creased the intensity of phalloidin staining, Rho-kinase appears to induce actin polymerization to a small extent. The cells stimulated by LPA in the presence of staurosporine showed randomly arranged actin filaments, but the cells injected with CAT in the presence of staurosporine did not form stress fibers, indicating that there are additional pathways (such as PIP5-K) that induce actin polymerization downstream of Rho.

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23. The cDNAs encoding the catalytic domain (amino acids 6 to 553) and catalytic domain mutated at the ATP-binding site (Lys^{121} → Gly) were inserted into the Bam HI site of pAcYM1-GST to produce GST-CAT and GST-CAT-KD, respectively. GST-CAT and GST-CAT-KD were produced in Sf9 cells with a baculovirus system [Y. Matsuura *et al.*, *J. Gen. Virol.* **68**, 1233 (1987)] and purified on a glutathione-Sepharose column (*10*). The cDNAs encoding the coiled-coil domain (amino acids 421 to 701), Rho-binding domain (amino acids 941 to 1075), and pleckstrin-homology domain (amino acids 1125 to 1388) were inserted into the Bam HI site of pGEX-2T to produce COIL, RB, and PH, respectively. COIL, RB, and PH were produced and purified from *E. coli* as described (*10*). The pEF-BOS-myc mammalian expression plasmids encoding CAT, CAT-KD, COIL, RB, and PH were constructed.
24. Binding of Rho to Rho-kinase was determined by overlay assay as described (*10*). Purified Rho-kinase (0.25 μg) or GST-RB (2.5 μg) were separated on an SDS-polyacrylamide gel (12%), transferred to nitrocellulose membrane, and probed with [^{35}S]GTP-γ-S·GST-RhoA or [^{35}S]GTP-γ-S·GST-$RhoA^{A37}$. The labeled bands were visualized by an image analyzer (Fuji).
25. Rho-kinase activity was assayed in 50 μl of the reaction mixture [40 mM tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 6.5 mM $MgCl_2$, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 0.1 μM calyculin A, 100 μM [γ-^{32}P]ATP (14 to 540 mCi/mmol), 4 μg of myosin light chain, and 20 ng of Rho-kinase or 8 ng of GST-CAT with or without 1.5 μM GTP-γ-S·GST-RhoA (*13*). After incubation for 10 min at 30°C, the reaction mixtures were boiled in SDS–sample buffer and subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The labeled bands were visualized by an image analyzer (Fuji).
26. Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%). Cells were seeded at a density of 8×10^3 to 10×10^3 cells onto 12-mm glass cover slips. After 4 days, the cells were deprived of serum for 24 hours in DMEM. Recombinant proteins were microinjected along with a marker protein (rabbit immunoglobulin G, 1 mg/ml) into the cytoplasm of cells. After microinjection, the cells were incubated at 37°C for 30 min. Actin and vinculin were visualized by tetramethylrhodamine B isothiocyanate (TRITC)–labeled phalloidin and an antibody to vinculin, respectively, as described (*2*). Nuclei were visualized by bisbenzimide.
27. MDCK cells were cultured in minimum essential medium supplemented with fetal bovine serum (10%). Cells were seeded at a density of 2×10^3 cells onto 12-mm glass cover slips and cultured for 1 day. Various plasmids were injected into the nucleus as described [A. Ridley *et al.*, *Cell* **70**, 401 (1992)]. After microinjection, the cells were incubated at 37°C for 3 hours. Actin was visualized with TRITC-labeled phalloidin as described (*2*).

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APC-Mediated Proteolysis of Ase1 and the Morphogenesis of the Mitotic Spindle

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The molecular mechanisms that link cell-cycle controls to the mitotic apparatus are poorly understood. A component of the *Saccharomyces cerevisiae* spindle, Ase1, was observed to undergo cell cycle–specific degradation mediated by the cyclosome, or anaphase promoting complex (APC). Ase1 was degraded when cells exited from mitosis and entered G_1. Inappropriate expression of stable Ase1 during G_1 produced a spindle defect that is sensed by the spindle assembly checkpoint. In addition, loss of *ASE1* function destabilized telophase spindles, and expression of a nondegradable Ase1 mutant delayed spindle disassembly. APC-mediated proteolysis therefore appears to regulate both spindle assembly and disassembly.

Cell cycle–specific proteolysis was first discovered as a mechanism for inactivation of mitotic cyclins. B-type cyclins are degraded through the ubiquitin-proteasome pathway (*1–3*). The cell-cycle specificity of this process comes from the ubiquitination reaction and not from the degradation by the proteasome. Ubiquitination requires the activation of ubiquitin by an E1 enzyme and its subsequent transfer to one of a family of ubiquitin-conjugating enzymes (E2 enzymes). Often, a third activity, the E3, is also required and is a determinant of substrate specificity (*1*). The E3 for proteolysis of mitotic cyclins is a multiprotein complex termed the cyclosome or anaphase promoting complex (*4–7*). Ubiquitination of mitotic cyclins requires a sequence motif termed the destruction box that is thought to be recognized by the APC (*2*, *8*).

Although the role of the APC in cyclin proteolysis is well established, there is now mounting evidence that the APC has other cell-cycle functions. A requirement for the APC during sister chromatid separation was deduced from experiments in *Xenopus laevis* egg extracts and in yeast (*5*, *9*, *10*). Recently, two APC substrates required for sister chromatid separation have been identified: Cut2, in fission yeast, and Pds1, in budding yeast (*11*). A role for the APC in some aspect of DNA replication has also been inferred from the finding that alleles of *Saccharomyces cerevisiae CDC16* and *CDC27* allow more than one complete round of DNA replication during a single cell cycle (*12*).

We found that the APC regulates the mitotic apparatus by targeting a component

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of the yeast spindle, Ase1, for degradation. *ASE1* (for anaphase spindle elongation) encodes a yeast nonmotor microtubule-binding protein [MAP (*13*)] that localizes to the anaphase spindle midzone, where spindle fibers from opposite poles overlap. Together with another nonmotor MAP, Ase1 is required for anaphase B, the elongation of the spindle and separation of the spindle poles (*13*).

Consistent with our genetic analysis that suggests a function for *ASE1* late in mitosis, the pattern of *ASE1* mRNA expression during the cell cycle closely paralleled that of the mitotic cyclin, *CLB2* (Fig. 1A) (*14*). The amount of Ase1 protein is also regulated during the cell cycle. Like Clb2, Ase1 is not detected during G_1 and is present in largest amounts during mitosis. Although Clb2 appears to be degraded during mid-anaphase, Ase1 persists throughout mitosis and then is abruptly lost as cells undergo cytokinesis (*13*, *15*). The general similarities between the pattern of expression of Ase1 and Clb2 prompted us to determine if Ase1 is rapidly degraded during G_1. The *ASE1* coding sequence was placed under the control of the inducible *GAL1* promoter, and Ase1 turnover in logarithmically growing cells and in G_1 cells was then compared. Ase1 is rapidly degraded in G_1 cells but is stable in cycling cells, with half-lives of 5 and 50 min, respectively [Fig. 1, B and C (*16*)]. Therefore, like mitotic cyclins, Ase1 expression is regulated by both transcriptional and proteolytic mechanisms.

At least four of the APC subunits, CDC16, CDC23, CDC27, and BIME/APC1, are highly conserved between yeast and vertebrate cells (*5–7*, *15*, *17*). In yeast, mutations in *CDC16*, *CDC23*, *CDC26*, or *APC1* block the rapid degradation of Clb2 in G_1 cells. To determine if the G_1-specific degradation of Ase1 requires the yeast APC, we measured the half-life of Ase1 in G_1-arrested *CDC23* and temperature-sensitive *cdc23* strains at the nonpermissive temperature. Ase1 was stable in the G_1-arrested *cdc23* strain but not in the *CDC23* strain (Fig. 2, A and B). Flow cytometry confirmed that these strains remained arrested in the G_1 phase throughout the experiment (Fig. 2B).

The Ase1 polypeptide contains five sequences with similarity to the cyclin destruction box (*2*, *18*). To determine if any of these sequences is a functional destruction box, all of the conserved residues in each of these sequences were mutated to alanine. Mutation of only one of these sequences (amino acids 760 to 768, Arg-Gln-Leu-Phe-Pro-Ile-Pro-Leu-Asn, changed to Ala-Gln-Leu-Ala-Pro-Ile-Pro-Leu-Ala) produced an Ase1 protein that was stable in G_1 cells (Fig. 2, C and D). This mutant, hereafter referred to as Ase1-db, is apparently functional because it both complements *ase1*Δ mutant phenotypes and displays normal localization. The fact that Ase1 degradation requires both *CDC23* and a destruction box sequence suggests that Ase1 is an APC substrate.

Because of the localization of Ase1 to the spindle midzone, we reasoned that Ase1 might have a role in maintaining the interaction between the two half-spindles and further, that Ase1 degradation might have a role in spindle disassembly. Therefore, we tested whether loss of Ase1 altered the structure of the spindle in a mutant, *cdc15*, that blocks cell-cycle progression at the end of mitosis. *ASE1 cdc15* and *ase1*Δ *cdc15* strains were arrested at the nonpermissive temperature, and spindle structures were visualized by tubulin immunofluorescence. The *ASE1 cdc15* strain arrested in telophase, with long spindles and segregated chromosomes (94% of cells, Fig. 3A, a and c). In contrast, although chromosome segregation was completed, the spindles in the *ase1*Δ *cdc15* strain fell apart, leaving structures resembling the astral microtubules observed in G_1 cells (91% of cells, Fig. 3A, b and d). These *ase1*Δ *cdc15* cells remained arrested and did not undergo cytokinesis. This experiment demonstrates that in the absence of Ase1, telophase spindles disassemble.

Because Ase1 is required for the stability of telophase spindles, we tested whether the expression of nondegradable Ase1 at the end of mitosis would block or delay spindle disassembly. The expression of Ase1 or Ase1-db was induced in *cdc15*-arrested cells, and spindle morphology was determined at intervals after release to the permissive temperature in medium containing the mating pheromone α-factor to produce a G_1 block. Expression of Ase1-db caused a delay in spindle disassembly (Fig. 3B). *GAL1*-driven expression of Ase1 had no effect on spindle disassembly (*19*, *20*). Protein immunoblotting confirmed that Ase1 but not Ase1-db was degraded after release from the *cdc15* block (Fig. 3C).

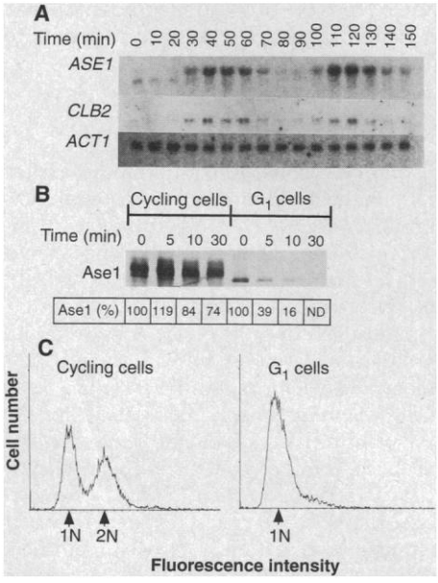

Fig. 1. Transcription and cell cycle–specific proteolysis of Ase1. (**A**) Northern analysis of *ASE1*, *CLB2*, and *ACT1* (actin) mRNA at intervals after release of cells from arrest in G_1 with the mating pheromone, α-factor (*25*). (**B**) Stability of ^{35}S-labeled Ase1 in cycling cells and in α-factor–arrested G_1 cells (*26*). The relative amount of Ase1 is indicated below each lane. ND indicates a time point where Ase1 was not detected. (**C**) Flow cytometry of cells at the last time point from (B) (*27*).

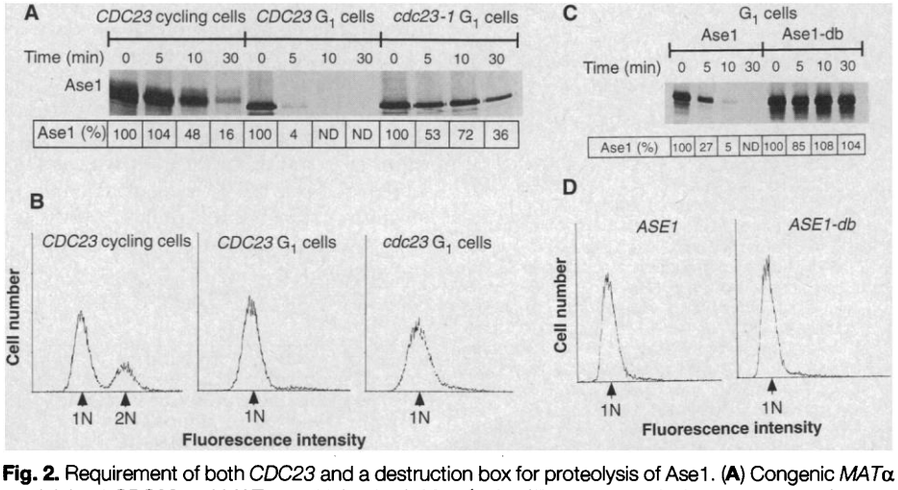

Fig. 2. Requirement of both *CDC23* and a destruction box for proteolysis of Ase1. (**A**) Congenic *MAT*α *ase1* Δ *bar1 CDC23* and *MAT*α *ase1*Δ *bar1 cdc23-1* strains (*5*) were grown in the presence or absence of α-factor at 24°C, then shifted to 36°C for 30 min, and the half-life of Ase1 was determined (*26*, *28*). (**B**) Flow cytometry of cells from the last time point in (A). (**C**) Stability of Ase1 and Ase1-db expressed from the *GAL1* promoter in *MAT*α *bar1 ase1*Δ cells arrested in G_1 with α-factor (*29*). (**D**) Flow cytometry of cells from the last time point in (C).

Next, we studied the effect of expression of Ase1-db in G_1, when it is normally not expressed. *ase1Δ* strains containing either *GAL1::ASE1*, *GAL1::ASE1-db*, or a control vector were arrested in G_1, expression from the *GAL1* promoter was transiently induced, and the cells were released from the G_1 block. In comparison with the cells expressing the control vector or Ase1, expression of Ase1-db in G_1 delayed cell-cycle progression through mitosis. The cells transiently expressing Ase1-db accumulated early in mitosis as large budded cells containing undivided nuclei, short mitotic spindles, and 2N DNA content [Fig. 4A (*21*)]. This phenotype is similar to that observed when either wild-type Ase1 or the destruction box mutant is induced for long periods of time in unsynchronized cells (*19*).

A checkpoint mechanism has been identified that blocks or delays mitosis in the presence of abnormal spindles (*22*). To determine if the mitotic delay induced by the expression of Ase1-db is due to a spindle defect, we expressed Ase1-db in a strain lacking a component of the checkpoint, *MAD1*. Expression of Ase1-db in G_1-arrested *mad1Δ* cells did not cause a mitotic delay (Fig. 4B). However, the absence of this delay resulted in decreased viability. Only 20% of *mad1Δ* cells transiently expressing *ASE1-db* were viable, whereas transient expression of *ASE1* or the control vector had no effect on viability. This demonstrates that the expression of Ase1-db during G_1 leads to spindle damage when cells subsequently enter mitosis. These observations provide an additional rationale for the surprising finding that APC-mediated proteolysis remains active during G_1. APC-mediated proteolysis during G_1 may prevent inappropriate expression of Clb2 that would inhibit budding (*5*, *23*) and may also prevent inappropriate expression of Ase1, which would interfere with spindle assembly.

Our findings suggest that the APC may have two important roles in regulating the function of the mitotic spindle: it may mediate the disassembly of the spindle and prevent proteins that are normally assembled onto the spindle late from accumulating too early. Because the nondegradable Ase1 protein delays but does not block spindle disassembly, we expect that other proteins that contribute to the stability of the spindle are targeted for degradation by the APC. Indeed, two components of the mammalian mitotic apparatus, CENP-E and CENP-F, are degraded at the end of mitosis (*24*). The localization of APC subunits to

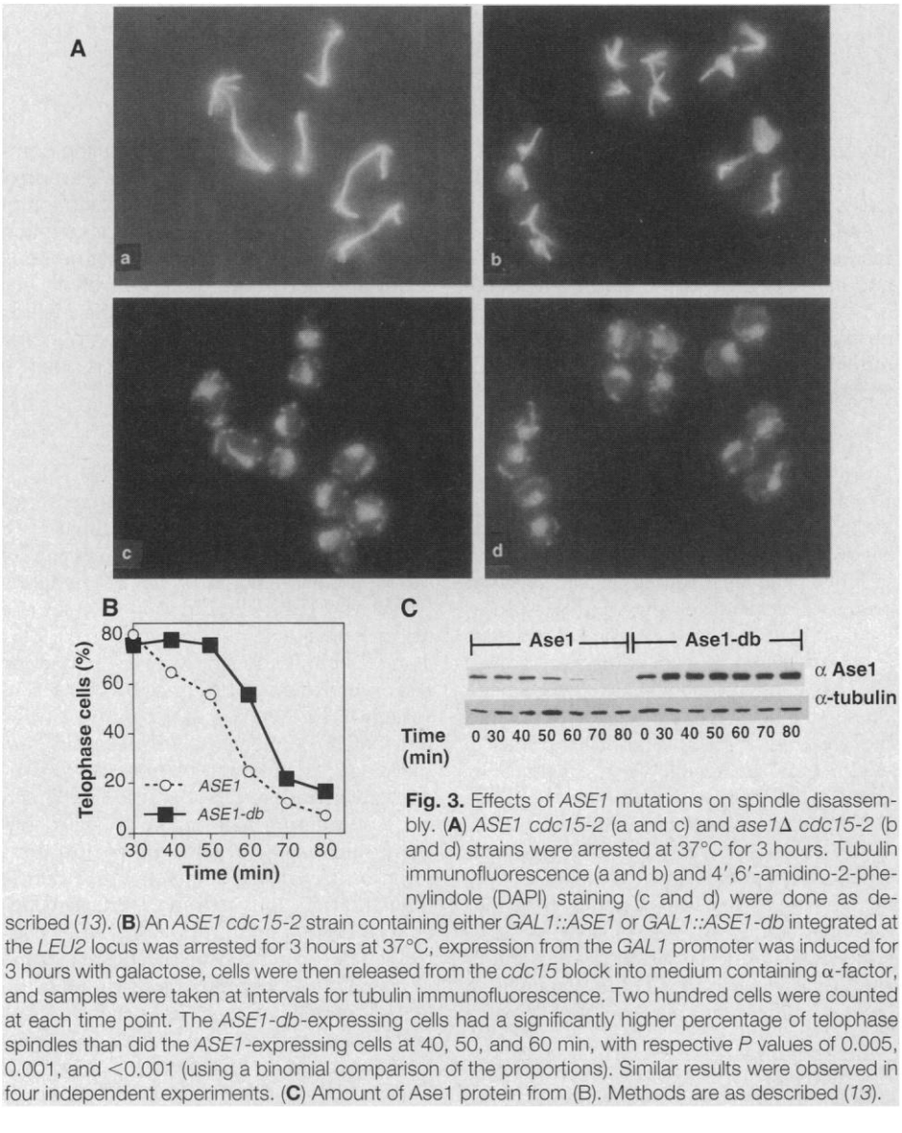

Fig. 3. Effects of *ASE1* mutations on spindle disassembly. (**A**) *ASE1 cdc15-2* (a and c) and *ase1Δ cdc15-2* (b and d) strains were arrested at 37°C for 3 hours. Tubulin immunofluorescence (a and b) and 4′,6′-amidino-2-phenylindole (DAPI) staining (c and d) were done as described (*13*). (**B**) An *ASE1 cdc15-2* strain containing either *GAL1::ASE1* or *GAL1::ASE1-db* integrated at the *LEU2* locus was arrested for 3 hours at 37°C, expression from the *GAL1* promoter was induced for 3 hours with galactose, cells were then released from the *cdc15* block into medium containing α-factor, and samples were taken at intervals for tubulin immunofluorescence. Two hundred cells were counted at each time point. The *ASE1-db*-expressing cells had a significantly higher percentage of telophase spindles than did the *ASE1*-expressing cells at 40, 50, and 60 min, with respective *P* values of 0.005, 0.001, and <0.001 (using a binomial comparison of the proportions). Similar results were observed in four independent experiments. (**C**) Amount of Ase1 protein from (B). Methods are as described (*13*).

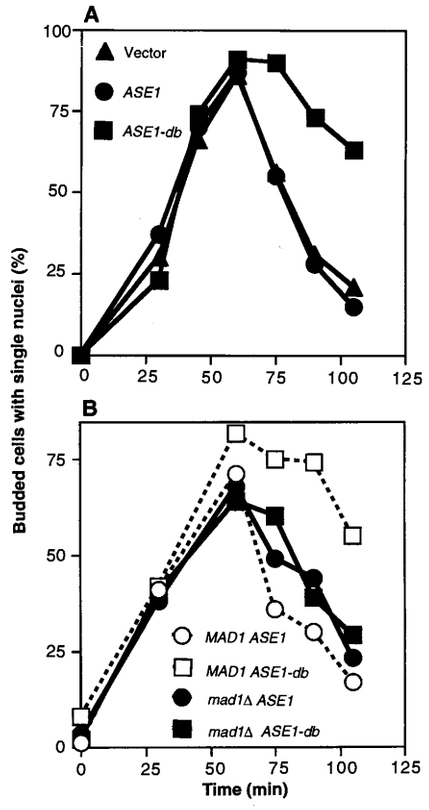

Fig. 4. Activation of the spindle assembly checkpoint from transient expression of Ase1-db in G_1 cells. (**A**) *MATa ase1Δ bar1* strains containing *GAL1::ASE1*, *GAL1::ASE1-db*, or a 2μ control vector were arrested with α-factor, and *GAL1* expression was induced for 3 hours with galactose. *GAL1* expression was then repressed with glucose, the cells were released from the G_1 block by washing into fresh medium, and samples were taken at intervals for DAPI staining and differential interference contrast microscopy. The percentage of cells that are budded and contain a single nucleus (early mitosis) is indicated. (**B**) The transient expression experiment from (A) was repeated for strains with the following genotypes: *GAL1::ASE1 MAD1*, *GAL1::ASE1-db MAD1*, *GAL1::ASE1 mad1Δ*, and *GAL1::ASE1-db mad1Δ*.

the spindle also supports a general role for the APC in regulating the mitotic apparatus (*7*).

Our findings also broaden the scope of cellular processes under APC control. In addition to controlling the abundance of mitotic cyclins, the APC regulates sister chromatid cohesion, the cellular DNA content, and the function of the mitotic spindle (*5*, *10*–*12*). The APC proteolytic system may therefore be a global cell-cycle regulator much like the cyclin-dependent kinases.

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16. We have also found that there is no difference between the half-life of Ase1 in cycling cells and cells arrested in G_2 with the microtubule depolymerizing agent, nocodozole, suggesting that the stability of Ase1 during mitosis is not due to co-assembly with microtubules (M. McLaughlin and D. Pellman, unpublished results).
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20. The rate of spindle disassembly was not different between cells containing *GAL1::ASE1* and the control vector (*19*).
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25. All strains used in this report are isogenic or congenic to W303a. Cells were arrested in G_1 with α-factor, either 5 μM for *BAR1* strains or 60 nM for *bar1* strains. A *MATa BAR1* W303a strain was used for Northern (RNA) analysis. Probes for Northern analysis are: an antisense riboprobe spanning base pairs 1077 to1739 of the *ASE1* coding sequence, a DNA probe from bases 1 to 1085 of *ACT1*, and a DNA probe containing the entire coding sequence of *CLB2*.
26. A fully functional myc-tagged *ASE1* construct (*13*) was placed under the control of the *GAL1* promoter on a 2μ vector and introduced into a *MATa bar1 ase1Δ* strain. ^{35}S-labeling and measurement of protein stability was done as described [D. Kornitzer, B. Raboy, R. G. Kulka, G. R. Fink, *EMBO J.* **13**, 6021 (1994)]. Ase1 levels were quantitated with a phosphorimager and normalized to the zero time point. Experiments using Ase1 polyclonal antibodies demonstrated that the myc epitope did not alter the half-life of Ase1 or Ase1-db (*19*).
27. Methods are as described (*13*). Cultures for pulse-chase analysis and flow cytometry were split before labeling, and the sample for flow cytometry was grown in parallel in identical medium containing unlabeled methionine.
28. The half-life of Ase1 in cycling cells at 36°C is somewhat shorter than at 30°C (compare Fig. 1 B with Fig. 2A).
29. The Ase1-db mutant was constructed by site-directed mutagenesis using an oligonucleotide of sequence 5′-CATGCAGTAAAACCAGCTCAGCTGGCTCCTA-TCCCGCTGGCTAAAGTCGACACTAAG-3′.

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Combinatorial Control Required for the Specificity of Yeast MAPK Signaling

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In yeast, an overlapping set of mitogen-activated protein kinase (MAPK) signaling components controls mating, haploid invasion, and pseudohyphal development. Paradoxically, a single downstream transcription factor, Ste12, is necessary for the execution of these distinct programs. Developmental specificity was found to require a transcription factor of the TEA/ATTS family, Tec1, which cooperates with Ste12 during filamentous and invasive growth. Purified derivatives of Ste12 and Tec1 bind cooperatively to enhancer elements called filamentation and invasion response elements (FREs), which program transcription that is specifically responsive to the MAPK signaling components required for filamentous growth. An FRE in the *TEC1* promoter functions in a positive feedback loop required for pseudohyphal development.

Because common signaling components such as the MAPK cascade respond to a large number of different stimuli, it is not clear how specific signals are produced. In *Saccharomyces cerevisiae*, elements of the MAPK pathway required for the mating pheromone response are also required for haploid invasive growth and diploid pseudohyphal development. These shared factors include Ste20, Ste11, Ste7, and Ste12 (*1*, *2*). The first three act in sequence and are homologs of the mammalian kinases PAK (p21-activated kinase), MEKK (MAP kinase kinase kinase), and MEK (MAP kinase kinase), respectively (*3*). The transcription factor Ste12 is a terminal component of these signaling cascades, functioning downstream of the kinases to drive either sexual differentiation or filamentous and invasive growth (*3*). In mammalian cells, myriad stimuli activate MAPK pathways, yet only a handful of target transcription factors have been identified (*4*). Therefore, we addressed the question of how a common target of MAPK signaling pathways, Ste12, can direct more than one distinct developmental program.

Ste12 binds cooperatively to pheromone response elements (PREs) of the consensus sequence TGAAACA (*5*, *6*), and two or more of these elements are necessary and sufficient to program pheromone-responsive transcription (*7*). Because Ste12 can act alone during mating, we thought that there might exist a pathway-specific transcription factor that retargets Ste12 during filamentation and invasion through cooperative DNA binding (combinatorial control). The expression of the reporter gene *FG(TyA)::lacZ* depends specifically on the MAPK signaling components that promote filamentous and invasive growth (*8*). Transcription of *FG(TyA)::lacZ* is driven by a fragment of the retrotransposon *Ty1*, whose

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