Cell, Vol. 103, 375–386, October 27, 2000, Copyright ©2000 by Cell Press

Cleavage of Cohesin by the CD Clan Protease Separin Triggers Anaphase in Yeast

Frank Uhlmann,*‖ Dominik Wernic,† Marc-André Poupart,† Eugene V. Koonin,‡ and Kim Nasmyth*§
*Research Insitute of Molecular Pathology
Dr. Bohr-Gasse 7
A-1030 Vienna
Austria
†Boehringer Ingelheim (Canada) Ltd.
2100 Cunard Street
Laval H7S 2G5
Canada
‡National Center for Biotechnology Information
National Insitutes of Health
Bethesda, Maryland 20894

Summary

In eukaryotic cells, replicated DNA strands remain physically connected until their segregation to opposite poles of the cell during anaphase. This "sister chromatid cohesion" is essential for the alignment of chromosomes on the mitotic spindle during metaphase. Cohesion depends on the multisubunit cohesin complex, which possibly forms the physical bridges connecting sisters. Proteolytic cleavage of cohesin's Scc1 subunit at the metaphase to anaphase transition is essential for sister chromatid separation and depends on a conserved protein called separin. We show here that separin is a cysteine protease related to caspases that alone can cleave Scc1 in vitro. Cleavage of Scc1 in metaphase arrested cells is sufficient to trigger the separation of sister chromatids and their segregation to opposite cell poles.

Introduction

Accurate chromosome segregation is crucial for the proliferation of somatic cells and for the production of haploid gametes during meiotic divisions. Mistakes during the chromosome segregation process give rise to cells with too many or too few chromosomes. This state, known as aneuploidy, is associated with most malignant tumor cells and is the leading cause of mental retardation (Down's syndrome) and spontaneous fetal abortion (Griffin, 1996; Lengauer et al., 1997).

The segregation of chromosomes to opposite poles of the cell requires their prior alignment on the mitotic spindle. During this process, sister chromatids condense, partially but not completely separate from each other, and attach to microtubules that extend to opposite poles of the cell. This culminates in a state called metaphase, during which sister chromatids come under tension from spindles attempting to pull them in opposite directions. Soon after all pairs of sister chromatids come under tension, chromosomes suddenly split and sister chromatids segregate to opposite halves of the cell; this period of mitosis is called anaphase (reviewed in Nasmyth et al., 2000). The metaphase to anaphase transition is one of the most dramatic and highly regulated steps in the process by which eukaryotic cells disseminate their genomes during cell proliferation. Its molecular basis has long remained elusive.

It has long been recognized that metaphase is a state of equilibrium (Mazia, 1961) during which sister chromatid splitting due to traction exerted by microtubules on kinetochores is counteracted by connections that hold sisters together. It is also clear that sister separation is an autonomous process that frequently still occurs even when poisons destroy the spindle (Mole-Bajer, 1958). These observations have led to the notion that chromosome segregation during anaphase might be triggered not by any change in the force exerted by microtubules, but rather by the sudden loss of cohesion holding sister chromatids together. Evaluation of this hypothesis, however, has had to await discovery of the apparatus that holds sisters together during metaphase.

Recent studies have identified a multisubunit complex, called cohesin, which is essential for holding sister chromatids together in organisms as diverse as yeast and *Xenopus* and is a good candidate for the substance that forms the bridge between sisters (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Toth et al., 1999). Cohesin functions from S phase until anaphase. It is both necessary for establishing sister chromatid cohesion during DNA replication (Uhlmann and Nasmyth, 1998) and for maintaining connections between sisters during metaphase (Ciosk et al., 2000).

In yeast, cohesin is tightly associated with chromosomes from DNA replication until the metaphase to anaphase transition (Michaelis et al., 1997; Toth et al., 1999), whereupon proteolytic cleavage of its Scc1 subunit causes cohesin to dissociate from chromosomes (Uhlmann et al., 1999). Scc1 cleavage occurs within two related sequence motifs. Mutation of both sequences abolishes cleavage and blocks the separation of sister chromatids. These results demonstrate that Scc1 cleavage is essential for sister chromatid separation. They also raise the possibility that Scc1 cleavage might be the long sought after anaphase trigger.

Scc1's cleavage and dissociation from chromosomes depends both in vitro and in vivo on a protein called Esp1 (Uhlmann et al., 1999). Because of their key role in separating sister chromatids, Esp1 and its homologs in other organisms have been called separins (Funabiki et al., 1996a; Ciosk et al., 1998). For most of the cell cycle, separins are bound by inhibitory proteins called securins (called Pds1 in budding yeast) (Yanagida, 2000). Shortly before the metaphase to anaphase transition, ubiquitination of securin by the anaphase-promoting complex (APC) in conjunction with the Cdc20 protein causes securin to be degraded by the 26S proteosome (Irniger et al., 1995; Cohen-Fix et al., 1996; Funabiki et al., 1996b; Yamamoto et al., 1996). This activates separin

§To whom correspondence should be addressed (e-mail: nasmyth@nt.imp.univie.ac.at).
‖Present address: Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom.

and enables it to promote Scc1 cleavage and sister separation (Ciosk et al., 1998; Uhlmann et al., 1999).

Previous studies have left unanswered whether separin is itself the protease that cleaves Scc1, whether Scc1 cleavage during metaphase is sufficient to trigger chromosome segregation, and whether separin has functions besides Scc1 cleavage that help orchestrate chromosome movement during anaphase. We show here that separin purified from yeast can cleave pure Scc1 protein prepared from insect cells. We also demonstrate that separin's active site resembles that of the CD clan of cysteine proteases, a group of related proteases that includes caspases. To address whether cleavage of Scc1 is separin's main if not sole function, we replaced one of Scc1's cleavage sites by that of a foreign protease and showed that cleavage by this protease is sufficient to trigger chromosome segregation in metaphase arrested cells.

Results

Mitosis-Specific Phosphorylation of Scc1 Correlates with Efficient Cleavage

In our previous study, Scc1 was presented to crude extracts containing separin in the form of a crude chromatin preparation. To address whether separin is the protease responsible for cleaving Scc1, we needed to prepare a better defined substrate. To do this, we first characterized the state of Scc1 protein at the time of its cleavage. We used a yeast strain whose sole source of the APC activator protein Cdc20 was under control of the galactose inducible *GAL1* promoter. Cells were arrested in metaphase by incubation in medium lacking galactose and subsequently induced to undergo anaphase by readdition of galactose (Uhlmann et al., 1999) (Figure 1A).

Using Western blotting after SDS-PAGE, we noticed that a large fraction of Scc1 prepared from metaphase arrested cells migrated more slowly during electrophoresis than Scc1 from asynchronous cells (Figure 1B). The slower migrating form of Scc1 disappeared as Scc1 cleavage products appeared, whereas the amount of the faster migrating form remained unchanged (Figure 1B, 10 min). This suggests that modification of a fraction of Scc1 during metaphase decreases its electrophoretic mobility and that modified but not unmodified Scc1 might be destined for cleavage during anaphase. Phosphatase treatment of Scc1 from chromatin showed that its decreased mobility when isolated from metaphase cells was due to phosphorylation (Figure 1C).

To address whether Scc1's phosphorylation affects its cleavability in vitro, chromatin from metaphase arrested cells was treated either with phosphatase or with a control buffer. The chromatin was subsequently washed to remove the phosphatase and used as substrate in an Scc1 cleavage reaction. While hyperphosphorylated Scc1 from the control chromatin was efficiently cleaved, Scc1 from the phosphatase treated chromatin was almost completely resistant to cleavage (Figure 1C). This suggests that mitosis-specific phosphorylation might make Scc1 a better substrate for cleavage. However, phosphatase treatment of the chromatin might have reduced the phosphorylation of other cohesin subunits and/or other chromosomal proteins, which may also have contributed to the reduced Scc1 cleavage.

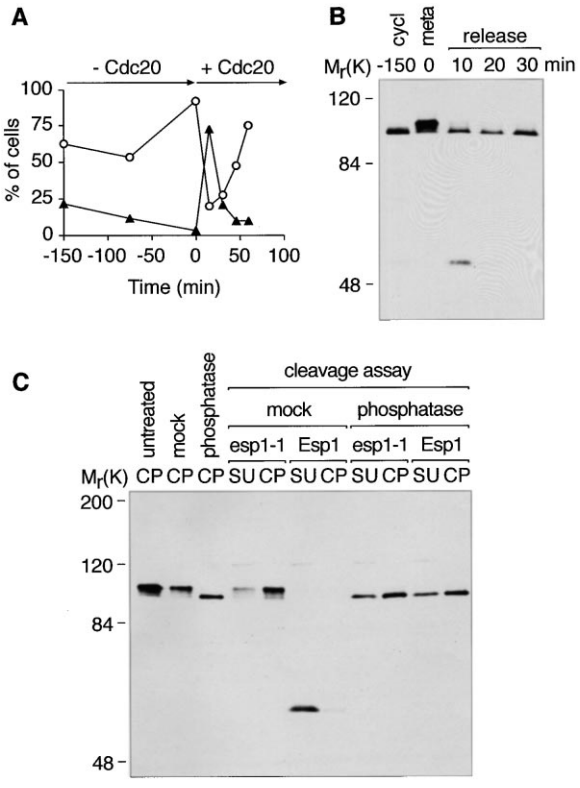

Figure 1. Mitotic Phosphorylation Makes Scc1 a Substrate for Esp1-Dependent Cleavage

(A) Metaphase arrest and release of strain K7677 (*MATa cdc20Δ GAL-CDC20 SCC1-HA3 TetR-GFP TetOs*) was performed as in (Uhlmann et al., 1999). The percentage of cells with Scc1 bound to chromosomes was determined on chromosome spreads (open circles), cells with separated sister chromatids were identified by the appearance of two separated CenV-GFP signals (filled triangles).

(B) SDS-PAGE analysis and Western blot of whole cell extracts prepared from cells at the indicated time points. Scc1-HA3 was detected with monoclonal antibody 16B12 (Babco).

(C) Chromatin was prepared from cells of strain K7677 arrested in metaphase. Chromatin was treated with lambda phosphatase, or mock treated, and used as substrate in an Scc1 cleavage assay as described (Uhlmann et al., 1999). Aliquots of the chromatin preparations (CP), as well as aliquots of the supernatant fractions (SU) and remaining chromatin (CP) after incubation in the cleavage assay were analysed by SDS-PAGE and Western blotting as in (B).

Phosphorylated Recombinant Scc1 Is a Cleavage Substrate

To establish whether Scc1 can serve as a cleavage substrate when separated from chromatin and other cohesin subunits, we purified Scc1 from insect cells expressing a Flag-epitope-tagged yeast *SCC1* gene from a recombinant baculovirus (Figure 2A). We also purified yeast Scc1 from baculovirus infected insect cells after they had been treated with Okadaic acid. This is a phosphatase inhibitor that induces a metaphase-like state in which many mitotic proteins are hyperphosphorylated (Kramer et al., 2000). Okadaic acid treatment caused

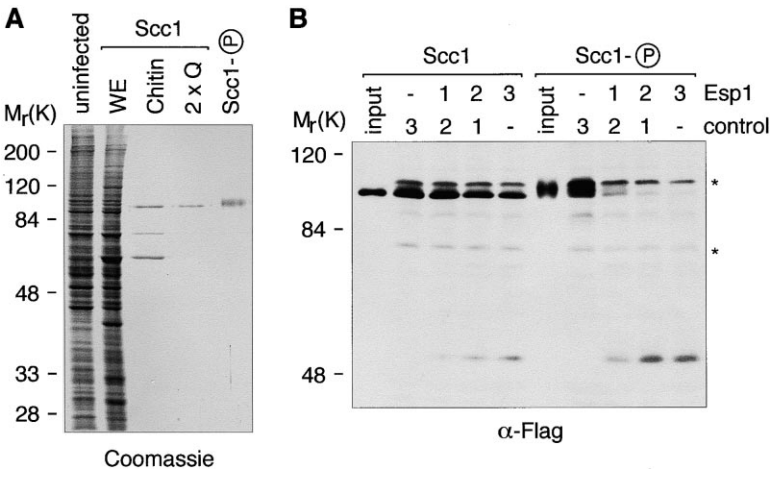

Figure 2. Purified Recombinant Scc1 in a Mitotically Phosphorylated Form Is a Cleavage Substrate

(A) Purification of Scc1 after expression from a recombinant baculovirus in insect cells. Whole cell extracts from uninfected and infected (WE) insect cells were analyzed by SDS-PAGE and staining of the gel with Coomassie brilliant blue. Fractions after chitin affinity chromatography (Chitin) and after two chromatographic steps on a MonoQ column (2 × Q) are shown. The final fraction after purifiction of Scc1 from insect cells treated with Okadaic acid (Scc1-P) is also shown.

(B) Purified Scc1 and Scc1-P were incubated in cell extracts from yeast strain K7287 *(MATα, esp1-1, GAL-ESP1 × 3, TetR-GFP TetOs).* Extract from a culture that had been induced to express Esp1 for 4 hr before harvesting (Esp1) was mixed with extract from uninduced cells (control) in the indicated ratios. After incubation, aliquots of each reaction were analyzed by SDS-PAGE followed by Western blotting against the Flag epitope at the C terminus of Scc1 using monoclonal antibody M2 (Sigma). Asterisks indicate Flag crossreacting bands present in the yeast cell extracts.

yeast Scc1 to migrate more slowly during SDS-PAGE (Figure 2A) and this was reversed by phosphatase treatment (data not shown). We called this hyperphosphorylated form Scc1-P. Scc1 and Scc1-P were incubated in yeast extracts containing varying amounts of separin (Figure 2B). Some underphosphorylated Scc1 was cleaved by extracts with the highest Esp1 concentration, but most remained uncleaved (Figure 2B, left half). In contrast, all Scc1-P was cleaved by extracts containing intermediate levels of Esp1 separin (Figure 2B, right half). We conclude that soluble recombinant Scc1 can be cleaved in an Esp1-dependent reaction and that mitosis-specific phosphorylation of Scc1 increases its susceptibility to cleavage.

Affinity-Purified Esp1 Separin Cleaves Scc1-P

Scc1 proteolytic activity has thus far only been detected in crude extracts prepared from yeast cells overexpressing Esp1 separin. The protease activity might be due entirely to separin or due to a protease that is merely activated by separin. To distinguish between these two alternatives, we expressed from the *GAL* promoter a version of Esp1 separin containing a chitin binding domain fused to its C terminus and used chitin beads to affinity purify the Esp1 fusion protein from yeast extracts (Figure 3A). This protein was also tagged at its N terminus with a Flag epitope.

Ponceau S staining detected only two abundant polypeptides in the affinity purified material. Western blotting against the Flag epitope showed that the 180 kDa band corresponded to the Esp1 fusion protein (Figure 3A, right panel), whereas mass spectrometry showed that a 70 kDa band was a mixture of 4 members of the Hsp70 family of chaperone proteins (data not shown). Copurification of heat shock proteins indicates that a sizable fraction of the overproduced Esp1 separin might be in a nonnative conformation. It is likely that silver staining would have detected several other proteins in this preparation, albeit at low abundance compared to the Esp1 fusion protein.

Remarkably, Scc1-P was efficiently cleaved when incubated with chitin beads loaded with Esp1 separin but not with beads prepared from uninduced extracts (Figure 3B). Thus, Esp1 separin might indeed be the protease that cleaves Scc1. However, we cannot eliminate at this stage the possibility that the Scc1 cleavage activity is due to a substoichiometric protease that copurifies with separin.

The Conserved Separin Domain Contains a Region that Resembles the Catalytic Site of CD Clan Cysteine Proteases

All known Esp1 separin homologs possess a conserved C-terminal "separin" domain. The recent arrival in ge-

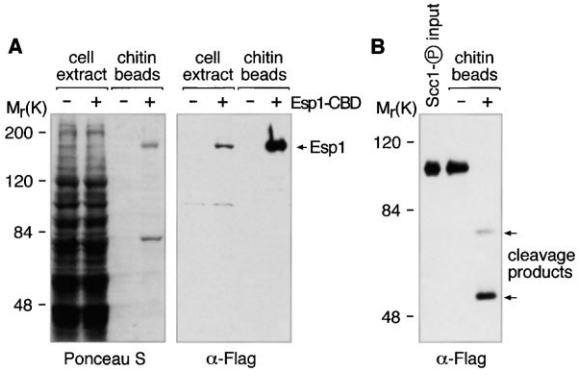

Figure 3. Affinity Purified Esp1 Cleaves Scc1-P

(A) Cell extracts from strain K8965 *(MATα, esp1-1, GAL-Flag-ESP1-CBD × 6, TetR-GFP TetOs)* were prepared without or with induction of Esp1 from the galactose inducible *GAL* promoter. Esp1 was affinity purified on a chitin resin as described in Experimental Procedures. Aliquots of the cell extracts and the chitin beads were analyzed by SDS-PAGE followed by transfer of the gel to a nitrocellulose membrane and Ponceau S staining (left panel). Esp1 was identified by probing the membrane with antibodies against the Flag epitope at the N terminus of Esp1 (right panel).

(B) Scc1-P was added to the chitin beads after the purification procedure and incubated. After incubation, the supernatant above the chitin beads was recovered and analyzed by SDS-PAGE and Western blotting against the Flag epitope on Scc1.

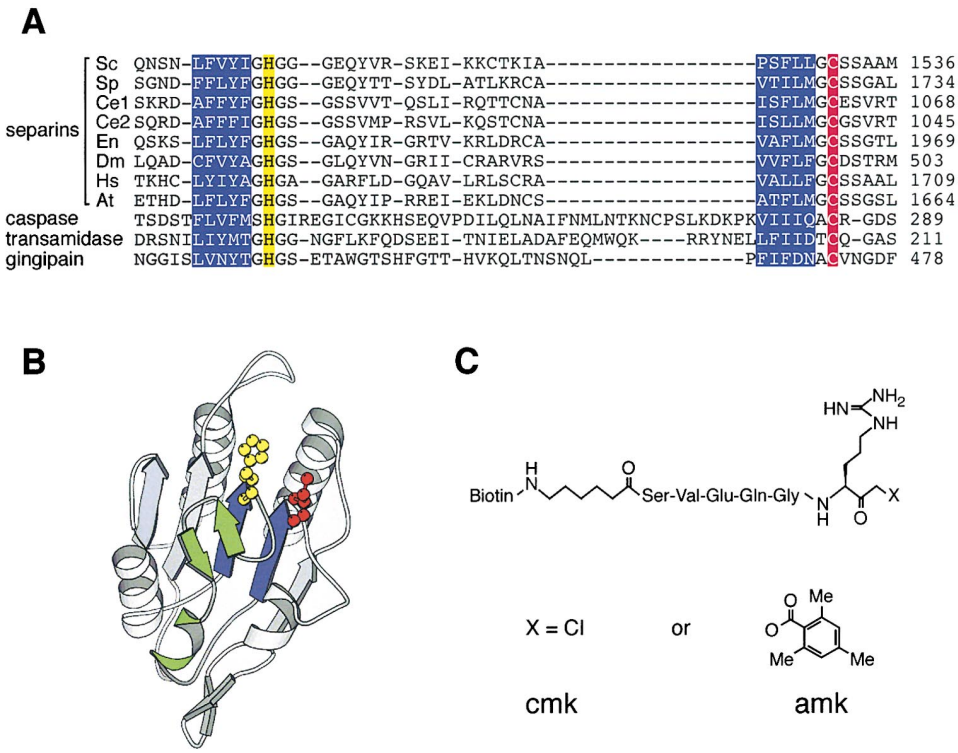

Figure 4. Esp1 and the CD Clan of Cysteine Proteases

(A) Sequence conservation between separins in the region of the proposed proteolytic catalytic site. Sequence alignment in the region of the strictly conserved His and Cys residues is shown together with the sequences of human caspase 1, the transamidase Gpi8, and gingipain. Key to sequences: separins from *S. cerevisae* Esp1 (AAB03897), *S. pombe* Cut1 (A35694), two proteins from *C. elegans* (AAA83576 and the WormPep-Entry CE22098), *E. nidulans* BimB (P33144), *D. melanogaster* (conceptual translation of AC0114811), *H. sapiens* (BAA11482), *A. thaliana* (CAA19812), human caspase 1 (P29466), human Gpi8 (CAA68871), and *P. gingivalis* gingipain (P95493).

(B) The catalytic site of gingipain (Eichinger et al., 1999) is shown as a model for that of separin. The histidine and cysteine catalytic residues are colored yellow and red respectively, whereas hydrophobic parallel β strands on the N-terminal side of these catalytic residues are shown in blue. The polypeptide chain between the two strands is in green. Residues within separins that are proposed to correspond to these structural motifs are marked with the same color scheme in the alignment in A. The figure was drawn with MOLSCRIPT (Kraulis, 1991).

(C) Structure of the chloromethyl ketone (cmk) and acyloxymethyl ketone (amk) derivatives of the Scc1 cleavage site peptide, synthesized as inhibitors against Esp1.

nome databases of several new members of this family have highlighted the most conserved amino acid residues within the separin domain. They include a conserved histidine and cysteine residue, which are hallmarks of cysteine proteases (Figure 4A)(Barrett et al., 1998). These two residues are invariably surrounded by small residues (glycine or serine), and each is preceded by amino acid sequences that are predicted to form hydrophobic β sheets. This pattern is characteristic of cysteine endopeptidases of the CD clan, which includes caspases, legumains, gingipains, clostripains, and GPI-anchor amidases (Chen et al., 1998; Eichinger et al., 1999). In the case of caspase and gingipain, whose crystal structures are known, the two hydrophobic β sheets associate with each other in parallel, bringing the histidine and cysteine residues into juxtaposition to form the catalytic dyad (Figure 3B)(Eichinger et al., 1999). This predicts that the conserved histidine in Esp1 at amino acid position 1505 and the cysteine at position 1531 should be essential for its proteolytic activity (see below).

An Esp1 Inhibitor

If separins are proteases whose mechanism resembles that of caspases, then it should be possible to create peptide based inhibitors (Nicholson et al., 1995; Faleiro et al., 1997) that form a covalent bond with their active site cysteine. Our yeast separin inhibitors were based on the hexapeptide sequence "SVEQGR", which precedes the (more efficiently used) cleavage site in Scc1 at amino acid position 268 (Uhlmann et al., 1999). The C terminus corresponding to the P1 position of the Scc1 cleavage site was modified to a chloromethyl ketone (cmk) or an acyloxymethyl ketone, (amk) (Aplin et al., 1983; Krantz, 1994). Meanwhile, a biotin moiety was linked to the N terminus of the peptide to allow identification of proteins to which the inhibitor might bind (Figure 4C).

Both inhibitors were effective at inhibiting cleavage of Scc1 (presented on yeast chromatin) by crude extracts containing separin (Figure 5). Preincubation with 10 μM Bio-SVEQGR-amk or 100 μM Bio-SVEQGR-cmk inhibited most cleavage activity. We could not detect labeling of any specific protein in the extracts treated with 10 μM Bio-SVEQGR-amk, presumably due to a high background level of naturally biotinylated proteins (data not shown). The same was true for the cmk derivative, whose less specific reactivity might have been responsible for its lower efficiency as a separin inhibitor in crude extracts. Crucially, Bio-SVEQGR-amk failed to inhibit, even at 1 mM, the ability of the TEV protease (Parks et

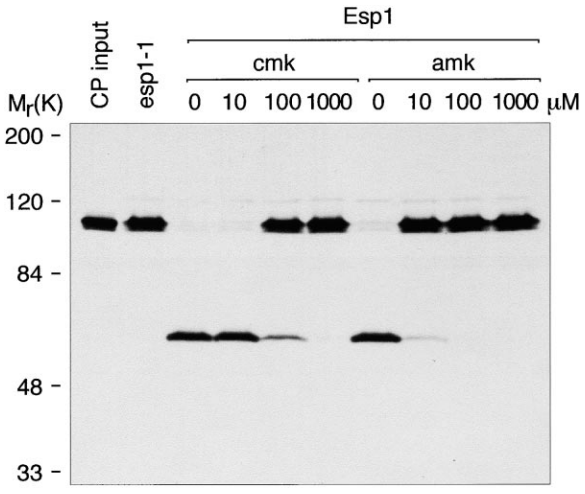

Figure 5. Inhibition of Scc1 Cleavage by the Covalent Inhibitors

An Scc1 cleavage assay was performed as described (Uhlmann et al., 1999), using chromatin prepared from strain K7563 *(MATα, SCC1-HA6)*, arrested in metaphase by treatment with nocodazole, as the substrate. Cell extract used for the reaction from strain K7287 *(MATα, esp1-1, GAL-ESP1 × 3, TetR-GFP TetOs)* was preincubated with the indicated concentrations of either inhibitor. After the reaction, chromatin and supernatant were not separated again, but an aliquot of the reaction mix was analyzed by SDS-PAGE and Western blotting against the HA epitope on Scc1.

al., 1995), which is also a cysteine protease, to cleave a version of Scc1 engineered to contain a TEV protease recognition site (see below and data not shown). We conclude that Bio-SVEQGR-amk selectively inhibits the Scc1 cleaving protease in crude extracts containing Esp1 separin.

The Effect of Catalytic Dyad Mutations on Protease Activity and Inhibitor Binding

We next mutated the histidine 1505 and cysteine 1531 residues that are predicted to form separin's catalytic dyad (Figure 4A). Both wild-type and the two mutant forms of Esp1 separin were purified after overexpression in yeast. SDS-PAGE analysis followed by Coomassie blue staining (Figure 6A) and Western blotting (Figure 6B) of the material bound to chitin beads showed that they contained similar amounts of wild-type or mutant separin protein. Beads containing wild-type protein but not those containing the mutant proteins fully cleaved Scc1-P (Figure 6C). Underphosphorylated Scc1 isolated from insect cells that had not been treated with Okadaic acid was a poorer substrate (last two lanes in Figure 6C). These results imply that histidine 1505 and cysteine 1531 are crucial for the protease activity associated with Esp1 separin and that phosphorylation of Scc1 facilitates its recognition and/or cleavage. It seems unlikely that two independent single amino acid changes in Esp1 would abolish the copurification of a contaminating protease. Both point mutations also abolished Esp1's ability, when expressed from the *GAL* promoter, to rescue the temperature sensitive lethality of a strain carrying the *esp1-1* mutation (data not shown). This indicates that both histidine 1505 and cysteine 1531 are essential for Esp1 function in vivo.

The Scc1-P cleavage activity associated with wild-type protein was abolished by preincubation of the beads in 10 μM Bio-SVEQGR-amk (Figure 6C). To detect the proteins to which the inhibitor had bound, beads containing either wild-type, H1505A, or C1531A Esp1 were incubated in the presence or absence of 10 μM Bio-SVEQGR-amk. Proteins from the beads were then separated by SDS-PAGE and analyzed by anti-biotin affinity blotting. Trace amounts of a highly abundant biotinylated cellular protein (asterisk in Figure 6D) were detected by this method on untreated beads containing either wild-type or mutant proteins. In the case of wild-type Esp1 and H1505A mutant Esp1, two other proteins with apparent molecular weights of around 180 kDa and 80 kDa were labeled with biotin only upon incubation with Bio-SVEQGR-amk (Figure 6D). Western blotting showed that the 180 kDa protein comigrated with the Flag-tagged Esp1 fusion protein. Neither the 180 kDa protein nor the 80 kDa protein was labeled by the inhibitor when the beads contained C1531A mutant protein. These results suggest that the 180 kDa protein is full-length Esp1 separin, whereas the 80 kDa protein is a cleavage product of Esp1 that lacks its N-terminal Flag epitope tag but contains its C-terminal catalytic cysteine.

The Bio-SVEQGR-amk inhibitor was designed to covalently modify the cysteine within separin's potential active site. The dependence of Esp1 labeling with Bio-SVEQGR-amk on cysteine 1531 is therefore consistent with the assignment of this residue to separin's active site. The histidine residues within the catalytic dyads of cysteine proteases are essential for their action on natural polypeptide substrates but they are dispensable for the binding of these covalent inhibitors. Thus, both the labeling of Esp1 by Bio-SVEQGR-amk and the dependence of this labeling on cysteine 1531 confirm that Esp1 separin is indeed the target of this inhibitor. Esp1 separin must therefore be the protease responsible for Scc1 cleavage.

Scc1 Cleavage Triggers Anaphase

Our finding that Esp1 separin is the Scc1 protease raises the possibility that cleavage of Scc1 might be Esp1's sole function and that this event alone triggers chromosome movement during anaphase. It is also possible that the Esp1 protease has several other important substrates whose cleavage is necessary for chromosome movement. Esp1 might even have activities unrelated to proteolysis, given that its protease domain is situated at the C terminus of a rather large protein.

If cleavage of Scc1 by Esp1 separin were sufficient to trigger anaphase, then cleavage of Scc1 by a protease other than Esp1 should be sufficient to trigger sister chromatid segregation in cells that have been arrested in metaphase. To test this, we took advantage of the fact that Scc1 has two separate cleavage sites, either of which can be mutated without blocking sister chromatid separation as long as the other site remains unchanged (Uhlmann et al., 1999). This enabled us to exchange the cleavage site at position 268 with the recognition sequence for the tobacco etch virus (TEV) protease (Dougherty et al., 1989) (Figure 7A). This Scc1 variant, called Scc1-TEV268, was capable of supporting the proliferation of cells lacking any other form of Scc1. Western blot analysis confirmed that the remaining separin rec-

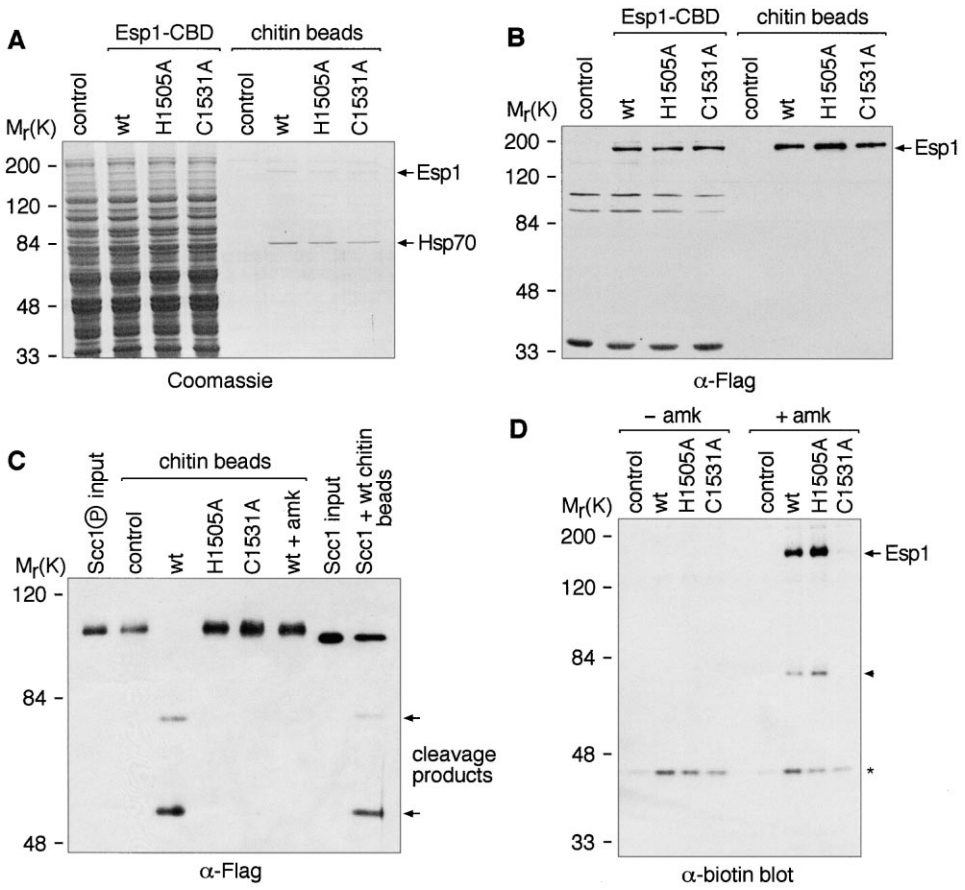

Figure 6. Mutants in the Predicted Esp1 Catalytic Active Site and Affinity Labeling of the Protease.

(A) Cell extracts were prepared from strains K8965 *(MATα, esp1-1, GAL-Flag-ESP1-CBD × 6, TetR-GFP TetOs)* before and after induction, and from strains K8966 *(MATα, esp1-1, GAL-Flag-ESP1-CBD(H1505A) × 6, TetR-GFP TetOs)* and K8967 *(MATα, esp1-1, GAL-Flag-ESP1-CBD(C1531A) × 6, TetR-GFP TetOs)* after induction of Esp1 expression for 5 hr. Esp1 was affinity purified from these extracts as described in Experimental Procedures. Aliquots of the cell extracts and of the material bound to the chitin beads after purification were analyzed by SDS-PAGE followed by staining of the gel with Coomassie brilliant blue.

(B) As (A), but proteins were analyzed by SDS-PAGE followed by Western blotting against the Flag epitope on Esp1. Three Flag crossreacting bands are visible in the uninduced control extract.

(C) Purified recombinant Scc1-P was incubated with affinity purified wild-type and mutant Esp1 on chitin beads. A reaction with Scc1-P incubated with chitin beads after a mock purification from extracts without induced Esp1 is shown (control), as well as a reaction where wild-type Esp1 was treated with the Bio-SVEQGR-amk inhibitor before adding Scc1-P. The last two lanes show a reaction using underphosphorylated Scc1 as the substrate on chitin beads with bound wild-type Esp1.

(D) Control chitin beads, and wild-type and mutant Esp1 bound to chitin beads were incubated without or with addition of 10 μM Bio-SVEQGR-amk. After incubation, proteins bound to the chitin beads were analyzed by SDS-PAGE followed by anti-biotin affinity blot (Faleiro et al., 1997).

ognition site at position 180 was cleaved in cycling cells (Figure 7B, cycl).

We next introduced TEV protease (Parks et al., 1995) under control of the inducible *GAL* promoter. Nuclear localization signals were attached to the protease to prevent its accumulation solely in the cytoplasm and myc epitopes were added to detect its expression in yeast. The myc-tagged TEV protease was induced in nocodazole arrested cells containing Scc1-TEV268. The protease accumulated within the nuclei of most cells after two hours and caused cleavage of Scc1-TEV268 at the expected position (Figure 7B). Analysis of chromosome spreads showed that Scc1-TEV268 cleavage was accompanied by its dissociation from chromosomes (Figure 7C). This demonstrates that cleavage of Scc1 is sufficient to induce its dissociation from chromosomes. Induction of the TEV protease in nocodazole arrested cells expressing only wild-type Scc1 caused neither cleavage nor dissociation from chromosomes (Figure 7C and data not shown).

To address whether cleavage of Scc1 induced by the TEV protease is sufficient to trigger anaphase, we used cells whose sole *CDC20* gene was under control of the *MET3* promoter (Yeong et al., 2000). We arrested these cells in metaphase by transferring them from minimal medium lacking methionine to complete medium containing methionine. Cells accumulated with large buds, replicated chromosomes, and a short bipolar mitotic spindle spanning a single DNA mass at the bud neck. TEV protease synthesis was then induced by the addition of galactose. Induction was less efficient than in nocodazole arrested cells but TEV protease neverthe-

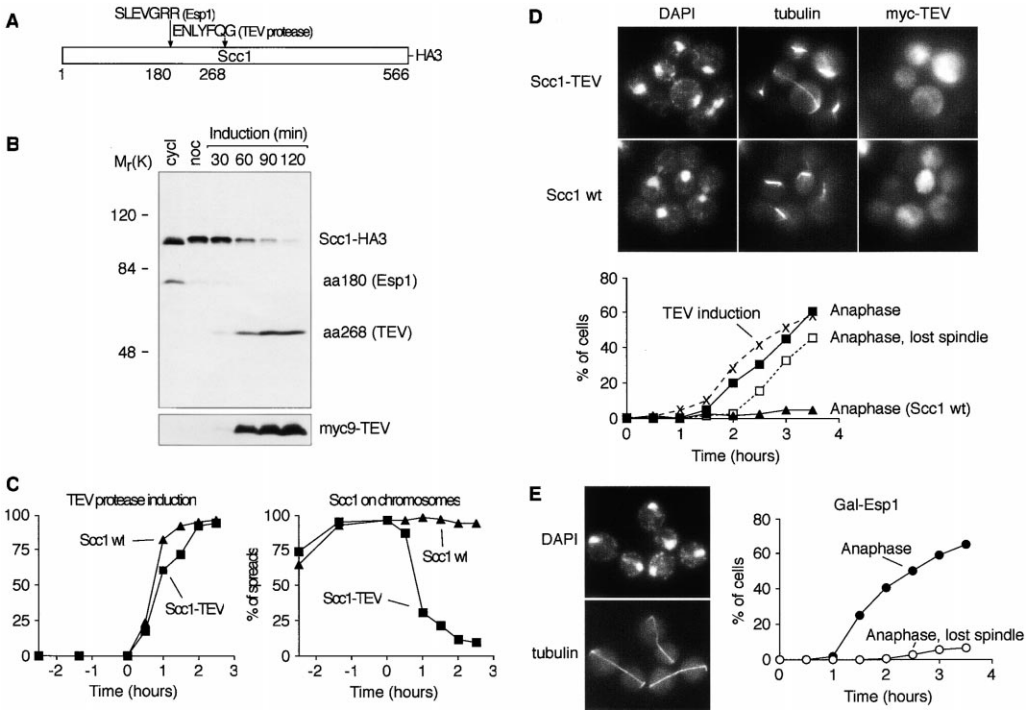

Figure 7. Scc1 Cleavage by TEV Protease Triggers Anaphase

(A) Schematic of the two cleavage sites in Scc1. The wild-type cleavage site at amino acid postion 180, and the TEV protease consensus recognition site that replaces the cleavage site at position 268.

(B) Strain K8758 *(MATα, scc1Δ, SCC1-TEV268-HA3, GAL-NLS-myc9-TEV-NLS2 × 10)* was grown in YEP medium with raffinose and arrested with nocodazole for 2.5 hr. Then, 2% galactose was added to induce TEV protease expression. Aliquots of the culture were harvested at the indicated times and whole cell extract was prepared and analyzed by SDS-PAGE followed by Western blotting against the HA epitope on Scc1.

(C) As (B), but also strain K9127 *(MATα, SCC1-HA3, GAL-NLS-myc9-TEV-NLS2 × 10)* was used in a parallel experiment. TEV protease expression was scored by in situ immunostaining against the myc epitope using monoclonal antibody 9E10. Scc1 association with chromosomes was seen on chromosome spreads stained for the HA epitope on Scc1 using monoclonal antibody 16B12 (Babco).

(D) Cells of strains K9027 *(MATα, MET3-CDC20, scc1Δ, SCC1-TEV268-HA3, GAL-NLS-myc9-TEV-NLS2 × 10, tetOs, tetR)* and K9128 *(MATα, MET3-CDC20, SCC1-HA3, GAL-NLS-myc9-TEV-NLS2 × 10, tetOs, tetR)* were arrested in metaphase as described in Experimental Procedures. At 30 min intervals, samples were retrieved from the culture and processed for immunostaining against tubulin using monoclonal antibody YOL1/34 (Serotec) and against the myc epitope on TEV protease using monoclonal antibody 9E10. DNA was stained with DAPI. The pictures shown are from cells at 3 hr after induction of TEV protease by galactose addition.

(E) As (D), but strain K9129 *(MATα, esp1-1, MET3-CDC20, SCC1-HA3, GAL-Flag-ESP1-CBD × 6, tetOs, tetR)* was used for the experiment.

less accumulated in 40% of the cells within two hours of galactose addition. Remarkably, most cells that had accumulated TEV protease after two hours possessed an elongated anaphase spindle and two equal DNA masses at opposite poles of the cell (Figure 7D, Scc1-TEV). In contrast, cells that had not accumulated TEV protease still possessed short metaphase spindles and a single DNA mass close to the bud neck. In cells with separated DNA masses, sister DNA sequences at the *URA3* locus, which were marked by GFP (Michaelis et al., 1997), were found in opposite halves of the cell (data not shown), indicating that sister chromatids had been properly segregated. We repeated this experiment with cells that expressed wild-type Scc1 and found that cells which had accumulated TEV protease still possessed short spindles and unseparated DNA masses at the bud neck (Figure 7D, Scc1 wt). This shows that cleavage of Scc1 in metaphase arrested cells is sufficient to trigger the movement of sister chromatids to opposite poles. They are presumably pulled there by the mitotic spindle, whose elongation is also triggered by Scc1 cleavage (Figure 7D).

At later time points after TEV protease induction, we observed an increasing number of cells whose elongated mitotic spindles had become weaker or had even disappeared. Such cells had accumulated high levels of TEV protease and had therefore expressed the protease for longer than other cells (Figure 7D). These cells invariably contained two spindle poles at opposite ends of the cell. Figure 7D shows that cells with fully separated DNA masses (with or without anaphase spindles) started to accumulate 60 min after galactose addition, whereas cells with fully separated DNA masses and faint or disassembled anaphase spindles did so 30 min later. These results suggest that TEV protease triggers both chromatid segregation and expansion of pole to pole microtubules but that the highly elongated spindles produced under these circumstances are unstable and break down approximately 30 min after their formation.

Disassembly of the mitotic spindle is not observed in Cdc20 deprived cells which have been permitted to undergo anaphase by deletion of the *PDS1* gene (Shirayama et al., 1999). This suggests that the spindle instability of Cdc20 deprived cells induced to undergo ana-

phase by the TEV protease cannot be due to the lack of Cdc20 function per se and suggests that it might be due to the lack of separin activity. To test whether spindles would be more stable in the presence of Esp1 separin, we analyzed spindle stability in Cdc20 depleted cells induced to undergo anaphase by expressing high levels of Esp1 from the *GAL* promoter. Induction of Esp1 triggered anaphase in a large fraction of cells and did so somewhat more rapidly than the TEV protease. Crucially, the extended anaphase spindles produced by Esp1 persisted even after long periods in all cells that had fully segregated their chromosomes (Figure 7E). We conclude that while cleavage of Scc1 in metaphase triggers chromatid segregation, it might not be sufficient to bring about a stabilization of the mitotic spindle that is necessitated by the movement of spindle poles to opposite ends of the cell. We cannot at this stage completely exclude the possibility that destabilization of anaphase spindles is an artifact of TEV protease expression. However, this seems unlikely because cells containing wild-type Scc1 proliferate normally even when high levels of TEV protease are continuously expressed (data not shown).

Discussion

Our previous experiments demonstrated that Scc1 cleavage is crucial for sister chromatid separation at the onset of anaphase. We also described a crude cell-free system that recapitulated cleavage of Scc1 and its dissociation from chromosomes (Uhlmann et al., 1999) and showed that both events were dependent on Esp1 separin. However, these experiments neither identified the protease responsible for Scc1 cleavage nor addressed whether cleavage actually triggers anaphase. The experiments described here show that Esp1 separin is the Scc1 protease and that Scc1 cleavage is the event that triggers the segregation of sister chromatids to opposite poles of the cell at the metaphase to anaphase transition.

Reconstitution of Scc1 Cleavage with Purified Components

Identification of the Scc1 protease required two separate tasks: first to prepare a fully defined substrate and second to purify the protease. We found that pure yeast Scc1 is a substrate for Esp1-dependent cleavage as long as it is hyperphosphorylated either during mitotic arrest in yeast cells or by the treatment of insect cells with Okadaic acid. Though our experiments do not directly address the physiological significance of Scc1 phosphorylation, they nevertheless raise the possibility that Scc1 cleavage is controlled by its cell-cycle-regulated phosphorylation as well as by cell cycle dependent destruction of the Esp1 inhibitor Pds1 securin.

Our identification of the Scc1 protease started from the premise that Scc1 cleavage is an Esp1-dependent reaction. We therefore set out to purify Esp1 and to characterize its activity. Our first attempts yielded virtually homogeneous Esp1 preparations which lacked any proteolytic activity on their own and also failed to stimulate Scc1 cleavage when added back to yeast cell extracts (data not shown). We found that the Scc1 cleaving activity from yeast cell extracts overexpressing Esp1 was unstable, with a half-life of a few hours. We therefore used a rapid single step purification scheme in which Esp1 fused to a chitin binding domain was bound to a chitin resin. Remarkably, such beads efficiently cleaved pure phosphorylated Scc1. This result suggested that Esp1 separin might alone be capable of cleaving Scc1 but it did not exclude the possibility that a substoichiometric protease bound to Esp1 was responsible for the proteolytic activity. The final line of argument that Esp1 is indeed the Scc1 protease was inspired by the discovery that separins contain a highly conserved domain at their C terminus that resembles the catalytic dyad of an established class of cysteine proteases.

Separins and the CD Clan of Cysteine Proteases

Sequence similarity between separins from different organisms is largely confined to a C-terminal region spanning some 400 residues, with the highest conservation within the 100 C-terminal residues. When the conserved amino acid pattern hhhhx[GS]HGx(4)hx(14,32)hhx[GA]Cx[GS] (h indicates a hydrophobic residue; x indicates any residue; letters in brackets indicate alternative residues; numbers in parentheses indicate the number of residues in an interval; and numbers separated by a comma mean "from–to") was used to search the complete protein sequence database, 175 sequences were retrieved, all of them representing either the separin family or the CD clan of cysteine proteases. Some of the latter were missed, but no false-positives were detected. A notable difference between separins and the CD clan proteases is the much shorter distance that separates the catalytic histidine and cysteine in the former. However, because the length, sequence, and arrangement of structural elements within this spacer are variable within the CD clan itself, this difference is not incompatible with the hypothesis that separins contain a cysteine protease domain that is structurally similar and evolutionarily related to CD clan proteases. It is remarkable that sequence-specific proteases of the same fold and likely common origin are involved in a crucial aspect of chromosome mechanics and in the control of programmed cell death.

As demanded by this model, the cysteine and histidine residues predicted to be part of separin's active site were found to be essential for Esp1's proteolytic activity. There is one other cysteine residue that is conserved in the separin domains of most species (cysteine 1556 in Esp1), except for *Arabidopsis* and *Aspergillus*, where it is replaced by a serine. This residue might also qualify as a putative catalytic amino acid. However, mutation of cysteine 1556 to alanine had no effect on Esp1's protease activity (data not shown). We therefore conclude that histidine 1505 and cysteine 1531 likely form Esp1's catalytic dyad.

Inspired by separin's similarity to caspases, we devised peptide based inhibitors that were designed to bind covalently to the active site cysteine of the Scc1 protease. Modification of the C-terminal arginine in the hexapeptide SVEQGR, which precedes the second Scc1 cleavage site, to an acyloxymethyl ketone created a potent and specific inhibitor of the Scc1 cleavage activity associated with purified Esp1. The inhibitor bound

covalently to full length Esp1 and to a minor Esp1 cleavage product but to no other protein in our purified Esp1 preparations. Furthermore, this binding was dependent on the proposed active site cysteine 1531. These results imply that Esp1 is the sole target of our inhibitor and that Esp1 must therefore be the Scc1 protease. It will nevertheless be important to confirm the presence of the CD clan like protease domain within separins by atomic resolution structural studies of Esp1 and its homologs.

Triggering Chromosome Movement in Anaphase

It has been a longstanding question what initiates the segregation of sister chromatids to opposite poles of the cell at the metaphase to anaphase transition. Activation of separin through destruction of their inhibitory securin chaperone by the APC is clearly a crucial step. Our previous finding that Scc1 cleavage is essential for sister chromatid separation together with the discovery that separin is the protease responsible for Scc1 cleavage suggest that Scc1 cleavage alone could trigger anaphase. To test this, we engineered a yeast strain in which one of Scc1's cleavage sites was replaced by that of the foreign TEV protease. Remarkably, induction of TEV protease in cells arrested in metaphase due to Cdc20 depletion was sufficient to cleave Scc1, to cause its dissociation from chromosomes, and to trigger sister chromatid separation and movement to opposite poles of the cell.

The TEV protease experiment also demonstrates that the yeast mitotic spindle is prepared to drive spindle poles to opposite halves of the cell during metaphase but that it is prevented from doing so by cohesin, connecting sister chromatids. To determine more precisely whether the kinetics of spindle extension due to a TEV protease-induced anaphase are similar to that in wild-type cells, it will be necessary to visualize these movements in real time. It is, however, unclear whether accumulation of TEV protease within nuclei following galactose induction is sufficiently instantaneous to make a meaningful comparison at the moment. Remarkably, expression of the TEV protease in yeast cells was harmless to wild-type cells. Thus, the introduction of TEV protease recognition sites to other proteins could also be used to study the consequences of their deliberate cleavage. This technique might be useful also in other organisms and particularly for applications where specific cleavage reactions are involved, e.g., signaling via the notch pathway (Blaumueller et al., 1997).

We noticed that the mitotic spindles of cells which had been induced to undergo anaphase by TEV protease were thinner than normal anaphase spindles and frequently broke after chromosome segregation. This phenotype was not observed when anaphase was triggered by inducing high levels of Esp1 in metaphase arrested cells. This suggests that separin might have a second function besides cleaving Scc1: to stabilize mitotic spindles during anaphase. It has previously been suggested that separin might have a role in spindle function on the basis of its association with mitotic spindles during anaphase (Funabiki et al., 1996a; Ciosk et al., 1998; Kumada et al., 1998). Whether Esp1 performs this function by cleaving other proteins or by a different mechanism remains to be investigated. It is even conceivable that Scc1 cleavage products have a role in anaphase spindle stabilization and that the products produced by TEV protease cleavage are altered in this regard.

There are good reasons to believe that proteolytic cleavage of cohesins by separin might trigger anaphase in all eukaryotic organisms. The catalytic site of yeast Esp1 is conserved in all known separins. Furthermore, an immunopurified Esp1 fraction from human cells posesses Scc1 cleavage activity and Scc1 is both cleaved and disappears from centromeres at the metaphase to anaphase transition in human cells (Waizenegger et al., 2000 [this issue of *Cell*]). Given their conservation and similarity to caspases, we suggest that separins might be better known as "chromatid separases" (Ostergren and Anderson, 1973), or simply "separases".

Experimental Procedures

Dephosphorylation of Scc1 on Chromatin

A crude chromatin fraction containing Scc1 tagged with HA epitopes was obtained as described (Liang and Stillman, 1997). Chromatin obtained from 50 μl cell lysate was resuspended in 200 μl phosphatase buffer (50 mM Tris/HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 2 mM $MnCl_2$, 5 mM dithiothreitol, 0.01% Brij 35, and 0.1% Triton X-100), and 2000 units lambda protein phosphatase (New England Biolabs) was added. Incubation was for 12 min at 30°C, then the chromatin was recovered by centrifugation and washed once before it was used for the Scc1 cleavage assay as described (Uhlmann et al., 1999). To exclude that the effect observed after phosphatase treatment of the chromatin was due to residual amounts of phosphatase carried into the subsequent cleavage reaction, a control reaction was carried out in which 200 units of lambda phosphatase were added to the cell extract. This had no effect on the efficiency of Scc1 cleavage in the assay.

Purification of Scc1 Expressed in Baculovirus Infected Insect Cells

The Scc1 coding sequence was cloned into the baculovirus transfer vector pFastBac1 (Gibco Life Technologies). At the C terminus, a Flag epitope tag was added followed by a cassette containing the yeast VMA intein and a chitin binding domain (New England Biolabs). Recombinant baculoviruses were obtained, and HiFive insect cells (Invitrogen) grown in monolayers to confluency were infected at a multiplicity of infection of 2. To obtain metaphase-like phosphorylation, 0.1 μM Okadaic acid was added 40 hr after infection. Forty three hours after infection, cells were harvested. Cytoplasmic and nuclear extracts were obtained as described (Cai et al., 1996). When Scc1 was purified after treatment of the cells with Okadaic acid, 5 mM NaF and 5 mM sodium pyrophosphate was added to the extraction buffer. Combined cytoplasmic and nuclear extracts from cells in 10 T250 flasks (8 ml, 100 mg protein) were loaded onto 1 ml bead volume of chitin beads (New England Biolabs), equilibrated in buffer H_{200} (50 mM HEPES/KOH [pH 7.5], 200 mM NaCl, 1.5 mM $MgCl_2$, and 0.01% Nonidet P-40). Loading was for 2 hr by rocking at 4°C . The chitin resin was then washed in a column with 20 column volumes each of buffer H_{600}, H_{1000}, and H_{200} (index indicates the concentration of NaCl). The column was then flushed with 3 column volumes of buffer H_{200} containing 50 mM dithiothreitol and closed for cleavage of the intein overnight. The eluate containing Scc1 released from the column was directly loaded onto a MonoQ HR 5/5 column (Amersham Pharmacia), and the column was developed with a gradient from H_{200} to H_{1000} in 10 ml. Fractions containing Scc1 were detected by SDS-PAGE followed by Coomassie staining and Western blotting against the Flag epitope. Pooled fractions were diluted to 200 mM NaCl by adding buffer H_0, and chromatography on the MonoQ column was repeated. This step was necessary to remove traces of a contaminating protein that copurified with Scc1 and was identified as Hsp70 from the insect host cells by mass spectrometry. The final eluate was dialyzed against buffer H_{100} and concentrated by

ultrafiltration. This protocol yielded 25 μg of purified Scc1 or purified metaphase-like phosphorylated Scc1.

Cleavage of Purified Scc1 in Yeast Extracts

Scc1 cleavage-competent yeast extracts after overexpression of Esp1 and control extracts were obtained as described (Uhlmann et al., 1999). Six microliters (20 ng/μl) purified Scc1 or Scc1-P in H_{100} was added to 12 μl of control or Esp1 containing extracts, or to mixtures at different rations between these extracts. Incubation was for 10 min at 25°C. The reaction was stopped by adding SDS-PAGE loading buffer, and aliquots were analyzed by SDS-PAGE and Western blotting against the Flag epitope.

Overexpression and Purification of Esp1 from Yeast Cells

The Esp1 coding sequence was cloned under control of the *GAL* promoter into the yeast vector YIp204 (Gietz and Sugino, 1988). At the N terminus of the protein, a Flag epitope tag was added; at the C terminus a chitin binding domain was added (New England Biolabs). Site directed mutagenesis of the residues His1505, Cys1531, and Cys1556 to alanine was performed using PCR. The linearized vectors were integrated into the yeast genome at the *TRP1* locus, and transformants were identified by Southern blotting that had integrated 6–8 copies of the respective constructs. Two-hundred milliliter cultures of these strains were grown in YEP medium with 2% raffinose as the carbon source (Rose et al., 1990) at 25°C. At a density of 2.5×10^6 cells/ml, the *GAL* promoter was induced by the addition of 2% galactose for 5 hr before cells were harvested. One culture was left uninduced as a control for the purification. Cells were resuspended in 6 ml 100 mM PIPES/KOH (pH 9.3), 10 mM dithiothreitol, and 0.1% sodium azide, and incubated at room temperature for 10 min. Cell were collected again by centrifugation and resuspended in 4 ml of 50 mM potassium phosphate (pH 7.5), 0.6 M sorbitol, 0.5 mM $MgCl_2$, and 10 mM dithiothreitol. Zymolase T-100 (ICN Biochemicals) was added to a final concentration of 40 μg/ml and spheroplasting was at 37°C for 10 min. Cells were collected again, washed in 2 ml of 50 mM HEPES/KOH (pH 7.5), 100 mM KCl, 2.5 mM $MgCl_2$, and 0.4 M Sorbitol, and resuspended in 350 μl buffer FB (50 mM HEPES/KOH [pH 7.5], 250 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM dithiothreitol, plus protease inhibitors as described [Liang and Stillman, 1997]). Cells were lysed by the addition of 0.25% Trition X-100 for 3 min on ice, and the lysate was cleared by centrifugation. The extracts were loaded onto 50 μl bead volume of chitin beads and equilibrated in buffer FBX (FB plus 0.05% Triton X-100) for 2 hr on a rotating wheel. Beads were transferred into a chromatography column and washed with 5 ml each of FBX, FBX_{1000}, and FBX_{100}, containing 1 M and 100 mM NaCl, respectively. Beads were then resuspended in a total volume of 100 μl FBX_{100} and 20 μl aliquots of the suspension were distributed. Beads were sedimented by gravity and 10 μl supernatant was retrieved and discarded. For detection of purified Esp1, 10 μl of SDS-PAGE loading buffer was added to the beads, boiled for 5 min, and 10 μl of the SDS eluate was loaded for SDS-PAGE.

Scc1 Cleavage by Esp1 Bound to Chitin Affinity Beads

Ten microliters (20 ng/μl) of purified Scc1 or Scc1-P in FBX_{100} supplemented with 0.1 mg/ml BSA was added to 10 μl of chitin beads obtained as described above. The reaction was incubated for 15 min at 25°C with continuous shaking. Beads were sedimented by brief centrifugation and 10 μl supernatant was retrieved and loaded for SDS-PAGE. In experiments where the peptide inhibitor was used, 10 μl of a 20 μM dilution of the inhibitor (see below) was added to 10 μl of chitin beads and incubated 10 min at 25°C. Then, 10 μl of supernatant was retrieved again, and Scc1 was added and incubated as above. To detect the proteins on the beads labeled by the inhibitor, the beads were boiled with 10 μl of SDS-PAGE loading buffer and the eluate was loaded for SDS-PAGE. After electrophoresis, the gel was transferred to a nitrocellulose membrane and biotinylated proteins were detected as described (Faleiro et al., 1997).

Sequence Analysis

Searches of the nonredundant protein sequence database and of nucleotide sequence databases at the National Center for Biotechnology Information (NIH, Bethesda) were performed using the PSI-BLAST program (Altschul et al., 1997). Additional database searches combining BLASTP with a pattern analysis were performed using the PHI-BLAST program (Zhang et al., 1998). Database screening for amino acid patterns was performed using the PATTINPROT program at the NPS2 server (Combet et al., 2000). Multiple sequence alignment construction and analysis, with statistical evaluation of the significance of motif conservation, were performed using the GIBBS sampling option of the MACAW program (Schuler et al., 1991; Neuwald et al., 1995). Multiple-alignment-based protein secondary structure prediction was performed using the PHD (Rost and Sander, 1994) and PSIPRED (Jones, 1999) programs.

Peptide Based Covalent Inhibitors for Esp1-Dependent Scc1 Cleavage

A biotinylated peptidyl chloromethyl ketone (cmk) and a peptidyl (2,4,6-trimethylbenzoyloxy)methyl ketone (acyloxymethyl ketone, amk) of the sequence Ser-Val-Glu-Gln-Gly-Arg were synthesized. The side chain protected peptide fragment Biotinyl-6-aminohexanoyl-Ser-Val-Glu-Gln-Gly-OH was obtained by solid phase synthesis on an acid sensitive 2-chlorotrityl resin. H-Arg(Z)2-cmk or H-Arg(Z)2-amk fragments were coupled in solution (Aplin et al., 1983; Krantz, 1994), and the final peptides were deprotected by a TFA-trifluoromethanesulfonic acid-thioanisole mixture (Yaima and Fujii, 1981). The compounds were purified to homogeneity by preparative reversed phase HPLC. One hundred millimolar stock solutions of these inhibitors were prepared in DMSO and stored at −80°C, dilutions were prepared in 50 mM HEPES/KOH (pH 7.5), 100 mM KCl, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol. To assess the effect of the inhibitors on Esp1-dependent Scc1 cleavage, a standard cleavage assay was performed as described (Uhlmann et al., 1999), but the cell extracts used for the cleavage reaction were preincubated for 10 min at 25°C with the inhibitors.

Cleavage of Scc1 by TEV Protease and Esp1 In Vivo

The sequence Ser-Val-Glu-Gln-Gly-Arg-Arg around the cleavage site in Scc1 at amino acid position 268 was changed into the consensus recognition sequence for TEV protease Glu-Asn-Leu-Tyr-Phe-Gln-Gly, (Dougherty et al., 1989) by site-directed mutagenesis. The resulting modified *SCC1-TEV268* gene was cloned together with 1150 nt of upstream promoter sequence into YIp211 (Gietz and Sugino, 1988), and a triple HA epitope tag was added at the C terminus. This *SCC1* construct was integrated at the *LEU2* locus, and correct integration was confirmed by Southern blotting. The endogenous copy of *SCC1* was then disrupted by replacement with the *HIS3* marker gene (Wach et al., 1994). The TEV protease coding sequence was cloned into YIp204 (Gietz and Sugino, 1988) under control of the *GAL* promoter. At the N terminus, the consensus sequence of the SV40 nuclear localization signal (Kalderon et al., 1984) was added together with a tandem repeat of nine myc epitopes. At the C terminus, a repeat of two SV40 nuclear localization signals was added. The resulting construct was integrated into the yeast genome at the *TRP1* locus, and strains with 10 copies of the vector integrated were selected by Southern blot analysis (integrated into the strain containing Scc1-TEV268 only: strain K8758, or containing wild-type Scc1: strain K9127). A strain containing the sole source of Cdc20 under control of the *MET3* promoter was constructed by integrating a modified version of plasmid pUS1278 (Yeong et al., 2000) into strain K7100, containing GFP marked chromosome V (Michaelis et al., 1997). The centromere and ARS region of pUS1278 were replaced by a fragment spanning nucleotides −806 to −212 upstream of *CDC20*. Linearization and integration of this plasmid at the *CDC20* locus results in replacement of the *CDC20* promoter with the *MET3* promoter (Strain K9022). Strains K8758 and K9022 were crossed to yield strain K9027 used for the experiments. A control strain (K9128) was constructed similarly, but only wild-type Scc1 was present in the cells, tagged at the C terminus with a triple HA epitope (Knop et al., 1999). The strain for induction of Esp1 from the *GAL* promoter in metaphase arrest due to depletion of Cdc20 was obtained by crossing strain K8965 (see above) with K9022. For arrest of *MET3-CDC20* cells in metaphase, cells were grown in synthetic minimal medium lacking methionine (Rose et al., 1990) at 23°C. Cells were filtered and transferred into YEP medium containing 2% raffinose supplemented with 2 mM methionine for

arrest in metaphase for 2.5 hr. Then, 2% galactose was added to induce expression of TEV protease or Esp1. The metaphase arrest due to Cdc20 depletion was stable until at least 4 hr after all cells had reached metaphase. In the case of overexpression of Esp1 in the metaphase arrest, a small percentage of cells (less than 20%) formed additional buds after 3.5 hr, probably because cyclin-dependent kinases were partly inactivated under these conditions (Tinker-Kulberg and Morgan, 1999). Cytokinesis or rereplication of the DNA, however, did not occur during the time of observation.

Acknowledgments

We thank G. Stier for providing the TEV protease clone. We also thank K. Mechtler for protein sequencing; W. Bode for providing the coordinates of the gingipain crystal structure; F. Eisenhaber and A. Schleiffer for sequence alignments and the gingipain figure; Y. Lazebnik and T. Skern for helpful discussions; A. Barrett, M. Glotzer, and I. Waizenegger for comments on the manuscript; and I. Waizenegger, S. Hauf, and J.-M. Peters for communicating unpublished results. This research was supported by Boehringer Ingelheim and the Austrian Industrial Research Promotion Fund. F. U. acknowledges support through an EMBO long-term fellowship.

Received June 21, 2000; revised August 18, 2000.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.

Aplin, R.T., Christiansen, J., and Young, G.T. (1983). Amino-acids and peptides, part 48. Synthesis of bradykinyl-chloromethane. Int. J. Peptdide Protein Res. *21*, 555–561.

Barrett, A.J., Rawlings, N.D., and Woessner, J.F. eds. (1998). Handbook of proteolytic enzymes (London, Academic Press).

Blaumueller, C.M., Qi, H., Zagouras, P., and Artavanis-Tsakonas, S. (1997). Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell *90*, 281–291.

Cai, J., Uhlmann, F., Gibbs, E., Flores-Rozas, H., Lee, C.-G., Phillips, B., Finkelstein, J., Yao, N., O'Donnell, M., and Hurwitz, J. (1996). Reconstitution of human replication factor C from its five subunits in baculovirus-infected insect cells. Proc. Natl. Acad. Sci. USA *93*, 12896–12901.

Chen, J.M., Rawlings, N.D., Stevens, R.A., and Barrett, A.J. (1998). Identification of the active site of legumain links it to caspases, clostripain and gingipains in a new clan of cysteine endopeptidases. FEBS Lett. *441*, 361–365.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998). An Esp1/Pds1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell *93*, 1067–1076.

Ciosk, R., Shirayama, M., Shevchenko, A., Tanaka, T., Toth, A., Shevchenko, A., and Nasmyth, K. (2000). Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol. Cell *5*, 1–20.

Cohen-Fix, O., Peters, J.-M., Kirschner, M.W., and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev. *10*, 3081–3093.

Combet, C., Blanchet, C., Geourjon, C., and Deléage, G. (2000). NPS@: network protein sequence analysis. Trends Biochem. Sci. *25*, 147–150.

Dougherty, W.G., Cary, S.M., and Parks, T.D. (1989). Molecular genetic analysis of a plant virus polyprotein cleavage site: a model. Virology *171*, 356–364.

Eichinger, A., Beisel, H.G., Jacob, U., Huber, R., Medrano, F.J., Banbula, A., Potempa, J., Travis, J., and Bode, W. (1999). Crystal structure of gingipain R: an Arg-specific bacterial cysteine proteinase with a caspase-like fold. EMBO J. *18*, 5453–5462.

Faleiro, L., Kobayashi, R., Fearnhead, H., and Lazebnik, Y. (1997). Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. EMBO J. *16*, 2271–2281.

Funabiki, H., Kumada, K., and Yanagida, M. (1996a). Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. EMBO J. *15*, 6617–6628.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T., and Yanagida, M. (1996b). Cut2 proteolysis required for sister-chromatid seperation in fission yeast. Nature *381*, 438–441.

Gietz, R.D., and Sugino, A. (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six base pair restriction sites. Gene *74*, 527–534.

Griffin, D.K. (1996). The incidence, origin, and etiology of aneuploidy. Int. Rev. Cytol. *167*, 263–296.

Guacci, V., Koshland, D., and Strunnikov, A. (1997). A direct link between sister chromatid cohesion and chromosome condensation revealed through analysis of MCD1 in S. cerevisiae. Cell *91*, 47–57.

Irniger, S., Piatti, S., Michaelis, C., and Nasmyth, K. (1995). Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast. Cell *81*, 269–278.

Jones, D.T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. *292*, 195–202.

Kalderon, D., Roberts, B.L., Richardson, W., and Smith, A. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499–509.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast *15*, 963–972.

Kramer, E.R., Scheuringer, N., Podtelejnikov, A.V., Mann, M., and Peters, J.-M. (2000). Mitotic Regulation of the APC Activator Proteins CDC20 and CDH1. Mol. Biol. Cell *11*, 1555–1569.

Krantz, A. (1994). Peptidyl (Acyloxy)methanes as Quiescent Affinity Labels for Cysteine Proteinases. Methods Enzymol. *244*, 656–671.

Kraulis, P.J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Crystallog. *24*, 946–958.

Kumada, K., Nakamura, T., Nagao, K., Funabiki, H., Nakagawa, T., and Yanagida, M. (1998). Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. Curr. Biol. *8*, 633–641.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1997). Genetic instability in colorectal cancers. Nature *386*, 623–627.

Liang, C., and Stillman, B. (1997). Persistent initiation of replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev. *11*, 3375–3386.

Losada, A., Hirano, M., and Hirano, T. (1998). Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. Genes Dev. *12*, 1986–1997.

Mazia, D. (1961). Mitosis and the Physiology of Cell Division, Vol III (New York, Academic Press).

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: Chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35–45.

Mole-Bajer, J. (1958). Cine-micrographic analysis of C-mitosis in endosperm. Chromosoma *9*, 332–358.

Nasmyth, K., Peters, J.-M., and Uhlmann, F. (2000). Splitting the chromosome: cutting the ties that bind sister chromatids. Science *288*, 1379–1384.

Neuwald, A.F., Liu, J.S., and Lawrance, C.E. (1995). Gibbs motif sampling: detection of bacterial outer membrane protein repeats. Protein Sci. *4*, 1618–1632.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature *376*, 37–43.

Ostergren, G., and Anderson, L. (1973). Staining the Kinetochore

in Plant Chromosomes, Vol *23*, T. Caspersson and L. Zech, eds. (Stockholm, Nobel Foundation).

Parks, T.D., Howard, E.D., Wolpert, T.J., Arp, D.J., and Dougherty, W.G. (1995). Expression and purification of a recombinant tobacco etch virus NIa proteinase: biochemical analyses of the full-length and a naturally occuring truncated proteinase form. Virology *210*, 194–201.

Rose, M.D., Winston, F., and Hieter, P. (1990). Laboratory Course Manual for Methods in Yeast Genetics, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Rost, B., and Sander, C. (1994). Combining evolutionary information and neural networks to predict protein secondary structure. Proteins *19*, 55–72.

Schuler, G.D., Altschul, S.F., and Lipman, D.J. (1991). A workbench for multiple alignment construction and analysis. Proteins *9*, 180–190.

Shirayama, M., Toth, A., Galova, M., and Nasmyth, K. (1999). APC^{Cdc20} promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. Nature *402*, 203–207.

Tinker-Kulberg, R.L., and Morgan, D.O. (1999). Pds1 and Esp1 control both anaphase and miotic exit in normal cells and following DNA damage. Genes Dev. *13*, 1936–1949.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleifer, A., and Nasmyth, K. (1999). Yeast Cohesin complex requires a conserved protein, Eco1p (Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev. *13*, 320–333.

Uhlmann, F., and Nasmyth, K. (1998). Cohesion between sister chromatids must be established during DNA replication. Curr. Biol. *8*, 1095–1101.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister chromatid separation at anaphase onset is triggered by cleavage of the cohesin subunit Scc1p. Nature *400*, 37–42.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast *10*, 1793–1808.

Waizenegger, I.C., Hauf, S., Meinke, A., and Peters, J.-M. (2000). Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. Cell *103*, this issue, 399–410.

Yaima, H., and Fujii, N. (1981). Studies on peptides. 103. Chemical synthesis of a crystalline protein with the full enzymatic activity of Ribonuclease A. J. Am. Chem. Soc. *103*, 5867–5871.

Yamamoto, A., Guacci, V., and Koshland, D. (1996). Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathways. J. Cell Biol. *133*, 99–110.

Yanagida, M. (2000). Cell cycle mechansims of sister chromatid separation; roles of Cut1/separin and Cut2/securin. Genes Cells *5*, 1–8.

Yeong, F.M., Lim, H.H., Padmashree, C.G., and Surana, U. (2000). Exit from mitosis in budding yeast: biphasic inactivation of the Cdc28-Clb2 mitotic kinase and the role of Cdc20. Mol. Cell *5*, 501–511.

Zhang, Z., Schaffer, A.A., Miller, W., Madden, T.L., Lipman, D.J., Koonin, E.V., and Altschul, S.F. (1998). Protein sequence similarity searches using patterns as seeds. Nucleic Acids Res. *26*, 3986–3990.