Regulation of carbohydrate metabolism upon osmostress

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PhD Thesis UPF

Barcelona, 2021

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Viure vol dir aprendre,
Viure vol dir respecte.

ACKNOWLEDGMENTS

Després de tots aquests anys ja ha arribat el moment de tancar aquesta etapa de la meva vida. No ha sigut fàcil, segurament molt més complicada de l'esperat i per motius inesperats, però de la qual he après moltíssimes coses tan professionalment com personal.

En primer lloc, voldria donar les gràcies al Francesc i la Laia per haver-me donat l'oportunitat de treballar al seu laboratori. Gràcies a això he pogut explorar la ciència al costat d'uns companys de feina magnífics i en uns entorns immillorables (tot i que amb el canvi a l'IRB hem perdut les vistes a la platja...).

El dia a dia al laboratori no hauria sigut el mateix sense aquells als que he vist més que a la meva família i que, mica en mica, també s'han convertit en amics. A la Maria només li puc dir que no podria haver tingut millor companya d'aventures en aquest camí, que quan una no podia més sempre hi era l'altra, quina sort haver coincidit! Al David agrair-li les converses sobre tot allò que no era ciència i tota la paciència quan he vingut a demanar ajuda científica. A la Mariona (tot i que crec que *marrons* t'escau més!) gràcies pels consells i les tardes de xerrar de la vida i la ciència, han sigut oasis en el desert! La Silvia i la Berta han sigut com les meves germanes grans al lab i us he trobat tant a faltar! A la MariaQ, la petita del grup que serà tot allò que vulgui ser, perquè ha sigut fantàstic compartir aquest any (i la xocolata!) amb tu! Al Cesc per haver posat una mica d'alegria aquest últim any i ensenyar-nos en qui ens hem d'emmirallar en el futur. Al Pablo que encara que últimament no el veiem gaire sempre està quan se'l necessita (i quan no tenim ni idea d'estadística). Al Nico, per les seves històries surrealistes amb les que descobrim un món paral·lel!

Al Gerard, per ser el nostre PhD student sènior i xef de referència, aviat em tens per NY! A l'Ivan pels memes i per haver fet que tots tornem al lab a mirar si hem apagat el foc (Health and Safety t'hauria de pagar un plus!). A l'Alba perquè encara que no tingui temps, sempre té un moment per escoltar-te i ajudar-te, mil gràcies per tots els consells! A la Carme per ajudar-me a entendre els problemes de bioquímica i per fer més senzilla la vida al lab. A l'Aida que si no hi fos... ai si no hi fos! En dos dies hauríem cremat el lab... gràcies per fer-ho fàcil. A l'Adri al que li he cedit un despatx més gran i crec que no em troba a faltar. Al Pedja que ens ensenya que la ciència es pot fer a un altre ritme. A l'Arturo amb el que vaig compartir els meus inicis i el seu final. A la Montse, com et trobem a faltar, per sempre saber què, quan i on. A la Laia S per fer-nos riure tant cada dia. Al Santi i al Jorge, no us puc separar i crec que la vida tampoc ho farà mai, gràcies per posar xispa a la vida. Al Pedro per tots els guants que em va arribar a llençar i totes les boles que no vaig col·locar bé jugant a vòlei. Al Guillem al que li desitjo tota la sort del món en aquest camí que comença. I a tota la resta de companys amb els que he compartit hores i hores: Hansol, Rado, Manel, Jana, René, Cate, Ramon, ...

També a tots aquells amb els que hem compartit la vida científica i han fet que tot fos més senzill. Als millors companys de passadís: el Marc (tenim una empresa pendent!), la Leire, la Gemma... sabem que ens trobeu a faltar jaja. Als millors companys de vòlei que podríem haver trobat, l'Hèctor i la Laia, per les hores a la sorra i perquè ja sabeu que tot és culpa de *Tu madre!* Als fisios que van viure els meus inicis a la ciència: A l'Anna (mai t'estaré prou agraïda per tota la

confiança), la Vicky, el Carlos, la Cris, la Bius... Als pombe: Clàudia, Luis, Rodri, Sònia, Cris... A l'Ali per compartir penes i misèries pels passadissos de l'IRB, quina sort haver coincidit al *retreat*. A la resta de persones que han fet que l'arribada a l'IRB fos més fàcil: Cris, Marcos, Sara, Raquel, Enric, Paloma...

Jo no se si avui estaria aquí si amb 16 anys no hagués tingut algú que m'expliqués la biologia amb la passió i l'amor que ho va fer la Paquita. Moltes gràcies per fer-me descobrir un món apassionant!

I encara que en alguns moments no ho semblava, fora del lab he tingut amics que m'han fet oblidar aquell Phostag que no havia sortit. A l'Anna, l'Aina, l'Alba, el Mario, el Ferran, la Judit i el Xito (i als saballuts en general) gràcies per ser-hi sempre i per riure tant, pels assajos i les actuacions, les festes, els sopars i els pàdels, us estimo! A la Patri, que sap que sempre sempre sempre ens podrem trucar quan ho necessitem, encara que estiguem a milers de kilòmetres de distància. A la Laia i l'Expo per compartir escapes on oblidar la realitat durant una estona! A l'Eduard per ensenyar-me que la vida és millor si el cap no va tan ràpid. Als de la uni (Paula, Anna, Carla C, Carla M, Isa, Guillem i Alberto) pels anys compartits i pel millor *hit* que s'ha fet mai a Campus Mar.

A la meva mare per sempre confiar en mi, per donar-nos ales i deixar-nos ser nosaltres mateixos, per ensenyar-nos que la vida és millor si la compartim. Al meu pare per ser-hi sempre encara que no *vulgo* que se'l noti, per ensenyar-me aquesta curiositat per les ciències i per emocionar-se amb els meus passos. A la Maria i el Joan per fer de mi

la germana més mimada i putejada, perquè sempre tindrem un gintònic a punt per parlar de la vida. Al Roc, l'Ona, el Miquel i la Jana per ser els millors *quitapenas* del mon, quina sort tinc de veure-us créixer. A la Paula, la Núria, l'Oriol i el Marcos per ser part del pilot de vespes. A la tieta Rosa i la Bebel. Us estimo!

A l'Àngel, perquè tot i que se que en alguns moments haurà buscat el tiquet per tornar-me, ha sapigut trobar el què, quan, on, com i perquè en cada moment. Per confiar en mi més que ningú, per fer-me riure cada dia i per tenir una paciència infinita. Perquè jo no hagués arribat fins aquí sense el seu suport. Ho hem aconseguit, hem arribat al final! T'estimo molt!

SUMMARY

This thesis describes a novel mechanism for the regulation of carbohydrate metabolism through phosphofructokinase 27 (Pfk27) in response to osmostress by the High Osmolarity Glycerol (HOG) pathway.

Yeast cells deal with continuous environmental perturbations that compromise cell fitness. To overcome osmostress, the Stress Activated Protein Kinase (SAPK) Hog1 leads a specific and coordinated cellular response.

Upon osmostress, HOG pathway is activated and modulates gene expression, protein translation, cell cycle and cellular metabolism. Although many Hog1 functions have been already described, there are still some roles that remain unraveled. Specifically, when yeast cells encounter a sudden increase in extracellular osmolarity, they compensate the osmotic imbalance by accumulating intracellular glycerol. The HOG pathway is in charge of modulating this cellular response through different mechanisms. First, the glycerol export channel, Fps1, closes and glycerol synthesis is promoted via the activation of Gpd1. Next, a long-term adaptation response initiates by the transcription of genes related with glycerol synthesis.

Albeit the role of Hog1 in glycerol accumulation upon stress is widely understood, the regulation of other steps of the carbohydrate metabolism remains unknown. It has been stablished the interaction between Hog1 and Pfk26, a phosphofructokinase in charge of synthetizing fructose 2,6-bisphosphate together with Pfk27. The fructose 2,6-bisphosphate serves as a potent allosteric activator for glycolysis regulation by activating Pfk1 and Pfk2. Upon osmostress,

Pfk26 is phosphorylated and activated by Hog1 and glycolysis is promoted to maintain a metabolic homeostasis (Dihazi et al., 2004). In this thesis, we identified 169 novel Hog1 potential targets through an unbiased biochemical *in vitro* screening and selected Pfk27 as one of the most interesting candidates. We further studied the role of the HOG pathway in the regulation of carbohydrate metabolism via Pfk27. We characterized how Pfk27 is phosphorylated and activated by Rck2 in a Hog1-dependent manner. Furthermore, we described the importance of Pfk27 in the proper metabolic response to osmostress by activating glycolysis. Finally, we studied how Pfk27 mutations affect cellular fitness both in basal and stress conditions. In summary, this thesis establishes a new mechanism by which the HOG pathway modulates the initial steps of glycolysis by regulation of Pfk27 upon osmostress.

Aquesta tesis descriu un nou mecanisme de regulació del metabolisme dels carbohidrats a través de la fosfofructoquinasa 27 (Pfk27) en resposta a estrès osmòtic, per la via de senyalització de HOG.

Les cèl·lules de llevats han de conviure amb continus canvis ambientals que comprometen la seva viabilitat cel·lular. Per tal de superar l'estrès osmòtic, la Proteïna Quinasa Activada per l'Estrès (SAPK) Hog1 lidera una resposta cel·lular específica i coordinada. En presència d'estrès osmòtic, la via de senyalització de HOG s'activa i modula l'expressió gènica, la traducció de proteïnes, el cicle cel·lular i el metabolisme de la cèl·lula. Tot i que moltes de les funcions de Hog1 ja han estat descrites, encara hi ha alguns rols que estan per descobrir. Específicament, quan les cèl·lules de llevat experimenten un increment sobtat de l'osmolaritat extracel·lular, compensen el desequilibri osmòtic acumulant glicerol intracel·lularment. La via de senyalització de HOG s'encarrega de modular aquesta resposta cel·lular mitjançant diversos mecanismes. Primer, el canal d'exportació de glicerol, Fps1, es tanca i la síntesi de glicerol es promou a través de l'activació de Gpd1. Després, s'inicia la transcripció de gens relacionats amb la síntesis de glicerol per tal de donar una resposta a llarg termini. Encara que el rol de Hog1 en l'acumulació de glicerol en resposta a estrès osmòtic estigui ben estudiat, la regulació d'altres passos del metabolisme dels carbohidrats és desconeguda. S'ha establert la interacció entre Hog1 i Pfk26, una fosfofructoquinasa encarregada de sintetitzar fructosa 2,6-bisfosfat juntament amb Pfk27. La fructosa 2,6-bisfosfat actua com un potent activador al·lostèric de la glicòlisis, activant Pfk1 i

Pfk2. En estrès osmòtic, Pfk26 és fosforilada i activada per Hog1 i promou un major flux glicolític per tal de mantenir una homeòstasi metabòlica (Dihazi et al., 2004).

En aquesta tesis, hem identificat 169 nous substrats potencials de Hog1 a través d'un cribratge bioquímic *in vitro* i hem seleccionat Pfk27 com un dels candidats més interessants. Hem estudiat en major profunditat el rol de la via de senyalització de HOG en la modulació del metabolisme dels carbohidrats a través de la regulació de Pfk27. Hem caracteritzat com Pfk27 és fosforilat i activat per Rck2 manera depenent de Hog1. A més, hem descrit la importància de Pfk27 en la resposta metabòlica a l'estrès osmòtic mitjançant l'activació de la glicòlisis. Finalment, hem estudiat com mutacions en Pfk27 afecten la supervivència cel·lular en condicions basals i d'estrès. En resum, aquesta tesis estableix un nou mecanisme a través del qual la via de senyalització de HOG modula els passos inicials de la glicòlisis regulant Pfk27 en estrès osmòtic.

PREFACE

Rewiring of the cellular metabolism is crucial for cell survival in response to growth, development or stress. However, understanding of the mechanisms behind metabolism deregulation in pathological conditions remains highly unknown. Cells are often confronted with external and internal insults that alter their metabolic network and essential processes, such as glycolysis or gluconeogenesis that should be tightly regulated.

Of note, in cancer cells, metabolism is in command of the oncogenes and its deregulation helps the metastatic development. Tumour cells tend to favour a specialized fermentation (or aerobic glycolysis) over the aerobic respiration that the other body cells usually do. This reaction is known as the Warburg effect and it is comparable to the Crabtree effect that occurs in yeast. In aerobic conditions and high glucose concentrations, *Saccharomyces cerevisiae* performs fermentation to generate ethanol rather than aerobic respiration. This suggests that the study of yeast metabolism could serve as a mirror to better understand cancer metabolism.

Particularly, upon osmostress, yeast cells must rewire their metabolism to overcome the stress situation. Once cells sense the stress, the HOG pathway is activated and cells rapidly increase the intracellular glycerol levels to compensate the osmotic imbalance. This glycerol accumulation is driven not only by the direct activation of its synthesis enzymes but also by the transcriptional increase of the genes that are involved in this process (reviewed in Brewster and Gustin, 2014). Moreover, the HOG pathway targets other enzymes

involved in the metabolic response to osmostress (Dihazi et al., 2004; Petelenz-Kurdziel et al., 2013a).

In this thesis, we performed a high throughput screen to identify novel candidates under the control of the SAPK. The main functions of the substrates of Hog1 include cell cycle progression, transcription and translation regulation and vesicle trafficking as well as many components of the carbohydrate metabolism. Here we provide evidence for a new target of the HOG pathway related with the carbohydrate metabolism. Our results indicated that regulation of Pfk27 upon osmostress is required to rewiring the carbohydrate metabolism to successfully overcome osmostress. The results shown in this thesis will be useful to further study the deregulation of phosphofructokinases in pathological conditions in higher eukaryotes.

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INTRODUCTION

1. YEAST RESPONSE TO OSMOSTRESS

The budding yeast *Saccharomyces cerevisiae* lives in nature as saprophyte growing in animal or plant tissues. Throughout evolution, yeasts have developed complex and specific mechanisms to respond and adapt to highly variable environments, maintaining their internal homeostasis. Sudden alterations in osmolarity, nutrients availability, temperature, oxidizing agents, toxic chemicals and others are commonly known as environmental stresses. Yeast cells can sense any of these changes and the signal is intracellularly transduced by signaling pathways giving rise a coordinated cellular response. Cell adaptation to environmental stresses is driven by dramatic intracellular changes to overcome the stress situation allowing cells to return to basal conditions. Yeast cells have to remodel their genetic program, modulate cell cycle, adjust metabolic routes and repress unnecessary functions such as protein biogenesis upon stress (Fig.1). (Gasch and Werner-Washburne, 2002; Gasch et al., 2000; Hohmann, 2002; Morano et al., 2012; Richter et al., 2010)

1.1 Osmostress Signaling

S. cerevisiae, in its natural habitat, decomposes fruits where sugar is abundant to produce nutrients for its survival. Despite that, this process increases substantially the niche's osmolarity, generating unfavourable living conditions. In general situations, an increase in extracellular osmolarity produces a water flux from inside to outside the cell to compensate for both intracellular and extracellular osmolyte concentrations. This flux induces immediate physical

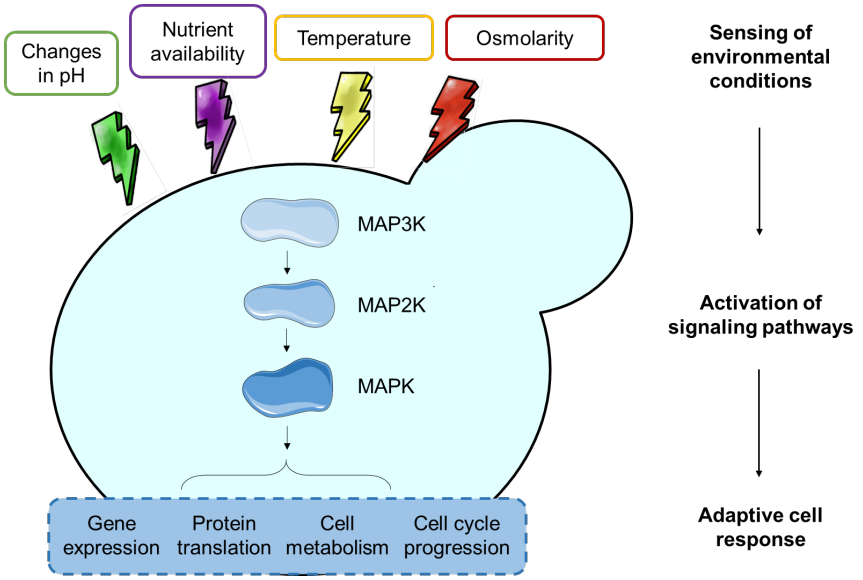


Figure 1. Yeast cells respond to environmental stresses through the activation of signaling cascades that set up the stress response. Environmental stresses such as changes in pH, nutrient availability, temperature or osmolarity, among others, are sensed by yeast cells and signals are transduced intracellularly leading to activation of signaling pathways that regulate many aspects of cell physiology to give rise to adaptation responses.

chances on the cell structure, such as a cell volume reduction and a decrease in cell membrane pressure (Mager and Varela, 1993). All these events compromise essential biochemical reactions inside the cell and put cell viability at risk.

The main pathway in charge to sense and transduce osmostress signals is the HOG (High Osmolarity Glycerol) signaling pathway. The Hog1 MAPK (Mitogen-Activated Protein Kinase) acts as the core effector of this response. The upper part of the HOG pathway senses osmostress by two, functionally redundant but mechanistically different, branches: Sln1 and Sho1-branches (Fig. 2) (Brewster et al., 1993; Saito and Posas, 2012). The Sln1 branch is composed by the

histidine kinase Sln1 that acts as an osmosensor. In the absence of stress, Sln1 remains active and phosphorylates Ypd1, which transfers the phosphate to Ssk1 (Posas et al., 1996). The Ssk1 phosphorylation inhibits its activity, hampering the activation of Ssk2/Ssk22, the MAPK kinase kinases (MAPKKK or MAP3K), preventing the downstream activation of the HOG pathway. In contrast, upon osmostress, Sln1 is inhibited as a result of the loss of membrane turgor and, subsequently, Ssk1 inactivating-phosphorylation by Ypd1 is abolished. In this new scenario, active Ssk1 is now able to activate Ssk2/Ssk22 promoting the osmoadaptive response (Posas and Saito, 1997).

The Sho1 branch presents two extracellular sensors, Msb2 and Hkr1, which activate the GTPase Cdc42 (Maeda et al., 1995; Yang et al., 2009) Parallelly, Sho1 and Opy2 anchor the downstream components of the pathway to the plasma membrane (Reiser et al., 2000; Tatebayashi et al., 2006). Activated Cdc42 associates with Cla4 and Ste20 PAK-like kinases, activating these MAPKKK kinases (MAPKKKK or MAP4K). Then Cla4/Ste20 phosphorylate the MAPKKK Ste11 (van Drogen et al., 2000; Lamson et al., 2002; Raitt et al., 2000)

After the activation of their respective MAP3K, both branches converge (Fig. 2). The Pbs2 MAPK kinase (MAPKK or MAP2K) is activated by the binding of Ssk2/Ssk22 and Ste11 on their docking sites. Once bound, any of these MAP3K can phosphorylate Pbs2 on Ser154 and Thr518. Activated Pbs2 in turn phosphorylates and activates the core protein of the pathway, the Hog1 MAPK, at Thr174 and Thr176 (Brewster et al., 1993; Posas and Saito, 1997).

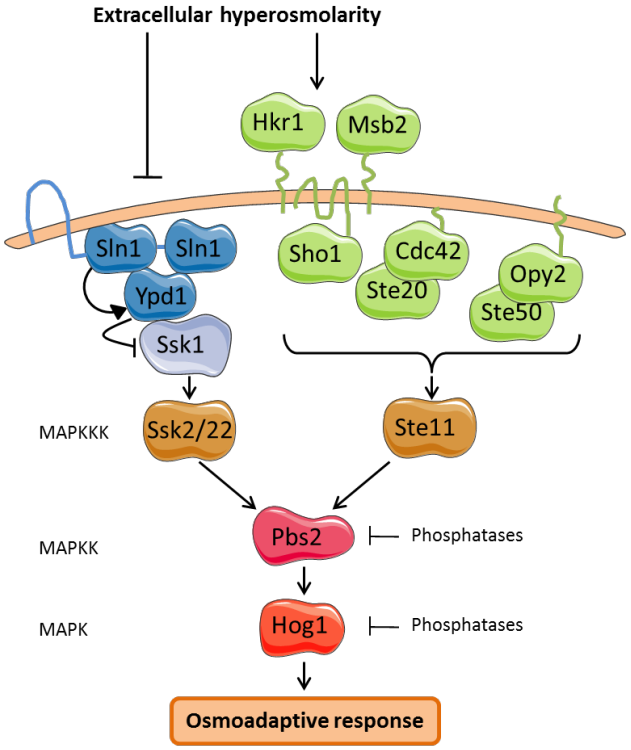


Figure 2. Outline of the HOG pathway. Yeast cells sense osmostress and activate the HOG pathway via two different sensor branches, Sln1 and Sho1. Both branches converge in the activation of the MAP2K Pbs2 by the Ssk2/22 and Ste11 MAP3Ks, respectively. Pbs2 activates the Hog1 MAPK that coordinates the intracellular osmostress response.

Hog1 is the main orchestrator of the osmostress response. At short-term, it activates the accumulation of glycerol inside the cell, to compensate the osmotic imbalance between the extracellular media and the cytosol (Brewster and Gustin, 2014; Nevoigt and Stahl, 1997) by inhibiting glycerol export and promoting new glycerol synthesis at the same time (reviewed in Saito and Posas, 2012; Brewster and Gustin, 2014; and further discussed in section 3).

Upon stresses such as DNA damage, oxidative stress, heat shock or osmostress, *S. cerevisiae* induces the transcription of a set of genes

that shape the environmental stress response (ESR). The ESR extent and kinetics depends on the severity of the stress. Besides the ESR response, each type of stress elicits its own defined transcriptional response (Gasch et al., 2000; Causton et al., 2001; Capaldi et al., 2008; Berry and Gasch, 2008). Right after its phosphorylation, Hog1 translocates into the nucleus to activate the specific transcriptional response that is required for a proper long-term osmoadaptation (Ferrigno et al., 1998).

Moreover, Hog1 controls the cell cycle progression upon osmostress. Hog1 regulates all the phases of the cell cycle (G1, S, G2, M) to delay cell division upon osmostress; an essential aspect for cell survival is to adapt first to osmostress and, afterwards, proceed with cell cycle progression. These controlled and organized mechanisms in each cell cycle phase ensure a safe cell cycle progression (Adrover et al., 2011; Canal et al., 2018; Chang et al., 2017; Clotet et al., 2006; Duch et al., 2013a; Escoté et al., 2004; Nadal-Ribelles et al., 2014; Tognetti et al., 2020; Zapater et al., 2005).

Besides glycerol metabolism, transcriptional process and cell cycle progression, osmostress also compromises other basic cell functions such as protein synthesis and catabolism. It is clear that osmotic stress puts yeast cells out of their comfort zone. Nevertheless, once yeast cells are adapted to the new situation, the stress adaptation response has to be attenuated to reestablish the previous basal cellular functions. The down-regulation of the HOG pathway is triggered by internal negative feedbacks that diminish Hog1 signaling by phosphorylating upstream HOG pathway components (Hao et al., 2007, 2008). On the other hand, Hog1 promotes the expression of

Ptc1, Ptp2 and Ptp3 phosphatases that dephosphorylate Hog1 modulating the activity of the pathway (Jacoby et al., 1997; Warmka et al., 2001). Finally, physiological cell volume and membrane tension levels are recovered by the accumulation of glycerol inside the cell (Nevoigt and Stahl, 1997; Lee et al., 2013).

1.2 Rck2, a novel osmotic signal spreader

1.2.1 Rck2 and osmostress

Rck2 is a member of the calmodulin-protein kinase family and, together with its paralog Rck1, it was discovered by reverse genetics studying the Ca^{2+} signaling in yeast. Nevertheless, Rck2 does not respond to Ca^{2+} input signals (Melcher and Thorner, 1996). Instead, upon osmostress, the C-terminus of Rck2 is strongly bound to the Hog1 MAPK as showed by two-hybrid analyses and coprecipitation assays (Bilsland-Marchesan et al., 2000; Teige et al., 2001a). Moreover, Rck2 is phosphorylated and activated directly by Hog1 in its potential auto inhibitory domain, at Ser519 (Bilsland-Marchesan et al., 2000). Although the deletion of *RCK2* does not show osmosensitivity, its overexpression has the ability to attenuate the osmosensitive phenotype of cells lacking *HOG1* or *PBS2*, suggesting that Rck2 is acting downstream of Hog1 (Dahlkvist and Sunnerhagen, 1994; Melcher and Thorner, 1996; Bilsland-Marchesan et al., 2000; Bilsland et al., 2004).

Once active, Rck2 phosphorylates itself and its substrates, controlling a subset of the osmotic stress response. As mentioned before, protein translation is one of the cell physiology aspects that is regulated by the HOG pathway upon osmostress. Regarding this, Elongation

factor 2 (EF-2) was the first Rck2-dependent substrate reported after an osmotic shock. Upon osmostress, Rck2 phosphorylates and attenuates EF-2 function, producing a decrease of translation elongation by inhibiting ribosome translocation (Teige et al., 2001). Specifically, EF-2 is phosphorylated at Thr56 and Thr58 by Rck2 and the mutation of those sites prevents the ability of EF-2 to bind to ribosomes *in vivo* (Bartish et al., 2007).

Upon osmostress, there is a regulation of mRNAs turnover and translation efficiencies in order to optimize the activity of stress-related gene products during all the stress response phases. For instance, destabilization of stress-induced mRNAs when they reach their maximum steady-state level prepares the cell for the next step, the recovery. It has been shown that Hog1 controls both the steady-state levels and the stability of stress-responsive transcripts, while Rck2 only affects mRNAs steady-states levels (Molin et al., 2009). Interestingly, Rck2 also shows a surprisingly transcriptional regulation role although this is a cytoplasmic kinase (Warringer et al., 2010). This function could be explained by the negatively influence that Rck2 exerts on Hog1 nuclear localization (Bilsland et al., 2004b). It has also been reported that Pde2 physically interacts with Rck2 stablishing a connection needed to reduce the phosphorylation of the cAMP-dependent protein kinase (PKA)-targeted stress-regulated transcription factors. (MacGilvray et al., 2018).

During many years, Hog1 has been considered the main signal spreader protein upon osmotic stress. However, in 2017, Romanov *et al*. discovered that the Serine / Threonine kinase Rck2 acts also as a central hub for many Hog1-dependent secondary phosphorylation

events. At least 16 kinases have been found to be targeted by Rck2, unraveling a complex protein network encompassing a large number of distinct cellular functions (Romanov et al., 2017).

1.2.2 Rck2 during oxidative stress

However, Rck2 has not only being involved in osmostress but also in oxidative stress responses. Hog1 and Rck2 are phosphorylated under oxidative stress in a Pbs2-dependent manner. Cells lacking *RCK2* gene show a mild sensitivity to oxidative stress. As in osmostress conditions, Hog1 nuclear localization partially depends on Rck2 presence, *rck2* mutant cells show more nuclear Hog1 when compared to *wild-type* cells upon oxidative stress. Thanks to two-hybrid screenings, the Zn^{2+} transporter Zrc1 has been identified as Rck2 target upon oxidative stress (Bilsland et al., 2004b).

Moreover, during oxidative stress, there is a dissociation of actively translating ribosomes (polysomes). The polysomes are lost in a higher degree in *rck2* mutant cells when compared to *wild-type* and all the polysome-associated mRNAs suffer a major deregulation in *rck2* mutant cells. This translational deregulation causes the inability of these mutant cells to cope with oxidative stress(Swaminathan et al., 2006). These findings remark the importance of Rck2 activity in Hog1-dependent stress responses.

2. YEAST CARBOHYDRATE METABOLISM

In all organisms, metabolism comprises the processes of assimilation (anabolism) and dissimilation (catabolism) of nutrients by a cell. The cell synthetizes new material through anabolic pathways, which are reductive processes. While it obtains energy by removing electrons from cell substrates or intermediates through catabolic pathways, that are oxidative. All these events are carried out by tightly regulated enzymatic reactions.

S. cerevisiae employs glucose and fructose as its major carbon sources and, subsequently, energy source. When glucose is not available, yeast can use a wide variety of alternative carbon sources, such as galactose, sucrose, glycerol, ethanol, maltose and acetate. It can also assimilate simply nitrogen sources to synthetize amino acids and proteins.

Yeast switches between respiratory and fermentative metabolism depending on the oxygen and glucose availability. Respiration occurs in the presence of oxygen and low glucose availability and generates cell biomass. In contrast, fermentation takes place in high glucose and anaerobic conditions and cell finally synthetizes ethanol. Both processes share the first steps (glycolysis) and differ on the pyruvate treatment: in respiration, pyruvate enters in the mitochondrial matrix and is degraded in the tricarboxylic acid (TCA) cycle; while, into fermentation, pyruvate is transformed to ethanol through diverse enzymatic reactions that reoxidize NADH to NAD.

During osmostress, Hog1 controls some early glycolysis steps and the glycerol synthesis branch (Alepuz et al., 2001; Ferreira et al., 2005; Lee et al., 2012; Rep et al., 1999, 2000). In the following

section, I will focus on these different steps and branches influenced by Hog1: the glycolysis and, its reverse pathway, gluconeogenesis, as well as in the trehalose and glycerol synthesis pathways.

2.1 Metabolic pathways of central carbon metabolism

As mentioned before, glucose is the major carbon source for yeast cells. In order to process this glucose to generate either cell biomass or ethanol, cell must first convert glucose to pyruvate through the glycolysis pathway. Nevertheless, in some conditions, yeast needs to synthetize glucose and other sugars to generate macromolecules, such as polysaccharides. The conversion of pyruvate to sugars is called gluconeogenesis (Figure 3).

2.1.1 Glycolysis

The glycolysis pathway is common in all cells, both prokaryotic and eukaryotic. The internal regulation of glycolysis is mainly shared among species while the external stress-dependent regulation can differ.

As shown in Figure 4, in yeast glucose enters into the cell through specific transporters and is rapidly phosphorylated by hexokinases (Hxk1, Hxk2, Glk1) to form glucose 6-phosphate (step 1). This first step is crucial to hampers glucose diffusion through plasma membrane and facilitates its further metabolism. For this reason, hexokinases are tightly regulated at several levels, both allosterically and transcriptionally (Ahuatzi et al., 2007; Blázquez et al., 1993; Fernández-García et al., 2012; Pelaez et al., 2010).

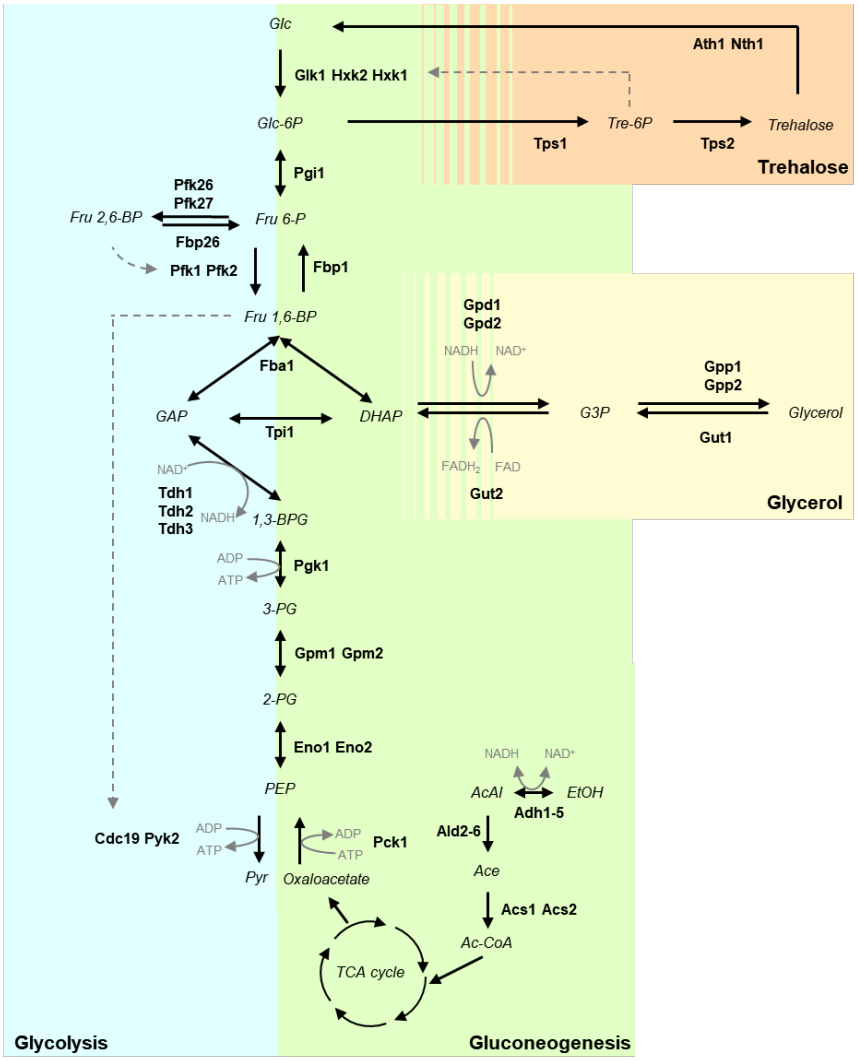


Figure 3. Schematic representation of the glycolysis, gluconeogenesis, trehalose and glycerol synthesis pathways. Every pathway is colored differently. The pathway is depicted with the enzymes in bold and metabolites in italic. Black arrows indicate enzymatic reactions, grey lines correspond to generation of secondary metabolites and grey dashed lines are regulation of the enzymes.

The following step of the glycolysis pathway is the synthesis of fructose 6-phosphate from glucose 6-phosphate, carried out by the phosphoglucose isomerase (Pgi1) (step 2).

One of the critical and limiting steps of glycolysis is the one performed by phosphofructokinases 1 and 2 (Pfk1 and Pfk2) (step 3). These enzymes are in charge of phosphorylating fructose 6-phosphate resulting in fructose 1,6-bisphosphate (Fru-1,6-P_2). In parallel, Pfk26 and Pfk27 also use fructose 6-phosphate as substrate to generate fructose 2,6-bisphosphate (Fru-2,6-P_2) (step 4). In the opposite direction, the fructose 2,6-bisphosphatase (Fbp26) reverses this reaction, converting Fru-2,6-P_2 to fructose 6-phosphate (step 5). Fru-2,6-P_2 is an allosteric activator of Pfk1 and Pfk2 and, subsequently, it increases the glycolytic flux. Phosphofructokinase enzymes are tightly regulated upon several situations as it is further discussed in section 2.3.

In the next stage of glycolysis, Fru-1,6-P_2 is split into glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), by the fructose 1,6-bisphosphate aldolase (Fba1) (step 6). GAP continues the glycolysis pathway while DHAP is used to synthetize glycerol. Interestingly, these two molecules can be interconverted by the triose phosphate isomerase (Tpi1), through a rapid and reversible reaction (step 7).

So far, one molecule of glucose has been converted into two molecules of GAP by consuming two ATP molecules. The sequence continues with the conversion of GAP into 1,3-bisphosphoglycerate (1,3-BPG) catalyzed by the triose phosphate dehydrogenases (Tdh1, Tdh22 and Tdh3) (step 8).

During the last steps of glycolysis, Phosphoglycerate kinase (Pgk1) catalyzes the dephosphorylation of 1,3-BPG generating 3-phosphoglycerate (3-PG) and ATP (step 9). Next, 3-PG is converted to 2-phosphoglycerate (2-PG) by the glycerate phosphomutase (Gpm1 and Gpm2) (step 10). Subsequently, 2-PG is dehydrated by enolases (Eno1 and Eno2) producing phosphoenolpyruvate (PEP) (step 11). Finally, the pyruvate kinase (Cdc19 and Pyk2) forms pyruvate from PEP generating a new ATP molecule (step 12). This essentially irreversible reaction act as the final control point of glycolysis. Cdc19 is the main isoform and is regulated allosterically by Fru-1,6-P_2 and by PKA-dependent phosphorylation (Boles et al., 1997; Jurica et al., 1998; Morris et al., 1986; Portela et al., 2006).
Once pyruvate is generated, it can either enter in the TCA cycle or serve as a substrate for ethanol synthesis.

2.1.2 Gluconeogenesis

In situations where non-sugar carbon sources are available, yeast survival may depend on the biosynthesis of glucose. Like in other organisms, gluconeogenesis is an ATP-dependent process and needs NADH as a reducing power.
As shown in Figure 5, gluconeogenesis starts with pyruvate as first substrate and shares with glycolysis many of its enzymatic reactions. Almost all glycolysis steps are reversible except for two enzymatic reactions: (i) Phosphoenol pyruvate is generated from oxaloacetate (instead from pyruvate) by phosphoenol pyruvate carboxykinase (Pck1) (step 1); (ii) Fructose bisphosphatase (Fbp1) is in charge of dephosphorylating Fru-1,6-P_2, giving rise to fructose 6-phosphate (step 2).

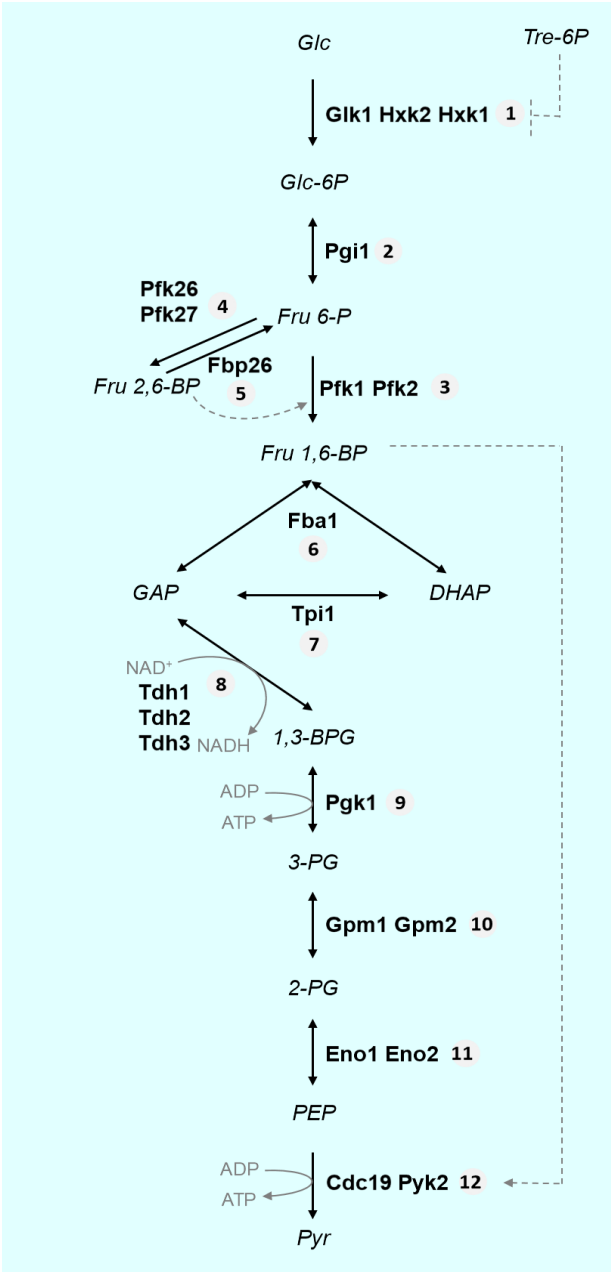


Figure 4. Schematic representation of the glycolysis pathway. The pathway is depicted with the enzymes in bold and metabolites in italic. Black arrows indicate enzymatic reactions, grey lines correspond to generation of secondary metabolites and grey dashed lines are regulation of the enzymes. Numbers in grey dots are the steps detailed in the text.

When glucose is available there is a tight regulation of the gluconeogenic enzymes: there is an enzyme allosteric regulation, mRNA degradation of *FBP1* and *PCK1* and targeted proteolytic degradation (Berg et al., 2002; Braun et al., 2011; Lin et al., 2009; Tripodi et al., 2015; Young et al., 2012).

Gluconeogenesis can also be performed from other carbohydrate metabolites than pyruvate such as ethanol, lactate or glycerol. In yeast, ethanol is the most used gluconeogenic substrate. This pathway begins with the dehydrogenation of ethanol by the alcohol dehydrogenase II (Adh2), generating acetaldehyde (AcAl) (step 3). Then, the aldehyde dehydrogenase converts the AcAl into Acetate (Ace) (step 4). Finally, acetyl-CoA is produced by the ligation of acetate to coenzyme A (CoA) (step 5). CoA enters into TCA cycle forming oxaloacetate that will be transformed to PEP by Pck1 (step 6).

2.2 Other pathways involved in carbon metabolism

Yeast carbohydrate metabolism encompasses not only the glucose anabolism and catabolism, but also synthetizes and degrades many other carbohydrate metabolites such as trehalose, glycerol or glycogen. All of them connect with the glycolytic / gluconeogenic pathways at some point. Yeast needs to synthetize or degrade these carbohydrate metabolites to produce energy when growing in non-fermentable carbon sources, store energy as glycogen or generate other metabolites for cellular adaptation in response to stress conditions.

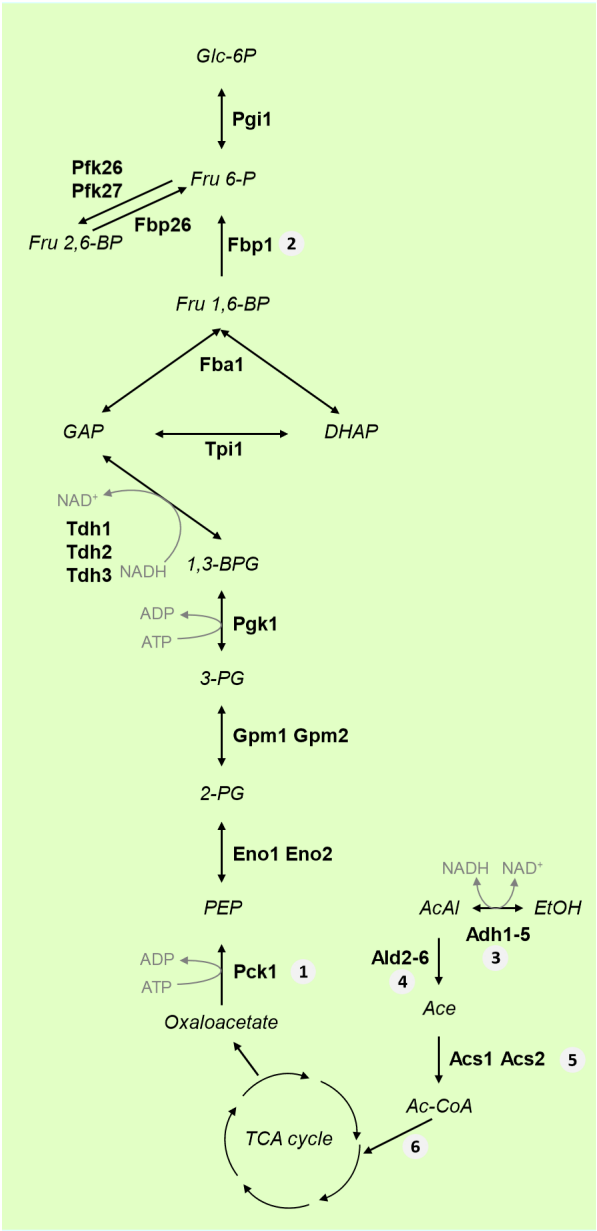


Figure 5. Gluconeogenesis and ethanol synthesis pathway. Schematic representation of gluconeogenesis and ethanol synthesis pathway. Enzymes are in bold and metabolites in italic. Black arrows indicate enzymatic reactions, grey lines correspond to generation of secondary metabolites. Numbers in grey dots are the steps detailed in the text.

2.2.1 Trehalose pathway

Trehalose is a sugar with a rare configuration, with two glucoses linked by their 1-carbon. Trehalose serves as a storage compound and as a protective metabolite in front of several stress conditions.

During the eighties, the correlation between the concentration of trehalose in the cell and the ability to cope with freezing was described (Coutinho et al., 1988), suggesting that trehalose has also a protective role inside the cell. Some stress conditions, such as glucose starvation or osmostress, can lead to variation ranging from 1 % to 20 % in trehalose concentration inside the cell (Hottiger et al., 1987; Lillie and Pringle, 1980; Panek, 1963).

Trehalose is synthesized from glucose 6-phosphate by trehalose 6-phosphate synthase (Tps1), generating trehalose 6-phosphate (T6P). Afterwards, the trehalose 6-phosphate phosphatase (Tps2) dephosphorylates the T6P to obtain trehalose (Cabib and F, 1958)(Cabib and Leloir, 1958). Tps1 and Tps2 form a four-subunit complex, named TPS complex, composed by the two enzymes and two regulatory subunits, Tsl1 and Tps3, suggesting a complex regulation of the trehalose synthesis (Reinders et al., 1997; Trevisol et al., 2014). The reason for the regulation on trehalose synthesis could be due to the regulatory effect of trehalose 6-phosphate on glycolysis and fermentation by inhibiting the Hxk1 and Hxk2 activities (Noubhani et al., 2000). Moreover, yeast can transport trehalose through a high-affinity H^+-trehalose symporter (Agt1) that its expression is repressed in a glucose-dependent manner and, therefore, it is highly present during stationary phase (Plourde-Owobi et al., 1999)n(Fig. 6).

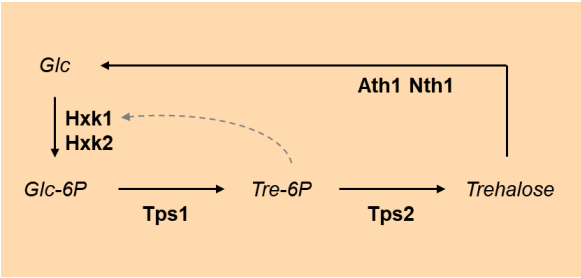


Figure 6. Trehalose pathway. Schematic representation of trehalose synthesis and degradation pathways. Enzymes are in bold and metabolites in italic. Black arrows indicate enzymatic reactions and grey dashed lines are regulation of the enzymes.

Yeast shows two distinct types of trehalases, the enzymes in charge of hydrolyzing trehalose in two glucose molecules: (i) the acidic trehalase (Ath1) is optimally active at pH 4.5-5.0 while (ii) the neutral trehalase (Nth1) has an optimal activity at pH 6.8-7.0 (App and Holzer, 1989; Mittenbühler and Holzer, 1991).

Under non stress conditions, yeast cells do not synthetize trehalose because the TPS complex is not completely formed. However, in response to stress conditions, such as heat, cold or osmostress, Tps1 and Tps2 activities increase due to the induction of expression of TPS complex genes, mainly *TSL1* (François et al., 1991; Nadal-Ribelles et al., 2014; Trevisol et al., 2014). Both TPS complex and *NTH1* genes contain a variable number of stress-responsive elements on their promoters (de Mesquita et al., 2003; Winderickx et al., 1996; Zähringer et al., 2000). Nevertheless, Nth1 needs to be phosphorylated by PKA to become active and hence trehalose is intracellularly accumulated upon stress. During the stress recovery phase, trehalose is not required and must be degraded, Nth1 becomes activated, forms a complex together with 14-3-3 proteins, Bmh1 and Bmh2, and degrades trehalose into glucose and, consequently,

provides also the energy necessary for stress recovery (Ortiz et al., 1983; Schepers et al., 2012; Singer and Lindquist, 1998; Veisova et al., 2012).

Specifically, in response to osmostress, when glucose is the main carbon source, glycerol accumulates inside the cell to overcome the osmotic imbalance. Instead, when the carbon source is ethanol, trehalose is the solute that is majorly accumulated. Nevertheless, the cell volume recovery observed in ethanol-grown cells is low in comparison with glucose-grown cells (Babazadeh et al., 2017).

2.2.2 Glycerol pathway

Glycerol is a biomolecule ubiquitous in nature, therefore it is not surprising that many organisms possess mechanisms to use this organic compound as a source of carbon and energy.

The synthesis of glycerol starts with the reduction of DHAP to glycerol 3-phosphate (G3P) thanks to the oxidation of NADH by the glycerol 3-phosphate dehydrogenases (Gpd1 and Gpd2) (Albers et al., 1996; Gancedo et al., 1968; Meikle, 1991). Then, the glycerol 3-phosphatases (Gpp1 and Gpp2) dephosphorylates the G3P to obtain glycerol as a final product (Gancedo et al., 1968; Norbeck et al., 1996) (Fig. 7).

Glycerol catabolism share the same intermediary metabolite as the anabolism, the G3P. The glycerol kinase (Gut1) is the enzyme in charge of phosphorylate glycerol in the cytosol, resulting in G3P. Then G3P enters in the mitochondria where the glycerol 3-phosphate dehydrogenase (Gut2) generates DHAP concomitantly with $FADH_2$. DHAP is then returned to cytosol where it can enter to glycolysis or gluconeogenesis pathways (Sprague and Cronan, 1977). *GUT1* and

GUT2 genes are carbon source regulated, expressed when non-fermentable carbon sources, such as glycerol or ethanol, are available and repressed in fermentable ones (Grauslund and Rønnow, 2000; Grauslund et al., 1999) (Fig. 7).

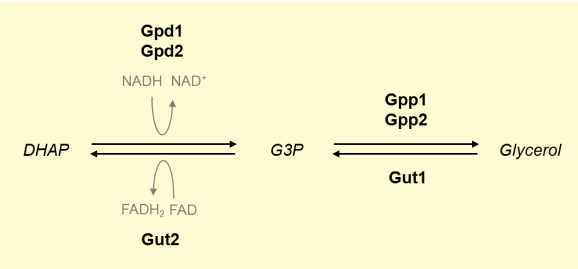


Figure 7. Glycerol pathway. Schematic representation of glycerol synthesis and degradation pathways. Enzymes are in bold and metabolites in italic. Black arrows indicate enzymatic reactions, grey lines correspond to generation of secondary metabolites.

There are many yeast species that use glycerol as a protectant molecule by accumulating it inside the cell, particularly in response to heat and osmotic stresses. As mentioned in section 1, glycerol plays an essential role during osmostress to restore the osmotic imbalance produced between the inside and the outside of the cell. Cells defective in glycerol production cannot cope with the increase in external osmolarity, therefore acquiring an osmosensitive phenotype (Albertyn et al., 1994; Nevoigt and Stahl, 1997). Detailed regulation of glycerol synthesis by HOG pathway during osmostress is further discussed in section 3.

Glycerol synthesis also plays an important role during anaerobiosis because, as a NADH-consuming process, it is essential for balancing the redox potential by oxidizing the excess of NADH. Yeast cells cannot grow in anaerobic conditions if glycerol synthesis pathway is affected (Nissen et al., 2000; Påhlman et al., 2001). During ethanol

fermentation, glycerol become its major resulting product and it can be used as a sole carbon source in aerobic conditions (Cronwright et al., 2002).

2.3 The phosphofructokinases

As mentioned in previous sections, phosphofructokinases 1 and 2 (Pfk1 and Pfk2) regulate one of the limiting steps of glycolysis, which is tightly regulated. Fructose 2,6-bisphosphate (Fru-2,6-P_2) that acts as an allosteric activator of Pfk1 and Pfk2. Fru-2,6-P_2 is synthetized from F6P by Pfk26 and Pfk27, enzymes that are also highly regulated.

2.3.1 Pfk1 and Pfk2, limiting steps in glycolysis

During glycolysis, Pfk1 and Pfk2 enzymes convert F6P and an ATP molecule into Fru-1,6-P_2 and ADP. Like the pyruvate kinases Cdc19 and Pyk2, both Pfk1 and Pfk2 only function in the forward direction, hence these enzymes are glycolysis specific and are not involved in gluconeogenesis.

Phosphofructokinase is a hetero oligomeric complex formed by four alpha-subunits (Pfk1) and four beta-subunits (Pfk2) (Fig. 8). Electron microscopy studies showed that the four alpha-subunits form the central core of the octamer (KOPPERSCHLÄGER et al., 1977; Ruiz et al., 2001). However, a single point mutation either in alpha- or beta-subunit can destabilize the whole complex, with all subunits tending to aggregate and subsequently degrade (Rgen Kirchberger et al., 1999). Both Pfk1 and Pfk2 subunits have a 55 % of sequence homology and a 20 % between N-terminal and C-terminal halves of each subunit. The N-terminal half is the one retaining the catalytic

function while the C-terminus is the one containing the allosteric binding sites (Poorman et al., 1984). Deletion mutants of either *PFK1* or *PFK2* still ferment glucose while doble mutant *PFK1 PFK2* is not able to grow in the presence of glucose (Arvanitidis and Heinisch, 1994).

In glucose-rich environment, expression of *PFK1* and *PFK2* genes is highly induced and, therefore, facilitates the switch from gluconeogenesis to glycolysis. Phosphofructokinase genes, together with Pdc1 and Pyk1, are highly induced when changing from a non-fermentable to a fermentable carbon source (Gonçalves et al., 1997; Moore et al., 1991).

Interestingly, ATP not only functions as a substrate for phosphofructokinases, but also as the principal regulatory mechanism acting as an allosteric inhibitor at millimolar range (KOPPERSCHLÄGER et al., 1977; Reuter et al., 2000). Each phosphofructokinase subunit presents 2 independent ATP binding sites, one in the catalytic site of the enzyme (N-terminus) and the other in the allosteric regulatory site (C-terminus). Single point mutation in the C-terminal half of Pfk1 and Pfk2 produces the loss of ATP inhibition. The aforementioned site is the same in both enzymes, Pro728, confirming that eukaryotic phosphofructokinases evolved from a gene duplication (Poorman et al., 1984; Rodicio et al., 2000). ATP inhibition is reversed by an independent activation through AMP and Fru-2,6-P_2. The effect of Fru-2,6-P_2 contributes to the coordination between glycolysis and gluconeogenesis, while AMP is involved in ATP homeostasis. It is described that Fru-2,6-P_2 enhances the enzyme affinity for AMP and, subsequently, AMP increases the

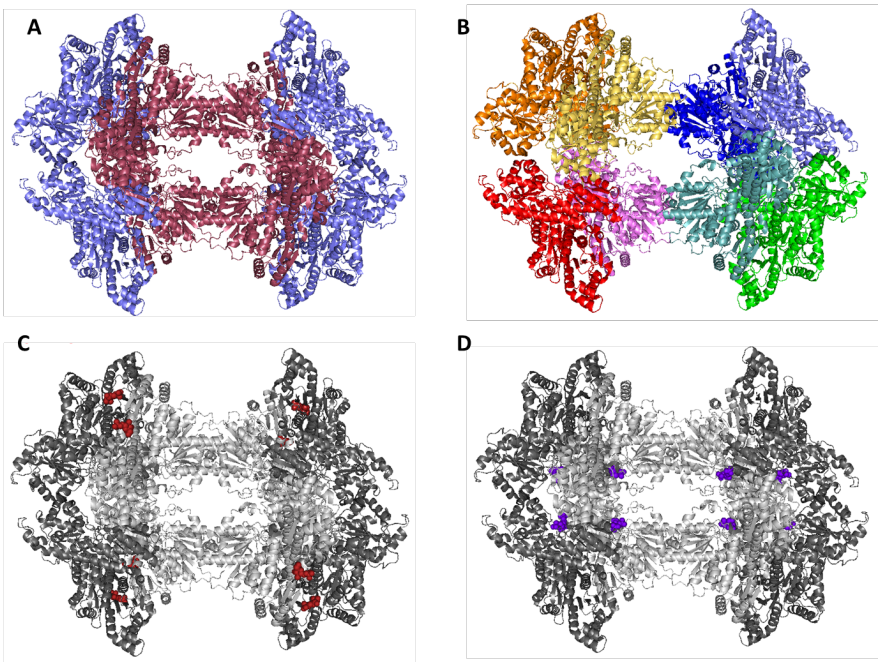


Figure 8. Pfk1 and Pfk2 protein structure. Pfk1 and Pfk2 hetero oligomeric crystal structures modified from PDB structure number 3o8o (Banaszak et al., 2011). **A)** Pfk1 is colored in red and Pfk2 in purple. **B)** Each of the eight chains are colored distinctly. **C and D)** Pfk1 is colored in light grey and Pfk2 in dark grey and the substrate, **C)** F6P is highlighted in red and **D)** Fru-2,6-P_2 in purple.

affinity of the phosphofructokinase for the F6P (Avigad, 1981; Nissler et al., 1983).

The Fru-2,6-P_2 binding site is located at the C-terminal half of the phosphofructokinases, and it evolves from a former F6P binding site (the substrate of the enzyme) thanks to gene duplication (Poorman, 1984). Two-point mutations in Ser724 and His859 of Pfk1 and Pfk2 lead to a loss of phosphofructokinase activation by Fru-2,6-P_2 (Boles et al., 1997). The effect of the allosteric regulator Fru-2,6-P_2 to Pfk1 and Pfk2 is no needed to sustain a glycolytic flux under fermentative conditions; however, it is important to maintain a correct homeostasis of metabolite concentrations (Müller et al., 1997).

2.3.2 Pfk26 and Pfk27, glycolytic activators

Cells require a tight control of the amount of Fru-2,6-P_2 in order to regulate glucose consumption and glycolysis flux. For this reason, both Pfk26 and Pfk27 are regulated at different levels and under different scenarios. Pfk26 shows a high sequence similarity with Pfk1 and Pfk2, conserving both the N-terminal catalytic and the C-terminal regulatory domains. However, Pfk27 is a truncated version of the common phosphokinase structure, conserving only the catalytic domain (Boles et al., 1997; FRANÇOIS et al., 1988) (Fig. 9).

Increases in extracellular glucose concentration activates the Ras-cAMP pathway. The cAMP-dependent protein kinase (PKA) is stimulated by the production of cAMP, which in turns regulates post-translational modifications of the key enzymes of glycolysis and gluconeogenesis. PKA phosphorylates and activates Pfk26 upon increase of extracellular glucose levels (Dihazi et al., 2003).

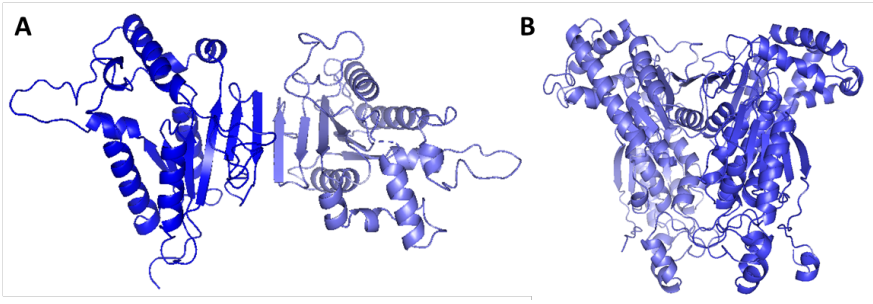


Figure 9. Pfk27 and Pfk26 model protein structure. There is no crystal structure of Pfk27 and Pfk26. **A)** Pfk27 protein structure model generated from crystal structure of human PFKFB3 sequence (PDB: 5ak0.1.B; Boyd *et al*., 2015) with SWISS-MODEL (Bertoni et al., 2017; Bienert et al., 2017; Guex et al., 2009; Studer et al., 2020; Waterhouse et al., 2018). **B)** Pfk26 protein structure model obtained from SWISS-MODEL repository (P40433).

Moreover, when cells are subjected to a change from non-fermentable carbon sources to glucose, the expression of *PFK26* gene, highly expressed in ethanol-growing cells, is repressed. In contrast, *PFK27* mRNA levels are very low in cells growing in ethanol but the gene is rapidly induced upon addition of a fermentable carbon source. Mutations in components of the Ras-cAMP pathway thoroughly affect the transcription regulation of *PFK26* and *PFK27* genes (Gonçalves et al., 1997).

Upon glucose removal, Snf1 kinase becomes active and phosphorylates Pfk27 in its N-terminal domain. The Pfk27 phosphorylation allows its association with the F-box of Grr1, a substrate specificity factor that interacts with SCF to form SCF^{Grr1} complex. The SCF complex acts as a ubiquitin ligase and targets its substrates to degradation. Pfk27 turnover leads to a reduction on Fru-2,6-P_2 levels. The expression of a non-phosphorylatable Pfk27 mutant by Snf1 inhibits cell growth on glycerol, pointing out the importance of Pfk27 turnover (Benanti et al., 2007).

In hypotonic stress, the Pkc1 MAPK becomes active to help in the remodeling of yeast cell wall. Pkc1 also phosphorylates and inhibits Pfk26 under these conditions. The inhibition of Pfk26 leads to the reduction of the glycolytic flux and an accumulation of glucose 6-phosphate. This metabolite is used to synthetize glucane, one of the main constituents of the cell wall (Davenport et al., 1995; Dihazi et al., 2001). In addition, Pfk26 also becomes acetylated in its N-terminus under hypotonic conditions. This post-translational modification is important for the phosphorylation by Pck1 upon hypo

osmotic stress: a Pfk26 mutant unable to be acetylated does not show the aforementioned phosphorylation (Dihazi et al., 2005).
Interestingly, Pfk26 has also been reported to be regulated under hyperosmotic stress and it is further discussed in section 3.

3. CARBOHYDRATE METABOLISM REGULATION UPON OSMOSTRESS

Upon osmostress, yeast cells need to recover cell volume and membrane tension to adapt to the new environmental situation. It does so by rapidly accumulating glycerol inside the cell by closing its export and promoting its synthesis. The Hog1 MAPK controls several mechanisms from carbohydrate metabolism and, particularly, glycerol metabolism (Fig. 10).
The aquaglyceroporin Fps1 is a member of the Major Intrinsic Protein (MIP) channel family and facilitates the glycerol transport through the plasma membrane (Luyten et al., 1995; Sutherland et al., 1997). In basal conditions, the Regulator of Glycerol Channel (Rgc2) maintains Fps1 in the open state by binding to its C-terminal cytoplasmic domain through a PH-PH domains interaction (Beese et al., 2009; Lee et al., 2013). Upon osmostress, glycerol export rate needs to be decreased in a subminute scale to rapidly increase the intracellular glycerol levels to compensate the osmolyte imbalance. For this reason, Fps1 gating is regulated through Hog1-dependent phosphorylations on both Rgc2 and Fps1. Moreover, upon activation, Hog1 is recruited to the N-terminal cytoplasmic domain of Fps1 and uses it as a platform to increase Hog1-dependent Rgc2 and Fps1 phosphorylations producing the eviction of Rgc2 from the channel

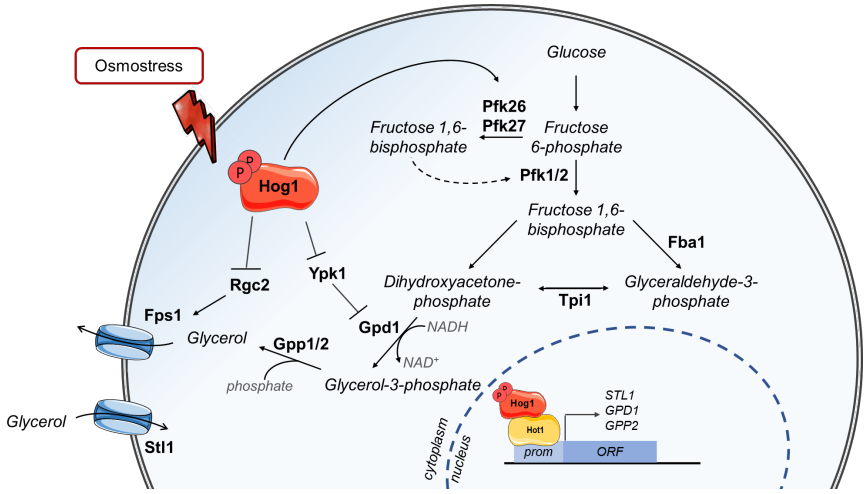


Figure 10. Carbohydrate metabolism regulation through Hog1 upon osmostress. Glycerol rapidly accumulates upon osmostress in a Hog1-dependent manner. Hog1 inhibits Rgc2 producing the closure of Fps1 channel. The MAPK also activates glycerol synthesis via the inhibition of Ypk1 and the subsequent activation of Gpd1. Finally, Hog1 also activates Pfk26 which generates more Fru-2,6-P_2 that activates glycolysis.

and, subsequently, its closure (Babazadeh et al., 2014; Lee et al., 2013).

Yeast also increases the glycerol levels by regulating the glycerol synthesis both at protein and transcriptional level. Under no stress conditions, Gpd1 and Gpd2 are negatively regulated by phosphorylation of different stress-dependent kinases. In the case of Gpd1, is a target of the TORC2-dependent kinases Ypk1 and Ypk2. Ypk1 basally phosphorylates and inhibits Gpd1 at Ser24; but when Hog1 is activated by osmostress, the MAPK phosphorylates Ypk1 at Thr622 inactivating the TORC2-dependent kinase and promoting the synthesis of glycerol by the dephosphorylated and activated Gpd1 (Lee et al., 2012; Roelants et al., 2011). This Gpd1 dephosphorylation is essential for rapid osmostress adaptation. As well, Hog1 also enhances the transcription levels of *GPD1* and *GPP2* genes in order

to maintain high glycerol intracellular concentration after Hog1 phosphorylation decay. Here, both *GPD1* mRNA transcription and Gpd1 dephosphorylation are essential for long-term adaptation to osmostress (Ferreira et al., 2005; Rep et al., 1999, 2000).

Even though, the Fps1 channel closure is the fastest way to increase intracellular glycerol levels, yeast also promotes glycerol uptake thanks to *STL1* gene expression, which encodes for the glycerol/H^+ symporter. In this case, Hog1 phosphorylates and activates the Hot1 transcription factor that regulates a subset of Hog1-responsive genes, such as *STL1, GPD1* and *GPP2* (Alepuz et al., 2001; Ferreira et al., 2005).

Moreover, Hog1 is also suggested to control the first steps of glycolysis through the regulation of Pfk activity. Dihazi *et al*., proposed that Hog1 directly phosphorylates Pfk26 increasing the amount of Fru-2,6-P_2, which is essential to increase glycerol levels upon osmostress (Dihazi et al., 2004). Nevertheless, other studies have proposed that the role of Pfk26 and Pfk27 is maintaining part of the carbon flux towards the glycolytic pathway rather than helping directly with osmoadaptation. Moreover, deletion of *PFK26* and *PFK27* genes produce a delay on Hog1 dephosphorylation upon osmostress, indicating that both enzymes are important for cell recovery after osmotic stress (Petelenz-Kurdziel et al., 2013b).

Carbohydrate metabolism regulation and, subsequently, glycerol accumulation is crucial for yeast cells to adapt to osmostress. In this thesis, I am going to focus on unraveling novel Hog1 interactions with the carbohydrate metabolism upon osmostress.

OBJECTIVES

Our group is interested in understanding the cell response to environmental stress. In this thesis, we aimed to identify novel substrates for Hog1 and the regulation of carbohydrate metabolism upon osmostress. We focused this thesis on the study of the contribution of Pfk27, as a new target of the HOG pathway, to rewire carbohydrate metabolism upon stress.

Specifically, the main objectives of this PhD were:

1. To perform an *in vitro* unbiased biochemical screening to identify novel Hog1 targets.

2. To characterize Pfk27 as a new target of the HOG pathway.

3. To study the role of Pfk27 in carbohydrate metabolism upon osmostress.

EXPERIMENTAL PROCEDURES

Growth media.

E.coli strains were grown at 37ºC in LB (Luria-Bertani) broth containing 1% bactoryptone, 0.5% yeast extract, and 1% NaCl. Plasmids were amplified using DH5α-competent cells. Each specific strain was grown in LB medium supplemented with ampicillin (100ug/ul) and/or chloramphenicol (25 ug/ml).

Yeast cells were grown at 30ºC in yeast extract peptone dextrose (YPD) or synthetic defined (SD) selective media lacking specific amino acids.

Plasmids.

The GST-Hog1 and GST-Pbs2^{DD} (PBS2 with S514D and T518D mutations) were cloned in pGEX4T as described in Bilsland-Marchesan *et al*., 2000. The *RCK2* gene was amplified by PCR from BY4741 genomic DNA and cloned in pGEX-6P-1 into *BamHI / XhoI* sites.

The GST-Pfk27 was obtained using the BY4741 genomic DNA as a template by PCR amplification and subcloned into *BamHI / XhoI* sites of the pGEX-6P-1. The different mutants of Pfk27 were generated by site directed mutagenesis using pGEX-6P-1-Pfk27 as a template were S or T were mutated to A or E: S144A, S158A, S144A S158A, S144A T157A S158A S159A, S144E T157E S158E S159E.

Yeast strains.

The strains used in this work were derived from BY4741 (MATa his3-D1 leu2-D0 met15-D0 ura3-D0).

In the Hog1-as screening, all the TAP-tagged proteins were obtained from the yeast TAP-tagged collection (Open Biosystems).

To assess the *in vivo* phosphorylation experiments, it was used *pfk27-TAP::HIS* (Open Biosystems) and derived from this *pfk27-TAP hog1::hph (*YAP1*)* and *pfk27-TAP rck2::URA* (YAP10). The Pfk27 point mutations were integrated on top of the *pfk27::56ΔURA* (YAP50) and then tagged with TAP::HIS: *pfk27 T278A-TAP::HIS* (YAP192), *pfk27 S144A-TAP::HIS* (YAP152), *pfk27 S158A-TAP::HIS* (YAP170a), *pfk27 S144A S158A-TAP::HIS* (YAP166), *pfk27 S144A T157A S158A S159A-TAP::HIS* (YAP171).

For Hog1 phosphorylation and metabolites measurements the following strains were used: *hog1::KAN* (Open Biosystems), *rck2::KAN* (Open Biosystems), *pfk26::KAN* (Open Biosystems), *pfk27::KAN* (Open Biosystems), *pfk26::KAN pfk27::KAN* (YAP55) *pfk27 S144A S158A* (YAP153), *pfk26::KAN pfk27 S144A S158A* (YAP160), *pfk27 S144A T157A S158A S159A* (YAP169), *pfk26::KAN pfk27 S144A T157A S158A S159A* (YAP173).

To study the growth phenotype by competition assays, the following strains were generated from the previous ones by tagging *ENO1* gene with YFP or mCherry. The strains were: *eno1-YPF::HPH* (YAP102), *rck2::KAN eno1-mCherry::HPH* (YAP98), *pfk26::KAN eno1-mCherry::HPH* (YAP99), *pfk26::KAN pfk27::KAN eno1-mCherry::HPH* (YAP101), *pfk27 S144A T157A S158A S159A eno1-mCherry::HPH* (YAP187), *pfk26::KAN pfk27 S144A T157A S158A S159A eno1-mCherry::HPH* (YAP188).

Antibodies.

The antibodies used are as follows: anti- Phospho p38 MAPK (Thr180 / Tyr 182) Rabbit mAb (Cell Signaling, 92155), anti-Hog1 D3 (Santa Cruz, SC-165978), anti-GST (GE Healthcare, 27-4577-01), anti-G6PDH (Sigma, A9521) and Peroxidase Anti-Peroxidase Soluble Complex Antibody (Sigma, P1291).

TAP-tagged yeast collection purification.

Pools of 10 different TAP-tagged proteins from the TAP collection (Open Biosystems) were grown in 1L of YPD until OD_{660} = 2.0, cells were centrifuged 5 minutes at 3200 rpm at 4ºC and pellets were stored at -80ºC until further processing. Pellets were resuspended in 5ml of Buffer A (50 mM Tris HCl pH 8.0, 15 mM EDTA pH 8.0, 15 mM EGTA pH 8.0, 0.1% Triton X-100) supplemented with 2 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine, 2 mg/ml leupeptin and 2 mg/ml of pepstatin. Glass beads were added and cells were lysated by vortexing, 10 rounds of 30 seconds at 4ºC. Cell extracts were cleared by centrifugation 15 minutes at 4000 rpm at 4ºC, supernatant was transferred to a 15 ml tubes. Next, 75 µl of previously equilibrated IgG-sepharose (Sigma) was added to each sample and rotated for 2h at 4ºC. Samples were spin 1 minute at 1200 g at 4ºC and supernatant was removed. IgG beads were recovered in 1 ml of Buffer A and transferred to a new eppendorf. Beads were washed 4 times with Buffer A and 5 times with Tris-DTT (50 mM Tris HCl pH 8.0, 2 mM DTT). Samples containing a minimum volume of Tris-DTT were stored at -80ºC until used for kinase assay.

Phenyl ethyl ATP radiolabeling.

First, Co^{2+} resin was equilibrated with HBS (150 mM NaCl, 10 mM HEPES pH 7.4) and mixed with 200 µg of resin with nucleoside diphosphate kinase (NDPK) (The NDPK was kindly given by Kevan M. Shokat and purified as described in (Kraybill et al., 2002) to obtain a bed volume of 100 µl. Aliquots of ready-to-use NDPK can be stored at -80ºC. 100 µl of NDPK were diluted in 1 ml of HBS and incubated 5 minutes on ice. The resin was placed in a Mobicol mini-column of 800 µl and washed with 1.5 ml of HBS + 5 mM $MgCl_2$. 2 mCi (7000 Ci/mmol ICN) of [γ^{32}P]-ATP were added to the column and incubated for 5 minutes at 30ºC. Washed with 2.5 ml HBS + 2.5 mM $MgCl_2$. Then, 8 µl of phenyl ethyl-ADP 1 mM were diluted in a final volume of 40 µl of HBS + 2.5 mM $MgCl_2$ and incubated 15 minutes at 30ºC. [γ^{32}P]-Phenyl ethyl ATP ([γ^{32}P]-PE-ATP) was eluted with 450 µl of HBS + 2.5 mM $MgCl_2$ and stored at -20ºC.

Hog1-as purification from yeast.

The strain PFY180 (TM233 *hog1*Δ) was transformed with the plasmid pX214-HOGAS. 2 L of culture were grown in Ura^- SD medium and collected at OD_{660} = 2 by centrifugation at 4000 rpm during 10 minutes at 4ºC. Cells were disrupted using Mortar Grinder for 10 minutes. Next, cell pellets were resuspended in 10 ml of Buffer A (50 mM Tris HCl pH 8.0, 15 mM EDTA pH 8.0, 15 mM EGTA pH 8.0, 0.1 % Triton X-100, 150 mM NaCl) supplemented with 2 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine, 2 mg/ml leupeptin and 2 mg/ml of pepstatin. Cell extracts were centrifuged at 14000 rpm for 5 minutes at 4ºC and

supernatant was incubated 4 hours at 4ºC with 170 µl of equilibrated Glutathione Sepharose 4B beads (GE Healthcare) per liter of culture. Beads were washed 4 times with Buffer A supplemented with protease inhibitors and 4 times with Tris-DTT buffer (50 mM Tris HCl pH 8.0, 2 mM DTT). Finally, GST-Hog1-as was eluted 2 times in 200 µl of elution buffer (10 mM reduced glutathione (GSH), 20 mM Tris pH 9.5, 500 mM DTT) by 30 minutes of ration at 4ºC. Purified protein was stored at -80ºC until further use.

Hog1-as screening kinase assay.

1.5 µg of GST-Hog1-as was pre-activated with 0.5 µg of GST-Pbs2^{DD} in kinase buffer (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 20 mM DTT) and 100 µM ATP for 30 min at 30ºC. In the meantime, TAP purification tubes were thawed on ice. Then, 5 µl of a 1/5 dilution of [γ^{32}P]-PE-ATP were deposit into the TAP purification tubes. The reaction was started by adding 25 µl of the pre-activated kinase to the substrates tubes and incubated for 20 minutes at 30ºC. Next, the reaction was stopped by the addition of 10 µl of 5x SDS loading buffer and boiled 5 minutes at 95ºC. Finally, proteins were separated by SDS-PAGE, blotted into a PVDF membrane and radioactivity was detected with Kodak X-OMAT films (Sigma) and proteins with Peroxidase Anti-Peroxidase Soluble Complex Antibody (Sigma, P1291).

Expression and purification of recombinant proteins.

Recombinant GST tagged proteins were expressed in *E. coli* BL21 strain. First, bacteria was grown at 37ºC to OD_{600} = 0.6 and then

induced by the addition of 1 mM isopropylthiogalactoside (IPTG) for 5 hours at 25ºC. Cells were collected by centrifugation 8 minutes at 6.000 g at 4ºC and lysed with STET buffer (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 5 % Triton X-100, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2 g/ml leupeptin and 2 g/ml pepstatin), and sonicated. Cleared extracts were obtained by centrifugation 10 minutes at 7,000 g at 4ºC. Recombinant GST-proteins were immunoprecipitated from the supernatant using glutathione-sepharose beads (GE Healthcare) for 1:30 hours rotating at 4ºC. Beads were washed with STET buffer and Tris-DTT (50 mM Tris pH 8.0, 2 mM DTT). Finally, bound proteins were eluted in elution buffer (10 mM reduced glutathione (GSH), 50 mM Tris-HCl pH 9.5, and 1mM DTT). Recombinant proteins were stored at -80ºC until further use.

***In vitro* kinase assays.**

1µg of GST-Hog1 was pre-activated with 0.5 µg of GST-Pbs2^{DD} in kinase buffer (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 20 mM DTT) and 50 µM ATP for 30 minutes at 30ºC. Then, 4 µg of each GST-Pfk27 *wild-type* or mutant protein were included in the kinases mix with radiolabeled γ-^{32}P ATP (5 µCi / sample, Perkin Elmer), and incubated 30 minutes at 30ºC. The reaction was stopped by the addition of 5X SDS loading buffer. Samples were resolved on 10 % SDS-PAGE, blotted into PVDF membrane and detected by autoradiography. Signals were quantified using Amersham Typhoon

Biomolecular Imager (GE Healthcare). GST-proteins were detected with Naphthol Blue (Sigma Aldrich).

Western blotting.

For cellular extracts, 1 ml of yeast culture at OD_{660} = 0.4 was stopped with 100 µl of STOP solution (200 mM Sodium Fluoride, 200 mM Sodium Azide) and pelleted by centrifugation 1 minute at 10000 rpm. Cell pellets were resuspended in 50 µl of 1x SDS loading buffer with 50 µl of glass beads, lysated 1 minute with FastPrep and boiled 5 minutes at 95ºC.

Protein extracts were resolved using SDS-PAGE and then blotted onto a PDVF membrane. Following incubation of the blots with the indicated antibodies, signals were detected using the ECL detection reagent (BioRad). Except for α- Phospho p38 MAPK (Thr180 / Tyr 182) and α-Hog1 D3 where secondary fluorescence antibodies were used (IRDye 680RD donkey anti-rabbit IgG secondary antibody and IRDye 800CW goat anti-mouse IgG secondary antibody from LI-COR) and detected with Odyssey IR 9120 (Li-COR). All the images were quantified using GelAnalyzer (version 19.1).

Phostag gels for *in vivo* phosphorylation assessment.

For resolving Pfk27-TAP electromobility shift, 35 µM of Phos-tag (Wako) and 70 µM $MnCl_2$ was added to the 8 % SDS-PAGE gels. Gels were run 30 minutes at 100 V at room temperature (RT) and 2:15 hours at 30 mA on ice. Proteins were transferred to a PVDF membrane for 2 hours at 200 mA on ice. Pfk27-TAP mobility shift was detected by western blot using Peroxidase anti-Peroxidase

antibody (sigma-Aldrich, 1:2000 in TBS-T supplemented with 5% milk).

ADP-Glo *in vitro* activity assay.

0.5 µg of recombinant GST-Pfk27 versions were incubated 30 minutes at 30ºC with kinase buffer (40 mM Tris HCl pH 7.5, 20 mM $MgCl_2$, 0.1 mg/ml Bovine Serum Albumin), 5 mM ATP (ADP-Glo Kinase Assay, Promega) and 5 mM Fructose 6-phosphate (Sigma). Next, concentration of the ADP generated was measured with ADP-Glo Kinase Assay kit (Promega). 5 µl of ADP-Glo Reagent was added and incubated 40 minutes at room temperature to stop the reaction and deplete the unconsumed ATP. Then, by adding 10 µl of Kinase Detection Reagent the ADP was converted to ATP and luciferase and luciferin are introduced to detect ATP. Luminescence was read after an incubation of 30 minutes at room temperature and protected from light. An EnSight –alpha lector (Perkin Elmer) was used to measure luminescence.

Metabolite measurements.

Yeast cells were grown until OD_{660} = 0.4 and collected by centrifugation 30 seconds 10,000 rpm 4 ºC (1 ml for glycerol and pyruvate, 2 ml for trehalose and 5 ml for protein concentration measurements). Cell pellets were washed once with PBS 1X. Finally, samples were frozen in liquid nitrogen and stored at – 20ºC until further use. Protein concentration measurement, cell pellets were resuspended in H_2O milliQ and assayed with Bradford reagent (BioRad). For trehalose measurements, manufacturer protocol

(Megazyme, K-TREH) was used with minor modifications; reaction volume was escalated to 100 µl. Glycerol and pyruvate concentrations were measured following the supplier protocols (Sigma Aldrich, MAK117 and MAK071). Colorimetric assays were red with Synergy HTX (BioTek).

Growth curves.

For growth curves analysis, cells were diluted at OD_{660} = 0.05 in YPD or YPD + 0.8 M NaCl. Growth was monitored at 30ºC every 30 minutes for 48 h in a 96-well plate using Synergy H1 Multi Mode Reader (Biotek). The figure plots the average and the standard deviation of three biological replicates, each replica consisting of at least two technical replicates.

Competition Assays.

Cells were grown overnight at 30ºC shaking in YPD. Cells were mixed (Cherry/YFP) as needed at initial OD_{660nm} = 0.05 in YPD and YPD + 0.8 M NaCl. Cells were diluted every morning and evening in the indicated media and never reach the stationary phase. For each time point, 1 ml of cells was harvested and stored at 4ºC until further processing. Cells were centrifuged for 3 minutes at 3,000 rpm at RT and resuspended with 1 ml 1X PBS (Phosphate buffered saline). Then 150 µl of cells were transferred into a sterile 96 well-plate (Thermo Fisher). Samples were analyzed with Sony SA3800 (Sony). A total of 10,000 events were analyzed using FlowJo (V10) software.

RESULTS

1. IDENTIFICATION OF NEW POTENTIAL HOG1 TARGETS BY A BIOCHEMICAL SCREENING

Several cellular functions have been associated to proper adaptation to stress such as the regulation of metabolism, protein folding, translation, gene expression and cell cycle progression. There are a number of targets already identified for p38 and Hog1, which have facilitated the unravelling of the role of these kinases in the regulation of physiological functions (see Introduction). However, the identification of the cellular functions and their underlying molecular mechanisms required for stress adaptation have not been systematically addressed. Such a systematic approach is essential to specifically study some of the functions downstream of these kinases as well as the role of additional signaling pathways in stress adaptation. For this reason, we performed an *in vitro* unbiased biochemical screening to discover new Hog1 targets.

1.1 Generation of a Hog1 analogue-sensitive mutant

With the aim of identifying Hog1 direct substrates, we took advantage of a previously described Hog1 analogue sensitive (Hog1-as) mutant version which carries the mutation T100G (Kim and Shah, 2007; Westfall and Thorner, 2006). The T100 site is located inside the ATP binding pocket of Hog1. The change of Threonine to Glycine produces a reduction in size of the amino acid side chain and, consequently, the ATP binding pocket becomes larger (Fig. 11). In the ATP binding pocket of Hog1-as can fit the bulky ATP analogue N(6)-2-phenyl ethyl ATP (PE-ATP), which cannot be used by non-mutated kinases (Allen et al., 2007). The combination of the use of the Hog1-

as version and PE-ATP served to specifically uncover Hog1 direct targets.

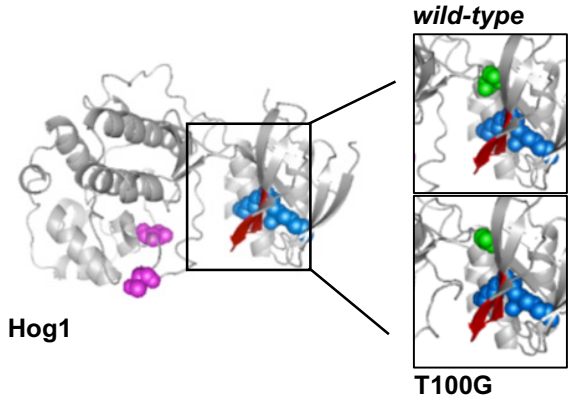


Figure 11. Generation of a Hog1-as mutant. Hog1 protein model generated from crystal structure of human p38α sequence (PDB: 5o8u; (Bührmann et al., 2017). The Hog1 phosphosites are colored in pink, the catalytic sites in blue and the ATP binding region in red. Right panel: upper image corresponds to *wild-type* Hog1 (T100) while the bottom image is the Hog1-as mutant (T100G), where the T100 or G100 site is highlighted in green.

1.2 Identification of new Hog1 targets

To test the entire yeast proteome, a simple but efficient approach was designed using the yeast TAP-tagged collection (Ghaemmaghami et al., 2003) (Fig. 12A and Experimental Procedures section). Briefly, in the TAP collection each ORF (open reading frame) is tagged with TAP tag in the C-terminal side and expressed from its natural chromosomal location. The TAP epitope consists of a modified version of the Tandem Affinity Purification (TAP) tag, which includes the calmodulin binding peptide, a TEV cleavage site and two IgG binding domains from *Staphylococcus aureus* (Gavin et al., 2002; Rigaut et al., 1999) and thus can be used for a two steps purification system. To screen more than 4,000 proteins, we first

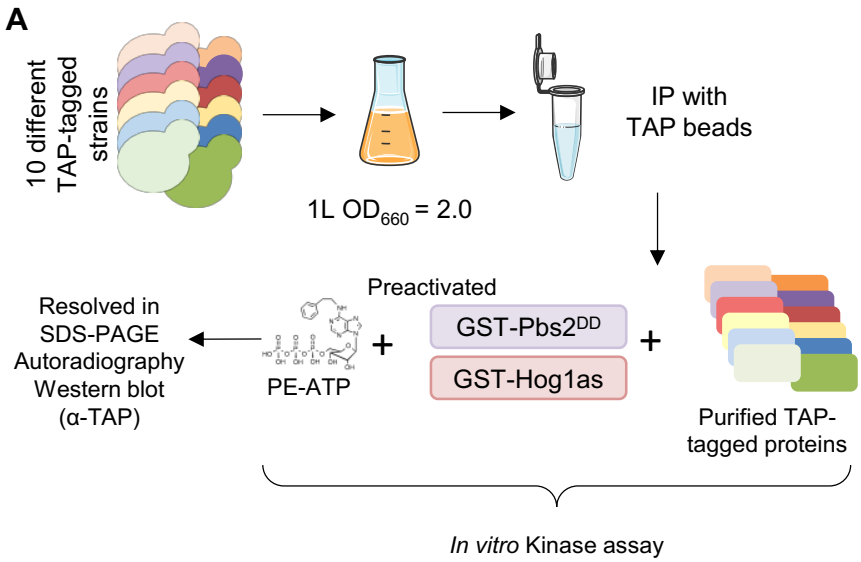
created pools of ten different TAP-tagged proteins with similar protein expression level but differing in their molecular weight. Next, the ten strains of each pool were grown together in rich medium (YPD) until they reach an OD_{660} = 2.0, cells were pelleted and stored at -80ºC until further processing. To purify the TAP-tagged proteins from each pool, cell extracts were incubated with IgG Sepharose beads. Finally, an *in vitro* kinase assay was performed with GST-Hog1-as activated by the genetically active version of the Pbs2 MAP2K (GST-Pbs2^{DD}; S514D, T518D), purified TAP-tagged proteins and radiolabeled PE-ATP (Fig. 12A). These samples were resolved by SDS-PAGE, phosphorylation was detected by autoradiography and TAP-proteins by blotting with α-TAP antibody (Fig. 12B).

Once a positive was detected, the corresponding TAP strain was grown isolated and the phosphorylation of the purified protein was validated in an *in vitro* kinase assay as described before.

Thus, using chemical genetics in yeast, we have performed a systematic biochemical assay and tested more than 4,000 purified proteins in an *in vitro* kinase assay with Hog1. From those studies, we were able to define 169 positive candidates phosphorylated by GST-Hog1-as *in vitro*, representing 4% of the total yeast proteome (Supplementary Table 1). Some of these proteins were already known Hog1 substrates, such as Sic1 (Escoté et al., 2004), Hsl1 (Clotet et al., 2006), Mrc1 (Duch et al., 2013b), Whi5 (González-Novo et al., 2015) and Sko1 (Gomar-Alba et al., 2013), affording validity to the screening results. Furthermore, an 80% of the 169 targets of Hog1

are conserved in mammals, highlighting the potential complexity of the role of the SAPKs in stress adaptation.

Clustering of the positive candidates by their cell function showed some enriched cellular functions (Supplementary Table 1 and Fig. 13). In the positive hits list, there were 23 proteins related with cell cycle, 15 required for replication and 24 involved in transcription, emphasizing the role of Hog1 in these cellular functions (see Introduction). Moreover, there was a cluster of 24 proteins that are related with translation and ribosome biogenesis processes, pointing



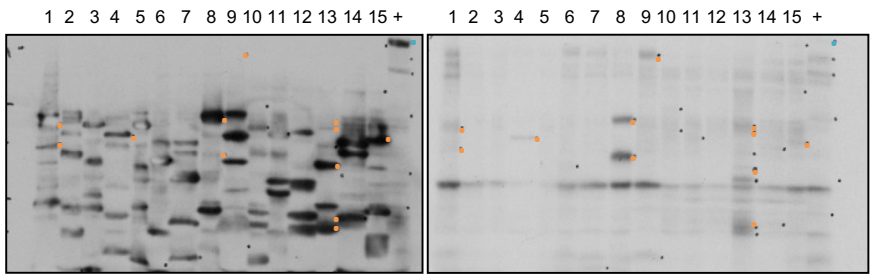


Figure 12. Unbiased biochemical screening to unravel new Hog1 targets. (A) Scheme of the Hog1-as screening protocol. The whole yeast proteome was divided into pools each one containing ten proteins of different molecular weights but similar protein expression levels. Each of these batches was grown together in 1L of YPD to an OD_{660} = 2.0 and then cells were pelleted by centrifugation. TAP-tagged proteins were purified using IgG Sepharose beads. For the *in vitro* kinase assay, GST-Hog1-as was activated with GST-$Pbs2^{DD}$. Once activated, they were incubated together with the TAP-tagged purified proteins and with radiolabeled ATP. Samples were resolved in SDS-PAGE, phosphorylation signals were detected by autoradiography and TAP-tagged proteins by blotting using TAP antibody. **(B)** Example of one blot to detect TAP-tagged proteins and the corresponding autoradiography to detect phosphorylated candidates. Numbers 1 to 15 represent different batches analyzed in this membrane, also a positive control (Ede1) (+) was always run in parallel. Orange dots indicate those proteins detected to be phosphorylated; blue dot points out the positive control.

out the importance that Hog1 could have in these mechanisms. It is worth mentioning that the clustering also revealed 25 proteins involved in vesicle trafficking, which has not yet been related with Hog1 signaling, and it is currently investigated in our group. Last, the functional analyses showed that 13 Hog1 target candidates were related to carbohydrate metabolism (Fig. 14). Although Hog1 regulation of glycerol synthesis upon osmostress is well established, it is not fully understood how it modulates other aspects of carbohydrate metabolism. Thus, this thesis focused on further study one of the candidates of the screening, the phosphofructokinase 27 enzyme (Pfk27), one of the metabolism-related Hog1 targets.

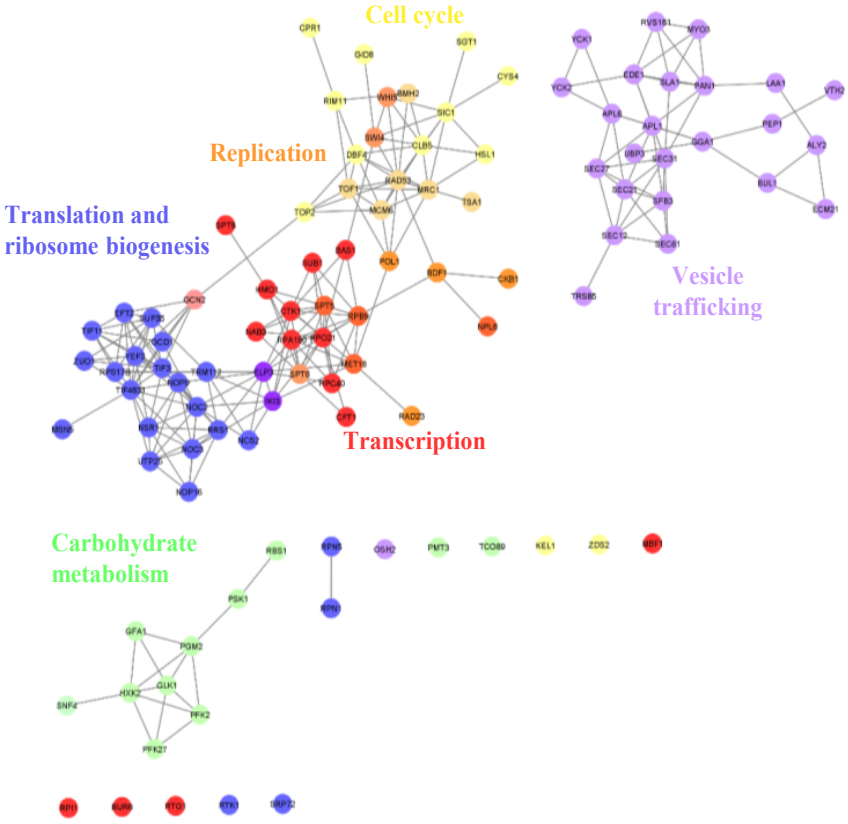


Figure 13. Functional network of potential Hog1 targets. The functional network of each cell function (cell cycle, transcription, replication, translation and ribosome biosynthesis, vesicle trafficking and carbohydrate metabolism) was performed using STRING. Then, the different networks were merged using Cytoscape software.

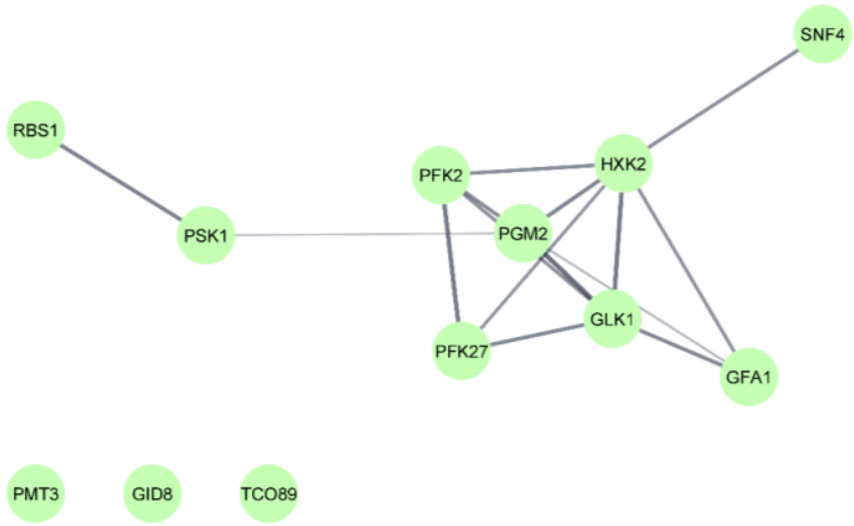


Figure 14. Functional network of the candidates related with carbohydrate metabolism. The functional network of carbohydrate metabolism-related candidates was performed using STRING.

2. PFK27 AS A NEW TARGET OF THE HOG PATHWAY

Upon osmostress, yeast cells need to increase the intracellular levels of glycerol to compensate the osmotic imbalance. The role of Hog1 in glycerol synthesis has been well studied, nevertheless some studies suggest that Hog1 regulates other enzymes of carbohydrate metabolism to overcome the osmostress situation (Dihazi et al., 2004; Petelenz-Kurdziel et al., 2013b). Previously, it was described how Hog1 regulates Pfk26 in order to modulate carbon flux upon osmostress (Dihazi et al., 2004) (see Introduction). Pfk27, the enzymatic partner of Pfk26, arose from our screening as one of the putative candidates to be a Hog1 substrate.

2.1 Hog1 indirectly modulates Pfk27 phosphorylation upon osmostress

To validate the biochemical screening, we first assessed whether Pfk27 was phosphorylated *in vivo* upon stress by Phos-tag assays. *Wild-type* and *hog1* mutant Pfk27-TAP cells were grown to exponential phase and exposed or not to stress (0.4 M NaCl) for the indicated time points (Fig.15B). After cell lysis, samples were resolved in a Phos-tag gel to compare the phosphorylation status of Pfk27 under the different conditions and time points. In *wild-type* cells, Pfk27-TAP was phosphorylated 10 minutes after osmostress and returned to the unphosphorylated form after 30 minutes. In contrast, in *hog1* cells, this phosphorylation shift was not present confirming that Hog1 regulates Pfk27 phosphorylation upon osmostress.

Pfk27 contains a kinase domain (85 – 305 amino acids) known as 6-phosphofructo-2-kinase domain. In this domain, there is the only potential Hog1 phosphorylation site (S/TP motif), T278 (Fig. 15A). Thus, we created a mutant strain containing the mutation of T278 to A278. To our surprise, the non-phosphorylatable alanine substitution of Pfk27 (*Pfk27*^{T278A}) did not prevent the gel-shift observed upon stress, and thus, additional phosphorylation sites should exist that mediate this gel shift in the Phos-tag gels (Fig. 15B). In conclusion, these results suggest that Pfk27 phosphorylation upon osmostress depends on Hog1 but that may be through an indirect regulation.

To determine if Hog1 was phosphorylating Pfk27 *in vitro*, recombinant GST-Pfk27 purified from bacteria was incubated with previously activated GST-Hog1 by GST-Pbs2^{DD} in the presence of radiolabeled ATP for 30 minutes at 30ºC. The *in vitro* kinase assay

did not show any phosphorylation signal for GST-Pfk27 in these conditions (Fig. 15C), pointing out that Pfk27 is not directly phosphorylated by Hog1.

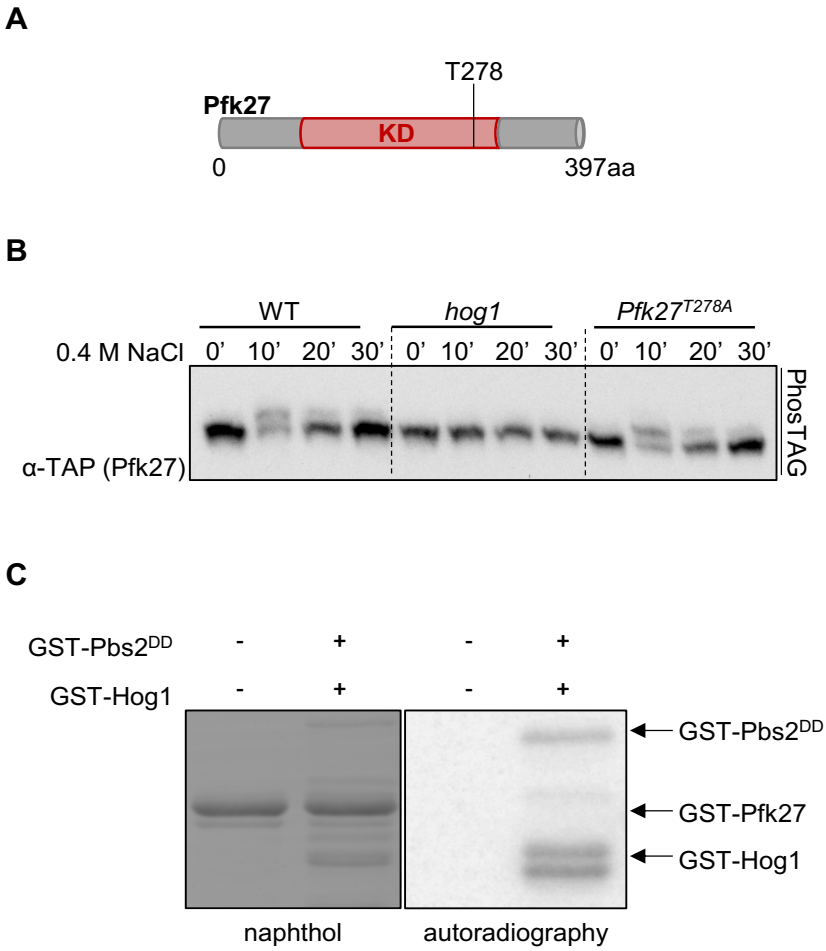


Figure 15. Hog1 modulates Pfk27 phosphorylation upon osmostress. (A) Scheme of Pfk27 protein with the S/TP site highlighted. KD indicates the kinase domain of Pfk27. **(B)** Pfk27-TAP tagged cells of the indicated strains were exposed to stress (0.4 M NaCl) and samples were collected at the indicated time points. Protein extracts were analyzed in 8% polyacrylamide gel supplemented with 35 µM Phos-tag, and Pfk27 mobility was followed by Western blot (α-TAP antibody). **(C)** Bacterially expressed GST-tagged Pfk27 was used as substrate for the *in vitro* kinase assay with GST–Hog1 preactivated with GST-Pbs2^{DD}.

2.2 Rck2 is the kinase in charge of Pfk27 phosphorylation *in vivo*

Rck2 is a Serine / Threonine kinase involved in the cellular response to osmostress and oxidative stress. Rck2 was initially described as a kinase downstream of Hog1 which was required for the control of translation (Bilsland-Marchesan et al., 2000; Teige et al., 2001). Moreover, in 2017, Romanov and colleagues described Rck2 as a new hub for spreading the osmostress signal response (Romanov et al., 2017). To test whether if Rck2 could be the kinase in charge of Pfk27 phosphorylation, *wild-type* and *rck2* mutant cells were subjected to osmostress (0.4 M NaCl), samples were collected at the indicated time points and resolved in a Phos-tag gel. As shown in Figure 16, mutation of *RCK2* fully abolished the Pfk27 shift upon stress in comparison to *wild-type* cells (Fig. 16), indicating that Rck2 is required for Pfk27 phosphorylation.

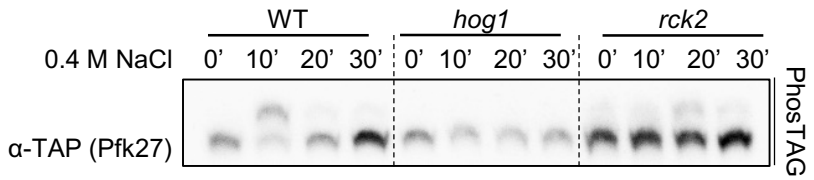


Figure 16. Rck2 regulates Pfk27 phosphorylation *in vivo*. Pfk27-TAP tagged cells of the indicated strains were subjected to stress (0.4 M NaCl) and samples were collected at the indicated time points. Protein extracts were analyzed in 8% polyacrylamide gel supplemented with 35 μM Phos-tag, and Pfk27 mobility was followed by Western blot (α-TAP antibody).

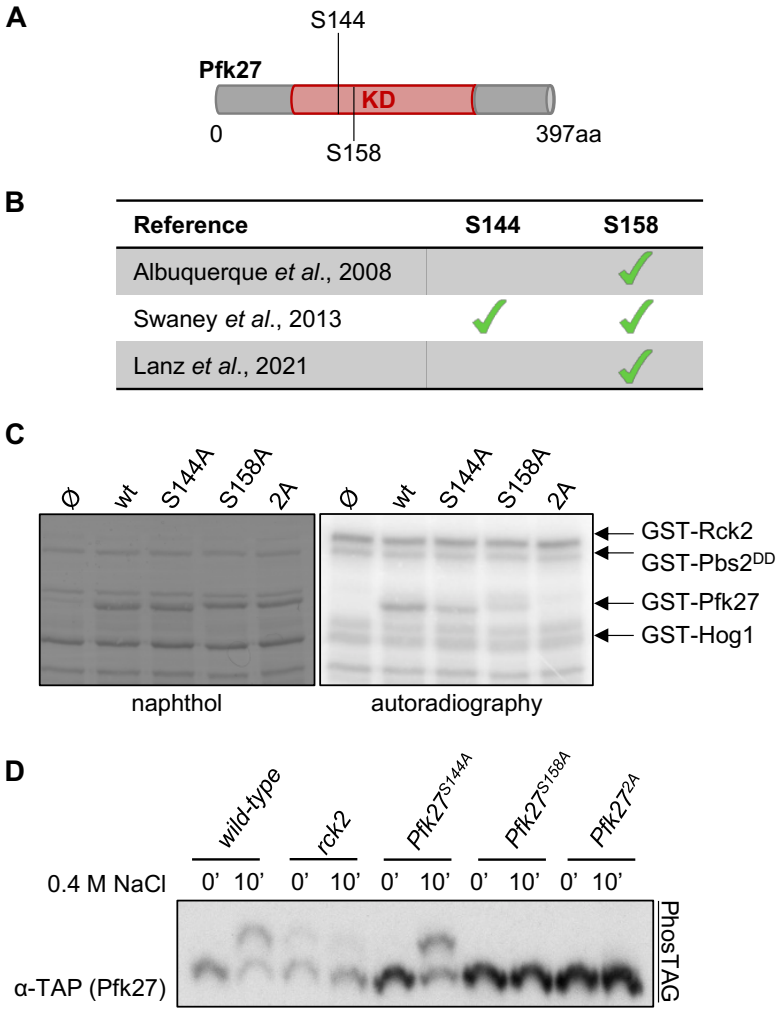


Figure 17. Rck2 phosphorylates Pfk27 at S144 and S158. (A) Scheme of Pfk27 indicating the Rck2 potential phosphorylation sites S144 and S158. **(B)** Table showing the previously described phosphorylation of the S144 and S158 sites. **(C)** *In vitro* kinase assay of recombinant GST-Pfk27 versions (*wild-type*, S144A, S158A and 2A) were incubated with radiolabeled ATP and GST-Rck2 preactivated with GST-Hog1 and GST-Pbs2^{DD}. **(D)** Pfk27-TAP versions (*wild-type,* S144A, S158A, 2A) cells were exposed to stress (0.4 M NaCl) and samples were collected at the indicated time points. Protein extracts were analyzed in 8% polyacrylamide gel supplemented with 35 µM Phos-tag, and Pfk27 mobility was followed by Western blot (α-TAP antibody).

To assess whether Rck2 is directly phosphorylating Pfk27, we performed an *in vitro* kinase assay with recombinant GST-Rck2 previously activated in the presence of GST-Pbs2^{DD} and GST-Hog1 an then incubated with GST-Pfk27 as a substrate together with radioactive ATP. As shown in Fig. 17C, Rck2 phosphorylates Pfk27 *in vitro* (Fig. 17C). Then, we scanned for potential Rck2 phosphorylation motifs (RXXS, SXXXL) in the Pfk27 protein sequence (Romanov et al., 2017) to identify Rck2-phosphorylation site(s). From eight motifs, we focused on those Pfk27 sites previously reported to be phosphorylated by mass spectrometry assays; S144 and S158 (Albuquerque et al., 2008; Lanz et al., 2021; Swaney et al., 2013) (Fig. 17A and 17B). We then assessed whether the single or double non-phosphorylatable mutants of Pfk27 were phosphorylated in *in vitro* kinase assays (Fig. 17C). Single mutation of both Pfk27 sites (S144A or S158A) showed a reduction of Pfk27 phosphorylation, being clearer in the S158A mutant. Moreover, the double mutation of Pfk27 sites (S144A S158A, namely *Pfk27^{2A}*) reduced the phosphorylation by Rck2 *in vitro* (Fig. 17C).

To assess whether S144A and S158A mutations in Pfk27 affected the phosphorylation pattern of Pfk27 *in vivo* upon osmostress, we tested *Pfk27^{S144A}, Pfk27^{S158A}, Pfk27^{2A}* strains by Phos-tag assays. Strikingly, the phosphorylation mobility shift was reduced when both mutations were present or in the presence of the single S158A (Fig. 17D). Together, these results suggested that Rck2 phosphorylates Pfk27 *in vitro* and *in vivo* and that these phosphorylations at least reside in the S144 and S158 sites of Pfk27.

2.3 The *Pfk27*^{2A} mutant does not show a delay in Hog1 phosphorylation upon osmostress

Once we identified the Rck2-dependent Pfk27 phosphosites, we aimed to determine the biological relevance of the phosphorylation on these sites. It was previously described that deletion of the *PFK26* and *PFK27* genes produces a delay in Hog1 dephosphorylation upon osmostress (Petelenz-Kurdziel et al., 2013).

Thus, we checked whether Hog1 phosphorylation was affected in the *pfk27*^{2A} mutant alone or in combination with *PFK26* gene deletion.

Deletion of the *RCK2* gene produced a significant delay in Hog1 phosphorylation (Fig. 18A), suggesting the important role of this kinase in the osmostress response. In addition, we confirmed the delay in Hog1 dephosphorylation in the *pfk26 pfk27* double deletion strain. Nevertheless, Hog1 phosphorylation in the strains carrying point mutations in *PFK27* (*pfk27*^{2A}), alone or in combination with the *PFK26* deletion, did not significantly differ from *wild-type* (Fig. 18B).

In conclusion, the Rck2-dependent osmostress response and, particularly, the deletion of *PFK26* and *PFK27,* delays Hog1 dephosphorylation suggesting an important role in cell recovery upon stress. Even so, the mutation of S144 and S158 Rck2-dependent sites on Pfk27 are not sufficient to mimic the *PFK27* gene deletion.

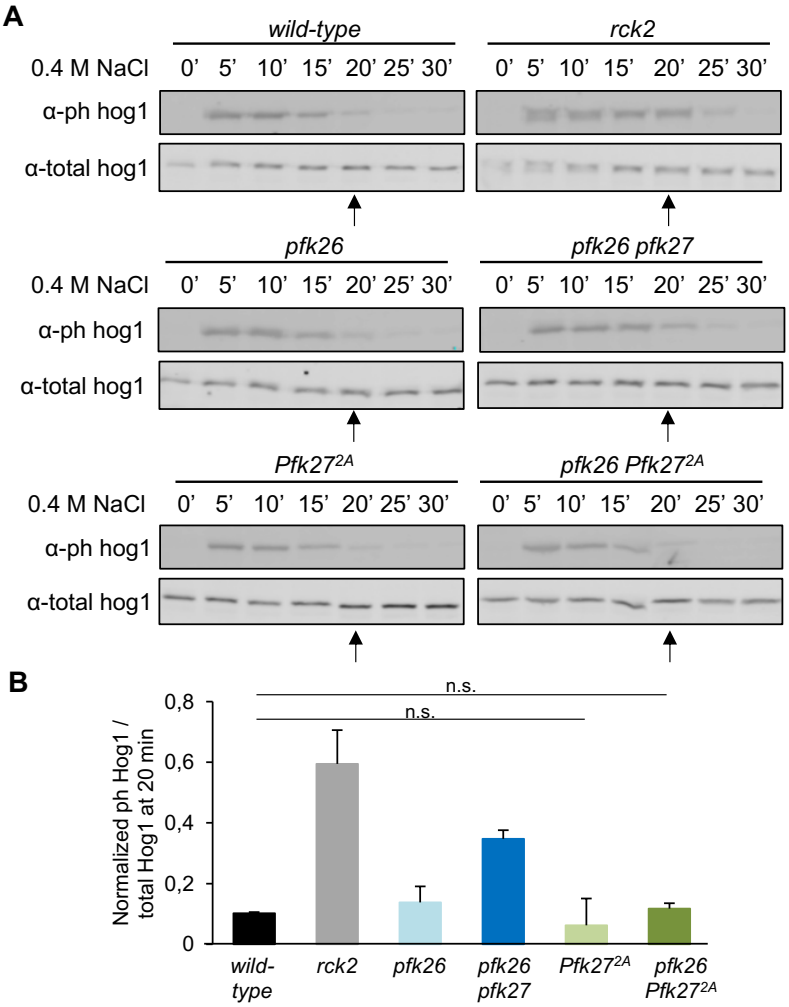


Figure 8. *Pfk27^{2A}* does not show a delay in Hog1 phosphorylation upon osmostress. (A) Cells of the indicated strains were exposed to stress (0.4 M NaCl) and samples were collected at the indicated time points. Protein extracts were resolved in 10 % SDS-PAGE gel and Hog1 was detected by Western Blot (α-ph Hog1 and α-total Hog1 antibodies). Arrows mark the time point quantified in panel B. **(B)** Quantification of Hog1 phosphorylation in the different strains at 20 minutes after stress. Hog1 phosphorylation was normalized by the total amount of Hog1 protein. Data represent the mean and standard deviation of biological triplicates. Asterisks indicate the Student's t test assuming unequal variance analysis comparing the indicated strains (n.s., no significance, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.005$)

2.4 The Pfk27 S144A T157A S158A S159A (*Pfk27^{4A}*) mutant displays a delay in Hog1 dephosphorylation upon osmostress

As the *Pfk27^{2A}* mutant strain did not show a differential phenotype in Hog1 dephosphorylation, we explored other mutant combinations. Browsing in the bibliography, there are two other Pfk27 sites previously reported to be phosphorylated under basal and stress conditions (Swaney et al., 2013). Interestingly, these sites surround the S158 site, namely T157 and S159. Thus, we created a Pfk27 mutant version containing the four sites mutated: S144A, T157A, S158A, S159A (*pfk27^{4A}*) (Fig. 19A and 19B).

Next, we tested the *in vitro* and *in vivo* phosphorylation status of this new mutant. First, we purified GST-Pfk27^{4A} mutant protein from *E. coli* and used it as a substrate to perform an *in vitro* kinase assay with previously activated GST-Rck2 by GST-Hog1 and GST-Pbs2^{DD}, in the presence of radiolabeled ATP. As expected, *Pfk27^{4A}* did not exhibit any phosphorylation signal as it contains the S144A and S158A mutations (Fig. 19C). To check the *in vivo* phosphorylation status of *Pfk27^{4A}* mutant, cells were treated as in previous sections and resolved in a Phos-tag gel. Again, the *Pfk27^{4A}* mutant strain presented the same phenotype as the *Pfk27^{2A}* mutant strain (Fig. 19D).

Next, the phosphorylation pattern of Hog1 was studied in the *Pfk27^{4A}* mutant strain to determine the biological relevance of these sites (Fig. 20). Cells were exposed to stress (0.4 M NaCl) for 30 minutes and samples were collected at the indicated time points. Then, Hog1 phosphorylation was assessed by Western Blot.

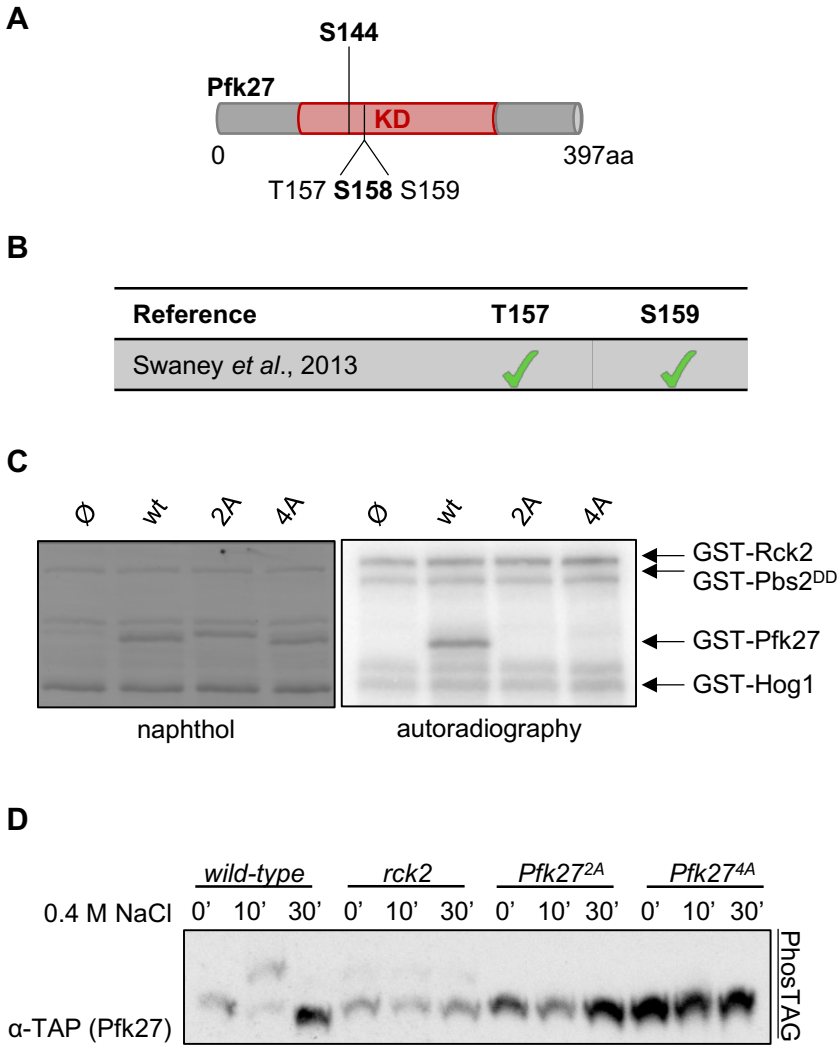


Figure 19. Generation of a four amino acid mutant of Pfk27. (A) Scheme of the *Pfk27^{4A}* protein with (S144, T157, S158, S159) sites mutated. **(B)** Table showing the previously described phosphorylations of the S144 and S158 sites. **(C)** *In vitro* kinase assay of recombinant GST-Pfk27 versions (*wild-type*, *Pfk27^{2A}* and *Pfk27^{4A}*) were incubated with radiolabeled ATP and GST-Rck2 activated with GST-Hog1 and GST-Pbs2^{DD}. **(D)** Pfk27-TAP tagged cells were stressed (0.4 M NaCl) and samples were collected at indicated time points. Protein extracts were resolved in 8% polyacrylamide gel supplemented with 35 µM Phos-tag, and Pfk27 mobility was followed by Western blot (α-TAP antibody).

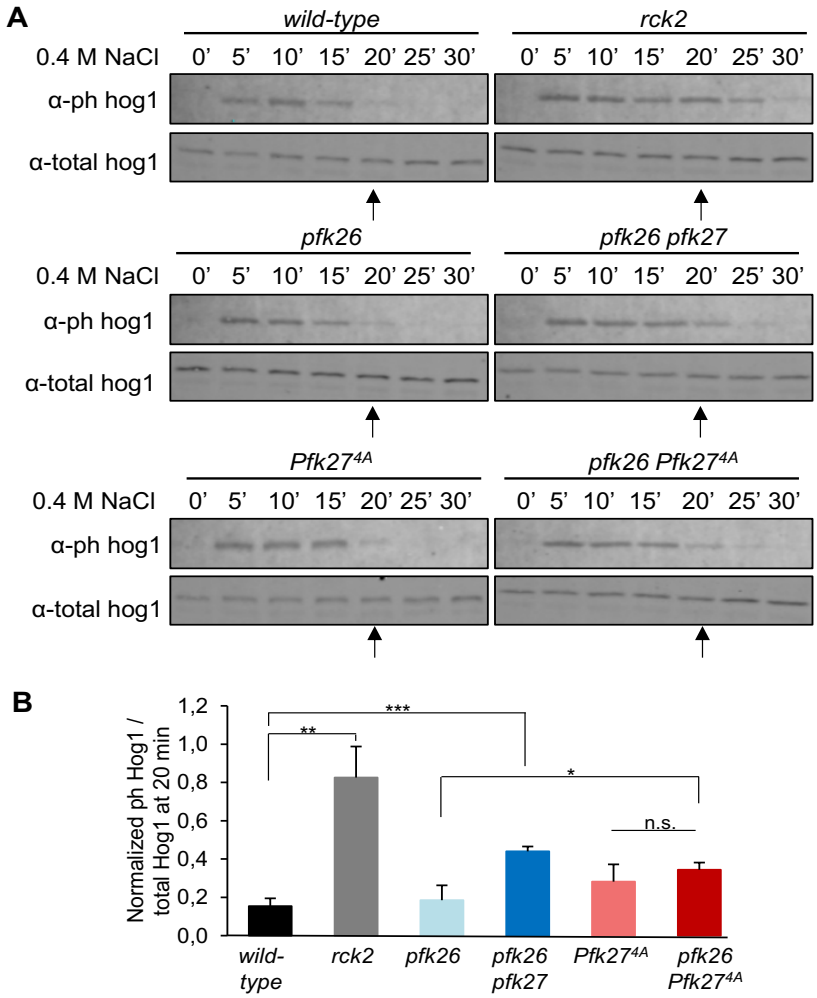


Figure 20. *Pfk27*^{4A} delays Hog1 dephosphorylation. (A) Cells of the indicated strains were exposed to stress (0.4 M NaCl) and samples were collected at the indicated time points. Protein extracts were resolved in 10 % SDS-PAGE gel and Hog1 was detected by Western Blot (α-ph Hog1 and α-total Hog1 antibodies). Arrows mark the time point quantified in panel B. **(B)** Quantification of Hog1 phosphorylation in the different strains at 20 minutes after the stress. Hog1 phosphorylation was normalized by the total amount of Hog1 protein. Data represent the mean and standard deviation of biological triplicates. Asterisks indicate the Student's t test assuming unequal variance analysis comparing the indicated strains (n.s., no significance, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$).

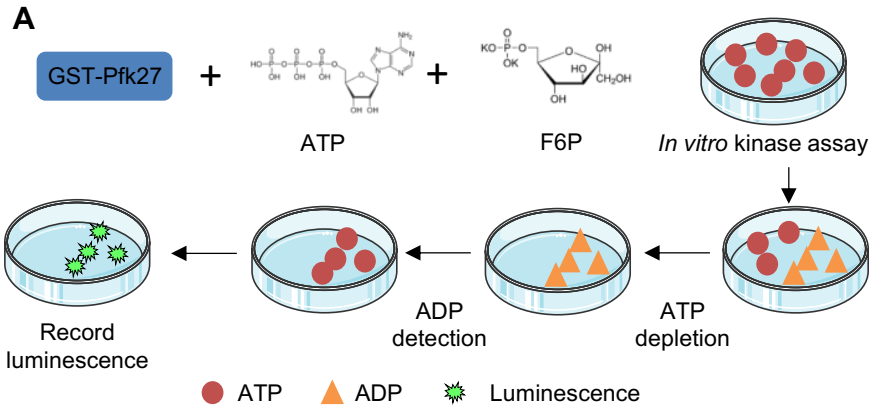
Pfk27^{4A}, alone or in combination with the *PFK26* deletion, showed a statistically significant delay in Hog1 phosphorylation at 20 minutes in comparison with *wild-type* and *pfk26* strains (Fig. 20B), suggesting that the phosphosites S144, T157, S158, S159 of Pfk27 are required for proper cell adaptation to osmostress.

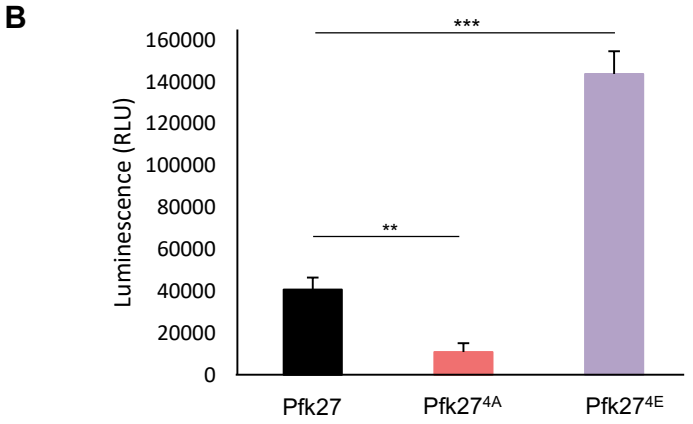
2.5 A phosphomimetic mutant of Pfk27 increases its *in vitro* activity

Next, we assessed whether the phosphorylation of Pfk27 by Rck2 affects its activity. To do so, an *in vitro* kinase assay of recombinant GST-Pfk27 together with its substrate (Fructose 6-Phosphate; F6P) was coupled to the detection of the amount of ADP generated from the reaction. Besides the non-phosphorylatable *Pfk27*^{4A} allele, a phosphomimetic mutant of Pfk27 was produced by mutation of the 4 sites (S144, T157, S158, S159) to glutamic acid (E), generating the *Pfk27*^{4E} version.

Briefly, 0.5 μg of the GST-Pfk27 versions were incubated for 30 minutes at 30ºC with 3 μM ATP and 5 mM F6P. Then, the amount of ADP generated from this kinase reaction was measured with the ADP-Glo™ Kinase Assay kit (Promega). First, the addition of ADP-Glo™ Reagent stopped the kinase reaction and depleted the remaining ATP. Finally, the Kinase Detection reagent from the same kit was added to the samples to convert ADP to ATP and measure this newly synthesized ATP via a luciferase-luciferin reaction. The luminescence signal was measured using an EnSight reader (Fig. 21A). This assay illustrated a markedly increase in *Pfk27*^{4E} activity in comparison with the *wild-type* version (Fig. 21B). Moreover, the

phosphomutant unable to be phosphorylated (*Pfk27*^{4A}) presented a reduced activity compared with the *wild-type* protein (Fig. 21B).
In conclusion, these data showed that mimicking the phosphorylation of the four S144, T157, S158, S159 residues of Pfk27 increases its activity.





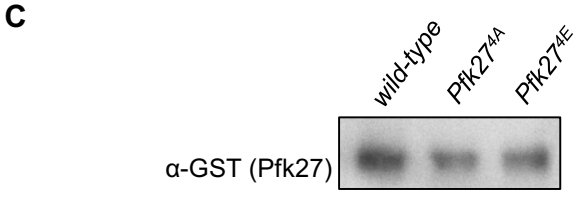


Figure 21. The Pfk27 phosphomimetic mutant increases its activity *in vitro*. **(A)** Scheme of the *in vitro* kinase assay coupled with the ADP-Glo Kinase assay kit (Promega) used to measure Pfk27 activity. 0.5 µg of recombinant GST-Pfk27 versions (*wild-type*, *Pfk27^{4A}*, *Pfk27^{4E}*) were incubated 30 minutes at 30ºC in the presence of 3 µM of ATP and 5 mM F6P. Then, the reaction was stopped and the unused ATP was depleted. Next, the ADP generated from the kinase assay was detected, transformed to ATP and converted to light by the luciferase reaction. Luminescence was measured using a luminescence reader. **(B)** Pfk27 *in vitro* activity. Data represent the mean and standard deviation of biological triplicates. Asterisks indicate the Student's t test assuming unequal variance analysis comparing the indicated strains (n.s., no significance, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$). **(C)** Western blot showing the loading control of the Pfk27 used for the *in vitro* activity assay. Samples from the kinase assay were taken and resolved in SDS-PAGE and blotted against α-GST antibody (Pfk27).

2.6 Rewiring of the carbohydrate metabolism upon osmostress

Under basal conditions, Pfk26 and Pfk27 activation ends up with an increase in Fru-2,6-P_2 concentration, which in turns allosterically activates Pfk1 and Pfk2, increasing the glycolysis flux (see Introduction). Upon osmostress, it has been proposed that Pfk26 and Pfk27 are activated to maintain the metabolic flux towards glycolysis (Dihazi et al., 2004; Petelenz-Kurdziel et al., 2013). After demonstrating that emulating the phosphorylation of Pfk27 increases its *in vitro* activity, we then aimed to test its relevance *in vivo*. As measuring Fru-2,6-P_2 is not trivial due to its similarity and low abundance compared with Fru-1,6-P_2, we monitored other metabolites concentrations as indirect measure of the glycolytic flux. Thus, we analyzed the concentration of trehalose, glycerol and pyruvate as readouts of carbohydrate metabolism at different steps upon osmostress (Fig. 22).

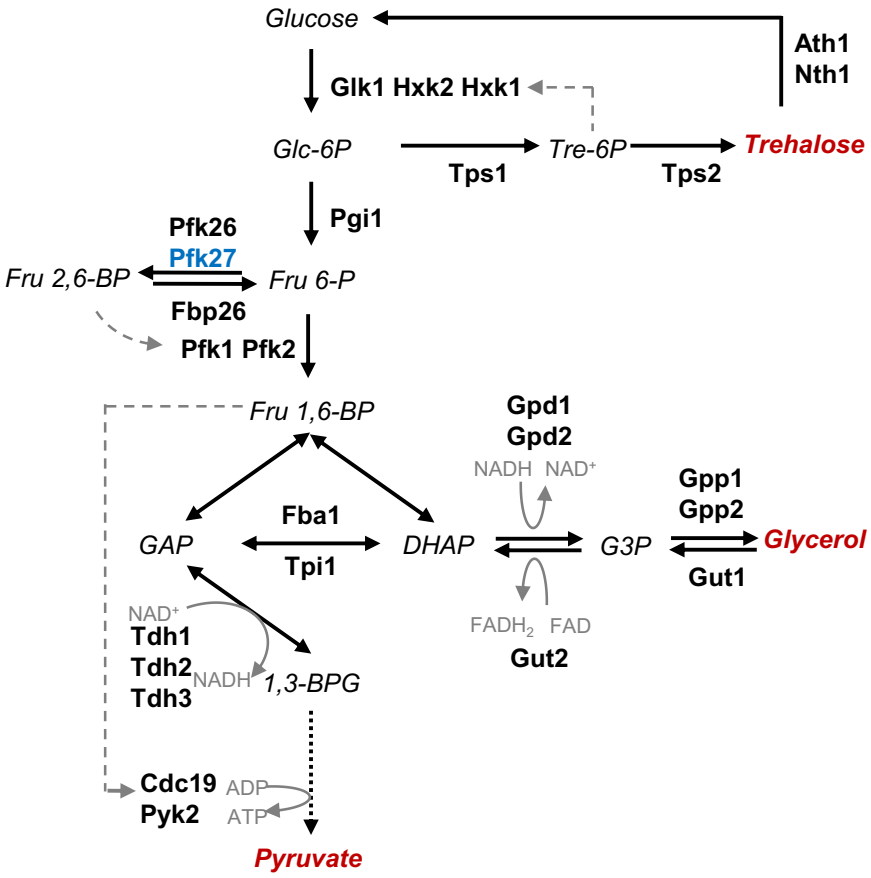


Figure 22. Trehalose, glycerol and pyruvate were used as readouts of carbohydrate metabolism pathways. Schematic view of glycolysis, trehalose and glycerol pathways. Measured metabolites are highlighted in dark red. Other metabolites are in italics. Pfk27 protein is shown in blue. Other enzymes are shown in bold. Black arrows indicate enzymatic reactions, grey lines correspond to generation of secondary metabolites and grey dashed lines are regulation of the enzymes.

2.6.1 The Pfk27^{4A} mutant accumulates higher levels of trehalose as osmoprotectant

Trehalose acts as protectant upon a diverse set of stress conditions, such as freezing, glucose starvation or even osmostress (see Introduction).

To determine whether mutations in Pfk27 modulated trehalose levels upon osmostress, cells were grown in YPD to exponential phase and then stressed (0.4 M NaCl). Samples were collected at the indicated time points and trehalose concentration was assessed using a commercial kit (KTREH, Megazyme) (Fig. 23). This kit uses a coupled enzyme assay involving trehalase, hexokinase and glucose 6-phosphate dehydrogenase to finally generate NADPH and measures its concentration with an absorbance reader at 340 nm. There was no trehalose accumulation upon stress in *wild-type* cells, nor in the *hog1* strain. However, the lack of Rck2 caused an increase in intracellular trehalose levels of up to 143 μg/ml trehalose / mg/ml total protein at 45 minutes, while *wild-type* cells only accumulate 34 μg/ml trehalose / mg/ml total protein. When analyzing the *pfk26* strain, a slight trehalose accumulation upon stress was detected but this was only statistically significantly when both phosphofructokinases were deleted, reaching the same levels as the *rck2* strain. The four non-phosphorylatable point mutations in Pfk27 showed an intermediate phenotype between *wild-type* and *pfk26 pfk27* cells (104 μg/ml trehalose / mg/ml total protein at 45 minutes). Of note, the *pfk26 Pfk27^{4A}* strain resembled the phenotype of the *rck2* and *pfk26 pfk27* strains (Fig. 23). Thus, trehalose accumulation in *Pfk27^{4A}* mutant strains suggested the importance of the

phosphorylation in the Pfk27 enzymatic activity and its role for the correct glycolytic flux.

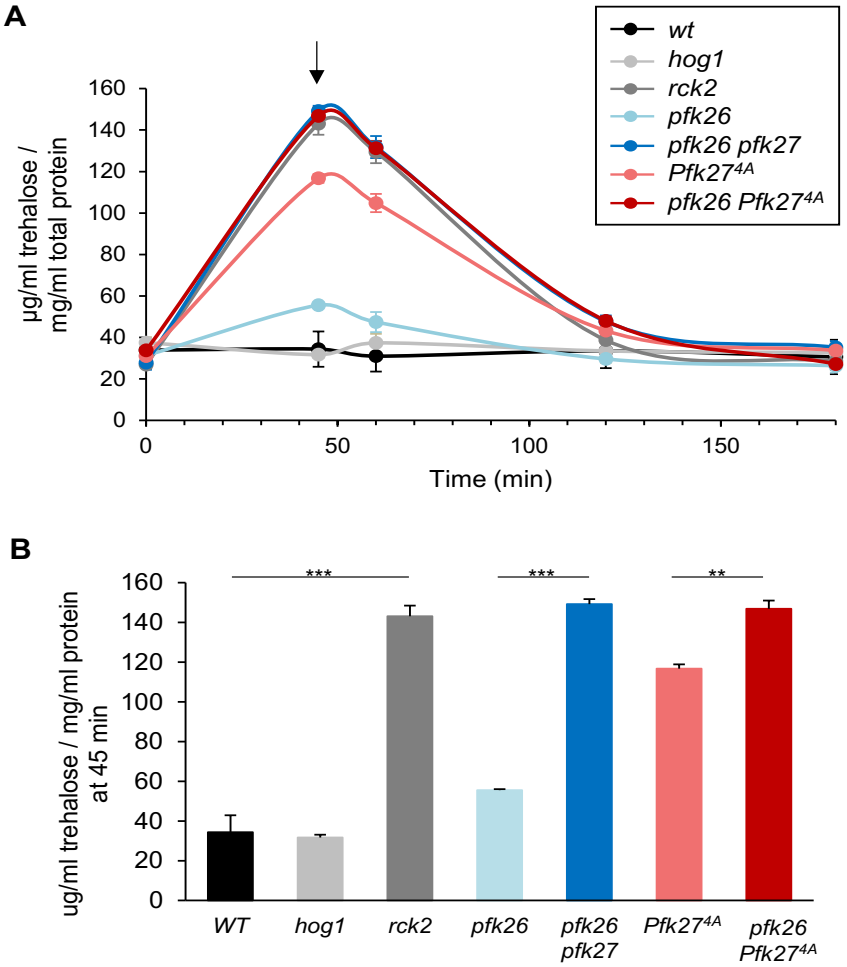


Figure 23. Pfk27 mutants accumulate trehalose upon osmostress. (A) Cells growing at exponential phase were exposed to stress (0.4 M NaCl) and samples were collected at the indicated time points. Trehalose was measured using a commercial kit (KTREH, Megazyme). Samples were normalized by the total protein amount. Arrows mark the time point quantified in panel B. **(B)** Trehalose measurements at 45 minutes after stress exposure are shown. Data represent the mean and standard deviation of biological triplicates. Asterisks indicate the Student's t test assuming unequal variance analysis comparing the indicated strains (n.s., no significance, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$).

2.6.2 The Pfk27^{4A} mutant displays a sustained glycerol concentration upon osmostress

When yeast cells grow in glucose, they transiently accumulate glycerol upon osmostress. Glycerol levels return to basal conditions when cells are already adapted to stress.

Thus, we examined the intracellular glycerol concentration in *PFK27* mutants upon osmostress. Samples were taken as described in the previous section and glycerol concentration was measured using a commercial kit (MAK117, Sigma) (Fig. 24). This kit uses a coupled enzyme assay using glycerol kinase and glycerol phosphate oxidase resulting in a colorimetric (570 nm) product.

As expected, upon stress, glycerol concentration increased at early time points and decreased after adaptation in the *wild-type* strain, almost returning to initial concentrations 180 minutes after the stress. In contrast, there was almost no glycerol accumulation in the *hog1* strain (Fig. 24A). Although, the deletion of *RCK2* produced an accumulation of glycerol at 45 minutes as in the *wild-type* strain, this concentration did not decrease to the same extent and it was maintained over time, showing a statistically significant difference when compared to the *wild-type* at 180 minutes (Fig. 24B). The deletion of *PFK26* also displayed a slightly greater increase in glycerol accumulation upon stress than *wild-type*. However, this glycerol accumulation was higher when both *PFK26* and *PFK27* genes were deleted, pointing out the importance of Pfk27 in the response to osmostress. Of note, the glycerol accumulation in the strains carrying the non-phosphorylatable point mutations in Pfk27

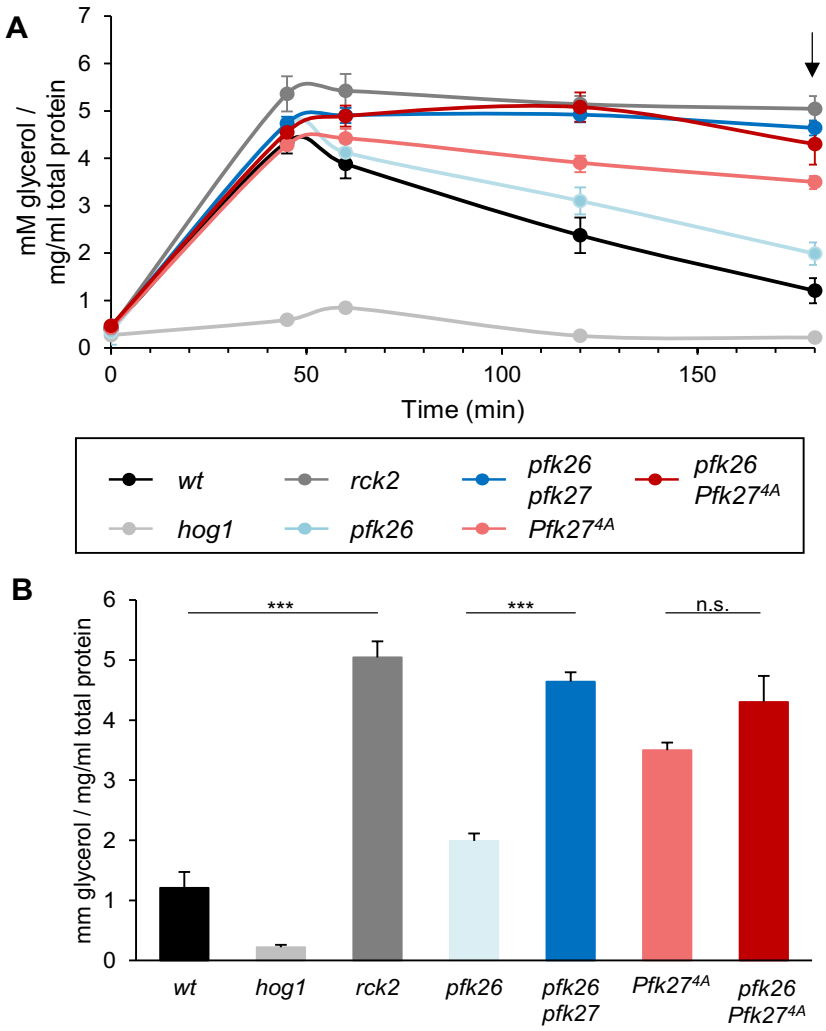


Figure 24. Alteration of Hog1 downstream targets results in an accumulation of glycerol upon osmostress. (A) Cells growing at exponential phase were subjected to stress (0.4 M NaCl) and samples were collected at the indicated time points. Glycerol was measured using a commercial kit (MAK117, Sigma). Samples were normalized by the total protein amount. Arrows mark the time point quantified in panel B. **(B)** Glycerol measures at 180 minutes after stress exposure are shown in this graph. Data represent the mean and standard deviation of biological triplicates. Asterisks indicate the Student's t test assuming unequal variance analysis comparing the indicated strains (n.s., no significance, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$).

(*Pfk27*^{4A} and *pfk26 Pfk27*^{4A}) resembled *pfk26 pfk27* strain (Fig. 24), demonstrating that the S144, T157, S158 and S159 sites in Pfk27 are required for the Pfk27 function in the regulation of carbohydrate metabolism during adaptation (Fig. 24).

2.6.3 Pyruvate levels do not vary upon osmostress

Finally, to investigate whether glycolysis was affected upon osmostress in the different mutant strains, we measured pyruvate levels as the final glycolytic product. Cells were treated and collected as previously described for the other metabolites and pyruvate concentration was measured with a commercially available kit (MAK071, Sigma). This kit uses a coupled enzymatic assay which results in a colorimetric product (570 nm), proportional to the pyruvate present in the sample. Pyruvate levels in *wild-type* cells did not change after cells were subjected to osmostress. Moreover, in *RCK1, PFK26* and *PFK27* mutants or *PFK27* punctual mutations did not affect pyruvate levels in neither basal nor stress conditions (Table 1).

Table 1. Pyruvate concentrations are not altered upon osmostress. Cells growing at exponential phase were exposed to stress (0.4 M NaCl) and samples were collected at the indicated time points. Pyruvate was measured using a commercial kit (MAK071, Sigma). Samples were normalized by the total protein amount. Data represent the mean and standard deviation of biological triplicates. Units are nM pyruvate / mg/ml total protein.

	0 min	45 min	60 min	120 min	180 min
wild-type	0.35 ±SD 0.03	0.41 ±SD 0.07	0.36 ±SD 0.09	0.39 ±SD 0.06	0.29 ±SD 0.04
hog1	0.43 ±SD 0.08	0.31 ±SD 0.05	0.29 ±SD 0.06	0.38 ±SD 0.03	0.27 ±SD 0.01
rck2	0.38 ±SD 0.07	0.34 ±SD 0.09	0.26 ±SD 0.02	0.26 ±SD 0.04	0.34 ±SD 0.03
pfk26	0.33 ±SD 0.03	0.33 ±SD 0.06	0.33 ±SD 0.07	0.32 ±SD 0.07	0.37 ±SD 0.05
pfk26 pfk27	0.31 ±SD 0.01	0.37 ±SD 0.07	0.36 ±SD 0.02	0.29 ±SD 0.04	0.35 ±SD 0.05
Pfk27^{4A}	0.36 ±SD 0.06	0.38 ±SD 0.05	0.38 ±SD 0.01	0.38 ±SD 0.05	0.38 ±SD 0.04
pfk26 Pfk27^{4A}	0.42 ±SD 0.03	0.41 ±SD 0.04	0.44 ±SD 0.05	0.41 ±SD 0.05	0.39 ±SD 0.1

All together, these results suggested that phosphorylation of Pfk27 by Rck2-Hog1 pathways is necessary to maintain the glycolytic flux and thus not accumulate metabolites such as trehalose. Furthermore, deficiencies in Pfk27 activation produced a glycerol accumulation, resulting in a longer adaptation to osmostress.

2.7 Mutations in the Rck2-dependent osmostress response alter cell growth upon stress

After directly establishing the role of Pfk27 in carbohydrate metabolism, we aimed to determine whether the mutations in *PFK27* had an impact on cell fitness in basal or osmostress conditions. We performed viability assays where cells were grown in a 96-well plate in YPD or YPD + 0.8 M NaCl at 30ºC and cell density was measured every 30 minutes. Under basal conditions, we did not observe any differences in *rck2* and *pfk26 Pfk27*^{4A} cells, although *pfk26 pfk27* cells exhibited a slower growth compared to *wild-type* (Fig. 25A). Nevertheless, we did not detect differences in cellular growth when cells were assayed under osmostress conditions (Fig. 25B).

Next, we performed competition cell assays to assess whether small defects on adaptation could lead to cells slightly reduced fitness that would be outcompeted in cellular consortia. Briefly, *wild-type* cells were tagged with *ENO1-YFP* and mutant cells with *ENO1-mCherry*. Then, we mixed the cells in an equal proportion and grew them together at 30ºC in either YDP or YPD + 0.8 M NaCl. We collected the samples at the indicated time points. Cells were diluted daily to never reach the stationary phase. Finally, samples were analyzed by flow cytometry to determine the relative abundance of each strain.

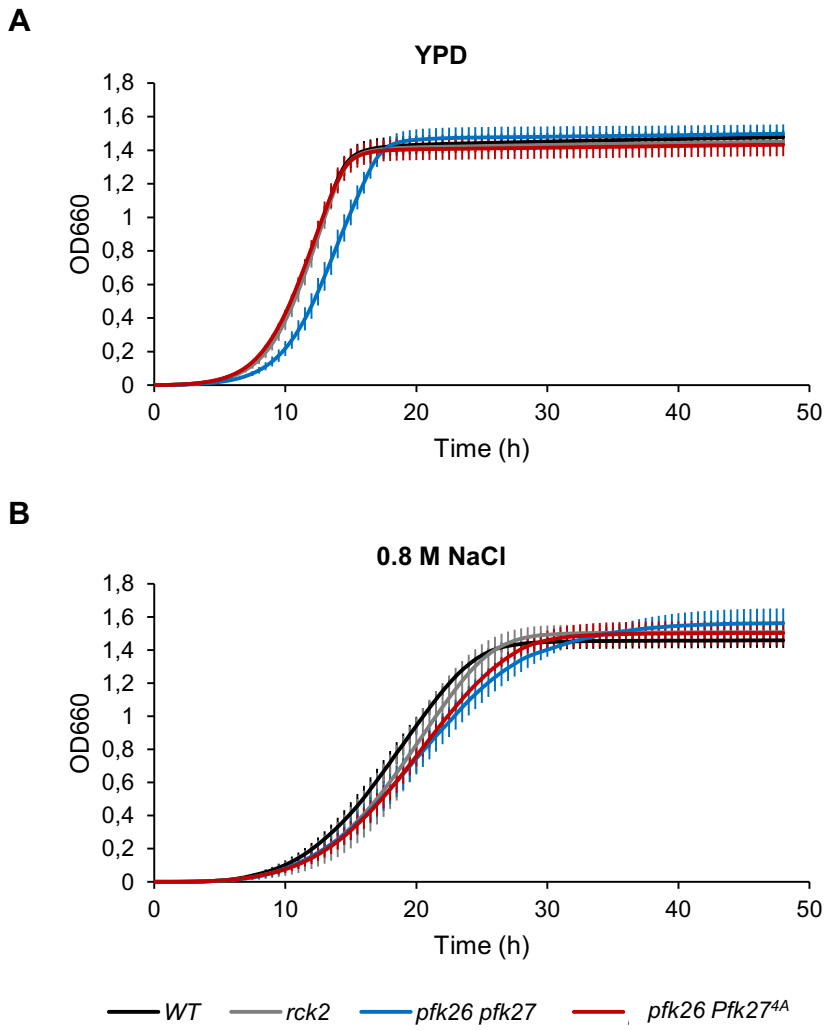


Figure 25. The Pfk27^{4A} mutant does not affect cell growth. Cells of the indicated strains were growth in YPD **(A)** or YPD + 0.8 M NaCl **(B)** at 30ºC and growth was monitored every 30 minutes in a 96-well plate using a Synergy H1 MultiMode Reader. Data represent mean and standard deviation from biological triplicates.

First, we observed that the *rck2* mutant cells grew as well as the *wild-type* strain under basal conditions but, surprisingly, it displayed a better cell fitness than the *wild-type* when grown under osmostress (Fig. 26A). Regarding the phosphofructokinase mutants, *PFK26* mutant cells showed a slight delay in cell growth under basal conditions compared with the *wild-type* cells (Fig. 26B). This delay is even higher when both *PFK26* and *PFK27* genes were deleted (Fig. 26C). As previously seen in the *RCK2* mutant cells, deletion of phosphofructokinases produced an advantage when cells were growing at 0.8 M NaCl (Fig. 26B and C). Finally, $Pfk27^{4A}$ and *pfk26* $Pfk27^{4A}$ mutant cells grew better under osmostress conditions than basal when compared to *wild-type* (Fig. 26D and E). To sum up, mutations in the Rck2-dependent osmostress response produces a fitness advantage when cells are subjected to osmostress for a long period.

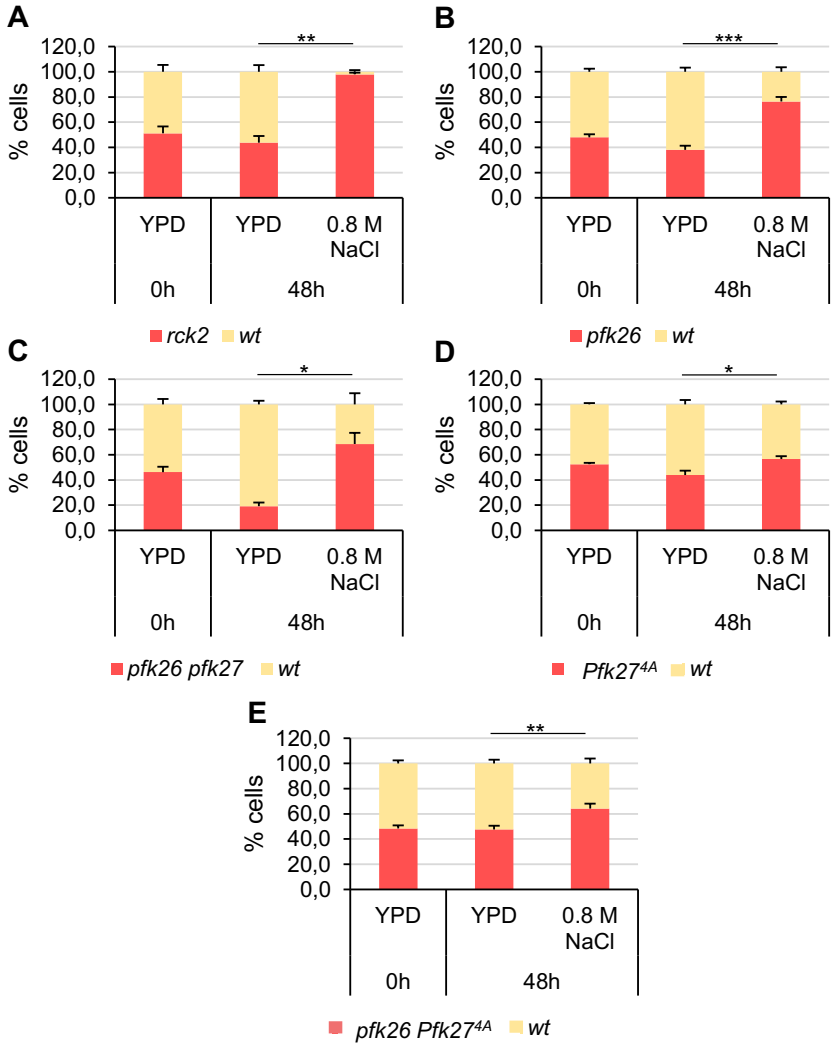


Figure 26. Mutations in the Rck2-dependent osmostress response favor cell growth upon osmostress. *Wild-type ENO1*-YFP cells were mixed with *rck2 ENO1*-mCherry **(A),** *pfk26 ENO1*-mCherry **(B)**, *pfk26 pfk27 ENO1*-mCherry **(C)**, *pfk27^{4A} ENO1*-mCherry **(D)** or *pfk26 pfk27^{4A} ENO1*-mCherry **(E).** Mixed cells were grown together in either YDP or YPD + 0.8 M NaCl. Samples were collected at the indicated time points and analyzed by flow cytometry. Data represent the mean and standard deviation of biological triplicates. Asterisks indicate the Student's t test assuming unequal variance analysis comparing the indicated strains (n.s., no significance, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$).

DISCUSSION

When cells suffer stress, regulation of several cellular functions is crucial for maximizing their survival. Cells display a specific transcriptional program for each stress condition, they also stop cell cycle progression to ensure a proper cell division and regulate their metabolism to suitably adapt to the new plight, among others. The aim of this thesis was to identify novel roles of Hog1 during stress adaptation and we have focused on the study of the role of the HOG pathway in carbohydrate metabolism regulation upon osmostress.

Many Hog1 roles in osmostress adaptation have been previously reported (see Introduction), but there are still several functions that remain unknown. Here, by using an unbiased biochemical approach, we identified 169 potential targets of Hog1 after testing the entire yeast proteome. In addition to detecting previously known Hog1 targets, such as Sic1 (Escoté et al., 2004) and Mrc1 (Duch et al., 2013b), we revealed new potential targets involved in cell cycle, transcription, translation, vesicle trafficking and carbohydrate metabolism. This global approach served to define the targets for a MAPK in a eukaryotic organism and opens new avenues on the physiological role for those enzymes in eukaryotic cells.

One of the most represented functional clusters among the new potential Hog1 targets were proteins related to carbohydrate metabolism. We detected 13 new metabolism-related candidates phosphorylated by Hog1. The function of Hog1 in the glycerol synthesis regulation has been widely understood, but its role in the modulation of other carbohydrate metabolism steps has not been well-defined (Ferreira et al., 2005; Lee et al., 2012; Rep et al., 1999, 2000; Roelants et al., 2011). Some of the identified candidates act in

the first steps of glycolysis, such as Hxk2, Glk1, Pfk2 or Pfk27, pointing out that Hog1 could possess an important role not only in the regulation of glycerol synthesis but also in glycolysis modulation. Previous studies reported that Hog1 regulates Pfk26 upon osmostress, the enzymatic partner of Pfk27 (Dihazi et al., 2004). Considering the importance of glycolysis regulation by phosphofructokinases we focused on the study of the regulation and function of Pfk27 upon osmostress.

We validated that Hog1 controls the *in vivo* phosphorylation of Pfk27 upon osmostress, but this is an indirect regulation. Despite Pfk27 being identified as a potential direct substrate of Hog1, the mutation of the single S/TP site of Pfk27 (T278) did not suppress the *in vivo* phosphorylation of Pfk27 upon stress. Moreover, Pfk27 was not significantly phosphorylated by Hog1 *in vitro*. These results indicated that Pfk27 is regulated upon osmostress in a Hog1-dependent manner, but the MAPK is not directly targeting the metabolic enzyme. We hypothesized that during the purification of the TAP-tagged proteins for the screening assay, the kinase that targets Pfk27 was co-immunoprecipitated and could use the PE-ATP as a substrate, explaining why Pfk27 emerged as a positive candidate in the screening.

Rck2 was identified as a kinase under the control of Hog1 upon osmostress (Bilsland-Marchesan et al., 2000; Teige et al., 2001). The Elongation factor 2 (EF-2) was the first Rck2 target identified in osmostress conditions, involving Rck2 with translation regulation (Teige et al., 2001). Rck2 has also been related with transcriptional processes by its negative influence on Hog1 nuclear localization

(Bilsland et al., 2004) and its indirect regulation of PKA-dependent transcription factors through its interaction with Pde2 (MacGilvray et al., 2018). In 2017, Romanov and colleagues suggested Rck2 as a new hub for osmostress response signal spreading (Romanov et al., 2017). Using mass-spectrometry, the authors identified several candidates that were phosphorylated upon osmostress in a Rck2-dependent manner. Furthermore, they also described potential Rck2 phosphorylation motifs (RXXS and SXXXL) (Romanov et al., 2017). Taking into account these findings, we hypothesized that Rck2 could be the kinase in charge of Pfk27 phosphorylation upon osmostress. The loss of the Phos-tag phosphorylation shift of Pfk27 in *rck2* mutant cells pointed out that this kinase is involved in Pfk27 regulation upon osmostress. Moreover, previously activated Rck2 was able to phosphorylate Pfk27 *in vitro*. These results lead us to conclude that Rck2 is directly phosphorylating Pfk27 upon osmostress in a Hog1-dependent manner.

As mentioned before, Rck2 was previously related with translation and transcription regulation upon osmostress. However, no previous functions of Rck2 in metabolism had been reported. Thus, we identified Pfk27 as the first metabolism-related target of Rck2.

We determined eight RXXS or SXXL Rck2 motifs in Pfk27. Two (S144 and S158) out of these eight sites had been previously identified to be phosphorylated under diverse treatments by mass-spectrometry (Albuquerque et al., 2008; Lanz et al., 2021; Swaney et al., 2013).

The single mutation of Pfk27 S144 or S158 to alanine reduced Pfk27 phosphorylation by Rck2 *in vitro*. Interestingly, the single S158A

mutation was required to abolish the Pfk27 phosphorylation shift *in vivo*.

It is known that the double deletion of the *PFK26* and *PFK27* genes produces a slight delay in Hog1 phosphorylation (Petelenz-Kurdziel et al., 2013). To investigate the contribution of Pfk27 phosphorylation by the Hog1-Rck2 pathway upon osmostress, we followed Hog1 phosphorylation upon osmostress as a readout of cell adaptation. Interestingly, we observed a significant delay in Hog1 phosphorylation when analyzing the *rck2* strain. This fact indicates the importance of Rck2 function in the cell adaptation response to osmostress. However, non-phosphorylatable Pfk27 doble point mutant (*Pfk27*^{2A}), alone or in combination with the *PFK26* deletion, did not show any difference in Hog1 phosphorylation kinetics when compared to *wild-type* cells, suggesting that there should be other Pfk27 phosphorylation sites by Rck2 involved in the osmostress response. The Pfk27 sites T157 and S159, were previously reported to be phosphorylated by mass spectrometry, albeit they are not typical potential Rck2 phosphorylation motif (Swaney et al., 2013). Moreover, they surround the S158 site. Interestingly, the non-phosphorylatable mutant of Pfk27 where S144, T157, S158 and S159 sites were substituted by alanines (*Pfk27*^{4A}), alone or in combination with *PFK26* deletion, delayed Hog1 phosphorylation upon osmostress. Moreover, the Hog1 phosphorylation delay seen in the *pfk27*^{4A} or *pfk26 pfk27*^{4A} cells resembled that produced in the *pfk26 pfk27* mutant strain (Petelenz-Kurdziel et al., 2013). Thus, the particular sites in Pfk27 (S144, T157, S158, S159) are required for proper Hog1 signaling and response to osmostress.

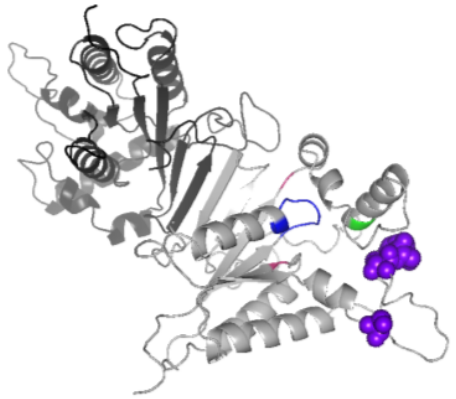


Figure 27. Pfk27 phosphosites location could explain their function. There is no crystal structure of Pfk27 and protein structure model was generated from crystal structure of human PFKFB3 sequence (PDB: 5ak0.1.B; (Boyd et al., 2015) with SWISS-MODEL (Bertoni et al., 2017; Bienert et al., 2017; Guex et al., 2009; Studer et al., 2020; Waterhouse et al., 2018). Pfk27 phosphosites (S144, T157, S158, S159) are highlighted in purple. ATP-binding sites are colored in blue, F6P-binding site is highlighted in green and active sites in pink.

When examining the modelled structure of Pfk27 (Fig. 27), we observe that Pfk27 has a pocket where reside the ATP-binding sites (G103-S111), the F6P-binding site (R269) and the active sites (D197 and C235). Interestingly, the Pfk27 sites phosphorylated by Rck2 upon osmostress are in close contact between them and located in an unstructured loop. Moreover, this loop is near the active pocket of Pfk27 and we hypothesize that phosphorylation of Pfk27 sites (S144, T157, S158, S159) may lead to a conformational change of the protein modulating the affinity of the enzyme to its substrate, F6P. We hypothesized that the phosphorylation of Pfk27 by Rck2 upon osmostress could increase its enzymatic activity, generating more

Fru-2,6-P_2 to facilitate maintenance of the glycolytic flux and cope with the stress insult. Indeed, a phosphomimetic mutant of Pfk27 (*Pfk27*^{4E}) exhibited more enzymatic activity than the *wild-type* version. We also tested the non-phosphorylatable *Pfk27*^{4A} mutant, not expecting to see any differences compared to the *wild-type* allele due to both recombined Pfk27 proteins were not being previously incubated with Rck2. However, *Pfk27*^{4A} had less *in vitro* activity than the *wild-type*. It might be possible that recombinant *wild-type* Pfk27 was slightly phosphorylated by some bacterial kinases, while the *Pfk27*^{4A} version could not be phosphorylated. It would be interesting to perform the *in vitro* activity assay of Pfk27 previously phosphorylated by Rck2 and compare it with the *Pfk27*^{4E} mutant.

Monitoring the *in vivo* activity of Pfk27 under basal and stress conditions over time would be informative. The best option to measure Fru-2,6-P_2 is using a reverse-phase ion pair HPLC technique that allows separation of the two isomers, Fru-1,6-P_2 and Fru-2,6-P_2 (Coulier et al., 2006; Han et al., 2013; Henderson and Henderson, 1986; Lu et al., 2010). As measuring Fru-2,6-P_2 was not trivial, we assessed the levels of other metabolites such as trehalose, glycerol and pyruvate as readouts of carbohydrate metabolism upon osmostress.

Trehalose has been studied as a stress protectant metabolite under several conditions, such as freezing, glucose starvation or osmostress (Coutinho et al., 1988; Hottiger et al., 1987; Lillie and Pringle, 1980; Panek, 1963). Indeed, when yeast cells grow in ethanol as the carbon source and face osmostress, trehalose, instead of glycerol, accumulates inside of the cells as an osmoprotectant (Babazadeh et

al., 2017). Nevertheless, in rich medium and upon osmostress, yeast cells do not usually accumulate trehalose. Our results showed that the trehalose concentrations rose 45 minutes after stress when the *RCK2* gene was deleted. Moreover, we observed the same phenotype in both *pfk26 pfk27* and *pfk26 pfk27*^{4A} mutant cells, pointing out that this trehalose accumulation is due to the loss of function of Pfk27 and that the identified sites of Pfk27 are required in this process. We hypothesized that the decrease in Pfk27 activity probably produces an accumulation of metabolites from the first glycolytic steps. Glucose 6-phosphate is one of these metabolites and serves as a substrate for the TPS complex to synthetize trehalose. Interestingly, the TPS complex components (Tps1, Tps2, Tps3 and Tsl1) are highly expressed under osmostress conditions (Nadal-Ribelles et al., 2014). The accumulation of trehalose precursors and the expression of trehalose synthesis enzymes lead to an accumulation of trehalose upon osmostress when cells are not able to process glucose as fast as required.

One of the most important readout of osmostress adaptation is glycerol accumulation. When yeast cells encounter osmostress, they need to compensate the osmotic imbalance between the inside and the outside of the cell. For this reason, cells start to accumulate glycerol by increasing its synthesis but also by closing the glycerol export channel, Fps1. Once cells become adapted to the stress situation, glycerol concentration decreases to basal levels. A prolonged accumulation of glycerol indicates the inability of the cell to overcome the osmostress (Alepuz et al., 2001; Babazadeh et al., 2014; Beese et al., 2009; Ferreira et al., 2005; Lee et al., 2013, 2012;

Rep et al., 1999, 2000; Roelants et al., 2011). Here we showed that *RCK2* deletion gene produced a sustained accumulation of glycerol over time, indicating that cells were not able to adapt to stress. Of note, Rck2 does not regulate glycerol synthesis as Hog1 does, because the loss of function of Hog1 impedes the accumulation of glycerol (Brewster and Gustin, 2014; Nevoigt and Stahl, 1997). This fact could be one of the main reasons why *rck2* cells did not show osmosensitivity when growing in the presence of osmostress (Bilsland-Marchesan et al., 2000; Dahlkvist and Sunnerhagen, 1994; Melcher and Thorner, 1996), because the inability to accumulate glycerol produces a reduction in cell fitness. However, the inability of the *rck2* mutant cells to restore glycerol basal levels implies that Rck2 has a significant role, at least in the first hours in the presence of stress.

Similar to *rck2* cells, mutations in *PFK27* also provoked an accumulation of glycerol upon stress. It has been proposed that, in osmostress, Pfk26 and Pfk27 are part of a rerouting mechanism involved in the maintenance of the metabolic flux to adjust the ATP production demand downstream of pyruvate, rather than being important for glycerol synthesis (Petelenz-Kurdziel et al., 2013). Our results support the idea of Pfk27 not being essential for glycerol synthesis, nevertheless its loss of function causes a slower glycolysis flux leading to a metabolic imbalance. The inability of the cells to produce enough ATP during glycolysis could lead to a slower adaptation of the *pfk26 pfk27* mutant strain during the initial hours of exposure to stress. This is also the case when Pfk27 cannot be phosphorylated in a Rck2-dependent manner. In conclusion,

phosphorylation of Pfk27 by Rck2 might be required for the maintenance of the glycolytic flux under stress conditions to rapidly overcome the stress situation.

As expected, we did not observe significant variations in pyruvate levels among time nor between strains upon osmostress. This is due to cells need to maintain a proper energy and redox balance by controlling the glycerol and pyruvate production ratio. Cells are able to increase glycerol production and maintain pyruvate levels by decrementing biomass production (Petelenz-Kurdziel et al., 2013).

When cells encounter stress situations, they need to display a correct metabolic flux to obtain sufficient ATP to promote an accurate adaptation response. Metabolic imbalances could lead to changes in cell sensitivity to stress conditions. We described that in basal conditions, *pfk26 pfk27* mutant cells exhibited a slower cell growth. As mentioned before, Pfk26 and Pfk27 are in charge of synthetizing one of the most powerful glycolytic activators, Fru-2,6-P_2. The elimination of this allosteric activator decreases the metabolic flux and cells carrying *PFK26* and *PFK27* deletions display a slower glucose consumption (Petelenz-Kurdziel et al., 2013). This could explain the growth phenotype observed in *pfk26 pfk27* mutant cells. However, this growth delay was not detected when cells where subjected to osmostress. We hypothesized that *pfk26 pfk27* cells could be subjected to a basal internal stress due to the deletion of the phosphofructokinases, putting the cells in a suboptimal condition. These pre-stressed cells could have a better predisposition to tolerate other types of stresses, a phenomenon called “cross-protection” (Komatsu et al., 1990; Kronberg et al., 2008; Lewis et al., 1995;

Siderius and Mager, 1997). For this reason, we believe that *pfk26 pfk27* mutant cells showed a delay in cell growth in basal conditions, but they adapt as good as the *wild-type* upon stress.

Previous studies described that deletion of the *RCK2* gene does not produce osmosensitivity (Bilsland-Marchesan et al., 2000; Dahlkvist and Sunnerhagen, 1994; Melcher and Thorner, 1996). However, others showed a clear osmosensitive phenotype for *rck2* cells (Romanov et al., 2017). Our results supported that *RCK2* deletion does not produce osmosensitivity. Furthermore, competition assays performed in our laboratory indicated that *rck2* mutant cells possess a cell growing advantage in comparison to *wild-type* cells under stress (0.8 M NaCl). For the first time, we reported the growth behaviour of *rck2* mutant cells in liquid medium and in competition assays with *wild-type* cells, while published data was based in spotting assays. Our approaches provide more accurate data than previous studies by following cell growth over time. In summary, these data suggested that Rck2 has a role in controlling cell growth only in the presence of stress. The reason why *RCK2* deletion produces an unexpected cell growth advantage upon osmostress is unknown but we hypothesized that Rck2 could target several proteins related with cell cycle progression and its deregulation could lead to this growing phenotype. Other hypothesis could be in line with the "cross-protection" phenomenon produced in pre-stressed cells as we explained before for *pfk26 pfk27* mutant cells.

As previously seen in the growth curves, when *pfk26 pfk27* cells competed with *wild-type* cells, the mutant cells exhibited a slower growth in YPD. Furthermore, our results disclosed that Pfk26 is the

main factor for this phenotype. However, the non-phosphorylatable point mutations in Pfk27 also promoted a slight delay in growth when compared to *wild-type*. Of note, this growth disadvantage was not observed upon osmostress. Indeed, some mutant strains displayed a better cell fitness in stress conditions. Particularly, cells carrying the *PFK26* deletion presented a more pronounced phenotype. Nevertheless, it is reported that the double *pfk26* pfk27 deletion mutant grows worse that *wild*-type cells upon osmostress (doubling time of 121 minutes *wild-type vs* 135 minutes *pfk26 pfk26* cells, respectively) (Petelenz-Kurdziel et al., 2013). However, our experiments were designed to analyze cell adaptation to a continuous osmostress exposure while those cited analyzed the acute response to osmostress. Moreover, it is worth mention that the cited article uses W303 cells exposed to 0.4 M NaCl while our studies were performed in a BY4741 background subjected to 0.8 M NaCl stress. It has been shown that there is an osmostress tolerance difference between W303 and BY4741 strains (Petrezselyova et al., 2010) that, as mentioned, it could explain part of the differences observed.

The reason why mutations in phosphofructokinases lead to a growth advantage upon osmostress remains unclear. One possible explanation could be a sustained expression of the *HXT1* gene in the mutant strains (Tomás-Cobos et al., 2004). Hxt1 is a low affinity glucose transporter, whose expression is regulated by both glucose signaling and the HOG pathway. *HXT1* gene expression is upregulated upon osmostress to increase glucose uptake and synthetize more glycerol (Tomás-Cobos et al., 2004). A longer Hog1 activation in *PFK27* mutant cells could lead to a higher *HXT1*

expression and a more sustained glucose uptake, therefore facilitating cell growth under osmostress conditions.

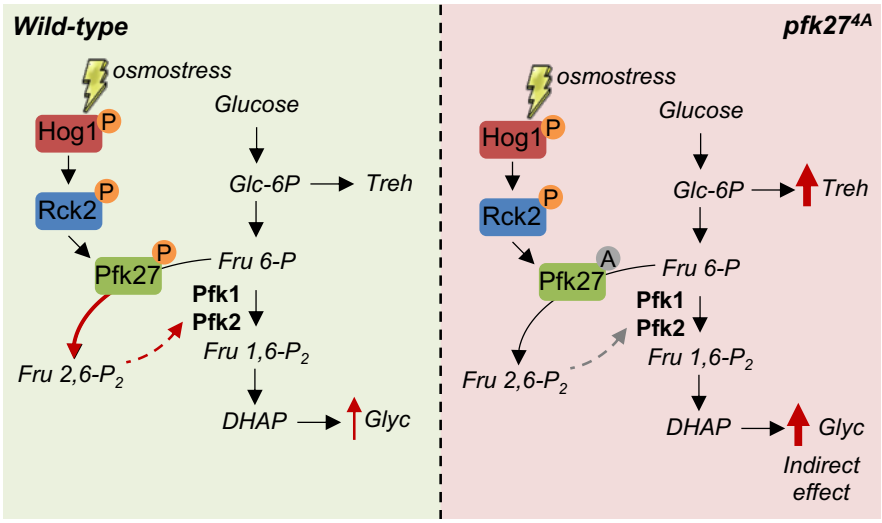


Figure 28. Pfk27 helps to maintain a metabolic balance upon osmostress. Work model of Pfk27 function upon osmostress. Phosphorylations are marked in orange circles while the Pfk27 alanine mutant is shown as a grey circle. Metabolites are in italics. Black arrows indicate enzymatic reactions and grey dashed lines indicate regulation of the enzymes. Red arrows indicate activated reactions or accumulation of metabolites.

In conclusion, this thesis describes a novel Rck2-dependent substrate, Pfk27, and its role and relevance in the osmostress adaptation response. We also demonstrated the importance of Rck2 in the metabolic rewiring of carbohydrate metabolism, confirming Rck2 as a hub for the stress response. Upon osmostress, Hog1-Rck2 pathway phosphorylates and activates Pfk27 increasing the synthesis of Fru-2,6-P_2 and facilitating the metabolic rewiring needed to accumulate intracellular glycerol. However, cells containing a non-phosphorylatable mutant version of Pfk27 (*Pfk27^{4A}*) display a lower

glycolytic flux ending up with an accumulation of metabolites from the initial steps of the glycolysis and a delay in cell adaptation response, accumulating glycerol over time (Fig. 28).

Personal contribution to this work: I have been involved in all the steps of this work except for Hog1-as screening, where I only validated some of the candidates.

CONCLUSIONS

This PhD thesis describes a novel role of the phosphofructokinase Pfk27 in rewiring the carbohydrate metabolism to maintain the metabolic flux upon osmostress.

More specifically, the results obtained during this PhD thesis led to the following conclusions:

1. An unbiased biochemical screening identified 169 potential novel Hog1 substrates.
2. Pfk27 is *in vivo* phosphorylated upon osmostress in a Hog1-dependent manner.
3. Phosphorylation of the sites S144, T157, S158 and S159 in Pfk27 are required for proper Hog1-Rck2 signaling upon osmostress conditions.
4. The loss of function of Pfk27 protein causes a delay in the cellular acute response to osmostress.
5. The phosphomimetic mutant of Pfk27 (*Pfk27*^{4E}) increases its enzymatic activity.
6. Cells carrying *Pfk27*^{4A} mutation increase a trehalose and glycerol accumulation upon osmostress.
7. Mutations in phosphofructokinases generate a growth advantage upon osmostress.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. List of the 169 positive candidates from the Hog1-as screening, alphabetically ordered. ORF: systematic name of yeast genes. Gene name: standard name of yeast genes. Cluster: cell functional cluster where this gene belong, only most represented clusters are listed. NA, not assigned. Known substrate: previously known Hog1 substrates; if the substrate was discovered by high or low throughput experiments is shown.

ORF	Gene Name	Cluster	Known substrate (low/high)	Reference
YGR204W	*ADE3*	NA		
YGR061C	*ADE6*	NA	High	MacGilvray *et al.*, 2018
YLR109W	*AHP1*	NA	High	MacGilvray *et al.*, 2018
YJL084C	*ALY2*	Vesicle trafficking		
YJR005W	*APL1*	Vesicle trafficking		
YGR261C	*APL6*	Vesicle trafficking		
YNL059C	*ARP5*	NA		
YMR068W	*AVO2*	NA		
YKR099W	*BAS1*	Transcription		
YLR399C	*BDF1*	Replication		
YER155C	*BEM2*	NA		
YDR099W	*BMH2*	Cell cycle / Replication	High	MacGilvray *et al.*, 2018
YMR275C	*BUL1*	Vesicle trafficking	High	Romanov *et al.*, 2017
YER159C	*BUR6*	Transcription		
YDL143W	*CCT4*	NA		
YJR076C	*CDC11*	NA		
YDR301W	*CFT1*	Transcription		
YGL019W	*CKB1*	Replication		
YPR120C	*CLB5*	Cell cycle		
YFR014C	*CMK1*	NA		
YDR155C	*CPR1*	Cell cycle		
YKL139W	*CTK1*	Transcription		
YGR003W	*CUL3*	NA		
YGR155W	*CYS4*	Cell cycle		
YDR052C	*DBF4*	Cell cycle		
YOL087C	*DUF1*	NA		

YBL101C	*ECM21*	Vesicle trafficking		
YBL047C	*EDE1*	Vesicle trafficking		
YDR385W	*EFT2*	Translation		
YGR162W	*EIF4G1*	Translation		
YPL086C	*ELP3*	Transcription / Translation		
YLR056W	*ERG3*	NA		
YOR133W	*ETF1*	NA		
YDR373W	*FRQ1*	NA		
YOR260W	*GCD1*	Translation		
YDR283C	*GCN2*	Cell cycle / Replication / Translation		
YOR375C	*GDH1*	NA		
YKL104C	*GFA1*	Carbohydrate metabolism		
YDR358W	*GGA1*	Vesicle trafficking		
YMR135C	*GID8*	Cell cycle / Carbohydrate metabolism		
YNL255C	*GIS2*	NA		
YML006C	*GIS4*	NA		
YCL040W	*GLK1*	Carbohydrate metabolism		
YDR108W	*GSG1*	Vesicle trafficking		
YPL127C	*HHO1*	NA		
YDR174W	*HMO1*	Transcription		
YOR267C	*HRK1*	NA		
YKL101W	*HSL1*	Cell cycle	Low	Clotet *et al.*, 2006
YGL253W	*HXK2*	Carbohydrate metabolism		
YLR384C	*IKI3*	Transcription / Translation		
YJR016C	*ILV3*	NA		
YLL027W	*ISA1*	NA		
YJL034W	*KAR2*	NA	High	MacGilvray *et al.*, 2018
YHR158C	*KEL1*	Cell cycle		
YPL263C	*KEL3*	NA		
YGL173C	*KEM1*	NA		
YKL168C	*KKQ8*	NA		

YJL207C	*LAA1*	Vesicle trafficking		
YKL143W	*LTV1, YKL2*	Translation		
YDL131W	*LYS21*	NA		
YDR234W	*LYS4*	NA		
YOR298C-A	*MBF1*	Transcription		
YGL201C	*MCM6*	Cell cycle / Replication		
YGR100W	*MDR1*	NA		
YIL128W	*MET18*	Replication / Transcription		
YMR002W	*MIC17*	NA		
YCL061C	*MRC1*	Cell cycle / Replication	Low	Duch *et al.*, 2012
YDR335W	*MSN5*	Transcription		
YKL129C	*MYO3*	Vesicle trafficking		
YPL190C	*NAB3*	Transcription		
YML117W	*NAB6*	NA		
YNL119W	*NCS2*	Translation		
YJR132W	*NMD5*	NA		
YOR206W	*NOC2*	Translation		
YLR002C	*NOC3*	Translation		
YER002W	*NOP16*	Translation		
YJL010C	*NOP9*	Translation		
YMR091C	*NPL6*	Replication / Transcription		
YOR209C	*NPT1*	NA		
YGR159C	*NSR1*	Translation	High	Breitkreutz *et al.*, 2010
YLR335W	*NUP2*	NA	Low	Regot *et al.*, 2013
YDL019C	*OSH2*	Vesicle trafficking		
YIR006C	*PAN1*	Vesicle trafficking	Low	Reiter *et al*, 2012
YIL050W	*PCL7*	Cell cycle / Carbohydrate metabolism		
YBL017C	*PEP1*	Vesicle trafficking		
YMR257C	*PET111*	NA		
YMR205C	*PFK2*	Carbohydrate metabolism	High	MacGilvray *et al.*, 2018

YOL136C	*PFK27*	Carbohydrate metabolism		
YMR105C	*PGM2*	Carbohydrate metabolism		
YBR106W	*PHO88*	NA		
YPL268W	*PLC1*	NA		
YOR321W	*PMT3*	Carbohydrate metabolism		
YNL102W	*POL1*	Replication		
YDR436W	*PPZ2*	NA	High	Romanov *et al.*, 2017
YER013W	*PRP22*	NA		
YAL017W	*PSK1*	Carbohydrate metabolism		
YML017W	*PSP2*	NA		
YDR032C	*PST2*	NA		
YEL037C	*RAD23*	Replication		
YPL153C	*RAD53*	Cell cycle / Replication		
YDL189W	*RBS1*	Carbohydrate metabolism		
YOR285W	*RDL1*	NA		
YMR139W	*RIM11*	Cell cycle		
YOR341W	*RPA190*	Transcription		
YGL070C	*RPB9*	Replication / Transcription		
YPR110C	*RPC40*	Transcription	High	MacGilvray *et al.*, 2018
YIL119C	*RPI1*	Transcription		
YHR027C	*RPN1*	Translation		
YDL147W	*RPN5*	Translation		
YDL140C	*RPO21*	Transcription	Low	Chasman *et al.*, 2014
YDR447C	*RPS17B*	Translation		
YDR341C	*RRS1*	Translation		
YOL067C	*RTG1*	Transcription		
YDL025C	*RTK1*	Translation		
YCR009C	*RVS161*	Vesicle trafficking		
YKL117W	*SBA1*	NA		
YJL080C	*SCP160*	NA	High	MacGilvray *et al.*, 2018
YNR026C	*SEC12*	Vesicle trafficking		
YNL287W	*SEC21*	Vesicle trafficking		

YGL137W	*SEC27*	Vesicle trafficking	High	MacGilvray *et al.*, 2018
YDL195W	*SEC31*	Vesicle trafficking		
YLR378C	*SEC61*	Vesicle trafficking		
YOR165W	*SEY1*	NA		
YHR098C	*SFB3*	Vesicle trafficking	High	MacGilvray*et al.*, 2018
YOR057W	*SGT1*	Cell cycle		
YLR079W	*SIC1*	Cell cycle	Low	Klein *et al.*, 2011
YHR149C	*SKG6*	NA		
YBL007C	*SLA1*	Vesicle trafficking		
YGL115W	*SNF4*	Transcription / Carbohydrate metabolism		
YML010W	*SPT5*	Replication / Transcription	Low	Silva *et al*, 2017
YGR116W	*SPT6*	Cell cycle / Transcription	Low	Silva *et al.*, 2017
YLR055C	*SPT8*	Transcription		
YLR082C	*SRL2*	NA		
YPL210C	*SRP72*	Translation		
YCR073C	*SSK22*	NA		
YMR039C	*SUB1*	Transcription		
YDR172W	*SUP35*	Translation	High	MacGilvray *et al.*, 2018
YDR297W	*SUR2*	NA		
YDR346C	*SVF1*	NA		
YER111C	*SWI4*	Transcription		
YIL129C	*TAO3*	NA		
YPL180W	*TCO89*	Carbohydrate metabolism		
YIL144W	*TID3*	NA		
YMR260C	*TIF11*	Translation		
YPR163C	*TIF3*	Translation		
YNL273W	*TOF1*	Cell cycle Replication		
YNL088W	*TOP2*	Cell cycle		
YNR046W	*TRM112*	Translation		
YML028W	*TSA1*	Cell cycle / Replication	High	MacGilvray *et al.*, 2018
YJL197W	*UBP12*	NA		
YMR304W	*UBP15*	NA		

YER151C	*UBP3*	Vesicle trafficking	Low	Sole *et al.*, 2011
YIL091C	*UTP25*	Replication		
YJL222W	*VTH2*	Vesicle trafficking		
YOR083W	*WHI5*	Cell cycle / Transcription	Low	Gonzalez-Novo *et al.*, 2015
YER024W	*YAT2*	NA		
YHR135C	*YCK1*	NA		
YNL154C	*YCK2*	Vesicle trafficking		
YLR249W	*YEF3*	Translation		
YFR016C	*YFR016C*	NA		
YGL036W	*YGL036W*	NA		
YJL107C	*YJL107C*	NA		
YML081W	*YML081W*	NA		
YOR052C	*YOR052C*	NA		
YOR059C	*YOR059C*	NA		
YPL260W	*YPL260W*	NA		
YML109W	*ZDS2*	Cell cycle		
YGR285C	*ZUO1*	Translation		

REFERENCES

Adrover, M.À., Zi, Z., Duch, A., Schaber, J., González-Novo, A., Jimenez, J., Nadal-Ribelles, M., Clotet, J., Klipp, E., and Posas, F. (2011). Time-dependent quantitative multicomponent control of the G 1-S network by the stress-activated protein kinase Hog1 upon osmostress. Science Signaling 4, ra63–ra63.

Ahuatzi, D., Riera, A., Peláez, R., Herrero, P., and Moreno, F. (2007). Hxk2 Regulates the Phosphorylation State of Mig1 and Therefore Its Nucleocytoplasmic Distribution. Journal of Biological Chemistry 282, 4485–4493.

Albers, E., Larsson, C., Lidén, G., Niklasson, C., and Gustafsson, L. (1996). Influence of the nitrogen source on Saccharomyces cerevisiae anaerobic growth and product formation. Applied and Environmental Microbiology 62, 3187–3195.

Albertyn, J., Hohmann, S., Thevelein, J.M., and Prior, B.A. (1994). GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. Molecular and Cellular Biology 14, 4135–4144.

Albuquerque, C.P., Smolka, M.B., Payne, S.H., Bafna, V., Eng, J., and Zhou, H. (2008). A multidimensional chromatography technology for in-depth phosphoproteome analysis. Molecular and Cellular Proteomics 7, 1389–1396.

Alepuz, P.M., Jovanovic, A., Reiser, V., and Ammerer, G. (2001). Stress-induced MAP kinase Hog1 is part of transcription activation complexes. Molecular Cell 7, 767–777.

Allen, J.J., Li, M., Brinkworth, C.S., Paulson, J.L., Wang, D., Hübner, A., Chou, W.H., Davis, R.J., Burlingame, A.L., Messing, R.O., et al. (2007). A semisynthetic epitope for kinase substrates. Nature Methods 4, 511–516.

App, H., and Holzer, H. (1989). Purification and characterization of neutral trehalase from yeast ABYS1 mutant. Journal of Biological Chemistry 264, 17583–17588.

Arvanitidis, A., and Heinisch, J.J. (1994). Studies on the function of yeast phosphofructokinase subunits by in vitro mutagenesis. The Journal of Biological Chemistry 269, 8911–8918.

Avigad, G. (1981). Stimulation of yeast phosphofructokinase activity by Fructose 2,6-bisphosphate. Biochemical and Biophysical Research Communications 102, 985–991.

Babazadeh, R., Furukawa, T., Hohmann, S., and Furukawa, K. (2014). Rewiring yeast osmostress signalling through the MAPK network reveals essential and non-essential roles of Hog1 in osmoadaptation. Scientific Reports 4.

Babazadeh, R., Lahtvee, P.J., Adiels, C.B., Goksör, M., Nielsen, J.B., and Hohmann, S. (2017). The yeast osmostress response is carbon source dependent. Scientific Reports 7.

Banaszak, K., Mechin, I., Obmolova, G., Oldham, M., Chang, S.H., Ruiz, T., Radermacher, M., Kopperschläger, G., and Rypniewski, W. (2011). The Crystal Structures of Eukaryotic Phosphofructokinases from Baker's Yeast and Rabbit Skeletal Muscle. Journal of Molecular Biology 407, 284–297.

Bartish, G., Moradi, H., and Nygård, O. (2007). Amino acids Thr56 and Thr58 are not essential for elongation factor 2 function in yeast. FEBS Journal 274, 5285–5297.

Beese, S.E., Negishi, T., and Levin, D.E. (2009). Identification of positive regulators of the yeast Fps1 glycerol channel. PLoS Genetics 5.

Benanti, J.A., Cheung, S.K., Brady, M.C., and Toczyski, D.P. (2007). A proteomic screen reveals SCFGrr1 targets that regulate the glycolytic-gluconeogenic switch. Nature Cell Biology 9, 1184–1191.

Berg, J., Tymoczko, J., and Stryer, L. (2002). Biochemistry (New York: Freeman).

Berry, D.B., and Gasch, A.P. (2008). Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. Molecular Biology of the Cell 19, 4580–4587.

Bertoni, M., Kiefer, F., Biasini, M., Bordoli, L., and Schwede, T. (2017). Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology. Scientific Reports 7.

Bienert, S., Waterhouse, A., de Beer, T.A.P., Tauriello, G., Studer, G., Bordoli, L., and Schwede, T. (2017). The SWISS-MODEL Repository-new features and functionality. Nucleic Acids Research 45, D313–D319.

Bilsland, E., Molin, C., Swaminathan, S., Ramne, A., and Sunnerhagen, P. (2004a). Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. Molecular Microbiology 53, 1743–1756.

Bilsland, E., Molin, C., Swaminathan, S., Ramne, A., and Sunnerhagen, P. (2004b). Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. Molecular Microbiology 53, 1743–1756.

Bilsland-Marchesan, E., Arino, J., Saito, H., Sunnerhagen, P., and Posas, F. (2000). Rck2 Kinase Is a Substrate for the Osmotic Stress-Activated Mitogen-Activated Protein Kinase Hog1. Molecular and Cellular Biology 20, 3887–3895.

Blázquez, M.A., Lagunas, R., Gancedo, C., and Gancedo, J.M. (1993). Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Letters 329, 51–54.

Boles, E., Schulte, F., Miosga, T., Freidel, K., Schlüter, E., Zimmermann, F.K., Hollenberg, C.P., and Heinisch, J.J. (1997). Characterization of a glucose-repressed pyruvate kinase (Pyk2p) in Saccharomyces cerevisiae that is catalytically insensitive to fructose-1,6- bisphosphate. Journal of Bacteriology 179, 2987–2993.

Boyd, S., Brookfield, J.L., Critchlow, S.E., Cumming, I.A., Curtis, N.J., Debreczeni, J., Degorce, S.L., Donald, C., Evans, N.J., Groombridge, S., et al. (2015). Structure-based design of potent and selective inhibitors of the metabolic kinase PFKFB3. Journal of Medicinal Chemistry 58, 3611–3625.

Braun, B., Pfirrmann, T., Menssen, R., Hofmann, K., Scheel, H., and Wolf, D.H. (2011). Gid9, a second RING finger protein contributes to the ubiquitin ligase activity of the Gid complex required for catabolite degradation. FEBS Letters 585, 3856–3861.

Brewster, J.L., and Gustin, M.C. (2014). Hog1: 20 years of discovery and impact. Science Signaling 7, re7–re7.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., and Gustin, M.C. (1993). An osmosensing signal transduction pathway in yeast. Science 259, 1760–1763.

Bührmann, M., Hardick, J., Weisner, J., Quambusch, L., and Rauh, D. (2017). Covalent Lipid Pocket Ligands Targeting p38α MAPK Mutants. Angewandte Chemie International Edition 56, 13232–13236.

Cabib, E., and F, L.L. (1958). The biosynthesis of trehalose phosphate. Journal of Biological Chemistry 231, 259–275.

Canal, B., Duch, A., Posas, F., and de Nadal, E. (2018). A novel mechanism for the prevention of transcription replication conflicts. Molecular and Cellular Oncology 5.

Capaldi, A.P., Kaplan, T., Liu, Y., Habib, N., Regev, A., Friedman, N., and O'shea, E.K. (2008). Structure and function of a transcriptional network activated by the MAPK Hog1. Nature Genetics 40, 1300–1306.

Causton, H.C., Ren, B., Sang Seok Koh, Harbison, C.T., Kanin, E., Jennings, E.G., Tong Ihn Lee, True, H.L., Lander, E.S., and Young, R.A. (2001). Remodeling of yeast genome expression in response to environmental changes. Molecular Biology of the Cell 12, 323–337.

Chang, Y.L., Tseng, S.F., Huang, Y.C., Shen, Z.J., Hsu, P.H., Hsieh, M.H., Yang, C.W., Tognetti, S., Canal, B., Subirana, L., et al. (2017). Yeast Cip1 is activated by environmental stress to inhibit Cdk1-G1 cyclins via Mcm1 and Msn. Nature Communications 8.

Clotet, J., Escoté, X., Adrover, M.Á., Yaakov, G., Garí, E., Aldea, M., de Nadal, E., and Posas, F. (2006). Phosphorylation of Hsl1 by Hog1 leads to a G2 arrest essential for cell survival at high osmolarity. EMBO Journal 25, 2338–2346.

Coulier, L., Bas, R., Jespersen, S., Verheij, E., van der Werf, M.J., and Hankemeier, T. (2006). Simultaneous quantitative analysis of metabolites using ion-pair liquid chromatography-electrospray ionization mass spectrometry. Analytical Chemistry 78, 6573–6582.

Coutinho, C., Bernardes, E., Félix, D., and Panek, A.D. (1988). Trehalose as cryoprotectant for preservation of yeast strains. Journal of Biotechnology 7, 23–32.

Cronwright, G.R., Rohwer, J.M., and Prior, B.A. (2002). Metabolic control analysis of glycerol synthesis in Saccharomyces cerevisiae. Applied and Environmental Microbiology 68, 4448–4456.

Dahlkvist, A., and Sunnerhagen, P. (1994). Two novel deduced serine/threonine protein kinases from Saccharomyces cerevisiae. Gene 139, 27–33.

Davenport, K.R., Sohaskey, M., Kamada, Y., Levin, D.E., and Gustin, M.C. (1995). A second osmosensing signal transduction pathway in yeast: Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway. Journal of Biological Chemistry 270, 30157–30161.

Dihazi, H., Kessler, R., and Eschrich, K. (2001). Phosphorylation and inactivation of yeast 6-phosphofructo-2-kinase contribute to the regulation of glycolysis under hypotonic stress. Biochemistry 40, 14669–14678.

Dihazi, H., Kessler, R., and Eschrich, K. (2003). Glucose-induced stimulation of the Ras-cAMP pathway in yeast leads to multiple phosphorylations and activation of 6-phosphofructo-2-kinase. Biochemistry 42, 6275–6282.

Dihazi, H., Kessler, R., and Eschrich, K. (2004). High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. Journal of Biological Chemistry 279, 23961–23968.

Dihazi, H., Kessler, R., Müller, G.A., and Eschrich, K. (2005). Lysine 3 acetylation regulates the phosphorylation of yeast 6-phosphofructo-2-kinase under hypo-osmotic stress. Biological Chemistry 386, 895–900.

van Drogen, F., O'Rourke, S.M., Stucke, V.M., Jaquenoud, M., Neiman, A.M., and Peter, M. (2000). Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo. Current Biology 10, 630–639.

Duch, A., de Nadal, E., and Posas, F. (2013a). Dealing with transcriptional outbursts during s phase to protect genomic integrity. Journal of Molecular Biology 425, 4745–4755.

Duch, A., Felipe-Abrio, I., Barroso, S., Yaakov, G., García-Rubio, M., Aguilera, A., de Nadal, E., and Posas, F. (2013b). Coordinated control of replication and transcription by a SAPK protects genomic integrity. Nature 493, 116–121.

Escoté, X., Zapater, M., Clotet, J., and Posas, F. (2004). Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1. Nature Cell Biology 6, 997–1002.

Fernández-García, P., Peláez, R., Herrero, P., and Moreno, F. (2012). Phosphorylation of Yeast Hexokinase 2 Regulates Its Nucleocytoplasmic Shuttling. Journal of Biological Chemistry 287, 42151–42164.

Ferreira, C., van Voorst, F., Martins, A., Neves, L., Oliveira, R., Kielland-Brandt, M.C., Lucas, C., and Brandt, A. (2005). A member of the sugar transporter family, Stl1p is the glycerol/H + symporter in Saccharomyces cerevisiae. Molecular Biology of the Cell 16, 2068–2076.

Ferrigno, P., Posas, F., Koepp, D., Saito, H., and Silver, P.A. (1998). Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. The EMBO Journal 17, 5606–5614.

François, J., van Schaftingen, E., and Hers, H. -G (1988). Characterization of phosphofructokinase 2 and of enzymes involved in the degradation of fructose 2,6-bisphosphate in yeast. European Journal of Biochemistry 171, 599–608.

François, J., Neves, M. -J, and Hers, H. -G (1991). The control of trehalose biosynthesis in Saccharomyces cerevisiae: Evidence for a catabolite inactivation and repression of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. Yeast 7, 575–587.

Gancedo, C., Gancedo, J.M., and Sols, A. (1968). Glycerol Metabolism in Yeasts: Pathways of Utilization and Production. European Journal of Biochemistry 5, 165–172.

Gasch, A.P., and Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. Functional and Integrative Genomics 2, 181–192.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., and Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Molecular Biology of the Cell 11, 4241–4257.

Gavin, A.C., Bösche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 415, 141–147.

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature 425, 737–741.

Gomar-Alba, M., Alepuz, P., and del Olmo, M. (2013). Dissection of the elements of osmotic stress response transcription factor Hot1 involved in the interaction with MAPK Hog1 and in the activation of transcription. Biochimica et Biophysica Acta - Gene Regulatory Mechanisms 1829, 1111–1125.

Gonçalves, P.M., Griffioen, G., Paul Bebelman, J., and Planta, R.J. (1997). Signalling pathways leading to transcriptional regulation of genes involved in the activation of glycolysis in yeast. Molecular Microbiology 25, 483–493.

González-Novo, A., Jiménez, J., Clotet, J., Nadal-Ribelles, M., Cavero, S., de Nadal, E., and Posas, F. (2015). Hog1 Targets Whi5 and Msa1 Transcription Factors To Downregulate Cyclin Expression upon Stress. Molecular and Cellular Biology 35, 1606–1618.

Grauslund, M., and Rønnow, B. (2000). Carbon source-dependent transcriptional regulation of the mitochondrial glycerol-3-phosphate dehydrogenase gene, GUT2 , from Saccharomyces cerevisiae . Canadian Journal of Microbiology 46, 1096–1100.

Grauslund, M., Lopes, J.M., and Rønnow, B. (1999). Expression of GUT1, which encodes glycerol kinase in Saccharomyces cerevisiae, is controlled by the positive regulators Adr1p, Ino2p and Ino4p and the negative regulator Opi1p in a carbon source-dependent fashion. Nucleic Acids Research 27, 4391–4398.

Guex, N., Peitsch, M.C., and Schwede, T. (2009). Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. Electrophoresis 30.

Han, J., Tschernutter, V., Yang, J., Eckle, T., and Borchers, C.H. (2013). Analysis of selected sugars and sugar phosphates in mouse heart tissue by reductive amination and liquid chromatography-electrospray ionization mass spectrometry. Analytical Chemistry 85, 5965–5973.

Hao, N., Behar, M., Parnell, S.C., Torres, M.P., Borchers, C.H., Elston, T.C.C., and Dohlman, H.G. (2007). A Systems-Biology Analysis of Feedback Inhibition in the Sho1 Osmotic-Stress-Response Pathway. Current Biology 17, 659–667.

Hao, N., Zeng, Y., Elston, T.C., and Dohlman, H.G. (2008). Control of MAPK specificity by feedback phosphorylation of shared adaptor protein Ste50. Journal of Biological Chemistry 283, 33798–33802.

Henderson, S.K., and Henderson, D.E. (1986). Reversed-phase ion pair HPLC analysis of sugar phosphates. Journal of Chromatographic Science 24, 198–203.

Hohmann, S. (2002). Osmotic Stress Signaling and Osmoadaptation in Yeasts. Microbiology and Molecular Biology Reviews 66, 300–372.

Hottiger, T., Schmutz, P., and Wiemken, A. (1987). Heat-induced accumulation and futile cycling of trehalose in Saccharomyces cerevisiae. Journal of Bacteriology 169, 5518–5522.

Jacoby, T., Flanagan, H., Faykin, A., Seto, A.G., Mattison, C., and Ota, I. (1997). Two protein-tyrosine phosphatases inactivate the osmotic stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. Journal of Biological Chemistry 272, 17749–17755.

Jurica, M.S., Mesecar, A., Heath, P.J., Shi, W., Nowak, T., and Stoddard, B.L. (1998). The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. Structure 6, 195–210.

Kim, S., and Shah, K. (2007). Dissecting yeast Hog1 MAP kinase pathway using a chemical genetic approach. FEBS Letters 581, 1209–1216.

Komatsu, Y., Kaul, S.C., Iwahashi, H., and Obuchi, K. (1990). Do heat shock proteins provide protection against freezing? FEMS Microbiology Letters 72, 159–162.

Kopperschläger, G., BÄR, J., Nissler, K., and Hofmann, E. (1977). Physicochemical Parameters and Subunit Composition of Yeast Phosphofructokinase. European Journal of Biochemistry 81, 317–325.

Kraybill, B.C., Elkin, L.L., Blethrow, J.D., Morgan, D.O., and Shokat, K.M. (2002). Inhibitor scaffolds as new allele specific kinase substrates. Journal of the American Chemical Society 124, 12118–12128.

Kronberg, M.F., Nikel, P.I., Cerrutti, P., and Galvagno, M.A. (2008). Modelling the freezing response of baker's yeast prestressed cells: A statistical approach. Journal of Applied Microbiology 104, 716–727.

Lamson, R.E., Winters, M.J., and Pryciak, P.M. (2002). Cdc42 Regulation of Kinase Activity and Signaling by the Yeast p21-Activated Kinase Ste20. Molecular and Cellular Biology 22, 2939–2951.

Lanz, M.C., Yugandhar, K., Gupta, S., Sanford, E.J., Faça, V.M., Vega, S., Joiner, A.M.N., Fromme, J.C., Yu, H., and Smolka, M.B. (2021). In-depth and 3-dimensional exploration of the budding yeast phosphoproteome. EMBO Reports 22.

Lee, J., Reiter, W., Dohnal, I., Gregori, C., Beese-Sims, S., Kuchler, K., Ammerer, G., and Levin, D.E. (2013). MAPK Hog1 closes the S. cerevisiae glycerol channel Fps1 by phosphorylating and displacing its positive regulators. Genes and Development 27, 2590–2601.

Lee, Y.J., Jeschke, G.R., Roelants, F.M., Thorner, J., and Turk, B.E. (2012). Reciprocal Phosphorylation of Yeast Glycerol-3-Phosphate Dehydrogenases in Adaptation to Distinct Types of Stress. Molecular and Cellular Biology 32, 4705–4717.

Lewis, J.G., Learmonth, R.P., and Watson, K. (1995). Induction of heat, freezing and salt tolerance by heat and salt shock in Saccharomyces cerevisiae. Microbiology 141, 687–694.

Lillie, S.H., and Pringle, J.R. (1980). Reserve carbohydrate metabolism in Saccharomyces cerevisiae: responses to nutrient limitation. Journal of Bacteriology 143, 1384–1394.

Lin, Y. yi, Lu, J. ying, Zhang, J., Walter, W., Dang, W., Wan, J., Tao, S.C., Qian, J., Zhao, Y., Boeke, J.D., et al. (2009). Protein Acetylation Microarray Reveals that NuA4 Controls Key Metabolic Target Regulating Gluconeogenesis. Cell 136, 1073–1084.

Lu, W., Clasquin, M.F., Melamud, E., Amador-Noguez, D., Caudy, A.A., and Rabinowitz, J.D. (2010). Metabolomic analysis via reversed-phase ion-pairing liquid chromatography coupled to a stand alone orbitrap mass spectrometer. Analytical Chemistry 82, 3212–3221.

Luyten, K., Albertyn, J., Skibbe, W.F., Prior, B.A., Ramos, J., Thevelein, J.M., and Hohmann, S. (1995). Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. EMBO Journal 14, 1360–1371.

MacGilvray, M.E., Shishkova, E., Chasman, D., Place, M., Gitter, A., Coon, J.J., and Gasch, A.P. (2018a). Network inference reveals novel connections in pathways regulating growth and defense in the yeast salt response. PLoS Computational Biology 14.

Maeda, T., Takekawa, M., and Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science 269, 554–558.

Mager, W.H., and Varela, J.C.S. (1993). Osmostress response of the yeast Saccharomyces. Molecular Microbiology 10, 253–258.
Meikle, A. (1991). The osmotic responses of Saccharomyces cerevisiae in K+-depleted medium. FEMS Microbiology Letters 78, 89–93.

Melcher, M.L., and Thorner, J. (1996). Identification and characterization of the CLK1 gene product, a novel CaM kinase-like protein kinase from the yeast Saccharomyces cerevisiae. Journal of Biological Chemistry 271, 29958–29968.

de Mesquita, J.F., Panek, A.D., and de Araujo, P.S. (2003). In silico and in vivo analysis reveal a novel gene in Saccharomyces cerevisiae trehalose metabolism. BMC Genomics 4, 1–6.

Mittenbühler, K., and Holzer, H. (1991). Characterization of different forms of yeast acid trehalase in the secretory pathway. Archives of Microbiology 155, 217–220.

Molin, C., Jauhiainen, A., Warringer, J., Nerman, O., and Sunnerhagen, P.E.R. (2009). MRNA stability changes precede changes in steady-state mRNA amounts during hyperosmotic stress. RNA 15, 600–614.

Moore, P.A., Sagliocco, F.A., Wood, R.M., and Brown, A.J. (1991). Yeast glycolytic mRNAs are differentially regulated. Molecular and Cellular Biology 11, 5330–5337.

Morano, K.A., Grant, C.M., and Moye-Rowley, W.S. (2012). The response to heat shock and oxidative stress in saccharomyces cerevisiae. Genetics 190, 1157–1195.

Morris, C.N., Ainsworth, S., and Kinderlerer, J. (1986). The regulatory properties of yeast pyruvate kinase. Effect of fructose 1,6-bisphosphate. Biochemical Journal 234, 691–698.

Müller, S., Zimmermann, F.K., and Boles, E. (1997). Mutant studies of phosphofructo-2-kinases do not reveal an essential role of fructose-2,6-bisphosphate in the regulation of carbon fluxes in yeast cells. Microbiology 143, 3055–3061.

Nadal-Ribelles, M., Solé, C., Xu, Z., Steinmetz, L.M., deNadal, E., and Posas, F. (2014). Control of Cdc28 CDK1 by a Stress-Induced lncRNA. Molecular Cell 53, 549–561.

Nevoigt, E., and Stahl, U. (1997). Osmoregulation and glycerol metabolism in the yeast Saccharomyces cerevisiae. FEMS Microbiology Reviews 21, 231–241.

Nissen, T.L., Hamann, C.W., Kielland-Brandt, M.C., Nielsen, J., and Villadsen, J. (2000). Anaerobic and aerobic batch cultivations of Saccharomyces cerevisiae mutants impaired in glycerol synthesis. Yeast 16, 463–474.

Nissler, K., Otto, A., Schellenberger, W., and Hofmann, E. (1983). Similarity of activation of yeast phosphofructokinase by AMP and fructose-2,6-bisphosphate. Biochemical and Biophysical Research Communications 111, 294–300.

Norbeck, J., Påhlman, A.K., Akhtar, N., Blomberg, A., and Adler, L. (1996). Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from Saccharomyces cerevisiae: Identification of the corresponding GPP1 and GPP2 genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. Journal of Biological Chemistry 271, 13875–13881.

Noubhani, A., Bunoust, O., Rigoulet, M., and Thevelein, J.M. (2000). Reconstitution of ethanolic fermentation in permeabilized spheroplasts of wild-type and trehalose-6-phosphate synthase mutants of the yeast Saccharomyces cerevisiae. European Journal of Biochemistry 267, 4566–4576.

Ortiz, C.H., Maia, J.C.C., Tenan, M.N., Braz-Padrão, G.R., Mattoon, J.R., and Panek, A.D. (1983). Regulation of yeast trehalase by a monocyclic, cyclic AMP-dependent phosphorylation-dephosphorylation cascade system. Journal of Bacteriology 153, 644–651.

Påhlman, A.K., Granath, K., Ansell, R., Hohmann, S., and Adler, L. (2001). The Yeast Glycerol 3-Phosphatases Gpp1p and Gpp2p Are Required for Glycerol Biosynthesis and Differentially Involved in the Cellular Responses to Osmotic, Anaerobic, and Oxidative Stress. Journal of Biological Chemistry 276, 3555–3563.

Panek, A. (1963). Function of trehalose in Baker's yeast (Saccharomyces cerevisiae). Archives of Biochemistry and Biophysics 100, 422–425.

Pelaez, R., Herrero, P., and Moreno, F. (2010). Functional domains of yeast hexokinase 2. Biochem. J 432, 181–190.

Petelenz-Kurdziel, E., Kuehn, C., Nordlander, B., Klein, D., Hong, K.K., Jacobson, T., Dahl, P., Schaber, J., Nielsen, J., Hohmann, S., et al. (2013a). Quantitative Analysis of Glycerol Accumulation, Glycolysis and Growth under Hyper Osmotic Stress. PLoS Computational Biology 9, e1003084.

Petrezselyova, S., Zahradka, J., and Sychrova, H. (2010). Saccharomyces cerevisiae BY4741 and W303-1A laboratory strains differ in salt tolerance.

Plourde-Owobi, L., Durner, S., Parrou, J.L., Wieczorke, R., Goma, G., and François, J. (1999). AGT1, encoding an α-glucoside transporter involved in uptake and intracellular accumulation of trehalose in Saccharomyces cerevisiae. Journal of Bacteriology 181, 3830–3832.

Poorman, R.A., Randolph, A., Kemp, R.G., and Heinrikson, R.L. (1984). Evolution of phosphofructokinase - Gene duplication and creation of new effector sites. Nature 309, 467–469.

Portela, P., Moreno, S., and Rossi, S. (2006). Characterization of yeast pyruvate kinase 1 as a protein kinase A substrate, and specificity of the phosphorylation site sequence in the whole protein. Biochem. J 396, 117–126.

Posas, F., and Saito, H. (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: Scaffold role of Pbs2p MAPKK. Science 276, 1702–1708.

Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C., and Saito, H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 “two-component” osmosensor. Cell 86, 865–875.

Raitt, D.C., Posas, F., and Saito, H. (2000). Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. 19, 4623–4631.

Reinders, A., Bürckert, N., Hohmann, S., Thevelein, J.M., Boller, T., Wiemken, A., and de Virgilio, C. (1997). Structural analysis of the subunits of the trehalose-6-phosphate synthase/phosphatase complex in Saccharomyces cerevisiae and their function during heat shock. Molecular Microbiology 24, 687–696.

Reiser, V., Salah, S.M., and Ammerer, G. (2000). Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. Nature Cell Biology 2, 620–627.

Rep, M., Reiser, V., Gartner, U., Thevelein, J.M., Hohmann, S., Ammerer, G., and Ruis, H. (1999). Osmotic Stress-Induced Gene Expression in Saccharomyces cerevisiae Requires Msn1p and the Novel Nuclear Factor Hot1p. Molecular and Cellular Biology 19, 5474–5485.

Rep, M., Krantz, M., Thevelein, J.M., and Hohmann, S. (2000). The transcriptional response of Saccharomyces cerevisiae to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. Journal of Biological Chemistry 275, 8290–8300.

Reuter, R., Naumann, M., Bär, J., Haferburg, D., and Kopperschläger, G. (2000). Purification, molecular and kinetic characterization of phosphofructokinase-1 from yeast Schizosaccharomyces pombe: evidence for an unusual subunit composition. Yeast 16, 1273–1285.

Rgen Kirchberger, J., Edelmann, A., Ger, K., Rgen, J., and Heinisch, J. (1999). A single point mutation leads to an instability of the hetero-octameric structure of yeast phosphofructokinase.

Richter, K., Haslbeck, M., and Buchner, J. (2010). The Heat Shock Response: Life on the Verge of Death. Molecular Cell 40, 253–266.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nature Biotechnology 17, 1030–1032.

Rodicio, R., Strauß, A., Rgen, J., and Heinisch, J. (2000). Single Point Mutations in Either Gene Encoding the Subunits of the Heterooctameric Yeast Phosphofructokinase Abolish Allosteric Inhibition by ATP* □ S. Journal of Biological Chemistry 275, 40952–40960.

Roelants, F.M., Breslow, D.K., Muir, A., Weissman, J.S., and Thorner, J. (2011). Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America 108, 19222–19227.

Romanov, N., Hollenstein, D.M., Janschitz, M., Ammerer, G., Anrather, D., and Reiter, W. (2017). Identifying protein kinase-specific effectors of the osmostress response in yeast. Science Signaling 10.

Ruiz, T., Kopperschläger, G., and Radermacher, M. (2001). The first three-dimensional structure of phosphofructokinase from Saccharomyces cerevisiae determined by electron microscopy of single particles. Journal of Structural Biology 136, 167–180.

Saito, H., and Posas, F. (2012). Response to hyperosmotic stress. Genetics 192, 289–318.

Schepers, W., van Zeebroeck, G., Pinkse, M., Verhaert, P., and Thevelein, J.M. (2012). In vivo phosphorylation of Ser21 and Ser83 during nutrient-induced activation of the yeast protein kinase A (PKA) target trehalase. Journal of Biological Chemistry 287, 44130–44142.

Siderius, M., and Mager, W. (1997). General stress response: In search of a common denominator. In Yeast Stress Responses, H. S, and M. WH, eds. (Mol. Biol. Intelligence Units, R.G. Landes Company), pp. 213–230.

Singer, M.A., and Lindquist, S. (1998). Thermotolerance in Saccharomyces cerevisiae: The Yin and Yang of trehalose. Trends in Biotechnology 16, 460–468.

Sprague, G.F., and Cronan, J.E. (1977). Isolation and characterization of Saccharomyces cerevisiae mutants defective in glycerol catabolism. Journal of Bacteriology 129, 1335–1342.

Studer, G., Rempfer, C., Waterhouse, A.M., Gumienny, R., Haas, J., and Schwede, T. (2020). QMEANDisCo—distance constraints applied on model quality estimation. Bioinformatics 36, 1765–1771.

Sutherland, F.C.W., Lages, F., Lucas, C., Luyten, K., Albertyn, J., Hohmann, S., Prior, B.A., and Kilian, S.G. (1997). Characteristics of Fps1-dependent and -independent glycerol transport in Saccharomyces cerevisiae. Journal of Bacteriology 179, 7790–7795.

Swaminathan, S., Masek, T., Molin, C., Pospisek, M., and Sunnerhagen, P. (2006). Rck2 is required for reprogramming of ribosomes during qxidative stress. Molecular Biology of the Cell 17, 1472–1482.

Swaney, D.L., Beltrao, P., Starita, L., Guo, A., Rush, J., Fields, S., Krogan, N.J., and Villén, J. (2013). Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. Nature Methods 10, 676–682.

Tatebayashi, K., Yamamoto, K., Tanaka, K., Tomida, T., Maruoka, T., Kasukawa, E., and Saito, H. (2006). Adaptor functions of Cdc42, Ste50, and Sho1 in the yeast osmoregulatory HOG MAPK pathway. EMBO Journal 25, 3033–3044.

Teige, M., Scheikl, E., Reiser, V., Ruis, H., and Ammerer, G. (2001a). Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast. Proceedings of the National Academy of Sciences of the United States of America 98, 5625–5630.

Tognetti, S., Jiménez, J., Viganò, M., Duch, A., Queralt, E., de Nadal, E., and Posas, F. (2020). Hog1 activation delays mitotic exit via phosphorylation of Net1. Proceedings of the National Academy of Sciences of the United States of America 117, 8924–8933.

Tomás-Cobos, L., Casadomé, L., Mas, G., Sanz, P., and Posas, F. (2004). Expression of the HXT1 low affinity glucose transporter requires the coordinated activities of the HOG and glucose signalling pathways. Journal of Biological Chemistry 279, 22010–22019.

Trevisol, E.T.V., Panek, A.D., de Mesquita, J.F., and Eleutherio, E.C.A. (2014). Regulation of the yeast trehalose-synthase complex by cyclic AMP-dependent phosphorylation. Biochimica et Biophysica Acta - General Subjects 1840, 1646–1650.

Tripodi, F., Nicastro, R., Reghellin, V., and Coccetti, P. (2015). Post-translational modifications on yeast carbon metabolism: Regulatory mechanisms beyond transcriptional control. Biochimica et Biophysica Acta - General Subjects 1850, 620–627.

Veisova, D., Macakova, E., Rezabkova, L., Sulc, M., Vacha, P., Sychrova, H., Obsil, T., and Obsilova, V. (2012). Role of individual phosphorylation sites for the 14-3-3-protein-dependent activation of yeast neutral trehalase Nth1. Biochemical Journal 443, 663–670.

Warmka, J., Hanneman, J., Lee, J., Amin, D., and Ota, I. (2001). Ptc1, a Type 2C Ser/Thr Phosphatase, Inactivates the HOG Pathway by Dephosphorylating the Mitogen-Activated Protein Kinase Hog1. Molecular and Cellular Biology 21, 51–60.

Warringer, J., Hult, M., Regot, S., Posas, F., and Sunnerhagen, P. (2010). The HOG pathway dictates the short-term translational response after hyperosmotic shock. Molecular Biology of the Cell 21, 3080–3092.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L., et al. (2018). SWISS-MODEL: Homology modelling of protein structures and complexes. Nucleic Acids Research 46, W296–W303.

Westfall, P.J., and Thorner, J. (2006). Analysis of Mitogen-Activated Protein Kinase Signaling Specificity in Response to Hyperosmotic Stress: Use of an Analog-Sensitive HOG1 Allele. EUKARYOTIC CELL 5, 1215–1228.

Winderickx, J., de Winde, J.H., Crauwels, M., Hino, A., Hohmann, S., van Dijck, P., and Thevelein, J.M. (1996). Regulation of genes encoding subunits of the trehalose synthase complex in Saccharomyces cerevisiae: novel variations of STRE-mediated transcription control? Molecular and General Genetics MGG 252, 470–482.

Yang, H.Y., Tatebayashi, K., Yamamoto, K., and Saito, H. (2009). Glycosylation defects activate filamentous growth Kss1 MAPK and inhibit osmoregulatory Hog1 MAPK. EMBO Journal 28, 1380–1391.

Young, E.T., Zhang, C., Shokat, K.M., Parua, P.K., and Braun, K.A. (2012). The AMP-activated protein kinase Snf1 regulates transcription factor binding, RNA polymerase II activity, and mRNA stability of glucose-repressed genes in Saccharomyces cerevisiae. Journal of Biological Chemistry 287, 29021–29034.

Zähringer, H., Thevelein, J.M., and Nwaka, S. (2000). Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: Variations of PKA effect during stress and growth. Molecular Microbiology 35, 397–406.

Zapater, M., Clotet, J., Escoté, X., and Posas, F. (2005). Control of cell cycle progression by the stress-activated Hog1 MAPK. Cell Cycle 4, 6–7.