Here we report functional characterization of the essential fission yeast Skp1 homologue. We have created a conditional allele of skp1 (skp1–3f) mimicking the mutation in the budding yeast skp1–3 allele. Although budding yeast skp1–3 arrests at the G/S transition, skp1–3f cells progress through S phase and instead display two distinct phenotypes. A fraction of the skp1–3f cells arrest in mitosis with high Cdc2 activity. Other skp1–3f cells as well as the skp1-deleted cells accumulate abnormal thick septa leading to defects in cell separation. Subsequent identification of 16 fission yeast F-box proteins led to identification of the product of pof6 (for pombe F-box) as a Skp1-associated protein. Interestingly, cells deleted for the essential pof6 gene display a similar cell separation defect noted in skp1 mutants, and Pof6 localizes to septa and cell tips. Purification of Pof6 demonstrates association of Skp1, whereas the Pcu1 cullin was absent from the complex. These findings reveal an essential non-Skp1-Cdc53/Cullin-F-box protein function for the fission yeast Skp1 homologue and the F-box protein Pof6 in cell separation.

 Degradation of targets critical for the G1/S transition is mediated by the Skp1-Cdc53/Cullin-F-box protein (SCF) ubiquitin ligase complex. The prototype SCFcdc42 (where the superscript denotes the identity of the variable F-box protein subunit) was defined in budding yeast by both genetic analysis and in vitro reconstitution. Mutants of cdc34, cdc4, cdc53, and skp1 have overlapping phenotypes and are compromised in their ability to degrade the cyclin-dependent kinase inhibitor Sic1 (1, 2). Degradation of Sic1 through ubiquitination is required for the G1/S transition and is mediated by the SCF components Cdc4, Cdc53, and Skp1 together with E1, the E2 enzyme Cdc34p, ATP, and ubiquitin (3, 4). Phosphorylation of Sic1 on a set of cyclin-dependent kinase consensus sites is a prerequisite for its recognition by the ubiquitin ligase (5, 6), and phosphorylation appears to be a general requirement for the recognition by a SCF ubiquitin ligase (7).

In addition to its role in G1, budding yeast Skp1 also belongs to at least three non-SCF type complexes. It is part of the centromere-binding CBF3 complex (8). On the budding yeast kinetochore, Skp1 is required for the activation of Ctf13 (9). Together with the F-box protein Rcy1, Skp1 plays a role in the recycling of the SNARE Snc1 (10, 11). Finally, Skp1 has also been reported to form a complex called RAVE with Rav1 and Rav2 to promote assembly of the vacuolar-ATPase holoenzyme (12, 13).

The 40-residue F-box domain is required (1) but not sufficient (14) for the interaction between a given F-box protein and Skp1. F-box proteins are implicated in the regulation of many cellular processes including transcription, signal transduction, development, metabolism, and cell cycle control (15–17) reflected by the abundance of F-box proteins in budding yeast and mammalian cells (16, 18).

In the fission yeast Schizosaccharomyces pombe, four F-box proteins have been characterized. The Cdc4-related Pop1 and Pop2/Sud1 (19–22) participate in the formation of SCF as either hetero- or homodimers (21–23). In pop mutants, both the short-lived Cdc2-Cdc13 inhibitor Rum1 and the S-phase regulator Cdc18 were stabilized (20, 21). Rum1 is the functional homologue of Sic1 (24), and Cdc18 is the homologue of budding yeast Cdc6 (25). Both budding yeast proteins are degraded through SCFcdc4 (26). pop mutations also result in constitutive transcription of cig2 (27) encoding a B-type cyclin required for normal S-phase (28, 29).

Two other non-essential F-box proteins Pof10 and Pof3 have been reported. Although the function of Pof10 remains to be characterized (30), Pof3 is required for genome integrity and control of telomere length (31).

In this study we have identified and characterized functions of the fission yeast Skp1 homologue. We have also identified a novel essential F-box protein Pof6 that associates with Skp1 in a non-SCF complex. Interestingly, both Skp1 and Pof6 appear to be essential for cell separation of fission yeast cells.

EXPERIMENTAL PROCEDURES

Yeast Strains and Techniques—S. pombe strains used: h α ade6–210 ura4D18 leu1–32, h α ade6–216 ura4D18 leu1–32, h α leu1–32 (Stratagene), h α cdc25–22 ade6–216 leu1–32 (lab stock), and h α cdc2–311 leu1–32 his2 (lab stock). Classical S. pombe techniques were performed as described (32).
Cloning of skp1. Genomic Insert and cDNA—A small-scale library of S. pombe genomic DNA was constructed and probed. Southern blot analysis revealed that when genomic DNA is digested with EcoRI-HindIII, a probe corresponding to the partial skp1 DNA hybridizes with a band of about 1.7 kb. Genomic DNA was digested with EcoRI and HindIII, and the fragments ranging from 1.5 to 2 kb were cloned in pBR322. The resulting library was probed with the same probe. Positive clones were shown to contain 1.7 kb in size. Full-length cDNA of skp1 was cloned by PCR on an S. pombe cDNA library (a kind gift of M. Minet and F. Lacroix) with the following primers: 5'-AGC-GGC-GGC-GTC-GAC-GAA-TTG-ATG-GG-C-3' and 5'-TGCG-GCC-GCG-CCA-TTC-CTC-GAGC-CTG-CTG-CTG-TCC-3'.

Deletion of skp1 and pof6—700 bp upstream (PCR1) and 400 bp downstream (PCR2) regions of the skp1 open reading frame were amplified using the following primers: 5'-CCG-GAA-TTC-TAG-TAA-CTC-CAC-TAA-CA and 5'-CCC-AAG-CTT-TGT-TGA-ATG-TAG-TAA-GTA-ATG-GAT-3' for PCR1 and 5'-CCC-AAG-CTT-TTT-GTG-GCT-TTT-TGT-GTA-ATG-5' and 5'-CCG-GTC-GAG-TAA-GAT-GCA-TTT-TTA-AAG-GTA-ATA-TAT-3' for PCR2. The EcoRI-HindIII-digested PCR1 fragment and the HindIII-Xhol-digested PCR2 fragments were cloned together in a pSK vector. A HindIII fragment containing the 5' skp1 ura4 gene was inserted between the PCR1 and PCR2 fragments to yield the pSK-skp1Δ.

A diploid strain was transformed with an EcoRI-XhoI fragment of the pSK-skp1Δ, and transformants were selected on plates lacking uracil and checked by Southern blot analysis. Deletion of pof6 was performed as described by Tasto et al. (33) using the following oligonucleotides: 5'-GAT-GTC-GTC-ATT-TTA-TGA-ATG-CTG-TAT-CAT-AAT-ATC-ATG-TTC-GCT-AGA-ACC-AAA-TAG-ATT-TC-3' and 5'-CTC-GAG-TGG-TGG-ACT-ATT-TTA-TGA-AGT-ATG-CGG-AAT-ACC-TG-CAC-CAT-C-3' for PCR1 and 5'-GGA-TAT-GAA-GCA-GAT-AAG-CAT-GAC-TTA-TTA-AAG-TGT-ATG-GTA-ATA-TAT-3' and 5'-ATG-GAT-3'.

Construction and Integration of skp1 Mutants—A two-step PCR approach was used to generate the skp1–3f and skp1–4f alleles using the following primers: external oligos, 5'-CCG-GAA-TTC-TAG-TAA-CTC-CAC-TAA-CA and 5'-CCC-AAG-CTT-TGT-TGA-ATG-TAG-TAA-GTA-ATG-GAT-3' for PCR1 and 5'-CCC-AAG-CTT-TTT-GTG-GCT-TTT-TGT-GTA-ATG-5' and 5'-CCG-GTC-GAG-TAA-GAT-GCA-TTT-TTA-AAG-GTA-ATA-TAT-3' and 5'-ATG-GAT-3'. The EcoRI-HindIII-digested fragments of the final PCR products were cloned in pSK digested in EcoRI and HindIII, and the fragments ranging from 1.5 to 2 kb were cloned in pRS306. The resulting library was probed with the same probe. Positive clones were shown to contain 1.7 kb in size. The DNA of these clones was sequenced. Subsequently, the PCR products were cloned in pSK digested in EcoRI and HindIII, and the fragments ranging from 1.5 to 2 kb were cloned in pRS306. The resulting library was probed with the same probe. Positive clones were shown to contain 1.7 kb in size. The DNA of these clones was sequenced.

Deletion of skp1 and pof6—700 bp upstream (PCR1) and 400 bp downstream (PCR2) regions of the skp1 open reading frame were amplified using the following primers: 5'-CCG-GAA-TTC-TAG-TAA-CTC-CAC-TAA-CA and 5'-CCC-AAG-CTT-TGT-TGA-ATG-TAG-TAA-GTA-ATG-GAT-3' for PCR1 and 5'-CCC-AAG-CTT-TTT-GTG-GCT-TTT-TGT-GTA-ATG-5' and 5'-CCG-GTC-GAG-TAA-GAT-GCA-TTT-TTA-AAG-GTA-ATA-TAT-3' and 5'-ATG-GAT-3'.

Construction and Integration of skp1 Mutants—A two-step PCR approach was used to generate the skp1–3f and skp1–4f alleles using the following primers: external oligos, 5'-CCG-GAA-TTC-TAG-TAA-CTC-CAC-TAA-CA and 5'-CCC-AAG-CTT-TGT-TGA-ATG-TAG-TAA-GTA-ATG-GAT-3' for PCR1 and 5'-CCC-AAG-CTT-TTT-GTG-GCT-TTT-TGT-GTA-ATG-5' and 5'-CCG-GTC-GAG-TAA-GAT-GCA-TTT-TTA-AAG-GTA-ATA-TAT-3' and 5'-ATG-GAT-3'.
for temperature sensitivity skp1–4f cells did not differ from wild type cells (Fig. 1D). By contrast, the skp1–3f allele was unable to grow at 37 °C (Fig. 1D) and was chosen for further analysis. At the permissive temperature (25 °C), exponentially growing skp1–3f cells were indistinguishable from wild type cells, but after 4 h at the restrictive temperature (37 °C),
skp1–3f cells arrested cell cycle progression with a 2C DNA content as shown by FACS (Fig. 1E). Cytological analysis revealed that a fraction of the arrested cells are elongated, resulting in a slight drift of the FACS 2C peak. Beside this phenotype, cells with multiple or very thick septum were also observed (Fig. 1E, calcofluor).

The 2C content of skp1–3f-arrested cells was unexpected considering the G1 arrest observed with the identical budding yeast mutant (8). To analyze G1/S progression in more detail, cells were synchronized in G1 by nitrogen starvation. Simultaneously, cells were shifted to 36°C (restrictive temperature for the nda3–311 allele encoding a cold-sensitive allele of beta-tubulin (40). Therefore a strain was generated combining nda3–311 (restrictive temperature 20°C) and skp1–3f (restrictive temperature 37°C). This would potentially allow a successive restriction of first nda3–311 and then skp1–3f functions leading to an increase in the number of cells with the septum defect. To this end, control nda3–311 cells and double mutant skp1–3f nda3–311 cells were synchronized for 6 h at 20°C followed by a rapid shift (in a PCR machine) to 36°C after which fraction of cells with septa was monitored (Fig. 2A). The septation index in the nda3–311 control cells peaked at 75% at 10 min and had decreased to 47% at 20 min. In the double-mutant skp1–3f nda3–311 cells, the septation index rose similarly to the controls, but no decrease was noted at 20 or 30 min (Fig. 2A), consistent with impaired septum processing.

A second approach to increasing the number of cells displaying aberrant septa involved a “block-release-block” experiment. This was initiated by incubating skp1–3f and control cultures at 36°C for 4 h in the presence of 15 μM hydroxyurea (HU). This led to an early S arrest in control cells (Fig. 2B, 240 min, control) with a 1C DNA content, whereas the skp1–3f cells displayed a predominantly 2C DNA content (Fig. 2B, 240 min, skp1–3f), suggesting they were blocked after S-phase, which is consistent with our previous results (Fig. 1).

Subsequently, control cells (blocked at G1/S) and skp1–3f cells (mostly blocked in mitosis) were shifted to 25°C (the permissive temperature for skp1–3f) with simultaneous removal of HU and incubated in fresh medium at 25°C for 20 min. Cells were then re-shifted to 36°C and followed by FACS analysis. In the skp1–3f cells a 4C DNA peak appeared at 30 min and increased until the last time point (Fig. 2B), whereas the 1C wild type culture completed S-phase and subsequently continued a normal cycle.

Cytological analysis of the 4C skp1–3f cells revealed accumulation of pronounced thick septa (Fig. 2C). Based on this, it is likely that the 4C DNA content of these cells is due to a 2C content in both daughter nuclei still attached by the thick septum, indicating that both nuclei have undergone replication. The possibility that the peak would be due to re-replication of unséptated cells was ruled out by unchanged levels of Cdc18 levels throughout the experiment (data not shown). The completion of DNA synthesis in the two daughter nuclei in the septated cells is consistent with the results above (Fig. 1), demonstrating that Skp1 is not required for replication.

The results described above indicate that skp1–3f arrest with a pleiotropic phenotype with elongated cells and with the majority of cells arresting in mitosis with high Cdc2 activity, but with other cells arresting just prior to cell separation with a septum defect. These pleiotropic phenotypes are likely to reflect the various functions Skp1 mediates in distinct SCF com-
plexes with various F-box proteins. For example, elongation of skp1–3f cells at 37 °C is likely to reflect inactivation of SCFPof3 as pof3 deletion has a similar phenotype (31).

A number of F-box proteins can be identified by similarity searches from the fission yeast genome. We have also performed two-hybrid screening with Skp1, leading to the isolation of several F-box proteins partly overlapping with those identified through similarity searches.2 One of the F-box proteins identified in this screen was Pof6.

Pof6 Is an Essential F-box Protein Required for Septum Processing—As described above, Pof6 was identified through a two-hybrid interaction with Skp1 (Fig. 3A). The predicted Pof6 protein encodes a 872-amino acid protein with the F-box region between amino acids 33 and 75 (Fig. 3B). Pof6 also has a CAAX-motif at its C terminus, suggesting that Pof6 may be modified through prenylation (41). Pof6 does not have any closely related sequences in fission yeast, and it is most closely related to Yarrowia lipolytica Sls2p (28% identity between amino acids 45 and 872 in Pof6 (42)) and budding yeast Rcy1p proteins (24% identity between amino acids 150 and 872 in Pof6 (10)). Sls2p has been implicated in secretion and Rcy1p is involved in recycling. In addition, significant sequence similarity was detected between Pof6 and the exocyst subunit Sec10 involved in exocytosis (43). Using reiterations on PSI-BLAST this similarity was noted between amino acids 147 and 853 of Pof6 (13% identity, 30% similarity with fission yeast Sec10). As septum processing and cell separation require exocyst function (44), Pof6 was chosen for further studies as a candidate F-box protein mediating the septum processing phenotype noted in skp1 mutants.

Pof6 protein was found to be constantly expressed during the cell cycle (Fig. 3C). pof6 is an essential gene as determined by the formation of two viable spores from sporulation of diploid S. pombe cells in which one copy of pof6 was deleted (Fig. 3D). Germinated spores form highly branched structures corresponding to unseparated cells (Fig. 3E), and in some cases, a thick septum between two cells was observed (Fig. 3F). This phenotype is highly reminiscent of what we observed in skp1–3f except that no branching was noted with skp1–3f, which is likely due to the fact that cells lacking Skp1 cannot proceed through several cell cycles because Skp1 is also required for other essential steps (see Fig. 1).
izes at the Septum—To test if the Pof6 F-box protein is part of an SCF, a TAP fusion protein was expressed from the endogenous pof6 locus. After tandem affinity purification (45), the eluate (Pof6-TAP) and an eluate from the control strain pcu1-myc (TAP Ctr) were separated by lithium dodecyl sulfate-PAGE and the gel was silver-stained. The gel was blotted and probed with anti-Psh1 (left panel) (= Skp1, a gift of D. Wolf) or anti-myc (right panel). For each Western blot, the original lysate used for the purification (Extr.) was also separated and probed as indicated. B, anti-GFP Western blot analysis of diploid strains expressing either GFP-Pof6 (wt) or mutants lacking the F-box (∆F-box) or the CAAX box (∆CAAX box) under control of the nmt1 promoter. Cells were grown in presence or absence of thiamine to repress or de-repress the promoter, respectively. C, GFP-Pof6 (wt), GFP-Pof6-∆F-box (∆F-box), or GFP-Pof6-∆CAAX-box (∆CAAX box) strains from B were sporulated, and tetrads were dissected as indicated. The 2:2 segregation obtained with the ∆F-box mutant indicates F-box is essential for growth. D, fluorescence micrographs of GFP-Pof6 (wt), GFP-Pof6-∆F-box (∆F-box), or GFP-Pof6-∆CAAX-box (∆CAAX box) strains grown in the presence of thiamine (low expression) and observed under fluorescence. Arrows point to GFP-Pof6 localization to the septum and the arrowhead to cell tips. (Bar = 10 μm).

Fig. 4. Pof6 belongs to a non-SCF complex and localizes at the septum. A, TAP purification was performed on a pof6-TAP pcu1-myc strain exactly as described (45). The eluate (Pof6-TAP) and an eluate from the control strain pcu1-myc (TAP Ctr) were separated by lithium dodecyl sulfate-PAGE and the gel was silver-stained. The gel was blotted and probed with anti-Psh1 (left panel) (= Skp1, a gift of D. Wolf) or anti-myc (right panel). For each Western blot, the original lysate used for the purification (Extr.) was also separated and probed as indicated. B, anti-GFP Western blot analysis of diploid strains expressing either GFP-Pof6 (wt) or mutants lacking the F-box (∆F-box) or the CAAX box (∆CAAX box) under control of the nmt1 promoter. Cells were grown in presence or absence of thiamine to repress or de-repress the promoter, respectively. C, GFP-Pof6 (wt), GFP-Pof6-∆F-box (∆F-box), or GFP-Pof6-∆CAAX-box (∆CAAX box) strains from B were sporulated, and tetrads were dissected as indicated. The 2:2 segregation obtained with the ∆F-box mutant indicates F-box is essential for growth. D, fluorescence micrographs of GFP-Pof6 (wt), GFP-Pof6-∆F-box (∆F-box), or GFP-Pof6-∆CAAX-box (∆CAAX box) strains grown in the presence of thiamine (low expression) and observed under fluorescence. Arrows point to GFP-Pof6 localization to the septum and the arrowhead to cell tips. (Bar = 10 μm).

To test if the Pof6 F-box protein is part of an SCF, a TAP fusion protein was expressed from the endogenous pof6 locus. After tandem affinity purification (45), the eluate was separated on lithium dodecyl sulfate-PAGE and silver-stained revealing several bands (Fig. 4A). The 19-kDa band represents Skp1 as demonstrated by Western blot analysis (Fig. 4A). The identity of several other bands is unknown and will be subjected to further study. However, interestingly, no bands were detected at the expected size of Pcu1-myc (112 kDa, the only essential cullin in S. pombe (21)) or Pip1 (13 kDa, the Rbx1 homologue). Furthermore, the absence of Pcu1 from the Pof6 complex could also be demonstrated by Western blot analysis, showing that although the characteristic doublet representing Pcu1-myc (46) was clearly detected in the extract used for the purification, it did not co-purify with Pof6. Separate co-immunoprecipitation analysis confirmed this result (data not shown).

To localize Pof6 in the cell, three GFP-Pof6 constructs corresponding to wild type, a mutant lacking the F-box, or a mutant lacking the CAAX box have been integrated in diploid cells at the pof6 locus under the control of the thiamine-regulated nmt1 promoter. Fig. 4B shows anti-GFP Western blots performed on extracts from these strains grown in the presence or absence of thiamine. The diploid strains were subsequently sporulated, and tetrads were analyzed indicating that only the disruption of the F-box of Pof6 was detrimental to fission yeast survival based on the 2:2 segregation (Fig. 4C). Germinated spores on the plate have a phenotype identical to the pof6 deletion (not shown).

To analyze the subcellular localization of GFP-Pof6, GFP-Pof6-∆F-box, or GFP-Pof6-∆CAAX, the strains were grown in the presence of thiamine (low expression, see Fig. 4B). The wild type GFP-Pof6 displays a nuclear enrichment but is excluded from a nuclear structure that could correspond to the nucleolus. GFP-Pof6 is also localized at cell tips and on both sides of the septum in septated cells (Fig. 4D, wt). Localization of the GFP-Pof6-∆F-box and GFP-Pof6-∆CAAX were similar to wild type (Fig. 4D, ∆F-box, ∆CAAX) except that in the CAAX box mutant, localization at the tips was less pronounced. This indicates that the F-box, and by extension association with Skp1, is not required for proper localization of Pof6, though it is essential for Pof6 function. It also shows that the CAAX box is not required for the essential function of Pof6.

The localization of Pof6 to the septum is consistent with a function in septum processing and cell separation. It is interesting to note that this subcellular localization is similar to that noted for the Sec8p exocyst component (44).

From these studies we conclude that Pof6 and Skp1 are physically associated in a non-SCF complex. Both Skp1 and Pof6 are required for normal septum processing and cell sepa-
ration, a phenomenon that has not been characterized in much detail. However, recently the Sec8 subunit of the exocyst complex was also found to be required for septum formation and cell separation (44), and several other exocyst proteins in addition to Sec3p were found at the septa. Thus it is interesting to speculate that Pof6 and Skp1 functions in cell separation would be related to exocyst function. In this regard it is very interesting to note that the apparent homologues of Pof6 in Y. lipolytica (Sla2p) and budding yeast (Rcy1p) have been implicated in transport and recycling, respectively (10, 11, 42).

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