase. Previously, proteins with important roles in mitosis were found to be modified with SUMO. One of these is Top2, DNA topoisomerase II. It was shown that modification of Top2 is required for the control of chromosome cohesion at centromeric regions (Bachant *et al*., 2002). SUMO-modified Top2 was unable to promote centromeric cohesions, and this may indicate a regulatory role for SUMO in dissolving the cohesion between sister chromatids. However, there is no evidence that Top2 modification may affect securin stability or APC/C activity. Other recently identified yeast substrates known to be modified by SUMO are the septins Cdc3, Cdc11 and Sep7, which form a ring at the yeast bud neck (Johnson and Blobel, 1999; Takahashi *et al*., 1999). As yeast strains expressing septins lacking SUMO conjugation sites did not display defects in cell cycle progression, it is rather unlikely that a failure to modify these septins contributes to the mitotic defects of Ubc9- and Smt3-depleted cells. It remains to be shown whether factors directly involved in APC/C-mediated proteolysis are modified by SUMO. Using an HA-tagged version of *SMT3*, we did not detect modifications of immunoprecipitated APC/C subunits Cdc16 and Cdc23 or of the substrates Pds1 and Clb2 (data not shown).

Various different functions for SUMO have been identified in the last few years, and it is tempting to speculate that one of these is also required for promoting APC/C-mediated proteolysis. For example, SUMO has been shown to modulate nucleocytoplasmic transport, subcellular localization or protein–protein interactions (Müller *et al*., 2001; Wilson and Rangasamy, 2001). As functional nucleocytoplasmic transport is needed for proteolytic destruction of various APC/C substrates (Loeb *et al*., 1995; Bäumer *et al*., 2000; Hildebrandt and Hoyt, 2001), we tested whether the nuclear localization of APC/C or its substrates is defective when SUMOylation is disturbed. We found that the APC/C subunit Cdc16, the activator protein Cdc20 and the substrates Pds1 were localized in the nucleus in *ubc9-2* and *smt3-331* mutants similarly to wild-type strains, indicating that nuclear import of these factors is not distinctly affected. Furthermore, it is conceivable that defects in nucleocytoplasmic transport would also interfere with Gcn4 proteolysis in these mutants. SCF^{Cdc4} activity is restricted to the nucleus (Blondel *et al*., 2000), and nuclear localization of Gcn4 is required for its efficient degradation (Pries *et al*., 2002). We suggest that SUMOylation rather affects APC/C inside the nucleus.

A possible function of SUMO may be the proper localization of APC/C or its substrates to specific subcellular elements. APC/C is known to be associated with centrosomes and mitotic spindles (Tugendreich *et al*., 1995). Substrates such as securins and cyclins were also localized to spindles (Jensen *et al*., 2001; Raff *et al*., 2002), and it is conceivable that the association of APC/C with the mitotic spindle is important for the destruction of spindle-associated substrates. As SUMOylation has been implicated in regulating protein–protein interactions, a further possibility could be that this modification is critical for the association of APC/C with regulatory proteins or for substrate recognition.

Proteomics may be a promising approach to reveal the SUMO targets essential for mitosis and APC/C function. Then, it will be interesting to elucidate whether SUMOylation is a novel mechanism for the regulation of this ubiquitin ligase.

Experimental procedures

Yeast strains and plasmids

All strains used in this study are derivatives of the *Saccharomyces cerevisiae* W303 strain and are listed in Table 1. The *ubc9* deletion strain containing the temperature-sensitive *ubc9-2* allele was created by C. Michaelis. Initially, a *ubc9* deletion strain (*ubc9Δ*) was constructed by transformation of a deletion cassette containing the *TRP1* gene inserted between a 500 bp fragment containing sequences immediately upstream of the *UBC9* open reading frame (ORF) start codon and a 280 bp fragment containing sequences of the 3′ region. The temperature-sensitive *ubc9-2* allele was created by polymerase chain reaction (PCR) mutagenesis of the *UBC9* gene. Plasmids from colonies growing at 25°C, but not at 37°C were isolated and cloned into the integrative plasmid YIplac128 (containing the *LEU2* marker).

ubc9 deletions strains kept alive by the *GAL-UBC9* gene fusion were created by transformation of a *UBC9/ubc9Δ* heterozygous diploid with a centromeric plasmid containing *GAL-UBC9* and *URA3* as selection marker. *ubc9Δ GAL-UBC9* segregants were obtained by sporulation and tetrad dissection. The *GAL-UBC9* construct was isolated from a *GAL-cDNA* library (Liu *et al*., 1992) during a genetic screening. It contains the entire *UBC9* ORF fused 24 bp upstream of the ATG start codon to the *GAL1-10* promoter. *The GAL-HA3-SMT3* strain, containing a replacement of the endogenous *SMT3* promoter by the *GAL1-10* promoter, and the *smt3-331* mutant strain were received from S. Biggins and have been described previously (Biggins *et al*., 2001).

Table 1. Yeast strains used in this study.

Strain	Relevant genotype
S001	W303-1A wild-type strain
	*MAT*a, *ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, GAL*
S056	*MAT*a, *GAL-CLB3-HA3/URA3, bar1::HisG*
S057	*MAT*a, *GAL-CLB2-HA3/URA3, bar1::HisG*
S088	*MAT*a, *GAL-CDC5-HA3/URA3, bar1::HisG*
S099	*MAT*α, *ubc9::TRP1, ubc9-2/ΛEY2*
S185	*MAT*a, *PDS1-HA3/URA3*
S206	*MAT*a, *GAL-PDS1-HA/URA3, bar1::HisG*
S234	*MAT*a, *CDC16-MYC6/URA3*
S365	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, GAL-PDS1-HA3/URA3, bar1::HisG*
S390	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, Gal-CLB3-HA3/URA3, bar1::HisG*
S396	*MAT*a, *GAL-IME2-HA3/TRP1*
S406	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, PDS1-HA3/URA3*
S425	*MAT*a, *ubc9::TRP1, pRS316-GAL-UBC9/URA3*
S481	*MAT*a, *MYC18-CDC20/TRP1*
S483	*MAT*a, *ubc9::TRP1, pRS316-GAL-UBC9/URA3, pds1::LEU2*
S487	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, GAL-CDC5-HA3/URA3, bar1::HisG*
S527	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, MYC18-CDC20/TRP1*
S528	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, CDC16-MYC6/URA3*
S533	*MAT*a, *GAL-HA3-SMT3/HIS3*
S534	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, GAL-IME2-HA3/TRP1*
S535	*MAT*a, *smt3-331, GAL-PDS1-HA3/URA3, bar1::HisG*
S537	*MAT*a, *PDS1-MYC18/LEU2*
S542	*MAT*α, *smt3-331*
S543	*MAT*a, *smt3-331, GAL-CLB2-HA3/URA3, bar1::HisG*
S544	*MAT*a, *smt3-331, GAL-CLB3-HA3/URA3, bar1::HisG*
S545	*MAT*a, *smt3-331, PDS1-MYC18/LEU2*
S546	*MAT*a, *ubc9::TRP1, ubc9-2/LEU2, PDS1-MYC18/LEU2*
S547	*MAT*a, *smt3-331, MYC18-CDC20/TRP1*
S548	*MAT*a, *smt3-331, CDC16-MYC6/URA3*
S550	*MAT*α, *GAL-HA3-SMT3/HIS3, PDS1-MYC18/LEU2*
S552	*MAT*a, *ubc9::TRP1, pRS316-GAL-UBC9/URA3, PDS1-MYC18/LEU2*
S566	*MAT*a, *GAL-HA3-SMT3/HIS3, mad2::URA3, PDS1-MYC18/LEU2*
S568	*MAT*α, *ubc9::TRP1, ubc9-2/LEU2, mad2::URA3, PDS1-MYC18/LEU2*

Growth conditions and cell cycle arrests

Cells were grown in YEP medium (2% bactopeptone, 1% yeast extract, 0.005% adenine sulphate) supplemented with either 2% glucose (YEPD) or 2% raffinose. Plasmid-carrying strains were grown in appropriate synthetic minimal medium containing 0.8% yeast nitrogen base, supplemented with amino acids and adenine. The *GAL1-10* promoter was induced by the addition of galactose (2% final concentration). Before gene expression from the *GAL1-10* promoter was induced, cells were grown in raffinose medium. To turn off the *GAL1-10* promoter, cells were filtered and resuspended in medium containing 2% glucose. To arrest cells in G1 with α-factor pheromone, cultures were incubated for 2.5 h in the presence of 5 μg ml^{-1} α-factor.

Immunoblotting

Preparation of yeast cell extracts and protein immunoblot analysis were performed as described previously (Surana *et al*., 1993). The enhanced chemiluminescence detection system was used. Antibodies were used in 1:1000 (Clb2), 1:2000 (Cdc28), 1:100 (HA, 12CA5) and 1:100 (MYC) dilutions.

Immunofluorescence and FACS analysis

For indirect immunofluorescence, cells were fixed in 3.7% formaldehyde, and spheroblasts were prepared as described previously (Pringle *et al*., 1991). Staining with 4,6-diamidino-2-phenylindole (DAPI) and anti-tubulin antibodies were used for visualization of nuclei and spindles respectively. The DNA content was determined by FACS analysis as described previously (Epstein and Cross, 1992).

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