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Involvement of Kinases Sch9, Pkh1 and PKA in Start-up of Growth in *S. cerevisiae*

Ines COTTIGNIE

Supervisor: Prof. Dr. Johan Thevelein

Co-supervisor: Dr. Griet Van Zeebroeck

Mentor: Zhiqiang Zhang & Michaela Conrad

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I wish them all the best in their future career and personal lives!

Please enjoy the reading!

Ines

II. Summary

In all kind of cells, re-addition of nutrients to starved cells triggers start-up of protein synthesis, and consequently cell growth. Although this is generally known, how exactly nutrient sensing is linked to protein synthesis still remains an unanswered question.

The MCB laboratory suggests a possible direct connection between amino acid induced activation of growth via protein kinase A and translation initiation in *Saccharomyces cerevisiae*. This is among others proposed via protein-protein interactions of the Gap1 amino acid transporter-receptor with eIF2 and eIF2B subunits, necessary for protein synthesis initiation.

The current work aims to examine the involvement of kinases in this suggested signalling cascade. It is investigated by studying interactions of Gap1 with kinases Sch9, Pkh1 and the catalytic subunits of PKA (Tpk1-3). Also interactions between these kinases and eIF2B subunits Gcd1 and Gcd6 are analysed.

For the first time, *in vitro* GST pull-down experiments show possible interactions of Gap1 with kinases Sch9, Pkh1 and Tpk1. These kinases also show interactions between at least one eIF2B subunit studied using the same technique. The interaction between Gap1 and Sch9 is most likely confirmed by the *in vivo* split citrine BiFC technique. Noteworthy, repetitions of the experiments are needed to confirm the findings. In addition, future experiments will have to demonstrate whether the physical interactions between the proteins are also found to be regulatory.

To conclude, the results of the current work suggest that kinases might be involved in linking nutrient availability to cell growth.

III. Samenvatting

In alle soorten cellen leidt het opnieuw toevoegen van nutriënten aan gestarveerde cellen tot de start van eiwitsynthese en bijgevolg celgroei. Ondanks het feit dat dit algemeen geweten is, blijft het een onbeantwoorde vraag op welke wijze de gewaarwording van nutriënten gelinkt is aan eiwitsynthese.

Het MCB laboratorium suggereert een mogelijks direct verband tussen aminozuur-geïnduceerde activatie van de groei, via PKA en translatie-initiatie in *Saccharomyces cerevisiae*. Deze hypothese wordt onder andere ondersteund door de vaststelling van eiwit-eiwitinteracties tussen Gap1, een transporter-receptor van aminozuren, en eIF2 en eIF2B subeenheden, noodzakelijk voor initiatie van eiwitsynthese.

De huidige scriptie beoogt de betrokkenheid van kinasen te onderzoeken in deze gesuggereerde signalisatiecascade. Dit wordt nagegaan door het bestuderen van interacties tussen Gap1 en de kinasen Sch9, Pkh1 en de katalytische subeenheden van PKA (Tpk1-3). Verder worden de interacties tussen bovenstaande kinasen en eIF2B subeenheden Gcd1 en Gcd6 geanalyseerd.

Voor het eerst tonen *in vitro* GST pull-down experimenten mogelijke interacties tussen Gap1 en kinasen Sch9, Pkh1 en Tpk1 aan. Met dezelfde technologie zijn ook interacties waargenomen tonen deze kinasen een tenminste één eIF2B subeenheid. De *in vivo* split citrine BiFC technologie bevestigde de interactie tussen Gap1 en Sch9 hoogst waarschijnlijk. Vanzelfsprekend zijn herhalingen van bovenstaande experimenten nodig ter confirmatie van de bevindingen. Daarnaast dienen toekomstige experimenten aan te tonen of de fysische interacties tussen de genoemde eiwitten ook instaan voor een regulatiefunctie in de cel.

Ter conclusie, de resultaten van de huidige scriptie suggereren dat kinasen betrokken kunnen zijn bij het linken van nutriëntenbeschikbaarheid met celgroei.

A. Problem Posing

In all kind of cells, re-addition of nutrients to starved cells triggers start-up of protein synthesis, and consequently cell growth [info protein synthesis; see *section B.3.*]. Although this is generally known, how exactly nutrient sensing is linked to protein synthesis still remains an unanswered question (Conrad, et al., 2014).

Nutrient sensing is extensively studied in *Saccharomyces cerevisiae* (*S. cerevisiae*) [info *S. cerevisiae*; see *section B.1.*]. Researchers discovered that during nutrient starvation, high-affinity nutrient-specific transporters are upregulated. Those transporters are found to additionally possess a signalling function: Activating protein kinase A (PKA) in the presence of their ligand. They are therefore called transporter-receptors or transceptors [info transceptors; see *section B.2.3.*] (Van Zeebroeck, *et al*., 2011). Since the PKA pathway is correlated with growth rate (Lillie & Pringle, 1980), it is interesting to study the molecular mechanism behind transceptor signalling to PKA to be able to unravel the mystery behind the start-up of growth [info PKA pathway; see *section B.2.2.*].

In *S. cerevisiae,* physical interactions both *in vitro* and *in vivo* have been shown between the transceptors and both the subunits of the eukaryotic initiation factor 2 (eIF2) and its guanine nucleotide exchange factor (eIF2B). Both eIF2 and eIF2B are necessary for protein synthesis initiation [info eIF2 and eIF2B; see *section B.3.*]. The *in vivo* experiment using intragenomically bimolecular fluorescence complementation (BiFC) with split citrine shows a concentrated fluorescent spot in the cell, a focus. This transporter/eIF2(B)-focus is localised in a, so far unidentified, specific region within the cytoplasm, indicating that the interaction between the transceptor and eIF2(B) is happening locally. Using the same *in vivo* technique for studying the Gnp1-Gcd1-interaction, a similar transporter/eIF2(B)-focus is observed in exponentially growing cells, with Gnp1 being a constitutively expressed amino acid transporter and Gcd1 an eIF2B subunit. These results indicate that not only starvation-induced transceptors, but also constitutively expressed transporters may signal through eIF2(B)(Conrad *et al*., in preparation).

In independent research, it was found that eIF2B and eIF2 subunits intragenomically fused with a full-length fluorescent protein, also localise in a specific cytoplasmic fluorescent focus, named the eIF2(B)-focus. Although, an evenly localized signal in the cytoplasm is expected. It is shown that this region is a place for the activation of eIF2 by eIF2B. Additionally, the cycling of eIF2 in and out of the fluorescent focus seems to be correlated with the activity of protein synthesis in nitrogen deprived versus exponential cells (Campbell, *et al*., 2005).

The eIF2(B)-focus is proven to be identical to the transporter/eIF2(B)-focus. Since transporters are integral membrane proteins, it was suggested that the protein interaction site is membrane enclosed. Studies with mutants in the vesicular trafficking, protein secretion and endocytosis pathways as well as co-localization studies with marker proteins for all known organelles

indicate that the transporter/eIF2(B)-focus is not present in a known organelle (Conrad *et al*., in preparation).

Both *in vitro* and *in vivo* interactions are observed between the eIF2B subunits and one of the catalytic subunits of PKA (Kimpe, 2012). Additionally, it is found that the deletion of *GCN2*, encoding a protein kinase, inhibitor of eIF2, leads to increased PKA signalling of the amino acid starvation induced Gap1 transceptor (Van Zeebroeck, *et al*., 2011). All together, these results indicate that eIF2 and eIF2B are in some way involved in PKA signalling.

The transceptors are thus possible candidates for linking the nutrient availability to protein synthesis. The suggested model is that starvation-induced high-affinity transceptors sequester eIF2(B), inhibiting protein synthesis, while low-affinity transporters in exponential phase stimulate growth by eIF2(B) in the presence of their substrate.

The Laboratory of Molecular Cell Biology at the KULeuven (MCB lab) called this regulatory system, visualised in the transporter/eIF2(B)-focus, the 'startosome', a putative novel organelle for nutrient regulation of protein synthesis (Conrad *et al*., in preparation). However, future experiments will have to proof whether the physical interactions also reflect regulatory interactions and whether the 'startosome' is also connected to the PKA pathway.

B. Introduction

1. *Saccharomyces cerevisiae*

1.1. Model Organism

S. cerevisiae is a unicellular eukaryotic species, commonly known as the brewer's or baker's yeast. As the names suggest, this organism is widely used for the production of alcoholic beverages and dough rising of bread (Mortimer, 2000). This ascomycotic fungus is naturally abundant among others in vineyards, oak trees, flowers and the soil of deserts (Greig & Leu, 2009). It has a cell size in between that of bacteria and human cells: About 3 to 7 µm in width and 5 to 15 µm in length (Satyanarayana & Kunze, 2009).

Due to a series of practical advantages, yeast has become an important organism in fundamental research as well as in the industry. Advantages are among others its non-pathogenicity, its well-defined haploid and diploid life cycle and its short regeneration time of 90 minutes under optimal lab conditions in the presence of glucose (Satyanarayana & Kunze, 2009).

The haploid genome of *S. cerevisiae* contains only 12.5 mega bases (Mb) ordered in 16 chromosomes, whereas the (healthy) haploid genome of humans contains 3200 Mb, organised in 23 chromosomes. Additionally, only approximately 4 % of the yeast genes have introns, resulting in a very dense genome, making it easier to study. At the moment researchers claim that baker's yeast has 6,604 open reading frames with few repeated sequences[1], whereas we have around 25,000 genes. Still, yeast has a very similar cellular organization to higher eukaryotic organisms, with a high conservation of proteins. The genome properties of *S. cerevisiae* have led to the development of systematic whole genome analyses using genome data and microarrays. Yeast is also known to have a very high level of homologous recombination. This makes it possible to replace genes in the genome without influencing the sequence at another location, facilitating gene disruption, deletion and modification. Together with its ability to be easily transformed, yeast is an interesting tool (Hartwell, *et al*., 2011).

S. cerevisiae is unique among eukaryotes to be able to grow fast under aerobic but also under strictly anaerobic conditions. In other words: It is growing rapidly in both the presence and the complete absence of oxygen, respectively, growing both by cellular respiration and fermentation (Visser, *et al*., 1990).

After getting to know its properties, it is not unimaginable that yeast is a very important microorganism in biotechnology applications and is the preferred model organism for studying eukaryotic cell biology. It is not only used for the production of alcoholic beverages and bread, but also for bioethanol production for transport fuel. Yeast is utilised in fundamental research, studying for example the cell cycle, DNA repair, signal transduction, differentiation, organelle biogenesis and protein interactions (Lodish, *et al*., 2007).

[1] http://www.yeastgenome.org/genomesnapshot

1.2. Life Cycle

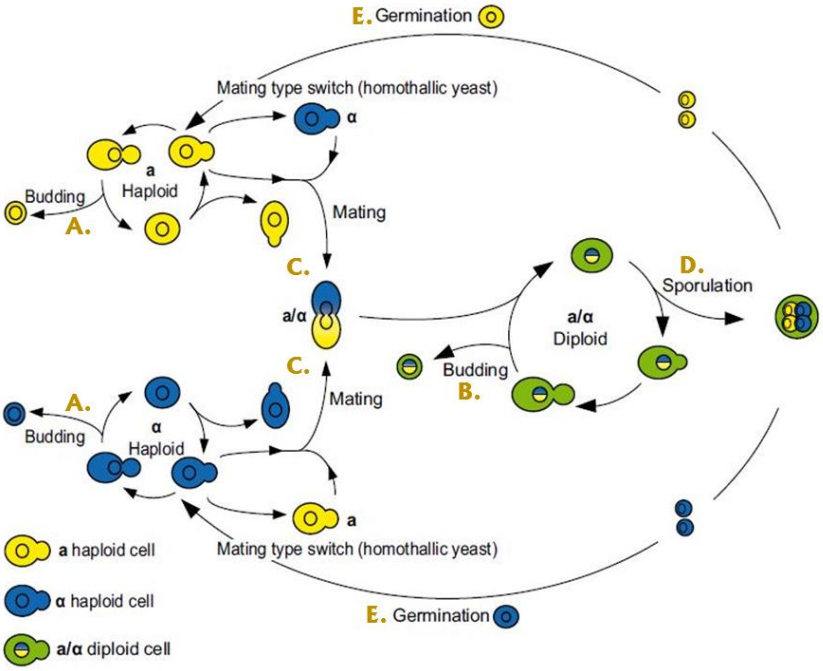

Figure 1: Schematic representation of the *S. cerevisiae* life cycle *(Steensels, et al., 2014).*

The life cycle of *S. cerevisiae* consists of different processes: budding, mating, sporulation and germination, represented in *Figure 1*, reviewed by (Steensels, et al., 2014)

Yeast can exist in a haploid, aneuploid and polyploid state, possibly ranging from diploid to octoploid (Hansen & Kielland-Brandt, 1996). They are reproducing asexually by budding, indicated by 'A' and 'B' in *Figure 1* for haploid and diploid cells respectively, giving *S. cerevisiae* its common name 'budding yeast'. The series of events that lead to bud formation by mitosis are referred to as the cell cycle, discussed in *section B.1.3*. Throughout almost the whole cell cycle, the parent cell carries a bud. Finally, a small bud is pinched off, called the daughter cell (Herskowitz, 1988). *S. cerevisiae* undergoes multilateral budding, meaning that the buds do never arise at the same site on the surface of the parent cell (Streiblová & Beran, 1963).

When adequate nutrients such as sugar, nitrogen and phosphate are no longer present, haploid yeast cells will not continue to divide asexually, but will reproduce sexually (Madhani, 2007). When haploid cells of opposite mating types (*MATa* and *MATα*) come into contact they will mate, also called conjugate (Duntze, *et al*., 1970), shown by 'C' in *Figure 1*. The cells grow in each other's direction in a process called shmooing. During conjugation the haploid cells first merge and subsequently their nuclei fuse, resulting in the formation of a diploid cell (MacKay & Manney, 1974).

Shmoo cell formation and subsequent conjugation is only possible by the presence of specific environmental responses: The mating factors. The α-factor secreted by a *MATα* cell binds to a specific pheromone receptor on *MATa* cells, whereas the a-factor of *MATa* cell binds to a

distinct pheromone receptor on a *MATα* cells (Duntze, *et al*., 1970). These pheromone receptors activate a heterotrimeric G protein activating the downstream MAP kinase cascade (Dietzel & Kurjan, 1987).

If *MATa/α* diploid cells with a normal a-α activity are starved for nitrogen and utilize a poor carbon source such as acetate, they undergo sporulation, consisting of meiosis followed by spore formation (Roman, *et al*., 1955; Kassir & Simchen, 1976), pinpointed in *Figure 1* by 'D'. The sporulation process of one diploid cell results in one ascus encapsulating four haploid ascospores, two of each mating type. These four spores are surrounded by a cell wall that is very resistant to environmental stress, giving the spore the capability to survive for a long period of time. When the conditions are more favourable, the spores are able to germinate (Esposito & Klapholz, 1981), marked by 'E' in *Figure 1*.

Some *S. cerevisiae* strains are able to undergo a mating type switch, they are known as homothallic strains. This process is mediated by an endonuclease which is encoded by the *HO* gene (Winge & Roberts, 1948).

1.3. Cell Cycle

The organization and the control of the cell cycle are well conserved over all eukaryotic organisms. The working mechanism of the cell cycle is studied extensively using two yeast species: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Forsburg & Nurse, 1991).

Conventionally, the eukaryotic cell cycle is divided into four phases: G1, S, G2, and M sequentially. The interphase, the G1, S and G2 phases together, has a growth activity, whereas the M phase a division activity (Pringle & Hartwell, 1981).

Major control points occur both at the bridge between the phases G2 and M ('mitotic entry' checkpoint) and phases G1 and S ('Start'). These checkpoints can be seen as internal regulatory systems, causing arrest of the cell cycle if the progression requirements are not met. The prerequisites that apply for both checkpoints are obtaining completely replicated and undamaged chromosomes and reaching a critical cell size. This is a survival mechanism, avoiding DNA damage and cell death. If the cells are in the periods in between the checkpoints, they are no longer sensible for external factors (Murray, 1995; Forsburg & Nurse, 1991).

The yeast 'Start' is equivalent to the restriction point in mammalian cells. It determines the commitment of the cell, either it progresses or exits the cell cycle. Exit from the cell cycle can be caused by different factors. Firstly, if cells are starved for certain nutrients they will enter the stationary (G0) phase with the consequences of arresting growth, decreasing protein synthesis and increasing stress resistance (Walker G. M., 1998). 99.9 % of all microbes exist

in this quiescent state, also called dormancy phase, waiting for the right environment to grow again (Kaeberlein T, 2002). Secondly, in the case of haploid cells, the presence of mating factors (pheromones) can cause cell cycle arrest, resulting in conjugation (Gupta, 2005). Lastly, and as mentioned in *section B.1.2.*: If *MATa/α* diploid cells are starved for nitrogen and carbon, sporulation is induced (Esposito & Klapholz, 1981).

1.4. Growth Curve

In among others *S. cerevisiae*, the absorbance (optical density, OD) of a liquid cell culture at wave length 600 nm gives indications about the amount of cells present and the growth phase cells are in. Each phase corresponds with a specific growth rate, an important characteristic of the yeast population. In *Figure 2,* a typical growth curve of *S. cerevisiae* is shown (Held, 2010).

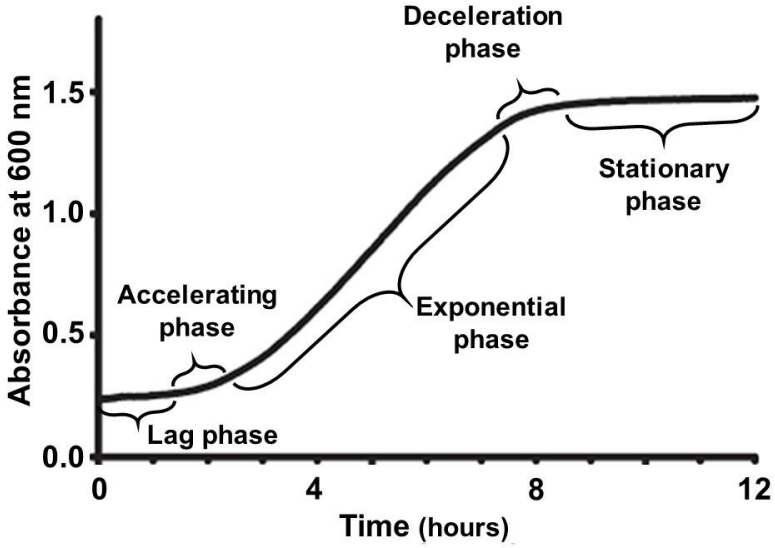

Figure 2: Typical growth curve of *S. cerevisiae.* The data points are obtained by measuring the absorbance with a spectrophotometer at a wavelength of 600 nm (OD_{600}) every two minutes for 12 hours after inoculation of a *S. cerevisiae* liquid YPD culture (complete medium for yeast) which is continuously incubated at 30 °C. Adapted from Held, 2010.

Directly after the inoculation of yeast cells, the lag phase is induced. In this phase the number of individuals in the population does not increase, cells are only growing in volume. As soon as cells initiate active cell division, they are in the accelerating phase. In the exponential phase a logarithmic cell doubling is seen with a maximum specific growth rate. If certain substrates are reaching a limiting concentration, the rate of growth is decreasing, entering the deceleration phase. When the absorbance is reaching its maximum (Absorbance 1.5 in the conditions mentioned in *Figure 2*), cells are in the stationary phase. The amount of cells is remaining constant, i.e. having a specific growth rate of zero. This refers to the fact that a population of cells is able to survive for months without adding nutrients (Walker G. M., 1998). A cell culture with OD_{600} of 1.0 consists of about 70 million cells per ml (Sherman, 1991).

Not all types of substrates that are reaching limiting concentrations lead to yeast populations in the deceleration phase and subsequently the stationary phase. Certain limiting nutritional conditions give rise to sporulation and the development of pseudomycelia. Not only nutrient limitation, but also physiological factors may stimulate the entrance into stationary phase. Examples are certain oxygen levels, high carbon dioxide levels, toxic metabolites like ethanol, low pH as well as elevated temperatures (Walker G. M., 1998).

2. Nutrient Sensing & Signalling

2.1. General Introduction

Changes in the environment like the absence of nutrients or other stress factors are triggering responses in all cells, also in *S. cerevisiae* (Gasch, et al., 2000). Nutrients are generating building blocks as well as energy for cells, besides that, they result in a regulatory effect (Conrad, et al., 2014).

Before nutrients can enter the cytoplasm of the baker's yeast cell, they have to cross the cell wall, periplasm and plasma membrane subsequently. The cell wall serves as an obstacle for big molecules. It will retain molecules greater than around 700 Da, depending on the shape, permitting most nutrients to pass (Walker G. M., 1998). On the other hand, only some nutrients will be able to diffuse through the plasma membrane without the help of transporter proteins, like water and ethanol. Those nutrients are only able to pass the membrane down the concentration gradient of the compound, following the diffusion rules. The protein-free membrane is impermeable for large uncharged polar molecules and charged molecules, being a barrier for e.g. glucose, fructose, amino acids, proteins, adenosine monophosphate (ATP), nucleic acids and ions. The plasma membrane is the place where extracellular nutrients are sensed and transported in a controlled way, leading to signalling cascades in the cell (Lodish, *et al*., 2007).

Yeast cells are able to sense nutrients both extracellularly and intracellularly. Possibly, these pathways exist in parallel, but being more or less interconnected. Researchers also distinguish 'specific' and 'general' nutrient signalling pathways. The former is a pathway responding to a certain nutrient or class of related nutrients, while the latter is responding to multiple types of nutrients. The different pathways lead to distinct downstream effects. A specific nutrient signalling pathway regulates mainly the uptake and metabolism of the nutrient(s), whereas the general pathway is coordinating changes in developmental and physiological traits like cell cycle control, stress tolerance, pseudohyphal growth and aging (Conrad, et al., 2014).

One of the processes requiring the most energy in cells is ribosome biogenesis. Consequently, the ribosome content, tightly connected to the growth rate, should be optimally adjusted to the

nutrient quality and quantity (Mager & Planta, 1991). Accordingly, the activities of the three RNA polymerases are securely regulated: Pol I for ribosomal RNA, Pol II for ribosomal protein genes, and Pol III for transfer RNA and small nuclear RNA synthesis (Lempiainen & Shore, 2009). Ribosome biogenesis is found to be regulated by the Target of Rapamycin protein kinase (TOR) as well as the protein kinase A (PKA) signalling cascades. These are two prominent, nutrient-responsive, growth-controlling pathways which are evolutionary conserved. They couple nutrient availability to the regulation of diverse cell processes that drive cell growth and proliferation (Zurita-Martinez & Cardenas, 2005). The TOR pathway is only sensing nitrogen sources, whereas the PKA pathway is influenced by the availability of fermentable carbon sources -being glucose, fructose or sucrose- as well as all other nutrients essential for yeast growth [info PKA pathway; see *section B.2.2.*] (Thevelein, et al., 2000). It is suggested that the ribosome biogenesis would be controlled by TOR via the PKA pathway (Martin, Soulard, & Hall, 2004). Ribosome gene expression is thus a cellular target regulated by multiple types of nutrients, regulated by 'general' nutrient signalling pathways (Mager & Planta, 1991).

A minor change in nutrient availability will trigger mostly a 'specific' and a 'general' nutrient signalling pathway. For example, if *S. cerevisiae* is starved for iron, high-affinity iron transporters will be upregulated, being a 'specific' response. At the same time, a 'general' response is induced. The growth rate declines, the cells arrest in the G1 phase and enter the G0 phase, obtaining stress tolerance properties. If there is a re-addition of iron, the high-affinity transporters are endocytosed (specific response) and the ribosomal gene expression is induced so that growth can start again (general response) (Conrad, et al., 2014).

2.2. The PKA Pathway

2.2.1. PKA Activity and Targets

PKA has a large number of targets, regulating characteristics associated with rapid fermentative growth, slow respiratory growth and stationary phase (Thevelein & de Winde, 1999).

PKA is a heterotetrameric protein, consisting of two regulatory and two catalytic subunits. The regulatory subunits are encoded by the *BCY1* gene and the catalytic subunits by three genes: *TPK1*, *TPK2* and *TPK3*, the resulting subunits can be combined in a complex. The binding of the classic second messenger 3',5'-cyclic adenosine monophosphate (cAMP) to the regulatory subunits causes dissociation of the complex, generating one regulatory homodimer and two active catalytic monomers (Toda, *et al*., 1987; Toda *et al*., 1987b). Upon dissociation, PKA is activated. The catalytic monomers are directly phosphorylating cytosolic enzymes and regulating gene expression at the level of transcription (Cameroni, *et al*., 2004). It is shown that the PKA catalytic subunit Tpk1 is accumulated in the nucleus during deprivation of cAMP, while it is rapidly relocalized to the cytoplasm after cAMP re-addition. The Bcy1 subunit, on the other hand, remains localized in the nucleus either in the absence or presence of cAMP (Griffioen, *et al*., 2000). The PKA activation is shown to be dependent on both the amplitude and frequency of the cAMP oscillations (Gerisch & Hess, 1974).

The three types of catalytic PKA subunits have different characteristics. They are all functionally redundant for cell viability, but seem to coordinate different cellular processes. More than 87 % of all PKA targets are phosphorylated by only one of the catalytic PKA subunits (Ptacek, et al., 2005). The C-terminal 320 amino acids of the subunits share mutually sequence similarity of 75 % or more, while there is no sequence similarity seen in the N-terminal regions. Additionally, the N-termini are different in length (Toda, *et al*., 1987).

When *S. cerevisiae* grows on a fermentable carbon source together with a complete growth medium -presence of nitrogen, phosphorus and sulfur sources-, a high PKA activity is seen *in vivo*. In this condition, cells are fermenting, they are also called glucose-repressed. On the other hand, when they live in a fermentable carbon source lacking one of the essential nutrients, they have a low PKA activity, being in the stationary phase. A low PKA activity is also observed if cells are growing on a non-fermentable carbon source like ethanol, acetate or glycerol. The cells in this state are in slow respirative growth and are named glucose-derepressed. Totally different physiological properties are thus observed for cells with low versus high PKA activity (Thevelein, et al., 2000).

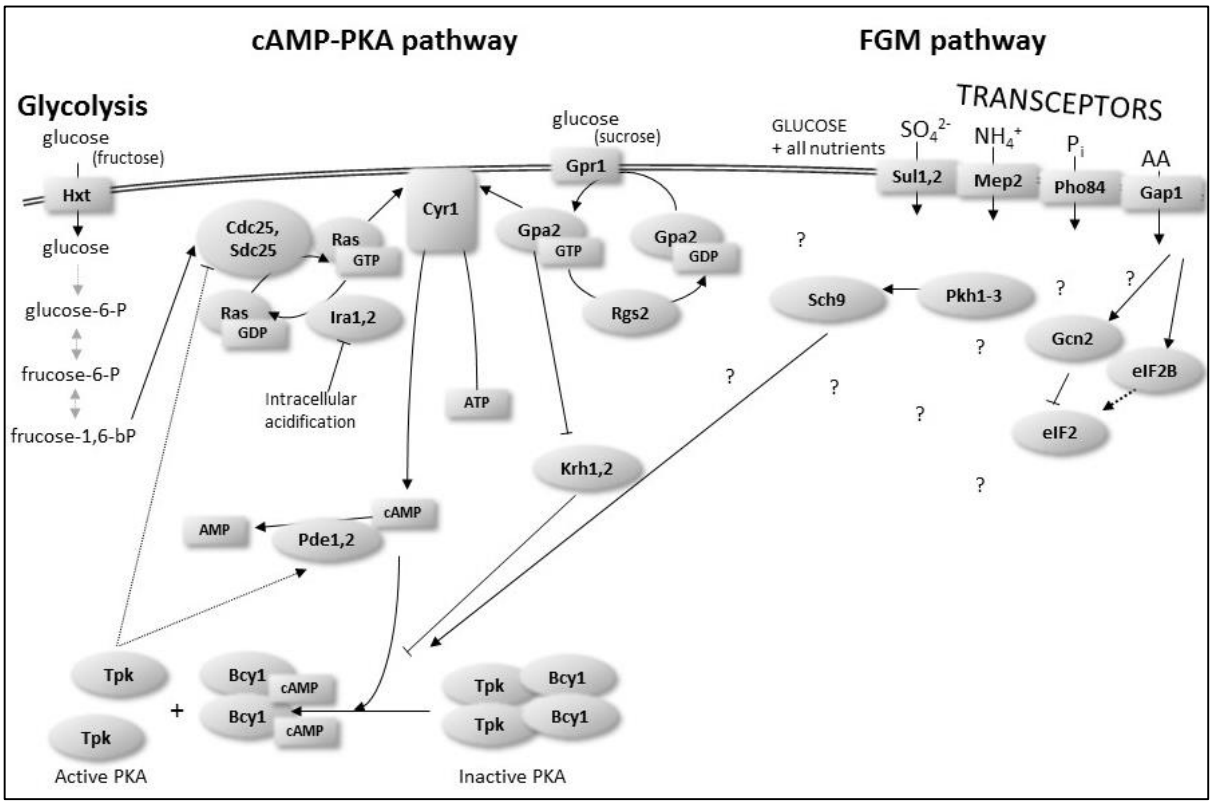

Figure 3: Regulation of PKA activity by the cAMP-PKA pathway and the FGM pathway. Metabolic reactions are depicted in grey, regulatory and signalling interactions in black. Dotted lines represent feedback loops (Figure of the current work).

In cells with low PKA activity, genes for fermentation and ribosome biogenesis are repressed. Furthermore, genes for respiratory growth or stationary phase are induced. The high expression of PDS-controlled (post-diauxic shift) and STRE-controlled (stress resistance) genes, like heat shock protein genes, is a property associated with low PKA activity. This can be linked with the activation of developmental pathways that leads to high sporulation capacity or pseudohyphal growth. Also a strong cell wall is found in this status, making them more resistant against the enzyme lyticase. In addition, the reserve carbohydrates -trehalose and glycogen- are accumulated. The physiological characteristics opposite to the ones of a low PKA activity are true for high PKA activity (Thevelein, 1994; Holsbeeks, *et al.*, 2004).

At least two pathways are causing and regulating a high PKA phenotype in *S. cerevisiae*: The cAMP-PKA pathway [info cAMP-PKA pathway; see *section B.2.2.2.*] and the fermentable growth medium induced pathway (FGM pathway) [info FGM pathway; see *section B.2.2.3.*], visualised in *Figure 3*. cAMP accumulation is triggered by the cAMP-PKA pathway, upon re-addition of glucose to glucose-derepressed cells. If no complete growth medium is present, the effect of this pathway is only transient. The FGM pathway is able to maintain the effect on the PKA targets if glucose combined with complete growth medium is present (Thevelein, et al., 2000).

2.2.2. The cAMP-PKA Pathway

If glucose is added to cells in exponential phase, growing on a non-fermentable carbon source, or to cells in stationary phase without glucose, there is a cAMP peak observed within one minute. Together with this fast increase and subsequent decrease of cAMP, the PKA activity increases, inducing a phosphorylation cascade and the associated phenotypic changes, all regulated by the cAMP-PKA pathway, visualised in *Figure 3*. This pathway mediates an extensive part of glucose sensing and signalling in *S. cerevisiae* (Hirimburegama, et al., 1992).

The conversion of ATP into pyrophosphate and cAMP is catalysed by the adenylate cyclase enzyme, encoded by *CYR1* in *S. cerevisiae* (Kataoka, *et al*., 1985). This enzyme is regulated by two G-protein systems that each are mediating one section of the glucose sensing pathway. The first section of this dual glucose-induced cAMP signalling is a G protein-coupled receptor (GPCR) system for extracellular glucose sensing [see Gpr1 - Gpa2 - Rgs2 GPCR system], in the second one Ras proteins -small GTPases- are involved [see Cdc25/Sdc25 - Ras1/2 - Ira1/2 system] (Thevelein & de Winde, 1999).

Gpr1 - Gpa2 - Rgs2 GPCR system

Extracellular glucose addition in glucose-deprived cells is sensed through a GPCR system consisting of Gpr1, a 7-transmembrane receptor, and its associated G-alpha protein, Gpa2. Gpr1 is stimulating guanine nucleotide exchange on Gpa2 and the resulting Gpa2-GTP is activating adenylate cyclase, inducing cAMP synthesis. The Rgs2 protein is encouraging the intrinsic GTPase activity of Gpa2, Rgs2 thus acts as a GTPase-activating protein (GAP) on Gpa2, being an inhibitor of the glucose activation of cAMP synthesis. This system was the first nutrient-sensing GPCR system identified in eukaryotes, before, ligands of GPCRs found were signalling molecules like hormones (Kraakman, *et al*., 1999; Rolland, *et al*., 2000; Colombo, *et al*., 1998; Versele, *et al*., 1999).

Only if adenylate cyclase is made responsive by activated Ras proteins (GTP-bound Ras), the Gpr1 - Gpa2 - Rgs2 GPCR system is able to activate the enzyme. The activation of the Ras proteins themselves requires transport and phosphorylation of glucose, resulting in the fact that the extracellular glucose sensing by the GPCR system is relying on the glucose metabolism (Colombo, *et al*., 2004).

Cdc25/Sdc25 - Ras1/2 - Ira1/2 system

Activated Ras proteins trigger adenylate cyclase by direct binding or via adenylate cyclase associated proteins (Srv2/CAP) (Gourlay & Ayscough, 2006). The regulation of the activity of the Ras proteins is well known. They depend on the adjustment of their intrinsic GTPase activity by a GAP and the exchange of GDP in GTP by a guanine nucleotide exchange factor (GEF). In *S. cerevisiae*, Cdc25 and its homolog Sdc25 act as GEFs, and Ira1 and Ira2 as GAPs for Ras1 and Ras2 (Broach & Deschenes, 1990). Fructose-1,6-bisphosphate, a glucose intermediate in glycolysis, is a potent activator of Ras, acting through Cdc25 (Peeters K. , 2013). Ras thus serves an intracellular system dependent on the uptake of glucose (via hexose transporters) and hexokinase-mediated phosphorylation, eventually activating adenylate cyclase (Rolland, *et al*., 2000; Thevelein & de Winde, 1999).

This G-protein system can, next to glucose sensing, be activated by intracellular acidification, acting in part through Ira1/2 inhibition, promoting Ras activation and subsequently activating adenylate cyclase (Thevelein, et al., 2000).

PKA activation bypassing adenylate cyclase stimulation

The Krh1 and Krh2 proteins, two Gpa2-associated Kelch repeat proteins, function in an adenylate cyclase bypass pathway, an alternative cAMP-independent PKA activation pathway (Lu & Hirsch, 2005; Peeters *et al*., 2007). The Kelch repeat proteins physically connect with the catalytic PKA subunits, stimulating the association of the catalytic and the regulatory PKA subunits, leading to a reduction in the PKA activity. GTP-bound Gpa2, activated due to glucose sensing by Gpr1, is able to inactivate Krh1 and 2, consequently reducing the amount of cAMP needed to activate PKA. In this way, a mechanism is created for the PKA activation without the need for a cAMP level change. Additionally, this pathway can function synergistically after an increase in the cAMP concentration, stimulating the PKA activation even more. This alternative activation of PKA can only act if a certain level of cAMP is present, as a result of - as described before- the necessity of cAMP for the dissociation of the PKA subunits, leading to PKA activation (Harashima & Heitman, 2002).

Feedback mechanisms

cAMP synthesis is tightly regulated to avoid over-activation of PKA. A proposed feedback inhibition system of PKA is through the phosphorylation of Cdc25 (Munder & Küntzel, 1988). An additional layer of regulation of the Ras signalling system, serving as feedback mechanism, is the compartmentalization of among others Ras1/2, Ira1/2, Cdc25/Sdc25 and adenylate cyclase. The localization of these proteins is not limited to the plasma membrane, they are additionally present in the cytosol and/or (sometimes indirectly) associated with membranes of the organelles (Belotti, et al., Localization of Ras signaling complex in budding yeast, 2012). Other feedback systems on the cAMP synthesis are proposed, but not proven yet.

As mentioned before, the cAMP level in budding yeast is influenced by activating adenylate cyclase by the two given separate G-protein systems. Additionally, the amount of cAMP present is determined by its degradation. Pde1 and Pde2, respectively low- and high-affinity phosphodiesterases (Pde), are able to catabolise the degradation of cAMP into adenosine monophosphate (AMP) (Sass, *et al*., 1986). Because the amount of cAMP is tightly influencing the PKA activity, a negative feedback loop exists to regulate the activity of those Pde enzymes (Ma, *et al*., 1999).

Additionally, upon glucose limitation, both the abundance and phosphorylation state of Bcy1 increase in a Krh-dependent manner. Tpk1-3 phosphorylates Bcy1, targeting its degradation unless it is protected by Krh1 protein, functioning as a negative feedback loop (Budhwar, *et al*., 2010).

2.2.3. The Fermentable-Growth-Medium-Induced Pathway

Regulating the entrance to and exit from the stationary phase is only possible in a complete growth medium along with a fermentable carbon source. Because this regulation is different from the cAMP-PKA pathway, the term 'fermentable growth medium induced' (FGM) pathway was introduced (Thevelein, 1994). Both pathways are represented in *Figure 3*.

If cells get depleted for a single essential nutrient in the presence of a fermentable carbon source, they will not pass 'Start', arrest in the G1 phase, and enter the G0 phase [info cell cycle phases: *section B.1.3.*]. A drop in PKA activity is observed, together with the expression of distinct nutrient high-affinity transceptors localized at the plasma membrane [info transceptors: *section B.2.3.*].

If the lacking essential nutrient is added back to the cells, the corresponding nutrient-specific transceptor signals to PKA, leading to a rise in PKA activity. This results in a phosphorylation cascade and rapid phenotypic changes starting fermentative growth. In contrast to the re-addition of glucose as described in *section B.2.2.2*, no spike of cAMP is seen. Still, a basal level of cAMP is necessary to dissociate the catalytic PKA subunits, activating PKA, consequently enabling the integration of nutrient signals (Hirimburegama, et al., 1992).

The exact mechanism of the FGM pathway is not revealed yet. However, there is some scientific evidence for the involvement of certain proteins discussed in the following paragraphs. A possible regulator of the FGM pathway is the Sch9 kinase (Zabrocki, et al., 2002), a yeast homologue of the mammalian protein kinase B (PKB/Akt) and S6 kinase. Sch9 is found to regulate the phosphorylation and localization of Bcy1 (Zhang, *et al*., 2011). The kinase acts as a PKA activator when cells are glucose-repressed, but on the other hand as a PKA inhibitor in glucose-derepressed cells. In nitrogen starved cells, the Sch9 kinase is required for PKA activation by amino acid and ammonium re-addition (Crauwels, et al., 1997). However, if phosphate is added to phosphate starved cells, the absence of Sch9 does not prevent the PKA activation, leading to the conclusion that Sch9 is not a general requirement for PKA activation via transceptor signalling (Giots, *et al*., 2003).

Sch9 also functions as a target of Tor complex 1 (TORC1), one of the two distinct structural and functional TOR complexes. Serine and threonine residues in the C-terminus of Sch9 are phosphorylated by TORC1, required for the Sch9 activity. It is suggested that Sch9 is dephosphorylated by protein phosphatases 2A, also direct targets of TORC1 and glucose sensing, downregulating Sch9. The dephosphorylation and thus inactivation of Sch9 happens in response to the addition of rapamycin -a TOR inhibitor- and if cells are starved for carbon, nitrogen, certain amino acids or phosphate (Urban, et al., 2007). With Sch9 being a possible regulator of the FGM pathway, there is a putative connection between the TOR and the FGM pathway (Zabrocki, et al., 2002).

Furthermore, the Pkh1, 2 and 3 kinases, homologues of the mammalian 3-phosphoinositide-dependent protein kinase 1 (PDK1), are modulating the Sch9 activity by phosphorylation. Pkh1-3 are also found to be regulating PKA activity, suggesting that they are involved in the FGM pathway (Voordeckers, *et al*., 2011; Haesendonckx, *et al*., 2012).

2.3. Transceptors

Membrane proteins able to take up nutrients and additionally sense extracellular nutrients are called transceptors (Hirimburegama, et al., 1992).

The most established examples of eukaryotic transceptors are the amino acid transceptor Gap1, the ammonium transceptors Mep1 and Mep2, the phosphate transceptor Pho84 and the sulphate transceptors Sul1 and Sul2. Putative transceptors for micronutrients are Frt1 for iron, Zrt1 for zinc and Crt1 for copper. All of them are transceptors of *S. cerevisiae* and are activating the PKA pathway upon re-addition of the missing nutrient in order to rapidly induce respirative growth, stimulating protein synthesis and start-up of growth (Donaton, *et al*., 2003; Van Nuland, *et al*., 2006; Giots, *et al*., 2003; Kankipati, *et al*., 2015).

The expression of all these transceptors is regulated by the presence of their specific nutrients. Most of them are strongly induced under starvation for their substrate, while other non-sensing transporters are downregulated. After re-addition of the limiting nutrient, the transceptors are internalized by a process called substrate-induced endocytosis (Ghaddar, et al., 2014). This rapid downregulation probably contributes to prevent overstimulation of the PKA signalling pathway (Kriel, *et al*., 2011). Just as other plasma membrane transporters, they undergo constitutive endocytosis (Gao & Kaiser, 2006; Lauwers, *et al*., 2010) to a sorting endosome from which they are recycled back to the plasma membrane.

The best established example of a eukaryotic transceptor is Gap1. It contains twelve transmembrane domains and is a proton symporter (Gilstring & Ljungdahl, 2000). One proton is required for the transport of one substrate, as a consequence, one ATP molecule is indirectly needed for the transport. Gap1 is proven to be regulated through substrate-induced internalization via the endocytic pathway (substrate-induced endocytosis). Thereby, after re-addition of amino acids, Gap1 is ubiquitinated by the Rsp5/Bul1/Bul2 ubiquitin ligase complex, leading to endocytosis and subsequent transport through early endosomes, the multi-vesicular body and late endosomes. Lastly the transceptor is degraded in the vacuole (Lauwers, *et al*., 2010).

Based on a screening of amino acid analogues for their functionality towards the Gap1 transceptor, it is concluded that transport of the substrate is not required to trigger signalling or induction of endocytosis. Only the induction of a specific occluded conformational change

is obligatory for those processes. This is perfectly in line with classical receptors, which do not transport, but are still able also to signal (Van Zeebroeck, *et al*., 2014).

Transceptor Gap1 does not only have the L-amino acids as substrates, but also the D-amino acids as well as certain amino acid analogues as beta-alanine. The D-amino acids and analogues are not metabolised by the cell, but still trigger the signalling pathway. Subsequently, one can conclude that metabolism is not required for transceptor signalling (Donaton, et al., 2003).

S. cerevisiae is not the only example in which transceptors are found, the mechanism seems to be conserved in higher eukaryotes as well. The PATH amino acid transporters in *Drosophila*, the mammalian SNAT2 and GLUT2 , the Nrt1 nitrate transporter and the Sultr1-2 sulphate transporters in *Arabidopsis thaliana* are found to also have an additional signalling function (Goberdhan, *et al*., 2005; Hyde, *et al*., 2007; Stolarczyk, *et al*., 2010; Gojon, *et al*., 2011; Zhang, *et al*., 2013).

3. Protein Synthesis

3.1. Overview

In *S. cerevisiae*, protein synthesis happens as universally: Transcription followed by translation. During transcription, RNA polymerase is transcribing mRNA from DNA. The mRNA code is translated into the peptide sequence during translation. This process is divided into three sequential steps: Initiation, elongation and termination. Translation initiation results in a complex consisting of a ribosome and initiation factors onto the mRNA start codon. During elongation, amino acids matching the mRNA codons are connected to each other. Subsequently, at the mRNA stop codon the peptide chain and the ribosomal subunits are released in the termination process. Finally, post-translational modifications of the peptide can facilitate the increase in stability and/or enhance the activity of the protein and/or its sorting to the organelles.

To allow rapid responses to nutrient deprivation and environmental stresses, the translational process is tightly regulated. The translation initiation is most regulated compared to the other translation steps. This occurs both by changing the conformation and activity of the initiation factors and by altering the mRNA (Sonenberg & Hinnebusch, 2009; Jackson, *et al.*, 2010).

3.2. Translation Initiation

3.2.1. Process

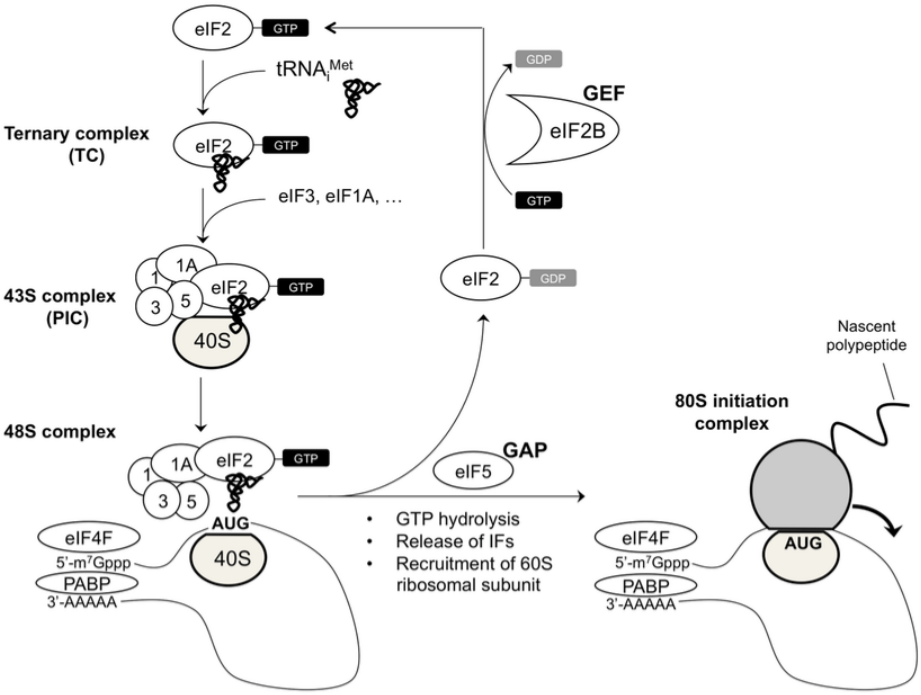

Figure 4: Schematic representation of translation initiation, adapted from *Sonenberg & Hinnebusch, 2009, and Merrick, 2010* by Dr. Kimpe (PhD Thesis).

In *Figure 4*, translation initiation is schematically represented.

The main eukaryotic initiation factor of protein synthesis is the heteropentameric protein eIF2B, comprising of five subunits: α, β, γ, δ and ε, encoded by *GCN3*, *GCD7*, *GCD1*, *GCD2* and *GCD6* respectively. This factor is catalysing the activation of the G-protein eIF2, consisting of three subunits: α, β and γ, encoded by *SUI2*, *SUI3* and *GCD11* respectively. eIF2B is thus the GEF of eIF2. Both eIF2 and eIF2B proteins are highly conserved among eukaryotes. The presence of GTP-bound eIF2 is required for translation initiation: It interacts with the initiator methionyl tRNA, resulting in the formation of the ternary complex (TC), being the rate-limiting step of initiation (Jackson, *et al*., 2010).

The 43S or pre-initiation complex (PIC) is formed by binding of the 40S ribosomal subunit and several eIFs -being 1, 1A, 3 and 5- to the TC. In the meantime, at both sides of the mRNA, different protein complexes are formed. At the 5' cap, the heterotrimeric cap complex eIF4F is localized, consisting of eIF4E, eIF4A and multi-adaptor eIF4G, while at the 3' poly A tail, poly(A)-binding proteins (PABPs) are attached. The eIF4F-PABPs-interaction results in a circular mRNA structure. This together with the eIF3-eIF4G interaction facilitates the PIC to bind the 5 prime cap, forming the 48S complex. Subsequently, the complex is scanning for the start codon (AUG), sliding over the mRNA strand, leading to the dissociation of eIF4E, eIF4A and eIF4G. As soon as the start codon is found and bound to the peptidyl site, the GTP-bound eIF2 will be hydrolysed, catalysed by its GAP eIF5B. The 60S ribosomal subunit is recruited to the complex, resulting in the 80S ribosomal complex, initiating the GTP hydrolysis of eIF5B. The complex can now recruit the respective tRNA into its aminoacyl site to catalyse the formation of the first peptide bond, starting the next step: Translation elongation (Pestova & Kolupeva, 2002; Sonenberg & Hinnebusch, 2009).

What exactly the role is of the specific eIF2 and eIF2B subunits, is described in this paragraph. The eIF2α/Sui2 is engaged in the start codon identification. The phosphorylation of eIF2α/Sui2 on Ser51 changes its function, it is competitively inhibiting the exchange of GDP into GTP. Hence, this subunit is essential for the function of eIF2, being the major regulator of eIF2B. In other words, unphosphorylated eIF2 is a substrate of eIF2B, resulting in eIF2-GTP, whereas phosphorylated eIF2 is a competitive inhibitor of eIF2B, leading to an eIF2 unable to form a ternary complex. eIF2β/Sui3, on the other hand, interacts with eIF5 and RNA. Curiously, it binds with eIF2B and can be phosphorylated by PKA on Ser218 (Welsh, *et al*., 1994; Laurino, *et al*., 1999). On the other hand, eIF2γ/Gcd11 is the primary guanine nucleotide binding subunit and can also interact with RNA. Furthermore, the recognition of the eIF2α/Sui2 phosphorylation happens through the collaboration of eIF2Bα/Gcn3, eIF2Bβ/Gcd7 an eIF2Bδ/Gcd2, forming the eIF2B regulatory domain. The eIF2Bγ/Gcd1 and eIF2Bε/Gcd6 together form the catalytic subcomplex, performing nucleotide exchange. A region of the eIF2Bε/Gcd6 alone is sufficient for eIF2 binding (Pavitt, 2005).

3.2.2. Regulation

Translation initiation in among others *S. cerevisiae* can be regulated via eIFs or ribosomes, inducing changes on initiation overall, or via mRNAs, potentially on the contrary only targeting specific mRNA subsets (Raught & Gingras, 1999).

Certain stress factors are influencing the phosphorylation state of eIF2α/Sui2. In *S. cerevisiae*, during amino acid starvation for example, the nutrient-regulated protein kinase Gcn2 phosphorylates eIF2α/Sui2. Gcn2 is activated by lowering of both TOR inhibition and aminoacyl-tRNA levels. Phosphorylated eIF2α/Sui2 (eIF2α-P) leads to a general reduction in translation, due to the fact that a stable TC cannot be formed. At the same time, a selective increase of the translation of the *GCN4* gene is seen, a transcriptional activator of 57 amino acid biosynthetic genes (Hinnebusch A. , 1997; Kimball, 1999; Mohammad-Qureshi, *et al*., 2008). Additionally, TORC1 is signalling to eIF2α/Sui2 via Sch9 and Tap42-PPase. The inhibition of Sch9 results in a phosphorylated eIF2α/Sui2 via a non-characterised pathway, whereas Tap42-PPase dephosphorylates eIF2α/Sui2 on Ser577 (Loewith & Hall, 2011). In line with what is said before, if cells are not starved for amino acids, high levels of the TC are seen due to the presence of unphosphorylated eIF2α/Sui2.

On the other hand, fusel alcohols are directly inhibiting eIF2B in yeast, for the reason that fusel alcohols are breakdown products of amino acids, indicating amino acid scarcity (Pavitt, 2005).

Another suggested level of protein synthesis regulation via eIFs in yeast is the subcellular localization of eIF2 and eIF2B. Under stress conditions, both factors are localized in a defined region of the cytoplasm. While eIF2B remains present in that region, eIF2 shuttles continuously between that region and the cytoplasm. It is therefore suggested that nucleotide exchange is happening in that region to generate active eIF2 factors (Campbell, *et al*., 2005).

Translation initiation regulation via mRNAs happens through an interaction of specific mRNA-binding proteins at the untranslated regions (UTR). The interaction is more common to take place at the 3’ UTR than at the 5’ UTR. An inhibitory loop is formed that hinders the access for eIF4F, by a protein that cross-links through an intermediate protein to a cap-binding protein (Raught & Gingras, 1999; Jackson, *et al*., 2010). Accordingly, the secondary structure of the UTRs is important for regulation. Secondly, mRNA decapping leads to mRNA degradation, inhibiting translation initiation. The regulation of yeast proteins like Pat1, Scd6, Edc3 and Dhh1 that promote decapping thus can neutralize the inhibition (Coller & Parker, 2005; Nissan, *et al*., 2010).

C. Aim

The ultimate goal of the current work is to reveal more information about linking nutrient availability to protein synthesis, and consequently cell growth. More specifically, the involvement of specific kinases in the signalling pathways is considered.

This research objective will be investigated by studying protein-protein interactions of the amino acid transceptor Gap1 with certain kinases, in particular Sch9, Pkh1, Tpk1, Tpk2 and Tpk3. Also interactions between these kinases and eIF2B subunits will be examined, particularly eIF2Bγ/Gcd1 and eIF2Bε/Gcd6.

eIF2B is relevant in this study since it is catalysing the activation of the G-protein eIF2, which is in turn found to be involved in PKA signalling. Moreover, the PKA pathway, including proteins Tpk1-3, is correlated with growth rate. As mentioned in *section A*, the Laboratory of Molecular Cell Biology at the KULeuven already found interactions between transceptors and the eIF2 and eIF2B subunits, making the transceptors possible candidates for linking nutrient availability to protein synthesis. Also Pkh1 and Sch9 are appropriate as protein of interest, since Pkh1 is found to regulate both Sch9 and PKA activity by phosphorylation. In addition, Sch9 is required for PKA activation by amino acid and ammonium re-addition in nitrogen starved cells in an undefined way.

Both *in vitro* and *in vivo* techniques to study protein-protein interactions will be utilised in the current work. GST pull-down is the selected *in vitro* approach, whereas split citrine BiFC is used as *in vivo* technique.

D. Materials & Methods

1. Strains

1.1. *Saccharomyces cerevisiae*

The *S. cerevisiae* strains provided for the current work are given in *Table 1*. The strains used are BY4741[2], BY4742[3] and Sigma 1278b[4], all laboratory strains [Risk Assessment *S. cerevisiae*: see *Addendum 1*]. They all have a stable mating type, making them heterothallic, due to a mutation in the *HO* gene (Hartwell, *et al*., 2011), facilitating to work with stable haploid strains (Ravishankar, 2016). All provided strains are among others sensitive for the antibiotic geneticin (G418). When they are transformed with DNA containing a *KanMX* marker, they acquire resistance to geneticin and kanamycin (Yörük & Albayrak, 2015).

BY4741 and BY4742 are derived from the S288C budding yeast, whereas Sigma 1278b has 75 genes that are not present in S288C (Dowell, et al., 2010). Sigma 1278b is prototrophic, while BY4741 and BY4742 have some auxotrophic mutations, given in *Table 1*, meaning that the latter two strains are unable to synthesize certain organic compounds required for growth (Brachmann, et al., 1998). On a selective medium without the particular compound, the mutation can thus be complemented after the transformation of a piece of DNA including the particular wild type biosynthesis gene. This makes it possible to select for the presence of the DNA that is to be transformed. Both strains are among others auxotrophic for histidine and uracil. The *HIS3* gene[5] encodes the enzyme imidazoleglycerol-phosphate dehydratase, catalysing the 6th step in histidine biosynthesis. The *URA3* gene[6] on the other hand encodes the orotidine-5'-phosphate decarboxylase enzyme, catalysing the 6th enzymatic step in the *de novo* biosynthesis of pyrimidines.

[2] http://www.yeastgenome.org/strain/BY4741/overview
[3] http://www.yeastgenome.org/strain/BY4742/overview
[4] http://www.yeastgenome.org/strain/Sigma1278b/overview
[5] http://www.yeastgenome.org/locus/S000005728/overview
[6] http://www.yeastgenome.org/locus/S000000747/overview

Table 1: The name and genotype of the *S. cerevisiae* strains used in the current work, provided by the MCB lab. More information about the construction of the Sigma strains can be found in *Suppl. Table 8*.

Name	**Genotype**
BY4741	*Mat **a** his leu met ura*
BY4742	*Mat α his leu lys ura*
BY proto Gcd1-CC:Gap1-NC	BY4741/BY4742 *Mat **a** his LEU LYS MET ura* Gcd1-C-Citrine (*SpHIS5*) Gap1-N-Citrine (*CaURA3*)
BY proto Gcd6-CC:Gap1-NC	BY4741/BY4742 *Mat **a** his LEU LYS MET ura* Gcd6-C-Citrine (*SpHIS5*) Gap1-N-Citrine (*CaURA3*)
BY4741 leu$^+$ met$^+$ Gcd1-CC	BY4741 *Mat **a** his LEU MET ura* Gcd1-C-Citrine (*SpHIS5*)
BY4741 leu$^+$ met$^+$ Gcd6-CC	BY4741 *Mat **a** his LEU MET ura* Gcd6-C-Citrine (*SpHIS5*)
BY4741 Gcd6-CC	BY4741 *Mat **a** his leu met ura* Gcd6-C-Citrine (*SpHIS5*)
BY4742 proto Gap1-NC	BY4742 *Mat α HIS LEU LYS ura* Gap1-N-Citrine (*CaURA3*)
BY4742 Gap1-NC	BY4742 *Mat α his leu lys ura* Gap1-N-Citrine (*CaURA3*)
BY4742 leu$^+$ lys$^+$	BY4742 *Mat α his LEU LYS ura*
Sigma GCD6-HA	Sigma 1278b Gcd6-3HAX (*KanMX6*)
Sigma GCD1-HA	Sigma 1278b Gcd1-3HAX (*KanMX6*)
Sigma GAP1-HA	Sigma 1278b YCplac33 *GAP1-3HA*

Legend: '-CC' and '-C-Citrine' stand for 'protein C-terminally tagged with the C-terminus of citrine', '-NC' and '-N-Citrine' for 'protein C-terminally tagged with the N-terminus of citrine'.

1.2. *Escherichia coli*

The *Escherichia coli* (*E. coli*) strain DH5α™[7] (Invitrogen™) is used for multiplying plasmids, whereas One Shot® BL21 Star™[8] (DE3, Invitrogen™) is used for protein expression [Risk Assessment *E. coli*: see *Addendum 1*]. The latter one is optimized for protein expression, being deficient for two important proteases Lon and OmpT. Both DH5α™ and One Shot® BL21 Star™ (DE3) are chemically competent and among others sensible for ampicillin, facilitating the transformation of plasmids containing an ampicillin resistance gene.

[7] https://www.thermofisher.com/order/catalog/product/18265017
[8] https://www.thermofisher.com/order/catalog/product/C601003

2. Growth Conditions and Media

In the laboratory, budding yeast is grown at 30 °C. To enable a good aeration, a liquid cell culture is grown in a shaking incubator at 200 rpm (revolutions per minutre) in an Erlenmeyer flask with a lid where gases can pass, respecting a liquid to air volume ratio of 1:2. Cells growing on agar plates -consisting of a solid medium- are put upside-down in a non-shaking incubator (Rose, *et al*., 1990).

E. coli at the other hand is grown at 37 °C in the laboratory. Just as yeast, a liquid culture is put in a shaking incubator at 200 rpm, while shaking is not needed for agar plates.

Growth media are made by first weighing the ingredients and subsequently adding distilled or Milli-Q water. Next, the pH of each type of yeast Synthetic Complete (SC) medium [*Suppl. Table 1*] is adjusted until pH 5.5 for liquid and 6.5 for solid purposes, obtained by using 4 M KOH. For Luria-Bertani (LB) medium [*Suppl. Table 1*] the pH is adjusted to 7.5 for liquid and 6.5 for solid purposes by addition of 4 M NaOH [Risk Assessment 4 M NaOH: see *Addendum 1*]. The pH of sporulation medium is modified to pH 6 with 4 M HCl [Risk Assessment HCl: see *Addendum 1*]. Thereafter, if the medium is used to make agar plates, 1.5 % (w/v) agar granulated (Difco) is combined in the solution.

After the preparations, the media are autoclaved for sterilization. Especially selection media for yeast are autoclaved immediately, because a contaminating yeast can grow on the medium, leading to the unwanted presence of the lacking amino acid(s) in the medium.

After autoclaving the prepared medium, a separately autoclaved concentrated glucose or galactose solution can be added, if required. If an antibiotic is needed, it is added afterwards to the cooled-down medium. Antibiotic solutions are filter sterilized because some are sensitive to heat (Aldrich). Media with respectively 0.02 % (w/v) and 0.01 % (w/v) geneticin or ampicillin are used. For making agar plates, the ready medium is pored into Petri dishes at the laminar flow cabinet, each with approximately 25 ml medium.

The content of the *S. cerevisiae* and *E. coli* media used in the current work is described in *Suppl. Table 1*.

For long-term storage a -80 °C glycerol stock is prepared. A 2 ml YPD (yeast extract peptone dextrose) [*Suppl. Table 1*] preculture in a glass tube is grown overnight (O/N) in a 30 °C shaking incubator. A screw cap tube is filled with a 1 ml solution containing the O/N culture with a final proportion of 30 % sterilised glycerol. The tube is directly put in a -80 °C freezer for storage. After adding 5 µl of the stock solution on a sterile agar plate and growing O/N in a 30 °C incubator, the strain can be used again.

3. Molecular Biology Techniques

3.1. Determination of DNA Concentration

A NanoDrop® ND-1000 Spectrophotometer (Isogen Life Sciences) is used to determine the concentration of DNA in a sample, it is done in the current work for both linear DNA and plasmids. The concentration is obtained by measuring the absorbance at 260 nm in a 10 mm path[9].

3.2. Polymerase Chain Reaction

A polymerase chain reaction (PCR) has the aim to amplify of a specific DNA region. A PCR reaction mixture needs to contain a DNA template which partially consists of the DNA sequence that is to be amplified, two DNA strands (primers) that are each homologous to one end of your DNA region of interest, deoxynucleotides (dNTPs), a DNA polymerase enzyme and a buffer in which the enzyme is able to function optimally[10]. Every time, a negative control of the reaction is prepared, comprising everything except the template. Milli-Q is added instead of the template. The PCR reaction mixtures are added to separate PCR tubes.

The reaction is carried out in a cyclic repetition of three steps namely 'denaturation', 'annealing' and 'elongation'. In the current work, the steps are repeated 30 times. The steps are executed at different temperatures. Firstly, single stranded DNA molecules are formed by disrupting the base stacking interactions and hydrogen bonds between the complementary bases. Secondly primers hybridise with the part of the single stranded DNA (ssDNA) template which is complementary. Finally the DNA polymerase synthesizes a new DNA strand by adding dNTPs in the 5' to 3' direction[11]. A PCR machine (Thermocycler Biometra®) is able to change temperature fast and precisely, to allow performing a PCR reaction.

The enzymes used in the current work for PCR are TaKaRa Ex Taq™ DNA Polymerase and Q5® High-Fidelity DNA Polymerase. The former has an extension time of 1 min per kilo base (kb)[12] while the latter 30 sec/kb[13]. Both have a high fidelity[14,15], the use of those enzymes is preferred when obtaining a correct PCR fragment is important, thinking of the usage of the PCR fragment for transformation or sequencing. The content of the PCR reaction mixtures with the different enzymes are given in *Suppl. Table 2*.

To have an idea of the annealing temperature that will lead to a PCR product, there is chosen for a calculation by the online tool New England BioLabs® Inc. Tm Calculator v.1.8.1[16]. It computes the annealing temperature (Ta) based on the sequences of your primer pair and the

[9] http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf
[10] http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechPCR.shtml
[11] https://www.neb.com/applications/dna-amplification-and-pcr/pcr
[12] http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_ManualsProductInstructions_Ex_Taq_Properties_and_Reaction_Conditions.pdf
[13] https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491
[14] http://www.clontech.com/SV/Products/PCR/High_Yield_PCR/Ex_Taq_DNA_Polymerase
[15] https://www.neb.com/products/pcr-polymerases-and-amplification-technologies/q5-high-fidelity-dna-polymerases/q5-high-fidelity-dna-polymerases
[16] http://tmcalculator.neb.com/#!/

primer concentration by the method of SantaLucia (SantaLucia, 1998). Different DNA polymerases result in a different Ta, since corrections for each enzyme and its buffer are made.

If multiple PCR reactions are performed, a mix of the common reagentia is made to reduce the pipetting error rate. Big volumes are pipetted first so that the small volumes are added inside the liquid to prevent the small volumes from sticking at the borders of the PCR tube, leading to incorrect concentration ratios.

The volume of the template that needs to be added to the reaction mix is based on the determination of the DNA concentration by the NanoDrop ND-1000 Spectrophotometer as written before [*section D.3.1.*].

The PCR reaction mixture containing DNA polymerase is kept on ice. At room temperature, annealing of primers at undesirable DNA regions can occur (non-specific annealing) and the polymerase can extend the duplexes since it has some activity at these temperatures, leading to unwanted secondary PCR products[17,18].

3.3. DNA Agarose Gel Electrophoresis

Agarose gel electrophoresis (AGE) is used to separate DNA utilising an electrical current applied to an agarose matrix. Due to the negative charge of DNA, it is directed through the matrix towards the positive pole (anode). Smaller fragments of DNA can travel faster in the matrix.

The 1 % (w/v) agarose gel is used for the efficient separation (DNA size resolution) of about 1 to 5 kb linear double stranded DNA molecules (Lewis). A 1 % (w/v) agarose solution is prepared in 0.5X TAE electrophoresis buffer [*Suppl. Table 3*] (TAE: Tris-acetate-EDTA) in an Erlenmeyer flask. The solution is heated using the microwave oven until it is transparent, showing that the agarose is completely dissolved. Subsequently, 1X SYBR® Safe DNA Gel Stain (Invitrogen™, 1:10 000 dilution of 10 000X Gel Stain) is added [Risk Assessment SYBR® Safe DNA Gel Stain: see *Addendum 1*]. The solution is gently shaken to distribute the stain evenly and the solution is poured into a gel rack with a gel support. The well creating comb is inserted at one side of the gel rack and the solution is allowed to cool down at room temperature, leading to polymerisation, resulting in a solid gel. After removing the comb, the gel with its gel support is inserted into the Mupid®-exU gel electrophoresis machine (Eurogentec). The electrophoresis tank is filled with 0.5X TAE buffer in such a way that the gel is covered to a depth of about 1 mm. Bromophenol Blue DNA Loading Dye 1X [*Suppl. Table 3*] is added to the PCR product samples and then loaded on the gel in separate wells. Also a

[17] http://separations.co.za/fileadmin/themes/default/pdf/2.%20PRODUCT%20LINES/X.%20TAKARA/PCR_Guide.pdf
[18] https://www.neb.com/products/pcr-polymerases-and-amplification-technologies/q5-high-fidelity-dna-polymerases/q5-high-fidelity-dna-polymerases

1 kb DNA Ladder (Thermo Scientific™ O'GeneRuler™, Ready-to-Use- 250-10,000 bp) is applied in a separate well. The ladder is used to investigate the molecular weight of the PCR product. This is possible because the DNA ladder contains DNA of known molecular weights. After closing the lid of the electrophoresis machine, the device is turned on for about 20 minutes at 130V. Visualization is possible by exposing the gel to UV light.

3.4. Sequencing

To acquire the sequence of a certain piece of DNA, first a PCR reaction is performed as described before [*section D.3.2.*] using the reaction mixture with TaKaRa Ex Taq™ DNA Polymerase given in *Table 2* and the PCR program indicated in *Suppl. Table 4*. A longer extension step is needed since a larger PCR product is to be obtained.

The presence of the PCR product with the wanted size is confirmed by performing DNA AGE as specified before [*section D.3.3.*]. Only a small part of the retrieved PCR reaction mix is used for the gel. The other part is used for the sequencing itself. First the DNA is purified with the kit Wizard® SV Gel and PCR Clean-Up System from Promega, the resulting DNA is sent for sequencing by the VIB Genetic Service Facility. Fragments of around 1000 base pairs (bp) are sequenced, taken into account that the first and last 50 base pairs of the fragment are less accurate due to the technical limitations of the technique.

The sequencing results are analysed using the program CLC Main Workbench 7 with the tool to assemble the obtained sequences to a reference sequence.

3.5. Replica Plating

An autoclaved replica plating velvet pad is used together with the replica plating apparatus to transfer yeast colonies from one agar plate to the other plate(s) without changing their orientation. This technique is convenient for screening auxotrophies or making copies of plates.

3.6. Determination of Growth Phase of Cell Culture

As mentioned in *section B.1.4.*, measuring the absorbance with a spectrophotometer at a wavelength of 600 nm (OD_{600}) gives indications about the growth phase cells are in. This does not only account for *S. cerevisiae*, but also for *E. coli*. A spectrophotometer BioPhotometer® D30 (Eppendorf) is used in the current work to determine the OD_{600} of a cell culture.

3.7. Techniques For *S. cerevisiae*

3.7.1. Transformation

The transformation steps performed in the current work are adapted from the GIETZ Transformation Super Protocol (Gietz & Schiestl, 2007). It is a heat shock lithium acetate transformation method, having as characteristics that intact yeast cells are used and that consequently the recovery can happen on the surface of agar plates. Lithium acetate (LiAc), ssDNA and polyethylene glycol (PEG) are used to improve the transformation efficiency. LiAc acts through its chaotropic effect of denaturing DNA, RNA and proteins (Norcum, 1991), ssDNA is used as a carrier (Zheng, et al., 2005) and PEG prevents cell rupture (Schiestl & Gietz, 1989). In the current work, the transformation method is both used to bring a PCR product and plasmid DNA into *S. cerevisiae* strains.

To avoid contamination glass ware and tubes are autoclaved before use and the handlings are done in the laminar flow cabinet in a sterile way.

- A preculture is made by adding a yeast colony (that needs to be transformed) to a glass tube with 3 ml YPD [*Suppl. Table 1*] and is grown O/N in a shaking incubator at 30 °C.
- The O/N preculture is used to inoculate a larger culture: A small amount of the preculture is added to an Erlenmeyer flask with 50 ml fresh YPD medium [*Suppl. Table 1*], diluting it in such a way that an OD_{600} of 0.01 is obtained so that after growing O/N in a shaking incubator at 30 °C an OD_{600} between 1 and 2 is reached, i.e. exponential phase [Determination of Growth Phase, see *section 3.6*].
- The yeast culture is kept on ice for 10 to 15 minutes, stopping the growth of the cells.
- The culture is centrifuged at 3,000 rpm for 5 min at 4 °C.
- After removing the supernatant, the pellet is resuspended in 40 ml sterilised Milli-Q and centrifuged again at 3,000 rpm for 5 min at 4 °C.
- After removing the supernatant, the pellet is resuspended in 1 ml of 100 mM LiAc and centrifuged at 5,000 rpm for 3 min at 4 °C.
- The supernatant is removed again and the pellet resuspended in 200 µl 100 mM LiAc.
- Salmon sperm DNA is heated on a heating block for 10 min on 99 °C to obtain single stranded DNA. Subsequently it is put on ice.
- In a 1 ml tube, 50 µl cells, 10 µl of heated salmon sperm DNA and approximately 10 µg PCR product or 1 µg plasmid are combined and shortly vortexed. The volume of the DNA that needs to be added is based on the determination of the DNA concentration by the NanoDrop ND-1000 Spectrophotometer [*section D.3.1.*].
- To the 1 ml tube, 300 µl of freshly prepared PLI is added, containing 0.1 M LiAc and 40 % PEG(6000) (w/v) in Milli-Q. The PLI is prepared freshly to increase the transformation efficiency.

- The tube is placed in a shaking incubator for 30 min at 30 °C and subsequently put in a water bath of 42 °C for 20 min as a heat shock.
- The solution is centrifuged at 14,000 rpm for 30 seconds.
- The supernatant is removed, the pellet is resuspended in 3 ml YPD [*Suppl. Table 1*] and thereafter incubated in a 30 °C shaking incubator for at least four hours for a PCR product transformation and two hours for a plasmid transformation.
- The solution is centrifuged at 3,000 rpm for 3 min.
- The supernatant is removed, the pellet is resuspended in 50 µl Milli-Q and plated out on an agar plate using a glass spreader. The agar consists of a selective medium with the idea that only cells that have undergone a successful transformation are able to survive. To make selection possible, there is chosen to transform DNA which in part codes for a selectable marker, both in the PCR product and the plasmid. After PCR product transformation, some *S. cerevisiae* cells will have replaced a part of their genome by the PCR product after homologous recombination. This is made possible by minimum 40 bp homologous flanking sequences in the PCR product. Only the sequence in between the crossing over sites is exchanged, this makes a controlled gene disruption, deletion or modification possible.
- Colonies appear after growth for 3 to 4 days on an agar plate in a 30 °C incubator.

3.7.2. Mating

For mating, haploid yeast cells of the opposite mating type need to be brought into each other's proximity (Duntze, *et al*., 1970). In the lab under laminar flow cabinet a bit of cells of one strain is transferred from its agar plate to a fresh sterile YPD agar plate [*Suppl. Table 1*] with a sterile pipet tip. Subsequently, the other strain is added with another sterile tip to the same spot on the YPD plate [*Suppl. Table 1*]. The cells are mixed well with the tip. They are put in a 30 °C incubator for about ten hours or more to obtain diploid cells. The shmoo formation is visualised under the microscope, adding 5 µl Milli-Q on a microscopy slide containing a bit of cells covered by a cover slip.

3.7.3. Sporulation

To induce sporulation, diploid cells are transferred on a sterile sporulation medium (SPM) agar plate [*Suppl. Table 1*] at 23 °C for minimum 4 days. Some strains need more time before asci are formed. Because the plates are remaining for a longer period in the incubator, the opening of the Petri dish is covered with parafilm to prevent dehydration.

Under the microscope, the formation of asci (tetrads) are visible by adding 5 µl of Milli-Q on a microscopy slide containing a bit of cells covered by a cover slip.

3.7.4. Tetrad Dissection

In tetrad dissection, the ascospores formed during sporulation [*section D.3.5.2*] are separated from each other. This is done by using a micromanipulator MSM 300 Systems (Singer Instruments).

- 45 µl of sterilised Milli-Q and a small amount of cells are combined in a sterile Eppendorf tube. The clump of cells added are barely visible by eye.
- 5 µl lyticase stock solution (10 000 U/ml, 1.187 mg/ml) is added to the mixture to remove the ascus wall (sac) without destroying the cells and spores.
- After an incubation of 3 minutes at room temperature, 7 µl of the mixture is added to a sterile YPD plate [*Suppl. Table 1*]. The droplet is guided by gravity to form a line.
- When the liquid is absorbed by the agar plate, the plate is put on the micromanipulator. The focus of its implemented microscope is adapted so that the surface of the plate is visible. If the needle of the micromanipulator is directly localized next to an ascospore, it sticks to it, since the ascospore walls are very hydrophobic (Briza, *et al*., 1986). This makes it possible to transfer the spores from one position on the plate to the other. For about 14 asci, the four ascospores are separated.

The ascospores form a nice haploid colony after growing for about four days in a 30 °C incubator. This is also called spore germination. The fact that the four products of meiosis can be recovered easily in yeast gives an important benefit for this model organism.

3.7.5. Determination of Mating Type

Figuring out whether the yeast strain is mating type a or alpha is possible by performing a mating type PCR (Illuxley, *et al*., 1990).

A PCR reaction [as described in *section D.3.2.*] is performed using the PCR reaction mixture stated in *Suppl. Table 5* with a tip of cells as template, the three primers given in *Suppl. Table 6* and the PCR program described in *Suppl. Table 7*. Subsequently, DNA AGE [as described in *section D.3.3*] is performed. A PCR product of 404 bp is obtained for *MAT*α and 544 bp for *MAT*a, while diploid lab strains result in two bands on the gel.

3.7.6. Mating Type Switch

As said before, the strains used in the lab are heterothallic, meaning that they are not able to undergo a mating type switch themselves (Hartwell, *et al*., 2011). However, to switch the mating type of a lab strain in a controlled way, a plasmid containing the wild-type *HO* gene behind a galactose inducible promotor (pFL39 *GAL1 HO KanMX*) is used [plasmids: *see Suppl. Table 17*].

After transforming the strain with the plasmid using the protocol as before [*section D.3.7.1.*], the cells are selected on a sterile YPD geneticin plate [*Suppl. Table 1*], since the plasmid contains the *KanMX* selectable marker. After incubating the plate for about three days in 30

°C, a fat streak of transformed yeast strains is put on a sterile YPG agar plate [*Suppl. Table 1*] for about one hour. This leads to a transient expression of the HO enzyme inducing the exchange in *MAT*-locus. Thereafter, the strains are transferred to a sterile YPD plate [*Suppl. Table 1*], now in a zigzag to obtain single colonies after incubating at 30 °C. The success of the mating type switch is verified by mating type PCR [*section D.3.7.5.*].

3.7.7. Genomic DNA Extraction

Genomic DNA of a yeast strain is obtained by performing the following extraction protocol. The DNA is used as template for PCR reactions.

- One PCR vial full of glass beads, 150 µl TE buffer pH 8.0 100X (10 mM Tris-HCl, 1 mM EDTA) and a tip of yeast cells from an agar plate are combined in a FastPrep tube. Subsequently, 150 µl of the lower phase of PCI is added under the laboratory hood wearing gloves (Phenol/Chloroform/Isoamyl Alcohol 25:24:1 v/v pH 8.0) [Risk Assessment PCI: see *Addendum 1*].
- The yeast cells are broken down by using the FastPrep®-24 Instrument (MP Biomedicals) for 20 seconds at 4M/S.
- The tubes are centrifuged for 10 min at 14,000 rpm.
- The upper transparent fluid is carefully transferred into an Eppendorf tube, leaving the cell debris in the FastPrep tube.
- After adding 200 µl isopropanol to the Eppendorf tube, it is put into a -20 °C freezer for one hour.
- The tube is centrifuged at 14,000 rpm for 5 min.
- The isopropanol is removed and 100 µl ethanol 70 % is added.
- Again, it is centrifuged at 14,000 rpm for 5 min.
- As much fluid as possible is pipetted off and the pellet is dried using the SpeedVac® System AES 2010 for 15 min.
- The resulting extracted DNA is resuspended in 200 µl TE buffer pH 8.0 100X. It is used immediately or stored in a -20 °C freezer.

3.8. Techniques For *E. coli*

3.8.1. Making Competent Cells

E. coli cells are made competent by the $CaCl_2$ method described below. The steps are performed in a sterile way.

- A single colony is picked to grow a 3 ml LB preculture [*Suppl. Table 1*] in a glass tube O/N in a 37 °C shaking incubator.
- The O/N preculture is used to inoculate a larger culture: A small amount of the preculture is added to an Erlenmeyer flask with 100 ml fresh sterile LB medium [*Suppl. Table 1*], diluting it in such a way that an OD_{600} between 0.5 and 0.8 is reached after 2-3 hours in a shaking incubator at 37 °C [Determination of Growth Phase, see *section 3.6*].
- The sample is centrifuged at 3,000 rpm for 20 minutes at 4 °C to collect the bacterial cells.
- The pellet is resuspended in ice-cold sterile water and centrifuged again with the same settings.
- The obtained pellet is resuspended in 40 ml ice-cold sterile 0.1 M $CaCl_2$ and kept on ice for at least one hour. The sample is centrifuged again with the same settings.
- The obtained pellet is resuspended in 1 ml ice-cold sterile 0.1 M $CaCl_2$.
- The cells are immediately used or kept in a -80 °C freezer after addition of sterilised glycerol so that a final concentration of 15 % is reached.

3.8.2. Transformation

Also *E. coli* cells can be transformed by a heat shock method, described below. The steps are performed in a sterile way.

- 100 µl of competent cells are combined in an Eppendorf tube with 500 ng plasmid DNA and kept on ice for at least 15 minutes. The volume of plasmid that needs to be added to the reaction mix is based on the determination of the DNA concentration by the NanoDrop ND-1000 Spectrophotometer as written before [*section D.3.1.*].
- A heat shock is performed for 1 min in a 42 °C water bath.
- They are put on ice for 5 min.
- 1 ml sterile liquid LB medium [*Suppl. Table 1*] is added to the tube and kept in a shaking incubator at 37 °C for 40 min.
- The cells are centrifuged at 5,000 rpm for 3 min and resuspended in 100 µl sterile Milli-Q.
- 100 µl of the solution is plated out using a spreader on an LB agar plate [*Suppl. Table 1*] containing the antibiotic corresponding with the antibiotic marker present on the plasmid.
- The plate is incubated O/N at 37 °C. The colonies that appear on the plate contain the plasmid (or are the result of contamination of an antibiotic resistant strain). The plate is removed from the incubator since the antibiotic is destroyed at high temperatures. The plate is stored in the fridge.

3.8.3. Plasmid Isolation

- A preculture is prepared in a glass tube, containing 3 ml sterile LB liquid [*Suppl. Table 1*] with the antibiotic corresponding with the antibiotic marker present on the plasmid and a single colony from the plate resulting from the transformation [*section D.3.8.2.*]. It is grown in a 37 °C shaking incubator O/N (16 h).
- The plasmids multiplied by *E. coli* in the current work are isolated by the kit NucleoSpin® Plasmid EasyPure of Macherey-Nagel.
- The plasmids are store in a -20 °C freezer.

4. *In Vitro*: GST Pull-Down

GST (Glutathione S-transferase) pull-down is an *in vitro* technique to study physical interactions between two (or more) proteins[19].

One yeast protein to be studied is expressed by an *E. coli* strain. The protein is tagged with the GST-tag so that it can be purified using glutathione sepharose beads. The other yeast protein is produced by *S. cerevisiae* itself and fused with the HA-tag (Human influenza hemagglutinin tag). After lysing the cells, the proteins are given the possibility to interact. The GST-tagged proteins are purified by the beads and the presence of the HA-tagged protein in the purified solution can accordingly be detected by the anti-HA-antibody.

As a negative control, the GST-tag (without a protein fused to it) is expressed in *E. coli*. This is used to examine the interaction between the HA-tagged protein and the GST-tag itself. Ideally, this does not result in a signal, since the interaction between the two proteins of interest is aimed to be studied.

Two proteins that are known to interact are used as a positive control, along with the proteins to be studied.

4.1. Expression of HA-tagged Proteins by *S. cerevisiae*

Sigma 1278b strains are used for the expression of HA-tagged proteins, containing either an intragenomically *HA*-tagged gene of interest (Sigma GCD6-HA and Sigma GCD1-HA) or a plasmid encoding the gene of interest adjacent to the *HA*-tag (Sigma GAP1-HA). Details of the strains are described in *Suppl. Table 8*. The protocol of expression is given below. Only the two first steps are performed in a sterile way.

- A preculture is made by adding a yeast colony to a glass tube with 3 ml sterile YPD [*Suppl. Table 1*] and is grown O/N in a shaking incubator at 30 °C. If the yeast was transformed with a plasmid, the corresponding SC drop-out medium [*Suppl. Table 1*] is used to control the presence of the plasmid.
- The O/N preculture is used to inoculate a larger culture: A small amount of the preculture is added to an Erlenmeyer flask with 50 ml fresh sterile YPD medium [*Suppl. Table 1*], diluting it in such a way that an OD_{600} of 0.3 is obtained so that after growing for about 3 hours in a shaking incubator at 30 °C an OD_{600} between 1 and 2 is reached, i.e. exponential phase [Determination of Growth Phase, see *section 3.6*].

If the protein is only expressed in nitrogen starvation, an additional step is subsequently performed: The exponential culture is centrifuged at 4,500 rpm for 5 min and the remaining pellet is transferred to 50 ml sterile Nitrogen Starvation Medium (NSM) [*Suppl. Table 1*] for growing for 24 h in a 30 °C shaking incubator.

[19] https://www.thermofisher.com/be/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/pull-down-assays.html

- The culture is placed on ice for 15 min.
- It is centrifuged at 4,500 rpm for 5 min at 4 °C.
- The supernatant is discarded and the pellet is resuspended in 7 ml ice-cold PBS buffer 1X (PBS: Phosphate buffered saline) [*Suppl. Table 9*].
- Seven ice-cold screw cap tubes are filled with 1 ml of the cell suspension.
- They are centrifuged at 14,000 rpm for 1 min at 4 °C.
- The supernatant is discarded.
- The screw cap tubes containing yeast cells with HA-tagged yeast proteins are flushed with liquid nitrogen and directly stored in a -80 °C freezer [Risk Assessment liquid nitrogen: see *Addendum 1*].

4.2. Expression of GST-tagged Proteins by *E. coli*

To obtain *E. coli* strains expressing GST-tagged yeast proteins, the MCB lab generated pGEX-4T-1 plasmids containing genes coding for the proteins of interest of the current work. By PCR, the open reading frame of each gene was amplified including the stop codon. Subsequently the PCR products were cloned into the pGEX-4T-1 plasmid[20] using the restriction sites *Bam*HI with *Xho*I or *Xma*I with *Xho*I. This eventually leads to proteins tagged N-terminally with GST. The pGEX backbone is an *E. coli* plasmid optimized for protein expression, containing an ampicillin resistance gene[21]. The seven distinct plasmids used contain the genes coding for the fusion proteins Pkh1-GST, Sch9-GST, Tpk1-GST, Tpk2-GST and Tpk3-GST, Sui3-GST and free GST [info plasmids: *see Suppl. Table 17*]. In the current work, the corresponding plasmids mentioned are used for expressing the proteins of interest in *E. coli*, as described in the protocol below. The seven first steps are performed in a sterile way.

- Chemically competent DH5α™ *E. coli* cells optimised for plasmid multiplication are transformed with a plasmid containing the gene coding for the GST-tagged yeast protein of interest, following the protocol [*section D.3.8.2.*], using a sterile LB ampicillin agar plate [*Suppl. Table 1*] for selection.
- The plasmid is isolated as described before [*section D.3.8.3.*].
- *E. coli* One Shot® BL21 Star™ (DE3) cells are made competent, according to the protocol [*section D.3.8.1.*].
- The chemically competent One Shot® BL21 Star™ (DE3) is transformed with a plasmid containing the gene coding for the GST-tagged yeast protein of interest, following the protocol [*section D.3.8.2.*], using a sterile LB ampicillin agar plate [*Suppl. Table 1*] for selection.

[20] http://www.gelifesciences.com/webapp/wcs/stores/servlet/ProductDisplay?categoryId=11737&catalogId=10101&productId=17049&storeId=11754&langId=-1
[21] https://www.addgene.org/vector-database/2876/

- A preculture is made by adding a One Shot® BL21 Star™ (DE3) colony to a glass tube with 3 ml sterile LB ampicillin medium [*Suppl. Table 1*] and is grown O/N in a shaking incubator at 37 °C.
- The O/N preculture is used to inoculate a larger culture: A small amount of the preculture is added to an Erlenmeyer flask with 100 ml sterile Terrific Broth medium with ampicillin [*Suppl. Table 1*], diluting it in such a way that an OD_{600} of 0.2 is obtained so that after growing for about 1 h 30 min in a shaking incubator at 37 °C an OD_{600} of about 1 is reached [Determination of Growth Phase, see *section 3.6*].

The Terrific Broth medium [*Suppl. Table 1*] is used instead of LB since it results in an extended growth phase due to the higher amounts of tryptone and yeast extract (Amresco).

- The plasmid gene expression is induced by adding 600 µM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and incubating for 2 to 3 hours at 30 °C in a shaking incubator, as yeast protein is expressed.
- The culture is placed on ice for 20 min.
- It is centrifuged at 4,500 rpm for 10 min at 4 °C.
- The supernatant is discarded and the pellet is resuspended in 6 ml ice-cold PBS buffer 1X [*Suppl. Table 9*].
- Six ice-cold screw cap tubes are filled with 1 ml of the cell suspension.
- They are centrifuged at 6,000 rpm for 2 min at 4 °C.
- The supernatant is discarded.
- The screw cap tubes containing *E. coli* cells with GST-tagged yeast proteins are flushed with liquid nitrogen and directly stored in a -80 °C freezer [Risk Assessment liquid nitrogen: see *Addendum 1*].

4.3. GST Pull-Down Assay

With this assay the physical interaction between every possible combination of the prepared GST-tagged proteins with the HA-tagged proteins is studied.

During the whole protocol the proteins are kept cold to minimise their degradation and inactivation, increasing the probability of interactions in their native conformation[22]. All tubes and buffers are kept on ice, a cold centrifuge is used and a roller drum is localized in the fridge.

All buffers [*Suppl. Table 9*] are prepared at the beginning of experiment, increasing the quality of the assay.

[22] http://www.indiana.edu/~lchenlab/protocol_files/protein_storage.pdf

Isolation of HA-tagged Proteins Expressed by *S. cerevisiae*

- A yeast glutathione sepharose bead suspension is prepared as follows. An equal volume of ice-cold Yeast Lysis Buffer [*Suppl. Table 9*] is added to the ice-cold glutathione sepharose beads. Firstly, it is mixed well and centrifuged for 1 min at 2,000 rpm at 4 °C. Then the supernatant is removed. This is repeated three times. Finally, an equal volume of ice-cold Yeast Lysis Buffer [*Suppl. Table 9*] is added.
- The screw cap tubes containing yeast cells with HA-tagged yeast proteins [made in *section D.4.1.*] are taken from the -80 °C freezer and placed on ice.
- To each tube, two PCR vials full of glass beads are added together with 500 µl of ice-cold Yeast Lysis Buffer [*Suppl. Table 9*].
- The yeast cells are broken down by using the FastPrep®-24 Instrument (MP Biomedicals) twice on 4M/S for 20 seconds. Both the machine and the samples need 5 min in between to be able to cool down, the samples are put back on ice during this waiting time.
- The samples are centrifuged for 15 min at 14,000 rpm at 4 °C and subsequently the supernatants of the same strain are transferred into the same fresh tube. 50 µl of the supernatant is set aside as 'Input'. This is used as an extra positive control, confirming the expression of the HA-tagged protein.
- 200 µl ice-cold Binding Buffer [*Suppl. Table 9*] and 200 µl of the ice-cold yeast glutathione sepharose bead suspension is added to the supernatant.
- The tubes are incubated for 1 h 30 min at 4 °C on a roller drum.
- They are centrifuged for 1 min at 2,000 rpm at 4 °C. The supernatant is kept for the protein-protein interaction assay. By only using the supernatant, the proteins that were interacting with the beads are removed, eliminating nonspecific interactions of the yeast proteins with the beads.

Isolation of GST-tagged Proteins Expressed by *E. coli*

- An *E. coli* glutathione sepharose bead suspension is prepared as follows. An equal volume of ice-cold *E. coli* Wash Buffer [*Suppl. Table 9*] is added to the ice-cold glutathione sepharose beads. Firstly, it is mixed well and centrifuged for 1 min at 2,000 rpm at 4 °C. Then the supernatant is removed. This is repeated three times. Finally, an equal volume of ice-cold *E. coli* Wash Buffer [*Suppl. Table 9*] is added.
- The screw cap tubes containing *E. coli* cells with GST-tagged yeast proteins [made in *section D.4.2.*] are taken from the -80 °C freezer and placed on ice.
- The pellets of the same strain are resuspended in 4 ml ice-cold *E. coli* Lysis Buffer [*Suppl. Table 9*] in the same fresh test tube.
- The mixture is sonicated two times for 15 seconds (Settings: Amplitude of 75 and cycle 1) and are put on ice in between.

- The sonicated mixture is centrifuged for 10 min at 12000 g at 4 °C. The supernatant is transferred into a fresh 15 ml falcon tube.
- 200 µl of the ice-cold *E. coli* glutathione sepharose bead suspension is combined with the supernatant.
- The tubes are incubated for 1 h 30 min at 4 °C on a roller drum so that the GST-tagged proteins can bind with the beads.
- They are centrifuged for 1 min at 2,000 rpm at 4 °C. The fluid above the beads is discarded.
- The beads are resuspended in 1 ml ice-cold *E. coli* Wash Buffer [*Suppl. Table 9*], are centrifuged for 1 min at 2,000 rpm at 4 °C and the supernatant is removed. This is repeated three times.
- The beads are resuspended in 200 µl of ice-cold Binding Buffer [*Suppl. Table 9*] and are used for the protein-protein interaction assay.

Protein-Protein Interaction Assay

- 75 µl of *E. coli* beads are combined with 250 µl of yeast extract and 200 µl ice-cold Binding Buffer [*Suppl. Table 9*] in fresh tubes.
- The tubes are incubated for 1 h 30 min at 4 °C on a roller drum.
- They are centrifuged for 1 min at 2,000 rpm at 4 °C. The fluid above the beads is discarded. This is done carefully so that no beads are pipetted off.
- The beads are resuspended in 1 ml PBS-T [*Suppl. Table 9*], centrifuged for 1 min at 2,000 rpm at 4 °C and the supernatant is removed. This is repeated three times.
- The residual liquid in between the beads is removed with a syringe.
- 45 µl of the SDS Sample Buffer (2X) [*Suppl. Table 9*] (SDS: sodium dodecyl sulphate) is added to each sample and to the yeast input (put aside in the beginning) [Risk Assessment SDS and β-mercaptoethanol: see *Addendum 1*].
- All tubes are heated at 65 °C for 10 min using the heating block and stored in the -20 °C freezer until they are visualised.

4.4. Visualisation

The GST pull-down interactions are visualised by first performing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) twice. Subsequently, one gel is used for western blotting and immunodetection and the other for Coomassie staining. During the whole procedure of visualisation gloves are worn, as a result of the skin toxicity of most solutions used for the procedure [Risk Assessment: see *Addendum 1*].

SDS-PAGE

This technique is widely used to separate proteins based on their molecular weight. SDS, a protein denaturant is added to the protein samples. This results in linearized peptide chains since it is disrupting tertiary and quaternary structures via adding negative charges. Consequently, after adding the SDS-containing samples to the polyacrylamide gel and applying the electric field, the proteins migrate towards the positive pole (anode). The removal of the 3D structure of the proteins also influences their migration rate through the gel (Walker & Rapley, 2008).

- The ready made polyacrylamide gel (Invitrogen™: NuPAGE® Novex® Bis-Tris 4-12 % gel) is inserted into the electrophoresis chamber [Risk Assessment NuPAGE® gel: see *Addendum 1*].

- The chamber is filled with Running Buffer [*Suppl. Table 9*] and the comb is removed cautiously.
- 7.5 µl ladder (Invitrogen™: SeeBlue®Plus2 Pre-Stained Standard) is added to first well. It is used as a reference to assist protein identification based on their molecular weight.
- The protein samples are taken from the -20 °C freezer and heated again at 65 °C for 10 minutes. 17 µl of each sample is loaded into the adjacent wells of the polyacrylamide gel.
- 150 V is applied (constant voltage) using Bio-Rad PowerPac 300 Electrophoresis Power Supply until the dye reached the base of the gel, this takes approximately 90 min.
- The ready made gel is removed from the electrophoresis chamber and the gel is detached from its cover by a spatula. The upper and lower parts of the gel are carefully separated and trashed. The remaining part is used for either western blotting / immunodetection [A] or for Coomassie staining [B].

A1. Western Blotting

To be able to perform the immunodetection, the proteins need to be present on a membrane binding the eluted macromolecules (Walker & Rapley, 2008). This is possible by applying an electrical current, transferring proteins from a polyacrylamide gel to a nitrocellulose membrane.

- A nitrocellulose membrane (GE Healthcare Life Sciences: Amersham™ Hybond™ ECL) is laid on the gel.
- The gel is sandwiched into a blotting holder by different materials that are all soaked in ice-cold Blotting Buffer [*Suppl. Table 9*]. One by one the materials are added so that no air bubbles remain in between the layers: First a sponge, then two pieces of Whatmann paper, subsequently the gel with the nitrocellulose membrane, again two pieces of Whatmann paper and finally another sponge.

- The blotting holder is placed into the blotting chamber oriented in such a way that the current guides the proteins onto the membrane. Ice-cold Blotting Buffer [*Suppl. Table 9*], a magnetic stirrer and an ice block is added to the electrophoresis chamber to prevent temperature fluctuations and overheating due to the electrical current[23].
- 300 mA (constant electrical current) is applied for 1 h using Bio-Rad PowerPac 300 Electrophoresis Power Supply.
- The resulting protein-blotted nitrocellulose membrane is removed from the blotting chamber and holder.

A2. Immunodetection

- To block nonspecific sites on the membrane, i.e. sites which do not contain proteins, the membrane is incubated in Blocking Buffer [*Suppl. Table 9*] for 1 h.
- The Blocking Buffer [*Suppl. Table 9*] is discarded and Antibody Buffer [*Suppl. Table 9*] is added. It is incubated for 2 h at room temperature or O/N at 4 °C on a rocking plate. In this process, the anti-HA antibody is binding to the HA-tagged proteins.
- The membrane is washed three times for 15 minutes with TBS-T [*Suppl. Table 9*] (TBS: Tris-buffered saline) on a rocking plate with the goal to remove the excess antibody.
- A 1:1 mixture of WesternBright™ Quantum™ (Advansta) and WesternBright™ Peroxide (Advansta) is spread over the membrane. The peroxidase enzyme conjugated to the anti-HA antibody reacts with its substrate provided by this mixture. The chemiluminescence resulting from the reaction is detected by the LAS imager (Fujifilm ImageQuant™ Luminescent Image Analyzer 4000 mini). Also the ladder is visualised with the LAS imager, using white light.

B. Coomassie Staining

The detection of the proteins present in the polyacrylamide gel is achieved by using the Coomassie dye. Under acidic conditions it adheres to basic and hydrophobic residues and gives a blue colour (Hayworth), thus staining all the proteins present. The ladder used makes it possible to identify proteins based on their molecular weight. The staining also gives indications about the relative amounts of the proteins present in the sample. It is expected that only *E. coli*-expressed proteins will be visible in the Coomassie stained gel, since the yield of yeast expression is low.

- The polyacrylamide gel is incubated in the Coomassie Solution [*Suppl. Table 9*] for 30 min on a rocking plate.
- The spaces in the matrix of the gel where no proteins are present, are destained by soaking the gel into the Destain Solution [*Suppl. Table 9*] on a rocking plate. Every 20 min fresh Destain Solution is added until the blue bands are seen clearly in a gel matrix that is almost colourless.
- A computer scanner is used to make a picture of the resulting Coomassie stained gel.

[23] http://www.bio-rad.com/LifeScience/pdf/Bulletin_2895.pdf

5. *In Vivo*: Fluorescence Microscopy

In fluorescence, light of a certain wave length is absorbed and emission of another wavelength is taking place[24]. In the current work the expression and correct folding of the fluorescent protein citrine, Red Fluorescent Protein (RFP) or Green Fluorescent Protein (GFP) gives rise to fluorescence. If a fusion protein is engineered combining a protein of interest and a fluorescent protein (in-frame), it is possible to study the location of the protein of interest in the cell *in vivo*. This is done in the current work by tagging a protein C-terminally with RFP or GFP, by an insertion in the genome.

Fluorescent proteins can also be used to study *in vivo* protein-protein interactions. The technique used in the current work to investigate this is split citrine Bimolecular Fluorescence Complementation, split citrine BiFC in short. Citrine is a mutant of the Yellow Fluorescent Protein (Chalfie & Kain, 2005), resulting in a yellowish-green emission (Kodama & Hu, 2012). If a peptide consisting of the first 155 amino acids of citrine is brought together with a peptide with amino acids 155 to 238 of citrine, it results in fluorescence after proper folding (Shyu, *et al*., 2006). This process is called fluorophore fragment complementation. The two proteins of which the interaction is to be studied in the current work, are each tagged C-terminally with one of the mentioned fragments of citrine, resulting in two fusion proteins. This is done by an intragenomical insertion. If these proteins are coexpressed and interacting with each other, green fluorescence might be observed. The protein-protein interaction thus facilitates fluorophore fragment complementation. Bimolecular fluorescence complementation is represented in *Figure 5*.

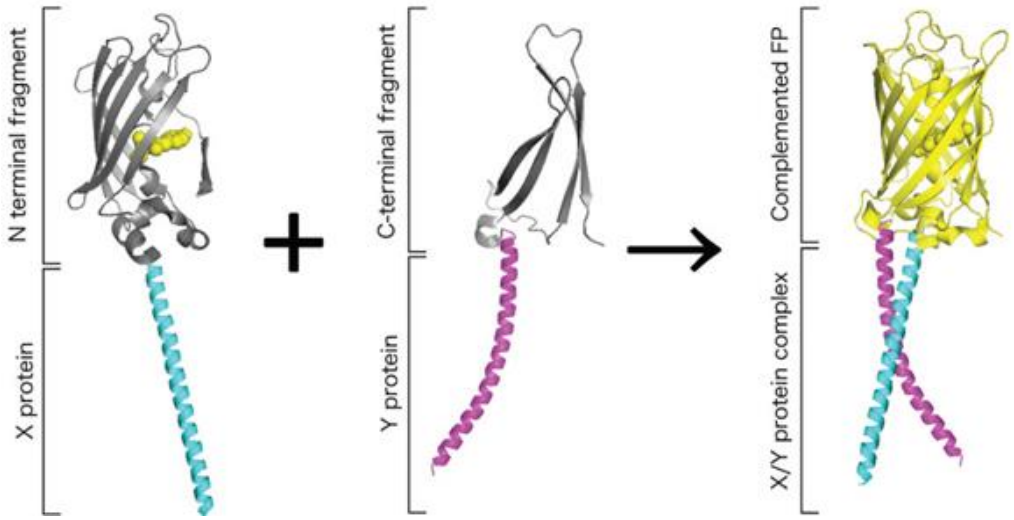

Figure 5: Schematical representation of bimolecular fluorescence complementation *(Kodama & Hu, 2012)*.

5.1. Construct Strains With Fusion Protein

In this section yeast strains with a fusion protein are obtained, described in *Table 2*. The strains are made by inserting a new sequence in the genome directly in front of the stop codon of the open reading frame of the gene of interest. This insertion is obtained by the transformation of a PCR product and the subsequent homologous recombination carried out by yeast.

The PCR product consists of a partial or an entire fluorescent tag together with a selectable marker. The latter can be either an auxotrophic or a dominant marker, making selection possible after transformation for example for histidine prototrophy or geneticin resistance respectively. Both ends of the PCR product contain an adaptor and a sequence homologous to the genomic region where the crossing over is planned to take place.

To obtain the PCR product, a PCR is performed using a plasmid as template containing a fluorescent tag and a selectable marker, flanked by adaptors. The primers used are tailed-primers, since they include sequences non-complementary to the template DNA at their 5' ends. This sequence is homologous to the genomic region where the crossing over is planned to take place. The primers hybridise to the adaptors of the template plasmid. The strategy of using tailed-primers facilitate that for tagging different genes, the same plasmid is used together with different primers. The 5' side of the forward primer (F primer) consists of the homologous region at the end of the gene of interest (before stop codon) of approximately 50 base pairs. Its 3' side of about 20 bp is the adaptor upstream of the tag. On the other hand, the +/- 50 bp at the 5' side of the reverse primer (R primer) consists of the homologous region right downstream the stop codon of the gene of interest. Its 3' side of about 20 bp is the adaptor downstream the selectable marker.

A schematic representation of the plasmid, the PCR product and homologous recombination in the genome of the transformed strain is given in *Figure 6*.

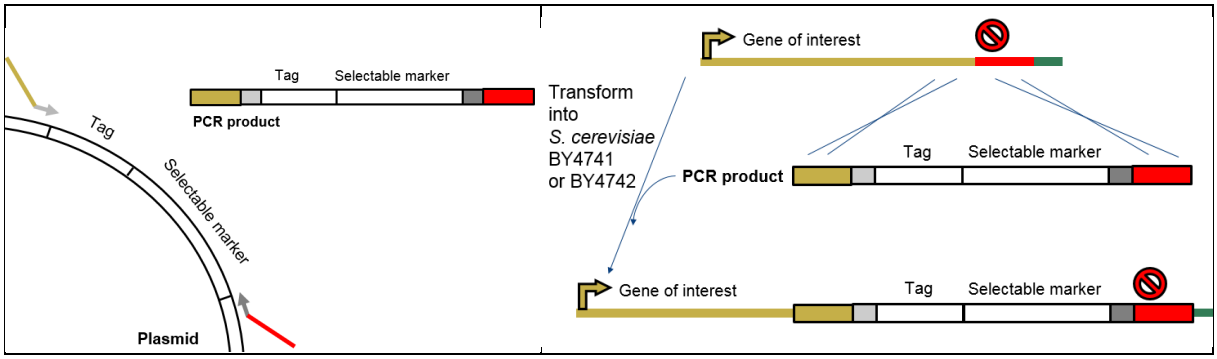

Figure 6: Schematic representation of the plasmid, the PCR product and homologous recombination in the genome of the transformed strain. The adaptors are represented in grey, the terminator region in red.

[24] http://www.olympusmicro.com/primer/lightandcolor/fluorescencehome.html

Table 2: List of the different strains constructed in the current work by transforming the mentioned BY- strain with a fusion protein cassette made by a PCR reaction using the corresponding forward primer and reverse primer and plasmid. Also the growth medium used for selection of the transformants and the name of the resulting strain is given.

Fusion Protein	F Primer	R Primer	Plasmid	Strain	Growth Medium	Resulting Strain
Tpk1 fused with GFP	A3499	A3500	pFA6a-*GFP-KanMX6*	BY4741	YPD geneticin	BY4741 Tpk1-GFP
Tpk2 fused with GFP	A3501	A3502	pFA6a-*GFP-KanMX6*	BY4741	YPD geneticin	BY4741 Tpk2-GFP
Tpk3 fused with GFP	A3503	A3504	pFA6a-*GFP-KanMX6*	BY4741	YPD geneticin	BY4741 Tpk3-GFP
Sch9 fused with RFP	B7605	B7606	pFA6a-*RFP-KanMX6*	BY4741	YPD geneticin	BY4741 Sch9-RFP
Pkh1 fused with RFP	B4180	B4179	pFA6a-*RFP-KanMX6*	BY4741	YPD geneticin	BY4741 Pkh1-RFP
Tpk1 fused with C-terminus of citrine	B2674	B2675	γC-*HIS*	BY4741	SC –his	BY4741 Tpk1-CC
Tpk2 fused with C-terminus of citrine	B2676	B2677	γC-*HIS*	BY4741	SC –his	BY4741 Tpk2-CC
Tpk3 fused with C-terminus of citrine	B2678	B2679	γC-*HIS*	BY4741	SC –his	BY4741 Tpk3-CC
Pkh1 fused with C-terminus of citrine	B2682	B2683	γC-*HIS*	BY4741	SC –his	BY4741 Pkh1-CC
Sch9 fused with C-terminus of citrine	B2702	B2703	γC-*HIS*	BY4741	SC –his	BY4741 Sch9-CC
Gap1 fused with C-terminus of citrine	B2642	B2643	γC-*HIS*	BY4741	SC –his	BY4741 Gap1-CC
Sul1 fused with C-terminus of citrine	B7357	B7358	γC-*HIS*	BY4741	SC –his	BY4741 Sul1-CC
Mep2 fused with C-terminus of citrine	B7168	B7169	γC-*HIS*	BY4741	SC –his	BY4741 Mep2-CC
Tpk1 fused with N-terminus of citrine	B2674	B2675	γN-*URA*	BY4742	SC –ura	BY4742 Tpk1-NC
Tpk2 fused with N-terminus of citrine	B2676	B2677	γN-*URA*	BY4742	SC –ura	BY4742 Tpk2-NC
Tpk3 fused with N-terminus of citrine	B2678	B2679	γN-*URA*	BY4742	SC –ura	BY4742 Tpk3-NC
Pkh1 fused with N-terminus of citrine	B2682	B2683	γN-*URA*	BY4742	SC –ura	BY4742 Pkh1-NC
Sch9 fused with N-terminus of citrine	B2702	B2703	γN-*URA*	BY4742	SC –ura	BY4742 Sch9-NC
Gcd1 fused with N-terminus of citrine	B2668	B2669	γN-*URA*	BY4742	SC –ura	BY4742 Gcd1-NC

Legend: '-GFP' stands for 'protein C-terminally tagged with the GFP', '-RFP' stands for 'protein C-terminally tagged with the RFP', '-CC' stands for 'protein C-terminally tagged with the C-terminus of citrine', '-NC' for 'protein C-terminally tagged with the N-terminus of citrine'.

The plasmid is isolated according to the protocol [*section D.3.8.3.]* starting from a transformed colony provided by the MCB lab. Subsequently, a PCR is performed [*section D.3.2.*] using the reaction mixture with Q5® High-Fidelity DNA Polymerase or TaKaRa Ex Taq™ DNA Polymerase given in *Table 2* and the PCR program indicated in *Suppl. Table 10* and *Suppl. Table 11* respectively. The primers and plasmids used for constructing the different strains are

displayed in *Table 2*, more information about the plasmids is provided in *Suppl. Table 17.* The PCR reaction results in the amplification of a PCR product, in the current work also called a fusion protein cassette. A small volume of the resulting PCR mixture is used for checking the presence of the wanted PCR product by AGE [*section D.3.3.*].

The successful PCR mixture is transformed into yeast [*section D.3.7.1.*]. The BY4742 strain is used for the transformation of PCR products including the N-terminal part of the citrine gene, while the BY4741 strain is transformed with PCR products including the C-terminal part of the citrine gene, the RFP- or GFP-gene. By homologous recombination, the fusion protein cassette is able to be introduced into the genome. Agar plates with the corresponding selection medium are used as given in *Table 2*.

The integration of the protein fusion cassette at the correct place in the genome is verified by performing a PCR [*section D.3.2.*] and/or sequencing [*section D.3.4.*] utilising AGE [*section D.3.3.*], after extracting the genomic DNA of the yeast colony [*section D.3.7.7.*]. Five different colonies are used for verification. This is necessary since false positive colonies can appear. The cassette with the selectable marker can be inserted into another locus in the genome, resulting in the survival on the selection medium. Yeast can also gain a spontaneous mutation that facilitates survival (McCusker & Haber, 1990). The agar plate can also be contaminated by another strain which is naturally able to survive on the selection medium.

A PCR is performed [*section D.3.2.*] using the reaction mixture TaKaRa Ex Taq™ DNA Polymerase given in *Table 2*, the primers as shown in *Suppl. Table 12* and the PCR program indicated in *Suppl. Table 4*. The F primer is homologous with a region of the gene of interest while the R primer is homologous with a region downstream the gene of interest. If the protein fusion cassette is successfully integrated, a higher band is present on the agarose gel since the PCR product is bigger, if not, a lower band is seen.

Sequencing is performed as described in *section D.3.4.* using the primers mentioned in *Suppl. Table 13*. Primers for sequencing are chosen in such a way that the end of the gene and the start of the downstream tag is sequenced.

5.2. Combining Traits of Strains

The new strains that are created in the current work by performing the steps described below are given in *Table 3*.

In this section, strains are obtained that contain genes in their genome coding for two different proteins of interest each tagged with a different fluorescent tag. This is achieved by mating two haploid strains with a different mating type and a gene coding for a distinct tagged protein of interest [mating; *section D.3.7.2.*]. If appropriate, the mating type is checked by mating type PCR [*section D.3.7.5.*] and a mating type switch is performed [*section D.3.7.6.*]. The mating type switch protocol is performed for the strains BY proto Gcd1-CC:Gap1-NC and BY proto Gcd6-CC:Gap1-NC.

After mating, the sporulation procedure is completed [*section D.3.7.3.*] and subsequently tetrad dissection [*section D.3.7.4.*] is performed. The resulting haploid strains are transferred to agar plates with selective medium by replica plating [*section D.3.5.*] and grown for one day. The resulting strains given in *Table 3* with '-CC' in their name are obtained by selecting on L-histidine single drop-out SC agar plates [*Suppl. Table 1*]. Additionally, if '-NC' is a part of their name, they are selected on uracil single drop-out SC agar plates as well [*Suppl. Table 1*]. Furthermore, the strains obtained via crossing with a strain which is prototrophic for leucine are selected on agar plates with leucine single drop-out SC medium [*Suppl. Table 1*]. In the current work, the strains created for studying interactions are made prototrophic for leucine, where possible. This strategy is chosen since strains with certain auxotrophic mutations studied under nitrogen starvation, especially leucine auxotrophic strains, result in unusual signalling to PKA. This possibly makes interactions found in auxotrophic strains irrelevant for wild type strains.

The strains growing on the wanted selective plates are stored [*section D.2.*]. The presence of the correct insertion of the tag in the genome is verified by performing a PCR [*section D.3.2.*] using the reaction mixture TaKaRa Ex Taq™ DNA Polymerase given in *Table 2*, the primers as shown in *Suppl. Table 7* and the PCR program indicated in *Suppl. Table 4*. Additionally, the verification is also done for most of the strains [*Suppl. Table 15*] by sequencing as described in *section D.3.4.* using the primers mentioned in *Suppl. Table 8*. Primers for sequencing are chosen in such a way that the end of the gene and the start of the downstream tag is sequenced.

Table 3: New strains created in the current work by mating two haploid strains, subsequently inducing sporulation, performing tetrad dissection and selecting for the wanted traits. The name and origin of each haploid strain is given together with the name of the new strain that is obtained.

Name Strain 1	**Source Strain 1**	**Name Strain 2**	**Source Strain 2**	**Resulting Strain**
BY4741 leu$^+$ met$^+$ Gcd1-CC	MCB lab	BY4742 Tpk1-NC	Current work	BY leu$^+$ Gcd1-CC:Tpk1-NC
BY4741 leu$^+$ met$^+$ Gcd1-CC	MCB lab	BY4742 Tpk2-NC	Current work	BY leu$^+$ Gcd1-CC:Tpk2-NC
BY4741 leu$^+$ met$^+$ Gcd1-CC	MCB lab	BY4742 Tpk3-NC	Current work	BY leu$^+$ Gcd1-CC:Tpk3-NC
BY4741 leu$^+$ met$^+$ Gcd1-CC	MCB lab	BY4742 Pkh1-NC	Current work	BY leu$^+$ Gcd1-CC:Pkh1-NC
BY4741 leu$^+$ met$^+$ Gcd1-CC	MCB lab	BY4742 Sch9-NC	Current work	BY leu$^+$ Gcd1-CC:Sch9-NC
BY4741 Tpk1-CC	Current work	BY4742 proto Gap1-NC	MCB lab	BY leu$^+$ Tpk1-CC:Gap1-NC
BY4741 Tpk2-CC	Current work	BY4742 proto Gap1-NC	MCB lab	BY leu$^+$ Tpk2-CC:Gap1-NC
BY4741 Tpk3-CC	Current work	BY4742 proto Gap1-NC	MCB lab	BY leu$^+$ Tpk3-CC:Gap1-NC
BY4741 Pkh1-CC	Current work	BY4742 proto Gap1-NC	MCB lab	BY leu$^+$ Pkh1-CC:Gap1-NC
BY4741 Sch9-CC	Current work	BY4742 proto Gap1-NC	MCB lab	BY leu$^+$ Sch9-CC:Gap1-NC
BY4741 Gap1-CC	Current work	BY4742 leu$^+$ lys$^+$	MCB lab	BY leu$^+$ Gap1-CC
BY4741 Mep2-CC	Current work	BY4742 Gap1-NC	MCB lab	BY Mep2-CC:Gap1-NC

Legend: '-CC' stands for 'protein C-terminally tagged with the C-terminus of citrine', '-NC' for 'protein C-terminally tagged with the N-terminus of citrine'.

5.3. Culturing Transformed Strains

The cells are grown in the wanted growth phase for observation with the fluorescence microscope. SC medium [*Suppl. Table 1*] is used instead of YPD, since it gives a lower background fluorescence (Weissman, *et al*., 2010). The steps below are performed in a sterile way.

- A preculture is made by adding a yeast colony of interest to a glass tube with 3 ml sterile SC medium (or the appropriate drop-out medium) [*Suppl. Table 1*] and is grown O/N in a shaking incubator at 30 °C.
- The O/N preculture is used to inoculate a larger culture: A small amount of the preculture is added to an Erlenmeyer flask with 50 ml fresh sterile SC medium (or the appropriate drop-out medium) [*Suppl. Table 1*], diluting it in such a way that an OD_{600} of 0.01 is obtained so that after growing O/N in a shaking incubator at 30 °C an OD_{600} between 1 and 2 is reached, i.e. exponential phase [Determination of Growth Phase, see *section 3.6*].

If a strain is to be visualised in nitrogen starvation, an additional step is subsequently performed: The exponential culture is centrifuged at 4,500 rpm for 5 min and the remaining pellet is transferred to 50 ml sterile NSM [*Suppl. Table 1*] for growing for 24 h in a 30 °C shaking incubator.

- Before visualising the cells with the fluorescence microscope, the OD_{600} is again inspected to see if the strain has grown as expected [Determination of Growth Phase, see *section 3.6*].

5.4. Visualisation

The cells are visualised with an Olympus Fluoview FV1000 confocal laser scanning microscope with the accompanying FV10-ASW 2.0 software.

The confocal microscope uses point illumination and point detection. Specifically, it consists of a focused laser beam for excitation and a pinhole aperture in the image plane in front of a photon detector. This is done to eliminate the out-of-focus signal, leading to an improved resolution. The presence of a dichromatic mirror, an emission filter and a point detector is crucial for this type of microscope. Depending on the fluorophore, different laser wave lengths and emission filters are used[25].

Dying cells produce a strong background signal (autofluorescence) and cannot be confused with the signal from the fluorescent proteins.

5 µl of the culture is pipetted onto a microscopy slide. Subsequently, a cover slip is added. The slide is placed on the stage of the microscope together with emersion oil. An objective lens of 60X together with an ocular lens of 10X is used for the observation.

[25] http://www.microscopyu.com/articles/confocal/confocalintrobasics.html

If the nuclei are to be stained, an extra step is added before pipetting 5 µl of the culture onto a microscopy slide. Two droplets of NucBlue® Live ReadyProbes® Reagent from Thermo Fisher are combined in a 1 ml culture in a non-transparent Eppendorf tube and incubated for about 10 minutes.

Contrast can be rendered in transparent specimens using Differential Interference Contrast (DIC) microscopy[26]. Simultaneously, both fluorescence and DIC microscopy are used to visualise the position of the fluorescence in the cells.

5.5. Growth Recovery after Nitrogen Starvation

The recovery of a nitrogen starved *S. cerevisiae* culture in a complete growth medium is studied by measuring the OD_{600} of the culture growing at 30 °C for 48 hours. This experiment is performed to investigate the possible influence of auxotrophies and fluorescent tags on the growth of strains.

The strains are prepared as followed, performed in a sterile way.
- A preculture is made by adding the yeast colony of interest to a glass tube with 3 ml sterile YPD medium [*Suppl. Table 1*] and is grown O/N in a shaking incubator at 30 °C.
- The O/N preculture is used to inoculate: A small amount of the preculture is added to a glass tube with 3 ml fresh sterile YPD medium [*Suppl. Table 1*], diluting it in such a way that an OD_{600} between 1 and 2 is reached after growing in a shaking incubator at 30 °C [Determination of Growth Phase, see *section 3.6*].
- The exponential culture is centrifuged at 4,500 rpm for 5 min and the remaining pellet is transferred to 3 ml sterile NSM [*Suppl. Table 1*] in a fresh glass tube for growing for 24 h in a 30 °C shaking incubator.
- The nitrogen starved culture is diluted in such a way that an OD_{600} of 0.5 is obtained in sterile YPD medium [*Suppl. Table 1*]. A sterile Greiner 24-well plate is filled with 1 ml samples in adjacent wells. The plate is covered for sterility reasons and put in the Synergy H1 Hybrid Reader. The settings of the machine are adapted using the Gen5 program as followed. The plate is shaken continuously in a double orbital with the frequency of 425 cpm (3 mm) to prevent the cells from sinking to the bottom. The temperature is set at 30 °C and the OD_{600} is measured every 30 minutes for 48 hours.

[26] http://www.microscopyu.com/articles/dic/index.html

E. Results

***In vitro* Analysis Indicates Protein-Protein Interactions Using GST Pull-Down**

A GST pull-down experiment was successfully performed, studying *in vitro* physical interactions of Gap1 with the kinases Pkh1, Sch9 and Tpk1. In the same assay, also interactions between these kinases and eIF2B subunits Gcd1 and Gcd6 were analysed. GST pull-down is used as a first indication of protein-protein interactions. An *in vivo* method should confirm the *in vitro* physical interactions found.
As mentioned in *section D.4.,* Gap1, Gcd1 and Gcd6 were expressed in yeast strains Sigma GAP1-HA, Sigma GCD1-HA, Sigma GCD6-HA respectively, resulting in HA-tagged proteins. The *E. coli* strains containing the plasmids pGEX-4T-1 Pkh1-GST, pGEX-4T-1 Sch9-GST and pGEX-4T-1 Tpk1-GST were used to express the yeast kinases Pkh1, Sch9 and Tpk1 respectively. To obtain a positive and negative control, the expression of Sui3 and the GST protein was induced in the *E. coli* strains transformed with the plasmids pGEX-4T-1 Sui3-GST and pGEX-4T-1 correspondingly. In the yeast expression system, the proteins were collected in the state in which they are present in the highest levels. Consequently, Gcd1 and Gcd6 were obtained in the exponential phase, whereas Gap1 was collected after nitrogen starvation. All yeast proteins expressed in *E. coli* are gathered after IPTG induction, resulting in a high yield.

The result of the GST pull-down experiment is given in *Figure 7*. The Coomassie gel shows that all proteins expressed by *E. coli* were present in adequate levels. If the proteins are not detectable with Coomassie staining, one cannot draw conclusions about the interactions. Bands at other heights were visible on the gel as well, showing that not only the *E. coli*-expressed protein of interest was present in the sample of the assay. This is intrinsically not a problem for drawing conclusions.

The Western blot after visualization with the peroxidase coupled anti-HA antibody results in black bands if the HA-tagged protein is present in the sample. Subsequently, if no band is observed, the HA-tagged protein is absent. The ‘Input’ is the sample containing yeast-expressed proteins without the presence of beads and *E. coli*-expressed proteins. It is used for showing that the HA-tagged protein is not absent. In this approach, one can confirm that all three proteins of interest were successfully expressed in yeast. Protein Sui3 is known to interact with Gap1, Gcd1 and Gcd6 and therefore serves in this assay as a positive control. For all three proteins, the positive control was successful, since a band was visible at the Sui3 lane. The GST lane on the other hand represents the interaction between the GST-tag itself and the HA-tagged protein of interest. If a band is visible in the GST lane, one cannot interpret the results of the investigated protein-protein interactions. For both Gap1-HA, Gcd1-HA and Gcd6-HA, no band was present. Consequently, it is verified that there was no interaction between the HA-tagged proteins and the GST-loaded beads.

A band was present at the Western blot of Gap1-HA for Pkh1-GST, Sch9-GST as well as for Tpk1-GST. One can conclude that an interaction was found between the respective proteins of interest via GST pull-down. In the Western blot of Gcd1-HA, interactions were found of Gcd1 with both Pkh1 and Tpk1. On the other hand, the Gcd6-HA Western blot showed interactions of Gcd6 with Pkh1, Sch9 and Tpk1. To conclude, the GST pull-down experiments of the current work resulted in scientific evidence about *in vitro* physical interactions of Gap1 with kinases Sch9, Pkh1 and Tpk1. The kinases mentioned also showed interactions between at least one eIF2B subunit studied using the same technique.

It should be noted that the experiment was also tried with proteins Tpk2-GST and Tpk3-GST. Unfortunately, the presence of the proteins was not detectable in Coomassie staining, so that no results were deducible.

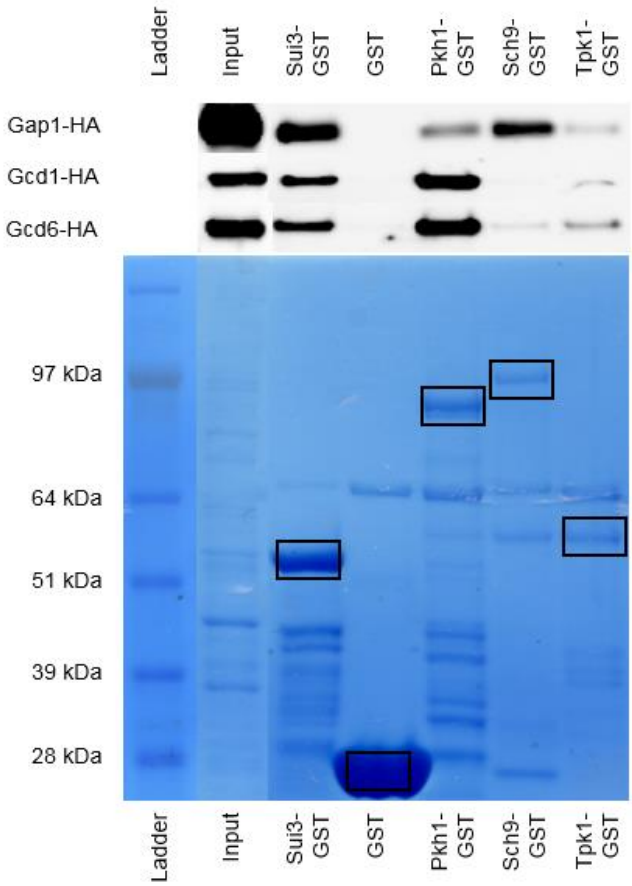

Figure 7: Result of performance of the GST pull-down experiment. The upper panel (grey) shows the result of the immunodetection after western blotting. The presence of HA-tagged proteins in the samples containing GST-tagged proteins (or the GST-tag itself) was detected by a peroxidase coupled anti-HA. The HA-tagged proteins were either Gap1, Gcd1 or Gcd6 respectively, whereas the GST-tagged proteins examined were Sui3, Pkh1, Sch9 or Tpk1 correspondingly. For every HA-tagged protein a separate polyacrylamide gel was used to obtain this panel of the figure. The lower panel (blue) represents the Coomassie stained gel, showing the proteins expressed by *E. coli* present in the samples after purification with glutathione sepharose beads. Since the same amount of each *E. coli*-expressed protein mixture is added to every yeast sample, the Coomassie stained gels of the three different yeast samples give the same result. In this figure one of them is shown. The 'Ladder' lane is comprising SeeBlue®Plus2 Pre-Stained Standard. The molecular weight of the known proteins are indicated. Based on the ladder, the proteins of interest were identified. The black rectangles on the Coomassie outcome display at which height the band corresponding to the GST-tagged protein is expected to be present.

In vivo **Analysis of Localisation of Proteins Using Fluorescent Tags**

In order to study the subcellular localization of the proteins of interest, being Tpk1, Tpk2, Tpk3, Sch9 and Pkh1, strains were engineered so that they contain a gene encoding a fusion protein of the protein of interest and a fluorescent protein (in-frame). The correct genomic insertion of the PCR product was confirmed by sequencing.

The resulting strains were cultured for fluorescence microscopy observation in two conditions, both exponentially and after 24 hours of nitrogen starvation. The results of the visualisation are displayed in *Figure 8* and *Figure 9* and summarised in *Table 4*. The wild type strain was used as a negative control. The power of the excitation laser was adapted in such a way that there was no background fluorescence in the control strain. These settings were applied for visualising all samples.

Table 4: Results of fluorescence microscopy visualisation for strains used for studying the localization of the protein of interest.

Strain	Exponential	Nitrogen Starved
BY4741 Tpk1-GFP	Yes: Localized	Yes: Localized
BY4741 Tpk2-GFP	Yes: Localized	Yes: Localized
BY4741 Tpk3-GFP	Yes: Localized	Yes
BY4741 Sch9-RFP	No	No
BY4741 Pkh1-RFP	No	No
BY4741	No	No

Legend: When 'Yes' is written, fluorescence was observed in the cells, being thus more than just background fluorescence. On the other hand, 'No' means that the same level of fluorescence was seen as the negative control. 'Localized' indicates that the fluorescence was not spread over the whole cytosol of the cell, but only in a strictly defined region in the cells.

The localization of proteins Tpk1-GFP, Tpk2-GFP and Tpk3-GFP in exponential phase and after nitrogen starved were studied successfully. In all cases, there was more fluorescence observed in the cytosolic compared to the negative control. In the case of Tpk1-GFP, there was clearly one distinct region with very strong fluorescence, both in exponential and nitrogen starvation conditions. It seems that there is one place in the cell where Tpk1 is more likely to be present. Also additional local fluorescence was observed for Tpk2-GFP in both conditions. However, this was less explicitly compared to Tpk1-GFP. Tpk1 and Tpk2 are therefore possibly present both randomly in the cytosol as well as in a distinct space in the cytoplasm. Tpk3-GFP on the other hand gave a partial localised signal in exponential phase cells, but not in nitrogen starvation. In the latter condition it was present everywhere in the cytosol.

Visualisation of fluorescently tagged Sch9 and Pkh1 did not work as expected. No fluorescence was observed, although, the genes contain the fluorescent tag according to sequencing. Therefore, it seems that there is an expression or folding problem.

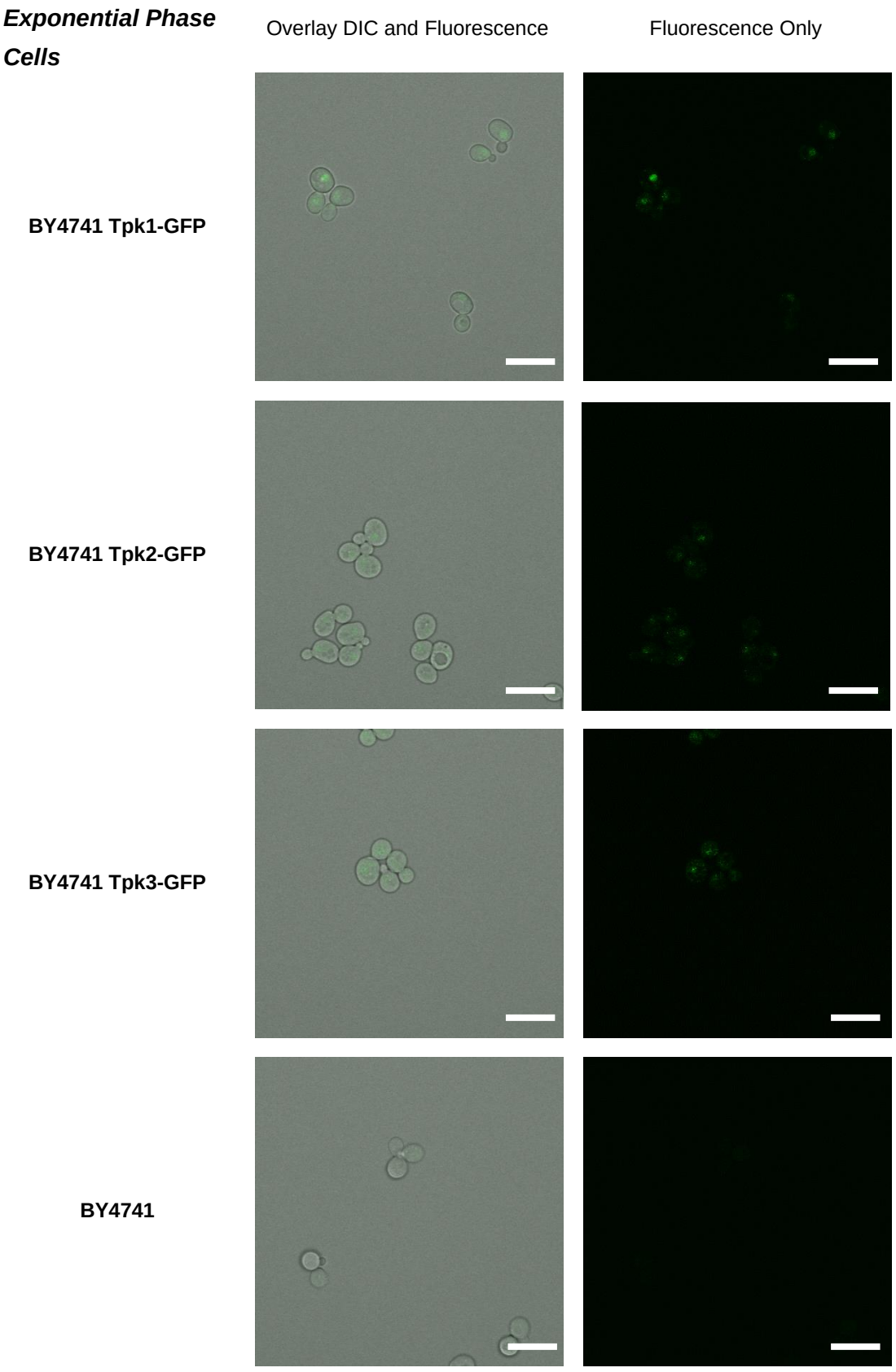

Figure 8: Localization of Tpk1-GFP, Tpk2-GFP and Tpk3-GFP in exponential phase cells. On the left hand side a picture with the overlay of the DIC and fluorescence channel is given, whereas on the right hand side a picture is shown obtained by the fluorescence channel only. Scale: the length of the stripe represents 10 µm.

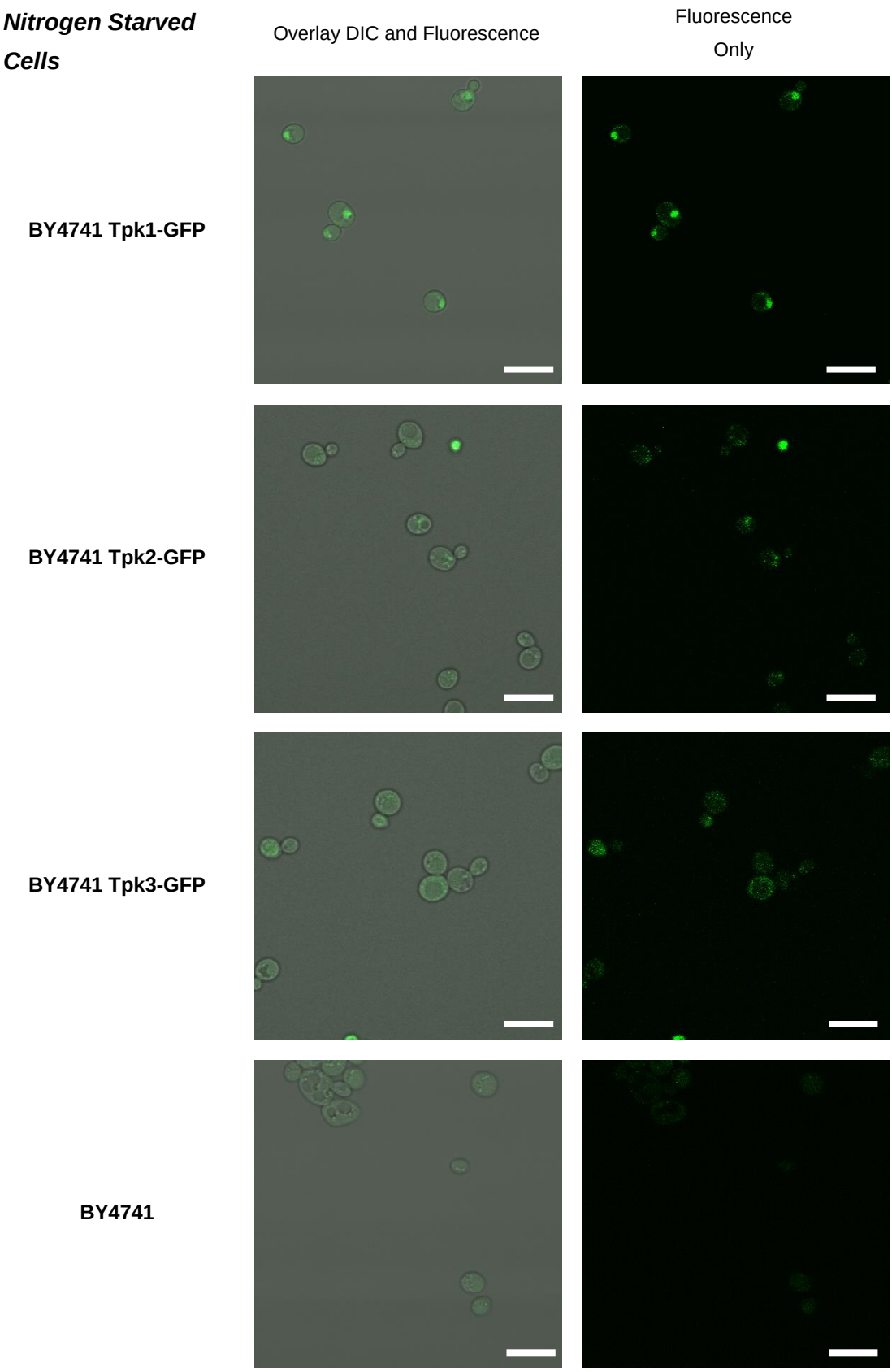

Figure 9: Localization of Tpk1-GFP, Tpk2-GFP and Tpk3-GFP in nitrogen starved cells. On the left hand side a picture with the overlay of the DIC and fluorescence channel is given, whereas on the right hand side a picture is shown obtained by the fluorescence channel only. Scale: the length of the stripe represents 10 µm.

In vivo **Analysis Indicates Protein-Protein Interaction Using Split Citrine BiFC**

In vivo protein-protein interactions in the current work were studied using split citrin BiFC. In order to visualise the interactions, several strains with fusion proteins have been created. Two proteins were each tagged C-terminally with one of the fragments of citrine, resulting in two fusion proteins in the same cell. For that, cells expression one fusion protein were engineered first, subsequently, cells were mated. The interactions between Gap1 and the kinases Sch9, Pkh1 and Tpk1-3 were studied *in vivo*. In addition, *in vivo* interactions of the kinases with Gcd1 were examined.
The correct construction of the genes of the resulting strains were confirmed by sequencing. Only the sequencing result for the gene encoding protein Tpk2 fused with the C-terminus of citrine of strain BY leu^+ Tpk2-CC:Gap1-NC was not interpretable. The sequencing procedure should be repeated for this strain.

Subsequently, the strains were cultured for fluorescence microscopy observation. To partially exclude false positive results in BiFC, the strains containing only protein fusion construct were visualised in nitrogen starvation. These include strains with genes encoding Tpk1-CC, Tpk2-CC, Tpk3-CC, Pkh1-CC, Sch9-CC, Tpk1-NC, Tpk2-NC, Tpk3-NC, Pkh1-NC, Sch9-NC, Gcd1-CC, Gcd1-NC, Gcd6-CC, Gap1-NC and Gap1-CC. As expected, since only one half of the fluorophore is expressed, no fluorescence was observed. To study the strains containing two fusion proteins, the power of the excitation laser was adapted in such a way that there was no background fluorescence in the control strains.
Additionally, positive control strains were taken along to confirm that the right growth conditions were used and the correct settings of the fluorescence microscope were applied. Both strains BY proto Gcd1-CC:Gap1-NC and BY proto Gcd6-CC:Gap1-NC served as a positive control. In previous unpublished work of the MCB lab, these two strains were found to result in transporter/eIF2(B)-focus with the split citrine BiFC technique in the nitrogen starved condition. Corresponding to what is described before, the positive control strains indeed showed fluorescence. *Figure 10* among others displays a picture of these strains.

Eventually, the strains to study the interaction between the proteins of interest were visualised. The analysis was performed both in the exponential phase and/or after 24 hours of nitrogen starvation. The results of visualisation with the fluorescent microscope are summarised in *Table 5*. Only strain BY leu^+ Sch9-CC:Gap1-NC resulted in fluorescence in the nitrogen starvation condition. A concentrated fluorescent spot was observed in the cells (a focus), this result was repeatable. It shows that it is very likely that an interaction occurs between Sch9 and Gap1 in a local region within the cell. All the other strains mentioned in *Table 5* did not show fluorescence in the split citrine BiFC approach under the studied conditions. *Figure 10* displays pictures obtained from some strains in nitrogen starvation.

In the positive control strains and BY leu$^+$ Sch9-CC:Gap1-NC the majority of the cells showed usually one, sometimes two, distinct localized fluorescent regions in the nitrogen starvation condition. The subcellular localization of this earlier described transporter/eIF2(B)-foci seemed to be different from the Sch9-Gap1 foci observed in the current work. To obtain an indication about the localization of the signal, nuclear staining was performed with the NucBlue® Live ReadyProbes® Reagent. The results of visualisation with the fluorescent microscope after staining are given in *Figure 11*. Using this dye, the nuclei were successfully stained. In addition to nuclear staining, a green signal appeared at the plasma membrane. This additional fluorescence was found to be an artefact, since strains without tagged proteins showed the same membrane-localised fluorescence. The foci of strains BY proto Gcd6-CC:Gap1-NC and BY proto Gcd1-CC:Gap1-NC were clearly not always located near the nucleus. In contrast, the foci of the BY leu$^+$ Sch9-CC:Gap1-NC strain were always near the nucleus. In some cases, even an overlap of the nuclear staining and the fluorescent focus was seen, whereas in other cases, the focus was just in contact with the nucleus.

For future use, the mating type of strain BY proto Gcd1-CC:Gap1-NC and BY proto Gcd6-CC:Gap1-NC was successfully switched from *MATa* to *MATα*. Also additional strains were constructed for future experiments, including BY4741 Sul1-CC, BY4741 Mep2-CC, BY4742 Gcd1-NC, BY leu$^+$ Gap1-CC and BY Mep2-CC:Gap1-NC.

Table 5: Results of fluorescence microscopy for strains used for studying protein-protein interactions.

Strain	**Exponential**	**Nitrogen Starved**
BY proto Gcd1-CC:Gap1-NC	No	Yes: Localized
BY proto Gcd6-CC:Gap1-NC	/	Yes: Localized
BY leu$^+$ Gcd1-CC:Tpk1-NC	No	No
BY leu$^+$ Gcd1-CC:Tpk2-NC	No	No
BY leu$^+$ Gcd1-CC:Tpk3-NC	No	No
BY leu$^+$ Gcd1-CC:Pkh1-NC	No	No
BY leu$^+$ Gcd1-CC:Sch9-NC	No	No
BY leu$^+$ Tpk1-CC:Gap1-NC	/	No
BY leu$^+$ Tpk2-CC:Gap1-NC	No	No
BY leu$^+$ Tpk3-CC:Gap1-NC	/	No
BY leu$^+$ Pkh1-CC:Gap1-NC	/	No
BY leu$^+$ Sch9-CC:Gap1-NC	No	Yes: Localized

Legend: When 'Yes' is written, fluorescence was observed in the cells, being thus more than just background fluorescence. On the other hand 'No' means that the same level of fluorescence was seen as the negative control. 'Localized' indicates that the fluorescence was not spread over the whole cytosol of the cell, but only in a strictly defined region in the cells. '/' means that the respective condition was not studied in the given strain.

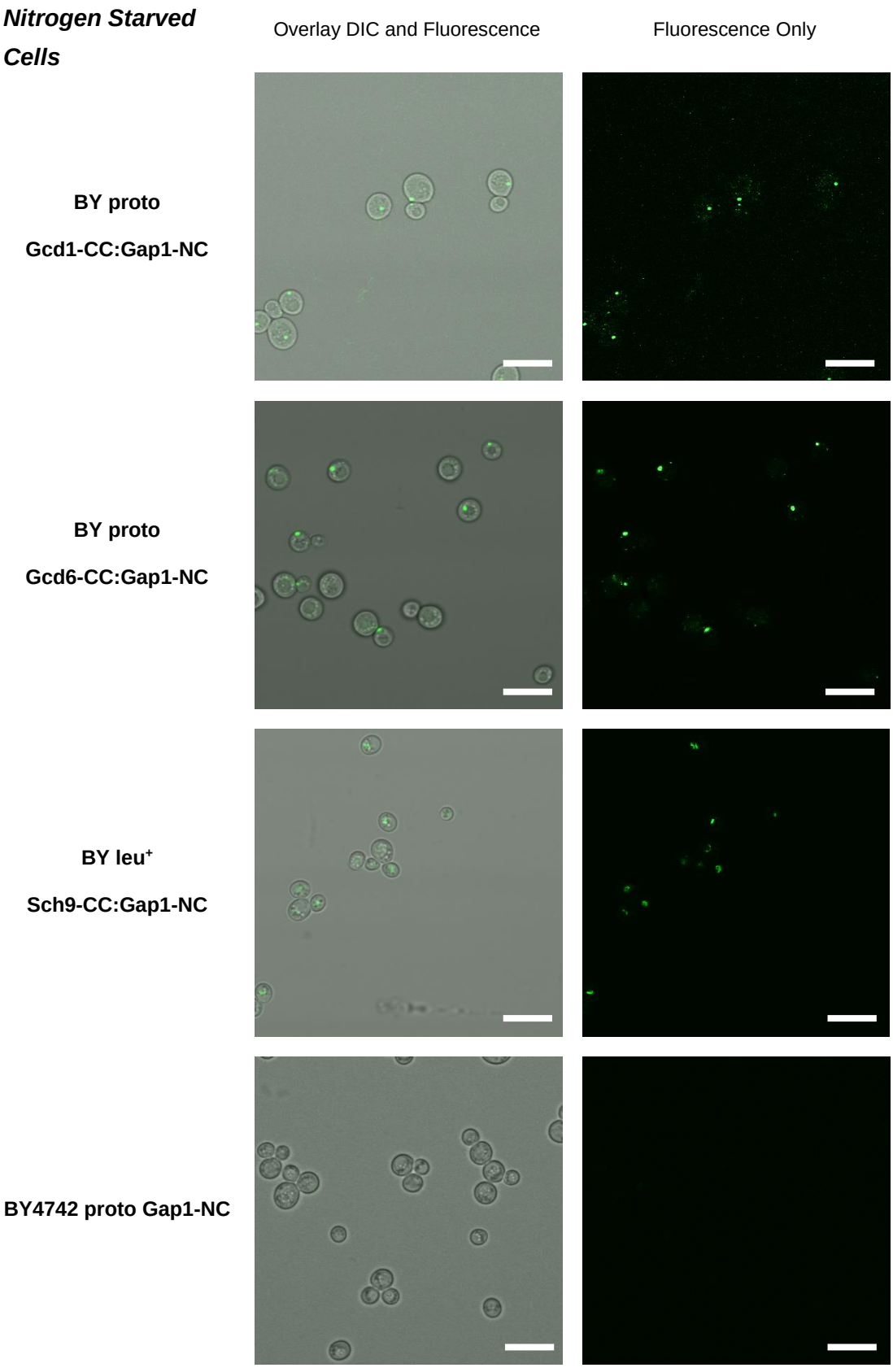

Figure 10: Split citrine BiFC interaction observed in nitrogen starved cells between Gap1-N-Citrine and Gcd1-C-Citrine, Gcd6-C-Citrine and Sch9-C-Citrine respectively. On the left hand side a picture with the overlay of the DIC and fluorescence channel is given, whereas on the right hand side a picture is shown obtained by the fluorescence channel only. Scale: the length of the stripe represents 10 µm.

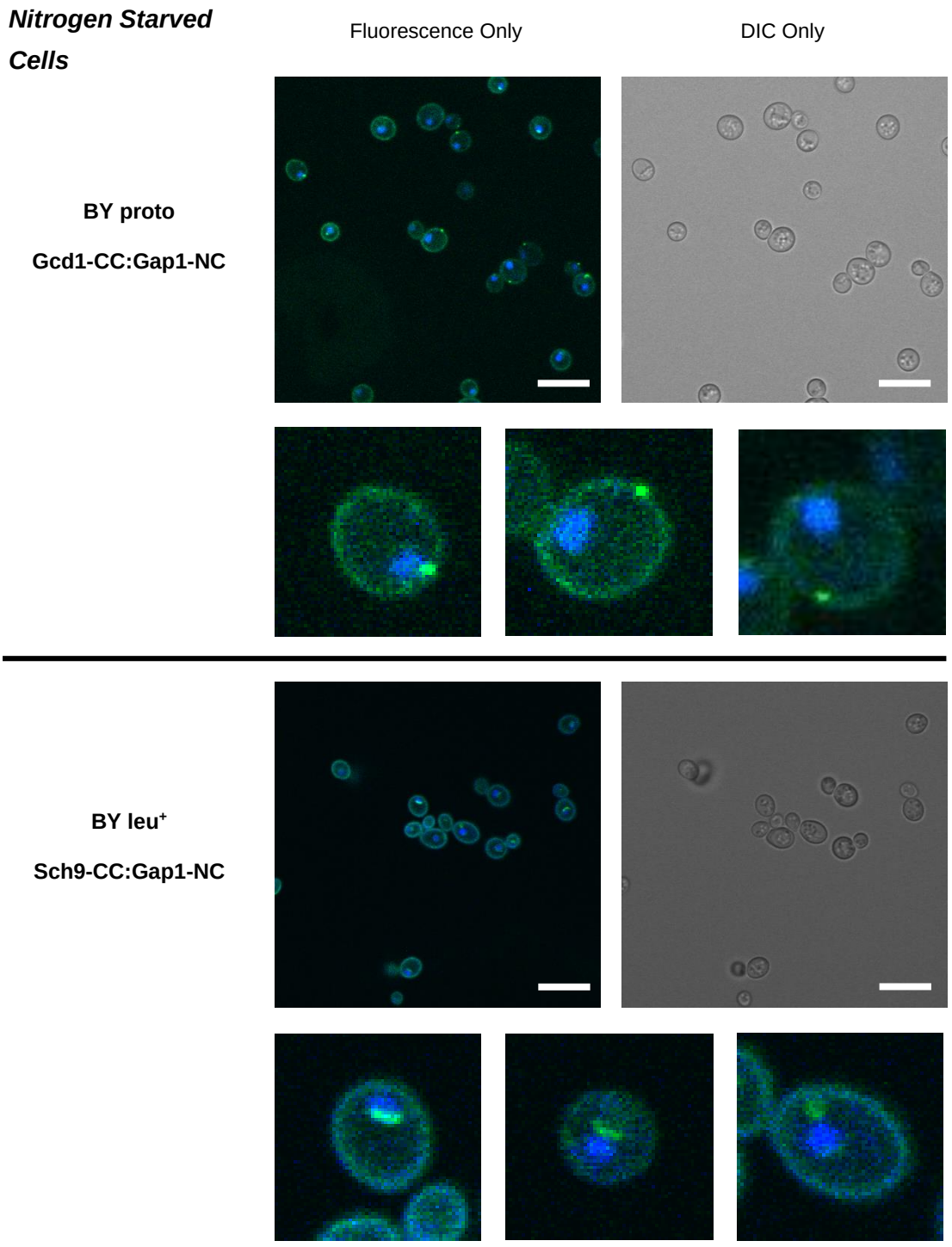

Figure 11: Split citrine BiFC interaction observed in nitrogen starved cells between Gap1-N-Citrine and Gcd1-C-Citrine, Gcd6-C-Citrine and Sch9-C-Citrine respectively, together with nuclear staining using NucBlue® Live ReadyProbes® Reagent. On the left hand side a picture is given obtained by the fluorescence channel only, whereas on the right hand side a picture is shown obtained by the DIC channel. Three magnified views (4X enlarged) of the fluorescence channel pictures are given at the bottom each time. Scale: the length of the stripe represents 10 µm.

Protein Functionality Studies of Split Citrine BiFC Strains

After creating *S. cerevisiae* strains with tagged proteins for split citrine BiFC, it is advised to examine the functionality of the fusion proteins. After all, a tag can among others lead to a lower mobility and activity of the protein of interest. Additionally, for strains containing two proteins with each one fragment of citrine, it should be noted that the complementation of citrine leads to an irreversible binding of the fluorescent protein. Consequently, the proteins are kept together, possibly interfering with their functionality (Kodama & Hu, 2012).

In the current work, protein functionality was studied by measuring growth recovery of different nitrogen starved cultures in a complete growth medium. This test is relevant for the proteins of interest, since the proteins Gcd1, Gcd6, Sch9, PKA and Gap1 are all important for growth, as described in *section B*. Strains co-expressing two fusion constructs were compared with strains expressing only one fusion construct. As a reference, non tagged wild type strains of the corresponding background were used, in particular the strains BY4741 and BY4742 leu^+ lys^+. Both strains were taken along since the influence of auxotrophies on growth is not elucidated yet. Since the complete experiment was performed once successfully with two technical replicates, it should be stated that drawing strong conclusions are constrained. The replicates were obtained by inoculating the same growth culture twice in a separate well of the same 24-well plate. The result was made visible using graphs, illustrated in *Suppl. Figure 1*.

The growth of strain BY leu^+ Gcd1-CC:Sch9-NC (still auxotrophies) was compared with its parents and the references in Panel A. Strains BY4741 leu^+ met^+ Gcd1-CC (ura^-) and BY4741 showed the same slow recovery, reaching a lower stationary level compared to the others. On the other hand strain BY4742 Sch9-NC (his^- leu^- met^-) and BY4742 leu^+ lys^+ (his^- ura^-) showed the same fast recovery and a high end OD_{600}. BY leu^+ Gcd1-CC:Sch9-NC had grown with a rate in between those mentioned, reaching the same high level of strains BY4742 Sch9-NC and BY4742 leu^+ lys^+ after 36 hours.

The recovery of strain BY leu^+ Sch9-CC:Gap1-NC (still auxotrophies), its parents and the reference strains were put next to each other in Panel B. All the strains showed a higher OD_{600} than reference BY4741 starting from 12 hours after inoculation. At the end of the experiment strains BY leu^+ Sch9-CC:Gap1-NC and BY4742 leu^+ lys^+ (his^- ura^-) appeared to have a slightly higher OD_{600} compared to BY4742 proto Gap1-NC and BY4741 Sch9-CC (leu^- met^- ura^-). The latter one needed more time to reach a high OD_{600}.

Panel C is a visualisation of the recovery of strain BY proto Gcd1-CC:Gap1-NC (no autotrophies), its parents and the references. Strains BY4741 and BY4741 leu^+ met^+ Gcd1-CC (ura^-) showed the same growth curve (taking into account the error bars). BY proto Gcd1-CC:Gap1-NC had the same high rate and stationary OD_{600} as BY4742 leu^+ lys^+. Also BY4742 proto Gap1-NC (no autotrophies) displayed the same rate, but leaded to a lower stationary OD_{600}.

Also the growth recovery of BY proto Gcd6-CC:Gap1-NC (no autotrophies) was analysed against its parents and the references, this is demonstrated in Panel D. BY4741 leu^+ met^+ Gcd6-CC (ura^-) and BY4741 showed a similar growth profile. BY proto Gcd6-CC:Gap1-NC and BY4742 leu^+ lys^+ (his^- ura^-) exhibited the same high growth rate and high stationary OD_{600}, which is similar to that of BY4742 proto Gap1-NC (no autotrophies).

In Panel E the diversity of the reference strains was studied in combination with strains expressing one fusion construct and strains co-expressing two fusion constructs. No group distinction was found between reference and single-fusion construct strains. Remarkably, all studied strains expressing two fusion constructs were showing a high OD_{600} at the end of the experiment. To conclude, the contrast in growth curves cannot clearly be elucidated by the strains expressing either two, one or no fusion constructs.

Panel F of *Suppl. Figure 1* shows the comparison in recovery of strains that are either auxotrophic or prototrophic for leucine. There was no general trend observed that enables to distinguish the two groups. Consequently, the differences between the growth curves cannot be explained by the strains being either prototrophic or auxotrophic for leucine. Noteworthy, there was a big difference in growth rate and stationary phase OD_{600} between BY4741 and BY4742 leu^+ lys^+ (only auxotrophic for histidine and uracil) as seen in the other panels. An additional experiment with the same settings was performed for comparing strains BY4741 with BY4742 (both auxotrophic for histidine, leucine and uracil). No significant difference in the growth curves was found between the two latter strains, indicating that the mating type and the difference in auxotrophy for methionine versus lysine was not responsible for the difference in growth curve between BY4741 and BY4742 leu^+ lys^+.

To summarise, strains expressing two fusion constructs resulted in the same or similar growth curve as the reference BY4742 leu^+ lys^+, showing no deleterious effect of the tags on the functionality of the proteins. However, BY4741 leu^+ met^+ Gcd1-CC (ura^-), BY4741 leu^+ met^+ Gcd6-CC (ura^-), BY4741 Sch9-CC (leu^- met^- ura^-) and BY4741 showed the slowest recovery from nitrogen starvation. It is noteworthy that all studied strains containing one fusion construct with the C-terminus of citrine showed a slower recovery. The data indicate that the functionality of the proteins Gcd1, Gcd6 and Sch9 were influenced by the tag. However, the tag with the C-terminus of citrine consists of 84 amino acids while the N-terminus of citrine is composed of 155 amino acids (Kodama & Hu, 2012). Probably, the conformation of the tag is more important than the size itself.

In addition, it is very likely that the possible negative influence of the tag on the functionality of the protein of interest is dependent on the function of the protein. This could explain the variability.

As a conclusion, the differences between the growth curves cannot easily be explained. Repetitions of the experiment are needed to define if these results were obtained by coincidence or not.

F. Discussion, Conclusion and Perspectives

The GST pull-down experiments of the current work resulted in scientific evidence about *in vitro* physical interactions of Gap1 with kinases Sch9, Pkh1 and Tpk1. Additionally, the kinases mentioned also showed interactions between at least one eIF2B subunit studied using the same technique. Since GST pull-down is an *in vitro* technique with its own limitations [more info*: see Addendum section 4*], it is used as a first indication of protein-protein interactions. An *in vivo* method should confirm the *in vitro* physical interactions found. The interaction between Gap1 and Sch9 was preliminary confirmed with split citrine BiFC in the current work, studying the strain BY leu^+ Sch9-CC:Gap1-NC.

None of the mentioned protein-protein interactions of the current work were already proven and given on the BioGRID website, comprising the entire literature for *S. cerevisiae*. The possibility exists that a protein pair is not studied yet, or investigated without a positive result. However, protein-protein interactions between the eIF2B subunits (Gcd1 & Gcd6) and kinases (Pkh1, Sch9 and Tpk1) of the current work were already explored in the PhD thesis of Dr. Marlies Kimpe (unpublished). A summary of these findings obtained by different *in vitro* and *in vivo* techniques are described in *Table 6*. The techniques used are GST pull-down, co-immunoprecipitation, Yeast two Hybrid and a kinase assay. The only interactions demonstrated were HA-Pkh1 with GST-Gcd1, and HA-Pkh1 with GST-Gcd6 in the *in vitro* GST pull-down assay. *Table 7* summarises the protein pairs explored in the current work which were also studied in the PhD thesis of Dr. Marlies Kimpe. The results highlighted in grey, both in *Table 6* and *Table 7*, are the experiments of the current work which were a repetition of the work of Dr. Marlies Kimpe. In the current work interactions were observed between those, in contrast with no interactions found by Dr. Kimpe. This is possibly explained by a different visualisation method for immunodetection. In the current work a more sensitive substrate for the peroxidase is used: WesternBright™ Quantum™ and Peroxide (Advansta) instead of SuperSignal® West Pico Chemiluminescent Substrate, Luminol/Enhancer Solution and Stable Peroxide Solution (ThermoFisherScientific) used by Dr. Kimpe.

Table 6: Summary of the protein-protein interactions explored in the PhD thesis of Dr. Marlies Kimpe.

	Gcd1					Gcd6				
	GST P.D.		Co-IP	Y2H	KA	GST P.D.		Co-IP	Y2H	KA
	H-G	G-H				H-G	G-H			
Pkh1	Y	N	n. s.	N	/	Y	N	N	N	/
Sch9	N	N	n. s.	N	N	N	N	N	N	N
Tpk1	N	N	n. s.	N	N	N	N	N	N	N

Legend: 'Y': interaction; 'N': no interaction; n. s.: nonspecific interaction, so not interpretable; '/': not studied; GST P.D.: *in vitro* GST pull-down experiment; 'H-G': HA-tagged kinase vs. GST-tagged eIF2B subunit; 'G-H': GST-tagged kinase vs. HA-tagged eIF2B subunit; Co-IP: *in vitro* co-immunoprecipitation with HA-tagged kinase; Y2H: *in vivo* Yeast Two Hybrid; KA: *in vitro* kinase assay.

Table 7: Summary of protein-protein interactions explored in the current work which are also studied in the PhD thesis of Dr. Marlies Kimpe.

	Gcd1			Gcd6		
	GST P.D.		BiFC	GST P.D.		BiFC
	H-G	G-H		H-G	G-H	
Pkh1	/	Y	N	/	Y	N
Sch9	/	N	N	/	Y	N
Tpk1	/	Y	N	/	Y	N

Legend: 'Y': interaction; 'N': no interaction; '/': not studied; GST P.D.: *in vitro* GST pull-down experiment; 'H-G': HA-tagged kinase vs. GST-tagged eIF2B subunit; 'G-H': GST-tagged kinase vs. HA-tagged eIF2B subunit; 'BiFC': *in vivo* split citrine bimolecular fluorescence complementation.

In the current work, also the localisation of Tpk1-GFP, Tpk2-GFP and Tpk3-GFP was observed both in cells in the exponential phase and starved for nitrogen.
The results of the current work are compared with two other studies, being the work of Huh *et al.* and Tudisca *et al*. The former studied the localization of GFP fusion proteins, among others Tpk1, Tpk2 and Tpk3, in mid-logarithmic phase in synthetic medium using strain EY0986 (ATCC 201388: Mat **a** *his leu met ura*). This strain is related to BY4741 and BY4742 since they are engineered from the same strain: S288C. In the work of Huh *et al.* strains were transformed so that they contain a fusion protein intragenomically with the GFP C-terminal to the protein of interest, just as in the current work. After co-localization experiments using mRFP fusion proteins, they defined the subcellular localization. Tudisca *et al.* studied Tpk1, Tpk2 and Tpk3 using strains with the same construct as described by Huh *et al*. In this work they examined different growth phases (Huh, *et al*., 2003; Tudisca, *et al*., 2010).
In the mid-logarithmic phase, Huh *et al*. described that Tpk1 and Tpk3 proteins were found both in the cytoplasm and nucleus, whereas Tpk2 was only located in the nucleus. In the work of Tudisca *et al.* during exponential growth on glucose, Tpk2 was found to be localized in the nucleus, while Tpk1 and Tpk3 were both found to be present in the nucleus as well as in the cytoplasm. This is perfectly in line with what was seen in the work of Huh *et al*. in the mid-logarithmic phase. In contrast, in the current work, Tpk1, Tpk2 and Tpk3 were found to be present all over the cell with locally additional fluorescence. It is not proven yet if this brighter spot is co-localizing with the nucleus. Future work of the MCB lab will provide additional information.
However, in the stationary phase, Tudisca *et al*. showed that Tpk1-3 are only localized in the cytoplasm. Additionally, both Tpk2 and Tpk3 were found to be associated to EGP bodies and P-bodies. The latter are found to be sites for repression of translation and mRNA degradation during glucose starvation and the stationary phase. On the other hand, EGP bodies are shown to be analogous to mammalian stress granules. They are described to originate during deprivation of glucose. It is not clear yet if they are the same as P-bodies (Brengues & Parker, 2007; Buchan, *et al*., 2008; Hoyle, *et al*., 2008). In contradiction to what was described before, the current work reveals that in nitrogen starvation Tpk1, Tpk2 and Tpk3 were present all over the cell with Tpk1 and Tpk2 showing additional fluorescence locally. Especially Tpk1 shows a very bright local signal. Future experiments have to declare if the results of the current work are repeatable and if Tpk1 and Tpk2 are localised in a known organelle.

In the future, the exact subcellular localization of the fluorescent signal of Tpk1-GFP, Tpk2-GFP and Tpk3-GFP in both exponential growth and nitrogen starvation can be elucidated by using localisation tags. Nuclear staining can be performed using NucBlue® Live ReadyProbes® Reagent. Co-localisation in the conditions of interest with EGP bodies and P-bodies can be performed as described in Tudisca *et al*. It is also interesting to test for the

research question if the catalytic subunits of PKA are co-localising with the earlier mentioned transporter/eIF2(B)-focus and/or the Sch9-Gap1 focus. This can be examined by engineering strains expressing either Tpk1, Tpk2 or Tpk3 fused with RFP. The strains can then be mated with the strains resulting in the BiFC foci to obtain a strain expressing both an RFP-fusion protein and two fusion proteins with each one fragment of citrine. Ultimately, the strains can be visualised using lasers and detectors for both citrine and RFP.

Studying the subcellular localization of Sch9-RFP and Pkh1-RFP was not successful in the current work. In the future, an optimised fusion construct with a fluorescent protein needs to be engineered. Different fluorophores can be tried in combination with different linker sequences and/or lengths for obtaining a fluorescent signal. An additional strategy can be tagging the protein at the other end (N-terminal versus C-terminal). Via the website of ThermoFisher, a strain expressing a C-terminally GFP-tagged Sch9 can be bought if preferred[27].

The growth recovery after nitrogen starvation experiment was performed in the current work to examine a possible influence of citrine tags and/or auxotrophies of the engineered strains for BiFC. Out of the results, one can conclude that the differences between the growth curves cannot be easily explained. Repetitions of the experiment are needed to define if the results of the current work were obtained by coincidence or not. No clear difference between the auxotrophic and prototrophic strains was observed. In the future, also the other proteins can be tested for their functionality.

The distinct functions and expression levels of the proteins that can potentially explain the observed differences in growth recovery are listed here. Depending on the function of the protein, the extent of the possible deleterious influence of the tag could be declared.

A normal growth of the strains containing a tagged Gap1 was observed in the current work. This is in line with the fact that Gap1 will not be present anymore fast after being present in the complete growth medium, resulting in no possible influence of the tag. *GAP1* is namely induced by starvation for nitrogen, the corresponding protein is thus present in the nitrogen starved condition, whereas it is downregulated after re-addition of amino acids, thus absent after transfer to YPD medium (Donaton, et al., 2003).

The BY4741 Sch9-CC strain showed a higher OD_{600} in the stationary phase compared to BY4741, which could indicate an influence of the tag on the function of Sch9. In literature, it is stated that deleting the Sch9 protein among others results in slow exponential growth, an arrested cycle progression in G1 and an increased survival and entry in the stationary phase[28].

[27] https://clones.thermofisher.com/cloneinfo.php?clone=yeastgfp
[28] www.uniprot.org

This reasoning indicates a possible influence of the tag on the functionality of Sch9. In the future, the functionality of tagged Sch9 protein can for example be tested by observing the cell size of the strain via flow cytometry, as a strain with a non-functional Sch9 results in a decreased cell size. Also observing growth via measuring OD_{600} over time gives information about the Sch9 functionality, since *SCH9* deletion strains show a slower exponential growth.
In the current work, the BY4741 leu^+ met^+ Gcd1-CC and BY4741 leu^+ met^+ Gcd6-CC strains were showing the same growth curve as BY4741, which reaches an OD_{600} way lower than BY4742 leu^+ lys^+. The activity of eIF2B is constrained as a reaction on starvation or stress by phosphorylation of eIF2α/sui2 (Pavitt, 2005). During exponential phase and recovery from stress, the activity of eIF2B is important, so an influence by the tag of Gcd1 or Gcd6 is possible in the conditions studied in the current work. Since deletion of *GCD1* or *GCD6* results in a lethal phenotype and the strains with fusion proteins of Gcd1 and Gcd6 are successfully constructed, one can conclude that proteins Gcd1 and Gcd6 are at least partially functional. For the strains used in the current growth experiment, no clear conclusion can be given of the influence of the tag since it is not clear if there is an influence of the auxotrophies. In a future experiment for studying the functionality of the tagged proteins, the OD_{600} over time in exponential growth cells can be followed, since mutations in Gcd1 and Gcd6 also result in slow growth (Giaever, 2002; Bushman, *et al*., 1993; Abastado, *et al*., 1991; Hinnebusch, 1985; Niederberger, *et al*., 1986).
The influence of tagging Pkh1 or Tpk1 was not studied in the current work. In the future, a strain containing a tagged Pkh1 can be tested for its sensitivity to certain drugs, a. o. fluoconazole, since deleting *PKH1* displays drug sensitivity. Testing the functionality of the catalytic subunits of PKA is harder. It is shown that none of the *TPK* genes is essential, but that at least one of them needs to be present for a normal growth (Toda, *et al*., 1987).

An interaction between Sch9 and Gap1 was observed in the current work via split citrine BiFC in the nitrogen starvation condition. Important to comment is that this technique can result in false positives. This occurs if the two fragments are brought in close proximity (7 nm) due to the presence of the proteins in a small subcellular compartment. This nonspecific complementation is therefore dependent on the proteins and their location (Kerppola, 2006). Without doubt, the next step for proving the interaction of Gap1 with Sch9 is implementing mutation(s) in the protein(s) which disrupt(s) the interaction between the proteins and consequently abolishes the fluorescent signal. This is the only relevant negative control for BiFC. Consequently, site-directed mutagenesis is to be performed in strains. Since no information is available yet about the presence and the site of interaction, this negative control was not directly constructed in the current work. Bio-informatics docking analyses can be performed to predict the site of interaction.

The fluorescence observed in strain BY leu$^+$ Sch9-CC:Gap1-NC was found to be restricted to a certain location in the cell, called the Sch9-Gap1 focus. After staining, it was found to be localized near the nucleus. Notably, the formation of citrine is irreversible. This means that the tagged proteins will be kept together after the formation of the fluorophore. Correspondingly, it is possible that the formation of the fluorophore results in a lower functionality of the proteins. To obtain more information about the location of the Sch9-Gap1 focus co-localisation with the ER and Golgi can be performed. If it co-localises with the organelles mentioned, it indicates that the BiFC focus is observed possibly due to a sorting problem caused by the fluorescent tags. The hypothesis would than be that Sch9 interacts immediately with Gap1 when made in the ER, resulting in the fact that no fluorescence is found in the plasma membrane, where Gap1 normally resides during nitrogen starvation.

Interesting for this research question as well is to get know if Sch9 is generally involved in linking external nutrient sensing to protein synthesis in the FGM pathway, or is only functioning in nitrogen sources. Protein-protein interactions of Sch9 with the different yeast transceptors, being Mep2, Sul1, Pho84, Ftr1 and Zrt1 can be studied with split citrine BiFC.

The subcellular localization of the earlier described transporter/eIF2(B)-foci (BY proto Gcd1-CC:Gap1-NC and BY proto Gcd6-CC:Gap1-NC) seemed to be different from the Sch9-Gap1 foci observed in the current work. Still, Sch9 can be involved in linking Gap1 with Gcd1. Future experiment may reveal if
Sch9 functions together with the eIF2(B)-subunits. Since Gcd6 shows an *in vitro* interaction with Sch9, also BY4741 leu+ met+ Gcd6-CC can be mated with Sch9 for studying the *in vivo* split citrine BiFC interaction. Additionally, to obtain information about Sch9 linking Gap1 and Gcd1, split citrine BiFC interactions between truncated Gap1 and Gcd1 and Sch9 can be studied. If the same truncations disrupt the binding of both Gcd1 and Sch9, one can conclude that the proteins are interacting at the same site at the Gap1 protein. Another way to obtain more information about the site of the interaction between proteins is the peptide mapping experiment[29].

The minority of the strains resulted in fluorescence by split citrine BiFC. In contrary, a lot of interactions were found via GST pull-down. This is expected since BiFC has a very high false negative rate. There are a lot of possible explanations why the fluorescence does not occur. For example, it is possible that the citrine tags of both interacting proteins are too far from each other to reconstitute an active fluorescent protein due to the 3D structure of the proteins of interest. Consequently, a negative result does not imply per definition that the proteins of interest do not interact in the wild type cell. In these cases, no conclusions can be drawn.

[29] www.pepperprint.com

Future experiments with optimised constructs will reveal whether or not there is an interaction between the protein partners. Optimisation is possible by constructing new strains containing another combination of the citrine halves or by constructing strains with the tags at the N-terminus of the proteins.

Without doubt, also other techniques can be used in the future to confirm the protein-protein interaction findings and to reveal the function of the proteins. An *in vitro* technique that is able to show a direct interaction is for example surface plasmon resonance spectroscopy (Ueki, *et al*., 2011).
Another interesting technique for studying *in vivo* protein-protein interactions is Bioluminescence Resonance Energy Transfer (BRET). One protein of interest is fused with Nanoluc® Luciferase (19 kDa), whereas the other one is fused with HaloTag®. One advantage is the small size (19 kDa) of Nanoluc® Luciferase, being smaller than fluorescent proteins used in Förster Resonance Energy Transfer (FRET). Additionally, BRET avoids the consequences of fluorescence excitation, since it is using bioluminescence[30].

In this paragraph, the possible applications of the current work are discussed. The type of research performed in the current work initially aims to make the understanding of processes in the cell more profound. Obtaining elementary knowledge in connecting nutrient sensing with growth in eukaryotic cells leads to important industrial applications, both for medicine and biotechnology.
Firstly, the knowledge mentioned is relevant for diseases in which a disturbed balance is seen in nutrient sensing and growth, including diabetes and cancer. After revealing the equivalent pathways in higher eukaryotes, novel drug targets can be found. The mammalian counterparts of the proteins studied in the current work are also involved in specific human diseases, listed below. As a consequence, information obtained from yeast is possibly useful for increasing the knowledge about the specific diseases. The vanishing white matter disease, being a fatal inherited brain disease, is caused by mutations in any of the eIF2B subunits (Leegwater, et al., 2001). On the other hand, Pkh1 is found to be the homologue of the mammalian PDK1 (Casamayor, *et al*., 1999), being a potential target for tackling Alzheimer's and prion disease, both neurodegenerative diseases (Checler, 2013). Sch9 is a yeast homologue of mammalian PKB (Voordeckers, et al., 2011), involved in among others the development of type 2 diabetes mellitus (Dufour & Clavien, 2009). Tpk1 in yeast is conserved in mammals as the cAMP-dependent protein kinase (Denis, *et al*., 1991). For example, the type 1 alpha-regulatory subunit of this mammalian kinase is involved in the Cushing's syndrome (Sahut-Barnola, et al., 2010). Additionally, PKA and Sch9 are promoting longevity through regulation of autophagy-

[30] http://pubs.acs.org/doi/abs/10.1021/acschembio.5b00143

related genes and pro-aging sphingolipids like ceramide (Hernández-Corbacho, *et al*., 2011; Sampaio-Marques, *et al*., 2011). Consequently, applications in extending lifespan are evident. Secondly, the elementary knowledge about nutrient sensing is valuable for biotechnology. Superior industrial yeast strains can be optimised for nutrient-dependent control of growth, for example leading to a higher speed and yield of fermentation. To stress the industrial importance of this subject, it is relevant to mention that a patent is filed for the use of the nutrient transporter-eIF2B/eIF2 complex (EP15183895.0. Filing date: 4 Sept. 2015 at EPO).

How the protein interaction findings of the current work can be connected with literature and our research question is stated below.

As mentioned (s*ection B.3.2.2.*), translation initiation can be regulated via eIFs or ribosomes, inducing changes on initiation overall, or via mRNAs, potentially on the contrary only targeting specific mRNA subsets. In the current work, there is given attention to eIFs, leading to a general response.

To rehearse, GTP-bound eIF2 is required for translation initiation, since it interacts with the initiator methionyl tRNA, resulting in the formation of the ternary complex, the rate-limiting step of initiation. eIF2B, including Gcd1 and Gcd6, are catalysing the activation of eIF2, being GTP-bound eIF2. It is hypothesised that a kinase would be able to regulate translation initiation if it phosphorylates eIF2B. A prerequisite of this hypothesis is that phosphorylation of eIF2B results in activation. In the mammalian eIF2Bε, corresponding with Gcd6 in yeast, a mutation of site Ser525 results in abolishing the regulation of eIF2B and protein synthesis by amino acids. Consequently, it seems that phosphorylation at that site is crucial for the function for eIF2B (Xuemin & Christopher, 2008) . This finding supports the stated prerequisite of the hypothesis of the current work.

As stated in the literature section, Sch9 is required in nitrogen starved cells for PKA activation by amino acid and ammonium re-addition. Additionally, Sch9 is activating PKA when cells are glucose-repressed, whereas acts as an inhibitor of PKA in glucose-derepressed cells. Since translation initiation is also induced in the conditions where Sch9 is activating PKA, Sch9 is a good candidate as kinase for directly regulating eIF2B by phosphorylation. Another option is that active PKA, being free Tpk1, Tpk2 and/or Tpk3, is phosphorylating eIF2B. Implicating that Sch9 is indirectly regulating eIF2B. Also the existence of both direct and indirect regulation of eIF2B by Sch9 is not excluded.

The Pkh1 kinase is proven to regulate both Sch9 and PKA activity by phosphorylation. If the above suggestions are correct, Pkh1 is thus indirectly regulating eIF2B. However, the *in vitro* GST pull-down experiments in the current work show an interaction between both Pkh1, Sch9 and Tpk1 with eIF2B, suggesting that they act in parallel. Taking into account the limitations of GST pull-down, the hypothesis of Pkh1 indirectly regulating eIF2B is not ruled out.

While interpreting the results, one should take into account that evidence is found for the fact that translation initiation factors themselves are inducing PKA activity. In particular, eIF2 and eIF2B seem to be involved in PKA signalling, since mutations in eIF2 and eIF2B affect amino acid-induced activation of the FGM pathway. Moreover, the kinase Gcn2, an inhibitor of eIF2, is found to be required for start-up of growth after re-addition of certain amino acids to cells starved for nitrogen. Additionally, it is shown *in vitro* that Pkh1 is able to phosphorylate Gcn2, possibly enhancing translation levels. Most probably, some feedback regulations make the interpretations harder.

The fact that in the current work also *in vitro* interactions are seen between the kinases Sch9, Pkh1 and Tpk1 and both Gap1 and eIF2B, strengthen the hypothesis for linking nutrient sensing to translation initiation in the FGM pathway. The interaction between Gap1 and Sch9 is confirmed by the *in vivo* split citrine BiFC, however additional control experiments are required to make this conclusion strong. Notably, future research will have to demonstrate whether the physical interactions between the proteins are also found to be regulatory, elucidating the molecular mechanisms.

Interesting to discuss as well is that another suggested level of protein synthesis regulation via eIFs in yeast is the subcellular localization of eIF2 and eIF2B. Under stress conditions, both factors are localized in a defined region of the cytoplasm. It is suggested that nucleotide exchange is happening in that region to generate active eIF2 factors. Also the BiFC interaction between the transceptors and eIF2(B) is found to be present in a specific unidentified region within the cytoplasm. To rehearse, this has lead to the identification of the ‘startosome’, a putative novel membrane system. In the current work, Sch9 and Gap1 also show a BiFC focus, being bigger compared to the transporter/eIF2(B)-focus and is always localized close to the nucleus. Future experiments should reveal if the two foci are correlated, if Sch9 is linking eIF2(B) with Gap1 and if Sch9 is also involved in the ‘startosome’.

To summarise, in the current work more information is obtained about linking nutrient availability to cell growth. The results of the *in vitro* and *in vivo* experiments indicate that several kinases might be involved in the proposed nutrient signalling. The *in vitro* GST pull-down experiments show interaction between Gap1 and the kinases Sch9, Pkh1 and Tpk1. These kinases moreover also show an interaction with at least one eIF2B subunit. Especially the interactions between Tpk2 and Tpk3 with Gap1, Gcd1 and Gcd6 need to be investigated in the future. The interaction between Gap1 and Sch9 is confirmed by the *in vivo* split citrine BiFC. Noteworthy, repetitions of the experiments are needed to confirm the findings. For the *in vivo*

BiFC, a relevant negative control for the interaction between Sch9 and Gap1 in the *in vivo* experiment needs to be executed.

The current study resulted in an improved understanding of the connection between translation initiation in S*. cerevisiae* and amino acid induced activation of growth via protein kinase A. However, many new questions have been raised and several questions remain. Future research will have to demonstrate whether the physical interactions between the proteins are also found to be regulatory, elucidating the molecular mechanisms behind start-up of growth.

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Addendum

1. Risk Assessment

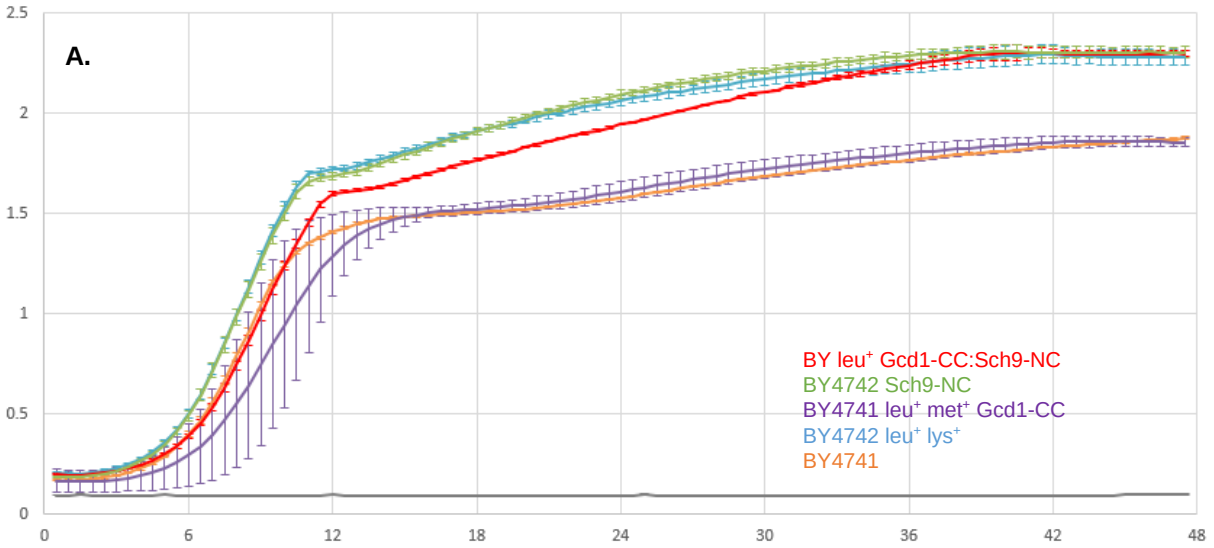
When working in the laboratory, personal protective equipment is indispensable. A laboratory coat is worn to protect the skin and personal clothing. It is used as a removable barrier in case of splash or spill. Before leaving the laboratory, hands are washed with soap. Additionally, gloves are worn in cases where the chemical is causing skin corrosion, burns and/or irritation. Gloves are also obligatory for compounds which have presumably genetic altering and cancer inducing characteristics. The hazardous chemicals which can cause headache, are deadly upon inhalation or have a pungent odour, are used in the laboratory fume hood to minimize their exposure and inhalation. The special precautions are taken for the substances listed in the table. All substances used in the current work are disposed according to the regulations to minimise the impact on the environment.

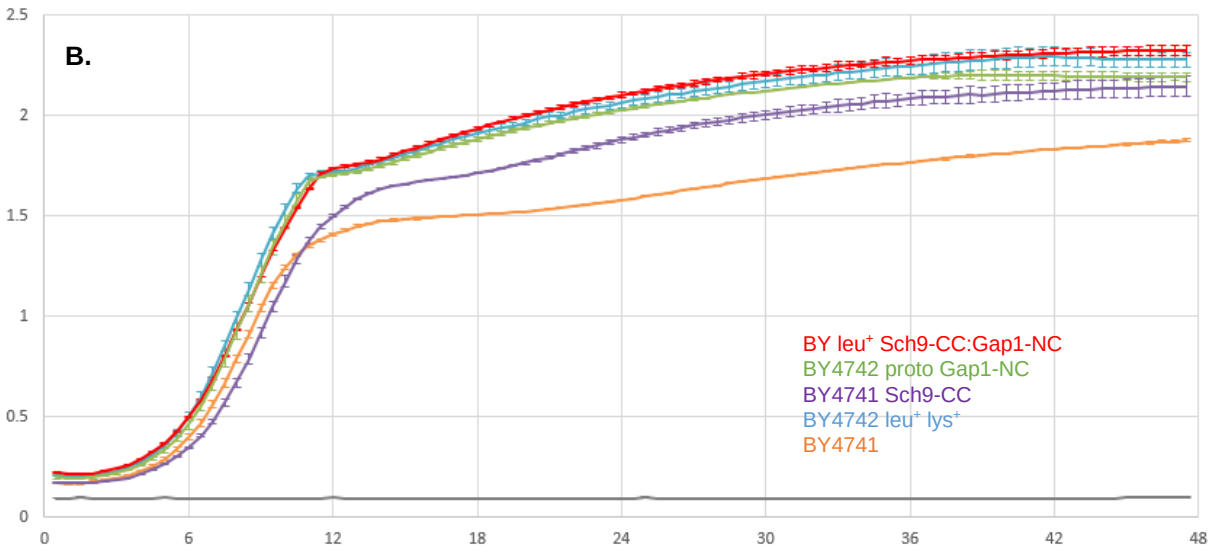
Acetic acid	Can cause skin corrosion and serious eye damage.
HCl	Can cause severe skin burns, serious eye damage and respiratory irritation.
Liquid nitrogen	Can cause cold burns and blurred vision. When the container is exposed to heat, it may explode or rupture. → Special safety requirements: wear insulating gloves and safety glasses.
Methanol	Can cause skin irritation, headache and blindness. Liquid and vapour are highly inflammable.
NaOH 4 M	Can cause severe skin burns, serious eye damage and respiratory irritation. After ingestion it will cause burns of the mouth, stomach and gastrointestinal tract.
NuPAGE® Bis-Tris 4-12 % gel	Since the packaging buffer includes residual acrylamide monomer and 0.02 % sodium azide, gloves are worn.
Phenol/Chloroform/Isoamyl Alcohol 25:24:1 v/v pH 8.0 (PCI)	Can cause severe skin burns and eye damage. Deadly upon inhalation, toxic upon swallowing. Presumably genetic altering and cancer inducing characteristics. Systemic toxicity if repeated exposure. → Special safety requirements: store in a closed container.
Sodium dodecyl sulphate (SDS)	Can cause skin and eye irritation. Harmful upon swallowing.
SYBR® Safe DNA Gel Stain 10,000X	Can cause respiratory irritation. Presumably genetic altering and cancer inducing characteristics.
β-Mercaptoethanol	Can cause severe skin and eye irritation. Pungent odour.

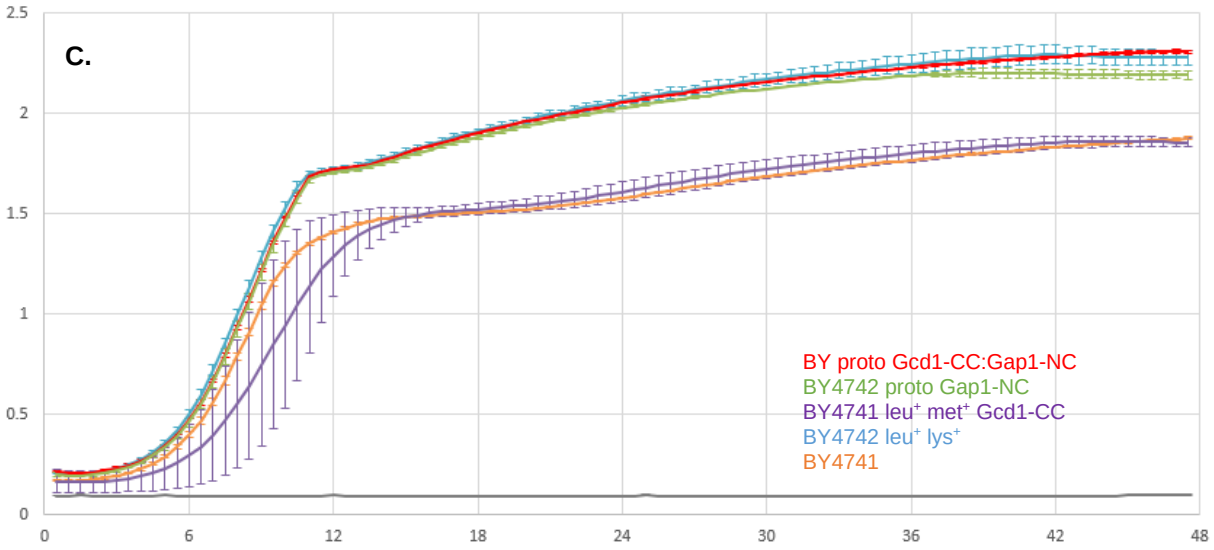
The *S. cerevisiae* and *E. coli* strains used in the current work are classified as Class of Risk I according to the Belgian classification for micro-organisms, due to the minimal biological risk of the laboratory strains. The Genetically Modified Organisms created and used in the current work contain a vector and insert which does not confer a phenotype which is able to cause a disease in man, animal or plant or is damaging the environment. Consequently, also the modified strains are classified as Risk Class I of the GMO regulation. As a consequence, containment level 1 is sufficient for all strains used, meaning that the basic laboratory practices and facilities are adequate for providing safety to men and environment. The cells are disposed in a container for hazardous medical waste or inactivated by autoclaving.

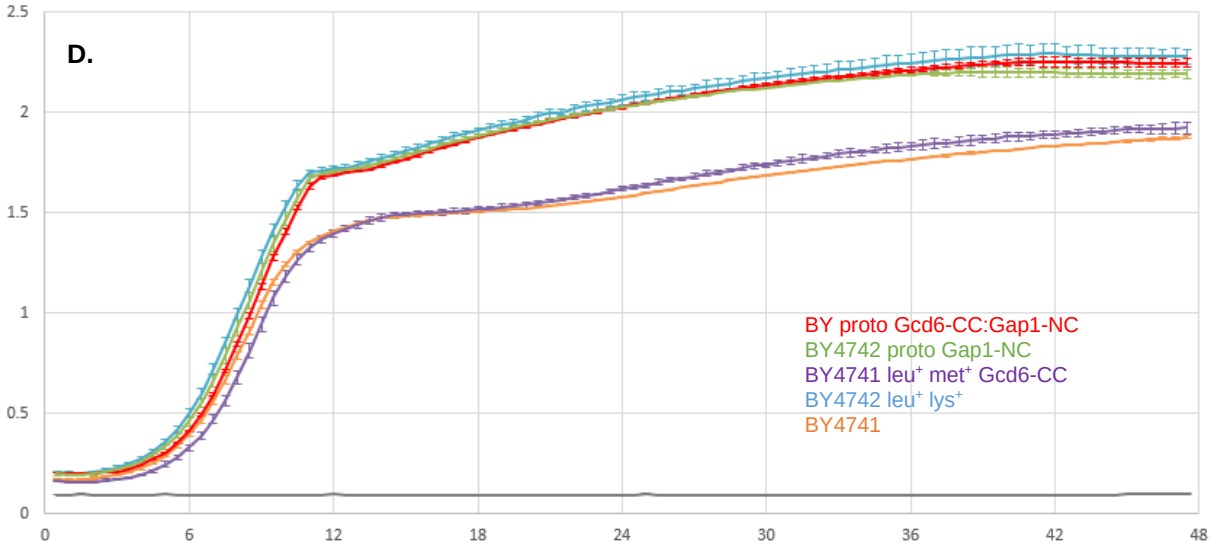
2. Supplementary Figure and Tables

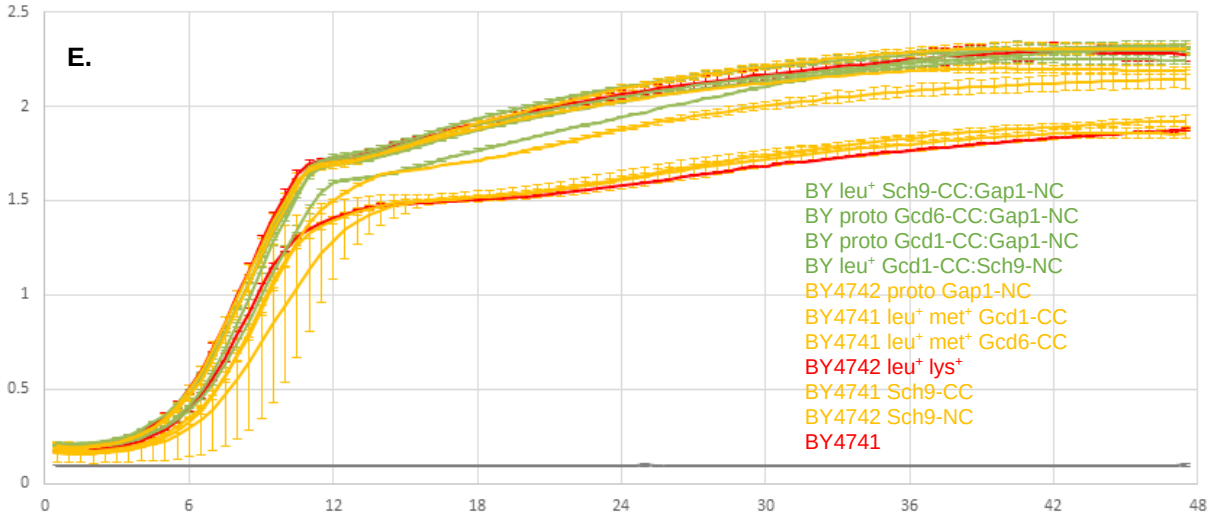
Suppl. Figure 1: Growth recovery of nitrogen starved cells on rich medium: The OD_{600} was followed over time (in hours). In all graphs, the negative control is given in grey, being YPD without cells. The error bars depicted in all graphs are standard deviations achieved from two technical replicates. These are obtained by inoculating the same growth culture twice in a separate well of a 24-well plate. All graphs are obtained from the same experiment, but results are graphically given separately for clarity.

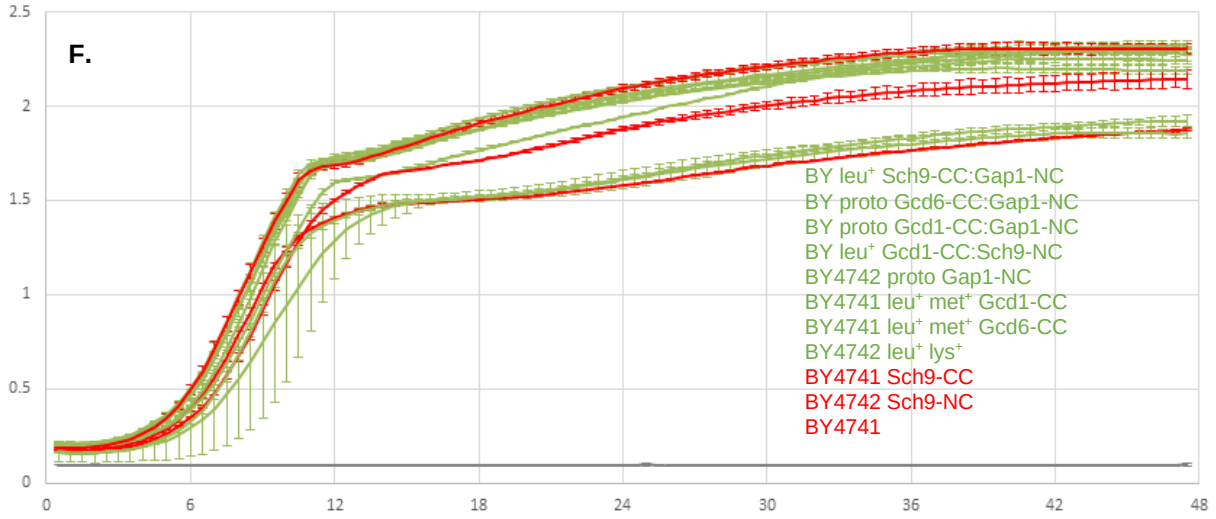












Panel **A**: BY leu$^+$ Gcd1-CC:Sch9-NC vs. its parents vs. references. Panel **B**: BY leu$^+$ Sch9-CC:Gap1-NC vs. its parents vs. references. Panel **C**: BY proto Gcd1-CC:Gap1-NC vs. its parents vs. references. Panel **D:** BY proto Gcd6-CC:Gap1-NC vs. its parents vs. references. Panel **E**: Reference strains (all red) vs. strains expressing one fusion construct (all orange) vs. strains co-expressing two fusion constructs (all green). Panel **F**: Strains at least prototrophic for leucine (all green) vs. auxotrophic (all red) strains.

Suppl. Table 1: The composition of *S. cerevisiae* and *E. coli* media.

Medium	Composition
SC medium = Synthetic complete medium	0.079 % (w/v) CSM (MP) 0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 0.5 % (w/v) ammonium sulphate (Sigma-Aldrich) 2 % (w/v) D-(+)glucose In Milli-Q
SC –his medium = Single drop-out of synthetic complete medium, w/o L-histidine	0.077 % (w/v) CSM –HIS (MP) 0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 0.5 % (w/v) ammonium sulphate (Sigma-Aldrich) 2 % (w/v) D-(+)glucose In Milli-Q
SC –ura medium = Single drop-out of synthetic complete medium, w/o uracil	0.077 % (w/v) CSM –URA (MP) 0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 0.5 % (w/v) ammonium sulphate (Sigma-Aldrich) 2 % (w/v) D-(+)glucose In Milli-Q
SC –his –ura medium = Drop-out of synthetic complete medium, w/o L-histidine and uracil	0.075 % (w/v) CSM –HIS -URA (MP) 0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 0.5 % (w/v) ammonium sulphate (Sigma-Aldrich) 2 % (w/v) D-(+)glucose In Milli-Q
SC –met medium = Drop-out of synthetic complete medium, w/o methionine and lysine	0.077 % (w/v) CSM –MET (MP) 0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 0.5 % (w/v) ammonium sulphate (Sigma-Aldrich) 2 % (w/v) D-(+)glucose In Milli-Q
SC –lys medium = Drop-out of synthetic complete medium, w/o methionine and lysine	0.074 % (w/v) CSM –lys (MP) 0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 0.5 % (w/v) ammonium sulphate (Sigma-Aldrich) 2 % (w/v) D-(+)glucose In Milli-Q

SC –leu medium = Single drop-out of synthetic complete medium, w/o leucine	0.069 % (w/v) CSM –LEU (MP) 0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 0.5 % (w/v) ammonium sulphate (Sigma-Aldrich) 2 % (w/v) D-(+)glucose In Milli-Q
YPD medium = yeast extract peptone dextrose medium	1 % (w/v) yeast extract granulated (Merck) 2 % (w/v) bacteriological peptone (OXOID) 2 % (w/v) D-(+)glucose In demi-H_2O
YPG medium = yeast extract peptone galactose medium	1 % (w/v) yeast extract granulated (Merck) 2 % (w/v) bacteriological peptone (OXOID) 2 % (w/v) galactose In demi-H_2O
NSM = Nitrogen Starvation Medium	0.17 % (w/v) Bacto-yeast nitrogen base without amino acids & without ammonium sulphate (Difco) 4 % (w/v) D-(+)glucose In Milli-Q
SPM = Sporulation medium	1 % (w/v) KAc (Riedel-de Häen) 0.05 % (w/v) $KHCO_3$ (Sigma-Aldrich) In Milli-Q
Terrific Broth Medium	1.2 % (w/v) tryptone 2.4 % (w/v) yeast extract 0.4 % (w/v) glycerol 0.231 % (w/v) KH_2PO_4 1.645 % (w/v) $K_2HPO_4.3H_2O$ In demi-H_2O
LB medium = Luria-Bertani medium	1 % (w/v) Bacto-tryptone (OXOID) 0.5 % (w/v) yeast extract (Merck) 1 % (w/v) NaCl (Fisher Scientific) In demi-H_2O

Suppl. Table 2: The content of PCR reaction mixtures: the reaction mixture with the TaKaRa Ex Taq™ DNA Polymerase on the left and the one with Q5® High-Fidelity DNA Polymerase on the right.

Reagens	**Final Concentration**	**Reagens**	**Final Concentration**
10X TaKaRa Ex Taq™ Reaction Buffer (incl. 20 mM Mg^{2+} plus)	1X	5X Q5® Reaction Buffer	1X
dNTPs	200 µM each	dNTPs	200 µM each
Forward Primer	0.4 µM	Forward Primer	0.5 µM
Reverse Primer	0.4 µM	Reverse Primer	0.5 µM
Template DNA	0.05-10 ng	Template DNA	< 1,000 ng
TaKaRa Ex Taq™ DNA Polymerase	0.025 U/µl	Q5® High-Fidelity DNA Polymerase	0.020 U/µl
Nuclease-Free Water		Nuclease-Free Water	

Suppl. Table 3: Solutions used in DNA Agarose Gel Electrophoresis.

Solution	**Composition**
TAE buffer 10X	0.4 M Tris pH 8.0 0.2 M acetic acid 10 mM EDTA pH 8.0
Bromophenol Blue DNA Loading Dye 6X	0.25 % (w/v) bromophenol blue 0.25 % (w/v) xylene cyanol FF 30 % glycerol

Suppl. Table 4: The thermocycling conditions used for sequencing and verification of correct genomic integration with TaKaRa Ex Taq™ DNA Polymerase. The temperature of the lid is set at 90 °C. The annealing temperature is decided based on the primer pair used.

Step	**T (°C)**	**Time (s)**
Initial denaturation	94	30
Denaturation	94	20
Annealing 30 x	**x**	30
Extension	72	240
Final extension	72	600
Hold	10	∞

Suppl. Table 5: The content of the PCR reaction mixture for mating type PCR with the TaKaRa Ex Taq™ DNA Polymerase.

Reagens	**Final Concentration**
10X TaKaRa Ex Taq™ Reaction Buffer (incl. 20 mM Mg^{2+} plus)	1X
dNTPs	200 µM each
Primer 1648	0.2 µM
Primer 1649	0.2 µM
Primer 1650	0.2 µM
TaKaRa Ex Taq™ DNA Polymerase	0.025 U/µl
Nuclease-Free Water	

Suppl. Table 6: The three primers used for mating type PCR [Sequence: see *Suppl. Table 16*].

Primer n°	**Locus**
1648	Corresponds with sequence at the right of and directed towards the *MAT* locus
1649	Corresponds with sequence within the α-specific DNA located at *MAT*α and *HML*α
1650	Corresponds with sequence within the a-specific DNA located at *MAT*a and *HMR*a

Suppl. Table 7: The thermocycling conditions used for the mating type PCR with TaKaRa Ex Taq™ DNA Polymerase. The temperature of the lid is set at 90 °C.

Step		**T (°C)**	**Time (s)**
Initial denaturation		95	240
Denaturation	30 x	92	45
Annealing		58	45
Extension		72	30
Final extension		72	600
Hold		10	∞

Suppl. Table 8: Specifications of strains containing an HA-tagged protein of interest: their name, the procedure used for constructing them, the resulting position of the HA-tag relative to the protein of interest and the growth medium that is used for the respective strains [info plasmids: *see Suppl. Table 17*].

Name	Procedure of Construction	Position of HA-tag	Growth Medium
Sigma GCD6-HA	HA-tag intragenomically fused to Gcd6 protein, created by homologous recombination in Sigma 1278b after transforming PCR product obtained from plasmid pFA6a-*3HAX-KanMX6*	C-terminal of protein of interest	YPD
Sigma GCD1-HA	HA-tag intragenomically fused to Gcd1 protein, created by homologous recombination in Sigma 1278b after transforming PCR product obtained from plasmid pFA6a-*3HAX-KanMX6*	C-terminal of protein of interest	YPD
Sigma GAP1-HA	Strain Sigma 1278b *Matα ura GAP1*::*KanMX* (JT20867) transformed with plasmid pFL38-*HA-GAP1* containing gene coding for the Gap1 protein adjacent to the HA-tag	N-terminal of protein of interest	SC –ura & NSM

Suppl. Table 9: Solutions used in *in vitro* GST pull-down.

Solution	**Composition**
Yeast Lysis Buffer	1X PBS 8.7 % glycerol 0.1 % Triton X-100 2.5 mM $MgCl_2$ 1 mM EDTA pH 8.0 2 mM DTT (1,4-dithiothreitol) 10 mM NaF 0.4 mM $Na_3VO_4^-$ 0.1 mM β-glycerol phosphate 1 mM PMSF (phenylmethylsulfonyl fluoride) In Milli-Q
E. coli Wash Buffer	1X PBS 0.1 %Triton X-100 2 mM $MgCl_2$ 1 mM EDTA pH 8.0 1 mM DTT In Milli-Q
E. coli Lysis Buffer	1X PBS 0.4 % Triton X-100 2 mM $MgCl_2$ 1 mM EDTA pH 8.0 2 mM DTT 0.2 % (w/v) lysozyme 1 mM PMSF In Milli-Q
PBS-T	1X PBS 0.1 % Triton X-100 In Milli-Q
Binding Buffer	1X PBS 0.05 % Triton X-100 0.1 mM DTT In Milli-Q

PBS (pH 7.3) 10X	1.4 M NaCl 27 mM KCl 0.1 M Na_2HPO_4 0.1 M KH_2PO_4 In demi H_2O
SDS Sample Buffer 4X	250 mM Tris-HCl pH 6.8 40% β-mercaptoethanol [Risk Assessment: see *Addendum 1*] 10 % SDS [Risk Assessment: see *Addendum 1*] 0.008 % bromophenol blue 40 % glycerol In demi H_2O
Running Buffer	5% NuPAGE® MOPS SDS Running buffer (1X) (Invitrogen™) In demi H_2O
Blotting Buffer	5% NuPAGE® MOPS SDS Running buffer (1X) (Invitrogen™) 20 % methanol [Risk Assessment: see *Addendum 1*] In demi H_2O
Blocking Buffer	5 % (w/v) milk powder In TBS-T
Antibody Buffer	2.5 % (w/v) milk powder 1:000 Rat monoclonal Anti-HA-Peroxidase High Affinity clone 3F10 (Roche Applied Science) In TBS-T
Coomassie Solution	0.25 % Coomassie Brilliant Blue 30 % methanol [Risk Assessment: see *Addendum 1*] 10 % acetic acid [Risk Assessment: see *Addendum 1*] In demi H_2O
Destain Solution	30 % methanol [Risk Assessment: see *Addendum 1*] 10 % acetic acid [Risk Assessment: see *Addendum 1*] In demi H_2O
TBS-T (pH 8.0)	0.15 M NaCl 0.025 M Tris 0.005 % Tween-20 In demi H_2O

Suppl. Table 10: The thermocycling conditions used for PCR for fluorescent tagging with Q5® High-Fidelity DNA Polymerase. The temperature of the lid is set at 90 °C. The annealing temperature is decided based on the primer pair used.

Step	T (°C)	Time (s)
Initial denaturation	94	240
Denaturation	98	20
Annealing 30 x	x	30
Extension	72	210
Final extension	72	300
Hold	10	∞

Suppl. Table 11: The thermocycling conditions used for PCR for fluorescent tagging with TaKaRa Ex Taq™ DNA Polymerase. The temperature of the lid is set at 90 °C. The annealing temperature is decided based on the primer pair used.

Step	T (°C)	Time (s)
Initial denaturation	98	30
Denaturation	98	10
Annealing 30 x	**x**	30
Extension	72	180
Final extension	72	600
Hold	10	∞

Suppl. Table 12: Primers used for verifying the integration of the protein fusion cassette at the correct place in the genome by PCR for the strains containing one fusion protein [Sequence: see *Suppl. Table 16*].

Resulting Strain	F Primer	R Primer
BY4741 Tpk1-GFP	A769	A3509
BY4741 Tpk2-GFP	5290	A3509
BY4741 Tpk3-GFP	A2988	A3509
BY4741 Sch9-RFP	A2761	A3509
BY4741 Pkh1-RFP	A1848	A3509
BY4741 Tpk1-CC	A769	A2098
BY4741 Tpk2-CC	5290	A2100
BY4741 Tpk3-CC	A2988	4366
BY4741 Pkh1-CC	A1848	A1846
BY4741 Sch9-CC	A2761	A1631
BY4741 Gap1-CC	B3354	A2697
BY4741 Sul1-CC	A3350	A3351
BY4741 Mep2-CC	6593	A91
BY4742 Tpk1-NC	A769	4054
BY4742 Tpk2-NC	5290	4054
BY4742 Tpk3-NC	5276	4054
BY4742 Pkh1-NC	A1848	4054
BY4742 Sch9-NC	A2761	4054
BY4742 Gcd1-NC	A2915	4054

Suppl. Table 13: Primers used for verifying the integration of the protein fusion cassette at the correct place in the genome by sequencing for the strains containing one fusion protein [Sequence: see *Suppl. Table 16*].

Resulting Strain	F Primer	R Primer
BY4741 Tpk1-GFP	7052	A3509
BY4741 Tpk2-GFP	A1258	A3509
BY4741 Tpk3-GFP	A2987	A3509
BY4741 Sch9-RFP	A2761	A3509
BY4741 Pkh1-RFP	A1644	A3509

Suppl. Table 14: Primers used for verifying the integration of the protein fusion cassette at the correct place in the genome by PCR of strains made by the procedure of mating two haploid strains, subsequently inducing sporulation, performing tetrad dissection and selecting for the wanted traits [Sequence: see *Suppl. Table 16*].

	C-terminal Tagged Gene of Interest		N-terminal Tagged Gene of Interest	
	F Primer	R Primer	F Primer	R Primer
BY leu$^+$ Gcd1-CC:Tpk1-NC	B8383	B6503	A769	4054
BY leu$^+$ Gcd1-CC:Tpk2-NC	B8383	B6503	5290	4054
BY leu$^+$ Gcd1-CC:Tpk3-NC	B8383	B6503	5276	4054
BY leu$^+$ Gcd1-CC:Pkh1-NC	B8383	B6503	A1848	4054
BY leu$^+$ Gcd1-CC:Sch9-NC	B8383	B6503	A2761	4054
BY leu$^+$ Tpk1-CC:Gap1-NC	A769	A2098	B3354	A2697
BY leu$^+$ Tpk2-CC:Gap1-NC	5290	A2100	B3354	A2697
BY leu$^+$ Tpk3-CC:Gap1-NC	A2988	4366	B3354	A2697
BY leu$^+$ Pkh1-CC:Gap1-NC	A1848	A1846	B3354	A2697
BY leu$^+$ Sch9-CC:Gap1-NC	A2761	A1631	B3354	A2697
BY leu$^+$ Gap1-CC	B3354	A2697	/	/
BY Mep2-CC:Gap1-NC	6593	A91	B3354	A2697

Suppl. Table 15: Primers used for verifying the integration of the protein fusion cassette at the correct place in the genome by sequencing of strains made by the procedure of mating two haploid strains, subsequently inducing sporulation, performing tetrad dissection and selecting for the wanted traits [Sequence: see *Suppl. Table 16*].

	C-terminal Tagged Gene of Interest		**N-terminal Tagged Gene of Interest**	
	F Primer	**R Primer**	**F Primer**	**R Primer**
BY leu⁺ Gcd1-CC:Tpk1-NC	B8383	B6503	7052	A2098
BY leu⁺ Gcd1-CC:Tpk2-NC	B8383	B6503	A1258	A2100
BY leu⁺ Gcd1-CC:Tpk3-NC	B8383	B6503	A2987	4366
BY leu⁺ Gcd1-CC:Pkh1-NC	B8383	B6503	A1644	A1846
BY leu⁺ Gcd1-CC:Sch9-NC	B8383	B6503	A2761	A1631
BY leu⁺ Tpk1-CC:Gap1-NC	7052	A2098	B3354	A2697
BY leu⁺ Tpk2-CC:Gap1-NC	A1258	A2100	B3354	A2697
BY leu⁺ Tpk3-CC:Gap1-NC	A2987	4366	B3354	A2697
BY leu⁺ Pkh1-CC:Gap1-NC	A1644	A1846	B3354	A2697
BY leu⁺ Sch9-CC:Gap1-NC	A2761	A1631	B3354	A2697

Suppl. Table 16: Sequences of all primers used in the current work.

Primer n°	**Sequence**
1648	AGTCACATCAAGATCGTTTATGG
1649	GCACGGAATATGGGACTACTTCG
1650	ACTCCACTTCAAGTAAGAGTTTG
4054	CAGTGACACCATGAGCATTGG
4366	GGCACCCATATGTCCATTGT
5276	GAACGCCGAATTGAAGTTCC
5290	CCAGATTATGAGAACGCTTGG
6593	CTGGTGCAGGATGTAACCTT
7052	ATCTTGCTTGATAAAAACGGG
A1258	TTGGGTTCGCCAAAGAGGTAC
A1631	CTCACTTATGGCATCCC
A1644	CGAGCCTCCTACTGATAGTGC
A1846	ACCTGACTCGGTGATGGACA
A1848	GATGTTGTCGACCCAGCTAC
A2098	TGAGAATGTGCTTGGTCCTG
A2100	ATCGAGGGATTTTGGACCTT
A2697	CGCAGTGAAAGTTAATAACG
A2761	GGTGCCATTGATGATGGAAGAG

A2915	CAATGTCAAGACAAGCTTCATTC
A2987	CAGAGGTATGCTTAGCGTTG
A2988	GAACCATCCGTGGTTTAACG
A3350	TTTGCCGGAATTATATCTCCATG
A3351	CATGTCTTTTCAGCGCCTTAAAC
A3499	TCAAGGTGAAGACCCATATGCTGATCTTTTCCGGGACTTCCGGATCCCCGGGTTAATTAA
A3500	AATATAGATACGAGAGGAAAATACAACAAAACATTAGTCAGAATTCGAGCTCGTTTAAAC
A3501	TCAAGGCGATGATCCATATGCTGAATACTTTCAAGATTTCCGGATCCCCGGGTTAATTAA
A3502	GTACTTGAAAATTGTTTTTGTGTTTTTTGGTTCATGGAACGAATTCGAGCTCGTTTAAAC
A3503	TCAAGGGGAGGATCCATATATGGATTTAATGAAAGAATTTCCGGATCCCCGGGTTAATTAA
A3504	TTTTTTTTTTTTTCAATTACAATTATCCCACTGAACCTCCGAATTCGAGCTCGTTTAAAC
A3509	CCGTCAGCCAGTTTAGTCTGA
A769	CATTCAACAGGGACAAGGTGAC
A91	TGGACCAAGCCTTGTTTGTG
B2642	GGCAATTATGGCCACAAAGCCAAGATGGTATAGAATCTGGAATTTCTGGTGTGGTGACGGTGCTGGTTTA
B2643	ATGATTATCTAAAAAATAAAGTCTTTTTTTGTCGTTGTTCGATTCATTATCGATGAATTCGAGCTCG
B2668	GTGAATATACCGACGAGTACGAGTACGAAGATGACGGATTATTTGAGCGTGGTGACGGTGCTGGTTTA
B2669	ACTTACTTGGTCTCTATTAAAGAGACTGAAGGAATATACATAAGTTTATATCGATGAATTCGAGCTCG
B2674	ACGGTGTTCAAGGTGAAGACCCATATGCTGATCTTTTCCGGGACTTCGGTGACGGTGCTGGTTTA
B2675	AAAAAAAAAAAATATAGATACGAGAGGAAAATACAACAAAACATTAGTCATCGATGAATTCGAGCTCG
B2676	ATTATGGTATTCAAGGCGATGATCCATATGCTGAATACTTTCAAGATTTCGGTGACGGTGCTGGTTTA
B2677	GTGAGAGAAAGTACTTGAAAATTGTTTTTGTGTTTTTTGGTTCATGGAACTCGATGAATTCGAGCTCG
B2678	ACTATGGAATTCAAGGGGAGGATCCATATATGGATTTAATGAAAGAATTTGGTGACGGTGCTGGTTTA
B2679	TTTTATTGATTTTTTTTTTTTTTCAATTACAATTATCCCACTGAACCTCCTCGATGAATTCGAGCTCG
B2682	TGGCAAGATCGACACAAATGCGGAAAAACATGACACGGACAGATGAAAAAGGTGACGGTGCTGGTTTA
B2683	CCCTTCACCATGTCTTACATATGCATATATATATATTATCAAGCACAGTTTCGATGAATTCGAGCTCG

B2702	ACGGCGACCAACACATGGATGACGAATTTGTCAGTGGAAGATTCGAAATAGGTGACGGTGCTGGTTTA
B2703	ATAAAAAGAAAAGGAAAAGAAGAGGAAGGGCAAGAGGAGCGATTGAGAAATCGATGAATTCGAGCTCG
B3354	CCTTCCCACTTGTTATGGTT
B4179	CCCTTCACCATGTCTTACATATGCATATATATATATTATCAAGCACAGTTGAATTCGAGCTCGTTTAAAC
B4180	TGGCAAGATCGACACAAATGCGGAAAAACATGACACGGACAGATGAAAAACGGATCCCCGGGTTAATTAA
B6503	GAAGCAACTGCGCCATTG
B7168	TCATGAAAACATTGATGATAAGATTGTGGGTAACACAGACGCAGAAAAGAATTCTACGCCTTCCGACGCTTCTTCTACTAAGAACACTGACCATATAGTAGGTGACGGTGCTGGTTTA
B7169	TATCAAAAAAAGAAAATATATCAAATATTAAAAATAAAATTATTAAATGAAGCGTTACATAAAGATTAAACATAAAATCATAGTCTGCTTGAGTATATCATCGATGAATTCGAGCTCG
B7357	AAGCACATCTTACAGTAACTATGAAACATTATGTGCTGCAACTGGGACAAATTTACCGTTTTTTCATATCGATATACCCGATTTTTCTAAATGGGACGTTGGTGACGGTGCTGGTTTA
B7358	ATTCCAGCTATTTGATGGTAATCTACATTAATAACACAATTATCTTTTCATGTATAACTAAAAACAGGGGGGGTAATTAAAGTACTTACTATCTACTACATCGATGAATTCGAGCTCG
B7605	ACGGCGACCAACACATGGATGACGAATTTGTCAGTGGAAGATTCGAAATACGGATCCCCGGGTTAATTAA
B7606	ATAAAAAGAAAAGGAAAAGAAGAGGAAGGGCAAGAGGAGCGATTGAGAAAGAATTCGAGCTCGTTTAAAC
B8383	ATACAGATTCAATCTGCCGC

Suppl. Table 17: Specifications of all plasmids used: their name, their application, the antibiotic or prototrophy used for their selection and their origin.

Name	Application	Selection	Source
pFL39 *GAL1 HO KanMX* (plasmid n° 555)	Mating type switch	Geneticin	(Connolly, *et al*., 1988)
pFA6a-*3HAX-KanMX6*	Construction of strain with fusion protein with HA-tag	Geneticin	(Longtine, et al., 1998)
pFL38-*HA-GAP1*	Expression of HA-tagged Gap1 protein	Uracil drop-out	MCB lab
pGEX-4T-1 Pkh1-GST	Expression of GST-tagged Pkh1 protein	Ampicillin	MCB lab
pGEX-4T-1 Sch9-GST	Expression of GST-tagged Sch9 protein	Ampicillin	MCB lab
pGEX-4T-1 Tpk1-GST	Expression of GST-tagged Tpk1 protein	Ampicillin	MCB lab
pGEX-4T-1 Tpk2-GST	Expression of GST-tagged Tpk2 protein	Ampicillin	MCB lab
pGEX-4T-1 Tpk3-GST	Expression of GST-tagged Tpk3 protein	Ampicillin	MCB lab
pGEX-4T-1 Sui3-GST	Expression of GST-tagged Sui3 protein	Ampicillin	MCB lab
pGEX-4T-1	Expression of GST protein	Ampicillin	GE Healthcare Life Sciences
γN-*URA* (pFA6a–link–split-yECitrine–*caURA3*)	Construction of strain with fusion protein with first 155 amino acids of citrine (N-terminus)	Uracil drop-out	Geovani Lopez Ortiz (Mexico City, Mexico)
γC-*HIS* (pFA6a–link–split-yECitrine–*SpHIS5*)	Construction of strain with fusion protein with amino acids 155-238 of citrine (C-terminus)	Histidine drop-out	Geovani Lopez Ortiz (Mexico City, Mexico)
pFA6a-*RFP-KanMX6* (pFA6a-*mRFP1-KanMX6*)	Construction of strain with fusion protein with entire RFP	Geneticin	Erin O'Shea (Cambridge, USA) (Bähler, et al., 1998)
pFA6a-*GFP-KanMX6* (pFA6a-*GFP*(S65T)-*KanMX6*)	Construction of strain with fusion protein with entire GFP	Geneticin	Jurg Bähler and John Pringle (Bähler, et al., 1998)

Suppl. Table 18: Properties of the proteins studied in GST pull-down.[31]

Protein of interest	Tag	Protein Size (AA)	Pho	Other PTMs	Subcellular Location
Gap1	HA	602	V	Glycyl lysine isopeptide (Lys-Gly) at position 76 (interchain with G-Cter in ubiquitin)	-Plasma Membrane -Endosome -ER to Golgi transport vesicles -Vacuole lumen -Multi-vesicular body
Gcd1	HA	578	V		-Cytoplasm
Gcd6	HA	712	V		-Cytoplasm
Sui3	GST	285	V		-Cytoplasm
Pkh1	GST	766	V		-Cell cortex -Cytosol
Sch9	GST	824	V		-Nucleus -Cytoplasm -Vacuole membrane
Tpk1	GST	397	V	Initiator methionine removed => N-acetylserine at AA 2 (acetylation)	-Nucleus -Cytoplasm
Tpk2	GST	380	V	N-acetylmethionine AA 1 (acetylation)	-Nucleus -Cytosol
Tpk3	GST	398	V		-Nucleus -Cytoplasm

Legend: 'V': required; 'AA': amino acids; 'Pho': phosphorylation; 'PTMs': posttranslational modifications.

[31] www.uniprot.org/
www.yeastgenome.org/

3. Strengths and Limitations of the Techniques

***In Vitro*: GST Pull-Down**

GST pull-down is a widely used *in vitro* technique. It is applied for various research purposes, including screening unknown protein-protein interactions, validating the existence of protein-protein interactions predicted by other techniques and revealing the activation status of proteins[32].

As for all research techniques, one should take into account its limitations while interpreting the results. Important to mention is that the assay works perfectly for examining stable or strong interactions. On the other hand, transient or weak interactions are not easily reproduced[33]. Studying interactions with insoluble proteins like membrane proteins with GST pull-down is not advised, since the protein needs to be solubilised in the buffer (Mackay, *et al*., 2007).

GST pull-down is a semi-quantitative technique. As a consequence, the presence or absence of an interaction can be concluded, but not the strength of the interaction. In the current work, an equal amount of the same protein sample was added to the tubes for the different interaction partners in the same batch of experiments. However, not every protein sample contained the same amount of proteins due to a different expression levels in the cells. If more information about the quantity needs to be obtained, the concentration of the protein sample added can be determined by the Lowry method before combining the proteins in the assay (Lowry, *et al*., 1951). Since the Tpk2-GST and Tpk3-GST proteins were not observable after Coomassie staining, the Lowry method could have shown if the protein concentration was either low or absent. This information could be used for trouble shooting. A low expression can be solved in the future by optimising the expression of the GST-tagged proteins via revising the IPTG concentration and the length of induction. On the other hand, a yeast protein expression problem in *E. coli* can possibly be solved by optimising the codons. *E. coli* uses the same codons as *S. cerevisiae*, but with a different frequency (Zhang, *et al*., 1991). Adapting the codons for expression in *E. coli* can influence the protein synthesis levels[34].

It should be noted that the HA-tag has a length of eight amino acids (Field, et al., 1988), whereas the GST-tag is 220 amino acids long. Consequently, sterical hindrance caused by the GST-tag can interfere with the protein-protein interaction (Walker & Rapley, 2008). All proteins expressed by *E. coli* for GST pull-down in the current work were bigger than 220 amino acids long [‘Protein Size (AA)’ in *Suppl. Table 18*], nevertheless Sui3, Tpk1, Tp2 and Tpk3 are relatively small.

[32] https://www.thermofisher.com/be/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/pull-down-assays.html
[33] https://www.thermofisher.com/be/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/pull-down-assays.html#/legacy=www.piercenet.com
[34] https://www.embl.de/pepcore/pepcore_services/cloning/choice_expression_systems/codons6/ index.html

Normally, no interaction is observed between GST and HA-tagged proteins. If an a band is present after immunodetection of the Western blot membrane in the GST lane, one could conclude that the beads were not thoroughly washed after binding so that some HA-tagged proteins remained in the tube, resulting in bands after immunodetection.

E. coli is used as expression system since it is easy to work with, quick and cheap. However, eukaryotic proteins expressed in *E. coli* can possibly form insoluble aggregates. This problem was already counteracted in the current work by expressing at a lower temperature (30 °C instead of 37 °C) and having a fusion protein with GST, a highly soluble partner (Mackay, *et al*., 2007). However, a yeast protein expressed by *E. coli* is potentially not existing in its native conformation due to the lack of certain posttranslational modifications (PTMs). All proteins used in the current work require one or more PTMs ['Pho' and 'Other PTMs' in *Suppl. Table 18*]. Both *E. coli* and *S. cerevisiae* are able to remove N-terminal methionine residues, so this is not a problem for the expression of the Tpk1 protein in *E. coli*. On the contrary, more complex modifications are only carried out by eukaryotic cells. In the current work all proteins of interest are phosphorylated and some acetylated. The absence of this PTMs can result in different conformations, resulting in different interactions with other proteins[35].

A drawback of GST pull-down is that the proteins that show an interaction in the assay are not per se in the proximity of each other *in vivo*. The proteins may be separated by the compartments of the cell. In the current work all proteins studied are at least present in the cytoplasm, with Gap1 being the only protein which is partially present in cytoplasm since it is a transmembrane protein ['Subcellular Location' in *Suppl. Table 18*]. This limitation of GST pull-down was thus not an issue for the current work.

False positive results can be obtained by the adherence of nucleic acids to basic areas of the proteins. Incidentally, nucleic acids can also inhibit an interaction. These two misleading processes can be prevented by adding ethidium bromide or micrococcal nuclease to the samples (Nguyen & Goodrich, 2006). Additionally, false positive results can be obtained by having other proteins linking the two proteins of interest, producing an indirect interaction (Walker & Rapley, 2008). In the majority of the lanes of the Coomassie gel given in *Figure 7*, a lot of bands are seen in addition to the desired band. This means not only the desired GST-tagged protein is purified, but probably also some the degraded or truncated protein of interest combined with distinct proteins from *E. coli*. These proteins are possibly linking the GST-tagged protein with the HA-tagged protein. The problems mentioned in the paragraph can be prevented by working with completely pure protein samples.

[35] https://www.embl.de/pepcore/pepcore_services/cloning/choice_expression_systems/comparison_expression_systems/index.html

***In Vivo*: Fluorescence Microscopy**

Studying the function of a protein by engineering a fusion construct with a fluorescent protein tag or using split citrine BiFC has a lot of advantages. Both techniques make a direct visualisation of the subcellular localization of the protein(s) possible *in vivo*, with a minimal disturbance of the normal cellular environment. Another valuable property is that the protein(s) of interest are expressed in the cell at concentrations comparable to their endogenous equivalents. Moreover, the fluorescence observed is of quantifiable meaning (Citovsky, 2006; Kerppola, 2006; Kodama & Hu, 2012).
Additionally, the techniques do not require staining with exogenous molecules and can be both used for the study of soluble and insoluble proteins (Zamyatnin, et al., 2006). Another property that influenced the popularity of the tools is that the fluorescent signal is detectable by a regular commercially available fluorescence microscope.

As expected, these techniques also have disadvantages. The construction of a strain with a fusion protein can require some time and sometimes optimisation. Different fluorophores can be tried in combination with different linker sequences before obtaining a fluorescent signal. Investigating the localisation of Sch9 and Pkh1 proteins in the current work was not possible, since no signal was obtained. Before optimising, expression can be examined. If the protein of interest is expressed in the sample but does not result in fluorescence, another fluorescent protein which is more tolerant to different environments can be chosen as a tag. Also a linker between the protein of interest and the fluorescent protein can be chosen differently, a linker of two to ten amino acids is recommended. This space can be required for the formation of the fluorophore and the correct folding of the protein of interest. An additional strategy can be tagging the protein at the other end (N-terminal versus C-terminal).
Additionally, the fluorescent tag can possibly affect the biological function of the protein of interest. This is caused by an inappropriate conformation. A small remark for microscopy in general is that not all the cells are per se in focus at the same time (Kerppola, 2006).

Additional strengths and limitations of split citrine BiFC specifically are given below. As mentioned before, this technique facilitates the detection of a protein-protein interaction and its location. Also weak protein-protein interacts result in a fluorescent signal. Noteworthy, a signal is obtained at lower protein concentrations and is less influenced by adapting cellular conditions compared to Förster Resonance Energy Transfer (FRET).
Important to mention is that the binding of the two citrine halves is irreversible. This can be interpreted both as a strength and a limitation. Fluorescence accumulates over the time, but dynamics can consequently not be observed (Citovsky, 2006; Kerppola, 2006; Kodama & Hu, 2012).

Also this technique can result in false positives. This occurs if the two fragments are brought in close proximity (7 nm) due to the presence of the proteins in a small subcellular compartment. This nonspecific complementation is therefore dependent on the proteins and their location. On the other hand, BiFC has a very high false negative rate. There are a lot of possible explanations why the fluorescence does not occur (Kerppola, 2006).

The growth recovery after nitrogen starvation experiment performed in the current work is a simple approach that enables to follow the recovery of the strains after nitrogen starvation. The only drawback that can be reported is the limitation of measuring OD_{600} itself. As mentioned in *section B.1.4.*, it provides indications about the amount of cells present and the growth phase cells are in. However, if the cells have a different size, the OD_{600} can be influenced, making comparisons hard.

4. List of Abbreviations

AA	Amino acids
AGE	Agarose gel electrophoresis
AMP	Adenosine monophosphate
BiFC	Bimolecular fluorescence complementation
bp	Base pair(s)
BRET	Bioluminescence Resonance Energy Transfer
cAMP	3',5'-cyclic adenosine monophosphate
DIC	Differential Interference Contrast
dNTPs	Deoxynucleotides
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
eIF	Eukaryotic initiation factor
F primer	Forward primer
FGM pathway	Fermentable growth medium induced pathway
FRET	Förster Resonance Energy Transfer
G0 state/phase	Stationary state/phase
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GOI	Gene of interest
GPCR	G protein-coupled receptor
GST	Glutathione S-transferase
HA	Human influenza hemagglutinin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo base
LB	Luria-Bertani
LiAc	Lithium acetate
MATa	Mating type a
MATα	Mating type alpha
Mb	Mega bases
MCB lab	Laboratory of Molecular Cell Biology at the KULeuven
NSM	Nitrogen Starvation Medium
O/N	Overnight
OD	Optical density
OD_{600}	Optical density at a wavelength of 600 nm
PABPs	Poly(A)-binding proteins
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCI	Phenol/Chloroform/Isoamyl Alcohol
PCR	Polymerase chain reaction
Pde	Phosphodiesterase

PDK1	Mammalian 3-phosphoinositide-dependent protein kinase 1
PEG	Polyethylene glycol
PIC	Pre-initiation complex
PKA	Protein kinase A
PKB/Akt	Mammalian protein kinase B
PMSF	Phenylmethylsulfonyl fluoride
PTMs	Posttranslational modifications
R primer	Reverse primer
RFP	Red fluorescent protein
rpm	Revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SC	Synthetic Complete
SDS	Sodium dodecyl sulphate
SPM	Sporulation medium
ssDNA	Single stranded DNA
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TC	Ternary complex
TE	Tris-EDTA
TOR	Target of Rapamycin protein kinase
TORC1	Target of Tor complex 1
Tpk1-3	Catalytic subunits of PKA, being Tpk1, Tpk2 and Tpk3
UTR	Untranslated region
YPD	Yeast extract peptone dextrose
YPG	Yeast extract peptone galactose

DEPARTMENT OF BIOLOGY, LABORATORY OF MOLECULAR CELL BIOLOGY
Kasteelpark Arenberg 31, box 2438
3001 HEVERLEE (LEUVEN), BELGIË
tel. + 32 (0)16 32 15 07
fax + 32 (0)16 32 19 79
Johan.Thevelein@mmbio-vib-kuleuven.be
bio.kuleuven.be/mcb/
www.kuleuven.be