ACE2, CBK1, and BUD4 in Budding and Cell Separation

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Mutations in the RAM network genes, including CBK1, MOB2, KIC1, HYM1, and TAO3, cause defects in bud site selection, asymmetric apical growth, and mating projections. Additionally, these mutants show altered colony morphology, cell separation defects, and reduced CTS1 expression, phenotypes also seen by mutating the Ace2 transcription factor. We show that an ACE2 multicopy plasmid suppresses the latter three defects of RAM network mutations, demonstrating that Ace2 is downstream of the RAM network and suggesting that these phenotypes are caused by reduced expression of Ace2 target genes. We show that wild-type W303 strains have a bud4 mutation and that combining bud4 with either ace2 or cbk1 in haploids results in altered colony morphology. We describe a timed sedimentation assay that allows quantitation of cytokinesis defects and subtle changes in budding pattern and cell shape. Experiments examining budding patterns and sedimentation rates both show that Ace2 and Cbk1 have independent functions in addition to their common pathway in transcription of genes such as CTS1. SWI5 encodes a transcription factor paralogous to ACE2. Additive effects are seen in cbk1 swi5 strains, and we show that activation of some target genes, such as EGT2, requires either Swi5 or Ace2 with Cbk1. The relative roles and interactions of Ace2, Cbk1, and Bud4 in bud site selection, polarized growth, and cell separation are discussed.

Cells are innately asymmetric, and even the most basic internal polarization can be exploited to program division patterns which lead to organized structures. The morphology of multicellular organisms or any multicellular assemblage is based chiefly on the shapes which cells adopt, the patterns of cell division, and the retention or loss of cellular contacts after division. Changes in these processes correlate with morphogenetic transitions in organismal development, as well as in resource exploitation and pathogenesis in so-called unicellular organisms. Saccharomyces cerevisiae has been shown to regulate these phenomena, in part by controlling intrinsic cell polarity as well as reorganization of polarity in response to external cues, the selection of sites at which budding initiates depending on cell type and environment, and by controlling separation of divided cells after mitosis (7, 8, 23, 27).

The yeast Cbk1 protein is a member of the AGC serine-threonine protein kinase family, which also includes S. cerevisiae Dbf2, Schizosaccharomyces pombe Orb6 and Sid2, Ndr1, WARTS/LATS, and others (15, 28, 35). Cells lacking Cbk1 show defects in a number of processes, including bud site selection, asymmetric apical growth of the emerging bud, response to mating pheromone, morphogenesis of mating projections, and cell separation after mitosis (3, 28). CBK1 is a component of the RAM (regulation of Ace2 activity and cellular morphogenesis) network, which includes the Cbk1 binding partner MOB2 and KIC1, HYM1, and TAO3, all strains of which have several phenotypes similar to cbk1 mutants when mutated (25). cbk1 mutant cells lack the normal ability to select budding locations near the polar sites of previous budding and instead form buds at random locations across the cell surface. Random budding is symptomatic of a more general loss of axis in cbk1 cells, which also fail to display the usual ellipsoid cell shape which arises from apically oriented growth in the emerging bud. Instead, cbk1 mutants grow isotropically to form more spherical cells. This lack of polarity is also evident as reduced development of mating projections in cbk1 and other RAM mutant cells, a consequence of loss of organization in the underlying actin cytoskeleton. Strains mutant in CBK1 also possess defects in colony formation, with a loss of the normal hemispherical shape and smooth opalescent surface, instead appearing irregular and rough. Mutations in cbk1 or any other RAM network gene are not tolerated in some genetic backgrounds, including those used for global knockout or any other RAM network gene are not tolerated in some genetic backgrounds, including those used for global knockout and expression studies. This strain-dependent difference in lethality is due to polymorphism at the SDD1 locus; thus lack of a RAM network gene is tolerated only if SDD1 is also mutated, and most studies of RAM network genes have been carried out in sdd1 strain backgrounds, such as W303 (20).

The zinc finger transcription factor Ace2 activates a set of genes in G1, many of which code for enzymes responsible for locally specific cell wall degradation (11, 12, 14). Loss or reduction in Ace2 activity causes daughter cells to remain attached by the cell wall to mothers at the bud site septum, but with no delay in subsequent nuclear or cytoplasmic cell cycles. This results in the accumulation of multicellular clusters or clumps. In diploids, or in haploids in some strain backgrounds, ace2 mutant strains also show the rough colony morphological defects similar to those in cbk1 strains (22). Ace2 activity and concomitant breakage of the mother-daughter connection is tightly controlled with respect to the cell cycle and to cell type. Several transcriptional and posttranscriptional mechanisms act through Ace2 to ensure that septum separation occurs only

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after the completion of cytokinesis and only on the daughter-proximal side of the chitinous budding ring. The ability of Ace2 to activate transcription is regulated by Cbk1 and other mem-
bers of the RAM network in ways that are not fully under-
stood, but deletion of any member of the network results in cell separation and target gene activation phenotypes similar to ace2 mutations without changes in Ace2 protein levels.

More than 70 genes have been identified which are necessary for yeast’s normal budding pattern programs (26). These genes have been classified into several groups based on the type of altered budding pattern seen in the mutant. Vegeta-
tively growing haploids follow an axially budding program, in which subsequent buds form in close apposition to the initial bud site, itself adjacent to the birth scar, resulting in a chain of bud scars visible around the long axis of the cell. Mutation of the BUD4 gene gives rise to haploids which fail in axial budding but instead execute an otherwise normal bipolar program typical of diploids, in which subsequent rounds of budding are directed to alternating poles of the mother cell (9). There are several other genes in which mutation causes this same axial-to-bipolar change in haploids, including AXL1 (18). Bud4 pos-
sesses pleckstrin homology and GTP-binding domains and fa-
cilitates axial budding by functioning as a molecular tag marking the position of the bud neck from previous budding event. Bud4 is modeled as an activator of Axl1, which is itself a repressor of the bipolar programming proteins Rax1 and Rax2 and the bipolar tags Bud8 and Bud9 (17). In diploids, while BUD4 is still expressed, its action on Axl1 is obviated by the a1/a2-dependent repression of AXL1 transcription, which allows the bipolar landmarks Bud8 and Bud9 to supercede the axial pattern (17).

Here we report that the widely used W303 strain back-
ground has a bud4 mutation, and we investigate the relative roles of BUD4, ACE2, and CBK1 in controlling cell separation. A bud4 mutation shows additive effects with either ace2 or cbk1, resulting in an altered colony morphology. We find that the random budding pattern associated with cbk1 haploids (28) is only seen in combination with a bud4 mutation but that a cbk1 diploid buds randomly irrespective of BUD4 genotype. Thus, the random budding seen in a cbk1 mutant is only ob-
served in a strain that would otherwise exhibit bipolar budding, but not in an axial budding strain. We describe a sedimentation assay that allows us to quantify defects in cell separation and also detects modest changes in budding pattern and cell shape.

**MATERIALS AND METHODS**

All yeast strains used are listed in Table 1 and are isogenic in the W303 background (37). Standard genetic methods were used for strain construction.
Plasmid Description Source or reference
M4213 BUD4 in Ylp-URA3 plasmid This work
M4214 BUD4 in YCp-URA3 plasmid This work
pRS426 YEp-URA3 vector 4
pGB1-1 ACE2 in YEp-URA3 plasmid 6
M4104 CBK1 in YEp-URA3 plasmid This work
M4105 HYM1 in YEp-URA3 plasmid This work
M4298 MOB2 in YEp-URA3 plasmid This work
M4102 KIC1 in YEp-URA3 plasmid This work

TABLE 3. Multicopy ACE2 suppresses RAM network mutants

| Plasmid | ace2 | cbk1 | hym1 | mob2 | kic1 |
|---------|------|------|------|------|------|
| YEp vector | +   |   |   |   |   |
| YEp-AE2 | +   | +  | +  | +  | +  |
| YEp-CBK1 | +   |   |   |   |   |
| YEp-HYM1 | +   |   |   |   |   |
| YEp-MOB2 | -   | *  | +  | +  | +  |
| YEp-KIC1 | -   | -  | -  | -  | -  |

...these mutants have two phenotypes in common: an altered colony morphology (see below) and reduced expression of a CTS1- lacZ reporter. We performed multicopy suppression experiments to investigate the relationships between these factors. As shown in Table 3, a multicopy plasmid with the ACE2 gene can suppress the colony morphology and CTS1-lacZ expression phenotypes of the cbk1, hym2, mob2, and kic1 mutants. These results suggest that CBK1, MOB2, HYM1, and KIC1 all act upstream of ACE2, consistent with the report that multicopy ACE2 suppresses the cell separation defects in RAM mutants (25, 26). However, we do not have a clear understanding of why mutating either ACE2 or upstream factors should affect repression by LexA-Sin3.

Genetic interactions between Ace2 and BUD4. An altered colony morphology is seen in an ace2 or cbk1 mutant strain (Fig. 1), and a similar phenotype is seen in mob2, hym1, and kic1 mutants. Colonies of these mutants lose the typical hemispherical shape and smooth opalescent surface and instead exhibit scalloped colony edges and a roughened surface with many branched channels and invaginations. Interestingly, these rough colonies are seen in ace2 W303 but not in ace2 S288c strains (18; W. P. Voth and D. J. Stillman, unpublished observations). When we used a visual screen for complementation of the rough morphology to clone CBK1, MOB2, HYM1, and ACE2, we identified several plasmids that complemented the colony morphology of all of the mutants. These plasmids contained BUD4 and several adjacent genes, and subcloning demonstrated that the BUD4 gene was sufficient for complementation. These results suggested that the parent W303 strain has a bud4 mutation and that two mutations are required for the colony phenotype. To test this hypothesis, we constructed a YIp-URA3-BUD4 plasmid which was integrated at the bud4 locus, creating a bud4::URA3::BUD4 allele. Growth on 5-fluoroorotic acid allowed us to recover W303 BUD4 strains, without the URA3 marker. In crosses between ace2 bud4 and ace2 bud4::URA3::BUD4 strains, the smooth colony phenotype always segregated with URA3. Additionally, we used the genetic linkage of the IME1 and BUD4 genes to demonstrate that the bud4::URA3::BUD4 allele is at the bud4 locus. A strain with this bud4::URA3::BUD4 allele was crossed to a strain with an ime1::TRP1 allele, and bud4::URA3::BUD4 showed tight linkage to IME1 in this cross. As shown in Fig. 1A, a smooth colony phenotype is seen in strains with a single mutation in either CBK1 or BUD4. Importantly, roughened colonies are
seen only when both \(CBK1\) and \(BUD4\) are mutant. Similarly, the \(ace2\) \(bud4\) double mutant has the altered colony morphology (Fig. 1A).

To investigate the roles of \(BUD4\) and \(CBK1\) on budding patterns, isogenic strains were stained with Calcofluor, which stains bud scars. As shown in Table 4 (top 12 strains), wild-type W303 haploid strains display a mixture of axial and bipolar budding patterns (strains DY150 and DY151), due to their natural \(bud4\) defect, while the \(BUD4\) derivatives are exclusively axial (strains DY6604 and DY6605). A \(cbk1\) mutation has no effect on budding pattern in \(BUD4\) haploid cells (compare strains DY6604 and DY6605 to strains DY9259 and DY9260). Interestingly, combining the \(bud4\) and \(cbk1\) mutations in haploid cells (strains DY5794 and DY5795) causes essentially all cells to bud randomly, but this is not seen in either single mutant. A \(bud4\) mutation does not alter budding pattern in diploids (compare strains DY8497 and DY1640), as reported previously (9). We also note that in diploids a \(cbk1\) mutation results in exclusively random budding, even when the wild-type \(BUD4\) gene is present (strain DY9216). We conclude that \(Cbk1\) is required for normal budding in both haploid and diploid cells and that the combination of \(cbk1\) with loss of \(Bud4\) activity, either through mutation or cell-type-specific repression, results in random budding.

It has been previously noted that \(bud4\) \(cbk1\) haploid cells are very round (28). We find that \(BUD4\) \(cbk1\) haploid cells are equivalently round and that a \(cbk1\) mutation affects cell shape in both haploids and diploids. Budding defects are often associated with a loss of cell polarity (3, 26), and \(bud4\) \(cbk1\) haploid

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**TABLE 4. \(cbk1\) causes a random budding phenotype**

| Strain   | Genotype        | Axial (%) | Bipolar (%) | Random (%) |
|----------|-----------------|-----------|-------------|------------|
| DY6604   | \(MATa\) \(BUD4\) \(CBK1\) | 99.5 ± 0.9 | 0.3 ± 0.6  | 0.2 ± 0.3  |
| DY6605   | \(MATa\) \(BUD4\) \(CBK1\) | 99.8 ± 0.3 | 0.2 ± 0.3  | 0.0 ± 0.0  |
| DY8497   | \(MATa\) \(MATa\) \(BUD4\) \(CBK1\) | 2.3 ± 1.9  | 94.9 ± 5.9 | 2.8 ± 4.0  |
| DY150    | \(MATa\) \(bud4\) \(CBK1\) | 32.1 ± 2.9 | 64.5 ± 3.7 | 3.3 ± 0.8  |
| DY151    | \(MATa\) \(bud4\) \(CBK1\) | 41.7 ± 5.6 | 51.5 ± 7.1 | 6.8 ± 1.8  |
| DY1640   | \(MATa\) \(MATa\) \(bud4\) \(CBK1\) | 1.8 ± 0.8  | 89.7 ± 0.3 | 8.2 ± 1.0  |
| DY9259   | \(MATa\) \(BUD4\) \(cbk1\) | 99.3 ± 0.3 | 0.2 ± 0.3  | 0.5 ± 0.5  |
| DY9260   | \(MATa\) \(BUD4\) \(cbk1\) | 100 ± 0.0  | 0.0 ± 0.0  | 0.0 ± 0.0  |
| DY9216   | \(MATa\) \(MATa\) \(BUD4\) \(cbk1\) | 0.3 ± 0.6  | 0.0 ± 0.0  | 99.7 ± 0.6 |
| DY5794   | \(MATa\) \(bud4\) \(cbk1\) | 1.5 ± 0.5  | 0.0 ± 0.0  | 98.5 ± 0.5 |
| DY5795   | \(MATa\) \(bud4\) \(cbk1\) | 1.0 ± 0.5  | 0.0 ± 0.0  | 99.0 ± 0.5 |
| DY8567   | \(MATa\) \(MATa\) \(bud4\) \(cbk1\) | 0.0 ± 0.0  | 0.0 ± 0.0  | 100 ± 0.0  |
| DY9197   | \(MATa\) \(BUD4\) \(ACE2\) | 96.0 ± 1.7 | 3.7 ± 1.2  | 0.3 ± 0.6  |
| DY8497   | \(MATa\) \(MATa\) \(BUD4\) \(ACE2\) | 2.3 ± 1.5  | 85.0 ± 2.0 | 12.7 ± 0.6 |
| DY2396   | \(MATa\) \(bud4\) \(ACE2\) | 22.3 ± 5.5 | 62.0 ± 4.6 | 15.7 ± 2.3 |
| DY1640   | \(MATa\) \(MATa\) \(bud4\) \(ACE2\) | 1.7 ± 0.6  | 98.0 ± 0.6 | 0.7 ± 0.6  |
| DY9190   | \(MATa\) \(BUD4\) \(ace2\) | 99.3 ± 0.6 | 0.3 ± 0.6  | 0.3 ± 0.6  |
| DY9399   | \(MATa\) \(MATa\) \(BUD4\) \(ace2\) | 7.1 ± 2.6  | 86.0 ± 4.2 | 6.7 ± 2.9  |
| DY3923   | \(MATa\) \(bud4\) \(ace2\) | 1.7 ± 0.6  | 96.0 ± 1.0 | 2.3 ± 0.6  |
| DY8566   | \(MATa\) \(MATa\) \(bud4\) \(ace2\) | 1.0 ± 0.0  | 93.0 ± 1.2 | 5.7 ± 1.2  |

*Log-phase cultures were sonicated, fixed with 3.7% formaldehyde, and stained with Calcofluor to visualize bud scars. For each strain, at least 200 cells were examined.*
between the initial and the final lower plateau values. The rapid sedimentation in these mutants is not reversed by addition of EDTA, and thus it is not due to the Ca$^{2+}$-dependent flocculation described in some mutants (36).

As shown in Fig. 3, the bud4 cbk1 strains sediment rapidly, with sedimentation half-times of 16 min for haploids and 4 min for diploids. In contrast, there is little change in the optical density for the haploid BUD4 CBK1 strain until after 50 min. The results from this assay allow us to make several conclusions. First, the bud4 and cbk1 mutations each separately cause an increase in sedimentation. Second, the bud4 cbk1 double mutant shows an additive increase in sedimentation, compared to the single mutants. Moreover, the effect of other RAM mutations in combination with bud4 is identical to that of cbk1 in the sedimentation assay (data not shown). Finally, we note that diploid cells show a faster sedimentation rate than haploids for all four genotypes tested. This could be because of the incomplete penetrance of the bipolar phenotype in bud4 haploids relative to diploids and also because of the larger overall size of diploids.

**Correlation between budding, sedimentation, and colony morphology.** In the sedimentation assay, we noted that the cbk1 diploid cells sedimented rapidly while cbk1 haploids sedimented slowly. Based on this difference, we examined the colony morphology of these strains and observed an altered morphology in the BUD4 cbk1 diploid that is different from that in the BUD4 cbk1 haploid (Fig. 1B). Examining the three genotypes, colony morphology, sedimentation rate, and budding pattern, we noted an important correlation. All of the strains listed in Table 5 showing axial or bipolar budding are smooth and sediment slowly, while the strains with a random budding pattern display the altered colony morphology and sediment rapidly. The correlation in these results suggests that the random budding pattern is responsible for the changes in colony appearance and sedimentation rate.

To investigate this idea further, we examined sedimentation rates and budding patterns in ace2 mutants, since an ace2 mutation has a similar effect on colony morphology as cbk1. Figure 4 shows the results of sedimentation assays for isogenic strains differing at the ACE2, CBK1, and BUD4 loci. In BUD4 strains, either an ace2 or a cbk1 mutation causes a considerable increase in sedimentation. Importantly, the ace2 and cbk1 mutations in BUD4 strains show a significantly increased sedimentation rate compared to the ace2 BUD4 and cbk1 BUD4 strains. Thus, ace2 and cbk1 are similar in that both show additive effects when combined with a bud4 mutation. There is a very modest increase in sedimentation in the ace2 cbk1 double mutant compared to the rate in each single mutant. This increased sedimentation in the ace2 cbk1 strain is seen in both the BUD4 or bud4 genotypes but is more significant in the BUD4 strains. In diploid cells, a similar additive increase in sedimentation rate is also seen in ace2 bud4 double mutants compared to that in the single mutants (data not shown). We conclude that while ace2 and cbk1 mutations are similar in both showing strong additive effects when combined with a bud4 mutation, the additive effect of combining ace2 with cbk1 suggests they have independent as well as overlapping functions. As described in the Discussion, we believe the independent functions of Ace2 and Cbk1 are distinct from their overlapping functions in cell separation.
The measurements of budding patterns show a marked difference between ace2 and cbk1 mutants (Table 4). Haploid bud4 ace2 cells show bipolar budding, while haploid bud4 cbk1 cells bud randomly (compare DY3923 to DY5794 and DY5795). Additionally, while a cbk1 mutation causes random budding in diploids (DY9216 and DY8567), diploid ace2 and bud4 ace2 strains show the typical bipolar pattern (DY9399 and DY8566). We conclude that an ace2 mutation does not affect budding pattern. This marked difference between ace2 and cbk1 phenotypes suggests independent functions.

Table 6 summarizes the effects of ace2 and bud4 mutations on three phenotypes: budding, sedimentation, and colony morphology. Importantly, the rough colony morphology does correlate with a rapid sedimentation rate. However, unlike the situation with cbk1 mutants (Table 5), none of the ace2 mutants shows random budding morphology. We conclude that a random budding pattern is not sufficient for the rough colony appearance and the rapid sedimentation, as the ace2 bud4 strain with bipolar budding also shows these phenotypes.

**Additive effects between cbk1 and swi5 mutations.** We used the sedimentation assay to investigate the genetic interactions between ACE2, SWI5, and CBK1. We have previously observed qualitatively increasing severity of cell separation defects in swi5, ace2, and ace2 swi5 double-mutant strains (12), and here we see a corresponding decrease in sedimentation half-time, with swi5 at 33 min, ace2 at 18 min, and ace2 swi5 at 6 min (Table 7). Interestingly, the 16-min sedimentation half-time in the cbk1 single mutant is similar to that seen in the ace2

**FIG. 3. bud4 and cbk1 affect sedimentation rates.** (A) Cells were grown in liquid YEPD medium, and the sedimentation rate at $1 \times g$ was determined as described in Materials and Methods. Strains DY8567 (diploid bud4 cbk1), DY9216 (diploid BUD4 cbk1), DY5794 (haploid MATa bud4 cbk1), DY9195 (haploid MATa BUD4 cbk1), DY1640 (diploid bud4), DY8497 (diploid BUD4), DY2396 (haploid MATa bud4), and DY9197 (haploid MATa BUD4) were used. Similar results were seen with haploid MATa strains. (B) From the data in panel A, the sedimentation half-time is the time for the optical density (O.D.) to fall to one-half of the difference between the initial and the final lower plateau values.

![Graph showing sedimentation rates](image)

**TABLE 5. Budding, sedimentation, and colony morphology in cbk1 mutants**

| Mutant     | Haploid Colony appearance | Sedimentation rate | Budding pattern | Diploid Colony appearance | Sedimentation rate | Budding pattern |
|------------|---------------------------|--------------------|-----------------|----------------------------|--------------------|-----------------|
| BUD4 CBK1  | Smooth                    | Slow               | Axial           | Smooth                     | Slow               | Bipolar         |
| bud4 CBK1  | Smooth                    | Slow               | Bipolar         | Smooth                     | Intermediate      | Bipolar         |
| BUD4 cbk1  | Smooth                    | Intermediate       | Axial           | Rough                      | Rapid              | Random          |
| bud4 cbk1  | Rough                     | Rapid              | Random          | Rough                      | Rapid              | Random          |

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single mutant, consistent with the cbk1 mutation affecting Ace2 activity. We see a strong additive effect on sedimentation in the ace2 swi5 double mutant, compared to that in the single mutants, consistent with the fact that transcriptional activation of some cell separation and other genes requires either Ace2 or Swi5.

To investigate the additive effect of cbk1 and swi5 mutations on sedimentation, we examined the effect of these mutations, along with ace2 and bud4, on transcription of known Ace2 target genes. RNA was prepared from 16 isogenic strains differing at the ACE2, BUD4, CBK1, and SWI5 loci, and S1 protection assays were performed to measure mRNA levels for EGT2, SCW11, DSE2, and CTS1 (Fig. 5) (data not shown). First, we can conclude that a bud4 mutation has no effect on expression of these genes. Additionally, Western blots probed with antibody against Ace2 show the same protein level in BUD4 or bud4 extracts, indicating that there is also no post-transcriptional effect of Bud4 on Ace2 (data not shown). Second, the pattern of regulation is different for EGT2 from those for SCW11, DSE2, and CTS1. EGT2 expression is only modestly reduced when there is a single mutation in ACE2, SWI5, or CBK1, but expression is reduced in the ace2 swi5 or the cbk1 swi5 double mutant. Importantly, EGT2 is still expressed in the ace2 cbk1 double mutant, consistent with Ace2 and Cbk1 functioning in the same pathway, separate from the Swi5 pathway. We conclude that EGT2 expression can be activated by either of the two related DNA-binding proteins, Ace2 or Swi5. In contrast, expression of SCW11, DSE2, and CTS1 is unaffected.

![Figure 4](https://example.com/figure4.png)

**FIG. 4.** bud4 and ace2 affect sedimentation rates. (A) Cells were grown in liquid YEPD medium, and the sedimentation rate at 1 × g was determined as described in Materials and Methods. Strains DY5796 (bud4 ace2 cbk1), DY5794 (bud4 cbk1), DY3923 (bud4 ace2), DY9188 (BUD4 ace2 cbk1), DY9190 (BUD4 ace2), DY9195 (BUD4 cbk1), DY2396 (bud4), and DY9197 (BUD4) were used. (B) From the data in panel A, the sedimentation half-time is the time for the optical density (O.D.) to fall to one-half of the difference between the initial and the final lower plateau values.

| Mutant | Colony appearance | Sedimentation rate | Budding pattern | Colony appearance | Sedimentation rate | Budding pattern |
|--------|-------------------|--------------------|-----------------|-------------------|--------------------|-----------------|
| BUD4 ACE2 | Smooth | Slow | Axial | Smooth | Slow | Bipolar |
| bud4 ACE2 | Smooth | Slow | Bipolar | Smooth | Intermediate | Bipolar |
| BUD4 ace2 | Smooth | Intermediate | Axial | Rough | Rapid | Bipolar |
| bud4 ace2 | Rough | Rapid | Bipolar | Rough | Rapid | Bipolar |

**TABLE 6.** Budding, sedimentation, and colony morphology in ace2 mutants
by an swi5 mutation and these genes require both ACE2 and CBK1 for full expression.

**DISCUSSION**

A cbk1 mutation sharply reduces expression of genes activated by the Ace2 transcription factor, and both cbk1 and ace2 mutants display an altered colony phenotype. A multicopy ACE2 plasmid suppresses both the transcriptional defects and the altered colony morphology of the cbk1 mutant and other mutants in the RAM pathway, demonstrating that Ace2 is downstream of Cbk1. Many of the Ace2 target genes, such as CTS1, DSE2, and SCW11, facilitate cytokinesis, and ace2 and cbk1 mutants both display defects in cell separation. In addition to their common role in transcriptional activation, we show that Ace2 and Cbk1 have distinct functions in cellular morphology and cell division. The W303 strain background has a mutation in the BUD4 gene, required for axial budding in haploid cells. Combining a bud4 mutation with either ace2 or cbk1 has an additive effect, seen as a rough colony morphology. The random budding pattern resulting from overall loss of polarity in cbk1 mutants is evident only due to genetic interaction between cbk1 and bud4. In addition, we also used a sedimentation assay to show that the bud4 cbk1 double mutant has quantitatively additive effects on multicellular morphology.

A mutation in SWI5, an Ace2 paralog, has additive effects when combined with cbk1 on both sedimentation rate and EGT2 expression, suggesting that Cbk1 has functions distinct from that of Ace2. In summary, we conclude that Ace2 and Cbk1 have independent as well as overlapping functions.

The RAM network comprises CBK1, HYM1, KIC1, MOB2, and TAO3. Mutations in any of these genes have similar phenotypes, including decreased CTS1 expression, failure in cell separation, altered colony morphology, and defects in polarized cell growth (3, 25, 28). Additionally, RAM gene products show many mutual physical and functional interactions (16, 19, 25, 28). Similar effects on CTS1 expression, cell separation, and colony morphology are seen in an ace2 mutant. We show that multicopy plasmids with ACE2 can suppress the effects of cbk1, hym1, kic1, and mob2 RAM network mutations with respect to the rough colony morphology and failure of cell separation after mitosis, as well as the defect in transcriptional activation of genes necessary for that cell separation. This multicopy suppression shows that Ace2 is functionally downstream of the RAM pathway components, consistent with other studies (3, 25, 28, 40). Strains lacking Cbk1 show a loss of expression in CTS1 and SCW11 to an extent that is similar to, but slightly less severe than, that in ace2 mutants. This suggests that Ace2 may have some residual function in RAM network-mutated strains, and elevating levels of Ace2 with a multicopy plasmid may provide enough active Ace2 to restore some function. Finally, polarized cell growth is normal in ace2 mutants, demonstrating that the RAM network has functions independent of Ace2.

**W303 strains have a bud4 mutation.** Although ace2 gene disruptions cause a failure of cell separation in all genetic backgrounds tested, there is a difference in colony morphology between strain backgrounds. S288c ace2 strains have a smooth, wild-type colony surface, while W303 ace2 strains show the same altered colony morphology seen in W303 strains with RAM network mutations. This difference can be explained by the fact that W303 strains contain a bud4 mutation. While

![FIG. 5. cbk1 and swi5 mutations have an additive effect on EGT2 expression.](image)

(A) S1 nuclease protection assays were performed using probes specific for EGT2, SCW11, and CMD1 internal control. RNAs were prepared from the following strains: DY2396 (bud4), DY9197 (BUD4), DY3923 (bud4 ace2), DY9190 (BUD4 ace2), DY3925 (bud4 swi5), DY9194 (BUD4 swi5), DY4653 (bud4 ace2 swi5), DY9186 (BUD4 ace2 swi5), DY5794 (bud4 cbk1), DY9195 (BUD4 cbk1), DY5796 (bud4 ace2 cbk1), DY9188 (BUD4 ace2 cbk1), DY5798 (bud4 swi5 cbk1), DY9192 (BUD4 swi5 cbk1), DY5800 (bud4 ace2 swi5 cbk1), and DY9184 (BUD4 ace2 swi5 cbk1). (B) mRNA levels were determined by PhosphorImager for EGT2 and SCW11 (from the data in panel A) and for DSE2 and CTS1 (data not shown). mRNA values are normalized to the wild-type strain. For this experiment, results are only shown for bud4 strains, but similar results were seen with BUD4 strains. (C) EGT2 activation requires either Swi5 or both Ace2 and Cbk1, while SCW11, DSE2, and CTS1 expression requires both Ace2 and Cbk1 but not Swi5.
S288c strains display axial budding in haploids and bipolar budding in diploids. W303 cells show bipolar bud site selection in haploids as well as diploids. Low-copy plasmids bearing BUD4 derived from S288c genomic DNA convert the W303 haploid pattern from bipolar to axial, and genetic crosses with a marked BUD4+ allele show complete 2:2 segregation with respect to the W303 bipolar haploid phenotype. W303 ace2 strains converted to BUD4+ now show a smooth colony morphology similar to S288c ace2 cells. Thus, combining a mutation that reduces cell separation, such as ace2 or cbk1, with a mutation that causes bipolar budding, such as bud4 or adl1, causes a change in colony morphology. Importantly, ace2/ace2 a/a diploids show rough colonies in either strain background, even though all ace2 strains still show the same cell separation defect regardless of cell type or BUD4 dosage. This indicates that the rough colony appearance is caused by the combination of two effects: a bipolar budding pattern and cells remaining attached postmitotically. We suggest that this combination results in more extended assemblages of cells than for unseparated cells budding axially, thus affecting the colony morphology.

Mutations in genes of the RAM network are lethal in certain genetic backgrounds, including the S288c-derived strain used by the Yeast Genome Deletion Consortium (41). However, these gene disruptions are viable in the W303 background. This strain-dependent difference in lethality is due to polymorphism at the SSD1 locus, with S288c possessing the dominant SSD1-v allele and W303 bearing the recessive ssd1-d allele encoding a truncated protein (20). Additionally, S288c strains tolerate RAM network gene disruptions if an ssd1 mutation is introduced (16, 20). Thus, most studies of RAM network genes have necessarily been carried out in the ssd1 mutant W303 background. Several lines of evidence point to a physical interaction of Ssd1 with members of the RAM network (19, 28), and the molecular role of Ssd1 is hypothesized to be as a regulator of protein dephosphorylation through protein phosphatases (34).

The fact that viable RAM network mutations were isolated primarily in W303, which also bore a bud4 mutation, has made analysis of the effects of RAM network genes on bud site selection, cell separation, and colony morphology problematic. Microscopic observation is especially challenging, considering the large clusters of cells due to defects in cell separation. We have constructed W303 strains with restored BUD4 function such that both diploids and haploids now display the normal bipolar and axial patterns, respectively. W303 haploid BUD4+ strains with a cbk1 mutation are still fully viable and show completely normal nonrandom axial budding. While BUD4+ CBK1+ diploids show the normal bipolar budding pattern, BUD4+ cbk1− diploids bud randomly. This is reflected in colony morphology and sedimentation rate due to the cell separation defect and occurs because the BUD4+ (axial) function is dispensable in diploids. Thus the cytoskeletal cell polarity cues programmed by Cbk1 and other RAM factors are necessary only for the bipolar pattern, in which budding machinery is deposited at alternating, antipolar termini of the long axis of the cell in each successive cycle, but not for the axial pattern in which localized tags must only be retained proximal to the site of previous budding (8). Without the polarity axis dependent on Cbk1, undirected budding machinery randomly locates to medial sites in the absence of the axial program.

**Budding pattern and cell separation effect colony morphology and sedimentation.** Mutations in genes of the RAM network, including CBK1, MOB2, KIC1, and HVM1, as well as a mutation in the downstream transcription factor ACE2, cause a rough colony morphology in the W303 genetic background with a bud4 mutation. This defect consists of scalloped rather than smooth colony margins and a surface with many branched channels and invaginations resulting in an eroded appearance. At the microscopic level, these rough colony mutants exhibit incomplete scission of the mother-daughter cell wall septum after cytokinesis. Since all other aspects of the cell cycle including cytokinesis continue in an apparently unaffected way, this loss of cell separation leads to the formation of multicellular aggregates in liquid medium, called clusters or clumps. The failure of cell separation is distinct from flocculent phenotypes caused by aggregation due to expression of cell surface agglutinins (36). Unlike flocculent strains, the clustered phenotype of RAM network and ace2 mutants cannot be restored to unicellularity by chelation of Ca2+ from the medium and thus is a stable morphogenetic state of the cell wall. Importantly, a multicopy plasmid with ACE2 suppresses the unseparated state of RAM mutants, demonstrating that the decreased expression of ACE2 target genes is responsible for the phenotype.

The sedimentation rate assay was developed as a way to quantify phenotypes which were normally observed in only a qualitative way as colony morphology defects. Several factors probably contribute to the end measurement. The obvious one is the average size of cell clusters brought about by incomplete scission after cytokinesis. Such clumping will lead to a lower aggregate frontal surface area for a given number of cells and thus less overall resistance to movement through a viscous fluid. Additionally, changes in the overall compactness or clusters of a given size would affect the sedimentation rate. For instance, bipolar bud site selection should result in a more extended filament-like structure than an axial program, in which daughter buds are formed in close apposition to previous daughters. Random bud site selection, in which a large number of medial buds form, might result in an intermediate density for a clump of a given cell number. In contrast, axially budding clumpy cells would exhibit the densest clusters. However, our observations indicate that clumpy cells with a bipolar or random pattern actually sediment more rapidly than those with an axial pattern (Fig. 4), and yet there is no evidence for differences in cell number per cluster due to bud site selection pattern differences (Fig. 2) or in expression of cell separation genes in response to BUD4 genotype (Fig. 5). To explain the more rapid sedimentation rates of bipolar clumpy cells relative to axial clumpy cells, we suggest that the bipolar clusters have longer, narrower, and more rod-like clusters, leading to more rapid sedimentation. In contrast, axially budded clusters, although not larger in any one dimension than bipolar clusters of the same cell number, are more uniform in all dimensions and are unable to adopt any orientation with a low frontal surface area that would speed sedimentation.

One phenotype of cbk1 mutants is that the cells themselves are rounder, having a smaller length-to-width ratio than wild-type cells (28). Rounder cells may form a more compact clump...
than the typical ellipsoid cells, since any resulting chain-like structures of a given number of cells would be shorter in contour length and thus manifested in changes in the sedimentation assay. Coupled with microscopic observations of budding patterns and cell shape, the sedimentation assay allows measurements of relatively subtle differences in phenotype in a quantifiable way.

Additive genetic effects. Strong additive effects are seen when a bud4 mutation is combined with either ace2 or cbk1, resulting in altered colony morphology and more rapid sedimentation. Slight additive effects are also seen in the sedimentation assay when ace2 and cbk1 mutations are combined (Fig. 4). One interpretation of this ace2 cbk1 additivity is that the Cbk1 kinase also directs cell separation, independently of Ace2, by modifying other transcriptional activators for cell separation enzymes or specific cell wall components. Previous quantification of the extent of cell separation defects indicated a small increase in the average clump size in ace2/ace2 diploids relative to cbk1/cbk1 diploids, irrespective of cell shape or budding pattern differences (3). Additionally, although expression of Ace2 target genes is more reduced in an ace2 mutant than in a cbk1 mutant, ace2 is epistatic to cbk1, since there is not an additive effect in the ace2 cbk1 double mutant (Fig. 5) (data not shown). Thus, the similar sedimentation rates in ace2 bud4 and cbk1 bud4 strains could be a product of the slightly reduced clumpiness of cbk1 cells in combination with the randomised budding pattern. The round cell phenotype seen in cbk1 mutants could also contribute to a more compact average cluster with reduced frontal area and less resistance to sedimentation relative to the total cell number. While cbk1 BUD4 haploid cells bud in the wild-type axial manner, these cells still possess a shortened long axis due to overall polarity defects.

This may also lead to a more compact cluster due to shorter chains of unseparated cells, which is again quantitatively indistinguishable from ellipsoidal ace2 strains in sedimentation measurements, due to their slightly larger clump size. The slight additive effect in ace2 cbk1 strains could be due to the slightly increased clump size caused by ace2, coupled with the round cell phenotype and/or budding pattern change caused by the cbk1 mutation, depending on the allele of BUD4 present.

Most of the target genes identified to date that require Ace2 for activation are cell wall-degrading enzymes or putative cell wall components (11, 12, 14). This is consistent with the incomplete cell wall scission phenotypes of ace2 mutants, as well as for the phenotypes of mutations in CBK1, necessary for full Ace2 activity. However, some genes which are directly activated by Ace2 can also be activated by Swi5, a transcription factor paralogous to Ace2 (14, 24). ACE2 gene itself is expressed in the late G2 and early M phase of the cell cycle (12), as are other genes required for cytokinesis, which are part of the CLB2 cell cycle group (10, 33). This timing leads to protein production at the time needed for cell division. BUD4 is part of this CLB2 group, and temporally the Bud4 protein localizes to the bud neck region similarly to Cbk1, along with many other morphogenetic factors (30). BUD9 encodes one of the localized tags for the bipolar pattern (31), and bud9 mutant diploids adopt a unipolar budding pattern (43). BUD9 expression is reduced in a cbk1 mutant (25) and also in an ace2 mutant (W. P. Voth., Y. Yu, and D. J. Stillman, unpublished observations). Most laboratory strains of yeast grow in a predominantly unicellular manner, but yeasts freshly isolated from their wild habitats are naturally clumpy (42). This difference has been attributed to the fact that laboratory strains of yeasts are mutated for the AMN1 gene, while the wild isolates bear a functional version of this gene (42). Genetically, Amn1 behaves as a repressor of Ace2 function (42). Further work is needed to understand this relationship, as AMN1 is reported to be expressed only in daughter cells (11), but AMN1 expression can be activated by either Swi5 or Ace2 (14). Thus, it is tempting to speculate that facultative modulation of Cbk1 and other RAM gene activity could cause alterations in bud site selection, cell polarity, and cell separation, which could be a normal part of coordinated regulation of morphogenetic transitions due to the influence of environmental factors.

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