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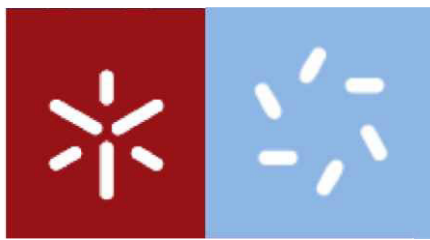
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Heterologous expression of human *KRAS*wt cDNA in Saccharomyces cerevisiae and its mutants from the Ras signalling pathway and phenotype screening

Tese de Mestrado
Mestrado em Genética Molecular

Trabalho realizado sob a orientação da
Professora Doutora Cândida Lucas
Doutora Célia Ferreira

janeiro 2015

DECLARAÇÃO

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Título da Tese: "Heterologous expression of human *KRAS*^{wt} cDNA in *Saccharomyces cerevisiae* and its mutants from the Ras signalling pathway and phenotype screening"

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Ano de conclusão: 2015

Designação do mestrado: Mestrado em Genética Molecular

Universidade do Minho, 30 de Janeiro de 2015

Assinatura: _____

Agradecimentos

Termino uma etapa importante da minha vida. E apesar de todo o trabalho e dedicação que lhe prestei, esta não teria sido alcançada sem a contribuição directa ou indirecta de todos. Deixo aqui o meu sincero agradecimento:

Em primeiro lugar às minhas orientadoras, Professora Doutora Cândida Lucas e Doutora Célia Ferreira, pelos conhecimentos transmitidos e por me proporcionarem a entrada neste "mundo" das leveduras. Garanto que a aprendizagem foi de todo enriquecedora.

Aos meus colegas de laboratório, pelo espírito de partilha e auto-ajuda que demonstramos sempre uns para com os outros, pelos momentos de descontração e pela alegria. Quando a companhia é boa o trabalho corre melhor!

Em especial à Joana Tulha pela imensa ajuda, incentivo, paciência e calma transmitida nos momentos mais difícies; à Giulia pelo apoio constante, companheirismo e troca de conhecimentos; e à Ana Sofia, a minha companheira de "guerra", obrigada por todos os momentos que passamos juntas... Assim se criou uma amizade!

A todos os funcionários, técnicos, e colegas do CBMA pela disponibilidade prestada e por toda a simpatia.

A todos os meus colegas de mestrado, com especial atenção a alguns, vocês sabem quem são, por todos os momentos e pela amizade. Levo-vos comigo!!!

Aos meus amigos por fazerem parte da minha vida, e acima de tudo pela amizade.

O meu maior agradecimento vai para os meus pais, pelo amor incondicional e pelos sacríficios que fizeram para o alcance dos meus objectivos. Para o Pedro podiam ir mil palavras, obrigada por nunca me teres faltado, obrigada por tudooo! Dedico este trabalho aos três.

Obrigada a todos!

Abstract

Most of our knowledge about basic cellular processes has originated from model organisms. *Saccharomyces cerevisiae* is consider a model system, representing the simplest eukaryotic organism, whose genome can be easily manipulated allowing suitable analysis and efficient assessment of gene products from humans. RAS genes encode low molecular weight, GTP-binding, GTP-hydrolysing proteins that are highly conserved throughout all eukaryotic species. RAS pathway has attracted attention because of its importance in malignant transformation of human cells. *KRAS* is a human RAS isoform, expressed in almost all cell types and essential for the normal cellular development, in addition it is the isoform most frequently mutated in many cancer types. The *S. cerevisiae* has two RAS genes, *RAS1* and *RAS2* that are essential, once their double deletion renders yeast unviable. These genes are structurally and functionally homolog of the mammalian *RAS* proto-oncogenes. This conservation allows the use of yeast genetics to the study of *KRAS*. Considering this, the first aim of this work was to build a set of *S. cerevisiae* strains (using haploid BY4741 wild type, *ras1Δ*, and *ras2Δ* as a basis) expressing the human *KRAS^wt^* cDNA, using a plasmid-based expression. The second aim was the phenotype screening of these *humanized* yeasts, as well as of the correspondent recipient strains. The effect of *KRAS^wt^* expression was evaluate on the cell stress response, growth, chronological aging, cell cycle progression and haploid invasive growth.

According to the results, the *KRAS^wt^* heterologous expression in yeast (1) had a negative or null effect on the resistance to the temperature, pH, osmotic and oxidative stresses; (2) decreased growth on non-fermentable carbon sources; (3) increased the adhesion capacity; (4) stimulated the haploid invasive growth in the *ras2Δ* strain; (5) modified the budding pattern of the wild type cells; (6) changed the cellular proliferation in a strain-dependent way; and (7) decreased the chronological lifespan. The results indicated that the expression of *KRAS^wt^* in the wild type strain, possibly leads to a hyperactivaction of the RAS/cAMP/PKA signalling pathway, which in turn triggers a decrease of stress resistance and longevity. This study also highlighted the relevance of the yeast background, to the possible achievement of different results, regardless the functional conditions used. The present work contributed to gain insight into KRAS mechanism of action using yeast as a model organism. Furthermore, all obtained results will constitute part of the development of a yeast-based high throughput phenotype platform for future pharmacological testing.

Resumo

Grande parte do nosso conhecimento acerca dos processos celulares fundamentais provêm de organismos modelo. A levedura *Saccharomyces cerevisiae* é considerada um sistema modelo, representando um organismo eucariota simples, cujo genoma pode ser facilmente manipulado, permitindo uma análise adequada e uma avaliação eficiente das proteínas humanas. Os genes RAS codificam proteínas de baixo peso molecular, de ligação e hidrólise de GTP, que estão amplamente conservadas em todas as espécies eucariótas. O estudo da via RAS tem atraído a atenção devido à sua importância na transformação maligna das células humanas. O *KRAS* é uma das isoformas RAS humanas, expressa em quase todos os tipos de células e essencial para o normal desenvolvimento celular, para além disso, é também a isoforma que se encontra frequentemente mutada em diversos tipos de cancro. *S. cerevisiae* tem dois genes RAS, *RAS1* e *RAS2*, essenciais uma vez que a sua dupla deleção é letal para a levedura. Estes genes são estruturalmente e funcionalmente homólogos dos proto-oncogenes RAS dos mamíferos. Esta conservação permite a utilização da levedura para o estudo do *KRAS*. Deste modo, o primeiro objectivo deste trabalho foi construir um conjunto de estirpes de *S. cerevisiae* (BY4741 haplóide *wild type*, *ras1Δ* e *ras2Δ* como receptoras) a expressar o $KRAS^{wt}$ humano, usando um plasmídeo de expressão. O segundo objectivo foi a realização de um *screening* fenotípico das leveduras humanizadas, bem como das estirpes não transformadas. O efeito da expressão do $KRAS^{wt}$ foi avaliado na resposta ao stress celular, crescimento, envelhecimento cronológico, progressão do ciclo celular e no crescimento invasivo haplóide.

De acordo com os resultados, a expressão heteróloga do $KRAS^{wt}$ na levedura (1) teve um efeito negativo, ou nulo, na resistência aos diferentes stresses (temperatura, pH, osmótico e oxidativo); (2) reduziu o crescimento em fontes de carbono não fermentáveis; (3) aumentou a capacidade de adesão; (4) estimulou o crescimento invasivo haplóide na estirpe *ras2Δ*; (5) modificou o padrão de divisão das células *wild-type*; (6) alterou a proliferação celular; e (7) diminuiu a longevidade cronológica. Os resultados indicaram que a expressão do $KRAS^{wt}$ na estirpe *wild type*, levou a uma possível hiperativação da via de sinalização RAS/cAMP/PKA, responsável pela diminuição da resistência ao stress e da longevidade. Este estudo também demonstrou a relevância do *background* da levedura para a possível obtenção de diferentes resultados, independentemente das condições usadas. O presente trabalho contribuiu para o conhecimento do mecanismo de ação do KRAS através da utilização da levedura como organismo modelo. Além disso, todos os resultados obtidos farão parte de uma plataforma de fenótipos de levedura para futuros testes farmacológicos.

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Chapter I

Introduction

1. Introduction

1.1 *Saccharomyces cerevisiae* as a model system in biological research

The *Saccharomyces cerevisiae* genome was the first eukaryotic genome to be fully sequenced in April 1996 [1], and over the years the data thus generated fuelled whole genome scale screening methods, including microarrays, two-hybrid analysis and the application of deletion and overexpression libraries [2]. The whole yeast nuclear genome contains a haploid set of 16 well-characterized chromosomes, ranging in size from 200 to 2,200 kb. In contrast to the genomes of multicellular organisms, the yeast genome is highly compact, with genes representing 72% of the total sequence (<2% in the human genome). *S. cerevisiae* has therefore a genome with a small size and low degree of complexity as compared with higher Eukaryotes. Furthermore, as all other eukaryotic organisms, the yeast *S. cerevisiae* also contains an additional extranuclear mitochondrial genome [3].

A considerable amount of knowledge about basic cellular processes originated from model organisms that include the simpler eukaryote *S. cerevisiae* [4]. The increasing amount and complexity of work performed in this organism, which occurred during the last decades, solidified yeast position at the forefront of eukaryotic cellular and molecular biology, facilitating the establishment of new fields of study like systems biology. This goes beyond the functions of individual genes and proteins, focusing on how these interact and work together to determine major properties underlying life and major biological properties and behaviours [5]. The reasons underlying include the many technical advantages that yeasts present over other systems for using in research and biotechnology, namely the short generation time and the high amenability to genetic modifications, as well as the ability to control its growth and division by adjusting environmental conditions [6]. Importantly, it accrues the fact of yeast being a GRAS organism, inexpensive to maintain and propagate, and easy to cultivate and store [3, 7, 8].

Yeast has been recognised the status of model organism for research of complex processes, as a simple eukaryotic organism whose genome can be easily manipulated allowing suitable analysis and efficient assessment of gene products from other Eukaryotes, including man [6, 9]. Although important aspects of human diseases lie beyond the reach of *S. cerevisiae* due to organisms' multicellular nature and

complexity, yeast is still used for unveiling molecular processes involved in some diseases based on their high degree of conservation [2, 7]. These are mostly basic cellular processes such as cell cycle control, DNA replication, recombination, repair, protein folding, trafficking, programmed cell death, and metabolic and regulatory mechanisms [10-12].

Moreover, many human genes that are mutated or that have their expression changed in tumour cells have yeast orthologues (approximately 30% of genes according to Françoise Foury) [13]. The fact that a gene codifying for a protein implied in a disease is conserved in yeast, opens the possibility to directly study its function by expressing it in yeast, or by integrating it into the yeast genome [5], namely to complement the correspondent yeast gene deletion [9, 14]. Yeast was used to analyse molecular mechanisms of several human diseases, sometimes of rather unexpected nature like neurological diseases [15-18], or cancer related signalling pathways [7, 8]. As said above, this was achieved by directly studying an endogenous protein orthologue of a human involved in the disease [15-18] or through the heterologous expression of human disease associated proteins [16, 19]. The use of yeast, expressing human genes or not, is therefore also of great value for high-throughput phenotyping and pharmacological assaying [6].

1.1.1 The yeast life cycle

The yeast life cycle (Fig. 1) comprehends two possible reproductive cycles, a mitotic asexual cycle in which cells reproduce by budding, and a sexual-like meiotic cycle in which cells sporulate, germinate, mate and then reproduce by budding. Mating cells can be genetically different from each other - heterothallic yeast – or identical – homothallic yeast. *S. cerevisiae* is heterothallic and produces two haploid mating types, MAT*a* and MAT*α*. After conjugation, diploid cells can reproduce asexually in identical fashion to haploid cells (by budding), or can suffer sporulation entering meiosis and consequently producing four haploid spores, which in turn can also germinate and reproduce asexually (by budding), or can mate returning the culture to diploid state [20].
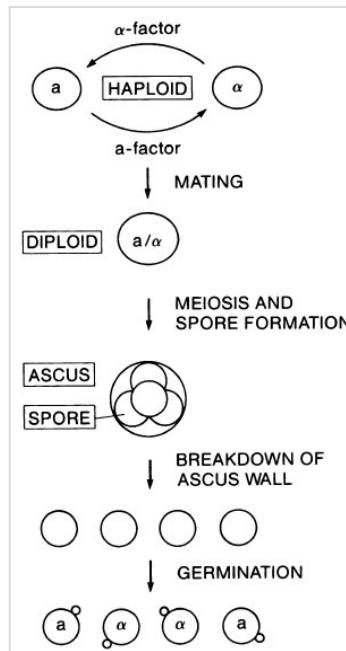
Figure 1. Scheme of yeast life cycle showing haploid and diploid states. Withdrawn from Herskowitz I (1988) [20].

Inversely to humans, *S. cerevisiae* lives mostly as a unicellular organism, yet as all other microbes, it can form multicellular aggregates, such as colonies or biofilms that are complex, organized, multicellular structures [20, 21]. Additionally, in certain stress conditions such as carbon or nitrogen starvation or oxygen limitation, *S. cerevisiae* cells can suffer a morphological switch characterized by changes in cell polarity and shape with consequences in cell adhesion and invasive behaviour [22, 23]. Cells become elongated, exhibit a polar budding and remain attached after cytokinesis, switching from growth as independent yeast-form cells on the surface of medium to branching chains of cells, that spread over and into the agar to forage for nutrients, known as filamentous growth [22-24]. In diploid strains this dimorphic switch is characterized by *pseudohyphae* formation (Fig. 2), while the haploid strains have an more subtle switching displaying only limited changes in cell morphology but an increased cell-cell adhesion, known as haploid invasive growth phenotype (Fig. 2) [23]. In addition, the pseudohyphal growth is a behaviour characteristic of diploids starved for nitrogen, whereas the similar haploid invasive growth is induced by fermentable carbon limitation [25]. Accordingly, stress-induced signalling has an important role in filamentous growth [23, 26].

Like all eukaryotic cells, yeasts reproduce while they are young, age and die when they are old. There are two ways of describing aging in yeasts. Replicative aging quantifies aging by the number of daughter cells produced by each mother cell before senescence, and chronological aging, quantifies aging by the length of time that a yeast cell can survive in a non-dividing state [27]. Finally, death can happen *via* necrose or apoptose, which can also happen upon different *stimuli* regardless to the yeast age [28].
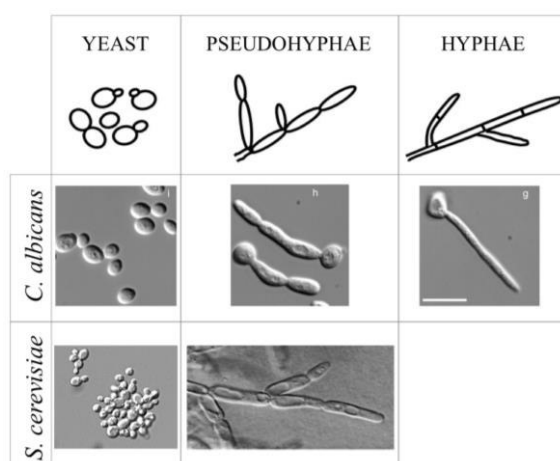
Figure 2. Morphological forms possible in *S. cerevisiae* as compared with the human commensalist and opportunistic pathogen *Candida albicans*. Adapted from Gancedo J (2001) [24], Cullen *et al.* (2000) [25], William *et al.* (2012) [29], Wightman *et al.* (2004) [30] and www.uni-goettingen.de/en/425121.html.

S. cerevisiae asexual cycle, called mitotic cell cycle initiates with an unbudded cell (mother cell) in the G1 checkpoint (Fig. 3). The end of G1 checkpoint is marked by the occurrence of the spindle plaque duplication and the beginning of DNA synthesis and bud emergence (daughter cell). At this stage, the unbudded cell starts polarized growth, predominantly at its apex, ensuring correct assembly of a new bud and septum formation before cell division [31]. Cells then enter S phase - DNA synthesis - and plaque separation occurs. The plaques separate until the complete spindle is formed and the bud size continues to grow along the remaining cycle. The finish of G2 checkpoint is marked by the nucleus migration to the cell neck where the spindle elongation occurs and the nuclear division begins, which in turn finishes during M phase followed by cytokinesis. The final event is the cell wall separation with the consequent formation of two unbudded cells, which does not occurs during filamentous growth [32]. This polarized cell division is characterized by two genetically programmed different spatial patterns, axial for haploid cells, and bipolar for diploid cells [33].

The major cell cycle control point is in the regulatory G1 time lapse called START. At start, yeast cell integrates many intra and extracellular signals, being then committed to continue proliferation, being switched to differentiation pathways, or entre stationary phase [34]. At START point the yeast cell needs to achieve a critical size in order to bud and progress into S phase, being the G1 phase delayed until this critical mass is reached [35]. The critical cell mass required for budding relates to the nutritional conditions of the cells, which also modulate the degree of asymmetry of cell division. For example, in poor medium usually the parent cells are large and the daughters are very small, whereas in rich medium the asymmetry between parent and daughter cells is reduced [35, 36]. The switch between yeast and pseudohyphae forms also involves important changes in the pattern of progression through the cell cycle. In pseudohyphal growth, there is an extended G2/M period and the daughter cell reaches the same size of the mother cell. So that both cells can start a new cycle synchronously, while in the yeast form, G1 phase presents itself as the extended cell cycle period, and the bud separates from the mother before reaching considerable size, starting both cells a new cell cycle independently [24].
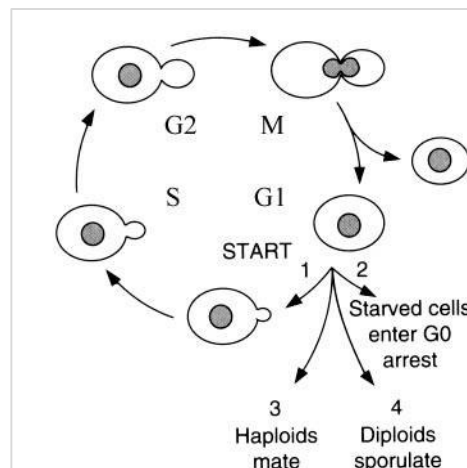
Figure 3. Phases of *S. cerevisiae* mitotic cell cycle. Adapted from Hartwell L.H (1974) [32].

1.1.2 The signalling pathways

1.1.2.1   Mitogen activated protein kinases (MAPK) pathways

MAPK pathways are highly conserved signalling operating units, required in all the eukaryotic cells to properly activate specific or general responses, which allow cells to

cope with different external *stimuli*, like all types of stress, nutrients availability, growth factors and cytokines, ensuring cell survival [37]. In *S. cerevisiae* MAPKs cascades participates in transmitting several extracellular signals controlling mating, morphogenesis, proliferation and cellular integrity in response to nutrients limitation, or osmotic, oxidative, temperature and pH stresses [38]. *S. cerevisiae* has several MAP kinases, allocated to six distinct MAPK pathways [39, 40] (Fig. 4):

- Fus3 – from the mating pheromone response pathway,
- Kss1 – common to the filamentous development and the cell wall integrity pathways,
- Hog1 – from the high osmolarity glycerol (HOG) pathway,
- Slt2/Mpk1 – cell wall integrity (CWI)/ protein kinase C (PKC) pathway, and
- Smk1 – from the spore wall assembly pathway.
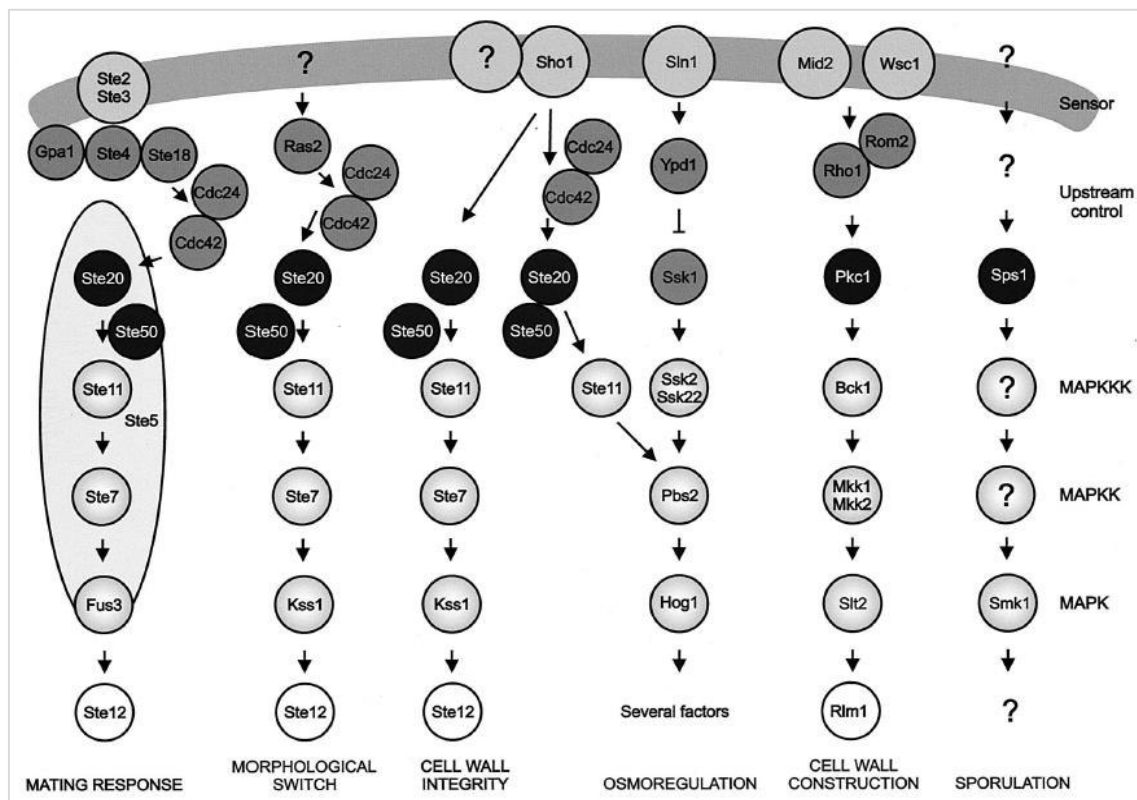
Figure 4. Brief outline of the yeast MAPK pathways. Withdrawn from Hohmann S (2002) [40].

These are upstream controlled by MAPKKs and MAPKKKs that may ensure some degree of crosstalk and a common answer to different *stimuli*. An example is the stimulation of the synthesis and subsequent function of the general stress response

transcription factors Msn2 and Msn4 which fine tuning allows the cell to respond to a series of different environmental constraints, including osmotic, heat and oxidative stresses [41].

1.1.1.1   Nutrient signalling pathways

In yeast, a major signalling pathway activated by glucose is the cAMP/PKA (cyclic adenosine monophosphate/ protein kinase A) pathway, which regulates many aspects of cellular physiology, according to nutrients availability [36]. cAMP is synthetized from ATP from adenylate cyclase, which activity is controlled by two distinct G-protein systems, the Ras1/Ras2 and the Gpr1/Gpa2 proteins. Ras1/Ras2-dependent adenylate cyclase activity is further controlled by the exchange factors Cdc25 as well as the GTPase-activating proteins Ira1 and Ira2 (Fig. 5).
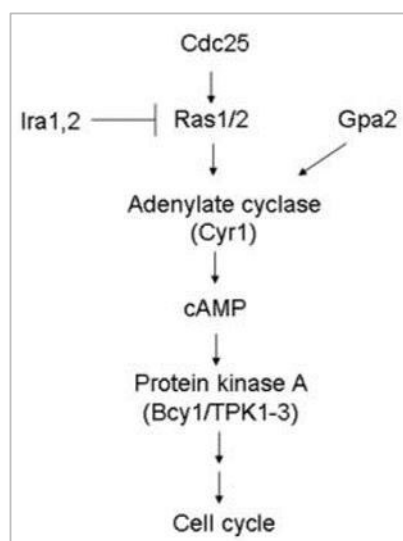
Figure 5. RAS/cAMP/PKA signalling pathway in *S. cerevisiae*. Adapted from Tamanoi F (2011) [42].

The activation of cAMP/PKA pathway can be triggered by transferring yeasts from a poor to a rich carbon source environment [43], which favours rapid growth and cell proliferation by stimulating the glycolytic flux, and repressing the stress response and the expression of genes required for respiratory metabolism [36, 44]. Alternatively, intracellular acidification can have an identical effect [43]. Gpr1/Gpa2 G-protein-coupled receptor system is required for glucose activation of cAMP synthesis, while Ras1/Ras2, in particular Ras2, may stimulate cAMP synthesis in response to either glucose or intracellular acidification [36, 43].

In addition to cAMP/ PKA pathway, the yeast also has other two nutrient signalling pathways, TOR (target of rapamicyn) and the less well-known Sch9, partially conserved in higher Eukaryotes [39, 45]. When glucose or other nutrients are present, the three pathways are active conveying signals that promote cell growth and division. In contrast, if the nutrients are scarce, the reduction of the Sch9, TOR, and cAMP/PKA signalling activity, causes cell division arrest and the activation of mechanisms responsible for cellular protection [46]. TOR and Sch9 act cooperatively with PKA to regulate multiple downstream processes, including autophagy, protein synthesis, mitochondrial function and stress resistance, in response to nutrient availability but also to other environmental signals [47]. Studies of chronological aging in *S. cerevisiae* led to the discovery that Sch9, TOR, and cAMP/PKA pathways are pro-aging pathways, negatively influencing the life span [48]. In particular, the nutrient-responsive Sch9 kinase, adenylate cyclase (Cyr1) and Tor1 are negative regulators of chronological life span (CLS). Accordingly, the deletion of *SCH9* and *TOR1* increased CLS [49]. This extension involves multiple stress-responsive transcription factors, namely the above mentioned Msn2 and Msn4, which once up-regulated lead to an increase of the levels of superoxide dismutase and catalase, thereby minimizing oxidative stress and cellular damage [50]. This demonstrates the association between longevity extension and ability to respond to stress, which has been observed in several organisms [27, 51].

1.1.1.2 Signalling crosstalk in particular cell responses

1.1.1.2.1 MAPK cascade and RAS/cAMP/PKA pathway in filamentous growth response

Evolutionarily conserved signalling pathways encompassing crosstalk and feedback mechanisms regulate the filamentous growth in *S. cerevisiae.* In particular, the morphogenetic switch requires the cooperation of two different signalling pathways, the filamentous MAPK cascade and the RAS/cAMP/PKA pathway [24]. PKA stimulates the expression of the MAPK cascade–controlled genes - *TEC1* [52] and *FLO11* [53] possessing a Filamentation Response Element (FRE) in their promoters. *FLO11* encodes a cell-surface flocculin required for filamentous growth in haploid and diploid strains [53]. As above referred, filamentous growth involves changes in the budding and elongation patterns, and invasive ability. Although these cellular processes are interconnect, they are not always controlled by the same elements. For instance,

Ste12p of MAPK cascade is required for cell elongation, while Tpk2p of cAMP/PKA pathway controls the pattern of budding, namely the budding switch, and both are involved in the invasive process [24].

1.1.1.2.2 CWI/PKC, TOR and RAS/cAMP/PKA pathways in oxidative stress response

Oxidative stress has been implicated in a large variety of biological processes including aging and apoptosis. Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), are generated endogenously in many cells as a consequence of metabolic processes, mostly at mitochondrial level [54]. By contributing to mitochondrial damage, ROS are also important in redox signalling from the organelle to the rest of the cell. As a result, aerobic organisms sense redox perturbations and develop several different adaptive mechanisms in order to acquire survival capacity [55]. *S. cerevisiae* was used to study the signal transduction pathways involved in the response to oxidative stress. In this yeast, TOR, CWI/PKC and RAS/cAMP/PKA, are the best characterized routes crosstalking in transducing the oxidative signal [38, 56]. They further react to glucose starvation in a similar manner [57] (Fig. 6). Mtl1 is a cell surface protein required for survival under oxidative stress [58]. Mtl1 transmits the oxidative signal to the CWI/PKC pathway's Rom2, which then activates the Rho1, this way inducing the activation of Slt2 [59]. Furthermore, in conditions of nutrient depletion and oxidative stress Mtl1 negatively influences CWI/PKC through the inhibition of Rom2, which is also inhibited indirectly via Tor1 and Ras2 inhibition through the inhibition by the same stimuli (Fig. 7). This repression eventually has several outputs, such as the decrease of cAMP, the activation of a wide subset of genes potentially regulated by the general stress response dual transcription factor Msn2/Msn4, and the ribosomal gene repression [38, 56]. This extensive crosstalk between culminates in the further interaction with the RAS/cAMP/PKA pathway in processes that ultimately lead to aging (Fig. 8).
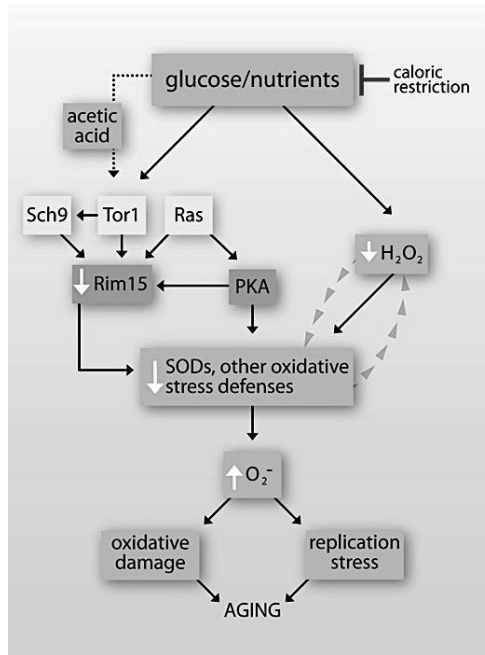
Figure 6: Schematic representation of the integrative yeast cellular response to glucose depletion and oxidative stress. Withdrawn from Weinberger *et al.* (2010) [57].
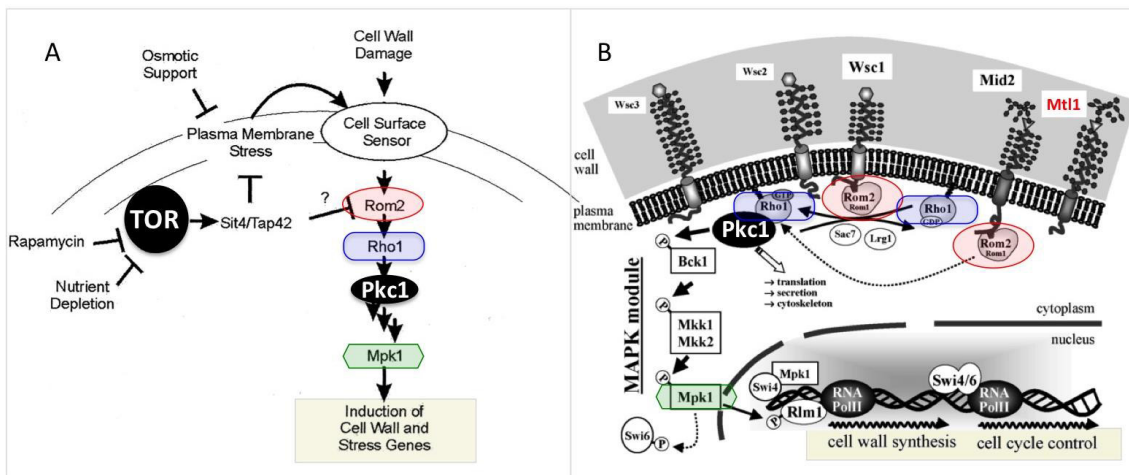
Figure 7: Schematic representation of the influence of TOR over WCI/PKC pathway via Rom2/Rho1 that also react to the effect of cell surface sensors of cell wall integrity, namely Mtl1. A) Adapted from Torres *et al.* (2002) [60]; B) adapted from Heinisch (2014) https://www.biologie.uni-osnabrueck.de/forschung/sfb_944/sfb_944_home/projects.html.
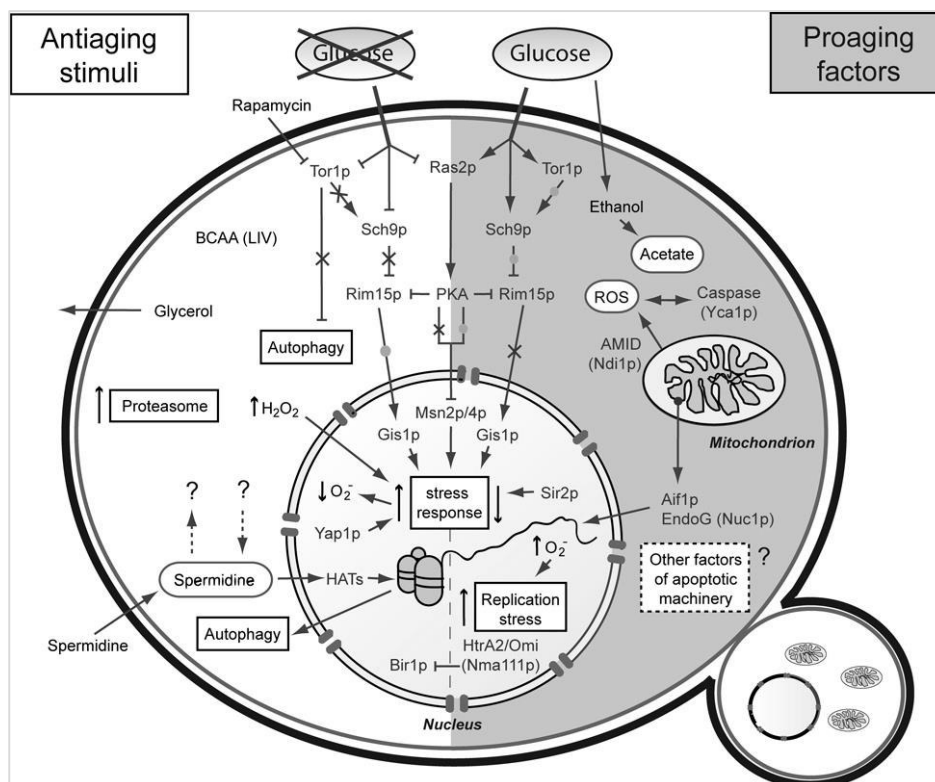
Figure 8: Schematic representation of the balance between different pathways that contributes to opposite effects depending on external stimuli, namely TOR, RAS/ cAMP/PKA. Withdrawn from Ruckenstuhl *et al.* (2010) [61].

1.2 Mammalian RAS pathway

Ras proteins are the founders of a large superfamily of GTPases, very conserved throughout all eukaryotic species, including 150 human members distributed into 5 distinct families, RAS, RHO, RAB, ARF and RAN [62, 63]. Each family is responsible for the control of certain cellular processes. For example, the RAS family controls cell growth, having thus a potential role in the cancer development [64]. Ras proteins are guanine nucleotide-binding proteins that function as GDP/GTP molecular switches. They are activate in response to the activation of a growth factor receptor by the Guanine Nucleotide Exchange Factors (GEFs). These stimulate the intrinsic GDP/GTP exchange activity of Ras proteins promoting the formation of the active RAS-GTP form. Otherwise, GTPase-Activating Proteins (GAPs) stimulate the intrinsic GTP hydrolysis activity of Ras proteins promoting the formation of the inactive RAS–GDP form. Consecutively, the active Ras protein will work as an adaptor protein recruiting

effectors from cytosol to the plasma membrane, where they are activated through the interaction with proteins and lipids, producing intracellular signals to modulate cellular behaviour [62, 65, 66].

In mammalian cells, there are at least three functional RAS genes, *KRAS, HRAS and NRAS*, encoding 21 kD proteins with 189 (KRas4B) and 188 (KRas4A, HRas and NRas) amino acids. KRas4A and 4B are alternative splice variants of *KRAS* at the level of C-terminal, which is important for post-translational modification [67]. These human RAS isoforms are particularly homologous between each other, sharing nearly 85% sequence homology in the first 165 amino acids (the N-terminal) and having a significant variation in 25 remaining amino acids (the C-terminal), except for the C-terminal CAAX box hypervariable region [67, 68]. This region is useful to distinguish between different Ras proteins [69] and does not implicate significant structural differences, since RAS family members, although functionally different, are closely related at structurally level [70].

Ras proteins regulate cell fates by coupling receptor activation to downstream effector pathways that control several cellular responses including proliferation, differentiation, survival and apoptosis [71]. To regulate signal transmission from cell surface receptors to intracellular signalling cascades, Ras proteins were firstly considered to necessarily localize in the plasma membrane [64, 66]. Nevertheless, Ras proteins localized in different cellular membranes could also signal, recruiting distinct upstream and downstream partner proteins and activating different signalling pathways [62, 65, 70]. Exception is the role of Ras proteins in oncogenesis-related processes, which apparently require their association with the inner face of plasma membrane [72].

The above-mentioned "hypervariable" region of RAS promotes the traffic of Ras proteins to the plasma membrane and their specific localization. Thus, the interactions of Ras proteins with the plasma membrane can differ from one RAS isoform to the other due to their different structure of the hypervariable domain [69]. All Ras proteins undergo a series of posttranslational modifications at their C-terminus namely in their CAAX motif. These modifications, which comprise farnesylation, cleavage, methyl esterification and palmitoylation, are essential for protein localization and biological activity. In particular, palmitoylation is essential for the tight association of Ras proteins with the plasma membrane, since after the first three modification steps (farnesylation, proteolysis, and methylation), Ras proteins remains largely cytosolic. For Kras, which is not palmitoylated, a stretch of basic residues (lysine) located just upstream of the

CAAX motif is required for plasma membrane localization [73]. RAS-membrane interactions can also depend on the activation state of specific RAS isoforms [72].

In mammalian cells, activated Ras proteins stimulate various pathways, being the Raf-mitogen-activated kinase (MAPK) and PI3K the best characterize Ras-controlled effectors (Fig. 9) [71]. The RAS/Raf/MEK/ERK signalling pathway has been shown to play key role in the transmission of proliferative signals originating from membrane-bound receptors [74]. Raf stimulates cell proliferation and differentiation by the MAPK pathway, while PI3K pathway is involved in the regulation of cell survival, cell cycle progression, cell size and cellular proliferation through numerous downstream effectors including Akt [65, 75, 76]. Furthermore, the survival signals induced by some receptors are mostly mediated by PI3K/Akt and it is known that mutations in this pathway are frequent in many cancers, namely, activating mutations of the PI3K catalytic subunit, and loss-of-function mutations of the PTEN tumour suppressor [75, 76].
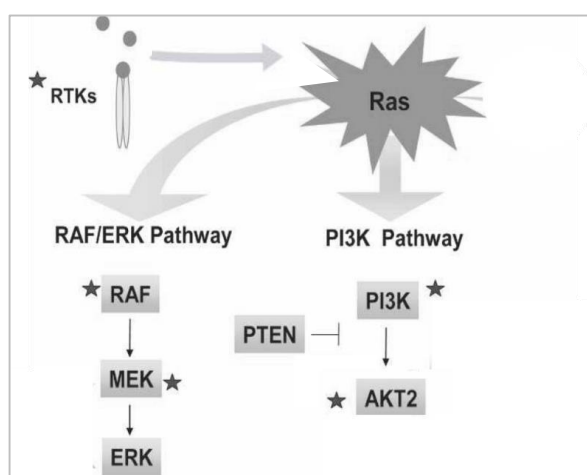
Figure 9. A general view of RAS signalling pathway in human cells. Adapted from Hezel A.F (2006) [63].

The HRas, KRas and NRas proteins are widely expressed, being KRas expressed in almost all types of eukaryotic cells [64]. *KRAS* is essential for normal cellular development while neither *N-* nor *HRAS* are, therefore having a well recognize role in tumorigenesis [77, 78]. The most prevalent oncogenic RAS-associated mutations in *KRAS* are point mutations causing amino acids substitution in codons 12, 13 and 61 [65, 67, 79]. These mutations hamper the capacity of GEFs to interact with KRas protein, producing constitutively activated KRas, and consequently, an improper activation of the pathway, ultimately generating a malignant phenotype [65, 71]. Concurrently, the duration and the intensity of RAS signalling regulate the

developmental programs in specific cellular types [71]. Furthermore, *KRAS* locus in humans encodes two splice variants, for which mutations in *KRAS* have the potential to interfere in splicing events [69].

1.3 RAS/cAMP/PKA pathway in *Saccharomyces cerevisiae*

1.3.1 *RAS* genes

S. cerevisiae has two isoforms of *RAS* genes, expressing extremely homologous small GTPases moderately redundant at phenotype level [26, 80]. Moreover, *RAS1* and *RAS2* are structurally and functionally homologs of the mammalian RAS proto-oncogenes [81]. *RAS1* and *RAS2* genes constitute an essential yeast gene family, since the deletion of both is lethal to the yeast cells [82]. *RAS1* is located on chromosome XV, 7 cM from *ADE2* and 63 cM from *HIS3*, and encodes a protein of 309 amino acid residues. On the other hand, *RAS2* is located on chromosome XIV, 2 cM from *MET4*, encoding proteins of 322 amino acid residues [42, 83]. Ras1 and Ras2 are nearly 90% homologous in the region corresponding to the N-terminal first 180 amino acids, but diverge in the remaining amino acid sequence. The N-terminal domain is also the region of greatest similarity with the mammalian Ras proteins, with nearly 90% homology between positions 10 - 90 and 3 - 83 of, respectively, the yeast and the mammalian Ras proteins. On the other hand, another major difference between these proteins lies in the sequence and size of their C-terminal, particularly in the hypervariable region including CAAX box, which in the case of Ras1 and Ras2 is much more extended [73, 84].

As mammalian Ras proteins, also yeast Ras proteins undergo a series of post-translational modifications of their C-termini, including farnesylation and palmitoylation that are required for targeting the proteins to the cytoplasmic face of the plasma membrane and for biological activity of the protein. Ras proteins biological activity requires farnesylation, while palmitoylation is required for their localization to the plasma membrane. The essential role of the farnesyl moiety on yeast Ras1/2 proteins is to enable the efficient activation of adenylyl cyclase and response to membrane-associated GEFs, *i.e.,* nucleotide exchange activity mediated by membrane bound full-length Cdc25 and Sdc25 exchange factors [73, 84]. In the N-terminal domain positions 12, 13, 59, 61, or 63, amino acid substitutions can activate the transforming potential of

the mammalian Ras proteins. At the equivalent positions, the yeast *RAS* genes encode the same amino acids. This homology could reflect both a highly conserved biochemical function and a mechanism for regulating that function [83, 85].

As mentioned above, Ras1 and Ras2 proteins are biologically equivalent, although transcriptional and translational controls determine under what physiological conditions either Ras protein will be expressed [86]. As a consequence of a fluid transcriptional and translational regulation, no clear-cut phenotype was ever apparent for *ras1Δ* mutant strains [86]. The following examples allow a deeper understanding of the resulting phenotyping conundrum:

a) Ras2 is the major regulator of adenylate cyclase, while Ras1 is only a minor one. Accordingly, *ras2Δ* mutants present low levels of intracellular cAMP [87];

b) *ras2Δ* mutants are defective for growth on non-fermentable carbon sources [80], however this growth defect can be suppressed by overproduction of the *RAS1* gene product [82];

c) *ras1Δ* mutants with a further temperature-sensitive *RAS2* mutation, accumulate unbudded cells at non-permissive temperatures because they arrest at G1 phase [42];

d) *ras2Δras2Δ* diploids an increase in storage carbohydrates, sporulation and an high heat shock resistance was observed, even in rich medium. On the contrary, an activating mutation of *RAS2* such as *RAS2^{Val19}*, causes a reduction of the glycogen storage level, and an increase in sensitivity to nutrient starvation and to heat shock [42, 51, 88];

e) *ras2Δ have* increased longevity and resistance to oxidative stress [89];

f) strains carrying hypo-active *RAS2* alleles exhibit a delay in recovery from glucose starvation [90].

1.3.2 RAS/cAMP/PKA signalling pathway

Similar to the mammalian cells, in the yeast *S. cerevisiae* RAS pathway is activated by growth signals, importantly glucose [91]. In *S. cerevisiae*, Ras proteins connect nutrient availability to cell growth through regulation of PKA activity, being thus an element of cAMP/PKA pathway [92] as above mentioned. RAS/cAMP/PKA is involved in cell adaptation to environmental changes, responding to nutrient status and various

types of stress, such as, oxidative, osmotic and heat shock stresses [28]. RAS/cAMP/PKA pathway performs a key role in the modulation of growth, metabolism, aging, stress resistance, morphogenesis (stimulates filamentous and invasive growth), and cell cycle progression, to ensure that growth occurs only when overall conditions are favourable [36, 42, 92]. This pathway negatively regulates cellular physiology characteristic of stationary phase/nutrient starvation. Consistently, cells with constitutive RAS/cAMP/PKA signalling fail to adapt their growth program in response to nutrient starvation and rapidly lose viability [93].

Ras1 and Ras2 are two small monomeric GTP-binding proteins capable to switch between an active GTP-bound state and an inactive GDP-bound form. In yeast as in mammals, the guanine adenosine phosphate (GAPs) encoded by *IRA1* and *IRA2* in yeast, down-regulate Ras1 and Ras2 through GTP hydrolysis, resulting in the accumulation of the inactive Ras protein form. Otherwise, the Cdc25 and Sdc25 GEFs promote GDP re-charging to GTP. When Ras1 and Ras2 are in their active conformation (phosphorylated), their hyper-variable domain binds directly to the adenylate cyclase (Cyr1p), which is the only RAS effector protein identified in *S. cerevisiae* and the main component of this signalling transduction pathway (Fig. 5-8) [92]. Cyr1 catalyses the synthesis of cAMP, that binds to the regulatory subunit of the PKA (Bcy1p) triggering the PKA activity by inducing its dissociation from the PKA catalytic subunits (encoded by *TPK1*, *TPK2*, and *TPK3*) [94]. In turn, PKA signals to the nucleus to carry out many functions of which the essential one is G1 cell cycle progression [95]. Ras proteins are required to maintain basal adenylate cyclase activity, being thus essential for cell viability [36, 73].

The constitutive activation of the RAS/cAMP/PKA pathway prevents several rapamycin-induced TOR responses, such as the nuclear translocation of the transcription factor Msn2 and the consequent induction of stress genes, the accumulation of storage carbohydrates, the induction of autophagy, and the down-regulation of ribosome biogenesis [93]. The constitutive activation of the RAS pathway also can suppress a TOR deficiency [93]. Moreover, many of TOR-mediated responses are signalled through the RAS/cAMP/PKA pathway, independently of TOR effectors, and acting upstream of Ras proteins to regulate the PKA activity [36] (Fig. 7, 8). This relationship between these two pathways could go as far as suggesting RAS pathway as a novel TOR effector branch [93]. This is emphasised by the fact that enhanced sensitivity to rapamycin is often a result of the hyperactivation of RAS/cAMP/PKA signalling [96].

1.3.2.1 RAS/cAMP/PKA pathway in response to different stresses

Many pathways, including the RAS/cAMP/PKA pathway, converge on the related transcription factors Msn2 and Msn4, which induce stress genes in response to a wide variety of environmental conditions including nutritional, osmotic, acidic, heat shock and oxidative stresses, as well as diauxic transition [31, 38].

The RAS/cAMP/PKA pathway is a negative regulator of the these stress response pathway genes, once in optimal growth conditions, RAS/cAMP/PKA pathway is activated and repress the function of the general stress transcription factor Msn2/Msn4 [97], also negatively regulating the Msn2/Msn4 nuclear localization, which is mandatory under stress condition [38, 40]. On the contrary, the nutrient starvation, oxidative stress, heat shock and entry into stationary phase demand RAS/cAMP/PKA repression [56]. In *S. cerevisiae*, the RAS/cAMP/PKA pathway must be shut down to allow cell cycle exit, and a full stress response [98]. Concurrently, mutants with high PKA activity display a low tolerance to stress usually associated to optimal growth conditions, while mutants with low PKA activity have high stress tolerance, which in turn is related to suboptimal growth conditions [40].

As above mentioned, in response to oxidative stress, CWI/PKC, TOR and RAS/cAMP/PKA pathways crosstalk through Rom2/Rho1 (Fig. 7). Two possible models were proposed to this crosstalk: (a) Rho1p signals simultaneously but independently of Tor1 and Ras2 inactivation; and (b) Rho1 first inactivates Tor1 protein and then this transmits the signal to inhibit Ras2. Moreover, crosstalk also occurs in a reverse flow, from TOR and RAS to the CWI/PKC pathway. In this case the signal flows from Ras2 and Tor1 inactivation to induce the phosphorylation of Slt2, activating the CWI/PKC pathway in the absence of the Mtl1 protein, assuring the proper adaptive response to oxidative and glucose deprivation [38, 56]. In addition, the hyperactivation of RAS/cAMP/PKA pathway decreases the cell tolerance to various stress conditions, including wall-damaging high-temperature [99-102].

1.3.2.2 RAS/cAMP/PKA pathway in filamentous growth

Ras proteins are central regulators, activators of the two pathways involved in the filamentous growth response [23, 26]. However, Ras1 and Ras2 have distinct importance in the regulation of invasive growth:

a) Ras2, not Ras1, appears to be the central element of the morphogenetic switch;

b) *RAS2* is needed to induce invasive growth, since *RAS1* expression in a *ras2Δ* mutant allows growth but not invasive growth;

c) The expression of specific *RAS2* alleles can uncouple the two effectors pathways;

d) The loss of *RAS2* prevents invasion, while the hyperactivation of RAS pathway by integration of the *RAS2^{V19}* allele, causes hyperfilamentation.

The activation of Ras2-dependent pathway is therefore required for filamentous growth [22, 23, 26]. Ras2 activates invasive growth using either of two downstream signalling pathways, the filamentation MAPK (Cdc42p/Ste20p/MAPK) cascade or the cAMP dependent protein kinase (Cyr1p/cAMP/PKA) pathway, which were above mentioned [22, 23, 26]. Surprisingly, although Ras2 has been placed upstream of pathways responsible for the morphogenetic switch, there is at present no information on the mechanisms by which Ras2 might act. Ras2 activity also decreases the activity of the stress responsive transcription factors Msn2p and Msn4p by an overactive Ras2/cAMP/PKA cascade, which is essential for invasive growth phenotype [91]. In addition, Ras2 is also involved in the yeast life span [26].

1.3.2.3   RAS/cAMP/PKA pathway in cellular aging

In fact, Ras2 downstream pathway has been argued to be a key regulator of aging in yeast, and to share similarities with the insulin/IGF1-(like) longevity pathway of mammals [27]. RAS constitutes a pro-aging pathway, negatively regulating the longevity. Mutations that decrease the activity of the RAS/Cyr1/PKA pathway extend longevity and increase stress resistance by activating the above-mentioned transcription factors Msn2/Msn4 (Fig. 8) [103, 104]. It is also remarkable that mutation or overexpression of several other components of the PKA cascade like *RAS1*, *CDC25*, *CYR1* affect lifespan, suggesting that in fact, this pathway modulates the life span [105].

RAS1 and *RAS2* show opposite roles in replicative aging. The deletion of *RAS1* extends, whereas the deletion of *RAS2* shortens replicative longevity, in turn the induced expression of the *RAS2* extends, while the overexpression of *RAS1* does not affect replicative longevity [87] [51]. Moreover, *RAS* genes also play opposite roles in the regulation of CLS. The deletion of *RAS2* causes CLS extension, while the constitutive activation of RAS signalling pathway (e.g. *RAS2^{val19}*) causes a decrease of CLS [27]. These opposite effects show that the two aging models are somehow related, but that at the same time each has particular characteristics [44].

1.3.2.4 **The expression of human Ras isoforms in *S. cerevisiae***

S. cerevisiae has been use as model system to study mammalian *RAS* genes via expression of their correspondent cDNAs in strains deleted for either yeast *RAS* gene. The mammalian *HRAS* cDNA was expressed for the first time in yeast using a clever construction strategy that enabled the deletion of both yeast RAS genes without the yeast losing viability [106]. Reversely, a modified *RAS1* transformed NIH-3T3 cells [107]. The functional interchangeability between yeast and mammalian *RAS* genes [42] indicates a profound conservation not only of the amino acid sequence, but also of detailed biological function.

1.4 Rationale and aims of the present work

This master thesis was develop within the scope of Glycopharm - Marie Curie Initial Training Network, which scientific objectives are: the development and testing of selective galectin-blocking compounds and the development and testing of galectin-mimetic peptides with respective target selectivity. This project impacts enormously on a wide variety of diseases in need for novel therapeutic solutions, namely through the aim of developing a rapid and easy-to-use tool for primary pharmacological testing. This thesis is enclose in the wide objectives of the network and aims at building and validating a high throughput-screening platform of yeast strains displaying phenotypes that can enable the survey of putative galectin-related ligands/inhibitors. This platform was designed to consist of two types of strains, the ones expressing *tout court* human galectins (in particular Gal3 and Gal1), and the ones expressing these human proteins together with the human *KRAS^{wt}* cDNA. An additional set of yeast strains expressing *KRAS^{wt}* without galectin will also be use, in order to study the effect of *KRAS^{wt}* alone and compare it with the effect resulting from the co-expression of *KRAS^{wt}* and galectins. The rationale behind this relates with the putative dialogue between galectins and RAS signalling pathway in mammals.

RAS1 and *RAS2* genes encode low molecular weight, GTP-binding/hydrolysing proteins that are highly conserved throughout all eukaryotic species [108]. In human cells, the RAS proto-oncogenes encode proteins that are involved in the control of cell growth, differentiation and survival, having thus a potential role in cancer development [109]. In humans as in *S. cerevisiae*, Ras proteins are signal switch molecules that regulate cell fates by coupling receptor activation to downstream effector pathways that control diverse cellular responses including proliferation and survival [42, 110, 111].

Beyond the protein structural similarities, also the genes from both organisms are functionally homologous and carry out similar functions [112]. Among all human *RAS* genes, *KRAS* is expressed in most cellular types, and is the one most frequently mutated in diverse types of cancer [110].

S. cerevisiae harbours one functional RAS pathway, mediated by *RAS1* and *RAS2* genes [113]. RAS signalling in yeast controls similar biological processes as in higher eukaryotes in response to environmental changes, namely cell proliferation, growth, metabolism, aging, morphogenesis, and cell cycle progression [42]. This functional conservation allows the use of yeast as a model organism to study mammalian proteins and molecular processes [5], including RAS signalling [108]. Moreover, in the last decades, the yeast *S. cerevisiae* has been identified as a powerful tool to study the relationship between genotype and phenotype in eukaryotic cells, due to an easy experimental tractability, allowing inference of individual gene functions or of network structures through various kinds of experiments [5].

The principal aim of this thesis was the construction of $KRAS^{wt}$ expressing strains of *S. cerevisiae,* and the subsequent phenotype screening, as part of the development of a yeast-based phenotypic platform for future pharmacological testing. Human $KRAS^{wt}$ cDNA will be express in *S. cerevisiae* BY4741 genetic background deleted for *RAS1* and *RAS2*. This genetic background was chosen according with the genetic marks availability, and for phenotyping control identical strains from the W303-1A background (also haploid and from the same mating type - *a*) will be used (Brito A.S., unpublished work).

The subsequent phenotypic characterization of the $KRAS^{wt}$ expressing strains will focus on assaying growth-influencing conditions, like carbon source, temperature, pH and different types of environmental stress, as well as proliferation, chronological aging, cell cycle progression and haploid invasive growth. The data to be obtain are expect to contribute to gain insight into the heterologous expression of *KRAS* in yeasts to allow the future utilization of the above-mentioned platform. The results of this thesis will be complemented with identically obtained phenotypes using BY4741 harbouring the chromosomal insertion of $KRAS^{wt}$ (Cazzanelli J., unpublished work), and using the control strains from *S. cerevisiae* W303-1A background*,* (Brito A.S., unpublished work). Altogether, this data will serve for the development of the yeast-based high throughput phenotype platform. This knowledge is expect to be subsequently validated in human derived cell lines, paving the way for the utilization of the platform in the development of new diagnostic/prognostic tests and new bioactive ligands/inhibitors.

Chapter II

Material and Methods

2. **Material and Methods**

2.1 Strains and growth conditions

The *Saccharomyces cerevisiae* and *Escherichia coli* strains used in this work are listed on Table 1. *E. coli* XL1-Blue strain was used for plasmid propagation.

Table 1. The *S. cerevisiae* BY4741 and *E.coli* strains used in present work.

Strains	Genotype	Origin
BY4741	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0*	Euroscarf single deletion strain collection
BY4741 *ras1∆*	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0; YOR101w::kanMX4*	Euroscarf single deletion strain collection
BY4741 *ras2∆*	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0; YNL098c::kanMX4*	Euroscarf single deletion strain collection
BY4741 p426GPD	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0; p426GPD (URA2)*	this study
BY4741 *ras1∆* p426GPD	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0; YOR101w::kanMX4; p426GPD (URA2)*	this study
BY4741 *ras2∆* p426GPD	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0; YNL098c::kanMX4; p426GPD (URA2)*	this study
BY4741 *KRAS*^{wt}	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0*	this study
BY4741 *ras1∆ KRAS*^{wt}	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0; YOR101w::kanMX4; p426GPD/KRAS*^{wt} *(URA2)*	this study
BY4741 *ras2∆ KRAS*^{wt}	MAT*a*; *his3∆1; leu2∆0; met15∆0; ura3∆0; YNL098c::kanMX4; p426GPD/KRAS*^{wt} *(URA2)*	this study
E.coli XL1-Blue	*endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq ∆(lacZ)M15] hsdR17(rK- mK+)*	Department of Biology Minho University

Yeast strains were grown and maintained in rich medium (YPD - yeast extract (1% w/v), peptone (2% w/v), glucose (2%, w/v)) or in minimal medium (YNB – yeast nitrogen base without amino acids and ammonium sulphate (0.17% w/v) [Difco], glucose (2% w/v), ammonium sulphate (0.5% w/v)). When appropriate, YNB was supplemented with adequate quantities of amino acids for auxotrophic

complementation (0.1 g/l leucine, 0.02 g/l histidine, 0.02 g/l methionine and 0.02 g/l uracil). All strains were batch-grown aerobically in liquid medium, at 30 ºC with orbital shaking at 200 rpm and air/liquid ratio of 3/1. The strains maintenance was done in solid medium (YNB or YPD supplemented with 2% agar), grown at 30 ºC until colonies were observed, and then kept at 4 ºC.

Bacterial cells were grown in liquid Luria-Bertani (LB) medium (yeast extract (0.5%, w/v); tryptone (1%, w/v); NaCl (1%, w/v)), overnight at 37 ºC with shaking at 200 rpm in an orbital shaker. Strains were maintained in LB medium supplemented with 2% agar, previously grown overnight at 37 ºC and kept/storage at 4 ºC. For the selection of transformants, LB was supplemented with 100 µg/ml ampicillin.

2.2 Plasmids

Two plasmids, p416GPD and p426GPD [114], were used in this work to clone human *KRAS^{wt}* (Fig. 10). These are episomal shuttle plasmids, containing the strong GPD promoter and selective marks for yeast (uracil) and bacteria (ampicillin). p416 is centromeric, while p426 harbours the 2µ element and produces 10-30 copies/cell. Both plasmids have their multicloning site (MCS) adjacent to a strong promoter (originating from *GPD1*), and have an identical terminator (originating from *CYC1*). The pUC19 vector was used as a positive control of *E. coli* transformation, serving to quantify the competence of *E. coli* cells. The cDNA from human *KRAS^{wt}* was received in a pLenti/*KRAS^{wt}* plasmid, provided by IPO-Porto.
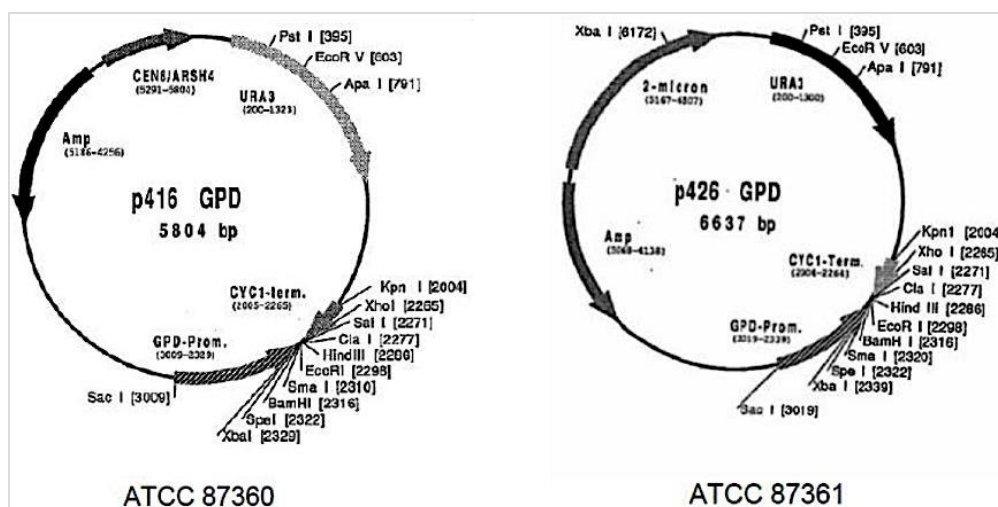
Figure 10. Schematic representation of p416GPD and p426GPD expression plasmids.

2.3 Preparation of *E. coli* competent cells

Competent cells of *E.coli* XL1-Blue strain were prepared using $CaCl_2$ (calcium chloride) and $MgCl_2$ (magnesium chloride), as previously described [115]. Briefly, cells were inoculated in 100 ml LB and cultivated at 37 ºC and 200 rpm for 4 hours (O.D$_{600}$ of 0.6). The culture was chilled in ice for 10 min. Cells were collected by centrifugation at 4 000 rpm (Sigma 4-16K) and 4 ºC for 10 min. Supernatant was discarded and the pellet resuspended in 20 ml of 0.1 M $MgCl_2$, followed by an identical centrifugation. The pellet was gently resuspended in 2 ml of 0.1 M $CaCl_2$ and incubated on ice for 2 hours, and finally distributed by 200 µl aliquots. Dimethyl sulfoxide (DMSO) was added at 7% (v/v) final concentration, and the cells were frozen in liquid nitrogen and storage at -80 ºC.

2.4 Polymerase chain reaction (PCR)

KRAS^{wt} gene inserted in pLenti plasmid, was amplified by PCR using the primers listed on Table 2. The primers were designed to include the *Hind*III and *Xho*I restriction sites to enable the cloning. The PCR reaction mix contained: 1X Taq reaction buffer (Fermentas), 0.2 mM dNTPs (deoxynucleotide triphosphates) (Biology Dept. of Minho University), 2 mM $MgCl_2$ (Fermentas), 0.2 µM of each primer (Eurofins Genomic), 200 ng DNA template (pLenti/*KRAS^{wt}*), 1.25 units/ 50 µl PCR of Taq DNA polimerase (Biology Dept. of Minho University) and ultrapure autoclaved water up to 20 µl final volume. As negative amplification control, a PCR reaction mixture without DNA template was used. Amplifications were made in PCR T100 Thermal Cycler (Biorad) all following the program: initial denaturation at 94 ºC for 5 min; 35 cycles of denaturation at 94 ºC for 30 sec, primers annealing at 55 ºC for 30 sec and extension at 68 ºC for 90 sec, and a final elongation step at 72 ºC for 10 min. To confirm the *KRAS^{wt}* amplification, the PCR products were analysed by electrophoresis in 1% (w/v) agarose gel, pre-stained with 2 µl of gel red (Biotium).

2.5 DNA electrophoresis in agarose gel

Agarose gels for DNA electrophoresis were prepared with 50 ml of TAE 1X buffer (1l of TAE 50X; 242g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8)) to a final concentration of 1% agarose. 2 µl of Gel Red (Biotium) dye was

used for gel pre-staining. Each 3-5 µl DNA sample was added 5 µl DNA loading buffer 6X (100 ml: 0,125 mg of bromofenol blue, 0.125 mg of Xylene cyanol, 12 ml 0.5 M EDTA [pH 8,0], and 30 ml of glycerol). 2 µl of DNA molecular ladder λ DNA/Eco-471 (MBI Fermentas) was used as reference. The electrophoresis performed in a Mini-SubCell GT (BioRad) system. The gel run in TAE 1X buffer at 75-100 V. The DNA visualized by UV illumination (245 nm) at UV- transilluminator GenoSmart (VWR). The extraction and purification of DNA fragments from agarose gel electrophoresis was done using *GenElute Gel Extraction Kit* (Sigma Aldrich), following the protocol of the manufacturer.

Table 2. Primers used for amplification of human *KRAS^wt* fragment.

Primer	Primer sequence (5´- 3')	Commentary
FW_11044 KRAS	GCG AAG CTT ATG ACT GAA TAT AAA CTT GTG GTA GTT GGA	Includes the *Hind*III restriction site (A^AGCTT)
RV_11044 KRAS	GCG CTC GAG CAT AAT TAC ACA CTT TGT CTT TGA CTT CTT	Includes the *Xho*I restriction site (C^TCGAG)

2.6 DNA enzymatic digestion and ligation

Each restriction mixture contained: 300 ng of human *KRAS^wt* cDNA (amplified by PCR) to be digested or 300 ng of either p416 or p426 vectors, 1X buffer R (Fermentas), 1X *Hind*III (Fermentas), 1X *Xho*I (Fermentas), and ultrapure water till complete the final 20 µl volume. The ratio of volumes for this mixture was calculated using the *Fermentas double digestion* tool (http://www.fermentas.com/en/tools/doubledigest). The appropriate buffer for the enzyme was checked in the correspondent company webpage devoted to enzyme/buffer compatibility. Digestion was allowed to occur for 4 hours at 37 ºC after which the enzymes were inactivated by heating at 75 ºC for 20 min. The final products of digestions were verify by DNA electrophoresis as described above. The ligation reaction mix contained: 20 ng of vector, 100 ng of insert, 2X T4 DNA ligase buffer (Roche), 5U of T4 DNA ligase (Roche) and ultrapure water up to the final volume of 20 µl. All ligations were allowed to occur overnight at room temperature.

2.7 Transformation of *E.coli* and plasmid extraction

The transformation of *E. coli* XL1-Blue competent cells with plasmid DNA was made following the heat shock method as previously described [116]. Transformants selection was performed based on ampicillin resistance. Approximately 200 ng of plasmid DNA was added to 200 µl of competent cells and maintained on ice for 40 minutes. This was subject to a 2 min heat shock at 42 ºC, followed again by 2 min cooling on ice. In order to allow cells to recover, 500 µl of LB medium was added and the cell suspension was incubated 1 h at 37 ºC with orbital shaking at 200 rpm. Volumes of 100 µl and 300 µl of each transformation mixture, were platted on selective medium (solid LB medium supplemented with 100 µg/ml of ampicillin). Cells were allowed to grow overnight at 37 ºC.

In order to extract plasmid DNA, *E. coli XL1Blue* transformed cells were inoculated in 5 ml of LB medium with ampicillin (100 µg/ml) and incubated overnight at 37 °C with 200 rpm orbital shaking. DNA extraction was performed using the *GenElutePlasmidMiniprep* kit (Sigma-Aldrich), according to the manufacturer instructions. The integrity of plasmid DNA was verify by double enzymatic digestion, with the restriction enzymes *Hind*III and *Xho*I, and a subsequent DNA electrophoresis in 1% (w/v) agarose gel.

2.8 Transformation of *S. cerevisiae* and verification by PCR

S. cerevisiae was transformed by the lithium acetate method, as previously described [116]. Cells were cultured overnight at 30 ºC in 5 ml of YPD with 200 rpm orbital shaking up to O.D$_{600}$ = 1. The cells were collected by centrifugation during 5 min at 3 000 rpm (Sigma 4-16K), washed twice with sterile deionized water and one with LiAc (0.1 M)/ TE (1X) for 2 min at 8 000 rpm (miniSpin, Eppendorf). The pellet was resuspended in 200 µl of LiAc (0.1 M)/ TE (1X) and placed at 4º C overnight. Each transformation mix was prepared with 100 µl of yeast competent cells, 20 µl of ssDNA carrier (2.5 mg/ml) (Sigma), pre-heated at 90 ºC during 5 min, 600 µl of LiAc (0.1 M)/TE (1x) – PEG 50%, and ~0.2 µg of plasmid DNA (except for the negative control). The cells were incubated at 30 ºC with orbital shaking during ~1 h, then submitted to a 15 min heat shock at 42 ºC and placed at 4 ºC for 10 min. The cells were harvested by centrifugation, 2 min at 8 000 rpm (miniSpin, Eppendorf), ressuspended in 200 µl of

sterile deionized water. The cellular suspension was plated on selective medium, YNB URA⁻, and incubated at 30 ºC until colonies appeared (around 3 days).

The verification of yeast transformants was done by PCR. Individual colonies grown on selective medium were picked and streaked onto a new plate of YNB URA⁻ and incubated 2 days at 30 ºC. For DNA extraction and precipitation, yeast biomass was picked from the selective medium plate, suspended in 100 µl of 200 mM LiAc/ 1% SDS solution and incubated for 5min at 70 ºC. After adding 300 µl of 100% ethanol and vortexing, the mixture of DNA and cell debris was spun down at 15 000 rpm (Sigma 4-16K) for 3 min. The pellet was washed with 70% ethanol, dissolved in 100 µl of ultrapure water, and cell debris was again spun down at 15 000 rpm (Sigma 4-16K) for 15sec. PCR reaction was done using 1 µl of the obtained supernatant, and it was performed as described above. The PCR products were checked by agarose gel electrophoresis.

2.9 Western Blot analysis

2.9.1 Yeast protein extraction and precipitation

For Western Blot (WB) analysis, yeast cell proteins were extracted using the trichloroacetic acid (TCA) method. Cells were grown in 5 ml YNB, supplemented with respective amino acids when appropriate, at 30 ºC and 200 rpm until mid-exponential phase. Cultures were diluted to $OD_{600}= 1$ and cells were collected by centrifugation at 4 000 rpm (miniSpin, Eppendorf) for 5 min. When necessary the pellet was stored at -20 ºC. The pellet, was ressuspended in 200 µl of 0.2 M NaOH/2% β-mercaptoethanol and incubated at 4 ºC for 10 min. 400 µl of 20% TCA was added to each tube and incubated on ice for 10 min. Cells were harvested by centrifugation, at 13 000 rpm (minispin, Eppendorf) during 5 min, the pellet was washed with 500 µl cold acetone and again collected by identical centrifugation. Acetone was completely removed by evaporation, and the pellet resuspended in 100 µl of Laemmli buffer 2X (SDS 4%, 120 mM Tris HCl, 20% glycerol, 0.1% bromophenol blue) with 3 µl of DTT 0.01 M. Protein suspensions were stored at -20 ºC.

2.9.2 SDS-PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis)

Polyacrylamide gels were prepared at 15% (w/v) for resolution gel, and 5% (w/v) for concentration gel. Prior to gel loading the protein extracts were denatured at 95 ºC during 10 min, and 10 µl of each total protein extract sample was loaded per well. NZYColour Protein Marker II (Nzytech) (2 µl) was used as reference. The gels were inserted into the electrophoresis tank and submerged with Running Buffer (19.2 mM glycin, 2.5 mM Tris base, 0.01% SDS). Electrophoresis was performed in Mini Protean Tetra Cell I system (BioRad), at 20 mA.

2.9.3 Western Blot assay

SDS-PAGE gels were electro blotted onto PVDF membrane (Roche) in a Trans-Blot Electrophoretic Transfer Cell System (BioRad) immersed in a transfer buffer (19.2 mM glycin, 2.5 mM Tris, 20% ethanol, 0.05% SDS, pH 8.3) at $1mA/cm^2$ of membrane, during 90 min. Membrane was blocked in 5% of blocking agent (GE Healthcare), dissolved in TBST (TBS 10X (Tris-base; NaCl) pH 7.5 and 0.1% Tween 20) and incubated overnight at 4 ºC with gentle rotation, in a roller mixer SRT1 equipment (Stuart®). The membrane was subsequently incubated in 5% blocking agent containing primary antibody anti-KRAS (Sigma) at dilution 1:1000, during 1 hour at room temperature with mild agitation in a roller mixer SRT1 equipment (Stuart). To remove the excess of primary antibody, the membrane was washed 3 times with TBST, 5 min each. The incubation with the secondary antibody - rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Sigma) - at dilution 1:10 000, for 1 hour at room temperature and mild agitation. The membrane was washed 6 times with TBST, 10 min each. HRP conjugated rabbit anti-mouse IgG was detected with ECL Plus Western Blotting Detection system (Amersham Biosciences) and the result was visualized in a ChemiDoc XRS image analysis system (BioRad Laboratories Inc.).

2.10   Phenotype Screening

2.10.1   Growth parameters in yeast batch cultures

Overnight yeast cultures grown were diluted to an OD_{600} of 0.1, and used to incubate fresh YNB batch cultures. Incubation was performed at 30 ºC, in an orbital

shaker (200 rpm), with an air/liquid ratio of 3:1. Growth was monitored spectrophotometrically (OD_{600nm}) in a Spectrophotometer Genesis 20 (Thermo Spectronic), every 2 hours up to ±30 h. Specific growth rate was calculated within the logarithmic growth part of the curve.

2.10.2   Growth assays in solid medium

To test sensibility/ resistance to different types of stress, as also growth in different carbon sources, drop tests assays were performed. Inoculum consisted of overnight YNB cultures at OD_{600} =1, containing approximately 1×10^7 cells/ml. Four serial dilutions of 1:10 (5 µl of each) were plated. Media are listed in Table 3. Results were score after 48 h incubation at 30 °C or other (Table 3). In some exceptional cases, results were just visible after a longer incubation period – mentioned in the Results. Plates were photographed in a ChemiDoc XRS image analysis system (BioRad Laboratories Inc.).

Table 3. Growth conditions tested using YNB as base-medium.

Carbon sources	• 2% glucose (optimal - control) • 2% ethanol • 2% glycerol
pH	• pH 6 (optimal - control) • pH 4 • pH 7
High temperature stress	• 30ºC (optimal - control) • 37ºC
Oxidative stress	• 0.5 mM • 1 mM • 2 mM
High osmotic stress	• 0.5 M NaCl (0.980 a_w) • 1 M NaCl (0.965 a_w) • 1 M Sorbitol (0.980 a_w) • 1.5 M Sorbitol (0.97 a_w)

2.10.3   Adherence to agar and Invasion

Exponential YNB cultures were diluted to an O.D$_{600}$ =1, and 200 µl were spotted onto YNB without glucose, glucose absence induce the filamentous invasive growth [25]. Controls were performed in YNB with 2% (w/v) glucose. Cells were allowed to grow at 30 ºC for 7 and 20 days. Adhesion to agar was tested washing the plates in running water with constant pressure during 30 sec, adapted from [33]. Plates were visualized and photographed in a ChemiDoc XRS image analysis system (BioRad Laboratories Inc.), before and after washing. The invasion capacity was inspected, the agar plates were subsequently cut longitudinally and the slice was inspected for invasive growth and photographed in a stereo microscope (Leica EZ4HD). Images were processed with LAS AF Leica Microsystems software.

2.10.4   Filamentous growth

Filamentous growth was observed according to the previous described [25], and adapted by [117]. Exponential YNB cultures were diluted and used to inoculate either liquid or solid medium. These were incubated at 30 ºC in filamentous growth-inducing condition YNB without glucose [25]. Controls were performed in YNB with 2% (w/v) glucose.

Liquid medium: Overnight cultures were diluted to an OD$_{600}$ of 0.2, and incubated at 30 ºC with orbital shaking at 200 rpm during 5 days. At several time points, 10 µl of the suspension was collected, observed by light microscopy and photographed (see below).

Solid medium: Cultures were inoculated using identical inoculum and were allowed to grow for 1-3 weeks. After washing assay, cells were collected with a loop and placed on microscopic slide with 10 µl of deionized water visualized by light microscopy and photographed. Microscopy assessment was done in a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings and using a 40x objective. Images were acquired by a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

2.10.5 Chronological aging assay

Chronological aging was tested using the methodology adapted from [118]. Overnight cultures on YNB were used to start 80 ml YNB batch cultures at OD_{600nm} of 0.1. Cells were allowed to grow for 48 h. This time-point was considered as a measure of 100% culture survival - T0. Cell viability was quantified by Colony Forming Units (CFU) in YPD during 13 further incubation days. For CFU assay, cells were counted on a Neubauer chamber and diluted in PBS (137 mM NaCl, 2.7 mM , 10 mM Na_2HPO4, 1.8 mM KH_2PO4) to achieve ~100 cells in 240 µl [118]. Six drops (~100 cells) of 40 µl each were plated on YPD medium per time sample and incubated at 30 °C for 48 h. Colonies formed were counted.

2.10.6 Cell cycle analysis

Cell cycle analysis was performed at different growth phases (exponential and late-stationary) and the cell treatment was adapted from [119]. For each assay, 500 µl of cells ($\pm 10^6$ cell/mL) were fixed overnight in 500 µl of 70% ethanol. The cells were then collected by centrifugation at 13 000 rpm (minispin, Eppendorf), during 2min, and washed 1x in 1 mL of 50 mM sodium citrate buffer at pH 7.5. After a new identical centrifugation, the pellet was resuspended in 400 µl of RNase A (2mg/mL in Tris-EDTA, pH 8.0) and incubated overnight at 37 ºC. After a new centrifugation, the pellet was resuspended in 200 µl of proteinase K at 5mg/mL diluted in H_2O (pH 7.5), and incubated at 37 ºC for 45 min, and again centrifuged. The pellet was resuspended again in 500 µl of 50 mM sodium citrate buffer at pH 7.5, added SYTOX®-Green (Life Tecnologies) 5 mM 100x diluted in Tris-EDTA, pH 8.0 (20µl per 100µl sample), and incubated overnight at 4 ºC in the dark. Finally, 600 µl of Triton X-100 (0.25% (v/v) in 50 mM sodium citrate buffer, pH 7.5) was mixed with the sample and vortexed. The final mixture was briefly sonicated (High intensity ultrasonic processor GEX 400), three times at 30 W, 2 sec each, incubated on ice between each sonication, for the reduction of cellular aggregates. At least 20 000 cells from each sample were analysed in an Epics® XL™ (Beckman Coulter) flow cytometer, with an excitation of 497 nm and an emission of 520 nm according to SYTOX®-Green manufacturer instructions. Flow cytometry data was processed using Flowing Software 2.

Chapter III

Results and Discussion

3. Results and Discussion

3.1 Heterologous expression of human *KRAS^wt* cDNA in *Saccharomyces cerevisiae*

In view of genetic, molecular and functional conservation, the low eukaryotic organism, *S. cerevisiae*, was been used as model to study several human cellular processes and the related signalling pathways [7, 8]. This is also due to the fact that *S. cerevisae* genome can be easily engineered, allowing suitable analysis and efficient assessment of gene products from human cells [9, 120]. *S. cerevisiae* has two *RAS* genes, the *RAS1* and *RAS2,* which proteins have amino acid sequences very similar to the proteins encoded by the mammalian *RAS* proto-oncogenes, the *HRAS*, *NRAS* and *KRAS* [81]. Both in mammals and in yeast, Ras proteins are signal switch molecules that regulate cell fate by coupling receptor activation to downstream effector pathways that control several cellular responses including proliferation and survival [42, 110]. In *S. cerevisiae,* the two RAS genes are essential and their double deletion is lethal [82]. The proteins Ras1 and Ras2 are extremely similar small GTPases that although biological equivalent both differ in the expression pattern under different physiological conditions [86]. In humans, the RAS isoforms (HRas, NRas a KRas) are also very similar to each other at sequence / structural level, but not functionally, since they generate different signal outputs [70]. From all RAS isoforms, *KRAS* is the more ubiquitously expressed, and it is essential for normal cellular development of many cell types [64]. The similarity between amino acid sequence of RAS in yeast and in mammals suggests homology at functional level [112]. This could enable the utilization of yeasts as genetic and molecular models for the study of *KRAS* expression. For this reason, *S. cerevisiae* mutants deleted for either *RAS1* or *RAS2* in the genetic background of the EUROSCARF Collection (BY4741) were used to express the wild type form of the human *KRAS* (*KRAS^wt*) cDNA as follows.

3.1.1 *KRAS^wt* plasmid harbouring construction

Human *KRAS^wt* cDNA was initially cloned into a pair of p4X6 series plasmids, with different expression strength - p416 and p426 [114]. Both p416GDP and p426GPD are considered useful tools for cloning procedures in yeast, the only distinction between them is the different expression strength: p426GPD is multicopy, ensuring high

expression, while p416GD only produces one copy of the recombinant protein per cell [114].

Human *KRAS^wt* cDNA was already inserted in a pLenti plasmid (kindly provided by Manuel Teixeira from IPO Research Centre - Porto). A restriction analysis was performed in order to select the appropriate restriction enzymes to be used during the cloning steps, considering the receptor plasmid MCS and the *KRAS^wt* fragment. The selected enzymes were *Hind*III and *Xho*I. To obtain appropriated amounts of the *KRAS^wt* fragment, this was amplified from pLenti/*KRAS^wt* using primers FW_11044 KRAS and RV_11044 KRAS (Table 2), which introduced a *Hind*III and an *Xho*I restriction site at the 5' and 3' ends of the *KRAS^wt*, respectively. The gene amplification was confirmed by electrophoresis (Fig. 11) and the *KRAS^wt* fragment was extracted and purified from the gel.
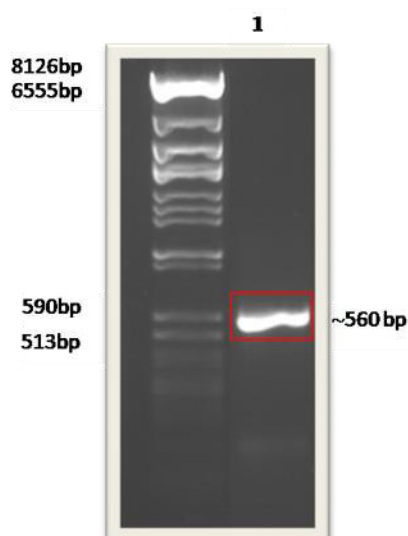
Figure 11. Electrophoretic analysis of PCR amplified fragment originating from pLenti/*KRAS^wt* (lane 1). Molecular weight marker: λ DNA/Eco47I (Fermentas). Red box: putative amplified *KRAS^wt*.

The purified fragment (insert) and the empty plasmid p416GPD, were double digested by *Hind*III and *Xho*I. The correct double digestion of the empty plasmid p416GPD was confirmed by electrophoresis (not shown). The insert was then ligated to the vector and the resultant construction, the p416GPD/*KRAS^wt*, was used to transform *E. coli* XL1-Blue competent cells for plasmid propagation. The *E. coli* XL1-Blue transformants were selected in LB medium supplemented with 100 µg/ml of ampicillin. Transformation was confirmed by choosing randomly 8 colonies for extracting the plasmidic DNA and digesting again with *Hind*III and *Xho*I. The expected restriction fragments restriction results should be two, one correspondent to the plasmid

p416GPD of app. 6.6 kb, and another to the *KRAS^wt* fragment of app. 560 bp. Electrophoresis revealed a unique band corresponding to the linearized plasmid (not shown).

A second attempt was made using a different plasmid - p426GPD that has a higher expression level comparatively to the p416GPD. The already amplified *KRAS^wt* fragment was again double digested with *Hind*III and *Xho*I restriction enzymes, as well as the p426GPD plasmid. The p426GPD correct enzymatic digestion was confirmed by electrophoresis (not shown). The double digested *KRAS^wt* was then ligated to the double digested vector and the resultant construction was transformed in *E. coli* XL1-Blue. Again, to verify the *E. coli* XL1-Blue positive transformants, the plasmidic DNA was extracted from 2 transformants and double digested with *Hind*III and *Xho*I restriction enzymes (Fig. 12 lanes 1-2). A band with ≈560 bp (indicated by the red rectangle), most likely corresponded to the *KRAS^wt* fragment. One positive clone was selected to extract plasmidic DNA to transform the *S. cerevisiae*.
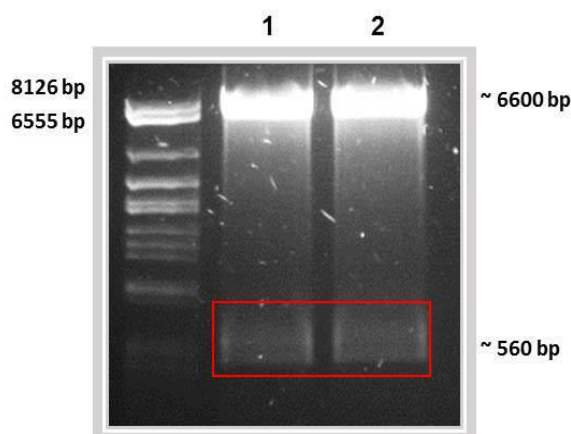
Figure 12. Electrophoretic analysis of the p426/*KRAS^wt* plasmid DNA extracted from *E.coli* XL1-Blue clones and double digested with *Hind*III and *Xho*I – Lanes 1 and 2. Molecular weight marker: λ DNA/Eco-471 (Fermentas). Red box: Digested *KRAS^wt* fragment.

3.1.2 Transformation of *S. cerevisiae* BY4741 with p426/*KRAS^wt*

Considering the purpose of evaluating the capacity of *KRAS^wt* to complement the yeast *RAS* deletions, the BY4741 strains harbouring a deleted *RAS1* or *RAS2*, were used for the expression of *KRAS^wt*. The two mutants and the mother strain were transformed with (a) the plasmid p426GPD (empty plasmid - ø), serving as a control, and (b) the corresponding plasmid containing a copy of *KRAS^wt*, under control of the GPD constitutive promotor (p426GPD/*KRAS^wt*).

The BY4741 wild-type (wt), *ras1Δ* and *ras2Δ* strains transformed with p426GPD/*KRAS^wt*, were all selected in minimal medium YNB supplemented with the required amino acids, except uracil – selective marker of the plasmid. Some clones of each transformation were selected for PCR amplification of *KRAS^wt* fragment. A sample of *KRAS^wt* amplified fragment from pLenti/*KRAS^wt*, was used as positive control. The PCR products verification by electrophoresis demonstrated the presence of the *KRAS^wt* corresponding band in 6 tested samples (Fig. 13 lanes 2-7). These positive transformants (clones containing the *KRAS^wt*) were stored at -80ºC in glycerol, and propagated in the solid selective medium for daily use.
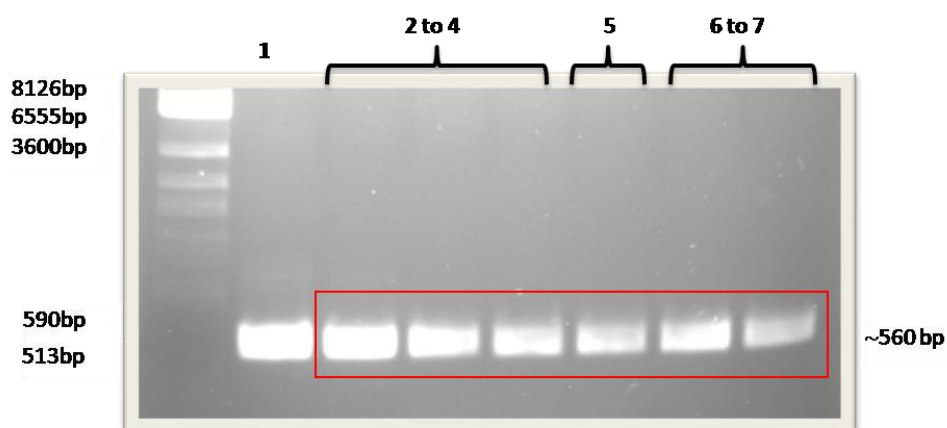
Figure 13. Electrophoretic analysis of PCR amplified *KRAS^wt* originating from *S. cerevisiae* BY4741 transformed with the p426GPD/*KRAS^wt*: wt (lane 2 to 4), *ras1Δ* (lane 5) and *ras2Δ* (lane 6 to 7). The *KRAS* insert corresponds to a band with a molecular weight of approximately 560 pb. As a positive control the pLenti/*KRAS^wt* was identically amplified by PCR (lane 1). Molecular weight marker: λ DNA/Eco47I (Fermenta). Red box: Bands corresponding to *KRAS^wt* fragment.

3.1.3 Confirmation of *KRAS^wt* expression in the BY4741 positive transformants

The total protein extracts from one positive transformant of each recombinant strain, including the strains transformed with the empty p426GPD, were isolated from exponential young batch cultures (OD_{600} =1) and were analysed by Western Blot, using the antibody anti-KRas (see Materials and Methods). In the first attempt, the antibody was used at dilution of 1:500 as recommended by the fabricant, however the signal was too strong. A workable dilution was 1:1000 (Fig. 14). WB analysis confirmed the expression of *KRAS^wt* in p426GPD-derived transformants.

	(A) BY4741 wt		(B) BY4741 *ras1Δ*		(C) BY4741 *ras2Δ*	
	p426 ø	KRAS^wt	p426 ø	KRAS^wt	p426 ø	KRAS^wt
KRas (21 KDa)	-	+	-	+	-	+

Figure 14. Western Blot analysis of KRAS^wt expression in *S. cerevisiae* BY4741. Total protein extracts of wt (A), *ras1Δ* (B) and *ras2Δ* (C), transformed with the empty p426GPD (Ø) and p426GPD/*KRAS^wt*, were probed against anti-KRas antibody (Sigma). Each column shows the result of blotting positive (+) or negative (-) transformants.

3.2 Phenotypic effects of the *KRAS^wt* expression in *S. cerevisiae* and functional complementation of the RAS genes deletion

In *S. cerevisiae*, the RAS/cAMP/PKA pathway is involved in the regulation of cell growth and proliferation in response to several environmental and metabolic cues, including nutritional, osmotic, heat, acid and oxidative stresses [31, 38, 121]. In these conditions, RAS signalling exerts a negative regulation, since cells are less stress-tolerant when RAS pathway is activated [56]. Moreover, although the Ras1 and Ras2 proteins are moderately redundant at phenotypic level, the Ras2 is the main responsible for the cellular functions controlled by RAS pathway [87]. In order to elucidate the effect of expressing *KRAS^wt* in *S. cerevisiae*, as well as its ability to functionally complement the *RAS1* and the *RAS2* single deletions, the wt and the *ras1Δ*, *ras2Δ* strains from the genetic background BY4741 were transformed with

p426GPD/*KRAS^wt* and submitted to phenotype screening. As controls, the recipient strains were also transformed with the empty plasmid. This way, the phenotypic screens were done using the recipient strains, the recipient strains transformed with the p426GPD/*KRAS^wt* plasmid, and transformed with p426GPD empty plasmid.

According to the roles attributed to the RAS genes in the response to different environmental cues, the yeast strains were incubated in YNB with 2% glucose and the required amino acids, and submitted to several types of stress:

- High temperature (37 ºC);
- Osmotic pressure/low water activity (a_w) (0.5 M and 1 M of NaCl; 1 M and 1.5 M of sorbitol) at 30 ºC;
- Oxidative stress (0.5 mM, 1 mM and 2 mM H_2O_2) at 30 ºC;
- pH (pH 4, pH 6, and pH 7) at 30 ºC.

Cells were also cultured in 2% ethanol and 2% glycerol to verify their ability to grow under glucose derepression/respiration conditions. All these environmental conditions were tested by drop test assay. Additionally, the cells were also tested as to their ability to adhere and invade agar surface and form filaments in function of the presence/absence of glucose, *i.e.,* in glucose starvation conditions. Finally, these strains were studied as to the ability to thrive into post-diauxic and stationary phase and survive, *i.e.,* chronological aging by c.f.u. assay, as well as the distribution of cells through the several cell cycle-associated phases by flow cytometry.

3.2.1 Stress phenotypes associated with *KRAS^wt* expression

3.2.1.1 High-temperature stress

RAS signalling is involved in response to high-temperature stress. Cells with a temperature-sensitive *RAS2* deletion are blocked in G1 phase of the cell cycle and accumulate as unbudded cells at non-permissive temperatures [42]. Moreover, cells exhibiting elevated levels of PKA are sensitive to high temperatures, whereas attenuated PKA activity renders an otherwise wt strain more resistant to heat shock [122]. According to this and to the fact that the RAS pathway leads to the PKA activation, it can be assumed that RAS signalling should be involved in the response to temperature stress.

The effect of *KRAS^wt^* expression in the *ras1Δ*, *ras2Δ* and wt cells on the response to the high temperature stress (37 ºC) was tested on glucose-based minimal medium (Fig. 15). As control, cells were identically cultivated at 30 °C (not shown).

Figure 15. Phenotypic evaluation of the sensitivity to high temperature of BY4741 *S. cerevisiae* set of strains expressing human *KRAS^wt^*. Strains were pre-grown on YNB w/ glucose and used for drop-out assay (ten-fold serial dilutions, 5 µl/ drop) on YNB w/ 2% glucose and incubated for 2 days at 37 °C. These results are representative of independent triplicates.

The obtained results show that the deletion of either yeast *RAS* genes had no effect on temperature resistance as compared to wt. The non-phenotype of the *ras2Δ* strain to high-temperature stress appears to contradict the literature, which predicted a growth defect caused by *RAS2* deletion [42, 82, 123]. Moreover, these results were observed in other than BY4741 genetic backgrounds, including the diploid EG81 strain [82], the 112 strain [80] and DC5/6 strain [88, 124]. Compared with these strains, BY4741 strain genome (Table 4) harbours a mutation on *MET15* that is not shared by any of the other strains. The putative influence of this mutation, alternative to *lys2* or *ade2* remains for the moment unknown. On this line of thought, Cohen and Engelberg [113] demonstrated that the *leu2* mutation on the BY4741 background could influence the experimental results. The authors verified an effect in the auxotrophic phenotype using cells grown in complete synthetic medium containing all the amino acids, however they indicated the possibility that the *leu2* deletion affects other biochemical and molecular activities in the cell.

The expression of *KRAS^wt^* had no effect on the observed resistance to high-temperatures, which in view of the above is not surprising. The exception was BY4741 wt strain (Fig. 15). This harbours three RAS alleles - human *KRAS^wt^* and the

endogenous *RAS1* and *RAS2* – leading to a probably increased amount of intracellular Ras proteins, which could conduct to the hyperactivation of the RAS/cAMP/PKA pathway. This is associated with the decrease of tolerance to various stress conditions including thermotolerance [99-102]. This being the case, the tests on other type of stresses made during the present work with the same strains should yield identical results.

3.2.1.2 High osmotic stress

Yeast cells respond to a shift to higher osmolarity by increasing the cellular content of the osmolyte glycerol. In addition to the HOG pathway also RAS/cAMP/PKA has been shown to affect the expression of genes upon an osmotic upshift [125]. PKA activity influences negatively the expression of response proteins to the osmotic stress [125]. In addition, the *ras2Δ* mutants demonstrated resistance to the hyperosmolarity [126]. The osmotic stress can be generated by high concentrations of salts, or neutral solutes like sorbitol or other sugar alcohols at concentrations causing similar decrease in a_w [40, 127]. To assess the strains sensitivity to hyperosmotic stress, NaCl was used at 0.5 M (0.980 a_w) and 1 M (0.965 a_w). Alternatively, identical a_w values were produced using sorbitol at final concentrations of 1 M (0.980 a_w) and 1.5 M (0.970 a_w) [127].

Results (Fig. 16) revealed identical results to the ones obtained with high temperature: all strains grew well in the presence of either NaCl or sorbitol concentrations, except the strain expressing the three RAS alleles – BY4741 wt *KRAS^{wt}* (Fig. 16). These results strengthen the suggestion above-mentioned of the difference imposed by genetic background on RAS-mediated stress responses. Similarly to what happened in high-temperature, the fact that *ras1Δ* and *ras2Δ* strains (expressing or not the *KRAS^{wt}*) and wt strain (not expressing *KRAS^{wt}*) had no visible phenotype in response to osmotic stress, suggested that the stress responses in BY4741 background might be highly increased.
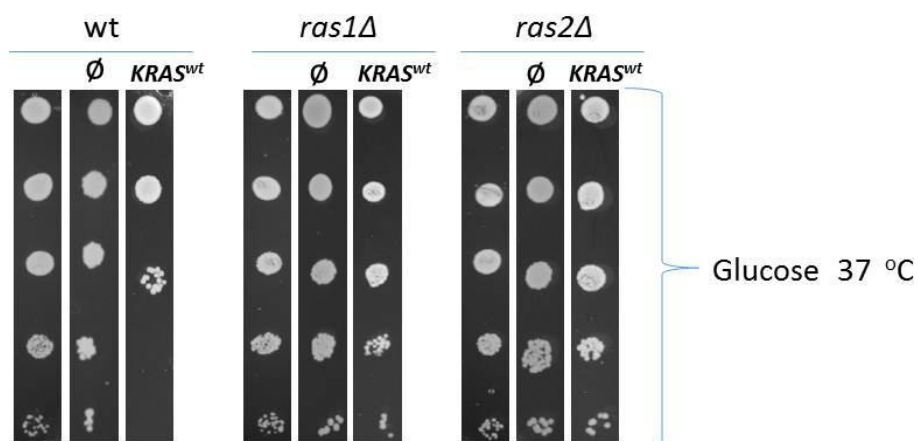
Figure 16. Phenotypic evaluation of the sensitivity to osmotic stress of BY4741 *S. cerevisiae* set of strains expressing human *KRAS^wt*. Strains were pre-grown on YNB w/glucose and used for drop-out assay (ten-fold serial dilutions, 5 µl/ drop) on YNB w/ 2% glucose with NaCl or sorbitol and incubated for 2 days at 30 °C. These results are representative of independent triplicates.

3.2.1.3   Oxidative stress

All aerobically growing organisms are continuously exposed to oxidants. Moreover, most reactive oxygen species (ROS) are generate as by-products during respiration. Oxidative stress therefore, occurs when the concentration of these oxidants increases beyond the antioxidant buffering capacity of the cell [128]. H_2O_2 is one of the most common reactive oxygen species. The RAS/cAMP/PKA pathway negatively affects the response to H_2O_2 stress [97, 129]. High intracellular cAMP levels induce hypersensitivity to H_2O_2 concomitant with a defective induction of H_2O_2 stress responsive genes [121]. Therefore, the RAS signal transduction pathway (responsible for regulating intracellular cAMP levels) is clearly important in the regulation of oxidative stress response [128], and mutations in *RAS* genes, namely the *RAS2* deletion, should increase survival and resistance to oxidative stress [89].

In the present work the oxidative stress response of wt, *ras1Δ* and *ras2Δ* strains, and further the effect of *KRAS^wt* expression, were assessed using different concentrations of H_2O_2 (0.5 mM, 1 mM and 2 mM).

Figure 17. Phenotypic evaluation of the sensitivity to osmotic stress of BY4741 *S. cerevisiae* set of strains expressing human *KRAS^wt*. Strains were pre-grown on YNB w/ glucose and used for drop-out assay (ten-fold serial dilutions, 5 µl/ drop) on YNB w/ 2% glucose and H_2O_2 and incubated for 3 days at 30 °C. These results are representative of independent triplicates.

As can be seen in Fig. 17, the pattern of response obtained with high temperature and osmotic stresses was repeated. wt and *RAS* mutant strains, as also the same strains harbouring the empty plasmid, were resistant to the presence of either 0.5 mM or 1 mM of H_2O_2. On the other hand, all the strains expressing *KRAS^wt*, were sensitive to 0.5 mM H_2O_2 but not to 1 mM H_2O_2, with the exception of wt expressing *KRAS^wt*, which was sensitive to both conditions. An additional experiment at 2 mM H_2O_2 killed all the strains (not shown).

According to the literature, *ras2Δ* mutant was described as more resistant to oxidative stress than wt strain [27]. Once again, this was described in a totally different genetic background as in Table 4 - [109]. The fact that BY4741 wt strain grew as well as the strains deleted in *RAS* genes could indicate, as mentioned above, that it is due to the different genetic background. Still, this is not straightforward since the further expression of *KRAS^wt* induced sensitivity to oxidative stress, most probably an effect caused by the overexpression *KRAS^wt* gene leading to the hyperactivation of the RAS pathway above discussed. Nevertheless, as described in the Introduction, full oxidative stress responsive is subject to crosstalk between the CWI/PKC, RAS and TOR signalling pathways, as well as HOG pathway. A miss-regulation at the top part of RAS

pathway could influence indirectly responses to stresses that depend on all or several of these pathways. This remains to be seen in the future.

3.2.1.4   High and low pH

Generally, eukaryotic cells maintain their intracellular pH within a narrow range, despite wide variations that may occur in the extracellular pH. As the gap between the extracellular and the intracellular pH widens, greater stress applies to the cells and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of the yeast [130]. The ability of the strains to growth on YNB medium at pH 4 (low pH) and at pH 7.0 (high pH) was tested. As control, the strains were also grown at pH 6, the optimal pH for growth. The results (not shown) clearly show that none of the pH values tested affected growth of any of the strains, suggesting that RAS genes could not important for intracellular pH homeostasis.

3.2.2 Effect of *KRAS^wt* expression on growth on non-fermentable carbon sources

Ras2 is expressed with roughly equal efficiency in any carbon source, whereas Ras1 is highly expressed on glucose and poorly expressed in non-fermentable carbon sources. As consequence, the *ras2Δ* strain is defective on non-fermentable carbon sources [80]. Thus, growth ability was tested on YNB medium with 2% (v/v) ethanol or 2% (v/v) glycerol. Controls were done on minimal medium with 2% (w/v) glucose. As expected [80], all strains tested grew better on glucose than on glycerol or ethanol (Fig. 18). As above, no phenotypic differences were visible between the wt and the mutant strains, indicating that the absence of *RAS2* in BY471 did not affect the growth in non-fermentable conditions. This result is supported by the study of *Tatchell et al.* [82], in which no phenotypic changes were observed when haploid strains containing the disruptions of either *RAS1* or *RAS2* were used. Instead, these authors found an impaired growth on the respiratory conditions when they used the diploid yeast cells with *ras2-530 disrupted RAS2* allele. On the other hand, the results in Fig. 18 show that the expression of *KRAS^wt* affected negatively growth on ethanol or glycerol, more strongly in the wt strain expressing the *KRAS^wt*. This result also indicates that the

hyperactivation of the RAS/cAMP/PKA pathway, already mentioned, interfered with the yeast ability to grow on non-fermentable carbon sources [131].
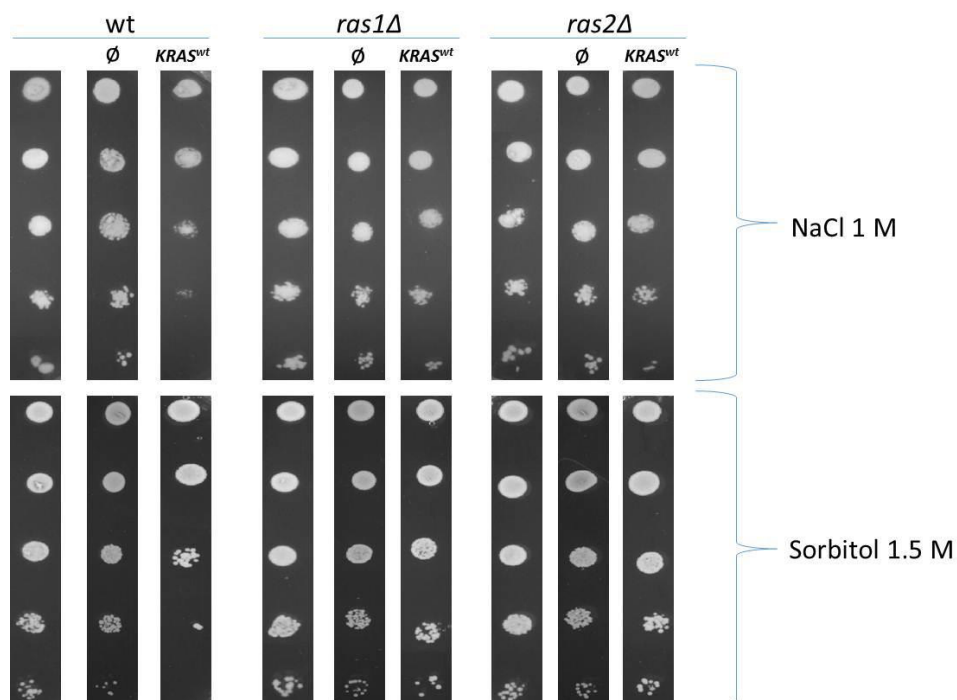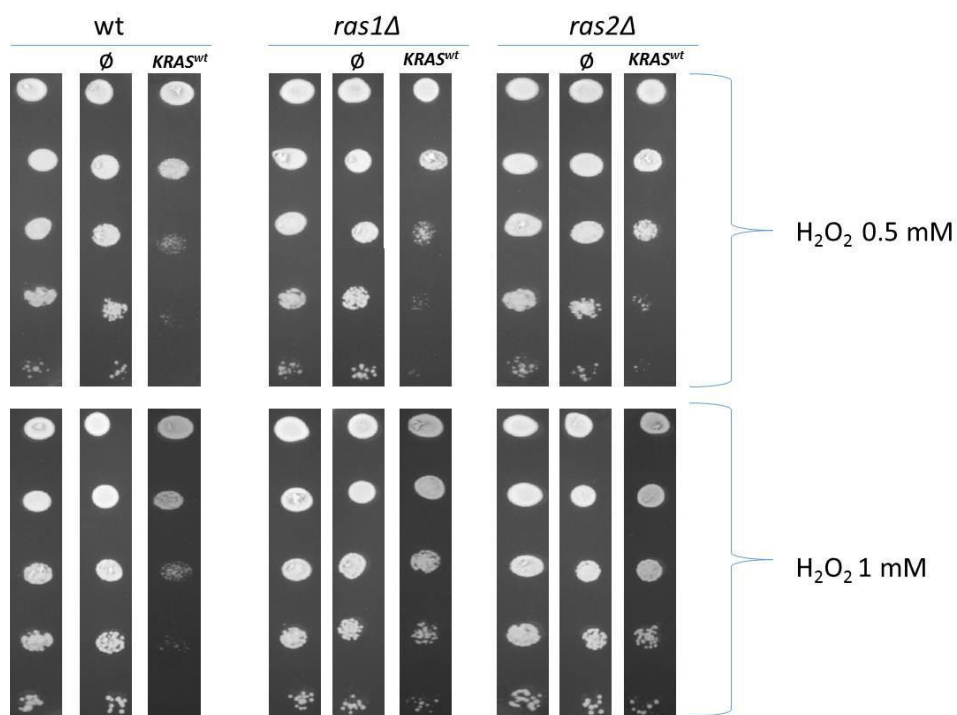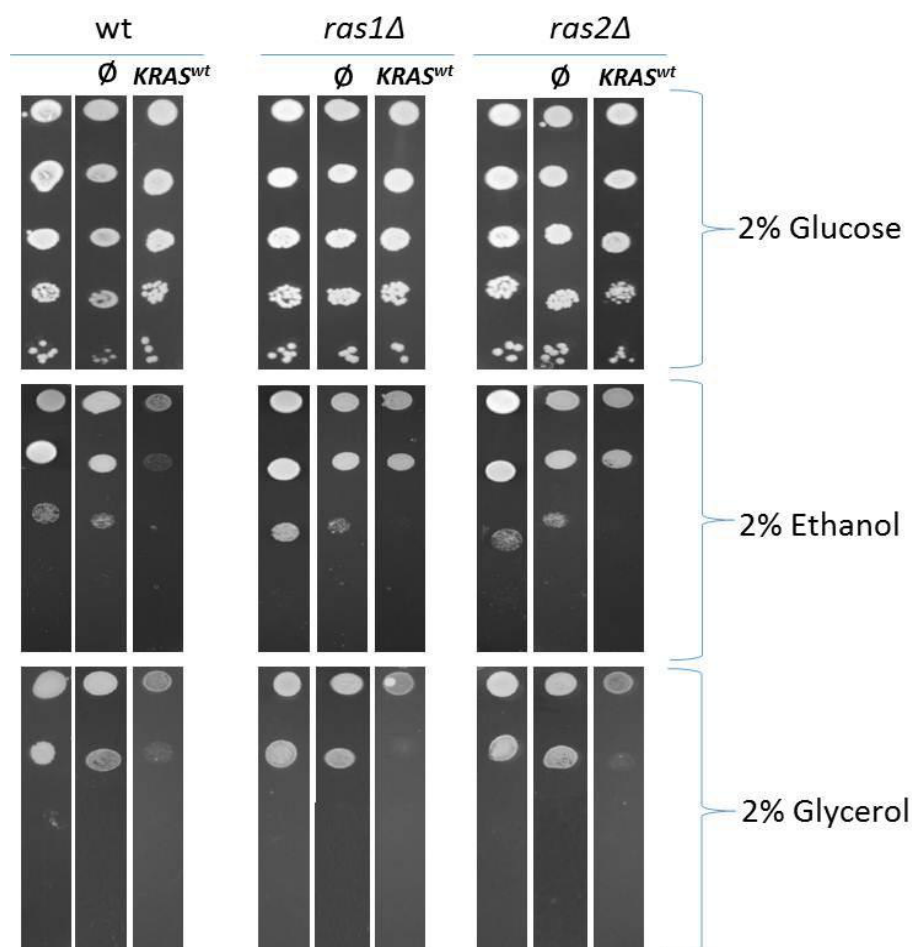

Figure 18. Phenotypic evaluation of the sensitivity to osmotic stress of BY4741 *S. cerevisiae* set of strains expressing human *KRAS^wt^*. Strains were pre-grown on YNB w/ glucose and used for drop-out assay (ten-fold serial dilutions, 5 µl/ drop) on YNB w/ 2% ethanol or glycerol, or 2% glucose for control. Results show cultures incubated at 30 °C for 2 days in the case of glucose, and 10 days for the other carbon sources. These results are representative of independent triplicates.

3.2.3 Effect of *KRAS^wt^* expression in the haploid invasive growth

Filamentous growth is a nutrient-regulated growth response. In conditions of nutrient limitation, *S. cerevisiae* haploid cells can suffer a subtle dimorphic switch, characterized by a slight elongation of cells, an enhanced cell-cell adhesion, and a change in cell polarity [25]. These modifications can culminate in the appearance of a filamentous form, which allows the cells a moderate penetration in agar medium

surface (invasive growth) [132]. RAS2/cAMP/PKA signalling is known to be required for filamentous growth and invasion [133]. Accordingly, previous studies demonstrated that the *RAS2* deletion prevents invasion [23, 26].

Adhesion to agar and invasive growth was tested in the same yeast strains group as above. Cell adhesion was assessed using the standard plate washing assay [33], followed by inspection of agar slices for invasive growth (see materials and Methods). Filamentation was verified by microscopic observation of cells recovered from the surface of agar. These assays were done using glucose grown and starved cells. This is due to the previous observation that a *S. cerevisiae* haploid strain (L5528 – Table 4) was capable of invasive behaviour and filament formation while growing on glucose [132]. All tested strains adhered to the surface of the medium without glucose (Fig. 19).
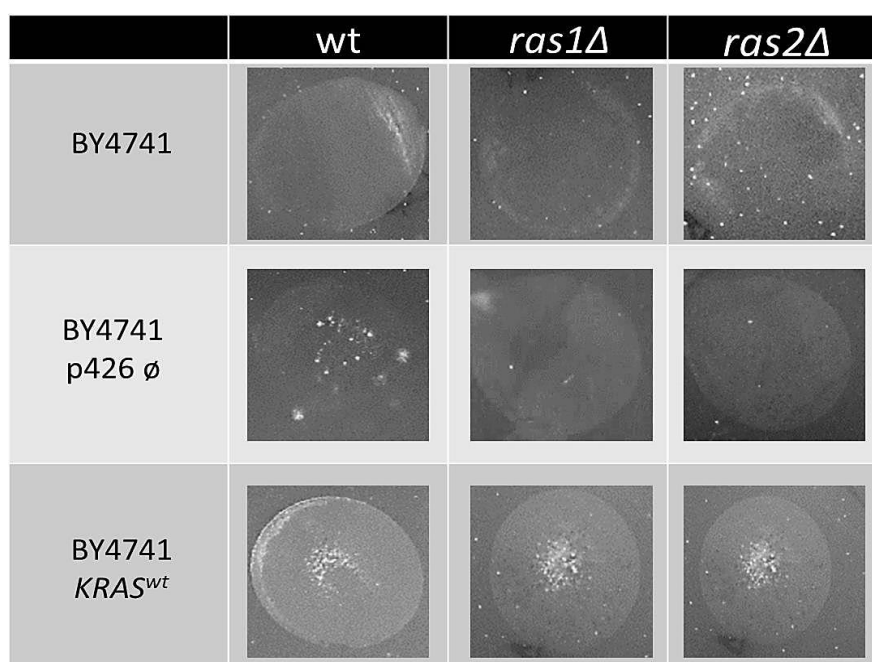
Figure 19. Assaying adherence to the agar of BY4741 *S. cerevisiae* set of strains expressing human *KRAS^wt^*. Strains were pre-grown on YNB w/ glucose and equal concentrations of cells were spotted onto plates of YNB w/o glucose, and incubated for 7 days at 30 °C. The adherence was verified after washing the surface of the agar with a gentle stream of running water. These results are representative of triplicates.

The strains expressing *KRAS^wt^* presented a stronger adherence than all other, in particular the wt strain. This increased adherence might suggest higher invasive growth ability, nevertheless, none of the strains presented agar invasion (not shown). Filamentous growth also did not occur in any of the strains (not shown). These results,

with the exception of the invasion phenotype, were confirmed in longer incubation periods (up to 3 weeks), and in glucose growing cells (not shown). Due to the *RAS2* expression (in the wt and *ras1Δ* strains), it was expected that these strains displayed filamentous and invasive behaviour. However, this is not unexpected considering that BY4741 background derives from S288C, which is defective in Flo8p, a protein required for filamentous growth [134, 135].

The expression of the *KRAS^{wt}* in the wt and *ras1Δ* mutant strains did not change the non-invasive phenotype of these strains on glucose-based medium. On the contrary, the expression of *KRAS^{wt}* in the *ras2Δ* strain led to an invasive phenotype (Fig. 20 – Panel I). Nevertheless, this invasive pattern did not present filaments as expected, but rather a cellular mass growing deep into agar. In the literature, some other haploid mutant strains, namely *bud1*, *bud2,* and *bud5*, were shown unable to form filaments but still penetrating agar in a similar fashion [132].
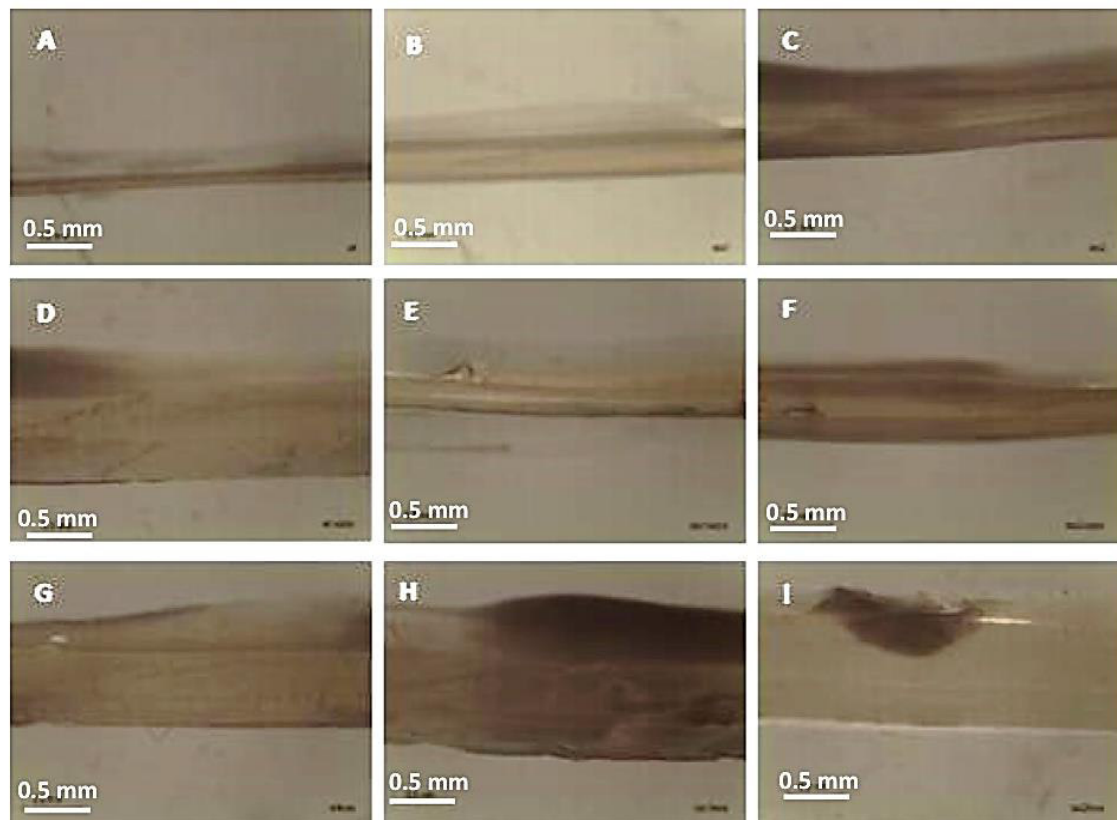
Figure 20. Assaying agar invasion of BY4741 *S. cerevisiae* set of strains expressing human *KRAS^{wt}*: wt (A); *ras1Δ* (B); *ras2Δ* (C); wt p426ø (D); *ras1Δ* p426ø (E); *ras2Δ* p426ø (F); wt *KRAS^{wt}* (G); *ras1Δ* *KRAS^{wt}* (H) and *ras2Δ* *KRAS^{wt}* (I). Assays were performed as in Fig.19 except that plates were incubated for 3 weeks. Agar was sliced with a scalpel, and sections were set perpendicular to the plan of invasion for visualization.

Table 4: Compared genetic backgrounds of BY4741 and the different strains used in the literature to describe Ras-related phenotypes.

Strain	Genetic background								
BY4741	MATa	*leu2Δ0*	*his3Δ1*	*ura3Δ0*	*met15Δ0*	---	---	---	---
EG81 [1]	MATa	---	*his4*	*ura3*	---	*lys2*	---	---	---
112 [2]	MATα	*leu2-3-112*	*his3*	*ura3-1*	---	---	*can1-100*	*ade2-1*	---
YPH98 [3]	MATα	*leu2-Δ1*	---	*ura3-52*	---	$lys2\,801^{amber}$	---	$ade2\,101^{ochre}$	*trp1-Δ1*
DC5/6 [4]	MATa/MATα	*leu2/leu2*	*his3/+ his3/+*	---	---	---	*can1/can1*	---	---
L5528 [5]	MATa	---	*his3:hisG*	*ura3–52*	---	---	---	---	---

[1] [68], [2] [69], [3] [109], [4] [78, 112], [5] [121]

Filamentation was further studied in glucose-starved cells grown in liquid medium batch cultures. Identically to what was observed in solid media, none of the strains displayed filamentation up to 5 days incubation (Fig. 21). Interestingly, the budding pattern of BY4741 wt *KRAS^wt* strain was different (Fig. 21 panel G – white arrows). A bipolar budding pattern was observed in the vast majority of dividing cells. Although, this is usually characteristic of diploid cells [132], it has also been associated with haploid invasive growth [136]. Additionally, the bud site selection has been suggested not to be a fixed parameter determined by cell type, but rather a dynamic process sensitive to environmental cues like nutrient deprivation [132]. According to the literature, haploid cells can switch their budding pattern from axial to bipolar and consequently form filaments through a series of polarized cell divisions [132], and these last should be enough to penetrate agar as was seen in the *ras2Δ KRAS^wt* strain (Fig. 20). The present results appear to indicate that although the occurrence of the budding pattern switching is necessary to find filamentous growth, it might not be sufficient. There is the further possibility that a rare event like might have happened, like homothallic *a/a* diploidy [137], or even of an *a/α* switch allowing mating [138]. Importantly, the BY4741 is MAT*a,* but is not deleted for *HO*, the site-specific endonuclease that is required for homothallic switching [139]. Its expression is under the control of Swi4p, Swi5p, Swi6p and Ash1p [140]. The Swi4p and 6 proteins work in the dependence of the TOR/PKC pathways and act as transcription regulators allowing parts of the chromosomes to become available for expression. As mentioned above, the crosstalk between TOR, RAS and PKC could lead to miss-regulation of genes under their interdependence including *HO,* and allow mating type switching and the consequent diploidization of the culture.
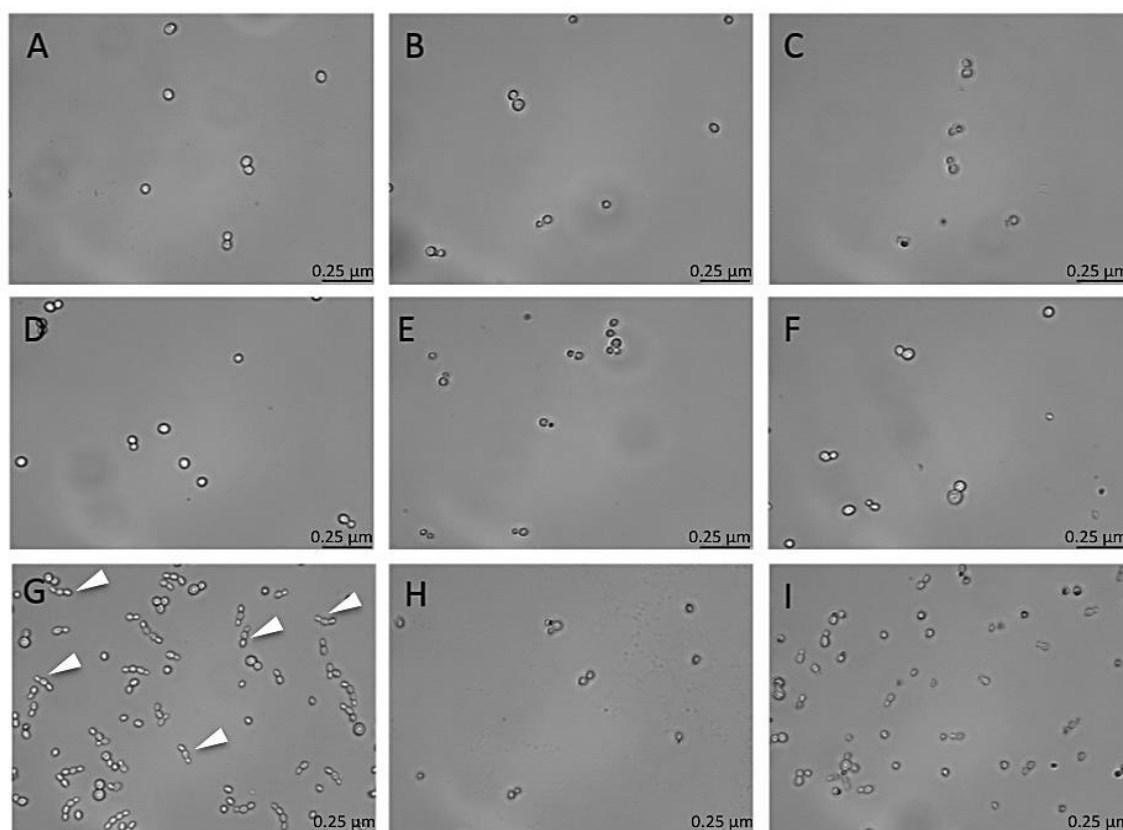
Figure 21. Morphological appearance of cells of the BY4741 *S. cerevisiae* set of strains expressing human *KRAS^wt^:* wt (A); *ras1Δ* (B); *ras2Δ* (C); wt p426ø (D); *ras1Δ* p426ø (E); *ras2Δ* p426ø (F); wt *KRAS^wt^* (G); *ras1Δ KRAS^wt^* (H) and *ras2Δ KRAS^wt^* (I). Cells were *s*tarved in YNB w/o glucose for 5 days at 30ºC and examined by light microscopy. White arrows in panel G indicate bipolar budding.

Genetically, the processes of cell adhesion, bud site selection and cell morphogenesis, which together are required for filament formation in *S. cerevisiae*, can be dissected from each other [141], generating possible discrepant results of adhesion, invasion and differentiation. Although the filament formation and invasion depend on signalling through elements of the MAP kinase cascade that controls filamentous growth, each process is ultimately controlled by distinct downstream morphological pathways [132, 142]. Moreover, for cells to be able to invade, they need to adhere to the growth medium surface, which nevertheless does not implicate invasion. Actually, adhesion, invasion and differentiation are all processes related to haploid invasive growth, and the absence of filament structures partially compromises the invasive growth [142]. In sum, we can consider that the *ras2Δ KRAS^wt^* strain from BY4741 background grown in YNB with glucose demonstrated a haploid invasive growth phenotype characterized by non-filamentous growth. Moreover, the putatively hyperactivated BY4741 wt *KRAS^wt^* strain, showed bipolar growth without invasive or filamentous growth.

3.2.4 Effect of *KRAS*[wt] expression on chronological life span and cell cycle

In *S. cerevisiae* growing on glucose-based medium, the activated RAS/cAMP/PKA pathway negatively regulates the entry into stationary phase through Ras2 activity [143, 144]. Loss of RAS/cAMP/PKA signalling, namely through the deletion of *RAS2*, leads to stationary phase-like growth arrest [143], accompanied by a decreased metabolic activity, increased resistance to a variety of environmental stress conditions, and an altered pattern of gene expression, essential for the long-term survival of these cells [145, 146]. Consequently, the deletion of *RAS2* causes the decrease of growth rate but the extension of chronological life span [27, 89]. These two processes were analysed using all the strains as above.

3.2.4.1 Chronological life span

Batch cultures on YNB with 2% glucose supplemented with the necessary amino acids were followed throughout exponential growth phase by the measuring of the optical density at 600nm. The values estimated for the correspondent growth rates (Table 5) indicate that all strains grow identically on glucose (mean μ_g 0.191±0.031 h^{-1}) except for *ras2Δ KRAS*[wt] strain that presented a slower specific growth rate (±60% the mean value of the other strains). Additionally, the O.D. achieved at T_{30h} was identical for all strains, including the *ras2Δ KRAS*[wt] (mean O.D. 0.167±0.082), while the wt *KRAS*[wt] presented a long lag phase of ±10h that is not observed in any other strain (not shown). All taken, the absence of significant differences in growth on glucose indicate identical fitness throughout glucose consumption as expected. Otherwise, the chronological life span (CLS) should be affected in view of the involvement of Ras proteins in aging [27, 104].

Table 5. Specific growth rates (μ_g) of *S. cerevisiae* BY4741 wt, *ras1Δ* and *ras2Δ* untransformed, harbouring p426ø, and expressing *KRAS*[wt] strains, grown in YNB medium with 2% glucose. Each value is the mean of three independent experiments.

	μ_g (h^{-1})		
	Wt	*ras1Δ*	*ras2Δ*
---	**0,206 ±0.084**	**0,177 ±0.092**	**0,182 ±0.094**
p426ø	**0,184 ±0.048**	**0,237 ±0.095**	**0,165 ±0.089**
KRAS[wt]	**0,230 ±0.041**	**0,158 ±0.091**	**0,120 ±0.098**

Cellular viability was assessed by c.f.u assay throughout the stationary phase until almost complete death of culture, according to the methodology described in the literature [118] (see Materials and Methods). The survival curves (Fig. 22) showed that, surprisingly, the expression of the empty p426ø conferred both *ras1Δ* and ras2Δ an extended survival rate compared with wt also expressing the empty plasmid: at day 2, they had lost, respectively, approximately 40, 20 and 60% viability. Nevertheless, complete death of the culture was several days faster for *ras1*Δp426ø. These differences are not too notorious and require confirmation in the future but suggest that the predicted interference of auxotrophic marks in RAS mutants phenotypes might actually be true since the empty plasmid is only conferring complementation for uracil.
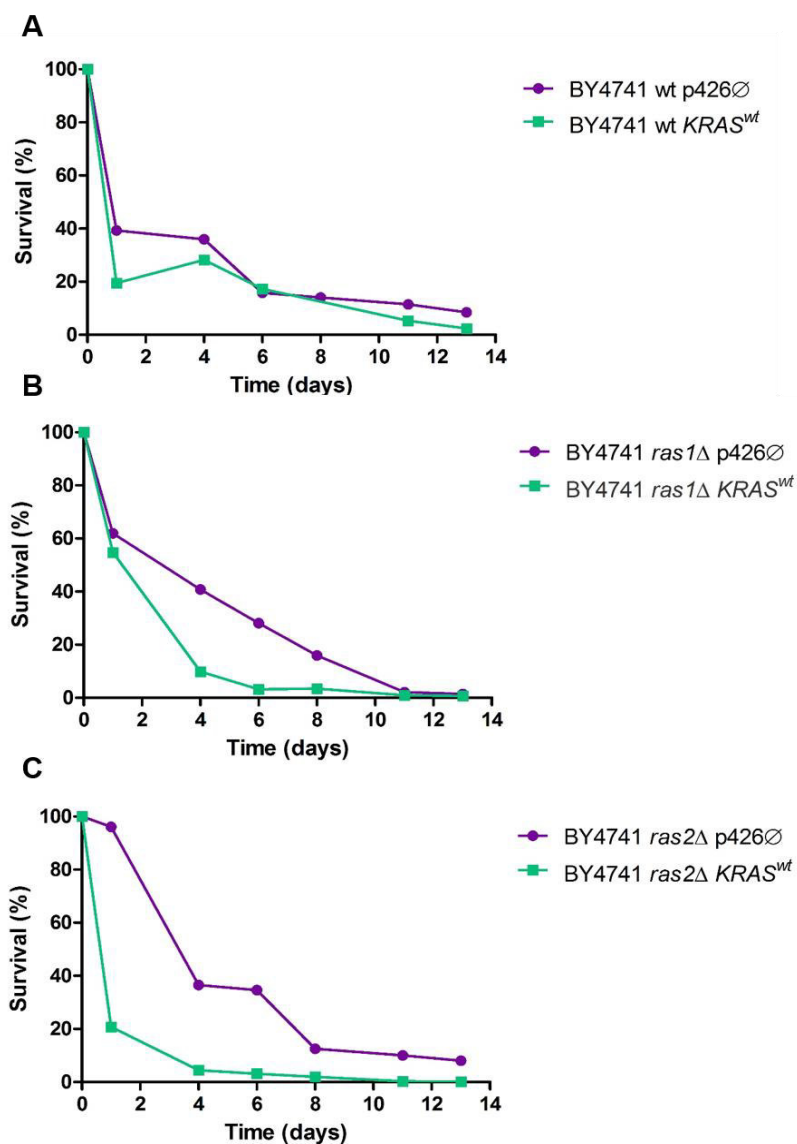
Figure 22. Survival of the BY4741 *S. cerevisiae* set of strains expressing human *KRAS*^{wt} during chronological aging phase. Cells were inoculated in YNB w/ 2% glucose and survival was monitored by c.f.u. Assays took place after exponential phase ended: T$_0$ corresponds to 100% survival. Results correspond to one (preliminary) experience.

On the other hand, the expression of *KRAS^wt* decreased cell survival of all strains. This loss of viability was stronger for *ras1KRAS^wt* and *ras2ΔKRAS^wt*. At day 6, almost all of their cultures were death while wt*KRAS^wt* still presented almost 20% of the culture alive. The results made evident that the *ras2Δ KRAS^wt* had a clear more pronounced loss of viability than other strains.

3.2.4.2   Cell cycle analysis

To clarify the effect of *KRAS^wt* expression during yeast growth and aging, the cell cycle of yeast was evaluated by flow cytometry. The division into different cell cycle phases is based on the amount of DNA detected with a specific probe, SYTOX Green. Four peaks can be observed: (1) at G1, when the DNA is in single copy; (2) at G2/M, when the DNA has already been duplicated but the cell is still not divided; (3) at the S phase, when DNA is being duplicated, but the duplication is not complete; and (4) at Sub-G0 phase corresponding to fragmented and or condensed DNA.

Cultures growing exponentially on glucose were used to assess the cell cycle progression during the exponential growth/replicative phase (Fig. 23). Identical cultures were allowed to grow onto late stationary phase up to 3 and 6 days and were then used to analyse the cell cycle during aging (Fig. 24).

Figure 23. Cell cycle analysis by flow cytometry of the BY4741 *S. cerevisiae* set of strains expressing human *KRAS^wt*. Strains were grown on YNB w/ 2% glucose up to mild-exponential phase. Results correspond to one (preliminary) experience.

Exponentially growing cells of almost all strains showed an equilibrated subdivision of the population between the three main phases of the cell cycle, having the majority of cells in G1 or G2/M and a smaller amount in S. The empty plasmid did not cause any effect on the distribution of cells through the several DNA replicative cycle of wt or *ras1*Δ cells. Conversely, it appeared to have an effect on *ras2*Δ strain, which presented a high percentage of cells in G0/G1. This should indicate a non-dividing culture, but is apparently not compatible with the results of growth above presented (Table 5) that did not differ from the other strains. Yet, it cannot be disregarded the possibility that the cells in the culture might yield a similar O.D. by having a very different size, therefore masking a slow-growing phenotype. The *ras2*Δ mutant is known to have a large cell size, almost doubling the size of wt (Brito A.S. (unpublished results) and [147]).

The expression of *KRAS*wt in the mutants caused an accumulation of cells in G0/G1, compared to the respective untransformed strains, while no effect was seen on the BY4741 wt *KRAS*wt. These results suggest that the expression of *KRAS*wt in *ras1*Δ causes a delay in the cell cycle that nevertheless does not match this strain growth rates (Table 5). On the other hand, the *ras2*Δ G1/G0 arrest caused by the empty plasmid was not significantly changed by the expression of *KRAS*wt.

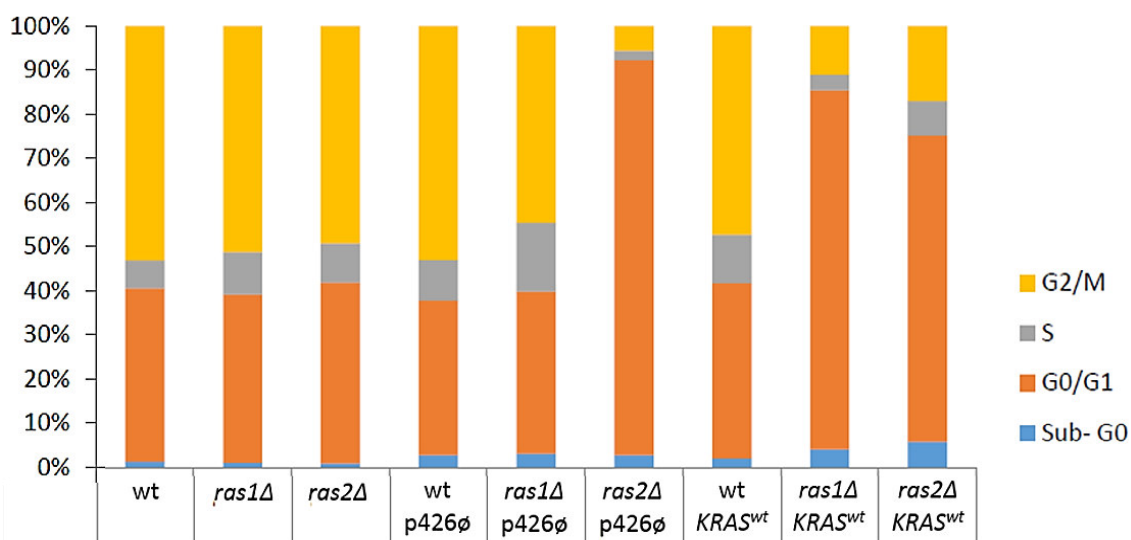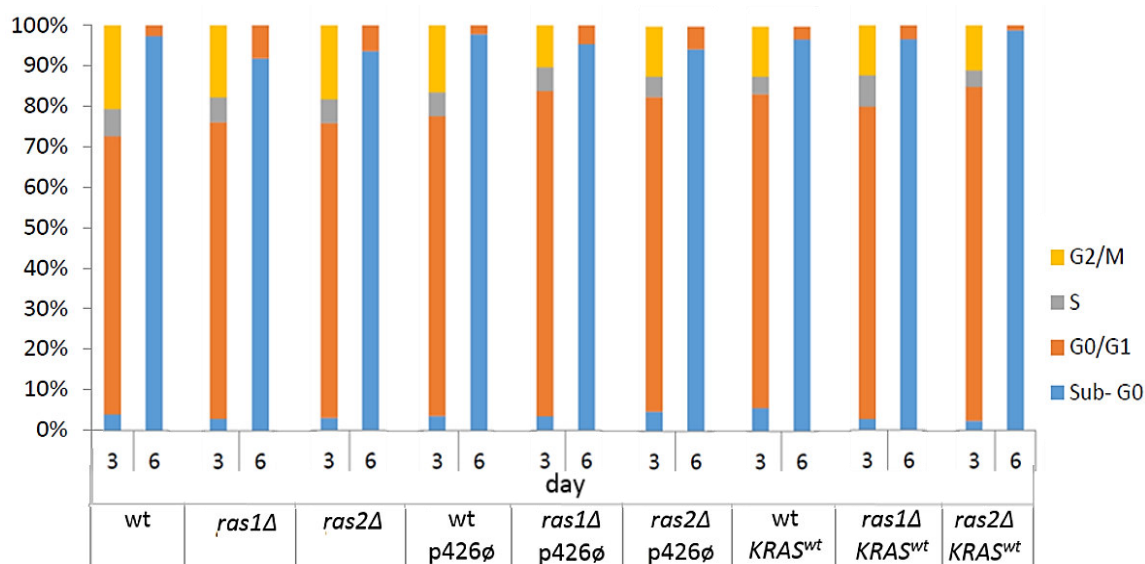**Figure 24.** Cell cycle analysis by flow cytometry of the BY4741 *S. cerevisiae* set of strains expressing human *KRAS*wt. Strains were grown on YNB w/ 2% glucose up to late-stationary phase. Results correspond to one single (preliminary) experience.

Corroborating the absence of effect that the above results suggests identical assays after 3 days of incubation - stationary phase - yielded identical results (Fig. 24). As expected, all strains had a higher percentage of cells in the G0/G1 phase, indicating a proper arrest in G0/G1, which is characteristic of non-dividing and chronological aging cells. Further, along the stationary phase, after 6 days, the strains were presenting almost all cells in sub-G0 indicating large DNA fragmentation as expected according to the loss of viability of the culture above described. Therefore, these results show a strong correlation with the faster loss of cell viability verified during yeast chronological aging (Fig. 22).

3.2.5 Comments on *KRAS^wt*-associated phenotyping

In the previous studies, the phenotypic complementation of the *RAS* double mutant, which is usually unviable, was achieved with the human *HRAS* isoform, but not with *KRAS* isoform (in the 112-699 and JR25-3C strains background) [123, 148]. Moreover the complementation of the single *RAS2* deletion, using the *KRAS^wt* isoform was also not achieved (in the W303-1B background) [149, 150]. This may be due to the fact that the two human isoforms are functionally different [70]. In addition, the correct functional expression of a foreign gene in yeast, in particular a human cDNA, requires that a complex multi-step process from gene to mature protein [151] occur in the correct manner through conserved mechanisms. RAS signalling is a rather conserved pathway bearing much in common in several organisms including yeast and -human [108]. Therefore, chances are good that *KRAS^wt* might work appropriately in yeast. The phenotypes detected in this work suggest that the RAS pathway might be hyperactivated through the heterologous expression of *KRAS^wt* in a wt yeast. Otherwise, the complementation of the *RAS* deletions appears less evident. Finally, it is important to advertise that in the literature, to the best of our knowledge, there are no works using the EUROSCARF background to the phenotype screening of RAS genes. The putative impact of the amino acid metabolic pathways on RAS-associated phenotypes is not to be discarded since feedback regulation is known to exist between nitrogen sensing, amino acid pathways, TOR and RAS/cAMP/PKA pathways [152, 153].

Chapter IV

Final Remarks

4. **Final Remarks**

The aim of this thesis was the construction of *KRAS^wt* expressing strains of *S. cerevisiae*, and the subsequent phenotype screening, as part of the development of a yeast-based phenotypic platform for future pharmacological testing. The heterologous expression of *KRAS^wt* cDNA was successfully obtained in BY4741 wt and the derived *ras1Δ* and *ras2Δ* mutants. The presence of the gene was confirmed by PCR of the DNA extracted from transformants, and the production of protein was proven by Western Blot assay with anti-KRas antibody. These and the correspondent recipient strains alone, or harbouring the empty plasmid, constituted a set of strains that were used for phenotyping. The cloning procedures were repeated using p426GPD and p416GPD but were only successful with the former. The collection of strains obtained is:

- BY4741 wt
- BY4741 wt p426
- BY4741 wt p426 *KRAS^wt*

- BY4741 *ras1Δ*
- BY4741 *ras1Δ* p426
- BY4741 *ras1Δ* p426 *KRAS^wt*

- BY4741 *ras2Δ*
- BY4741 *ras2Δ* p426
- BY4741 *ras2Δ* p426 *KRAS^wt*

The second objective of this work was to use this collection of strains to study the effect of *KRAS^wt* expression, including the verification of the ability of *KRAS^wt* to complement the deletion of yeast homologue genes. The literature predicts that the *RAS2* deletion leads to defective growth on non-fermentable carbon sources, and impaired invasion capacity, as well as an increase of stress resistance and chronological lifespan [27, 80]. The cellular processes assessed took into account the literature: growth rate, chronological aging, cell cycle, haploid invasive growth, growth on non-fermentable carbon sources, as well as the cell resistance to different stresses (high temperature, osmotic stress, low and high pH and oxidative stress). Summarizing results:

- *KRAS^wt* expression did not affect the resistance that RAS mutants have to high-temperature (37 ºC), osmotic stress, low and high pH, and oxidative stress caused by 1 mM H_2O_2. *KRAS^wt* expression otherwise increased the sensitivity to other H_2O_2 concentration (0.5 mM H_2O_2), and decreased the stress resistance of the wt strain to different stresses except to the pH.
- All strains adhered to the YNB solid medium with and without glucose, and adherence was enhanced by *KRAS^wt* expression, while only *ras2Δ KRAS^wt*

strain was able to invade the medium (YNB with glucose). None of the strains presented filamentous growth.

- Extensive bipolar budding was observed in *S. cerevisiae* wt *KRAS^wt* cells growing on YNB liquid medium without glucose. This might derive from a *a/α* switch allowing mating and consequent diploidy.

- *KRAS^wt* decreased chronological life span, namely in *ras2Δ* strain.

- All strains presented decreased growth in ethanol and glycerol, intensified with the expression of *KRAS^wt*, more so in the wt strain.

In the BY4741 yeast genetic background used in this work, the deletion of *RAS1* or *RAS2* had a phenotypic impact much less significant than described in the literature. This was surprising but understandable considering that BY4741 and derived strains have not been used before to study RAS-associated biological processes. This emphasises a recurrent problem, so often observed in the literature, of results' generalization between different in yeast species, which is also important between different yeast stains, in particular when these have cumulative numbers of mutations affecting metabolism like auxotrophic marks [154]. Accordingly, the functional complementation of either *ras1Δ* or *ras2Δ* by *KRAS^wt* could not be demonstrated in any of the phenotypes tested in solid medium assays since the recipient mutant and wt strains often behaved identically. This raises considerable questions as to (1) the true roles attributed to, and molecular regulation of RAS pathway in yeast, or (2) the masking/interfering effect of amino acid metabolic pathways on RAS-derived yeast phenotypes.

Results further showed that the heterologous expression of *KRAS^wt* on yeast RAS mutants (1) decreased growth on non-fermentable carbon sources, (2) had a negative or null effect on the resistance to stress, (3) increased the adhesion capacity, (4) stimulated the haploid invasive growth in the *ras2Δ* strain, and (5) decreased the chronological lifespan. Importantly, the expression of *KRAS^wt* on the wt strain therefore harbouring native *RAS1* and *RAS2* caused phenotypic changes that could be explained by the hyperactivation of the RAS pathway, leading to increased cell proliferation and decreased longevity and stress resistance. This result is compatible with the notion that the hyperativation of the RAS pathway is typical of cancer cells, which present uncontrolled growth [91, 110]. Finally, this wt *KRAS^wt* strain also showed a bipolar budding pattern that might derive from abnormal diploidy.

Altogether, the results from the phenotyping assays using the set of strains generated within the present thesis open novel routes for investigating the roles of Ras

proteins and pathway in yeast. Importantly, on the process of developing a yeast-based high throughput platform for pharmacological testing, the hereby-described results will be confront with other strains' phenotyping (a) expressing *KRAS^wt* from a chromosomal instead of plasmidic construction, and (b) having a different genetic background.

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5. References

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