Regulation of Growth and Development by the Small GTPase Cdc42p and the Transcription Factor Tec1p in *Saccharomyces cerevisiae*

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Summary

The yeast *Saccharomyces cerevisiae* undergoes a dimorphic filamentous transition in response to particular nutrient cues. Upon nitrogen starvation, diploid cells switch from the yeast form (YF) to a filamentous pseudohyphal form (PH), leading to the formation of chains of elongated cells that radiate away from the colony border and penetrate the agar surface. A related but less exuberant process called invasive growth occurs in haploids after prolonged incubation on rich medium. The signals triggering pseudohyphal or invasive growth are transmitted via a conserved mitogen activated protein kinase (MAPK) cascade. Interestingly, elements of this cascade are also required to relay pheromone signals that lead to mating of haploid yeast cells.

The roles of two proteins for yeast cellular development were investigated in this work: The small GTPase Cdc42p and the transcription factor Tec1p. Cdc42p is an essential protein that acts as a molecular switch, cycling between a GTP-bound, active conformation and a GDP-bound, inactive conformation. In the active state, Cdc42p interacts with a large number of effector proteins to transduce upstream signals. Using a PCR-mutagenesis approach, mutant *cdc42* alleles encoding proteins with single amino acid substitutions were isolated that separate functions of Cdc42p required for pseudohyphal and invasive growth from those required for cell division.

Tec1p contains a conserved DNA-binding motif named TEA/ATTS domain, which is shared by a group of eukaryotic transcription factors. Tec1p was previously reported to function exclusively in conjunction with another transcription factor, Ste12p. Tec1p and Ste12p bind cooperatively to combined sequence elements consisting of a Ste12p-binding site and an adjacent Tec1p-binding site (TCS) present in the promoters of target genes. Here, we could demonstrate that Tec1p also activates target gene expression and cellular development via single or combined TCS elements in the absence of Ste12p.

The regulation of *TEC1* by the MAP kinases Kss1p and Fus3p was further analyzed. Whereas Kss1p has been regarded as filamentation/invasion specific MAPK, Fus3p is considered to be mating specific. However, both MAPKs were found to physically interact with Tec1p. Moreover, Tec1p was phosphorylated by Fus3p in response to pheromone signals, and Tec1p protein levels were downregulated in a Fus3p dependent manner. These data suggest that phosphorylation of Tec1p by Fus3p serves to target Tec1p for degradation, representing a mechanism that might contribute to maintenance of signaling specificity.

Zusammenfassung

Die Bäckerhefe *Saccharomyces cerevisiae* ist ein dimorpher Pilz, der in verschiedenen Wachstumsformen vorkommen kann. Stickstoffmangel löst in diploiden Zellen den Übergang vom Wachstum in der einzelligen Hefeform zu einer multizellulären, filamentösen Form aus. Diese Wuchsform wird als pseudohyphales Wachstum bezeichnet. Ein ähnlicher Differenzierungsprozess tritt bei haploiden Zellen auf Vollmedium auf. Allerdings sind die zellulären Veränderungen beim sogenannten haploid invasiven Wachstum weniger tiefgreifend als bei der Pseudohyphenbildung. Die Signale, welche filamentöses/invasives Wachstum auslösen, werden über eine konservierte Mitogen-aktivierte Protein-Kinase (MAPK) Kaskade übertragen. Erstaunlicherweise werden einige Komponenten dieses Weges ebenfalls für die Konjugation haploider Hefezellen benötigt.

In dieser Arbeit wurden die Funktionen von zwei Proteinen, Cdc42p und Tec1p, für die Zelldifferenzierung in der Hefe untersucht. Die kleine GTPase Cdc42p ist ein essentielles Protein. Es fungiert als molekularer Schalter, der zwischen einer aktiven, GTP-gebundenen und einer inaktiven, GDP-gebundenen Konformation wechselt. In der aktiven Form interagiert Cdc42p mit einer Reihe von Effektorproteinen, um empfangene Signale weiterzuleiten. In dieser Arbeit wurden durch PCR-Mutagenese *cdc42* Mutantenallele isoliert, die für neue Varianten von Cdc42p kodieren, welche die essentiellen Zellteilungsfunktionen von Cdc42p von solchen Funktionen trennen, die spezifisch für die Pseudohyphenbildung benötigt werden.

Der Transkriptionsfaktor Tec1p enthält eine konservierte DNA-Bindedomäne. Dieses sogenannte TEA/ATTS-Motiv wurde in einer Reihe weiterer Transkriptionsfaktoren in unterschiedlichen eukaryotischen Organismen identifiziert. Bisher ging man davon aus, dass Tec1p nur im Verbund mit einem weiteren Transkriptionsfaktor, Ste12p, als Heterodimer die Expression filamentspezifischer Zielgene auslösen kann. Hier konnte hingegen gezeigt werden, dass Tec1p prinzipiell auch ohne Ste12p funktionell ist.

In einem weiteren Projekt wurde die Regulation von *TEC1* durch die MAP Kinasen Kss1p und Fus3p untersucht. Fus3p gilt als konjugationsspezifische MAP Kinase, während Kss1p als filament-/invasionsspezifische MAP Kinase angesehen wird. Überraschenderweise interagiert Tec1p jedoch mit beiden Proteinkinasen, obwohl es für die Konjugation nicht benötigt wird. Durch Pheromonbehandlung wurde Tec1p von Fus3p phosphoryliert, und die Tec1p Proteinmenge wurde deutlich herunterreguliert. Dies deutet auf eine Funktion von Tec1p als Spezifitätsfaktor hin, der verhindert, dass ein Pheromonsignal gleichzeitig filament-/invasionsspezifische Zielgene aktiviert.

Chapter 1

Introduction

1. Cellular differentiation of *Saccharomyces cerevisiae*

1.1 Filamentous and invasive growth in yeast

1.1.1 Pseudohyphal development as a model to study dimorphism

The budding yeast *Saccharomyces cerevisiae* is a dimorphic fungus that can switch between a unicellular and a multicellular filamentous growth mode (Fig. 1) (Mösch, 2000; Mösch, 2002). The unicellular growth form is the preferred mode of reproduction of yeasts, and it has several advantages over complex growth forms like hyphae. The spherical form is more economical with cell wall materials, confers a better resistance to osmotic ruptures and is easier dispersed by water, air or insects. However, under certain conditions of nutrient starvation, diploid yeast cells respond by switching to a filamentous growth form consisting of chains of elongated cells called pseudohyphae. This growth form is considered an important adaptive response that allows cells in a starving colony to forage for nutrients (Gimeno *et al.*, 1992). The switch from the yeast form to pseudohyphal growth is accompanied by changes in several distinct cellular processes (Fig. 2) (Gimeno *et al.*, 1992; Kron *et al.*, 1994). The budding pattern of cells changes from bipolar to unipolar distal, resulting in linear filamentous chains of cells. Cell morphogenesis is altered from ellipsoidal-shaped yeast form cells to long, thin pseudohyphal cells. Pseudohyphal cells, in contrast to yeast form cells, exhibit invasive growth behavior, resulting in direct substrate invasion. Cell separation switches from complete to incomplete scission, leading to multicellular growth, where cells remain attached to each other.

A related phenomenon called invasive growth is observed in haploids (Roberts and Fink, 1994). Despite of the different terms used for these developmental options in diploids and haploids, both processes are closely related and share many similarities with respect to cellular morphology, substrate invasion/adhesion, cell polarity and the underlying signal transduction machinery. However, dimorphic switching in haploid *S. cerevisiae* is more subtle, leading to increased cell-cell adhesion and agar penetration but only limited

changes in cell morphology. Moreover, haploids growing invasively display a bipolar rather than unipolar distal budding pattern. As a consequence, haploid filaments consist of only moderately elongated cells and fail to spread over the substrate surface.

Similar to the apathogenic yeast *S.cerevisiae*, many pathogenic fungi are dimorphic. Typically, the dimorphic switch in pathogenic organisms is tuned such that cells elongate or increase cell-cell adherence when exposed to their host (San-Blas *et al.*, 2000; Sanchez-Martinez and Perez-Martin, 2001). Such regulated dimorphism has been established as an important virulence factor, determining invasion and colonization by pathogenic fungi like *Candida albicans*, *Magnaporthe grisea* and *Ustilago maydis* (Lengeler *et al.*, 2000; Sanchez-Martinez and Perez-Martin, 2001). Because the signal transduction machinery regulating filamentous growth is highly conserved in diverse fungi, studies of *S. cerevisiae* may reveal fundamental molecular principles common to all fungi.

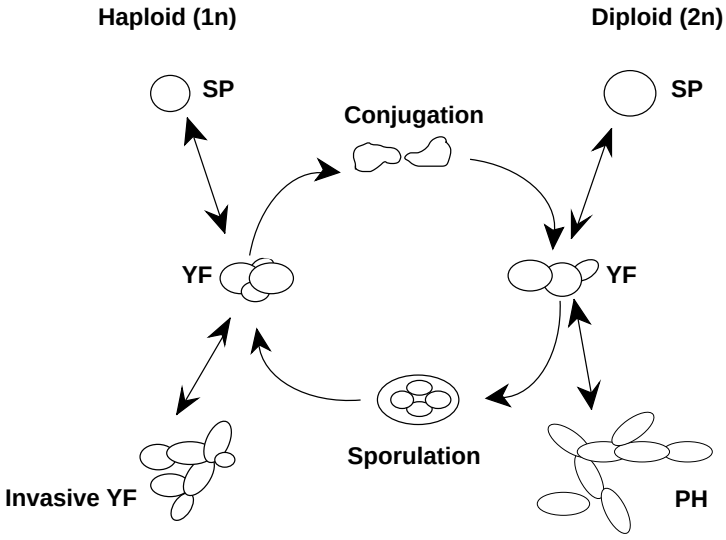

Fig. 1. Life cycle of *S. cerevisiae*. Both haploids and diploids can grow vegetatively in the yeast form (YF), arrest growth in the stationary phase (SP) or switch to an invasive growth mode. In diploids, the latter option leads to the formation of multicellular, filamentous networks of cells called pseudohyphae (PH), whereas haploids produce only microfilaments. Haploids (1n) of opposite mating types can conjugate to form diploids (2n), and these diploids can sporulate to form haploids.

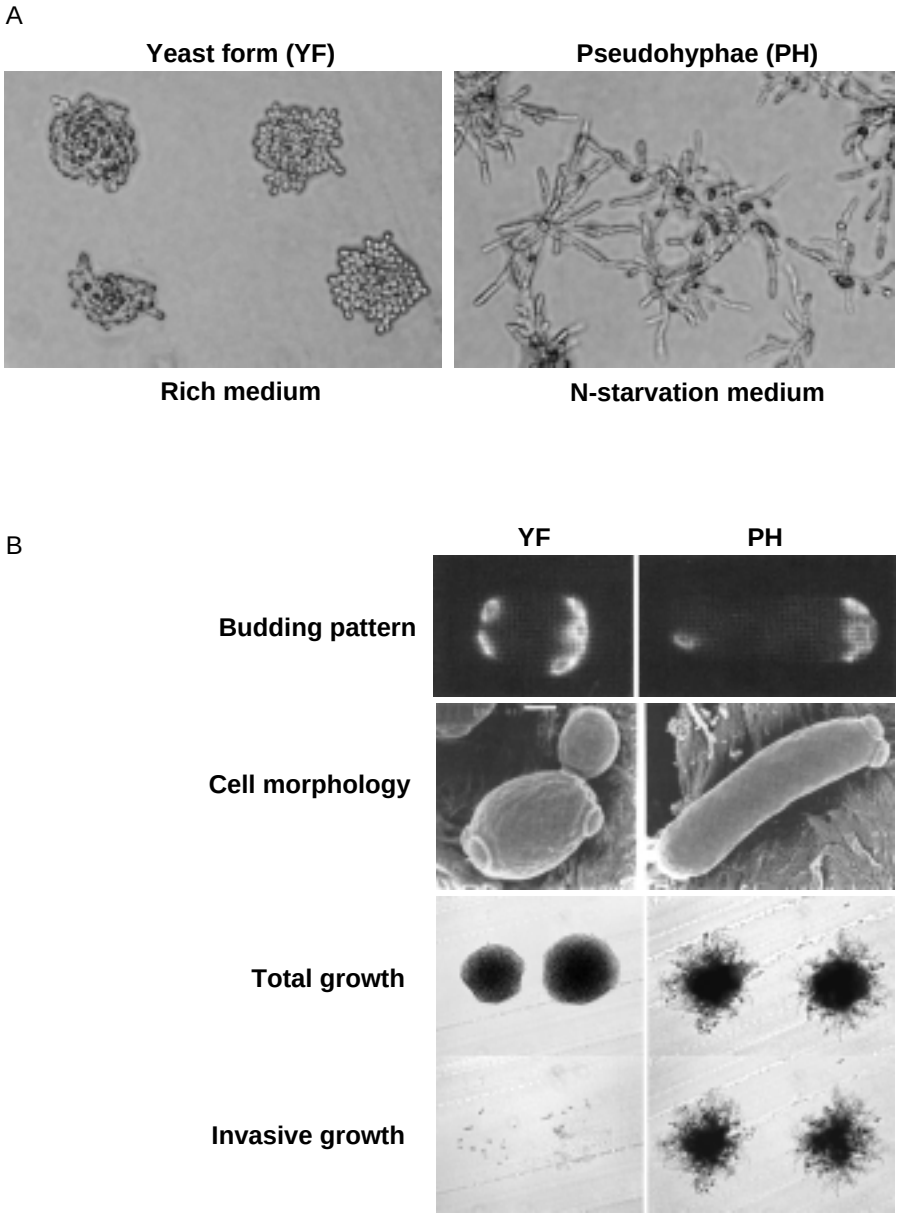

Fig. 2. Pseudohyphal development of *S. cerevisiae.* (A) Pseudohyphal growth is induced by nitrogen starvation. Diploid strains were streaked for single colonies on either nitrogen rich medium or nitrogen starvation medium. Shown are microcolonies of cells growing as yeast form (YF) or as pseudohyphae (PH). (B) Comparison of budding pattern, cell morphology and growth behaviour of YF and PH. For determination of budding patterns, bud scars were stained with calcofluor and visualized by fluorescence microscopy. YF cells show a bipolar distribution of bud scars, whereas PH cells bud preferentially from the distal cell pole. Differences in cellular morphology are demonstrated by SEM analysis of typical YF and PH cells. Invasive growth behavior is demonstrated by a wash test. Growth of yeast form cells is restricted to the surface of the agar plate, whereas PH cells invade the substrate.

1.1.2 Environmental sensing mechanisms

Yeast cellular development is tightly controlled by the nutrients available in the environment (Fig. 1). Laboratory culture conditions for *S. cerevisiae* have been developed to optimize cell growth. Typically, yeast is cultured in media that provide an excess of all required nutrients, and yeast cells adopt a fermentative yeast form growth mode in such rich media containing abundant nitrogen sources and a fermentable carbon source. Once depletion of the media results in sufficiently low nutrient concentrations to limit cell growth, yeast undergo divergent responses. Two nutrients have turned out to be of major importance for regulation of yeast cellular development: presence of a fermentable carbon source (e.g. glucose) and supply with nitrogen. Starvation for either of these nutrients triggers different cellular responses. At the diauxic shift, when fermentable carbon sources are exhausted, cells transiently arrest and then reprogram their metabolic patterns to utilize ethanol and continue slow, vegetative growth (Russell *et al.*, 1993). Complete carbon or nitrogen source starvation causes growth arrest in stationary phase, a G_0 exit from the cell cycle. During conditions of moderate nitrogen starvation and when nonfermentable carbon sources are available, diploid cells can enter meiosis and perform sporulation. In response to nitrogen limitation in the presence of glucose or another fermentable carbon source, diploids perform a dimorphic switch and start filamentous growth. In contrast to diploid pseudohyphal growth, haploid invasive growth is not triggered by nitrogen starvation but occurs in response to glucose depletion or general nutrient limitation after prolonged incubation on rich medium (Cullen and Sprague, 2000).

Among the fermentable sugars that promote filamentous growth under nitrogen starvation conditions are glucose, galactose, sucrose, maltose and raffinose (Gimeno *et al.*, 1992; Kron *et al.*, 1994; Lorenz *et al.*, 2000b). Nutrient signals might be sensed by Gpr1p, a cell surface G-protein coupled receptor that binds to the heterotrimeric GTP-binding protein alpha subunit Gpa2p (Xue *et al.*, 1998; Yun *et al.*, 1998). Gpr1p has at least two functions in nutrient sensing. It plays a role in nitrogen detection, and the *GPR1* mRNA is strongly induced by nitrogen starvation (Xue *et al.*, 1998). However, the primary function of Gpr1p seems to be glucose sensing (Kraakman *et al.*, 1999; Lorenz *et al.*, 2000a; Yun *et al.*, 1998). Therefore, Gpr1p may serve to integrate glucose and nitrogen signals in the pseudohyphal pathway. The interaction between Gpr1p and Gpa2p is dependent on the phosphatidylinositol-specific phospholipase C (Ansari *et al.*, 1999). The Gpr1p-Gpa2p-Plc1p complex regulates pseudohyphal growth via the cAMP pathway (see below). Further

proteins that are important for regulation of filamentation under nitrogen starvation conditions are the small GTP-binding protein Ras2p and its regulators, the guanine nucleotide exchange factor Cdc25p and the GTPase-activating proteins Ira1p and Ira2p (Broach, 1991; Broach and Deschennes, 1990; Jiang *et al*., 1998). The Ras2p module is responsive to changes in glucose availability, but a glucose sensor protein activating Ras2p has not been identified so far. Although at least two further mechanisms are known in yeast to play a role in glucose sensing (Gancedo, 1998; Ozcan and Johnston, 1999), their function for pseudohyphal growth has not been investigated. However, one of the mechanisms involves the Ser/Thr protein kinase Snf1p which has recently been demonstrated to be required for expression of glucose-repressed genes responsible for invasive growth in the absence of glucose in haploids (Cullen and Sprague, 2000).

Among the different nitrogen sources tested, low ammonium levels or standard concentrations of histidine, proline or uracil were permissive for diploid filamentous growth. The membrane localized high-affinity ammonium permease Mep2p is a receptor used by the cells to detect nitrogen compounds. Strains lacking the *MEP2* gene do not form filaments in response to ammonium limitation (Lorenz and Heitman, 1998a), suggesting that Mep2p senses extracellular ammonium starvation and creates a signal transduced to the interior of the cell, ultimately leading to filamentation. Mep1p, a low-affinity permease, may also play a role in nitrogen sensing (Lorenz and Heitman, 1998b). Filamentous growth is also affected by amino acid concentrations. Two proteins involved in amino acid uptake, the Ssy1p amino acid permease and the Ptr3p peptide permease regulator, are required for sensing of extracellular amino acids (Klasson *et al.*, 1999). Moreover, mutations in *SHR3*, which is involved in processing amino acid permeases, enhance filamentous growth (Gimeno *et al.*, 1992).

Apart from glucose abundance and nitrogen limitation, some other environmental stimuli have been described to induce filamentous growth, but their influence is less well investigated. Oxygen limitation may affect dimorphic switching in both haploids and diploids (Wright *et al.*, 1993). Additionally, the product of sugar fermentation, ethanol, and some fusel alcohols like 1-butanol or isoamyl alcohol have been shown to stimulate filamentation (Dickinson, 1996; Lorenz *et al.*, 2000a). Moreover, certain types of stress like mild thermal stress, high salt concentration or high osmolarity seem to affect filamentation (O'Rourke and Herskowitz, 1998; Zaragoza and Gancedo, 2000). Interestingly, filamentation and invasion is regulated in haploids by mating pheromones.

Genome-wide transcriptional profiling studies have revealed that several genes known to be induced during filamentous growth are stimulated by mating pheromone (Madhani *et al.*, 1999; Roberts *et al.*, 2000). Low concentrations of mating pheromones were found to increase agar-invasive growth and filament formation in haploids (Erdman and Snyder, 2001; Roberts *et al.*, 2000). The physiological significance of this observation might be to enhance the ability of haploid yeast cells to forage for mating partners along a gradient of pheromone.

In summary, the upstream sensing systems for detection and integration of the multiple environmental stimuli inducing filamentous and invasive growth are complex and only partially understood.

1.1.3 Signal transduction pathways and transcriptional control

Considerably more is known about the signal transduction pathways than the sensors and effectors of pseudohyphal differentiation. The regulatory machinery is complex and involves several parallel pathways that are interconnected (Fig. 3). The two best characterized pathways regulating filamentous growth are the cAMP-dependent protein kinase A (PKA) and a conserved MAPK pathway (recently reviewed by Banuett, 1998; Borges-Walmsley and Walmsley, 2000; D'Souza and Heitman, 2001; Gancedo, 2001; Gustin *et al.*, 1998; Kronstad *et al.*, 1998; Lengeler *et al.*, 2000; Mösch, 2000; Mösch, 2002; Palecek *et al.*, 2002; Pan *et al.*, 2000; Posas *et al.*, 1998).

A central regulator controlling pseudohyphal growth is the small GTP-binding protein Ras2p, which transmits signals for filamentation through both pathways. Ras2p is required for pseudohyphal growth, and a dominant active $RAS2^{Val19}$ allele induces filamentation even in the absence of nitrogen starvation (Gimeno *et al.*, 1992). Activation of Ras2p stimulates the adenylate cyclase Cyr1p and elevates intracellular cAMP levels, which in turn activate the yeast protein kinase A (PKA). A number of studies emphasize the importance of cAMP and PKA for pseudohyphal growth. Hyperfilamentation caused by dominant active $RAS2^{Val19}$ can be suppressed by overexpression of the cAMP-hydrolyzing phosphodiesterase Pde2p (Ward *et al.*, 1995). Defects in pseudohyphal growth of strains that lack the *RAS2* and *GPA2* genes can be attributed to reduced intracellular cAMP levels and can be rescued by addition of exogenous cAMP (Kübler *et al.*, 1997; Lorenz and Heitman, 1997). The Gpr1p receptor and Gpa2p G-protein alpha subunit also activate Cyr1p, but they do not appear to function upstream of Ras2p. The yeast protein kinase A is

composed of one of three catalytic subunits, Tpk1-3p, that form a complex with a single cAMP-binding regulatory subunit, Bcy1p (Broach, 1991). The three Tpk proteins are redundant for viability (Toda *et al.*, 1987), but only Tpk2p is required for pseudohyphal development (Robertson *et al.*, 2000; Robertson and Fink, 1998). In fact, mutations in *TPK1* and *TPK3* enhance filament formation, suggesting that Tpk1p and Tpk3p act as inhibitors of pseudohyphal growth, possibly by a feedback loop that inhibits cAMP production (Nikawa *et al.*, 1987; Pan and Heitman, 1999). Putative targets of PKA are the two transcription factors Sfl1p and Flo8p. Both proteins regulate transcription of *FLO11*, a gene that encodes a glycosyl-phosphatidylinositol (GPI)-linked cell surface flocculin which is indispensable for diploid pseudohyphal growth and haploid invasive growth (Lambrechts *et al.*, 1996; Lo and Dranginis, 1998). Flo8p is required for filamentous growth and positively regulates *FLO11* expression (Pan and Heitman, 1999; Rupp *et al.*, 1999). In this context, it is noteworthy that the commonly used laboratory strain S288C harbors a naturally occuring *flo8* mutation that prevents filamentous differentiation (Liu *et al.*, 1996). Sfl1p is negatively regulated by Tpk2p and represses *FLO11* transcription (Robertson and Fink, 1998).

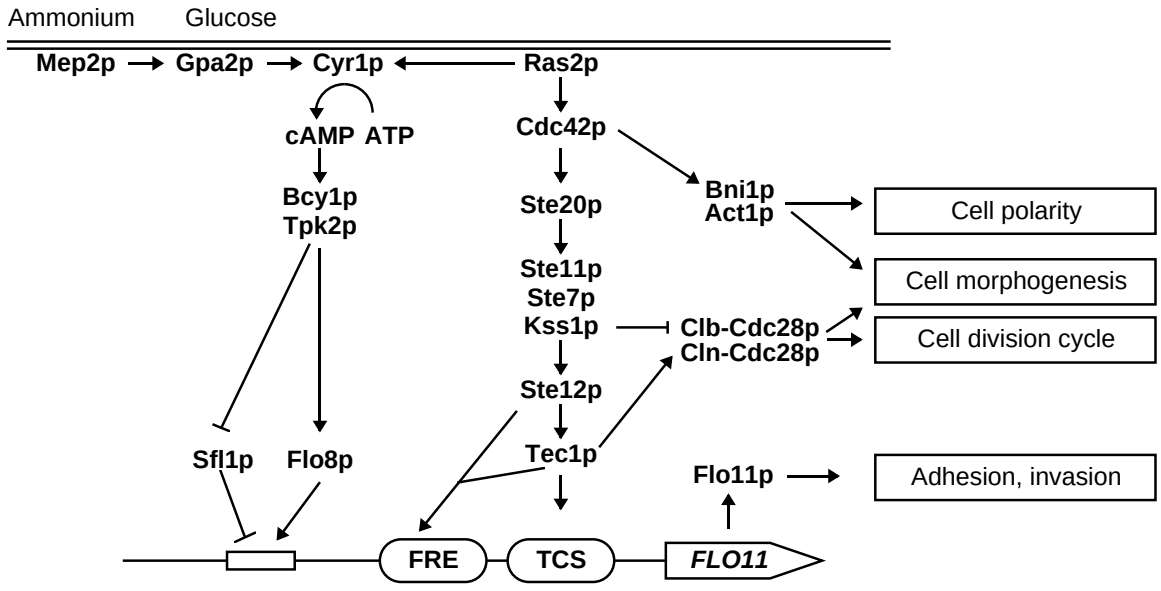

Fig. 3. Model of signaling pathways regulating pseudohyphal growth in *S. cerevisiae* (see text for details).

In addition to stimulating PKA activity, Ras2p also affects filamentous growth via a MAPK (mitogen-activated protein kinase) signaling cascade (Fig. 3.) (Mösch *et al.*, 1999). In general, MAPK modules are highly conserved and consist of a series of protein kinases that are sequentially activated by phosphorylation, finally leading to activation of transcription factors that in turn induce target gene expression. Five different MAPK pathways are known in yeast, and they are required for diverse developmental processes, i.e. mating, filamentous growth, growth on high osmolarity medium, cell integrity and spore wall assembly (Banuett, 1998; Gustin *et al.*, 1998; Herskowitz, 1995; Posas *et al.*, 1998). Remarkably, the filamentation MAPK cascade shares many components with the mating or pheromone response MAPK pathway that mediates conjugation of haploid yeast cells (see below). However, the pheromones, pheromone receptors, and subunits of the pheromone-activated heterotrimeric G-protein are dispensable for filamentous growth and are not expressed in diploids (Liu *et al.*, 1993). Ras2p is linked to the pseudohyphal MAPK cascade via another small GTP-binding protein, Cdc42p (Mösch *et al.*, 1996). Both GTPases are important for pseudohyphal development and require the pseudohyphal MAPK cascade for their functions (Mösch *et al.*, 2001; Mösch *et al.*, 1999; Mösch *et al.*, 1996). This MAPK cascade contains the protein kinases Ste20p (MAPKKKK), Ste11p (MAPKKK), STE7p (MAPKK) and Kss1p (MAPK) (Liu *et al.*, 1993; Madhani *et al.*, 1997). Kss1p was previously thought to be redundant with Fus3p in the mating pathway, but several lines of evidence now suggest that Kss1p is indeed the MAPK of the filamentation pathway (Cook *et al.*, 1997; Madhani *et al.*, 1997). Kss1p plays a dual role in the pseudohyphal MAPK pathway. A putative positive function was derived from the finding that haploid *kss1Δ* strains show strongly reduced invasive growth (Cook *et al.*, 1997; Roberts and Fink, 1994). More is known about the inhibitory function of Kss1p. In the absence of signals permissive for filamentous growth, Kss1p is unphosphorylated. In this form, it binds to the transcription factor Ste12p and prevents it from activating target gene (e.g. *FLO11*) expression (Bardwell *et al.*, 1998). When activated by the upstream kinase Ste7p, Kss1p relieves its inhibition of Ste12p, thereby allowing Ste12p to activate target gene transcription. Two additional proteins, Dig1p and Dig2p, are required for Kss1p-mediated repression of Ste12p (Bardwell *et al.*, 1998; Cook *et al.*, 1996). For activation of target gene transcription, Ste12p acts in combination with Tec1p, a protein that was originally identified as a regulator of expression of *Ty1* transposon insertions (Laloux *et al.*, 1990). Tec1p is also required for pseudohyphal growth (Gavrias *et al.*,

1996; Mösch and Fink, 1997), and it contains the conserved TEA/ATTS DNA-binding domain, which is shared by several eukaryotic transcription factors, including *Aspergillus nidulans* AbaAp (Andrianopoulos and Timberlake, 1991; Bürglin, 1991). Ste12p and Tec1p together form heterodimers and bind in a cooperative manner to specific target sequences called filamentation and invasion response elements (FREs), which are present in the promoter regions of target genes including *TEC1* itself (Madhani and Fink, 1997) and *FLO11* (Lo and Dranginis, 1998). Recent studies have clearly demonstrated that regulation of filamentous growth by Ste12p and Tec1p is not only executed by combinatorial control, but involves additional control mechanisms in which Ste12p activates *TEC1* expression via clustered Ste12p binding sites (pheromone response elements or PREs) in the *TEC1* promoter and where Tec1p regulates expression of target genes by binding to Tec1p binding sites (TCS elements) (Köhler *et al.*, 2002; Oehlen and Cross, 1998).

The pseudohyphal MAPK pathway is not a straight linear pathway reaching from Ras2p down to the transcription factors Ste12p and Tec1p, but it integrates multiple signals at the level of the MAPKKKK Ste20p and the MAPKKK Ste11p. The yeast 14-3-3 proteins Bmh1p and Bmh2p associate with Ste20p and are essential for pseudohyphal growth (Roberts *et al.*, 1997). Likewise, Ste50p associates with Ste11p to regulate pseudohyphal development (Jansen *et al.*, 2001; Ramezani Rad *et al.*, 1998).

A number of further regulators of pseudohyphal growth are known that cannot be placed directly within the MAPK or cAMP pathways. *ELM1* encodes a protein kinase that seems to inhibit pseudohyphal growth, and its absence leads to a constitutive pseudohyphal morphology (Blacketer *et al.*, 1993; Blacketer *et al.*, 1995). The fork-head transcription factors Fkh1p and Fkh2p also regulate filament formation and invasive growth. A single deletion of either fork-head gene alone has no effect, while a double deletion of *FKH1* and *FKH2* induces filamentous growth (Hollenhorst *et al.*, 2000). Fkh1p and Fkh2p regulate transcription of *CLB2* and other genes involved in mitosis that antagonize filamentation, further demonstrating the link between the cell cycle and pseudohyphal growth (Zhu *et al.*, 2000). Other genes controlling pseudohyphal growth include *PHD1* and *SOK2* (Gimeno and Fink, 1994; Mösch and Fink, 1997; Ward *et al.*, 1995). Sok2p appears to antagonize pseudohyphal growth, whereas Phd1p appears to promote it. Two further proteins, Mss10p and Mss11p, regulate both filamentous growth and *FLO11* expression (Gagiano *et al.*, 1999; Lambrechts *et al.*, 1996). Ash1p, a transcription factor that is asymmetrically

distributed to the nuclei of daughter cells, is also required for pseudohyphal growth and might regulate interactions between mother and daughter cells (Chandarlapaty and Errede, 1998).

1.1.4 Cell cycle regulation

The dimorphic transition between growth in the yeast form and pseudohyphal growth in *S. cerevisiae* involves important changes in the pattern of cell cycle progression. Although a large number of studies have contributed to unravel the molecular mechanisms underlying the cell division cycle, most of these studies have addressed analysis of growth in the yeast form. One of the key regulatory proteins for cell cycle control is the cyclin dependent kinase (CDK) Cdc28p, which mediates cell cycle progression by association with distinct groups of cyclins. The various cyclin/Cdc28p complexes control different aspects of cell cycle progression, including the cell size checkpoint START at G_1/S transition and mitosis (Nasmyth, 1993). Once a yeast cell has achieved a critical mass, it enters a new cell cycle by passing START. At that time, DNA replication is onset, the microtubule-organizing center (MTOC) is duplicated and a new bud emerges. The START event depends upon a peak activity of Cdc28p complexed with the G_1 cyclin Cln1p. G_1 cyclins are also required for morphological aspects of the cell cycle. Activation of Cdc28p by the G_1 cyclins Cln1p, Cln2p and Cln3p is required for polarization of the actin cytoskeleton towards the tip of the bud, promoting apical growth of the daughter cell. In contrast, activation of Cdc28p by the mitotic cyclins Clb1p and Clb2p triggers depolarization of the actin cytoskeleton, causing the cell to switch from apical to isotropic growth (Lew and Reed, 1993). The yeast form and the pseudohyphal cell cycle show some striking differences when compared to each other (Fig. 4) (Kron *et al.*, 1994). The yeast form cell cycle is controlled at the G_1/S transition before START. Yeast form cells divide asymmetrically, producing small daughters from full-sized mothers. As a result, mothers and daughters bud asynchronously. Mothers bud immediately, but daughters grow in G_1 until they achieve a critical cell size. By contrast, pseudohyphal cells divide symmetrically, restricting mitosis by a supposed G_2 cell-size checkpoint until the bud grows to the size of the mother. Thus, mother and daughter bud synchronously in the next cycle, without a G_1 delay before START.

Some recent studies suggest that the antagonistic activities of Cln and Clb cyclins are targets of the bifurcated pseudohyphal signal transduction pathway. Whereas *cln1Δ* mutants are deficient for filamentous growth (Loeb *et al.*, 1999; Madhani *et al.*, 1999),

clb2Δ or *cln1Δ clb2Δ* double mutants are hyperfilamentous (Loeb *et al.*, 1999). The activity of the Clb2p-Cdc28p complex as a regulator of the apical-isotropic switch may be repressed by at least two different mechanisms. MAPK signaling might inhibit Clb2p nuclear export and sequester active Clb2p-Cdc28p in the nucleus, thereby preventing mitotic exit (Miller and Cross, 2001; Rua *et al.*, 2001). Moreover, the cAMP-dependent branch might contribute to pseudohyphal cell cycle control by activation of Cln1p-Cdc28p, which is thought to stabilize the G_2/M cell morphogenesis checkpoint kinase Swe1p. Activated Swe1p in turn might downregulate cytoplasmic Clb2p-Cdc28p complexes (Ahn *et al.*, 2001). The positive role of G_1 cyclin function in pseudohyphal growth is further supported by the finding that *CLN1* expression is transcriptionally upregulated upon overexpression of *TEC1* (Madhani *et al.*, 1999). It is not yet known whether inhibition of Clb2p-Cdc28p requires a cyclin-dependent kinase (CKI) inhibitor analogous to Far1p in pheromone signal transduction.

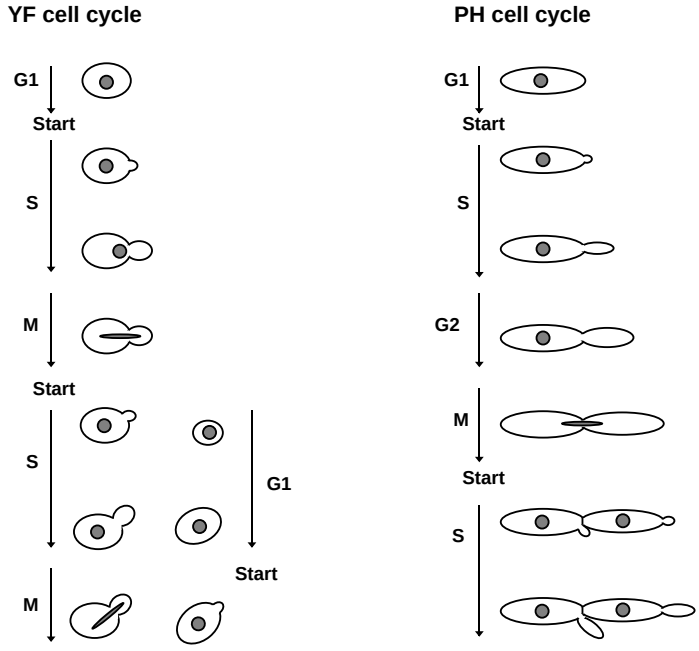

Fig. 4. Comparison of yeast form (YF) and pseudohyphal (PH) cell division cycles. YF cells divide asymmetrically, producing small daughters from full-sized mothers. As a result, mothers and daughters bud asynchronously. By contrast, PH cells bud symmetrically, restricting mitosis until the bud grows to the size of the mother. Thus, mother and daughter bud synchronously.

1.1.5 Regulation of cell polarity and morphology

Polarized cell growth and directional cell division are fundamental processes that are essential for the development of eukaryotes. The budding yeast *S. cerevisiae* undergoes polarized growth during several stages of its life cycle, and growth occurs at defined positions on the cell surface. Selection of bud sites is determined by the mating type of the cell and whether it was last a mother or daughter cell (Madden and Snyder, 1998). Haploid cells bud axially (i.e. at the proximal pole); mother cells form a new bud adjacent to the previous bud site, and daughter cells bud next to the birth site. Diploid cells have a different budding pattern. Diploid daughters bud opposite the birth site (distal pole), and diploid yeast form mother cells bud in a bipolar pattern, where new buds form equally either at the proximal or at the distal pole (Chant and Pringle, 1995; Freifelder, 1960). Yeast cell polarity is affected by external cues, e.g. nutrients or stress conditions. Diploids exposed to nitrogen starvation switch their budding pattern from bipolar to unipolar, where most of the buds emerge from the distal pole (Gimeno *et al.*, 1992; Kron *et al.*, 1994). The unipolar distal budding pattern facilitates the production of the long chains of cells resembling hyphae that spread over and invade into the growth substrate.

Establishment of cell polarity can be divided into three basic steps, which can be separated genetically from each other (Bähler and Peter, 2000). First, the cell has to choose a spatial site of polarization on its surface, the landmark. Second, the landmark is recognized by a series of proteins called polarity establishment proteins. Finally, these proteins recruit the machinery required to organize and polymerize the actin cytoskeleton. The polarized cytoskeleton then targets exocytosis or secretion towards the landmark, leading to polarized growth and cell morphogenesis.

The proteins involved in bud site selection can be divided into three classes (Madden and Snyder, 1998). One class of proteins is specifically required for haploid axial budding, but leaves the bipolar budding pattern of diploids unaffected. Genes of this class include *AXL1*, *BUD10/AXL2*, *BUD3*, and *BUD4* (Chant and Herskowitz, 1991; Fujita *et al.*, 1994; Halme *et al.*, 1996; Roemer *et al.*, 1996; Sanders and Herskowitz, 1996). A second class of genes is essential for both axial budding in haploids and bipolar budding in diploids. This class includes *RSR1/BUD1*, *BUD2*, and *BUD5* (Bender and Pringle, 1989; Chant *et al.*, 1991; Chant and Herskowitz, 1991; Park *et al.*, 1993). The corresponding gene products constitute a GTPase signaling module which is thought to couple the landmark to the machinery required for bud emergence. A final class of genes affects the bipolar budding

pattern of diploids, but is not required for haploid axial budding. *AIP3/BUD6*, *BUD7*, *BUD8*, *BUD9*, *BNI1*, *PEA2*, and *SPA2* belong to this class (Snyder, 1989; Valtz and Herskowitz, 1996; Zahner *et al.*, 1996). Several genes from this class have been shown to be required for pseudohyphal development (Mösch and Fink, 1997). Whereas mutations in most of these genes cause a random budding pattern in diploids, only two genes, *BUD8* and *BUD9*, shift the budding pattern from bipolar to unipolar when they are mutated (Zahner *et al.*, 1996), suggesting that Bud8p and Bud9p might act as bipolar landmark proteins.

The current knowledge about how nutritional signals lead to changes in cell polarity is very limited, because most of the studies addressing questions of cell polarity were performed under nutrient-rich conditions with cells growing in the yeast form. One study investigated the roles of Bud8p and Bud9p for regulation of bud site selection in both cell types, the yeast form and pseudohyphae (Taheri *et al.*, 2000). Both proteins are asymmetrically localized at the distal cell pole and appear to play opposing roles. Bud8p might serve to trigger bud initiation at the distal pole, whereas Bud9p might interfere with Bud8p and inhibit budding from the distal pole. Upon nitrogen starvation, the localization of Bud9p to the distal cell pole is prevented, causing the cells to bud preferentially from this pole.

Once the cell has integrated spatial cues from the budding landmarks, this information is fed to the polarity establishment machinery, which is responsible for polarization of the cytoskeleton and cellular compounds along the chosen axis. A central component for regulation of polarized growth is the actin cytoskeleton (Hall, 1998; Madden and Snyder, 1998; Pruyne and Bretscher, 2000a; Pruyne and Bretscher, 2000b). Filamentous actin is primarily organized into cortical patches and actin cables. Actin patches are localized at the cortex, primarily in the bud, and actin cables run along the length of the cell and intersect actin patches at their ends. Analysis of various temperature-sensitive actin mutants (Gabriel and Kopecka, 1995; Novick and Botstein, 1985; Novick *et al.*, 1989; Shortle *et al.*, 1984; Wertman *et al.*, 1992) has clearly demonstrated that the actin cytoskeleton promotes polarized growth mainly by guiding secretory vesicles to the sites of polarization, thereby promoting the incorporation of new cell wall material.

Actin localization is very dynamic throughout the cell cycle (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Actin localizes as a ring at the incipient bud site, and at the bud tip in apically growing cells, where it guides secretory vesicles to the cell surface. Later in the cell cycle, actin cables and patches redistribute randomly within the bud in

vegetatively growing cells. Cell growth is still restricted to the bud, but now it expands isotropically, giving rise to the typical ellipsoid shape. Interestingly, yeast form and pseudohyphal cells differ markedly with respect to actin distribution (Kron *et al.*, 1994). In pseudohyphal cells, actin patches remain polarized at the distal tip of the daughter, prolonging apical growth to generate highly elongated cells.

Several cytoskeletal proteins have been found to be required for pseudohyphal cell morphogenesis, including the cyclase-associated protein Srv2p, the fimbrin protein Sac6p, the actin-binding protein Tpm1p (tropomyosin) and the formin homology protein Bni1p (Cali *et al.*, 1998; Mösch and Fink, 1997).

It is still largely unknown how pseudohyphal signaling is coupled to the cytoskeletal machinery to induce cell elongation. Undoubtedly, the Rho-type GTPase Cdc42p together with its multiple effector proteins plays an extraordinary role in polarized growth processes (Hall, 1998; Johnson, 1999; Pruyne and Bretscher, 2000a). The roles of Cdc42p for growth and development will be discussed in a following section.

1.1.6 Substrate adhesion and invasion

An important issue in diploid filamentous and haploid invasive growth concerns cell-cell and cell-substrate interactions. Yeast form cells are completely separated after mitosis and grow as single cells. In contrast, pseudohyphal cells remain attached to each other, promoting the formation of filamentous networks of cells. Pseudohyphal cells not only show enhanced cell-cell adhesion, but they also stick to solid substrates and even invade them. Attachement of the cells to each other may provide the mechanical integrity to penetrate the surrounding matrix. Cell-cell adhesion is mediated in part by flocculins, a family of highly glycosylated glycosylphosphatidylinositol-anchored proteins. Flo11p appears to be the most important flocculin for adhesion and invasion (Lo and Dranginis, 1998). Whereas Flo11p is required for filamentous growth, related flocculins like Fig2p are not, although they can partially substitute a *flo11Δ* deletion when they are overexpressed (Guo *et al.*, 2000). The *FLO11* promoter spans at least 2.8 kb and is one of the largest promoters in the genome of *S. cerevisiae*, indicating that substrate invasion is regulated in a very complex manner. Many upstream activation and repression sequences have been identified on the *FLO11* promoter (Rupp *et al.*, 1999), and it is thought to integrate multiple inputs generated by the distinct signaling pathways.

In addition to cell-cell adhesion, a lack of mother-daughter separation following cytokinesis may contribute to filament formation. Although a complete septum is formed between mother and daughter cells at cytokinesis even in pseudohyphal filaments, it persists rather than being cleaved, maintaining a stable connection (Kron *et al.*, 1994).
In addition to Flo11p-mediated adhesion, secreted enzymes that break down structural compounds in the growth substrate might contribute to invasive growth. Expression of Pgu1p, a protein that degrades polygalacturonic acid (Madhani *et al.*, 1999), has been shown to be positively controlled by the filamentous growth MAPK pathway, and other matrix-degrading enzymes might exist as well.

1.2 Mating of haploid yeast cells

1.2.1 Pheromone response as a model to study cell fusion

Yeast cells can exist as either haploid or diploid cells. As an alternative to vegetative (mitotic) proliferation, haploid cells of the opposite mating type (**a** or α) are able to conjugate (mate) to form diploids (**a**/α cells) (Fig. 1). The mating process is initiated by small peptide pheromones that are constitutively secreted into the medium by haploid yeast cells. *MAT**a*** cells release **a**-factor, whereas *MAT*α cells release α-factor, and the two kinds of pheromone act only on cells of the opposite mating type. The mating process is complex and involves various aspects including altered transcription, cell recognition and aggregation, cell cycle arrest, polarity and morphology changes, cell fusion, nuclear fusion, and adaptation and recovery. The yeast mating-pheromone response is one of the best characterized signal transduction pathways in eukaryotic organisms (Fig. 5) (Banuett, 1998; Gustin *et al.*, 1998). As during filamentous/invasive growth, pheromone signals are transmitted via a conserved MAP kinase module. Strikingly, many of the components required for filamentous/invasive growth signaling are part of the pheromone pathway as well. These shared factors include the MAPK module, composed of Ste20p (MAPKKKK), Ste11p (MAPKKK), Ste7p (MAPKK) and Fus3p and Kss1p, two partially redundant MAPKs. Whereas the latter MAPK is specific for the filamentation-invasion pathway (Cook *et al.*, 1997; Madhani *et al.*, 1997), Fus3p appears to play the predominent role in the mating pheromone response. However, both MAPKs have been shown to share an overlapping role in mating, because cells lacking either *KSS1* or *FUS3* are able to mate (although the mating efficiency of a *fus3Δ* mutant is reduced to 10% compared to a wild-

type), whereas cells lacking both MAPKs are sterile (Cherkasova *et al.*, 1999; Elion *et al.*, 1991b; Farley *et al.*, 1999). Compared to filamentous/invasive growth, where the cells reproduce vegetatively (mitotic proliferation) and no sexual processes occur, mating is far more complex and has more drastic consequences. Although cells remain attached to each other during filamentous/invasive growth, they retain their autonomy. By contrast, during mating two cells and nuclei fuse, giving rise to a diploid cell from two haploid daughters. This process also involves genetic recombination.

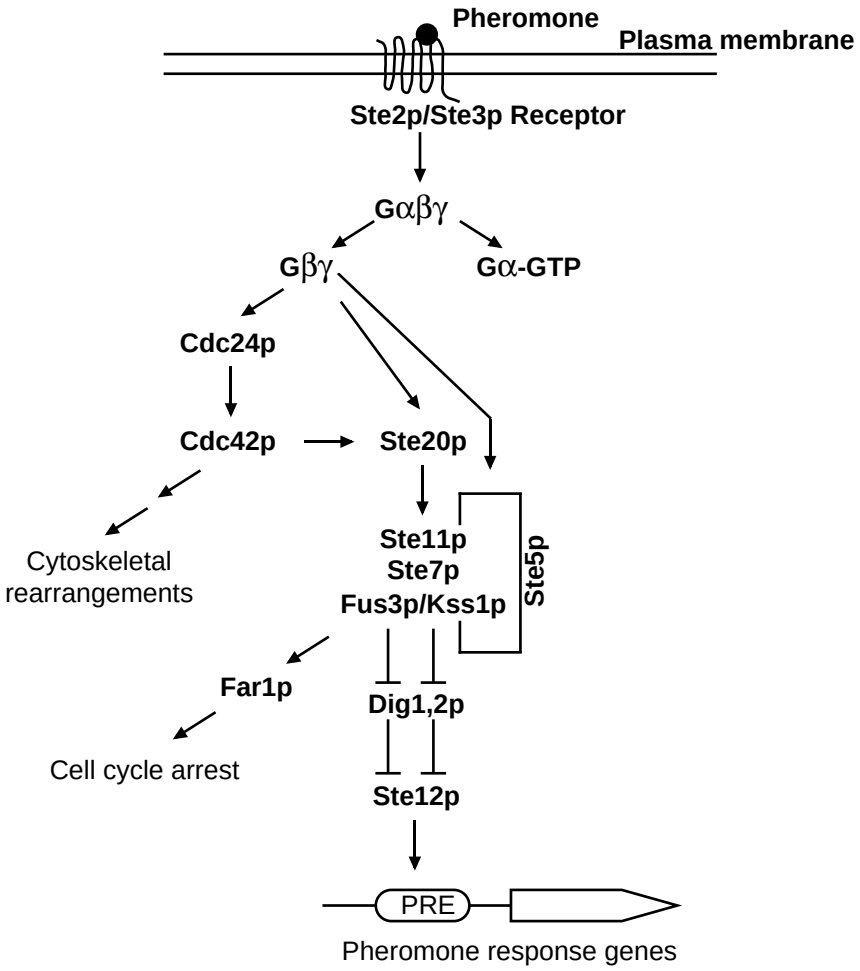

Fig. 5. Pheromone response pathway of *S. cerevisiae* (see text for details).

1.2.2 Pheromone sensing and signal transduction

The receptors for **a**- and α-factor are encoded by *STE3* and *STE2*, respectively. (Herskowitz, 1988). Both receptors belong to the seven transmembrane family, and they are both coupled to a heterotrimeric G-protein which is identical in both cell types. It consists of Gα, Gβ, and Gγ subunits, encoded by *GPA1*, *STE4*, and *STE18*, respectively (Kurjan, 1993; Marsh *et al.*, 1991). Binding of pheromone to the receptor results in exchange of GTP for GDP in the Gα subunit. Subsequently, the heterotrimeric G-protein dissociates into Gα-GTP and Gβγ subunits. The latter one is the activator of the downstream components, as a *gpa1Δ* deletion results in constitutive activation of the mating pathway and cell cycle arrest, whereas *ste4Δ* or *ste18Δ* mutants are sterile. After dissociation, the Gβγ subunit exposes a site that binds to Ste5p, a scaffold protein that interacts with and is thought to assemble Ste11p, Ste7p, Fus3p (and maybe Kss1p) into a mating-specific MAPK complex (Choi *et al.*, 1994; Kranz *et al.*, 1994; Marcus *et al.*, 1994; Printen and Sprague, 1994). The Ste5p scaffold is essential for mating-pathway activation and operates at multiple steps of the pathway. A second regulatory protein, Ste50p, plays a more ancillary role that involves the MAPKKK Ste11p and is not specific to the mating pathway or essential for signaling (Ponting, 1995; Wu *et al.*, 1999; Xu *et al.*, 1996). Ste5p is solely required for the mating MAPK cascade pathway and is expressed only in haploid cells. Therefore, Ste5p is regarded as a major specificity determinant that prevents signal spillover by tethering the kinases that constitute the mating MAPK cascade. The mechanism by which the MAPK cascade is activated is not well understood and remains an intensive area of research. Binding of Gβγ to Ste5p occurs via Gβ (Ste4p) and recruits the scaffold to the membrane where it encounters the active MAPKKKK Ste20p. Thus, Ste5p appears to play an important spatial role assembling the kinases at Gβγ dimers at the plasma membrane. Ste5p forms homo-oligomers, and there is strong evidence that oligomerization is important for signal relay from Ste11p to Ste7p (Feng *et al.*, 1998; Inouye *et al.*, 1997; Yablonski *et al.*, 1996). Ste4p binds to Ste20p and Ste5p through distinct domains (Dowell *et al.*, 1998). In contrast to Ste5p, which is rapidly recruited to the plasma membrane by the pheromone stimulus (Mahanty *et al.*, 1999), a pool of Ste20p is already at the cell cortex prior to the pheromone stimulus. Ste20p enrichement at the cell cortex depends on its interaction with Cdc42p, an essential Rho-type GTPase (Johnson and Pringle, 1990; Ziman *et al.*, 1991). Ste20p binds Cdc42p through its CRIB (Cdc42/Rac interactive binding) domain (Leberer *et al.*, 1997; Moskow

et al., 2000; Peter *et al.*, 1996; Simon *et al.*, 1995; Ziman *et al.*, 1993). Gathering of Gβγ, Ste20p, and the kinases tethered by Ste5p at the cell cortex triggers a chain reaction: Ste20p phosphorylates and thereby activates Ste11p; Ste11p, in turn, activates Ste7p; and Ste7p activates the MAPKs Fus3p and Kss1p at the bottom of the cascade. Finally, Fus3p (and maybe Kss1p) find a number of targets (transcriptional activators, cytoskeleton organizers and cell cycle inhibitors) to produce the cellular responses culminating in cell fusion and diploid formation. For many years, Fus3p has been regarded as the mating specific MAPK. This view is founded on the observation that Fus3p but not Kss1p is required for cell cycle arrest (see below) and efficient mating (Elion *et al.*, 1991a). However, recent experiments indicate that both MAPKs are *bona fide* pheromone signal transduction components. First, treatment of wild-type cells results in immediate phosphorylation and activation of both Fus3p and Kss1p (Breitkreutz *et al.*, 2001; Cherkasova and Elion, 2001; Sabbagh *et al.*, 2001). Second, pheromone stimulation induces similar overall transcription profiles in each MAPK deletion strain, so the mating defect of a *fus3Δ* strain cannot be attributed to reduced transcriptional activation (Breitkreutz *et al.*, 2001).

1.2.3 Transcriptional control

The mating MAPK pathway is important for sending signals from the pheromone receptors in the plasma membrane to gene targets in the nucleus, and pheromone signals finally lead to profound changes in the transcriptional program of a cell. Among the products of genes stimulated by pheromone are proteins that activate (e.g. Fus3p) or inhibit (e.g. Msg5p) signaling on the pheromone response pathway (Doi *et al.*, 1994; Elion *et al.*, 1990; Zhan *et al.*, 1997) and proteins needed for cell fusion (e.g. Fus1p), nuclear fusion (e.g. Kar4p) and other mating related functions (Kurihara *et al.*, 1996; McCaffrey *et al.*, 1987; Trueheart *et al.*, 1987). All these genes contain PREs (pheromone response elements) in their 5´regulatory regions, elements which are necessary and sufficient for pheromone regulated transcription (Dolan *et al.*, 1989; Hagen *et al.*, 1991; Kronstad *et al.*, 1987). Ste12p associates with pheromone-inducible promoters as a homomultimer or as a heterodimer with the Mcm1p protein if the PRE is juxtaposed to a distinct target sequence, the P box (Madhani and Fink, 1998). The MAPK cascade mediates pheromone induction of transcription of PRE-containing genes through phosphorylation and activation of at least three nuclear proteins: Dig1p, Dig2p and Ste12p (Cook *et al.*, 1996; Song *et al.*, 1991;

Tedford *et al.*, 1997). Dig1p and Dig2p are related proteins with overlapping and partially redundant functions that act as negative regulators of Ste12p and together efficiently repress the transcription of pheromone responsive genes. In unstimulated cells, Dig1p and Dig2p form a complex containing Fus3p, Kss1p and Ste12p (Cook *et al.*, 1996; Pi *et al.*, 1997; Tedford *et al.*, 1997), thereby preventing transcriptional activation by Ste12p. Pheromone stimulation increases phosphorylation of Dig1p, Dig2p and Ste12p by Fus3p and Kss1p, resulting in complex dissociation (Elion *et al.*, 1993; Tedford *et al.*, 1997). Liberated Ste12p is then competent to activate target gene transcription.

1.2.4 Cell cycle regulation

In addition to transcriptional regulation, the mating MAPK cascade is important for mediation of cell cycle arrest in response to pheromone. Pheromone-treated cells arrest in G_1 phase as unbudded cells with a 1N DNA content, and this pheromone-induced cell cycle arrest depends on the CKI (cyclin dependent kinase inhibitor) Far1p (Chang and Herskowitz, 1990; Gartner *et al.*, 1998; Peter *et al.*, 1993; Tyers and Futcher, 1993). Far1p causes cell cycle arrest by inhibiting the activity of G_1 cyclin-Cdc28p complexes which are important for G_1/S transition (Jeoung *et al.*, 1998; Peter and Herskowitz, 1994). This function of Far1p is dependent on the MAPK Fus3p, but not on Kss1p, because Far1p is much more efficiently phosphorylated by Fus3p both *in vivo* and *in vitro* (Breitkreutz *et al.*, 2001; Peter *et al.*, 1993). The mechanism by which Far1p mediates cell cycle arrest is not well understood (Gartner *et al.*, 1998). Interestingly, Far1p is not only phosphorylated (and thereby activated) by Fus3p, but also by its target G_1 cyclin-Cdc28p, and this phosphorylation seems to trigger ubiquitin-dependent degradation of Far1p (Henchoz *et al.*, 1997; Peter *et al.*, 1993; Tyers and Futcher, 1993). The preferential phosphorylation of Far1p by Fus3p contrasts with the transcriptional targets Ste12p and Dig1p that are phosphorylated by Fus3p and Kss1p to a similar extent. Because *far1Δ* strains have a severe mating defect, the inability to activate Far1p might partly explain the reduced mating efficiency of *fus3Δ* strains.

1.2.5 Regulation of cell polarity and morphology

In contrast to the predetermined budding patterns exhibited during vegetative growth, mating cells initiate projection ("shmoo") formation at novel points on the cell surface in response to pheromone signals. Therefore, the mating MAPK cascade must be tightly

coupled to the cytoskeleton. The cytoskeleton-signal transduction relationships during mating have been extensively studied in yeast. In this regard, two proteins have a central function: the small GTPase Cdc42p and Bem1p. The essential GTPase Cdc42p is required to orient the actin cytoskeleton for polarized growth during vegetative growth, for cell division, and to form mating projections (see section 2). Cdc42p interacts with a variety of different proteins that regulate actin cytoskeleton function. Like other small GTPases, Cdc42p can exist in two different conformations depending on the type of guaninnucleotide bound. GTP-bound and GDP-bound forms of Cdc42p exist in a dynamic equilibrium, and regulatory proteins control the cycling between both conformations. Exchange of GDP for GTP on Cdc42p is triggered by the GEF (guaninnucleotide exchange factor) Cdc24p (Zheng *et al.*, 1994), placing Cdc42p in an activated state. Hydrolysis of the Cdc42p-bound GTP to GDP is predicted to be regulated by the GAPs (GTPase activating proteins) Bem3p and Rga1p (Stevenson *et al.*, 1995; Zheng *et al.*, 1994). Cdc42p appears to have multiple functions in the mating response. Cdc42p interacts with Ste20p, and this interaction is necessary for proper localization of Ste20p at the shmoo tip. The Gβ subunit Ste4p interacts not only with Ste5p and Ste20p to activate the MAPK cascade, but also with Cdc24p (Nern and Arkowitz, 1998; Zhao *et al.*, 1995), and mutations in Cdc24p that block interaction with Ste4p also block chemotropic growth. Thus, the interaction of Ste4p with Cdc24p appears to locally activate Cdc42p and Cdc42p-dependent polarization functions in the vicinity of pheromone-occupied receptors. Cdc42p is linked to the actin cytoskeleton via the formin homology protein Bni1p (Evangelista *et al.*, 1997; Ozaki-Kuroda *et al.*, 2001), which in turn interacts with the actin monomer-binding protein profilin (Imamura *et al.*, 1997).

Bem1p, like Cdc42p, interacts with a large number of proteins important for the function of the actin cytoskeleton in polarized growth. Proteins found to interact with Bem1p include actin, Ste5p and Ste20p (Leeuw *et al.*, 1995; Lyons *et al.*, 1996). Bem1p colocalizes with Cdc24p and Cdc42p to growth sites, and it is thought to serve as a scaffold that promotes coupling between polarity determinants and Cdc24p-Cdc42p by directly binding both Cdc24p and shmoo-selection proteins (Ayscough and Drubin, 1998; Bender and Pringle, 1991; Chenevert *et al.*, 1992). Recruitment of Bem1p to sites of polarized growth also depends on the polarity determinant Far1p, which interacts with liberated Gβγ and helps to localize the Cdc24p-Cdc42p module (Butty *et al.*, 1998). Far1p

has a second function as a key regulator of pheromone-induced cell-cycle arrest (Chang and Herskowitz, 1990) (see above).

1.2.6 Cell-cell adhesion and fusion

Compared to the aspects described so far, much less is known about the mechanism of cell fusion. Prior to cell fusion, cells bind or clump with one another via agglutinins, glycosylphosphatidylinositol (GPI)-linked glycoproteins related to the adhesins of pathogenic fungi. The *S. cerevisiae* genome contains a family of genes encoding such cell wall proteins. In wild-type cells, each of these has a unique function in different developmental processes (mating, invasive growth, cell-cell adhesion, or filamentation). However, even distant family members can compensate for each other in diverse morphogenetic events if localized and expressed appropriately (Guo *et al.,* 2000). Mating of yeast cells is potentially a problem in a liquid environment, since cells need to stay stuck together long enough to initiate cell fusion. *MAT*α cells produce α-agglutinin and *MAT****a*** cells **a**-agglutinin. The latter one consists of two subunits, the anchorage protein Aga1p and the soluble peptide Aga2p, which are linked via disulfide bonds (Roy *et al.,* 1991; Watzele *et al.,* 1988). The **a**- and α-agglutinins bind tightly to each other in a highly species-specific manner (Crandall *et al.,* 1977; Lipke and Kurjan, 1992). Following cell-cell adhesion, cells become deformed in the region of contact and the cell wall thins. A conjugation tube develops in the region of fusion. Dissolution of the cell wall septum in a localized region of cell-cell contact permits membrane fusion and cytoplasmic mixing. A number of genes important for the fusion step of mating were identified by isolation of fusion-defective mutants. Those mutants can be divided into two classes. One class of mutants including *spa2*, *bni1*, and *tpm1* grossly disrupts the morphology and polarity of the pheromone responding cells and of vegetative cells, whereas the second class, *fus1* and *fus2*, does not. Fus1p and Fus2p are partially redundant proteins that appear to function during membrane fusion. Expression of both genes is highly inducible by mating pheromones (Elion *et al.,* 1990; McCaffrey *et al.,* 1987; Trueheart *et al.,* 1987). Mating defects are most severe when a *fus1 fus2* double mutant is crossed with another *fus1 fus2* double mutant (bilateral defect). Although the functions of Fus1p and Fus2p remain unclear, they have been proposed to mediate cell fusion indirectly as scaffolds to direct the assembly of other proteins that do directly mediate cell fusion. Alternatively, they might be

involved in targeting or controlling exocytic or endocytic processes required for cell fusion.

1.3 Comparison of pheromone response and filamentous/invasive growth: a paradigm to study signaling specificity

In contrast to the conserved MAPK module which participates in both pathways, mating and filamentation/invasion, the upstream components required for detection and transmission of the corresponding stimuli in both pathways are completely different. It is a central question how different stimuli use common proteins to elicit distinct outcomes, or, in other words, how signaling specificity is maintained and inappropriate crosstalk between both cascades is avoided. At least four different mechanisms appear to contribute to specificity (Breitkreutz and Tyers, 2002; Pryciak, 2001; Ptashne and Gann, 2003). (i) Scaffold proteins provide local activation platforms to insulate different MAPK pathways. (ii) MAPKs actively contribute to pathway specificity. (iii) Negative feedback loops restrict MAPK cascades. (iv) MAPKs possess intrinsic substrate specificity.

As described above, Ste5p serves as a scaffold for the mating MAPK cascade. Similarly, the MAPKK Pbs2p functions as a scaffold in the HOG (high-osmolarity glycerol) pathway (Posas and Saito, 1997). For the invasive/filamentous growth MAPK cascade, a scaffold protein has not been identified until now. Artificially constructed fusions between Ste11p (the MAPKKK of both the pheromone and the HOG pathway) and either of the scaffold proteins Ste5p or Pbs2p differentially induce either a mating or a high osmolarity response by specifically activating the corresponding MAPK, Fus3p or Hog1p, respectively (Harris *et al.*, 2001). Thus, scaffolds actively channel signals towards the appropriate MAPK. Moreover, scaffolds recruit MAPK complexes to specific subcellular sites (see above) and contribute to the spatial organization of MAPK signaling.

Scaffolding is not the only mechanism to ensure pathway specificity. The MAPKs themselves seem to prevent signal spillover to other pathways. Fus3p and Kss1p are equally activated by exposure to pheromone (Breitkreutz *et al.*, 2001; Sabbagh *et al.*, 2001). Nevertheless, Kss1p does not induce filamentation genes in response to α-factor unless the *FUS3* gene is deleted, indicating that Fus3p prevents crosstalk. Recent biochemical experiments have clearly demonstrated that Fus3p directly interferes with activation of Kss1p, although the mechanism is still unclear. Kss1p activation by α-factor was significantly increased in a *fus3Δ* strain, so Fus3p appears to limit activation of Kss1p,

thereby blocking the filamentation pathway after prolonged pheromone treatment (Sabbagh *et al.*, 2001).

Further contributions of the MAPKs to maintenance of signaling specifity involves negative feedback loops within the MAPK modules. For instance, Fus3p phosphorylates the upstream components Ste7p and Ste11p (Breitkreutz *et al.*, 2001; Zhou *et al.*, 1993), and Ste11p is degraded in a MAPK-dependent manner after pheromone induction (Esch and Errede, 2002). Possibly, Fus3p-mediated degradation of Ste11p prevents it from spilling over into other pathways. Moreover, this negative feedback mechanism might ensure a transient biological response, allowing the cells to recover from pheromone exposure.

Finally, inherent specificities of the MAPKs towards particular substrates (e.g. in case of Fus3p towards Far1p, see above) appear important for MAPK signaling specificity. All MAPKs phosphorylate very similar motifs with the minimal consensus sequence Ser/Thr-Pro (Countaway *et al.*, 1989), so additional specificity determinants must exist. Many substrates interact with MAPKs through conserved docking sites (Sharrocks *et al.*, 2000) which recruit the kinase to the correct substrate and enhance their fidelty and efficiency of action. However, the knowledge on these domains in yeast MAPKs is very limited.

2. Regulation of cell growth and cellular differentiation by the essential Rho-type GTPase Cdc42p

2.1 General properties of small GTP-binding proteins

Small GTP-binding proteins (also referred to as small G proteins or GTPases) are monomeric proteins with a molecular mass of 20-30 kDa. They are highly conserved and exist in all eukaryotes from yeast to human. They constitute a superfamily of GTPases that all act as molecular switches, yet they regulate an astonishing diversity of cellular functions (Takai *et al.*, 2001). Based on structural relationships, they can be clustered into five main groups: Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf, and Ran subfamilies. Members of the Ras subfamily mainly regulate gene expression; the Rho/Rac/Cdc42 subfamily members regulate both cytoskeleton reorganization and gene expression; the Rab and Sar1/Arf family members regulate intracellular vesicle trafficking; and the Ran family members regulate nucleocytoplasmic transport during the G_1, S, and G_2 phases of the cell cycle and microtubule organization during the M phase (Matozaki *et al.*, 2000).

All small GTPases function as molecular switches that can be present in either of two interconvertable forms, being inactive when bound to GDP and active when bound to GTP (Fig. 6). An upstream signal stimulates the dissociation of GDP from the GDP-bound form, which is followed by the binding of GTP. Exchange of GTP for GDP induces a conformational change, which allows the effector domain to interact with downstream effectors to induce the appropriate cellular response. The GTP-bound form is converted by the action of the intrinsic GTPase activity to the GDP-bound form, which then releases the bound downstream effector(s). The rate-limiting step of the GDP/GTP exchange reaction is the dissociation of GDP from the GDP-bound form. This reaction is extremely slow on its own, but can be stimulated by guanine-nucleotide exchange factors (GEFs), one of three classes of regulatory proteins identified so far that control the nucleotide state of small GTPases. The activity of the GEF is often stimulated by an upstream signal. The GTPase activating proteins (GAPs) constitute another family of regulators, which increase the intrinsic GTPase activity, thus favoring the conversion from the GTP- to the GDP-bound form. Whereas regulation of GTP/GDP-cycling by GEFs and GAPs is common to all small GTPases, only the Rho/Rac/Cdc42 and Rab proteins are furthermore controlled by a third type of regulators, named guanine-nucleotide dissociation inhibitors (GDIs). They bind

preferentially to the GDP-bound form and prevent nucleotide release, thereby maintaining the GTPase in the inactive state.

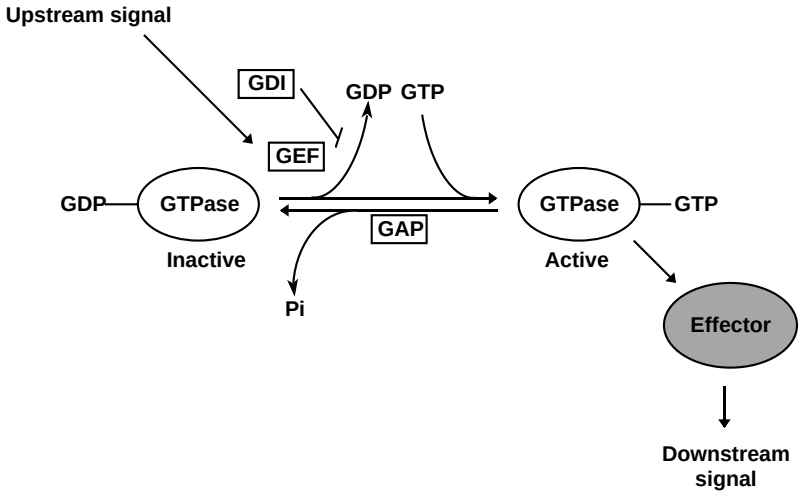

Fig. 6. Regulation of small G protein (GTPase) activity. Small G proteins function as molecular switches, being inactive when bound to GDP and active when bound to GTP. Three types of regulatory proteins are known to control the interconversion. Upon receipt of an upstream signal, the GEFs (**g**uanine nucleotide **e**xchange **f**actors) promote the release of GDP from the GTPase, being immediately replaced by GTP. The activated G protein specifically interacts with downstream effctors to transduce the signal. By stimulating the intrinsic GTPase activity of the small G protein, GAPs (**G**TPase **a**ctivating **p**roteins) participate in the conversion of the G protein to its inactive GDP-bound form. GDIs (**g**uanine nucleotide **d**issociation **i**nhibitors) bind predominantly to the GDP-bound conformation, thereby inhibiting the GDP/GTP exchange and keeping the G protein inactive.

2.2 Functions of the Rho-type GTPase Cdc42p in *S. cerevisiae*

2.2.1 Structure and functional domains of Cdc42p

Cdc42p belongs to the Rho subfamily of the Ras superfamily of small GTPases. Together with the other Rho-type GTPases belonging to the same class (Rho1-4p), Cdc42p plays an essential role in regulating the signal transduction pathways that control the generation and maintenance of cell polarity. The major functions of Cdc42p seem to be in regulating the rearrangements of the actin cytoskeleton in response to extracellular and intracellular

signals as well as in modulating protein kinase cascades that result in the transcriptional activation of genes required for growth control and other processes. The Cdc42p GTPase was initially identified from a *S. cerevisiae* mutant strain carrying a temperature-sensitive (*ts*) mutation, *cdc42-1*^{ts}, that affected bud formation at the restrictive temperature and resulted in formation of greatly enlarged, unbudded cells (Adams *et al.*, 1990). Staining of those mutants with rhodamine phalloidin showed that the cells had severe defects in polarized organization of the actin cytoskeleton, whereas isotropic growth was not affected. Due to its significant role in cell polarization, *S. cerevisiae cdc42* null mutants are inviable (Johnson and Pringle, 1990). Structural and functional Cdc42p homologs have been identified in a large number of eukaryotes (Johnson, 1999), and the high degree of identity (80 to 95%) in the predicted amino acid sequences highlights the conservation and significance of Cdc42p proteins among different eukaryotic organisms (Fig. 7). The similarity of Cdc42p to other GTPases of the Ras superfamily is only ~40%, but it is clustered in several important domains. Mutational analysis of Cdc42p and determination of the solution structure of Cdc42 from *Homo sapiens* (Cdc42Hs) by nuclear magnetic resonance (NMR) spectroscopy (Feltham *et al.*, 1997), along with the determination of the X-ray crystal structure of Cdc42Hs in complex with various regulatory or effector proteins (Garrard *et al.*, 2003; Hoffman *et al.*, 2000; Nassar *et al.*, 1998; Rossman *et al.*, 2002) have greatly aided in defining functional domains within Cdc42p (Fig. 7). Four domains are implicated in binding and hydrolysis of GTP, and their structure is highly conserved among GTPases of the different subfamilies. Two *cdc42* point mutations analyzed early in *S. cerevisiae* (Ziman *et al.*, 1991) were mapped to these sites, *cdc42*^{G12V} and *cdc42*^{Q61L}. They are both lethal to yeast because they reduce the intrinsic GTPase activity, thereby shifting the mutant protein to an activated, GTP-bound form which constitutively interacts with downstream effectors. According to current knowledge, GTPases bind to GEFs when in the nucleotide-free or GDP-bound state and bind to GAPs and downstream effectors when in the GTP-bound state. Two domains of Cdc42p called switch I (also referred to as effector domain) and switch II undergo the most significant conformational changes upon binding the different guanine nucleotides, suggesting that these regions are important specificity determinants required for differential interaction with downstream effectors and regulatory proteins. This hypothesis has been corroborated by the analysis of interaction of many different point-mutated Cdc42p variants with downstream effectors (Davis *et al.*, 1998; Mösch *et al.*, 2001; Richman and Johnson, 2000; Richman *et al.*, 1999). However,

mutations in the switch I domain not only affect binding to downstream effectors of Cdc42p, but also interaction with the GEF of Cdc42p, Cdc24p (Davis *et al.*, 1998).

Another important domain of Cdc42p is the Rho insert domain, which is unique to members of the Rho subfamily of Ras GTPases. This domain mediates interaction with one of the Cdc42p effectors, Iqg1 (Osman and Cerione, 1998). Moreover, the Rho-insert domain is, at least in the human homologue, important for interaction with its GDI (Wu *et al.*, 1997b).

Fig. 7. Sequence alignment of Cdc42p from *S. cerevisiae* (Cdc42Sc) and from human (Cdc42Hs). Vertical lines indicate identical residues. Known GTP-binding/hydrolysis domains (GTP/GDP), switch I and switch II domains, and the Rho-insert domain are underlined.

With two exceptions, all Cdc42p proteins identified so far contain the C-terminal Cys-Xaa-Xaa-Leu sequence. This conserved domain is necessary for proper membrane anchorage. Therefore, Cdc42p is prenylated with a C_{20} geranylgeranyl isoprene group at its C-terminal Cys residue (Cys^{188}). This modification is dependent on geranylgeranyltransferase β-subunit encoded by *CDC43* (Finegold *et al.*, 1991). Membrane anchorage of Cdc42p after geranylgeranylation on Cys^{188} is thought to be followed by proteolytic cleavage of the last three amino acids and carboxyl methylation of the now C-terminal Cys residue. A second localization determinant is a polylysine region found next to the Cys^{188} residue. This

positively charged stretch might interact with negatively charged components of the membrane. Cdc42p is present in two cellular pools in yeast (Miller and Johnson, 1994; Ziman *et al.*, 1993). The majority of Cdc42p was found in the particulate fraction, but up to ~20% were found in a soluble pool. This fraction is supposed to be either nonprenylated or complexed with the Rho-GDI Rdi1p (Koch *et al.*, 1997; Masuda *et al.*, 1994). Immunofluorescence and immunoelectron microscopy studies revealed that Cdc42p localized to the plasma membrane at sites of polarized growth, i.e. to the tips of growing buds and of mating projections in pheromone-treated cells (Ziman *et al.*, 1991; Ziman *et al.*, 1993). Moreover, a GFP-Cdc42p fusion also localized to internal (vacuolar, nuclear) membranes and to the mother-bud neck region, suggesting that Cdc42p also plays a role in cytokinesis and/or septation (Richman *et al.*, 2002).

2.2.2 Cell cycle functions of Cdc42p

A central issue regarding regulation of cell polarity concerns the temporal and spatial control of Cdc42p localization at the cell cortex throughout the cell cycle. Cytoskeletal polarity is guided in yeast by the distribution of Cdc42p and its GEF Cdc24p on the plasma membrane. Cdc42p seems to function at multiple stages of the cell cycle (Fig. 8) (Johnson, 1999). Newly synthesized Cdc42p is geranylgeranylated by the Cdc43p-Ram2p geranylgeranyltransferase I. It is bound by the Rho-GDI Rdi1p in the cytosol, thereby preventing membrane localization. In contrast, its GEF Cdc24p is already localized at the plasma membrane, suggesting that Cdc42p is in the inactive, GDP-bound state when retained in the cytosol. Initiation of bud emergence is guided by preexisting cortical cues established during previous budding events by the *BUD* gene products. These cues allow a Ras-related protein, Rsr1p (Bud1p), to bind to Cdc24p at a discrete region of the plasma membrane during early G_1 (Chant, 1999; Park *et al.*, 1999; Zheng *et al.*, 1995). Binding of Cdc24p by activated (GTP-bound) Rsr1p is supposed to activate Cdc24p, which in turn recruits Cdc42p to the nascent bud site and allows bud emergence to begin. Polarization of Cdc24p and Cdc42p depends strongly on Bem1p, which serves as a scaffold and promotes coupling between polarity determinants and the Cdc24p-Cdc42p module (Bender and Pringle, 1991; Chenevert *et al.*, 1992). Recruitment of Cdc42p to the membrane by Cdc24p frees the GDI Rdi1p, allowing membrane tethering of Cdc42p via its hydrophobic geranylgeranyl anchor. Upon activation of Cdc24p, it catalyzes the dissociation of GDP from Cdc42p, being immediately replaced by GTP. Cdc24p is released and can recycle to

the bud site or become available for nucleotide exchange later in the cell cycle. Activated, GTP-bound Cdc42p now interacts with a couple of specific effectors to form a complex that binds to the scaffold protein Bni1p, which in turn interacts with a large number of polarity-determining proteins like e.g. Pfy1p, Sph1p, Spa2p, Pea2p and Bud6p. This complex is termed polarisome (Sheu *et al.*, 1998) and mediates apical actin organization by linking Rho-GTPase signaling to actin filament assembly. Cdc42p effectors implicated in this early polarization event include Gic1p and Gic2p and at least one of the p21-activated kinases (PAKs) Ste20p, Cla4p and Skm1p. All these proteins contain a CRIB (for Cdc42/Rac interactive binding) domain which mediates high-affinity interaction with the effector domain of GTP-bound Cdc42p (Burbelo *et al.*, 1995; Thompson *et al.*, 1998). Deletion analysis has revealed that Gic1p and Gic2p play partially redundant roles in cellular morphogenesis. Whereas deletion of either gene alone did not produce an abnormal phenotype, a *gic1Δ gic2Δ* mutant displayed severe morphological abnormalities at elevated temperatures (Brown *et al.*, 1997; Chen *et al.*, 1997). Similarly, the three PAKs Ste20p, Cla4p and Skm1p seem to have partially overlapping functions in regulating actin-dependent growth during the cell cycle. While *ste20Δ* and *cla4Δ* single mutants did not die, the *ste20Δ cla4Δ* double mutant was inviable (Cvrckova *et al.*, 1995). Skm1p seems to be less important, because its loss did not show synthetic lethality with *ste20Δ* or *cla4Δ* mutations. Ste20p is the most likely PAK involved in coupling Cdc42p to the cortical actin cytoskeleton at this stage, because it is the only PAK found so far to localize to the bud tips dependent on Cdc42p (Leberer *et al.*, 1997; Peter *et al.*, 1996). The interaction of Cdc42p with Ste20p brings the PAK in close proximity to the class I myosins Myo3p and Myo5p, the only cytoskeletal substrates of PAKs identified so far in yeast (Wu *et al.*, 1997a). These molecular motors interact with the Bni1p-polarisome complex and are necessary for proper cytoskeletal organization (Geli and Riezman, 1996; Goodson *et al.*, 1996). After bud emergence, Gic2p is phosphorylated and ubiquitinated, finally leading to its degradation.

After DNA replication, apical growth of the bud switches to isotropic growth. This switch depends on activation of the Clb1p-2p/Cdc28p kinase complex and on the Cla4p PAK. Cells lacking *CLA4* generate highly elongated buds, indicating that they are deficient in switching to isotropic growth (Cvrckova *et al.*, 1995). Cla4p kinase activity peaks during G_2-M (Benton *et al.*, 1997), and the apical-isotropic switch correlates with Clb-dependent phosphorylation of Cla4p (Tjandra *et al.*, 1998). Cortical actin and Cdc42p are distributed to the sides of enlarging buds. Cdc42p is converted to the inactive conformation by the

intrinsic GTPase activity and by action of one or more GAPs (Bem1p, Rga1p, Rga2p) (Smith *et al.*, 2002). Subsequently, GDP-bound Cdc42p is again extracted from the membrane by the GDI Rdi1p. Finally, Cdc42p and its GEF Cdc24p localize to the mother-bud neck region in large-budded cells (Richman *et al.*, 2002; Toenjes *et al.*, 1999). There, Cdc42p becomes activated and interacts again with one or more of the PAKs, most likely Cla4p, as well as another effector, the IQGAP homolog Iqg1p (Epp and Chant, 1997; Lippincott and Li, 1998; Osman and Cerione, 1998). In contrast to the Gic1,2p proteins and the PAKs, Iqg1p contains no CRIB domain, but is thought to bind to Cdc42p through a GAP homology domain (GRD). Iqg1p serves as a scaffold mediating Cdc42p-actin interactions during cytokinesis. The activated PAK might now phosphorylate a number of myosin proteins (Myo1p, Myo2p, Myo4p), as well as the septins that comprise the 10 nm filament ring present at the mother-bud neck region. These interactions lead to formation of a septin-dependent actomyosin ring at the mother-bud neck region. Following anaphase, the ring contracts, leading to cytokinesis, septum formation and cell separation.

A number of further proteins have been shown to interact with Cdc42p, but the significance of those interactions is less well understood. Bee1p (Li, 1997) is a homolog of the mammalian WASP (Wiskott-Aldrich syndrome protein) and presumably serves as another scaffold linking Cdc42p and actin cytoskeleton. Zds1p and Zds2p (Bi and Pringle, 1996; Yu *et al.*, 1996) are negative regulators of Cdc42p functions that are together required for cell cycle progression. Bem4p (Hirano *et al.*, 1996; Mack *et al.*, 1996) interacts with both GDP-bound and GTP-bound Cdc42p. Moreover, it also interacts with other GTPases of the Rho-subfamily (Rho1p, Rho2p, and Rho4p). Boi1p and Boi2p bind to Cdc42p and to the scaffold Bem1p (Bender *et al.*, 1996). Far1p is a CKI (Cyclin-dependent kinase inhibitor) that is required for G_1 arrest of haploid yeast cells exposed to mating pheromone (see above) (Butty *et al.*, 1998). Msb3p and Msb4p are homologous proteins that localize to sites of polarized growth in a Cdc42p-dependent manner and seem to function downstream of Cdc42p in a pathway leading to actin organization (Bi *et al.*, 2000).

Cdc42p does not only regulate proper progression through the cell cycle by mediating rearrangements of the actin cytoskeleton. A further primary function of Cdc42p leads to transcriptional induction of target genes required during specific developmental processes (mating, filamentous/invasive growth) (see above).

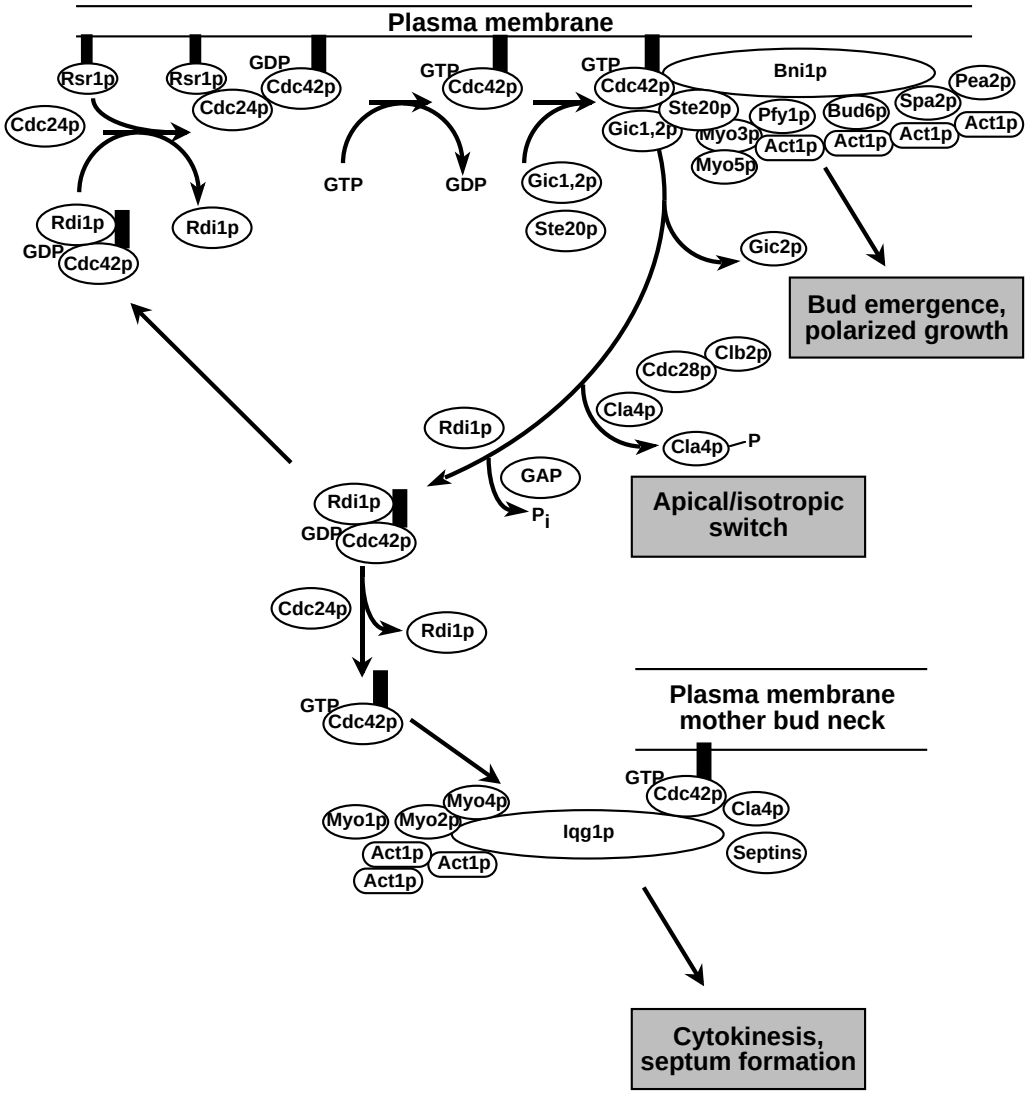

Fig. 8. Molecular model for Cdc42p-dependent processes during the *S. cerevisiae* cell cycle (see text for details).

2.2.3 Regulation of cell polarity and morphology by Cdc42p

Cell polarity is involved in almost every aspect of cell and developmental biology and is functionally important for differentiation, proliferation, and morphogenesis in single-cell as well as multicellular organisms. Establishment of cell polarity is a multistep process. First, a specific site at the cell cortex, the landmark, is chosen as the future site of polarization. This can either be a future bud site in vegetatively growing cells, or in haploid cells in response to pheromone a site where the mating projection will be formed. Secondly, specific polarity establishment components required for actin or microtubule polymerization are recruited to this site. Finally, the polarized cytoskeleton targets exocytosis or secretion towards the landmark, leading to various forms of polarized growth

and cell morphogenesis. The polarization machinery must be disassembled and inactivated to end the polar growth phase. Cdc42p is a central component of the polarity establishment machinery, and a key polarizing event is the recruitment of Cdc42p to growth sites on the plasma membrane, where the GTPase activates effectors that signal to the actin cytoskeleton (Ziman *et al.*, 1993). An overview of Cdc42p- and actin distribution at the cell cortex during the mitotic cell cycle is given in Figure 9 (Pruyne and Bretscher, 2000a).

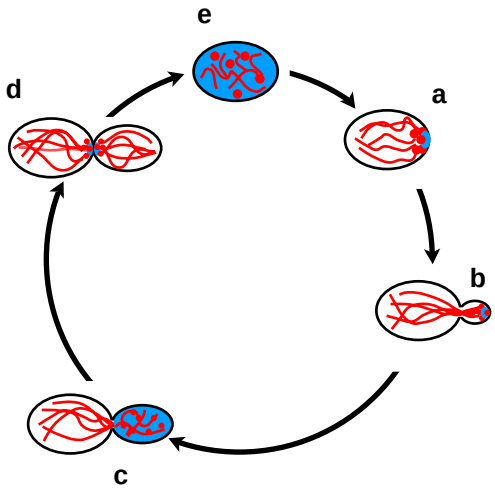

Fig. 9. Temporally and spatially regulated membrane recruitment of the Rho GTPase Cdc42p triggers polarization of the actin cytoskeleton throughout the cell division cycle. Cdc42p is shown in blue; the major actin structures, cortical patches and cables, are shown in red. (a) After the cell passes the START checkpoint, Cdc42p is recruited to the nascent bud site and orients the actin cytoskeleton. (b) The actin cytoskeleton guides secretory vesicles to the cell surface at the bud tip, where they accumulate and lead to apical bud growth. Cdc42p and the actin cytoskeleton remain polarized during apical growth phase. (c) Cdc42p redistributes over the bud surface, redirecting bud growth isotropically. When bud growth is completed, patches and cables disorganize, a cytokinetic ring forms, then contracts and disassembles after mitosis (not shown). (d) Cdc42p localizes to the mother bud neck region and reorients actin and growth between the two cells to generate new cell walls. (e) The new daughter undergoes a period of undirected growth until budding is resumed.

After entry into a new cell cycle, yeast cells select a nonrandom bud site in G_1. Clustering of Cdc42p and proteins related to its function to this site directs cortical actin patches and actin cables towards the nascent bud site. In turn, the actin cytoskeleton guides secretory vesicles to the cell surface, where they accumulate and fuse, thus polarizing growth. At the beginning, the new formed bud grows apically (from the tip). Later on during G_2-M, Cdc42p and the actin cytoskeleton are redistributed over the bud surface, thus redirecting growth isotropically, finally giving rise to the ellipsoidal bud shape. After completion of bud growth, actin patches and cables disorganize and redistribute randomly in the mother and bud while a cytokinetic F-actin ring assembles at the bud neck, contracts and disassembles. (Field *et al.*, 1999). Following cytokinesis, actin and growth are repolarized by Cdc42p to the former bud neck to direct synthesis of cell walls between the two new cells. Following cell separation, the mother cell resumes budding immediately, whereas the daughter undergoes a period of undirected growth until it reaches a critical size to enter a new cell cycle. In addition to vegetative growth, yeast cells also polarize growth in a Cdc42p-dependent manner during mating to produce the characteristic mating projections.

2.2.4 Regulation of cellular differentiation by Cdc42p

Besides its general functions for cell polarity and morphology, Cdc42p has some specialized signaling functions critical for cellular differentiation. During the filamentous/invasive growth response, Cdc42p serves to couple the Ras2p small GTPase to the filamentation/invasion MAPK cascade, and activated alleles of *CDC42* (*CDC42*^{Val12} and *CDC42*^{Leu61}) induce filamentous growth and expression of an FRE reporter gene (Mösch *et al.*, 1996). Ste20p mutants defective for binding of Cdc42p are impaired for pseudohyphal growth and agar invasion (Peter *et al.*, 1996). Recently, novel Cdc42p mutant proteins with single amino acid substitutions were isolated that uncoupled essential Cdc42p functions from regulatory functions required for pseudohyphal development and invasive growth (Mösch *et al.*, 2001).

The views on the question whether Cdc42p actually participates in pheromone signal transduction are not uniform. Initial studies pointed to Cdc42p as a part of this pathway, because temperature sensitive *cdc42* mutants have defects in maintaining G_1 arrest at the restrictive temperature and in α-factor stimulated transcription of *FUS1*, a mating-specific gene (Simon *et al.*, 1995; Zhao *et al.*, 1995). Later on, the participation of Cdc42p in pheromone signaling was called in question, because mutant Ste20p variants lacking the

CRIB domain and therefore defective in interaction with Cdc42p were reported to be wild-type with regard to typical mating responses (Leberer *et al.*, 1997; Peter *et al.*, 1996). Recent evidence suggests that the CRIB domain in PAK family kinases is part of an autoinhibitory domain, leading to a model in which Cdc42p activates Ste20p (or other PAK family kinases) by relief of autoinhibition (Bagrodia and Cerione, 1999; Tu and Wigler, 1999; Zenke *et al.*, 1999). Moreover, the role of Cdc42p within pheromone signal transduction is supported by the isolation of novel viable pheromone-resistant *cdc42* alleles that retain the ability to perform other, polarity-related functions (Moskow *et al.*, 2000).

3. Regulation of cellular differentiation by the TEA/ATTS family transcription factor Tec1p

3.1 General properties of TEA/ATTS family transcription factors

Many of the transcriptional regulatory proteins known so far can be grouped into distinct classes based on conservation of their primary sequence. Various structural motifs have been identified in these factors that mediate binding to specific DNA sequences. These motifs include zinc finger, bZIP, bHLH, helix-turn-helix, homeobox and POU domains. In 1991, two groups independently realized that the human SV40 and papillovirus-16 enhancer factor TEF-1 (Xiao *et al.*, 1991), the developmental regulator AbaAp from *Aspergillus nidulans* (Mirabito *et al.*, 1989; Sewall *et al.*, 1990), and the yeast regulator of *Ty1* transcriptional activity Tec1p (Laloux *et al.*, 1990) share a sequence motif with significant similarity (Andrianopoulos and Timberlake, 1991; Bürglin, 1991). This previously unidentified DNA-binding motif was termed TEA domain (TEF-1, Tec1p, AbaAp) and ATTS domain (AbaAp, Tec1p, TEF-1 sequence), respectively.

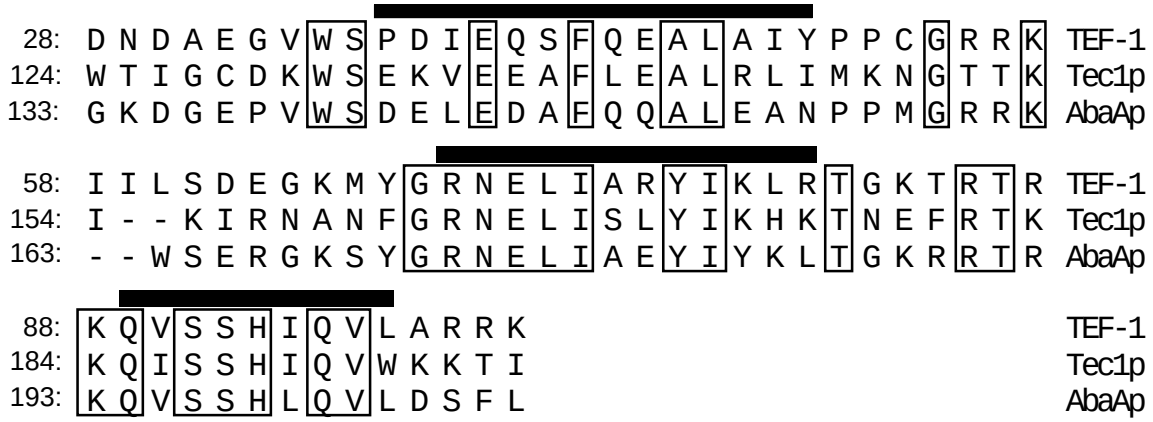

Fig. 10. Protein sequence alignment of the TEA/ATTS DNA-binding domains of human TEF-1, *S. cerevisiae* Tec1p, and *A. nidulans* AbaAp. Identical amino acids are boxed. Predicted α-helices are shown above the sequence as black bars.

The regions of highest sequence conservation span about 70 amino acid residues and are located toward the amino terminus in all three proteins (Fig. 10). Identities are between 44% and 65% in this region. Outside the TEA/ATTS motif is little or no sequence similarity. A crystal structure of a TEA/ATTS domain protein has so far not been determined. Many DNA-binding domains use an α-helix to contact DNA, and three α-helices have been predicted for the TEA/ATTS domain (Bürglin, 1991).

Since the initial characterization of the TEA/ATTS motif, several novel members from vertebrates, nematodes, insects and fungi have been identified. Most of these transcription factors regulate the expression of stage-specific genes. For instance, *Drosophila melanogaster* Scalloped protein is required for sensory organ differentiation (Campbell *et al.*, 1992). M-CAT binding factor (MCBF) from chicken is a regulator of cardiac troponin T gene expression (Farrance *et al.*, 1992). AbaAp from *A. nidulans* is required for asexual spore formation (Mirabito *et al.*, 1989), and a homologous protein from *Penicillium marneffei* participates in conidiation and dimorphic growth (Borneman *et al.*, 2000).

These transcription factors recognize and bind short DNA sequences. Two distinct target sites are known for human TEF-1, the GT-IIC site (5′-GTGGAATGT-3′) and the similar Sph-I and Sph-II sites (5′-AAGCATGCA-3′ and 5′-AAGCATGCA-3′) (Davidson *et al.*, 1988). Chicken MCBF binds to the sequence 5′-CATTCCT-3′ (Stewart *et al.*, 1994), and the TEA consensus sequence (TCS) in a fungal TEA/ATTS transcription factor target promoter has been defined as 5′-CATTCCY-3′, were Y is a pyrimidine (Andrianopoulos and Timberlake, 1994).

3.2 Functions of Tec1p in *S. cerevisiae*

3.2.1 Regulation of *Ty* expression

Tec1p (*Ty 1* enhancement control) was originally identified as a regulator of *Ty1* retrotransposon insertions (Laloux *et al.*, 1990). Retrotransposons are a class of repetitive mobile elements which transpose via the reverse transcription of an RNA intermediate (Boeke *et al.*, 1985). These eukaryotic elements are abundant, widespread and hypothesized to be of major evolutionary significance. There are five families of retrotransposons in yeast, *Ty1*-*Ty5*. Whereas Tec1p is required as trans-acting factor for full *Ty1* expression and *Ty1*-mediated gene activation, it had only little impact on *Ty2* transcript levels (Laloux *et al.*, 1990). Tec1p alone is not sufficient to activate *Ty1*-

mediated transcription of adjacent genes, but requires cooperative interaction with a second transcription factor, Ste12p. The sequences in *Ty1* required for *TEC1*- and *STE12*-dependent activation were defined by replacing the UAS element of a *TDH3::lacZ* reporter by different portions of *Ty1*. By this means, a sequence called SRE (sterile response element) which contained a Ste12p-binding site (PRE) and an adjacent TCS element was identified to be sufficient for transcriptional activation (Baur *et al.*, 1997; Laloux *et al.*, 1994). The combination of a PRE and a TCS element was later termed FRE (**f**ilamentation and invasion **r**esponse **e**lement, see below).

3.2.2 Transcriptional control of filamentous and invasive growth

In *S. cerevisiae*, filamentous and invasive growth are activated via a conserved MAPK signal transduction cascade. Signaling through this cascade leads to activation of two transcription factors, Tec1p and Ste12p, which bind as heterodimer in a cooperative manner (also termed combinatorial control) to FRE sites composed of a TCS and an adjacent PRE (Madhani and Fink, 1997). FREs have been found in the promoters of genes involved in filamentous and invasive growth and include *FLO11* encoding a cell surface flocculin (Lo and Dranginis, 1998; Rupp *et al.*, 1999) and *TEC1* itself. *TEC1* and *STE12* are both required for proper execution of these developmental programs (Gavrias *et al.*, 1996; Liu *et al.*, 1993; Mösch and Fink, 1997; Roberts and Fink, 1994). The concerted action of Tec1p together with Ste12p for regulation of differentiation distinguishes *S. cerevisiae* from other fungi. The Tec1p homologue from *C. albicans* (CaTec1p) regulates target genes, serum-induced hyphal growth, and virulence primarily by a mechanism that does not require the Ste12p homologue CaCph1p (Lane *et al.*, 2001; Liu, 2001; Schweizer *et al.*, 2000) Likewise, *A. nidulans* AbaAp activates target gene expression and conidiophore development independently of SteAp, a protein with similarity to Ste12p (Vallim *et al.*, 2000). Instead, SteAp is required for sexual development. *A. nidulans* AbaAp is the best studied fungal TEA/ATTS domain transcription factor with respect to DNA-binding and the mechanism of transcriptional activation (Andrianopoulos and Timberlake, 1994). *In vitro* binding studies revealed that a single AbaAp target sequence was sufficient for efficient binding of recombinant AbaAp. In a heterologous *S. cerevisiae* expression system, AbaAp clearly activated a reporter construct containing AbaAp binding sites fused to an enhancerless *CYC1-lacZ* reporter. However, there was a two-site requirement for efficient reporter activation whereas a single site had been sufficient to

mediate *in vitro* DNA binding. Although AbaAp contains a potential leucine zipper dimerization domain (Mirabito *et al.*, 1989), it does not appear to function as a dimer.

The view that Tec1p functions in *S. cerevisiae* exclusively in cooperation with Ste12p to regulate target gene expression and differentiation is partly due to the fact that *TEC1* expression is controlled by Ste12p via a cluster of PREs present in the *TEC1* promoter. This leads to a 20-fold reduction of *TEC1* transcription in strains lacking *STE12* (Oehlen and Cross, 1998). However, a number of genes reported to be transcriptionally controlled by Tec1p (Madhani *et al.*, 1999) do not contain FREs in their promoter region. Instead, they often contain several TCS elements without adjacent PREs, raising the possibility that Tec1p also functions by a mechanism distinct from combinatorial control, just like other TEA/ATTS family members.

4. Aim of this work

The aim of this work was to characterize the roles of two conserved proteins, the essential small GTPase Cdc42p and the TEA/ATTS domain transcription factor Tec1p, for regulation of cellular differentiation in the yeast *S. cerevisiae*. By random mutagenesis, a pool of *cdc42* alleles was created and introduced into haploid and diploid yeast strains lacking a wild-type *CDC42* copy. Subsequently, these strains were screened for developmental mutants to explore whether it is possible to uncouple essential functions of Cdc42p required for cell cycle progression from regulatory functions required for pseudohyphal development and invasive growth. The isolated *cdc42* alleles and the corresponding yeast strains were analysed in detail by sequencing, cell biological, genetical, and biochemical methods to elucidate how they affect cellular differentiation.

The transcription factor Tec1p shares the conserved TEA/ATTS DNA-binding domain with several other eukaryotic transcription factors, like e.g. AbaAp from the filamentous fungus *Aspergillus nidulans*. Here, the question whether the mode of activation of target genes and cellular differentiation by Tec1p is also conserved was addressed. Moreover, the regulation of *TEC1* expression by the MAPKs Kss1p and Fus3p on transcriptional and postranscriptional levels was analysed, revealing a surprising link between the pheromone and filamentation/invasion signal transduction pathways.

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Chapter 2

Different domains of the essential GTPase Cdc42p required for growth and development of *S. cerevisiae*

ABSTRACT

In budding yeast, the Rho-type GTPase Cdc42p is essential for cell division and regulates pseudohyphal (PH) development and invasive growth. Here, we isolated novel Cdc42p mutant proteins with single amino acid substitutions that are sufficient to uncouple functions of Cdc42p essential for cell division from regulatory functions required for pseudohyphal development and invasive growth. In haploid cells, Cdc42p is able to regulate invasive growth dependent and independent of *FLO11* gene expression. In diploid cells, Cdc42p regulates pseudohyphal development by controlling PH cell morphogenesis and invasive growth. Several of the Cdc42p mutants isolated here block PH cell morphogenesis in response to nitrogen starvation without affecting morphology or polarity of yeast-form cells in nutrient rich conditions, indicating that these proteins are impaired for certain signaling functions. Interaction studies between developmental-specific Cdc42p mutants and known effector proteins indicate that apart from the p21-activated (PAK)-like protein kinase Ste20p, the Cdc42p/Rac-interactive-binding (CRIB) domain containing Gic1p and Gic2p proteins and the PAK-like protein kinase Skm1p might be further effectors of Cdc42p that regulate pseudohyphal and invasive growth.

INTRODUCTION

The Rho-type GTPase Cdc42p is a member of the Ras superfamily of small GTP-binding proteins that play an essential role in regulating proliferation and differentiation in all eukaryotes (reviewed by Johnson, 1999; Mackay and Hall, 1998). In *Saccharomyces cerevisiae*, Cdc42p has been implicated in the regulation of diverse processes essential both for cell division and cellular development, and a great number of genetic and biochemical studies have shown that the major roles of Cdc42p in these processes are regulation of actin rearrangements and modulation of protein kinase cascades (reviewed by Johnson, 1999).

Cdc42 proteins act as molecular switches that by exchange of GTP for GDP are placed in an activated, signaling state, which is terminated by the hydrolysis of GTP to GDP (Mackay and Hall, 1998). Cdc42p must interact with a variety of regulators and downstream effector proteins to constitute a functional GTPase signaling module (Johnson, 1999). Regulation is mediated by guanine nucleotide exchange factors (GEFs), which in *S. cerevisiae* are represented by Cdc24p (Sloat *et al.*, 1981; Zheng *et al.*, 1994), and by GTPase activating proteins (GAPs) that in yeast comprise Bem3p and Rga1p (Chen *et al.*, 1996; Stevenson *et al.*, 1995; Zheng *et al.*, 1994). A growing number of Cdc42p downstream effector proteins are known that interact with the GTP-bound (activated) form and thereby mediate numerous downstream events. In *S. cerevisiae*, Cdc42p effectors include the p21-activated (PAK) family protein kinases Ste20p, Cla4p, and Skm1p (Benton *et al.*, 1997; Cvrckova *et al.*, 1995; Eby *et al.*, 1998; Leberer *et al.*, 1997; Martin *et al.*, 1997; Peter *et al.*, 1996; Simon *et al.*, 1995; Zhao *et al.*, 1995), the formin homology (FH) proteins Bni1p and Bnr1p (Evangelista *et al.*, 1997; Fujiwara *et al.*, 1999; Fujiwara *et al.*, 1998; Imamura *et al.*, 1997; Jansen *et al.*, 1996; Umikawa *et al.*, 1998; Zahner *et al.*, 1996), the Wiskott-Aldrich syndrome protein (WASP) family member Bee1p/Las17p (Li, 1997; Naqvi *et al.*, 1998), the IQGAP homologue Iqg1p/Cyk1p (Epp and Chant, 1997; Lippincott and Li, 1998; Osman and Cerione, 1998; Shannon and Li, 1999), and the novel Gic1p and Gic2p proteins that share the conserved Cdc42p/Rac-interactive-binding (CRIB) domain (Brown *et al.*, 1997; Chen *et al.*, 1997).

The molecular mechanisms by which Cdc42p temporally and spatially discriminates between the distinct effectors are largely unknown. Helpful tools for dissecting the distinct functions of Cdc42p are effector mutant proteins that have lost the ability to bind and activate certain effectors but not others. So far, only a few such alleles have been uncovered (Li *et al.*, 1999; Owen *et al.*, 2000; Richman *et al.*, 1999). Most Cdc42p mutants that have been studied to date display defects in functions of Cdc42p that are essential for cell division, as based on their lethal or temperature sensitive growth phenotypes (Davis *et al.*, 1998; Johnson, 1999; Kozminski *et al.*, 2000; Miller and Johnson, 1997; Richman *et al.*, 1999; Ziman *et al.*, 1991). No alleles of *CDC42* have been described that separate the functions of Cdc42p required for

cell division from developmental functions, e.g. appropriate morphological and signaling responses to changes in the environment.

Pseudohyphal development is a process in which *S. cerevisiae* alters its morphology in response to nutritional signals. When starved for nitrogen, diploid *S. cerevisiae* strains undergo a developmental transition from growth as single yeast form (YF) cells to a multicellular form consisting of filaments of pseudohyphal cells (PH) (Gimeno *et al.*, 1992). This dimorphic switch, referred to as pseudohyphal development, is a composite of genetically dissectable cellular changes including alterations in the budding pattern, cell morphology and invasive growth behavior (Gimeno *et al.*, 1992; Mösch and Fink, 1997). A related phenomenon, invasive growth, occurs in haploid cells (Roberts and Fink, 1994). Pseudohyphal development and invasive growth are under the control of at least two signaling pathways. One of the routes involves the GTP-binding proteins Ras2p and Gpa2p and the cAMP-dependent protein kinase (Kübler *et al.*, 1997; Lorenz and Heitman, 1997; Ward *et al.*, 1995). Activation of the cAMP pathway stimulates expression of *FLO11* encoding a cell wall protein required for invasive growth and pseudohyphal development (Lo and Dranginis, 1998; Rupp *et al.*, 1999). A second pathway that regulates pseudohyphal development and invasive growth involves Cdc42p (Mösch *et al.*, 1999; Mösch *et al.*, 1996; Roberts *et al.*, 1997). In this pathway, Ras2p is thought to signal via Cdc42p and Ste20p to the Kss1p MAPK cascade that shares several components with the MAPK cascade required for mating (Liu *et al.*, 1993). The role of Cdc42p in this signaling pathway was deduced from several studies. Dominant activated $Cdc42^{G12V}$ and $Cdc42^{Q61L}$ proteins not only induce pseudohyphal development, but also stimulate expression of genes controlled by the Kss1p MAPK cascade (Mösch *et al.*, 1996). Expression of the dominant negative $Cdc42^{D118A}$ mutant protein inhibits Ras2p-dependent activation of pseudohyphal development, and expression of $Cdc42^{G12V}$p or $Cdc42^{Q61L}$p rescues defective invasive growth of haploid *ras2Δ* mutant strains, placing Cdc42p downstream of Ras2p (Mösch *et al.*, 1999). Dominant active $Cdc42^{G12V}$p and $Cdc42^{Q61L}$p require Ste20p for activation of the Kss1p MAPK pathway, and Cdc42p-Ste20p interactions depend on the CRIB domain of Ste20p, placing Cdc42p upstream of Ste20p (Mösch *et al.*, 1996; Peter *et al.*, 1996). However, all studies investigating the function of Cdc42p in pseudohyphal development involved dominant active or inactive variants that also affect functions essential for cell division.

In this study, we isolated novel mutant alleles of *CDC42* with the goal to separate functions of Cdc42p required for pseudohyphal and invasive growth from those required for cell division. We find that single amino acid substitutions within Cdc42p are sufficient to uncouple functions required for cell division from that regulating cellular development. Several of the Cdc42p mutants isolated here block pseudohyphal cell morphogenesis in response to nitrogen starvation, but do not affect morphology or polarity of the yeast-form in nutrient rich conditions, indicating that these proteins are signaling rather than general morphological mutants. Interaction studies between these developmental-specific Cdc42p mutants and an

array of known effectors indicate that apart from Ste20p, the Gic1p and Gic2p proteins and the protein kinase Skm1p might be further effectors of Cdc42p that are important for pseudohyphal and invasive growth.

MATERIALS AND METHODS

Yeast strains and growth conditions.

All yeast strains used in this study are congenic to the Σ1278b genetic background (Table I). The *cdc42Δ::HIS3* deletion mutation was introduced using deletion plasmid pME1758 (Table II). RH2197 and RH2442 are segregants of RH2441, and RH2199 was obtained by mating of RH2197 with RH2442. Standard methods for genetic crosses and transformation were used and standard yeast culture medium was prepared essentially as described (Guthrie and Fink, 1991). Synthetic complete medium (SC) lacking appropriate supplements was used for scoring invasive growth or β-galactosidase assays. Invasive growth tests were performed as described previously using solid SC medium lacking appropriate supplements (Roberts and Fink, 1994). Low ammonium medium (SLAD) was prepared as described (Gimeno *et al.*, 1992). When required, uracil was added to SLAD medium to a final concentration of 0.2 mM to make SLAD+Ura.

Plasmid constructions.

Plasmid pME1758 was created by replacement of the *CDC42*-coding sequence with the *HIS3*-selectable marker using a PCR-based 3-step cloning strategy. Plasmid pME1534 was constructed by subcloning of a 1.7-kb *CDC42* *Bam*HI-*Hin*dIII fragment from YCp(CDC42Sc) (Ziman *et al.*, 1991) into plasmid pME1533 (pTF27, from J. Hegemann, Heinrich-Heine-University, Düsseldorf, Germany). Plasmid pME1533 is a derivative of pRS316 (Sikorski and Hieter, 1989) and contains a 24 bp deletion in the *CEN6*-region that decreases mitotic stability of the plasmid (Fiedler and Hegemann, unpublished). Plasmids pME1759 and pME1760 both expressing GFP-Ste20p from the *STE20*-promoter were constructed as follows. (i) A 4.9-kb *Kpn*I-*Not*I fragment carrying *STE20* was subcloned from pRS426-STE20 (Mösch *et al.*, 1996) into pRS316 to yield plasmid pRS316-STE20. (ii) A 750-bp DNA fragment containing part of the *STE20*-promoter was amplified from pRS316-STE20 by PCR introducing a *Bgl*II site after the ATG translational start site of *STE20* and cloned into the *Eco*RV site of pBluescriptKS (Stratagene). (iii) A 510-bp fragment coding for the N-terminal portion of Ste20p was amplified by PCR introducing a *Bgl*II site in front of the second codon of *STE20* and inserted as *Bgl*II-*Xba*I fragment into the *Bgl*II and *Xba*I site of construct (ii). (iv) A 375-bp *Bam*HI-*Sph*I fragment of (iii) was then exchanged for the corresponding *Bam*HI-*Sph*I fragments in both pRS316-STE20 and pRS426-STE20 yielding plasmids pRS316-STE20-BglII and pRS426-STE20-BglII, respectively, each containing a single *Bgl*II site between the ATG translational start site and the second codon of *STE20*. (v) A 750-bp fragment containing the GFPuv variant of GFP was amplified from plasmid pBAD-GFPuv (Clontech) by PCR introducing *Bgl*II sites before the ATG translational start and

before the translational stop codon of GFPuv. After restriction digestion with *Bgl*II, the amplified GFPuv-fragment was inserted into the *Bgl*II site of both plasmid pRS316-STE20-BglII and pRS426-STE20-BglII yielding plasmid pME1759 and pME1760, respectively. Plasmid pJG4-5(*STE20*) was constructed by (i) introducing a *Bgl*II site into the multiple cloning site of pJG4-5 and (ii) insertion of a 3.1 kb *Bgl*II-*Kpn*I *STE20* fragment from plasmid pRS426-STE20-BglII into the modified version of pJG4-5 from (i).

Library of *CDC42* mutants.

CDC42 was mutagenized by PCR amplification of a 1.7-kb fragment from YCp(CDC42Sc) (Ziman *et al.*, 1991) using *Taq* DNA polymerase and primers T3 and T7 in the presence of 0.24 mM $MnCl_2$. The resulting DNA was digested with *Bam*HI and *Hin*dIII and exchanged for the 1.7-kb *Bam*HI-*Hin*dIII fragment carrying the wild-type version of *CDC42* in plasmid YCp(CDC42Sc) (Ziman *et al.*, 1991). A library of approximately 130'000 recombinants was obtained. Following identification of mutants (see below), both DNA strands of the *CDC42* coding sequences were sequenced using the ABI PRISM™ BigDye terminator sequencing kit and an ABI 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems GmbH, Weiterstadt, Germany).

Screen for *CDC42* alleles.

For isolation of haploid invasive growth mutants, strain RH2197 was transformed with the *CDC42* mutant library described above. A pool of approximately 26'000 transformants was obtained by growth selection on SC-Leu medium and subsequent growth on medium containing 0.1% 5-fluoroorotic acid to remove plasmid pME1534 carrying the wild-type *CDC42* gene by counterselection. This pool was plated on YPD medium at a density of ~500 colonies per plate and mutants were identified by an invasive growth test (Roberts and Fink, 1994). Phenotypes were confirmed by isolating the *CDC42*-containing plasmids and reintroducing them into the parental strain by plasmid shuffling. For isolation of pseudohyphal developmental mutants, strain RH2199 was transformed with the *CDC42* mutant library and a pool of approximately 60'000 transformants was obtained as described above. This pool was plated on SLAD medium supplemented with uracil. Pseudohyphal mutants were identified by the appearance of colonies visualized with a dissecting microscope. Phenotypes were confirmed by isolating the *CDC42*-containing plasmids and reintroducing them into the parental strain by plasmid shuffling using either centromere-based or integrative versions.

Pseudohyphal development assays.

Qualitative and quantitative assays for pseudohyphal development including determination of substrate invasion and cell shape as well as determination of bud site selection patterns were performed as described previously (Mösch and Fink, 1997). Pseudohyphal colonies were

viewed with a Zeiss axiovert microscope and photographed using a digital camera.

β-Galactosidase assays.

FLO11-lacZ activity: strains carrying the *FLO11-lacZ* plasmid B3782 were grown to exponential growth phase and extracts were prepared and assayed for β-galactosidase activity as described previously (Mösch *et al.*, 1996). Specific β-galactosidase was normalized to the total protein in each extract and equals $(OD_{420} \times 1.7)/(0.0045$ X protein concentration $\times$ extract volume $\times$ time). Assays were performed on at least three independent transformants, and the mean value is presented. Standard deviation did not exceed 15%.

Northern blot analysis.

Total RNA was prepared from cultures grown on solid media for 30 h according to the method described by (Cross and Tinkelenberg, 1991). Total RNA was separated on a 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes as described earlier (Mösch *et al.*, 1992). *CDC42* and *ACT1* transcripts were detected using gene specific ^{32}P-radiolabeled DNA probes. Hybridizing signals were quantified using a BAS-1500 Phosphor-Imaging scanner (Fuji, Tokyo, Japan).

Cell extracts and Western blot analysis.

Preparation of total cell extracts and subsequent western blot analysis was performed essentially as described (Roberts *et al.*, 1997). Cdc42p and Cdc28p proteins were detected using ECL technology (Amersham, Buckinghamshire, United Kingdom) after incubation of nitrocellulose membranes with polyclonal anti-Cdc42p (Santa Cruz Biotechnology, Santa Cruz, California) or anti-Cdc28p antibodies and a peroxidase-coupled goat anti-rabbit IgG secondary antibody (Dianova, Hamburg, Germany). Cdc42p and Cdc28p signals were quantified using the Molecular Analyst software (Bio-Rad, Munich, Germany).

Photomicroscopy.

Staining of bud site chitin rings and actin cytoskeleton was performed essentially as described (Adams and Pringle, 1991; Pringle, 1991). Briefly, cells were grown to mid-log phase in liquid YNB+Ura medium, fixed in 3.7% formaldehyde for 1 h and stained in dark with calcofluor (Fluorescent Brightener 28, F-3543, Sigma) or with rhodamine-phalloidin (R-415, Molecular Probes). Morphology of cells and stained bud scars or actin cytoskeleton were visualized on a Zeiss axiovert microscope by either differential interference contrast (DIC) microscopy or fluorescence microscopy using appropriate filter sets. Cells harboring plasmids encoding GFP-Ste20p were grown to exponential growth phase and immediately

viewed *in vivo* using Nomarski optics (DIC) or a GFP filter set (AHF Analysentechnik AG, Tübingen, Germany). All cells shown were photographed using a Xillix Microimager digital camera and the Improvision Openlab software (Improvision, Coventry, England).

Two-hybrid protein interactions.

All *CDC42* alleles tested in two-hybrid protein interaction assays were subjected to site-directed PCR mutagenesis in order to introduce the C188S mutations avoiding membrane localization of the proteins. *CDC42* alleles were amplified by PCR and checked by sequence analysis before introduction into vector pEG202 as described (Richman *et al.*, 1999). The methods for performing two-hybrid analysis have been described before (Gyuris *et al.*, 1993). Reporter strain EGY48-p1840 was cotransformed pairwise with the various pEG202(*CDC42*) and pJG4-5 constructs (Table II), and transformants were selected on SC-His-Trp medium. Transformants were grown at 23ºC in liquid SC-His-Trp medium containing 2% galactose and 2% raffinose to an OD_{600} between 1 and 2, and ß-galactosidase liquid assays were performed as described previously (Guarente, 1983). ß-Galactosidase activities were normalized to the activities obtained for $Cdc42^{C188S}p$ (wt) and the different effectors with values set to 100. Absolute values for $Cdc42^{C188S}p$ were 383 Miller units when interacting with Gic1p, 323 units for Gic2p, 227 units for Bni1p, 39 units for Ste20p, 78 units for Cla4p and 22 units for Skm1p. All assays were performed in triplicate on at least four independent transformants for each combination of plasmids.

Computer modeling.

A three-dimensional structure model of *S. cerevisiae* Cdc42p was obtained by homology modeling of the primary structure of *S. cerevisiae* Cdc42p using the SWISS-MODEL service (Guex and Peitsch, 1997) and the WebLab™ Viewer software (Molecular Simulations Inc., San Diego, California).

Table I. Strains used in this study

Strain	Genotype	Plasmid	Reference
RH2441	*MAT***a**/*MATα cdc42Δ::HIS3/CDC42 ura3-52/ura3-52 his3::hisG/his3::hisG*	pTF27-CDC42	This study
RH2197	*MATα cdc42Δ::HIS3 ura3-52 his3::hisG leu2::hisG*	pTF27-CDC42	This study
RH2442	*MAT***a** *cdc42Δ::HIS3 ura3-52 his3::hisG leu2::hisG*	pTF27-CDC42	This study
RH2199	*MAT***a**/*MATα cdc42Δ::HIS3/cdc42Δ::HIS3 ura3-52/ura3-52 his3::hisG/his3::hisG leu2::hisG/leu2::his*G	pTF27-CDC42	This study
RH2488	*MAT***a**/*MATα bni1::LEU2/bni1::LEU2 ura3-52/ura3-52 leu2::hisG/leu2::his*G *trp1::hisG/TRP1*		(Mösch and Fink, 1997)

Table II. Plasmids used in this study

Plasmid	Description	Reference
pME1758	Cassette for full deletion of *CDC42*	This study
pME1533	Derivative of pRS316 with mutated *CEN*-region	J. Hegemann
pME1534	*CDC42* in pTF27	This study
YCp(CDC42Sc)	*CDC42* in pRS315	(Ziman *et al.*, 1991)
pME1552	$CDC42^{N26I}$ in pRS315	This study
pME1555	$CDC42^{A30T}$ in pRS315	This study
pME1538	$CDC42^{I46M}$ in pRS315	This study
pME1557	$CDC42^{D65N}$ in pRS315	This study
pME1548	$CDC42^{R68S}$ in pRS315	This study
pME1536	$CDC42^{S71P}$ in pRS315	This study
pME1560	$CDC42^{N92D}$ in pRS315	This study
pME1559	$CDC42^{E95K}$ in pRS315	This study
pME1551	$CDC42^{E100G}$ in pRS315	This study
pME1545	$CDC42^{S158T}$ in pRS315	This study
pME1546	$CDC42^{L160P}$ in pRS315	This study
pME1759	P_{STE20}-*GFP-STE20* fusion in pRS316	This study
pME1760	P_{STE20}-*GFP-STE20* fusion in pRS426	This study
B3782	*FLO11-lacZ* reporter construct	(Rupp *et al.*, 1999)
pEG202	Vector for construction of LexA-fusion proteins	(Gyuris *et al.*, 1993)
pME1913	*LexA-*$CDC42^{C188S}$ in pEG202	(Richman *et al.*, 1999)
pME1914	*LexA-*$CDC42^{N26I,C188S}$ in pEG202	This study
pME1915	*LexA-*$CDC42^{A30T,C188S}$ in pEG202	This study
pME1916	*LexA-*$CDC42^{I46M,C188S}$ in pEG202	This study
pME1917	*LexA-*$CDC42^{D65N,C188S}$ in pEG202	This study
pME1918	*LexA-*$CDC42^{R68S,C188S}$ in pEG202	This study
pME1919	*LexA-*$CDC42^{S71P,C188S}$ in pEG202	This study
pME1920	*LexA-*$CDC42^{N92D,C188S}$ in pEG202	This study
pME1921	*LexA-*$CDC42^{E95K,C188S}$ in pEG202	This study
pME1922	*LexA-*$CDC42^{E100G,C188S}$ in pEG202	This study
pME1923	*LexA-*$CDC42^{S158T,C188S}$ in pEG202	This study
pME1924	*LexA-*$CDC42^{L160P,C188S}$ in pEG202	This study
pJG4-5	Vector for construction of B42 transcription activation domain fusions	(Gyuris *et al.*, 1993)
pJG4-5(*GIC1*)	*B42AD-GIC1*	(Chen *et al.*, 1997)
pJG4-5(*GIC2*)	*B42AD-GIC2*	(Chen *et al.*, 1997)
pJG4-5(*BNI1 1-1214 aa*)	*B42AD-BNI1(1-1214 aa)*	(Evangelista *et al.*, 1997)
pJG4-5(*STE20*)	*B42AD-STE20*	This study
pJG4-5(*CLA4*)	*B42AD-CLA4*	(Cvrckova *et al.*, 1995)
pJG4-5(*SKM1*)	*B42AD-SKM1*	(Richman *et al.*, 1999)

RESULTS

Functions of Cdc42p required for cell division and cellular development can be uncoupled by single amino acid substitutions.

We tested whether the different functions of Cdc42p for regulation of distinct processes can be separated by specific amino acid substitutions within the Cdc42 protein. Specifically, we wanted to uncouple functions of Cdc42p required for cell division from those regulating pseudohyphal and invasive growth development. We created a library of PCR-mutagenized *CDC42* genes and introduced them on a centromeric vector into both a haploid *cdc42Δ* and a diploid *cdc42Δ/cdc42Δ* null strain. Because genomic *cdc42Δ* null mutations are lethal, *CDC42* mutant genes were introduced by plasmid shuffling (Guthrie and Fink, 1991). Mutants of *CDC42* causing defective haploid invasive growth on rich medium were isolated from the haploid *cdc42Δ* background. Mutant alleles of *CDC42* leading to both reduced (nonfilamentous) as well as enhanced (hyperfilamentous) pseudohyphal development on low ammonium medium were identified from the diploid pool. In all cases, one or more amino acid exchanges in Cdc42p were found, but only mutants with single amino acid substitutions were further investigated. The $CDC42^{I46M}$ and $CDC42^{S71P}$ mutant alleles were isolated from haploids that conferred defective invasive growth (Table III; Fig. 1A). Five *CDC42* mutants causing a nonfilamentous phenotype - $CDC42^{N26I}$, $CDC42^{R68S}$, $CDC42^{E100G}$, $CDC42^{S158T}$ and $CDC42^{L160P}$ - as well as four alleles of *CDC42* conferring hyperfilamentation - $CDC42^{A30T}$, $CDC42^{D65N}$, $CDC42^{N92D}$ and $CDC42^{E95K}$ - were identified in diploid strains on low ammonium medium (Table III; Fig. 1B). Importantly, none of the isolated *CDC42* mutant genes caused significant defects in the growth rate (Table III), demonstrating that they did not affect functions essential for cell division. Moreover, strains harboring the mutant alleles did not display a detectable temperature-sensitive growth phenotype when grown at 37°C.

These results demonstrate that specific single amino acid exchanges within Cdc42p are sufficient to separate the function of this Rho-type GTPase required for cell division from its regulatory function in cellular development.

Table III. Regulation of growth and development by different *CDC42* alleles

Mutation	Developmental phenotype	Growth rate (h^{-1})[c]
	Haploid invasive growth[a]	
none	+	0.34
I46M	-	0.34
S71P	-	0.33
	Diploid pseudohyphal growth[b]	
none	+	0.37
N26I	-	0.39
A30T	++	0.32
D65N	++	0.38
R68S	+/-	0.39
N92D	++	0.36
E95K	++	0.38
E100G	-	0.36
S158T	-	0.33
L160P	-	0.38

[a]Haploid invasive growth was determined by expression of *CDC42* alleles in a haploid *cdc42Δ* mutant strain and growth on solid SC-Leu medium for 4 days before performing a wash assay. A value of + indicates the degree of invasiveness of a control strain and – indicates a complete loss of invasive growth.

[b]Pseudohyphal growth was determined by expression of *CDC42* alleles in a diploid *cdc42Δ/cdc42Δ* mutant strain and growth on solid SLAD + Ura medium for 5 days. The degree of filamentation is given by values of + for a control strain, ++ for enhanced, +/- for reduced and – for non-detectable filamentous growth.

[c]Growth rate was determined in liquid SC-Leu medium.

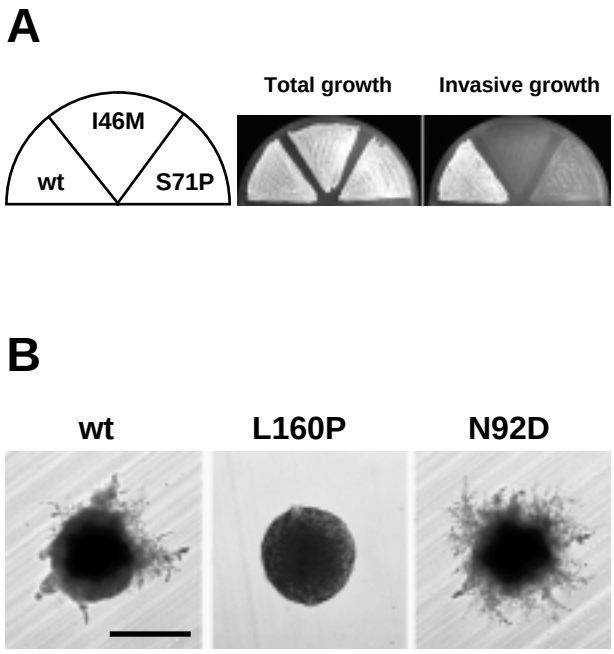


Fig. 1. Cdc42p mutants causing specific defects in yeast development. (A) Haploid invasive growth of strains expressing wild-type *CDC42* (wt), $CDC42^{I46M}$ (I46M) or $CDC42^{S71P}$ (S71P). Plasmids carrying the different *CDC42* alleles were introduced into the haploid *cdc42Δ* strain RH2197 (*MATα*) by plasmid shuffling. Resulting strains were patched on SC-Leu medium for 4 d. After incubation, the plate was photographed before (Total growth) and after (Invasive growth) washing cells off the agar surface. (B) Pseudohyphal development of diploid strains expressing wild-type *CDC42* (wt), $CDC42^{L160P}$ (L160P) or $CDC42^{N92D}$ (N92D). Plasmids carrying the different *CDC42* alleles were introduced into the diploid *cdc42Δ/cdc42Δ* strain RH2199. Resulting strains were streaked to obtain single colonies on nitrogen starvation medium (SLAD+Ura). Plates were incubated at 30° C and representative colonies were photographed after 3 d of growth. Scale bar = 100 µm.

Cdc42p regulates haploid invasive growth by mechanisms both dependent on and independent of *FLO11* expression.

Expression of dominant activated forms of *CDC42*, $CDC42^{G12V}$ or $CDC42^{Q61L}$, not only induces pseudohyphal growth in diploids (Mösch *et al.*, 1996) but also suppresses defective invasive growth caused by loss of *RAS2* in haploid cells (Mösch *et al.*, 1999). This suggests that the functions of *CDC42* in regulating haploid invasive growth and diploid pseudohyphal development are overlapping. Therefore, we tested the effects of all eleven isolated *CDC42* alleles on invasive growth when expressed in a haploid background. *CDC42* mutant genes were introduced into a haploid *MAT***a** *cdc42Δ* strain by plasmid shuffling and invasive growth was assayed on medium rich in nitrogen (Fig. 2A). We found that with the exception of R68S all amino acid exchanges in Cdc42p causing reduced pseudohyphal development in diploid

cells also lead to a suppression of invasive growth in haploids. All amino acid substitutions conferring hyperfilamentation in diploids supported invasive growth in haploid cells. *CDC42* mRNA and intracellular Cdc42 protein levels were measured in all strains to exclude that the invasive growth phenotypes found were due to altered expression or stability of Cdc42p mutant proteins (Fig. 2B and 2C). No significant differences were found between wild-type and any of the mutant forms of *CDC42*, demonstrating that developmental phenotypes are caused by altered function and not intracellular amounts of the different Cdc42p mutant proteins.

Invasive growth development in haploid cells is regulated by at least two signaling pathways, the Kss1p MAPK cascade and the cAMP/PKA pathway. Both pathways regulate expression of *FLO11*, encoding a cell surface flocculin required for invasive growth (Lo and Dranginis, 1998; Rupp *et al.*, 1999), and *FLO11* expression is well correlated to the invasiveness of yeast cells. Therefore, we determined expression of the *FLO11-lacZ* reporter gene (Rupp *et al.*, 1999) in all haploid strains containing the isolated *CDC42* mutant genes (Fig. 2D). We found that *FLO11* expression was clearly reduced in strains carrying either the $CDC42^{I46M}$ or the $CDC42^{S71P}$ mutant alleles. The I46M mutation caused a reduction to 45% when compared to a wild-type control whereas expression of *FLO11* was reduced to 26% in strains expressing the S71P mutant protein. An increase of *FLO11* expression to 175% was found in strains expressing the N92D mutant protein. No significant changes in the *FLO11* expression levels were detected in strains carrying any of the other *CDC42* mutant alleles, although most of them caused clearly detectable changes in the invasive growth behavior. These measurements indicate that in haploid cells, Cdc42p might regulate invasive growth by different mechanisms: one that affects expression of *FLO11* by acting via the Kss1p MAPK cascade and a second one that must operate independently of changes in *FLO11* expression levels.

We further tested whether mating of *MAT***a** haploid cells is affected by the amino acid exchanges in Cdc42p that we found to affect haploid invasive growth. However, none of the mutations significantly influenced pheromone-induced growth arrest, induction of *FUS1-lacZ* expression, formation of mating projections or formation of diploid cells (data not shown). This is in agreement with earlier studies showing that Cdc42p does not appear to directly regulate pheromone mediated gene expression (Leberer *et al.*, 1997; Oehlen and Cross, 1998; Peter *et al.*, 1996).

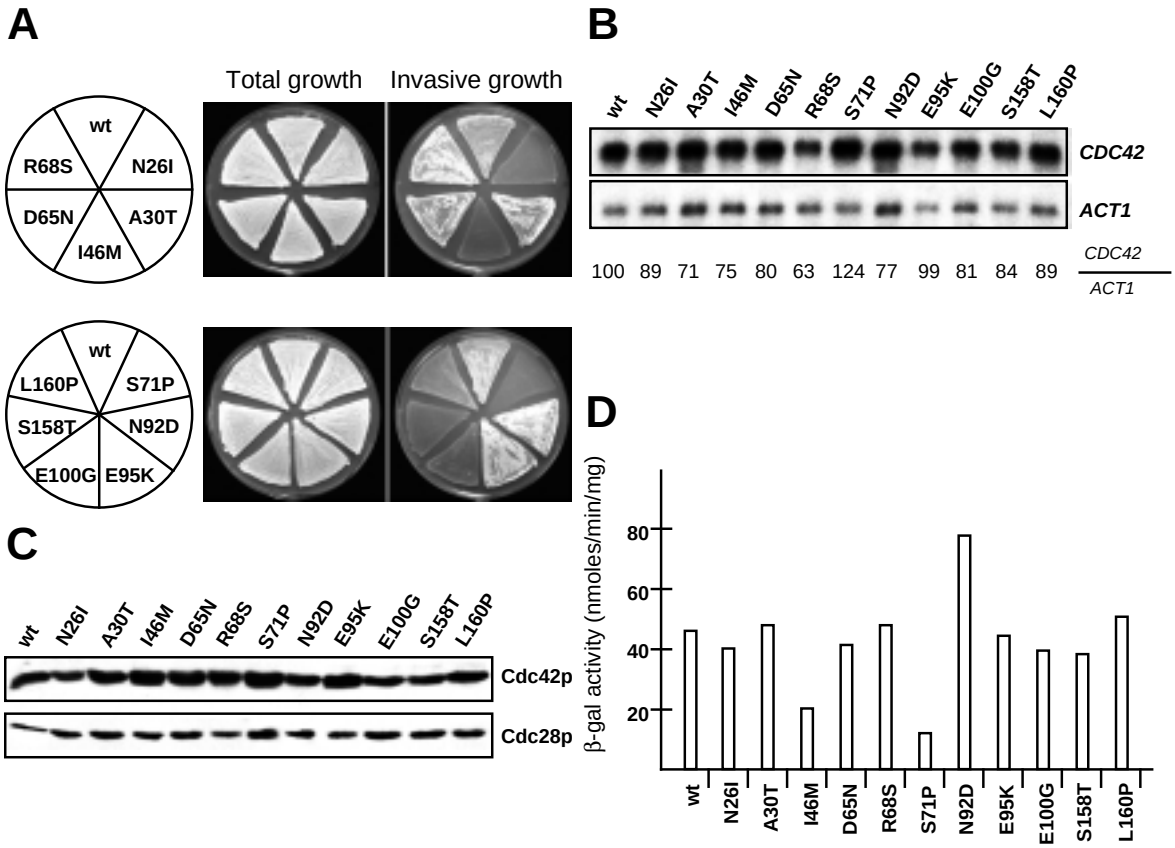


Fig. 2. Regulation of haploid invasive growth and *FLO11* expression by *CDC42* alleles. (A) Haploid invasive growth of strains expressing wild-type *CDC42* (wt) or *CDC42* alleles encoding proteins with single amino acid substitutions as indicated. Plasmids carrying the different *CDC42* alleles were introduced into the haploid *cdc42Δ* strain RH2442 (*MAT***a**) and resulting strains were patched on SC-Leu medium for 4 d. After incubation, plates were photographed before (Total growth) and after (Invasive growth) washing cells off the agar surface. (B) Autoradiogram showing steady-state mRNA levels of *CDC42* alleles in strains described in (A). *ACT1* gene expression was used as internal standard. Relative expression levels of *CDC42* alleles (*CDC42/ACT1*) are shown and were obtained using a Phosphor-Imaging scanner. Numbers represent mean values of three independent measurements and were obtained by normalizing *CDC42* transcript levels with respect to *ACT1* levels and to wild-type *CDC42* (wt). Standard deviation was below 20%. (C) Expression levels of Cdc42 proteins were determined in extracts from strains described in (A) by Western blot analysis using a polyclonal anti-Cdc42p antibody. As an internal control, expression levels of Cdc28p were measured in the same extracts using a polyclonal anti-Cdc28p antibody (lower panel). (D) *FLO11::lacZ* expression levels. β-Galactosidase activity was measured in strains described in (A) carrying *FLO11::lacZ* on a plasmid (B3782). Units are nmol mg^{-1} min^{-1}. Bars depict means of three independent measurements with a standard deviation not exceeding 15%.

Cdc42p regulates pseudohyphal development by affecting PH cell morphogenesis and invasive growth.

Pseudohyphal development is a composite of several cellular changes including alterations in cell morphogenesis and invasive growth behavior. We tested the effects of all eleven *CDC42* mutant alleles on diploid pseudohyphal growth in more detail. Mutant alleles were introduced into a diploid *cdc42Δ/cdc42Δ* strain by plasmid shuffling and diploid pseudohyphal development was assayed on nitrogen starvation medium (Fig. 3).

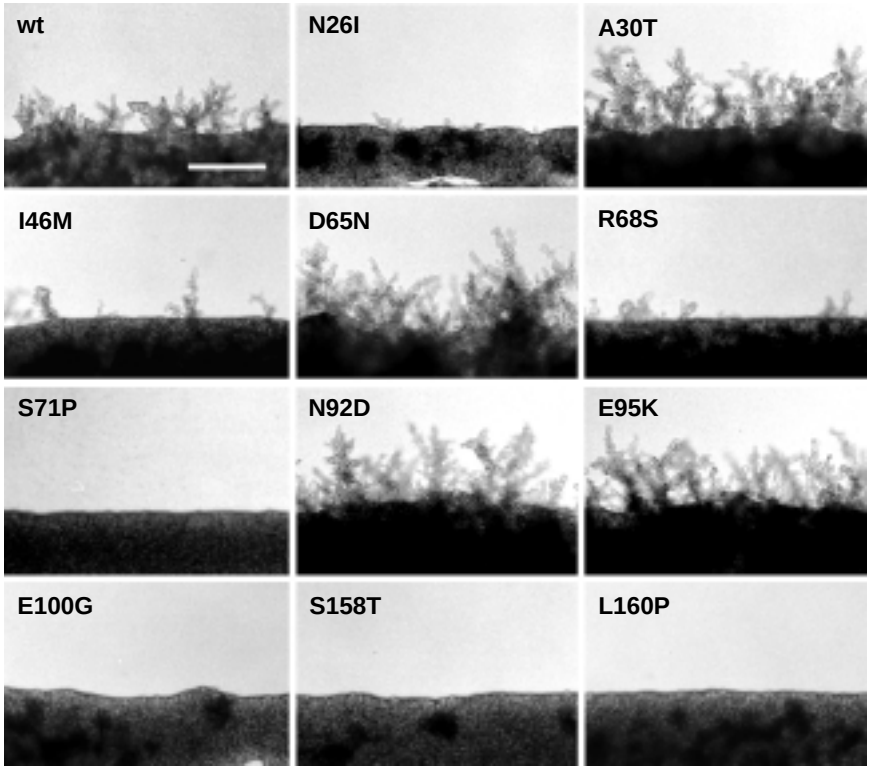


Fig. 3. Regulation of pseudohyphal development by *CDC42* alleles. Shown is the growth on nitrogen starvation medium of diploid strains expressing wildtype *CDC42* (wt) or mutant *CDC42* alleles leading to single amino acid substitutions as indicated. Plasmids carrying the different *CDC42* alleles were introduced into the diploid *cdc42Δ/cdc42Δ* strain RH2199 by plasmid shuffling. Pseudohyphal development of resulting strains was photographed after 4 d of growth on SLAD+Ura medium. Scale bar = 100 µm.

We found that both alleles of *CDC42* that were isolated from haploids to suppress invasive growth, $CDC42^{I46M}$ and $CDC42^{S71P}$, also lead to reduced pseudohyphal development in diploids. We further characterized each of the diploid *CDC42* mutant strains with respect to changes in cell shape upon nitrogen starvation and the ability to invade agar as described earlier (Mösch and Fink, 1997). The shape of cells was determined by defining three morphological groups: long pseudohyphal cells (PH), oval yeast form (YF) cells and round YF cells (Table IV). Substrate invasion was measured by determining the ratio of invasive vs. noninvasive cells after growth on nitrogen starvation medium. Characterization of all *CDC42* mutant alleles by these criteria defined three different classes.

Table IV. Budding pattern, morphology, and invasive growth behavior of diploid *cdc42* mutants

	Budding pattern[a]			Cell shape[b]						
				Long PH		Oval YF		Round YF		
Genotype	Bipolar (%)	Random (%)	Unipolar (%)	+N (%)	-N (%)	+N (%)	-N (%)	+N (%)	-N (%)	Invasive Growth[c]
CDC42	72	8	20	4	29	54	57	42	14	+
$CDC42^{N26I}$	75	6	19	1	3	48	61	51	36	-
$CDC42^{A30T}$	70	5	25	6	55	46	39	48	6	+
$CDC42^{I46M}$	74	5	21	1	6	49	49	50	45	-
$CDC42^{D65N}$	51	8	41	8	40	66	55	26	5	+
$CDC42^{R68S}$	67	2	31	12	33	59	55	29	12	+
$CDC42^{S71P}$	81	5	14	0	2	37	35	63	63	-
$CDC42^{N92D}$	57	8	35	20	61	59	33	21	6	+
$CDC42^{E95K}$	60	10	30	11	45	73	40	16	15	+
$CDC42^{E100G}$	69	6	25	1	6	43	55	56	39	-
$CDC42^{S158T}$	70	6	24	0	2	44	34	56	64	-
$CDC42^{L160P}$	73	7	20	0	1	51	37	49	62	-
bni1	13	77	10	0	0	2	6	98	94	+

[a]Budding patterns of strains were determined after growth to logarithmic phase in nitrogen rich medium.

[b]Cell shape patterns were determined after growth in nitrogen rich medium (+N) or on nitrogen starvation plates (-N).

[c]Invasive growth was determined after growth on nitrogen starvation plates.

A first class includes six alleles of *CDC42* - *CDC42*^{N26I}, *CDC42*^{I46M}, *CDC42*^{S71P}, *CDC42*^{E100G}, *CDC42*^{S158T} and *CDC42*^{L160P} - that are impaired for pseudohyphal development (Fig. 3). These mutants are unable both to produce regular amounts of long PH cells and to invade the agar in response to nitrogen starvation (Table IV). A second class comprises four *CDC42* alleles, *CDC42*^{A30T}, *CDC42*^{D65N}, *CDC42*^{N92D} and *CDC42*^{E95K}, that display a hyperfilamentous growth phenotype (Fig. 3). The most prominent phenotype of mutants in this class is that they produce significantly more cells of the long PH type. A single *CDC42* allele, *CDC42*^{R68S}, defines a third class of mutations. Although expression of this allele impairs filament formation of diploids, PH cell morphogenesis is normal and cells invade the agar almost indistinguishable from cells expressing wild-type *CDC42* (Fig. 3; Table IV). This result correlates with our finding that haploids expressing the *CDC42*^{R68S} allele display a normal invasive growth behavior (Fig. 2A). Thus, regulation of cellular functions other than PH morphogenesis or invasiveness appears to be impaired in the R68S mutant protein that causes a nonfilamentous phenotype.

Mutations in Cdc42p blocking pseudohyphal cell morphogenesis in response to nitrogen starvation do not severely affect morphology or polarity of the yeast-form.

Several mutations in actin or in proteins associated with the actin cytoskeleton have been found to affect pseudohyphal development, including Bni1p, Tpm1p, Srv2p or certain variants of Act1p (Cali *et al.*, 1998; Mösch and Fink, 1997). However, these mutation not only suppress pseudohyphal cell morphogenesis in response to nitrogen starvation, but additionally cause characteristic morphological and polarity defects in the yeast-form when grown in nutrient rich medium. Typical phenotypes include a random budding pattern, partially depolarized actin and consequently a very high proportion of YF cells with round shape. Therefore, we characterized all *CDC42* mutations with respect to their effects on bud site selection, actin distribution and cell morphology when strains were grown in nutrient rich medium. Bud site selection patterns were determined by staining bud scars of exponentially growing yeast form cells with calcofluor (Fig. 4) and dividing them into three groups: bipolar, random and unipolar (Table IV). Localization of actin was visualized by staining yeast-form cells with rhodamine-phalloidin (Fig. 5). Cell shape patterns of exponentially growing cells in nitrogen-rich medium were determined as described above and were directly compared to the patterns obtained by growth under nitrogen starvation conditions (Table IV).

This analysis revealed that none of the Cdc42p mutations suppressing pseudohyphal development and invasive growth (N26I, I46M, S71P, E100G, S158T or L160P) significantly affected morphology or polarity of cells grown in the yeast-form. None of these mutations caused a random budding pattern as found for a *bni1* mutant strain but led to regular bipolar budding (Fig. 4; Table IV). Actin distribution in the N26I, I46M, E100G, S158T and L160P mutants was indistinguishable from the *CDC42* wildtype strain (Fig. 5). A higher

percentage of mother cells containing actin patches at early stages of the cell division cycle were found in the S71P mutant. However, this phenotype was clearly less pronounced when compared to a *bni1* mutant strain (Fig. 5). Cell morphology patterns of the N26I, I46M, E100G, S158T or L160P mutants were normal with 43% - 51% of oval shaped YF cells (Table IV; Fig. 4). The S71P mutation had a slight but distinct effect on the yeast-form morphology with 37% of cells having the oval YF. In contrast, only 2% of *bni1* mutant cells were oval and 98% were circular round shaped. Thus, with respect to cell polarity, distribution of actin patches and yeast-form morphology the N26I, I46M, E100G, S158T and L160P mutants do not resemble any of the *bni1*, *tpm1* or certain *act1* mutant strains. However, these *CDC42* mutants have a specific block in their morphological response to nitrogen starvation conditions and are unable to grow invasively (Table IV). In contrast, *bni1* or *tpm1* mutations do not affect agar invasion (Table IV; (Mösch and Fink, 1997).

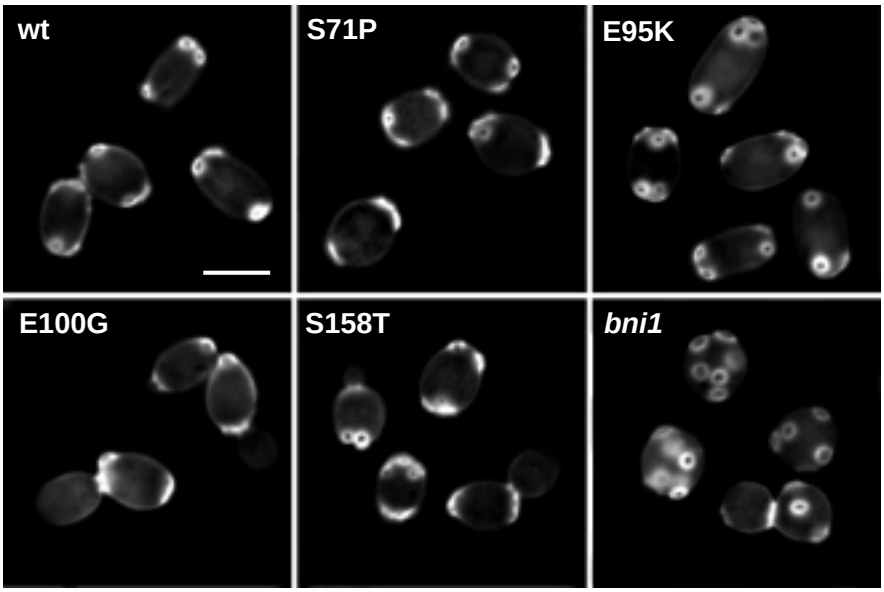


Fig. 4. Chitin localization. Shown are representative YF cells of diploid strains expressing wild-type *CDC42* (wt) or *CDC42* mutant alleles (S71P, E95K, E100G, and S158T) or carrying a *bni1* mutation (RH2488). Strains were grown to logarithmic growth phase in nutrient rich medium, fixed and stained with calcofluor before fluorescence imaging. Scale bar = 5 µm.

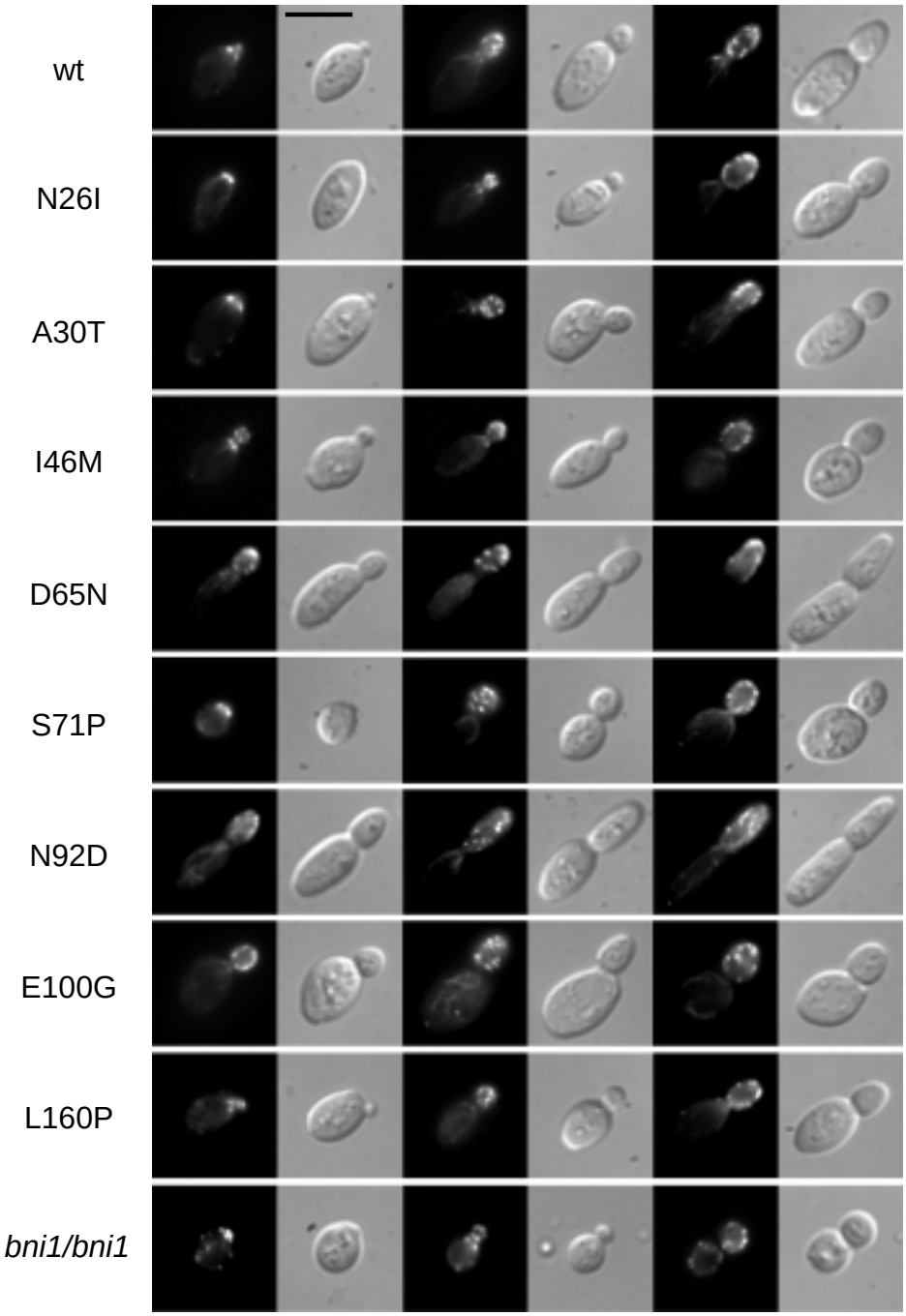


Fig. 5. Actin localization. Shown are representative YF cells at different stages of the cell cycle of diploid strains expressing wildtype *CDC42* (wt) or *CDC42* mutant alleles (N26I, A30T, I46M, D65N, S71P, N92D, E100G, L160P) or carrying a *bni1* mutation (RH2488). Strains were grown to logarithmic growth phase in nutrient rich medium, fixed and stained with the actin fluorescent stain rhodamine-phalloidin before observation using either fluorescence microscopy or by differential interference contrast microscopy (DIC). Scale bar = 5 µm.

Cdc42p mutations causing hyperfilamentous growth (A30T, D65N, N92D and E95K) did not significantly alter the bud site selection patterns of the yeast-form (Table IV, Fig. 4). Alterations in cell morphology patterns could be detected in case of the D65N, N92D and E95K mutants, where higher proportions of elongated cells were found when strains were grown in nutrient rich medium (Table IV). No changes in the yeast-form morphology pattern was found for the A30T mutation although this mutant produces a much higher amount of long PH cells under nitrogen starvation conditions (Table IV). Actin staining patterns of the A30T, D65N, N92D and E95K mutants were not significantly different from a *CDC42* wildtype strain (Fig. 5). In summary, the D65N, N92D and E95K mutants exhibit hyperpolarized growth under both nutrient rich and starvation conditions, partly explaining their hyperfilamentous growth phenotype. In contrast, the A30T mutant is hyperinducible for morphology changes specifically in response to nitrogen starvation.

Localization of Ste20p to the bud tip by Cdc42p is not sufficient for pseudohyphal cell morphogenesis.

Several lines of evidence suggest that the Ste20p kinase is necessary for the Cdc42p-dependent induction of pseudohyphal development and that interactions between Cdc42p and the CRIB domain of Ste20p are necessary for this induction (Leberer *et al.*, 1997; Mösch *et al.*, 1999; Mösch *et al.*, 1996; Peter *et al.*, 1996). Moreover, Ste20p is localized to the sites of polarized growth in emerging buds, while the CRIB-deleted Ste20p shows a general cytoplasmic staining (Peter *et al.*, 1996). These results led to the view that Cdc42p is necessary for proper localization of Ste20p to the sites of polarized growth and that this localization of Ste20p during cell growth might be important for the development of PH cells. Therefore, we determined the localization of a GFP-Ste20p fusion protein in strains expressing *CDC42* mutant alleles causing specific defects in pseudohyphal development. Diploid N26I, I46M, S71P, E100G, S158T or L160P mutant strains were transformed with a plasmid carrying a *GFP-STE20* fusion gene under the control of the endogenous *STE20* promoter. Expression of this *STE20prom-GFP-STE20* fusion gene is sufficient to restore the filamentous growth defect of a *ste20Δ/ste20Δ* mutant strain (data not shown). Resulting strains were analyzed for localization of the GFP-Ste20p protein by fluorescence microscopy (Fig. 6). We predicted that if localization of Ste20p to the tip of the emerging bud was necessary for PH cell morphogenesis, all *CDC42* mutant alleles causing a nonfilamentous growth phenotype should display a general cytoplasmic staining. However, we found that only the S71P mutation in Cdc42p causes a general cytoplasmic staining of cells by GFP-Ste20p (Fig. 6). In all other strains expressing either of the N26I, I46M, E100G, S158T or L160P mutants, GFP-Ste20p was found to be localized to the tip of emerging cells (for L160P see Fig. 6), although these strains are unable to form pseudohyphae. Thus, localization of Ste20p to the bud tip of emerging daughter cells alone is not sufficient to induce PH cell morphogenesis.

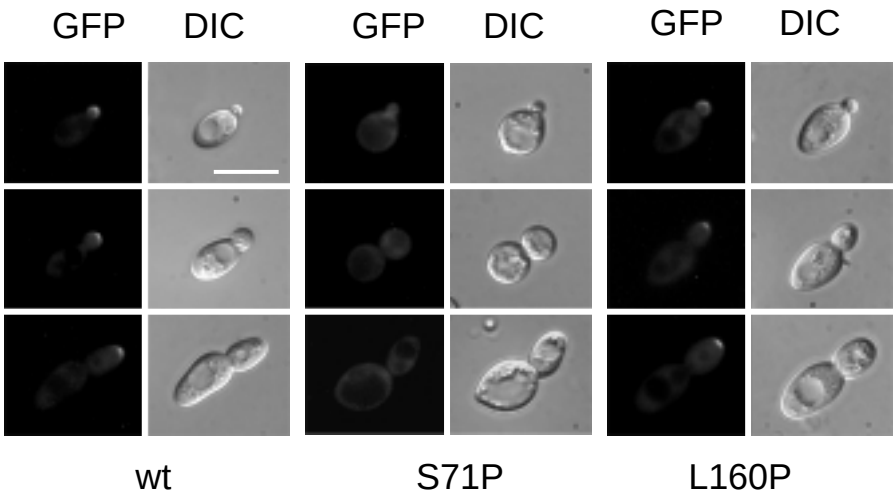


Fig. 6. Subcellular localization of GFP-Ste20p in *CDC42* pseudohyphal mutants. Shown are cells of diploid strains that express *CDC42* (wt), $CDC42^{S71P}$ (S71P) or $CDC42^{L160P}$ (L160P) and harbor plasmid pME1760 encoding GFP-Ste20p under control of the *STE20* promoter. Living cells at different stages of the cell cycle were chosen for photography according to their bud size and were viewed by either fluorescence microscopy (GFP) or by differential interference contrast microscopy (DIC). Identical results were obtained with centromere-based plasmid pME1759, although with markedly decreased fluorescence signals due to lower expression of GFP-Ste20p. Scale bar = 10 µm.

Cdc42p developmental mutations alter interaction patterns with several downstream effectors.

The specific developmental defects caused by the novel mutations in *CDC42* obtained here suggested that these Cdc42p mutants do not display general biochemical defects, but might have altered interaction patterns with downstream effectors. Indeed, analysis of the mutations in a three-dimensional structure model of *S. cerevisiae* Cdc42p revealed that most mutations were located on the surface of Cdc42p predicting altered interactions with other proteins (Fig. 7). To test this prediction, we performed a two-hybrid analysis between all Cdc42p mutants and the Gic1p, Gic2p, Bni1p, Ste20p, Cla4p and Skm1p effector proteins. The C188S mutation was additionally introduced in all mutants to prevent localization of the proteins to the plasma membrane. Interaction of mutant proteins with the diverse effectors was then measured and compared to $Cdc42^{C188S}$p as control (Fig. 8).

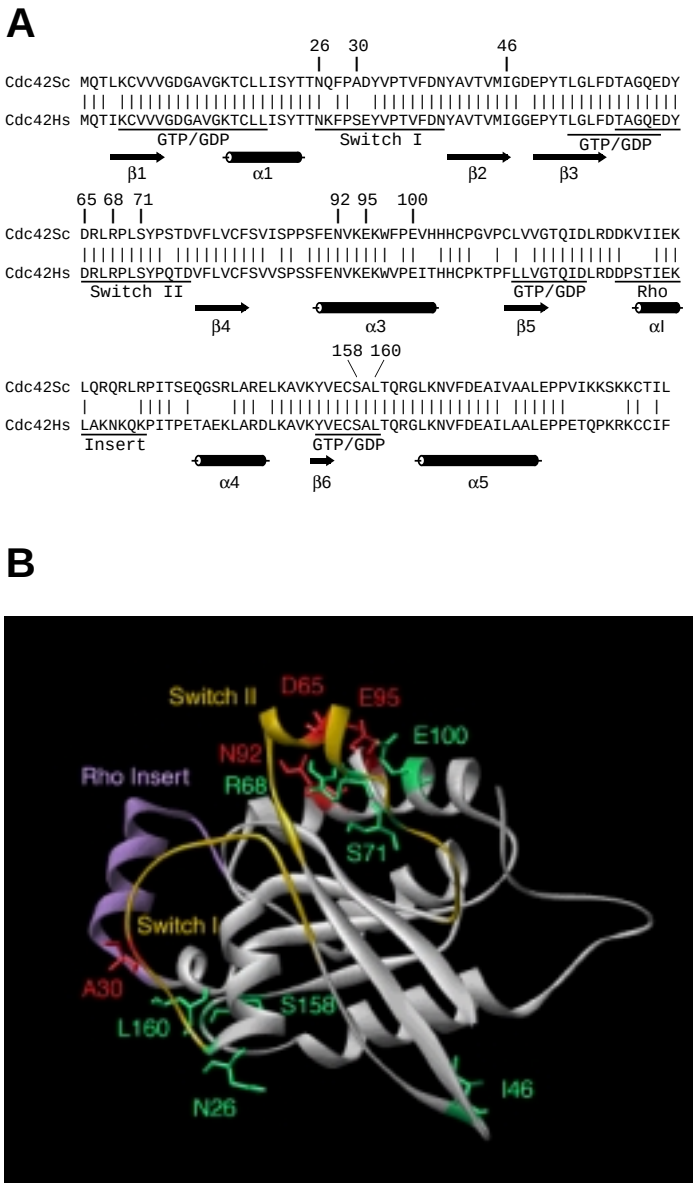
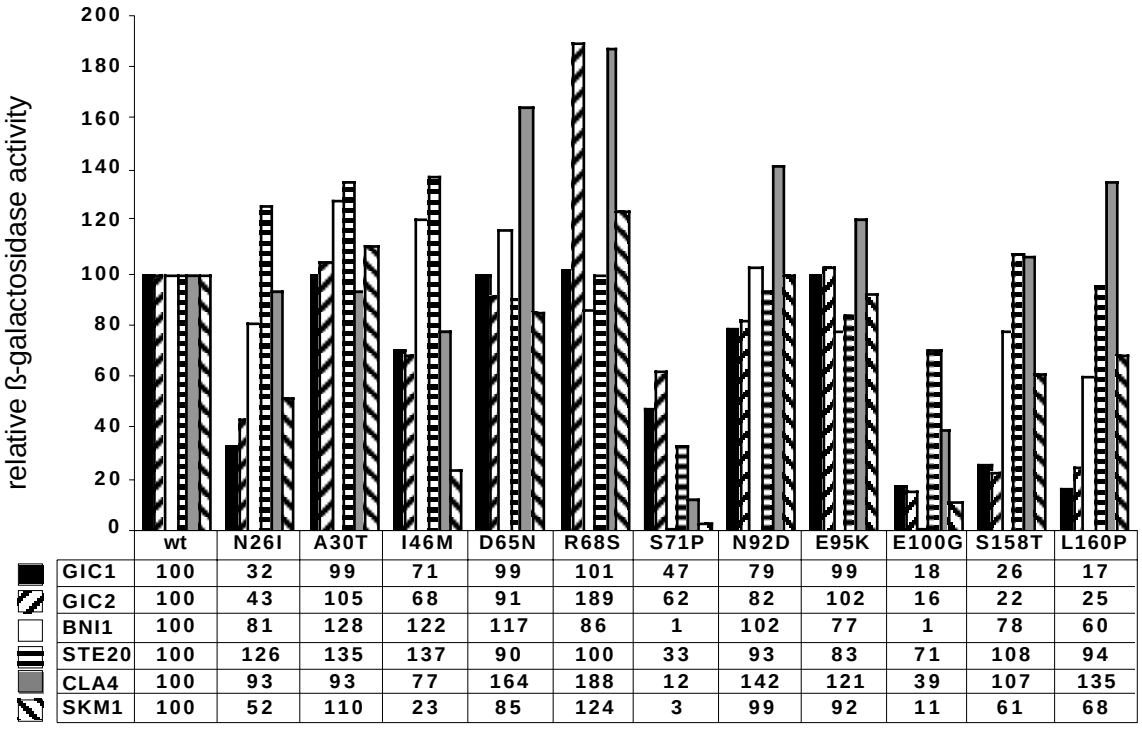


Fig. 7. Cdc42p developmental mutations and structural model of Cdc42p. (A) Sequence alignment of Cdc42p from *S. cerevisiae* (Cdc42Sc) and from human (Cdc42Hs). Vertical lines indicate identical residues. Known GTP binding/hydrolysis domains (GTP/GDP), Switch I and Switch II domains, and the Rho insert domain are underlined. Numbers indicate residues that were identified by mutations in the yeast Cdc42p sequence in this study. (B) A three-dimensional structure model of *S. cerevisiae* Cdc42p was obtained by homology modeling of the primary structure of *S. cerevisiae* Cdc42p using the SWISS-MODEL service (Guex and Peitsch, 1997) and is based on the X-ray crystal structure of Cdc42Hs (Nassar *et al.*, 1998; Rudolph *et al.*, 1999). Amino acid residues identified in this study are indicated in different colors based on the phenotypes caused by their exchange. Substitutions of green residues were found to suppress pseudohyphal or invasive growth and exchanges of red residues enhanced pseudohyphal development. Switch I and Switch II domains are colored yellow and the Rho Insert domain is shown in purple.



	wt	N26I	A30T	I46M	D65N	R68S	S71P	N92D	E95K	E100G	S158T	L160P
GIC1	100	32	99	71	99	101	47	79	99	18	26	17
GIC2	100	43	105	68	91	189	62	82	102	16	22	25
BNI1	100	81	128	122	117	86	1	102	77	1	78	60
STE20	100	126	135	137	90	100	33	93	83	71	108	94
CLA4	100	93	93	77	164	188	12	142	121	39	107	135
SKM1	100	52	110	23	85	124	3	99	92	11	61	68

Fig. 8. Two-hybrid protein interactions between Cdc42 proteins and effector proteins Gic1p, Gic2p, Bni1p, Ste20p, Cla4p and Skm1p. All Cdc42p proteins measured (wt or mutant proteins N26I, A30T, I46M, D65N, R68S, S71P, N92D, E95K, E100G, S158T, L160P) carry the C188S mutation to prevent plasma membrane localization. Bars and numbers show relative ß-galactosidase activities normalized to the activities obtained for wild-type Cdc42p (wt) and the different effectors with values set to 100 (for absolute units see Materials and Methods). The value shown for each interaction represents the average of at least four independent ß-galactosidase assays each measured in triplicate.

This complex analysis of 72 different combinations revealed specific changes in the effector interaction patterns for the six Cdc42p mutants causing defects in pseudohyphal development and invasive growth (N26I, I46M, S71P, E100G, S158T, L160P). Four distinct interaction patterns were found. In case of N26I, S158T and L160P, binding to Gic1p was clearly reduced (between 17% and 32% of the control) and interaction with Gic2p was reduced to 22% - 43%. In addition, interaction of these mutants with Skm1p and Bni1p was partially reduced (between 52% and 81% of the control). However, no alterations in binding to Ste20p and Cla4p were found. The I46M mutant protein was specifically reduced in its interaction with Skm1p with a binding efficiency of only 23% compared to the control. No strong alterations were detected for the binding of I46M to the other effectors. The most dramatic changes for effector binding were measured for the

S71P and E100G mutant proteins. Whereas interaction of S71P with Gic1p and Gic2p was only partially altered, binding to Bni1p, Ste20p, Cla4p and Skm1p was strongly reduced. A yet different pattern was found for the E100G mutant protein. Binding of E100G to Gic1p, Gic2p, Bni1p and Skm1p was strongly reduced, whereas interaction with Ste20p and Cla4p was only partially affected. In case of the non-filamentous R68S mutant no reduction in effector binding was measured but a somewhat higher affinity to Gic2p and Cla4p was found. No significant alterations in effector binding were detectable for any of the hyperfilamentous A30T, D65N, N92D or E95K variants.

In summary, the effects on pseudohyphal development and invasive growth of the non-filamentous N26I, I46M, S71P, E100G, S158T, and L160P Cdc42p variants can be correlated with alterations in the binding to distinct subsets of downstream effectors. This suggests that several of the Cdc42p mutants isolated here are specific effector mutants.

DISCUSSION

Evidence for specific Cdc42p signaling mutations.

Here, we have isolated novel Cdc42p mutant proteins that have lost the ability to confer PH cell morphogenesis and invasive growth in response to nitrogen starvation. A crucial finding of our study is that many of these mutants do not affect polarity or cell morphogenesis during yeast-form proliferation as measured by morphology indices, actin staining and bud scar distribution. The developmental defects caused by the N26I, I46M, E100G, S158T or L160P mutations are very specific and are clearly different from previously identified mutations in Cdc42p that either cause lethality (e.g. G12V, K16A, T35A, D38A, Y40K, Q61L, D118A) or temperature sensitivity (e.g. K5A, Y32K, V36T, V44A, D76A, W97R) and that lead to significant changes in actin distribution or cell morphology and polarity (Davis *et al.*, 1998; Johnson, 1999; Kozminski *et al.*, 2000; Miller and Johnson, 1997; Richman *et al.*, 1999; Ziman *et al.*, 1991). Mutations in Cdc42p identified here also differ from mutations in actin-associated proteins such as Bni1p or Tpm1p. Diploid *bni1* or *tpm1* mutants display altered cell morphology already under yeast-form growth conditions (and consequently are suppressed for PH morphogenesis), but unlike the N26I, I46M, E100G, S158T or L160P mutants have no significant invasive growth defects (Mösch and Fink, 1997). Phenotypically, *CDC42* mutants isolated here much more resemble mutants in the known signaling pathways that control pseudohyphal growth. For instance, mutations in components of the pseudohyphal and invasive growth Kss1p-MAPK cascade - e.g. Ste20p, Ste11p, Ste7p, Ste12p, Tec1p - as well as mutations in Ras2p, Gpa2p or Tpk2p specifically suppress PH morphogenesis and invasive growth. Much like the N26I, I46M, E100G, S158T and L160P mutations isolated here, Kss1p-MAPK or cAMP signaling mutants do not affect cell polarity or morphology during yeast-form proliferation. Expression of *FLO11*, a gene reporting activities of both the Kss1p-MAPK and the cAMP pathway, is also reduced in some of these Cdc42p mutants, whereas activated expression of *FUS1* or formation of mating projections in response to pheromone is not affected. Thus, several of the Cdc42p mutants uncovered in this study appear to be specific signaling mutants rather than general morphological mutants, because they display transcriptional and morphological defects only in response to certain nutritional signals.

We also found several mutations in Cdc42p that cause alterations in cell morphology independently of the growth conditions. These include the hyperfilamentous D65N, N92D and E95K and to some degree the non-filamentous S71P mutations. However, these mutations also differ from other morphological mutations in Cdc42p, as they do not cause lethality or temperature sensitivity. Because these mutants also affect invasive growth they might cause both general morphological alterations and signaling defects.

Novel Cdc42p effector mutations point to Gic1p, Gic2p, and Skm1p as developmental-specific Cdc42p effectors.

A surprising outcome of our study is the finding that several of the Cdc42p mutants that suppress pseudohyphal and invasive growth display specific defects in binding to Gic1p and Gic2p (S158T and L160P) or Skm1p (I46M). These effectors of Cdc42p have not yet been described to be involved in regulation of pseudohyphal and invasive growth. Gic1p and Gic2p are required for cell polarization, but are dispensable for MAPK signal transduction and thus have been suggested to link Cdc42p to dynamic rearrangements of the actin cytoskeleton (Brown *et al.*, 1997; Chen *et al.*, 1997). Interestingly, Gic1p and Gic2p act in a pathway for signaling from Cdc42p to the actin cytoskeleton that operates in parallel with a pathway that includes Bni1p, Msb3p and Msb4p (Bi *et al.*, 2000). Because the Gic1p/Gic2p pathway and the Bni1p/Msb3p/Msb4p pathways are largely redundant in function, it has been proposed that each of the pathways may be optimized for distinct growth conditions. One interpretation of our results is that the Gic1p/Gic2p pathway might be optimized for cell polarization in response to the nutritional signals that induce pseudohyphal and invasive growth. Whether interaction of Cdc42p with the Bni1p/Msb3p/Msb4p pathway is also essential for pseudohyphal development cannot be concluded from our study, because the two mutations blocking interaction with Bni1p (S71P and E100G) also diminish binding to other effectors. However, these mutants allow the conclusion that binding of Cdc42p to Bni1p is not essential for all functions of this effector. Both, the S71P and E100G mutations completely abolish Bni1p binding, yet these mutants do not display the polarity or morphology defects caused by a *bni1* mutation. This finding is not unexpected due to the fact that Bni1p interacts with several other proteins such as Rho1p, Rho3p, Rho4p, Pfy1p or Spa2p (Evangelista *et al.*, 1997; Fujiwara *et al.*, 1998; Imamura *et al.*, 1997). Yet, S71P and E100G are novel mutations as to defining residues of Cdc42p that are essential for binding to Bni1p.

Our results indicate that interaction between Cdc42p and Gic1p/Gic2p is required for pseudohyphal development. However, this function of Cdc42p alone cannot be sufficient for a full developmental response. This interpretation can be made based on the finding that the I46M mutant displays only minimal alterations in Gic1p/Gic2p binding, but has a much more pronounced defect in binding to Skm1p. This points towards Skm1p as a further effector of Cdc42p that affects pseudohyphal and invasive growth.

Our study did not uncover mutations in Cdc42p that exclusively block binding to Ste20p. The only mutant showing significantly reduced interaction with Ste20p is S71P. This finding correlates with the fact that S71P is the only mutant found here that affects both localization of GFP-Ste20p to the bud tip and expression of the Kss1p-MAPK target gene *FLO11*. However, although reduced binding of Cdc42p to Ste20p causes some of the phenotypes that would be expected based upon previous studies defining Ste20p as an important pseudohyphal effector, interpretation of this data is complicated by the fact that S71P also affects interaction of Cdc42p with other effectors.

All together, effector mutants isolated here are very helpful for dissecting the distinct functions of Cdc42p, although we cannot exclude that some of the functions that are impaired in these mutants are due to reduced binding or activation of further effectors of Cdc42p. This might also explain the fact that none of the hyperfilamentous A30T, D65N, N92D or E95K variants display altered binding patterns to the effectors tested here. However, the novel mutants found in this study point towards Gic1p/Gic2p and Skm1p as effectors that, apart from Ste20p, might be important for morphological responses to nutritional signals. Whether each of these effectors acts independently or whether and how functions of Ste20p, Gic1p/Gic2p and Skm1p are connected to control pseudohyphal development remains to be investigated.

New insights into Cdc42p structure and effector binding.

A growing compendium of mutations in *CDC42* together with both the solution and the crystal structures of human Cdc42Hs have defined several functional domains within Cdc42p (Feltham *et al.*, 1997; Johnson, 1999; Nassar *et al.*, 1998; Rittinger *et al.*, 1997; Rudolph *et al.*, 1999). Four domains have been implicated in the binding and hydrolysis of GTP, and three regions - Switch I, Switch II and Rho Insert – are involved in protein-protein interactions with downstream effector proteins (Fig. 7). With the exception of Rho Insert, all of these domains are highly conserved between human Cdc42Hs and *S. cerevisiae* Cdc42p (Fig. 7A). Further important information on Cdc42p structure and effector binding domains comes from studies using specific effector mutants that are selectively impaired for binding to certain effectors but not to others. For instance, the V44A mutation selectively blocks binding of Cdc42p to Gic1p, Gic2p and Cla4p, but not binding to Bni1p, Skm1p or Ste20p (Richman *et al.*, 1999). Our study has identified several residues of Cdc42p previously unknown to be required for selective effector binding. Although specificity is not absolutely clear-cut in all mutants, significant differences can be observed. Residues S158 and L160 show selectivity for interaction with Gic1p and Gic2p. These two residues map to a conserved region of Cdc42p that has been implied in GTP-binding and hydrolysis. However, the S158T and L160P mutations are not likely to inhibit GTPase activity of the protein, because neither binding to Bni1p, Ste20p or Cla4p nor functions essential for cell division are affected. A more likely explanation is that residues S158 and L160 are involved in effector binding. N26 is a further residue that is required for binding to Gic1p/Gic2p (and to a certain extent to Skm1p), but not for interaction with Bni1p, Ste20p or Cla4p. N26 maps to the end of Switch I, a region that together with Switch II is among the regions of Cdc42p that display the most significant flexibility in NMR measurement of the protein (Loh *et al.*, 1999). The three-dimensional structure model of Cdc42p shows that N26, S158 and L160 are in close proximity on the surface of Cdc42p (Fig. 7), defining this region of the protein to be important for binding of Gic1p/Gic2p. A further residue important for discrimination between distinct effectors is I46 due to the selective binding pattern of the I46M mutant that is impaired for binding to Skm1p. I46 is located in the loop between Switch I and Switch II, a region that

also includes residue V44 required for selective binding to Gic1p/Gic2p and Cla4p (Richman *et al.*, 1999). Our study has further identified S71, located within the Switch II region, and E100, residing within the helix α3, as residues that are crucial for binding to diverse effector proteins. These residues do not appear to be selective for a single effector. However, they still define a part of the protein that appears to be highly important for binding to Bni1p. Effects of the S71P and E100G mutations on binding of Cdc42p to Gic1p/Gic2p (S71P) or Ste20p and Cla4p (E100G) are by far less pronounced than effects measured on interaction with Bni1p (with 1% of binding capacity left). Because both residues are in very close vicinity on the Cdc42p surface (Fig. 7), this region might be an important binding site for Bni1p.

Taken together, our results help to improve our understanding of the functions and functional domains of the essential GTPase Cdc42p in *S. cerevisiae*. Given the high degree of conservation of Cdc42p throughout the eukaryotic kingdom, mutations identified here might help to dissect the distinct functions of Cdc42p in other organisms.

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Chapter 3

Dual role of the yeast TEA/ATTS family transcription factor Tec1p in regulation of gene expression and cellular development

ABSTRACT

In *Saccharomyces cerevisiae*, the transcription factors Tec1p and Ste12p are required for haploid invasive and diploid pseudohyphal growth. Tec1p and Ste12p have been postulated to regulate these developmental processes primarily by cooperative binding to filamentous and invasion responsive elements (FREs), which are combined enhancer elements that consist of a Tec1p-binding site (TCS) and a Ste12p-binding site (PRE). They are present in the promoter region of target genes, e.g. *FLO11*. Here, we show that Tec1p efficiently activates target gene expression and cellular development in the absence of Ste12p. We further demonstrate that TCS elements alone are sufficient to mediate Tec1p-driven gene expression by a mechanism termed TCS control that is operative even when Ste12p is absent. Mutational analysis of *TEC1* revealed that TCS control, *FLO11* expression and haploid invasive growth require the C-terminus of Tec1p. In contrast, the Ste12p-dependent FRE control mechanism is sufficiently executed by the N-terminal portion of Tec1p that contains the TEA/ATTS DNA-binding domain. Our study suggests that regulation of haploid invasive and diploid pseudohyphal growth by Ste12p and Tec1p is not only executed by combinatorial control, but involves additional control mechanisms in which Ste12p activates *TEC1* expression via clustered PREs, and where Tec1p regulates expression of target genes, e.g. *FLO11*, by TCS control.

INTRODUCTION

Saccharomyces cerevisiae is a dimorphic fungus that interconverts between unicellular and multicellular filamentous growth modes (Gimeno *et al.*, 1992). Upon nitrogen starvation, diploid cells switch from growth as single yeast form (YF) cells to a filamentous form consisting of chains of elongated cells called pseudohyphae (PH). A related phenomenon called invasive growth is observed in haploid cells (Roberts and Fink, 1994). In contrast to PH growth, haploid invasive growth is not triggered by nitrogen starvation but occurs on rich medium in response to glucose depletion (Cullen and Sprague, 2000). In addition, low concentrations of α-factor mating pheromone induce haploid invasive growth (Erdman and Snyder, 2001; Roberts *et al.*, 2000).

Both haploid invasive growth and diploid pseudohyphal development require the transcription factors Tec1p and Ste12p (Gavrias *et al.*, 1996; Liu *et al.*, 1993; Madhani and Fink, 1997; Mösch and Fink, 1997; Roberts and Fink, 1994). Tec1p was originally identified as a regulator of expression of *Ty1* transposon insertions (Laloux *et al.*, 1990). Tec1p contains an evolutionary conserved DNA-binding domain that has been named TEA (TEF-1, Tec1p, and AbaAp) or ATTS (AbaAp, TEF-1, Tec1p, and Scalloped) motif (Bürglin, 1991). The TEA/ATTS domain is shared by a group of eukaryotic transcription factors including Tec1p, human TEF-1, which regulates SV40 and papillovirus-16 gene expression, and *Aspergillus nidulans* AbaAp, a development specific transcription factor required for asexual spore formation (Andrianopoulos and Timberlake, 1991; Bürglin, 1991; Xiao *et al.*, 1991). These transcription factors recognize and bind the conserved sequences CATTCC and CATTCT that have been termed TEA/ATTS consensus sequences or TCS elements (Andrianopoulos and Timberlake, 1994; Baur *et al.*, 1997; Hwang *et al.*, 1993; Madhani and Fink, 1997). Ste12p was initially identified as a regulator of mating factor responsive genes that contain pheromone response elements (PREs) matching the consensus sequence TGAAACA in their promoter regions (Fields and Herskowitz, 1985; Kronstad *et al.*, 1987; Van Arsdell *et al.*, 1987). Ste12p binds poorly to a single PRE but binds cooperatively to multiple PREs leading to efficient transcriptional activation (Dolan *et al.*, 1989; Yuan and Fields, 1991). Clustering of PRE elements is common to mating factor responsive genes (e.g. *FUS1* and *SST2*), and confers not only mating factor- but also cell cycle-regulated transcription (Oehlen and Cross, 1998a; Oehlen and Cross, 1994; Oehlen *et al.*, 1996). Single PREs confer transcriptional activation only if juxtaposed by recognition sites for other transcription factors, e.g. Mcm1p or Tec1p that bind cooperatively with Ste12p to combined enhancer elements (Baur *et al.*, 1997; Errede and Ammerer, 1989; Madhani and Fink, 1997).

Combination of one PRE and one TCS element creates an enhancer element that has been termed filamentation and invasion response element or FRE (Madhani and Fink, 1997).

Ste12p and Tec1p have been demonstrated to bind cooperatively to FREs and to activate gene expression in a synergistic manner (Madhani and Fink, 1997). FREs are present in the promoter regions of genes involved in haploid invasive growth and diploid pseudohyphal development and include *FLO11* encoding a cell surface flocculin (Lo and Dranginis, 1998; Rupp *et al.*, 1999), and *TEC1* itself (Madhani and Fink, 1997). These findings have led to the view that FRE-mediated gene expression is the major control mechanism, by which Tec1p regulates haploid invasion and diploid pseudohyphal growth, and that confers specificity to the distinct Ste12p-regulated developmental programs (Madhani and Fink, 1997). However, several lines of evidence indicate that FRE control might not be the sole mechanism by which Ste12p and Tec1p control expression of genes required for haploid invasive and pseudohyphal growth. First, the *TEC1* promoter contains several clustered PREs that mediate control of *TEC1* transcription by mating pheromone, by Ste12p and other elements of the mating factor signal transduction pathway and by the cell cycle (Oehlen and Cross, 1998a). Second, microarray analysis has identified several genes that are under control of Ste12p and Tec1p but that do not contain FRE enhancer elements in their promoter regions, e.g. *PGU1* encoding a secreted endopolygalacturonase that degrades the plant-specific polysaccharide pectin (Madhani *et al.*, 1999). Instead, the promoter regions of these genes often contain several TCS elements that are not neighbored by PRE sites. Third, the TEA/ATTS family transcription factor AbaAp from *A. nidulans* activates gene expression by binding on its own to either single or multiple TCS elements present in the promoter region of target genes (Andrianopoulos and Timberlake, 1994). Finally, ectopic expression of the Tec1p-related transcription factors AbaAp from *A. nidulans* and CaTec1p from the human pathogen *Candida albicans* induce *S. cerevisiae* haploid invasive and pseudohyphal growth in strains that lack *STE12* (Gavrias *et al.*, 1996; Schweizer *et al.*, 2000).

These findings prompted us to examine the possibility that Ste12p and Tec1p control gene expression and cellular development by mechanisms distinct from FRE-mediated combinatorial control. We provide evidence that *TEC1* gene expression requires Ste12p, but is largely independent of Tec1p autoregulation. As a consequence, Tec1p levels drop 20-fold in strains lacking Ste12p. When restored to high levels, Tec1p activates haploid invasive growth and expression of the *FLO11* and *PGU1* genes even in the absence of *STE12*. Tec1p efficiently activates gene expression mediated by synthetic single or combined TCS elements inserted upstream of an UAS-less reporter gene. This Tec1p-mediated transcriptional control mechanism, termed TCS control, is operative even when Ste12p is absent. Mutational analysis of *TEC1* reveals that the C-terminus of Tec1p is required for haploid invasive growth and TCS-mediated gene expression, but is dispensable for the FRE control mechanism. Based on our results, we propose that Ste12p and Tec1p control gene expression and cellular development by several distinct mechanisms.

MATERIALS AND METHODS

Yeast strains and growth conditions.

All yeast strains used in this study are congenic to the ∑1278b genetic background (Table I). The *tec1Δ::HIS3* and *ste12Δ::TRP1* deletion mutations were introduced using deletion plasmids ptec1Δ::HIS3 (Mösch *et al.*, 1999), and pste12Δ::TRP1. RH2757 was derived from RH2756 by transformation with a linear fragment containing the *TRP1* gene. RH2758 was obtained by mating of RH2500 with RH2757, and RH2759 resulted from mating of RH2501 with RH2778. Standard methods for genetic crosses and transformation were used, and standard yeast culture medium was prepared essentially as described (Guthrie and Fink, 1991). When required, synthetic complete medium (SC) lacking appropriate supplements was used. Invasive growth tests were performed as described previously (Roberts and Fink, 1994). Pseudohyphal development was induced by growth on synthetic low-ammonium medium (SLAD) (Gimeno *et al.*, 1992).

Plasmid constructions.

All of the plasmids used in this study are listed in Table II. Plasmids pME2044, pME2045 and pME2047 were constructed by subcloning of a 3.1 kb *Pst*I-*Hin*dIII fragment carrying *TEC1* from B3366 (Mösch and Fink, 1997) into YCplac33, YCplac111 and YEplac181, respectively. For regulated expression of *TEC1* from the inducible *GAL1-10* promoter, a 2.5 kb *Bsp*120I/*Sac*I fragment containing a *GAL1(p)::TEC1* cassette was subcloned from pME2071 (Mösch *et al.*, 1999) into pRS315 yielding plasmid pME2049. A *Sal*I-site was inserted after the ATG start codon of *TEC1* by a two step strategy. (i) The *TEC1* promoter region was amplified by PCR using primers TEC1-3 (ACGCGTCGACCATGGTTAAACAGGTATCAGAATTGTTG) and TEC1-4 (CTTCAGGCAAGAGTACGTTCTTCGCTGG) and plasmid B3366 (Mösch and Fink, 1997) as template, yielding a 1.05 kb PCR product that was digested with *Pst*I and *Sal*I and inserted into plasmid YCplac33 to obtain plasmid YCplac33-TEC1(p). (ii) A 2.1 kb fragment containing the *TEC1* open reading frame was amplified from B3366 using primers TEC1-5 (ACGCGTCGACAGTCTTAAAGAAGACGACTTTGGCAAGG) and TEC1-6 (CGCGGATCCGGCCCCGACTTGAATGATTTTCAAGGTAGG), introducing a *Sal*I site in front of the second codon of *TEC1* and a *Bam*HI site 650 bp downstream of the TAA stop codon. This fragment was inserted into the *Sal*I and *Bam*HI sites of YCplac33-TEC1p from (i) to obtain plasmid pME2068, carrying a functional *TEC1(p)::TEC1* gene with a *Sal*I restriction site after the ATG start codon. Plasmids pME2289 and pME2294 were obtained by subcloning of the *TEC1(p)::TEC1* cassette from pME2068 into the *Pst*I and *Bam*HI sites of YCplac111 and YCplac181, respectively. Plasmids pME2279 and pME2280, both expressing a triple myc epitope-tagged version of Tec1p under the control of the *TEC1*

promoter, were obtained by the following cloning strategy: (i) a 3.1 kb *Pst*I-*Bam*HI fragment containing *TEC1(p)::TEC1* was subcloned from pME2068 into YEplac195. (ii) A synthetic *Bgl*II linker was ligated into the *Sal*I site following the *TEC1* translational start codon. (iii) Finally, a 120 bp *Bam*HI fragment carrying the triple myc epitope (myc^3) was inserted into the *Bgl*II site to yield plasmid pME2280. The complete *TEC1(p)::*myc^3*-TEC1* cassette was isolated by *Pst*I-*Sma*I digestion and inserted into YCplac33 to yield pME2279. In addition, plasmids pME2295 and pME2296 were constructed, which differ from pME2280 and pME2279 only by the copy number of myc epitope-tags. Plasmid pME2295 consists of a *TEC1(p)::*myc^6*-TEC1* cassette in YCplac33, and plasmid pME2296 carries the same cassette in YEplac195. Plasmids carrying the different *tec1* mutant alleles were identified in a mutant allele library screen (see below). A subset of these *tec1* alleles were subcloned as 3.1 kb *Pst*I-*Bam*HI fragments into YCplac111 to obtain plasmids pME2290 to pME2293. The different *tec1* mutant alleles were myc epitope-tagged by isolation of 2.1 kb *Sal*I-*Bam*HI fragments from the respective plasmids and exchange for the corresponding wild-type *TEC1* fragment in plasmids pME2280 and pME2279 to yield plasmids pME2281 to pME2288.

For expression of *A. nidulans* derived *abaA* in *S. cerevisiae*, the *abaA* ORF was placed behind the *S. cerevisiae TEC1* promoter using the following cloning strategy: (i) The intron-less *abaA*-open reading frame was amplified by PCR from plasmid pAA35 (Andrianopoulos and Timberlake, 1991) using primers ABAA-1 (ACGCGTCGACGCTACTGACTGGCAACCCGAGTGTATGG) and ABAA-2 (ACGCGTCGACCTAGACAGCCTCAACCGCAGTATGTTC), introducing *Sal*I sites at both ends. The resulting 2.4 kb PCR fragment was placed downstream of the *TEC1* promoter by insertion into the *Sal*I site of YCplac33-TEC1(p) to yield pME2043. The whole *TEC1(p)::abaA* cassette of pME2043 was released by *Pst*I-*Sma*I digestion and inserted into YCplac111 and YEplac181 to yield pME2046 and pME2048, respectively. Plasmids pME2050 and pME2070 were obtained by subcloning of a 3.1 kb *GAL1(p)::abaA* expression cassette from plasmid pAA35 (Andrianopoulos and Timberlake, 1991) into YCplac111 and pRS316, respectively.

Plasmid pME1108 was constructed by deletion of a 430 bp *Xho*I fragment containing the *CYC1* UAS sequence of plasmid pLI4 (Sengstag and Hinnen, 1988). Plasmids pME2051 to pME2058 carrying various combinations of TCS elements upstream of the *CYC1-lacZ* reporter gene were constructed by replacing the *Xho*I fragment of pLI4 for one or several copies of a synthetic linker containing a single TCS element and *Xho*I-cohesive ends. The linker was prepared by annealing of primers TCS1 (TCGAGTCA<u>CATTCT</u>TCTGC) (the TCS element is underlined) and TCS2 (TCGAGCAGA<u>AGAATG</u>TGAC) (the reverse complement of the TCS element is underlined). The number and orientation of the inserted TCS elements were determined by DNA sequence analysis and are shown in Fig. 5. The integrative *FRE*(*Ty1*)-*lacZ* reporter plasmid pME2066 was obtained by subcloning a *Sal*I-

*Bam*HI fragment from plasmid *FRE(Ty1)::lacZ* (Madhani and Fink, 1997) into pLI4. The *TEC1-lacZ* reporter plasmid pME2065 was constructed by subcloning of the *TEC1* promoter as a 1.05 kb *Pst*I-*Sal*I fragment from pME2041 into YEp356R (Myers *et al.*, 1986), and plasmid pME2300 was obtained by subcloning the *TEC1-lacZ* expression cassette from pME2065 into YCplac33.

Library of *TEC1* mutants.

TEC1 was mutagenized by PCR amplification of a 2.2 kb fragment of pME2068 containing the *TEC1* ORF using *Taq* DNA polymerase in the presence of 0.24 mM $MnCl_2$. The resulting DNA was digested with *Sal*I and *Bam*HI and exchanged for the corresponding *Sal*I-*Bam*HI fragment in pME2068 yielding a library of more than 20,000 independent recombinants. Following identification of mutants (see below), *TEC1* alleles were sequenced using the ABI Prism Big Dye terminator sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany).

Screen for *TEC1* invasive growth mutants.

For isolation of mutants with altered invasive growth and *FRE-lacZ* reporter induction, strain RH2499 carrying a chromosomal deletion of *TEC1* and an integrated *FRE*(*Ty1*)::*lacZ* reporter was transformed with the *TEC1* mutant library described above. A pool of approximately 30,000 transformants was plated on solid medium lacking uracil (SC-Ura) at a density of ~500 colonies per plate. Invasive growth mutants were isolated by employing an invasive growth test (Roberts and Fink, 1994), and expression of the *FRE*(*Ty1*)::*lacZ* reporter gene was measured by a qualitative filter assay (Breeden and Nasmyth, 1985). Initial mutant phenotypes were confirmed by isolation of the *TEC1*-containing plasmids and reintroduction into the parental strain.

Pseudohyphal development assay.

Qualitative assays for pseudohyphal development were performed as described previously (Mösch *et al.*, 1996). After 3 days of growth on solid SLAD medium, pseudohyphal colonies were viewed with a Zeiss Axiovert microscope and photographed using a Kappa DX30 digital camera and the Kappa ImageBase software (Kappa opto-electronics, Gleichen, Germany).

ß-Galactosidase assays.

Strains carrying plasmid-borne or integrated *lacZ* reporters were grown in selective liquid SC medium to exponential growth phase, and extracts were prepared and assayed for ß-galactosidase activity as described previously (Mösch *et al.*, 1996). Specific ß-galactosidase activity was normalized to the total protein in each extract and equals [the optical density at

420 nm (OD_{420}) × 1.7]/[0.0045 × protein concentration × extract volume × time]. Assays were performed on at least three independent transformants, and the mean value is presented. Standard deviations did not exceed 20%.

Northern blot analysis.

Total RNAs were prepared from exponentially growing liquid cultures as described earlier (Cross and Tinkelenberg, 1991). Total RNAs were separated on an 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes as described earlier (Mösch *et al.*, 1992). *TEC1*, *FLO11* and *ACT1* transcripts were detected using gene-specific ^{32}P-radiolabeled DNA probes. Hybridizing signals were quantified using a BAS-1500 Phospho-Imaging scanner (Fuji, Tokyo, Japan).

Protein analysis.

Yeast strains harboring plasmids encoding myc-Tec1p were grown to exponential phase in liquid SC medium. Preparation of total cell extracts and subsequent Western blot analysis were performed essentially as described (Roberts *et al.*, 1997). myc-Tec1p fusion proteins were detected using enhanced chemiluminescence technology (Amersham, Buckinghamshire, United Kingdom) after incubation of nitrocellulose membranes with monoclonal mouse anti-myc antibodies (9E10) together with a peroxidase-coupled goat anti-mouse IgG secondary antibody (Dianova, Hamburg, Germany). For detection of Cdc42p, the membranes were incubated with polyclonal anti-Cdc42p antibodies and a peroxidase-coupled goat anti-rabbit IgG secondary antibody (Dianova, Hamburg, Germany). Tec1p and Cdc42p signals were quantified using a sanner and the Molecular Analyst software (Bio-Rad, Munich, Germany).

Indirect immunofluorescence microscopy.

Yeast strains harboring plasmids encoding myc-Tec1p were cultured to exponential growth phase in liquid YNB medium supplemented with appropriate amino acids. Cells from 1 ml of the cultures were harvested by centrifugation and fixed in 3.7% formaldehyde. Spheroblasts were prepared as described previously (Pringle *et al.*, 1991). 4´,6-diamidino-2-phenylindole (DAPI) staining and monoclonal mouse anti-myc antibodies (9E10) together with an Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, OR, USA) were used for visualization of nuclei and myc epitope-tagged proteins, respectively. Cells were viewed on a Zeiss Axiovert microscope by either differential interference contrast microscopy (DIC) or fluorescence microscopy using standard DAPI and fluorescein isothiocyanate (FITC) filter sets. Cells were photographed using a Xillix Microimager digital camera and the Improvision Openlab software (Improvision, Coventry, UK).

Table I. Strains used in this study

Strain	Relevant genotype[b]	Reference
RH2754	*MAT**a** ura3-52 leu2::hisG his3::hisG trp1::hisG*	This study
RH2500	*MAT**a** tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG*	This study
RH2755	*MAT**a** ste12Δ::TRP1 ura3-52 leu2::hisG his3::hisG trp1::hisG*	This study
RH2501	*MAT**a** tec1Δ::HIS3 ste12Δ::TRP1 ura3-52 leu2::hisG his3::hisG trp1::hisG*	This study
RH2756	*MATα tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG*	This study
RH2757	*MATα tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG*	This study
RH2778	*MATα tec1Δ::HIS3 ste12Δ::TRP1 ura3-52 leu2::hisG his3::hisG trp1::hisG*	This study
RH2758	*MAT**a**/MATα tec1Δ::HIS3/tec1Δ::HIS3 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1Δ::hisG/TRP1*	This study
RH2759	*MAT**a**/MATα tec1Δ::HIS3/tec1Δ::HIS3 ste12Δ::TRP1/ste12Δ::TRP1 ura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1Δ::hisG/trp1Δ::hisG*	This study
RH2499	*MATα tec1Δ::HIS3 FRE(Ty1)-lacZ::LEU2 ura3-52 leu2::hisG his3::hisG trp1::hisG*	(Mösch *et al.*, 1999)
RH2767[a]	as RH2500 except *CYC1(ΔUAS)-lacZ::URA3*	This study
RH2765[a]	as RH2500 except *TCS^{fw}-CYC1-lacZ::URA3*	This study
RH2766[a]	as RH2500 except *TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2764[a]	as RH2500 except *TCS^{fw}-6-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2763[a]	as RH2500 except *TCS^{fw}-11-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2760[a]	as RH2500 except *TCS^{fw}-14-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2761[a]	as RH2500 except *TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2762[a]	as RH2500 except *TCS^{bw}-13-TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2768[a]	as RH2500 except *TCS^{fw}-14-PRE^{fw}-CYC1-lacZ::URA3*	This study
RH2776[a]	as RH2501 except *CYC1(ΔUAS)-lacZ::URA3*	This study
RH2774[a]	as RH2501 except *TCS^{fw}-CYC1-lacZ::URA3*	This study
RH2775[a]	as RH2501 except *TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2773[a]	as RH2501 except *TCS^{fw}-6-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2772[a]	as RH2501 except *TCS^{fw}-11-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2769[a]	as RH2501 except *TCS^{fw}-14-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2770[a]	as RH2501 except *TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2771[a]	as RH2501 except *TCS^{bw}-13-TCS^{bw}-13-TCS^{bw}-CYC1-lacZ::URA3*	This study
RH2777[a]	as RH2501 except *TCS^{fw}-14-PRE^{fw}-CYC1-lacZ::URA3*	This study

[a] Strains were obtained by targeted integration of corresponding reporter plasmids (see Table II) into RH2500 or RH2501.

[b] *TCS^{fw}* indicates forward orientation of TCS elements, and *TCS^{bw}* indicates backward orientation with respect to the orientation of the TCS element in *FRE(Ty1)* (Madhani and Fink, 1997).

Table II. Plasmids used in this study (1 of 2)

Plasmid	Description	Reference
YCplac33	*URA3*-marked centromere vector	(Gietz and Sugino, 1988)
YEplac195	*URA3*-marked *2µm* vector	(Gietz and Sugino, 1988)
YCplac111	*LEU2*-marked centromere vector	(Gietz and Sugino, 1988)
pRS315	*LEU2*-marked centromere vector	(Sikorski and Hieter, 1989)
YEplac181	*LEU2*-marked *2µm* vector	(Gietz and Sugino, 1988)
YEp356R	*URA3*-marker *2µm* vector for *lacZ*-fusions	(Myers *et al.*, 1986)
pME2044	3.1 kb fragment containing *TEC1* in YCplac33	This work
pME2045	3.1 kb fragment containing *TEC1* in YCplac111	This work
pME2047	3.1 kb fragment containing *TEC1* in YEplac181	This work
pME2049	2.5 kb *GAL1(p)::TEC1* fusion in pRS315	This work
pME2071	2.5 kb *GAL1(p)::TEC1* fusion in pRS316	(Mösch *et al.*, 1999)
pME2077	*tec1-102* in YCplac33	This work
pME2083	*tec1-103* in YCplac33	This work
pME2085	*tec1-104* in YCplac33	This work
pME2086	*tec1-105* in YCplac33	This work
pME2102	*tec1-201* in YCplac33	This work
pME2096	*tec1-202* in YCplac33	This work
pME2101	*tec1-203* in YCplac33	This work
pME2103	*tec1-204* in YCplac33	This work
pME2290	*tec1-102* in YCplac111	This work
pME2291	*tec1-105* in YCplac111	This work
pME2293	*tec1-201* in YCplac111	This work
pME2292	*tec1-202* in YCplac111	This work
pME2068	*TEC1(p)::TEC1* in YCplac33	This work
pME2289	*TEC1(p)::TEC1* in YCplac111	This work
pME2294	*TEC1(p)::TEC1* in YEplac181	This work
pME2279	*TEC1(p)::myc^3-TEC1* fusion in YCplac33	This work
pME2295	*TEC1(p)::myc^6-TEC1* fusion in YCplac33	This work
pME2280	*TEC1(p)::myc^3-TEC1* fusion in YEplac195	This work
pME2296	*TEC1(p)::myc^6-TEC1* fusion in YEplac195	This work
pME2281	*TEC1(p)::myc^3-tec1-102* fusion in YEplac195	This work
pME2282	*TEC1(p)::myc^3-tec1-103* fusion in YEplac195	This work
pME2283	*TEC1(p)::myc^3-tec1-104* fusion in YEplac195	This work
pME2284	*TEC1(p)::myc^3-tec1-105* fusion in YEplac195	This work
pME2287	*TEC1(p)::myc^3-tec1-201* fusion in YEplac195	This work
pME2285	*TEC1(p)::myc^3-tec1-202* fusion in YEplac195	This work
pME2286	*TEC1(p)::myc^3-tec1-203* fusion in YEplac195	This work

Table II. Plasmids used in this study (2 of 2)

Plasmid	Description[a]	Reference
pME2288	*TEC1(p)::myc*^{3}*-tec1-204* fusion in YEplac195	This work
pME2043	3.5 kb *TEC1(p)::abaA* fusion in YCplac33	This work
pME2046	3.5 kb *TEC1(p)::abaA* fusion in YCplac111	This work
pME2048	3.5 kb *TEC1(p)::abaA* fusion in YEplac181	This work
pME2050	3.1 kb *GAL1(p)::abaA* fusion in YCplac111	This work
pME2070	3.1 kb *GAL1(p)::abaA* fusion in pRS316	This work
pME2300	*TEC1-lacZ* fusion in YCplac33	This work
pME2065	*TEC1-lacZ* fusion in YEp356R	This work
B3782	*FLO11-lacZ* fusion in YEp356R	(Rupp *et al.*, 1999)
pME2064	*PGU1-lacZ* fusion in YEp356R	(Madhani *et al.*, 1999)
pLI4	*CYC1-lacZ* fusion in *URA3*-marked integrative vector	(Sengstag and Hinnen, 1988)
pME1108	pLI4 with *CYC1(ΔUAS)-lacZ*	This work
pME2051	pLI4 with *TCS*^{fw}*-CYC1-lacZ*	This work
pME2052	pLI4 with *TCS*^{bw}*-CYC1-lacZ*	This work
pME2056	pLI4 with *TCS*^{fw}*-6-TCS*^{bw}*-CYC1-lacZ*	This work
pME2055	pLI4 with *TCS*^{fw}*-11-TCS*^{bw}*-CYC1-lacZ*	This work
pME2053	pLI4 with *TCS*^{fw}*-14-TCS*^{bw}*-CYC1-lacZ*	This work
pME2057	pLI4 with *TCS*^{bw}*-13-TCS*^{bw}*-CYC1-lacZ*	This work
pME2058	pLI4 with *TCS*^{bw}*-13-TCS*^{bw}*-13-TCS*^{bw}*-CYC1-lacZ*	This work
pME2066	pLI4 with *TCS*^{fw}*-14-PRE*^{fw}*-CYC1-lacZ*	This work
ptec1Δ::HIS3	Cassette for full deletion of *TEC1* open reading frame	(Mösch *et al.*, 1999)
pste12Δ::TRP1	Cassette for full deletion of *STE12* open reading frame	This work

[a] *TCS*^{fw} indicates forward orientation of TCS element, and *TCS*^{bw} marks backward orientation with respect to the orientation of the TCS element in *FRE(Ty1)* (Madhani and Fink, 1997). *TCS*^{fw}*-14-PRE*^{fw} corresponds to the sequence of *FRE(Ty1)* (Madhani and Fink, 1997).

RESULTS

Tec1p and AbaAp induce haploid invasive growth and PH development in the absence of Ste12p.

We tested whether Tec1p is able to induce haploid invasive growth and diploid pseudohyphal development of *S. cerevisiae* in the absence of Ste12p. Previous work had shown that the Tec1p homologue AbaAp from *A. nidulans* induces *S. cerevisiae* pseudohyphal development in strains lacking *STE12*, but only when expressed from the highly inducible *GAL1*-promoter (Gavrias *et al.*, 1996). We expressed *TEC1* and *abaA* from the endogenous *TEC1*-promoter on either low copy (*CEN*) or high copy (*2µm*) plasmids in both haploid and diploid *tec1Δ* and *tec1Δ ste12Δ* mutant strains. In addition, both genes were expressed from the *GAL1*-promoter. Haploid strains were assayed for invasive growth by a wash test (Fig. 1), and diploid PH development was measured by growth on nitrogen starvation medium (Fig. 2).

As expected, haploid *tec1Δ* and *tec1Δ ste12Δ* mutant strains failed to grow invasively when harboring the empty vectors as control plasmids (Fig. 1). Expression of *TEC1* from the low copy plasmid was sufficient to restore invasive growth in the *tec1Δ* strain, but not in the *tec1Δ ste12Δ* background. However, defective agar invasion of the *tec1Δ ste12Δ* strain was fully restored when *TEC1* was expressed from either a high copy plasmid (Fig. 1A) or from the *GAL1*-promoter (Fig. 1B). Identical results were obtained for strains expressing AbaAp instead of Tec1p. Expression of a single copy of *abaA* driven by the *TEC1*-promoter restored agar invasion in the *tec1Δ* strain, but not in the *tec1Δ ste12Δ* double mutant. As found for *TEC1*, defective invasive growth of the *tec1Δ ste12Δ* strain was suppressed by expression of *abaA* under control of the *TEC1*-promoter from a high copy plasmid or when driven by the *GAL1*-promoter (Fig. 1A and 1B). These experiments show that both Tec1p and AbaAp can induce haploid invasive growth in the absence of Ste12p.

PH development assays of diploid strains led to results similar to that obtained for invasive growth in haploids. PH growth was virtually absent in diploid *tec1Δ/tec1Δ* and *tec1Δ/tec1Δ ste12Δ/ste12Δ* mutant strains carrying control plasmids. Low copy expression of *TEC1* restored defective PH growth of the *tec1Δ/tec1Δ* mutant, but not of the *tec1Δ/tec1Δ ste12Δ/ste12Δ* double mutant. Again, PH growth defects of strains lacking *STE12* could be suppressed by expression of *TEC1* from the high copy plasmid, although suppression was only partial when compared to the *tec1Δ/tec1Δ* single mutant (Fig. 2). Low copy expression of *abaA* driven by the *TEC1*-promoter only weakly stimulated pseudohyphal growth in the *tec1Δ/tec1Δ* mutant. However, expression of *abaA* driven from a high-copy plasmid was sufficient to induce pseudohyphal growth in the *tec1Δ/tec1Δ* and *tec1Δ/tec1Δ ste12Δ/ste12Δ* mutants. In summary, Tec1p and AbaAp are able to at least partially induce PH development in the absence of Ste12p.

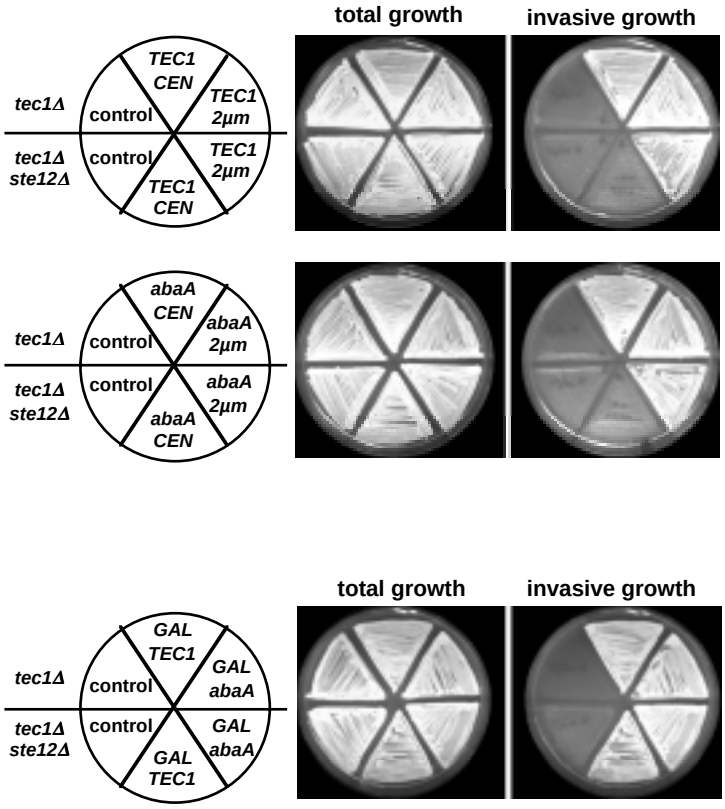


Fig. 1. Haploid invasive growth of yeast strains expressing *TEC1* or *A. nidulans abaA* driven by the *TEC1* promoter. (A) Haploid strains RH2500 (*tec1Δ*) and RH2501 (*tec1Δ ste12Δ*) carrying plasmids YCplac111 (control), pME2045 (*TEC1 CEN*), pME2047 (*TEC1 2µm*), pME2046 (*abaA CEN*) or pME2048 (*abaA 2µm*) were grown on SC-Leu medium containing 2% glucose for 4 days. Plates were photographed before (total growth) and after (invasive growth) cells were washed off the agar surface. (B) Haploid strains RH2500 (*tec1Δ*) and RH2501 (*tec1Δ ste12Δ*) carrying plasmids YCplac111 (control), pME2049 (*GAL-TEC1*) or pME2050 (*GAL-abaA*) were grown on SC-Leu medium containing 2% galactose to induce expression from the *GAL1*-promoter. Invasive growth was measured as described for panel *A*.

Expression of *TEC1* requires Ste12p, but is largely independent of Tec1p itself.

Haploid yeast wild-type, *tec1Δ*, *ste12Δ* and *tec1Δ ste12Δ* strains were constructed that express a myc epitope-tagged version of Tec1p (myc^6-Tec1p) from either low copy or high copy plasmids, to measure intracellular amounts of Tec1p. Strains were tested for invasive growth behavior and found to be indistinguishable from strains expressing untagged Tec1p (data not shown), demonstrating that the epitope-tagged version is fully functional. When expressed from a low copy plasmid, myc^6-Tec1p levels were identical in wild-type and *tec1Δ*

strains (Fig. 3A). In contrast, myc[6]-Tec1p levels dropped roughly 20-fold in strains lacking *STE12* (*ste12Δ* and *tec1Δ ste12Δ*), which consequently were unable to grow invasively. Thus, Ste12p is required for expression of Tec1p to levels required for induction of invasive growth. When expressed from a high copy plasmid, almost identical amounts of myc[6]-Tec1p were detectable in all strains, even in the *ste12Δ* mutants (Fig. 3A). Levels of myc[6]-Tec1p expressed from high copy number plasmids were not more than 2.7-fold higher than levels obtained by low copy expression of myc[6]-Tec1p in wild-type or *tec1Δ* strains. Thus, expression of *TEC1* from the high copy number plasmid in *ste12Δ* mutant strains reflects a situation, where in the absence of Ste12p the amount of Tec1p is restored and therefore allows invasive growth.

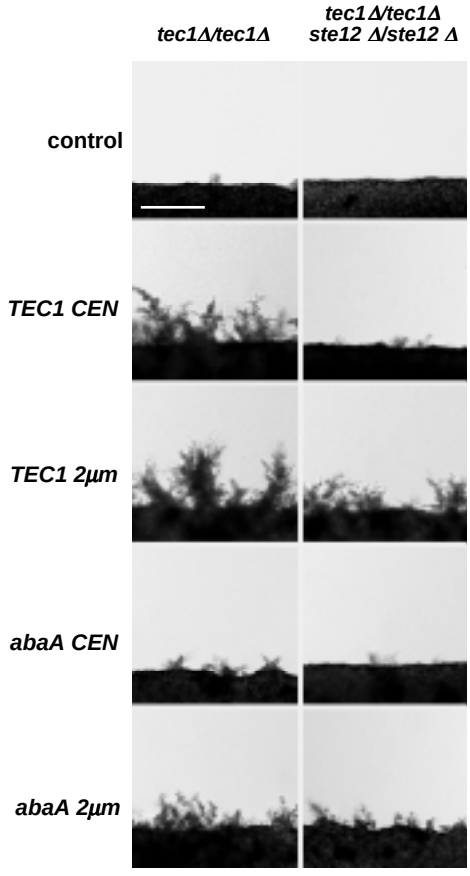


Fig. 2. Pseudohyphal growth of diploid yeast strains expressing *TEC1* or *A. nidulans abaA* driven by the *TEC1* promoter. Diploid strains RH2758 (*tec1Δ/tec1Δ*) and RH2759 (*tec1Δ/tec1Δ ste12Δ/ste12Δ*) carrying plasmids YCplac111 (control), pME2045 (*TEC1 CEN*), pME2047 (*TEC1 2µm*), pME2046 (*abaA CEN*) or pME2048 (*abaA 2µm*) were transformed with YCplac33 to obtain uracil prototrophy. Transformants were grown on solid nitrogen starvation medium for 4 days before pseudohyphal development was visualized under a microscope and photographed. Bar, 100 µm.

Expression of a translational *TEC1-lacZ* fusion gene from low copy and high copy plasmids was measured in haploid wild-type and *tec1Δ*, *ste12Δ* and *tec1Δ ste12Δ* mutant strains, to quantify regulation of *TEC1* gene expression by Ste12p and by Tec1p itself. Previous studies had shown that *TEC1* transcript levels drop at least 6-fold when Ste12p is absent and that activation of *TEC1* transcription by Ste12p is predominantly mediated by several PRE sites present in the *TEC1* promoter and only to a minor extent by its single FRE site (Laloux *et al.*, 1990; Oehlen and Cross, 1998b). We found that *TEC1* is not required for expression of a *TEC1-lacZ* fusion gene expressed from the low copy plasmid and only to a minor extent for expression of the high copy version (Fig. 3B). This indicates that Tec1p does not greatly contribute to its own expression, a conclusion that is supported by the previous finding that high-copy expression of Tec1p induces its own expression not more than twofold (Madhani and Fink, 1997). In contrast, expression of *TEC1-lacZ* was reduced between 4.5-fold (low copy *TEC1-lacZ*) and 11-fold (high copy *TEC1-lacZ*), when Ste12p was absent. High copy expression of *TEC1-lacZ* in the *ste12Δ* background was only 3.8-fold higher than levels obtained by low copy expression in strains carrying a functional *STE12* gene, corroborating the results obtained by measuring Tec1p protein levels (Fig. 3A). However, several differences between *TEC1-lacZ* expression and Tec1p protein levels were found. In *STE12*-carrying strains (wild-type or *tec1Δ*), expression of *TEC1-lacZ* from the high copy plasmid was 42-fold higher than from the low copy version (Fig. 3B). In contrast, Tec1p protein levels in *STE12* strains are only 2.7-fold higher when *TEC1* is expressed from the high copy plasmid than when expressed from the low copy version (Fig. 3A). Furthermore, *TEC1-lacZ* expression dropped between 4.5- and 11-fold when *STE12* was deleted, whereas Tec1p protein levels decrease by a factor of 20 in the absence of Ste12p. These discrepancies could be explained by a difference in protein stability between Tec1p and β-galactosidase.

In summary, Ste12p appears to control the expression of *TEC1* to a larger extent than Tec1p itself.

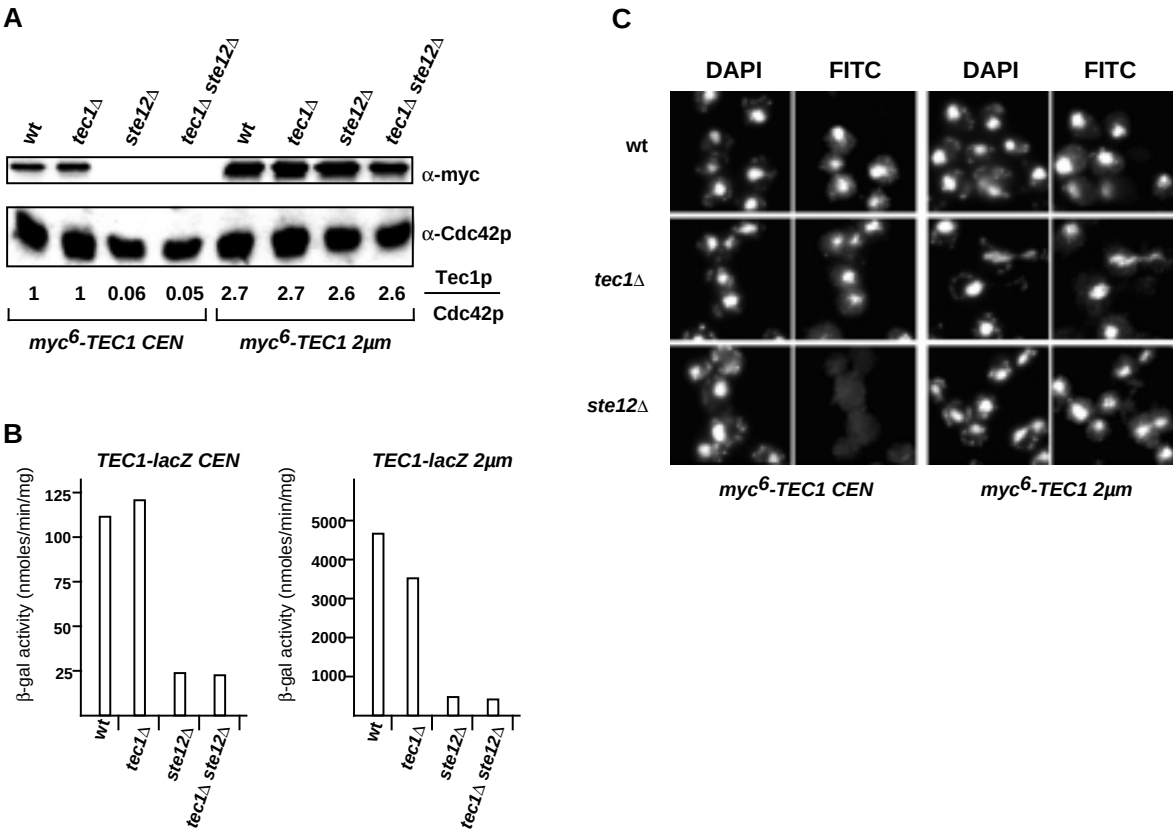


FIG. 3. Expression and localization of Tec1p in *S. cerevisiae*. (A) Expression of myc^6-Tec1p. Protein extracts were prepared from haploid yeast strains RH2754 (wt), RH2500 (*tec1Δ*), RH2755 (*ste12Δ*) and RH2501 (*tec1Δ ste12Δ*) expressing *myc^6-TEC1* on low copy plasmid pME2295 (*CEN*) or high copy plasmid pME2296 (*2μm*), and levels of myc^6-Tec1p were determined by Western blot analysis using a monoclonal anti-myc antibody. As internal control, expression levels of Cdc42p were measured in the same extracts using a polyclonal anti-Cdc42p antibody (lower panel). Relative expression levels of Tec1p (Tec1p/Cdc42p) are shown in arbitrary units and were obtained by normalizing Tec1p signals to Cdc42p signals and to levels measured in strain RH2500 (*tec1Δ*) expressing *TEC1* from a low copy plasmid. (B) *TEC1-lacZ* expression levels. Specific ß-galactosidase (ß-gal) activity was measured in haploid strains carrying *TEC1-lacZ* on low copy plasmid pME2300 (*CEN*) or high copy plasmid pME2065 (*2μm*) and in the presence or absence of *TEC1* and *STE12* in different combinations. Bars depict means of three independent measurements, with standard deviations not exceeding 15%. (C) Localization of myc^6-Tec1p. Haploid strains RH2754 (wt), RH2500 (*tec1Δ*) and RH2755 (*ste12Δ*) expressing *myc^6-TEC1* on low copy plasmid pME2295 (*CEN*) or high copy plasmid pME2296 (*2μm*) were grown to exponential phase and prepared for anti-myc immunofluorescence. Shown are representative cells that were viewed for nuclear DNA with DAPI imaging (DAPI) or for anti-myc immunofluorescence (FITC). Bar, 5 μm.

Tec1p enters the yeast nucleus without Ste12p.

Intracellular localization of Tec1p has not yet been determined directly, although indirect evidence suggested that Tec1p is a nuclear protein (Madhani and Fink, 1997). Here, subcellular localization of Tec1p was determined, to analyze whether Ste12p is required for nuclear transport. Wild-type, *tec1Δ* and *ste12Δ* strains that express myc epitope-tagged Tec1p from low copy or high copy plasmids were used for indirect immunofluorescence microscopy. Specific myc^6-Tec1p signals could be detected in the nucleus of wild-type and *tec1Δ* strains in the case of both low and high copy expression (Fig. 3C). In the *ste12Δ* strain, specific nuclear signals were absent when myc^6-Tec1p was expressed from the low copy plasmid, corroborating the results obtained by Western blot analysis (Fig. 3A). Importantly, nuclear localization of myc^6-Tec1p was restored in the *ste12Δ* strain when expressed from the high copy plasmid, suggesting that Tec1p is a nuclear protein that enters the nucleus even in the absence of Ste12p.

Tec1p and AbaAp activate expression of *FLO11* and *PGU1* in the absence of Ste12p.

Our finding that Tec1p can induce invasive growth independently of Ste12p prompted us to measure expression of *FLO11* and *PGU1* in *ste12Δ* strains, in which Tec1p levels had been restored by using high copy number plasmids. Expression of both the *FLO11* and *PGU1* genes is strongly reduced in the absence of either Tec1p or Ste12p (Lo and Dranginis, 1998; Madhani *et al.*, 1999; Rupp *et al.*, 1999). We reasoned that if expression of *FLO11* and *PGU1* could be activated in strains lacking Ste12p but expressing Tec1p at sufficient levels, activation of *FLO11* and *PGU1* by Tec1p would have to involve a Ste12p-independent control mechanism.

FLO11 transcript levels were measured in *tec1Δ* and *tec1Δ ste12Δ* strains expressing *TEC1* or *A. nidulans* derived *abaA* from either the endogenous yeast *TEC1*-promoter on low and high copy plasmids or from the *GAL1*-promoter (Fig. 4A). In the *tec1Δ* genetic background, introduction of single copies of either *TEC1* or *abaA* into yeast induced *FLO11* transcription 4.2-fold (*TEC1*) and 4.7-fold (*abaA*) when compared to a control strain expressing no Tec1p. High copy expression of *TEC1* or *abaA* or expression from the inducible *GAL1*-promoter led to an increase in *FLO11* transcript levels of 4.9-fold (high copy *TEC1*), 8.5-fold (high copy *abaA*), 13-fold (*GAL1-TEC1*) or 5.6-fold (*GAL1-abaA*), when compared to strains expressing a single copy of *TEC1*. Thus, expression of *FLO11* is depending on the dosage of Tec1p and AbaAp. In the *tec1Δ ste12Δ* strain, introduction of a single copy of *TEC1* or *abaA* was not sufficient to activate normal *FLO11* expression. However, high copy expression of Tec1p or AbaAp restored *FLO11* transcription to levels detected in the *tec1Δ* strain expressing single copies of *TEC1* or *abaA* (corresponding to a wild-type situation).

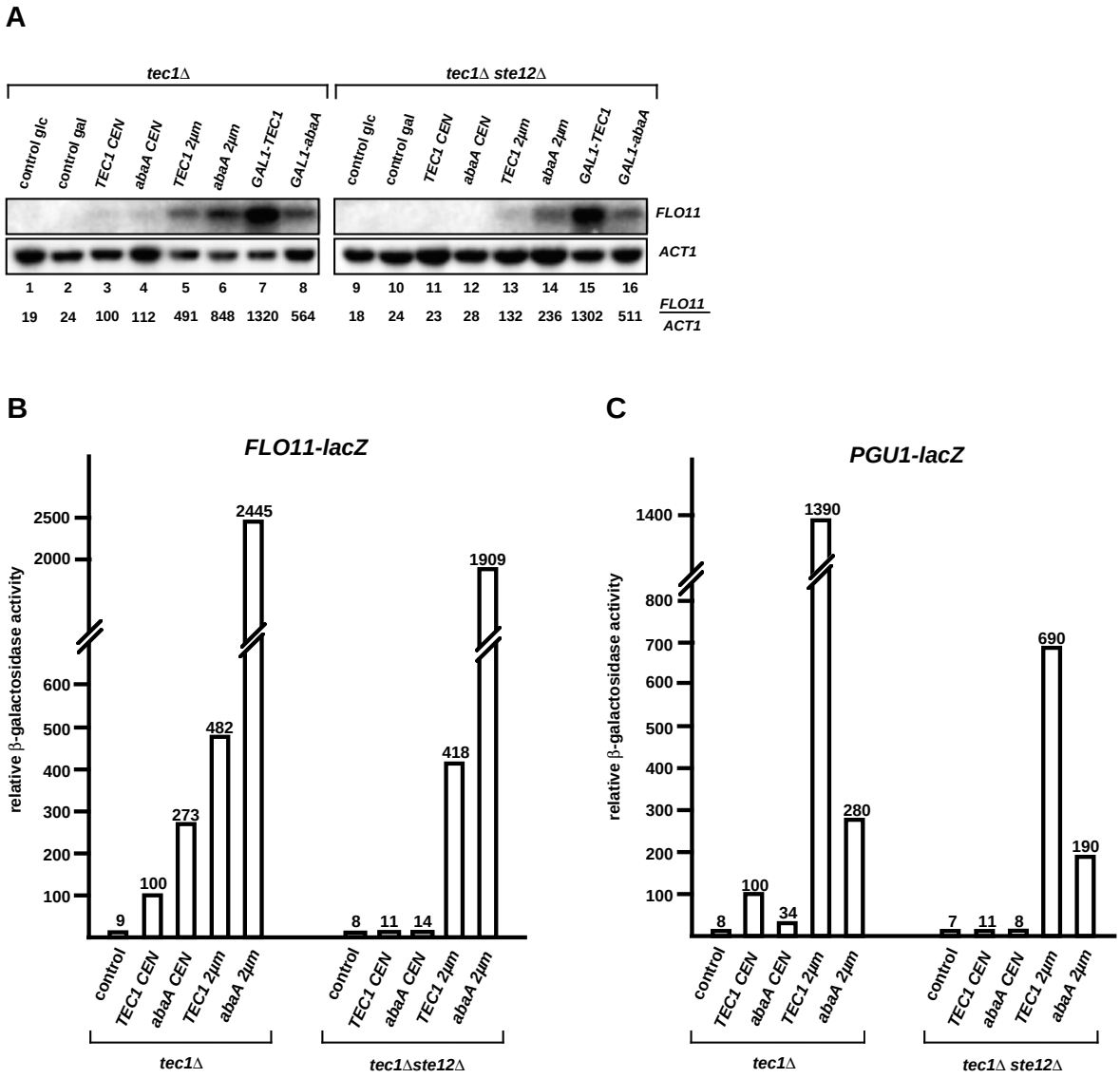


Fig. 4. *TEC1* and *abaA* activate expression of *FLO11* and *PGU1* in the absence of *STE12* in yeast. (A) *FLO11* transcript levels. Strains RH2500 (*tec1Δ*) and RH2501 (*tec1Δ ste12Δ*) carrying the plasmids YCplac111 (control), pME2045 (*TEC1 CEN*), pME2046 (*abaA CEN*), pME2047 (*TEC1 2µm*), pME2048 (*abaA 2µm*), pME2049 (*GAL1-TEC1*) or pME2050 (*GAL1-abaA*) were grown in SC-Leu medium containing 2% glucose (lanes 1, 3, 4, 5, 6, 9, 11, 12, 13, 14) or 2 % galactose (lanes 2, 7, 8, 10, 15, 16) to exponential phase before total RNA was prepared and used for Northern analysis. *ACT1* gene expression served as internal standard. Relative expression levels of *FLO11* (*FLO11/ACT1*) were obtained using a Phospho-Imaging scanner by normalizing *FLO11* transcript levels to *ACT1* levels. All values are given in percentage of *FLO11* expression measured in control strain RH2500 (*tec1Δ*) expressing *TEC1* from a low copy plasmid (lane 3). (B) *FLO11-lacZ* expression levels. Strains described in panel *A* were transformed with plasmid B3782 carrying the *FLO11-lacZ* reporter (Rupp *et al.*, 1999) and grown to exponential phase before specific β-galactosidase activity was measured. Bars depict relative values normalized to the activity measured in strain RH2500 (*tec1Δ*) expressing *TEC1* from a *CEN*-plasmid set to 100. (C) *PGU1-lacZ* expression levels. β-Galactosidase activity was measured in strains carrying the *PGU1-lacZ* reporter plasmid pME2064

(Madhani *et al.*, 1999) and expressing *TEC1* and *A. nidulans abaA* driven by the *TEC1* promoter at different levels. Activities are given as relative values normalized to strain RH2500 (*tec1Δ*) expressing *TEC1* from a *CEN*-plasmid, which was defined as 100. Bars in panel *B* and *C* depict means of three independent measurements, with standard deviation not exceeding 20%.

An even stronger induction of *FLO11* transcription was measured for *GAL1-TEC1* (13-fold) and *GAL1-abaA* (5.1-fold). These results demonstrate that in the absence of Ste12p both Tec1p and AbaAp are able to activate expression of *FLO11* to levels sufficient for invasive growth.

Results obtained by analysis of *FLO11* transcript levels were further corroborated by β-galactosidase assays using a *FLO11-lacZ* reporter gene (Rupp *et al.*, 1999) that was introduced into *tec1Δ* and *tec1Δ ste12Δ* yeast strains expressing *TEC1* and *abaA* at different levels (Fig. 4B). In the *tec1Δ* strain, single copies of *TEC1* or *abaA* increased *FLO11-lacZ* reporter activity 11.1-fold (*TEC1*) or 27.3-fold (*abaA*) in comparison to a control strain lacking *TEC1* (Fig. 4B). High copy expression of *TEC1* induced the expression of *FLO11-lacZ* 4.8-fold when compared to low copy *TEC1*, whereas high copy *abaA* led to a 24.4-fold higher reporter activity. Again, a single copy of *TEC1* or *abaA* was not sufficient to activate *FLO11-lacZ* expression in the absence of *STE12* (*tec1Δ ste12Δ* background), whereas high copy expression of both *TEC1* and *abaA* led to high *FLO11-lacZ* reporter activities that were comparable to activities measured in the presence of *STE12* (*tec1Δ* background).

Regulation of *PGU1* was measured by use of a *PGU1-lacZ* reporter gene (Madhani *et al.*, 1999) that was expressed in *tec1Δ* and *tec1Δ ste12Δ* strains containing no, single or high copies of *TEC1* or *abaA* (Fig. 4C). Expression of *PGU1-lacZ* was stimulated 12.5-fold by introduction of a single *TEC1* copy into the *tec1Δ* background and further 13.9-fold by high copy expression of *TEC1*. A 3.4-fold induction was obtained by single copy and a further 8.2-fold induction by high copy *abaA*. In the absence of Ste12p (*tec1Δ ste12Δ* strain), single copies of *TEC1* or *abaA* were not able to significantly stimulate *PGU1-lacZ* reporter activity beyond basal levels, but high copy *TEC1* or *abaA* activated reporter expression 99-fold (*TEC1*) and 27-fold (*abaA*), respectively.

In summary, Tec1p and AbaAp significantly activate expression of *FLO11* and *PGU1* in the absence of Ste12p, a fact that might explain why these transcription factors are able to induce invasive growth in *ste12Δ* strains.

Tec1p activates gene expression via TCS elements in the absence of Ste12p.

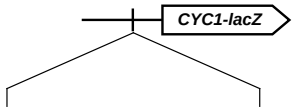
The sole mechanism by which Tec1p has been proposed to activate gene expression is

cooperative binding together with Ste12p to FRE sites present in the promoter region of target genes (Baur *et al.*, 1997; Madhani and Fink, 1997; Madhani *et al.*, 1999). In contrast, AbaAp activates target gene expression by binding to repeated TCS elements (Andrianopoulos and Timberlake, 1994). By computer search, we found that the promoter regions of both *FLO11* and *PGU1* contain several TCS sites without neighboring PRE sites as found in a typical FRE site. The *FLO11*-promoter region contains four single TCS elements and a sequence composed of two TCS elements in close neighborhood, whereas the *PGU1*-promoter region contains three single TCS sites in different orientation, but does not contain a typical FRE element. This suggested that Tec1p might activate gene expression not only via FRE sites but also via TCS elements. To test this hypothesis, we constructed a series of TCS-driven reporter genes by insertion of TCS elements in different numbers and orientations upstream of an enhancerless *CYC1-lacZ* gene (Guarente and Ptashne, 1981). Expression of these reporters was measured in *tec1Δ* and *tec1Δ ste12Δ* yeast strains expressing *TEC1* at different levels and compared to *CYC1-lacZ* reporter genes containing either no UAS element or a single FRE site (Fig. 5). Without a functional UAS element, the *CYC1-lacZ* reporter could not be activated beyond basal levels under any condition. A single FRE element in front of *CYC1-lacZ* led to a 375-fold induction of reporter activity that was dependent on *TEC1* and *STE12*. In the presence of *STE12* alone (control plasmid in the *tec1Δ* strain), no activation of the FRE-driven reporter beyond basal levels was found, corroborating that Tec1p and Ste12p cooperatively activate FRE-driven gene expression (Madhani and Fink, 1997). However, *TEC1* alone was sufficient to activate the FRE-driven reporter up to 7-fold even without *STE12* (*TEC1* on *2µm* plasmid in the *tec1Δ ste12Δ* strain), suggesting that the TCS element present in the FRE site can be used by Tec1p mediating significant activation of gene expression. This finding is in agreement with the fact, that single TCS elements in forward or backward orientation were sufficient to mediate at least 10-fold activation of the *CYC1-lacZ* reporter by Tec1p, even in the absence of Ste12p. In contrast to the FRE-reporter, activation mediated by TCS elements was Tec1p dosage-dependent, because induction was between 2- to 3-fold higher (up to 116-fold) in strains containing a 2- to 3-fold higher amount of Tec1p (*TEC1* expressed from *2µm* plasmid in *tec1Δ* background), when compared to activation (up to 46-fold) measured in strains expressing lower levels of Tec1p (*TEC1* expressed from *CEN* plasmid; compare Fig. 3A). Dosage-dependence was also observed when *STE12* was absent. TCS-reporters were not induced in *ste12Δ* strains expressing *TEC1* from low copy plasmids (*TEC1 CEN*), a finding that is explained by the fact that Ste12p is required for *TEC1* expression and therefore in these strains only low levels of Tec1p can be detected by Western analysis (Fig. 3A). However, restoration of intracellular amounts of Tec1p (*TEC1* expressed from *2µm* plasmid in *tec1Δ ste12Δ* strains) also restored activation of the TCS-driven reporters (up to 10-fold) comparable to a wild-type situation. Double TCS elements led to activation of *CYC1-lacZ* reporter expression between 61- and 265-fold depending on the spacing between

the TCS elements and the Tec1p levels. Optimal activation was observed when spacing between TCS elements was 11 bp. Stimulation of gene expression was almost 3-fold better than obtained by single TCS elements, pointing to a synergistic effect of the double element. In contrast, double TCS elements separated by 6 bp led to an only 1.3-fold better activation (61 units) than single TCS elements (46 units). As found for single TCS-driven reporters, activation mediated by double TCS elements was Tec1p dosage-dependent. Again, restoration of Tec1p protein levels in *ste12Δ* strains by expressing *TEC1* from the *2µm* plasmid led to activation of the double TCS reporters comparable to a wild-type situation. We further tested a reporter construct composed of three TCS elements in backward orientation, each separated by 13 bp. In comparison to the reporter with two backward oriented TCS elements, this triple TCS-reporter could be further activated (261-fold versus 171-fold), when Tec1p was present at high levels.

In summary, these results demonstrate that Tec1p is able to activate gene expression not only via FRE elements (FRE control), but also via single or multiple TCS elements (TCS control). Interestingly, TCS control depends on Ste12p to some degree, because we found a consistent 2- to 3-fold drop in expression of all TCS-reporters in *ste12Δ* strains compared to *STE12* strains (which contain comparable levels of Tec1p). However, Tec1p alone is sufficient to significantly activate TCS-dependent gene expression up to 20-fold even in the absence of Ste12p.

FRE- and TCS-driven reporters were further tested for activation by *abaA*. We found that *abaA* activated the FRE reporter 26-fold when expressed from the endogenous *TEC1* promoter on a low copy plasmid. Interestingly, AbaAp appears to activate the FRE-reporter cooperatively with Ste12p, because activation was up to 70-fold (*2µm* plasmid), whereas activation mediated by single TCS elements was not more than 22-fold. This suggests an even higher similarity in the mechanism of Tec1p and AbaAp function as previously assumed. Double-TCS-driven reporters were activated by AbaAp between 31- and 72-fold depending on the spacing between the TCS elements. The best activation (72-fold) was found for double TCS elements oriented as inverted repeats and separated by 14 bp. This finding is in agreement with an earlier study showing that inverted TCS repeats with a 13 bp spacing are optimal for activation (Andrianopoulos and Timberlake, 1994). As found for Tec1p, TCS-mediated activation by AbaAp was functional even in strains lacking Ste12p.



	β-galactosidase activity (nmoles/min/mg)									
	*tec1*Δ					*tec1*Δ *ste12*Δ				
	control	*TEC1 CEN*	*TEC1 2μm*	*abaA CEN*	*abaA 2μm*	control	*TEC1 CEN*	*TEC1 2μm*	*abaA CEN*	*abaA 2μm*
no insert	1	1	1	1	1	3	3	3	3	3
14 bp TCS PRE	1	375	379	26	70	3	6	20	3	11
TCS	1	46	106	4	22	3	3	32	4	19
TCS	1	42	116	4	20	3	3	29	3	16
6 bp TCS TCS	1	61	178	6	46	4	6	79	3	30
11 bp TCS TCS	1	132	265	8	67	5	6	105	6	30
14 bp TCS TCS	1	84	213	16	72	4	5	86	6	55
13 bp TCS TCS	1	72	171	3	31	5	8	80	6	28
13 bp 13 bp TCS TCS TCS	3	103	261	6	32	9	11	107	9	29

Fig. 5. Activation of FRE- and TCS-dependent gene expression by *TEC1*, *abaA*, and *STE12* in yeast. An enhancerless *CYC1-lacZ* fusion gene carrying no UAS element (no insert), FRE (TCS + PRE), or TCS elements in different combinations was integrated as a single copy into the genomes of strains RH2500 (*tec1Δ*) and RH2501 (*tec1Δ ste12Δ*), and β-galactosidase specific activity was measured with *TEC1* and *abaA* being absent (control) or present at a low (*CEN*) or high (*2μm*) copy level. Activities are expressed in nmoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute times milligrams of protein and are means of four measurements with two independent transformants. The standard deviation was less than 20 %.

The C-terminal region of Tec1p is required for haploid invasive growth and TCS-mediated reporter gene expression, but is dispensable for FRE-driven gene expression

We isolated novel variants of the *TEC1* gene, to characterize functional domains of the Tec1p protein. A library of PCR-mutagenized *TEC1* alleles was created and introduced into a haploid *tec1Δ* yeast strain that carried a chromosomally integrated *FRE-lacZ* reporter gene. Resulting transformants were screened for invasive growth and *FRE-lacZ* reporter activity yielding two classes of mutants (Table III, Fig. 6A). Class I mutants were defective for invasive growth but still promoted induction of the *FRE-lacZ* reporter. Class II mutants were enhanced for both invasive growth and *FRE-lacZ* expression. No class of mutants could be isolated that grew invasively but failed to activate *FRE-lacZ*. Mutants being suppressed for

both phenotypes were excluded. Sequence analysis revealed that each of the class I mutant alleles encoded a C-terminally truncated protein and that all of the class II alleles coded for proteins with single or double amino acid substitutions (Table III).

Table III. Invasion-defective and hyperinvasive *TEC1* mutants

Class and allele	Mutation(s)	Invasive Growth[a]	Fold *FRE-lacZ* expression[b]
Control			
tec1Δ	Full deletion	-	<1
TEC1	None	+++	100
Class I			
tec1-102	Δ(K281-Y486)	+	67
tec1-103	E222G, Δ(Y257-Y486)	+	89
tec1-104	F93L, Δ(P335-Y486)	+	104
tec1-105	Δ(I353-Y486)	+	98
Class II			
tec1-201	T273M	++++	166
tec1-202	P274S	++++	157
tec1-203	K154R, S453P	++++	127
tec1-204	K154R, S262G	++++	105

[a] Invasive growth was measured by a plate-washing assay and quantified as described in Mösch and Fink, 1997.

[b] Induction of an *FRE-lacZ* reporter by the different *TEC1* alleles was measured in strain RH2499. The values shown are relative β-galactosidase activities normalized to the activity obtained with wild-type *TEC1*, which was set at 100.

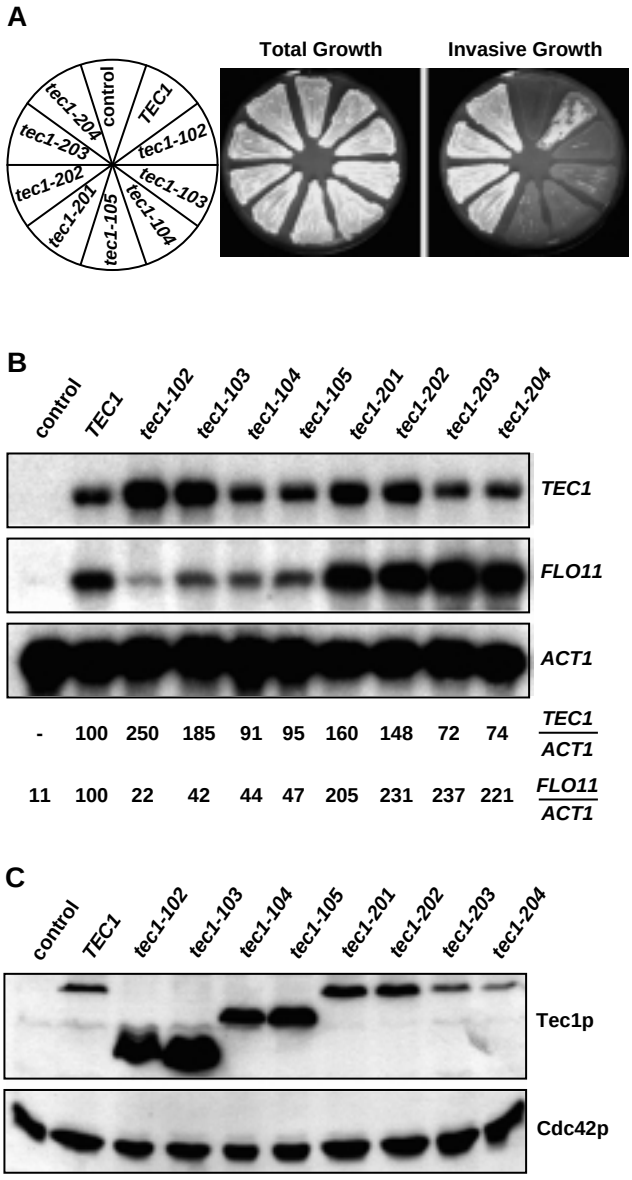


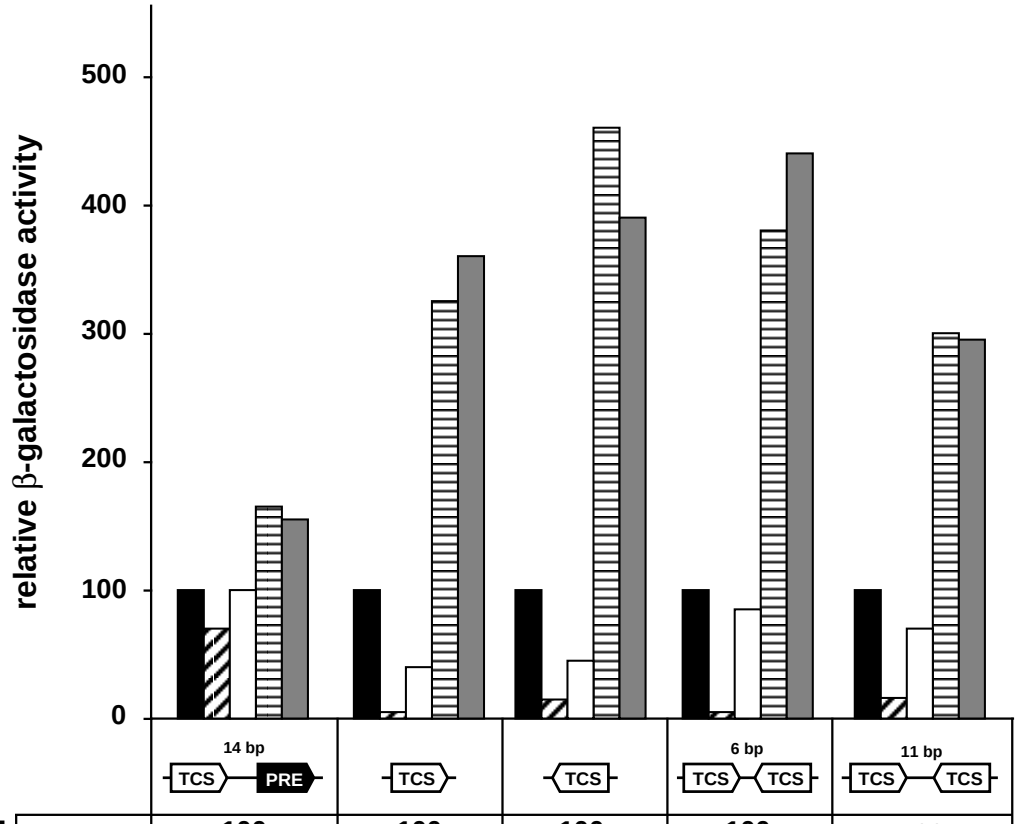
Fig. 6. Characterization of novel yeast *TEC1* alleles. (A) Regulation of haploid invasive growth. Strain RH2499 (*tec1Δ*) carrying a control plasmid, wild-type *TEC1*, or different *TEC1* mutant alleles on low-copy plasmids was patched on SC-Leu-Ura medium and grown for 4 days before the plate was photographed before (total growth) and after (invasive growth) cells were washed off the agar surface. (B) Expression of *FLO11* depending on different *TEC1* alleles. Shown are transcript levels of *TEC1*, *FLO11* and *ACT1* measured in strain RH2499 (*tec1Δ*) expressing different *TEC1* alleles. *ACT1* transcripts served as internal standard. Relative expression levels of *TEC1* alleles (*TEC1/ACT1*) and *FLO11* (*FLO11/TEC1*) are indicated below and were obtained using a Phospho-Imaging scanner followed by normalization of *TEC1* and *FLO11* transcript levels to *ACT1* levels and to a strain expressing wild-type *TEC1*. (C) Western blot analysis. Shown are steady state levels of the various myc-tagged Tec1p proteins obtained by expression of the indicated *TEC1* alleles in strain RH2499. Proteins were detected using an anti-myc monoclonal antibody, and expression of Cdc42p was measured in the same extracts as an internal control.

A triple-myc epitope tag was inserted just after the start codons of all *TEC1* mutant genes, to further characterize mutant proteins. No phenotypic differences were detected between the epitope-tagged and nontagged versions (data not shown). *TEC1* mRNA and Tec1p protein levels were measured in all mutants, to exclude that reduced expression or stability of Tec1p mutant proteins might account for the phenotypes observed (Fig. 6B and 6C). No significant decrease in mRNA levels was found for any of the *TEC1* mutant alleles. However, an increase in transcript levels was detected in case of *tec1-102* (2.5-fold), *tec1-103* (1.9-fold), *tec1-201* (1.6-fold) and *tec1-202* (1.5-fold). Protein levels of Tec1-102p, Tec1-103p, Tec1-104p and Tec1-105p were markedly higher than that of the wild-type protein, excluding the possibility that low expression of these variants was responsible for reduced invasiveness of corresponding mutant strains. In contrast, reduced protein levels were detected for Tec1-203p and Tec1-204p, ruling out that increased expression of these mutants was the cause for enhanced invasive growth found for these class II mutants.

FLO11 transcripts levels were measured in the different *TEC1* mutant strains (Fig. 6B). All strains expressing class I mutant alleles showed a significant decrease in *FLO11* expression with levels varying between 22% and 47% with respect to the wild-type control, whereas values between 205% and 237% were obtained for strains expressing the class II alleles. Thus, the degree of invasive growth correlates well with expression of *FLO11* in both classes of *TEC1* mutants.

Each two *TEC1* mutants from class I and class II were further tested for activation of four different *TCS-lacZ* reporters and compared to *FRE-lacZ* reporter activation (Fig. 7). From class I, the severely truncated Tec1-102p mutant protein (ΔK281-Y486) was almost deficient to activate any of the *TCS-lacZ* reporters with values reaching not more than 16% compared to the wild-type control. In contrast, Tec1-102p was still able to efficiently activate expression of *FRE-lacZ* to levels corresponding to 67% of wild-type Tec1p. The less severely truncated Tec1-105p variant (ΔI353-Y486) activated *FRE-lacZ* indistinguishable from wild-type Tec1p , whereas expression of the single *TCS-lacZ* reporters dropped to 41% (forward orientation of TCS) or 45% (backward orientation). Expression of double *TCS-lacZ* reporters by Tec1-105p was found to be reduced less significantly, with values reaching either up to 83% of the wild-type control. From class II, Tec1-201p and Tec1-202p strongly activated all *TCS-lacZ* reporters between 3.0-fold and 4.6-fold in comparison to wild-type Tec1p. In contrast, these variants stimulated *FRE-lacZ* not more than 1.6-fold.

In summary, these data suggest that distinct domains of Tec1p are required for activation of gene expression mediated by either TCS or FRE sites (Fig. 8). The N-terminal region of Tec1p appears to be sufficient for transcriptional activation via FRE but not via TCS sites. TCS-mediated activation by Tec1p additionally requires the C-terminal part of the protein.



TEC1	100	100	100	100	100
tec1-102	67	5	15	4	16
tec1-105	98	41	45	83	70
tec1-201	166	321	461	378	298
tec1-202	157	358	388	439	297

Fig. 7. Activation of FRE- and TCS-dependent *CYC1-lacZ* reporter genes by *TEC1* mutant alleles in yeast. Wild-type *TEC1* and *tec1-102*, *tec1-105*, *tec1-201*, and *tec1-202* mutant alleles were expressed in *tec1Δ* mutant strains carrying a *CYC1-lacZ* fusion gene driven by an FRE (TCS + PRE) or TCS elements in different combinations, and strains were assayed for β-galactosidase specific activity. Bars and values represent relative activities normalized to the value obtained by expression of wild-type *TEC1* (black bars), which was set at 100. All values are means of three independent measurements. Standard deviation was less than 20%.

DISCUSSION

Ste12p and Tec1p regulate gene expression and cellular development by several distinct mechanisms.

The role of the TEA/ATTS family transcription factor Tec1p from *S. cerevisiae* in regulating gene expression and cellular development was investigated. So far, Tec1p was thought to regulate these processes primarily by cooperative binding together with Ste12p to FRE sites present in the promoter region of target genes and of *TEC1* itself. This combinatorial model for Tec1p regulation is based on the observation that strains lacking either *TEC1* or *STE12* or both are equally suppressed for haploid invasive and diploid pseudohyphal growth (Gavrias *et al.*, 1996; Liu *et al.*, 1993; Madhani and Fink, 1997; Mösch and Fink, 1997; Mösch *et al.*, 1999; Roberts and Fink, 1994). The finding that FRE enhancer elements are bound *in vitro* by Tec1p and Ste12p in a cooperative manner further supports this model (Madhani and Fink, 1997). The assumption that combinatorial control is the sole mechanism for Tec1p-regulated processes implies that presence of either Tec1p or Ste12p alone is not sufficient to activate target gene expression and development. However, this prediction has not been tested experimentally prior to our study. Here, we have shown that strains containing sufficient amounts of Tec1p but lacking Ste12p are able to activate natural target genes and reporter genes that are driven by TCS elements. As a consequence, these strains undergo significant cellular development, although they do not contain any Ste12p. We further present evidence that Ste12p controls expression of *TEC1* to a larger extent than Tec1p itself suggesting the involvement of mechanisms other than combinatorial control. Our data suggest that Ste12p and Tec1p control gene expression and cellular development by several distinct mechanisms (Fig. 9). We propose a model in which Ste12p regulates expression of *TEC1* by both combinatorial control together with Tec1p via FRE sites and without Tec1p by acting via clustered PRE sites present in the *TEC1* promoter. In turn, Tec1p regulates gene expression by two distinct mechanisms, the FRE control in combination with Ste12p and the TCS control that is operative even without Ste12p.

TCS control is an efficient mechanism for activation of developmental target genes by Tec1p.

A central finding of our study is that single TCS elements inserted upstream of a reporter gene are sufficient to mediate activation of gene expression by Tec1p. Activation is up to 100-fold and independent of the orientation of the TCS. This finding correlates well with the distribution and arrangement of TCS elements found in natural *S. cerevisiae* promoter regions. *In silico* analysis of the complete *S. cerevisiae* genome reveals a group of more than 100 genes that contain at least 4 TCS elements in their promoter region (our unpublished results). Most of these TCS elements are in single arrangement, that is they are

separated from each other by at least 20 bp, and are not neighbored by a Ste12p-binding site. Remarkably, this group includes *FLO11*, *FLO8*, *PHD1*, *DFG10*, *CLN1*, *CDC25*, and *PGU1*, which are all genes that have previously been implicated in invasive and pseudohyphal growth (Gimeno and Fink, 1994; Gimeno *et al.*, 1992; Liu *et al.*, 1996; Lo and Dranginis, 1998; Loeb *et al.*, 1999; Madhani *et al.*, 1999; Mösch and Fink, 1997; Rupp *et al.*, 1999). Here, we have shown that two representatives of these genes, *FLO11* and *PGU1*, are activated by Tec1p even when Ste12p is absent. This suggests that Tec1p, much like other TEA/ATTS family transcription factors, can activate target genes and cellular development also without Ste12p by TCS control. The view that TCS control is evolutionary conserved is supported by several observations. (i) We demonstrated that in *S. cerevisiae*, the TEA/ATTS family transcription factor AbaAp from *A. nidulans* not only activates TCS-driven reporter genes, but also *FLO11* and *PGU1* even when Ste12p is absent. In *A. nidulans*, AbaAp activates target genes by binding to AREs (AbaAp responsive elements) that are identical to TCS elements (Andrianopoulos and Timberlake, 1994). As found for TCS elements in *S. cerevisiae* genes, multiple AREs are distributed predominantly in single arrangement in the promoter region of known target genes of AbaAp, such as *brlA*, *wetA*, *yA*, *rodA* and *abaA* itself (Andrianopoulos and Timberlake, 1994). Moreover, AbaAp activates target genes and conidiophore development independently of SteAp, a protein with similarity to Ste12p that regulates sexual but not AbaAp-dependent asexual development (Vallim *et al.*, 2000). In this context, it should be noted that *A. nidulans* asexual spore formation involves a process, which is reminiscent of yeast pseudohyphal development (Gimeno and Fink, 1994). (ii) In the human pathogen *C. albicans*, the TEA/ATTS transcription factor CaTec1p regulates target genes, serum-induced hyphal growth and virulence primarily by a mechanism that does not require the Ste12p-like protein CaCph1p (Lane *et al.*, 2001; Liu, 2001; Schweizer *et al.*, 2000). (iii) In mammals, members of the transcriptional enhancer factor (TEF-1) family, which share the TEA/ATTS DNA-binding domain, activate target genes by binding to the consensus sequence GGAATG that matches the consensus of TCS elements in reverse orientation (Hwang *et al.*, 1993; Jacquemin *et al.*, 1996; Jiang *et al.*, 2000).

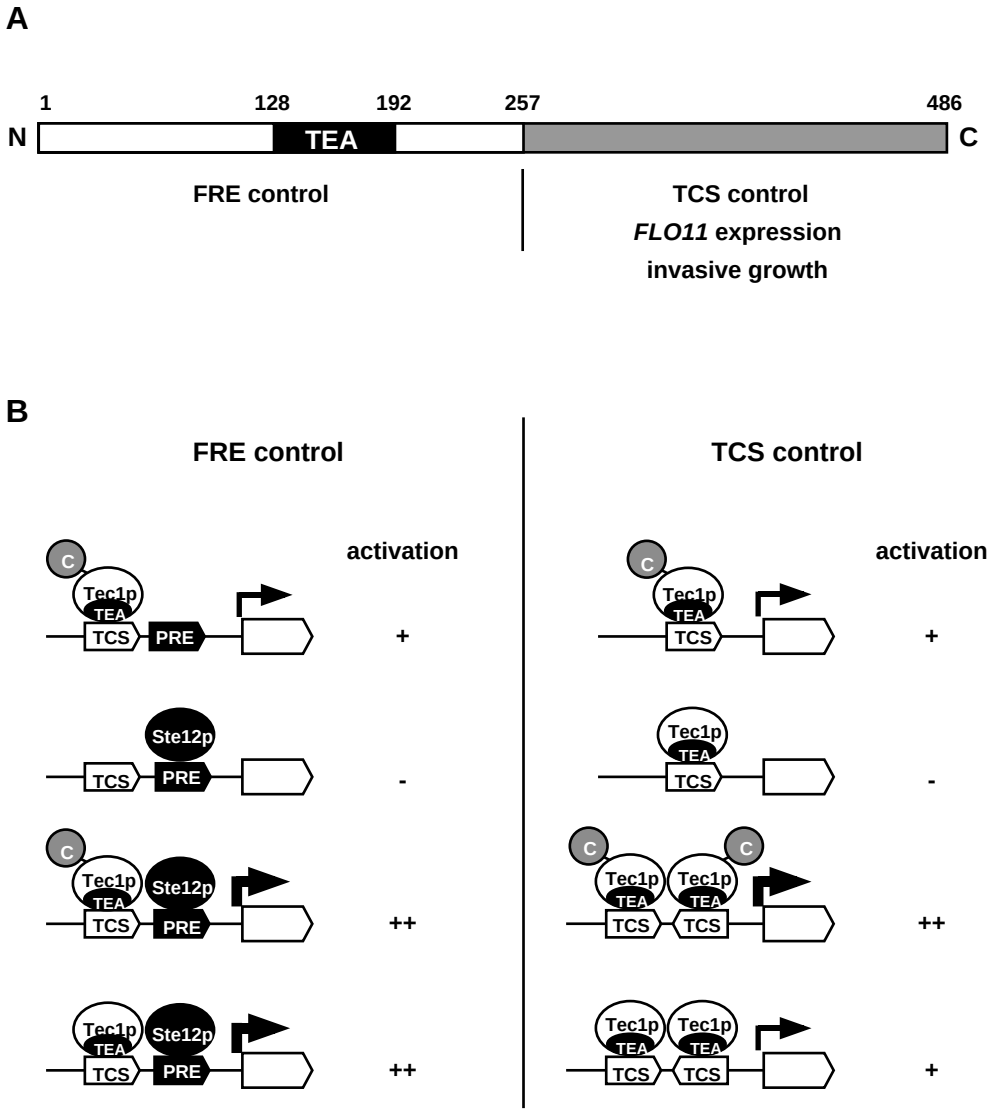


Fig 8. Domains and functions of yeast Tec1p. (A) Domain structure of Tec1p. The N-terminal part of Tec1p encompassing amino acids 1 to 257 includes the conserved TEA DNA-binding domain and is sufficient for activation of FRE-dependent gene expression together with Ste12p (FRE control). The C-terminal domain spanning amino acids 258 to 486 is required for the activation of gene expression via TCS elements (TCS control). (B) FRE and TCS control mechanisms. High-level activation of gene expression (++) via combined TCS and PRE elements (FRE control) depends on cooperative binding of Tec1p and Ste12p and does not require the C-terminal part of Tec1p. The presence of Ste12p is not sufficient for activation (-). In contrast, the presence of Tec1p alone is sufficient for significant activation (+) of FRE-driven expression via the single TCS site. TCS control depends on the number of TCS elements and the C-terminal part of Tec1p. Single TCS elements mediate significant activation (+) of gene expression by Tec1p depending on its C-terminal part. High-level activation (++) comparable to FRE-mediated expression is achieved by combination of multiple TCS elements but also depends on the C-terminus of Tec1p.

Functions and functional domains of Tec1p.

The hallmark of TEA/ATTS family transcription factors is their DNA-binding domain that consists of three putative alpha-helices, which in the case of human TEF-1 have been demonstrated to be required for DNA binding (Bürglin, 1991; Hwang *et al.*, 1993; Jacquemin *et al.*, 1996). DNA-binding and transcriptional activation have been proposed to involve either homodimer formation, as found in the case of *A. nidulans* AbaAp (Andrianopoulos and Timberlake, 1994), or heterodimer formation together with a second transcription factor, e.g. Ste12p, as shown for *S. cerevisiae* Tec1p (Madhani and Fink, 1997), or serum response factor (SRF), as found in the case of human TEF-1 (Gupta *et al.*, 2001). Here, we have performed a functional analysis of *S. cerevisiae* Tec1p. Our results suggest that TEA/ATTS family transcription factors are able to regulate gene expression by at least two separate modes of action that involve distinct functional domains of the proteins. In the case of Tec1p, we found that gene expression is activated either by FRE control or by TCS control and that these two mechanisms are separable with respect to the Tec1p protein structure. We have shown that the N-terminal 257 amino acids of Tec1p containing the conserved TEA/ATTS DNA-binding domain are sufficient to confer cooperative FRE control together with Ste12p. This finding suggests that the N-terminal part of Tec1p might contain a Ste12p-interacting domain (Fig. 8). Although this assumption remains to be tested biochemically, it is supported by the observation that *A. nidulans* AbaAp activates FRE sites in *S. cerevisiae* in a cooperative manner that depends on Ste12p (Fig. 5). Because AbaAp and Tec1p do not share any regions showing significant similarity other than the TEA/ATTS DNA-binding domain, cooperativity might be conferred by this conserved part of AbaAp. A previous study has shown that the N-terminal TEA/ATTS DNA-binding domain of mammalian TEF-1 is sufficient for physical interaction with serum response factor, a MADS box family transcription factor that confers combinatorial control together with TEF-1 in gene activation (Gupta *et al.*, 2001). Therefore, one might speculate that the evolutionary conserved TEA/ATTS domain not only governs the DNA-binding activity but also the domain for heterodimer formation with binding partners that confer combinatorial control and cooperative activation of gene expression.

Tec1p possesses an additional domain in the C-terminal part that is required for gene activation by a second mechanism, the TCS control. What is the mechanism by which Tec1p executes this function? An important observation is that TCS control does not appear to involve cooperativity. Our study shows that multiple TCS elements mediate activation in a predominantly additive manner, because no significant cooperative effects can be observed in case of double or triple TCSs (Fig. 5). A slight cooperative effect is observed when two inverted TCS elements are separated by 11 bp. However, cooperativity is not very pronounced (1.4-fold) and not comparable to that observed for a FRE site when Tec1p acts together with Ste12p (19-fold). Remarkably, Tec1p can activate an FRE-driven reporter gene also in the absence of Ste12p. Obviously, Tec1p can bind to the TCS element within

the FRE and activate transcription without Ste12p. In this case, however, cooperativity is lost and TCS control depends on the dosage of the Tec1p protein, much like observed for TCS-driven reporters. Whether Tec1p binds to single TCS elements as a monomer or as a homodimer can not be concluded form our study. However, biochemical studies with AbaAp suggest that it binds to single TCS elements predominantly as a monomer and not as a homodimer (Andrianopoulos and Timberlake, 1994). Taken together, these findings support a mechanism, by which Tec1p binds as a monomer to single TCS elements and activates transcription by involvement of its C-terminal part (Fig. 8).

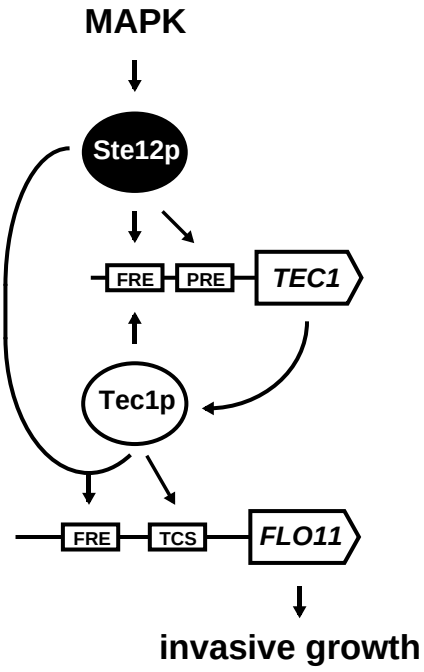


Fig. 9. Model for regulation of *FLO11* expression and invasive growth by Tec1p. The filamentation/invasion mitogen-activated protein kinase (MAPK) cascade controls Ste12p, which activates *TEC1* expression either without Tec1p by binding to PRE sites or in combination with Tec1p by binding to a single FRE site present in the *TEC1* promoter. Tec1p can activate expression of *FLO11* and invasive growth either in combination with Ste12p via the single FRE site or without Ste12p via the TCS sites present in the *FLO11* promoter.

What is the function of the C-terminal part of Tec1p and why is it required for TCS but not for FRE control? A simple answer to this questions is that the C-terminal portion of Tec1p provides the same function(s) for the TCS control mechanism as does Ste12p for FRE control. These functions might include nuclear transport, regulation of DNA-binding activity or interaction with the transcriptional apparatus. Whether the C-terminal part of Tec1p fulfills these functions on its own or by interaction with further partners remains to be elucidated. Interestingly, Tec1p has been found to be physically associated not only with Ste12p, but also with the MAP kinase Kss1p (Ho *et al.*, 2002). This opens the possibility that FRE control and TCS control might involve association of Tec1p with Kss1p. In case of FRE control this interaction might be established by the TEA/ATTS domain and involve Ste12p, whereas in case of TCS control the C-terminal part of Tec1p could associate with Kss1p independently of Ste12p. However, Ste12p might also be involved in TCS control mediated by the C-terminal part, because TCS-driven expression was found to be 2- to 3-fold less efficient in the absence of Ste12p. In this scenario, non-DNA bound Ste12p would contribute to TCS control by interaction with the C-terminus of Tec1p in a manner similar to interaction of Ste12p with the alpha-1 protein at alpha-specific genes (Yuan *et al.*, 1993).

In conclusion, our study shows that the TEA/ATTS transcription factor Tec1p fulfills more than one function in regulation of gene expression and cellular development. These functions are executed by distinct domains of Tec1p and require combination with additional factors, e.g. Ste12p or maybe Kss1p. It will be interesting to resolve the exact temporal and spatial interactions between the different domains of Tec1p and these factors in the future.

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Chapter 4

Domain-specific regulation of the transcription factor Tec1p by the MAP kinases Fus3p and Kss1p determines signaling specificity during cellular development

ABSTRACT

In *Saccharomyces cerevisiae,* two distinct developmental processes, mating and haploid invasive growth, are controlled by the same conserved MAP kinase signal transduction cascade. Signaling specificity is thought to arise in part from activation of pathway-specific MAPKs for each program, with Fus3p being specific for the mating process and Kss1p for invasive growth. At the level of gene expression, specificity is conferred by activation of different transcription factors. Ste12p is sufficient to activate mating specific genes, and Tec1p is required to induce invasion specific genes. Because *TEC1* expression is under control of Ste12p, and both MAPKs activate Ste12p, it is a mystery how specificity is achieved to elicit the mating or invasive growth program. Here, we show that Tec1p itself is an important specificity determinant that prevents cross-activation of the invasion pathway in response to pheromone signals. We provide evidence that Tec1p interacts with Fus3p and Kss1p through distinct domains. In naive cells, Tec1p is weakly phosphorylated by Fus3p and Kss1p in an *in vitro* kinase assay. In contrast, pheromone stimulation resulted in an elevated phosphorylation of Tec1p only by Fus3p. Activation of the pheromone response pathway leads to a drop in Tec1p protein levels, but only when Fus3p is present. Downregulation of Tec1p is blocked in hyperinvasive mutants expressing Tec1p variants carrying single amino acid substitutions in a conserved MAP kinase phosphorylation site. These data suggest that phosphorylation of Tec1p by activated Fus3p targets the protein for degradation, thereby preventing erroneous activation of the invasive growth response by pheromone.

INTRODUCTION

Cellular development often requires transmission of extracellular signals from the cell surface into the cytoplasm and nucleus to elicit a cellular response. A common mechanism for intracellular signal transduction in all eukaryotes involves the sequential phosphorylation of three protein kinases that act in a linear pathway and constitute a so-called mitogen activated protein kinase (MAPK) cascade (Gustin *et al.*, 1998). The main targets of MAPK cascades are transcription factors that control expression of specific gene sets (Treisman, 1996). Most MAPK cascades are activated by a specific signal and mediate a distinct cellular response. However, a single MAPK cascade might also be activated by different signals to generate different outputs. For example, cultured neuroendocrine PC12 cells treated with nerve growth factor (NGF) or epidermal growth factor (EGF) differentiate into neurons or start to proliferate, respectively, although both signals activate the same extracellular signal-regulated kinase (ERK) pathway (Vaudry *et al.*, 2002). In *Drosophila melanogaster*, JNK and p38 pathways both are required for polarity establishment during normal development and for regulation of the immune response in the fly (Stronach and Perrimon, 1999). How the same MAPK pathway can evoke the proper cellular responses dependent on different stimuli is only partially understood. Expression of tissue-specific transcription factors as well as differences in signal strength and amplitude appear to maintain signaling specificity in these cases.

In the yeast *Saccharomyces cerevisiae*, five distinct MAPK cascades have been described (Gustin *et al.*, 1998). Four of these pathways are present in vegetatively growing cells and mediate mating, filamentous/invasive growth, high osmolarity responses and cell integrity. The remaining pathway mediates spore wall assembly. Remarkably, the mating (pheromone) response and the filamentous/invasive growth signaling pathways share several proteins including the MAPK kinase Ste7p, the MAPKK kinase Ste11p and the upstream kinase Ste20p (Roberts and Fink, 1994). Mating of haploid yeast cells is triggered by small, cell specific peptide pheromones that are constitutively produced and secreted into the medium. *MAT**a*** cells produce only **a**-factor and *MAT*α cells produce only α-factor. Both pheromones act exclusively on cells of the opposite mating type. The peptides bind to the cognate G-protein coupled receptor present on the cell surface (Ste2p in case of *MAT**a*** cells and Ste3p in case of *MAT*α cells) and stimulate dissociation of the coupled heterotrimeric G-protein. Liberated G$\beta\gamma$ then activates via the upstream kinase

Ste20p the mating MAPK module. Signal transduction finally leads to phosphorylation and activation of two MAPKs, Fus3p and Kss1p, which in turn phosphorylate several target proteins that mediate various responses (e.g. cell cycle arrest, mating-specific gene expression, cell and nuclear fusion) finally culminating in formation of a diploid zygote.

A different developmental option that requires the same MAPK cascade is induced by nutritional signals. *S. cerevisiae* is a dimorphic fungus that interconverts between unicellular and multicellular filamentous growth modes (Gimeno *et al.*, 1992). When starved for nitrogen, diploid cells undergo a developmental transition from growth in the yeast form to a multicellular form consisting of chains of elongated cells that radiate away from the colony borders and penetrate the substrate surface. A related phenomenon called invasive growth is observed in haploids upon glucose depletion after prolonged incubation on rich medium (Cullen and Sprague, 2000; Roberts and Fink, 1994).

Much of the research over the past decade has been dedicated to the questions how different external signals stimulate the same MAPK cascade to trigger two contrasting differentiation programs and how inappropriate crosstalk between both processes is prevented. One mechanism to ensure signaling specificity is the use of scaffold proteins that serve to insulate different MAPK pathways (van Drogen and Peter, 2002). Ste11p serves as MAPKK kinase not only during pheromone and invasive/filamentous growth signaling, but also for the high-osmolarity glycerol (HOG) pathway. In the mating pathway, the MAPK cascade components are tethered by the scaffold Ste5p (Choi *et al.*, 1994; Kranz *et al.*, 1994; Marcus *et al.*, 1994; Printen and Sprague, 1994), whereas they associate with Pbs2p in the osmolarity cascade (Posas and Saito, 1997). In contrast, no scaffold protein for the filamentation/invasion MAPK cascade has been identified so far. Fusion proteins generated between Ste11p and either of the scaffolds Ste5p or Pbs2p specifically activated the proper MAPKs, demonstrating that attachement of Ste11p to a pathway-specific scaffold is sufficient to actively channel signals towards the appropriate MAP kinase (Harris *et al.*, 2001).

Another mechanism that is regarded as a major specificity determinant is the use of different MAPKs for each program. Fus3p seems to be more important for mating. A *fus3Δ* deletion reduces mating efficiency by 90% compared to a wild-type, whereas the absence of *KSS1* has no effect. A strain lacking both MAPKs is completely sterile, suggesting that Kss1p can partially substitute for Fus3p in the pheromone response (Elion *et al.*, 1991a; Gartner *et al.*, 1992). Since pheromone stimulation induces similar overall transcription

profiles in strains deleted for either of the MAPK genes, the mating defect of a *fus3Δ* strain is possibly not due to altered target gene expression (Breitkreutz *et al.*, 2001; Roberts *et al.*, 2000). However, the MAPKs display different substrate specificities, because only Fus3p is able to phosphorylate and activate the CKI (**c**yclin-dependent **k**inase **i**nhibitor) Far1p, which mediates G1 cell cycle arrest by inhibition of Cln-Cdc28p complexes (Breitkreutz *et al.*, 2001; Peter *et al.*, 1993). This different substrate specificity towards Far1p contrasts with the common transcriptional target Ste12p and its general repressors Dig1p and Dig2p, proteins that are phosphorylated by either MAP kinase.

In contrast to mating, Kss1p seems to be the principal MAPK for regulation of invasive/filamentous growth (Madhani and Fink, 1997). Initial studies had suggested that invasive growth does not require a MAPK at all, because cells lacking both MAPKs invaded the agar like a wild-type (Roberts and Fink, 1994). Deletion of either *FUS3* or *KSS1* alone had opposite effects: a *kss1Δ* strain is hypoinvasive, whereas a *fus3Δ* strain invades the agar even more vigorously than a wild-type, demonstrating that Kss1p is actually required for invasive growth and Fus3p plays an antagonistic role. Subsequent studies helped to explain this apparent paradoxon and proved that Kss1p is in fact the MAPK that regulates invasive and filamentous growth (Cook *et al.*, 1997; Madhani *et al.*, 1997). Kss1p has both kinase-dependent activating and kinase-independent inhibitory functions. In its inactive (unphosphorylated) form, Kss1p binds the target transcription factor Ste12p, thereby preventing target gene activation (Bardwell *et al.*, 1998). The MAPK kinase Ste7p acts to relieve this negative regulation by switching Kss1p from an inhibitor to an activator.

The fact that Fus3p and Kss1p are principally both proficient for pheromone signal transduction raises the question why stimulation with pheromone does not cross-activate the filamentation/invasion MAPK cascade. According to the physical occlusion model (Madhani *et al.*, 1997), Fus3p sterically prevents Kss1p from gaining access to mating signaling complexes. Upon deletion of *FUS3*, Kss1p could then substitute for Fus3p and undertake its mating function. This model was recently conflicted by the finding that pheromone induction equally activates Fus3p and Kss1p (Sabbagh *et al.*, 2001), arguing against sterical hindrance of Kss1p by Fus3p during pheromone response. Moreover, a catalytically inactive Fus3p variant behaved like a *fus3Δ* deletion strain and could not block the erroneous activation of a filamentous specific *FRE-lacZ* reporter. Instead, active Fus3p appears to limit the magnitude and duration of Kss1p phosphorylation, leading to

only transient activation of Kss1p by pheromone (Sabbagh *et al.*, 2001). Fus3p also phosphorylates the upstream components Ste7p and Ste11p (Breitkreutz *et al.*, 2001; Zhou *et al.*, 1993). This is thought to serve a negative feedback mechanism to ensure transient activation or to prevent signal spillover.

Another mechanism contributing to signaling specificity is the use of different transcription factors for both differentiation programs. Both processes, mating and filamentation/invasion, require the transcription factor Ste12p. Ste12p binds cooperatively to pheromone response elements (PREs) matching the consensus sequence TGAAACA. Those PREs are clustered in the promoters of mating-factor responsive genes, and at least two of these elements are required for efficient transcriptional activation by Ste12p (Dolan *et al.*, 1989; Fields and Herskowitz, 1985; Hagen *et al.*, 1991; Kronstad *et al.*, 1987; Van Arsdell *et al.*, 1987; Yuan and Fields, 1991). Ste12p can associate with pheromone-inducible promoters as a homomultimer or, if a distinct target site called P-box is present adjacent to the PRE, as a heteromultimer with the Mcm1p transcription factor (Errede and Ammerer, 1989; Johnson, 1995). Filamentation and invasion require another transcription factor called Tec1p (Gavrias *et al.*, 1996; Köhler *et al.*, 2002; Mösch and Fink, 1997). Tec1p was originally identified as an activator of Ty1 transposons and Ty1-mediated gene expression (Laloux *et al.*, 1990). It contains a conserved DNA-binding domain named TEA/ATTS motif (Andrianopoulos and Timberlake, 1991; Bürglin, 1991), which is shared by a number of transcription factors from other eukaryotic organisms. These factors bind to the DNA sequence CATTCY (with Y being a C or a T) which has been termed **T**EA/ATTS **c**onsensus **s**equence (TCS) (Andrianopoulos and Timberlake, 1994; Baur *et al.*, 1997; Hwang *et al.*, 1993). Ste12p and Tec1p bind cooperatively to distinct promoter elements called FREs (**f**ilamentation and invasion **r**esponse **e**lements) which consist of a PRE and an adjacent TCS element (Madhani and Fink, 1997). FREs have been identified in the promoter regions of at least two genes specifically required for the filamentous/invasive growth program, *TEC1* itself (Madhani and Fink, 1997) and *FLO11* encoding a cell surface flocculin (Lo and Dranginis, 1998; Rupp *et al.*, 1999). Combinatorial control by cooperative binding of Ste12p and Tec1p to FREs is not the only way how filamentation/invasion-specific gene expression is controlled. Principally, Tec1p can activate target gene expression and invasive growth even in the absence of Ste12p via single or clustered TCS elements by a mechanism termed TCS control (Köhler *et al.*, 2002).

Because Tec1p is the only known downstream component of the filamentation/invasion MAPK pathway that is not required for the mating process, we tested the possibility whether Tec1p itself might serve as a specificity factor. Therefore, we analyzed regulation of invasive growth and of the Tec1p protein itself by the MAPKs Fus3p and Kss1p under conditions permissive for either invasive growth or mating. Genetic analysis revealed that functions of both MAPKs for invasive growth are completely dependent on Tec1p. We further show that Tec1p physically interacts with both MAPKs, Kss1p and Fus3p through different domains. The N-terminal part of Tec1p is sufficient for interaction with Fus3p but dispensable for interaction with Kss1p and vice versa. We found that Tec1p is phosphorylated by Fus3p but not by Kss1p after pheromone stimulation, whereas both MAPKs confer a weak basal phosphorylation in the absence of pheromone. Analysis of Tec1p protein levels in cell extracts revealed that pheromone exposure substantially reduces Tec1p steady state protein levels. This decrease of Tec1p was dependent on *FUS3*, suggesting that active Fus3p destabilizes Tec1p. Two Tec1p variants that confer hyperinvasive growth were unresponsive to pheromone. Interestingly, these mutants each contain single amino acid substitutions in a putative MAPK phosphorylation site, suggesting that phosphorylation of Tec1p by Fus3p in response to pheromone could target Tec1p for degradation. On the basis of our results, we establish a model in which Fus3p prevents inappropriate crosstalk into the filamentation/invasion MAPK pathway by inhibition of Tec1p.

MATERIALS AND METHODS

Yeast strains and growth conditions.

All yeast strains used in this study are congenic to the ∑1278b genetic background (Table II). The *ste12Δ::ble* deletion mutation was introduced by using deletion plasmid pME2518, and the *kss1Δ::kanMX4* deletion was introduced directly after PCR-amplification from the corresponding strain constructed within the scope of the *Saccharomyces* genome deletion project (Winzeler *et al.*, 1999). The *fus3Δ::TRP1* and *tec1Δ::HIS3* deletions are described elsewhere (Madhani and Fink, 1997; Mösch *et al.*, 1999). To obtain strains carrying all possible combinations of the *ste12Δ::ble, kss1Δ::kanMX4, tec1Δ::HIS3* and *fus3Δ::TRP1* deletions, strains RH2949 and RH2950 were crossed following tetrad dissection. Strains expressing integrated versions of myc epitope-tagged *TEC1* were constructed by targeting the corresponding plasmids to the *leu2::hisG* locus. Likewise, the various *TCS*- and *FRE-lacZ* reporters were targeted to the *ura3-52* locus. Correct integrations were verified by Southern blot analysis. Standard methods for genetic crosses and transformation were used, and standard yeast culture medium was prepared essentially as described (Guthrie and Fink, 1991). When required, synthetic complete medium (SC) lacking appropriate supplements was used. For all pheromone induction experiments performed in liquid media, cultures were first incubated in the absence of α-factor to exponential growth phase, followed by a 1 hour treatment with 0.5 to 1 μM synthetic α-factor (Novabiochem, Switzerland) (stock solution: 2 mM dissolved in methanol) before cell extracts were prepared. Invasive growth tests were performed as described previously (Roberts and Fink, 1994).

Plasmid constructions.

All plasmids used in this study are listed in Table I. Plasmids pME2509 and pME2514 were constructed by subcloning of a 3.2 kb *Pst*I/*Sma*I fragment containing a *TEC1(P)::myc*^{3}*-TEC1* cassette from pME2280 (Köhler *et al.*, 2002) into YEplac181 and YIplac128, respectively. To obtain myc epitope-tagged *tec1-102, tec1-201 and tec1-202* alleles on an integrative plasmid, the alleles were isolated as 2.1 kb *Sal*I/*Bam*HI fragments from plasmids pME2281, pME2287 and pME2285 (Köhler *et al.*, 2002) and exchanged for the corresponding wild-type fragment from pME2514. pME2440 for high-copy expression of a myc-tagged N-terminal part of Tec1p (Tec1$^{M1\text{-}E280}$p) was obtained by subcloning of a

3.2 kb *Pst*I/*Sma*I fragment containing the *TEC1(P)::myc*^{3}*-tec1-102* cassette from pME2281. For expression of a myc-tagged C-terminal part of Tec1p (Tec1$^{K281\text{-}Y486}$p), the following cloning strategy was applied: (i) A 3.4 kb *TEC1(P)::myc*^{6}*-TEC1* cassette was isolated by *Pst*I/*Sma*I digestion from pME2295 (Köhler *et al.*, 2002) and inserted into YEplac181, yielding pME2439. Following *Bam*HI cleavage, a 2.2 kb *myc*^{3}*-TEC1* fragment was removed, leaving behind the *TEC1* promoter fused to a single *myc*^{3} copy (*TEC1(P)::myc*^{3}) with *Bam*HI cohesive ends. (ii) A fragment encoding the C-terminal part of Tec1p was amplified by PCR using primers TKTEC1-9 (CGCGGATCCAAAAAAATTGAAAATTTCATAAAAACTAATGC) and TEC1-6 (CGCGGATCCGGCCCCGACTTGAATGATTTTCAAGGTAGG), introducing *Bam*HI sites at both ends. The resulting 1.3 kb PCR fragment was placed downstream of the *TEC1(P)::myc*^{3}-cassette into pME2439 lacking the *myc*^{3}*-TEC1* part, yielding pME2510. For construction of pME2435 and pME2436, the *KSS1* and *FUS3* open reading frames were amplified as *Bam*HI fragments and inserted in frame downstream of the *GST* ORF in pYGEX-2T (Schlenstedt *et al.*, 1995). For expression of the corresponding kinase-dead MAPKs fused to GST, *KSS1*^{K42R} and *FUS3*^{K42D} were amplified from plasmids B3744 (Madhani *et al.*, 1997) and GA1905 (Gartner *et al.*, 1992), respectively. Plasmids pME2511 to pME2513 for expression of LexA-Tec1p, LexA-Tec1^{T273M}p and LexA-Tec1^{P274S}p fusion proteins were constructed by amplification and subcloning of the corresponding *TEC1* alleles as *Eco*RI/*Bam*HI fragments in pEG202 using pME2068, pME2102 and pME2096 as templates (Köhler *et al.*, 2002).

β-Galactosidase assays.

Strains carrying plasmid-borne or integrated *lacZ* reporters were grown in selective liquid SC or YNB medium to exponential growth phase, and extracts were prepared and assayed for β-galactosidase activity as described (Mösch *et al.*, 1996). β-Galactosidase specific activity was normalized to the total protein in each extract and equals (optical density at 420 nm × 1.7]/[0.0045 × protein concentration × extract volume × time]. Assays were performed on at least two independent transformants, each done in triplicate, and the mean value is presented. Standard deviations did not exceed 20%.

Halo assays.

α-Factor induced growth inhibition was measured by a halo assay as described previously (Elion *et al.,* 1990) with minor modifications. Briefly, an overnight culture of yeast cells grown in selective SC medium was diluted 10-fold, and 200 µl cells were plated with glass beads on an agar plate. Small filter disks (Whatman) soaken with 5 µl of 2mM α-factor in methanol were laid on top of the plates. Plates were incubated at 30 °C for two days before photographing.

Northern blot analysis.

Total RNAs were prepared from exponentially growing liquid cultures as described earlier (Cross and Tinkelenberg, 1991). Total RNAs were seperated on a 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes as described earlier (Mösch *et al.,* 1992). *TEC1*, *FLO11* and *ACT1* transcripts were detected using gene-specific ^{32}P-radiolabeled DNA probes. Hybridizing signals were quantified using a BAS-1500 Phosphor-Imaging scanner (Fuji, Tokyo, Japan).

Protein analysis.

(a) Whole-cell extracts. Yeast strains harbouring integrated versions of myc epitope-tagged *TEC1* were grown to late exponential phase (OD_{600} 1.5 – 2.0) in liquid SC medium. Cultures were split into two halfs, one treated with 1µM α-factor, the other with methanol for one hour. Cells were chilled, washed in ice-cold buffer R (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM dithiothreitol), lysed with glass beads in 200 µl of buffer R containing a cocktail of protease inhibitors (complete tablets purchased from Roche Diagnostics, Mannheim, Germany), and spun down to remove glass beads and cell debris. Extracts (10 µl) were removed to determine total protein concentration using a protein assay kit (Bio-Rad, München, Germany). SDS loading dye was added to the remaining total extracts and proteins were denatured by heating at 75 °C for 10 min. Equal amounts of proteins were then subjected to SDS-PAGE and transferred to nitrocellulose membranes. myc^3-Tec1p fusion proteins were detected using enhanced chemiluminescence technology (Amersham, Buckinghamshire, United Kingdom) after incubation of nitrocellulose membranes with monoclonal mouse anti-myc antibodies (9E10) together with a peroxidase-coupled goat anti-mouse IgG secondary antibody (Dianova, Hamburg, Germany). For detection of Cdc42p, the membranes were incubated with polyclonal anti-Cdc42p antibodies and a

peroxidase-coupled goat anti-rabbit IgG secondary antibody (Dianova, Hamburg, Germany).

(b) Protein purification from E. coli. Plasmids expressing the MBP-FLAG (B3672) and MBP-TEC1-FLAG (B3513) (Madhani and Fink, 1997) fusion proteins were transformed into *E. coli* BL21(DE3)-RIL cells (Stratagene). Cells were grown in rich medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose) containing standard concentrations of ampicillin and chloramphenicol to OD_{600} 0.5. Fusion proteins were induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37° C. Cells were collected and homogenized in lysis buffer (20 mM Tris-Cl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) by sonification. The fusion proteins were purified by affinity chromatography with amylose resin (New England Biolabs, Frankfurt, Germany)

(c) Affinity purification of proteins binding to GST fusion proteins from yeast cells. Extracts of cells expressing GST fusion proteins together with myc-tagged versions of various Tec1p variants were prepared essentially as described (Roberts *et al.*, 1997). Strains were incubated in 50 ml liquid selective SC medium containing 2% glucose to OD_{600} 0.6. Cells were spun down, resuspended in SC medium containing 2% galactose to induce GST fusions and further incubated at 30 °C for 5 h until cells were harvested. Cells were chilled, washed in buffer B (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA) and lysed with glass beads in 300 µl buffer B + complete protease inhibitors. Following cell lysis, 300 µl buffer B + complete protease inhibitors + 0.08% Triton X-100 were added and mixed. Glass beads and cell debris were removed by centrifugation and extracts were incubated with glutathione-agarose in 1 ml buffer B + complete protease inhibitors + 0.08% Triton X-100 + 10% glycerol overnight at 4 °C. The beads were repeatedly washed and collected to purify GST fusions and any associated proteins. Samples were denatured by heating in SDS loading dye, and equal amounts of each sample were analysed by western blot analysis as described above using either polyclonal anti-GST antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) or the monoclonal mouse anti-myc antibody (9E10).

(d) *in vitro Phosphorylation assay*. The Kss1p and Fus3p kinases were purified as GST fusion proteins from yeast cells as described above except that a part of the cultures cultures were treated with 0.5 µM α-factor for 30 min prior to cell harvest. Moreover, buffer B was supplemented with phosphatase inhibitor cocktails 1 and 2 available from

Sigma (catalogue numbers P 2850 and P 5726). After collection, the glutathione-agarose beads with the associated proteins were repeatedly washed with and finally collected in 100 µl kinase buffer (50 mM Tris-Cl pH 7.5, 20 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM Na_3VO_4). 25 µl of the slurry were incubated together with 200 ng of the substrate protein purified from *E. coli* in a total volume of 50 µl. Phosphorylation reactions were started by the addition of 1 µCi of [γ-^{32}P]ATP, incubated for 20 min at 30 °C, resolved by SDS-PAGE and exposed to film.

Table I. Plasmids used in this study

Plasmid	Description	Reference
YEplac181	*LEU2*-marked 2µm vector	(Gietz and Sugino, 1988)
YIplac128	*LEU2*-marked integrative vector	(Gietz and Sugino, 1988)
pYGEX-2T	*URA3*-marked 2µm *GAL1(p)-GST* fusion vector	(Schlenstedt *et al.*, 1995)
pME2436	*GAL1(p)-GST-FUS3* fusion in pYGEX-2T	This study
pME2507	*GAL1(p)-GST-FUS3*^{K42D} fusion in pYGEX-2T	This study
pME2435	*GAL1(p)-GST-KSS1* fusion in pYGEX-2T	This study
pME2508	*GAL1(p)-GST-KSS1*^{K42R} fusion in pYGEX-2T	This study
pME2509	*TEC1(p)-myc*^{3}*-TEC1* fusion in YEplac181	This study
pME2440	*TEC1(p)-myc*^{3}*-TEC1*$^{M1-E280}$ fusion in YEplac181	This study
pME2510	*TEC1(p)-myc*^{3}*-TEC1*$^{K281-Y486}$ fusion in YEplac181	This study
B3672	*MBP*-polylinker-*FLAG* fusion in pMAL-c2 (New England Biolabs)	(Madhani and Fink, 1997)
B3513	*MBP-TEC1-FLAG* fusion in pMAL-c2 (New England Biolabs)	(Madhani and Fink, 1997)
pEG202	Vector for construction of LexA fusion proteins	(Gyuris *et al.*, 1993)
pME2511	*LexA-TEC1* in pEG202	This study
pME2512	*LexA-tec1-201* in pEG202	This study
pME2513	*LexA-tec1-202* in pEG202	This study
pME2514	*TEC1(p)-myc*^{3}*-TEC1* fusion in YIplac128	This study
pME2515	*TEC1(p)-myc*^{3}*-tec1-102* fusion in YIplac128	This study
pME2516	*TEC1(p)-myc*^{3}*-tec1-201* fusion in YIplac128	This study
pME2517	*TEC1(p)-myc*^{3}*-tec1-202* fusion in YIplac128	This study
pLI4	*CYC1-lacZ* fusion in *URA3*-marked integrative vector	(Sengstag and Hinnen, 1988)
pME1108	pLI4 with *CYC1(ΔUAS)-lacZ*	(Köhler *et al.*, 2002)
pME2051	pLI4 with *TCS*^{fw}*-CYC1-lacZ*	(Köhler *et al.*, 2002)
pME2055	pLI4 with *TCS*^{fw}*-11-TCS*^{bw}*-CYC1-lacZ*	(Köhler *et al.*, 2002)
pME2066	pLI4 with *TCS*^{fw}*-14-PRE*^{fw}*-CYC1-lacZ*	(Köhler *et al.*, 2002)
pME2518	*ste12Δ::ble* cassette for full deletion of *STE12* ORF	This work

Table II. Strains used in this study

Strain	Relevant genotype	Reference
YM106	*MAT**a** ura3-52 leu2::hisG his3::hisG trp1::hisG fus3Δ::TRP1*	(Madhani and Fink, 1997)
RH2949	Same as YM106 except *ste12Δ::ble*	This study
RH2500	*MAT**a** tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG*	(Köhler *et al*., 2002)
RH2950	Same as RH2500 except *kss1Δ::kanMX*	This study
RH2951	Same as RH2500 except *TEC1(p)::myc^3-TEC1::LEU2 TRP1*	This study
RH2952	Same as RH2500 except *TEC1(p)::myc^3-tec1-102::LEU2*	This study
RH2953	Same as RH2500 except *TEC1(p)::myc^3-tec1-201::LEU2*	This study
RH2954	Same as RH2500 except *TEC1(p)::myc^3-tec1-202::LEU2*	This study
RH2955	Same as RH2500 except *LEU2 TRP1*	This study
RH2501	*MAT**a** tec1Δ::HIS3 ste12::TRP1 ura3-52 leu2::hisG his3::hisG trp1::hisG*	(Köhler *et al*., 2002)
RH2956	*MAT**a** tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG fus3Δ::TRP1*	This study
RH2957	Same as RH2956 except *LEU2*	This study
RH2958	Same as RH2956 except *TEC1(p)::myc^3-TEC1::LEU2*	This study
RH2959	Same as RH2956 except *TEC1(p)::myc^3-tec1-102::LEU2*	This study
RH2960	Same as RH2956 except *TEC1(p)::myc^3-tec1-201::LEU2*	This study
RH2961	Same as RH2956 except *TEC1(p)::myc^3-tec1-202::LEU2*	This study
RH2962	*MAT**a** tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG kss1Δ::kanMX*	This study
RH2963	Same as RH2962 except *LEU2 TRP1*	This study
RH2964	Same as RH2962 except *TEC1(p)::myc^3-TEC1::LEU2 TRP1*	This study
RH2965	Same as RH2962 except *TEC1(p)::myc^3-tec1-102::LEU2*	This study
RH2966	Same as RH2962 except *TEC1(p)::myc^3-tec1-201::LEU2*	This study
RH2967	Same as RH2962 except *TEC1(p)::myc^3-tec1-202::LEU2*	This study
RH2968	*MAT**a** tec1Δ::HIS3 ura3-52 leu2::hisG his3::hisG trp1::hisG fus3Δ::TRP1 kss1Δ::kanMX*	This study
RH2969	Same as RH2968 except *LEU2*	This study
RH2970	Same as RH2968 except *TEC1(p)::myc^3-TEC1::LEU2*	This study
RH2971	Same as RH2968 except *TEC1(p)::myc^3-tec1-102::LEU2*	This study
RH2972	Same as RH2968 except *TEC1(p)::myc^3-tec1-201::LEU2*	This study
RH2973	Same as RH2968 except *TEC1(p)::myc^3-tec1-202::LEU2*	This study

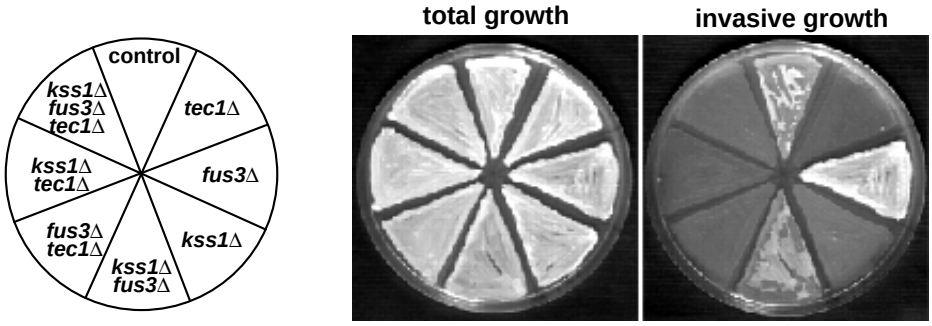
RESULTS

Regulation of invasive growth by *KSS1* and *FUS3* depends on *TEC1*.

We performed a detailed analysis of invasive growth regulation by the MAP kinases Fus3p and Kss1p in conjunction with the transcription factor Tec1p. All experiments were performed with haploid strains expressing a single copy of myc epitope-tagged *TEC1*. This construct complemented the invasive growth defect of a *tec1Δ* strain (Fig. 1A), demonstrating its functionality. Consistent with previous results (Roberts and Fink, 1994), we found the two MAPKs Fus3p and Kss1p play opposing roles in regulation of haploid invasive growth. Whereas a *kss1Δ* strain showed remarkably reduced agar invasion, a *fus3Δ* mutant invaded the agar even more vigorously than a wild-type. The *fus3Δ kss1Δ* double mutant exhibited wild-type invasive growth behaviour. However, agar invasion was completely abolished in *fus3Δ tec1Δ*, *kss1Δ tec1Δ* and *fus3Δ kss1Δ tec1Δ* strains (Fig. 1A), suggesting that the two MAPKs Fus3p and Kss1p might control invasive growth by directly regulating activity of Tec1p. We have previously demonstrated that Tec1p regulates invasive growth not only by combinatorial control together with Ste12p via FRE elements, but also by a second mechanism termed TCS control which is operative even in the absence of Ste12p (Köhler *et al.*, 2002).

We now wanted to investigate whether strains deleted for one or both of the MAPK genes show changes in FRE- or TCS reporter induction. We integrated two different *TCS-lacZ* reporters, containing either a single or a combined double TCS element, along with an FRE reporter and an enhancerless *CYC1-lacZ* control into the chromosome of strains carrying all possible combinations of *fus3Δ*, *kss1Δ* and *tec1Δ* deletions. Induction of the various reporters in the different strain backgrounds was measured and compared (Fig. 1B). Deletion of *TEC1* blocked activation of all reporters almost completely in each strain, consistent with our result that regulation of invasive growth by *KSS1* and *FUS3* depends on *TEC1*. The highest activation was observed for the FRE reporter in the wild-type background (302 U). The single TCS reporter reached 9% and the double TCS reporter 23% of this value in the same strain, reinforcing the notion that activation of TCS reporters by Tec1p is an additive process, increasing in parallel to the number of TCS elements present. *FRE-lacZ* reporter induction remained constantly high in *fus3Δ* or *kss1Δ* strains. Only the *fus3Δ kss1Δ* double mutant showed a moderate reduction of *FRE-lacZ* activity to 67% of the wild-type value.

A



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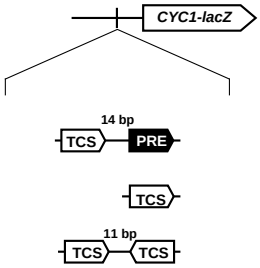

β-galactosidase activity (nmoles/min/mg)

wt	*tec1*Δ	*fus3*Δ	*kss1*Δ	*fus3*Δ *kss1*Δ	*fus3*Δ *tec1*Δ	*kss1*Δ *tec1*Δ	*fus3*Δ *kss1*Δ *tec1*Δ
302	4	282	263	206	4	10	2
27	1	50	16	14	0	0	1
69	10	112	51	25	8	7	8

Fig. 1. Regulation of invasive growth and gene expression by *FUS3*, *KSS1* and *TEC1*. (A) Regulation of substrate invasion by *FUS3* and *KSS1* depends on *TEC1*. Haploid strains RH2951 (wild-type), RH2955 (*tec1Δ*), RH2958 (*fus3Δ*), RH2964 (*kss1Δ*), RH2970 (*fus3Δ kss1Δ*), RH2956 (*fus3Δ tec1Δ*), RH2962 (*kss1Δ tec1Δ*) and RH2968 (*fus3Δ kss1Δ tec1Δ*) were grown on YEPD medium for 3 days. Plates were photographed before (total growth) and after (invasive growth) cells were washed off the agar surface. (B) Activation of FRE- and TCS-dependent gene expression by the MAPKs and Tec1p. An enhancerless *CYC1-lacZ* fusion gene carrying no UAS element (no insert), an FRE (TCS plus PRE), a single TCS element or a combined double TCS element was integrated as a single copy into the genomes of strains RH2951 (wild-type), RH2955 (*tec1Δ*), RH2958 (*fus3Δ*), RH2964 (*kss1Δ*), RH2970 (*fus3Δ kss1Δ*), RH2957 (*fus3Δ tec1Δ*), RH2963 (*kss1Δ tec1Δ*), and RH2969 (*fus3Δ kss1Δ tec1Δ*). β-galactosidase specific activities were determined and are expressed in nanomoles of o-nitrophenyl-β-D-galactopyranoside hydrolized per minute times milligrams of protein. Background activities obtained for strains with UAS-less *CYC1-lacZ* reporters have already been substracted. Values shown are the mean of four measurements with two independent transformants. The standard deviation did not exceed 20%.

Expression of the single- or double *TCS-lacZ* reporters was affected to a larger extent. In the *fus3Δ* strain, both constructs were 1.9-fold (single TCS) or 1.6-fold (double TCS) better activated than in the wild-type. In contrast, activation of both reporters was reduced to 59%

(single TCS) or 74% (double TCS) of the wild-type in the *kss1Δ* genetic background. Oddly, TCS reporter activation was even further reduced to 52% (single TCS) or 36% (double TCS) of the wild-type activity in the *fus3Δ kss1Δ* double mutant. All other strains carrying combinations of *tec1Δ* together with a deletion of one or both MAPK genes equally blocked activation of all reporters to the basal level, again demonstrating that *TEC1* is indispensable for invasion-specific gene activation. In summary, our plate washing and reporter gene assays suggest that the MAPKs Fus3p and Kss1p require Tec1p for regulation of invasive growth and affect TCS- and FRE-mediated gene expression.

Tec1p physically associates with Kss1p and Fus3p through distinct domains.

Our finding that Fus3p and Kss1p regulate invasive growth in a Tec1p-dependent manner suggested that one or both of the MAPKs might regulate Tec1p by physical interaction with the protein. Indeed, a recent large scale approach using mass spectrometry has identified Tec1p and Kss1p to be present in a complex (Ho *et al.*, 2002). To test physical interactions between Tec1p and both MAPKs, we constructed in-frame fusions between glutathione S-transferase (GST) and *FUS3* or *KSS1*. The resulting fusions were expressed in yeast together with a myc epitope-tagged version of *TEC1*. Fusion proteins were purified with glutathione beads to isolate each fusion and any associated proteins. Proteins purified by glutathione-agarose were analysed by western blot analysis using polyclonal anti-GST antibodies or monoclonal anti-myc antibodies. We found that myc^3-Tec1p co-purifies with both GST-Kss1p and GST-Fus3p, but not with GST alone (Fig. 2). Thus, Tec1p appears not only to be associated with Kss1p, but in addition with Fus3p. In a previous work, we had analyzed Tec1p with respect to invasive growth regulation and gained some new insights into the functions of Tec1p subdomains (Köhler *et al.*, 2002). The conserved TEA/ATTS DNA-binding domain is located near the amino terminus and comprises amino acids 126 to 193 (Bürglin, 1991). We found that the N-terminal 280 amino acids of Tec1p including the TEA/ATTS domain were sufficient for execution of FRE-control together with Ste12p, whereas efficient induction of invasive growth additionally required the remaining C-terminal portion of Tec1p. Moreover, we identified five sites conforming to the minimal consensus sequence Ser/Thr-Pro (S/TP) for MAP kinase phosphorylation sites (Sharrocks *et al.*, 2000) and at least two putative MAP kinase docking sites in Tec1p. To further characterize the interaction between Tec1p and both MAPKs, the N- and C-terminal portions of Tec1p (Tec1$^{M1\text{-}E280}$p and Tec1$^{K281\text{-}Y486}$p) were

expressed seperately and tested for interaction with GST-Fus3p, GST-Kss1p, or GST alone (Fig. 2). Strikingly and unexpectedly, Tec1$^{M1\text{-}E280}$p was co-purified with Fus3p, but not with Kss1p, suggesting that the N-terminal half of Tec1p mediates interaction with Fus3p, but not with Kss1p. Vice versa, myc^3-Tec1$^{K281\text{-}Y486}$p was co-purified with Kss1p, but not with Fus3p. This finding suggests that different domains of Tec1p are required for interaction with the MAPKs Fus3p and Kss1p. The N-terminus, encompassing amino acids 1 to 280, is required and sufficient for interaction with Fus3p, whereas the C-terminus (amino acids 281-486) is required and sufficient for interaction with Kss1p.

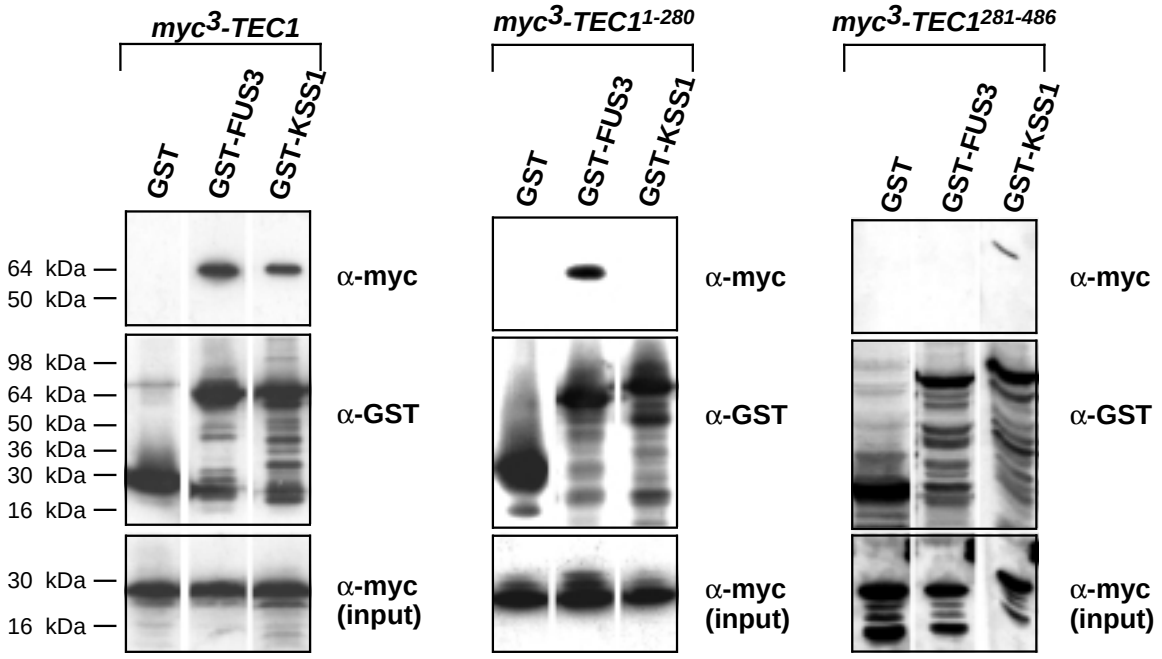

Fig. 2. Co-purification of myc^3-Tec1p with GST-Fus3p and GST-Kss1p. Total protein extracts were prepared from strain RH2500 (*tec1Δ*) carrying each possible combination of one plasmid encoding a GST fusion (pYGEX-2T: *GST*, pME2436: *GST-FUS3*, pME2435: *GST-KSS1*) together with another plasmid encoding a myc epitope-tagged Tec1p variant (pME2509: *myc^3-TEC1*, pME2440: *myc^3-TEC1$^{M1\text{-}E280}$*, pME2510: *myc^3-TEC1$^{K281\text{-}Y486}$*). GST and GST fusion proteins were purified as described. Equivalent amounts of each sample were subjected to SDS-PAGE, transferred to nitrocellulose and probed with a monoclonal anti-myc antibody (α-myc) or a polyclonal anti-GST antibody (α-GST). The SeeBlue pre-stained protein standard (Invitrogen, Karlsruhe, Germany) was used for determination of protein sizes.

Kss1p and Fus3p from vegetative cells confer weak phosphorylation of Tec1p *in vitro*. Our finding that Tec1p interacts with Fus3p and Kss1p *in vivo* prompted us to investigate whether Tec1p is also phosphorylated by any of the two MAPKs. First, we examined whether Tec1p is phosphorylated by the MAPKs isolated from vegetatively growing cells. We purified Fus3p and Kss1p as fusions to GST with glutathione beads from yeast strain RH2500 (*tec1Δ*). Tec1p was purified from *Escherichia coli* as protein fusion with N-terminal maltose-binding protein (MBP) and C-terminal FLAG tag (Madhani and Fink, 1997). Phosphorylation of MBP-Tec1p by GST-Fus3p or GST-Kss1p was determined by ^{32}P incorporation. Both MAPKs, Fus3p and Kss1p, caused a moderate phosphorylation of MBP-Tec1p (Fig. 3A), and this phosphorylation occured specifically on the Tec1p part of the MBP-Tec1p fusion, because MBP alone was virtually not phosphorylated. When compared to each other, the basal kinase activities of Kss1p (7.5-fold induction over background) and Fus3p (6.4-fold induction) towards Tec1p were nearly identical, indicating that both kinases might equally contribute to basal phosphorylation under conditions permissive for invasive growth.

Pheromone highly stimulates phosphorylation of Tec1p by Fus3p but not by Kss1p. We next determined phosphorylation of recombinant MBP-Tec1p by GST-Fus3p and GST-Kss1p isolated from pheromone-treated yeast cells to maintain the MAPKs in a highly activated state. We included catalytically inactive variants for each of the MAPKs, GST-Fus3^{K42D}p and GST-Kss1^{K42R}p, as controls (Gartner *et al.*, 1992). Unexpectedly, MBP-Tec1p signals were overlaid by signals from another protein that was co-purified together with GST-Kss1p and GST-Fus3p and that we suspected from its size to be Ste12p (not shown). Purification of the different MAPKs from a *ste12Δ* mutant strain (RH2501) led to disappearance of this non Tec1p-specific signal indicating that Ste12p is co-purified along with GST-Kss1p and GST-Fus3p and is phosphorylated in response to pheromone treatment. In contrast, when activated by pheromone only GST-Fus3p substantially phosphorylated MBP-Tec1p (Fig. 3B). This phosphorylation was nearly abolished when the kinase-dead version of Fus3p (Fus3^{K42D}p) was applied, demonstrating that MBP-Tec1p phosphorylation depends on Fus3p kinase activity. GST-Kss1p, on the other hand, did not significantly phosphorylate MBP-Tec1p, neither in its wild-type form nor when the kinase-dead version was applied. We therefore conclude that Fus3p, but not Kss1p, is the MAP kinase that specifically phosphorylates Tec1p in response to pheromone stimulation.

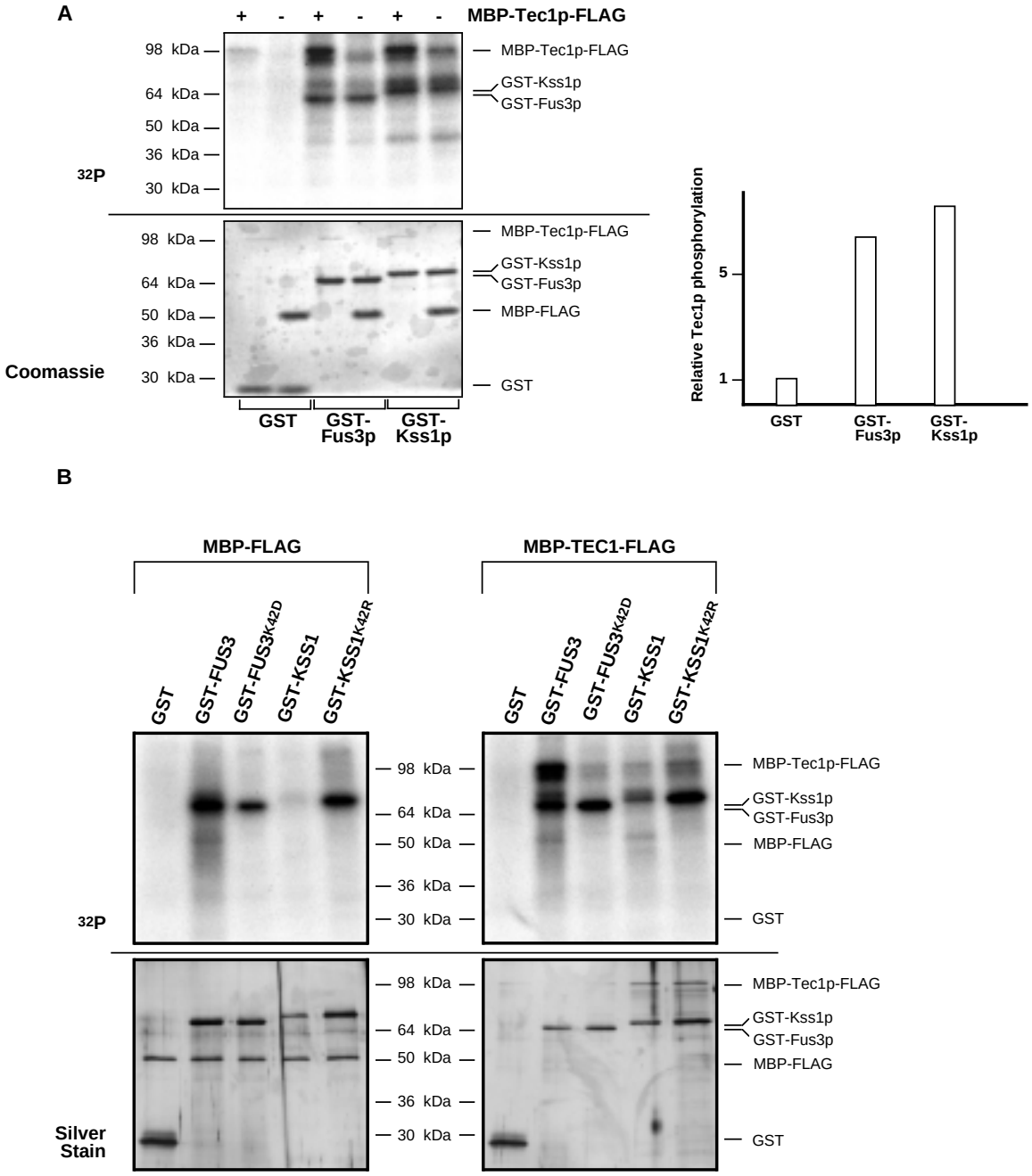

Fig. 3. *In vitro* phosphorylation assay. (A) Basal phosphorylation of Tec1p by unstimulated MAPKs. Bacterially purified MBP-Tec1p-FLAG (+) or MBP-FLAG (-) were tested for ^{32}P incorporation in the presence of GST, GST-Fus3p or GST-Kss1p purified from yeast strain RH2500 (*tec1Δ*). Plasmids used for expression of GST fusions were pYGEX-2T (*GST*) (Schlenstedt *et al.*, 1995), pME2436 (*GST-FUS3*) and pME2435 (*GST-KSS1*). Portions of the reaction mixtures were subjected to SDS-PAGE and exposed to film for autoradiography (top). Afterwards, the gel was stained (Coomassie) (bottom) to show that equal amounts of GST- and MBP-fusions were applied in each reaction. Phosphorylation of MBP-Tec1p by GST-Fus3p and GST-Kss1p was quantified using a Phospho-Imaging scanner. (B) Phosphorylation of Tec1p by activated MAPKs. MBP-FLAG (left) or MBP-Tec1p-FLAG (right) were tested for ^{32}P incorporation in the presence of GST, GST-Fus3p, GST-Fus3^{K42D}p, GST-Kss1p, or GST-Kss1^{K42R}p purified from yeast strain RH2501 (*tec1Δ*

ste12Δ) treated with α-factor. pME2507 (*GST-FUS3*^{K42D}) and pME2508 (*GST-KSS1*^{K42R}) were included as controls for expression of kinase-dead MAPKs fused to GST. Portions of the reaction mixtures were subjected to SDS-PAGE and exposed to a film for autoradiography (top). Afterwards, the gel was stained with $AgNO_3$ (bottom) to show that equal amounts of GST- and MBP-fusions were applied in each reaction.

Activation of Fus3p but not Kss1p by pheromone downregulates Tec1p protein and *FLO11* transcript levels.

The reasons for the different invasive growth phenotypes of *fus3Δ, kss1Δ* or *fus3Δ kss1Δ* strains are only partially understood. Principally, both MAPKs regulate expression of *TEC1* by relief of inhibition of Ste12p, the upstream transcription factor finally controlling *TEC1* mRNA formation. However, this model cannot explain the differences in substrate invasion observed. Our findings that both MAPKs differentially interact with Tec1p suggest that more direct control mechanisms might exist. Therefore, we examined the effect of the MAPKs Fus3p and Kss1p on the amount of *TEC1* mRNA and Tec1p protein levels without and with stimulation by pheromone (Fig. 4). We found that *TEC1* transcript levels were only weakly affected in the various MAPK deletion strains under both conditions (Fig. 4A). Consistent with previous studies (Oehlen and Cross, 1998; Roberts *et al.*, 2000) the *TEC1* transcript level was elevated in a control strain treated with α-factor when compared to the same strain cultured without α-factor. All strains deleted for any one or both of the MAPK genes showed relatively constant *TEC1* transcript levels on either condition. Only the *fus3Δ* strain displayed an increase of *TEC1* mRNA to 184% in vegetative cells, whereas basal *TEC1* transcription was reduced to 72% of the wild-type level in the *fus3Δ kss1Δ* double mutant. These results suggest that regulation of *TEC1* transcription is not the primary mechanism by which the MAPKs Fus3p and Kss1p regulate yeast invasive growth.

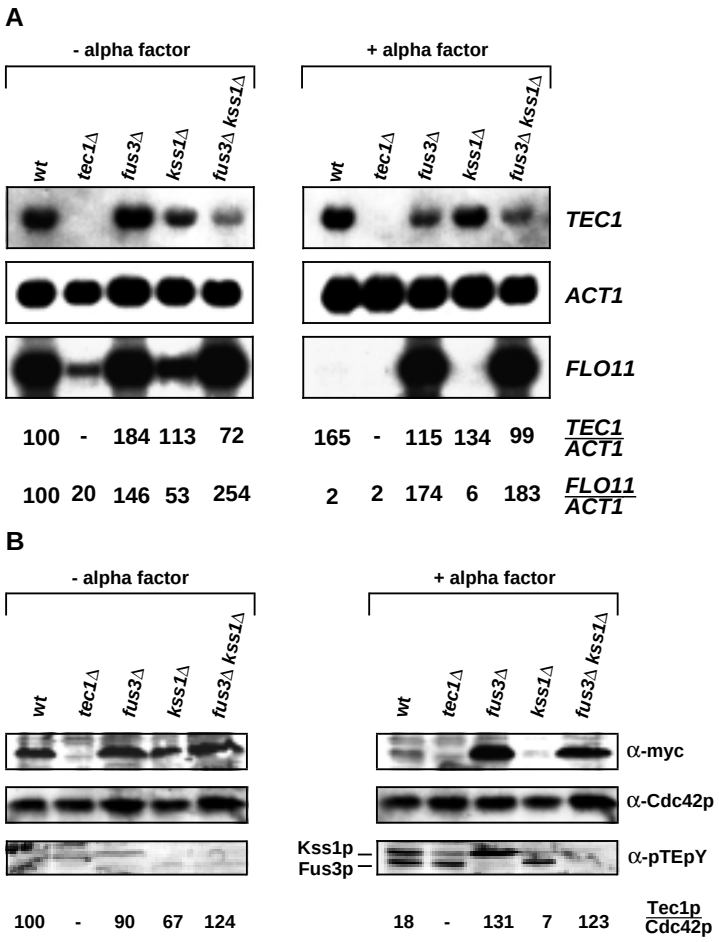

Fig. 4. Regulation of *TEC1* and *FLO11* expression by *FUS3, KSS1* and α-factor. (A) *TEC1* and *FLO11* transcript levels in various MAP kinase deficient strains. Strains RH2951 (wild-type), RH2955 (*tec1Δ*), RH2958 (*fus3Δ*), RH2964 (*kss1Δ*) and RH2970 (*fus3Δ kss1Δ*), with the exception of RH2955 all expressing a myc epitope-tagged integrated *TEC1* version, were grown in liquid YEPD medium to exponential phase. Cultures were divided into two halfs, one of it treated with α-factor and the other with methanol, and further incubated for one hour before total RNA was prepared and used for Northern analysis. *ACT1* expression served as an internal standard. Relative *TEC1* (*TEC1*/*ACT1*) and *FLO11* (*FLO11/TEC1*) expression levels were obtained by using a Phospho-Imaging scanner and normalizing *TEC1* and *FLO11* transcript levels to *ACT1* levels. (B) Expression of myc^3-Tec1p. The same strains as in (A) were cultured in liquid SC-Leu medium and treated with α-factor as described above. Protein extracts were prepared and the levels of myc^3-Tec1p were determined by Western blot analysis with an anti-myc monoclonal antibody. As an internal control, expression levels of Cdc42p were measured in the same extracts by using an anti-Cdc42p polyclonal antibody. Relative Tec1p (Tec1p/Cdc42p) levels are shown in arbitrary units and were obtained by normalizing Tec1p signals to Cdc42p signals and to levels measured in strain RH2951 (wt) cultivated in the absence of pheromone. In addition, the membranes were reprobed with a phosphorylation-state specific antibody (α-pTEpY) (New England Biolabs, catalog number 9101) to demonstrate the effect of pheromone treatment on MAPK phosphorylation.

Next, we determined the amount of myc epitope-tagged Tec1p (myc^3-Tec1p) in the same strains by immunoblotting (Fig. 4B). With the exception of the *tec1Δ* control strain, specific myc^3-Tec1p signals of comparable levels were detectable in all strains grown in the absence of α-factor. Quantification of the myc^3-Tec1p signals yielded levels between 67% (*kss1Δ*) and 124% (*fus3Δ kss1Δ*) of the wild-type reference. Unexpectedly, treatment of cells with α-factor led to a strong decrease of the myc^3-Tec1p signals in all strains containing the *FUS3* gene (Fig. 4B). Tec1p levels were reduced to 18% in the control strains and to 7% in the *kss1Δ* strain. In contrast, myc^3-Tec1p signals remained constantly high in strains deleted for *FUS3* (*fus3Δ* and *fus3Δ kss1Δ*) upon α-factor exposure. These findings suggest that activation of Fus3p by pheromone (Fig. 4B) induces a significant drop in Tec1p protein levels by a posttranscriptional mechanism. This function of Fus3p is not shared by Kss1p, because myc^3-Tec1p remains stable in a *fus3Δ* single mutant in response to α-factor treatment.

The finding that α-factor causes reduced Tec1p levels in a *FUS3* dependent manner made us investigate whether expression of *FLO11*, the most prominent invasion specific gene, is affected by α-factor as well. Therefore, we reprobed the membranes used before to determine *TEC1* transcript levels with a *FLO11* specific gene probe (Fig. 4A). Indeed, we found that *FLO11* expression was dramatically reduced in response to α-factor treatment in strains carrying the *FUS3* gene, with *FLO11* transcript levels reduced to 2% – 6% of the level obtained for the untreated wild-type. In contrast, *FLO11* transcript levels did not decline in *fus3Δ* or *fus3Δ kss1Δ* strains. Concluding, our expression analysis suggests that control of yeast invasive growth by the MAPKs Fus3p and Kss1p is dependent on Tec1p. Altered *TEC1* transcription is not the reason for the differences in substrate invasive growth behaviour displayed by wild-type, *fus3Δ*, *kss1Δ* and *fus3Δ kss1Δ* strains. Rather, Fus3p might play a role in regulation of Tec1p stability/degradation as revealed by our α-factor induction experiments.

Amino acid substitutions in a conserved MAPK phosphorylation site of Tec1p confer hyperinvasive growth, stability against active Fus3p, and higher tolerance against pheromone.

We have previously isolated variants of *TEC1* confering an increased *FLO11* transcription and a hyperinvasive phenotype (Köhler *et al.*, 2002). Remarkably, the amino acids substituted in the proteins encoded by *tec1-201* and *tec1-202* are located directly adjacent

to each other. We found that both residues, T273 and P274, together build up a site that conforms to the minimal consensus sequence Ser/Thr-Pro (S/TP) for MAP kinase phosphorylation sites (Sharrocks *et al.*, 2000). We integrated myc epitope-tagged versions of these alleles, along with an allele causing reduced agar invasion (*tec1-102*), into wild-type, *fus3Δ*, *kss1Δ* and *fus3Δ kss1Δ* strains to measure interactions between the MAPKs and these Tec1p mutants. (Fig. 5A). As expected, the *tec1-102* allele caused reduced agar invasion compared to a control strain expressing *TEC1* in the wild-type strain background, whereas *tec1-201* and *tec1-202* stimulated agar invasion. Consistent with previous studies (Roberts and Fink, 1994), a *fus3Δ* strain expressing wild-type *TEC1* was hyperinvasive, a *kss1Δ* strain showed reduced invasion and the *kss1Δ fus3Δ* double mutant was as invasive as a wild-type. The *tec1-102* allele in combination with the *fus3Δ* deletion resulted in the same noninvasive phenotype as the *fus3Δ tec1Δ* control strain. In contrast, all strains expressing the *tec1-201* or *tec1-202* alleles were hyperinvasive, no matter if expressed in a wild-type, *fus3Δ*, *kss1Δ* or *fus3Δ kss1Δ* background. This suggested that *tec1-201* and *tec1-202* might no longer be under control of the MAPKs and α-factor.

We therefore investigated whether Tec1^{T273M}p and Tec1^{P274S}p show altered sensitivity towards α-factor (Fig. 5B). We expressed myc epitope-tagged versions of *TEC1*, *tec1-102*, *tec1-201* and *tec1-202* in a wild-type background. Cultures were grown to mid-exponential phase and divided into two parts. One part was treated with α-factor, the other with methanol for one hour until protein extracts were prepared and used for immunoblotting. As already described above (Fig. 5B), we found that the amount of wild-type Tec1p was drastically reduced (10-fold) after α-factor treatment. However, we found that all our mutant proteins were present in equal amounts in extracts prepared from α-factor treated or mock treated cultures. This suggests that Tec1^{T273M}p, Tec1^{P274S}p and even Tec1$^{M1\text{-}E280}$p are no longer downregulated by α-factor and Fus3p.

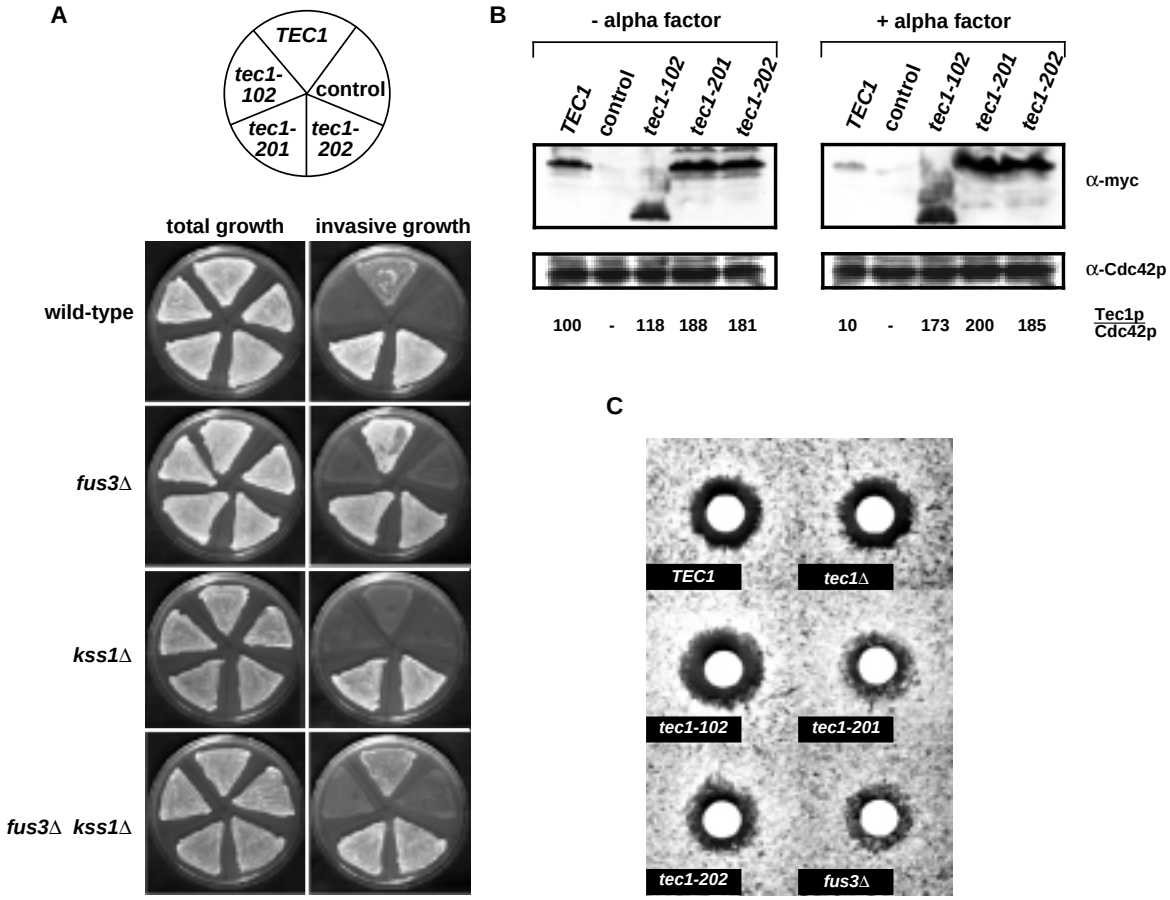

Fig. 5. *In vivo* and *in vitro* effects of amino acid substitutions in a conserved MAPK phosphorylation site of Tec1p. (A) Haploid invasive growth of MAPK deficient strains expressing different *tec1* alleles. Single copies of the indicated myc epitope-tagged *tec1* alleles were integrated into the chromosome of strains RH2500 (*tec1Δ*), RH2956 (*tec1Δ fus3Δ*), RH2962 (*tec1Δ kss1Δ*) or RH2968 (*tec1Δ fus3Δ kss1Δ*). Strains were grown on YEPD medium for 3 days. Plates were photographed before (total growth) and after (invasive growth) cells were washed off the agar surface. (B) Expression of different myc^3-Tec1p variants after treatment with α-factor. Strains RH2951 (*myc^3-TEC1*), RH2955 (*tec1Δ*, control), RH2952 (*myc^3-tec1-102*), RH2953 (*myc^3-tec1-201*) and RH2954 (*myc^3-tec1-202*) were grown in SC-Leu medium to exponential phase, divided into two parts and treated with α-factor as described. Protein extracts were prepared and the levels of myc^3-Tec1p were determined by Western blot analysis with a monoclonal anti-myc antibody. As an internal control, expression levels of Cdc42p were measured in the same extracts by using a polyclonal anti-Cdc42p antibody. Relative Tec1p (Tec1p/Cdc42p) levels are shown in arbitrary units and were obtained by normalizing Tec1p signals to Cdc42p signals and to levels measured in the wild-type strain RH2951 (*TEC1*) cultivated in the absence of pheromone. (C) Growth arrest in response to α-factor. The same strains as in (B) plus a *fus3Δ* mutant (RH2958) were used for halo assays performed as described in materials and methods.

The Fus3p MAP kinase has an important function which is not shared by Kss1p in mediating G_1 cell cycle arrest in response to α-factor. Fus3p phosphorylates the CKI Far1p (Elion *et al.,* 1993; Peter *et al.,* 1993), which in turn associates with and inhibits Clnp/Cdc28p complexes. Moreover, Fus3p and Kss1p together promote G_1 arrest by repressing transcription of G_1/S-phase cyclin genes (Cherkasova *et al.,* 1999; Elion *et al.,* 1991b; Valdivieso *et al.,* 1993; Wittenberg *et al.,* 1990). Because Tec1p positively regulates transcription of the *CLN1* gene (Madhani *et al.,* 1999), we speculated that our *tec1-201* and *tec1-202* mutants might interfere with α-factor induced cell cycle arrest. To test this prediction, we performed halo assays with strains expressing either wild-type or mutant *TEC1* alleles (Fig. 5C). Indeed we found that the diameter of the halo of strains expressing *tec1-201* or *tec1-202* alleles was significantly reduced compared to all other strains, even the *fus3Δ* mutant. Thus, proper regulation of the Tec1p protein by Fus3p during pherome signaling seems to be important for cell cycle arrest in G_1.

Tec1^{T273M}p and Tec1^{P274S}p are better transcriptional activators than wild-type Tec1p.
Our data suggest that phosphorylation of Tec1p by active Fus3p at the 273/274 phosphorylation site causes destabilization of the protein. However, deletion of *FUS3* does not lead to a strong increase in Tec1p protein level, yet *fus3Δ* strains are hyperinvasive. We wondered, whether phosphorylation/dephosphorylation of Tec1p at the 273/274 phosphorylation site might affect not only Tec1p protein stability, but also the transactivation capacity of the protein. We tested this hypothesis by using the one-hybrid system. Wild-type Tec1p, when expressed as a fusion to the heterologous DNA binding protein LexA, only very weakly activates transcription of a synthetic *LexAop-lacZ* reporter gene (Laloux *et al.,* 1994). We constructed in frame fusions of wild-type *TEC1* and our *tec1-201* and *tec1-202* alleles to the gene encoding the LexA DNA binding domain using the two hybrid plasmid pEG202 (Gyuris *et al.,* 1993). The resulting plasmids were transformed into the yeast strain EGY48-p1840 which carries an integrated *LexAop-lacZ* reporter, and ß-galactosidase activities were determined (Fig. 6). The wild-type *LexA-TEC1* construct weakly activated the reporter beyond the basal level, but both mutant alleles further induced ß-galactosidase activity by 39 or 25-fold, respectively. This finding indicates that the alleles *tec1-201* and *tec1-202* indeed possess an enhanced intrinsic capacity to activate target gene transcription.

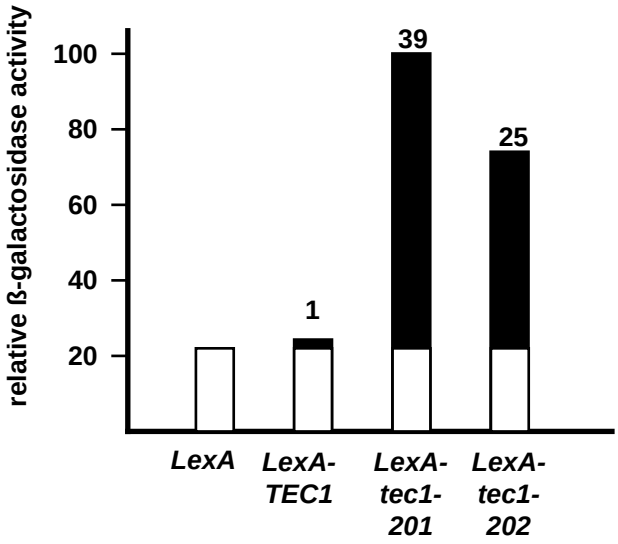

Fig. 6. Transcriptional activation of a *LexAop-lacZ* reporter by different Tec1p variants. Yeast strain EGY48-p1840 carrying an integrated *LexAop-lacZ* reporter was transformed with plasmids pEG202 (*LexA*), pME2511 (*LexA-TEC1*), pME2512 (*LexA-tec1-201*) or pME2513 (*LexA-tec1-202*). Strains were grown to exponential phase before β-galactosidase specific activity was measured. Bars depict are the mean of three independent measurements. Given are relative values with the highest activity set to 100. The white part of the bars refers to activation of the reporter by LexA. The black-coloured part indicates reporter activation that can be attributed to the Tec1p moiety. The numbers above the bars indicate the extent of reporter activation over basal activation by LexA alone, with activation caused by wild-type Tec1p set to 1. Standard deviations were below 20%.

DISCUSSION

Differential regulation of Tec1p by the MAPKs Fus3p and Kss1p confers signaling specificity during yeast development.

Here, we investigated the regulation of yeast cellular development (mating and haploid invasive growth) by the MAPKs Fus3p and Kss1p in conjunction with the TEA/ATTS family transcription factor Tec1p. Whereas it was previously assumed that both MAPKs have specialized functions for yeast cellular development with Fus3p being specific for mating and Kss1p for invasive/filamentous growth, a growing number of publications indicate that either of the two MAPKs is actually involved in regulation of both processes. For instance, Fus3p and Kss1p have recently been demonstrated to be both activated by pheromone exposure (Sabbagh *et al.*, 2001). Moreover, analysis of the corresponding deletion mutants revealed that *KSS1* and *FUS3* are both important for invasive growth (Roberts and Fink, 1994). These and other findings are incompatible with the view that each of the MAPKs serves specifically a single purpose. A central question to answer is how specificity is imposed on the system and how the same MAP kinase can differentiate between pheromone or nutritional signals to initiate only the appropriate developmental program. Many studies dealing with MAP kinase signaling specificity in yeast have helped to identify at least four mechanisms contributing to specificity: (i) differences in signal duration, amplitude or frequency, (ii) scaffold proteins, (iii) activation of different transcription factors, and (iv) different specificities of the MAPKs toward substrates. At least one substrate, the cyclin-dependent kinase inhibitor Far1p, is much more efficiently phosphorylated by Fus3p than by Kss1p (Breitkreutz *et al.*, 2001; Peter *et al.*, 1993), but other substrates that are differentially regulated are likely to exist. Since Tec1p becomes active only during invasive growth, but plays no role for mating specific gene expression, it is a good candidate as a further specificity determinant that might be differentially regulated by Fus3p and/or Kss1p.

Several findings presented in this work support that Tec1p indeed plays a crucial role not only for execution of the invasive growth program, but also for the specificity aspect. We found that Tec1p is associated not only with Kss1p (as previously described), but also with Fus3p. Intriguingly, Tec1p interacts with both MAPKs through distinct domains. Whereas the N-terminal half of Tec1p ($Tec1^{M1\text{-}E280}p$) was necessary and sufficient for interaction with Fus3p, the C-terminal portion ($Tec1^{K281\text{-}Y486}p$) was necessary and sufficient for

interaction with Kss1p. By using an *in vitro* kinase assay, we could show that both MAPKs also differentially phosphorylate Tec1p. When isolated from naive cells, they equally conferred a weak phosphorylation of Tec1p. In contrast, Tec1p phosphorylation by Fus3p but not by Kss1p was highly stimulated when the MAPKs were isolated from pheromone-treated cells. This pattern correlates well with the observation that active Fus3p limits the extent of Kss1p activation (Sabbagh *et al.*, 2001).

What might be the biological significance of phosphorylation of Tec1p by Fus3p and Kss1p? In case of Kss1p, it is tempting to speculate that phosphorylation confers activation of Tec1p or allows assembly into transcription activation complexes to promote FRE- or TCS-mediated gene expression. In this context, it is interesting that the supposed activating function of Kss1p has so far not clearly been defined and is founded largely on genetic studies (Cook *et al.*, 1997; Madhani *et al.*, 1997), whereas the inhibitory function (retention of Ste12p by the unphosphorylated MAP kinase) is well known and biochemically proven (Bardwell *et al.*, 1998). Other proteins that have been shown to be substrates for Kss1p in kinase reactions are Ste12p and Dig1/2p (Breitkreutz *et al.*, 2001; Cook *et al.*, 1996; Tedford *et al.*, 1997), so it is possibly a combination of different phosphorylation events that contributes to mediate the activating function of Kss1p. Because *fus3Δ* strains are hyperinvasive, it is more likely that phosphorylation of Tec1p by Fus3p has an inhibitory effect on Tec1p. When we assayed Tec1p protein levels in naive or pheromone-treated cells, we found that steady state Tec1p levels were dramatically reduced under the latter condition, and this reduction was clearly dependent on Fus3p. On the basis of our new results, we can extend the present model of Tec1p regulation by Kss1p and Fus3p and insulation of the invasive growth pathway from the pheromone signaling pathway (Fig. 7). Nutrient signals like e. g. glucose depletion trigger invasive growth. They activate the MAPKs Fus3p and Kss1p at a low, but constitutive level. Both MAPKs interact with Tec1p and phosphorylate the protein on different residues. Based on our pulldown experiments, interaction and phosphorylation of Tec1p by Fus3p occurs on the N-terminal half of Tec1p ($Tec1^{M1\text{-}E280}p$). In contrast, interaction and phosphorylation by Kss1p occurs on the C-terminal half of Tec1p ($Tec1^{K281\text{-}Y486}p$). We suppose that phosphorylation of Tec1p by Fus3p and Kss1p has opposite effects. However, the activating function of Kss1p must get the upper hand of Fus3p, and the activity of Fus3p is possibly too weak to inhibit/destabilize Tec1p substantially. Tec1p in turn activates invasion specific gene expression.

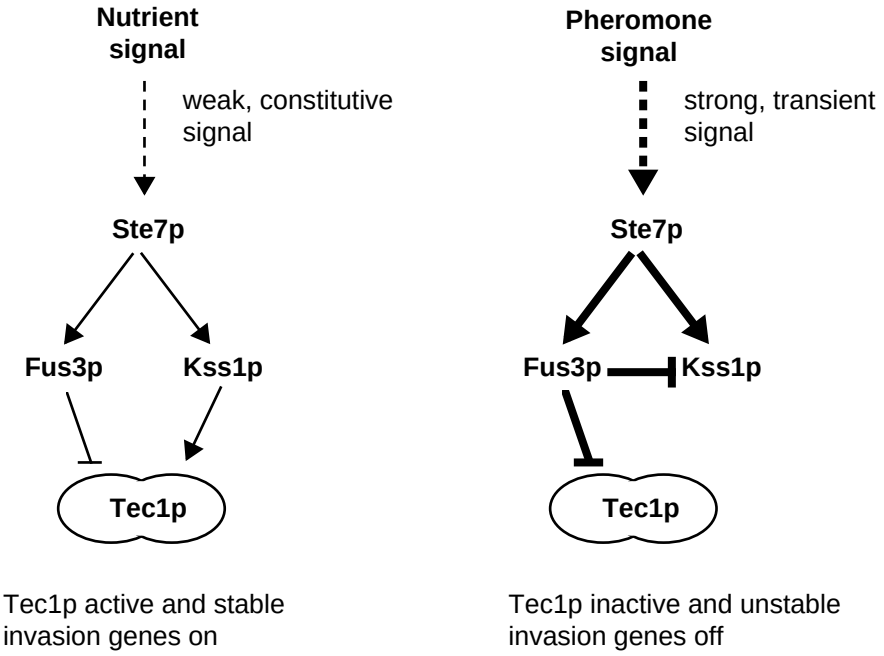

Fig. 7. Model for Tec1p as a specificity factor that prevents cross-activation of the filamentation/invasion pathway upon pheromone induction. Nutrient signals (left) permissive for invasive growth cause a weak but sustained activation of the MAPKs Fus3p and Kss1p. Both MAPKs phosphorylate Tec1p, Fus3p in the N-terminal half and Kss1p in the C-terminal half. Phosphorylation by Fus3p is thought to inhibit the protein or mediate its degradation, whereas phosphorylation by Kss1p possibly activates Tec1p. The weak activity of Fus3p under conditions permissive for invasive growth is too low to inhibit Tec1p substantially. By contrast, pheromone signals (right) cause a strong (indicated by bold arrows), but only transient activation of Fus3p and Kss1p. Active Fus3p in turn inhibits Tec1p and Kss1p, thereby preventing initiation of invasive growth specific gene expression. See text for details.

When the same MAPK cascade is activated by pheromone instead of nutrient signals, Fus3p and Kss1p are activated to a considerably higher extent (Sabbagh *et al.*, 2001). Enhanced phosphorylation of Tec1p by Fus3p leads to reduced Tec1p protein levels, possibly by activation of ubiquitin-dependent proteolysis of Tec1p. This is an exclusive function of Fus3p and not shared by Kss1p, because Tec1p remains stable in a *fus3Δ* strain upon pheromone induction. Tec1p is not the only substrate that is inhibited by active Fus3p. Recently, Fus3p has been shown to interfere with Kss1p activity in response to pheromone signals. Although both MAPKs are activated by pheromone, Fus3p limits the extent and duration of Kss1p activation. Possibly, both negative influences of Fus3p on Tec1p and Kss1p together efficiently prevent a signal spillover from the mating into the

invasive growth signaling pathway. Further support for our model comes from the finding that the rate of *Ty1* retrotransposition, which depends on Tec1p and Ste12p, is highly elevated in a *fus3Δ* mutant (Conte *et al.*, 1998; Conte and Curcio, 2000), possibly because Tec1p cannot be downregulated in the absence of *FUS3*.

Downregulation of Tec1p by Fus3p in response to pheromone could also have a second biological significance and contribute to G_1 cell cycle arrest. Fus3p mediates G_1 arrest by Far1p-dependent and -independent processes. Far1p-independent arrest functions operate by an unknown mechanism through repression of G_1/S cyclins *CLN1*, *CLN2* and *CLB5* (Cherkasova *et al.*, 1999; Elion *et al.*, 1991b). Remarkably, Tec1p has been shown to regulate transcription of *CLN1* positively (Madhani *et al.*, 1999). Therefore, it appears likely that downregulation of Tec1p protein upon pheromone stimulation contributes actively to cell cycle arrest by reducing *CLN1* expression.

Specific MAPK docking sites in Tec1p could mediate differential interaction with Fus3p and Kss1p.

As all MAP kinases phosphorylate very similar motifs (see above), and as many potential substrates contain this motif, further specificity determinants must be inherent in the substrates to recruit the correct kinases. In this context, specific docking domains play a crucial role. MAP kinase docking domains have two general purposes: they impart specificity, and they enhance efficiency of phosphorylation. A comparison of the many known and predicted MAP kinase docking sites in transcription factors from mammals (Sharrocks *et al.*, 2000) revealed no clear overall consensus, although certain general principles emerged. Generally, the docking sites are bipartite and contain a cluster of at least two basic residues (Lys, Arg, rarely His) seperated by a spacer of 2-6 residues from a hydrophobic-X-hydrophobic (or hydrophobic-X-hydrophobic-X-hydrophobic) sequence, where the hydrophobic residues are long-chain aliphatics (Leu, Ile, sometimes Val). Immediately C-terminal to the hydrophobic element and also in the spacer is a high propensity for the presence of Pro, Asn, and/or Gly. Recently, a MAP kinase docking motif was discovered in Ste7p, the MAPK kinase that activates Fus3p and Kss1p (Bardwell *et al.*, 2001), and this docking site comprising the N-terminal 25 amino acids of Ste7p was sufficient to target heterologous proteins to Kss1p. Moreover, putative MAP kinase docking sites were identified in 5 other proteins known to interact with Fus3p, including Gpa1p, Dig1p and Far1p (Metodiev *et al.*, 2002). When we scanned the primary sequence

of Tec1p, we found two putative MAP kinase docking sites meeting the criteria defined above: RKNEVPNISV (amino acids 98-107) and KKQISSHIQV (amino acids 182-191). Both sequences are present in the N-terminal part of Tec1p that interacted exclusively with Fus3p. Whether they actually mediate interaction with Fus3p remains to be determined. By contrast, we could not find a corresponding sequence in the C-terminal half of Tec1p that was necessary and sufficient for interaction with Kss1p. This indicates that classical bipartite sequence motifs are not a general prerequisite for binding of the proper MAP kinase, and distinct elements might work as well. In this regard, it is a future challenge to identify the sequence of Tec1p that makes contact to Kss1p.

Evidence for destabilization of Tec1p by phosphorylation on Thr273.

Although our hypothesis that phosphorylation of Tec1p by Fus3p triggers Tec1p for degradation was not directly proven, several clues point in this direction. As we could demonstrate, Fus3p phosphorylates Tec1p *in vitro* at an elevated level when activated by pheromone. Moreover, strains containing a functional *FUS3* gene displayed a 5- to 10-fold drop in Tec1p steady state protein levels. Concomitantly, these strains showed a large decrease in *FLO11* expression, one of the main target genes of Tec1p for induction of invasive growth. We previously observed that it is impossible to raise Tec1p protein levels in the cell beyond a 2- to 3-fold excess over normal levels, even when expressed from a high-copy number plasmid. Obviously, the protein is relatively instable and subject to rapid turnover. Two *tec1* alleles isolated earlier in a screen for hyperinvasive *tec1* mutants (Köhler *et al.*, 2002) encode proteins with single amino acid substitutions. Strikingly, both substitutions (T273M and P274S) occur at adjacent amino acid residues that together constitute a putative MAP kinase phosphorylation motif. This motif conforms to the minimal consensus sequence Ser/Thr-Pro (S/TP) which is phosphorylated by all MAP kinases throughout the eukaryotic kingdom (Sharrocks *et al.*, 2000). Our finding that the amino acid substitutions T273M and P274S render the protein completely insensitive to pheromone-stimulated degradation and diminish sensitivity to α-factor is a strong yet indirect evidence that phosphorylation occurs at T273 in the wild-type protein. Actually, both mutant proteins were readily phosphorylated by Fus3p, but we found 5 putative MAP kinase phosphorylation sites in the primary sequence of Tec1p and can therefore not exclude the possibility that these sites are phosphorylated as well. How could phosphorylation of Tec1p lead to degradation? Protein phosphorylation is often a

prerequisite for ubiquitin mediated proteolysis. Another component of the mating MAP kinase cascade, the MAPKK kinase Ste11p, was recently demonstrated to be degraded through a MAP kinase feedback and ubiquitin-dependent mechanism (Esch and Errede, 2002), and the same mechanism might account for Tec1p turnover after pheromone stimulation.

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Chapter 5

Conclusions and perspectives

Cdc42p assembles into different signaling complexes to control various aspects of growth and development.

In yeast, distinct developmental programs, mating and invasive/filamentous growth, are regulated by signal transduction through the same conserved MAPK cascade (Roberts and Fink, 1994). How identical proteins can participate in different pathways and manage to elicit only the proper developmental program depending on the stimulus is a field of intense research, yet it is only beginning to be understood. In this work, the role of two proteins for yeast cellular development was studied: the small GTP-binding protein Cdc42p and the transcription factor Tec1p. The highly conserved small GTPase Cdc42p is a key regulator of cell polarity and cytoskeletal organization in eukaryotic cells (Hall, 1998; Johnson, 1999). In yeast, Cdc42p is essential not only for vegetative growth, but also for mating and filamentous/invasive growth. Cdc42p has been implicated in a large number of cellular processes including polarization of the actin cytoskeleton (Li *et al.*, 1995; Rohatgi *et al.*, 1999; Zigmond *et al.*, 1998), septin ring assembly and cytokinesis (Cid *et al.*, 2001; Gladfelter *et al.*, 2002; Gladfelter *et al.*, 2001; Richman *et al.*, 2002), transcriptional activation of filamentation and mating specific genes (Mösch *et al.*, 1996; Moskow *et al.*, 2000; Simon *et al.*, 1995; Zhao *et al.*, 1995), and exocytosis and membrane fusion (Adamo *et al.*, 2001; Eitzen *et al.*, 2001; Müller *et al.*, 2001). How can Cdc42p manage to regulate so many diverse cellular processes? Multiple regulatory proteins and effectors of Cdc42p have been identified, and it is thought that Cdc42p, depending on its activation-state and subcellular localization, preferentially interacts with distinct effectors to promote the appropriate response. It is of particular interest to elucidate how Cdc42p interacts differentially with the multiple effectors, and which Cdc42p-effector interaction regulates a specific process. One way to assess the contribution of different effector pathways downstream of small GTPases has been the use of specific mutants that selectively disrupt the interaction with only a subset of their effectors. In case of yeast Cdc42p, a large number of mutant variants containing single amino acid substitutions have been analyzed with regard to interaction behaviour and phenotypic consequences (Davis *et*

al., 1998; Gladfelter *et al.*, 2001; Kozminski *et al.*, 2000; Moskow *et al.*, 2000; Richman and Johnson, 2000; Richman *et al.*, 1999). Characterization of these mutants has provided valuable insight into how Cdc42p functions. One region of Cdc42p called switch I or effector domain consisting of amino acids 26 to 50 turned out to mediate interactions with most known Cdc42p effectors. However, most of these mutants were either lethal or displayed severe morphological abnormalities. In the work presented here, a number of Cdc42p mutants were isolated and characterized that specifically block cellular development in response to nutritional signals (pseudohyphal/invasive growth) but do not affect essential functions required for general cell polarity and cell division. Mapping of the mutations on a structural model of yeast Cdc42p revealed that they were predominantly located on the surface of Cdc42p, suggesting that these sites are important for interaction with effector proteins. Indeed, subsequent two-hybrid analysis between our novel Cdc42p mutant proteins and a set of known Cdc42p effectors revealed that the amino acid substitutions differentially affected interaction patterns. Moreover, most of the amino acids substituted in our mutants reside outside of the switch I (effector) domain, suggesting that other domains of Cdc42p are important for interaction with effectors as well. In particular, two regions of Cdc42p were identified to be specifically critical for binding to the PAK-family kinase Skm1p or Gic1p and Gic2p, respectively (Fig. 1A). These effectors were as yet unknown to be required for pseudohyphal/invasive growth. Mutations in another region of Cdc42p nearly abolished interaction with the actin-associated protein Bni1p. However, these mutations were less specific, because the interaction with other effectors was also affected, although less dramatically. Binding to further effectors not tested here might also be impaired and be critical for filamentous growth. Large-scale biochemical approaches have led to the identification of a growing number of proteins that are associated with Cdc42p (Drees *et al.*, 2001; Ho *et al.*, 2002; Uetz *et al.*, 2000), and analysis of a series of Cdc42p effector loop mutants indicates that several as yet unknown effectors must exist (Gladfelter *et al.*, 2001). Considering the limited size (21 kDa) of Cdc42p, it is hardly imaginable how it can cope with the multitude of upstream regulators and downstream effectors to fulfill its various functions. Obviously, we have to change our view of Cdc42p as being just a simple ″on-off″ switch that interacts with a single target at a given time to elicit a particular response. Rather, Cdc42p could serve as a signal transduction switchboard that assembles into different signaling complexes in a temporally and spatially regulated manner (Fig. 1B). Analysis of complex formation of our novel Cdc42p

developmental mutants by means of tandem affinity purification (TAP) tagging (Puig *et al.*, 2001) in combination with mass spectrometry could help to elucidate which Cdc42p complexes are actually required for filamentous growth regulation.

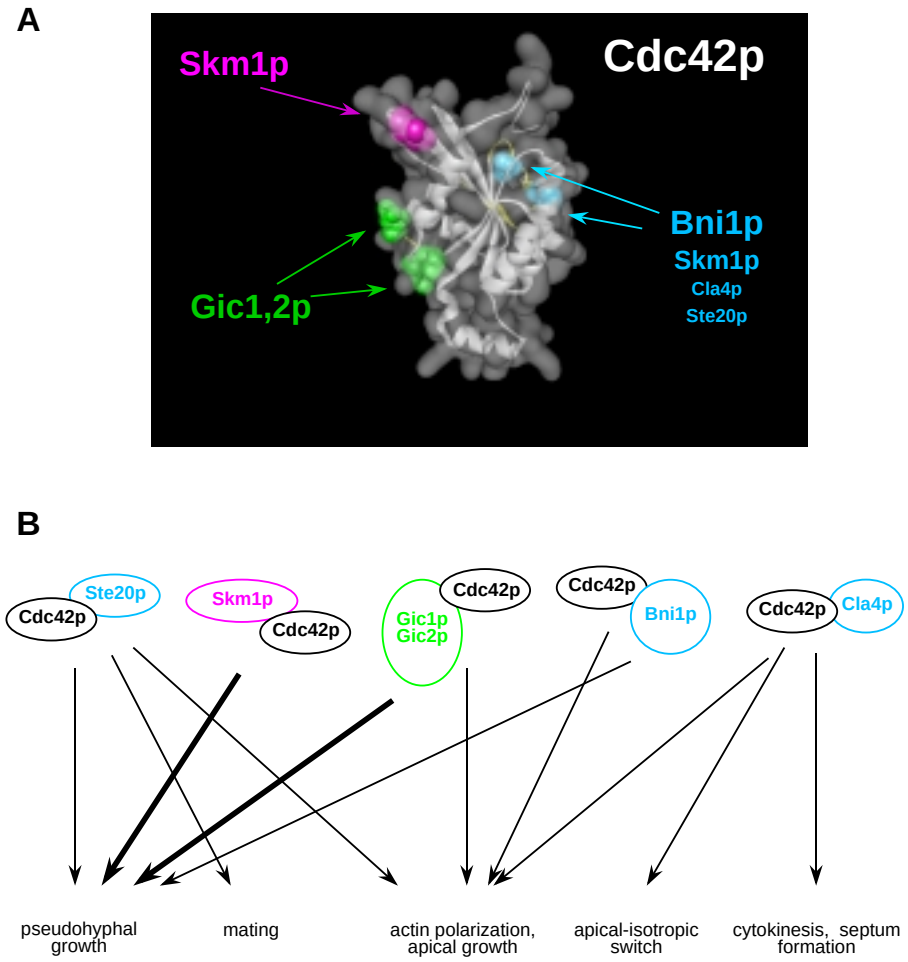

Fig. 1. Specific surface areas of Cdc42p mediate interaction with distinct effectors and assembly into various signaling complexes. (A) Three-dimensional structure model of *S. cerevisiae* Cdc42p obtained by homology modeling of the primary structure of *S. cerevisiae* Cdc42p using the Swiss-Model service (Guex and Peitsch, 1997) and the WebLab Viewer software (Molecular Simulations Inc., San Diego, Calif.). Amino acid residues important for interaction with specific effectors are marked in different colours. Substitution of residues depicted in blue concomitantly reduced interaction with a number of effectors, and the size of the letters reflects the relative degree of reduction for each Cdc42p-effector interaction. (B) Hypothetical model for Cdc42p acting as a platform for assembly of different signaling complexes with different cellular functions. Roles of Skm1p and Gic1,2p in pseudohyphal development (bold arrows) were previously unknown and concluded from reduced binding of the corresponding mutant Cdc42p variants with these effectors.

Tec1p is a dual function protein that regulates invasive growth specific gene expression and maintains signaling specificity.

The transcription factor Tec1p was previously regarded to act solely together with Ste12p to induce target gene expression by cooperative binding to FRE elements consisting of a Tec1p binding site (TCS) and an adjacent Ste12p binding site (PRE) (Madhani and Fink, 1997). Here, we could extend this view and provide evidence that Tec1p can also induce target gene expression and invasive growth in the absence of Ste12p by binding to single or combined TCS elements which are accumulated in the promoters of target genes (TCS control). This function of Tec1p had not been recognized so far because the *TEC1* gene itself is regulated on the transcriptional level by Ste12p, causing a 20-fold reduction of *TEC1* transcription in cells lacking *STE12* (Köhler *et al.*, 2002; Oehlen and Cross, 1998). Our model of TCS control by Tec1p is largely based on studies of target gene expression and the use of synthetic *TCS-lacZ* reporter constructs. Whereas the combinatorial control model has already been proven biochemically by gel retardation and Deoxyribonuclease I footprint analysis (Madhani and Fink, 1997), this remains to be done with purified Tec1p and synthetic TCS oligos to further corroborate our model. Although we found that Tec1p does not require the assistance of Ste12p to drive gene expression via TCS elements, it is unlikely to do this alone, because a LexA-Tec1p chimera was not capable of activating a *LexA(op)-lacZ* reporter, indicating that Tec1p has no transcriptional activation domain. A potential binding partner of Tec1p that could provide this function has so far not been identified, although a number of novel Tec1p binding partners were identified in a two hybrid screen (our unpublished results). Our work gives some initial information about the organization of Tec1p into distinct functional domains. In a genetic screen designed to separate different functions of Tec1p, we isolated a number of interesting *tec1* alleles. As a result of this screen, the N-terminal part of Tec1p including the conserved TEA/ATTS DNA-binding domain turned out to be sufficient to mediate gene activation via FREs together with Ste12p, whereas TCS control and full induction of invasive growth additionally required the C-terminus of Tec1p.

So far, only two processes were known to be regulated by Tec1p: invasive/filamentous growth and *Ty1* transposition. Here, we found evidence that Tec1p additionally serves as a specificity factor that prevents cross-activation of the invasive growth pathway by pheromone signals. The mechanisms by which Fus3p and Kss1p regulate *TEC1* expression and Tec1p protein activity are summarized in Fig. 2A. Specific nutritional signals trigger

haploid invasive growth. They activate the MAPKs Fus3p and Kss1p at a low, but constitutive level. Ste12p is released from complexes with Fus3p and/or Kss1p and activates expression of Tec1p. In addition to this activation by relief of repression, Ste12p might be activated by phosphorylation through Kss1p. Tec1p in turn activates invasion specific gene expression (e.g. *FLO11*), either as a heterodimer together with Ste12p by binding to FREs or without Ste12p via TCS elements. In contrast to the invasive growth program, mating specific genes (e. g. *FUS1*) require a higher level of active Ste12p and are therefore not induced. Likewise, activation of the CKI Far1p to mediate G_1 cell cycle arrest requires a higher level of active Fus3p. Activation of the same MAPK cascade by pheromone instead of nutrient signals causes stronger activation of Fus3p and Kss1p (Sabbagh *et al.*, 2001). However, this strong activation is only transient. Fus3p and/or Kss1p itself participate in downregulation of the mating signal by feedback phosphorylation of its activator Ste7p (Zhou *et al.*, 1993) and Ste11p (Breitkreutz *et al.*, 2001; Esch and Errede, 2002). These negative feedback mechanisms might be important for recovery from pheromone induced cell cycle arrest. High levels of active Fus3p now are sufficient to activate Far1p and induce cell cycle arrest. Moreover, the elevated amount of active Ste12p exceeds the threshold level for activation of mating genes. Concomitantly, transcription of the *TEC1* gene is induced by mating factor (Oehlen and Cross, 1998; Roberts *et al.*, 2000). Thus, one could expect invasive growth specific genes to be induced as well. However, this is not the case because Fus3p downregulates Tec1p protein levels. Evidence for this model comes from our finding that Tec1p interacts not only with the MAP kinase Kss1p as previously described (Ho *et al.*, 2002), but also with the mating-specific MAP kinase Fus3p. This interaction seems to be physiologically relevant because both MAPKs were able to phosphorylate purified Tec1p in an *in vitro* kinase assay. Whereas both MAPKs phosphorylated Tec1p at a low level when isolated from naive cells, only Fus3p caused an elevated phosphorylation of Tec1p after pheromone treatment. The question why phosphorylation of Tec1p by Kss1p was not stimulated likewise by pheromone might be answered with an inhibitory action of Fus3p on Kss1p, because activated Fus3p limits the magnitude and duration of Kss1p phosphorylation (Sabbagh *et al.*, 2001). Interestingly, Fus3p and Kss1p interact with Tec1p through distinct domains, suggesting that different target sites are phosphorylated and that these phosphorylation events might serve different purposes.

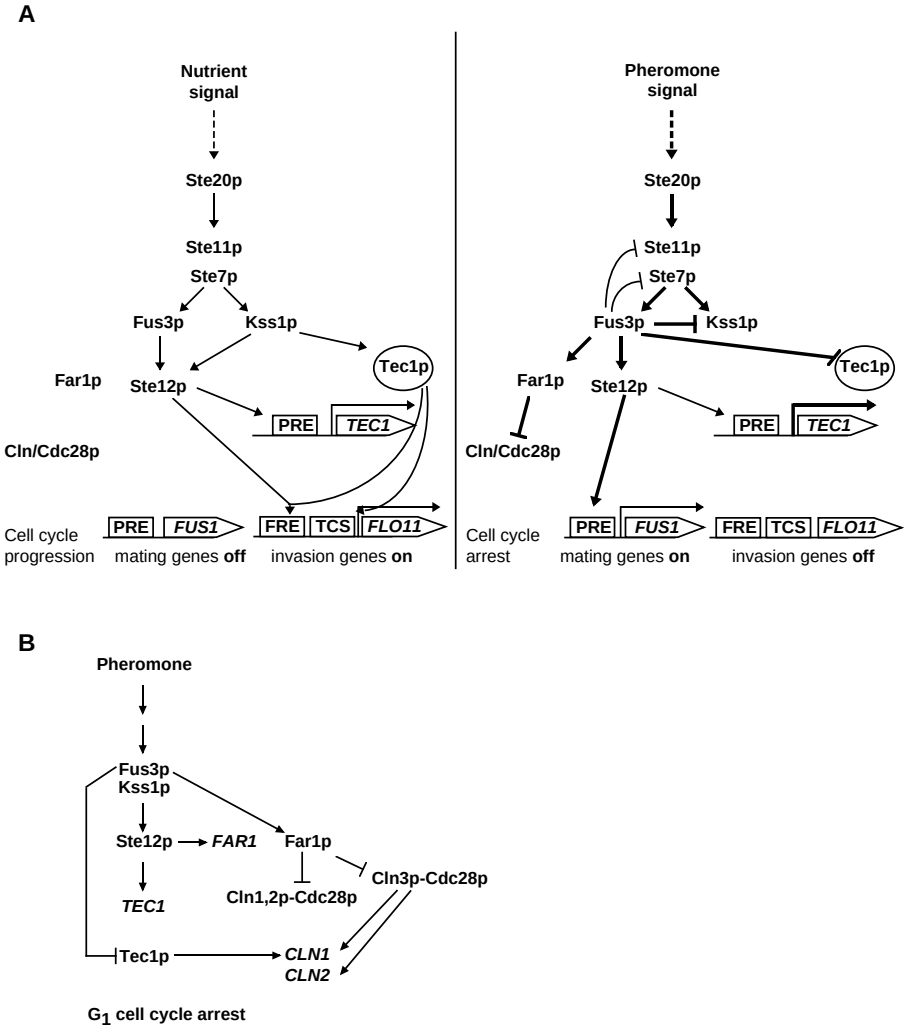

Fig. 2. Proposed functions of Tec1p during yeast developement. (A) Tec1p might serve as specificity factor that prevents cross-activation of the filamentation/invasion pathway upon pheromone induction. Nutrient signals (left) permissive for invasive growth cause a weak but sustained activation of the MAPKs Fus3p and Kss1p. This leads to a moderate activation of Ste12p, which is sufficient to drive Tec1p expression and initiate the invasive growth program. However, the activation of Fus3p and Ste12p is too weak to mediate cell cycle arrest (via Far1p and Cln/Cdc28p complexes) or mating specific gene expression. By contrast, pheromone signals (right) cause a strong (indicated by bold arrows), but only transient activation of Fus3p and Kss1p. Active Fus3p in turn inhibits Tec1p and Kss1p, thereby preventing initiation of invasive growth specific gene expression. High levels of active Ste12p are sufficient to mediate cell cycle arrest and activate mating specific genes. Fus3p attenuates pheromone signals by feedback phosphorylation of upstream kinases. (B) Model for regulation of G1 arrest by Far1p and Tec1p. Pheromone signals activate the MAPKs Fus3p and Kss1p, which in turn activate the transcription factor Ste12p. Ste12p induces transcription of the *FAR1* and *TEC1* genes. Far1p becomes activated by Fus3p and inhibits the activity of G_1 cyclin (Cln)/Cdc28p complexes. Inhibition of Cln3p/Cdc28p reduces transcription of the *CLN1* and *CLN2* genes. Inhibition of Tec1p by Fus3p reinforces the suppression of *CLN1* transcription. Reduced G1 cyclin activities finally promote cell cycle arrest to prepare the cells for mating. See text for details.

Presumably, phosphorylation of Tec1p by Kss1p activates the protein to induce target gene expression, whereas phosphorylation by Fus3p has a negative influence. Indeed, Tec1p steady state levels drop by a factor of 10 in cells treated with α-factor, and this drop was dependent on *FUS3*. By contrast, Tec1p levels were insensitive to pheromone in mutant strains expressing Tec1p variants that contain single amino acid substitutions (T273M and P274S, respectively) in a putative MAP kinase phosphorylation site. This finding suggests that phosphorylation of Tec1p by activated Fus3p upon a pheromone stimulus leads to rapid turnover of Tec1p, possibly by a ubiquitin-dependent process. As a consequence, activation of FRE- and TCS-dependent target genes is prevented and signaling specificity is guaranteed.

In addition to control of Tec1p turnover, the protein itself might actively contribute to the mating process (Fig. 2B). A prerequisite for mating of haploid yeast cells is arrest of growth in G_1 phase before passage of START, the commitment point of the cell cycle, to synchronize growth of both mating partners. This is achieved by a *FUS3* dependent inhibition of G_1 cyclins which occurs at two different levels: repression of transcription of *CLN1* and *CLN2* (Elion *et al.*, 1991; Wittenberg *et al.*, 1990) and direct inhibition of the three different Clnp/Cdc28p kinases by Far1p (Jeoung *et al.*, 1998; Peter *et al.*, 1993; Peter and Herskowitz, 1994; Tyers and Futcher, 1993). *CLN1* and *CLN2* transcription has been shown to be stimulated by Cln3p (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991), and posttranscriptional inhibition of Cln3p by Fus3p accounts for part of *CLN1* and *CLN2* repression. Because Tec1p has been shown to regulate transcription of *CLN1* positively (Madhani *et al.*, 1999), a downregulation of Tec1p levels after pheromone stimulation could contribute to reduced Cln1p levels, thereby promoting G_1 arrest.

In summary, our analysis of the Tec1p protein revealed several new findings about Tec1p, the mechanism how it regulates target gene expression and invasive growth, and the mechanism how it prevents crosstalk from the mating pathway to the filamentation/invasion pathway.

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