UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali

Dipartimento di Biotecnologie e Bioscienze

Corso di Dottorato di Ricerca

in Biotecnologie Industriali, XXIII ciclo

Yeast cell size control: an interplay among ribosome biogenesis, protein synthesis and MAPK routes.

Tesi di: Matteo Viganò

Tutor: Prof.ssa Marina Vai

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"The critical size model is undeniably elegant.
However, evolutionary mechanisms may
favor complexity over elegance (Laabs et al. 2003)"

...to myself, to my family and those who love me...

Contents

Abstract

Cell-size homeostasis requires that proliferating cells coordinate growth and cell cycle, such that each division is matched by a doubling of mass. Size homeostasis is a universal but poorly understood feature of the cell cycle control. In the unicellular budding yeast *Saccharomyces cerevisiae*, the coordination of division with growth occurs at Start, a short interval during late G1 phase, after which cells are committed to division. A prerequisite for the passage through Start is the attainment of a critical cell size, whose value is set by ploidy and growth conditions.

The critical-size threshold maintains uniform the cell size over many generations, and under minimal nutrient conditions forces cells to accumulate the energy stores required to complete the division cycle.

Nutrients modulate the critical cell-size threshold according to the proliferation rate. Generally, cells growing slowly on a poor medium pass Start at a smaller size than fast-growing cells on a rich medium.

In *S. cerevisiae* mutants that subvert the size control process have two phenotypes: small (*whi)* and large (*lge)*. The former undergo Start a smaller cell size and the latter at a larger. Moreover, a systematic determination of cell-size distributions for all yeast deletion strains identified many new potential Start regulators. Many of the genes encoding potential Start repressors are implicated in ribosome biogenesis, suggesting the existence of a link between these two seemingly disparate processes. One of the smallest *whi* mutant is linket to *SFP1* gene deletion. *sfp1Δ* cells display a disproportionate effect on size relative to the change in growth rate.

SFP1 gene encodes a zinc-finger protein that is a key transcriptional regulator of ribosome biogenesis whose function is required for normal yeast growth. Nuclear localization of Sfp1, requires active TORC1 and it is highly stress sensitive. In addition, Sfp1 interacts directly with and is phosphorylated by TORC1. In contrast to Sch9 kinase, a major downstream target of TORC1, TORC1 phosphorylation of Sfp1 is unaffected by either osmotic or nutritional stresses, suggesting a different mode of regulation. Significantly, Sfp1, through its transcriptional activation function, exerts a negative feedback control on TORC1 activity toward the Sch9 kinase. Sfp1 also interacts with Mrs6, a conserved Rab escort protein that in turn

regulates Sfp1 nuclear localization. The Mrs6 interaction with Sfp1 and TORC1 is related to a still poorly understood connection between TOR signaling and vesicle transport.

The aim of this work has been to better characterized the relationship among Sfp1, the cell size control and some signalling pathways involved in the coordination of division with growth.

In order to better elucidate the role of Sfp1 as a negative regulator of Start, we analyzed the level of some of the key players of the G1 to S transition, the G1 cyclins (Cln1-3) and the Cki Sic1, in a *sfp1Δ* mutant. *sfp1Δ* cells are characterized by a *whi* phenotype, slow growth, decreased budding index elongation of G1 phase and reduction of G2/M transition. Accordingly with some aspects of this phenotype, the Cln1-2 level resulted decreased while Cln3 levels were unaffected in agreement with data reporting that the mechanism through which Sfp1 couples ribosome biogenesis to Start is independent of Cln3. Interestingly, the main effect of the *SFP1* deletion is on Sic1 that resulted entirely nuclear, all linked to Clb5 and stabilized by phosphorylation on threonine 173 (Thr173). Phosphorylation that is well known to induce Sic1 accumulation by preventing its degradation. In the *sfp1Δ* mutant, Sic1 stabilization is required for both the elongation of the G1 phase and the reduction of the G2/M transition. A similar situation that involves Sic1 stabilization by phosphorylation on Thr173 but leading to a G1 arrest is observed after inhibition of TORC1 by rapamycin where Sic1 accumulates in the nucleus to avoid improper Clb5/6-Cdc28-driven DNA replication under conditions of poor nutrient availability. This parallelism is in line with the fact that Sfp1 associated with Tor1 kinase and that this binding is essential for a correct localization of TORC1 together with Sfp1 at the RP promoters.

A condition of poor nutrient availability can be considered as a stress condition for a cell. Similarly, the activation of the Hog1 MAP kinase after osmotic stress also induces a cellular response where the stabilization of Sic1, always via Thr173 phosphorylation is involved. Moreover, in this context, the cellular response to *SFP1* inactivation appears more similar to the response to osmotic stress than that to rapamycin. In fact, the latter induces a G1 arrest linked to a Sic1 stabilization but subsequently a decrease of Cln3 accumulation takes place; such a decrease is essential for maintaining a prolonged G1 arrest. On the contrary, after the osmotic stress

Sic1 is stabilized, Cln1 and Cln2 are low, Cln3 levels are unaffected as in the mutant. In addition, the stress response do not always provoke a cell cycle arrest, but is often a slowdown of cell cycle progression, necessary for cell adaptation to new conditions. Only if the stress is too intense, cells arrest growth. Yeast cells modulate stress response via the activation of mitogen-activated protein kinases (MAPKs) which respond to different conditions such as pheromone signals (mediated by the MAPK Fus3), osmolarity (mediated by the MAPK Hog1), nutrient deprivation (mediated by the MAPK Kss1) and cell wall stress (mediated by the MAPK Slt2). Since the first three MAPKs pathways use basically the same signaling machinery, when one of the three pathways is activated, the others are suppressed (cross-talk). The stress response linked to *SFP1* inactivation involves a complex cross-talk between the Hog1 and Kiss1 pathways. Both pathways are activated but only Kiss1 is phosphorylated. Kss1 is the MAP kinase that primarily functions under conditions of nutrient deprivation such as the lack of nitrogen and/or glucose in the growth medium. Under these conditions the signal mucin Msb2 regulates the activation of the filamentous growth (FG) pathway that induces the phosphorylation of Kss1, necessary to guarantee cell survival. The lack of Sfp1 is sensed by the cell as a condition of nutrient scarcity. In fact, under optimal growth conditions, Sfp1 localizes to the nucleus, where it promotes the RP and RiBi genes expression. In response to changes in nutrient availability, Sfp1 is released from RP and RiBi gene promoters and exits from the nucleus; thus, the ribosome biogenesis is down-regulated. Moreover, since Msb2 is also required for activation of the Hog1 pathway a reciprocal inhibitory loop takes place between the Hog1 and Kss1 pathways allowing stable activation of the latter. We found that once activated Kiss1 is able to stabilized Sic1.

We hypothesize that the activation of the FG pathway following Sfp1 lost of function involves a glycosylation defective-like response. In fact, activation of FG pathway by inhibition of N-glycosylation combined with a specific O-glycosylation defect induces activation of both Hog1 and Kss1 pathways and only Kss1 is phosphorylated. We found that *SFP1* inactivation induces some defects that are also observed following the inhibition of glycosylation such as alterations in cell wall permeability, activation of the cell wall integrity pathway and alteration in the secretory pathway.

All our data indicate that not only Sfp1 is regulated by stress and nutrients (both affecting its localization), but that Sfp1 can, in turn, regulate the stress response. The linker between Sfp1 and stress response pathway is the secretory pathway. We can hypothesize that a reduction of ribosome biogenesis may induce a defect in the secretory pathway leading to the activation of Msb2 and thus of the FG pathway. The exit from the nucleus of Sfp1, necessary for the reduction of ribosome biogenesis, allows the release of the Rab GTPase that is essential to switch off the defect in the secretory pathway. Consequently, the inactivation of *SFP1* induces a complex activation of the MAPKs pathway that is responsible of the regulation of different aspects that characterized the mutant. The main of these is the regulation of the G1-S transitions by the stabilization of Sic1. Finally we showed that consequently to the *SFP1* inactivation (probably due to the alteration in the secretory pathway), the mutant cells are characterized by an alteration of Cytoplasmic volume/ Protein content linked to an increase in the cytoplasmic volume. This let us to speculate that growth might be composed of two elements: the Size that is the growth in cell volume and the Mass that is the increase in the protein content. Consequently, alterations of cell growth in response to changes in the environmental conditions imply a coordinate regulation of Size and Mass with the aim of maintaining their ratio constant. One of the key elements necessary to maintain this balance is Sfp1.

Chapter 1:

Nutrient sensing in *Saccharomyces cerevisiae*

All living organisms have evolved complex signal transduction networks that ensure the fast and optimal adaptation of cellular metabolism to changes in the environmental conditions. Since signal transduction components and mechanisms are highly conserved among all eukaryotes, the unicellular *Saccharomyces cerevisiae* is often used as a model organism to study cell signaling. For yeast cells, the constantly fluctuating nutrient content the environment is a key determinant of cell cycle progression and for growth, stress resistance and metabolism. In general, a nutrient is sensed by the signaling network (i) externally, via a receptor protein in the plasma membrane, which after binding of the nutrient adopts a new conformation activity a downstream signaling cascade, or (ii) internally, after the uptake of the nutrient, and generally after being metabolized its intacellular concentration changes and this modulates downstream signaling.

Yeast can use a wide variety of substances as nutrient source. Nevertheless, some nutrients are preferred over others and nutrient metabolism is regulated in such a way that the preferred nutrient source is consumed first. Especially, the carbon source has a high impact on *S. cerevisiae* metabolism. In contrast to most yeast species, when all other essential nutrients are present in adequate amounts, *S. cerevisiae* cells will preferably ferment glucose and other rapidly fermentable sugars to ethanol and acetate, although respiration would be energetically more favourable. It is believed that this phenomenon, called the Crabtree effect, gives a competitive advantage, as the ethanol produced during fermentation inhibits growth of other micro organisms to yeast cells. When glucose becomes limiting yeast enters the diauxic shift, during which its metabolism shifts from fermentation to respiration allowing to use ethanol and acetate, which were accumulated during the fermentative growth phase. Finally, also when these carbon sources have been exhausted, cells enter the stationary phase (G0). Importantly, when another essential nutrient becomes limiting before glucose, yeast cells directly enter the stationary phase without passing through all other growth phases.

The main pathways responsible for the regulation of cell growth in response to nutrients are the TORC1 and PKA pathway. TOR and PKA are mainly activated by nitrogen and carbon sources, respectively. Inactivation of both pathway arrests cells in G1 [1,2] and triggers multiple aspects of the starvation response [3], suggesting that these two pathways coordinately drive cell growth and proliferation by promoting G1 progression [Fig.1].

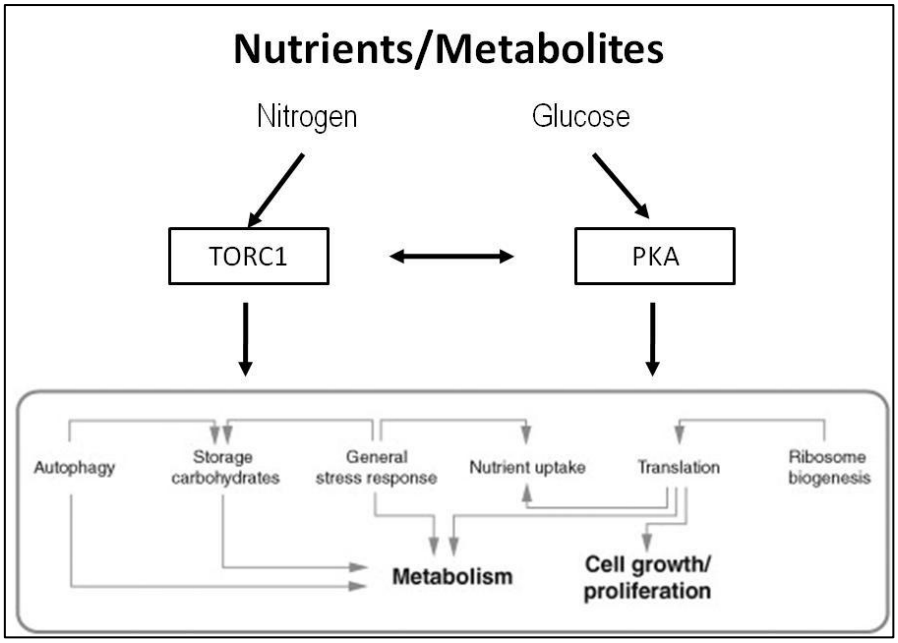

Fig.1: TOR and PKA promote cell growth in response to nutrients. Schematic representation of the TORC1 and PKA signaling pathways in budding yeast. TORC1 and PKA regulate metabolism and cell growth by affecting cellular physiology through multiple, highly interconnected downstream processes [4].

1.1 The TORC pathway

The Target Of Rapamycin (TOR), a highly conserved Ser/Thr protein kinase, is the central component of a major regulatory signaling network that controls cell growth in diverse eukaryotic organisms. The TOR proteins were first identified in yeast as the targets of the antifungal and immunosuppressive agent rapamycin, hence their name [5]. In contrast to most eukaryotes, yeast contains two TOR homologues, Tor1 and Tor2. Moreover, two functionally and structurally distinct TOR multiprotein complexes exist: TOR complex 1 (TORC1) and TOR complex 2 (TORC2) [6]. Both Tor1 as well as Tor2 can be found in TORC1 complex together

with Lst8, Kog1 and Tco89. A separate pool of Tor2 also associates with Lst8, Avo1, Avo2, Avo3, Bit61 and Bit2 to form TORC2 [6]. The precise function of these TOR-interacting proteins is not known yet. They might play a role in the binding of the TOR complexes to their substrates, be the receivers of upstream signals and/or determine the localization of the complexes. Both TOR complexes are essential for viability, since deletion of *TOR2* (inactivation of TORC2) or deletion of both *TOR1* and *TOR2* are lethal for yeast [5]. Deletion of TOR1 alone, however, is not lethal, indicating that Tor1 and Tor2 have a redundant role in TORC1 signaling.
While TORC1 drives cell growth through its effects on protein synthesis and cellular metabolism, TORC2 functions mainly in the organization of the spatial aspects of growth, such as the control of actin cytoskeleton [7]. Similarly, rapamycin treatment results in inactivation of TORC1, but not of TORC2 [7]. Indeed, the addition of rapamycin induces dramatic phenotypic changes such as cell cycle arrest and entry into G0, general downregulation of protein synthesis, accumulation of the reserve carbohydrate glycogen and the stress protectant trehalose, upregulation of stress response genes, autophagy and alterations in nitrogen and carbon metabolism. The mechanisms regulating TORC2 are still poorly understood, but do not appear to be directly connected to nutrient levels [7].

1.1.1 Localization of the TOR protein complexes

Many studies investigated the localization of TORC1 and TORC2. Different localization patterns were observed, which are probably due to the fact that the TOR signaling controls a multitude of processes. In general, the TOR complexes were found associated with membranes, ranging from the plasma membrane to the vacuolar and internal over of the protein secretory pathway [8]. TORC2 appears to be predominantly localized in discrete dots at the plasma membrane, while TORC1 is mainly found the vacuolar membrane; it is intriguing to reveal that the vacuole is a reservoir of nutrients and that TORC1 signaling is believed to be regulated by nutrients [8]. According to a recent study, TORC1 is also targeted to the nucleus where it induces 35S rRNA synthesis under favourable growth conditions [9].

1.1.2 Upstream regulators of TORC1

Rapamycin treatment, transfer of yeast cells from good- to poor-quality carbon or nitrogen sources, or starvation for carbon or nitrogen all elicit very

similar responses indicating that TORC1 is regulated by the abundance and/or quality of the available carbon and nitrogen sources. While it is still largely unknown which metabolite(s) may regulate TORC1, glutamine appears to play a particularly important role in TORC1 activation [6]. Understanding of how nutrients (including amino acids such as glutamine) are sensed and how this information is transmitted to TORC1 still remains one of the major challenges in the TORC1 field research.

In this context, the vacuolar membrane-associated EGO (exit from rapamycin-induced growth arrest) protein complex (EGOC), which consists of Ego1/Meh1/Gse2, Ego3/Nir1/Slm4/Gse1, Gtr1, and Gtr2, has been proposed to function as a critical hub that directly relays an amino acid signal to TORC1[6]. This initial idea has recently been bolstered by the finding that the EGOC subunit Gtr1, which is homologous to mammalian Rag GTPases, directly interacts with and activates TORC1 in an amino acid-sensitive and nucleotide-dependent manner [10].

Interestingly, a genome-wide synthetic genetic interaction screen revealed that *tor1Δ* cells were particularly sick, or not viable, in the absence of individual subunits of either of both complexes, namely the EGO complex and the homotypic fusion and vacuole protein sorting (HOPS/class C-Vps) complex [11]. These and additional genetic data indicate that the class C-Vps/HOPS complex may, like EGOC, directly or indirectly control TORC1 signaling in response to amino acids. Notably, the HOPS complex is thought to facilitate the transition from tethering to trans-SNARE pairing during fusion at the vacuole in part by nucleotide exchange on the GTPase Ypt7, which is exerted by the HOPS complex subunit Vam6. Intriguingly, recent genetic and biochemical data indicate that Vam6 may in fact control TORC1 function directly by regulating the nucleotide-binding status of the EGOC subunit Gtr1 [10]. This suggests that Vam6 may actually integrate amino acid signals to coordinate the control of TORC1 activity and vacuolar fusion events. While the discovery of the EGOC as an activator of TORC1 signaling represents an important step in deciphering the molecular events that signal nutrient availability to TORC1, an interesting question that remains unsolved is how amino acid availability is sensed and communicated to Vam6 and/or the EGOC.

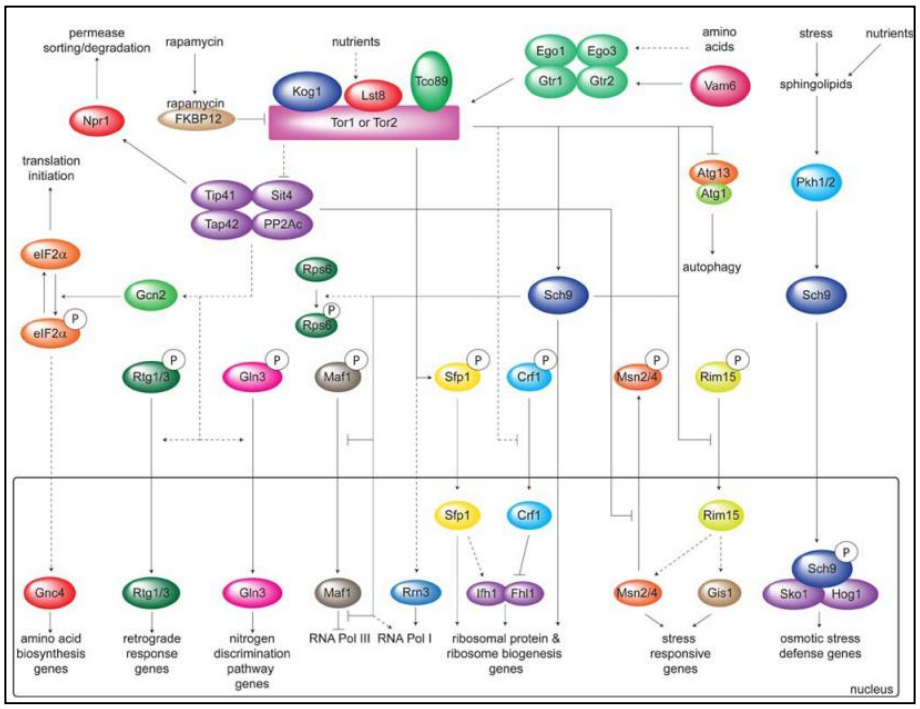

Fig.2: The TORC1 pathway in *S. cerevisiae.* Nutrients activate TORC1, resulting in the stimulation of protein synthesis and the inhibition of stress response genes, autophagy and several pathways that allow growth on poor nitrogen sources. The majority of these processes is regulated by the rapamycin-sensitive TORC1 complex either via the Tap42-Sit4/PPA2c or the recently identified Sch9 branch. See text for further details. Arrows and bars represent positive and negative interactions, respectively. Dashed lines represent putative or indirect interaction [12].

In agreement with data obtained in mammalian cells [13], recent evidence indicates that the yeast AMPK homologue, Snf1, phosphorylates the TORC1 component Kog1/Raptor in response to glucose starvation. This potentially identifies a mechanism for glucose regulation of the TORC1 complex [4]. Finally, the Golgi Ca2+/Mn2+ ATPase Pmr1 has been recently implicated in the TORC1 regulation [14].

1.2.3 Targets of TOR pathway

The precise mechanism how TORC1 regulates its downstream effectors are not completely well understood. Several TORC1-mediated processes involve the protein kinase Sch9 [15]. Others appear to be regulated via the PP2a and the PP2a-related protein phosphatases [16]. TORC1 controls the activity of these phosphatases via Tap42. In actively growing cells, the Tap42-

associated phosphatase complexes reside mainly in membranes where they associate and are inactive, with TORC1. Rapamycin treatment or nitrogen starvation abrogate the TORC1 association and release the Tap42-associated phosphatase complexes into the cytosol [17]. Several studies reveal an important role for another player in TORC1-dependent regulation of PP2A and Sit4, i.e. Tip41. However, both Tip41 and Tap42 may cooperate in determining the substrate specificity of PP2A and Sit4, and may fulfil essentially a similar function in TORC1 signaling [18].

One of the first described examples where TORC1 signaling involves the regulation of PP2A and Sit4 is the control of nitrogen metabolism. Yeast cells adapt their metabolism to the available nitrogen sources via the nitrogen catabolite repression pathway (NCR) also known as the nitrogen discrimination pathway (NDP) [19]. TORC1 inhibits transcription of NDP genes by controlling Gln3 and Gat1, two activators of this pathway. Thus, it appears that, through inhibition of the Tap42-Sit4 phosphatase complex, TORC1 promotes Gln3 phosphorylation, inhibiting its activity [20]. Nevertheless, recent results indicate that PP2A phosphatase activity is also necessary for Gln3 nuclear import upon rapamycin treatment, although the mechanistic details remain elusive [21].

TORC1 further controls negatively the general amino acid control (GAAC) pathway. The central component of this pathway is Gcn4, a transcription factor important for activating transcription of genes needed for amino acid biosynthesis in response to amino acid starvation [22]. This pathway is induced by uncharged tRNAs, which presumably activate the kinase Gcn2. In turn, Gcn2 phosphorylates the α subunit of eIF2 and although this results in a reduction of the general translation initiation, it specifically stimulates the translation of GCN4 mRNA [23]. Note that Gcn4 is also a target of the NDP, suggesting that TORC1, via inhibition of NDP gene expression, also inhibits GCN4 transcription.

A third pathway involved in nitrogen metabolism and that is subject to TORC1 control is the retrograde response pathway (RTG). Among other functions, this pathway induces the expression of genes whose products are required for the biosynthesis of α-ketoglutarate as precursor for glutamate synthesis in cells grown on poor nitrogen sources as well as in respiration-

deficient cells [24]. Expression of these genes requires the transcriptional activators Rtg1 and Rtg3. TORC1 controls the cytoplasmic sequestration of these factors through phosphorylation of Mks1, which thereby forms a complex with the 14-3-3 proteins Bmh1/2 to provide the cytoplasmic anchor for Rtg1 and Rtg3. Genome-wide expression analysis revealed that Tap42 is probably also involved in this regulatory mechanism [25].

Moreover, TORC1 also appears to control the turnover of several amino acid permeases. Depending on the quality and quantity of the nitrogen sources in the medium, yeast cells activate a different set of amino acid permeases. Under nutrient rich conditions, the so-called constitutive permeases, such as the high-affinity tryptophan permease Tat2, are targeted to the plasma membrane, whereas the nitrogen-responsive ones, such as the general amino acid permease Gap1, are sorted to the vacuole for degradation. During periods of nitrogen limitation, opposite sorting occurs and Gap1 is allowed to reach the plasma membrane, while Tat2 is endocytosed and delivered to the vacuole [26]. The protein kinase Npr1 plays a major role in the sorting of these two classes of permeases. It is required for Gap1 stabilization at the plasma membrane and induces the degradation of Tat2, possibly by regulating their ubiquitination [27]. TORC1 activity, through control of the Tap42–Sit4 phosphatase complex, promotes the phosphorylation of Npr1 [28]. Recently, it has been shown that mutants affected in Lst8 display vacuolar targeting of Gap1 under conditions where the permease should normally be sorted to the plasma membrane. Lst8 is a component of TORC1 and TORC2 and, consistently, the impairment of TOR signaling by treatment with low, sublethal doses of rapamycin triggers a similar missorting of Gap1. As both mutation of Lst8 and rapamycin-induced impairment of TOR signaling cause a significant increase in intracellular amino acid pools, it was suggested that this could act as a signal that directs the vacuolar sorting of Gap1[29].

Apart from its controlling function on nitrogen metabolism, TORC1 has a major regulatory role in protein synthesis as it promotes expression of the rRNA and the ribosomal proteins (RP) genes as well as of the so-called ribosome biogenesis (Ribi) regulon [30]. Recent data suggest that TORC1 promotes recruitment of the RNA polymerase I to the rDNA locus in a Rrn3-independent way, via a signaling route that requires the TORC1-effector protein kinase Sch9 [31]. Tor1 itself binds to the 35S and 5S rDNA region

under favourable nutrient conditions and this seems to be essential for the synthesis of 35S and 5S rRNA via, respectively, RNA polymerase I and III [9]. In addition, the Tor1 association with 5S rDNA chromatin is also required for TORC1 to stimulate the expression of tRNAs by RNA polymerase III [32][Fig.3].

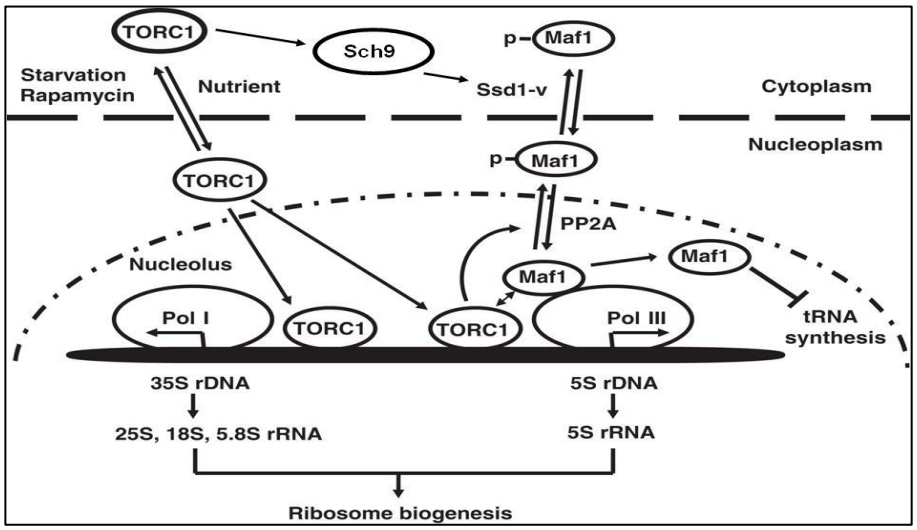

Fig.3: A working model for TORC1-dependent regulation of transcription by Pol I and Pol III. TORC1 is localized in the nucleus and is associated with both 5S and 35S rDNA regions, promoting their transcription by Pol I and Pol III, respectively, in a nutrient-dependent and rapamycin-sensitive manner. The concomitant TORC1 association/dissociation with 5S rDNA and 35S promoter provides a simple yet efficient mechanism to coordinate the synthesis of ribosomal RNAs in response to environmental changes. TORC1 at 5S rDNA and possibly also 35S rDNA promoter regulates the Maf1 phosphorylation, and subsequently Pol III-transcribed genes, including 5S rRNA and tRNA genes [32].

This regulation of RNA polymerase III expression involves, at least in part, the inhibition of Maf1, a repressor of RNA polymerase III transcription that is also inhibited by PKA[32]. Interestingly, most recent data suggest that TORC1 mediates phosphorylation of Maf1 also indirectly via Sch9 [32].
Concerning the regulation of RP gene transcription by RNA polymerase II, it has been found that TORC1 promotes complex formation between Fhl1, a forkhead-like transcription factor that binds to the promoter of RP genes, and its co-activator Ifh1 [33,34]. Then this Fhl1–Ifh1 complex promotes the expression of RP genes. How TORC1 exactly interferes with Fhl1 complex formation and activity is unclear. One mechanism seems to involve the stress- and nutrient-sensitive transcription factor Sfp1. TORC1 regulates,

probably via direct phosphorylation, the nucleo-cytoplasmic distribution and the promoter binding of Sfp1 (see below). Another protein involved in TORC1-mediated transcriptional control of ribosome biosynthesis is Hmo1. This protein is a member of the HMG protein family that encompasses architectural proteins that bind to DNA with low sequence specificity. Hmo1 associates with RP gene promoters and with the rDNA region and this association requires TORC1 activity [35]. TORC1 also regulates protein synthesis at has been post-transcriptional level. It recently shown that TORC1 controls the nucleo-cytoplasmic shuttling of Dim2 and Rrp12, two 40S ribosome synthesis factors that are involved in ribosome assembly and the nucleo-cytoplasmic translocation of pre-ribosomes. Furthermore, TORC1 was found to be essential for translation initiation [2] and to have a positive effect on the stability of translation initiation factor eIF4G and eIF2α regulation [2].

Next, TORC1 exerts a major impact on the transcription of stress response genes. Here, TORC1 has a dual control. On the one hand, by Sch9 it prevents nuclear translocation of the protein kinase Rim15, a protein kinase required in yeast for the proper entry into stationary phase (G0) [36]. On the other hand, TORC1 inhibits the transcription of stress-responsive genes via a Rim15-independent, but Tap42–PP2A-dependent route, thereby promoting the phosphorylation and cytoplasmic retention of Msn2 a transcription factor necessary for the stress answer. [18].

Finally, TORC1 is also a known negative regulator of autophagy [28]

1.2 The PKA pathway

PKA have an important role in glucose signaling pathway and is essential for viability and for driving cell growth. Indeed, high levels of PKA activity cause inhibition of stress responses and induction of pseudohyphal and invasive growth. PKA consists of a regulatory subunit, Bcy1, and three catalytic subunits encoded by the *TPK1*, *TPK2* and *TPK3* genes. Glucose stimulates adenylate cyclase (Cyr1) to produce cellular cyclic AMP (cAMP) that, by binding the regulatory subunit of PKA (Bcy1), induces the release of active catalytic subunits [37].

cAMP is an integrator of intracellular and extracellular glucose signals [38]. Extracellular glucose triggers adenylate cyclase activity through a G protein-coupled receptor (GPCR) system, composed of Gpr1 and Gpa2. Gpr1 is a G protein-coupled seven-transmembrane receptor (GPCR) present on the cell

surface and appears to function as a low-affinity glucose receptor responding to high concentrations of glucose in the extracellular environment. Gpa2 is a member of the heterotrimeric G protein α subunit (Gα) protein family [39]. Addition of glucose to derepressed cells activates Gpr1, which in turn stimulates the exchange of GDP for GTP on Gpa2 which associates with adenylate cyclase stimulating its activity [Fig.4].

In parallel to the GPCR system, the small G-protein Ras, encoded by *RAS1* and *RAS2* in yeast, is required for adenylate cyclase activation in response to intracellular glucose signals. Ras is a small G protein that demonstrates its biological functions through a cycle of GDP/GTP exchange and GTP hydrolysis. The GTP-bound form is active while the hydrolysis of the bound GTP to GDP inactivates it. The GDP/GTP exchange on the Ras protein is catalyzed by Cdc25 and the GTP hydrolysis reaction is stimulated by the hydrolases Ira1 and Ira2 [40]. Despite its key role in the regulation of cell growth, the molecular mechanisms of glucose mediated Ras activation remain poorly understood.
In a screen for putative regulators of Ras-dependent PKA activation [Fig.4], the protein Tfs1 has been isolated as a multicopy-suppressor of a mutation in the RasGEF, Cdc25 [41]. *In vivo*, Tfs1 binds to Ira2 and inhibits its function towards Ras [42]. In addition, Tfs1 interacts with and thereby inhibits the vacuolar protease Carboxypeptidase Y [43].

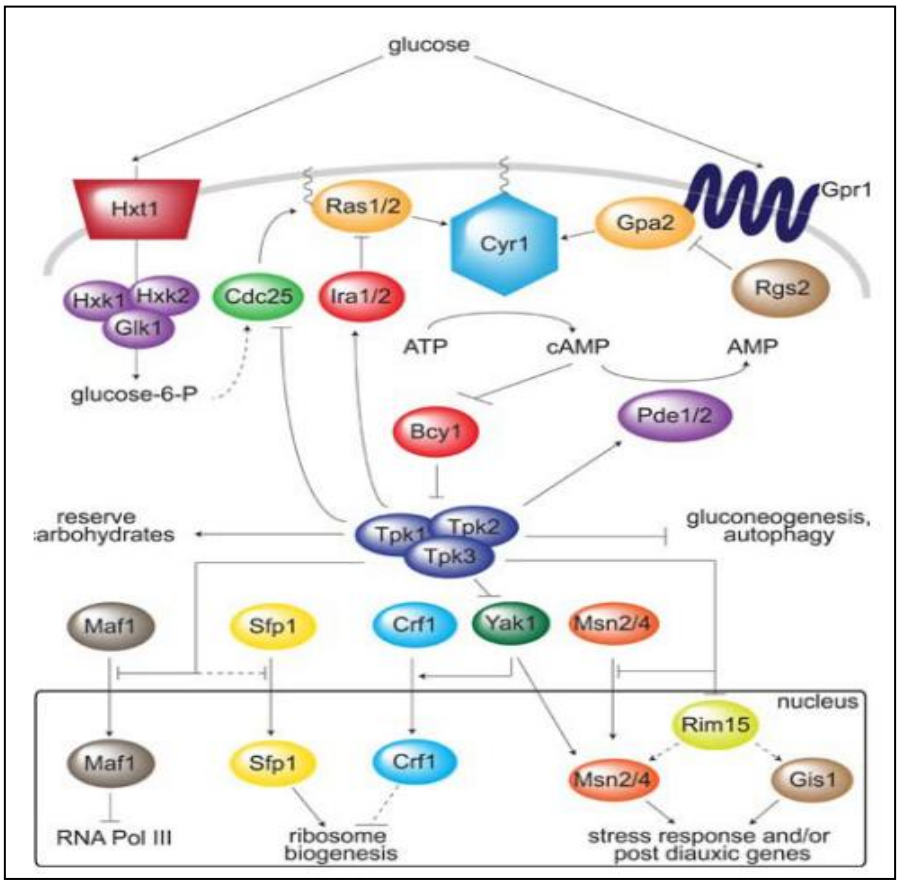

Fig.4: The cAMP-PKA pathway in *S. cerevisiae.* Addition of glucose to glucose-starved, respiring cells triggers the rapid synthesis of cAMP and, subsequently, the activation of PKA. Glucose-induced cAMP synthesis requires two sensing systems: (i) extracellular detection of glucose via the Gpr1–Gpa2 system and (ii) intracellular detection of glucose, which requires uptake and phosphorylation of the sugar. The intracellular sensing system probably transduces signals via the GEF protein Cdc25 and the Ras proteins. Activated PKA mediates the fast transition from respiratory to fermentative growth via the modulation of numerous downstream targets. Arrows and bars represent positive and negative interactions, respectively. Dashed lines represent putative or indirect interactions. See text for further detail [12]

A strong negative feedback mechanism ensures that the glucose-induced increase in cAMP levels and PKA activity are transient and can only be triggered in glucose-derepressed cells. PKA itself is involved in this mechanism since basal cAMP levels are dramatically increased in strains with reduced activity of the kinase. cAMP is hydrolyzed by the low- and high-affinity phosphodiesterases, respectively, encoded by *PDE1* and *PDE2*. The high-affinity phosphodiesterase Pde2 control a basal cAMP levels,

which is important to prevent undesirable PKA activity during the stationary phase [44]. The low-affinity phosphodiesterase Pde1, however, was shown to be specifically involved in the feedback inhibition of glucose-induced cAMP signaling and is probably activated by PKA itself [45].

1.2.1 Targets of cAMP-activated PKA

cAMP-activated PKA has a major impact on gene expression, which is well-illustrated by the observation that 90% of the transcriptional changes upon glucose addition to glucose-starved cells could be mimicked by artificial activation of PKA [46]. Accordingly, several of the known PKA targets affect gene transcript levels, either directly or indirectly [Fig. 4]. Two of these are the transcription factors Msn2 and Msn4, which mediate the transcription of the so-called stress response element (*STRE*)-controlled genes. *STRE* genes are involved in a wide variety of processes, including protection against diverse types of stress such as heat, oxidative and osmotic stresses, carbohydrate metabolism and growth regulation. Msn2 and Msn4 are inhibited by PKA. Moreover, PKA seems to additionally inhibit the function of Msn2 and Msn4 via the protein kinases Yak1 and Rim15 [47]. During growth on glucose, Msn2 and Msn4 are phosphorylated and reside in the cytosol. Upon glucose exhaustion, they are hyperphosphorylated and translocated to the nucleus, where they induce the expression of the *STRE*-controlled genes.

Furthermore, PKA activates the transcription of ribosomal protein genes as well [48]. It was reported that PKA promotes nuclear localization and binding of the transcriptional activator Sfp1 to the promoters of ribosomal protein genes [49]. In addition, PKA to induces transcription of ribosomal protein genes also by inhibition of Yak1, which in turn is required to promote the activity of the transcriptional corepressor Crf1 [50] [Fig.4].

In addition to the control of gene expression and protein synthesis, PKA directly modulates the activity of metabolic enzymes. The PKA modifications result in the stimulation of glycolysis and the inhibition of gluconeogenesis when glucose is added to glucose-starved cells [51].

Finally, PKA is a known inhibitor of autophagy, a degradative process that recycles non-essential proteins and organelles during periods of nutrient starvation .The key players involved in autophagy are the Atg proteins and three of these, Atg1, Atg13 and Atg18, contain a PKA consensus phosphorylation site. At least for Atg1, this site appears to be functional

since data confirmed that PKA phosphorylation negatively controls the recruitment of Atg1 to the sites of autophagosome formation upon nutrient limitation [52].

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Chapter 2:

MAPK routes in *Saccharomyces cerevisiae*

A fundamental property of living cells is the ability to sense and respond appropriately to changing environmental conditions and various other stimuli. One frequently utilized molecular device for eliciting these responses is the three-tiered cascade of protein kinases known as the mitogen-activated protein kinase (MAPK) module. The canonical MAPK pathway contains a key, three-component signal relay in which an activated MAPK kinase kinase (MAPKKK or MEKK) activates a MAPK kinase (MAPKK or MEK), which then activates a MAPK (or ERK, for extracellular signal-regulated kinase) [Fig.1]. MAPKKKs contain an N-terminal regulatory domain and a C-terminal serine/threonine protein kinase domain. Upon activation, a MAPKKK phosphorylates two serine or threonine residues at conserved positions in the activation loop of its target MAPKK, which is a dual-specificity (serine/threonine and tyrosine) protein kinase. The activated MAPKK then proceeds to phosphorylate both the threonine and tyrosine residues of a conserved -Thr-X-Tyr- motif in the activation loop of its target MAPK. These phosphorylations activate the MAPK by causing substantial conformational changes; once active MAPKs phosphorylate a diverse set of well-characterized substrates, including transcription factors, translational regulators, MAPK-activated protein kinases (MAPKAP kinases), phosphatases, and other classes of proteins, thereby regulating metabolism, cellular morphology, cell cycle progression, and gene expression in response to a variety of extracellular stresses and molecular signals [1].

The MAPK cascades are found in mamalians [2], plants [3] and fungi [4]. Many extracellular and intracellular signals modulate transcription of specific genes through activation or inhibition of MAPK cascades[Fig. 1].

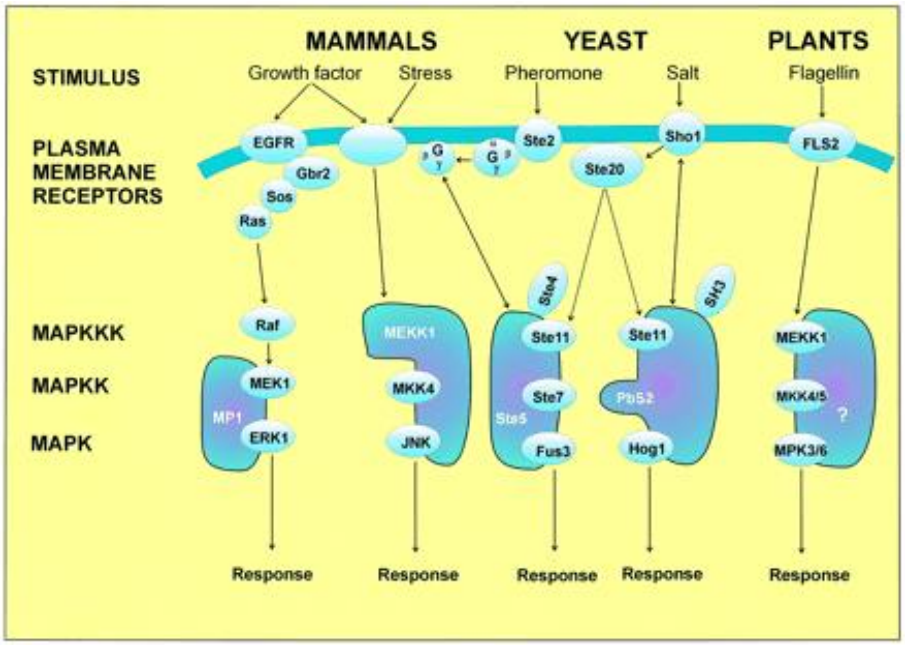

Fig. 1: Scheme of distinct MAPK signalling pathways in mammals, yeast and plants. Note the general similarity in the organization of MAPK pathways in all three eukaryotic systems. MAPKKK, mitogen activated protein kinase kinase kinase; MAPKK, mitogen activated protein kinase kinase; MAPK, mitogen activated protein kinase. Scaffolding proteins (depicted in dark blue) are integrating signalling pathways. [5]

The understanding of the *Saccharomyces cerevisiae* MAPK pathways is more complete than that of MAPK pathways in other organisms. Extensive genetic and biochemical analyses plus the complete sequencing of its genome have revealed that budding yeast contains five MAPKs on five functionally distinct cascades [6]. Four of these pathways, the mating pathway, the filamentation-invasion pathway, the cell integrity pathway, and the high-osmolarity growth pathway, are present in growing cells [Fig.2]. The Smk1p MAPK, part of the spore wall assembly pathway, is not present in growing cells but appears during sporulation and regulates that developmental process.

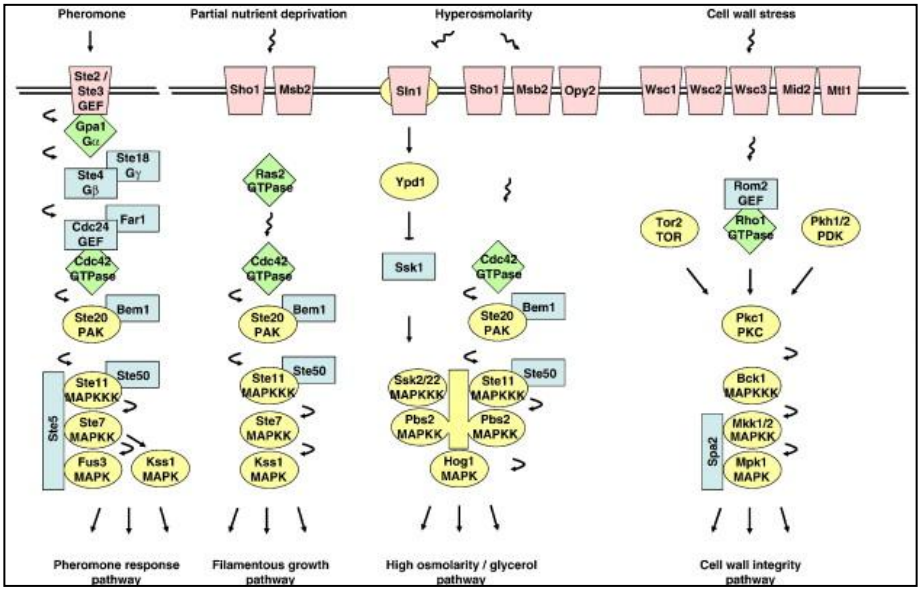

Fig. 2: Schematic diagrams of the MAPK signaling pathways in *S. cerevisiae*. Symbols are: protein kinases, ovals; GTP-binding proteins, diamonds; scaffold, adaptor, and activating proteins, rectangles; cell surface proteins, trapezoids; activation, arrows; inhibition, T-bars; direct action, smooth lines; indirect action (or unknown molecular mechanism), squiggly lines. For clarity, not all factors and interactions are shown, connections to other pathways and processes upstream of the MAPKs are omitted, and direct targets of the MAPKs are not included (Chen, *et. al.*).

2.1 Pheromone response pathway

Yeast can exist in haploid or diploid states. Haploid cells identify mating partners by responding to pheromone gradients generated by cells of the opposite sex or mating type (a or α). Both pheromones and the receptors are mating type-specific, ensuring that cells only respond to signals appropriate to their mating type. Cells form a mating projection (shmoo) toward the highest concentration of pheromone and arrest their cell cycle in G1 in preparation for mating. Once mating partners are in contact, the intervening cell walls are dissolved, permitting fusion of the plasma membranes. Nuclear fusion ensues, resulting in the formation of a zygote, which resumes its cell cycle to grow as a stable a/α diploid cell.

The intracellular response to mating pheromone has been well characterized [7] [Fig.2]. Pheromone from the opposite mating type binds and activates a seven transmembrane G protein–coupled receptor (Ste2 or Ste3). Receptor

activation causes a Gβγ complex (Ste4 and Ste18) to be released from inhibition by a Gα subunit (Gpa1), but they remain at the plasma membrane due to lipid modifications on the Gα (S-palmitoylation and N-myristoylation) and Gγ (farnesylation and S-palmitoylation) subunits. Gβγ interacts with a host of different proteins involved in mating and colocalizes relevant proteins to facilitate action of the pathway. In particular, it binds the scaffold protein Ste5, recruiting it to the plasma membrane where the bound kinase complex, consisting of the Ste11 MEKK, the Ste7 MEK, and the Fus3 MAPK, is activated [8]. The Rho family protein Cdc42, a small GTPase localized to the plasma membrane, activates the p21-activated kinase (PAK) Ste20 upon GTP binding through an interaction with a CRIB motif in Ste20 [9]. Ste20 also displays an interaction between its C-terminal tail and the Gβγ complex [10]. Upon recruitment of the Ste5 complex to the plasma membrane by Gβγ, Ste20 is activates scaffold-bound Ste11 by direct phosphorylation . Activation likely occurs through relief of inhibition of an intramolecular interaction. Ste11 activation is assisted by the Ste50 protein, which binds to the N-terminal noncatalytic domain of Ste11 via interactions between SAM (Sterile Alpha Motif) oligomerization domains present in Ste50 and in Ste11 [11]. Active Ste11 phosphorylate MEK Ste7 on the Ste5 scaffold. Ste7 in turn activates the MAPK Fus3 by dual phosphorylation of the TEY motif present in its activation loop. Phosphorylated Fus3 activates mating gene transcription and promotes cell cycle arrest.

2.1.1 Key players of Pheromone response pathway

Ste5: The scaffold Ste5 has no obvious homologues outside fungi, but at least 18 mammalian MAPK signaling scaffolds have been described, which themselves are unrelated to each other [12]. Thus, although the primary structures of MAP kinase signaling scaffolds have not been conserved, evolution has apparently turned to scaffolding multiple times. Ste5 functions specifically in the mating pathway; it is dispensable for filamentous growth and for the response to high osmolarity [13]. Ste5 was among the first signaling scaffolds to be identified. A combination of genetic, two-hybrid, coimmunoprecipitation, and cosedimentation studies have demonstrated that Ste5 is a modular protein that can bind each of the kinases in the cascade (Ste11, Ste7, and Fus3), as well as Gβ through distinct binding domains (Elion 2001). Although it is presumed that the scaffold binds all components simultaneously, this has yet to be demonstrated rigorously. A MAP kinase

highly related to Fus3, Kss1, is required for mating when Fus3 is inactivated by mutation [14]. However, because Kss1 is the MAP kinase for the filamentous growth pathway (see below), we consider it as a pathway-specific component of this pathway [15]. Two-hybrid and coimmunoprecipitation studies suggest that Ste5 can form dimers and/or higher-order oligomers. An intriguing study reported that in order to be competent for plasma membrane localization and binding to Ste11, Ste5 must first shuttle through the nucleus [16]. The current model is that shuttling enhances oligomerization, and only oligomers are competent for signaling, possibly by disrupting an intramolecular inhibitory interaction and allowing the Ste4 and Ste11 binding sites to become free. Although the details of this mechanism remain to be elucidated, it may provide for tight regulation of signaling.

Ste5 is thought to promote signaling efficiency by concentrating and perhaps even orienting and aligning the relevant components. Results of a recent study suggest that concentration of the binding partners may be sufficient for signaling (56). This study exploited previously identified point mutations that decrease the affinity of Ste5 for either Ste11 or Ste7. The defects associated with these mutations could be partially suppressed by appending artificial cognate binding domains to Ste5 and the kinase target. While signaling efficiency is reduced, these artificial interactions supported signal transduction, suggesting that the exact orientation of the kinases on the scaffold may not be essential for signaling. However, since the kinase binding domains of Ste5 were not deleted, the suppression of the missense mutations by artificial recruitment might not reflect a fundamentally different geometry of binding to the scaffold.

Fus3: Fus3 is a homolog of the mammalian ERK-type MAPKs, which are activated in response to diverse stimuli, including mitogens. Fus3 plays a central role in mating because it controls not only pheromone-dependent gene expression, but also promotes cell cycle arrest and mating projection formation [7]. Fus3 presumably has many direct phosphorylation targets; however, few have been characterized. A key function of Fus3 is to activate the transcription factor Ste12, which is required for pheromone-dependent gene induction. Fus3 apparently activates Ste12 indirectly, by inactivating (likely by direct phosphorylation) two redundant inhibitors of Ste12, Dig1/Rst1 and Dig2/Rst2 [17]. A second key function of Fus3 is to promote

cell cycle arrest by phosphorylating the Cdk inhibitor Far1 [18] (See chapter 4).
Finally Fus3 is required for specificity between the mating and filamentation pathways. In *fus3Δ* cells or in cells harboring a kinase-dead allele of *FUS3*, the pheromone response pathway can activate the filamentation MAPK pathway [4].

2.2 Filamentous Growth MAPK Pathway

In response to nitrogen starvation and other signals, diploid a/α yeast cells undergo a developmental change and switch to a filamentous form of growth called pseudohyphal development [19]. This transition includes cell elongation, a switch to a unipolar budding pattern, maintenance of attachment between mother and daughter cells, and the consequent ability to invade semisolid media. This morphological change is likely a foraging response allowing cells to scavenge for nutrients. Filamentous growth, although ubiquitous in wild yeast, has been lost from most recombinant inbred laboratory strains. Consequently, studies of filamentation have been largely restricted to filamentation-competent strains such as Σ1278b. In *S. cerevisiae*, haploid mating-type a or α cells can also switch to a filamentous growth form. Glucose starvation [20] and some alcohols such as isoamyl alcohol (a product of normal yeast metabolism) [21] promote this switch, which is termed haploid invasive growth. A close kinship between haploid invasive growth and diploid pseudohyphal growth is suggested by the observation that both are controlled by a common set of conserved signaling pathways.
The MAP kinase signaling cascade required for filamentous growth shares many components with the mating pheromone response pathway and is discussed further below. A pathway that uses cAMP as a second messenger senses environmental nutrient levels and also controls filamentous growth [22]. Both the MAPK and PKA pathways control the expression of *FLO11*, a cell surface adhesion molecule required for cell-cell attachment during filamentous growth. The *FLO11* upstream region is complex and integrates information from multiple signaling pathways [23]. These pathways are likely to have numerous other targets that mediate this critical developmental switch.

Cdc42 and Ste20 are components of the MAPK pathway that controls filamentous growth pathway [Fig.2/3].

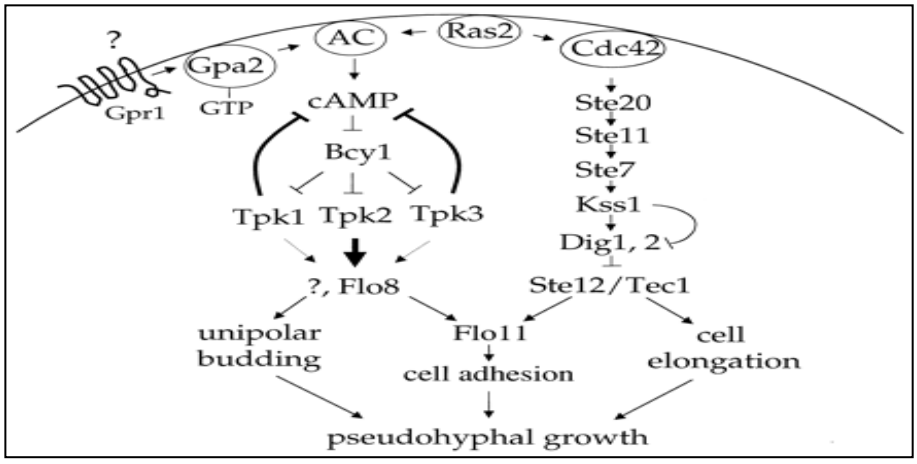

Fig. 2: A model for the regulation of pseudohyphal growth by the PKA and MAP kinase pathways. The three catalytic subunits of PKA play distinct roles in regulating yeast pseudohyphal growth. The Tpk2 catalytic subunit plays a positive role to activate filamentous growth, whereas the Tpk1 and Tpk3 catalytic subunits play negative roles to inhibit filamentous growth. Epistasis analysis indicates that PKA signals downstream of the Gpr1 receptor and G protein Gpa2. PKA and the MAP kinase cascades function independently to regulate budding pattern and cell elongation, respectively, during filamentous growth. In contrast, PKA (via Flo8) and the MAP kinase cascade (via Ste12 and Tec1) coordinately regulate the cell surface flocculin Flo11, agar invasion, and cell adhesion [23].

As in the pheromone response pathway, these proteins act upstream of a cascade involving the Ste11 MEKK and the Ste7 MEK. The MAP kinase for the filamentous growth pathway is Kss1. Dig1 and Dig2 act as inhibitors in this pathway as well, but they do not appear to be completely redundant as they are in the pheromone response pathway. Remarkably, even the transcription factor for the pheromone response pathway, Ste12, is required for filamentous growth [24]. However, at filamentation promoters, Ste12 acts with the filamentation-specific factor Tec1 [25] [25]. These two transcription factors bind cooperatively to promoter elements, termed filamentation response elements (*FREs*), that have been defined in the promoters of *TEC1* and the Ty1 retrotransposon [25].

2.2.1 Environmental sensing mechanisms

Several distinct transmembrane proteins that reside in the plasma membrane and are exposed to the cell surface are necessary (in some cases, in haploids,

and in other cases, in diploids) for initiation of filamentous growth. These transmembrane proteins include: Sho1 (four transmembrane segments); Msb2 (one transmembrane segment); Mep2 (ten transmembrane segments); and, Gpr1 (seven transmembrane segments) [26] [21].

Sho1 can also form hetero-oligomeric complexes with another single-pass transmembrane protein, Opy2, a protein which is necessary for growth under hyperosmotic conditions (see Section 2.3).
Sho1 serves as a common subunit of two different membrane sensors allowing cells to respond to two different stimuli. The role of Sho1 is reminiscent of what is seen for several classes of cell surface receptors in mamnalian cells, such as the common gamma chain (γc) shared by different multi-chain cytokine receptor.

Msb2 possesses a large highly O-glycosylated exocellular domain that is related to the so-called mucin family of mammalian transmembrane proteins. Strikingly, deletions within the extracellular mucin-homology domain of Msb2 cause significant constitutive activation of the filamentous growth response in haploids. This observation leads to the simple model that glucose limitation leads to ipo-glycosylation of Msb2, alleviating some negative structural constraint and promoting the events necessary to trigger downstream signal propagation. In this regard, it has also been reported that the short C-terminal cytosolic tail of Msb2 can bind to Cdc42 directly [27].

Mep2 is a high-affinity ammonia permease that also acts as a nitrogen sensor and is required for diploid pseudohyphal growth. Loss of the related, but lower affinity, ammonia permeases, Mep1 and Mep3, has no effect on diploid filamentation. Activated Ras2 bypasses the need for Mep2 in diploid pseudohyphal growth, suggesting that this is the level at which the function of Mep2 is connected to stimulation of the MAPK cascade that activates Kss1 and PKA [29].

Gpr1 is a glucose (and sucrose)-binding GPCR that associates with a distinct Gα subunit, Gpa2, and is serves as a carbon sensor [28]. Intriguingly, expression of the *GPR1* gene is also induced under conditions of nitrogen limitation; thus, under limiting nitrogen, the cell presumably becomes more acutely "aware" of the status of its carbon supply.

The mechanisms by which the signals transduced by all of these transmembrane proteins are coordinated to achieve an optimal filamentous growth response are not known.

In this same regard, it has also been observed that filamentous growth can be stimulated by fusel alcohols and aromatic olies. These small molecules are, of course, potential membrane perturbants and may thus act via effects on one or more of the membrane proteins discussed above. Nevertheless, the fact that these small molecules are secondary metabolites generated by the yeast itself and released into the surrounding milieu has led to the proposal that these compounds could provide a quorum-sensing mechanism for regulating the onset of filamentous growth (Nelson 2004).

2.2.2 Key players of Filamentous Growth MAPK pathway

Kss1: Kss1, the MAPK for the filamentous growth pathway, is an ERK-type MAPK highly homologous to Fus3. Its identity as the MAPK for this pathway was difficult to elucidate because a null mutant in *KSS1* still undergoes pseudohyphal development and haploid invasive growth. The resolution of this apparent paradox is that Kss1 can both positively and negatively regulates the filamentation transcription factor Tec1-Ste12 [25]. In the absence of signaling, Kss1 inhibits Tec1-Ste12 and filamentous growth in a kinase-independent manner. Once phosphorylated and activated by Ste7, Kss1 switches into an activator of filamentation gene expression [25]. The precise mechanisms by which Kss1 inhibits and activates filamentous growth are not understood. Current evidence suggests that unphosphorylated Kss1 acts as an inhibitor by direct binding to Ste12 as well as by promoting the inhibitory functions of Dig1 and Dig2, which also bind directly to Ste12. The active form of Kss1 may function by inactivating the Dig1 and Dig2 inhibitors by phosphorylation and/or by phosphorylation of Tec1-Ste12 [7].

As mentioned above, Kss1 was originally assigned to the pheromone response pathway because it is required for mating in cells lacking Fus3. One way of reconciling this observation with the role of Kss1 as the MAPK for the filamentous growth pathway is to propose that Kss1 functions only in mating when Fus3 is inactive. However, mating pheromone treatment of cells causes phosphorylation and activation of a population of Kss1 [31]. This population appears to be small because a much larger amount of phosphorylation occurs in cells lacking Fus3. Nevertheless, this phosphorylation does not lead to transcriptional activation of a filamentation reporter (*FRE*-LacZ), suggesting that the consequences of the small amount of Kss1 activation in response to mating pheromone seen in wild-type cells

is suppressed before it can lead to the activation of the filamentation transcriptional program.

Tec1: Tec1 was originally identified as a transcription factor required for the expression of the yeast copia-like retrotransposon, Ty1. Subsequent work showed that was important for pseudohyphal development and haploid invasive growth [32]. In contrast to Ste12, Tec1 is dispensable for the pheromone response. Analysis of *FRE*-dependent reporters and genetic experiments demonstrate that Tec1 acts downstream of the filamentation MAPK pathway ([25]. It harbors a TEA/ATTS DNA binding domain that recognizes the same target sequence, CATTCY, as its homologs in *Aspergillus nidulans* (AbaA) and humans (TEF-1) ([25]. *In vitro,* purified recombinant full-length derivatives of Tec1 and Ste12 bind cooperatively to *FRE* elements derived from the *TEC1* and Ty1 promoters ([25].
Genome-wide chromatin immunoprecipitation experiments have defined the direct targets of Ste12 and Tec1 under conditions that promote mating (pheromone treatment) or filamentous growth (butanol treatment). These studies demonstrate that Ste12 redistributes across the genome in a manner that depends on the environmental conditions. Ste12 tends to be bound to mating promoters under conditions of pheromone treatment, but shifts to a distinct set of genes, including many known to be involved in filamentous growth, upon treatment of cells with butanol [33]. This redistribution to filamentation promoters requires Tec1, suggesting that earlier studies using isolated *FREs* as a model for the cooperative role of Tec1 with Ste12 apply to a significant set of endogenous genes.

2.3 High osmolarity/glycerol pathway

The HOG pathway is a branched MAPK (Mitogen Activated Protein Kinase) signal transduction system [Fig.2]. The MAP kinase Hog1 is the yeast orthologue of mammalian p38. The physiological role of the HOG pathway is to orchestrate the adaptation of yeast cells to increased osmolarity of the surrounding medium [34].Such increased medium osmolarity leads to water loss and cell shrinking. The cell needs to counteract these effects in order to maintain shape and turgor and to ensure appropriate water and ion concentration in the cytosol and its organelles for optimal functioning of

biochemical reactions. In addition, it has been shown that Hog1 is also required for adaptation to other stress conditions, such as oxidative stress, arsenite, cold stress and acetic acid stress [35]. Orthologues of Hog1 appear to be involved in osmoadaptation and other types of stress responses in probably all eukaryotes, although the specific sensing and regulatory mechanisms as well as the molecular targets of these pathways certainly differ between organisms.

The phosphorylation and hence activity of the Hog1 MAPK is controlled by two branches, the Sln1 and the Sho1 branch, which converge on the MAP kinase kinase (MAPKK) Pbs2. At present, it is not entirely clear why Hog1 is controlled by two branches, because i) either branch alone can activate Hog1 in response to hyperosmotic stress, ii) the Sln1 branch has a far more prominent role in pathway control as it is more sensitive to osmotic changes and supports the full pathway activation even in the absence of the Sho1 branch [36], and iii) the Sho1 branch does not seem to be connected to the Hog1 MAPK cascade in of other fungi.

Sln1: The Sln1 branch is controlled by the plasma membrane-localised sensor Sln1, which spans the membrane twice. Sln1 is related to the two-component osmosensor EnvZ in bacteria and might sense changes in membrane tension and/or turgor, although the physical mechanism of osmosensing is still unknown. Sln1, Ypd1 and Ssk1 form a phosphorelay system, the eukaryotic version of the two-component system. Sln1 is active under ambient conditions and inactivated upon hyperosmotic shock. Active Sln1 is a dimer that performs auto-phosphorylation on a histidine. This phospho group is then transferred to a receiver domain on Sln1, further to Ypd1 and eventually to the receiver domain on Ssk1. Phospho-Ssk1 is the inactive form and hence does not activate the downstream MAP kinase cascade. Phospho-Ssk1 is intrinsically unstable or dephosphorylated by an unknown phosphatase. Upon hyperosmotic shock, the level of unphosphorylated Ssk1 rapidly increases. Ssk1 binds to the regulatory domain of the Ssk2 and Ssk22 MAPKKKs, allowing Ssk2 and Ssk22 to autophosphorylate and activate themselves. Active Ssk2 and Ssk22 then phosphorylate and activate Pbs2, which in turn phosphorylates (on Thr174 and Tyr176) and activates Hog [37].

Sho1: The Sho1 branch is controlled by two mucin-like transmembrane sensors, Msb2 and Hkr1. Mucins connect the cell interior with the extracellular matrix (in fungi, the cell wall) and hence monitor movements

between the cell wall and the plasma membrane. Sho1 is an additional transmembrane protein in this branch of the HOG pathway. It has long been believed to be the sensor in the system. However, it plays a role as membrane-localised scaffold protein that recruits components to the cell surface at places of active cell surface growth and remodelling. Many molecular details of the activation mechanism remain unknown at this point. It is clear, however, that stimulation of the sensors by hyperosmotic shock results in recruitment to the plasma membrane of Pbs2, which not only serves as MAPKK but also as scaffold for the Sho1 branch. Probably, Pbs2 carries along the Ste11 MAPKKK, which thereby is brought into vicinity of the Ste20 and Cla4 kinases. These are located at the plasma membrane in association with the Cdc42 G-protein. Phosphorylation of Ste11 by Ste20 and/or Cla4 activates Ste11, which then phosphorylates Pbs2, which phosphorylates and activates Hog1 [38].

2.3.1 Transcriptional regulators of the HOG pathway

Acute hyperosmotic stress leads to a rapid (sub-minute) increase in the amount of phospho-Hog1, the active form of the kinase. Under such conditions, phosphorylation is accompanied (and required for) import of Hog1 into the nucleus, where the kinase participates on target promoters in the control of gene expression. Unlike action of Fus3 and Kss1 through Ste12, and Slt2/Mpk1 is largely through Rlm1, Hog1 influences the expression of genes driven by a wide variety of transcription factors (those grouped together are sequence-related), including: Hot1 and Msn1 (activators); Msn2 and Msn4 (activators); Sko1 (repressor); and Smp1 (activator; very related to Rlm1, but is similarity mainly confined to their N-terminal MADS box-type DNA-binding domains) All of these factors interact with Hog1 at the promoters of the respective target genes, and the role of Hog1 at some of them has been well characterized [35] [Fig. 4].

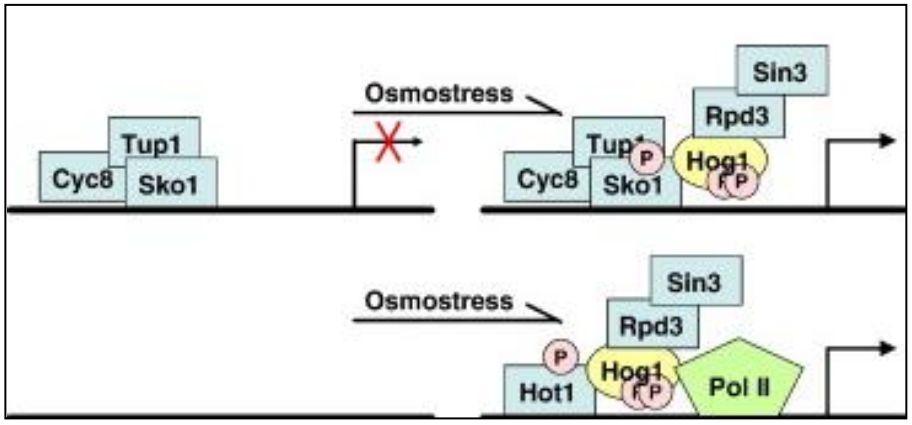

Fig. 4: Inactive Hog1 resides mainly in the cytosol and Pbs2-dependent dual phosphorylation activates Hog1 and promotes its translocation into the nucleus, where it stimulates transcription at some promoters, in part, by binding to and converting a transcriptional repressor, the Sko1–Cyc8/Ssn6–Tup1 complex, into a transcriptional activator, and, in part, by affecting the state of local chromatin modification via recruitment of a specific histone deacetylase, the Sin3–Rpd3 complex. Active Hog1 can also stimulate transcription at other promoters by phosphorylating and binding to a transcriptional activator, such as Hot1, and thereby serving as an adaptor or mediator that also binds to and recruits RNA polymerase II holoenzyme. See the text for additional details. [35]

Smp1: Phosphorylation of Smp1 by Hog1 is required for its activator function.

Sko1: this DNA-binding protein acts as a repressor by binding the Tup1–Ssn6/Cyc8 complex, which, in turn, recruits the ISW2 chromatin-remodeling and nucleosome-positioning complex and several histone deacetylases, including Hda1 and thereby prevents RNA polymerase II access to chromatin. Hog1-mediated phosphorylation of Sko1 somehow converts it into an activator, perhaps by causing it to jettison some or all of its repressive co-factors.

Hot1: the Hog1 action stimulates Hot1 function. Although Hog1 phosphorylates Hot1, this modification is not necessary for the activator function of Hot1; rather, active Hog1 is required for Hot1 binding at some promoters, and it is active Hog1 that serves as an adaptor to recruit RNA polymerase II to the promoter-bound Hog1–Hot1 complex [35].

Curiously, and contrary to the dogma that histone deacetylation is correlated with repression of gene expression, at many osmo-responsive promoters, it has been reported that Hog1 interacts with and thereby recruits another class of histone deacetylase, the Sin3–Rpd3 complex. At these genes, Rpd3 action somehow enhances, rather than prevents, recruitment of RNA polymerase II,

thus promoting osmostress-induced gene expression by a different another mechanism [39] [fig.4].

Two different groups have recently reported that the Hog1 MAPK may function not only as an integral component of several different types of transcriptional initiation complexes, but may also serve as a co-factor for transcriptional elongation, beyond interacting with regulators of transcription initiation [40] [Fig. 5].

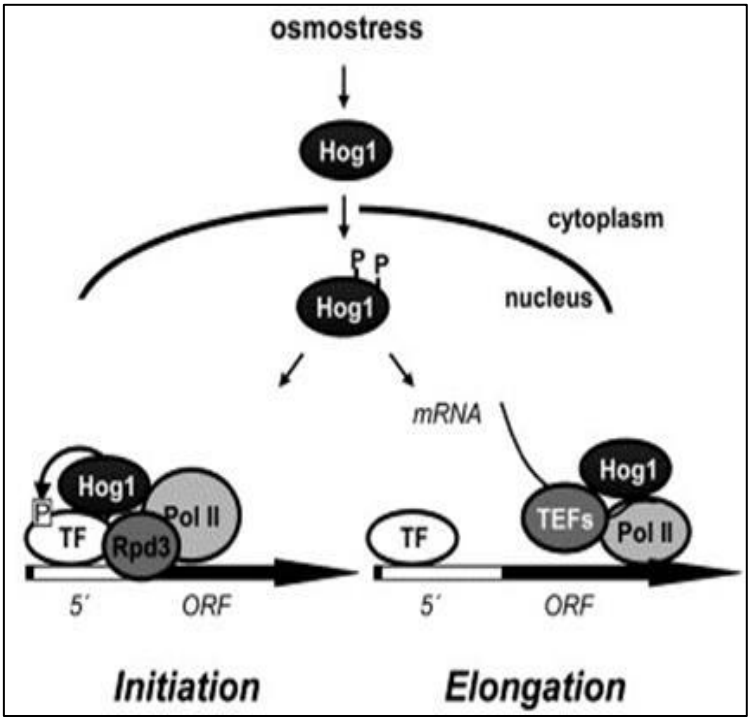

Fig. 5: Schematic diagram depicting the role of Hog1 in the transcription cycle. Upon osmostress, Hog1 is activated and concentrates into the nucleus, where it regulates several aspects of the transcription. There are several mechanisms by which Hog1 modulates transcription initiation: the direct regulation of transcription factor activity, direct stimulation of the recruitment of the Pol II at osmoresponsive promoters, recruitment of the Rpd3 histone deacetylase complex, and modification of chromatin. In addition, Hog1 is also involved in the process of elongation. [40].

2.4 Cell wall integrity (CWI) pathway

The MAPK Slt2/Mpk1 becomes activated under a number of different conditions that affect the structure and function of the yeast cell wall,

including hypotonic medium, treatment of cells with glucanases (e.g. Zymolyase), exposure to chitin-binding agents (e.g. Calcofluor White and Congo Red), as well as oxidative stress, depolarization of the actin cytoskeleton, and pheromone-induced morphogenesis [41]. The genes under control of this response pathway include many involved in the synthesis and modification of the major components of the yeast cell wall (glucans, mannans, and chitin) [42], and lack of an Slt2/Mpk1-dependent response causes cell lysis in the absence of an osmotic support in the medium.

This pathway is controlled by the Pkc1 (Protein Kinase-C-1)-mediated signal. Pkc1 is an essential activator (MAPKKKK) of the MAPK cascade required for CWI signalin: Bck1 (MAPKKK), Mkk1 and Mkk2 (two semi-redundant MAPKKs), and Slt2/Mpk1 (MAPK) [Fig.2]; the MAPKKs and MAPK in this pathway are bound by the scaffold protein Spa2 [43].

Five plasma membrane proteins (each containing a single transmembrane region), Wsc1, Wsc2, Wsc3, Mid2, and Mtl1, have been identified as important for activation of the CWI pathway, although the precise mechanisms by which they sense their direct signals/stressors are unclear. Information is then transduced via the GDP/GTP exchange factor Rom2 to the small GTPase Rho1. The latter, like all small GTPases, is considered active in its GTP-bound and inactive in its GDP-bound state. Sac7 and Lrg1 act as GAPs (GTPase-Activating Protein) for Rho1 and thus function as negative regulators. Further GAP functions have been assigned to Bag7 and Bem2 but are less crucial for Rho1 function. Similar to many other small GTPases, Rho1 has a set of different target proteins in respect to different signals ensuring cellular integrity but the main effectors of Rho1 is Pkc1 [44].

As mentioned, cell integrity pathway responds not only to cell wall damage but also respond to different signals including cell cycle regulation, growth temperature, changes in external osmolarity, and mating pheromone It is thought that the common element sensed in all of these cases is stretching of the plasma membrane and/or alterations of its connections to the cell wall. For this reason the cell integrity pathway is not a single straight cascade but rather a network of interacting signaling routes that diverge from or converge to Pkc1 and the G-protein Rho. Pathways that interact with these central components of the CWI, physically and/or genetically, are: the TORC2 pathway, the Hog pathway, a Phosphatidylinositol pathway,

CDC28-dependent control of the cell cycle, and probably other additional pathways [42] [Fig.6].

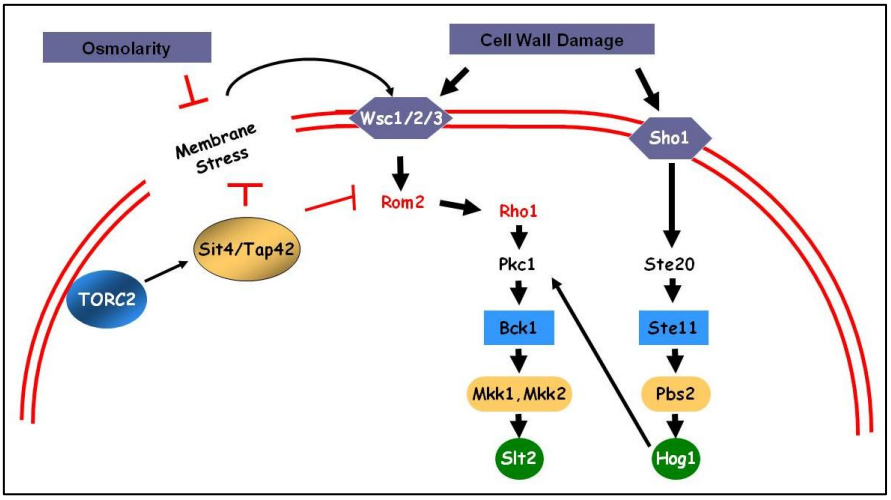

Fig. 6: Cell integrity pathway of *S. cerevisiae*. This pathway is be regulated by several different signals listed at the top: nutrients, temperature, osmolarity, pheromone, and cyclin-dependent kinase (CDK). Where these different upstream signals feed in to the pathway is currently unknown [45].

2.4.1 Regulators of the CWI pathway

Slt2/Mpk1 is responsible for stimulating the expression of some genes codinf for enzymes and other factors involved in cell wall biosynthesis [46]. Slt2/Mpk1 stimulates the expression of cell wall biosynthesis genes via phosphorylation of the transcription factors, Rlm1 and Swi4 [47]. It has also been reported that Slt2/Mpk1 interacts physically with Swi4 (a subunit of the heterodimeric Swi4–Swi6 transcription factor, termed SBF). Moreover, the recruitment of Swi4 to promoters is reduced in strains lacking Slt2/Mpk1, and Slt2/Mpk1 and Swi4 share a set of target genes that are independent of Swi6, including the Pho85/CDK5-specific cyclin, Pcl1, and the 1,3-β-glucan synthase, Gsc2 [48].

Additionally, Slt2/Mpk1 activation is necessary for stimulation of calcium influx through a plasma membrane Ca2+ channel (Cch1–Mid1), a response that, in turn, activates calcineurin (a heterotrimeric enzyme comprising two Ca2+-binding regulatory subunits, Cmd1/calmodulin and Cnb1, associated with either of two semi-redundant catalytic subunits, Cna1 and Cna2) [49]. Activated calcineurin dephosphorylates a transcription factor, Crz1, permitting its retention in the nucleus and thereby its ability to stimulate

expression of genes involved in dealing both with cell wall stress and with ER stress [49]. Finally, another interesting direct substrate of Slt2/Mpk1 is Sir3, a protein required for the maintenance and spreading of heterochromatin. Mutation of the Slt2/Mpk1 phosphorylation site on Sir3 increases yeast lifespan [50]. Sir3 is also reportedly a substrate for Fus3. These observations suggest molecular connections among the sensing of extracellular conditions, gene silencing, and cellular senescence [50].

2.5 Signal specificity and cross-pathway interactions

The MAPK routes often utilize the same molecular components. For example, Sho1, Msb2, Cdc24, Cdc42, Bem1, Ste20, Ste50, Ste11, Ste7, Kss1, and Ste12 are involved utilized in at least two MAPK-mediated signal response pathways [Fig.2 e Fig.7]. Differently to differentiated cells of metazoan where signal fidelity can be maintained by separating alternative downstream targets in yeast is more difficult to maintain specificity. In fact in yeast the potentially confounding factors are all expressed together and are, relatively speaking, readily accessible to each other, as demonstrated by the fact that rather straightforward genetic mutations can give rise to abnormal cross-talk on pathways.

Several general mechanisms have been proposed to explain how undesirable cross-talk between MAPK pathways might be prevented [Fig. 7].

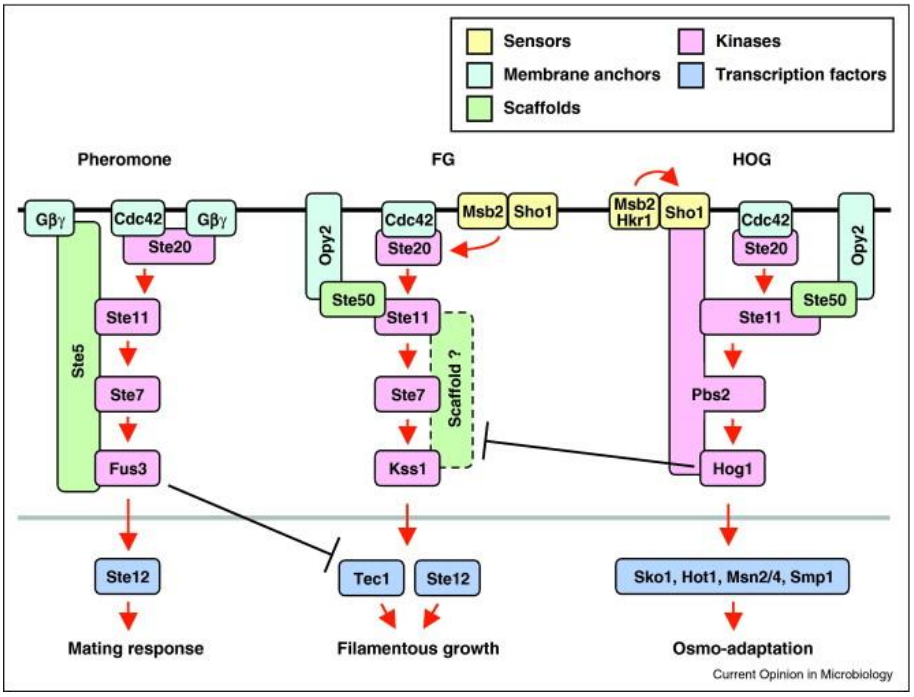

Fig.7: Signal flow through the three MAPK pathways in yeast that share the Ste11 MAPKKK. For details, see the text. Components are color-coded based on their functions (note that Pbs2 is both a kinase and a scaffold). The scaffold protein in the FG pathway is hypothetical. Red arrows indicate signal flow, whereas black T-shaped bars indicate inhibition. Black horizontal line: plasma membrane; gray horizontal line: nuclear membrane [51]

2.5.1 Cross-talk between pheromone and FG MAPK modules

The mating and filamentation pathways have two levels of shared components. The first level includes PAK, MEKK, and MEK, which then activates a specific MAPK (Fus3 versus Kss1). The next downstream components in the hierarchies are the inhibitors Dig1 and Dig2, and the transcription factor Ste12, which are shared between the mating and filamentous pathways.

The reason why pheromones predominantly activate Fus3, but not Kss1, is because the scaffold protein Ste5 tethers Ste11, Ste7, and Fus3 together. Recent studies have shown that the mechanism is not or simple. In fact pheromone activates both Fus3 and Kss1, but with different time courses and dose responses providing an example of the kinetic insulation strategy for cross-talk inhibition [51]. Thus, Kss1 activation is more transient than that of Fus3. Furthermore, Fus3 is activated only when the external pheromone

concentration is above a certain threshold, similar to an on–off switch, whereas Kss1 is activated more in a graded rheostat manner[52]. These differences are due to an interesting characteristic of the Fus3 binding domain (Fus3BD) on Ste5. Indeed Fus3 can bind to this domain only when it is phosphorylated by active Fus3. This interplay underlies the strong ultrasensitivity (step-like dose response) of pheromone-induced Fus3 activation.

By contrast, activation of Kss1 by Ste7 is neither enhanced nor inhibited by Ste5, which explains the graded dose response of pheromone-induced Kss1 activation. In support of this model, Fus3 behaves like Kss1 in the presence of a Ste5 mutant that lacks the Fus3BD region or the Ste5-ms region [52]. Thus, under these conditions, very low concentrations of pheromone actually activates Kss1 more strongly than Fus3, and induce FG-like cell elongation [52], which might help cells to reach distant mating partners.

Although Kss1 activation by pheromone is weaker and more transient than Fus3 activation, these differences cannot fully explain the observed dominance of pheromone-specific gene expression in pheromone-treated cells. Indeed, there is another mechanism that ensures that only the Fus3-dependent pheromone-specific gene expression pattern is realized when both Fus3 and Kss1 are activated. Thus, pheromone-specific gene expression is controlled by a homodimer of the transcription factor Ste12, whereas FG-specific gene expression requires a heterodimer of Ste12 and Tec1. In unstimulated cells, these transcription factors are inhibited by the transcription repressors Dig1/Dig2 as well as by binding to nonactivated Kss1. Activated Fus3 or Kss1 phosphorylates Dig1/Dig2 and relieves their inhibition of the transcription factors. Activated Fus3, but not Kss1, also phosphorylates Tec1, and thereby induces Tec1 ubiquitination and degradation. In this manner, activated Fus3 prevents FG-specific gene expression, which requires the Tec1/Ste12 heterodimer, even if Kss1 is also activated in the same cells.

Expression of the *MSB2* gene is induced under glycosylation-defective and/or nutrient-limiting conditions, thus further enhancing the FG response. The FG signal is then transmitted to the Ste11 → Ste7 MAPK module through a mechanism that involves Sho1, Opy2, Ste50, Cdc42, and Ste20, but, notably, not Ste5 [53].

In this case, only Kss1 is activated, because Kss1 can be activated by Ste7 without help of Ste5, also ensuring that Tec1 is not degraded, and that Ste12/Tec1-dependent FG-specific gene expression is induced.

2.5.2 Cross-talk between pheromone and HOG MAPK modules

As we have seen, activation of the pheromone MAPK module (Ste11 → Ste7 → Fus3) is dependent on the presence of the Ste5 scaffold. In a similar manner, activation of the HOG MAPK module (Ste11 → Pbs2 → Hog1) is dependent on the presence of the Sho1 and Pbs2 co-scaffolds [54]. Furthermore, Ste7 cannot activate Hog1, as the docking sites in Ste7 have no affinity to Hog1. Thus, these two MAPK modules are securely insulated from each other by specific scaffolds and docking interactions, making it unlikely that any cross-pathway inhibition is necessary to prevent inappropriate activation of Fus3 by osmostress or of Hog1 by pheromone. Unexpectedly, however, using fluorescent protein probes it was shown that the HOG and the pheromone pathways are bistable in a single cell, that is, cells respond to only one stimulus even when exposed to both osmostress and pheromone [55]. Furthermore, in *fus3Δ kss1Δ* mutant cells, Hog1 is activated by pheromone. MAPK pathways occurred over a broad range of stimulant concentrations. Thus, insulation by scaffolds and docking interactions, not cross-inhibition, may be sufficient to prevent inappropriate cross-talk between these two MAPK pathways [51].

2.5.3 Cross-talk between HOG and FG MAPK modules

The HOG MAPK module (Ste11 → Pbs2 → Hog1) and the FG MAPK module (Ste11 → Ste7 → Kss1) share only the MAPKKK Ste11. However, the HOG and the FG pathways additionally share many components upstream of Ste11. The current model of HOG pathway activation is that signal transduction is initiated by the redundant osmosensors Hkr1 and Msb2, which are both highly O-glycosylated mucin-like transmembrane proteins [53]. The FG pathway is also initiated by Msb2, but not by Hkr1. In the HOG pathway, the Msb2 or Hkr1 sensor interact with, and activate the membrane protein Sho1, which recruits the Pbs2 MAPKK to the plasma membrane.

However, despite a shared upstream components, there is no significant cross-talk between the two pathways in wild-type cells: osmostress activates

the Kss1 MAPK only very weakly and transiently [56], and glycosylation defects that activate Kss1 do not activate Hog1 [53]. In the absence of Pbs2 or Hog1, however, osmostress robustly activates Kss1, and induces FG-like polarized cell growth [57]. Conversely, activation of Hog1, either by osmostress or by overexpression of Pbs2, inhibits FG responses. However, a membrane-tethered version of Hog1, which cannot enter the nucleus, can prevent cross-talk, implying that a cytoplasmic substrate, rather than a nuclear substrate, is responsible for diversion of the signal from osmostress to Kss1 [58].

Finally HOG MAPK pathway was indeed inhibited when the FG MAPK pathway was activated by glycosylation defects.

Glycosylation defects activate the Ste11 MAPKKK, which can activate, if uninhibited, both the Ste7-Kss1 and the Pbs2-Hog1 kinase cascades. However, only Kss1 appears to be activated because its activation prevents, by the phosphatase Ptp2, the activation of the Hog1. In the absence of Ste7/Ptp2 or Kss1 glycosylation defects activate Hog1, indicating that the FG pathway also cross-inhibits the HOG pathway [53].

Perhaps, both the FG and the HOG MAPK pathways are initially activated to limited extents, but, depending on the intensity and lengt of the stimulus, cross-inhibition in one or the other direction eventually dominates, resulting in activation of only one pathway.

2.5.4 Cross-talk between HOG and CWI MAPK modules

It has been found recently that Slt2/Mpk1 becomes activated in response to hyperosmotic shock in a manner that depends primarily on the O-glycosylated, integral plasma membrane protein Mid2 (rather than on any of the other CWI sensors) [Fig. 2] and also requires activated Hog1. Mid2 is also required for the activation of Slt2/Mpk1 that is observed when the extracellular medium is rapidly acidified (low pH stress), but the role of Hog1 in this process was not explored. Similary to hyperosmotic stress, perturbation of cell wall β-1,3-glucans by digestion with Zymolyase also activates lt2/Mpk1 in a Hog1-dependent manner, but requires Sho1 to do so [59].

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Chapter 3:

Ribosome biogenesis in *Saccharomyces cerevisiae*

The ribosome is a complex molecular machine that is composed of a small 40S and a large 60S subunit. Despite their conserved molecular function, eukaryotic and prokaryotic ribosomal subunits differ significantly in size and complexity (*S.cerevisiae*: 40S [18S rRNA, 33 RPs]; 60S [25S, 5.8S, 5S rRNA, 46 RPs]–*Escherichia coli*: 30S [16S rRNA, 21 RPs]; 50S [23S, 5S rRNA, 34 RPs]) [1].

In all growing cells, ribosome biogenesis is a major energy-consuming process that accounts for a significant fraction of total transcriptional output. Ribosome biogenesis faces the challenge to coordinate the processing and modification of ribosomal RNA (rRNA) with its correct structural assembly with the assist of a large number of non-ribosomal factors (> 200) and snoRNAs with ribosomal proteins (RP) [2]. Furthermore this process has to be regulated according to the cellular environment (see below, Warner 1999), hence ribosome biogenesis is tightly coupled to growth rate: actively dividing cells, including cancer cells, depend on active ribosome biogenesis, whereas arrested or starving cells halt the production of new ribosomal subunits.

Due to its easy experimental accessibility by genetic, biochemical, and cellulers biological methods, *S. cerevisiae* represents a suitable eukaryotic model organism to study ribosome biogenesis and assembly.

If cell size depends on some aspects of ribosome synthesis, it is important to consider what features distinguish the control of ribosome synthesis from other control pathways:

i) Ribosome synthesis requires the coordinated activities of all three RNA polymerases: Pol I for rRNA, Pol II for the RP genes, and Pol III for 5S RNA.

ii) Quantity matters. The cell needs precisely equimolar amounts of rRNA and each of the 79 RPs. Excess of rRNA will not be properly assembled into a ribosome with insufficient RPs. Insufficiency of any one of the 79 RPs will, in most cases, lead to aberrant processing and insufficient ribosomal subunits.

iii) The processing of rRNA and the assembly of ribosomes requires an army of nearly 200 proteins[4], whose synthesis is regulated in tandem with, but not identically to, that of the RPs (the ribi regulon) [5,6].

3.1 The rRNA processing pathway

Nucleoli, the sites of ribosome biogenesis, form around clusters of rRNA gene repeats at the nucleolar organiser regions (NORs) on one or more chromosomes moreover, their existence depends on active rRNA gene transcription (Nomura 2001). The 18S, 5.8S and 28S rRNAs, the scaffold and catalytic heart of the eukaryotic ribosome [8], are transcribed as a single pre-rRNA by the dedicated RNA PolI.

The first detectable intermediate (35S in yeast) contains 5′ and 3′ external transcribed region (ETS) as well as the mature 18S rRNA, 5.8S and 25S rRNA interspersed with non coding sequences ITS1 and ITS2. The 18S rRNA will be the rRNA component of the small 40S subunit whereas the 5.8S and 25S rRNA together with the 5S rRNA, synthesized independently by RNA PolIII, will constitute the RNA component of the 60S subunit.

The 35S pre-rRNA is successively cleaved in the 5′-ETS at site A0 (generating the 33S pre-rRNA), at site A1, the 5′ end of the mature 18S rRNA (generating the 32S pre-rRNA) and at site A2 in ITS1 (generating the 20S and 27SA2 pre-rRNAs) [Fig. 1].

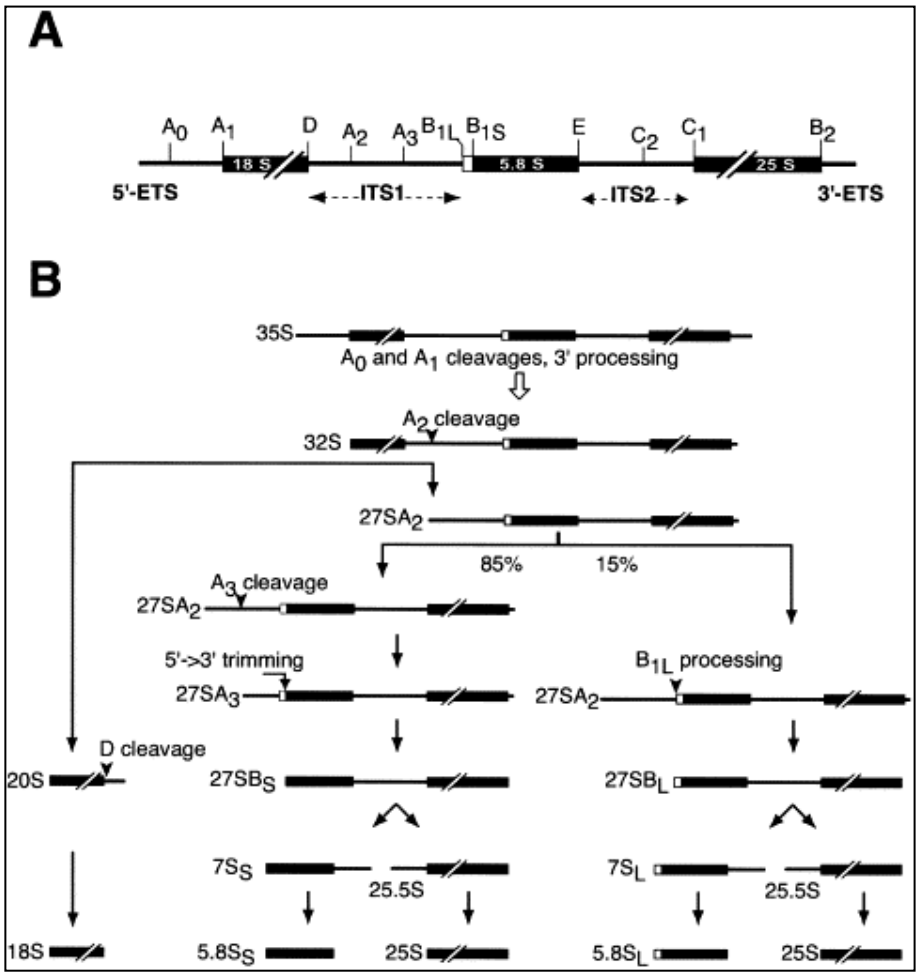

Fig. 1. Pre-RNA processing in *S. cerevisiae*. (A) Structure of the pre-rRNA 35S containing the mature rRNA, 18S, 5.8S and 25S. (B) Schematic representation of the rRNA processing pathway described in details in the text. [9]

Further processing steps of the 20S rRNA occur in the cytoplasm by cleavage at site D to generate the mature 18S rRNA whereas processing of the 27SA2 pre-rRNAs continues in the nucleus. The 27SA2 is matured to the 5.8S and 25S rRNAs by two alternative pathways: about 85% of the 27SA2 population is cleaved at site A3 in ITS1 by RNase MRP, rapidly followed by 5′ to 3′ trimming to site B1S by Rat1; 15% of 27SA2 is cleaved directly at site B1L. Cleavage at site B2, at the 3′end of the 25S, occurs concomitantly with cleavage at site B1. The two forms of 27SB (27SBS and 27SBL) are

matured following identical pathways involving processing at sites C2→C1 and 3′ to 5′ exonucleolytic digestion to site E by the exosome complex [9].

3.1.1 Gene activation

Since, the rRNA genes form a key component of the signaling network controlling cell growth and proliferation, the growth regulation of the rRNA genes is rapid [5]. Most eukaryotes contain a hundred or more chromosomal rRNA genes arranged in one or more tandem repeats. The rRNA gene promoter in mammals, amphibia, and yeasts consists of around 150 bp of DNA containing two sequence elements, the upstream control element (UCE or UE) and the core [10].

The upstream element is required for a high level of transcription, but is dispensable for basal transcription, whereas the core promoter is essential for accurate transcription initiation (4–6). Initiation of yeast rDNA transcription by Pol I uses four factors in addition to Pol I: upstream activating factor (UAF), associated with the TATA-box binding protein (TBP), and core factor (CF) [7] [Fig.2]

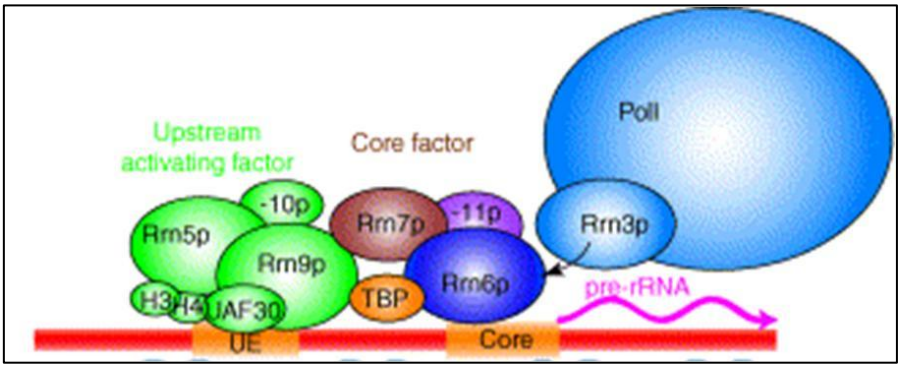

Fig. 2. The PolI initiation complex. in yeast and: The various polypeptides that have been associated with UAF, CF, SL1 and PolI are indicated as ellipsoids. The individual HMG1boxes of UBF are also shown as ellipsoids. Common colours indicate potential homologs among yeast and mammalian TAFs. [10]

UAF is a multiprotein transcription factor containing three Pol I-specific proteins, Rrn5, Rrn9, and Rrn10, histones H3 and H4, and uncharacterized protein p30, while the CF consists of Rrn6, 7, and 11. Assembly of a preinitiation complex in yeast starts with the recruitment of the UAF to the UE. Subsequently, the CF and TBP and finally the polymerase are recruited. TBP is probably able to provide a bridge between the UAF and CF

complexes, and it is tempting to suggest that this bridging might occur via a TBP dimerization. CF is released from the promoter at each round of initiation. Mutations that inactivate PolI also prevent CF recruitment, suggesting that these factors may normally be recruited together . Thus, it is the UAF and the upstream promoter element that are required for stable promoter commitment in yeast [11].

In fact the genes encoding Pol I-specific subunits of *UAF*, *RRN5*, *RRN9*, and *RRN10*, are not absolutely required for cell growth [12]. In each mutant two alternative reversible states exist for rDNA transcription: one favoring transcription by Pol II (the polymerase-switched state or PSW) and the other suitable for Pol I transcription (non-PSW). The presence of UAF in normal cells appears to stabilizes the second state, thus achieving a stringent silencing of rDNA transcription by Pol II.

3.1.2 Regulation by Silencing

Regulation of rRNA gene transcription could then logically occur by modulating the activity of the transcription machinery, by changing the number of active genes or both. Differential accessibility of the rRNA genes to the DNA crosslinker psoralen led to the surprising conclusion that, in both higher and lower eukaryotes, no more than 50% of chromosomal ribosomal genes are active at any given time [13]. In stationary phase, yeast reduces pre-rRNA synthesis by 10 or more times. Concomitantly it also reduces the proportion of its rRNA genes that are actively transcribed [14].

Modification of chromatin has become a key theme in our understanding of gene regulation. Thus, it was not surprising to find that loss of histone acetylation, in this case at lysines (K) -5 and -12 of H4, was correlated with yeast rRNA gene inactivation. These data implicate the histone deacetylase Rpd3 (homolog of HDAC1,2) and perhaps the opposing acetyl-transferase Esa1 (TIP60) [15]. However, although Rpd3 deacetylase is responsible for rRNA gene inactivation in the stationary growth phase in yeast, loss of Rpd3 does not lead to the re-activation of the 50% of silent genes (Sandmeier 2002). Thus, at least two distinct states of rRNA gene inactivation exist in yeast, a facultative one mediated by Rpd3 and a constitutive one of unknown origin. Only a long-term constitutive silencing of the rRNA genes, referred to nucleolar dominance, has been recognised in higher eukaryotes. In these cases, silencing is of a complete NOR, but whether this type of constitutive silencing is responsible for maintaining 50% of the rRNA genes inactive at

any given moment is still far from being clear. In some cases, DNA methylation has been suggested to be responsible, but in other cases methylation *per se* is not the answer [16].

Gene silencing at the yeast NOR refers not to the inactivation of rRNA genes but rather to the suppression of recombination, to the inactivation of PolII genes transposed into the NOR and to the inhibition of PolII transcription of the rRNA genes themselves [17]. PolII silencing requires the NAD-dependent deacetylase Sir2, the PolI promoter binding factor UAF [Fig. 3] and is unidirectionally spread along the *locus* by active PolI transcription (Buck *et al.* 2002; Cioci *et al.* 2003). Sir2, a regulator of aging and part of the nucleolar RENT complex bound to PolI [20], is responsible for deacetylating K16 of H4 and K9,14 of H3 at the NOR [20], events all that are essential for heterochromatin formation in eukaryotes.

3.2 Ribi an RB regulon

In yeast, the 137 RP genes as well as the large set of Ribi genes (200 protein-encoding genes plus 75 small nucleolar RNA genes) that arerequired for ribosome biogenesis and/or translation [6] display very similar mRNA profiles in response to nutrient and stress conditions [5,6]. Regulation of RP and Ribi gene expression has been best studied in *Saccharomyces cerevisiae,* where several proteins have been implicated in RP gene activation, including Forkhead-like 1 (Fhl1), Rap1, Sch9, and Sfp1 [6,21-23] Among these factors, the AGC kinase Sch9,(Urban *et al.* 2007), and the zinc (Zn)-finger protein Sfp1 also appear to be intimately involved in the activation of Ribi genes. Epistasis analysis suggests that the two proteins act in parallel (Jorgensen *et al.* 2004).
In addition to Sch9 and Sfp1, RP and RIBI gene expression depends on several other transcription factors and chromatin modifiers (Jorgensen *et al.* 2004) [Fig.4].

3.2.1 Role of the Forkhead Transcription Factor 1 (FHL1)

A central transcription factor driving RP genes is Fhl1 [23], In addition to Fhl1 RP transcription, involves two other proteins, Ifh1 and Crf1, which turn out to act as a coactivator and a corepressor of Fhl1, respectively. Thus, in

growing cells Ifh1 binds to Fhl1 at RP gene promoters to activate gene expression [Fig.3]. By contrast, in stationary cells, Crf1 displaces Ifh1 and binds instead to Fhl1, forming a Crf1-Fhl1 complex that is unable to support transcription. The physical basis for mutually exclusive binding of Fhl1 to either Ifh1 or Crf1 is due to the presence of a common binding domain within the latter two proteins, termed a Forkhead binding domain (FHB), which competes for binding to a specific region of Fhl1. In summary, Fhl1 binds constitutively to RP gene promoters and acts as either an activator or repressor of transcription, depending on whether it is associated with Ifh1 or Crf1.

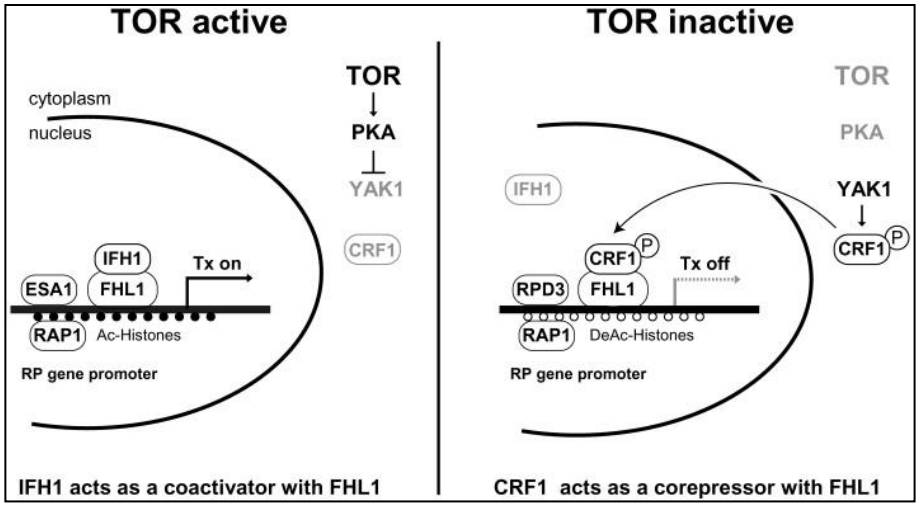

Fig. 3. Model of RP Gene Regulation by TOR and the Fhl1. See Discussion for details.[23]

Crf1 represents the crucial point of regulation in this system indeed in growing cells Crf1 is dephosphorylated and sequestered in the cytoplasm where it is unable to interact with Fhl1; following TORC1 inactivation, Yak1 phosphorylates Crf1 that enters in the nucleus displacing Ifh1 from Fhl1 (Martin *et al.*, 2004). (Martin *et al.*, 2004). Several studies have linked Yak1 to both TOR and ras-cAMP-PKA signaling. Thus, in total, an entire pathway has been outlined that links nutrients to Crf1 regulation via a TOR/PKA/YAK1 cascade (Martin *et al.*, 2004).

3.2.2 Role of Rap1

For a long time Rap1 has been an intriguing protein because of the number and variety of its roles in the cell, from coating telomeres to silencing the MAT loci and to activating both glycolytic and RP genes (perhaps 50% of the Pol II transcripts of the cell). There is an ample evidence that Rap1 acts by clearing nucleosomes from a region of chromatin, this alteration of the structure of the DNA by Rap1 is a prerequisite for recruiting Fhl1p and Ifh1 [23]

Although a recent bioinformatic study has implicated two motifs as characteristics of genes regulated as RP genes are [24], these motifs are rather degenerate and seem not to be universal among the RP genes. Indeed, minor changes in the sequence context of the Rap1 sites can lead to drastic changes both in the occupancy by Ifh1p as measured by ChIP and in the level of transcription of the adjacent gene.

3.2.3 Role of Sch9

One major role of Sch9 is to regulate translation in function of nutrient availability and the growth potential. As such, Sch9 controls the expression of RP genes and the Ribi regulon, by interfering with the transcriptional processes conducted by the RNA Pol I, II and III [21,25,26]. Concerning RNA Pol I transcription, Sch9 is required to maintain the optimal activity of the polymerase, presumably by promoting the recruitment of the catalytic subunit Rpa190 to the rDNA locus. In addition, Sch9 is essential for the proper processing of the 35S transcript into the 25S, 18S and 5.8S rRNA and at least of one component of the processome, (Rps6), was shown to be phosphorylated by Sch9. The latter led to the conclusion that Sch9 should be considered as the orthologue of the mammalian kinase S6K1, rather than being the yeast counterpart of PKB [25,26]. Probably, Sch9 may still combine the functions of S6K and PKB and thus represent the ancestor from which both kinases have evolved. For processes mediated by the RNA Pol II, Sch9 phosphorylates and inhibits the activity of the transcriptional repressors Stb3 and Dot6/Tod6, which, respectively, bind the *RRPE* and *PAC* elements in the promoters of ribi genes (Huber *et al.* 2009). For RNA Pol III-dependent transcription, the downstream target of Sch9 is Maf1, the repressor that is also regulated by PKA and TORC1 [Fig.4].

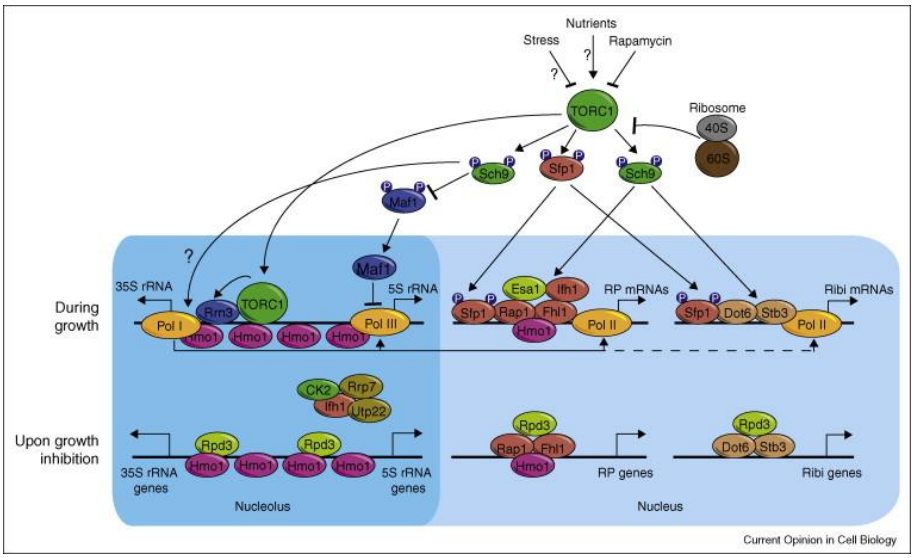

Fig. 4. Regulatory network from TORC1 to ribosomal gene expression in *S. cerevisiae*. Major transcriptional regulators of rRNA, RP, and Ribi gene expression, and their regulation by the nutrient-sensitive and stress-sensitive TORC1 kinase under optimal growth conditions are indicated (upper panel). In addition, the RNA Pol I-dependent regulation of RNA Pol II and III activities is highlighted. Lower panel shows the promoter architecture under growth inhibiting conditions. The Ifh1 containing 'CURI' (CK2, Utp22, Rrp7, and Ifh1) complex, presumably localized to the nucleolus, is shown. Also the feedback mechanism from ribosome function to the regulation of TORC1 activity is illustrated. For simplification, not all pathway components and connections are illustrated. Question marks indicate signaling connections that are poorly understood. For further details see the main text.(Lempiäinen and Shore 2009)

3.3 The Split finger protein 1 (Sfp1)

The split zinc-finger protein Sfp1 is a key transcriptional regulator of ribosome biogenesis (Cipollina, *et al.* 2008a; Jorgensen *et al.* 2004; Marion *et al.* 2004; Lempiäinen, Uotila, *et al.* 2009) that function is required for normal yeast growth.

SFP1 gene encodes a protein that is a unique member of the Cys_2His_2 zinc-finger family of DNA-binding proteins because its two zinc-finger domains are separated from one another by 40 amino acids (aa) as opposed to the usual spacing of 7 or 8 aa for Cys_2His_2 proteins [28]. Sfp1 was initially identified in a screen for genes that altered import of nuclear proteins when present on high-copy-number plasmids [28]. Overexpression of *SFP1* was found to result in the mislocalization of several endogenous nucleolar proteins, although the null mutant did not appear to be altered in nuclear

import or protein localization [28]. These results suggested that Sfp1 played some uncharacterized role in nuclear localization. Then other data indicated that Sfp1 could be a negative regulator of the G2/M transition during the normal cell cycle after DNA damage [29]. In fact *sfp1Δ* cells did not arrest at the G2/M border after treatment with methyl methan sulphonate (MMS) and the overexpression of *SFP1*, caused the accumulation of budded cells with a G2 DNA content [29] differntly to the *sfp1Δ* cells that had a short length of G2/M transition.

Additionally, *sfp1Δ* cells were found to be more sensitive to ionizing radiation and alkylating agents than wild type, consistent with the presence of a defect in DNA repair. Finally, *sfp1* mutant cells were significantly smaller than wild-type cells and showed a significant defect in their growth rate [29]. This phenotype was further detected in a screen for mutations that affected critical cell size at Start [21].Its characterization indicated that Sfp1 plays a key role in yeast ribosome biogenesis.

The role of Sfp1 as a transcriptional activator of genes involved in ribosome biogenesis explained most of the phenotypes previously described. It was shown that *SFP1* overexpression interfered with proper nuclear localization of a mitochondrial protein containing a nuclear localization sequence [28]. The high levels of Sfp1 in the cell likely stimulated ribosome biogenesis. Since ribosomal subunits and r-proteins are continuously shuttled in and out of the nucleus, abnormally high levels of ribosomal subunit trafficking may interfere with the localization of other nuclear proteins or prevent complete assembly of ribosomal subunits. This model is supported by the finding that the *sfp1Δ* mutant showed no defect in localization of nuclear proteins [28]. The role of Sfp1 in transcriptional regulation of ribosome biogenesis genes may also explain the DNA damage and checkpoint phenotype previously observed in an *sfp1* mutant strain [29]. The inability of *sfp1Δ* cells to arrest at the G2/M checkpoint may be due to the inability of the cell to properly synthesize the proteins required to respond to DNA damage. Furthermore, the induction of *SFP1* transcription in response to treatment with MMS may be linked to the induction of stress response proteins. Since a large amount of proteins must be present to respond to DNA damage, Sfp1 may stimulate ribosome biogenesis in order to synthesize the proteins needed to respond to such a stress. Alternatively, it is possible that this can be the result of an adaptative response. *SFP1* expression was shown to be induced after prolonged exposure to MMS. Since many cellular stresses cause reduction in

the ribosome biogenesis, over time this process is likely to be restored as the cells adapt to these stresses.

3.3.1 Regulation of Sfp1

Sfp1 function is regulated, at least in part, at the level of subcellular localization [Fig.4].

In glucose medium, Sfp1 is localized in the nucleus where it promotes both RP and Ribi gene expression, but upon nutrient limitation or exposure to various stresses, Sfp1 relocalizes to the cytoplasm within few minutes (Jorgensen *et al.* 2004; Marion *et al.* 2004). Nutrient-responsive localization of Sfp1 depends on TOR and PKA signaling (Jorgensen *et al.* 2004; Marion *et al.* 2004).). A recent study (Lempiäinen, *et al.* 2009) showed that Sfp1 interacts directly with and is phosphorylated by TORC1. In contrast to Sch9 kinase, a major downstream target of TORC1 (Urban *et al.* 2007), TORC1 phosphorylation of Sfp1 is unaffected by either osmotic or nutritional stresses, suggesting a different mode of regulation. Significantly, Sfp1, through its transcriptional activator function, exerts a negative feedback control on TORC1's activity toward Sch9 kinase (Lempiäinen, *et al.* 2009). Sfp1 also interacts with Mrs6, a conserved Rab escort protein that regulates Sfp1 nuclear localization (Lempiäinen, *et al.* 2009; Singh *et al.* 2009). The Mrs6 interaction with Sfp1 and TORC1 may be related to a still poorly understood connection between TOR signaling and vesicle transport revealed by genetic studies [33,34].

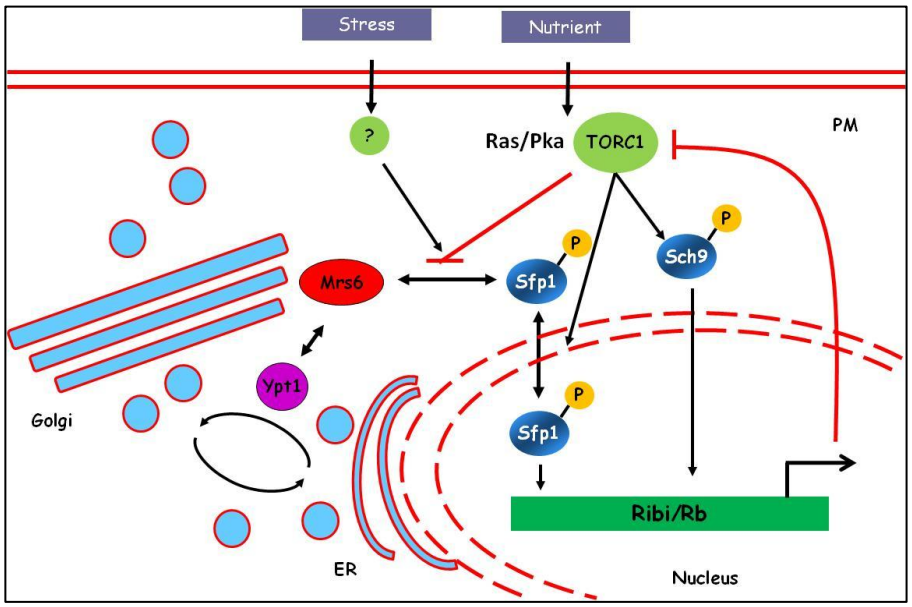

Fig. 4. Schematic model of connections between TORC1, Sfp1, Sch9, and Mrs6 signaling. Competition between Sfp1 and Rab GTPases for Mrs6 may couple activity of the secretory system to ribosome biogenesis. (ER) Endoplasmic reticulum; (PM) plasma membrane

3.3.2 Regulation of RiBi and RP genes by Sfp1

Sfp1 controls a large cohort of >200 genes implicated in the complex pathway that assembles mature ribosomes, termed the Ribi regulon; Sfp1 also directly or indirectly activates the RP regulon (Jorgensen *et al.* 2002).

The involvement of Sfp1 in transcriptional regulation of RiBi and RP genes has so far been a controversial issue. Most of the data derived from shake-flask cultures indicated that during growth on glucose, Sfp1 is involved in transcriptional regulation of both RiBi and RP genes (Jorgensen *et al.*, 2004; Marion *et al.*, 2004). The former gene cluster is characterized by the presence of RRPE and PAC elements in the promoter regions. The latter is particularly enriched for the promoter elements recognized by the transcription factors Rap1 and Fhl1. All attempts to demonstrate a physical interaction between Sfp1 and the RiBi gene promoters have failed, while some weak interactions have been observed between Sfp1 and the promoters of some RP genes [6,21,22,35,36]. Recently it has been shown that the protein Stb3 binds the RRPE elements *in vivo* and is required for the proper regulation of RRPE-containing genes in response to glucose [37]. It has been suggested that Sfp1 acts upstream of Stb3 in modulating the transcription of

RiBi genes (Cipollina *et al.* 2008a; Cipollina *et al.* 2008b). Moreover, it has been proposed that the main target of Sfp1 is the RiBi cluster, and that the downregulation of RP genes observed in the *sfp1Δ* mutant during growth might be a secondary effect. Since the transcriptional regulation of RP and RiBi genes has been shown to be tightly co-ordinated (Griffioen *et al.* 1994), thus the down regulation of RP genes observed in the mutant is due to the adjustment of RP expression levels to the low expression of RiBi and RNA Pol I genes. In fact, consequently to the deletion of *SFP1* the nucleolar relocalization of Fhl1 and Ifh necessary for the repression of RP gene synthesis was observed [41]. This relocalization can be due both directly to the lack of Sfp1 and also indirectly to the reduced expression of the RIBI regulon because of the lack of Sfp1.

3.3.3 Sfp1 linking growth metabolism and cell size

Sfp1 is a regulator of cell size at Start and a key element in the connection between growth and cell cycle progression. In fact besides its role in ribosome biogenesis, Sfp1 is involved in the modulation of cell-size setting. Cell-size setting is well known to be strictly dependent on growth rate. Indeed cells growing slowly on a poor medium pass Start at a smaller size than fast-growing cells on a rich medium (Carter and Jagadish, 1978; Jagadish and Carter, 1977; Vanoni *et al.*, 1983).

Budding yeast mutants that provided key insights into the size control mechanism fall into two distinct groups: large (lge) and small (whi) mutants (Jorgensen *et al.*, 2002). The former undergo Start at a larger cell size and the latter at a smaller one. Interestingly, the deletion of *SFP1* results to one of the smallest *whi* mutants, displaying a disproportionate effect on size relative to the change in growth rate (Jorgensen *et al.*, 2002); *sfp1Δ* cells are much smaller than other deletion mutants with the same proliferation rate (Jorgensen *et al.*, 2002). Conversely, increasing *SFP1* expression level causes a *lge* phenotype and slow growth (Jorgensen *et al.*, 2002, Vai unpublished data). Moreover the reduction in cell size of the mutant is tightly linked to the cellular metabolism.

It has been proposed that Sfp1 may have a role in cell-size modulation during growth on fermentable carbon sources (glucose) while being localized in the nucleus. During growth on non-fermentable carbon sources (ethanol or glycerol) the localization of Sfp1 is mostly cytoplasmic and its role in cell size regulation is less apparent (Cipollina *et al.*, 2005; Jorgensen

et al., 2004; Marion *et al.*, 2004). These findings are in line with ribosome biogenesis being a central element in the nutrient-dependent control of cell cycle progression and size setting (Jorgensen *et al.*, 2004).

This evidence on the possible function of *SFP1* in the regulation of cell size principally derived from the comparison of *sfp1Δ* with the isogenic reference strain growing in shake flask batch cultures (Cipollina *et al.*, 2005; Fingerman *et al.*, 2003; Jorgensen *et al.*, 2002, 2004). In this condition, the slow-growing phenotype of an *sfp1Δ* mutant during growth in shake flasks 'per se' generates a number of effects that are difficult to uncouple from those directly generated by the absence of *SFP1*. In fact transcriptional activity of a cell, ribosome biogenesis, cell size control and cell cycle progression are dependent on the specific growth rate (Regenberg *et al.*, 2006). Since a regulatory role for Sfp1 has been suggested for all these processes (Cipollina *et al.*, 2005; Fingerman *et al.*, 2003; Jorgensen *et al.*, 2002, 2004) the study of a slow-growing *sfp1* mutant could lead to misleading conclusions. Thus the role of *SFP1* has been studied by growing the *sfp1Δ* mutant and the isogenic reference strain in continuous cultures under different carbon limitations where the specific growth rate was equal for both strains. These studies revealed that the role of *SFP1* in the cell size is independent of the growth rate, the supplied carbon source and the metabolism of the cell. Conversely, the involvement of Sfp1 in RiBi transcriptional regulation appears to be strongly related to the carbon source and the growth regime (Cipollina, *et al.* 2008a; Cipollina *et al.* 2008b). This suggests that the control of size setting might act through a different pathway compared to the transcriptional control of the RiBi cluster that is promoted by Sfp1 mainly during glucose-limited growth (Cipollina *et al.* 2008b).

Then Sfp1 may act at two regulatory levels by modulating ribosome biogenesis and size setting. This is further supported by the observation that the presence of defects in ribosome biogenesis are not enough to explain the generation of a *whi* phenotype (Rudra *et al.*, 2005). Whether the setting of cell size is a readout of the growth events or it is dictated upstream by ribosome biogenesis still remains an open question [21].

In addition, always by studying the role of *SFP1* in continuous cultures, it has been shown that the deletion of *SFP1* affects not only the induction of RiBi genes but also glycolytic activity in response to glucose (Cipollina *et al.* 2008a) further underlining Sfp1 involvement in the network that links ribosome biogenesis and cell metabolism.

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Chapter 4:
Cell Cycle and Cell Growth

The coordinated production of new cells (cell cycle) and growth (the increase in biomass of individual cells) is essential for both development of multicellular organisms and fitness of microorganisms. In order to maintain long-term size homeostasis, on average, cells must double in size before they divide. In most organisms, this is accomplished by establishing a dependency on growth for cell cycle entry. Cells typically initiate a new cell cycle in late G1 phase only once a critical cell size, a function of protein synthesis rate, is achieved [1,2] At least in simpler eukaryotes, nutrient conditions determine both growth rate and the critical size threshold, so that slower growing cells are smaller than faster growing cells [3]. In the budding yeast *S.s cerevisiae*, coordination of division with growth occurs at Start, where cells must reach a critical cell size to enter a new cell cycle [1,4].

4.1 Cell Cycle

The cell cycle can be defined as the period between division of a mother cell and subsequent division of its daughter progeny. The regulatory mechanisms that order and coordinate the progress on the cell cycle have been intensely studied [5]. The eukaryotic cell cycle involves both continuous events (cell growth) and periodic events (DNA synthesis and mitosis). The periodic events can be divided into four phases: DNA synthesis (S phase); a post-synthetic gap (G2 phase); mitosis (M phase); and a pre-synthetic gap (G1 phase). Downstream events are driven by the cell-cycle engine, but they do not contribute directly to engine function. These include budding and morphogenesis, spindle pol body (SPB) duplication, DNA replication, mitotic spindle formation, orientation and elongation, and activation of the mitotic exit network (MEN). Numerous proteins that have been characterized through a characterization of mutants are the cell division cycle (Cdc) proteins; among these an important role during cell cycle are played by cyclins.

4.1.1 Cyclin roles during the cell cycle

Cyclin-dependent kinases (Cdks) control progression of cell cycle in all eukaryotic cell. They are serine and threonine kinases, and their activity are dependent on associations with their activating subunits, cyclins. Cyclin abundance is regulated by protein synthesis and degradation; the activity of Cdks is therefore regulated to a large degree by the presence of different cyclins. In *S. cerevisiae*, a single Cdk, Cdc28 (which is equivalent to Cdk1 in other organisms), associates with different cyclins to regulate the cell cycle. By contrast, mamalians cells possess multiple cell-cycle-regulatory Cdks, which are each regulated by different cyclins. The rationale for possessing different cyclins is not fully understood; probably different cyclins are advantageous because they allow for flexible control of the cell cycle. Different cyclins are independently regulated transcriptionally and post-transcriptionally, providing regulatory flexibility at the input level. Also, cyclins possess overlapping, but distinct, functional activities, allowing further refinement of control.

Cyclin specificity can be deduced from genetic requirements for a specific subset of cyclins for a cell-cycle event to occur [Fig. 1].

For example, advancement through G1 phase of the cell cycle, which involves bud emergence, SPB duplication and the activation of subsequently expressed cyclins, requires at least one of the G1-phase cyclins — Cln1-3. In the absence of all *CLN1–3*, G1 arrest occurs. Following Cln activities, efficient initiation of DNA replication and progression through S phase requires the early-expressed B-type cyclins Clb5 and Clb6. In their absence, the B-type cyclins Clb1-4 will drive a late initiation of DNA replication. These cyclins are required for mitotic events, such as spindle morphogenesis, and they also prevent mitotic exit and cytokinesis. Therefore, mitotic cyclin activity must be downregulated for cell division to be completed.

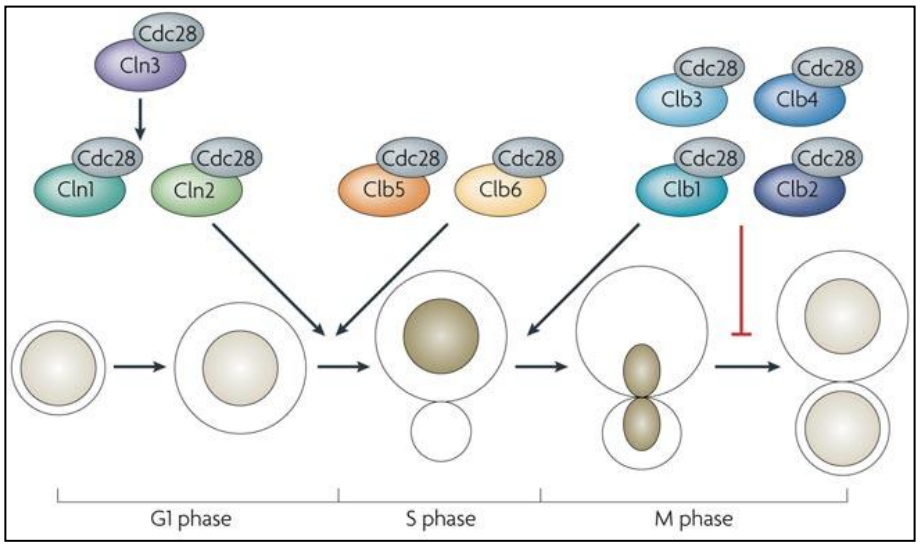

Fig. 1: Budding yeast cyclins activate a single cyclin-dependent kinase (Cdc28). The G1-phase cyclins (Cln1, Cln2 and Cln3) promote bud emergence, spindle pole body duplication (not shown) and activation of the B-type cyclins. The S-phase cyclins (Clb5, Clb6) advance DNA replication (shaded nucleus), and the M-phase cyclins (Clb1, Clb2, Clb3 and Clb4) promote spindle formation and the initiation of mitosis. Mitotic cyclins inhibit mitotic exit and cell division. Following cytokinesis, a mother and daughter cell are generated. [6]

Mechanisms that contribute to cyclin specificity in the yeast cell-cycle engine include the differential transcriptional activation of cyclins, their degradation, the cyclins localization and the association of cyclin–Cdk complexes with different Cdk inhibitors,

Transcriptional regulation of cyclins. A crucial mechanism for cyclin specificity is the differential regulation of G1 and B-type cyclins at the level of transcription during the cell cycle. Transcription of the *CLN3* gene is detectable throughout all the cell cycle, but peaks in late M–early G1, whereas the transcription of the *CLN1*,*CLN2*, *CLB5* and *CLB6* genes peaks during G1–S, followed by the transcription of the *CLB1-4* genes. Transcription of early-expressed cyclins is largely controlled by the heterodimeric transcription factor SBF and the related MBF transcription factor. SBF and MBF are composed of related DNA-binding proteins Swi4 and Mbp1, respectively, which interact with a common regulatory subunit Swi6 to drive expression of a massive suite of ~200 genes. *CLN1* and *CLN2* expression has been primarily attributed to SBF, whereas the transcriptional activation of the *CLB5* and *CLB6* genes is primarily attributed to MBF.

However, recent evidence indicates that there is a high degree of overlapping for these transcription factors in gene activation [7].

After Start, Clb2 can specifically inactivate SBF-mediated gene expression, which correlates with the ability of Clb2 to bind to Swi4 [8]. Clb2 also positively regulates its own expression. The transcription factor Mcm1 recruits the forkhead transcription factor Fkh2 and the co-activator Ndd1 to regulate the expression of *CLB2*. Clb2–Cdc28 phosphorylates Ndd1, which is important for its recruitment to the *CLB2* gene promoter and phosphorylates Fkh2, which enhances the interaction of Fkh2 with Ndd1. Transcriptional control of *CLB2* is highly specific because Clb2 is probably specialized in Ndd1 phosphorylation and because *CLB2* is also a specific cyclin target of this circuit [Fig. 2].

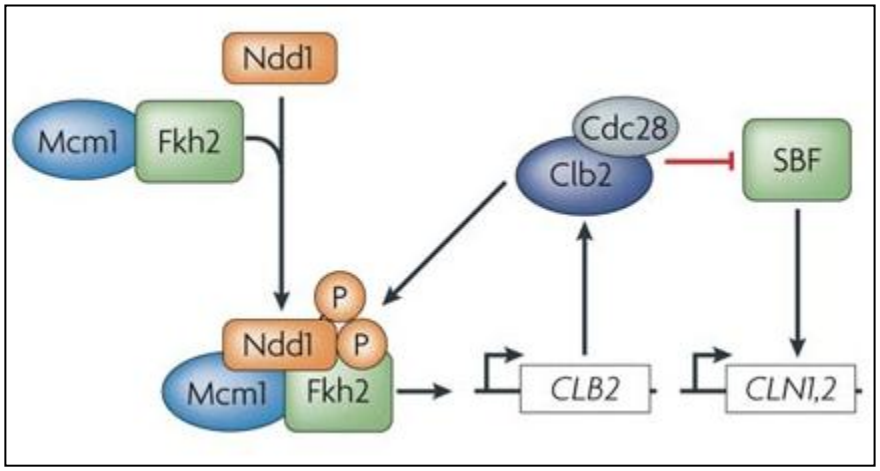

Fig.2: The co-activator Ndd1 recruits minichromosome maintenance-1 (Mcm1) and the transcription factor Fkh2 for the activation of the gene that encodes Clb2. Phosphorylation of Ndd1 and Fkh2 by Clb2–Cdc28 promotes Ndd1-dependent recruitment of Mcm1–Fkh2 to the promoter of CLB2. Clb2–Cdc28 also phosphorylates and inhibits SBF to repress the transcription of G1-phase cyclins. P, phosphate. [6]

Degradation of cyclins. Cyclins are also regulated at the level of protein degradation, by ubiquitin-mediated proteolysis. Proteins tagged with a chain of ubiquitin molecules are degraded by the 26S proteasome. The sensitivity of different cyclins to different ubiquitin ligases constitutes an important mechanism for cyclin specificity in controlling the cell-cycle. The ubiquitination and degradation of Cln1 and Cln2 is mediated by an SCF complex that contains the F-box protein Grr1 (SCFGrr1) [9]. Clb6 is the only B-type cyclin that has been shown to be degraded in an SCF-dependent

manner in yeast; it is degraded earlier than Clb5 by an SCF complex that contains the F-box protein Cdc4 (SCFCdc4) [Fig. 3].

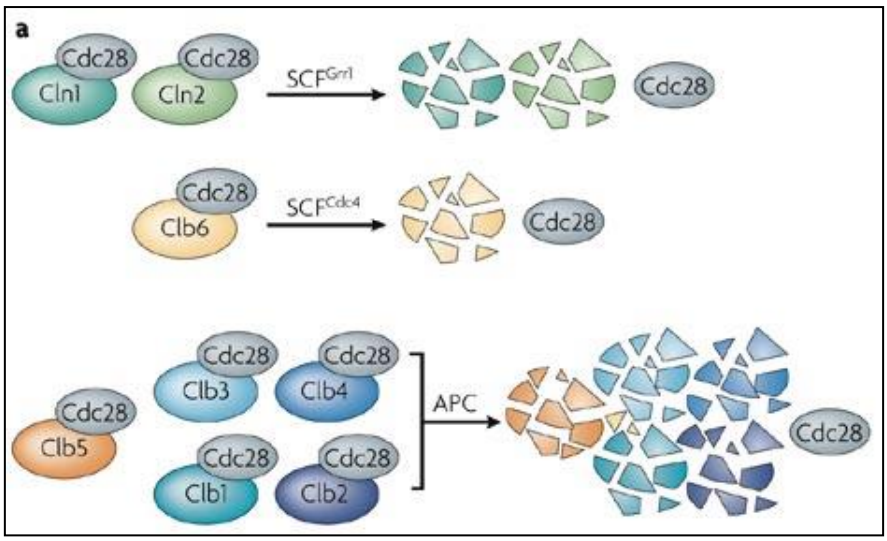

Fig.3: Cyclins are ubiquitynated by different ubiquitin ligases and degraded by 26S proteasome. The G1-phase cyclins (Cln1 and Cln2) are ubiquitynated by SCFGrr1, Clb6 is ubiquitylated by SCFCdc4, and the other B-type cyclins (Clb1, Clb2, Clb3, Clb4 and Clb5) are ubiquitylated by the anaphase promoting complex (APC). Sic1 inhibits the activity of Clb–Cdc28 complexes. Cln–Cdc28 phosphorylates Sic1, which promotes SCFCdc4-mediated ubiquitynation and subsequent degradation of Sic1, allowing for Clb–Cdc28 activation and S-phase entry. Clb–Cdc28 complexes also phosphorylate Sic1 induces its proteolysis. P, phosphate. [6]

The other B-type cyclins are ubiquitynated by the APC [10] [Fig.3a]. During metaphase, APC is bound to Cdc20 and targets Clb5 and mitotic B-type cyclins for degradation. Later in mitosis, APC that is bound to the adaptor protein Cdh1 completes the degradation of mitotic B-type cyclins, including the main mitotic cyclin, Clb2. APC/Cdc20 and APC/Cdh1 are differentially regulated by Cdc28-mediated phosphorylation.

Cyclin localization. The localization of individual cyclins to different subcellular compartments provides a means for cyclin-specific targeting. The G1 cyclin Cln3 is primarily nuclear, whereas Cln2 is primarily cytoplasmic and can localize to sites of polarized growth. This localization pattern contributes to the abilities of Cln2 and Cln3 to regulate different substrates. Indeed, cytoplasmic Cln2, but not nuclear Cln2, can rescue the phenotypes

of mutations in proteins that are involved in budding, which indicates that cytoplasmic Cln2 might be involved in this pathway. The localization of Cln2 is regulated by Cdc28-mediated phosphorylation. When the Cdc28 *consensus* phosphorylation sites in Cln2 are mutated, Cln2 is exclusively nuclear, which indicates that phosphorylation can conceal an NLS or expose a nuclear export signal.
The role of Cln3 in promoting transcription by phosphorylation and nuclear exclusion of the transcriptional repressor Whi5 (see later) is probably promoted by the nuclear localization of Cln3. In fact, the introduction of a nuclear export signal into *CLN3* sequence renders the Cln3–Cdc28 complex largely non-functional for this role [11,12].

Furthermore, Clb1–4 are localized primarily to the nucleus, with regions associated with the mitotic spindle and SPBs. Clb2 is the only mitotic cyclin that is also distributed to the bud neck . Localization of Clb2 to the bud neck is independent of its binding to Cdc28, but is dependent on its hydrophobic patch. Sequences deletion of the gene that encodes the bud-neck protein Bud3 eliminates bud-neck-localized Clb2 and delays cytokinesis, which indicates that Bud3 is important for targeting Clb2 to the bud neck, and this might positively regulate cell division [13].

4.1.2 Inhibition of cyclin–Cdk complexes.

Specific cyclin–Cdk complexes are negatively regulated by binding to stoichiometric inhibitors. Clb–Cdc28 complexes, but not Cln–Cdc28 complexes, are blocked when bound to Sic1 [14]. Sic1 is synthesized at the end of the cell cycle at when its Cdk inhibitory activity initially potentiates mitotic exit [Fig.4] [15]. *SICl* expression depends on Swi5, that begins to transcribe *SICl* during telophase. However, in the absence of Swi5, part of the residual gene expression depends on the related transcription factor Ace [15].

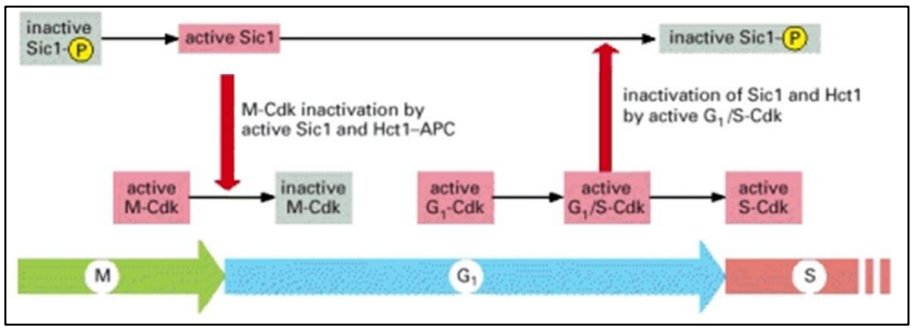

Fig.4: The control of G1 progression by Cki: As cells exit from mitosis and inactivate M-Cdk, the resulting increase in Sic1 activities results in stable Cdk inactivation during G1. When conditions are positive for entering a new cell cycle, the increase in G1-Cdk and G1/S-Cdk activities leads to the inhibition of Sic1 by phosphorylation, allowing S-Cdk activity to increase. From: Intracellular Control of Cell-Cycle Events

Sic1 is stable until the G1–S transition [14]. During this phase Sic1 function seems to be the inhibition of the Clb5–Cdk1 complex, which is responsible for initiation of DNA replication. Clb5–Cdk1 complexes accumulate progressively during the latter part of G1 but are maintained in an inactive pool to prevent the premature initiation of DNA replication. At the G1/S boundary, the entire pool of Sic1 is subjected to concerted and complete ubiquitin-mediated proteolysis [14]. This confers simultaneous activation to the entire resident pool of Clb5–Cdk1 and presumably also robust and decisive initiation of DNA replication [Fig.5]. This is in line with the observation that mutants deleted for *SIC1*, although viable, are prone to genomic instability [16].

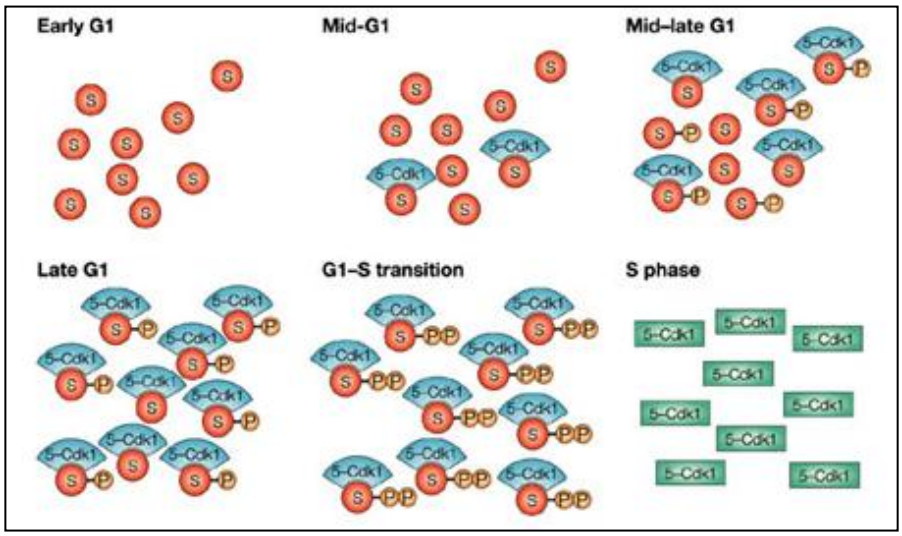

Fig. 5: Sic1 regulation of Clb5–Cdk1 at t population level. A pool of Sic1 molecules (S, red) is present in early in G1. In mid-G1, Clb5–Cdk1 molecules (5–Cdk1, blue) begin to accumulate but are kept inactive by Sic1 binding. As cells progress from mid- to late-G1, Clb5–Cdk1 complexes accumulate but are kept in an inactive Sic1-bound pool. At the same time, active Cln–Cdk1 complexes accumulate and progressively phosphorylate Sic1. In late G1, a saturated pool of Sic1–Clb5–Cdk1 complexes is hyperphosphorylated as Cln–Cdk1 levels become maximal. This triggers the concerted destruction of Sic1 and concomitant activation of the entire pool of Clb5–Cdk1 (5–Cdk1, green), leading to the G1–S-phase transition.[17]

In the context of the yeast cell cycle, phosphorylation of Sic1 occurs progressively late in G1, as G1 cyclins accumulate and activate Cdk1. However, Sic1 ubiquitynation and destruction do not occur until Cln–Cdk1 levels are maximal and the cell is ready to commit to the G1–S transition, [14] [Fig.6].

The importance of this built-in delay is underscored by creating a Sic1 derivative that contains the efficient cyclin-E degron. Yeast cells express of this mutated version of Sic1 enter S phase prematurely and experience genomic instability [10].

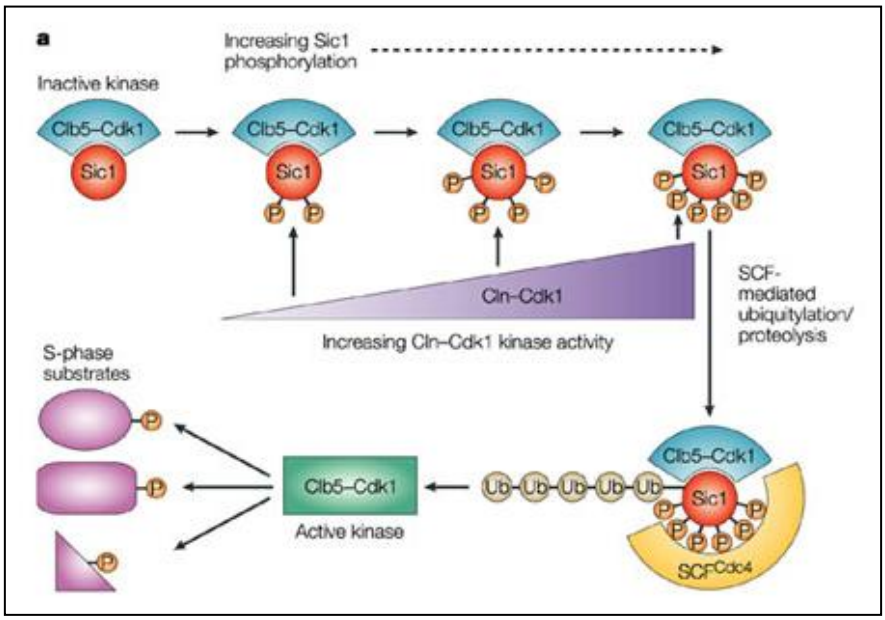

Fig. 6: Sic1 regulation of Clb5–Cdk1 at molecular level. Clb5–Cdk1 complexes are held inactive as they accumulate in G1 by virtue of their binding to Sic1. Increasing accumulation of G1-specific cyclin-dependent-kinase (Cdk) activity (Cln–Cdk1) leads to progressive phosphorylation of Sic1 molecules. Recognition of Sic1 by Skp1/Cullin/F-box protein (SCF)Cdc4 occurs only when Sic1 becomes hyperphosphorylated, which occurs when Cln–Cdk1 levels are maximal. Ubiquitin-mediated proteolysis of Sic leads to activation of Clb5–Cdk1 and phosphorylation of S-phase substrates.[27]

4.1.3 Start

As mentioned above Start is the short interval during late G1 phase after which cells are committed to division. Passage through Start has several requirements as follows: i) growth to a critical cell size, ii) nutrient sufficiency , iii) attainment of a critical translation rate , and for haploids, iv) absence of mating pheromone [1]. The first three conditions are likely to be interrelated. The critical size requirement and minimum translation rate explain why slowing growth rate increases the length of G1 phase, whereas the time required to transit the rest of the cell cycle is relatively constant . The critical-size threshold maintains uniform cell size over many generations, and under minimal nutrient conditions forces cells to accumulate the energy stores required to complete the division cycle. At constant nutrient levels, the critical-size requirement couples growth and division only in daughter cells, as mother cells by definition have already attained critical size.

The molecular basis of Start has been extensively studied over the last two decades, and many of its key molecular players and their interactions have now been identified [Figure 5].

Start depends on activation of the SBF and MBF transcription factor complexes that bind the promoters of G1/S-regulated genes [18]. Among these, the key transcripts are the G1 cyclins *CLN1* and *CLN2* and the B-type cyclins *CLB5* and *CLB6*. Cln1 and Cln2 activate Cdc28 in order to trigger bud emergence and inactivate Sic1 and Cdh1, two key inhibitors of B-type cyclin–Cdc28 activity. Once unleashed, Clb5/6–Cdc28 complexes initiate DNA replication [19]. Therefore, Start can be viewed as the short interval required to accumulate sufficient Cln1/2–Cdc28 activity to phosphorylate Cdh1 and Sic1, and perhaps other substrates.

The G1 cyclin Cln3 is the most upstream activator of the Start transition[20-22] and the main regulator of the size-sensing module; its activity is important for SBF-mediated gene activation, indeed overexpression or deletion of *CLN3* result in small or large cell size, respectively [20-22], presumably reflecting early or late activation of the transcriptional program ("early" or "late" relative to the cell size "clock"). Regulation of Start by Cln3–Cdc28 is dependent on the phosphorylation of Whi5, a transcriptional repressor of SBF, to induce its nuclear export and allows for *CLN1* and *CLN2* transcription [22,23]. The phosphorylation of Whi5 by Cln3–Cdc28 early in the cell cycle probably reflects, at least in part, the fact that Cln3 is the only cyclin that is expressed at this time. In fact, in the absence of *CLN3*, Cln1 and Cln2 are sufficient for transcriptional activation via positive feedback. A basal level of Cln1,2 inactivates Whi5 and/or directly activates SBF/MBF, thus driving transcription of SBF/MBF target genes including *CLN1*,2 [Fig.7] [20-22]

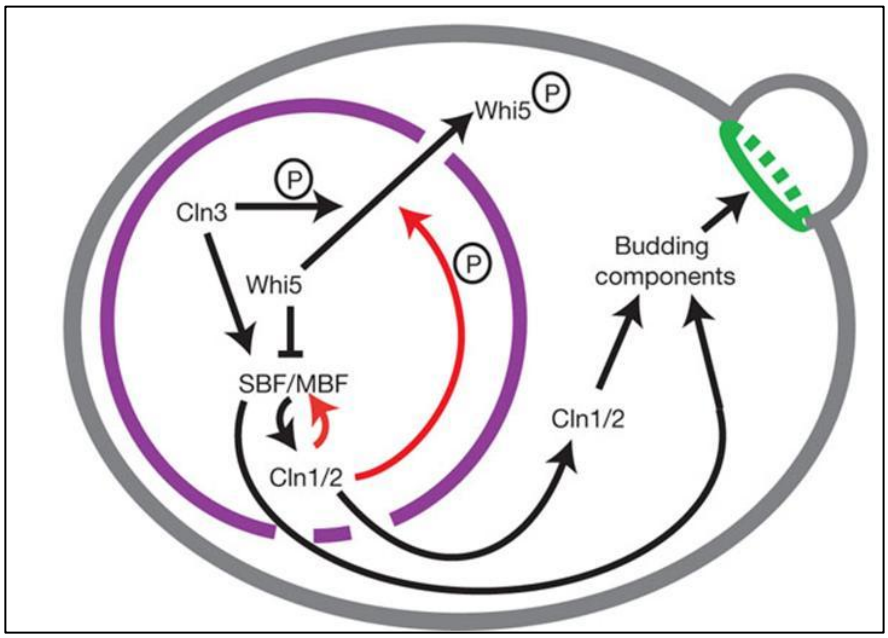

Fig.7: Model for regulon activation and bud emergence; red lines indicate pathways generating positive feedback. [24]

Recently, Di Talia and coworkers [25], using single cell imaging techniques, decomposed G1 into two independent steps, separated by Whi5 nuclear exit: a size-sensing module and a size-independent module. The former step depends on both Cln3 and cell size, and the latter step depends only on Cln2, and neither on cell size or Cln3 [Fig.8]. Temporal variability in the former step is due to the natural variability in cell size at birth coupled with size control, as well as molecular noise, possibly due to variability in *CLN3* expression. The duration of the latter step is cell-size independent; its variability is affected by the expression of the G1 cyclin *CLN2*, one of the primary final effectors of Start . This can be interpreted in the classical framework of sizers and timers [26] by defining the point at which cells switch from efficient size control to a timer control as “critical size”.

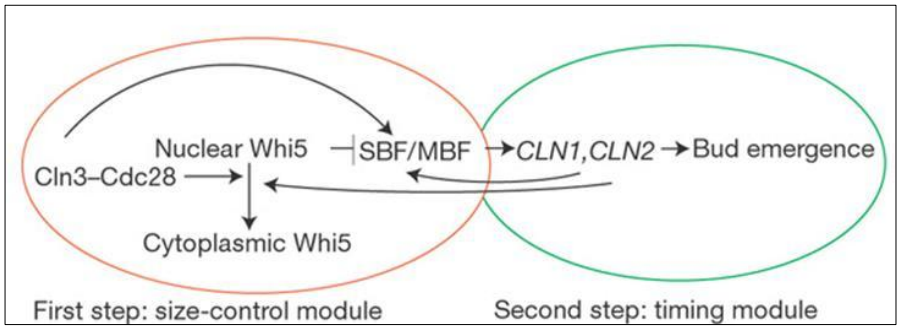

Fig. 8: Model decomposing Start into a size-control module and an independent timing module unaffected by cell size. [25]

4.2 Connecting growth to Cell Cycle

Nutrients modulate the critical cell-size threshold in proportion to the proliferation rate [27,28]. The effects of nutrients on critical cell size are conveyed rapidly, as cells are not committed to Start at a given size threshold until just before the threshold is reached. Thus, shifting cells from poor to rich medium temporarily increases the fraction of unbudded G1-phase cells as these cells grow to the new threshold.

Systematic determination of cell-size distributions for all yeast deletion strains has identified many potential Start regulators [29,30]. Many of the genes that encode potential Start repressors are implicated in ribosome biogenesis. Therefore control of critical cell-size threshold at Start by nutrient is communicated by rates of ribosome production. Ribosome biogenesis thus underlies the cell's capacity to grow. Indeed ribosome biogenesis is a chief occupation of growing cells, accounting for >50% of total transcription in yeast and mammalian cells [31,32]. In yeast, the rate of ribosome synthesis is dictated by the rate of transcription of the RNA and protein subunits of the ribosome (Warner 1999). rRNA and ribosome protein (RP) gene transcription is exquisitely sensitive to the growth potential of the cell and is rapidly repressed in response to a wide variety of internal and external stresses [32,33]. The 137 RP genes, referred to here as the RP regulon, are tightly coregulated. The vast majority of genes in the RP regulon have promoter-binding sites for Rap1, whereas a few have sites for Abf1 [34].

A large number (>200) of other genes, which we term the Ribi regulon, show nearly identical transcriptional responses as RP genes to environmental or genetic perturbations. The promoters of these coexpressed genes are strongly enriched for the presence of two motifs, termed RRPE and PAC, and therefore appear to constitute a distinct regulon [29,31,33]. Most of these genes encode proteins involved in ribosome biogenesis, a process involving more than 100 accessory factors that assemble and modify rRNA and RPs in the nucleolus [35]. A number of additional functional categories are present in this regulon, including subunits of RNA Polymerase I and III, enzymes involved in ribonucleotide metabolism, tRNA synthetases, and translation factors[29,33]. The Ribi regulon thus consists of non-RP genes that boost translational capacity. Two central nutrient-signaling conduits, the Ras/PKA and TOR signaling pathways, can activate rRNA, RP, and Ribi transcription.

The partial uncoupling of growth from division by specific mutations in ribosome biogenesis pathways suggests that the critical cell size threshold is not set simply as a passive downstream readout of protein synthetic rate [36]. Instead, commitment to division may be dynamically linked to signals that stimulate ribosome biogenesis, which is the predominant biosynthetic activity of a growing yeast cell. That is, the cell may anticipate changes in its protein synthetic rate by adjusting the critical cell size threshold before any actual change in ribosome content. This interpretation is consistent with the observation that the critical cell size threshold increases in nutrient-rich conditions, as do the rates of ribosome biogenesis and protein synthesis [34].

The most potent size regulators involved in ribosome biogenesis, identify by a screen for nonessential genes whose disruption reduces cell size at budding yielded are Sfp1 and Sch9.

These proteins activate expression of proteins involved in the RiBi and RP regulons [2,29]. Yeast sense nutrients and stress via the Ras/PKA and TOR signaling pathways which signal to Sfp1 and Sch9 to alter ribosome synthesis. Sfp1, Sch9, and Ras/PKA function in a nonlinear network that dictates both critical cell size and expression of the Ribi and RP regulons. Critical cell size at Start is decreased when any of these components is crippled, whereas either constitutive activation (Ras/PKA) or inactivation (Sfp1, Sch9) renders cell size impervious to carbon source control [5,37,38]. Each component of this trio is needed for proper RP and Ribi gene expression. Like Ras/PKA signaling, Sfp1 and Sch9 are sensitive to nutrient

conditions, at the level of localization and abundance, respectively. In addition, strains deleted for numerous genes implicated in the actual events of ribosome biogenesis, as well as ribosome structural genes, are similarly, if less dramatically, uncoupled for growth and division [29].
The mechanism of how Sfp1 and Sch9 couple ribosome biogenesis to Start through the SBF/MBF complexes is unknown, but it is thought that decreasing the rate of ribosome biogenesis lowers the critical size setpoint needed for budding. It has been hypothesized that these effects are independent of Cln3 and Whi5 because the cell size setpoint can be changed independently of *CLN3*/*WHI5* [2].
Recently Bernstein and coworkers have found that ribosome biogenesis can also positively promote Start [39]. They have found that adequate ribosome biogenesis is needed to promote Start and that this effect is mediated, at least in part, by Whi5. Therefore, the initial response to ribosome biogenesis defects may occur through a Whi5-dependent mechanism. Alternatively, increased nuclear Whi5 could be a result, and not the cause, of the cell cycle defects observed. These observations have suggest that Whi5 is involved in coordinating adequate ribosome synthesis to G1 cell cycle progression. Together these results suggest that ribosome biogenesis can have negative and positive regulatory roles on Start. Although the nature of the repressive influence of ribosome biogenesis on Start remains to be determined, the interplay between ribosome assembly and cell cycle progression appears to be a conserved feature of eukaryotic cell division [40-42]. Indeed pathways that control critical cell size at Start in budding yeast may provide further insight into mechanisms that couple growth and division in higher eukaryotes.

4.2.1 Actin polarization limits cell growth

It is well-established that cell growth controls cell division in budding and fission yeast [29]. Recently it has been observed that in budding yeast, the reciprocal relationship also holds. Indeed Goranov studying the growth rates of cdc mutants after the shift at the non permissive temperature concluded that cell cycle stage are able to dictates cell growth. In particular, the ability of cells to grow is higher in anaphase and G1 than in other cell cycle stages [43]. These data support the idea that the G1 phase can be divided into two independent steps: a size-sensing module and a size-independent module [25].

One mechanism whereby cell cycle events affect growth is through changes in the actin cytoskeleton. Indeed during the normal cell cycle, passage through the G1/S-phase boundary drives a transition from an isotropic to an apical pattern of growth; correspondingly, a decrease in growth rate at this boundary is consistent with the notion that actin polarization regulates growth.

In order to grow in size, cells must fuse new lipid vesicles with the cell membrane. The vesicles are transported to sites of fusion on actin cables by Myosin V [44-46].The actin cytoskeleton undergoes dramatic changes during the cell cycle [Fig.9] In G1 phase, actin cables are evenly distributed throughout the cytoplasm, resulting in uniform vesicle deposition, isotropic growth and spherical cell morphology [Fig,9i]. As cells enter the cell cycle, the actin cytoskeleton becomes polarized through the action of the Cln cyclin G1 CDKs [Fig.9ii] and vesicle deposition occurs apically at the site of bud emergence. After initial bud emergence, growth remains limited to the developing daughter cell, but becomes isotropic to create a spherical bud [Fig. 9 iv] [44-46]

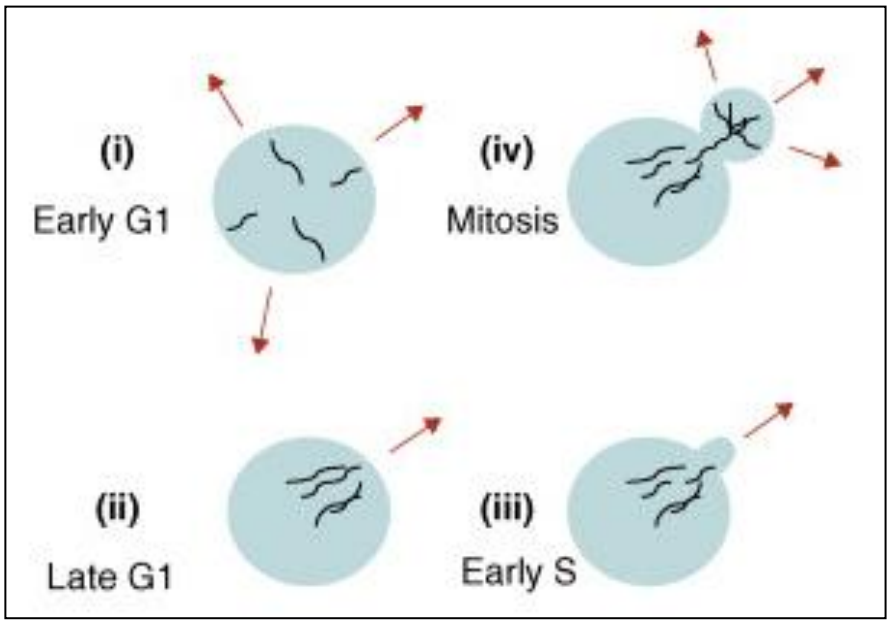

Fig 9. Changes in actin organization during the cell cycle of S. cerevisiae. Black wavy lines represent actin cables and red arrows indicate the locations of cell growth (vesicle deposition). See text for detailed explanation. [47]

Polarization of the actin cytoskeleton requires the concerted action of many proteins. Cortical cues established in the prior cell cycle cause the Rho-like GTPase Cdc42 to accumulate at the site of bud emergence where it activates the formins Bni1 and Bnr1 to nucleate actin cable formation [48,49]. Cell cycle position dictates the pattern of actin polarization and growth. The Cln-CDKs phosphorylate and activate components of the actin polarizing machinery in late G1 phase . The B-type cyclin (Clb)-CDKs, which are activated subsequently to Cln-CDKs and promote S phase and mitosis, mediate the switch from polarized growth to isotropic growth, likely through down-regulation of the Cln-CDK activity [50][51]. Whether these dramatic changes in the cell architecture affect growth has not been studied.

The actin cytoskeleton not only affects growth but also appears to influence cellular density. Mitchison and others predicted that cell density peaks around the time of budding [52]. How do changes in the actin cytoskeleton affect cell growth and density and are these two events connected? A simple hypothesis is that at the time of budding, the cell surface increases at a lower rate but initially protein synthesis continues at the same rate. This results in a temporary (<30 min) uncoupling of cell surface growth and protein synthesis

and hence increased cell density. The basis for this behavior could be the properties of apical growth: there is limited space at the bud tip and only few vesicles are incorporated into the membrane to contribute to cell surface growth [Fig.9]. Initially protein synthesis continues unabated causing a transient increase in cell density. Sometimes thereafter feedback mechanisms are activated to downregulate protein synthesis in response to actin hyperpolarization.

How actin polarization leads to the downregulation of protein synthesis is not understood, but the analysis of mutants defective in secretion provides a framework for how to think about this signaling mechanism. In fact, in cells defective for secretion, unincorporated vesicles accumulate. Secretion mutants also show decreased protein synthesis rates [53]. The CWI (Pkc1) pathway, which senses cell wall stress [54], down regulates protein synthesis in these secretion mutants [55,56]. Polarized growth may create a similar situation as that observed in secretion mutants. The area of membrane where vesicles can be deposited is limited [Fig.9 ii and 9 iii], which may lead to the accumulation of unincorporated vesicles. This could cause activation of the Pkc1 pathway and hence a downregulation of protein synthesis.

Other cell cycle events could also regulate growth in budding yeast. Cells arrested in metaphase grow relatively little compared to G1 arrested cells, although the actin cytoskeleton is not highly polarized driving this arrest. Regulators of anaphase entry, that is the ubiquitin ligase APC, could accelerate growth as cells exit from mitosis and resume rapid growth. Like in *S. pombe*, duplication of the DNA content could also accelerate growth. However, given the poorly defined nature of G2 in *S. cerevisiae* detecting this growth rate change may be difficult.

4.3 Control of cell cycle progression by MAPK

4.3.1 Control of cell cycle by pheromone response pathway

Pheromone stimulation leads to cell cycle arrest in the G1 phase in preparation for the formation of mating projections and eventual cell and nuclear fusion of the haploid partners. This cell cycle arrest is dependent upon a function of Far1 that is independent of its role in delivering the GEF (Cdc24) for GTP loading of Cdc42, which is, in turn, essential for both MAPK activation (via the PAK, Ste20) and cell polarization (via Bni1 and

other effectors). Upon pheromone stimulation, Fus3 phosphorylates Far1, which then is then able to associate with and inhibit the function of cyclin–CDK complexes (Cln1- and Cln2-bound Cdc28). Whether this inhibitory effect is due, mechanistically, to direct inhibition of the catalytic activity of the Cdc28 CDK is controversial [57] . Additionally, Fus3 and Kss1 can impose pheromone-induced cell cycle arrest in a Far1-independent manner, although the molecular basis for this effect seems to be indirect, namely via reducing expression of genes (*CLN1*, *CLN2* and *CLB5*) encoding cyclins necessary for the G1-S phase transition [58] [Fig. 11].

4.3.2 Control of cell cycle progression by hyperosmotic stress

Like pheromone stimulation, hyperosmotic stress also causes MAPK-mediated cell cycle arrest. Although this arrest is only transient, it seems important for osmoresistance. Unlike pheromone-imposed arrest, osmostress leads to cell cycle delays in both G1 and G2 [59] and regulation the exit from mitosis [51] [Fig.10 and Fig. 11].

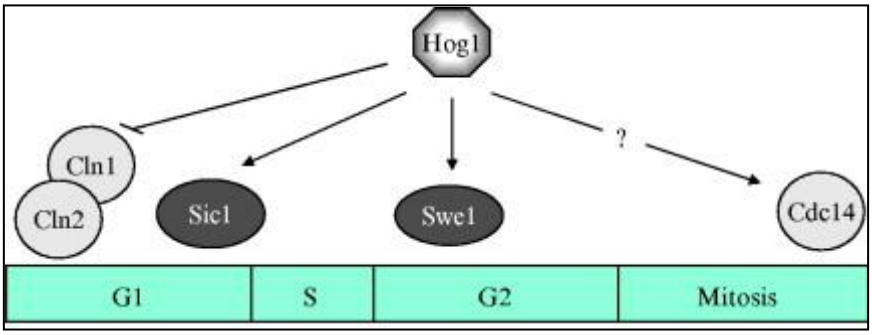

Fig. 6: The osmocheckpoint modulates cell cycle transitions through SAPK activation. SAPKs from different yeasts act on several factors that control cell cycle progression positively (clear circles) or negatively (dark ellipses). Question marks indicate that the exact mechanism of action is not known. This figure suggests that SAPKs are able to protect cells from osmoinsults in any phase of the cell cycle. (Clotet, et. al.).

Control G1 phase: Hog1-mediated G1 arrest is partially due to the downregulation of Cln1, Cln2, and Clb5 expression. The exact nature of the mechanism that represses the expression of SBF and MBF promoters under osmotic stress is still not known. Therefore, the downregulation of Cln production might explain, at least in part, the delay in S-phase entry as a consequence of an increased accumulation of Sic1.

Hog1 arrests cells in G1 by an alternative mechanism: the direct phosphorylation of Sic1[60]. Hog1 interacts physically with Sic1 in *vivo* and in *vitro* and, upon osmostress, phosphorylates a single residue threonine 173 (Thr173) at the carboxyl terminus of Sic1, which results in Sic1 stabilization and inhibition of cell cycle progression. Phosphorylation at This residues interferes somehow with the binding of Sic1 with Cdc4.
Therefore, Sic1 is targeted by Hog1 through two independent mechanisms: downregulation of Cln levels and direct phosphorylation of Thr 173.
This dual mechanism is required because the only increase of Sic1 stability by Cln1,2/Cdc28 downregulation under osmotic stress is not sufficient to ensure the correct entry into S phase. Then direct Hog1-mediated phosphorylation of Sic1 could offer this extra mechanism to ensure the exact timing of G1 /S transition [60].

Control G2 phase: The efficiency of passage from G2 to M is regulated, in part, by a morphogenesis checkpoint where assembly of the septin collar at the bud neck leads to recruitment of an AMPK-related protein kinase, Hsl1. Hsl1 promotes entry into mitosis by recruiting and phosphorylating another protein, Hsl7. Together these factors act to stimulate degradation of Swe1, a protein kinase that phosphorylates and negatively regulates the M phase-specific B-type cyclin (Clb1 and Clb2)-bound form of Cdc28. During osmostress, Hog1 reportedly phosphorylates Hsl1 at a site within its Hsl7-interacting domain, thereby preventing Hsl7 recruitment, these stabilizes Swe1 and causing a delay in exiting G2 and entering M phase [59] Regulation of Exit from Mitosis: Exit from mitosis is a key step in regulation of the cell cycle and could be targeted by the action of SAPK under osmotic stress. This idea is sustained by results shown by Amon and colleagues, who found that under hypertonic stress MEN mutants exit from mitosis in a manner dependent on the Hog1 stress-activated kinase. In such MEN mutants, the HOG pathway drives exit from mitosis by promoting activation of the MEN effector, the protein phosphatase Cdc14, although the exact mechanism of such an effect remains unclear [61].

4.3.3 Control of cell cycle progression after CWI induction

Perturbation of the actin cytoskeleton (for example, by exposure to the actin monomer-binding drug, latrunculin-B) activates Slt2/Mpk1 and causes an Slt2/Mpk1-dependent G2 arrest. Unlike Fus3- and Hog1-mediated regulation

of the cell cycle via effects on CKIs (Far1 and Sic1, respectively), cell cycle arrest by Slt2/Mpk1 seems to occur via blocking the Mih1 function, the phosphatase that must act to reverse the inhibitory phosphorylation installed by Swe1. In the absence of Mih1 function, Swe1 action is sufficient to hold Clb-bound Cdc28 in check, preventing mitotic entry. However, how Slt2/Mpk1 acts to prevent Mih1 function has not been determined at the molecular level [62][Fig.11].

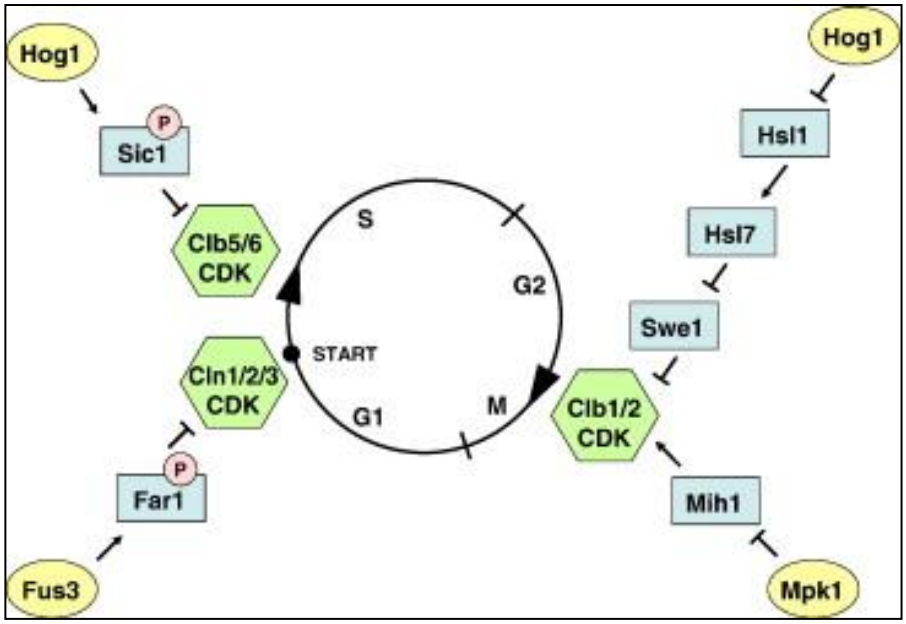

Fig 11. Mechanisms of MAPK regulation of yeast cell cycle progression. Fus3 (in response to pheromone) and Hog1 (in response to hyperosmotic stress) impose cell cycle arrest in the G1 phase via their direct phosphorylation of two different proteins (Far1 and Sic1, respectively) that act as direct inhibitors of yeast CDK1 (Cdc28). Hog1 also imposes cell cycle arrest in the G2 phase via blocking the activity of a protein kinase (Hsl1) necessary for initiating the ubiquitin- and proteasome-mediated destruction of a protein kinase, Swe1 (mammalian ortholog, Wee1), that is a specific antagonist of cyclin B (Clb)-bound CDK1. Slt2/Mpk1 (in response to cell wall stress) imposes G2 cell cycle arrest via inhibition (direct or indirect) of the phosphoprotein phosphatase, Mih1 (mammalian ortholog, Cdc25C), that is necessary to reverse the inhibitory tyrosine-specific phosphorylation installed on CDK1 by Swe1. See the text for further details.[63]

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Chapter 5
The stabilization of the Cdk inhibitor Sic1 is essential for G1 elongation in the *sfp1Δ* mutant

and

Activation and cross-talk of MAPK pathways following *SFP1* inactivation

5.1 Materials and Methods

5.1.1 Yeast strains and growth conditions

All *S. cerevisiae* haploid strains constructed in this work and those received from other sources are listed in Table 1. Gene disruption and tagging were performed using PCR-based standard techniques [1,2]. The tagged strains were undistinguishable from the congenic untagged ones with respect to different cell cycle parameters, duplications times, DNA content, budding index etc. Standard methods were used for DNA manipulation and yeast transformation [3]. Following appropriate selection, the accuracy of all gene replacements and correct integration and tagging were verified by PCR with flanking and internal primers. Primer sequences are available upon request. The pCM189 plasmid carrying the $SIC1^{T173A}$ gene was obtained has been described in Zinzalla *et al.* (2007). Yeast cells were grown in batches at 30°C in rich medium YEPD (1% yeast extract, 2% Bacto-peptone 2% (w/v) glucose) and in Difco Yeast Nitrogen Base without amino acid (YNB-aa, 6,7 g/l) medium containing 2% glucose. Supplements were provided at a final concentration of 50 mg/l execpt for adenine at 100mg/l.

D-sorbitol, NaCl (Sigma) and KCl (Sigma) were used at the indicated concentration. Stock solutions (1 mg /ml) of rapamycin and of doxicycline (Sigma) were prepared in the drug vehicle DMSO and 50% ethanol respectively, and were added to plates or liquid media to specified final concentration. Cell number, duplication time (TD), cell volumes, percentage of budded cells and length of cell cycle phases were determined as previously described [4,5].

5.1.2 Co-Immunoprecipitation

Cells were harvested and lysed in ice-cold NP40 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 15 mM MgCl2, and 1% Nonidet P-40) plus proteases inhibitor mix (Complete EDTA free Protease Inhibitor Cocktails Tablets, Roche) and phosphatase inhibitor mix (Sigma). Typically 2×10^8 cells were collected by centrifugation. An equal volume of acid-washed glass beads (Sigma) was added, cells were broken by 12 vortex/ice cycles of 1 min each. Extracts were transferred to new tubes and then clarified by centrifugation. Protein concentration was determined using the BCA™ Protein Assay Kit (Pierce). Briefly, 1 mg of total protein extracts were diluted to 0.5 ml with NP40 buffer and incubated for 16 h at 4 °C on a rocking wheel in the presence of 3 μl of Myc-Tag antibody (Cell Signaling), following treatment with 30 μl of Protein A–Sepharose (1:1 suspension purchased from Amersham Biosciences) to precipitate the immunocomplexes (2 h). Then the immunocomplexes were washed 10 times with NP40 buffer and four times with 25 mM MOPS (pH 7.5).

5.1.3 Preparation of cell extracts and immunoblotting analyses

Total protein extracts from *S. cerevisiae* cells were obtained as described [6] except for the analysis of Slt2 phosphorylation; in this case cell extracts were prepared by mild alkaline treatment [7]. Nuclear and cytoplasmic extracts were prepared using the NE-PER Extraction Kit (Pierce Biotechnology) with minor modification [8].

Primary antibodies used were: anti-TAP antibody (Open Biosystems), anti-Myc-Tag antibody (Cell Signaling), anti-HA mAb (12CA5; Roche), anti-Nop1 mAb (EnCor Biotechnology), anti-3-phosphoglycerate kinase (Pgk) mAb (22C5, Invitrogen), anti p44/42 MAP Kinase antibody (Cell Signaling) for Slt2, anti phospho-p44/42 MAP Kinase antibody (Cell Signaling) for phospho-Kss1, phospho-Fus3 and phospho-Slt2, anti-Hog1 antibody (Santa Cruz), anti phospho-p38 MAP Kinase antibody (Cell Signaling) for phospho Hog1, anti-eIF2α phospho-specific antibody (Biosource), anti-eIF2α antibody kindly provided by T. Dever (Institute of Health & Human Services Bethesda).The secondary antibodies used were anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase purchased from Amersham.

Binding was visualized using the ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech) according to the manufacturer's

instructions. Immunoblots were stripped by incubating them in a buffer (glycine 0,2M/NaCl 0,5M pH 2,8) at room temperature with gentle shaking for 10 minutes and then washed twice with TBS (0.01 M Tris-0.9% NaCl, pH 7.4). Correct loading was confirmed by staining filters with Ponceau Red (Sigma) before immunodecoration. After ECL detection films were scanned on a Bio-Radd GS-800 calibrate imaging densitometer and quantified with Scion Image software.

5.1.4 Flow cytofluorimetric analysis and Reverse Transcription (RT)-PCR analysis.

Protein and DNA contents were determined on cells fixed in ethanol 70% (v/v) as previously described [5]. Total proteins were stained with fluorescein isothiocianate (FITC) and total DNA with propidium iodide (PI). FITC-fluorescence and PI-fluorescence signal intensities were acquired from a FACStarplus (Becton & Dickinson) equipped with an argon-ion laser (excitation wavelength 488 nm, laser power 200mW).

Total RNA extraction, RNA cleanup and DNAse I treatment were performed as previously described [9]. The access RT-PCR system (Promega) was used. RT-PCR was carried out to amplify the *CIT2*, *GLN1*, *GLN3*, *HSP26* mRNAs. *ACT1* mRNA was used for normalization. Experiments were repeated at least twice with different RNA preparations and with sample prepared from different wild type and *sfp1Δ* strains. Primers sequences used are available upon request.

5.1.4 Sensitivity assay

To determine the sensivity of the different strains to, Calcofluor White (CW). Yeast exponentially growing were dropped (5µl from concentrated suspension of 10^7 cell/ml and from serial tenfold dilutions) onto glucose rich medium plates supplemented with 50 µg/ml of CW. Plates were incubated at 30°C for three days. Cells were also dropped onto plates without CW to control cell growth.

5.1.5 Microscopy and image processing.

Yeast cells were photographed using a Nikon Eclipse E600 microscope equipped with a Leica DC 350F ccd camera. Digital images were acquired using FW 4000 software (Leica)

5.1.6 Reporter assays

Reporter assays were performed using the FG reporter plasmid pBHM746 (2µ, *pFRE(TEC1)::lacZ URA3*) [10] and HOG reporter plasmid pMP253 (*2µ, CYC1prom-(2xCREENA1)::lacZ TRP1*) [11]. pBHM746 was a kind gift of H. Madhani (Departement of Biochemistry and Biophysics San Francisco), while pMP253 was a kind gift of F. Posas, (Universitat Pompeu Fabra Barcelona).

β-galactosidase activity was determined as described [12]. All reporter assays were at least performed on triplicate using independent cultures. Activity of β-galactosidase in cell extracts was normalized against protein content cell densities and expressed as Miller units (Miller, 1972). Error bars represent standard deviations

Table 1. Strains used in this work.

Strain	Relevant Genotype	Source
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
sfp1Δ	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sfp1Δ::KanMX4*	Euroscarf
rsa1Δ	(BY4741) *rsa1::KanMX4*	Euroscarf
Cln2-Tap	(BY4741) *CLN2*-TAP::*HIS3*	Open Biosystems
Clb5-Tap	(BY4741) *ClB5*-TAP::*HIS3*	This study
Sic1-Tap	(BY4741) *SIC1*-TAP::*HIS3*	Open Biosystems
YVM2	(BY4741) *CLN1*-TAP::*HIS3 sfp1Δ::KILEU2*	This study
YVM3	(BY4741) *CLN2*-TAP::*HIS3 sfp1Δ::KILEU2*	This study
YVM5	(BY4741) *ClB5*-TAP::*HIS3 sfp1Δ::KILEU2*	This study
YVM6	(BY4741) *SIC1*-TAP::*HIS3 sfp1Δ::KILEU2*	This study
YVM13	(BY4741) *SIC1*-TAP::*HIS3 CLB5*-9MYC::*URA3*	This study
YVM14	(BY4741) *SIC1*-TAP::*HIS3 sfp1Δ::KILEU2 CLB5*-9MYC::*URA3*	This study
YVM15	(BY4741) *CLN2*-TAP::*HIS3* [pcm188: *CLN2-3*HA *URA*)]	This study
YVM16	(BY4741) *CLN2*-TAP::*HIS3 sfp1Δ::KILEU2* [pcm188: *CLN2-3HA URA3*]	This study
YVM21	(BY4741) *CLN2*-TAP::*HIS3 sic1Δ::KanMX4* [pcm189: *SIC1-4HA URA3*]	This study
YVM22	(BY4741) *CLN2*-TAP::*HIS3 sfp1Δ::KILEU 2 sic1Δ::KanMX4* [pcm189: *SIC1-4HA URA3*]	This study
YVM25	(BY4741) *CLN2*-TAP::*HIS3 sic1Δ::KanMX4* [pcm189 *SIC1T173A-4HA URA3*]	This study
YVM26	(BY4741) *CLN2*-TAP::*HIS3 sfp1Δ::KILEU2 sic1Δ::KanMX4* [pcm189: $SIC1^{T173A}$*-4HA URA3*]	This study
YVM29	(BY4741) *SIC1::HIS3 hog1Δ::URA3*	This study
YVM30	(BY4741) *SIC1::HIS3 sfp1Δ::KILEU2 hog1Δ::URA3*	This study
YVM40	(BY4741) [pBHM746: 2µ, *pFRE(TEC1)::lacZ URA3*]	This study
YVM42	(BY4741) *sfp1Δ::KanMX4* [[pBHM746: 2µ, *pFRE(TEC1)::lacZ URA3*]	This study
YVM59	(BY4741) *ptp2::KILEU2* [pBHM746: 2µ, *pFRE(TEC1)::lacZ URA3*]	This study
YVM60	(BY4741) *sfp1Δ::KanMX4 ptp2::KILEU2* [pBHM746: 2µ, *pFRE(TEC1)::lacZ URA3*]	This study
W303-1A	*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can 1-100,*	Lab Collection
Cln3-15myc	(W303-1A) *CLN3*-15MYC::*HIS3*	Zinzalla *et al.* (2007)
YVM4	(W303-1A) *sfp1Δ::KILEU2*	This study
CEN.PK	MATa *ura3-52 trp1-289 leu2-3_112 his3 Δ1 MAL2-8C SUC2*	Lab Collection
YVM46	(CEN.PK) *sfp1Δ::KILEU2*	This study
YVM47	(CEN.PK) [pMP253: 2µ, *CYC1prom-(2xCREENA1)::lacZ TRP1*]	This study
YVM48	(CEN.PK) *sfp1Δ::KILEU2* [pMP253: 2µ, *CYC1prom-(2xCREENA1)::lacZ TRP1*]	This study
YVM62	(CEN.PK) *ptp2::URA3* [pMP253: 2µ, *CYC1prom-(2xCREENA1)::lacZ TRP1*]	This study
YVM63	(CEN.PK) *sfp1Δ::KILEU2 ptp2::URA3* [pMP253: 2µ, *CYC1prom-(2xCREENA1)::lacZ TRP1*]	This study

Plasmids are indicated by square brackets

5.2 Results

5.2.1 Effect of *SFP1* inactivation on G1 cyclins and Sic1 levels

In order to better elucidate the role of Sfp1 as a negative regulator of Start, we analyzed the effect of *SFP1* deletion on the protein level of some of the key players of the G1 to S transition: the G1 cyclins, Cln1-3, and the Cki Sic1.

To this purpose we inactivated the *SFP1* gene in yeast strains harboring functional TAP/myc-tagged alleles of the genes encoding the three G1 cyclins and Sic1 to facilitate detection of these proteins by Western analysis. For all the *sfp1Δ* strains obtained a detailed analysis of the resulting phenotype was performed. In fact the main phenotype trait due to *SFP1* deletion is a very reduced cell size (*whi* phenotype), accompanied by a slow growth rate [13,14]. Thus, we evaluated i) cell size by determining cellular volume and protein content and ii) growth by measuring TD, length of budded and G1 phases, budding index and DNA content (Table 1). As shown in Table 1, all the mutants had the same cell cycle parameters and all displayed a *whi* phenotype. Moreover, the phenotype of tagged *sfp1Δ* strains was indistinguishable from the untagged one (data not shown) and perfectly in agreement with published data [5,13].

A

Wild-tipe					
Strain	**Volume (fL)**	**Budding Index**	***μ (h^{-1})**	***TG1 (h)**	***TB (h)**
CLN1-TAP	40	0,65	0,339	0,87	1,18
CLN2-TAP	41	0,62	0,329	0,86	1,25
CLB5-TAP	40	0,65	0,364	0,81	1,10
SIC1-TAP	40	0,67	0,319	0,88	1,29
CLN3-15myc	59	0,66	0,370	0,78	1,09

sfp1Δ					
Strain	**Volume (fL)**	**Budding Index**	***μ(h^{-1})**	***TG1(h)**	***TB (h)**
YVM2	33	0,35	0,169	2,32	1,77
YVM3	32	0,34	0,164	2,43	1,78
YVM5	30	0,36	0,168	2,29	1,82
YVM6	32	0,33	0,170	2,39	1,67
YVM4	51	0,34	0,192	2,32	1,28

*μ (specific growth rate), *TG1* and *TB* (the lengths of the G_1 and budded phases, respectively) have been determined as described in Materials and methods.

B

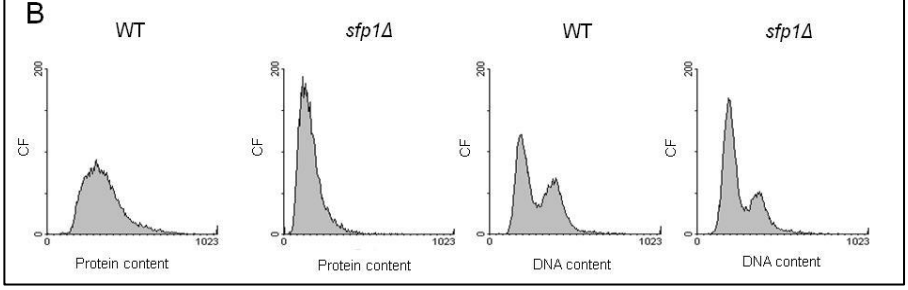


Table 1: A) Cell cycle parameters of wild type (W.T) and *sfp1Δ* exponentially growing cells on YNB-aa, 2% glucose. B) Representative flow cytometric distributions of protein and DNA contents (expressed as channel number) vs. cell frequency (CF) of asynchronous exponentially growing cells.

Then, all strains were grown in rich medium containing 2% glucose (YPD) and samples during the exponential phase were taken to monitor the level of the different tagged proteins by Western analysis [Fig.1].

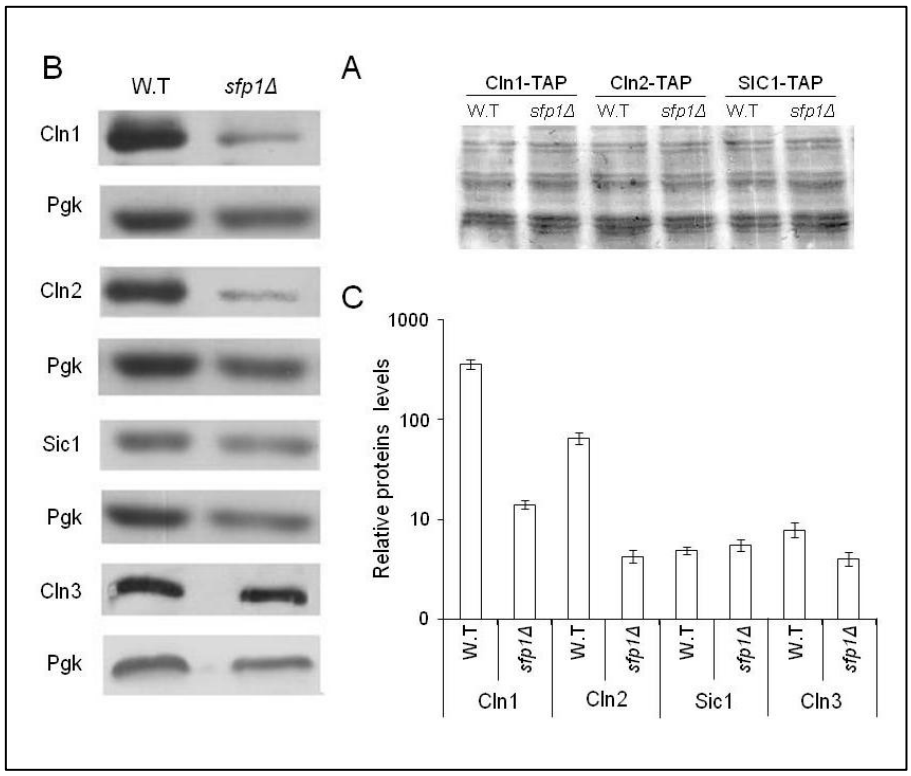


Fig.1 Start-related proteins levels in cells lacking *SFP1* gene: A) Representative filters stained with Ponceau used as control for total protein loading. B) Immunoblot of TAP/myc tagged proteins in cell extracts from wild type (W.T), *sfp1*Δ cells exponentially growing on glucose**.** Pgk was used as housekeeping loading control. C) The level of each protein relative to Pgk was determined by densitometric analysis. Data are expressed as arbitrary units. Standard deviations are shown. Quantification was done with ImageJ (avaible at http://rsbweb.nih.gov/ij/)

The level of cyclins Cln1 and Cln2 were dramatically reduced in *sfp1Δ* cells compared to the wild type ones [Fig.1B and C]. Since these cyclins are responsible for budding induction [15,16], their lower level is in agreement with the observation that *sfp1* null mutants show a low budding index as a consequence of a G1 extension. On the contrary, in the mutant strain, Cln3 levels were only slightly affected [Fig.1B and C]. Cln3 is the most upstream activator of the Start transition and the main regulator of the size-sensing module; indeed, overexpression or deletion of *CLN3* result in small or large cell size, respectively [17,18]. In agreement with our results, the introduction of *CLN3* deletion into *sfp1Δ* cells results in an additive interaction phenotype indicating that the effects of Sfp1 on Start may be independent of Cln3 [19].

Moreover, Sic1 levels were quite similar between the wild type and the mutant strains [Fig.1B and C]. Sic1 is a negative regulator of Start, whose degradation is required for entry into S phase and we decided to investigate more in detail its regulation in *sfp1Δ* cells.

5.2.2 In *sfp1Δ* cells Sic1 is nuclear and bound to Clb5.

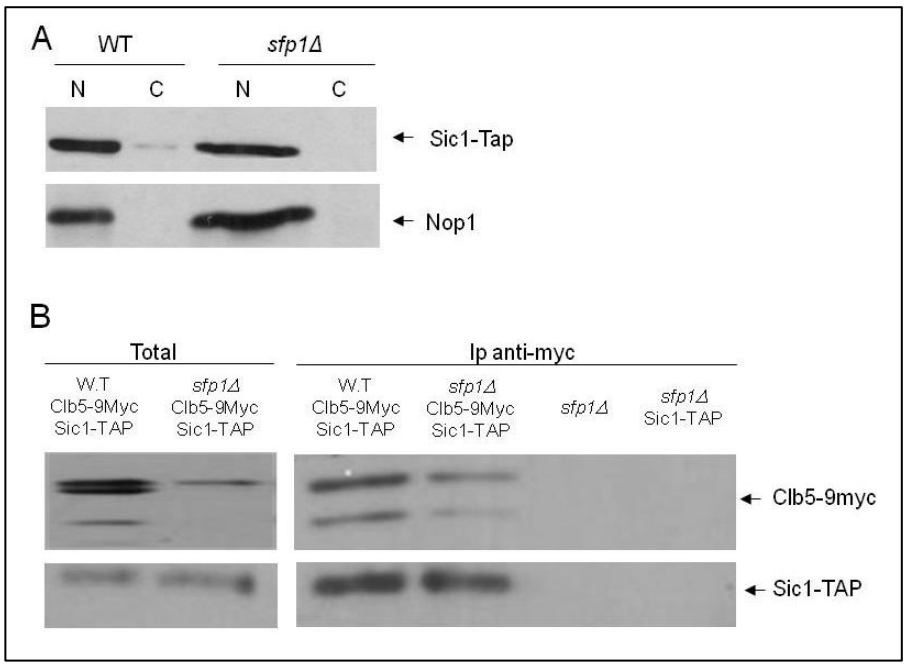


Fig.2 Analysis of Sic1 localization in *sfp1Δ* cells: A) Cytoplasmic (C) and nuclear (N) fractions obtained from Sic1-TAP cells. The filter was first probed with anti-Tap antibody to detect Sic1, and subsequently with an antibody that recognizes the nuclear protein Nop1. B) Wild type and *sfp1*Δ strains expressing Sic1-Tap and Clb5-9Myc were immunoprecipitated using anti-myc antibody under non-denaturing conditions. Immunoprecipitated proteins were denatured and then subjected to Western analysis with anti-myc and anti-TAP antibodies. *sfp1*Δ and *sfp1*Δ /Sic1-Tap cells are two negative controls of immunoprecipitation. Filters stained with Ponceau used as control for total protein loading are not shown.

Sic1 activity is controlled by its cellular localization and by the regulation of its half-life strictly linked to a multistep phosphorylation by Cln1,2-Cdc28 kinase complex [20,21] .

Since the nuclear import of Sic1 is essential for its degradation and for the correct cell cycle progression, we analyzed its cellular localization in *sfp1Δ* cells. As shown in Fig.2A, Sic1 localized mainly in the nucleus in the wild type in agreement with [8], while in the mutant cells is detected exclusively in the nucleus similarly to what has been observed in rapamycin treated cells

[22]. In these G1 arrested cells Sic1 accumulates in the nucleus where it avoids improper Clb5,6-Cdc28 driven initiation of DNA replication under scarce availability of nutrients [23].

Consequently we performed a co-immunoprecipitation experiment to evaluate the association between Sic1 and Clb5 in the *sfp1Δ* cells. Thus, we used wild type and *sfp1Δ* strains expressing Sic1-TAP and Clb5-9myc whose phenotypes were indistinguishable from the untagged ones (data not shown). Whole-cell extracts from exponentially growing cells were immunoprecipitated with anti-Myc antibody; co-precipitated Sic1 (if present) was determined by immunodection with anti-TAP antibody.

Total amounts of Clb5 were reduced in mutant cells compared to the wild type ones [Fig.2B]. This result is perfectly in line with the low level of Cln1-2 proteins that we observed in the *sfp1Δ* strains [Fig.1B]. In fact, transcription of *CLB5* is dependent on Cln proteins and occurs in parallel with the transcription of *CLN1-2* [20].

As shown in Fig.2B in the immunoprecipitated fraction, Sic1 level was similar in the wild type and the mutant strains, despite the low level of Clb5 immunoprecipitated in the mutant indicating that much more Sic1 is associated with Clb5. Since in the *sfp1Δ* cells Sic1 has an exclusive nuclear localization, this results in a greater association of Sic1 with Clb5.

Thus, probably the *SFP1* inactivation induces a mechanism to avoid the initiation of DNA replication mediated by Sic1 similar to what observed in rapamycin-treated cells.

5.2.3 In *sfp1Δ* mutant Sic1 levels are not affected by Cln2 overexpression

Sic1 levels are strictly dependent upon Cln1,2 ones. In fact in addition to their involvement in bud formation and spindle pole body duplication, these cyclins trigger the degradation of Sic1 allowing DNA replication to occur [23]. In order to analyze whether Sic1 levels observed in *sfp1Δ* cells were linked to decreased Cln2 ones, the effects on Sic1 in *sfp1Δ* cells ectopically expressing *CLN2* under the control of a tetracycline (doxycycline)-repressible promoter were evaluated. The wild type and *sfp1Δ* strains ectopically expressing *CLN2* were grown to exponential phase in YPD medium in the presence of doxycycline. After removal of the drug and consequent overexpression of Cln2, samples were taken at different time points to monitor the level of Sic1 and Cln2 by Western analysis [Fig.3B].

Collection times were calculated considering the differences in the Td of each strain. The scheme of the experiments was shown in Fig 3A. As expected, in wild type cells Cln2 overexpression induced a rapid decrease of Sic1 level [Fig.3] [24]. On the contrary, in *sfp1Δ* cells the pattern of Sic1 accumulation was unaffected by Cln2 overexpression [Fig.3 B] indicating that Sic1 seems to be insensitive to the dosage of Cln2 and stabilized in some way.

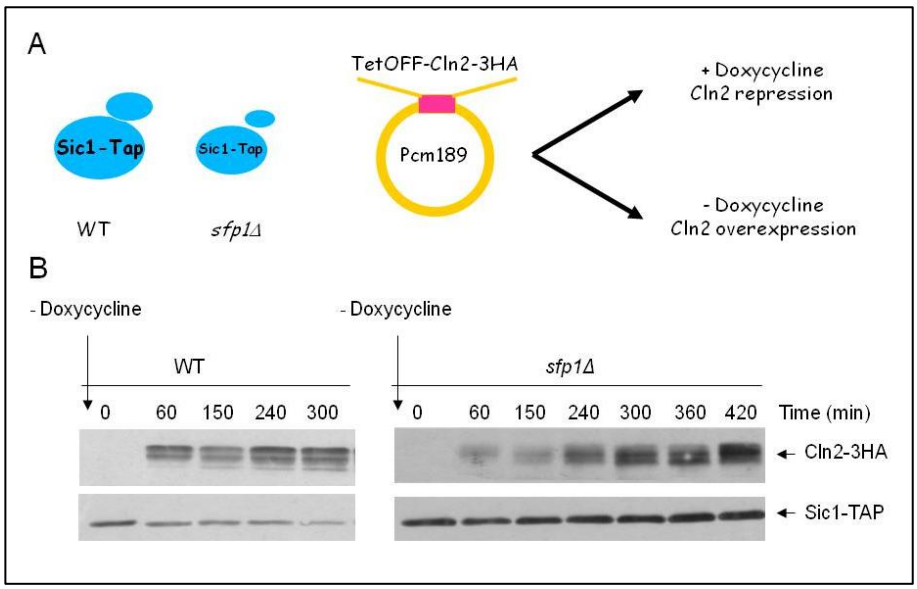


Fig.3 Sic1 accumulates in response to *SFP1* inactivation even when *CLN2* is ectopically overexpressed: A) Scheme of the experiment; B) Wild type and *sfp1*Δ Sic1-TAP strain expressing HA-tagged Cln2 under the control of a tetracycline-repressible promoter (pTet-Cln2) were grown to exponential phase in YPD in the presence of doxycycline (5 µg/ml), Then cells were collected, washed and released in fresh medium. Western analysis with anti-TAP antibody for the Sic1 level at different times is shown. The same blot was stripped and re-probed with anti-HA antibody to detect the presence of Cln2-3HA.

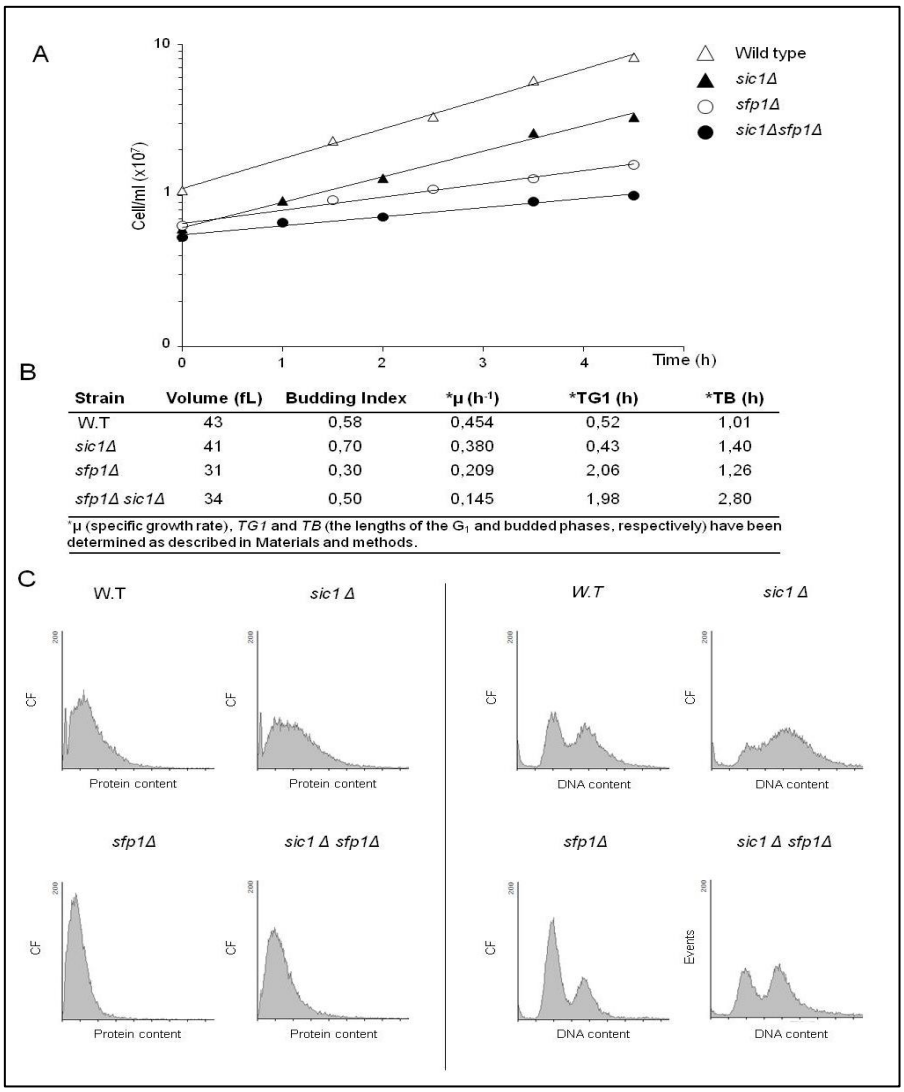
5.2.4 Sic1 is required for the G1 elongation in *sfp1Δ* cells

Sic1 is a B-type Cki. It inactivates both the S phase Clbs-Cdk complex, preventing the entrance in S phase, and the mitotic Clbs-Cdk complex allowing the exit from mitosis. Consequently, cells lacking Sic1 initiate DNA replication from fewer origins, prolong S phase and accumulate in early mitosis [23]. On the contrary, the expression of an unphosphorylable and thus stable Sic1 is not lethal, but results in a lengthened G1 and in a budded phase significantly shorter [25]. Since *sfp1Δ* in addition to G1 elongation showed a reduction of the length of G2/M transition [27] the

results reported in the previous paragraph suggest that both the G1 elongation [Table 1] and the G2/M reduction characteristic of the *sfp1* null cells can be probably linked to Sic1 stabilization. To evaluate this, we studied the effect of *SIC1* inactivation on *sfp1Δ* phenotype (TD, length of budded and G1 phases, budding index, cell volume, DNA and protein content) [Fig.4].

As shown in Fig. 4A-C inactivation of *SIC1* gene in a wild type strain induced a cell growth slowdown associated with a reduction in the G1 length, increase in the budded phase and accumulation of G2 cells in agreement with previous data [23], while the protein content is unaffected [Fig.4C].

The double mutant *sfp1Δsic1Δ* displayed the same trend of phenotypic changes observed following *SIC1* deletion alone. In fact as shown in Fig.4A-C, exponentially growing *sfp1Δsic1Δ* cells were characterized by an increase of TD associated with a reduced G1 phase, an increase in the TB phase length, and accumulation of G2 cells compared with *sfp1Δ* cells. No significant effect was observed on protein content [Fig.4C]. This result supports the hypothesis that both the elongation of G1 phase and the reduction in the length of G2/M transition in the mutant can be a consequence of Sic1 stabilization.



Strain	Volume (fL)	Budding Index	*μ (h^{-1})	*TG1 (h)	*TB (h)
W.T	43	0,58	0,454	0,52	1,01
sic1Δ	41	0,70	0,380	0,43	1,40
sfp1Δ	31	0,30	0,209	2,06	1,26
sfp1Δ sic1Δ	34	0,50	0,145	1,98	2,80

*μ (specific growth rate), *TG1* and *TB* (the lengths of the G_1 and budded phases, respectively) have been determined as described in Materials and methods.

Fig.4 Sic1 is involved in the G1 elongation in sfp1Δ cells: A) Growth kinetics of wild type, sic1Δ, sfp1Δ and sfp1Δsic1Δ cells. Cell number was determined over time during growth in YNB-aa glucose. B) Cell cycle parameters of sic1Δ, sfp1Δ and sfp1Δsic1Δ cells exponentially growing cells. B) Representative flow cytometric distributions of protein and DNA contents (expressed as channel number) vs. cell frequency (CF).

5.2.5 Sic1 is stabilized in *sfp1Δ* cells

Activation of the SAPK Hog1 by high osmolarity stress and TORC1 inhibition by rapamycin both induce Sic1 accumulation, which is mediated by phosphorylation on Thr173 [22,28].

In order to analyze Sic1 stability, we expressed either Sic1-4HA or Sic1^{T173A}-4HA proteins under the control of a tetracycline-repressible promoter (pTet-Sic1) in the *sic1Δ* and *sfp1Δsic1Δ* strains. These cells were grown in YPD medium to early exponential phase and Sic1 stability was tested after treatment with doxycycline (0.2 µg/ml) to shut off *SIC1* expression [Fig. 5A].

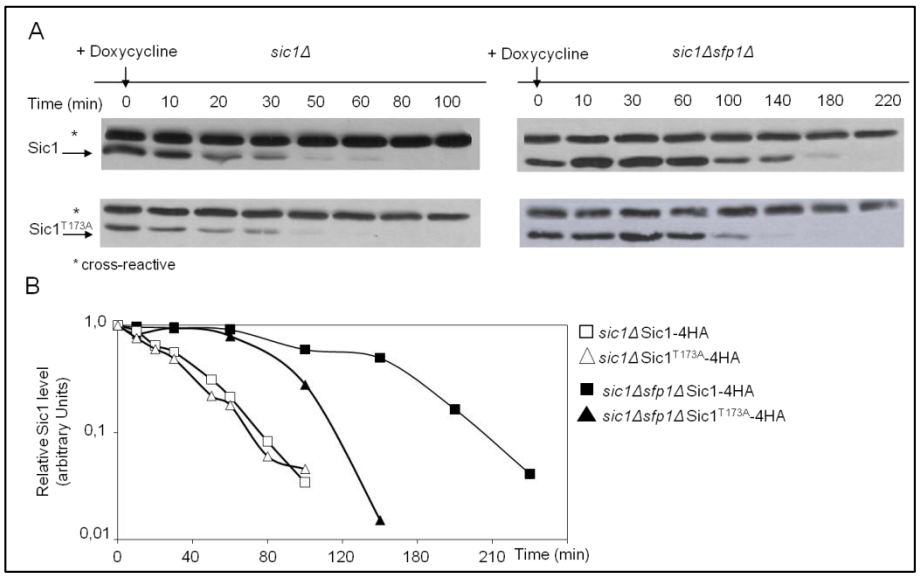


Fig. 5 Sic1 stabilization in *sfp1Δ* cells involves phosphorylation of Thr173: Time course of Sic1 protein level As for the experiments reported in Fig.3 the time course analysis was performed considering the differences in TD of each strain. A) *sic1Δ* and *sic1Δ sfp1Δ* pTet-Sic1 strains (upper panel) and *sic1Δ* and *sic1Δ sfp1Δ* pTet-Sic1^{T173A} strains (lower panel) were grown to early exponential phase. At time 0, *SIC1* expression was switched off with doxycycline (0.2 µg/ml). Samples were taken at the time point indicated for Western analysis with anti-HA antibody. B) The Sic1 level relative to a cross-reactive band (*) was determined by densitometry and the decrease due to doxycicline addition was plotted. The curves are representative of three independent experiments.

In *sic1Δ* cells, the Sic1^{T173A} stability was similar to the wild type protein, in line with published data [22,28]. On the contrary, in *sic1Δsfp1Δ* cells, the mutant protein is degraded more rapidly than the wild type form[Fig.5], suggesting that the phosphorylation of Thr173 is involved in the Sic1 stabilization observed in the *sfp1Δ* cells.

5.2.6 *SFP1* deletion induces a Rpd3-mediated gene expression

In addition to Sic1, another common mediator regulated after hyperosmotic stress and rapamycin treatment is Rpd3 [29]. Rpd3 is a class I histone deacetylase (HDAC) that modulates the expression of a large number of genes [30]. In particular, it acts as a repressor of genes involved in ribosome biogenesis and as an activator of those genes whose products are necessary for cell survival to stress. Then, we investigated whether *SFP1* inactivation could trigger Rpd3 activation. We used RT-PCR analysis to compare mRNA levels of specific genes in wild type and *sfp1Δ* strains during exponential growth and after rapamycin treatment. In particular, we analyzed the expression of two genes whose transcription depends on Rpd3 (*HSP26* and *ARO9*), and of two other genes that are induced after rapamycin treatment independently of Rpd3 (*GLN1* and *GLN3*) [31] . As shown in Fig.6 in wild type cells following rapamycin treatment all the four genes were upregulated at different levels as expected. Interestingly, we observed that mRNA levels of *HSP26* and *ARO9* were higher in the mutant than in the wild type even in the absence of rapamycin and were not induced any more after drug treatment. On the contrary the other two genes (Rpd3 indipendent) displayed a similar expression pattern in the *sfp1Δ* cells compared to wild type ones indicating that Sfp1 loss of function induces a Rpd3 mediated genes expression. This result indicates that the *SFP1* inactivation induces the activation of the stress response pathway.

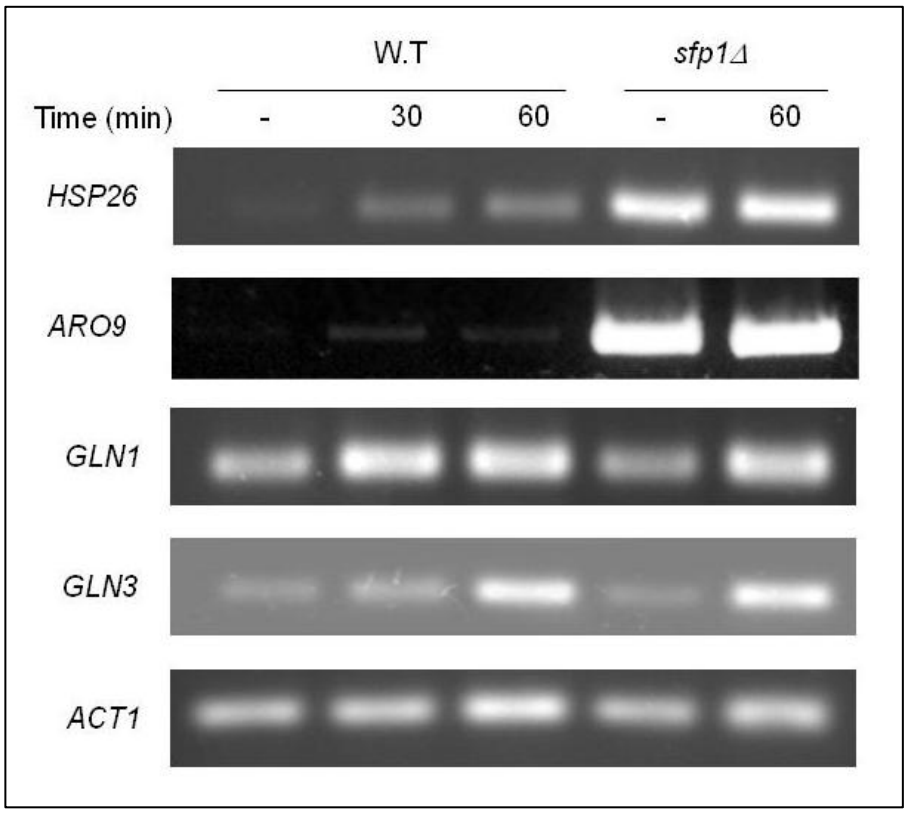


Fig. 6 *SFP1* deletion induces Rpd3-mediated gene expression: Total RNA was prepared from exponentially growing *sfp1Δ* and from W.T cells in the absence or in the presence of rapamycin (0,2µg/ml). mRNA levels were analyzed by RT-PCR. *ACT1* mRNA was used as a normalization control. Representative examples of RT-PCR amplifications are shown.

5.2.7 Hog1 is activated in *sfp1Δ* cells

It is known that after hyperosmotic stress the active form of the MAPK Hog1 (Hog1-P) is necessary for Sic1 phosphorylation on Thr173 [28]; moreover, the recruitment in the nucleus of Rpd3 is mediated both dependently and independently by Hog1 active form [32]. However, no information is available on Hog1 involvement in the mechanism of Sic1 phosphorylation on Thr173 and Rpd3 recruitment after rapamycin treatment. So, on the basis of our results, we asked whether, in the *sfp1Δ* mutant, Hog1 is required for Rpd3 activation and/or Sic1 phosphorylation.

A dual approach to detect the presence of Hog1 activation in *sfp1Δ* cells was used: one employing specific antibodies against the Hog1-P form and the other employing a reliable and very sensitive method based on a highly

osmoresponsive *LacZ* reporter gene under the control of a specific promoter (*2XCRE*) that is activated only when active Hog1 localizes into the nucleus [11]. NaCl treatment provided a control for Hog1 activation. As for the experiments reported in Fig.3 the time course analysis was performed considering the differences in TD of each strain.

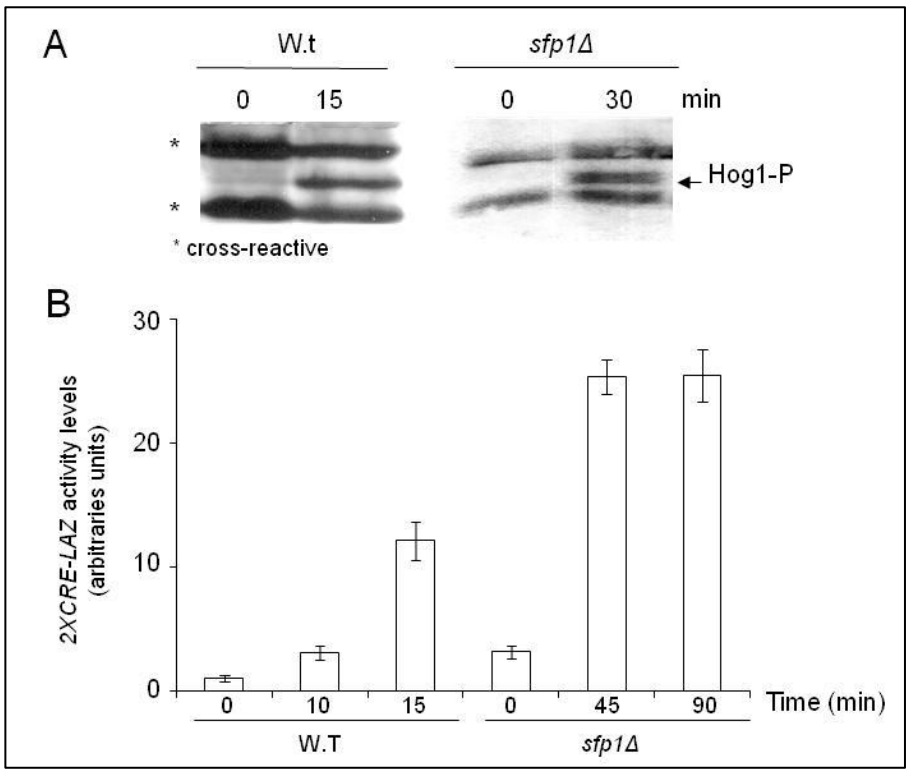


Fig. 7 *SFP1* inactivation induces low activation of Hog1: A) Immunoblot analysis of Hog1 phosphorylation: cellular extracts were prepared for wild type and *sfp1Δ* cells grown in YEPD and treated with 0.4M NaCl. The presence of activated Hog1 (Hog1-P) was visualized by probing filters with an antibody anti-phospho Hog1. B) Wild type and *sfp1Δ* cells carrying the *2XCRE-lacZ* plasmid were grown exponentially, treated with 0,4M NaCl for the times indicated, and assayed for β-galactosidase activity. Activity of β-galactosidase in cell extracts was normalized using protein content and expressed as Miller units (Miller, 1972). Both values obtained after treatment with NaCl and values obtained for the mutant were normalized to wild type at time zero which was arbitrary set to 1. All values are the average of at least two independent experiments. Standard deviations are indicated.

In exponentially growing untreated *sfp1Δ* cells, Hog1 is present at the same level than the wild type one (data not shown) and appeared phosphorylated only upon NaCl treatment [Fig.7A]. On the contrary, the higher sensitivity β-galactosidase assay allowed to detect a low induction of the expression of the reporter gene in the mutant also in the absence of stress [Fig.7B].

Induction that increased after the salt treatment at higher level than that obtained for the wild type cells [Fig.7B]

5.2.8 The *sfp1Δhog1Δ* mutants show a shmoo-like morphology

Since the reporter assay indicated that *SFP1* inactivation is accompanied with a partial activation of the Hog1 pathway, we decided to examine/confirm more in detail such activation. To this purpose we deleted *HOG1* in the *sfp1*mutant and we analyzed the effect.

As expected *HOG1* deletion in wild type cells had no effect on cellular morphology [Fig.8A]. Unexpectedly, the double mutant displayed strong morphological defects. Cells lose their ellipsoidal shape and became elongated with a shmoo-like morphology. Interestingly alteration in cellular morphology can be also obtained by exposing *hog1Δ* cells to 1M sorbitol [Fig. 8B]. In fact under high osmolarity conditions, Hog1 activation is known to be necessary to limit the activation (cross talk) of the low nitrogen response pathway (mediated by the MAPK Kss1) and of the pheromone response pathway (mediated by the MAPK Fus3) [33] [Fig.8A].

Since these MAPKs pathways use basically the same signaling machinery, when one of the three pathways is activated, the others are suppressed [34] [Fig.8B]. Consequently osmostress does potently activate the Kss1 and Fus3 MAPKs in *hog1Δ* strain [33] [Fig.8A and B]. In the same way the appearance of shmoo-like cells following Hog1 loss of function in a *sfp1Δ* mutant suggests that in the single mutant Hog1 pathway is activated and inhibits directly or indirectly the other pathways.

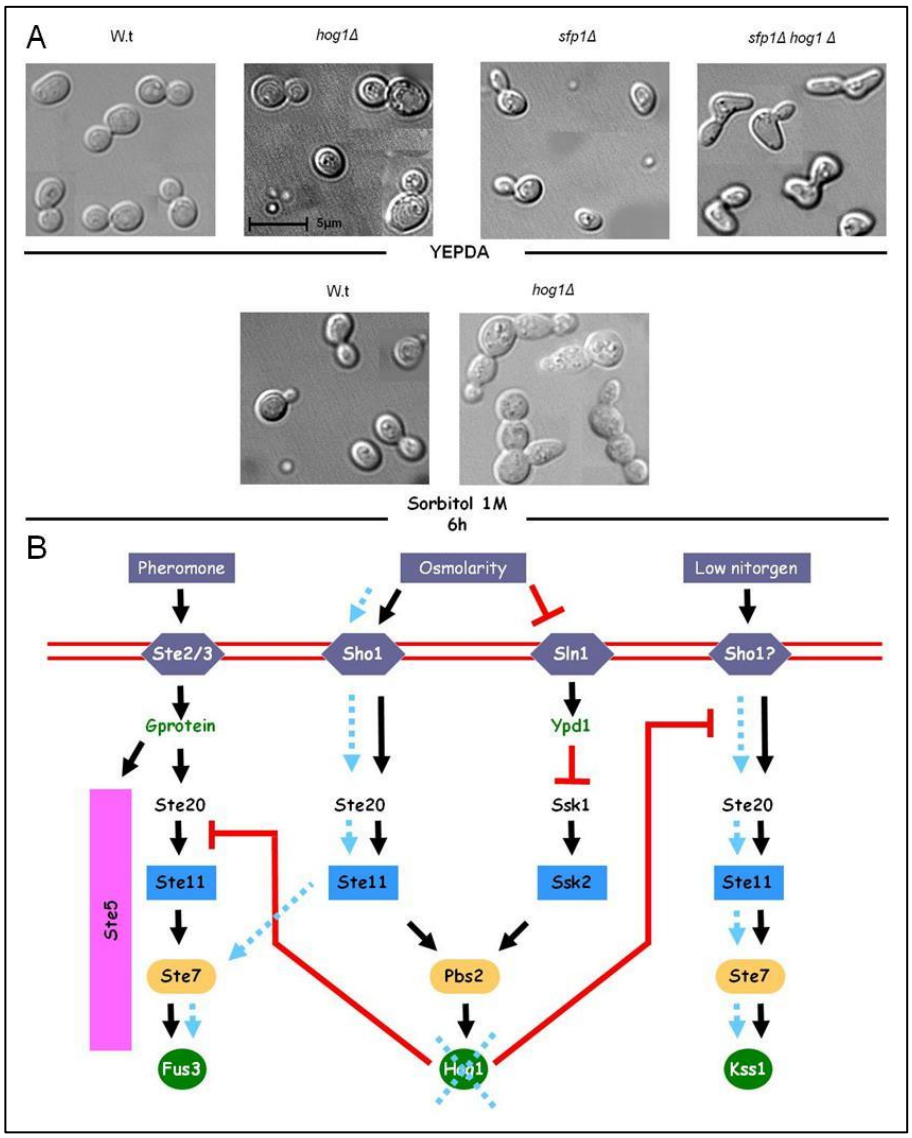


Fig. 8 The Hog1 pathway is activated in *sfp1Δ* mutants: A) Nomarski images of the indicated strains in exponential phase and after sorbitol treatment. B) Scheme of the MAPK cross-talk pathway.

5.2.9 Kss1 is phosphorylated in *sfp1Δ* cells.

Thus, we performed a Western analysis in order to evaluate if the morphological changes observed in the double mutant *sfp1Δhog1Δ* were linked to the activation of the other MAPK pathways. As shown in Fig.9 *HOG1* inactivation in *sfp1Δ* cells resulted in the phosphorylation of both

Fus3 and Kss1, similarly to what happened in *hog1Δ* cells after sorbitol treatment [Fig.9A]. In the *sfp1Δhog1Δ* mutant, the level of Fus3 phosphorylation was lower than that of sorbitol-treated *hog1Δ* cells. Levels that increased after sorbitol treatment [Fig.9A]. Surprisingly, the inactivation of *SFP1* alone induced Kss1 phosphorylation [Fig.9A].

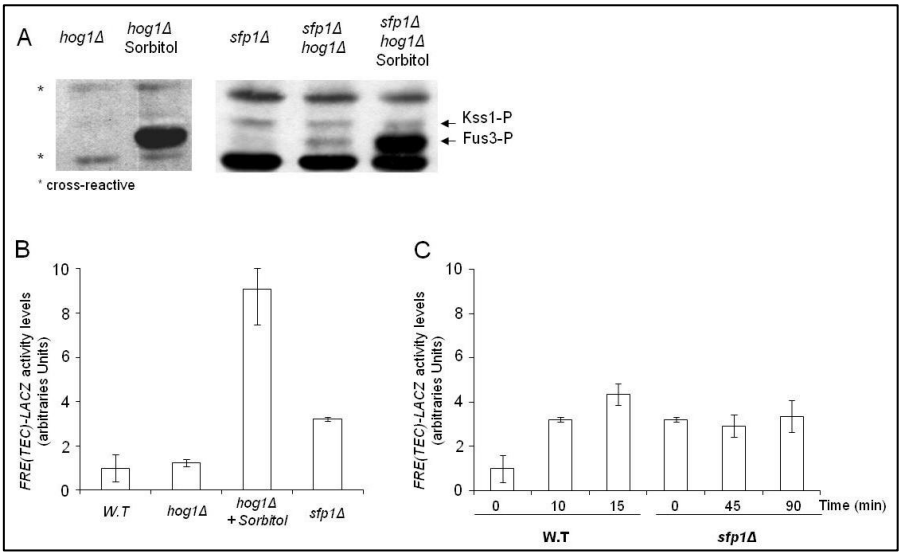


Fig.9 Kss1 is activated and phosphorylated *sfp1Δ* mutants: A) Protein extracts were probed for phosphorylated (activated) forms of Fus3 (Fus3-P) and Kss1 (Kss1-P) by immunoblotting using anti-phospho-p44/42 antibody. B-C) Exponentially wild type, *sfp1Δ* and *hog1Δ* cells carrying the *pFRE(TEC)-lacZ* plasmid were treated with KCl (1 M) or Sorbitol (1M) and assayed for β-galactosidase activity. Activity of β-galactosidase in cell extracts was normalized using protein content and expressed as Miller units (Miller, 1972). Both values obtained after osmotic stress and values obtained for the mutants were normalized to wild type at time zero which was arbitrary set to 1. All values are the average of at least two independent experiments. Standard deviations are indicated.

To confirm Kss1 phosphorylation and to determine if this is associated with the activation of the SAPK, we examined Kss1-mediated gene induction using a filamentous growth-responsive reporter (*pFRE(TEC1)::lacZ*) [10]. As shown in Fig.9B, the *FRE(TEC)*-lacZ expression was more induced in *sfp1Δ* cells compared with that in wild type and the *hog1Δ* mutant before sorbitol treatment, supporting the results obtained in Fig.9A that indicates Kss1 phosphorylation in *sfp1Δ* cells. The greater induction of reporter gene expression in the *hog1Δ* cells after sorbitol treatment that we observed in Fig. 9B is due to the presence of the high level of activated Fus1 [Fig.9A]. Indeed the Filamentous and invasive growth Response Element (*FRE*), used as a promoter for *LACZ*, is a combination of TEA/ATTS Consensus

Sequence (TCS) and the Pheromone Response Element, (PRE) that can be also recognized by activated Fus1 [35].
Then, we compared the induction of *FRE(TEC*)-lacZ expression after KCl treatment. KCl is an osmotic stress that can activate Kss1. It is known that KCl osmotic stress activates Kss1 as well as Hog1. The former activation is more transient, but when *HOG1* is deleted, activation of Kss1 is sustained [36].
As shown in Fig.9C, in wild type cells KCl induced *FRE(TEC*)-lacZ expression while, even in the absence of the salt stress, *SFP1* inactivation induced a high reporter expression. Expression that did not increase significantly after KCl treatment indicating that Kss1 activation cannot be stimulated anymore.
To test whether the effects of *SFP1* inactivation on MAPKs were specific and did not derive on a more general effect of growth rate reduction/ribosome biogenesis defect, we repeated these experiments in a *rsa1Δ* mutant strain. In the Yeast Deletion Project, a cluster analysis of the growth rates of different mutant strains on fermentable (glucose) and non-fermentable substrates (ethanol, glycerol and lactate) grouped into class II, together with *sfp1Δ* mutant the *rsa1Δ* one (mutants with a fermentable defect) (see http://www-deletion.stanford.edu/YDPM/YDPM_index.html).
Rsa1 is a protein involved in the assembly of 60S r-subunits.*RSA1* deletion leads to a slow growth phenotype similar to the *sfp1Δ* phenotype and decrease in the pool of free 60S r-subunits [37]. *rsa1Δ* strain did not show a high basal level of β-galactosidase activity and it responded to KCl stimulus in a way similar to the wild type (data not shown) indicating that Kss1 activation is a strictly linked to Sfp1 loss of function.

5.2.10 Kss1 is able to mediate Sic1 Thr 173 phosphorylation.

We found that in the *sfp1Δ* strain there was a stabilization of Sic1 mediated by Thr173 phosphorylation [Fig.5] which correlated with a Kss1 phosphorylation and activation [Fig.9] but not with Hog1 phosphorylation [Fig.8]. This makes us ask whether, in addition to Hog1, also Kss1 could mediate phosphorylation, and thus stabilization of Sic1. To assess this hypothesis we analyzed if Sic1 accumulated in response to Kss1 activation by investigating the level of Sic1 in Sic1-TAP and Sic1-TAP *hog1Δ* strains after treatment with KCl (a condition that determines a Kss1 sustained activation). Experiments were performed on cells synchronized in G1 phase

after α-factor treatment. In this condition in both strains a high level of Sic1 was observed due to G1 arrest [Fig.10A and B]. Then, cells were resuspended in fresh medium without α-factor and after 15 min of release cells were then treated or not with KCl; the scheme of the experiment is reported in Fig. 10A. The release was followed by determining the budding index [Fig.10C] and cell number (data not shown). For both strains, the removal of α-factor determined, as expected, exit from G1 arrest and resumption of cell growth. In line with the increase in the budding index, Sic1 levels decreased in parallel [Fig.10B and C]. Moreover, in *hog1Δ* cells, we could observe a delay in the exit after α-factor release respect to the wild type. In fact, in the mutant strain, both a less increase in the budding index and a slower reduction of Sic1 levels could be observed. Apart from this difference, both strains reached the maximum budding index value after 45 minutes from the release, correlated with the disappearance of Sic1. After 60 minutes, cells lose their synchrony and consequently Sic1 could again be detected.

In response to KCl addition, both strains showed a delayed exit from G1 arrest compared with non-treated cells [10B and C]. In fact, after osmostress treatment, the budding index did not increase in the same way as it did in untreated cells [Fig.10C]. Moreover, KCl addition led to Sic1 stabilization: after 45 minutes from α-factor release, in the untreated cells, Sic1 was not detectable while it was still present in treated cells [Fig.10C]. This effect is due to a major delay in the exit from G1 arrest compared with the untreated cells. In fact, after 75 minutes in the treated strains it was possible to observe both a disappearance of Sic1 and a corresponding increase in the budding index.

Since in *hog1Δ* mutant strain the KCl stress response is mediated uniquely by Kss1 and Sic1 can be stabilized this suggested that also Kss1 may mediate Sic1 stabilization [Fig.10B].

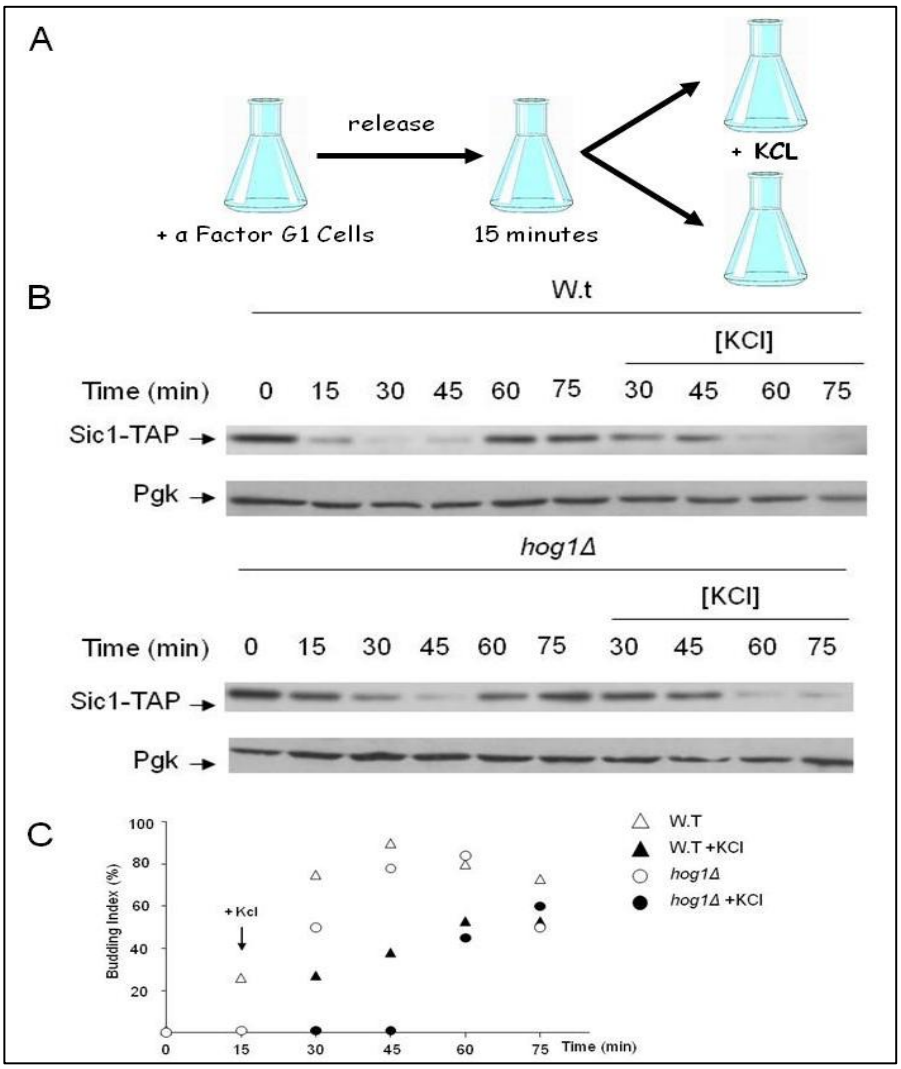


Fig.10 Activation of Kss1 results in a stabilization of Sic1: A) Scheme of the experiment; B) Wild type and *hog1Δ* cells carrying Sic1-TAP were synchronized in G1 phase with α-factor and then released into fresh media. Cells were then treated or not with KCl after 15 min from the release. Cells were taken at the indicated times and the level of Sic1 was analyzed using monoclonal antibodies against TAP. Pgk was used as a loading control. C) Budding index of cells during the release.

5.2.11 The deletion of *SFP1* induces activation of cell wall integrity (CWI) pathway

We showed that the inactivation of *SFP1* induces the activation of Hog1 and Kss1 pathways, but only Kss1 is phosphorylated. A similar situation has

been described in cells with specific glycosylation defects: these defects activate only Kss1, that once activated inhibits Hog1 phosphorylation [38].
Glycosylation is essential for normal cell wall assembly. Alterations in such a process can result in defects in the cell wall permeability. In fact, yeast o-glycosylation mutants, such as *pmt4Δ* cells, are more sensitive to Calcofluor White (CW) [39]. This compound is generally used (together with SDS, Congo Red for example) to select cell wall mutants. CW is a compound that interferes with the synthesis/assembly of one of the cell wall components such as chitin.
Thus, we investigated possible defects in cell wall permeability in the *sfp1Δ* mutants, by studying the sensitivity to CW.

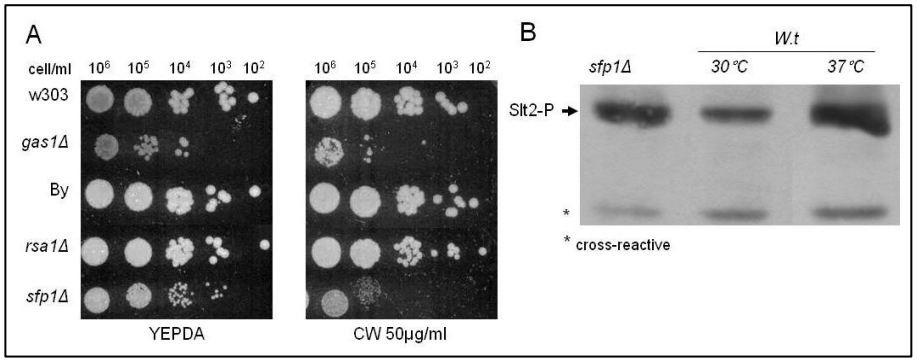


Fig.11 Activation of cell wall integrity (CWI) pathway in *sfp1Δ* cells A) Sensitivity to C.W of *sfp1Δ* cells: exponentially growing wild type, *gas1Δ, rsa1Δ* and *sfp1Δ* strains were spotted on YPD plates with and without 50 µg/ml of CW (5µl from a concentrated suspension of $1x10^7$ cell/ml and from tenfold dilutions). *gas1Δ* was used as positive control of sensitivity. B) Immunoblot analysis of Slt2 phosphorylation: cellular extracts were prepared for wild type and *sfp1Δ* cells grown at 30°C and for wild type shifts at 37°C for 2 hours. The presence of activated form of Slt2 (Slt2-P) was visualized by probing filters with anti-phospho-p44/42 antibody.

In addition to the *rsa1Δ* mutant as a negative control (as explained in the previous paragraph), also a well characterized cell wall mutant was used as a positive control for sensitivity. In fact *gas1Δ* mutant lacking the β1-3 glucanosyltransferase responsible for a correct formation of β,1-3 glucan chains, has a high sensitivity to CW [40]. Interestingly, also *sfp1Δ* cells were more sensitive to CW, while the *rsa1Δ* cells behaved as wild type cells. This indicates that the *sfp1Δ* sensitivity is not a consequence of the diminished ribosomal synthesis or the slow growth rate that characterized both these mutants but can be a specific consequence of the lack of Sfp1.

The yeast cell wall is a dynamic structure that determines the cell shape and integrity of the yeast during growth and cell division. It must provide the cell with sufficient mechanical strength to withstand changes in osmotic pressure imposed by the environment. Consistent with this notion, cell wall damage induced by wall-perturbing drugs, such as CW, caffeine, SDS or zymolyase, or by mutations in cell wall-related genes (*gas1* mutant), activates a so-called cell wall integrity (CWI) pathway (Levin 2005). The final effectors of this pathway is the MAPK Slt2/Mpk1 whose signal transduction is essential for cell wall integrity maintenance (Levin 2005).

Therefore, we analyzed the effect of *SFP1* inactivation on Slt2 activation [42]. As positive control wild type cells shocked at 37°C were used. All cells grown at 30°C or after the increase in the temperature had the same level of Slt2 (data not shown). In wild type cells the increase in the temperature determined, as expected, increase in the level of Slt2 phosphorylation (Fig.11B). Phosphorylated form that is already detectable at 30°C due to a partial activation linked to the temperature (30°C instead of 25°C), As shown in Fig.11B, in the mutant cells growing at 30°C the level of phosphorylated Slt2 was higher than that of wild type cells grown at the same temperature and was comparable with the level in wild type cells after thermal shock, indicating a CWI activation.

5.2.12 *SFP1* inactivation induces an alteration of the cytoplasmic volume/protein content ratio

The glycosylation of proteins and the synthesis of some important constituents of the cell wall (for example all the mannoproteins, the β-1,6 glucans, Gas1) have in common the secretory pathway [43] . Alterations in such a pathway result in defects in the glycosylation of proteins and in the cell wall assembly[44-47]. Another effect induced by the alteration in the secretory pathway is the attenuation of translation initiation, independently of both glycosylation and CWI activation. eIF2α phosphorylation has been identified as one of the two mediator of the translation attenuation response following secretory defects [48]. Consequently, we checked whether levels of eIF2α-P were also increased in *sfp1Δ* cells. Phosphorylations of Ser 51 in eIF2α were detected by Western analysis with antibodies specific for eIF2α phosphorylated on Ser51 (eIF2α-P) and compared with the total amount of eIF2α protein. Wild type cells after rapamycin treatment were used as a

control; in fact rapamycin is a drug that has been reported to enhance Gcn2 mediated phosphorylation of eIF2α [49].

As shown in Fig.12, although we loaded the same amount of total protein (see Pgk levels), the amount of eIF2α was dramatically reduced in *sfp1Δ* cells compared to the wild type one before and after rapamycin treatment (Fig.12A). As expected, the eIF2α-P level increased in wild type cells after rapamycin treatment (Fig.12B). Conversely, in *sfp1Δ* cells, despite the reduction in the total level of eIF2α this was more phosphorylated compared with the level in wild type cells before rapamycin treatment [Fig.12B]. These results indicate that probably the *SFP1* inactivation somehow affects the secretory pathway.

Recently it has emerged that the secretory pathway is needed to maintain nuclear shape under conditions of membrane proliferation [47]. This means that when vesicle trafficking is alterated, nuclei change their morphology increasing the nuclear envelope (NE) surface area but without affecting the Nuclear/Cytoplasm (N/C) volume ratio. In wild-type growing cells, nuclear size increases, and this increase is more closely correlated with cell size than DNA content [50]. This means that yeast maintains a constant N/C volume ratio [50] . *sfp1Δ* cells are the only *whi* mutants where the N/C ratio somehow decreases [50]. As a final refinement of our characterization, we analyzed the C volume/protein content (C/P) ratio in wild type and *sfp1Δ* cells growing both in fermentable and non-fermentable conditions. It has been shown that in *sfp1Δ* mutants the reduction in cell size is not only a consequence of the reduced growth rate, but it is tightly linked to the cellular metabolism [5]. Indeed in respiro-fermentative growth conditions (ethanol or glycerol) *sfp1* mutant cells enter S phase with a critical cell size higher than that of cells grown on glucose [5].

Thus, wild type and *sfp1Δ* strains were grown in YNB-aa medium with different carbon sources such as glucose, ethanol and glycerol which is also the only condition of growth where all Sfp1 is cytoplasmic. During the exponential phase, samples were taken to monitor the cell volume measured with a Coulter particle analyzer and the protein content measured with FACS (see Materials and Methods).

As shown in Fig. 12C both in the wild type and the *rsa1Δ* cells (used as a control as described in previous paragraphs) the C/P ratio remained constant in all growth conditions. Differently in *sfp1Δ* strains this ratio was increased under fermentative growth conditions while in respiro-fermentative growth

conditions was comparable to the ratio of wild type cells. taken together all the results indicate that the inactivation of the *SFP1* gene is accompanied with an increase in the cytoplasmic volume. This increase is not due to vacuole increase (data not shown) and is under investigation (secretory membrane proliferation?) .

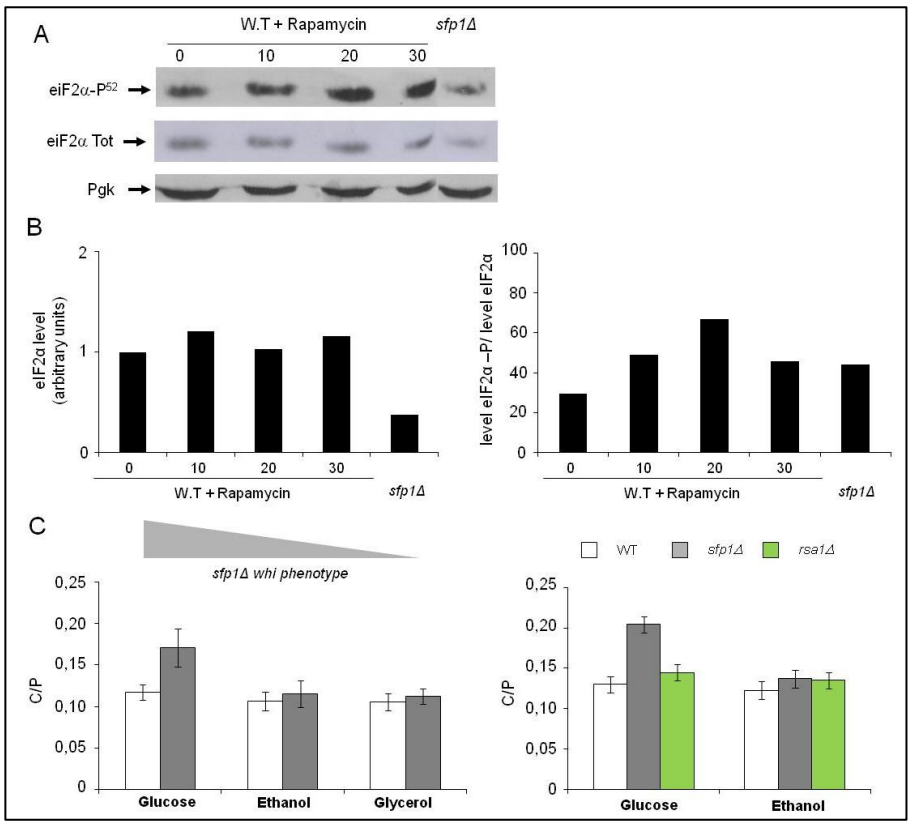


Fig.12 The lacking of *SFP1* gene induce alteration cell volume. A) Immunoblot of cell extracts from wild type (W.T), *sfp1*Δ cells exponentially growing on glucose**.** Immunodecoration was performed with specific antibodies against SLT2α and its phosphorylated form.Pgk was used as housekeeping loading control. B) (Left): The level of eIF2α relative to Pgk was determined by densitometric analysis. The values obtained after rapamycin treatment and values obtained for the mutants were normalized to wild type at time zero which was arbitrary set to 1. All values are the average of at least two independent experiments. (Right): level of eiF2α-P respect to the total. C) Cytoplasmic volume/protein content (C/P) of wild type, *sfp1Δ* and *rsa1Δ* cells grown in glucose, ethanol and glycerol. Cell volume was measured with a Coulter particle analyzer while the protein content was determined with FACS analysis (see Material and Methods). Standard deviations are shown.

5.2.13 Kss1 is involved in the rapamycin response (preliminary data)

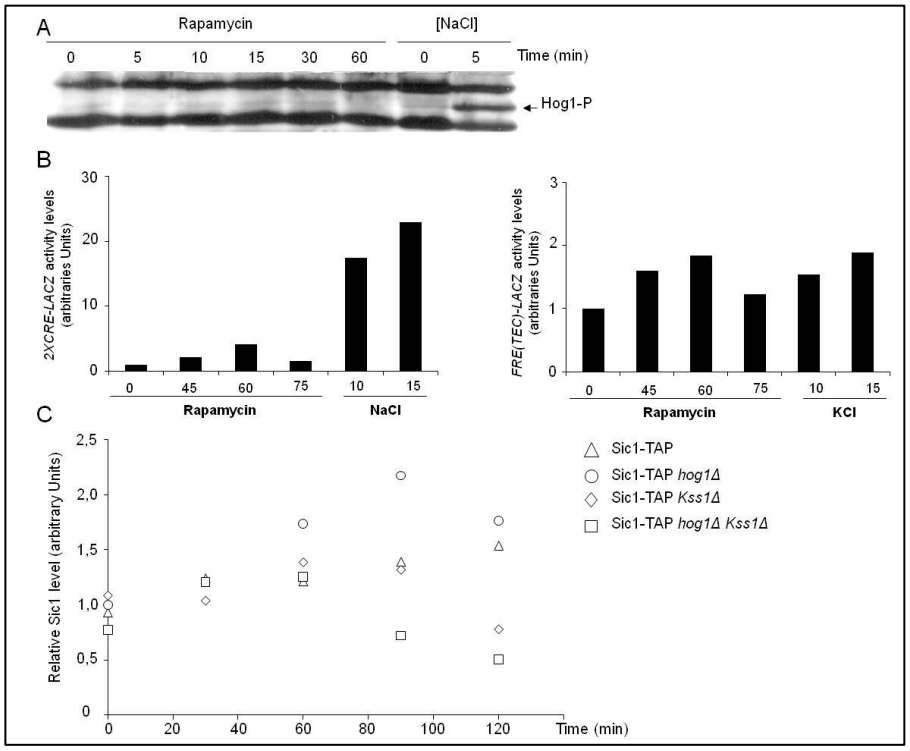


Fig. 13 Rapamycin treatment induces activation of Kss1: A) Immunoblot analysis of Hog1 phosphorylation: cellular extracts were prepared for wild type cells grown in YEPD and treated with rapamycin (0,2µg/ml) or with 0.4M NaCl for the times indicated. Hog1-P was visualized by probing filters with an antibody anti-phospho Hog1. B) (Left) Wild type cells carrying the 2XCRE-lacZ plasmid (Left) and wild type cells carrying the pFRE(TEC)-lacZ plasmid (Right) were grown exponentially and treated with rapamycin (0,2µg/ml) and with NaCl (0,4M Left) or with KCl (1M Rigth) for the times indicated and assayed for β-galactosidase activity. Activity of β-galactosidase in cell extracts was normalized using protein content and expressed as Miller units (Miller, 1972). Values obtained after treatment were normalized to wild type at time zero which was arbitrary set to 1. C) Sic1-TAP, kss1Δ Sic1-TAP, hog1Δ Sic1-TAP, kss1Δhog1Δ Sic1-TAP were grown to early exponential phase and treated with rapamycin (0,2µg/ml) Samples were taken at the time point indicated for Western analysis with anti-TAP and anti-Pgk antibody (data not shown). The Sic1 level relative to Pgk band was determined by densitometry for each lane and the values obtained after treatment were normalized to at time zero for each strains which was arbitrary set to 1.

5.3 Discussion

Here, we provide evidence that G1-S elongation that affects the *sfp1Δ* mutant is a consequence of the stabilization of the Cki Sic1. Furthermore we show that the inactivation of *SFP1* induces a complex activation of the MAPKs pathway. Finally, we provide evidence that the inactivation of *SFP1* gene induces an alteration in the cytoplasmic volume/protein content ratio of the cell. Models summarizing all the results are presented in Fig. 14-16.

5.3.1 Regulation of Sic1 stability in *sfp1Δ* cells.

In yeast, cells must reach a critical cell size to progress through Start. Cells sense if ribosome biogenesis, and not the number of mature ribosomes, is adequate for cell cycle progression at Start. Indeed, decreasing the rate of ribosomal biogenesis lowers the critical size setpoint needed for budding [51]. *SFP1* has been identified in a screening for mutants involved in the size control mechanism at Start. In fact, SFP1 deletion gives rise to one of the smallest *whi* mutants, displaying a disproportionate effect on size relative to the change in the growth rate. *Whi* mutants undergo Start at a smaller cell size [13] . Sfp1 is an unusual zinc finger transcription factor that activates the expression of genes whose product are involved in the Ribi and RP regulons [13,14,19]. The mechanism of how Sfp1 couples ribosome biogenesis to Start is unknown.

In order to better elucidate the role of Sfp1 as a negative regulator of Start, we analyzed the level of some of the key players of the G1 to S transition, the G1 cyclins (Cln1-3) and the Cki Sic1, in the *sfp1Δ* mutant [Fig.1]. Cln1 and Cln2 cyclins are responsible, on the one side, for the activation of B-type cyclins together with Cln3 [52] and on the other side, responsible for bud formation [53,54]. As previously reported, *sfp1Δ* cells are characterized by a *whi* phenotype, slow growth, decreased budding index and elongation of G1 phase. Accordingly with this phenotype, the Cln1-2 level resulted decreased in the mutant strain respect to the wild type one [Fig.1B and C]. On the contrary the levels of Cln3 in the mutant strain were only slightly affected [Fig.1B and C], in agreement with genetic analysis of Jorgensen and coworkers. By analyzing the phenotype of various mutants they concluded that the mechanism through which Sfp1 couples ribosome biogenesis to Start is independent of Cln3 and Whi5, because the cell size setpoint can be

changed independently of *CLN3* and *WHI5* [19]. Sic1 is a negative regulator of Start whose Cln1/2-dependent degradation is required for entry into S phase. After binding the Clb5-Cdc28 complexes, it inhibits their function so that DNA replication cannot occur anymore. Our results indicated that *SFP1* inactivation is accompanied by a stabilization of Sic1 [Fig.1-4]. In fact i) in the *sfp1Δ* growing cells, Sic1 levels were the same as (or only slightly higher) than in the wild type cells [Fig.1B and C]. ii) Sic1 was entirely nuclear while in wild type exponentially growing cells, it localized both in the nucleus and in the cytoplasm [Fig.2A]. Moreover, iii) in the mutant cells all Sic1 was linked to Clb5, even if Clb5 levels were lower than in the wild type [Fig.2B]. Finally, iv) Sic1 level in the mutant was independent from Cln2 level [Fig.3]. In fact, while overexpression of Cln2 in a wild type strain reduced Sic1 level, in the mutant no changes were observed.

Furthermore, the stabilization of Sic1 in the mutant cell was found to be dependent on threonine 173 (Thr173). It is well known that the phosphorylation of this residue induces Sic1 accumulation by interfering with the binding of ubiquitin-conjugating enzymes [28]. Substitution of Sic1 Thr173 residue by alanine resulted in a less stable Sic1 in the *sfp1Δ* cells.

A Sic1 stabilization is in according with the *sfp1Δ* phenotype. In fact, for some aspects the phenotype of *sfp1Δ* is similar to *SIC1-0P* cells. These cells expressed an unphosphorylable (*SIC1-0P*, all nine phosphorylation sites mutated) and thus stable Sic1. Similarly to *SFP1* inactivation, replacement of *SIC1* with *SIC1-0P* resulted in a lengthened G1 while the budded period is significantly shorter [25] . The evidence that both the elongation of G1 phase and reduction of the length of G2/M transition in the mutant is a consequence of Sic1 stabilization is supported by the fact that inactivating *SIC1* in the *sfp1Δ* cells, resulted in a premature entrance in S-phase and a slow G2/M transition [Fig.4].

A similar situation but leading to a G1 arrest is that observed after inhibition of TORC1 by rapamycin where there is a initial accumulation of Sic1 to restrict entry into S-phase. This inhibitor accumulates in the nucleus to avoid improper Clb5/6-Cdc28-driven DNA replication under conditions of poor nutrient availability [22]. Sic1 stabilization by phosphorylation on Thr173 and localization in the nucleus to avoid uncorrect initiation of DNA replication take place also after inhibition of TORC1 by rapamycin[22]. This parallelism between the response induced by *SFP1* inactivation and what has been observed after rapamycin treatment is in according with the

fact that Sfp1 associated with Tor1 kinase [55], and that this binding is essential for a correct localization of TORC1 together with Sfp1 at the RP promoters [55].

Furthermore, a similar mechanism has been also described in response to osmotic stress. In fact, the activation of the Hog1 MAP kinase after NaCl exposure, induces the stabilization of Sic1, always via Thr173 phosphorylation [28]. In this context, the cellular response to *SFP1* inactivation that we observed is more similar to the response to osmotic stress than the response to rapamycin. In fact, the latter induces a G1 arrest linked to a Sic1 stabilization and, subsequently, a decrease of Cln3 accumulation takes place. This decrease is essential for maintaining a prolonged G1 arrest [22]. After the osmotic stress Sic1 is stabilized, Cln1 and Cln2 are low and Cln3 levels are unaffected [28] as in our mutant. In addition, the stress response do not always provoke a cell cycle arrest, but is often a slowdown of cell cycle progression, necessary for cell adaptation to new conditions [56]. Only in case the stress is too intense, cells arrest growth. Our observations that *SFP1* inactivation results in a Sic1 stabilization can be resumed in the following model of G1-S transition. Cross and coworkers hypothesized that G1 phase is made up of two temporally uncorrelated processes in G1 which are separated by the molecular event of Whi5 inactivation and nuclear exit [57] [Fig.14]. The upstream module (size-dependent) is responsible for cell cycle control and depends on *CLN1-2*-dependent positive feedback more than on a linear Cln3-Whi5-SBF pathway, whereas the downstream (size-independent) module actuates cell cycle progression. The downstream module is dependent on Cln-kinase activity in order to modulate many events among which the main is Sic1 phosphorylation and degradation leading to DNA replication. We hypothesize that this second module is also regulated by stress [Fig.14].

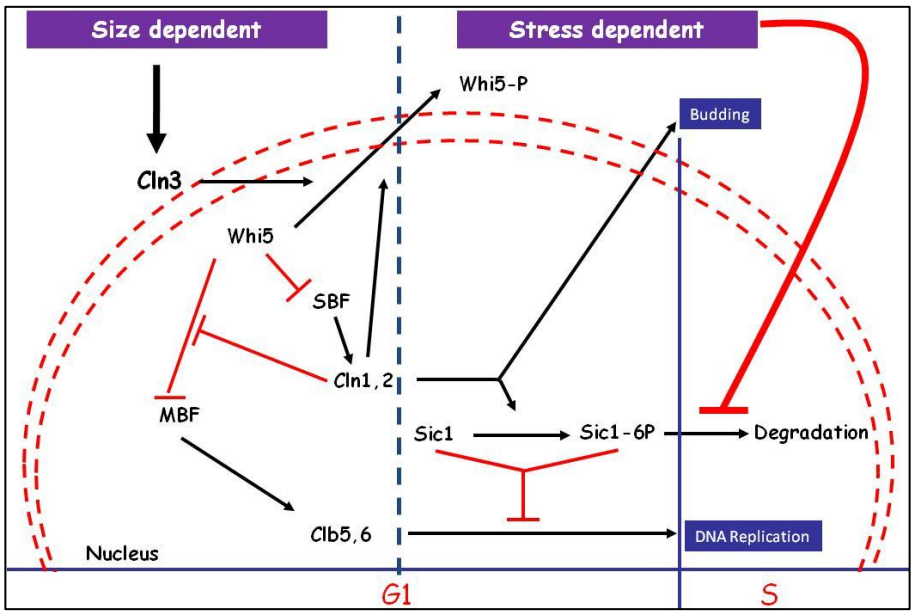


Fig. 14. Schematic representation of the model proposed

In this context, initially stress induces Sic1 stabilization leading to a G1 elongation, but only if the stimulus is intense cells modulate the first module by reducing the cell size in a Sfp1-dependent way. This model is also coherent with cell response to rapamycin treatment. In fact, as reported, the first event that characterizes the cell response to rapamycin is Sic1 stabilization [22]. Thus, the first cellular response to TORC1 inactivation is stress-like. Only subsequently by inhibition of ribosome biogenesis, the first module can be regulated by Cln3 reduction. This induces a G1 arrest.

According to this model, it follows that the lack of Sfp1 alone is not enough to affect the first module as the inhibition of TOR pathway can do. However this is in agreement with the fact that TOR inhibition induces both the exit of Sfp1 from the nucleus (and thus inactivation) and the dephosphorylation (and thus inactivation) of Sch9, another substrate of TOR that acts in parallel with Sfp1 in the expression of the Ribi and RP regulons [13,19]. Differently, after the *SFP1* deletion the transcription of RiBi and RB genes is not completely abrogated. In fact Sfp1, negatively regulates the phosphorylation of Sch9 by TORC1 necessary for its activity. Consequently, the *SFP1* inactivation induces Sch9 hyperphosphorylation which is therefore partly able to compensate the lack of Sfp1. Moreover, the regulation of the second

module via Sic1 in the *sfp1Δ* cells can also explain the difference in the critical cell size observed in the *sfp1Δ* mutant. Indeed Laabs and coworkers suggested that *S. cerevisiae* cells may not have a size checkpoint at all [58]. Instead, they suggested that yeast cell maintains its size by coordinating the rate of cellular growth with the rate of progression through G1 [58]. In this frame, *SFP1* inactivation affecting cell size could interrupt the balance between growth, by reducing the synthesis of ribosomes, and the progression through the cell cycle, by the stabilization of the G1 Cki Sic1.

5.3.2 *SFP1* inactivation induces a complex activation of the MAPKs pathway.

The results exposed in the previous paragraph suggest that the *SFP1* inactivation induces the activation of the stress response pathway. Cell modulates stress response via the activation of mitogen-activated protein kinase (MAPK). Yeast cells use different MAP-kinases, which respond to different conditions such as pheromone signals, osmolarity, cell wall stress and nutritional status [59]. Their activation results in the generation of a set of adaptive responses that leads to the modulation of several aspects of cell physiology essential for cell survival, such as gene expression, translation, morphogenesis, and regulation of cell cycle progression.

Modulation of gene expression by stress activates a program called the Environmental Stress Response (ESR) that consists of approximately 600 repressed genes and approximately 300 induced genes. The Rpd3-Large complex is required for proper expression of both induced and repressed ESR genes under multiple stress conditions [60]. Moreover, the inhibition of the TOR pathway by rapamycin leads to the recruitment of Rpd3 to gene promoters [61]. We showed by RT-PCR that similar to a stress the Sfp1 lost of function induces an Rpd3 mediated genes expression [Fig.6].

Thus, we decided to study if in the stress response linked to *SFP1* inactivation the Hog1 pathway was involved. In fact, the Sic1 phosphorylation of Thr173 and the transcriptional response mediated by Rpd3 detected in the *sfp1Δ* cells [Fig.5 and 6] are both mediated by Hog1.

The transient osmostress-induced activation of Hog1 correlates with its phosphorylation mediated by the MAPKK Pbs2 [62], and with its nuclear translocation [63]. Once activated Hog1 can directly induce the transcriptional response to osmostress.

By analyzing the activation of Hog1 in *sfp1Δ* cells, we showed that as a consequence of *SFP1* inactivation there is a basal level of Hog1-dependent transcription but Hog1 is not phosphorylated [Fig.7]. Nevertheless, in the *SFP1* mutant the pathway responsible for Hog1 phosphorylation is constitutively active. In fact, after *HOG1* deletion *sfp1Δ* cells lose their ellipsoidal shape and became elongated with a shmoo-like morphology [Fig.8]. It is known that the same morphological alterations can be induced in *hog1Δ* cells only after a high induction of the pathway by osmostress [33]. In the budding yeast the Fus3, Kss1, and Hog1 MAPK pathways share a series of components, yet cross-talk between these pathways is avoided [Fig.8B]. One function of activated Hog1 is to evade the activation of Kss1 and Fus3 after osmostress stimulation [64]. Fus3 is responsible for the formation of shmoo morphology and Kss1 for the pseudohyphal morphology. Consequently, the simultaneous activation of these two MAPK explains the morphological alterations that occur in *hog1Δ* cells following osmostress [64].

Interestingly, in the *sfp1Δ* cells Kss1 results both phosphorylated and able to induce the activation of a filamentous growth-responsive reporter [Fig.9]. Kss1 is the MAP kinase that primarily functions under conditions of nutrient deprivation such as the lack of nitrogen and/or glucose in the growth media [65]. Under these conditions the signal mucin Msb2 regulates the activation of the filamentous growth (FG) pathway that induces the phosphorylation of Kss1, necessary to guarantee cell survival [66]. Consequently, the presence of the active form of Kss1 in our mutant may be due to the fact that the lack of Sfp1 is felt by the cell as a condition of nutrient scarcity. In fact, under optimal growth conditions, Sfp1 localizes to the nucleus, where it promotes the RP and RiBi genes expression. In response to changes in nutrient availability, Sfp1 is released from RP and RiBi gene promoters and exits from the nucleus; thus, the ribosome biogenesis is down-regulated [67]. Similarly to glucose and nitrogen limiting conditions, *sfp1Δ* cells also display a decrease both in rRNA and protein content.

It was also reported that, following scarce nutrient availability, the reduction of the tRNA modification complexes can induce Kss1 activation [68]. It follows that Kss1 activation in the mutant might be due either to a direct effect of Sfp1 lack o to diminished ribosomal/protein synthesis. Experiments

performed on a slow growth mutant with defects in ribosomal assembly lead us to exclude the latter hypothesis

The activation of Kss1 in *sfp1Δ* cells can also explain the absence of a high activation of Hog1 [Fig.7]. In fact, since Msb2 is also required for activation of the Hog1 pathway a reciprocal inhibitory loop exists between these pathways allowing stable activation of only one under various stress conditions [69]. Finally, we showed that Kss1 is able to stabilized Sic1 [Fig.10]. This stabilization event may happen through the phosphorylation of Thr173. In fact, the MAPK consensus sequence are not characterized by high stringency (Serine or Threonine followed by Proline). This means that if one of the MAPKs is not present the others can phosphorylate and thus act on the same substrates. So cells need a strong stringency mechanism of cross-talk to avoid improper activation of substrates.

Thus, we can conclude that not only Sfp1 is regulated by stress and nutrients (both affecting its localization), but that Sfp1 can in turn regulate the stress response. This observation is in agreement with the fact that the stress response is transient and, after cell adaptation, it has to be switched off.

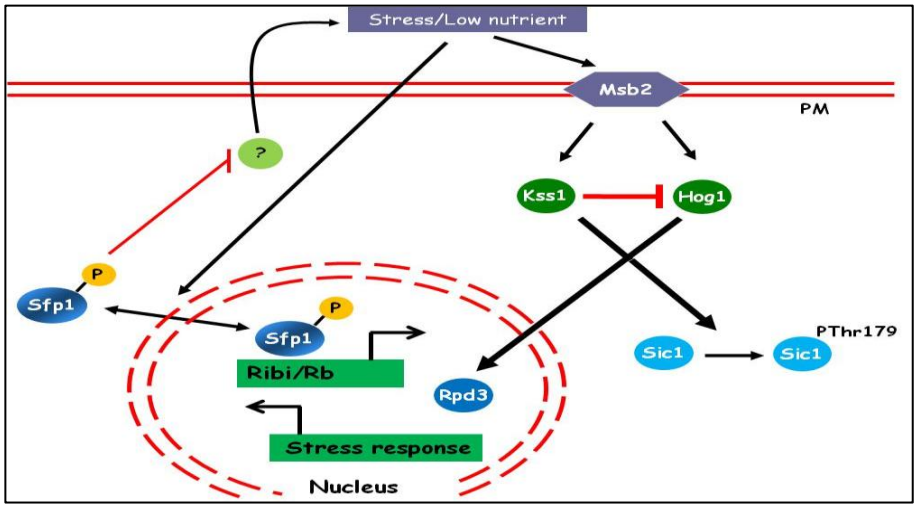


Fig. 15. Schematic representation of the model proposed

5.3.3 *SFP1* inactivation induces an alteration in the cytoplasmic volume/protein content ratio.

The results exposed in the previous paragraph suggest the deletion of *SFP1* induce the activation of FG pathway. It has been reported that FG pathway

can be also activated either by defective glycosylation of Msb2 [70] or by Msb2 proteolytic cleavage [71]. On the basis of our results, we hypothesize that *SFP1* inactivation might be accompanied by a glycosylation defective-like response [Fig.11-12]. In fact i) it was seen that (in a way similar to what we observed in the mutant cells) activation of FG pathway by inhibition of N-glycosylation combined with a specific O-glycosylation defect induces activation of both Hog1 and Kss1 pathways and Kss1 is also phosphorylated [70][Fig.7-9]. We showed ii) that *SFP1* inactivation induces defects in cell wall permeability [Fig.11]. In the same way, alterations in the glycosylation can also result in defects in the cell wall permeability due to the essential role of the glycosylation in the formation of some components of the cell wall. Furthermore, as a result of the cell wall alterations *sfp1Δ* cells activate the cell wall integrity (CWI) pathway (Levin 2005). This is a pathway that regulates the cellular responses to cell wall and membrane stress by MAPK Slt2/Mpk1 whose signal transduction is essential for cell wall integrity maintenance[41].

The induction of a glycosylation defective-like response in the mutant can be linked to alterations in the secretory pathway. In fact, *sfp1Δ* cells are also characterized by an attenuation of translation initiation (mediated by the phosphorylation of eIF2α): an effect described following secretory defects [48] [Fig.12]. Moreover, recently Shore and Tyers laboratories have found a link between Sfp1 and the secretory pathway that involves the importin Mrs6 [55,72] . Mrs6 is an essential escort protein that senses vesicle flux by virtue of its essential interactions with the Rab GTPases, including Ypt1, Sec4, Ypt6, Vps21, and other family members that control vesicle trafficking at different stages in the secretory system [73-75].

Mrs6 is required for the cytoplasmic relocalization of Sfp1 under poor nutrient conditions through a direct binding. The binding site on Mrs6 for Sfp1 is shared with an essential Rab GTPase. In this frame, we can hypothesize that a reduction of ribosome biogenesis may induce a defect in the secretory pathway leading to the activation of Msb2 and thus of the FG pathway [Fig.16]. The exit from the nucleus of Sfp1, necessary for the reduction of ribosome biogenesis, allows the release of the Rab GTPase that is essential to switch off the defect in the secretory pathway. According to this model *rsa1Δ* cell didn't show activation of FG pathway. In fact, since the transcriptional regulation of RP and RiBi genes is tightly co-ordinated [76], the lack of Rsa1 probably only induces a reduction of RiBi

transcription mediated by the exit of Sfp1. Moreover, when conditions are critical (poor nutrient conditions) and the cells cycle has to be stopped, the stress signal that follows the ribosome reduction is higher than the switch off signal due to Sfp1. In fact, we observed an activation of the Kss1 pathway after inhibiting TOR pathway by rapamycin treatment [Fig.13 preliminary data].

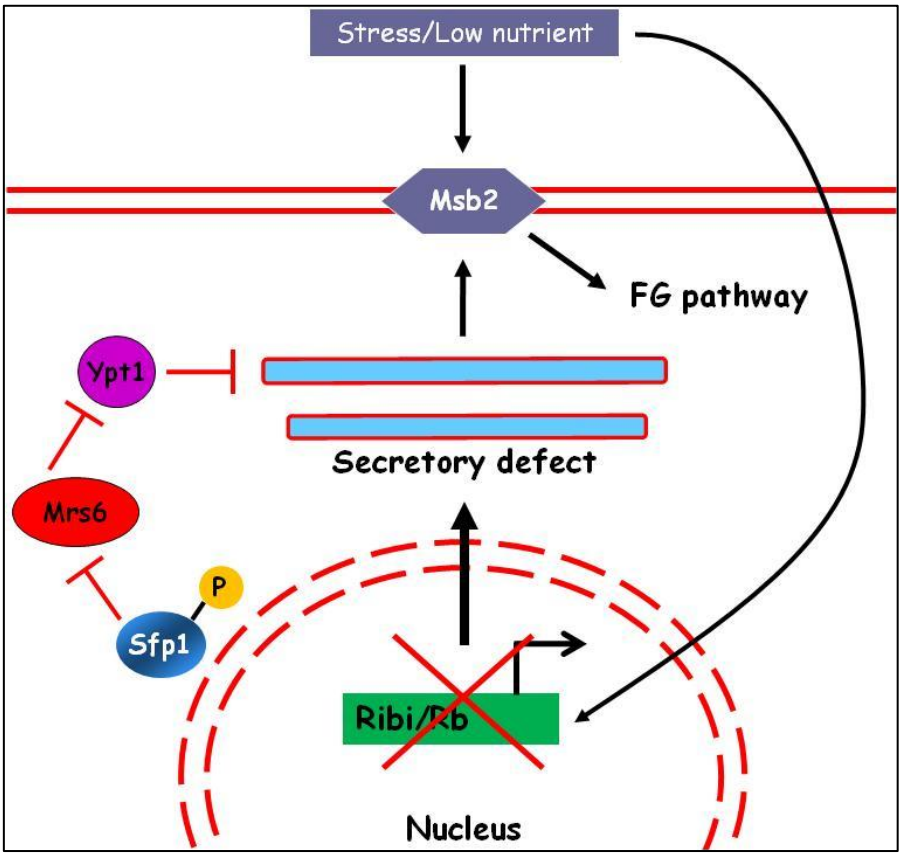


Fig. 15. Schematic representation of the model proposed

Finally, we showed that similarly to the Nuclear/Cytoplasmic (N/C) volume ratio [50] also the Cytoplasmic/Protein content (C/P) volume is maintained constant in budding yeast [Fig.12]. Both these values result alterated in the *sfp1Δ* cells due to an increase in the cytoplasmic volume.

This let us to speculate that growth might be composed of two elements: the Size that is the growth in cell volume and the Mass that is the increase in the protein content. Consequently, alterations of cell growth in response to changes in the environmental conditions imply a coordinate regulation of

Size and Mass with the aim of maintaining their ratio constant. This is in agreement with a recent work showing that the rate of cell growth is governed by cell cycle stage [77]. In fact, this work showed that i) the cell growth is not constant during the cell cycle and that this change ii) reflects a change in actin cytoskeleton that is connected to the growth in Size. In fact, vesicles that are required for the increase of the cell membranes are transported to sites of fusion on actin cables.

Moreover, the presence of a mutually regulation between these two aspects of cell growth has been recently described in mammalian cells. One of the principal regulator of the actin cytoskeleton dynamics is the mTORC2 complex whose activity is linked to ribosomal biogenesis [78].

This can suggest that, similarly to mammalian cells, also in yeast might exist a regulation of ribosomal biogenesis by TORC2 and consequently a link to Mass and Size.

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Riassunto

Per poter mantenere l'omeostasi delle dimensioni cellulari è richiesto che le cellule in attiva proliferazione coordino la crescita con il ciclo cellulare cosi che ad ogni divisione corrisponda un raddoppio della massa. Il mantenimento delle dimensione è una caratteristica universale, ma allo stesso tempo, il meccanismo di controllo sul ciclo cellulare risulta essere poco chiaro.

Nel lievito gemmante *Saccharomyces cerevisiae* la coordinazione fra crescita e divisione avviene durante lo "Start", un breve periodo durante la tardiva fase G1 dopo il quale la cellula inizia a dividersi. Un prerequisito necessario per il passaggio attraverso Start è il raggiungimento di una massa critica, il cui valore è determinato dalle condizioni ploidia e di crescita.

Il raggiungimento di una massa critica permette di mantenere uniformi le dimensioni cellulari anche dopo molte generazioni e garantisce che in presenza di scarsi nutrienti la cellula inizi a dividersi solo dopo aver accumulato abbastanza energia per poter sostenere il completamento della divisione. I nutrienti modulano la soglia di massa critica necessaria per iniziare la divisione a seconda della velocità di proliferazione. Generalmente cellule che crescono lente in terreno povero passano lo Start con una massa minore rispetto a quella di cellule che stanno crescendo rapidamente in un terreno ricco. In *S. cerevisiae* i mutanti che esibiscono un disaccoppiamento tra crescita e divisione cellulare posso essere caratterizzati da due fenotipi: mutanti small (*whi*) o large (*lge*). I primi superano Start, con dimensioni minori mentre i secondi con dimensioni maggiori. Inoltre mediante lo studio sistematico delle dimensioni cellulari associate alla delezione di ognuno dei geni di lievito ha permesso l'identificazione di nuovi potenziali regolatori di Start. Molti dei geni che codificano per potenziali repressori dello Start sono risultati essere implicati nella biogenesi dei ribosomi suggerendo l'esistenza di un legame tra questi due processi. Uno dei fenotipi *whi* più piccolo e risultato essere dovuto alla inattivazione di *SFP1*.

L'inattivazione di *SFP1* una variazione sulle dimensione sproporzionato rispetto a quella che si osserva sulla velocità di crescita.

Il gene *SFP1* codifica per un fattore trascrizionale che appartiene alla famiglia delle proteine caratterizzate dalla presenza di un dominio "Zinc-

Finger". Sfp1 è un regolatore chiave nella trascrizione dei geni coinvolti nella biogenesi dei ribosomi la cui funzione è richiesta per la normale crescita. La localizzazione nucleare di Sfp1 richiede che il complesso TORC1 sia attivo ed è inoltre sensibile agli stress ambientali. Inoltre, Sfp1 risulta interagire direttamente ed essere target di TORC1. Diversamente da Sch9, che e il maggiore effettore a valle di TORC1, la fosforilazione di TORC1 su Sfp1 risulta non essere influenzata ne da stress osmotici ne da stress dovuti alla scarsa presenza di nutrienti, suggerendo un differente modo di regolazione di Sfp1. Significativamente Sfp1, attraverso la sua funzione di attivatore trascrizionale, esercita un controllo a feedback negativo sulla attività di TORC1 verso la chinasi Sch9.

Sfp1 interagisce anche con MRS6, una proteina conservata della famiglia delle "Rab escort" la cui funzione e quella di regolare la localizzazione nucleare di Sfp1. L'interazione con MRS6 di Sfp1 e TORC1 è legata a una connessione ancora poco compresa tra il signaling di TOR e il trasporto vescicolare.

Lo scopo di questo lavoro e stato quello di caratterizzare meglio le relazioni tra Sfp1 e il controllo delle dimensioni cellulari e alcune vie di trasduzione del segnale coinvolte nella coordinazione tra crescita e divisione.

Per poter meglio chiarire il ruolo di Sfp1 come regolatore negativo dello Start abbiamo analizzato i livelli di alcuni elementi chiave della transizione G1-S ossia le cicline di fase G1 (Cln1-3) e la proteina Cki Sic1 nei mutanti *sfp1Δ*. Le cellule mancanti il gene *SFP1* sono caratterizzati da un fenotipo di tipo *whi*, crescita lenta, riduzione dell'indice di gemmazione, allungamento della fase G1 e riduzione della transizione G2/M. In accordo con alcune di queste caratteristiche fenotipiche i livelli di Cln1 e Cln2 sono risultati diminuiti mentre i livelli di Cln3 non hanno subito variazioni. I livelli di Cln3 sono accordo con quanto riportato che indica che il meccanismo attraverso il quale Sfp1 accoppia la biogenesi dei ribosomi con lo Start risulta indipendente da questa ciclina. Il maggior effetto della delezione di Sfp1 si è osservato essere su Sic1 che risulta essere interamente nucleare tutta legato a Clb5 e stabilizzata dalla fosforilazione sulla treonina 173 (Thr173). Fosforilazione che è nota'indurre l'accumulo di Sic1 prevenendo la sua degradazione. Nel mutante *sfp1Δ* la stabilizzazione di Sic1 e richiesta sia per l'allungamento della fase G1 che per la riduzione della transizione G2/M. Una situazione simile che coinvolge la stabilizzazione di Sic1, sempre mediante la fosforilazione della treonina 173, ma che porta ad un

arresto nella fase G1 è quella che si osserva dopo l'inibizione di TORC1 con la rapamicina. In questo caso si osserva un accumulo Sic1 necessario per impedire ai complessi Clb5/6-Cdc28 di iniziare la replicazione del DNA in una condizione di scarsa disponibilità di nutrienti. Questo parallelismo e in linea con il fatto che Sfp1 è associato con la chinasi Tor1 e che questo legame è essenziale per la corretta localizzazione di TORC1 assieme a Sfp1 a livello dei promotori dei geni RP.
Una condizione di scarsa disponibilità di nutrienti per la cellula può essere considerata una situazione di Stress. Allo stesso modo anche l'attivazione della MAP chinasi Hog1, dopo stress osmotico induce la stabilizzazione Sic1 sempre attraverso la treonina 173. La risposta cellulare alla inattivazione di *SFP1* appare molto più simile alla risposta alla stress osmotico rispetto alla risposta alla rapamicina. Infatti la seconda induce arresto in G1 legata alla stabilizzazione di Sic1 e successivamente alla riduzione di Cln3 necessaria per mantenere un arresto prolungato in G1. Al contrario dopo stress osmotico Sic1 e stabilizzato, i livelli di Cln1 e Cln2 sono ridotti mentre quelli di Cln3 non sono alterati come nel mutante. Inoltre la risposta allo stress non sempre provoca un arresto del ciclo cellulare ma spesso e associato a un rallentamento della progressione del ciclo cellulare necessaria per l'adattamento alle nuove condizioni. Solo se lo stress e troppo intenso le cellule arrestano la crescita. Le cellule di lievito modulano la risposta allo stress attraverso le MAPK che rispondono a differenti condizioni quali ferormoni (mediata dalla MAPK Fus3), osmolarità (mediata dalla MAPK Hog1), scarsi nutrienti (mediati dalla MAPK Kss1) e stress da alterazioni della parete cellulare (mediata dalla MAPK Slt2). Dato che la le prime tre MAPK condividono alcuni elementi della via di trasduzione del segnale quando una delle tre vie è attiva le altre risultano soppresse (cross-talk). La risposta legata alla inattivazione di *SFP1* coinvolge un complesso cross-talk tra il pathway di Hog1 e quello di Kss1. Entrambi i pathway sono risultati attivi ma solo Kss1 e fosforilato. Kss1 è la MAP chinasi che funziona principalmente in condizioni di scarsità disponibilità di nutrienti quali la mancanza di azoto e/o glucosio nel terreno di crescita. In queste condizioni la proteina Msb2 regola l'attivazione del pathway di crescita filamentosa (FG) che induce la fosforilazione di Kss1 necessaria per garantire la sopravvivenza cellulare. La mancanza di Sfp1 e sentita dalla cellula come una condizione di scarsa disponibilità di nutrienti. Infatti in presenza di ottime condizioni di crescita Sfp1 localizza nel nucleo dove promuove

l'espressione dei geni RB e RiBi. In risposta ai cambi di disponibilità di nutrienti Sfp1 e rilasciato dai promotori ed esce dal nucleo e conseguentemente la biogenesi dei ribosomi viene ridotta. Inoltre, poiché Msb2 è richiesta anche per l'attivazione del pathway Hog1 esiste un meccanismo di reciproca inibizione tra le vie Hog1 e Kss1 che garantisce, in condizione di scarsi nutrienti, l'attivazione stabile solo di Kss1. Abbiamo scoperto che una volta attivata Kss1 è in grado di stabilizzare Sic1. Abbiamo ipotizzato che l'attivazione dell'FG pathway dovuta all'inattivazione di Sfp1 coinvolge una risposta simile a quella che si osserva in seguito a difetti nella glicosilazione. Infatti l'attivazione del FG pathway dovuta alla inibizione della N-glicosilazione combinata con una specifico difetto nella O-glicosilazione induce l'attivazione sia del pathway di Hog1 che di Kss1 ma anche in questo caso solo Kss1 risulta essere fosforilata. Inoltre l'inattivazione di *SFP1* induce anche alcuni difetti che sono stati osservati seguire l'inibizione della glicosilazione quali l'alterazione nella permeabilità della parate cellulare, l'attivazione del pathway di integrità e alterazioni nel pathway secretivo. Tutti i nostri dati indicano che non solo Sfp1 e regolato dagli stress e nutrienti (entrambi influenzando la sua localizzazione) ma che a sua volta Sfp1 può regolare la risposta agli stress. Il legame tra Sfp1 e la risposta agli stress e il pathway secretorio. Abbiamo ipotizzato che la riduzione della biogenesi dei ribosomi può indurre un difetto nel pathway secretivo che porta alla attivazione di Msb2 e quindi alla attivazione dell'FG pathway. L'uscita dal nucleo di Sfp1 necessaria per la riduzione della biogenesi dei ribosomi consente il rilascio del Rab GTPasi che è essenziale per lo spegnimento del difetto nel pathway secretorio. Conseguentemente l'inattivazione di *SFP1* induce una complessa attivazione dei pathway delle MAPK che sono responsabili per la regolazione di differenti aspetti che caratterizzano il mutante. Il principale e la regolazione della transizione G1-S mediante la stabilizzazione di Sic1. In fine abbiamo visto che come conseguenza dell'inattivazione di *SFP1* (probabilmente dovuta ad alterazioni nel pathway secretorio) i mutanti sono caratterizzati da una alterazione del rapporto volume citoplasmatico/contenuto proteico legato ad un aumento del volume citoplasmatico. Questo ci ha portato speculare sul fatto che la crescita cellulare può essere composta da due elementi: le Dimensioni intese come la crescita nel volume cellulare e la Massa intesa invece come l'aumento nel contenuto proteico. Di conseguenza alterazioni nella crescita in risposta a variazioni nelle condizioni ambientali

portano a una coordinazione regolata tra le Dimensioni e la Massa con lo scopo di mantenere il rapporto costante. Uno degli elementi chiave necessario a mantenere questo bilanciamento è Sfp1.

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