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ABSTRACT

Fundamental cellular processes appear to be highly conserved between *Saccharomyces cerevisiae* and other more complex Eukaryotic species, including humans. “Humanized yeast systems” emerged as a tool to study molecular aspects of human pathologies. The present work aimed at contributing to build and validate a large high throughput platform of yeast strains displaying phenotypes that can enable further testing galectin-related drugs and peptides. This platform was designed to consist of two types of strains, the ones expressing human galectins and the ones expressing these together with the human *KRAS* cDNA. The rationale behind this relates with the putative dialogue between Galectins and RAS signaling pathway in mammals. Considering that EGFR mediates KRAS signaling and that yeast also harbors a RAS signaling pathway, the “humanized yeasts” expressing KRAS were used to identify the yeast target of anti-EGFR. Furthermore, it was also used for phenotyping the most well-known biological processes known to be controlled by RAS pathway. On the other hand, considering that the deletion of *GUP1* in *S. cerevisiae* increases the resistance to the oncological drug Imatinib, the similarities between the phenotypes associated to the deletion of RAS and GUP genes were also verified.

Two Hsp70, Ssa2p and Ssb2p and one glyceraldehyde-3-phosphate dehydrogenase Tdh3p, were identified as EGFR-like proteins. The subsequent alignments analysis between EGFR and these proteins revealed that Ssb2p and its very close homologue Ssa2p present some homology with EGFR sequence, namely at the level of three EGFR conserved amino acids known to be responsible for the interaction with the anti-EGFR antibody Cetuximab used in cancer treatment. This and other lines of evidence support Ssb2p and/or Ssa2p as good candidates for EGFR homology. The phenotypic tests revealed that both the deletions of GUP and RAS genes promote a reduction in chronological life span and cell size, except in the case of Δ*ras2* strain, whose cells were bigger than wild type control. Nutrient depletion (carbon) promoted replication stress in Δ*ras2* cells that failed to enter into G1 arrest, and were blocked in S phase, concurring with the bigger size of Δ*ras2* cells and their short lifespan. Moreover, the cells with GUP genes deleted, in opposition to RAS mutants, showed ability to adhere to solid nitrogen-deficient medium. Neither RAS nor GUP mutants were able to invade or filament under these conditions.

With this work we were able to determine the possible homologue of EGFR, many times associated with cancer pathologies, and contributed to gain insights on RAS and GUP genes common phenotypes. In conclusion, the present work opens doors to future discovery of new pathways in yeast, in addition to showing that *S. cerevisiae* is a suitable model to create a platform to explore therapeutic drugs/antibodies.

RESUMO

Vários processos celulares fundamentais encontram-se conservados entre a levedura *Saccharomyces cerevisiae* e outras espécies eucariotas mais complexas, incluindo humanos. A "Levedura humanizada" surgiu como uma ferramenta de estudo sobre aspectos moleculares de patologias humanas. Com este trabalho pretendeu-se contribuir para a construção e validação de uma plataforma de estirpes de levedura que exibam determinados fenótipos, permitindo o teste de drogas e péptidos relacionados com as galectinas. Esta foi planeada para incluir duas estirpes a expressar galectinas humanas, assim como o cDNA do *KRAS* humano. O propósito desta plataforma advém de uma possível interação entre as Galectinas e a via de sinalização dos RAS em mamíferos. Tendo em conta que o EGFR medeia a cascata de sinalização KRAS, e que também a levedura possui uma via de sinalização Ras, usou-se as leveduras humanizadas a expressar o *KRAS* para identificar o alvo do anti-EGFR. Para além disso, estas foram usadas para a fenotipagem de processos biológicos controlados pela cascata RAS. Por outro lado, tendo em conta que a deleção do *GUP1* aumenta a resistência à droga oncológica Imatinib, verificou-se também as semelhanças fenotípicas entre as deleções RAS e GUP.

Foram identificadas duas proteínas Hsp70, Ssa2p e Ssb2p, e uma gliceraldeído-3-fosfato desidrogenase Tdh3p, como sendo os alvos do anti-EGFR. Subsequentemente, a análise dos alinhamentos entre o EGFR e estas proteínas revelaram que a Ssb2p e a sua homóloga Ssa2p apresentam similaridade com a sequência do EGFR, nomeadamente ao nível de três aminoácidos responsáveis pela interação com o anticorpo anti-EGFR, Cetuximab, usado no tratamento do cancro. Esta informação suporta a hipótese das proteínas Ssb2p e/ou Ssa2p serem boas candidatas a homólogas do EGFR. Os testes fenotípicos revelaram que as deleções dos genes GUP e RAS promovem uma redução da longevidade cronológica e da área celular, com excepção para a estirpe Δ*ras2* cujas células se revelaram maiores do que a wt. A depleção de nutrientes (carbono) induziu stress replicativo nas células Δ*ras2*, que por sua vez falharam a entrada na fase G1, ficando bloqueadas na fase S, o que está de acordo com o aumento da área celular e a baixa longevidade cronológica das células Δ*ras2*. Além disso, as células com a deleção nos genes GUP, contrariamente aos mutantes RAS, mostraram habilidade para aderir a um meio deficiente em nitrogénio. Nenhum dos mutantes RAS ou GUP foram capazes de invadir ou filamentar nas condições anteriormente descritas.

Com este trabalho fomos capazes de determinar o possível homólogo do EGFR, muitas vezes associado a patologias relacionadas com o cancro, assim como contribuir para melhor compreender os fenótipos comuns associados aos genes RAS e GUP. Em conclusão, o presente trabalho abre portas para futuras descobertas de novas vias de sinalização em levedura, além de reforçar a utilização da *S. cerevisiae* como um bom modelo para criar uma plataforma de exploração de drogas/anticorpos.

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LIST OF ABBREVIATIONS

AC	Adenylate cyclase
Adh	Alcohol dehydrogenase
AIF	Apoptosis-inducing factor
APAF	Apoptotic protease activating factor
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BLAST	Basic local alignment search tool
BRAF	v-RAF murine sarcoma viral oncogene homologue B1
cAMP	Adenosine 3', 5'-cyclic monophosphate
CDK	Cyclin dependent kinase
CFU	Colony Forming Units
CFW	Calcofluor white
CLS	Chronological life span
CRC	Colorectal cancer
CREB	cAMP response element binding protein
Ctx	Cetuximab
DMSO	Dimethyl sulfoxid
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR variant III
ER	Endoplasmatic reticulum
ERK	Extracellular regulated kinase
Fab	Fragment antigen binding
Fc	Fragment constant
FDA	Food and drug administration
FGF	Fibroblast growth factor
Fv	Fragment variable
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor

GFP	Green fluorescence protein
GPI	Glycosyl phosphatidylisositol
GRB2	Growth factor receptor bound protein 2
GTP	Guanosine triphosphate
HER	Human epidermal growth factor receptor
HHATL	Hedgehog acyltransferase-like protein
HOG	High osmolarity glycerol
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IGF	Insulin growth factor
JAK	Janus kinase
KRAS	v-Ki-ras2 Kristen rat sarcoma viral oncogene homologue
LB	Luria-Bertani medium
LGT	Lateral gene transfer
mAb	Monoclonal antibody
MALDI	Matrix assisted laser desorption ionization
MAPK	Mitogen-activated protein kinase
MBOAT	Membrane bound O-acetyltransferase
MCS	Multi cloning site
miRNA	micro RNA
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MWM	Molecular weight marker
$Na_2HPO_4 \bullet 7H_2O$	Sodium monohydrogen phosphate heptahydrate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
$NaH_2PO_4 \bullet H_2O$	Sodium dihydrogen phosphate hydrate
Native-PAGE	Native polyacrylamide gel electrophoresis
NBD	Nucleotide binding domain
NCBI	National center for biotechnology information
NF-kB	Nuclear factor kappa B
NGF	Nerve growth factor
NK	Natural killer
OD	Optical density
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline Tween 20
PCR	Polymerase chain reaction

PDC	Pyruvate decarboxylase
PDGF	Platelet derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology
pH	Potential of hydrogen
PI3K	Phosphotidylinositol 3-kinase
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phosphoinositide phospholipase C
PVDF	Poly vinylidene difluoride
RLS	Replicative life span
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SBD	Substrate binding domain
SC	Synthetic complet
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sEGFR	soluble EGFR
SGD	*Saccharomyces* genome database
SH1	Src homology domain 1
SH2	Src homology domain 2
SHH	Sonic hedgehog
SLAD	Synthetic low ammonium sulfate and dextrose
STAT	Signal transducer and activator of transcription
TCA	Trichloroacetic acid
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitors
TOR	Target of rapamycin
tRNA	transfer RNA
VEGF	Vascular endothelial growth factor
WB	Western Blot
YNB	Yeast nitrogen base
YPD	Yeast extract, peptone, dextrose

CHAPTER I

Introduction

1. Introduction

1.1 Yeast as Eukaryotic Model

To date, the yeast model has allowed identifying new targets and novel therapeutic opportunities. Several features make *Saccharomyces cerevisiae* an ideal model system for the study of human diseases, one of which is the simplicity of yeast genome that comprises only 6,000 genes (1) compared to about 25,000 for the human genome (2). Studies of yeast have been essential for understanding fundamental cellular processes such as metabolism (3), DNA replication and recombination (4), the regulation of cell cycle (5), cell death (6) and for elucidating many mechanisms of several diseases (7). Yeast also presents many practical advantages over human cells. It is well suited to high throughput methods because its life cycle is quick (±90 minutes), it can grow in liquid or on solid media forming suspended cells or colonies respectively, and its culture requires neither elaborate sterile technique nor expensive media (8). Yeast reproduces and dies old or by apoptosis provoked by several stimulus. It lives as individual cells or in colonies, the cells can differentiate into pseudo or true hyphae in response to environmental events, this differentiation is related with the capacity to invade and adhere (9). Moreover, it is a genetically modifiable organism, amenable to changes such as gene deletion, gene marking, mutation or gene dosage effects (7, 10). It has been possible to vary the level of expression of essentially every individual yeast gene and to assemble collections of mutant strains with genome-wide coverage (8).

The use of yeast as a model organism was extended to the analysis of the molecular mechanisms of human diseases, sometimes of rather unexpected nature (11, 12). This was achieved by directly studying an endogenous protein orthologue of a human involved in the disease (11, 13) or through the heterologous expression of human disease associated proteins (14, 15). Although several aspects of the disease in high Eukaryotes are beyond the extend of a unicellular organism like yeast, many processes and pathways are greatly conserved in this organism, namely, cell cycle checkpoint controls, mitochondria biogenesis, protein quality control, vesicular trafficking, apoptosis and autophagic pathways (7, 14, 16). When establishing related protein models, different approaches are used according to the degree of conservation of the protein under study. If the gene codifying for the protein is conserved in yeast, it is possible to directly study its function. If the gene has no orthologue in yeast, the

heterologous expression of the human gene in this organism (then designated humanized yeast) can be highly advantageous because yeast conserve protein interactions that give information to its function (14). An example of this strategy is the expression of the tumor suppressor p53 in yeast (17), or of tyrosine kinase receptors, well known to interfere in numerous types of cancer (15). Furthermore the expression of the human gene in yeast often leads to disease relevant phenotypes because yeasts and mammalian cells can respond alike to the appearance of such mutant genes (18).

The yeast genome was the first eukaryotic genome to be sequenced (1) and it has allowed pioneer genome scale screening methods, including microarrays (19), two-hybrid analysis (20) and the use of deletion and overexpression libraries (21). Moreover, advances in yeast technology have stimulated the use of this model organism for the creation of high throughput screening platforms for new biologically active compounds, namely through haplo-insufficiency and synthetic lethality screening, or fitness profiling (10). Generally speaking, yeast is considered an excellent model for understanding cellular and molecular processes underlying many diseases. Yeasts harbor well conserved pathways, like TOR, PKC, Calcineurin, stress responsive, secretory, protein sorting pathways and the RAS/cAMP/PKA (22). The MAPK cascades have as principal function regulate transcription factors by MAPK-mediated phosphorylation. Presently, the budding yeast *S. cerevisiae* has five recognized MAPK pathways, the mating-pheromone response (23), the filamentation-invasion pathway (24), the high osmolarity glycerol (HOG) stress response (25), and the cell integrity pathway (26) (Fig. 1). All of them are operate in vegetative cells during sporulation and regulates the correspondent developmental process (27).

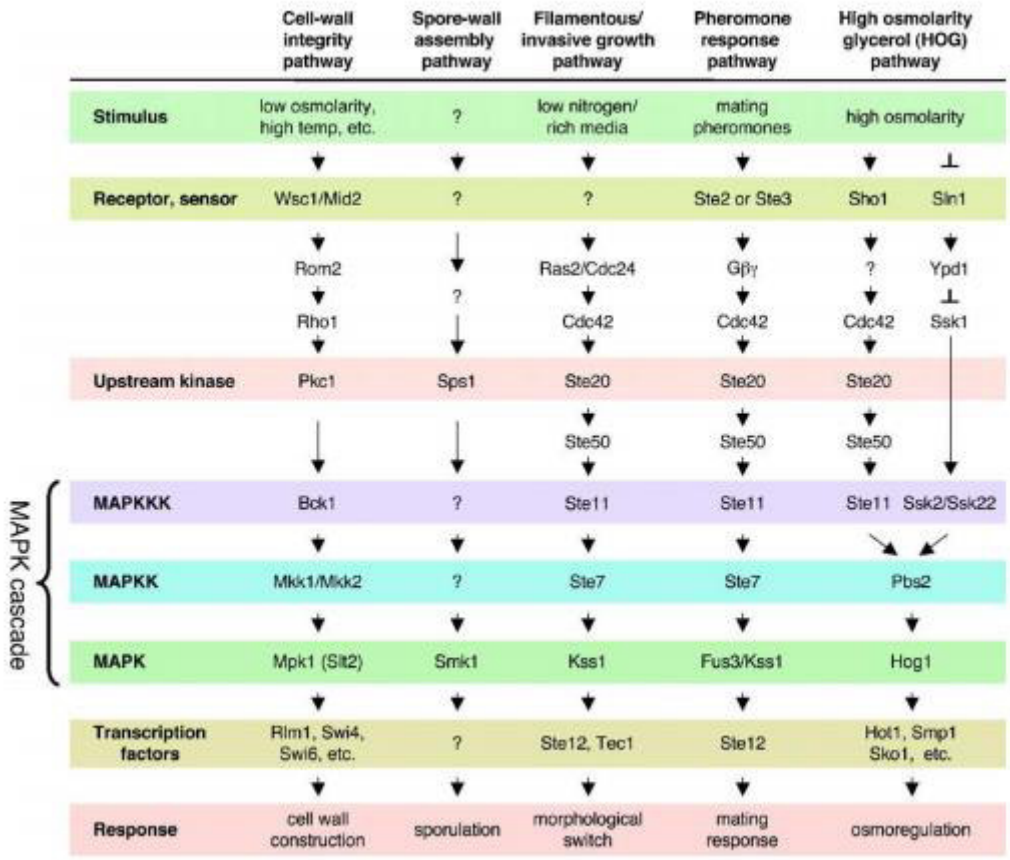

Figure 1. MAPK pathways in yeast *S. cerevisiae*. Withdrawn from (28).

The majority of the cancer-causing mutations were discovered in non-human species, such as yeast, before their role in human cancer was realized. Many of the genes that are frequently altered in tumors have structural or functional orthologues in model genetic systems, including the yeast *S. cerevisiae* (29). Actually, yeast presents a considerable degree of homology to the human proteome (30). For example, one homology particularly relevant for this work is the one between the oncogenes of the RAS family in human and the two RAS genes *RAS1* and *RAS2* in yeast (31). Hartwell won 2001 Nobel Prize in Physiology or Medicine for identified in yeast more than 100 genes involved in cell cycle control checkpoints, generally known as the cdc genes (from cell division cycle). The same genes that control the cell cycle in baker's yeast, identically control cell cycle progression in human cells and malfunction in tumor cells (7, 32).

1.2 Yeast as a Cell Aging Model

In the last years, the yeast *S. cerevisiae* has been used as a model to study a range of factors affecting cellular aging, as well as genes involved in pathways controlling life span (33). In view of the specificities of life cycle of yeasts, two types of ageing processes have been identified and can be studied separately: replicative life span (RLS) and chronological life span (CLS). The number of times that a single mother cell, before senescence, originates daughter cells was defined as RLS. On the other hand, the length of time that yeast in non-dividing phase remains viable defined the CLS (34). Several studies using yeast as a model reveled a relation between the longevity and availability of nutrients, thus is now know that the calorie restriction increase the RLS as well as the CLS (34, 35). In others eukaryotic model organisms (worms, flies, zebra fish) the reduction of growth hormones factors promotes longevity as improves overall health by decreasing the probability of developing diseases of diverse types, like cancer, heart attack and diabetes, all related with aging (36).

To date, three signaling pathways have been described as regulators of life span: RAS/cAMP/PKA, TOR and Sch9 (37-39) (Fig. 2). In yeast, these pathways are regulated by the availability of nutrients, being activated in presence of glucose and others nutrients inducing cells to proliferate. In opposition, in conditions of nutrient exhaustion, the reduction of signaling of these pathways arrests cell cycle and cells enter a quiescent state (40). Accordingly, the deletions of the genes *RAS2*, *SCH9* and *TOR1*, as well as the inactivation of other proteins of TOR pathway, increase the yeast CLS and promotes stress resistance (41). The association between these two types of phenotype suggests that increasing cellular protection against damage, and concomitantly increasing the cell repair, can be a strategy to retard aging (40). On the other hand, Tor1p forms a complex with other proteins known as TORC1, which phosphorylates and consequently promotes the activation of the serine threonine kinase Sch9p (42). The deletion of *SCH9* triples life span and increases resistance to oxidative and temperature stress (43). The role of Tor1p on longevity could therefore be due to activation of Sch9p (39). Additionally, also mutations decreasing the activity of the RAS/cAMP/PKA pathway extend longevity and increase oxidative stress resistance. This occurs because general stress responsive transcription factors Msn2/Msn4 are activated and induce the transcription of SOD2 the mitochondrial antioxidant enzyme

superoxide dismutase (43) and catalase levels, consequently promoting the decrease in oxidative stress and cellular damage (44). Accordingly, growth signaling promotes chronological aging by inducing superoxide anions that inhibit quiescence (45).

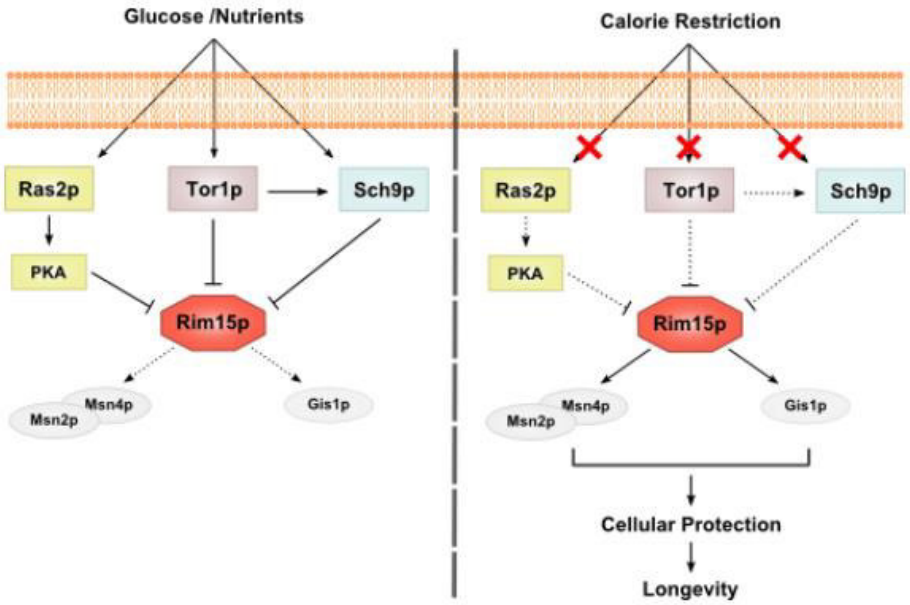

Figure 2. RAS/PKA, TOR and Sch9 pathways regulators of life span in *S. cerevisiae*. Glucose and others nutrients activate the three pathways which promote the repression of Rim15p and consequently the down regulation of dependent stress resistance system Msn2p/Msn4p and Gis1p. In condition of nutrients restriction the down regulation of RAS/PKA, TOR and Sch9 pathways promotes de activation of Rim15p as well as the protection system Msn2p/Msn4p and Gisp. Withdrawn from (46).

The extension of CLS promoted by the deletions of *RAS2*, *TOR1* and *SCH9* or by nutrient restriction is dependent on the activity of a serine/threonine kinase, Rim15. Its deletion causes the reversion of the CLS extension phenotype observed on any of the three mentioned mutants. This suggests that the aging pathways controlled by Sch9p, Tor1p, and Ras2p converge on the protein Rim15p (41), which major role is the activation of the above mentioned stress resistance transcription factors Msn2p, Msn4p and Gis1p (44).

1.3 RAS/cAMP/PKA Pathway

The RAS/cAMP/PKA pathway regulates of other processes besides chronological life span. These include cell cycle (47), the polarity of actin cytoskeleton (48), spore morphogenesis (49), the activity of the general amino acid permease Gap1p (50) and DNA damage checkpoint (51). The genome of *S. cerevisiae* has two RAS genes, *RAS1* and *RAS2*, this the latter more expressed than the former (31). Ras1p and Ras2p are small GTPases with respectively 309 and 322 amino acid residues, which N-terminal portions have high homology to the mammalian Ras proteins, namely KRAS (see section 1.7 of Chapter 1). This region contains G1 to G5 boxes, short stretches of amino acids that are involved in the recognition of guanine nucleotide and phosphate (52). Conversely, it is in the C-terminal that yeast Ras proteins diverge from mammalian Ras. The sequence close to the C-terminus including the 4 terminal amino acids that constitute the CAAX motif (C is cysteine, A is aliphatic amino acid, and X is the C-terminal amino acid) is important for post-translational modifications that facilitate their association with the membrane (53).

The RAS genes are essential for growth, so Δ*ras1*Δ*ras2* mutants are nonviable (54, 55). *RAS1* is repressed when cells are grown on non-fermentable carbon sources like as glycerol and pyruvate. Therefore the Δ*ras2* mutants should not grow on a non-fermentable carbon source, because in those conditions the strain is defective for both Ras1p and Ras2p. Cells with a temperature sensitive *RAS2* mutation or Δ*ras1* deletion are blocked in the G1 phase of the cell cycle and accumulate as unbudded cells at nonpermissive temperatures (54). Mutations in *RAS2* promote accumulation of carbohydrates and increase the sporulation. On the other hand, yeast cells expressing an activating mutant of Ras2p, $Ras2^{val19}$ exhibit decreased sporulation ability as detected by a reduced glycogen storage level, and are sensitivity to heat shock and nutrient starvation. Also, it is known that the amount of cAMP inside the cell is decreased in the Δ*ras* mutants, and increased in the activated mutant expressing $Ras2^{val19}$ (54). Ras1p and Ras2p activate the adenylate cyclase Cyr1p (55) which is associated with a protein called CAP (cyclase-associated protein) promoting the production of cAMP. cAMP binds with the Bcy1 protein that induces its dissociation from the PKA catalytic subunits (encoded by *TPK1*, *TPK2* and *TPK3*) and consequently activate PKA (Fig. 3). Subsequently, the phosphorylation of several substrates leads to the control of a large

variety of functions including cell cycle progression (47). The synthesis of cAMP is also regulated by the Gα protein called Gpa2p that is activated by glucose (56). The activation of PKA pathway enhances activities related with proliferation. The inactivation of cAMP is regulated by Pde1 and Pde2 phosphodiesterases, these enzymes act as antagonists in yeast signaling as well as represents the main control of feedback in PKA pathway. This regulation decreases rapidly the pathway activity. Accordingly, yeast strains harboring mutations in which this type of feedback is inactive, may accumulate high quantities of cAMP (57).

Yeast Ras1p and Ras2p are inhibited by two Ira proteins (Ira1p and Ira2p) (58). They have two very similar genes, *IRA1* and *IRA2*. A region of approximately 360 amino acids called GAP domain is responsible for the intrinsic activation of GTPase activity from Ras (59). Ira1p and Ira2p have similar functions, consequently mutations in *IRA1* and *IRA2* result in similar phenotypes, and the double mutant has more pronounced phenotypes (60). Apparently, Ira proteins are regulated by Kelch proteins Gpb1p and Gpb2p that bind to a C-terminal domain of Ira1p or Ira2p (61). Gpb is a Gβ mimic that does a protein complex with Gpa2p (62). Gpb1p also has been identified as a binding partner of Ira2p that regulates negatively Ira2p by promoting its ubiquitin-dependent proteolysis (63). On the other hand, Gpb2p regulates positively Ira2p (64). Other important gene in RAS signaling is the *CDC25* that encodes an activator of Ras1p and Ras2p, which acts as a GEF (Guanine nucleotide Exchange Factor) that facilitate the exchange of bound GDP with GTP (65, 66). *CDC25* is also reported as a gene that is essential for cAMP production. The Sdc25p was been reported to also contain a GEF domain (67). Ras proteins are synthesized in the cytoplasm with a process very similar to the mammalian RAS. The removal of methionine at the N-terminus is the first step in the synthesis, which probably occurs co-translationally. The C-terminal modifications is the next step that include farnesylation, deletion of C-terminal 3 amino acids, carboxyl methylation and, finally, addition of palmitic acid (60).

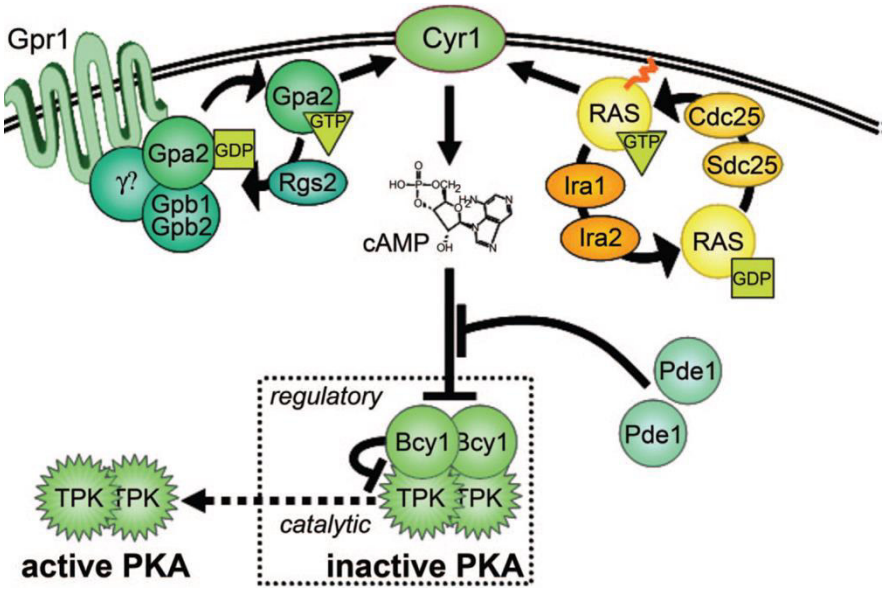

Figure 3. RAS signaling pathway in *S. cerevisiae*. Ras and Gpa2p (GTP bound G proteins) bind to adenylate cyclase (Cyr1p) and promote its production of cAMP. Cdc25p and Sdc25p (Ras GEFs) and Ira1p and Ira2p (Ras GAPs) are represented in Ras-Cyr1 complex because they regulate adnylate cyclase by controlling the Ras switch. Gpr1p, a member of the G protein coupled receptor, acts upstream of Gpa2p. Gpa2p was very similar with the mammalian G subunits of heterotrimeric G proteins. Phosphodiesterases (Pde1p and Pde2p) antagonize the signaling via enzymatic inactivation of cAMP. The PKA tetramer is the regulatory target of cAMP. The regulatory Bcy1p subunits keep PKA in an inactive state. cAMP activates the catalytic subunits by binding to Bcy1p subunits and promoting dissociation of the complex. Withdrawn from (68).

1.4 TOR Pathway

In addition to the RAS/cAMP/PKA signaling pathway, the other major nutrient responsive, growth controlling pathway in yeast is the TOR (Fig. 4). Tor (Target of rapamycin) serine/threonine kinases belong to the phosphatidylinositol-3 kinase (PI3K) family, and exert their functions in two distinct multiprotein complexes: TOR Complex 1 (TORC1), which controls many aspects of yeast growth and cell proliferation, and TORC2, which regulates cell polarity and actin cytoskeleton organization (69, 70). The main function of TORC1 is to respond to nutritional status, where its major function appears to be the regulation of translation capacity in response to environmental signals by promoting ribosome biogenesis, amino acid availability, and translation efficiency. Inhibition of TORC1 by rapamycin mimics nutrient starvation and causes G1 arrest, inhibition of protein synthesis, glycogen accumulation, induction of autophagy and entry into quiescence (69, 70). TOR also controls other aspects of ribosome biogenesis,

such as the Pol I- and Pol III-dependent transcription of the rDNA and tRNA genes via phosphorylation of dedicated transcription factors (71). Tor1p itself may activate rDNA transcription in rich nutrient conditions by entering the nucleus and binding directly to promoters (72), however, in other studies, Tor1p has been localized to internal membrane structures but not the nucleus (73, 74). TORC1 is also intimately implicated in vesicular trafficking (75). On the other hand, TORC2 signaling is rapamycin insensitive and it is required for the organization of the actin cytoskeleton. Upstream regulators of TORC2 are not known yet (76).

Rapamycin and nitrogen starvation treatment shows very similar responses in *S. cerevisiae*, suggesting that TORC1 is regulated by the availability of nitrogen source (77). The control of nitrogen metabolism involves the regulation of PP2A and the PP2A-like phosphatase, Sit4p. Yeast cells can adapt the metabolism to the nitrogen sources through the nitrogen catabolite repression pathway (NCR) also known as the nitrogen discrimination pathway (NDP) (78). Two activators, Gln3 and Gat1, and two repressors Dal80 and Gzf3 are the transcription factors that are involved in the regulation of selective use of the nitrogen via NCR (79). Under rich nitrogen sources, Gln3 is phosphorylated and sequestered in the cytoplasm. On the other hand, rapamycin treatment or poor nitrogen sources rapidly triggers the dephosphorylation of Gln3 in a Tap42-phosphatase-dependent manner. The Gln3 enters into the nucleus activating NCR genes (70, 80).

Many functional interactions between TOR and the RAS/cAMP/PKA pathway have been showed (69). It was demonstrated that the activation of PKA signaling pathway confers resistance to rapamycin. So, the activation of the PKA pathway prevents several rapamycin-induced responses. It is also known that TOR controls the subcellular localization of both PKA catalytic subunit Tpk1p and the Ras/cAMP signaling-related kinase Yak1p. However, the detailed relationship between the TOR and RAS/PKA networks is still not understood. Several possibilities have been suggested. On one hand, it was proposed that the TOR and PKA signaling cascades independently coordinated the expression of several genes. On the other hand, it has been proposed that TOR may work upstream of Rasp to regulate PKA activity, thus the RAS/PKA pathway can be a novel TOR effector branch (69, 70, 81).

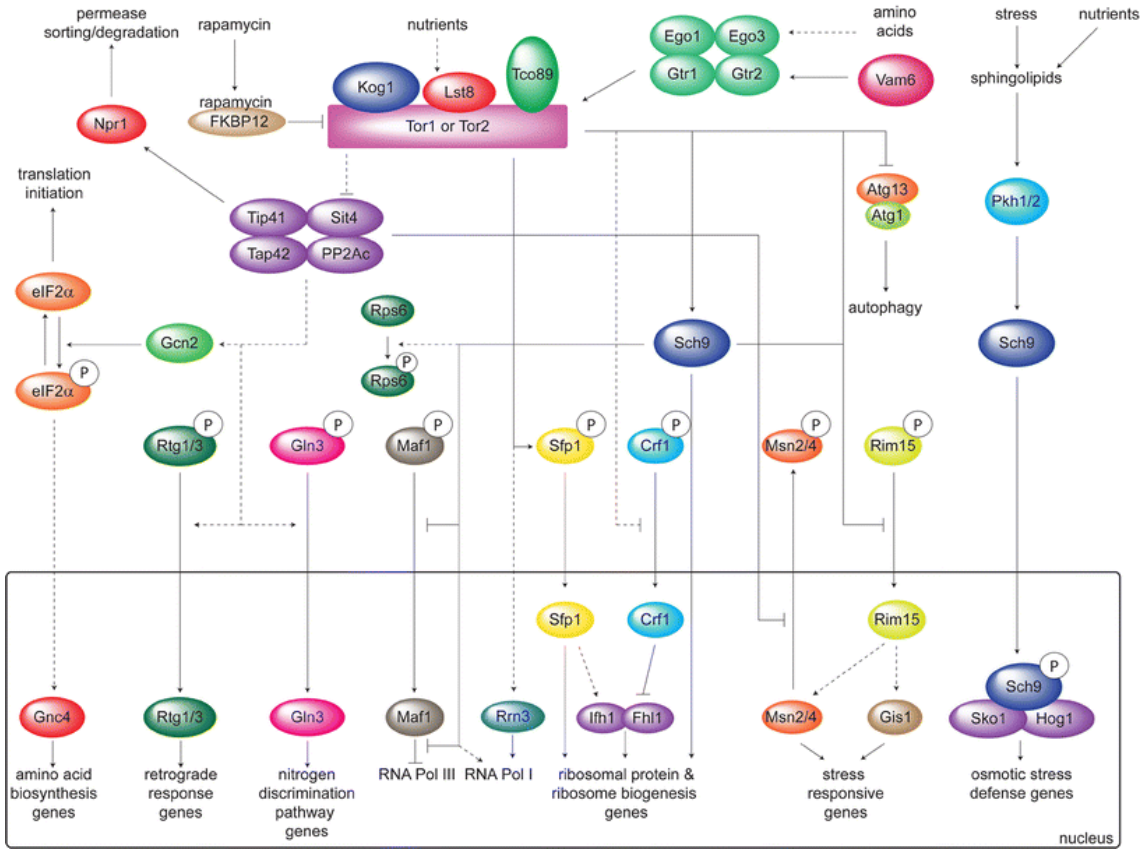

Figure 4. The TOR pathway in *S. cerevisiae*. The activation of TORC1 by nutrients results in the stimulation of protein synthesis and the inhibition of stress response genes, autophagy and several pathways that allow growth on poor nitrogen sources. These processes are regulated by the rapamycin sensitive TORC1 complex via the Tap42-Sit4/PPA2c or the Sch9 branch. Withdrawn from (70).

1.5 Sch9 Pathway

Like PKA and TOR, the less well-known Sch9 pathway plays a role in nutrient-mediated signaling in yeast (70). In parallel with the PKA pathway, Sch9p is phosphorylated by TORC1, regulating many of the TORC1 processes. However, Sch9p also acts independently of TORC1, promoting adaptation to stress (70). The main functions of Sch9p are regulation of cell size, activation of ribosomal biogenesis (82), action as a negative regulator of both CLS and RLS and regulation of mitochondrial respiration (37, 83). It was demonstrated that the deletion of *SCH9* up-regulates electron transport chain which is associated with an increase in mitochondrial respiration (84). Additionally, it has also been shown that yeast Sch9 is an important component of a network that controls genes involved in a metabolic switch from the TCA cycle and respiration to glycolysis and glycerol biosynthesis. During chronological aging, the

Δsch9 exhausts extracellular ethanol and reduces stored lipids, but synthesizes and releases glycerol, suggesting in this way that glycerol production enhances life span (85).

1.6 Gup protein in *Saccharomyces cerevisiae*

Gup1p and its close homologue Gup2p are members of the membrane-bound O-acetyltransferase (MBOAT) superfamily (86-88). Gup1p was firstly described in *Saccharomyces cerevisiae* as involved in glycerol metabolism and transport and accordingly included in the major facilitator superfamily (88). Nevertheless, Gup1p is now well known for other aspects of cell physiology that do not relate directly to glycerol active transport, which protein was identified as the Stl1p member of the HXT family of hexose transporters (89). The actual influence of Gup1p on Stl1p activity was found to be indirect through the influence of Pma1 H^+ATPase miss localization and consequent defective active transport-driving proton motive force (90). Gup1p is localized in the plasma membrane, more precisely oriented across the membrane plasmatic where the N-terminus is located in periplasmic space, and the C-terminus located intracellularly (88, 91). However, it also co-localizes with cytochrome *c* oxidase from mitochondria and with NADPH-cytochrome *c* redutase from the endoplasmatic reticulum (88). These several sub-cellular localizations suggest complex regulation and roles. Gup1p was associated with the integrity and biogenesis of cell wall and plasma membrane (90, 92), and relatedly, the deletion of *GUP1* impaired growth under anaerobic conditions and sterol uptake (93). Additionally, this deletion also induced phenotypes on cytoskeleton polarization (94) and bud site selection (95), secretory and endocytic pathway (96), as well as telomere length (97). At the level of cellular morphology, Δ*gup1* presents aberrant vacuole morphology (96), while in *C. albicans* it induces the absence of hyphae formation and consequently defective invasive growth/biofilm formation (98). The extracellular matrix (ECM) of *S. cerevisiae* is also affected by GUP genes deletion, both at the level of protein and sugar fractions. Many proteins involved in cellular arrangement, carbon metabolism, cell defense and protein fate are not present in *S. cerevisiae* ECM from Δ*gup1* mutant (99). Moreover, also the sugar fractions from *S. cerevisiae* and *C. albicans* differ (100). Finally, Tulha *et al.,* (101) reveled the sensitivity of Δ*gup1* cells to acetic acid, leading to cell death. This

displayed non-apoptotic characteristics and seemed to undergo instead a necrotic death process, Δ*gup1* cells presenting a reduced chronological life span. The deletion of *GUP1* is further associated with the resistance to complex chemicals like ergosterol synthesis inhibitors, which indicated an interference of Gup1p on sphingolipid and ergosterol synthesis (90), and conversely with the increased sensitivity to sphingolipid synthesis inhibitors, which, together with other evidences, suggested the involvement of Gup1p on the glycosylphosphatidylinositol (GPI) remodeling system (90, 102). Additionally, it was also involved on the resistance to the anti-cancer drug Imatinib (103), together with proteins that regulate the vacuolar pH. Imatinib, marketed as Glivec/Gleevec® by Novartis is a tyrosine kinase inhibitor specific for cancerous cells, namely some types of leukemia (104). Yeasts do not have recognized tyrosine kinases or tyrosine kinase receptors, though the broad sensitivity of *S. cerevisiae* to this drug (103) suggests otherwise.

Gup1p multiple localizations, and numerous associated processes and phenotypes implies a crucial role for this protein in cellular survival and successful progression through cell cycle. *GUP1/2* genes have counterparts in higher Eukaryotes, including mammalians. Abe and co-workers (105) described the mousse homologue of *GUP1* as a negative regulator for N-terminal palmitoylation of sonic hedgehog (SHH) protein (Fig. 5). This protein is responsible for the control of morphogenesis, patterning and differentiation during embryogenesis, as well as cellular morphology and proliferation during that process and wound healing. Accordingly, the mammalian Gup1 protein was named Hedgehog acyltransferase-like protein (HHATL) while Gup2, based on amino acid sequence homology was named as Hedgehog acyltransferase protein (HHAT), and these two proteins supposedly exert opposite roles in hedgehog extracellular signal activation prior to export into the outer space. These roles are in accordance with the above-mentioned functions in yeast, suggesting the putative existence of SHH-like pathway in yeast (105).Some evidences suggest the role of SHH pathway in tumor development, because an existence of high expression levels of this protein in neuroblastoma cell lines. When SHH protein is inhibited it promotes apoptosis and stopped proliferation (106). The above-described resistance to an oncologic drug, such as Imatinib of the *GUP1* deleted strains concurs. For the time being, no relation was found or searched for that matter between the Gup related processes and phenotypes and the RAS/cAMP/PKA's above described. Yet, in view of

the data available, it is predictable that this relation exists. Importantly, as referred for the hyperactivation of the RAS pathway (107), Δ*gup1* is also resistant to rapamycin (108).

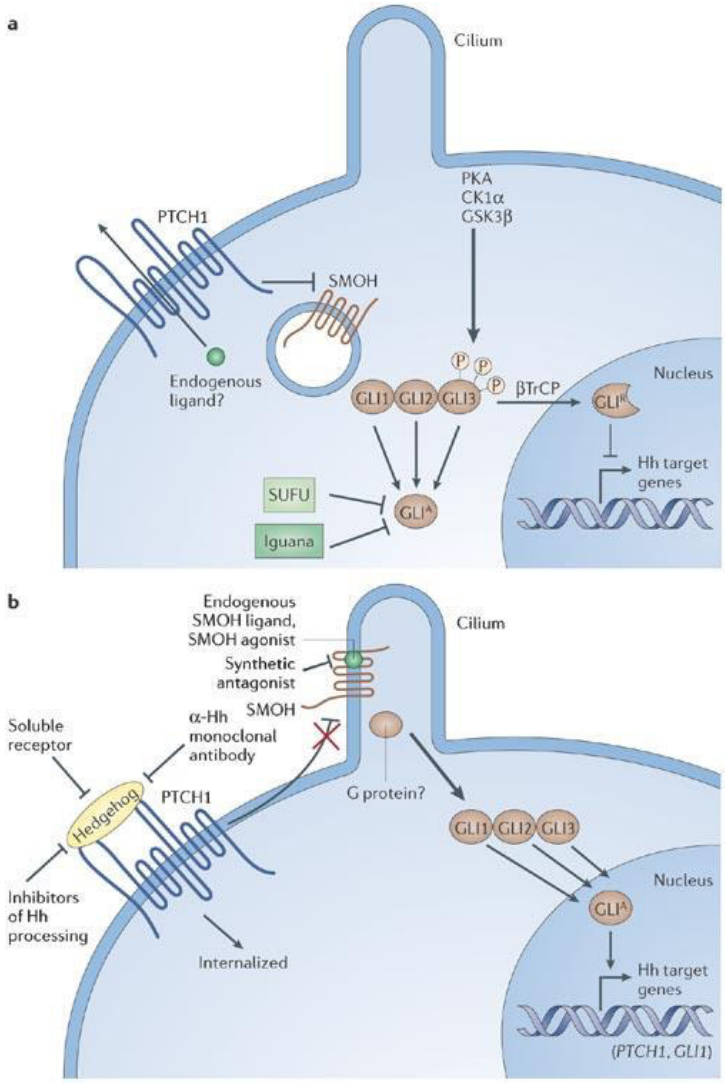

Figure 5. The vertebrate Sonic Hedgehog signaling pathway in the absence or presence of Hh ligands.
In absence of Hh (a),PTCH1, a 12-transmembrane domain protein, is located on the plasma membrane, and the protein GPCR-like receptor Smoothened (SMOH) is located in the membrane of intracellular endosomes. It is proposed that an intracellular small molecule that acts as an agonist for SMOH is transported outside the cell by PTCH1 so that it is not able to bind to SMOH. Under these circumstances, different kinases phosphorylate GLI2/3, creating a repressor form of this transcription factor. Iguana and SUFU prevent the active form of GLI from transactivating Hh-responsive genes in a manner that is still not completely understood.
In presence of Hh ligand (b), PTCH1 is internalized so that it can no longer transport the endogenous agonist molecules outwards. This allows them to accumulate intracellularly and activate SMOH, which itself translocates to the plasma membrane, apparently concentrating in cilia in at least some types of cells. Culminating in the appearance of activator forms of GLI that then regulate the expression of Hh

target genes. The known synthetic small-molecule SMOH agonists and antagonists bind to the same site as the putative endogenous ligand. Withdrawn from (109).

1.7 The Ras family of small GTPases

The Ras is a component of the broad family of small GTPases. The Ras genes are transforming oncogenes that have primarily been recognized as murine sarcoma viruses by Jennifer Harvey (Harvey-Ras or HRas) and Werner Kristen (Kristen-Ras or KRas) in 1960 (110, 111). Subsequent studies led to the identification of a third human Ras gene, designated as *NRAS* in human neuroblastoma cells. So, the three human Ras proteins are designated as HRas, KRas and NRas, which regulate intracellular signaling pathways involved in important cellular processes such as proliferation, cell polarity, differentiation, migration, adhesion, apoptosis and cytoskeletal dynamism (112, 113).

Ras proteins have as principal function the conversion of extracellular stimuli into intracellular signaling cascades, which eventually evoke changes in cellular activities. Thus, in normal mammalian cells, Ras proteins demonstrated functions as molecular switches for critical changes in cellular activities, namely cell proliferation and survival, and their proper regulation is indispensable to maintain the homeostasis of cells. On the other hand, uncontrolled activity of the Ras proteins, or the molecular components of their downstream pathways, can result in cancer or other diseases (113). Approximately 30% of human tumors are estimated to harbor activating mutations in one of the three Ras isoforms. KRAS is most frequently mutated, its mutation rate in all tumors being estimated to lie between 25 and 30%. KRAS mutation is especially frequent in colorectal carcinoma (35–45%), non-small cell lung cancer (16–40%) and pancreatic ductal carcinoma (69–95%) (114). In contrast, activating mutations of NRAS and HRAS are less common (8% and 3%, respectively) (115). The activating oncogenic mutations commonly occur in the GTPase catalytic domains, in codons 12, 13 and 61 (116). All these activating mutations render Ras proteins resistant to GTP hydrolysis, and consequent Ras inactivation stimulated by GTPase activating proteins (GAPs). These constitutively activated oncogenic Ras mutant proteins, therefore, initiate intracellular signaling cascades without the input of extracellular stimuli, resulting in uncontrolled cell proliferation and abnormal cell survival (113).

Ras activates several pathways, including the RAF-MEK-ERK/MAPK cascade, which transmits signals downstream and results in the transcription of genes involved in controlling several cellular mechanisms (117). Ras proteins are anchored in the cytoplasmic membrane by carboxylterminal farnesylation but, in some cases, the Ras proteins are bound by Ras-escort proteins which include galectin-1 and galectin-3 that have strong binding affinity to GTP-HRas and GTP-KRas, respectively (117, 118). Ras-escort proteins stabilize the Ras proteins in the GTP-bound state. Disruption of the interaction between these escort proteins and Ras has been exploited as a strategy to modulate aberrant Ras signaling (119). Ras communicates external cellular signals to the nucleus, and its altered activation leads to inappropriate cellular activities including enhanced cell growth, differentiation and survival of the cells (120, 121). The RAS-RAF-MEK-ERK pathway is activated by several known growth factors and cytokines that act through receptor tyrosine kinase signals and by activating mutations in the RAS and RAF genes (120).

The Ras intrinsic GTPase activity, is to hydrolyze the GTP into GDP (122). Ras is therefore a single GTPase molecule that like the other G proteins act as molecular switches and timers that cycle from inactive GDP-bound to active GTP-bound states (123). In normal quiescent cells, Ras is bound to GDP and is inactive (*off* state), while upon extracellular stimuli, Ras bind to GTP (*on* state), which has an extra phosphate group than GDP. This extra phosphate holds the two switch regions in a "loaded-spring" configuration. Upon the release of this phosphate, the switch regions relax leading to conformational modifications and return to the inactivate state (Fig. 6). Therefore, a cycling switching between the active/inactive GDP-bound forms controls the activation/inactivation of Ras and several other small G proteins. The cyclic process of GDP/GTP is facilitated by guanine GEFs and the GTPase activating proteins (GAPs) (122).

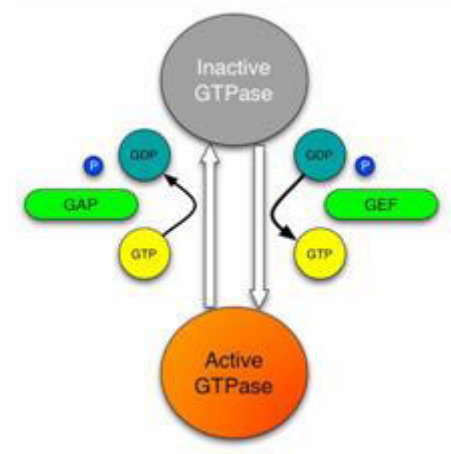

Figure 6. GTPase signaling. GTPase is off when bound to GDP, then a GEF removes GDP and allows GTP to bind to the GTPase, turning it on. All GTPases can hydrolyze GTP to GDP and turn themselves off, though GAPs accelerate this process. Withdrawn from (124).

Normally, ligand binding to receptor tyrosine kinases (RTK) induces dimerization of the receptor and autophosphorylation of specific tyrosine residues in the C-terminal region. This generates binding sites for adaptor proteins like the growth factor receptor-bound protein 2 (GRB2), that recruit the GEF Sos at the plasma membrane, and in turn activates the membrane bound Ras by catalyzing the conversion of GDP into GTP. In its GTP bound conformation, Ras combines with Raf and mobilizes the inactive protein from the cytoplasm recruiting the Raf kinases to the plasma membrane (112, 125). Once the Ras-Raf complex is translocated to the cell membrane, Ras activates the serine/threonine kinase function of Raf isoforms. Upon activation of Ras, Raf acts as a MAP kinase kinase kinase (MAPKKK) to activate MEK1 and MEK2, which, in turn, catalyze the activation of the effector ERK1 and ERK2 kinases, and their translocation into the nucleus. Once activated ERK1/ERK2 broadly phosphorylates several nuclear and cytoplasmic effector proteins involved in diverse cellular responses, such as proliferation, survival and differentiation (Fig. 7) (126, 127).

Although RAF can also be activated by RAS-independent activators (128). Some data have clearly shown that Ras can activate other downstream signaling pathways including phosphatidylinositol 3-kinase (PI3K) and Rac and Rho proteins, associated with the regulation of the cytoskeleton and invasiveness of tumor cells (129).

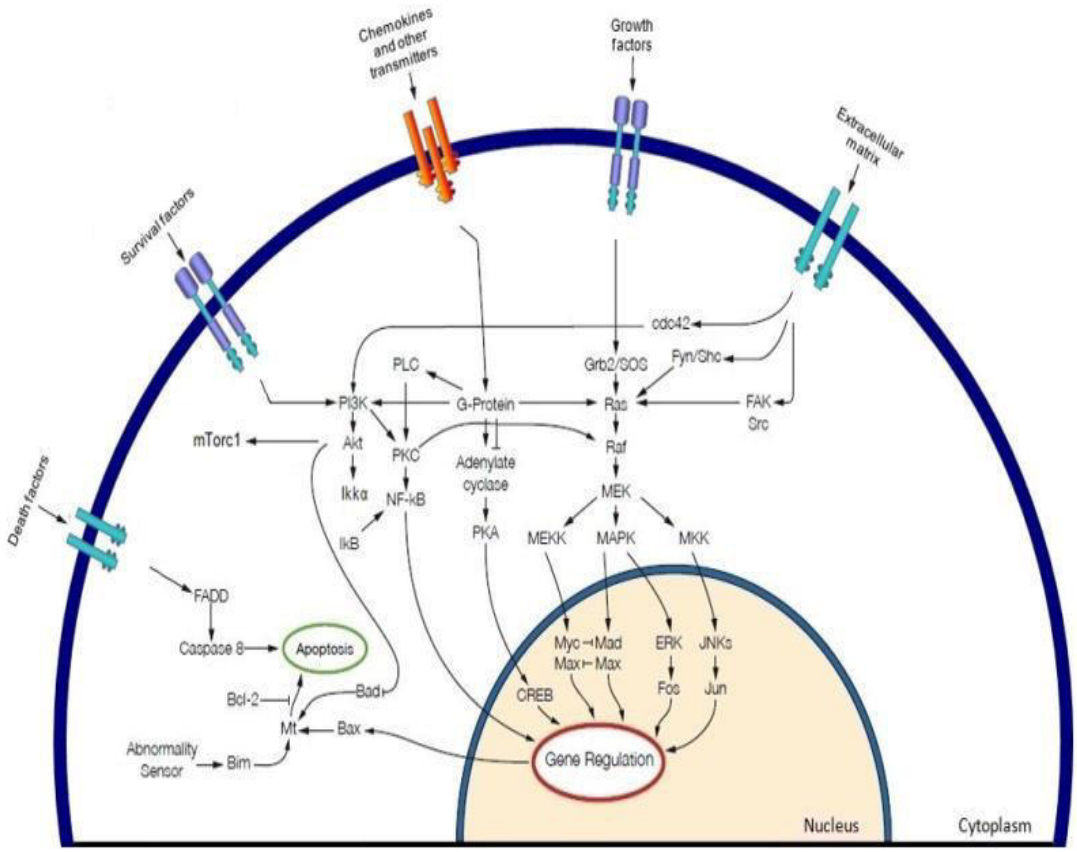

Figure 7. MAPK cascade activation and potential cross talk signals. In the MAPK cascade the growth factors binding and consequently promotes activation of tyrosine kinase receptors, the activation of the RAS GTPase promotes the kinase activity of the RAF serine/threonine protein kinases. Activated RAF phosphorylates MEK in the cytoplasm, which in turn phosphorylates ERKs that translocates to the nucleus where they phosphorylate and regulate various nuclear and cytoplasmic substrates involved in diverse cellular responses, such as cell proliferation, survival, differentiation, motility, and angiogenesis. RAS may cross-talk with different pathways, such as PI3K. Withdrawn from (112).

The embryonic lethality of KRas knockout mice illustrated the importance of KRas expression during development as a result of liver defects and anemia. In opposition, mice with HRAS or NRAS knockouts are completely viable (130). In another study the expression of oncogenic HRas or KRas under tissue-specific promoters induces various types of malignancies in multiple transgenic mouse models (131).

1.8 Metabolic Similarities between Cancer Cells and Yeast

It was supposed that cancer cells suppress mitochondrial metabolism. The early discoveries from Otto Warburg pointed out that cancer cells display a decreased respiration along with an enhanced lactate production, suggesting that they depend mainly on fermentative metabolism for ATP generation (132). The spite of the decrease in energy yield as a consequence of the glycolytic phenotype seems to allow an increase in cell proliferation and be applicable to other fast growing cells (133). In this case, the repression of oxidative metabolism occurs even in the presence of oxygen, this metabolic phenomenon is known as "aerobic glycolysis" or the "Warburg effect". Moreover, it has been showed that vary cancer cells can reversibly switch between fermentation and oxidative metabolism, depending on the absence or the presence of glucose and the environmental conditions (134, 135). More recently, it was proposed that the "glycolytic" cells could establish a metabolic symbiosis with the "oxidative" ones through lactate shuttling (136). A well defined feature of some cancer cells is the glucose-induced suppression of respiration and oxidative phosphorylation (137, 138). This is a reversible event that is called as "Crabtree effect". This event might represent an advantage of cancer cells in vivo, as it would allow them to adapt their metabolism to the rather heterogeneous microenvironments in malignant solid growths (139).

The yeast *S. cerevisiae* is a respiro-fermentative organism, moreover it is a Crabtree positive yeast because upon glucose addition, respiration is inhibited despite the presence of oxygen (140, 141). When glucose amount is high, the yeast uses as main metabolic pathway fermentation, and when this carbon source becomes scarce it can switch to oxidative metabolism (142). In relation to energy metabolism, there are similarities between the glucose-induced repression of oxidative metabolism of yeast and the "aerobic glycolysis" of tumor cells. In both cells, the downregulation of oxidative metabolism is observed with an enhanced fermentation despite the presence of oxygen. Additionally, *S. cerevisiae* shares with cancer cells the same metabolic features that are identified as the main causes of the above-mentioned Warburg effect. For example, like cancer cells, yeasts overexpress glycolysis enzymes in response to glucose (143, 144). Moreover, the activity and expression pattern of the glycolysis key enzymes, such as hexokinase, phosphofructokinase and pyruvate kinase, are also modified in yeast (144, 145).

Although, yeast lacks the genetic defects identified in cancer cells, *S. cerevisiae* has homologues with genes related with cancer such as p53, cyclin D and Ras (29). Therefore, an interesting approach would be to use "tumourized yeasts" through the introduction of muted genes related with cancer and apply this as a model for anti-cancer drug screening and for metabolic studies.

1.9 The Role of Cell Surface Receptors

Cell signalling requires not only extracellular signal molecules, but also a set of receptor proteins in each cell that enable it to bind and respond to the signal molecules in a characteristic way. These cell surface receptor proteins act as signal transducers. They convert an extracellular ligand-binding event into intracellular signals that alter the behaviour of the target cell (146, 147). The extracellular signal molecules often act at very low concentrations and the receptors that recognize them usually bind them with high affinity. In most cases, the receptors are transmembrane proteins on the target cell surface. When these proteins bind an extracellular signal molecule, they become activated and generate various intracellular signals. In other cases, the receptor proteins are inside the target cell, and the signal molecule needs to enter the cell to bind to them, this process requires that the signal molecule be sufficiently small and hydrophobic to diffuse across the target cell's plasma membrane (148). This knowledge is common to high and low Eukaryotes. Nevertheless, the presently recognized players at the level of signal reception/sensing are quite different in both types of organisms.

In higher Eukaryotes, the RTKs are a large superfamily of receptors with function as the receptors for a wide array of growth factors, including epidermal growth factor (EGF), nerve growth factor (NGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin and the insulin-like growth factors (IGF), and the ephrins and angiopoietins (149). RTKs are essential components of cellular signalling pathways that are activated during embryonic development and adult homeostasis. Because of their roles as growth factor receptors, many RTKs have been implicated in the onset or progression of various cancers, either through receptor gain-of-function mutations or through receptor/ligand overexpression (150). Consequently, cell surface receptors are essentials to the mechanism of many chemical toxicants and serve as targets for the development of

drugs (149, 150). Growth factors modulate signaling pathways, which control cell proliferation and death in both normal and malignant cells. The EGF was one of the first growth factors to be discovered and is the prototype of a large family of closely related growth factors, which includes TGF, amphiregulin, heparin binding EGF, and betacellulin. Among these growth factors, TGF has been identified as a key modulator in the process of cell proliferation in both normal and malignant epithelial cells. TGF binds to the receptor, the epidermal growth factor receptor (EGFR), which promotes the activation of the EGFR tyrosine kinase enzymatic activity that triggers the intracellular signaling pathway (151). The EGFR is part of a subfamily of four closely related receptors: EGFR (or ErbB-1), HER-2/neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4). The receptors exist as inactive monomers, which dimerize after ligand activation. This causes homodimerization or heterodimerization between EGFR and another member of the Erb receptor family. After ligand binding, the tyrosine kinase intracellular domain of the receptor is activated, with autophosphorylation of the intracellular domain, which initiates a cascade of intracellular events (152, 153). The signaling pathway involves activation of Ras and mitogen activated protein kinase, which activates several nuclear proteins, including cyclin D1, a protein required for cell cycle progression from G1 to S phase (154). EGFR signaling is not only essential for cell proliferation. Several studies have demonstrated that EGFR signaling also mediates other processes that are crucial to cancer progression, including angiogenesis, metastatic spread and the inhibition of apoptosis (153-156). Activation of the TGF-EGFR autocrine growth pathway in cancer cells can be attributed to several mechanisms, such as overexpression of the EGFR, increased concentration of ligand, decreased phosphatase activity, decreased receptor turnover, and the presence of aberrant receptors, including EGFR gene alterations. In this context, the most common EGFR mutant found in human cancer is EGFRvIII (157).

TGF and/or EGFR are overexpressed in many different solid human cancers, including breast, head and neck, gastric, prostate, ovarian, colorectal carcinomas, and glioblastomas, in which it is generally associated with advanced disease and poor prognosis (156, 158, 159). Human EGFR gene locates at chromosome 7p11-13 and the mature protein is synthesized from a 1,210 residues polypeptide precursor. This originates a 170 kDa protein containing approximately 20% of carbohydrate of its molecular mass and is heavily N-glycosylated (160-163). Glycosylation is important in

case of protein-protein interactions that occur between protein ligand and their receptors, because it plays a role in determining protein structure and known to affect the three-dimensional configuration of proteins (164).

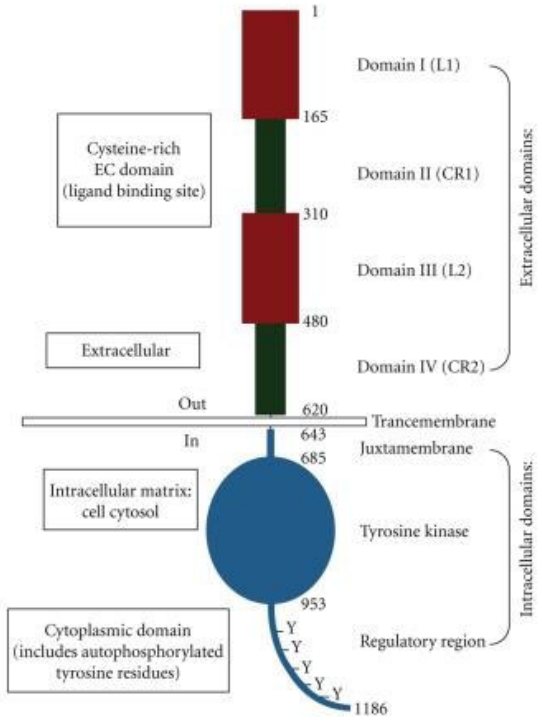

Figure 8. Basic structure of EGFR displaying the relevant domains. (1) The extracellular domains: domain I/L1; domain II/CR1; domain III/L2; domain IV/CR2. (2) Transmembrane domains. (3) The intracellular domains: juxtamembrane domain; tyrosine kinase domain; regulatory region domain. The phosphorylation of several substrates by the tyrosine kinase domain of the EGFR receptor is responsible for activating of various signaling cascades. Withdrawn from (163).

Like all RTKs EGFR is characterized by three main domains. The extracellular domain of the mature receptor contains 621 amino acids, followed by a single transmembrane domain and a juxtamembrane domain (Fig. 8) (160, 162). Crystallographic studies of the EGFR extracellular domain complexed to its ligands have shown that the domains I, II and III form a ligand-binding pocket (165, 166). In the absence of ligand, EGFR exist as monomers on the cell surface. Binding of ligand to EGFR leads to the formation of receptor homo and heterodimers, depending on whether EGFR dimerizes with another EGFR or with other ErbB family members, respectively (167). EGFR dimerization is entirely receptor-mediated, with no contacts between the two growth factor molecules in the dimeric complex (165). By binding simultaneously

to two sites (within domains I and III) in the extracellular region of the receptor, the growth factor alters the special arrangement of the domains (as shown schematically in Fig. 9) (166). Phosphorylation of the EGFR activation loop in contrast to other kinases is not necessary for its activation (168). The EGFR kinase is activated by an asymmetric dimer in which the C-terminal lobes of two-kinase domain bind with each other in a manner analogous to cyclin in activated CDK/cyclin complexes. Thus, ligand binding brings two receptor monomers together and allows for the dimerization and subsequent activation of the kinase domain (169). Ligand induced EGFR dimerization leads to autophosphorylation of several key tyrosine residues in the cytoplasmic domain of each receptor monomer (170). These phosphorylated tyrosine residues then serve as binding sites for a number of adapter and signaling molecules leading to the activation of several intracellular signaling pathways downstream of the receptor. Some of the best characterized EGFR effector pathways are the RAS-RAF-MEK-ERK, PI3K/Akt, JAK/STAT and the PLCγ-PKC pathways, which upon activation lead to cell proliferation, motility and survival (Fig. 10) (170, 171).

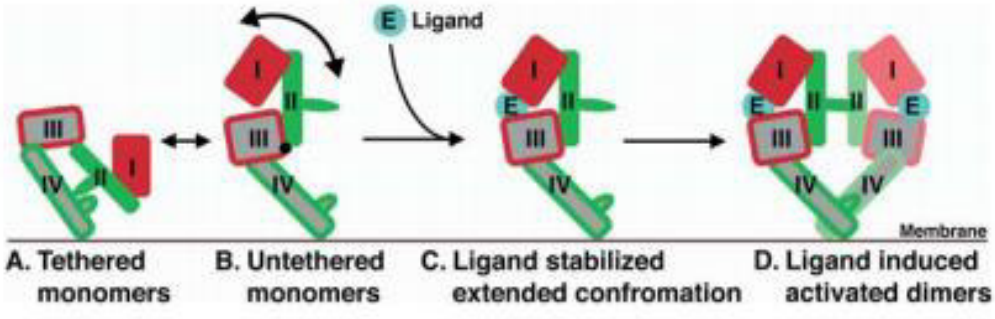

Figure 9. Mechanism of ligand-induced EGFR dimerization. About 95% of the unliganded EGFR exists in a compact auto-inhibited or tethered conformation, in which domains II and IV form an intra-molecular interaction or tether (A). In 5% of the unliganded molecules, this tether is broken, and the soluble extracellular region of EGFR (sEGFR) can adopt a range of untethered conformations (B). Ligand binds preferentially to untethered molecules, and interacts simultaneously with domains I and III, stabilizing the particular extended form in which domain II is exposed and the receptor can dimerize (C). Dimerizations entirely receptor mediated and dominated by domain II interactions (D). Withdrawn from (166).

The Ras/extracellular signal regulated kinase (ERK) pathway is a critically important route that regulates cell proliferation and survival in yeasts (see above) as in

mammalian cells (see above) (172). In these last, GRB2 is an SH2/SH3 domain containing protein that binds EGFR either directly or through the association with the adaptor molecule Shc, and acts as a common adapter protein in a majority of growth factor related signaling events (173, 174).

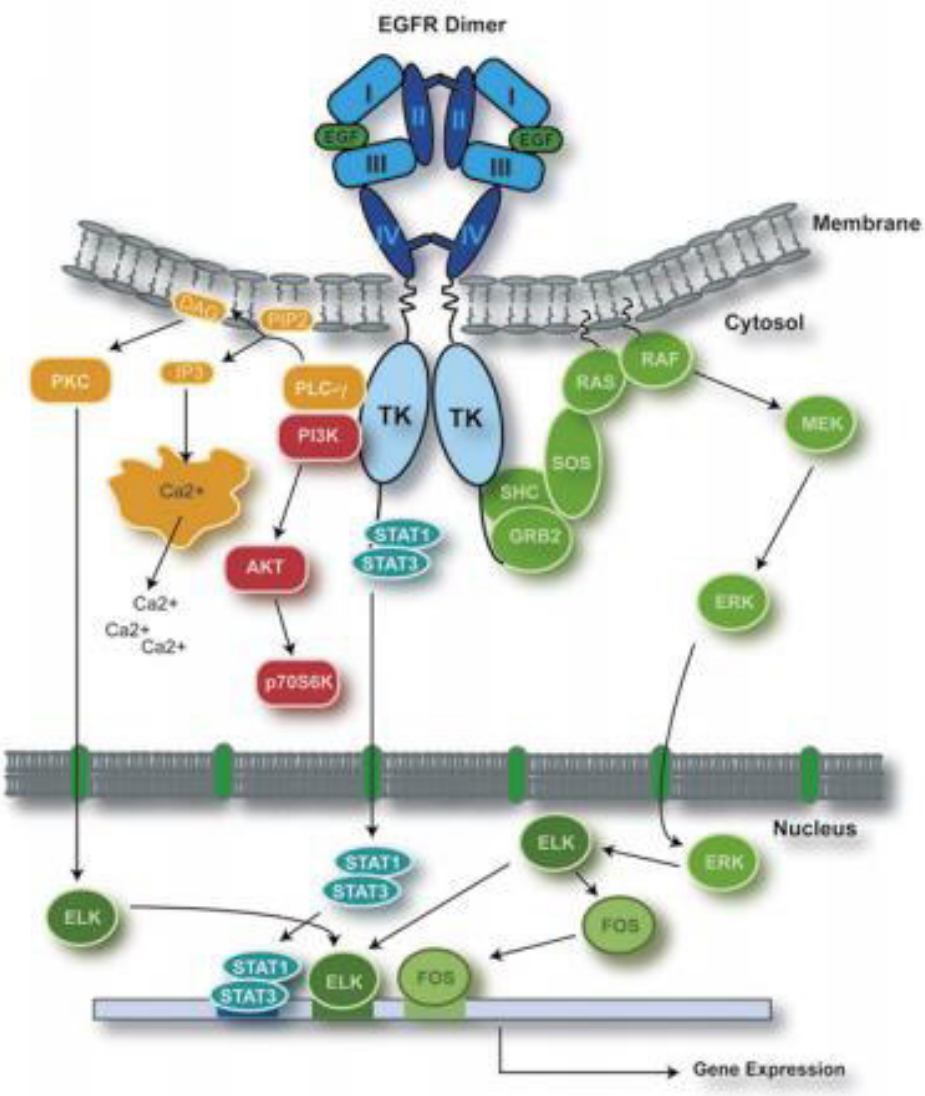

Figure 10. EGFR signaling. Binding of ligand to EGFR leads to receptor dimerization, autophosphorylation and activation of several downstream signaling pathways. Only selected pathways and transcription factors are presented. Withdrawn from (161).

The PI3K/Akt signaling pathway also affects many cellular processes including cell proliferation, apoptosis and invasion (175, 176). PI3K is recruited to the membrane by directly binding to phosphotyrosine consensus residues of growth factor receptors or

adaptors through one or both SH2 domains in the adaptor subunit (177). This leads to allosteric activation of the catalytic subunit. Activation results in the production of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). The lipid product of PI3K, PIP3, recruits a subset of signaling proteins with pleckstrin homology (PH) domains to the membrane, including PDK1 and Akt. Once activated, Akt mediates the activation and inhibition of several targets, resulting in cellular survival, growth and proliferation (178). The interlinked Ras/MAPK and PI3K/Akt signaling pathways play an important role in tumourigenesis via phosphorylation of various proteins and transcription factors. Furthermore, mutation in *KRAS*, *BRAF*, or *PIK3CA* results in continuous activation of the downstream Ras/MAPK or PI3K pathways, regardless of whether the EGFR is activated or pharmacologically blocked (179-181). EGFR ligands are not only responsible for stimulation of pathways that positively regulate EGFR, but also stimulate pathways that negatively regulate the EGFR coupling to malignant phenotypes and this balance between these positive and negative regulators of EGFR coupling to malignant phenotypes may be altered in tumor cells (169). Generally, $1x10^5$ EGFR per cell are expressed by normal cells, but tumor cells can express more than $2x10^6$ receptors per cell (182). It was reported that the hypoxic microenvironment of tumors can also induce overexpression of EGFR by increasing EGFR mRNA translation, since it was considered that receptor overexpression commonly develops due to gene amplification (183). Further the EGFR overexpression can result in high levels of autocrine signaling (184), autocrine production of TGF-α or EGF reduces the chances of cancer survival (185).

Inactivation of the EGFR can be mediated either by receptor dephosphorylation by phosphotyrosine phosphatases or receptor downregulation. Receptor downregulation is the most prominent regulator of EGFR signal attenuation and involves the internalization and subsequent degradation of the activated receptor in the lysosomes (161, 186).

1.9.1 EGFR as a Therapeutic Target

A large body of experimental and clinical work supports the view that the EGFR is a relevant target for cancer therapy. Two therapeutic approaches have been shown most promising and are currently being used to inhibit the EGFR in clinical studies: (a) monoclonal antibodies (MAbs) like Cetuximab (Erbitux®) used in colorectal cancer (CRC) therapy (187, 188), and (b) small molecule inhibitors of the EGFR tyrosine kinase enzymatic activity (TKIs) like Imatinib (Gleevec®) used in leukemia therapy (104, 169). Small-molecule TKIs compete reversibly with adenosine 5'triphosphate to bind to the intracellular catalytic domain of EGFR tyrosine kinase and inhibit the EGFR autophosphorylation and downstream signaling (169). MAbs are generally directed at the external domain of the EGFR to block ligand binding and receptor activation (165, 169). Cetuximab (Ctx) was approved by the FDA in 2004 for squamous cell carcinoma of the head and neck and advanced stage of CRC overexpressing EGFR (189). Ctx is a 152 KDa chimeric monoclonal antibody of the immunoglobulin G1 subclass produced in mammalian cell culture by mouse myeloma cells. It was constructed by attaching the variable regions of the murine monoclonal antibody M225 against EGFR to constant regions of the human IgG1. It has two identical heavy chains consisting of 449 amino acids each and two light chains of 214 amino acids each (Fig. 11) (190, 191).

Cetuximab has a 5-10 fold higher affinity for EGFR than the native ligand, resulting in inhibition of the receptor function (192, 193). It is also able to mediate antibody dependent cell mediated cytotoxicity (194), and receptor downregulation leading to a mitigation of EGFR activity that does not affect other HER family receptors (195). Ctx induces inhibition of EGFR signaling, prevents heterodimerization and leads to downregulation of downstream targets (Fig. 12) (166). It avoids several cell signaling pathways, including the Ras–Raf–MAPK, PI3K/Akt, PKC, STAT and SRC, all of which play important roles in tumor cell proliferation, invasion and inhibition of apoptosis (196). Further, Ctx blocks cell cycle progression by inducing G1 arrest (197-200) as well as the transport of EGFR into the nucleus (201), and also has the potential to kill targets cells by mediating antibody-dependent cell-mediated cytotoxicity (194).

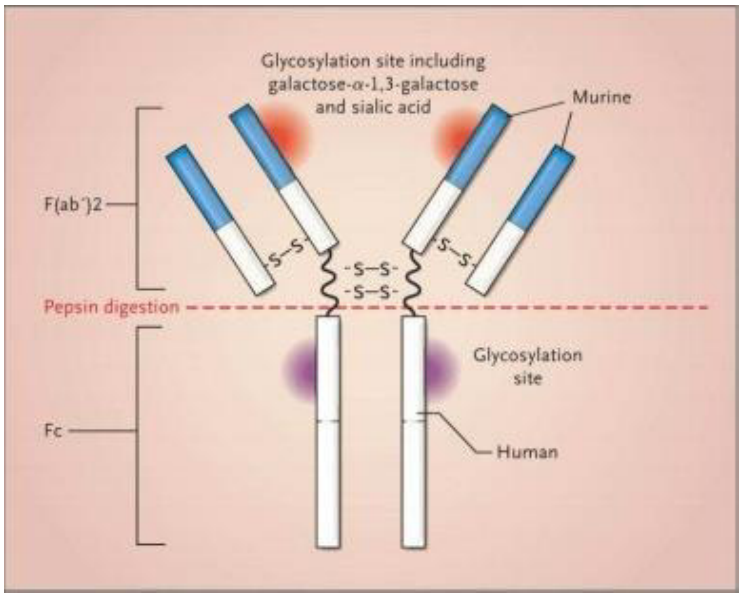

Figure 11. Structure of Cetuximab. Cetuximab is a chimeric IgG1 monoclonal antibody composed by the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions. The sugars on the Fab portion include galactose-α-1,3-galactose and the sialic acid N-glycolylneuraminic acid, the glycosylation site of the Fc portion includes only oligosaccharides that are commonly present on human proteins. Withdrawn from (202).

Antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity belong to the most important processes allowing IgG1 antibodies to destroy microorganisms and cancer cells. Antibodies can bind specifically to epitopes of cancer antigens. If the antigen is a receptor, as in anti-EGFR therapy, the intracellular transduction pathway is blocked but also cytotoxic cells become activated. After coating the cancer cell, antibodies bind to NK cells and other immune cells, which have receptors for the antibody Fc fragment on their surface. NK cells bound to the target cell become degranulated releasing perforins, granulysins and granzymes, which induce apoptosis of cancer cells. Similarly, the membrane of the target cell can become lysed as a result of activation of components of the complement system (203, 204). The net effects of Ctx are inhibition of tumor growth (197, 205), invasion and angiogenesis (206-208), metastasis, and DNA damage repair (200, 205, 206, 209). The Ctx-mediated potentiation of apoptosis is correlated with the induction of Bax and the increase in expression of caspases (205, 210). The inhibition of tumor induced angiogenesis is probably due to reduced tumor production of angiogenic factors, including TGF-α, VEGF, interleukin-8 and basic fibroblast growth factor, leading to reduced tumor

microvessel density, and inhibition of invasion and metastases by inhibiting extracellular matrix metalloproteinases (206, 207, 211, 212).

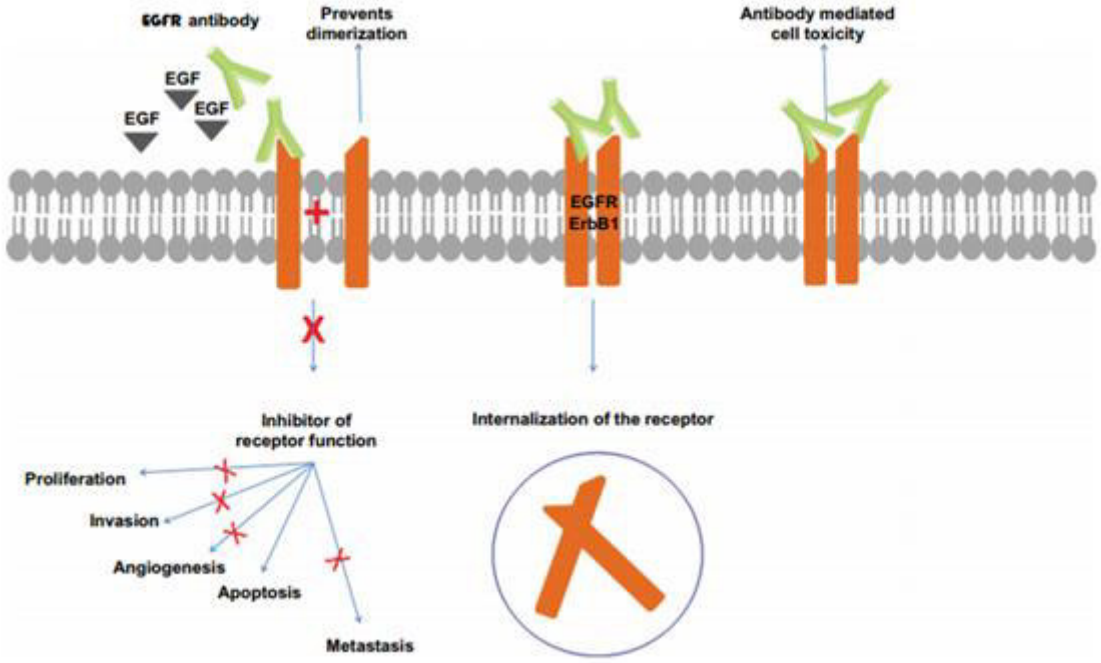

Figure 12. The mechanism of anti-EGFR antibodies action. The antibody binding to EGFR prevents receptor dimerization, leading to inhibition of receptor function. Anti-EGFR binding also fosters receptor internalization and promotes antibody-dependent cell cytotoxicity. The resulting outcomes include disruption of angiogenesis, invasion, proliferation, metastasis and the promotion of apoptosis. Adapted from (213).

In the abrogation of the EGFR function, it became evident that in addition to EGFR, other key downstream molecules were equally important. Several reports have shown that constitutive activation of key downstream components renders the EGFR blockade by antibodies and/or TKI ineffective (214-216). One of these essential downstream factors is the small G protein proto-oncogene KRAS. Thereby, besides the detection of EGFR expression, mutation of the KRAS gene is an important predictive marker of resistance to treatment with Ctx. Studies have demonstrated that Ctx efficacy is confined only to tumors without the KRAS gene mutation, i.e. KRAS wild-type tumors (217). Although, patients may acquire resistance-mediating mutations within the extracellular EGFR domain, consequently the exact binding sites of EGFR targeting antibodies may help to predict treatment responses (218).

Mutations in codon 12 (82%), 13 (17%) and 61 (4%) of KRAS protein have been implicated in resistance to treatment for CRC (219). Although the KRAS without mutations (wild type) seems to be a condition for response, however most patients with KRAS codon 12 and 13 wild-type tumors do not respond to anti-EGFR monoclonal antibodies (217). Mutations in other downstream effectors of the EGFR signaling pathway, such as BRAF, NRAS, and PI3K, might also have a negative effect on response to anti-EGFR antibodies (220). These mutations result in continuous activation of the downstream Ras/MAPK or PI3K pathways, regardless of whether the EGFR is activated or pharmacologically blocked. Such activation in turn enhances transcription of various oncogenes, including MYC, CREB, and NF-κB (179-181). *KRAS* mutation is thought to be an early event in tumourigenesis, being accordingly the most commonly mutated gene (35%-45% of CRC patients), while mutations in *PIK3CA* (≤ 20%) and *BRAF* (<15%) are less common (220, 221). Patients with a colorectal tumor bearing mutated KRAS cannot benefit from Ctx. However, the prognostic role of *KRAS* mutation in CRC remains uncertain (217). Therefore, the identification of each patient KRAS mutation status is crucial for disease and life expectancy prognosis, as well as avoiding a costly and potentially toxic administration of this treatment in non-responder patients. The importance of these facts becomes more prominent when it is acknowledged CRC is the third most frequent cancer in men, after prostate and lung, and the second most common in women, after breast (222). The incidence of cancer is augmenting in developed countries as a result of an increase in population life span/age, and principally caused by adoption of cancer associated lifestyle choices including smoking, not balanced diets and sedentary lifestyle. Genetic factors such as familial traits and genetic predispositions also promote this augment (222).

Successful accomplishments have been made in cancer therapy strategy by inhibiting some oncogenes achieving tumor cell death, differentiation or senescence. Drugs targeting protein kinase oncogenes such as the above-mentioned anti-BCR-ABL Imatinib, and anti-EGFR Cetuximab, as well as others like the anti-HER2 Transtuzumab, have been used in a variety of cancers, including CRC (223, 224). Despite the advances in medical practices and the progresses obtained with the introduction of new cytotoxic agents, there are still a high number of cancer patients for whom treatment is not effective due to the development of resistance to anticancer drugs as a result of host factors or a result of genetic and epigenetic changes in cancer

cells (225). Importantly and finaly, there are suggestions that therapy resistance can also be a consequence of survival pathways activation during carcinogenesis by oncogenic transformation, being some examples of oncogenes that can activate survival pathways Ras, Raf, HER2 and EGFR (226).

1.10 Rationale and Aims of the thesis

Glycopharm is a Marie Curie Initial Training Network that has as scientific objectives the development and testing of selective galectin-blocking compounds and the development and testing of galetin-mimetic peptides with respective target selectivity. This impacts enormously on a wide variety of diseases in need for novel therapeutic solutions and rapid and easy to use tools for primary pharmacological testing. This thesis is enclosed in the wide objectives of the network and aims at building and validating a large high throughput platform of yeast strains displaying phenotypes that enable testing galectin-related drugs and peptides. This platform was designed to be made of two types of strains, the ones expressing *tout court* human galectins (in particular Galectin 3 and Galectin 1) and the ones expressing these human proteins together with the human *KRAS* cDNA. The rationale behind this relates to the putative dialogue between Galectins and RAS signaling pathway in mammals. For this purpose, two genetic backgrounds were chosen according to the genetic marks available to ease constructions: BY4741 and W303, both haploids and from the same mating type *a*. Therefore, BY4741 wt and RAS mutants were used for express *KRAS* from within a plasmid construction (this thesis), and from within a chromosomal insertion (Cazzanelli, unpublished work), while W303 RAS derived mutants were used in a first phase as phenotyping controls (this thesis). Phenotyping using W303 is to be confronted with the one simultaneously obtained with BY4741 background (Carneiro, unpublished work). If considered necessary, a subsequent step of KRAS cloning in W0303 will follow.

This way, the present thesis covers a group of tasks complementary to the work of two other students for the development of a yeast-based high throughput platform for human KRAS and galectin 3 or galectin 1 phenotyping:

1. To build a set of yeast strains expressing human *KRAS* cDNA in *S. cerevisiae* BY4741 RAS deficient background,

2. To identify protein(s) from *S. cerevisiae* putatively interacting with KRAS in an identical fashion as EGFR in mammalian cells,
3. To test the phenotypes associated with adhesion, invasiveness and filamentation in the *S. cerevisiae* W303 RAS deficient background chosen as control of the constructions in point 1,
4. To get insights about the *S. cerevisiae* pathways putatively correlating RAS and GUP genes.

This data is expected to contribute to gain insight into KRAS mechanism of action using yeast as a model organism, as well as to determine the possible homologue of EGFR in *S. cerevisiae*. On the other hand, it will also contribute to gain insight into RAS and GUP genes and their functions in signaling considering, their phenotype overlapping. This knowledge is also expected to be subsequently validated in human derived cell lines and applied to define clinical correlation, paving the way to the development of new diagnostic/prognostic tests and new bio-active ligands/inhibitors.

CHAPTER II

Material and Methods

2. Material and Methods

2.1 Strains and Growth Conditions

The strains of *Saccharomyces cerevisiae* and *Escherichia coli* used in this study are listed in Table 1. *E. coli* XL1-Blue was used for DNA propagation and plasmid cloning.

Table 1. Yeast and bacteria strains used in the present work.

Strain	Genotype	Origin
S.cerevisiae BY4741wt	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf collection
S.cerevisiae BY4741 Δ*ras1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YOR101w::KanMX4*	Euroscarf collection
S.cerevisiae BY4741 Δ*ras2*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YNL098c::KanMX4*	Euroscarf collection
S.cerevisiae BY4741 $KRAS^{wt}$	Isogenic to BY4741 but transformed with p426GPD+*hsKRAS*	This study
S.cerevisiae BY4741 Δ*ras1* $KRAS^{wt}$	Isogenic to BY4741 Δ*ras1* but transformed with p426GPD+*hsKRAS*	This study
S.cerevisiae BY4741 Δ*ras2* $KRAS^{wt}$	Isogenic to BY4741 Δ*ras2* but transformed with p426GPD+*hsKRAS*	This study
S.cerevisiae W303-1A	*MATa leu2Δ3 leu2Δ112 ura3Δ1 trp1Δ1 his3Δ11 his3Δ15 ade2Δ1 can1Δ100*	(227)
S. cerevisiae W303-1A Δ*gup1*	Isogenic to W303-1A but $gup1::HIS5^{+}$	(88)
S. cerevisiae W303-1A Δ*gup2*	Isogenic to W303-1A but *gup2::KanMX*	(88)
S. cerevisiae W303-1A Δ*gup1/2*	Isogenic to W303-1A but $gup1::HIS5^{+}$ and *gup2::KanMX*	(88)
S. cerevisiae W303-1A Δ*ras2*	Isogenic to W303-1A but *ras2::KanMX4*	(228)
S. cerevisiae W303-1A Δ*ras1*	Isogenic to W303-1A but *ras1::HIS3*	(228)
E. coli XL1-Blue	*endA1 gyrA96 (nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK-mK+)*	-

The p426 plasmid (Fig. 13) is a yeast multicopy expression vector, harboring the T3 and T7 promoters and the AmpR marker for bacterial expression, the GPD promoter, the selectable marker URA3 and the 2μ origin of replication for expression in yeast. The p416 yeast centromeric plasmid (Fig. 13) was also used in this study. This plasmid is

identical to p426 except for the CEN6/ARS4 element that replaces the 2μ origin of replication (229).

E. coli strains were cultured in Luria-Bertani medium (LB) - 1% (w/v) of NaCl, 1% (w/v) of tryptone and 0.5% (w/v) of yeast extract with agar 2% (w/v) for solid growth. Selection of transformants was done on LB with 100μg/ml of ampicillin. Cultures were incubated at 37 °C and 200 rpm orbital shaking in the case of liquid growth. *S. cerevisiae* strains were batch-grown on rich medium - YPD (1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose), or minimal medium - YNB (0.67% (w/v) YNB without amino acids and nitrogen source (Difco)), supplemented with 2% (wt/v) glucose, 0.5% (w/v) ammonium sulfate, and adequate quantities of auxotrophic requirements, adding 2% agar for solid growth. Auxotrophic requirements were: leucine, methionine and histidine 10g/L, uracil and adenine 2g/L, tryptophan 5g/L. In the case of liquid growth, yeasts were cultured at 30 ºC and 200 rpm orbital shaking with an air/liquid ratio of 5:1. All yeast and bacteria strains were conserved at 4 ºC on week lasting solid media for the full time of the thesis. Yeast growth was monitored spectrophotometrically (OD_{600nm}) in a Spectrophotometer Genesys 20.

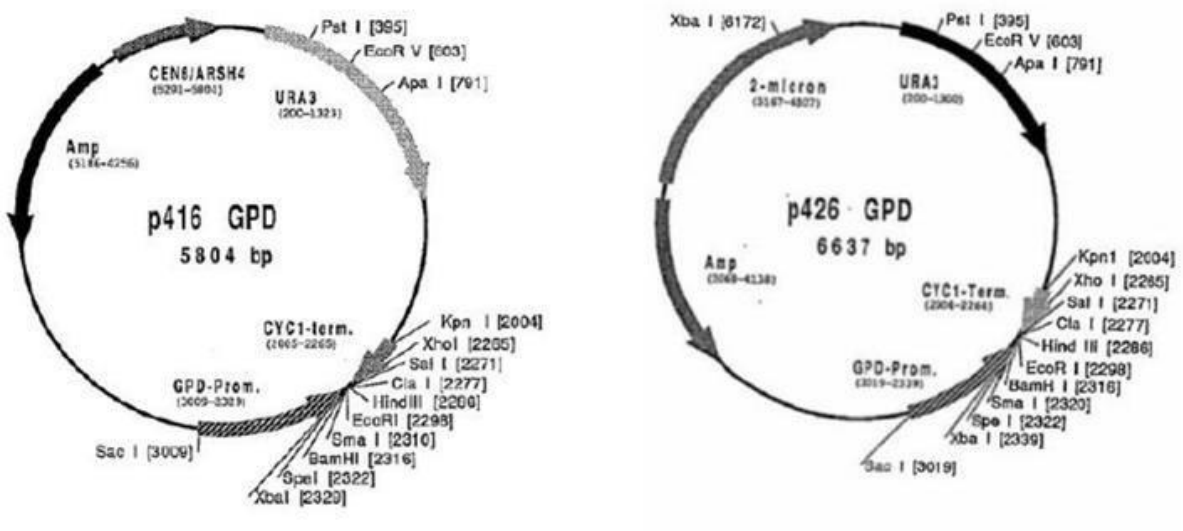

Figure 13. Plasmids p426GPD and p416GPD.

2.2 Competent *E. coli* cells

The competent cells of *E.coli* XL1-Blue were prepared using $CaCl_2$ and $MgCl_2$ (230). The cells were pre-inoculated in LB medium and grown overnight at 37 ºC and 200 rpm, and then inoculated in 100ml fresh medium until reaching an OD_{600} of 0.6. The cells were cooled in ice during 10min and centrifuged at 4,000 rpm (Sigma 4-16K centrifuge) for 10min at 4 ºC. The supernatant was discarded and the pellet resuspended in 20ml of $MgCl_2$ 0.1M (sterilized and refrigerated at 4 ºC). This step was followed by a centrifugation identical to the previous one. The pellet was resuspended in 2ml of 0.1M $CaCl_2$ (sterile and kept at 4 ºC) and cooled on ice during 2h. Finally, DMSO to a final concentration of 7% was added to the cells. They were portioned in 200µl cells aliquots, frozen in liquid nitrogen and stored at -80 ºC.

2.3 Construction of *E. coli* p426*KRAS*^{wt}

2.3.1 DNA amplification by Polymerase Chain Reaction (PCR)

The cDNA from human *KRAS* was obtained from IPO-Porto in a pLenti-*KRAS*^{wt} plasmid. This gene was amplified by PCR directly from the plasmid using the specific primers listed in Table 2.

Table 2. Primers used to amplify the human *KRAS* fragment.

Primer	Sequence (5' → 3')	Comments
1	GCG AAG CTT ATG ACT GAA TAT AAA CTT GTG GTA GTT GGA	Foward_11044KRAS
2	GCG CTC GAG CAT AAT TAC ACA CTT TGT CTT TGA CTT CTT	Reverse_11044KRAS

The primers were designed adding the recognition sequences of the restriction enzymes HindIII and XhoI at the border of the sequence complementary to the *KRAS* gene, in order to enable the cloning in the plasmid p426 and p416 above mentioned. The PCR reaction mix of 20µl was prepared with:

- 2mM $MgCl_2$ (Fermentas),
- 1X Taq reaction buffer (Fermentas),

- 1.25U Taq DNA polymerase/50µl PCR reaction,
- 200µM dNTPs,
- 0.2µM of each primer (Eurofins Genomic),
- 200ng of DNA,
- upH_2O to complete the final volume of 20µl.

The amplification was performed in the thermocycler T100 Thermal Cycler (Bio-Rad) programmed for:

- 1 cycle - initial denaturation 5min at 94 ºC,
- 35 cycles - denaturation 30sec at 94 ºC + annealing 30sec at 55 ºC + extension 1.5 min at 68 ºC,
- 1 cycle - final extension 10min at 72 ºC.

To confirm the DNA amplification, the PCR products were visualized in 1% (w/v) agarose gel, pre-stained with Gel-Red (Biotium). The products of PCR reaction were stored at 4 ºC.

2.3.2 DNA electrophoresis

DNA agarose gels were prepared with 50ml 1X TAE buffer (50X; 242g Tris base, 57.1ml glacial acid acetic, 100ml 0.5M EDTA pH8) to a final concentration of 1% agarose. Gel Red (Biotium) dye (2µl) was used for gel pre-staining. DNA ladder (λ DNA/Eco47I from Fermentas) (3µl mixed with 7µl of loading buffer) was used as reference. The gels were run in a Mini-SubCell GT system (BioRad) at 75V. The gels were visualized in the UV-transilluminatorGenoSmart (VWR). The extraction of the DNA fragment of interest from a gel was done using *GenElute Gel Extraction Kit* (Sigma-Aldrich) strictly following the manufacturer recommendations.

2.3.3 DNA digestion and ligation

KRAS cDNA (amplified by PCR) and plasmids were both digested with HindIII and XhoI restriction enzymes (Fermentas). The restriction mix of final volume of 20µl was composed of:

- 1x buffer R (Fermentas),
- 1x HindIII (Fermentas),
- 1x XhoI (Fermentas),
- 300ng of human *KRAS* cDNA,
- 300ng of p416 or p426 vectors (these digestions were performed separately),
- upH_2O up to the final volume of 20 µl.

The ratio between the two enzymes and the buffer were verified using the Fermentas double digestion tool (http://www.fermentas.com/en/tools/doubledigest). The restriction mix was incubated 3h at 37 ºC. The enzymes were inactivated by heating 20min at 75 ºC. The final product of the digestions was run in a 1% (w/v) agarose gel, and visualized as mentioned above.

Ligation was performed using T4 DNA ligase (Roche) as follows:

- 20ng of vector,
- 100ng of fragment,
- 2x T4 DNA ligase buffer (Roche),
- 5U of T4 DNA ligase (Roche)
- upH_2O to perform the final volume of 20µl.

The reaction was incubated overnight at room temperature.

2.3.4 Plasmid amplification in *E. coli*

E. coli transformation was done with the heat shock method (231). A mix was prepared with 200µl of competent cells XL1-Blue and 0.2mg of plasmid DNA, and incubated on ice 40min. The mixture was subsequently incubated for 2min at 42 ºC and cooled again on ice for 2min. LB medium (500µl) was added and incubated 1h with shaking (200 rpm) at 37 ºC. At last, different amounts of the transformation solution (100µl and 300µl) were plated on LB-Amp and incubated overnight at 37 ºC. For the extraction of plasmidic DNA, a single colony grown on LB plate with selective marker was cultured overnight in 5ml of LB-Amp. The DNA was extracted using the *GenElutePlasmidMiniprep* kit (Sigma-Aldrich) according to the manufacturer instructions. The presence of the *KRAS* insert in the plasmidic DNA was checked by

digestion with the restriction enzymes HindIII and XhoI and run in a 1% (w/v) agarose gel.

2.3.5 Transformation of *S. cerevisiae* and colony PCR

S. cerevisiae BY4741 was transformed using the method of litium acetate (231). Cells were cultured at 30 °C, 200 rpm to an OD_{600} of 1.0 in 5ml of YPD. The cells were harvested by centrifuging 5min at 3,000 rpm (Sigma 4-16K centrifuge), and washed twice with sterile dH_2O and then with LiAc(0.1M)/TE(1X). Finally, the cells were resuspended in 200µl of LiAc(0,1M)/TE(1x). For each transformation were used 100µl of competent cells.

The competent cells were placed at 4 ºC overnight. Each transformation mix contained:

- 20µl of ssDNA carrier (2.5mg/ml) (Sigma-Aldrich),
- 100µl of competent cells,
- 0.2µg of plasmidc DNA (except for the negative control) and
- 600µl of LiAc(0.1 M)/TE(1X)-PEG 50%.

The tubes were incubated 1h at 30 ºC with shaking, and then subjected to a heat-shock for 15min at 42 ºC, followed by cooling for 10min at 4 ºC. The cells were harvested centrifuging 2min at 8,000 rpm (miniSpin centrifuge, Eppendorf), resuspended in 200µl sterile dH_2O, plated on selective medium YNB URA^- and incubated at 30 ºC until the colonies appeared (around 3 days).

The verification of yeast transformants was done by colony PCR. Individual colonies grown on selective medium were picked and re-strain 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 plate, suspended in 100µl of 200mMLiAc/1%SDS solution and incubated for 5min at 70 ºC. After adding 300µl of 100% ethanol and vortexing, the mixture of DNA and cell debris was spun down at 15,000 rpm (Sigma 4-16K centrifuge) for 3min. The pellet was washed with 70% ethanol, dissolved in 100µl upH2O, and cell debris was again spun down at 15,000 rpm (Sigma 4-16K centrifuge) for 15sec. PCR was done using 1µl of the obtained supernatant, and it was performed as described above.

2.4 Western Blot Analysis

2.4.1 Yeast protein extraction and precipitation

Total protein content from *S. cerevisiae* was extracted using the trichloroacetic acid (TCA) method. Cells were grown in YNB until exponential phase (OD_{600}=1) and harvested by centrifuging 5min at 4,000 rpm (miniSpin centrifuge, Eppendorf). The pellet was resuspended in 200µl of 0.2M NaOH/2%β-mercaptoethanol and incubated 10min at 4 ºC, then 400µl of TCA 20% was added, the mixture was left in ice during at least 10min, and then centrifuged 5min at 13,000 rpm (miniSpin centrifuge, Eppendorf). The pellet was washed with 500µl of cold acetone and centrifuged 5min at 13,000 rpm (miniSpin centrifuge, Eppendorf). The acetone was allowed to evaporate, and the precipitate was resuspended in 100µl of Laemmli sample buffer 2X (SDS 4%, 120Mm Tris-HCl, 20% glycerol, 0.1% Bromophenol Blue) and 3µl DTT 0.01M. The samples were stored at -20 ºC.

2.4.2 SDS-PAGE (Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis)

The protein extracts were heated for 10min at 95 ºC before application onto 10% polyacrylamide gel. A two-part polyacrylamide gel, containing one 10% running gel and one 5% stacking gel was loaded with 10µl total protein extract per well. Nzycolour protein marker II (Nzytech) (2µl) was used as reference. The gels were submerged with Running Buffer (19.2mM glycin, 2.5mM Tris base, 0.01% SDS). The electrophoresis run 1h at 20mA/gel. The apparatus used was a Mini Protean Tetra Cell I system (Bio-Rad). When needed, staining was performed with Coomassie Brilliant Blue (50% ethanol, 10% acetic acid, 0.25% Coomassie R258).

2.4.3 Western blot assay

The SDS-PAGE gel was electro blotted in a Trans-Blot Electrophoretic Transfer Cell System (Bio-Rad), onto PVDF membrane (Roche). The transfer was performed in a transfer buffer (19.2mM glycin, 2.5mM Tris, 20% ethanol, 0.05% SDS, pH8.3) for 2h

at 154mA/cm^2 membrane. The membrane was then incubated in agitation in blocking solution (5% of blocking agent (GE Healthcare) in phosphate buffer saline Tween 20 (PBST)) for 2h at room temperature to block the nonspecific sites, and then incubated with the primary antibody, overnight at 4 ºC with gently rotation (roller mixer SRT1, Stuart®). To remove the excess of this antibody, the membrane was washed 3 times with PBST, 5 min/wash, and then incubated with the secondary antibody 2h at room temperature with gentle rotation (roller mixer SRT1, Stuart®). To remove residual antibodies, the membrane was washed 6 times with PBST, 10min/wash, and developed with 500µl of ECL Plus Western Blotting Detection System (Amersham Biosciences) in an Image Analysis System ChemiDoc XRS (Bio-Rad, Laboratories Inc.). Antibodies were stored at -20 ºC. The following antibodies were used:

- Monoclonal anti-KRas antibody (Sigma-Aldrich) – dilution 1:1000 (in PBST),
- Monoclonal anti-EGFR antibody (Santa Cruz Biotechnology) – dilution 1:500 (in PBST),
- Monoclonal anti-EGFR antibody Cetuximab, kindly provided by Merck, USA in its clinical formulation Erbitux® 5mg/mL – dilutions from 1:1000 to 1:1 (in PBST),
- Secondary antibody for anti-KRas and also anti-EGFR Cetuximab detection, polyclonal rabbit anti-mouse antibody coupled with horseradish peroxidase (Sigma-Aldrich) – dilution 1:10,000 (in PBST);
- Secondary antibody for anti-EGFR detection, polyclonal goat anti-Rabbit IgG – Peroxidase (Sigma-Aldrich) – dilution 1:10,000 (in PBST).

Erbitux is a sterile, clear, colorless liquid of pH7.0, containing a small quantity of white visible Cetuximab particulates. Erbitux is composed of a preservative free solution of NaCl, $Na_2HPO_4 \bullet 7H_2O$, $NaH_2PO_4 \bullet H_2O$ and water. Concentrations of these basic components, as well as of the antibody within Erbitux® are commercial secret and were not supplied.

2.5 Native-PAGE

Native protein electrophoresis in opposition to SDS-PAGE relies on separation according to the charge, size and conformation of each protein, which depends on the

amino acid sequence of the protein (isoelectric point) and the pH during electrophoresis. One major advantage of the native-PAGE is that the biological activity of the proteins remains intact (232). Proteins are prepared in a non-reducing, non-denaturing sample buffer 2x (62.5Mm Tris-HCl, pH6.8; 25% glycerol; 1% Bromophenol Blue), which maintains the proteins' secondary structure and native charge. A 10% polyacrylamide gel without SDS was loaded with 5µl sample mixed with 10µl sample buffer (2x). As for SDS-PAGE, 2µl of Nzycolour protein marker II (Nzytech) was used as reference. The running buffer, running conditions and staining procedures were identical as for SDS-PAGE (above) except for the absence of SDS in the Running Buffer formula.

2.6 Yeast Physiology Assays

2.6.1 Chronological life span (CLS)

To follow the chronological aging of yeast on batch cultures, pre-inoculum cultures were allowed to grow overnight until exponential phase (OD_{600nm} 0.4-0.8), and then diluted to an OD of ±0.1 in YNB liquid medium supplemented appropriately. Cellular viability was assessed by Colony Forming Units (C.F.U.) assay, as previously described (233). After 72 hour of growth (time 0 for the aging experiment), culture aliquots were diluted to an OD of 1.0 (OD_{600nm}), corresponding to a cell density of about $1x10^7$cells/ml, and sequentially diluted 1:10 four times in sterile water, to a final dilution of 1:10,000. The last dilution was used to plate 6 drops of 40µl each on YPD agar plates, incubated for 2 days at 30 °C. Colonies formed were counted.

The 3 days cultures were considered to be totally alive and therefore the number of colonies therein obtained was established as 100% of viability. Subsequent older samples were quantified in relation to day 3. Aging was followed until colonies were no longer produced. Statistical analysis was performed as described below.

2.6.2 Cell size analysis

Cells grown on YNB liquid medium were collected in exponential growth phase and observed with a Leica Microsystems DM-5000B epifluorescence microscope, with

appropriate filter settings and a 100x/1.3 oil-immersion objective, as previously described (41). Images were acquired by a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software. Area of 80 to 100 cells was measured using ImageJ freeware (http://imagej.nih.gov/ij/). Statistical analysis was performed as described below.

2.6.3 Cell cycle analysis

Cell cycle analysis was performed as described in the literature (234), with modifications. Initially, 500µl of cells (±10^6cells/ml) were harvest by centrifugation and resuspended in 500µl of 70% ethanol and fixed, overnight at 4 ºC. The cells were then collected by centrifugation 2min at 13,000 rpm (miniSpin centrifuge, Eppendorf), followed by washing 1x in 1mL of 50mM sodium citrate buffer pH7.5. The pellet was resuspended in 400µl of RNase A (2mg/ml in Tris-EDTA, pH8.0) and incubated at 37 ºC overnight. After an identical centrifugation, the pellet was resuspended in 200µl of proteinase K diluted in H_2O (pH7.5 with HCl) to 5mg/ml and incubated 45min at 37 ºC and again centrifuged. The pellet was resuspended in 500µl of 50mM sodium citrate buffer pH7.5 and to 100µl cell suspension were added 20µl of SYTOX®-Green (Life Technologies) (5mM 100x diluted in Tris-EDTA, pH8.0), and incubated overnight at 4 ºC in the dark. Finally, 600µl of Triton X-100 (0.25% v/v in 50mM sodium citrate buffer, pH7.5) was added and vortexed. The final suspension was briefly sonicated three times at 30W, each time for 1-2 seconds, incubated in ice between sonication, to reduce cellular aggregates. The samples were analyzed in an Epics® XL™ (Beckman Coulter) flow cytometer, with an excitation of 497nm and an emission of 520nm according to SYTOX®-Green manufacturer instructions. Flow cytometry data were processed using Flowing Software 2.

2.6.4 Adherence to and invasion of agar

Equal volumes of young yeast cultures were diluted to 1×10^7cells/ml (OD_{600nm}=1), and 200µl of cells suspension was spotted onto SLAD medium agar (2% glucose, 1.67% YNB without amino acids and ammonium sulfate, 0.05mM $(NH_4)_2SO_4$, 2% agar) (235). Cultures were allowed to grow at 30 °C for 7 days. The cells on the

surface of the culture were then removed by washing under running water during 60sec and the remaining culture visualized in a magnifying glass (236). The images were captured in an Image Analysis System ChemiDoc XRS (Bio-Rad, Laboratories Inc.).

Inspection of agar invasion was performed by visualization of longitudinal cuts displaying the aerial and internal agar/growth boundaries by light microscopy (98).

2.7 Statistical Analysis

Data are reported as mean values of at least three independent assays and presented as mean ± SD. The arithmetic means are given with SD with 95% confidence value. Statistical analyses were carried out using Two-way ANOVA. *P <0.05 was considered statistically significant.

CHAPTER III

Results and Discussion

3. Results and Discussion

3.1 Heterologous expression of *KRAS*^{wt} in *Saccharomyces cerevisiae*

The molecular mechanism and basic machinery of fundamental cellular processes appear to be highly conserved between the yeast *S. cerevisiae* and other eukaryotic species, including humans. Into this context, "humanized yeast systems" emerged as a tool to study molecular aspects of different biological processes (14). Here, we describe the construction of one such humanized yeast model expressing the human oncogene *KRAS*. *S. cerevisiae* has two orthologues of the human RAS proteins, Ras1p and Ras2p (31). Therefore, we individually expressed the human KRas^{wt} protein in yeast wild type haploid strain BY4741 (Euroscarf), as well as the strains deleted in *RAS1* or *RAS2* from the same genetic background (Table 1). The double deletion of *RAS1* and *RAS2* renders yeast unviable (54). Previous attempts to sequentially delete the two *RAS* genes using *KRAS* gene to complement of one of the deletions by to ensure survival failed (228). For this reason the double mutant was not considered.

In order to express *KRAS*^{wt}, the gene was amplified (Fig. 14) from pLenti-*KRAS*^{wt} plasmid, kindly offered from IPO-Porto, and afterwards extracted from agarose gel electrophoresis and cloned into the yeast plasmids p426GPD and p416GPD. These plasmids have different origins of replication, a centromeric (p416) and a 2μ (p426) and respectively differ in the number of copies produced (1 and 10-30 copies per cell). They both, share a strong GPD promoter and a CYC1 terminator (229). HindIII and XhoI enzymes were chosen to be included into the primers that amplify the *KRAS* gene, since their restriction sequences are present in the multi cloning site (MCS) of both plasmids and do not cut the *KRAS* cDNA. The cDNA were inserted in the p426GPD plasmid through the action of a T4 ligase (Roche). Plasmid amplification in *E. coli* XL1-Blue was performed as described in Material and Methods. Plasmidic DNA extracted from *E. coli* XL1-Blue p426GPD-*KRAS* clones was double digested with HindIII and XhoI and the resulting product analyzed by electrophoresis (Fig. 15). The positive clones were confirmed by the presence of two expected bands: a band of approximately 6,637bp corresponding to the empty plasmid and a band of approximately 567pb corresponding to the *KRAS*^{wt} fragment.

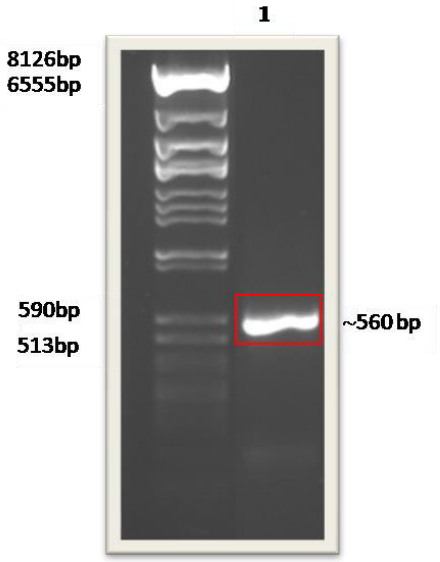

Figure 14. Electrophoretic analysis in 1% agarose gel of *KRAS*^{wt} amplified by PCR from pLenti-*KRAS*^{wt} plasmid (lane 1). Molecular weight marker λ DNA/Eco47I by Fermentas was used as ladder. In red: the fragment corresponding to *KRAS*.

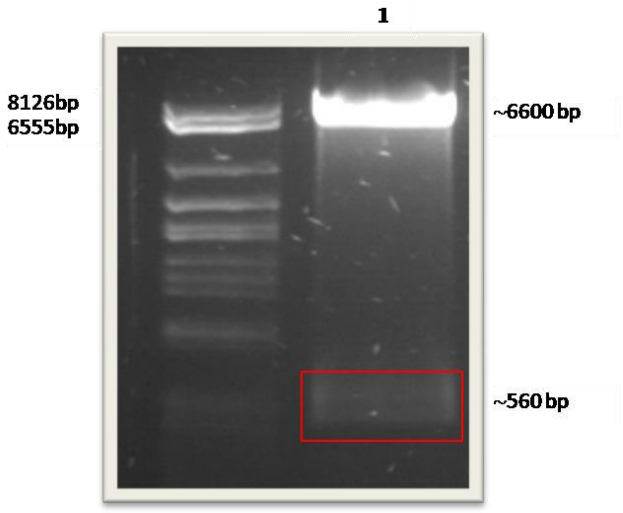

Figure 15. Electrophoretic analysis of p426GPD-*KRAS* DNA from *E. coli* XL1-Blue double digested with HindIII and XhoI (lane 1). Molecular weight marker λ DNA/Eco47I by Fermentas was used as ladder. In red: the fragment corresponding to *KRAS*.

Cloning procedures in *E.coli* XL1-Blue were repeated using the low copy plasmid p416. The DNA bacteria clones, transformed with the p416GPD-*KRAS*, were double digested with HindIII and XhoI enzymes and the resulting product analyzed by electrophoresis (Fig. 16). The positive clones should yield two bands: a band of

approximately 5,804bp corresponding to the empty plasmid and a band of approximately 567pb corresponding to the *KRAS* fragment, but the result of electrophoresis only showed one band of approximately 5,804 bp, revealing problems in DNA ligation into the p416GPD plasmid.

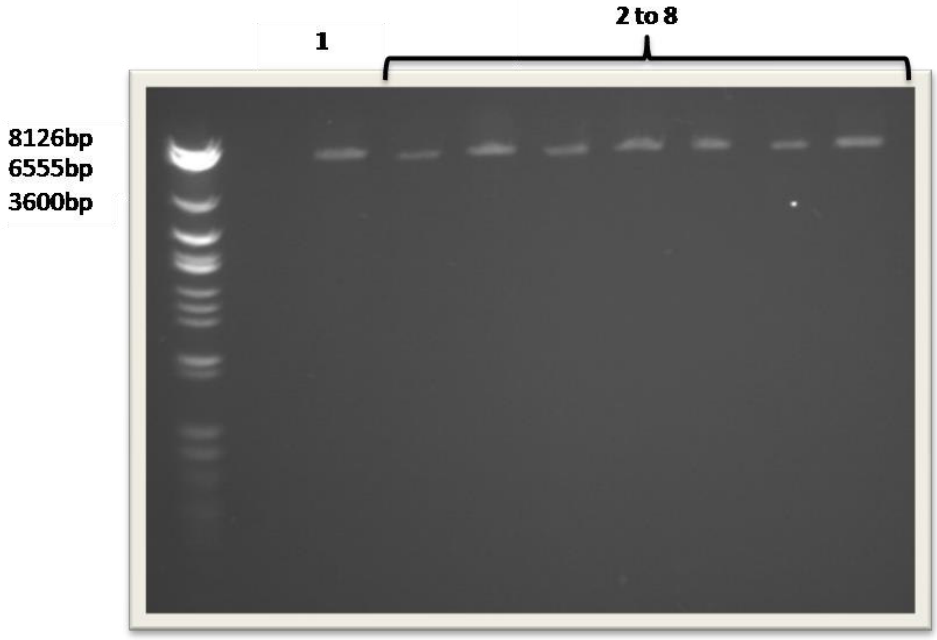

Figure 16. Electrophoretic analysis of p416GPD-*KRAS* DNA from *E.coli* XL1-Blue double digested with HindIII and XhoI (lane 2 to 8). Double digestion of p416GPD with the enzimes HindIII and XhoI (lane 1). Molecular weight marker λ DNA/Eco47I by Fermentas was used as ladder.

Thereby, we proceeded to the yeast transformation with p426GPD-$KRAS^{wt}$. The presence of p426GPD-$KRAS^{wt}$ in yeast strains BY4741wt, BY4741 *Δras1* and BY4741 *Δras2* transformants was tested by colony PCR (Fig. 17). Clones testing positive presented a band of approximately 567pb corresponding to the $KRAS^{wt}$ fragment.

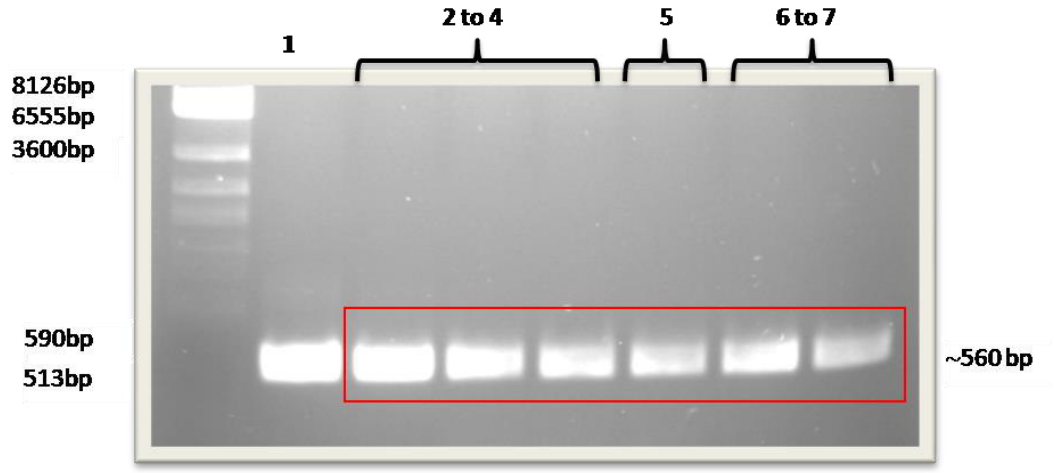

Figure 17. Colony PCR of *S. cerevisiae* BY4741wt (lane 2 to 4), BY4741 Δ*ras1* (lane 5) and BY4741 Δ*ras2* (lane 6 to 7) transformed with the plasmid p426GPD-*KRAS*. The *KRAS* insert corresponds to a band with a molecular weight of approximately 560pb. As positive control the DNA plasmid harboring *KRAS*^{wt} was identically amplified by PCR (lane 1). Molecular weight marker λ DNA/Eco47I by Fermentas was used as ladder. In red: the fragment corresponding to *KRAS*.

Moreover, the expression of *KRAS* in yeast was tested by Western blot (WB) using a monoclonal antibody. For that purpose, the protein extracts of all the strains transformed with p426GPD-*KRAS* (Table 1) were isolated from exponential cultures in YNB without uracil (selectable marker) and subjected to WB using the anti-KRas antibody as described in the Materials and Methods. Representative results shown in figure 18, confirm the expression of KRas protein in yeast.

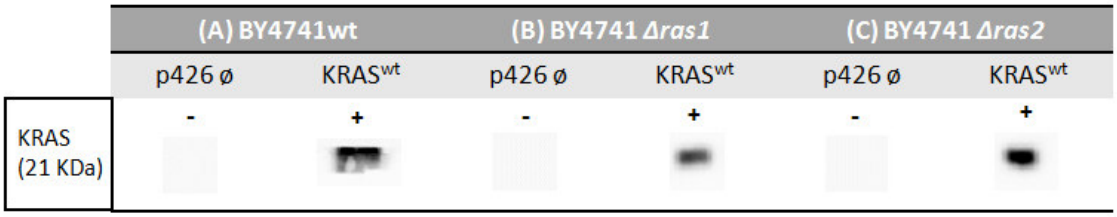

Figure 18. WB of protein extracts from the strains BY4741wt (A) BY4741 Δ*ras1* (B) and BY4741 Δ*ras2* (C) transformed with the plasmid p426GPD-*KRAS*^{wt} blotted with KRas antibody. Negative controls with transformants harbouring empty plasmids are shown.

These strains are part of a large *platform* consisting of yeast strains deleted in the yeast RAS genes in two genetic backgrounds, and complemented with the human *KRAS* cDNA. The platform has been built with the purpose of creating a tool for pharmacological testing programmed within the Glycopharm ITN Marie Curie Network. The two yeast backgrounds are the BY4741 (Euroscarf) above mentioned and the W303, both from the same mating type BY4741 Mat*a* and W303-1A. Both strains deleted in *RAS1* and *RAS2* were already available. The BY4741 was used to complement with the *KRAS* cDNA. Depending on the results coming from the compared phenotypic responses, the W303 will also be used to express *KRAS*. For now it provides a phenotype control. Importantly, the chromosomal insertion of *KRAS* cDNA was also achieved in BY4741 within the scope of a complementary work, as was the phenotypic screening of the BY4741 set of strains.

3.2 Identification of the yeast target of anti-EGFR (Cetuximab) by Western Blot

Erbitux® is an oncological therapeutic cocktail commercially exploited by Merck (Darmstadt, Germany) that has Cetuximab (Ctx) as active ingredient and is used in the treatment of colorectal cancer (CRC) (187). Ctx is a monoclonal antibody (mAb) that blocks the Epidermal Growth Factor Receptor (EGFR), a protein highly present on the surface of mammalian cells and responsible for recognition and signaling of the EGF growth hormone. Ctx is highly specific for EGFR (165, 169). Still, its therapeutic efficacy is confined to the so-called *KRAS* wild-type tumors (217). Mutations at the level of KRAS, one of the proteins downstream the EGFR signaling cascade, preclude its appropriate effect (219). Unfortunately, these mutations are present in a large fraction of the population, reducing these patients' therapeutic alternatives. Additionally, CRC patients may also acquire resistance-mediating mutations within the extracellular EGFR domain, which alter the affinity for Ctx (218). Therefore, modulating the exact binding sites of anti-EGFR antibodies may help to predict the response to EGFR-targeting treatment.

Taking into account the high specificity of Ctx against EGFR, and considering that it mediates a KRAS-including signaling cascade controlling proliferation and that yeast also harbors a RAS/cAMP/PKA signaling cascade, the yeast constructions generated in the first part of this work were used to test the ability of Ctx to bind to yeast proteins. If yeast possesses an EGFR-like protein, it should complex with Ctx. This was attempted before with success (237). One single band was obtained identified as pyruvate decarboxylase 1 (Pdc1). The present work sought to confirm this identification. For this purpose, Ctx was used as primary antibody in WB against the whole yeast proteome of *S. cerevisiae* BY4741, BY4741 Δ*ras1*, BY4741 Δ*ras2* strains. This was repeated using also the transformants BY4741 p426GPD-*KRAS*, BY4741 Δ*ras1* p26GPD-*KRAS* and BY4741 Δ*ras2* p426GPD-*KRAS*.

As mentioned in the Introduction, Ctx is a recombinant protein, which contains the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions. The anti-human secondary antibody recognized the human and free constant regions (Fc) of the heavy and light chains of Ctx that match to 2/3 of the antibody. The region that is recognized by the anti-mouse antibody is the variable

region (Fv) of the heavy and light chains, both from a mouse anti-EGFR monoclonal antibody. This variable region of Ctx is responsible for binding the antigen and only corresponds to 1/3 of the antibody. A complex of the anti-mouse antibody, with HRP was used for primary antibody binding recognition. Standard protocols for WB were used without success (not shown). Therefore an optimization of the protocol took place:

1) the primary antibody dilution was decreased up to 1:1,
2) the secondary antibody dilution was decreased up to 1:5000,
3) the amount of protein extract was increased up to 20µl,
4) several blocking agents for different incubation times, between 30 minutes and 4 hours were used,
5) increased times of exposition up to 480sec were tried, and
6) virgin Ctx ampoules were used to freeze-dry and resuspended the lyophilized antibody in 5ml of ultra-pure water or phosphate buffer saline (PBS), causing an increase in its concentration.

None of these optimization steps yielded a positive result (not shown). A final approach was attempted. The aggregation of monoclonal antibodies is a crucial problem that results not only in the fast loss of activity but also affects product safety for therapeutic utilization (238, 239). Aggregation of monoclonal antibodies can be induced by chemical alterations, namely the modification of covalent bonds through deamidation, oxidation or disulfide bridge shuffling (240). Aggregation may also be caused by freeze–thawing or elevated temperature, typical stress factors during production, transport and storage. Physical instability includes protein unfolding, adsorption or non-covalent interaction of native protein with itself or other proteins (241, 242). Mechanically induced aggregation of the monoclonal antibody Ctx can be removed either by filtration (243) (e.g. 0.22 mm filter) or by centrifugation (244). Several Ctx samples were therefore centrifuged at 12,000 rpm (miniSpin centrifuge, Eppendorf) during 10min and subsequently observed by PAGE.

SDS-PAGE is commonly used to test recombinant monoclonal antibody purity or stability and detect antibody fragments and aggregates (243). In theory, when analyzing an antibody by PAGE under non-denaturing conditions (Native-PAGE), there should be only one band of the intact molecule (243), while in SDS-PAGE there should be two bands corresponding to the light and heavy chains of the antibody. Ctx is a 148

kDa glycoprotein composed of two light and two heavy chains of, respectively, 23 kDa and 50 kDa each (243, 245). A centrifuged Ctx sample was subjected to SDS-PAGE (Fig. 19) and Native-PAGE (not shown). In SDS-PAGE, Ctx presented more than the two expected bands of 23 and 50 kDa. The bands with higher molecular mass probably correspond to aggregates non-separated by centrifugation, while the bands with lower molecular mass could correspond to fragmented peptides. On the other hand, the Native-PAGE did not yield any band (not shown). Based on these two results, and the absence of hybridization detected by WB above described, the samples of Ctx were not considered for further use.

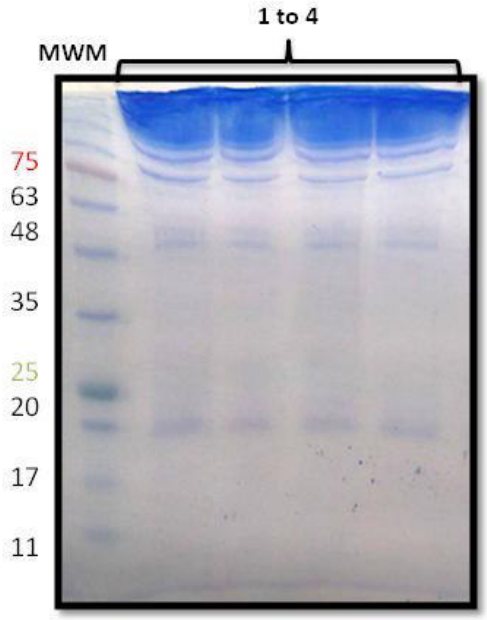

Figure 19. SDS-PAGE of mAb Cetuximab, after centrifugation at 12,000 rpm, during 10min (Lane 1 to 4). As molecular weight marker (MWM) were used 2µl of NZYCOLOUR protein marker II.

In alternative, an anti-EGFR commercial antibody from Santa Cruz Biotechnologies was used for WB the full proteomes of the yeast strains above mentioned (Fig. 20). Up to three immunoreactive bands appeared (red boxes) of molecular weights around 70kDa, 40kDa and ≤19kDa. All the strains showed bands at the same molecular weights. A negative control was made using the same samples and only the secondary antibody produced in goat anti-Rabbit IgG–Peroxidase to verify whether any of the bands in figure 20 was a result of the secondary antibody hybridization and not directly due to anti-EGFR recognition. The control showed no

bands as due (data no shown). Therefore, the bands obtained in WB using the anti-EGFR primary antibody should be specific to EGFR-like protein(s).

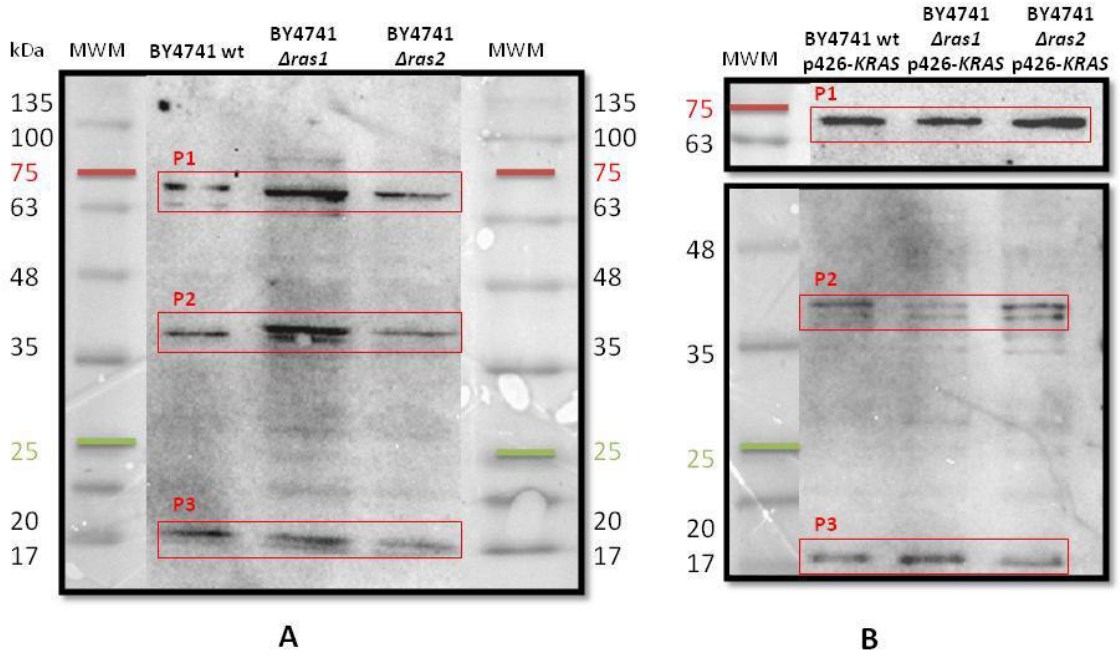

Figure 20. Identification of the yeast target of anti-EGFR. SDS-PAGE followed by immunoblotting of *S. cerevisiae* BY4741wt , BY4741 Δ*ras1* and BY4741 Δ*ras2* (A) and the same strains transformed with p426-$KRAS^{wt}$ (B) with the anti-EGFR as primary antibody and anti-Rabbit IgG – Peroxidase as secondary antibody. The 3 immunoreactive bands were named, from top to bottom, as "P1", "P2" and "P3"

The 3 immunoreactive bands (red boxes in Fig. 20) were named, from top to bottom, as "P1", "P2" and "P3". The proteins corresponding to "P1", "P2" and "P3" bands were excised from the SDS-PAGE gel and sent for identification at the Unidad de Proteómica-Moncloa, Parque Tecnológico de Madrid/Univ. Complutense, Madrid, Spain. There, the protein bands were reduced and alkylated with carbamidomethylated, and subsequently digested with trypsin that cleaves specific sites located after lysine and arginine residues. The resulting peptides were concentrated on a ZipTip micropurification column and eluted onto an anchorchip target for analysis on a Bruker Autoflex Speed MALDI TOF/TOF instrument. The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination. MALDI MS/MS was performed on 15 peptides for peptide fragmentation analysis. The MS and MS/MS spectra were combined and used for database searching using the Mascot software. The

data were finally BLASTED against protein databases downloaded from NCBI, including the NRDB database containing more than 17 million known non-redundant protein sequences. The results from protein identification are shown in Table 3. The higher molecular mass proteins identified are discussed below while the lower one was ignored due to the very low score that suggests an unreliable identification.

Table 3. Protein identification by MS peptide mapping and sequencing analysis.

Sample name	Protein found in database	MW	Score	Sequence coverage
P1	Heat shock protein SSA2 (*S.cerevisiae* S288c)	69599	99	19%
P1	Heat shock protein SSB2 (*S.cerevisiae* S288c)	66668	94	13%
P2	Glyceraldehyde-3-phosphate dehydrogenase 3 TDH3 (*S.cerevisiae* S288c)	35838	263	30%
P3	Peroxiredoxin type-2 AHP1 (*S.cerevisiae* S288c)[1]	19274[1]	58[1]	22%[1]

Note: 1. The identification of this protein is uncertain because the score is outside the 95% confidential level.

P1 - Heat shock proteins Ssa2p and Ssb2p

SSA genes encode chaperone proteins that are comprised in the *S. cerevisiae* SSA subfamily of the large and evolutionarily conserved family of cytosolic Hsp70 proteins (246). Hsp70 proteins were classified based on their induction by heat shock, and their approximate size of 70kDa (247). The Hsp70 proteins show a highly conserved structure which is composed by different domains (Fig. 21): a domain highly conserved that exhibits ATPase activity of aproximately 44 kDa called N-terminal nucleotide-binding domain (NBD), a middle flexible linker region, a substrate binding domain (SBD) of aproximately 15 kDa, which interacts with stretches of hydrophobic

aminoacids in peptides and, finally, a 10 kDa α-helical C-terminal domain. The C terminus of this protein is the least conserved (248, 249).

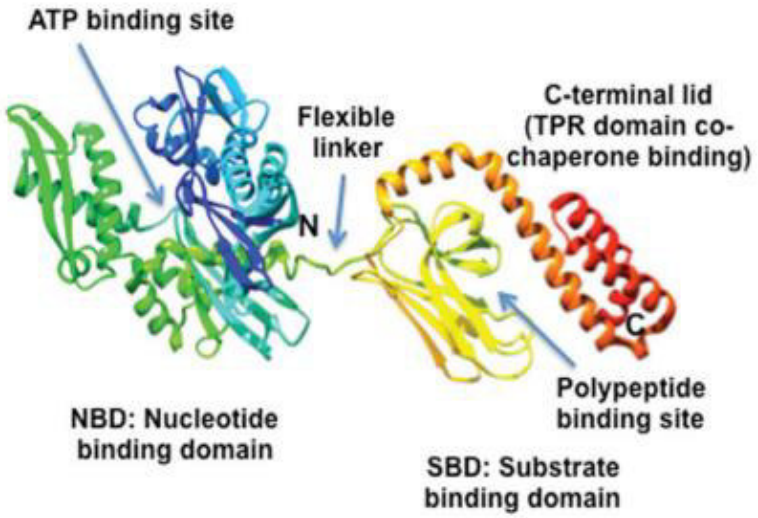

Figure 21. The structure of Hsp70 protein, with ATP binding domain, polypeptide binding domain and C-terminal helical. Withdraw from (250).

The main function of these proteins is to serve as molecular chaperones, binding newly translated proteins, to assist in proper folding (247, 251). *S. cerevisiae* has at least 9 cytosolic forms of Hsp70: Ssa1-4, Ssb1 and 2, Sse1 and 2, and Ssz1 (247, 251). These proteins are involved in the disassembling of misfolded proteins aggregates, in the translocation of proteins into the mitochondria and endoplasmatic reticulum, and in the regulation of the expression of other heat shock proteins. Moreover, they are involved in protein refolding after stress, and in the control of the activity of regulatory proteins from signal transduction pathways (252, 253). All these important activities of Hsp70 are regulated by its capacity to interact with hydrophobic stretches of proteins in an ATP dependent mode, avoiding non-productive interactions that would cause aggregation and encourage protein refolding (254). The 4 *S. cerevisiae* SSA genes are closely related, with Ssa2p sharing 99%, 84%, and 85% amino acid identity with Ssa1p, 3 and 4, respectively (255). Ssa2p is the only member of the SSA subfamily whose transcription is not inducible by heat or stress (256). Ssa2p fused with GFP was observed to relocate from the cytosol to the outer surface of mitochondria upon

oxidative stress (257). Additionally, Ssa2p has been implicated in DNA damage as it has been identified as members of Rad9 DNA checkpoint complexes (258). Although the majority of Ssa proteins are found in the cytosol, Ssa1p and Ssa2p can also be detected in the cell wall (256). In recent times, it was demonstrated that Ssa1 play a role as signal transducer controlling growth through G1 cyclin quantity and activity, a procedure mainly dependent on Ssa phosphorylation at a very conserved threonine residue in the NBD (259). Given the importance of the Hsp70 family in essential cellular functions Hsp70 can be a potential therapeutic target for a variety of human diseases.

The recent data indicate that cancer cells become 'addicted' to Hsp70 through their chaperone activity on multiple cell signaling and survival pathways. Three of these cancer-relevant activities of Hsp70 are: apoptosis, senescence and autophagy (Fig. 22) (250). Overall, the Hsp70 family of proteins can be thought as a potent buffering system for cellular stress, on which cancer cells rely heavily for survival (250). Accordingly, the vast majority of human tumors over-express Hsp70 family members, and expression of these proteins is typically a marker for poor prognosis (260). Recently, inhibitors of the Hsp90 chaperone have emerged as important anticancer agents, and probably also Hsp70 can be a potential target as inhibitor of carcinogenesis (261). Jäättelä was the first to prove that silencing of Hsp70 with antisense RNA resulted in massive cell death in breast cancer cell lines, but it was demonstrated to be non-toxic for normal cells (262, 263). If the present results in yeast are confirmed, the Hsp70 could be a target that inhibited, identically induces cell death upon a stimulus by an EGFR-like antibody. It would be interesting to verify the expression of the Hsp70p in these strains transformants after treatment with Ctx and, afterwards, assess the apoptosis, senescence and autophagy activities. Other interesting experiment would be the identification of the yeast target of the anti-EGFR (Ctx) in the strains transformed with the mutated *KRAS*.

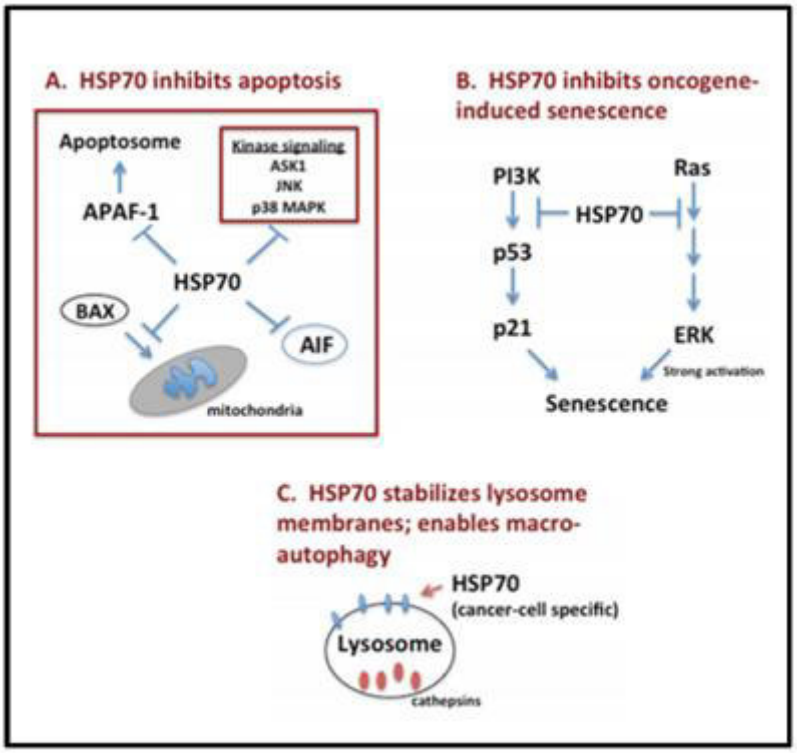

Figure 22. Cancer pathways associated with Hsp70. A) Hsp70 inhibits the intrinsic and extrinsic apoptosis pathways, by inhibiting BAX translocation to mitochondria, the recruitment of APAF-1 to the apoptosome, the activity of stress-induced kinases and the function of AIF-1. B) Hsp70 inhibits oncogene induced senescence. C) Hsp70 localizes to lysosome membranes in cancer cells, stabilizes lysosome function and allows autophagy, a key cancer survival pathway. Adapted from (250).

P2 - Glyceraldehyde-3-phosphate dehydrogenase 3 (Tdh3p)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been documented as an important enzyme for energy metabolism, the production of ATP and pyruvate, in the cytoplasm, through anaerobic glycolysis (264). In addition, GAPDH has recently been implicated in several non-metabolic processes, including DNA repair (265), tRNA export (266), regulation of mRNA stability (267), membrane fusion and transport (268), cytoskeletal dynamics (269) and initiation of apoptosis (270). The multifunctional properties of GAPDH are likely to be controlled by its oligomerization, posttranslational modifications and subcellular localization (271). GAPDH is a tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bi-phosphoglycerate and consumes inorganic phosphate to harness the energy into the reduced form of nicotinamide adenine dinucleotide (NADH) (272). Three genes, *TDH1-3*, encode

related but not identical polypeptides that form catalytically active homo-tetramers with different specific activities (273). Fascinatingly, these catalytically active enzymes are found in the cytoplasm and cell wall. Tdh2p and Tdh3p are detected in cells growing exponentially while Tdh1p is principally detected during stationary phase (274). GAPDH activity is necessary during gluconeogenesis, the process in which glucose is produced from non-carbohydrate precursors. This process enables yeast cells to grow on ethanol, glycerol or peptone. The reactions of gluconeogenesis mediate conversion of pyruvate to glucose (275).

GAPDH is a ubiquitous enzyme of approximately 37kDa that is found ubiquitously located in the cytoplasm, vesicles, mitochondria and nuclei of cells. In yeast it has also been found in the outer part of the cell wall, i.e., the external surface of the cell (276, 277). Its detection by an externally added antibody is therefore not impossible. The results suggest that GAPDH, more precisely Tdh3 can be a yeast target of anti-EGFR.

Similarity between human EGFR and the yeast targets of anti-EGFR

Amino acid sequence alignments between EGFR, Tdh3p, Ssa2p and Ssb2p were generated using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/) (Fig. 23). This program produces biologically significant multiple sequence alignments of divergent sequences and it calculates the greatest match for the selected sequences. The alignment between the human EGFR and the yeast Ssa2p, Ssb2p and Tdh3p proteins was studied. The three yeast proteins were compared. As expected, Ssa2p and Ssb2p are highly similar to each other, while Tdh3p is very different.

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TDH3                        ----------------------------------------------------
SSA2                        -------------------------MSKAVGIDLGTTYSCVAHFSNDRVD 25
SSB2                        --------------------MAEGVFQGAIGIDLGTTYSCVATYESS-VE 29
EGFR                        MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLS 50


TDH3                        --------MVRVAINGFGRIGR----------------------LVMRIA 20
SSA2                        IIANDQGNRTTPSFVGFTDTER----------------------LIGDAA 53
SSB2                        IIANEQGNRVTPSFVAFTPQER----------------------LIGDAA 57
EGFR                        LQRMFNNCEVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIP 100
                                     .  .   :    *                     :   .
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TDH3                            LSRPNVEVV------------ALNDPFITNDYAAYMFKYDSTH------- 51
SSA2                            KNQAAMNPANTVFDAKRLIGRNFNDPEVQGDMKHFPFKLIDVD------- 96
SSB2                            KNQAALNPRNTVFDAKRLIGRRFDDESVQKDMKTWPFKVIDVD------- 100
EGFR                            LENLQIIRGNMYYENSYALAVLSNYDANKTGLKELPMRNLQGQKCDPSCP 150
                                 ..  :                :      .     ::  . .

TDH3                            -----------------------------GRYAGEVSHDDKHIIVD-GKK 71
SSA2                            -----------------------------GKPQIQVEFKGETKNFT-PEQ 116
SSB2                            -----------------------------GNPVIEVQYLEETKTFS-PQE 120
EGFR                            NGSCWGAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQCAAGCTGPRE 200
                                                             *.   :  .          .:

TDH3                            IATYQE---RDPANLPWGSSNVDIAIDSTGVFKELDTAQKHIDAGAKK-- 116
SSA2                            ISSMVLGKMKETAESYLG-AKVNDAVVTVPAYFNDSQRQATKDAGTIAGL 165
SSB2                            ISAMVLTKMKEIAEAKIG-KKVEKAVITVPAYFNDAQRQATKDAGAISGL 169
EGFR                            SDCLVCRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCVKKC 250
                                        :: *        :     :.         :   .*

TDH3                            ---VVITAPSSTAPMFVMG-------------------------VNEEKY 138
SSA2                            NVLRIINEPTAAAIAYGL-------------DKKGKEEHVLIFDLGGGTF 202
SSB2                            NVLRIINEPTAAAIAYGLGA-----------GKSEKERHVLIFDLGGGTF 208
EGFR                            PRNYVVTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGIGEF 300
                                    ::.   : .                                :.   :

TDH3                            TSDLKIVSNASCTTN---CLAPLAKVINDAFG------------------ 167
SSA2                            DVSLLSIEDGIFEVK---ATAGDTHLGGEDFDNR---------------- 233
SSB2                            DVSLLHIAGGVYTVK---STSGNTHLGGQDFDTN---------------- 239
EGFR                            KDSLSINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDIL 350
                                  .*          :   . :   ::    *

TDH3                            ----------IEEGLMTTVHSLTATQ------------------------ 183
SSA2                            ----------LVNHFIQEFKRKNKKD------------------------ 249
SSB2                            ----------LLEHFKAEFKKKTGLD------------------------ 255
EGFR                            KTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNI 400
                                          : :            :

TDH3                            KTVDGPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLP------------- 220
SSA2                            LSTNQRALRRLRTACERAKRTLSSSAQTSVEIDSLFEGIDFYTSITRARF 299
SSB2                            ISDDARALRRLRTAAERAKRTLSSVTQTTVEVDSLFDGEDFESSLTRARF 305
EGFR                            TSLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRG 450
                                 : .  : :    .              :    .::

TDH3                            -ELQGKLTG-------------------MAFRVPTVDVSVVDLTVKLNKE 250
SSA2                            EELCADLFRSTLDPVE------------KVLRDAKLDKSQVDEIVLVGGS 337
SSB2                            EDLNAALFKSTLEPVE------------QVLKDAKISKSQIDEVVLVGGS 343
EGFR                            ENSCKATGQVCHALCSPEGCWGPEPRDCVSCRNVSRGRECVDKCNLLEGE 500
                                 :                             :  . . . :*    :  .

TDH3                            TT------------------------------------------------ 252
SSA2                            TR------------------------------------------------ 339
SSB2                            TR------------------------------------------------ 345
EGFR                            PREFVENSECIQCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPHCVKTC 550
                                .

TDH3                            ----YDEIKKVVKAAAEGKLKGVLGYTEDAVVS------SDFLGDSHS-- 290
SSA2                            ----IPKVQKLVTDYFNGKEPNRSINPDEAVAYGAAVQAAILTGDESS-- 383
SSB2                            ----IPKVQKLLSDFFDGKQLEKSINPDEAVAYGAAVQGAILTGQSTSD- 390
EGFR                            PAGVMGENNTLVWKYADAGHVCHLCHPNCTYGCTGPGLEGCPTNGPKIPS 600
                                      : :.::    :.        .: :         .   .

TDH3                            ------------SIFDASAGIQLS-----------------PKFVKLVSW 311
SSA2                            ----KTQDLLLLDVAPLSLGIETAG-GVMTKLIPRNSTIPTKKSEVFSTY 428
SSB2                            ----ETKDLLLLDVAPLSLGVGMQG-DIFGIVVPRNTTVPTIKRRTFTTV 435
EGFR                            IATGMVGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQERELVEPLTPS 650
                                             :   . *:                         : .

TDH3                            YDNEYGYSTRVVDLVEHVAKA------------------------------ 332
SSA2                            ADNQPGVLIQVFEGERAKTKDNNLLGKF---------------------- 456
SSB2                            SDNQTTVQFPVYQGERVNCKENTLLGEF---------------------- 463
EGFR                            GEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAIK 700
                                 :        : .  .

TDH3                            -------------------------------------------------
SSA2                            ELSGIPPAPRGVPQIEVTFDVDSNGILNVS--------------------- 486
SSB2                            DLKNIPMMPAGEPVLEAIFEVDANGILKVT--------------------- 493
EGFR                            ELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPF 750
```

```
TDH3                            ---------------------------------------------------
SSA2                            -------------------------------------AVEKGTGKSNKIT 499
SSB2                            -------------------------------------AVEKSTGKSSNIT 506
EGFR                            GCLLDYVREHKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLV 800

TDH3                            ---------------------------------------------------
SSA2                            ITNDKGRLSKEDIEKMV-AEAEKFKEEDEKESQRIASKN----------- 537
SSB2                            ISNAVGRLSSEEIEKMV-NQAEEFKAADEAFAKKHEARQ----------- 544
EGFR                            KTPQHVKITDFGLAKLLGAEEKEYHAEGGKVPIKWMALESILHRIYTHQS 850

TDH3                            ---------------------------------------------------
SSA2                            ----------QLESIAYSLKNTISE--AGDKLEQADKDA----VTKKAEE 571
SSB2                            ----------RLESYVASIEQTVTDPVLSSKLKRGSKSK----IEAALSD 580
EGFR                            DVWSYGVTVWELMTFGSKPYDGIPASEISSILEKGERLPQPPICTIDVYM 900

TDH3                            ---------------------------------------------------
SSA2                            TIAWLDSNTTATKEEFDDQLKELQEVANPIMSKLYQAG------------ 609
SSB2                            ALAALQIED-PSADELRKAEVGLKRVVTKAMSSR---------------- 613
EGFR                            IMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGDERMHLPSPTDS 950

TDH3                            ---------------------------------------------------
SSA2                            --------------------------GAPEGAAPGGFPGGAPPAPEAEGP 633
SSB2                            ---------------------------------------------------
EGFR                            NFYRALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLLSSLSATSNNS 1000

TDH3                            ---------------------------------------------------
SSA2                            TVEEVD------------------------------------------- 639
SSB2                            ---------------------------------------------------
EGFR                            TVACIDRNGLQSCPIKEDSFLQRYSSDPTGALTEDSIDDTFLPVPGEWLV 1050

TDH3                            -------------------------------------------
SSA2                            -------------------------------------------
SSB2                            -------------------------------------------
EGFR                            WKQSCSSTSSTHSAAASLQCPSQVLPPASPEGETVADLQTQ 1091
```

Figure 23. Alignment between human EGFR and *S. cerevisiae* protein Ssa2p and its close homologue Ssb2p, and Tdh3p. EGFR protein sequence of 1091 amino acids was obtained from NCBI (accession: AAH94761.1 GI: 63101670). Ssa2p with 639 amino acids, Ssb2p with 613 amino acids and Tdh3p with 332 amino acids were obtained from SGD.

Ssb2p is predicted to interact physically with yeast Ras1p (http://www.yeastgenome.org/ (278)). Ras proteins are also very conserved, KRAS sharing with Ras1p and Ras2p 63% and 64% identity respectively (Fig. 24). Therefore, the probability that Ssb2p and the very similar Ssa2p might be able to interact with KRAS, makes them better candidates for EGFR homology than Tdh3p.

```
RAS1                             MQGNKSTIREYKIVVVGGGGVGKSALTIQFIQSYFVDEYDPTIEDSYRKQ 50
RAS2                             MPLNKSNIREYKLVVVGGGGVGKSALTIQLTQSHFVDEYDPTIEDSYRKQ 50
KRAS                             -------MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQ 43
                                       : ***:****.***********: *.:****************
```

```
RAS1                            VVIDDKVSILDILDTAGQEEYSAMREQYMRTGEGFLLVYSVTSRNSFDEL 100
RAS2                            VVIDDEVSILDILDTAGQEEYSAMREQYMRNGEGFLLVYSITSKSSLDEL 100
KRAS                            VVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDI 93
                                ****.:..:****************:****.***** *::::. .*::::

RAS1                            LSYYQQIQRVKDSDYIPVVVVGNKLDLENERQVSYEDGLRLAKQLNAPFL 150
RAS2                            MTYYQQILRVKDTDYVPIVVVGNKSDLENEKQVSYQDGLNMAKQMNAPFL 150
KRAS                            HHYREQIKRVKDSEDVPMVLVGNKCDLPS-RTVDTKQAQDLARSYGIPFI 142
                                  * :** ****:: :*:*:**** ** . : *. ::.  :*:. . **:

RAS1                            ETSAKQAINVDEAFYSLIRLVRDDGGKYNSMNRQLDNTNEIRDSELTSSA 200
RAS2                            ETSAKQAINVEEAFYTLARLVRDEGGKYN--KTLTENDNSKQTSQDTKGS 198
KRAS                            ETSAKTRQRVEDAFYTLVREIR----QYR----------LKKISKEEKTP 178
                                *****   .*::***:* * :*    :*.           : *:  . .

RAS1                            TADREKKNNGSYVLDNSLTNAGTGSSSKSAVNHN------------GETT 238
RAS2                            GANSVPRNSGGHRKMSNAANGKNVNSSTTVVNARNASIESKTGLAGNQAT 248
KRAS                            GCVKIKK------------------------------------------- 185
                                 .    :

RAS1                            KRTDEKNYVNQNNNN----EGNTKYSSNGNGNRSDISRGNQNNALNSRSK 284
RAS2                            NGKTQTDRTNIDNSTGQAGQANAQSANTVNNRVNNNSKAGQVSNAKQARK 298
KRAS                            ---------------------------------------------------


RAS1                            QSAEPQKNSSANARKESSGGCCIIC 309
RAS2                            QQAAPGGN-TSEASKSGSGGCCIIS 322
KRAS                            ---------------------CIIM 189
                                                     ***
```

Figure 24. Alignment between human oncogene KRAS and *S. cerevisiae* protein Ras1p and its close homologue Ras2p. KRAS protein sequence of 189 amino acids was obtained from NCBI (accession: AGC09594.1 GI: 440503003). Ras1p with 309 amino acids and Ras2p with 322 amino acids were obtained from SGD.

The alignment with EGFR (Fig. 25) showed that Ssa2p and Ssb2p proteins aligned in the region of EGFR domain I (L1) (green), followed by domain II (CR1), domain III (L2) (yellow), domain IV (CR2), the transmembrane (red) and juxtamembrane (blue) domains and the tyrosine kinase domain. Ssa2p residues 1-379 and Ssb2p residues 9-385, that corresponds to ATP binding domain, align with EGFR domains I, II, III and IV as well as with critical residues involved in Ctx binding (grey). The EGFR transmembrane and juxtamembrane domains align with some residues of the peptide binding domain of Ssa2p (384-540) and Ssb2p (301-547). The grey residues correspond to critical residues involved in Ctx binding according to the literature, since their mutation resulted in a decrease of antibody binding capacity (166, 279).

```
SSA2                            ------------------------MSKAVGIDLGTTYSCVAHFSNDRVD 25
SSB2                            -------------------MAEGVFQGAIGIDLGTTYSCVATYESS-VE 29
EGFR                            MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLS 50
                                                     .  :  . ... : :. :..  :.
```

```
SSA2                    IIANDQGNRTTPSFVGFTDTER-----------------LIGDAAKNQAA 58
SSB2                    IIANEQGNRVTPSFVAFTPQER-----------------LIGDAAKNQAA 62
EGFR                    LQRMFNNCEVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIP 100
                        :    :. ... . : :*  :*                 **.  : :: .

SSA2                    MN-----PANTVFDAKRLIGRNFNDPEVQGDMKHFPFKLIDVDGKP---- 99
SSB2                    LN-----PRNTVFDAKRLIGRRFDDESVQKDMKTWPFKVIDVDGNP---- 103
EGFR                    LENLQIIRGNMYYENSYALAVLSNYDANKTGLKELPMRNLQGQKCDPSCP 150
                        ::       *  :: .  :.   :    : .:*  *:: :: :

SSA2                    ------QIQVEFKGETKNFTPEQISSMVLGK------MKETAESYLGAKV 137
SSB2                    ------VIEVQYLEETKTFSPQEISAMVLTK------MKEIAEAKIGKKV 141
EGFR                    NGSCWGAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQCAAGCTGPRE 200
                               : :    ** : .:: *.    *       :: * .  * :

SSA2                    NDAVVT--------------------VPAYFNDSQRQATKDAGTIAGLNV 167
SSB2                    EKAVIT--------------------VPAYFNDAQRQATKDAGAISGLNV 171
EGFR                    SDCLVCRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCVKKC 250
                        ...::                      .:*  * : :.. . *: .  :

SSA2                    LR--IINEPTAAAIAYGL--DKKGKE-----------EHVLIFDLGGGTF 202
SSB2                    LR--IINEPTAAAIAYGLGAGKSEKE-----------RHVLIFDLGGGTF 208
EGFR                    PRNYVVTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGIGEF 300
                         *  ::.:  :.. * *    :  ::            : :  .:* * *

SSA2                    DVSLLSIEDGIFEVK---ATAGDTHLGGEDFDNRLVNHFIQEFKR----- 244
SSB2                    DVSLLHIAGGVYTVK---STSGNTHLGGQDFDTNLLEHFKAEFKK----- 250
EGFR                    KDSLSINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDIL 350
                        . **     .:  .*   : :*: *:    *    . *      :

SSA2                    KNKKDLS------------TNQRALRRLRTACERAKRT------LSSSAQ 276
SSB2                    KTGLDIS------------DDARALRRLRTAAERAKRT------LSSVTQ 282
EGFR                    KTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNI 400
                        *.  :::             : :*:..*.    *:*:       : *

SSA2                    TSVEIDSLFEGID------------FYTSITRARFEELCADLFRSTLDPV 314
SSB2                    TTVEVDSLFDGED------------FESSLTRARFEDLNAALFKSTLEPV 320
EGFR                    TSLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRG 450
                        *:: : ** :  *            : .::.  ::    .   :   :

SSA2                    EK--------------------------VLRDAKLDKSQVDEIVLVGGS 337
SSB2                    EQ--------------------------VLKDAKISKSQIDEVVLVGGS 343
EGFR                    ENSCKATGQVCHALCSPEGCWGPEPRDCVSCRNVSRGRECVDKCNLLEGE 500
                        *:                           ::.. .:. :*:  *: *.

SSA2                    TR------------------------------------------------ 339
SSB2                    TR------------------------------------------------ 345
EGFR                    PREFVENSECIQCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPHCVKTC 550
                        .*

SSA2                    ----IPKVQKLVTDYFNGKEPNRSINPDEAVAYG-AAVQAAILTG----D 380
SSB2                    ----IPKVQKLLSDFFDGKQLEKSINPDEAVAYG-AAVQGAILTG----Q 386
EGFR                    PAGVMGENNTLVWKYADAGHVCHLCHPNCTYGCTGPGLEGCPTNGPKIPS 600
                            : : :.*: .: :. .  :  :*: : .   ..::..  .*    .
SSA2                    ESS-KTQDLLLLDVAPLSLGIETAG----------------GVMTKLIP- 412
SSB2                    STSDETKDLLLLDVAPLSLGVGMQG----------------DIFGIVVP- 419
EGFR                    IATGMVGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQERELVEPLTPS 650
                         ::  .  **** *..*.:*:                        :.  : *

SSA2                    ----RNSTIPTKKSEVFSTYADNQPGVLIQVFEGERAKTKD--NNLLGKF 456
SSB2                    ----RNTTVPTIKRRTFTTVSDNQTTVQFPVYQGERVNCKE--NTLLGEF 463
EGFR                    GEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAIK 700
                            .:: :   *   *..     . .   *::*      :  :  :.

SSA2                    ELSGIPPAPRGVPQIEVTFDVDSNG------------------------- 481
SSB2                    DLKNIPMMPAGEPVLEAIFEVDANG------------------------- 488
EGFR                    ELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPF 750
                        :*   .    .   ::  : : : .

SSA2                    --------------------------------ILNVSAVEKGTGKSNKIT 499
SSB2                    --------------------------------ILKVTAVEKSTGKSSNIT 506
EGFR                    GCLLDYVREHKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLV 800
                                                       : .   *.:. .  . :.

SSA2                    ITNDKGRLSKEDIEKMVAEAEKFKEED----------EKESQRIASKN- 537
SSB2                    ISNAVGRLSSEEIEKMVNQAEEFKAAD----------EAFAKKHEARQ- 544
EGFR                    KTPQHVKITDFGLAKLLGAEEKEYHAEGGKVPIKWMALESILHRIYTHQS 850
                         :    :::.  : *::   *:    :           *   ::  :::
```

```
SSA2                            ----------QLESIAYSLKNTISE--AGDKLEQADKD------------ 563
SSB2                            ----------RLESYVASIEQTVTDPVLSSKLKRGSKS------------ 572
EGFR                            DVWSYGVTVWELMTFGSKPYDGIPASEISSILEKGERLPQPPICTIDVYM 900
                                          .* :   .  : :.    .. *::..:

SSA2                            ---------------------AVTKKAEETIAWLDSNTT-------ATKE 585
SSB2                            ---------------------KIEAALSDALAALQIED--------PSAD 593
EGFR                            IMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGDERMHLPSPTDS 950
                                                      .     :.   *  :         .: .

SSA2                            EFDDQLKELQEVANPIMSKLYQAG--GAPEGAAPGGFPGGAPPAPEAEGP 633
SSB2                            ELRKAEVGLKRVVTKAMSSR------------------------------ 613
EGFR                            NFYRALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLLSSLSATSNNS 1000
                                ::       : :     :.

SSA2                            TVEEVD--------------------------------------------- 639
SSB2                            ---------------------------------------------------
EGFR                            TVACIDRNGLQSCPIKEDSFLQRYSSDPTGALTEDSIDDTFLPVPGEWLV 1050


SSA2                            ----------------------------------------
SSB2                            ----------------------------------------
EGFR                            WKQSCSSTSSTHSAAASLQCPSQVLPPASPEGETVADLQTQ 1091
```

Figure 25. Alignment between human EGFR and *S. cerevisiae* protein Ssa2p and its close homologue Ssb2p. EGFR protein sequence of 1091 amino acids was obtained from NCBI (accession: AAH94761.1 GI: 63101670). Ssa2p with 639 amino acids, Ssb2p with 613 amino acids were obtained from SGD. Green – EGFR L1/I domain; Yellow – EGFR L2/III domain; Red – EGFR transmembrane domain; Blue – EGFR juxtamembrane domain; Grey – critical residues involved in Cetuximab binding. The sequences were aligned using ClustalW2 coupled with the default parameters. An alignment will display by default the following symbols denoting the degree of conservation observed in each column: * - the residues in that column are identical in all sequences in the aligment; : - conserved substitutions have been observed; . - semi-conserved substitutions are observed.

Interestingly, three (F_{331}, Q_{387}, K_{444}) of eight amino acid residues critical for the binding of Ctx to EGFR are present in Ssa2p and Ssb2p, or a conserved substitution has been observed. This is also true for Tdh3p in which F_{331} is conserved. Therefore, it is possible to explain the reason why the anti-EGFR antibody recognized all the three proteins, even if Ssb2p and Ssa2p appear better candidates for an EGFR-like role. Neither Ssb2p, Ssa2p nor Tdh3p have transmembrane domains allowing to predict their localization in the plasma membrane while EGFR has two (not shown). This is though not to prove wrong the protein recognition and identification since yeast, in opposition to animal cells, has wall around the cell and the receptor of an antibody may reside at the wall or its surface. Proteins well known for their function in the cytosol have already been found on the cell surface as is the case of glycolytic enzymes in *C. albicans* (276) or in *S. cerevisiae* extracellular matrix (99, 277).

These results reinforce the parallelism between the RAS signaling pathway in humans and yeasts and make conceivable suggesting Ssb2p, Ssa2p or Tdh3p, as possible EGFR-like proteins signaling to the Ras proteins (Fig. 26). The further use of the *Δssa2*, *Δssb2*, *Δtdh3* and the construction of a triple mutant *Δssa2Δssb2Δtdh3* would be ideal to clarify by WB which protein, or if all, are recognized by anti-EGFR.

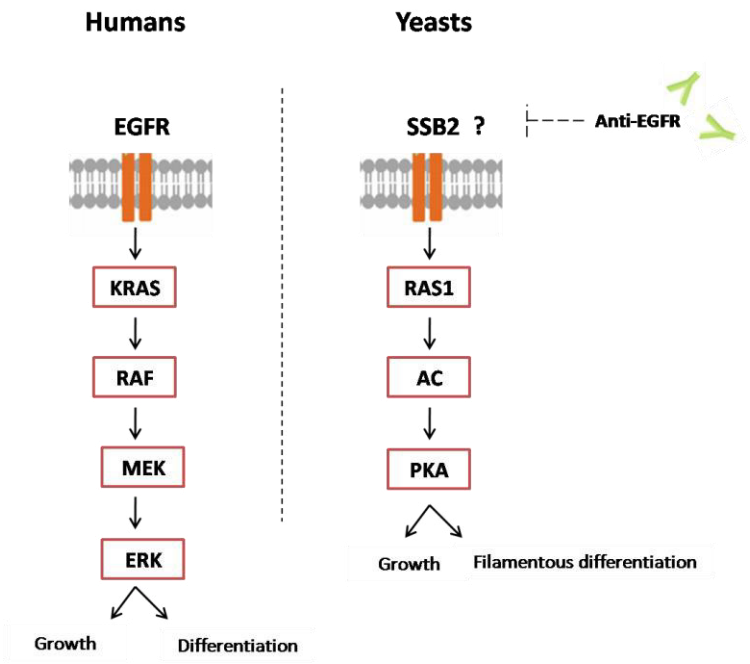

Figure 26. Schematic representation of the results obtained in BY4741 yeast cells with anti-EGFR as a protein targeting and a possible signaling downstream, showing a parallelism between RAS signaling pathways in human and yeasts. (—) stimulated signaling; (---) inhibited signaling.

3.3 Insights on RAS and GUP genes in the W303-1A strain

The RAS/cAMP/PKA pathway regulates a variety of processes such as life span (280), cell cycle (47), polarity of actin cytoskeleton (48), spore morphogenesis (49), regulation of the activity of amino acid transporter Gap1 permease (50) and DNA damage checkpoint (51). The genome of *S. cerevisiae* contains two RAS genes, *RAS1* and *RAS2*, the last exhibiting higher levels of expression compared to its homologue *RAS1* (31). *GUP1* is another gene involved in important cellular processes, such as secretory and endocytic pathway (96), bud site selection (95), telomere length (97), cytoskeleton polarization (94), vacuole morphology (96), and anaerobic growth (93). Additionally, resistance to the oncological drug Imatinib (Glivec®), a tyrosine kinase inhibitor blocking signaling, was also found associated with the deletion of *GUP1* in *S. cerevisiae* (103). Gup1p has a paralogous gene in *S. cerevisiae,* Gup2p, considerable less studied. Additionally, *GUP1* has orthologous in higher eukaryotes known with to a different nomenclature derived from their recognized role as negative regulators of the Hedgehog family of extracellular signaling molecules: HHATL (Hedgehog acyl transferase-like protein) (105). The two regulatory pathways driven by RAS and Hedgehog are known to interact in mammalian and other higher eukaryotic models to control morphogenesis in relation to cell cycle regulation and proliferation (105, 106). In yeast though, a morphogenic pathway like the one of human Hedgehog has not been recognized so far. Taking into consideration the overlapping between the phenotypes associated to the deletion of either RAS or GUP genes, we decided to check some further phenotypes for these genes. The *S. cerevisiae* W303-1A strain deleted in *Δras1*, *Δras2*, *Δgup1*, *Δgup2* and *Δgup1/2* were subjected to common physiology tests, such as chronological life span (CLS), cell size analysis, cell cycle regulation, adherence and invasion to agar. The results are meant to be compared with identical testing using the BY4741 *KRAS* complemented set of strains built in the first part of this work.

3.3.1 Growth and CLS assessments

3.3.1.1 RAS and GUP deletions affect growth on minimal media

To follow yeast growth on batch cultures including aging experiments, pre-inoculum cultures were allowed to grow overnight until exponential phase (OD_{600nm}0.4-0.8), and then diluted to an OD of ±0.1 and used to inoculate YNB liquid medium supplemented appropriately. Growth was monitored spectrophotometrically at stipulated points at 600nm, in a Spectrophotometer Genesys 20. Each culture growth was followed throughout exponential and post-diauxic phases. As control, the W303-1A wt was used. Results showed that W303-1A *Δras2* presented a small delay at the beginning of the growing phase compared to W303-1A wt and the *Δras1* mutant (Fig. 27A). On the other hand, the growth curves of GUP mutants was indistinguishable from the control and between themselves, however when the growth rates were measure they showed significant differences (Fig. 27B, Table 4).

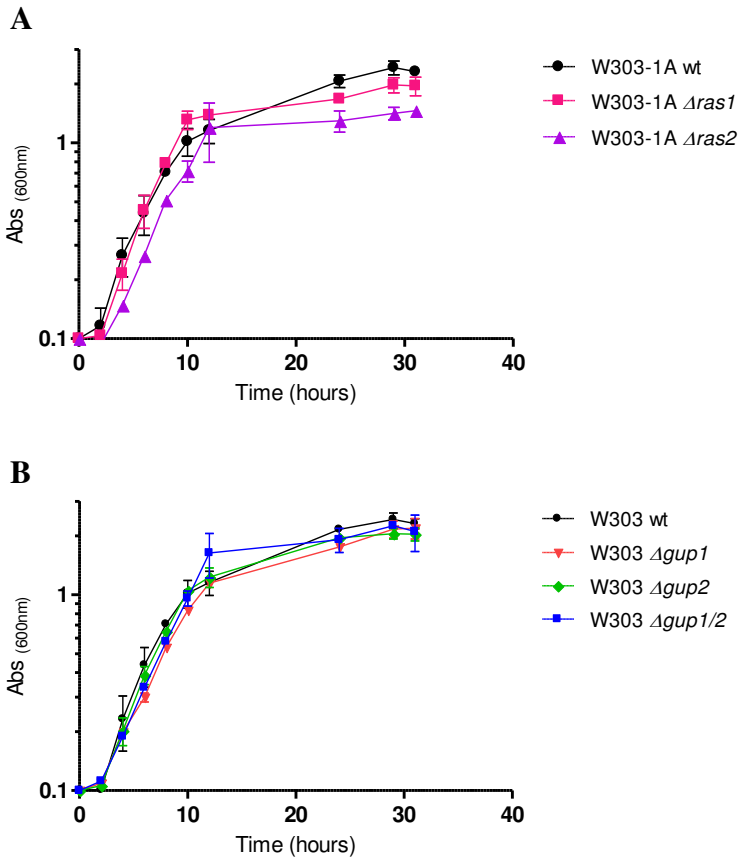

Figure 27. Effect of RAS and GUP genes on yeast growth. Growth curves of parental strain W303-1A, and mutants Δ*ras1* (A) and Δ*ras2* (A), and Δ*gup1* (B), Δ*gup2* (B) and Δ*gup1/2* (B). Growth in YNB

medium at 30 ºC was followed by measuring Abs_{600nm}. The results are representative of at least three independent experiments.

The values corresponding to the specific growth rate of these curves (medium values were used) are presented in Table 4. All the mutations produced a considerable effect on μ_g, which in *Δras1* and *Δras2* and in GUP mutants were respectively 35%, 37% and 50% slower than in wt strain. The results agree with what is described in the literature for both the RAS (34) and GUP (88) mutants.

3.3.1.2 The RAS and GUP mutants exhibit a reduced chronological life span

Loss of Ras2/cAMP/PKA signaling activity leads to stationary phase-like growth arrest (31), 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 (281). Moreover, the deletion of *RAS2* gene decreases the growth rate but extends the chronological life span (CLS) (34). Yeast CLS is described as the period of time a population remains viable in the post-diauxic and stationary growth phases in batch cultures (34). The survival of RAS and GUP mutants on glucose-based medium was monitored continuously for 25 days throughout stationary phase until complete death of the culture. The 3 days cultures were considered to be totally alive and therefore the number of colonies therein obtained was established 100% of viability. Both RAS and GUP mutants die sooner than wt, and the former sooner than the later. The deletion of either *RAS1* or *RAS2* yielded identical results, while in the case of GUP mutants, the *Δgup1* was the most sensitive, although all the three mutants ultimately reached full death at the same time point (Fig. 28, Table 4).

Analyzing the CLS results for the *Δras1* and *Δras2* mutant cells, a high decrease on CLS is visible in the mutants as compared to wt strain, μ_d being higher (Fig. 28A, Table 4). After 3 days the survival rate of RAS mutants started to decrease reaching 25% at day 6 (Fig. 28A). Accordingly, the μ_d of the mutants was more than double of the wt one (Table 4).

Fabrizio and Longo (34) describe in DBY746 background the doubling of yeast life span in association with the deletion of *RAS2*. Ras2p acts as an upstream regulator of Cyr1, the adenylate cyclase responsible for the production of cAMP, which is involved in cell cycle arrest in G1 phase (68). However, an opposite phenotype was observed. The same was observed in BY4741 *Δras2* (unpublished results from Carneiro). To the best of our knowledge, no results have been published so far using either W303 or BY4741 backgrounds. The three strains differ in methionine, adenine and tryptophan auxotrophy as well as in mating type:

- BY4741: *MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*
- W303-1A: *MATa leu2Δ3 leu2Δ112 ura3Δ1 trp1Δ1 his3Δ11 his3Δ15 ade2Δ1 can1Δ100*
- DBY746: *MATα leu 2-3, 112 his3Δ1 trp1-289 ura 3-52 GAL*$^{+}$

Considering the large interplay recognized between the RAS and TOR pathways, an interference of the amino acids / nitrogen biosynthesis pathways could be predicted. Whether this might be the explanation for the contradictory results remains to be verified. Thus, RAS may play similar roles in the regulation of chronological longevity since the deletion of *RAS1* and *RAS2* shorten the chronological life span.

Concerning the GUP mutants, the viability started to decrease at day 3, reaching 50% around day 6 for *Δgup1*, and day 9 for *Δgup2* and *Δgup1/2* (Fig. 28B). The wt reached 50% survival at day 12 and on day 20 the percentage of viable cells was almost zero (Fig. 28). Accordingly, *Δgup1*, *Δgup2* and *Δgup1/2* μ_d was respectively 54%, 25% and 46% higher than wt (Table 4). Therefore, it can be concluded that the GUP mutants, in particular *Δgup1*, die sooner than the wt which is in accordance with the literature (101). The fact that the *GUP2* and the double deletion identically increased the death rate could imply that both genes have a redundant role in that matter, which is important since so far, and in contrast with the *Δgup1,* very few phenotypes have been associated with this gene deletion. Importantly, both the RAS and the GUP genes identically affected the chronological life span, Ras more strongly than Gup suggesting a common path in signaling which was expected, but has yet to be understood.

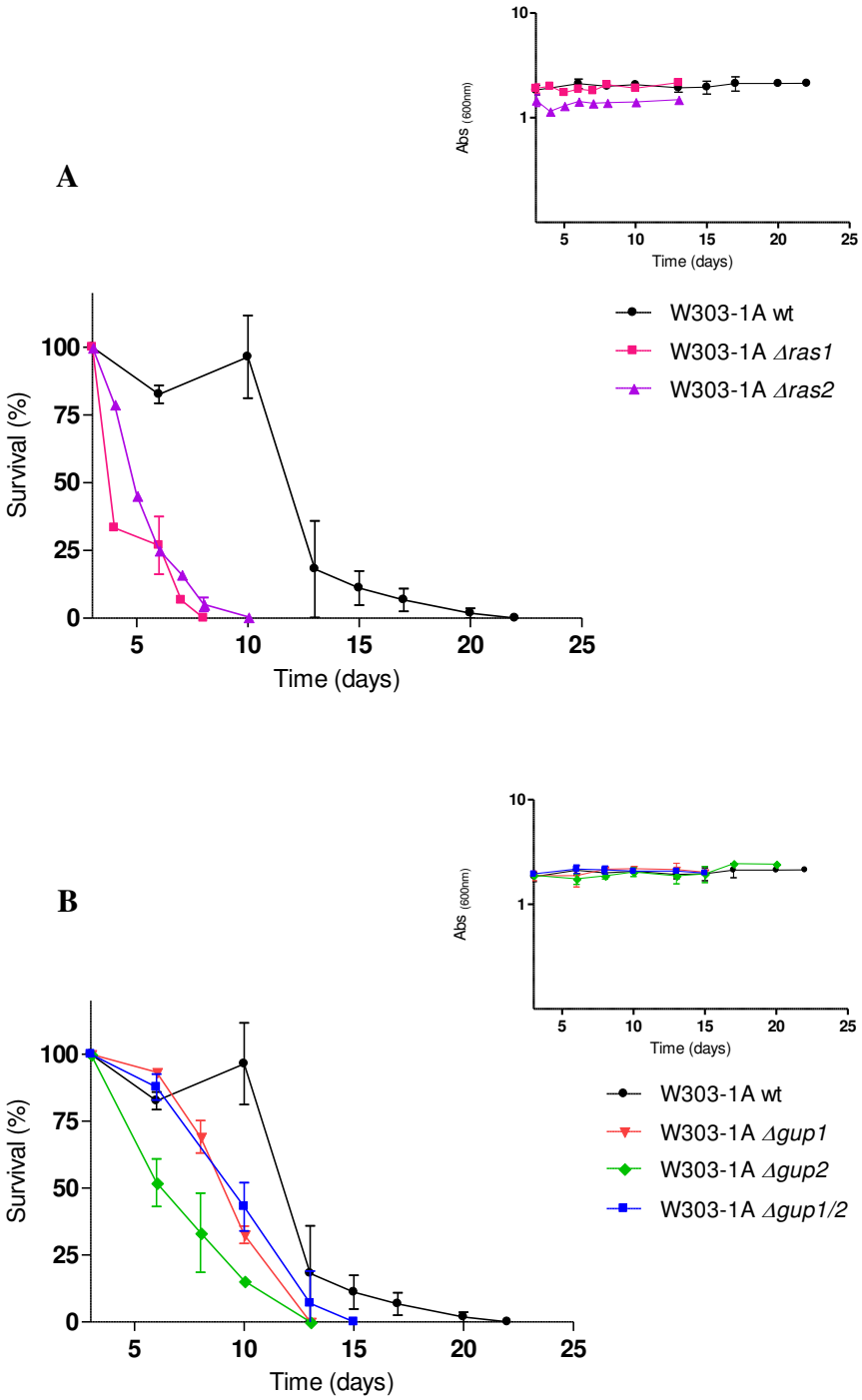

Figure 28. Deletion of RAS (A) and GUP (B) genes decreases chronological life span. The wt and the mutants were inoculated in YNB medium and survival monitored by C.F.U. for 25 days, after exponential phase (100% represents the number of C.F.U obtained in time 0). The growth curve in YNB, after the exponential phase, is presented in the insert. Data represent mean ± SD of at least three independent experiments.

Table 4. Growth and death rates for W303-1A wt, RAS and GUP mutants in YNB medium.

	Strains					
	W303-1A	**Δ*ras1***	**Δ*ras2***	**Δ*gup1***	**Δ*gup2***	**Δ*gup1/2***
μ_g (h^{-1})	0.49 R^2=0.999	0.32 R^2=0.992	0.31 R^2=0.998	0.26 R^2=0.994	0.30 R^2=0.997	0.27 R^2=0.999
OD_{600} (stationary phase)	2.3±0.1	2.0±0.2	1.5±0.01	2.2±0.3	2.0±0.2	2.1±0.5
μ_d (h^{-1})	-0.24 R^2=0.999	-0.69 R^2=0.999	-0.73 R^2=0.995	-0.37 R^2=0.999	-0.30 R^2=0.976	-0.35 R^2=0.895
100% death obtained at (days)	22	8	10	13	13	15

Some studies previously demonstrated that wild type cells nutrient depleted in synthetic complete medium (SC) showed a decreased CLS rather than in rich (YPD) medium (282, 283). Although the reason for the decreased CLS in SC medium is not clear, nutrient depleted cells cultured in this medium reveal a higher metabolic rate, which has been correlated with the shorter CLS (284). These effects were detected in different genetic backgrounds as demonstrated by Weinberger (285). The same mechanism could occur in the minimal medium YNB used in this study since these media like SC shares a media based upon YNB only differing from SC in amino acid composition. Either or both strain background and medium composition might underlie these results.

3.3.2 The GUP mutants and *Δras1* are smaller, while *Δras2* is bigger than wt

There is a close and frequently intricate relationship between shape and function in living organisms. Moreover, another basic and essential characteristic of an organism is size, which represents a significant determinant of cellular physiology (286). In yeasts, cell size is described to affect the duration of G1 cell cycle phase, because cells that are born small stay more time in this phase to gain the appropriate size to start the DNA replication and budding. This narrow interval is called currently START (287). Moreover, cell size is often described as a complex relationship between division and growth, involving several cellular pathways. Furthermore, it is supposed that the G1 cyclin named Cln3 allows the cell size communication (288). The connection between

RAS signaling and cell size has been noted, so mutant cells with reduced cAMP signaling usually exhibit a decrease in cell size, as well as a mutant deleted in *cdc25* temperature sensitive. In contrast, the over-activation of the PKA pathway results in large cells, and accordingly, $RAS2^{V19}$, an activated allele of *RAS2*, increases the cell size (289). RAS/cAMP/PKA therefore regulates a transcriptional network that ensures the equilibrium between division and cell growth (289, 290).

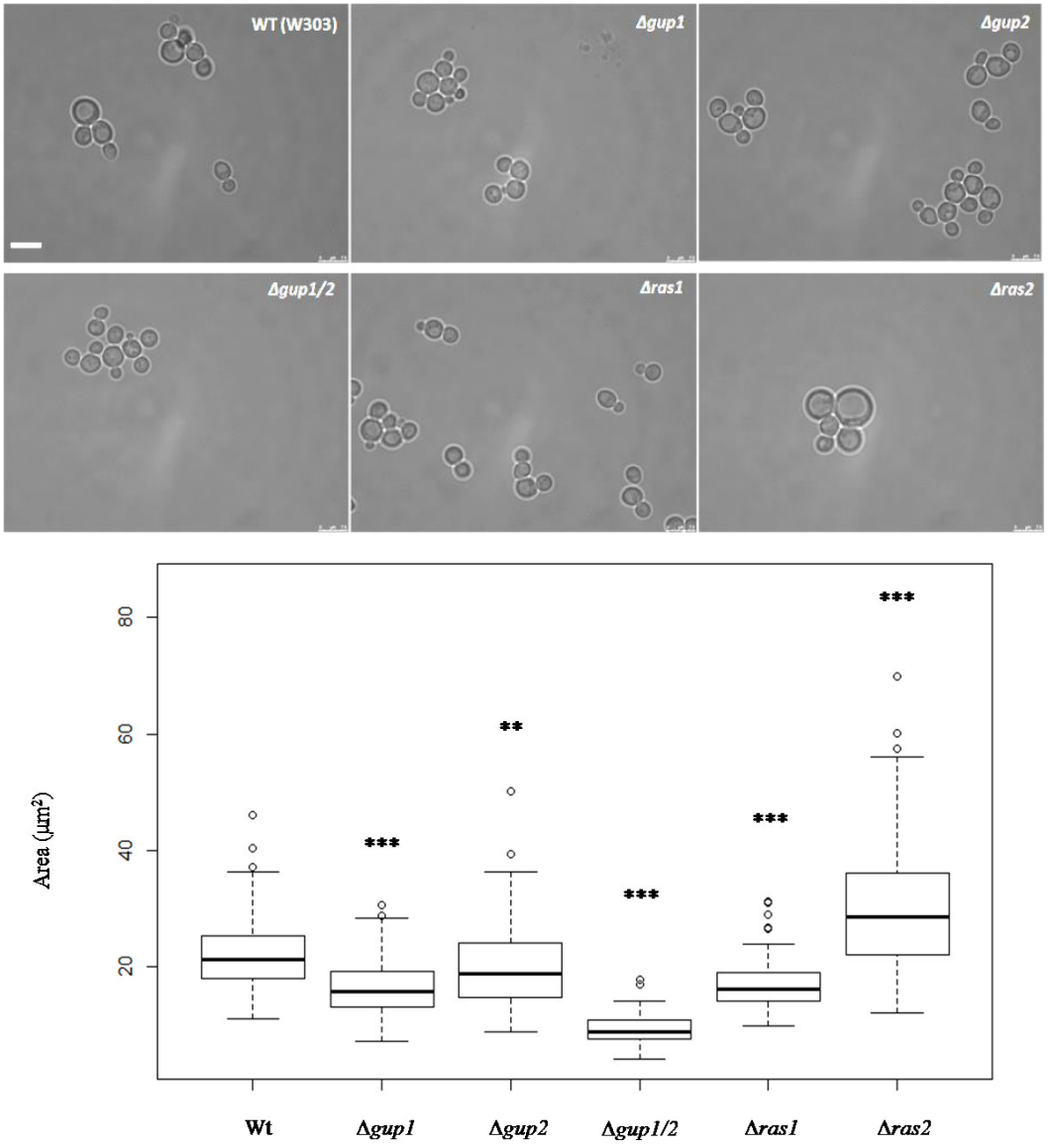

Figure 29. Cell size analyses. Top: Images of cells from W303-1A wt, Δ*ras1*, Δ*ras2*, Δ*gup1*, Δ*gup2* and Δ*gup1/2* were captured in a Leica DCF350FX digital camera in a Leica Microsystems DM-5000B epifluorescence microscope (Bar, 7.5µm). Bottom: Scatter plot of cell areas with bar indicating median and standard deviation. Eighty to 100 cells were measured per genotype. *,p≤0.05; **,p≤0.01; ***, p≤0.001, two-tailed t-test.

The size of the RAS and GUP mutant cells was estimated using a microscopic assessment methodology (41). Cells growing on YNB liquid medium were collected in exponential growth phase and observed with a Leica Microsystems DM-5000B epifluorescence microscope. The area of 80 to 100 cells was measured using ImageJ freeware. Results showed that all the mutations of RAS and GUP produced a considerable effect on cell size (Fig. 29). Cells lacking *GUP1*, *GUP2* or both are respectively 27%, 11% and 59% smaller than wt. Also, the *Δras1* cells exhibited a decreased in size of 25% compared to wt. In opposition, cells lacking *RAS2* demonstrated a 34% increase. Wei *et al.,* (2003), using the same technique, also showed an increase in cell size for *RAS2*-null cells compared with DBY746 wild type strain though smaller (10% decreased compared to *Δras2*) (41). On the other hand, other authors using cytometry-based methodologies observed a small decrease in cell volume in Δ*ras2* mutants. This is conceivable considering that *RAS2* deletion associated with a decrease in cAMP production and therefore there should be a decrease in size (289).

3.3.3 Cell Cycle analysis

Microorganisms like the budding yeast *S. cerevisiae* demonstrate some mechanisms that regulate the capacity to growth and to progress in cell cycle according to nutrient availability. Thus when large amounts of nutrients are available the proliferation is rapid, on other hand the exhaustion of the resources stop the capacity to grow. Into this context, glucose is an important nutrient that produces signals that govern growth and cell cycle progression. In *S. cerevisiae* cell cycle progression onto G1 and S phase is regulated by a narrow interval known as START (291). At this checkpoint, the cells evaluate some environmental and internal signals including critical cell size (above), nutrient availability and metabolism. These commands whether they can progress onto a new cell cycle, enter stationary phase, or assume an alternative differentiation such as sporulation or pseudo-hyphal growth. Progression through START commits to a new mitotic cell cycle and requires the activation of the cyclin-dependent kinases (292), and an efficiently coordination (293). This contributes to ensure that DNA replication, and consequently the starting of cell division, only happens when cells have acquired an optimal cell size (69). cAMP pathway is implicated in the control of cell cycle progression as well as in cell size regulation for

the cells entry into the S phase (294). Concomitantly, the addition of cAMP to an exponentially growing culture largely increases cell size. Furthermore, cAMP delayes the G1/S transition in small cells, but is ineffective in large cells (290). On the other hand, inactivation of the cAMP signaling arrests cell cycle at START, followed by entry into stationary phase (G0) (68). Thereby, cells with impaired PKA activity show numerous features representative of G0 phase cells, including improved stress resistance, impaired filamentous differentiation and sporulation efficient capacity (69). Moreover, as mentioned above, Gup1 is described to interfere in telomere length (97). Short telomeres decrease life expectancy of the cells interfering in cell cycle progression (295).

Considering the results described above, the cell cycle for the RAS and GUP mutants was analyzed by flow cytometry. The division into different cell cycle phases is based on the amount of DNA, detected with a probe specific for it, in this case was SYTOX® Green. Therefore, there is one peak corresponding to G1, when the DNA is single copy, one to G2/M, when the DNA has already been duplicated, but the cell is still not divided. Also an intermediate phase in which the DNA is being duplicated, but the duplication is not complete, the S phase, and finally a phase in which the amount of DNA detected is less than the normal condition, the sub-G0 phase, corresponding to fragmented and/or condensated DNA. Yeast strains were analyzed throughout exponentially growth into late stationary phase, when the chronological life span assay was assessed (above). Cells were thus collected at defined times points:

- Exponential growth (8h),
- Late stationary phase (3days),
- Dying cells (5 days for *Δras1* and *Δras2*; 6 days for *Δgup1*; 8 days for *Δgup2* and *Δgup1/2*).

The time points for death phase were determined through the survival curve for each mutant (Fig. 28). The wt strain after 8h of exponential growth still presented more than 50% of cells in G0/G1 phase and approximately 35% in G2/M phase. After 3 days (stationary growth phase) the amount of cells in G2/M decreased and a considerable percentage of cells appeared in Sub-G0 (Fig. 30A). The sub-G0 peak reveals the fragmentation and condensation of DNA that can be an indication of an apoptotic cell death process. Thus, it is not surprising that this percentage was more pronounced when

the cells were collected in the death phase (Fig. 30A). The cells of Δ*ras1* and Δ*ras2* after 8h of growth showed a high percentage (>50%) of G2/M and a low amount in G0/G1 (Fig. 30B/C). After 3 days, the amount of the cells in G2/M decreased and a high percentage of cells in sub-G0 appear (Fig. 30B). In opposition to Δ*ras1*, the Δ*ras2* cells showed a high amount of cells in S phase (Fig. 30C). *Δras1* and *Δras2* did not allow the assessment of the cell cycle in the death phase, probably because the staining was insufficient or because of the quick loss of viability in this cells, in spite of the fact that the time points for collecting the cells were based on previous results (Fig. 28). Exponentially growing Δ*gup1* mutants have more cells in G2/M than wt. At day 3 this amount diminished and the percentage of cells in G0/G1 increased, as well as the one of cells in sub-G0 (Fig. 30D). The *Δgup2* presented a cell cycle similar to wt throughout the whole period (Fig. 30E). Finally, the double deleted strain behaved similarly to Δ*gup1* (Fig. 30F).

We verified that during the exponential growth, Δ*ras1*, Δ*ras2* and both *Δgup1* and *Δgup1/2* cells in a less extended manner, were more abundant in G2/M phase, that may cause a delay in growth, as we observed in the grown rate (Table 4). In our results, the Δ*ras2* cells in stationary phase, in opposition to what has been described in the literature (69), did not arrest in the G1 phase of cell cycle. Accordingly, we didn't observe an extension of life span. The results showed that nutrient depletion causes replication stress in Δ*ras2* cells that fails to enter into G1 arrest and instead arrests after DNA replicates in S phase. These results might explain the increased cell size of Δ*ras2* cells together with their short lifespan. Growth arrest of cells in S phase could be caused by a reduction in the levels of nucleotides and other factors required for efficient DNA synthesis, which would lead to replication stress (285). As in other eukaryotes, replication stress promotes genome instability and apoptosis in budding yeast (296). Anyway, to have a better understanding of these results, it would be indicated to follow the cells through the cell cycle and not only to observe them only at one point. This would be possible synchronizing the cells and taking samples along an extended period of time. Smaller cells spend more time in G1 which allow them to grow more than larger cells (297). This is the case of all the GUP mutants. In the stationary growth phase Δ*gup2* cells demonstrated a sub-G0 peak, that reveal the fragmentation of DNA, this same peak did not appear in Δ*gup1* and Δ*gup1/2*, which lead us to think that, Δ*gup2* cells can die by apoptosis, but Δ*gup1* (101) and Δ*gup1/2* cannot.

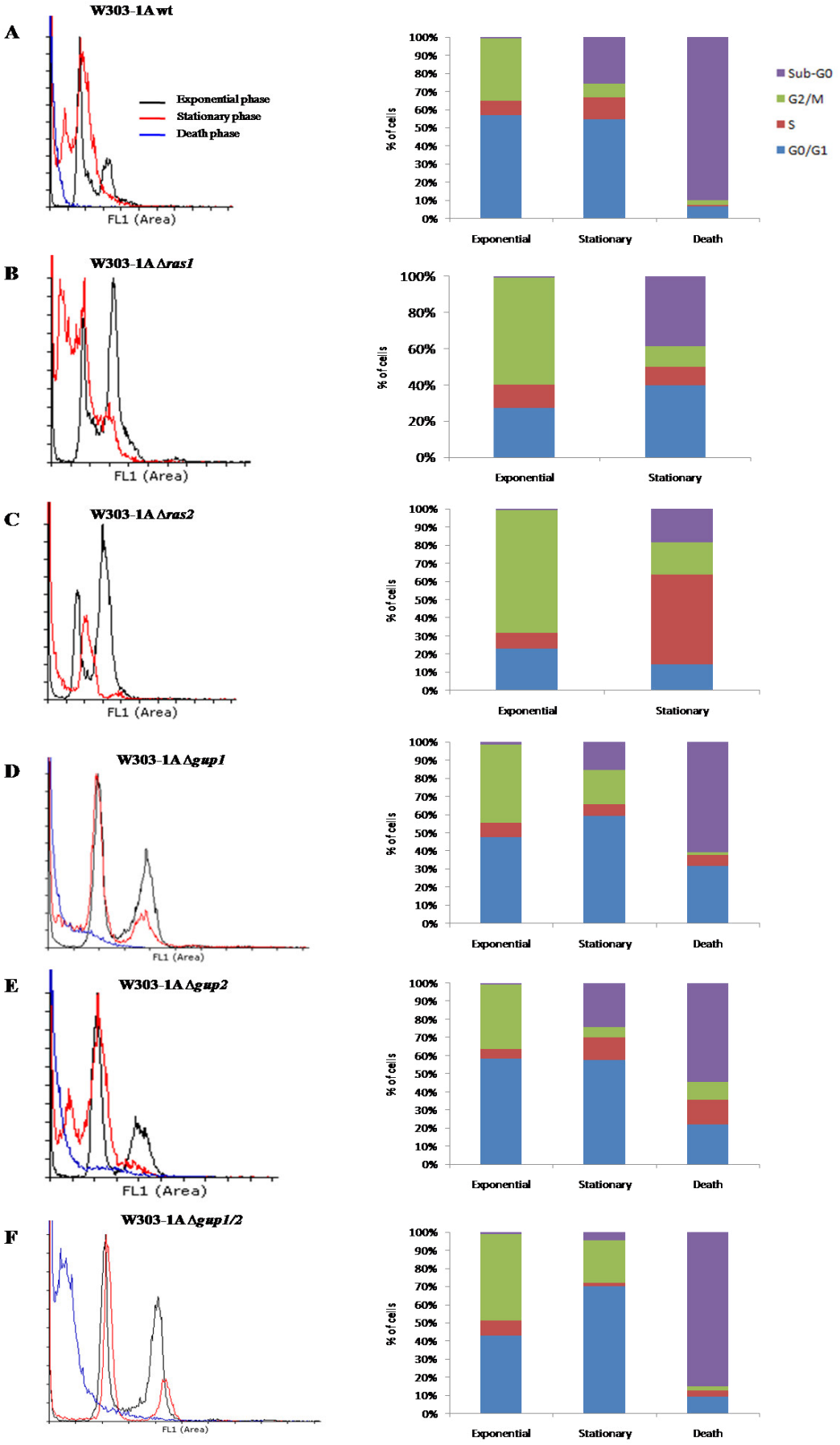
A
W303-1A wt
Exponential phase
Stationary phase
Death phase
FL1 (Area)
% of cells
Exponential
Stationary
Death
Sub-G0
G2/M
S
G0/G1
B
W303-1A Δras1
C
W303-1A Δras2
D
W303-1A Δgup1
E
W303-1A Δgup2
F
W303-1A Δgup1/2

Figure 30. Cell cycle histograms (left) and corresponding percentages of the different phases (right), of wild type W303-1A and Δ*ras1*, Δ*ras2*, Δ*gup1*, Δ*gup2* and Δ*gup1/2* mutants. In each histogram, the left-most peak represents the G0/G1 population and the right-most peak represents the G2/M, the space between both correspond the S population. When some peak appears before G0/G1, this area represents the sub-G0 population. The samples to analyze were collected in three different points of growth curve, respectively exponential, stationary and death phases. Results represent one preliminary experience.

3.3.4 Adherence to and Invasion of agar

As mentioned above, START checkpoint in *S. cerevisiae* commands whether the cells progress into the full mitotic cell cycle or alternatively enter stationary phase, or undergo differentiation namely into pseudo-hyphae (69, 291). Hyphal growth is a morphological behavior many times associated with yeast pathogens, namely *C. albicans*. Yet, budding yeasts like *S. cerevisiae* can also filament growing into pseudo-hyphae. This is described as a process by which the cells divide by cytokinesis but keep connected to each other through the cell wall (298). Nutrient limitation is a promoter of filamentous growth in yeast. Pseudo-hyphae formation is frequently described as associated with diploidy. However, filamentation and associated invasive growth has been showed in haploid cells invading agar (298, 299). Filamentous growth is related to nitrogen levels (235), as well as to the absence of fermentable carbon source (299). *RAS2* was found to be required for filamentous growth regulation (235). Previous work showed that *Δgup1* mutant from *C. albicans* was unable to adhere to and invade agar (98). Therefore, the GUP and RAS mutants were studied on capacity to adhere and to invade agar on nitrogen-deficient medium SLAD agar plates as described in the Materials and Methods. According to the literature (235, 298), the RAS mutants did not adhere to SLAD plates (Fig. 31). The same was true for the wt strain, which was also expected when considering the W303 genetic background (unpublished results/personal communications from several groups). In opposition, the GUP mutants adhered to SLAD plates, more evident in the case of the *Δgup1/2* mutant and less evident in the case of *Δgup2* (Fig. 31).

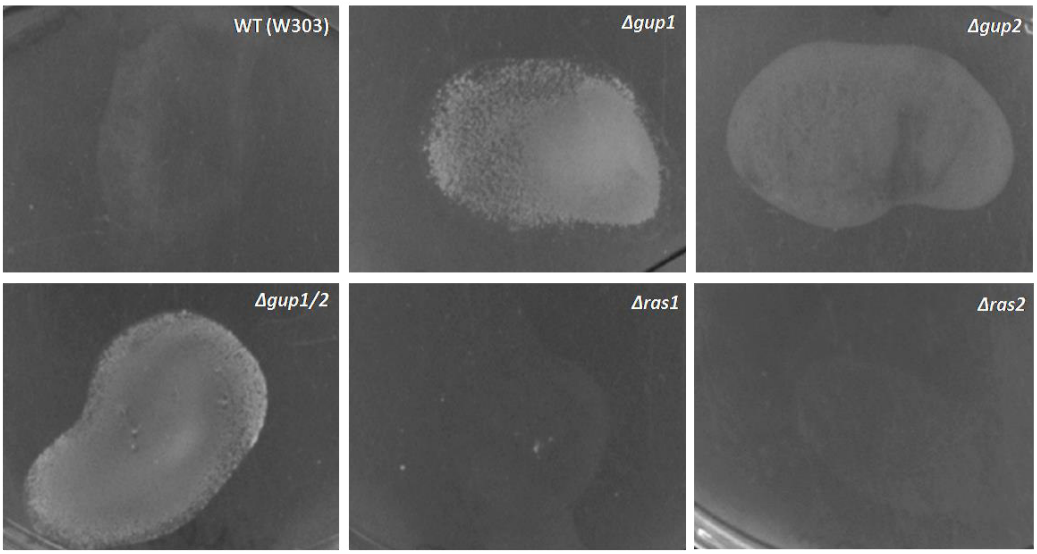

Figure 31. Adherence to agar of wild-type W303-1A and Δ*ras1*, Δ*ras2*, Δ*gup1*, Δ*gup2* and Δ*gup1/2* mutants, after 7 days of growth on a nitrogen depleted medium (SLAD). Cells were pre-cultured overnight in YNB media and diluted to 1×10^7cells/ml prior to plating. Adherence to agar was assayed by washing the cells off the surface of the agar.

The agar invasion of these strains was assessed as described in Material and Methods. None of the strains was able to invade agar in the conditions tested (data not shown). Moreover, none underwent filamentation (not shown).

Adhesion phenomenon is not only dependent on filamentation, but also on cell wall hydrophobicity, cell wall charge, and cell wall composition (300). GUP mutants unlike RAS mutants are well documented to have an altered cell membrane and wall composition (90, 92), which could account for this phenotype of adherence without invasion or filamentation. This could also derive from the absence of nitrogen in the medium. Although this condition is recommended to assay filamentation in wt strains, it could affect mutants phenotypes.

TOR pathway controls cell growth in response to nutrient signals such as nitrogen, amino acids and carbon starvation. Rapamycin has been described to cause effects similar to those promoted by nutrient starvation (75, 301). Nitrogen-limiting conditions promote cell cycle arrest in G1 caused by nuclear accumulation of Gln3 (80). This protein regulates the activation of genes involved in nitrogen metabolism in response to nitrogen deprivation. On the other hand, RAS pathway controls filamentation morphological switch and invasive growth in concert with TORC1

complex, although the mechanism of interdependence between the two is not well understood (302). The TOR pathway might control indirectly the cell cycle by controlling the subcellular localization of the polo-like kinase Cdc5, which indirectly acts upon Swe1, a kinase that inactivates the mitotic cyclin-dependent kinase (Cdc28) (302). These proteins are well-known players of RAS pathway involved in the suppression of many rapamycin-induced phenotypes by the hyper-activation of RAS/PKA (81). The deletion of *GUP1* alone or together with the deletion of *GUP2* increased the resistance to rapamycin while the single deletion of *GUP2* did not (108). Both adherence under nitrogen starvation and resistance to rapamycin associate with TOR function, which relates with the RAS signaling. In either mutants strain the absence of filamentation indicates the RAS pathway might be inactive and the fact that the GUP mutants share this and all the other above-mentioned phenotypes indicates a close relationship between Gup and the two upstream TOR and RAS pathways, which has to be confirmed in the future. Finally, as mentioned, previous results showed that the deletion of *GUP1* alone or together with the deletion of *GUP2* increased resistance to rapamycin while the single deletion of *GUP2* did not. This was considered an epistatic relation between deletions, which was also found in other conditions (89, 92) including the above results with μ_g, μ_d (Table 4), cell size (Fig. 29) and cell cycle (Fig. 30). In the adherence to nitrogen-deficient medium agar this was again observed.

CHAPTER IV

Final Remarks and Future Perspectives

4. Final Remarks and Future Perspectives

S. cerevisiae is a powerful eukaryotic model organism due to its simplicity and to the availability of numerous genetic manipulation tools (14). The present thesis covers a group of tasks complementary to the work of two other students for the development of a yeast-based high throughput platform for human KRAS and galectin 3 or galectin 1 phenotyping. Therefore, responding to the main aims of this project we can conclude that all the 4 points were achieved with success. The major comments and remarks to the obtained results are described below.

Task one:

- As expected, this study described a new "*KRAS*^{wt} humanized yeast", heterologously expressing human wild-type *KRAS*. With this cell system, it was possible to express *KRAS* without the genetic and molecular complexity of of human cells and their RAS-related pathways, namely the interference of the other human RAS isoforms *HRAS* and *NRAS*. Briefly, the p426GPD-*KRAS*^{wt} construction was used to transform the yeast strain BY4741wt. However, since the yeast Ras proteins (Ras1p and Ras2p) are globally speaking structurally and functionally complementary to the human RAS proteins, the study was also performed in the BY4741 deleted in *RAS1* and *RAS2* genes to avoid possible interferences. These single deletions additionally allow different levels of expression of the RAS/cAMP/PKA pathway since the yeast genes are not physiologically redundant. Yeast transformation with *KRAS* cDNA was confirmed by colony PCR, and the expression of KRas protein in yeast was confirmed by Western blot with anti-KRas against total yeast proteome.

Task two:

- Probing total proteins extracts from *S. cerevisiae*, BY4741wt, BY4741 *Δras1*, BY4741 *Δras2* and the *KRAS*^{wt} humanized yeasts, with the monoclonal antibody anti-EGFR lead to the identification of the Hsp70 proteins Ssa2p and Ssb2p, and of the glyceraldehyde-3-phosphate dehydrogenase 3 Tdh3p, as putative EGFR-like proteins.
- The amino acid sequence alignments between EGFR and the above mentioned proteins revealed that Ssb2p, and its close homologue Ssa2p, present two

regions with considerable homology with EGFR amino acid sequence. Therefore, Ssa2p and Ssb2p seem to be the most probable EGFR-like protein.

- The alignment revealed that three out of the eight amino acid residues of EGFR known to be critical for the binding of Cetuximab are present in both Ssa2p and Ssb2p : F_{331}, Q_{387}, $K_{444.}$
- Ssb2p is predicted to interact physically with yeast Ras1p (278), which presents 63% similarity with the KRAS human protein.

These results reinforce the parallelism between the RAS signaling pathway in humans and yeasts. Similarly to what happens in mammalian cells with EGFR and the downstream regulation of Ras/Raf/MAPK pathway, other molecules may work in yeast as upstream regulators of the this pathway, controlling growth, differentiation, stress resistance, cell cycle and transcription. It is known that cancer cells become 'addicted' to Hsp70 through its activity on cell signaling and survival pathways. Three of these cancer relevant activities of Hsp70 are: apoptosis, senescence and autophagy (250). If the present results in yeast are confirmed, the inhibition of Hsp70 could identically induce cell death upon the action of an EGFR-like antibody. Additionally, the recognition of a heat shock protein opens doors to a whole new range of possible functions for these and other yeast heat shock proteins whose function remains poorly understand.

Tasks three and four:

The RAS/cAMP/PKA pathway and the Gup1p protein regulate a variety of important processes in yeast, some of which are common. This apparent sharing underlies the choice of compared physiologic assays performed using the Ras and Gup mutants:

- The deletion of RAS and GUP genes produced a considerable decrease on specific growth rate.
- The GUP and RAS mutant cells exhibited a reduction in chronological life span, Ras more strongly than Gup.
- The GUP and *Δras1* mutant cells showed a reduction in the cell size, while *RAS2* deletion resulted in bigger cells.
- During exponential growth, RAS mutants presented a higher G2/M phase, accordingly with the observed delay in growth.

- The nutrient depletion (carbon) caused replicative stress in Δ*ras2* cells that failed to enter into G1 arrest, instead arrested growth in S phase. These results may explain the increased cell size of Δ*ras2* cells and also the short lifespan.
- RAS mutants did not adhere to SLAD agar plates while GUP mutants did. RAS are therefore essential genes to promote this ability, which could relate to low nitrogen, and consequently associate with TOR pathway.
- None of the GUP and RAS deleted strains was able to invade agar, and none showed filamentation capacity.

In either mutant strain the absence of invasion indicates that the RAS pathway might be inactive. The fact that the GUP mutants share this and all the other above-mentioned phenotypes indicates a close relationship between Gup1/2 and TOR and RAS pathways, which has to be confirmed in the future. In a future work, to confirm an involvement of Gup proteins with RAS/cAMP/PKA and/or TOR, it will be important to further delete RAS genes on the GUP set of mutants (or *vice versa*), and/or use *KRAS* to complement yeast cells deleted in GUP genes. These strains will then be used for phenotyping, using as controls the already existent set of humanized yeasts built on GUP mutants expressing higher eukaryotes *HHATL* and/or *HHAT*.

One more time, yeast provided a helpful contribution to reveal the molecular mechanisms underlying the KRAS function, as well as to determine the possible homologue of EGFR, many times associated with cancer, particularly in colorectal carcinoma. On the other hand, it also contributed to gain insights about RAS and GUP genes and their functions in a possible shared pathway. This knowledge is expected to pave the way to the identification of new targets/inhibitors as well as the development of new diagnostic/prognostic tests.

CHAPTER V

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