TÉCNICO LISBOA

The role of nitrogen in cytosolic pH regulation in *Saccharomyces cerevisiae*

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...’”

Isaac Asimov

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ABSTRACT

pH regulation inside the cell has become a matter of research since the science community became aware that it is not constant throughout growth. Orij et al. identified cytosolic pH (pHc) as a signal that controls growth rate in the yeast baker model *Saccharomyces cerevisiae* (Orij et al. 2011). Since most of biological relevant molecules are weak acids or bases, their protonation state can change in response to the intracellular pH behavior. cAMP-dependent Protein Kinase A (PKA) pathway controls cellular growth and can regulate cytosolic pH based on glucose availability.

Target of Rapamycin (TOR) pathway also controls growth and protein genesis but in response to nitrogen, thus we asked whether this nutrient has also a role on pH_c regulation. To assess this question we monitored growth and pH_c of cells growing in different amounts of nitrogen and different nitrogen sources. Along this study, we identified that the concentration of nitrogen present during growth determines the extension of cytosol acidification after cells naturally depleted glucose: cells growing in higher concentrations of nitrogen tend to have a lower pH_c.

We also performed artificial starvations and in most cases glucose starvation experiments mimicked growth curves. We tried to link nitrogen availability to pH_c regulation via TORC1 pathway performing treatments with rapamycin, an immunosuppressant that inhibits TORC1 activity. However based on our results we discarded the hypothesis that this complex mediates the nitrogen effect on pH_c regulation.

Keywords: cytosolic pH, glucose, nitrogen, starvation, TORC1, rapamycin

RESUMO

Desde que a comunidade científica se apercebeu que o pH intracelular não é constante ao longo do crescimento cellular, que o tema da regulação do pH se tornou num tema de investigação. Orij *et al.*, identifica o pH citosólico como sendo um sinal que controla a velocidade de crescimento, estudado no modelo de leveduras *Saccharomyces cerevisiae*. A maioria das moléculas biológicas são ácidos ou bases fracas, logo o seu estado de protonação pode variar consoante o comportamento do pH intracelular. A Proteína Quinase A dependente de AMP cíclico (PKA_c) é responsável por controlar o crescimento celular e regula o pH citosólico com base na disponibilidade de glucose.

TOR (Target of Rapamycin, "alvo da rapamicina") é uma quinase que, tal como a PKA, controla o crescimento e a síntese de proteínas, mas em resposta à disponibilidade de azoto. Desta forma surgiu a questão se também este nutriente desempenha um papel na regulação do pH_c. Para responder a esta questão, monitorizámos o crescimento e o pH_c de células a crescer em diferentes quantidades e fonts de azoto. Ao longo do estudo conseguimos identificar que a concentração de azoto presente durante o crescimento determina a acidificação no citoplasma depois das células consumirem toda a glucose: leveduras a crescer em maiores quantidades de azoto tendem a ter o pH_c mais baixo.

Expusemos as leveduras a deprivações artificiais de glucose e na maioria dos casos este protocolo replicou o efeito do consumo da glucose durante o crescimento. Tentámos ligar a disponibilidade de azoto com a activação de TORC1 e a regulação do pH_c realizando tratamento com a rapamicina. Esta droga é um imunossupressor, que inibe a actividade de TORC1. Contudo, com base nos resultados obtidos essa hipótese foi descartada.

Palavras-chave: pH citosólico, glucose, azoto, deprivação, TORC1, rapamicina

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ACRONYMS

AA amino acids

AC Adenylate cyclase

AS Ammonium Sulphate

EGOC EGO Complex

GA Glutamic Acid

H Histidine

kDa kilo Dalton

L Leucine

LiAc Lithium Acetate

Loflo Low fluorescence medium

M Methionine

N Nitrogen

NCR Nitrogen Catabolite Repression

nM nanomolar

nm nanometre

n.s. no significance

OD Optical Density

pH_c cytosolic pH

pH_i intracellular pH

PKA Protein Kinase A

Pr Purine

SC Synthetic Complete

SD Synthetic Defined

SD-AA Synthetic Defined without amino acids

SEACAT SEA sub complex activating TORC1

SEACIT SEA sub complex inhibiting TORC1

SEM Standard Error of the Mean

ser/thr serine/threonine

TOR Target of Rapamycin

TORC1 Target of Rapamycin Complex 1

TORC2 Target of Rapamycin Complex 2

Ur Urea

w/o without

WT wild type

YPD Yeast Peptone Dextrose

1 LITERATURE REVIEW

1.1 Yeast cell as a model organism

Some of the biochemical information relevant to humans can be obtained by studying simple organisms, as many of the regulating proteins and cellular pathways are evolutionary conserved. The yeast baker model *Saccharomyces cerevisiae* has been recognized as a favourite organism to study fundamental biochemical processes, like nutrient-sensing and signalling mechanisms (Rødkaer & Faergeman 2014) (Conrad et al. 2014). Nowadays, there are available a lot of molecular and genetic tools to study yeast, thus making this organism an undoubtedly contributor for understanding cellular processes in higher eukaryotes (De Virgilio & Loewith 2006).

Nutrient sensing and signalling pathways are commonly deregulated in human metabolic diseases, like cancer (Efeyan et al. 2015). Studying how these pathways are regulated at a unicellular level helps to find solutions for multicellular organisms. In multicellular organisms, most cells are not directly exposed to changes in environmental nutrient levels like unicellular organisms that are able to sense both intracellular and environmental fluctuations of nutrients. Nevertheless, intercellular nutrient sensing mechanisms are also present in multicellular organisms, since nutrients levels can also fluctuate. The conservation of these pathways has allowed extrapolating what happens in yeast to address issues in more complex organisms, such as cancer metabolism or growth control (Broach 2012).

1.2 Biological pH

The classical definition of the pH of a solution as the negative logarithm of the free protons activity in water does not simply apply to the pH inside of a yeast cell. This is mainly because of all the metabolites (weak acids or bases) inside the cell that can take up or donate a proton, maintaining the cytosol as a complex buffer (Orij et al. 2011). Thus making it difficult to achieve a steady concentration of protons. Almost all the biosynthetic intermediates are ionized at neutral intracellular pH (pH_i). Most of them are phosphorylated or carboxylated, and their molecular behaviour can change with respect to pH_i, which makes pH_i very relevant for cell function. For example, a change in the protonation state in the active site of an enzyme can change the interaction with the residue and, therefore, the affinity with the substrate or its catalytical activity, influencing the enzymatic reaction (Milletti et al. 2009). Besides this, cytosolic pH (pH_c) on yeast cells is maintain around neutrality during growth, despite the pH value of each organelle (Orij et al. 2009) (Preston et al. 1989) (Brett et al. 2005) (Martínez-Muñoz & Kane 2008). Indeed, Young *et al.*, found that intracellular pH can influence the binding of proteins to

the signalling lipid phosphatidic acid, because it changes the protonation state of the phosphate head group (Young et al. 2010).

To determine intracellular pH in yeast cells requires a determination of the relative protonation state of a particular molecule and this would require knowing all the pK_a values of the acidic and basic groups in their context (Preston et al., 1989). However methods for this measurement are unavailable, thus it was necessary to discover methods that measure the intracellular pH of cells. Some of classical methods for intracellular pH determination require extension manipulation of cells like, ^{31}P nuclear magnetic resonance (NMR) (Gillies et al., 1981) (Ogino et al. 1983), probing cells with pH-sensitive dyes like C.SNARF-1 (Bracey et al., 1998) or equilibrium distribution of weak acids by radiolabelling (Krebs et al., 1983) (Siegumfeldt et al., 2000), 9-aminoacridine and fluorescein. The recently discovered use of pH-sensitive fluorescent proteins (like the ratiometric pHluorin) has made it possible to determine cytosolic pH without perturb the cells, in a time-resolved and organelle specific manner (Orij et al. 2009).

Most organelles have their specific pH value and ion distribution over the plasma membrane and in the membranes of these organelles, creating H^+ gradients and membrane potentials (Orij et al. 2009). Among those gradients, H^+ are often used for the transport of nutrients by symport or antiport and pH_i is highly dependent on nutrient availability (Orij et al. 2009).

pH_i values are a highly dynamic property of yeast cells and besides being sensitive to changes in environmental conditions yeast can withstand substantial shifts on pH, without loss of viability. It is well established that in yeast cells cytosolic pH responds to the availability of glucose (Dechant & Peter 2010). Earlier reports have linked cytosolic pH to cell proliferation and therefore, associating pH_c as a cellular signal that controls growth (Orij et al. 2011) (Orij et al. 2012). Some studies in yeast and also in higher eukaryotes have shown that cytosolic pH of tumour cells was higher than normal cells, suggesting that a rather alkaline cytosol is a prerequisite for cell proliferation (Webb et al. 2011), while a decrease in pH has shown to trigger apoptosis (Balgi et al. 2011). The relevance of changes in intracellular pH is apparent but unclear: are these changes a signal that triggers cellular responses or the response of the cell itself, a way to create the perfect cell environment for certain processes to occur?

1.2.1 pH homeostasis in yeast

Different organelles have different pH depending on the processes that take place in those compartments. The gradients created by the ion concentration differences between them are essential for cell functioning (Nishimura et al. 1998). For example, vacuolar proteases have optimal activity at low pH and the other way around for mitochondria, that is alkaline compared to the cytosol. The energy that these gradients create (the membrane potential) is the main driving force for ion and nutrient translocation (Nishimura et al. 1998) (Ohsumi & Anraku, 1981).

The overall pH homeostasis is actively coordinated by the function of two different types of ATP-dependent H^+ pumps: P-types ATPases that are in the plasma membrane and V-types in the vacuolar membrane, both known to be electrogenic pumps (Martínez-Muñoz & Kane 2008). Pma1p is one of the main regulators of pH_c, pumping protons out of the cell at a stoichiometry of 1 H^+ translocated per ATP hydrolysed. The phosphorylation of Pma1 is induced by glucose availability. The pump activity itself is pH-sensitive and increases with cytosolic acidification (Orij et al. 2011). Vacuolar-type H^+-ATPases reside in the membranes of various organelles, such as the vacuole, endosomes and late Golgi apparatus, pumping protons out of the cytosol and are very highly conserved among eukaryotes (Martínez-Muñoz & Kane 2008). They consist of fourteen subunits arranged in two sub complexes: V_1 peripheral sub complex that contains the sites for ATP hydrolysis and V_o sub complex that comprises the proton pore (Orij et al. 2011).

Both proton pumps coordinate in parallel to regulate pH homeostasis and both are activated by glucose (Figure 1-1) (Orij et al. 2011). Besides being both regulated by glucose that is converted by glycolysis producing protons used to produce ATP and NADPH; Pma1 and V-ATPases pump H^+ with the energy of ATP hydrolysis out of the cytosol and are pH-sensitive (Martínez-Muñoz & Kane 2008). Pumping protons out of the cytosol, once their concentration increase because of all the metabolic pathways, is the way they maintain the concentration of H^+ stable and pH around 7. In the absence of glucose ATP is not produce, thus both pumps are inactive and protons remain inside the cell, lowering pH_c.

The combined efforts of both H^+-pumps gives yeast the ability to be viable under various stresses, such as low pH or absence of nutrients. However, V-ATPases are not essential for growth at optimal growth conditions (Orij et al. 2011), like Pma1, but they are able to affect pH_c without affecting Pma1 localization (Orij et al. 2009).

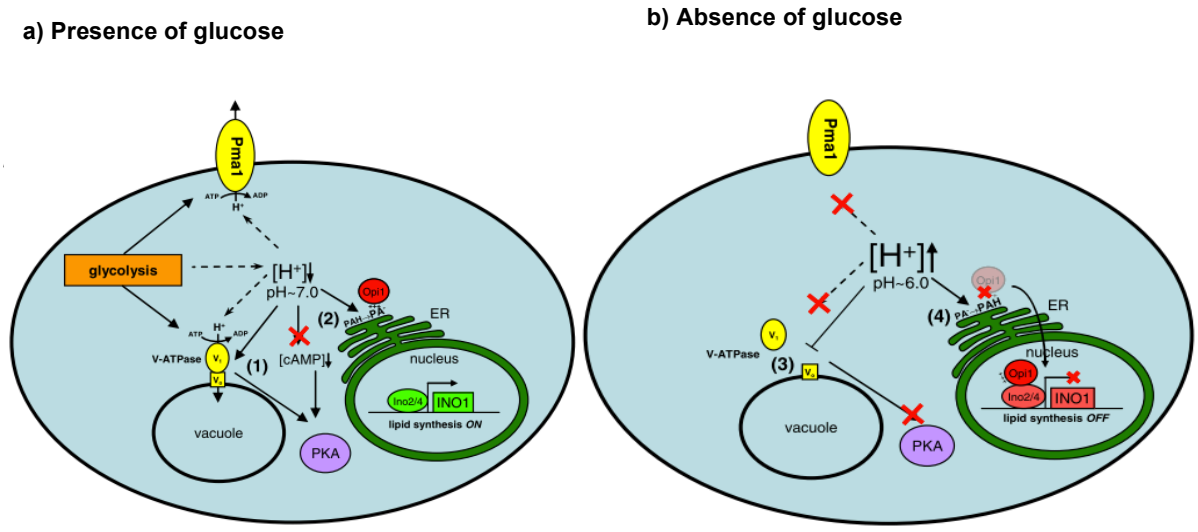


Figure 1-1 – *pH_c signalling in yeast adapted from (Orij et al. 2011), dependent on glucose availability.* a) In the presence of glucose, pH_c is maintained around 7 by the combination of the two activated proton pumps: Pma1 and V-ATPase. They affect (1) the activity of PKA pathway and (2) the transition to the deprotonated form of the

phosphatidic acid in the Endoplasmatic Reticulum (ER) membrane. b) In the absence of glucose there is an acidification of the cytosol; (3) V-ATPase disassembles from the vacuole and (4) there is a protonation of the phosphatidic acid of the deprotonated form, in the ER. Source: (Orij et al. 2011)

Most of the nutrients uptake depends on the proton gradient over the plasma membrane (Efeyan et al. 2015); therefore yeast cells prefer an acidic external pH to a neutral one or above.

Orij *et al.*, followed growth of yeast cells and monitored cytosolic pH and observed a decrease of pH_c after glucose depletion (Figure 1-2 a)). pH_c is not static but dynamic during growth. In Figure 1-2 a) it is clear that intracelullar pH is maintain around 7 during exponential growth and that this is the optimal pH for cells to grow; once they start to depleted glucose, an immediate drop in pH_c can be observed as weel as in the growth rate. In this study they had also evidences that pH_c controls growth rate (Figure 1-2 b) based on glucose availability (Orij et al. 2012).

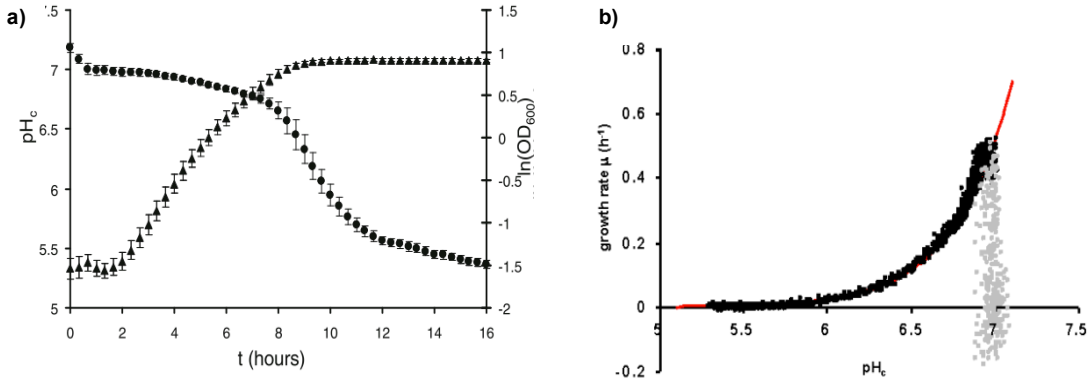


Figure 1-2 – *pH_c is dynamic during growth and controls growth rate.* a) Decrease in pH_c after glucose depletion. Growth and pH_c were monitored during growth on glucose: pH_c is around neutrality during lag and exponential phases and once glucose is depleted and an acidification occurs and pH_c drops around 1 unit. b) pH_c controls growth rate: lower pH leads cells to stop growing. Source: (Orij et al. 2012).

These studies contributed to highlight the importance of nutrient availability in the cell survival and how a simple physiological parameter can influence growth and dead.

1.3 Nutrient signalling pathways in yeast

Availability of key nutrients dictates the development and growth rates of yeast cells. Nutrients such as sugars, amino acids, and nitrogen compounds (Broach 2012) provide energy and essential building blocks for the synthesis of biomolecules, such as proteins, lipids, and nucleotides (Shimobayashi & Hall 2016). But nutrients supply not only the substrates but also the signals for growth, exerting crucial regulatory roles (Conrad et al. 2014). In *S. cerevisiae*, nutrient availability is sensed by a number of overlapping signalling and metabolic pathways that influence cells translational, posttranslational,

metabolic and transcriptional profiles (Broach 2012). Understanding nutrient regulation in yeast requires taking into consideration the roles of nutrient as both metabolites and as signalling molecules and their interconnection.

Yeast cells can respond to nutrient availability and they adjust to nutrient limitation by developing alternative processes based on the particular nutritional circumstances. These adaptations allow the reversible transition from rapid mitotic growth in rich media to distinct quiescent states that shut down the cell activity in response to starvation for one or more nutrients (Broach 2012) (Klosinska et al. 2011).

Understanding how yeast takes up these nutrients, what are the metabolic pathways involved in their regulation and the processes and metabolites that are influence by them becomes a must.

1.3.1 Glucose signalling in yeast

S. cerevisiae can grow on a wide variety of carbon sources, but like most organisms, uses glucose as the preferred carbon and energy source. Glucose is the main contributor to the carbon skeleton of most biological molecules synthetized in the cell (Rødkaer & Faergeman 2014) and dictates the aerobic ethanol fermentation characteristic of yeast (the Crabtree effect) (Kim et al. 2013). The Crabtree effect consists on the cell repression of respiration by fermentation, since yeast consumes glucose mainly by aerobic fermentation (Deken 1966). Yeast prefers any fermentable source, namely glucose or fructose, over any that has to be catabolized by oxidative phosphorylation, such as glycerol, ethanol or acetate. This hierarchical pattern of carbon sources consumption is established by (1) allosteric regulation of enzymes responsible for glycolysis and glycogenesis and (2) repression of transcription of genes that are required for catabolism of less favourable sugars and that encodes components for the electron transport chain on mitochondrial proteins (Broach 2012).

Glucose signalling in *S. cerevisiae* is a complex process because of the multiple and parallel glucose-responsive pathways involved. In yeast there are two main glucose-sensing pathways based on glucose availability: Gpr1 and HXT. Yeast has six different types of glucose transporter (HXT) genes and expresses them depending on the affinity for glucose (Kim et al. 2013)

There are three main glucose response pathways in yeast and the aerobic fermentation is in part regulated by two: (1) the Rgt2/Snf3 glucose induction pathway and by (2) the Snf1/Mig1 repression pathway. The first is responsible for glucose uptake and the latter responsible for regulating negatively the genes responsible for the use of non preferred sugars and the ones involved in glucose oxidation (Kim et al. 2013). The other is (3) Protein Kinase A (PKA) pathway, that is responsible for mediating stress responses and the glucose effects on the cell biosynthetic capacity (Zaman et al. 2009). The three pathways converge at multiple points, for instance on the induction of the glucose transport genes, depending on glucose availability (Kim et al. 2013).

In this work we want to understand how PKA pathway works mainly because it regulates cytosolic pH in yeast and because it controls fermentative growth (Orij et al. 2011). Its activity is both necessary and sufficient to induce the majority of the transcriptional responses in response to glucose availability. Besides this PKA is involved in cell growth, stress responses and metabolism (Kim et al. 2013) since inactivation of this pathway eliminates most of these genes response (Zaman et al. 2009). It exists has a tetrameric holoenzyme comprising two catalytic subunits encoded by three related genes, *TPK1*, *TPK2*, and *TPK3*, and two regulatory subunits encoded by *BCY1* (Figure 1-3). PKA activity is coordinated by cAMP. This second messenger binds to Bcy1 liberating the catalytic subunits. Glucose activates adenylate cyclase (AC) which synthetizes cAMP, via two branches (Gpr1/Gpr2 and the Ras1/Ras2). The mechanisms by which glucose affects Ras GTP levels are still unclear.

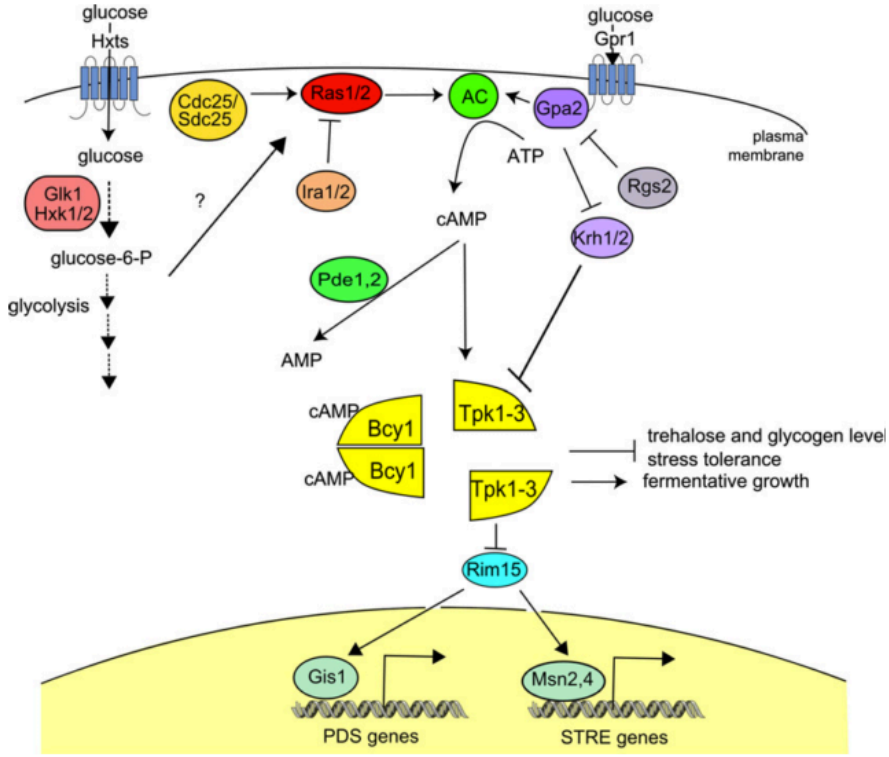


Figure 1-3 – *Activation of the cAMP-dependent PKA pathway by both extracellular glucose sensing systems: GPCR system and hexokinase-mediated phosphorylation.* Gpr1 and the complex Cdc25/Sdc25 act as GEF towards Gpa2 and Ras1,2 respectively. Rgs2 and Ira1/2 act as GAP to Gpa2 and Ras1/2 respectively. AC is activated by the activated forms of Gpa2 and Ras1/2 and produces cAMP after ATP hydrolysis. cAMP binds to the catalytic subunits of the PKA pathway causing activation of the catalytic sub-units. The activation of the PKA pathway supress stress tolerance of yeast cells and inhibits Rim15, known as a positive regulator of the transcription factors that activate post diauxic growth and stress-response gene expression. Source: (Conrad et al. 2014)

As described above, pH_c is a crucial signal for glucose availability and glucose is a known signal of PKA pathway (Orij et al. 2011). Yeast cells starved for glucose are no longer able to maintain cytosolic acidification around neutrality and a decrease in pH is observed. However, re-addition of glucose to

starved cells leads to a rapidly acidification, followed by an alkalinisation to neutral pH before growth recommences, likely by the coordination of both proton pumps (Dechant & Peter 2014) (Martínez-Muñoz & Kane 2008). This response of intracellular pH to glucose availability was one of the firt suggestions that pH_c may be under the control of glucose response.

Recent work indicates that PKA activity prior to glucose starvation is essential to set cytosolic pH once this nutrient is absent. Addition of cAMP prior to glucose starvation helped to induce a stronger cytosol acidification once glucose was depleted, compared to cells not treated. This suggested that cells have memory of the preconditioning conditions. Also, the increase in proton concentration was almost as big as the one induced by the absence of glucose itself ((Edo n.d.), data not published). How PKA sets pH_c or if it is pH that functions as a signal to regulate the pathway is still not clear.

Nothing is known about the effect of other nutrients, such as nitrogen, in cytosolic pH regulation or if they also have an effect on pH_c like glucose. But PKA pathway is known to regulate growth via glucose availability and studies indicate that it also regulates pH (Orij et al. 2012) (Edo *et al*., data not published). This pathway, along side with Target of Rapamycin (TOR) pathway, control cellular growth and maybe the latter has also a role in pH regulation. TOR pathway is activated by nitrogen availability. Thus the first question is whether it is possible that nitrogen availability also affects cytosolic acidification in the presence or absence of glucose.

1.3.2 Nitrogen signalling in yeast

Like glucose, nitrogen is a crucial nutrient for yeast metabolism, specially due to its essential role as a constituting part of cellular building blocks, such as amino acids and nucleic acids (Rødkaer & Faergeman 2014). *S. cerevisiae* can uptake via permeases up to 30 nitrogen sources (Godard et al. 2007), ranging from amino acids, urea or ammonium, to nitrogen bases. Besides being used for biosynthesis reactions these sources can also be catabolized to release nitrogen via deamination, via transamination or both, in the form of ammonium or glutamate, respectively. When yeast cells assimilate nitrogen from other sources than glutamate they synthetize glutamine by the condensation of glutamate to ammonium. These three nitrogen sources form the nitrogen metabolism hub by an interlinked of specific enzyme systems (Godard et al. 2007).

Yeast cells adapt to nitrogen availability in their surroundings and its accessibility regulates metabolism, growth, transcription, post-transcriptional protein sorting and turnover. They adjust their transcriptional metabolic and biosynthetic capabilities based on the quality and quantity of the nitrogen compounds (Broach 2012). Also for this nutrient, yeast exhibits a hierarchical preference, distinguishing between favoured/rich and alternative/poor nitrogen sources (Rødkaer & Faergeman 2014). Godard *et al.*, complemented the traditional classification of nitrogen sources based on the doubling time (Cooper 1982) with the expression data of nitrogen-regulated genes, versus urea. Thus classifying the nitrogen sources in two main groups: group of the good nitrogen sources, including

asparagine, glutamine and ammonium, supporting a fast growth with a generation time of about 2 hours; and with a generation time of more than 3 hours, the poor nitrogen sources group, containing GABA, methionine and proline, for example (Godard et al. 2007).

Cells sense nitrogen via several mechanisms like the Nitrogen Catabolite Repression (NCR) pathway and the Target of Rapamycin (TOR) pathway. NCR establishes this preference by repressing the expression of genes required for uptake and catabolism of less preferred ones, upon addition of glutamine or ammonia to cells growing in poor nitrogen sources (Broach 2012). These genes are regulated by an interplay of two transcriptional activators of GATA-type zinc finger, Gln3 and Gat1, and two transcriptional repressors, Dal 80 and Gzf3 (Conrad et al. 2014). The latter two are also activated upon addition of rapamycin to nitrogen rich-medias (Godard et al. 2007).

In the presence of limiting amounts of nitrogen, yeast cells slow their growth through reduction of ribosome biogenesis and induce cell cycle arrest to consequently reduce their cell size, and induce catabolite processes to compensate for the loss of nitrogen. When cells are deprived for nitrogen they enter in a nitrogen-specific quiescent, non growing state (Broach 2012). Quiescent cells have the ability to maintain viability for an extended period and to resume mitotic growth once growth conditions are restored (Klosinska et al. 2011).

Growth in response to changes in nitrogen availability and quality are mainly mediated through the Target of Rapamycin Complex 1 (TORC1) pathway. This complex is a serine/threonine kinase conserved from yeast to humans (Huber et al. 2009). It is responsible for coordinating growth with cell cycle arrest (Moreno-Torres et al. 2015), making sure that different genes are activated accordingly to the needs of the cell regarding nutrient, stress or hormones cues (Hughes Hallett et al. 2014).

1.4 Nitrogen signalling pathway: Target of Rapamycin

1.4.1 Target of what?

The history of Target of Rapamycin (TOR) pathway started in the 1960s with a Canadian expedition to Easter Island (in the native language, Rapa Nui) to gather plant and soils samples for analyses. In one of these soil samples they found a metabolite with a potent antifungal activity, now called rapamycin, in the bacterium *Streptomyces hygroscopicus* (Loewith 2011). This characteristic along side with the anti-cancer and its immunosuppressive potential raised the question of what would be its target inside the cell. Three classes of mutants could grew after plating a selection of mutants of the budding yeast *Saccharomyces cerevisiae* in plates containing a cytostatic concentration of rapamycin. These mutants had defects in the *FPR1*, *TOR1* or *TOR2* genes, being the first the one that encodes a non-essential proline isomerase required for rapamycin toxicity (Loewith 2011). After biochemical purification of the last two from yeast, they were found to function in two distinct multi protein

complexes: Target of Rapamycin Complex 1 (TORC1) with Tor1 and Tor2, and Target of Rapamycin Complex 2 (TORC2) with only Tor2 (Wullschleger et al. 2006).

Rapamycin is an antibiotic macrolide that targets FK506-binding protein 12, a cytosolic peptidyl-prolyl *cis-trans* isomerase (Loewith & Hall 2011). The complex binds to TOR, blocking its downstream effectors (Torres et al. 2002). However, rapamycin only binds to TORC1 in the TOR pathway, thus only its activity is inhibited following acute treatment with this drug (Soulard et al. 2010). Because of this, knowledge on TORC2 is scarce compared to TORC1, because there is no tool that can help to interrogate its function. However, genetic studies have suggested that this complex plays a role in actin polymerization (Hall 1996) and it was found to regulate migration responses and organelle distribution in human tissue culture cells (Loewith 2011).

The use of this drug and its inhibitory role on TORC1 is a major advantage to study how this complex behaves in different conditions. However it is important to know how the pathway signals nitrogen and what are the downstream processes influenced by its activation.

1.4.2 TOR pathway and its upstream and downstream effectors

TOR1 and TOR2 are 67% identical and are 282 kDa in size each, however they are functionally different (Barbet et al. 1996). Both have similar domain structures compose by the two HEAT repeats, the FAT domain, the FRB domain that is the FKBP-rapamycin binding region, the kinase domain and the FATC domain, represented in the Figure 1-4. In this figure, there is also a representation of TORC1 (Figure 1-4 B) and TORC2 (Figure 1-4 C) compositions. Both have Tor2 and Lst8 and have every protein twice, however TORC1 comprises also Tor1, HEAT repeat, Kog1 and Tco89; TORC2 contains also Avo1, Avo2 and Avo3, Bit61/2, PH and RasGEFN. Previous studies showed that disruption of *TOR1* alone has little to no effect and that of *TOR2* alone causes cell to arrest growth (Wullschleger et al. 2006). This led to model that TOR2 has two functions: one shared with TOR1 in TOR Complex 1 and the other unique to TOR complex 2 (Loewith & Hall 2011).

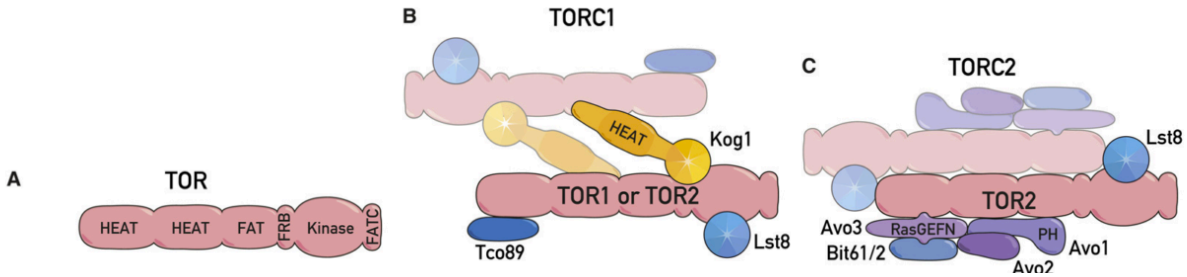


Figure 1-4 – *Target of Rapamycin composition.* (A) Structure of TOR: N-terminal composed by two HEAT repeats with ~40aa; the FAT domain with ~500aa; ~100aa the FRB domain with missense mutations that confer complete resistance to rapamycin; the kinase domain that phosphorylates Ser/Thr residues in protein substrates; and the

FATC domain with ~35aa in the C-terminal. (B) Composition of TORC1: Kog1, Tco89, Lst8 and Tor1 and Tor2. (C) Composition of TORC2: Lst8, Avo1, Avo2 and Avo3, Bit2 and Tor2. In both complexes is likely that each component is present in two copies. Source: (Loewith & Hall 2011).

Rapamycin inhibits TORC1 activity and this causes the cells to behave as if they were starved for nutrients, especially nitrogen. This nitrogen starvation-like phenotype was one of the firsts suggestions that TOR pathway was able to control growth in response to nutrients (Cutler et al. 2001). Which was highlighted after some studies where they observed the phosphorylation of enzymes involved in mobilization of metabolic reserves, characteristic of the cell response to starvation: accumulation of energy, carbohydrates and lipids (Loewith 2011) (Oliveira, Ludwig, et al. 2015) (Klosinska et al. 2011).

The whole pathway controls growth, but each complex controls it in two different branches, one responsible for temporal (TORC1) and the other for spatial (TORC2) growth control (Loewith & Hall 2004). TORC2 upstream branch is poorly characterized but, on the contrary of TORC1, there is no evidence that this complex is controlled by nutrients. However the first studies in mammalian cells suggested that TORC1 is activated by direct association with the ribosome (Zinzalla et al. 2011) and by environmental stress cues (Mulet et al. 2006).

The first observation of the role of TOR pathway was made in yeast with the demonstration that TORC1 promotes protein biogenesis, when in the presence of favourable nutrient conditions. Deletion of TOR1 and TOR2 leads to cell cycle arrest in the early G1 phase and to an early inhibition of translation initiation, which suggested that this pathway has the ability to control growth (Barbet et al. 1996). Also, reduced activity of this complex increases yeast lifespan (Maruyama et al. 2016). All of these observations highlight the importance of TOR pathway in cellular growth control. For this reason in this work we are focusing on TORC1 because is the complex of TOR pathway responsible for sensing nutrient cues.

TORC1 is responsible for controlling anabolic processes like protein and lipid synthesis, ribosome biogenesis and inhibiting catabolic processes like autophagy through phosphorylation of its downstream effectors (Hughes Hallett et al. 2014). Deregulation of this complex is linked to many cancers and metabolic disorders, thus understanding how TORC1 is regulated, what acts upstream and what are the functions of its substrates is highly important (Binda et al. 2009).

TOR complex 1 integrates signals from many extracellular and intracellular cues that are transmitted to the complex by a coordinated interaction of many kinases, GTPases, their modulators and substrates (Dokudovskaya & Rout 2015). TORC1 activation by nitrogen availability occurs via Rag GTPases, regulated by their guanine nucleotide-loading status, in the EGO complex (EGOC), which consists of Ego1, Ego3, Gtr1 and Gtr2 (being active in the $Gtr1^{GTP}$-$Gtr2^{GDP}$ form) (Powis et al. 2015) (Zhang et al. 2012) (Panchaud et al. 2013a). A SEA complex has also been identified as an upstream regulator of TOR pathway and part of this GTPases effectors' web (Dokudovskaya & Rout 2015).

The SEA complex can be divided in two sub complexes with opposite effects on TORC1 and it was first characterized has being associated with trafficking and regulatory events (Dokudovskaya et al.

2011). SEA sub complex inhibiting TORC1 (SEACIT) is compose by Iml1/Sea1, Npr2 and Npr3 and SEA sub complex activating TORC1 (SEACAT) that contains Sea2, Sea3, Sea 4, Seh1 and Sec13 (Figure 1-5). The way they interact to regulate TORC1 is still unclear and depends on the presence or absence of nitrogen and the quality of the nitrogen source (Dokudovskaya & Rout 2015).

In starved cells the inhibiting SEA sub complex of TORC1 act as a GAP (GTPase activating protein) of Gtr1, keeping the heterodimer in its inactive form: $Gtr1^{GDP}$-$Gtr2^{GTP}$ (Dokudovskaya & Rout 2015). Panchaud *et al.*, stated that is the protein Iml1 that functions as a GAP towards Gtr1 in amino acid starvation, linking the complex Iml1-Npr2-Npr3 to the EGOC (Panchaud et al. 2013a) (Wu & Tu 2011). Upon amino acid or nitrogen stimulation SEACAT acts as an inhibitor of SEACIT (thus being the 'inhibitor of an inhibitor'(Dokudovskaya & Rout 2015)) and the small GTPases Gtr1 and Gtr2 are in their active form: $Gtr1^{GTP}$-$Gtr2^{GDP}$ leading to TORC1 activation. The GEF activity of Vam6 and LeuRS on Gtr1 and the effect of Lst4-Lst7 in Gtr2 promote the formation of the active form of the heterodimer (Péli-Gulli et al. 2015). Still the way these upstream regulators' act on TORC1 activity need better understanding.

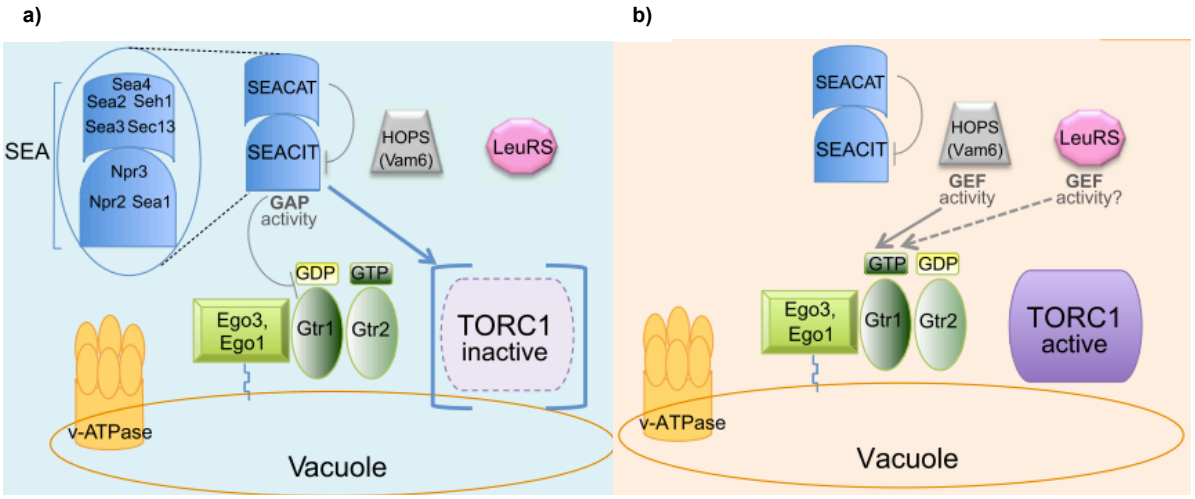


Figure 1-5 – *Simplistic schematic representation of TORC1 activation.* a) In the absence of nitrogen sources, particularly amino acids, SEACT inhibits SEACIT that act as a GAP towards Gtr1 in the EGOC. This inactivation inhibits TORC1 activity. b) In the presence of amino acids however the whole SEA complex is inhibited and Vam6 senses the amino acid intracellular signal acting as a GEF to Gtr1. The heterodimer becomes active in the form: $Gtr1^{GTP}$-$Gtr2^{GDP}$ activating TORC1 at the vacuole membrane. Adapted from (Dokudovskaya & Rout 2015).

Some studies state that the EGO complex is vital for amino-acid dependent activation of TORC1 (Zurita-Martinez et al. 2007) and that this branch is conserved amongst all eukaryotes (Powis et al. 2015). However it is not clear the way this complex activates the GTP activated form of Gtr1 and some say that there is probably another branch responsible for amino acid signalling to TORC1 (Binda et al. 2009). Besides the unclear SEA complex, Binda *et al*., suggests it is through the conversed guanine exchange factor (GEF) Vam6 (Binda et al. 2009) and others suggest a complex that acts as a GAP for Gtr2 anchoring it to the vacuole membrane, in the absence of amino acids, the Lst4-Lst7 complex (Péli-Gulli et al. 2015) (Bonfils et al. 2012).

The localization of the EGOC at the vacuole membrane is essential for TORC1 activation and Powis *et al.*, demonstrated that Ego2 is crucial for this (Powis et al. 2015). Ego1 is necessary to anchor the whole complex to the membrane and Ego3 is necessary for the vacuolar localization of the heterodimer Gtr1-Gtr2 (Zhang et al. 2012). TORC1 localization at the vacuolar membrane suggests that maybe there is an interaction between the complex and V-ATPase proton pump, which can lead to a role of TORC1 on pH_c regulation.

An active TORC1 acts through a variety of downstream effectors (Hughes Hallett et al. 2014), being the S6 kinase 1 (Sch9) and the type 2A-related phosphatases well characterized ones (Powis et al. 2015). Others include Gln3, the regulator of the transcription of nitrogen metabolism and Atg13 and Atg1, both autophagy regulators (Oliveira, Ludwig, et al. 2015). The relationship of the complex with Sch9 is straightforward compared with PP2A. The complex directly phosphorylates Sch9 that is responsible for activating protein genesis and growth, at multiple C-terminal sites, when is active (Urban et al. 2007). However, for PP2A to be active TORC1 needs to be inactive: an active TORC1 has anchored to it a phosphorylated Tap42 that only binds to PP2A, activating this branch when TORC1 is inhibited (Yan et al. 2012). Upon starvation or treatment with rapamycin, TORC1 no longer phosphorylates Tap42 and the protein binds to PP2A after being released in the cytoplasm (Yan et al. 2013).

An interaction between the two signalling pathways TORC1 and PKA has been suggested since the discovery that they have common targets, activating or inhibiting the same biological processes (Soulard et al. 2010). Also, both are recognized as central regulators of nutrient sensing and response, together with Sch9 (Rødkaer & Faergeman 2014). Glucose is vital for PKA activation and also induces Sch9 phosphorylation and regulates it by increasing its level in the cell (Jorgensen et al. 2004) and both appear to perform similar functions in the cell by targeting overlapping substrates (Huber et al. 2009).

Soulard *et al.*, investigated the effect of TORC1 inhibition on PKA and come up with a model where the first inhibits BCY1 phosphorylation via Sch9 deactivating PKA pathway (Soulard et al. 2010) (Sundaram et al. 2015). Since PKA regulates intracellular pH we wondered whether TORC1 also plays a role in this regulation via PKA and Sch9.

Some studies in mammalian cells have already linked mTORC1 (mammalian TORC1) to pH_c regulation (Fonseca et al. 2012). Balgi *et al.*, suggested that mTORC1 down regulates protein genesis and growth once it senses an acidic intracellular environment, in order for the cell to adapt and respond to stress cues (Balgi et al. 2011). Because TORC1 is highly conserved amongst all eukaryotes, as previously stated, this study is another indication that in yeast this down regulation also occurs.

2 MOTIVATION

When the subject is the cell, “the structural, functional and biological unit of all organisms”, the more the understanding on how it works the better. Knowing how cell regulates growth, protein genesis, or even, autophagy and what are the key regulators and pathways involved are a must in order to know how to overcome several problems such as human diseases like cancer. Signalling pathways such as cAMP-dependent Protein Kinase A (PKA) pathway or Target of Rapamycin (TOR) pathway regulate and maintain normal growth conditions in response to nutrient availability and inhibit it in the presence of stress.

In eukaryotic cells, nutrient availability is a major determinant for regulation of cell growth, yet mechanisms of nutrient sensing are still unclear. In yeast, in the presence of all essential nutrients, the two highly interconnected nutrient signalling pathways, PKA and TOR, promote growth and anabolic processes like ribosome biogenesis while inhibiting catabolic processes and stress (Dechant *et al*, 2014). However there are more physiological parameters inside the cell that respond to extracellular conditions. For instance, intracellular pH also responds to nutrient availability. Considering that almost all the processes that occur in living cells are pH dependent, since the protonation state of cellular metabolites can vary with it, leading to different responses and functions, one cannot simply ignore this physiological parameter.

The present project was developed in the Department of Molecular Biology and Microbial Food Safety in the Swammerdam Institute for Life Sciences in the University of Amsterdam. Our research group is interested in studying how pH dynamics affect cellular function and how processes in living cells regulate it, by using as a model organism the baker yeast *Saccharomyces cerevisiae*. Since the biophysical properties of the signal are universal amongst all living organisms, having a better understanding on this subject can provide key answers to diseases related to higher eukaryotes. Intracellular pH was identified as a signal for growth control in yeast (Orij et al. 2011) and it responds to nutrient availability. It is well established that glucose levels have an effect on pH: an acidification inside the cell cytosol occurs as the fast fermentative growth ceases because of glucose depletion (Martínez-Muñoz & Kane 2008) (Dechant et al. 2014) (Orij et al. 2011). Previous work in the group showed that this cytosolic acidification is regulated by the PKA pathway in response to glucose availability inside the cell (Edo *et al.*, data not published).

How all of these signals: nutrients, pH_i, TOR and PKA pathways interact in order to properly regulate growth rate in response to environmental cues is the main question. Glucose has been established has a pH effector and a growth controller, but maybe other nutrients also play a role in pH regulation. This work will focus on nitrogen. Do cells growing in different concentrations of nitrogen or in different nitrogen sources have different extends of acidification in their cytosol? Does nitrogen have any role in pH_c regulation? Since TOR pathway responds to nitrogen availability inside the cell and is known to control cellular growth, as well as PKA pathway, can it also regulate pH? Does TOR pathway play any

role in pH_c regulation depending on nitrogen? If so, do PKA and TOR pathways regulate pH inside the cell in response to nutrients availability? Does pH regulate growth via PKA and TOR? What is the relationship between pH, nutrient availability and signalling pathways inside the cell?

2.1 Main Objective/Hypothesis

In a general way, the goal of this thesis is to analyse what is the effect of nitrogen availability, quality and source on cytosolic pH in *S. cerevisiae* and discover if this signal is mediated through TORC1 affecting cellular growth and pH_c (Figure 2-1). To achieve this goal we divided the general aim in two and both in the following partial objectives:

1 – Assess the effect of nitrogen in pH_c:
- 1.1 – Monitor growth and measure pH_c of cells growing in minimal media with different nitrogen sources and concentrations.
- 1.2 – Check if glucose starvation experiments mimic growth curve effects.
- 1.3 – Understand if there is any difference in pH_c in cells growing in synthetic complete or synthetic defined media with different nitrogen sources and concentrations.
- 1.4 – Assess the effect on pH_c of nitrogen starvation in the presence or absence of glucose.

2 – Dissect TORC1 role on pH_c:
- 2.1 – Rapamycin addition during growth to cells growing in complete media or in minimal media with the different nitrogen sources.
- 2.2 – Treatments with rapamycin prior to nutrient starvations.
- 2.3 – Monitor growth of different mutants involved in the upstream part of TORC1 pathway.

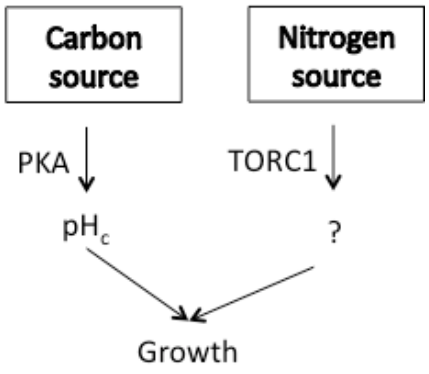


Figure 2-1 – *Schematic representation of the main goal of this project*.

3 MATERIALS AND METHODS

3.1 Strains and Growth Conditions

Strains used in this project are listed in Table 3-1. Unless stated otherwise, the auxotrophic yeast strain BY4741 and mutants derived from it were used with the plasmid pHluorin (*pYES-PACT1-pHluorin (URA3)*) (Orij et al. 2012). Cultures were cultured in synthetic complete (SC) media lacking uracil (for composition see Table 3-2). In order to test the effects of single nitrogen sources we needed to make these strains prototrophic. We did so by inserting the plasmid pHLM. These were pre-cultured in synthetic defined (SD) medium without amino acids or nitrogen bases and the indicated nitrogen source. Both media were buffered at pH 5 with 25mM sodium citrate.

Strains were pre-cultured in liquid medium in glass tubes on a rotary stove at 30°C. For all experiments cultures were grown overnight in Erlenmeyer flasks on a rotary shaker at 200 r.p.m. on low fluorescence media with the same composition as they were inoculated.

Table 3-1 – Strains used in this project.

Strain	Genotype	Source
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Wild type
*bcy1*Δ	BY4741 *bcy1*Δ::kanMX	(Casado et al. 2011)
*npr2*Δ	[YL515]MATα; *npr2*Δ::kanMX4	(Panchaud et al. 2013b)
*npr3*Δ	[YL515]MATα; *npr3*Δ::kanMX4	(Panchaud et al. 2013a)
*sch9*Δ	BY4741 *sch9Δ*::NatMX2	(Swinnen et al. 2014)

Compositions of the stocks and culture medias used in this project are listed in Table 3-2. We used Milli-Q water to prepare all solution. Unless stated otherwise culture medias and stocks were autoclaved at 120°C for 20 minutes.

Table 3-2 – Composition of the culture medias and stocks used in this study.

Name	Composition
YPD (rich medium)	2% bacto peptone; 2% glucose and 1% yeast extract
SD (synthetic defined)	6,7 g/L YNB (yeast nitrogen base) without amino acids; 2% glucose
SC (synthetic complete)	6,7 g/L YNB without amino acids; 2% glucose; 2 g/L dropout mix without uracil (see supplemental data)

Low fluorescence (Loflo) medium (10x stock)	1,7 g/L YNB (without amino acids, ammonium sulphate, folic acid and riboflavin); 0,1% NaGlutamate; 0,1% dropout mix w/o uracil;
Low fluorescence (Loflo) medium w/o nitrogen (10x stock)	1,7 g/L YNB (without amino acids, ammonium sulphate, folic acid and riboflavin)
Glucose (10x stock)	200 g/L
NaCitrate (10x stock)	250 mM

Yeast culture were grown in low fluoresce medium in order obtain a proper fluorescence signal, necessary for accute pH_c determination. This media was prepared as a 10x stock and then filter sterilized, since it cannot be autoclaved. The carbon or the nitrogen sources when necessary were added from the 10x or the 5x stocks. Because to study the influence of the different nutrients in cytosolic pH regulation we needed to have medias with different conditions regarding glucose and nitrogen presence or absence.

To study the effect of nitrogen in pH regulation we used four different nitrogen sources in different concentrations: glutamic acid (GA), ammonium sulphate (AS), proline (Pr) and urea (Ur).

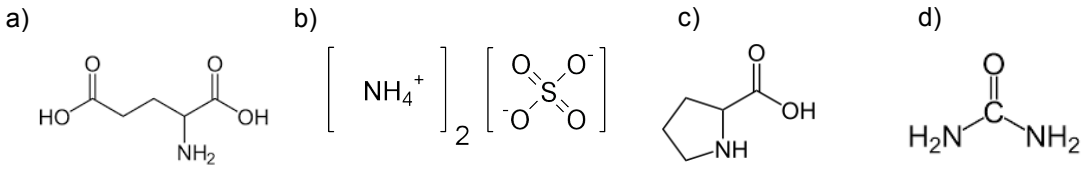


Figure 3-1– *Chemical formula of the different nitrogen sources used in this study.* a) glutamic acid, b) ammonium sulphate, c) proline and d) urea.

As it can be seen in Figure 3-1 ammonium sulphate and urea both have two N in their structure, while glutamic acid and urea only have one. In order to provide to the cells the same amount of N within each nitrogen source in study, we doubled the amount of GA and Pr in solution. All of the four sources were prepared as 5x concentrated stocks and filter sterilized. Each nitrogen source was used in a SD media, as the sole nitrogen source present, or in SC. The same medias were also made in the absence of glucose, for the starvations in SD-AA.

To compare different concentrations of nitrogen, we prepared SD-AA medias containing two different concentrations: 10mM of molecular N and 5mM of molecular N. (Godard et al. 2007) Therefore the medias prepared were 10mM and 5mM for GA and Ur; and 5mM and 2,5mM for AS and Pr.

Rapamycin, unless stated otherwise, was always used with concentration of 200nM, previously described by Huber *et al*. (Huber et al. 2009). Rapamycin was stored prepared as a 1mM stock in DMSO and kept at -20^{o}C.

3.2 Yeast transformation

In order to measure the cytosolic pH of yeast cells, the plasmid *pYES-PACT1-pHluorin (URA3)* was transformed in the WT strain. We also constructed a BY4741 prototrophic strain by transforming the strain containing the *pYES-PACT1-pHluorin (URA3)* plasmid with the plasmid pHLM, which provided the strain the ability to produce histidine (H), leucine (L) and methionine (M) by complementing the deletions present in the genome of the wildtype.

For the transformation procedure we used an adaptation of the High Efficiency Yeast Transformation Protocol, from Gietz and Woods, 2002 (Gietz et al. 2002). Overnight cultures of the WT strain BY4741 were inoculate to an OD_{600} of 0,5 and grown for 4-5 hours until OD_{600} 2, in 5 mL of YPD per transformation. Cells were washed with 1mL of 0,1M Lithium Acetate (LiAc) and ressuspended again in 50µL of 0,1M LiAc per number of plasmid transformed. Cells were then incubated for 10 minutes at room temperature after centrifuged for 4 minutes at 3000 r.p.m.. We then added the transformation mix containing 500ng of DNA, 5µL of ssDNA (pre boiled) and 300µL PLi (solution with 80% of PEG50% and 10% of 1M LiAc), mixed and subjected to a heat shock for 22 minutes at 42°C. Transformations were then plated in the appropriate selection plates.

To check if pHluorin was properly expressed in the cytosol of the transformed cells, we imaged our transformants using an Axiovert 40 CFL microscope (Carl Zeiss) with a Plan Neofluoar 100x/NA 1.3 oil objective on an Endow GFP and Cy3 narrow-band excitation filter sets for fluorescent images.

3.3 Determination of cytosolic pH

As mentioned above, to measure pH_c we used a method based on the expression of the ratiometric pH-sensitive protein pHluorin in the yeast cytosol. This method allows us direct monitoring of pH_c in a time-resolved manner in living cells, thus being able to monitor real time responses is the major advantage of this method (Orij et al. 2009).

To perform pH_c measurements, we monitored OD_{600} and fluorescence emission at 510nm after excitation at 390nm and 470nm with a FLUOstar OPTIMA microplate reader (BMG labtech, Ortenberg, Germany). The fluorescence signal measured is a sum of fluorescence of the media, the auto fluorescence of yeast cells and the pHluorin signal. Thus, to determine the pH_c we subtracted the background from the media and the auto fluorescence of the cells from the total measured fluorescence signal.

3.3.1 Determination of the autofluorescence corrections

The auto fluorescence of the cells at 390nm and 470nm was calculated using the correction curves (equation 1 and 2) below, being x the subtracted OD values. To obtain these two correction curves we grew cells without pHluorin. We measured OD and fluorescence, at the appropriated wavelengths, in a set of dilutions covering the whole OD range. Fluorescence values were then plotted versus OD and adjusted to a polynomial trend line of order two, after subtraction of the media.

$$y_{390nm} = 912{,}69x^2 - 170{,}77x + 92{,}301 \qquad r^2 = 0{,}9936 \qquad (1)$$

$$y_{470nm} = 420{,}74x^2 - 18{,}51x + 58{,}964 \qquad r^2 = 0{,}997 \qquad (2)$$

After subtraction is important to check if the signal was high enough, establishing a threshold of the cell signal measured being two times higher than the total background, before further calculations.

After the background subtraction we can calculate the ratio between the two-excitation wavelengths:

$$ratio_{390nm/470nm} = \frac{raw\ data\ 390nm - total\ background}{raw\ data\ 479nm - total\ background} \qquad (3)$$

With this ratio we can then calculate the pH_c using the calibration curve (4), that was constructed as described in (Orij et al. 2009).

3.3.2 Determination of the calibration curve

BY4741 strain with the pHluorin vector was grown in Erlenmeyer flasks until mid-log phase. Cells were centrifuged for 3 minutes at 4000 r.p.m. and the media was removed. Then, the pellets were ressuspended with a 100 µg/mL digitonin in PBS and incubated for 10 minutes at room temperature. This step is critical since too long treatments will lead to pHluorin leakage from the cells, while too short won't permeabilize them enough. The first will not provide the cells fluorescence enough for a proper measurement and the latter will not let intracellular pH to equilibrate with the extracellular one. Cells were then placed on ice, washed with PBS and concentrated 10x in PBS.

They were then transferred to a CELLSTAR black polystyrene clear-bottom 96-well microtitre plate (Greiner Bio-One) filled with a set of citric acid/Na_2HPO_4 buffers covering the pH range from 4 to 9

(20µL of cells to 180µL of media). OD and the pHluorin fluorescence were measured at 510nm using a FLUOstar OPTIMA microplate reader (BMG labtech, Ortenberg, Germany) in two excitation lengths: 390nm and 470nm.

Background was then subtracted to the signal values, as previously explained and the ratio of emission intensity ($R_{390nm/470nm}$) was plotted against the corresponding buffer pH in order to remove the points that are out of range. This being the ones from the tails, in the begging and end of the curve. pH was then plotted against the remaining ratio points and adjusted to a polynomial trend line of 4th order, which lead to the following calibration curve

$$y = -0{,}0516x^4 + 0{,}6785x^3 - 3{,}1186x^2 + 6{,}4805x + 1{,}7859 \qquad r^2 = 0{,}992 \qquad (4)$$

3.4 Growth curve

In growth curve experiments, overnight stationary phase pre-cultures were diluted in a CELLSTAR black polystyrene clear-bottom 96-well microtitre plate (Greiner Bio-One) to an OD_{600} of 2 in the media of study. Optical Density at 600nm (OD_{600}) and fluorescence was measured every 10-20 minutes, for 48-72 hours. pH was calculated as described in 3.3.

After subtraction of the media to the total OD signal measured, OD values were corrected to OD real (OD_{real}) using (Orij et al. 2012):

$$OD_{real} = OD^2 \cdot 1{,}1476 + OD \cdot 0{,}8072 + 0{,}0096 \qquad (5)$$

For rapamycin treatments, we added the drug 4 hours after inoculation and DMSO was used as a control.

To average the starvation pH_c of biological replicates we first averaged one hour of data after 15 hours of cells being in the post-diauxic phase with a growth rate around 0, for each set of data of technical replicates to avoid single point outliers. With those values we average and performed statistical analysis (t-test).

3.5 Starvation experiments

In order to study the effect of the availability of nutrients we performed starvation experiments. Strains were pre-cultured overnight in the medium in study until exponential phase with an OD_{600} about 5-7, which was measured using a Lightwave II UV/Visible Spectrophotometer.

Cell aliquots were transferred to 1,5 mL tubes, centrifuged for 1 minute at 10000 r.p.m. and washed twice with fresh media lacking glucose and/or nitrogen. For control treatments we performed washes with fresh media containing glucose and nitrogen. pH_c was monitored for 60-120 minutes and calculated as described in 3.4.

When necessary 200nM of rapamycin was added to cells as a pre-treatment and DMSO used as a control. After addition of the drug or the solvent, cells were incubated at 30°C for 90 minutes in glass tubes or Erlenmeyer flasks. Starvation experiments were then performed as previously described.

4 RESULTS AND DISCUSSION

Cells need nutrients, such as carbon and nitrogen sources to be present in the culture media to grow and maintain a normal cellular function. Different signalling pathways that regulate different cellular responses sense each of these nutrients. Intracellular pH (pH_i) is tightly controlled in growing cells since changes on pH_i can essentially affect any biological process (Orij et al. 2011). Cytosolic pH (pH_c) affects the protonation state of biologically relevant molecules, which may lead to different functions and physiological responses.

As shown by Orij *et al.* pH_c is dynamic during growth and when yeast depletes glucose and enters the post diauxic phase, pH_c decreases almost one pH unit (Orij et al. 2011). Starvation of yeast cells for glucose also causes a similar acidification (Figure 4-1) suggesting that artificial glucose deprivation in yeast mimics the effect of glucose depletion during growth.

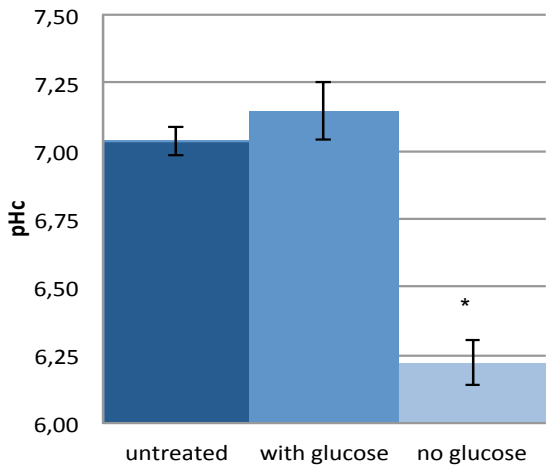


Figure 4-1 – *pH_c after glucose starvation mimics the effect after glucose is depleted during growth.* Cells were grown in SC-U and starved with the same media with and without glucose as described in Materials and Methods. Data was determined with the average of pH_c during one hour of measurement. Error bars represent the standard error of the mean of at least three independent experiments. *p-value<0,05

Orij *et al.* showed that pH_c controls growth rate (Orij et al. 2011). In yeast, growth is mainly controlled by the nutrient signalling pathways Protein Kinase A (PKA) and Target of Rapamycin (TOR), which respond to carbon and nitrogen availability, respectively (Soulard et al. 2010). These pathways do not act independently but they are known to interact to ensure a proper growth control (Dechant et al. 2014). This suggests that they may also interact with pH_c. Indeed, we have also observed that PKA controls cytosolic pH, upon glucose depletion, and others showed that PKA is pH_c-sensitive ((Edo n.d.), data not published). The goal in this work is to determine whether nitrogen and the nitrogen responsive growth-controlling pathway TORC1 have a role on cytosolic pH regulation.

4.1 Study of the effect of nitrogen quality and quantity on pH_c

4.1.1 Nitrogen quality and concentration present in the media affects pH_c upon natural glucose depletion

Besides providing energy and building blocks to cells, nutrients also exert crucial regulatory roles. Glucose is now known as a key regulator of cytosol acidification (Orij et al. 2011). Once cells consume all glucose from the culture media and reach the end of exponential growth, their cytosol acidifies and there is a drop on pH_c. This parameter is known to control growth rate and it is regulated by PKA pathway (Orij et al. 2011). Because TORC1 controls cellular grow like the PKA pathway we asked whether TORC1 would also have a role in pH_c regulation. The first step was then assed whether nitrogen availability has an effect on this physiological parameter, since TORC1 activity depends on the presence of this nutrient.

To assess if nitrogen regulates cytosolic pH, we grew cells in minimal media (SD-AA) with a single nitrogen source. This way we could study whether the quality of the nitrogen source present in the culture media mattered for pH_c regulation, during growth. Also if nitrogen indeed affects pH_c and changes it in which conditions: presence or absence of glucose.

We used two rich nitrogen sources (glutamic acid or ammonium sulphate) and two poor ones (proline or urea). During growth, in the presence of glucose, we did not see a difference in pH_c between the different sources and for all it was around 7. However, as expected, we could see a drop in pH_c at the time cells consumed all glucose in the media, yet no difference in pH_c was observed between the two rich nitrogen sources or the two poor ones. This was the first suggestion that the quality of nitrogen where cells are growing sets the acidification inside the cell, once glucose is absent.

Cells grow in the presence of nitrogen sources and carbon sources, so to study the effect of the quantity of nitrogen we then grew them in two different concentrations, of each nitrogen source. We used equimolecular nitrogen concentrations when comparing the nitrogen sources with 1 or 2 assimilable N molecules. 10mM of nitrogen was used as a reference (Godard et al. 2007). This way the concentration used for the nitrogen sources were: ammonium sulphate and urea was 5mM, because both have 2 assimilable N and glutamic acid and proline only have 1 assimilable N thus the concentration used was 10mM.

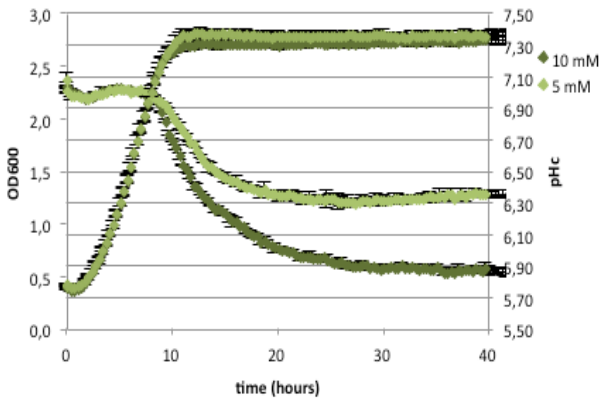
Growth and pH_c of cells growing in 10mM and 5mM of GA and Pr and in 5mM and 2,5mM of AS and Ur were monitored. During growth, in the presence of the carbon source, for all the different nitrogen conditions cells presented a neutral acidification, which suggests that nitrogen only affects pH_c in the absence of glucose.

Cells cultivated in the presence of proline, as a sole N source, did not grew, while the ones in urea did not depleted glucose during the time course of the experiment (see Figure S1 in Supplemental data).

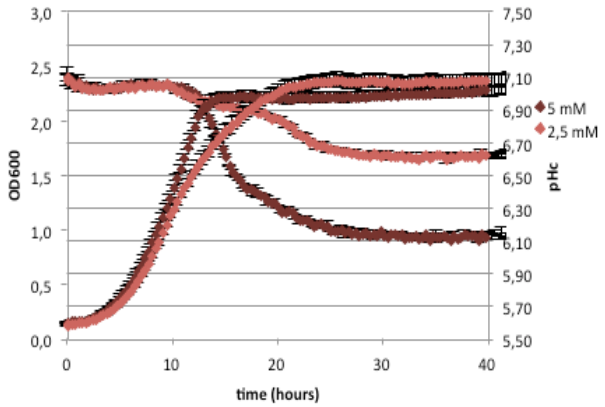
Specific growth rate can be reduce by a variety of stresses like less preferred nitrogen sources (Conrad et al. 2014). For this reason, we focused our studies in assessing the effect on pH_c of cells growing in minimal media with rich nitrogen sources.

For the rich nitrogen sources, ammonium sulphate and glutamic acid, cells depleted glucose between 10 and 20 hours after growth. There was no difference in pH_c between the different nitrogen concentrations in study during growth and cytosolic pH was maintained around 7 till the end of the exponential phase. This suggests that in the presence of glucose there is no effect of the amount of nitrogen on pH_c. Upon glucose depletion pH_c rapidly decreased as previously described, for all the conditions analysed. Interestingly, this acidification was dependent on the concentration of N inside the culture media: higher concentration led to a higher cytosol acidification and therefore to a lower pH (Figure 4-2). This difference in pH_c values between the two concentrations is significant (Figure 4-2 c) and about 0,5 in pH units.

a)



b)



c)

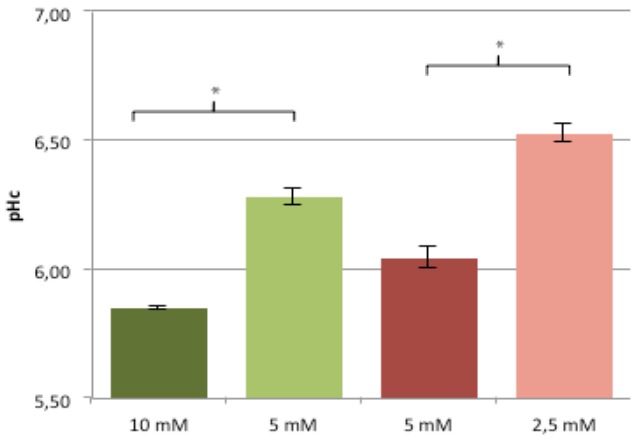


Figure 4-2 – *Nitrogen affects pH_c during growth, upon glucose depletion.* OD_{600} (line) and pH_c (symbols) were monitored during growth in microplates, in SD media with (a) glutamic acid (GA) or (b) ammonium sulphate (AS) as nitrogen sources. In both (a) and (b) darker colours represent 10mM N and the lighter 5mM of nitrogen. Data show a representative result and error bars represent the standard error of three technical replicates. (c) Starvation pH_c of cells growing in GA (green) or AS (red), calculated as described in Materials and Methods. Data represent averages of at least three independent biological replicates. Error bars represent the standard error of the mean (SEM). *p-value < 0,0001.

These results suggest that indeed nitrogen quality and quantity are important and crucial to regulate cytosolic pH, however only once glucose is absent. During exponential phase, pH_c maintained neutral as required for the cell normal growth, for all the different nitrogen conditions, which implies that the amount of nitrogen is not relevant for cytosolic pH regulation in the presence of glucose. However, once glucose was depleted high nitrogen concentrations lead to a stronger acidification of the cytosol, leading to a lower pH_c. This data highlights the importance of the nutrient conditions present in the culture media.

4.1.2 Effect of glucose starvation on pH_c in yeast cultured in minimal media

We could see a higher acidification in the cytosol because of the higher amount of nitrogen present in the culture media where cells were growing, after they naturally depleted glucose. Artificial glucose starvations in synthetic complete media could replicate the natural depletion of glucose in growing cells.

The next step was to check if glucose starvation in minimal media would also mimic the effect observed on pH_c after growth. Starvations experiments have the advantage to be faster than letting the cells grow and deplete glucose naturally. To do so, exponentially growing cells in synthetic defined medias with GA (10mM and 5mM) or AS (5mM and 2,5mM) were washed with the same media that they grew in and starved for glucose.

Since different N concentrations or sources did not affect pH_c during growth (Figure 4-2), no difference in pH_c was expected in cells washed with media that contained glucose. As in the growth curve, we expected that cells starved for glucose with the 10mM of N in minimal media had a lower pH than the ones starved with the 5mM of N. Since stronger acidifications in the cytosol were expected to occur to cells that depleted glucose in the presence of the higher amounts of nitrogen.

For both nitrogen sources no difference on pH_c was apparent in the presence of glucose, as expected from previous data. However, in the absence of glucose some differences were observed for the two nitrogen sources used.

For cells growing in ammonium sulphate as the sole nitrogen source, pH_c was not affected after glucose starvation by the amount of nitrogen present. pH_c was around 6,5 for both 5mM and 2,5mM of ammonium sulphate present (Figure 4-3 a). This was not the effect observed in growth curves where glucose is naturally depleted, as we can observed from the previous Figure 4-2 b): right at the moment when exponential phase ends an accentuated drop in pH_c of the cells growing 5mM occurs to pH_c around 6,10, while the pH_c of the ones growing in 2,5mM drop to 6,6.

Interestingly this effect of different concentrations of AS on starvation pH were the first data in which starvation did not mimic the effect on pH_c of glucose natural depletion during growth. In this case, starvation of glucose to cells cultured in 5mM or in 2,5mM of AS led to the same cytosolic acidification. This could suggest that the amount of nitrogen does not have a role on pH_c regulation. But there is a gap in understanding the link between the long-term adaptations to a specific nutrient and the rapid response to a stress at the molecular level (Conrad et al. 2014). And this result goes in disagreement with the previous described growth curves of the same nitrogen source. These differences between the starvation experiment and growth curves can be explained by the stage of growth cells are in: in the first cells may have adapted to stationary phase entry leading, for example, to more carbohydrate production that can influence pH (Orij et al. 2011) (Klosinska et al. 2011). Also, during growth, cells consume glucose and start producing ethanol (Deken 1966), secrete proteins (Efeyan et al. 2015) and the media is not fresh and all of these parameters can have some influence in pH_c, that in the case of the starvation experiment does not happen: we always wash cells with fresh media.

On the contrary, the starvation experiments in SD media with glutamic acid as the sole nitrogen source were in agreement with the growth curve data (Figure 4-3 b). Cells pre cultured and starved with media with the same amount of nitrogen had the expected effect in the end of the starvation. Upon glucose starvation, 10mM of GA led to a lower pH_c than the ones starved in the presence of 5mM of GA. The higher the concentration of glutamic acid, at the moment of the starvation, the higher the cytosolic acidification. This can suggest that there is a long-term adaptation to the amount of nitrogen present in the media that is maintain even after glucose depletion. We then asked whether these effects were a result of the preconditioning during growth or an immediate consequence of the N availability during the starvation itself.

To answer this question, exponentially growing cells in the synthetic defined medias with 10mM and 5mM of GA were washed with the same nitrogen concentration medium or with the other, in the

presence or absence of glucose. With this experiment we could assess if there is a rapid response of the amount of nitrogen present at the time of the starvation on pH_c regulation and if a downshift of nitrogen contradicts the effect on pH_c of the high concentration of nitrogen in the inoculum and vice versa. If the amount of nitrogen present at the time of the starvation was the cause of the difference in pH_c values, the long term adaptation to the amount of nitrogen would not have any effect. So, if this was the case we would expect pH_c to be lower in cells in the presence of the lowest amount of nitrogen at the time of the starvation, independently of the nitrogen concentration at which they were grown. Cells cultured on 10mM of GA and starved with 5mM would have less acidification in their cytosol compared to the ones cultured and starved with 10mM. On the other hand, cells cultured in 5mM of GA and starved for glucose with 10mM would have a lower pH_c than the ones cultured and starved with 5mM of GA. On the contrary, if nitrogen concentrations affect pH_c as a pre treatment, cells grown at 5mM of GA would lead to a higher cytosol pH value independently of the amount of nitrogen present in the starvation media.

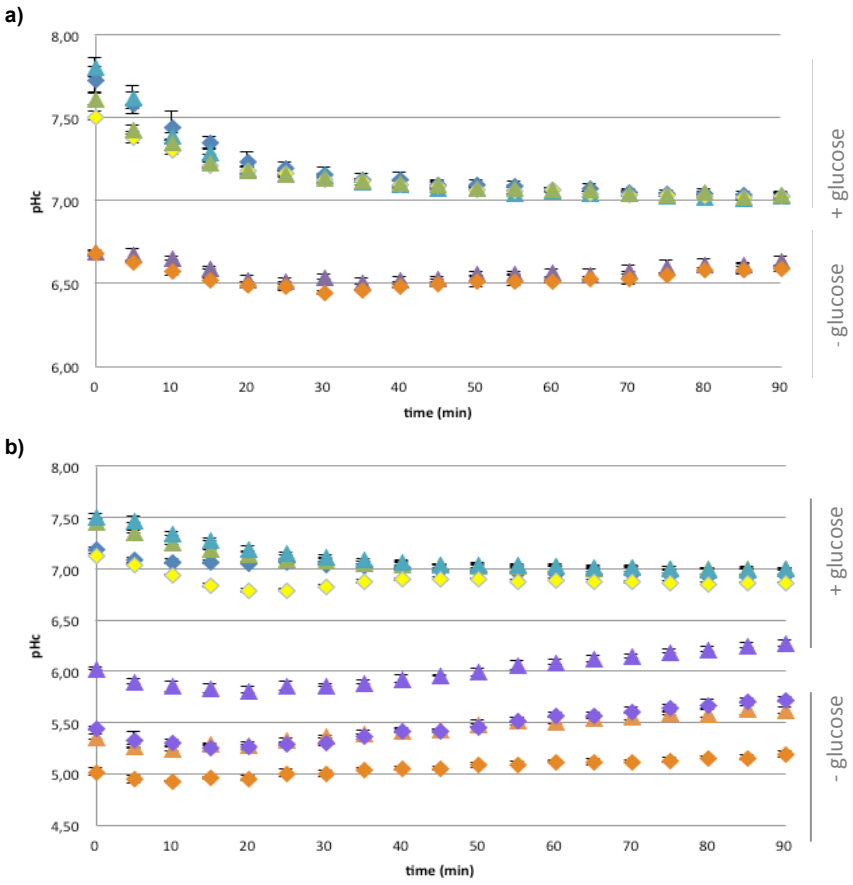


Figure 4-3 – *The amount of nitrogen present at the time of the starvation influences pH_c.* pH_c values measured for 90 minutes after glucose starvation in (a) ammonium sulphate and (b) glutamic acid. Triangles represent cells pre cultured in 10mM of N and diamonds the ones in 5mM of N. As expected, there is no difference in cytosolic pH values when glucose is present in the media. In the absence of glucose, purple data points represent cells washed with the medium with the full nitrogen concentration and orange the ones with the half. Data show a representative result. Error bars represent the standard deviation of three technical replicates.

When looking at the results of this N switch starvation experiment we could observe that: (1) the final pH_c of the cells grown on 10mM of GA and washed with 5mM (orange triangles in Figure 4-3 b) was about 0,5 units higher than the ones washed with 10mM of GA, as they grew in. (2) The ones starved with 10mM of GA after being pre cultured in 5mM of GA decreased pH_c about the same amount (purple diamonds), overlapping with former pH_c value. Thus, both switches lead to the same pH_c, about 5,5. These results suggest an intermediate situation between the two hypothesis considered: that pH_c upon glucose depletion is influenced both by the amount of nitrogen present where they were grown and by the amount of nitrogen present at the time of glucose withdrawal.

The data from the starvation in glutamic acid minimal media experiments indicate that switching the amount of nitrogen present in the media somehow helps cells to recovery the acidification from the pre conditioning effect on pH_c resulted from the initial amount of N present while growing. This result suggest that the amount of nitrogen present in the media is important for pH regulation and therefore for cellular growth.

Given that cells seem to have a rapid response to the amount of glutamic acid present in the media but not to the amount of ammonium sulphate suggest thats the nitrogen uptake mechanism may interfer with pH_c signal. Nitrogen sources enter in the cell via H^+-dependent import and maybe there is a metabolic pathway that by being responsible for nitrogen uptake is also responsible for setting pH_c upon stress.

4.1.3 Different nitrogen sources and concentrations in SC-U media do not affect pHc during growth

In the experiments shown so far yeast cells where grown in minimal media, thus they have to be prototrophic. Therefore, a plasmid with *URA*, *HIS*, *LEU* and *MET*1 genes had to be inserted for the strain to have the ability to grow in the presence of a single nitrogen source. One aspect that we observed during the course of the experiments was that in this minimal media cells grew slower than in a media with supplements present. In this media we observed and acidification of the cytosol upon glucose depletion. We then asked if this effect of the amount of nitrogen present in the media would still be maintained in synthetic complete media. Thus, in this section we monitored cell growth and pH_c in SC-U medias, which contained a supplement mix composed by amino acids and nitrogen bases besides the main nitrogen source (glutamic acid, ammonium sulphate, proline or urea). We wondered if the same effect on pH_c was observed after glucose depletion in cells growing in this type of media: lower pH_c for cells growing in higher amounts of nitrogen.

First we monitored growth and pH_c in SC-U with the rich nitrogen sources present in the two concentrations in study (10mM or 5mM of GA and 5mM and 2,5mM of AS). We notice that cells grew faster in this media, supporting the idea that amino acid production may reduce the nutrients available for growth. Cells growing in 5mM of GA or in 2,5mM of AS led to a small delay in glucose depletion

and to a slightly higher OD compared with the 10mM of GA and with the 5mM of AS, respectively. Glucose depletion occurred around 8 hours after inoculation and we did not observe a significant difference between the starvation pH_c on two concentrations of nitrogen used (Figure 4-4 b).

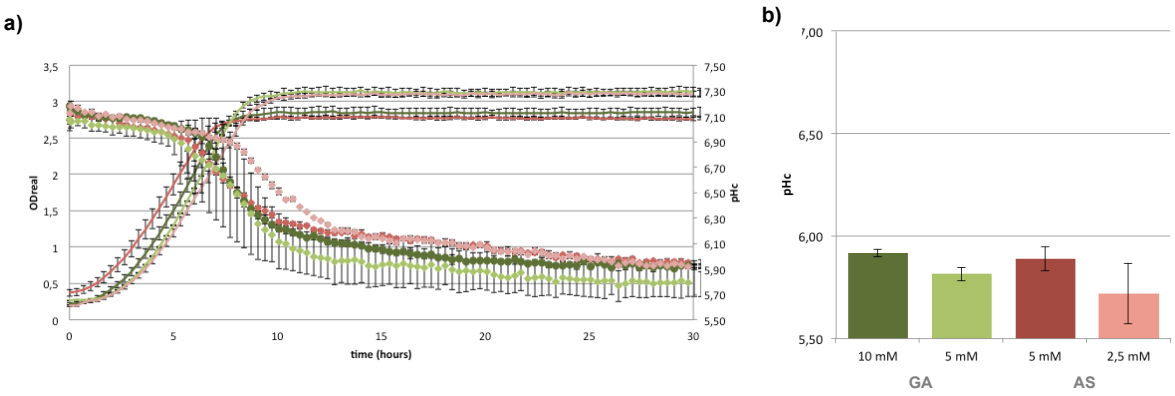


Figure 4-4 – *pH_c is unaffected by the concentration of N after glucose depletion in SC-U media, in rich nitrogen sources*. In both graphics green is for glutamic acid (GA) conditions and red is for ammonium sulphate (AS) (dark colour represents the higher concentration used and the light one for the half concentration). (a) OD was monitored during growth. Data show a representative result and error bars represent the standard error of three technical replicates. (b) pH_c values after glucose depletion, calculate as described in Materials and Methods. Error bars represent the standard error of the mean of two biological replicates.

Growth was also measured in cells cultured in the presence of proline or urea as the main nitrogen sources in SC-U (Figure 4-5). In these conditions, cells were able to grow normally, depleting glucose after less than 10 hours, in contrast with our observations in minimal media where they did not deplete glucose during the time of the measurement (Figure S1 in Supplemental data). Again, no difference in pH_c between nitrogen sources or concentrations was observed, neither during growth nor when glucose was depleted. The fast growth rate in the presence of these two poor nitrogen sources in this case can be likely explained by the supplements present in this media, which may be also used as nitrogen sources.

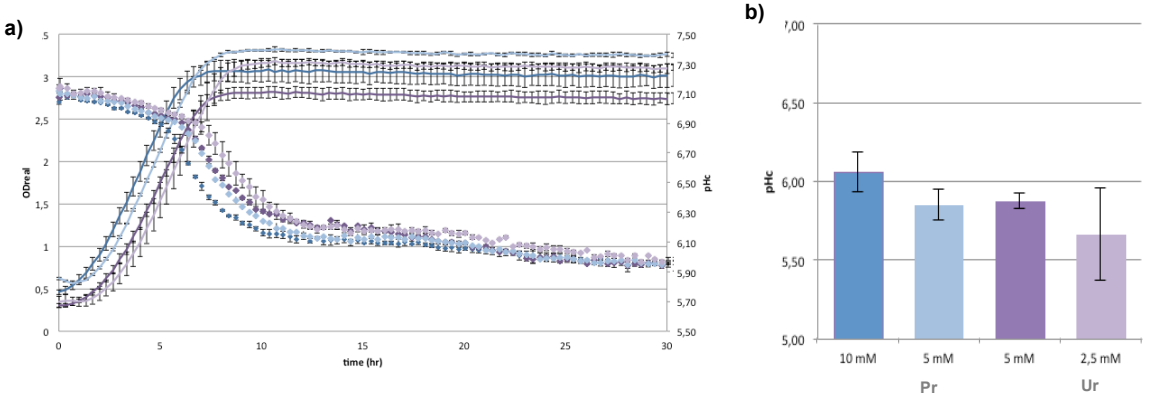


Figure 4-5 - *pH_c is unaffected by the concentration of N after glucose depletion in SC-U media, in poor nitrogen sources.* In both graphs blue is for proline (Pr) conditions and purple is for urea (Ur) (dark colour represents the

higher concentration used and the light one for the lower concentration). (a) OD was monitored during growth. Data show a representative result and error bars represent the standard error of three technical replicates. (b) pH_c values after glucose depletion, calculate as described in Materials and Methods. Error bars represent the standard error of the mean of two biological replicates.

These observations indicate that synthetic defined media is more appropriate to study the effect of nitrogen quality and quantity availability in pH_c regulation, in order to avoid interferences with other nitrogen compounds. However, these results are also strong indicators of the crucial role of the amount of nitrogen present during growth: cells that grow in favourable nutrient conditions have their cytosol acidified at a higher extend then cells that grow in the presence of only one nitrogen source. This can indicate that cells are already growing in stress and adapting to it during growth, which leads to a better adaptation once glucose is depleted and therefore the a higher pH_c.

4.1.4 Nitrogen absence affects pH_c once cells are starved for glucose

Above observations in SD indicate that the amount of nitrogen present in the media has an effect on cytosolic pH in the absence of glucose. Culture cells in 5mM of N led to an acidification of the cytosol upon glucose deprivation but to a lower extend when compared to cells cultured in 10mM of nitrogen. In the case of GA this effect was dependent on the concentration of the nitrogen source present both during growth and during the starvation experiments. We could not reproduce these effects in SC-U medias with these specific nitrogen sources, likely because of the amount of nitrogen sources present in the media. To test if nitrogen would still have a role on setting the starvation pH_c, we measured pH_c in combined glucose and nitrogen starvations in SC-U media. This way we could also test whether pH_c of cultures grown in SC-U media was influenced by nitrogen availability.

Cells were washed with media containing glucose and nitrogen, glucose and no nitrogen and no glucose with or without nitrogen. In the presence of glucose, there was no significant difference on pH_c between nitrogen presence or absence (Figure 4-6). This result supports our previous observations in which cytosolic pH stayed neutral independently of the nitrogen availability. However, in the absence of glucose, pH_c was influenced by the presence or absence of nitrogen: we observed a reduced cytosolic acidification in the absence of nitrogen when compared to cells deprived only for glucose that is maintain throughout measurement (see Figure S2 in Supplemental data).

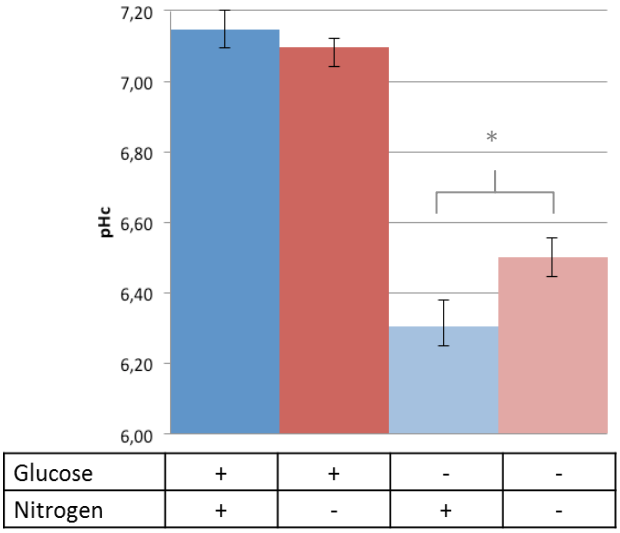


Figure 4-6 – *Nitrogen absence leads to a smaller acidification of the cytosol after glucose starvation*. Blue bars represent nitrogen present conditions and red bars the ones without nitrogen. Error bars represent the standard error of the mean of at least three independent experiments. * p-value <0.05

These results are in agreement with the N-regulation of pH_c in SD-AA medias: pH_c is not regulated by nitrogen while glucose is present in the media, but only upon glucose deprivation. The acidification upon glucose depletion was reduced when cells were also starved for nitrogen. This experiment confirms that the amount of nitrogen present in the culture media determines the extend of the cytosolic acidification, independently of the media used, since this experiment was perform in SC-U media with the supplement mix and a difference in pH_c is observed.

4.2 Is pH_c regulated by nitrogen via TORC1?

Our observations indicate that nitrogen availability regulates the extension of the acidification of cytosol, upon glucose deprivation, both in glucose natural growth and in starvation experiments. Target of Rapamycin Complex 1 (TORC1) regulates cellular growth in response to nitrogen quality (Stracka et al. 2014) and quantity (Neklesa & Davis 2009). The absence of this nutrient inhibits TORC1 activity (Li & Guan 2009). In this section we evaluated if the nitrogen effect on pH_c would be regulated via TORC1 pathway.

4.2.1 TORC1 inhibition by rapamycin present a nitrogen starvation-like phenotype on pH_c

To check if the lower cytosolic acidification reached upon glucose and nitrogen starvation would be regulated via TORC1, we asked if artificial inactivation of the pathway could recapitulate the effects of nitrogen starvation. For such purpose we performed treatments with rapamycin. This drug is known to inhibit TORC1 (Wullschleger et al. 2006) and therefore can be used to study specially the effect of TORC1 inhibition. Rapamycin is considered to mimic TORC1-dependent effects of nitrogen starvation (Soulard et al. 2010) (Stracka et al. 2014). Hence, we hypothesized that nitrogen availability may regulate pH_c via TORC1. If this is the case, it is expected that rapamycin treatment to glucose-starved cells would lead to higher pH_c compared to the untreated control, as nitrogen starvation does.

Our previous unpublished observations suggested that rapamycin addition to glucose-starved cells had no effect on pH_c. We then thought that maybe TORC1 inactivation before glucose depletion is determinant to regulate pH in the absence of glucose, similarity to what occurs in PKA ((Edo n.d.), data not published). For this reason we performed rapamycin pre treatments in order to evaluate the effect of TORC1 inhibition on the starvation pH_c.

In the presence of glucose, rapamycin pre-treated cells showed a slightly decreased on pH_c, still the difference is gradually lost during the 60 minutes of measurement. Cells pre-treated with rapamycin showed a higher pH_c after glucose starvation, compared to the ones treated with DMSO (control) (Figure 4-7). This data is in accordance with previous described pH_c values after glucose and nitrogen starvation in cells cultured in SC-U, showing the similarity of effects caused by TORC1 inhibition. This data, suggests that TORC1 inhibition increases pH_c upon glucose starvation, suggesting that nitrogen availability may control pH_c via TORC1.

To further test the effect of the immunosuppressant in the cells, growth and pH_c were monitored in SC-U media upon rapamycin or mock treatment in their exponential phase. As expected, rapamycin addition caused a slight growth rate decrease. This caused cells to deplete glucose later than the control. Rapamycin also lead to a slightly higher cytosolic pH_c once cells depleted glucose (Figure 4-8), which supports the hypothesis that TORC1 may have a role in setting cytosolic pH when glucose is absent.

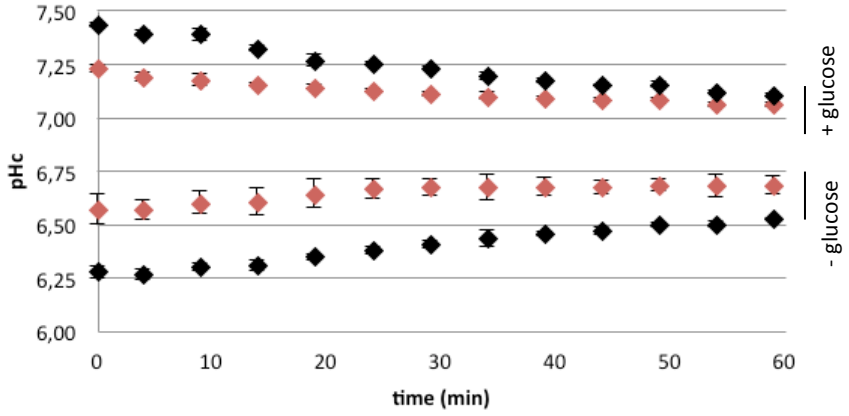


Figure 4-7 – *Rapamycin treated cells present a nitrogen starvation-like phenotype, once glucose is absent*. Red data points represent rapamycin pre-treated cells and black the control with DMSO. Data show a representative result and error bars represent the standard deviation of three technical replicates.

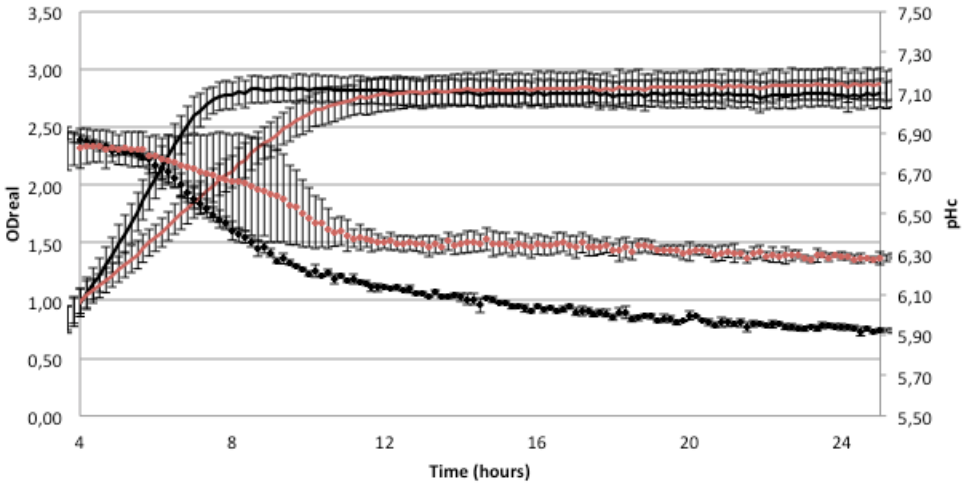


Figure 4-8 – *Effect of rapamycin, once added during growth, on pH_c, in SC-U.* OD (line) and pH_c (diamonds) of cells growing in SC-U were measured with addition of rapamycin after 4 hours of inoculation. Red data points represent cells treated with rapamycin and black data points represent cells with the mock treatment. Data show a representative result and error bars represent the standard deviation of three technical replicates.

Rapamycin addition during growth led to the same effect on pH_c as the treatment prior to glucose starvation. In both experiments TORC1 inhibition induced a decrease on the acidification inside the cell. This observation also supports the idea that artificial glucose starvations are proper experiments to test how different conditions influence pH_c once cells are deprived for glucose.

Both experiments present above were made in SC-U, therefore in the presence of a supplement mix lacking uracil but containing a whole range of amino acids and nitrogen bases. Thus, these pH_c values are a result of TORC1 inhibition by rapamycin in the presence of nitrogen, the activator of this pathway.

As stated before, once glucose is absent, both inhibition effects led to a lighter acidification of the cytosol compared to cells where TORC1 was active. That led us to question what would be the effect of the two inhibitions combined: are they additive or not in pH_c control? If both, rapamycin and the absence of nitrogen would inhibit TORC1 at the same extend as only one inhibitor would, upon glucose depletion, pH_c would be the same as the effect of only one inhibitor. On the other hand, if in the absence of glucose, pH_c would be higher than the previous described, this could suggest that (1) TORC1 is not completely inhibit by any of the treatments or that, (2) nitrogen absence may be regulating pH_c independently of TORC1 inhibition. We pre treated cell with rapamycin and mock and starve them for one or both nutrients (see Figure S3 in Supplemental data for pH_c during measurement), this way we could dissect the role of nitrogen and TORC1 on cytosolic pH regulation.

Assuming that nitrogen starvation and rapamycin addition both regulate pH_c via TORC1, it is expected that those treatments would not be additive in their effect on pH_c.

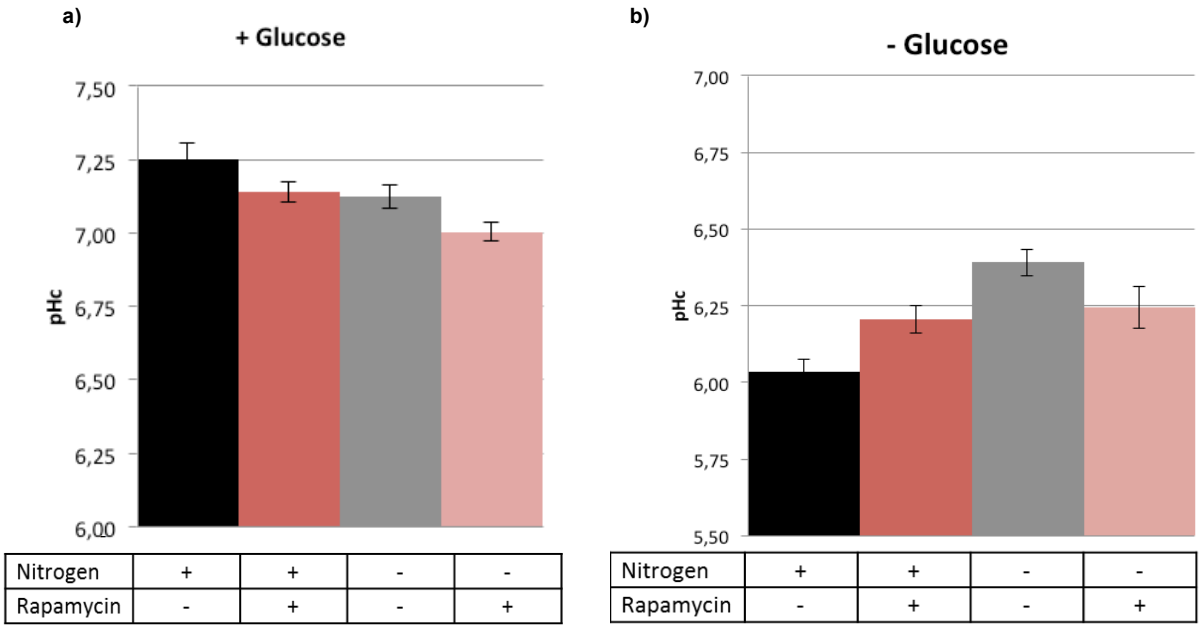


Figure 4-9 – *pH_c values after glucose and nitrogen starvation on rapamycin pre-treated cells, in SC-U*. In (a) data represents conditions in the presence of glucose. In (b), data represent conditions in the absence of glucose; black and red bars represent cells washed in the presence of nitrogen, without and with the pre-treatment, respectively; grey and light red bars represent cells starve for nitrogen, respectively without and with rapamycin pre-treatment. Data shown is an average of two biological replicates. Error bars represent the standard error.

In the presence of glucose (Figure 4-9 a), there was a small decrease in the pH_c value for cells with the mock treatment and starved for nitrogen compared with the not starved cultures. Like in the previous observations, rapamycin treatments prior to the washes with fresh media containing both nutrients led to a slight decrease in cytosolic pH. Interestingly, this decrease was to the same value as the ones treated with mock and starved for nitrogen. This suggest that TORC1 may be the pathway involved in pH_c regulation since both ways of inhibiting the complex lead to the same effect on pH_c, in the presence of glucose. When both the absence of nitrogen and the addition of rapamycin were

combined, we could observe a drop of 0,25 in pH compared with the control (no rapamycin in the presence of nitrogen). Thus, pH_c in this case was lower than any of the treatments individually.

However, in the presence of glucose effects seem additive: a higher acidification was observed when both TORC1 inhibitors were combined. This may indicate that the inhibition of the TORC1 complex was not complete in the presence of only one treatment. One way to seek deeply in this subject would be to repeat these starvation experiments but pre-treat the cells with a higher concentration of rapamycin. The goal would be to understand if a higher concentration of rapamycin would inhibit TORC1 to a higher extend leading to a lower pH_c in the presence of glucose.

On the other hand in the absence of glucose interesting results come up. The results from the cells pre-treated with mock were as the ones previous described: in the absence of both nutrients the extend of the cytosol acidification is shorter when compared to cells only starved for glucose. The opposite effect on pH_c was observed for cells in the absence of both nutrients between cells pre-treated with rapamycin or mock. Rapamycin pre-treated cells had the expected increase in pH_c in the presence of nitrogen but to our surprise, a decrease in its absence. Interestingly, the value of both rapamycin pre-treatments in the presence or absence of nitrogen is about the same (red bars in Figure 4-9 b). Overall, this can suggest that rapamycin inhibits TOR Complex 1 in a way that it becomes deaf to the nitrogen signal inside the cell. The way TORC1 senses the signal of nitrogen is still unclear and rapamycin is known to bind to TORC1 after hijacking Fpr1 protein and block it's signalling to downstream effectors (Torres et al. 2002). The Fpr1-rapamycin complex binds to the FRB subunit of TORC1 complex and maybe this subunit is also important for the nitrogen signalling branches upstream of TORC1. Since when the inhibitory complex is anchored to FRB no signalling of nitrogen availability seems to be sensed by TORC1, at least when it comes to pH_c control.

This data contradicts literature about rapamycin inhibition of TORC1 reproducing nitrogen starvation effects, at least in what pH_c is concern. We cannot say that in the absence of glucose the combination of the two inhibitors of TORC1 had an additive effect on pH_c, since this was not the highest value. In the absence of glucose nitrogen absence had a slightly stronger effect on cytosolic pH when compared to the addition of the drug. Along with this, the effect of rapamycin in all nutrient conditions was not significant, suggesting that pH_c is not regulated via TORC1.

All the above experiments were performed using rapamycin as a pre-treatment, thus TORC1 was first inhibit by rapamycin. After glucose starvation, pH_c was about the same for both nitrogen starved and not starved cells. This result raised the question whether nitrogen starvation prior to rapamycin treatment would have the same effect on cytosolic pH, after glucose deprivation. If we starved cells for nitrogen before rapamycin treatment, three different outcomes could be considered comparing the pH_c value with the one for cells starved for both nutrients without the treatment: (1) equal pH_c value would suggest that once TORC1 is deactivated by the absence of nitrogen it becomes deaf to other inhibitors; (2) higher pH_c would mean that the inhibitory effect of rapamycin would deactivate TORC1 to a higher extend combined with the nitrogen starvation and that both inhibitions are additive in their

effect on cytosolic acidification or (3) lower pH would be the same interesting result as seen in Figure 4-9 b).

To understand if higher amounts of rapamycin would provoke an even lower acidification of the cytosol than the one observed pH_c (Figure 4-10) and growth (Figure 4-11) were monitored to cells with the addition of different concentrations of rapamycin. We observed a relationship between increasing concentrations of this drug and higher pH_c values once glucose was deprived, compared to the ones treated with mock. This suggests that higher amounts of rapamycin deactivate TORC1 at a higher extend, which leads to a lower acidification of the cytosol once cells consume all the glucose present in the media.

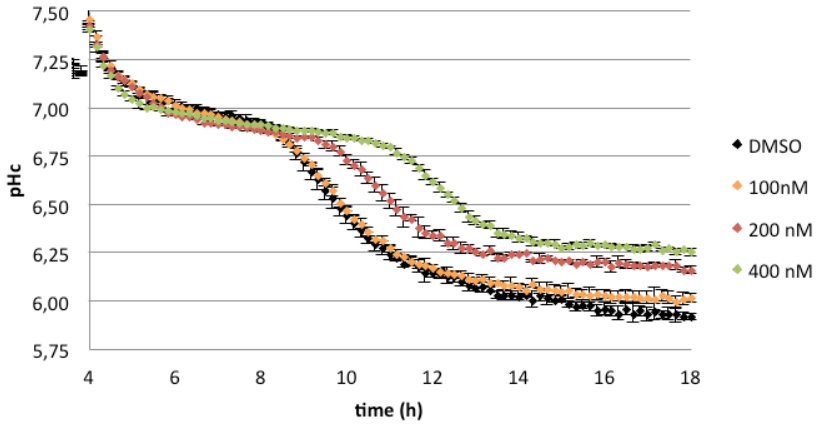


Figure 4-10 – *pH_c is influenced by the amount of rapamycin added to the cells*. Higher amounts of rapamycin led to a small decrease in pH_c once glucose is depleted. Green data points represent 400nM, red 200mM and orange 100nM. Rapamycin was added 4 hours after inoculation along side with mock (black data points). Data show a representative result and error bars represent the standard deviation of three technical replicates.

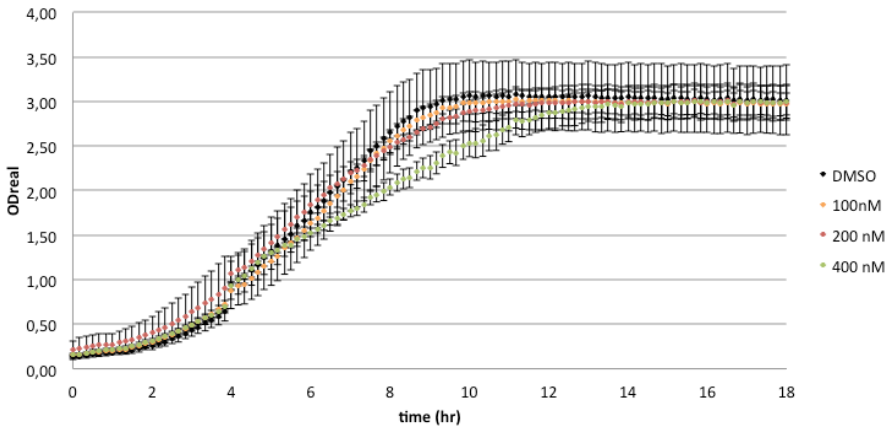


Figure 4-11 – *Higher the amount of rapamycin later cells reach post diauxic phase.* Black data points represent the control; orange 100nM of rapamycin; red represent 200nM and green 400nM of rapamycin. It is clear that cells take longer to deplete glucose when in the present of higher amounts of rapamycin. This highlights the immunosuppressant ability of this drug. Data shows a representative result and error bars represent the standard error of three technical replicates.

The role of TORC1 in growth control is clear in the figures above, where higher amounts of rapamycin lead to slower growth rates, highlighting the inhibitory role on growth of this drug. With this data it seems that TORC1 plays a role in pH_c regulation, since its activation somehow keeps the cytosol acid.

4.2.2 Rapamycin addition during a course of a growth curve does not affect pH_c in cells growing in SD-AA media

As described before, the amount of nitrogen present in the media where cells are growing influences the acidification extend in the cytosol and therefore its pH value once they reach post diauxic phase during growth. If TORC1 would be the responsible for this acidification, linking nitrogen signals to cytosol pH regulation, we expected a difference in pH_c between cells treated with the drug, TORC1 inhibitor. All the observations so far suggested a clear effect of nitrogen on pH_c regulation, but whether this effect was regulated via TORC1 pathway was unclear, from our experiments in SC-U medias. In this section we wanted to study what would be the effect of TORC1 inhibition to cells growing in minimal media in the presence of only one nitrogen source. This way we could study the effect of rapamycin inhibition without having a high amount of nitrogen compounds in the media that can affect TORC1 activity, If this was the pathway that regulates pH_c, based on nitrogen availability, than addition of rapamycin to cells growing in the presence of only 5mM or to 10mM of nitrogen would lead to the same cytosol acidification, after glucose depletion.

We monitored growth and pH_c of cells growing in SD-AA medias with 10mM or 5mM of glutamic acid or 5mM or 2,5mM of ammonium sulphate and added rapamycin or mock 4 hours after inoculation (see Figure 7-2 in Supplemental Data). Interesting, at the moment rapamycin is added to the cells an immediate but slightly drop in pH_c can be observed, as previously described in SC-U medias, in the presence of glucose. In this case the difference between pH values for cells pre-treated and not with the drug is not maintain during the time of the measurement.

As expected, rapamycin indeed inhibited growth. At the time cells consumed all glucose present in the media it was clear that TORC1 inhibition did not led cells to the same cytosolic acidification, for both GA and AS conditions (Figure 4-12). The difference in pH_c once cells depleted glucose is not significant between cells treated or not (with DMSO). Upon glucose depletion a decrease in pH_c was observed for treated or not treated cells' and about the same value for the same amount of nitrogen condition. For both nitrogen sources, the cytosolic acidification reached after glucose depletion between different concentrations of nitrogen present was significant with a p-value < 0,0001: lower concentration of nitrogen present in the media where cells are growing led to a lower acidification and therefore to a higher cytosolic pH.

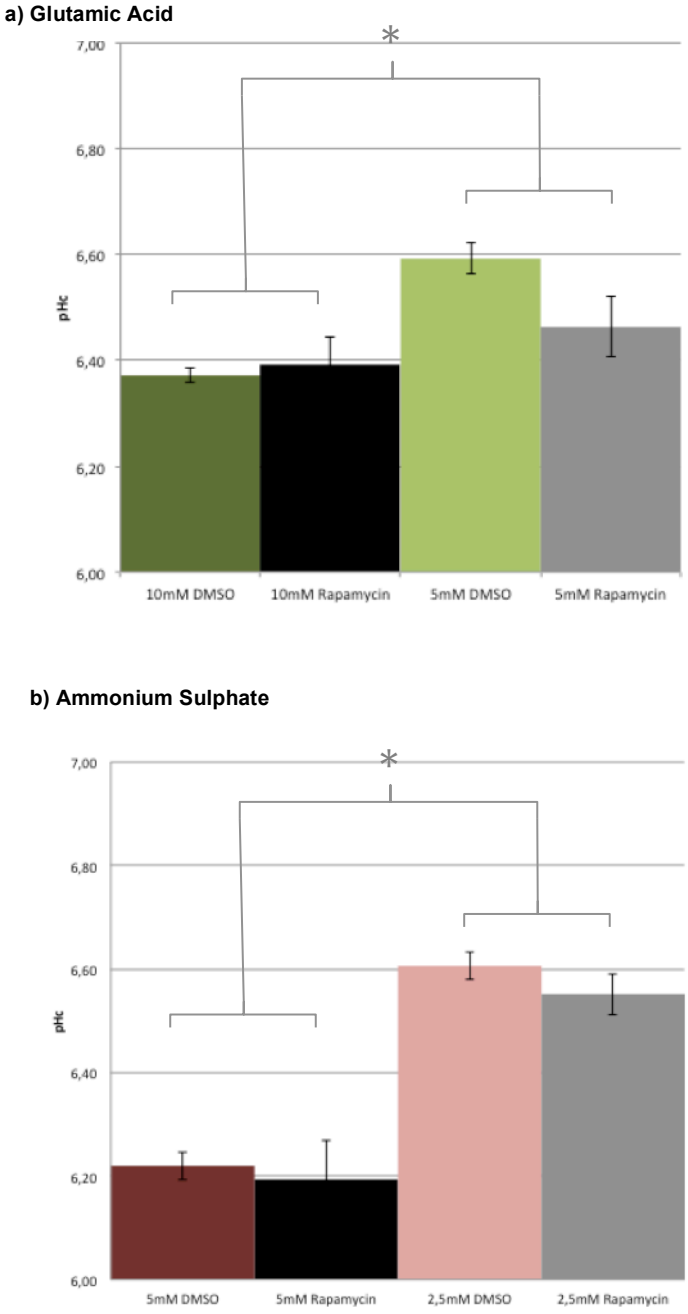


Figure 4-12 – *Rapamycin does not affect pH_c in minimal media.* pH_c values after rapamycin addition during growth in SD-AA with a) glutamic acid and b) ammonium sulphate. Data was measured as described in Materials and Methods, after glucose depletion. There is a significant difference between concentrations with DMSO but not with rapamycin. Erros bars represent the standard error of the mean of three biological replicates. *n.s.

This experiment highlights the nitrogen availability effect on pH_c regulation and supports the idea that TORC1 is not involved, since rapamycin inhibition leads to not significant changes on cytosolic pH, whereas different amounts of nitrogen do. Inhibition of TORC1 did not led to the same cytosol acidification extend between different amounts of nitrogen present for both GA and AS. These results do not go in agreement with the data showed in in Figure 4.9 b) with glucose and nitrogen starvation

after rapamycin pre-treatment, where the availability of nitrogen does not seem to be sensed once cells are pre-treat with rapamycin.

4.2.3 TORC1 and its upstream regulators Npr2 and Npr3

As described in the introduction, TOR pathway is a complex pathway and its upstream regulation is still unclear. It is known that its activity depends on nitrogen availability but the way the pathway senses nitrogen signals is not properly defined. In the majority of the literature found about TORC1, studies were made on a specific nitrogen source effect on TORC1 activity, making it difficult to question and extrapolate the data found to others. So far, three regulators that act upstream of TORC1 have been uncovered: Vam6 (Binda et al. 2009), Lst4-Lst7 complex (Péli-Gulli et al. 2015) and SEAC (Dokudovskaya & Rout 2015). All of them are responsible for activating the heterodimer in $Gtr1^{GTP}$-$Gtr2^{GDP}$ form, part of the EGOC that activates TORC1. This activation process only happens when the EGOC anchors TORC1 at the membrane of the vacuole (Powis et al. 2015) which may also suggests a role between the proton pump V-ATPase and TORC1.

We focused on studying deletion mutants of the SEACIT complex because these lead to overactive TORC1 mutants, providing us with a complementing approach to TORC1 inactivation. We expected that mutants with an overactive TORC1 had a lower pH_c than WT, upon glucose depletion.

Npr2 and Npr3 are both regulators of nitrogen permeases that down regulate TORC1 in response to amino acid starvation (Neklesa & Davis 2009), as part of the SEA sub complex inhibiting TORC1 (SEACIT) (Dokudovskaya & Rout 2015). Literature shows that both single and double deletion of *NPR2* and *NPR3* resulted in increased activity of TORC1, in the presence and absence of amino acids (Panchaud et al. 2013a). This indicates that *npr2*Δ; *npr3*Δ or *npr2*Δ*npr3*Δ cells present overactive TORC1, suggesting that they cannot signal the amount of nitrogen inside the cell (Neklesa & Davis 2009). Thus, if these mutants still show a difference in pH_c values after glucose starvation in the presence or absence of nitrogen or when comparing different nitrogen concentrations, growing them in SD-AA with GA or AS, we could be able confirm that TORC1 is not involved in pH_c regulation.

We inoculated *npr2*Δ in SC-U and starved it for glucose and nitrogen to test the effect of the absence or presence of nitrogen in pH_c value. As expected, in the presence of glucose no significant differences in pH_c values were observed (data not shown). Surprisingly, the starvation pH_c after glucose depletion was higher for *npr2*Δ than for WT. This contradicts our hypothesis that overactive TORC1 mutants would lead to higher acidification after glucose withdrawal compared to WT (Figure 4-13). Besides, starving *npr2*Δ for nitrogen did not seem to have an effect on pH_c, since the values for both presence or absence of nitrogen were not significantly different. However in this experiment we could not see a big effect in pH_c for WT either, comparing both nitrogen conditions. Therefore we cannot conclude that the *npr2*Δ mutant does not really sense nitrogen availability.

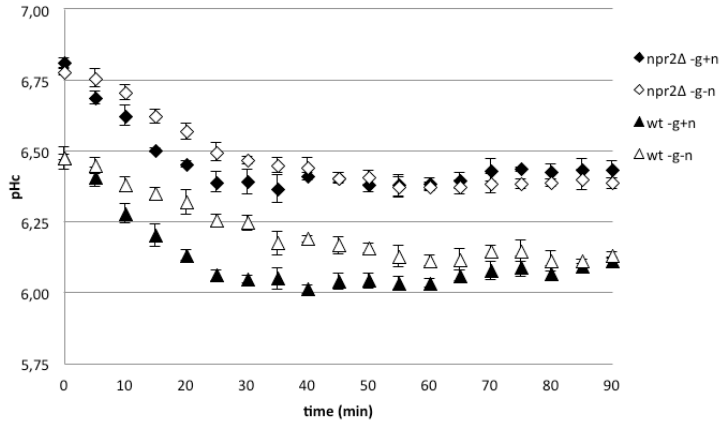


Figure 4-13 – *pH_c after glucose starvation to npr2Δ and WT*. Diamonds represent *npr2*Δ and triangles WT. Filled data points represent cells that were starved for glucose in the presence of nitrogen and empty ones represent the ones starved for both nutrients. Data represents one representative result and error bars are the standard deviation of three technical replicates.

This data contradicted the hypothesis that TORC1 mediates cytosolic pH regulation by nitrogen availability, since overactive TORC1 led to the opposite effect of what we expected comparing with all the data gathered before with TORC1 inhibition experiments. Since this was a single experiment it could be an outlier. We should have also performed starvations experiments with other SEACIT mutants to discard specific effects of *NPR2* deletion mutants. We should also keep in mind that deletion mutants have suffered some adaptations resulting from the mutation, which could lead to unreliable data.

Because we could not conclude that *npr2*Δ did not signal the amount of nitrogen present at the time of the starvation we performed growth curves. Growth and pH_c of *npr2*Δ and *npr3*Δ, single deletions, and of WT was monitored in SD-AA with 5mM or 2,5mM of ammonium sulphate (Figure 4-14). This way we could study if different amounts of nitrogen present in the media would lead to the same pH_c, confirming they do not sense nitrogen availability. *npr2*Δ pH_c behaviour was as the WT which was unexpected, because this suggests that the mutant senses the amount of nitrogen present and behaves accordingly to it. With *npr3*Δ we could observe a higher pH_c in the presence of glucose compare to WT. The *npr3*Δ growing in 2,5mM of AS took longer to deplete glucose, pH_c value in the end was the same as the ones growing in 5mM and about the same as WT growing in 2,5mM of AS. Thus, for this mutant both nitrogen concentrations led to the same pH_c, which agrees with the statement that these deletion mutants do not signal nitrogen availability (Neklesa & Davis 2009). This supports the idea that Npr3 may be involved in N-dependent pH_c regulation. However we cannot make proper conclusions since we could not grew the double deletion strain that would help us discard the outlier result.

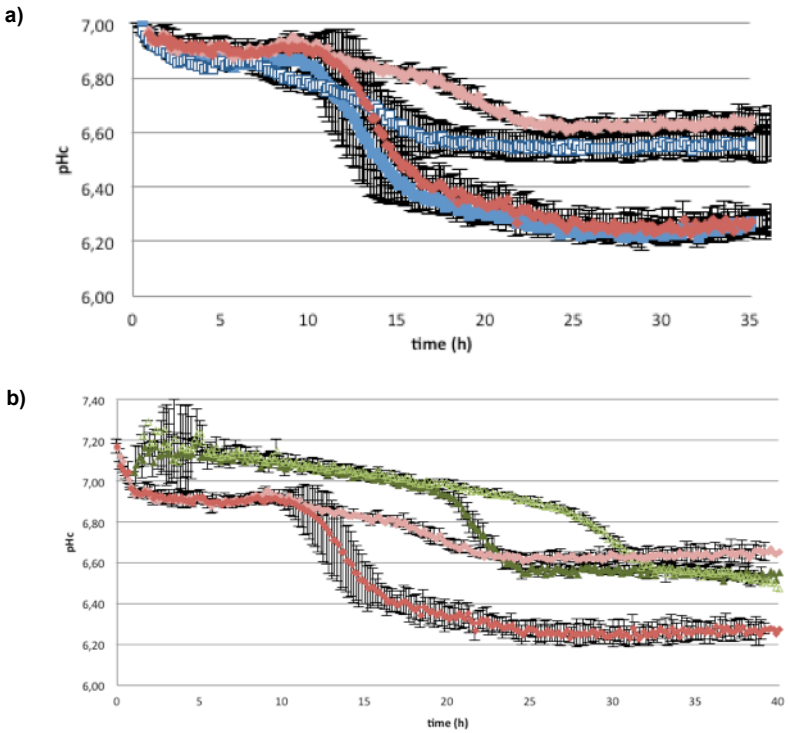


Figure 4-14 – *Overactive TORC1 mutants have different pH_c behaviours.* pH_c of a) *npr2*Δ (blue squares) and b) *npr3*Δ (green triangles) and WT (red diamonds) growing in SA-AA with AS in 5mM (filled blue square, filled green triangle and dark red diamond) or 2,5mM of AS (empty square, empty triangle and light red diamond). Data represents one representative result and error bars are the standard deviation of three technical replicates.

All of these data led us to the conclusion that the key to answer the question "How nitrogen regulates cytosolic pH?" may be at a metabolic level, thus more knowledge is required in on the way cells uptake the different nitrogen sources and what are the pathways involved in each signalling. TORC1 pathway does not seem to be the one involve mediating pH regulation by nitrogen availability.

5 TAKE HOME MESSAGE AND FUTURE PERSPECTIVES

With this project is clear that nutrient availability is essential for cytoplasmic pH regulation in yeast. The presence of glucose is essential for intracellular neutrality and for cells to maintain their normal function and growth. During the course of every experiment no significant changes in pH_c were observed in the presence of the carbon source, neither when we monitored growth or in glucose starvations experiments. Literature suggests that pH_c is dynamic and indeed, when cells consume all the glucose present in the culture media and exponential phase ends, an intracellular acidification occurs (Orij et al. 2012). Our work focused on first understanding if nitrogen availability would provoke any changes in cytosolic acidification and if it did, trying to place TORC1 as the pathway that links nitrogen to pH_c regulation.

On one hand we revealed the importance of the quality and quantity of the nitrogen source present in the cells' inoculum in setting the starvation pH_c. Growing a BY4741 prototrophic strain in SD-AA medias with one rich nitrogen source present in two different concentrations led to different extends of acidification, only seen in the absence of glucose. Higher amounts of nitrogen present in the media led to lower pH_c once cells depleted glucose.

Oliveira *et al.*, revealed that shifts in nitrogen quality would change the cellular network and identified TORC1 downstream targets to be involved (Oliveira, Dimopoulos, et al. 2015). In the future, yeast growth and pH_c should be monitored in a range of different nitrogen concentrations to see whether there is a minimum or a maximum amount of this nutrient that can provoke any effect on cytoplasmic pH regulation. Because cells uptake the different nitrogen sources by distinct pathways based on them being considered rich or poor sources and on their size (Cooper 1982), via H^+-dependent transport. An asset to the continuity of this study would be to seek more into how this uptake is regulated and how these proton interactions would influence pH_c. Maybe this adaption could explain why glucose starvations in minimal media with AS did not mimicked growth curve while with GA do. Also, in the future, chronological life span experiments in minimal media would be important to understand if cells adapt to the quiescence state easily (Klosinska et al. 2011), surviving to nutrient starvation longer.

The availability of nitrogen is responsible for TORC1 pathway activation. Thus, in the second part of the project we tried to discover whether the nitrogen effect discovered on pH_c would be regulated by TORC1. To assess this question we performed treatments with rapamycin that has an immunosuppressive potential. This drugs binds to TORC1 leading to its inactivation and in many cases leads to nitrogen starvation-like phenotypes (Loewith 2011). Indeed, glucose starvation after cells were treated with rapamycin led to a small cytosolic acidification similar to nitrogen starvation, compared with cells treated with the control.

Despite most of glucose starvation experiments mimicked the effects of natural glucose depletion during growth we had some issues working with rapamycin before starving the cells, since we could

not always see this difference. This is why adding rapamycin during growth and study how cells behave naturally is a must, because starvation experiments sometimes are not reliable on their own.

Once we combined the two inhibitors of TORC1: nitrogen starvation after treatment with rapamycin we had surprising results in the absence of glucose conditions. We could observe the same pH_c for cells treated with rapamycin in the presence or absence of nitrogen. This suggested that once rapamycin inhibits the complex it becomes deaf to nitrogen availability inside the cell. This pH_c value was lower than cells treated with mock after nitrogen and glucose starvations, which goes against rapamycin inhibition on TORC1 having nitrogen-starved phenotype. However, these differences on rapamycin treated cells were not significant, suggesting that TORC1 does not mediate the signal of nitrogen availability in pH_c regulation. Addition of rapamycin during growth to cells inoculated in minimal media led to the same conclusion: differences on pH_c between drug or mock treatment in the same conditions were not significant, upon glucose depletion. In the future, repeat nitrogen starvation experiments before rapamycin treatment could help to understand how TORC1 inhibition is regulated, because maybe one of the inhibitors blocks the other's activity.

In order to fully establish that TORC1 does not play a role in cytosolic acidification more studies with deletion mutations of the pathway should be made. Deletion strains for all the components that act upstream or downstream of TORC1 should be cultured in SD-AA culture medium and pH_c monitored alongside with rapamycin treatments. This way we could figure out what is the upstream branch of TORC1 responsible for nitrogen signals. We tried to work with overactive TORC1 mutants: *npr2*Δ and *npr3*Δ single deletion strains, to try a different approach on understanding the pathway, however data was not conclusive. Another aspect of TORC1 is that the complex is active at the vacuole, V-ATPase proton pump also regulates pH_c (Dechant & Peter 2010), and thus maybe V-ATPase proton pump has a role on TORC1 and its role on pH_c regulation. This interaction between V-ATPase and TORC1 should be a focus subject for studies in the future.

As in every research project new answers result in even more questions to be investigated. The yeast baker model *Saccharomyces cerevisiae* used in this project provides an important model to study nutrient control of eukaryotic cell growth in general. There is still a long way to go, but the findings of this study contribute to a better understanding of the role of nutrients in pH regulation in yeast: breakthrough ideas emerge from small discoveries.

Thus, all together, the main conclusion of this project is that nitrogen plays a role on cytosolic acidification that is probably not mediated via TORC1 pathway.

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SUPPLEMENTAL DATA

1) Supplement mix composition of the synthetic complete media

Table S1 – Recipe drop out mix

(as CSH http://cshprotocols.cshlp.org/content/2006/1/pdb.rec8585.full?text_only=true)

Reagent	**Amount to add (g)**
Adenine	0.5
Alanine	2.0
Arginine	2.0
Asparagine	2.0
Aspartic acid	2.0
Cysteine	2.0
Glutamine	2.0
Glutamic acid	2.0
Glycine	2.0
Histidine	2.0
Inositol	2.0
Isoleucine	2.0
Leucine	10.0
Lysine	2.0
Methionine	2.0
para-Aminobenzoic acid	0.2
Phenylalanine	2.0
Proline	2.0
Serine	2.0
Threonine	2.0
Tryptophan	2.0
Tyrosine	2.0
Uracil	2.0
Valine	2.0
Combine the appropriate ingredients, minus the relevant supplements, and mix in a sealed container. Turn the container end-over-end for at least 15 min; add several clean marbles to help mix the solids.	

2) OD_{real} and pH_c of cell growing in proline or urea in SD-AA

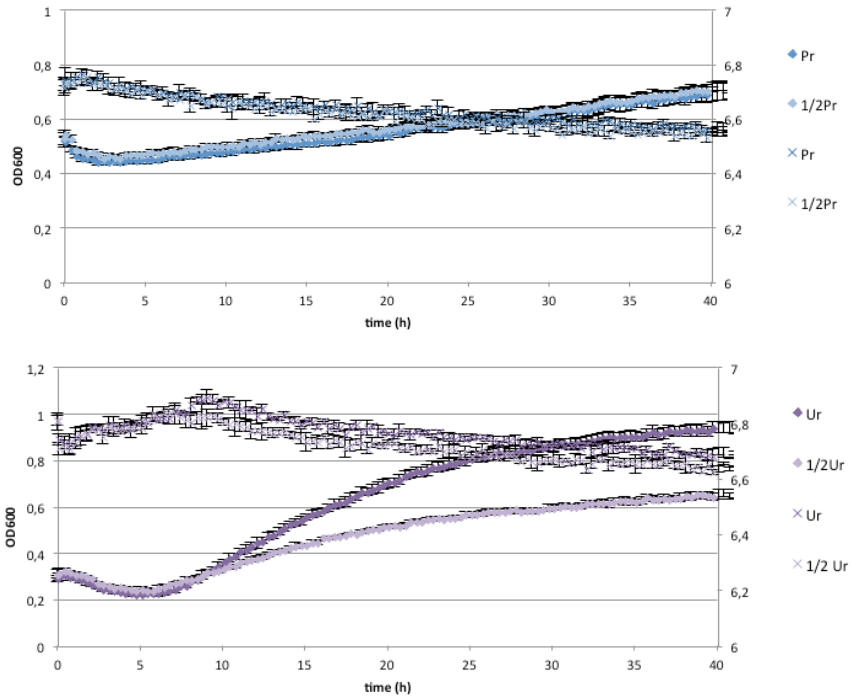

Figure S1 – *Poor nitrogen sources do not have an effect on pH_c during growth.* OD_{600} (diamonds) and pH_c (cruxes) were monitored during growth in microplates, in SD media with proline (Pr) (on the top) or urea (Ur) (on the bottom) as nitrogen sources. In both (a) and (b) darker colours represent 10mM N and the lighter 5mM of nitrogen (in the figure as ½). Cells growing in Pr did not grew, while cells growing in Ur did not deplete glucose during the course of the measurement and no difference in pH_c can be observed between the two concentrations in study. Data shows a representative result and error bars represent the standard error of three technical replicates.

3) pH_c after nutrient starvation in SC-U

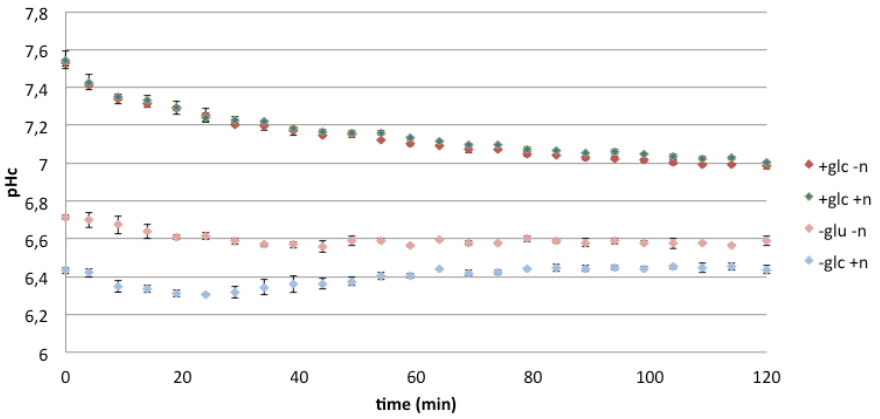

Figure S2 – *pH_c after nutrient starvation.* The absence of nitrogen does not affect pHc in the presence of glucose. However, in the absence of glucose, cells also starved for nitrogen (light red diamonds) have a higher pHc than

the ones starved in the presence of this nutrient (light blue data points). Data shows a representative result and error bars represent the standard error of three technical replicates.

4) Nutrient starvation after rapamycin treatment

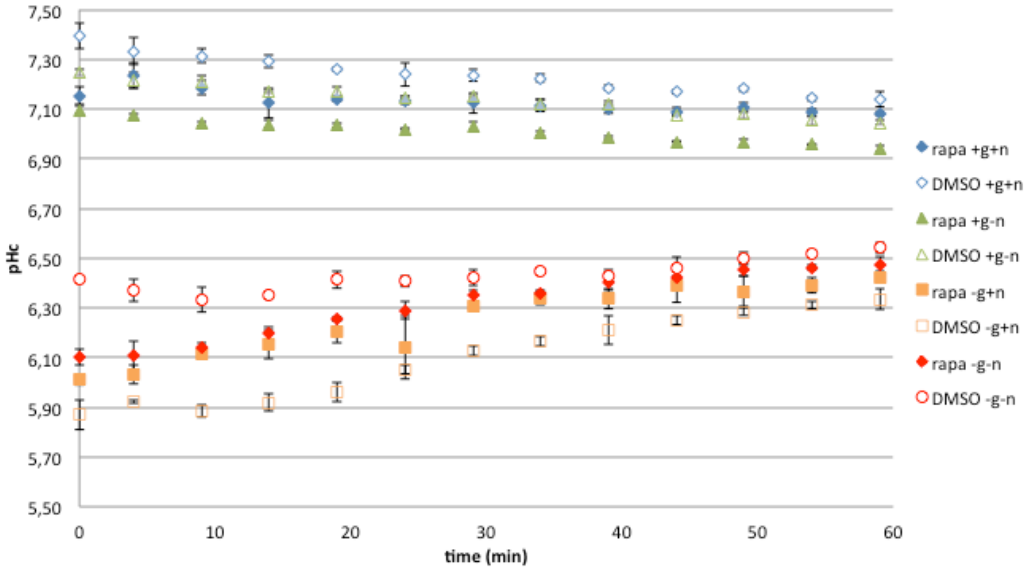

Figure S3 – *Rapamycin treatment prior to glucose starvation seems to block nitrogen availability signal of cells in setting* pH_c. Filled data points are for rapamycin treated cells and empty data points are for control. rapa means rapamycin; conditions with + mean they are in the presence of that nutrient and conditions with – mean they are in the absence of the specific nutrient. Data shows a representative result and error bars represent the standard error of three technical replicates

5) pH_c during growth with addition of rapamycin and DMSO in SD-AA

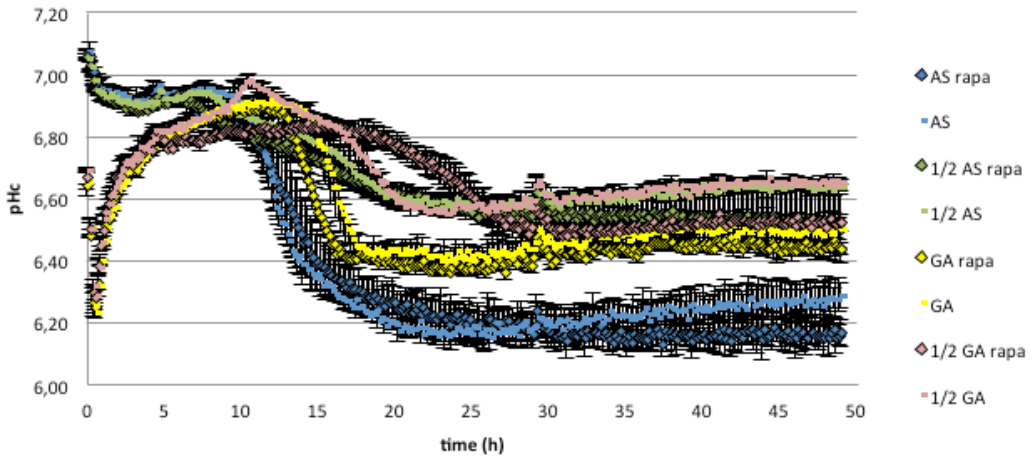

Figure S4 – *Rapamycin effect on pH_c during growth to cells growing in SD-AA with glutamic acid or ammonium sulphate.* AS represents 5mM of ammonium sulphate and ½ AS, 2,5mM. GA represents 10mM of glutamic acid and ½ 5mM. Lines represent cells with mock treatment and diamonds cells with rapamycin. In the presence of glucose we could see a slight decrease on pH_c right after rapamycin addition (4 hours after inoculation), consistent with the starvation experiment. Data shows a representative result and error bars represent the standard error of three technical replicates.

6) Preliminary data on the relationship between PKA, TORC1 and pH_c

In some conditions, TORC1 pathway is also known to regulate PKA (Zhang et al. 2011). In turn, PKA has been show to regulate pH_c upon glucose depletion (Edo *et al*., unpublished). We also aimed to check if both signalling cascade pathways respond to nutrients and regulate cytosolic acidification together.

Sch9 is a direct substrate for TORC1 and one of the main branches of the TORC1 signalling pathway in yeast. The activity of Sch9 is dependent on TORC1 phosphorylation (Urban et al. 2007). This branch of TORC1 is responsible for the coordinated expression of the protein synthesis machinery (Huber et al. 2009). Soulard *et al.*, also placed Sch9 as an upstream regulator of PKA pathway (Soulard et al. 2010). We wondered whether Sch9 was the link between the two pathways and if TORC1 acted upstream of PKA through Sch9 to regulate pH_c.

We monitored both growth and pH_c of *sch9*Δ mutant and, as expected an acidification in the cytosol occurred after the mutant consumed all the glucose in the media. However it was stronger compared to WT and pH_c was lower: phenotype resembled to an overactive PKA after been glucose deprived (Edo *et al*., unpublished). In this mutant TORC1 is active and this result suggested that PKA was overactive and it was not elucidative answering the question whether Sch9 is placed in between both

pathways, but if the link exists is likely to be indirect. If TORC1 is active and there is no Sch9 to phosphorylate then the *sch9*Δ has also PKA overactive, based on this hypothesis. However for this to be true, pH_c values once cells reached the post diauxic should be the same. pH_c of *sch9*Δ was between WT and *bcy1*Δ and this is not enough to say that TORC1 acts upstream of PKA. On the contrary, it seems that Sch9 does not mediate the signal through TORC1 to regulate cytosolic pH.

PKA has a catalytic subunit encoded by *TPK1, TPK2* and *TPK3* genes and a regulatory subunit encoded by *BCY1* that controls PKA activity in response to cAMP (Soulard et al. 2010). cAMP binds to Bcy1 releasing it from TPK and activating PKA.

We also monitored *bcy1*Δ growth and pH_c and as expected, because this mutant presents the phenotype of an overactive PKA, once cells depleted glucose pH_c was lower compared to WT, in SC-U medium. The best way to check TORC1 involvement is through rapamycin pre treatments. We could not get proper results once we perform them in *bcy1*Δ. Because of this we starved this mutant for nitrogen and glucose in order to study the effect of TORC1 inhibition on this mutant. As expected, in the presence of glucose no difference was observed between pH_c values on cells in the presence or absence of nitrogen. With this mutant, starvation of glucose also mimicked the growth curve effect and once glucose was depleted pH_c was the lowest, compared with WT. Starvation of both nutrients (inactive TORC1) led to an even lower pH_c. Inactive TORC1 should result in a higher value if this was the pathway involved in cytosolic pH regulation.

These preliminary data suggest that both pathways work in parallel to regulate pH_c and not trough the same phosphorylation route. However much more work needs to be done with this set of mutants.