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Studies on active Ras proteins localization and evidences for nuclear active Ras2 involvement in invasive growth in *Saccharomyces cerevisiae*

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ABSTRACT

In the yeast *Saccharomyces cerevisiae,* the Ras proteins are part of the cAMP/PKA signalling pathway, which plays a fundamental role in the control of many cellular processes including cells proliferation, stress resistance, metabolism, and growth. They belong to the super-family of the small GTPases that act as molecular switches by cycling between an inactive GDP-bound form and an active GTP-bound form. This process is controlled by two classes of regulatory proteins: the GEFs promote the activation of Ras by catalyzing the GDP-GTP exchange, whereas the GAPs turn off the Ras proteins by stimulating the hydrolysis of GTP to GDP.

In the first section of this thesis, we investigated the localization of active Ras proteins in wild type cells and in mutants in several components of the cAMP/PKA pathway to understand how the proteins involved in this pathway influence the localization of active Ras. To this aim we used a probe in which the eGFP (enhanced green fluorescent protein) is fused to a trimeric Ras binding domain (RBD3) of the human Ras effector, c-Raf1. This RBD directly binds to the active Ras with a much higher affinity than the inactive Ras. We also investigated the influence of PKA activity on active Ras localization analyzing different mutants with either high or low/absent PKA activity. The cells of the different strains expressing the eGFP-RBD3 probe growing on glucose medium were observed under the microscope. In wild type cells, Ras-GTP was mainly localized at the plasma membrane and surprisingly in the nucleus. In *cyr1Δ* and *gpr1Δ* cells, the probe showed a similar localization as in wild type cells. In *gpa2Δ*, *hxk2Δ* and *hxk1Δhxk2Δ* cells, the fluorescence accumulated in internal membranes and mitochondria. However, in the *hxk1Δhxk2Δ* cells transformed with the centromeric plasmid YCp*HXK2* expressing Hxk2, the eGFP-RBD3 probe was mainly localized at the plasma membrane and in the nucleus. These results suggest that Gpa2 and Hxk2 play a role in the localization of active Ras. We also observed that the localization of active Ras is dependent on PKA activity. Indeed, in the *bcy1Δ* mutant, showing high PKA activity, there was a clear relocalization of active Ras to the cytoplasm and to the nucleus, while no active Ras was localized at the plasma membrane anymore. In a strain with either reduced PKA activity, the *tpk1*^{w1} *tpk2Δ tpk3Δ* strain or

absent PKA activity, the *tpk1Δ tpk2Δ tpk3Δ yak1Δ* strain, active Ras was mainly localized at the plasma membrane.
In the second section of this thesis, we investigated the role played by active Ras in the nucleus. To this aim, a fusion was made between the Ras2 protein and the Nuclear Export Signal (NES) from the HIV virus (HIV virus Rev protein NES) (Henderson *et al*., 2000), generating the NES-RAS2 strain. Our results showed that the exclusion of Ras2 protein from the nucleus did not cause a growth defect neither on fermentable nor non fermentable carbon sources and did not influence the PKA related phenotypes analyzed in our work. Cells expressing the fusion protein were only defective for the invasive growth, suggesting that nuclear active Ras2 is involved in this cellular process. These results were confirmed using also the Tlys86 strain, that is commonly used to test this phenotype. We also demonstrated that the nuclear localization of Cdc25, the main GEF of Ras proteins, is required for invasive growth and that PKA activity controls invasive growth influencing the localization of active Ras.

Data in literature (Cazzaniga *et al*., 2008; Pescini *et al*., 2012) show the presence in silico of cAMP levels oscillations. In the last section of this thesis, we tested two different FRET sensors, previously used in mammalian cells, to monitor the cAMP levels (CFP-Epac1-YFP probe) and PKA activity in single cells *in vivo* (AKAR3 probe). We inserted the sequences coding for the CFP-Epac1-YFP sensor and for the AKAR3 sensor in a multicopy yeast expression vector and the sensors were expressed under the control of the TPI promoter in several yeast strains. We used a two-photon confocal microscope system to measure the FRET efficiency. Our preliminary results showed that in a wild type strain expressing either the Epac sensor or the AKAR3 sensor there was respectively an increase of cAMP level and PKA activity in a single yeast cell after glucose addition to glucose-starved cells.

AIM OF THE THESIS

The Ras proteins, both in yeast and in mammals, play an essential role in controlling the activity of many signal transduction pathways, which regulate cell proliferation, growth and differentiation. Their different subcellular locations could regulate distinct functions.

In this work we studied the spatio-temporal localization of active Ras proteins in wild type cells and in mutants in different components of the cAMP/PKA pathway to understand how the proteins involved in this pathway influence the localization of active Ras. To this aim we used a probe consisting of a eGFP fusion with a trimeric Ras Binding Domain of c-Raf1 (eGFP-RBD3), which binds active Ras with a much higher affinity than inactive Ras. Our results showed that the probe accumulated mainly at the plasma membrane and in the nucleus in wild type cells growing on glucose medium and that Gpa2 and Hxk2 play a role in the localization of active Ras. Since data in literature show that PKA regulates the localization of certain cAMP/PKA signaling pathway components, like the *PDE2*-encoded high-affinity cAMP phosphodiesterase (Hu *et al*., 2010), Cdc25 (Belotti *et al*., 2011) and the Ras2 protein (Dong and Bai, 2011), we also investigated the influence of PKA activity on active Ras localization.

The nuclear localization was unexpected and could be indicative of a specific nuclear function of active Ras. Consequently, in this thesis we have also investigated the role played by active Ras in the nuclear compartment. To this aim we generated a strain where Ras2 was fused to the Nuclear Export Signal (NES) from the HIV virus (HIV virus Rev protein NES), in order to completely exclude Ras2 from the nucleus. Our results showed that nuclear active Ras2 is involved in invasive growth.

INTRODUCTION

1.1 The yeast *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* is a unicellular fungus. It is commonly known as baker's, brewer's or budding yeast. It has been extensively used for many years in food industry, i.e. for the production of beer, wine and bread, and in pharmaceutical industry, i.e. for the production of recombinant proteins like human interferon, small hepatitis B surface proteins for use in hepatitis B vaccines and insulin. Thanks to its various properties, it is one of the most widely studied eukaryotic model organisms in molecular biology and in genetic research. It is non pathogenic and it has been classified as a GRAS organism (Generally Regarded As Safe).

In 1996, the genome of *S. cerevisiae* was the first eukaryotic genome to be completely sequenced. The sequence of 12068 kilobases is organized in 16 linear chromosomes and defines 5885 potential protein-encoding genes, approximately 140 genes specifying ribosomal RNA, 40 genes for small nuclear RNA molecules and 275 transfer RNA genes (Goffeau *et al*., 1996). It grows rapidly and genetic modifications are relatively easy to perform.

Unlike most other microorganisms, the budding yeast *S. cerevisiae* can stably exist in either haploid or diploid state. In both conditions, proliferation occurs by vegetative cell division or budding, in which a daughter cell is initiated as an outgrowth from the mother cell, followed by nuclear division, cell-wall formation, and finally cell separation (Sherman, 1998). After separation, the cells start growing until they reach a critical mass again. The gap between two generations can vary in size depending on the genetic background of the strain and the growth medium. Each mother cell usually forms no more than 20-30 buds, and its age can be determined by the number of bud scars left on the cell wall.

The haploid cells can express one of two different mating types, *a* or *α*, determined by a mating-type locus MAT and can conjugate generating a diploid cell with one nucleus, the zygote. The whole process is arranged by pheromones, specific signaling molecules *a* and *α* which are secreted respectively by MAT*a* and MAT*α* cells (Herskowitz, 1988). Diploid *a/α* cells can undergo sexual differentiation or meiosis under conditions of nutritional stress, notably carbon and nitrogen sources starvation. The meiotic divisions generate four haploid spores, specialized cells highly

resistant to environmental stress, that can be isolated and individually analyzed (Yamamoto *et al.*,1997). The four products of a single meiosis are held together in a sac called ascus. When conditions improve, the spores germinate and become haploid vegetative cells (fig.1). In yeast cells, optimal growth occurs at 30°C in a rich medium. They can grow on solid or in liquid media under aerobic conditions (oxidative metabolism) or anaerobic conditions (fermentative metabolism). Glucose and fructose are preferred as carbon sources but *S.cerevisiae* is also able to grow on other carbon sources (Schüller, 2003), through a process called cellular respiration. In the presence of oxygen the organic molecules will be oxidized to carbon dioxide and water. Some of the energy produced by this oxidation is stored in the chemical bonds of 38 adenosine triphosphates (ATP). Without oxygen, yeast cells undergo fermentation which produces 2 ATP molecules during the breakdown of a fermentable sugar, like glucose, into ethanol and carbon dioxide. Even though respiration has higher energy yields, *S. cerevisiae* catabolyzes glucose mainly by a fermentative process, even under aerobic conditions. This effect was presented as the Crabtree effect. When the amount of glucose is limited, a metabolic switch will occur to allow the cell to use ethanol as alternative energy source.

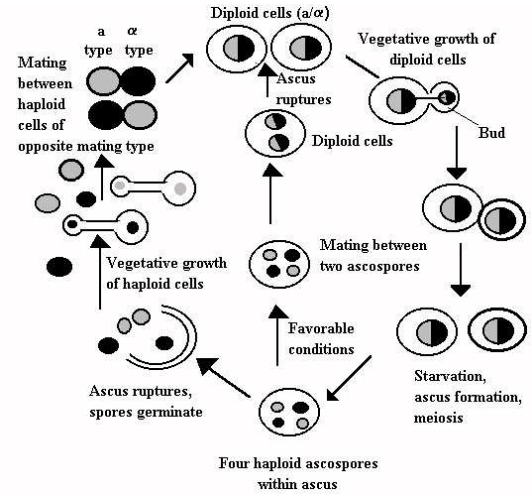


Figure 1. Life cycle of *Saccharomyces cerevisiae*

1.1.1 *Saccharomyces cerevisiae* cell cycle

The cell cycle of *S. cerevisiae* is divided into four phases: G1 phase, during which the cell grows and prepares for cell cycle entry; S phase, during which DNA synthesis takes place; G2 phase, during which cells prepare for M phase; and M phase, in which chromosomes segregate and cells divide (fig.2). Another phase named G0 can also exist, representing a quiescent state with a seriously impaired metabolic activity that cell undergoes in adverse environmental conditions such as nutrients starvation. In *S. cerevisiae* regulation of cell cycle progression is achieved predominantly during a narrow interval in the late G1 phase known as START. At START, yeast cells integrate many environmental and intracellular signals and determine if to proceed in the cell cycle or if to block it by entering in G0 quiescent phase or by conjugating (Pringle and Hartwell, 1981). START point passing is an irreversible process: once cell cycle is began, it has to be concluded until the next G1 phase.

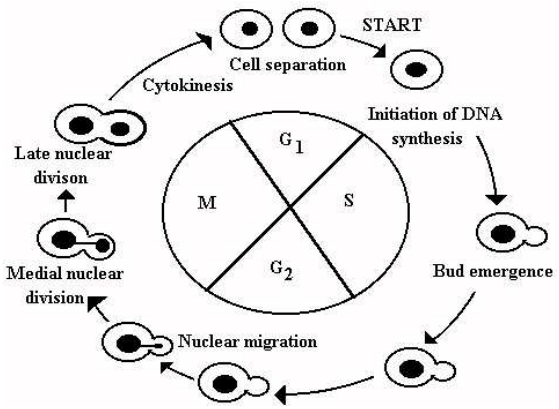


Figure 2. Cell cycle of *Saccharomyces cerevisiae*

The cell cycle is controlled at the molecular level by cyclin-dependent kinases (Cdks). The Cdks are a family of serine/threonine kinases whose action depends on association with their activating subunits, cyclins. Cyclins abundance is regulated by protein synthesis and degradation; the activity of Cdks is therefore regulated to a large degree by the presence of different cyclins (Bloom and Cross, 2007). In the budding yeast *S. cerevisiae*, a single

Cdk, Cdc28, associates with multiple cyclins to regulate the cell cycle. In *S. cerevisiae* there are nine different cyclins which can associate with Cdc28 and thereby can confer stage-specific functions: the G1-phase cyclins (Cln1, Cln2 and Cln3) promote bud emergence, spindle pole body duplication and activation of the B-type cyclins and are necessary for START; the S-phase cyclins (Clb5 and Clb6) advance DNA replication; and the M-phase cyclins (Clb1, Clb2, Clb3 and Clb4) promote spindle formation and the initiation of mitosis. Mitotic cyclins inhibit mitotic exit and cell division (fig.3).
Crucial mechanisms that contribute to cyclins specificity include the differential regulation of G1 and B-type cyclins at the level of transcription, the degradation by ubiquitin-mediated proteolysis, the association of cyclin-Cdk complexes with different Cdk inhibitors, the localization of cyclins and the inhibitory phosphorylation of Cdk.

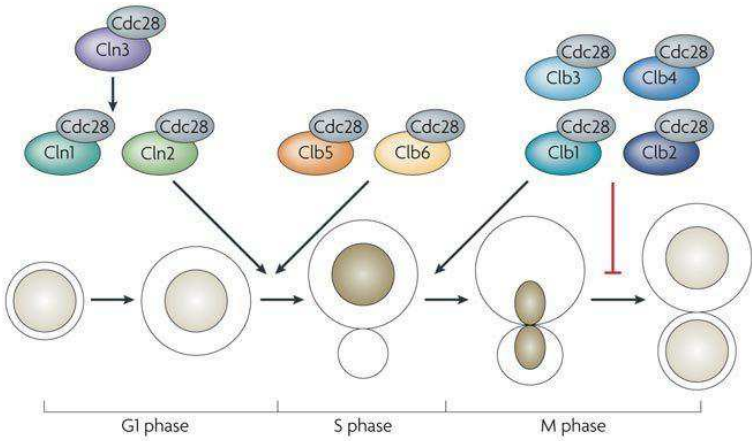


Figure 3. Cyclins in the budding yeast cell cycle (Bloom and Cross, 2007).

To maintain a constant size during cell proliferation cells need to grow and double in mass during each round of the chromosome cycle (Dirick *et al*., 1995). The coordination of mass accumulation with cell cycle progression relies on a size mechanism, so that DNA replication and/or cell division start only when cells have reached a critical cell size (Wells, 2002). The critical cell size required for the G1/S transition in budding yeast is usually indicated as Ps (protein content per cell at the onset of DNA replication).

Nutrients and growth rate modulate Ps, that is smaller in slow-growing cells than in fast-growing cells (Wells 2002; Neufeld and Edgar 1998).
A key role in the Ps regulation is played by the cyclin Cln3 which binds and activates the Cdk Cdc28. The Cln3-Cdc28 complex activates the transcription factors SBF and MBF. SBF and MBF control the expression of a large number of genes including those encoding Cln1, Cln2, Clb5 and Clb6 that regulate many events required to enter in S phase. Cln3 is much less abundant than the other cyclins, its level is regulated by nutrients and its concentration remains constant during G1 phase. Experimental data suggest that Cln3p amount increase promotes S phase entry, considering that Cln3p level alteration affects Ps (Tyers *et al*., 1993). In *S. cerevisiae* the cyclin-Cdk activity is negatively regulated by Cdk inhibitors (Ckis), like Sic1 and Far1.

1.2 The cAMP/PKA pathway

In *S. cerevisiae* the cAMP/PKA signalling pathway plays a central role in the control of metabolism, stress resistance and proliferation, in particular in connection with the available carbon source (Thevelein *et al.,* 1999). The addition of glucose or other rapidly fermentable sugars to derepressed cells (carbon starved or growing on a non-fermentable carbon source) triggers a remarkable variety of regulatory phenomena, including many rapid changes at the post-translational and transcriptional level. Both addition of glucose to glucose-deprived (derepressed) cells and intracellular acidification trigger the activation of the cAMP/PKA pathway (Colombo *et al*.,1998; Colombo *et al*.,2004). The cAMP/PKA pathway has also been implicated in aging, thermotolerance, bud site selection, actin repolarization, glycogen accumulation and sporulation; it may also regulate pseudoyphal differentiation in response to nutrient limitation (Santangelo, 2006). The activation of the cAMP/PKA signalling pathway causes a rapid, but transient increase in the intracellular cAMP level. The cyclic AMP synthesis from ATP is catalyzed by adenylate cyclase, encoded by the *CYR1/CDC35* gene, which activity is controlled by two different systems: the G-protein-coupled receptor system or GPCR, acting through the G-protein Gpa2 (Colombo *et al*.,1998), and the Ras system (Toda et al., 1985). Ras proteins are members

of the small GTPase superfamily, which are active in the GTP-bound form and inactive in the GDP-bound form. In *S. cerevisiae* there are two different Ras proteins, Ras1 and Ras2, encoded by the *RAS1* and the *RAS2* gene. The Ras activation state is positively controlled by the guanine nucleotide exchange factor (GEFs) encoded by *CDC25* and *SDC25*, which stimulate the GDP-GTP exchange on Ras (Broek *et al*., 1987; Camonis and Jacquet 1988; Jones *et al*., 1991; Camus *et al*., 1994), and negatively regulated by the two GTPase-activating proteins (GAPs) encoded by *IRA1* and *IRA2*, which promote intrinsic Ras GTPase activity (Tanaka *et al*., 1989, 1990 a, 1990 b, 1991).

cAMP is one of the most important second messengers in yeast and other eukaryotic cells, activating the cAMP-dependent protein kinase A (PKA) by binding to its regulatory subunits (encoded by *BCY1*), thereby releasing and activating the catalytic protein kinase subunits (encoded by *TPK1*, *TPK2* and *TPK3*) (Toda *et al*., 1987).

PKA activity is counteracted by phosphodiesterase activity; actually cAMP is hydrolyzed by the low-affinity and high-affinity phosphodiesterases, respectively encoded by *PDE1* and *PDE2*. In addition, a very potent system down-regulating cAMP levels in yeast is feedback inhibition of cAMP synthesis by PKA (fig.4).

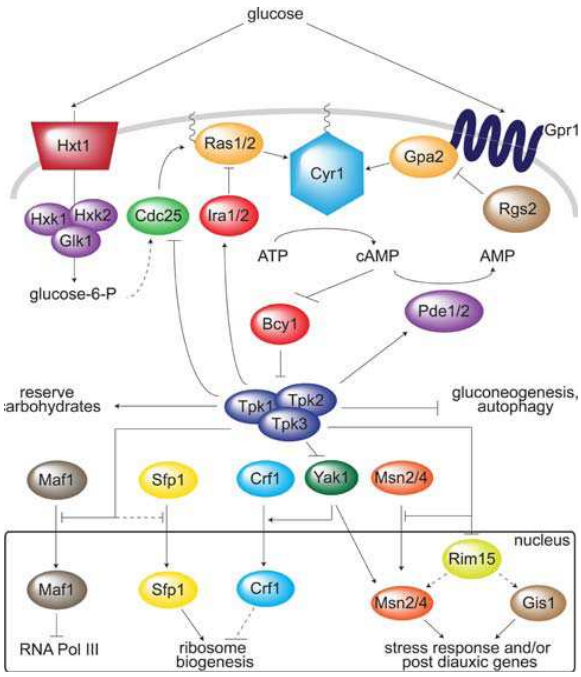


Figure 4. The CAMP/PKA pathway in *S. cerevisiae* (Smets *et al*., 2010).

1.2.1 Ras proteins

Yeast monomeric Ras proteins, Ras1 and Ras2, belong to the superfamily of small GTP-binding proteins that regulate cell growth and differentiation and cycle between an inactive GDP-bound form and an active GTP-bound form in response to a nutrient signal. In the active form they are able to activate its downstream effectors. The GEFs, Cdc25 and Sdc25, promote the GDP-GTP exchange (Martegani *et al*., 1986; Boy-Marcotte *et al*., 1996), while the GAPs, Ira1 and Ira2, promote the hydrolysis of GTP to GDP stimulating Ras intrinsic GTPase activity and their inactivation (Tanaka *et al*., 1990 a and b). The *RAS1* and *RAS2* genes (located respectively on chromosome XV and XIV) encode for a 34 kDa and 35 kDa polypeptide of respectively 309 and 322 amino acids. These proteins are about 70% homologous to mammalian Ras, distributed over the first 180 amino acids at the N-terminal of both of them (Temeles *et al.*, 1984). In both *Saccharomyces cerevisiae* and mammals, Ras proteins play an important functional role in regulating cell

division and growth (Barbacid, 1987). A loss or mutation in either *RAS1* or *RAS2* has no effect on growth in glucose, whereas a loss of function of both genes is lethal (Kataoka *et al*., 1984). Ras proteins are differentially expressed, *RAS1* being repressed during growth on non-fermentable carbon sources (Breviario *et al*., 1986). Overexpression of Ras1 completely suppresses the failure of *ras2*Δ cells to grow on this medium (Tatchell *et al.,* 1985).Ras proteins are essential regulatory elements of adenylate cyclase activity.

Ras proteins are synthesized as cytosolic precursors and then they undergo a series of post-translational lipid modifications essential for their binding to membranes and regulators and for their activation of adenylate cyclase. These modifications are initiated by a 'CAAX' motif present at the C-terminal of Ras proteins (C is cysteine, A is an aliphatic amino acid, and X is any amino acid) (Chiu *et al.*, 2002). The modifications include farnesylation of the CAAX-box Cys, proteolytic removal of the -AAX residues, carboxy methylation, and palmitoylation of a second cysteine adjacent to the CAAX box (Casey *et al*., 1989;Deschenes *et al*., 1989; Hancock *et al*., 1990; Stimmel *et al*., 1990). The first step in the modification pathway, farnesylation, has been shown to be sufficient to target Ras to the endoplasmic reticulum (ER) and Golgi membranes, where they encounter processing enzymes (Chiu *et al.*, 2002) like the Ras-converting enzyme Rce1, an endoprotease, which removes the 'AAX' amino acids. At this point, the new C-terminal becomes a substrate for a specific isoprenylcysteine carboxylmethyltransferase (Icmt), in *S. cerevisiae* Ste14, that methyl esterifies the α- carboxyl group of the isoprenylcysteine. Finally, there is the translocation of Ras from the ER to the plasma membrane. Palmitoylation serves as the second signal for mammalian H-ras, N-ras, and the yeast Ras proteins, whereas a stretch of basic residues (polybasic) provides the signal for K-ras-4B (Choy et al., 1999; Hancock *et al*., 1990). Hancock and colleagues have demonstrated that palmitoylated H-ras protein localizes to the plasma membrane via the classical secretory pathway. In yeast, palmitoylation is required for the plasma membrane localization of Ras1p and Ras2p (Bhattacharya *et al.*, 1995). Yeast Ras2 is normally modified by a thioester linkage of farnesyl to cysteine 319, a thioester linkage of palmitate

to cysteine 318, proteolytic removal of the carboxyl-terminal three amino acids, and metyl esterification of the revealed carboxyl terminal cysteine 319 (Bhattacharya *et al.*, 1995). One mutation, C318S, prevented palmitoylation of the protein without affecting farnesylation, proteolytic cleavage, or methyl esterification. A second mutation, C319S, blocked farnesylation of the protein and, since farnesylation is required for all subsequent modifications, precluded palmitoylation, cleavage, and methyl esterification as well (Hancock *et al*., 1989). Farnesylation enables Ras to recruit its effector molecule to the membrane (Bhattacharya *et al.*, 1995) and it is also a prerequisite for stimulation of the nucleotide exchange by full-length Cdc25 and Sdc25. Farnesylation is also required for proper plasma membrane localization of Ras, indeed blocking addition of palmitate via the single mutation C318S causes mislocalization of the mutated Ras2 protein to the cytoplasm (Jiang *et al*., 1998). Palmtoylation, in contrast to farnesylation, seems not to be essential for normal growth (Bhattacharya *et al.*, 1995). Blocking both modifications with the double point mutation C318S C319S results in a non functional molecule (Dong et al., 2003). Plasma membrane localization of Ras2 is unaffected by disruption of the classical secretory pathway, suggesting the existence of an alternative or non classical pathway for Ras translocation from ER to plasma membrane (Dong *et al*., 2003; Zhao *et al*., 2002).

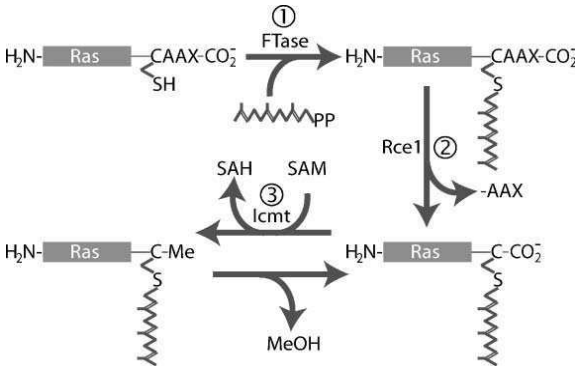


Figure 5. Post-translational modifications of Ras proteins (Wright *et al*., 2006).

1.2.2 GEFs and GAPs

The level of activated Ras is controlled by two types of regulatory proteins. First, the guanine exchange factors (GEF), Cdc25 and Sdc25, promote the active GTP-bound state of Ras. On the other hand, the Ras proteins have intrinsic GTPase activity, thereby inhibiting their own activity. Additionally, this GTPase activity is greatly stimulated by two regulatory proteins, Ira1 and Ira2.

1.2.2.1 Guanine nucleotide exchange factors: Cdc25 and Sdc25

The dissociation of GDP from the GDP-bound form is extremely slow and therefore stimulated by a GEF. The *S. cerevisiae CDC25* gene is an essential gene and it was originally isolated as a suppressor for the growth defect of a temperature-sensitive cell cycle deficient *cdc25* mutation (Martegani *et al*., 1984; Martegani *et al*., 1986). Mutant *cdc25ts* cells have reduced intracellular level of cAMP (Martegani *et al*., 1986; Camonis *et al*., 1986; Broek et al., 1987) and have reduced adenylate cyclase activity (Broek et al., 1987). Disruption of *CDC25* is lethal, but can be suppressed by additional deletion of *IRA1*, expression of the activated *RAS2*^{Val19} allele or by overespression of either adenylate cyclase or the catalytic subunits of PKA (*TPK1*) (Broek et al., 1987; Tanaka *et al*., 1989). The *CDC25* gene encodes for a protein of 180 kDa, which is characterized by a C-terminal catalytic domain and a large N-terminal regulatory domain. The C-terminal region (aa 1121-1573) contains the guanine nucleotide exchange domain, highly homologous to Ras guanine nucleotide exchange factors from higher eukaryotes (Quilliam *et al*., 1995), and it is essential for normal growth and viability of *cdc25Δ* and *cdc25ts* mutants (Lai *et al*., 1993; Coccetti *et al*., 1995). The large N-terminal region (aa 1-1121) contains a cyclin destruction box (CDB) that drives the ubiquitin-dependent degradation of Cdc25 , that regulates the Cdc25 levels inside the yeast cell and therefore is important for the short half-life of the protein (Kaplon and Jacquet, 1995), and also a SH3 motif (aa 60-130) that binds adenylate cyclase (Freeman *et al*., 1996). Two-hybrid analysis demonstrated that the N-terminal of Cdc25 could interact with either the N- or C-terminus of the protein itself (Chen et al.,

2000). The N-terminal was also found to exert an inhibitory effect on the catalytic activity of Cdc25 itself (Chen et al., 2000; Belotti *et al*., 2006). This region is involved in the regulation of cell size in presence of different carbon sources (Belotti *et al*., 2006), and it is essential for Ras activation upon glucose addition (Paiardi *et al*., 2007). It is known that Cdc25 is required for the glucose-induced Ras-GTP increase and it was suggested that the intracellular levels of GTP, which quickly respond to nutrient availability, could be the metabolic signal that regulates Cdc25 activity in response to glucose (Rudoni *et al*. 2001; Colombo *et al*. 2004; Cazzaniga *et al*. 2008). The *S. cerevisiae* GEF Cdc25 has a nuclear localization (Tisi *et al*., 2008). This localization seems to rely on sequences present in the central region of the protein, from aa 353 to aa 1100, which is a largely uncharacterized region of this protein (Tisi *et al*., 2008).

The C-terminal part of Cdc25 is homologous to the second Ras guanine nucleotide exchange factor encoded by *SDC25* gene (Boy-Marcotte *et al*., 1996). Although *SDC25* is functionally homologous to *CDC25*, it is not essential for cell growth and only expressed in stationary phase or on non-fermentable carbon sources and cannot suppress a *cdc25Δ* mutation, which is lethal (Damak *et al.*, 1991). In the W303-1A strain background, the activity of Cdc25 is not necessary for growth in glucose, but is essential for growth in galactose and non fermentable carbon sources (Folch-Mallol J.L., *et al.*, 2004)**.**

1.2.2.2 GTPase activating proteins: Ira1 and Ira2

The GTPase activity of each small G-protein is variable, but relatively very slow and it is stimulated by GAPs. In yeast *S. cerevisiae* there are two different GAP proteins, Ira1 and Ira2 (Tanaka *et al.*, 1989,1990). *IRA1* and *IRA2* genes encode for two big proteins of respectively 2938 and 3079 amino acid residues (Tanaka *et al.*,1990). Ira1 and Ira2 negatively regulate cellular levels of cAMP. Despite the high degree of homology (about 45% identity) between them, the functions of these two proteins are similar, but not exactly the same (Tanaka *et al.*, 1990). Ira2 appears to act more efficiently on Ras1 than on Ras2 (Tanaka *et al.*, 1990 b).

Both *ira1*Δ and *ira2*Δ mutants are sensitive to a heat shock and nitrogen starvation. Deletion of *IRA* genes suppresses the lethality of the *CDC25* mutation and, in particular deletion of *IRA2* gene, ensures an increased level of cAMP (Tanaka *et al.*, 1989, 1990; Colombo *et al*., 2004). These results reveal a role for the Ira proteins as Ras-GTPase activating proteins.
Ira1 and Ira2 also share homology with mammalian p120-GAP and NF1 (Tanaka *et al.*, 1990).
Mitts *et al.* (1991) proposed that Ira1 has also a structural role in anchoring adenylate cyclase to the membrane. The authors suggest that these proteins are involved, together with Ras, in an oligomeric complex localized in the membranes of the yeast cell.

1.2.3 The G-protein-coupled receptor system

The G-protein-coupled receptor system (GPCR) consists of the receptor-like protein Gpr1 and the Gα protein Gpa2. Gpr1 belongs to the G protein-coupled seven-transmembrane receptor (GPCR) superfamily (Yun *et al*., 1997; Xue *et al*., 1998) and Gpa2 is a member of the heterotrimeric G protein α subunit (Gα) protein family (Nakafuku *et al*., 1988). This heterotrimeric G-protein has been shown to participate in glucose signalling in *S. cerevisiae*. This system acts as a sensor for external glucose and specifically controls glucose-induced activation of cAMP synthesis. Addition of glucose to derepressed cells activates the low affinity seven-transmembrane receptor Gpr1, which in turn stimulates the exchange of GDP for GTP on Gpa2. The concentration of glucose required for activation of the GPCR system appears to be approximately the level at which cells fully switch from respiration to fermentation. Hence, the GPCR system seems to sense and signal the availability of an optimal concentration of glucose for fermentation. GTP-bound Gpa2 activates the cAMP/PKA pathway and this is most probably through stimulation of adenylate cyclase (Nakafuku *et al*. 1988; Kubler *et al*. 1997; Lorenz and Heitman 1997; Colombo *et al*. 1998; Rolland *et al*. 2000; Peeters *et al*. 2006).Indeed, adenylate cyclase binds directly only to active, GTP-bound Gpa2. (Peeters *et al*. 2006).

Gpa2 interacts with Rgs2, a member of the family of regulators of G protein signalling (RGS), that negatively regulates the Gpa2-GTP signal by stimulating the intrinsic GTPase activity of Gpa2 (Versele *et al*., 1999). Gpa2 was originally identified by screening a yeast genomic library with cDNA probes encoding mammalian Gα subunits (Nakafuku *et al*., 1988). It is unclear whether the Gα protein Gpa2 also associates with canonical Gβ and Gγ subunits. A recent report suggests that Asc1 functions as the Gβ subunit for Gpa2 (Zeller *et al*. 2007). Asc1 has the typical 7-WD domain structure of a canonical Gβ protein, interacts directly with Gpa2 in a guanine nucleotide-dependent manner and inhibits Gpa2 guanine nucleotide exchange activity. In addition, Asc1 binds to adenylate cyclase and diminishes the glucose-induced production of cAMP. Another hypothesis states that the kelch-repeat proteins Krh1/Gpb2 and Krh2/Gpb1 serve as Gβ subunit and Gpg1 as Gγ for Gpa2 (Harashima and Heitman, 2002; Batlle *et al*., 2003). Deletion of *GPA2* gene is not lethal and confers the typical phenotype associated with a reduced cAMP level: higher heat resistance, a higher level of trehalose and glycogen and elevated expression of STRE-controlled genes. Furthermore, Gpa2 is involved in the cAMP- dependent pseudohyphal growth, that is induced by nitrogen starvation (Lorentz and Heitman, 1997).

A two-hybrid protein interaction screen with *GPA2* as the bait led to the identification of the *GPR1* gene, which encodes a member of the G protein coupled seven-transmembrane receptor superfamily (fig.6).

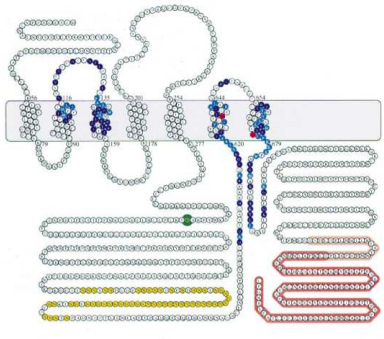


Figure 6. Gpr1 receptor structure (Kraakman *et al*., 1999).

Gpr1 is located on the yeast cell surface (Xue *et al*., 1998). Genetic analysis strongly suggests that Gpa2 acts downstream from Gpr1 in the same signalling pathway: the low growth rates of *gpr1*Δ *ras2*Δ, *gpa2*Δ *ras2*Δ, and *gpa2*Δ *gpr1*Δ *ras2*Δ strains are essentially identical, and introduction of either multicopy or activated *GPA2* suppresses the defect of the *gpr1*Δ *ras2*Δ strain (Xue *et al*., 1998). Deletion of *GPR1* diminishes (although does not eliminate) genome-wide transcriptional induction and repression in response to glucose (Wang et al., 2004). The seven-transmembrane receptor Gpr1 may detect extracellular glucose and thereby activate Gpa2, but it is not required to signal Ras; Gpr1 or Gpa2 deletion does not prevent the glucose-induced increase in Ras2-GTP levels (Colombo *et al*., 2004).

The *RGS2* gene was isolated as a multi-copy suppressor of glucose-induced loss of heat resistance in stationary phase cells. The N-terminal half of the Rgs2 protein consists of a typical RGS (Regulators of heterotrimeric G protein signalling) domain (Versele *et al*., 1999). Deletion and overexpression of the *RGS2* gene, respectively, enhances and reduces glucose-induced accumulation of cAMP. Overexpression of *RGS2* generates phenotypes consistent with low activity of cAMP-dependent protein kinase A (PKA), such as enhanced accumulation of trehalose and glycogen, enhanced heat resistance and elevated expression of STRE-controlled genes. Deletion of *RGS2* causes opposite phenotypes (Versele *et al*., 1999). Rgs2 functions as a negative regulator of glucose-induced cAMP signalling through direct GTPase activation of Gpa2.

1.2.4 Adenylate cyclase

Adenylate cyclase is an enzyme, encoded by CDC35/*CYR1* gene, of 200 kDa that converts ATP into cyclic AMP. *cdc35Δ* cells grow only in presence of extracellular cAMP. Adenylate cyclase consists of 2026 amino acid residues (Kataoka *et al*., 1985a) and includes three different domains: a domain containing a well-conserved catalytic region at the C-terminal, a central part characterized by leucine-rich repeats and a N-terminal regulatory domain.

The N-terminal region of adenylate cyclase is reported to act as an inhibitor of the catalytic activity of the protein (Heideman *et al*., 1987; Uno *et al*., 1987).
The central leucine-rich repeat domain consists in a repetition of a 23-amino acid amphipatic leucine-rich motif and it was shown to be important for interaction with Ras proteins. It contains a primary binding site for GTP-bound Ras, the Ras-associating domain (Kido *et al.*, 2002), and also seems to be indirectly involved in anchoring adenylate cyclase to the membrane, promoting the interaction with other proteins (Mitts *et al.*, 1991). A second Ras binding site is formed at the extreme C-terminal of adenylate cyclase by association with the N-terminal of the CAP (cyclase activating protein) protein. This GTP independent interaction strictly requires post-translational modification, in particular farnesylation, of Ras (Kuroda *et al.,* 1993).

1.2.5 Phosphodiesterases: Pde1 and Pde2

In yeast *S. cerevisiae* cells, cAMP is hydrolyzed by the low-affinity and high-affinity phosphodiesterases, respectively, encoded by *PDE1* and *PDE2*. While the high-affinity phosphodiesterase Pde2 appears to control basal cAMP level, which is important to prevent undesirable PKA activity during stationary phase (Park *et al*., 2005), the low-affinity phosphodiesterase Pde1 was shown to be specifically involved in the feedback inhibition of glucose-induced cAMP signalling. It was demonstrated that Pde1 activity is regulated by PKA, indeed, upon addition of glucose to glycerol-growing cells, Pde1 is rapidly phosphorylated at serine 252 (Ma *et al*., 1999). The phosphorylation doesn't change Pde1 intrinsic activity or affinity for cAMP but appears to be important for protein-protein interaction and for targeting Pde1 to a specific subcellular location (Ma *et al*., 1999).

1.2.6 The cAMP-dependent protein kinase A (PKA)

Activation of the cAMP/PKA pathway will activate protein kinase A that triggers a phosphorylation cascade (Broach, 1991). PKA is a hetero-tetramer

composed of two catalytic subunits, encoded by *TPK1*, *TPK2* and *TPK3* and two regulatory subunits, encoded by *BCY1* (Toda *et al*., 1987). cAMP activates PKA by binding two cAMP molecules to each regulatory subunit, thereby releasing and activating the catalytic subunits.

PKA activity is critical for yeast since at least one of the three catalytic subunits is necessary for viability (Toda *et al*. 1987). This implies as well that there is probably a large overlap in the functions of Tpk1-3, which is further consistent with the high level of protein sequence similarity between the different catalytic subunits (Toda *et al*. 1987). Cells lacking the regulatory subunit Bcy1 display a pleiotropic phenotype due to hyperactivation of the Tpk subunits, which includes the inability to grow on carbon sources other than glucose, an abnormally high heat shock sensitivity, the lack of a proper G1-arrest during nutrient starvation and defects in DNA replication and sporulation (Matsumoto *et al*., 1983). Overexpression of any of the *TPK* genes, or deletion of *BCY1* restores growth of a *cyr1Δ* mutant as well as the lethal phenotype of a *ras1Δ ras2Δ* double deletion (Toda et al., 1987; Santangelo, 2006).

In *S. cerevisiae* the Tpk subunits phosphorylate target proteins preferentially on serine or threonine residues of the consensus sequence RRX(S/T) (Ptacek *et al*., 2005; Denis *et al*., 1991).

TPK1, *TPK2* and *TPK3* genes codify for three proteins respectively of 397, 380 and 398 amino acid residues (Toda *et al*., 1987). *TPK1* gene was cloned by complementation screening for *cdc25ts* mutation and then it was use as probe to isolate the other *TPK* genes (Toda *et al*., 1987). Tpk1, Tpk2 and Tpk3 share a partially redundant function, although there are several examples of separate, specific functions for the different subunits, such as the induction of pseudohyphal growth, the expression control of genes involved in iron uptake or branched amino acid biosynthesis, or the regulation of mitochondrial enzymes (Robertson and Fink, 1998; Pan and Heitman, 1999; Robertson *et al*., 2000; Zhu *et al*., 2000; Singh *et al*., 2004; Chevtzoff *et al*., 2005; Ptacek *et al*., 2005). For example, the three Tpk have dramatically different roles in pseudohyphal development: Tpk2 is essential, whereas Tpk3 inhibits it. Tpk1 has no discernible effect on pseudohyphal growth (Robertson and Fink, 1998; Pan and Heitman 1999; Chen and Thorner, 2010).

Bcy1, the regulatory subunit of cAMP-dependent protein kinase in yeast, encoded by the BCY1 gene, binds to the catalytic subunits when cAMP concentrations are low and dissociates from the catalytic subunits when cAMP concentrations are high (Edelman *et al*., 1987; Taylor *et al.,* 1990).In cells in stationary phase there are different isoforms of Bcy1, derived from a large number of different post-translational modifications particularly phosphorylations, that could be important for the control of the PKA activity (Werner-Washburne *et al*., 1991). Bcy1 is necessary for growth on non-fermentable carbon sources, for entry into stationary phase, for thermotolerance and for sporulation (Werner-Washburne *et al*., 1991).
Moreover, its subcellular distribution is carbon source dependent, indeed In glucose-grown cells, Bcy1 is almost exclusively nuclear, while it appears more evenly distributed between nucleus and cytoplasm in carbon source-derepressed cells (Griffioen *et al*., 2000). It has also been demonstrated that the Bcy1 N-terminus plays a crucial role in its localization (Griffioen *et al*., 2000).

1.2.6.1 Targets of PKA

The cAMP/PKA pathway plays a central role in regulating many important aspects of the yeast cell life: mutants with an hyperactive pathway or with too low activity of the pathway display specific and strong phenotypic defects. Mutants with an hyperactive pathway show low levels of storage carbohydrates, a high sensitivity to heat shock and nutrient starvation, failure to arrest in G1 phase upon nutrient limitation, inability to growth properly on non-fermentable carbon sources (Thevelein, 1994). Instead, depletion of cAMP or inactivation of PKA causes enhanced level of glycogen and trehalose, enhanced stress resistance, constitutive expression of heat shock genes and other genes only expressed in stationary phase in wild type cells. Moreover, reduced PKA activity causes cells arrest in the G1 phase of the cell cycle at the same point as nutrient starved cells, and then they enter in G0 phase (Thevelein, 1994).
The cAMP/PKA pathway negatively influences the capacity to sporulate and stimulates pseudohyphal growth (Toda *et al*., 1987).

The PKA downregulates a large number of genes through some elements in their promoter called STRE elements (stress responsive elements). The transcription factors Msn2 and Msn4 mediate the transcription of the so-called STRE-controlled genes. STRE genes are involved in a wide variety of processes, including protection against diverse types of stress such as heat, oxidative and osmotic stress, carbohydrate metabolism and growth regulation (Mai and Breeden, 1997; Moskvina *et al*., 1998; Smith *et al*., 1998; Gash *et al*., 2000). Msn2 and Msn4 are inhibited by PKA and, interestingly, their cellular localization is strongly influenced by stress conditions and PKA activity (Gorner *et al*., 1998; Garmendia-Torres *et al*., 2007). In mutants with reduced PKA activity, indeed, these factors were constitutively located in the nucleus. During growth on glucose, Msn2 and Msn4 are phosphorylated and reside in the cytosol. Upon glucose exhaustion, they are hyperphosphorylated and translocated to the nucleus, where they induce expression of the STRE-controlled genes. PKA inhibits nuclear import of Msn2/4, probably through direct phosphorylation of their nuclear localization signal (Gorner *et al*., 1998, 2002; Garreau *et al*., 2000), and controls their function also via the protein kinases Rim15 and Yak1, that are under negative control of PKA, probably by direct phosphorylation. Lee *et al*. (2008) suggest that Yak1 phosphorylates and thereby activates Msn2 through a still unknown mechanism, which apparently does not implicate the control of Msn2 subcellular localization (Fig.7). Since *YAK1* gene itself is induced by Msn2/4, this would generate a positive feedback loop (Smith *et al*., 1998). Yak1 was also found to stimulate the activity of Hsf1, another transcriptional activator of stress response genes that was recently shown to be under negative control of PKA (Hahn *et al*., 2004; Ferguson et al., 2005; Lee et al., 2008). It has been demonstrated that Yak1 directly phosphorylates Hsf1 *in vitro*, leading to the increase in DNA binding activity of Hsf1 (Lee *et al*., 2008). Yak1 phopshorylates Msn2 *in vitro*, but this phosphorylation does not affect its DNA binding activity or nuclear localization upon glucose depletion (Lee *et al*., 2008).

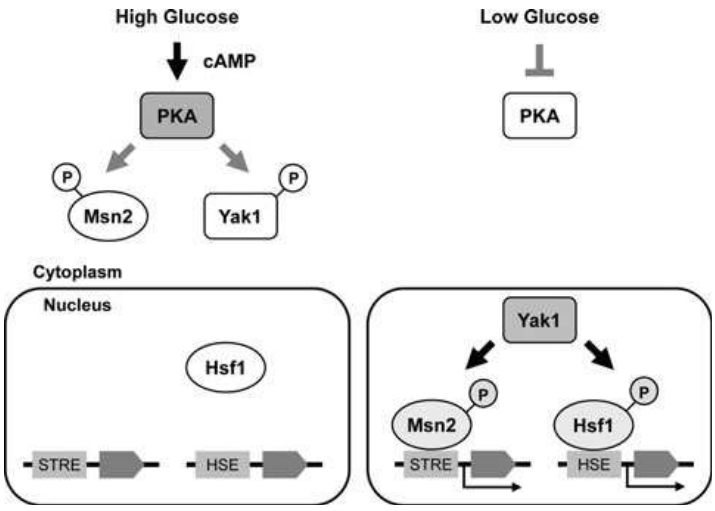


Figure 7. Model of Yak1-dependent regulation of Hsf1 and Msn2 (Lee *et al*., 2008)

Rim15 was initially identified as an activator of meiotic gene expression (Vidan and Mitchell, 1997). Rim15 is essential for proper entry into G0 in stress conditions and is inhibited by PKA-mediated phosphorylation under conditions of nutrient abundance (Reinders *et al*., 1998); in nutrient starvation PKA is downregulated, Rim15 is not phosphorylated and can drive the entry of the cells into G0. Interestingly, deletion of Rim15 suppresses the lethality caused by PKA inactivation, suggesting that some of the stationary phase-induced gene may be inhibitory for growth. In yeast *S. cerevisiae* Rim15 integrates signals derived from several different nutrient-sensory kinases (i.e. PKA, TORC1, Sch9 and Pho85-Pho80) that transmit information on the availability of different nutrients, and acts via Msn2 and Msn4 and Gis1, a zinc finger transcription factor that induces transcription of post-diauxic shift (PDS) genes (Fig.8). Overexpression of *GIS1* prevents growth and loss of Gis1 partially relieves the dependence on PKA function, indicating that Gis1 activates one or more genes whose products inhibit cell proliferation (Pedruzzi *et al*., 2000).

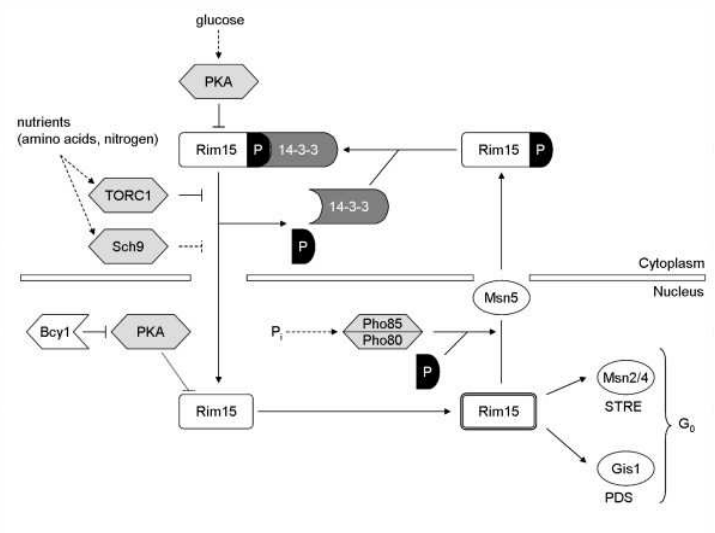


Figure 8. Integration of nutrient signals by Rim15 (Swinnen *et al*., 2006).

In addition to the control of gene expression, PKA directly modulates the activity of metabolic enzymes. PKA-dependent phosphorylation inhibits the activity of fructose-1,6-bisphosphatase (Fbp1) and stimulates the activity of 6-phosphofructo-2-kinase (Pfk2) and of both isoforms of pyruvate kinase (Pyk1 and Pyk2) (Gancedo *et al*., 1983; Rittenhouse *et al*., 1987; Cytrynska *et al*., 2001; Vaseghi *et al*., 2001). Together, these modifications result in the stimulation of glycolysis and the inhibition of gluconeogenesis when glucose is added to glucose-starved cells (Smets *et al*., 2010). *In vitro* phosphorylation by PKA also modulates the activity of enzymes involved in the metabolism of trehalose (Tps1 and Nth1) and glycogen (Gsy2 and Gph1), but it remains to be established whether these enzymes are direct *in vivo* substrates of PKA (Uno *et al.*, 1983; Wingender- Drissen and Becker, 1983; Panek *et al*., 1987; Hardy and Roach, 1993; Smets *et al*., 2010).

Another important target of the PKA pathway is cAMP synthesis itself. It is known that cAMP synthesis in yeast is strongly regulated by PKA that exerts a very strong feedback inhibition, indeed mutants with reduced activity of the protein kinase display hyperaccumulation of cAMP, while mutants with hyperactivated PKA display a reduced cAMP level (Nikawa *et al*., 1987b).

Some proteins of the cAMP/PKA pathway has been demonstrated to be target of the feedback mechanism, i.e. Pde1 (Ma *et al*., 1999), Pde2 (Hu *et al.,* 2010) and Ras2 (Bai and Dong, 2010). The deletion of the two phosphodiesterases genes in a $Ras2^{Val19}$ strain causes a significant increase in the cAMP level, but in a strain with attenuated PKA activity there is a similar high cAMP level in spite of the presence of the phosphodiesterases (Nikawa *et al*., 1987a). This indicates that high PKA activity in some way is required for efficient breakdown of cAMP by phosphodiesterases (Thevelein 1992). Pde1, the low affinity phosphodiesterase is rapidly phosphorylated *in vivo* upon addition of glucose to glycerol-grown cells by PKA at serine 252 (Ma *et al*., 1999) and its deletion results in much higher glucose- and acidification- induced cAMP accumulation. Recently, it was demonstrates that the protein level of Pde2 is positively correlated with PKA activity (Hu *et al*., 2010) suggesting that the high-affinity cAMP phosphodiesterase Pde2 may also play an important role in the PKA-governed feedback inhibition of intracellular cAMP level. Both Ras1 and Ras2 proteins are phosphorylated *in vivo* (Sreenath *et al*., 1988; Cobitz *et al*., 1989), furthermore Whistler and Rine (1997) identified the serine 214 residue on Ras2 protein as the most preferred PKA phosphorylation site. A serine to alanine substitution at this position in Ras2 resulted in enhanced sensitivity to heat shock, reduced levels of storage glycogen and enhanced both basal cAMP level and glucose-induced cAMP signal (Bai and Dong 2010). The serine 214 of Ras2 plays an important role in the feedback regulation of the cAMP/PKA pathway, indeed the $Ras2^{Ala214}$ had a higher GTP-binding capability than wild type Ras2 (Bai and Dong 2010).

1.3 The Ras pathway in mammalian cells

The Ras pathway in mammalian cells plays a central role in regulating many cellular processes such as proliferation, differentiation and apoptosis (Satoh et al., 1992). This pathway is activated by the tyrosine kinase receptors located on the cellular surface. The activation of these receptors rise to a signal that through the Ras proteins arrives at the nucleus, using a cytoplasmatic kinases cascade. In mammalian cells there are three *RAS* genes, *H-RAS*, *K-RAS* and *N-RAS* codifying four proteins: H-Ras, K-RasA, K-

RasB and N-Ras. These proteins show high homology in the first 85 residues at the N-terminal domain, where reside all the structural determinants of the active site, the mechanism of hydrolysis, the domains that interact with the downstream effectors and with the positive and negative modulators. Their C-terminal domain is an hypervariable region (Taparowsky *et al*., 1983). H-RAS has been identified as a retroviral oncogene of rat sarcomas (Dhar *et al*., 1982); subsequently have been identified N-ras and K-RAS.

Also in mammalian cells Ras proteins are small GTPases that cycle between an inactive GDP-bound form and an active GTP-bound form. The GDP-GTP exchange is controlled by different GEFs, such as Sos (Chardin *et al*., 1992), p140 Ras-GFR1 and C3G. Ras proteins can be activated by the EGF (epidermal growth factor): binding of the ligand to the receptor leads to tyrosine kinase receptor dimerization and autophosphorilation of some tyrosine residues on its cytosolic domain. Grb2 (a SH2- and SH3- containing protein) binds to the active receptor via its SH2 domain and binds to the proline-rich sequence in the C-terminus of Sos via its SH3 domain. In this way Sos is recruited to the close proximity of Ras in the plasma membrane and can activate Ras. Grb2 interacts also with the insulin receptor (Skolnick *et al.,* 1993), the PDGF receptor (Bennet *et al*., 1994; Li *et al*., 1994), the cytokine receptor (Cutler *et al*., 1993) and the T-cell receptor (Ravichandran *et al*., 1993). At this point active Ras interacts with the N-terminal region of Raf (serine/threonine protein kinase) and recruits it to the plasma membrane. There are three different isoforms of Raf: c-Raf1, B-Raf and A-Raf (Leevers *et al*., 1994) (Marais *et al*., 1995). It has been discovered by mutagenesis the existence in c-Raf1 of some sites specific for the interaction with Ras proteins. The RBD (Ras Binding Domain) of Raf binds Ras-GTP with a binding affinity 10^4 times higher than Ras-GDP. Activated Raf in turn activates MEK1 and MEK2 (Mitogen-activated extracellular receptor-regulated kinase 1 and 2) by phosphorylation on two conserved serine residues. Activated MEK can phosphorylate and activate the MAPKs, ERK1 and ERK2. Active MAPKs can phosphorylate a number of substrates in the plasma membrane and in the cytoplasm or can translocate into the nucleus where they can phosphorylate some nuclear transcription factors, such as Elk-1 (Fig.9).

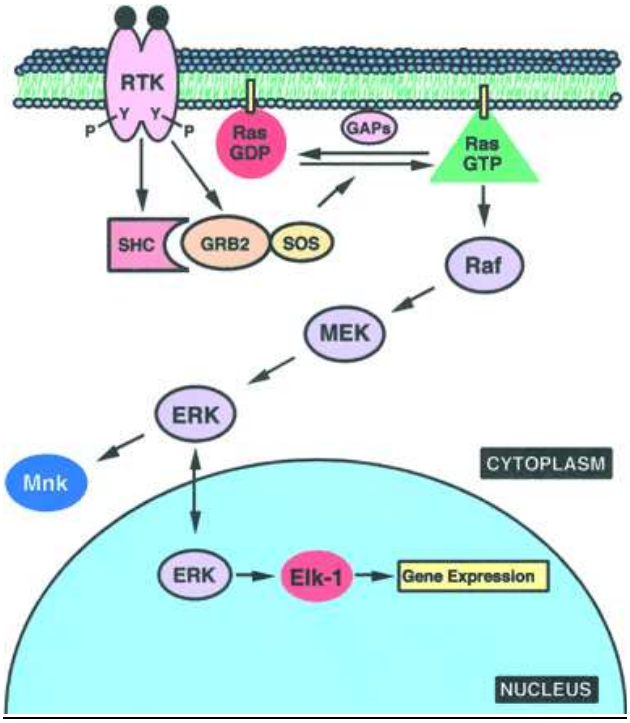


Figure 9. Ras/MAPK signaling pathway in mammals (Vojtek e Der, 1998).

1.4 Ras-Raf interactions

Sequence analysis of the different isoforms of Raf and their homologues showed the presence of three highly conserved regions, named CR1, CR2 and CR3 (Heidecker *et al.,* 1992). CR1 is located between aa 53 and 200 of the N-terminal domain. In this region there is a cysteine rich sequence that can act as a zinc finger. CR2 is a short N-terminal sequence rich in serine and threonine residues. This region contains several phosphorylation sites (Morrison *et al*., 1993). CR3 contains the kinase domain. The N-terminal region of the molecule is very important for the regulation of c-Raf1 activity, indeed mutations in this region activate the oncogenic potential of this protein (Ishikawa *et al*., 1988). It has been shown that the minimal region of interaction between Ras and Raf is the Ras Binding Domain (RBD) between aa 55 and 131. The RBD binds active Ras with an affinity 10^4 times higher than inactive Ras (Bivona *et al*., 2006).

Mutations in the RBD which prevent the interaction between c-Raf1 and Ras prevent also the association of c-Raf1 to the membrane, and then its activity (Marais *et al*., 1995).
There is another site of interaction between Ras and c-Raf1, the Cysteine Rich Domain (CRD), between aa 152 and 184 (Drugan *et al*., 1996; Hu *et al*., 1995). It has been shown that mutations in the CRD and in the Ras domain that interact with the CRD prevent the binding Ras-c-Raf1 and decrease the kinase activity of c-Raf1 (Drugan et al., 1996).

1.5 The MAP kinase pathways in the yeast *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* has five MAPKs, Fus3, Kss1, Hog1, Slt2/Mpk1 and Smk1, involved in the pherormone, filamentous and invasive growth, high osmolarity (HOG), cell wall integrity (CWI) and spore wall assembly pathways, respectively (Chen and Thorner, 2007) (Fig.10). Four of these pathways are present in growing cells: the mating pathway, the filamentation-invasion pathway, the cell integrity pathway and the high-osmolarity growth pathway. The Smk1p MAPK, part of the spore wall assembly pathway, is not present in growing cells but appears during sporulation and regulates that developmental process. The biochemical mechanisms mediating signal transduction in MAPK cascades are fairly well understood (Robinson and Cobb, 1997). MEKK has a regulatory domain at the NH2 terminus and a protein kinase domain at the COOH terminus. When activated, MEKK phosphorylates both a serine and a threonine residue in a conserved domain in the NH2-terminal portion of MEK. The activated MEK then phosphorylates MAPK on a threonine and a tyrosine residue, separated by a single amino acid, within the activation loop (Johnson *et al*., 1996) of the conserved kinase domain, thereby activating the kinase activity.

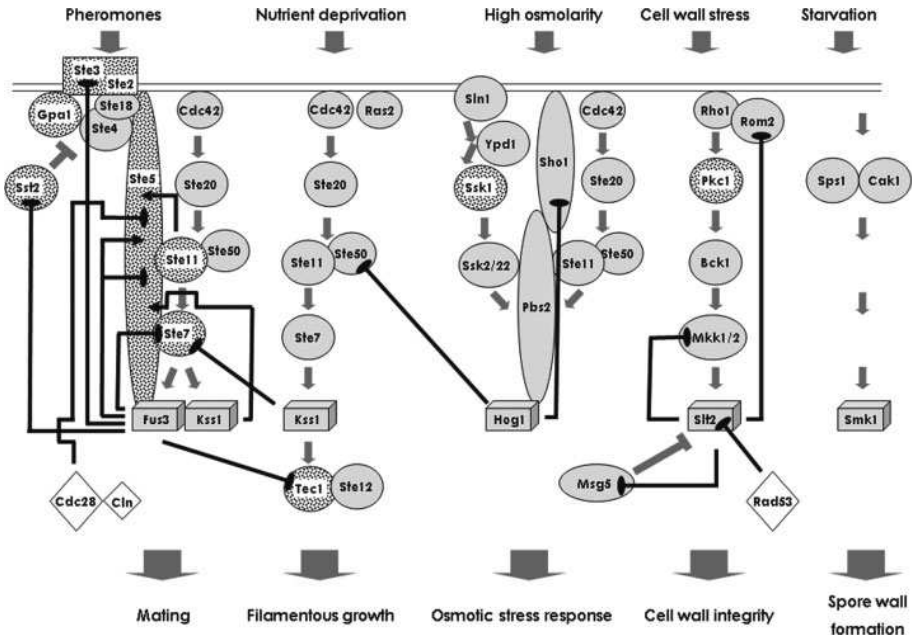


Figure 10. MAPK cascades of *S .cerevisiae* (Molina *et al*., 2010).

1.5.1 The mating-pherormone response pathway

The mating-pherormone response pathway is the best understood of all eukaryotic MAPK pathways. Yeast *S. cerevisiae* has two mating types, **a** and α (genotypes *MAT***a** and *MAT*α, respectively). *MAT***a** and *MAT*α cells are haploid, and the result of a successful mating will be that two haploid cells of opposite mating type fuse to form a *MAT***a**/*MAT*α diploid. *MAT*α cells secrete α- Factor pheromone and respond to **a**-Factor. *MAT***a** cells secrete **a**-Factor and respond to α-Factor. When a yeast cell is stimulated by pheromone secreted by a nearby cell of the opposite mating type, it undergoes a series of physiological changes in preparation for mating. Cellular responses to mating pherormone include polarized growth toward a mating partner, cell cycle arrest in G1 and increased expression of proteins needed for cell adhesion, cell fusion, and nuclear fusion (Gustin *et al.*, 1998). Pheromone binds to and activates a seven transmembrane domain receptor that in turn is thought to induce the dissociation of a heterotrimeric G protein (Gustin *et al*., 1998). As show in figure 11, the liberated Gβ(Ste4p)- Gγ(Ste18p) complex then activates downstream

proteins Ste5p and Ste20p, and these in turn stimulate the Ste11p-Ste7p-Fus3p MAPK cascade. The MAPK Fus3p phosphorylates several downstream targets, e.g., Far1p, Dig1p, Dig2p, and Ste12p, that mediate various responses required for successful mating (Gustin *et al*., 1998).

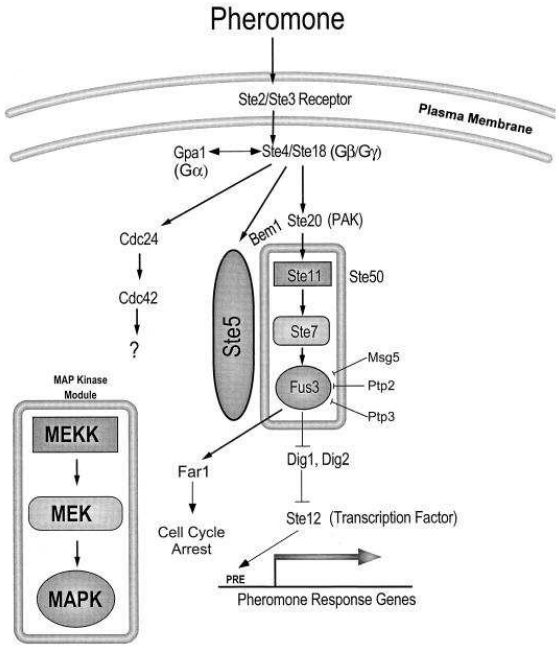


Figure 11. Pherormone response pathway of *S. cerevisiae* (Gustin *et al*., 1998).

1.5.2 The cell integrity pathway

A second MAPK cascade is found in budding yeast as part of the cell integrity pathway, that mediates cell cycle-regulated cell wall synthesis and responds to different signals including cell cycle regulation, growth temperature, changes in external osmolarity and mating pherormone (Gustin *et al*., 1998). This pathway includes different proteins: the GTP binding protein Rho1p (Kamada *et al*., 1996; Nonaka *et al.,* 1995), the protein kinase C homologue Pkc1p (Levin *et al*., 1990), the MEKK Bck1p (Lee and Levin, 1992) (also called Slk1p (Costigan et al., 1992)) the redundant pair of MEKs Mkk1p and Mkk2p (Irie *et al*., 1993), the MAPK Slt2p (Torres *et al*., 1991), and the transcription factor targets Rlm1p (Dodou and

Treiasman, 1997) and SBF (Madden *et al*., 1997), the latter being composed of the polypeptides Swi4p and Swi6p (Fig.12). This pathway has the general function to positively regulate growth and cell proliferation.

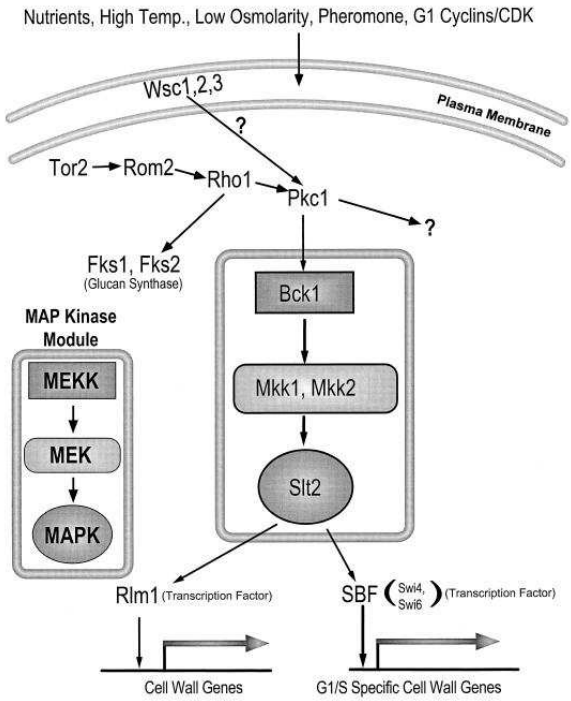


Figure 12. Cell integrity pathway of *S. cerevisiae* (Gustin *et al*., 1998).

1.5.3 The HOG pathway

Another pathway mediates the response to hyperosmotic conditions through the MAP kinase pathway homologs Pbs2 and Hog1, which stimulate glycerol accumulation. This MAP kinase cascade consists of five protein kinases. The MEKKs Ssk2p, Ssk22p and Ste11p activate a single downstream MEK, Pbs2p, that in turn activates a single MAP kinase, Hog1p. The upstream part of the HOG pathway can be considered to have two incoming and partially redundant branches of signaling proteins that regulate the MEK Pbs2p. On one branch are the putative membrane protein Sho1p and the MEKK Ste11p. The other upstream branch of the HOG pathway contains a three-component signaling protein complex composed

of Sln1p, Ypd1p, and Ssk1p (Gustin *et al*., 1998) (Fig.13). The HOG pathway is required for part of the osmoregulatory response to an increase in osmolarity (Gustin *et al*., 1998).

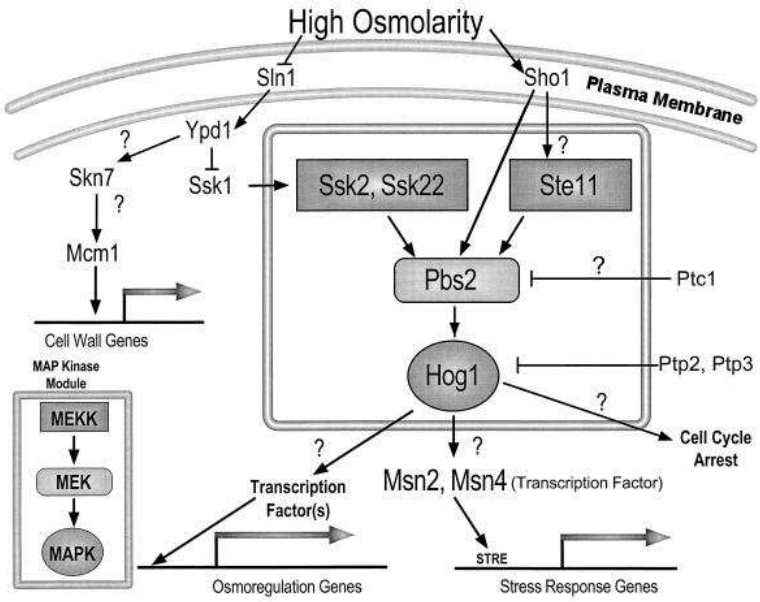


Figure 13. HOG pathway of *S. cerevisiae* (Gustin *et al*., 1998)

1.5.4 The filamentation-invasion pathway

In budding yeast *Saccharomyces cerevisiae,* nutrient limitation induces morphological changes that depend on the cAMP/PKA pathway and the Kss1 MAPK pathway. These changes in growth characteristics are termed "pseudohyphal growth" in diploids and "invasive growth" in haploids, or generally "filamentous growth" (Chen and Thorner, 2010). Pseudohyphal growth is induced by nitrogen source limitation in diploids, while invasive growth is induced by carbon source limitation in haploids. Filamentous growth is a composite of several distinct and genetically dissectable processes: cell elongation, filament formation and agar penetration (Gimeno *et al.,* 1992). The MAPK cascade that promotes the filamentous growth consist of Ste20, Ste11, Ste7 and Kss1, that is the MAP kinase (Figs.10, 14 and 15). Fus3, another MAP kinase activated by Ste7 in

response to a different stimulus (mating pherormone), inhibits the filamentous growth (Cook *et al*., 1997; Madhani *et al*., 1997) at least in part by targeting a transcription factor (Tec1) required for filamentous growth gene expression for ubiquitin- and proteasome- mediated destruction (Bao *et al*., 2004; Chou *et al*., 2004). The three yeast catalytic subunit isoforms of PKA have dramatically different roles in pseudohyphal development. Deletion of *TPK2* abolished filamentous growth, whereas deletion of *TPK1* had no effect. Deletion of *TPK3* caused hyperfilamentous growth, suggesting that Tpk3 may function in an inhibitory capacity (Robertson and Fink, 1998; Pan and Heitman, 1999; Cullen and Sprague, 2012). The three Tpks induce different target genes that regulate diverse metabolic outputs ranging from trehalose metabolism to iron uptake (Robertson *et al*., 2000). Among the substrates of Tpk2 is the transcription factor Flo8. Phosphorylation of Flo8 by Tpk2 results in its activation and expression of filamentation target genes (Pan and Heitman, 1999). The PKA pathway regulates filamentous growth not only by regulating the transcription factor Flo8, via Tpk2, but also by regulating the dual-specificity tyrosine-regulated kinase Yak1, via Tpk1. Yak1 has a positive role in regulating filamentous growth (Zhang *et al.*, 2001). Specifically, Yak1 is phosphorylated by Tpk1, which inactivates the protein (Deminoff *et al*., 2006). In its nonphosphorylated (active) form, the protein regulates Flo11 expression through the transcription factors Sok2 and Phd1 (Malcher *et a*l., 2001; Cullen and Sprague, 2012).

The MAPK and the PKA pathways share a common upstream activator, the GTPase Ras2 (Mosch *et al*., 1999) (Fig.15). Ras2 not only activates trough the PKA the Flo8 transcription factor, but also stimulates the activation of the guanine nucleotide exchange factor for Cdc42, Cdc24. Activated Cdc24, in turn, activates Cdc42 that interacts directly with the protein kinase Ste20 (Peter *et al*., 1996; Leberer *et al.,* 1997), and displaces the negative regulator Hsl7 (Fujita *et al*., 1999). In this way Ste20 can activate the MAPK cascade formed by Ste11, Ste7 and Kss1 (Liu *et al*., 1993; Madhani *et al*., 1997). The MAP kinase Kss1 in its unphosphorylated form is able to interact with the transcription factor Ste12 and with the negative regulators Dig1(Rst1) and Dig2(Rst2), thereby potentiating the Dig mediated block of Ste12 activity (Cook *et al*., 1996; Bardwell *et al*., 1998). When Kss1 is

phosphorylated by Ste7, both on a threonine and on a tyrosine residue, it is able to phosphorylate Ste12 and Dig1/2; the Dig proteins dissociate and this allows derepression of the target genes to which Ste12 may bind (Bardwell *et al*., 1998). Flo8 and Ste12 seem to coactivate the transcription of genes, i.e. *FLO11*, which are essential for invasive growth and pseudohyphal formation. Elements called FRE (for Filamentation and invasive Response Element) have been identified in the promoters of several genes, including *FLO11* (Madhani *et al*, 1997) that is regulated by unusually large promoter where multiple signaling pathways converge (i.e. the cAMP/PKA pathway and the MAPK cascade).

One change that is associated with filamentous growth is cell-cell adhesion. Filamentously growing yeast cells undergo cytokinesis but remain attached to each other through protein and polysaccharide attachments (Cullen and Sprague, 2012). The adhesion/flocculin Flo11 is the major cell adhesion molecule that controls the filamentous growth (Lambrechts *et al*. 1996; Lo and Dranginis 1998; Guo *et al*. 2000) and changes in this gene or in its promoter causes dramatic effects on cell adhesion. Flo11 contains a putative N-terminal signal sequence and transmembrane domain, an external Ser/Thr/Pro-rich repeat region that is heavily glycosylated, and a C-terminal glycosylphosphatidylinositol anchor (Cullen and Sprague, 2012). It is a member of the *FLO* gene family and is the major expressed between the different flocculins.

Strains lacking Ras2 are incapable to invade the agar medium and are restored for invasive growth by expression of a constitutively Ras2p ($Ras2^{Val19}$) (Stanhill *et al*., 1999), by high expression of Ras1 (Mösch *et al*., 1999), by deletion of *BCY1* or overexpression of the catalytic A-kinase subunit encoding *TPK1*, *TPK2* or *TPK3* genes (Mösch *et al*., 1999). Moreover, activated alleles of *CDC42* ($CDC42^{Val19}$ and $CDC42^{Leu61}$) induce diploid filamentous growth, and also increase transcription of the FRE reporter gene (Mösch *et al.,* 1996). The function of *RAS1,* the second *RAS* gene in *S. cerevisiae,* in regulating pseudohyphal development or haploid invasive growth is not known (Mösch *et al*., 1999). How Ras2 action promotes membrane recruitment and activation of Cdc24 to stimulate GTP loading on Cdc42 is not clear (Chen *et al*., 2007). Furthermore, production of activated Ras2 presumably demands that something about the conditions that

promote filamentous growth also stimulates recruitment and activity of the GEF for Ras2, Cdc25. The precise mechanisms by which Cdc25 and Ras2 become activated in this pathway are also unclear. However, several distinct transmembrane proteins that reside in the plasma membrane and are exposed to the cell surface are necessary (in some cases, in haploids, and in other cases, in diploids) for initiation of filamentous growth. These transmembrane proteins include: Sho1 (Cullen *et al*., 2004) (four transmembrane segments); Msb2 (Cullen *et al*., 2004) (one transmembrane segment); Mep2 (Lorentz and Heitman, 1998) (ten transmembrane segments); and, Gpr1 (Tamaki *et al*., 2000; Lorentz *et al*., 2000) (seven transmembrane segments). Sho1 can form hetero-oligomeric complexes with Msb2, and the absence of either protein blocks Kss1 activation and prevents filamentous growth in haploids (Cullen *et al*., 2004).
Lorenz and Heitman (1997) found that also the G protein GPA2 regulates pseudohyphal differentiation, indeed cells lacking GPA2 have a defect in filamentous growth. Moreover, a dominant active *GPA2* allele stimulates pseudohyphal differentiation, even on nitrogen-rich media.

Pseudohyphal growth and invasive growth are observed in a very few laboratory strains, in particular those of the ∑1278b genetic background. Strains of other genetic backgrounds commonly used in research, such as the SP1 and the W303-1A, exhibits a weaker invasive activity than the ∑1278b strains (Stanhill *et al*., 1999). The observations that ∑1278b strains are more competent than most laboratory strains for filamentous growth has been related with the fact that the usual laboratory strains have a defect in the *FLO8* gene (Kron, 1997). It has also been reported that the cAMP/PKA pathway is overactive in the ∑1278b genetic background (Stanhill *et al*., 1999).

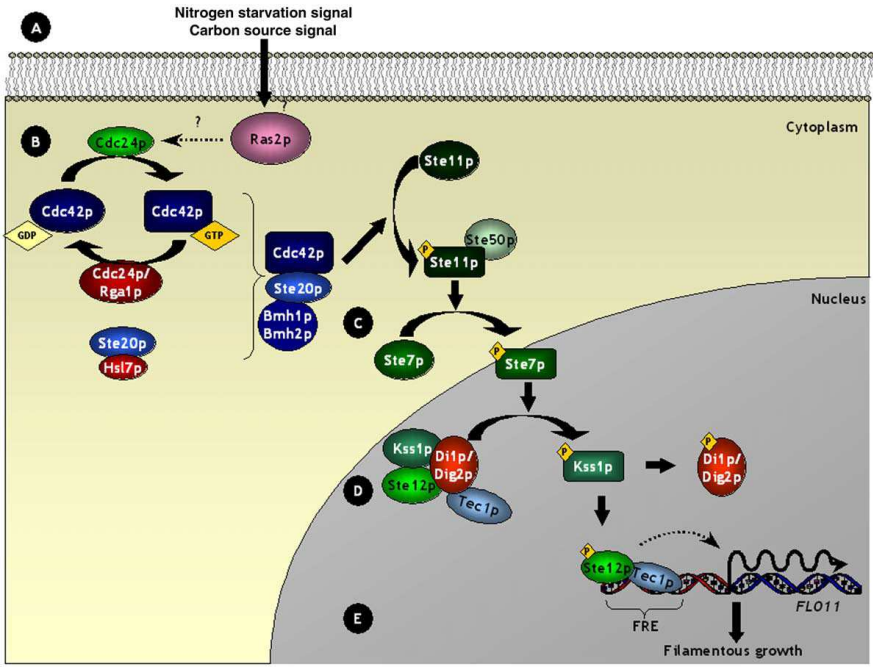


Figure 14. The components of the mating pherormone MAPK cascade and filamentous growth (Gagiano *et al*., 2002).

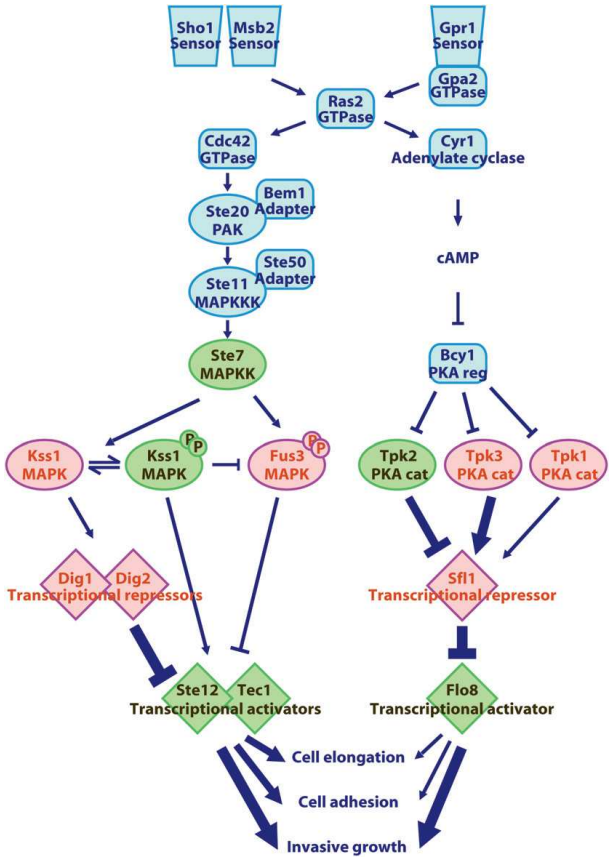


Figure 15. Signaling circuitry involved in the response of haploid cells to nutrient limitation. (Chen and Thorner, 2010).

1.6 Computational model of the cAMP/PKA pathway in yeast

Nowadays, computational analysis represents an indispensable tool to better understand the complexity of cellular systems, since the use of experimental analysis alone is typically not able to unravel the whole picture of the spatiotemporal cascade of molecular interactions and of their emerging dynamics (Pescini *et al.,* 2012). Indeed, in living cells many processes are regulated by means of negative and positive feedback mechanisms, which function to either attenuate or amplify molecular noise (Rao *et al.,* 2000). These elementary control mechanisms are usually

interlaced in complex regulatory networks involving multiple feedback loops, which can determine diverse macroscopic phenomena as oscillations. By exploiting mathematical modeling and computer simulation tools, integrated with the current biological knowledge and any available experimental data, it's possible to make predictions on the unknown behaviors of many cellular systems, therefore gaining insights into their working principles and their organization (Klipp *et al*., 2009; Szallasi *et al.,* 2006). In *S. cerevisiae* the cAMP/PKA signalling pathway plays a central role in the control of metabolism, stress resistance and proliferation, in particular in connection with the available carbon source (Thevelein *et al.,* 1999). A reduction of its activity, related to a decrease of intracellular cAMP, results in the accumulation of storage carbohydrates (glycogen and trehalose), high stress tolerance and arrest of growth and cell cycle (Thevelein *et al.,* 1999). The complex interplay between all the components of this pathway leads to many challenges in both the computational and experimental investigations. In fact, the Ras/cAMP/PKA signaling cascade is tightly regulated (Pescini *et al*., 2012). The cAMP is synthesized by the adenylate cyclase Cyr1 that is controlled by the Ras proteins. Ras proteins are positively controlled by the activity of Cdc25, that stimulates the GDP-GTP exchange, and negatively controlled by Ira1 and Ira2, that stimulate the GTPase activity of Ras. Cazzaniga *et al*. (2008) presented a stochastic discrete model for the cAMP/PKA pathway (Fig.16) based on an accurate description of the mechanisms of Ras activation, cAMP production and PKA activity. By using this model, they showed that the intracellular GTP/GDP ratio could represent a relevant metabolic signal for the regulation of the pathway, as also suggested by Haney and Broach (1994) and Rudoni *et al*. (2001).

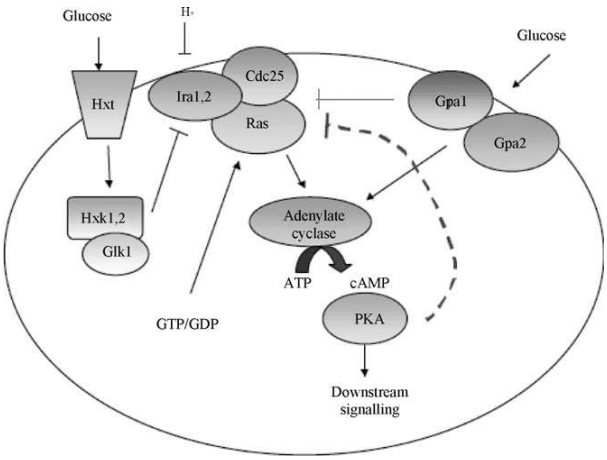


Figure 16. Model of cAMP/PKA pathway. (Cazzaniga *et al.,* 2008)

But experimental evidences indicate that other negative feedback mechanisms, not considered in the previous model, operate within the pathway through the generation of a still more complex interplay between the cascade components (Colombo *et al.,* 2004). This is suggested also by some recent evidences that oscillations related to this pathway can be observed *in vivo* under specific stress conditions (i.e. nucleocytoplasmatic oscillations of the transcription factor Msn2 in low glucose) (Garmendia-Torres *et al*., 2007; Medvedik *et al*., 2007). Pescini *et al*. (2012) generated a new model (Fig.17), based on the previous model, including the feedback mechanisms that operate directly at the level of Ras2 regulation: the phosphorylation of Cdc25 and Ira2 exerted by PKA. These feedback result in a decrease of the activity of adenylate cyclase. By using this model, stable oscillatory regimes for the dynamics of cAMP were observed *in silico*.

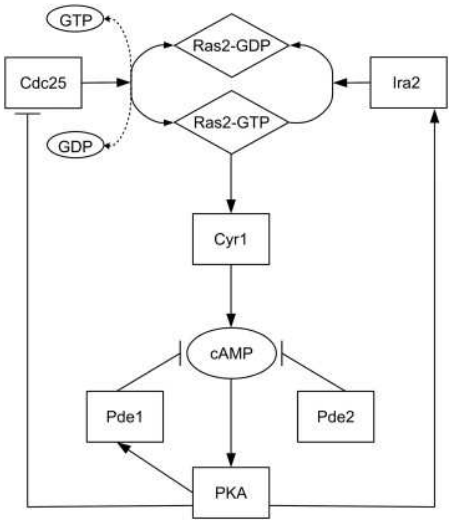


Figure 17. positive and negative regulations in the Ras/cAMP/PKA pathway in *S. cerevisiae* (Pescini *et al*., 2012).

1.6.1 Probes for monitoring cAMP levels *in vivo*

In the yeast *S. cerevisiae* the second messenger cyclic AMP (cAMP) plays a central role in the control of cell metabolism, stress resistance and proliferation in response to available nutrients. A basal level of cAMP is required for growth, while a transient increase of cAMP induced by addition of glucose is required for transition from respiratory to fermentative metabolism (Thevelein and De Winde, 1999). Although the cAMP/PKA pathway has been extensively studied in yeast and both upstream and downstream elements are known, data on the spatiotemporal variation of cAMP in single cells are till now lacking.

Ponsioen *et al*. (2004) proposed a system based on a FRET (Fluorescence Resonance Energy Transfer) sensor to monitor the changes in cAMP level in mammalian cells, based on the mammalian protein EPAC, originally developed by Nikolaev *et al*. (2004), in which Epac1 is fused amino terminally to CFP and carboxy terminally to YFP (Fig.18). Epac1 is a guanine nucleotide exchange factor for Rap1, a GTPase that cycle between an inactive GDP-bound form and an active GTP-bound form, that is activated by direct binding of cAMP. Epac1 consists of a C-terminal catalytic domain characteristic of exchange factors for Ras family GTPases and an N-terminal

regulatory domain. *In vitro* studies have shown that cAMP is absolutely required for the activation of Epac (de Rooij *et al*., 1998). FRET, the radiationless transfer energy from a fluorescent donor to a suitable acceptor fluorophore, depends on fluorophore orientation and on donor-acceptor distance at a molecular scale (Poinsioen *et al*., 2004). In mammalian cells the CFP-Epac1-YFP construct shows significant energy transfer, which rapidly diminishes following a rise in intracellular cAMP and increases again in response to a fall in cAMP (Poinsioen *et al*., 2004). This indicates that cAMP causes a significant conformational change *in vivo* (Poinsioen *et al*., 2004). CFP-Epac1-YFP in mammals is a highly sensitive cAMP indicator *in vivo*.

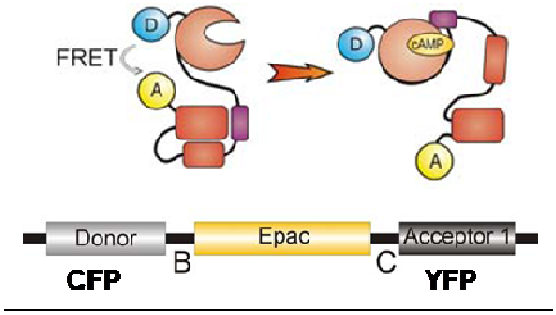


Figure 18. Model for the conformational change following binding of cAMP to the regulatory domain of Epac (Krogt *et al*., 2006).

1.6.2 Probes for monitoring spatio-temporal oscillations of PKA activity *in vivo*

The involvement of PKA in regulating diverse cellular processes (i.e. gene expression, cellular metabolism, cell growth and proliferation) requires that its activity is regulated precisely within the cellular context (Allen and Zhang, 2003). PKA is compartimentalized, localized to specific cellular regions and recruited to specific substrates via interaction with A-kinase anchoring proteins (AKAPs) (Wong *et al*., 2004; Tasken *et al*., 2004). For monitoring the spatiotemporal dynamics of PKA in mammalian cells, it has been proposed a FRET sensor, based on AKAR3 (A-kinase activity reporter)

(Allen and Zhang, 2006). AKAR3 is a recombinant protein composed of a phosphoamino acid binding domain and PKA-specific substrate sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (Zhang *et al*., 2001b and 2005). When phosphorylated by PKA, intramolecular binding of the substrate by the phosphoamino acid domain drives a conformational reorganization, leading to an increase in FRET between CFP and YFP (Fig.19). The AKAR-3 probe in mammals is a highly sensitive indicator of spatiotemporal oscillation of PKA activity *in vivo*.

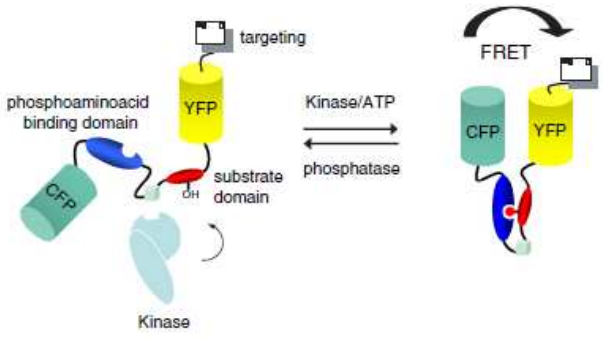


Figure 19. Basic structure of a kinase activity reporter (Ni *et al*., 2006)

MATERIALS AND METHODS

2.1 Plasmids

2.1.1 pYX yeast expression vectors

Yeast expression vectors of the pYX series allow maximum flexibility for expression of a particular gene in a chosen strain of *Saccharomyces cerevisiae*. They all vary from each other in different promoters, selectable markers and the presence or absence of the 2 micron plasmid origin.

pYX212 (Fig.1A) and pYX242 (Fig.1B) are vectors both containing the 2 micron origin of replication (*2μ ori*) sequence which guarantees the stable maintenance of a multicopy number of the plasmid in yeast. They also have in common a selectable marker that encodes for antibiotic resistance in *E. coli* (*AmpR*), a multiple cloning site with unique restriction sites used to clone the DNA of interest into the vector, and a TPI promoter (Triose Phosphate Isomerase) providing the control of the gene transcription. The two vectors differ from each other in the choice of auxotrophic selectable marker that makes a selection in *S. cerevisiae* possible. As pYX212 has a *URA3* yeast selectable marker, pYX242 on the other hand has a *LEU2* marker.

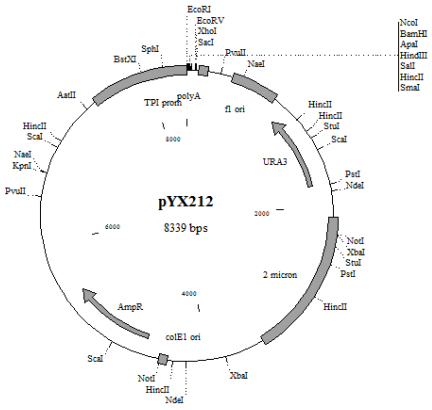


Figure 1A. Map of expression vector pYX212

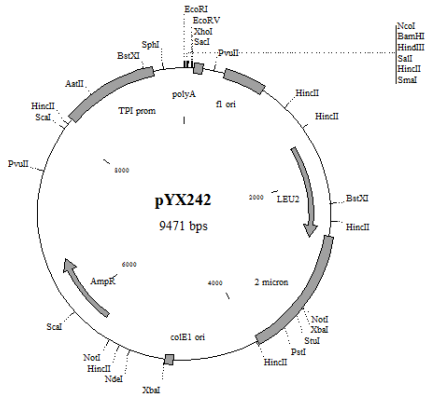


Figure 1B. Map of expression vector pYX242

2.1.2 pYX212-EGFP-RBD3 and pYX242-EGFP-RBD3

To obtain these plasmids the peGFP-C2 construct expressing the fusion eGFP-RBD3 (Augsten *et al.,* 2006) was digested with *Nhe*I, and the pYX expression vector, pYX212 and pYX242, were digested with *Eco*RI. Filling in of the 3' recessing ends was done using the Klenow fragment of DNA polymerase I (New England Biolabs, MA, United States). The blunted DNA was then digested with *Sal*I and ligated with the linearized vectors. (Fig.2).
The pYX212-EGFP-RBD3 and pYX242-EGFP-RBD3 plasmids are used to detect *in vivo* the localization of active Ras proteins, indeed in the probe eGFP-RBD3 the Enhanced Green Fluorescent Protein EGFP is fused to a trimeric Ras-binding domain (RBD3) of human Ras effector c-Raf-1, which binds active-Ras with a much higher affinity than inactive Ras.

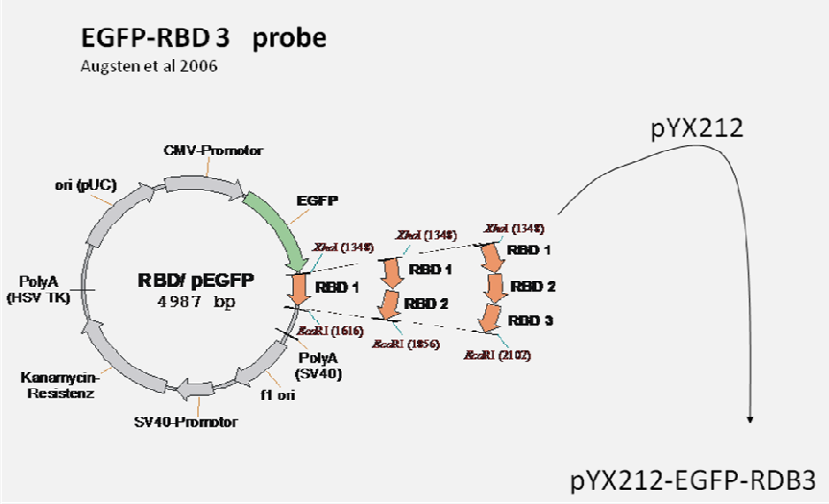


Figure 2. Map of the plasmid containing the eGFP-RBD3 probe (Augsten *et al*., 2006) and synthetic scheme of the cloning.

2.1.3 pYX212-YFP-EPAC1-CFP

To obtain this plasmid the pcDNA3 construct expressing the YFP-Epac1-CFP probe (Nicolaev *et al*., 2004) and the pYX expression vector pYX212 were

digested with *Hind*III and *Xho*I. The fragments obtained were then ligated (Fig.3). The pYX212-YFP-Epac1-CFP plasmid is used to measure the level of cAMP in a single yeast cell and to test directly the presence of cAMP oscillations *in vivo.*

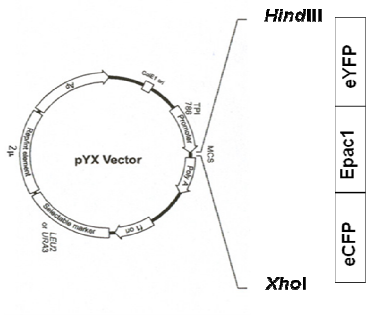


Figure 3. Synthetic scheme of pYX212-YFP-Epac1-CFP cloning.

2.1.4 pYX212-YFP-AKAR3-CFP

To obtain this plasmid the pcDNA3 construct expressing the AKAR3 probe (Allen and Zhang, 2006) was digested with *Bam*HI and *Xba*I, and the pYX expression vector pYX212 was digested with *Bam*HI and *Nhe*I. The fragments obtained were then ligated (Fig.4).

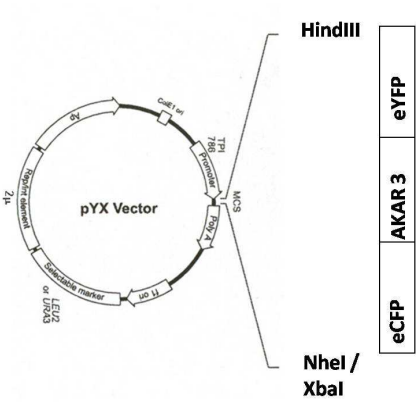


Figure 4. Synthetic scheme of pYX212-AKAR3 cloning.

The pYX212-AKAR3 plasmid is used to monitor the oscillations of PKA activity in a single yeast cell *in vivo.*

2.1.5 pGEX2T-RBD

This plasmid encode Ras Binding Domain of human Raf1 (aa 51-131) fused to GST.

2.1.6 pNESHIS5

pNESHIS5 is a plasmid of 5013 base pairs which include over 40 restriction sites. The plasmid contains an Ampicillin Resistance gene (Amp^R) for *E.coli*, a Yellow Fluorescent Protein (*YFP*) gene, the *NES3* sequence, the *SpHIS5* yeast selective marker and two multiple cloning sites.

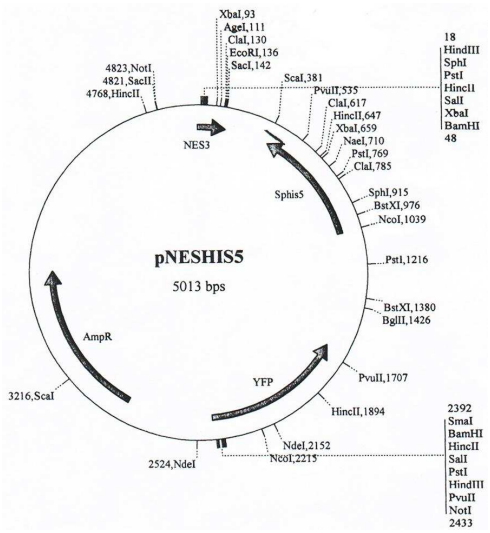


Figure 5. Map of the plasmid pNESHIS5.

2.1.7 YCp50Ras2

The YCp50RAS2 plasmid consists of 9111 base pairs and contains an *URA3* selection marker. It is a plasmids that was constructed by inserting the *RAS2* sequence in a YCp50 plasmid, at the *Hpa*I restriction site.

2.2 Strains

2.2.1 *Escherichia coli*

Strain	**Genotype**
Top10	F´ *mcr*AΔ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*Kλ- *rps*L *end*A1 *nup*G

2.2.2 *Saccharomyces cerevisiae*

Strain	Genotype	Reference
W303-1A	*MAT* **a** *ade2-1 can1-100 his3-11,15 leu2-31 12 trp1-1 ura3-1*	Thomas and Rothstein (1989)
W303-1A(YCpRas2^{Val19})	W303-1A + YCpRas2^{Val19} (URA marker)	Colombo *et al.* (2004)
KT98	*MAT* **a** W303 expressing a fusion of Nup49 protein with the fluorescent protein t-dimer	Sheff and Thorn (2004)
GG104	*MAT* **a** W303-1A *cyr1::kanMX2 pde2::TRP1 msn2::HIS3 msn4::TRP1*	Roosen *et al.* (2005)
GG104 *cdc25Δ*	GG104 with *cdc25::URA3*	This study
PM731	*MAT* **a** W303-1A with *gpa2::URA3*	Colombo et al. (1998)
LK42/RL5	*Mat a* W303 with *gpr1::LEU2*	Kraakman *et al.* (1999)
YSH327	*Mat a* W303 with *hxk1::HIS3 hxk2::LEU2*	De Winde *et al.* (1996)
YSH297	*Mat a* W303 with *hxk1::HIS3*	De Winde *et al.* (1996)

YSH310	*Mat a* W303 with *hxk2::LEU2*	De Winde *et al.* (1996)
JT2083	*Mat a W303 with hxk1::HIS3, hxk2::TRP1*	J.Thevelein
JT2083 (YCpHXK2)		This study
Tlys86	*Mat* a *leu2::hisG ura3-52 trp1::hisG*	G.R. Fink
NepH8*gpa2::*	*Mat* α *gpa2::LEU2*	G.R. Fink
W303-NES-Ras2	*Mat a* W303 with NES-Ras2	This study
Tlys86-NES-Ras2	*Mat a* Tlys86 with NES-Ras2	This study
W303-4xHA-Cdc25NES		Tisi *et* al. (2008)
WΔN1	*Mat α* W303 with *cdc25::CDC25 (aa 907-1589)/URA3*	Belotti *et al*. (2006)
WΔCdc25^{Mm}	*Mat α* W303 with *cdc25::ADH prom-Cdc25Mm (aa 976 - 1262)/URA3*	Belotti *et al*. (2006)
WΔhSos1	*Mat α* W303 with *cdc25::ADHprom-hSos1 (aa 549-1333)/URA3*	Belotti *et al*. (2006)
RT990	*Mat a* with *cyr1::KanMX pde2::TRP1 yak1::LEU2*	Görner *et al*. (2002)
JT 122	*Matα leu2 his3 trp1 ade8 ura3* ***tpk1^{w1}*** *tpk2::HIS3 tpk3::TRP1*	M. Wigler (1988)
	Mat a SP1 with *tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*	

SP1	*Mata leu2 his3 trp1 ade8 can1 ura3*	M. Wigler (1988)
JT105 B	SP1 with *bcy1::LEU2*	
JW01072	W303 with *bcy1 ::URA3*	
2455	*MAT α ura3 trp1 his3 ras1::HIS3 ras2^{C318S} leu2*	Broach J., Princeton University

2.3 Growth conditions and cultural medium composition

Escherichia coli

E.coli strains were grown in LB (LuriaBroth) medium (1% NaCl, 1% Bacto-peptone, 0.5% yeast extract, 0.1% glucose) at 37°C. The solution is autoclaved at 120°C – 1 atm for 30 min. For Amp^R selection, after cooling down, ampicillin is added from a stock of 50 mg/ml to a final concentration of 100 µg/ml. The plates are prepared as mentioned above, with 2% agar.

E. coli strains can be conserved for many years at -80°C in cultural medium or in an aqueous solution containing 50% glycerol.

Saccharomyces cerevisiae

Yeast cells were grown in YP (1% yeast extract, 2% Bacto-peptone) supplemented with 50 mg/l adenine and 2% w/v glucose (YPDA).
Yeast strains carrying a plasmid were grown in minimal medium (SC) containing 0.67% w/v yeast nitrogen base w/o amino acids, synthetic amino acid mixture required and the appropriate sugar (2% glucose, 2% galactose, 2% fructose, 3% glycerol, 3% ethanol, 3% glycerol with 0.1% glucose). Solid medium contained 2% agar. The solutions are autoclaved at 120°C – 1 atm for 30 min. Solid medium contained 2% agar.
Carbon sources were prepared and autoclaved separately and were added to the different media after the autoclave. The carbon sources stocks have different concentration: glucose 40%, glycerol 87%, galactose 20%,

raffinose 20%, fructose 40%, saccarose 40%. The stock solution of Yeast Nitrogen Base has a concentration of 6,7%.
All amino acids are stored with a concentration of 5 g/l, except for the stock solutions of uracil and adenine which are 1,25 g/l. YNB and the individual amino acids were sterilized by filtration and added to the medium after the autoclave.
For plates containing 0.5 M NaCl and H_2O_2 (2 mM or 6mM), NaCl and H_2O_2 were sterilized by filtration and then added to the autoclaved medium.
In all conditions cells were grown at 30°C.

S. cerevisiae strains can be conserved for many years at -80°C in an aqueous solution containing 15% glycerol.

2.4 Creation of the W303-NES-RAS2 strain

2.4.1 Creation of the plasmid pNESRAS2

- The pNESHIS5 plasmid was digested with *EcoR*I and *Sac*I.

- The *RAS2* sequence was obtained from the YCp50Ras2 plasmid by PCR. The primers used for the PCR contain the restriction sites (sequence underlined) for *EcoR*I in the forward (FW) primer, and for *Sac*I in the reverse (RW) primer.

  Ras2FW:
  CCGGAATTC**ATGCCTTTGAACAAGTCGAAC**

  Ras2RW:
  CGCGAGCTC**TTAACTTATAATACAACAGCCACCCG**

DNA (YCp50Ras2)	40 ng
Phusion Buffer HF	1X
dNTP	0.2 mM
Ras2FW	0.5 µM
Ras2RW	0.5 µM
Phusion DNA polymerase	1 u

(BioLabs M0530S)	
H2O a volume	to 50 µl

The following PCR reaction program was used:

Step 1 98°C 2 min
Step 2 98°C 10 sec
Step 3 66°C 20 sec
Step 4 72°C 45 sec

Repeat steps 2 to 4 for 25 cycles

Step 5 72°C 10 min
Step 6 4°C 24 hrs

- The PCR product (about 1000bps) was digested with *EcoR*I and *Sac*I and then ligated with the pNESHIS5 plasmid previously digested with the same enzymes.

2.4.2 PCR to amplify the NES-RAS2-HIS5 cassette

With this PCR we amplified from the pNESRAS2 construct the cassette that will be used to substitute the *RAS2* gene in the yeast genome. The primers used in this reaction both contain a piece of the yeast genome, 54 bases upstream (FW) and 54 bases downstream (RW) of the *RAS2* sequence, followed by 20 nucleotides of the pNESHIS5.

NESRas2FW b:
TAAAAAAACCAAGTTAACCGTTTTCGAATTGAAAGGAGATATACAGAAAAAAAA**ATGACCATGATTACGCCAAG**

underlined: 1-54 nucleotides upstream from the *RAS2* ATG start codon
in bold: nucleotides 1 – 20 of the pNESHIS5 plasmid

NESRas2RW:
CCGGCAACCATATGATATTGCCCAAAGTTTCCAATGTTAACAAGAATGAAACGG**C GCCACTTCTAAATAAGCGA**

underlined: 137–191 nucleotides downstream from the *RAS2* TAA stop codon

in bold: nucleotides 1645 – 1664 of the pNESHIS5 plasmid

DNA (pNESRAS2)	40 ng
Phusion Buffer HF	1X
dNTP	0.2 mM
NESRas2FWb	0.5 µM
NESRas2RW	0.5 µM
Phusion DNA polymerase (BioLabs M0530S)	1 u
H2O a volume	to 50 µl

The following PCR reaction program was used:

Step 1 98°C 2 min
Step 2 98°C 10 sec
Step 3 66°C 20 sec
Step 4 72°C 135 sec

Repeat steps 2 to 4 for 4 cycles

Step 5 98°C 10 sec
Step 6 72°C 20 sec
Step 7 72°C 135 sec

Repeat steps 5 to 7 for 4 cycles

Step 8 72°C 10 min
Step 9 4°C 24 hrs

The PCR product (about 2750 bps) was used to transform the yeast W303-1A cells. The presence of the *NES-RAS2* gene instead of the wt *RAS2* gene was controlled by colony PCR.

2.4.3 Colony PCR

The primers used in this experiment are small primers, which consist of 20 nucleotides: FW = 198 – 218 nucleotides upstream from the *RAS2* ATG start codon and RW = complementary to nucleotides 700 – 719 of the *RAS2* sequence.

Colonies that were unsuccessfully transformed will show a band of about 937 bps, the successful ones will show a band that is larger (about 1084 bps), containing the *NES* sequence.

cntNESRas2FW:
CGTTGTCTTCTCTTATCGCC

cntNESRAS2RW
GTCTTACTCTCTATGCTTGC

Each colony was suspended in a seperate tube in 10 µl H_2O. Then 2 mix were prepared for each colony.

Mix A for each colony:

10X Buffer	1x
5µM primer cntNESRas2FW	0.5µM
5µM primer cntNESRas2RW	0.5µM
50mM $MgCl_2$	2.5 mM
H_2O	to 12.2 µl
Final volume	12.2 µl

Mix B for each colony:

2.5mM dNTP	0.2 mM
@Taq polymerase (Euro Clone, cod. EME013500)	2u

PCR program

Step 1 95°C 5min
Step 2 92°C 50 sec
Step 3 54°C 1 min
Step 4 74°C 1 min 30 sec

Repeat steps 2 to 4 for 33 cycles

Step 5 74°C 10 min
Step 6 4°C 24 hrs

2.5 Creation of the Tlys86-NES-RAS2 strain

2.5.1 Creation of the pYX212-NESRAS2 plasmid

The sequence coding for the fusion protein NESRAS2 was obtained by digestion of the pNESRas2 plasmid with *Hpa*I and *Sac*I. The fragment containing this sequence was ligated with the pYX212 plasmid digested with *Sma*I and *Sac*I.

2.5.2 PCR to amplify the NES-RAS2-URA3 cassette

With this PCR we amplified from the pYX212-NESRAS2 construct the cassette that will be used to substitute the *RAS2* gene in the yeast genome of Tlys86 cells. The primers used in this reaction both contain a piece of the yeast genome, 54 bases upstream (FW) and 54 bases downstream (RW) of the *RAS2* sequence, followed by 20 nucleotides of the pYX212-NESRas2.

NESRas2FW b:
TAAAAAAACCAAGTTAACCGTTTTCGAATTGAAAGGAGATATACAGAAAAAAAA**ATGACCATGATTACGCCAAG**

underlined: 1-54 nucleotides upstream from the *RAS2* ATG start codon
in bold: nucleotides 1 – 20 of the pYX212-NESRas2 plasmid

pYXNESRas2RW:
CCGGCAACCATATGATATTGCCCAAAGTTTCCAATGTTAACAAGAATGAAACGG**TACTGAGAGTGCACCATACC**

underlined: nucleotides downstream from the *RAS2* TAA stop codon
in bold: nucleotides 3023-3042 of the pYX212-NESRas2 plasmid downstream of the *URA3* sequence

DNA (pYX212-NESRas2)	40 ng
Phusion Buffer HF	1X
dNTP	0.2 mM
NESRas2FWb	0.5 µM
NESRas2RW	0.5 µM
Phusion DNA polymerase (BioLabs M0530S)	1 u

H2O a volume	to 50 µl

The following PCR reaction program was used:

Step 1 98°C 2 min
Step 2 98°C 10 sec
Step 3 66°C 20 sec
Step 4 72°C 155 sec

Repeat steps 2 to 4 for 4 cycles

Step 5 98°C 10 sec
Step 6 72°C 20 sec
Step 7 72°C 155 sec

Repeat steps 5 to 7 for 4 cycles

Step 8 72°C 10 min
Step 9 4°C 24 hrs

The PCR product (about 3150 bps) was used to transform the yeast Tlys86 cells. The presence of the *NES-RAS2* gene instead of the wt *RAS2* gene was controlled by colony PCR.

2.5.3 Colony PCR

The primers used in this experiment are small primers, which consist of 20 nucleotides: FW = 198 – 218 nucleotides upstream from the *RAS2* ATG start codon and RW = complementary to nucleotides 700 – 719 of the *RAS2* sequence.

Colonies that were unsuccessfully transformed will show a band of about 937 bps, the successful ones will show a band that is larger (about 1084 bps), containing the *NES* sequence.

cntNESRas2FW:
CGTTGTCTTCTCTTATCGCC

cntNESRAS2RW
GTCTTACTCTCTATGCTTGC

Each colony was suspended in a seperate tube in 10 µl H_2O. Then 2 mix were prepared for each colony.

Mix A for each colony:

10X Buffer	1x
5µM primer cntNESRas2FW	0.5µM
5µM primer cntNESRas2RW	0.5µM
50mM $MgCl_2$	2.5 mM
H_2O	to 12.2 µl
Final volume	12.2 µl

Mix B for each colony:

2.5mM dNTP	0.2 mM
@Taq polymerase (Euro Clone, cod. EME013500)	2u

PCR program

Step 1 95°C 5min
Step 2 92°C 50 sec
Step 3 54°C 1 min
Step 4 74°C 1 min 30 sec

Repeat steps 2 to 4 for 33 cycles

Step 5 74°C 10 min
Step 6 4°C 24 hrs

2.6 Trasformation of *E. coli*

The cells are made competent so the uptake of the plasmid is made possible. The successfully transformed cells will be able to multiply very fast in a selective medium. This means that a large quantity of the plasmid will be generated.

SOLUTIONS

- ✓ Liquid LB medium
- ✓ MOPS1 pH 7.0:
  - 10 mM MOPS
  - 10 mM RbCl
- ✓ MOPS2 pH 6.5:
  - 100 mM MOPS
  - 10 mM RbCl
  - 50 mM $CaCl_2$

PROTOCOL

- Dilute an overnight bacterial culture 1:50 in LB medium and grown at 37°C for 2-3 hours (OD 600= 0.2-0.3).
- Collect the cells at 4000 rpm for 10 minutes. Discard the supernatant.
- Resuspend the pellet in ½ of the original culture volume with cold MOPS 1 solution.
- Centrifuge at 4000 rpm for 10 minutes. Discard the supernatant.
- Resuspend the pellet in ½ of the original culture volume with cold MOP 2 solution. Keep cells on ice for 15 minutes.
- Centrifuge at 4000 rpm for 10 minutes. Discard the supernatant.
- Resuspend the pellet in 1/10 of the original culture volume with cold MOPS 2 solution.
- Transfer 200 µl of cell suspension in new tubes. Add 3µl of DMSO to each transformations.
- Add DNA of interest (1-200 ng) to cells. In parallel way it's useful transform one aliquot with 1-10 ng of purified DNA as positive control.

Use one aliquot without DNA as negative control to test bacteria sensibility to antibiotic.

- Keep cells on ice for 30 minutes.
- Heat shock cells at 43°C for exactly 30 seconds.
- Add 1 ml of LB medium to each samples and incubate at 37°C for 1 hour.
- Centrifuge at 4000 rpm for 5 minutes at 4°C.
- Spread the cells on LB + ampicillin plates and incubate at 37°C overnight.

2.7 "MIDI-prep" extraction of plasmid DNA from *E. coli*

SOLUTIONS

- ✓ Lysis buffer :
  - 50mM glucose
  - 10 mM EDTA
  - 25 mM Tris-HCl (pH 8)
- ✓ Hypertonic Saline Solution (HSS):
  - 120 ml of potassium acetate 5 M
  - 23 ml of acetic acid
  - 57 ml of water MilliQ
- ✓ TE buffer:
  - 10mM Tris-HCl, pH8.0
  - 1mM EDTA

PROTOCOL

- Inoculate 50 ml of E. coli culture at 37°C overnight.
- Harvest cells at 4000 rpm for 10 min.
- Resuspend the bacterial pellet in 4ml of ice-cold Lysis buffer and incubate the tube on ice for 10 min.
- Add 8 ml of freshly prepared 0.2 N NaOH/ 1% SDS solution, mix and store the tube 10 min. at room temperature.
- Add 6 ml of ice-cold 5 M HSS and mix the content by vortexing. Store the tube on ice for 10 min.
- Centrifuge at 4000 rpm at 4°C for 10 min.

- Pour the supernatant into a clean tube through a small two-ply square of cheesecloth placed in the centre of a funnel.
- Precipitate the nucleic acids by adding equal volume of isopropanol to the tube and store it at -20°C for 15-20 min. Centrifuge at 4000 rpm at 4°C for 30 min.
- Discard supernatant and dry the pellet as well as possible.
- Dissolve the pellet in 1 ml of TE buffer, add 1 ml of 5 M LiCl and mix the content by inverting the tube.
- Incubate the tube 30 min. on ice.
- Centrifuge at 13000 rpm at 4°C for 10-15 min.
- Pour off the supernatant containing plasmid DNA into a clean tube. Add 0.1 volume of 3 M NaAc pH 5.2 and a volume of isopropanol.
- Precipitate the nucleic acids at -20°C for 10-20 min.
- Centrifuge at 13000 rpm at 4°C for 20 min. and dry the pellet.
- Resuspend the pellet in 500 µl of TE containing DNAse-free pancreatic RNAse (40 µg/ml). Vortex briefly. Store at 37°C for 20 min.
- Extract proteins from the plasmid DNA adding an equal volume of phenol/chloroform/isoamyl alcohol. Vortex vigorously for 30 seconds. Centrifuge at full speed for 5 min. at room temperature.
- Remove upper aqueous layer containing the plasmid DNA carefully and repeat the previous step.
- Add 5 M KAc (final 0.3 M) and 2 volumes of absolute ethanol to precipitate the plasmid DNA.
- Store at -80°C for 15-30 min or overnight. Centrifuge at full speed for 15 min. at 4°C. Dry the pellet.
- Dissolve the pellet in 50-100 µl of TE or H_2O.
- Measure the concentration of the plasmid DNA by gel electrophoresis.

2.8 DNA gel electrophoresis

Gel electrophoresis allows to separate a mixture of molecules through a stationary material (gel) in an electric field. Agarose gel is usually used as a support for separation of the DNA fragments.

SOLUTIONS

- ✓ TAE Buffer pH8:
  - Tris-HCl 242 g
  - glacial acetic acid 57.1 ml
  - EDTA 18.61 g
  - dH2O to 1 L

PROTOCOL

Agarose powder was dissolved in TAE buffer. Gels were run in the same buffer at 4-6 Volt/cm for 1-2 hours. DNA samples were prepared by mixing with a "loading buffer" containing 50% glycerol and 0.25% bromophenol blue.

Molecular weight markers, a mixture of DNA fragments with known molecular weights, are used to estimate the sizes of DNA fragments in the sample. DNAs were visualized with UV light by staining the gel with ethidium bromide 5 µg/ml.

2.9 Counting of yeast cells

In some experiments, like fluorescence microscopy and the preparation of the crude extract, it is necessary to determine the cell concentration. Therefore, 1 ml is taken from an overnight culture and transferred into a tube. These cell samples are sonicated to avoid the formation of cell aggregates. Of each sample a certain volume is taken and diluted into 10 ml of ISOTON. The density of the culture is measured with the Coulter Counter ZBI. This instrument measures the amount of cells in 0,5 ml diluted sample, which allows to calculate the density of the culture.

Sometimes the cells density was determined by measuring optical density at 600 nm (OD600).

2.10 Trasformation of *S. cerevisiae* with LiAc method

This procedure, according to the Gietz method, allows the introduction and expression of plasmid DNA and obtains a very high transformation efficiency of yeast cells.

SOLUTIONS

- ✓ Liquid YPDA medium
- ✓ PEG 6000 (50%)
- ✓ 1 M LiAc
- ✓ 100 mM LiAc
- ✓ Salmon sperm DNA (10 mg/ml)

PROTOCOL

- Preinoculate the desired yeast strains in 5 ml of liquid YPDA and incubate overnight with vigorous shaking at 30°C.
- Inoculate 25 ml of YPDA medium to an OD600 of 0.4.
- Incubate the culture at 30°C with vigorous shaking until the OD600 reaches about 1.5. This will take 4 to 6 hours. This culture will give sufficient cells for 6 transformations.
- Harvest the cells by centrifugation at 4000rpm for 10 minutes.
- Remove the supernatant and resuspend the pellet in 12.5 ml of sterile H_20. Centrifuge again at 4000 rpm for 10 minutes.
- Remove the supernatant, resuspend the pellet in 0.5 ml 100 mM LiAc and transfer the suspension in a 1.5 microfuge tube.
- Centrifuge the cells at 13000 rpm for 15 seconds. Remove the LiAc.
- Resuspend the pellet with 100 mM LiAc to a final volume of 250 µl.
- Mix the cell suspension well and transfer 50 µl into labelled microfuge tubes. Each transformation requires one sample extra to use it as negative control.
- Centrifuge the cells at 13000 rpm for 15 seconds. Remove the LiAc completely.
- Add to each pellet the following components:

  240 µl PEG 6000 (50%)

  36 µl 1 M LiAc

  5 µl Salmon sperm DNA (10 mg/ml)

  50 µl sterile H_20 in which the plasmid DNA (0,1-10 µg) is resuspended.

The order is important because PEG 6000 shields the cells from the detrimental effects of the high concentration of LiAc.

- Resuspend and vortex each tube vigorously until the cell pellet has been completely mixed. Incubate at 30°C for 30 minutes.
- Incubate at 42°C for 20 minutes.
- Centrifuge at 7000 rpm for 15 seconds and remove the transformation mix.
- Wash cells with 1 ml of sterile water.
- Resuspend the pellet in 100 µl of sterile water, spread the cells on selective medium plates and incubate for 2-3 days at 30°C.

2.11 Fluorescence microscopy

Due to expression of the Enhanced Green Fluorescent Protein (EGFP) a fluorescent signal is visible *in vivo* using fluorescence microscopy.

2.11.1 Observation of glucose growing cells

SOLUTIONS

- ✓ Concanavaline A (100µg/ml in H_2O)
- ✓ Opportune minimal synthetic media

PROTOCOL

- The cover glasses are covered completely with concanavaline A (100 µg/ml) and are left like that for at least one hour. Then, the concanavaline A is recuperated and the cover glasses are preserved in water.
- Inoculate the desired strains in 5 ml of minimal medium with 2% glucose and incubate it overnight at 30 °C with vigorous shaking.
- The next day transfer 1 ml of the cellular culture into a tube and sonicate the cells 2 times for about 15 sec. Then count the cells using the Coulter Counter ZBI.

- When the density of the culture is about $5*10^6$-10^7 cells/ml wash four times the concanavaline A coated cover glasses with 1 ml of the proper medium.
- Put on the cover glass 40 µl of the cellular culture and wait 5 min. The concanavaline fixates the yeast cells on the coverglass.
- Wash the coverglass four times with the 1 ml of the proper medium and put it – face down – on the Toma chamber. Dry the glass and the chamber. If necessary, insert a little medium under the cover glass so the cells will not dehydrate.

Images were acquired with a Nikon Eclipse E600 fluorescence microscope equipped with a 60X, 1.4 oil Plan-Apochromat objective and a standard FITC filter set (Nikon, EX 450-490, DM 505, BA 520) for GFP fluorescence and a Cy3 filter set for t-dimer, RFP and Rhodamine fluorescence. Images were recorded digitally using a Leica DC 350F camera and processed using Adobe Photoshop (Adobe Systems, Inc.).

For confocal microscopy, a Leica TCS SP2 confocal microscope equipped with an inverter Leica DMIRE2 microscope and a PL APO 63 x oil immersion objective (numerical aperture: 1.4) was used. An average of 10 optical sections were acquired for every single cell (Z scan: 0.9 µm).

2.11.2 Determination of the spatio-temporal localization of active Ras in vivo in single cells after addition of either glucose or fructose to glucose-starved cells

SOLUTIONS

- ✓ Concanavaline A (100µg/ml in H_2O)
- ✓ MES buffer (25 mM 2-(N-morpholino)ethanesulfonic acid) pH 6.0
- ✓ Opportune minimal synthetic media
- ✓ 3% glucose or fructose in MES

PROTOCOL

- The cover glasses are covered completely with concanavaline A (100 µg/ml) and are left like that for at least one hour. Then, the concanavaline A is recuperated and the cover glasses are preserved in water.
- Inoculate the desired strains in 5 ml of minimal medium with 2% glucose and incubate it overnight at 30 °C with vigorous shaking.
- The next day transfer 1 ml of the cellular culture into a tube and sonicate the cells 2 times for about 15 sec. Then count the cells using the Coulter Counter ZBI.
- When the density reaches about 10^7 cells/ml, harvest by centrifugation for 5 minutes at 3000 rpm 4 ml of cells are. Carefully remove the medium.
- Wash the cells two times with MES buffer.
- Resuspend the cells in MES buffer at $5*10^7$ cells/ml. Incubate the cells for about 1 hour and 40 minutes.
- Wash four times the concanavaline A coated cover glasses with 1 ml of MES buffer.
- Put 40 µl of the cellular resuspension and wait 5 min. The concanavaline fixates the yeast cells on the coverglass.
- Wash the coverglass four times with the 1 ml of MES and put it – face down – on the Toma chamber. Dry the glass and the chamber. If necessary, insert a little MES under the cover glass so the cells will not dehydrate.
- Several domains are photographed before addition of 100mM glucose.
- Add 20 µl of glucose/fructose (3% in MES) and keep making pictures for 15 min (or more) of the same domain in the green light, to follow the reaction.

Images were acquired with a Nikon Eclipse E600 fluorescence microscope equipped with a 60X, 1.4 oil Plan-Apochromat objective and a standard FITC filter set (Nikon, EX 450-490, DM 505, BA 520) for GFP fluorescence and a Cy3 filter set for t-dimer, RFP and Rhodamine fluorescence. Images were recorded digitally using a Leica DC 350F camera and processed using Adobe Photoshop (Adobe Systems, Inc.).

2.11.3 Staining of yeast cells with Rhodamine B hexyl ester perchlorate

SOLUTIONS

Rhodamine B hexyl ester perchlorate (Molecular Probes, Eugene, Oregon) stock 1 mM in DMSO diluted in water at time of use to a final concentration of 10 µM. Added to the cells to a final concentration of 100 nM.

PROTOCOL

Incubate the cells with the mitochondrial marker Rhodamine B hexyl ester perchlorate (100 nM final) for 5-10 min at 30 °C in the dark while shaking, the signal from the mitochondria will become very strong. Images were acquired with Nikon Eclipse E600 fluorescence microscope equipped with a 60X, 1.4 oil Plan-Apochromat objective.

2.12 Invasive growth test

SOLUTIONS

- ✓ YPDA

PROTOCOL

- Inoculate the desired strains in 5 ml of YPDA and incubate it overnight at 30 °C with vigorous shaking.
- The day after count the cells with the Coulter Counter ZBI.
- Spot on YPD agar plates three times 5 µl of cells.
- Incubate 3 days at 30°C.
- Wash gently the plates from the top down with water, using a pipette. For the Tlys86 background the plates were washed directly under the running water.

2.13 Heat Shock sensitivity assay

SOLUTIONS

✓ YPDA or opportune selective liquid medium

PROTOCOL

- Inoculate the desired strains in 5 ml of complete or selective medium containing 2% glucose and incubate overnight at 30 °C with vigorous shaking.
- When the density reaches about $5*10^6$ cells/ml dilute the cells in fresh medium to a concentration of $1.25*10^6$ cells/ml.
- Put 100 µl of the diluted cultures in 6 new microfuge tubes.
- Shock the cells at 51°C for 1, 2, 3, 4 and 5 minutes. Remember to include an untreated sample as control.
- Spot 5 µl of cells in complete or selective agar medium and incubate at 30°C.

2.14 Osmotic and oxidative growth test

SOLUTIONS

✓ YPDA

PROTOCOL

- Inoculate the desired strains in 15 ml of YPDA and incubate overnight at 30 °C with vigorous shaking.
- When the density reaches about $5*10^6/10^7$ cells/ml, harvest by centrifugation the cells.
- Wash three times the cells with sterile water.
- Resuspend the cells in sterile water to a concentration of $1*10^7$ cells/ml and prepare three 10-fold serial dilutions.
- Spot 5µl of the concentrated suspension and of the different 10-fold serial dilutions on the different agar plates: YPD agar plates, YPD agar plates containing 0.5M NaCl, YPD agar plates containing 2 mM H_2O_2, YPD agar plates containing 6 mM H_2O_2.
- Incubate at 30°C.

2.15 Growth test on different carbon sources

SOLUTIONS

✓ YPDA

PROTOCOL

- Inoculate the desired strains in 15 ml of YPDA and incubate overnight at 30 °C with vigorous shaking.
- When the density reaches about $5*10^6/10^7$ cells/ml, harvest cells by centrifugation.
- Wash three times the cells with sterile water.
- Resuspend the cells in sterile water to a concentration of $1*10^7$ cells/ml and prepare three 10-fold serial dilutions.
- Spot 5µl of concentrated suspension and of the different 10-fold serial dilutions on the different agar plates: YPD agar plates, YPEtOH (Ethnanol) agar plates, YPGly (Glycerol) agar plates.
- Incubate at 30°C.

2.16 Preparation of the crude extract of yeast cells

This technique is used to extract the total amount of proteins of *S. cerevisiae* cells.

SOLUTIONS

✓ Shalloway buffer (Tayler and Shalloway, 1996)

Components	Final concentration	Stock solution	Volume
Hepes pH 7.5	25 mM	200 mM	5 ml
NaCl	150 mM	5 M	1.2 ml
NP-40	1%	10%	4 ml
Na- deoxycholate	0.25%	5%	2 ml

Glycerol	10%	87%	4.6 ml
NaF	25 mM	0.5 M	2 ml
$MgCl_2$	10 mM	75 mM	5.3 ml
EDTA	1 mM	0.5 M	80 µl
Prot. Inhibitor cocktail	1 X	25 X	1.6 ml
Na-Vanadate	1 mM	100 mM	0.4 ml
H2O			13.8 ml
Final volume			**40 ml**

PROTOCOL

- Inoculate the cells in 20 ml of selective minimal medium and incubate at 30 °C with shaking until the density of the culture is about 5×10^6 - 10^7 cells/ml.
- Harvest about 3×10^8 yeast cells by centrifugation at 3000 x *g* for 5 min at 4°C. Remove the supernatant, add 1 ml H20 to wash the cells, resuspend and transfer them to a 1.5 ml Fastprep tube.
- Centrifuge at 13000 rpm for 2 minutes at 4°C.
- Remove the supernatant and eventually store the pellet at -80°C until use.
- Add to the cells 300 µl of Shalloway buffer and 300 µl of glass beads.
- Disrupt the cells using a Fastprep instrument, set on speed 6: 3 cycles, 20 sec each, with a 1 min cooling period between each cycle.
- Centrifuge for 1 min at 13.000 rpm at 4 °C to pellet the cellular debris. Transfer the supernatant to a clean tube.
- Centrifuge at 13.000 x *g* for 10 min at 4°C and transfer the supernatant to a clean tube.
- Centrifuge at 13.000 x *g* for 20 min at 4°C and transfer the supernatant to a clean tube.
- Store in ice until use.

2.17 Bradford assay

The Bradford protein assay is an analytical procedure for determining the concentration of protein in solution. It involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. The absorbance maximum of the dye increases from 465 nm in acidic conditions to 595 nm after binding to proteins. At this wavelength the dye is in a stable, blue form which can be detected at 595 nm with the spectrophotometer. There is a linear relationship between the absorbance and the total protein concentration of the sample within the range defined by the standard, bovine serum albumin (BSA). The more protein present in the sample, the more blue the Coomassie gets. There is a linear relationship between the absorbance maximum and the protein concentration, which enables interpolation of unknown samples. Disadvantages of this assay are the interference with some reagents and detergents, and the protein-to-protein variation because Coomassie binds primarily to basic aromatic amino acid residues.

SOLUTIONS

- ✓ Bradford reagent: Biorad protein assay reagent diluted 1:5 in milliQ H2O. Store at 4°C
- ✓ Bovine serum albumin (BSA): 1 mg/ml. Store at -20°C.

PROTOCOL

- Prepare standards in plastic cuvettes twice containing 2, 4, 6 and 8 µl BSA in 1 ml diluted BioRad reagent. Cover the cuvettes with Parafilm and mix very well.
- Incubate at room temperature for at least 5 minutes. Samples should not be incubated longer than 1 hour.
- Measure the absorbance at 595 nm with the spectrophotometer. Set up a standard curve of absorbance versus concentration of BSA. Make sure the curve is linear to be able to interpolate unknown amounts of proteins.
- Dilute the samples by adding 1 ml of the same diluted BioRad reagent to 2-3 µl of sample in plastic cuvettes so that the absorbance falls within

the range of them standard curve. Prepare each sample twice. Cover and mix very well.

- Measure after 5 minutes the absorbance at 595 nm. Calculate the amount of proteins in the original sample by interpolating the average of the two values into the standard curve.

2.18 Determination of the Ras2-GTP/Total Ras2 Ratio *in vivo*

2.18.1 *E. coli* culture growth for preparative purification

SOLUTIONS

- ✓ LB medium
- ✓ Ampicillin (500x): 50 mg/ml in water (store in small aliquots at – 20°C)
- ✓ Isopropyl 1-thio-β-D-galactopyranoside (IPTG) (1000x): 100 mM in water (store in small aliquots at – 20°C)

PROTOCOL

- Inoculate the JM109 strain carrying the plasmid pGEX2T-RBD in 40 ml of LB broth containing ampicillin (100μg/ml) and grow the culture at 37°C overnight with vigorous shaking.
- Inoculate 1 liter of LB broth containing ampicillin (100μg/ml) 1:25 with the non-induced overnight culture and grow at 37°C with vigorous shaking until an OD_{600} of 0.5 is reached (about 2-3 hours).
- Induce expression by adding IPTG to a final concentration of 0.1 mM and incubate the culture for 4-5 h, at 30°C with vigorous shaking.
- Harvest the cells by centrifugation at 4000 x *g* for 20 min at 4°C.
- Store cell pellet at -80°C or proceed immediately to protocol for preparation of cleared *E. coli* lysates.

2.18.2 Preparation of cleared *E. coli* lysates under native conditions

SOLUTIONS

- ✓ PBS 10X pH 7.4

Components	Final concentration
NaCl	1.37 M
KCl	0.027 M
$Na_2HPO_4 \cdot 7H_2O$	0.043 M
KH_2PO_4	0.014 M

✓ Lysis buffer A

Components	Final concentration
EDTA	1 mM
DTT	0.5 mM
PBS	1X
Prot. Inhibit. cocktail	1 table/50 ml

✓ 10% (w/v) Triton X-100 in PBS 1X

✓ Glycerol

PROTOCOL

- Thaw the cell pellet and resuspend the cells in 40 ml Lysis buffer A.
- Separate the cell suspension into two 50 ml tubes.
- Put the two 50 ml tubes at -80°C for 15 min and defrost the cell suspensions in H_2O.
- Separate the cell suspension into four 50 ml tubes (about 10 ml cell suspension each tube) and add 4 ml glass beads to each tube.
- Brake the cells using a vortex: 5 cycles, 1 min each, with a 1 min cooling period between each cycle.
- Add TritonX-100 to a final concentration of 1% (use 10% TritonX-100 in PBS 1x).
- Incubate at 4°C for 30 min, mixing gently on a rotary shaker.

- Centrifuge lysate at 10.000 x *g* for 20 min at 4°C to pellet the cellular debris. Save supernatant.
- Add Glycerol to a final concentration of 10%.
- Store in aliquots at -80°C or proceed immediately to protocol for preparation of GST-RBD pre-bound to Glutathione Sepharose 4B.

2.18.3 Preparation of GST-RBD pre-bound to Glutathione Sepharose 4B

SOLUTIONS

- ✓ Glutathione Sepharose 4B (GE Healthcare)
- ✓ PBS 10X pH 7.4
- ✓ Phosphate buffered saline containing 1 mM EDTA (PBSE)
- ✓ Shalloway buffer
- ✓ PBS buffer

Components	Final conc.	Stock solution	Volume
Triton X-100	1%	10%	1 mL
Glycerol	10%	87%	1,150 mL
EDTA	1 mM	0,5 M	20 µL
DDT	0,5 mM	0,5 M	10 µL
Prot.inhibit.cockt.	1 X	25 X	0,4 mL
Na-vanadate	1 mM	100 mM	100 µL
PBS 1X		1 X	7,32 mL
Final volume			**10 mL**

PROTOCOL

- Sediment 300 µl of bed volumes by centrifugation at 500 x g for 5 min at 4°C and discard the supernatant.
- Wash the matrix with 10 bed volumes of cold PBSE.

- Centrifuge the suspension at 500 x g for 2 min at 4°C to sediment the matrix. Discard the wash.
- Repeat the wash twice for a total of three washes.
- Wash the matrix with 10 bed volumes of ice-cold PBS buffer.
- Repeat the wash once for a total of two washes.
- Completely remove the wash (you may help yourself using a proper Hamilton syringe).
- Add 600 µl of cleared *E. coli* [pGEX2T-RBD] lysate each 20 µl of bed volume (each sample) and mix gently on a rotary shaker at 4°C for 60 min.
- Centrifuge the suspension at 500 x g for 2 min at 4°C to sediment the matrix. Remove the supernatant.
- Wash the Glutathione Sepharose 4B pellet with 10 bed volumes of ice-cold PBS buffer.
- Centrifuge the suspension at 500 x g for 2 min at 4°C to sediment the matrix. Discard the wash.
- Repeat the wash once for a total of two washes.
- Wash the Glutathione Sepharose 4B pellet with 10 bed volumes of ice-cold Shalloway buffer.
- Centrifuge the suspension at 500 x g for 2 min at 4°C to sediment the matrix. Discard the wash.
- Repeat the wash twice for a total of three washes and completely remove the wash (you may help yourself using a proper Hamilton syringe).
- Resuspend the GST-RBD pre-bound to Glutathione Sepharose 4B in ice-cold Shalloway buffer to a final concentration of 20%.
- An aliquot of 100 µl of the 20% GST-RBD pre-bound to Glutathione Sepharose 4B slurry is transferred into each 1.5 ml tubes.
- Store the 20% GST-RBD pre-bound to Glutathione Sepharose 4B slurry aliquots in ice until use.

2.18.4 Purification of Ras2-GTP

SOLUTIONS

- ✓ Glutathione S-transferase (GST)-RBD fusion protein pre-bound to glutathione-Sepharose 4B (20% slurry)
- ✓ Yeast crude extract preparation diluted 0.4 μg/μl
- ✓ Shalloway buffer
- ✓ SDS-sample buffer 2X

PROTOCOL

- Add 500 μl of yeast crude extract preparation to 100 μl of the 20% GST-RBD pre-bound to Glutathione Sepharose 4B slurry. Take 40 μl of yeast crude extract preparation, add 40 μl 2x SDS sample buffer and boil for 10 min. This is the Total Ras protein sample. Freeze until use (until the SDS-PAGE will be performed). Incubate at 4°C for 1 hour, mixing gently on a rotary shaker.
- Centrifuge at 500 x *g* for 2 min at 4°C to sediment the matrix. Discard the supernatant.
- Wash the matrix with 0.5-1 ml of ice-cold Shalloway buffer.
- Centrifuge at 500 x g for 5 min at 4°C to sediment the matrix. Discard the wash.
- Repeat the wash twice for a total of three washes.
- Completely remove the wash (you may help yourself using a proper Hamilton syringe).
- Add 80 μl 1.25x SDS sample buffer and boil for 10 min. This is the Ras-GTP protein sample. Freeze until use (until the SDS-PAGE will be performed).

2.19 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used for molecular weight analysis of proteins. The purpose is to separate proteins according to their size. Therefore the proteins are denaturated by the presence of the detergent sodium dodecyl sulfate (SDS) into the gel and

the presence of SDS and β- mercaptoethanol into the sample buffer. SDS is a detergent that can dissolve hydrophobic molecules and due to the sulfate group it will negatively charge each protein in proportion to its mass. β-mercaptoethanol is able to cleave the disulfide bonds of a protein so it will further denature them. To ensure complete denaturation the samples are boiled before loading them on the gel.

SOLUTIONS

- ✓ Sample buffer 4 X:10
  - 200 mMTris-HCl pH 6.8
  - 4 % β–mercaptoethanol
  - 0.4 % bromophenol blue
  - 8 % SDS
  - 40 % glycerol
- ✓ Running buffer 5 X:
  - 15.1 g Tris
  - 94 g glycine
  - 5 g SDS
  - Add H_2O to 1 l
- ✓ Lower gel buffer 4 X:
  - 1.5 M Tris-HCl pH 8.8
  - 0.4 % SDS
- ✓ Upper gel buffer 4 X:
  - 0.5 M Tris-HCl pH 6.8
  - 0.4 % SDS
- ✓ Running gel 10 % (8 ml):
  - 2 ml acrylamide + bisacrylamide 40 % (Biolabs)
  - 2 ml lower gel buffer 4 X
  - 10 µl N,N,N',N'-tetramethylethylenediamine (TEMED)
  - 67 µl ammonium persulfate (APS) 10 %
  - 3.923 ml H_2O
- ✓ Stacking gel 4 % (3.5 ml):
  - 350 µl acrylamide + bisacrylamide 40 % (Biolabs)
  - 875 µl upper gel buffer 4 X

5 µl TEMED
10.5 µl APS 10 %
2.216 ml H_2O

PROTOCOL

- Clean the glass plates and assemble the casting stand with a gap of 1,5 mm.
- Prepare the running and the stacking gel. Add TEMED just before pouring the gel to avoid polymerization. Pour the running gel until 1 cm beneath the wells of the comb.
- Add some H_20 on top of the gel to make the surface flat and so the gel stays moist.
- Remove all the water after polymerizing. Pour the stacking gel on top of the running gel and insert the comb. Remove the bubbles.
- After polymerization, remove the comb and wash the wells with H_20.
- Take the glass plates with the gel out of the casting stand, place them in the upper chamber and put this in the electrophoresis tank. Fill the upper chamber with running buffer 1X and the lower chamber until the bottom of the gel is immersed.
- Centrifuge the samples at 13000 rpm for 3 minutes and load in every well the same amount of proteins.
- Close the electrophoresis tank and apply a constant electric current of 20 mA/gel until the BBF front reaches the bottom of the gel.

2.20 Western blot analysis

Western blotting is used to transfer proteins from a SDS-PAGE to a nitrocellulose membrane. The membrane is then treated with blocking solution, which covers all of the membrane, except where the proteins are blotted. This way, binding of primary antibodies will only occur at the proteins and there will be no background interference due to aspecific binding of the antibodies. Next, secondary antibodies will bind to the primary antibodies. These antibodies are tagged with Horse Radish Peroxidase (HRP). The membrane is soaked in an Enhanced

Chemiluminiscence (ECL) solution. The HRP oxidates the luminol in the ECL solution and a light signal is generated.

SOLUTIONS

- ✓ Transfer buffer
  - 200 ml running buffer 5 X
  - 200 ml methanol
  - 600 ml H2O
  - Store at 4 °C
- ✓ 5 X Tris-buffered saline (TBS)(1 l)
  - 6 g Tris-HCl pH 7.4
  - 45 g NaCl
  - H_2O
- ✓ Washing buffer
  - 1 X TBS
  - 0.05% Tween-20
- ✓ Blocking solution
  - 1 X TBS
  - 0.05 % Tween-20
  - 5 % milk
- ✓ Primary antibodies: goat anti-Ras2 polyclonal IgG (yC-19) – diluted 1:200 in blocking solution
- ✓ Secondary antibodies: donkey anti-goat IgG-HRP (sc-2020) – diluted 1:5000 in blocking solution
- ✓ Enhanced Chemiluminiscence (ECL) detection reagent E and P (GENESPIN). Store at 4 °C
- ✓ Developer solution (Kodak) diluted 1:4 in H_2O
- ✓ Fixer solution (Kodak) diluted 1:4 in H_2O

PROTOCOL

- Cut 4 pieces of Whatman filter paper and 1 nitrocellulose membrane (Protan) with the size of the gel. Wet these in transfer buffer together with 2 sponges.

- A stack is put together in the blotting cassette in the following order from anode to cathode: 1 sponge, 2 filter papers, gel on top of membrane, 2 filter papers, 1 sponge.
- Remove bubbles in between the layers with a Pasteur pipette.
- Close the cassette and place it in the electrophoresis tank with the membrane, on which the negatively charged proteins will migrate, faced to the anode.
- Fill the tank with cold transfer buffer. Run for 1h 30 min at 100 V with an ice-cooling unit inside the tank and a stir bar to maintain temperature and ion distribution.
- Rinse the membrane with washing buffer and incubate in blocking solution for 1 hour at room temperature with agitation. Every washing step or incubation a sufficient amount of solution is used to cover the membrane.
- Incubate with primary antibodies diluted in blocking solution overnight at 4°C with agitation.
- Rinse the membrane and wash three times for 10 minutes with washing buffer.
- Incubate with secondary antibodies diluted in blocking solution for 1-2 hours at room temperature with gentle shaking.
- Rinse the membrane and wash three times for 10 minutes with washing buffer.
- Drain the excess buffer from the membrane on filter paper. Prepare a 1:1 mixture of ECL detection reagents 1 and 2. The final volume required is 0,125 ml/cm².
- Add the detection reagent to the membrane and incubate for 1,5 minute at room temperature with little agitation.
- Dry the membrane on filter paper and place it, protein side up, in the film cassette between two plastic films. Fix them so they can't move.
- Place an autoradiography film on top of the membrane in the dark room.
- Develop the autoradiography film using developer and fixer solution.
- Rinse the film with water, dry and put marks at the signs of the Protein Marker.

RESULTS

3.1 Description of the eGFP-RBD3 probe to investigate the localization of active Ras proteins *in vivo* in *Saccharomyces cerevisiae* and evaluation of its specificity

Data in literature well report the specificity of interaction between Ras-GTP and the Ras-binding domain (RBD) of Raf-1 to detect activated Ras proteins (de Rooij and Bos, 1997). Since there is a high degree of homology between yeast and mammalian Ras proteins, the yeast Ras proteins are able to interact with the RBD of Raf-1, given the possibility to differentiate between active and inactive Ras in this microorganism (Rudoni *et al*., 2001; Colombo *et al*., 2004). In the past, to investigate the localization of active Ras *in vivo* in *S. cerevisiae*, in my lab they made a probe consisting of a GFP fusion with the Ras Binding Domain of Raf-1 (eGFP-RBD), which showed the inability to report endogenous Ras activation. Recently, a probe consisting of a GFP fusion with a trimeric Ras Binding Domain of Raf-1 (eGFP-RBD3) has proven to be a useful live-cell biosensor for Ras-GTP in mammalian cells (Augsten *et al*., 2006). At the beginning of the work, we inserted the eGFP-RBD3 fusion protein in an integrative plasmid and we expressed it in a wild type W303-1A strain, but the level of expression in glucose-growing cells was very low and it was too difficult to clearly discriminate the localization signal (Fig. 1a). Therefore, we inserted the eGFP-RBD3 fusion in a multicopy plasmid and the fusion protein was expressed in the wild type W303-1A strain under the control of the TPI promoter. Cells were grown on synthetic complete medium containing 2% glucose at 30°C until exponential phase and observed under the microscope. In the majority of the wild type cells the eGFP-RBD3 probe accumulated at the plasma membrane and possibly in the nucleus (Fig. 1b). To doubtless prove the nuclear localization of active Ras we used cells of the strain KT98, isogenic to W303-1A, expressing both a fusion of the nuclear envelope protein Nup49 with the fluorescent protein t-dimer and the eGFP-RBD3 probe (data not shown). In order to better investigate the localization of active Ras, we analyzed W303-1A wild type cells expressing both the eGFP-RBD3 probe and RFP at the mitochondria with a confocal microscope (Fig. 2). The optical sections of cells growing on glucose medium showed doubtless the nuclear and plasma membrane

localization of Ras-GTP (Fig. 2A) and mitochondria were clearly visible close to the plasma membrane (Fig. 2B). *S. cerevisiae* has two Ras proteins, Ras1 and Ras2, which are differentially regulated, being the latter the more abundant (Breviario *et al*., 1988). Consequently, we believe that the fluorescent signal that we observed at the plasma membrane and in the nucleus was mainly due to Ras2, since in glucose growing medium this proteins is at least ten times more expressed then Ras1.

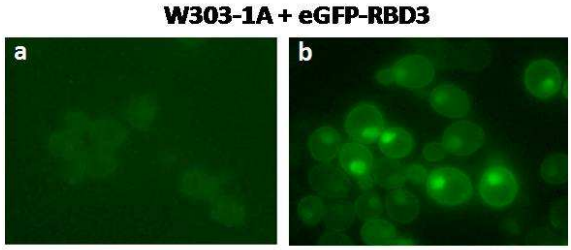


Figure 1. The eGFP-RBD3 probe is specific for Ras-GTP and reveals a nuclear and plasma membrane localization. (a) Wild type cells transformed with an integrative plasmid containing the eGFP-RBD3 probe. The level of expression of the eGFP-RBD3 probe inserted in an integrative plasmid is too low and is very difficult to clearly discriminate the localization signal. (b) Wild type cells transformed with a multicopy plasmid containing the eGFP-RBD3 probe. Cells were grown in medium containing 2% glucose at 30°C and then photographed with a Nikon fluorescence microscope.

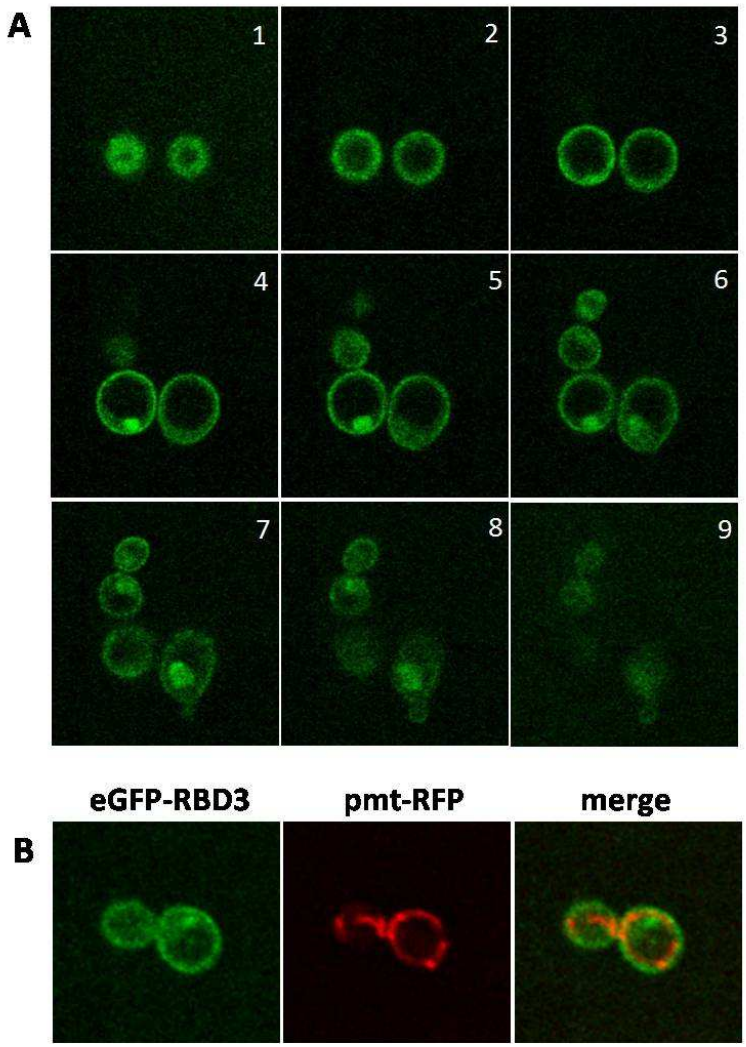


Figure 2. Nuclear and plasma membrane localization of active Ras in the W303-1A wild type strain observed at the confocal microscope. Cells transformed with both YEpeGFP-RBD3 and pmt-RFP were grown in 2% glucose medium at 30°C until exponential phase and then were photographed with the confocal microscope. (A) Optical sections showing the nuclear and plasma membrane localization of Ras-GTP. (B) Single optical section showing mitochondria close to the plasma membrane.

As a control, we investigated the wild type strain expressing the only eGFP, using epi-fluorescence and confocal microscopy; as expected, we observed a diffuse and uniform labeling of the cells (Figs. 3 and 4). To evaluate the specificity of the eGFP-RBD3 probe we assessed its localization in a strain deleted for *CDC25* and rescued by deletion of *MSN2* and *MSN4* (GG104 *cdc25*Δ strain); as expected, we observed a diffuse and uniform labeling of the cells, consistent with the fact that almost all Ras proteins should be present in the inactive form (Figs. 3 and 5). Finally, we investigated the localization of active Ras proteins in the wild type W303-1A strain expressing the dominant and active *RAS2*^{Val19} allele, which was reported to show a high level of Ras2-GTP (Colombo *et al*., 2004). In this cells growing on glucose medium the probe accumulated mostly in the plasma membrane and in the nucleus (Figs. 3 and 6), indicating that the increased amount of activated Ras2 protein did not influence its localization.

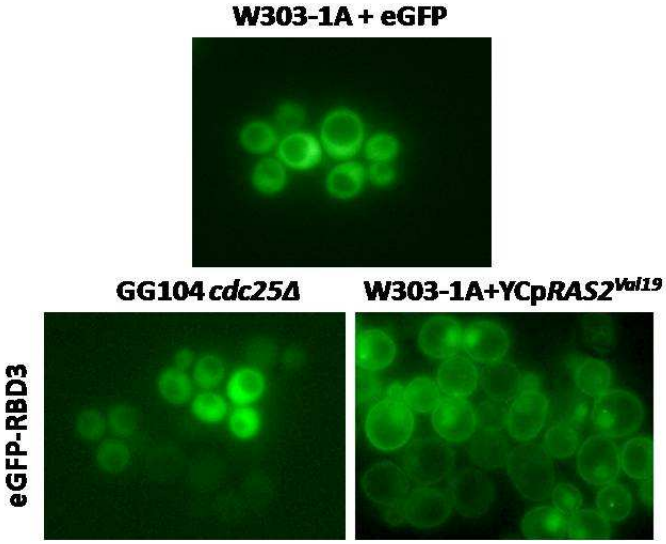


Figure 3. Localization of the eGFP-RBD3 probe in GG104 *cdc25*Δ strains and in the wild type W303-1A strain expressing the dominant and active *RAS2*^{Val19} allele and localization of eGFP in W303-1A wild type cells. Cells of the indicated strains were grown in medium containing 2% glucose at 30°C and then photographed with a Nikon fluorescence microscope.

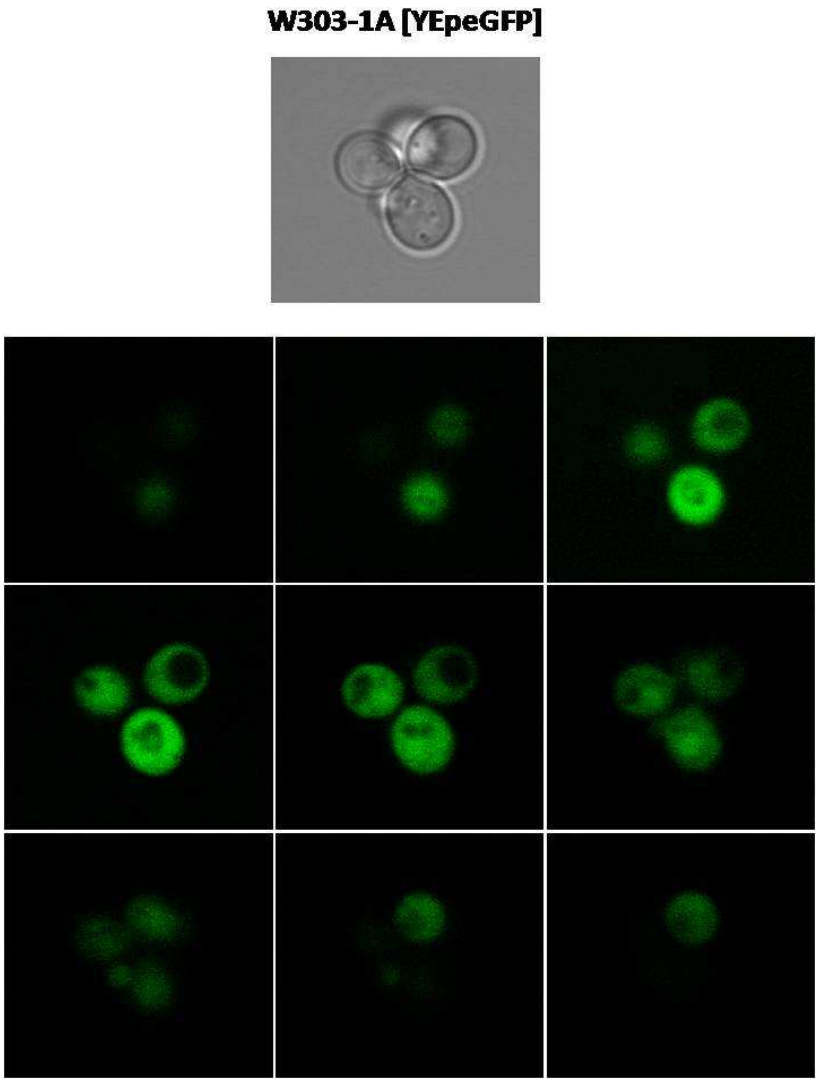


Figure 4. Localization of eGFP in the W303-1A wild type strain expressing eGFP observed at the confocal microscope. Cells transformed with YEpeGFP were grown in 2% glucose medium at 30°C until exponential phase and then eGFP fluorescence was photographed with the Leica TCS SP2 confocal microscope. Optical sections showing a diffuse and uniform labelling of the cells and transmission image.

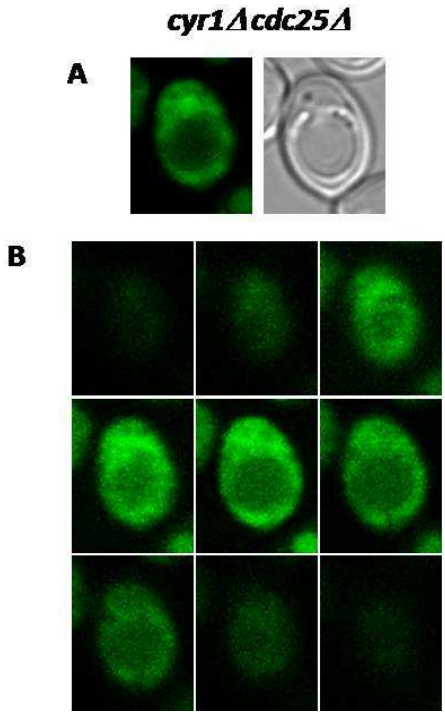


Figure 5. Localization of active Ras in a strain deleted for *CDC25* and rescued by deletion of *MSN2* and *MSN4* (GG104 *cdc25*Δ strain) observed at the confocal microscope. Cells were transformed with YEpeGFP-RBD3 and grown in 2% glucose medium at 30°C until exponential phase and then eGFP fluorescence was photographed with the Leica TCS SP2 confocal microscope. (A) Average sum of the different Z-scan and transmission image. (B) Optical sections showing a diffuse and uniform labeling of the cells.

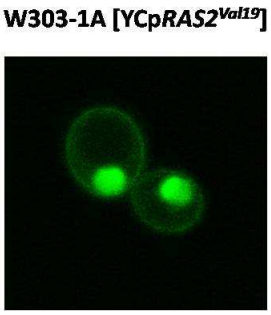


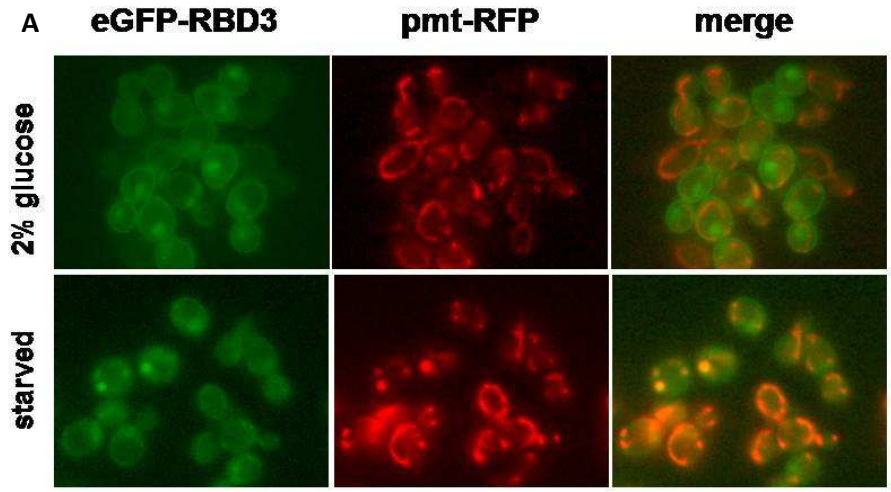
Figure 6. Nuclear and plasma membrane localization of active Ras in the W303-1A wild type strain expressing the dominant and active *RAS2*^{Val19} allele observed at the confocal microscope. W303-1A cells transformed with both YEpeGFP-RBD3 and YCp*RAS2*^{Val19} were

grown in 2% glucose medium at 30°C until exponential phase and then eGFP fluorescence was photographed with the Leica TCS SP2 confocal microscope.

3.2 Localization of active Ras in the wild type strain

3.2.1 Localization of active Ras proteins in wild type glucose-growing and glucose-starved cells

With the aim to investigate in details the localization of active Ras in wild type glucose growing and glucose-starved cells, we identified four groups of cells. Our results showed that in glucose growing cells, approximately 30% showed plasma membrane staining only, 42% exhibited plasma membrane and nuclear staining, 20% a diffuse and uniform labeling and only 8% showed mitochondrial staining (Fig. 7). On the contrary, in approximately 60% of starved wild type cells the eGFP-RBD3 accumulated in internal membranes and mitochondria, approximately 20% showed a diffuse and uniform labeling, 13% showed nuclear staining only and 5% exhibited plasma membrane and nuclear staining (Fig. 7).



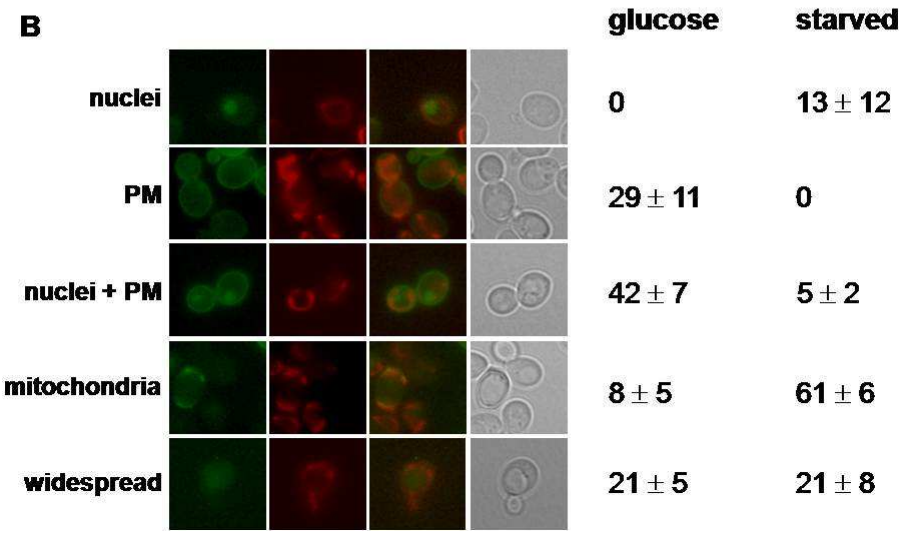


Figure 7. Localization of active Ras in glucose-growing and glucose-starved W303-1A cells. (A) Co-localization of eGFP fluorescence and RFP in W303-1A cells transformed with both YEpeGFP-RBD3 and pmt-RFP. Cells were grown in 2% glucose medium at 30°C until exponential phase, collected by centrifugation and resuspended in MES buffer for about 1 h and 30 min (starved). Glucose-growing and starved cells were then photographed with a Nikon fluorescence microscope. (B) Subcellular distribution of eGFP fluorescence in glucose-growing and glucose-starved cells. The percentages of cells with: nuclear, plasma membrane (PM), simultaneously nuclear and plasma membrane, mitochondrial and widespread fluorescence were calculated. A representative example of cells for each class is shown.

3.2.2 Localization of active Ras after addition of glucose to glucose-starved cells

Colombo *et al*. (2004) demonstrated that addition of glucose to glucose-starved cells triggers a fast increase in the GTP loading of Ras2, concomitant with the glucose induced increase in cAMP. In order to study the spatiotemporal localization of Ras-GTP *in vivo* in single cells after addition of 100 mM glucose, cells of the W303-1A wild type strain were grown on complete synthetic medium containing 2% glucose until exponential phase, harvested by centrifugation and resuspended to the density of $5*10^7$ cells/ml using MES buffer. After 1 hour and 40 minutes at 30°C glucose-starved cells were immobilized on a cover glass previously pre-treated with concanavalin and the cover glass was put on top of a Thoma chamber. We

took pictures at the fluorescence microscope before and after addition of the sugar and we calculated the percentage of cells showing different subcellular localization of the fluorescent probe. We observed upon addition of glucose a rapid recruitment of the probe back to the plasma membrane only and simultaneously to the plasma membrane and to the nucleus (Fig. 8).

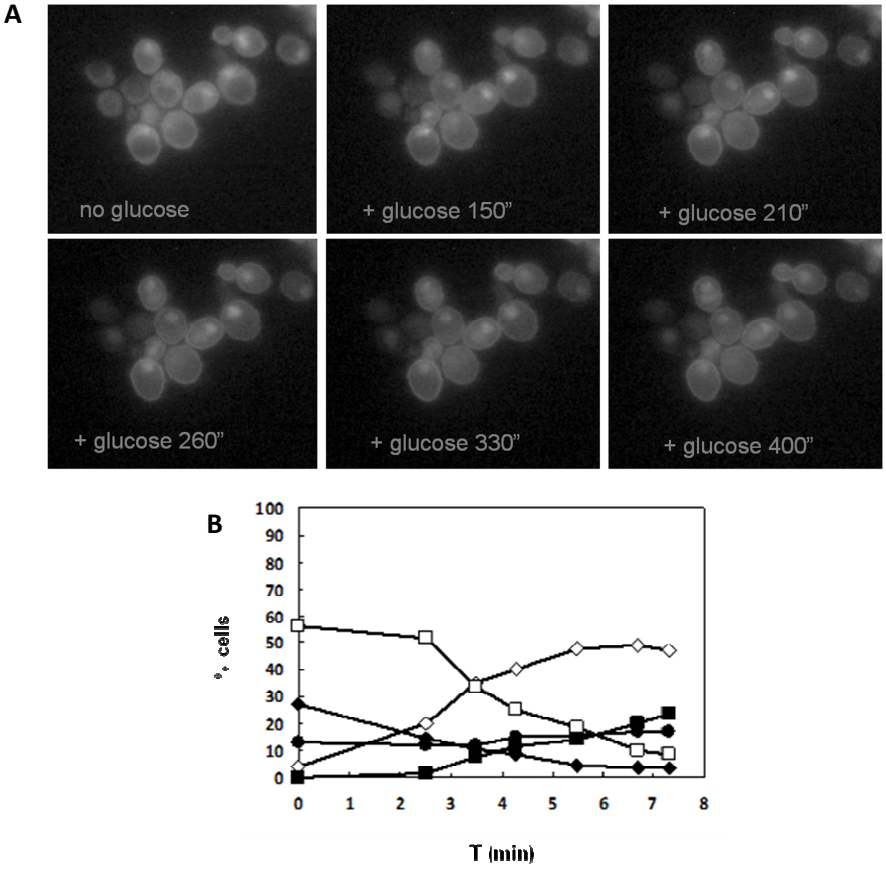


Figure 8. Relocalization of active Ras to the plasma membrane and to the nucleus in the W303-1A wild type cells following addition of glucose to starved cells. (A) W303-1A cells were grown in 2% glucose medium at 30°C until exponential phase, collected by centrifugation and resuspended in MES buffer for about 1 h and 30 min. At time zero glucose was added and images were captured after the indicated time periods using a Nikon fluorescence microscope. (B) Effect of addition of 100mM glucose to glucose-starved cells: cells with plasma membrane (■), nuclear(♦), simultaneously nuclear and plasma membrane (◊), mitochondrial (□) and widespread (●) localized fluorescence.

3.2.3 Influence of post-translational modifications on active Ras localization

Since data in literature (Bhattacharya *et al*., 1995) show that post-translational modifications of the C-terminal domain of Ras proteins are very important for their proper localization and biological activity, to understand if post-translational modifications could influence the localization of active Ras, we investigated the localization of the probe in a *RAS2*^{C318S} strain expressing a *RAS2* allele which can be farnesylated but not palmitoylated. We observed that in cells growing on 2% glucose medium the probe was localized in the nucleus like in the wild type W303-1A cells, while no active Ras was localized at the plasma membrane, in agreement with the absence of the palmitoylation (Fig. 9).

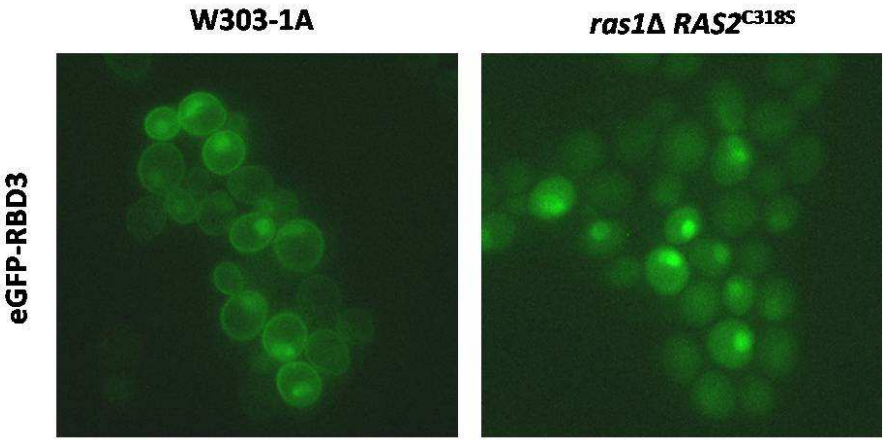


Figure 9. Localization of active Ras in WT and *RAS2*^{C318S} cells growing on glucose medium. Cells were transformed with YEpeGFP-RBD3 and grown in synthetic complete medium containing 2% glucose at 30 °C until exponential phase and then photographed.

3.3 Localization of active Ras in mutants of the cAMP/PKA pathway

In order to establish whether the nuclear and the plasma membrane localization of Ras-GTP observed in the wild type strain during growth on synthetic complete medium containing 2% glucose was dependent on

specific components of the cAMP/PKA pathway, we investigated the distribution of the probe in mutants of this signal transduction pathway.

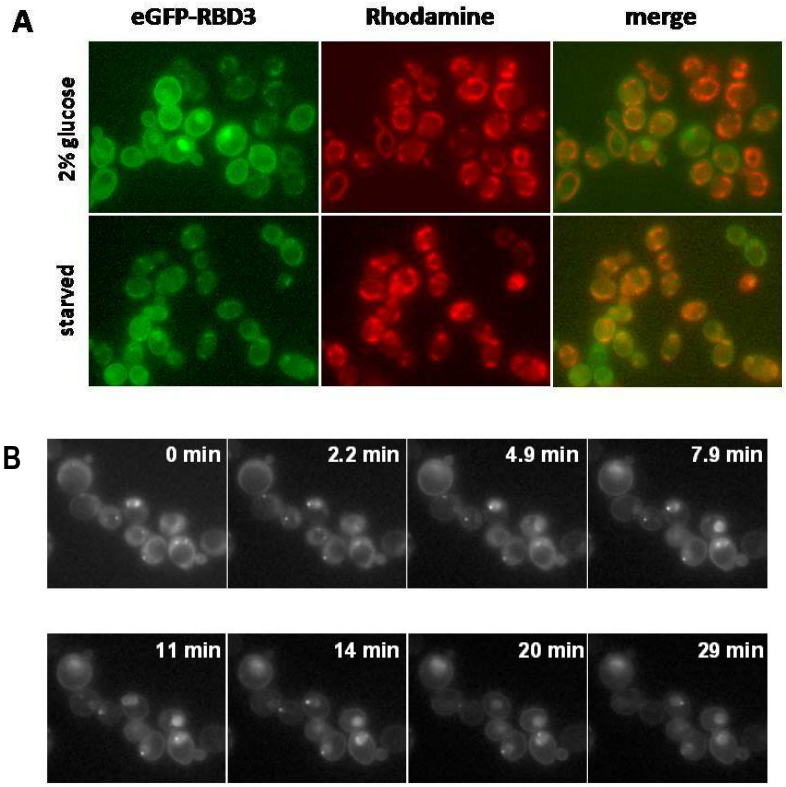
3.3.1 Localization of active Ras in the *cyr1*Δ *pde2*Δ *msn2Δ msn4*Δ strain

We observed that in the *cyr1*Δ strain growing on medium containing 2% glucose the probe accumulated mostly in the plasma membrane (about 43%), being the simultaneous nuclear and plasma membrane localization less pronounced compared with the wild type strain (19% compared with 42%) (Fig. 10), indicating that the absence of adenylate cyclase only partially influenced the localization of Ras-GTP. In starvation, most of the cells showed a mitochondrial staining, while after addition of glucose to glucose-starved cells a rapid recruitment of the probe back to the plasma membrane and to the nucleus was observed (Fig. 10).

3.3.2 Localization of active Ras in *hxk1*Δ, *hxk2*Δ and *hxk1*Δ *hxk2*Δ strains

In *S. cerevisiae* three isoenzymes mediate glucose phosphorylation, Hxk1, Hxk2 and Glk1. The hexokinases Hxk1 and Hxk2 phosphorylate glucose as well as fructose, whereas the glucokinase Glk1 can only phosphorylate glucose. Any one of the three isoenzymes can sustain glucose-induced cAMP signalling, whereas specifically one of the two hexokinases is required for fructose-induced cAMP signalling (Beullens *et al*., 1988; Rolland *et al*., 2000). Recently it has been shown that glucose phosphorylation did not influence the basal Ras2-GTP loading state but it is required for the glucose-induced increase in Ras2-GTP and that glucokinase alone can sustain the increase (Colombo *et al*., 2004). In order to investigate the role played by each of the two hexokinases in active Ras localization, we analyzed under the microscope the *hxk1*Δ, the *hxk2*Δ and the double deletion mutant *hxk1*Δ *hxk2*Δ growing exponentially on glucose medium. We observed that deletion of *HXK1* did not impair the proper nuclear and plasma membrane localization of active Ras, while deletion of *HXK2* caused a mitochondrial localization of the probe, indicating that the absence of Hxk2 is involved in

the proper localization of Ras-GTP (Fig. 11). In line with this result, the double deletion mutant *hxk1*Δ *hxk2*Δ showed a pattern comparable with that observed for the *hxk2*Δ strain. Moreover, we transformed the *hxk1*Δ *hxk2*Δ strain with the centromeric plasmid YCp*HXK2* expressing Hxk2. We observed that the eGFP-RBD3 probe was mainly localized in the plasma membrane and in the nucleus, reinforcing the hypothesis that Hxk2 is actually the kinase involved in the proper localization of Ras-GTP (Fig. 11).



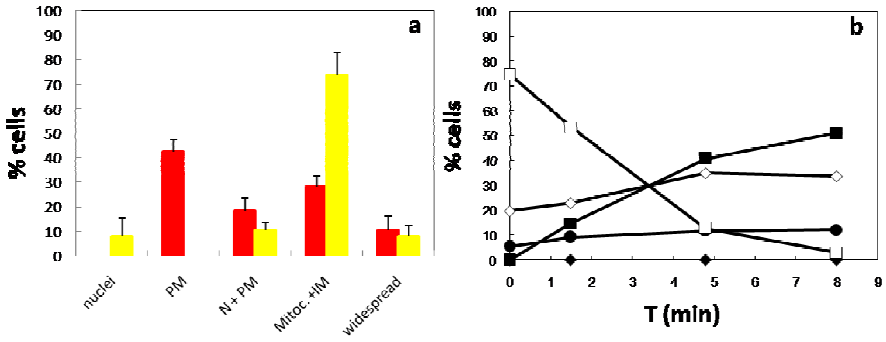


Figure 10. Localization of active Ras in a *cyr1Δ* strain. (A) Co-localization of eGFP fluorescence and red-fluorescent rhodamine B hexyl ester in *cyr1Δ* cells transformed with YEpeGFP-RBD3. (B) Localization of active Ras following addition of glucose to starved cells. (C) Panel a: subcellular distribution of eGFP fluorescence in glucose-growing (**red**) and glucose-starved (**yellow**) cells; panel b: effect of addition of 100 mM glucose to glucose-starved cells: cells with plasma membrane (■), nuclear(♦), simultaneously nuclear and plasma membrane (◊), mitochondrial (□) and widespread (●) localized fluorescence.

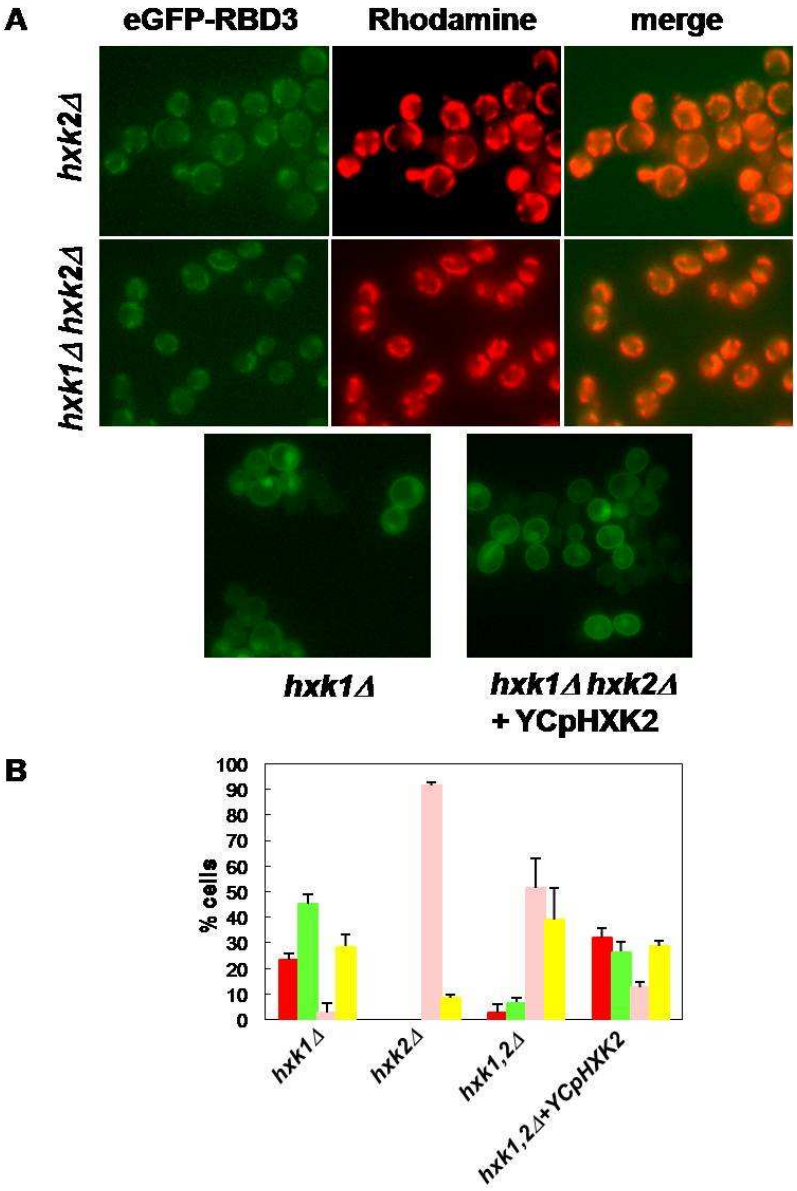


Figure 11. Localization of active Ras in mutants of the cAMP/PKA pathway. (A) hxk1Δ, hxk2Δ, hxk1Δhxk2Δ cells transformed with YEpeGFP-RBD3 and hxk1Δhxk2Δ cells transformed with both YCpHXK2 and YEpeGFP-RBD3 growing on glucose medium at 30°C. Mitochondria were visualized using rhodamine B hexyl ester. (B) Subcellular distribution of eGFP fluorescence: cells with plasma membrane (**red**), simultaneously nuclear and plasma membrane (**green**), mitochondrial (**pink**) and widespread (**yellow**) localized fluorescence.

3.3.3 Localization of active Ras in cells lacking Gpa2 and Gpr1

It is well known that adenylate cyclase activity in budding yeast *Saccharomyces cerevisiae* is controlled by the Ras proteins and by the G-protein-coupled receptor system, composed of Gpr1 and its Gα protein Gpa2 (Colombo *et al*., 1998; Kraakman *et al*.,1999; Rolland *et al*., 2001; Xue *et al*., 1998). The deletion of *GPR1*, or especially of *GPA2*, enhanced the basal level of Ras2-GTP compared with that observed in the wild type strain and the glucose-induced activation of Ras2 did not require the Gpa2-Gpr1 system (Colombo *et al*., 2004). In order to investigate the role played by the Gpr1-Gpa2 G-protein-coupled receptor system in the localization of active Ras, we used the *gpr1*Δ and the *gpa2*Δ strains. We observed that in the *gpr1*Δ strain, growing exponentially on synthetic complete medium containing 2% glucose, the probe accumulated mostly in the plasma membrane (41%), while the simultaneous plasma membrane and nuclear localization was less pronounced similar to the *cyr1*Δ strain (Fig. 12), indicating that the absence of the G-protein coupled receptor only partially impaired the localization of Ras-GTP. Surprisingly, in cells lacking Gpa2 the probe accumulated in internal membranes and mitochondria, indicating that Gpa2, but not Gpr1 is required for the recruitment of Ras-GTP at the plasma membrane and in the nucleus (Fig. 13). In both *gpa2*Δ and *gpr1*Δ glucose-starved cells the eGFP-RBD3 accumulated mostly in internal membranes and mitochondria, although in the latter strain a nuclear and plasma membrane localization was also detectable in a minor percentage of cells (Figs. 12 and 13). However, only in the *gpr1Δ* but not in the *gpa2Δ* mutant, the eGFP-RBD3 probe rapidly relocated to the plasma membrane and to the nucleus after addition of glucose (Fig. 12). To reinforce the hypothesis that the proper relocation of active Ras to these cellular compartments was due to the presence of Gpa2 itself and not to the activation state of this protein, we investigated the localization of Ras-GTP in a wild type strain after addition of 100 mM fructose, a sugar unable to activate the GPCR system (Rolland *et al.*, 2000). We observed a rapid relocation of the probe from the mitochondria back to the plasma membrane and to the nucleus when 2% fructose was added to starved wild

type cells (Fig. 14), indicating that Gpa2 protein and not its activation state, is required for a proper localization of active Ras.

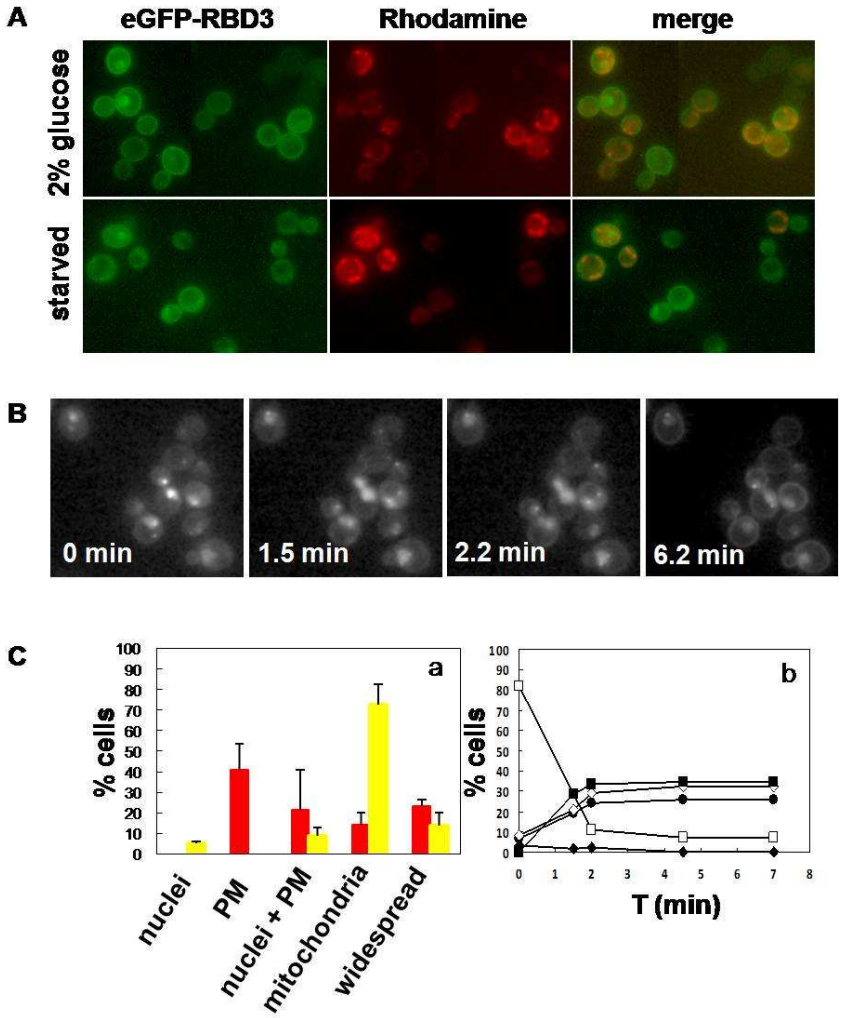


Figure 12. Localization of active Ras in a *gpr1Δ* strain. (A) Co-localization of eGFP fluorescence and red-fluorescent rhodamine B hexyl ester in *gpr1Δ* cells transformed with YEpeGFP-RBD3. (B) Localization of active Ras following addition of glucose to starved cells. (C) Panel a: subcellular distribution of eGFP fluorescence in glucose-growing (**red**) and glucose-starved (yellow) cells; panel b: effect of addition of 100 mM glucose to glucose-starved cells: cells with plasma membrane (■), nuclear(♦), simultaneously nuclear and plasma membrane (◊), mitochondrial (□) and widespread (●) localized fluorescence.

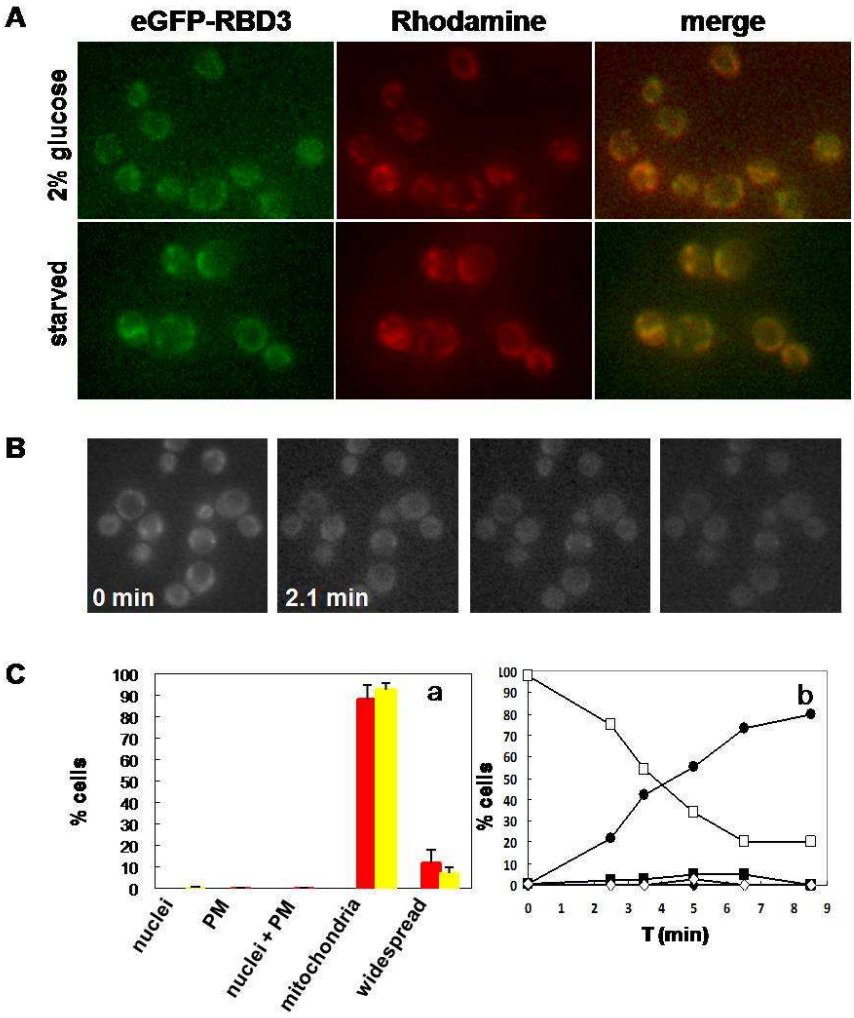


Figure 13. Localization of active Ras in a *gpa2Δ* strain. (A) Co-localization of eGFP fluorescence and red-fluorescent rhodamine B hexyl ester in *gpa2Δ* cells transformed with YEpeGFP-RBD3. (B) Localization of active Ras following addition of glucose to starved cells. (C) Panel a: subcellular distribution of eGFP fluorescence in glucose-growing (**red**) and glucose-starved (**yellow**) cells; panel b: effect of addition of 100 mM glucose to glucose-starved cells: cells with plasma membrane (■), nuclear(♦), simultaneously nuclear and plasma membrane (◊), mitochondrial (□) and widespread (●) localized fluorescence.

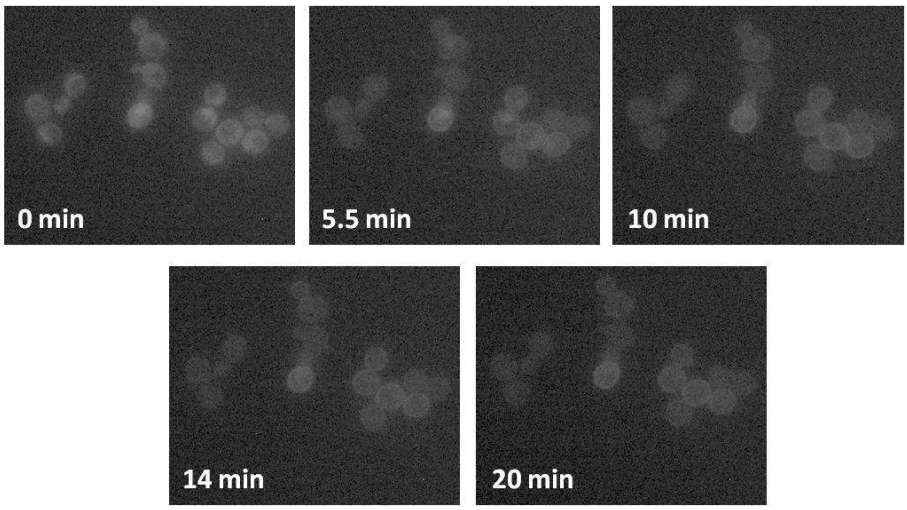


Figure 14. Relocalization of active Ras to the plasma membrane and to the nucleus in the W303-1A wild type cells following addition of fructose to starved cells. W303-1A cells were grown in 2% glucose medium at 30°C until exponential phase, collected by centrifugation and resuspended in MES buffer for about 1 h and 30 min. At time zero fructose was added and images were captured after the indicated time periods using a Nikon fluorescence microscope.

3.4 Effect of the expression of the eGFP-RBD3 probe on the growth rate of the different yeast strains analyzed

We next investigated whether the expression of the eGFP-RBD3 probe would influence the growth rate of the different strains analyzed in our work. The cells of the strains, either expressing and not expressing the probe, were gown at 30°C in minimal synthetic medium until early exponential phase. Every hour, one ml of the cellular culture was taken and transferred into a tube. The cell samples were sonicated to avoid the formation of cell aggregates. Of each sample, a certain volume was taken and diluted into 10 ml of ISOTON. The density of the culture was measured with the Coulter Counter ZBI.

We showed that the expression of the eGFP-RBD3 probe did not impair cell growth of the wild type, the *cyr1Δ* and the *hxk1Δ hxk2Δ* strains (Table A, Fig. 15). Interestingly we found that the eGFP-RBD-3 expression did not

impair cell growth of the *gpr1*Δ mutant, while it caused a consistent decrease in growth rate in the *gpa2*Δ mutant (Table A, Fig. 16)

Strain (main genotype)	Td (h), strains with eGFP-RBD3 (A)	Td (h), strains w/o eGFP-RBD3 (B)	ratio (A)/(B)
W303-1A	**1.9**	**1.8**	**1.1**
GG104 (*cyr1*Δ)	**1.7**	**1.8**	**0.9**
YSH327 (*hxk1*Δ *hxk2*Δ)	**2.6**	**2.3**	**1.1**
LK42/RL5 (*gpr1*Δ)	**1.9**	**1.9**	**1.0**
PM731 (*gpa2*Δ)	**3.4**	**1.6**	**2.1**

Table A. Doubling time of wild type and mutants of the cAMP/PKA pathway grown in the appropriate medium containing 2% glucose.

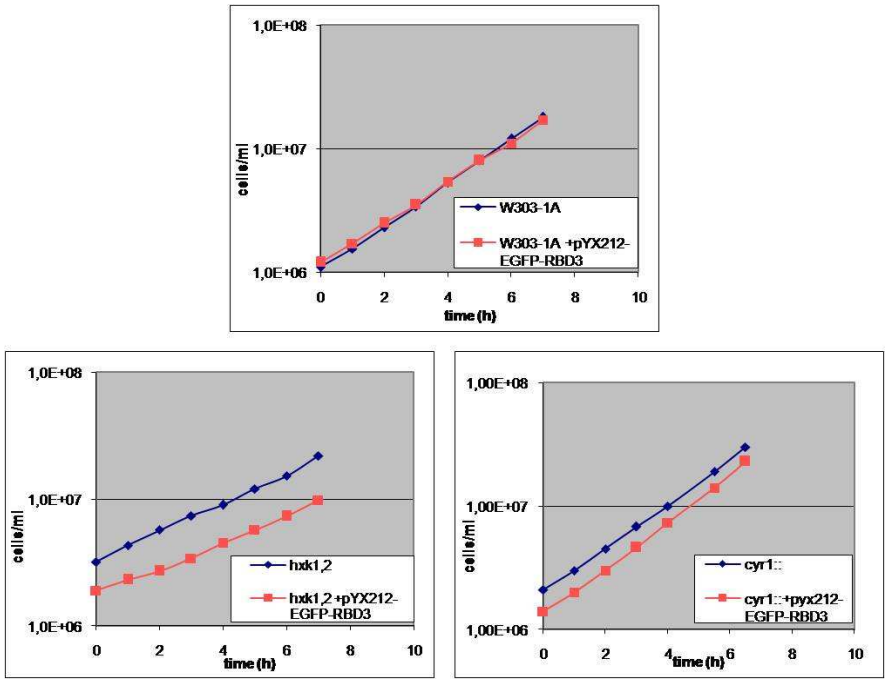


Figure 15 Growth curves of wild type (W303-1A), *cyr1Δ* and *hxk1*Δ *hxk2*Δ strains growing on glucose medium.

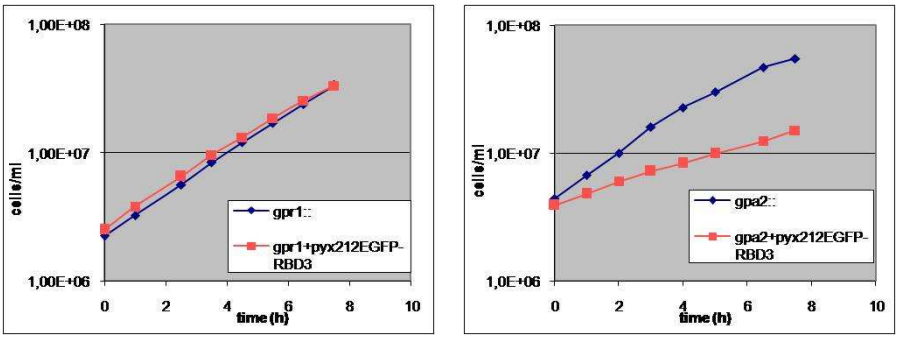


Figure 16 Growth curves of *gpr1Δ* and *gpa2*Δ strains growing on glucose medium.

3.4.1 The expression of the eGFP-RBD3 probe in a *gpa2Δ* strain does not influence the intracellular Ras2-GTP level

To investigate the different effect of the expression of the probe on the growth rate of the *gpr1*Δ and *gpa2*Δ mutants, we performed a Ras2-GTP/GST-RBD pull-down assay to measure the intracellular Ras2-GTP level in the W303-1A and in the *gpa2*Δ strains growing in glucose-medium, either expressing or not expressing the probe. Colombo *et al*. (2004) previously showed that the intracellular level of Ras2-GTP was enhanced in the *gpa2*Δ mutant compared with the wild type strain. Interestingly, we now found that the level of Ras2-GTP in the *gpa2*Δ mutant was not influenced by the expression of the eGFP-RBD3 probe, while it was increased in wild type cells (Fig. 17). The ability of the wild type strain expressing the probe to increase the level of Ras2-GTP might compensate the sequestration of Ras2-GTP by the eGFP-RBD3 probe, allowing the cells to grow at a normal growth rate. Conversely, the inability of the *gpa2*Δ mutant to do this could be one of the reason of the decrease in growth rate observed in this strain.

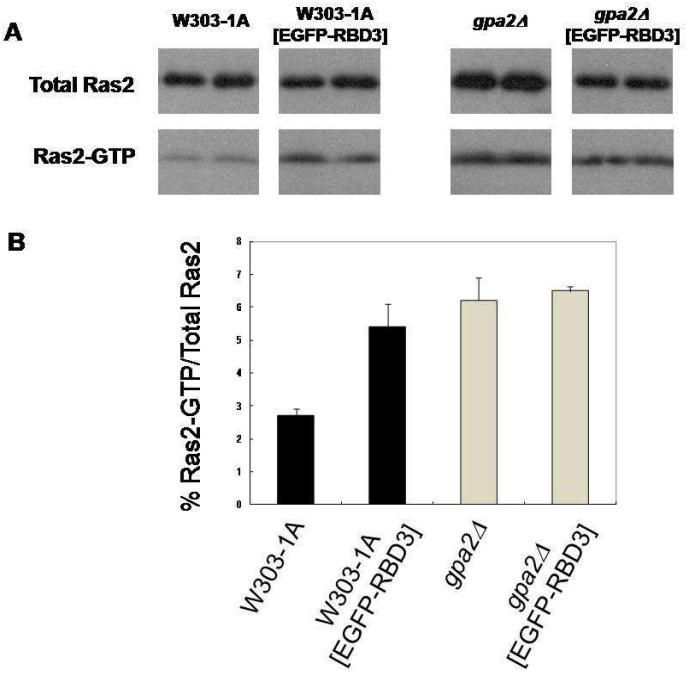


Figure 17. Ras2-GTP content during exponential growth on glucose. (A) Immunoblots of Ras2 in cell lysate (1.5 µg of total protein) (total Ras2) and Ras2 bound to GST-RBD eluted with SDS-PAGE sample buffer (Ras2-GTP) of the indicated strains during exponential growth on synthetic medium containing 2% glucose. (B) Quantitative analysis of Ras2-GTP levels. The ratio Ras2-GTP/Total Ras2 in the W303-1A strain with or without YEpeGFP-RBD3 (black) and in the *gpa2Δ* strain with or without YEpeGFP-RBD3 (light grey) was calculated by densitometry analysis and the fraction of Ras2-GTP was estimated taking into account the amounts of proteins loaded. Values are reported as the mean of two independent experiments.

3.5 Influence of PKA activity on active Ras localization

3.5.1 Localization of active Ras in mutants with either high or low PKA activity

The cAMP-dependent protein kinase A (PKA) is a central component of the cAMP/PKA pathway. Mutants with attenuated PKA activity display hyperaccumulation of cAMP, whereas mutants with an hyperactivated PKA pathway display reduced cAMP level. Data in literature show that PKA

regulates the localization of certain cAMP/PKA signaling pathway components, like the *PDE2*-encoded high-affinity cAMP phosphodiesterase (Hu *et al*., 2010), Cdc25 (Belotti *et al*., 2011) and the Ras2 protein (Dong and Bai, 2011). We investigated whether PKA activity plays a role in the localization of active Ras using the eGFP-RBD3 probe, both in a SP1 background and in a W303-1A background. Cells were grown on glucose medium until exponential phase at 30°C and then observed under the microscope. Our results showed that the localization of the probe was actually dependent on PKA activity. In particular, concerning the SP1 background, in the *bcy1Δ* mutant showing high PKA activity, there was a clear relocalization of active Ras to the cytoplasm and to the nucleus, while no active Ras was localized at the plasma membrane anymore (Fig. 18A and B). In a strain with either reduced PKA activity, the *tpk1*^{w1} *tpk2Δ tpk3Δ* strain or absent PKA activity, the *tpk1Δ tpk2Δ tpk3Δ yak1Δ* strain, more than 60% of the cells showed a strong plasma membrane localization of active Ras, while the nuclear localization was dramatically reduced compared with the wild type strain (Fig. 18A and B).

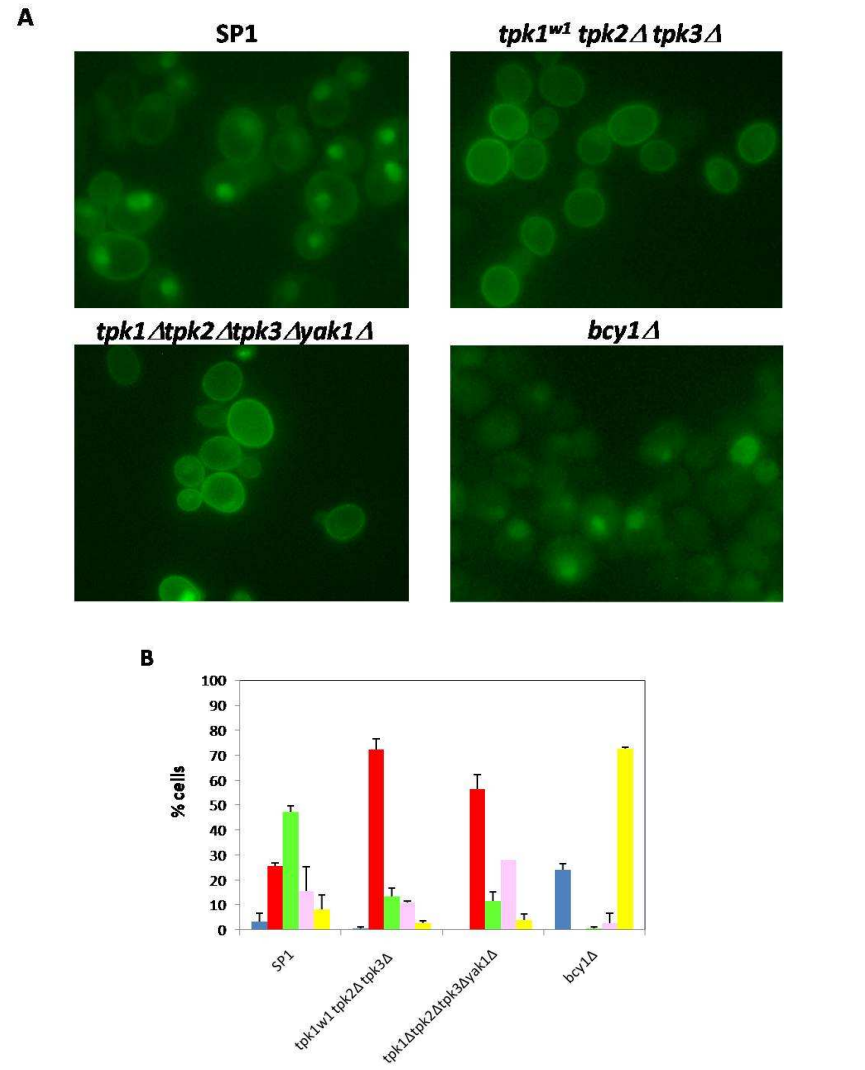


Figure 18. Effect of PKA activity on the localization of active Ras in strains of the SP1 background. (A) Cells of the indicated strains were grown in synthetic complete medium containing 2% glucose at 30 °C until exponential phase and then photographed. (B) Subcellular distribution of eGFP fluorescence: cells with nuclear (**blue**), plasma membrane (**red**), simultaneously nuclear and plasma membrane (green), mitochondrial (pink) and widespread (yellow) localized fluorescence.

The same results were obtained with PKA mutants made in the W303-1A background. Indeed in the *cyr1Δ pde2Δ yak1Δ* strain, with absent PKA

activity, active Ras localized mainly at the plasma membrane (Fig. 19A and B), while in a *bcy1Δ* strain active Ras did not localize in this cellular compartment (Fig. 19A and B), indicating that the localization of active Ras is dependent on PKA activity.

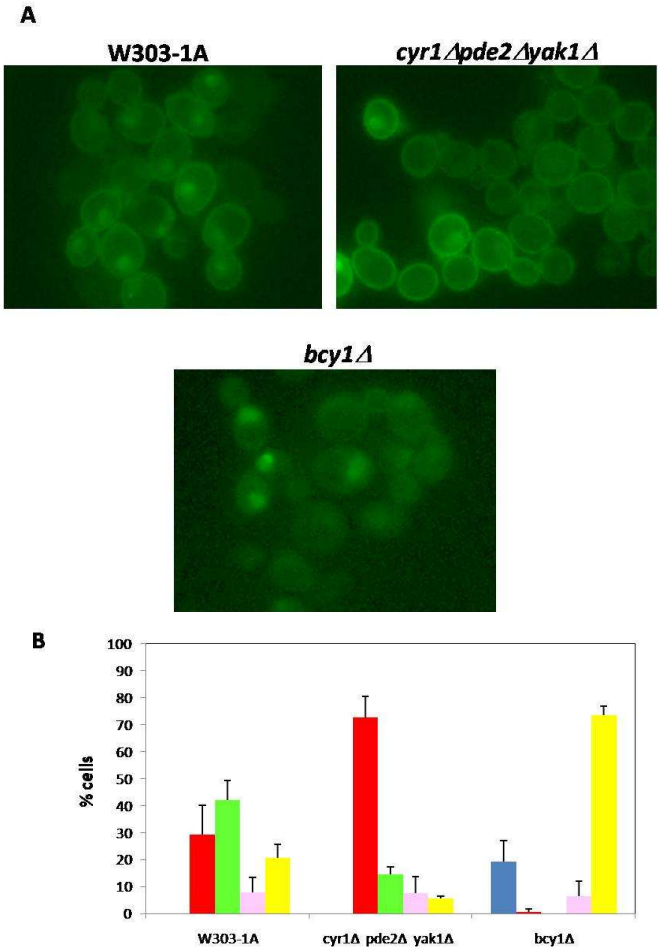


Figure 19. Effect of PKA activity on the localization of active Ras in strains of the W303-1A background. (A) Cells of the indicated strains were grown in synthetic complete medium containing 2% glucose at 30 °C until exponential phase and then photographed. (B) Subcellular distribution of eGFP fluorescence: cells with nuclear (**blue**), plasma membrane (**red**), simultaneously nuclear and plasma membrane (green), mitochondrial (pink) and widespread (yellow) localized fluorescence.

3.5.1.1 The level of total Ras2 and Ras2-GTP is dependent on PKA activity

We next investigated whether PKA activity regulates Ras2p and Ras2-GTP levels in different PKA mutants. In line with published data (Colombo *et al*., 2004; Dong and Bai, 2011; Paiardi *et al*., 2007), our results showed that the level of Ras2p and Ras2-GTP were dependent on PKA activity. In particular, as expected, cells with high PKA activity, like the *bcy1Δ* cells, showed a lower Ras2-GTP/total Ras2 ratio than the wild type strain, while cells with either low or absent PKA activity, like the *tpk1*^{w1} *tpk2Δ tpk3Δ* and the *tpk1Δ tpk2Δ tpk3Δ yak1Δ* cells, showed a higher Ras2-GTP/total Ras2 ratio than the wild type strain (Fig. 20B). Moreover, cells with high PKA activity showed lower levels of Ras2p than wild type cells, while cells with either low or absent PKA activity showed higher levels of Ras2p than the wild type cells (Fig. 20A).

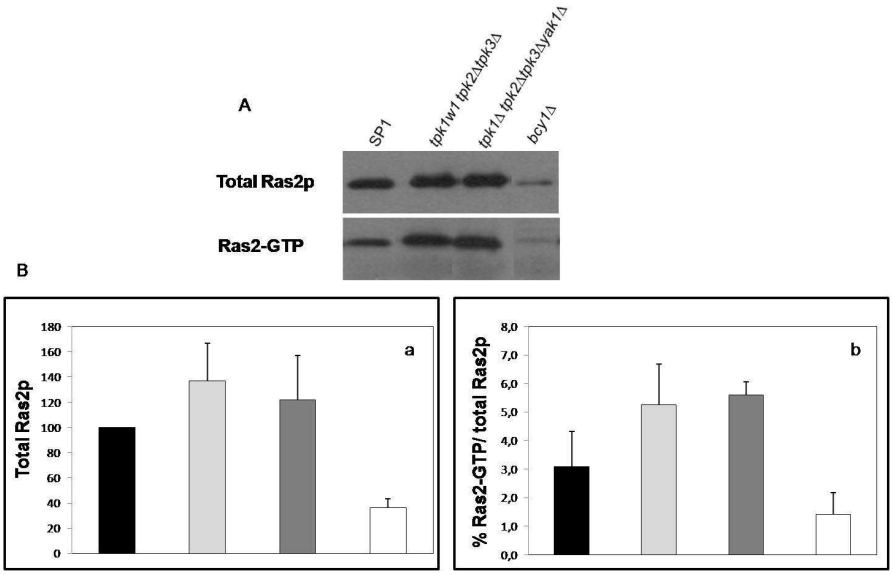


Figure 20. Effect of PKA activity on the intracellular level of total Ras2 and Ras2-GTP. PKA mutant cells were incubated on glucose medium to log phase and subjected to Ras2-GTP/RBD pull-down assay. Protein samples were separated on 10% SDS-PAGE gels. (A) Immunoblots of total Ras2p and Ras2-GTP bound to GST-RBD. (B) Panel a: intracellular level of total Ras2 in SP1 wild type cells (black), *tpk1*^{w1} *tpk2Δ tpk3Δ* cells (light grey), *tpk1Δ tpk2Δ tpk3Δ yak1Δ* cells (dark grey) and *bcy1Δ* cells (white). Panel b: intracellular level of Ras2-GTP

in SP1 wild type cells (black), *tpk1*^{w1} *tpk2Δ tpk3Δ* cells (light grey), *tpk1Δ tpk2Δ tpk3Δ yak1Δ* cells (dark grey) and *bcy1Δ* cells (white).

3.5.2 Effect of cAMP on the localization of active Ras in *cyr1Δ pde2Δ msn2Δ msn4Δ* and *cyr1Δ pde2Δ yak1Δ* cells growing on glucose

Since our results showed that in a strain with high PKA activity, the *bcy1Δ* strain, there was a clear relocalization of the active Ras to the cytoplasm and to the nucleus, we decided to better investigate the effect of PKA hyperactivation on active Ras localization. To this aim, we investigated the localization of the probe in strains *cyr1Δ pde2Δ msn2Δ msn4Δ* and *cyr1Δ pde2Δ yak1Δ,* before and after addition of 2mM cAMP. In fact, in these strains, PKA activity is not required for growth, but PKA can be activated by addition of exogenous cAMP.

Strains *cyr1Δ pde2Δ msn2Δ msn4Δ* and *cyr1Δ pde2Δ yak1Δ* are deleted in the gene encoding adenylate cyclase. Deletion of *PDE2* allows these strains to use cAMP added to the medium, bypassing lethality caused by deletion of *CYR1* (Mitsuzawa *et al*., 1993*;* Wilson *et al.,* 1993). Moreover, deletion of *CYR1* can be overcome either by deletion of the genes encoding the transcriptional factors Msn2 and Msn4 (Smith *et al*., 1998) or by deletion of *YAK1*, which allow these strains to grow even in the absence of cAMP (Roosen *et al*., 2005).

Cells were grown on glucose medium until exponential phase and then 2mM cAMP was added directly to the cell cultures. We observed that 45 minutes after addition of cAMP, the probe localized mainly in the nucleus (Fig. 21) in both these mutants, supporting the idea that the localization of active Ras is influenced by PKA activity and in particular that high PKA activity promotes the relocalization of the probe from the membrane to the nucleus.

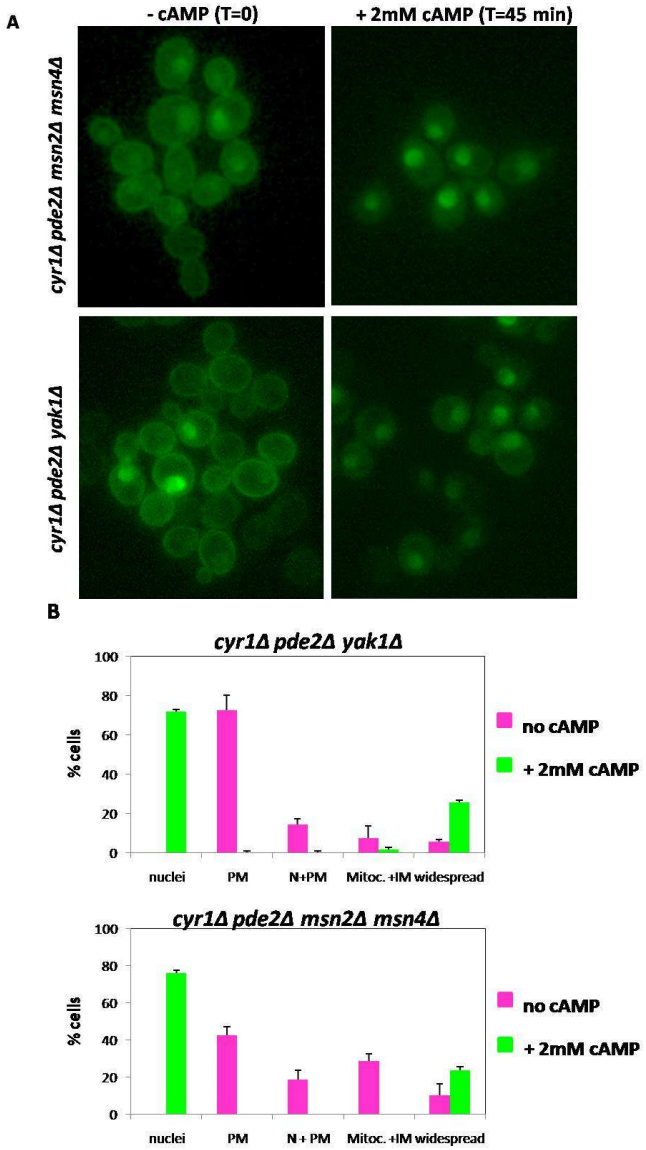


Figure 21. Effect of addition of 2mM cAMP to *cyr1Δ pde2Δ msn2Δ msn4Δ* and the *cyr1Δ pde2Δ yak1Δ* glucose-growing cells. (A) Cells of the indicated strains were grown on glucose medium until exponential phase, 2mM cAMP was added to the different cultures and after 45 minutes pictures were taken. (B) Subcellular distribution of eGFP fluorescence before (**pink**) and after (**green**) cAMP addition: cells with nuclear (nuclei), plasma membrane (PM), simultaneously nuclear and plasma membrane (N+PM), mitochondrial and internal membranes (mitoc+IM) and widespread (widespread) localized fluorescence.

3.5.3 Effect of alkalinization on active Ras localization

Since our results showed that in a strain with either reduced PKA activity or absent PKA activity there was a clear relocalization of the probe mainly at the plasma membrane, we decided to better investigate the effect of downregulation of PKA activity on active Ras localization.

Data in literature show that alkalinization of the medium causes a transient downregulation of the cAMP/PKA pathway and therefore a transient decrease in cAMP levels in the first 5-15 minutes, followed by a recovery to the initial levels after 30 minutes of stress. Moreover, the alkalinization results also in a rapid nuclear translocation of the transcriptional factor Msn2, which mediates a general stress response by binding with STRE (stress response element) sequences in the promoter of different genes (Casado *et al.,* 2011).

To investigate the effect of the alkaline pH stress on the localization of active Ras proteins, cells of the W303-1A wild type strain transformed with the eGFP-RBD3 probe were grown in 2% glucose medium until exponential phase. At this point we divided the culture in different subcultures. We added to the subcultures an amount of KOH sufficient to reach pH 8 and we incubated them for 5 and 30 minutes at 30°C. We added to the control cells the same amount of KCl. In wild type cells growing on glucose medium, the probe localized, as expected, at the plasma membrane and in the nucleus and the addition of KCl for different times did not impair the proper localization of active Ras. The addition of KOH caused within 5 minutes the delocalization of active Ras almost exclusively to the plasma membrane. But this was a transient relocalization, indeed after 30 minutes the probe again localized also in the nucleus (Fig. 22). These results reinforced the hypothesis that the localization of active Ras proteins is actually regulated by PKA activity.

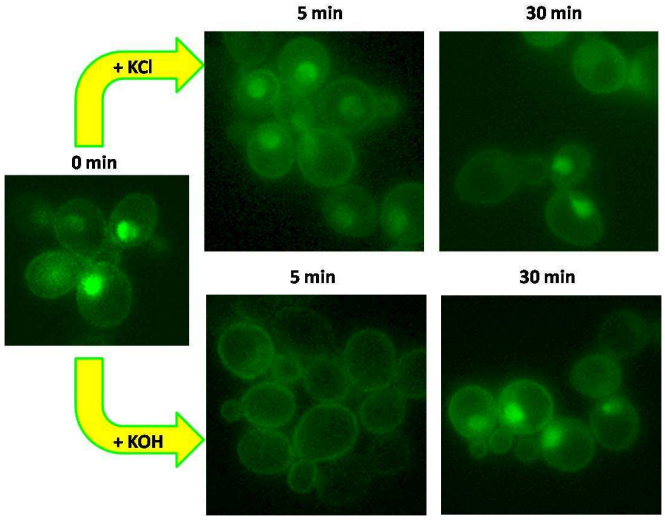


Figure 22. Effect of alkalinization on active Ras localization. Cells of the W303-1A wild type cells transformed with the eGFP-RBD3 probe grown on 2% glucose medium without KCl or KOH (0min) and incubated with KCl or KOH for 5 minutes and 30 minutes.

3.5.3.1 Effect of alkalinization on Ras2-GTP/Ras2 total levels

In line with published data (Colombo *et al*., 2004; Dong and Bai, 2011; Paiardi *et al*., 2007), in section 3.5.1.1 we showed that the levels of Ras2-GTP are dependent on PKA activity and in particular that in cells with either low or absent PKA activity, the Ras2-GTP/total Ras2 ratio was higher than in the corresponding wild type strain. Using a pull-down assay, we decided to investigate whether alkaline pH stress, causing a transient downregulation of the cAMP/PKA pathway, could positively influence the Ras2-GTP-loading state. Cells of the wild type strain expressing the eGFP-RBD3 probe were grown until the exponential phase at 30°C and collected by centrifugation before and 2, 5 and 30 minutes after addition of KOH. As expected, our result showed that addition of KOH to glucose-growing cells increased the Ras2-GTP levels already within 2 minutes (Fig. 23).

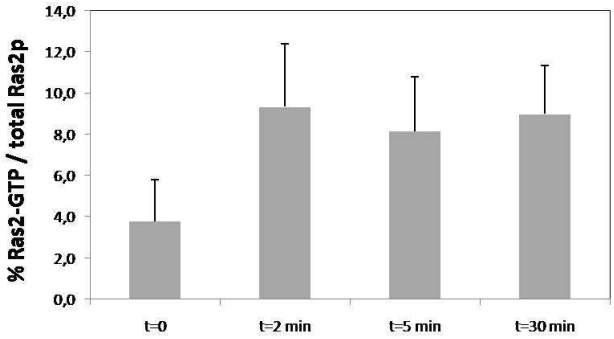


Figure 23. Effect of alkalinization on Ras2-GTP levels in the W303-1A wild type cells.

3.6 Nuclear active Ras2 is involved in invasive growth in the W303-1A background

3.6.1 The expression of the NES-Ras2 protein does not cause a growth defect either on fermentable or non fermentable carbon sources

As previously showed (section 3.1), the eGFP-RBD3 probe was localized to the plasma membrane in wild type W303-1A cells growing exponentially on glucose medium, indicating the presence of active Ras in this cellular compartment. Surprisingly the probe was also found to accumulate within the nucleus and only marginally in mitochondria. To investigate the nuclear function of the Ras2 protein, we generated a strain where Ras2 was completely excluded from this cellular compartment. To this aim a fusion was made between the Ras2 protein and the Nuclear Export Signal (NES) from the HIV virus (HIV virus Rev protein NES) (Henderson *et al*., 2000), generating the W303-1A-NES-RAS2 strain. The expression of NES-Ras2 in W303-1A wild type cells resulted in a polypeptide of the correct size (Fig. 24A) and no obvious defects in growth were observed in these cells growing on glucose-medium (Fig. 24B).

To investigate the localization of active Ras in the W303-1A cells expressing the NES-Ras2 fusion protein, the eGFP-RBD3 probe and epi-fluorescence

microscopy were used; as expected, the insertion of Rev NES sequence completely excluded the NES-Ras2 protein from the nucleus (Fig. 25).

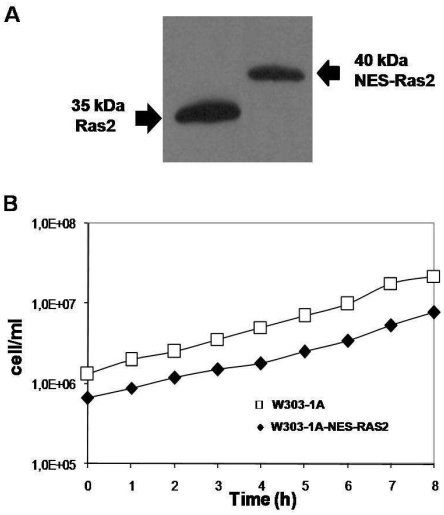


Figure 24. Expression level of Ras2 and NES-Ras2 proteins and growth rate of W303-1A wild type and W303-1A-NES-RAS2 strains growing on glucose medium. (A) Analysis of the expression of the NES-Ras2 fusion protein using a SDS-PAGE and western blotting analysis. The fusion protein was expressed as a polypeptide of about 40 kDa, indicating that Ras2 was actually expressed as a fusion with the NES sequence. The Ras2 protein had a size of about 35 kDa. (B) The W303-1A-NES-RAS2 strain grew in minimal medium containing 2% glucose at a rate comparable to that of the W303-1A wild type strain.

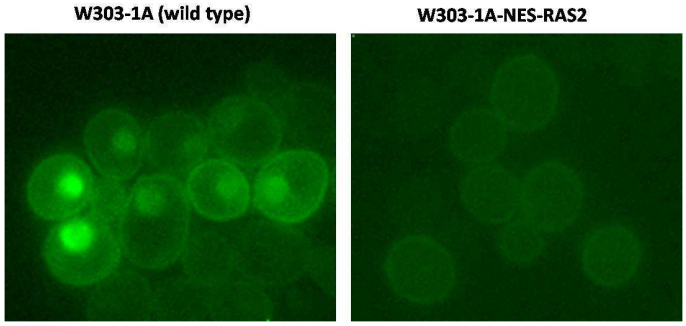


Figure 25. Localization of active Ras in glucose-growing W303-1A and W303-1A-NES-RAS2 cells. The Rev NES sequence completely excluded the Ras2 protein from the nucleus. Cells were grown in medium containing 2% glucose at 30°C and then photographed with a Nikon fluorescence microscope.

To characterize the nuclear function of Ras2, we first investigated the ability of W303-1A-NES-RAS2 cells to grow on media containing different carbon sources. Our results show that the W303-1A-NES-Ras2 strain grew in YPD, YPGly and YPEtOH media at a rate comparable to that of the corresponding wild-type strain, indicating that the expression of the NES-Ras2 protein did not cause a growth defect neither on fermentable nor non fermentable carbon sources (Fig. 26).

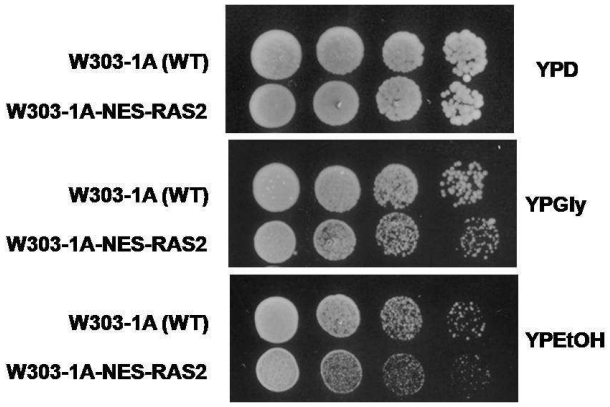


Figure 26. W303-1A-NES-Ras2 mutant cells grow either on fermentable or non fermentable carbon sources at a rate comparable to that of the W303-1A wild type cells. Cells of the indicated strains were incubated in YPD medium and grown till to exponential phase. Then cells were harvested by centrifugation, washed three times with sterile water and resuspended in sterile water at 10^7 cells/ml. 5 µl from the concentrated suspension and from 10–fold dilutions were spotted on agar plates containing different fermentable (Glucose) and non fermentable (Glycerol and Ethanol) carbon sources. Pictures were taken after 3 days at 30°C. One significant experiment out of 3 is shown.

3.6.2 The expression of the NES-Ras2 protein does not influence the PKA activity related phenotypes

A possible consequence of altered localization of the active Ras2 protein could be a change of PKA activity. In order to investigate this aspect, phenotypic properties usually related to PKA activity were considered. In particular, sensitivity to heat shock, osmotic and oxidative stress have been tested. Our results showed that the expression of the NES-Ras2 protein had no effect on heat shock, osmotic and oxidative stress (Fig. 27), indicating

that the delocalization of active Ras2 does not interfere with PKA activity and consequently does not influence the PKA activity related phenotypes analyzed.

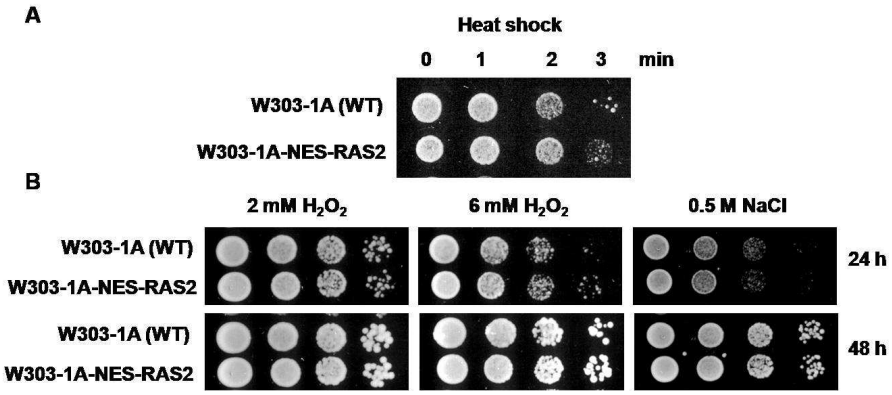


Figure 27. Analyses of PKA-activity-related phenotypes in the W303-1A-NES-RAS2 mutant strain. (A) Heat-shock resistance in exponentially growing cells. Cells of the indicated strains were incubated in YPD medium to exponential phase and then exposed to heat shock at 51 °C for 0, 1, 2 and 3 min. Approximately 10^4 cells were spotted on YPD agar and incubated at 30 °C for 24 hours. (B) Oxidative and osmotic stress resistance in exponentially growing cells. Cells of the indicated strains were incubated in YPD medium until exponential phase. Then cells were harvested by centrifugation, washed three times with sterile water and resuspended in sterile water at 10^7 cells/ml. 5 µl from the concentrated suspension and from 10–fold dilutions were spotted on glucose agar plates containing respectively 2 mM H_2O_2 and 6 mM H_2O_2 (oxidative stress) and 0.5 M NaCl (osmotic stress). After 24 hours and 48 hours at 30°C pictures were taken. One significant experiment out of 3 is shown.

3.6.3. The expression of the NES-Ras2 protein impairs the invasive growth phenotype in the W303-1A background

Data in literature show that in *S. cerevisiae* the small GTPase Ras2, but not Ras1, activates invasive growth using either of two downstream signalling pathways, the filamentation MAPK (Cdc42p/Ste20p/MAPK) cascade or the cAMP/PKA pathway, indicating that a crosstalk exists between these signalling pathways (Mösch *et al*., 1999) and this could happen in the

nucleus. To investigate the possible involvement of nuclear Ras2 in invasive growth, we performed an invasive growth test, including the *gpa2Δ* strain as a control strain, since disruption of Gpa2 activity was previously reported to impair invasive growth (Lorenz M. 1997 and 2000). Cells were grown on YPDA liquid medium at 30°C until exponential phase and then spotted on YPD agar plates. After 3 days of incubation at 30°C the plates were washed gently with water from top down. As shown in Fig. 28, the weak invasive activity of the W303-1A strain was completely eliminated in *gpa2Δ* cells and in cells expressing the NES-Ras2 fusion protein, indicating that active Ras2 in the nucleus is actually required for invasive growth in this cellular background.

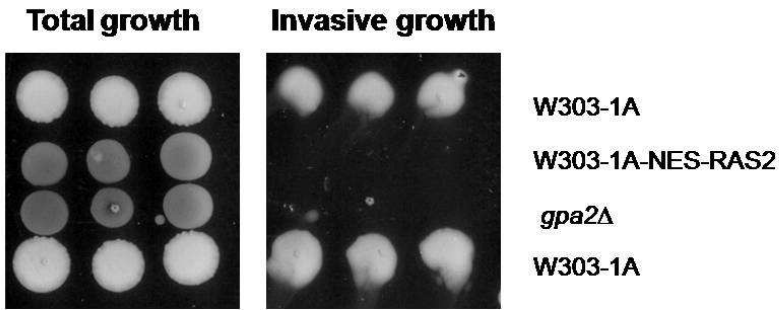


Figure 28. Nuclear active Ras2 is required for invasive growth in W303-1A background. YPD exponentially growing cells of the indicated strains were spotted on YPD agar plates. After 3 days at 30°C the plates were gently washed with water from top down and pictures were taken before (Total growth) and after (Invasive growth) washing.

3.7 Nuclear active Ras2 is involved in invasive growth in the Tlys86 background

3.7.1 The expression of the NES-Ras2 protein does not cause a growth defect either on fermentable or non fermentable carbon sources and does not influence the PKA related phenotypes

To confirm the inability of cells lacking active Ras2 in the nucleus to perform invasive growth, we made a fusion between the Ras2 protein and the Rev NES sequence in the strain Tlys86 commonly used to test this phenotype,

generating the strain Tlys86-NES-RAS2. Also in this background, expression of NES-Ras2 resulted in a polypeptide of the correct size (Fig. 29A), the mutant strain grew in minimal medium containing glucose at a rate comparable to that of the wild-type strain (Fig. 29B) and the insertion of the Rev NES sequence completely excluded the NES-Ras2 protein from the nucleus (Fig. 29C). We verified that, also in this background, the expression of the NES-Ras2 protein did not cause a growth defect neither on fermentable nor non fermentable carbon sources and that the expression of the NES-Ras2 protein had no effect on heat shock, osmotic and oxidative stress (Fig. 30).

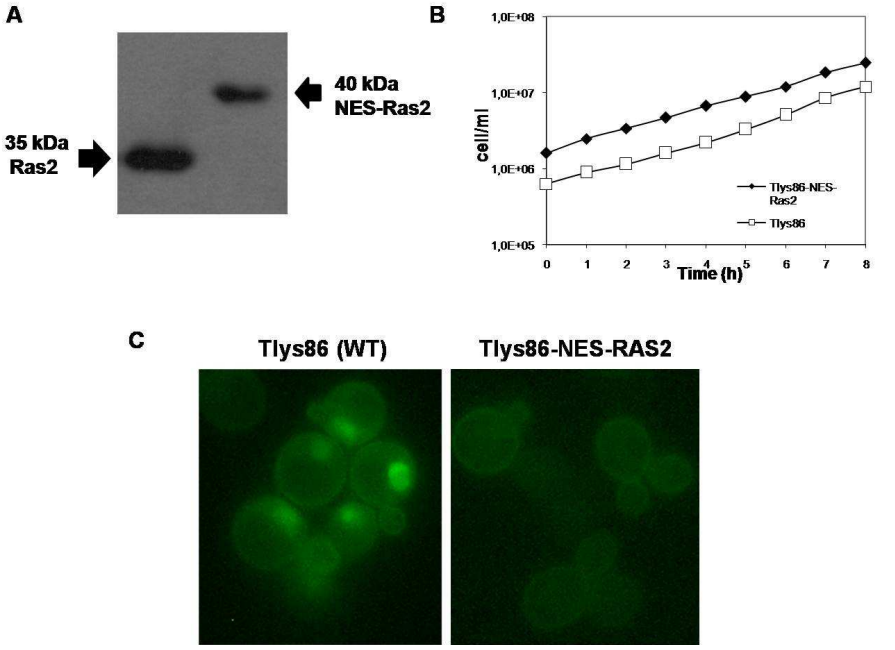


Figure 29. Expression level of Ras2 and NES-Ras2 proteins and growth rate of Tlys86 wild type and Tlys86-NES-RAS2 strains growing on glucose medium. (A) Analysis of the expression of the NES-Ras2 fusion protein using a SDS-PAGE and western blotting analysis. The NES-Ras2 fusion protein was expressed as a polypeptide of about 40 kDa, indicating that Ras2 was actually expressed as a fusion with the NES sequence. (B) The Tlys86-NES-RAS2 strain grew in minimal medium containing 2% glucose at a rate comparable to that of the Tlys86 wild type strain. (C) Localization of active Ras in glucose-growing Tlys86 and Tlys86-NES-RAS2 cells. The Rev NES sequence completely excluded the Ras2 protein from the nucleus. Cells

were grown in medium containing 2% glucose at 30°C and then photographed with a Nikon fluorescence microscope.

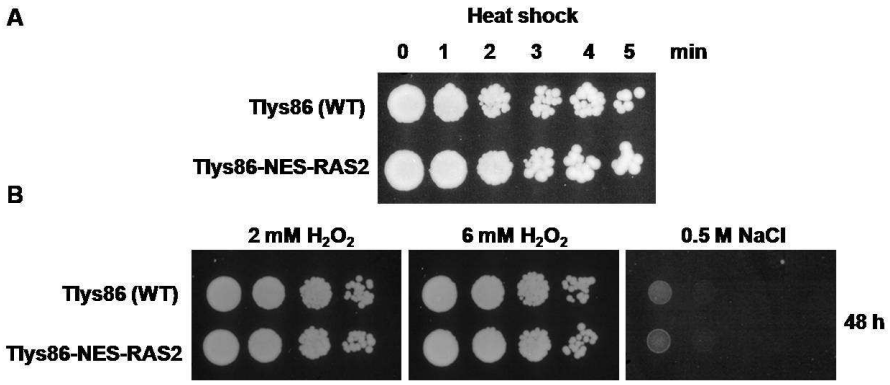


Figure 30. Effect of expression of the NES-Ras2 fusion protein on PKA-activity-related phenotypes. (A) Heat-shock resistance in exponentially growing cells. Cells of the indicated strains were incubated in YPD medium to exponential phase and then exposed to heat shock at 51 °C for 0, 1, 2, 3 , 4 and 5 min. Approximately 10^4 cells were spotted on YPD agar and incubated at 30 °C for 24 hours. (B) Oxidative and osmotic stress resistance in exponentially growing cells. Cells of the indicated strains were incubated in YPD medium to exponential phase. Then cells were harvested by centrifugation, washed three times with sterile water and resuspended in sterile water at 10^7 cells/ml. 5 µl from the concentrated suspension and from 10–fold dilutions were spotted on glucose agar plates containing respectively 2 mM H_2O_2 and 6 mM H_2O_2 (oxidative stress) and 0.5 M NaCl (osmotic stress). After 24 hours and 48 hours at 30°C pictures were taken. One significant experiment out of 3 is shown.

3.7.2 The expression of the NES-Ras2 protein impairs the invasive growth phenotype in the Tlys86 background

To verify if actually nuclear localization of active Ras2 is involved in the invasive growth, we performed an invasive growth test also in this background. As shown in Fig. 31, the invasive activity of the Tlys86 strain was completely eliminated in cells expressing the NES-Ras2 fusion protein, confirming that active Ras2 in the nucleus is actually required for invasive growth.

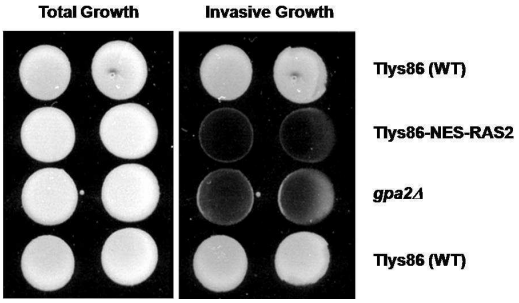


Figure 31. Nuclear active Ras2 is required for invasive growth in Tlys86 background. YPD exponentially growing cells of the indicated strains were spotted on YPD agar plates. After 3 days at 30°C the plates were washed directly under the water and pictures were taken before (Total growth) and after (Invasive growth) washing. One significant experiment out of 3 is shown.

3.8 Nuclear Cdc25 is required for invasive growth

Data in literature show that Cdc25, the main guanine nucleotide exchange factor (GEF) that controls the GDP/GTP exchange on Ras proteins, localizes in internal membranes and also in the nucleus in exponentially growing cells (Tisi *et al.,* 2008; Belotti *et al.,* 2011). To investigate whether the nuclear localization of Cdc25 is involved in the invasive growth phenotype, we performed an invasive growth test using a mutant where the Cdc25 protein contained the HIV Rev protein NES sequence (Tisi *et al.,* 2008). Our results showed that Cdc25-NES cells were not able to invade the agar (Fig. 32A), indicating that nuclear Cdc25 is required for invasive growth. In particular, this result suggests that Cdc25 could have the function to activate the Ras proteins in the nucleus and promote invasive growth. Consequently, we investigated the localization of active Ras in the Cdc25-NES strain growing on glucose medium. Our results showed that the fluorescent signal was mainly localized at the plasma membrane and in the nucleus, like in the corresponding wild type strain (Fig. 32B), suggesting that the role of nuclear Cdc25 is not to activate the Ras proteins in this cellular compartment.

To better investigate the role played by the catalytic domain of Cdc25 in invasive growth, we performed the invasive growth test in three different

Cdc25 mutant strains: the WΔN1 strain that lacks most of the Cdc25 N-terminal domain and express the sequence comprised between the aa 907 and 1589; the WΔCdc25^{Mm} strain in which the entire *CDC25* ORF was deleted and substituted with an expression cassette containing the Ras-exchange domain of the mouse GEF RasGRF1/Cdc25Mm; the WΔhSos1 strain in which the entire *CDC25* ORF was deleted and substituted with an expression cassette containing the Ras-exchange domain of human GEF Sos1 (Belotti *et al*., 2006). In these mutants the catalytic domain of Cdc25 localizes in the nucleus (Tisi *et al*., 2008). Preliminary results showed that these strains are not able to invade the agar medium, indicating that only the expression of the Ras exchange domain of Cdc25 is not enough to promote the invasive growth in absence of the N-terminal domain (Fig. 33) and probably is not the catalytic activity of Cdc25 that promotes the invasive growth.

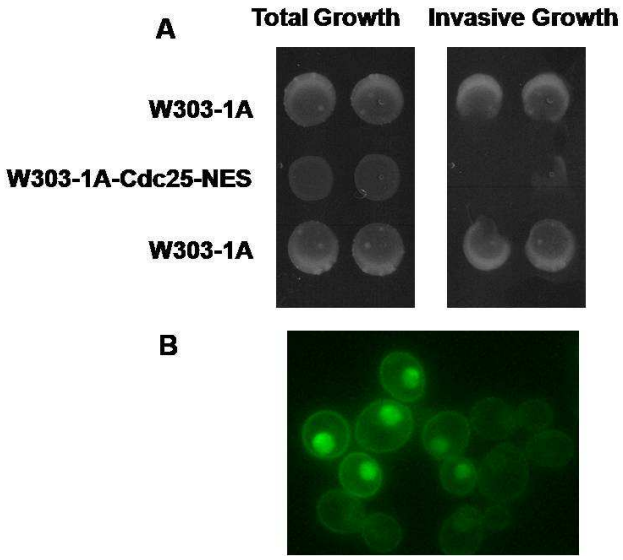


Figure 32. Nuclear Cdc25 is required for invasive growth. (A) YPD exponentially growing cells of the indicated strains were spotted on YPD agar plates. After 3 days at 30°C the plates were gently washed from top down with water and pictures were taken before (Total growth) and after (Invasive growth) washing. (B) Active Ras localized at the plasma membrane and in the nucleus in Cdc25-NES cells growing on glucose medium. Cells were grown in medium containing 2% glucose at 30°C until exponential phase and then photographed with a Nikon fluorescence microscope.

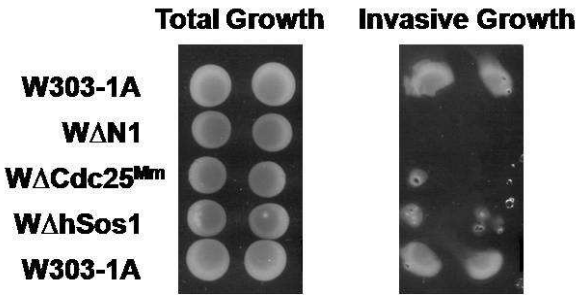


Figure 33. The expression of the Ras exchange domain of the yeast (WΔN1), mouse (WΔCdc25^{Mm}) and human (WΔhSos1) GEF does not induce invasive growth in cells lacking the Cdc25 N-terminal domain. YPD exponentially growing cells of the indicated strains were spotted on YPD agar plates. After 3 days at 30°C the plates were gently washed with water and pictures were taken before (Total growth) and after (Invasive growth) washing.

3.9 PKA activity controls invasive growth influencing the localization of active Ras proteins

Results previously shown (section 3.5.1) indicated that in a strain with either reduced or absent PKA activity, active Ras was mainly localized to the plasma membrane, while the nuclear localization was drastically reduced compared with the corresponding wild type strain. On the contrary, in a strain with high PKA activity, the plasma membrane accumulation was almost completely absent and active Ras localized mainly in the nucleus (Figs. 18 and 19), indicating that the localization of active Ras was dependent on PKA activity. To investigate if PKA could affect invasive growth by influencing the localization of active Ras proteins, we analyzed the ability of different PKA mutant cells to invade the agar medium. Our results showed that both in SP1 and W303-1A background, cells with high PKA activity (*bcy1Δ*) were able to invade the agar, while mutants with either low (*tpk1*^{w1} *tpk2Δ tpk3Δ*) or absent PKA activity (*cyr1Δ pde2Δ yak1Δ*) did not invade the agar anymore (Fig. 34), indicating that PKA activity controls invasive growth influencing the active Ras localization.

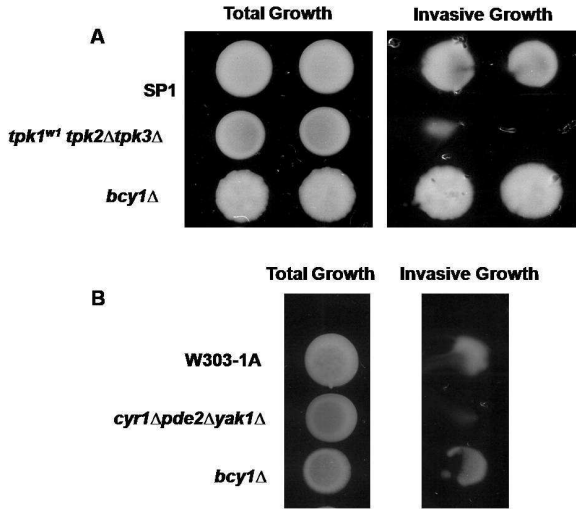


Figure 34. PKA activity controls invasive growth influencing the localization of active Ras. YPD exponentially growing cells of the indicated strains were spotted on YPD agar plates. After 3 days at 30°C the plates were gently washed with water from top down and pictures were taken before (Total growth) and after (Invasive growth) washing. (A) Cells of SP1 background; (B) cells of W303-1A background.

3.10 Studies on cAMP/PKA pathway oscillations in single yeast cell *in vivo*

3.10.1 Analysis of cAMP levels in a single yeast cell *in vivo*

In the yeast *S. cerevisiae* the second messenger cyclic AMP (cAMP) plays a central role in the control of cell metabolism, stress resistance and proliferation in response to available nutrients. A basal level of cAMP is required for growth, while a transient increase of cAMP induced by addition of glucose is required for transition from respiratory to fermentative metabolism (Thevelein and De Winde, 1999). Although the cAMP/PKA pathway has been extensively studied in yeast and both upstream and downstream elements are known, data on the spatiotemporal variation of cAMP in single cells are till now lacking. Data in literature (Cazzaniga *et al*., 2008; Pescini *et al*., 2012) show the presence in silico of cAMP levels

oscillations. Ponsioen *et al*. (2004) proposed a system based on a FRET (Fluorescence Resonance Energy Transfer) sensor to monitor the changes in cAMP level in mammalian cells, based on the mammalian protein EPAC, originally developed by Nikolaev *et al*. (2004). Epac1 is the GEF for Rap1 that is activated by direct binding of cAMP. The GEF Epac1 consist of a C-terminal catalytic domain characteristic of exchange factors for Ras family GTPases and a N-terminal regulatory domain. The latter domain contains a cAMP-binding site similar to those of protein kinase a (PKA) (de Rooij *et al*., 1998; Rehmann *et al*., 2003). In the CFP-Epac1-YFP probe used in mammalian cells, Epac1 is fused amino terminally to cyan fluorescent protein and carboxy terminally to yellow fluorescent protein. We inserted the sequence coding for the sensor in a multicopy yeast expression vector and the sensor was expressed under the control of the TPI promoter in several yeast strains: two wild type strains (SP1 and W303-1A), a strain which is not able to produce cAMP (*cyr1Δ*) and a strain deleted for the phosphodiesterase-1 gene (*pde1Δ*). We used a two-photon confocal microscope system to measure in a single cell the CFP and YFP fluorescence before and after addition of 100mM glucose to glucose-starved cells and the relative FRET efficiency was determined from the CFP/YFP fluorescence ratio. In wild type strains (W303-1A and SP1) the CFP/YFP ratio increased immediately after glucose addition to derepressed yeast cells, according to the predictions made with the mechanistic model of the Ras/cAMP/PKA pathway (Cazzaniga *et al*., 2008; Pescini *et al*., 2012), no changes were observed when glucose was added to strain that is not able to produce cAMP (*cyr1Δ*), while a sustained increase was observed in a strain bearing a deletion in the phosphodiesterase-1 gene (*pde1Δ*) (Fig. 35). However, there was a high noise related to the FRET evaluation and it was impossible to point out long term oscillations of cAMP levels in single cells.

3.10.2 Analysis of PKA activity in a single yeast cell *in vivo*

Since Epac did not seem to be a highly sensitive cAMP sensor in yeast cells , parallely we tried to use another system to investigate the oscillations in PKA activity *in vivo* in a single yeast cell. Recently, in mammalian cells was used a new FRET sensor to monitor the spatiotemporal dynamics of PKA

based on AKAR3 (A-kinase activity reporter) probe. AKAR3 is a recombinant protein composed of a phosphoamino acid binding domain and PKA-specific substrate sandwiched between CFP and YFP. We inserted the sequence coding for the sensor in a multicopy yeast expression plasmid and the sensor was expressed in several yeast strains: a wild type strain (SP1), a strain without PKA activity (*tpk1Δ tpk2Δ tpk3Δ msn2Δ msn4Δ*) and a strain that is not able to produce cAMP (*cyr1Δ*). We used a two-photon confocal microscope system to measure in a single cell the CFP and YFP fluorescence before and after addition of 100 mM glucose to derepressed cells and the relative FRET efficiency was determined from the YFP/CFP fluorescence ratio. In a wild type strain (SP1) the YFP/CFP ratio increases immediately after glucose addition to glucose-starved yeast cells, no changes were observed when glucose was added to strain that is not able to produce cAMP (*cyr1Δ*) or in a strain without PKA activity (*tpk1Δ tpk2Δ tpk3Δ msn2Δ msn4Δ*) (Fig. 36).

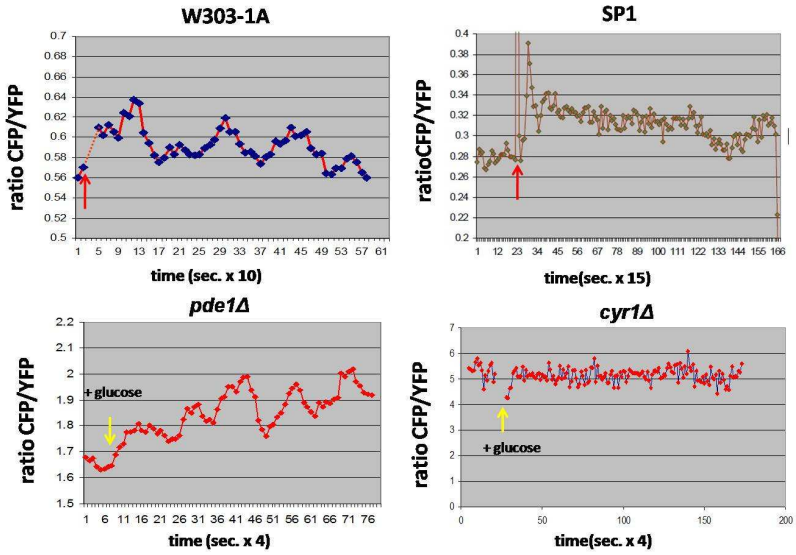


Figure 35. cAMP changes in single yeast cells after addition of glucose to derepressed cells. Cells of indicated strains were grown in 2% glucose medium at 30°C until exponential phase, collected by centrifugation and resuspended in MES buffer for about 30 min. At time zero (red or yellow arrow) 100mM glucose was added.

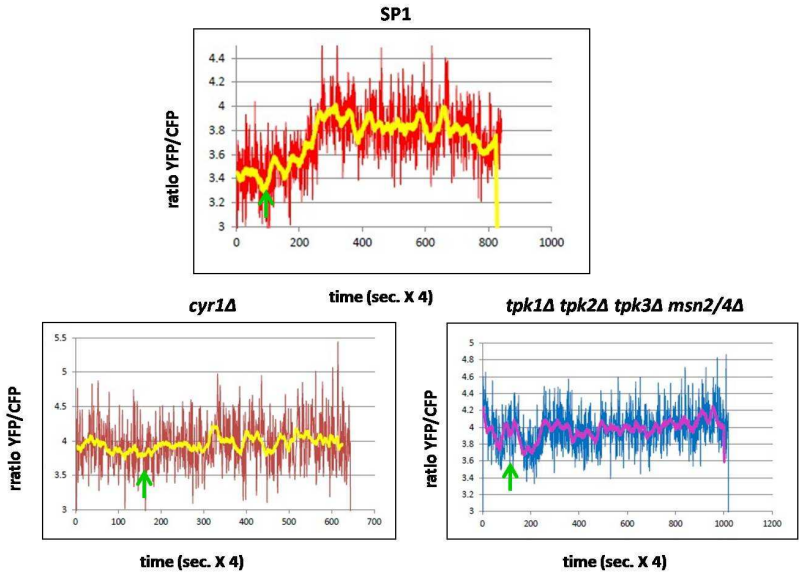


Figure 36. PKA changes in single yeast cells after addition of glucose to derepressed cells. Cells of indicated strains were grown in 2% glucose medium at 30°C until exponential phase, collected by centrifugation and resuspended in MES buffer for about 30 min.

DISCUSSION

The spatial regulation of signalling events at the subcellular level is increasingly recognized as an important aspect of signal transduction. Quite some evidence points to Ras playing distinct signaling roles depending on its subcellular location (Casar *et al*., 2008). However, the understanding of Ras trafficking and targeting is still incomplete. In our laboratory, using a pull-down assay, it was previously demonstrated that glucose addition to derepressed yeast cells triggers a fast increase in the GTP loading state of Ras2 (Colombo *et al*., 2004; Rudoni *et al*., 2001) and that this increase is dependent on Cdc25 and on active sugar kinases. They also showed that the deletion of *GPR1* or *GPA2* enhances basal and induced Ras2-GTP level. But this assay measures only the "when" of activation and does not report anything about the "where" of signalling. Methods have been developed that use the green fluorescent protein (GFP) to report when and where Ras become activated in living cells. Here, we describe the use of eGFP-RBD3 as a useful live-cell biosensor to report Ras activation in *Saccharomyces cerevisiae*. As a first approach, in our laboratory a monomeric Ras Binding Domain expressed as a fusion with the eGFP was used. The fluorescence was distributed homogeneously throughout the cytoplasm and nucleoplasm in both wild type and mutant strains of the cAMP/PKA pathway, indicating the inability of such a probe to report endogenous Ras activation. In mammalian cells as well, Ras-GTP imaging studies using the monomeric RBD of the Ras effector c-Raf1 have produced discrepant results about a possible activation of Ras at a Golgi apparatus, while the RDB3 oligomerization provided a better probe, showing that endogenous Golgi-located Ras was not under the control of growth factors and arguing for the plasma membrane as the predominant site of agonistic-induced Ras activation (Chiu *et al*. 2002). Consequently, we made a new probe that expressed eGFP fused to three sequential RBD domains starting from the construct used as a live-cell biosensor for Ras-GTP in mammals (Augsten *et al.*, 2006). We show that, as expected, the eGFP-RBD3 probe was localized to the plasma membrane in wild type cells growing exponentially on glucose medium, indicating the presence of active Ras in this cellular compartment. To test the specificity of the probe we expressed the eGFP in wild type cells and the eGFP-RBD3 probe in GG104 *cdc25*Δ cells. In both

cases we observed a diffuse staining, indicating that the plasma membrane staining occurs as a result of activated forms of Ras. Surprisingly the probe was also found to accumulate within the nucleus. However active Ras is not the only component of the cAMP/PKA pathway found to be localized in the nucleus. In cells growing on glucose, the PKA holoenzyme was found almost exclusively nuclear, whereas, in respiring or in stationary phase cells, Bcy1 and Tpk1 were more evenly distributed over both nuclear and cytoplasmic compartments (Griffioen *et al*., 2000). Also Cdc25, the main exchange factor for Ras, and Ira1 were recently found to accumulate in the nucleus (Tisi *et al.,* 2008; Belotti *et al.,* 2011). On the other end, Cyr1, the well characterised effector of Ras, was not found in the nuclear compartment, but it was mainly localized in internal membranes (Belotti *et al*., 2011), indicating that nuclear import could actually have the meaning to subtract the Ras proteins from adenylate cyclase. Finally, *S. cerevisiae* is not the only organism showing a nuclear localization of Ras, since also in mammalian cells and in *Dictostelium* active Ras has been seen accumulating in this cellular compartment, although the significance is still unclear (Sutherland *et al*, 2001; Fuentes-Calvo *et al*., 2010; Contente *et al*., 2011).

The nuclear localization of active Ras in wild type cells exponentially growing on glucose medium was unexpected, but it could be indicative of a specific nuclear function of Ras2-GTP. The cAMP/PKA pathway is involved in the regulation of cell growth in response to available nutrients and in the adaptation to glucose, but it also affects morphogenesis and development, including pseudohyphal and invasive growth and sporulation capacity. To investigate the role played by active Ras in the nucleus, we generated a strain where Ras2 is fused to the Nuclear Export Signal (NES) from the HIV virus (HIV virus Rev protein NES), in order to completely exclude Ras2 from the nucleus. Our results show that the exclusion of the Ras2 protein from the nucleus does not cause a growth defect neither on fermentable nor non fermentable carbon sources and does not influence the stress resistance of the cells. Cells expressing the fusion protein were only defective for the invasive growth, suggesting that nuclear active Ras2 actually is involved in this process. In particular, data in literature show that Ras2, but not Ras1, activates invasive growth using either of two downstream signalling

pathways, the filamentation MAPK (Cdc42p/Ste20p/MAPK) cascade or the cAMP/PKA pathway, indicating a crosstalk between both signalling pathways and this could happen in the nucleus (Pokholok *et al*., 2006; Mosch *et al*., 1999). This hypothesis is substantiated by the observation that in a stain deleted in *GPA2*, which has a defect in pseudohyphal growth (Lorentz and Heitman, 1997), active Ras does not accumulate in the nucleus, but is mainly localized in mitochondria. Ras2 is the common upstream activator of the MAPK and PKA pathways and it seems reasonable that activation of one pathway might promote activation of the other, but the connection between the two pathways is still not completely clear. Evidence obtained in some studies suggest that the MAPK and PKA pathways function independently, at least for regulation of diploid pseudohyphal growth (Pan and Heitman 1999), whereas other findings suggest that, for haploid invasive growth, the PKA pathway regulates the MAPK pathway (Mosch *et al.,* 1999). However, another study conclude the converse, namely that the MAPK pathway regulates the PKA pathway in haploids (Cherkasova *et al.* 2003), whereas Chen and Thorner (2010) suggest that the MAPK and PKA pathways contribute additively to promote invasive growth. In this thesis we clearly show that PKA activity regulates the ability of the cells to perform invasive growth by influencing the localization of active Ras2. In particular, we show that in a strain with either reduced or absent PKA activity (e.g. *cyr1Δ pde2Δ msn2/4Δ* cells or *tpk1Δ tpk2Δ tpk3Δ yak1Δ* cells) active Ras localizes mainly at the plasma membrane and cells do not invade the agar anymore. On the contrary, in a strain with high PKA activity, active Ras localizes to the nucleus and cells are able to invade the agar. Data in literature show that PKA regulates the localization of other components of the cAMP/PKA pathway, like the *PDE2*-encoded high-affinity cAMP phosphodiesterase (Hu *et al*., 2010), Cdc25 (Belotti *et al*., 2011) and the Ras2 protein (Dong and Bai, 2011), in particular it has been demonstrated that PKA influence the localization of Cdc25 and Ras2 through their direct phosphorylation. We can speculate that PKA could regulate the nuclear localization of active Ras2 proteins by phosphorylating these GTPases. Active Ras in the nucleus could promote invasive growth by activating specific targets. It has been shown that Cdc24, the guanine

nucleotide exchange factor for Cdc42, which is a potent regulator of filamentous growth, localizes into the nucleus and Cdc42 itself, that has been shown to act downstream Ras2, (Mosch *et al.,* 1996), localizes to the periphery of the nucleus (Richman *et al*., 2002). Consequently, we cannot exclude that active Ras2 in the nucleus could promote invasive growth by acting directly either on Cdc42 or on Cdc24.
In this thesis we clearly show that the localization of active Ras depends on PKA activity. This result is substantiated by the fact that high PKA activity, due to the addition of cAMP to strains unable to synthesizes it, promotes the relocalization of the probe from the membrane to the nucleus, while the downregulation of the cAMP/PKA pathway, due to the alkalinization of the medium, causes in wild type cells a transient delocalization of active Ras exclusively to the plasma membrane.

Our findings show that also the nuclear localization of Cdc25, the main GEF of Ras proteins, is required for invasive growth. Cells where this protein is forced out of the nucleus by the NES sequence are not able to invade the agar medium, suggesting that Cdc25 could have the function to activate the Ras proteins in the nucleus and promote invasive growth. However, in the Cdc25-NES strain, the fluorescent signal of the probe was mainly localized at the plasma membrane and in the nucleus, like in the corresponding wild type strain, suggesting that the effective role of nuclear Cdc25 is not to activate the Ras proteins in this cellular compartment. This hypothesis is substantiated by the fact that in mutants lacking Cdc25, the expression of the catalytic domain of this GEF (either the yeast catalytic domain or murine catalytic domain or human catalytic domain) is not sufficient to restore the invasive growth phenotype, although the catalytic domain of Cdc25 has been shown to localize inside the nucleus (Tisi *et al*., 2008).

In this thesis we also demonstrate that both in *cyr1*Δ and *gpr1*Δ strains growing on glucose medium the probe accumulated mostly in the plasma membrane, indicating that the absence of either adenylate cyclase or the G-protein coupled receptor substantially did not impair the localization of Ras-GTP. In glucose starved wild type cells as well as in *cyr1*Δ and *gpr1*Δ

cells the probe accumulated mainly in mitochondria and relocalized to the plasma membrane following glucose addition, arguing for the plasma membrane as the predominant site of agonistic-induced Ras activation. Surprisingly, in cells deleted in the *GPA2* gene the probe accumulated in internal membranes and mitochondria, both when cells were growing on glucose medium or were starved, indicating that Gpa2, independently on Gpr1 and consequently on its activation state, was required for the recruitment of Ras-GTP at the plasma membrane and in the nucleus. This result points to an uncoupling between Gpa2 and its G-protein coupled receptor Gpr1, uncoupling which has also been previously reported using a global transcriptional analysis approach in combination with genetics to obtain a nearly complete view of glucose signalling in yeast (Zaman *et al*., 2009). Pull-down assay and co-purification experiments have been done to investigate either a direct or indirect physical interaction between Gpa2 and the Ras proteins, but apparently, supposed that this interaction exist, it was too weak to be shown. Nevertheless, we proved that it was actually the presence of Gpa2 itself and not its activation state important for a proper localization of active Ras. Moreover, we show that the nuclear and the plasma membrane localization of Ras-GTP observed in the wild type strain during growth on glucose medium was also dependent on the hexokinase 2, since in the absence of this protein the fluorescence was mainly located in mitochondria. Interestingly, we show that the expression of Hexokinase 2 in the *hxk1Δ hxk2Δ* mutant was able to restore the nuclear and plasma membrane localization of the probe, indicating that Hxk2 is actually involved in the proper localization of Ras-GTP. The significance of the mitochondrial localization of the probe is unclear and currently under investigation. However, other reports have found Ras associated with mitochondria, both in mammals and lower eukaryotes. Leadsham *et al*. (Leadsham *et al.,* 2009) showed that cells lacking Whi2p exhibit an aberrant accumulation of activated Ras at the mitochondria in response to nutritional depletion. In this mutant, the failure to address Ras to the vacuole and consequently the failure to shut down Ras signaling would lead to mitochondrial dysfunction, the accumulation of damaging ROS and cell death. Disruption of class C *VPS* genes, encoding for proteins playing a role

in endosome and vacuole membrane fusion, results in mitochondrial defects and an accumulation of total Ras proteins on mitochondrial membranes (Wang *et al*., 2006). Ras proteins have been shown to fractionate with the outer mitochondrial membrane also in wild-type cells, pointing to a role of mitochondria in the nonclassical pathway of Ras trafficking (Dong et al., 2002; Wang et al., 2006). Our data clearly show that active Ras proteins localize in mitochondria in a variety of strains. Such a localizations has been observed in starved wild type (around 60%), *cyr1Δ* (around 75%) and *gpr1Δ* (75%) cells. Both glucose growing and starved *gpa2Δ, hxk2Δ* and *hxk1Δ hxk2Δ* cells also showed a mitochondrial localization of the probe. The functional involvement of Ras proteins in mitochondrial function is currently under investigation. A mitochondrial localization of Ras has been reported also in higher eukaryotes. In the human kidney, H-Ras localizes with mitochondria in proximal and distal convoluted tubules (Kocher *et al*., 2005). Bcl-2 and Ras proteins interact at mitochondria in the murine T-cell line TS1_ß, and the mitochondrial association of the three Ras proteins is differentially regulated by interleukin-2 supplementation (Rebollo *et al*., 1999). Several downstream effectors of Ras proteins were also found in mitochondria in mammalian cells and lower eukaryotes. Grb10 was detected at mitochondria and interacts with the mitochondrion associated Raf-1 pool (Nantel *et al*., 1999). A homolog of the yeast prenylcyteine methyltransferase, Ste14, has been reported to be part of the mitochondrial proteome of *Neurospora crassa* (Schmitt *et al*., 2006). Finally, Philips recently showed that phosphorylation results in translocation of K-Ras from the plasma membrane to the mitochondria, where it appears to play a role in apoptosis (Philips, 2005).

Data in literature show that at intermediate intensities, stress induces nucleocytoplasmic oscillations of the yeast transcription factor Msn2 and these are evidence for periodic PKA activation (Garmendia-Torres et al., 2007). Moreover, Cazzaniga *et al*. (2008) and Pescini *et al*. (2012) show the presence in silico of cAMP levels oscillations. Ponsioen *et al*. (2004) suggested the use of a Epac-based FRET (Fluorescence Resonance Energy

Transfer) probe to monitor the cAMP levels *in vivo* in mammalian cells. Epac1 is a guanine nucleotide exchange factor for Rap1 that is activated by direct binding of cAMP. In their study the authors monitored Epac1 activation *in vivo* by using a CFP-Epac1-YFP fusion construct. When expressed in mammalian cells this probe shows significant FRET and a better signal-to-noise ratio. In our work we tried to use the same sensor inserted in a yeast expression vector under the control of the TPI promoter to monitor changes in cAMP levels *in vivo* in single yeast cells. We show that in wild type strains (W303-1A and SP1) the CFP/YFP ratio increases immediately after glucose addition to derepressed yeast cells, according to the predictions made with the mechanistic model of the Ras/cAMP/PKA pathway (Cazzaniga *et al*., 2008; Pescini *et al.,* 2012), however since there is a high background noise it's impossible to monitor if there are oscillations of cAMP levels in the long term. To overcome the problem of background noise it will be necessary to develop new experiments using strains of different genetic background or different experimental conditions. Recently, in mammalian cells Allen and Zhang (2006) used a new FRET sensor to monitor the spatiotemporal dynamics of PKA based on AKAR3 (A-kinase activity reporter) probe, previously evolved and optimized by Ni *et al.* 2006. The authors also targeted the sequence encoding the FRET sensor to subcellular locations via various localization signals. To examine PKA activities in subcellular compartments, several AKAR3 derivates were generated with specific targeting motifs. In particular the reporter was targeted to the plasma membrane, to the cytoplasm and to the mitochondria. Since we had some problems with the Epac probe, in parallel we try to use this probe to monitor the spatiotemporal changes in PKA activity also in single yeast cells *in vivo*. Our preliminary results show that in a wild type strain (SP1) the YFP/ CFP ratio increases immediately after glucose addition to derepressed yeast cells. But the FRET efficiency also in this case is not like the efficiency in mammalian cells and it will be necessary to test other strains and other conditions to determine whether AKAR3 could be use to visualize the subcellular dynamics of PKA in yeast cells.

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