The EMBO Journal Vol.17 No.2 pp.498–506, 1998

Budding yeast *RSI1/APC2*, a novel gene necessary for initiation of anaphase, encodes an APC subunit

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***SIC1* is a non-essential gene encoding a CDK inhibitor of Cdc28-Clb kinase activity. Sic1p is involved in both mitotic exit and the timing of DNA synthesis. To identify other genes involved in controlling Clb-kinase activity, we have undertaken a genetic screen for mutations which render *SIC1* essential. Here we describe a gene we have identified by this means, *RSI1/APC2*. Temperature-sensitive *rsi1/apc2* mutants arrest in metaphase and are unable to degrade Clb2p, suggesting that Rsi1p/Apc2p is associated with the anaphase promoting complex (APC). This is an E3 ubiquitin-ligase that controls anaphase initiation through degradation of Pds1p and mitotic exit via degradation of Clb cyclins. Indeed, the anaphase block in *rsi1/apc2* temperature-sensitive mutants is overcome by removal of *PDS1*, consistent with Rsi1p/Apc2p being part of the APC. In addition, like our *rsi1/apc2* mutations, *cdc23-1*, encoding a known APC subunit, is also lethal with *sic1*Δ. Thus *SIC1* clearly becomes essential when APC function is compromised. Finally, we find that Rsi1p/Apc2p co-immunoprecipitates with Cdc23p. Taken together, our results suggest that *RSI1/APC2* is a subunit of APC.**

Keywords: APC/cell cycle/SIC1/yeast

Introduction

Orderly progression through the eukaryotic cell cycle is controlled by the regulated association of specific cyclins with a CDK (cyclin-dependent kinase). In the budding yeast, *Saccharomyces cerevisiae*, Cdc28 is the major CDK and is largely responsible for controlling cell cycle progression (reviewed in Nasmyth, 1996). G_1 cyclins Clns 1, 2 and 3 are active during G_1 up until S phase while B-type cyclins Clbs 1–6 control DNA synthesis (Schwob *et al.*, 1994) and mitosis (Surana *et al.*, 1991). The specific association of the appropriate cyclin with Cdc28 is achieved by cell cycle-controlled synthesis as well as controlled degradation of the cyclin at key stages of the cycle (reviewed in King *et al.*, 1996; Deshaies, 1997). The mitotic B-type cyclin Clb2p is active from late S phase until the end of mitosis when it is rapidly degraded by ubiquitin-mediated proteolysis (Surana *et al.*, 1991; Irniger *et al.*, 1995; Amon, 1997; Irniger and Nasmyth, 1997). Exit from mitosis and entry into the G_1 phase of the next cell cycle requires the inactivation of the Clb2 protein; over-production of Clb2p which has been stabilized by removal of its destruction box causes cells to arrest in telophase with divided chromatin and an elongated spindle (Surana *et al.*, 1993).

E3 ubiquitin-protein ligases play an important role in specifying the selection of proteins for ubiquitin-mediated degradation (Ciechanover, 1994). Recently, studies in human, *Xenopus* and yeast systems have converged to reveal the E3 ubiquitin-protein ligase activity which confers specificity for cell cycle-regulated degradation of mitotic cyclin by the ubiquitin pathway (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995). This E3 is a large complex of at least seven proteins, and has been called the cyclosome or anaphase promoting complex (APC). The APC is part of the essential cell cycle machinery whose components are evolutionarily conserved (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995; Peters *et al.*, 1996; Zachariae *et al.*, 1996). In yeast *CDC16*, *CDC23*, *CDC26*, *CDC27* and *APC1* have been identified as genes coding for some of these components (Lamb *et al.*, 1994; Irniger *et al.*, 1995; Zachariae *et al.*, 1996). As its name suggests, an active APC is required for cells to initiate anaphase; APC-defective yeast strains arrest in metaphase with replicated DNA but with unseparated sister chromatids (Lamb *et al.*, 1994; Zachariae *et al.*, 1996).

While Clb2p degradation is normally initiated at anaphase onset (Surana *et al.*, 1991; Irniger *et al.*, 1995), its degradation is not required for the metaphase to anaphase transition (Surana *et al.*, 1993) and some Clb2 protein persists until telophase (Irniger *et al.*, 1995). Thus, the APC clearly has other critical targets. One of the early events required for the metaphase to anaphase transition is sister chromatid separation. Several proteins whose proteolysis is required for sister chromatid separation have recently been identified; the *pimples* product in *Drosophila* (Stratmann and Lehner, 1996), Cut2 in fission yeast (Funabiki *et al.*, 1996) and Pds1p in budding yeast (Cohen Fix *et al.*, 1996; Yamamoto *et al.*, 1996a,b). Interestingly, yeast strains lacking the *PDS1* gene are viable but in APC-deficient strains show a mixed terminal arrest phenotype with a large proportion of the cells in telophase, that is, with divided chromatin (Yamamoto *et al.*, 1996a). This suggests that the critical requirement for the APC at the metaphase to anaphase transition is to ensure the proper separation of sister chromatids. Other known APC targets include Ase1p, a budding yeast protein involved in spindle elongation (Juang *et al.*, 1997) whose destruction is required for proper spindle disassembly after the metaphase to anaphase transition. The final essential role of the APC in mitotic exit is the degradation of Clb2p (Surana *et al.*, 1993; Irniger *et al.*, 1995). However, there

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Table I. Yeast strains[a]

Strain name	Genotype	Source
CG378	*MATa ura3 leu2 trp1 ade5*	C.Giroux, (Wayne State University, Detroit, Michigan)
CG379	*MATα ura3 leu2 trp1 his7*	C.Giroux
JD100	*MATα sic1Δ::TRP1 leu2 ura3 his7*	Donovan *et al.* (1994)
KTM100U	*MATα rsi1::LEU2*[b] *sic1Δ::TRP1 his7* p*SIC1U*[c]	this study
KTM100H	*MATα rsi1::LEU2 sic1Δ::TRP1* p*SIC1H*[d]	this study
KTM100sΔH	*MATα rsi1::LEU2 sic1Δ::TRP1 ura3* p*sic1ΔH*[e]	this study
KTM110	*MATa rsi1::LEU2 trp1 ura3 ade5*	this study
KTM208	*MATa rsi1Δ::HIS7 ura3 leu2* p*rsi1-8*[f]	this study
CG378CH	*MATa CLB2HA3 ura3 leu2 trp1 ade5*	this study
KTM208CH	*MATa CLB2HA3 rsi1Δ::HIS7 ura3 leu2* p*rsi1-8*[f]	this study
CG378GCH	*CG378* p*GAL-CLB2HA3*[g]	this study
KTM208GCH	*MATa rsi1Δ::HIS7 ura3 leu2* p*rsi1-8*[f] *pGAL-CLB2HA3*	this study
2351	*MATa cdc23-1 ura3 leu2 ade2 his3*	K.Nasmyth, IMP, Vienna
2351W	*MATa cdc23-1 ura3 leu2 trp1 ade2 his3*	this study
KTM308	*MATa rsi1Δ::HIS7 pds1Δ::URA3 leu2* p*rsi1-8*	this study
CG378 RSI1Myc3	*CG378* p*RS306-RSI1myc3*[h]	this study
CG378 RSI1Myc3, CDC23HA2	*CG378* p*RS306-RSI1myc3 pRS329-CDC23HA2*[i]	this study
CG378 CDC23HA2	*CG378* p*RS329-CDC23HA2*	this study

[a]All strains except *cdc23-1* are in a CG378/CG379 background. *cdc23-1* is in a W303 background.
[b]*rsi1::LEU2* is referred to as *rsi1-1* in the text.
[c]p*SIC1U* is a CEN-based plasmid containing *SIC1* and *URA3*.
[d]p*SIC1H* is an ARS-based plasmid containing *SIC1* and *HIS7*.
[e]p*sic1ÆH* is as p*SIC1H* except *SIC1* is deleted.
[f]p*rsi1-8* is a CEN-based plasmid containing *TRP1* and a temperature-sensitive mutation in *RSI1*.
[g]p*GAL-CLB2HA3* is a CEN-based plasmid containing *CLB2HA3* under control of the the *GAL10* promoter and *URA3*.
[h]pRS306-*RSI1Myc3* is an integrative plasmid containing *RSI1Myc3* and *URA3*.
[i]pRS329-*CDC23HA2* is a CEN-based plasmid containing *TRP1* and expressing *CDC23HA2* under its own promoter (a gift from the Phil Hieter laboratory).

are clearly other factors which contribute to the control of Clb2p inactivation since the APC becomes active at metaphase but Clb2p levels persist until telophase.

Another protein controlling Clb2p activity is Sic1p, a CDK inhibitor of both S phase and mitotic Clb–Cdc28 kinases (Donovan *et al.*, 1994; Schwob *et al.*, 1994; Amon, 1997). Sic1 protein regulates the timing of DNA synthesis by binding to and inhibiting S phase kinase Cdc28–Clb5 early in the cell cycle (Schwob *et al.*, 1994). Low Clb-kinase activity at this stage of the cell cycle is critical for enabling replication proteins such as Cdc6 and Mcm2-7 to be loaded onto replication origins making the DNA competent for replication (Donovan *et al.*, 1997), a process that is inhibited by Clb kinase activity (Dahmann *et al.*, 1995; Tanaka *et al.*, 1997). Shortly before S phase, Sic1p is degraded by the Cdc4/Cdc34/Cdc53-dependent ubiquitin-mediated proteolytic pathway, thereby activating Cdc28–Clb5 kinase. Sic1p is then resynthesized in late mitosis (Donovan *et al.*, 1994; Toyn *et al.*, 1997).

Three separate lines of evidence suggest a role for Sic1p in late mitosis. The first is the synthetic lethality between *sic1Δ* and *hct1Δ*. *HCT1* is a recently described non-essential gene that directly controls Clb2p proteolysis (Schwab *et al.*, 1997); *hct1Δ* mutants have high levels of Clb2p throughout the cell cycle and overexpression of *HCT1* induces ectopic activation of Clb2 proteolysis. Second, *SIC1* interacts genetically with a variety of late mitotic arrest mutants (for review see Deshaies, 1997) and multiple copies of *SIC1* rescue temperature-sensitive mutants of the *DBF2* group of kinases which all arrest in telophase with large budded cells, divided chromatin, an elongated spindle and high mitotic kinase activity (Toyn and Johnston, 1994; Deshaies, 1997). Third, *sic1Δ dbf2Δ* is a lethal combination and the double mutant has a late mitotic arrest terminal phenotype (Toyn *et al.*, 1997). Taken together, these observations argue strongly that *SIC1* and various other genes such as *DBF2* and *HCT1* are involved in functionally overlapping pathways controlling exit from mitosis.

Because control of mitotic exit clearly involves redundant pathways, isolating mutations that are synthetically lethal with *sic1Δ* should identify other genes involved in mitotic exit. Accordingly, we carried out a screen for such mutants and describe here the isolation of a gene we call *RSI1* (Requires Sic1 CDK Inhibitor). Although *RSI1* is essential, we have characterized a particular allele which is viable but becomes lethal in a *sic1Δ* background. We show that Rsi1p is required for the metaphase to anaphase transition and also for Clb2p degradation at key cell cycle stages. Finally we show that Rsi1p interacts *in vivo* with the APC component Cdc23p. We conclude that *RSI1* codes for an APC subunit and propose alternative models accounting for the *rsi1 sic1Δ* synthetic lethality.

Results

A genetic screen for mutants requiring SIC1

Mutagenesis was carried out in strain JD100 (Table I), a *sic1Δ::TRP1* haploid strain containing *SIC1* on a low copy number *URA3* plasmid (p*SIC1U*). We exploited the toxicity of 5-fluoroorotic acid (FOA) in *URA3* strains (see Materials and methods; Ota and Varshavsky, 1992) to isolate mutants requiring *SIC1* for growth; such mutants will not be able to lose p*SIC1U* and therefore will be unable to grow on FOA. For the mutagenesis, we used a yeast genomic library, generously provided by Dr M.Snyder, Yale Univer-

sity (Burns *et al.*, 1994), in which wild-type sequences are randomly disrupted by *LEU2* insertions. Disrupted yeast sequences are cut out of the library vector and integrated into the target genome by transplacement. This effectively replaces wild-type genomic DNA with *LEU2* disrupted sequences. We felt disruption to be an appropriate mutagenesis since late mitotic pathways are clearly redundant (see Introduction), hence, at least some genes are expected to be dispensable in a wild-type background. We screened 80 000 LEU^+ transformants and only a single mutation lethal with *sic1*Δ was identified. This lay in a previously uncharacterized gene we are calling *RSI1* (see above). We refer to this mutant allele as *rsi1-1*.

*sic1*Δ *rsi1-1* synthetic lethality

The *rsi1-1* mutant was isolated by its inability to grow on FOA plates in a *sic1*Δ *pSIC1U* genetic background (KTM100U, Table I and Figure 1A). Providing wild-type *SIC1* on a plasmid vector with *HIS7* as a selectable marker (KTM100H, Table I) allowed growth on FOA whereas *sic1*Δ on the same plasmid vector (KTM100sΔH, Table I) did not. To confirm the synthetic lethality of *rsi1-1* and *sic1*Δ, we crossed KTM100U with wild-type CG378 (Table I). Out of eight tetrads dissected, all LEU^+ (*rsi1-1*) TRP^+ (*sic1*Δ) spore clones were also URA^+ (p*SIC1U*) and unable to grow on FOA. Furthermore, a subsequent cross between an *rsi1-1 SIC1* haploid (KTM110, Table I) and JD100, the original *RSI1 sic1*Δ strain used for the mutagenesis, yielded no LEU^+ (*rsi1-1*) TRP^+ (*sic1*Δ) viable spore clones from 18 tetrads dissected, confirming that *rsi1-1* requires a wild-type copy of *SIC1* for viability. Non-viable spores had germinated but arrested as large budded cells after one or several divisions.

The sequences around the *LEU2* insertion site in *rsi1-1* were cloned by integrating a single copy of a bacterial/yeast shuttle vector at that site in KTM100H (see Materials and methods). Sequence analysis revealed that the *LEU2* insertion in *rsi1-1* is in the promoter of a gene on chromosome XII (Figure 1B) we have named *RSI1* (see above). *RSI1* encodes a 853 amino acid protein of 99 kDa (DDBJ/EMBL/GenBank Z73299; YPD YLR127C) containing several potential Cdc28 phosphorylation sites. Database searches reveal a domain of weak homology (23–28% identity, 49–55% similarity) to *Drosophila* and and human genes belonging to the cullin family (Jackson, 1996; Kipreos *et al.*, 1996) as well as to a *Schizosaccharomyces pombe* hypothetical protein (Figure 1C). Cullin family members appear to have a role in cell cycle regulation and include *S.cerevisiae CDC53*, which controls G_1 cyclin degradation (Jackson, 1996; Willems *et al.*, 1996).

A null allele of *RSI1* was created by replacing one copy of the *RSI1* coding sequences with the *HIS7* gene (Figure 1B) in diploid strain CG378×CG379 (Table I). Sporulation followed by dissection of 18 tetrads gave a segregation pattern of 2 viable:2 dead in all cases and the viable spores were all his^- (*RSI1*). *RSI1* is therefore an essential gene. The non-viable spores had germinated, entered the

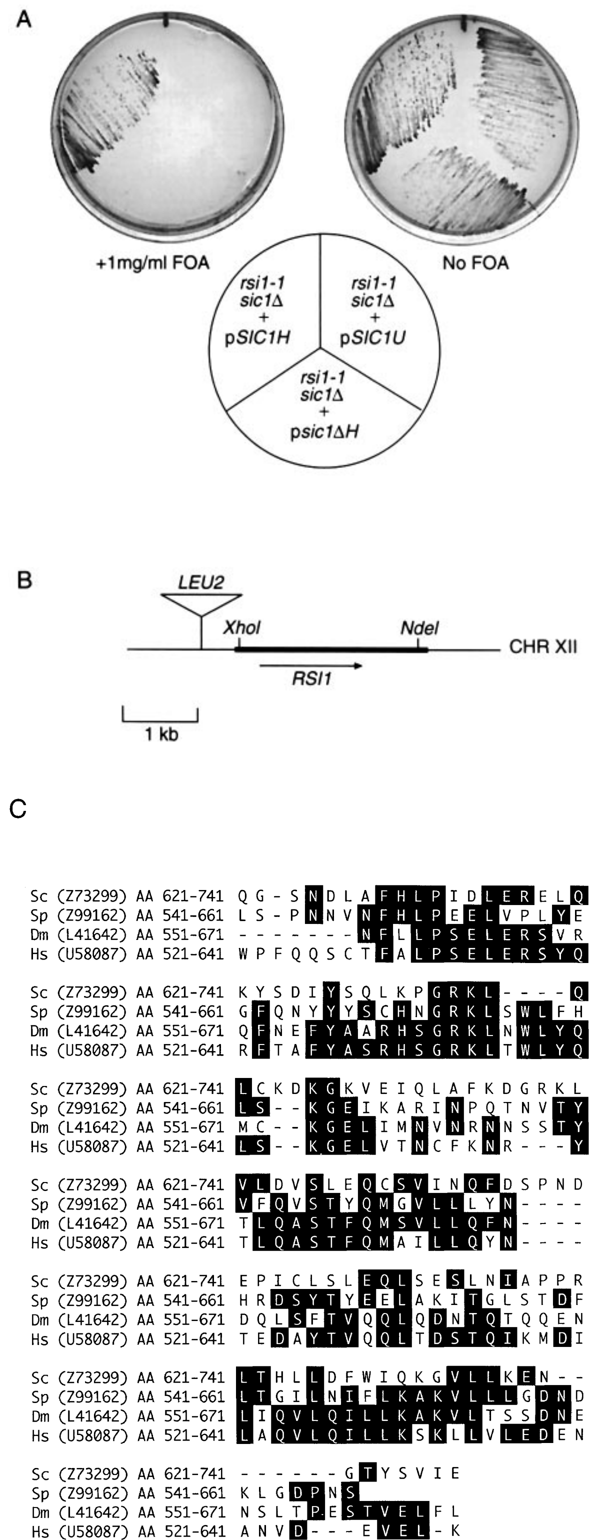


Fig. 1. (**A**) *sic1*Δ *rsi1-1* synthetic lethality. Cells were streaked onto plates containing either no FOA or 1mg/ml FOA and incubated for 3 days at 30°C. The strain *sic1*Δ *rsi1-1* p*SIC1U* carries *SIC1* on a *URA3* marked plasmid (Table I). Strains *sic1*Δ *rsi1-1* p*SIC1H* and *sic1*Δ *rsi1-1* p*sic1*Δ*H* are identical to *sic1*Δ *rsi1-1* p*SIC1U* but carry either *SIC1* or *sic1*Δ on a *HIS7* marked plasmid. (**B**) The heavy line indicates the *RSI1* 2.5 kb open reading frame located on the left arm of CHR XII. The *rsi1-1* mutation is a *LEU2* -transposon insertion 425 bp upstream of the initiating ATG. The *Xho*I–*Nde*I fragment was replaced by the *HIS7* gene in *rsi1*Δ strains. (**C**) Sequence alignment showing the homologous region between Rsi1 and cullin family members. DDBJ/EMBL/GenBank accession numbers are indicated in brackets. Sc, *S.cerevisiae* Rsi1; Sp, *S.pombe* hypothetical protein; Dm, *D.melanogaster* Lin-19; Hs, *Homo sapiens* Cul-1.

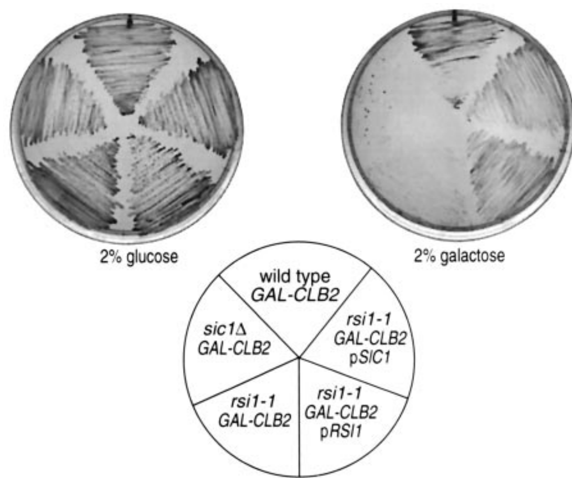


Fig. 2. The *rsi1-1* mutant is hypersensitive to *CLB2* levels. A single copy of *GAL-CLB2* was integrated at the *URA3* locus in the *rsi1-1* mutant as well as in congenic *sic1*Δ and wild-type strains. Cells were streaked onto plates containing either 2% glucose or 2% galactose and incubated for 3 days at 30°C.

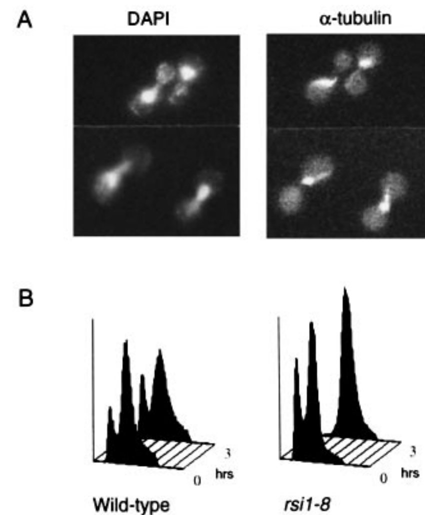


Fig. 3. Terminal arrest phenotype of *rsi1-8* temperature-sensitive mutants. *rsi1-8* cells were grown to log phase at 25°C then shifted to 37°C for 3 h. (**A**) Chromatin was visualized by DAPI staining and spindles were visualized by anti-tubulin staining. (**B**) FACS analysis. Cells were stained with propidium iodide and 20 000 were analyzed for each sample.

cell cycle and arrested as large budded cells after only a few divisions.

The rsi1-1 strain is hypersensitive to Clb2p levels

Strains defective in mitotic exit, including *sic1*Δ strains, are hypersensitive to increased levels of Clb2p (Shirayama *et al.*, 1994; Toyn *et al.*, 1997); a single copy of *GAL-CLB2* prohibits growth on galactose. We reasoned that if the *sic1*Δ *rsi1-1* synthetic lethality is due to a defect in Clb2p kinase inactivation, then the *rsi1-1* mutation on its own might be sensitive to increased levels of Clb2p. To examine this, a single copy of *GAL-CLB2* was integrated at the *URA3* locus in the *rsi1-1* mutant and also in congenic wild-type cells. The copy number of the integrated *GAL-CLB2::URA3* was confirmed by Southern analysis (data not shown). Figure 2 shows that while all strains can grow on 2% glucose, the *rsi1-1* mutation, like *sic1*Δ, prohibits growth on 2% galactose. Furthermore, sensitivity to *GAL-CLB2* expression in the *rsi1-1* strain is overcome by providing either *RSI1* or *SIC1* on a low copy plasmid. These results taken together with the *sic1*Δ *rsi1-1* synthetic lethality strongly suggest that Rsi1p and Sic1p function in parallel pathways controlling the inactivation/degradation of Clb2p.

Cell cycle phenotype of rsi1 temperature-sensitive mutants

To characterize the function of *RSI1* in more detail, we generated a bank of temperature-sensitive alleles using mutagenic PCR and *in vivo* 'gap repair' (Connelly and Hieter, 1996). All of the mutant alleles showed a similar terminal arrest phenotype as exemplified by the *rsi1-8* allele in strain KTM208 (Table I) described below. When a log phase culture of *rsi1-8* cells was shifted from growth at 25°C to 37°C for 3 h, the cells displayed a cell cycle phenotype, arresting with large buds, an undivided nucleus positioned at the neck of the bud and a short mitotic spindle (Figure 3A). FACS analysis showed them to contain fully replicated DNA (Figure 3B). This phenotype is identical to that reported for *cdc23-1*, a temperature-sensitive allele of *CDC23* which encodes an APC subunit (Sikorski *et al.*, 1993).

The rsi1-8 strain is defective in Clb2p degradation

cdc23-1 mutants are defective for Clb2p degradation, therefore we assessed the Clb2-Cdc28-specific H1 kinase activity as well as Clb2p stability in the *rsi1-8* mutant. The H1 kinase activity was measured using anti-HA immunoprecipitated Clb2HA3. First, we examined the kinase activity from a log phase culture of *rsi1-8* cells containing an integrated copy of *CLB2HA3*, that had been shifted from growth at 25°C to 37°C for 3 h. In this experiment the tagged *CLB2* replaced the endogenous gene and was fully functional. This was compared with the same activity from congenic wild-type cells also containing *CLB2HA3* and treated for 3 h either in nocodazole (a microtubule-depolymerizing drug), to induce an M phase arrest where Clb2–Cdc28-specific H_1 kinase activity is high (Amon *et al.*, 1994), or with α-factor to induce an early G_1 arrest where the kinase activity is undetectable. FACS analysis confirmed that the predicted arrests had occurred (Figure 4A). As expected, the α-factor arrested wild-type cells had no detectable Clb2–Cdc28-specific H1 kinase activity (Figure 4A, lane 1). In marked contrast, the extracts from *rsi1-8* mutant cells had a high Clb2–Cdc28-specific H_1 kinase activity at 37°C (Figure 4A, lane 3), comparable with the nocodazole arrested wild-type cells (Figure 4A, lane 2). Moreover, Clb2p was readily detectable in the *rsi1-8* cells at 37°C (Figure 4A, lane 3) suggesting that it was stabilized.

To confirm this we examined Clb2p stability in *rsi1-8* cells that had been arrested in G_1 with α-factor. Clb2p normally has a half-life of <1 min in G_1 (Amon *et al.*, 1994) but is stabilized in G_1-arrested *cdc23-1* cells (Irniger *et al.*, 1995). We used a *GAL-CLB2* fusion construct with a triple HA epitope tag, p*GAL-CLB2HA3*, to examine Clb2p stability in G_1-arrested *rsi1-8* and isogenic wild-type cells. Log phase cultures were exposed to the mating

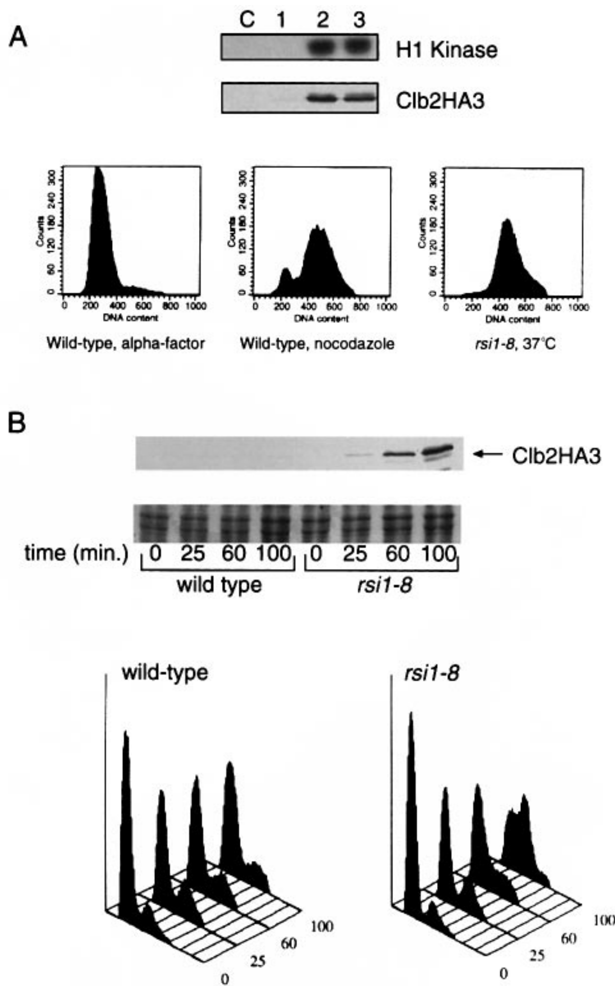


Fig. 4. *rsi1-8* temperature-sensitive mutants arrest with high Clb2p kinase activity and have a defect in Clb2p degradation at the restrictive temperature. (**A**) Strain KTM 208CH, *rsi1-8*, congenic wild-type CG378 and strain CG378CH containing integrated *CLB2HA3* were grown to log phase at 25°C. The wild-type cells were treated for 3 h with α-factor or with nocodazole. The *rsi1-8* cells were shifted to 37°C for 3 h. Extracts were prepared and Clb2p levels and associated H1 kinase activity was measured. Lanes 1 and 2, wild-type *CLB2HA3* cells arrested with α-factor and nocodazole, respectively; lane 3, 37°C arrested *rsi1-8 CLB2HA3* cells; lane C, nocodazole-arrested wild-type cells that do not contain *CLB2HA3*. (**B**) Strain KTM208GCH, *rsi1-8*, and a congenic wild-type strain, CG378GCH, both containing *GAL-CLB2HA3* on a low copy number plasmid, were grown to log phase at 25°C in 2% raffinose medium. α-factor was added and after 4 h galactose was added to 2% and the culture was simultaneously shifted to 37°C. Extracts were prepared from cells after 0, 25, 60 and 100 min growth at 37°C. Additional α-factor was added at intervals to ensure continued G_1 arrest. Equal amounts of protein were analyzed by SDS–PAGE and Western blotting using a monoclonal anti-HA antibody (12CA5, upper panel). The nitrocellulose membrane was stained with Ponceau S and is shown as a loading control (lower panel). At the same time points, cells were also removed for FACS analysis.

pheromone α-factor during growth in raffinose at 25°C. Once the cells were arrested in G_1, as determined by the absence of buds and the formation of mating projections, galactose was added to 2% to induce high levels of ectopic Clb2p expression and simultaneously the cultures were shifted to 37°C. In the strain carrying the *rsi1-8* mutation, Clb2 protein was detectable by Western blotting after 25 min with Clb2p levels increasing throughout the 100 min induction (Figure 4B). In contrast, in the wild-type strain, we observed no such Clb2 protein accumulation. Thus, in α-factor-arrested cells, Rsi1p is required for Clb2p degradation.

It is significant that in the *rsi1-8* mutant after 100 min of galactose-induced *CLB2* expression at 37°C, FACS analysis showed that the cells had entered S phase (Figure 4). In contrast, the wild-type cells maintained a G_1 DNA content throughout the experiment. It is important to note that the *rsi1-8* cells had not initiated DNA synthesis due to escape from the α-factor holding; they retained the mating projection induced by α-factor and remained unbudded. Clb kinase is required for entry into S phase (Schwob *et al.*, 1994) but is kept low during G_1 by the presence of the Sic1p CDK inhibitor and persistent APC function (Irniger and Nasmyth, 1997). In the mutant, however, sufficient levels of Clb kinase had accumulated to initiate S phase despite the presence of α-factor. This phenomenon has been previously observed in mutants of APC components (Zachariae *et al.*, 1996; Irniger and Nasmyth, 1997) and supports a direct association of Rsi1p with the APC.

cdc23-1 is also synthetically lethal with sic1Δ

The phenotypic similarities between *rsi1-8* and *cdc23-1* led us to look for a possible genetic interaction between *CDC23* and *SIC1*. We crossed a *cdc23-1* mutant with a *sic1Δ::TRP1* strain and examined segregation of temperature sensitivity (*cdc23-1*) with the *TRP1* (*sic1Δ*) marker. Out of 27 tetrads dissected, no spore clones were temperature-sensitive (*cdc23-1*) and TRP^+ (*sic1Δ*), indicating that the *sic1Δ cdc23-1* combination is lethal in haploids. We also examined spores where the *sic1Δ cdc23-1* combination could be predicted based on the genotype of the viable spore clones. Of 16 such spores, all but one had germinated and arrested as large budded cells after one or a few divisions. Because this is identical to the phenotype observed in the *sic1Δ rsi1-1* spores described above we considered the possibility that Rsi1p is an APC subunit and thus shares a common role in cell cycle progression with Cdc23p.

Rsi1p interacts with the APC component Cdc23p in vivo

As a conclusive indication that *RSI1* encodes a subunit of APC we looked for a direct association of Rsi1p with Cdc23p *in vivo*. A triple Myc epitope-tagged version of *RSI1*, *RSI1Myc3,* was integrated in wild-type haploid CG378 at the *RSI1* locus. In this strain the only copy of *RSI1* with a promoter is the *RSI1Myc3* version of the gene; hence this is the only source of Rsi1p. RsiMyc3p was detected as a 100 kDa protein by Western blotting of crude extracts from log phase cells (Figure 5B). The *RSI1Myc3* strain and the corresponding isogenic parental strain were transformed with a low copy plasmid containing a double HA epitope-tagged *CDC23, CDC23HA2*. Extracts prepared from cells expressing various combinations of these tagged proteins were subjected to immunoprecipitation with anti-Myc antibodies and probed with an anti-HA antibody to look for co-precipitation of Cdc23p with Rsi1p (Figure 5A). As expected, Cdc23HA2p was specifically co-precipitated with Rsi1Myc3p. This *in vivo* interaction between Cdc23p and Rsi1p strongly supports our evidence that Rsi1p is indeed a newly identified

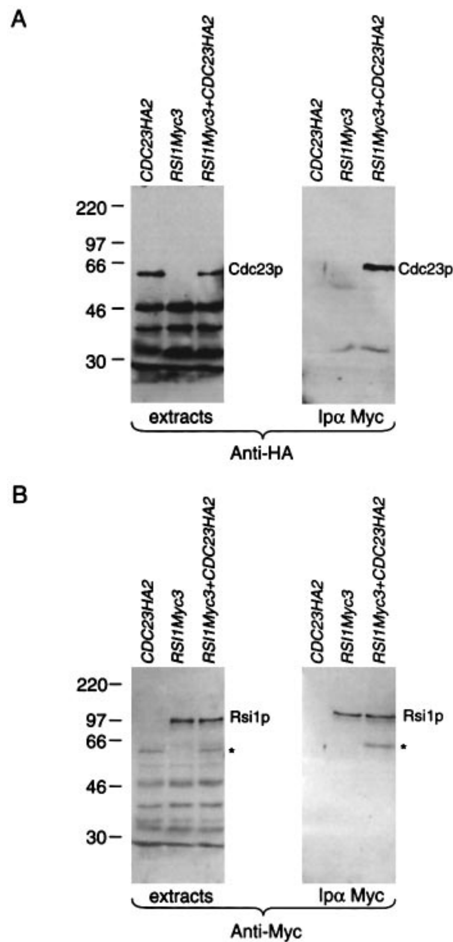
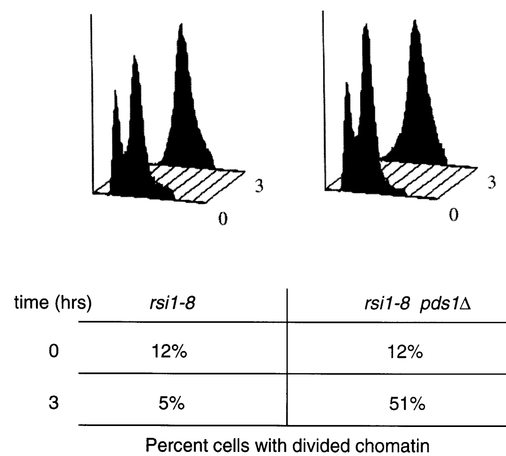


Fig. 5. The *in vivo* interaction between Rsi1p and Cdc23p. Isogenic strains containing either *RSI1Myc3* or *CDC23HA2* alone or with both constructs together were grown to log phase and cell extracts were prepared. (**A**) Protein from crude extracts (left panel) and from extracts immunoprecipitated using a monoclonal anti-Myc antibody (9E10, right panel) were electrophoresed and probed with a monoclonal anti-HA antibody (12CA5) to detect Cdc23HA2p. (**B**) The anti-HA antibody was stripped from the blot shown in (A) and it was re-probed with monoclonal anti-Myc antibody to detect Rsi1Myc3p. *indicates remaining traces of the previous HA probing.

component of APC. Rsi1p is likely to be the previously detected 100 kDa component of APC (Zachariae *et al.*, 1996).

The pre-anaphase block in rsi1-8 mutants can be overcome in the absence of Pds1p

A unique and characteristic phenotype of mutations in APC components is manifested in strains lacking Pds1p. Because APC-mediated proteolysis of Pds1p is essential for sister chromatid separation (Cohen Fix *et al.*, 1996; Yamamoto *et al.*, 1996a,b), a high proportion of APC-deficient *pds1Δ* cells enter anaphase and arrest in telophase with divided chromatin (Yamamoto *et al.*, 1996a). Accordingly, we examined the terminal arrest phenotype in a *rsi1-8 pds1Δ* double mutant compared with a *rsi1-8 PDS1* control strain. Log phase cultures of both strains growing at 25°C were shifted to growth at 37°C for 3 h. FACS analysis showed that after 3 h both strains had stopped growing and had arrested with fully replicated DNA (Figure 6). However, examination of chromosome segre-



time (hrs)	*rsi1-8*	*rsi1-8 pds1Δ*
0	12%	12%
3	5%	51%

Percent cells with divided chomatin

Fig. 6. Deletion of *PDS1* relieves the anaphase block in *rsi1-8* arrested cells. *rsi1-8* and *rsi1-8 pds1Δ* isogenic strains were grown to log phase at 25°C and then shifted to 37°C for 3 h. Cells harvested at 0 and 3 h were prepared for FACS analysis and stained with DAPI to visualize the chromatin. The DAPI-stained cells were examined for the presence of divided chromatin and the figures are presented in Table I.

gation in DAPI stained cells showed a dramatic difference in the arrest phenotype. The number of cells with divided chromatin in the *rsi1-8 pds1Δ* double mutant had increased from 12% in the log phase culture (0 h, Figure 6) to 51% after 3 h at 37°C. In contrast, the number of cells with divided chromatin in the *rsi1-8 PDS1* control strain showed a significant decrease from 12% in log phase to 5% after 3 h at 37°C, these cells having accumulated in metaphase. The percentage of cells with divided chromatin in the *rsi1-8 pds1Δ* double mutant after 3 h at 37°C is in good agreement with the previously observed percentage in terminally arrested *cdc16 pds1* and *cdc23 pds1* double mutants (Yamamoto *et al.*, 1996a). Thus, rsi1 behaves in the same way as *cdc16* and *cdc23* in a *pds1Δ* genetic background. The high proportion of cells with divided chromatin in the *rsi1-8 pds1Δ* double mutant shows that those cells which were able to initiate anaphase had subsequently arrested in telophase demonstrating, in common with APC, a critical requirement for Rsi1p at two cell-cycle transition points, the metaphase to anaphase transition and mitotic exit.

Discussion

Rsi1p is an APC component

We have used a wide range of criteria including genetic interactions, cytology, cell physiology and biochemistry to demonstrate that *RSI1* codes for a previously uncharacterized component of the APC. Our first indication that Rsi1p might be an APC component was that the *rsi1-8* temperature-sensitive mutant showed the same metaphase arrest phenotype as a temperature-sensitive allele of *CDC23* (Sikorski *et al.*, 1993). All of our subsequent results support this; *RSI1* is an essential gene and the *rsi1-8* mutant shows a metaphase arrest; Rsi1p controls proteolysis of two known APC targets, namely Clb2p and Pds1p; Rsi1p interacts with Cdc23p *in vivo*. While it could be argued that Rsi1p is merely involved in controlling APC function and is not itself an APC subunit, there are several lines of evidence against this argument. First, we have shown that Rsi1p interacts physically with Cdc23p,

a known APC component. Second, Rsi1p controls both Clb2p and Pds1p degradation. We showed this directly in the case of Clb2p; in the *rsi1-8* mutant Clb2p is stabilized at the cell-cycle arrest point and when expressed in G_1. Regarding Pds1p we find that in the *rsi1-8* mutant, the metaphase arrest is dependent on the presence of the anaphase inhibitor Pds1p. In the absence of Pds1p, a high proportion of *rsi1-8* cells are able to enter anaphase but subsequently arrest in telophase, a phenotypic characteristic that has been reported for other APC mutants in a *pds1*Δ background (Cohen Fix *et al.*, 1996; Yamamoto *et al.*, 1996a). We can contrast this with Hct1p which is a regulator specifically of Clb2p proteolysis (Schwab *et al.*, 1997). Although *hct1* mutants share some phenotypic characteristics with *rsi1* mutants, one of the most important features of Hct1p is that it acts specifically in Clb2p degradation but has no apparent effect on Pds1p.

The APC isolated from yeast contains at least seven proteins; Cdc16p, Cdc23p, Cdc26p, Cdc27p, Apc1p, a 100 kDa protein and an 80 kDa protein (Zachariae *et al.*, 1996). In all probability, Rsi1p corresponds to the 100 kDa protein which was previously detected in an immunoprecipitate from a *CDC16-myc6* strain (Zachariae *et al.*, 1996). Although it is known that the APC functions as an E3 ubiquitin-protein ligase (see Introduction), it is not clear what the function of each component might be or how these functions are co-ordinated. Cdc16p, Cdc23p and Cdc27p all contain TPR repeat protein–protein interaction domains (Lamb *et al.*, 1994; Zachariae *et al.*, 1996). On the other hand, Rsi1p has no recognized protein–protein interaction domains. The significance of the two to four potential Cdc28 phosphorylation sites in Rsi1p is unclear but they may be important for the regulation of APC. Rsi1p function is not controlled by cell cycle-regulated *RSI1* expression according to our Northern analysis (data not shown). On the other hand, it is thought that Clb–Cdc28 kinase controls the APC (Irniger *et al.*, 1995; Amon, 1997).

We have recently learned that, in agreement with our results, Rsi1p has been identified as an APC component under the name Apc2 in other laboratories (K.Nasmyth, personal communication). As the nomenclature *APC2* reflects the cross species conservation of APC members we will henceforth refer to *RSI1* as *APC2*.

Rsi1p/Apc2p may be a cullin family member

The homology between Rsi1p and members of the cullin family of proteins is intriguing. Cullins were first defined as a family of proteins by homology to *Caenorhabditis elegans cul-1* (Kipreos *et al.*, 1996) which was identified as a mutant failing to properly exit the cell cycle during *C.elegans* post-embryonic development. Yeast database searches identify several related cullin proteins, including Cdc53p. It has been proposed that Cdc53p is a component of an E3 ligase which mediates G_1 cyclin as well as Sic1p degradation (Willems *et al.*, 1996). The functional similarity between Cdc53p, Rsi1p/Apc2p and (perhaps) Cul-1 suggests that Rsi1p/Apc2p may be a cullin family member, despite the rather limited homology with other cullin family members. It can hardly be coincidental that structurally related proteins occur in two yeast E3 ubiquitin ligases. The role of individual proteins within these complexes is at present unknown but it is tempting to speculate that Rsi1p/Apc2p and Cdc53p may perform similar functions.

The role of Sic1p in cell-cycle control

The genetic interaction between *RSI1* and *SIC1* is a striking result. Sic1p clearly has no role in anaphase initiation since the protein is only present in cells from the end of mitosis into G_1. Sic1p is known to act late in the cell cycle, presumably facilitating mitotic exit by inhibition of Clb2p kinase activity (Donovan *et al.*, 1994; Toyn *et al.*, 1997) whilst during G_1 Sic1p binds to and inhibits Clb5 allowing the formation of functional replication complexes before S phase initiation (Schwob *et al.*, 1994; Dahmann *et al.*, 1995; Tanaka *et al.*, 1997). Since the only known function of Sic1p is as a CDK inhibitor of Clb kinases and since Clb2p is an APC target, we assume that in the *sic1*Δ *rsi1-1* double mutant there is a lethal excess of Clb2p at some stage of the cell cycle. This is supported by the sensitivity of the *rsi1-1* mutation to elevated levels of Clb2p and by the ability of *SIC1* in low copy number to suppress this sensitivity. As mentioned above (see Introduction) cells overexpressing non-degradable Clb2p are unable to exit mitosis and arrest in telophase. Thus, one possible explanation for the *sic1*Δ *rsi1-1* synthetic lethality is that in an APC-compromised strain lacking Sic1p there is an accumulation of Clb2p which causes a telophase arrest. Supporting this model is the fact that *rsi1-8 pds1*Δ cells are able to initiate anaphase but cannot exit mitosis. This implies an essential function for APC in mitotic exit. We assume that this function is the targeting of Clb2p for proteolysis; however, it is possible that another currently unknown protein also needs to be degraded before cells can exit mitosis. A second possibility is that even with elevated levels of Clb2p, the *sic1*Δ *rsi1-1* double mutant is able to exit mitosis but cannot then form functional replication complexes because of high levels of inhibitory Clb2p and Clb5p kinase activity. This would result in a late G_1 cell cycle block. Clearly, it will be critical to examine the terminal phenotype displayed by the *sic1*Δ *rsi1-1* double mutant. We have attempted this by shutting off *SIC1* expression in the double mutant using a variety of regulatory promoters but have been unable to reproduce the lethal phenotype. Evidently, even the very low basal levels of Sic1p expressed from these promoters is sufficient to drive the cell cycle in the *rsi1-1* mutant.

Our screen for mutations lethal in a *sic1*Δ background was clearly not saturated since we did not recover other genes known to be synthetically lethal with *sic1*Δ such as *DBF2* and *HCT1*. Nevertheless, given that *RSI1* is associated with the APC, the question arises as to why we did not recover mutations in any other genes coding for APC components. The probable reason for this is that all known APC components are essential and therefore most *LEU2* insertions in APC genes would be lethal events. We believe the *RSI1* mutation we recovered, *rsi1-1*, was fortuitous because the insertion of *LEU2* in the promoter (Figure 1B) resulted in a compromised but not an inactive APC. In fact, a deletion of *RSI1* promoter sequences just 125 bp closer to (but still 300 bp away from) the initiating ATG is lethal (data not shown). The *sic1*Δ *cdc23-1* synthetic lethality clearly indicates that the absence of other APC genes recovered from this screen is due to the

nature of the screen itself and is not an indication that the *RSI1 SIC1* genetic interaction is specific to Rsi1p as an APC component. Instead, our results suggest that *SIC1* becomes essential whenever APC function is sufficiently compromised. According to this interpretation, elevated levels of Clb2p at inappropriate stages of the cell cycle augment the requirement for Sic1p. Support for this model comes from the *sic1*Δ *hct1*Δ as well as the *sic1*Δ *cdc23-1* synthetic lethality; both *hct1*Δ and *cdc23-1* are severely defective for Clb2p degradation, the *cdc23-1* defect being manifested even at the permissive temperature (Irniger *et al.*, 1995; Schwab *et al.*, 1997). On the other hand, in *CDC16* temperature-sensitive mutant *cdc16-1*, Clb2p is degraded in α-factor-arrested cells even at the restrictive temperature (Irniger *et al.*, 1995) and a cross between *cdc16-1* and *sic1*Δ haploids showed no synthetic lethality (data not shown). Thus, the synthetic lethality of *sic1*Δ with mutants of APC components clearly depends upon the severity of the mutant allele.

We are currently extending the screen for mutations that are lethal in a *sic1*Δ background using EMS mutagenesis. This will allow us to recover essential as well as non-essential genes. We expect to recover other APC components, genes regulating APC activity and possibly genes involved in the G_1 to S phase transition.

Materials and methods

Strains and media

Relevant yeast strain genotypes are indicated in Table I. All yeast strains were in a CG378 or congenic CG379 background except for *cdc23-1* strain 2531W (Table I). 2531W was generated by crossing *cdc23-1* haploid 2531 (Table I) to W303 to obtain the *cdc23-1* allele in a *trp1* background. All plasmids were propagated in DH5α cells. Yeast transformations were as described (Schiestl and Gietz, 1989). Yeast was grown in rich broth supplemented with 2% glucose, YPD, (Sherman *et al.*, 1986) or with the appropriate carbon source at 2%. Minimal medium was yeast nitrogen base (Difco), with appropriate supplements and a carbon source at 2%. FACS analysis was done using a FACStar Becton Dickinson flow cytometer. For cell cycle arrests, 10 µml α-factor or 10 µg/ml nocodazole were used.

Plasmids and constructs

The *RSI1* null allele was a deletion of the open reading frame made by removing the 2.5 kb internal *Xho*I–*Nde*I fragment and replacing it with the *HIS7* gene. This construct retains only eight 5′ amino acids and eleven 3′ amino acids.

To measure sensitivity of *CLB2* overexpression, *CLB2* was expressed from YIp*G7CLB2* (Shirayama *et al.*, 1994) as previously described (Toyn *et al.*, 1997). For *CLB2* overexpression during α-factor arrest we transformed pWS945 (a gift from B.Futcher, Cold Spring Harbor), a YCplac33 (Gietz and Sugino, 1988) -based plasmid containing *CLB2HA3* under control of the *GAL10* promoter, into KTM208 and CG378.

The *rsi1-8* temperature-sensitive allele was generated using mutagenic PCR and *in vivo* gap repair (Connelly and Hieter, 1996). The mutagenic PCR product was synthesized from *RSI1* containing Ycplac111 (Gietz and Sugino, 1988), a *TRP1* marked vector. The oligonucleotides used, ACCGCCTCTCCCCGCGCG and TCTCTATGGTGAGACAGC were to opposite sides of the YCplac111 multiple cloning site and generated a product with ~200 bp of vector sequences at either end. The PCR products were co-transformed along with YCplac111 vector, that had been linearized by cutting with *Sma*I, into a *rsi1*Δ strain which was kept alive by an ARS-based, *RSI1 URA3* plasmid. The *RSI1 URA3* plasmid was removed by growth on FOA and the remaining TRP^+ transformant were tested for temperature sensitivity.

The *pds1*Δ construct was made by digesting pOC52 (Cohen Fix *et al.*, 1996), a pUC19-based plasmid carrying *PDS1::HA* and *URA3*, with *Bgl*II. This removed part of the promoter and the first 522 base pairs of the open reading frame. This construct was used to replace the wild-type genomic *PDS1* sequences by transplacement. The *pds1*Δ*::URA3* disrupt was confirmed by Southern hybridization analysis.

Screen for synthetic lethality with sic1Δ

JD100 (Table I) was transformed with p*SIC1U*, a YCplac33 (Gietz and Sugino, 1988) -based plasmid carrying *SIC1* on a *Eco*RI–*Ssp*I fragment and the *URA3* gene. The object of the screen was to find mutants unable to grow in the absence of *SIC1* and we used sensitivity to FOA to indicate the inability to lose p*SIC1U*. The JD100 p*SIC1U* strain was transformed with DNA from a yeast genomic library whose inserts had been randomly disrupted with *lacZ::LEU2* as described (Burns *et al.*, 1994) (this library was a generous gift from M.Snyder, Yale University). LEU^+ transformants were then screened for sensitivity to FOA. To test if the FOA sensitivity was *SIC1*-dependent, we provided transformants with a second copy of *SIC1*. FOA-sensitive mutants were thus transformed with either p*SIC1H* or p*sic1*Δ*H*. Both of these plasmids are ARS-based plasmids derived from YCplac33 with CEN4 deleted. *pSIC1H* carries *SIC1* and the *HIS7* gene whilst p*sic1*Δ*H* is as p*SIC1H* but with the *SIC1* ORF deleted from the *Spe*I site at the N-terminus. Transformed strains were again tested for FOA sensitivity. Only one strain was found from ~80 000 LEU^+ transformants that was FOA-sensitive in a *SIC1*-dependent manner. The site of the *LEU2* insertion was cloned as described (Burns *et al.*, 1994). Briefly, a bacterial/yeast shuttle vector, YIp5 (Struhl *et al.*, 1979), was integrated by transformation into the *LEU2* insertion site by targeting it to bacterial vector sequences contained within the *LEU2* insert. The genomic sequences surrounding the *LEU2* insertion were recovered by digesting then self-ligating the genomic DNA and recovering the *LEU2* bearing plasmid by transformation into *Escherichia coli*. Sequencing was done as described (Burns *et al.*, 1994) using a fmol Cycle Sequencing Kit (Promega).

Cloning RSI1 and epitope tagging

A DNA fragment containing the *RSI1* ORF and flanking promoter sequences was generated by PCR using the following oligonucleotides: ATTGAATTGTTTGCCTGACTAGTG and CGGGATCCTCTGATAT-ACGTACGACC. The resulting PCR product was digested with *Bam*HI and *Spe*I and cloned into a *URA3* bearing integrative plasmid vector. The plasmid was cut at a unique *Nru*I site and integrated upstream of *RSI1*. Genomic DNA from the transformant was digested with *Hin*dIII, self-ligated and transformed into *E.coli*. The resulting *URA3* plasmid also carries 4.5 kb of *RSI1* sequences including the complete open reading frame and 1.4 kb of upstream sequences. This construct was able to rescue a lethal open reading frame deletion.

The triple Myc epitope tagged *RSI1Myc3* was constructed as follows: the *RSI1* ORF was amplified using PCR with the oligonucleotides RSI1-5 and RSI1-3 (5′ GGCTCTAGATTTGGTATTGTTCAAAATTTTC 3′ and 5′ GGCTCTAGAATGTCATTTCAGATTACCCCA 3′) and *Pfu Taq* polymerase (Stratagene). The PCR product was digested with *Xba*I and cloned into *Xba*I digested pRS306-3Myc vector (unpublished). In frame insertion was confirmed by sequencing the resulting pRS306-RSI1-3Myc construct. For integration at the *RSI1* locus, this construct was cut at a unique *Spe*I site 240 bp downstream from the initiating ATG.

Cytology

Chromatin and spindles were visualized as described (Toyn and Johnston, 1994) and references therein.

Preparation of yeast crude extracts and protein analysis

Strains were grown to mid-log phase, cells were pelleted, washed once with saline buffer and frozen. Cell pellets (2×10^8 cells) were resuspended in 150 µl breaking buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 5 mM EDTA, 1% NP-40, 10 µg/ml each protease inhibitors (leupeptin, pepstatin, chymotrypsin, aprotinin, antipain) and 1 volume of acid-washed glass beads was added. For the co-immunoprecipitation experiment, extracts were prepared as described (Lamb *et al.*, 1994). Lysis was achieved by vortexing four times (30 s vortexing and 30 s cooling on ice). A volume of 100 µl breaking buffer was added and the lysate was separated from the beads by centrifugation. The lysate was collected and protein concentration was determined using the Bio-Rad Bradford protein assay. For Western blot analysis, 25 µg of protein in cracking buffer (Printen and Sprague, 1994) was resolved by PAGE–SDS, transferred to Protran Nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and detected using chemiluminescence detection (ECL, Amersham) with primary antibodies 12CA5 (HA) (NIMR, London) or 9E10 (MYC) (ICRF, London).

For the immunoprecipitation, 200 µg (H1 kinase assay) or 1 mg (co-immunoprecipitation experiment) of protein extract in 50 µl breaking buffer was immunoprecipitated with 2 µg of anti-HA or anti-Myc antibody for 1 h at 4°C. Protein A/G beads (15 µl) (50% slurry) was added and incubated on a rotating wheel for 30 min at 4°C. For the H_1

kinase assay, protein A/G beads–Immune complex were collected by centrifugation and washed three times with breaking buffer, twice with kinase buffer (25 mM MOPS pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA) and incubated for 20 min at room temperature with 15 µl kinase buffer containing 1 mg/ml histone H1 and 0.1 µl [γ-^{32}P]ATP (10 mCi/ml). The reaction was stopped by adding 1 volume of cracking buffer. Phosphorylated histone H1 were analysed by PAGE–SDS and autoradiography. For the co-immunoprecipitation experiment, protein A/G beads–immune complex were collected, washed three times with breaking buffer and co-precipitating proteins were released by boiling in cracking buffer. The supernatant was processed for Western blotting analysis as detailed above.

Acknowledgements

We wish to thank A.Page, P.Hieter, D.Koshland, W.Zachariae, and K.Nasmyth for reagents; S.Tournier for help with microscopy; L.Frenz for sequencing the *RSI1Myc3* construct; N.Bouquin, J.Millar and E.Schwob for valuable suggestions and discussions. K.M.K. was supported by a fellowship from the Human Frontiers Science Program (LT225/95). D.F. was supported by a grant from Fondation pour la recherche Medicale.

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Received October 1, 1997; revised November 10, 1997; accepted November 11, 1997