The *Saccharomyces cerevisiae* 14-3-3 proteins are required for the G1/S transition, actin cytoskeleton organization and cell wall integrity

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14-3-3 proteins are highly conserved polypeptides participating in many biological processes by binding phosphorylated target proteins. In *S. cerevisiae*, two functionally redundant 14-3-3 isoforms are encoded by the *BMH1* and *BMH2* genes, whose concomitant deletion is lethal. To gain insights into the essential function(s) shared by these proteins, we searched for high dosage suppressors of the growth defects of temperature-sensitive *bmh* mutants. Both the protein kinase C1 (Pkc1) and its upstream regulators Wsc2 and Mid2 were found as high dosage suppressors of *bmh* mutants’ temperature-sensitivity, indicating a functional interaction between 14-3-3 and Pkc1. Consistent with a role of 14-3-3 proteins in Pkc1-dependent cellular processes, *bmh* mutants turned out to be severely impaired at restrictive temperature in initiation of DNA replication, polarization of the actin cytoskeleton and budding, as well as in cell wall integrity. Since Pkc1 acts in concert with the Swi4-Swi6 (SBF) transcriptional activator to control all these processes, the defective G1/S transition of *bmh* might be linked to impaired SBF activity. Consistently, the levels of the G1 cyclin *CLN2* transcripts, which are positively regulated by SBF, were dramatically reduced in *bmh* mutants. Remarkably, budding and DNA replication defects of *bmh* mutants were suppressed by *CLN2* expression from an SBF-independent promoter, suggesting that 14-3-3 proteins could contribute to regulating the late G1 transcriptional program.
INTRODUCTION

The 14-3-3 proteins are a large family of highly conserved, ubiquitously expressed acidic polypeptides of 28-33 kDa that are found in all eukaryotes. There are at least seven isoforms in mammals and up to 15 isoforms in plants, while two isoforms have been identified in yeast, *Drosophila melanogaster* and *Caernohabditis elegans* (reviewed in Hermeking, 2003; Dougherty and Morrison, 2004). They form homo- and heterodimers able to bind protein ligands that are usually phosphorylated on serine/threonine residues of consensus binding motifs (Jones et al., 1995; Muslin et al., 1996; Yaffe et al., 1997; Chaudhri et al., 2003). By inducing conformational changes or steric hindrance in protein ligands, 14-3-3 proteins can activate/repress their enzymatic activity, prevent degradation, modulate localization and/or facilitate/inhibit protein modifications and interactions (reviewed in Hermeking, 2003; Dougherty and Morrison, 2004). Targets of 14-3-3 family members are found in all subcellular compartments and include transcription factors, biosynthetic enzymes, cytoskeletal proteins, signaling molecules, checkpoint and apoptosis factors and tumor suppressors. This plethora of interacting proteins allows 14-3-3 to play important roles in a wide range of regulatory processes such as cell cycle control, mitogenic signal transduction and apoptotic cell death, and to be implicated in cancerogenesis and some human diseases (reviewed in Dougherty and Morrison, 2004). However, since multiple 14-3-3 isoforms are present in mammals and 14-3-3 proteins have several binding targets, the mechanisms underlying 14-3-3 functions are not fully understood.

The two *S. cerevisiae* members of the 14-3-3 protein family, which share 93% amino acid identity, are encoded by the *BMH1* and *BMH2* genes. While single *bmh1Δ* and *bmh2Δ* mutants do not show detectable growth defects compared to wild type, the *bmh1 bmh2* double
disruption is lethal in most laboratory strains (van Heusden et al., 1992, 1995; Gelperin et al., 1995; Roberts et al., 1997).

Although their essential functions are not well understood, budding yeast Bmh proteins appear to be involved in many cellular processes. For example, they modulate the activity of some transcription factors. In fact, loss of function mutations in the SIN4 gene, encoding a global transcriptional regulator, and in the RTG3 gene, encoding a basic helix-loop-helix transcription factor, suppress the temperature sensitive phenotype of a bmh1 bmh2 mutant (van Heusden and Steensma, 2001). Moreover, Bmh1 physically interacts with phosphorylated Rtg3, suggesting that 14-3-3 proteins inhibit Rtg3 transcriptional activation function by binding its phosphorylated form (van Heusden and Steensma, 2001). Finally, Bmh1 physically interacts with Msn2 and Msn4, two transcription factors required to activate a large number of stress-related genes, and retains their phosphorylated forms in the cytoplasm (Beck and Hall, 1999).

Also vesicular transport and cortical actin network organization likely involve 14-3-3 proteins (Gelperin et al., 1995; Roth et al., 1999). In fact, S. cerevisiae cells overproducing the carboxy-terminal region of Bmh2 fail to polarize vesicular transport and show a disrupted actin cytoskeleton (Roth et al., 1999). Moreover, 14-3-3 proteins interact with many proteins involved in cytoskeletal regulation in both yeast and mammals (Jin et al., 2004). In particular, two-hybrid interactions have been reported for Bmh2 with Msb3 (Mayordomo and Sanz, 2002), which is involved in actin cytoskeleton organization (Bach et al., 2000; Bi et al., 2000), and with Gic2 (Mayordomo and Sanz, 2002), which is required together with Gic1 for cytoskeletal polarization during bud emergence (Brown et al., 1997; Chen et al., 1997). Both Bmh1 and Bmh2 interact also with the p21-activated kinase (PAK) Ste20, and this interaction appears to be specifically required for Ras/MAPK cascade signaling during pseudohyphal development (Roberts et al., 1997). Finally, mammalian 14-3-3 proteins regulate actin
dynamics by stabilizing phosphorylated cofilin, a family of proteins essential for high rates of actin filament turnover through regulation of the actin polymerization/depolymerization cycles (Gohla and Bokoch, 2002).

By interacting with various regulatory proteins, 14-3-3 proteins participate in diverse signal transduction pathways. In fact, hyperactivation of the Ras/cAMP-dependent protein kinase A (PKA) pathway by overproducing Tpk1, the catalytic subunit of PKA, suppresses cell lethality caused by Bmh depletion (Gelperin et al., 1995). Consistent with a link between 14-3-3 and PKA, Bmh proteins are dispensable for yeast cell viability in Σ1278b background (Roberts et al., 1997), where the Ras/cAMP signaling pathway is hyperactivated (Stanhill et al., 1999). However, $bmh1\Delta$ $bmh2\Delta$ Σ1278b derivative cells exhibit osmo-remediable temperature sensitivity and sensitivity to high osmolarity (Roberts et al., 1997), suggesting that some functions of Bmh proteins are still required at 37°C even in this background. Moreover, 14-3-3 proteins have been implicated also in Ras/MAPK cascade signaling in vertebrates (Fantl et al., 1994; Li et al., 1995) and during pseudohyphal development in $S$. $cerevisiae$ (Roberts et al., 1997). Finally, vertebrate 14-3-3 proteins were shown to inhibit or activate Protein Kinase C (PKC), which is involved in many signaling processes (Toker et al., 1990; Isobe et al., 1992; Tanji et al., 1994), and to stimulate the interaction between PKC and the mitogen-stimulated Raf1 kinase that controls cell growth (Van Der Hoeven et al., 2000).

In a previous study we isolated 4 $bmh1$ alleles, whose presence in the cell as the sole 14-3-3 source caused temperature-sensitive growth (Lottersberger et al., 2003). In order to provide new insights into the essential functions of the $S$. $cerevisiae$ 14-3-3 proteins, we have carried out a detailed phenotypic characterization of these mutants and searched for high dosage suppressors of their temperature sensitivity. We provide evidence that hyperactivation of the protein kinase C1 (Pkc1)-dependent pathways suppresses the growth defects of these $bmh$ mutants, suggesting that 14-3-3 proteins functionally interact with Pkc1. Accordingly, $bmh$
mutants are impaired in Pkc1-regulated processes at the G1/S transition, such as budding, initiation of DNA replication, actin cytoskeleton polarization and cell wall integrity. Our data suggest that both the temperature-sensitivity and the G1/S transition defects of our \textit{bmh} mutants might be ascribed to an impaired activity of the SBF transcription factor, which is known to act in concert with Pkc1 to control all the above processes.
RESULTS

High dosage of *MID2, WSC2, PKC1* and *GIC1* suppresses *bmh* mutants’ temperature-sensitivity: We previously generated *bmh1-103*, *bmh1-221*, *bmh1-266* and *bmh1-342* yeast temperature-sensitive mutants (Lottersberger et al., 2003 and Fig. 1). In each of our mutant strains, the *bmh1* mutant allele was the sole 14-3-3 source, since all of them carried a *BMH2* deletion, which did not cause by itself any of the phenotypes described during this study (data not shown). Importantly, overproduction of the catalytic subunit of Protein Kinase A (PKA), Tpk1, was unable to suppress the growth defects of these *bmh* mutants at 37°C (data not shown), in contrast to its ability to suppress the cell lethality caused by Bmh depletion at 25°C (Gelperin et al., 1995), indicating that our *bmh* mutants are not solely impaired in the activation of the Ras/cAMP-dependent PKA pathway.

In order to identify cellular partners for 14-3-3 proteins, we searched for high dosage suppressors of the temperature-sensitivity of the *bmh1-266* mutant. To this purpose, *bmh1-266* cells were transformed with a *S. cerevisiae* genomic library constructed in the YEp24 2µ vector (Carlson and Botstein, 1982), and 40,000 Ura<sup>+</sup> transformants were screened for the ability to form colonies on YEPD plates at 37°C (see Materials and Methods). Besides 190 *BMH2*- and 20 *BMH1*-bearing plasmids, the screen allowed the recovery of ten plasmids carrying different ORFs. Subcloning of the several ORFs carried by three of these plasmids in the YEplac195 2µ vector revealed that high copy number of the *GIC1, WSC2* or *MID2* genes could partially suppressed the temperature-sensitivity of *bmh1-266*, *bmh1-103* and *bmh1-221* cells (Fig. 1A). Unfortunately, we were unable to assess this suppressing ability in *bmh1-342* cells, due to their high frequency of 2µ plasmid loss.

While the *GIC1* and *GIC2* genes encode two homologous proteins required for actin polarization and bud formation (Brown et al., 1997; Chen et al., 1997), *WSC2* and *MID2* gene
products are transmembrane cell surface sensors. They have been proposed to perform partially overlapping functions in cell wall remodeling during vegetative growth and under stress conditions (Verna et al., 1997; Marcoux et al., 1998; Rajavel et al. 1999; Ketela et al., 1999; Philip and Levin, 2001), and to detect and transmit the cell wall status to the Protein Kinase C1 (Pkc1). The latter is involved in a multiplicity of pathways, including those related to bud emergence, cell wall integrity, and organization of the actin cytoskeleton in response to heat shock, pheromone, low osmolarity, nutrient starvation and cell cycle progression (Heinisch et al., 1999). This prompted us to examine whether an excess of Pkc1 could also suppress the temperature-sensitivity of our bmh mutants. Indeed, high copy number of PKC1 ameliorated the ability of bmh1-103, bmh1-221 and bmh1-266 cells to form colonies at 37°C (Fig. 1B), indicating that hyperactivation of a Pkc1-dependent cascade may compensate for defects in 14-3-3 proteins.

Pkc1 is believed to possess multiple functions (Lee and Levin, 1992; Verna et al., 1997; Delley and Hall, 1999; Ketela et al., 1999; Andrews and Stark, 2000; Zanelli and Valentini, 2005), only one of which is to regulate the activity of the MAP kinase cascade that ultimately regulates cell wall integrity, bud emergence, response to hypotonic shock and actin reorganization (reviewed in Levin and Errede, 1995). Since high levels of Wsc2, Mid2 or Pkc1, which are predicted to lead to increased signaling through Pkc1, suppressed the temperature-sensitive growth defects of our bmh mutants, we asked whether the latter were defective in activating the Pkc1-dependent MAPK cascade. To this end, we monitored Mpk1 phosphorylation, which is an established marker for activation of the Pkc1-MAPK pathway (Lee et al., 1993; Zarzov et al., 1996; de Nobel et al., 2000). As shown in figure 1C, the amount of phosphorylated Mpk1 in bmh mutants was higher than in wild type already at the permissive temperature. Moreover, heat shock, which is known to induce a rapid transient depolarization of the actin cytoskeleton and cell wall weakening (Delley and Hall, 1999),
increased the amount of Mpk1 phosphorylated forms in both wild type and bmh mutants after shift to 37°C for 30 minutes (Fig. 1C). However, phosphorylated Mpk1 level was significantly decreased in wild type cells after 3 hours at 37°C, due to adaptation to the high temperature, whereas it remained high in bmh cells under the same conditions (Fig. 1C). Thus, the growth defects of our bmh mutants are unlikely due to faulty MAPK kinase signaling, since the latter appears to be instead hyperactivated in these mutants. Rather, these mutants could be deficient in some Pkc1-regulated pathway that parallels the one involving MAP kinases. In this view, the MAPK cascade could be hyperactivated in bmh mutants as a compensatory mechanism to maintain cell viability in the absence of 14-3-3 function. Accordingly, we found that MPK1 deletion was lethal for the bmh1-103, bmh1-221, bmh1-266 and bmh1-342 mutants (data not shown). Therefore, an excess of Wsc2, Mid2 or Pkc1 may suppress temperature-sensitivity of bmh mutants by acting through a Pkc1-dependent MAPK-independent pathway.

**Temperature-sensitive bmh mutants are defective in the G1/S transition and actin polarization:**

Since enhanced Pkc1 signaling contributes to cell viability in the absence of 14-3-3 function, we asked whether bmh mutants were impaired in Pkc1-regulated cellular processes, such as bud formation, actin reorganization, cell wall remodeling, and cell cycle progression (Heinisch et al., 1999). To investigated whether defects in 14-3-3 functions may affect bud formation at the G1/S transition, exponentially growing cultures of wild type, bmh1-103, bmh1-221, bmh1-266 and bmh1-342 cells were arrested in G1 with α-factor at 25°C and then released into fresh medium at 37°C. As shown in figure 2A, most of bmh1-103, bmh1-221, bmh1-266 and bmh1-342 cells were still largely unbudded after 1 hour at 37°C, when bud emergence had already occurred in 90% of similarly treated wild type cells. After 3 hours at
37°C, most of bmh1-221 and bmh1-342 mutant cells were still unbudded, while approximately 50% of bmh1-103 and bmh1-266 cells managed to bud (Fig. 2A and B). However, their buds appeared mostly mis-shaped and kept elongating upon further incubation at 37°C (Fig. 2A and B). Moreover, some elongated budded cells appeared also in bmh1-221 and bmh1-342 mutants at later time points. Thus, bmh mutants might be impaired in the switch between apical to isotropic growth.

Delayed bud formation in bmh mutants paralleled with defects in DNA synthesis initiation. In fact, all bmh mutants severely delayed initiation of DNA replication, although to different extents, after shift to 37°C (Fig. 2C). While wild type cells initiated DNA replication 45-60 minutes after release at 37°C from the G1 block, the onset of DNA replication took place in bmh1-221 and bmh1-342 cells only around 150 minutes after release under the same conditions (Fig. 2C). In addition, a major fraction of cells in both mutants was unable to replicate DNA by 240 minutes (Fig. 2C). The bmh1-103 and bmh1-266 cells started DNA replication about 120 and 75 minutes, respectively, after release at 37°C and again only a fraction of these mutant cells managed to complete DNA replication by 240 minutes at 37°C (Fig. 2C). As shown in figure 2D, initiation of DNA replication upon release at 25°C of the same G1-arrested cell cultures was delayed by 15-30 minutes in bmh mutant cell cultures compared to wild type. Altogether, these data indicate that 14-3-3 proteins are required for a timely G1/S transition.

Both bud emergence and its subsequent surface growth require the polarization of the actin cytoskeleton, such that cortical patches and actin cables converge at the bud site (reviewed in Pruyne and Bretscher, 2000). Since 14-3-3 proteins have been previously linked to actin cytoskeleton organization (Gelperin et al., 1995; Roth et al., 1999), impaired bud formation in the above bmh mutants might be related to defects in this process. To address this issue, we analyzed actin polarization upon Alexa-Fluor 546 phalloidin staining of wild type and bmh
mutant cells that were arrested in G1 by α factor and then released at 37°C for 1 hour. As shown in figure 3, actin cortical patches, which normally clustered at the bud tips of wild type cells, were completely missing in bmh1-221 and bmh1-342 cells and appeared only in a small fraction of bmh1-103 and bmh1-266 cells. Therefore, organization of the actin cytoskeleton at the future bud emergence sites is perturbed in bmh mutants, thus affecting bud formation at the G1/S transition.

We then combined the different bmh1 alleles, together with the BMH2 deletion, with the temperature-sensitive cdc42-1 or cdc24-1 alleles, altering the essential Cdc42 GTPase and its guanine-nucleotide-exchange factor (GEF) Cdc24 (Adams et al., 1990; Johnson and Pringle, 1990; Van Aelst and D’Souza-Schorey, 1997), which are both required to establish actin cytoskeleton polarity (reviewed in Pruyne and Bretscher, 2000). As shown in figure 4, the ability to form colonies at 32°C of all bmh1 bmh2Δ cdc24-1 and bmh1 bmh2Δ cdc42-1 triple mutants was severely impaired compared to that of the parental mutants. This synthetic effect between bmh and cdc24 or cdc42 mutant alleles further support a role for budding yeast 14-3-3 proteins in actin polarization and bud formation.

**Bmh defects cause sensitivity to cell wall stress and their effects on the G1/S transition can be relieved by osmotic support:**

Pkc1 controls cell wall metabolism by regulating β-glucan synthesis at the site of wall remodeling, as well as expression of cell wall biosynthesis genes necessary for maintaining cellular integrity both during bud formation and in response to heat shock, pheromone and nutrient starvation (reviewed in Levin, 2005). We therefore asked whether defects in 14-3-3 functions might result in impaired cell wall integrity, by analyzing the ability of bmh mutants to grow at permissive temperature in the presence of compounds such as the chitin antagonist calcofluor white and SDS, which have both proved to be powerful tools for revealing yeast
cell wall defects (Ram et al., 1994). As shown in figure 5A, bmh1-103, bmh1-221, bmh1-266 and bmh1-342 cells were unable to grow on YEPD plates supplemented with 0.01% SDS, which did not affect wild type cell growth. Moreover, growth of all bmh mutants on YEPD was compromised, although to different extents, by addition of 0.01 mg/ml calcofluor white (Fig. 5B). Finally, microscopic examination of the bmh mutant cell cultures revealed accumulation of cell debris at 37°C (data not shown), suggesting that cell lysis frequently occurred. Thus, 14-3-3 proteins appear to be required for a stable cell wall structure.

We therefore examined whether osmotic stabilization of the medium might relieve the temperature sensitivity and the G1/S transition defects of our bmh mutants. As shown in figure 5C, addition of the osmotic stabilizer sorbitol restored the ability of bmh1-103, bmh1-221, bmh1-266 and bmh1-342 cells to grow on YEPD plates at 37°C. Moreover, the presence of sorbitol in the medium largely rescued the defects in bud emergence (Fig. 5D) and initiation of DNA replication (Fig. 5E) displayed by bmh mutants upon G1 release at 37°C. Thus, slow-growth and delayed G1/S transition that are caused by defective 14-3-3 proteins are osmo-remediable, consistent with a primary defect of bmh mutants in cell wall biogenesis.

**High dosage of Wsc2, Mid2 or Pkc1 can partially suppress the G1/S transition defects of bmh mutants:**

Since enhanced Pkc1-dependent signaling by high copy number WSC2, MID2 or PKC1 suppressed the temperature sensitivity of bmh mutants, we asked whether it suppressed also their G1/S transition defects. As shown in figure 6A, bmh1-103, bmh1-221 and bmh1-266 cells carrying WSC2, MID2 or PKC1 on a 2µ plasmid and released from α-factor at 37°C underwent budding more efficiently than the same mutant cells carrying the empty vector. Moreover, an excess of Wsc2, Mid2 or Pkc1 attenuated the abnormal bud morphology of bmh1-103 and bmh1-266 cells after three hours at 37°C (Fig. 6A). Similarly, bud emergence
took place more efficiently in *bmh1-221*, *bmh1-103* and *bmh1-266* cells containing an excess of Gic1, although this, as expected, caused bud elongation even in wild type cells due to sustained polarized growth (Fig. 6A) (Brown et al., 1997; Chen et al., 1997).

Suppression of *bmh* defects in DNA replication initiation were also apparent upon *WSC2*, *MID2* or *PKC1* increased dosage. In fact, *bmh1-103*, *bmh1-221* and *bmh1-266* cells carrying high copy number *WSC2*, *MID2*- or *PKC1*-bearing plasmids initiated DNA replication at 37°C earlier and more efficiently than the same mutants with the empty vector (Fig. 6B). Thus, an excess of Wsc2, Mid2 or Pkc1 can partially suppress the G1/S transition defects of *bmh* mutants.

**Low G1 cyclin-Cdk1 levels may account for the G1/S transition defects of *bmh* mutants:**
During the G1/S transition, Pkc1 acts in concert with the SBF transcription factor to control the actin cytoskeleton, cell cycle progression and transcription of cell wall biosynthesis genes (reviewed in Levin, 2005). SBF is composed of the Swi6 and Swi4 subunits, and is responsible for transcriptional activation of the *CLN1* and *CLN2* cyclin genes, whose products associate with the Cyclin-dependent kinase 1 (Cdk1) to promote bud morphogenesis and DNA replication (reviewed in Levin et al., 1995; Nasmyth, 1996). Since Pkc1 hyperactivation was shown to partially compensate for the lack of SBF activity (Gray et al., 1997; Igual et al., 1996), we asked whether the G1/S transition defects of *bmh* mutants might be related to impaired formation of G1 Cyclin/Cdk1 complexes. We therefore measured the levels of *CLN2* mRNA in the *bmh1-221* and *bmh1-342* mutants, which showed the most severe G1/S transition defects at 37°C compared to the other *bmh* mutants (Fig. 2).

Exponentially growing cultures of wild type, *bmh1-221* and *bmh1-342* cells were arrested in G1 with α-factor and released into the cell cycle at 37°C. At different time points after release total RNA was analysed by Northern blot with a *CLN2* probe. As shown in figure 7A, *CLN2*
mRNAs started to appear in wild type cells 30-45 minutes after release, right before bud emergence (data not shown) and initiation of DNA replication. Conversely, their amount was dramatically reduced in both \textit{bmh1-221} and \textit{bmh1-342} mutant cells that remained arrested with 1C DNA contents for at least 180 minutes after release at 37°C (Fig. 7A). If the G1/S transition defects of our \textit{bmh} mutants were due to low amounts of G1 cyclin/Cdk1 complexes caused by the reduced \textit{CLN1} and \textit{CLN2} mRNA levels, \textit{CLN2} expression from an ectopic promoter might suppress the G1/S transition defects of our \textit{bmh} mutants. To test this hypothesis, cultures of wild type, \textit{bmh1-103}, \textit{bmh1-221}, \textit{bmh1-266} and \textit{bmh1-342} strains, carrying or lacking a galactose-inducible \textit{GAL1-CLN2} construct, were grown in YEP+raffinose at 25°C, arrested in G1 with \textit{α}-factor and then released at 25°C or 37°C in galactose-containing medium to induce \textit{CLN2} expression. \textit{GAL1-CLN2} induction significantly rescued the G1/S defects of most \textit{bmh} mutants. In fact, both bud emergence (data not shown) and initiation of DNA replication (Fig. 7B) were advanced upon galactose induction in all \textit{bmh GAL1-CLN2} strains compared to the isogenic \textit{bmh} strains, both at 25°C and 37°C. In particular, S phase entry took place in \textit{GAL-CLN2}, \textit{bmh1-103 GAL-CLN2}, \textit{bmh1-221 GAL-CLN2} and \textit{bmh1-342 GAL-CLN2} strains at 30, 45, 30 and 60 minutes, respectively, after release at 37°C in galactose-containing medium, while similarly treated \textit{bmh1-103}, \textit{bmh1-221} and \textit{bmh1-342} cells neither budded (data not shown) nor initiated DNA replication up to 4 hours after release (Fig. 7B). Conversely, ectopic \textit{CLN2} expression had only a marginal effect on \textit{bmh1-266} cells, allowing only 30% of them to initiate DNA replication by 4 hours at 37°C (Fig. 7B). This suggests that functions other than activation of Cln1, 2/Cdk1 might be affected in this mutant. It worth noting that expression of \textit{CLN2} from the \textit{GAL1} promoter caused cytokinesis defects at late time points in most strains, leading to accumulation of cells with elongated buds (data not shown) and more than 2C DNA contents (Fig. 7B), as previously reported (Lew and Reed, 1993). Altogether these data indicate that
reduced amounts of G1 cyclin/Cdk1 complexes may partially account for the G1/S transition defects of our bmh mutants.
DISCUSSION

In order to understand the essential function(s) of S. cerevisiae 14-3-3 proteins, we searched for high dosage suppressors of the temperature-sensitivity of bmh mutants, carrying a bmh1 mutant allele as the sole 14-3-3 source (Lottersberger et al., 2003). We found that the growth defects of bmh1-103, bmh1-221 and bmh1-266 at 37°C can be rescued by overproducing Pkc1 or its transmembrane cell surface sensors Wsc2 and Mid2, which have been proposed to perform partially overlapping functions in cell wall remodeling during vegetative growth and under stress conditions by detecting and transmitting cell wall status to Pkc1 (Verna et al., 1997; Philip and Levin, 2001; Rajavel et al., 1999; Ketela et al., 1999). Pkc1 is believed to possess multiple functions (Lee and Levin, 1992; Verna et al., 1997; Delley and Hall, 1999; Ketela et al., 1999; Andrews and Stark, 2000; Zanelli and Valentini, 2005), one of which is to regulate the MAP kinase cascade involved in cell wall construction and polarized growth (reviewed in Levin and Errede, 1995). Based on Mpk1 phosphorylation, the Pkc1-dependent MAPK cascade appears to be hyperactivated both at 25°C and 37°C in our bmh mutants, suggesting that defects in 14-3-3 proteins affect a pathway regulated by Pkc1 other than that Mpk1-dependent. Thus, Wsc2, Mid2 or Pkc1 may act as high dosage suppressors by stimulating the former pathway, whereas the hyperactivation of the MAPK cascade in our mutants could be the result of a compensatory mechanism that contributes to their cell viability at the permissive temperature. Accordingly, deletion of MPK1 was lethal for our bmh mutants, indicating that 14-3-3 proteins and Mpk1 act in different branches of the Pkc1 pathway to sustain cell viability.

Pkc1, together with its upstream regulators Wsc1-3 and Mid2, controls actin cytoskeleton reorganization, cell cycle progression and transcription of cell wall biosynthesis genes involved in synthesis and assembly of cell wall components at the bud (reviewed in Levin,
Since enhanced Pkc1-dependent signaling can partially suppress the temperature sensitivity of bmh mutants, some of the above Pkc1-regulated processes might be impaired in these mutants. Indeed, we found that all our temperature-sensitive bmh alleles cause defects in G1/S transition, actin polarization at the pre-bud site and cell wall integrity at 37°C. In fact, shift to the restrictive temperature severely impairs bud formation and initiation of DNA replication in bmh1-221 and bmh1-342 mutants and significantly slows down the same processes in bmh1-103 and bmh1-266 cells. When entry into S phase and bud emergence eventually take place in the latter mutants, buds are elongated, suggesting a defective apical-isotropic switch in bud growth. Consistent with a function for 14-3-3 proteins in bud formation and actin polarization, bmh mutant alleles also cause synthetic effects at semipermissive temperature when combined with the cdc42-1 and cdc24-1 temperature-sensitive alleles, altering the Rho-family GTPase Cdc42 and its guanine-nucleotide-exchange factor (GEF), respectively, that are essential for polarizing the actin cytoskeleton (reviewed in Pruyn and Bretscher, 2000). Moreover, high levels of the Cdc42 effector Gic1, which binds to the activated GTP-bound form of Cdc42 and is required for cytoskeletal polarization during bud emergence (Chen et al., 1997; Brown et al., 1997), can partially suppress the temperature-sensitivity of bmh1-103, bmh1-221 and bmh1-266 mutants. Finally, our bmh mutants undergo cell lysis at restrictive temperature and are hypersensitive to calcofluor and SDS at permissive temperature, suggesting that they are impaired in cell wall integrity. In agreement with a 14-3-3 role in cell wall biogenesis, both the growth defects and the G1/S transition delay at 37°C of our bmh mutants can be rescued by the addition of the osmo-stabilizer sorbitol.

Both initiation of DNA replication and bud morphogenesis require activation of G1 cyclin/Cdk1 complexes (reviewed in Nasmyth, 1996). In particular, when cells reach a critical size, Cln3-Cdk1 activates the Swi6/Swi4 transcription factor (SBF) that induces transcription of the CLN1 and CLN2 genes (Nasmyth and Dirick, 1991; Ogas et al., 1991; Dirick et al.,
The Pkc1-dependent cascade acts in concert with SBF to control the actin cytoskeleton and transcription of cell wall biosynthesis genes involved in maintaining cellular integrity during bud formation (Levin and Bartlett-Heubusch, 1992; Lew and Reed, 1993; Mazzoni et al., 1993; Igual et al., 1996; Marini et al., 1996; Zarzov et al., 1996; Gray et al., 1997; Madden et al., 1997; Delley and Hall, 1999). Accordingly, \(swi4\Delta\) and \(swi6\Delta\) mutants are sensitive to cell wall stresses and the growth defects of \(swi4\Delta\) cells can be partially relieved by osmotic stabilization, supporting a role for SBF in cell wall biogenesis (Igual et al., 1996; Gray et al., 1997). Moreover, \(swi4\) and \(pkc1\) mutations are synthetically lethal (Madden et al., 1997), whereas the temperature-sensitive growth of \(swi4\Delta\) cells can be suppressed by overproduction of Pkc1 or Wsc1, the latter belonging to the Wsc1-3 family of transmembrane proteins required for heat stress activation of the Pkc1-MAPK cascade (Gray et al., 1997; Igual et al., 1996). Finally, Pkc1 seems to play redundant functions with G1 cyclins, since deletion of \(PKC1\) causes cell death in a \(cln1\Delta cln2\Delta\) double mutant (Gray et al., 1997).

The partially redundant function of Pkc1 and SBF-depending pathways and the similarities in the behaviour of \(bmh\) and \(swi4\) or \(swi6\) mutants raise the possibility that the phenotypes of \(bmh\) mutants might be due to defective SBF activity. In agreement with this hypothesis, Cln1, 2/Cdk1 complexes appear to be limiting for execution of the G1/S transition in \(bmh\) mutants. In fact, the amount of \(CLN2\) mRNA is dramatically reduced in both \(bmh-103\) and \(bmh1-342\) mutants at 37°C compared to wild type. Moreover, expression of \(CLN2\) from an SBF-independent promoter can partially suppress \(bmh\) mutant defects in budding and DNA replication. Consistent with the possibility that defective 14-3-3 proteins may impair SBF-dependent accumulation of Cln1, 2/Cdk1 complexes, a mutation in the \(SIN4\) gene, whose lack of function bypasses the requirement for Swi4 and Swi6 to transcribe the \(HO-LacZ\) reporter gene (Nasmyth et al., 1987; Lycan et al., 1994; Li et al., 2005), was shown to suppress the
temperature sensitivity of a bmh2 bmh1Δ mutant (van Heusden and Steensma, 2001). Since SBF-dependent induction of Cln1, 2/Cdk1 triggers both entry into S phase by turning on proteolysis of the cyclin B-Cdk1 inhibitor Sic1 and cytoskeleton polarization for bud formation (Lew and Reed, 1993; Schwob et al., 1994; Tyers, 1996), impaired SBF-dependent Cln1, 2/Cdk1 complex formation may account for both budding and DNA replication defects of our bmh mutants. It should in fact be noted that, unlike in most of the other laboratory strains, simultaneous deletion of CLN1 and CLN2 is lethal in the W303 genetic background that we used for all our experiments (Cvrckova et al., 1995). Therefore, low levels of Cln1, 2/Cdk1 complexes in bmh mutants can be the cause of their G1/S transition defects at high temperatures.

In any case, since SBF and Pkc1 play a partially redundant role in allowing bud formation and cell integrity (Gray et al., 1997; Igual et al., 1996; Madden et al., 1997), hyperactivation of the Pkc1-dependent cascades by high levels of Wsc2, Mid2 or Pkc1 might suppress the growth and G1/S transition defects of bmh mutants by partially compensating defects in SBF activity and G1 cyclin/Cdk1 complex accumulation.

Taken together, these data indicate that S. cerevisiae 14-3-3 proteins play essential functions in regulating processes that occur at the G1/S transition. Since 14-3-3 proteins are highly conserved in evolution, our studies may help to elucidate their essential functions also in higher eukaryotes.
MATERIALS AND METHODS

**Yeast strains and media:** The relevant genotypes of all the yeast strains are listed in Table 1. All the strains used during this study were derivatives of W303 (MATa or MATα, ade2-1, can1-100, his3-11,15, leu2-3,112, trpl-1, ura3, ssd1).

Strains YLL1082, YLL1081, YLL1120 and YLL1092 were previously described (Lottersberger et al., 2003). Wild-type, bmh1-103 bmh2Δ, bmh1-221 bmh2Δ, bmh1-266 bmh2Δ and bmh1-342 bmh2Δ strains carrying either the 2µ vector, or 2µ WSC2 or 2µ MID2 or 2µ GIC2 plasmids were constructed by transforming strains W303, YLL1082, YLL1081, YLL1120 and YLL1092 with plasmids YEplac195 (2µ URA3), pML489 (2µ WSC2 URA3), pML490 (2µ MID2 URA3) and pML493 (2µ GIC2 URA3), respectively.

A MATα strain, carrying the GAL-CLN2 construct integrated at the CLN2 chromosomal locus and obtained after sporulation of the diploid L96 kindly provided by L. Dirick (Montpellier, France), was crossed to strains YLL1082, YLL1081, YLL1120 and YLL1092 to obtain DMP4370/2D, DMP4372/3B, DMP4373/7C and DMP4465/3B strains, respectively. Strain DMP4357/1B was obtained after sporulation of the diploid L96. Strain YLL1906, carrying the deletion of the PKC1 gene, was kindly provided by R. Tisi (University of Milano-Bicocca, Italy).

Strains DMP4436/3C, DMP4439/6B, DMP4440/5C and DMP4441/5A were meiotic segregants from crosses of strains YLL1082, YLL1081, YLL1120 and YLL1092, respectively, with a MATα cdc24-1 strain. Strains DMP4430/7A, DMP4433/10B, DMP4434/7B and DMP4435/4B strains were meiotic segregants from crosses of strains YLL1082, YLL1081, YLL1120 and YLL1092, respectively, with a MATα cdc42-1 strain.

The accuracy of all gene replacements and integrations was verified by Southern blot analysis or PCR. Standard yeast genetic techniques and media were according to Rose et al. (1990).
Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP+raf) or 2% raffinose and 1% galactose (YEP+raf+gal). Transformants carrying the \textit{KANMX4} cassette were selected on YEPD plates containing 400 \(\mu\)g/ml G418 (US Biological).

\textbf{Plasmids:} To obtain plasmid pML490, containing a \textit{MID2} fragment spanning from 663 bp upstream of the coding region start codon to 425 bp downstream of the stop codon, a 2219 bp \textit{MID2} fragment was amplified by PCR using yeast genomic DNA as template and the oligonucleotides PRP551 (5'-CGG GAT CCC GAT TGA GAG ATC TCA CGG AAA TG-3') and PRP552 (5'-CGG GAT CCC GTC ACA GAA CTC GGT AAG TTT TC-3') as primers. The PCR amplification product was then cloned into the \textit{BamHI} site of plasmid YEplac195 (Gietz and Sugino, 1988).

To obtain plasmid pML489, containing the \textit{WSC2} ORF flanked by 459 bp upstream of the start codon and 293 bp downstream of the stop codon, a 2264 bp \textit{WSC2} fragment was amplified by PCR using yeast genomic DNA as template and the oligonucleotides PRP543 (5'-CGG GAT CCC GCT ACG GTA AAC ATG CCT GAT GG-3') and PRP544 (5'-CGG GAT CCC GTG TGA TCT AGC ACT TCT CCC AG-3') as primers. The PCR amplification product was then cloned into the \textit{BamHI} site of plasmid YEplac195.

To obtain plasmid pML493, containing the \textit{GIC1} ORF flanked by 343 bp upstream of the start codon and 282 bp downstream of the stop codon, a 1570 bp \textit{GIC1} fragment was amplified by PCR using yeast genomic DNA as template and the oligonucleotides PRP561 (5'-GGG GTA CCC CGT TGT CTG AGC AGG AAT AAA GAG-3') and PRP562 (5'-GGG GTA CCC CGG GTA GTA GAC ATC GCT A TT ATC-3') as primers. The PCR amplification product was then cloned into the \textit{KpnI} site of plasmid YEplac195.

Plasmids YEplac112 (Gietz and Sugino, 1988), carrying the \textit{PKCI} gene, was kindly provided by R. Tisi (University of Milano-Bicocca, Italy).
Search for high dosage suppressors: To search for high dosage suppressors of the temperature-sensitivity caused by the \textit{bmh1-266} mutation, strain YLL1120 was transformed with a \emph{S. cerevisiae} genomic library based on the multicopy 2\,µ plasmid YEp24 (Carlson and Botstein, 1982). Ura\(^+\) transformants were tested for their ability to grow at 37°C on YEPD plates, which inhibited the untransformed strain. Plasmids from transformants showing co-segregation of the thermo-resistance with the \textit{URA3} vector marker were recovered and introduced again into the YLL1120 strain, to confirm their ability to suppress \textit{bmh1-266} temperature-sensitivity. Restriction analysis allowed us to identify several classes of plasmids containing different yeast genomic fragments. The nucleotide sequences of both ends of the smallest DNA insert of each plasmid class were determined and compared with the whole \textit{Saccharomyces cerevisiae} genomic sequence in the \textit{Saccharomyces} Genome Database (SGD). Since most inserts contained several ORFs, the suppressor genes were identified by cloning subfragments of the inserts into the 2\,µ plasmid YEplac195 and testing the derivative plasmids for their ability to suppress the temperature-sensitivity of the \textit{bmh1-266 bmh2\textDelta} mutant strain.

Other techniques: Synchronization experiments were performed as described in Lotterberger et al., 2003. Flow cytometric DNA analysis was determined on a Becton-Dickinson FACScan. To stain actin cytoskeleton, cells were treated 2’ with Rhodamine-phalloidin 20U/ml (Sigma-Aldrich) in PBS and then washed three in PBS buffer. Digital images were taken with a CCD camera and software (CoolSNAP; Photometrics). For Western blot analysis, native protein extracts were prepared in 0.1% SDS, 1% Triton, 50mM Tris pH7.5, 1mM sodium deoxycolate, 120mM \(\beta\)-glicerophosphate, 1,72mM sodium orthovanadate, 10mM DTT, 1mM AEBSF, 15mM para-nitrophenilphosphate and a protease inhibitor cocktail (Boehringer Mannheim). To detect phosphorylated Mpk1 and Mpk1, polyclonal anti-phospho p42/p44 (Cell Signaling) and anti-Mpk1 (Santa Cruz Biotechnology) antibodies
were used, respectively, after 1:1000 dilution in BSA-TBS. Secondary antibodies were purchased from Amersham and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer.
ACKNOWLEDGEMENTS

We thank L. Dirick and R. Tisi for providing yeast strains and plasmids, M. Vai and all the members of our laboratory for useful discussions and criticisms. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro to M.P.L. and S.P., Cofinanziamento 2005 MIUR/Università di Milano-Bicocca to M.P.L. and Fondo per gli investimenti della Ricerca di Base (FIRB) to G.L.. F.L. was supported by a fellowship from Associazione Italiana per la Ricerca sul Cancro.
FIGURE LEGENDS

FIGURE 1. High dosage suppressors of the bmh temperature-sensitive growth defects. (A and B) Exponentially growing cell cultures (selective media at 25°C) of wild type (W303), bmh1-103 bmh2Δ (YLL1082), bmh1-221 bmh2Δ (YLL1081) and bmh1-266 bmh2Δ (YLL1120) strains transformed with 2µ plasmids, either empty or carrying the GIC1, MID2, WSC2 (A) or PKC1 (B), were spotted on YEPD plates and incubated at 25°C or at 37°C for 3 days. (C) Cell cultures of wild type (K699), bmh1-103 bmh2Δ (YLL1082), bmh1-221 bmh2Δ (YLL1081), bmh1-266 bmh2Δ (YLL1120) and bmh1-342 bmh2Δ (YLL1092), exponentially growing in YEPD at 25°C, were shifted to 37°C. Aliquots were withdrawn at time zero (25°C) and 30 minutes or 3 hours after shift at 37°C to prepare protein extracts, which were subjected to western blot analysis with anti-phospho-p44/p42 antibodies (Cell Signaling) to detect Mpk1 phosphorylation (top, P-Mpk1). The two faster migrating bands were likely P-Mpk1 degradation products, and they were not detected by polyclonal antibodies raised against a C-terminal Mpk1 peptide (Santa Cruz Biotechnology), which were used to measure total Mpk1 levels in the same samples (bottom, Mpk1). Specificity of the antibodies was checked by using protein extract prepared from a mpk1Δ (YLL1906) strain incubated 30 minutes at 37°C.

FIGURE 2. Temperature-sensitive bmh mutants are defective in bud emergence and initiation of DNA replication. Cell cultures of wild type (W303), bmh1-103 bmh2Δ (YLL1082), bmh1-221 bmh2Δ (YLL1081), bmh1-266 bmh2Δ (YLL1120) and bmh1-342 bmh2Δ (YLL1092) strains, exponentially growing at 25°C in YEPD, were arrested in G1 with α-factor for 2 hr and released at time zero in YEPD at 25°C or 37°C. (A) 200 cells for each strain were analyzed to determine the frequency of cells with no, small, large or elongated buds at 25°C.
(0 hr) and at the indicated time points after shift at 37°C. (B) Photographs were taken 3 hours after shift at 37°C. (C and D) To determine DNA contents by fluorescence-activated cell sorting (FACS) analysis, samples were withdrawn at the indicated times after release in YEPD at 37°C (C), or 25°C (D).

FIGURE 3. Actin organization in the temperature-sensitive bmh mutants. Cell cultures of wild type (W303), bmh1-103 bmh2Δ (YLL1082), bmh1-221 bmh2Δ (YLL1081), bmh1-266 bmh2Δ (YLL1120) and bmh1-342 bmh2Δ (YLL1092) strains, exponentially growing at 25°C in YEPD, were synchronized in G1 with α-factor and released at time zero in YEPD at 37°C. Cells were fixed 1 hour after the release at 37°C, stained with fluorochrome-conjugated phalloidin, and scored for the presence of cells with polarized actin by fluorescence microscopy. Differential interference contrast (left) and epi-fluorescence (right) images are shown as examples.

FIGURE 4. Synthetic effects between bmh and polarization mutant alleles. The following strains were used: wild type (W303), cdc24-1, cdc42-1, bmh1-103 bmh2Δ (YLL1082), bmh1-103 cdc24-1 bmh2Δ (DMP4436/3C), bmh1-103 cdc42-1 bmh2Δ (DMP4430/7A), bmh1-221 bmh2Δ (YLL1081), bmh1-221 cdc24-1 bmh2Δ (DMP4439/6B), bmh1-221 cdc42-1 bmh2Δ (DMP4433/10B), bmh1-266 bmh2Δ (YLL1120), bmh1-266 cdc24-1 bmh2Δ (DMP4440/5C), bmh1-266 cdc42-1 bmh2Δ (DMP4434/7B), bmh1-342 bmh2Δ (YLL1092), bmh1-342 cdc24-1 bmh2Δ (DMP4441/5A) and bmh1-342 cdc42-1 bmh2Δ (DMP4435/4B). Serial dilutions of cell cultures, exponentially growing in YEPD at 25°C, were spotted on YEPD plates and incubated at the indicated temperatures for 3 days.
FIGURE 5. Temperature-sensitive \textit{bmh} mutants are sensitive to cell wall stress. (A) Serial dilutions of cell cultures of wild type (W303), \textit{bmh1-103 bmh2\Delta} (YLL1082), \textit{bmh1-221 bmh2\Delta} (YLL1081), \textit{bmh1-266 bmh2\Delta} (YLL1120) and \textit{bmh1-342 bmh2\Delta} (YLL1092), exponentially growing in YEPD at 25°C, were streaked on SD plates with or without SDS (0.01%). (B) The same cultures in A were spotted on YEPD plates with or without Calcofluor (0.01mg/ml). Plates were incubated at 25°C for 4 days. (C) Serial dilution of wild type (W303), \textit{bmh1-103 bmh2\Delta} (YLL1082), \textit{bmh1-221 bmh2\Delta} (YLL1081), \textit{bmh1-266 bmh2\Delta} (YLL1120) and \textit{bmh1-342 bmh2\Delta} (YLL1092) cell cultures, exponentially growing in YEPD at 25°C, were spotted on YEPD plates in the absence or presence of 1M sorbitol and incubated at the indicated temperatures for 3 days. (D and E) Cell cultures of wild type (W303), \textit{bmh1-103 bmh2\Delta} (YLL1082), \textit{bmh1-221 bmh2\Delta} (YLL1081), \textit{bmh1-266 bmh2\Delta} (YLL1120) and \textit{bmh1-342 bmh2\Delta} (YLL1092) strains, exponentially growing at 25°C in YEPD, were arrested in G1 with \textit{\alpha}-factor for 2 hr, and released at time zero in YEPD at 25°C or 37°C in the absence or presence of 1M sorbitol. Samples were withdrawn at the indicated times after \textit{\alpha}-factor release to analyze the kinetics of bud emergence (D) and DNA contents by FACS analysis (E).

FIGURE 6. \textit{WSC2}, \textit{MID2} and \textit{PKC1} overexpression can suppress the G1/S transition delay of \textit{bmh} mutants. Exponentially growing (selective media at 25°C) cell cultures of wild type (W303), \textit{bmh1-103 bmh2\Delta} (YLL1082), \textit{bmh1-221 bmh2\Delta} (YLL1081) and \textit{bmh1-266 bmh2\Delta} (YLL1120) strains transformed with 2µ plasmids, either empty or carrying the \textit{WSC2}, \textit{MID2}, \textit{PKC1} or \textit{GIC1} genes, were arrested in G1 with \textit{\alpha}-factor and released at time zero in YEPD at 25°C or 37°C. (A) 200 cells for each strain were analyzed to determine the frequency of cells with no, small, large or elongated buds after 3 hours at 37°C. (B) Samples were withdrawn at the indicated times after \textit{\alpha}-factor release to analyze DNA contents by FACS analysis.
FIGURE 7. CLN2 mRNA levels and CLN2 ectopic expression in bmh mutants. Cell cultures of wild type (W303), bmh1-221 bmh2Δ (YLL1081) and bmh1-342 bmh2Δ (YLL1092) strains, exponentially growing in YEPD at 25°C, were synchronized in G1 with α-factor and released at time zero into YEPD at 37°C. Samples were taken at the indicated times after the release into the cell cycle to analyze CLN2 mRNA by Northern analysis (left) and to determine DNA contents by FACS analysis (right). Loading control of the northern blot is a methylene blue-stained filter of ribosomal RNAs (rRNAs). (B) Cell cultures of wild type (W303), GAL-CLN2 (DMP4357/1B), bmh1-103 bmh2Δ (YLL1082), bmh1-103 bmh2Δ GAL-CLN2 (DMP4370/2D), bmh1-221 bmh2Δ (YLL1081), bmh1-221 bmh2Δ GAL-CLN2 (DMP4372/3B), bmh1-266 bmh2Δ (YLL1120), bmh1-266 bmh2Δ GAL-CLN2 (DMP4373/7C), bmh1-342 bmh2Δ (YLL1092) and bmh1-342 bmh2Δ GAL-CLN2 (DMP4465/3B) strains, exponentially growing in YEP+raffinose at 25°C, were synchronized in G1 with α-factor for 2 hr. Galactose was added 30 minutes before release. Synchronized cells were then released at time zero into YEP+raf+gal at 25°C (top) or at 37°C (bottom). Samples were taken at the indicated times after release to determine DNA contents by FACS analysis.
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% cells with polarized actin after 1hr at 37°C

- **wt**: 90%
- **bmh1-221**: 1%
- **bmh1-342**: 1%
- **bmh1-103**: 12%
- **bmh1-266**: 15%
A

B

C

D

E

SD SDS (0.01%)

wt bmh1-103 bmh1-221 bmh1-266 bmh1-342

25°C 25°C+sorbitol 37°C+sorbitol 37°C

YEPD calcofluor

wt

bmh1-103 bmh1-221 bmh1-266 bmh1-342

0 30 60 90 120 150 180 240

37°C 37°C + sorbitol

Budded cells (%)

0 30 60 90 120 150 180 210 240 270 300

0 20 40 60 80 100

37°C

37°C + sorbitol

Time after α-factor release (min.)

D

E

SD SDS (0.01%)

wt bmh1-103 bmh1-221 bmh1-266 bmh1-342

0 30 60 90 120 150 180 210 240 270 300

0 30 60 90 120 150 180 210 240 270 300

Budded cells (%)

0 30 60 90 120 150 180 210 240 270 300

0 30 60 90 120 150 180 210 240 270 300

37°C

37°C + sorbitol

Time after α-factor release (min.)

Time after α-factor release (min.)

Time after α-factor release (min.)

Time after α-factor release (min.)
A

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B

|                | 0 | 15 | 30 | 45 | 60 | 75 | 90 | 105 | 120 | 135 | 150 | 165 | 180 | 195 | 210 | 225 | 240 |
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| 37°C           | ![Graph](image5) |

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- wt [2µ WSC2]
- wt [2µ MID2]
- wt [2µ PKC1]

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CLN2

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