A contribution of budding yeast to unravel aging. Entry into quiescence relies on the transceptor Mtl1 by sensing nutrients and regulation of signaling pathways

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A contribution of budding yeast to unravel aging. Entry into quiescence relies on the transceptor Mtl1 by sensing nutrients and regulation of signaling pathways.

Ph.D Dissertation

For the fulfillment of Doctoral Degree

By

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Under the supervision of

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Maria Angeles de la Torre-Ruiz, Ph.D in Biological Sciences and Professor, Department of Basic Medical Sciences of University of Lleida, as supervisor of this thesis,

Hereby certify that,

Venkatraghavan Sundaram (Passport number: F3156264)**,** with Bachelor of Pharmacy (B.pharm) and Master of Pharmacy (M.pharm) in Pharmacy Practice (Tamil Nadu Dr. M.G.R. Medical Univeristy, Chennai India), has completed the experimental work entitled **"A contribution of budding yeast to unravel aging. Entry into quiescence relies on the transceptor Mtl1 by sensing nutrients and regulation of signaling pathways"** under my direct supervision**.**

This work, including the thesis has been completed to my level of satisfaction to meet the requirements for the presentation before the corresponding defence tribunal and, so be the case, to obtain **Doctor of Philosophy degree (Ph.D)** from the University of Lleida.

Signed:

Dr. Maria Angeles de la Torre-Ruiz

Lleida, 19th of December 2014

"Whosoever offers me, with love or devotion,

a leaf, a flower, a fruit or water,

that offering of love of the pure and self controlled man

is willingly and readily accepted by me"

-Lord Shri Krishna
In Bhagwad Gita, IX-26

To

Shri Sai Baba

The Antaryami

This work with myself

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INDEX

ABBREVIATIONS

AAP	Amino acid Permeases
ACO	Aconitase
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ATG	Autophagy
ATP	Adenosine Triphosphate
Cdk	cyclin-dependent kinase
CIT2	Citrate synthase II
CLS	Chronological Life Span
CWI	Cell Wall Integrity
CWP	Cell Wall Glycoproteins
DIC	Diffrential Interfrence Contrast
DiO6	3,3'-dihexyloxacarbocyanine iodide
ERC's	Extra Chromosomal rDNA Circles
FADH	Flavin adenine Dinucleotide
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
GABA	Gamma Aminobutyric Acid
GAP	GTPase activating proteins
GDP	Guanosine Di Phosphate
GEF	GTPase Exchange Factor
GFP	Green Fluorescent Protein
GPI	Glycosyl Phosphatidyl Inositol
GTF	General Transcription Factor
GTP	Guanosine Tri Phosphate
HAP	The heme-responsive transcription factor
HOG	High Osmolarity Glycerol

HSF	Heat Shock Factor
HSP	Heat Shock Protein
H2-DCFDA	Dihydrofluoresceindiacetate
IP	Immunoprecipitation
MAPK	Mitogen-Activated Protein Kinase
MMP	Mitochondrial Membrane potential
NAD	Nicotinamide adenine dinucleotide- oxidised
NADH	Nicotinamide adenine dinucleotide- reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NCR	Nitrogen Catabolite Repression
NF-κB	Nuclear Factor kappa-B
OXPHOS	Oxidative phosphorylation
OXR	Oxidative Stress Respone
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered Saline
PDS	Post Diauxic Shift
PFY1	Profilin
PIK	Phosphatidyl Inositol Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC1	Protein Kinase C
PoL I	RNApolymerase I
Pol II	RNApolymerase II
Pol III	RNApolymerase III
Polyp	Linear chain phosphate polymer
Por1	Porin

PP2A	Type 2A Phosphatase
PVDF	Polyvinyledenedifluoride
RiBi	Ribosomal Biogenesis
ROS	Reactive Oxygen Species
RTG	Retrograde Response
SAGA	Spt-Ada-Gcn5-acetyltransferase
SLIK	SAGA like
SC	Synthetic Complete media
SD	Synthetic minimum media
SDS	Sodium Dodecyl Sulphate
ST's	Steryl Esters
STREE	Stress Response Element
SNF	Sucrose Non Fermenting
TET	Triethyltin Bromide
TG's	Triglycerides
$tetO_7$	Tetracycline promoter
TOR	Target of Rapamycin
TORC1	Target of Rapamycin Complex 1
YPD	Yeast extract-Peptone-Dextrose
wt	Wild type

ABSTRACTS

Abstract

Saccharomyces cerevisiae is a premier model system to study ageing in post mitotic cells. In this study, we show that Mtl1, member of the Cell Wall Integrity pathway (CWI), plays a positive role in Chronological Life Span (CLS). The absence of Mtl1 shortens CLS whereas its overexpression extends it. In *mtl1* cells, we observe mitochondrial dysfunction during diauxic shift that is reflected in i) increase in uncoupled respiration associated to low oxygen consumption; ii) significant increase in mitochondrial membrane potential; iii) ROS (Reactive Oxygen Species) accumulation and as well as iv) a descent in aconitase activity. We demonstrate that this mitochondrial dysfunction observed in *mtl1* mutant is a consequence of improper regulation of key signaling pathwaysrequired for entry in quiescence. *TOR1/SCH9* deletion and less effectively PKA (Protein Kinase A) inactivation in *mtl1* not only suppressed the mitochondrial defects but also increased the CLS. Mtl1 links mitochondrial dysfunction with TOR (Target of Rapamycin) and PKA pathways in quiescence.

In the absence of Mtl1, the stability of the inhibitory subunit of PKA, Bcy1 is severely reduced, mainly as a consequence of a high PKA activity. Mtl1 regulates PKA inactivation through Bcy1 phosphorylation, both in diauxic conditions and mainly in response to glucose depletion. In these conditions, Mtl1 negatively regulates Tor1/Sch9 function to phosphorylate Bcy1 and thus to inhibit PKA through phosphorylation of Bcy1 by Slt2 MAPK, although additional kinases might be also involved in this signal. We also show that Mtl1 function in CLS does not totally depend on CWI pathway since *mtl1slt2* double mutant presents synthetic lethality. Mtl1 acts as a glucose sensor thereby it regulates both NCR (Nitrogen Catabolite Repression) and RTG (Retrograde Response Mitochondrial-Nucleus) pathways through Snf1 kinase upon glucose depletion, although independent of TORC1 and PKA functions. Again, additional targets are not completely ruled out.

In conclusion, Mtl1 is an efficient transceptor involved in sensing and signaling nutrient availability in quiescence, mainly glucose, and in regulating multiple pathways involved in life extension.

Resumen

Saccharomycescerevisiae es un sistema modelo muy adecuado para el estudio de los procesos de envejecimiento en células posmitóticas. En el presente estudio se muestra que Mtl1, un miembro de la ruta de integridad celular (CWI), tiene una función positiva en el proceso de extensión cronológica de la vida (CLS). La ausencia de Mtl1 provoca un acortamiento de la CLS mientras que su superexpresión la prolonga. En el mutante *mtl1* se observa una disfunción mitocondrial durante el shiftdiauxico, lo que se evidencia tras la observación de los siguientes parámetros: i) incremento de la respiración desacoplada ligado a un bajo consumo de oxígeno; ii) aumento significativo del potencial de membrana; iii) acumulación de ROS (Especies Reactivas de Oxígeno); así como iv) un descenso en la actividad de la aconitasa.Nosotros demostramos que la disfunción mitocondrial observada en el mutante *mtl1*ocurre como consecuencia de ladesregulación de las rutas de señalización que se necesitan para la correcta entrada en la fase de quiescencia. La delección de los genes *TOR1* y *SCH9* y con menor eficiencia la inactivación de PKA (Protein Quinasa A),suprimen los defectos mitocondriales de *mtl1* y provocan un aumento sustancial de su CLS. Mtl1 es el nexo de unión entre la disfunción mitocondrial y las vías TOR (Target of Rapamycin) y PKA en la quiescencia. La estabilidad de Bcy1, una subunidad inhibitoria de PKA, está muy reducida en ausencia de Mtl1. Esta proteína regula la inactivación de la función PKA a través de la fosforilación de Bcy1 durante el shiftdiauxico y principalmente en respuesta a la depleción de glucosa. En ambas condiciones, Mtl1 regula negativamente a la función Tor1/Sch9, lo que activa a la MAP quinasa Slt2, la cual fosforila a Bcy1, hecho que inhibe la función PKA. Sin embargo, no se descarta la posibilidad de que otras quinasas estén también involucradas en este proceso. También se muestra que la función de Mtl1 en la CLS no depende totalmente de la ruta CWI puesto que los genes *MTL1* y *SLT2* presentan letalidad sintética en estas condiciones.Mtl1 actúa como un sensor de glucosa y como tal también regula la función de Snf1 en respuesta a la depleción de glucosa, y como consecuencia a los genes regulados por los sistemas NCR (Represión Catabólica Nitrogenada) y RTG (Respuesta Retrógrada Núcleo-Citoplasma), además, este proceso es independiente de TORC1 y PKA. En conclusión, Mtl1 es un transceptor que detecta y señaliza la disponibilidad de nutrientes (principalmente glucosa) en quiescencia para regular la función de múltiples vías de señalización relevantes para la extensión de la vida.

Resum

Saccharomyces cerevisiae és un sistema model molt adecuat pels procesos d'envelliment en cèl.lules postmitòtiques. En aquest estudi es demostra que Mtl1, un membre de la vía d'integritat cel.lular (CWI), té una funció positiva en el procés d'extensió cronológica de la vida (CLS). L'absència de Mtl1 redueix la CLS mentre que la seva sobre expressió l'allarga. En el mutant *mtl1* s'observa una disfunció mitocondrial durant el shift diàuxic, que es fa evident al observar els següents paràmetres: i) increment de la respiració desacoplada unit a un baix consum d'oxígen; ii) augment significatiu del potencial de membrana; iii) acumulació de ROS (Espècies Reactives d'Oxígen); així com iv) un descens en l'activitat de l'aconitasa. Nosaltres demostrem que la disfunció mitocondrial observada en el mutant *mtl1* succeeix com a conseqüència de la desregulació de les vies de senyalització necessaries per a la correcta entrada a la fase de quiescencia. La delecció dels gens *TOR* y *SCH9* i amb menor eficàcia, la inactivació de PKA (Protein Kinasa A), suprimeixen els defectes mitocondrials de *mtl1* i indueixen una augment significatiu de la seva CLS. Mtl1 es el nexe d'unió entre la disfunció mitocondrial i les vies TOR (Target of Rapamycin) y PKA en quiescència. L'estabilitat de Bcy1, una subunitat inhibitoria de PKA, està molt reduïda en absència de Mtl1. Aquesta proteïna regula la inactivació de la funció de PKA mitjançant la fosforilació de Bcy1 durant el shift diàuxic i principalment en resposta a la depleció de glucosa. En ambdues condicions, Mtl1 regula negativament la funció Tor1/Sch9, fet que indueix l'activació de la MAP quinasa Slt2, la qual fosforila a Bcy1, procés que inhibeix la funció PKA. No obstant, no es descarta la possibilitat de que altres quinases estiguin participant en aquest procés. També es mostra que la funció de Mtl1 en la CLS no depen totalment de la vía CWI, donat que els gens *MTL1* y *SLT2* presenten sintètica letalitat en aquestes condicions. Mtl1 actua con un sensor de glucosa i com a tal, també regula la funció de Snf1 en resposta a la depleció de glucosa, i en conseqüència als gens regulats pels sistemes NCR (Repressió Catabòlica Nitrogenada) y RTG (Resposta Retrògrada Nucli-Citoplasma), a més a més, aquest procés es independent de TORC1 y PKA. Concluïnt, Mtl1 es un transreceptor que detecta i senyalitza la disponibilitat de nutrients (principalment glucosa) en quiescència per a regular la funció de múltiples vies de senyalització rellevants per l'extenció de la vida.

INTRODUCTION

1.Yeast A powerful organism for ageing and biomedical research

Aging studies have gained prime importance in biomedical research. Our understanding of the ageing process has seen fundamental advances over the last decade and raised optimism to build interventions to slow ageing may be on the horizon (reviewed in Kaeberlein, 2010; Petralia et al., 2014) .

Researchers have to understand in detail about aging mechanism and their consequence in health care. A thorough knowledge in ageing and modifying its rate would reduce incidence or progression of disease. These interventions will possibly lead to increased life span and also allowing elderly people to work with minimal health care costs. Yeast longevity factors have now been shown to modulate ageing in invertebrate and mammalian models. The potential of interventional approaches targeted at aging has yet to be realized and recently studies of yeast have resulted in some of the best candidates for anti-ageing drugs currently in development (reviewed in Kaeberlein, 2010; Longo et al., 2012).

In almost two and a half decades yeast has risen from position as a premier model for eukaryotic cell biology to become a powerful organism that has facilitated the establishment of diverse fields of study called “Functional genomics” and “Systems biology”(reviews from Botstein and Fink, 2011; Duina et al., 2014). We need simple model systems for the ageing process because human genetics has not resulted in sufficient evidence to pinpoint a generally recognized mechanism for the ageing process (Petralia et al., 2014).

Over the years, the field of ageing in *Saccharomyces cerevisiae* has been growing rapidly with numerous studies now indicate that the budding yeast as an essential model, and multiple findings relate mechanisms of ageing in this unicellular organism to those present in more complex life forms (Barros et al., 2010; reviewed in Kaeberlein, 2010).

S. cerevisiae is an optimal model to study the ageing for the following reasons:

i) a high degree of homology is presented by budding yeast genome with the human genome;
ii) there are many proteins which although not homologous in sequence, show an elevated functional homology with specific human proteins;
iii) it has reasonably very simple genetic manipulation system and whose cultivation is easy and regarded to be inexpensive compared to higher eukaryotic models;

iv) study of multiple processes is possible by working with an haploid state;

v) databases are upto date and the most complete of all the eukaryotic models.

S. cerevisiae has been regarded as the leading partner generating a wealth of biological knowledge on eukaryotic cells, especially in the study of mitochondrial genetics and biogenesis. For ageing research *S. cerevisiae* is a particularly well-suited model, to elucidate the central role of mitochondria in diseases. Five decades back, in 1956 Harman considered the involvement of the toxic side products of respiratory oxygen metabolism in ageing and in a number of human diseases (Harman, 1998) when he formulated the 'free radical theory of ageing' that also became known as the 'oxygen theory of ageing' (reviewed in Pan, 2011). According to this theory aging is caused by the cellular damage produced by highly reactive oxygen species with unpaired electrons, which are generated mostly in the mitochondrion at complexes I and III of the electron transport chain (reviewed in Longo and Fabrizio, 2012). Off late, studies on yeast have recently helped to uncover important new aspects of mitochondrial diseases as well as the role of mitophagy (Bolotin-Fukuhara et al., 2010).

However, other yeasts have also contributed to the knowledge bank specifically, such as *Schizosaccharomyces pombe* in the understanding of the cell cycle, *Kluyveromyces lactis* on the respiro-fermentative metabolism and *Hansenula polymorpha* on peroxysome biogenesis would be few examples (reviewed in Bolotin-Fukuhara et al., 2010).

2. Mitochondrial Models for Aging

The budding yeast, *Saccharomyces cerevisiae*, is a well-established model for studying mitochondrial function and aging. Unlike many higher organisms, budding yeast can endure lack of oxygen and do not require mitochondria for ATP production. Thus, it is an ideal system for exploring mitochondria's important roles besides energy production (Pan, 2011). Aging in yeast is described either as chronological or replicative aging. Chronological aging is defined as the time nondividing cells can survive and is typically measured as viability of cells during stationary phase. Replicative aging is defined as the number of daughter cells a mother cell can produce (Longo et al., 2012; Parrella and Longo, 2008). Stationary phase begins at the end of the postdiauxic phase between days 2 and 7, depending on the medium used in the experiment, and is characterized by lower metabolic rates and upregulation of stress resistance pathways. The yeast Choronological life span (CLS) assay was developed to complement the Replicative life span (RLS) assay,

by providing the ability to model aging of nondividing cells of higher organisms (Longo et al., 2012). A special case of chronological lifespan represents longtime survival under conditions with very low metabolic activity, as found at low temperature (Breitenbach et al., 2013). Using the yeast CLS system, researchers confirmed the importance of mitochondrial ROS in aging. Like other aging models, ROS detoxifying enzymes have important but complicated roles in CLS (Pan, 2011). Downregulating the PKA, TORC1, and Sch9 pathways extents chronological as well as replicative life span (Fabrizio et al., 2001; Longo et al., 1999; Powers et al., 2006), partially by increasing cellular protection against oxidative stress through activation of *SOD2*, encoding mitochondrial superoxide dismutase (Fabrizio et al., 2003). These results indicate that the longevity of yeast cells is regulated by the signaling and transcription mechanisms that are instrumental for transition to quiescence.

3. Yeast : Cell Growth

S. cerevisiae is a versatile organism because it can be grown both in batch and continuous cultures under a variety of conditions that allow modulation of its physiological response. In any given growth medium supporting cellular proliferation, a time window can be defined in batch cultures in which macromolecular syntheses and cell division are coordinated, so that any given intracellular parameter, such as protein or DNA distribution in the population, is constant. This so called "balanced exponential growth" or "Logarithimic phase", is usually preceded by a lag phase and followed by a transient phase or diauxic shift leading to stationary phase (Fig 1) (Busti et al., 2010).

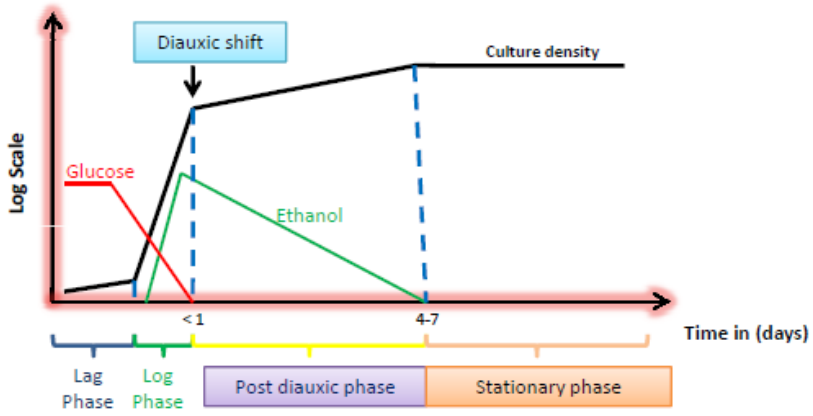


Figure 1: **Growth curve of *S. cerevisiae***. Growth phases of *S. cerevisiae* cultivated in rich medium supplemented with glucose. When quiescent, stationary phase cells are inoculated in fresh medium, they

exhibit an initial lag phase of variable length. During the subsequent exponential phase or also called log phase; cells proliferate rapidly by fermenting glucose to ethanol. When glucose becomes limiting, cells transiently arrest growth to adjust their metabolism from fermentative to the respiratory mode (diauxic shift): after the switch to respiration, cells restart growing at a reduced rate by slowly consuming the ethanol accumulated in the medium. When ethanol is also exhausted, cells cease dividing and enter into a quiescent state known as stationary phase that becomes deeper and deeper as cells spend more time in this state. Image adapted from (Busti et al., 2010; Gray et al., 2004; Herman, 2002)

4. Yeast -Stationary Phase

All living cells appear to be capable of exiting the normal cell cycle (proliferating state) and entering an alternative (resting) state termed quiescence or $G_{(0)}$. Almost two thirds of Earth bio mass represents quiescent microbes. Like all microorganisms, yeast cells spend most of their natural lifetime in a reversible, quiescent state. When nutrients become depleted then the orderly cessation of cell cycling and arrest in stationary phase is an important and necessary action. The biochemical changes which accompany stationary phase arrest have been known for many years. Cells originally growing on glucose switch from a fermentative metabolism using mainly glycolysis and forming ethanol, to a respiratory metabolism in which the ethanol formed in the earlier stages of growth are consumed using the TCA and glyoxylate cycles and the mitochondrial electron transport chain (reviewed in De Virgilio, 2012; Galdieri et al., 2010; Gray et al., 2004).

4.1 Definition

In laboratory conditions, quiescent yeast cells obtained by growing liquid cultures to saturation in rich media, usually for 5 to 7 days at 30°C.

The term “stationary phase” has been used to describe the state of saturated liquid cultures and the state of the constituent cells. Currently the nomenclature state that “stationary phase” is used to refer to the state of a saturated culture and the term “quiescence” is used to refer to the state of the constituent cells in such a saturated culture. Since it is not known if all cells in a stationary phase culture are quiescent, but the general assumption is that a substantial proportion are, including the daughter cells that were produced during the final doublings in the post-diauxic phase of culture growth (reviewed in Gray et al., 2004). Therefore the definition is reinstated as the state of a subpopulation of dense, unbudded, G_0-arrested daughter cells formed after glucose exhaustion (Allen et al., 2006).

4.3 Cell Quiescence Cycle

For years, the process of entering into quiescence has been represented as a reversible reaction, with exit from quiescence being simply the reverse of entry. Entry into quiescence is triggered when the cell senses for starvation for various nutrients such as carbon, ammonia, sulfate, phosphate, or biotin (reviewed in De Virgilio, 2012). In contrast, exit is triggered by quiescent cell sensing the presence of a carbon source. The processes of entry into and exit from quiescence to share any common intermediate states of the cell are still questionable. The entry, survival in, and exit from the quiescent state is now considered as a developmental process that, by analogy to the proliferative cell cycle, can be called the cell quiescence cycle. Hence entry into and exit from quiescence are distinct processes (reviewed in Gray et al., 2004).

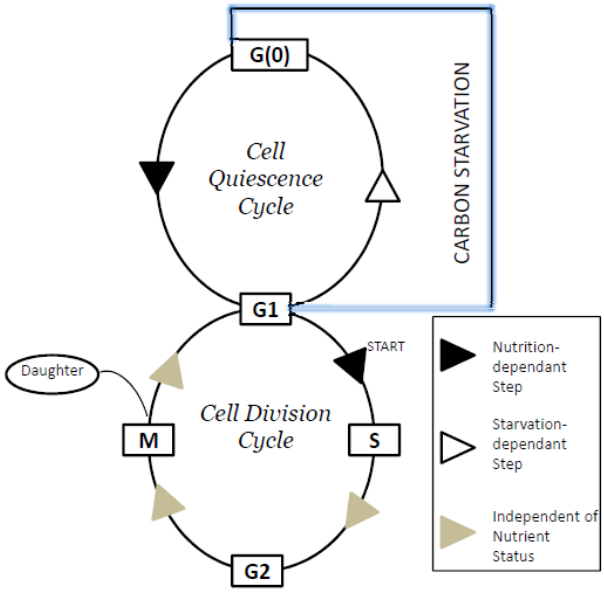


Figure 2: The relationship between cell quiescence cycle and cell division cycle. The cell quiescence cycle is the process by which nutrient limitation (e.g., carbon starvation in our reference case) causes exit from active proliferation (the cell division cycle) and triggers entry into the stable non-proliferating state, quiescence/G_0. Only after a favorable change in nutrient availability will a turn of the quiescence cycle be completed, since nutrient availability triggers exit from quiescence/G_0. The cell quiescence cycle and the cell division cycle intersect at the G_1 phase, where a cell has not yet committed to the cell division cycle. In the presence of sufficient nutrients and with no other influences, a G_1 cell will pass START, after which it is committed to completing a turn of the cell division cycle with production of a daughter cell. Slow depletion of an essential nutrient such as carbon will allow the completion of an ongoing cell division cycle but will

not allow passage through START. In this case of insufficient nutrient availability, the cell will enter the cell quiescence cycle. Image adapted from (Gray et al., 2004)

Once yeast cells have passed Start, they usually traverse all phases of the cell cycle until reaching Start again. If a decision is made not to proliferate, haploid cells preferentially arrest at Start and enter quiescence. In reality, each turn of either cycle changes the state of the starting cell: in the case of the cell cycle, the mother cell becomes one generation older (i.e., has reduced replicative capacity); in the case of the quiescence cycle, the cell also becomes older, again with respect to loss of replicative capacity (Fig 2).

The cell cycle results in a doubling of cell the number, whereas the quiescence cycle does not. The cell division cycle and the cell quiescence cycle intersect at the G_1 phase. In this phase, a cell can enter either the cell division cycle or the quiescence cycle. In the presence of ample food supplies (and other conditions permitting), a G_1 cell passes START and enters the proliferative cell cycle (Fig 2). The subsequent removal of nutrients does not generally hamper completion of the ongoing cell cycle, which is driven by internally controlled fluctuations in cyclin-dependent protein kinase activity. In the absence of a sufficient carbon source, a G_1 cell fails to pass START and enters the quiescence cycle. Unlike the proliferative cell cycle, the quiescence cycle does not turn under its own steam but cycles with changes in the environment: the lack of nutrients triggers entry into quiescence; the resupply of nutrients triggers exit (reviewed in Gray et al., 2004; Hartwell, 1974; Werner-washburne et al., 1996).

4.4 Signal transduction pathways regulating entry into the diauxic shift and stationary phase

The entry of yeast cells to the diauxic shift and stationary phase from the exponential phase is regulated by protein kinase A (PKA), TOR apparent negative regulators of the transition into quiescence; while Snf1, Protein Kinase C (PKC) signaling pathways apparent positive activators of the transition (Fig 3). These pathways signal changes in availability of nutrients during transition from the exponential phase when glucose is abundant to the diauxic shift when cells utilize ethanol and to the stationary phase when no carbon source is available (reviewed in Galdieri et al., 2010; Gray et al., 2004). In both these transitions cells use an oxidative metabolism producing high amounts of ROS, in turn suffering oxidative stress (reviewed in De Virgilio, 2012; Gray et al., 2004).

However, a correct and tight regulation of specific signaling pathways and their target transcriptional factors, ensure a prolonged life extension. Entering diauxic shift and stationary phase induces a genetic program by which, TOR activity is inhibited as the type 2A phosphatase (PP2A) is dissociated from TORC1 complex, (Di Como and Arndt, 1996; Jacinto et al., 2001) and Sch9 gets inactivated. These processes trigger the inactivation of ribosomal biogenesis and the translocation to the nucleus of the transcription factor Rim15 (Pedruzzi et al., 2003; Urban et al., 2007).

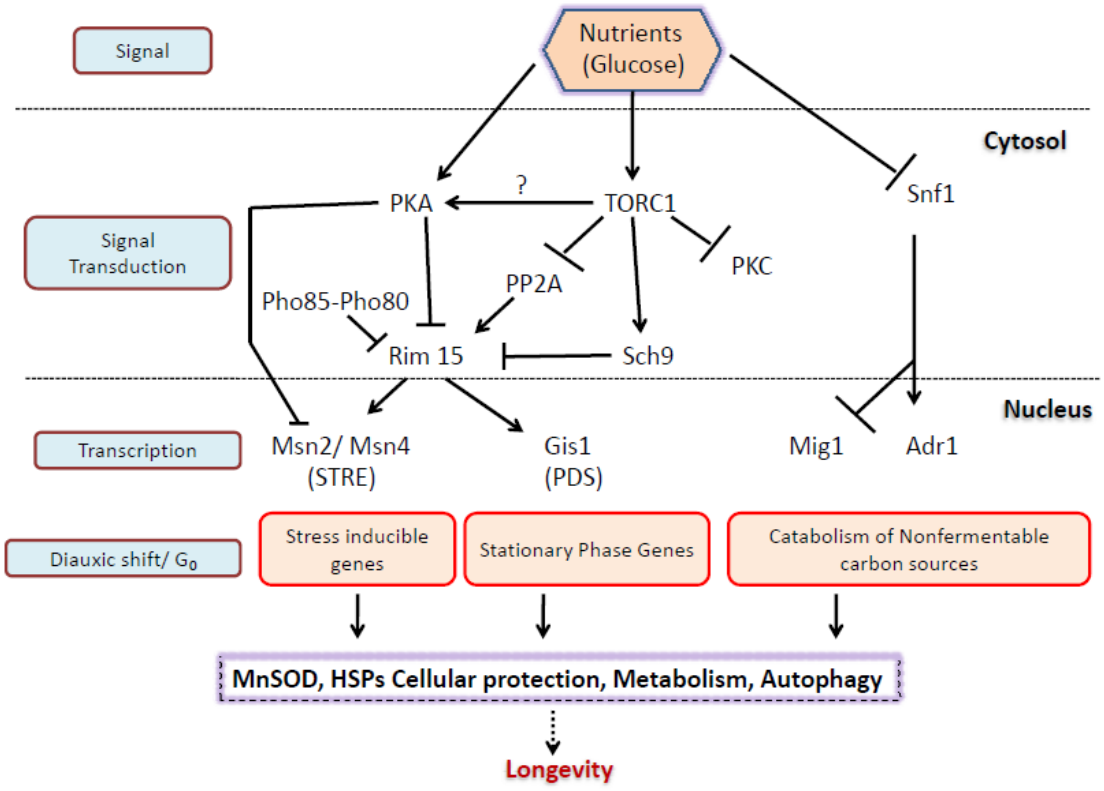


Figure 3: Overview of signaling pathways that control transcription during transition from exponential growth to diauxic shift and stationary phase. PKA and TORC1 are active in the presence of nutrients and act to repress aspects of quiescence. When cells are starved of carbon, both pathways are downregulated and they converge to negatively regulate Rim15 for transition to diauxic shift and quiescence. The key targets of this pathway are transcriptional factors Msn2/Msn4 and Gis1. Inactivation of the TORC1 causes activation of PKC, leading to some characteristics of quiescence such as a remodeled cell wall. Snf1 is active when glucose is exhausted and regulates expression of genes involved in catabolism of non-fermentable carbon sources and contributes to the switch from fermentative to respiratory metabolism that is essential for entry into quiescence. Abbreviations used, STRE- Stress Response Element and PDS- Post Diauxic Shift. Image apated from (Galdieri et al., 2010; Kaeberlein, 2010; Longo et al., 2012)

Mutations in RAS and Sch9 signaling pathways extend the CLS and also the resistance to oxidative stress in *S. cerevisiae*, in part through the activation of the general stress response Msn2/Msn4 and Gis1 by the protein kinase Rim15 (Fabrizio et al., 2001; Fabrizio et al., 2003; Wei et al., 2008).

When cells enter into quiescence PKA/cAMP pathway is inactivated, converging in the inhibition of ribosomal genes and ribosomal biogenesis (Powers et al., 2006) and upregulation of both Msn2/Msn4 and Rim15 (Cardenas et al., 1999; Hasan et al., 2000; Powers et al., 2006). In fact, cells with high PKA activity fail to enter properly into stationary phase in part because they are unable to activate the stress response. The activity of the PKC pathway is essential for cells to enter in quiescence, this is a mechanism tightly connected to TORC1 inhibition. Yeast Pho85 is a cyclin-dependent kinase (Cdk), involved in the regulation of phosphate metabolism in function of external phosphate availability and also other cellular processes, including the sensing of other environmental changes and cell cycle control (Huang et al., 2007a and Huang et al., 2007b). Downstream targets of Pho85 include the Pho4 transcription factor controlling expression of the PHO genes, the Gcn4 transcription factor controlling amino acid biosynthesis genes, the cyclin Cln3 involved in cell cycle control, and the Rim15 protein kinase involved in different nutrient regulated pathways, controlling among others the activity of the transcriptional activators, Msn2 and Msn4 (Conrad et al., 2014). The Snf1 kinase (orthologous of mammalian AMPK) is a positive regulator of quiescence and is required for cell growth in conditions of glucose depletion because SNF complex activates genes involved in the metabolism of alternative carbon sources (Broach, 2012).

4.5 Physiological changes and characteristics during diauxic shift and stationary phase

Stationary cells are also more resistant to heat and osmotic shocks, as well as to treatment with toxic drugs. The cellular responses initiated at the diauxic transition include the transcriptional induction of genes whose products are involved in respiration, fatty acid metabolism, and glyoxylate cycle reactions, and, likely as a consequence of the on-setting respiratory activity, of genes encoding antioxidant defences that allow scavenging and/or the destruction of reactive oxygen species (ROS) (Galdieri et al., 2010).

It is shown that stationary phase yeast cultures (defined as 47 days old) exhibit a complex, heterogeneous community structure, composed of least two different cell types: quiescent and non-quiescent cells (Allen et al., 2006). Quiescent cells are dense, unbudded, replicatively younger, and are able to synchronously re-enter mitotic cell cycle. Non-quiescent cells are less dense, rapidly lose their ability to reproduce or in other terms they have reduced ability to re-enter mitotic cell cycle; gradually accumulate ROS, exhibit genomic instability, and become senescent or apoptotic (Allen et al., 2006; Davidson et al., 2011; De Virgilio, 2012; Galdieri et al., 2010).

The final characteristics of quiescent cells reflect their integrated responses and adaptations triggered by progression through distinct, sequential physiological phases, which critically affect the cells' life span and their ability to withstand environmental stresses. The earliest of these phases begins when nearly half of the initial glucose has been consumed and they start accumulating storage molecules glycogen, trehalose, triacylglycerols, and probably also polyphosphate and induce autophagy (Davidson et al., 2011; De Virgilio, 2012).

4.6 Transcription

Like other eukaryotes, three distinct DNA-dependent RNA polymerases that transcribe different sets of genes is also contained in yeast cells. RNApolymerase I (Pol I) transcribes rDNA into 35S precursor ribosomal RNA. RNA polymerase II (Pol II) transcribes protein-encoding genes, and RNA polymerase III (Pol III) synthesizes 5S ribosomal RNA and tRNAs (Galdieri et al., 2010). At least 25% of the yeast genome is involved in transcriptional reprogramming during the diauxic shift, postdiauxic shift (PDS), and stationary phases and is controlled by various signaling pathways (De Virgilio, 2012).
The transcription by all three RNA polymerases is under the control of the signal transduction pathways such as TOR, PKA, and Snf1, in diauxic shift and especially during stationary phase transcription significantly decreases. During the diauxic shift, the cells reprogram their transcription machinery, which results in increased expression of hundreds of genes (De Risi et al., 1997; Radonjic et al., 2005); where majority of them are targets of Msn2p/Msn4. When cells reach the stationary phase, the number of upregulated genes decreases to about 100, whereas the majority of genes are significantly downregulated (Radonjic et al., 2005). Most of the genes that are upregulated in the stationary phase

experience increased transcription already at the diauxic shift, and only very few genes display increased transcription only after passage through diauxic shift.

Rim15

As addressed above, TORC1 and Sch9 inhibit translocation of Rim15 from the cytoplasm to the nucleus. To further inhibit Rim15 function, PKA has also been demonstrated to effectively reduce Rim15 activity (Pedruzzi et al., 2003). Inhibition of Rim15 by TORC1, Sch9, or PKA activity prevents the cells from entering the G_0 program as long as the nutrient conditions are favorable (Pedruzzi et al., 2003). The targets of Rim15 include three transcriptional factors: Msn2, Msn4, and Gis1. These factors mediate the Rim15 effect on transcription during a diauxic shift. The functions of Msn2/Msn4 and Gis1 partially overlap, and deletion of all three genes causes synthetic growth defect during growth on nonfermentable carbon sources (Galdieri et al., 2010)**.**

Msn2/Msn4

Msn2, together with its partially redundant homolog Msn4, are zinc-finger transcription factors that play an important role in transcriptional response to starvation and other forms of environmental stress (Estruch, 2000; Estruch and Carlson, 1993; Martinez-Pastor et al., 1996). The Msn2/Msn4 transcription factor activates genes containing the stress response element (STRE: CCCCT), in response to several stresses. Transcriptional activation of Msn2 dependent genes (like *HSP12, CTT1, TPS2, SSA3* and *HSP26*) is very complex. Msn2 is regulated by nuclear translocation (Beck and Hall, 1999; Chi et al., 2001; Görner et al., 1998; Görner et al., 2002) or by increased binding of Msn2 to the STRE elements in the promoters of stress-responsive genes (Hirata et al., 2003).

Genetic or environmental conditions that decrease the activity of the RAS/cAMP pathway increase resistance to oxidative stress, in part, by the concomitant induction of Msn2/Msn4 function (Hasan et al., 2002). Recent evidence suggest that apart from RAS/PKA, Msn2/Msn4 is tightly regulated by several signaling pathways where it is down regulated by TOR, CWI, HOG and positively regulated by Snf1 (reviewed in Conrad et al., 2014; Petkova et al., 2010). Msn2/Msn4 also favors life extension by inducing the expression of longevity genes (Medvedik et al., 2007). In conditions of caloric restriction Msn2/Msn4 mediates life extension in conjunction with TOR inactivation (Fabrizio et al., 2001).

Gis1

GIS1 was first identified as a high copy number suppressor of the defect to express SSA3 in *rim15* cells. Epistasis analysis indicates that Gis1 acts in the PKA pathway downstream of Rim15. Gis1 binds to the postdiauxic shift (PDS) element that is present in promoters of

genes that are induced by glucose exhaustion at the diauxic shift (Pedruzzi et al., 2000). Msn2p/Msn4p and Gis1p are not functionally equivalent and are able to differentiate between similar STRE and PDS promoter elements (Pedruzzi et al., 2000). Expression of Gis1 dependent genes is regulated by the PKA, Sch9, and TORC1 pathways and depends on Rim15 (Cameroni et al., 2004; Pedruzzi et al., 2000, Pedruzzi et al., 2003; Roosen et al., 2005; Zhang et al., 2009). The mechanism of Gis1 regulation by these or other pathways has not been elucidated yet. Recent data indicate that the activity of Gis1 is regulated by proteasome-mediated proteolysis to ensure that all Gis1 dependent transcription is under Rim15 control (Zhang and Oliver, 2010).

4.7 Translation

In exponentially growing yeast cell, protein synthesis consumes a huge amount of the energy. ≈60% of the total transcription is respresented by rRNA transcription while ribosomal protein synthesis represents ≈15% of total translation. During the transition into the quiescent state, is coordinated downregulation of genes that and global shutdown of the transcription of genes coding for the proteins in both subunits of the ribosome. Quiescent cells maintain excess translational capacity and protein synthesis continues, despite this shutdown of ribosomal protein biosynthesis, at much reduced rates ≈ 0.3% of the rate in proliferating cells. A few proteins have so far been identified that are selectively synthesized after entry into quiescence such as Hsp26 or of the Sno and Snz families as they are required for pyridoxine biosynthesis and also for two reasons one may be important cofactors for metabolism in quiescent cells and another may function as antioxidant compounds, providing a defense against endogenously generated reactive oxygen species (De Virgilio, 2012; Galdieri et al., 2010; Gray et al., 2004).

It has been reported that some of the molecular pathways that couple nutrient availability to translation initiation in yeast converge on Ser^{51} of the α-subunit of the eukaryotic translation initiation factor 2 (eIF2 α) (De Virgilio, 2012). The eIF2α –kinase, Gcn2 and eIF2 α -phosphatases (eIF2α-PPs) that include the type I protein phosphatase (PP1) Glc7 and the type 2A protein phosphatase (PP2A) related Sit4 tightly control the levels of eIF2α -Ser^{51} phosphorylation (Wek et al., 1992; Cherkasova et al., 2010). Additionally, several major nutrient signaling kinases including Gcn2, Snf1, and TORC1 have be known to regulating the levels of eIF2α phosphorylation and hence contribute to the fine tuning of translation initiation (De Virgilio, 2012).

4.8 Exit from quiescence

Resuspending quiescent cells in media containing all necessary nutrients such as carbon sources stimulates exit from quiescence and completion of the quiescence cycle. Stimulated i.e, refed; quiescent cells lose thermo tolerance, become sensitive to cell wall-degrading enzymes, and display increased rates of RNA and protein synthesis. Internal carbohydrate stores such as glycogen and trehalose are also mobilized. Ultimately, stimulated cells resume cell growth and begin proliferation, i.e., enter the proliferative cell cycle (Werner-Washburne et al., 1993). The question that always remains is how quiescent cells sense the presence of nutrients. One possibility is that cells sense the presence of essential nutrients that may initiate the exit from quiescence or another possibility is that cell may sense to a selective list of nutrient which render them to exit from quiescent phase. However, the mechanism that targets RNA Pol II to the promoters of genes needed for exit from the stationary phase is not known (Radonjic et al., 2005). Following the sensing of nutrients there is wide range of transcriptional changes; proteome changes (see nutrient sensing detailed below).

5. Mitochondria, Respiration and redox balance

The postdiauxic phase is the period that begins approximately 24 hr after initial inoculation when cells deplete extracellular glucose, dramatically reduce growth, and switch to a mitochondrial respiratory mode of metabolism dependent on the ethanol generated during fermentation (Werner-washburne et al., 1996). Unlike proliferative cells, quiescent yeast cells cannot dilute out damage to proteins and DNA by rapid synthesis of new macromolecules and cell division. Hence quiescent cells are potentially more vulnerable to internal and external stresses than are proliferating cells (Gray et al., 2004). Mitochondria is regarded as “the powerhouses of the cell” because they link the energy releasing activities of electron transport and proton pumping with the energy conserving process of oxidative phosphorylation, to harness the value of foods in the form of ATP. Mitochondrial respiration appears to be a major source of energy for quiescent cells. Unfortunately, respiration produces large amounts of reactive oxygen species, whose toxic effects must be countered if viability is to be maintained (Gray et al., 2004; Guerrero-Castillo et al., 2011). The control of mitochondrial biogenesis is complex, requiring coordinated transcription of a large number of nuclear and mitochondria encoded genes. In

addition, there must be coordinated control of the synthesis, import, and incorporation of proteins and lipids to existing mitochondria alongside appropriate replication of the mitochondrial DNA (mtDNA) (Leadsham and Gourlay, 2010).

Over the time, the picture of the respiratory chain/oxidative phosphorylation system (OXPHOS) has been viewed out by the description of the four major protein complexes that facilitate this flow of electrons and protons. It is known that both complexes I (an NADH dehydrogenase) and II (a succinate dehydrogenase) transfer electrons from TCA cycle-generated NADH and $FADH_2$ to ubiquinone (also called coenzyme Q), then to complex III (ubiquinone oxidoreductase), then to cytochrome *c*, then to complex IV (cytochrome *c* oxidase), and finally to molecular oxygen, ultimately producing water. At the same time, protons cross the inner membrane at complexes I, III, and IV to generate the electrochemical proton gradient that will be used by the F_1F_0-ATP synthase (called complex V, but technically not a member of the respiratory chain) (Schon and Dencher, 2009). This so called mitochondrial membrane potential is dissipated through ATP synthase to generate ATP.

The efficiency of this system varies when electrons enter or exit the respiratory chain at different enzymes or when the H^+ gradient is used by secondary pumps for the active transport of proteins, ions and metabolites (Guerrero-Castillo et al., 2011). Three of the four respiratory complexes in an orthodox respiratory chain are proton pumps. These enzymes oxidize substrates, transferring electron(s) to the next acceptor in the chain and expelling H^+(s) to the intermembrane space. Recycling of the electron within a given pump often results in H^+/e^- stoichiometries higher than 1 (Brandt 2006; Hosler et al., 2006). This high efficiency comes at an expense, as redox reactions involve several steps where incomplete reductions transiently convert coenzymes into reactive free radicals (Drose and Brandt 2008; Kushnareva et al. 2002). Therefore, when the mitochondrial ADP concentration drops, the rate of electron flux through the respiratory chain decreases (State IV respiration) and mitochondria become an important source of superoxide and other reactive oxygen species (ROS) (Chen et al. 2003).

Mitochondria convert nutritional energy more effectively into readily available energy, i.e. ATP, than non-oxidative metabolism of carbohydrates and some amino acids does. E.g., while glycolytic metabolism of one mol of glucose generates 4 mols of ATP only, its oxidative metabolism generates 30 mols of ATP. Hence, and as indicated by findings in

yeast (Lin et al., 2001) and *C. elegans* (Schulz et al., 2007), decreased glucose availability would induce mitochondrial metabolism to increase OXPHOS, aiming to maintain intracellular ATP supply. In addition to ATP, mitochondria play an essential role in the synthesis of lipids and membrane compounds such as ceramides (Aerts et al., 2009; Salminen et al., 2012).

As seen above, one of the markers of the cellular energy state is the mitochondrial membrane potential, whose changes may reflect changes in the respiration rate, ATP production, ROS generation (Laun et al., 2001), and which plays also an important role in apoptosis as its dissipation is one of the crucial steps of apoptosis (Ricci et al., 2003). Another important functional marker, the mitochondrial respiration rate, was observed to change in different eukaryotic cell types during ageing. Mitochondrial morphology changes during cell ageing. Mitochondria frequently undergo fission and fusion that regulate their shape, size and number. They are very dynamic especially in the exponential phase of yeast growth where fission and fusion events occur every few minutes (Nunnari et al., 1997). The two processes must be balanced for optimal mitochondrial function. It was observed that replicatively older cells (10–12 generations) contain mainly fragmented mitochondrial network, while the tubular morphology dominates in younger cells (Scheckhuber et al., 2007).

As mentioned earlier, during mitochondrial respiration, variety of reactive oxygen species (ROS) are generated within cells which damages cellular constituents like DNA, lipids and proteins. Yeast cells respond to oxidizing agents by inducing a series of specific antioxidant mechanisms including the synthesis of glutathione and the production of enzymes (example: superoxide dismutase, catalases, glutathione peroxidases etc) which detoxify oxidants or repair the damage caused by them (Jamieson, 1998). The same mechanism is responsible for retaining some capacity of responding to oxidative stress in quiescent cells (Cyrne et al., 2003) via their adaptive response to mitochondrial respiratory metabolism-derived ROS production (Greetham et al., 2010). This is in agreement with the fact, that respiratory deficient stationary-phase yeast cells are hypersensitive to oxidants (Jamieson, 1992). Despite the apparent negative effects of mitochondrial respiration-derived ROS, efficient respiration appears to play a positive role in life span extension in certain mutant backgrounds (Bonawitz and Shadel, 2007; Lavoie and Whiteway, 2008) and may be critical for the survival of quiescent cells, possibly by maintaining the redox balance and/or NAD^+/$NADP^+$ pools (Davidson et al., 2011).

Accordingly, enhanced expression of the cytosolic copper, zinc-superoxide dismutase (Cu,Zn-SOD) Sod1, and the mitochondrial manganese-superoxide dismutase (Mn-SOD) Sod2 during adaptation to efficient respiratory metabolism (for instance during the diauxic shift phase) is critical for maximal stationary-phase survival (or CLS) (Weinberger et al., 2010).

Recent evidence suggests that calorie restriction (CR) and specifically reduced glucose metabolism induces mitochondrial metabolism to extend life span in various model organisms, including *Saccharomyces cerevisiae, Drosophila melanogaster, Caenorhabditis elegans* and possibly mice. In conflict with Harman's free radical theory of aging (FRTA), these effects may be due to increased formation of ROS within the mitochondria causing an adaptive response that culminates in subsequently increased stress resistance assumed to ultimately cause a long-term reduction of oxidative stress. This type of retrograde response has been named mitochondrial hormesis or mitohormesis, and may in addition be applicable to the health-promoting effects of physical exercise in humans and, hypothetically, impaired insulin/IGF-1-signaling in model organisms (Ristow and Zarse, 2010).

6. Nutrient sensing

S. cerevisiae has the ability to control growth in response to changes in nutrient availability. Stationary cells, unlike exponential cells, are able to survive for extended periods of time without nutrients; however, they are able to mount a similar transcriptional response to different stresses. In order for cells entering the stationary phase to develop these ''resistance'' characteristics, they have to properly sense imminent depletion of a key nutrient and uniformly arrest in the unbudded state. Not all nutritional stresses elicit this developmental pathway into quiescence. When auxotrophic cells are starved for the required amino acid, they fail to arrest uniformly as unbudded cells and rapidly lose viability (Saldanha et al., 2004).

Yeast cells grow on a wide variety of compounds as sources of energy and as carbon containing precursors of anabolic metabolism and biomass accumulation. Yeast cells preferentially consume glucose or fructose to other mono-, di-, and trisaccharides, such as sucrose, raffinose, or trehalose. They also prefer any fermentable carbon source over any other source, such as glycerol, ethanol, or acetate, that has to be catabolized by oxidative

phosphorylation (Broach, 2012). Glucose has been shown to represses transcription of genes required for initial catabolism of less favorable sugars and of genes encoding components of the electron transport chain and other mitochrondrial proteins. Glucose repression of mitochrondrial function is the basis of the Crabtree effect, where by *S. cerevisiae* ferments glucose to produce ethanol even under aerobic conditions (Broach, 2012). The shift of cells from glycerol to glucose have shown changes in transcription of 40% of genes required for mass accumulation, such as ribosomal protein and ribosomal biogenesis genes, by more than two fold activation and also in repression of genes associated with stress response or required for use of alternate carbon sources (Broach, 2012; Santangelo, 2006). When yeast are starved for macronutrients, such as glucose, nitrogen, or phosphorous, they produce a stable arrest in growth and cell division and become quiescent, with cell wall thickening, reduced transcription and translation, and increased stress tolerance (Conway et al., 2012). Restoration of the limiting nutrient quickly restores growth. Moreover, during glucose deprivation, not only the expression of many genes involved in protein synthesis declines, but also the entire translational machinery is inhibited within minutes after initiation of glucose starvation (Rødkaer and Faergeman, 2014; Smets et al., 2010). In addition, glucose depletion/repletion very rapidly alters the levels of more than 2000 transcripts by at least 2-fold, a large portion of which are involved with either protein production in growth or stress responses in starvation (Conway et al., 2012; Gray et al., 2004). There are numerous specialized cellular receptors in yeast which continuously sense and react to the availability of glucose. When these receptors are stimulated and also along with the transcriptional and metabolic effect of glucose involves various signalling networks such as the Ras /PKA, Gpr1/PKA, Snf3/Rgt2, Snf1/Mig1, Hap and Sch9 which in concert constitute a complex regulatory network (Conrad et al., 2014). The signal is highly specific for which these pathways and networks cross communicate with each other and are also regulated at several steps and by numerous different regulators (Conrad et al., 2014; Rødkaer and Faergeman, 2014). For example, glucose effects on biosynthetic capacity and stress responses are mediated by the protein kinase A pathway, while repression of genes involved in use of alternative carbon sources are mediated predominantly by Snf1 and tuning of the glucose uptake machinery to match glucose levels is effected through the Rgt/Snf3 circuit (Broach, 2012). Eventhough the core components of most of these pathways have presumably been identified, the mechanism by which glucose activates some of these pathways is poorly understood.

7. Cell Wall Intergrity Pathway

7.1 Overview of CWI signaling

During growth and morphogenesis, and in response to environmental challenges, the cell wall is remodeled in a highly regulated and polarized manner, a process that is principally under the control of the cell wall integrity (CWI) signaling pathway (Levin, 2011).

The cell wall integrity pathway in budding yeast involves a MAP kinase cascade that participates in transducing extracellular signals for cell-wall, heat shock, hypo-osmotic, nutritional, oxidative, pH stresses and DNA damage (Heinisch and Lorberg, 1999; Levin, 2005; Serrano et al., 2006; Vilella et al., 2005). The PKC1-MAPK pathway is integrated by several cell-wall proteins that are putative cell membrane receptors of different stimuli – Wsc1-3, Mid2 and Mtl1 (Fig 4).

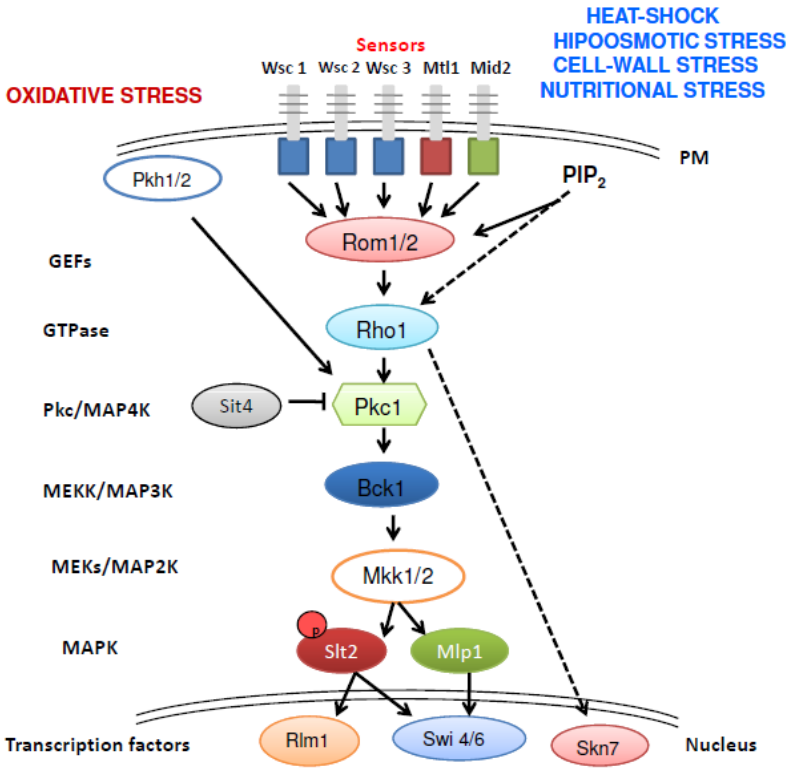


Figure 4: Cell wall integrity (PKC1-MAPK) pathway. Extracellular signals for cell-wall, heat shock, hypoosmotic, nutritional, pH and oxidative stresses are sensed at the plasma membrane by a number of cell surface sensors: Wsc1-3, Mid2 and Mtl1 that are highly glycosylated. Together with PIP_2, recruit Rom2, the

GEF to the plasma membrane, the sensors stimulate nucleotide exchange of the GTPase Rho1 and thereby activate it. Rho1 activates five effectors, including the Pkc1-MAP kinase cascade. Pkc1 activates a MAP kinase module composed of: Bck1, Mkk1-2, Slt2/Mpk1and Mlp1. Sit4 protein phosphatase negatively modulates the activity of Pkc1. Several MAP kinase phosphatases downregulate Slt2/Mpk1. Two transcription factors, Rlm1 and SBF (Swi4-Swi6), are targets of the Slt2/Mpk**.** Image adapted from (Levin, 2005; Levin, 2011; Vilella et al., 2005)

They transmit signals to Rom2, which activates the G protein Rho1. In turn, Rho1 activates, among others, the protein kinase C Pkc1 (Levin, 2011) (Fig 4). Pkc1 activates a mitogen-activated protein kinase module composed of: the MAPKKK Bck1, which phosphorylates the redundant MAPK kinases Mkk1 and Mkk2, and they together activate the last kinase of the module, Slt2/Mpk1. Slt2/Mpk1 dual phosphorylation correlates to activation of the CWI pathway (de Nobel et al., 2000; Martín et al., 2000). Slt2/Mpk1 regulates the function of Swi6 (a transcription factor involved in cell cycle progression) and Rlm1 (a transcription factor that regulates the expression of a subset of genes involved in the cell-wall remodelling) (Fig 4).

7.4 CWI Pathway Architecture in *S.cerevisiae*

7.4.1 Cell surface sensors: Wsc1-3, Mid2 and Mtl1

Members of a family of cell-surface sensors, which detect and transmit cell wall stress to Rho1 through a set of GEFs, are principally responsible for activation of CWI signaling (Rodicio and Heinisch 2010). These sensors include Wsc1 (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998), Wsc2, Wsc3 (Verna et al., 1997), Mid2, and Mtl1 (Ketela et al., 1999; Rajavel et al., 1999). Mtl1 shows 50% aminoacid sequence identity with Mid2 (Rajavel et al., 1999; Ketela et al., 1999). All five are plasma membrane proteins whose overall structures are similar in that they possess short C-terminal cytoplasmic domains, a single transmembrane domain, and a periplasmic ecto domain rich in Ser/Thr residues (Ketela et al., 1999; Lodder et al., 1999; Rajavel et al., 1999; Philip and Levin, 2001). The Ser/Thr-rich regions are highly *O*-mannosylated, probably resulting in extension and stiffening of the polypeptide. Protein *O*-mannosylation is a vital type of glycosylation that is conserved among fungi, animals, and humans and its defects interfere with cell wall integrity and ER homeostasis in yeast (Loibl and Strahl, 2013). In *S. cerevisiae*, the analysis of viable *pmt* mutants revealed that *O*-mannosyl glycans are vital for the localization stability, and/or function of various secretory and membrane proteins and

therefore affect diverse cellular processes such as cell wall integrity, cell polarity, mating and filamentation (Loibl and Strahl, 2013; Petkova et al., 2012). Therefore, these proteins have been proposed to function as mechanosensors that act as rigid probes of the extracellular matrix (Rajavel et al., 1999; Philip and Levin, 2001). *O*-mannosylation of the Mid2 and Wsc1 ecto-domains requires either Pmt2 or Pmt4 (Philip and Levin, 2001; Lommel et al., 2004), members of a seven isoform family of proteins that catalyze the first step in protein *O*-mannosylation (Strahl-Bolsinger et al. 1999). Consistently, a double *pmt2pmt4* mutant undergoes cell lysis in the absence of osmotic support (Gentzsch and Tanner 1996). This defect is suppressed by overexpression of Pkc1, Wsc1, or Mid2 (Lommel et al., 2004), revealing that *O*-mannosylation of the sensors, although important, can be bypassed. Recently, Mtl1 is also found to be highly *O*-mannosylated and *N*-glycosylated transmembrane protein (Petkova et al., 2012). Pmt2 is the main protein *O*-mannosyltransferase isoform capable of specifically modifying Mtl1. While Pmt1 and Pmt6 affect as well Mtl1 *O*-mannosylation. Protein *O*-mannosylation is essential in oxidative stress response, through at least Mtl1. Mtl1 *O*-mannosylation by Pmt2 is required to elicit cellular responses to TOR inhibition (Petkova et al., 2012). In addition these authors also describe, Mtl1 *O*-mannosylation confers its proper localisation to the cell periphery, the septum and the tip of the shmoo. Similarly, Mid2, unlike Wsc1, is additionally *N*-glycosylated near its N terminus (Hutzler et al., 2008). In contrast to *O*-mannosylation, Mid2 signaling is affected by this modification, rather than its stability or localization.

Aside from the gross structural similarities between the two subfamilies of cell-wall sensors, their sequences are not conserved. The Wsc proteins possess an N-terminal cysteine-rich region, termed the WSC domain, which is absent from Mid2 and Mtl1. Mutation of the conserved cysteine residues in Wsc1 destroys its function (Heinisch et al., 2010). Wsc 1-3 proteins are required for the viability of yeast cells during vegetative growth under various stress conditions, including heat, treatment with DNA-damaging drugs. Among the WSC family members, Wsc1 plays the major role in maintaining cell wall integrity. Deletion of *WSC1* results in cell lysis at elevated growth temperatures, whereas deletion of *WSC2* and *WSC3* does not cause obvious cell lysis but exacerbates the defect when combined with *wsc1* (Gray et al., 1997; Verna et al., 1997). The phenotype is suppressed by overexpression of Rho1, Rom2, or Pkc1. Consistent with the importance of Wsc1 for survival of thermal stress, the *wsc1* is deficient in Slt2/Mpk1 activation upon

heat shock (Gray et al., 1997; Verna et al., 1997). Like most other components of the CWI, during vegetative growth Wsc1 localizes to sites of polarized cell growth (Delley and Hall, 1999; Huh et al., 2003; Straede and Heinisch, 2007). In contrast to this, Mid2 (*M*ating *I*nduced *D*eath *2*) is uniformly distributed across the plasma membrane during growth (Ketela et al. 1999; Rajavel et al. 1999; Straede and Heinisch 2007).

However, consistent with the importance of Mid2 during pheromone-induced morphogenesis, this sensor becomes enriched at the tips of mating projections (Hutzler et al. 2008). Upon treatment with mating pheromone *mid2* fails to activate the CWI at the onset of morphogenesis and consequently dies (Ketela et al., 1999; Rajavel *et al.,* 1999). Additionally, *mid2* is not temperature sensitive for growth but is partially impared for Slt2/Mpk1 activation in response to heat shock, particularly in double deletion with *mtl1*. *MID2* was isolated as a multicopy activator of a Skn7-LexA-dependent transcriptional reporter, suggesting that activation of CWI signaling can stimulate transcriptional activation by Skn7 (Ketela et al., 1999). Despite their discrete functions Wsc1 and Mid2 serve partially overlapping roles in CWI signaling. Overexpression of *WSC1* partially rescues the pheromone-induced cell death of *mid2* (Rajavel et al., 1999) and conversely, overexpression of *MID2* suppresses the temperature sensitivity of *wsc1* (Ketela et al., 1999; Rajavel et al., 1999). In addition, the simultaneous deletion of *MID2* and *WSC1* results in severe cell lysis at all temperatures in the absence of osmotic support (Ketela et al., 1999; Rajavel et al., 1999).

For the first time, Mtl1 (*M*id2 *T*wo *L*ike *1*) has been described as a cell-wall sensor of oxidative stress based on the following evidence: the induction of Slt2/Mpk1 phosphorylation is severely impared in *mtl1,* the *mtl1* mutant cells are sensitive to diamide and *mtl1* cell viability is rescued by increasing the level and the activity of the Pkc1 (Vilella et al., 2005). *MTL1* was identified as a suppressor of *rgd1* mutants, with Rgd1 being a GTPase-activating protein of Rho3 and Rho4 (De Bettignies et al., 2001) and also as a multicopy suppressor of Rho1 (Sekiya-Kawasaki et al., 2002). The mutant *mtl1* cells display no growth defect at temperatures up to 39°C (Rajavel et al., 1999). Loss of *MTL1* does not result in pheromone-induced cell death, nor enhance the sensitivity of *mid2*. However, overexpression of *MTL1* partially suppresses the pheromone sensitivity of *mid2* mutant (Rajavel et al., 1999; Ketela et al., 1999). While *mtl1* mutant is sensitive to caffeine to relative extent, *mid2* cells are mildly more susceptible than wild-type cells, the double *mtl1mid2* mutants show strong sensitivity to this drug. The phenotype is

suppressed by addition of 1M sorbitol in the growth media or by overexpression of Wsc2. However, multicopy *WSC1, RHO1, PKC1, BCK1,* and *MPK1/SLT2* do not bypass this phenotype (Ketela et al., 1999). Recently Mtl1 was found to be involved in oxidative stress as its function was vital in transcriptional activity of Msn2/Msn4 to activate general stress response for cell tolerance against the applied stress (Petkova et al., 2010a).

7.4.2 The GEF Rom2, and regulators of Rho1

It has been demonstrated that the cytoplasmic C-terminal domains of Wsc1 and Mid2 interact with Rom2 GEF (Philip and Levin, 2001) and these interactions stimulate the nucleotide exchange of the GTPase Rho1, thereby activating it. It is unclear whether interaction of Rom2 with a cell wall sensor is solely to further facilitate its recruitment near membrane-anchored Rho1 or has additional activating functions. At least with regard to Wsc1, this interaction is mediated by the Rom2 N-terminal domain. This domain is distinct from the Rho1-interacting domain, suggesting that the GEF can interact simultaneously with a sensor and with Rho1 (Philip and Levin, 2001). Mutational analysis of the cytoplasmic domain of Wsc1 has identified two regions important for Rom2 interaction. These regions are separated by an inhibitory region, which includes a cluster of seryl residues that appear to be phosphorylated. Wsc1 interaction with Rom2 is negatively regulated by phosphorylation, however that is not the only mean of Wsc1 regulation (Vay et al., 2004). Mtl1 exhibits two-hybrid interactions with Rom2, through its cytoplasmic domain (Petkova et al., 2012). These authors confirm with Co-immunoprecipitation experiments to demonstrate that Mtl1 physically interacts with Rom2. They propose a model in which the signalling of oxidative stress from Mtl1 is basically transmitted to the upper elements of the CWI pathway and from there to the general stress response (Petkova et al., 2012).

The Rho1 cycle is regulated both by GTPase-activating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs) acting in opposition. *S. cerevisiae* possesses 11 Rho-GAPs. Out of which, 4 have been shown to act on Rho1 both in vitro and in vivo: Bem2, Sac7, Bag7, and Lrg1 (Peterson et al., 1994; Schmidt et al., 1997; Cid et al., 1998; Martín et al., 2000; Roumanie et al., 2001; Watanabe et al., 2001; Schmidt et al., 2002) (Fig 5). For example, Lrg1 is dedicated to regulation of GS, Bem2 and Sac7 are the only GAPs that regulate the Pkc1-MAPK pathway, Bag7 and Sac7 collaborate to control the actin cytoskeleton.

Rho1 is stimulated primarily through the action of the Rom1 and Rom2 GEFs, which provide a redundant function in the activation of Rho1 (Ozaki et al., 1996). Loss of *ROM2* function results in temperature-sensitive growth, whereas loss of both *ROM1* and *ROM2* is lethal (Ozaki et al., 1996). A *rom2* mutant has been shown to retain 60% of GTP binding to Rho1, which suggests that the coordinated action of other GTPase exchange factors (such as Rom1 and Tus1) is responsible for the CWI activation (Philip and Levin, 2001). Rom2 has a Dbl homology (DH) domain, found in other GEFs for Rho family G-proteins, which interacts with GDP-bound Rho1 and possesses the nucleotide exchange activity (Ozaki et al., 1996). It also possesses pleckstrin homology (PH) domain, which binds phosphatidylinositol-4,5-biphosphate ($PI4,5P_2$) (Fig 4) and is responsible for proper localisation of Rom2 to the plasma membrane (Audhya et al., 2002). An N-terminal domain of Rom2 that is separate from either DH or PH domains is responsible for associating with Wsc1 and Mid2 and likely other cell surface sensors (Philip and Levin, 2001).

Rom2 is involved in determining the intrinsic stress tolerance of yeast to a broad range of stresses including freezing and thawing, long term starvation, oxidative stress, hyper-osmotic stress, and metabolic stress (Park et al., 2005). Rom2 is required for heat shock stress-induced actin cytoskeleton depolarization (Delley and Hall, 1999) as well as for repolarisation of the actin cytoskeleton in response to oxidative stress (Vilella et al., 2005). The expression of constitutively active allele *RHO1* suppresses the sensitivity of the *rom2* mutant to reactive oxygen species, cobalt ions but not to NaCl and caffeine, suggesting the existence of other ways of Rom2 activation independently on Slt2/Mpk1. *ROM2* restores temperature-sensitive growth in a $tor2^{ts}$ (Schmidt et al., 1997), in a *ira2* (a GAP negatively regulating Ras) (Park et al., 2005), in *tus1* (Schmelzle et al., 2002).

7.4.3 Rho1: Master regulator of CWI signalling

Members of the Rho (Ras-homologous) family of GTPases play a central role in polarized growth in animal and fungal cells (Drubin and Nelson 1996; Heasman and Ridley 2008). Rho1 is highly conserved when compared with mammalian RhoA protein. Like other G-proteins, Rho1 cycles between the active GTP-bound state and the inactive GDP-bound state. *S. cerevisiae* possesses six Rho-type GTPases, named Rho1 to Rho5 and Cdc42 (reviewed in Perez and Rincón, 2010). They reside at the plasma membrane and serve related but distinct roles in cell polarity establishment and maintenance (Johnson et al.,

1999; Helliwell et al., 1998b; Matsui and Tohe, 1992; Ridley et al., 1995; Schmitz et al., 2002). Rho proteins are C-terminally prenylated, (either farnesyl or geranylgeranyl) a modification that increases their hydrophobicity and allows their association with membranes (Schafer et al., 1992). The absence of prenylation renders Rho1 soluble and unable to activate or even interact with glucan synthase (Inoue et al., 1999).

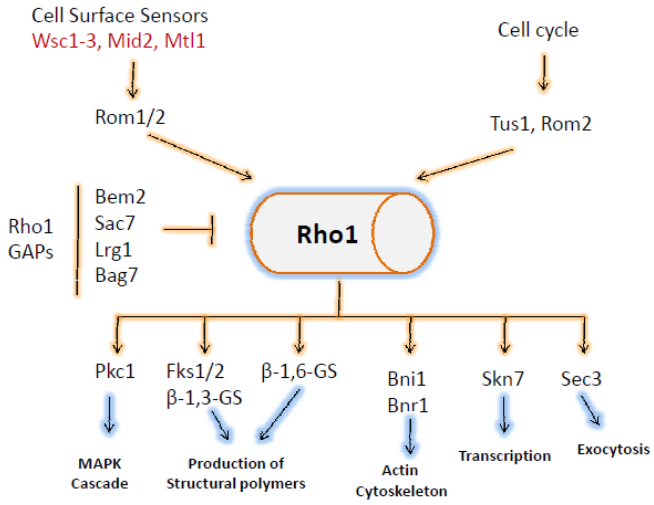


Figure 5: Rho1 regulators and down stream targets. Rho1 localization and activity are regulated through the cell cycle and in response to cell wall stress by cell- surface sensors, a family of GEFs (Rom1, Rom2, and Tus1), and a set of GAPs (Bem2, Sac7, Lrg1, and Bag7). Six known Rho1 effectors control cell wall biogenesis through polymer synthesis, polarization of the actin cytoskeleton, directed secretion, and transcription. Image adapted from (Levin, 2011)

Rho1 is considered as the master regulator of CWI signalling not only because it receives the major inputs from the cell surface but also because it regulates a variety of outputs (Fig 5). In total six effectors for Rho1 have been described: the Pkc1 protein kinase, the GS, β-1,6-glucan synthase activity, the Bni1 and Bnr1 formin proteins, the Sec3 exocyst component, and the Skn7 transcription factor (Fig 5). Together, these effectors coordinate synthesis of cell wall glucans and chitin, polarization of the actin cytoskeleton, expression of genes important for cell wall biogenesis, and polarized exocytosis. Rho1binds and activates Pkc1, which in turn controls the actin cytoskeleton and transcription of cell-wall biosynthesis genes via a mitogen-activated protein (MAP) kinase cascade (Igual et al., 1996; Kamada et al., 1996; Helliwell et al., 1998; Zhao et al., 1998). Rho1 also binds and activates the integral plasma membrane protein Fks1 (β-1,3-glucan synthase) and thereby controls cell wall synthesis directly (Qadota et al., 1996). Rho1 interacts with Bni1 and Bnr1, two formins involved in nucleating actin filament formation, and thus controls actin

cytoskeleton organisation via them (Evangelista et al., 2002). Rho1 activates Skn7, the transcription factor of the two-component signalling response regulator family, and which appears to be multifunctional as reflected by its ability to partner with variety of other transcriptional regulators at distinct promoter sites under different conditions, including oxidative stress. Rho1 is responsible for spatial regulation of Sec3, a subunit of the secretory vesicle-associated exocyst complex (Guo et al., 2001). Rho1 is localized to sites of polarised cell growth in a manner dependent on the actin cytoskeleton (Qadota et al., 1996) and may activate only a subset of its effectors in response to a particular input.

7.4.4 The protein kinase C: Pkc1

The genome *of Saccharomyces cerevisiae* encodes only a single homolog of mammalian protein kinase C, which is at least 10 isoforms in mammalian cells (Levin et al., 1990). It was the first component of the CWI pathway discovered. Deletion of *PKC1* is lethal under normal growth conditions, but the viability of *pkc1* can be rescued by osmotic support (1M sorbitol) (Levin and Bartlett-Heubusch, 1992). Loss of *PKC1* results in a more severe growth defect than that displayed by deletion of any of the members of the MAP kinase cascade under the control of Pkc1, prompting the suggestion that Pkc1 regulates multiple pathways (Lee and Levin, 1992).

Pkc1 associates with and is activated by GTP-bound Rho1, which confers upon the protein kinase the ability to be stimulated by phosphatidylserine as a lone cofactor (Nonaka et al., 1995; Kamada et al., 1996). Pkc1 resides at sites of polarised cell growth (Andrews et al., 2000). Early in the cell cycle Pkc1 is detected at the prebud site and at the bud tips. Later in the cell cycle, it becomes delocalised and finally relocalised at the mother-bud neck. The neck localisation of Pkc1 requires an intact septin ring.

It has been demonstrated that Pkc1 which is normally localised in bud tips and cell necks translocates to the cell periphery upon diamide treatment (Vilella et al., 2005). Pkc1 overexpression confers cells with more resistance to oxidising agents and Pkc1 is needed to repolarise and restore the actin cytoskeleton in response to oxidative stress provoked by hydrogen peroxide (Vilella et al., 2005). Pkc1, like Rho1, appears to control both depolarisation and repolarisation of the actin cytoskeleton (Delley and Hall, 1999). These authors proposed actin depolarisation to be mediated by a branch of the CWI pathway that includes Rho1 and Pkc1, but not the MAP kinase cascade. Null mutant in *PKC1* abrogates

the repression of ribosomal protein and rRNA genes caused by the *sec1-1* mutation (Nierras et al., 1999), thus implicating Pkc1, but not the MAP kinase cascade in the arrest-of-secretion response. Pkc1 is also a target of the Pkh1 and Pkh2 protein kinases (Inagaki et al., 1999; Friant et al., 2001) (Fig 4). Pkh1 and 2 serve an essential but overlapping function in the maintenance of cell wall integrity, and their function is required for full activation of Pkc1 in response to heat shock. Regulation of Pkc1 by Pkh1/ 2 is exerted by phosphorylation of an activation loop residue within the catalytic domain of Pkc1 (Thr983). It is not yet clear if Pkh1/2 activity functions as a regulatory input to Pkc1 or is merely required to establish basal activity of the latter kinase (Jesch et al., 2010).

7.4.5 The CWI MAP kinase cascade

A linear protein kinase cascade is responsible for amplification of the CWI signal from Rho1 (Fig 4). MAPK cascades serve both to amplify a small signal initiated at the cell surface and to convert a graded input to a highly sensitive, switch-like response (Ferrell 1996; Huang and Ferrell 1996). Briefly, the MAPK cascade for CWI signaling is composed of Pkc1 (Levin et al., 1990), a MEKK (Bck1) (Costigan et al., 1992; Lee and Levin 1992), a pair of redundant MEKs (Mkk1/2; Irie et al. 1993), and a MAPK (Mpk1/Slt2) (Lee et al., 1993; Martín et al., 1993). Mpk1 is a functional ortholog of human ERK5 (Truman et al. 2006), a MAPK that is activated in response to growth factors, as well as to hyper- osmotic, oxidative, and fluid sheer stresses (Abe et al., 1996; Yan et al., 2001).

Through, genetic and biochemical studies researchers have established that Pkc1 activates Bck1, which activates Mkk1 and 2, which in turn activate Mpk1. Pkc1 phosphorylates Bck1 in vitro at several sites in a hinge region between its putative regulatory domain and its catalytic domain (Levin et al., 1994; Levin 2005) that is also the site of activating mutations (Lee and Levin 1992). Bck1 is presumed to phosphorylate and activate Mkk1/2 on the basis of genetic epistasis studies, two-hybrid interactions, and its requirement for activation of Mpk1 (Irie et al. 1993; Kamada et al. 1995; Paravicini and Friedli 1996; Ho et al. 2002). Mkk1 and 2 phosphorylate Mpk1 on neighboring tyrosyl and threonyl residues in a T-X-Y motif within the activation loop conserved among MAPKs. Loss of function of any protein kinase below Pkc1 (or both Mkk1 and Mkk2) results in cell lysis at 37ºC. The growth defects of these mutants are remediated by elevated extracellular osmolarity (e.g., 1M sorbitol), consistent with a primary defect in cell wall biogenesis.

However *pkc1* mutants in the presence of 1M sorbitol lose viability upon starvation to a greater extent than do *mpk1* cells (Krause and Gray, 2002). Slt2/Mpk1 resides predominantly in the nucleus under normal conditions but rapidly relocates to the cytoplasm in response to cell wall stress (Kamada et al., 1995). A small pool of Slt2/Mpk1 localises to sites of polarised cell growth and shuttles constitutively between these sites and the nucleus (van Drogen and Peter, 2002). Mkk1 and Mkk2 are mainly cytoplasmic proteins, but, like Slt2/Mpk1, they can be detected at sites of polarised growth in a Spa-dependent manner. Bck1 has a cytoplasmic localisation (van Drogen and Peter, 2002). One likely target of Mpk1 at the cell surface is the Rom2 GEF for Rho1, which is phosphorylated and delocalized from the bud tip in an Mpk1-dependent manner in response to cell wall stress (Guo et al., 2009).

7.5 Downstream targets of the MAP kinase cascade

At present, only a few targets have been established for the Slt2/Mpk1, including two transcription factors, a protein phosphatase that reciprocally regulates Slt2/Mpk1 and a cell surface Ca^{2+} channel. Genetic evidence has implicated Slt2/Mpk1 in the control of several additional substrates, including the actin cytoskeleton.

The Rlm1 transcription factor (resistant to the lethality of constitutive Mkk1) is responsible for the majority of the transcriptional output of CWI signalling. Rlm1 displays two-hybrid interaction with Slt2/Mpk1 (Watanabe et al., 1995) and is phosphorylated by Slt2 (Yan and Lennarz, 2002). Rlm1 regulates the expression of at least 25 genes, most of which encode cell wall proteins or have been somehow implicated in cell wall biogenesis (Jung and Levin, 1999), and acting as either a transcription activator or a repressor depending on the context. Unlike loss of components of the MAP kinase cascade, deletion of *RLM1* does not result in temperature-sensitive cell lysis. The modest phenotypic defect of an *rlm1* compared with *slt2* suggests that Slt2/Mpk1 phosphorylates additional targets.

Based on genetic and biochemical evidence, Slt2/Mpk1 has been proposed to regulate SBF (binds SCB regulatory sequence during G1 phase and regulates both the transition from G1 to S and cell morphogenesis). This transcription factor has a regulatory subunit, Swi6, and a DNA-binding protein, Swi4. Whi5 has been identified as an inhibitory subunit (Breeden, 2003). Swi6 is phosphorylated in Slt2/Mpk1-dependent manner in response to cell wall stress (Madden et al., 1997). Since Swi6 is required for the cell cycle-dependent

binding of Swi4 to DNA (Harrington and Andrews, 1996), Slt2/Mpk1 may regulate SBF activity in part by driving Swi6 out of the nucleus both during periods of the cell cycle in which SBF is inactive and under conditions of wall stress. Swi4 associates with Slt2/Mpk1 and may form an alternative transcription complex for the regulation of some cell-wall and morphogenesis-related genes, notably *FKS2* and *PCL1* (Ki-Young et al., 2008).

7.6 PROTEIN REGULATORS OF THE CWI PATHWAY

7.6.1 Protein kinases Pkh1/2/3 and Ypk1/2/3

Pkh1 and Pkh2, the yeast homologues of mammalian 3'-phosphoinositide-dependent kinase 1 (PDK1), serve an essential but overlapping function in the maintenance of the cell wall integrity (Inagaki et al., 1999). They phosphorylate and thereby activate Pkc1 (Fig 4). A *pkh1* (Ts) *pkh2* mutant exibits both actin polarisation and osmoremedial cell lysis defects at the restrictive temperature. Growth is partially restored by constitutive activation of Pkc1, Bck1, or Mkk1.

Pkh1/2 have also been proposed to act upstream of the essential pair of homologous kinases Ypk1 and Ypk2 (Casamayor et al., 1999; Inagaki et al., 1999). The *YPK1/2* genes encode functional analogs of mammalian SGK and have been implicated in CWI signalling (Casamayor et al., 1999; Schmelzle et al., 2000). YPK-deficient cells are defective in cell wall integrity signalling (Schmelzle et al., 2000). Mutants lacking either YPKs or only *YPK1* display a randomized distribution of the actin cytoskeleton and severely reduced activation of Slt2/Mpk1 in response to heat stress. Upregulation of Rho1 or Pkc1 effector MAP kinase pathway suppresses the growth and actin defects of *ypk* cells. The finding that *ypk* lethality is suppressed by Rho1, more precisely by overexpression of Rho1 GEFs Rom2 and Tus1, suggests that YPKs are upstream of Rho1. PKHs and YPKs act downstream of a sphingolipid-derived signal, linking sphingolipid signaling and the CWI pathway (Schmelzle et al., 2002).

7.6.2 MAP kinase phosphatases

Inactivation of MAPK pathways occurs through both constitutive and induced (negative feedback) mechanisms. Slt2/Mpk1 is down-regulated by protein phosphatases of the tyrosine or dual-specifity (Tyr and Ser/Thr) classes. Interestingly, two of these, Msg5 and Ptp2, appear to undergo reciprocal regulation by Slt2/Mpk1. The Ptp2 and Ptp3 tyrosine

phosphatases, which have been shown to dephophorylate the Fus3 and Hog1 MAP kinases, act also on Slt2/Mpk1 in vivo and in vitro (Mattison et al., 1999).

The dual-specificity protein phosphatase Sdp1 appears to target Slt2/Mpk1 specifically (Collister et al., 2002). Expression of *SDP1* is under the control of Msn2/Msn4 stress-activated transcription factors. Thus, although Sdp1 may be the only protein phosphatase dedicated solely to the regulation of Slt2, its regulation appears to be independent of Slt2.

The dual-specifity protein phosphatase Msg5 has a significant role in down regulating the activity of Fus3 to promote recovery from pheromone stimulation. For Slt2/Mpk1, however, Msg5 seems more important for maintaining the low basal phosphorylation of Slt2 and is not much involved in down-regulating Slt2 after its stress-induced activation. Slt2/Mpk1 phosphorylates Msg5 in response to CWI pathway activation (Flandez et al., 2004).

Sit4 is a Ser/Thr protein phosphatase member of the PPP phosphatase family that is closely related to the PP2A family (Arndt et al., 1989). Sit4 participates in a number of cellular processes such as the TOR-mediated response to nutrients (Beck and Hall, 1999) and the regulation of monovalent ion homeostasis and intracellular pH (Masuda et al., 2000). Sit4 also plays an important role in cell cycle regulation, as it is required for the proper G1 to S phase transition (Sutton et al., 1991). De la Torre-Ruiz *et al.* (2002) have demonstrated that Sit4 is required for down-regulation of Pkc1 activity, and is consequently needed for a number of functions that depend on this kinase, such as Slt2/Mpk1 activity, cytoskeleton organisation, ribosomal gene expression.

Ppz1 and Ppz2 represent another subset of Ser/Thr protein phosphatases, which plays opposite role to Sit4 in cell cycle regulation. Genetic evidence indicates that Ppz1/2 phophatases act independently of the PKC1-MAPK pathway (Lee et al., 1993). Their role seems to be different from that of other phosphatases, such as Ptp2/3 and Msg5, which are known to dephosphorylate and negatively regulate Slt2/Mpk1.

7.7 Activation and functions of CWI signalling

CWI signaling is induced in response to a variety of cell wall stresses. CWI signaling is stimulated by oxidative stress, high and low pH, and DNA-damaging agents, as measured by Mpk1 activation. The protein kinase activity of epitope-tagged Mpk1 can be measured

in an immune complex using bovin emyelin basic protein as a substrate (Kamada et al., 1995; Zarzov et al., 1996). Alternatively, because Mpk1 is activated by phosphorylation of neighboring threonyl and tyrosyl residues within its activation loop, residues that are analogous to Thr^{202}/Tyr^{204} of mammalian p44/p42 MAPK (Erk1/2), commercially available antibodies against phospho-p42/p44 are very effective at detecting activated Mpk1 (De Nobel et al., 2000; Martín et al., 2000).

7.7.1 Cell cycle regulation

At times when growth is polarised to a single site on the cell surface, the cell experiences the greatest wall stress. By contrast, during G1 and mitosis, cell surface growth becomes isotropic. CWI signalling is regulated periodically through the cell cycle, peaking at the time of bud emergence, the time at which growth is most highly polarised (Zarzov et al., 1996). Although Slt2/Mpk1 activity is regulated through the cell cycle, its activation is not strictly dependent on Cdc28. Cyclic Slt2/Mpk1 activity probably reflects the level of cell wall stress signalled during different parts of the cell cycle. Consistent with this, the localisation of CWI pathway components reveals that most follow a cell cycle-dependent pattern of localisation to sites of polarised cell growth.

7.7.2 Heat stress

CWI signaling is activated persistently in response to growth at elevated temperatures (e.g.,37ºC–39 ºC) (Kamada et al., 1995; Zarzov et al., 1996). Reports indicate that the Mpk1 activation state is restored to normal after 2 hr at elevated temperature (Schmelzle et al., 2002; Guo et al., 2009). However a *pkc1* mutant is kept alive by a constitutive allele of *BCK1* is capable of activating Slt2 in response to mild heat shock. Likewise, heat shock activates Slt2 in a *bck1* mutant expressing a constitutive form of Mkk1. These findings led to the proposal that the protein kinase cascade is required in a passive way to provide basal signal to Slt2 and that heat shock activation is achieved by inhibition of the protein phosphatases that act on Slt2 (Sdp1, Msg5, Ptp2, and Ptp3). It seems likely that there are two inputs driving the response to this particular form of wall stress – one through the sensors at the top of the pathway to send an activating signal to Slt2, and a second that inhibits the MAP kinase phosphatases and appears to be specific to heat shock activation of Slt2. A *wsc1* was found to suppress the heat shock phenotype of *ira2* mutant. A *ras2* mutant rescues the heat shock sensitivity of *wsc* and a *wsc* overexpressing *IRA2* is not

sensitive to heat shock (Verna et al., 1997). It was found that Wsc1, but not Wsc2, functions in conjunction with RAS signaling and Wsc1 has opposing effects on a downstream target. Thus, Wsc1 facilitates cross talk with cAMP-PKA pathway through the downstream activation of Slt2 and negative regulation of downstream target of RAS in response to thermal stress. Additional cross talk with cAMP-PKA pathway occurs at the Slt2/Mpk1 position of the CWI. Slt2/Mpk1 is negatively regulated by Msn2/Msn2-dependent Sdp1 from cAMP-PKA pathway under heat shock conditions (Hahn and Thiele, 2002). The CWI sensors are important for thermal activation of Mpk1, supporting the conclusion that this stress is ultimately transmitted to the cell surface (Gray et al., 1997; Ketela et al., 1999; Rajavel et al., 1999; Martín et al., 2000).

7.7.3 Hypo-osmotic shock

Hypo-osmotic shock induces a rapid, but transient, activation of CWI signaling (Davenport et al., 1995; Kamada et al., 1995). Mpk1 is activated within 15 sec of an osmotic downshift, but basal activity is restored after ≈30 min. The Sln1 cell-surface osmosensor is also stimulated by hypo-osmotic shock, which results in activation of the Skn7 transcription factor in support of cell wall biogenesis. Further, the resulting *pkc1, bck1* and *mkk1mkk2* lyse in the absence of osmotic stabilisers (Lee and Levin, 1992). In addition, Skn7 transcription factor is activated through Sln1 in support of cell wall biogenesis. By contrast, the HOG MAP kinase is activated in response to hyper-osmotic shift (a result of Sln1 inactivation) which is possible by a secondary consequence of the increased intracellular glycerol generated by the HOG pathway (García-Rodríguez et al., 2005).

7.7.4 Pheromone-induced morphogenesis

Mating pheromones are constitutively secreted by haploid cells. Activation of the mating pheromone response pathway induces cell cycle arrest in G1 phase followed by the formation of a mating projection toward the source of pheromone (Elion 2000). Projection formation constitutes a cell wall stress because it requires polarization of the actin cytoskeleton and the secretory pathway to mobilize remodeling of the cell surface. Treatment with mating pheromone stimulates CWI signaling through Mid2 as a cell surface sensor. Mutants defective in CWI signaling undergo cell lysis during pheromone-induced morphogenesis (Errede et al., 1995), reflecting the major re-organization of the

cell wall associated with projection formation. It is considred that pheromone induced CWI signaling is an indirect event linked to the morphogenesis. Both Rho1 and Pkc1 localize to projection tips of cells treated with pheromone (Ayscough and Drubin 2003; Bar et al., 2003). As seen above, the Mid2 sensor is also recruited to mating projections (Hutzler et al. 2008).

7.7.5 Response to pH stress

The CWI pathway also functions to facilitate tolerance to pH changes. Alkaline stress is sensed by Wsc1. Other genes important to alkaline tolerance are *BCK1* and *SLT2/MPK1* (Serrano et al., 2006). Deletion of *WSC1* does not completely abolish the phophorylation of Slt2/Mpk1 in alkaline stress, suggesting the involvement of a different sensor or through proteins outside the linear CWI. Alkaline activated Wsc1 leads to transcription of SBF-dependent genes. Under low pH Stress, mid2 exhibits a decrease in the transcription of *PST1*, which is Rlm1-dependent. The CWI receives lateral influence under acidic conditions from Rgd1 and it appear to be in a HOG-dependent manner (Claret et al., 2005).

7.7.6 Oxidative stress

Pkc1 and the upper elements of this pathway are needed for cell survival and adaptation in response to oxidising agents, such as diamide and hydrogen peroxide. Diamide depletes glutathione and oxide thiol groups. Hydrogen peroxide promotes lipid peroxidation, protein oxidation and DNA damage. Among the transmembrane proteins, only Mtl1 has been characterized as a cell surface sensor for oxidative stress (Vilella et al., 2005). However, the CWI elements downstream Pkc1 seem to be dispensable for this mechanism. Pkc1 overexpression confers cells with more resistance to oxidising agents. Exposure of *rom2* to oxidizing agent results in diminished Slt2/Mpk1 phosphorylation. It has been demonstrated that upon oxidative stress Pkc1 translocates to the cell periphery. Pkc1 transmits the signal to Slt2/Mpk1 if cells have intact secretory machinery (Vilella et al., 2005). Oxidative stress depolarises the actin cytoskeleton. None of the CWI elements are required to mediate this depolarisation mechanism, however, Pkc1 is essential in order to restore the organization of the actin cytoskeleton in oxidative conditions. Moreover, Pkc1 overexpression induces an early polymerisation of the actin cytoskeleton and cable formation in response to hydrogen peroxide treatment, concomitantly with an increase in

cell survival (Pujol et al., 2009; Vilella et al., 2005). *mtl1* mutant cells are sensitive to oxidising agents and also impairs Slt2 phosphorylation and consequently the activation of the CWI pathway (Petkova et al., 2010a; Vilella et al., 2005). It is found that the cells are deficient in the transcriptional response mediated by Msn2/Msn4, in the absence of *MTL1*. The RAS2-PKA pathway appears upregulated in *mtl1* mutant. Mtl1 is required in order to signal to TOR1 and RAS2 inhibition in response to oxidative stress and glucose deprivation (Petkova et al., 2010a). Oxidative stress also provokes the repression of ribosomal genes and ribosomal biogenesis. Huge quantity of ATP is required for ribosome biogenesis, therefore, it is necessary to downregulate the process of oxidative damage. In response to oxidative stress, Pkc1 signals the downregulation of ribosomal gene expression (Petkova et al., 2010a).

7.7.8 Cell wall-stressing agents

Chemical agents that induce cell wall stress such as the chitin antagonists Calcofluor white and β 1,3-glucan binding dye Congo red, enchinocandin inhibitors of β 1,3-Glucan Synthase, the cell wall lytic enzyme zymolyase, and caffeine activate CWI signaling (Kopecka and Gabriel 1992; Ketela et al., 1999; De Nobel et al., 2000, Martín et al., 2000; Jung et al., 2002; Reinoso-Martín et al., 2003; Garcia et al., 2004; Garcia et al., 2009; Kuranda et al., 2006; Bermejo et al., 2008).

A recent genome profiling study suggests the drug caffeine may target the Tor protein kinase complexes (Lum et al., 2004; Kuranda et al., 2006). Mutations that impair cell wall biosynthesis similarly activate CWI signaling (De Nobel et al., 2000; Terashima et al., 2000; Bulik et al., 2003; Lagorce et al., 2003). Activation of CWI signaling in response to treatment with zymolyase is largely independent of Wsc1 and Mid2 as well as all three of the Rho1-GEFs (Bermejo et al., 2008). Instead, activation of CWI signaling requires the components of the Sho1 branch of the HOG pathway, including Hog1, suggesting sequential activation of the two pathways by zymolyase (Bermejo et al., 2008, Bermejo et al., 2010; Garcia et al., 2009). This unusual activation route for CWI signaling requires Pkc1 and the MAPK cascade (Bermejo et al., 2008). In addition, loss of Pkc1, Bck1, or Mpk1 causes zymolyase sensitivity and drastically reduces cell viability following carbon or nitrogen starvation, suggesting that CWI pathway-controlled cell wall remodeling is an important aspect of the quiescence program (Krause & Gray, 2002; Torres et al., 2002).

7.7.9 Actin cytoskeleton depolarization

As earlier descriced, when cells are subjected to heat stress, the actin cytoskeleton becomes redistributed from a polarized state to a more uniform localization around the cell periphery (Lillie and Brown 1994; Desrivieres et al., 1998). The CWI pathway, including Mpk1, is required for repolarization of the actin cytoskeleton after cell wall stress (Delley and Hall 1999). The observation that the requirement for Mpk1 in actin repolarization can be overcome by artificial downregulation of Pkc1 (Guo et al., 2009), suggesting that signaling through the upper part of the pathway must be terminated to re-establish actin polarity. Depolarization of the actin cytoskeleton by treatment with the actin antagonist latrunculin-B activates Mpk1 (Harrison et al., 2001). However, Mpk1 activation in response to actin depolarization was blocked by the presence of osmotic support (Harrison et al. 2001; Torres et al. 2002), suggesting that the CWI pathway senses actin depolarization as a cellwall stress. This may arise as a consequence of disrupting polarized secretion. Similarly, rapamycin treatment, which depolarizes the actin cytoskeleton by inhibiting the shared function of the Tor1/2 protein kinases, also induces Mpk1 activation (Krause and Gray 2002; Torres et al., 2002). Apparently, both cells entering stationary phase and cells treated with rapamycin exhibit enhanced phosphorylation of Mpk1/Slt2 at sites required for its activation (Krause & Gray, 2002; Torres et al., 2002). The Tor2 unique function is important for organisation of actin cytoskeleton (Schmidt et al., 1996). The actin organisation defect was connected genetically to Rho1 signaling by the finding that the *tor2*^{ts} growth arrest and actin depolarization defects were suppressed by overexpressing CWI pathway components at any level (Helliwell et al., 1998). Rom2 restores growth in a *tor2*^{ts}. However, Tor2 overexpression does not repair the growth defect of *rom2* (Schmidt et al., 1997). Further evidence that Tor influences Rom2 is demonstrated by the reduction in Rom2 GEF activity in a *tor2*^{ts} mutant strain. Thus Rom2 likely functions downstream Tor2 (Schmidt et al., 1997).

7.7.10 ER stress

There is an intimate inter-relationship between CWI signaling and ER stress. Several studies have shown that ER stress induced by tunicamycin, 2-deoxyglucose, or dithiothreitol activates CWI signaling and that Mpk1 is an important determinant of ER stress survival (Bonilla and Cunningham 2003; Chen et al., 2005; Babour et al., 2010).

Genetic analyses revealed that ER stress activation of CWI signaling is independent of the unfolded protein response (UPR), the classic ER stress response pathway controlled by Ire1 and Hac1 (Chen et al., 2005). Mpk1 activation in response to tunicamycin treatment appears to be triggered principally by the Wsc1 sensor (Babour et al., 2010), but in some manner dependent on the Hos2/Set3 histone deacetylase complex (Cohen et al., 2008).

7.7.11 Mitophagy

In a recent genome-wide yeast mutant screen for mitophagy defective strains it was found that Bck1 is required for mitophagy (Kanki et al., 2009). Two MAPKs, Slt2 and Hog1, in the yeast *S. cerevisiae*, and showed that both are required for mitophagy (Mao and Klionsky, 2011; Mao et al., 2011). Both Slt2 and Hog1 are phosphorylated and remain in the cytosol during mitophagy(Mao and Klionsky, 2011). In addition, these authors show that Slt2 is required for pexophagy and mitophagy, but not bulk autophagy while Hog1 is a positive regulator only required for mitophagy, but not other types of selective autophagy or bulk autophagy (Mao et al., 2011).

8. TOR SIGNALING NETWORK

Rapamycin is a natural secondary metabolite with lipophilic macrolide propertirs was produced by *Streptomyces hygroscopicus*, a bacterium isolated from a soil sample collected in Rapa-Nui (Easter Island) in 1965-hence the name rapamycin. Rapamycin was originally purified in the early 1970s as an antifungal agent (reviewed in Loewith and Hall, 2011).

The Target Of Rapamycin (TOR), is a highly conserved Ser/ Thr protein kinase, and plays as the central component of a major regulatory signalling network that controls cell growth in diverse eukaryotic organisms, ranging from yeast to man. The TOR proteins were first identified in *Saccharomyces cerevisiae* as the targets of the antifungal and immunosuppressive agent rapamycin, hence their name (Heitman et al., 1991). The rapamycin acts by binding the highly conserved intracellular cofactor FKBP12 and the complex then binds and inhibits the TOR function (encoded by the homologous *TOR1* and *TOR2* genes in *S. cerevisiae*) (De Virgilio and Loewith, 2006).

In contrast to most eukaryotes, yeast contains two TOR homologues, Tor1 and Tor2. Two functionally and structurally distinct TOR multiprotein complexes exist: TOR complex 1

(TORC1) and TOR complex 2 (TORC2) (De Virgilio and Loewith, 2006; Smets et al., 2010). In addition it is known that only TORC1 is specifically inhibited by rapamycin. TORC1 and TORC2 in general, function as multimers and the structural components of both these TORCs are highly conserved from yeast to mammals (Takahara and Maeda, 2013). The addition of rapamycin induces dramatic phenotypic changes such as cell cycle arrest and entry into G_0, general downregulation of protein synthesis, accumulation of the reserve carbohydrate glycogen and the stress protectant trehalose, upregulation of stress response genes, autophagy and alterations in nitrogen and carbon metabolism (De Virgilio and Loewith 2006a, b; Rohde et al. 2008). Hence, it appears that TORC1 signaling controls the temporal aspects of cell growth in response to the quality of the available nitrogen and carbon sources. On the other hand, TORC2, which is insensitive to rapamycin and is less well characterized in comparison to TORC1, is thought to regulate the spatial aspects of growth, such as the control of actin polarization (De Virgilio and Loewith 2006a, De Virgilio and Loewith 2006b; Rohde et al., 2008)

Although TORs resemble lipid kinases, they actually belong to a family of Ser/Thr protein kinases known as phosphatidylinositol kinase-related kinases (PIKKs; Keith and Schreiber, 1995). Eukaryotic TORs are large proteins (~280kDa) that share 40-60% identity in their primary sequence. PIKK family members contain a carboxy-terminal Ser/Thr protein kinase domain that resembles the catalytic domain of phosphatidylinositol-3-kinases (PI3Ks) and PI4Ks. Amino-terminal to the kinase domain is the FKBP12-rapamycin binding domain (FRB). Single amino acid substitutions in this domain yield TOR1-1 and TOR2-1 that are no longer bound and inhibited by the FKBP12-rapamycin complex. Located amino-terminal to the FRB domain and carboxy terminal to the kinase domain is the FAT and FATC domains, respectively. These domains are found in all PIKKs and always together, suggesting that they may interact. The amino-terminal half of TOR contains tandem HEAT repeats providing large interfaces for protein-protein interactions. But FKBP-rapamycin can only bind TORC1, apparently because in TORC2 the FRB domain, to which it binds, is protected by Avo1 (Loewith et al., 2002; Wullschleger et al., 2005). TOR-shared and TOR2-unique functions correspond to two separate signalling branches that regulate numerous aspects of cell growth and metabolism. The signalling specificity and differential sensitivity of TOR to rapamycin in these two branches is explained by the observation that TOR operates in each branch as a component of distinct multiprotein complex (Loewith et al., 2002).

8.1 Two TOR complexes – TORC1 and TORC2

8.1.1 TORC1 is sensitive to rapamycin and regulates temporal aspects of cell growth

TORC1 contains Lst8, Kog1, Tco89 and either Tor1 or Tor2 (Fig 6) (Loewith et al., 2002). FKBP12-rapamycin binds TORC1, and TORC1 disruption mimics rapamycin treatment, suggesting that TORC1 mediates the rapamycin-sensitive temporal control of cell growth (Loewith et al., 2002). Lst8 is found in both TORC1 and TORC2. Lst8 is essential, 34kDa protein composed entirely of seven WD40 repeats and is conserved from yeast to man. Lst8 acts positively with TOR in TORC1. Several alleles of *LST8* have been generated with differing effects of these alleles on various TORC1 readouts (Chen and Kaiser, 2003). Kog1 possesses four HEAT repeats, seven WD40 repeats and a caspase-like domain. Kog1 acts positively in TORC1, demonstrated by using temperature-sensitive alleles of *KOG1*. Tco89, rich in Ser/Thr residues, potential sites for phosphorylation, is conserved only among fungi. Cells lacking *TCO89* are hypersensitive to rapamycin and caffeine, and in other ways resemble cells lacking *TOR1*, suggesting that Tco89 acts positively in TORC1 (De Virgilio and Loewith, 2006).

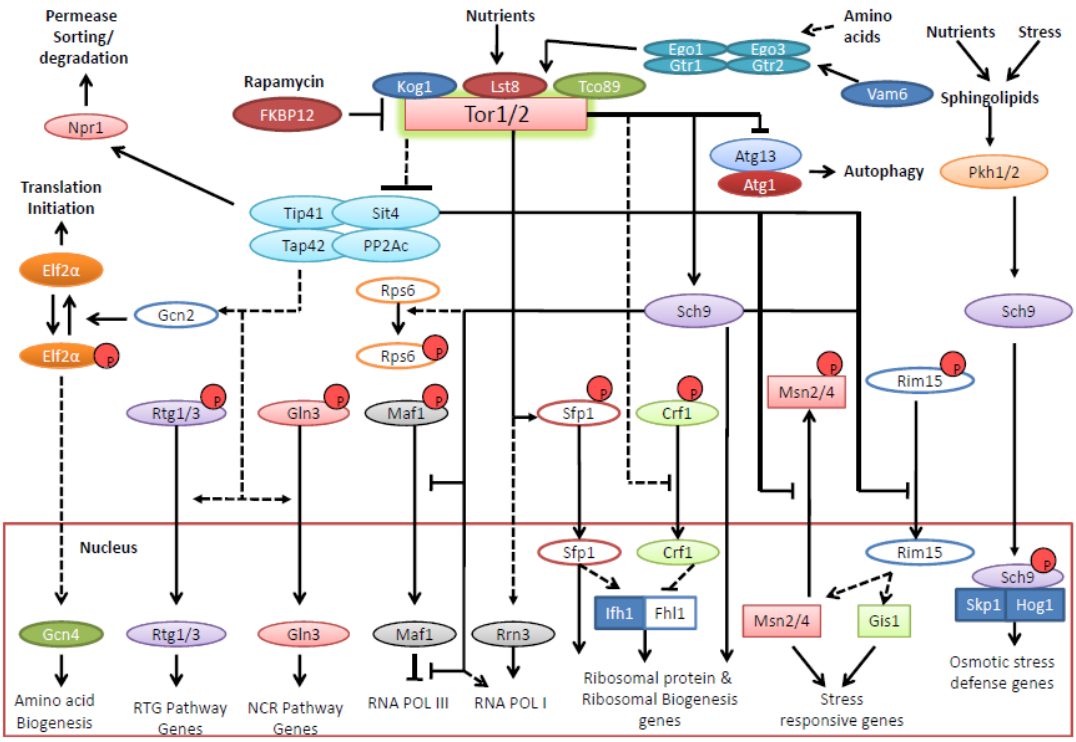


Figure 6: The TORC1 pathway in *S. cerevisiae*. Nutrients activate TORC1, resulting in the stimulation of protein synthesis and the inhibition of stress response genes, autophagy and several pathways that allow

growth on poor nitrogen sources. High glutamine levels as well as other amino acids stimulate the vacuolar/endosome membrane-located, EGO complex. This complex is composed of the two Ras-like GTPases, Gtr1, Gtr2, and the Ragulator-like, Ego3 and Ego1. Activation of EGO is stimulated by GTP-bound Gtr1 and GDP-bound Gtr2. GTP loading of Gtr1 is stimulated by the guanine nucleotide-exchange factor (GEF) activities of Vam6 and the L-Leu-tRNA synthetase. TORC1 also phosphorylates Sch9 and Tap42, the latter leading to the inhibition of several protein phosphatases (PPA2, Sit4, etc.). As a result, the protein phosphatases can no longer dephosphorylate the Ure2 complexes with Gln3 and Gat1, reinforcing their hyperphosphorylation. Synthesis of glutamine and glutamate occurring via anaplerotic reactions shared with the TCA cycle is also downregulated. Active TORC1 stimulates growth and inhibits stress responses largely by activating Sch9 and inhibiting Tap42, a regulatory subunit of PP2A and PP2A-like phosphatases. Upon nutrient starvation or the presence of noxious stresses, the small GTPase Rho1 outcompetes Tap42 for binding to Kog1, leading to release and activation of Tap42–phosphatase complexes. At the same time, Rho1 inhibits the activity of Tor1/2, which results in the dephosphorylation and inactivation of Sch9.The activity of Sch9 is additionally regulated by Pkh1 and Pkh2. Image adapted from (De Virgilio and Loewith, 2006; Rødkaer and Faergeman, 2014; Smets et al., 2010; Swinnen et al., 2014)

In yeast, activity of TORC1 is regulated at the vacuolar membrane through the interplay of four proteins: Ego1, Ego3, Gtr1, and Gtr2, which together form the EGO complex, EGOC (Fig 6) (Dubouloz et al., 2005). Gtr1 and Gtr2 are Ras-family GTPases represented in metazoans by the orthologous Rag GTPases, Rag A-D (Sancak et al., 2008; Efeyan et al., 2012). Ego1 and 3 are functional homologs of the Ragulator complex in vertebrates (Kogan et al., 2010; Sancak et al., 2010). Ego1 is N-terminally myristoylated and palmitoylated, attaching EGOC to the vacuolar membrane (Dubouloz et al., 2005; Binda et al., 2009; Zhang et al., 2012). The function of Ego3 in the complex is still unclear (Conrad et al., 2014).

TORC1 promotes anabolic processes such as protein synthesis and ribosome biogenesis, while antagonises catabolic processes such as autophagy and inhibits stress-response processes (Crespo and Hall, 2002; Wullschleger et al., 2006; De Virgilio and Loewith, 2006). TORC1 activity appears to be sensitive to both nutrient and stress cues. Nutrients, especially amino acids, regulate TORC1 signalling (Fig 6 and 7). Glutamine may play a particularly important role as a regulator of TOR (Crespo et al., 2002). It is a key intermediate in nitrogen metabolism but also an important indicator of cell's general nutrient status. Starvation for glutamine causes nuclear localisation and activation of Gln3 and Rtg1/Rtg3 (Fig 7 and 8), but other TORC1 readouts such as Msn2 and Gat1 (detailed below) remain unaffected, suggesting that TORC1 may process different nutrient signals

to elicit nutrient-specific responses. In addition, growth factors and energy regulate mTORC1 in higher eukaryotes (reviewed in Wullschleger et al., 2006).

8.1.2 TORC2 is insensitive to rapamycin and regulates spatial aspects of cell growth.

TORC2 contains Avo1, Avo2, Avo3, Lst8, Bit61 and Tor2, but not Tor1 (Loewith et al., 2002). Avo1, Avo3 and Lst8 are essential conserved proteins required for kinase activity. By contrast Avo2 and Bit61 are not essential and no clear homologous counterparts have been identified in higher eukaryotes. TORC2 is a multimeric super complex that is likely a TORC2-TORC2 dimer assembled via a Tor2-Tor2 interaction (Wullschleger et al., 2005). Multimerisation may be a general property of TOR complexes (TORC1 are also multimeric). TORC2 is known to regulate the cell-cycle dependent polarisation of actin cytoskeleton (see below). Upstream regulators of TORC2 remain unknown.

8.2 TORC1 EFFECTORS

TORC1 exerts most of its control via two major effector branches: the mammalian S6 kinase (S6K) ortholog, AGC kinase Sch9, and the Tap42–PPase complex (Broach, 2012; Loewith and Hall, 2011) (Fig 6). Through these two proximal effectors, TORC1 modulates distal readouts to positively regulate ribosome biogenesis and translation and to inhibit stress responses that are incompatible with growth and are typically induced in quiescent cells (De Virgilio, 2012).

8.2.1 The Sch9 effector pathway

Sch9 is homologous to the mammalian protein kinase B (PKB/Akt). Alignment of Sch9 and PKB revealed 44% identity and 68% similarity over 397 residues and showed that the homology between Sch9 and PKB is most pronounced at the catalytic domain and the C-terminus (Geyskens et al., 2001). Furthermore, Sch9 and PKB are not only structurally, but also functionally related since expression of PKB rescues the slow growth and small colony phenotype of a *sch9* deletion mutant. Yeast TORC1 phosphorylates serine and threonine residues in the C-terminus of Sch9, and this phosphorylation is necessary for Sch9 activity and is used as a fast read-out for TORC1 activation (Urban et al., 2007). This kinase was originally identified through a screen aiming to isolate multi-copy suppressors of the growth defect at high temperatures of a cAMP-PKA signalling- deficient $cdc25^{ts}$ strain (Toda et al., 1988).

Sch9 is rapidly dephosphorylated not only in response to rapamycin but also in response to carbon, nitrogen, phosphate, or specific amino acid starvation (Urban et al., 2007; Binda et al., 2009). Sch9 functions in coordination with other nutritional sensor pathways, beyond TORC1. One of the main TORC1 regulated roles of Sch9 is to modulate translation and cell size attained before cell division (Jorgensen et al., 2002, Jorgensen et al., 2004). Although it is mainly localized at the vacuolar limiting membrane, consistent with its TORC1 related function, Sch9 is also associated with chromatin where it could exert more direct effects in the control of transcription factors like Gis1 (Pascual-Ahuir & Proft, 2007). This role seems to take place independently from TORC1. One major role of Sch9 is to regulate translation in function of nutrient availability and the growth potential. As such, Sch9 controls the expression of RP genes and of the Ribi regulon, by interfering with the transcriptional processes conducted by the RNA polymerases I, II and III (Crauwels et al., 1997a, Crauwels et al., 1997b; Jorgensen et al., 2004; Roosen et al., 2005; Urban et al., 2007; Smets et al., 2008; Huber et al., 2009).

8.2.2 TORC1 regulated protein phosphatases

Some of the TORC1 readouts are mediated by the type 2A and 2A-related protein phosphatases. Protein phosphatase 2A (PP2A) exists predominantly as a heterotrimeric complex consisting of a catalytic (PP2Ac – Pph21, Pph22, Pph3) subunit, one or two regulatory subunits (Cdc55, Rts1) and a scaffold subunit (Tpd3). Several PP2A-related catalytic subunits, including Sit4 exist. Four regulatory subunits (Sap155, Sap185, Sap190 and Sap4) individually associate with Sit4. Like PP2A, Sit4 has a range of cellular targets. In addition to these heterotrimeric PP2A and Sit4-Sap complexes, a fraction of PP2Ac and Sit4 can be found in distinct complexes containing Tap42, and either Rrd1 or Rrd2 (Di Como and Arndt, 1996; Santhanam et al., 2004). It is these Tap42-containing complexes that appear to be regulated by TORC1. Under optimal conditions, Tap42 is phosphorylated and tightly associated with both Sit4-Rrd1 and PP2Ac-Rrd2. Carbon or nitrogen starvation or rapamycin treatment results in dephosphorylation of Tap42 and a reduced association of Tap42 with Sit4-Rrd1 and PP2Ac-Rrd2 (Di Como and Arndt, 1996) (Fig 6). TORC1 may directly phosphorylate Tap42 (Düvel and Broach, 2004). Alternatively, TORC1 may regulate Tap42 via Tip41 (Jacinto et al., 2001). Tip41 dephosphorylation following rapamycin treatment correlates with increased association of Tip41 with Tap42. The phosphorylation status of Tap42 and/or its association with Tip41 alters the substrate

specificity of associated phosphatases (Düvel and Broach, 2004). Readouts, including autophagy, transcription of *RP, RiBi, rRNA and tRNA* genes appear to be independent of PP2A and Sit4 (Schmelzle et al., 2004). In contrast, TORC1 promotes nuclear export of Msn2 via Tap42-dependent inhibition of Pph21 and Pph22 (Gorner et al., 2002; Santhanam et al., 2004). Similarly, PP2Ac and/or Sit4 are required for rapamycin-induced dephosphorylation of NPR1 (Schmidt et al., 1998), Gcn2, Rtg1/3 (Düvel and Broach, 2004), and Gln3 (Beck and Hall, 1999) (Fig 8). On the contrary, the inhibition of Sit4 by TORC1 and Tap42 maintains Slt2/Mpk1 activity at basal levels (Torres et al., 2002).

8.3 FUNCTIONS OF TORC1 SIGNALING

Nitrogen Metabolism

TORC1 signalling involves the regulation of PP2A and the PP2A-like phosphatase Sit4 is the control of nitrogen metabolism. Yeast cells adapt their metabolism to the available nitrogen sources via the nitrogen catabolite repression pathway (NCR) also known as the nitrogen discrimination pathway (NDP) (Magasanik and Kaiser 2002). This pathway ensures that genes encoding proteins required for the usage of poor nitrogen sources are repressed when rich nitrogen sources, such as glutamine, are present in sufficient quantities (See nitrogen catabolite repression pathway (NCR) explained in **section 9** of introduction) (Fig 6 and 7).

Initiation of protein synthesis in eukaryotes is a highly regulated process and TORC1 appears to regulate translation initiation at multiple levels. TORC1 negatively controls the general amino acid control (GAAC) pathway (Hinnebusch, 2005). The central component of this pathway is Gcn4, a transcription factor important for activating transcription of genes needed for amino acid biosynthesis in response to amino acid starvation (Natarajan et al., 2001). The pathway is induced by uncharged tRNAs, which presumably activate the kinase Gcn2. In turn, Gcn2 phosphorylates the α subunit of eIF2 and although this results in a reduction of the general translation initiation, it specifically stimulates the translation of *GCN4* mRNA (Dever et al., 1992). TORC1 inhibits Gcn2 activity by promoting its phosphorylation at Ser^{577}. This occurs indirectly and involves the inhibition of the PP2A-like phosphatase Sit4 via Tap42. As such, TORC1 enhances translation initiation and antagonizes *GCN4* mRNA translation (Valenzuela et al., 2001; Cherkasova and Hinnebusch, 2003; Kubota et al., 2003; Rohde et al., 2004). However, *GCN4* is also a target of the NCR (Godard et al., 2007), suggesting that TORC1, via inhibition of NCR

gene expression, also inhibits *GCN4* transcription. The G_1 cell cycle arrest observed upon TOR inhibition is a consequence of a translation defect, emphasising that growth-induced synthesis of cell cycle machinery components, such as the G_1 cyclin Cln3, plays important role in activating and allowing progression of the cell cycle (Barbet et al., 1996).

A third pathway that is involved in nitrogen metabolism and that is subject to TORC1 control is the retrograde response pathway (RTG). TORC1 promotes cytoplasmic retention of a heterodimeric bZip/HLH transcription factor composed by Rtg1 and Rtg3, which are central elements of mitochondria-to-nucleus signaling pathway (also known as retrograde response pathway) that activate genes whose products (mitochondrial and peroxisomal enzymes) are required for biosynthesis and homeostasis of glutamate and glutamine (Butow and Avdhani, 2004). (See Retrograde Response (RTG) Pathway explained in **section 10** of introduction) (Fig 6 and 7).

Finally, TORC1 also appears to control the turnover of several amino acid permeases. TORC1 positively regulates the activity of high-affinity permeases which are expressed under optimal growth conditions thus coupling nutrient cues with posttranslational sorting of these transporters (Schmelzle et al., 2004). TORC1 may regulate the sorting of amino-acid transporters via the protein kinase NPR1 by mediating its phosphorylation (Schmidt et al., 1998; Beck and Hall, 1999). Nutrient starvation or rapamycin treatment strongly induces the expression of the ammonia permease gene *MEP2,* and the general amino acid permease gene *GAP1*, via the two GATA transcription factors, Gln3 and Gat1, which are negatively regulated by TORC1. (See nitrogen catabolite repression pathway (NCR) explained in **section 9** of introduction) (Fig 7).

Protein Synthesis

Apart from its controlling function on nitrogen metabolism, TORC1 has a major regulatory role in protein synthesis as it promotes expression of the rRNA and the ribosomal proteins (RP) genes as well as ribosome biogenesis (Ribi) regulon (Jorgensen et al., 2004). TORC1 positively affects the interaction between Rrn3 and RNA polymerase I, which is necessary for proper recruitment of the polymerase to the 35S rDNA promoter (Claypool et al., 2004). TORC1 promotes recruitment of the RNA polymerase I to the rDNA locus in a Rrn3-independent way, via Sch9 (Huber et al., 2009). Apparently, 5S rDNA-associated TORC1 phosphorylates Maf1, thereby inhibiting the nucleoplasm-to-nucleolus translocation of Maf1 and the concomitant binding of Maf1 with RNA

polymerase III-transcribed genes (Fig 6) (Wei et al., 2009). In addition, TORC1 mediates phosphorylation of Maf1 indirectly via Sch9 (Huber et al. 2009). Several transcription factors regulate RP gene expression in a TORC1 dependent manner. FHL1 contains a fork head DNA-binding domain and is found localised to the promoters of most *RP* genes. FHL1 binding to *RP* promoters appears to be constitutive, and is facilitated by Hmo1, a high-mobility group protein, and Rap1, a protein required for expression of *RP* genes among many other activities (Hall et al., 2006). The interaction of FHL1 with two other proteins, IFH1 and CRF1, appears to be regulated by TORC1 activity (Martin et al., 2004). IFH1-FHL1 complexes stimulate, whereas CRF1-FHL1 complexes suppress expression of RP genes (Fig 6). The phosphorylation state of both IFH1 and CRF1 is dictated by TORC1 activity. CRF1 is phosphorylated by PKA-regulated kinase YAK1 (Martin et al., 2004), suggesting that TOR regulates RP gene expression via PKA and FHL1.

TOR-dependent regulation of RP genes is still observed in the absence of FHL1/IFH1/CRF1 system, suggesting the existence of other mechanisms by which TOR regulates RP gene expression. One candidate is the transcription factor Sfp1 (Fig 6). Under favourable growth conditions, Sfp1 localises to many *RP* and *RiBi* promoters and activates RP gene expression. Sfp1 nuclear localisation (and concentration) responds rapidly to nutrient and stress, including oxidative stress conditions, and is regulated by TOR and cAMP-PKA. For the ribosomal gene regulation it has been proposed that TOR acts upstream of PKA controlling its activity (Schmelzle et al., 2004). However, as for Sfp1 regulation, there is a branch from TOR that signals independently on PKA, since in response to osmotic or oxidative stress, Sfp1 cellular localisation does not appear to be dependent on PKA (Marion et al., 2004).

Translation Initiation

TORC1 was found to be essential for translation initiation (Barbet et al., 1996). Deletion of *CDC33*, encoding for the translation initiation factor eIF4E, results in a similar phenotype as observed when TORC1 is inactivated, it was proposed that TORC1 might control translation initiation via eIF4E (Barbet et al., 1996; Danaie et al., 1999). In addition, TORC1 was shown to have a positive effect on the stability of translation initiation factor eIF4G that binds to eIF4E (Berset et al., 1998). A third translation initiation factor that is regulated by TORC1 is eIF2. However, the regulation of eIF2 by TORC1 is indirect and mediated through the kinase Gcn2, of which the phosphorylation

status is controlled via the Tap42 effector branch (Fig 6). Phosphorylated Gcn2 in turn prevents phosphorylation of the α-subunit of eIF2 and thereby the inhibition of translation initiation (Cherkasova and Hinnebusch 2003; Kubota et al. 2003; Hinnebusch 2005) (Fig 6).

Autophagy

TORC1 is also a known negative regulator of autophagy (Chang et al., 2009). TORC1 activity controls the phosphorylation status of Atg13 (Fig 6). When TORC1 is active, Atg13 is hyperphosphorylated, whereas rapamycin addition induces a rapid dephosphorylation of Atg13 (Kamada et al., 2000). The latter apparently stimulates the affinity of Atg13 for Atg1 and promotes Atg1–Atg13 complex formation which is a requirement for autophagy (Funakoshi et al. 1997; Kamada et al. 2000). TOR controls not only bulk protein degradation by macroautophagy, but also the ubiquitination, internalization, and turnover of specific nutrient transporters. It is possible that PP2A is involved in TORC1-dependent regulation of Atg13 phosphorylation, since it was shown that autophagy is negatively regulated by the Tap42-PP2A pathway (Yorimitsu et al., 2009).

8.4 TOR signaling , oxidative stress and life extension.

The connection between mitochondrial translation, oxidative stress and aging, has been proposed in the model system *S. cerevisiae* (Bonawitz et al., 2006). TOR has been demonstrated to play a role in the general response to stress. With respect to the response to oxidative stress, Bonawitz and coworkers (Bonawitz et al., 2007) have presented evidence demonstrating that deletion of *TOR1* activates respiration by increasing mitochondrial translation and perhaps ribosome biogenesis. This increases the concentration of OXPHOS complexes, thus generating less ROS accumulation in the cells. As a consequence, cells become more resistant to oxidative stress. These authors also highlight the fact that TOR inhibition downregulates the general cytoplasmic translation (Barbet et al., 1996; Gingras et al., 2001; Schmelzle and Hall, 2000), in contrast with the effect that *TOR1* deletion provokes in mitochondrial translation.

Contrary to this model Powers et al. (2006), propose that the adquisition of stress resistance observed in the *tor1* mutant is partly due to the activation of the Msn2/Msn4 transcription factor.

A model proposes that TOR signals to *SCH9* downregulating Rim15 activity (Wei et al., 2008). Signals for Rim15 inhibition come from RAS2/PKA, SCH9 and TOR (Vidan et al., 1997; Swinnen et al., 2006; Weinberger et al., 2010) (Fig 3). In support of this model, (Pan et al., 2009), present evidence demonstrating that Sch9 functions downstream of Tor1. In fact, TOR inhibition also requires down-regulation of Sch9, in order to extend the chronological life span and to increase resistance to oxidative stress. (Madia et al., 2009) have demonstrated that Sch9 activates respiration in quiescence. This notably increases ROS accumulation and also the oxidative DNA damage. Sch9 inactivates Gis1 by an independent pathway; therefore, deletion of *SCH9* attenuates age-dependent mutagenesis as a consequence of Gis1 activation and subsequent induction of *SOD2* (Fabrizio et al., 2003). This process prevents DNA oxidation and therefore prevents DNA damage that occurs in the normal process of aging. However, the life extension induced by mutants in TOR, SCH9 or RAS signalling is not totally explained by Rim15/Msn2/Gis1, suggesting that other independent unknown mechanisms are also important to extend life when these signalling pathways are not working. (See also Transcription detailed in **Section 4.6** of Introduction)

Interestingly, *tor1* deletion makes cells transitorily sensitive to hydrogen peroxide (Bonawitz et al., 2007). TOR controls several downstream processes in order to increase cell resistance to oxidative stress. Other genes subjected to TORC1 negative regulation via Sch9 are involved in mitochondrial function (Smets et al., 2010), sphingolipid homeostasis and signaling (Swinnen et al., 2013, Swinnen et al., 2014; Huang et al., 2013), autophagy and longevity (Sampaio-Marques et al., 2011). Recent work in the latter field has shown that abrogation of the conserved TOR, Ras/ cAMP-dependent PKA and/or Sch9 proteins, all negative regulators of autophagy, promotes longevity (Swinnen et al., 2013, Swinnen et al., 2014). Inhibiting the synthesis of proaging sphingolipids, including ceramides, has a positive effect on longevity by promoting autophagy (Hernandez-Corbacho et al., 2011; Swinnen et al., 2013, Swinnen et al., 2014).

8.5 TORC2 READOUTS

It has been demonstrated that TORC2 activates Ypk2 via direct phosphorylation (Kamada et al., 2005). Recent results indicate that TORC2 activity is also required for Pkc1 phosphorylation (Facchinetti et al., 2008), although is not clear whether this phosphorylation is direct. A constitutive active mutant of Ypk2 suppresses the lethality

provoked by the complete loss of TORC2 (Kamada et al., 2005), suggesting that most of TORC2-mediated functions, including actin remodeling, are mediated via Ypk2 (Cybilski and Hall, 2009). TORC2 function also controls the sphingolipid biosynthetic pathway (Aronova et al., 2008).

TORC2 signalling is less well-characterized than TORC1 signalling due to the absence of a rapamycin equivalent for TORC2. Even before the two TOR complexes were identified, Tor2 (but not Tor1) was known to regulate the cell cycle-dependent polarisation of the actin cytoskeleton (Schmidt et al., 1996), thereby implicating Tor2 (and hence later TORC2) in the spatial control of yeast cell growth. Further studies showed that the abberant depolarisation of the actin cytoskeleton in *tor2*^{ts} mutants could be suppressed by hyperactivation of the CWI pathway. Several genetic studies revealed that Pkc1, via Rom2 and Rho1, mediates TORC2 signaling to actin organisation (Schmidt et al., 1996; Helliwell et al., 1998; Loewith et al., 2002; Schmidt et al., 1997). Overexpression of components of the CWI pathway could restore viability and actin polarization defects of mutants compromised in their TORC2 function (Schmidt et al., 1996; Bickle et al., 1998; Helliwell et al., 1998). These results suggest that TORC2 functions upstream or in parallel to the CWI pathway to regulate actin polarization. Additionally, it was found that the protein kinases, Ypk1 and Ypk2, and the PH domain proteins, Slm1 and Slm2, which also play a role in actin polarization, are downstream targets of TORC2 signalling (Audhya et al., 2004; Fadri et al., 2005; Kamada et al., 2005). Interestingly, Ypk1/2 and Slm1/2 appear to be upstream regulators of the CWI pathway and might, therefore, provide a mechanistic link between TORC2 and the CWI pathway (Schmelzle et al., 2002; Ho et al., 2008). Since both Ypk1/2 and Slm1/2 were also shown to be regulated by sphingolipids, these proteins also couple TORC2 to sphingolipid metabolism and signalling (Aronova et al. 2008).

9. Nitrogen Catabolite Repression (NCR) pathway

In the presence of preferred nitrogen sources, that is, nitrogen compounds that can be easily converted into the main amino acid precursors; ammonia, glutamate, and glutamine, yeast activates the NCR pathway.

9.1 Components of NCR pathway

It represses the expression of genes involved in the use of alternative, less preferred nitrogen sources, such as proline, urea, allantoin, GABA. The expression of NCR genes results from the interplay of four transcription factors, two activators, Gln3 and Gat1/Nil1, and two repressors, Gzf3/Nil2/Deh2 and Dal80/Uga3, which bind to GATA sequences in the promoters (Cooper, 2002; Magasanik & Kaiser, 2002). Gat1 dependent activation of NCR depends on Gln3 activating Gat1 expression, whereas Gln3 dependent activation can promote transcription in the absence of the other transcription factors (Georis et al., 2009). TORC1 inhibits transcription of NCR genes by controlling Gln3 and Gat1 function (Beck et al., 1999; Cardenas et al., 1999; Hardwick et al., 1999; Shamji et al., 2000) (Fig 7). Normally, during growth on rich nitrogen sources, Gln3 is phosphorylated and sequestered in the cytoplasm through its binding with the cytoplasmic anchor protein Ure2 (Fig 7).

Rapamycin treatment, however, rapidly triggers, in a Sit4-dependent manner, the dephosphorylation of Gln3, dissociation from Ure2 and entry into the nucleus, where Gln3 can exert its transcriptional activator function on NCR genes (Beck et al., 1999). Nevertheless, PP2A phosphatase activity is also necessary for Gln3 nuclear import upon rapamycin treatment, although the mechanism behind remain elusive (Tate et al., 2009) (Fig 7). Additionally, TORC1 promotes via an unknown mechanism the phosphorylation of Ure2 and this might further modulate the interaction between Gln3–Ure2 (Cardenas et al., 1999; Hardwick et al., 1999). Rapamycin treatment also triggers nuclear import of Gat1 (Beck et al., 1999). How TORC1 regulates this process is unclear, yet it appears to be different from TORC1 dependent Gln3 control and does not involve Ure2 or Sit4 (Kuruvilla et al., 2001; Crespo et al., 2002; Georis et al., 2008). Both transcription factors have overlapping but also specific effects on NCR genes (Kuruvilla et al., 2001).

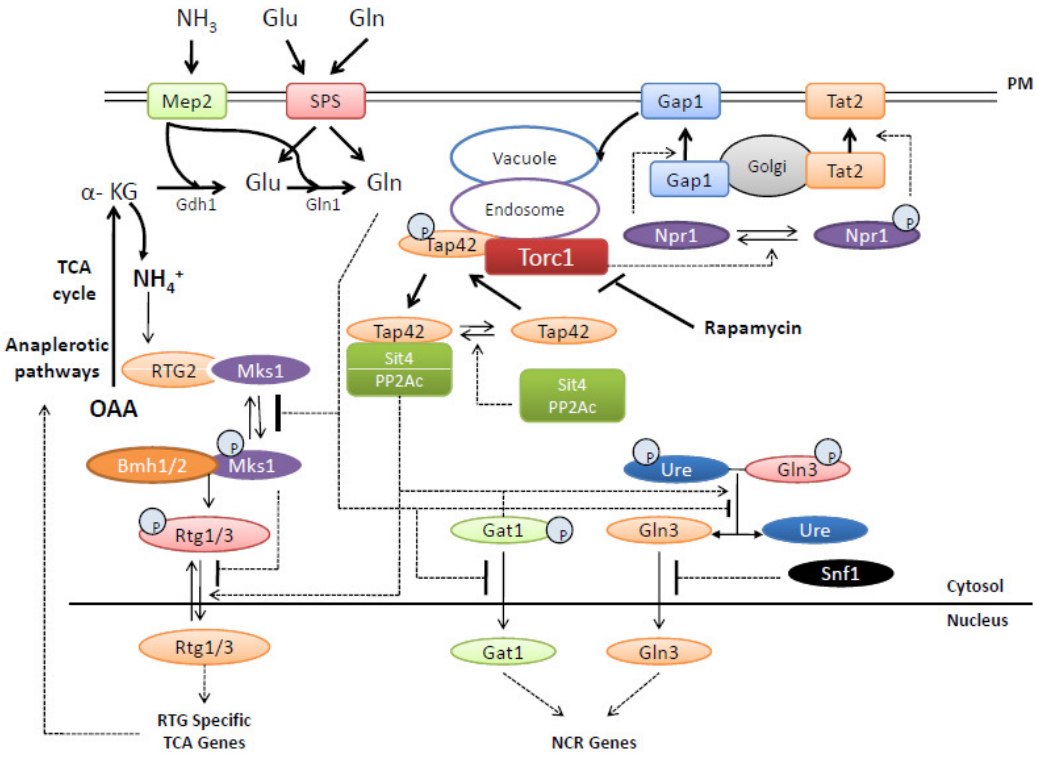


Figure 7: Nitrogen regulation and the TORC1 network. Nitrogen availability, manifested through intracellular glutamate (Glu) and glutamine (Gln) levels, affects the activity of the Tor Complex 1 (TORC1) as well as expression of the Nitrogen Catabolite Repression Pathway (DAL/NCR) and retrograde signaling (RTG) genes. TORC1 also regulates these sets of genes through modulation of Tap42-PP2A phosphatase activity in response to, and likely in parallel with, nitrogen availability. The SPS (Ssy1-Ptr3-Ssy5) signaling system regulates amino acid permease gene expression. The general amino acid (GAP) permease and a collection of amino-acid-specific permeases, including Tat2, are inversely regulated by TORC1, at least in part through control of vesicular trafficking by the Npr1 kinase. Snf1 aids this derepression by phosphorylating Gln3. Image adapted from (Broach, 2012; Conrad et al., 2014; Rødkaer and Faergeman, 2014; Zaman et al., 2008)

As mentioned earlier, retrograde response pathway (RTG) is also involved in nitrogen metabolism controlled by TORC1 function (Fig 6 and 7). Apart from having other functions, this pathway induces the expression of genes whose products are required for the biosynthesis of α-ketoglutarate as precursor for glutamate synthesis in cells grown on poor nitrogen sources as well as in respiration-deficient cells (Liu and Butow, 2006). Expression of these genes requires the transcriptional activators Rtg1 and Rtg3. TORC1 controls the cytoplasmic sequestration of these factors through phosphorylation of Mks1, which thereby forms a complex with the 14-3-3 proteins Bmh1/2 to provide the cytoplasmic anchor for Rtg1 and Rtg3. Rapamycin treatment induces dephosphorylation

of Mks1 and causes disassembly of the complex. This directs Mks1 to bind to the positive regulator of the pathway, i.e. Rtg2, thereby relieving the cytoplasmic sequestration and promoting nuclear translocation of Rtg1 and Rtg3 and the induction of the target genes of RTG pathway (Komeili et al., 2000; Dilova et al., 2002, 2004; Tate et al., 2002; Liu et al., 2003). Genome-wide expression analysis revealed that Tap42 is probably also involved in this regulation mechanism (Duvel et al., 2003) (See also the RTG pathway in the following section)

9.2 Regulation of Gln3

Gln3 has a total of 146 Ser/Thr putative phosphorylation sites, and its phosphorylation state determines its exclusion from the nucleus (Rai et al., 2013). Gln3 and Gat1 phosphorylation/dephosphorylation and shuttling in/out the nucleus seem to respond separately to TORC1 and nitrogen limitation. Rapamycin treatment causes Gat1 and to a lesser extent Gln3 nuclear localization independently of TORC1 controlled phosphatase activity (Georis et al., 2011). In contrast, nitrogen limitation such as growth on proline or addition of the glutamine synthetase inhibitor, methionine sulfoximine (Msx), causes Gln3 but not Gat1 nuclear localization. Changes in Gln3 phosphorylation also do not seem to correlate with the activity of a single pathway upstream upstream of TORC1. For example, Gln3 phosphorylation is reduced upon rapamycin addition but not under nitrogen limitation, while both conditions inhibit TORC1 activity. Inactivation of TORC1 can affect Gln3 phosphorylation status via activation of the Tap42–PP2A and Tap42–Sit4 complexes (Beck & Hall, 1999). Gln3 is peripherally associated with membranes, and this localization may facilitate its control by TORC1-dependent phosphorylation/dephosphorylation events (Puria et al., 2008). Additional evidence for nitrogen limitation acting in parallel rather than as part of the TORC1-mediated control of NCR genes includes the fact that rapamycin cannot activate NCR when Tap42 is inactivated, while Tap42 inactivation has no effect on the response to nitrogen limitation (Duvel et al., 2003).

Hence, nitrogen cues seem to control NCR gene expression via at least two parallel signaling branches, only one of them conveying TORC1 dependent signals. In further support for this observation, a very recent study has analyzed the effect of the five different conditions regularly used to affect the regulation of NCR gene expression: nitrogen starvation, Msx addition, nitrogen limitation, rapamycin addition, and leucine

starvation (Tate and Cooper, 2013). This study has demonstrated that Sit4 and PP2A are not required in all cases to trigger Gln3 nuclear localization. Leucine starvation or treatment with leucyl-tRNA synthetase inhibitors also did not elicit increased nuclear Gln3 levels. As result of this work, the authors have proposed that rather than a single TORC1-dependent pathway, different pathways are involved in NCR regulation, which converge in a common regulatory branch where glutamine and/or a related metabolite act as the metabolic signal.

9.3 Control of Nitrogen uptake

One important level of NCR regulation takes place through control of the uptake of alternative nitrogen sources. This occurs both at the transcriptional and at post-transcriptional level. Under nutrient-rich conditions, SPS (Ssy1-Ptr3-Ssy5) controlled expression of amino acid permeases (AAPs) specific for different amino acid subsets takes place, whereas in the presence of poorer nitrogen sources or total absence of nitrogen, expression of most of these permeases is replaced by expression of the general AAP, Gap1 (Fig 7) (Ljungdahl & Daignan-Fornier, 2012). The TORC1 responsive kinase Npr1 is responsible for the stabilization of Gap1 at the plasma membrane and vacuolar sorting of specific AAPs like Tat2 in nitrogen-derepressed conditions (Schmidt et al., 1998; Springael & Andre, 1998). Increasing evidence has shown instead that Npr1 controls endocytosis of the permeases by phosphorylation of ubiquitin ligase adaptors, also known as arrestins, for example Aly1,2, Bul1,2 (O'Donnell et al., 2010; MacGurn et al., 2011; Merhi & Andre, 2012). It is known that inactivation of TORC1 leads to Tap42-Sit4-dependent dephosphorylation of Npr1 (Schmidt et al., 1998; Jacinto et al., 2001; Gander et al., 2008). Most recent work links Tap42-Sit4-dependent Npr1 dephosphorylation to its activation and subsequent phosphorylation of arrestin like Bul proteins, which in turn inhibits endocytosis of Gap1 under nitrogen limitation (Merhi & Andre, 2012).

10. Retrograde Signaling (RTG) Pathway

In budding yeast, retrograde signaling is a pathway which transmits signal from mitochondria to the nucleus and other cellular compartments, which helps cells in monitoring and adjusting to the changes in the functional state of their mitochondria (reviewed in Butow and Avadhani, 2004). In addition, it can be seen during the reduction of mitochondrial respiratory functioning or the disturbance in the key metabolic pathways

like krebs cycle (McCammon et al., 2003). These fluctuations lead to changes in the expression of a subset of nuclear genes, which, in turn, result in a recasting of carbohydrate and nitrogen metabolism. The cells induce anaplerotic pathways to supply key intermediates–oxaloacetate, acetyl-CoA and citrate to the Krebs cycle to maintain the synthesis of α-ketoglutarate, the direct precursor for glutamate (Liu and Butow, 1999). This integration of carbohydrate and nitrogen metabolism is also reflected in the finding that retrograde signaling can be activated in cells in which the TOR pathway, one of whose functions is nutrient sensing, is inhibited by the immunosuppressant, rapamycin (Komeili et al., 2000; Shamji et al., 2000). Three retrograde response genes: *RTG1, RTG2*, and *RTG3* have been identified by genetic screening, which are essential for the transduction of the retrograde signal. (Sekito et al., 2000; Liu et al., 2003)

10.1 Overview of the RTG response

RTG pathway initiates upon encountering a signal from the dysfunctional mitochondria (Fig 8, reviewed in Jazwinsky, 2013). Until now, several positive and negative regulators of the RTG pathway have been identified, which have given insights into the signaling from mitochondria nucleus and its regulation (Fig 8). This signal is received and transmitted by Rtg2 to the heterodimeric transcription factor *RTG1-RTG3*, which partially dephosphorylates Rtg3 leading Rtg1-Rtg3 complex to the nuclear translocation, so as to induce the expression of an array of retrograde target genes (Fig 8).

Basic helix-loophelix Leucine zipper-(bHLH/Zip) type transcription factors are encoded by *RTG1* and *RTG3,* which form a heterodimer and bind to the promoter region of target genes. The transcriptional activation domain of the Rtg1/3 complex lies within Rtg3 (Rothermel et al., 1997; Sekito et al., 2000). Unlike most of other bHLH-type transcription factors, Rtg1/3 binds to an unusual site, GTCAC (R box) (Jia et al., 1997; Liao and Butow, 1993). Rtg3 activation corresponds to its partial dephosphorylation followed by nuclear translocation along with Rtg1p (Sekito et al., 2000).

Rtg2 is a cytoplasmic protein and a member of actin/Hsp70/sugar kinase superfamily, with a N-terminal ATP (Adenosine Triphosphate) binding domain (essential for Rtg2 function; Bork et al., 1992; Koonin, 1994). Structures of these ATP binding domains offer a similarity with two subdomains and a pseudodyad symmetry with the active site predicted to be in the cleft formed between the two subdomains (reviewed in Liu and Butow, 2006). This nuclear translocation is inhibited by Mks1, which in association with the 14-3-3

protein Bmh1/2 promotes Rtg3 hyperphosphorylation (Dilova et al., 2004; Sekito et al., 2002). 14-3-3 proteins (Bmh1/Bmh2), which are conserved in all the eukaryotic organisms examined, are involved in a diverse array of cellular functions via interaction with different partner proteins (often phosphorylated proteins).

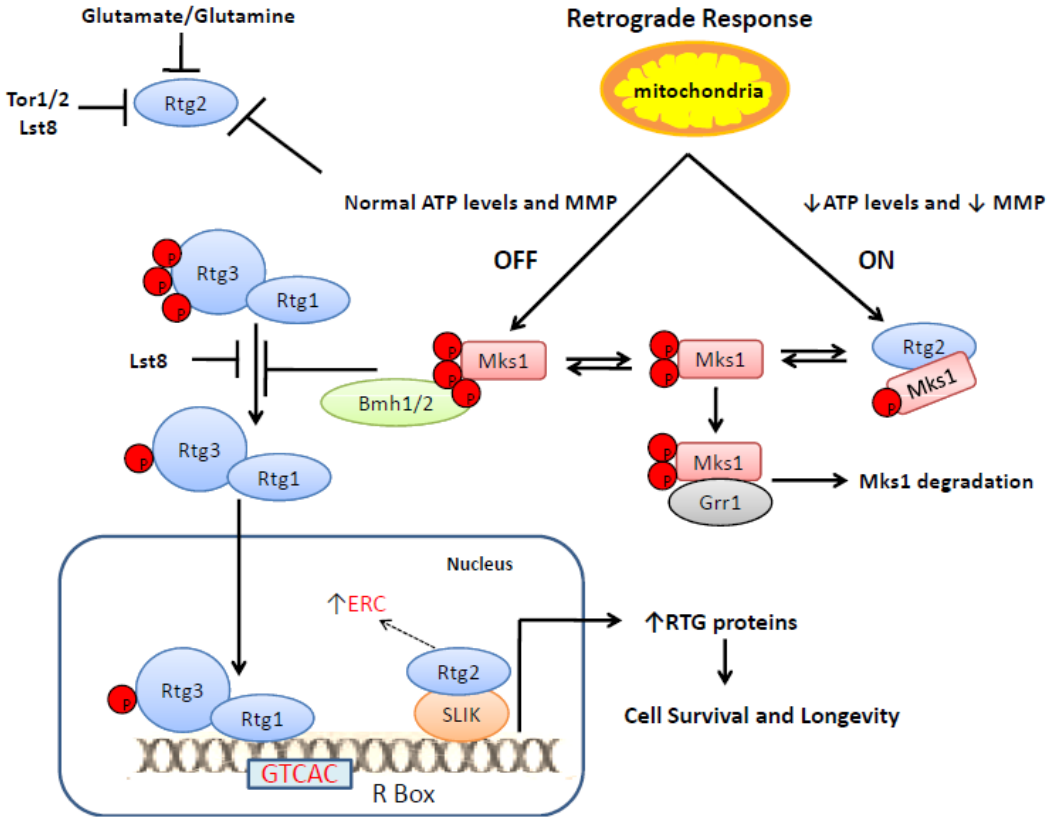


Figure 8: Yeast retrograde response. Positive and negative regulators of the RTG pathway are shown. Lst8 functions at two sites, with one upstream and the other downstream of Rtg2. Activation of the RTG pathway leads to a higher level of glutamate and glutamine, which in turn inhibit the RTG pathway. This constitutes a feedback control for retrograde signaling. External glutamate and glutamine are sensed through the SPS amino acid sensing pathway to inhibit Rtg2.Under normal conditions, ATP competitively binds to Mks1, a negative regulator of the retrograde response (RTG), releasing Rtg2 and allowing Mks-1 to bind to the 14-3-3 protein Bmh1/2, another negative regulator of the RTG. Consequently, this inhibits the nuclear translocation of the Rtg1/3 complex. Rtg2 has also been shown to suppress the formation of extrachromosomal rDNA circles (ERCs). Under conditions of reduced mitochondrial membrane potential or mitochondrial stress, Rtg2 is stabilized by Mks1. Stabilized Rtg2 promotes the dephosphorylation of Rtg3. Subsequently, Rtg1/3 complex translocates to the nucleus where it turns on the transcription of retrograde genes. Rtg2 can also modulate the retrograde response by interacting with the transcriptional co-activator SAGA-like (SLIK) complex. As a result, cell survival is promoted. Grr1-dependent degradation of free Mks1 ensures an efficient switch between the Rtg2-Mks1 complex and Bmh1/2-Mks1 complex. Image adapted from (Ferreira Júnior et al., 2005; Hill and Van Remmen, 2014; Jazwinski, 2013; Liu and Butow, 2006).

The two functionally unessential 14-3-3 proteins in yeast are encoded as *BMH1* and *BMH2* (Gelperin et al., 1995; van Heusden et al., 1995). Another possible mechanism, by which these proteins regulate the RTG pathways, is by binding to Rtg3 and keeping it in an inactive state (van Heusden and Steensma, 2001) (Fig 8). Grr1 acts as a positive regulator of the RTG pathway by leading Mks1p to ubiquitin-mediated degradation (Liu et al., 2005). This ubiquitination process is catalyzed through a cascade of enzymatic reactions mediated by enzymes known as E1, E2 and E3. Grr1 has the F box component of the SCF^{Grr1} (Skp1- Cdc53/Cullin-F box protein) E3 ubiquitin ligase, serves as an adaptor complex to bring its substrate to the vicinity of the E2 ubiquitin-conjugating enzyme Cdc34 (Deshaies, 1999; Willems et al., 1999) (Fig 8).

Lst8 was originally identified through a mutation synthetically lethal with the *sec13-1* mutation (Liu et al., 2001). Lst8 is an essential protein comprised of seven WD-repeats (tryptophan-aspartate repeats) (Roberg et al., 1997; Liu et al., 2001). As earlier mentioned, Lst8 is an intrinsic component of two TOR kinase complexes: TORC1 and TORC2 (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003). The negative regulation of the RTG pathway by Lst8 is achieved with its one site upstream and other site downstream to Rtg2 (Chen and Kaiser, 2003; Liu et al., 2001) (Fig 8).Wd-protein Lst8 (one subunit of nutrient sensor TORC1), is a known retrograde response regulator, inhibits retrograde signaling both upstream as well as downstream of Rtg2, by regulating Rtg2 and Mks1 in negative and positive manner respectively (Fig 8).

TORC1 antagonises Rtg1/Rtg3 function by promoting their association with Mks1 and the 14-3-3 proteins Bmh1 and Bmh2 (Dilova et al., 2004). Dephosphorylation of Mks1, following TORC1 inactivation, or as a result of mitochondrial dysfunction, causes disassembly of this inhibitory complex, association of Mks1 with its inhibitor Rtg2, and subsequent translocation of Rtg1/Rtg3 to the nucleus (Liu et al., 2003). Activation of the RTG pathway via rapamycin treatment is strictly dependent on Rtg2. The mechanism by which TORC1 impinges on Mks1 (and/or Rtg2) is unclear but involves, at least in part, the TORC1 subunit Lst8. Lst8 has been identified as a negative regulator of the RTG pathway with multiple roles in the regulation of RTG and NCR sensitive genes (Giannattasio et al., 2005). These authors have indicated that the retrograde signalling due to mitochondrial dysfunction is separable from TOR regulation of RTG and NCR gene expression by comparing the rapamycin sensitivity of ρ° and ρ^{+} yeast cells.

10.3 Transcriptional Analysis of the Retrograde Response

There are two groups of *RTG*-target genes (Chelstowska et al., 1999; Liao and Butow, 1993; Liao et al., 1991; Liu and Butow, 1999), the first group includes *CIT2 (*encodes the peroxisomal isoform of citrate synthase) and *DLD3 (*encodes a cytoplasmic isoform of d-lactate dehydrogenase), which show a robust retrograde response, and the second group includes several tricarboxylic acid (TCA) cycle genes namely *CIT1* (encodes a mitochondrial isoform of citrate synthase), *ACO1* (encodes aconitase), and *IDH1/2* (encodes NAD^+ dependent isocitrate dehydrogenase) which do not show an obvious retrograde response.

Analysis of expression of these *RTG* target genes reveals both the mode of their regulation by Rtg1/3 and also provides the prototypical target gene of the RTG pathway, *CIT2 (*Liao and Butow, 1993; Liao et al., 1991*).* It is has been reported that *CIT2* contains an inverted repeat of R box sequences in its promoter region this acts as a binding site for the Rtg1/3 heterodimer. The binding of Rtg1/3 to the *CIT2* promoter is abolished when mutations in the R box sequences is observed, resulting in failure of both basal and retrograde expression of *CIT2* (Reviewed in Liu and Butow, 2006).

10.5 Functions of RTG pathway

10.5.1 Glutamate biosynthesis and homeostasis

As previously mentioned, RTG pathway involves in glutamate metabolism and hence *rtg* mutants are glutamate auxotrophs reflecting *RTG*-dependent expression of genes involved in glutamate biosynthesis, namely *CIT1*, *CIT2*, *ACO1*, *IDH1*, and *IDH2*. Glutamate has been shown to inhibit expression of these early TCA cycle genes as well as *CIT2* (Haselbeck and McAlister-Henn, 1993; Kim et al., 1986; Rosenkrantz et al., 1994; Vélot et al., 1996), which can now be viewed as a feedback control of glutamate biosynthesis. Glutamate needs to be maintained at a certain level to meet biosynthetic needs. Therefore, one main function of the RTG pathway is to ensure that a sufficient level of α-ketoglutarate for glutamate synthesis is made to meet the demand. (Reviewed in Liu and Butow, 2006) (Fig 7).

10.5.2 Mitochondrial Dysfunction

Expression of these four genes (*CIT1*, *ACO1*, and *IDH1*/2) switches from Hap2-5 control to Rtg1/3 in cells with compromised mitochondrial function. The switch ensures the supply of ATP through catabolic oxidative metabolism and provision of metabolic intermediates for anabolic biosynthesis functions of the TCA cycle. The heme-responsive transcription factor *HAP1* and the heme-independent *HAP2*-5 transcription factor complex collectively has the global control in the expression of the TCA cycle genes, and many genes involved in oxidative metabolism in cells grown under respiratory conditions. (Reviewed in Liu and Butow, 2006). The retrograde response can also be activated by defects in genes encoding TCA cycle enzymes. Genome-wide transcriptional profiling suggested that different retrograde responses are activated according to specific mitochondrial defects. It has been shown that genome responses to treatment of rho^+ (cells with mitochondrial DNA) cells with three different inhibitors of mitochondrial function, antimycin A (inhibitor of mitochondrial electron transport by blocking the reoxidation of reduced cytochrome b), CCCP (uncoupler of ATP synthesis by dissipating the proton gradient across the inner mitochondrial membrane), and oligomycin (a specific inhibitor of the F_0 component of the F_1-F_0 ATP synthase complex) were examined and compared to that elicited in rho^0 cells. In terms of changes in global gene expression, antimycin is most similar to rho^0 cells, whereas there is little overlap of up-regulated genes between CCCP or oligomycin treatment and rho^0 cells. There is also a significant overlap of up-regulated genes between CCCP and antimycin or oligomycin treatment (reviewed in Liu and Butow, 2006).

10.6 Regulatory Role of RTG pathway

10.6.1 Retrograde response and longevity

The relationship between the RTG response and longevity was first implicated in a long-lived rho° yeast strain. Specifically, deletion of Rtg2 was shown to reverse replicative life span extension in rho° cells (Kirchman et al., 1999). A hallmark phenotype of yeast ageing is the accumulation of Extra Chromosomal rDNA Circles (ERCs), which have detrimental effects on cellular homeostasis (Sinclair and Guarente, 1997) (Fig 8). It has been reported that activation of the RTG response is associated with the accumulation of ERCs, as in Rho ° cells which have a great accumulation of ERCs. Deletion of *FOB1,* a gene that

encodes for a protein required for ERC formation, eliminates accumulation of ERCs and extends lifespan by 50% (Borghouts et al., 2004). Rtg2 suppresses ERCs production (Fig 8) and disruption of the SLIK complex by *GCN5*. However, disruption of SLIK suppresses the RTG and lifespan extension of rho° cells (Kim et al., 2004). Together, these finding suggested that lifespan extension in rho° cells is mediated by RTG signaling, and not by ERC accumulation (Fig 8). In addition current research studies have established a relationship between the RTG pathway in yeast and the NF-κB pathway in higher organisms. Involvement of NF-κB in chromosomal stability, co-regulation of mitochondrial respiration, and cross talk with the TOR pathway points to a conserved mechanism also found in yeast (Srinivasan et al., 2010).

10.6.2 Mitophagy

Mitophagy is the term given to selective removal of mitochondria by autophagy. Mitophagy utilizes the same machinery as autophagy; however, the process becomes selective through the participation of Atg32, which tags mitochondria for elimination (Jazwinski, 2013). Retrograde signaling appears to be important for mitophagy, at least in stationary phase. In yeast stationary phase mitophagy requires the *AUP1* gene (Journo et al., 2009). It has been identified that a conserved mitochondrial protein phosphatase homolog, Aup1, is found to regulate the RTG pathway. Furthermore, deletion of *AUP1* prevents retrograde signaling and expression of retrograde target genes, and deletion of *RTG3* suppresses stationary phase mitophagy. In addition, the phosphorylation pattern on Rtg3 is dependent on Aup1 in stationary phase (Journo et al., 2009).

11. RAS-cAMP/PKA PATHWAY

The cAMP-PKA pathway plays a major role in the control of metabolism, stress resistance and proliferation, in particular in connection with the available carbon source. In response to a sudden availability of rapidly fermentable sugars, the pathway transiently induces the synthesis of cAMP to boost the activity of the cAMP-dependent protein kinase (PKA). In turn, PKA will affect several downstream targets thereby allowing cells to make the necessary adaptations for fermentative growth. These include the upregulation of glycolysis, the stimulation of cell growth and cell cycle progression, the downregulation of stress resistance and gluconeogenesis, and the mobilization of the reserve carbohydrate

glycogen and the stress protectant trehalose (Thevelein et al., 2000; Santangelo, 2006; Tamaki, 2007; Gancedo, 2008).

11.1 COMPONENTS

Protein kinase A (PKA) PKA is a hetero-tetramer composed of two catalytic subunits, redundantly encoded by TPK1, TPK2 and TPK3 and two regulatory subunits, encoded by BCY1 (Toda et al., 1987a; Toda et al., 1987b) (Fig 9). Binding of the secondary messenger cAMP to the regulatory subunit induces the dissociation of the hetero-tetramer and activation of the catalytic subunits (Kuret et al., 1988). PKA activity is critical for yeast since at least one of the three catalytic subunits is necessary for viability (Toda et al., 1987b). *bcy1* mutants exhibit a number of phenotypes, including heat-shock sensitivity, sensitivity to nutritional deprivation, and failure to arrest in G1 in response to starvation (Toda et al., 1987a). Nevertheless, there are several examples of separate, specific functions for the different subunits, such as the induction of pseudohyphal growth, the expression control of genes involved in iron uptake or branched amino acid biosynthesis, or the regulation of mitochondrial enzymes (Robertson and Fink, 1998; Pan and Heitman, 1999; Robertson et al., 2000; Zhu et al., 2000; Singh et al., 2004; Chevtzoff et al., 2005; Ptacek et al., 2005). Given their central and essential role, the activity of the catalytic subunits is tightly regulated.

11.2 Regulation of the cAMP-PKA pathway

A dual glucose-sensing system is involved in the activation of the cAMP-PKA pathway: On the one hand, extracellular glucose sensing occurs through the GPCR system composed of Gpr1 and its associated Gα protein, Gpa2, and on the other hand, an intracellular system dependent on glucose uptake and hexokinase-mediated phosphorylation that activates in some unknown way the Ras proteins (Rolland et al., 2000). Hence, yeast adenylate cyclase (AC) is controlled by two G proteins that each mediate one branch of a glucose-sensing pathway (Fig 9). In *S. cerevisiae*, there are three glucose phosphorylating enzymes (Hxk1, Hxk2 and Glk1) and any of these proteins can fulfil this phosphorylation requirement to activate the cAMP-PKA pathway. Moreover, the intracellular glucose phosphorylation signal is further transduced to the cAMP-PKA pathway via the Ras proteins, Ras1 and Ras2, which belong to the group of small G proteins. The GTP bound, active Ras proteins stimulate the activity of the adenylate

cyclase Cyr1 (also known as Cdc35), the enzyme which catalyses the synthesis of cAMP from ATP (reviewed in Smets et al., 2010) (Fig 9). Extracellular glucose detection occurs through a G protein-coupled receptor (GPCR) system, composed of Gpr1 and Gpa2 (Kraakman et al., 1999). Gpr1 belongs to the G protein-coupled seven-transmembrane receptor (GPCR) superfamily (Yun et al., 1997; Xue et al., 1998) and Gpa2 is a member of the heterotrimeric G protein a subunit (Gα) protein family (Nakafuku et al., 1988).

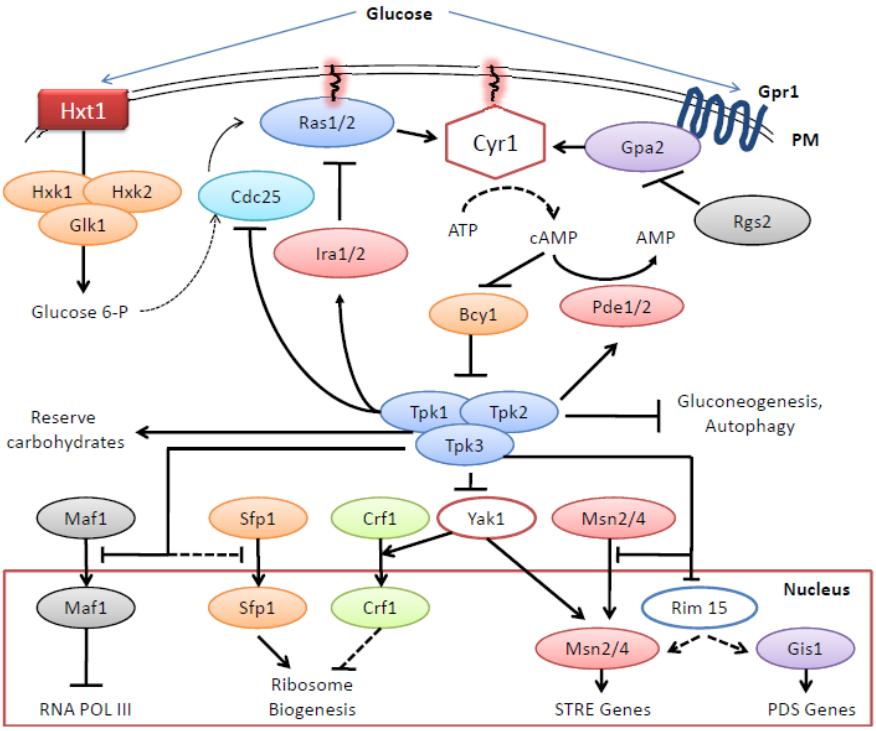


Figure 9: Glucose activation of the cAMP-PKA pathway. Adenyl Cyclase encoded by Cyr1 is activated by glucose through two different G-protein-coupled systems. The Gpr1-Gpa2-Rgs2 GPCR system senses extracellular glucose, while the Cdc25,-Ras1,2-Ira1,Ira2 system senses intracellular glucose through glucose catabolism in glycolysis. The glucose-sensing GPCR, Gpr1, and the Cdc25,Sdc25 proteins stimulate guanine nucleotide exchange on Gpa2 and Ras1,2, respectively. Rgs2 and Ira1,2 act as GAPs on Gpa2 and Ras1,2, respectively. cAMP binds to the Bcy1 regulatory subunits of PKA causing dissociation and activation of the catalytic subunits, Tpk1-3. Activated PKA mediates the fast transition from respiratory to fermentative growth via the modulation of numerous downstream targets. Arrows and bars represent positive and negative interactions, respectively. Dashed lines represent putative or indirect interactions. Image adapted from (Smets et al., 2010)

Addition of glucose to derepressed cells activates Gpr1, which in turn stimulates the exchange of GDP for GTP on Gpa2 (Kraakman et al., 1999). GTP-bound Gpa2 activates the cAMP-PKA pathway through stimulation of adenylate cyclase (Rolland et al., 2000; Peeters et al., 2006). Gpa2 interacts with Rgs2, a member of the family of regulators of G

protein signalling (RGS), that negatively regulates the Gpa2-GTP signal by stimulating the intrinsic GTPase activity of Gpa2 (Versele et al., 1999).

The kelch repeat proteins directly bind to the catalytic subunits of PKA and thereby stimulate the association of the catalytic and regulatory subunits of PKA, lowering PKA activity. Their inactivation thus reduces the amount of cAMP required to activate PKA, creating a mechanism for the activation of PKA without change in the cAMP level or for synergistic stimulation of PKA activation after an increase in the cAMP concentration. On the other hand it is shown that Krh1,2 affect both the abundance and phosphorylation state of Bcy1, such that its levels increase upon glucose limitation in a Krh-dependent manner. PKA establishes a negative feedback loop by phosphorylation of Bcy1 in Ser^{145}, which targets Bcy1 for degradation unless it is protected by Krh proteins (Budhwar et al., 2010; Budhwar et al., 2011).

A strong negative feedback mechanism ensures that the glucose induced increase in cAMP levels and PKA activity are transient and can only be triggered in glucose derepressed cells. A balance between cAMP synthesis catalysed by adenylyl cyclase, and cAMP degradation catalysed by phosphodiesterases, encoded by *PDE1* and *PDE2,* establishes the level of cAMP in the cell. PKA also regulates the localization and protein concentration of Pde2 (Hu et al., 2010). The small GTP-binding proteins, Ras1 and Ras2, stimulate adenylyl cyclase through direct interaction with the enzyme. Ras1 and Ras2 cycle between a GTP-bound and a GDP-bound state and can stimulate adenylyl cyclase only in the GTP-bound state. The level of Ras-GTP results from a balance between GTP loading and GTP hydrolysis, the former catalysed by the guanine nucleotide exchange factor, Cdc25, and the latter catalysed by the intrinsic GTPase activity of Ras, which can be dramatically stimulated by redundant GTPase activating proteins (GAPs), Ira1 and Ira2 (Fig 9). Activating mutations in Ras polypeptides increase GTP association in a GEF-independent manner and are responsible for many human cancers (Wilson et al., 1993). Glucose addition to starved cells results in a rapid but transient increase in intracellular cAMP levels through a process dependent on Ras. The Ras proteins are also involved in the negative feedback control of the activated cAMP-PKA pathway. The rapid increase in cAMP production matches the increase in glucose-stimulated Ras-GTP levels in the cell and the subsequent decrease in cAMP levels likely results from feedback inhibition of synthesis and perhaps stimulation of Pde activity (Fig 9) (Santangelo, 2006; Zaman et al., 2008).

11.3 FUNCTIONS OF RAS-cAMP-PKA SIGNALLING

PKA affects a wide variety of targets in yeast cells. In general, it acts positively on properties that are associated with rapid fermentative growth (e.g. rate of fermentation and growth) and acts negatively on properties associated with slow, respirative growth or stationary phase (e.g. accumulation of carbohydrate stores, stress tolerance, and other stationary-phase characteristics) (Fig 9). Active PKA is thought to phosphorylate proteins involved in transcription, energy metabolism, and cell cycle progression (Griffioen and Thevelein, 2002), thus transmitting the glucose regulatory signal (Fig 9). The Ras-induced changes in gene expression depend entirely on PKA, indicating that Ras affects transcription in response to glucose solely through modulation of PKA (Toda et al., 1987; Wang et al., 2004; Zaman et al., 2008). PKA activates the transcription of ribosomal protein genes as well (Herruer et al., 1987; Kraakman et al., 1993; Griffioen et al., 1994). It was reported that PKA promotes nuclear localization and binding of the transcriptional activator Sfp1 to the promoters of ribosomal protein genes (Marion et al. 2004) (Fig 9). In addition, PKA appears to induce transcription of ribosomal protein genes also by inhibition of Yak1, which in turn is required to promote the activity of the transcriptional corepressor Crf1 (Martin et al., 2004). PKA further stimulates protein synthesis indirectly by inhibiting nuclear import of Maf1, which represses 5S rRNA and tRNAs transcription by RNA Polymerase III (Moir et al., 2006; Willis and Moir, 2007). PKA is a known inhibitor of autophagy, during periods of nutrient starvation (Budovskaya et al., 2004; Schmelzle et al., 2004; Yorimitsu and Klionsky, 2005). PKA phosphorylation negatively controls the recruitment of Atg1 to the sites of autophagosome formation upon nutrient limitation (Budovskaya et al., 2005).

11.3.1 The role of RAS/PKA in the oxidative stress response and longevity

The genes involved in glucose signalling are associated to life extension. The Ras2-cAMP pathway is important to regulate aging in yeast and presents a certain degree of homology with the insulin/IGF1-(like) longevity pathway of worms and mammals (Fabrizio et al., 2003). Mutations in RAS and Sch9 signalling pathways extend the chronological life expand and also the resistance to oxidative stress in *Saccharomyces cerevisiae*, through the activation of the general stress response Msn2/Msn4 and the protein kinase Rim15 (Fabrizio et al., 2001; Fabrizio et al., 2003).

Sch9, Cyr1 and Ras2 have been demonstrated to play a role in oxidative stress resistance (Fabrizio et al., 2001; Fabrizio et al., 2003; Longo, 2003). The *ras2, sch9* and *cyr1* mutants are more resistant to oxidants than the correspondent wild type strain. The mutants *ras2, cyr1* and *sch9* increase oxidative stress resistance through the following mechanisms: 1) by preventing superoxide toxicity (in the former mutants, higher aconitase activity was detected compared to wild type cells); 2) by activating the Msn2/Msn4 transcription factor which in turn induces the expression of some genes that confer cells with more resistance to oxidation (*CTT1,*encoding for catalase or *SOD2,* encoding for superoxide dismutase); 3) other unknown mechanisms (Fabrizio et al., 2001; Longo and Fabrizio, 2002; Longo, 2004). Fabrizio et al., (2003) demonstrated that the oxidative resistance observed in either *ras2* or *cyr1* mutants was due to the elevated Msn2/Msn4 and *SOD2* activity.

A recent study highlights the contribution of the Ras/PKA signalling pathway to oxidative stress (Hlavatá et al., 2008). These authors demonstrate that Ras activity can favour the accumulation of ROS species through two different routes: A) One route is via PKA/cAMP, leading to the inhibition of genes with antioxidant capacity, this occurs by blocking the activity of the Msn2/Msn4 transcription. B) An independent route affects the mitochondrial ATP synthase and causes higher ROS production by the mitochondria. However, (Heeren et al., 2004) characterised certain dominant activated RAS mutants that produced superoxide in the absence of a complete mitochondrial electron transport chain. Therefore these authors discuss the possibility of the existence of a non-mitochondrial source for ROS generation in which RAS activity also plays an important role.

The Ras-cAMP pathway is related to the actin dynamics in response to stress (Ho et al., 2001; Hubberstey et al., 2002). Actin and the Ras-cAMP interact to regulate oxidative stress and cell viability as demonstrated by Gourlay and Ayscough (2005; 2006). They also show that in mutants with stabilised actin, Ras signalling is hyperactivated. The resultant increase in cAMP levels leads to the loss of mitochondrial membrane potential, accumulation of ROS, and cell death. The authors suggest that the effect of cAMP elevation in inducing actin-mediated apoptosis functions primarily through the Tpk3 subunit of PKA. In wild type cells growing in stationary phase, Ras pathway becomes inactivated, leading to a descent in cAMP activity resulting in the downregulation of PKA activity (Gourlay and Ayscough, 2006). Low PKA activity does not induce loss of mitochondrial membrane potential, therefore does not provoke ROS accumulation or loss

of cell viability. However, in actin stabilised cells, elements of the Ras pathway are localised in F-actin large aggregates at the stationary phase. The Ras/cAMP route becomes activated, leading to PKA high activity levels which induce mitochondrial ROS production and more actin stabilisation. Altogether these effects cause cell death. All these mechanisms have a logical biological explanation. ATP/ADP correct homeostasis is required not only for nutritional maintenance of cells but also for correct actin dynamics of the cytoskeleton. ATP production is thus essentially controlled by the Ras-cAMP activity. This pathway represents a define link between environmental sensing, actin remodelling, and apoptosis in *Saccharomyces cerevisiae* (Gourlay and Ayscough, 2006).

12. Snf1 pathway

Budding yeast preferentially uses glucose as a fermentable source of carbon and energy. As mentioned earlier in presence of preferential carbon source a complex regulatory network is activated that downregulates many components involved in transport and metabolism of alternative carbon sources, as well as respiratory function. These adaptations are primarily dependent on the AMP-activated kinase (AMPK)/sucrose nonfermenting 1 protein, Snf1. Snf1 was first identified by Celenza & Carlson (1984) in the budding yeast and it is regarded as the founding member of the SNF1/AMPK family of protein kinases, which is not only highly conserved in eukaryotes but also required for energy homeostasis in mammals, plants, and fungi (Hedbacker and Carlson, 2006). The central role of AMPKs is to sense and respond to conditions in which energy reserves are depleted, by promoting both generation and preservation of energy (Conrad et al., 2014). In *S. cerevisiae*, Snf1 mainly responds to decline in glucose levels, by promoting respiratory metabolism, glycogen accumulation, gluconeogenesis, autophagy, glyoxylate cycle, peroxisome biogenesis (Ashrafi et al., 2000; Lin et al., 2003; Hedbacker & Carlson, 2008; Usaite et al., 2009). In addition, Snf1 regulates acetyl CoA homeostasis and histone acetylation to increase fitness and stress resistance which is implicated in metabolic control of ageing (Friis et al., 2014). On the other hand, active Snf1 represses lipid and protein biosynthesis through inactivation of acetyl CoA carboxylase and several transcription factors respectively (Shirra et al., 2008; Chumnanpuen et al., 2012). Recently it has been reported that *SNF1* mutants affect cell wall strength and are hypersensitive to different cell wall stresses such as (Calcofluor white, Congo red, Zymolyase or the glucan

synthase inhibitor Caspofungin). They confirm that SNF1 complex and the CWI pathway independently affect yeast cell integrity by epistasis analyses (Backhaus et al., 2013).

12.1 Components of Snf1 complex

Snf1 works as part of a heterotrimeric protein complex (referred to as SNF1) (Amodeo et al., 2007) and comprised of Snf1 as the catalytic kinase (α) subunit, a regulatory (γ) subunit, Snf4, and one of three β-subunits, encoded by *GAL83*, *SIP1*, or *SIP2* which function as scaffold and localisation determinants (Jiang and Carlson, 1996) (Fig 10).

Snf1 catalytic α-subunit contains N-terminal kinase domain and a C-terminal autoinhibitory domain. Snf4 is required for SNF1 in yeast cells but deletion of autoinhibitory domain eliminates the function of Snf4 activity.The primary function of Snf4 is to bind to the C-terminus of Snf1 to alleviate Snf1 from autoinhibition (Celenza et al., 1989; Leech et al., 2003; Momcilovic et al., 2008). In mammalian cells, binding of AMP to the γ -subunit stimulate the kinase activity and releases the catalytic domain from the autoinhibitory domain (Chen et al., 2009).

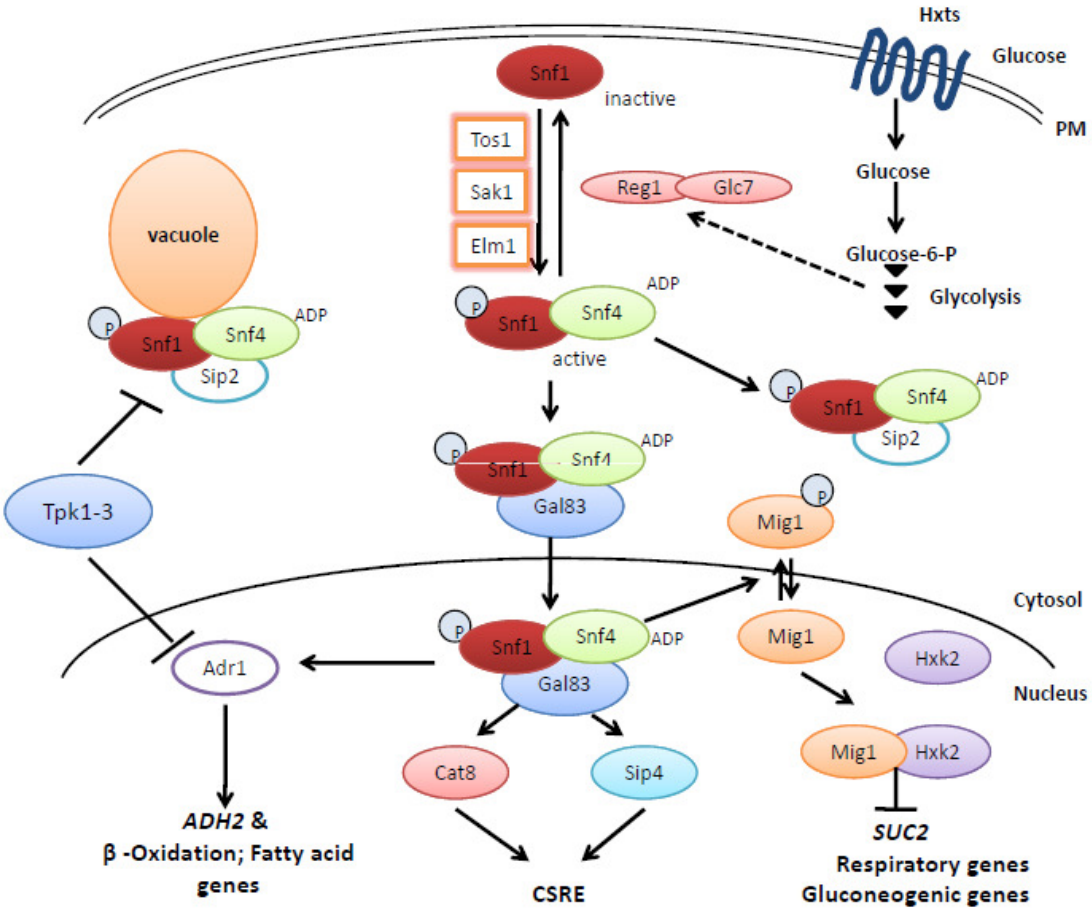


Figure 10: The Snf1 protein kinase glucose repression pathway. The Snf1 protein kinase orchestrates glucose repression of alternative carbon source utilization, and genes involved in respiration and gluconeogenesis. The Snf1 heterotrimeric complex consists of the catalytic subunit Snf1, the stimulatory subunit, Snf4, and one of the three b-subunits: Gal83, Sip1, or Sip2. Snf1 is active in phosphorylated form and the phosphorylation is performed by the three upstream protein kinases Sak1, Tos3, and Elm1, while the phosphatase Glc7 in conjunction with its regulatory subunit Reg1 is responsible for its dephosphorylation. The glucose sensing mechanism, Hxk2 plays an important role, to activate the Glc7-Reg1 protein phosphatase to trigger dephosphorylation of Snf1. In its active form, Snf1-Snf4 binds to each of the three β-subunits, acquiring differential specificity for localization and target phosphorylation. Upon glucose exhaustion, a major role is played by the Snf1–Gal83 complex, which enters the nucleus to trigger derepression. This is accomplished by activation of the transcription factors Adr1, Sip4, and Cat8 and inactivation of Mig1 by releasing its interaction with Hxk2 and promoting its cytosolic localization by phosphorylation. This leads to the expression of a wide range of carbon source-responsive element (CSRE) containing genes involved in the use of alternative carbon sources, gluconeogenesis, ethanol, and fatty acid metabolism. Image adapted from (Broach, 2012; Conrad et al., 2014; Smets et al., 2010)

However, Snf4-mediated protection of active Snf1 kinase in yeast seems to take place through allosteric interaction of other nucleotides, like ADP, with Snf4 (Mayer et al., 2011) (Fig 10). Snf4 contains two ADP-binding sites which bind to ATP, AMP, and ADP with varying strengths, while NADH competes for the stronger site. Upon glucose depletion and increase in ADP levels, ADP binds to the weaker site inducing a conformational change in Snf4 that protects active Snf1 (Wilson et al., 1996; Mayer et al., 2011). On the other hand, association with each β-subunit determines differential substrate and upstream kinase specificity as well as the localization of the different SNF1 sub-complexes (Schmidt & McCartney, 2000; Vincent et al., 2001). At high glucose levels, the sub-complexes are cytosolically located, regardless of the β-subunit. Upon glucose depletion, Sip1 containing SNF1 locates at the vacuolar membrane, Gal83-containing SNF1 at the nucleus, and Sip2-containing SNF1 at the cytosol (Vincent et al., 2001; Hedbacker et al., 2004a, Hedbacker et al., 2004b; Hedbacker & Carlson, 2006) (Fig 10).

12.2 Regulation of Snf1 activity

Increased phosphorylation of Thr210 residue in Snf1 (located within its activation loop), is generally associated with activation of Snf1. Thr210 phosphorylation happens in response to glucose limitation, for which the physiological role is well understood, but it is also stimulated by other stress conditions including high salinity, alkaline pH, oxidative stress, nitrogen starvation, and conditions causing the inactivation of Tor kinases (Nath et al.,

2003; Sutherland et al., 2003; Orlova et al., 2006; Hong & Carlson, 2007; Zhang et al., 2011b; Perez-Sampietro et al., 2013). Thr210 Phosphorylation is dependent on the activity of the three partially redundant upstream kinases Sak1, Tos3, and Elm1 (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003) and opposed by the Reg1-Glc7 protein phosphatase 1, PP1 (Fig 10, Tu & Carlson, 1995). Additionally, a possible role for the type 2A-like protein phosphatase, Sit4, and the Ptc1 protein phosphatase 2C, has been identified in Thr210 dephosphorylation (Ruiz et al., 2011, Ruiz et al., 2013). Of the three upstream kinases, Sak1 (Snf1-activating kinase 1) plays a major role in Snf1–Gal83 complex activation and the $Snf1^+$ phenotype. While the other kinases complement the function of Sak1 in its absence, the deletion of only *ELM1* or *TOS3* has little effect on Snf1 activity (Hedbacker et al., 2004a, Hedbacker et al., 2004b; Kim et al., 2005; McCartney et al., 2005). Moreover, it seem like three upstream kinases play additional roles in glucose regulation. Recently, it has been shown that Gpa1, the Gα subunit of the heterotrimeric G protein of the pheromone signaling pathway is phosphorylated by Sak1, Tos3, and Elm1, upon glucose limitation, resulting in reduced pheromone signaling and mating efficiency. Moreover, Reg1 was also found to dephosphorylate Gpa1 to maintain a strong mating response in the presence of glucose (Clement et al., 2013).

There is also a preference for specific substrates and upstream kinases depending on the β-subunit in the Snf1 oligomeric complex (Hedbacker et al., 2004a, Hedbacker et al., 2004b; McCartney et al., 2005). Gal83 is the most important isoform not only for growth on nonfermentable carbon sources but also for the regulation of sterol biosynthesis under glucose limiting conditions. Sip2, but not Sip1, can partially take over its function when *GAL83* is deleted. Sip1, on the other hand, seems sufficient for specific processes, such as the regulation of nitrogen metabolism and meiosis (Zhang et al., 2010). The Gal83 isoform of the Snf1 complex can be activated by all three upstream kinases, but there is a clear preference for Sak1 over the other two kinases and deletion of Sak1 also leads to cytoplasmic retention of Gal83 upon glucose depletion (Hedbacker et al., 2004a, Hedbacker et al., 2004b).

12.3 Transducing glucose signal to Snf1

An important issue about the glucose repression pathway that remains unclear is how the glucose signal is exactly transduced to the Snf1 kinase complex or its regulators: the kinases Sak1, Elm1 and Tos3 and the phosphatase complex Glc7–Reg1 (Fig 9).

Phosphorylation of a truncated form of Snf1 (1–309), unable to interact with Snf4 -γ and the β- subunits, or of a wild-type Snf1 in cells lacking Snf4- γ and β- subunits, still increased in response to glucose limitation, indicating that activation can happen independently of these regulatory subunits (Ruiz et al., 2011). This was contrary to the previous hypothesis that Snf4 exerts its regulation by limiting access of the phosphatases to Thr210 (Mayer et al., 2011). An alternative hypothesis is that Snf1 is constitutively phosphorylated by the upstream kinases and that the changes in its phosphorylation level as a function of glucose availability are due to changes in the activity or recruitment of the Reg1-Glc7 phosphatase. A recent report suggests that adenylate ligand binding to Snf4 and to the active site of Snf1 could trigger a conformational change, rendering Snf1 in the complex more resistant to phosphatase activity (Chandrashekarappa et al., 2013). As a result, the phosphorylation of Thr210 and activation of SNF1 would be enhanced even in the presence of high glucose levels. This, along with results showing that particular alterations in different parts of the SNF1 heterotrimeric complex result in increased phosphorylation of Thr20 and activation of SNF1 even in the presence of high glucose, indicates that a proper conformation of the SNF1 complex is crucial for its activity, at least for the maintenance of the inactive state during growth on high glucose, independent of the phosphorylation level of Thr210 (Momcilovic et al., 2008). In this respect, the glycogen binding domains in the β-subunits, which are required for interaction with Snf4 (Momcilovic et al., 2008), are known to be important for conferring glucose repression activity to the complex (Ruiz et al., 2011). Recent findings highlight the possibility that glucose activation of the cAMP-PKA pathway may play a role in the inactivation of Snf1 by dephosphorylation in the presence of glucose (Barrett et al., 2012).

12.4 Downstream target of Snf1

Active Snf1 regulate the expression of a variety of target genes through different mechanisms. During glucose limitation, induction of filamentous growth and derepression of genes involved in the metabolism of alternative carbon sources is mediated by Snf1-dependent inactivation of the transcriptional repressor, Mig1/2 (Treitel et al., 1998; Karunanithi & Cullen, 2012). Mig1 is localized in the nucleus and inhibits the expression of many glucose repressed genes in the presence of glucose, by binding to their promoter in association with the corepressor complex Cyc8/Ssn6–Tup1 (Smets et al., 2010). In association with Hxk2, Mig1/2 functions as a transcriptional repressor (Fig 10).

Phosphorylation of Mig1 and Hxk2 by Snf1 prevents their nuclear localization and thus prevents access to the target genes (Ahuatzi et al., 2004; Fernandez-Garcia et al., 2012). Under high glucose conditions, Mig1 interaction Hxk2 with might prevent the phosphorylation of Mig1 by Snf1 (Pelaez et al., 2010). Transcription factors responsible for the induction of gluconeogenesis genes such as Cat8, Sip4, and Rsd2 are activated by Snf1 (Vincent & Carlson, 1999; Roth et al., 2004). Snf1 phosphorylates transcription factors Hsf1 and Msn2 to regulate stress-response genes (Sanz, 2003; Hahn & Thiele, 2004; De Wever et al., 2005). Snf1-dependent activation of Adr1, for the induction of genes involved in the β-oxidation of fatty acids and ethanol metabolism, is counteracted by binding of the yeast 14-3-3 proteins to the phosphorylated Ser230 in the Adr1 regulatory domain (Fig 10, Parua et al., 2010; Ratnakumar & Young, 2010; Braun et al., 2013).

13. Cross talk among nitrogen sensing and glucose sensing pathways

As addressed above, cellular signaling also includes several cross-talk events that take place among different signaling pathways. By doing so, cells are able to precisely regulate central components and thereby achieve a more sophisticated response to the initial signal.

A major challenge in the field is to understand how the different signals transmitted by the TORC1, PKA, Snf1, and Pho85 pathways are integrated to ensure the induction of a quiescence program that allows the cells to survive starvation for any of the key nutrients. The existence of a cross talk between different signaling pathways such as the Pkc1-MAPK and RAS2-cAMP routes in response to oxidative stress has been reported (Petkova et al., 2010a; Petkova et al., 2010b; Park et al., 2005). That Mtl1 senses both glucose depletion and oxidative stress is not surprising, since the sudden absence of glucose would force cells to change from fermentative conditions to respiration, with the consequent increment of ROS released from mitochondria. Localization of the stress-responsive transcription factors, Msn2 and Msn4, are regulated by TORC1. In addition, nuclear cytoplasmic shuttling of Msn2/4 also depends on PKA and Snf1 mediated phosphorylation. Remarkably, phosphorylation of Msn2/4 by any of the three kinases results in reduced nuclear accumulation and hence diminished stimulation of stress-responsive gene expression (Petkova et al., 2010a; Boy-Marcotte et al., 1998; Gorner et

al., 2002; Mayordomo et al., 2002; Duvel et al., 2003; Santhanam et al., 2004). The nuclear entrance of Msn2/4, on the other hand, is regulated by dephosphorylation by the protein phosphatases PP1A and PP2A (Santhanam et al., 2004; De Wever et al., 2005).

At present, there are two different models describing the biological relationship between TOR and RAS2/PKA in budding yeast. One model proposes that TOR and PKA function independently (Zurita-martinez et al., 2005). The second model, proposes that under some conditions dependent on nutrient availability, TOR signals to RAS2/cAMP (Schmelzle et al., 2004). There exists a crosstalk between Mtl1 and Tor1 and the Ras-cAMP pathway through the Rho1 protein in *S. cerevisiae*. Whether or not, this crosstalk occurs via Mtl1-Rho1-Tor1-Ras2-Msn2/Msn4, or it occurs from Mtl1 to Rho1, and then independently to Tor1 and to Ras2 to converge in Msn2/Msn4, remains to be elucidated (de la Torre-Ruiz et al., 2010).

On the other hand, the many shared targets of PKA and TORC1 may at least partly be explained by the fact that TORC1 seems to stimulate PKA toward certain targets (Soulard et al., 2010). Bcy1 contains 17 phosphorylation sites which are divided in to three clusters (Fig 11). The cluster I is been situated in the extreme N-terminal of HA containing fragment of BCY1. The cluster II phosphorylation residues are related to Bcy1 function andanother cluster was identified to be located near the autoinhibitory domain where the catalytic subunit of PKA "autophosphorylates" the highly conserved S145 ($RRxS^{145}$) to inhibit Bcy1 (Fig 11).

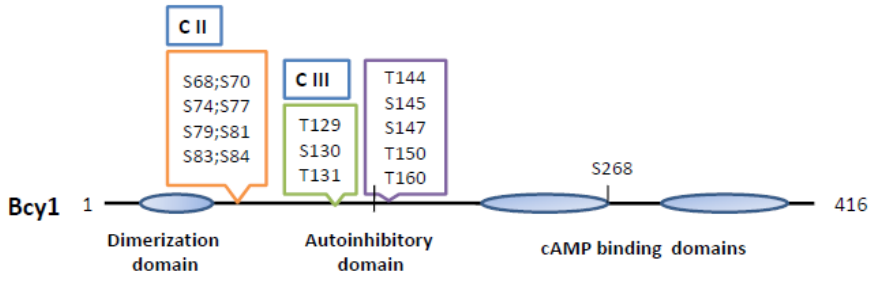


Figure 11: Bcy1 phosphorylation states: Graphical representation of the phosphorylated amino acids identified in Bcy1 phosphoproteome by Mass Spectrometry. CII/III, cluster II/III. Image adapted from (Soulard et al., 2010)

The novel and more important is the cluster III which is comprised of T129, S130 and T131 phosphorylation residues. Bcy1 inhibitory function on PKA is induced through the phosphorylation of T129 residue or also called RxxS/T motif. Rapamycin treatment

caused phosphorylation of Bcy1 at the RxxS/T motif and this effect was due to TORC1 inhibition (Budhwar et al., 2010; Soulard et al., 2010; Werner-Washburne et al., 1991).

As earlier mentioned the protein kinase Rim15, is found to be important for the entry into stationary phase, and has also been identified as a common downstream target of nitrogen and glucose signaling. Consequently, TORC1 may, possibly via Sch9 and/or Tap42–Sit4, impinge upon the CWI pathway, but whether this occurs at the level of the Cell wall sensors, Rom2, or the MAPK cascade is currently unknown (Torres et al., 2002; Reinke et al., 2004; Araki et al., 2005; Kuranda et al., 2006; Soulard et al., 2010).

Another instance is where Snf1 mediated phosphorylation promotes nuclear localization of Gln3, whereas TORC1-mediated phosphorylation inhibits this process (Fig 7). These observations suggest a convergence of Snf1 and TORC1 mediated signaling on nitrogen repressed gene expression (Bertram et al., 2002; Georis et al., 2011; Orlova et al., 2006; Rai et al., 2013). In keeping with this, recently it has been shown that in glucose starvation conditions, Snf1 with at least one other factor, push the TORC1 pathway into an off state, in which Sch9-branch signaling and PP2A-branch signaling are both inhibited (Hughes Hallett et al., 2014). In addition to this, these authors show that TORC1 pathway remains in the glucose starvation (PP2A inhibited) state even when cells are simultaneously starved for nitrogen and glucose. Finally, it is crucial to determine the localization of these converging signals in order to understand the overall regulatory network that serves to respond adequately toward changes in the presence of different nutrients.

OBJECTIVES

Main Objective:

To analyse the essential role of the transceptor Mtl1 in quiescence.

Specific Objectives:

1. To assess the role of *MTL1* in Chronological Life Span (CLS)
2. To determine the function that *MTL1* might have on mitochondria in diauxic shift and stationary phase.
3. To identify the functional relationship between *MTL1* and TORC1, *SCH9*, RAS, PKA & CWI pathways in quiescence and to establish the possible role of *MTL1* as a connector molecule between different signaling pathways thus promoting cell survival.
4. To establish the functional role of *MTL1* as a nutrient sensor especially to glucose and to identify the possible relationship with *SNF1* and downstream transcriptional regulators such as *RTG1/3* and *GLN3*

MATERIALS

and

METHODS

1. Yeast Strains and gene disruptions

The yeast strains used in this thesis are described in Table 1, which includes the relevant phenotype and respective comments. The yeast strains were disrupted either by one-step disruption method using the *kan*MX4 module or by *nat*MX4 module as previously described (Petkova et al., 2010a; Vilella et al., 2005). *SCH9* was disrupted using *nat*MX4 module in wt strain and in *mtl1* mutant respectively. *SLT2* was disrupted using *kan*MX4 in wt strain and *nat*MX4 module was used for disruption in *mtl1* mutant.

Table 1: Yeast strains employed in this thesis.

Strain	Relevant phenotype	Comment
CML128	MATa, leu2-3,112, ura3-52, trp1, his4, canI.r	wt type strain
GSL34	MATa*tor1::kanMX4* in CML128	(Petkova et al., 2010a)
GSL45	MATa*mtl1::kanMX4* in CML128	(Petkova et al., 2010a)
GSL48	MATa*mtl1::kanMX4 tor1::LEU2MX5* in CML128	(Petkova et al., 2010a)
GSL53	MATa*ras2::LEU2MX5* in CML128	(Petkova et al., 2010a)
GSL 54	MATa*mtl1::kanMX4 ras2::LEU2MX5*	(Petkova et al., 2010a)
GSL190	MATa, his, ura, *trp slt2::KanMX4* in CML128	This work
GSL196	MATa*slt2::NatMX4 mtl1::KanMX4* in CML128	This work
GSL205	MATa *sch9::NatMx4* in CML128	This work
GSL206	MATa *sch9::NatMX4 mtl1::KanMx4* in CML128	This work
GSL118	CML128 *tetMtl1HA-URA*	(Petkova et al., 2012)
GSL107	MATalpha *ura3Δ52 leu2-3,112 trp1-1 his4 can^R*	wt type strain from EG 123 From the

		laboratory collection
GSL108	MATalpha *slt2::TRIP1* in EG123	From the laboratory collection
GSL109	MATa *his3Δ1,leu2Δ,met15Δ,ura3Δ*	wt type strain from BY4741 (EUROSCARF)
GSL110	MATa *slt2::KanMX4* in BY4741	From the laboratory collection
W303-1A	MATa *ura3-1 ade2-1 leu2-3,112 trp1-1 his3-11,15*	wt type strain
W303 *mpk1*	MATa *mpk1::URA3* in W303-1A	From the laboratory collection
MML447	MATa *tor1::KanMX4 mpk1::URA* in CML128	From the laboratory collection
GSL 246	MATa *rtg2::NatMx4* in CML128	This work
GSL 252	MATa *mtl1::kanMX4 rtg2:: NatMx4* in CML128	This work
FY250	MATalpha *his3Δ200 leu2Δ1 trp1Δ63 ura3-52*	From the laboratory collection
snf1Δ	*snf1Δ10* derivate of FY250	From the laboratory collection

2. DNA Manipulation and Plasmids

The plasmids employed and the respective characteristics are described in Table 2.

Table 2: Plasmids employed in this study.

Plasmid name	Origin/Vector	Characterstics
pTP100	YCplac22	YCplac22 empty centromeric vector -Msg5myc having *TRIP1* marker. Gift sample from Maria Molina
pTP 267	pVT 100 U	Plasmid carrying GFP tagged to mitochondrial protein to visualize mitochondria having *URA3* as marker (Westermann and Neupert, 2000)
pTP266	pYX142	Plasmid carrying GFP tagged to mitochondrial protein to visualize mitochondria having *LEU2* as marker (Westermann and Neupert, 2000)
pTP268	Bcy1-HA.	Plasmid Bcy1 tagged with HA and having *HIS3* as marker from Francesca Randez
	pAG25	Includes the NAT cassette to disrupt genes from Goldstein & McCusker (1999)
	pHU10	Marker Swap plasmid , to change *HIS3* by *URA3* Gift sample from Francesca Randez (Cross, 1997)
pTP282	This work	Bcy1-HA containing *URA3* marker swapped from pTP268 with *HIS3* marker using pHU10 (marker swap plasmid).
pTP295	pUG35	Centromeric Vector having MET-25 promoter and Multicloning site containing YGFP in C-terminal. Gift sample from J.Hegemann
pTP313	pMM536	pFA6a-KanMX4 includes the KAN cassette to disrupt genes.
	pBCK1-20	*BCK1* hyper activated mutation containing TRIP as marker in pRS413 vector. (Petkova et al., 2010a)
pTP263	pTB395	C-terminally 3xHA tagged GLN3 expressed under control of its own promoter with *LEU3* as marker. GLN3 is on 2.8kb BamHI-Pst1 PCR fragment. Backbone pHAC181 Gift sample from Mike Hall

pTP264	pRS313	Snf1-GFP as pOV 84 containing *HIS3* as marker (Vincent et al., 2001)
pTP287	pRS313	Snf1-GFP swapped from pTp264 also called pOV84 containing *HIS3* marker using pHU10 (marker swap plasmid) to *URA3* marker was produced in this work.
pTP26	pRS416	C-terminally 3xHA tagged Mks1 under control of its own promoter with *URA3* as marker. Gift sample from Feng Zhang (Zhang et al., 2013)

3. Cell Culture

3.1 Growth conditions

S. cerevisiae and *E. coli* cells were respectively grown at 30°C and 37°C, unless otherwise indicated. The liquid cultures were incubated with continuous rotation at 165 rpm. Growth was measured with a spectrophotometer by determining Optical Density (OD) at 600 nm. Yeast samples for further analyses were taken from cultures growing exponentially at a concentration between 1.5 to 3 x 10^7 cells/ml, which is equivalent to 0.4-0.6 units of OD measured at 600 nm.

3.2 Growth medium for *E. coli* cells

For cultures of *E. coli* cells Liquid Broth (**LB)** medium was used with the following composition: triptone 1%, NaCl 1%, yeast extract 0.5%, at pH 7.5 adjusted with 1 M NaOH. For the selection of transformed cells the medium was supplemented with ampicillin at 50 µg/ml final concentration. The medium was solidified with 2% agar.

3.3 Growth medium for *S. cerevisiae* cells

I. **YPD:** glucose 2%, peptone 2%, yeast extract 1%. Depending on the required conditions, geneticin (selection with the *kanMX4* marker) or nourseothricin (selection with the *natMX4* marker) were added at a final concentration of 200 µg/ml respectively.

II. **SD:** The minimum media contains 0.67% yeast nitrogen base without amino acids (Difco™) and 2% glucose.

III. SC: The media contains 0.67% yeast nitrogen base without amino acids (Difco^{TM}), 2% glucose and 0.2%. *Drop out.* The *Drop out* is the combination in dry of nitrogenated bases, amino acids without Uracil, Leucine, Histidine and Tryptophan +/- 2% agar) when required (Kaiser et al., 1994). Its composition is detailed in Table 3.

Table 3: *Drop out* components and their respective quantities per litre of SC medium (Sherman, 2002).

Amino acid	(mg)	Amino acid	(mg)	Amino acid	(mg)
Adenine	20	Glycine	20	Tyrosine	30
Alanine	20	Isoleucine	30	Valine	150
Arginine	20	Lysine	30		
Asparagine	20	Methionine	20		
Aspartic acid	100	Phenylalanine	50		
Cysteine	20	Proline	20		
Glutamine	20	Serine	400		
Glutamic acid	100	Threonine	200		

4. Metabolic Treatments

4.1 Glucose depletion

For glucose depletion experiments we followed the procedure as described in (Petkova et al., 2010a). In glucose depletion, the cells were grown in SD medium plus amino acids to exponential phase and were subsequently centrifuged at 3000 rpm for 3 min at 4 °C. The cell pellet was washed in synthetic medium minus glucose and amino acids for 3-4 times at 4 °C. After the last wash, cells were suspended in SD medium plus amino acids without glucose and incubated at 30°C. Later cell aliquots were collected, centrifuged and processed for respective experiments for the times indicated in the Figures.

4.2 Nitrogen depletion

For nitrogen depletion the cells were grown in SD medium plus amino acids to exponential phase and were subsequently centrifuged at 3000 rpm for 3 min at 4 °C. The cell pellet was washed in synthetic medium without ammonium sulphate and amino acids for 3-4 times at 4 °C. After the last wash, cells were suspended in SD medium without

ammonium sulphate and amino acids and later cell aliquots were collected, centrifuged and processed for required experiment for the times indicated in the Figures.

4.3 Special treatments added to culture media

4.3.1 Glutamate

For glutamate treatment, cells were grown in SD medium plus amino acids and also in presence and absence of 0.1% L-glutamic acid hydrochloride (Sigma) for 16h at 30°C and treated as precultures. The cultures were diluted and subsequently allowed to grow to exponential phase. From these cultures, cells were harvested and subsequently centrifuged at 3000 rpm for 3 min at 4 °C. Cell pellet were processed for total mRNA extraction and analyzed by Northern Blot otherwise pellets were frozen in liquid nitrogen and stored at-80 °C until needed.

For usage in plates, 0.1% L-glutamic acid hydrochloride final concentration was used in respective medium (SD/SD/YPD) with 2% agar and appropriate auxotrophies.

4.3.2 Rapamycin

For treatment with rapamycin we followed the protocol as described in (Petkova et al., 2010a), cultures were grown in minimum medium (SD plus amino acids) or rich medium (YPD) to exponential phase and 200ng/ml Rapamycin (Sigma) final concentration was added from 1µg/µl (suspended in 90% ethanol and 1% TWEEN 20) stock solution and incubated at 30°C for the indicated times in the figure. Later cells were harvested as indicated in the figures and processed for total proteins extracts or total mRNA extracts as explained below otherwise samples were frozen in liquid nitrogen and stored at -80 °C until needed.

For usage in plates, 1ng/ml of Rapamycin (Sigma) final concentration was added by serial dilutions from 1µg/µl (suspended in 90% ethanol and 1% TWEEN 20) stock solution in respective medium (SD/SD/YPD) with 2% agar and appropriate auxotrophies.

4.3.3 N-acetyl cysteine

N-acetyl cysteine (NAC) (Sigma) was used in two concentrations 5 and 10mM from 0.5M stock solution. Preculturs of Yeast were grown in the specific media (SD, SC or YPD)

plus and minus NAC and subsequently allowed to grow to exponential phase. Cells were harvested by centrifugation at 3000 rpm for 3 min at 4 °C according to the required as per the experimental requirements indicated in the figures. Subsequently the cell pellet was processed for respective analysis or frozen in liquid nitrogen until further analysis. For Chronological Life Span (CLS) determination, cells were treated with NAC and subsequently assayed for survival analysis as described below.

4.3.4 Hydrogen peroxide

For treatment with Hydrogen peroxide (H_20_2) (Sigma) we followed the protocol as described in (Petkova et al., 2010a). Plates containing 1mM H_20_2 final concentration from 100mM stock solution was used in (SD/SC/YPD) medium with 2% agar respectively.

4.3.5 Ter-Butyl Hydro peroxide

For treatment with Ter-ButylHydroperoxide (t-*BOOH*) (Sigma), we used plates containing 1mM t-*BOOH* final concentration from 100mM stock solution was used in (SD/SC/YPD) medium with 2% agar respectively.

5. Transformation methods for *E. coli* and *S. cerevisiae*

Commercially available competent cells of *E. coli*; DH5-α (Invitrogen) and subsequent transformation were carried out according to manufacturer's instructions. For *S. cerevisiae* cells transformation, the lithium acetate (AcLi) method was employed as described in (Gietz et al., 1992).

6. Plasmid purification from *E. coli* cultures

Plasmids were purified from *E. coli* cultures grown at 37°C upto 16 hours in LB medium containing 50µg/ml ampicillin, using the NucleoSpin Plasmid QuickPure system (Macherey-Nagel) following the manufacturer's instructions.

7. Cell survival and Chronological Life Span

To assay cell viability cell were grown to mid log phase (O.D.600: 0.6) in SC medium supplemented with auxotrophic aminoacids. We measured the Chronological Life Span (CLS) in the different strains based on the survival of populations of non-dividing yeast

using the protocol described in (Parrella and Longo, 2008). Yeast cells grown in SC medium plus auxotrophic amino acids, stop dividing after 24-48 hours. According to the authors we also considered the 3rd day of growth as the starting point for the CLS experiment. To precisely register viability cells were serial diluted and plated by triplicate onto YPD plates. The viability was scored by counting the number of cells able to form colonies. Averages and standard deviations were subsequently plotted in histogram diagrams.

8. Assessment of ROS accumulation

We followed the protocol as described in (Owada et al., 2013). Yeast cells were grown in YPD at 30ºC to reach logarithmic phase, and subsequently allowed to grow for 24 hours to enter into diauxic shift. The post diauxic cells were treated with 5µg/ml, dihydrofluoresceindiacetate (H_2-DCFDA, Molecular Probes), for 4 hours in the dark in continuous shacking. Prior to analyses, cultures were diluted to a density of $1x10^8$ cells/ml in 50mM Tris-HCl, pH 7,5 and subsequently sonicated. ROS content was determined by registering the fluorescent data using a BD FACSCanto II Flow Cytometer. FACS parameters were: FITC-A: 350, SSC-A: 400 and FSC-A: 401 nm, respectively (filter FL 1 channel).

9. Mitochondrial Membrane potential

Mitochondrial membrane potential was determined in cells grown to diauxic shift (24 hours from a logarithmic culture OD600:0.6) in YPD as described in the determination of ROS accumulation. Cells were harvested and later were washed and suspended in phosphate-buffered saline (PBS). Later the cell suspension was treated with 20 ng/ml of membrane-potential-sensitive dye 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes) in PBS buffer as described in (Leadsham & Gourlay, 2008; Simon et al., 1997). Cell suspensions were incubated for 30 min at 30ºC, washed and suspended in PBS buffer and sonicated to be analysed by flow cytometry using the FL1 channel. Mitochondrial membrane potential was quantified by using all events FITC-A mean.

10. Mitochondrial Morphology

To observe mitochondrial morphology yeast cells were transformed with plasmid pVT100U (URA marker) or pYX142 (LEU marker) expressing a mitochondrial protein fused to GFP (Westermann and Neupert, 2000). Yeast cells were grown in YPD to logarithimic phase and subsequently allowed to grow upto 24 to reach diauxic shift. The cells were visualized in fluorescence microscope (Olympus BX-51) using 60X magnification to observe the mitochondrial morphology.

11. Aconitase activity

Aconitase activity was assayed by following the protocol described (Rodríguez-Manzaneque et al., 2002). Activities were expressed in units (nanomoles per minute) per milligram of cell protein. Cells were grown to exponential phase and also allowed to grown to stationary phase as per requirement. The cells were collected to the volume corresponding to 12 OD's and processed to form pellets as per time periods indicated. Extracts were prepared in 100 mMTris-HCl buffer, pH 8 plus protease inhibitors (Roche Complete Plus®) using glass beads to disrupt the cells. Aconitase reaction was measured in a buffer containing 50 mM Tris-HCl, pH 7.5 plus 2.4 mMNaCl and cis-aconitic acid (Sigma) as a substrate. The reaction was registered in a spectrophotometer Shimadzu UV-2401 at 240 nm and 25°C keeping 1 min reaction time. The aconitase assay was performed in triplicate and mean ± SD values were plotted using histograms.

12. Glycogen accumulation

Glycogen accumulation was determined following the protocol described (Petkova et al., 2010a; Swiegers et al., 2006). Cells are grown in specific medium plus amino acids to logarithmic phase then spotted on to solid SD medium plus amino acids and allowed to grow for 4-5 days. A solution of 0.2% Iodine/0.4% potassium iodide was poured over the spots and incubated for 3 min. Later photographs were taken and color development is compared among the strains. Brown color was indicative of glycogen accumulation.

13. Oxygen consumption and respiration

i. Cell cultures:

Yeast cultures were grown in YPD medium or unless specified for 24h at 30ºC from exponential growing cells to analyze the respiration rate in diauxic shift.

ii. Instrument Calibration:

To ascertain the respiratory capacity of the cells we used Oroboros Oxygraph-2krespirometer. Prior to the main experiment, the instrument was calibrated to obtain a standard oxygen concentration and correction for instrumental background oxygen flux was performed by following the protocol described (Boada et al., 2004; Hutter et al., 2004; Leadsham and Gourlay, 2010). DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition (2 s time intervals) and analysis. The instrument was calibrated using YPD media with chamber volumes set at 2 ml and a stirring speed of 500 rpm.

iii. Respiration Rate and oxygen consumption:

Oxygen flux of suspensions of yeast was measured by high-resolution respirometry. The experimental regime started with routine respiration or basal respiration, which is defined as respiration in cell-culture medium without additional substrates or effectors. After closing the chamber, steady-state respiratory flux was observed by allowing a time interval between 5 and 10 min. The respiration was analyzed by sequential addition of triethyltin bromide (TET; Sigma) at 1.75µM to test for the effect of inhibiting mitochondrial ATP synthase (leak respiration). The oxygen consumption begin to lower upon addition of TET. Later in order to revive back the respiration, the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP- Sigma) was added 1µM in a step wise addition until maximum respiration rate was observed by increase in oxygen flux. An average of 3µM of FCCP was added in 3-5 injections to determine maximum respiratory capacity. Finally, complex III activity was inhibited with Antimycin A (Sigma) at 1.8µM single addition to measure residual oxygen consumption, i.e. the respiration due to oxidative side reactions or non mitochondrial respiration. The titration method was completed within 30 min on an average from the routine respiration. In order to avoid oxygen limitations, all the experiments were performed above 50% oxygen saturation and all the inhibitors were prepared in 100% ethanol. The chambers were washed thrice with

distilled water which was then followed by three washes of 10 min interval with 100% ethanol prior to next set of sample evaluation.

14. RNA Preparation and Northern Blot Analyses

Total RNA purification from yeast, preparation of agarose gels and conditions for electrophoresis, Nylon membrane transfer and fixation of RNAs, hybridisation and probe labelling with digoxygenin were carried out as described (Gallego et al., 1997). Probes covering the entire open reading frame, without adjacent sequences, were generated by PCR from genomic DNA are listed in (Table 4) along with their corresponding oligonucleotide sequences. The PCR program used for generating probes is mentioned in (Table 5).

Table 4: Oligonucleotides utilized for obtaining cDNA probes

Oligonucleotides	Sequence
***CIT2* probe**	
GSLOL93	5′-CAGGTATATGGTGGTATGAG-3′
GSLOL94	5′-AAGACACCAGAGTGAGCATC-3′
***COX2* probe**	
GSLOL222	5′-ACTGGGATCCATGTTAGATTTATTAAGATT-3′
GSLOL223	5′-ACTGGAATTCTTCTTCATTTAATCATTCCA-3′
***COX4* probe**	
GSLOL220	5′-ACTGGGATCCATGCTTCACTACGTCAATC-3′
GSLOL221	5′-ACTGAAGCTTTTAGTGATGGTGGTCATCATTTG-3′
***MEP2* probe**	
GSLOL89	5′-ATGTCTTACAATTTTACAGG-3′
GSLOL90	5′-ATACTATATGGTCAGTGTTC-3′
***GAP1* probe**	

GSLOL91	5′-CTGAGTTTCTAACTCAGGAG-3′
GSLOL92	5′-AGATTTCTGGCAGAAATCTC-3′
***HSP12* probe**	
MMO588	5′-ATGTCTGACGCAGGTAGAA-3′
MMO589	5′-TTACTTCTTGGTTGGGTCTT-3′
***CTT1* probe**	
MMO346	5′-GATCAATCAGCTCAGCTTCA-3′
MMO347	5′-CTTGGCTGTTCGAAGTCTAG-3′
***RPL3* probe**	
MMO98	5′-CATGTCTCACAGAAAGTACG-3′
MMO99	5′-CAAAACTTCTTACAAGTCCTTC-3′
***U1* probe**	
CMO265	5′-CTAAGGCGACGAGTTTTC-3′
CMO266	5′-GATCCACCCGTTCCTACC-3′
***U2* probe**	
CMO263	5′-GATCAAGTGTAGTATCTGTTC-3′
CMO264	5′-GAACGACTCCACAAGTGCG-3′

Table 5: PCR program for probes labeled with digoxygenin

Initial denaturation	95ºC, 2min	
Denaturation	95ºC, 30 seconds	30 cycles
Annealing	24ºC, 1min	
Extension	72ºC, 30 seconds	
Final extension	72ºC, 10 min	
Hold	4ºC, ∞	

15. Bcy1 protein stability

To assess the Bcy1 stability we followed the protocol described by (Budhwar et al., 2010). Cycloheximide (Sigma) was used at 150 µg/ml from a stock of 100 mg/ml in DMSO to inhibit protein synthesis. Bcy1 half-life was determined by following the protocol described (Budhwar et al, 2010). The yeast cells were grown to reach log phase and cycloheximide was added to the cells to a final concentration of 150µg/ml for 30 minutes in continuous shacking at 30ºC. After the treated time, aliquots were removed at different time points (0 or no treatment then 30, 60, 90, 120, 150 minutes post cycloheximide addtion) and centrifuged to collects cells. The cells were washed with 1.5 ml of cold stop buffer (50 mM Tris-Cl, pH 7.5, 50mM NaF, and 0.1% NaN_3) prior to centrifugation at 3000 rpm for 3 minutes at 4 ºC. Pellets were frozen in liquid nitrogen or processed for protein extraction followed by immunoblot analysis to evaluate the endogenous Bcy1 protein using Polyclonal anti-Bcy1 (Yc-20) as described below. The same procedure was followed for cells which were continuously grown for 24 hours from lograthimic phase to check Bcy1 stability in diauxic shift.

16. Protein extraction and immunoblot analyses

16.1 Total Protein extraction

Total yeast protein extracts were prepared as previously described in (Torres et al., 2002). The yeast cells were boiled at 95°C for 3min with 5M urea and then total protein lysates were extracted by adding equal volumes of glass beads and subsequently broken using a ribolyser (5.5m/s 3-4 times at 4 °C). The cell lysates were boiled with 10% Sodium Dodecyl Sulphate (SDS) at 95°C for 3min and denatured proteins were quantified by Lowry's method at 620nm.

16.2 Immunoblotting by SDS- PAGE

Cell lysates were resolved on Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (usually 10% resolving gel and 5% stacking gel) using 1x TGS buffer (BIO-RAD) as running buffer. Proteins were electrotransferred from the gel to a polyvinyledenedifluoride (PVDF) Immobilon®-P transfer membrane (Millipore, Billerica, MA) using a semi-dry Trans-Blot apparatus (Hoefer, Amersham Pharmacia Biotech) and transfer buffer (Tris-Cl, methanol and SDS in sterile water). Membranes

were blocked with Tris-buffered saline with Tween 20 (TBS-T) (20mM Tris-HCl pH 7.4, 150mM NaCl and 0.1% Tween 20) containing 5% non fat dry milk or 0.3% I-Block® (TROPIX) for 1h at room temperature, washed with TBS-T and incubated overnight with the primary antibody. After overnight incubation, the membranes were washed with TBS-T for 3 times each 10 min time interval. Later the membranes are incubated with the corresponding secondary antibody as per the manufacturer's instructions at room temperature upto 1h slow shaking. The antibodies used in Western Blot are detailed in (Table 6).

Table 6: List of antibodies used for western blotting

S.no	Primary Antibody	Manufacturer / Reference	Dilution	Secondary Antibody	Manufacturer/ Reference	Dilution
1.	Monoclonal anti-HA (3F-10)	Roche / 11867423001	1:5000	Anti- rat	Millipore	1:10000
2.	Polyclonal anti-Bcy1- (yc-20)	Santa Cruz / SC6765	1:2000	Anti-goat	Vector / PI: 9500	1:15000
3.	Anti-tubulin	Sigma/ T5168	1:5000	Anti-mouse	GE Healthcare/ LNA931v/AG	1:10000
4	Anti-Porin	Invitrogen/ 459500	1:8000	Anti-mouse	GE Healthcare/ LNA931v/AG	1:10000
5	Anti-Phosphoglycerate kinase	Invitrogen/ 459250	1:12000	Anti-mouse	GE Healthcare/ LNA931v/AG	1:10000

6	Phospho - specific P44/42 MAPK antibody	Cell signalling/ 91016	1:2500	Anti-rabbit	GE Healthcare/ LNA934v/AG	1:10000

16.3 Chemiluminescent detection

After the incubation with secondary antibody, the membranes are washed at least 3-4 times at 10 min time interval each with TBS-T. The protein-antibody complexes were visualised by enhanced chemiluminescence using the Supersignal substrate (Thermoscientific) in a Chemidoc™-MP Imaging system (BIO-RAD). Later data acquisition and bands/image quantification were performed using Image Lab software (version 4.0.1 build 6- BIO-RAD Laboratories).

17. Immunoprecipitation

17.1 Cell cultures

For immunoprecipitation (IP) studies were performed on yeast cells grown for continuous 36h in YPD media from exponentially growing cells or cells previously depleted for glucose/nitrogen for 3 hours (see **section 3.1 and 3.2**) respectively. The protocol as described in (Petkova et al., 2012) was followed for IP's.

17.2 Materials

Beads-wash buffer: 20mM Tris-Cl pH 7.5, 140mM Nacl, 10mM EDTA, 10% Glycerol, and 0.1% Nonidet NP-40 (FLUKA).

Lysis buffer: 20mM Tris-Cl pH 7.5, 140mM Nacl, 10mM EDTA, 10% Glycerol, 1% Nonidet NP-40, 1mM Vanadat (Sodium orthovanadate Na_3VO_4) (Sigma), 25mM Sodium fluoride (Sigma), 40mM β-Glycerophosphate (Sigma), 1mM PMSF (Sigma), 1x Phosphatase Inhibitors (mixture) and 1x Complete® EDTA free Protease inhibitors cocktail (Roche)

Wash buffer: Lysis buffer without 10% Glycerol, 1mM PMSF and 1x Complete® EDTA free Protease inhibitors cocktail.

Loading buffer: 20mM Tris-Cl pH6.8, 2mM EDTA, 4% Sodium Dodecyl Sulphate (SDS (ISOGEN)), 0.2% Bromophenol blue (Sigma), 20% Glycerol and 5% β-Mercaptoethanol (BIORAD).

17.3 Procedure

Protein extracts were prepared from the cells collected in centrifuge tubes in volumes corresponding to 210 OD's and later total protein was extracted using lysis buffer and adding equal volume of washed glass beads. The cells were broken using a ribolyser at 5.5m/sec speed for 45sec 3-4 times. The mixture was subsequently centrifuged to obtain a clear lysate. The total protein was measured by Lowry method. A brief quantity of lysate was kept aside as crude extract. The remaining lysate were rocked at 4 degrees using Anti-HA Affinity Matrix Beads (Roche) equilibrated in advance using the beads-wash buffer. After the incubation time of 1-2h rocking, the beads were washed by centrifugation for 15-20 sec at 12000 rpm at 4°C using wash buffer (lysis buffer without protease inhibitors) for three times and once with lysis buffer. Later beads were treated with (2-2.5x the beads volume) of 1x loading buffer and boiled at 95°C for 3min. The samples were centrifuged at maximum speed for a min before immunoblotting or stored at -20°C until analysis. The samples were subjected for immunoblotingon SDS- PAGE and other steps were followed as previously described in **section 15** of this chapter. The antibodies used in IP's are detailed in (Table 7).

Table 7: The following antibodies were used for IP's.

S.no	Primary Antibody	Manufacturer/ Reference	Dilution	Secondary Antibody	Manufacturer/ Reference	Dilution
1.	Monoclonal anti-HA (3F-10)	Roche / 11867423001	1:5000	Anti rat	Millipore	1:10000
2.	Polyclonal anti-Bcy1 (yc-20)	Santa Cruz / SC6765	1:2000	Antigoat	Vector / PI: 9500	1:15000
3.	Phospho PKA substrate antibody RxxS/T*	Cell signalling / 9621S	1:2000	Anti rabbit	GE Healthcare/ LNA934v/AG	1:10000

* - Membranes treated with this antibody is first blocked in 0.3% I-Block™ (TROPIX)** in TBST (0.1%) for 1hour room temperature and both primary and secondary antibodies are incubated in 0.015% of I-Block™ diluted using TBST(0.1%)

**- 0.3% I-Block™ is prepared freshly by weighing the required quantity of I-Block™ and dissolved in preheated TBST(0.1%), mixed well and allowed to cool down to room temperature before consumption. Caution must be taken not to overheat or boil TBST(0.1%).

18. Fluorescence microscopy to detect Snf1 localisation

To observe Snf1 localisation we transformed cells with plasmid pTP287 harbouring Snf1-GFP. The cells containing pSnf1 GFP were grown in SD media plus amino acids to reach mid-logarithmic phase. The cells were visualised under the Fluorescence microscope (Olympus BX-51) using 60X magnification, to see the nuclear and cytoplasmic localisation of Snf1. Subsequently, cells were depleted for glucose as mentioned in **section 3.1** and later at indicated times in the figure, cells were visualised for Snf1 localisation.

19. Detection of Total and Phosphorylated form of Snf1

19.1 Cell cultures

Yeast cultures were grown to logarithmic phase in SD media plus amino acids at 30°C to get an average 3×10^6 cells/ ml. At this juncture, the cultures were subjected to glucose starvation as previously mentioned in **section 3.1** and cells were harvested at the times indicated in respective figure post glucose depletion and proteins were extracted as mentioned below.

19.2 Materials and reagents

- 1x T.E. : 10mM Tris-HCl and 1mM EDTA pH7.5
- 0.2 M NaOH
- Loading Buffer for SDS-PAGE: 80mM Tris-HCl pH 6.8, 2mM EDTA pH 7.5, 4% SDS, 20% glycerol, 0.2% Bromophenol blue and 5% β-Mercaptoethanol
- 0.2 M glycine

19.3 Procedure

We followed the protocol to extract proteins by Heat alkaline treatment as described by (Orlova et al., 2008). Heating the cell cultures at 100°C is very important to avoid environmental phosphorylation of Snf in Thr210 (T210) residue which denotes the active form of phosphorylation. The protein extracts can be stored at -20°C until further anaylsis.

*Culture boiling and protein extraction***:**

- Before the treating the cells, the heat resistant borosilicate flask is preheated in a water bath maintained at 100°C
- Approximately 10^8 cells per culture (3≈5ml) is transferred to the preheated flask and immediately submerged in a boiling water bath with vigorous swirling for first 20 seconds to ensure rapid and uniform heat transfer. The flask is left for an additional 3min in the boiling water bath.
- The flasks were then removed from the bath, allowed to air cool to room temperature, and the 'cooked' cells were harvested by centrifugation at 3000 rpm for 5 min.
- The supernatants were aspirated, and each pellet was resuspended in 150 µl 1xTE buffer and later the contents were transferred to a centrifuge tube.
- To the mixture 150 µl of 0.2M NaOH was added, followed by incubation for 5 min at room temperature.
- The tubes were then spun down in a microfuge at 10,000 rpm for 1 min.
- The supernatants were carefully aspirated, and the pellets were gently resuspended in an appropriate volume of SDS–PAGE loading buffer.
- The tubes were heated at 95°C for 5 min and later allowed to cool down to room temperature.
- The tubes were centrifuged to pellet the remnant and supernantant (10-15 µl) was resolved on a SDS- PAGE followed by Western Blotting. Else the tubes are stored at -20°C until further anaylsis.

Immunoblot and SDS –PAGE was performed as earlier described in **section 15** with 7.5-9% separating gel and as usual 5% stacking gel. The antibodies used for detection of phosphorylated and total Snf1 are detailed in (Table 8).

Table 8: List of antibodies employed in determination of Snf1-P (T-210) and total Snf1

S.no	Primary Antibody	Manufacturer/ Reference	Dilution	Secondary Antibody	Manufacturer/ Reference	Dilution
1.	Monoclonal anti-Phospho-Thr172 AMPK	Cell Signaling/ 2535S	1:1000	Anti rabbit	GE Healthcare/ LNA934v/AG	1:10000
2.	Polyhistidine anti-Snf1	Gift sample from P.Sanz (Institute de biomedicine de Valencia, CSIC)	1:1000	Antimouse	GE Healthcare/ LNA931v/AG	1:10000

If the blot had been already probed with anti-phospho-Thr172–AMPK, it was first washed twice with TBS-T (0.1%) at room temperature. Then the membranes were stripped by incubation for 5 min at room temperature with gentle shaking in a stripping solution (0.2M glycine). After stripping, the membranes were washed twice for 5 min interval with TBS-T (0.1%) at room temperature. This procedure was followed as described in (Bellí et al., 1998). Following which the protocol of western blot (detailed in **section 15**) was followed for probing with polyhistidine-anti-Snf1 antibody to detect total Snf1.

RESULTS

Chapter 1

Role of Mtl1 in chronological life span & mitochondrial function

Background

S. cerevisiae is an optimal eukaryotic model to unravel the machinery that controls the chronological life span (CLS) through the study of the stationary phase. When yeast cells are deprived from nutrients they enter into stationary phase (reviewed in De Virgilio, 2012; Dechant and Peter, 2008; Galdieri et al., 2010; Gray et al., 2004). A correct adaptation to nutrient deprivation through a tight and precise regulation of different signaling cascades is crucial in order to extend life (reviewed in Conrad et al., 2014; De Virgilio, 2012). Mtl1 is a transmembrane protein and member of Pkc1-MAPK pathway that senses oxidative stress and nutrient deprivation transmitting the signal to inactivate *TOR1* and *Ras2* in exponentially growing cells (Petkova et al., 2010a). Mtl1 physically interacts with the Rho1 GTPase Exchange Factor (Petkova et al., 2012). Mtl1 is highly N-glycosylated and O-mannosylated and this mannosylation seems to determine its role in the oxidative response and in TOR regulation (Petkova et al., 2012). It has been reported that Mtl1 is transcriptionally upregulated in stationary phase through Msn2/Msn4 and upon rapamycin treatment, both conditions represent different quiescent states (reviewed in De Virgilio, 2012; Petkova et al., 2012). In addition, upon rapamycin treatment and in stationary phase, have shown a significant increase in Mtl1 fluorescence in the periphery of the cells (Petkova et al., 2012). These observations made us to consider that Mtl1 could play a role in quiescent conditions.

1.1 Absence of Mtl1 shortens Chronological life span

In order to ascertain this we first analyzed *mtl1* Chronological Life Span (CLS) in comparison to wild type cells. To do this we followed the protocol described by (Parrella and Longo, 2008) (see Materials and Methods) and we observed a dramatic loss of viability in *mtl1* mutant compared to wild type (wt) cells (Fig 1A). In order to check if Mtl1 overexpression supports viability of budding yeast, we next over-expressed Mtl1 plasmid cloned under $tetO_7$ promoter in wt cells. We observed an increased CLS than that determined in wt cultures transformed with the empty plasmid (Fig 1A). Therefore, we concluded that Mtl1 plays a positive role in the life extension of budding yeast. Experiments of CLS were performed in Synthetic Complete (SC) medium and these results are the average of three independent experiments.

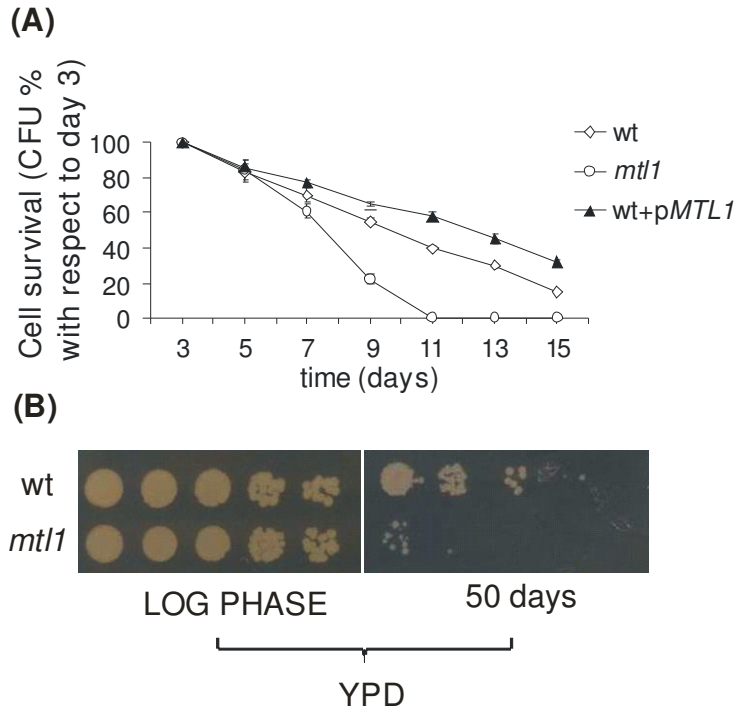


Figure1: **Mtl1 positively influences chronological life span**. **A**) Wild type, *mtl1* and wt expressing p*MTL1* under the $tetO_7$ were logarithmically grown in SC medium at 30˚C, samples were taken at the indicated times to determine CLS. These results are the average of three independent experiments and the error bars represents standard deviations. **B**) Cultures of wt and *mtl1* mutant were grown in YPD and subsequently allowed to grow upto 50 days. Cells were 10-fold serial-diluted and spotted on to YPD plates. The plates were then incubated at 30ºC for 2–3 days.

In order to circumvent the possibility of nutrient limitation in cell viability, we also analyzed *mtl1* viability in rich YPD medium. Wild type cells lived longer in rich medium. We noted that *mtl1* exhibited a clear loss of viability upon 50 days of incubation compared to wt cells (Fig 1B).

1.2 The absence of Mtl1 impairs mitochondrial function in quiescence

In budding yeast, diauxic shift and stationary phase are both conditions in which the metabolic state of the cells is mainly through respiration, due to glucose exhaustion. Mitochondria play a cardinal role in cell respiration and to produce energy for cell viability by Krebs cycle. It has been reported in mitochondria, oxidative phosphorylation results from the coupling between the redox-primary proton pumps in the respiratory chain and the F_1F_0-ATP synthase. The efficiency of this system varies when electrons enter or exit the respiratory chain at different enzymes or when the H^+ gradient is used by

secondary pumps for the active transport of proteins, ions and metabolites (Guerrero-Castillo et al., 2011) .

Taking this into account, we reasoned that *mtl1* loss of viability could be related to impaired mitochondrial function during the diauxic shift. In order to ascertain this, we performed several experiments with the objective of characterizing *mtl1* mitochondrial function.

First, we analyzed the respiratory capacity of *mtl1* cells compared to wt using a respirometer. Upon 24 hours post inoculation in YPD medium, when cultures enter in the postdiauxic shift and glucose is depleted, we measured the oxygen consumption in wt and *mtl1* cells. This respiration is termed as Routine or Basal respiration as cells are without external interference. The mutant *mtl1* presented a remarkable reduction in oxygen consumption compared to wt cells in routine respiration (Fig 2A). To identify the source of this respiratory problem we evaluated oxygen consumption in response to different respiratory inhibitors.

We first treated cells with triethyltin bromide (TET), a lipophilic inhibitor of the mitochondrial ATPase (Aguilaniu et al., 2001). Wild type responded by decreasing substantial oxygen consumption (Fig 2A) however *mtl1* mutant was minimally affected. This observation indicated that the contribution of coupled respiration (i.e. mitochondrial phosphorylative activity) to routine respiration in *mtl1* is very low. Moreover, we could observe that this mutant hardly respires in basal conditions. We next treated cell cultures with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore that activates oxygen consumption by allowing protons to re-enter the mitochondrial matrix. This treatment provokes proton leakage and consequently uncouples electron transport from ATP synthesis. We observed that wild type cells recovered their oxygen consumption capacity to values slightly higher than the basal ones whereas *mtl1* cells increased their oxygen consumption two fold with respect to the initial values, although maximal respiratory capacity remained lower than in wild type cells. It has been reported that non-phosphorylating conditions a high proton transmembrane gradient inhibits the rate of oxygen consumption mediated by the mitochondrial respiratory chain (state IV) (Guerrero-Castillo et al., 2011). This reveals the existence of a membrane potential in *mtl1* and confirms that respiration in this mutant is predominantly non-phosphorylating. Finally, we added Antymicin A to determine residual respiration by blocking the activity

of complex III. Both wild type and *mtl1* strains reduced their oxygen consumption to values near zero (Fig 2A). These results also indicated that *mtl1* oxygen consumption is predominantly mitochondrial.

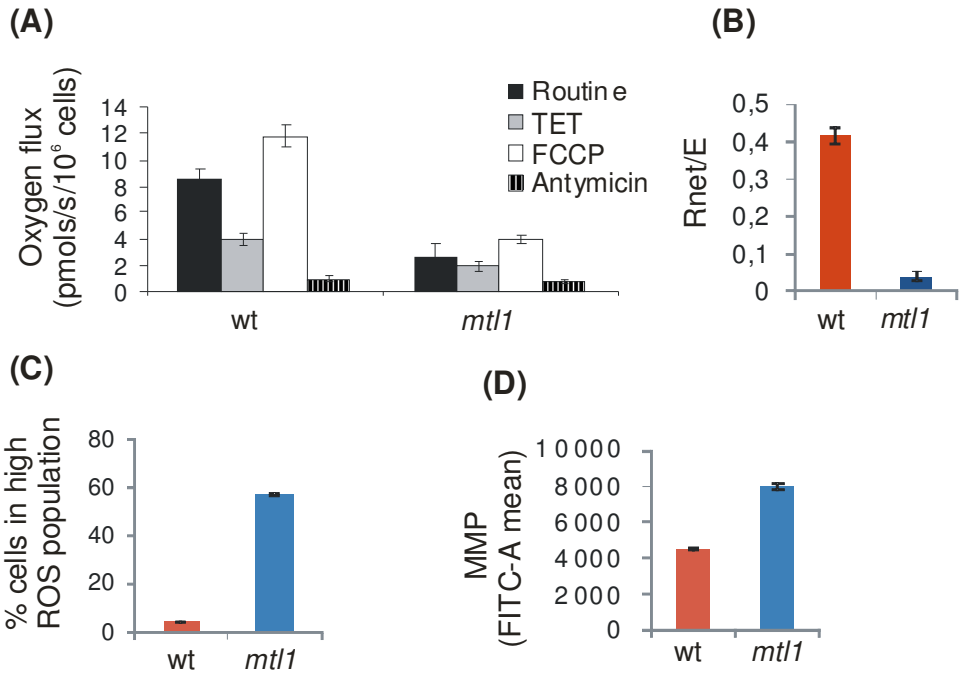


Figure 2: **Absence of Mtl1 causes ROS accumulation, high mitochondrial membrane potential and uncoupled respiration affecting the respiratory capacity. A)** Mitochondrial respiration was determined in a respirometer (See Materials and Methods**).** Cultures from wt and *mtl1* strains were grown in YPD at 30ºC to log phase (OD600: 0.6) and further allowed to grow for 24 hours to enter diauxic shift. Routine represents oxygen consumption from cells placed in the respirometer directly from the culture without any external interference. TET is an ATP synthase inhibitor that blocks respiration. The remaining respiration occurs as a consequence of protons leaking across the mitochondrial membrane. The ionophore FCCP restores mitochondrial respiration. Antymicin A, an inhibitor of complex III, blocks mitochondrial respiration; consequently the remaining oxygen consumption was not of mitochondrial origin. **B)** Rnet/E is the ratio between net routine respiration and the values obtained upon treatment with the uncoupler FCCP. Values were estimated in wt, and *mtl.* **C)** ROS accumulation was performed using H_2DCF-DA assay (See Material and Methods) in wt and *mtl1* cells in diauxic shift. **D)** Mitochondrial membrane potential (MMP) was performed in wt and *mtl1* in diauxic shift using $DiOC_6$ assay (See Materials and Methods).These experiments were performed in triplicate (the values are the means of three independent experiments and the error bars represent standard deviations) and the averages plus standard deviations were plotted in the histograms.

To study further changes in mitochondrial function associated to the absence of *mtl1*, mitochondrial coupling states were analyzed. Respiratory flux control ratios were

determined from the aforementioned titrations with the ATP synthase inhibitor TET and the uncoupler FCCP. When TET-resistant respiration is subtracted to routine respiration (net routine), an estimation of the coupled respiration is obtained. The ratio of net routine respiration to uncoupled respiration (Rnet/E) was significantly reduced in *mtl1* with respect to wild type cells (Fig 2B). From these observations we can take several conclusions: i) in the absence of Mtl1 the respiration coupled to oxidative phosphorylation is minoritary; ii) *mtl1* respiratory capacity is significantly diminished compared to wild type cells; and iii) *mtl1* respiration is predominantly uncoupled.

Since *mtl1* mutant hardly respirates, we assumed ROS accumulation could be a possible reason for this mitochondrial malfunctioning. Hence we next quantified ROS accumulation as an indicative of a respiratory chain impairment. We detected high ROS accumulation in *mtl1* cells during the diauxic shift while ROS were almost undetectable in wt cells (Fig 2C). These results reflected that Mtl1 was affecting the proper function of the respiratory chain. ROS accumulation and uncoupled respiration in *mtl1* suggest alterations in the mitochondrial membrane potential (MMP).The MMP was determined in cells according to (Leadsham et al., 2008, Simon et al., 1997). The membrane-potential-sensitive dye 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) was added to cell suspensions and analyzed by flow cytometry using the FL1 channel. MMP was quantified by using all events FITC-A mean. As we hypothesized there existed a marked increase in MMP in *mtl1* mutant compared to wt cells (Fig 2D).

Following these previous observations, we decided to investigate whether *mtl1* respiratory defects were due to variations in the mitochondrial content and/or mitochondrial function. We transformed wt and *mtl1* cells with a plasmid harboring a gene encoding a mitochondrial protein fused to GFP. Mitochondrial morphology was similar in both *mtl1* and wt strains growing exponentially. During the diauxic shift, wild type cells mitochondria presented the characteristic globular pattern homogeneously distributed throughout the cytoplasm (Breitenbach et al., 2013). However, in the *mtl1* mutant, mitochondria appeared grouped in bigger patches and the distribution was not completely homogeneous in the cytoplasm (Fig 3A) which correlated with *mtl1* respiratory deficiency.

We next decided to determine mitochondrial content by analyzing the transcriptional levels of *COX2*, a mitochondrial gene that encodes for a protein part of the respiratory

complex IV. *COX2* mRNA levels were equivalent in both *mtl1* and wt cells, both in exponential cultures (Fig 3B).

Since signaling processes play a major part in cells entering quiescence, maintenance and also exit from quiescence. We considered signaling of genes which regulate between nucleus to mitochondria would be necessary to determine the effects of mitochondrial dysfunction in *mtl1* mutant. Where *COX4* is a nuclear gene encoding for a protein which is also a part of the respiratory complex IV was used analyzed for its expression levels by Northern blot analysis. Interestingly, we observed a remarkable defect in *COX4* mRNA levels in *mtl1* cells compared to wt cells (Fig 3B). Since *COX4* is tightly regulated through several signaling pathways, this result suggested that *mtl1* defects were likely due to defects in specific signaling pathways.

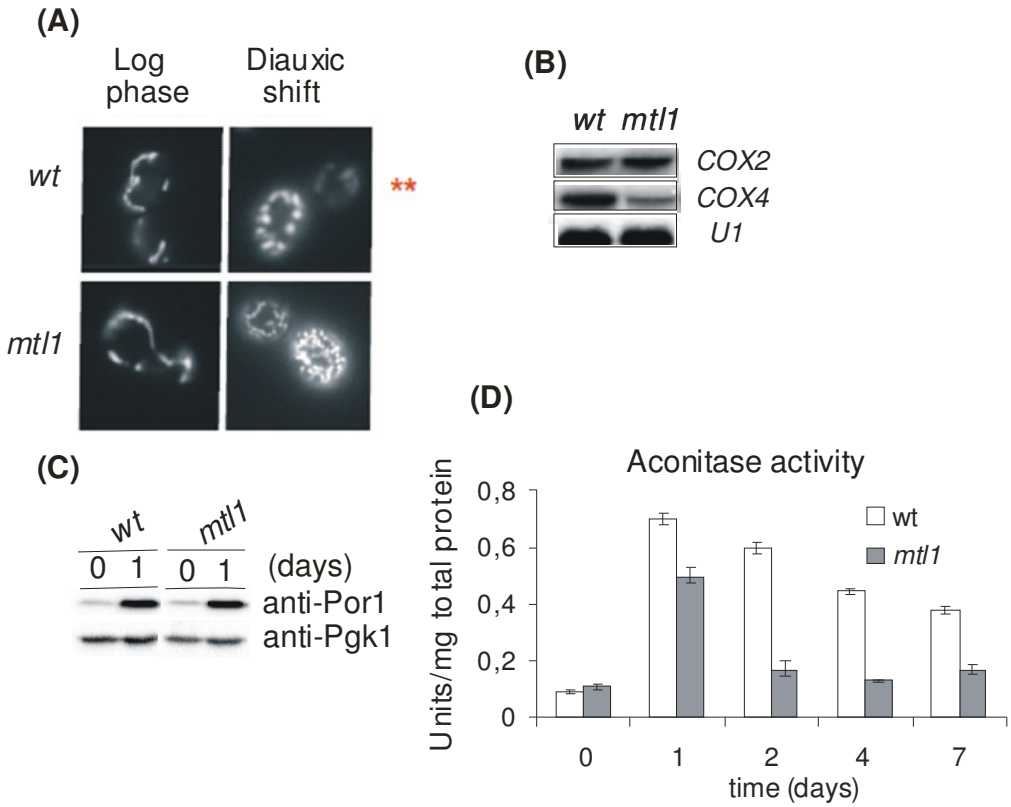


Figure 3:***mtl1* mutants are impaired in the mitochondrial function. A)** wt and *mtl1* cells transformed with pVT100U plasmid, harboring a gene encoding for a mitochondrial protein; fused to GFP, were grown in SC plus amino acids to log phase and grown susbequently to diauxic shift (36 hours of growth), aliquots were collected for their *in vivo* observation under the fluorescence microscope. **B)** Aliquots from wt and *mtl1* cultures growing exponentially in SD plus amino acids were collected and mRNA was extracted for northern blot analyses using *COX2*, *COX4* and *U2* as probes. *U2* was used as the loading control. **C)** Wild type and mtl1 cells were grown in YPD medium to logarithmic phase and subsequently to diauxic shift (1 day).

Samples were collected at both time points to prepare protein extracts to detect Por1 levels using anti-Por1 and anti-Pgk1 as loading control. **D)** wt and *mtl1* cultures were grown in YPD medium; samples were collected at different points to determine aconitase activity, from log phase (time 0 in the figure) to 7 days, as depicted in the histogram. Histograms represent the mean plus standard deviations of three repetitions.

In order to obtain more precise information regarding mitochondrial content we analysed the amount of Por1 in samples taken from cultures growing exponentially and upon 1 day from both wt and *mtl1* cells (Fig 3C). Por1 levels significantly increased upon 1 day of growth, indicating an active mitochondrial biogenesis as a consequence of the respiratory metabolism that is activated in the diauxic shift. Both wt and *mtl1* cells presented equivalent levels of Por1, indicating that *mtl1* did not present any defect in the mitochondrial content.

In order to obtain a quantitative characterization of the mitochondrial function we measured the aconitase activity. Aconitase operates in the TCA cycle and is needed for respiration, therefore aconitase activity is considered an indirect measurement of mitochondrial function (Gangloff et al., 1990).We monitored aconitase activity in *mtl1* and wt cells growing in YPD for several days and detected a severe reduction in this enzymatic activity in *mtl1* cells compared to wt cultures (Fig 3D). Taking altogether these results we concluded that absence of Mtl1 protein provoked structural and functional mitochondrial problems affecting respiratory capacity, inducing ROS accumulation and shortening CLS.

1.3 Reactive Oxygen Species are not the primary cause of mitochondrial dysfunction nor CLS shortage in *mtl1* mutant

We reasoned that *mtl1* mitochondrial impairment could cause abnormal ROS accumulation affecting *mtl1* cell viability and/or mitochondrial function, thus explaining the former described *mtl1* phenotypes. In order to ascertain this we used the antioxidant N-acetylcysteine in two concentrations 5mM and 10mM to perform CLS studies in wt and *mtl1* cells respectively (Fig 4A). Surprisingly, we observed a severe CLS reduction in *mtl1* cells upon addition of the antioxidant in a dose-dependent manner (Fig 4A). ROS accumulation was reduced to about 95% in *mtl1* cells during the diauxic shift. This result suggested that rather than being toxic, ROS production acted as signaling molecules promoting cell survival in a physiological state in where mitochondrial function is severely impaired. To check the role of ROS and mitochondrial function, we performed

aconitase activity measurements in addition with ROS scavenger N-acetyl cysteine. Aconitase activity was not affected by the addition of N-acetylcysteine (Fig 4B) in neither wild type nor *mtl1* cultures, suggesting that ROS accumulation was not the cause but a consequence of the mitochondrial dysfunction observed in the *mtl1* mutant.

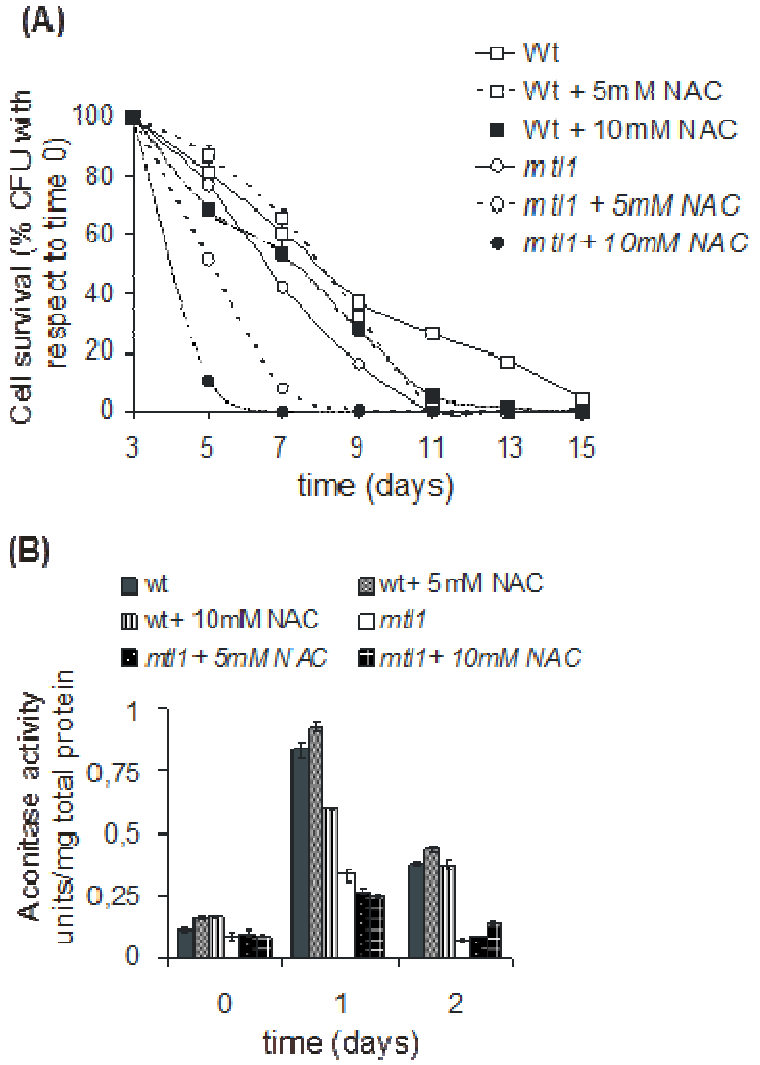


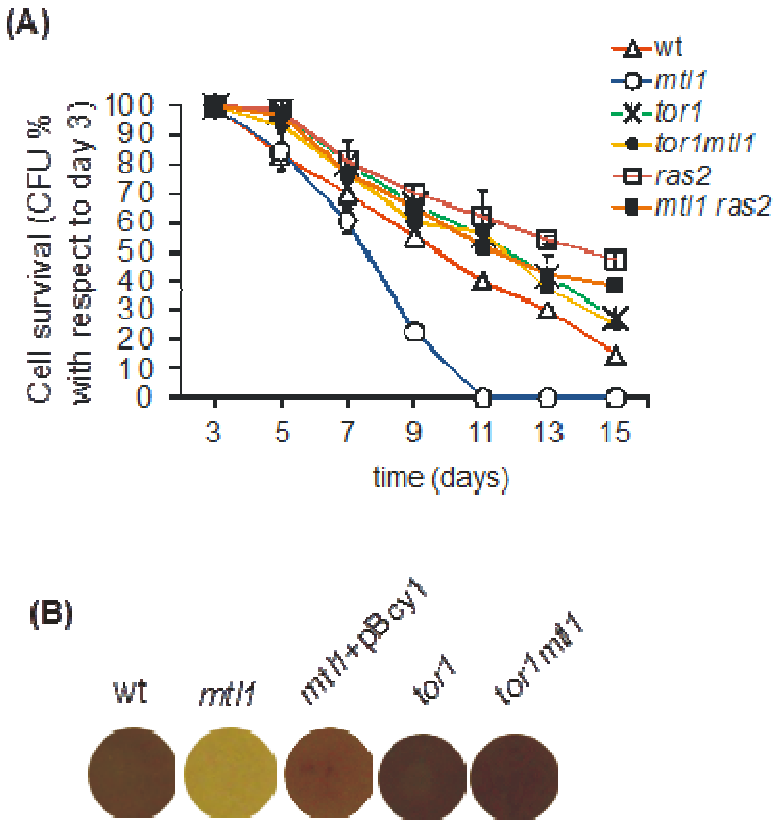
Figure 4**: N-acetylcysteine reduces CLS in wt and more importantly in *mtl1* cells, without affecting mitochondrial function. A)** wt and *mtl1* cells were grown as in Fig 1A to perform CLS experiments in different conditions: culture cells were either untreated or treated with 5 or 10mM N-acetyl cysteine. These results are the average of three independent experiments and the error bars represents standard deviations. **B)** Aconitase activity was determined in both wt and *mtl1* cultures treated or not with either 5 or 10mM N-acetylcysteine. Samples were taken at the times indicated in the histogram. Histograms represent the mean plus standard deviations of three repetitions.

These results supported the former hypothesis that *COX4* regulation reflected a misregulation of signaling pathways, may be due to the absence of *MTL1*. One possible

explanation is that when cells entered in quiescence, ROS accumulated in cells acted as signaling molecules to promote cell survival.

1.5 Mtl1 is required to extend life span through the inactivation of *TOR1* and PKA in stationary phase

There are several signaling pathways whose regulation is important to correctly progress into quiescence (De Virgilio, 2012; Gray et al., 2004). From the previous results we present evidence demonstrating that Mtl1 is required for proper mitochondrial function and progression through quiescence, but the mutant does not present any significant impairment in mitochondrial content. According to the former results, we reasoned that *mtl1* mitochondrial defect could be related to signaling misregulation rather than to a primary mitochondrial problem. In this line, we decided to investigate the possible interaction between *MTL1*and *TOR1*, *RAS2* and PKA. Firstly, we analyzed CLS in *mtl1tor1*, *mt1ras2*, *tor1*, *ras2* mutants and in both wt and *mtl1* transformed with a plasmid harbouring Bcy1.



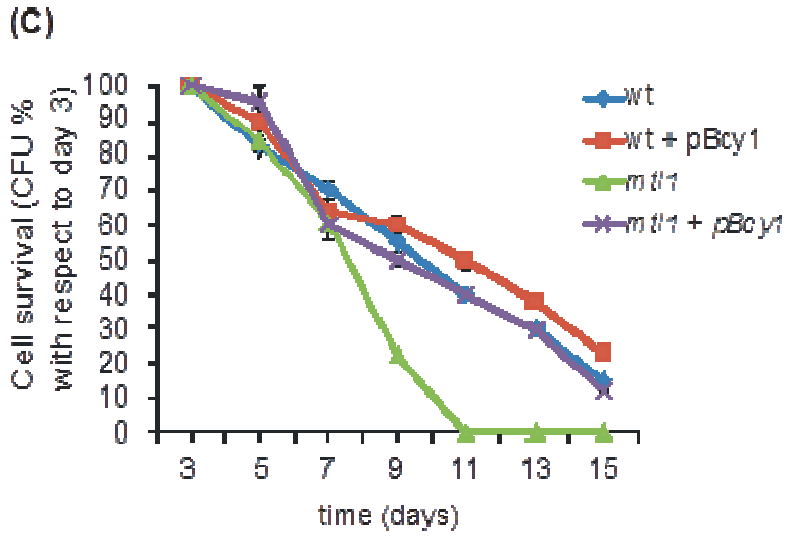


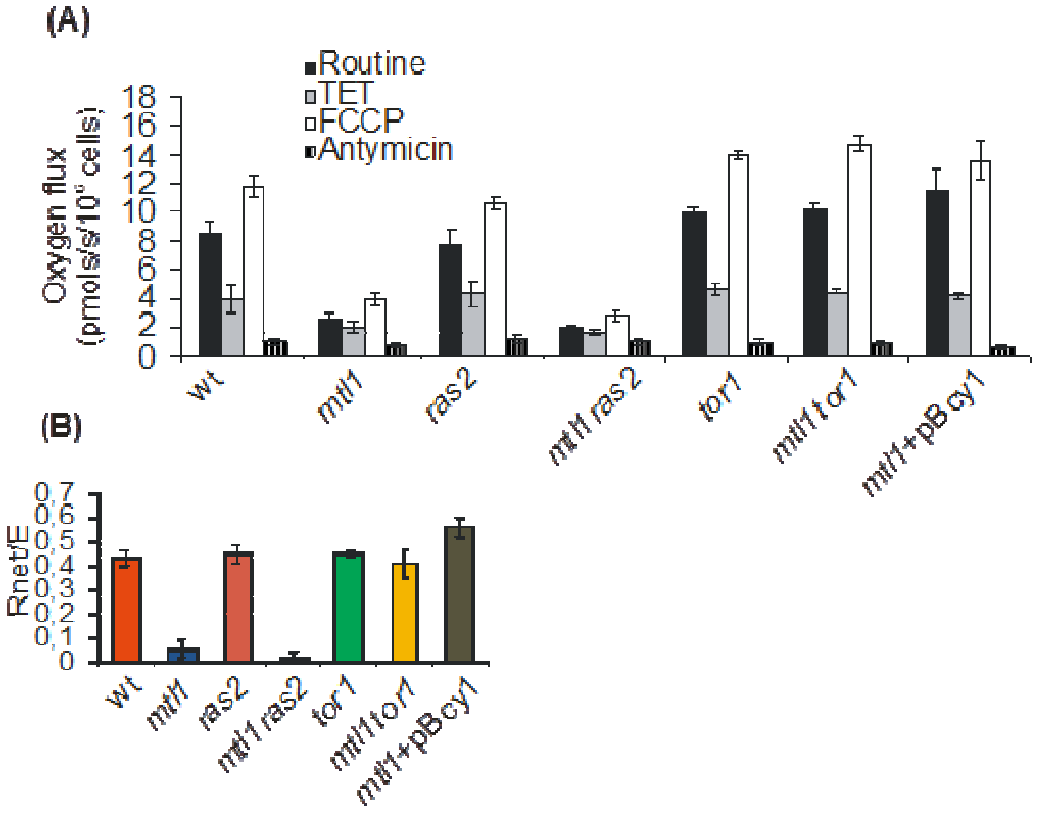
Figure 5**: Deletion of *TOR1* extends *mtl1* life span to *tor1* levels, whereas PKA inactivation restores *mtl1* CLS to wild type values. A)** CLS estimation in wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1* and *mtl1tor1* strains was carried out as in Fig 1A. These results are the average of three independent experiments and the error bars represent standard deviations. **B)** Glycogen accumulation was determined in cultures of wt, *mtl1, tor1, mtl1tor1* and *mtl1*+pBcy1 strains grown in YPD to diauxic shift (36 hours) (see Materials and Methods). **C)** CLS studies in wt, wt+pBcy1, *mtl1* and *mtl1*+pBcy1 were performed as in Fig 1A. These results are the average of three independent experiments and the error bars represents standard deviations.

We observed that *TOR1* deletion suppressed *mtl1* defects in chronological life span, moreover, CLS values in *mtl1tor1* mutant were epistatic to those determined in *tor1* (Fig 5A), suggesting that Mtl1 might function upstream of TORC1, negatively regulating Tor1 in the progression through stationary phase. Deleting *RAS2* also significantly increased *mtll1* CLS, compared to wt levels, although *mtl1ras2* double mutant presented a more reduced CLS than that determined in *ras2* single mutant, suggesting that Mtl1 and Ras2 might act in parallel pathways regarding cell survival in quiescence. We over expressed Bcy1 to mimic PKA inhibition, this is verified by the observation that *TOR1* deletion and increasing Bcy1 levels in *mtl1* both also enhanced glycogen accumulation nearly to wt levels (Fig 5B).

Bcy1 over expression restored *mtl1* CLS to wild type levels (Fig 5C). These results suggested that Mtl1 might have a role in the down regulation of both *TOR1* and PKA during stationary phase and this inactivation was required for cell viability.

1.6 *TOR1* deletion or PKA inactivation, both restore *mtl1* mitochondrial function independently on *RAS2*

We examined oxygen consumption in all the strains used in the former experiment and found out that both *TOR1* deletion and Bcy1 over expression not only restored the respiratory capacity of *mtl1* (Fig 6A) but also properly coupled *mtl1* respiration during the diauxic shift (Fig 6A). Interestingly, *RAS2* deletion did not suppress the *mtl1* respiratory defect in diauxic shift (Fig 6A).The ratio of net routine respiration to uncoupled respiration (Rnet/E) was evaluated to determine respiratory flux control ratios in all the strains used in (Fig 6B). The Rnet/E values are depicted in (Fig 6B) and the low respiratory flux ratios of *mtl1* mutant are rescued by *TOR1* deletion and also over expression of pBcy1 in diauxic shift. This result confirms *TOR1* down regulation induces coupled respiration in mitochondria for energy production and also survival in quiescence.



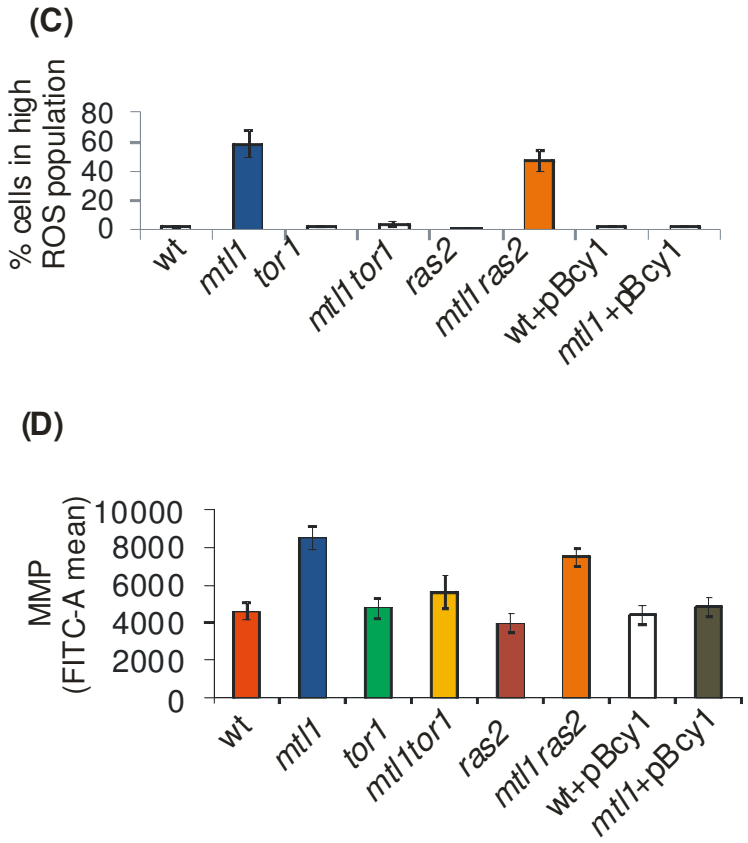


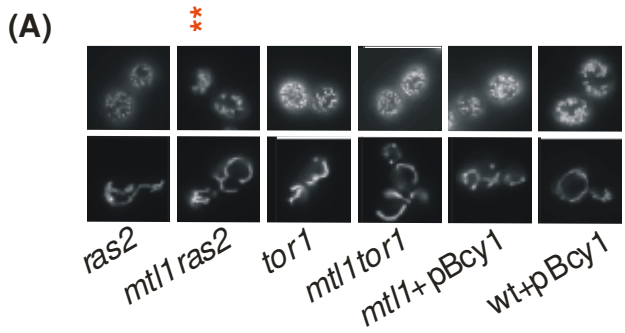
Figure 6**: Both *TOR1* deletion and PKA inactivation suppress *mtl1* defects in oxygen consumption, ROS accumulation and MMP independently on *RAS2*. A)** Mitochondrial respiration determined as in Fig 2A in cultures of wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1*, *mtl1tor1* and *mtl1*+pBcy1 strains grown for 24 hours in YPD to the diauxic shift. **B)** Rnet/E values were determined as in Fig 2B in the strains used in A**C)** ROS accumulation was quantified as described in Fig 2C in the strains used in A. **D)** Mitochondrial membrane potential (MMP) determined as in Fig 2D in the strains used in A. Histograms represent the mean plus standard deviations of three repetitions.

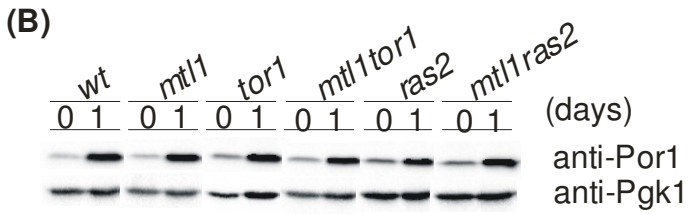
To further evaluate mitochondrial functional with respect to oxidants produced in respiratory conditions, ROS production was estimated in strains wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1*, *mtl1tor1* and *mtl1*+pBcy1 grown in YPD for 24h to diauxic shift. In one hand and in accordance to previous result the high accumulation of ROS in *mtl1* mutant was suppressed by *TOR1* deletion and also Bcy1 over expression. On the other hand, *RAS2* deletion did not suppress the ROS levels in *mtl1* mutant as the double mutant *mtl1ras2* accumulated sufficient ROS population in diauxic shift (Fig 6C)**.**

We next monitored the MMP in wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1*, *mtl1tor1* and *mtl1*+pBcy1 cultures grown in YPD for 24h to diauxic shift (Fig 6D). We observed restoration of high

MMP values of *mtl1*mutant to be lowered by *TOR1* deletion and Bcy1 overexpression, while *RAS2* deletion in *mtl1* did not significantly affect the membrane potential.

Finally to see the difference in morphological pattern change in mitochondria during diauxic shift we visualized mitochondrial morphology as previously done (Fig 2E) in *ras2*, *mtl1ras2*, *tor1*, *mtl1tor1* expressing pVT100U where a mitochondrial protein fused to GFP. We also performed the same in wt+pBcy1and *mtl1*+pBcy1 cultures co-transformed with pYX142 expressing a mitochondrial protein fused to GFP having Leucine as marker. The cultures were grown in YPD for 24h to diauxic shift (Fig 7A) and the cultures were visualized under fluorescence microscope. The mitochondrial morphology were abnormal globular distribution observed in *mtl1* mutant were restored upon *TOR1* deletion in *mtl1* to *tor1* levels, or upon PKA inhibition in *mtl1* to wt levels; however, *RAS2* deletion did not rescue any of the above *mtl1* mentioned phenotypes. We decided to check the mitochondrial protein, Por 1 expression to confirm the mitochondrial content evaluation, and so we had analyzed protein levels in logarithmic phase and diauxic shift in wt, *mtl1, tor1, mtl1tor1, ras2* and *mtl1ras2* strains (Fig 7B). The results in (Fig 7B) indicate that *mtl1* shows no difference in mitochondrial content in diauxic shift.





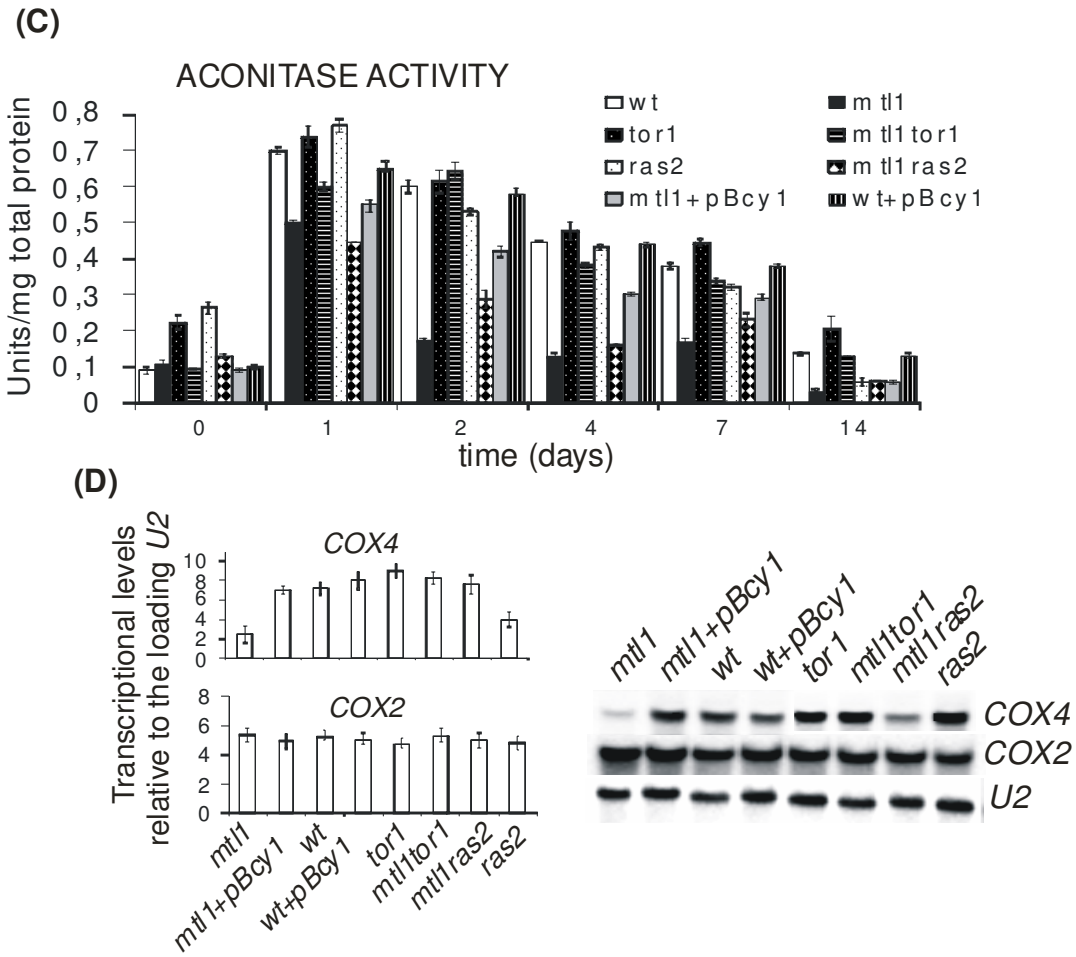


Figure 7**: Both *TOR1* deletion and PKA inactivation suppress *mtl1* defects in mitochondrial function independently on *RAS2*. A)** Mitochondrial morphology is depicted in cultures of *ras2*, *mtl1ras2*, *tor1*, *mtl1tor1,*wt+ pBcy1 and *mtl1*+ pBcy1 and determined as mentioned in Fig 2A. **B)** Samples from cultures of wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1*, *mtl1tor1* of the former experiment were collected in logarithmic phase and diauxic shift, later processed for western blot to detect Por1 levels using anti-Por1 and anti-Pgk1 was used as loading control. **C)** Aconitase was determined in wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1*, *mtl1tor1,* wt+ pBcy1 and *mtl1*+ pBcy1 as previously described Fig 2D at times indicated in the figure. Histograms represent the mean plus standard deviations of three repetitions. **D)** Samples from the strains used in C were grown in SD media plus aminoacids to logarithmic phase to perform northern blot analysis to detect *COX2* and *COX4* expression. *U1* probe was used as loading control for northern blot. The histograms represent the relative values of each *COX2* and *COX4* transcription with respect to the loading control.

To analyze the functional capability of mitochondria in quiescence we measured aconitase, a key enzyme involved in TCA cycle in cultures of wt, *mtl1*, *tor1*, *mtl1tor1*, *ras2*, *mtl1ras2*, wt + pBcy1 and *mtl1*+pBcy1. As seen in earlier results, the very low aconitase activity levels detected in *mtl1* during quiescence were also rescued upon *TOR1* deletion and after Bcy1 over expression (Fig 7C), whereas *RAS2* deletion did not increase aconitase levels in *mtl1* mutant. However, in stationary phase aconitase activity was

significantly higher in *tor1mtl1* than in *mtl1* harbouring Bcy1 (Fig 7C). In fact, *TOR1* deletion in *mtl1* mutant raised aconitase levels to wt levels whereas Bcy1 over expression was less efficient in this function.

The results analyzed indicate that both *TOR1* deletion and PKA inactivation suppress *mtl1* mitochondrial dysfunction. However, the suppression observed in *mtl1*upon *TOR1* deletion was epistatic to *tor1* whereas *mtl1* upon PKA inactivation was epistatic to wild type strain.

These results suggest that *mtl1* mitochondrial defects might be a consequence of the inability to inactivate Tor1 in quiescent conditions and that PKA inactivation could represent one of the outputs regulated by Tor1 in this response. These data are in agreement with the survival experiments in where *TOR1* deletion increased the CLS in *mtl1* more efficiently than Bcy1 over expression.

Finally, and in accordance with the results described above, we performed an experiment to analyze the signal between Mtl1 & Tor1 and Mt1l & PKA respectively by transcription pattern of *COX4*. We observed that *COX4* mRNA expression was recovered to wild type levels in *mtl1tor1* mutant and to a lower extent in *mtl1*+pBcy1 (Fig 7D). Again, in *mtl1 ras2; COX4* levels were lower than that those observed in wild type cells and similar to those determined in *mtl1* strain (Fig 7D).

Chapter 2

Role of Mtl1 in regulating CWI, TORC1 and PKA function in Quiescence

Background

In major eukaryotic model organisms, from the unicellular yeast to mice, pathways such as TOR/ Sch9, and RAS/PKA are involved in the regulation of metabolism and growth, and can also promote aging (Mirzaei et al., 2014). Wiring of the different signaling pathways is crucial for a proper onset of stationary phase and quiescence. TORC1 and PKA both negatively regulate the transition into quiescence whereas PKC1-MAPK and Snf1 are positive regulators in this signaling network (reviewed in De Virgilio, 2012; Gray et al., 2004).

The CWI pathway has been involved in the cellular responses to many stresses such as cell-wall, heat shock, hypo-osmotic, nutritional, pH and oxidative stresses (de la Torre-Ruiz et al., 2010; Heinisch and Lorberg, 1999; Levin, 2005; Petkova et al., 2010a; Petkova et al., 2012; Serrano et al., 2006; Vilella et al., 2005). Signal from Mtl1 is transferred to Rho1, GTpase of Pkc1-MAPK pathway and then later follows two simultaneous routes: (i) one leads to activation of the CWI pathway and (ii) the other is transduced through Rom2 and Rho1 to inactivate both the Tor1 and Ras2/cAMP functions (Petkova et al., 2010a; Petkova et al., 2010b). Slt2/Mpk1 activity is essential for cell survival under quiescence conditions (reviewed in Gray et al., 2004; Petkova et al., 2010a; Torres, 2002). In response to rapamycin treatment TORC1 is inhibited, thus releasing Slt2, inhibition by Sch9 leading to Bcy1 phosphorylation in residue T129 mediated by the MAPK; this specific phosphorylation inhibits PKA activity (Soulard et al., 2010). Under specific physiological conditions TORC1 and PKA antagonise each other (Ramachandran and Herman, 2011). In some circumstances TOR and PKA seem to work independently (Zurita-martinez et al., 2005), whereas in other conditions TORC1 signals to PKA/cAMP (Martin et al., 2000; Petkova et al., 2010b; Schmelzle et al., 2004; Soulard et al., 2010), demonstrating that the interconnection between these pathways is complex. Although the coexistence of crosstalk at different levels between the CWI, Ras2/cAMP/PKA and TOR pathways is already accepted, the precise demonstration of the different levels at which this occurs is still obscure.

2.1 The absence of Mtl1 prevents *TOR1* down regulation in the diauxic shift

In order to precisely determine the connection between Tor1 and Mtl1 we chose genes representative of TOR readouts Rtg1/3 (*CIT2*), Gln3 (*MEP2*) and Msn2/4 (*HSP12*) and performed transcriptional analysis in samples taken during progression through the diauxic shift. In (Fig 8), it can be observed that in wild type cells there is a clear induction of *CIT2*, *HSP12* and *MEP2* genes, indicative of TORC1 down regulation. However, the induction of the three reporter genes was severely impaired in *mtl1* mutant, meaning that during the transition between fermentative to respiratory metabolism, TORC1 down regulation is defective in the absence of Mtl1. These results strongly support our hypothesis that in the absence of *MTL1*, TORC1 is not properly down regulated to progress into stationary phase.

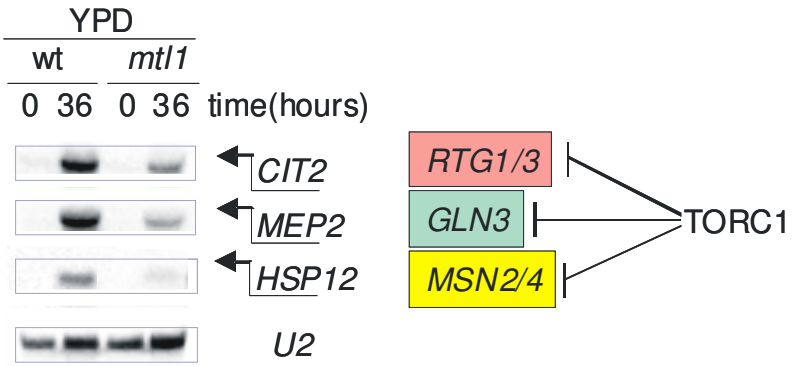


Figure 8**: TORC1 readouts RTG1/3, GLN3 and MSN2/4 are down regulated in diauxic shift in the *mtl1* mutant**. Wild type and *mtl1* cultures were grown to logarithmic phase and up to 36 hours in YPD. Samples were taken and processed for mRNA extraction and northern blot analysis. Genes representative of TOR readouts Rtg1/3 (*CIT2*), Gln3 (*MEP2*) and Msn2/4 (*HSP12*) were chosen to perform transcriptional analysis in samples taken during progression through the diauxic shift. *U2* probe was used as a loading control.

2.2 Mtl1 regulates Bcy1 stability through *TOR1* in quiescence, independently on *RAS2*

Bcy1 inhibitory effect on PKA activity occurs upon its phosphorylation in different residues. In order to investigate a possible relationship between Mtl1 and PKA we analyzed Bcy1 protein phosphorylation in both *mtl1* and wt cells in log phase and during the diauxic shift by expressing a plasmid harboring Bcy1HA (Fig 9A). We observed a

progressive phosphorylation of Bcy1 as wt cells entered into stationary phase, being evident a severe reduction in Bcy1 phosphorylation in *mtl1* cells compared to wild type levels (Fig 9A). More over treatment of 30 min with rapid Alkaline Phosphatase (rAP) to identify the phosphorylated band revealed that the upper band out of the two bands observed was found out to be the activated phosphorylated form of Bcy1 (Fig 9A).

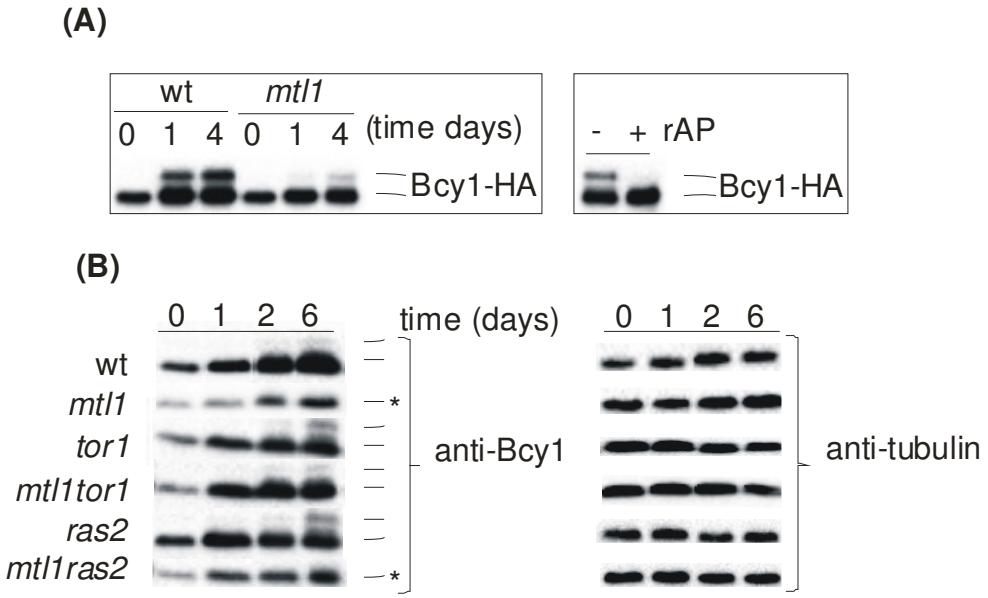


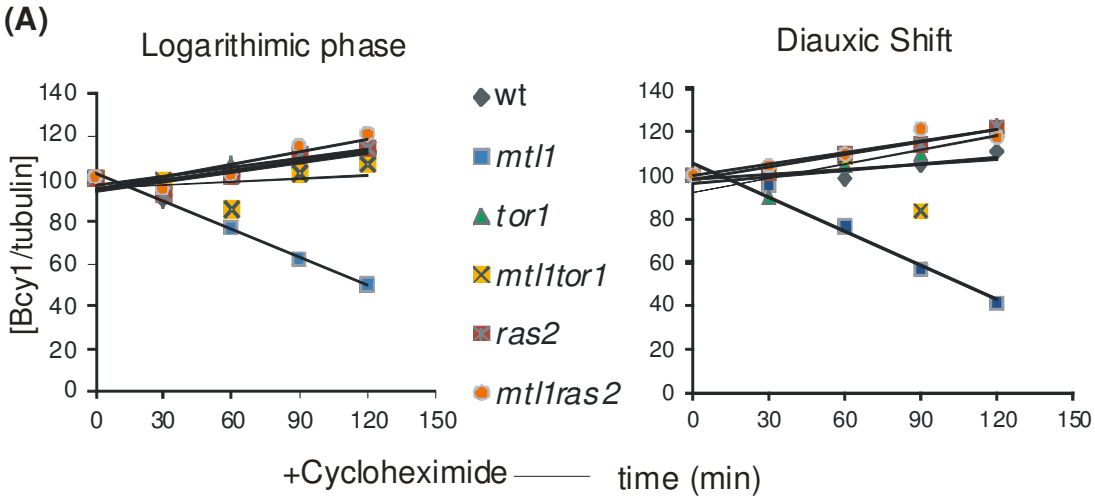
Figure 9**: Mtl1 regulates Bcy1 phosphorylation through Tor1 inactivation in stationary phase**. **A)** wt and *mtl1* cells transformed with a plasmid harbouring Bcy1HA were grown in YPD to log phase and after 1 and 4 days; after that samples were collected and processed for western blot analyses, two mobility forms of the Bcy1 protein were detected by using anti-HA antibody. Wild type cultures were grown to diauxic shift and later immunoprecipitated and dephosphorylated with Rapid Alkaline Phosphatase (rAP) to demonstrate that the upper mobility form corresponded to the Bcy1 phosphorylated form. **B)** Cultures of wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1* and *mtl1tor1* strains were exponentially grown in YPD medium, samples were collected at the times indicated in the Figure to perform western blot analyses in order to detect Bcy1 total protein, by using the specific anti-Bcy1 polyclonal antibody (Yc-20) (see Materials and Methods).

In addition, we tried in wt, *mtl1, tor1, mtl1tor1, ras2* and *mtl1ras2* cells to check the expression of the inhibitory subunit, Bcy1 of the PKA pathway. Using a specific anti-Bcy1 polyclonal antibody (Yc-20) we detected an increase in endogenous Bcy1 protein levels in wild type cells along with the appearance of a lower mobility band compatible with the phosphorylated band as cells progressed into stationary phase.

Bcy1 steady levels were lower in *mtl1* cells than in wt cells, at each time point analyzed (Fig 9B). Deletion of *TOR1* in *mtl1* cells not only raised Bcy1 protein levels but also the phosphorylation levels similar to those determined in either *tor1* or wt strains. The results

also showed *RAS2* deletion in *mtl1* did not provoke any substantial variation in Bcy1 phosphorylation with respect to *mtl1* (Fig 9B).

PKA activity is regulated through a feedback mechanism mediated by cAMP (Budhwar et al., 2010; Ma et al., 1999) this observation made us to consider the possibility that some of the defects detected in *mtl1* mutant related to Bcy1 phosphorylation could be an indirect consequence of high PKA activity. In order to explain whether the low levels of Bcy1 phosphorylation detected in *mtl1* were due to posttranslational effects, we studied Bcy1 stability both in cells growing exponentially and in the diauxic shift (Fig 10A). For this, we treated both exponential cultures or cultures grown to diauxic shift of *mtl1*, *tor1*, *mtl1tor1*, *ras2*, *mtl1ras2* and wild type cells with cycloheximide for specific periods of time (30 min) (see Material and Methods). Later samples were taken at different time intervals indicated in the figure to determine total amount of endogenous Bcy1 (Fig 10A). We have demonstrated in (Fig 10A) that after several repetitions and quantifications Bcy1 half-life in *mtl1* cultures was 95 to 100 min post cycloheximide addition, whereas in wt cells Bcy1 was highly stable during the course of the experiment. Moreover, *TOR1* deletion restored Bcy1 stability in *mtl1* cells to wild type values along with Bcy1 phosporylation, after 2 days of growth in YPD, although the corresponding band was more clearly visualized upon 6 days of incubation, as depicted in (Fig 10A). However, in the double mutant *mtl1ras2* we also observed an increase in Bcy1 stability but Bcy1 phosphorylation was not detectable upon 6 days of growth in YPD.



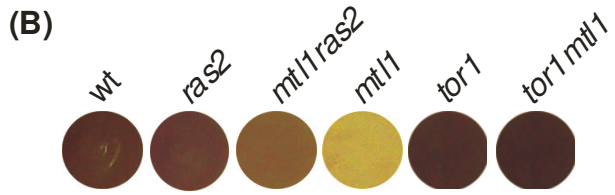


Figure 10**: Mtl1 regulates Bcy1 stability through Tor1 inactivation in both logarithmic phase and diauxic shift**. **A)** Cultures of wt, *mtl1, tor1, mtl1tor1, ras2* and *mtl1ras2* were logarithmically grown to OD600: 0.6 and subsequently allowed to grow for 24h in YPD to diauxic shift. Both logarithmic and diauxic cultures were independently treated with 150µg/ml of cycloheximide to terminate protein synthesis (See Materials and Methods). Cells were harvested at the indicated times in the figure for western blotting to analyze the expression of Bcy1 from total protein extracts by SDS-PAGE, using polyclonal anti-Bcy1 antibody. Quantifications of Bcy1 protein were performed relative to the total amount of tubulin used as loading control. Graphs represent the average of 3 independent experiments. **B)** Glycogen content was analyzed in wt, *mtl1, tor1, mtl1tor1, ras2* and *mtl1ras2* cultures grown in YPD to diauxic shift (see Materials and Methods).

To study the biological significance of these results we examined glycogen accumulation in these strains upon 36 hours of growth in YPD medium. We observed that *mtl1* was unable to accumulate glycogen; however *tor1* and *mtl1tor1* both accumulate glycogen whereas *MTL1* deletion diminished the accumulation of glycogen in *ras2* mutant (Fig 10B).

2.3 In quiescence and glucose depletion conditions, Mtl1 regulates Bcy1 activating phosphorylation through *TOR1*, independently on *RAS2*

Bcy1 contains multiple phosphorylation sites, out of which some of them determine Bcy1 activation, having the ability to inhibit PKA, whereas other phosphorylation sites have inhibitory role on Bcy1 function (Budhwar et al., 2010; Kuret et al., 1988; Soulard et al., 2010; Werner-Washburne et al., 1991). Bcy1 inhibitory function on PKA is induced through the phosphorylation of the residue T129 or RxxS/T motif is site specific. Rapamycin treatment induced phosphorylation of Bcy1 at the RxxS/T motif and this effect was due to TORC1 inhibition and also indicates TORC1 control over PKA function through some substrates (Budhwar et al., 2010; Soulard et al., 2010; Werner-Washburne et al., 1991).

In order to determine the mechanism underlying Mtl1 control of Bcy1 activity in quiescent conditions, we made use of the antibody anti-RxxS/T which detects the phosphorylation of the residue T129 of Bcy1. We immunoprecipitated Bcy1 in the strains of wt, *mtl1, tor1, tor1mtl1, ras2* and *mtl1ras2* grown in YPD for 36h to diuaxic shift, and observed that Bcy1 activating phosphorylarion was markedly reduced in *mtl1* mutant compared to wild type cells (Fig 11). These results suggest that the primary effect of *MTL1* deletion is to mediate the activating phosphorylation of Bcy1 in quiescence. Deletion of *TOR1* in *mtl1* cells restored Bcy1 activating phosphorylation (Fig 10).

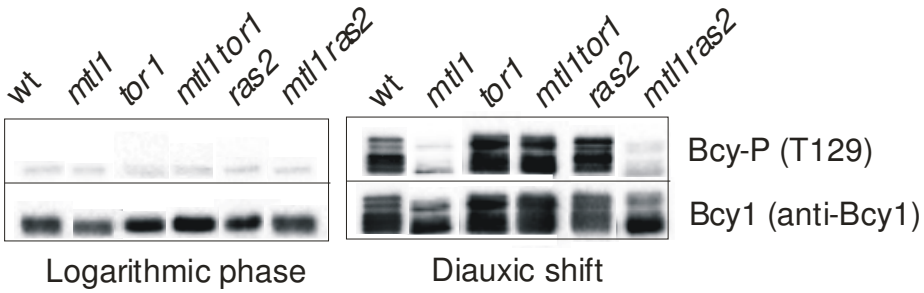


Figure 11**: Mtl1 mediates PKA inactivation through Bcy1-T129 phosphorylation through Tor1 in quiescence.** Cultures of wt, *mtl1, ras2, mtl1ras2, tor1*and *mtl1tor1* strains were exponentially grown in YPD medium and further allowed to grow for a total of 36 hours, corresponding to diauxic phase. Samples from both logarithmic phase and diauxic shift were collected to extract total protein and Bcy1HA was immunoprecipitated (See Materials and Methods). The IP lysates were resolved on SDS-PAGE and Bcy1 phosphorylated form was detected by Immunoblot analysis using polyclonal anti-RxxS/T antibody/phospho PKA antibody that specifically recognises the phosphorylated residue T129 of Bcy1. In parallel, total Bcy1 was detected with the polyclonal anti-Bcy1 antibody.

In addition, phosphorylated Bcy1 is present at much lower level in *mtl1ras2* mutant compared to single *ras2* mutant. Contrary to that observed with *TOR1* deletion, *RAS2* deletion did not efficiently restored Bcy1 phosphorylation in T129 residue in *mtl1* mutant cells. These observations allowed us to build the following hypothesis, in the diauxic shift, wt cells inactivate PKA, and however, in the absence of Mtl1 this activity is abnormally high, being *TOR1* deletion able to completely inactivate PKA in *mtl1* mutant, placing Mtl1 upstream TORC1. Finally, these results place *RAS2* in a different branch with respect to *MTL1* in the regulation of progression through stationary phase.

Another interesting conclusion derived from these results is that Bcy1 instability observed in *mtl1* cells is an indirect consequence of the abnormal high PKA activity detected in this mutant in diauxic conditions.

Our experimental work has been performed during the diauxic shift. Since this transition occurs when glucose is depleted we wondered whether Mtl1 was actually sensing glucose depletion to subsequently downregulate Tor1. In order to ascertain this hypothesis we depleted *mtl1* and wt cells for either glucose or nitrogen, at different time intervals and we performed western blot analysis upon Bcy1 immunoprecipitation with the objective of detecting Bcy1 activating phosphorylation.

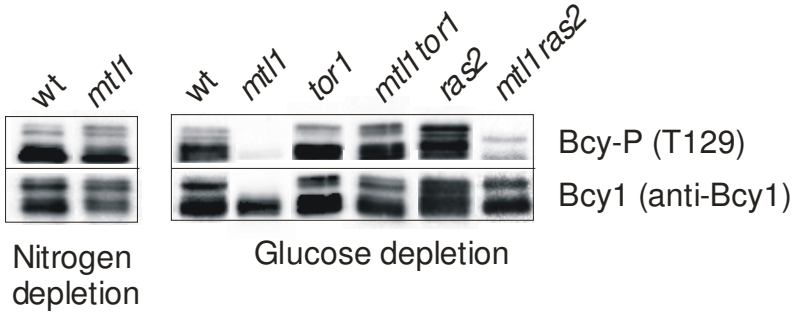


Figure 12**: Mtl1 mediates PKA inactivation through Bcy1-T129 phosphorylation through Tor1 in glucose depletion conditions** Cultures of wt, *mtl1, ras2, mtl1ras2, tor1* and *mtl1tor1* strains were exponentially grown in SD plus amino acids. After that, all the cultures were washed and transferred to minimum medium either minus nitrogen (left panel) or minus glucose (right panel) plus amino acids for 3 hours (see Materials and Methods). Samples were subsequently collected and processed for protein extraction and immunoprecipitation (see Materials and Methods). Total and phosphorylated Bcy1 was detected by immune blot analysis as described in Fig 11.

In (Fig 12) we have shown that upon glucose depletion, Bcy1 activating phosphorylation is clearly detected in wt cells whereas in *mtl1* and *mtl1ras2* mutants are almost completely absent. *TOR1* deletion, completely restored Bcy1 phosphorylation in *mtl1* cells to wild type levels under glucose depletion. However, upon nitrogen starvation, Bcy1 activating phosphorylation was highly and equivalently detected in both wt and *mtl1* (Fig 12). Similar results were obtained with *mtl1tor1* and *tor1* cells with respect to nitrogen depletion (these two not shown). It has been reported that nitrogen starvation also inactivates TORC1 complex (reviewed in Rødkaer and Faergeman, 2014), and our results clearly place Mtl1 upstream Tor1 for Bcy1 phosphorylation.

In accordance with these findings, when we analyzed *COX4* transcriptional pattern in *mtl1* cells depleted for glucose, we observed a marked deficiency in transcription. *TOR1* deletion in *mtl1* cells completely restored *COX4* expression to wild type levels, whereas Bcy1 overexpression also increased *COX4* transcriptional levels in *mtl1* cells, although

less efficiently than *TOR1* deletion (Fig 13). Once more, *RAS2* deletion did not suppress *mtl1* transcriptional deficiency in these conditions.

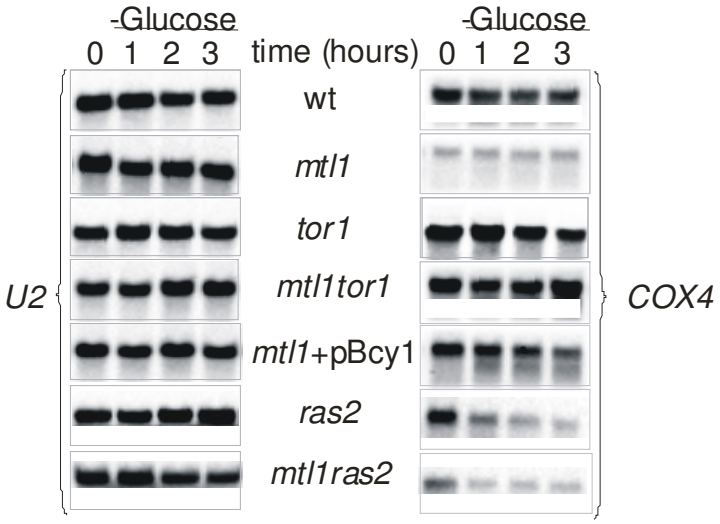


Figure 13: A**bsence of Mtl1 prevents *TOR1* down regulation in the diauxic shift**. Cultures from wt, *mtl1, mtl1*+pBcy1*, tor1, mtl1tor1, ras2* and *mtl1ras2* strains were grown in SD to logarithmic phase (time 0), then washed and transferred to minimum media depleted for glucose (see Materials and Methods). Samples were collected at indicated times in the Figure, to extract total mRNA and to perform northern blot analyses by probing with *COX4* and *U2* as a loading control.

2.4 SCH9 deletion in *mtl1* mutant restores viability, mitochondrial function in quiescence and glucose depletion conditions

Sch9 is a protein kinase belonging to the AGC family (Toda et al., 1988). It has been reported that Sch9 is a major target of TORC1 and it is primarily required for TORC1 to properly regulate ribosome biogenesis, translation initiation, and entry into G_0 phase (Urban et al., 2007). It has been reported that under rapamycin treatment inactivation of Sch9 by TORC1 not only phosphorylates Bcy1 T129 and inhibition of PKA but also activates MAPkinase Slt2 (Soulard et al., 2010). In order to ascertain the role of Mtl1 and Sch9 in cell viability we had performed CLS studies to ensure the signal from Mtl1 is through Tor1 and later to Sch9. The results of the CLS performed with wt, *mtl1*, *sch9* and *mtlsch9* is presented in (Fig 14A). We observed that *SCH9* deletion suppressed *mtl1* defects in chronological life span, moreover, CLS values in *mtl1sch9* mutant were epistatic to those determined in *sch9* (Fig 14A). After CLS studies, we had checked the viability of the strains utilsed in CLS experiment in glucose depleted conditions. It can be

observed from (Fig 14B) that *SCH9* deletion in *mtl1* mutant suppressed the defects of *mtl1* viability in glucose depletion conditions.

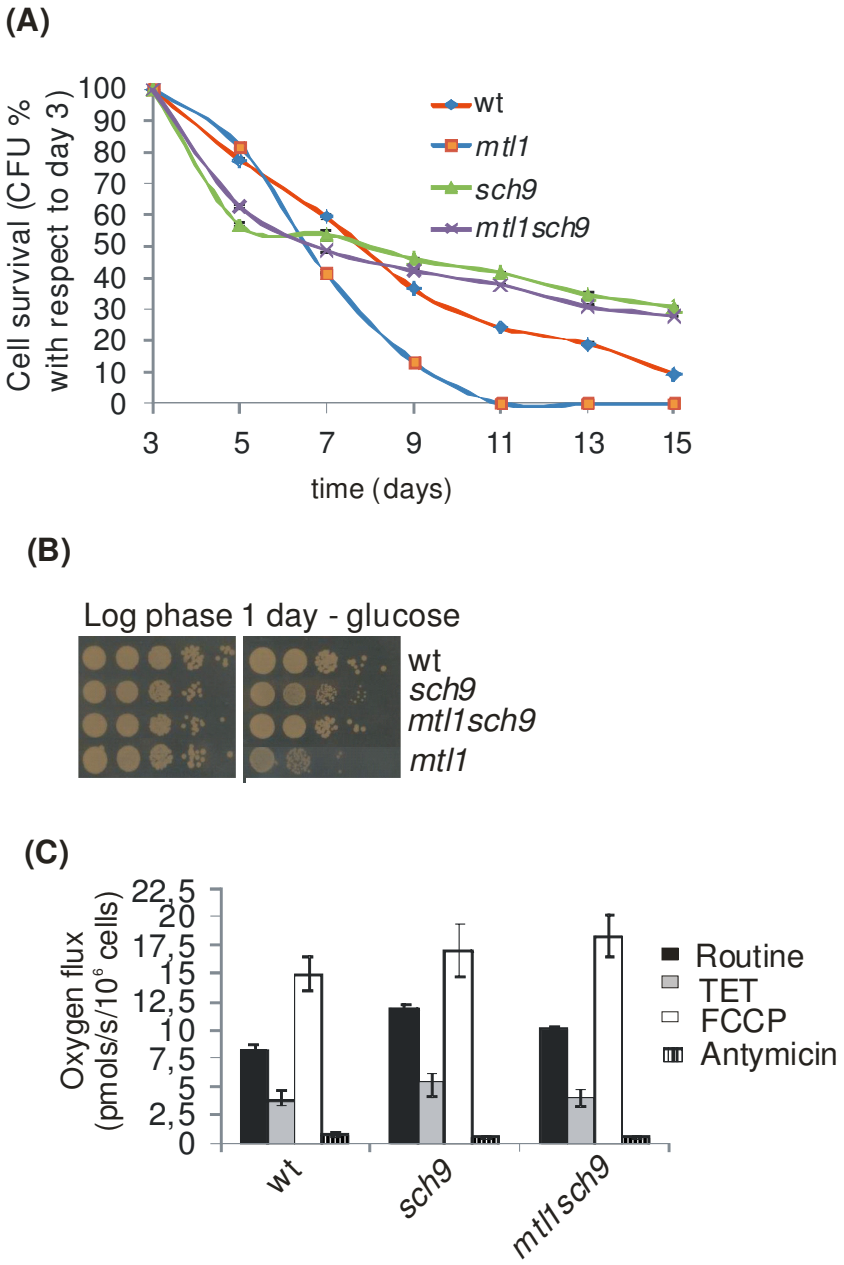


Figure 14**: *SCH9* deletion restores viability and respiration in *mtl1* mutant. A)** CLS studies in wt, *mtl1*, *sch9*, *mtl1sch9* were performed as in Fig 1A. These results are the average of three independent experiments and the error bars represents standard deviations. **B)** Cultures mentioned in A were grown in SD plus amino acids and subsequently depleted for glucose (see Materials and Methods). Cells were 10-fold serial-diluted and spotted on to YPD plates at indicated times in the figure. The plates were then incubated at 30ºC for 2–3 days. **C)** Oxygen consumptions in strains used in A as described previously in Fig 2A. Histograms represent the mean plus standard deviations of three repetitions.

It has been described that Sch9 plays an pivotol role in respiration and survival in stationary phase (Pan et al., 2009; Pan et al., 2011). To check role Sch9 in oxygen consumption under diauxic conditions, we had anlysed respiration in strains of wt, *sch9* and *mtl1sch9*. Deletion of *SCH9* in *mtl1* mutant restored the oxygen consumption (Fig 14C) upto *sch9* levels.

2.5 Mtl1 regulates Bcy1 activating phosphorylation through *TOR1*/Sch9 in quiescence and glucose depletion conditions

To determine whether *MTL1* controls PKA inhibition through *TOR1* via *SCH9* we analysed Bcy1 T129 phosphorylation in both *sch9* and *mtl1sch9* strains upon diauxic shift and glucose depletion conditions. As shown in (Fig 15) Bcy1 T129 phosphorylation was restored in *mtl1* mutant upon *sch9* deletion in both diauxic shift and also in glucose depletion conditions.

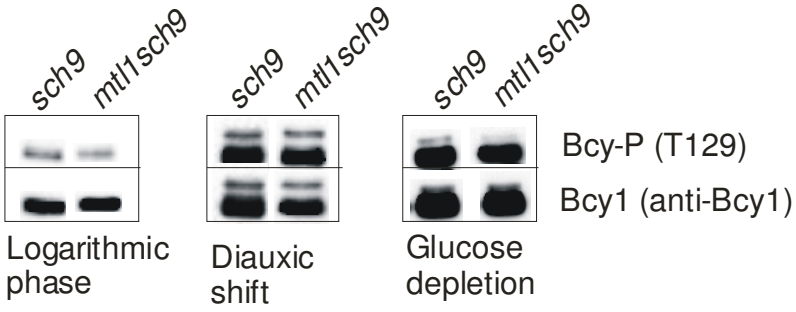


Figure15**: Mtl1 mediates PKA inactivation through Bcy1-T129 phosphorylation through Tor1/Sch9 in quiescence and glucose depletion.** Cultures of *sch9* and *mtl1sch9* strains were exponentially grown in YPD medium and subsequently allowed to grow for a total of 36 hours, corresponding to diauxic phase. Samples from logarithmic phase and diauxic shift were collected to extract total protein and Bcy1HA was immunoprecipitated and T129 residue of Bcy1 was detected using polyclonal anti-RxxS/T antibody. In parallel, total Bcy1 was detected with the polyclonal Bcy1 antibody. The strains used above were grown exponentially in SD plus amino acids. Later the cells were depleted for glucose (See Materials and Methods) and incubated in SD medium minus glucose for 3h. Samples were subsequently collected and processed for protein extraction and immunoprecipitation analyses. Total and phosphorylated Bcy1 was detected as mentioned above.

These findings indicated that *MTL1* signals *TOR1* and later to *SCH9* inhibition to inactivate PKA upon glucose depletion and quiescence. Deficiencies in this signaling process preclude both survival and mitochondrial function.

2.6 *SCH9* deletion activates CWI pathway in quiescent and glucose depletion states

To ascertain the relationship of Sch9 with respect to CWI pathway we had checked the activation of Slt2 MAPKinase in wt, *mtl1*, *sch9*, and *mtl1sch9* strains in both stationary phase and glucose depleted conditions. We checked Slt2 activation using anti-phospho MPK1 also known as phosphor p44/42 MAPK [Thr202/Tyr204] antibody by immunoblot analysis. The results observed in (Fig 16) showed that under quiescence and also in glucose depletion conditions *SCH9* deletion in *mtl1* induced progressive phosphorylation of Slt2 respectively in comparison to lack of Slt2 activation in *mtl1* mutant. *SCH9* deletion in *mtl1* suppressed the defects in Slt2 activation observed in *mtl1* single mutant in both stationary phase and also glucose depletion. The result indicated that signaling pathways are closely linked and the optimal regulation is required for maintaining sustained life.

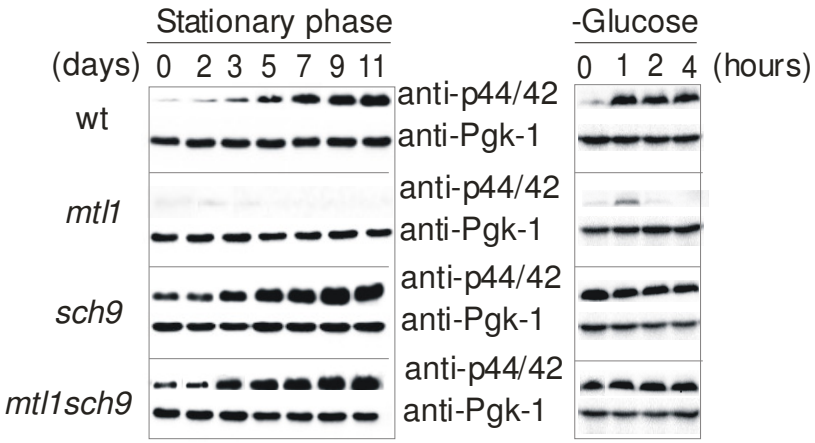


Figure 16**: *SCH9* deletion activates CWI pathway in both stationary phase and glucose starvation.** Cultures of wt, *mtl1*, *sch9* and *mtl1sch9* were exponentially grown (0) in SD medium plus amino acids. The cultures were either allowed to grow to stationary phase or alternatively were depleted for glucose (See Materials and Methods). Samples were taken at the times indicated from both stationary phase cells and glucose depleted cells. Immunoblot analysis was performed to analyse Slt2 phosphorylation using anti-phospho p44/42 antibody and anti-Pgk1 antibody as loading control.

2.7 The signal that flows from Mtl1 to *TORC1* in stationary phase and glucose depletion conditions converges in Sch9 inhibition, but is not only transmitted through Slt2 kinase activity

Bcy1-T129 phosphorylation mediated by Slt2 depends on TORC1 inactivation, as reported by (Soulard et al., 2010). To further investigate a role for Slt2 in PKA inhibition in the conditions used previously, we determined Bcy1 T129 phosphorylation in *slt2* and *mtl1stl2* mutants. In order to avoid the cell lethality due to cell-wall defects caused by the absence of Slt2, all the cultures were grown in the presence of an osmotic stabilizer, 0.4M Potassium chloride. From (Fig 17) we observed that Bcy1 T129 phosphorylation was partly defective in *slt2* mutant with respect to wt cells under quiescent conditions. However, Bcy1 phosphorylation was higher in *slt2* when compared to both *mtl1* and *mtl1slt2* strains; these two mutants presented equivalent levels of Bcy1 phosphorylation (Fig 17). Ou results indicated that in quiescence and upon glucose depletion, Mtl1 signals to Sch9 inactivation through Tor1inhibition, then releasing Slt2 activity. However, Slt2 kinase activity is not sufficient to fully phosphorylate Bcy1 in quiescent cells.

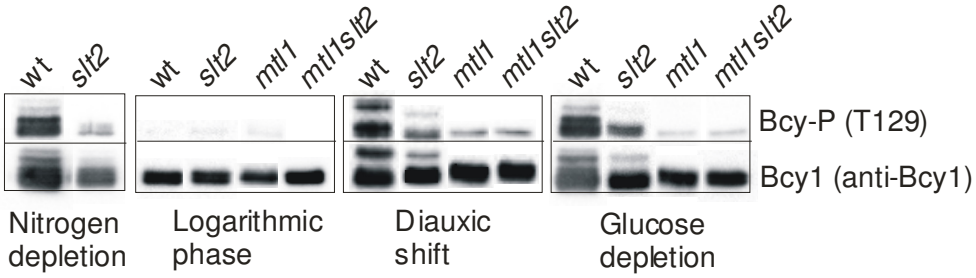


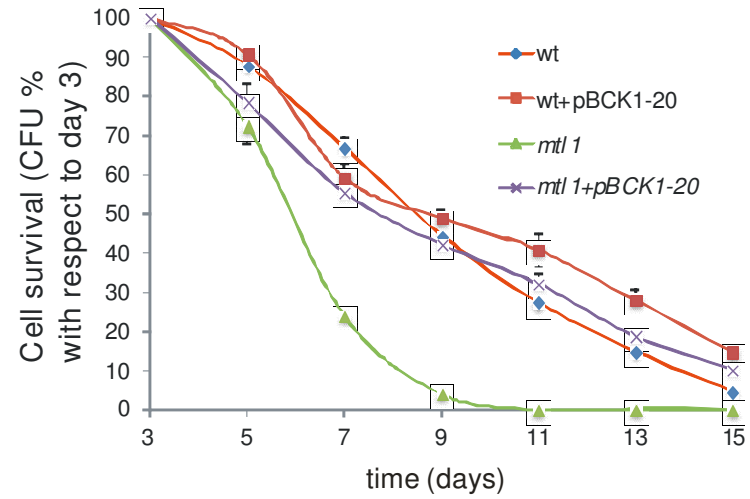
Figure 17**: Mtl1 mediates PKA inactivation through Bcy1-T129 phosphorylation via Tor1 and Sch9 in quiescence and upon glucose depletion and not only transmitted through Slt2 kinase activity.** Cultures from wt, *slt2* and *mtl1slt2* were grown and treated as in Fig 11 and 12 respectively for diuaxic shift and SD media plus amino acids. The osmotic stabilizer KCl was added to all the media at 0.4M final concentration. Samples exponentially growing in SD were also transferred to SD media without nitrogen or glucose respectively as in Fig. 12. Samples were subsequently collected and processed for protein extraction and immunoprecipitation analyses. Total and phosphorylated Bcy1 was detected as mentioned in Fig 11.

Altogether our results strongly suggest that Mtl1 specifically signals to TORC1 function in response to glucose depletion, regulating, among other substrates, Bcy1 activating phosphorylation to inhibit PKA activity.

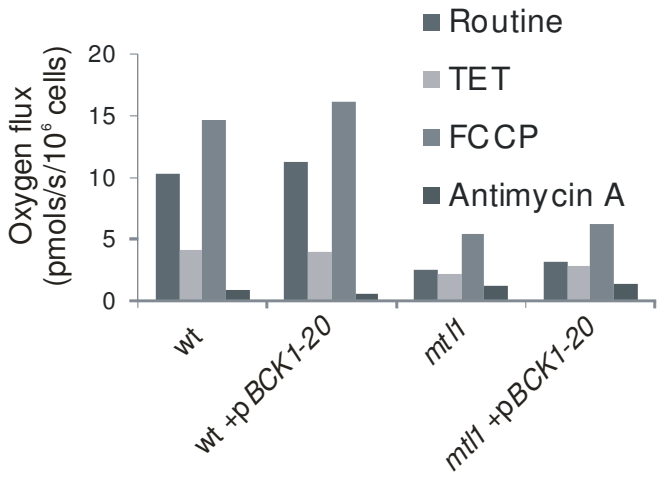
2.8 Mtl1 respiratory function is independent of the MAPk module.

In order to ascertain whether *mtl1* defects described in this thesis were a consequence of the inability to activate the CWI pathway in quiescent conditions we decided to constitutively activate that pathway by using a plasmid harbouring *BCK1-20* (a hyperactive allele of *BCK1*). The expression of *pBCK1-20* partly restored the chronological life span of *mtl1* mutant (Fig 18A). These results indicated that Mtl1 mitochondrial function is independent of the MAP kinase module of the CWI pathway.

(A)



(B)



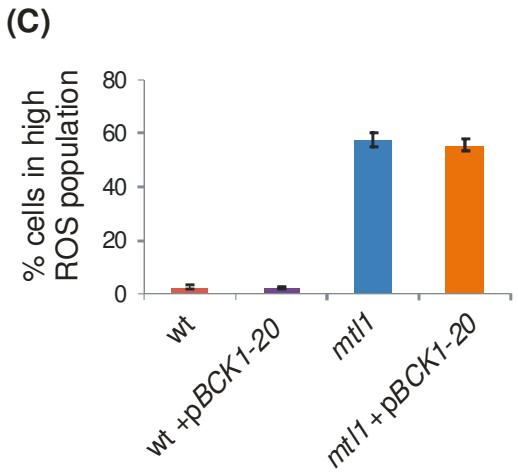


Figure 18**: PKC1-MAPK pathway activation partly restores *mtl1* survival in stationary phase. A)** CLS studies in cultures of wt, wt+ *pBCK1-20*, *mtl1* and *mtl1*+ *pBCK1-20* as performed in Fig 1A. These results are the average of three independent experiments and the error bars represents standard deviations. **B)** Cultures mentioned in A were grown in YPD to diauxic shift and respiration experiment was performed as described in Fig 2A. **C)** Cultures mentioned in A were grown in YPD to diauxic shift and ROS accumulation was analysed as described in Fig 2C. Histograms represent the mean plus standard deviations of three repetitions.

However, expression of *pBCK1-20* neither able to restore the respiratory function (Fig 18B) nor reduce ROS accumulation in *mtl1* (Fig 18C).

2.9 *mtl1* defects in CLS are not a consequence of cell-wall problems.

Some mutants in the PKC1- MAPK pathway are defective in the cell-wall synthesis and therefore they require an osmotic support to avoid the loss of viability. In order to discard the possibility that *mtl1* mutant's defect in viability during quiescence are due to cell-wall problems, we added an osmotic stabilizer, potassium chloride 0.4M final concentration and performed CLS along with wt cultures in presence of 0.4M KCl. The results showed that there was no considerable difference in viability of the *mtl1* mutant in comparison to *mtl1* mutant grown in presence of potassium chloride (Fig 19).

This experiment made us conclude that *mtl1* mutant does not exhibit cell-wall defects as presented by other mutants of the CWI pathway.

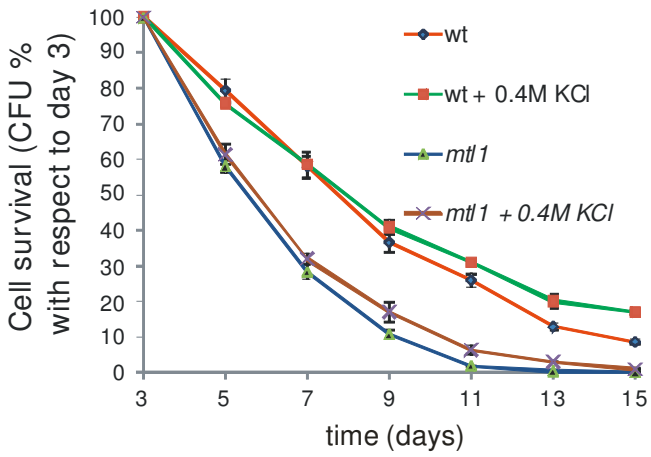


Figure 19**: Mtl1 mutant does not exhibit any cell wall defects.** CLS studies in wt, wt+ 0.2M KCl, wt+ 0.4M KCl, *mtl1*and*mtl1*+0.4M KCl were performed as in Fig 1A. These results are the average of three independent experimentsand the error bars represents standard deviations.

2.10 *SLT2* and *MTL1* mutants present a synthetic lethality in stationary phase and glucose depleted conditions.

Since Mtl1, a member of the CWI pathway and along with Slt2 is involved in PKA regulation in quiescence we decided to investigate the genetic relationship between *MTL1* and *SLT2* in the conditions of study. For this we constructed the double mutant *mtl1slt2* and observed a synthetic lethality during stationary phase (Fig 20A) and upon glucose depletion (Fig 20B).

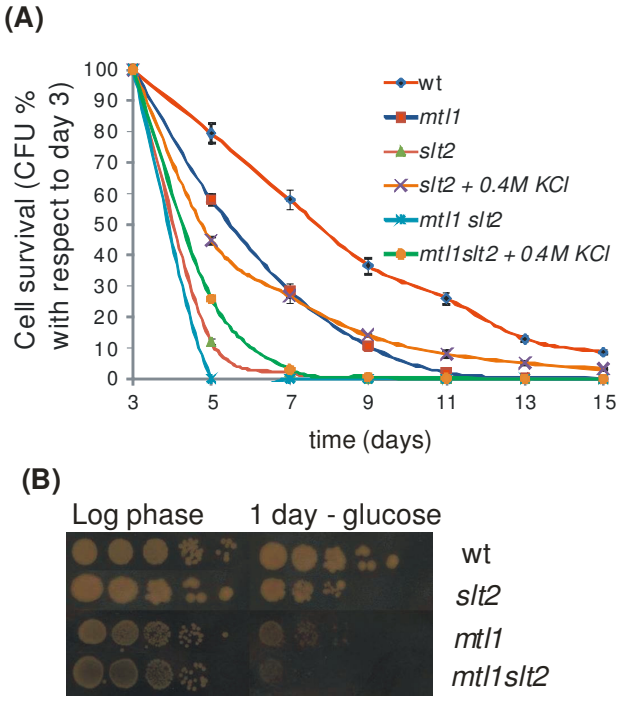


Figure 20**: *SLT2* deletion in *mtl1* is more lethal than *mtl1* in stationary phase and glucose depletion. A)** CLS studies in wt, *mtl1*, *slt2*, *slt2* + 0.4MKCl, *mtl1slt2* and *mtl1slt2* + 0.4MKCl were performed as in Fig 1A. These results are the average of three independent experiments and the error bars represents standard deviations. **B)** Cultures mentioned in A were grown in SD plus amino acids in presence of 0.4M KCl to logarithmic phase. The cultures were subsequently depleted for glucose (see Materials and Methods). Cells were 10-fold serial-diluted and spotted on to YPD plates containing 0.4M KCl at indicated times. The plates were then incubated at 30ºC for 2–3 days.

This phenotype was not suppressed upon addition of the osmotic stabilizer, 0.4M KCl. Our results allowed us obtain several conclusions: 1) Like *mtl1* mutant, the defects in CLS observed in s*lt2* are not totally explained because of cell-wall defects, indicating a specific role for both members of the pathway in life extension. 2) From the genetic approach we conclude that *MTL1* and *SLT2* act in different pathways with respect to their specific functions in quiescence.

2.11 Neither PKA inactivation nor *TOR1* deletion restores viability in *slt2* mutant in stationary phase.

Since Slt2 is required for life extension in quiescent conditions, we next tried to inactivate PKA through over expression of Bcy1 in *slt2* mutant from a different background EG 123 as the current *slt2* from CML 128 background used in previous experiments needed an osmotic stabilizer for their viability. We performed CLS experiments with the wt, wt +pBcy1, *slt2* and *slt2*+pBcy1 (Fig 21A).

In addition, we had performed the CLS experiment in the double mutant *slt2tor1* and wt (Fig 21B). The result (Fig 21A &B) showed that unlike *mtl1*, *TOR1* deletion or PKA inactivation do not suppress the *slt2* defects in chronological life span.

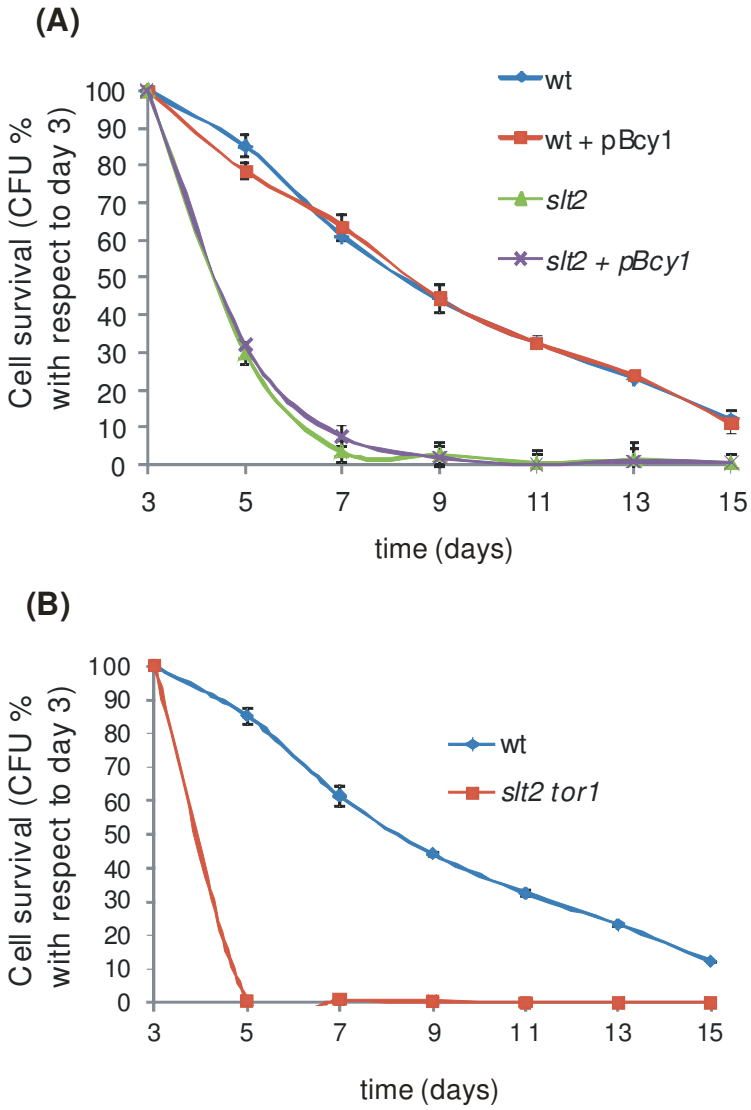


Figure 21**: Neither PKA inactivation nor *TOR1* deletion do not restore survival in *slt2* mutant in stationary phase. A)** CLS studies with wt, wt +pBcy1, *slt2* and *slt2*+pBcy1 of EG 123 background, performed as in Fig 1A. **B)** CLS studies with wt and *slt2tor1* from CML background, performed as in Fig 1A. These results are the average of three independent experimentsand the error bars represents standard deviations.

2.12 *SLT2* mutant provoke Bcy1 instability in log phase but not in diauxic shift.

Above we have shown results demonstrating that Slt2 partly regulates Bcy1 inhibitory function in stationary phase and glucose deprivation conditions. In order to elucidate whether this regulation occurred indirectly through the regulation of Bcy1 instability, we checked Bcy1 stability studies using cycloheximide in the absence of *SLT2* and corresponding wt strains from different backgrounds (EG 123, W303, BY4741) available

from the laboratory collection. Experiment was performed in both exponentially growing cells and cultures subsequently allowed to grow to diauxic shift.

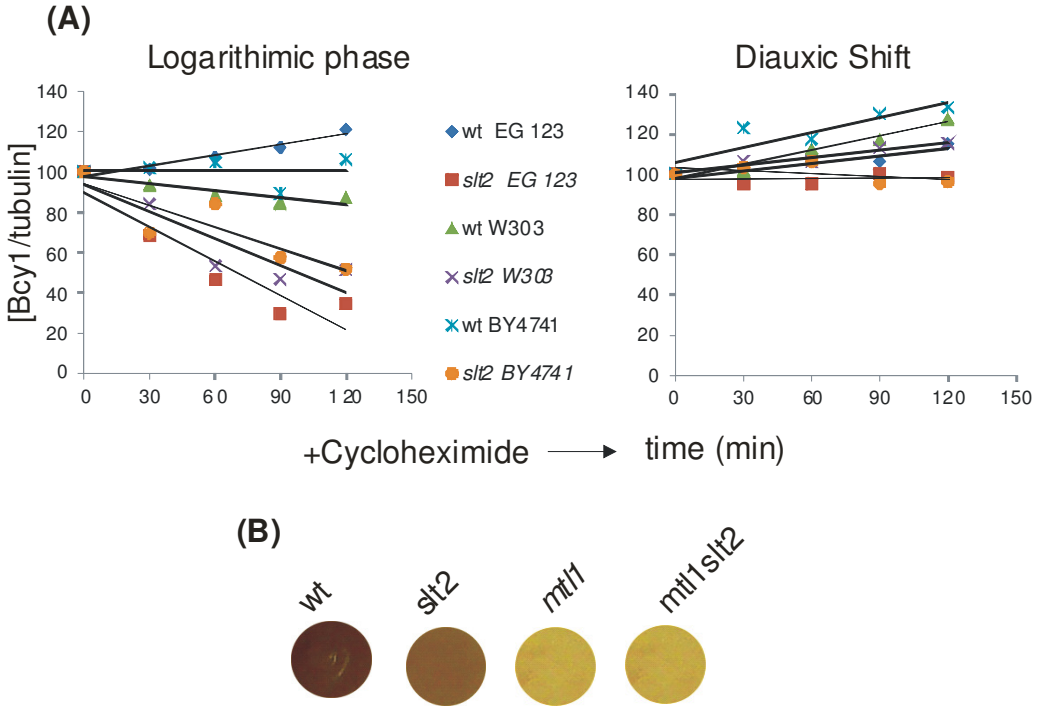


Figure 22**: Slt2 regulates Bcy1 instability in log phase but not in diauxic shift. A)** Cultures of wt and *slt2* from EG 123, W303 and BY4741 backgrounds respectively were used in the determination of Bcy1 stability as mentioned earlier in Fig10A. **B)** Glyogen accumulation was performed in cultures of wt, *mtl1*,*slt2* and *mtl1slt2* from CML128 background grown to diauxic shift in YPD plus 0.4M KCl as described (See Materials and Methods).

Similarly to that shown in (Fig 10A) and section **2.7** of this chapter of thesis, in the (Fig 22A) we have shown that Slt2 affects Bcy1 stability only in logarithimic phase conditions. The half-life of Bcy1 in *slt2* mutant from different backgrounds ranged between 70-90 min post cylohexymide addition in logarithmic phase (Fig 22A). However and as shown in (Fig 22A) this increase in Bcy1 stability in diauxic shift is independent on Bcy1 phosphorylation by Slt2.

In addition, we had performed accumulation of glycogen in wt, *slt2* and *mtl1slt2* mutant grown in YPD to diauxic shift in presence of an osmotic stabilizer, 0.4M KCl. The glycogen accumulation in *slt2* mutant (Fig 22B) was little higher in comparison to *mtl1* and *mtl1slt2* mutants which accumulated lower glycogen levels in stationary phase. We had obtained similar results from *slt2* mutant belonging to the other backgrounds used in

Bcy1 stability studies (data not shown). These results indicate that *slt2* mutant partially inhibit PKA function in stationary phase.

2.13 Addition of osmotic stabilizer improves the respiratory compliance in *slt2* mutant phenotypes

To elucidate the role of Slt2 in mitochondrial function, we next checked the mitochondrial functional capacity by performing respiration experiments in *slt2* and wt from different backgrounds used in previous section **2.12.** Results shown in (Fig 23A) indicate oxygen consumption is varied in different backgrounds of study. The routine respiration and subsequent addition of various respiratory inhibitors was lower in *slt2* mutant of EG 123 background compared to the respective wt strain. Whereas in W303 and BY4741 backgrounds there was no much significant change in oxygen consumption compared to their respective wt strains (Fig 23A).

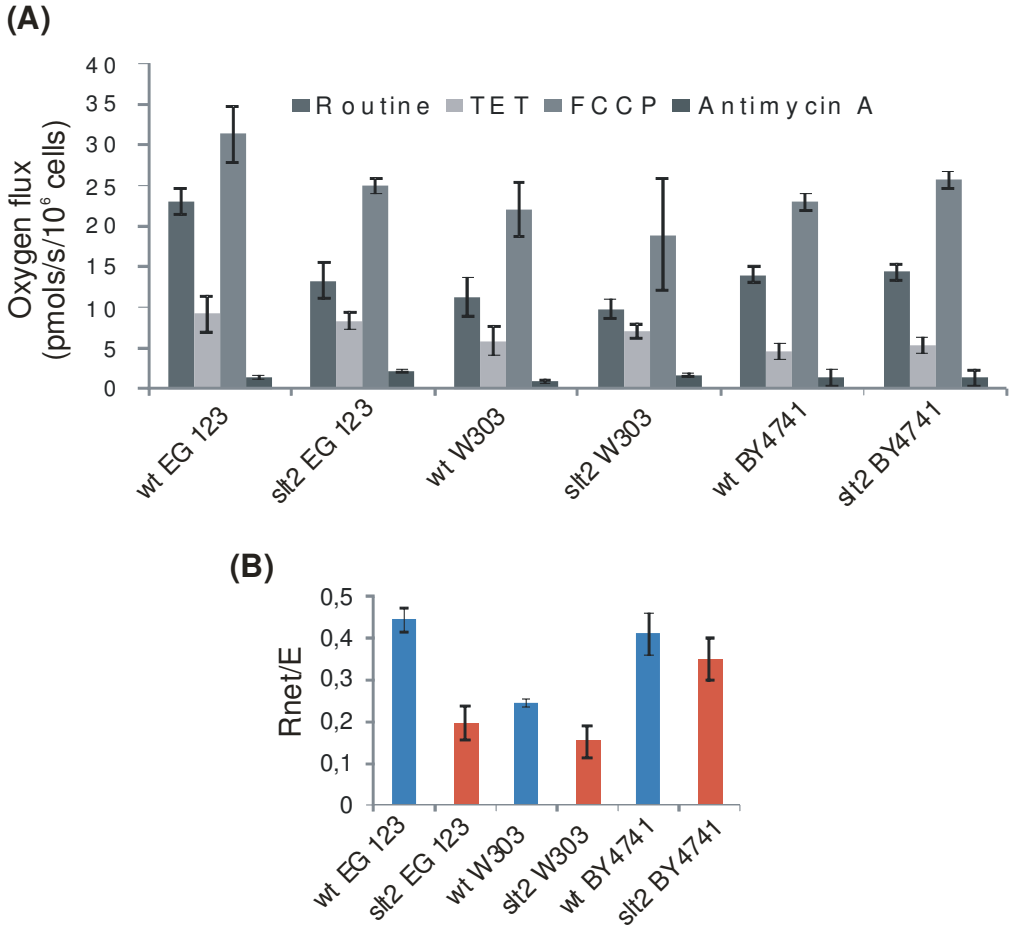


Figure 23**: *slt2* mutant have deficiency in oxygen consumption and respiration rate differs with respect to background. A)** Oxygen consumption was estimated as performed in Fig 2A in cultures of wt and *slt2*

from EG123, W303, and BY4741 backgrounds. **B)** Rnet values were calculated in the above mentioned strains as described in Fig 2B. Histograms represent the mean plus standard deviations of three independent repetitions.

Overall the respiration in *slt2* mutant is found to be uncoupled as the difference between the routeine and TET oxygen consumption was low and more over addition of the protonophore confirms the respiration to be non-phosphorylating. The results also suggest that mitochondrial functioning clearly depends on the background used in the study. The result also highlights that in EG 123 background there is much lower respiratory capacity as observed by Rnet values (Fig 23B) in *slt2* mutant with respect to the wt strain. The Rnet values of *slt2* mutants from other backgrounds (W303 and BY4741) used was little reduced in comparison to their respective wt strains (Fig 23B).

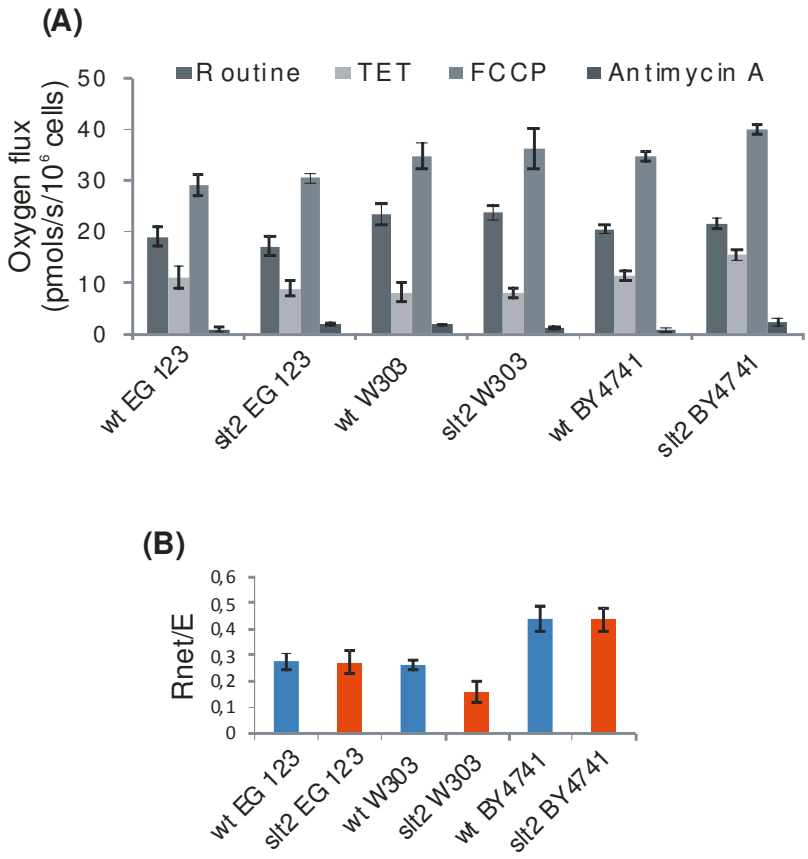


Figure 24**: Osmotic stabilisation improves respiratory compliance in *slt2* mutant phenotypes. A)** Oxygen consumption was estimated as performed in Fig 2A in cultures of wt and *slt2* from EG 123, W303, and BY4741 backgrounds in presence of 0.8M sorbitol. **B)** Rnet values were calculated in the above mentioned strains as described in Fig 2B. Histograms represent the mean plus standard deviations of three repetitions.

The values of Rnet ratios indicate an interim respiratory defect; this could be a possibility of cell wall defects presented by CWI pathway mutants. To overcome this scenario we had performed the experiment in presence of an osmotic stabilizer. We added 0.8M sorbitol to the above mentioned strains and performed the oxygen consumption experiment in diauxic shift (Fig 24A).

The results of Rnet ratios indicated in (Fig. 24B) show that adding sorbitol, an osmotic stabiliser to *slt2* mutant has suppressed the respiratory defects and restored the respiration upto the corresponding wt levels. The reason for this effect could be because of cell-wall defects presented by *slt2* mutants.

Chapter 3

Role of Mtl1 as a glucose sensor

Background

The yeast, *S cerevisiae* has been a favorite organism for pioneering studies on nutrient sensing and signaling mechanisms. In line with this, our lab has some unpublished data (Fig 25) were some of the transcripts involved in sensing glucose and nitrogen had been tested in wt and *mtl1* mutant in absence of glucose and nitrogen respectively. To determine the connection between glucose/ nitrogen sensing and Mtl1 we chose genes representative of Retrograde response pathway (RTG) transcription factor Rtg1/3 (*CIT2*); nitrogen catabolite-repressible (NCR) transcription factor Gln3 (*MEP2*); stress response transcription activator Msn2/4 (*HSP12 and CTT1*); and ribosomal protein biogenesis gene regulator Sfp1 (*RPL3*) were evaluated by transcriptional analysis in glucose and nitrogen starvation.

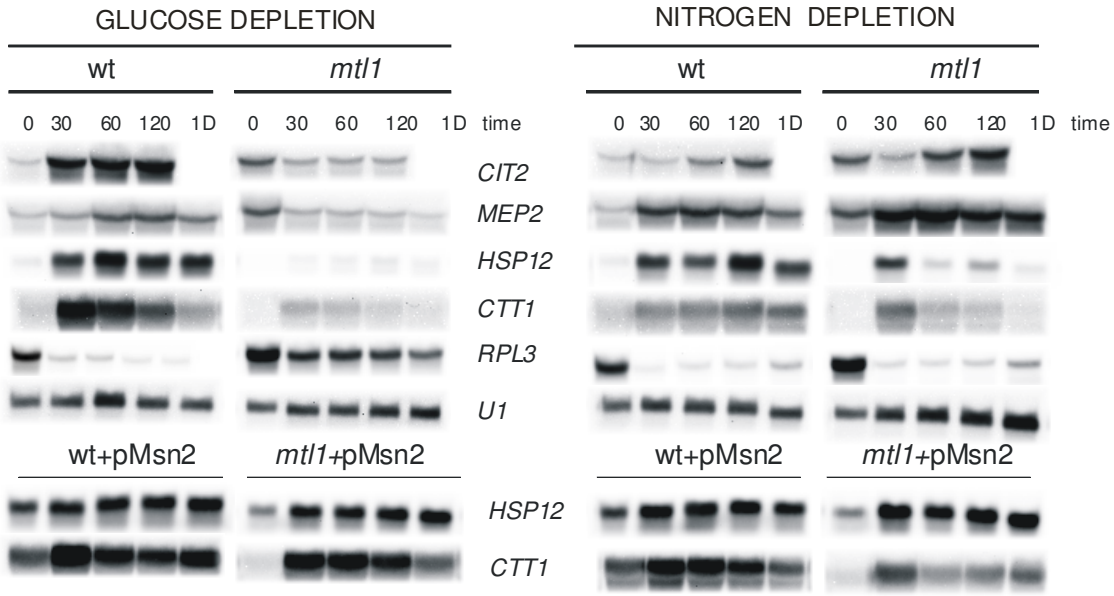


Figure 25**: *mtl1* mutant down regulates two TORC1 readouts upon glucose depletion but not upon nitrogen depletion.** Wildtype and *mtl1* cultures were grown in SD medium plus amino acids to logarithmic phase and subsequently depleted for glucose and nitrogen respectively (See Material and Methods). The cells were harvested at indicated times were then processed to extract total RNA and northern blot analysis was performed using *CIT2, MEP2, HSP12, CTT1, RPL3* probes and *U1* probe as loading control. In addition wt and *mtl1* were transformed with pMsn2 and depleted for glucose and nitrogen as mentioned above and later northern blot analysis were carried out to analyze *HSP12* and *CTT1* transcription levels.

The result in (Fig 25) showed that the above mentioned readouts of TOR pathway were misregulated in *mtl1* mutant in comparison to wt cells under glucose depletion. Poor to no nitrogen is known to down regulate TOR pathway, and hence under nitrogen starvation *mtl1* mutant recovered the transcriptional activity of the readouts of TOR pathway (Fig

25). Interestingly, it was evident that over expression of the antioxidant pMsn2 in *mtl1* mutant restored the transcriptional deficiency of *CTT1* and *HSP12* under glucose depletion (Fig 25). These results suggested that Mtl1 could play a functional role in glucose sensing through the regulation of two TORC1 readouts: *GLN3* and *RTG1/RTG3*. Our preliminary data also suggested that Mtl1 does not exert this specific function in presence of glucose.

Established reports suggest that cAMP/PKA, Snf1, Snf3 and Sch9 kinases are involved in glucose signaling in yeast. On the other hand, nitrogen signaling is controlled by the RTG, NCR and the TOR pathways (Broach, 2012; Conrad et al., 2014; Rødkaer and Faergeman, 2014; Tate and Cooper, 2013). Apart from this, cellular signaling also includes several crosstalk events that take place among different signaling pathways and it is crucial to determine the localization of these converging signals in order to understand the overall regulatory network (Rødkaer and Faergeman, 2014). It has been reported that there is a convergence of glucose and nitrogen signaling on *GLN3* by several authors (Bertram et al., 2002; Broach, 2012; Orlova et al., 2006; Sanz, 2003). With this information we tried to evaluate the role of Mtl1 in cellular response to glucose depletion through the regulation of *GLN3* and *RTG1*/*RTG3*. For which we followed the several experimental strategies as mentioned below.

3.1 *TOR1* deletion and PKA inactivation does not restore NCR induction in *mtl1* mutant upon glucose depletion.

In order to study *GLN3* regulation we chose one of its favorite targets *MEP2* which is known to function for in sensing ammonium ion and it is regulated by NCR transcription factors Gln3 and Gat1(Conway et al., 2012; Georis et al., 2009). For this experiment we grew cultures of wt, *mtl1*, *tor1*, *mtl1tor1*, *ras2*, *mtl1ras2* and *mtl1* +pBcy1 in SD medium plus aminoacids to logarithmic phase, later the cultures were depleted for glucose (see Materials and Methods) and incubated with SD medium plus amino acids and minus glucose. The cultures were incubated at 30ºC and cells were harvested at 0 (log phase), 50, 120 and 180 minutes post glucose depletion. All the samples were subjected to Northern blot analysis to see the expression of *MEP2* and *U2* was used as loading control. The results in (Fig 26A) revealed that *MEP2* expression in *mtl1* mutant was very low and not induced upon glucose depletion conditions with respect to the wt cells. Neither *TOR1* deletion nor *RAS2* deletion in *mtl1* mutant rescued *MEP2* transcription levels in glucose

depletion. Similarly constitutive PKA inactivation in *mtl1* also showed similar results in *MEP2* expression in response to glucose depletion. The result clearly suggests that NCR pathway is independent of TORC1 function in response to glucose sensing and depletion.

The next objective was to block TORC1 using rapamycin in wt and *mtl1* logarithmic cultures and to check the expression of NCR pathway reporter genes *MEP2* and *GAP1*. Rapamycin treatment mimics quiescent state by arresting the cell in G1/G0. Earlier studies reported that transcription of *MEP2* and *GAP1* has been known to be regulated upon rapamycin treatment and nitrogen starvation (Cardenas et al., 1999). The genes *MEP2* and *GAP1* expressions in (Fig 26B) indicated that blocking of TORC1 activated the NCR pathway in response to rapamycin treatment and also made us conclude that nitrogen starvation and also rapamycin treatment were independent of Mtl1 on NCR pathway induction.

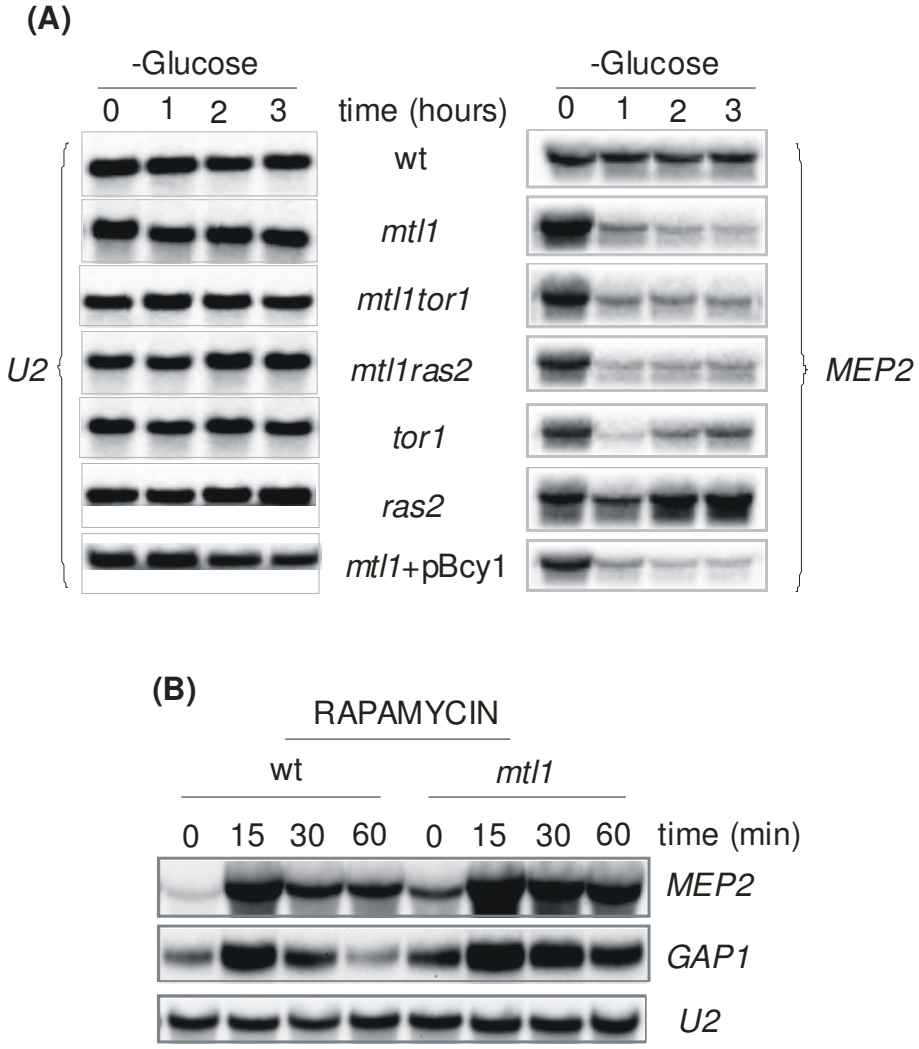


Figure 26**: Mtl1 regulates NCR pathway independently on both *TOR1* deletion and PKA inactivation upon glucose depletion. A)** Cutures of wt, *mtl1, mtl1tor1, mtl1ras2, tor1, ras2*, and *mtl1*+pBcy1 were grown in SD medium plus amino acids to exponential phase later depleted for glucose (see Materials and Methods) and incubated in SD medium containing amino acids and without glucose. Cells were harvested at indicated times and Northern blot analysis was performed in total RNA extracts using *MEP2* and *U2* probes , with *U2* as loading control. **B)** wt and *mtl1* cultures were grown in SD medium plus amino acids to exponential phase and treated with 200ng/ml Rapamycin to the cultures. Later cells were collected at 15, 30, and 60 min post rapamycin and northern blot analysis was performed using *MEP2*, *GAP1* and *U2* probes.

It is well known that Gln3, a GATA-type transcription factor of nitrogen catabolite-repressible (NCR) genes respond to the quality of nitrogen sources and which in turn controls the phosphorylation and cytoplasmic retention of Gln3 via TOR protein (Bertram et al., 2002; Georis et al., 2009). These authors have established that glucose and nitrogen signaling pathways converge onto Gln3. Nitrogen starvation or inhibition of TOR by rapamycin causes rapid dephosphorylation and nuclear accumulation of Gln3. On the contrary, reports have suggested that Gln3 gets phosphorylated independently on TORC1 function in response to glucose starvation (Bertram et al., 2002). In line with this we tried to see the phosphorylation of Gln3 tagged to HA epitope under glucose depletion in wt and *mtl1* mutant cells. The result in (Fig 27A) revealed that *mtl1* mutant is unable to phosphorylate Gln3 in comparison to wt upon 1 day glucose depletion suggesting a functional role of Mtl1 in regulation of NCR genes in response to glucose availability.

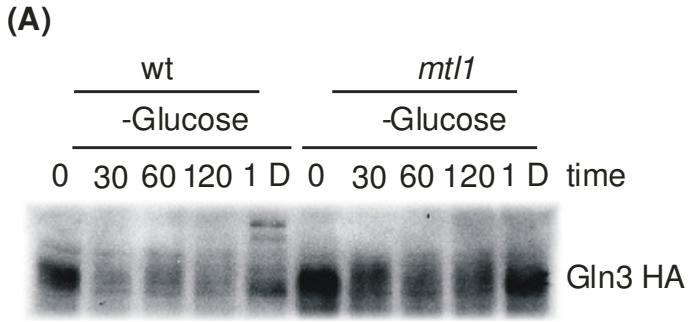
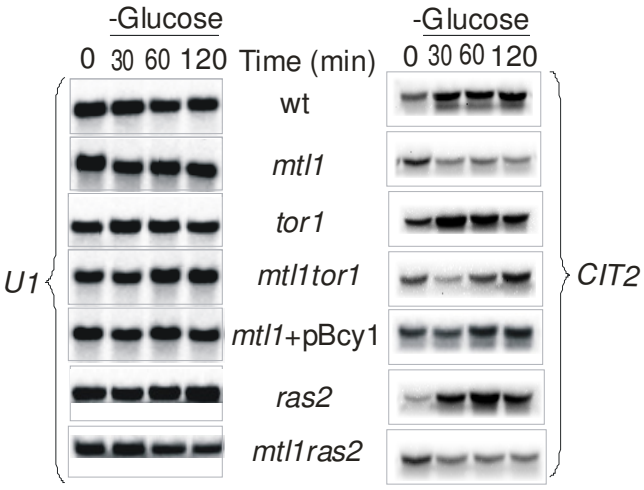


Figure 27**: NCR pathway is regulated by Mtl1 independently of TORC1 function under glucose depletion. A)** wt and *mtl1* strains were grown to exponential phase in SD medium and later depleted for glucose (See Materials and Methods). The cells were harvested at indicated times mention in figure and immunoblot analysis to analyse Gln3 HA was performed using anti-HA antibody.

From the above results we can draw a conclusion that Mtl1 functions as a nutrient sensor especially to glucose and NCR regulation in glucose depletion may be independent of TORC1 function.

3.2 *TOR1* deletion and PKA inactivation hardly restore RTG responses in *mtl1* mutant upon glucose depletion.

To continue with this satudy we next analysed the relationship between Mtl1 and Rtg1/Rtg3 in conditions when glucose is depleted, as we performed with Gln3. In this case we used *CIT2* as a prototypical readout of *RTG1/RTG3* (Liu and Butow, 2006)and analysed its transcription in wt and *mtl1*. We observed *CIT2* transcriptional induction upon glucose starvation in wt cells, however this transcription was absent in *mtl1* mutant (Fig 28). Deletion of *ras2* did not rescue *CIT2* transcription in *mtl1* cells and both *TOR1* deletion and Bcy1 overexpression only rescued very minimal the expression of *CIT2* upon glucose depletion. These results clearly suggest that Mtl1 acts as a nutrient sensor especially to glucose. The results falls in line with the idea that Mtl1 signals to NCR response under glucose depletion, independently on other signaling pathways analysed in this study.



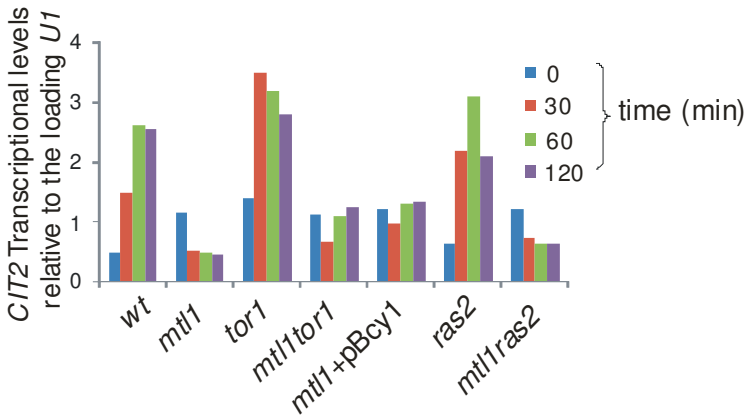


Figure 28**: *TOR1* deletion and PKA inactivation very partly restore RTG responses in *mtl1* mutant upon glucose depletion.** Cultures of wt, *mtl1, mtl1tor1, mtl1ras2, tor1, ras2*, and *mtl1*+pBcy1 were grown in SD medium plus amino acids to exponential phase later depleted for glucose (see Materials and Methods) and incubated in SD medium containing amino acids and without glucose. Cells were harvested at indicated times and Northern blot analysis was performed in total RNA extracts using *CIT2* and *U1* probes, with *U2* as loading control. The histograms represent the relative values of *CIT2* transcription with respect to the loading control.

In order to obtain more clear information about the possible cross-talk between TORC1, *MTL1* and the RTG response we decided to investigate *CIT2* expression in response to different treatments. Rapamycin as an inhibitor of TORC1, glutamate as a repressor of the RTG pathway independently of TORC1 (Giannattasio et al., 2005) and glucose depletion. We also combined these treatments to obtain a more complete picture about the possible interactions between pathways. We used wt and *mtl1* mutant in presence and absence (mock treatment) of three treatments rapamycin, glucose and glutamate respectively. The results depicted in (Fig 29) indicated interesting facts on RTG response as follows:

i. Glucose depletion induces *CIT2* transcription by feedback mechanism from mitochondria to nucleus and it is observed in wt in comparison to *mtl1* mutant which has very low *CIT2* transcription.
ii. When glutamate was added in addition to rapamycin treatment and in presence of glucose, it was observed that *CIT2* was still induced in both wt and *mtl1* mutant confirming that TORC1 downregulation directly induces RTG response independently of Mtl1 and of mitochondrial dysfunction.
iii. When rapamycin was added to *mtl1* samples depleted for glucose *CIT2* transcription was not further induced, suggesting that in the absence of glucose TORC1 does not signal to RTG activation.
iv. Similarly when glutamate was added in addition to rapamycin treatment and in absence of glucose, it was observed that *CIT2* transcription having low repression in wt in comparison to wt cells treated with rapamycin in presence of glucose and absence of glutamate.

These results clearly suggest that RTG response can be activated independently to TORC1 function.

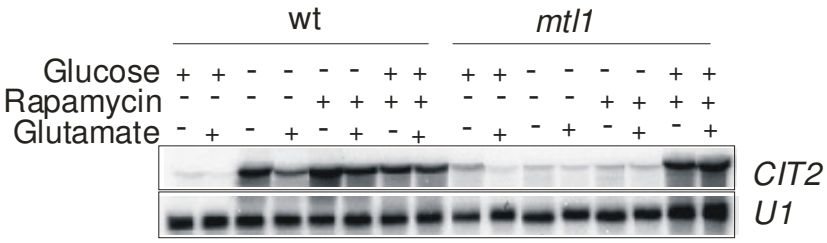
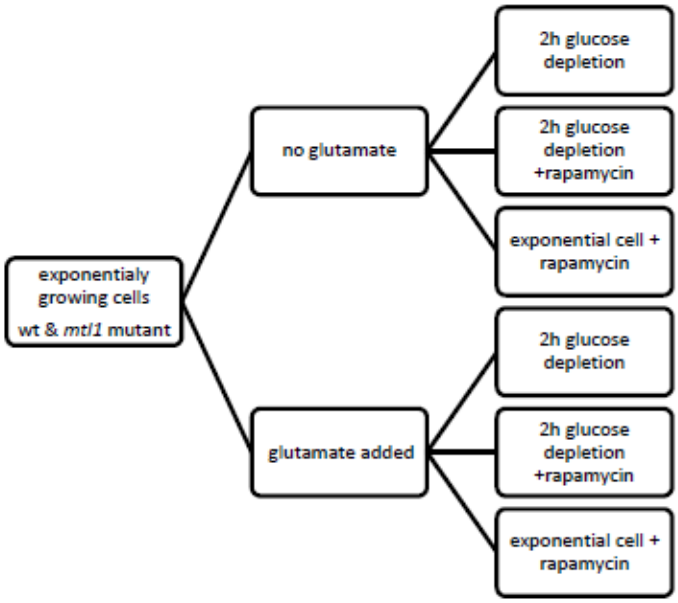


Figure 29**: RTG pathway can be regulated in glucose depletion independent of TORC1 function.** Cultures of wt and *mtl1* were exponentially grown in SD plus amino acids to logarithimic phase in the presence and absence of 0.1% glutamate (L-glutamic acid HCl). Later the samples were spilt as depicted in the flowchart given below. The treatment and its duration were: Rapamycin (200ng/ml, 2h) and/ or glucose depletion (2h) (See Materials and Methods). All the Samples were processed for northern blot analysis using *CIT2* and *U1* as probes.



3.3 In absence of glucose Mtl1 regulates RTG response

One of the negative regulators of *RTG1/RTG3* is Mks1. It is known that Mks1 binding to Rtg2 allows Rtg1/Rtg3 translocation to the nucleus to induce the expression of genes required for the mitochondrial to nucleus response (Ferreira Júnior et al., 2005). Regulation of Msk1 phosphorylation is complex, but its hyperphosphorylation has been associated to binding to Bmh1/2 proteins and inactivation of the RTG pathway. Hence to further ponder the connection between Mtl1 and the RTG response we checked the phosphorylation of Mks1 in wt and *mtl1* cells upon glucose depletion. Results shown in (Fig 30) indicated a progressive dephosphorylation of Mks1 in wt cells when starved for

glucose at indicated times, suggesting the activation of the RTG response. This is correlated with the former result (Fig 29) in which upon glucose depletion *CIT2* transcription was highly induced in wt cells. However, in *mtl1* mutant the phosphorylation of Mks1 remained unchanged and moreover in a hyperphosphorylated state suggesting deficiency to activate RTG response under glucose depletion as co stated in (Fig 30). Interestingly, in wt cells a clear decrease in Mks1 protein levels was observed concomitantly with a descent in Mks1 phosphorylation (Fig 30), but this effect was not detected in *mtl1* mutant.

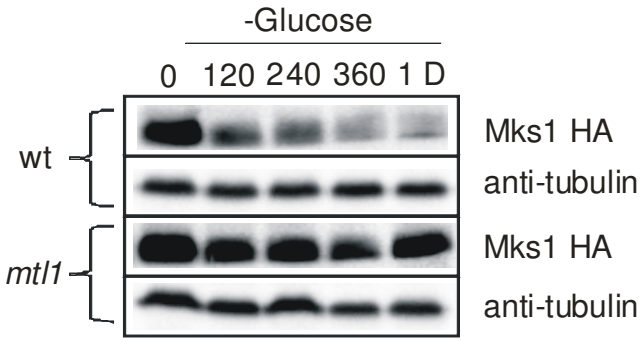


Figure 30**: *mtl1* mutant regulates RTG response acting as a sensor upon glucose starvation.** Cultures of wt+ pMks1HA and *mtl1*+pMks1HA, were grown in SD plus amino acids to logarithimic phase and later depleted for glucose (See Materials and Methods). Both exponential (0) and glucose depleted cells were collected at indicated times and processed for Immunoblot analysis using Anti-HA antibody to check Mks1 phosphorylation and anti-tubulin was used as loading control.

In view from above results, it can be summarized that Mtl1 plays a pivotal role in regulation of RTG response in absence of glucose. In line to these results we tried to establish the biochemical and functional role of Mtl1 with Rtg2. In has been reported that RTG response depends on Rtg2 which is a positive regulatory factor (Ferreira Júnior et al., 2005; Jazwinski, 2013). So to establish the link we deleted *RTG2* from wt and *mtl1* mutant to get single and double mutant respectively. To check RTG response under glucose limitation, we had analyzed *CIT2* transcription in wt, *mtl1*, *rtg2*, and *mtl1rtg2* mutants depleted for glucose from exponentially growing cells (Fig 31). The results suggested that the single mutant *rtg2* and double mutant *mtl1rtg2* are totally deficient in RTG response and RTG signal is mainly through Rtg2 (Fig 31). The experiment was performed in addition of 0.1% glutamate as *RTG* mutants are auxotrophic to glutamate (Jazwinski, 2005; Jazwinski, 2013). This result indicated that Rtg2 acts downstream Mtl1 in response to glucose starvation conditions.

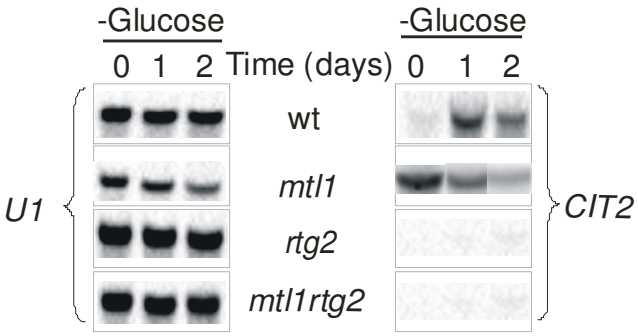


Figure 31**: RTG response signals solely through Rtg2 in minus glucose condtions.** Cultures of wt, *mtl1,rtg2,* and *mtl1rtg2* strains were grown in SD medium plus amino acids upto logarithimic phase and later glucose was depleted from exponential cells and cells were harvested at indicated times in the figure. The RTG mutants were grown in presence of 0.1% L-glutamic acid pre and post glucose depletion. Samples were processed for northern blot analysis using *CIT2* and *U1* as probes.

In addition to *CIT2* transcription, we had performed viability assays by serial dilutions in conditions of glucose depletion and oxidative stress in wt, *mtl1*, *rtg2* and *mtl1rtg2* strains. Cultures were grown in SD medium plus aminoacids to exponential phase. The RTG mutants were grown in presence of 0.1% L-glutamic acid. The exponential grown cultures were serially diluted and spotted on plates containing oxidative stress agents (Hydrogen peroxide (H_2O_2) or Ter-Butyl Hydrogen peroxide (*t*-BOOH)) or rapamycin in SD agar plates containing all auxotrophies (Fig 32). The exponential grown cultures were depleted for glucose and plated on SD agar plates containing all auxotrophies on day 1 and 2 post glucose depletion (Fig 32).

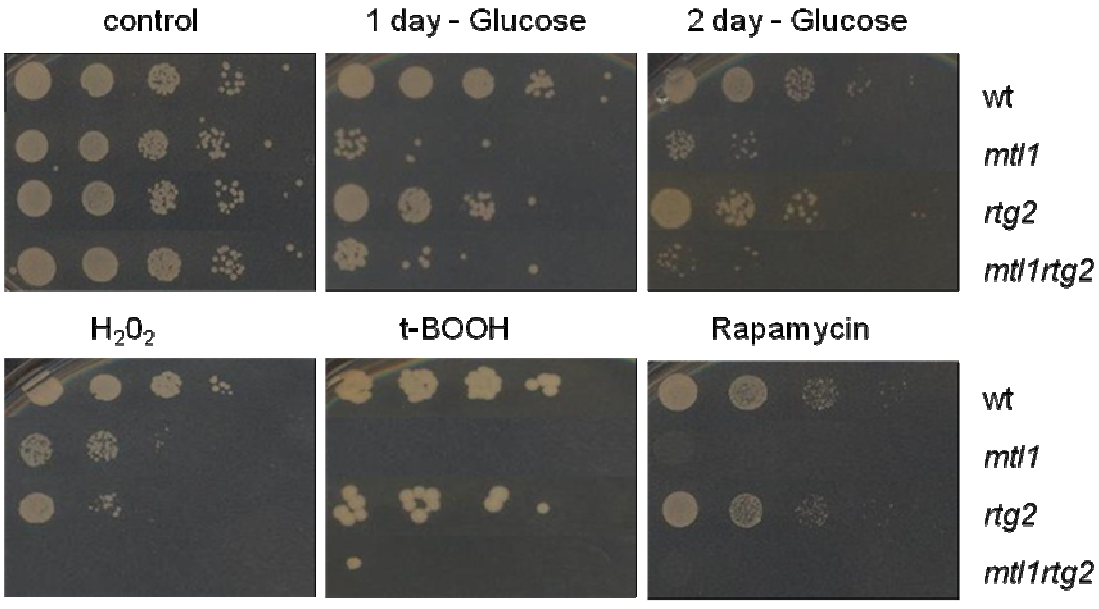


Figure 32**: *RTG2* deletion in *mtl1* mutant is synthetically lethal to oxidative stress and glucose depletion conditions.** Cultures of wt, *mtl1*, *rtg2*, and *mtl1rtg2* strains were exponentially grown in SD plus amino acids upto logarithmic phase and later glucose was depleted (See Materials and Methods). The RTG mutants were grown in presence of 0.1% L-glutamic acid pre and post glucose depletion conditions. The lograthimic cultures were spotted on SD agar plates containing 1mM hydrogen peroxide (H_2O_2) or 1mM Ter-Butyl hydrogen peroxide (t-BOOH) or 1ng/ml rapamycin plus all auxotrophies. The control plates contained no agents. The glucose depleted cultures were incubated at 30ºC and plated on SD agar plates plus auxotrophies on 1 and 2 days post glucose depletion. All the plates were done in triplicate and grown at 30ºC for two to three days and seen for growth.

The result in (Fig 32) indicated that the double mutant *mtl1rtg2* is more hypersensitive and synthetically lethal to glucose depletion and oxidative stress conditions in comparison to single mutants *rtg2* and *mtl1* respectively. The single mutant *rtg2* is found to be partly sensitive to oxidative stress conditions in comparison to wt. These results indicated that Mtl1 and Rtg2 may be acting on independent pathways for the common function of cell survival in response to different stress conditions.

3.4 In absence of glucose Mtl1 participates in the regulation of Snf1 activation

Our results regarding Mtl1 function in the absence of glucose, pointed to a connection with the AMP kinase Snf1. It has been reported that Snf1 (the yeast homologous to mammalian AMP-activated protein kinase) has major functional requirement in the adaptation of yeast cell to glucose limitation and for growth on alternative carbon source (Busti et al., 2010). In *S. cerevisiae*, the Snf1 protein kinase regulates a wide range of responses to stress caused by glucose deprivation (Orlova et al., 2006). In addition, established reports suggest that Snf1 possibly regulates Gln3 phosphorylation in glucose starvation condtions (Bertram et al., 2002; Sanz, 2003).

It has been reported that Snf1 is phosphorylated on Thr-210 in the activation loop (T-loop threonine) in response to glucose depletion and to other stresses and this pohpsohorylation correlates with activation of the kinase (Barrett et al., 2012; Hong and Carlson, 2007; Jiang and Carlson, 1996). Therefore and in order to ascertain whether in the absence of *MTL1,* Snf1 kinase activity was affected, we performed glucose depletion conditions in wt and *mtl1* cultures to detect the activating phophorylating form (T-210) of Snf1 through immunoblot analysis by using anti-phospho-Thr172–AMPK. Total Snf1 was detected with

anti-Snf1 antibody (See Material and Methods). The results in (Fig 33) indicated that Snf1 T-210 residue in *mtl1* was hyperphosphorylated in comparison to wt cells upon glucose starvation.

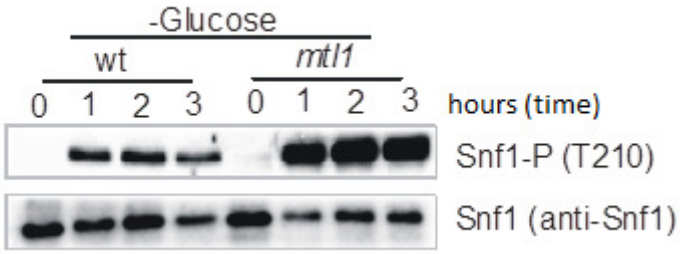


Figure 33**: *mtl1* mutant hyperphosphorylates Snf1 (T210) phosphorylation upon glucose depletion.** Wildtype and *mtl1* mutant strains were grown in SD medium plus amino acids to logarithmic phase and later cells were harvested at 0, 1, 2, and 3 hours post glucose depletion (See Materials and Methods). The proteins were extracted by heatt-alkaline treatment and later eluted by SDS-PAGE (See Materials and Methods). The immunoblot anaylsis was carried our using anti-phospho-Thr172–AMPK and total Snf1 protein, was detected using anti-Snf1 antibody.

3.5 Mtl1 regulates AMPKinase Snf1 nuclear localisation in glucose depletion conditions

Since the results were evident that in *mtl1* mutant, Snf1 was hyperphosphorylated, we tried to see the regulation process involved in localization of Snf1.

Prior reports suggested that depending on the stress type nuclear localization and activation of Snf1 may be independent to the T210 phosphorylation status of Snf1 (Hong and Carlson, 2007; Pérez-Sampietro et al., 2013). To ascertain this regulatory role in glucose depletion, we had transformed Snf1 expressing GFP plasmid in wt and *mtl1* mutant.

The strains were grown in SD medium plus amino acids to exponential phase and localization of Snf1-GFP was visualized using a fluorescence microscope. Subsequently, both the strains were subjected to glucose depletion and incubated in SD minus glucose medium plus amino acids. The cells were screened for Snf1GFP localization upon 10 mins and later up to 2h post glucose depletion. As expected, Snf1 was localized in the cytoplasm in wt and *mtl1* cultures growing exponentially in SD medium (not shown). Upon glucose depletion, Snf1 was translocated to the nucleus in wt cells (Fig 34),

however, in the absence of *MTL1*, we observed Snf1 retained in the cytoplasm (Fig 34) throughout the course of experiment.

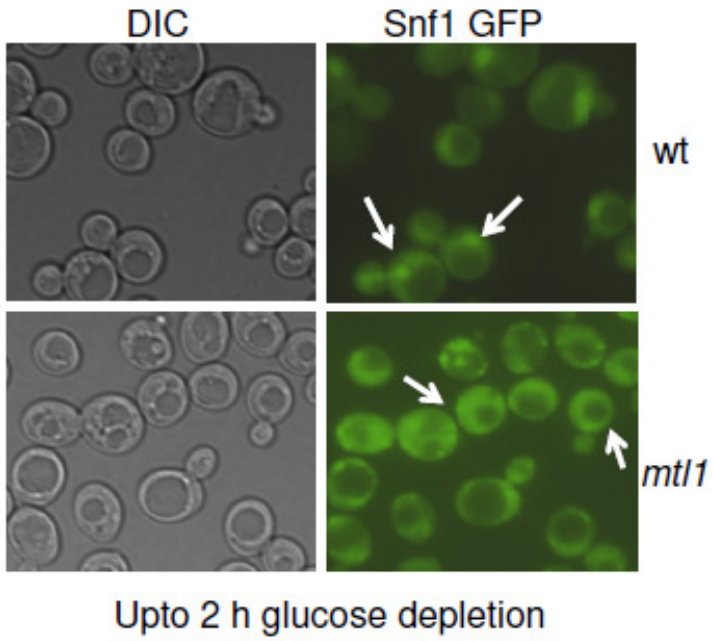


Figure 34**: *mtl1* mutant does not accumulate Snf1 in the nucleus upon glucose depletion.** Snf1-GFP were visualised for its localisation in 2h post glucose depleted cultures of wt and *mtl1* mutant in SD minus glucose media plus aminoacids. Abbreviations used DIC: Differential Interfrence Contrast; GFP: Green Fluoroscent Protein

In line with our former findings we decided to investigate whether Snf1 localisation defect in *mtl1* mutant was related to its known inability to inactivate TOR and RAS/PKA pathways. Consequently we transformed Snf1 expressing GFP plasmid in *tor1, mtl1tor1, ras2* and *mtl1ras2* mutants. Deletion of both *TOR1* and *RAS2* in *mtl1* very minimally restored nuclear localization of Snf1 upon 2h glucose depletion (data not shown). To further evaluate the degree of Snf1 nuclear localization we quantified the glucose depleted cells of wt, *mtl1*, *tor1*, *mtl1tor1*, *ras2*, and *mtl1ras2* having Snf1 GFP in the nucleus 10 min and upto 2h post glucose depletion respectively (Fig 35). The results had shown that *mtl1* exhibits very low Snf1 nuclear localization compared to wt. In glucose depleted conditions both *TOR1* and *RAS2* deletion in *mtl1* had more or less equal level distribution of nuclear localization of Snf1 GFP and hence very partially suppressing the defects in *mtl1* phenotypes.

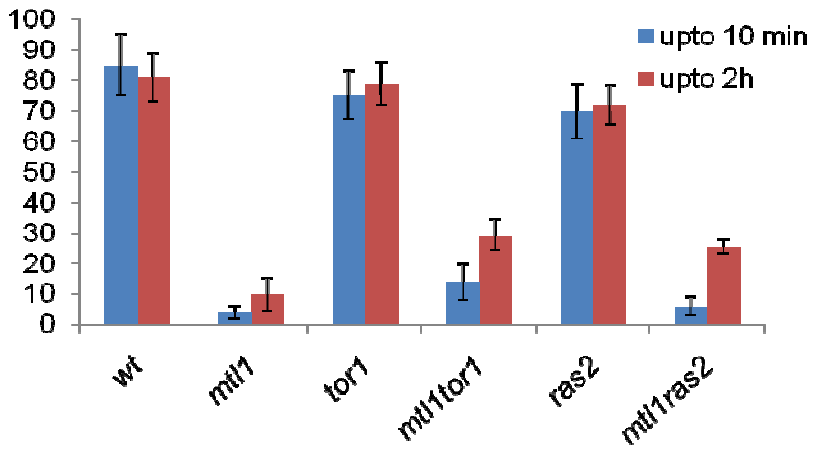


Figure 35**: *RAS2 and TOR1* deletion in *mtl1* very partially restore Snf1 nuclear localization in glucose depletion.** The graphs indicate the percentage of cells containing Snf1 GFP in the nucleus upon 10 min and upto 2h post glucose depletion respectively. We had counted on an average 1000 cells per strain coming from different images. Histogram represents mean % ± S.D. values calculated from total number of cells counted per strain.

3.6 Snf1 partly regulates RTG response in glucose starvation conditions.

From the aforementioned results it is evident that RTG response is separable from TORC1 function under glucose depletion conditions. Hence to answer the question whether Snf1 regulates RTG response in glucose depleted conditions, we had analyzed the transcription levels of *CIT2* in FY250 background's wt and *snf1* mutant. The result in (Fig 36) showed that *snf1* mutant was partially deficient in RTG response upon glucose depletion. This result gives an insight that Snf1 regulates RTG response; moreover carbon and nitrogen signaling pathways may also converge on RTG pathway. Further studies are required to establish this link for nutrient sensing.

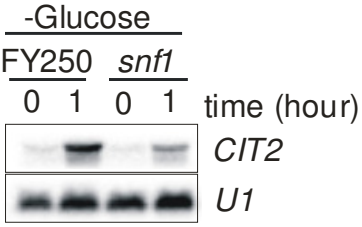


Figure 36**: *Snf1* mutants are deficient in RTG response under glucose starvation conditions**. Cells of wt (FY250) and *snf1* mutant from FY 250 background were grown in SD medium plus amino acids to logarithmic phase and later subjected to glucose depletion (See Materials and Methods). Cells were

harvested at the indicated times in the figure and samples were analyzed for *CIT2* transcription with *U1* probe as loading control.

Taking all the results of this chapter together, we propose a preliminary model in which Mtl1 signals NCR and RTG function under glucose deprivation conditions. This signal is independent or only very partly dependent of TOR and PKA pathways. Another preliminary and interesting data derived from our results is the involvement of Snf1 in RTG response and its functional relationship with Mtl1 regarding Gln3 and Rtg1/Rtg3 regulation when glucose is exhausted.

DISCUSSION

Compared to other animals, the human lifespan is fairly long, but is nevertheless limited with most people living about 80 years and rare individuals reaching 100 years. On the other hand, some animals, plants and fungi are found to survive for several hundred or even several thousand years, showing minimal senescence. The questions repeatedly asked is what could be the possible mechanisms underlying this great longevity? And is there a possibility to apply this knowledge to enhance the health and lifespan of humans? (Petralia et al., 2014).

Yeast cells and complex metazoans share numerous circumstances where genetic control of ageing and biochemical pathways lead to senescence. Over the years, the field of ageing has made *S. cerevisiae* a preferred choice as a model and studies in yeast has been growing rapidly, and many findings correlate mechanisms of ageing in this unicellular organism to those present in more complex life forms (Barros et al., 2010).

Diauxic shift and stationary phase are both conditions in which cells are not under catabolite repression, oxygen consumption is elevated and respiratory metabolism is induced. During these phases, cells are stressed by the depletion of nutrients and the accumulation of toxic metabolites (Gray et al., 2004; Santangelo, 2006; Valcourt et al., 2012). However, quiescent state reflects metabolic conditions opposite to those occurring during exponential growth in glucose (Santangelo, 2006). The starting point for this study came from the observation that Mtl1 is transcriptionally up regulated in the diauxic shift and in stationary phase, both quiescent conditions, in a manner dependent on the stress transcription factor Msn2/Msn4 (Petkova et al., 2012). In this study we present evidence demonstrating that the transmembrane protein Mtl1 plays an essential role in life extension in quiescence and upon glucose depletion through the down regulation of TORC1 and PKA signaling pathways via *SCH9* and partly through. In addition, we also demonstrate the role of Mtl1 as a glucose sensor and its relationship among glucose signaling pathway such as PKA, TOR and Snf1/AMPK.

1. Absence of Mtl1 affects chronological life span and mitochondrial dysfunction in diauxic shift & stationary phase.

Deletion of *MTL1* shortens chronological life span whereas its over-expression extends life span, demonstrating a direct role of Mtl1 in the chronological aging of budding yeast. After diauxic shift, cells use ethanol and in stationary phase, lack of carbon supply, make

mitochondrial respiration the major source of energy to cells. The respiratory metabolism provokes ROS generation that must be effectively countered for cell viability.

Consequently, signaling mechanisms have to be properly and tightly regulated in order to couple metabolism with detoxification and repair of oxidative damage. TOR and RAS/PKA-cAMP pathways have to be down regulated whereas CWI and *SNF1* are up-regulated in the diauxic shift (Galdieri et al., 2010; Gray et al., 2004). Mtl1 is a cell-wall protein, member of the CWI pathway, upstream of Pkc1(Levin, 2011; Vilella et al., 2005). Mtl1 was initially identified as a yeast homologue of Mid2 (Rajavel et al., 1999; Ketela et al., 1999). The CWI pathway is activated during chronological aging (Barbosa et al., 2012; Torres, 2002), being Pkc1 and the downstream elements necessary to extend life (Stewart et al., 2007).

The well known markers to assess the cellular energy state are mitochondrial membrane potential, respiration rate and ROS accumulation whose changes during ageing affect the ATP production and also play a vital role in cell senescence (Laun et al., 2001; Ricci et al., 2003). Apart from these markers, the genes associated with Electron Transport Chain (*CIT1*, *ACO1* and *IDH1*/2) are highly induced in glucose limitation conditions (Wang et al., 2010).

In this context, we demonstrate that the absence of *MTL1* affects the transition through the diauxic shift, provoking a severe descent in oxygen consumption with an increment of uncoupled respiration, high MMP and high ROS generation. A transition to a state 4 of respiration has been suggested to cause an increase in mitochondrial membrane potential, ROS accumulation and uncoupled respiration prone to abnormal ROS accumulation (Hlavatá and Aguilaniu, 2003; Hlavatá et al., 2008). Apart from yeast, it is also shown in higher eukaryotes, that the coupled mitochondrial respiration in old rats was approximately 45% lower than in cells from young adult animals (Lemieux et al., 2010). From above mentioned results, our data point towards mitochondrial dysfunction leads to shortening of life span.

In addition apart from uncoupled respiration, *mtl1* cells are not affected in mitochondrial content but its mitochondrial function is impaired, as revealed by the reduced aconitase activity and low transcription of *COX4*, a subunit of the respiratory complex IV involved in the oxidative respiration. Recently it was shown that, superoxide levels were increased in *cox4* cells when compared to wild-type, and this was further exacerbated by deletion of

SOD1 and also the overexpression of *SOD1* or *SOD2* did not alleviate ROS levels nor did they prevent loss of viability (Leadsham et al., 2013). These data in accordance to ours where its suggests that a reduction in COX activity promotes superoxide production which is further converted to hydrogen peroxide that accumulates to toxic levels (Leadsham et al., 2013). The observation that the expression of *COX2*, a mitochondrial gene also part of the respiratory complex IV, is not affected in *mtl1*, made us to suspect that *COX4* downregulation could be reflecting a deficient signaling mechanism caused by the absence of *MTL1*. During mitochondrial biogenesis, porin, is one of the most abundant mitochondrial outer membrane proteins expressed (Goldberg et al., 2009). We also show that porin levels are not affected in *mtl1* mutant during diauxic shift, which confirms that *mtl1* mutant is not affected in mitochondrial mass. Our result with respect to porin is comparable with the earlier published report (Goldberg et al., 2009) where they show that the intracellular levels of porin were very similar in chronologically aging calorie restricted and non- calorie restricted yeast.

Another important parameter which changes in parallel to ageing is the mitochondrial morphology. Highly dynamic mitochondrial fission and fusion processes which regulate their shape, size and number must be balanced for optimal mitochondrial function (Müller and Reichert, 2011). We show that *mtl1* mutant exhibited characteristic globular/dotted mitochondrial distribution in diauxic shift along with low oxygen consumption. Studies carried out on mitochondrial morphology and ageing have established that mitochondrial fission takes place as the cell becomes old and this depends on the mitochondrial functional capacity (Volejníková et al., 2013). These authors present that mitochondrial morphology changes on day 4 and gradually with time, on day11, the cell's mitochondrial filaments disappear with extensive fragmentation and appearing as small dots or larger patches along with very low levels of GFP staining. They also mention that progressing mitochondrial fragmentation on subsequent days is accompanied by a functional decline that apparently affects both respiration and mitochondrial membrane potential (Volejníková et al., 2013).

Other authors propose that replicatively older cells (10–12 generations) contain mainly fragmented mitochondrial network, while the tubular morphology dominates in younger cells (Scheckhuber et al., 2007). The yeast cells reform the normal network after the end of oxidative stress and continue growth. This is in contrast to the overall globular pattern of fragmented mitochondria in a terminally old cell, where the globular pattern is locked

in, as these cells are unable to dynamically restore the mitochondrial network (Breitenbach et al., 2013). These findings are consistent with our result and hence we speculate that *mtl1* mutant behaves like an aged cell, with extensive fragmentation of mitochondria which appears as small dots or large patches with severe loss in mitochondrial function. Furthermore, mitochondrial dynamics exhibited by *mtl1* mutant is thought to counteract aging and constitute an organellar quality control mechanism. Our data suggest that *mtl1* mutant having dysfunctional mitochondria can serve as a signaling platform to promote the loss of redox homeostasis, ROS accumulation, and accelerate aging in yeast.

2. ROS acts more of a signaling molecule rather than a central component for cell lethality in *mtl1* mutant

Here we show that ROS increase in *mtl1* cells is not the main cause of mitochondrial dysfunction or of cell lethality, on the contrary, here we demonstrate that ROS favors life span extension both in wt and especially in *mtl1* cells. However, reducing ROS levels by using N-acetylcysteine did not increase aconitase activity in *mtl1* mutant during stationary phase. It is because aconitase activity is directly related to mitochondrial function (Gangloff et al., 1990) and so we hypothesize that ROS accumulation is only a consequence but not the cause of mitochondria dysfunction in *mtl1* during the diauxic shift. ROS are signaling molecules not necessarily causing growth defects and it has been shown that when cellular damage surpasses a critical threshold, cells will die by executing apoptosis (Swinnen et al., 2014). These results point to an adaptive response more specifically named mitochondrial hormesis or mitohormesis. ROS are essential signaling molecules which are required to promote health and longevity. Built on hormetic extension of lifespan, the mitohormesis theory posits that ROS production by mitochondria under glucose restriction enhances stress resistance, thereby extending lifespan (Schulz et al., 2007). In line with our results, other studies show that antioxidant supplements may be disease-promoting and/or may even reduce lifespan in humans and other model organisms (Ristow and Schmeisser, 2011; Ristow and Zarse, 2010). Some authors report the possible mechanism of chronological life extension under caloric restriction or inactivation of catalases is through mitochondrial hormetic signal by regulating the levels of the reactive oxygen species especially hydrogen peroxide, which activate superoxide dismutases to inhibit the accumulation of superoxide anions in early stationary phase (Mesquita et al., 2010; Weinberger et al., 2010). Hence, our results point

to Mtl1 regulating mitochondrial hormetic signaling possibly through elevated ROS levels for cell survival in quiescence.

3. Downregulation of TOR and PKA extends life span in *mtl1* mutant

Our group has previously reported a connection between Mtl1 with TOR and RAS in log phase (Petkova et al, 2010a) and between Pkc1 and TOR in quiescence provoked by rapamycin (Krause and Gray 2002, Torres et al, 2002). Here we show that *tor1mtl1* CLS was equivalent to that determined in *tor1*. This epistatic relationship between Mtl1 and Tor1 suggests that Mtl1 is an upstream negative regulator of *TOR1* for some signals such as nutritional stress and aging. Moreover, deletion of *TOR1* in *mtl1* restored coupled respiration, mitochondrial dysfunction and reduced ROS accumulation to *tor1* levels. Reduced TORC1 signalling extends chronological life span and enhances oxidative phosphorylation (Bonawitz et al, 2007; Zid et al, 2009, Schieke and Finkel, 2007, Pan et al, 2009) by regulating mitochondrial respiratory coupling (Pan et al., 2009). In quiescent conditions *TOR1* down regulation is required to activate the OXPHOS system and to extend life span (Bonawitz et al., 2007). Earlier report show that TORC1 down regulation is required in the transition from fermentative to respiratory metabolism is the yeast cells. This is because rapamycin blocked TORC1 activity causes an immediate and widespread transcriptional response that result in metabolic reprogramming similar to that of diauxic shift (Hardwick et al., 1999). In addition, another possible mechanism is that *tor1* mutant generates superoxide during growth which serves as a hormetic signal (Pan et al., 2011). These authors show that the activation of OXPHOS electron flow with coupled respiration by reduction in TOR signaling, temporarily elevates mitochondrial membrane potential and enhances superoxide generation. The superoxide peak generated suppresses ROS production in stationary phase and prolongs CLS (Pan, 2011). In this context we speculate that, during diauxic shift, in the absence of *MTL1*, cells fails to respond to the mitochondrial hormetic signal due to the deregulation of TORC1 function. Hyper activation or non regulated activation of TOR can induce mitochondrial ROS production and also inhibit respiration (Shamji et al, 2000). These effects of activated TOR fairly explain the vast majority of *mtl1* phenotypes in diauxic shift and stationary phase. Our model of Mtl1 function is in accordance with the findings of (Bonawitz et al., 2007) that conclude that *TOR1* deletion favors life span extension through a mechanism related to the

regulation of proper mitochondrial respiration. Other authors have also reported that *TOR1* deregulation caused by the absence of *ISC1* in response to nutrient deprivation induces a premature aging (Teixeira et al., 2014). These findings put together, confirm that TOR signaling is closely associated with mitochondrial function and ROS accumulation converging on CLS extension (Parrella and Longo, 2008).

The PKA pathway has become of great interest for the study of aging, since mutations that cause a reduction in PKA signaling, extend lifespan in yeast, delay the incidence and severity of age-related disease, and promote longevity in mice (Enns and Ladiges, 2010). Reduction of cAMP/PKA activity extends life span in yeast cells (Longo 2003). According to Leadsham and Gourlay, (2010) the improper PKA activity also induces transcriptional changes promoting the increase of dysfunctional mitochondrial ROS. These authors also suggest that elevated PKA activity caused by the subunit Tpk3 results in non respiring mitochondria prone to produce high levels of ROS. Therefore PKA activity was another candidate to explain *mtl1* phenotypes, and turned out to be a good candidate since PKA inhibition by Bcy1 overexpression efficiently coupled respiration, suppressed ROS accumulation and restored CLS to wild type levels in the *mtl1* mutant. Hence, Bcy1 over-expression suppressed *mtl1* mitochondrial problems, RTG induction is reduced to wild type levels, and the aconitase activity became significantly increased in *mtl1* mutant, although at lower levels than those determined in wild type cells. These results strongly suggest that Mtl1 operates negatively regulating PKA activity in diauxic shift and stationary phase. In addition, we also show that *COX4* transcription and mitochondrial morphology is restored in *mtl1* upon *TOR1* deletion or Bcy1 over expression. Collectively our results also suggest that improper activation of TOR and PKA functions in *mtl1* cells, contribute to alter the respiratory capacity by inducing a blockade in the oxidative phosphorylation and by increasing mitochondrial membrane potential.

4. Mtl1 is required for the cell's proper entry into diauxic shift.

We show that in the absence of *mtl1*, the cell fails to regulate key transcription factors (*RTG1/3, GLN3, MSN2* and *MSN4*) who reciprocate stress signals for the entry to diauxic shift (De Virgilio, 2012). Cell's entry into diauxic shift is orchestrated by sensing nutrient deprivation especially glucose and nitrogen along with proper mitochondrial function.

In accordance with our results, CWI, PKA and TOR regulates stress responsive genes such as *HSP12* and *HSP26* through Msn2/4 (Petkova et al., 2010a; Zaman et al., 2008) and *MTL1* regulates *HSP12* transcription with respect to oxidative and nutritional stress signals (Petkova et al., 2010a). In addition, recent reports suggest that heat shock proteins belonging to DJ-1 family are required for cells to enter into diauxic shift, reprogramming and survival in quiescence (Miller-Fleming et al., 2014). In accordance with these reports, we speculate that in the absence of *MTL1*, cells fails to correctly enter into diauxic shit and further fail to activate the stress response system Msn2/Mns4. Furthermore, it is known that Msn2/Msn4 is tightly regulated by several signaling pathways, hence it is downregulated by TOR, RAS/PKA, CWI, HOG and by Snf1. Msn2/Msn4 also favors life extension by inducing the expression of longevity genes (Fabrizio et al., 2001; Wei et al., 2008). Improper regulation of the signaling pathways could be the possible reason why *mtl1* cells cannot properly enter into diauxic shift and loose viability in quiescence.

Nuclear gene expression can be influenced by signals coming from mitochondria, a process called retrograde communication (RTG) (Jazwinski, 2013; Liu and Butow, 2006). *CIT2* induction is considered to be the hallmark diagnostic gene for the RTG pathway activation (Jazwinski, 2005; Liu and Butow, 2006). We demonstrate that, *CIT2* transcription is downregulated in *mtl1* during diauxic shift. The retrograde response senses changes in the functional state of mitochondria and adjusts nuclear gene expression accordingly. These adaptations compensate to some degree the mitochondrial damage and slow the aging process (Jazwinski, 2005; Jazwinski, 2013). We hypothesize that RTG activation must be a compensatory mechanism in *mtl1* mutants (data not shown) in order to restore the mitochondrial function through the induction of anaplerotic pathways that resore the cellular pool of glutamate. Recently, it has been reported that Mks1, negative regulator of the RTG pathway gets dissociated from Rtg2 in a manner dependant on physiological concentrations of ATP (Zhang et al., 2013). These authors propose that the RTG response is an ATP homeostasis pathway coupling ATP production with ATP mediated repression of the retrograde response by releasing Mks1 from Rtg2. Hence, decrease in physiological levels of ATP induces the RTG response and also determines the mitochondrial functional state. Furthermore, it has been known that activation of Rtg1/3 upregulates expression of genes encoding enzymes catalyzing the first three reactions of the Krebs cycle, which is coupled to ATP synthesis through oxidative phosphorylation (Liu and Butow, 2006). In stationary phase experiments performed in SD media, *mtl1* cells

present higher *CIT2* levels (data not shown) and in these conditions we speculate that ATP levels are activating RTG response. As disussed earlier, in *mtl1* mutant there is impairment in CLS and in these conditions ATP levels are low due to mitochondrial dysfunction observed in *mtl1* cells. The low levels of ATP can induce RTG response independent on Mtl1 signaling only as a consequence of the physiological state of *mtl1*. Therefore this RTG activation (*CIT2* induction in *mtl1* compared to wt cells in stationary phase (result not shown)) helps to extend life span in *mtl1* cells. In line with these results, we also tried to elucidate the genetic relationship between Rtg2, a positive regulator of RTG response and Mtl1. In agreement with the result mentioned above, the double mutant *mtl1rtg2* shows synthetic lethality in all stress conditions observed. The synthetic lethality observed in deletion of *rtg2* in *mtl1* possibly explains the fact that RTG activation is important for cell survival in stationary phase. In addition, we also observed that *rtg2* cells exhibited lower sensitivity to rapamycin, glucose starvation and oxidative stress than either *mtl1* or double mutant *mtl1rtg2*. These observations suggest that Mtl1 and Rtg2 may function on independent pathways or as a complex in response to impending stress to promote cell survival. Whether or not RTG activation is also compensating *mtl1* defects in CLS will have to be demonstrated in future. Moreover we also show that in logarithimic phase *mtl1* cells present induction of *CIT2* while in glucose starvation there is a complete repression in *mtl1* mutant. The situation here is different, rather opposite and this suggests two possibilities:

a. ATP induced RTG expression can be TOR dependent.

b. RTG regulation in glucose deprivation conditions depend on signaling from Mtl1/Snf1 as suggested by Hughes et al. (2014) that TOR does not have a representative function in minus glucose condtions. (See section 9 and 10 of Discussion chapter of the thesis).

Recently, studies propose that there is an inverse relationship between MMP and RTG activation. Therefore lowering of the MMP leads to activation of the RTG pathway which results in lifespan increase (Miceli et al., 2012). This could be the reason where mitochondrial dysfunction causes increase in MMP in *mtl1* cells and as a consequence there is impairment in *CIT2* transcription. On the other hand, we speculate that uncoupled respiration as a possible cause of cell lethality in *mtl1* mutants. We came across this hypothesis because all the conditions that extended *mtl1* life span also induced coupled

respiration and restored the function of the electron respiratory chain in the mutant, as occurred in *mtl1tor1* mutant and in *mtl1* cells overexpressing Bcy1.

We also demonstrate that NCR reporter gene *MEP2* is downregulated in *mtl1* cells during diauxic shift in comparison to the observed of *MEP2* upregulation in wt cells. The yeast NCR pathway serves as a regulator to utilize alternative nitrogen sources (Cooper, 2002). The ammonium permease, *MEP2* is involved in ammonium activation of trehalase, to mark the entry to diuxic shift (Van Nuland et al., 2006). These findings correlate with our results where absence of *MTL1* inhibits inactivation of both TOR and PKA pathways along with low glycogen accumulation in diauxic shift. Overall, these findings suggest that *MTL1* is an essential gene required for cell's proper entry to diauxic shift by aiding in the down regulation of TOR and PKA pathways.

5. *RAS2* deletion in *mtl1* cells neither restores mitochondrial function nor mitochondrial morphology during diauxic shift.

TOR1 deletion and PKA inactivation were both capable to alleviate all the respiratory problems detected in *mtl1* (high membrane mitochondrial potential, ROS accumulation, uncoupled respiration, blockade in a respiratory state 4 (Boveris and Chance, 1973, Korshunov et al, 1997, Hlavata et al., 2003), low oxygen consumption and low *COX4* levels) supporting a model in which Mtl1 signals to both Tor1 and PKA inhibition in respiratory conditions such as the diauxic shift. On the other hand it is shown that excessive RAS signaling suppress genes involved in the regulation of stress that contain stress response (STRE) and postdiauxic shift (PDS) elements via the transcription factors *MSN2/4, RIM15*, and *GIS1* (Galdieri et al., 2010). It has been described in a similar situation when *RAS2* is hyperactivated, the cells were arrested in a respiratory state 4 blockade (Hlavatá et al., 2008). However, deleting *RAS2* in *mtl1* did not restore a wild type level of normal respiration. Therefore we discarded the possibility that Ras2 activity was the cause for *mtl1* mitochondrial dysfunction in diauxic shift. Recently it was shown that, loss of COX function leads to activation of mitochondria-localized RAS signaling to promote ROS accumulation (Leadsham et al., 2013). In addition they also that Ras2 and PKA participate through independent mechanisms during respiration and ROS production (Leadsham et al., 2013). Consistent to these findings, we have show that *RAS2* deletion in *mtl1* accumulated more ROS and as well low *COX4* transcription levels suggesting that

PKA inactivation independent of Ras2 functions plays an important role in mitochondrial functioning in quiescence.

6. TORC1 effector Sch9 restores *mtl1* life span along with proper mitochondrial functioning in quiescence.

Our observations indicated that *SCH9* is also genetically related to *MTL1* since *SCH9* deletion in *mtl1* mutant restores CLS, respiration and Bcy1 phosphorylation to levels equivalent to those observed in *sch9*. Recently it has been shown that, upon nutrient deprivation and obnoxious stress, Rho1GTPase successfully competes with Tap42 for binding to the Kog1 subunit of the TORC1 complex and causes major impact on the two key branches of TORC1 signaling by inducing both Sch9 dephosphorylation and the release of Tap42–phosphatases (Yan et al., 2012). This cellular response leads to increased cell resistance to environmental stress by detoxifying ROS through activation of Rim 15 orchestrated *MSN2*/4 and *GIS1* transcription and also proper mitochondrial function (Fabrizio et al., 2001; Kawai et al., 2011; Pan et al., 2011; Urban et al., 2007). Our group has recently shown that Mtl1 has a physical interaction in its cytoplasmic domain with Rom2, GEF of the CWI pathway (Petkova et al., 2012). In addition, we also published that Mtl1 senses nutritional and oxidative stresses and delivers the signal to Rom2 to activate Rho1 which in turn signals to Tor1 to initiate adaptative responses for survival (de la Torre-Ruiz et al., 2010; Petkova et al., 2010a; Petkova et al., 2010b). These results are consistent with a model in which Mtl1 signals to TORC1 inhibition and consequent inactivation of Sch9 in quiescence and in response to glucose deprivation. For over a decade it is observed that one of the well known phenotypes of *tor1* or *sch9* strains is the increase in CLS, compared with wild-type cells (Fabrizio et al., 2001; Powers et al., 2006). Moreover, overexpression of Sch9 leads to a rapid loss of viability in *tor1* mutant cells by abolishing stress resistance and shortening of life span (Wei et al., 2009). Apart from yeast, studies in mice indicate an increased lifespan after treating with rapamycin (Harrison et al., 2009) and also mice lacking S6K1 (human ortholog of yeast Sch9), (Powers, 2007) are long-lived with additional protection against age dependent effects (Selman et al., 2009).

Mechanisms described to date involved in lifespan extension by calorie restriction or downregulation of TORC1–Sch9 signaling are

1. Increased coupled respiration, due to increased translation of mtDNA-encoded OXPHOS complex subunits (Bonawitz et al., 2007; Pan et al., 2009), as well as an increased transcriptional activity of the nuclear respiratory regulon, via a Hap4-dependent mechanism (Lavoie and Whiteway, 2008). We also observed that *HAP4* transcription in *mtl1* cells was downregulated in stationary phase (data not shown).
2. Metabolic reprogramming involving glycerol production, accumulation and also transcriptional upregulation of glycerol metabolism (Wei et al., 2009) along with autophagy (Binda et al., 2009). We also observed that glycogen accumulation was restored up to *sch9* levels in *mtl1sch9* during stationary phase (data not shown).

Collectively our data point towards lifespan extension by Mtl1 modulating signaling through highly conserved TORC1 and cAMP/PKA pathways by apt entry into diauxic shift, correcting mitochondrial functioning and increasing stress resistance.

7. Mtl1 regulates PKA activity through Bcy1 activating phosphorylation in diauxic shift and glucose starvation conditions

As the concentration of glucose decreases and cells enter in stationary phase, Bcy1 becomes more phosphorylated in the amino acid T129. This site specific phosphorylation activates Bcy1 inhibitory function on Tpks, as recently described by (Soulard et al., 2010). We show that Mtl1 is required to properly phosphorylate Bcy1 when glucose is exhausted. It has been demonstrated that Bcy1 phosphorylation in residue T129 occurred when TORC1 was inactivated by rapamycin (Soulard et al., 2010). Mtl1 mediates Bcy1 activatory phosphorylation through TORC1 downregulation and indirectly influences Bcy1 protein stability, this later due to the high PKA activity in *mtl1*. Interestingly, Mtl1 behaves similarly to the kelch repeat proteins Gpb1 and Gpb2 (Budhwar et al., 2010; Budhwar et al., 2011) in the regulation of Bcy1 and consequently, in the regulation of PKA. The common feature is that either Gpb1/Gpb2 or Mtl1 proteins represent two types of PKA regulation that occur through a branch different to *RAS2*. The novel finding is that Mtl1 couples mitochondrial activity with TORC1 and PKA inhibition in respiratory conditions such as the diauxic shift. PKA inactivation alleviates very efficiently *mtl1* problems in diauxic shift, but to a lesser extent compared to effects provoked upon TOR1 inactivation, resulting in Bcy1 and PKA placed downstream TORC1. TORC1 phosphorylates and activates the kinase Sch9 that in turn inhibits Slt2/Mpk1

phosphorylation (Soulard et al, 2010). We also show that deletion of *SCH9* in *mtl1* restored Bcy1 activating phosphorylation upto *sch9* levels, demonstrating that it acts downstream TORC1 as an effector. Recently, it has been described that Sch9, inhibits PKA activity by regulating the localization and phosphorylation of Bcy1 via Yak1 kinase (Griffioen et al., 2001; Zhang and Gao, 2012).

Again, Soulard *et al.* (2010) described that, upon Sch9 inhibition, Slt2 kinase activity phosphorylates Bcy1 in the residue T129, thus inducing its inhibitory function on PKA. In agreement with these authors we have also observed that Slt2 plays a role in PKA inhibition upon glucose depletion and in quiescence. Constitutive activation of the CWI pathway partly restores *mtl1* CLS; however it does not restore mitochondrial function, suggesting that alternative substrates other than Slt2 might be regulated from *MTL1* through *TOR1* and *SCH9* in quiescence and glucose depletion conditions promoting life span. Here we show that Mtl1 regulates Bcy1 phosphorylation in response to glucose depletion whereas nitrogen deprivation induces Bcy1 inhibitory phosphorylation independently on Mtl1 but dependently on Slt2. *TOR1* deletion suppresses all *mtl1* phenotypes in diauxic shift showing an epistatic relationship, therefore we hypothesize that Mtl1 main function is to down regulate TORC1 in quiescence being glucose depletion the main signal that triggers this regulation. From TORC1 the signal flows to inhibit Sch9 and from here to activate Slt2 and possibly to other unknown kinase/s converging in PKA inhibition through the activation of Bcy1 phosphorylation and the increase in its stability. However our results suggest that PKA is not the only target to be regulated. Additional kinases Pkh1, 2, and 3, the yeast orthologs of mammalian 3-phosphoinositide-dependent protein kinase 1 (PDK1; Casamayor et al., 1999) have been reported to phosphorylate Sch9 and PKA. Phosphorylation of Sch9 by these kinases appears to be required for its activity, and mutagenesis of the PDK1 site in the catalytic Tpk1 subunit interferes with binding to the regulatory Bcy1 subunit (Voordeckers et al., 2011; Haesendonckx et al., 2012). Although we do not demonstrate this through experimental data, more studies are required to identify the additional targets.

8. In quiescent conditions Slt2 functions downstream TOR1 and PKA to extend cell survival in both stationary phase and glucose depletion.

In order to place Slt2 in the signaling cascade initiated by Mtl1, we performed epistatic experiments with *TOR1* and PKA. The observation that *TOR1* deletion or PKA inactivation did not suppress any of the Slt2 phenotypes such as loss in viability, Bcy1 phosphorylation and stability places Slt2 downstream *TOR1/SCH9* and PKA due to the following reasons

1. Slt2 has a functional role in cell survival in stationary phase. This result is in agreement with the established reports that describe CWI activation is required for survival in quiescence (Barbosa et al., 2012; Stewart et al., 2007; Torres, 2002). The loss of viability observed in *slt2* mutant was not due to cell-wall problems, since osmotic stabilization had not suppressed the effect in cell viability in quiescence and also glucose starvation. Recently comparable to our data, it has been reported that *slt2* mutant was hypersensitive to genotoxic stresses in the presence of sorbitol, which indicated that cell death was not related to cell wall defects (Soriano-Carot et al., 2012).

2. Slt2 is essential for PKA activity and we consider that Slt2 is partly required for Bcy1 phosphorylation and activation. However Slt2 is not the only kinase and does not fully explain Bcy1 phosphorylation, we would expect a minimal rescue in cell viability in Slt2 mutant harbouring Bcy1. Conversely, we see no change in CLS of *slt2* mutant over expressing Bcy1. This could be possibly because Slt2 is involved in other functions for life extension other than Bcy1 phosphorylation. We also show that *slt2tor1* double mutant is more lethal than the single mutant *slt2*. The possible reason could be *TOR1* deletion requires Slt2 and possibly other kinase/s to inhibit PKA function. If this is the case we would expect that in the CLS experiment TOR is downregulated first and then Slt2 activation; so therefore *slt2tor1* double mutant should display the same CLS as *slt2* single mutant. Conversely we observe *slt2tor1* las lower CLS than *slt2* leading to two important conclusions:

a. Slt2 is required for life extension mediated by TOR inhibition.

b. Life extension due to TOR inhibition is not fully explained through PKA inhibition mediated by Slt2 but to additional functions for which Slt2 are primarily required.

3. TORC1 may, possibly via Sch9 and/or Tap42–Sit4 phosphatases, impinge upon the CWI pathway, but whether this occurs at the level of the cell-wall sensors, Rom2, or the MAPK cascade is currently unknown (Torres et al., 2002; Reinke et al., 2004; Araki et al., 2005; Kuranda et al., 2006; Soulard et al., 2010). In agreement with Soulard et al. (2010), we demonstrate that during quiescence and glucose starvation, signal flows from Mtl1-Tor1-Sch9-Slt2.

The observation that *MTL1* and *SLT2* presented synthetic lethality during stationary phase suggested that even though they are in the same signaling pathway (CWI), they might operate in different pathways regarding the function of life extension. Another possible interpretation is that they both can be forming a complex for this function; however we do not have any evidence to support this latter hypothesis. In addition, we also show that in stationary phase, *slt2* mutant accumulated more glycogen in comparison to *mtl1* and *mtl1slt2* mutant respectively. This suggests a role of Slt2 in Bcy1 phosphorylation due to indirect PKA inactivation in diauxic shift possibly through *TOR1/SCH9* downregulation.

However, we demonstrate that during diauxic shift, oxygen consumption in *slt2* mutant is lower than that determined in the wild type cells being slt2 resipration partly uncoupled respiration. We reasoned that the *slt2* mutant respiratory problems were due to the consequence of cell-wall defects, as the addition of osmotic stabilizer restored the oxygen consumption up to wild type levels.

To summarize the total facts, we present a model to describe the role of Mtl1 in quiescence and aging process (Fig 1). Glucose availability and stationary phase stress signals are sensed by Mtl1 and it transmits the signal to downregulate Tor1 for the optimal entry to diauxic shift. Downregulation of Tor1 dephosphorylates its downstream effector Sch9. Down regulation of Sch9 causes two major effects. One it phosphorylates Bcy1 to inhibit PKA function and the other it also phosphorylates Slt2 MAPkinase to activate the CWI pathway. Moreover, Slt2 activation partly phosphorylates Bcy1 in quiescent conditions, although additional targets are yet to be identified. Downregulation of Tor1/Sch9 and PKA promote cell survival by regulating the mitochondrial function.

Proper mitochondrial function through RTG response leads to decrease in mitochondrial stress and also optimize ROS levels in quiescence. Additional studies are required to understand better the process of aging. During diauxic shift and decrease in glucose levels, in the absence of *MTL1* the cells fail to downregulate *TOR1* and PKA functions. This causes increased mitochondrial stress which inturn increases ROS to abnormal levels and also impairs RTG response. As a consequence of this, cells fail to enter properly into diauxic shift and also losses viability leading to decreased chornological life span in quiescence.

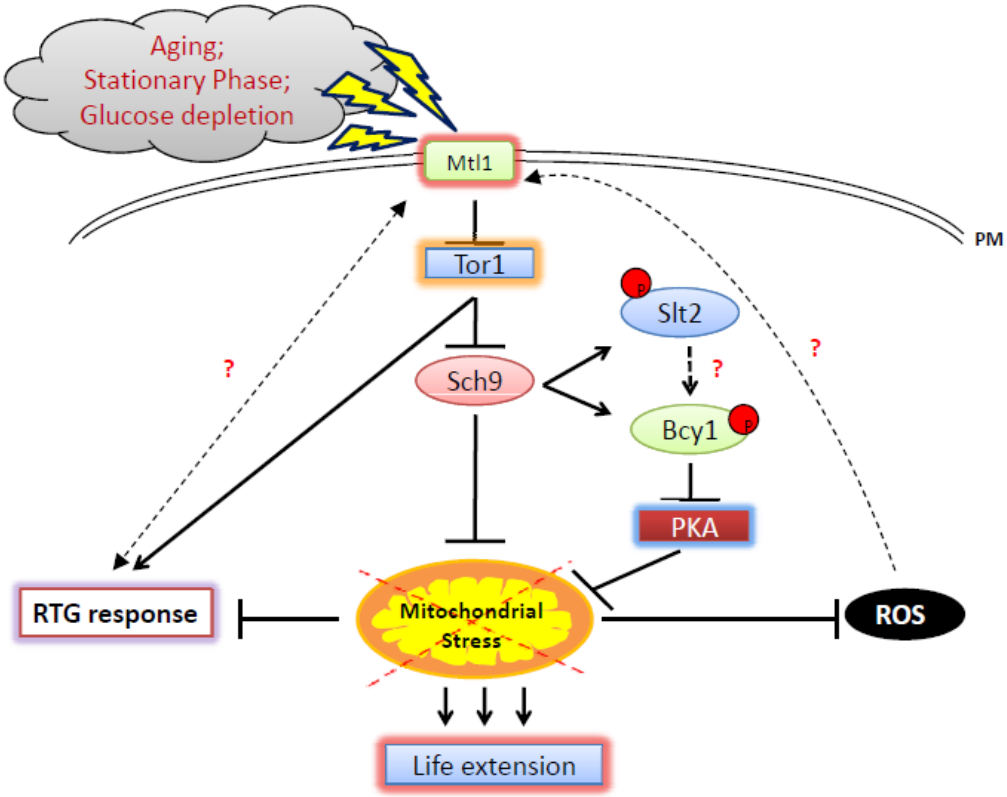


Fig 1: Role of Mtl1 in quiescence. Mtl1 senses glucose availability and stationary phase signals and transmits the signal to Tor1 and this downregulates *TOR1* function. Down regulation of TOR aids the cell's entry into diauxic shift. The downregulated Tor1 inhibits Sch9 to futher release Slt2 and activate the CWI pathway. The activated Slt2 partially phosphorylates Bcy1 to inhibit PKA activity. In addition, downregulated Sch9 phosphorylates Bcy1 to inhibit PKA activity. Downregulation of TOR/Sch9 and PKA kinases optimize the mitochondrial function and elleiviates mitochondrial stress. Proper mitochondrial function not only reduces ROS levels but also regulates RTG pathway for cell survival. In addition, downregulation of TOR leads to the activation of RTG pathway to maintain intracellular glutamate levels. Mtl1 sensing the availability of glucose also regulates RTG response; still further experiments are needed to explain this mechanism. Moreover, ROS levels are also sensed by Mtl1 to downregulate TOR and elicit adapatative signaling mechanism.

9. Upon glucose starvation, Mtl1 regulates both NCR and RTG pathways independently on *TOR1* and PKA

In this study we present evidence demonstrating that in conditions of glucose depletion Mtl1 regulates both NCR (*MEP2* and *GAP1*) and RTG (*CIT2*) gene expression. This regulation seems to be specific to glucose since upon nitrogen starvation we did not observe it. In the absence of nitrogen or rapamycin treatment, the nitrogen regulated genes, large number of permeases and other factors required for the assimilation of alternative nitrogenous sources are expressed in a TORC1 dependent manner via the Tap42 phosphatases branch (Crespo et al., 2002; Loewith and Hall, 2011). In line with these findings we show that rapamycin treatment, in presence of glucose caused induction of *MEP2*, *GAP1* and *CIT2* transcription in both wt and *mtl1* mutant (Fig 26B and 29). We also show that, during nitrogen starvation and rapamycin treatment, NCR and RTG response genes are directly regulated by TORC1 function independent of Mtl1. However, we show that in case of glucose starvation conditions *MEP2* and *CIT2* transcription were induced in wt cells but not in *mtl1* cells. In order to check this induction of NCR pathway and RTG response genes upon glucose depletion was TOR dependent as observed in the case on nitrogen starvation, we tried to elucidate the transcription of *MEP2* and *CIT2* in both single mutant *tor1* and double mutant *mtl1tor1* under glucose depreviation conditions. We show that deletion of *tor1* in *mtl1* cells did not significantly suppress the defects in the transcription of *CIT2* and *MEP2* upon glucose depletion. In agreement with our results, it has been reported that TORC1 does not participate in induction of NCR genes upon glucose starvation (Bertram et al., 2002; Hughes Hallett et al., 2014). Till date there is no concrete evidence to show how RTG response is induced in glucose starvation conditions. In addition, we also show that deletion of *RAS2* and over expression of Bcy1 in *mtl1* mutant did not significantly induce both *MEP2* and *CIT2* transcripion in glucose starvation conditions. This result indicates that NCR and RTG pathways are very minimally dependent on PKA function in glucose depletion conditions.

Firstly, to understand the possible relationship between Mtl1 and NCR in glucose starvation conditions, we checked the phosphorylation status of transcriptional activator of the NCR pathway, Gln3. Earlier reports suggest that in response to the quality of nitrogen source, TOR regulates expression of nitrogen regulated genes by sequestering the GATA binding transcription factors *GLN3* and *GAT1* in the cytoplasm (Crespo et al., 2002; Beck

and Hall, 1999). It is established that upon nitrogen limitation or rapamycin treatment, Gln3 is dephosphorylated by the type 2A like phosphatase Sit4 and translocated into the nucleus where it activates target genes (Loewith and Hall, 2011). In addition current evidences, now suggest that GATA transcription factor contains multiple phosphorylation sites and Gln3 regulation may occur by two separate pathways, one TORC1-dependent and the other NCR-sensitive (Rai et al., 2013; Tate and Cooper, 2013). NCR-sensitive, regulation was achieved through nitrogen-responsive control of the TORC1 serine/threonine kinase complex and its downstream effector branch, PP2A-type phosphatases. Nitrogen limitation induced nuclear GATA factor localization exhibits markedly different PP2A requirements than required by rapamycin treatment. Moreover, Gln3 and Gat1 phosphorylation/dephosphorylation and shuttling in/out the nucleus seem to respond separately to TORC1 and nitrogen limitation (Georis et al., 2009; Georis et al., 2011). In addition, recent evidences suggest that Gln3 phosphorylation/dephosphorylation does not always correlate with its nuclear localization (Bertram et al., 2002; Georis et al., 2011; Rai et al., 2013). Rapamycin provokes Gat1 and to a lesser extent Gln3 nuclear localization independently of TORC1-controlled phosphatase activity (Georis et al., 2011). These growing evidences not only suggest that Gln3 phosphorylation can be independent of TORC1 function but also phosphorylation does not necessarily affect its function and nuclear localization. In line with these evidences, we show that Gln3 phosphorylation status remained unchanged in *mtl1* upon glucose depletion and infact we also demonstrate phosphorylation of Gln3 upon one day glucose starvation in wt cells. Matching with our data, recently it has been reported that during glucose starvation Gln3 remains phosphorylated, or becomes more phosphorylated (Hughes Hallett et al., 2014). We show that Mtl1 regulates Gln3 phosphorylation upon glucose starvation occurring independently on *TOR1* function. In addition, impairment in Gln3 phosphorylation is the possible cause for down regulation of *MEP2* and *GAP1* transcription in *mtl1* cells upon glucose starvation condition.

In order to elucidate the possible relationship between Mtl1 and RTG response, we performed an experiment to see *CIT2* transcription in presence and absence of glucose, glutamate and rapamycin treatment in wt and *mtl1* cells resepctively. To date, it is known that glucose represses RTG response but the mechanism is still unclear (Jazwinski, 2013). The possible mechanism reported is that *MKS1* gene was isolated as a negative regulator

of the RAS signaling pathway and hence it is hypothesized that PKA function could be connected with RTG response with respect to glucose availability (Jazwinski, 2005).

In this study we present two major observations, in one hand, in the absence of glucose; TORC1 does not signals RTG activation and on the other hand, TORC1 downregulation by rapamycin directly induces RTG response independently of Mtl1. It is known that glutamate, glutamine, and TOR, inhibits the nuclear localization and activation of the transcription factors *GLN3*, *RTG1* and *RTG3* (Crespo et al., 2002; Liu and Butow, 2006). Comparable to our findings, Giannattasio *et al.* (2005) have reported that glutamate represses *CIT2* expression triggered by mitochondrial dysfunction, but not that elicited by TOR inhibition by rapamycin. These authors conclude that expression of RTG repsonse and NCR pathway genes is differentially regulated in response to mitochondrial dysfunction or rapamycin treatment, and as well by glutamine or histidine starvation. Recently, it has been reported that in the presence of glucose, rapamycin treatment triggers activation of the PP2A branch of the TORC1 pathway (Hughes Hallett et al., 2014). These authors also report that simultaneous rapamycin (induced nitrogen starvation) and glucose starvation inactivates the PP2A branch signaling (Hughes Hallett et al., 2014). Similarly, as discussed previously, our data clearly suggests that Mtl1 also regulates RTG response upon glucose availability and this signal is very minimally dependent on *TOR1*/PKA function.

Another important question still yet to be answered is “who dephosphorylates Rtg3 to elicit the RTG response (*RTG1/3*) in the nucleus?” Possible phosphatases which regulate this function could be Tap42-phosphatases (both PP2A and PP2A like Sit4) (Düvel et al., 2003; Hughes Hallett et al., 2014; Schneper et al., 2004; Zaman et al., 2008). On the other hand, Rtg2 itself could function as phosphatases to trigger the RTG response (Jazwinski, 2013; Liu and Butow, 2006). In addition, the WD-40 protein, Lst8, is also found as a negative regulator of the RTG pathway. Analysis of different mutant alleles of Lst8 that result in constitutive activation of RTG target gene expression reveals that Lst8 acts at two distinct sites, one upstream and the other downstream of Rtg2 (Chen and Kaiser, 2003; Giannattasio et al., 2005; Liu and Butow, 2006). The downstream effect of Lst8 and how it regulates RTG response is still unknown.

In addition, we also demonstrate the functional role of Mtl1 with RTG response where we present that Mks1, a negative RTG regulator is hypersphorylated upon glucose depletion

in *mtl1* cells in comparison to dephosphorylation of Mks1 in wt cells. In addition, *CIT2* repression observed in *mtl1* cells upon glucose depletion, clearly suggest that RTG pathway is turned off in the absence of *MTL1*. Moreover, it has been reported that free Mks1, unbound to either Rtg2 or Bmh1/2, is degraded through Grr1, a component of the SCF^{Grr1}E3 ubiquitin ligase. This causes dependent ubiquitination and degradation, and thereby would keep free Mks1 levels lower enough to tighten control of Mks1 on-off switch and finally to allow the full activation of the RTG pathway (Liu et al., 2005). Our results coincide with these findings, as we see dephosphorylation of Mks1 in wt cells through Grr1 upon glucose starvation and this effect is not seen in *mtl1* mutant.

As discussed earlier, we tried to elucidate the genetic relationship between *MTL1* and *RTG2*. In line with this, we performed glucose starvation experiments in *rtg2* single and *mtl1rtg2* double mutant and analyzed *CIT2* transcription. Here we demonstrate that RTG response is solely elicited through *RTG2* independent on glucose availability. We show that both *rtg2* and *mtl1rtg2* presented a complete *CIT2* repression upon glucose starvation. These results indicate that Rtg2 functions downstream Mtl1 because absence of *RTG2* completely abolishes RTG response. The possible reason why RTG response is completely dependent on *RTG2* is because Rtg2's cardinal function is to dephosphorylate Rtg1/3 and translocate the complex into the nucleas and also Rtg2 is found to be intregral part of the SLIK (SAGA-like) transcriptional activator complex which is required to induce RTG related gene expression in the nucleas (Jazwinski, 2013; Liu and Butow, 2006; Wang et al., 2010).

Collectively these results suggest that Mtl1 regulates NCR and RTG pathways in response to glucose availability and very minimally dependent on TORC1 and PKA function. We came across this hypothesis because deletion of *RAS2*, *TOR1* and also *mtl1* over expressing Bcy1 does not suppress the impairment of RTG response and NCR pathways observed in *mtl1* phenotypes upon glucose starvation. Our data fits the proposed model suggested by Hughes Hallett et al. (2014) that TORC1 goes into off state (PP2A and Sch9 inhibited state) during glucose starvation conditions and further more these authors suggest that TORC1 acts as a central processing hub for different stress responses. The next question asked is "how Mtl1 regulates both NCR and RTG pathways with respect to glucose availability and through whom this response is elicited?"

One of the possible candidates to regulate NCR pathway in response to glucose availability is Snf1 (Bertram et al., 2002).

10. Mtl1 plays a role in signaling the absence of glucose to both NCR and RTG outputs through Snf1

Maximal catalytic activation of Snf1 requires phosphorylation of its conserved activation loop Thr210 residue by upstream kinases, and cellular levels of phospho-Thr210-Snf1 increase dramatically upon glucose deprivation (Orlova et al., 2006). We show that in the absence of *MTL1*, Thr210-Snf1 is hyperphosphorylated upon glucose depletion. We also observe that Snf1 nuclear localization was much reduced in *mtl1* glucose depleted cells. Moreover to date, how glucose regulates SNF1complex activity through either phosphorylation or dephosphorylation or both is unclear (Conrad et al., 2014; Kim et al., 2013; Rødkaer and Faergeman, 2014). The dephosphorylation of Snf1 catalytic subunit in response to the glucose signal is partly mediated by the PP1 phosphatase Glc7 that acts in a complex with the Reg1 regulatory subunit (Hedbacker and Carlson, 2008). Recent research has also identified a possible role for the type 2A-like protein phosphatase, Sit4, and also for Ptc1 protein phosphatase 2C, in Thr210 dephosphorylation (Ruiz et al., 2011; Ruiz et al., 2013). Finally, a recent study revealed that glucose exerts a more direct effect on phosphatase action, as it activates PP1 and PP2A posttranslationally (Castermans et al., 2012). In line with these evidences, we hypothesize that Mtl1 senses glucose availability and positively regulates the active SNF1complex function. Although, more studies are awaited to explain the mechanism for this regulation.

In addition to Snf1's primary function of glucose sensing and metabolism, it has been reported that Snf1 protein kinase is regulated differently during adaptation of cells to NaCl and alkaline pH (Hong and Carlson, 2007). These stresses varied with respect to both temporal regulation of activation and subcellular localization of Snf1. Moreover, it was seen that Snf1 kinase becomes enriched in the nucleus in response to alkaline pH but not salt stress (Hong and Carlson, 2007). Similarly, a recent study was reported where Snf1 phosphorylation is linked to its essential function for selenite defence whereas its localisation was confined to be cytoplasmic (Pérez-Sampietro et al., 2013). We hypothesize that, Snf1 hyperphosphorylation in *mtl1* mutant could be the possible cause of impaired Snf1 nuclear localization.

We demonstrate that deletion of both *TOR1* and *RAS2* in *mtl1* very partially restore the nuclear localization of Snf1 upon glucose depletion. We also show that both *tor1* and *ras2* single mutants have higher Snf1 nucelar localization in glucose starvation conditions. In agreement with our findings, it has been established that Snf1 is negatively regulated by TOR kinase and also cAMP-PKA respectively (Barrett et al., 2012; Bertram et al., 2002; Orlova et al., 2006; Sanz, 2003). These results suggest that Mtl1 regulates both the Snf1 activation and also nuclear localisation hardly dependent of *TOR1* and *RAS2* function upon glucose depletion.

It has been established that glucose regulates Gln3 phosphorylation and its subcellular localization is mediated by Snf1 (Bertram et al., 2002; Orlova et al., 2006; Rødkaer and Faergeman, 2014). Active Snf1 kinase also interacts with and phosphorylates Gln3, promoting nuclear internalization of the NCR pathway's transcription factor. The effect of Snf1 kinase on Gln3 was only dependent on carbon starvation and independent on TORC1 function (Bertram et al., 2002; Sanz, 2003; Hedbacker and Carlson, 2008). In addition Bertram et al. (2002) have also reported that upon glucose starvation, Snf1 induced Gln3 phosphorylation occurs on a different phosphorylating residue other than the TORC1 dependent phosphorylation. Furthermore, recently it has been published that Snf1 is important for inactivation of TORC1-Sch9 signaling in glucose starvation conditions but not in nitrogen starvation conditions through PP2A phosphatases branch (Hughes Hallett et al., 2014). Moreover, it has been reported that not only regulation of amino acid biosynthesis but also fatty acid metabolism is coordinately regulated by Snf1 and TORC1 (Zhang et al., 2011). Taking these evidences together along with our data on Gln3 phosphorylation and *MEP2/ GAP1* transcription, we propose that Mtl1 positively regulates NCR pathway in response to glucose signal possibly through Snf1.

We also observed that the *snf1* mutant was deficient in RTG signaling upon glucose starvation. Recent evidence suggests that Snf1 is required for repression of the TORC1 dependent gene expression program in glucose starvation conditions (Hughes Hallett et al., 2014). In addition, Hughes Halett et al. (2014) reported that *snf1* cells do not activate the PP2A branch during simultaneous starvation of glucose and nitrogen and they conclude that AMPK regulates possibly TORC1 in yeast through an unknown factor which mediates this signal. Similarly in mammalian cells, AMPK inactivates TORC1 by phosphorylating the key regulatory subunit Raptor/Kog1 (Gwinn et al., 2008). The findings of Hughes Halett et al. (2014) contradict in some stress types to the previous

established reports which indicate that PP2A branch is active in different stress responses (Crespo et al., 2002; Santhanam et al., 2004; Yan et al., 2012).

Putting all facts together, we propose a preliminary model for Mtl1 which senses the absence of glucose and transmit the signal to the active Snf1 (Fig 2). This signal induces the Snf1 function involved in pushing TORC1 complex to an off-state. In this situation PP2A and PP2A like phosphatases have a low activity sufficient to dephosphorylate Snf1 to a certain level that maintains its activity to interact and phosphorylate Gln3 and possibly to activate RTG response. Alternatively, RTG regulation could be under the control of Sit4/ PP2A phosphatases (the low activity required for Rtg3 dephosphorylation, since TORC1 is in an off-state). In the absence of Mtl1, Snf1 is not properly signaled and moreover it is hyperphosphorylated, being unable to push TORC1 to an off-state. As a consequence of this, Sit4/PP2A phosphatases are inactive and unable to dephosphorylate Snf1 and Rtg3.

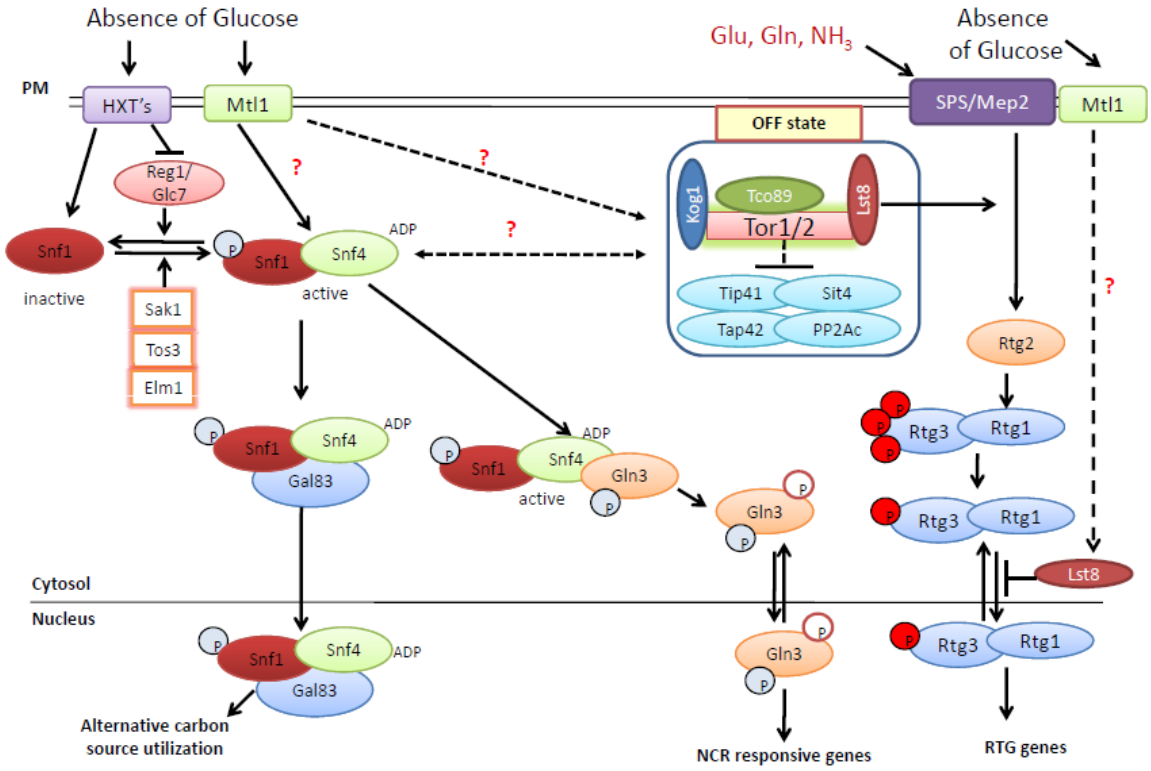


Fig 2: Role of Mtl1as a glucose sensor. In the absense of glucose, Hexokinases, Hxt's senses the signal and transmits to the Snf1's upstream kinases (Sak1, Elm1 and Tos3) to phosphorylate and activate Snf1 by binding to Snf4; in addition Hxt's inhibit Reg1/Glc7 phosphatase. Mtl1 senses no to low glucose levels signals and transmits the signal to active Snf1 and this signal sends TORC1 into an off-state. Along with

TORC1 the Tap42 phosphatases (PP2A and Sit4) are also inhibited or pertain a low activity. How this signal from active Snf1 is transmitted to both TORC1 and Tap42 phosphatases is yet to be indentified. In addition, Mtl1 also communicates to TORC1 in response to glucose and this mechanism is still open. Active Snf1 attaches to the Gal83 and forms complex for its nuclear localization and functions for utilization of alternative carbon sources. Active Snf1 phosphorylates Gln3 and aids in the nuclear localization of the GATA transcription factor to activate the NCR genes. The low level of Tap42 phosphatases in glucose limitation conditions causes *RTG1/3* nuclear localization and also maintains the cytoplasmic Snf1 phosphorylation. The model also describes the role of Mtl1 regulating Lst8 in response to glucose levels independent of TORC1 function. This mechanism is also yet to be indentified.

Improper Snf1 and Rtg3 phosphorylation blocks both their translocation to the nucleus in response to glucose deprivation. In addition, the Snf1 hyper-phosphorylation observed in the *mtl1* mutant might impair Gln3 phosphorylation and function upon glucose starvation. Still, in this model *snf1* mutant should have complete inactivation of RTG response in absence of glucose. Conversely, we demonstrate a partial RTG response in *snf1* mutant. The possible reason could be the because of Lst8 which is shown to regulate RTG response both upstream and downstream Rtg2 and could play a role in response to glucose availability independent on TORC1 function. We hypothesize that the effect from Lst8 could be Mtl1 dependent because we demonstrate that in the absence of *MTL1*, *CIT2* transcription is completely repressed in glucose starvation conditions. Nevertheless, this model includes additional targets which are yet to be identified in the signal process. Still daunting research is required to unravel the mechanism of how glucose regulates Snf1, RTG and NCR responses.

CONCLUSIONS

1. Mtl1 plays an essential role in the entry of cells into diauxic shift and is required for chronological life span. In its absence, cells suffer mitochondrial dysfunction leading to loss of viability in quiescence.

2. In the absence of *MTL1*, ROS act as signaling molecules for mitohormesis adaptative response.

3. Mtl1 negatively regulates TORC1 and PKA in diauxic shift promoting a correct mitochondrial function and cell survival.

4. *RAS2* and *MTL1* function on separate pathways in quiescence since *RAS2* deletion in *mtl1* does not restore mitochondrial functional defects.

5. Mtl1 regulates both Bcy1 stability and its activating form of phosphorylation to regulate PKA function in both diauxic shift and upon glucose depletion through Tor1/Sch9 but is not only transmitted through Slt2 kinase activity. This signal is independent on *RAS2* function

6. TORC1 signal impinges on CWI pathway via Sch9 shown by the phosphorylation of Slt2 in both diauxic shift and upon glucose starvation.

7. Slt2 is downregulated by Sch9 and partly activates PKA activity through the regulation of Bcy1 phosphorylation and stability in diauxic shift.

8. Both Mtl1 and Slt2 play additive roles in life extension meaning that for this function Mtl1 and Slt2 donot work in the same pathway or alternatively they form a complex.

9. Mtl1 positively regulates the response to glucose deprivation by modulating both NCR and RTG responses independently on both TORC1 and PKA but possibly in a manner dependent on Snf1, although we propose a preliminary mechanism by which Mtl1 regulates Snf1 nuclear localization.

10. Mtl1 regulates Snf1 nuclear localization very partly dependent on both *TOR1* and *RAS2* inhibition upon glucose deprivation conditions.

11. Snf1 partially regulates RTG response under glucose starvation conditions, resulting in the convergence of glucose and nitrogen signaling pathways on RTG along with NCR pathways.

BIBLIOGRAPHY

Abate, G., Bastonini, E., Braun, K.A., Verdone, L., Young, E.T. and Caserta, M. (2012). Snf1/AMPK regulates Gcn5 occupancy, H3 acetylation and chromatin remodelling at S. cerevisiae ADY2 promoter. *Biochim.Biophys.Acta.* 1819, 419–427.

Abe, F. and Iida, H. (2003). Pressure induced differential regulation of the two tryptophan permeases Tat1 and Tat2 by ubiquitin ligase Rsp5 and its binding proteins, Bul1 and Bul2. *Mol.Cell.Biol.* 23, 7566–7584.

Abe, J., Kusuhara, M. Ulevitch, R. J. Berk, B. C. and Lee, J. D. (1996). Big mitogen-activated protein kinase 1 (BMK1) is a redox sensitive kinase. *J. Biol. Chem.* 271, 16586–16590.

Aerts, A.M., Zabrocki, P., Govaert, G., Mathys, J., Carmona-Gutierrez, D., Madeo, F., Winderickx, J., Cammue, B.P. and Thevissen, K. (2009). Mitochondrial dysfunction leads to reduced chronological lifespan and increased apoptosis in yeast. *FEBS Lett.* 583, 113–117

Aguilaniu, H., Gustafsson, L., Rigoulet, M. and Nyström, T. (2001). Protein oxidation in G0 cells of *Saccharomyces cerevisiae* depends on the state rather than rate of respiration and is enhanced in pos9 but not yap1 mutants. *J. Biol. Chem.* 276, 35396–404.

Ahuatzi, D., Herrero, P., de la Cera, T. and Moreno, F. (2004). The glucose-regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent. *J.Biol.Chem.* 279, 14440–14446.

Allen, C., Büttner, S., Aragon, A. D., Thomas, J. A., Meirelles, O., Jaetao, J. E., Benn, D., Ruby, S. W., Veenhuis, M., Madeo, F., et al. (2006). Isolation of quiescent and nonquiescent cells from yeast stationary-phase cultures. *J. Cell. Biol.* 174, 89–100.

Amodeo, G.A., Rudolph, M.J. and Tong, L. (2007). Crystal structure of the heterotrimer core of *Saccharomyces cerevisiae* AMPK homologue SNF1. *Nature.* 449, 492–495.

Andrews, P. D. and Stark, M. J. (2000). Dynamic, Rho1p-dependent localization of Pkc1p to sites of polarized growth. *J. Cell Sci.* 113, 2685-2693.

Araki, T., Uesono, Y., Oguchi, T. and Toh, E.A. (2005). LAS24/KOG1, a component of the TOR complex 1 (TORC1), is needed for resistance to local anesthetic tetracaine and normal distribution of actin cytoskeleton in yeast. *Genes. Genet.Syst.* 80, 325–343

Arndt, K. T., Styles, C. A. and Fink, G. R. (1989). A suppressor of HIS4 transcriptional defect encodes a protein with homology to the catalytic subunit of protein phosphatases. *Cell.* 56, 527-537.

Aronova, S., Wedaman, K., Aronov, P. A., Fontes, K., Ramos, K., Hammok, B. D. and Powers, T. (2008). Regulation of ceramide biosynthesis by TOR complex 2. *Cell Metab.* 7, 148-158.

Ashrafi, K., Lin, S.S., Manchester, J,K. and Gordon, J.I. (2000). Sip2p and its partner snf1p kinase affect aging in *S. cerevisiae*. *Genes.Dev.* 14, 1872–1885.

Audhya, A. and Emr, S. D. (2002). Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev. Cell.* 2, 593-605.

Ayscough, K. R. and D. G. Drubin, (2003). A role for the yeast actin cytoskeleton in pheromone receptor clustering and signaling. *Curr. Biol.* 8, 927–930.

Babour, A., Bicknell, A. A., Tourtellotte, J. and Niwa, M. (2010). A surveillance pathway monitors the fitness of the endoplasmic reticulum to control its inheritance. *Cell.*142, 256–269.

Backhaus, K., Rippert, D., Heilmann, C. J., Sorgo, A. G., de Koster, C. G., Klis, F. M., Rodicio, R. and Heinisch, J. J. (2013). Mutations in SNF1 complex genes affect yeast cell wall strength. *Eur. J. Cell Biol.* 92, 383–95.

Bar, E. E., Ellicott, A. T. and Stone, D. E. (2003). Gbg recruits Rho1 to the site of polarized growth during mating in budding yeast. *J. Biol. Chem.* 278, 21798–21804.

Barbet, N.C., Schneider, U., Helliwell, S.B., Stansfield, I., Tuite, M.F. and Hall, M.N. (1996). TOR controls translation initiation and early G1 progression in yeast. *Mol. Biol. Cell.*7, 25–42.

Barbosa, A.D., Graça, J., Mendes, V., Chaves, S. R., Amorim, M. A., Mendes, M. V., Moradas-Ferreira, P., Côrte-Real, M. and Costa, V. (2012). Activation of the Hog1p kinase in Isc1p-deficient yeast cells is associated with mitochondrial dysfunction, oxidative stress sensitivity and premature aging. *Mech. Ageing. Dev.* 133, 317–30.

Barrett, L., Orlova, M., Maziarz, M. and Kuchin, S. (2012). Protein kinase A contributes to the negative control of Snf1 protein kinase in *Saccharomyces cerevisiae*. *Eukaryot. Cell.*11, 119–28.

Barros, M. H., da Cunha, F. M., Oliveira, G. A., Tahara, E. B. and Kowaltowski, A. J. (2010). Yeast as a model to study mitochondrial mechanisms in ageing. *Mech. Ageing Dev*. 131, 494–502.

Batiza, A. F., Schulz, T. and Masson, P. H. (1996). Yeast respond to hypotonic shock with a calcium pulse. *J.Biol. Chem.* 271, 23357–23362.

Bazzi, M. D., and Nelsestuen, G. L. (1990). Protein kinase C interaction with calcium: a phospholipid-dependent process. *Biochemistry.* 29, 7624-7630.

Beck, T., and Hall, M.N. (1999). The TOR signaling pathway controls nuclear localization of nutrient regulated transcription factors. *Nature.* 402, 689–692.

Bellí, G., Garí, E., Piedrafita, L., Aldea, M. and Herrero, E. (1998). An activator / repressor dual system allows tight tetracycline-regulated gene expression in budding yeast. *Nucleic.Acids.Res*. 26, 942–947.

Bermejo, C., Rodríguez, E., García, R., Rodríguez-Peña, J. M., Rodríguez de la Concepción, M.L. et al. (2008). The sequential acti- vation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. *Mol. Biol. Cell.*19,1113–1124.

Bermejo, C., García, R., Straede, A., Rodríguez-Peña, J. M., Nombela, C. et al. (2010). Characterization of sensor-specificstressre- sponse by transcriptional profiling of wsc1 and mid2 deletion strains and chimeric sensors in *Saccharomyces cerevisiae* . *OMICS*.14, 679–688.

Berset, C., Trachsel, H. and Altmann, M. (1998). The TOR (target of rapamycin) signal transduction pathway regulates the stability of translation initiation factor eIF4G in the yeast *Saccharomyces cerevisiae* . *Proc.Natl.Acad.Sci. USA*. 95, 4264–4269.

Bertram, P. G., Choi, J. H., Carvalho, J., Ai, W., Zheng, X. F. S. and Chan, T. (2002). Convergence of TOR-Nitrogen and Snf1-Glucose Signaling Pathways onto Gln3 Convergence of TOR-Nitrogen and Snf1-Glucose Signaling Pathways onto Gln3. *Mol. Cell. Biol.* 22, 1246–1252.

Bickle, M., Delley, P.A., Schmidt, A. and Hall, M.N. (1998). Cell wall integrity modulates RHO1 activity via the exchange factor ROM2. *EMBO.J*. 17, 2235–2245.

Binda, M., Peli-Gulli, M.P., Bonfils, G et al. (2009). The Vam6 GEF controls TORC1 by activating the EGO complex. *Mol Cell*. 35, 563–573.

Boada, J., Cuesta, E., Perales, J. C., Roig, T. and Bermudez, J. (2004). Glutathione content and adaptation to endogenously induced energy depletion in Mv1Lu cells. *Free. Radic. Biol. Med.* 36, 1555–65.

Bolotin-Fukuhara, M., Dumas, B. and Gaillardin, C. (2010). Yeasts as a model for human diseases. *FEMS.Yeast.Res*. 10, 959–60.

Bonawitz, N. D. and Shadel, G. S. (2007). Rethinking the Mitochondrial Theory of Aging The Role of Mitochondrial Gene Expression in Lifespan Determination ND ES Abbreviations RIB. *Cell.Cycle.* 6, 1574–1578.

Bonawitz, N. D., Chatenay-Lapointe, M., Pan, Y. and Shadel, G. S. (2007). Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. *Cell.Metab.* 5, 265–77.

Bonilla, M., and Cunningham, K. W. (2003). Mitogen-activated protein ki- nase stimulation of Ca^{2+} signaling is required for survival of endoplasmic reticulum stress in yeast. *Mol.Biol.Cell*. 14, 4296–4305.

Borghouts, C., Benguria, A., Wawryn, J., and Jazwinski, S.M. (2004). Rtg2 protein links metabolism and genome stability in yeast longevity. *Genetics*, 166(2), 765-77.

Bork, P., Sander, C., and Valencia, A. (1992). An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc.Natl.Acad.Sci. U S A*. 89(16), 7290-4.

Botstein, D. and Fink, G. R. (2011). Yeast: an experimental organism for 21st Century biology. *Genetics.*189, 695–704.

Boveris, A. and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* 134, 707-716.

Boy-Marcotte, E., Perrot, M., Bussereau, F., Boucherie H. and Jacquet, M. (1998). Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae* . *J.Bacteriol.*180, 1044–1052.

Brandt, U. (2006). Energy converting NADH:quinone oxidoreductase (complex I). *Annu.Rev.Biochem.* 75, 69–92.

Braun, K.A., Parua, P.K., Dombek, K.M., Miner, G.E. and Young, E.T. (2013). 14-3-3 (Bmh) proteins regulate combinatorial transcription following RNA polymerase II recruitment by binding at Adr1-dependent promoters in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 33, 712–724.

Breeden, L. L. (2003). Periodic transcription: a cycle within a cycle. *Curr. Biol.* 13, R31-38.

Breitenbach, M., Rinnerthaler, M., Hartl, J., Stincone, A., Vowinckel, J., Breitenbach-Koller, H. and Ralser, M. (2013). Mitochondria in ageing: there is metabolism beyond the ROS. *FEMS.Yeast. Res*. 14, 198–212.

Broach, J. R. (2012). Nutritional control of growth and development in yeast. *Genetics.*192, 73–105.

Budhwar, R., Lu, A. and Hirsch, J. P. (2010). Nutrient control of yeast PKA activity involves opposing effects on phosphorylation of the Bcy1 regulatory subunit. *Mol. Biol. Cell*. 21, 3749–58.

Budhwar, R., Fang, G. and Hirsch, J. P. (2011). Kelch repeat proteins control yeast PKA activity in response to nutrient availability. *Cell.Cycle*. 10, 767–770.

Budovskaya, Y.V., Stephan, J.S., Reggiori, F., Klionsky, D.J. and Herman, P.K. (2004). The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in *Saccharomyces cerevisiae* . *J.Biol.Chem.* 279, 20663–20671.

Budovskaya, Y.V., Stephan, J.S., Deminoff, S.J. and Herman, P.K. (2005). An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase. *Proc.Natl.Acad.Sci.USA*. 102, 13933–13938.

Bulik, D. A., Olczak, M., Lucero, H. A., Osmond, B. C., Robbins, P. W et al. (2003). Chitin synthesis in *Saccharomyces cerevisiae* in re- sponse to supplementation of growth medium with glucosamine and cell wall stress. *Eukaryot. Cell.* 2, 886–900.

Busti, S., Coccetti, P., Alberghina, L. and Vanoni, M. (2010). Glucose signaling-mediated coordination of cell growth and cell cycle in *Saccharomyces cerevisiae* . *Sensors (Basel).* 10, 6195–240.

Butow, R.A. and Avadhani, N.G. (2004). Mitochondrial signaling: the retrograde response. *Mol.Cell,* 14(1), 1-15.

Cameroni, E., Hulo, N., Roosen, J., Winderickx, J. and De Vir- gilio, C. (2004). The novel yeast PAS kinase Rim15 orchestrates G0-associated antioxidant defense mechanisms. *Cell.Cycle.* 3, 462–468.

Cardenas, M. E., Cutler, N. S., Lorenz, M. C., Di Como, C. J. and Heitman, J. (1999). The TOR signaling cascade regulates gene expression in response to nutrients. *Genes.Dev.* 13, 3271–3279.

Cassamayor, A., Torrance, P. D., Kobayashi, T., Thorner, J. and Alessi, D. R. (1999). Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr. Bi*ol. 9, 186-197.

Castermans, D., Somers, I., Kriel, J. et al. (2012) Glucose-induced posttranslational activation of protein phosphatases PP2A and PP1 in yeast. *Cell.Res*. 22, 1058–1077.

Chang, Y.Y., Juhasz, G., Goraksha-Hicks, P., Arsham, A.M., Mallin, D.R., Muller, L.K. and Neufeld. T.P. (2009) Nutrient-dependent regulation of autophagy through the target of rapamycin pathway. *Biochem.Soc.Trans*. 37, 232–236.

Chelstowska, A., Liu, Z., Jia, Y., Amberg, D. and Butow, R.A. (1999). Signalling between mitochondria and the nucleus regulates the expression of a new D-lactate dehydrogenase activity in yeast. *Yeast*, 15(13), 1377-91.

Chen, L., Jiao, Z.H., Zheng, L.S., Zhang,Y.Y., Xie, S.T., Wang, Z.X., and Wu, J.W. (2009). Structural insight into the autoinhibition mechanism of AMP-activated protein kinase. *Nature*. 459, 1146–1149.

Chen, E. J. and Kaiser, C. A. (2003). LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway. *J.Cell.Biol*. 161, 333–47.

Chen, Y., Feldman, D. E., Deng, C., Brown, J.A., DeGiacomo, A.F et al. (2005). Identification of mitogen-activated protein kinase signaling pathways that confer resistance to endoplasmic reticulum stress in *Saccharomyces cerevisiae* . *Mol.Cancer.Res*. 3, 669–677.

Cherkasova, V.A. and Hinnebusch, A.G. (2003). Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes.Dev*.17, 859–872.

Cherkasova, V.A, Qiu, H. and Hinnebusch, A.G. (2010). Snf1 promotes phosphorylation of the a subunit of eukaryotic translation initiation factor 2 by activating Gcn2 and inhibiting phosphatases Glc7 and Sit4. *Mol.Cell.Biol.* 30, 2862–2873.

Chevtzoff, C., Vallortigara, J., Averet, N., Rigoulet, M. and Devin, A. (2005). The yeast cAMP protein kinase Tpk3p is involved in the regulation of mitochondrial enzymatic content during growth. *Biochim.Biophys.Acta.* 1706, 117–125.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., et al. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes.Dev*. 15, 1078–1092.

Chumnanpuen, P., Zhang, J., Nookaew, I. and Nielsen, J. (2012). Integrated analysis of transcriptome and lipid profiling reveals the co-influences of inositol-choline and Snf1 in controlling lipid biosynthesis in yeast. *Mol.Genet. Genomics.* 287, 541–554.

Cid, V. J., Cenamor, R., Sanchez, M. and Nombela, C. (1998). A mutation in the Rho1-GAP-encoding gene BEM2 of Saccharomy- ces cerevisiae affects morphogenesis and cell wall functionality. *Microbiology*.144, 25–36.

Claret, S., Gatti, X., Doignon, X., Thoraval, D. and Crouzet, M. (2005). The Rgd1p Rho GTPase-activating protein and the Mid2p cell wall sensor are required at low pH for protein kinase C pathway activation and cell survival in *Saccharomyces cerevisiae. Eukaryot.Cell*. 4, 1375–1386.

Claypool, J.A., French, S.L., Johzuka, K., Eliason, K., Vu, L., Dodd, J.A., Beyer, A.L. and Nomura, M. (2004). Tor pathway regulates Rrn3p- dependent recruitment of yeast RNA polymerase I to the promoter but does not participate in alteration of the number of active genes. *Mol. Biol.Cell*. 15, 946–956.

Clement, S.T., Dixit, G. and Dohlman, H.G. (2013). Regulation of yeast G protein signaling by the kinases that activate the AMPK homolog Snf1. *Sci.Signal*. 6, ra78.

Cohen, T. J., Mallory, M. J., Strich, R. and Yao, T. P. (2008). Hos2p/Set3p deacetylase complex signals secretory stress through the Mpk1p cell integrity pathway. *Eukaryot. Cell*. 7, 1191–1199.

Collister, M., Didmon, M. P., MacIsaac, F., Stark, M. J., MacDonald, N. Q. and Keyes, S. M. (2002). YIL113w encodes a functional dual-specificity protein phosphatase which specifically interacts with and inactivates the Slt2/Mpk1p MAP kinase in S. cerevisiae. *FEBS.Lett*. 527, 186-192.

Conrad, M., Schothorst, J., Kankipati, H. N., Van Zeebroeck, G., Rubio-Texeira, M. and Thevelein, J. M. (2014). Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae* . *FEMS.Microbiol.Rev*. 38, 254–99.

Conway, M. K., Grunwald, D. and Heideman, W. (2012). Glucose, nitrogen, and phosphate repletion in *Saccharomyces cerevisiae* : common transcriptional responses to different nutrient signals. *G3 (Bethesda).* 2, 1003–17.

Cooper, T.G. (2002). Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. *FEMS.Microbiol.Rev.* 26, 223–238.

Costigan, C., Gehrung, S. and Snyder, M. (1992). A synthetic lethal screen identifies SLK1, a novel protein kinase homolog impli- cated in yeast cell morphogenesis and cell growth. *Mol.Cell.Biol*. 12, 1162–1178.

Crauwels, M., Donaton, M.C., Pernambuco, M.B., Winderickx, J., de Winde, J.H. and Thevelein. J.M. (1997a). The Sch9 protein kinase in the yeast *Saccharomyces cerevisiae* controls cAPK activity and is required for nitrogen activation of the fermentable growth-medium induced (FGM) pathway. *Microbiology*. 143(Pt 8), 2627–2637

Crauwels, M., Winderickx, J., de Winde, J.H. and Thevelein, J.M. (1997b).Identification of genes with nutrient-controlled expression by PCR-mapping in the yeast *Saccharomyces cerevisiae*. *Yeast*. 13, 973–984

Crespo, L., Powers, T., Fowler, B. and Hall, M. N. (2002). RTG1 , and RTG3 are regulated in response to intracellular levels of glutamine. *Proc. Natl. Acad. Sci. USA*. 99, 6784–6789.

Cross, F. R. (1997). "Marker swap" plasmids: convenient tools for budding yeast molecular genetics. *Yeast*.13, 647–53.

Cybulski, N. and Hall, M. N. (2009). TOR complex 2: a signaling pathway of its own. *Trends.Biochem.Sci*. 34, 620-627.

Cyrne, L., Martins, L., Fernandes, L. and Marinho, H.S. (2003). Regulation of antioxidant enzymes gene expression in the yeast *Saccharomyces cerevisiae* during stationary phase. *Free.Radical.Bio.Med*. 34, 385–393.

Danaie, P., Altmann, M., Hall, M.N., Trachsel, H. and Helliwell, S.B. (1999). CLN3 expression is sufficient to restore G1-to-S-phase progres- sion in *Saccharomyces cerevisiae* mutants defective in transla- tion initiation factor eIF4E. *Biochem.J*.340(Pt 1), 135–141.

Davidson, G. S., Joe, R. M., Roy, S., Meirelles, O., Allen, C. P., Wilson, M. R., Tapia, P. H., Manzanilla, E. E., Dodson, A. E., Chakraborty, S., et al. (2011). The proteomics of quiescent and nonquiescent cell differentiation in yeast stationary-phase cultures. *Mol.Biol.Cell*. 22, 988–98.

De Bettignies, G., Thoraval, D., Morel, C., Peypouquet, M. F. and Crouzet, M. (2001). Overactivation of the protein kinase C-signaling pathway suppresses the defects of cells lacking the Rho3/Rho4-GAP Rgd1p in *Saccharomyces cerevisiae* . *Genetics.* 159, 1435-1448.

De la Torre-Ruiz, M. A., Mozo-Villarías, A., Pujol, N. and Petkova, M. I. (2010). How budding yeast sense and transduce the oxidative stress signal and the impact in cell growth and morphogenesis. *Curr.Protein.Pept.Sci*. 11, 669–79.

De Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M. and Klis, F. M. (2000). Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2 mediated increase in *FKS2-lacZ* expression, glucanase resistance and thermotolerance. *Microbiology*.146, 2121-2132.

De Virgilio, C. and Loewith, R. (2006). The TOR signalling network from yeast to man. *Int.J.Biochem.Cell.Biol*. 38, 1476–81.

De Virgilio, C. and Loewith, R. (2006a). Cell growth control: little eukaryotes make big contributions. *Oncogene.* 25,6392–6415.

De Virgilio, C. and Loewith, R. (2006b). The TOR signalling network from yeast to man. *Int.J.Biochem.Cell.Biol.* 38, 1476–81.

De Virgilio, C. (2012). The essence of yeast quiescence. *FEMS.Microbiol.Rev*. 36, 306–39.

De Wever, V., Reiter, W., Ballarini, A., Ammerer, G. and Brocard, C. (2005). A dual role for PP1 in shaping the Msn2-dependent transcriptional response to glucose starvation. *EMBO.J* . 24, 4115–4123.

Dechant, R. and Peter, M. (2008). Nutrient signals driving cell growth. *Curr.Opin.Cell.Biol.* 20, 678–87.

Delley, P. A. and Hall, M. N. (1999). Cell wall stress depolarizes cell growth via hyperactivation of Rho1. *J.Cell.Biol*. 147, 163–174.

Denis, V. and Cyert, M. S. (2005). Molecular analysis reveals local- ization of *Saccharomyces cerevisiae* protein kinase C to sites of polarized growth and Pkc1p targeting to the nucleus and mitotic spindle. *Eukaryot.Cell*. 4, 36–45.

DeRisi, J.L., Iyer, V.R. and Brown, P.O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science.* 278, 680–686.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu.Rev.Cell.Dev.Biol*. 15, 435-67.

Desrivieres, S., F. T., Cooke, P., Parker,J. and Hall, M. N. (1998). MSS4, a phosphatidylinositol-4-phosphate 5-kinase re- quired for organization of the actin cytoskeleton in Saccharomy- ces cerevisiae. *J.Biol.Chem*. 273, 15787–15793.

Dever, T.E., Feng, L., Wek, R.C., Cigan, A.M., Donahue, T.F. and Hinnebusch, A.G. (1992). Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell*. 68, 585–596

Di Como, C.J. and Arndt, K.T. (1996). Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes.Dev*. 10, 1904–1916.

Dilova, I., Aronova, S., Chen, J. C. and Powers, T. (2004). Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of Rtg1 Rtg3p-dependent target genes. *J.Biol.Chem*. 279, 46527–46535.

Drose, S. and Brandt, U. (2008). The mechanism of mitochondrial superoxide production by the cytochrome bc1 complex. *J.Biol.Chem*. 283, 21649–21654

Dubouloz, F., Deloche, O., Wanke, V., Cameroni, E. and De Virgilio, C. (2005). The TOR and EGO protein complexes orchestrate microauto- phagy in yeast. *Mol.Cell*. 19, 15–26

Duina, A. A, Miller, M. E. and Keeney, J. B. (2014). Budding yeast for budding geneticists: a primer on the *Saccharomyces cerevisiae* model system. *Genetics*.197, 33–48.

Duvel, K., Santhanam, A., Garrett, S., Schneper, L and Broach, J.R. (2003). Multiple roles of Tap42 in mediating rapamycin-induced tran- scriptional changes in yeast. *Mol.Cell*. 11, 1467–1478.

Duvel, K. and Broach, J.R. (2004). The role of phosphatases in TOR signaling in yeast. *Curr.Top.Microbiol.Immunol*. 279, 19–38

Efeyan, A., Zoncu, R. and Sabatini, D.M. (2012). Amino acids and mTORC1: from lysosomes to disease. *Trends.Mol Med.* 18, 524–533.

Elion, E. A. (2000). Pheromone response, mating and cell biology. *Curr.Opin.Microbiol*. 3, 573–581.

Enns, L. C. and Ladiges, W. (2010). Protein kinase A signaling as an anti-aging target. *Ageing.Res.Rev*. 9, 269–72.

Errede, B., Cade, R. M., Yashar, B. M., Kamada, Y., Levin, D. E., Irie, K. and Matsumoto, K. (1995). Dynamics and organization of MAP kinase signal pathways. *Mol.Reprod.Dev*. 42, 477-485.

Estruch, F., and Carlson, M. (1993). Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae* . *Mol.Cell.Biol.* 13, 3872–3881.

Estruch, F. (2000). Stress-controlled transcription factors, stress- induced genes and stress tolerance in budding yeast. *FEMS.Microbiol.Rev.* 24, 469–486

Evangelista, M., Pruyne, D., Amberg, D. C., Boone, C. and Bretscher, A. (2002). Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nat.Cell.Biol.* 4, 32-41.

Fabrizio, P., Pozza, F., Pletcher, S. D., Gendron, C. M. and Longo, V. D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. *Science.* 292, 288–90.

Fabrizio, P., Liou, L., Moy, V. N., Diaspro, A., Selverstonevalentine, J., Gralla, E. B. and Longo, V. D. (2003). SOD2 Functions Downstream of Sch9 to Extend Longevity in Yeast. *Genetics*. 46, 35–46.

Fadri, M., Daquinag, A., Wang, S., Xue, T. and Kunz, J. (2005). The pleckstrin homology domain proteins Slm1 and Slm2 are required for actin cytoskeleton organization in yeast and bind phosphatidylinositol- 4,5-bisphosphate and TORC2. *Mol.Biol.Cell*. 16, 1883–1900

Feller, A., Georis, I., Tate, J.J., Cooper, T.G. and Dubois, E. (2013). Alterations in the Ure2 alphaCap domain elicit different GATA factor responses to rapamycin treatment and nitrogen limitation. *J.Biol.Chem*. 288, 1841–1855.

Fernandez-Garcia, P., Pelaez, R., Herrero, P. and Moreno, F. (2012). Phosphorylation of yeast hexokinase 2 regulates its nucleocytoplasmic shuttling. *J Biol.Chem.* 287, 42151–42164.

Ferreira Júnior, J. R., Spírek, M., Liu, Z. and Butow, R.A (2005). Interaction between Rtg2p and Mks1p in the regulation of the RTG pathway of *Saccharomyces cerevisiae* . *Gene.* 354, 2–8.

Ferrell, J. E. Jr. (1996). Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends.Biochem.Sci.* 21, 460-466.

Flandez, M., Cosano, I. C., Nombela, C., Martin, H. and Molina, M. (2004). Reciprocal regulation between Slt2 MAPK and isoforms of Msg5 dual-specificity protein phosphatase modulates the yeast cell integrity pathway. *J.Biol. Chem*. 279, 11027-11034.

Friant, S., Lombardi, R., Schmelzle, T., Hall, M. N. and Riezman, H. (2001). Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. *EMBO.J*. 20, 6783–6792.

Friis, R. M. N., Glaves, J. P., Huan, T., Li, L., Sykes, B. D. and Schultz, M. C. (2014). Rewiring AMPK and mitochondrial retrograde signaling for metabolic control of aging and histone acetylation in respiratory-defective cells. *Cell.Rep*. 7, 565–74.

Funakoshi, T., Matsuura, A., Noda, T. and Ohsumi, Y. (1997), Analyses of APG13 gene involved in autophagy in yeast, *Saccharomyces cerevisiae* . *Gene*. 192, 207–213.

Galdieri, L., Mehrotra, S., Yu, S. and Vancura, A. (2010). Transcriptional regulation in yeast during diauxic shift and stationary phase. *OMICS*. 14, 629–38.

Gallego, C., Garí, E., Colomina, N., Herrero, E. and Aldea, M. (1997). The Cln3 cyclin is down-regulated by translational repression and degradation during the G1 arrest caused by nitrogen deprivation in budding yeast. *EMBO.J*. 16, 7196–206.

Gancedo, J.M. (2008). The early steps of glucose signalling in yeast. *FEMS.Microbiol.Rev*.32, 673–704.

Gander, S., Bonenfant, D., Altermatt, P et al. (2008). Identification of the rapamycin-sensitive phosphorylation sites within the Ser/Thr-rich domain of the yeast Npr1 protein kinase. *Rapid.Commun.Mass.Spectrom.* 22, 3743– 3753.

Gangloff, S. P., Marguet, D. and Lauquin, G. J. (1990). Molecular cloning of the yeast mitochondrial aconitase gene (ACO1) and evidence of a synergistic regulation of expression by glucose plus glutamate. *Mol.Cell.Biol.* 10, 3551–61.

García, R., Bermejo, C., Grau, C., Perez, R., Rodríguez-Peña, J. M et al. (2004) The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. *J.Biol.Chem*. 279, 15183–15195.

García, R., Rodríguez-Peña, J. M., Bermejo, C., Nombela, C. and Arroyo, J. (2009). The high osmotic response and cell wall integ- rity pathways cooperate to regulate transcriptional responses to zymolyase-induced cell wall stress in *Saccharomyces cerevisiae* . *J.Biol.Chem*. 284, 10901–10911.

García-Rodríguez, L. J., Valle, R., Durán, A. and Roncero, C. (2005). Cell integrity signaling activation in response to hyperosmotic shock in yeast. *FEBS.Lett.* 579, 6186–6190

Garreau, H., Hasan, R.N., Renault, G., Estruch, F., Boy-Marcotte, E. and Jacquet, M. (2000). Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae* . *Microbiology*. 146(Pt 9), 2113–2120.

Garrett-Engele, P., Moilanen, B. and Cyert, M.S. (1995). Calcineurin, the Ca2+/calmodulin-dependent protein phosphatase, is essential in yeast mutantswith cell integrity defects and in mutants that lack a functional vacuolar H+-ATPase. *Mol.Cell.Biol*. 15, 4103–4114.

Gelperin, D., Weigle, J., Nelson, K., Roseboom, P., Irie, K., Matsumoto, K.. and Lemmon, S. (1995). 14-3-3 proteins: potential roles in vesicular transport and Ras signaling in *Saccharomyces cerevisiae* . *Proc.Natl.Acad. Sci.U S A*. 92(25), 11539-43.

Gentzsch, M. and W. Tanner. (1996). The PMT gene family: protein O-glycosylation in *Saccharomyces cerevisiae* is vital. *EMBO.J*. 15, 5752–5759.

Georis, I., Tate, J.J., Cooper, T.G. and Dubois, E. (2008). Tor pathway control of the nitrogen-responsive DAL5 gene bifurcates at the level of Gln3 and Gat1 regulation in *Saccharomyces cerevisiae* . *J.Biol.Chem*. 283, 8919–8929.

Georis, I., Feller, A., Tate, J. J., Cooper, T. G. and Dubois, E. (2009). Nitrogen catabolite repression-sensitive transcription as a readout of Tor pathway regulation: the genetic background, reporter gene and GATA factor assayed determine the outcomes. *Genetics* .181, 861–74.

Georis, I., Tate, J. J., Cooper, T. G. and Dubois, E. (2011). Nitrogen-responsive regulation of GATA protein family activators Gln3 and Gat1 occurs by two distinct pathways, one inhibited by rapamycin and the other by methionine sulfoximine. *J.Biol.Chem*. 286, 44897–912.

Geyskens, I., Kumara, S.H.M.C., Donaton, M.C.V., Bergsma, J.C.T., Thevelein, J.M. and Wera, S. (2001). Expression of mammalian PKB complements deletionof the yeastprotein kinase Sch9. *NatoSciSerA3*.16, 117–126

Giannattasio, S., Liu, Z., Thornton, J. and Butow, R. A (2005). Retrograde response to mitochondrial dysfunction is separable from TOR1/2 regulation of retrograde gene expression. *J.Biol.Chem*. 280, 42528–35.

Gietz, D., St Jean, A, Woods, R. A and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic.Acids.Res*. 20, 1425.

Gingras, A. C., Raught, B. and Sonenberg, N. (2001). Control of translation by the target of rapamycin proteins. *Genes. Dev*. 15, 807-826.

Godard, P., Urrestarazu, A., Vissers, S., Kontos, K., Bontempi, G., van Helden, J and Andre, B. (2007). Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. *Mol.Cell.Biol*. 27, 3065–3086.

Goldberg, A. A, Bourque, S. D., Kyryakov, P., Gregg, C., Boukh-Viner, T., Beach, A., Burstein, M. T., Machkalyan, G., Richard, V., Rampersad, S et al. (2009). Effect of calorie restriction on the metabolic history of chronologically aging yeast. *Exp.Gerontol*. 44, 555–71.

Goldstein, A. L. and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae* . *Yeast*. 15, 1541–53.

Gourlay, C. W. and Ayscough, K. R. (2004). Identification of an upstream regulatory pathway controlling actin-mediated apoptosis in yeast. *J.Cell. Sci.* 118, 2119-2132.

Gourlay, C. W. and Ayscough, K. R. (2005). A role for actin in aging and apoptosis. *Biochem.Soc.Trans.* 33, 1260-1264.

Gourlay, C. W., and Ayscough, K. R. (2006). Actin-induced hyperactivation of the Ras signaling pathway leads to apoptosis in *Saccharomyces cerevisiae* . *Mol.Cell. Biol*. 26, 6487-6501.

Gray, J. V., Ogas, J. P., Kamada, Y., Stone, M., Levin, D. E et al. (1997). A role for the Pkc1 MAP kinase pathway of Saccharomy- ces cerevisiae in bud emergence and identification of a putative upstream regulator. *EMBO.J*. 16, 4924–4937.

Gray, J. V, Petsko, G. A, Johnston, G. C., Ringe, D., Singer, R. A and Werner-Washburne, M. (2004). "Sleeping beauty": quiescence in *Saccharomyces cerevisiae* . *Microbiol.Mol.Biol.Rev*. 68, 187–206.

Greetham, D., Vickerstaff, J., Shenton, D., Perrone, G.G., Dawes, I.W. and Grant, C.M. (2010). Thioredoxins function as deglutathionylase enzymes in the yeast *Saccharomyces cerevisiae* . *BMC.Biochem.* 11, 3.

Griffioen, G., Mager, W.H. and Planta, R.J. (1994). Nutritional upshift response of ribosomal protein gene transcription in Saccharo- myces cerevisiae. *FEMS.Microbiol.Lett*. 123, 137–144.

Griffioen, G. and Thevelein, J. M. (2002). Molecular mechanisms controlling the localization of protein kinase A. *Curr. Genet*. 41, 199-207.

Griffioen, G., Branduardi, P., Ballarini, a, Anghileri, P., Norbeck, J., Baroni, M. D. and Ruis, H. (2001). Nucleocytoplasmic distribution of budding yeast protein kinase A regulatory subunit Bcy1 requires Zds1 and is regulated by Yak1-dependent phosphorylation of its targeting domain. *Mol.Cell.Biol*. 21, 511–23.

Guerrero-Castillo, S., Araiza-Olivera, D., Cabrera-Orefice, A., Espinasa-Jaramillo, J., Gutiérrez-Aguilar, M., Luévano-Martínez, L. a, Zepeda-Bastida, A. and Uribe-Carvajal, S. (2011). Physiological uncoupling of mitochondrial oxidative phosphorylation. Studies in different yeast species. *J.Bioenerg.Biomembr*. 43, 323–31.

Guo, S., Shen, X., Yan, G., Ma, D., Bai, X et al. (2009). A MAP kinase dependent feedback mechanism controls Rho1 GTPase and ac- tin distribution in yeast. *PLoS ONE*. 4, e6089.

Guo, W., Tamanoi, F. and Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat.Cell.Biol*. 3, 353-360.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A et al. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol.Cell*. 30, 214-226.

Görner, W., Durchslag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B et al. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes. Dev.* 12, 586–597.

Görner, W., Durchslag, E., Wolf, J., Brown, E.L., Ammerer, G., Ruis, H et al. (2002). Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *EMBO.J.* 21, 135–144.

Haesendonckx, S., Tudisca, V., Voordeckers, K., Moreno, S., Thevelein, J.M. and Portela, P. (2012). The activation loop of PKA catalytic isoforms is differentially phosphorylated by Pkh protein kinases in *Saccharomyces cerevisiae* . *Biochem. J*. 448, 307–320.

Hahn, J. S. and Thiele, D. J. (2002). Regulation of the *Saccharomyces cerevisiae* Slt2 kinase pathway by the stress-inducible Sdp1 dual specificity phosphatase. *J.Biol.Chem*. 277, 21278-21284.

Hahn, J.S. and Thiele, D.J. (2004). Activation of the *Saccharomyces cerevisiae* heat shock transcription factor under glucose starvation conditions by Snf1 protein kinase. *J.Biol.Chem*. 279, 5169–5176.

Hall, D.B., Wade, J.T. and Struhl, K. (2006). An HMG protein, Hmo1, associates with promoters of many ribosomal protein genes and throughout the rRNA gene locus in *Saccharomyces cerevisiae* . *Mol.Cell.Biol*. 26, 3672–3679.

Hardwick, J.S., Kuruvilla, F.G., Tong, J.K., Shamji, A.F. and Schreiber, S.L.(1999). Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc.Natl.Acad Sci.USA*. 96, 14866–14870.

Harrington, L. A. and Andrews, B. J. (1996). Binding to the yeast Swi4,6-dependent cell cycle box, CACGAAA, is cell cycle regulated *in vivo*. *Nucleic.Acids.Res*. 24, 558-565.

Harrison, D.E., Strong, R. and Sharp, Z.D et al. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature*. 460, 392–395.

Hartwell, L. H. (1974). *Saccharomyces cerevisiae* Cell Cycle. *Microbiol.Mol.Biol.Rev*. 38, 164–198.

Hasan, R. N., Renault, G., Estruch, F., Boy-marcotte, E. and Jacquet, M. (2000). Hyperphosphorylation of Msn2p and Msn4p in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae* . *Microbiology*.146, 2113–2120.

Hasan, R., Leroy, C., Isnard, A.D., Labarre, J., Boy-Marcotte, E. and Toledano, M.B. (2002). The control of the yeast H_2O_2 response by the Msn2/4 transcription factors. *Mol.Microbiol.*45, 233-241.

Haselbeck, R.J. and McAlister-Henn, L. (1993). Function and expression of yeast mitochondrial NAD- and NADP-specific isocitrate dehydrogenases. *J.Biol Chem*, 268(16), 12116-22.

Hedbacker, K., Hong, S.P. and Carlson, M. (2004a). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. *Mol. Cell.Biol* . 24, 8255–8263.

Hedbacker, K., Townley, R. and Carlson, M. (2004b). Cyclic AMP-dependent protein kinase regulates the subcellular localization of Snf1-Sip1 protein kinase. *Mol.Cell. Biol.* 24, 1836–1843.

Hedbacker, K. and Carlson, M. (2006). Regulation of the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase. *Eukaryot. Cell* .5, 1950–6.

Hedbacker, K. and Carlson, M. (2008). SNF1/AMPK pathways in yeast. *Front.Biosci.* 13, 2408-20.

Heeren, G., Jarolim, S., Laun, P., Rinnerthaler, M., Stolze, K., Perrone, G. G., Kohlwein, S. D., Nohl, H., Dawes, I. W. and Breitenbach, M. (2004). The role of respiration, reactive oxygen species and oxidative stress in mother cell-specific ageing of yeast strains defective in the RAS signalling pathway. *FEMS.Yeast. Res*. 5, 157–67.

Heinisch, È. J. and Lorberg, A. (1999). MicroReview The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae* . 32, 671–680.

Heinisch, J. J., Dupres, V., Wilk, S., Jendretzki, A. and Dufrêne, Y. F. (2010) .Single-molecule atomic force microscopy reveals cluster- ing of the yeast plasma-membrane sensor Wsc1. *PLoS ONE.* 5, e11104.

Heitman, J., Mowa, N. R. and Hall, M. N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science.* 253, 905-909.

Helliwell, S. B., Howald, I., Barbet, N. and Hall, M. N. (1998a). *TOR2* is part of two related signaling pathways coordinating cell growth in *Saccharomyces cerevisiae* . *Genetics.* 148, 99-112.

Helliwell, S. B., Schmidt, A., Ohya, Y. and Hall, M. N. (1998b). The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. *Curr.Biol.* 8, 1211-1214.

Herman, P. K. (2002). Stationary phase in yeast. 602–607.

Hermann, G.J. and Shaw, J.M. (1998). Mitochondrial dynamics in yeast. *Annu.Rev.Cell.Dev.Biol*. 1, 265–303.

Hernandez-Corbacho, M.J., Jenkins, R.W., Clarke, C.J., Hannun, Y.A., Obeid, L.M., Snider, A.J. and Siskind, L.J. (2011). Accumulation of long-chain glycosphingolipids during aging is prevented by caloric restriction. *PLoS ONE.* 6, e20411.

Herruer, M.H., Mager, W.H., Woudt, L.P., Nieuwint, R.T., Wassenaar, G.M., Groeneveld, P. and Planta, R.J. (1987) Transcriptional control of yeast ribosomal protein synthesis during carbon-source upshift. *Nucleic.Acids.Res*. 15, 10133–10144.

Hill, S. and Van Remmen, H. (2014). Mitochondrial stress signaling in longevity: A new role for mitochondrial function in aging. *Redox Biol*. 2, 936–944.

Hinnebusch, A.G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu.Rev. Microbiol.* 59, 407–450.

Hirata, Y., Andoh, T., Asahara, T. and Kikuchi, A. (2003). Yeast glycogen synthase kinase-3 activates Msn2p-dependent tran- scription of stress responsive genes. *Mol.Biol.Cell.* 14, 302–312.

Hlavatá, L. and Aguilaniu, H. (2003). The oncogenicRAS2val19 mutation locks respiration, independently of PKA, in a mode prone to generate ROS. *EMBO.J*. 22, 3337–3345.

Hlavatá, L., Nachin, L., Jezek, P. and Nyström, T. (2008). Elevated Ras/protein kinase A activity in *Saccharomyces cerevisiae* reduces proliferation rate and lifespan by two different reactive oxygen species-dependent routes. *Aging.Cell*. 7, 148–57.

Ho, H.L., Lee, H.Y., Liao, H.C. and Chen, M.Y. (2008). Involvement of *Saccharomyces cerevisiae* Avo3p/Tsc11p in maintaining TOR complex 2 integrity and coupling to downstream signaling. *Eukaryot.Cell*. 7, 1328–1343.

Ho, J. and Bretscher, A. (2001). Ras regulates the polarity of the yeast actin cytoskeleton through the stress response pathway. *Mol.Biol.Cell* 12, 1541-1555.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L. et al. (2002). Systematic identification of protein complexes in Saccha- romyces cerevisiae by mass spectrometry. *Nature*. 415, 180–183.

Hong, S.P., Leiper, F.C., Woods, A., Carling, D. and Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc. Natl.Acad.Sci.USA*. 100, 8839–8843.

Hong, S.P. and Carlson, M. (2007). Regulation of snf1 protein kinase in response to environmental stress. *J.Biol.Chem*. 282, 16838–45.

Honigberg, S.M. and Lee, R.H. (1998). Snf1 kinase connects nutritional pathways controlling meiosis in *Saccharomyces cerevisiae* . Mol.Cell.Biol. 18, 4548–4555.

Hosler, J.P., Ferguson-Miller, S. and Mills, D.A. (2006). Energy transduction: proton transfer through the respiratory complexes. *Annu.Rev.Biochem*. 75, 165–187

Hu, Y., Liu, E., Bai, X. and Zhang, A. (2010). The localization and concentration of the PDE2-encoded high-affinity cAMP phosphodiesterase is regulated by cAMP-dependent protein kinase A in the yeast *Saccharomyces cerevisiae* . *FEMS.Yeast.Res*. 10, 177–187.

Huang, C.Y. and Ferrell Jr, J. E. (1996). Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc.Natl. Acad.Sci.USA*. 93, 10078–10083.

Huang, X., Liu, J. and Dickson, R.C. (2012). Down-regulating sphingolipid synthesis increases yeast lifespan. *PLoS Genet.* 8, e1002493.

Huang, X., Withers, B.R. and Dickson, R.C. (2013). Sphingolipids and lifespan regulation. *Biochim.Biophys.Acta*. pii, S1388-1981 (13)00166-2. .

Hubberstey, A. V. and Mottilo, E. P. (2002). Cyclase-associated proteins: CAPacity for linking signal transduction and actin polymerization**.** *FASEB.J.* 16, 487-499.

Huber, A., Bodenmiller, B., Uotila, A., Stahl, M., Wanka, S., Gerrits, B., Aebersold, R. and Loewith, R. (2009). Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. *Genes.Dev*. 23, 1929– 1943.

Hughes Hallett, J. E., Luo, X. and Capaldi, A. P. (2014). State Transitions in the TORC1 Signaling Pathway and Information Processing in *Saccharomyces cerevisiae* . *Genetics*. 198, 773-786.

Huh, W. K., Falvo, J. V., Gerk, L. C., Carroll, A. S., Howson, R. W et al. (2003). Global analysis of protein localization in budding yeast. *Nature*. 425, 686–691.

Hutter, E., Renner, K., Pfister, G., Stöckl, P., Jansen-Dürr, P. and Gnaiger, E. (2004). Senescence-associated changes in respiration and oxidative phosphorylation in primary human fibroblasts. *Biochem. J.* 380, 919–28.

Hutzler, F., Gerstl, R., Lommel, M. and Strahl, S. (2008). Protein N-glycosylation determines functionality of the *Saccharomyces cerevisiae* cell wall integrity sensor Mid2p. *Mol.Microbiol*. 68, 1438–1449.

Igual, J. C., Johnson, A. L. and Johnston, L. H. (1996). Coordinated regulation of gene expression by the cell cycle transcription factor SWI4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO.J*. 15**,** 5001-5013.

Inagaki, M., Schmelzle, T., Yamaguchi, K.., Irie, K.., Hall, M. N. and Matsumoto, K. (1999). PDK1 homologs activate the Pkc1-mitogen-activated protein kinase pathway in yeast. *Mol.Cell Biol*. 19, 8344-8352.

Inoue, S. B., Qadota, H., Arisawa, M., Watanabe, T., and Ohya, Y. (1999). Prenylation of Rho1p is required for activation of yeast 1,3-beta-glucan synthase. *J.Biol.Chem*. 274, 38119-38124.

Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K. and Oshima, Y. (1993). *MKK1* and *MKK2*, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase kinase homologs, function in the pathway mediated by protein kinase C. *Mol.Cell Biol.* 13, 3076-3083.

Jacinto, E., Guo, B., Arndt, K. T., Schmelzle, T. and Hall, M. N. (2001). TIP41 Interacts with TAP42 and Negatively Regulates the TOR Signaling Pathway. *Mol.Cell*. 8, 1017–1026.

Jacoby, J. J., Nilius, S. M. and Heinisch, J. J. (1998). A screen for upstream components of the yeast protein kinase C signal transduction pathway identifies the product of the SLG1 gene. *Mol Gen Genet.* 258, 148–155.

Jamieson, D.J. (1992). *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. *J.Bacteriol,* 174, 6678–6681.

Jamieson, D.J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae* . *Yeas*t, 14, 1511–1527.

Jazwinski, S. M. (2005). Rtg2 protein: at the nexus of yeast longevity and aging. *FEMS.Yeast.Res*. 5, 1253–9.

Jazwinski, S. M. (2013). The retrograde response: when mitochondrial quality control is not enough. *Biochim.Biophys. Acta* 1833, 400–9.

Jesch, S. A., Gaspar, M. L., Stefan, C. J., Aregullin, M. A. and Henry, S. A. (2010). Interruption of inositol sphingolipid synthesis triggers Stt4p-dependent protein kinase C signaling. *J.Biol.Chem*. 285, 41947–41960.

Jia, Y., Rothermel, B., Thornton, J., and Butow, R.A. (1997). A basic helix-loop-helix-leucine zipper transcription complex in yeast functions in a signaling pathway from mitochondria to the nucleus. *Mol.Cell. Biol*, 17(3), 1110-7.

Jiang, R. and Carlson, M. (1996). Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. *Genes.Dev*. 10, 3105–3115.

Johnson, D. I. (1999). Cdc42: an essential rho-type GTPase controlling eukaryotic cell polarity. Microbiol. *Mol.Biol. Rev.* 63, 54-105.

Jorgensen, P., Nishikawa, J.L., Breitkreutz, B.J. and Tyers, M. (2002). Systematic identification of pathways that couple cell growth and division in yeast. *Science*. 297, 395–400

Jorgensen, P., Rupes, I., Sharom, J.R., Schneper, L., Broach, J.R. and Tyers M (2004) A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. *Genes Dev*. 18, 2491–2505

Journo, D., Mor, A. and Abeliovich, H. (2009). Aup1-mediated regulation of Rtg3 during mitophagy. *J.Biol.Chem*. 284, 35885–95.

Jung, U. S. and Levin, D. E. (1999). Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Mol. Microbiol*. 34, 1049-1057.

Jung, U.S., Sobering, A. K., Romeo, M.J. and Levin, D. E. (2002). Regulation of the yest Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase. *Mol.Microbiol*. 46, 781–789.

Kaeberlein, M. (2010). Lessons on longevity from budding yeast. *Nature*. 464, 513–519.

Kamada, Y., Jung, U. S, Piotrowski, J. and Levin, D. E. (1995). The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes.Dev*. 9, 1559-1571.

Kamada, Y., Qadota, H., Python, C. P., Anraku, Y., Ohya, Y. and Levin, D. E. (1996). Activation of yeast protein kinase C by Rho1 GTPase. *J.Biol.Chem*. 271, 9193-9196.

Kamada, Y., Fujioka, Y., Suzuki, N. N., Inagaki, F., Wullschleger, S., Loewith, R et al. (2005). Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. *Mol.Cell. Biol.* 25, 7239–7248.

Karunanithi, S. and Cullen, P.J. (2012). The filamentous growth MAPK pathway responds to glucose starvation through the Mig1/2 transcriptional repressors in *Saccharomyces cerevisiae* . *Genetics*. 192, 869–887.

Kawai, S., Urban, J., Piccolis, M., Panchaud, N., De Virgilio, C. and Loewith, R. (2011). Mitochondrial genomic dysfunction causes dephosphorylation of Sch9 in the yeast *Saccharomyces cerevisiae* . *Eukaryot.Cell*. 10, 1367–9.

Ketela, T., Green, R. and Bussey, H. (1999). *Saccharomyces cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the *PKC1-MPK1* cell integrity pathway. *J. Bacteriol.* 181, 3330-3340.

Kim, K.S., Rosenkrantz, M.S. and Guarente, L. (1986). *Saccharomyces cerevisiae* contains two functional citrate synthase genes. *Mol.Cell. Biol*, 6(6), 1936-42.

Kim, S., Ohkuni, K., Couplan, E. and Jazwinski, S.M. (2004). The histone acetyltransferase GCN5 modulates the retrograde response and genome stability determining yeast longevity. *Biogerontology*. 5(5), 305-16.

Kim, M.D., Hong, S.P. and Carlson, M. (2005). Role of Tos3, a Snf1 protein kinase kinase, during growth of *Saccharomyces cerevisiae* on nonfermentable carbon sources. *Eukaryot.Cell*. 4, 861–866.

Kim, J.H., Roy, A., Jouandot, D. and Cho, K. H. (2013). The glucose signaling network in yeast. *Biochim.Biophys.Acta* 1830, 5204–10.

Kirchman, P.A., Kim, S., Lai, C.Y. and Jazwinski, S.M. (1999). Interorganelle signaling is a determinant of longevity in *Saccharomyces cerevisiae* . *Genetics*. 152(1), 179-90.

Kirchrath, L., Lorberg, A., Schmitz, H. P., Gengenbacher, U. and Heinisch, J. J. (2000). Comparative genetic and physiological studies of the MAP kinase Mpk1p from Kluyveromyces lactis and *Saccharomyces cerevisiae* . *J. Mol.Biol.* 300, 743–758.

KiYoung, K., Truman, A. W. and Levin, D. E. (2008). Yeast mitogen-activated protein kinase activates transcription through Swi4/Swi6 by a noncatalytic mechanism that requires upstream signal. *Mol.Cell. Biol.* 28, 2579-2589.

Kogan, K.., Spear, E.D., Kaiser, C.A. and Fass, D. (2010). Structural conservation of components in the amino acid sensing branch of the TOR pathway in yeast and mammals. *J.Mol.Biol*. 402, 388–398.

Komeili, A., Wedaman, K.P., O'Shea, E.K.. and Powers, T. (2000). Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. *J.Cell.Biol*. 151(4), 863-78.

Koonin, E.V. (1994). Yeast protein controlling inter-organelle communication is related to bacterial phosphatases containing the Hsp 70-type ATP-binding domain. *Trends.Biochem.Sci*. 19(4), 156-7.

Kopecka, M., and Gabriel, M. (1992). The influence of Congo red on the cell wall and 1,3-b-D-glucan microfibril biogenesis in Sac- charomyces cerevisiae. *Arch.Microbiol*. 158, 115–126

Korshunov, S.S., Skulachev, V.P. and Starkov, A.A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS.Lett.* 416, 15-18.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A.W., Donaton, M.C., Van Dijck, P., Winderickx, J., de Winde, J.H. and Thevelein, J.M. (1999a). A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol.Microbiol*. 32, 1002–1012

Kraakman, L.S., Winderickx, J., Thevelein, J.M., De Winde, J.H. (1999b). Structure–function analysis of yeast hexokinase: structural requirements for triggering cAMP signalling and catabolite repression. *Biochem.J*. 343, 159–168.

Krause, S. A. and Gray, J. V. (2002). The protein kinase C pathway is required for viability in quiescence in *Saccharomyces cerevisiae* . *Curr.Biol.* 12, 588-593.

Kubota, H., Obata, T., Ota, K., Sasaki, T. and Ito, T. (2003). Rapamycin- induced translational derepression of GCN4 mRNA involves a novel mechanism for activation of the eIF2 alpha kinase GCN2. *J.Biol.Chem*. 278, 20457–20460.

Kuchin, S., Vyas, V.K. and Carlson, M. (2002). Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate FLO11, haploid invasive growth, and diploid pseudohyphal differentiation. *Mol.Cell.Biol*. 22, 3994–4000.

Kuranda, K.., Leberre, V., Sokol, S., Palamarczyk, G. and François, J. (2006). Investigating the caffeine effects in the yeast Saccharo- myces cerevisiae brings new insights into the connection be- tween TOR, PKC and Ras/cAMP signalling pathways. *Mol.Microbiol*. 61, 1147–1166.

Kuret, J., Johnson, E. and Zollerl, M. J. (1988). Mutagenesis of the Regulatory Subunit of Yeast CAMP-dependent Protein Kinase. *J.Biol.Chem*. 263, 9149–9154.

Kuruvilla, F.G., Shamji, A.F. and Schreiber, S.L. (2001). Carbon- and nitrogen-quality signaling to translation are mediated by distinct GATA-type transcription factors. *Proc.Natl.Acad.Sci.USA*. 98, 7283–7288.

Kushnareva, Y., Murphy, A.N. and Andreyev, A. (2002). Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. *Biochem. J.* 368, 545–553

Lagorce, A., Hauser, N. C., Labourdette, D., Rodriquez, C., Martin Yken, H et al. (2003). Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae* . *J.Biol.Chem.* 278, 20345–20357.

Laun, P., Pichova, A., Madeo, F., Fuchs, J., Ellinger, A., Kohlwein, S., Dawes, I., Frohlich, K.U. and Breitenbach, M. (2001). Aged mother cells of *Saccharomyces cerevisiae* show markers of oxidative stress and apoptosis. *Mol .Microbiol.* 39, 1166–1173.

Lavoie, H. and Whiteway, M. (2008). Increased respiration in the sch9Delta mutant is required for increasing chronological life span but not replicative life span. *Eukaryot.Cell*. 7, 1127–35.

Leadsham, J. E. and Gourlay, C. W. (2008). Cytoskeletal induced apoptosis in yeast. *Biochim.Biophys.Acta- Mol.Cell. Res.* 1783, 1406–1412.

Leadsham, J. E. and Gourlay, C. W. (2010). cAMP/PKA signaling balances respiratory activity with mitochondria dependent apoptosis via transcriptional regulation. *BMC.Cell.Biol*. 11, 92.

Leadsham, J. E., Sanders, G., Giannaki, S., Bastow, E. L., Hutton, R., Naeimi, W. R., Breitenbach, M. and Gourlay, C. W. (2013). Loss of cytochrome c oxidase promotes RAS-dependent ROS production from the ER resident NADPH oxidase, Yno1p, in yeast. *Cell.Metab*. 18, 279–86.

Lee, K. S. and Levin, D. E. (1992). Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. *Mol. Cell. Biol.* 12, 172-182.

Lee, K. S., Hines, L. K.. and Levin, D. E. (1993). A pair of functionally redundant yeast genes (PPZ1 and PPZ2) encoding type I-related protein phosphatases function within the PKC1-mediated pathway. *Mol.Cell.Biol.* 13, 5843-5853.

Lee, K. S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K.. and Levin, D. E. (1993). A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signaling by protein kinase C. *Mol.Cell. Biol.* 13, 3067-3075.

Leech, A., Nath, N., McCartney, R.R. and Schmidt, M.C. (2003). Isolation of mutations in the catalytic domain of the snf1 kinase that render its activity independent of the snf4 subunit. *Eukaryot.Cell*. 2, 265–273.

Lemieux, H., Vazquez, E. J., Fujioka, H. and Hoppel, C. L. (2010). Decrease in mitochondrial function in rat cardiac permeabilized fibers correlates with the aging phenotype. *J.Gerontol.A.Biol.Sci.Med.Sci*. 65, 1157–64.

Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M. and Thorner, J. (1990). A candidate protein kinase C gene, PKC1, is required for the *S. cerevisiae* cell cycle. *Cell.* 62, 213–224.

Levin, D. E. and Bartlett-Heubusch, E. (1992). Mutants in the S. cerevisiae PKC1 gene display a cell cycle-specific osmotic stability defect. *J.Cell.Biol*. 116, 1221–1229.

Levin, D. E., Bowers, B., Chen, C. Y., Kamada, Y. and Watanabe, M. (1994). Dissecting the protein kinase C/MAP kinase signalling pathway of *Saccharomyces cerevisiae* . *Cell.Mol.Biol.Res*. 40, 229–239.

Levin, D. E. (2005). Cell Wall Integrity Signaling in *Saccharomyces cerevisiae* Cell Wall Integrity Signaling in *Saccharomyces cerevisiae* . *Microbiol.Mol.Biol.Rev*. 69, 262–291.

Levin, D. E. (2011). Regulation of cell wall biogenesis in *Saccharomyces cerevisiae* : the cell wall integrity signaling pathway. Genetics 189, 1145–75.

Liao, X. and Butow, R.A. (1993). RTG1 and RTG2: two yeast genes required for a novel path of communication from mitochondria to the nucleus. *Cell*. 72(1), 61-71.

Liao, X.S., Small, W.C., Srere, P.A. and Butow, R.A. (1991). Intramitochondrial functions regulate nonmitochondrial citrate synthase (CIT2) expression in *Saccharomyces cerevisiae* . *Mol. Cell. Biol*. 11(1), 38-46.

Lillie, S. H. and Brown, S. S. (1994). Immunofluorescence local- ization of the unconventional myosin, Myo2p, and the puta- tive kinesin-related protein, Smy1p, to the same regions of polarized growth in *Saccharomyces cerevisiae J.Cell.Biol*. 125, 825–842.

Lin, S.S., Manchester, J.K. and Gordon, J.I. (2001). Enhanced gluconeogenesis and increased energy storage as hallmarks of aging in *Saccharomyces cerevisiae* . *J.Biol.Chem*. 276, 36000–36007.

Lin, S.S., Manchester, J.K. and Gordon, J.I. (2003). Sip2, an N-myristoylated beta subunit of Snf1 kinase, regulates aging in *Saccharomyces cerevisiae* by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing. *J. Biol.Chem.* 278, 13390–13397.

Liu, Z. and Butow, R.A. (1999). A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. *Mol.Cell.Biol*. 19(10), 6720-8.

Liu, Z., Sekito, T., Epstein, C.B. and Butow, R.A. (2001). RTG-dependent mitochondria to nucleus signaling is negatively regulated by the seven WD-repeat protein Lst8p. *EMBO.J*, 20(24), 7209-19.

Liu, Z., Sekito, T., Spírek, M., Thornton, J., and Butow, R.A. (2003). Retrograde signaling is regulated by the dynamic interaction between Rtg2p and Mks1p. *Mol.Cell*, 12(2), 401-11.

Liu, Z., Spírek, M., Thornton, J. and Butow, R.A. (2005). A novel degron-mediated degradation of the RTG pathway regulator, Mks1p, by SCFGrr1. *Mol.Biol.Cell*, 16(10), 4893-904.

Liu, Z. and Butow, R.A. (2006). Mitochondrial retrograde signaling. *Annu.Rev.Genet*, 40, 159-85.

Ljungdahl, P.O. and Daignan-Fornier, B. (2012). Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae* . *Genetics.* 190, 885–929

Lo, W.S., Duggan, L., Emre, N.C., Belotserkovskya, R., Lane, W.S., Shiekhattar, R. and Berger, S.L. (2001). Snf1–a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. *Science*. 293, 1142–1146.

Lodder, A. L., Lee, T. K. and Ballester, R. (1999). Characterization of the Wsc1 protein, a putative receptor in the stress response of *Saccharomyces cerevisiae* . *Genetics*. 152, 1487–1499.

Loewith, R. and Hall, M. N. (2011). Target of rapamycin (TOR) in nutrient signaling and growth control. *Genetics.* 189, 1177–201.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol.Cell.* 10, 457-468.

Loibl, M. and Strahl, S. (2013). Protein O-mannosylation: what we have learned from baker's yeast. *Biochim.Biophys. Acta*. 1833, 2438–46.

Lommel, M., Bagnat, M. and Strahl, S. (2004). Aberrant processing of the WSC family and Mid2p cell surface sensors results in death of *Saccharomyces cerevisiae* O-mannosylation mutants. *Mol.Cell.Biol.* 24, 46–57.

Longo, V. D. (1999). Mutations in signal transduction proteins increase stress resistance and longevity in yeast, nematodes, fruit flies, and mammalian neuronal cells. *Neurobiol.Aging*. 20, 479-486.

Longo, V. D. (2003). The Ras and Sch9 pathways regulate stress resistance and longevity. *Exp.Gerontology.* 38, 807-811.

Longo, V. D. (2004). Ras: the other pro-aging pathway. *Sci. Aging.Knowledge.Environ*. 36.

Longo, V. D. and Fabrizio, P. (2002). Regulation of longevity and stress resistance: a molecular strategy conserved from yeast to humans?. *Cell.Mol.Life.Sci.* 59, 903-908.

Longo, V. D., Liou, L. L., Valentine, J. S. and Gralla, E. B. (1999). Mitochondrial superoxide decreases yeast survival in stationary phase. *Arch.Biochem.Biophys*. 365, 131–142.

Longo, V.D., Shadel, G. S., Kaeberlein, M. and Kennedy, B. (2012). Replicative and chronological aging in *Saccharomyces cerevisiae* . *Cell.Metab*. 16, 18–31.

Lottersberger, F., Panza, A., Lucchini, G. and Longhese, M.P. (2007). Functional and physical interactions between yeast 14-3-3 proteins, acetyltransferases, and deacetylases in response to DNA replication perturbations. *Mol.Cell. Biol*. 27, 3266–3281.

Lum, P. Y., Armour, C. D., Stepaniants, S. B., Cavet, G., Wolf, M. K et al. (2004). Discovering modes of action for therapeutic com- pounds using a genome-wide screen of yeast heterozygotes. *Cell*. 116, 121–137.

Ma, P., Wera, S., Van Dijck, P. and Thevelein, J. M. (1999). The PDE1-encoded low-affinity phosphodiesterase in the yeast *Saccharomyces cerevisiae* has a specific function in controlling agonist-induced cAMP signaling. *Mol. Biol.Cell.* 10, 91–104.

MacGurn, J.A., Hsu, P.C., Smolka, M.B. and Emr, S.D. (2011). TORC1 regulates endocytosis via Npr1-mediated phosphoinhibition of a ubiquitin ligase adaptor. *Cell*. 147, 1104–1117.

Madden, K., Sheu, Y. J., Baetz, K., Andrews, B. and Snyder, M. (1997). SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science.* 275, 1781-1784.

Madia, F., Wei, M., Yuan, V., Hu, J., Gattazzo, C., Pham, P., Goodman, M. F. and Longo, V. D. (2009). Oncogene homologue Sch9 promotes age-dependent mutations by a superoxide and Rev1/Polzeta-dependent mechanism. *J. Cell.Biol.* 186, 509–23.

Magasanik, B. and Kaiser, C.A. (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene.* 290, 1–18.

Manning, B. D., Padmanabha, R. and Snyder, M. (1997). The Rho-GEF Rom2p localizes to sites of polarized cell growth and participates in cytoskeletal functions in *Saccharomyces cerevisiae* . *Mol .Biol. Cell.* 8, 1829-1844.

Mao, K. and Klionsky, D. J. (2011). MAPKs regulate mitophagy in *Saccharomyces cerevisiae* . *Autophagy.* 7, 1564–1565.

Mao, K., Wang, K., Zhao, M., Xu, T. and Klionsky, D. J. (2011). Two MAPK-signaling pathways are required for mitophagy in *Saccharomyces cerevisiae* . *J.Cell.Biol*. 193, 755–67.

Marion, R.M., Regev, A., Segal, E., Barash, Y., Koller, D., Friedman, N. and O'Shea, E.K. (2004). Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression. *Proc. Natl.Acad.Sci.USA.* 101, 14315–14322.

Martin, D.E., Soulard, A. and Hall, M.N. (2004). TOR regulates ribosomal protein gene expression via PKA and the Forkhead transcription factor FHL1. *Cell.* 119, 969–979.

Martin, H., Arroyo, J., Sanchez, M., Molina, M. and Nombela, C. (1993). Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C. *Mol.Gen. Genet.* 241, 177-184.

Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C. and Molina, M. (2000). Regulatory Mechanisms for Modulation of Signaling through the Cell Integrity Slt2-mediated Pathway in *Saccharomyces cerevisiae* . *J.Biol. Chem.* 275, 1511–1519.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler Bauer, A., Ruis, H. and Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO. J*. 15, 2227–2235.

Masuda, C. A., Ramirez, J., Pena, A. and Montero-Lomeli, M. (2000). Regulation of monovalention homeostasis and pH by the Ser-Thr protein phosphatase SIT4 in *Saccharomyces cerevisiae* . *J.Biol.Chem*. 275, 30957-30961.

Matsui, Y. and Toh,E. A. (1992). Yeast *RHO3* and *RHO4* ras superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes *CDC42* and *BEM1. Mol.Cell Biol*. 12, 5690-5699.

Mattison, C. P., Spencer, S. S., Kresge, K. A., Lee, J. and Ota, I. M. (1999). Differential regulation of the cell wall integrity mitogen-activated protein kinase pathway in budding yeast by the protein tyrosine phosphatases Ptp2 and Ptp3. *Mol.Cell. Biol*. 19, 7651-7660.

Mayer, F.V., Heath, R., Underwood, E et al. (2011) ADP regulates SNF1, the *Saccharomyces cerevisiae* homolog of AMP-activated protein kinase. *Cell. Metab.* 14, 707–714.

Mayordomo, I., Estruch, F. and Sanz, P. (2002). Convergence of the target of rapamycin and the Snf1 protein kinase pathways in the regulation of the subcellular localization of Msn2, a transcriptional activator of STRE (Stress Response Element)-regulated genes. *J.Biol.Chem*. 277, 35650–35656.

Mazzoni, C., Zarov, P., Rambourg, A. and Mann, C. (1993). The SLT2 (MPK1)MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae* . *J.Cell.Biol*. 123, 1821–1833.

McCammon, M.T., Epstein, C.B., Przybyla-Zawislak, B., McAlister-Henn, L. and Butow, R.A. (2003). Global transcription analysis of Krebs tricarboxylic acid cycle mutants reveals an alternating pattern of gene expression and effects on hypoxic and oxidative genes. *Mol.Biol. Cell*, 14(3), 958-72.

McCartney, R.R., Rubenstein, E.M. and Schmidt, M.C. (2005) Snf1 kinase complexes with different beta subunits display stress-dependent preferences for the three Snf1-activating kinases. *Curr.Genet*. 47. 335–344.

Measday, V., Moore, L., Retnakaran, R., Lee, J., Donoviel, M., Neiman, A.M. and Andrews, B. (1997). A family of cyclin-like proteins that interact with the Pho85 cyclin-dependent kinase. *Mol.Cell. Biol.* 17, 1212–1223.

Medvedik, O., Lamming, D.W., Kim, K.D. and Sinclair, D.A. (2007). MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in *Saccharomyces cerevisiae* . *PLoS.Biol.* 5(10).

Merhi, A. and Andre, B. (2012). Internal amino acids promote Gap1 permease ubiquitylation via TORC1/Npr1/ 14-3-3-dependent control of the Bul arrestin-like adaptors. *Mol.Cell.Biol*. 32, 4510–4522.

Mesquita, A., Weinberger, M., Silva, A., Sampaio-Marques, B., Almeida, B., Leão, C., Costa, V., Rodrigues, F., Burhans, W. C. and Ludovico, P. (2010). Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H2O2 and superoxide dismutase activity. *Proc.Natl.Acad.Sci.USA*. 107, 15123–8.

Miceli, M. V, Jiang, J. C., Tiwari, A., Rodriguez-Quiñones, J. F. and Jazwinski, S. M. (2012). Loss of mitochondrial membrane potential triggers the retrograde response extending yeast replicative lifespan. *Front.Genet*. 2, 102.

Miller-Fleming, L., Antas, P., Pais, T. F., Smalley, J. L., Giorgini, F. and Outeiro, T. F. (2014). Yeast DJ-1 superfamily members are required for diauxic-shift reprogramming and cell survival in stationary phase. *Proc.Natl.Acad.Sci. USA*. 111, 7012–7.

Mirzaei, H., Suarez, J. A. and Longo, V. D. (2014). Protein and amino acid restriction, aging and disease: from yeast to humans. *Trends .Endocrinol. Metab*. 1–9.

Moir, R.D., Lee, J., Haeusler, R.A., Desai, N., Engelke, D.R. and Willis, I.M. (2006). Protein kinase A regulates RNA polymerase III tran- scription through the nuclear localization of Maf1. *Proc.Natl.Acad.Sci.USA*. 103, 15044–15049.

Momcilovic, M., Iram, S.H., Liu, Y. and Carlson, M. (2008). Roles of the glycogen-binding domain and Snf4 in glucose inhibition of SNF1 protein kinase. *J.Biol.Chem*. 283, 19521–19529

Müller, M. and Reichert, A. S. (2011). Mitophagy, mitochondrial dynamics and the general stress response in yeast. *Biochem.Soc.Trans.* 39, 1514–9.

Nakafuku, M., Obara, T., Kaibuchi, K., Miyajima, I., Miyajima, A., Itoh, H., Nakamura, S., Arai, K., Matsumoto, K. and Kaziro, Y. (1988). Isolation of a second yeast *Saccharomyces cerevisiae* gene (GPA2) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. *Proc.Natl.Acad.Sci.USA*. 85, 1374–1378.

Nanduri, J., Mitra, S., Andrei, C., Liu, Y., Yu, Y., Hitomi, M. and Tartakoff, A. (1999). An unexpected link between the secretory path and the organization of the nucleus. *J.Biol.Chem.* 274, 33785-33789.

Natarajan, K., Meyer, M.R., Jackson, B.M., Slade, D., Roberts, C., Hinnebusch, A.G. and Marton, M.J. (2001). Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol.Cell.Biol*. 21, 4347–4368.

Nath, N., McCartney, R.R. and Schmidt, M.C. (2003). Yeast Pak1 kinase associates with and activates Snf1. *Mol.Cell. Biol*. 23, 3909–3917.

Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994). The ancient regulatory-protein family of WD-repeat proteins. *Nature.* 371(6495), 297-300.

Nierras, C. R. and Warner, J. R. (1999). Protein kinase C enables the regulatory circuit that connects membrane synthesis to ribosome synthesis in *Saccharomyces cerevisiae* . *J.Biol Chem.* 274, 13235-13241.

Nonaka, H., Tanaka, K.., Hirano, H., Fujiwara, T., Kohno, H. et al. (1995). A downstream target of RHO1 small GTP-binding pro- tein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevi- siae. *EMBO.J*. 14, 5931–5938.

Nunnari, J., Marshall, W.F., Straight, A., Murray, A., Sedat, J.W. and Walter, P. (1997). Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol.Biol.Cell*. 8, 1233–1242.

O'Donnell, A.F., Apffel, A., Gardner, R.G. and Cyert, M.S. (2010). Alpha-arrestins Aly1 and Aly2 regulate intracellular trafficking in response to nutrient signaling. *Mol.Biol.Cell*. 21, 3552–3566.

Orlova, M., Kanter, E., Krakovich, D. and Kuchin, S. (2006). Nitrogen availability and TOR regulate the Snf1 protein kinase in *Saccharomyces cerevisiae* . *Eukaryot. Cell*. 5, 1831–7.

Orlova, M., Barrett, L. and Kuchin, S. (2008). Detection of endogenous Snf1 and its activation state: application to Saccharomyces and Candida species. *Yeast.* 25, 745–54.

Otsuga, D., Keegan, B.R., Brisch, E., Thatcher, J.W., Hermann, G.J., Bleazard, W. and Shaw, J.M. (1998). The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J.Cell.Biol.*143, 333–349.

Owada, S., Shimoda, Y., Tsuchihara, K. and Esumi, H. (2013). Critical role of H2O2 generated by NOX4 during cellular response under glucose deprivation. *PLoS One*. 8, e56628.

Ozaki, K., Tanaka, K., Imamura, H., Hihara, T., Kameyama, T., Nonaka, H., Hirano, H., Matsuura, Y. and Takai, Y. (1996). Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for Rho1p small GTP binding protein in *Saccharomyces cerevisiae* . *EMBO.J.* 15, 2196-2207.

Pan, X. and Heitman, J. (1999). Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in Saccharomyces cere- visiae. *Mol.Cell.Biol.* 19, 4874–4887.

Pan, Y., Shadel, G. S., Shadel, C. G. S. and Box, P. O. (2009). Extension of chronological life span by reduced TOR signaling requires down - regulation of Sch9p and involves increased mitochondrial OXPHOS complex density. *Aging* (*Albany*, *NY*). 1, 131-145.

Pan, Y. (2011). Mitochondria, reactive oxygen species, and chronological aging: a message from yeast. *Exp.Gerontol.* 46, 847–52.

Pan, Y., Schroeder, E. A, Ocampo, A., Barrientos, A. and Shadel, G. S. (2011). Regulation of yeast chronological life span by TORC1 via adaptive mitochondrial ROS signaling. *Cell.Metab*. 13, 668–78.

Paravicini, G. and Friedli, L. (1996). Protein-protein interactions in the yeast PKC1 pathway: Pkc1p interacts with a component of the MAP kinase cascade. *Mol.Gen.Genet*. 251, 682–691

Park, J. I., Collinson, E. J., Grant, C. M. and Dawes, I. W. (2005). Rom2p, the Rho1 GTP/GDP exchange factor of *Saccharomyces cerevisiae ,* can mediate stress responses via the Ras-cAMP pathway. *J.Biol.Chem.* 280, 2529-2535.

Parrella, E. and Longo, V. D. (2008). The chronological life span of *Saccharomyces cerevisiae* to study mitochondrial dysfunction and disease. *Methods*. 46, 256–62.

Parua, P.K., Ratnakumar, S., Braun, K.A., Dombek, K.M., Arms, E., Ryan, P.M. and Young, E.T. (2010). 14-3-3 (Bmh) proteins inhibit transcription activation by Adr1 through direct binding to its regulatory domain. *Mol.Cell.Biol.* 30, 5273– 5283.

Pascual-Ahuir, A. and Proft, M. (2007). The Sch9 kinase is a chromatin- associated transcriptional activator of osmostress-responsive genes. *EMBO.J* 26, 3098–3108.

Pedruzzi, I., Burckert, N., Egger, P. and De Virgilio, C. (2000). *Saccharomyces cerevisiae* Ras/cAMP pathway controls post- diauxic shift element-dependent transcription through the zinc finger protein Gis1. *EMBO. J.* 19, 2569–2579.

Pedruzzi, I., Dubouloz, F., Cameroni, E., Wanke, V., Roosen, J., Winderickx, J. and De Virgilio, C. (2003). TOR and PKA Signaling Pathways Converge on the Protein Kinase Rim15 to Control Entry into G0. *Mol.Cell.* 12, 1607–1613.

Peeters, T., Louwet, W., Gelade, R., Nauwelaers, D., Thevelein, J.M. and Versele, M. (2006). Kelch-repeat proteins interacting with the Galpha protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. *Proc.Natl.Acad.Sci.USA*. 103, 13034–13039.

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

Perez, P. and Rincón, S. A. (2010). Rho GTPases: regulation of cell polarity and growth in yeasts. *Biochem. J*. 426, 243–53.

Pérez-Sampietro, M., Casas, C. and Herrero, E. (2013). The AMPK family member Snf1 protects *Saccharomyces cerevisiae* cells upon glutathione oxidation. *PLoS. One.* 8, e58283.

Peterson, J., Zheng, Y., Bender, L., Myers, A., Cerione, R et al. (1994). Interactions between the bud emergence proteins Bem1p and Bem2p and rho-type GTPases in yeast. *J.Cell.Biol*. 127, 1395–1406.

Petkova, M. I., Pujol-Carrion, N., Arroyo, J., García-Cantalejo, J. and Angeles de la Torre-Ruiz, M. (2010a). Mtl1 is required to activate general stress response through Tor1 and Ras2 inhibition under conditions of glucose starvation and oxidative stress. *J.Biol.Chem*. 285, 19521–31.

Petkova, M. I., Pujol-Carrion, N. and de la Torre-Ruiz, M. A. (2010b). Signal flow between CWI/TOR and CWI/RAS in budding yeast under conditions of oxidative stress and glucose starvation. *Commun.Integr.Biol*. 3, 555–7.

Petkova, M. I., Pujol-Carrion, N. and de la Torre-Ruiz, M. A. (2012). Mtl1 O-mannosylation mediated by both Pmt1 and Pmt2 is important for cell survival under oxidative conditions and TOR blockade. *Fungal.Genet.Biol*. 49, 903–14.

Petralia, R. S., Mattson, M. P. and Yao, P. J. (2014). Aging and longevity in the simplest animals and the quest for immortality. *Ageing.Res.Rev*. 16C, 66–82.

Philip, B. and Levin, D. E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. *Mol.Cell Biol.* 21, 271-280.

Piao, H. L.,.Machado, I. M. and Payne, G. S. (2007). NPFXD-mediated endocytosis is required for polarity and function of a yeast cell wall stress sensor. *Mol.Biol.Cell*. 18, 57–65.

Powers, R. W., Kaeberlein, M., Caldwell, S. D., Kennedy, B. K. and Fields, S. (2006). Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes.Dev*. 20, 174–84.

Powers, T. (2007). TOR signaling and S6 kinase 1: Yeast catches up. *Cell.Metab*. 6, 1–2.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X et al. (2005). Global analysis of protein phosphorylation in yeast. Nature. 438, 679-684.

Pujol, N., Bonet, C., Vilella, F., Petkova, M. I., Mozo-Villarías, A. and de la Torre-Ruiz, M. A. (2009). Two proteins from *Saccharomyces cerevisiae* : Pfy1 and Pkc1, play a dual role in activating actin polymerization and in increasing cell viability in the adaptive response to oxidative stress. *FEMS.Yeast.Res*. 9, 1196–207.

Puria, R., Zurita-Martinez, S.A. and Cardenas, M.E. (2008). Nuclear translocation of Gln3 in response to nutrient signals requires Golgi-to-endosome trafficking in *Saccharomyces cerevisiae* . *Proc.Natl.Acad.Sci.USA*. 105, 7194–7199.

Qadota, H., Anraku, Y., Botstein, D. and Ohya, Y. (1994). Conditional lethality of a yeast strain expressing human RHOA in place of RHO1. *Proc.Natl. Acad. Sci. USA*. 91, 9317-9321.

Radonjic, M., Andrau, J.C., Lijnzaad, P., Kemmeren, P. et al. (2005). Genome-wide analyses reveal RNA polymerase II located upstream of genes poised for rapid response upon S. cerevisiae stationary phase exit. *Mol.Cell.* 18, 171–183.

Rai, R., Tate, J. J., David, R., Cooper, T. G. and Nelson, D. R. (2013). gln3 Mutations Dissociate Responses to Nitrogen Limitation (Nitrogen Catabolite Repression) and Rapamycin Inhibition of TorC1. *J.Biol.Chem*. 288, 2789–2804.

Rajavel, M., Philip, B., Buehrer, B. M., Errede, B., and Levin, D. E. (1999). Mid2 is a putative sensor for cell integrity signaling in *Saccharomyces cerevisiae* . *Mol.Cell. Biol.* 19, 3969-3976.

Ramachandran, V. and Herman, P. K. (2011). Antagonistic interactions between the cAMP-dependent protein kinase and Tor signaling pathways modulate cell growth in *Saccharomyces cerevisiae* . Genetics. 187, 441–54.

Rapaport, D., Brunner, M., Neupert, W. and Westermann, B. (1998). Fzo1p Is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in *Saccharomyces cerevisiae* . *J.Biol.Chem*. 273, 20150–20155.

Ratnakumar, S. and Young, E.T. (2010). Snf1 dependence of peroxisomal gene expression is mediated by Adr1. *J.Biol. Chem*. 285, 10703–10714.

Reinke, A., Anderson, S., McCaffery, J.M., Yates, J.3rd., Aronova, S., Chu, S., Fairclough, S., Iverson, C., Wedaman, K.P., and Powers, T. (2004). TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in *Saccharomyces cerevisiae* . *J Biol Chem*, 279(15), 14752-62.

Reinoso-Martín, C., Schüller, C., Schuetzer-Muehlbauer, M. and Kuchler, K. (2003). The yeast protein kinase C cell integrity path- way mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. *Eukaryot.Cell*. 2, 1200–1210.

Ricci, J.E., Gottlieb, R.A. and Green, D.R. (2003). Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J.Cell.Biol*. 160, 65–75.

Ridley, A. J. (1995). Rho-related proteins: actin cytoskeleton and cell cycle. *Curr.Opin.Genet.Dev*. 5, 24-30.

Ristow, M. and Zarse, K. (2010). How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). *Exp.Gerontol*. 45, 410–8.

Ristow, M. and Schmeisser, S. (2011). Extending life span by increasing oxidative stress. *Free.Radic.Biol.Med*. 51, 327–36.

Roberg, K.J., Bickel, S., Rowley, N. and Kaiser, C.A. (1997). Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by SEC13, LST4, LST7 and LST8. *Genetics*. 147(4), 1569-84.

Robertson, L.S., Causton, H.C., Young, R.A. and Fink, G.R. (2000). The yeast A kinases differentially regulate iron uptake and respiratory function. *Proc.Natl.Acad.Sci.USA* 97, 5984–5988.

Robertson, L.S. and Fink, G.R. (1998). The three yeast A kinases have specific signaling functions in pseudohyphal growth. *Proc.Natl.Acad.Sci.USA*. 95,13783–13787

Rodicio, R. and Heinisch, J. J. (2010). Together we are strong: cell wall integrity sensors in yeast. *Yeast*. 27, 531–540.

Rødkaer, S. V and Faergeman, N. J. (2014). Glucose- and nitrogen sensing and regulatory mechanisms in *Saccharomyces cerevisiae* . *FEMS.Yeast.Res*. 14, 683–96.

Rohde, J.R., Campbell, S., Zurita-Martinez, S.A., Cutler, N.S., Ashe, M. and Cardenas, M.E. (2004). TOR controls transcriptional and transla- tional programs via Sap-Sit4 protein phosphatase signaling effectors. *Mol.Cell.Biol*. 24, 8332–8341.

Rohde, J.R., Bastidas, R., Puria, R., Cardenas, M.E (2008). Nutritional control via Tor signaling in *Saccharomyces cerevisiae* . *Curr.Opin.Microbiol* .11, 153–160.

Rolland, F., De Winde, J.H., Lemaire, K., Boles, E., Thevelein, J.M. and Winderickx, J. (2000). Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracel- lular glucose detection and a separable hexose kinase-dependent sensing process. *Mol .Microbiol*. 38, 348–358.

Roosen, J., Engelen, K., Marchal, K., Mathys, J., Griffioen, G., Cameroni, E et al. (2005). PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient avail- ability. *Mol.Microbiol.* 55, 862–880.

Rosenkrantz, M., Kell, C.S., Pennell, E.A., Webster, M. and Devenish, L.J. (1994). Distinct upstream activation regions for glucose-repressed and derepressed expression of the yeast citrate synthase gene CIT1. *Curr.Genet*. 25(3), 185-95.

Roth, S., Kumme, J. and Schuller, H.J. (2004). Transcriptional activators Cat8 and Sip4 discriminate between sequence variants of the carbon source-responsive promoter element in the yeast *Saccharomyces cerevisiae* . *Curr.Genet*. 45, 121– 128.

Rothermel, B.A., Thornton, J.L. and Butow, R.A. (1997). Rtg3p, a basic helix-loop-helix/leucine zipper protein that functions in mitochondrial-induced changes in gene expression, contains independent activation domains. *J.Biol. Chem*. 272(32), 19801-7.

Roumanie, O., Weinachter, C., Larrieu, I., Crouzet, M. and Doignon, F. (2001). Functional characterization of the Bag7, Lrg1 and Rgd2 RhoGAP proteins from *Saccharomyces cerevisiae* . *FEBS.Lett*. 506, 149–156.

Ruiz, A., Xu, X. and Carlson, M. (2011). Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. *Proc.Natl.Aca. Sci.USA.* 108, 6349–6354.

Ruiz, A., Xu, X. and Carlson, M. (2013). Ptc1 protein phosphatase 2C contributes to glucose regulation of SNF1/AMP-activated protein kinase (AMPK) in *Saccharomyces cerevisiae* . *J.Biol.Chem.* 288, 31052–31058.

Saldanha, A.J., Brauer, M.J. and Botstein, D. (2004). Nutritional homeostasis in batch and steady-state culture of yeast. *Mol.Biol.*Cell. 15, 4089–4104.

Salminen, A., Ojala, J., Kaarniranta, K. and Kauppinen, A. (2012). Mitochondrial dysfunction and oxidative stress activate inflammasomes: impact on the aging process and age-related diseases. *Cell.Mol.Life.Sci*. 69, 2999–3013.

Sampaio-Marques, B., Felgueiras, C., Silva, A., Rodrigues, F. and Ludovico, P. (2011). Yeast chronological lifespan and proteotoxic stress: is autophagy good or bad? *Biochem.Soc.Trans* . 39, 1466–1470.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S. and Sabatini, D.M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell.* 141, 290–303.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar- Peled, L. and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*. 320, 1496– 1501

Santangelo, G. M. (2006). Glucose signaling in *Saccharomyces cerevisiae* . *Microbiol.Mol.Biol.Rev*. 70, 253–82.

Santhanam, A., Hartley, A., Duvel, K., Broach, J.R. and Garrett, S. (2004). PP2A phosphatase activity is required for stress and Tor kinase regulation of yeast stress response factor Msn2p. *Eukaryot.Cell*. 3,1261–1271.

Sanz, P. (2003). Snf1 protein kinase: a key player in the response to cellular stress in yeast. *Biochem.Soc.Trans*. 31, 178–81.

Scheckhuber, C.Q., Erjavec, N., Tinazli, A., Hamann, A., Nystro, A.M. and Osiewacz, H.D. (2007). Reducing mitochondrial fission results in increased life span and fitness of two fungal ageing models. *Nat.Cell.Biol.* 9, 99–105

Schieke, S.M. and Finkel, T. (2007). TOR and aging: less is more. *Cell.Metab.* 5, 233-235.

Schmelzle, T., Beck, T., Martin, D.E. and Hall, M.N. (2004). Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Mol.Cell.Biol*. 24, 338–351.

Schmelzle, T. and Hall, M. N. (2000). TOR, a central controller of cell growth. *Cell.* 103, 256-262.

Schmelzle, T., Beck, T., Martin, D. E. and Hall, M. N. (2004). Activation of the RAS / Cyclic AMP Pathway Suppresses a TOR Deficiency in Yeast Activation of the RAS / Cyclic AMP Pathway Suppresses a TOR Deficiency in Yeast. *Mol.Cell.Biol*. 24, 338–351.

Schmelzle, T., Helliwell, S. B.,and Hall, M. N. (2002). Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast. *Mol.Cell. Biol*. 22, 1329-1339.

Schmidt, A., Beck, T., Koller, A., Kunz, J. and Hall, M.N. (1998). The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. *EMBO. J.* 17, 6924–6931.

Schmidt, M.C. and McCartney, R.R. (2000). beta-subunits of Snf1 kinase are required for kinase function and substrate definition. *EMBO.J.* 19, 4936–4943.

Schmidt, A., Bickle, M., Beck, T. and Hall, M. N. (1997). The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell*. 88, 531-542.

Schmidt, A., Schmelzle, T., and Hall, M. (2002). The RHO1-GAPs SAC7, BEM2, and BAG7 control distinct RHO1 functions in *Saccharomyces cerevisiae* . *Mol.Microbiol*. 45, 1433-1441.

Schmitz, H.P., Huppert, S., Lorberg, A. and Heinisch, J. J. (2002). Rho5p downregulates the yeast cell integrity pathway. *J.Cell. Sci*. 115, 3139-3148.

Schneper, L., Düvel, K. and Broach, J. (2004). Sense and sensibility: nutritional response and signal integration in yeast. *Curr.Opin.Microbiol*. 7, 624–630.

Schon, E. A. and Dencher, N. A. (2009). Heavy breathing: energy conversion by mitochondrial respiratory supercomplexes. *Cell.Metab*. 9, 1–3.

Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M. and Ristow, M. (2007). Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell. Metab*. 6, 280–93.

Sekito, T., Liu, Z., Thornton, J. and Butow, R.A. (2002). RTG-dependent mitochondria-to-nucleus signaling is regulated by MKS1 and is linked to formation of yeast prion [URE3]. *Mol.Biol.Cell*, 13(3), 795-804.

Sekito, T., Thornton, J. and Butow, R.A. (2000). Mitochondria-to-nuclear signaling is regulated by the subcellular localization of the transcription factors Rtg1p and Rtg3p. *Mol.Biol. Cell*, 11(6), 2103-15.

Sekiya-Kawasaki, M., Abe, M., Saka, A., Watanabe, D., Kono, K., Minemura-Asakawa, M., Ishihara, S., Watanabe, T., and Ohya, Y. (2002). Dissection of upstream regulatory components of the Rho1p effector, 1,3-beta-glucan synthase, in *Saccharomyces cerevisiae* . *Genetics.* 162, 663-676.

Selman, C., Tullet, J.M. and Wieser, D et al. (2009). Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* .326, 140–144.

Serrano, R., Martín, H., Casamayor, A. and Ariño, J. (2006). Signaling alkaline pH stress in the yeast *Saccharomyces cerevisiae* through the Wsc1 cell surface sensor and the Slt2 MAPK pathway. *J.Biol.Chem*. 281, 39785–95.

Shamji, A.F., Kuruvilla, F.G. and Schreiber, S.L. (2000). Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins. *Curr.Biol.* 10, 1574–1581.

Shaw, J.M. and Nunnari, J. (2002) Mitochondrial dynamics and division in budding yeast. *Trends.Cell.Biol.* 12, 178–184.

Sherman, B. F., Sherman, M. F. and Enzymol, M. (2003). Getting Started with Yeast . 41, 3–41.

Shirra, M.K., McCartney, R.R., Zhang, C., Shokat, K.M., Schmidt, M.C. and Arndt, K.M. (2008). A chemical genomics study identifies Snf1 as a repressor of GCN4 translation. *J.Biol.Chem*. 283, 35889–35898.

Simon, V. R., Karmon, S. L. and Pon, L. A (1997). Mitochondrial inheritance: cell cycle and actin cable dependence of polarized mitochondrial movements in *Saccharomyces cerevisiae* . *Cell.Motil.Cytoskeleton*. 37, 199–210.

Sinclair, D.A. and Guarente, L. (1997). Extrachromosomal rDNA circles--a cause of aging in yeast. *Cell*. 91(7), 1033-42.

Singh, K.K., Rasmussen, A.K. and Rasmussen, L.J. (2004). Genome-wide analysis of signal transducers and regulators of mitochondrial dysfunction in *Saccharomyces cerevisiae* . *Ann.NY.Acad.Sci*. 1011, 284–298.

Smets, B., De Snijder, P., Engelen, K., Joossens, E., Ghillebert, R., Thevissen, K., Marchal, K. and Winderickx, J. (2008). Genome-wide expression analysis reveals TORC1-dependent and -independent functions of Sch9. *FEMS .Yeast.Res*. 8, 1276–1288.

Smets, B., Ghillebert, R., De Snijder, P., Binda, M., Swinnen, E., De Virgilio, C. and Winderickx, J. (2010). Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae* . *Curr.Genet*. 56, 1-32.

Smith, T.F., Gaitatzes, C., Saxena, K., and Neer, E.J. (1999).The WD repeat: a common architecture for diverse functions. *Trends .Biochem.Sci.* 24(5), 181-5.

Soriano-Carot, M., Bañó, M. C. and Igual, J. C. (2012). The yeast mitogen-activated protein kinase Slt2 is involved in the cellular response to genotoxic stress. *Cell.Div*. 7, 1.

Soulard, A., Cremonesi, A., Moes, S., Schütz, F., Jenö, P. and Hall, M. N. (2010). The rapamycin-sensitive phosphoproteome reveals that TOR controls protein kinase A toward some but not all substrates. *Mol.Biol.Cell* 21, 3475–86.

Springael, J.Y. and Andre, B. (1998) Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae* . *Mol.Biol.Cell*. 9, 1253–1263.

Srinivasan, V., Kriete, A., Sacan, A. and Jazwinski, S. M. (2010). Comparing the yeast retrograde response and NF-κB stress responses: implications for aging. *Aging.Cell*. 9, 933–41.

Stewart, M. S., Krause, S. A., McGhie, J. and Gray, J. V (2007). Mpt5p, a stress tolerance- and lifespan-promoting PUF protein in *Saccharomyces cerevisiae* , acts upstream of the cell wall integrity pathway. *Eukaryot.Cell* . 6, 262–70.

Straede, A. and Heinisch, J. J. (2007) Functional analyses of the extra- and intracellular domains of the yeast cell wall integrity sensors Mid2 and Wsc1. *FEBS.Lett*. 581, 4495–4500

Strahl-Bolsinger, S., Gentzsch, M. and Tanner, W. (1999). Protein O-mannosylation. *Biochim.Biophys.Acta*. 1426, 297–307.

Sutherland, C.M., Hawley, S.A., McCartney, R.R., Leech, A., Stark, M.J., Schmidt, M.C. and Hardie, D.G. (2003) Elm1p is one of three upstream kinases for the *Saccharomyces cerevisiae* SNF1 complex. *Curr.Biol*. 13, 1299–1305.

Sutton, A., Immanuel, D. and Arndt, K. T. (1991). The SIT4 protein phosphatase functions in late G1 for progression into S phase. *Mol.Cell Biol.* 11, 2133-2148.

Swiegers, J. H., Pretorius, I. S. and Bauer, F. F. (2006). Regulation of respiratory growth by Ras: the glyoxylate cycle mutant, cit2Delta, is suppressed by RAS2. *Curr.Genet*. 50, 161–71.

Swinnen, E., Wanke, V., Roosen, J., Smets, B., Dubouloz, F., Pedruzzi, I., Cameroni, E., De Virgilio, C. and Winderikx, J. (2006). Rim15 and the crossroads of nutrient signalling pathways in *Saccharomyces cerevisiae* . *Cell.Div*.1, 3.

Swinnen, E., Ghillebert, R., Wilms, T. and Winderickx, J. (2013). Molecular mechanisms linking the evolutionary conserved TORC1-Sch9 nutrient signalling branch to lifespan regulation in *Saccharomyces cerevisiae* . *FEMS. Yeast.Res*. 17-32

Swinnen, E., Wilms, T., Idkowiak-Baldys, J et al. (2014) The protein kinase Sch9 is a key regulator of sphingolipid metabolism in *Saccharomyces cerevisiae* . *Mol.Biol.Cell*. 25, 196–211.

Kanki, T., Wang, K.., Cao, Y., Baba, M. and Klionsky, D.J. (2009). Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Developmental.Cell*. 17, 98–109.

Takahara, T. and Maeda, T. (2013). Evolutionarily conserved regulation of TOR signalling. *J.Biochem*. 154, 1–10.

Tamaki, H. (2007) Glucose-stimulated cAMP-protein kinase A pathway in yeast *Saccharomyces cerevisiae* . *J.Biosci. Bioeng*. 104, 245–250

Tate, J. J. and T. G. Cooper. (2003). Tor1/2 regulation of retrograde gene expression in *Saccharomyces cerevisiae* derives indirectly as a consequence of alterations in ammonia metabolism. *J.Biol.Chem*. 278, 36924–36933.

Tate, J. J., Georis, I., Feller, A., Dubois, E. and Cooper, T. G. (2009). Rapamycin-induced Gln3 dephosphorylation is insufficient for nuclear localization: Sit4 and PP2A phosphatases are regulated and function differently. *J.Biol. Chem.* 284, 2522– 2534.

Tate, J. J. and Cooper, T. G. (2013). Five Conditions Commonly Used to Down-regulate Tor Complex 1 Generate Different Physiological Situations Exhibiting Distinct Requirements and Outcomes . *J.Biol.Chem.* 288, 27243-262.

Teixeira, V., Medeiros, T. C., Vilaça, R., Moradas-ferreira, P. and Costa, V. (2014). Reduced TORC1 signaling abolishes mitochondrial dysfunctions and shortened chronological lifespan of Isc1p-deficient cells. *Microbial.Cell*. 1, 21–36.

Terashima, H., Yabuki, N., Arisawa, M., Hamada, K.and Kitada, K.. (2000). Up-regulation of genes encoding glycosylphosphatidyli- nositol (GPI)-attached proteins in response to cell wall damage caused by disruption of FKS1 in *Saccharomyces cerevisiae* . *Mol.Gen.Genet*. 264, 64–74.

Thevelein, J.M., Cauwenberg, L., Colombo, S., De Winde, J.H., Donation, M., Dumortier, F., Kraakman, L., Lemaire, K., Ma, P., Nauwelaers, D., Rolland, F., Teunissen, A., Van Dijck, P., Versele, M., Wera, S.and Winderickx, J. (2000). Nutrient-induced signal transduction through the protein kinase A pathway and its role in the control of metabolism, stress resistance, and growth in yeast. *Enzyme.Microb.Technol*. 26, 819–825

Thevissen, K., Yen, W.L., Carmona-Gutierrez, D., Idkowiak-Baldys, J., Aerts, A.M., François, I.E., Madeo, F., Klionsky, D.J., Hannun, Y.A. and Cammue, B.P. (2010). Skn1 and Ipt1 negatively regulate autophagy in *Saccharomyces cerevisiae* . *FEMS.Microbiol. Lett*, 303(2), 163-8.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K. and Wigler, M. (1985). In yeast, RAS proteins are controlling elements of adenylate cyclase. *Cell.* 40, 27-36.

Toda, T., Cameron, S., Sass, P. and Wigler, M. (1988). SCH9, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. *Genes.Dev*. 2, 517–527.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D et al. (1987). Cloning and characterization of BCY1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae* . *Mol. Cell.Biol*. 7, 1371-1377.

Torres, J. (2002). Regulation of the Cell Integrity Pathway by Rapamycin-sensitive TOR Function in Budding Yeast. *J. Biol.Chem*. 277, 43495–43504.

Treitel, M.A., Kuchin, S. and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae* . *Mol.Cell.Biol.* 18, 6273–6280.

Truman, A. W., Millson, S. H., Nuttall, J. M., King, V., Mollapour, M et al. (2006). Expressed in the yeast *Saccharomyces cerevisiae* , human ERK5 is a client of the Hsp90 chaperone that comple- ments loss of the Slt2p (Mpk1p) cell integrity stress-activated protein kinase. *Eukaryot.Cell* 5,1914–1924.

Tu, J. and Carlson, M. (1995). REG1 binds to protein phosphatase type 1 and regulates glucose repression in *Saccharomyces cerevisiae* . *EMBO.J.* 14. 5939–5946.

Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H et al. (2007). Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae* . *Mol.Cell*. 26, 663–74.

Usaite, R., Jewett, M.C., Oliveira, A.P., Yates, J.R. III., Olsson. L. and Nielsen, J. (2009). Reconstruction of the yeast Snf1 kinase regulatory network reveals its role as a global energy regulator. *Mol.Syst.Biol*. 5, 319.

Valcourt, J. R., Lemons, J. M. S., Haley, E. M., Kojima, M., Demuren, O. O. and Coller, H. A. (2012). Staying alive Metabolic adaptations to quiescence. *Cell.Cycle*. 11, 1680–1696.

Valenzuela, L., Aranda, C. and Gonzalez, A. (2001). TOR modulates GCN4- dependent expression of genes turned on by nitrogen limitation. *J.Bacteriol*. 183, 2331–2334.

Van Drogen, F. and Peter, M. (2002). Spa2p functions as a scaffold-like protein to recruit the Mpk1p MAP kinase module to sites of polarized growth. *Curr.Biol.* 12, 1698-1703.

van Heusden, G.P. and Steensma, H.Y. (2001). 14-3-3 Proteins are essential for regulation of RTG3-dependent transcription in *Saccharomyces cerevisiae* . *Yeast*. 18(16), 1479-91.

van Heusden, G.P., Griffiths, D.J., Ford, J.C., Chin-A-Woeng, T.F., Schrader, P.A., Carr, A.M., and Steensma, H.Y. (1995). The 14-3-3 proteins encoded by the BMH1 and BMH2 genes are essential in the yeast *Saccharomyces cerevisiae* and can be replaced by a plant homologue. *Eur.J.Biochem*. 229(1), 45-53.

Van Nuland, A., Vandormael, P., Donaton, M., Alenquer, M., Lourenco, A. et al. (2006). Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. *Mol.Microbiol*. 59, 1485–1505

Vay, H. A., Philip, B. and Levin, D. E. (2004). Mutational analysis of the cytoplasmic domain of the Wsc1 cell wall stress sensor. *Mol.Microbiol*. 150, 3281–3288.

Vélot, C., Haviernik, P., and Lauquin, G.J. (1996). The *Saccharomyces cerevisiae* RTG2 gene is a regulator of aconitase expression under catabolite repression conditions. *Genetics*, 144(3), 893-903.

Verna, J., Lodder, A., Lee, K., Vagts, A. and Ballester, R. (1997). A family of genes required for the maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae* . *Proc.Natl.Acad.Sci.USA.* 94, 13804-13809.

Versele, M., de Winde, J.H. and Thevelein, J.M. (1999). A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activa- tion of the cAMP pathway through direct inhibition of Gpa2. *EMBO.J.* 18, 5577–5591.

Vidan, S. and Mitchell, A.P. (1997). Stimulation of yeast meiotic gene expression by the glucose-repressible protein kinase Rim15p. *Mol.Cell. Biol.* 17, 2688-2697.

Vilella, F., Herrero, E., Torres, J. and de la Torre-Ruiz, M. A. (2005). Pkc1 and the upstream elements of the cell integrity pathway in *Saccharomyces cerevisiae* , Rom2 and Mtl1, are required for cellular responses to oxidative stress. *J.Biol.Chem*. 280, 9149–59.

Vincent, O. and Carlson, M. (1999). Gal83 mediates the interaction of the Snf1 kinase complex with the transcription activator Sip4. *EMBO. J.* 18, 6672–6681.

Vincent, O., Townley, R., Kuchin, S. and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes.Dev.* 15, 1104–14.

Volejníková, A., Hlousková, J., Sigler, K. and Pichová, A. (2013). Vital mitochondrial functions show profound changes during yeast culture ageing. *FEMS.Yeast.Res*. 13, 7–15.

Voordeckers, K., Kimpe, M., Haesendonckx, S., Louwet, W., Versele, M. and Thevelein, J.M. (2011). Yeast 3-phosphoinositide-dependent protein kinase-1 (PDK1) orthologs Pkh1-3 differentially regulate phosphorylation of protein kinase A (PKA) and the protein kinase B (PKB)/ S6K ortholog Sch9. *J.Biol.Chem*. 286, 22017–22027

Wang, J., Jiang, J. C. and Jazwinski, S. M. (2010). Gene regulatory changes in yeast during life extension by nutrient limitation. *Exp.Gerontol*. 45, 621–31.

Wang, L., Renault, G., Garreau, H. and Jacquet, M. (2004). Stress induces depletion of Cdc25p and decreases the cAMP producing capability in *Saccharomyces cerevisiae* . *Microbiol*. 150, 3383-3391.

Wanke, V., Cameroni, E., Uotila, A., Piccolis, M., Urban, J., Loewith, R. and De Virgilio, C. (2008). Caffeine extends yeast lifespan by targeting TORC1. *Mol.Microbiol.* 69, 277–285.

Watanabe, D., Abe, M. and Ohya, Y. (2001). Yeast Lrg1p acts as a specialized RhoGAP regulating 1,3-β-Glucan synthesis. *Yeast*. 18, 943-951.

Watanabe, M., Chen, C.Y. and Levin, D. E. (1994). *Saccharomyces cerevisiae PKC1* encodes a protein kinase C (PKC) homolog with a substrate specificity similar to that of mammalian PKC. *J.Biol Chem*. 269, 16829-16836.

Wedaman, K.P., Reinke, A., Anderson, S., Yates, J. 3rd., McCaffery, J.M. and Powers, T. (2003). Tor kinases are in distinct membrane-associated protein complexes in *Saccharomyces cerevisiae* . *Mol.Bio. Cell*. 14(3), 1204-20.

Wei, M., Fabrizio, P., Hu, J., Ge, H., Cheng, C., Li, L. and Longo, V. D. (2008). Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. *PLoS.Genet*. 4, e13.

Wei, M., Fabrizio, P., Madia, F., Hu, J., Ge, H., Li, L. M. and Longo, V. D. (2009). Tor1/Sch9-regulated carbon source substitution is as effective as calorie restriction in life span extension. *PLoS.Genet*. 5, e1000467.

Weinberger, M., Mesquita, A., Caroll, T., Marks, L., Yang, H., Zhang, Z., Ludovico, P. and Burhans, W. C. (2010). Growth signaling promotes chronological aging in budding yeast by inducing superoxide anions that inhibit quiescence. *Aging* (*Albany*. *NY*). 2, 709–26.

Wek, R.C., Cannon, J.F., Dever, T.E. and Hinnebusch, A.G. (1992). Truncated protein phosphatase GLC7 restores translational activation of GCN4 expression in yeast mutants defective for the eIF-2a kinase GCN2. *Mol.Cell. Biol.* 12, 5700–5710.

Werner-washburne, M., Braun, E. L., Crawford, M. E. and Peck, V. M. (1996). MicroReview Stationary phase in *Saccharomyces cerevisiae* . *Mol.Microbiol*. 19, 1159–1166.

Werner-Washburne, M., Brown, D. and Braun, E. (1991). Bcy1, the regulatory subunit of cAMP-dependent protein kinase in yeast, is differentially modified in response to the physiological status of the cell. *J.Biol.Chem*. 266, 19704–9.

Werner-Washburne, M., Braun, E., Johnston, G. C. and Singer, R. A. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae* . *Microbiol.Rev.* 57,383–401.

Westermann, B. and Neupert, W. (2000). Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae* . *Yeast*. 16, 1421–7.

Willems, A.R., Goh, T., Taylor, L., Chernushevich, I., Shevchenko, A., and Tyers, M. (1999). SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis. *Philos.Tran. R.Soc.Lond.B.Bio. Sci*, 354(1389), 1533-50.

Willis, I.M. and Moir, R.D. (2007) Integration of nutritional and stress signaling pathways by Maf1. *Trends.Biochem.Sci* 32, 51–53.

Wilson, W.A., Hawley, S.A. and Hardie, D.G. (1996). Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr.Biol*. 6, 1426–1434.

Woods, A., Munday, M.R., Scott, J., Yang, X., Carlson, M. and Carling, D. (1994). Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. *J.Biol.Chem*. 269, 19509–19515.

Wullschleger, S., Loewith, R., Oppliger, W. and Hall, M.N. (2005) Molecular organization of target of rapamycin complex 2. *J.Biol.Chem* .280, 30697–30704

Xue, Y., Batlle, M. and Hirsch, J.P. (1998). GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. *EMBO. J*. 17,1996–2007.

Yan, C., Luo, H., Lee, J. D., Abe, J. and Berk, B. C. (2001). Molecular cloning ofmouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains. *J.Biol.Chem*. 276, 10870–10878

Yan, G., Lai, Y. and Jiang, Y. (2012). The TOR complex 1 is a direct target of Rho1 GTPase. *Mol.Cell*. 45, 743-753.

Yan, Q. and Lennarz, W. J. (2002). Studies on the function of oligosaccharyl transferase subunits: a glycosylatable photoprobe binds to the luminal domain of Ost1p. *Proc.Natl Acad. Sci.USA*. 99, 15994-15999.

Yorimitsu, T. and Klionsky, D.J. (2005). Autophagy: molecular machinery for self-eating. *Cell.Death.Differ* 12(Suppl 2), 1542–1552.

Yorimitsu, T., He, C., Wang, K. and Klionsky, D.J. (2009). Tap42-associated protein phosphatase type 2A negatively regulates induction of autophagy. *Autophagy*. 5, 616–624.

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

Yun, C.W., Tamaki, H., Nakayama, R., Yamamoto, K. and Kumagai, H. (1997). G-protein coupled receptor from yeast *Saccharomyces cerevisiae*. *Biochem.Biophys.Res.Commun*. 240, 287–292.

Zaman, S., Lippman, S. I., Zhao, X. and Broach, J. R. (2008). How Saccharomyces responds to nutrients. *Annu.Rev. Genet*. 42, 27–81.

Zarzov, P., Mazzoni, C. and Mann, C. (1996). The SLT2 (MPK1) MAP kinase is activated during periods of polarized cell growth in yeast. *EMBO.J.* 15, 83-91.

Zhang, J., Olsson, L. and Nielsen, J. (2010). The beta-subunits of the Snf1 kinase in *Saccharomyces cerevisiae* , Gal83 and Sip2, but not Sip1, are redundant in glucose derepression and regulation of sterol biosynthesis. *Mol.Microbiol.* 77, 371–383.

Zhang, A. and Gao, W. (2012). Mechanisms of protein kinase Sch9 regulating Bcy1 in *Saccharomyces cerevisiae* . *FEMS.Microbiol.Lett*. 331, 10–6.

Zhang, A., Shen, Y., Gao, W. and Dong, J. (2012). Role of Sch9 in regulating Ras-cAMP signal pathway in *Saccharomyces cerevisiae* . *FEBS.Lett.* 585, 3026–3032.

Zhang, F., Pracheil, T., Thornton, J. and Liu, Z. (2013). Adenosine Triphosphate (ATP) Is a Candidate Signaling Molecule in the Mitochondria-to-Nucleus Retrograde Response Pathway. *Genes* (*Basel*). 4, 86–100.

Zhang, J., Vaga, S., Chumnanpuen, P., Kumar, R., Vemuri, G.N., Aebersold, R. and Nielsen, J. (2011). Mapping the interaction of Snf1 with TORC1 in *Saccharomyces cerevisiae* . *Mol.Syst.Biol*.7, 545.

Zhang, N. and Oliver, S.G. (2010). The transcription activity of Gis1 is negatively modulated by proteasome-mediated limited proteolysis. *J.Biol.Chem.* 285, 6465–6476.

Zhang, N., Wu, J. and Oliver, S.G. (2009). Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast. *Microbiology*. 155, 1690–1698.

Zhao, C., Jung, U. S., Garrett-Engele, P., Roe, T., Cyert, M. S., and Levin, D. E. (1998). Temperature-induced expression of yeast *FKS2* is under the dual control of protein kinase C and calcineurin. *Mol.Cell.Biol*. 18**,** 1013-1022.

Zhu, H., Klemic,J.F., Chang, S., Bertone, P., Casamayor, A., Klemic, K.G., Smith, D., Gerstein, M., Reed ,MA., Snyder, M. (2000). Analysis of yeast protein kinases using protein chips. *Nat.Genet*. 26, 283–289.

Zid, B.M., Rogers, A.N., Katewa, S.D., Vargas, M.A., Kolipinski, M.C., Lu, T.A., Benzer, S. and Kapahi, P. (2009). 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. *Cell.* 139, 149-160.

Zurita-martinez, S. A., Cardenas, M. E. and Carolina, N. (2005). Tor and Cyclic AMP-Protein Kinase A : Two Parallel Pathways Regulating Expression of Genes Required for Cell Growth Tor and Cyclic AMP-Protein Kinase A : Two Parallel Pathways Regulating Expression of Genes Required for Cell Growth. *Eukaryot.Cell* 4, 62–71.

Errata

1. Page 126: Figure 7 D : The histogram representation of the northern blot corresponding to *COX4* and as well as *COX2* mRNA, the last two strains have been interchanged. The correct order in the histogram is *mtl1 , mtl1+ pBcy1,* wt, wt +pBcy1, *tor1, mtl1tor1, ras2 and mtl1ras2*.

2. Page 143: The sentence "These results indicated that Mtl1 mitochondrial function is independent of the MAP kinase module of the CWI pathway" has to be placed at the end of the paragraph after (Fig 18 C).

3. Page 146: The conclusion for the result (Fig 21 A) is missing. The conclusion is "The result indicated that PKA inactivation does not change the CLS of *slt2* mutant and also PKA inactivation associated life span extension is independent to the function of Slt2 in CLS".

4. Page 167: Figure 35: The Y axis legend is missing. It is to be coined as "% Snf1 nuclear localization".

5. Page 171: Paragraph 3; lines 19-22 of the following sentence is incomplete "In this study we present evidence demonstrating that the transmembrane protein Mtl1 plays an essential role in life extension in quiescence and upon glucose depletion through the down regulation of TORC1 and PKA signaling pathways via *SCH9* and partly through **Slt2**".

6. Page 177: Paragraph 2; lines 21-24 of the following sentence is corrected as "We hypothesize that RTG activation **in logarithmic phase** must be a compensatory mechanism in *mtl1* mutants ~~(data not shown)~~ in order to restore the mitochondrial function through the induction of anaplerotic pathways that **restore** the cellular pool of glutamate"

6. Page 192: Figure legend number should be Fig 2 and not Fig 1.

7. Statistical data were performed and it has been included in the presentation. In addition quantification for immunoprecipitations/immunoblot analysis associated with Phosphorylated Bcy1 (T129) to Total Bcy1 has been shown in the presentation.