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Functional, genomic and molecular characterisation of Mtl1, an element of the CWI pathway of *Saccharomyces cerevisiae* with a role in the oxidative stress response

Mima Ivanova Petkova

Universitat de Lleida

Departament de Ciències Mèdiques Bàsiques

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Mima Ivanova Petkova

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Mª ANGELES DE LA TORRE RUIZ, Professora Agregada de Microbiologia i Parasitologia de la Facultat de Medicina de la Universitat de Lleida,

CERTIFICA:

Que **MIMA IVANOVA PETKOVA** ha realitzat en el Departament de Ciències Mèdiques Bàsiques de la Facultat de Medicina de Lleida, sota la direcció de la Dra. Mª Angeles de la Torre Ruiz, el treball que presenta per accedir al Grau de Doctora sota el títol:

"Functional, genomic and molecular characterisation of Mtl1, an element of the CWI pathway of *Saccharomyces cerevisiae* with a role in the oxidative stress response"

Per a que així consti, signo el present certificat:

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CONTENTS

SUMMARY

The eukaryotic microorganism *Saccharomyces cerevisiae* serves as a model system in which to study the signal transduction pathways involved in the oxidative stress response. Up to date, there is no evidence of any MAPK cascade which is specific to oxidative signals. Mtl1 is a member of the CWI pathway, which functions as a cell wall sensor for oxidative stress.

In the present study, we propose an essential role for Mtl1 in signalling oxidative stress and quiescence to the CWI pathway and to the general stress response through the inhibition of either Tor1 or Ras2 functions.

The Mtl1 function is required i) to induce ribosomal gene repression, ii) to induce the general stress response driven by the transcription factor Msn2/Msn4, and iii) to activate the CWI pathway in response to both oxidative stress and glucose starvation. The signalling from Mtl1 to Tor1 and/or Ras2 inhibition under these conditions occurs through Rom2 and Rho1, and probably through Pkc1, at least that signal which target is the ribosomal gene expression.

We demonstrate that the Mtl1 cytoplasmic domain physically interacts with the GEF Rom2. Our data indicate that Slt2 activity is really essential in terms of cell survival in quiescent conditions. However, in response to oxidative stress the contribution of Msn2/Msn4 function is more significant. In addition, we demonstrate that deletion of either *TOR1* or *RAS2* is sufficient to activate Slt2 upon the above mentioned stress conditions, independently on Mtl1. These data suggested that CWI, TOR and Ras-cAMP provide diverse cross talks in order to assure the cells to appropriately adapt metabolism and growth.

We demonstrate that Mtl1 is N-glycosylated and highly O-mannosylated mostly by Pmt2 protein O-mannosyltransferase. Mtl1 localises to the cell periphery, the bud, the septum, and to the tip of the shmoo. Mtl1 O-mannosylation confers its proper localisation. We provide evidence for the importance of protein O-mannosylation in oxidative stress response, through at least Mtl1. This is the first report suggesting a role of protein O-mannosylation in cell survival upon TOR blockage. Mtl1 O-mannosylation by Pmt2 is required to elicit cellular responses to TOR inhibition. Both Pmt2 and Mtl1 play positive roles in the chronological life span.

RESUMEN

Saccharomyces cerevisiae es un microorganismo eucariota que se utiliza como modelo de estudio de las vías de transducción de señal implicadas en la respuesta a estrés oxidativo. Hasta el momento no se ha descrito la existencia de una ruta específica de señales de oxidación. Mtl1 es un miembro de la ruta CWI (Cell Wall Integrity: vía de integridad celular) que funciona como un sensor transmembrana que detecta el estrés oxidativo.

En el presente estudio se demuestra que Mtl1 es esencial en el proceso de señalización del estrés oxidativo y quiescencia hacia la ruta CWI y hacia el factor general de respuesta a estrés Msn2/Msn4. En este último caso la señalización ocurre a través de Rom2 y Rho1 (y probablemente también a través de Pkc1) hacia la inhibición de las funciones Tor1 y Ras2.

La función Mtl1 se requiere para: i) la represión de la trascripción de genes ribosomales, ii) la inducción del factor transcripcional Msn2/Msn4 y iii) activar la ruta CWI en respuesta a estrés oxidativo y ayuno de glucosa.

En la segunda parte de la presente tesis se muestra que el dominio citoplasmático de Mtl1 interacciona físicamente con Rom2, la GEF (GTP Exchange Factor: factor intercambiador de GTP). Nuestros datos sugieren que la actividad Slt2 es importante para la supervivencia en condiciones de quiescencia. Sin embargo, Msn2/Msn4 contribuyen de manera más significativa a la supervivencia celular frente a condiciones oxidativas. Además, la ausencia de *TOR1* o *RAS2* es suficiente como para inducir la activación de Slt2 de manera independiente de Mtl1, en las condiciones de estrés mencionadas anteriormente. Todo ello sugiere que entre CWI, TOR y RAS-cAMP se establecen una serie de reacciones cruzadas encaminadas a asegurar que las células sean capaces de adaptar el crecimiento y su maquinaria metabólica de manera adecuada.

Mtl1 se N-glicosila y se O-manosila, principalmente por la manosil transferasa Pmt2. Mtl1 se localiza preferentemente y de manera homogénea en la periferia celular, yema, septo y en la punta del shmoo. La manosilación de Mtl1 es importante para la localización de Mtl1 de manera regular en la periferia y en la punta del shmoo. La O-manosilación catalizada por Pmt2 en general, y en particular la O-manosilación de Mtl1, poseen una gran relevancia en: a) la respuesta a estrés oxidativo; b) frente al bloqueo en la ruta TOR; y c) la extensión cronológica de la vida.

RESUM

Saccharomyces cerevisiae és un microorganisme eucariota que s'utilitza com a model per l'estudi de les vies de transducció de senyal implicades en la resposta a estrès oxidatiu. Fins l'actualitat, no s'ha descrit l'existència d'una via específica de senyals d'oxidació. Mtl1 es un membre de la via CWI (Cell Wall Integrity: via d'integritat cel.lular) que funciona com un sensor transmembrana que dectecta l'estrès oxidatiu.

En aquest estudi es demostra que Mtl1 es esencial en el procés de senyalització de l'estrès oxidatiu i quiescència cap a la via CWI i cap al factor general de resposta a estrès Msn2/Msn4. En aquest últim cas, la senyalització es duu a terme a través de Rom2 i Rho1 (i probablement de Pkc1) cap a l'inhibició de les funcions Tor1 i Ras2.

La funció de Mtl1 es necessaria per: i) la repressió de la transcripció de gens ribosomals, ii) l'inducció transcripcional Msn2/Msn4 i iii) l'activació la via CWI en resposta a estrès oxidatiu i dejú de glucosa.

En la segona part d'aquesta tesi, es demostra que el domini citoplasmàtic de Mtl1 interacciona físicament amb Rom2, la GEF (GTP Exchange Factor: factor intercanviador de GTP). Les nostres dades suggereixen que l'activitat de Slt2 es important per la supervivència en condicions de quiescència. No obstant, Msn2/Msn4 contribueixen de manera significativa a la supervivència cel.lular davant condicions oxidatives. A més a més, l'absència de *TOR1* o *RAS2* es suficient com per induir l'activació de Slt2 de manera independent de Mtl1, en les condiciones d'estrès anomenades prèviament. Tot això suggereix que entre CWI, TOR i RAS-cAMP s'estableixen un seguit de reaccions creuades encaminades a asegurar que les cèl.lules siguin capaces d'adaptar el seu creixement i la seva maquinaria metabòlica adecuadament.

Mtl1 es N-glicosila i es O-manosila, principalment per la manosil transferasa Pmt2. Mtl1 es localitza preferentment i de manera homogènia en la perifèria cel.lular, gemma, septe i extrem apical del shmoo. La manosilació de Mtl1 es important per la localització de Mtl1 de manera regular en la perifèria i en l'extrem apical del shmoo. La O-manosilació catalizada per Pmt2 en general, i en particular la O-manosilació de Mtl1, poseeixen una gran relevancia en: a) la resposta a estrès oxidatiu; b) davant el bloqueix de la via TOR; i c) en l'extensió cronològica de la vida.

INTRODUCTION

1. CELL WALL ARCHITECTURE AND DYNAMICS IN *SACCHAROMYCES CEREVISIAE*

The yeast cell wall is a highly dynamic structure with a key role in growth mode, the defense and the adaptation of the cells to environmental conditions (Klis *et al.,* 2006). *S. cerevisiae* spends a considerable amount of metabolic energy in cell wall construction. Depending on growth conditions, its mass in terms of dry weight may account for about 10-25% of the total cell mass.

It consists of an inner layer of glucan polymers and chitin (N-acetylglucosamine polymers), acting as a scaffold for a protective outer layer of mannoproteins that extend into the medium (Lipke and Ovalle, 1998). The major polysaccharide is a moderately branched 1,3-β-glucan (Fleet, 1991) (30-45% of wall mass). Due to the presence of side chains, 1,3-β-glucan molecules can only locally associate through hydrogen bonds, resulting in the formation of a continuous, three-dimensional network. This network is highly elastic and is considerably extended under normal osmotic conditions. The non-reducing ends of the 1,3-β-glucan molecules may function as attachment sites for covalent attachment of other polysaccharides. At the external face of the 1,3-β-glucan network, highly branched 1,6-β-glucan chains are found (5-10% of wall mass), which in turn may be connected to a GPI (glycosylphosphatidylinositol)- modified mannoprotein (Kollar *et al.,* 1997). At the inside of the 1,3-β-glucan network in the lateral walls, chitin chains may become attached (representing the smallest fraction 1,5-6% of wall mass), but only after cytokinesis has taken place (Shaw *et al.,* 1991). The lateral walls of the growing bud generally do not contain chitin, demonstrating that chitin is not essential for the mechanical strength of the lateral walls. Chitin may not become glycosidically linked to non-reducing ends of 1,3-β-glucan but also of 1,6-β-glucan chains, particularly in response to cell wall stress.

The outer cell wall layer is a lattice of highly glycosylated mannoproteins. Especially the high degree of O-linked and high-mannose type N-linked carbohydrate chains and their modification by negatively charged phosphate groups determine cell wall permeability (Klis *et al.,* 2006). Glycosylation determines specific features of individual proteins that are crucial for cell wall biogenesis and/or structure (see Postranslational modifications Section). In *pmt* mutants (deficient in O-linked mannosylation) the cell wall chitin content is significantly increased (Gentzsch and Tanner, 1996) as a result of triggered cell wall compensatory mechanism. In addition, the protein population is highly diverse and this diversity is believed to play an important role in adaptation of the cells to environmental conditions, in growth mode and in survival. Cell wall proteins allow the cells to flocculate, recognize mating partners, form a biofilm and grow pseudohyphally and invasively; they also help the cells to

retain iron and facilitate sterol uptake and are required for growth under anaerobic conditions (Klis *et al.,* 2006). Cell wall proteins may also strongly affect hydrophobicity of the cells (Reynolds and Fink, 2001). The majority of cell wall proteins are GPI-modified and they are indirectly linked to the 1,3-β-glucan network. GPI-CWPs are directed through the secretory pathway to the extracellular face of the plasma membrane by lipid anchors at their C-termini. GPI-proteins are liberated from the plasma membrane by cleavage of their anchors prior to attachment to the cell wall (Kollar *et al.,* 1997). A smaller group of proteins are directly linked to the 1,3-β-glucan network through an unidentified linkage that is sensitive to mild alkali. These proteins are called ASL (alkali-sensitive linkage)-CWPs and include the family of Pir-CWPs (Pir, proteins with internal repeats) (De Groot *et al.,* 2005). Whereas the GPI-CWPs are found in the outer layer of the wall, the Pir-CWPs seem to be uniformly distributed throughout the inner skeletal layer (Kapteyn *et al.,* 1999).

The main functions of the yeast cell wall are:

a) Stabilisation of the osmolarity of the cytoplasm of yeast and other fungi. Extension of the wall creates a counter-acting pressure by the wall, which stops water influx.
b) The combination of considerable mechanical strength high elasticity allows the wall to transmit and redistribute physical stresses, thus offering protection against mechanical damage.
c) Maintenance of cell shape, which is a precondition for morphogenesis.
d) The cell wall is a scaffold for proteins. These glycoproteins, and especially their N-linked carbohydrate chains, limit the permeability of the cell wall for macromolecules and allow the creation of microenvironment in the inner region of the wall, particularly in colonies. The high degree of glycosylation and the presence of negatively charged phosphate groups in their carbohydrate chains probably also contribute to water retention.

Cell wall construction is tightly controlled. Polysaccharide composition, structure and thickness of the cell wall vary considerably, depending on environmental conditions. Oxygen levels, for example, strongly affect the protein composition of the wall (Klis *et al.,* 2002). Cell wall formation is strictly coordinated with the cell cycle. Not surprisingly, the majority of the cell wall protein-encoding genes are cell-cycle regulated (Klis *et al.,* 2002). During periods of polarized cell growth, the wall is loosened by digestive enzymes (glucanases, chitinases) and expanded at a single point on the cell surface. Wall remodeling is carried out in a highly-regulated manner – the growth site is loosened enough to allow expansion but not so much to risk rupture. The wall of cells that grow exponentially in rich medium differ strongly from

post-diauxic shift cells entering the stationary phase. Post-diauxic cells possess less permeable walls and are more resistant to glucanases, accompanied by radical change in the protein profile (De Nobel *et al.,* 1990).

2. CELL WALL INTEGRITY (PKC1-MAPK) PATHWAY

The cell wall integrity is tightly controlled and precisely coordinated during both vegetative growth and mating pheromone-induced morphogenesis by the CWI pathway. (reviewed in Levin, 2005). The cell wall integrity pathway in budding yeast involves a MAP kinase cascade that participates in transducing extracellular signals for cell-wall, heat shock, hypoosmotic, nutritional, oxidative and pH stresses (Heinisch *et al.,* 1999; Vilella *et al.,* 2005; Levin, 2005; Serrano *et al.,* 2006). The PKC1-MAPK pathway is integrated by several cell-wall proteins that are putative cell membrane receptors of different stimuli – Wsc1-Wsc4, Mid2 and Mtl1. They transmit signals to Rom2, which activates the G protein Rho1. In turn, Rho1 activates, among others, the protein kinase C Pkc1 (reviewed in Levin, 2005). 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; Martin *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 1).

2.1. CWI PATHWAY ARCHITECTURE IN *SACCHAROMYCES CEREVISIAE*

2.1.1. Cell surface sensors: Wsc1-Wsc4, Mid2 and Mtl1

Environmental stresses are sensed at the plasma membrane by the cell surface sensors of WSC family (Wsc1 – Wsc4) (Gray *et al.,* 1997; Verna *et al.,* 1997; Lodder *et al.,* 1999), by Mid2 (Ketela *et al.,* 1999; Rajavel *et al.,* 1999; Philip and Levin, 2001), and by Mtl1 (Ketela *et al.,* 1999; Rajavel *et al.,* 1999; Villela *et al.,* 2005).

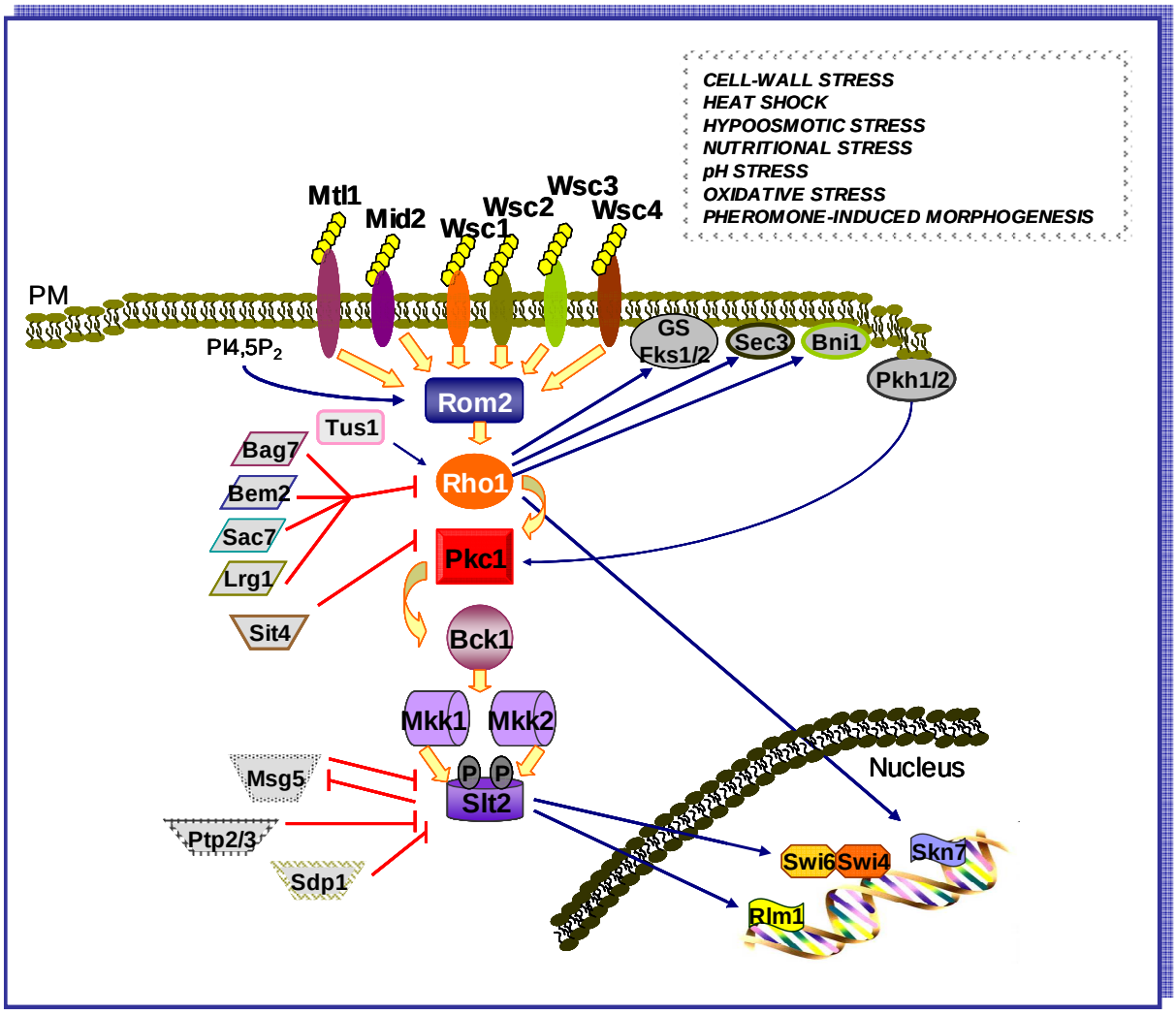

Figure 1. Cell wall integrity (PKC1-MAPK) pathway. Extracellular signals for cell-wall, heat shock, hypoosmotic, nutritional, pH and oxidative stresses are sensed at the plasma membrane by a number of cell surface sensors: Wsc1-4, Mid2 and Mtl1 that are highly glycosylated. Together with $PI4,5P_2$, which recruits the Rom2 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, the β1,3-glucan synthase (GS), the Bni1 formin protein, the exocyst component Sec3, and the Skn7 transcription factor. Additional regulatory inputs from Tus1 GEF, the inhibitory Rho1 GAPS, and the Pkh1-2 protein kinases are indicated. Pkc1 activates a MAP kinase module composed of: Bck1, Mkk1-2 and Slt2/Mpk1. 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/Mpk1 **(figure adapted from Levin, 2005).**

The Wsc family members, Mid2 and Mtl1 are type I transmembrane proteins with similar overall structures in that they possess small cytoplasmic domains highly charged, single transmembrane domains, and large extracellular protein domains rich in Ser/Thr residues, highly O-mannosylated (see Posttranslational modifications section). Mtl1 shows 50% aminoacid sequence identity with Mid2 (Rajavel *et al.,* 1999; Ketela *et al.,* 1999) (Fig 2).

Mid2 and Mtl1 do not contain an extracellular cysteine-rich motif that is characteristic of the Wsc proteins. Apart from the repetitive serine/threonine-rich region, there is no statistically significant amino acid residue sequence similarity between Mid2 and Wsc subfamilies. Wsc1 and Mid2 are shown to reside in the plasma membrane (Verna *et al.,* 1997; Lodder *et al.,* 1999; Ketela *et al.,* 1999; Rajavel *et al.,* 1999). The Mid2 and Wsc1-2 extracellular domains are crucial for sensing (Ketela *et al.,* 1999; Rajavel *et al.,* 1999; Lommel *et al.,* 2004 and see Posttranslational modifications section), however how they perceive cell wall structure and stresses is completely unknown. 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).

The WSC family in *S. cerevisiae* has four members, encoded by the *WSC1/HCS77/SLG1, WSC2, WSC3* and *WSC4* genes (Gray *et al.,* 1997; Verna *et al.,* 1997; Jacoby *et al.,* 1998). These 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). Residual signalling to the Slt2/Mpk1 is evident even in the triple *wsc1wsc2wsc3,* indicating that these signalling components serve a partially redundant function with another sensor (Verna *et al.,* 1997). Like most other components of the CWI, during vegetative growth Wsc1 localizes to sites of polarised cell growth (Delley and Hall, 1999). Bud tip localisation of Wsc1 is disrupted by the actin antagonist latrunculin, indicating that its positioning is dependent on actin cytoskeleton. Thus,

although Wsc1 controls actin polarisation through the action of Rho1, this sensor also responds to changes in actin cytoskeleton, similar to bidirectional signalling between integrin receptors and actin cytoskeleton in mammalian cells (Delley and Hall, 1999).

Mid2 (*M*ating *I*nduced *D*eath *2*) is required for the growth of yeast under various cell wall stresses and during pheromone-induced morphogenesis (Ono *et al.,* 1994; Ketela *et al.,* 1999; Rajavel *et al.,* 1999). 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). A *mid2* is not temperature sensitive for growth but is somewhat impared for Slt2/Mpk1 activation in response to heat shock, particularly in combination with *mtl1.* An interesting distinction between Mid2 and Wsc1 is the former's uniform distribution around the cell periphery (Huh *et al.,* 2003; Ketela *et al.,* 1999; Rajavel *et al.,* 1999). The diffuse distribution of Mid2 in the plasma membrane may reflect its role in signalling wall stress resulting from pheromone-induced morphogenesis, which may be initiated at any point on the surface of G1-arrested cells. Pheromone treatment causes Mid2 to accumulate at the site of emerging mating projection (shmoo). *MID2* was isolated as a multicopy activator of a Skn7-LexA-dependent transcriptional reporter, suggesting that activation of CWI signalling can stimulate transcriptional activation by Skn7 (Ketela *et al.,* 1999).

Despite their discrete functions Wsc1 and Mid2 serve partially overlapping roles in CWI signalling. 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).

Vilella *et al.* (2005) describe Mtl1 (*M*id2 *T*wo *L*ike *1*) for the first time 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. De Bettignies *et al.* (2001) identified *MTL1* as a suppressor of *rgd1* mutants, with Rgd1 being a GTPase-activating protein of Rho3 and Rho4. *MTL1* was also identified by Sekiya-Kawasaki *et al.* (2002) as a multicopy suppressor of Rho1. The mutant *mtl1* cells display no growth defect at temperatures up to 39℃ (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* single mutants are sensitive to caffeine

to some 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).

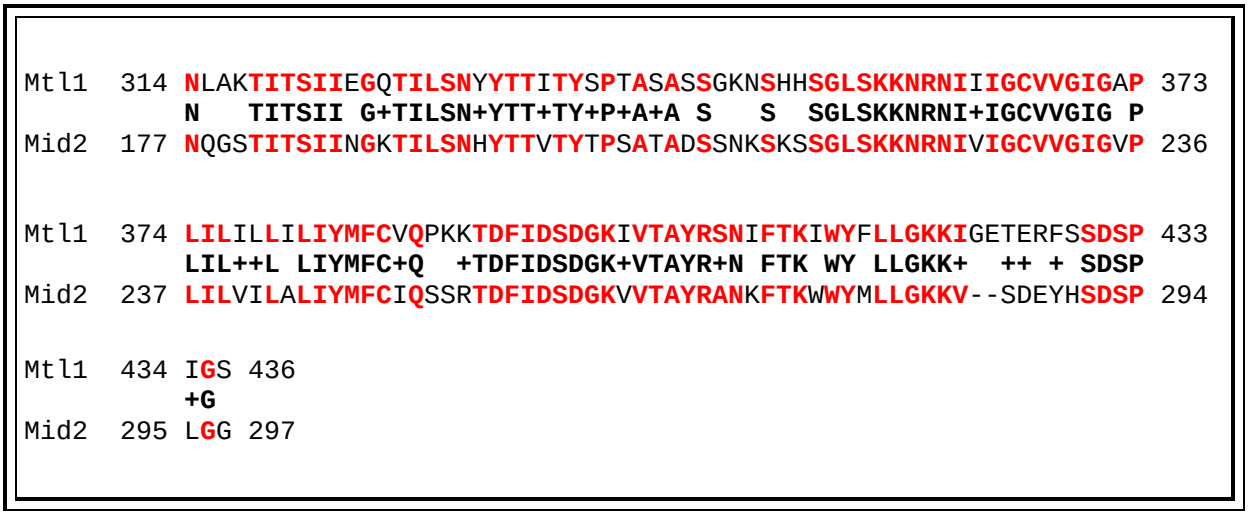

Figure 2. Sequence alignment of Mtl1 and Mid2 of *Saccharomyces cerevisiae*. Identical residues are in red bold. Mtl1 belongs to Mid2 supefamily.

2.1.2. The GEF Rom2, an essential regulator of Rho1

The Rho1 cycle is regulated both by GTPase-activating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs) acting in opposition. In *S. cerevisiae* four GAPs are found to act on Rho1 –Bem2, Sac7, Bag7, and Lrg1 (Roumanie *et al.,* 2001; Schmidt *et al.,* 2002; Watanabe *et al.,* 2001) and these GAPs appear to regulare Rho1 in a target-specific manner. 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). Like Rho1, Rom2 (and probably Rom1) resides at the sites of polarised cell growth (Manning *et al.,* 1997) and in response to environmental stresses, such as elevated temperature, redistributes to cell periphery where functions together with β-1,3-glucan syntase to remodel the cell wall (Audhya *et al.,* 2002; Delley and Hall, 1999). 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 ($PI4,5P_2$) 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).

Deletion mutants *rom2* indicate that 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 depolarisation (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).

2.1.3. Rho1: Master regulator of CWI signalling

Rho1 is a small GTP binding protein of the Rho subfamily of Ras-related proteins and is required for cell growth (Qadota *et al.,* 1994). 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. *Saccharomyces cerevisiae* possesses six Rho-type GTPases, named Rho1 to Rho5 and Cdc42. 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.,* 1998a; Matsui and Toh-e, 1992; Ridley *et al.,* 1995; Schmitz *et al.,* 2002). Rho proteins are C-terminally prenylated, 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).

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 involved in cell wall biogenesis, actin organisation, and polarised secretion (Fig 1). Rho1 has five direct downstream effectors. It binds 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.,* 2003). 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.

2.1.4. The protein kinase C: Pkc1

The *Saccharomyces cerevisiae* genome 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 (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 (Kamada *et al.,* 1996). Pkc1 possesses two homologous region (HR1) domains (HR1A and B) at its N-terminus, and the HR1A domain contributes to the interaction of the kinase with Rho1

(Schmitz *et al.,* 2002b). Pkc1 possesses a cysteine-rich domain, also known as C1 domain, which is defined by a pair of zinc finger motifs and appears to be a second site for interaction with Rho1 (Schmitz *et al.,* 2001). The C2 domain, also found in the conventional PKCs, is responsible for binding phospholipids in a Ca^{2+}-dependend manner (Bazzi *et al.,* 1990). Pkc1 possess the kinase domain itself and a pseudosubstrate site that regulate the protein kinase activity through an intramolecular interaction with the active site (Watanabe *et al.,* 1994). 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. A molecular dissection of Pkc1 suggested that each domain was responsible for localizing a pool of Pkc1 to various subcellular sites (Denis and Cyert, 2005). The existence of nuclear pool of Pkc1 may help to explain some of the functions of this protein that are not connected to cell wall integrity.

Vilella *et al.* (2005) demonstrate that Pkc1 which is normally localised in bud tips and cell necks translocates to the cell periphery upon diamide treatment. Pkc1 function is essential for cell viability under oxidative conditions on despite of the presence of sorbitol as osmotic stabiliser. 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. According to their model, the CWI pathway would drive repolarisation of actin through the MAP kinase cascade only after the wall damage had been repaired. Thus, different branches of the same pathway would mediate actin depolarisation and repolarisation in a sequential manner. Nierras *et al.* (1999) demonstrate that a null mutant in *PKC1* abrogates the repression of ribosomal protein and rRNA genes caused by the *sec1-1* mutation, thus implicating Pkc1, but not the MAP kinase cascade in the arrest-of-secretion response. This response is thought to couple nuclear activities, including transcription of genes encoding ribosomal components, to vesicular transport along the secretory pathway (Nanduri *et al.,* 1999; Mizuta *et al.,* 1994).

2.1.5. The CWI MAP kinase cascade

The hearth of the Pkc1 pathway is the Pkc1-activated MAPK cascade module consisting of a MAPKKK Bck1 (Lee and Levin, 1992), a pair of redundant MAPKKs, Mkk1 and Mkk2 (Irie *et al.,* 1993), and a MAPK Slt2/Mpk1 (Lee *et al.,* 1993; Martin *et al.,* 1993). It

is one of the five MAP kinase signalling pathways in yeast that regulate mating, response to high osmolarity, pseudohyphal/invasive growth, sporulation, and response to cell wall stress (reviewed in Gustin *et al.,* 1998).

Pkc1 phosphorylates Bck1 in vitro at several sites (Ser939, Thr1119, and Ser1134) in a hinge region between its putative regulatory domain and its catalytic domain. Bck1 is presumed to phosphorylate and activate the threonine/tyrosine kinases Mkk1/2 based on genetic epistasis studies, two-hybrid interaction, and its requirement for activation of Slt2/Mpk1. Mkk1/2 phosphorylate Slt2/Mpk1 on neighboring tyrosyl and threonyl residues in a T-X-Y motif that is diagnostic for MAP kinases. Such an arrangement serves both to amplify a small signal initiated at the cell surface and to convert a graded input to a highly sensitive, switch-like response (Ferrel, 1996). Loss of function of any protein kinase below Pkc1 (or both Mkk1 and Mkk2) results in cell lysis at elevated growth temperature. The growth defects of these mutants can be rescued by osmotic stabilisation (1M sorbitol), consistent with a primary defect in cell wall biogenesis (Errede *et al.,* 1995). Other phenotypes associated with mutants in the CWI MAP kinase cascade include sensitivity to mating pheromone and cell wall antagonists such as calcofluor white, Congo red, caffeine, and the wall lytic enzyme zymolyase (Errede *et al.,* 1995), and actin polarisation defects (Mazzoni *et al.,* 1993). The death of *mpk1* mutants upon nitrogen and carbon starvation is largely prevented by the presence of osmotic stabiliser. 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).

2.1.6. 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 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. Recent reports have identified Whi5 as an inhibitory subunit (Breeden, 2003). Swi6 is phosphorylated in Slt2/Mpk1-dependent manner in response to cell wall stress (Madden *et al.,* 1997). Because 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).

2.2. PROTEIN REGULATORS OF THE CWI PATHWAY

2.2.1. 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 1). 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.,* 2002). YPK-deficient cells are defective in cell wall integrity signalling (Schmelzle *et al.,* 2002). Mutants lacking both *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).

2.2.2. MAP kinase phosphatases

Inactivation of MAPK pathways occurs through both constitutive and induced (negative feedback) mechanisms. In addition to their roles in preventing inviability, however, these mechanisms are also well-suited and utilised for more finely tuned regulation that affects the quantitative profile of signal transduction activity spatially and temporally, as well as the qualitative nature of the output elicited. 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 (Fig 1).

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). Both genetic and biochemical evidence suggests that Ptp2 is more effective than Ptp3 against activated Slt2. Additionally, expression of *PTP2* is induced in response mild heat shock in an Slt2-dependent manner. One hypothesis is that Ptp2 and Ptp3 function to reestablish the resting state of Slt2/Mpk1 after stress-induced activation.

The dual-specifity protein phosphatase Sdp1 appears to target Slt2/Mpk1 specifically. It has been demonstrated by two-hybrid and coimmunoprecipitation analyses that Slt2 is the only one with which Sdp1 interacts (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). A decrease in the affinity between Msg5 and Slt2 was observed after activation of Slt2 by mild heat shock, suggesting that Slt2 phosphorylation of Msg5 interferes with association between the proteins. This would constitute a positive feedback loop for prolonged activation of Slt2/Mpk1, which has been observed in response to thermal upshift (Kamada *et al.,* 1995).

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 (Clotet *et al.,* 1999). 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.

2.3. ACTIVATION OF CWI SIGNALLING AND CROSS-TALK WITH OTHER SIGNALLING PATHWAYS

2.3.1. 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.

2.3.2. Heat stress

In *S. cerevisiae,* heat shock causes the activation of Slt2/Mpk1. Although null mutants in *PKC1, MKK1/2,* and *BCK1* are blocked for activation of Slt2 by all tested forms of wall stress, including heat shock (Harrison *et al.,* 2004), a *pkc1* mutant that 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. Deletion of *WSC1* results in termosensitive growth at 37℃ and impare d Slt2 activation (Verna *et al.,* 1997), indicating that Wsc1 plays a role as a sensor for heat shock. 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 signalling 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 termal 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) (Fig 3B).

2.3.3. Hypo-osmotic shock

Hypo-osmotic shock induces a rapid but transient activation of CWI signalling (Kamada *et al.* 1995). This response relies on components of the MAPK kinase module. Deletion of the components of this cascade module prevents Slt2/Mpk1 phosphorylation under hypo-osmotic conditions (Davenport *et al.,* 1995). 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). In addition to these pathways, the Cch/Mid1 Ca^{2+} chanel is activated by hypo-osmotic shock. That all three of these signalling pathways are involved in the cell wall response to hypo-osmotic shock is a testament to the chalenge posed by this particular stress to the survival of the yeast cell.

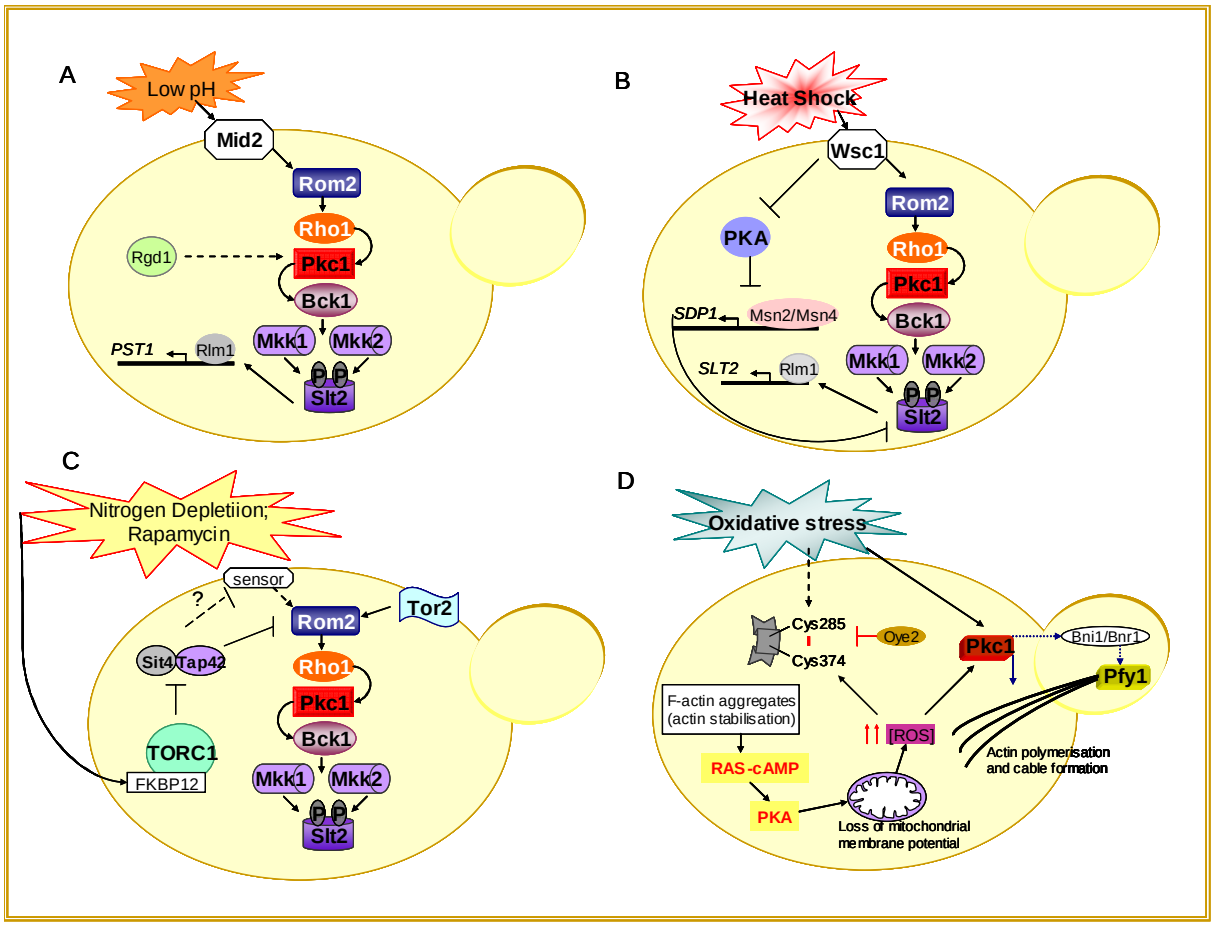

Figure 3. Activation of the CWI signalling. A) Extracellular low pH conditions activate the CWI via the cell surface sensor Mid2. The transmitted signal leads to stimulation of the MAP kinase module. A potential lateral cross talk with Rgd1 is indicated **(adapted from Fuchs and Mylonakis, 2009)**. B) Wsc1 functions as a cell surface sensor to activate the CWI in response to heat shock. Wsc1 is involved as well in the negative regulation of RAS-cAMP-PKA under the same conditions. Slt2/Mpk1 is negatively regulated by Msn2/Msn4 dependent Sdp1 **(adapted from Fuchs and Mylonakis, 2009).** C) Entry into stationary phase, nitrogen starvation or rapamycin treatment inhibits TORC1 signalling and elicits activation of the CWI pathway. Thus TORC1 negatively regulates the CWI pathway. Rapamycin-induced hyperactivation of the CWI is also manifested as depolymerisation of the actin cytoskeleton. The level at which TORC1 signals impinge upon the CWI is unclear **(figure adapted from Fuchs and Mylonakis, 2009; Torres *et al.*, 2002).** D) Oxidative stress activate the Pkc1-MAPK pathway, and hence the protein kinase Slt2/Mpk1. The activated Pkc1 in turn activates Pfy1 (probably through the action of both Bni1 and Bnr1 formins) to increase the actin polymerisation rate and actin cable formation. Further, there is indication that F-actin aggregates, caused by a decrease in actin dynamics, hyperactivate the RAS-cAMP-PKA leading to the loss of mitochondrial membrane potential. This process induces a substantial increase in the

accumulation of ROS. ROS oxidise actin molecules provoking the formation of disulphide bonds between Cys285 and Cys374. The Oye2 protein is in charge of reducing these disulphide bonds **(figure adapted from de la Torre-Ruiz *et al.,* 2010).**

2.3.4. Pheromone-induced morphogenesis

Haploid cells constitutively secrete mating pheromones. Detection of the presence of pheromone from cells of the opposite mating type triggers a developmental program through the mating pheromone response pathway. This pathway induces cell cycle arrest in G1 phase followed by the formation of a mating projection toward the source of pheromone. Projection formation requires polarisation of the actin cytoskeleton and the secretory pathway. Treatment with mating pheromone stimulates CWI signalling through Mid2 as a cell surface sensor. Mutants defective in CWI signalling undergo cell lysis during pheromone-induced morphogenesis (Errede *et al.,* 1995), reflecting the major reorganisation of the cell wall associated with projection formation. It is important to note that pheromone induced CWI signalling is an undirect event linked to the morphogenesis. Mid2, Rho1 and Pkc1 localise to the projection tips of cells treated with pheromone.

2.3.5. 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 conditions, *mid2* exibits 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) (Fig 3A).

2.3.6. Oxidative stress

Oxidative stress occurs in the natural environmental with expose to aerobic conditions and UV light. Oxide radicals are also the consequence of normal cellular metabolism. The major source of reactive oxygen species is the mitochondrial respiratory chain. ROS include a range of molecules that are either oxidants (such as H_2O_2, the superoxide anion O_2^-) or

reductants and are capable of affecting the redox homeostasis of the cell. In addition to the primary ROS, there are a number of toxic reactive species produced from their reaction with other compounds in cells. ROS can damage a wide range of molecules, including nucleic acids, proteins and lipids, and with this wide range of targets. The accumulation of oxidised proteins, DNA damage and the increased production of reactive oxygen species, concominant with a depletion of antioxidant defences, seem to be key factors in aging and cell death.

Different transcription factors regulate the adaptive response to oxidative stress conditions: the general stress response is mediated by the Msn2/Msn4 transcription factor, whereas specific responses are mediated by Yap1, Skn7 and Hsf1. Msn2/Msn4 nuclear localisation and activity are regulated by both TORC1 and PKA (detailed elsewhere in the introduction). For the induction of many antioxidant genes, Skn7 and Yap1 act cooperatively (Lee *et al.,* 1999; Brombacher *et al.,* 2006; He *et al.,* 2005). The contribution of Skn7 to the oxidative stress response does not occur through any of the cysteines of the protein but through Ser/Thr phosphorylation, therefore it is unlikely that Skn7 is a redox sensor. It has been proposed a model in which nuclearly localised Skn7 does not itself respond to oxidants but cooperates with Yap1 when it translocates to the nucleus. The association of Yap1 with Skn7 is a prerequisite for Skn7 phosphorylation and the activation of oxidative stress response genes (He *et al.,* 2009). Skn7 interacts also with Hsf1 and both cooperate to induce heat shock genes specifically in response to oxidative stress (Raitt *et al.,* 2000). Hsf1, like Msn2/Msn4, is negatively regulated by PKA via Yak1 kinase (Fig 7; see sections: TOR Signalling Network, RAS-cAMP Pathway).

In budding yeast, diamide and hydrogen peroxide exposure aid in the efforts to deduce the CWI pathway response to oxidative stress. 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 characterised as a cell surface sensor for oxidative stress (Vilella *et al.,* 2005). Exposure of *rom2* to oxidising agent results in diminished Slt2/Mpk1 phosphorylation. Pkc1 is also required but the MAP kinase module, downstream of Pkc1, seems to be dispensable for this mechanism. Pkc1 overexpression confers cells with more resistance to oxidising agents. 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).

The actin molecule is sensitive to oxidative stress (Dalle-Donne *et al.,* 2001). Upon oxidative conditions the actin molecule can be oxidised and a disulphide bond can be formed

between cysteins 285 and 374 (Dalle-Donne *et al.,* 2001; 2003). Actin oxidation accelerates cell death in yeast (Dalle-Donne *et al.,* 2001). Studies in eukaryotic model *S. cerevisiae* have allowed the identification of the oxidoreductase *OYE2* (*O*ld *Y*ellow *E*nzyme *2*) that is important to protect actin molecules from being oxidised in Cys284 and Cys373 (Haarer *et al.,* 2004). A deletion in the *OYE2* gene induces ROS accumulation and makes cells more sensitive to oxidation (Farah *et al.,* 2007; Odat *et al.,* 2007). Although Oye enzymes are placed in the signalling network that governs ROS, actin cytoskeleton and survival, it remains unknown at the molecular level which is the connection between any specific signal transduction pathway and Oye2 in response to the redox signal.

Vilella *et al.* (2005) describe a role for CWI pathway in connecting oxidative stress stimulus with the actin cytoskeleton. This study reveals that oxidative stress depolarises the actin cytoskeleton. None of the CWI elements is required to mediate this depolarisation, however Pkc1 is essential in order to restore the organisation of the actin cytoskeleton in oxidative conditions, concominantly with an increase in cell viability (Vilella *et al.,* 2005).

In a recent work (Pujol *et al.,* 2009) it has been demonstrated that actin polymerisation is a target of hydrogen peroxide. The authors develop an assay based on total protein extracts obtained from different strains of *S. cerevisiae*. These protein extracts are used as polymerisation seeds to study actin assembly. Actin filaments are detected by means of the technique of fluorescence recovery after photobleaching (FRAP). The rationale of this assay is that the association of small amounts of protein extracts with actin monomers could enhance or even inhibit actin nucleation/polymerisation. If the activity of certain protein extracts could promote actin polymerisation, then small oligomers of actin will be created, acting as polymerisation precursors than can accelerate or increase the extent of actin polymerisation (Haarer *et al.,* 1990). By means of this assay the authors demonstrate that Pkc1 plays an important role in promoting actin nucleation both under normal growth conditions and in response to treatment with hydrogen peroxide.

Profilin is an actin binding protein conserved in all eukaryotic systems studied so far. In budding yeast profilin is encoded by the *PFY1* genes. In *pfy1* mutant, actin cables are not detectable (Imamura *et al.,* 1997). Therefore profilin is required for the correct cable assembly. Rho1 and Rho4 protein members of the CWI pathway signal to the two formins identified in *S. cerevisiae*: Bni1 and Bnr1, which in turn interact with profilin to regulate the organisation of the actin cytoskeleton (Carlsson *et al.,* 1997). Profilin was identified as an inhibitor for the polymerisation of the actin cytoskeleton (Evangelista *et al.,* 2002). Strikingly, in budding yeast, the interaction between formins and profiling activates the assembly of

actin cables (Imamura *et al.,* 1997; Mosely *et al.,* 2005). Profilin can form a complex with actin and associate with filament barbed ends thus participating in their assembly. Pfy1 enhances actin filament dynamics by catalysing nucleotide exchange on actin (Wolven *et al.,* 2000). Pfy1 overexpression is rate limiting for actin assembly (Loisel *et al.,* 1999; Pujol *et al.,* 2009). One interpretation could be that profilin might be restricting the addition of actin monomers to filament barbed ends, or alternatively could be blocking addition to pointed ends, as discussed by Pollard and Cooper, (1984). The situation is completely different in oxidative conditions. According to Pujol *et al.* (2009), profilin overexpression increases the actin polymerisation rate, specifically upon hydroxide peroxide treatment. Interestingly, this effect also increases cell survival when exposed to oxidants (Pujol *et al.,* 2009). Other authors (Sagot *et al.,* 2002) also demonstrated that purified profilin induces actin assembly in the presence of formins. However, and in spite of that, Pfy1 overexpression is sufficient to activate actin polymerisation. In the absence of Pkc1, Pfy1 is unable to induce actin cable formation. Pujol *et al.* (2009) propose a model in which oxidative stress activates Pkc1. This kinase activates Pfy1 to promote actin nucleation and polymerisation, leading to an early restoration of actin cable remodelling. This enhancement of the actin dynamics is a defence mechanism against the deleterious effects of oxidative stress (Fig 3D).

Mitochondrial membrane potential and ROS production have been linked to the actin cytoskeleton dynamics in many eukaryotic systems, including yeast (Franklin-Tong *et al.,* 2008). The connection between mitochondria and actin is unclear. Two models have been proposed (Gourlay and Ayscough, 2006). In one of them, actin could interact with mitochondria to regulate the release of ROS. In the second model, actin could be sensor of cell-well being and indirectly would regulate the mitochondrial function and the Ras-cAMP signaling activity. Gourlay and Ayscough, (2006) have demonstrated that stabilisation of actin leads to hyperactivation of the Ras-cAMP pathway. The consequent rise in cAMP levels leads to the loss of mitochondrial membrane potential, accumulation of ROS and cell death (Fig 3D).

Further insight about actin dynamics in conditions of oxidative stress is given by the study of Pujol-Carrion and de la Torre-Ruiz, (2010) in which the authors describe role for Grx3 and Grx4 in actin cytoskeleton remodeling and in cellular defenses against oxidative stress caused by ROS accumulation. The Grx4 protein plays a unique role in the maintenance of actin cable integrity, which is independent of its role in the transcriptional regulation of Aft1. Grx3 plays an additive and redundant role, in combination with Grx4, in the organisation of the actin cytoskeleton, both under normal conditions and in response to oxidative stress. Each of the Grx domains of the two proteins plays a role in ROS

detoxification and cell viability. However, the Trx domain of each Grx4 and Grx3 protein acts independently of its respective Grx domain in a novel function that involves the polarisation of the actin cytoskeleton, which also determines cell resistance against oxidative conditions.

2.3.7. Cell wall-stressing agents

Agents that cause cell wall stress, such as the chitin antagonist calcofluor white, the β 1,3-glucan binding dye congo red, the cell wall lytic enzyme zymolyase, and the caffeine activate CWI signalling. A recent genome profiling study suggests the drug caffeine may target the Tor ptotein kinase complexes. Mutations that impare cell wall biosynthesis also activate signalling. A genome wide analysis of genes upregulated by mutations that affect the cell wall identified a group of genes possessing regulatory motifs for Rlm1, Swi4, Crz1 and Msn2/Msn4.

2.3.8. Actin cytoskeleton depolarisation

Rapamycin treatment which depolarises the actin cytoskeleton by specifically inhibiting the shared function of the Tor1/2 protein kinases induces Slt2/Mpk1 activation Krause and Gray, 2002; Torres *et al.,* 2002). The TOR inhibited-induced activation of Slt2 is dependent on components of the CWI. Slt2 activation is abrogated in *pkc1* and *bck1* strains. Deletion of *ROM2* also diminishes Slt2 phosphorylation (Torres *et al.,* 2002) (Fig 3C).

The Tor2- unique function is important for organisation of actin cytoskeleton (Schmidt *et al.,* 1996). The actin organisation defect was connected genetically to Rho1 signalling by the finding that the *tor2*^{ts} growth arrest and actin depolarisation defects were suppressed by overexpressing CWI pathway components at any level (Helliwell *et al.,* 1998a). 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) (Fig 3C).

3. POSTTRANSLATIONAL MODIFICATIONS OF THE CELL SURFACE SENSORS; GLYCOSYLATION

The CWI pathway sensor proteins – WSC family members, Mid2 and Mtl1 – have similar overall structure in that they possess small cytoplasmic domains, single transmembrane domains, and large extracellular protein domains rich in Ser/Thr residues. As demonstrated for Mid2 and Wsc1, the Ser/Thr-rich regions are highly O-mannosylated by at least three out of seven members of the PMT family of protein O-mannosyltransferases – Pmt1, Pmt2 and Pmt4 (Lommel *et al.,* 2004 ; Philip and Levin, 2001 ; Ketela *et al.,* 1999). This is not only structurally important modification, but it is required to prevent an aberrant posttranslational processing thus determining stability and ensuring signalling competence of Wsc1 and Mid2 proteins (Lommel *et al.,* 2004). Impared O-mannosylation in the double *pmt2pmt4* mutant causes cell lysis without osmotic stabilisation and cell death in response to mating pheromone or heat shock and these phenotypes are rescued by overexpression of the upper elements of PKC1-MAPK pathway leading to the finding that this pathway is defective in *pmt2pmt4* mutant (Lommel *et al.,* 2004). Induction of Slt2/Mpk1 phosphorylation does not occur in *pmt2pmt4* mutants during exposure to mating pheromone or elevated temperature and Lommel *et al.* demonstrate that O-mannosylation increases the activity of Wsc1, Wsc2 and Mid2. Truncated forms of Mid2 become localised to the plasma membrane (Ketela *et al.,* 1999; Rajavel *et al.,* 1999), although during mating-induced morphogenesis deficiency in O-mannosylation alters Mid2 localisation (Hutzler *et al.,* 2008).

It has been suggested that the modification of Ser/Thr-rich region by O-mannosyl glycans, which are short linear oligosaccharides consisting of 1-5 mannose residues (Willer *et al.,* 2003), cause the protein to adopt a rod-like structure to span the periplasmatic space and interact with the cell wall (Rajavel *et al.,* 1999; Philip and Levin, 2001).

In addition Mid2 is N-glycosylated and the extent of N-linked glycan and its distance from the plasma membrane affects the protein function (Hutzler *et al.,* 2008). In contrast to O-mannosylation, lack of N-linked glycan affects neither the stability, nor distribution at the plasma membrane. However, non-N-glycosylated Mid2 fails to perceive cell wall challenges (Hutzler *et al.,* 2008).

3.1. Protein O-mannosylation

Protein O-mannosylation represents a crucial posttranslational modification (reviewed in Lehle *et al.,* 2006; Lommel and Strahl, 2009). The initial mannosyltransfer reaction is

catalysed at the endoplasmatic reticulum by the essential family of dolichyl phosphate-D-mannose:protein O-mannosyltransferases (PMTs) evolutionary conserved from yeast to humans (Strahl-Bolsinger *et al.,* 1999 ; Willer *et al.,* 2003 ; Lommel and Strahl, 2009). O-mannosyl glucan biosynthesis has been greatly studied in *S. cerevisiae*. PMTs catalyse the transfer of the first of the several mannosyl residues (in α-1,2 and α-1,3 linkages) from dolichyl-phosphate activated mannose to the hydroxyl groups present in the side-chains of serine/threonine residues belonging to proteins entering the secretory pathway. After the first mannose has been added, the proteins move on to the Golgi apparatus for the oligomannose chain to be formed. GDP-Mann functions as mannosyl donor in the Golgi apparatus. The biosynthesis of sugar chains linked through O-mannoside proceeds in mammals in the same way as in yeast (Manya *et al.,* 2004). In yeast, neutral O-mannosyl glycans varying in length from one to several sugar residues are present. Man_{0-2}-Man-Ser/Thr is common to all yeasts studied. In mammals, O-mannosyl glycans vary in length and can be sialylated or sulphated. The $Gal\beta_{1-4}GlcNAc\beta_{1-2}$Man-Ser/Thr core structure is common to all mammalian O-mannosyl glycans isolated to date (Fig 4A).

PMT family is phylogenetically subdivided into PMT1, PMT2 and PMT4 subfamilies which differ in protein substrate specifity and whose members are closely related to *S. cerevisiae* Pmt1, Pmt2 and Pmt4, respectively (Girrbach *et al.,* 2000 ; Lehle *et al.,* 2006) (Fig). In *S. cerevisiae* and *C. albicans,* the PMT1 and PMT2 subfamilies are redundant (two or more members of each subfamily) whereas only a singe member of the PMT4 subfamily is present (Gentzsch and Tanner, 1996). In higher eukaryotes only two genes are present, one of which belongs to the PMT2 subfamily and the other to the PMT4 subfamily (Fig 4B).

Members of PMT1 subfamily form distinct heteromeric complexes with PMT2 subfamily members whereas PMT4 subfamily acts as homomeric complexes (Girrbach and Strahl, 2003). Members of Pmt family form homodimers as well as heterodimers as a mechanism to enhance substrate specificity (Hutzler *et al.,* 2007) and to ensure efficient O-mannosylaion in a wide range of target proteins (Lommel and Strahl, 2009). Hutzler *et al.* (2007) have identified that Pmt4 O-mannosylation signals are not just linear protein's primary structure sequences but rather are highly complex, being the membrane association a determinant for substrate recognition by Pmt4.

Analyses of single viable *pmt* and conditionally lethal double *pmt* mutants of *Saccharomyces cerevisiae* reveals that O-mannosylation affects important cellular processes such as maintenance of cell wall integrity, cell polarity and morphogenesis by being essential

for structural and functional aspects of proteins – stability, folding, localisation, solubilisation in ER, ligand interaction (reviewed in Lommel and Strahl, 2009).

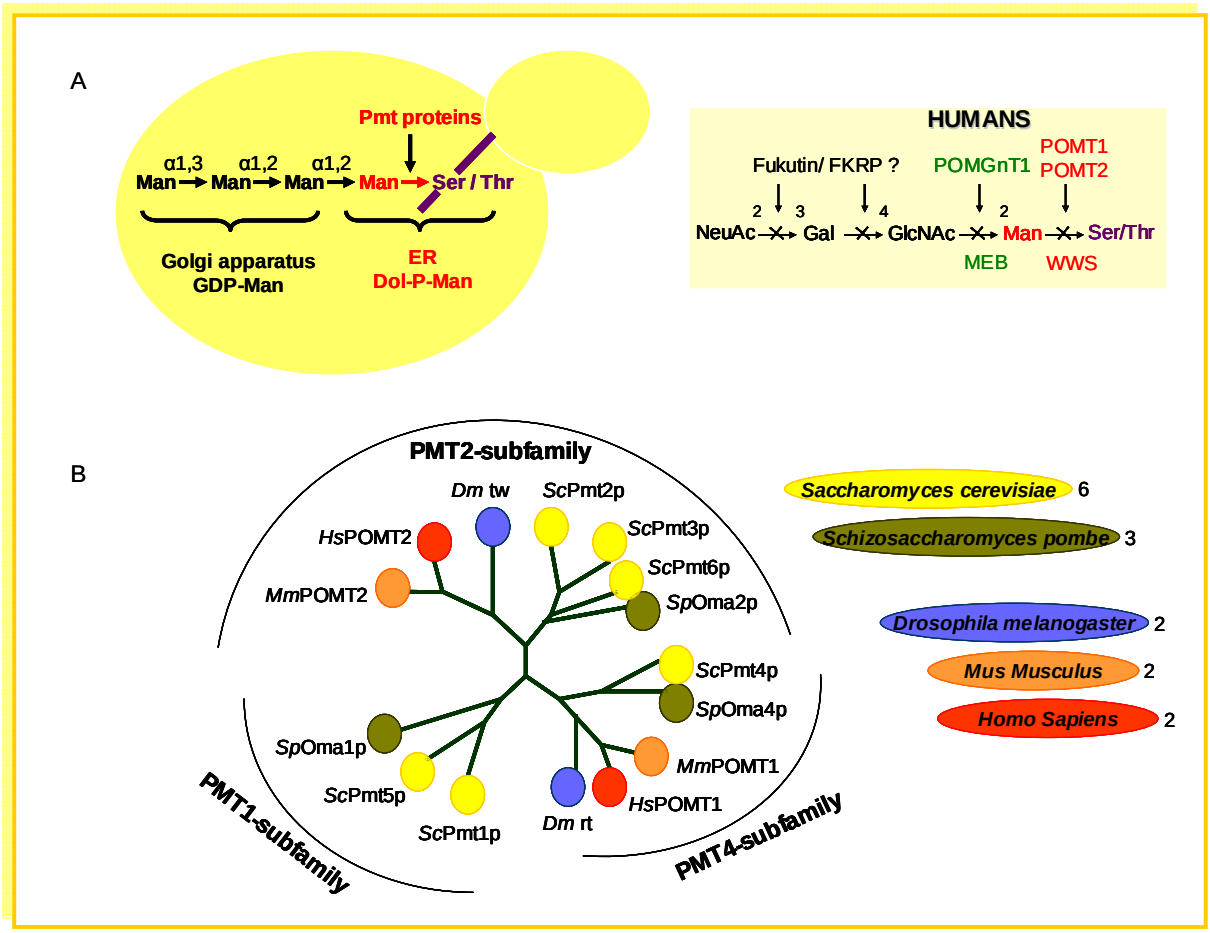

Figure 4. Protein O-mannosylation. A) The initial mannosyltransfer reaction is catalysed at the endoplasmatic reticulum by the essential family of protein O-mannosyltransferases (PMTs). PMTs catalyse the transfer of the first of the several mannosyl residues from dolichyl-phosphate activated mannose to Ser/Thr residues of secretory proteins. Further extension of the saccharide proceeds in the Golgi apparatus from GDP-Man (on the left, *Saccharomyces cerevisiae*). In the right panel, the O-mannosyl glycan chain of α-dystroglycan and the underlying congenital muscle dystrophy and neuronal migration defects caused by protein underglycosylations in humans, are depicted: MEB, *m*uscle-*e*ye-*b*rain disease; WWS, Walker-Warburg syndrome. In B), the evolutionary relationship of the protein O-mannosyltransferases is depicted. Yeast possess three PMT subfamilies; in all higher eukaryotes only two subfamilies exist. The *PMT* genes of higher eukaryotes are called *POMT*. The numbers indicate how many different transferases are present in the corresponding genome altogether **(figure adapted from Lehle *et al.*, 2006).**

Viable single *pmt* mutants adapt to the decreased O-mannosylation capacity and ensure the maintenance of glycosylation by evolving compensatory mechanisms – upregulation of other Pmt family members and formation of alternative Pmt complexes (Girrbach and Strahl, 2003). In addition, more general compensatory mechanisms, such as upregulation of chitin synthesis (Kapteyn *et al.,*1999), activation of metabolic and stress response pathways, in particular activation of the CWI pathway, are observed in glycosylation mutants (Lagorce *et al.,* 2003; Lommel *et al.,* 2004) or in response to general inhibition of O-mannosylation (Arroyo *et al.,* 2011). Arroyo *et al.* present a genome-wide transcriptional analysis of the response to general inhibition of O-mannosylation and demonstrate that many genes of the unfolded protein response (UPR) and ER-associated protein degradation (ERAD) are induced, mating and filametous growth are repressed.

O-mannosylation of specific secretory proteins of fungal pathogens contributes significantly to virulence (reviewed in Lengeler *et al.,* 2008). In mammals, Pmt proteins are essential for cellular differentiation and development, while deficiency in O-mannosylation causes congenital neuromuscular disorders (reviewed in Lehle *et al.,* 2006).

3.2. Protein N-glycosylation

The N-glycosylated proteins contain oligosaccharides that are N-glycosydically linked to γ-amido group of asparagines. Commonly, N-glycosylated proteins are secretory proteins. The formation of highly variable N-linked oligosachharides from different proteins begins with the transfer of the $GlcNAc_2Man_9Glc_3$ unit, the core oligosachharide, from the dolichol-pyrophosphate-activated state to the protein. This process is carried out by an oligosaccharyltransferase (OST) located in the luminal side of the Endoplasmic Reticulum. The protein is synthesized by the ribosomes associated to the Endoplasmatic Reticulum and it is then translocated to the luminal side of the ER by the Sec61 complex, a transmembrane chanel. The protein bound oligosaccharide is modified by disposing sugar residues and adding different ones. The protein-bound oligosaccharide is first trimmed by removing all three glucoses and one mannose while still in the ER. The correctly folded glycoprotein goes from the ER to the Golgi apparatus where several hydrolytic reactions take place (which follow a strict order determined by the specificity of the glycosyl transferase): demannosylation followed by transfer of a GlcNAc (N-acetylglucosamine), galactose, sialic acid and fucose residues in mammals. In yeast, the number of mannoses is not reduced and it may be further extended in the Golgi apparatus with more than 100 mannose units. In the terminal glycosylation phase, the mature glycan is built in a protein dependent, tissue and organ specific manner (only one pathway is depicted in the figure). In the case of soluble

lysosomal glycoproteins there will be a mannose-6-phosphate determinant generated which has a signalling function for targeting the protein to the lysosome. N-glycosylation reactions in the ER vary subtly whereas in the Golgi they differ considerably. This fact is due to the existence of several sugars with their adequate transferases in mammals which are absent in yeast. N-linked glycans play a crucial role in the folding and degradation of glycoproteins in the ER (Helenius and Aebi, 2004). As demonstrated for the cell surface sensor Mid2 (Hutzler *et al.,* 2008) N-glycosylation affects the protein function.

4. TOR SIGNALLING NETWORK

The target of rapamycin (TOR) was originally identified in *Saccharomyces cerevisiae* by mutations, *TOR1-1* and *TOR2-1,* which confer resistance to the growth inhibitory properties of rapamycin (Heitman *et al.,* 1991). The immunosuppressive and anticancer drug 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*). Since its discovery, TOR has been widely investigated and has been recognised as a central controller of cell growth in eukaryotes (Schmelzle and Hall, 2000; Wullschleger *et al.,* 2006). *TOR* homologues have been found in all eukaryotic genomes examined but, unlike yeast, which possess two *TOR* genes, nearly all other eukaryotic genomes possess only a single *TOR* gene (Crespo and Hall, 2002; Lee *et al.,* 2005; Crespo *et al.,* 2005). 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 are 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.

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). TOR complexes, and the signalling branches they regulate, are conserved from yeast to man (Wullschleger *et al.,* 2006).

4.1. TWO TOR COMPLEXES – TORC1 AND TORC2

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

TORC1 contains Lst8, Kog1, Tco89 and either Tor1 or Tor2 (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).

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) (Fig 5).

TORC1 activity appears to be sensitive to both nutrient and stress cues. Nutrients, especially amino acids, regulate TORC1 signalling. 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, 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).

4.1.2. 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 supercomplex 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.

4.2. FUNCTIONS OF TORC1 SIGNALLING

4.2.1. Translation initiation

Initiation of protein synthesis in eukaryotes is a highly regulated process and TORC1 appears to regulate translation initiation at multiple levels. The mechanisms by which TORC1 regulates translation initiation are now better characterised in mammalian cells. In yeast, TORC1 translation targets include eIF4E, eIF4G and IF2. The eIF4E is a cap-binding protein, encoded by *CDC33. cdc33* and *tor* mutants display similar phenotypes (Barbet *et al.,* 1996). eIF4G is an adaptor protein that binds to eIF4E and recruits additional initiation factors to the 5' cap. Rapamycin treatment or nutrient depletion results in enhanced degradation of eIF4G (Berset *et al.,* 1998). GTP-loaded IF2 is required to deliver an initiator methionyl-tRNA to the 40S ribosomal subunit and eventually to the translation initiation codon of mRNA. The α-subunit of IF2 is phosphorylated by Gcn2 and this inhibits general translation initiation. Gcn2 is negatively regulated by phosphorylation of Ser-577 which is promoted by TORC1 (Hinnebush, 2005).

The G1 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 G1 cyclin Cln3, plays important role in activating and allowing progression of the cell cycle (Barbet *et al.,* 1996).

4.2.2. Ribosome biogenesis

Ribosome biogenesis is a major consumer of cellular energy and its regulation is a key aspect of cell growth control. TOR signalling links nutrients availability to the byosynthesis of ribosomes. Rapamycin treatment, like nutrient limitation, results in dramatic reduction in expression of *RP, RiBi* (encode accessory factors that assemble and modify rRNA and RPs in the nucleolus, translation factors, tRNA synthetases, subunits of RNA Pol I and III, enzymes involved in ribonucleotide metabolism), *rRNA* and *tRNA* genes (Cardenas *et al.,* 1999), and TORC1 is also required for efficient processing of the 35S precursor *rRNA* (Powers and Walter, 1999).

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. 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 (Fig 5).

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. 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) (Fig 5).

The AGC-family kinase Sch9 also has been proposed to regulate *RP* and *RiBi* gene expression in a TORC1-dependent, but FHL1- and Sfp1-independent manner (Jorgensen *et al.,* 2004) (Fig 5). Additionally, histone modifying factors affect RP gene expression and have

been implicated as TOR effectors (Humphrey *et al.,* 2004). Although unclear, TORC1 regulates *rRNA* transcription by influencing the activity of Rrn3, an essential RNA Pol I initiation factor (Claypool *et al.,* 2004).

4.2.3. mRNA turnover

Nutrient limitation or rapamycin treatment accelerate the major mRNA decay pathway (deadenylation-dependent decapping pathway), resulting in an enhanced turnover of some but not all mRNA. The control of mRNA stability by TORC1 is unclear. TORC1 signals could promote mRNA flux out of P bodies or alternatively inhibit the exit of mRNAs from translation (Coller and Parker, 2005).

4.2.4. Amino acid permease activity

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). In contrast, TORC1 negatively regulates low-affinity transporters and this occurs primarily at transcriptional level. 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 (described below).

4.2.5. Autophagy

Starved cells degrade cytoplasmic contents, including organelles, and thereby recycle macromolecules to ensure survival under nutrient deprivation. This catabolic process, known as macroautophagy involves the enclosure of cytoplasm by double-membrane structure and its subsequent delivery to the vacuole. TOR negatively regulates macroautophagy through inhibition of the protein kinase Atg1 (Kamada *et al.,* 2000). TOR controls not only bulk protein degradation by macroautophagy, but also the ubiquitination, internalization, and turnover of specific nutrient transporters. TORC1 prevents the turnover of amino-acid and glucose transporters (Schmelzle *et al.,* 2004).

4.2.6. Transcription

TORC1 negatively controls transcriptional programs that normally operate under nutrient starvation and/or general stress conditions. The most striking set of genes whose expression is induced upon TORC1 inhibition includes those involved in assimilation of alternative nitrogen sources, in protein degradation and in general stress protection (Cardenas *et al.,* 1999; Shamji *et al.,* 2000; Chen and Powers, 2006). One common mechanism by which TORC1 controls the expression of nutrient- and stress-responsive genes is by sequestering specific transcription factors in the cytoplasm.

TORC1 inhibits transcription of genes normally activated upon nitrogen deprivation by promoting the association of the GATA transcription factor Gln3 with the cytoplasmic repressor protein Ure2 (Beck and Hall, 1999). The binding of Gln3 to Ure2 requires TOR-dependent phosphorylation of Gln3 by a mechanism that involves Tap42-mediated inhibition of the phosphatase Sit4 (see below; and Fig 5). TORC1 blockage causes dephosphorylation, dissociation from Ure2 and nuclear import of Gln3. Subsequent Gln3-dependent activation of nitrogen-catabolite repression (NCR)-sensitive genes enables the cells to import and catabolise poor nitrogen sources (Beck and Hall, 1999). TORC1 controls also the phosphorylation of Ure2 (Cardenas *et al.,* 1999). TORC1 prevents nuclear localisation of the GATA transcription factor Gat1 although the mechanism likely differs from the one observed for Gln3 (Beck and Hall, 1999; Crespo and Hall, 2002).

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). 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) (Fig 5). 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. Transcription factors regulated by TOR can be differentially regulated by multiple factors through parallel pathways, such as RTG pathway in the case of Rtg1/3 (Giannattasio *et al.,* 2005), and the ubiquitin-dependent signalling pathway in the case of Gln3 (Crespo *et al.,* 2004). A relationship between TOR and mitochondrial function has been described in mammalian cells in which perturbations of mitochondrial function leads to inhibition of TOR kinase activity (Kim *et al.,* 2002).

TORC1 further controls both the nucleocytoplasmic distribution and activity of the functionally redundant Zn^{2+}-finger transcription factors Msn2 and Msn4. Msn2/Msn4 regulate stress-responsive element (STRE)-dependent transcription in response to wide range of stresses (Martinez-Pastor *et al.,* 1996; Gorner *et al.,* 1998; Schmitt and McEntee, 1996), including carbon source limitation (Gorner *et al.,* 2002; Smith *et al.,* 1998). TORC1 regulates Msn2/Msn4 by promoting their phosphorylation and cytoplasmic accumulation, which may (Beck and Hall, 1999) or may not (Santhanam *et al.,* 2004) require cytoplasmic 14-3-3 anchor proteins. Upon nutrient limitation transcriptional activation by Msn2/Msn4, and Gis1, closely related transcription factor that activates expression of post-diauxic-shift (PDS) element-controlled genes, also requires the Ser/Thr kinase Rim15 (Reinders *et al.,* 1998; Swinnen *et al.,* 2006). The nucleocytoplasmic distribution of Rim15 is also controlled by TORC1 in a 14-3-3-dependent manner. Rim15 is required for proper entry into stationary phase (G_0) although the mechanisms by which it influences Msn2/Msn4 and Gis1 activity are not known (Swinnen *et al.,* 2006). TOR may control Msn2/Msn4 in conjunction with cAMP-PKA pathway as nuclear localisation of Msn2/Msn4 is also controlled negatively by PKA (Gorner *et al.,* 1998; see the sections below; and Fig 5). PKA-dependent phosphorylation of the nuclear localisation signal (NLS) of Msn2 has been demonstrated to inhibit nuclear import of Msn2 (Gorner *et al.,* 2002). On the other hand, PP2A phosphatase that is under the negative control of TOR has been shown to inhibit nuclear export of Msn2 upon TORC1 inactivation, or in response to heat and osmotic stress (Beck and Hall, 1999; Santhanam *et al.,* 2004). However, nuclear translocation seems not the only step for regulation of Msn2/Msn4 activity. It has been shown that Msn2 activity can also be regulated at the level of DNA binding (Hirata *et al.,* 2003), transactivation (Boy-Marcotte *et al.,* 2006) and degradation of nuclear Msn2 (Lallet *et al.,* 2004).

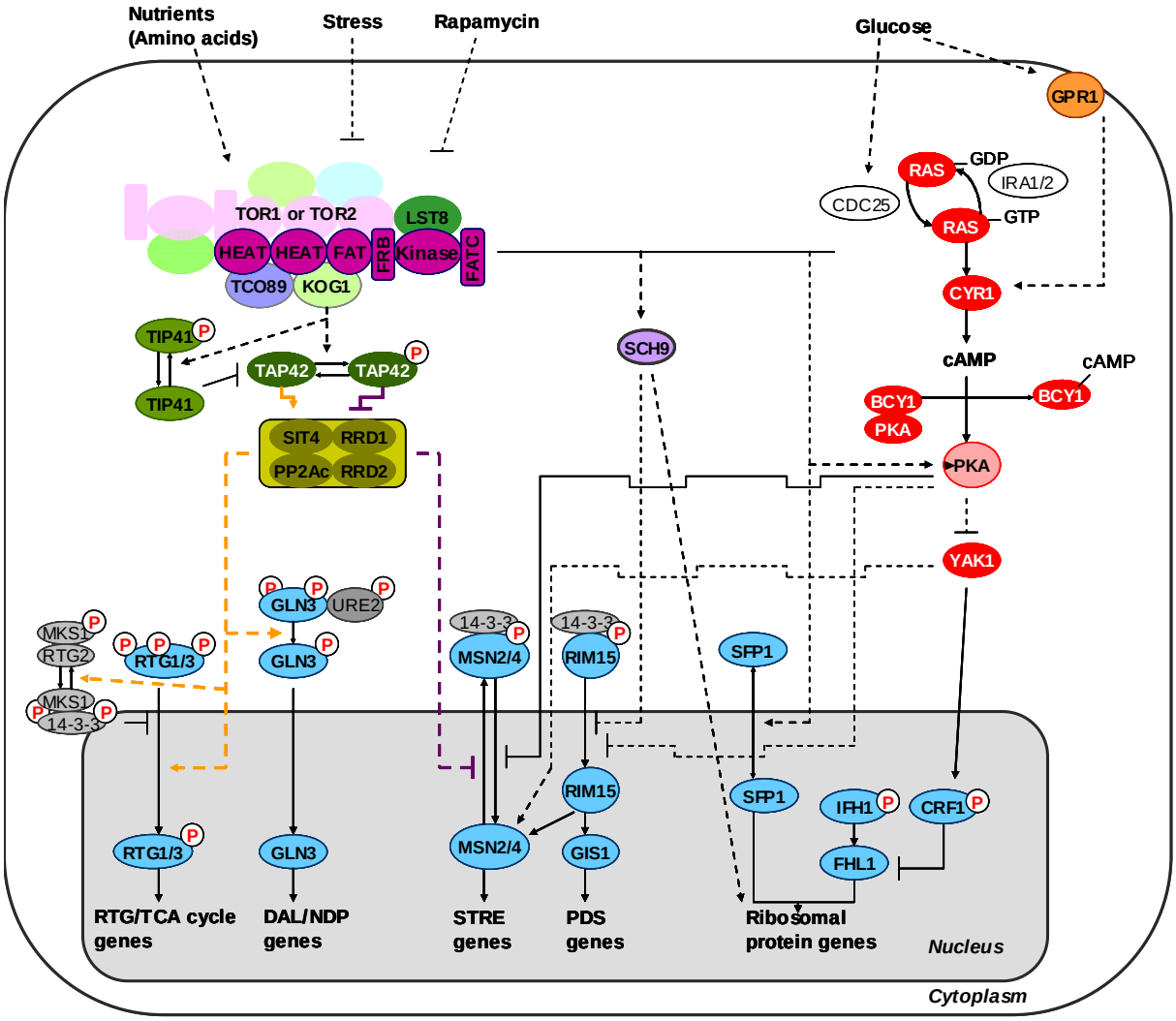

Figure 5. Schematic diagram of the TORC1 signalling and RAS-cAMP pathway in *S. cerevisiae.* Depicted are TOR-associated proteins in TORC1 complex and the domains found in TOR. TORC1 is multimer, likely dimer. TORC1 mediates the rapamycin-sensitive signalling branch that regulates temporal aspects of cell growth. TORC1 inhibits transcriptional stress responses mediated by RTG1/3, GLN3, MSN2/4. Some TORC1 readouts are also influenced by the protein kinase A (PKA) signalling pathway and SCH9. TORC1 controls other readouts via type 2A (PP2A) and/or the PP2A-like protein phosphatase SIT4. TORC1 controls nuclear import and/or export of SFP1. Glucose signalling is mediated by the G-proteins RAS through PKA to induce ribosome biogenesis and suppress the general stress response controlled by MSN2/4 and RIM15. GPR1 is a plasma membrane G-protein coupled receptor that interacts with the GPA2 (G protein α-subunit) and regulate adenylyl cyclase (CYR1) activity as well in response to glucose signals. Not depicted are the stimulation of the translation and the inhibition of autophagy by TORC1 and RAS-cAMP. Arrows and bars denote positive and negative interactions, respectively. Solid arrows and bars refer to direct interactions, dashed arrows and bars refer to indirect and/or potential interactions. Circles containing letter P depict phosphorylated amino acid residues. STRE, stress-responsive element; PDS, post-diauxic shift element; DAL, degradation of urea and allantoin; NDP, nitrogen discrimination pathway; RTG, retrograde regulation; TCA, tricarboxylic acid. See text for further details **(figure adapted from de Virgilio and Loewith, 2006).**

4.3. TOR SIGNALLING AND ITS RELATIONSHIP WITH OXIDATIVE STRESS AND LIFE SPAN.

Respiratory metabolism produces free radicals generating oxidative damage. It is generally accepted that aging and cell death are both a consequence of oxidative damage. The extension of life is therefore correlated with oxidative stress resistence (Moradas-Ferreira *et al.,* 2000). Recently, the existence of a connection between mitochondrial translation, oxidative stress and aging, has been proposed in the model system *S. cerevisiae* (Bonawitz *et al.,* 2006b). 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 (oxidative phosphorylation) 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. This model enters in discrepancy with that of Powers et al. (2006), who propose that the adquisition of stress resistance observed in the *tor1* mutant is partly due to the activation of the Msn2/Msn4 transcription factor.

Activation of the retrograde pathway signalling, a transcriptional response that has evolved to counter the consequences of reduced mitochondrial respiratory capacity, antagonises the negative effect of TOR signalling of mitochondrial respiratory capacity, thereby reducing ROS production and increasing life span (Jazwinski *et al.,* 2005; Liu and Butow, 2006).

Wei *et al*. (2008) propose a model in which TOR signals to *SCH9* downregulating Rim15 activity. Signals for Rim15 inhibition come from RAS2/PKA, SCH9 and TOR (Vidan *et al.,* 1997; Swinnen *et al.,* 2006). Rim15 upregulates the activity of Msn2/Msn4 and Gis1 (Cameroni *et al.,* 2004). Wei *et al.* (2008) have reported that Rim15 and the downstream stress response transcription factors Msn2/Msn4 are required for chronological life span extension in mutants in RAS2/cAMP/PKA or in mutants TOR/SCH9. These data suggest the existence of two regulatory pathways involved in the chronological life extension, both converging on Rim15. In support of this model, Pan and Shadel (Pan *et al.,* 2009) in a recent study, 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 (Fig 6). In a recent study (Madia *et al.,* 2010) the authors 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*. 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.

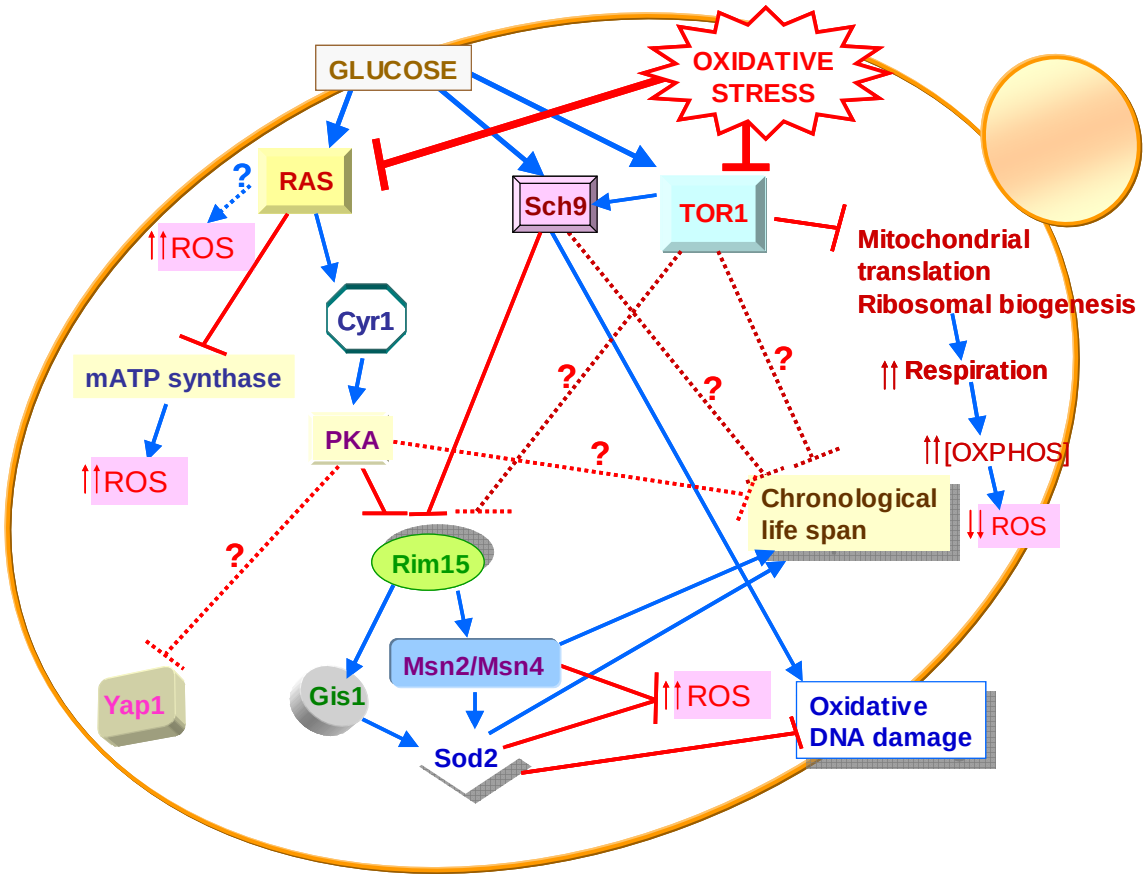

Figure 6. Oxidative stress inhibits Tor1 and Ras functions. Tor1 inhibition avoids the inhibition of mitochondrial translation and ribosomal biogenesis then the respiration is not activated and OXPHOS species are not accumulated, as a result of this process there is an increase in ROS. Tor1 inhibition also impairs the subsequent activation of Sch9; consequently Rim15 function will not be correctly blocked, leading to activation of

Msn2/Msn4 and Gis1 transcription factors with the subsequent increase in *SOD2* expression and reduction in both ROS accumulation and in DNA oxidative damage. Oxidative stress also reduces RAS activity what impairs Gis1 activation and consequently the blockage of Rim15 mediated by PKA does not take place. Again this mechanism leads to a lesser accumulation of ROS and oxidative damage. Discontinuous lines reflect a possible signal mechanism not yet demonstrated. Tor1 is likely to inhibit Rim15 and the three signal cascades mediated by each of the Tor1, Sch9 and Pka proteins impair the chronological life span. Blue lines indicate activation whereas red lines indicate repression **(figure taken from de la Torre-Ruiz *et al.,* 2010).**

Interestingly, *tor1* deletion makes cells transitorily sensitive to hydrogen peroxide (Bonawitz *et al.,* 2007). However, in the same study the authors demonstrate that the absence of Tor1 function confers cells with more resistance to oxidative stress. One possible explanation for this effect is the induction of the expression of the antioxidant *SOD2* observed in the *tor1* mutant. Nevertheless, disruption of TOR signalling increased the chronological life span also independently on *SOD2*. In conclusion, TOR controls several downstream processes in order to increase cell resistance to oxidative stress. However, some of the outputs remain to be elucidated.

4.4. TORC1 EFFECTORS

4.4.1. TORC1 regulated protein phosphatases

Some of the TORC1 readouts are mediated by the type 2A and 2A-related protein phosphatases (Fig 5). 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). 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. Presumably, 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 PP2Ac 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). Conversely, the inhibition of Sit4 by TORC1 and Tap42 maintains Slt2/Mpk1 activity at basal levels (Torres *et al.,* 2002).

4.4.2. Other signalling pathways acting in parallel to TORC1 for the regulation of common readouts.

TORC1 and the glucose-responsive PKA pathway provide diverse ways of cross-talk and regulate common readouts (Fig 5). For instance, TORC1 and PKA (via direct phosphorylation) independently regulate nuclear export and import, respectively, of Msn2/Msn4 (Gorner *et al.,* 1998; Santhanam *et al.,* 2004). TORC1 prevents nuclear accumulation of Rim15 by modulating the phosphorylation status within its 14-3-3-binding domain, whereas PKA-dependent phosphorylation inhibits Rim15 kinase activity (Pedruzzi *et al.,* 2003). The subcellular localisation of Sfp1 is regulated by PKA as well as by a PKA-independent TORC1 effector branch (Marion *et al.,* 2004). Seemingly conflicting models have been proposed about the interplay between the two signaling cascades as for the overlapping regulation by TORC1 and PKA. Transcriptional profile studies support a model in which TORC1 and PKA provide separate inputs in fine tuning control of various gene clusters (Zurita-Martinez and Cardenas, 2005). Nevertheless, these findings do not exclude the possibility that signal integration between TORC1 and PKA may rely on additional layers of regulation. Several findings suggest that TORC1 is an activator of PKA, placing both kinases in the same signaling pathway. First, hyperactivation of the RAS-cAMP-PKA pathway suppresses TORC1 deficiency, whereas downregulation of the PKA pathway leads to increased rapamycin sensitivity (Schmelzle *et al.,* 2004). Second, TORC1 controls RP gene expression via PKA (Martin *et al.,* 2004; Schmelzle *et al.,* 2004). Third, Sch9 kinase, which is a direct substrate of TORC1, was initially identified as a multicopy suppressor of mutations that reduce PKA activity (Toda *et al.,* 1988). Fourth, inactivation of TORC1 by rapamycin causes a rapid accumulation of the PKA catalytic subunit TPK1 in the nucleus (Schmelzle *et al.,* 2004). In a recent study it has been demonstrated that TORC1 inhibits BCY1

phosphorylation (the negative regulatory subunit of PKA) via Sch9 and the MAP kinase Slt2/Mpk1, suggesting that TORC1 activates PKA towards some substrates by preventing Slt2-mediated activation of BCY1 (Soulard *et al.,* 2010).

TORC1 also shares common target proteins with Snf1 kinase. The transcription factor Gln3 is independently and oppositely regulated by TORC1 and Snf1: TORC1 promotes cytoplasmic retention of Gln3, whereas Snf1 (via direct phosphorylation) promotes nuclear accumulation of Gln3 (Bertram *et al.,* 2002). In addition, TORC1 and Snf1 also converge on Msn2. TORC1 positively controls nuclear export of Msn2, whereas Snf1, likely as part of an adaptive response to prolonged glucose starvation, slow down nuclear import of Msn2 (Mayordomo *et al.,* 2002).

It has been proposed that TORC1-dependent phosphorylation is required for Sch9 activity (Urban *et al.,* 2007). Sch9 is required for TORC1 to properly regulate ribosome biogenesis, translation initiation, and entry in G_0 phase, but not expression of Gln3-dependent and Rtg1/3 target genes (Fig 5).

4.5. TORC2 READOUTS

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). However, further molecular details on the activation of this pathway required the identification of TORC2 substrates. 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).

5. RAS-cAMP PATHWAY

The Ras-cAMP-PKA network plays the primary role in cell's response to glucose and is involved in the regulation of cell growth, metabolism and stress resistance.

5.1. COMPONENTS

Protein kinase A (PKA) plays critical roles in growth, in response of cells to glucose and in coupling cell cycle progression to mass accumulation. PKA comprises a heterotetramer composed of two catalytic and two regulatory subunits. Three closely related genes, *TPK1, TPK2* and *TPK3,* redundantly encode the catalytic subunits, with each gene product capable of phosphorylating distinct but somewhat overlapping set of target proteins (Ptacek *et al.,* 2005). The PKA regulatory subunit is encoded by *BCY1.* This subunit acts as pseudo substrate for the catalytic subunits to bind and restrict their activity. Upon binding of cAMP to Bcy1, the catalytic subunits are released to perform their functions. *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.,* 1987).

A balance between cAMP synthesis catalysed by adenylyl cyclase, encoded by *CYR1*, and cAMP degradation catalysed by phosphodiesterases, encoded by *PDE1* and *PDE2,* establishes the level of cAMP in the cell. 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 5). 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 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 (Santangelo, 2006; Zaman *et al.,* 2008).

5.2. FUNCTIONS OF RAS-cAMP-PKA SIGNALLING

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). Ras activation results in induction of genes that are involved in ribosomal protein synthesis, ribosome biogenesis, glycolysis and repression of genes that are involved in stress response, in gluconeogenesis, in metabolism of storage carbohydrates. A large fraction of genes that are induced by glucose in a Ras2-dependent manner are involved in ribosome production (Wang *et al.,* 2004).

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. Mutations impairing specific components of the RNA Pol II transcriptional machinery are synthetically lethal with the hyperactive $Ras2^{Val119}$ allele, and PKA can phosphorylate a component of Srb (Ssn2) complex. The latter may be one of the Ras-cAMP-mediated modifications of nuclear factors needed to accomplish the broad spectrum of alterations to the yeast transcriptome upon glucose addition or depletion (Howard *et al.,* 2002; Chang *et al.,* 2004). Some of the PKA activities were discussed in the previous sections in the view of the cross-talk with other signaling cascades (Fig 5).

Ras-cAMP pathway has also been implicated in aging (Lin *et al.,* 2002), thermotolerance (Zhu *et al.,* 2000); bud site selection, actin repolarisation (Schneper *et al.,* 2004), glycogen accumulation (Smith *et al.,* 1998); stress resistance (Wang *et al.,* 2004); authophagy (Budovskaya *et al.,* 2005); and sporulation (Cameron *et al.,* 1988). It may also regulate pseudophyphal differentiation in response to nutrient limitation (Gimeno *et al.,* 1992). It is shown to regulate cell size in response to nutrient conditions (Baroni *et al.,* 1989).

5.3. 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).

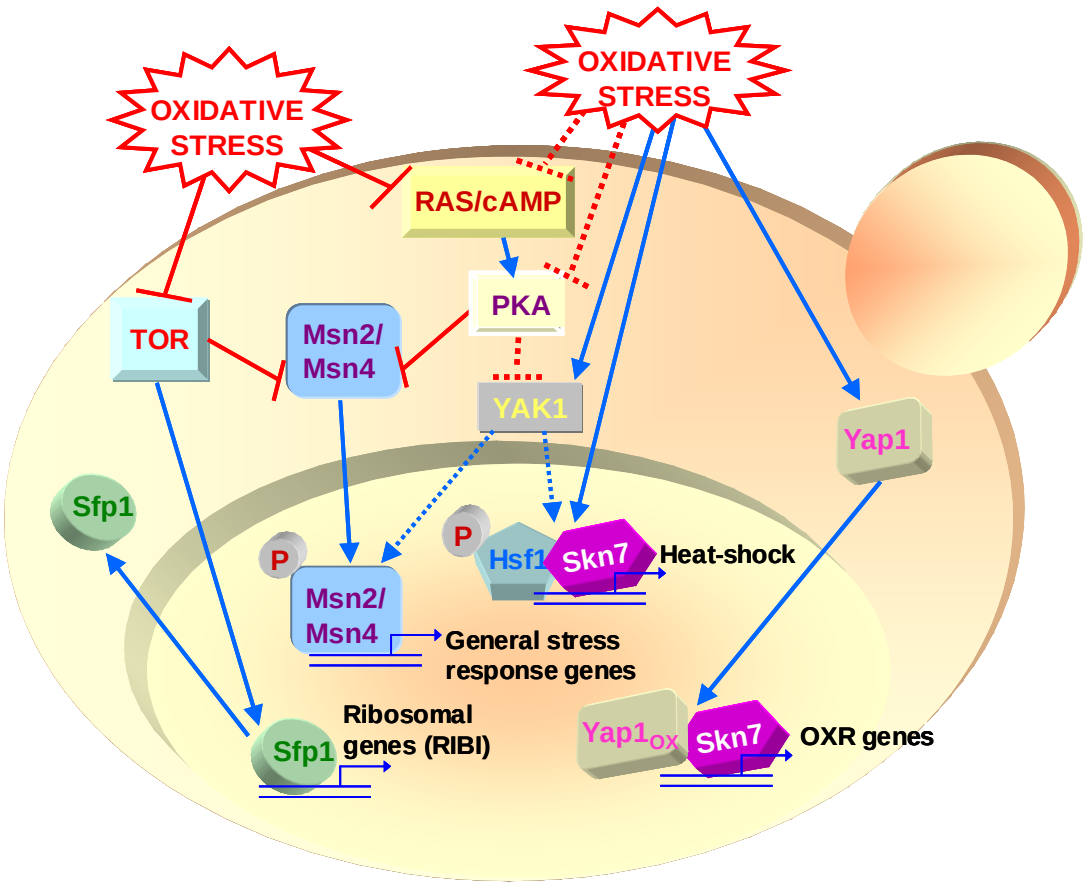

Figure 7. Signalling to the transcription factors involved in the oxidative stress response. Oxidative stress activates Yak1 which promotes the activation of the complex Hsf1/Skn7 inducing the expression of heat-shock genes. In addition Yak1 activates by phosphorylation Msn2/Msn4 which translocates to the nucleus inducing the expression of the general response genes. The activation of Hsf1/Skn7 in the nucleus can also occur directly upon oxidative stress. Oxidative stress oxidises cytoplasmic Yap1. Once Yap1 is oxidised, it is translocated to the nucleus where it forms a complex with Skn7 in order to induce the transcription of OXR (oxidative stress response) genes. Oxidative stress inhibits the Ras-cAMP activity then not activating PKA. Therefore, PKA can not block YAK1 function favouring the activation of the downstream transcription factors regulated by YAK1. Oxidative stress also blocks Tor1 function which affects Sfp1 localisation. Tor1 activity determines Sfp1 nuclear localisation and the subsequent activation of ribosomal genes. Absence of Tor1 function signals to Sfp1 translocation from the nucleus to the cytoplasm **(figure taken from de la Torre-Ruiz *et al.*, 2010)**.

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. Msn2/Msn4 is the general stress responsive transcription factor with a role in the regulation of a number of genes involved in the oxidative repair (Hasan *et al.,* 2002) (Fig 6 and Fig 7). Superoxide dismutase appears to be quite important in order to protect cells against free radicals (Cullota, 2000).

A recent study highlights the contribution of the Ras/PKA signalling pathway to oxidative stress (Hlavata *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).

OBJECTIVES

- **To characterise the essential role of Mtl1 for the oxidative stress response**
- **To characterise the of role of Mtl1 within the cell wall integrity pathway and the cross talk with other signalling pathways**
- **Searching for cellular targets of oxidative stress regulated by CWI components**
- **Functional characterisation of Mtl1 protein**

ARTICLE I

Objectives:

- Elucidating the essential role of Mtl1 in the oxidative stress response
- Characterisation of the functional relationship between Mtl1 and Msn2/Msn4, as well as the functional and genetic interaction between Mtl1 and either TOR or RAS-cAMP
- Characterisation of the functional relationship between Mtl1 and members of the CWI pathway

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Mtl1 Is Required to Activate General Stress Response through Tor1 and Ras2 Inhibition under Conditions of Glucose Starvation and Oxidative Stress*[S]

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Mima Ivanova Petkova[‡1], Nuria Pujol-Carrion[‡1], Javier Arroyo[§¶], Jesús García-Cantalejo[¶], and Maria Angeles de la Torre-Ruiz[‡2]

From the [‡]Departament de Ciències Mèdiques Bàsiques-IRBLleida, Facultad de Medicina, Universidad de Lleida, Lleida 25008, Spain, the [§]Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense, Madrid 28040, Spain, and the [¶]Unidad de Genómica-Campus Moncloa Universidad Complutense de Madrid/Parque Cientifico de Madrid (Campus Moncloa), Universidad Complutense, Madrid 28040, Spain

Mtl1 is a member of the cell wall integrity (CWI) pathway of *Saccharomyces cerevisiae*, which functions as a cell wall sensor for oxidative stress. Genome-wide transcriptional analysis revealed a cluster of genes that were down-regulated in the absence of Mtl1. Many of these genes were potentially regulated by the general stress response factor Msn2/Msn4. In response to rapamycin, caffeine, glucose starvation and oxidative stress provoked by H_2O_2, *mtl1* presents a significant loss of viability as well as a deficiency in the transcriptional response mediated by Msn2/Msn4. The Mtl1 function was required (i) to induce ribosomal gene repression, (ii) to induce the general stress response driven by the transcription factor Msn2/Msn4, and (iii) to activate the CWI pathway in response to both glucose starvation and oxidative stress. We also detected higher cAMP levels in the *mtl1* mutant than in wild type cells indicative of up-regulated RAS2-PKA activity. Disruption of *TOR1*, disruption of *RAS2*, or hyperactivation of Rho1 restored both the viability and the transcriptional function (both ribosomal and Msn2/Msn4-dependent gene expression) in the *mtl1* mutant to almost wild type levels when cells were starved of glucose or stressed with H_2O_2. Taking our results together, we propose an essential role for Mtl1 in signaling oxidative stress and quiescence to the CWI pathway and to the general stress response through Rho1 and the inhibition of either the *TOR1* or *RAS2* functions. These mechanisms would be required to allow cells to adapt to both oxidative and nutritional stresses.

Organisms living in aerobic conditions are exposed to reactive oxygen species that provoke cellular damage and, as a consequence, various human diseases. The eukaryotic microorganism *Saccharomyces cerevisiae* serves as a model system in which to study the signal transduction pathways involved in the response to oxidative stress. In budding yeast the MAPK[3] pathways are not well characterized for sensing and transmitting oxidative stress to the cytoplasm and nuclear elements. However, Mtl1 has already been characterized as a receptor for oxidative stress (1). Mtl1 was initially identified as a yeast homologue of Mid2 by Rajavel *et al.* (2) who observed that it performed a function in cell integrity signaling involved in vegetative growth. De Bettignies *et al.* (3) identified *MTL1* as a suppressor of *rgd1* mutants, with Rgd1 being a GTPase-activating protein of Rho3 and Rho4. *MTL1* was also identified by Sekiya-Kawasaki *et al.* (4) as a multicopy suppressor of Rho1.

The cell integrity pathway in budding yeast involves a protein kinase (MAPK) cascade that participates in sensing and transmitting several extracellular signals and stresses that include cell-wall, osmotic, mating, and nutritional stress (5, 6) and, more recently, oxidative (1) and pH (7) stresses. The PKC1-MAPK pathway is integrated by several cell-wall proteins that are putative cell-membrane receptors of different stimuli; they are the Wsc1-Wsc4 family, Mid2, and Mtl1. They transmit signals to Rom2, which activates the G protein Rho1, which in turn activates the kinase Pkc1 (a protein that has high degree of homology with other isoforms of PKC in eukaryotic cells). Pkc1 activates a mitogen-activated protein kinase module: Bck1 (that is the MAPKKK) phosphorylates the redundant MAPK kinases Mkk1 and Mkk2, and together they activate Slt2, the last kinase member of the pathway. Two downstream events correlate with Slt2 activation: transcriptional activity driven by Rlm1 and Swi6 phosphorylation (5, 6). The upper elements of the CWI pathway are involved in the organization of the actin cytoskeleton under different conditions that include cell-wall and nutritional stresses (8–10), oxidative stress (1), and pH (11).

The Pkc1 pathway is also related to the TOR pathway. Budding yeasts have two different TOR genes, *TOR1* and *TOR2*, that share 67% sequence identity and are partly redundant in function (12). Loewith *et al.* (13) have characterized two distinct TOR complexes, TORC1 and TORC2. TORC1 is sensitive to rapamycin, modulates translation initiation, inhib-

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[1] Both authors were supported by fellowships from the Generalitat de Catalunya (Spain).

[2] To whom correspondence should be addressed. Tel.: 34-973702410; E-mail: madelatorre@cmb.udl.es.

[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PKA, protein kinase A; CWI, cell wall integrity; wt, wild type; GFP, green fluorescent protein.

its protein turnover, and represses the transcription of genes related to nutrient starvation. TORC2 is closely related to the organization of the actin cytoskeleton and independent of rapamycin inhibition (13–16). Tor2 functions in both complexes, whereas Tor1 only participates in the TORC1 complex. The cellular function regulated by TOR contains a general mechanism; that is, the sequestration of the transcription factors Msn2/Msn4, Gln3 (17), and Rtg1/Rtg3 (18, 19) in its cytoplasm. The Tor function also regulates ribosomal protein expression in response to environmental conditions via PKA (20).

Ras signals via the cAMP-PKA pathway regulate the cellular metabolism in response to the carbon source (21, 22). Two Ras proteins are present in budding yeast and are encoded by the redundant small GTPases *RAS1* and *RAS2* (RAS) (23). The Ras-cAMP-PKA pathway also negatively regulates Msn2/Msn4 nuclear localization (24–26).

The Msn2/Msn4 transcription factor binds and activates genes containing the stress response element (CCCCT) in response to a wide variety of stresses, including nutritional, osmotic, acidic, and oxidative stress (17, 24, 27, 28).

In recent years several studies have been published that demonstrate the relationship between the TOR and cAMP-PKA pathways. Some authors have suggested that the RAS/cAMP pathway could be a novel TOR effector branch (29). Both the TOR and cAMP-PKA pathways regulate the expression of genes needed to overcome the diauxic and stationary phases (30, 31) and in whose regulation Msn2/Msn4 transcriptional activity has been reported to be essential (32). They also coregulate the expression of genes involved in fermentation and aerobic respiration (33). The CWI pathway is also required for viability in quiescence as Slt2 phosphorylation is necessary for cells to survive in stationary phase and upon rapamycin treatment (10, 34).

Here, we have employed a genomic approach to identify possible novel regulatory functions for the gene *MTL1* and have demonstrated that Mtl1 signals toward Msn2/Msn4 transcriptional function in response to various stresses such as rapamycin, oxidative stress, and glucose starvation. We have also presented evidence to demonstrate that the Mtl1 function is required to down-regulate Tor1 and Ras2 activity upon glucose starvation and oxidative stress. This down-regulation is necessary to ensure cell viability in response to both types of stress. We show that Mtl1 is the member of the CWI pathway that acts as a sensor for oxidative stress and glucose starvation by activating the kinase Slt2. However, Slt2 activation is only visibly important for cell viability in conditions of quiescence.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—Yeasts were grown in SD medium (2% glucose, 0.67% yeast nitrogen base, and the required amino acids) (35). Diamide, hydrogen peroxide, caffeine, and rapamycin were purchased from Sigma. Diamide was dissolved in DMSO. Hydrogen peroxide was prepared in sterile distilled water.

Yeast Strains and Gene Disruptions—The yeast strains used in this study are listed in Table 1. *MTL1* was disrupted either by the one-step disruption method using the *kan*MX4 module, as described in Vilella *et al.* (1), or by using the *nat*MX4 module (36). *RAS2* and *TOR1* were disrupted by the same procedure but using the *LEU* marker.

TABLE 1
Yeast strains used in this work

Strain	Relevant genotype	Reference
CML128	*MAT**a** leu2-3,112 ura3-52 trp1 his4 can1*^{r}	Gallego *et al.* (39)
GSL41	*MAT**a** mtl1::kan MX4*	This work[a]
GSL34	*MAT**a** tor1::kanMX4*	This work[a]
GSL48	*MAT**a** mtl1::kan MX4 tor1::LEU2MX5*	This work[a]
GSL53	*MAT**a** ras2::LEU2MX5*	This work[a]
GSL54	*MAT**a** mtl1::kan MX4 ras2::LEU2MX5*	This work[a]
W303-1A	*MAT**a** ade2-1, trp1-1, leu2-3,2-111, his3-11,75, ura3*	C. Gancedo
msn2msn4	*MAT**a** ade2 can1 his3 leu2 trp1 ura3 msn2-delta3::HIS msn4-1::TRP1*	F. Estruch

[a] CML128 background.

DNA Manipulation and Plasmids—Plasmid pPkc1 contains Pkc1 under the *tet*O7 promoter and is also tagged with the hemagglutinin epitope, as initially described in Angeles de la Torre-Ruiz *et al.* (37) under the name of pMM126. Plasmid pSlt2 is a YEP352 derivative containing the Slt2 ORF under its own promoter and tagged with hemagglutinin in the C terminus; this plasmid was described in Vilella *et al.* (1). In this study, we used the pSlt2 plasmid for overexpression analysis and also to determine total Slt2 protein using the anti-hemagglutinin monoclonal antibody.

The plasmid p*BCK1-20* is a pRS413 derivative bearing the constitutively activated *BCK1–20* allele (38). The plasmid pRho1* contains the *LEU* marker and bears the Rho1his368 allele, which is constitutively activated, bears the hemagglutinin epitope in the C-terminal position, and is cloned under the Gal1 promoter. This plasmid was kindly provided by Dr. Tobias Schmelzle.

The plasmid pMsn2-GFP contains the *LEU* marker, cloned under the ADH1 promoter in a centromeric plasmid, as originally described in Görner *et al.* (44). This plasmid was kindly provided by Dr. Francisco Estruch. This plasmid was originally described as functional, but we checked its functionality in an *msn2msn4* background. We demonstrated that it efficiently suppresses both the lack of viability and the deficiency in the transcription of *HSP12* and *CTT1* that were observed in the double mutant *msn2msn4* in response to the stresses used in this study.

Microarray Experiments and Data Analysis—For microarray analysis, wild type and *mtl1* cells were grown in SD minimum medium at 30 °C to a final A_{600} 0.6. Total RNA from *S. cerevisiae* was extracted from 25 ml of each culture by mechanical disruption following the instructions for the RNeasy Midi kit manufacturer (Qiagen). RNA concentration was measured at 260 nm, and sample quality was checked using RNA Nano Labchips in a 2100B Bioanalyzer (Agilent Technologies, Palo Alto, CA).

For each experimental condition (wt and *mtl1*Δ strain), two microarray experiments corresponding to two biological replicates were processed and analyzed. Double-stranded cDNA was synthesized from 5 μg of total RNA using a "One-cycle cDNA Synthesis Kit" (Affymetrix, Santa Clara, CA).

After cDNA purification using the "GeneChip Sample Cleanup Module" (Affymetrix), this DNA was used as a template for the *in vitro* transcription to obtain the biotin-labeled cRNA. The cRNA obtained was fragmented and hybridized to the Affymetrix GeneChip® Yeast Genome S98 array for 16 h at 45 °C. Hybridized microarrays were washed and stained with a streptavidin-phycoerythrin conjugate in a GeneChip® Fluidics Station 450. All these procedures were carried out as suggested by the manufacturer. Hybridized cRNA was finally identified by the fluorescence signal in a GeneChip® 3000 scanner.

After scanning, numerical data were obtained and processed with GCOS software (Affymetrix). We evaluated different parameters that accounted for the quality of the hybridization. The noise value was less than 2.51 (commonly between 1.5 and 3), the scaling factor was between 0.22 and 0.41, and the background was between 107 and 50.6. The arrays showed percentages of "Present" probe sets ranging between 72.3 and 79.5. The signals from the arrays were linearly scaled to an average signal value of 100.

Data from mutant and WT strains were compared in pairs, obtaining a total of four comparisons. For further analysis, a selection of significant results was carried for each comparison using the statistical criteria implemented by the GCOS software. Those genes labeled as "Absent" by the detection algorithm (using a p value of < 0.04) in both arrays of a comparison were discarded for the analysis. Those genes that gave "Increase" or "Decrease" values, according to the change algorithm (using a p value of <0.0025) in all four comparisons were used for further analysis. The signal log ratio was obtained as the mean of the signal log ratio obtained in each of the four comparisons; genes with signal ratios of >2 or <0.5 were selected for additional analysis. Probes on the yeast genome S98 chips that did not correspond to specific open reading frames were disregarded in this analysis.

RNA Preparation and Northern Blot Analyses—RNA purification, Northern blot, and probe labeling with digoxygenin were carried out according to Gallego *et al.* (39). Probes covering the entire open reading frame, without adjacent sequences, were generated by PCR from genomic DNA.

Yeast Extracts and Immunoblot Analyses—Both methods were performed as described in Angeles de la Torre-Ruiz *et al.* (37). The methodology for the use of both anti-phospho-p44/42 and anti-Swi6 antibodies was that previously described in Angeles de la Torre-Ruiz *et al.* (37). Horseradish peroxidase-linked anti-rabbit (NA931, Amersham Biosciences) was used at a 1:10,000 dilution in Tris-buffered saline-Tween buffer containing 1% milk fat. In all cases chemiluminescent detection was performed using the Supersignal substrate (Pierce) in a Lumi-Imager (Roche Applied Science).

cAMP Assay—The Amersham Biosciences cAMP Biotrak Enzyme-immunoassay system was used to determine intracellular levels of cAMP.

Strains wt, *mtl1*, *ras2*, *ml1ras2*, *tor1*, *mtl1tor1* were grown in SD selective media at 30 °C. We performed the experiment using exponentially growing cultures (A_{600} 0.6) and stationary cultures grown for up to 3 days. We basically followed the method described by Swiegers *et al.* (40) to obtain the samples. A total of 40 ml of each of the exponentially growing cultures (and the corresponding amount of cells for the stationary cultures) was harvested by centrifugation at 3000 rpm at room temperature for 4 min. After that, the wet weight was determined, the cells were subsequently resuspended in 300 μl of lysis buffer 1B (Amersham Biosciences), and 150 μl of glass beads were added. The suspension was vortexed for 30 min at 8 °C and then spun down for 10 min at 4 °C at 12,000 rpm. The supernatant (100 μl) was used for the protocol; that is, intracellular cAMP measurement using the non-acetylation enzyme-immunoassay procedure with the novel lysis reagents described in the kit.

RESULTS

The Mtl1 Function Is Required to Maintain the Basal Transcription of a Cluster of Genes Regulated by Msn2/Msn4—Our group previously demonstrated that Mtl1 functions as a cell-surface sensor for oxidative stress (1). To gain a further insight into the role that Mtl1 plays in cell integrity, we decided to analyze the genome-wide gene expression profile of the *mtl1* mutant strain. The transcriptional profile of this mutant growing in SD medium was compared with the wild type strain CML128 under the same conditions using Affymetrix GeneChip® Yeast Genome S98 arrays, as detailed under "Experimental Procedures." Microarray analysis of this profile revealed the presence of 102 genes that were repressed (-fold $\leq$0.5) (supplemental Table S1) and 13 that were induced genes (-fold $\geq$2) (supplemental Table S2) as a consequence of the *MTL1* deletion. A functional classification of these genes was carried out according to MIPS and GO Databases using FUNSPEC. This analysis revealed the presence of several statistically significant functional groups (p value less than 0.01) within the repressed response. These included electron transport and energy uptake, stress response, and sugar and aromatic compound utilization among others (supplemental Table S3). Interestingly, and as deduced from the YEASTRACT data base, an important group of the repressed genes was potentially regulated by Msn2/Msn4 (34 of 102 repressed genes); most of these related in one way or another to the stress response (Table 2).

In an initial characterization of Mtl1 we focused our efforts on studying the possible relationship between Mtl1 and Msn2/Msn4. First, and to validate our data, we performed Northern blot assays with samples taken from *mtl1* and wt strains growing exponentially. We then tested *HSP12*, *GRE1*, *TRX3*, and *DDR2* gene expression, as they are potentially regulated by the transcription factor Msn2/Msn4. In all cases we observed that the basal levels of these transcripts were lower in the *mtl1* mutant than in the wt cells; this confirmed the results obtained in the genomic study described above and validated our genomic analysis (Fig. 1*A*).

Mtl1 Regulates the Msn2 Transcriptional Function in Response to Rapamycin, Glucose Starvation, and Oxidative Stress—To ascertain the physiological significance of the previously mentioned results, we proceeded to screen *mtl1* survival under different conditions, inferred from *mtl1* global transcription analysis. We hypothesized that a possible cause of the gene

TABLE 2
Genes down-regulated in *mtl1* strain, which are potentially regulated by Msn2/Msn4

Gene/open reading frame	Function	Biological process
ALD3	Aldehyde dehydrogenase activity	Stress response
CTT1	Catalase activity	
DAK1	Glycerol kinase activity	
DDR2		
GAD1	Glutamate decarboxylase activity	
GRE1		
HSP12		
HSP26	Unfolded protein binding	
MCR1	Cytochrome-b_5 reductase activity	
SIP18	Phospholipid binding	
SSA4	Unfolded protein binding	
TRX3	Thiol-disulfide exchange intermediate activity	
PHM7		Unknown
SOL4	6-Phosphogluconolactonase activity	
NQM1	Transaldolase activity	
YHR033W		
YKL151C		
AIM33		
YMR090W		
AGX1	Alanine-glyoxylate transaminase activity	Amino acids
POT1	Acetyl-CoA C-acyltransferase activity	Lipid metabolism
TES1	Acyl-CoA thioesterase activity	
TKL2	Transketolase activity	Carbohydrates
YJR096W	Aldehyde reductase activity	
TSA2	Thioredoxin peroxidase activity	Homeostasis
ECM4	Glutathione transferase activity	Cell wall
FMP45		
TFS1	Lipid binding/protease inhibitor activity	Protein catabolism
VPS73	Substrate specific transmembrane transporter activity	Transport
SPS100		Sporulation
HBT1		Conjugation
YLR294C	Hydrogen-transporting ATP synthase	Respiration
GTT1	glutathione transferase activity	Others
YDL124W	α-Keto amide reductase activity, α-keto ester reductase activity	

down-regulation observed in the *mtl1* background was a defect in the Msn2/Msn4 function. As the Msn2/Msn4 is a transcription factor involved in the general stress response, we decided to screen the survival response of the *mtl1* mutant to various stresses in which Msn2/Msn4 is involved: rapamycin, hydrogen peroxide, and glucose deprivation. In Fig. 1*B* we show that Mtl1 is needed for cell survival in response to rapamycin, hydrogen peroxide, caffeine, and carbon deprivation, whereas in stationary phase (not shown), cell survival is undistinguishable from wt cell. All these phenotypes are consistent with a defect affecting the general stress response; they also resemble those already described for *msn2msn4* mutants (41, 42). We next analyzed the transcriptional regulation of *CTT1* and *HSP12* in wt and *mtl1* mutant cells growing exponentially (time 0) or treated either with rapamycin or hydrogen peroxide or deprived of glucose (Figs. 1, *C* and *D*). We observed that the *mtl1* mutant was deficient in the transcriptional induction of *HSP12* and *CTT1* upon treatment with rapamycin or peroxide or glucose deprivation. The regulation of these genes under these conditions was exerted by the Msn2 function because *HSP12* and *CTT1* genes were not induced in response to the three conditions in an *msn2msn4* mutant (Fig. 1*E*). Moreover, we overexpressed Msn2 in both wt and *mtl1* strains and observed that higher Msn2 levels were sufficient to complement the defects in *HSP12* and *CTT1* transcription (both regulated by Msn2/Msn4) in *mtl1* cells in response to all the treatments to almost wild type levels (Fig. 1, *C* and *D*). Msn2 overexpression was also able to rescue the defect in *mtl1* cell growth provoked by each of the previously mentioned stress types (Fig. 1*B*). Moreover, and in line with the previously mentioned results, Msn2 overexpression also restored the logarithmic levels of *HSP12* and *CTT1* transcription (Fig. 1, *C* and *D*). In view of these results and given that in the absence of Mtl1 function a wide cluster of genes regulated by Msn2 were down-regulated with respect to the wild type strain, we concluded that Mtl1 signals to Msn2 and regulates its function in response to the blockade of the TOR function, hydrogen peroxide, and glucose deprivation.

It is well known that Msn2 has a cytoplasmic localization when cells grow exponentially in rich medium and also that Msn2 translocates to the nucleus in response to several types of stress. We decided to investigate how the lack of Mtl1 function affected the subcellular localization of Msn2 to find an explanation for the transcriptional deficiency observed in the *mtl1* mutant. To do this, we studied *in vivo* GFP-Msn2 localization in *mtl1* mutant cells. In response to either hydrogen peroxide or rapamycin, Msn2 translocates to the nucleus in both wt and *mtl1* cells. However, in *mtl1* cells, we observed a significant delay in Msn2 translocation to the nucleus compared with wt cells (Fig. 2*A*). The timing of Msn2 translocation from the cytoplasm to the nucleus in response to stress has been attributed to RAS activity, whereas Msn2 translocation from the nucleus to the cytoplasm has been reported to be mainly dependent on TOR activity (30). PKA prevents the nuclear import of Ms2/Msn4, whereas the TOR function controls their nuclear export (43, 44). We also checked the mRNA levels of *MSN2* in wild type, *mtl1*, *ras2*, *mtl1ras2*, *tor1*, and *mtl1tor1* cells. In the mutant *mtl1* we were able to observe a reduction in *MSN2* basal transcription in exponentially growing cells with respect to wild type levels. However, deletion of either *RAS2* or *TOR1* in the *mtl1* background restored the steady state transcription of *MSN2* to wild type levels (Fig. 2*B*). These results together with the others described above led us to investigate the possible relationship between Mtl1 and the TOR and Ras pathways.

Mtl1 Is Genetically Related to Tor1 and Ras2 in the Regulation of the Msn2 Function—It has been reported that Tor signals to Ras regulating the Msn2 function (29). As the *mtl1* mutant presented high sensitivity to rapamycin (a macrolide that specifically inhibits the TORC1 function) and as the induction of both *HSP12* and *CTT1* provoked by this drug was dependent on Mtl1, we next decided to investigate the relationship between the TORC1 complex and Mtl1. In a first approach we constructed an *mtl1tor1* double mutant and observed that this strain exhibited greater sensitivity to rapamycin than either of the two single mutants: *tor1* or *mtl1* (Fig. 3*A*). However, when we tested for glucose starvation or hydrogen peroxide sensitivity, we observed that both the *tor1* and *mtl1tor1* strains presented similar levels of sensitivity, although both were less sensitive than the single mutant *mtl1* (Fig. 3*A*). We next analyzed the transcriptional pattern in these strains and observed that *tor1* deletion restored the induction of both *HSP12* and *CTT1* in *mtl1* to almost wild type levels in response to oxidative stress and rapamycin and glucose starvation (Figs. 3, *B–D*). Because the TORC1 function also regulates ribosomal gene

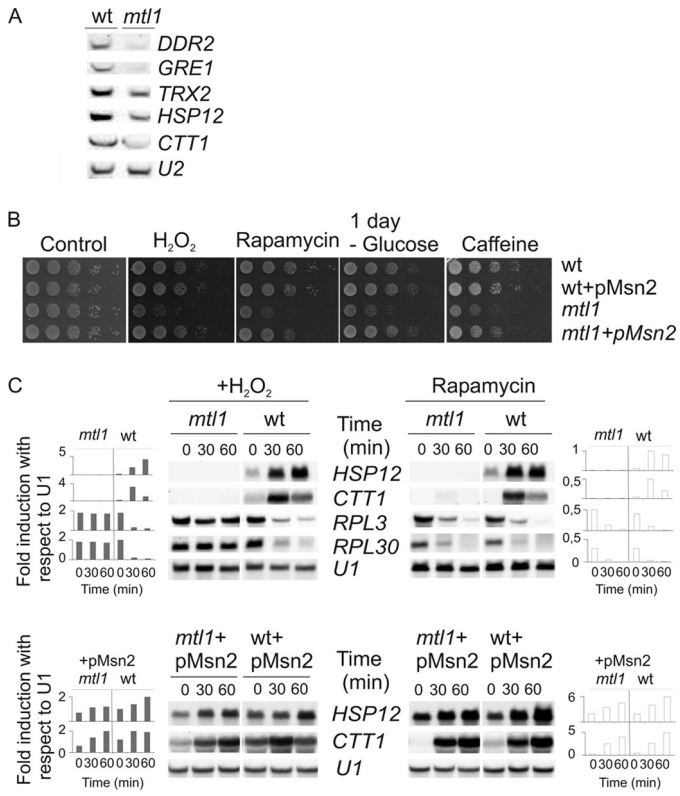


FIGURE 1. **Mtl1 is required for survival in response to various types of stress and it is essential for the regulation of the Msn2 transcription function.** *A*, shown is Northern blot analysis of wt and *mtl1* samples growing in SD medium at 30 °C to logarithmic phase using *DDR2*, *GRE1*, *TRX2*, *HSP12*, and *CTT1* probes and U2 as a loading control. *B*, exponentially growing cultures of wt (CML128), *mtl1*, wt + pMsn2, and *mtl1* + pMsn2 were serial-diluted and spotted onto plates containing 1 mM H_2O_2, 1 ng/ml rapamycin, and 5 mM caffeine. In parallel, aliquots were taken, washed four times with equivalent volumes of SD minus glucose, and incubated in fresh SD medium without glucose for 1 day before being serial diluted and spotted onto YPD plates. Growth on the plates was allowed to take place for 3 days at 30 °C. *C*, Northern blot analysis of wt, *mtl1*, wt + pMsn2, and *mtl1* + pMsn2 samples growing in SD medium at 25 °C to logarithmic phase and then treated with either 1 mM H_2O_2 or 200 ng/ml rapamycin is shown. Samples were collected and processed at the indicated times using *HSP12*, *CTT1*, *RPL3*, and *RPL30* probes and *U1* as a loading control. Histograms represent the -fold induction normalized with respect to the corresponding *U1* value for each of the genes analyzed. *D*, experiments were as in *B*, except that exponentially growing cells were washed four times with SD minus glucose and then transferred to fresh SD medium minus glucose. Samples were collected at the indicated times. Northern blot analyses of exponentially growing wt and *mtl1* cultures. *Histograms* represent the -fold induction normalized with respect to the corresponding *U1* value for each of the genes analyzed. *E*, Northern blot analyses of wt and *msn2msn4* growing exponentially and then treated either with H_2O_2 or rapamycin or starved of glucose as in *C* and *D*. *d*, day.

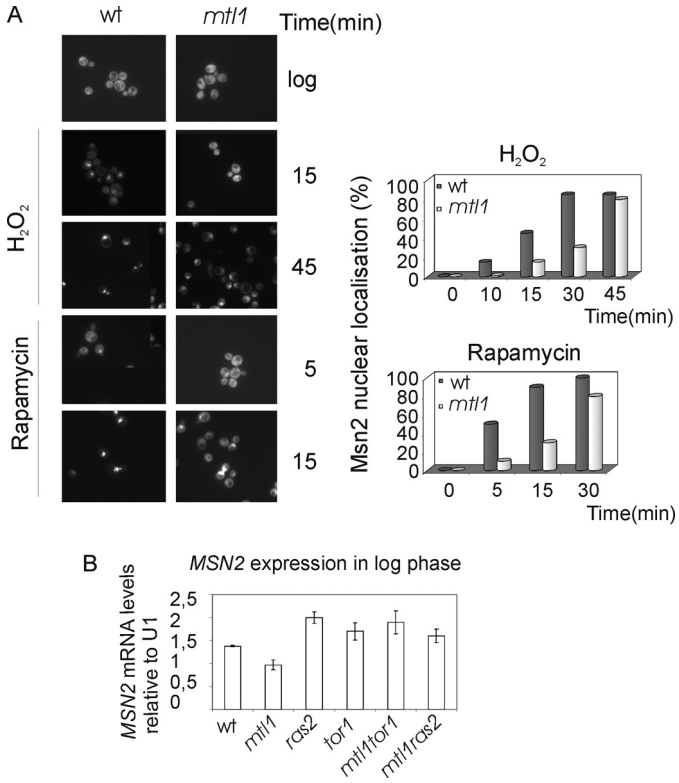


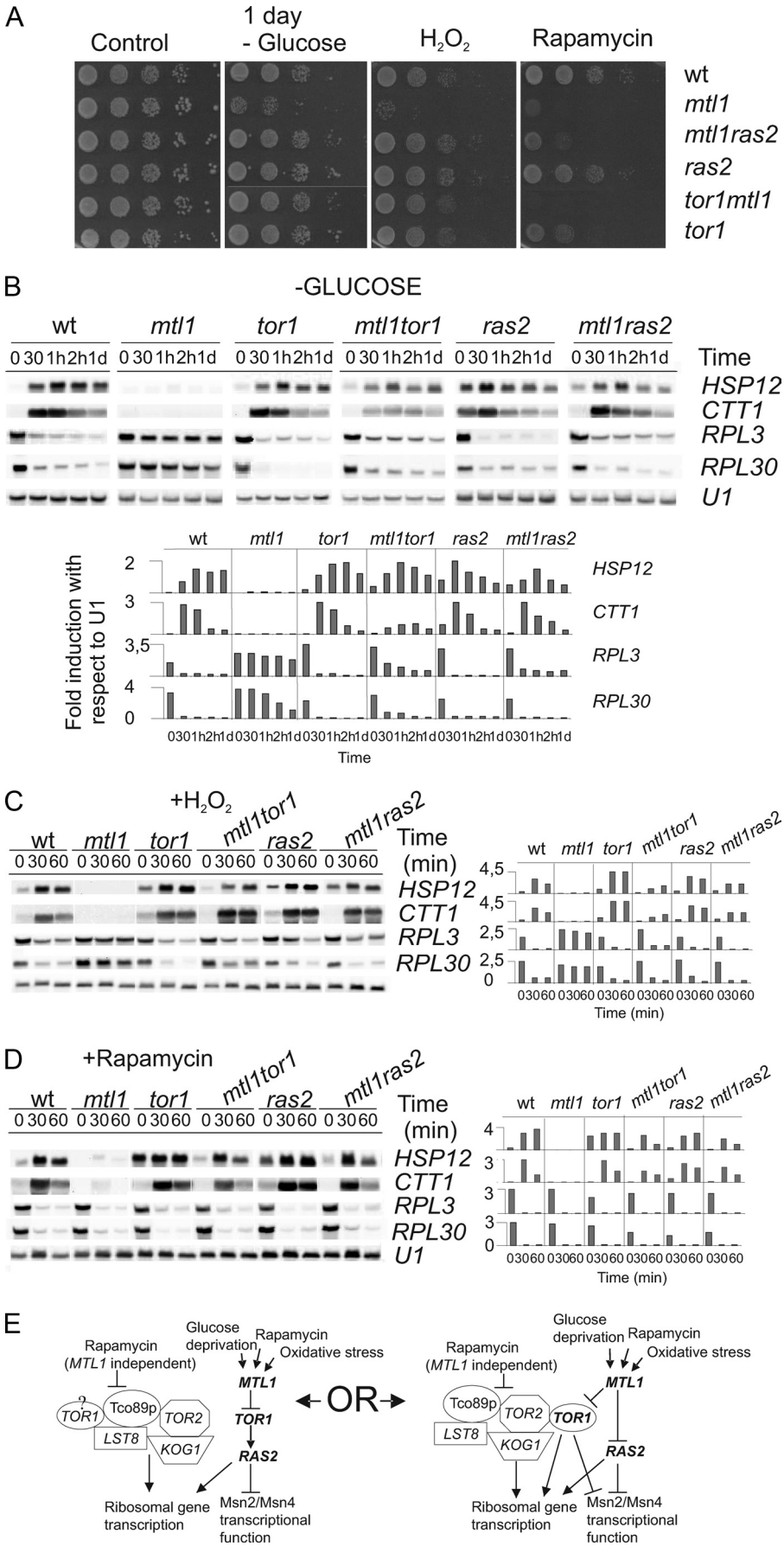
FIGURE 2. **Msn2 regulation in different mutants.** *A*, shown is the Msn2 location in response to hydrogen peroxide and rapamycin. wt and *mtl1* cells were transformed with a multicopy plasmid carrying Msn2 fused to GFP in the C terminus and under the control of the ADH1 promoter as described under "Experimental Procedures." Cultures were grown in SD minimum medium at 30 °C to logarithmic phase and then treated with rapamycin and hydrogen peroxide for the times indicated in the figures. *Histograms* represent the percentage of cells with Msn2 localized in their nuclei counted from a total of 300 cells. We have chosen a representative experiment from three repetitions that were significantly similar. *B*, *histograms* represent the *MSN2* mRNA levels calculated in wt, *mtl1*, *ras2*, *mtl1ras2*, *tor1*, and *mtl1tor1* cells growing exponentially in logarithmic phase and normalized with respect to the values obtained using a *U1* probe as a loading control. *Error bars* represent S.D. calculated from three repetitions.

expression, we decided to analyze the transcription of two ribosomal genes: *RPL3* and *RPL30* in wt, *mtl1*, *tor1*, and *tor1mtl1* cells under all the stress conditions used in the current study (Fig. 3, *B–D*). As expected and in accordance with previous studies, we observed clear ribosomal gene repression in wt,

tor1, *mtl1*, and *tor1mtl1* cells in response to the rapamycin treatment. Additionally, both the *mtl1* and *tor1mtl1* mutants presented similar responses to wild type cells; this indicated that Mtl1 was not required to repress ribosomal gene transcription when the TOR function was blocked by rapamycin (Fig. 3*E*). Our results suggest that both *TOR1* and *MTL1* regulate a common process in response to rapamycin and also that at least one of the two genes is required for cells to survive upon rapamycin treatment, although they are in different pathways. *MTL1* and *TOR1* signal to Msn2, suggesting that *MTL1* could signal *TOR1* inhibition for the regulation of the Msn2 function in response to the blockade of the TORC1 function (Fig. 3*E*). However, Mtl1 is not involved in the ribosomal gene repression mediated by rapamycin treatment.

In response to the other two stresses, oxidative stress and glucose depletion, the *mtl1* mutant was unable to induce ribosomal gene repression. Interestingly, in the double mutant *tor1mtl1*, the repression was restored to almost wild type levels (Fig. 3, *B–C*). These results, therefore, suggest that Mtl1 requires Tor1 repression to transmit the signal for glucose starvation and hydrogen peroxide stress to downstream effectors that regulate both ribosomal gene expression and the Msn2/Msn4 function (Fig. 3*E*).

Because it has been previously reported that TOR signals to Ras/cAMP for the regulation of ribosomal gene expression and the Msn2/Msn4 function (29), we further investigated the possible relationship between Mtl1 and Ras2. We constructed both *ras2* and *mtl1ras2* double mutants and obtained similar results to those already described above with respect to the interaction between Tor 1 and Mtl1. In the double mutant *mtl1ras2* we observed (i) restoration of *mtl1* viability after rapamycin treatment, oxidative stress or glucose deprivation, (ii) restoration of Msn2/Msn4 wild type activity (as



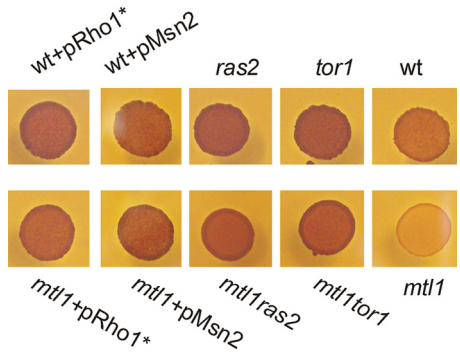


FIGURE 4. **Glycogen accumulation in the different strains: wt, wt+p*RHO1***, wt+pMsn2, *ras2*, *tor1*, *mtl1*+*pRHO1**, *mtl1*+pMsn2, *mtl1ras*, and *mtl1tor1*.** Cells were grown on SD medium to logarithmic phase then spotted onto SD medium plus amino acids to be grown for 5 days. A solution of 0.2% iodine/0.4% potassium iodide (Swiegers *et al.* (40)) was poured over the spots, and photographs were taken 3 min later. The darker the color, the more glycogen was accumulated, and the lighter the color, the less glycogen was present. Glycogen provides an indirect measurement of cAMP/PKA activity.

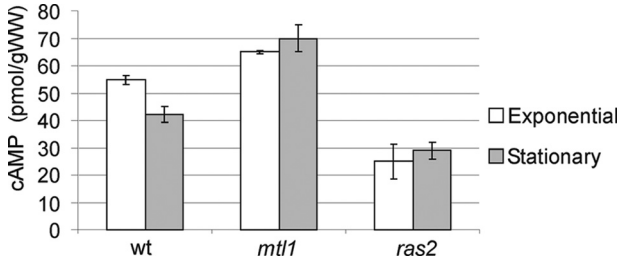


FIGURE 5. **Intracellular cAMP in different cultures.** wt, *mtl1*, and *ras2* strains were grown either exponentially or to stationary phase (3 days of incubation at 30 °C) in SD medium plus amino acids and harvested for cAMP extraction and detection as described under "Experimental Procedures." Values are the average of three independent experiments (*error bars* represent the S.D.). With respect to both conditions, exponential and stationary phases, the three strains (wt, *mtl1*, and *ras2*) were significantly different from one another, with a $0.01 < p < 0.02$. These values were calculated upon performing two-tailed *t* tests.

deduced from the levels of expression of both transcripts, *CTT1* and *HSP12*), and (iii) ribosomal gene repression similar to that associated with wild type and *ras2* cells (Fig. 3, *B–D*). Thus, the deletion of Ras2 restored the majority of the phenotypes exhibited by the *mtl1* cells to almost wild type levels in response to both oxidative stress and glucose depletion. Our results, therefore, suggest a role for Mtl1 in transmitting the signal for oxidative stress and glucose starvation to Msn2/Msn4 and also in the down-regulation of Ras2 functions (Fig. 3*E*).

The Absence of Either Tor1 or Ras2 Restores the Defects in the Accumulation of Glycogen Observed in the mtl1 Cells—Accumulation of glycogen occurs when cells are starved of nutrients. It has been demonstrated that both the TOR and Ras-cAMP pathways control glycogen homeostasis in cells (45–47). Part of this effect is due to the fact that Msn2/Msn4 regulates the transcription of a subset of genes required for glycogen synthesis (48, 49). Because the absence of the Mtl1 function impaired the activity of Msn2/Msn4, we tested whether *mtl1* cells presented any defects in the accumulation of glycogen. We confirmed our hypothesis and demonstrated that *mtl1* was defective in the accumulation of the carbon storage source glycogen (Fig. 4). Interestingly, *TOR1* and *RAS2* deletion and also Msn2 overexpression restored the capacity to accumulate glycogen in *mtl1* cells. This supports the hypothesis that Mtl1 signals the inhibition of Tor1 and Ras2 and consequently contributes to the activation of the Msn2 function in response to quiescence (Fig. 4).

Mtl1 Affects Intracellular Levels of cAMP—Mtl1 is genetically related to Ras2 function. This is a logical consequence of what would be an additional relationship with the PKA pathway that should be reflected in cAMP intracellular levels. We decided to determine the effect of deleting Mtl1 on cellular cAMP levels. The levels of cAMP were determined in wild type, *ras2*, and *mtl1* strains during exponential growth and in stationary phase. Under both conditions, *mtl1* cells showed higher levels of cAMP than those determined in wild type cells (Fig. 5). Deletion of *ras2* caused a significant decrease in cAMP levels compared with wild type cells. Taking all these results together we can conclude that Mtl1 regulates cAMP levels through the down-regulation of the *RAS2* function.

Involvement of the Pkc1-MAPK Pathway in the Stress Response Mediated by Mtl1—Mtl1 is a component of the cell integrity pathway and a sensor of oxidative stress. We wondered whether the effect that Mtl1 has on both Tor1 and Ras2 was also mediated by elements of the Pkc1-MAPK pathway. To ascertain this, we checked the functional relationship between Mtl1 and several members of the pathway. We first checked whether Rho1 was involved in this response. To do this we used a constitutively activated Rho1 allele in a centromeric plasmid under the control of the GAL1 promoter and transformed both wild type and *mtl1* cells. As described in legend to Fig. 6, glucose repressed Rho1 expression (Fig. 6*A*), whereas galactose, the sole carbon source, induced Rho1 overexpression (Fig. 6*A*, *middle panel* corresponding to galactose). As shown in Fig. 6, expression of the hyperactive *RHO1* allele in the *mtl1* mutant strain led to (*a*) an increase in cell-survival in response to hydrogen peroxide (Fig. 6*A*) but not to rapamycin, (*b*) the partial restoration of the transcriptional induction of *CTT1* and *HSP12* (Fig. 6, *B* and *C*), and the repression of ribosomal genes under conditions of oxidative stress (Fig. 6*B*). We could not perform these experiments under conditions of glucose depletion because the expression of the *RHO1* hyperactivated allele was regulated by the Gal1 promoter. However, we were able to detect that when wild type cells were transferred from glucose to galactose there was induction of the genes regulated by Msn2/Msn4 (Fig. 6*B*). Interestingly, when we transferred *mtl1* cells from glucose to galactose (as the sole carbon source), we did not observe any

FIGURE 3. **The absence of either *TOR1* or *RAS2* restores cell viability, ribosomal gene expression, and transcriptional regulation dependent on Msn2 in the *mtl1* mutant to wild type levels.** *A*, cell viability was as in Fig. 1 but using the following strains: wt, *mtl1*, *ras2*, *ras2mtl1*, *tor1*, and *tor1mtl1*. *B*, shown are Northern blot analyses of the above-mentioned strains growing exponentially and then starved of glucose or treated with either H_2O_2 (*C*) or rapamycin (*D*), as described in Fig. 1. *Histograms* represent the -fold induction normalized with respect to the corresponding *U1* value for each of the genes analyzed. *E*, the schematic diagram shows two possible models, which could explain how Mtl1 signals to both *TOR1* and *RAS2* to regulate Msn2/Msn4 function in response to various stresses. *d*, day.

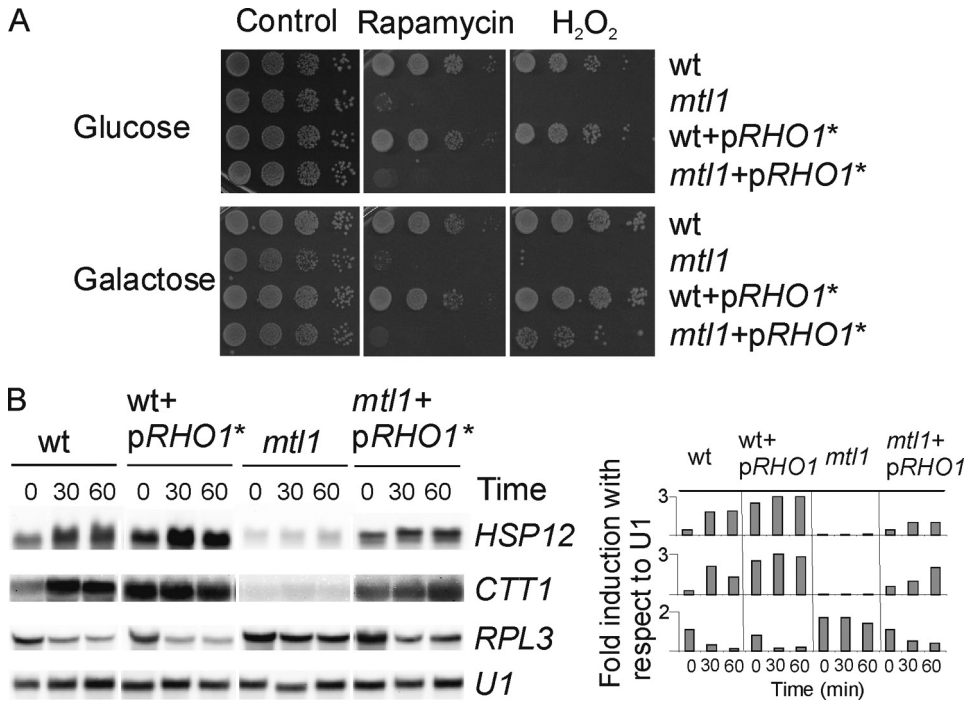


FIGURE 6. **Hyperactivation of Rho1 restores *mtl1* cell viability, transcriptional induction being dependent on Msn2 and the ribosomal gene transcription in *mtl1* cells in response to hydrogen peroxide but not in response to rapamycin.** *A*, serial dilutions of exponentially growing cultures of wild type, *mtl1*, wt+p*RHO1**, and *mtl1*p*RHO1** were grown in SD plus amino acids at 25 °C. Half of the cultures were serial-diluted and directly plated onto SD plates containing either hydrogen peroxide or rapamycin (*first line* in Fig. 5*A*). The other half was washed four times with two volumes of minimum medium containing galactose as the sole carbon source and then transferred to minimum medium containing galactose to induce the expression of *RHO1** for 12 h of induction. After this period cells were serial-diluted and plated onto SD plates containing hydrogen peroxide or rapamycin (*second line* in Fig. 5*B*). *B*, shown is a Northern blot analysis of the strains growing logarithmically in SD minimum medium (wt and *mtl1* strains) or in minimum medium containing galactose as a carbon source, as described in *A* (wt+p*RHO1**, *mtl1*+p*RHO1**) and then treated with 1 mM hydrogen peroxide. Samples were collected after the indicated times as in Fig. 2*B*. *Histograms* represent the -fold induction normalized with respect to the corresponding *U1* value for each of the genes analyzed.

significant induction of the genes used to report Msn2 activity (*CTT1* and *HSP12*) with respect to the wild type cells, in which this shift induced the transcription of both genes. Importantly, overexpression of the *RHO1* hyperactive allele in *mtl1* cells not only rescued the transcriptional levels of Msn2-dependent genes to almost wild type levels but also the hyperactivation of the MAPK (Slt2) of the CWI pathway (Fig. 8*A*) when cells were transferred from glucose to galactose-containing medium and were subsequently treated with hydrogen peroxide. Furthermore, Rho1 also suppressed the deficiency in glycogen accumulation in *mtl1* cells (Fig. 4). Taking these results together, we hypothesize that the signaling from Mtl1 to Tor1 and Ras2 that we have characterized occurred through Rho1. In addition we were able to observe that Rho1 presented stress specificity, as it did not rescue *mtl1* cell viability in response to rapamycin treatment (Fig. 6*A*). We performed the same experiments by overexpressing Rom2 (a GAP for Rho1) and obtained equivalent results to those shown for Rho1 (data not shown).

To further analyze the role of the downstream elements in the CWI pathway involved in this signaling process, we used a constitutively activated allele of the MAPKKK (MAPK kinase kinase) *BCK1*, the allele *BCK1–20*, and also checked for overexpression of the last kinase in the pathway, Slt2 (data not shown). The results shown in Fig. 7 indicate that upon treatment with hydrogen peroxide or upon glucose depletion, neither the expression of the *BCK1–20* allele nor Slt2 overexpression (data not shown) was able to restore the following functions in the mutant *mtl1*; (i) cell viability (Fig. 7*A*), (ii) Msn2/Msn4 transcriptional function (Fig. 7*B*), and (iii) repression of ribosomal genes (Fig. 7*B*). Moreover, as in the case of the *RHO1** allele, the *BCK1–20* allele did not suppress the lethality caused by rapamycin in *mtl1* cells. All these results indicate that there is cross-talk between Mtl1 and Tor1 and the Ras-cAMP pathways through the Rho1 protein. However, the other elements in the CWI pathway downstream of Rho1 did not seem to play any significant role in the signaling from Mtl1 to TOR and Ras/cAMP. However, the constitutive activation of the pathway at the level of the kinase *BCK1* did restore cell viability in response to glucose depletion (Fig. 7*A*). This result stresses the role that the CWI pathway plays in quiescence.

Mtl1 Is Required to Activate the Pkc1-MAPk Pathway in Response to Hydrogen Peroxide and Glucose Starvation—As Mtl1 is a cell-wall sensor and a component of the CWI pathway, we decided to investigate the role of this protein in signaling through this pathway in response to hydrogen peroxide and glucose starvation. To check this we checked the phosphorylation of the MAPK Slt2 by Western blotting in wild type and *mtl1* cells treated with hydrogen peroxide or starved of glucose. As detailed in Fig. 8, the MAPK Slt2 became notably activated in wt cells in response to both stresses. However, the hyperactivation of Slt2 was clearly abrogated in *mtl1*-stressed cells.

To further demonstrate that Mtl1 transmits the signal to Slt2 through the Pkc1 pathway, these experiments were carried out in *mtl1* cells alternatively transformed with the *RHO1* hyperactive allele and the constitutively active *BCK1–20* allele. As shown in Fig. 8, *A* and *B*, hyperactivation of the pathway downstream from Mtl1 compensated for the absence of the receptor in the activation of Slt2 upon both oxidative and glucose depletion stresses. These results indicate that Mtl1 is involved in signaling the absence of glucose or oxidative stress caused by hydrogen peroxide and transmits it to Slt2 through the classical elements of the CWI pathway.

DISCUSSION

We have used a genomic approach to identify other functions in which the cell-surface protein Mtl1 could play a role. Having observed a large cluster of genes that were potentially regulated by Msn2 and down-regulated in the absence of Mtl1, we iden-

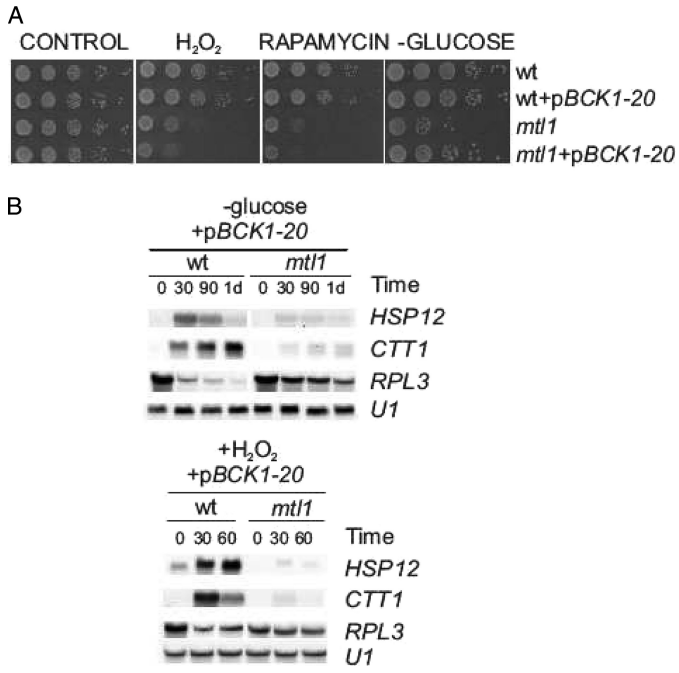


FIGURE 7. **Constitutive activation of *BCK1* only suppresses *mtl1* cell lethality upon glucose deprivation, but it does not suppress the transcriptional regulation of ribosomal genes or of the Msn2 genes that are dependent on *mtl1*cells in response to hydrogen peroxide or glucose deprivation.** *A*, shown are serial dilutions of exponentially growing cultures of wild type, *mtl1*, wt+*pBCK1-20*, and *mtl1*+p*BCK1-20* plated onto plates containing either hydrogen peroxide or rapamycin as in Figs. 1, 2, and 5. As in Fig. 1*B* and 2*A*, aliquots were taken, washed four times with equivalent volumes of SD minus glucose, and then incubated in fresh SD medium without glucose for 1 day to be subsequently serial diluted and spotted onto YPD plates. *B*, Northern blots with samples treated with hydrogen peroxide or starved of glucose as in Figs. 1 and 2 are shown. Samples were harvested at the indicated incubation times.

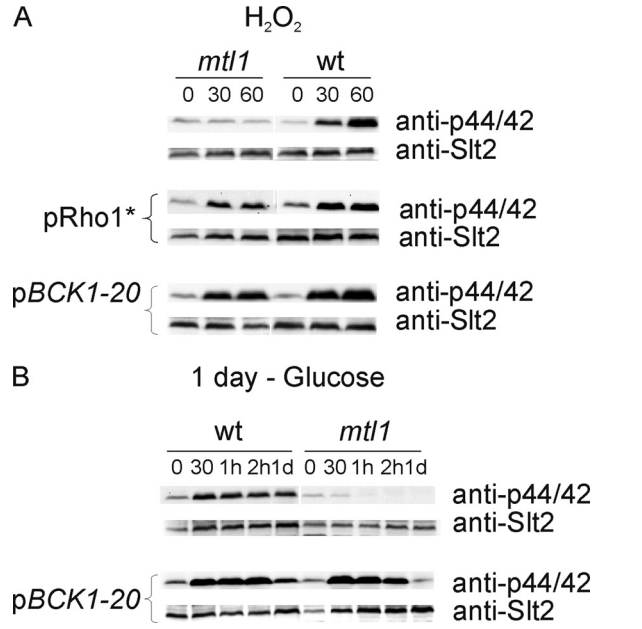


FIGURE 8. **Mtl1 is involved in signaling both oxidative stress caused by hydrogen peroxide and glucose starvation to the protein kinase Slt2 through elements of the Rho1 and Bck1 cell integrity pathways.** *A*, Western blot analysis of Slt2 activity using the p44/42 polyclonal antibody in samples from wt, *mtl1*, wt+p*RHO1**, *mtl1*+p*RHO1**, wt+p*BCK1-20*, and *mtl1*+p*BCK1-20* growing exponentially and treated with 1 mM hydrogen peroxide for the indicated times. For p*RHO1** overexpression we followed the same procedure as described in the legend to Fig. 5*A*, the previous hydrogen peroxide treatment. *B*, procedures were the same as in *A*, but the strains were exponentially grown in SD, washed four times with minimum medium without glucose, and then transferred to SD minus glucose to be incubated at 25 °C. Samples were collected at the indicated times for Western blot analysis. Anti-GSTSlt2 antibody was used to detect total Slt2 protein (not shown).

tified Msn2 as a possible target for Mtl1. The absence of Mtl1 makes cells sensitive to oxidizing agents, glucose starvation, and conditions in which the TORC1 function is compromised. This sensitivity is also associated with a notable defect in Slt2 activation. Msn2 overexpression was quite efficient at rescuing the loss of viability caused by the absence of the Mtl1 function in all the stress conditions studied here. It is very interesting to observe that a significant number of the Msn2-dependent genes that are down-regulated in the *mtl1* mutant are related to the oxidative stress response. This is perhaps not surprising as Mtl1 has previously been characterized as an oxidative stress sensor (1). However, how Mtl1 senses these stimuli currently remains unknown. Some authors (28) have reported that Msn2 transcriptional control is important for the response to hydrogen peroxide. This paper helps to extend our existing knowledge of this field by attributing Mtl1, a sensor for oxidative stress, with a novel and essential role in the transduction of the oxidative signal to Msn2. One possible explanation for this is that Msn2 controls the transcription of the genes required for the oxidative stress response. This occurs, for example, with *GAD1*, a gene required for cellular resistance to hydrogen peroxide, and with other oxidant agents (50).

Msn2 is known to be regulated by PKA activity (26, 45) and the TOR pathway (17). It is also generally accepted that sensitivity to rapamycin is specific to mutants in the TORC1 complex but not in the TORC2 complex (13). The mutant *mtl1* is sensitive to rapamycin, which is what connects it to the TORC1 complex. The observation that *TOR1* deletion restores the Msn2/Msn4 function in *mtl1* cells reinforces the hypothesis that Mtl1 acts as a possible negative regulator of *TOR1*. However, if this were the case, we should not expect any ribosomal gene repression to occur in the absence of Mtl1 as both TOR and Ras inhibition induce ribosomal gene repression (20, 34, 43, 51–53). The observation that rapamycin induced a marked repression of the ribosomal genes in *mtl1* mutant cells was not unexpected as rapamycin blocks the TORC1 function and not only *TOR1* activity. It has recently been reported that at least two independent branches of TORC1 are involved in the control of ribosomal protein genes (54). The presence of the other TORC1 components would be sufficient to signal to the ribosomal transcription independently of Mtl1. The synthetic lethality observed in the double mutant *tor1mtl1* upon rapamycin treatment suggests that the two proteins act in different pathways but probably perform a common regulatory function (see Fig. 3*E*). However, more studies will be required to elucidate the meaning of this genetic interaction.

Upon oxidative treatment or glucose starvation, Tor1 deletion was required to restore ribosomal gene repression and the Msn2/Msn4 function in *mtl1* cells. We consequently concluded that Mtl1 is a negative regulator of *TOR1* and acts specifically in response to glucose starvation and oxidative stress. We wondered whether Mtl1 also negatively regulates other TOR readouts such as Gln3 and Rtg1/Rtg3 transcriptional functions (17, 55).

We observed, however, that Mtl1 was not involved in such regulation in response to rapamycin (not shown).

It has been reported that RAS/cAMP may be a TOR effector branch (29). These authors reported that the activation of RAS causes 1) a deficiency in *HSP12* transcription in response to rapamycin and 2) no ribosomal transcriptional repression in response to TOR inactivation. One of our initial assumptions was, therefore, that the RAS/cAMP pathway could be improperly up-regulated in the *mtl1* mutant.

Deletion of Ras2 caused a significant decrease in cAMP levels, as expected, and as previously reported (by 56–58). We also obtained clear results indicating that cAMP levels were higher in *mtl1* cells than in those determined in wild type cells. It has been demonstrated that high PKA levels negatively regulate *MSN2* transcription. This could explain the lower transcription levels determined in *mtl1* mutants in comparison with wild type cells, and this would also be in accordance with the increase in the transcription of *MSN2* observed in both the *mtl1tor1* and *mtl1ras2* double mutants in the logarithmic phase. Taking all of these results together, we conclude that Mtl1 negatively affects cAMP levels through the regulation of the *RAS2* function and more specifically in response to both oxidative stress and glucose deprivation (as depicted in Fig. 3*E*).

Glucose starvation induced a change from a fermentative to a respiratory metabolism in *S. cerevisiae.* This sudden shift to respiratory growth would lead to the accumulation of reactive oxidative species derived from the electronic chain in the mitochondria. It is not, therefore, surprising that both oxidative and nutritional stresses induce a common pathway that is sensed primarily by a common sensor, Mtl1, which would transmit the signal for both Tor1 and Ras2 inactivation, thus reducing the accumulation of cAMP. In mammal cells, amino acid depletion inhibits TORC1 (59). Unrestrained activity in mammals is associated with several diseases such as inflammation, cancer, and diabetes (60). Ras inhibition also increases resistance to oxidative stress in neurons (61). The inhibition of Tor1 and Ras must, therefore, be a common mechanism used by cells to survive nutrient depletion and other specific types of stress. In this context and because of the existence of either homologous TOR or RAS in unicellular organisms, they constitute a good cellular model for studying these signaling processes, stressing the interest of the current study.

It was recently (62) proposed that the TOR and PKA signals function independently of each other. However, other authors (29) have proposed that nutrient availability signals to the TOR function and that from there, the signal could diverge into two separate branches, one of which is directed to Ras/cAMP, whereas the other is related to ribosome biogenesis, stress response, and glycogen accumulation. In our studies we observed that in response to rapamycin, Mtl1 displayed an independent genetic relationship with TOR. However, in response to oxidative stress and glucose starvation, our data support the hypothesis that a signal is transmitted from Mtl1 and Rho1 to Tor1 and Ras/cAMP, which converges (at least) on the stress transcription factor Msn2/Msn4. Glycogen accumulation is also controlled by TOR, PKA, and Msn2/Msn4. The observation that Rho1 activation, Msn2 overexpression, or the deletion of either Tor1 or Ras2 suppressed the deficiency in glycogen accumulation in *mtl1* mutants also supports our model.

The findings from our study were in line with a previous publication (63) that described a role for Rom2 in Ras2-cAMP down-regulation in response to several types of stress, including oxidative stress. These authors also indicate that this cross-talk between Ras-cAMP and the Pkc1-MAPK pathway could be mediated by Rho1. In the present study we have extended this information to Mtl1, as it is the cell-surface protein member of the cell integrity pathway that transmits the signal to Tor1 and Ras2 through Rom2 (data not shown) and Rho1. However, downstream elements of the cell integrity pathway do not participate in this cross-talk. This was deduced from the observation that the *BCK1–20* allele did not restore the Msn2/Msn4 function in *mtl1*cells in response to oxidative stress and glucose starvation. Moreover, and in accordance with Park *et al.* (63), we also observed a certain specificity of stress in this signaling, as the *RHO1** and *BCK1–20* alleles did not suppress the loss of viability that the *mtl1* cells experienced in response to other stress-inducing agents such as rapamycin.

In general, the cellular responses to oxidative stress or glucose starvation mediated by Mtl1 described in this paper were common ones. With only one exception, the constitutive activation of the Pkc1-MAPK pathway at the level of *BCK1* clearly increased cell survival in *mtl1* cells when they were starved of glucose. This result underlines the importance of cell integrity activity in quiescence (10, 34). The observation that Slt2 activation is not sufficient to recover cell viability in response to oxidative stress in an *mtl1* mutant is in line with a previous publication (1). In it, we demonstrated that the elements of the MAPK module of the CWI pathway are dispensable for cell viability in response to oxidative stress. However, more studies are required to look more deeply into the biological significance of these divergences.

In conclusion, our results suggest that the essential functions mediated by Mtl1 are to inactivate the Tor1 and Ras2 function to repress ribosomal gene transcription, to induce Msn2/Msn4 gene transcription, and to reduce the cAMP cellular levels (see Fig. 3*E*). We believe that the Msn2 function directly contributes to the maintenance of cell viability in *mtl1* mutants in response to oxidative stress, whereas Slt2 activity reflects an adaptive response. However, in response to glucose starvation, both Slt2 and Msn2 activity help to increase cell survival in the absence of Mtl1.

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Table S1. ORFs whose transcripts are repressed at least 2 fold in an *mtl1* strain compared to the wild-type. Functional annotation is based on the information provided by SGD.

ORF	Gene	Ratio mtl1Δ/wt	Gene description; Function
YBL098W	*BNA4*	0.500	Kynurenine 3-mono oxygenase, required for biosynthesis of nicotinic acid
YIR035C		0.500	Unknown Function
YKL141W	*SDH3*	0.500	Cytochrome b subunit of succinate dehydrogenase; respiratory chain complex II
YLR395C	*COX8*	0.500	Subunit VIII of cytochrome c oxidase; mitochondrial inner membrane electron transport chain
YNL333W	*SNZ3*	0.500	Member of a stationary phase-induced gene family
YOR065W	*CYT1*	0.500	Cytochrome c1, component of the mitochondrial respiratory chain; respiratory chain complex III
YAL063C	*FLO9*	0.491	Lectin-like protein with similarity to Flo1p, involved in flocculation
YDR377W	*ATP17*	0.491	Subunit f of the F0 sector of mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase complex
YPL078C	*ATP4*	0.491	Subunit b of the stator stalk of mitochondrial F1F0 ATP synthase, proton-transporting ATP synthase complex
YBR039W	*ATP3*	0.483	Gamma subunit of the F1 sector of mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase complex
YHR138C		0.483	Unknown function; Homologous to PBI2
YJR019C	*TES1*	0.483	Peroxisomal acyl-CoA thioesterase;likelyto be involved in fatty acid oxidation
YJR077C	*MIR1*	0.483	Mitochondrial phosphate carrier, imports inorganic phosphate into mitochondria
YDL124W		0.475	NADPH-dependent alpha-keto amide reductase
YLR231C	*BNA5*	0.475	Kynureninase, required for biosynthesis of nicotinic acid from tryptophan
YOL083W		0.475	Unknown Function
YDL004W	*ATP16*	0.467	Delta subunit of the central stalk of mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase
YDR148C	*KGD2*	0.467	Dihydrolipoyl transsuccinylase, a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex
YDR298C	*ATP5*	0.467	Subunit 5 of the stator stalk of mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase
YIL042C	*PKP1*	0.467	Mitochondrial protein kinase involved in negative regulation of pyruvate dehydrogenase complex activity
YLR294C		0.467	Unknown Function
YML087C	*AIM33*	0.467	Unknown Function
YPL271W	*ATP15*	0.467	Epsilon subunit of the F1 sector of mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase
YGL187C	*COX4*	0.459	Subunit IV of cytochrome c oxidase; member of the mitochondrial inner membrane electron transport chain
YKL148C	*SDH1*	0.459	Flavoprotein subunit of succinate dehydrogenase
YER158C		0.451	Uknown function
YGR035C		0.451	Uknown function
YLR295C	*ATP14*	0.451	Subunit h of the F0 sector of mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase complex
YPR191W	*QCR2*	0.451	Subunit 2 of the ubiquinol cytochrome-c reductase complex; mitochondrial inner membrane electron transport chain
YBL045C	*COR1*	0.443	Core subunit of the ubiquinol-cytochrome c reductase complex ; mitochondrial inner membrane electron transport chain
YIL099W	*SGA1*	0.443	Intracellular sporulation-specific glucoamylase involved in glycogen degradation
YCR083W	*TRX3*	0.435	Mitochondrial thioredoxin, required to maintain the redox homeostasis of the cell

YGL104C	*VPS73*	0.435	Mitochondrial protein of unknown function involved in vacuolar protein sorting
YKL150W	*MCR1*	0.435	Mitochondrial NADH-cytochrome b5 reductase, involved in ergosterol biosynthesis
YHR001W-A	*QCR10*	0.428	Subunit of the ubiqunol-cytochrome c oxidoreductase complex, involved in aerobic respiration
YLL053C		0.428	Uknown function
YML070W	*DAK1*	0.420	Dihydroxyacetone kinase, required for detoxification of dihydroxyacetone (DHA); involved in stress adaptation
YNL202W	*SPS19*	0.420	Peroxisomal 2,4-dienoyl-CoA reductase, auxiliary enzyme of fatty acid beta-oxidation
YJR079W		0.413	Unknown function; mutation results in impaired mitochondrial respiration
YKL016C	*ATP7*	0.413	Subunit d of the stator stalk of mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase,
YPR020W	*ATP20*	0.413	Subunit g of the mitochondrial F1F0 ATP synthase; proton-transporting ATP synthase complex
YDL130W-A	*STF1*	0.406	Protein involved in regulation of the mitochondrial F1F0-ATP synthase; proton-transporting ATP synthase complex
YBR230C	*OM14*	0.392	Integral mitochondrial outer membrane protein
YIR038C	*GTT1*	0.392	ER associated glutathione S-transferase capable of homodimerization
YLL052C	*AQY2*	0.386	Water channel that mediates the transport of water across cell membranes
YPL095C	*EEB1*	0.386	ethanol O-acyltransferase responsible for short-chain fatty acid ethyl ester production during fermentation
YKR049C	*FMP46*	0.372	Mitochondrial protein of unknown function
YOR107W	*RGS2*	0.372	Negative regulator of glucose-induced cAMP signaling
YDR059C	*UBC5*	0.366	Ubiquitin-conjugating enzyme that mediates selective degradation of abnormal proteins; stress response
YHR033W		0.366	Uknown function
YJL163C		0.366	Uknown function
YKR076W	*ECM4*	0.366	Uknown function
YLL041C	*SDH2*	0.366	Iron-sulfur protein subunit of succinate dehydrogenase
YKL093W	*MBR1*	0.347	Protein involved in mitochondrial functions and stress response
YLR178C	*TFS1*	0.342	Carboxypeptidase Y inhibitor
YPR127W		0.342	Uknown function
YGR052W	*FMP48*	0.336	Uknown function; localized in the mitochondria
YJR096W		0.336	Putative xylose and arabinose reductase
YLR356W	*ATG33*	0.330	Mitochondrial mitophagy-specific protein
YNL015W	*PBI2*	0.330	Cytosolic inhibitor of vacuolar proteinase B, required for efficient vacuole inheritance
YBR169C	*SSE2*	0.324	Member of the heat shock protein 70 (HSP70) family
YMR196W		0.319	Uknown function
YJR078W	*BNA2*	0.313	Tryptophan 2,3-dioxygenase
YKL151C		0.313	Uknown function
YBL043W	*ECM13*	0.292	Uknown function
YMR090W		0.292	Uknown function
YOR185C	*GSP2*	0.292	GTP binding protein involved in the maintenance of nuclear organization, RNA processing and transport
YBL015W	*ACH1*	0.282	Acetyl-coA hydrolase, primarily localized to mitochondria
YDR453C	*TSA2*	0.277	Stress inducible cytoplasmic thioredoxin peroxidase

YGR248W	*SOL4*	0.263	6-phosphogluconolactonase with similarity to Sol3p
YOR338W		0.263	Uknown function
YGL156W	*AMS1*	0.254	Vacuolar alpha mannosidase, involved in free oligosaccharide (fOS) degradation
YBR054W	*YRO2*	0.250	Putative plasma membrane protein of unknown function
YER103W	*SSA4*	0.237	Heat shock protein that is highly induced upon stress
YDL085W	*NDE2*	0.233	Mitochondrial external NADH dehydrogenase, catalyzes the oxidation of cytosolic NADH
YMR250W	*GAD1*	0.225	Glutamate decarboxylase; involved in response to oxidative stress
YOL155C	*HPF1*	0.221	Haze-protective mannoprotein
YML123C	*PHO84*	0.218	High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter
YDL222C	*FMP45*	0.203	Uknown function
YDR034W-B		0.186	Uknown function
YGR088W	*CTT1*	0.183	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide
YKL163W	*PIR3*	0.183	O-glycosylated covalently-bound cell wall protein required for cell wall stability
YOL084W	*PHM7*	0.183	Uknown function
YDL181W	*INH1*	0.180	Protein that inhibits ATP hydrolysis by the F1F0-ATP synthase; proton-transporting ATP synthase complex
YML128C	*MSC1*	0.174	Uknown function
YDL204W	*RTN2*	0.151	Uknown function
YFL014W	*HSP12*	0.151	Plasma membrane localized protein that protects membranes from desiccation
YOL053C-A	*DDR2*	0.151	Multistress response protein
YFL030W	*AGX1*	0.144	Glyoxylate aminotransferase, catalyzes the synthesis of glycine from glyoxylate
YIL160C	*POT1*	0.134	3-ketoacyl-CoA thiolase; beta-oxidation of fatty acids
YGR043C	*NQM1*	0.125	Transaldolase of unknown function
YMR107W	*SPG4*	0.119	Uknown function
YBR072W	*HSP26*	0.102	Small heat shock protein with chaperone activity
YMR169C	*ALD3*	0.098	Cytoplasmic aldehyde dehydrogenase, involved in beta-alanine synthesis
YHR139C	*SPS100*	0.093	Protein required for spore wall maturation
YGR256W	*GND2*	0.090	6-phosphogluconate dehydrogenase
YBR116C		0.084	Uknown function
YDL223C	*HBT1*	0.084	Substrate of the Hub1p ubiquitin-like protein that localizes to the shmoo tip
YDR070C	*FMP16*	0.076	Uknown function; mitochondrion
YPL223C	*GRE1*	0.061	Hydrophilin of unknown function; stress induced
YMR175W	*SIP18*	0.041	Protein of unknown function whose expression is induced by osmotic stress
YBR117C	*TKL2*	0.016	Transketolase, similar to Tkl1p; required for synthesis of aromatic amino acids

Table S2. ORFs whose transcripts are induced at least 2 fold in an *mtl1* strain compared to the wild-type. Functional annotation is based on the information provided by SGD.

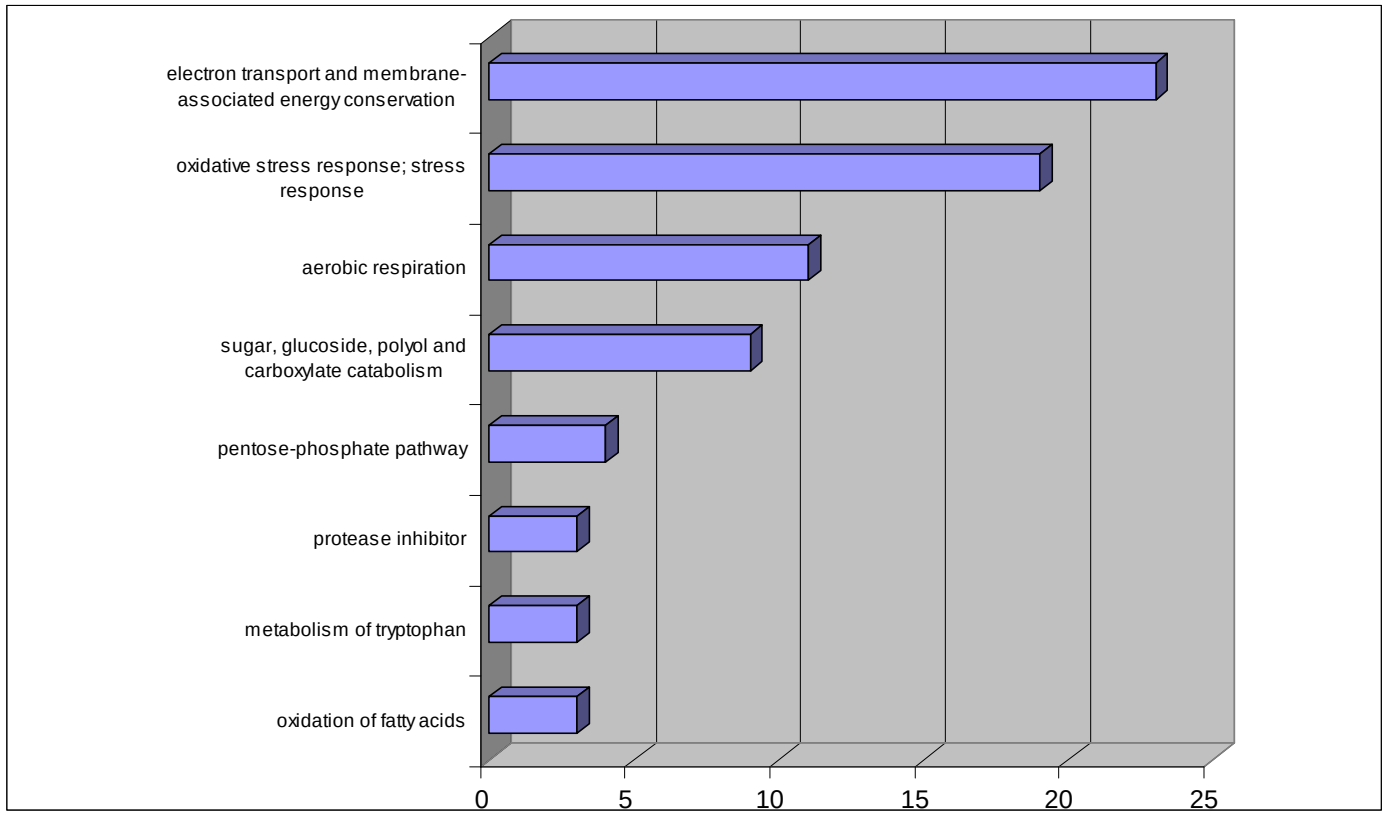
ORF	Gene	Ratio mtl1Δ/wt	Gene descrption; Function
YMR095C	*SNO1*	3.249	Protein involved in pyridoxine metabolism
YNR060W	*FRE4*	3.249	Ferric reductase;iron-siderophore transport
YPL250C	*ICY2*	3.138	Protein involved in chromatin organization and nuclear transport,
YMR011W	*HXT2*	2.878	High-affinity glucose transporter of the major facilitator superfamily
YIL121W	*QDR2*	2.828	Multidrug transporter
YBR145W	*ADH5*	2.685	Alcohol dehydrogenase isoenzyme V
YGL117W		2.685	Unknown Function
YBR244W	*GPX2*	2.594	Phospholipid hydroperoxide glutathione peroxidase; oxidative stress
YER081W	*SER3*	2.219	3-phosphoglycerate dehydrogenase
YBR115C	*LYS2*	2.107	Alpha aminoadipate reductase; biosynthesis of lysine
YPL111W	*CAR1*	2.107	Arginase, responsible for arginine degradation
YMR096W	*SNZ1*	2.000	Protein involved in vitamin B6 biosynthesis
YOL158C	*ENB1*	2.000	Endosomal ferric enterobactin transporter

Table S3. Classification of the collection of genes whose expression was lower in *mtl1* than in wild type cells. We present two different classifications. The first according to GO Biological Process and the second according to MIPS functional classification.

GO Biological Process

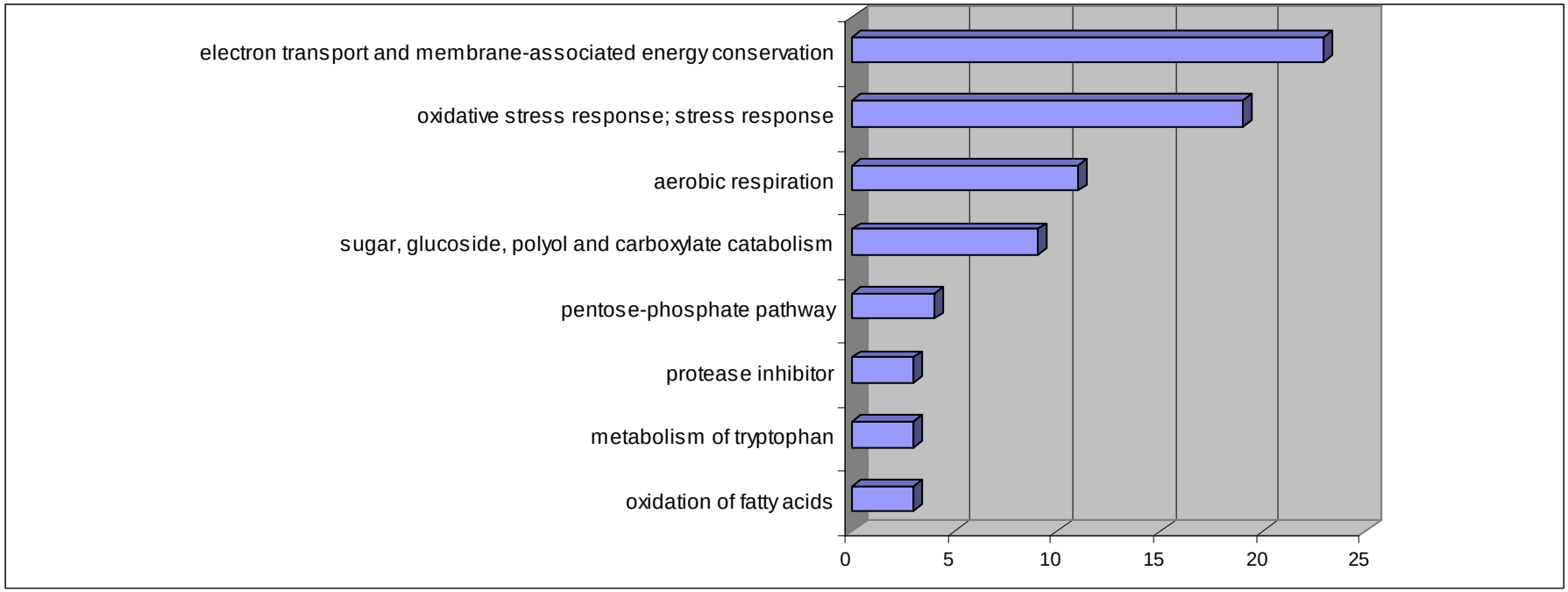
Category	p-value	In Category from Cluster	k
oxidation reduction	5,78E-13	BNA4 LYS2 ADH5 GPX2 NDE2 YDL124W TSA2 SER3 COX4 CTT1 GND2 YIR035C MIR1 BNA2 YJR096W SDH1 MCR1 FMP46 SDH2 COX8 AIM33 ALD3 SPS19 FRE4 ATP20 YPR127W	26
metabolic process	3,96E-05	BNA4 LYS2 TKL2 ADH5 INH1 KGD2 SER3 AGX1 AMS1 NQM1 GND2 SGA1 POT1 YIR035C YMR090W SNZ1 ALD3 SPS19 SNZ2	19
response to oxidative stress; response to stress		GPX2 TRX3 YDL124W TSA2 HSP12 CTT1 YJR096W MCR1 GAD1; HSP26 SSE2 UBC5 SSA4 DAK1 ALD3 DDR2 YOR338W GRE1	18
ATP synthesis coupled proton transport	4,81E-07	ATP3 ATP16 ATP5 ATP17 ATP7 ATP14 ATP4 ATP15 ATP20	9
mitochondrial electron transport		SDH3 SDH1 SDH2; QCR10 CYT1 QCR2	6
aromatic compound metabolic process	0,00298911	ADH5 YDL124W SER3 SNO1 ENB1	5
ascospore formation	0,00962488	FMP45 UBC5 SGA1 SPS19 YOR338W	5
pentose-phosphate shunt	0,00041566	TKL2 NQM1 SOL4 GND2	4
de novo NAD biosynthetic process from tryptophan	5,02E-05	BNA4 BNA2 BNA5	3
pyridoxine biosynthetic process	0,00076692	SNO1 SNZ1 SNZ2	3
cell death	0,00895324	COX4 CYT1 QCR2	3
proton transport [GO:0015992]	2,51E-07	ATP3 ATP16 ATP5 ATP17 ATP7 ATP14 ATP4 ATP15 ATP20	9
negative regulation of nucleotide metabolic process [GO:0045980]	0,00030074	STF1 INH1	2
ATP biosynthetic process [GO:0006754]	7,01E-10	ATP3 ATP16 ATP5 ATP17 ATP7 ATP14 ATP15 ATP20	8

pyridoxine metabolic process [GO:0008614]	0,00544529	SNO1 SNZ1 SNZ2	3
ion transport [GO:0006811]	7,21E-07	ATP3 YRO2 ATP16 ATP5 ATP17 ATP7 ATP14 FRE4 ENB1 ATP4 ATP15 ATP20	12
amino acid and derivative metabolic process [GO:0006519]	0,00596414	BNA4 SER3	2



MIPS Functional Classification		
Category	p-value	In Category from Cluster
electron transport and membrane-associated energy conservation		COR1 ATP3 ATP16 NDE2 STF1 INH1 ATP5 ATP17 COX4 QCR10 ATP7 SDH3 SDH1 MC SDH2 ATP14 COX8 ATP4 ATP15 ATP20 QCR2; TRX3 CYT1
oxidative stress response; stress response		GPX2 TRX3 TSA2 HSP12 CTT1 GTT1 MCR1 FMP46 GAD1 GRE1; YRO2 SSE2 PIR3 DA SNO1 SNZ1 ALD3 SNZ2 DDR2
aerobic respiration	7,03E-08	COR1 NDE2 COX4 QCR10 MBR1 SDH3 SDH1 SDH2 COX8 CYT1 QCR2
sugar, glucoside, polyol and carboxylate catabolism	9,83E-06	TKL2 KGD2 AMS1 NQM1 YJR096W SDH3 SDH1 SDH2 HPF1
pentose-phosphate pathway	0,000833	TKL2 NQM1 SOL4 GND2
protease inhibitor	5,02E-05	YHR138C TFS1 PBI2
metabolism of tryptophan	0,00027	BNA4 BNA2 BNA5
oxidation of fatty acids	0,000401	POT1 TES1 SPS19

Fe/S binding [16.21.08]	5,02E-05	SDH3 SDH1 SDH2
homeostasis of phosphate [34.01.03.03]	0,009994	MIR1 PHO84
tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle) [02.10]	0,001911	KGD2 SDH3 SDH1 SDH2
cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.) [20.01.01.01]	2,05E-05	ATP3 ATP16 ATP5 ATP17 ATP7 ATP14 ATP4 ATP15
homeostasis of protons [34.01.01.03]	1,18E-06	ATP3 ATP16 ATP5 ATP7 ATP14 PHO84 ATP4 ATP15


electron transport and membrane-associated energy conservation
oxidative stress response; stress response
aerobic respiration
sugar, glucoside, polyol and carboxylate catabolism
pentose-phosphate pathway
protease inhibitor
metabolism of tryptophan
oxidation of fatty acids
0
5
10
15
20
25

ARTICLE II

Objectives:

- Elucidating the cross-talk between CWI and either TOR or RAS-cAMP in conditions of oxidative stress and glucose deprivation

- To search for Mtl1 targets in the regulation of ribosomal biogenesis in response to oxidative stress

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Signal flow between CWI/TOR and CWI/RAS in budding yeast under conditions of oxidative stress and glucose starvation

Mima Ivanova Petkova, Nuria Pujol-Carrion and Maria Angeles de la Torre-Ruiz*
Departament de Ciències Mèdiques Bàsiques-IRBLleida; Facultad de Medicina; Universidad de Lleida; Lleida, Spain

The CWI pathway cross-talks with TOR and RAS in both the oxidative and glucose starvation responses. Mtl1 is the cell-wall protein in charge of sensing and regulating this response. Rom2 and Rho1, which are the upper elements in the pathway, mediate this signal. Several outputs are involved and required for this response, one of which, ribosomal gene expression, seems to be regulated by Sfp1, amongst other possible transcription factors. Moreover, cross-talk also occurs in a reverse flow from TOR and RAS to the CWI pathway. Thus Tor1 and Ras2 inhibition also activates Slt2 in the absence of the Mtl1 protein and assures the proper adaptive response to oxidation and glucose deprivation.

Key words: Mtl1, CWI, *RAS2*, *TOR1*, Sfp1, cross-talk, oxidative stress, glucose starvation, signalling pathway

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*Correspondence to: Maria Angeles de la Torre-Ruiz; Email: madelatorre@cmb.udl.cat

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Mtl1 is a cell-surface protein of *Saccharomyces cerevisiae* with a very high similarity to Mid2.[1] Mtl1 localization has not been described to date, however its sequence contains a transmembrane domain and a very long domain rich in serine-threonine residues.[2] This domain could be a suitable region of *O*-mannosylation, as it is similar to that described for Mid2.[3] Mtl1 has been described as a molecule required for survival under oxidative stress conditions.[4] Mtl1 is a putative sensor responsible for transmitting the oxidative signal to the CWI (Cell Wall Integrity pathway) and thereby inducing the activation of Slt2: the last kinase in the cascade; it is also responsible for the depolarization of the actin cytoskeleton.[4] We have recently demonstrated that Mtl1 is required to inactivate both the Tor1 and Ras2 functions in response to glucose depletion and oxidative stress provoked by hydrogen peroxide.[5] This is how Mtl1 transmits these signals to Rom2, the GAP (GTPase Activating Protein) of Rho1,[5] which then activates the Rho1 GTPase. From Rho1, the signal follows two simultaneous routes: (A) One leads to a cascade of activation Pkc1-Bck1-Mkk1/Mkk2, ending in Slt2 dual phosphorylation and the downstream events concomitantly with the activation of the pathway.[5] (B) The second signal, which is, in fact, a signal of repression, is transmitted to Tor1 and Ras2. This repression eventually has several outputs: ribosomal gene repression (an ATP housekeeping mechanism), cAMP descent and the activation of a wide subset of genes which are potentially regulated by the dual transcription factor Msn2/Msn4.[6] At this point, we do not know the exact nature of the connection between Rho1-Tor1-Ras2. We can speculate, however, that two models can be proposed: Rho1 first inactivates Tor1 protein from the TORC1 complex and Tor1 transmits the signal to Ras2 inactivation in response to glucose deprivation and hydrogen peroxide treatment. In the second model, Rho1 signals simultaneously, but independently, to Tor1 and Ras2 inactivation. According to our study, both models suit the results that we present in the paper. *Saccharomyces cerevisiae* is a model that facilitates study of the various cross-talks regulating the different signal transduction pathways. The relationship between Tor-Ras and the PKC1 pathway suggests that the three pathways are essential for survival in response to specific stresses. The repression of ribosomal gene transcription is a general defence mechanism in stress response.

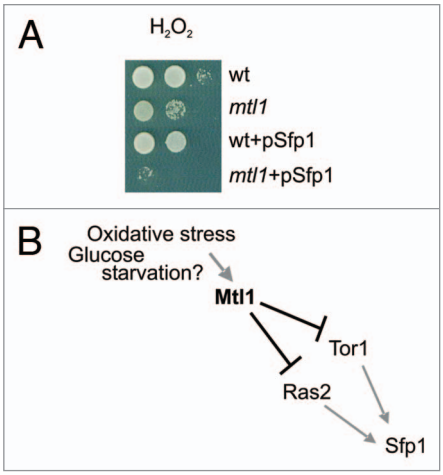

Figure 1. Sfp1 overexpression is toxic in the absence of Mtl1 when cells are exposed to hydrogen peroxide treatment. (A) Serial dilutions of wt, *mtl1*, wt^{+}pSfp1, *mt1l*$^{+}$pSfp1 were plated onto SD plates containing or not containing hydrogen peroxide 1 mM. Sfp1 was cloned in pUG35 plasmid containing the *URA3* gene. Sfp1 ORF is under the MET25 promoter in this plasmid. (B) A temptative model depicting the possible interactions between Mtl1, *TOR1*, *RAS2* and Sfp1.

One question which arose from our study was whether the transcription factor that regulates ribosomal gene expression in the signal was mediated by Mtl1. Preliminary results from our lab suggest that Sfp1 could be involved in this process and in oxidative response. Sfp1 is a transcription factor whose nuclear localization determines the transcriptional induction of both ribosomal and RiBi genes.[7,8] Sfp1 is positively regulated by TOR and RAS activity.[7,8] In response to several types of stress, including oxidative treatment, Sfp1 translocates to the cytoplasm, which provokes the repression of ribosomal gene transcription.[6] We observed that Sfp1 overexpression reduced cell viability upon hydrogen peroxide treatment, especially in *mtl1* mutants. As previously reported, ribosomal gene repression did not occur under these conditions.[6] The observation that Sfp1 overexpression severely impaired *mtl1* cell viability upon hydrogen peroxide treatment (**Fig. 1**) suggests that Sfp1 could be negatively regulated by Mtl1 in response to specific types of stress. An excess of ribosomal gene transcription under glucose starvation and oxidative stress conditions would constitute a waste of ATP and would be detrimental for cell viability. This result did not exclude the existence of other regulatory proteins in our system, but more studies of this mechanism are required before we can draw any further conclusions.

There is an information flow between different signal transduction pathways and the mechanisms that integrate information from different signal pathways in a common response.[9] However, how the different pathways talk is not completely understood. In a recent review, other authors have commented on CWI cross-talk with other pathways.[10] We described a process that connects the PKC1-MAPK pathway with TOR and RAS. In our study, the signal flowed from Mtl1, an element on the pathway to each of the other two routes: TOR and RAS. We also have data that suggest the existence of cross-talk in the opposite direction: from *RAS2* and *TOR1* to CWI. In both double mutants *ras2mtl1* and *tor1mtl1*, we therefore observed an example of Slt2 phosphorylation in response to peroxide treatment and glucose depletion, which contrasted with the absence of Slt2 activation determined in the single mutant *mtl1* (**Fig. 2**). The CWI activation observed in these mutants when stressed, correlated with an increase in cell viability that was similar to that determined in wild type cells.[6] These results demonstrate that both oxidative and nutritional stress provoked a simultaneous signalling flow from Mtl1 to several different pathways: CWI, TOR and RAS. *RAS2* and *TOR1* inhibition in the absence of Mtl1 also induced the activation of Slt2, the last kinase on the CWI pathway. These results indicate that the connection between the three pathways occurred at several different levels. On one hand, Mtl1 seemed to be the key regulator in response to external insults, as in the cases of oxidative and glucose depletion stress. In the absence of Mtl1, CWI was not activated and *TOR1* and *RAS2* were not inactivated; this severely impaired cell viability. However, according to the data shown here, another signal flowed from the inactivated *RAS2* and *TOR1* to activate CWI; this was independent of the cell wall receptor Mtl1 and assured that in response to certain specific types of stress, the downregulation of *RAS2* and/or *TOR1* guaranteed the activation of the CWI pathway. These mechanisms were necessary for cell survival and adaptation in the budding yeast system.

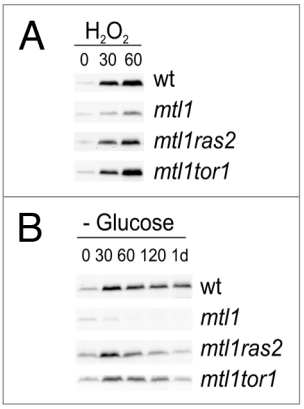

Figure 2. The simultaneous absence of Mtl1-Tor1 and Mtl1-Ras2 induces Slt2 phosphorylation in response to hydrogen peroxide treatment and in response to glucose depletion. (A) western blot analysis of Slt2 activity using the p44/42 polyclonal antibody in samples from wt, *mtl1*, *mtl1ras2* and *mtl1tor1* growing exponentially and treated with 1 mM hydrogen peroxide for the indicated times. (B) The same as in (A) but the strains were exponentially grown in SD, washed four times with minimum medium without glucose, and then transferred to SD minus glucose for incubation at 25°C. Samples were collected at the indicated times for western blot analysis. Anti-GSTSlt2 antibody was used to detect total Slt2 protein; this was equivalent in all the samples taken in the experiment (not shown for simplification).

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ARTICLE III

Objectives:

- Biochemical characterisation of Mtl1 protein. Analysis of posttranslational modifications of Mtl1; the role of Pmt2 protein O-mannosyltransferase in the oxidative stress response and upon TOR blockage
- Functional analysis of O-mannosylation in life extension in *Saccharomyces cerevisiae*
- To study possible interactions with upper elements of the CWI pathway
- To determine the cellular localisation of Mtl1

Mtl1 O-mannosylation mediated by Pmt2 is important for cell survival under oxidative conditions and TOR blockage, also favouring chronological life span

Mima Ivanova Petkova, Venus Sosa, Nuria Pujol-Carrion and Maria Angeles de la Torre-Ruiz*

[1]Departament de Ciències Mèdiques Bàsiques-IRBLleida, Facultad de Medicina, Universidad de Lleida, Lleida 25008, Spain.

Running title: Mtl1 O-mannosylation is required in quiescent and oxidative conditions to extend life

*Address correspondence to: Mª Angeles de la Torre-Ruiz. Departament de Ciències Mèdiques Bàsiques-IRBLleida, Facultad de Medicina, Universidad de Lleida, Lleida 25008, Spain..Phone number: (+34) 973702410. e-mail: madelatorre@cmb.udl.es

Key words: Mtl1, Pmt2, O-mannosylation, N-glycosylation, oxidative stress, rapamycin, Pkc1, TOR, chronological life span, Slt2, cell wall integrity (CWI).

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Summary

Mtl1 is a cell surface sensor and member of the Pkc1-MAPK pathway that senses oxidative stress and nutrient starvation. In this study we demonstrate that the Mtl1 cytoplasmic domain physically interacts with the GEF protein Rom2 in order to transmit a signal to downstream effectors. Mtl1 is N-glycosylated and highly O-mannosylated by Pmt1 and mostly O-mannosylated by Pmt2. Mtl1 localises to the bud, septum, the tip of the shmoo and the cell periphery, where it is homogenously distributed in a dotted pattern covering the entire surface. The O-mannosylation deficiency that occurs in the *pmt2* mutant adversely affects the distribution of Mtl1 on the cell surface and also impinges upon Mtl1 localisation in the tip of the shmoo. In this study we propose a relevant role for Pmt2 and Mtl1 in quiescence based on the following evidence: i) O-mannosylation and, more specifically, Mtl1 are required for cell survival in response to oxidative stress and quiescence conditions upon TOR blockade; ii) Slt2 activity is impaired in the stationary phase and upon rapamycin treatment in both *pmt2* and *mtl1* mutants; iii) Mtl1 is transcriptionally upregulated in response to rapamycin and during the stationary phase; iv) Mtl1 and Pmt2 both play a positive role in the chronological life span.

Introduction

The yeast cell wall is a highly dynamic structure and plays an essential role in the adaptive responses of cells to environmental cues (Klis *et al.,* 2006). The integrity of the cell wall is tightly controlled and precisely coordinated by the CWI pathway (Cell Wall Integrity pathway) throughout cell growth (reviewed in Levin, 2005). In budding yeast, the CWI pathway participates in transducing extracellular signals for cell-wall, heat shock, hypoosmotic, nutritional, oxidative and pH stresses (Heinisch *et al.,* 1999; Vilella *et al.,* 2005; Levin, 2005 and Serrano *et al.,* 2006). Environmental stresses are sensed at the plasma membrane by a large number of cell surface sensors: the WSC family (Wsc1, 2, 3 and Wsc4 proteins) (Gray *et al.,* 1997; Verna *et al.,* 1997), Mid2 (Ketela *et al.,* 1999; Rajavel *et al.,* 1999; Philip and Levin, 2001), and Mtl1 (Ketela *et al.,* 1999; Rajavel *et al.,* 1999; Villela *et al.,* 2005). It has been demonstrated that the cytoplasmic C-terminal domains of both Wsc1 and Mid2 interact with the GEF Rom2 (Philip and Levin, 2001) and that these interactions stimulate the nucleotide exchange of the GTPase Rho1 and thereby activate it. In turn, amongst others, Rho1 also activates the protein kinase C Pkc1 (reviewed in Levin, 2005). Pkc1 activates a mitogen-activated protein kinase module composed of: the MAPKKK Bck1, which phosphorylates the redundant MAPK kinases; Mkk1 and Mkk2 kinases. Together, they then activate the last kinase in the module: Slt2/Mpk1. Slt2 dual phosphorylation correlates with activation of the CWI pathway (de Nobel *et al.,* 2000; Martin *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 remodelling the cell-wall). The upper elements of the CWI pathway are involved in the organisation of the actin cytoskeleton under different conditions (Helliwell *et al.,* 1998; Delley and Hall, 1999; De la Torre *et al.,* 2002; Torres *et al.,* 2002; Villela *et al.,* 2005; Motizuki *et al.,* 2008).

Of the transmembrane receptors, only Mtl1 has been characterised as a cell-wall sensor for oxidative stress and glucose starvation (Petkova *et al.,* 2010a). Mtl1 is required to down-regulate both the Tor1 and Ras2 functions in response to glucose deprivation and oxidative stress provoked by hydrogen peroxide. From Rho1, the signal 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 and b). Slt2/Mpk1 activity is essential for cell survival under quiescence conditions (Torres *et al.* 2002; Krause and Gray, 2002; Petkova *et al.,* 2010a). However, the contribution of the Msn2/Msn4 function is more significant in response to oxidative stress (Petkova *et al.,* 2010a). This is in accordance with the role for Rom2 in signalling oxidative stress (and other stresses) through Ras2/cAMP down-regulation (Park *et al.,* 2005). The Wsc1 transmembrane receptor has been shown to be essential in Slt2/Mpk1

activation upon heat-shock and also in the negative regulation of Ras2/cAMP under the same conditions (Verna *et al.,* 1997; Sobering *et al.,* 2002). The coexistence of such cross-talk events with other stress-responsive pathways definitively expands the signalling capabilities of the CWI pathway (reviewed in Fuchs and Mylonakis, 2009; de la Torre- Ruiz *et al.,* 2010 and Petkova *et al.,* 2010b).

The TOR and RAS-cAMP signalling pathways are two major pathways that control cell growth in response to nutrient availability and in yeast they are important for stress tolerance (Wullschleger *et al.,* 2006; Santangelo, 2006). Two TOR kinases are found in budding yeast: Tor1 and Tor2. Although they both belong to the TORC1 complex, only Tor2 belongs to the TORC2 complex. Rapamycin specifically inhibits TORC1 (Loewith *et al.,* 2002). TORC1 promotes anabolic processes such as protein synthesis and ribosome biogenesis, while it antagonizes catabolic processes such as autophagy and inhibits stress response processes (Crespo and Hall, 2002). PKA kinase is part of the RAS-cAMP signalling cascade that controls glucose metabolism and also regulates translation, ribosome biogenesis, autophagy and stress responses (Santangelo, 2006). TORC1 and PKA provide various types of cross-talk and regulate common readouts such as the nuclear localisation of transcription factors associated with the general stress response transcription factor Msn2/Msn4 and other transcription factors involved in RP gene expression. According to some authors, TOR and RAS-cAMP function independently (Zurita-Martinez *et al.,* 2005), although others suggest that RAS-cAMP is a TOR effector branch (Schmelzle *et al.,* 2004; Martin *et al.,* 2004). Soulard *et al.* (2010) suggest that TORC1 activates PKA toward some - but not all - substrates, by preventing Slt2-mediated activation of the BCY1 regulatory subunit of PKA.

The CWI pathway sensor proteins: the WSC family members, Mid2 and Mtl1, all have similar overall structures, possessing small cytoplasmic domains, single transmembrane domains, and large extracellular protein domains rich in Ser/Thr residues. Mtl1 exhibits 50% amino acid sequence identity with Mid2 (Rajavel *et al.,* 1999; Ketela *et al.,* 1999). As demonstrated for Mid2 and Wsc1, Ser/Thr-rich regions are highly O-mannosylated by at least three of the seven members of the PMT family of protein O-mannosyltransferases: Pmt1, Pmt2 and Pmt4 (Lommel *et al.,* 2004; Philip and Levin, 2001; Ketela *et al.,* 1999). O-mannosylation is not only a structurally important modification, but it is also required to prevent aberrant posttranslational processing. It therefore determines the stability of Wsc1 and Mid2 proteins and ensures their signalling competence (Lommel *et al.,* 2004).

Protein O-mannosylation represents a crucial posttranslational modification which is evolutionarily conserved from bacteria to humans (reviewed in Lehle *et al.,* 2006; Lommel and Strahl, 2009). Protein O-mannosylation in *S.cerevisiae* - and in fungi in general - affects important cellular processes such as the maintenance of cell wall integrity, cell polarity and

morphogenesis (reviewed in Lommel and Strahl, 2009). O-mannosylation of the specific secretory proteins of fungal pathogens significantly contributes to virulence (reviewed in Lengeler *et al.,* 2008). In mammals, Pmt proteins are essential for cellular differentiation and development, while O-mannosylation deficiencies cause congenital neuromuscular disorders (reviewed in Lehle *et al.,* 2006).

In this study, we present data that show that Mtl1 is N-glycosylated and O-mannosylated and that it is localised to the septum, bud and cell surface. O-mannosylation partly determines this localisation. Mtl1 physically interacts with the GEF Rom2 through its cytoplasmic domain and is determinant for the performance of certain Mtl1 functions. We also demonstrate that protein O-mannosylation is important in the response to oxidative stress and quiescence and in the chronological life span. Mtl1, whose O-mannosylation is mainly catalysed by the Pmt2 protein O-mannosyltransferase isoform, is one of the most relevant protein sensors with a role in this function.

Results

Mtl1 is a highly O-mannosylated and N-glycosylated transmembrane protein.

In our previous study, we characterised Mtl1 as a putative cell surface sensor for oxidative stress, TOR inhibition and glucose starvation. To gain a better insight into Mtl1 function, we proceeded to characterize it.

Mtl1 encodes a type I membrane-spanning protein (551amino acids), containing an N-terminal secretion signal peptide (aa 1-35) that is followed by a domain rich in Ser/Thr residues; this is likely to be a recipient region for O-linked mannosylation (Tanner *et al.*, 1987; Ketela *et al*., 1999; Lommel *et al.,* 2004) (Fig. 1A-B). The C-terminal third of the protein is comprised by a single transmembrane domain (aa 362-384) and a small cytoplasmic domain (Fig. 1). Based on the biochemical characteristics deduced from the protein sequence, we decided to investigate posttranslational modifications of Mtl1.

To examine Mtl1 biochemically, we expressed a C-terminally HA epitope-tagged version of Mtl1 under an inducible *tet*O_7 promoter in wild-type yeast cells (pTP30). Total protein extracts were resolved on SDS-PAGE and Mtl1^{HA} proteins were analysed by Western blot, using monoclonal anti-HA antibodies. The molecular mass of Mtl1 was deduced to be 57 kDa, as shown in Fig 2A. We also detected a mature form of Mtl1 with a molecular mass of approximately >200 kDa. This band is compatible with O-linked polymannose chains. The initial O-mannosyl transfer reaction is catalysed by the essential family of dolichyl phosphate-D-mannose: protein O-mannosyltransferases (PMTs) at the endoplasmic reticulum (Gentzsch and Tanner, 1996; Strahl-Bolsinger *et al*., 1999; Willer *et al*., 2003). We therefore used various mutants that were deficient in O-linked mannosylation and consequently

transformed *pmt1, pmt2, pmt3, pmt5* and *pmt6* single mutants using a plasmid harbouring Mtl1 under the $tetO_7$ promoter, as described above.

In the *pmt6* mutant, we clearly observed a considerable reduction in the 200kDa band (Fig. 2A-B). In the *pmt1* mutant, the 200 kDa Mtl1 form was also reduced with respect to the wild type and a new band of 100 kDa was detected that was absent in the wt strain. In the *pmt2* cells, only the 57kDa Mtl1 protein form was clearly detected by the anti-HA antibody, although a very small amount of the 100 kDa and 200 kDa forms were also observed when the image was further contrasted (Fig. 2A, lane 12, fig 2B, lane 6). In the other *pmt* mutants, we did not detect any significant differences with respect to the wt cells. From these data, we conclude that Pmt6 and Pmt1 differentially affect the maturation of Mtl1 but that Pmt2 is the protein O-mannosyltransferase that makes the greatest contribution to Mtl1 posttranslational modification. We cannot discard the possibility that other protein O-mannosyltransferases may also contribute to Mtl1 maturation.

The pattern observed in Fig. 2A-B shows that Pmt1 and Pmt2 partly affect the maturation of Mtl1 in a qualitatively similar way. Even so, it is relevant to consider that Mtl1 was overexpressed and that the fully modified 200 kDa form of Mtl1 was almost absent from the *pmt2* mutant cells. We consequently suggest that Pmt2 could be the main protein O-mannosyltransferase isoform capable of specifically modifying Mtl1 *in vivo*. Fig. 2A shows that the total amount of $Mtl1^{HA}$ in *pmt2* was significantly decreased compared to *pmt1* and that it was also reduced in *pmt6* than in wt. The same results were obtained for *pmt2* in two different backgrounds: CML128 and BY4741 (not shown). These data suggest that impaired O-mannosylation affects Mtl1 stability.

Mtl1 has several potential N-glycosylation sites (NXS/T) at positions Asn-5, 42, 292, 479 and Asn-547. To test whether Mtl1 is in vivo N-glycosylated, we purified $Mtl1^{HA}$ proteins by immunoprecipitation. The resulting immunoprecipitates were subsequently treated with Endo H, an enzyme that catalyses the hydrolysis of asparagine-linked carbohydrate chains. Treatment with Endo H resulted in a change in the molecular mass of the Mtl1 from 200 to 170-180 kDa (Fig. 3A), which proved that Mtl1 was modified by an N-linked carbohydrate chain.

In exponentially growing cells, the fully modified (O-mannosylated and N-glycosylated) form of Mtl1 was abundant, although another faster migrating band was also detected, in low abundance, in wt, *pmt3*, *pmt5* and *pmt6* mutants. This band was the same size as the approx 180 kDa band that resulted from the digestion of Endo-H (see arrow plus asterisk in Fig. 3A). This form was not detectable in *pmt1* (Fig. 3A) see asterisk) or *pmt2* (not shown) mutants in which O-mannosylation was impaired. Analysis of $Mtl1^{HA}$ in the *sec1-1* mutant (Fig. 3B) revealed that upon blocking the last step of the secretion of Mtl1 protein was

hypoglycosylated. This suggests that Mtl1 is secreted to the plasma membrane through the secretory pathway.

Mtl1 interacts with Rom2, a GEF for Rho1, through its cytoplasmic domain.

If Mtl1 is a sensor for oxidative and nutrient starvation stress, it should signal to the intracellular components of the CWI pathway. As we previously described that Mtl1 signals to Rom2 to inhibit the TOR1 and RAS2 functions in response to oxidative stress and nutrient starvation, the most likely candidate for interaction with Mtl1 would be Rom2.

We examined an *in vivo* interaction involving Mtl1 and Rom2 through two-hybrid analysis. The sequence encoding the cytoplasmic domain of Mtl1 (aa 386-551) was fused to the *GAL4BD* (binding domain) of the pGBT9 vector. The sequence corresponding to the whole ORF of *ROM2* was fused to the *GAL4AD* (activating domain) of the pACTII vector. We cotransformed the AH109 recipient strain with both plasmids and detected Mtl1-Rom2 interaction using both the reporter genes: *HIS* and *ADE.* Our results clearly show that Mtl1 interacts with Rom2 through its cytoplasmic domain (Fig. 4A) and prove the importance of the cytoplasmic domain for the Mtl1 signalling function. We also performed functional analysis with both the cytoplasmic and extracellular Mtl1 domains. None of them was able to complement the *mtl1* phenotypes (not shown); this contrasted with the observation that full length Mtl1 was able to restore wild type phenotypes in *mtl1* cells (Fig 7C). These data demonstrate that both the Mtl1 domains are required for the protein function.

We also performed co-immunoprecipitation analyses between Mtl1 and Rom2 proteins. Mtl1 ORF was cloned in the integrative pMM351 plasmid containing three repetitions of HA epitope in the C-terminal position and expressed under the $tetO_7$ promoter. Rom2 ORF was cloned with GST as a fusion protein in the C-terminus under the control of *ADH1* promoter. $Mtl1^{HA}$ was immunoprecipitated with anti-HA antibody on Protein A-Sepharose beads and then analysed with SDS-PAGE and Western blotting, using anti-GST antibody to detect the protein partner of the interaction. In Fig. 4B we show that Mtl1 *in vitro* interacts with Rom2.

Mtl1 localises to the cell periphery, bud and septum and Msn2/Msn4 regulates its transcription in response to quiescence.

We constructed an Mtl1GFP protein in the chromosomal loci of Mtl1. GFP was fused in frame in the C-terminus of Mtl1 ORF. We analysed Mtl1 cellular localization in cells growing exponentially and upon their respective treatments with rapamycin, hydrogen peroxide and stationary phase. We observed that Mtl1localised to the cell periphery, bud and septum in exponentially growing cells (Fig. 5A). This localization did not significantly change

upon applying the treatments mentioned. In order to obtain more information regarding Mtl1 localisation, we synchronised the cells in G1 with alpha factor. We then washed the cultures and released cells into fresh medium in order to allow them to progress throughout the cell cycle. We observed that Mtl1 localised to the tip of the shmoo when synchronised in G1 (Fig. 5B). Upon washing with alpha factor and release into fresh medium, Mtl1 accumulated throughout the cell surface but it did so more intensely in emerging bud and in the area surrounding it. Once the bud was well grown, Mtl1 also localised to the septum (Fig. 5B). When we changed the focus, we were able to clearly observe how Mtl1 was arranged in small dots that were homogeneously scattered throughout the cell surface (Fig. 5C), independently of the phase of the cell cycle (not shown).

This localisation is consistent with the idea that Mtl1 is a homogeneously distributed cell-surface receptor that covers the entire cell surface. Mtl1 localisation also suggests a function in cell division and/or cytokinesis. Mtl1 localisation to the septum and bud is also consistent with the hypothesis that Mtl1 belongs to the Pkc1-family.

Hydrogen peroxide treatment did not significantly change the pattern of Mtl1 localisation (not shown). However, upon rapamycin treatment (not shown) and entering the stationary phase, we clearly detected a significant increase in Mtl1 fluorescence in the periphery of the cells (Fig. 6A). This result suggested that in response to TOR blockage or stationary phase - both of which are quiescent conditions - *MTL1* either experienced transcriptional induction or there was an increase in Mtl1 protein stability. In order to settle this question, we performed northern-blot analysis and demonstrated that either rapamycin treatment or stationary phase induced the transcription of Mtl1 (Fig. 6B). These data are consistent with the up-regulation of *MTL1* gene expression under quiescent conditions.

In silico analysis of the promoter region of *MTL1* revealed a theoretical DNA regulatory motif for the general stress response transcriptional factor, Msn2/Msn4. We overexpressed Msn2 and checked *MTL1* mRNA levels in order to ascertain whether Msn2/Msn4 was the transcription factor in charge of *MTL1* transcription regulation. Overexpression of Msn2 in wild type cells provoked notable *MTL1* transcriptional induction (Fig. 6B). However, in the *msn2msn4* double mutant, the levels of *MTL1* transcription were similar to those determined in wild type cells (Fig 6C). Our results suggest that Msn2/Msn4 regulates *MTL1* transcription in response to TORC1 inhibition by rapamycin and conditions of quiescence or nutrient starvation: the stationary phase. However, our data also suggest that Msn2/Msn4 is not the only regulator for *MTL1* transcription. In conclusion, we consider *MTL1* to be a stress-responsive gene.

Mlt1 O-mannosylation is required for appropriate localisation of Mlt1 to the septum during mitosis

To analyse whether glycosylation affects Mtl1 localization, we observed Mtl1GFP distribution in *pmt2* cells upon pheromone-induced synchronization in G1 (Fig. 5B). In *pmt2* mutant cells, Mtl1GFP largely failed to accumulate at the shmoo. Upon its release from alpha factor, Mtl1 sequentially localised to the cell periphery, bud and septum in wild-type cells. In contrast, septum localisation was clearly defective in the *pmt2* mutant (Fig. 5B). We observed Mtl1 *in vivo* localisation in stationary *pmt2* cells and also in cultures treated with rapamycin or hydrogen peroxide. We did not, however, detect any substantial differences with respect to wild type cells, other than that the absence of Pmt2 protein impinged Mtl1 localisation to the septum under all the conditions tested.

One interesting observation was that in *pmt2* mutants Mtl1 was not homogeneously distributed throughout the total cell surface. In Fig. 5C it is possible to observe how, in *pmt2* cells, the Mtl1 pattern of superficial distribution was altered with respect to wild type cells. This result suggests that Mtl1 protein maturation mediated by Pmt2 is also required to correctly localize Mtl1 on the cell surface.

One interpretation of the observation that Mtl1 is not localised to the septum in *pmt2* cells could be that the *pmt2* mutant had a defect in its septum organisation and it was therefore not possible to specifically preclude the localisation of any given protein. To check this possibility, we analysed the localisation of Cdc11, which is a septin that is naturally localised in the septum. We observed that Cdc11 was correctly localised in the septum both in wild type and *pmt2* cells (Fig 5D). These results suggest that protein O-mannosylation catalysed by Pmt2 is required for the correct localisation of Mtl1 to the septum and the tip of the shmoo.

Importance of protein O-mannosylation in response to oxidative stress and Tor blockage.

The absence of *MTL1* makes cells sensitive to oxidizing agents and to the conditions in which the TORC1 and RAS functions are compromised.

To test the importance of protein O-mannosylation in the context of the Mtl1 function, we decided to analyse in the *pmt1, pmt2, pmt3, pmt5* and *pmt6* mutants several phenotypes that are closely associated with the *mtl1* mutant, as described in Petkova *et al.,* (2010a). We first analysed cell viability. Fig 7A shows that *pmt1* and *pmt2* displayed greater sensitivity to rapamycin than wild type cells. However, *pmt2* was also most sensitive to oxidizing agents. In view of these results, we suggest that O-mannosylation is important for cell survival under conditions of oxidative stress and TOR blockade. Pmt2 was most relevant for cell survival in the oxidative stress response. In conclusion, the *pmt* mutants that were most sensitive to the

agents tested (*pmt1* and *pmt2*) were the ones that were most relevant for appropriate Mtl1 maturation.

In order to gain a better insight into *pmt2* sensitivity to hydrogen peroxide and rapamycin, we analysed Msn2-dependent gene expression and ribosomal gene transcription. In *pmt2* strain *HSP12* and *CTT1* transcriptional induction and *RPL3* and *RPL30* gene expression upon both hydrogen peroxide and rapamycin treatment were equivalent to that determined in wt cells (Fig. 7B). In view of these results, we suggest that impairment of the O-mannosylation - and more specifically, Mtl1 maturation catalysed by Pmt2 - does not affect signalling to Msn2/Msn4 or ribosomal gene expression.

Mtl1 signals through Rom2 and Rho1 to inactivate both Tor1 and Ras2. Here, we demonstrate that Mtl1 physically interacts with Rom2. In order to ascertain whether the observed *pmt2* sensitivity to rapamycin and oxidising agents was a consequence of a defect in signal transmission (due at least in part to impared O-mannosylation), we overexpressed Mtl1, Rho1, Rom2 and Slt2 in *pmt2* and wild type strains and looked for complementation to *pmt2* phenotypes. Overexpression of Mtl1 fully complements *mtl1* mutant sensitivity to both rapamycin and hydrogen peroxide (Fig 7C).

Overexpression of Mtl1 restored the cell growth defect found in *pmt2* in response to rapamycin (Fig 7D). We examined its ability to survive rapamycin treatment using different clones of *pmt2* that overexpressed Mtl1HA. Interestingly, in one of these clones, Mtl1 modification was totally abolished; this clone failed to suppress the sensitivity of *pmt2* in response to rapamycin (not shown). Neither the expression of the hyperactive allele Rho1 nor overexpression of Rom2 was able to restore the cell viability of *pmt2* following rapamycin treatment (Fig 7D). These results were consistent with our previously published findings in which neither Rho1 nor Rom2 rescued *mtl1* growth defect upon TOR blockage.

Overexpression of Mtl1 partially suppressed the defect in *pmt2* cell growth in response to hydrogen peroxide (Fig 7D). Moreover, the two *pmt2* clones overexpressing Mtl1, in which the hyperglycosylated form of Mtl1 was differently expressed, displayed the same degree of viability; this contrasted with the response to rapamycin (not shown). The expression of the hyperactive allele Rho1, or overexpression of Rom2 (Fig 7D), also led to an increase in the cell viability of the *pmt2* mutant following oxidative stress (again, in accordance with our previously published studies in which Rom2 and Rho1 overexpression suppressed *mtl1* cell lethality).

Rapamycin sensitivity and glycogen accumulation are two phenotypes that reflect PKA inactivity (Schmelzle *et al.,* 2004; Zurita-Martínez and Cardenas, 2005). Accumulation of glycogen occurs when cells are starved of nutrients. Both the TOR and Ras2-cAMP pathways control glycogen homeostasis in cells and part of the effect due to Msn2/Msn4 regulation of the transcription of genes required for glycogen synthesis. Since PKA activity is

regulated by Ras2 and the *pmt2* mutant is sensitive to rapamycin, we investigated glycogen accumulation in this mutant under several different conditions: stationary phase, rapamycin and hydrogen peroxide treatments. In all these cases, it was impossible to distinguish glycogen accumulation in the *pmt2* mutant from that determined in wild type cells (not shown). This demonstrated that O-mannosylation catalysed by Pmt2 does not affect PKA activity and that Msn2/Msn4 activity in the *pmt2* mutant is not impaired.

Taken together, these results suggest that protein O-mannosylation by Pmt2 does not affect signalling from Mtl1 to the TOR and RAS-cAMP pathways upon TORC1 inhibition or hydrogen peroxide treatment. However, protein O-mannosylation is important for correct cellular responses to oxidative stress and TORC1 inhibition and this is possibly mediated through Mtl1 and other protein receptors. We therefore suggest that other, as of yet uncharacterised, Mtl1 functions involved in oxidative stress and the TOR blockage response must be affected by Mtl1 O-mannosylation.

Protein O-mannosylation contributes to signal Slt2 activity upon TOR blockage

To ascertain whether Mtl1 O-mannosylation determines the activation of the cell wall integrity pathway, we decided to check Slt2 phosphorylation in *pmt2* mutant cells growing under different conditions. Upon applying oxidative stress or rapamycin treatments, Slt2/Mpk1 activity increases as a result of the dual phosphorylation of a threonine and a tyrosine residue present in the protein (Martin *et al.,* 2000). We therefore measured Slt2 phosphorylation in wt, *mtl1* and *pmt2* strains upon treatment with rapamycin and hydrogen peroxide, using phosphospecific p44/42 MAP kinase antibodies. In exponentially growing cultures, under non-induced conditions, the level of phospho-Slt/Mpk1 was higher in the *pmt2* mutant than in the wt cells; this was in agreement with a previous study by Lommel *et al.* (2004). Peroxide treatment induced Slt2 phosphorylation in both *pmt2* and wild type cells at similar rates. We have previously demonstrated an essential role for Mtl1 in signalling oxidative stress to the CWI and in the general stress response through Rho1 and the inhibition of either Tor1 or the Ras2 function. Here, we present data demonstrating that O-mannosylation is important for the oxidative stress response. Even so, this modification does not preclude Slt2 activity, since the kinase remains activated in *pmt2* mutants (Fig. 8A). This is in line with our previous finding that constitutive activation of CWI at the level of Bck1 was not sufficient to restore the defect in *mtl1* cell viability in response to hydrogen peroxide treatment. However, in response to rapamycin, the phosphorylation of Slt2/Mpk1 in the *pmt2* mutant turned out to be impaired; interestingly, it was impaired to the same extent as observed in the *mtl1* mutant cells (Fig. 8B).

We conclude that Pmt2 protein O-mannosyltransferase contributes to CWI signalling upon TORC1 inactivation by modifying Mtl1.

Importance of protein O-mannosylation in stationary phase cultures.

Starvation of nitrogen or carbon causes yeast to enter a quiescent state. Rapamycin has been reported to cause effects similar to those exhibited by nutrient-starved cells (Gray *et al.,* 2002; Shamji *et al.,* 2000; Di Como and Arndt, 1996). We demonstrated that the *pmt2* mutant is sensitive to rapamycin and that Mtl1 overexpression complements this phenotype independently of the downstream members of the CWI pathway. These results suggest that protein O-mannosylation is required for cell survival in quiescence mediated by a mature Mtl1 and possibly other cell-wall receptors. Stationary phase is another physiological model of quiescence (Di Como and Arndt, 1996). We decided to investigate whether Mtl1 and Pmt2 were required for cell survival during the stationary phase. In order to examine this, we grew wt, *mtl1* and *pmt2* cells in minimum medium to log phase and then monitored cell survival in stationary cultures. We observed that *pmt2* cells exhibited a significant loss of viability with respect to wild type cells throughout the course of the experiment; even so, the decrease in cell survival was more dramatic in the *mtl1* mutant (Fig. 9A). These results suggested a possible role for Mtl1 and protein O-mannosylation in quiescent cells and in the chronological life span. We therefore measured chronological life span in wt, *pmt2* and *mtl1* cell cultures based on the survival of populations of non-dividing yeast cells. According to Parrella and Longo (2008), yeast cells growing in synthetic complete glucose medium stop dividing after 24-48 hours. We followed the protocol described by these authors and considered the 3rd day of growth as the starting point for the chronological life span experiment (see Experimental procedures).

Both the *pmt2* and *mtl1* mutants experienced a significant reduction in life span during the stationary phase (Fig. 9B). However, the mean life span of *mtl1* was dramatically reduced with respect to wild type cells.

Slt2 phosphorylation is required for cells to survive in stationary cells (Torres *et al.,* 2002). We checked Slt2 phosphorylation in wild type, *mtl1* and *pmt2* stationary samples and observed no induction in the *mtl1* mutant and only a partial and defective activation in the *pmt2* mutant compared with wild type cells; this was similar to what had previously been observed in samples treated with rapamycin (Fig. 9C). Interestingly, Mtl1 presents a hyperglycosylated mature form in stationary phase (Fig. 9D). These results support the hypothesis that Mtl1, and in particular Mtl1 O-mannosylation, is required for cell survival in quiescence and also that Mtl1 is a receptor that signs quiescent conditions to the CWI pathway. Our results also suggest a function for protein O-mannosylation catalysed by Pmt2 in the chronological life span. We also conclude that the whole Mtl1 protein plays a direct role in the chronological life span.

Discussion

Mtl1 is a member of the PKC1 cell wall integrity pathway of *Saccharomyces cerevisiae* which functions as a cell surface sensor for oxidative stress and quiescence (Vilella *et al.,* 2005; Petkova *et al.,* 2010a). Our data indicate that Mtl1 is a highly O-mannosylated and N-glycosylated transmembrane protein. Here, we present evidence of the importance of both the extracellular highly-modified domains and the cytoplasmic domain for its function.

Analysis of the Mtl1 protein in *pmt* strains (deficient in O-linked mannosylation) reveals that Pmt2 isoform is capable of specifically modifying Mtl1. It should therefore not be unexpected that the O-mannosylation of Mtl1 could be impaired in both *pmt1* and *pmt2* mutants in a similar way, since Pmt1 and Pmt2 are thought to predominantly act as components in a heterodimeric protein complex (Gentzsch *et al*., 1997; Girrbach & Strahl, 2003). Members of the Pmt family form homodimers and heterodimers as a mechanism for enhancing substrate specificity (Hutzler *et al.,* 2007) and ensuring efficient O-mannosylation in a wide range of target proteins (Lommel & Strahl, 2009).

It is most notable, however, that the fully modified form of Mtl1 is almost totally absent from the single *pmt2* mutant. Furthermore, O-linked mannosylation by Pmt2 and Pmt6 affects Mtl1 protein stability. Mtl1 shares a high degree of homology with Mid2 (Rajavel *et al.*, 1999). An interesting observation relates to the finding that Mid2 is exclusively O-mannosylated by Pmt2 (Philip & Levin, 2001); this contrasts with the suggestion by Lommel *et al* (2004) that Mid2 is modified by both Pmt2 and Pmt4 at the least. The removal of N-linked carbohydrate chains by treatment with Endo-H reduces the molecular mass of Mtl1 isolated from wild type cells, which demonstrates that Mtl1 is N-glycosylated *in vivo.* A compensatory response between O-mannosylation and N-glycosylation has also been proposed (Arroyo *et al.,* 2011). Both the upregulation of the genes responsible for the biosynthesis of N-linked high mannose carbohydrate chains in *pmt* mutants (Arroyo *et al.,* 2011) and the enhanced transcription of *PMTs* under conditions of N-glycosylation inhibition (Travers *et al.,* 2000) suggest that the O- and N-linked glycans of cell wall mannoproteins may, at least partially, compensate for each other. Such a mechanism could be applicable to Mtl1 modification in *pmt1* and *pmt2* mutants because the band below 200 kDa is absent in these mutants, (Fig 2B). It is likely that N-linked glycans compensate for the formation of mannose outer chains under conditions of decreased O-mannosylation; this is in line with the proposed compensatory response between O-mannosylation and N-glycosylation (Arroyo *et al.,* 2011).

Mtl1 is secreted to the plasma membrane through the putative secretory pathway since the *sec1-1* mutant Mtl1 is hypoglycosylated.

Glycosylation is a highly abundant and essential posttranslational protein modification (Lehle *et al.,* 2006). Some combinations of *pmt* mutations result in lethality (Gentzsch &

Tanner, 1996); this indicates that protein O-mannosylation is a crucial protein modification. There is a degree of functional overlap among the different Pmt isoforms (Strahl-Bolsinger *et al*., 1993 and 1999). Analyses of viable single *pmt* and conditionally lethal double *pmt* mutants shows that O-mannosylation is not only a structurally important modification, but that it is crucial for functional aspects of a protein such as localisation, turnover, folding and ligand interaction (reviewed in Lommel & Strahl, 2009; Lehle *et al.,* 2006; Marth *et al.,* 2008). As such, it is therefore also indispensable for cell wall integrity, cell polarity and morphogenesis. Mannosylated threonine and serine residues in proteins constitute protein modifications that have been evolutionarily conserved from unicellular yeast to man (Chai *et al.,* 1999; Chiba *et al.,* 1997; Lehle *et al.,* 2006); it is also well known that deficiencies in protein O-mannosylation can cause serious developmental disorders in humans, such as congenital neuromuscular disorders.

In our study, which was based on the finding that the absence of Pmt2 makes cells sensitive to oxidising agents and to rapamycin, we proposed an essential role for protein O-mannosylation in both the oxidative stress response and in the response to TOR blockage, through at least Mtl1. Even so, we did not rule out the possible participation of other cell-wall receptors in this mechanism.

However, we consider that at least some of the *pmt2* defects observed in the presence of oxidative stress and rapamycin treatment are due to Mtl1 O-mannosylation defect. This conclusion is based on the evidence that Mtl1 overexpression is able to repair cell growth in *pmt2* mutant in response to rapamycin and also in response to oxidative stress. Moreover, overexpression of other members of the PKC1-MAPK pathway (such as Rho1 and Rom2) leads to an increase in *pmt2* cell survival in response to oxidative stress. Mtl1 O-mannosylation is important for transmitting the signal to Rom2 and Rho1. However, we believe that Slt2 activation does not play an essential role in the oxidative stress response; this conclusion is based on the fact that the activation of this kinase was not impaired in the *pmt2* mutant upon hydrogen peroxide treatment. This is consistent with our previously published data (Petkova *et al.,* 2010a) in which Rho1 and Rom2 rescued *mtl1* cell viability in response to oxidative treatment. Even more relevant is the evidence that this suppression presented stress specificity as Rom2 and Rho1 did not suppress *mtl1* loss of viability upon rapamycin treatment. The same effect was again observed with respect to the *pmt2* mutant in the present study. We also observed that the activation of Slt2/Mpk1 in *pmt2* was compromised upon rapamycin treatment and that, curiously, this occurred to the same extent as in the *mtl1* mutant cells. Our hypothesis is that Pmt2 and the mannosylated form of Mtl1 are both necessary to signal TOR function inhibition, but that Slt2 phosphorylation could reflect a read-out associated to signal impairment.

To the best of our knowledge, this is the first report to suggest a role for protein O-mannosylation in cell survival upon TOR blockage. In this mechanism, Slt2 phosphorylation is impaired; but curiously, the upper elements of the CWI pathway, Rom2 and Rho1, are not involved in signalling cell survival from Mtl1. In our previous report (Petkova *et al*., 2010a), we presented data showing that Mtl1 is required for cellular responses to rapamycin, although independently of the upper elements of the Pkc1 pathway. Here, we present additional data that suggest that Mtl1 O-mannosylation is required to elicit cellular responses to TOR inhibition.

The cytoplasmic domain of Mtl1 exhibits two-hybrid interactions with the GEF Rom2. Co-immunoprecipitation experiments also demonstrate the interaction between the two proteins. This is of a great importance for the model that we propose (Petkova *et al.,* 2010a) in which the signalling of oxidative stress and quiescence from Mtl1 is basically transmitted to the upper elements of the CWI pathway and from there to the general stress response. The data presented here support the model that Mtl1 O-mannosylation is a protein modification that is required to transmit the oxidative signal to Rom2 and Rho1. With respect to TOR blockage by rapamycin, we demonstrate that O-mannosylation plays an important role in cell survival through at least Mtl1; even so, the signal does not pass through either Rom2 or the upper elements of the CWI.

All these experimental results stress and reinforce the close connection between Mtl1 O-mannosylation, Mtl1 function and Pmt2.

Defects in glycosylation trigger a PKC1-MPK1-dependent cell wall compensatory mechanism (Lagorce *et al.,* 2003; Lommel *et al.,* 2004; Chen *et al.,* 2005; Arroyo *et al.,* 2011). These authors demonstrated that defects in O-mannosylation lead to the activation of the CWI pathway. Consistent with this, an increased basal level of Slt2/Mpk1 phosphorylation occurs in the *pmt2* mutant with respect to wild type cells. O- and N-glycosylation mutants show various cell wall-related phenotypes; glycosylation must therefore be indispensable for the cell wall structure and its stability (Strahl-Bolsinger *et al.,* 1993; Gentzsch and Tanner, 1996; Nakayama *et al.,* 1992; Lorberg *et al.,* 1999; Goto, 2007). Most of these phenotypes closely resemble those of mutants with defects in their PKC1-cell wall integrity pathways (Gray *et al.,* 1997; Lee *et al.,* 1993; Levin and Bartlett-Heubusch, 1992; Philip and Levin, 2001; Lommel *et al.,* 2004).

In the current study, we also demonstrate that under conditions of oxidative stress or TOR blockage, the *pmt2* mutant is not impaired in Msn2/Msn4 mediated transcriptional induction or in ribosomal gene expression. Glycogen accumulation is unaffected, which supports our hypothesis that protein O-mannosylation does not seem to be essential for the transmission of the signal from Mtl1 to Tor1 and Ras2 functions upon application of the previously mentioned stress treatments.

One feature shared by the cell-wall receptors Mtl1 and Mid2, which both belong to the CWI pathway, is the capacity of the Pmt2 isoform to modify them. Unlike Mid2, however, Mtl1 does not play a relevant role in maintaining the shmoo cell-wall integrity (Rajavel *et al.,* 2005). However, and somewhat surprisingly, Mtl1 also accumulates to the tip of the shmoo. Given their high degree of similarity, we hypothesize that Mtl1 and Mid2 could have diverged in their recent evolution. Mtl1 localisation to the tip of the shmoo must be a common characteristic that has been conserved throughout its evolution. Interestingly, Mtl1 localises to the septum, in a similar way to that observed in other Pkc1-MAPk members (Levin, 2005), and localises to the cell periphery, distributed in a dotted pattern that is homogeneously distributed throughout the cell surface. The latter localisation perfectly fits with the function of Mtl1 as a cell-surface receptor. Our data also suggest that protein O-mannosylation is important for maintaining appropriate cell surface localisation, which could determine correct functionality. This would, at least partially explain the *pmt2* and *mtl1* phenotypes.

Our results also suggest that the loss of viability experienced by *pmt2* under the conditions tested in this study: oxidative stress, TOR blockage and quiescence was mainly due to deficiencies in Mtl1 protein maturation. It is also evident that *pmt2* loses viability in stationary phase cultures. The observation that Pmt2 is required for life extension suggests that the maturation of protein receptors plays a substantial role in preventing early cell death. In a previous study, we demonstrated that Mtl1 was required to inactivate Ras2 and Tor1 functions under determined stress conditions (Petkova *et al.,* 2010a and b). Since *TOR1* and *RAS2* deletions prolong life span, one hypothesis is that protein O-mannosylation helps, or at least contributes to, signal starvation in order to correctly set quiescence. In this function, the role of some protein receptors, including Mtl1, may be required to correctly transmit the signal to downstream effectors. We previously proposed that the TOR function may maintain the signal (or signals) that prevents CWI activation under optimal growth conditions (Torres *et al.,* 2002). The evidence presented in this study is consistent with this hypothesis as Slt2 activation is impaired in *mtl1* and *pmt2* mutants in response to rapamycin treatment and also in the stationary phase; these are both conditions in which the TOR function is inhibited. In this case, both *PMT2* and *MTl1* (and consequently *MTL1* O-mannosylation) make important contributions to signalling.

Further data presented in this study supports the theory that Mlt1 O-mannosylation is relevant in quiescence and also to extend the chronological life: a) Mtl1 presents a hyperglycosylated mature form in stationary phase and O-mannosyl glycans appear to enhance protein stability under these conditions. b*) MTL1* expression specifically increases upon TOR blockade and during the stationary phase. C) Mtl1 is tightly regulated by the stress response transcription factor Msn2/Msn4; it is upregulated under conditions of nutrient starvation and the downregulation of the TORC1 function.

However, the mechanism by which Mtl1 transmits signals to Rom2 has yet to be elucidated. Other questions that also arose from this study include whether other mannosylated proteins could be involved in cell survival under quiescent conditions.

Experimental Procedures

Yeast Strains and Gene Disruptions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table1. *MTL1 and PMT2* were disrupted either by the one-step disruption method that uses the *kanMX4* module (Wach *et al.,* 1994) or by using the *natMX4* module (Goldstein and McCusker, 1999). These disruptions were verified by Northern blot analyses.

Mtl1GFP was constructed as follows: a PCR-generated *sGFP-kanMX4* module using pCYC86 (obtained from Dr. M. Aldea) was used as a template and the oligonucleotides MMO618 and MMO619 were designed to insert the product in-frame, just before the stop codon of the genomic sequence of *MTL1.*

DNA manipulation and plasmids

Plasmid pTP30 is a pCM265 derivative (*URA3/CEN;* Garí *et al.,* 1997) that contains *MTL1* under the $tetO_7$ promoter and is tagged with three copies of the HA epitope at the C-terminal position. The *MTL1*-coding sequence (1,657kb) was amplified from genomic DNA by using GSLOL55 and GSLOL56 and cloned into the *Not*I and *Pst*I vector sites. The cloning of *MTL1* was first carried out in the *E. coli* host DH10b (Invitrogen) or in SURE®2 (Stratagene). However, all of the trials that we conducted produced proteins that were not fully functional and which also impaired *E. coli* growth. We were only able to obtain a fully functional Mtl1 protein when it was expressed through a ligation reaction that was carried out inside the *S. cerevisiae* cells themselves. We cotransformed *S. cerevisiae* competent cells with a *Not*I-*Pst*I digested PCR fragment containing the *MTL1* ORF and the pCM265 vector that had been digested by the same enzymes. Yeast transformations were carried out following the method described by Gietz *et al.,* (1992).

The plasmid pMM351 is an integrative vector that contains three repetitions of the HA epitope in the C-terminal position and also carries the regulatable $tetO_7$ promoter. *Bst*EII was used to linearise this vector and to help integrate it at the *LEU3* locus. pTP55 is a derivative of the previously mentioned plasmid; it is obtained by cloning *MTL1* into the *Not*I-*Pst*I sites of pMM351.

We cloned genomic *ROM2* ORF amplified with GSLOL115 and GSLOL116 into *Bam*HI-*Sal*I sites of the pHW4 vector. This vector (a gift from Pascual Sanz) contains the ADH1 promoter and the GST epitope sequence. In this way, we obtained a Rom2GST fusion protein under the control of the ADH1 promoter (pTP84).

For two-hybrid analysis, we constructed the following plasmids: pTP184. The sequence encoding the C-terminal domain of *MTL1* (0.48 kb) was PCR amplified by using GSLOL179 and GSLOL118 and cloned in-frame into the *Eco*RI-*Bam*HI sites of the two-hybrid vector pGBT9. To construct pTP175, *ROM2* ORF was PCR amplified by means of GSLOL123 and GSLOL124 and cloned into the *Bam*HI-*Xho*I sites of pACTII.

The plasmid p*SLT2/MPK1* is a YEP352 derivative containing *SLT2/MPK1* ORF under its own promoter and tagged with HA epitope in the C terminus (a gift from M. Molina). In this study, we used the p*SLT2* plasmid for overexpression analysis and also to determine total Slt2 protein using the anti-HA monoclonal antibody.

The plasmid pAS106 expresses functional NH_2 terminally HA-tagged activated *RHO1* (H68) under the control of the *GAL1* promoter and the plasmid pAS48 expresses functional NH_2 terminally HA-tagged *ROM2* under the control of the *GAL1* promoter (both plasmids were kindly provided by T. Schmelzle).

The plasmid p*MSN2-GFP* contains the *LEU* marker, cloned under the ADH1 promoter in a centromeric plasmid, which was originally described in Görner *et al.,* (1998) and kindly provided by F. Estruch.

Media and Growth Conditions

Yeasts were grown in SD medium (2% glucose, 0.67% yeast nitrogen base that lacked the corresponding amino acids for plasmid maintenance) (Kaiser *et al.,* 1994). YPD (2% glucose, 2% peptone, 1% yeast extract and 2% agar) and SC dropout (2% glucose, 0.67% yeast nitrogen base, 0.2% dropout and 2% agar) plates were used when needed. Rapamycin (Sigma) was used at 200ng/ml in liquid cultures and at a final concentration of 1ng/ml (added from a 1mg/ml stock solution in 90% ethanol / 10% Tween20) in plates. Hydrogen peroxide and tert-butyl hydroperoxide were both diluted in sterile, distilled water (stock solutions from Sigma) for use in 1mM final concentrations at the indicated times. The pheromone α-factor concentration used in the G1 blockage experiments was 15µg/ml (from a stock solution in sterile water 5mg/ml, Sigma). To inactivate the temperature sensitive *sec1-1* allele, cells were shifted from 25 to 38˚C for 3 hours.

RNA preparation and Northern Blot Analyses

RNA purification, Northern blot, and probe labelling with digoxygenin were carried out according to Gallego *et al.,* (1997). Probes covering the entire open reading frame, without any adjacent sequences, were generated by PCR from genomic DNA.

Two-Hybrid Assays

Two-Hybrid analyses were carried out using the recipient yeast strain AH109 for co-transformation. The vectors used for cloning were: the pACTII plasmid containing the *ADH1* promoter and the *GAL4AD* (activating domain); the pGBT9 vector containing the *ADH1* promoter; and the *GAL4BD* (binding domain). As described above, to construct fusion proteins, the sequence of *ROM2* was cloned in-frame in the pACTII plasmid just after *GAL4AD* and the sequence encoding the cytoplasmic tail of *MTL1* was fused to the *GAL4BD* of the pGBT9 plasmid in the N-terminus. The targeting of each fusion protein was achieved by specific nuclear localisation sequences contained in the *GAL4* domain. The resulting cotransformants were tested for growth on selective SC plates without either histidine or adenine. The plates were incubated at 30℃ for 3-5 days.

Protein Extraction and Immunoblot Analyses

Total yeast protein extracts were prepared as previously described in Torres *et al.* (2002). The antibodies for Western blotting were as follows: anti-HA (mouse monoclonal, 12CA5) was used at a dilution of 1:1000 in 0.25% non-fat milk / TBST-0.1 (0.1%Tween20 final concentration) and the corresponding secondary anti-mouse HRP-linked antibody (Amersham Bioscience) at a dilution of 1:10000 in 0.25% non-fat milk / TBST-0.1 (as described in Vilella *et al.,* 2005). 3F10 (Roche Applied Science) and the corresponding anti-rat antibody coupled to HRP (Millipore) under the same conditions as for anti-HA. Anti-GST (goat polyclonal, GE Healthcare) at a dilution of 1:1000 in 5% non-fat milk / TBST-0.1 and the corresponding secondary anti-goat IgG-peroxidase at a dilution of 1:15000 in TBST-0.1 (Vector Laboratories). Anti-phospho-p44/42 (rabbit polyclonal, Cell Signalling) antibody was used (as described in de la Torre *et al.,* 2002) at a 1:2000 dilution in TBST-0.1 and the corresponding secondary ant-rabbit, HRP-linked antibody (Amersham Biosciences) was used at 1:10000 in TBST-0.1 containing 1% non-fat milk. The protein-antibody complexes were visualised by enhanced chemiluminescence using the Supersignal substrate (Pierce) in a Lumi-Imager (Roche Applied Science).

Immunoprecipitation

Protein extracts were prepared from exponentially growing cells. At least 150 OD cells were harvested and resuspended in 500 µl ice-cold IP buffer (50mM Tris-HCl [pH 7.5], 250mM NaCl, 5mM EDTA, 0.1% Triton X-100) containing phosphatase inhibitors (50mM NaF, 1mM β-glycerophosphate, 1mM EGTA, 25mM sodium pyrophosphate), protease inhibitors (1mM PMSF, 0.1mM TPCK, 5µg/ml chymostatin, 5µg/ml leupeptin, 5µg/ml antipain, 1µM pepstatin) and 1mM sodium orthovanadate. An equal volume of glass beads was added to the suspension and the cells were broken by vigorous vortexing at 4℃. The beads and

cell debris were removed by centrifugation at 10000rpm at a temperature of 4℃. HA-tagged Mtl1 proteins were immunoprecipitated from cell extracts with protein A-Sepharose beads coupled to anti-HA antibody for 2h at 4℃. The precipitates were washed three times with ice-cold IP buffer and the proteins were eluted in SDS sample buffer and then subjected to 7.5 – 10% SDS-PAGE. For co-immunoprecipitation, we preferentially used the following IP buffer: 20mM Tris-HCl [pH 7.5], 140mM NaCl, 10mM EDTA, 1% NP-40, 10% glycerol, 25mM NaF, 40mM β-glycerophosphate, 1mM sodium orthovanadate and 1x Roche protease inhibitor cocktail. For immunoblotting, we used anti-HA and anti-GST antibodies to detect the $Mtl1^{HA}$ and $Rom2^{GST}$ proteins, respectively, as described above.

Deglycosylation by Endo H digestion

Immunoprecipitates were suspended in 25 µl of endoglycosidase H buffer (50mM sodium phosphate buffer [pH 5.5] containing 50mM β-mercaptoethanol, 0.1% SDS and protease inhibitors, as described above) and digested overnight with 30mU Endo H (Calbiochem®) at 37℃. Mock incubations were also carried out without Endo H. The reactions were stopped by adding 10 µl of 5x SDS sample buffer.

In vivo cellular localisation of Mtl1

To study Mtl1 subcellular localization, we cloned Mtl1-GFP upon applying PCR to amplify the sGFP-kanMX4 cassette from the pCYC86 plasmid (as described in Vilella *et al.,* 2005). We used oligonucleotides designed to insert Mtl1 ORF in-frame just before the stop codon of the genomic *MTL1* sequence to obtain the fusion protein Mtl1-GFP.

Cell survival and chronological life span

To assay cell viability, cells were grown to mid-log phase O.D.600: 0.6 in SD medium supplemented with an excess of amino acids. We therefore measured the chronological life span in the wt, pmt2 and *mtl1* cell cultures based on the survival of populations of non-dividing yeast cells. According to Parrella and Longo (2008), yeast cells growing in synthetic complete glucose medium stop dividing after 24-48 hours. We followed the protocol described by these authors and considered the 3rd day of growth as the starting point for the chronological life span experiment. However, these authors also proposed reducing the starting point when the strains used lose their viability as the cells enter the stationary phase. The survival curves clearly show that the mtl1 mutant loses viability after the second day of culture. We have therefore presented an alternative histogram that considers the second day as the starting point for the chronological life span experiment. To precisely register viability, the cells were serial diluted and plated by triplicate onto YPD plates. Viability was scored by

counting the number of cells able to form colonies. Averages and standard deviations were subsequently plotted in the histogram diagrams.

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Figure Legends:

Fig. 1. Structural features of Mtl1. (A) A schematic delineation of the Mtl1 protein domains/motifs is shown. (B) Deduced amino acid sequence of Mtl1p (551aa). Protein regions are depicted as follows: bold: secretion signal peptide (1-35aa); highlighted in grey: Ser/Thr-rich region; underlined: sequence homologous to Mid2; underlined in bold: transmembrane domain (362-384aa); highlight black NXS/T – potential N-glycosylation sites.

Fig. 2. The Mtl1 protein is highly O-mannosylated, predominantly by the Pmt2 protein mannosyltransferase. (A) Western blot analysis of $Mtl1^{HA}$ isolated from the wild-type and *pmt* mutant cells harbouring the pTP30 plasmid and expressing $Mtl1^{HA}$ under the inducible $tetO_7$ promoter. Total protein extracts (40µg) from exponentially growing cells were resolved on 10% SDS-PAGE, followed by immunoblotting with anti-HA antibody. The unlabelled lanes were loaded with extracts from the same strains harbouring the empty pCM265 vector. The arrows indicate the migration pattern of $Mtl1^{HA}$ (200-, 100- and 57kDa protein variants). Molecular mass markers are given in kDa, on the left. (B) The same samples were separated on 7.5% polyacrylamide gels to increase the resolution of the mature $Mtl1^{HA}$ with the different degrees of glycosylation.

Fig. 3. The Mtl1 protein is modified by the N-linked carbohydrate chain and requires intact secretory machinery. (A) Wild-type cells expressing $Mtl1^{HA}$ under the inducible $tetO_7$ promoter were grown to exponential phase in SD at 30℃. After total protein extraction, HA-tagged Mtl1p was immunoprecipitated with monoclonal anti-HA antibody coupled to the A-

Sepharose protein. The immunoprecipitates were either treated with Endo H (+), or mock treated (-), overnight, at 37ºC, and Mtl1 ^{HA} proteins were detected by Western blotting with anti-HA antibodies. (B) Wild-type and *sec1-1* mutant cells expressing Mtl1^{HA} from the pTP30 plasmid were grown to logarithmic phase in SD at 25ºC and subsequently shifted to 38ºC for 3 hours to inactivate the thermosensitive *sec1-1* allele. Mtl1^{HA} was analyzed from total protein extracts by SDS-PAGE and Western blot, using monoclonal anti-HA antibody.

Fig. 4. Mtl1 interacts with Rom2 both in vivo and in vitro. (A) The cytoplasmic domain of Mtl1 displays two-hybrid interactions with Rom2. The yeast strain AH109 was co-transformed with the following pairs of plasmids: pTP184 (pGBT9 containing the C terminal tail of *MTL1*) and pTP175 (*ROM2* ORF cloned in pACTII); pTP184 and the empty pACTII; the empty pGBT9 and pTP175; the vectors pGBT9-*SNF1* and pACTII-*SNF4* as a positive control for interaction. The resulting transformants were tested for growth on SC plates without Histidine or Adenine, following an incubation of 3-5 days at 30°C. Three different clones from each transformation are shown in the figure.

(B) Co-immunoprecipitation reveals the physical interaction between Mtl1 and Rom2. Mtl1^{HA} (from the integrative pTP55) and Rom2^{GST} (from pTP84) were individually transformed or cotransformed in wt cells, as indicated. Wild type cells transformed with Mtl1^{HA} were grown exponentially and total protein extracted. Immunoprecipitation was performed using monoclonal anti-HA antibody coupled to the Protein A-Sepharose: Mtl1^{HA} was detected by Western blotting using anti-HA (3F10) antibody (left panel). In the right panel we show coimmunoprecipitation assays using monoclonal anti-HA antibody coupled to Protein A-Sepharose beads; Rom2^{GST} was detected by Western blotting using anti-GST antibody. Line 1: wild type cells transformed with Mtl1^{HA}; Line 2: wt cells transformed with Rom2^{GST}; Line 3: wild type cells cotransformed with both Mtl1^{HA} and Rom2^{GST}. Western blotting corresponding to the crude extract prepared from wild type cultures cotransformed with both Mtl1^{HA} and Rom2^{GST} and detected with anti-GST antibody is also shown in this panel. The same controls were performed with cells expressing only one of the two proteins (not shown, to simplify).

Fig. 5. Mtl1 cellular localisation. (A) GFP-Mtl1 localisation in exponentially growing cells. (B) wt and *pmt2* strains were transformed with the GFP-Mtl1 plasmid. Exponentially growing cells were treated with alpha factor for two hours at 30ºC. Once cells were synchronized in G1, photographs were taken using the fluorescence microscope. The cells were then washed four times and released into fresh medium without alpha factor. Mtl1 localisation was followed throughout the first cell cycle and photographed as shown in the images. (C) GFP-Mtl1 distribution throughout the cell periphery. (D) GFP-Cdc11 localisation to the septum in wt and *pmt2* cells.

Fig. 6. Mtl1 transcriptional upregulation under quiescent conditions. (A) GFP-Mtl1 fluorescence increases in stationary cells with respect to log phase cultures. (B) Northern blot analyses of mRNA samples obtained from: wt exponentially growing cells (0) treated with 1mM hydrogen peroxide or 200ng/ml rapamycin; wt and wt cells overexpressing Msn2 during the stationary phase. We used *MTL1* probe and *U1* as a loading control. (C) Northern blot analysis of mRNA samples taken from exponentially growing wt and *msn2msn4* cultures.

Fig. 7. Protein O-mannosylation catalysed by Pmt1 and Pmt2 is required for cells to survive the application of oxidants and rapamycin. Mtl1 complements *pmt2* survival deficiencies and makes it possible to achieve wild type levels. (A) The absence of *PMT2* or *PMT1* makes cells sensitive to oxidizing agents and rapamycin, which are characteristic phenotypes of *mtl1*. Exponentially growing cultures of wild-type, *pmt1, pmt2, pmt3, pmt5* and *pmt6* were prepared and a cell quantity corresponding to $3x10^7$ was serially 10-fold diluted and spotted onto SD plates containing 1mM tertbutylhydroperoxide or 2ng/ml rapamycin. The plates were then incubated at 30℃ for 2-3 days. (B) Impared O-manno sylation by Pmt2 does not affect the transcriptional induction of Msn2/Msn4-dependent genes or ribosomal gene repression in response to oxidative stress or rapamycin treatment. Northern blot analyses of exponentially growing cells treated either with hydrogen peroxide or rapamycin, as described in the legend to Fig 6. Blots were probed for *HSP12, CTT1,* and *RPL3.* A probe for *U2* was used as a loading control. (C) Wild-type and *mtl1* expressing p*MTL1* under the $tetO_7$ or harbouring the empty pCM265 vector were grown to exponential phase in SD plus amino acids, serial-diluted and spotted on SD plates containing 1mM hydrogen peroxide or 1ng/ml rapamycin. (D) Overexpression of members of the PKC1-MAPK pathway partially suppresses the cell growth defect of *pmt2* in response to oxidizing agent, but only overexpression of Mtl1 restores the *pmt2* cell viability upon rapamycin treatment. Exponentially growing cultures of wild-type, *pmt2, pmt2* transformed with pRho1*, pRom2, pSlt2 and pMtl11 in SD were prepared. In parallel, the log phase cultures of *pmt2*+pRho1* and *pmt2*+pRom2 were washed four times with equal volumes of minimum medium containing galactose as a sole carbon source and incubated for 12 hours in minimum medium containing galactose to induce the expression of either the hyperactive Rho1* allele or Rom2. The cells were serial-diluted and plated on SD plates containing either tert-butyl hydroperoxide or rapamycin (as in A).

Fig. 8. Impaired O-mannosylation, of at least Mtl1, by Pmt2 isoform affects signalling to Slt2/Mpk1 in response to rapamycin but not to oxidative stress. (A) Wild-type, *mtl1* and *pmt2* mutant cells were grown exponentially in SD at 25℃ and treated with 1mM H_2O_2. Samples were collected at the indicated times and processed for Western blotting. Double phosphorylation of Slt2/Mpk1 was detected by using anti-phospho-p44/42 antibody, whereas

total Slt2/Mpk1 was detected using anti-HA antibody. (B) Western blot analysis as described in (A), but the indicated strains were treated with 200ng/ml rapamycin. Histograms represent fold induction in Slt2 activity which is represented as the ratio between the quantitative levels of Slt2 phosphorylation obtained with anti-p44/42 antibody at either 30 or 60 min and the Slt2 phosphorylation level measured at time 0 (untreated cells). All the single quantified values were normalized with respect to their corresponding loading controls as determined with anti-HA antibody.

Fig 9. Mtl1 hyperglycosylation and Pmt2 are required for cell survival and CWI activation during stationary phase. (A) Survival curves of wild type, *mtl1* and *pmt2* strains in stationary phase. Wild-type cells, *mtl1* and *pmt2* mutant cells were grown in SD medium at 30ºC. At the indicated times, viability was measured by plating onto YPD plates. CFU were counted after 2-3 days at 30ºC. Approximately 500 cells were counted at each time point by adjusting the dilution factor and the volume plated according to the mortality rate. The values are the means of three independent experiments, each conducted in triplicate, and the error bars represent standard deviations. (B) Wild type, *mtl1* and *pmt2* chronological life span. In this study, the number of CFU at day 3 (after the log phase) was considered to provide the initial survival point (100% survival). As in (A), the values are means of three independent experiments, each conducted in triplicate, and the error bars represent standard deviations. (C) Aliquots taken from the former experiment were processed for total protein extraction and western blotting analysis, using anti p44/42 antibody to detect the double phosphorylated form of Slt2 kinase. (D) Mtl1 hyperglycosylation in the stationary phase. Western blot analysis of $Mtl1^{HA}$ isolated from exponentially and stationary phase cultures of wild-type cells expressing Mtl1 under the $tetO_7$ promoter. Total protein extracts (40µg) were subjected to 10% SDS-PAGE and $Mtl1^{HA}$ proteins were detected by immunoblotting with monoclonal anti-HA antibody. The unlabelled lanes were loaded with extracts from cells carrying empty pCM265 vector. Protein mass markers are given in kDa on the left-hand side.

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TABLE 1

Yeast strains used in this work

Strain	Relevant genotype	Reference
CML128	*MAT**a** leu2-3,112 ura3-52 trp1 his4 can1^r*	Gallego *et al,* (1997)
GSL41	*MAT**a** mtl1::kanMX4*	Petkova *et al,* (2010) [a]
GSL128	*MAT**a** pmt2::kanMX4*	This work [a]
GSL1	CML128 *MTL1GFP-kanMX4*	This work
GSL136	*MAT**a** pmt2::natMX4 MTL1GFP-kanMX4*	This work [a]
GSL103	CML128 *tetO$_7$MTL1HA-LEU3*	This work
BY4741	*MAT**a** his3d1 leu2d0 met17d0 ura3d0*	Obtained from J. Ariño
MML1017	*MAT**a** pmt1::kanMX4*	Obtained from J. Ariño [b]
MML1018	*MAT**a** pmt2::kanMX4*	Obtained from J. Ariño [b]
MML1019	*MAT**a** pmt3::kanMX4*	Obtained from J. Ariño [b]
MML1020	*MAT**a** pmt5::kanMX4*	Obtained from J. Ariño [b]
MML1021	*MAT**a** pmt6::kanMX4*	Obtained from J. Ariño [b]
AN3-5D	*MAT? ura3-52 trp1 leu2-3,113 sec1ts*	Obtained from L. Castillo
W303-1A	*MAT**a** ade2-1 trp1-1 leu2-3,2-111 his3-11,75 ura3*	Obtained from C. Gancedo
msn2msn4	*MAT**a** ade2 can1 his3 leu2 trp1 ura3 msn2-delta3::HIS msn4-1::TRP1*	Obtained from F. Estruch
AH109	*MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4delta gal80delta LYS2::GAL1UAS-GAL1TATA-HIS3 GAL2UAS-GAL2TATA-ADE2 URA3::MEL1UAS-MEL1TATA-lacZMEL1*	Obtained from E. Gari

[a] CML128 background
[b] BY4741 background

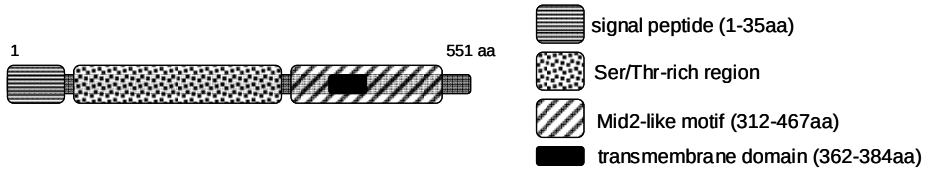
TABLE II

Oligonucleotides used in this work

Oligonucleotide	Sequence 5'-3'
GSLOL55	ACTAGGCGGCCGCATGGCAAGCTGCAATCCGAC
GSLOL56	ACTAGCTGCAGATAGTTCGTGATATTTAAGC
GSLOL115	CATGGGATCCTGAGCGAAACCAACGTTGACAG
GSLOL116	CATGGTCGACTTAACCCCAGAAATCTAACG
GSLOL118	CATGGGATCCTTAATAGTTCGTGATATTTA
GSLOL123	CATGGGATCCTGAGCGAAACCAACGTTGACAG
GSLOL124	CATGCCTCGAGTTAACCCAGAAATCTAACG
GSLOL179	ACGTGAATTCGATTTCATTGACTCTGACGG
GSLOL183	TTAGTCCACATCGCTACTGTCATTCTGTTTCGAACCG
	TGGTCCAGCAATCCGTACGCTGCAGGTCGAC
GSLOL184	AGTCCAAAACCAGTTTAAGTAGCGGAAGTTACTACTT
	GGTCCCTCCATACCATCGATGAATTCGAGCTCG
MMO618	CGAGAACTATTACACGAAACCAAACAACGGCTTAAAT
	ATCACGAACTATCCAGC TGAAGCTTCGTACGC
MMO619	GAGGGTGATTTTAAGAAGAAAAGTTATGGCAAAGCTG
	CTTTCGCTATGATGCATAGGCCACTAGTGGATC

Fig 1

A



B

```
  1  MASCNPTRKK SSASSLSMWR TILMALTTLP LSVLSQELVP ANSTTSSTAP
 51  SITSLSAVES FTSSTDATSS ASLSTPSIAS VSFTSFPQSS SLLTLSSTLS
101  SELSSSSMQV SSSSTSSSSS EVTSSSSSSS ISPSSSSSTI ISSSSSLPTF
151  TVASTSSTVA SSTLSTSSSL VISTSSSTFT FSSESSSSLI SSSISTSVST
201  SSVYVPSSST SSPPSSSSEL TSSSYSSSSS SSTLFSYSSS FSSSSSSSSS
251  SSSSSSSSSS SSSSYFTLST SSSSSIYSSS SYPSFSSSSS SNPTSSITST
301  SASSSITPAS EYSNLAKTIT SIIEGQTILS NYYTTITYSP TASASSGKNS
351  HHSGLSKKNR NIIIGCVVGI GAPLILILLI LIYMFCVQPK KTDFIDSDGK
401  IVTAYRSNIF TKIWYFLLGK KIGETERFSS DSPIGSNNIQ NFGDIDPEDI
451  LNNDNPYTPK HTNVEGYDDD DDDDANDENL SSNFHNRGID DQYSPTKSAS
501  YSMSNSNSQD YNDADEVMHD ENIHRVYDDS EASIDENYYT KPNNGLNITN
551  Y*
```

Fig 2

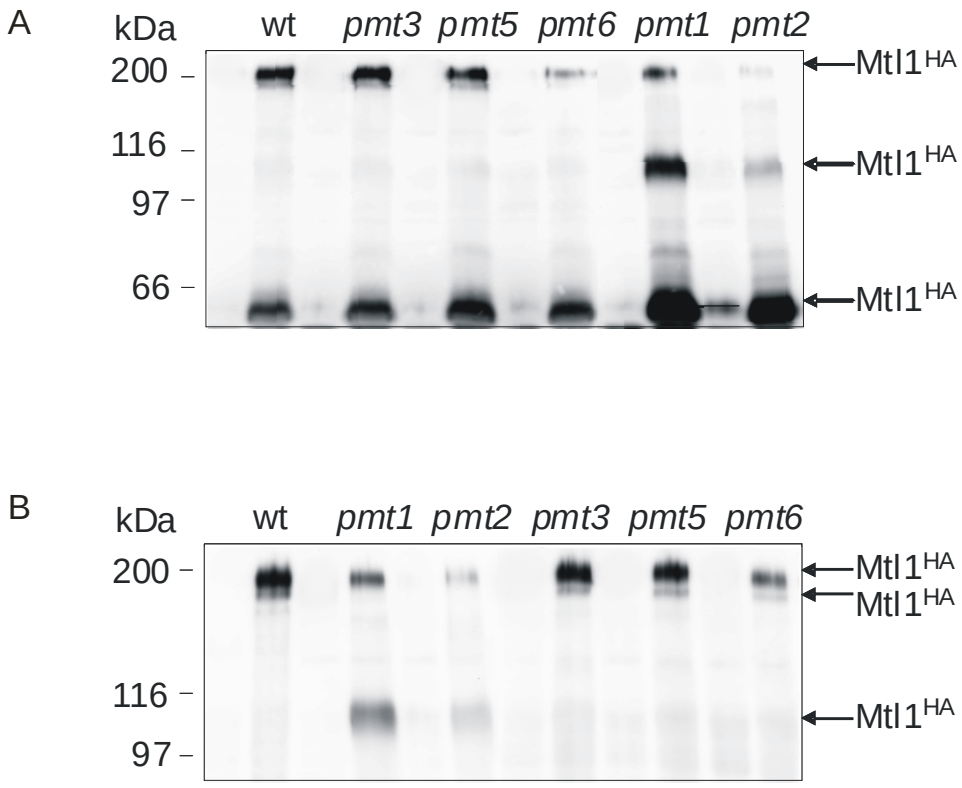
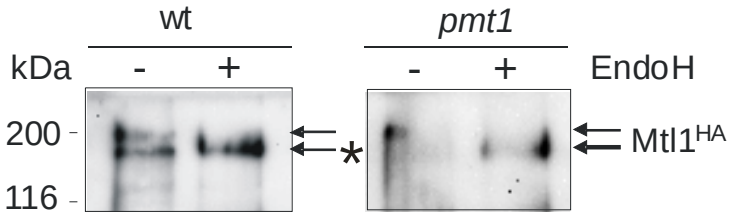

A
kDa
wt
pmt3
pmt5
pmt6
pmt1
pmt2
200
116
97
66
Mtl1HA
Mtl1HA
Mtl1HA
B
kDa
wt
pmt1
pmt2
pmt3
pmt5
pmt6
200
116
97
Mtl1HA
Mtl1HA
Mtl1HA

Fig 3

A


wt
pmt1
kDa
-
+
-
+
EndoH
200
116
*
Mtl1HA

B

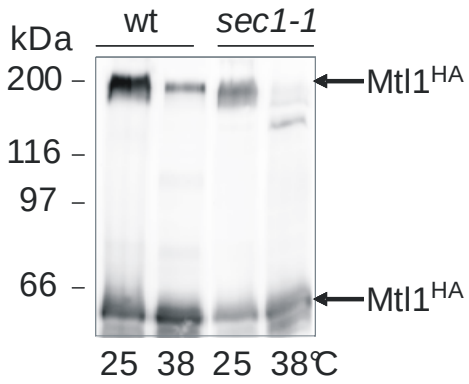
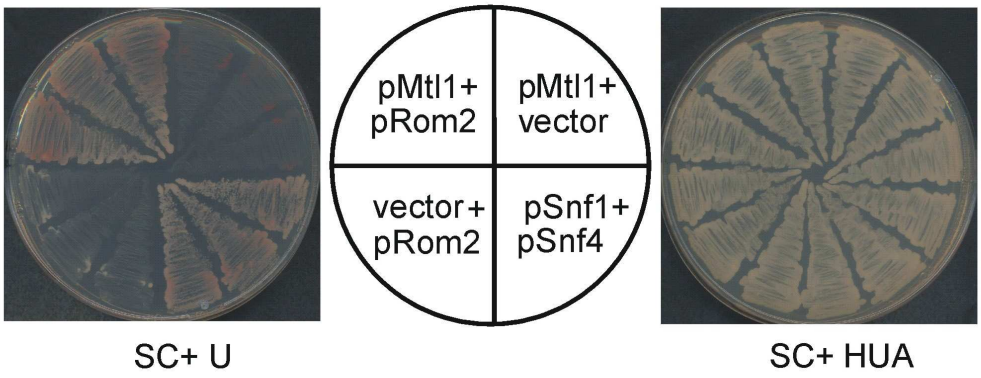

kDa
wt
sec1-1
200
116
97
66
Mtl1HA
Mtl1HA
25
38
25
38℃

Fig4

A


pMtl1+
pRom2
pMtl1+
vector
vector+
pRom2
pSnf1+
pSnf4
SC+ U
SC+ HUA

B

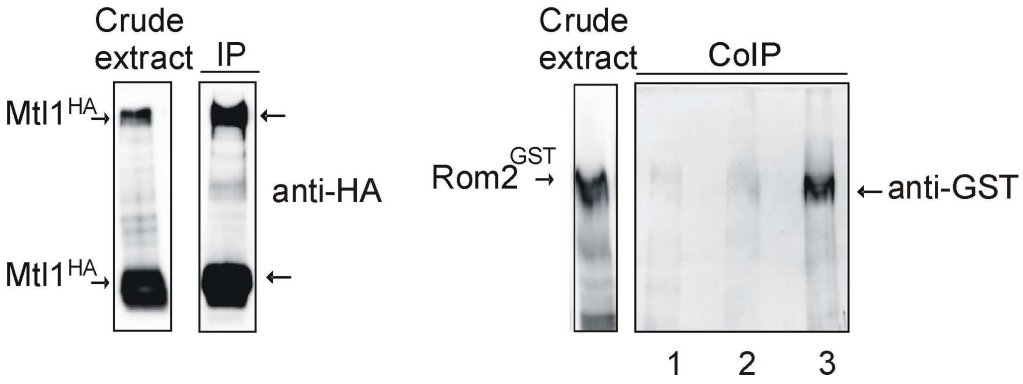

Crude
extract
IP
Mtl1HA
Mtl1HA
anti-HA
Crude
extract
CoIP
Rom2GST
anti-GST
1
2
3

Fig 5

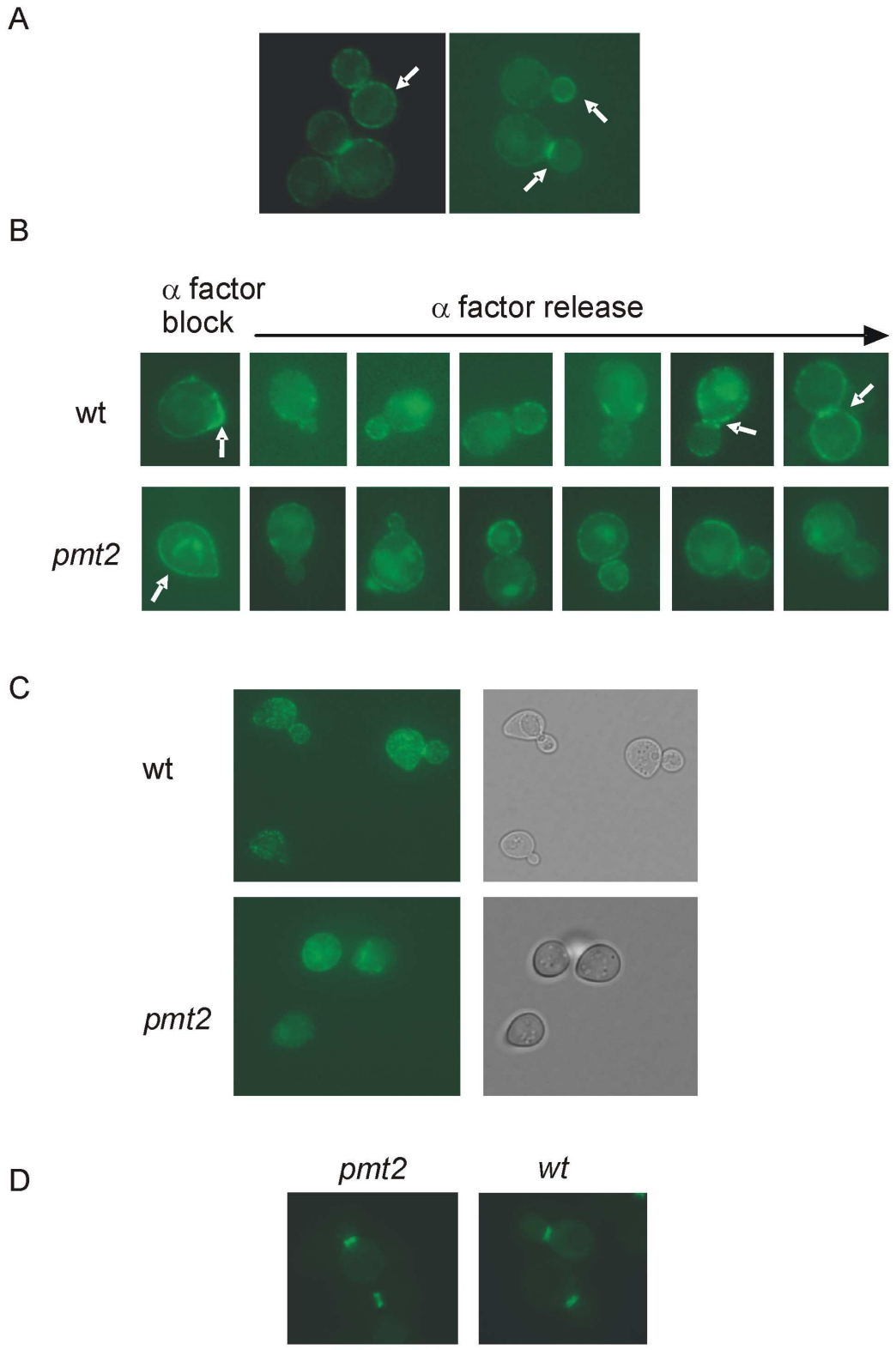


Fig 6

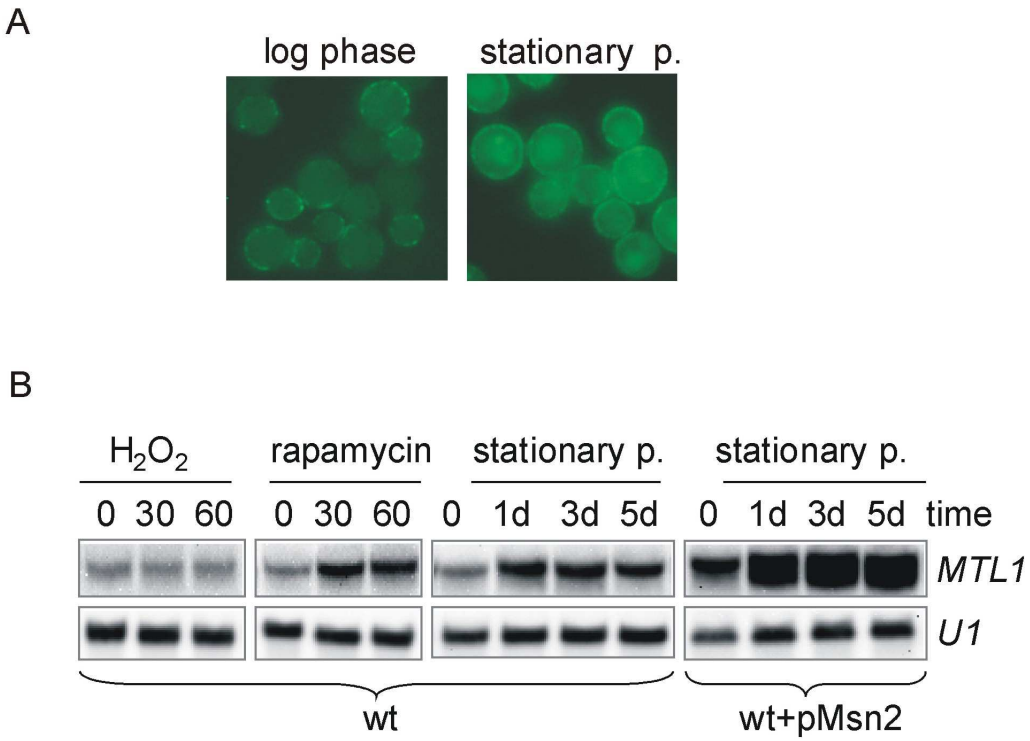

A
log phase
stationary p.
B
H2O2
rapamycin
stationary p.
stationary p.
0 30 60 0 30 60 0 1d 3d 5d 0 1d 3d 5d time
MTL1
U1
wt
wt+pMsn2
C
WT
msn2msn4
lg lg
MTL1
U1

Fig 7

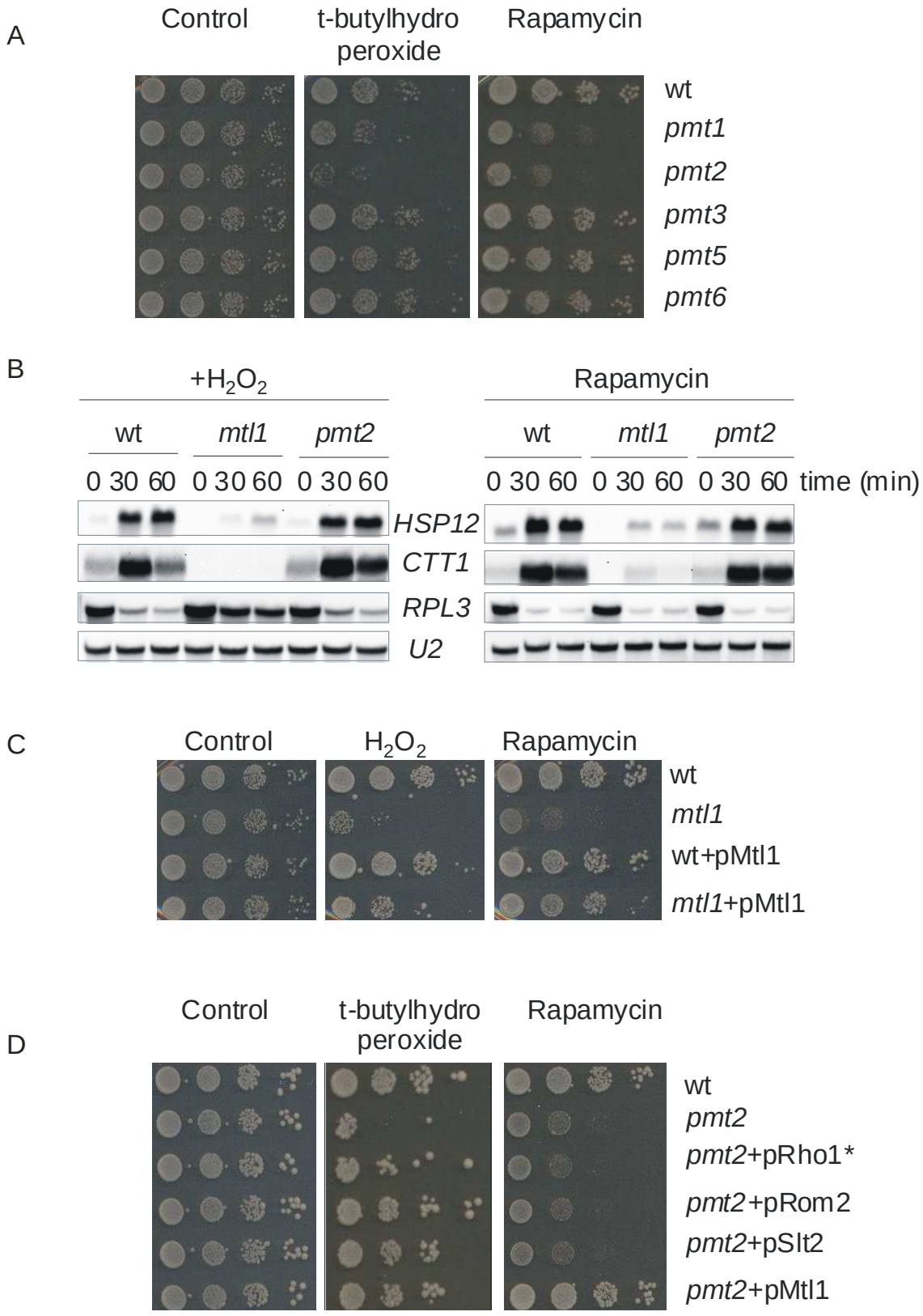
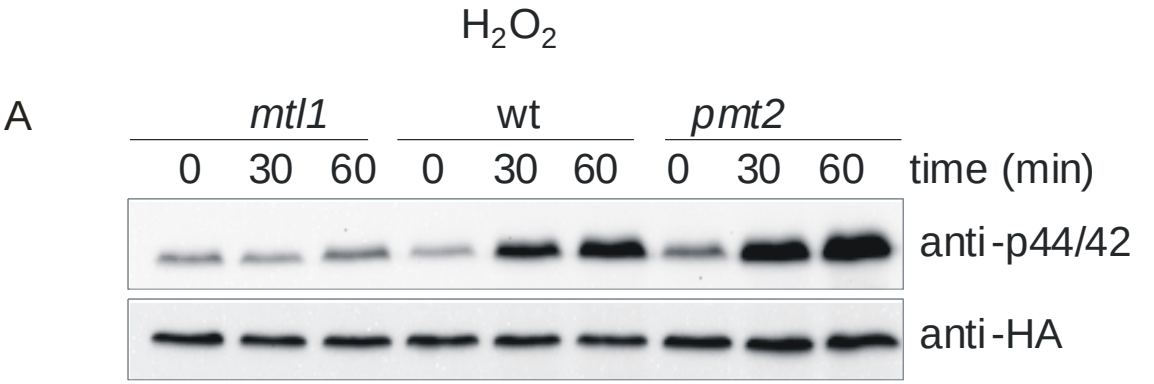
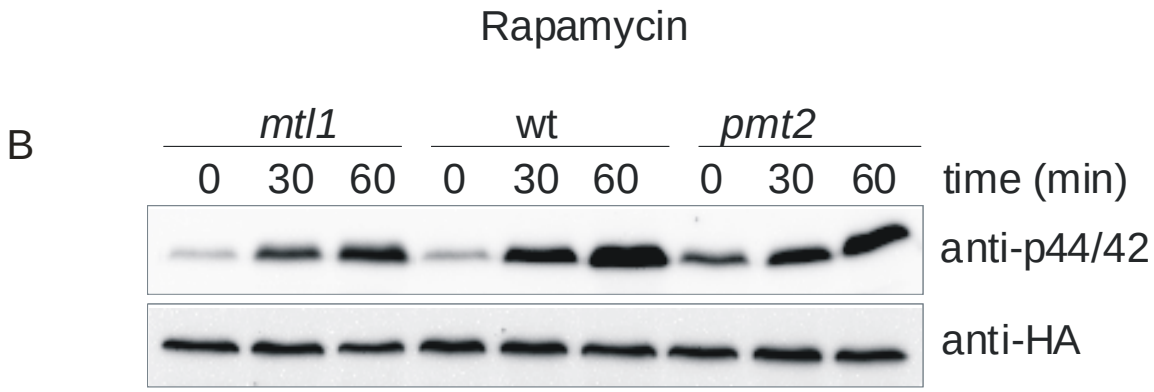

A
Control
t-butylhydro peroxide
Rapamycin
wt
pmt1
pmt2
pmt3
pmt5
pmt6
B
+H2O2
Rapamycin
wt
mtl1
pmt2
wt
mtl1
pmt2
0 30 60 0 30 60 0 30 60 0 30 60 0 30 60 0 30 60 time (min)
HSP12
CTT1
RPL3
U2
C
Control
H2O2
Rapamycin
wt
mtl1
wt+pMtl1
mtl1+pMtl1
D
Control
t-butylhydro peroxide
Rapamycin
wt
pmt2
pmt2+pRho1*
pmt2+pRom2
pmt2+pSlt2
pmt2+pMtl1

Fig 8


H_2O_2
A
mtl1
wt
pmt2
0 30 60 0 30 60 0 30 60
time (min)
anti-p44/42
anti-HA


Rapamycin
B
mtl1
wt
pmt2
0 30 60 0 30 60 0 30 60
time (min)
anti-p44/42
anti-HA

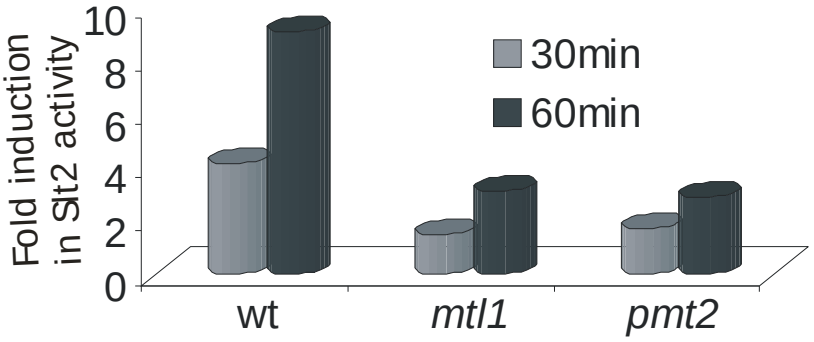
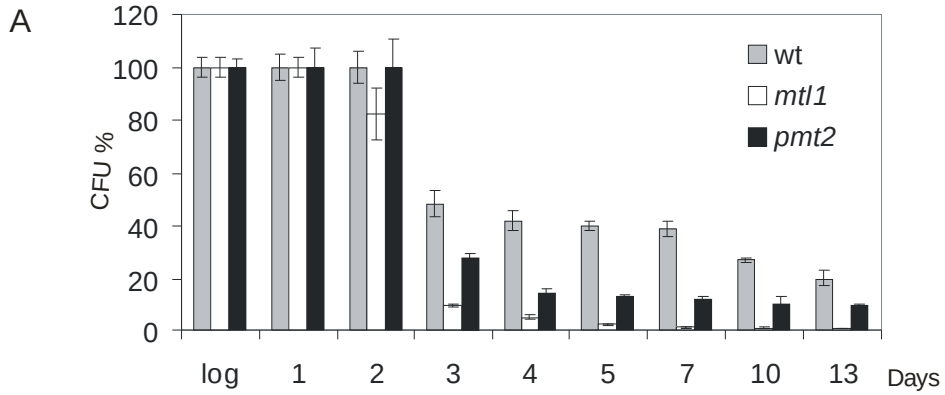
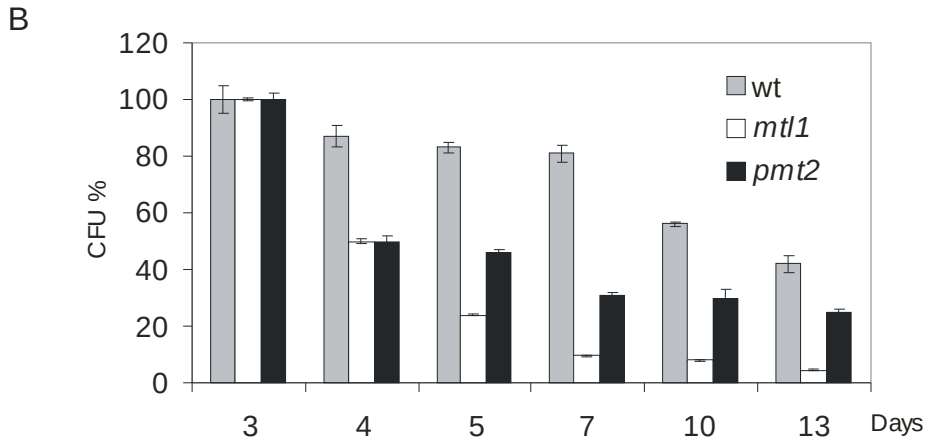
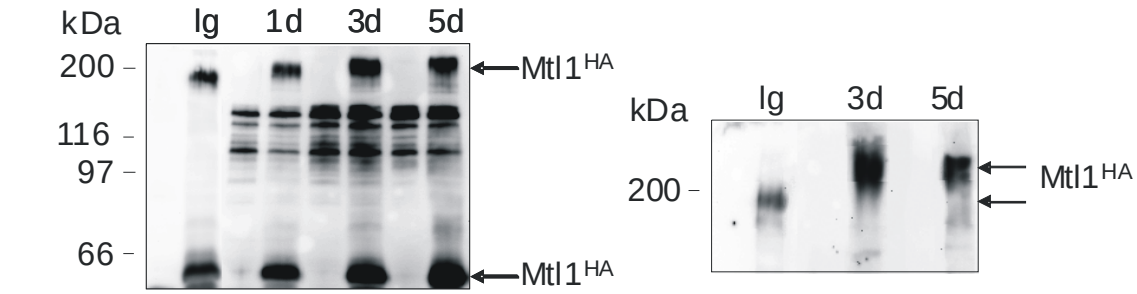

Fold induction in Slt2 activity
10
8
6
4
2
0
30min
60min
wt
mtl1
pmt2

Fig 9


A
120
100
80
60
40
20
0
CFU %
wt
mtl1
pmt2
log
1
2
3
4
5
7
10
13
Days


B
120
100
80
60
40
20
0
CFU %
wt
mtl1
pmt2
3
4
5
7
10
13
Days


C
log 1 2 3 4 time (days)
anti-p44/42
wt
pmt2
mtl1


D
kDa
lg
1d
3d
5d
200
116
97
66
Mtl1HA
Mtl1HA
kDa
lg
3d
5d
200
Mtl1HA

ARTICLE IV

Objectives:

- To study the role of Pkc1 protein and the actin cytoskeleton in ribosomal gene expression in the context of oxidative stress response

- Searching for cellular functions affected by oxidative stress in which Pkc1 is involved

Pkc1 and actin polymerisation activities play a role in ribosomal gene repression associated with secretion impairment caused by oxidative stress

Felipe Vilella Mitjana[1], Mima Ivanova Petkova [2], Nuria Pujol-Carrion[2] and Maria Angeles de la Torre-Ruiz[2]

[1]Prince Felipe Research Centre, Valencia 46012, Spain

[2]Departament de Ciències Mèdiques Bàsiques-IRBLleida, Facultad de Medicina, Universidad de Lleida, Lleida 25008, Spain.

Running title: Pkc1 and actin polymerisation repress ribosome biogenesis under oxidative conditions

Key words: Oxidative stress, Pkc1, CWI, actin polymerisation, ribosomal gene transcription, secretion, invertase, carboxypeptidase, FM4-64.

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Abstract

In *Saccharomyces cerevisiae*, the cell integrity pathway plays a role in the oxidative stress response. In this study we show that the Pkc1 protein mediates oxidative signalling by helping to downregulate ribosomal gene expression when cells are exposed to hydrogen peroxide. An active actin cytoskeleton is required for this function, since the cells blocked in actin polymerisation were unable to repress ribosomal gene transcription. Following the invertase secretion pattern, we hypothesise that oxidative stress induced by hydrogen peroxide could have affected the latter steps of secretion. This would explain why the Pkc1 function was required to repress ribosomal biogenesis.

We have recently demonstrated that oxidative stress induces transient and dosage-dependent ribosomal gene repression upon treatment with hydrogen peroxide (Petkova *et al.,* 2010). Other types of environmental stress are also known to regulate ribosomal gene transcription (Mizuka and Warner, 1994). The cells affected in the secretory pathway require the Pkc1 function to repress ribosomal gene expression (Nieras and Warner, 1999). Some cell-wall receptors are believed to sense the increase in turgor pressure caused by secretion impairment and to transmit the signal to Pkc1 (Li *et al*, 2000). In response to oxidative stress, Mtl1, a cell-wall receptor and component of the CWI (Cell Wall Integrity) or Pkc1-MAPK pathway, signals ribosomal gene repression through Tor1 and Ras2 inhibition (Petkova *et al.,* 2010). Mtl1 is also required for cell survival in response to oxidative stress. According to Vilella *et al*. (2005), of all the elements integrating the PKC1-MAPK pathway, only Rom2 and Pkc1 are essential for cell viability following treatment with different oxidants. Mtl1 also activates the Pkc1-MAPK pathway when cells are exposed to oxidants (Petkova *et al.,* 2010). Pkc1 is a Protein Kinase C and the key element in the CWI pathway. Given the close connection between Mtl1 and Pkc1 in the oxidative stress response, we wondered whether the Pkc1 protein also had a role in the ribosomal gene repression that occurs in response to oxidative stress. To check this, we grew wild type and *pkc1* cells to log phase and treated both cultures with hydrogen peroxide. As *pkc1* is lethal in the absence of the cell-wall stabiliser sorbitol, we added this reagent to the culture media to a final concentration of 0.8M. The presence of sorbitol in the culture media conferred wild type cells with more resistance to ribosomal gene repression in response to 1mM hydrogen peroxide treatment. This is probably due to the fact, to some extent, hydrogen peroxide affects the cell-wall integrity (not shown), even though the main target for hydrogen peroxide is not necessarily the cell-wall (Vilella *et al*., 2005 and Petkova *et al.*, 2010). We therefore increased the hydrogen peroxide concentration to 10mM in SD medium containing sorbitol and the required amino-acids, without affecting wild type cell viability (not shown). We took samples at several times (as shown in Figure 1) and processed them for subsequent northern blot analyses. We checked the *RPS6A, RPS28A* and *RPL30* ribosomal genes and also *U1* as a loading control. In the presence of 0.8M sorbitol and 10mM hydrogen peroxide, wild type did not exhibit any substantial loss of viability (not shown, cell viability was checked in plates by doing serial dilutions from exponentially growing cultures) whereas ribosomal gene transcription was dramatically repressed (Fig 1A) similarly to that described in Petkova *et al.,* (2010). However, in the case of *pkc1* strain, repression was almost absent (Fig 1A), while cell viability was seriously affected (not shown); this effect was similarly to one previously reported (Vilella *et al.,* 2005). These results demonstrate that Pkc1 is required to transmit the oxidative signal to ribosomal biogenesis in response to oxidative stress. Another of the functions also regulated by Pkc1 and affected by oxidative stress is the organisation of the actin cytoskeleton.

Oxidative stress induces actin depolarisation and depolymerisation (Vilella *et al.,* 2005 and Pujol *et al.,* 2009) upon hydrogen peroxide treatment. The actin cytoskeleton transiently depolarises upon hydrogen peroxide treatment. We observed that under the experimental conditions applied in this study, actin depolarisation was both qualitatively and quantitatively identical to that previously described (not shown, and Vilella *et al.*, 2005). This suggested that actin depolarisation and ribosomal gene repression upon oxidative treatment, are two functions that occur simultaneously. In order to ascertain whether actin polymerisation activity influences ribosomal gene expression in the oxidative stress response, wild type cells were treated with 150µM Latrunculin for 1hour to completely depolarise the actin cytoskeleton. Hydrogen peroxide was then added to the cultures. Samples were taken and processed for northern analyses. Fig 1A shows that Latrunculin A did not affect the ribosomal gene expression compared to wild type cells. However, wild type cells treated with 150µM Latrunculin A and subsequently with 10mM hydrogen peroxide were unable to repress the transcription of the ribosomal genes that were tested. In conclusion, actin polymerisation activity is required to repress ribosomal biogenesis under oxidative conditions. Three plausible possibilities could explain our findings: i) Pkc1 signals ribosomal gene repression and actin polymerisation divergently, in response to oxidative stress; ii) Pkc1 linearly signals actin polymerisation and then ribosomal gene expression and iii) actin is an oxidative stress sensor (as proposed by Gourlay and Ayscough, 2005) which acts upstream of Pkc1 in the signalling pathway. This point deserves further research in future studies.

It is well known that arresting secretion causes ribosomal gene repression. We wondered whether oxidative stress could also provoke secretion impairment. To answer this question, we decided to check both the early and late stages of the secretory pathway. Carboxypeptidase Y is a vacuole protein which is transported from ER to the Golgi apparatus and then via late endosomes, to the vacuole. The vacuolar form of CPY is the mature form (M) which displays the greatest electrophoretic mobility, whereas CPY localisation to the Golgi apparatus renders an immature or unprocessed form (P) which displays less electrophoretic mobility. *SEC18* has been reported to be required for the transport of carboxypeptidase Y through the yeast Golgi complex but not for the final delivery of CPY to the vacuole (Graham and Erm, 1991). Therefore, we used the *sec18-1* mutant to test whether hydrogen peroxide could have affected secretion from ER to the Golgi and analysed the CPY protein. A blockade in the first steps of secretion affects CPY transport (Gram and Erm, 1991). We grew cultures of the *sec18-1* thermosensitive mutant to exponential phase at 25ºC and then shifted them from 25ºC to 37ºC for two hours in order to block secretion from ER to the Golgi. In Fig 1B, we observe that in a *sec18-1* mutant growing at 25ºC, anti-CPY detected a single band corresponding to the mature processed form of CPY. When the cultures were shifted from 25ºC to 38ºC for two hours, anti-CPY detected two forms: a more

abundant band, corresponding to the mature or processed form (M), and a less abundant band, corresponding to the unprocessed form (P). Hydrogen peroxide treatment did not cause any defects in CPY processing, as can be observed in Fig 1B. (We used increasing concentrations of hydrogen peroxide and the result did not change, data not shown). We therefore concluded that hydrogen peroxide did not affect the first stages of secretion in *Saccharomyces cerevisiae*.

FM4-64 lipophilic styryl dye is a vital stain which is used to follow bulk membrane-internalisation and transport to the vacuole in yeast (Vida and Erm, 1995). These authors have described that whereas wild type showed significant vacuolar membrane staining, the *sec1-1* thermosensitive mutant was impaired in vesicle to plasma membrane secretion at 37ºC, but not at 25ºC. We followed FM4-64 internalisation in exponentially growing wild type cells that were both treated with hydrogen peroxide or untreated (Fig 1C). Whereas in wild type cells FM4-64 correctly stained vacuole membranes in exponential cultures, upon hydrogen peroxide treatment, this dye suffered impairment in both its capture and internalisation (Fig 1C). These results further strengthen the hypothesis that oxidative stress affects the secretory pathway.

In order to check the late stages of secretion we used anti-invertase to detect the glycosylated forms of the enzyme invertase. Invertase is an enzyme that catalyses the hydrolysis of sucrose into fructose and glucose. As depicted in Fig 1D, blocking secretion using the thermosensitive mutant *sec1-1,* induced a marked reduction in invertase secretion. When we treated cells with increasing concentrations of hydrogen peroxide, we observed an equivalent reduction in invertase secretion. Budding yeast express two different forms of invertase (Gascon and Ottolengui, 1967), being both of them encoded by *SUC2*. The biggest form which is of glycoproteic nature, is located in the cell-wall, and is known as heavy invertase. The second type is a free carbohydrate form which is located and accumulates intracellularly in an unglycosylated form. This form is the small or light invertase, which is constitutively synthesised. However, the heavy chain is synthesised under conditions of catabolic derepression, it is highly glycosylated and secreted to the cell periphery. We hypothesised that if hydrogen peroxide affected secretion, invertase would not be correctly glycosylated and as a consequence would not be properly secreted. We used an anti-invertase antibody to detect the unglycosylated form of invertase in wild type cells growing in rich media. Upon shifting to a low glucose concentration (0.3%), an elevated band was revealed which corresponded to different hyperglycosylated forms at 25ºC in a *sec1-1* mutant. When the cells were shifted to 38ºC for 2 hours, the wide band corresponding to the glycosylated forms almost disappeared, indicating a blockage in the later steps of secretion. We investigated the response to increasing concentrations of hydrogen peroxide and observed that the multiple bands of glycosylation gradually disappeared with increasing

concentrations of hydrogen peroxide. These results suggested that oxidative stress caused by hydrogen peroxide impaired invertase secretion to a level equivalent to that observed in the *sec1-1* mutant. Taking all the results shown in Fig 1 together we can conclude that oxidative stress affects the later steps of secretion and consequently causes ribosomal gene repression mediated by the Pkc1 function. This process also requires polymerisation of the actin cytoskeleton.

Acknowledgements:

We are very grateful to Dr Luis Rodriguez Dominguez from the Universidad de la Laguna (Spain) for providing with anti-invertase polyclonal antibody. Mima I. Petkova was funded by the Generalitat de Catalunya. This work was supported by the Spanish Ministry Education and Science (Spanish Government), through Grant BFU2009-11215.

Legend to Fig 1.

Effect of hydrogen peroxide on the secretory pathway ligated to the Pkc1 function and actin polymerisation activity. A) Northern blot analysis of wt, *pkc1* mutant and wt cells treated with 150µM Latrunculin A for two hours. All the cell cultures were grown to logarithmic phase (0) at 30ºC in SD medium containing 0.8M sorbitol and then treated with 10mM hydrogen peroxide for different times, as depicted in the Figure. *RPS6A*, *RPS28A* and *RPL30* were used as probes and *U1* was used as a loading control. B) *sec18-1* logarithmic cultures were grown in SD medium plus aminoacids at 25ºC, then treated (lines 3 to 6) or not treated (line 1) with 1mM hydrogen peroxide for the indicated times, or shifted to 38ºC for 2 hours (line 2). Samples were processed for western blot analyses and anti-CPY antibody was used to detect carboxypeptidase enzyme. C) FM4-64 capture in wt cells. Wild type cells were exponentially grown at 30ºC in SD medium and subsequently treated, or not treated with 1mM hydrogen peroxide for 1 hour. After that, 1.5 OD_{600} units of both cultures were harvested by centrifugation and resuspended in 50 µl of fresh SD medium containing 1.5 µl FM4-64 (Invitrogene). Samples were incubated for 45 min at 30ºC. Cells were washed four times and resuspended in fresh medium for observation under a microscope. D) *sec1-1* mutant cells were exponentially grown at 25ºC in SD medium, containing 4% glucose plus amino acids; one aliquot was collected for western blot analysis (first line). Cultures were subsequently washed and transferred to SD medium medium containing 0.3% glucose for 12 hours at 25ºC. After that period of time, one aliquot was collected for western blot analysis (second line in the Figure) and the cultures were split in two: one half of the culture was shifted at 38ºC for two hours and the other half was divided into two cultures and

subsequently treated either with 2.5mM or 5mM H_2O_2 for 3 hours at 25ºC. Anti-invertase polyclonal antibody was used to detect the different forms of invertase.

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Gourlay CW & Ayscough KR. (2005). A role for actin in aging and apoptosis. *Biochem Soc Trans.* 33:1260-1264.

Gram TR & Emr SD. 1991. Compartmental organisation of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (*NSF*) mutant. *J of Cell Sci*. 114: 207-218.

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Nierras CR & Warner JR. (1999). Protein kinase C enables the regulatory circuit that connects membrane synthesis to ribosome synthesis in *Saccharomyces cerevisiae. J Biol Chem.* 274: 13235-13241.

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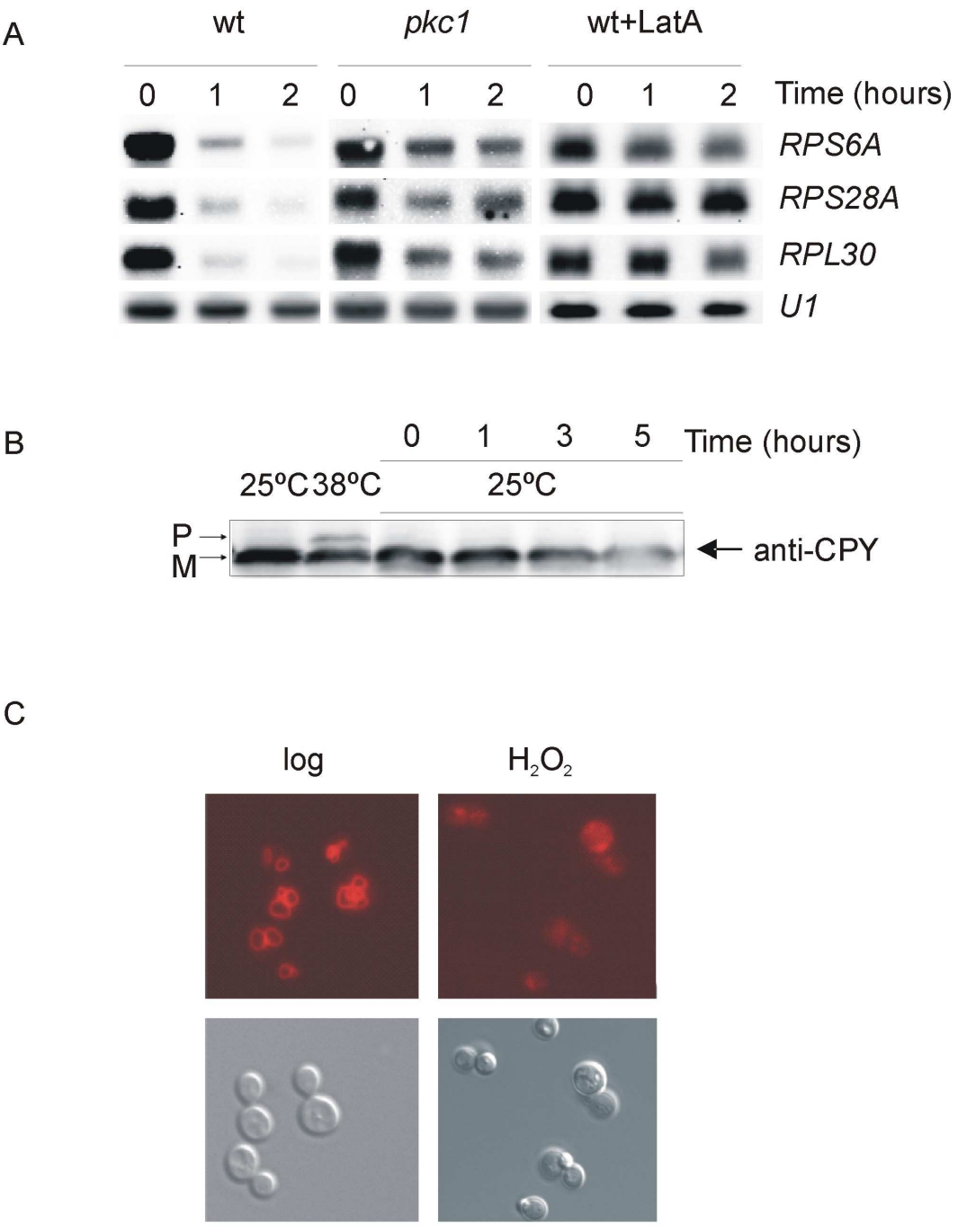
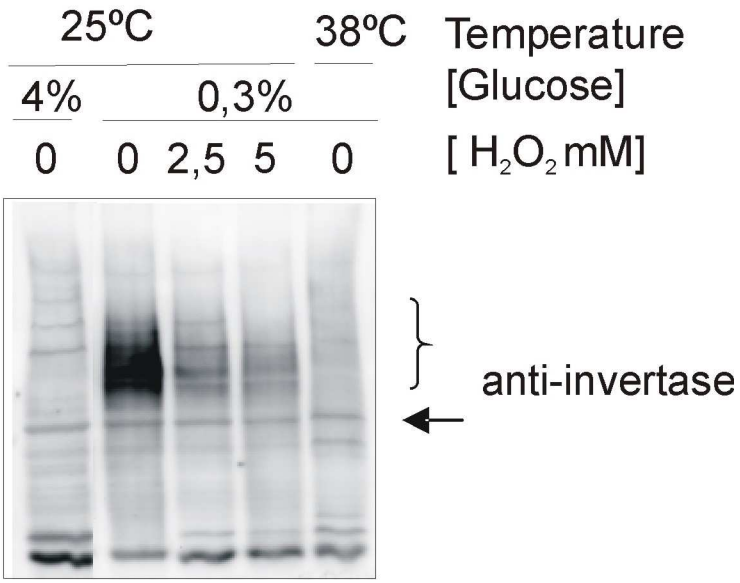
Vilella F, Herrero E, Torres J, & de la Torre-Ruiz MA. (2005). Pkc1 and the upstream elements of the cell integrity pathway in *Saccharomyces cerevisiae*, Rom2 and Mtl1, are required for cellular responses to oxidative stress. *J Biol Chem* 280: 9149-9159.

Fig 1


A
wt
pkc1
wt+LatA
0 1 2 0 1 2 0 1 2 Time (hours)
RPS6A
RPS28A
RPL30
U1
B
0 1 3 5 Time (hours)
25ºC 38ºC 25ºC
P
M
anti-CPY
C
log
H2O2

D


25ºC
38ºC
Temperature
4%
0,3%
[Glucose]
0
0
2,5
5
0
[H2O2 mM]
anti-invertase

GENERAL DISCUSSION

Organisms living in aerobic conditions are exposed to reactive oxygen species which provoke cellular damage, and as a consequence, various human diseases. The eukaryotic microorganism *Saccharomyces cerevisiae* serves as a model system in which to study the signal transduction pathways involved in the oxidative stress response. In budding yeast, specific MAPK pathways exist which are activated by the presence of certain stresses. Up to date, there is no evidence of any MAPK cascade which is specific to oxidative signals. Oxidative stress is related to aging, and in many model organisms has been experimentally demonstrated that longevity extension is associated to oxidative stress resistance (Longo, 1999; Finkel and Holbrook, 2000). Among other cellular processes, signal transduction pathways are associated to oxidative stress responses and cell longevity, and the existence of different levels of cross-talks between these signalling pathways maintains a safeguard mechanism assuring that the cells adapt metabolism and growth appropriately. Among the cell surface sensors belonging to the PKC1-MAPK pathway, only Mtl1 has been characterised as a receptor for oxidative stress (Vilella *et al.,* 2005). Mtl1 was initially identified as a yeast homologue of Mid2 (Rajavel *et al.,* 1999; Ketela *et al.,* 1999). In the present study we demonstate novel evidence about the functional relationship between Mtl1 and either TOR or RAS-cAMP, as well as the implication of other members of the CWI, in the context of oxidative stress response and quiescence.

1. Mtl1 is essential for cell survival in response to various types of stresses. Mtl1 regulates Msn2/Msn4 and is required for the proper ribosomal gene repression in response to oxidative stress and glucose starvation.

We have used a genomic approach in order to identify other functions in which the cell surface protein Mtl1 could play a role. As a consequence of *MTL1* deletion, a large cluster of genes that were potentially regulated by Msn2 were downregulated. We thus identified Msn2 as a possible target for Mtl1. The absence of Mtl1 makes cells sensitive to oxidising agents, glucose starvation and conditions in which the TORC1 function is compromised. Msn2 overexpression was quite efficient at rescuing the loss of viability caused by the absence of the Mtl1 function, as well as at complementing the defects in *HSP12* and *CTT1* transcription in *mtl1* mutant to wild-type levels, in all the stress conditions studied here. It is very interesting to observe that a significant number of the Msn2-dependent genes, which are downregulated in the *mtl1* mutant, are related to the oxidative stress response. Mtl1 was previously described to function as a cell-surface sensor for oxidative stress by our group (Vilella *et al.,* 2005). However, how Mtl1 senses these stimuli currently was unknown. Some authors (Hasan *et al.,* 2002) have reported that Msn2

transcriptional control is important for the response to hydrogen peroxide. The current study helps to extend our existing knowledge of this field by attributing Mtl1, a sensor for oxidative stress, with a novel and essential role in the transduction of the oxidative signal to Msn2. One possible explanation for this is that Msn2 controls the transcription of the genes required for the oxidative stress response. As a consequence of *MTL1* deletion, there is also improper ribosomal gene regulation upon both hydrogen peroxide treatment and glucose depletion. The sensitivity of *mtl1* mutant to the conditions studied here, is also associated with a notable defect in Slt2 activation.

2. Mtl1 signals the inhibition of Tor1 and Ras2 for the regulation of Msn2/Msn4 function and ribosomal gene expression in response to both oxidative stress and glucose starvation.

TORC1 and PKA provide various types of cross-talk and regulate common readouts such as the localisation and activity of transcription factors associated with the general stress response transcription factor Msn2/Msn4 and other transcription factors involved in RP gene expression (Schmelzle *et al.,* 2004; Zurita-Martinez *et al.,* 2005). It is also generally accepted that sensitivity to rapamycin is specific to mutants in the TORC1 complex but not in the TORC2 complex (Loewith *et al.,* 2002). The mutant *mtl1* is sensitive to rapamycin, therefore Mtl1 is somehow related to TORC1 function. The observation that *TOR1* deletion restores the Msn2/Msn4 function in *mtl1* cells reinforces the hypothesis that Mtl1 acts as a possible negative regulator of *TOR1* under specific conditions. However, if this were the case, we should not expect any ribosomal gene repression to occur in the absence of Mtl1 since both TOR and RAS inhibition induce ribosomal gene repression (Martin *et al.,* 2004). The observation that rapamycin induced a marked repression of the ribosomal genes in *mtl1* mutant cells was not unexpected since rapamycin blocks the TORC1 function and not only *TOR1* activity. It has recently been reported that at least two independent branches of TORC1 are involved in the control of ribosomal protein genes (Lee *et al.,* 2009). The presence of the other TORC1 components would be sufficient to signal to the ribosomal transcription, independently of Mtl1. The synthetic lethality observed in the double mutant *tor1mtl1* upon rapamycin treatment suggests that the two proteins act in different pathways, but probably perform a common regulatory function. However, more studies will be required in order to elucidate the meaning of this genetic interaction.

Upon oxidative treatment or glucose starvation, Tor1 deletion was required to restore ribosomal gene repression and the Msn2/Msn4 function in *mtl1* cells. We consequently

concluded that Mtl1 is a negative regulator of *TOR1* and specifically in response to glucose starvation and oxidative stress.

It has been reported that RAS-cAMP may be a TOR effector branch (Schmelzle *et al.,* 2004). These authors reported that the activation of RAS causes: 1) a deficiency in *HSP12* transcription in response to rapamycin 2) no ribosomal transcriptional repression in response to TOR inactivation. In a recent study Soulard *et al.* (2010) define a signalling cascade in which TORC1 is upstream of PKA toward at least some substrates. By using rapamycin-sensitive phosphoproteome analysis and targeted experiments, the authors propose a model in which TORC1 inhibits BCY1 phosphorylation (the negative regulatory subunit of PKA) by phosphorylating and activating Sch9 that in turn inhibits the MAP kinase Slt2/Mpk1. Slt2 phosphorylates BCY1 directly. Thus, TORC1 activates PKA toward some substrates by preventing Slt2-mediated activation of BCY1 (Soulard *et al.,* 2010).

One of our initial assumptions was therefore that the RAS-cAMP pathway could be improperly upregulated in the *mtl1* mutant. Deletion of Ras2 caused a significant decrease in cAMP levels, as expected, and as previously reported (Matsumoto *et al.,* 1985; Toda *et al.,* 1985). We also obtained clear results indicating that cAMP levels were higher in *mtl1* cells than those determined in wild type cells. It has been demonstrated that high PKA levels negatively regulate Msn2/Msn4 dependent gene transcription (Schmelzle *et al.,* 2004). This could explain the lower transcription levels determined in *mtl1* mutants in comparison with wild type cells and this would also be in accordance with the increase in the transcription of Msn2/Msn4 dependent genes observed in both the *mtl1tor1* and *mtl1ras2* double mutants in all the conditions studied here. Deletion of *RAS2* restored the cell growth defects and the deficiency in the ribosomal gene repression exhibited by *mtl1* mutant cells to almost wild-type levels in response to both oxidative stress and glucose deprivation. Taking all of these results together, we conclude that Mtl1 negatively affects cAMP levels through the regulation of the Ras2 function and more specifically in response to both oxidative stress and glucose deprivation.

Glucose starvation induces a change from a fermentative to a respiratory metabolism in *Saccharomyces cerevisiae.* This sudden shift to respiratory growth would lead to the accumulation of reactive oxidative species derived from the electronic chain in the mitochondria. It is not therefore surprising that both oxidative and nutritional stresses induce a common pathway which is sensed primarily by a common sensor, Mtl1, which would transmit the signal for both Tor1 and Ras2 inactivation, thus reducing the accumulation of cAMP. Unrestrained activity of TORC1 in mammals is associated with several diseases such

as inflammation, cancer and diabetes (Reiling and Sabatini). Ras inhibition also increases resistance to oxidative stress in neurons (Li *et al.,* 2008). In both yeast and mammalian cells, the expression of constitutively active Ras, $Ras^{ala18val19}$ and Ras^{val12}, respectively, results in the accumulation of ROS and a reduction of the life span (Heeren *et al.,* 2004; Serrano *et al.,* 1997; Longo, 2004). This correlates with i) the observed much higher levels of ROS in *mtl1* mutant cells especially in stationary phase cultures compared to wild-type cells, and their reduction by overexpressing Msn2 (data not published), ii) the loss of *mtl1* cell viability in stationary phase (see below), iii) the higher cAMP levels and iv) the deficiency in Msn2/Msn4 dependent gene induction, thus indicating improper Ras-cAMP inactivation in stationary phase in *mtl1*. The majority of defects exhibited by *mtl1* are suppressed by the deletion of either *RAS2* or *TOR1*. The inhibition of Tor1 and Ras must therefore be a common mechanism used by cells in order to survive nutrient depletion and other specific types of stress.

3. Rom2 and Rho1 participate in the stress response mediated by Mtl1.

The expression of hyperactive allele Rho1 in *mtl1* mutant led to an increase in cell survival in response to hydrogen peroxide, but not to rapamycin, the partial restoration of the transcriptional induction of *CTT1* and *HSP12*, and the repression of ribosomal genes under conditions of oxidative stress. Equivalent results we obtained by overexpressing Rom2. Taken these results together we concluded that the signalling from Mtl1 to Tor1 and Ras2 occurred through Rom2 and Rho1 and that the latest presented stress specificity. At this point we do not know the exact nature of the connection between Rho1 and Tor1/Ras2. According to some authors, TOR and RAS-cAMP function independently (Zurita-Martinez *et al.,* 2005), although others suggest that RAS-cAMP is a TOR effector branch (Schmelzle *et al.,* 2004; Martin *et al.,* 2004; Soulard *et al.,* 2010). In our studies, we observed that in response to rapamycin, Mtl1 displayed an independent genetic relationship with the TORC1 complex. However, in response to oxidative stress and glucose starvation we could speculate that two models might be proposed: Rho1 first inactivates Tor1 protein from the TORC1 complex and Tor1 transmits the signal to Ras2 inactivation in response to glucose deprivation and hydrogen peroxide treatment. In the second model, Rho1 signals simultaneously, but independently, to Tor1 and Ras2 inactivation and the signal converges (at least) on the general stress response transcription factor Msn2/Msn4. Glycogen accumulation is also controlled by TOR, PKA, and Msn2/Msn4. The observation that Rho1 activation, Msn2 overexpression, or the deletion of either *TOR1* or *RAS2* suppressed the deficiency in glycogen accumulation in *mtl1* mutants also supports both models.

The findings from our study were in line with a previous publication (Park *et al.,* 2005) in which the authors described a role for Rom2 in Ras2-cAMP downregulation in response to several types of stress, including oxidative stress. These authors also indicate that this cross-talk between Ras-cAMP and the PKC1-MAPK pathway could be mediated by Rho1. In the present study, we have extended this information to Mtl1, as it is the cell-surface protein member of the cell integrity pathway that transmits the signal to Tor1 and Ras2 through Rom2 (data not shown) and Rho1.

4. Implication of Sfp1 in the regulation of the ribosomal gene expression signalled by Mtl1 in oxidative stress conditions.

Sfp1 is a transcription factor whose nuclear localisation determines the transcriptional induction of both ribosomal and RiBi genes (Marion *et al.,* 2004; Jorgesen *et al.,* 2004). Sfp1 is positively regulated by TOR and RAS activity. Based on the observations that i) Sfp1 translocation to the cytoplasm was clearly defective in *mtl1* mutant upon oxidative stress, ii) overexpression of Sfp1 severely impared *mtl1* cell viability upon hydrogen peroxide treatment and iii) as discussed above ribosomal gene repression did not occur in *mtl1* under these conditions, we suggested that Sfp1 could be negatively regulated by Mtl1 under oxidative stress. Whether the signal is transmitted from TOR through RAS inhibition, or independently to TOR and RAS inactivation for the ribosomal gene repression would be speculative. 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 of TOR that signals to Sfp1 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). An excess of ribosomal gene transcription under glucose starvation and oxidative stress would constitute a waste of ATP and would be detrimental for cell viability. However, this result does not exclude the existence of other regulatory proteins in this system.

5. Pkc1 plays a role in the ribosomal gene repression upon oxidative stress, associated with actin polymerisation. Secretion impairment is caused by oxidative stress.

The repression of ribosomal gene transcription is a general defence mechanism in stress response. In addition to the described role of Mtl1 in the ribosomal gene repression

upon oxidative stress we demonstrate that Pkc1 is required to transmit the oxidative signal to ribosomal biogenesis. This event is closely related with active actin cytoskeleton, since cells blocked in actin polymerisation were not able to repress ribosomal gene transcription in oxidative conditions. Actin polymerisation is a target of hydrogen peroxide treatment and Pkc1 is required to restore this function upon oxidative stress as proved by Vilella *et al.* (2005) and Pujol *et al.* (2009). However, the exact nature of the signalling between Pkc1 and actin, and RP genes under these conditions is still speculative. Pkc1 could signal ribosomal gene repression and actin polymerisation divergently, in response to oxidative stress; or alternatively Pkc1 could linearly signal actin polymerisation and then ribosomal gene repression. Actin has been reported to act as well as a sensor of cell-well being (Gourlay and Ayscough (2006). It has been described that Pkc1 function is required to repress ribosomal gene expression when the secretory pathway is blocked (Nierras and Warner, 1999). Here, following invertase secretion pattern, we suggest that oxidative stress affects the latest steps of secretion and consequently cause ribosomal gene repression mediated by Pkc1 function. All these results taken together are in line with the role of Pkc1 for the cell survival and adaptation in response to oxidising agents.

6. Mtl1 is required to activate the PKC1-MAPK pathway in response to oxidative stress and glucose starvation.

We observed that the activation of Slt2 was clearly abrogated in *mtl1* stressed cells in conditions of hydrogen peroxide treatment and glucose depletion. Hyperactivation of the pathway downstream from Mtl1 compensated for the absence of the receptor in the activation of Slt2 under the mentioned conditions. However, the observation that Slt2 activation is not sufficient to recover cell viability in response to oxidative stress in an *mtl1* mutant is in line with a previous publication by our group (Vilella *et al.,* 2005). In it, we demonstrated that the elements of the MAPK module of the CWI pathway are dispensable for cell viability in response to oxidative stress. Here we report that these elements of the MAP kinase cascade do not participate in the cross-talk between Mtl1 and Tor1/Ras2. This was deduced from the observation that the *BCK1-20* allele did not restore the Msn2/Msn4 function in *mtl1*cells in response to oxidative stress and glucose starvation.

Constitutive activation of the Pkc1-MAPK pathway at the level of *BCK1,* however, clearly increased cell survival in *mtl1* cells when they were starved of glucose. This result underlines the importance of cell integrity activity in quiescence (Krause and Gray, 2002; Torres *et al.,* 2002). Slt2 activity is really essential in terms of cell survival in quiescence

conditions. However in response to oxidative stress the contribution of Msn2/Msn4 is more significant.

7. Bidirectional cross-talk signalling between CWI and TOR/RAS pathways.

We describe an information flow that connects the PKC1-MAPK pathway with TOR and RAS, in which the signal is transmitted from Mtl1, an element of the pathway to each of the other two routes: TOR and RAS. Interestingly, as a consequence of *TOR1* or *RAS2* deletion in the *mtl1* mutant background Slt2 phosphorylation was induced in response to oxidative stress and glucose starvation, which contrasted with the absence of Slt2 activation determined in the single *mtl1* mutant. Thus, on one hand, Mtl1 appears to be key regulator in response to external stresses, as in the case of oxidative stress and glucose depletion. Under these conditions Mtl1 function is required for activation of the CWI pathway, as well as for the inactivation of Tor1 and/or Ras2. However, as a consequence of Tor1 or Ras2 inactivation, another signal, independent on Mtl1, is transmitted to activate the CWI in the mentioned conditions. These mechanisms were necessary for *mtl1* cell survival and adaptation.

8. Mtl1 is a highly O-mannosylated and N-glycosylated transmembrane protein. Evidence for the importance of both extracellular highly-modified and cytoplasmic domains for its function.

Analysis of the Mtl1 protein in *pmt* strains (deficient in O-linked mannosylation) reveals that Pmt2 isoform is capable of specifically modifying Mtl1. It should therefore not be unexpected that the O-mannosylation of Mtl1 could be impaired in both *pmt1* and *pmt2* mutants in a similar way, since Pmt1 and Pmt2 are thought to predominantly act as components in a heterodimeric protein complex (Gentzsch *et al*., 1997; Girrbach & Strahl, 2003). Members of the Pmt family form homodimers and heterodimers as a mechanism for enhancing substrate specificity (Hutzler *et al.,* 2007) and ensuring efficient O-mannosylation in a wide range of target proteins (Lommel & Strahl, 2009). It is most notable, however, that the fully modified form of Mtl1 is almost totally absent in the single *pmt2* mutant. Furthermore, O-linked mannosylation by Pmt2 and Pmt6 affects Mtl1 protein stability. Mtl1 shares a high degree of homology with Mid2 (Rajavel *et al.*, 1999). An interesting observation relates to the finding that Mid2 is exclusively O-mannosylated by Pmt2 (Philip &

Levin, 2001); this contrasts with the suggestion by Lommel *et al* (2004) that Mid2 is modified by both Pmt2 and Pmt4 at the least.

The removal of N-linked carbohydrate chains by treatment with Endo-H reduces the molecular mass of Mtl1 isolated from wild type cells, which demonstrates that Mtl1 is N-glycosylated *in vivo.* A compensatory response between O-mannosylation and N-glycosylation has also been proposed (Arroyo *et al.,* 2011). Both the upregulation of the genes responsible for the biosynthesis of N-linked high mannose carbohydrate chains in *pmt* mutants (Arroyo *et al.,* 2011) and the enhanced transcription of *PMTs* under conditions of N-glycosylation inhibition (Travers *et al.,* 2000) suggest that the O- and N-linked glycans of cell wall mannoproteins may, at least partially, compensate for each other. Such a mechanism could be applicable to Mtl1 modification in *pmt1* and *pmt2* mutants because the band below 200 kDa is absent in these mutants. It is likely that N-linked glycans compensate for the formation of mannose outer chains under conditions of decreased O-mannosylation; this is in line with the proposed compensatory response between O-mannosylation and N-glycosylation (Arroyo *et al.,* 2011). Mtl1 is secreted to the plasma membrane through the putative secretory pathway since the *sec1-1* mutant Mtl1 is hypoglycosylated.

The cytoplasmic domain of Mtl1 exhibits two-hybrid interactions with the GEF Rom2. Co-immunoprecipitation experiments also demonstrate that Mtl1 physically interacts with Rom2. This is of a great importance for the model that we propose in which the signalling of oxidative stress and quiescence from Mtl1 is basically transmitted to the upper elements of the CWI pathway and from there to the general stress response.

9. Protein O-mannosylation by Pmt2 is essential in the oxidative stress response and in response to TORC1 inhibition, through at least Mtl1. Protein O-mannosylation contributes to signal Slt2 activity upon TOR blockage.

Glycosylation is a highly abundant and essential posttranslational protein modification (Lehle *et al.,* 2006). Some combinations of *pmt* mutations result in lethality (Gentzsch & Tanner, 1996); this indicates that protein O-mannosylation is a crucial protein modification. There is a degree of functional overlap among the different Pmt isoforms (Strahl-Bolsinger *et al*., 1993 and 1999). Analyses of viable single *pmt* and conditionally lethal double *pmt* mutants shows that O-mannosylation is not only a structurally important modification, but that it is crucial for functional aspects of a protein such as localisation, turnover, folding and ligand interaction (reviewed in Lommel & Strahl, 2009; Lehle *et al.,* 2006; Marth *et al.,* 2008).

As such, it is therefore also indispensable for cell wall integrity, cell polarity and morphogenesis. O- and N-glycosylation mutants show various cell wall-related phenotypes (Strahl-Bolsinger *et al.,* 1993; Gentzsch and Tanner, 1996; Nakayama *et al.,* 1992; Lorberg *et al.,* 1999; Goto, 2007). Most of these phenotypes closely resemble those of mutants with defects in their PKC1-cell integrity pathways (Gray *et al.,* 1997; Lee *et al.,* 1993; Levin and Bartlett-Heubusch, 1992; Philip and Levin, 2001; Lommel *et al.,* 2004). Mannosylated threonine and serine residues in proteins constitute protein modifications that have been evolutionarily conserved from unicellular yeast to man (Chai *et al.,* 1999; Chiba *et al.,* 1997; Lehle *et al.,* 2006); it is also well known that deficiencies in protein O-mannosylation can cause serious developmental disorders in humans, such as congenital neuromuscular disorders.

In our study, based on the finding that the absence of Pmt2 makes cells sensitive to oxidising agents and to rapamycin, we proposed an essential role for protein O-mannosylation in both the oxidative stress response and in the response to TOR blockage, through at least Mtl1. Even so, we did not rule out the possible participation of other cell-wall receptors in this mechanism. However, we consider that at least some of the *pmt2* defects observed in the presence of oxidative stress and rapamycin treatment are due to Mtl1 O-mannosylation defect. This conclusion is based on the evidence that Mtl1 overexpression is able to repair cell growth in *pmt2* mutant in response to rapamycin and also in response to oxidative stress. Moreover, overexpression of other members of the PKC1-MAPK pathway (such as Rho1 and Rom2) leads to an increase in *pmt2* cell survival in response to oxidative stress. Mtl1 O-mannosylation is important for transmitting the oxidative signal to Rom2 and Rho1. However, we believe that Slt2 activation does not play an essential role for the cell survival in the oxidative stress response; this conclusion is based on the fact that the activation of this kinase was not impaired in the *pmt2* mutant upon hydrogen peroxide treatment. This is consistent with our data in which Rho1 and Rom2 rescued *mtl1* cell viability in response to oxidative treatment. Even more relevant is the evidence that this suppression presented stress specificity as Rom2 and Rho1 did not suppress *mtl1* loss of viability upon rapamycin treatment. The same effect was again observed with respect to the *pmt2* mutant. We also observed that the activation of Slt2/Mpk1 in *pmt2* was compromised upon rapamycin treatment and that, curiously, this occurred to the same extent as in the *mtl1* mutant cells. Our hypothesis is that Pmt2 and the mannosylated form of Mtl1 are both necessary to signal TOR function inhibition, but that Slt2 phosphorylation could reflect a read-out associated to signal impairment.

To the best of our knowledge, this is the first report to suggest a role for protein O-mannosylation in cell survival upon TOR blockage. In this mechanism, Slt2 phosphorylation is impaired; but curiously, the upper elements of the CWI pathway, Rom2 and Rho1, are not involved in signalling cell survival from Mtl1. We suggest that Mtl1 O-mannosylation is required to elicit cellular responses to TOR inhibition.

Defects in glycosylation trigger a PKC1-MPK1-dependent cell wall compensatory mechanism (Lagorce *et al.,* 2003; Lommel *et al.,* 2004; Chen *et al.,* 2005; Arroyo *et al.,* 2011). These authors demonstrated that defects in O-mannosylation lead to the activation of the CWI pathway. Consistent with this, an increased basal level of Slt2/Mpk1 phosphorylation occurs in the *pmt2* mutant with respect to wild type cells.

We also demonstrate that under conditions of oxidative stress or TOR blockage, the *pmt2* mutant is not impaired in Msn2/Msn4 mediated transcriptional induction or in ribosomal gene expression. Glycogen accumulation is unaffected, which supports our hypothesis that protein O-mannosylation does not seem to be essential for the transmission of the signal from Mtl1 to Tor1 and Ras2 functions upon application of the previously mentioned stress treatments.

10. Mtl1 O-mannosylation confers its proper localisation to the cell periphery, the septum and the tip of the shmoo.

Interestingly, Mtl1 localises to the bud and the septum, in a similar way to that observed in other Pkc1-MAPK members (Levin, 2005), and localises to the cell periphery in a dotted pattern that is homogeneously distributed throughout the cell surface. The latter localisation perfectly fits with the function of Mtl1 as a cell-surface receptor. Our data also suggest that protein O-mannosylation is important for maintaining appropriate cell surface and septum localisation, which could determine correct functionality. This would, at least partially explain the *pmt2* and *mtl1* phenotypes. One feature shared by the cell-wall receptors Mtl1 and Mid2, which both belong to the CWI pathway, is the capacity of the Pmt2 isoform to modify them. Unlike Mid2, however, Mtl1 does not play a relevant role in maintaining the shmoo cell-wall integrity (Rajavel *et al.,* 2005). However, and somewhat surprisingly, Mtl1 also accumulates to the tip of the shmoo, and impared O-mannosylation by Pmt2 impinges upon Mtl1 localisation. Given their high degree of similarity, we hypothesize that Mtl1 and Mid2 could have diverged in their recent evolution. Mtl1 localisation to the tip of the shmoo must be a common characteristic that has been conserved throughout its evolution.

11. Mtl1 and, in particular, O-mannosylation catalised by Pmt2 are required in quiescence. Both Mtl1 and Pmt2 play positive role in the chronological life span.

Our results suggest that the loss of viability experienced by *pmt2* under the conditions tested: oxidative stress, TOR blockage and quiescence was mainly due to deficiencies in Mtl1 protein maturation. It is also evident that *pmt2* loses viability in stationary phase cultures. The observation that Pmt2 is required for life extension suggests that the maturation of protein receptors plays a substantial role in preventing early cell death. We already demonstrated that Mtl1 was required to inactivate Ras2 and Tor1 functions under determined stress conditions. Since *TOR1* and *RAS2* deletions prolong life span, one hypothesis is that protein O-mannosylation helps, or at least contributes to, signal starvation in order to correctly set quiescence. In this function, the role of some protein receptors, including Mtl1, may be required to correctly transmit the signal to downstream effectors. Our group has previously proposed that the TOR function may maintain the signal that prevents CWI activation under optimal growth conditions (Torres *et al.,* 2002). The evidence presented in this study is consistent with this hypothesis as Slt2 activation is impaired in *mtl1* and *pmt2* mutants in response to rapamycin treatment and also in the stationary phase; these are both conditions in which the TOR function is inhibited. In this case, both *PMT2* and *MTl1* (and consequently Mtl1 O-mannosylation) make important contributions to signalling.

Further data presented in this study supports the theory that Mlt1 O-mannosylation is relevant in quiescence and also to extend the chronological life: a) Mtl1 presents a hyperglycosylated mature form in stationary phase and O-mannosyl glycans appear to enhance protein stability under these conditions. b*) MTL1* expression specifically increases upon TOR blockage and during the stationary phase. c) Mtl1 is tightly regulated by the stress response transcription factor Msn2/Msn4; it is upregulated under conditions of nutrient starvation and the downregulation of the TORC1 function.

CONCLUSIONS

CONCLUSIONS

- **First:** Mtl1 is essential for cell survival in response to rapamycin, glucose depletion, and oxidative stress. Mtl1 regulates Msn2/Msn4 function under these conditions and is required for proper ribosomal gene repression in response to oxidative stress and glucose starvation.

- **Second:** Mtl1 signals the inhibition of Tor1 and/or Ras2 for the regulation of Msn2/Msn4 function and ribosomal gene expression in response to oxidative stress and glucose starvation.

- **Third:** Mtl1 regulates cAMP levels through down-regulation of Ras2 function.

- **Fourth:** The signalling from Mtl1 to Tor1 and/or Ras2 occurs through Rom2 and Rho1. Moreover Rom2 and Rho1 present oxidative stress specifity.

- **Fifth:** The elements of the MAP kinase cascade module of the CWI pathway do not participate in the signalling mediated by Mtl1 to Msn2/Msn4 dependent transcription and ribosomal gene expression. However Pkc1 mediates oxidative signalling by down-regulating ribosomal gene expression upon hydrogen peroxide treatment and the signal to the ribosomal gene repression requires actin polymerisation.

- **Sixth:** Sfp1, which is a transcriptional activator of ribosomal and RiBi genes downstream both TOR and Ras/PKA kinases, is negatively regulated by Mtl1.

- **Seventh:** CWI, TOR and Ras-cAMP pathways cross talk at different levels. First, a signal for oxidative stress or glucose depletion is transmitted from Mtl1 to Tor1 and/or Ras2 inhibition. Second, under these conditions CWI is activated as a consequence of *TOR1* or *RAS2* deletion independently on Mtl1.

- **Eight:** Mtl1 is required to activate the PKC1-MAPK pathway in response to oxidative stress and glucose deprivation. Slt2 activity is really essential in terms of cell survival in quiescence conditions. However, in response to oxidative stress the contribution of Msn2/Msn4 function is more significant.

- **Ninth:** Mtl1 interacts with Rom2, a GEF for Rho1, through its cytoplasmic domain.

- **Tenth:** Mtl1 is a highly O-mannosylated and N-glycosylated transmembrane protein. Pmt2 is the main protein O-mannosyltransferase isoform capable of specifically modifying Mtl1. Pmt1 and Pmt6 affect as well Mtl1 O-mannosylation.

- **Eleventh:** 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. Both Pmt2 and Mtl1 play positive role in the chronological life span.

- **Twelfth:** Mtl1 localises to the cell periphery, bud and septum. Mtl1 O-mannosylation confers its proper localisation.

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