Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms

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Abstract

The response of yeast cells to sudden temperature downshifts has received little attention compared with other stress conditions. Like other organisms, both prokaryotes and eukaryotes, in *S. cerevisiae* a decrease in temperature induces the expression of many genes involved in transcription and translation, some of which display a cold-sensitivity phenotype. However, little is known about the role played by many cold-responsive genes, the sensing and regulatory mechanisms that control this response or the biochemical adaptations at or near 0ºC. This review focuses on the physiological significance of cold-shock responses, emphasising the molecular mechanisms that generate and transmit cold signals. There is now enough experimental evidence to conclude that exposure to low temperature protects yeast cells against freeze injury through the cold-induced accumulation of trehalose, glycerol and heat shock proteins. Recent results also show that changes in membrane fluidity are the primary signal triggering the cold-shock response. Notably, this signal is transduced and regulated through classical stress pathways and transcriptional factors, the HOG MAP kinase pathway and Msn2/4p. Alternative cold-stress generators and transducers will also be presented and discussed.

Introduction

Diminutions in ambient temperature are common in almost every ecological niche. A drop in temperature may take place seasonally, daily or just unexpectedly, depending on the region, climate and environment. The effects of low temperature on life have been studied thoroughly, as cold influences the structural and functional properties of cellular components negatively, both physically and chemically. A decrease in membrane fluidity and diffusion rates, alterations in molecular topology or modifications in enzyme kinetics reportedly occur as a consequence of low temperatures (Gast *et al*., 1993; Thieringer *et al*., 1998; Inouye, 1999). To withstand such temperature downshifts, organisms have developed mechanisms that enable them to adapt and survive. These mechanisms (commonly referred to as the "cold-shock response"), have been extensively studied in bacteria and plants. In *Escherichia coli*, *Bacillus subtilis* and other prokaryotes, a certain group of proteins is specifically (but not uniquely) induced upon a cold shock (Thieringer *et al*., 1998; Gualerzi *et al*., 2003). These cold-shock proteins are involved in transcription, translation and other fundamental functions that play a role in maintaining nucleic acid structure. However, little is known about the mechanisms underlying the transcriptional activation of cold-responding genes. In plants, cold-inducible genes cover a wider range of functions, and some of them also respond to drought or high salinity (Shinozaki & Yamaguchi-Shinozaki, 1996; Seki *et al*., 2002a). Moreover, it is thought that there is some cross-talk between the signal transduction pathways responding to these stresses (Seki *et al*., 2002b; 2004), although an exclusive pathway for cold-induced gene expression has also been proposed (Shinozaki & Yamaguchi-Shinozaki, 1996).

Although the cold-shock response has been widely studied in bacteria and plants, relatively little attention has been paid to fungi, and specifically yeast. The budding yeast *Saccharomyces cerevisiae* has colonized different natural and artificial environments, all of which can be subject to low temperatures. Yeast growing on the ground or on the surface of grapes and other fruits are exposed to temperature downshifts like those described above. On the other hand, when these yeasts are used in certain brewing and wine fermentations, they are exposed to temperatures of around 10-12º Celsius, far below the physiological temperature corresponding to this organism (25º-30ºC). Moreover, yeasts, as well as other micro-organisms, are stored at very low temperature (4ºC) in industrial and laboratory conditions. This temperature is chosen because it is generally accepted that although it is growth-restrictive, viability is fully maintained over long periods of time.

All these features indicate that *S. cerevisiae* must have the necessary molecular machinery to survive and adapt to cold, and that this is probably one of the reasons why this species is so widespread. Therefore, the study of the cold-shock response in *S. cerevisiae* is of crucial interest for both basic and technological reasons (Randez-Gil *et al*., 2003). The growth in high throughput analytical techniques has led to a number of publications regarding this subject in very recent years. Our knowledge of cold response in yeast, though limited, is expanding and we are gaining new insight into other molecular mechanisms, signalling pathways and regulators which, until now, were apparently unrelated to low temperature. The state-of-the-art and some possible models for cold-signal generation, signal transduction and function of cold-responding genes, as well as the prospects for future research, are discussed in this article.

Time and temperature-dependent and -independent genetic responses to cold

On analyzing the different gene expression patterns in *S. cerevisiae* after a downshift in temperature, it becomes clear that there is a common response involving certain groups of genes (Sahara *et al*., 2002; Homma *et al*., 2003; Kandror *et al*., 2004; Schade *et al*., 2004;

Murata *et al*., 2006). Among these are the genes encoding members of the DAN/TIR family of putative cell-wall mannoproteins, *TIP1* (temperature-shock inducible protein), *TIR1/SRP1*, *TIR2* and *TIR4*, which are induced at 10ºC, 4ºC (Sahara *et al*., 2002; Homma *et al*., 2003; Schade *et al*., 2004; Panadero *et al*., 2006; Murata *et al*., 2006) and even at 0ºC (Kandror *et al*., 2004). Similarly, *TIR*-related DNA sequences, seripauperin (PAU) family proteins (*PAU1*, *PAU2*, *PAU4*, *PAU5*, *PAU6* and *PAU7*), which have been shown to display phospholipid-interacting activity (Zhu *et al*., 2001) are also up-regulated after cold shock (Homma *et al*., 2003; Murata *et al*., 2006).

Apart from these characteristic cold-stress markers, part of the genetic response to low temperature seems to be time-dependent. Examination of different microarray-based studies reveals sequential changes in the transcriptional profile during the time-course of exposure to 10ºC. Authors remark two (Schade *et al*., 2004) or even three (Sahara *et al*., 2002) stages, depending on the stress duration. Initial responses (0-2 h) include the enhanced expression of key genes involved in phospholipid synthesis, such as *INO1* and *OPI3,* and fatty-acid desaturation (*OLE1*) (Sahara *et al*., 2002; Schade *et al*., 2004). Other genes that are induced within this period are those related to transcription (RNA helicases like *DBP2*, RNA polymerase subunits like *RPA49* and RNA processing proteins like *NSR1*, among others), and an important number of ribosomal protein genes. Thus, 94 of the 323 genes found to be up-regulated after yeast was incubated for 2 h at 10ºC encode ribosomal proteins (Sahara *et al*., 2002).

Remarkably, most of these transcription-related and ribosomal genes are abruptly repressed after longer incubation periods (4-24 h) at 10ºC (Sahara *et al*., 2002; Schade *et al*., 2004) and also at 4ºC (Homma *et al*., 2003; Murata *et al*., 2006). This phase is rather characterised by the transcriptional activation of typical stress-marker genes. Indeed, some members of the main gene family involved in cellular protection, the *HSP* genes (for *Heat*

Shock Proteins), are induced at this stage. Genes encoding HSPs, namely *HSP12*, *HSP26, HSP42, HSP104*, *SSA4, SSE2* and *YRO2*, are up-regulated after 4 to 12 h of transfer to 10º C (Sahara *et al*., 2002; Schade *et al*., 2004), as well as at similar times at 4ºC (Homma *et al*., 2003; Murata *et al*., 2006), 0ºC (Kandror *et al*., 2004) and -80ºC (Odani *et al*., 2003). Other members of the HSP family (*i.e. CIS3*, *HMS2*, *HSC82*, *HSP30*, *HSP60*, *HSP78*, *HSP82*, *HSP150*, *SSA1*, *SSA2*) are repressed at 10ºC (Sahara *et al*., 2002), whereas at lower temperatures their mRNA content increases (Murata *et al*., 2006). This discrepancy indicates that different members of the HSP gene family are differentially regulated in both a time- and temperature-dependent manner. The inventory of genes that are induced after 4-6 h of cold incubation and beyond also includes genes involved in the metabolism of reserve carbohydrate glycogen (*GLG1*, *GSY1*, *GLC3*, *GAC1, GPH1* and *GDB1*), and trehalose (*TPS1*, *TPS2* and *TSL1*), and genes for detoxifying reactive oxygen species (ROS) and defence against oxidative stress, such as catalase (*CTT1*), glutaredoxin (*TTR1*), thioredoxin (*PRX1*) and glutathione transferase (*GTT2*).

Cold sensitive mutants and the cold ribosome adaptation hypothesis

In view of the available data, it appears that yeasts adapt to temperature downshifts in terms of the duration and the extent of the stress. This is especially evident if we consider the opposite regulation displayed by ribosomal and transcription-related genes between short and long incubation periods at 10ºC or 4ºC (Sahara *et al*., 2002; Schade *et al*., 2004; Murata *et al*., 2006), which is consistent with previous reports about cold growth defective mutants. Several of the 106 mutants displaying growth defects at low temperatures (Hampsey, 1997) (catalogued in http://mips.gsf.de/proj/yeast/CYGD/db/index.html) are affected in ribosomal proteins and translation (Winzeler *et al*., 1999; Zhang *et al*., 2001), or in proteins involved in pre-rRNA processing for ribosome biogenesis or assembly (Lee & Baserga, 1997), protein folding (Craig & Jacobsen, 1985), exocytosis (Lehman *et al*., 1999) and nucleus-cytosol exchange (Noguchi *et al*., 1997; 1999). These observations indicate the need for remodelling the translational machinery and secondary structure of nucleic acids compromised by cold-shock; moreover, this could support a model for cold ribosome adaptation in *S. cerevisiae* that is similar to that proposed for *E. coli* (Jones & Inouye, 1996). At temperatures that are low, but still permit growth, there is an up-regulation of genes that are essential for cold growth (Fig. 1). On the other hand, such genes are expressed minimally or repressed after prolonged periods of stress (when all the transcriptional and translational machinery has already been re-modelled) or under severe cold conditions like 4ºC (generally considered to be a growth-restrictive temperature, although minimal growth still occurs), where low or not *de novo* protein synthesis is needed (Fig. 1).

Even so, results from transcriptomic experiments reveal that several genes respond to cold conditions regardless of exposure time and temperature. This finding suggests that not all the cold regulated genes are involved in functions related to growth.

The freeze-protective function of cold response

Although some of the cold-responsive proteins might be essential for the cells to adapt and resume growth under the new unfavourable environmental conditions, some evidence suggests that the main aim of other common cold-shock responses is to protect cells against freeze injury (Fig. 1). For example, the stability of Ole1p, the only fatty-acid desaturase known in *S. cerevisiae* (Stukey *et al*., 1989; 1990), seems to be important for cold growth (Loertscher *et al*., 2006); however, overexpression of *OLE1* does not confer growth advantages at low temperatures (Kajiwara *et al*., 2000). By contrast, production of recombinant desaturases increased the unsaturation index and fluidity of the yeast membrane, and positively influenced freeze tolerance of baker's yeast cells (Rodríguez-Vargas *et al*., 2006). These observations suggest that changes in membrane composition after a downshift in temperature might be important in influencing cell survival upon freezing. Membrane organization and dynamic properties are main targets of freeze injury (Wolfe & Bryant, 1999). Hence, the cold-instigated induction of fatty acid desaturases, cell-wall mannoproteins and seripauperins are consistent with this idea. Similarly, it has been shown that, in addition to temperature downshifts (Zhang *et al*., 2003), freezing and thawing generate superoxide anions and free radicals (Park *et al*., 1997; Du & Takagi, 2005), which is consistent with the cold-provoked up-regulation of genes for ROS detoxification and defence against oxidative stress, and with genes encoding HSPs. In this sense, HSPs are molecular chaperones involved in the response to several kinds of stress, preventing protein denaturation and misfolding (Papp *et al*., 2003; Young *et al*., 2003).

A downshift in temperature also activates the metabolism of reserve carbohydrates. It has been proposed that glycogen turnover occurs in response to different kinds of stress, like heat shock or oxidative damage, because the genes for both its synthesis and degradation are simultaneously induced, with no net accumulation of this polysaccharide (Parrou *et al*., 1997). In spite of the similar transcriptional profile observed at low temperatures (Sahara *et al*., 2002; Schade *et al*., 2004; Murata *et al*., 2006), such recycling does not appear to be the case for cold stress, because glycogen is significantly accumulated in yeast after 12 h of treatment at 10ºC (Schade *et al*., 2004). Like glycogen, trehalose starts to accumulate after 12 h incubation at 10º, 4º or 0ºC, regardless of whether there is growth or not (Kandror *et al*., 2004; Schade *et al*., 2004). Consistent with this, the genes involved in the biosynthesis of this disaccharide are cold-induced after 4-8 hours incubation at 10ºC. The same activation occurs at lower temperatures, showing maximum induction levels at 0º C (Sahara *et al*., 2002; Kandror *et al*., 2004; Schade *et al*., 2004; Murata *et al*., 2006). Trehalose is known to accumulate at high levels in response to different stress conditions (Estruch, 2000; Blomberg, 2000), and several protecting roles have been proposed for this disaccharide, such as a membrane and protein stabilizer (Singer & Lindquist, 1998; Elbein *et al*., 2003; Gancedo & Flores, 2004). Moreover, high levels of trehalose have also been correlated with freezing resistance (Kim *et al*., 1996). Although trehalose is not needed for growth at 10ºC (Schade *et al*., 2004), the results obtained by Kandror *et al*. (2004) revealed that this compound protects cells against very cold conditions. In fact, viability of cells incubated at 0ºC for 5 to 20 days, correlated with the intracellular trehalose content, and cold-instigated accumulation of the disaccharide protected the cells from viability losses due to freezing. Similar behaviour has been observed with glycerol. *GPD1*, the gene encoding the main enzyme involved in glycerol synthesis (Albertyn *et al*., 1994) is activated upon a shift to low temperature, and cells begin to accumulate this osmolyte, displaying higher values at 4ºC than at 12ºC (Panadero *et al*., 2006). Like trehalose, glycerol accumulation provides freeze-protection, although it is not needed for growth at 12ºC (Panadero *et al*., 2006). Glycerol is the only osmoprotectant solute accumulated by yeast upon hyperosmotic stress, and is also a by-product of redox homeostasis. The protective role of glycerol against freezing is well known and has been explained in terms of the osmotic shrinkage resulting from freezing/thawing processes (Wolfe & Bryant, 1999; Panadero *et al*., 2006).

In view of the above results, there is a clear link between exposure to low temperatures and resistance to freezing. In natural environments, frosts are often preceded by periods of very low temperatures, so this hypothesis is meaningful from an evolutionary point of view. However, there are still some aspects that should be clarified. For example, there is no evidence about the possible function of glycogen in cold response, and a better explanation is needed for the protective role of trehalose at 0ºC, a temperature at which cells are not frozen. On the other hand, the function of DAN/TIR and PAU family proteins remains unclear. Future work will contribute to clarifying the dual aspects of cold-shock responses, as a way to survive, adapt and grow at low temperatures, as well as a protective mechanism for subsequent freezing.

The cold signal and cold-sensing mechanism

For transcriptional activation to be triggered, yeast cells must sense temperature downshifts. Evidence indicates that changes in the physical state of the membrane could be perceived as a primary signal of a variation in temperature (Vigh *et al.*, 1998). This concept was first put forward by Carratù *et al*. (1996), who demonstrated that a membrane lipid perturbation causes a signal to be triggered, inducing the transcription of heat-shock genes in yeast. Based on this finding and additional evidence, the group of Murata demonstrated that, in the cyanobacteria *Synechocystis*, a drop in temperature causes a reduction of the membrane fluidity, and this fact plays a role in the perception of cold temperatures and the subsequent signal transduction (Los & Murata, 2004; Inaba *et al*., 2003). Genetically-engineered *Synechocystis* cells, that lacked genes encoding fatty acid desaturases, displayed higher membrane rigidity than the wild type at both cold and physiological temperatures, showing enhanced cold inducibility of gene activation (Inaba *et al*., 2003). In *Synechocystis*, expression of these desaturase genes upon cold-shock is controlled by the histidine kinase Hik33 (Suzuki *et al*., 2000; 2001) an integral membrane protein that functions as a cold sensor. Similarly, DesK in *Bacillus subtilis* (Aguilar *et al*., 2001) and TRP-channels in the mammalian nervous system (McKemy *et al*., 2002; Peier *et al*., 2002), which are integral membrane proteins, act as cold sensors. Furthermore, the extent to which the DesK protein senses temperature is regulated by the rigidity of the membrane lipid bilayer (Mansilla & de Mendoza, 2005). Overall, these observations indicate that changes in the physical state of the membrane caused by a temperature downshift,are recognized by cold-sensors anchored in the membrane, generating the cold-signal.

Recently, Hik33 has been shown to regulate the expression of osmostress-inducible genes in *Synechocystis* (Mikami *et al*., 2002), suggesting that both osmotic and cold-stress could be perceived in this organism by common mechanisms and sensing elements. Like Hik33, Sln1p, the only known yeast histidine kinase sensor, has recently been reported in the cold sensing mechanism (Panadero *et al*., 2006). Sln1p, together with Ypd1p and Ssk1p forms a phosphorelay system, which transmit the osmostress signal through different elements of the HOG (for High Osmolarity Glycerol) pathway, the most important osmotic stress-responding cascade in *S. cerevisiae* (Hohmann, 2002; Westfall *et al*., 2004). Activation of the HOG pathway was also found to be induced by the membrane-rigidifier agent dimethylsulfoxide, DMSO (Panadero *et al*., 2006). Several lines of research in plants and cyanobacteria have shown that this chemical indeed mimics the changes in membrane fluidity caused by a sharp decrease in temperature, and triggers the activation of several cold-induced MAPKs (Örvar *et al*., 2000; Sangwan *et al*., 2002). Thus, Sln1p appears to function under either of these stressful conditions (Panadero *et al*., 2006; Hayashi & Maeda, 2006) and it is likely, therefore, that the basic mechanism of its activation by low temperature and osmotic pressure could be similar, or even identical.

The fluidity state of the cell membrane might be a key factor to integrate the sensing mechanism of cold and hyperosmolarity. There is evidence that different osmosensors, like EnvZ in *E. coli* or OpuA, a transmembrane transporter of *Lactobacilllus lactis* with osmosensor and osmoregulator properties, are stimulated by changes in the fluidity of membrane lipids (Tokishita & Mizuno, 1994; van der Heide & Poolman, 2000; van der Heide *et al*., 2001). Moreover, studies in *B. subtilis* and *S. cerevisiae* indicate that osmotic stress reduces cell-membrane fluidity (López *et al*., 2000; Laroche *et al*., 2001). Altogether, these observations suggest a model in which Sln1p monitors the changes in membrane fluidity caused by different stressors. Moreover, the fact that Sln1p responds to DMSO supports this view. However, more work is required to confirm this idea and to understand the exact molecular mechanism of cold-signal perception.

Cold-induced transduction pathways and transcription factors

Signal transduction pathways are the link between the sensing mechanism and the genetic response. As is the case for the cold signalling mechanism, to date there is not a signal transduction pathway or transcription factor known to respond exclusively to low temperatures. Although the existence of such a pathway cannot be ruled out, available data point to known signal transduction mechanisms, operating under other kinds of stimuli, which may also be triggered by cold stress. We shall now go on to describe putative and known regulatory mechanisms controlling cold-instigated gene expression.

The MOX factors

The coordinated expression of the *DAN/TIR* genes under anaerobic conditions reportedly depends on the transcriptional activator *MOX4*, whereas their repression, in conditions of oxygen availability, is mediated by the two repression factors *MOX1* and *MOX2* in a heme-dependent fashion (Abramova *et al.*, 2001a). All of these factors act through the consensus sequence AR1 (Cohen *et al.*, 2001). Mox4p, Mox1p and Mox2p (or some factors dependent upon them) are believed to form a heme-sensitive complex similar to the galactose-sensitive Gal4p-Gal80p (Zenke *et al.*, 1996; Platt & Reece, 1998). Like *GAL4* regulation, *MOX4* expression is autoregulated in anaerobic cells via its AR1 sequence. Interestingly, expression of *TIR* genes under cold-shock was eliminated in a *mox4* mutant strain (Abramova *et al.*, 2001b). Furthermore, *MOX4* mRNA was also more abundant in cold-shocked cells (Abramova *et al.*, 2001a), suggesting that induction of the activator contributes to up-regulation of *TIR* genes under this condition (Fig. 2).

Regulation of *OLE1* expression and RUP processing

Unlike *DAN*/*TIR* genes, the promoter of *OLE1* does not contain AR1 sequences. Unsaturated fatty acids (UFAs) mediate *OLE1* repression via the *cis* transactivation element FAR (Fatty Acid Regulated). Two genes for fatty acid synthesis (*FAA1* and *FAA4*), the transcriptional activator *HAP1* and the acyl-CoA-binding protein Acb1p, have also been reported to regulate *OLE1* expression (Choi *et al*., 1996). In addition, Spt23p and Mga2p, two functionally and structurally related factors, are also necessary for *OLE1* transcription (Zhang *et al*., 1999) (Fig. 2). Spt23p appears to play a major role in fatty acid regulation, whereas Mga2p is known to be essential for the hypoxic induction of *OLE1* through a consensus sequence named LORE (for Low-Oxygen Response Element) (Jiang *et al*., 2001). This sequence is also found in several genes induced by hypoxia, including *TIR1*. All these genes could, therefore, share regulation via LORE (Vasconcelles *et al*., 2001). The transcriptional induction of *OLE1* under cold-shock is also dependent on Mga2p (Nakagawa *et al*., 2002), suggesting that the LORE-binding complex could drive the expression of a subset, at least, of cold-responsive genes (Fig. 2).

Moreover, Mga2p could also play a role as a cold-sensor, as previously postulated (Nakagawa *et al*., 2002). This hypothesis was construed on the basis that a novel pathway has been described in *S. cerevisiae*, the so-called “RUP (for Regulated Ubiquitin/Proteasome-dependent) processing” pathway, which regulates the activation of Spt23p and Mga2p by UFAs (Hoppe *et al*., 2000; 2001). Spt23p and Mga2p are initially created as dormant precursors, anchored in the endoplasmic reticulum or nuclear envelope membranes by their C-terminal tails. The shortage of UFAs leads to the ubiquitination of Spt23p and Mga2p, releasing the N-terminal transcription factor domain into the cytosol by the action of the 26S proteasome in a dual way: first, processing the transcriptional factor into its active form and second, destroying it after the activation of genes such *OLE1* (Auld *et al*., 2006). In the processing of Mga2p, UFAs exert a modest influence, with hypoxia being the main signal that triggers its processing (Jiang *et al*., 2002). In this scenario, a change in the physical state of the membrane due to a downshift in temperature or to hypoxia conditions (Nakagawa *et al*., 2003) could activate Mga2p by starting off the RUP-processing (Fig. 2).

The cAMP-PKA and General Stress Response

Thirty-two protein phosphatase genes have been identified in the *S. cerevisiae* genome (Sakumoto *et al*., 1999). From all of them, only *YVH1* was found to be induced by cold-shock (Hampsey, 1997) and to play a role in growth at low temperatures (Sakumoto *et al*., 1999). *YVH1* encodes a dual-specificity protein phosphatase (DSPs), which exhibits a catalytic domain displaying protein-tyrosine phosphatase activity and a C-terminal cysteine-rich motif (Fauman & Saper, 1996; Muda *et al*., 1999). Yvh1p shares the control of some cellular processes like sporulation, growth, and glycogen accumulation with PKA. The cAMP-protein kinase A (PKA) pathway in *S. cerevisiae* plays a major role in the control of the genetic response to a wide variety of stresses (Thevelein & de Winde, 1999; Estruch, 2000; Wilson & Roach, 2002; Santangelo, 2006), through the phosphorylation of the Msn2p and Msn4p transcription factors (Griffioen & Thevelein, 2002) (Fig. 3, pathway 1). Upon stressful conditions, Msn2/4p migrates to the nucleus and promotes the transcription of an important number of stress-responding genes, which have the recognition sequence called STRE (for Stress Response Element) in their promoter (Martinez-Pastor *et al*., 1996). It has been suggested that Yvh1p could negatively modulate the cAMP level or a downstream component of the PKA signalling cascade. In fact, deletion of *YVH1* resulted in decreased expression of a multi-STRE reporter construction (Beeser & Cooper, 2000). Like Yvh1p, Mck1p, a Ser/Thr/Tyr protein kinase might also be involved in the cold-shock signalling via PKA.

Genetic data indicate that Yvh1p acts upstream of Mck1p, at least in sporulation signalling (Beeser & Cooper, 1999). Moreover, Mck1p is involved in the phosphorylation of Bcy1p (Griffioen *et al*., 2003), the negative regulator of PKA. This modification occurs under environmental stress conditions like heat shock, salt or oxidative stress and determines the distribution of Bcy1p between the nucleus and cytoplasm. The specific relocation of Bcy1p could help to drive the PKA activity towards certain substrates under these conditions. In addition, Mck1p is necessary for the proper stress response, due to its ability to indirectly promote a DNA-Msn2p complex (Hirata *et al*., 2003), without the need of Msn2p phosphorylation. Deletion of *MCK1* leads to a growth defect at low temperature (de Jesus Ferreira *et al*., 2001) and inhibition of STRE-dependent transcription (Hirata *et al*., 2003), similar to that found in the *yvh1* mutant (Sakumoto *et al*., 1999).

The cold signal transduction via the cAMP-PKA signalling pathway would, as anticipated, be essential in determining the genetic response to cold. Indeed, genes induced at the late phase of a cold-shock, like those encoding trehalose and glycogen metabolism enzymes, as well as *HSP12*, *HSP26*, *HSP42*, *SSA4* and the ROS-defensive genes *CTT1*, *PRX1* and *TTR1*, are known to be strictly dependent on Msn2/4p (Kandror *et al*., 2004; Schade *et al*., 2004). Expression of early responding genes like *DPB2*, *NSR1*, *OPI3* and *RPA49*, as well as *PAU* genes, whose cold-provoked induction is maintained over time, has also been reported to depend on a functional PKA pathway (Wang *et al*., 2004). Moreover, signalling through the cAMP-PKA pathway would play a major role in providing cold-adaptation and tolerance to freezing. Mutants lacking *MSN2*/*MSN4* die more rapidly at 0ºC or upon freezing (Kandror *et al*., 2004). Mutants in *RAS2*, the gene for a regulatory protein in the synthesis of cAMP, clearly showed a better freeze-thaw tolerance than the wild-type, while mutants in *BCY1* were sensitive to this stress (Park *et al*., 1997). In the same line, mutants from a commercial baker's yeast strain, showing partial inactivation in adenylate cyclase, were better at maintaining freeze resistance (van Dijck *et al.*, 2000). These results indicate than any event involving a decrease in the level of PKA activity improves cell tolerance to freeze stress. Consistent with this, deletion of *IRA2* encoding the Ras GTPase (an activator of the PKA pathway), led to a freeze-sensitivity phenotype. Similarly to this, mutants in *PDE2*, the high-affinity phosphodiesterase encoding gene, also showed impaired freezing tolerance (Park *et al.*, 2005a).

The PKC pathway

Signalling through the so-called PKC pathway (for a review see Levin, 2005) is triggered under conditions that jeopardize cell-wall stability, including heat-shock (Kamada *et al.*, 1995) and hypo-osmotic stress (Davenport *et al.*, 1995). Activation of the G-protein Rho1, the master regulator of the pathway, triggers a linear MAPK signalling cascade that starts with Pkc1p, a homologue to the mammalian protein kinase C, and finish with the Slt2p/Mpk1p kinase. The PKC pathway controls important cell functions, such as cell-wall maintenance and actin polarisation. Also, Pkc1p regulates additional targets that are separated from the MAPK cascade (Levin, 2005). Activation of Rho1p is stimulated by Rom2p, which has GDP/GTP exchanging activity. Disruption of *ROM2* leads to temperature-sensitive growth defects at both high (37ºC) and low (11ºC) temperature (Manning *et al.*, 1997). These defects include failure to form normal bud polarisation and sensitivity to the microtubule depolymerising drug benomyl. Although the activation of the PKC pathway by heat stress is well documented (Kamada *et al.*, 1995; Heinisch *et al.*, 1999), the phenotype of the *rom2* null mutant suggests a general function of PKC in perceiving and signalling changes in environmental temperature. Some collateral observations support this idea. For example, mutants in several genes encoding proteins related to actin polarisation are cold sensitive. Among them, Num1p (Revardel & Aigle, 1993) interacts with Bni1p, a formin that nucleates the assembly of linear acting filaments in response to activation by the PKC pathway (Levin, 2005). In addition, some mutants in Rho3p, a homologue of Rho1p which is involved in Bni1p activation during bud growth, also confer sensitivity to low temperatures (Imai *et al.*, 1996).

PKC may also play a role in connexion with the cAMP-PKA pathway. Evidence of this interaction has been provided by Park and co-workers (Park *et al.*, 2005b). These authors demonstrated that Rom2p can negatively regulate the Ras-cAMP pathway by controlling the cAMP levels. Thus, a *rom2* null mutant showed sensitivity to freeze stress, a phenotype that was suppressed by additional mutation of *RAS2* or in a *tpk1 tpk2* background. On the contrary, the absence of either *IRA2* or *PDE2* exacerbated the freeze-sensitivity phenotype of the single *rom2* mutant.

The TOR pathway

In plants, cold acclimation is associated with rapid and reversible changes in the phosphorylation status of specific pre-existing proteins that allow the expression of CAS (cold-acclimated specific) genes (Monroy *et al.*, 1993). The type 2A Ser/Thr protein phosphatase (PP2A) is involved in basic cellular processes, such as metabolism, transcription and signal transduction (Ariño *et al.*, 1993). One of the signals regulated by PP2A is a downshift in temperature (Monroy *et al.*, 1998). The composition of PP2A subunits determines its specificity, activity and subcellular localisation. In plants, *TAP46* codes for a PP2A regulatory subunit, and is induced by cold-shock, suggesting that Tap46p targets PP2A under this condition (Harris *et al.*, 1999). In *S. cerevisiae*, the homologue to plant *TAP46* is *TAP42*. As in plants, Tap42p associates to PP2A catalytic subunits, Sit4p or Pph21/22p (di Como & Arndt, 1996; Jiang & Broach, 1999). This association depends on the Tor proteins, phosphatidylinositol kinase-related kinases, which are members of the TOR signalling cascade, a pathway that controls cellular functions necessary for cell growth and metabolism in response to environmental cues (Schmelzle & Hall, 2000; Wullschleger *et al*., 2006). On the contrary, inhibition of yeast TOR activity leads to transcriptional activation of genes so they can adapt to nutrient depletion (di Como & Arndt, 1996; Jiang & Broach, 1999; Rohde *et al*., 2004). Recently, it has been shown that inactivation of Tap42p prolongs the up-regulation of several Msn2/4p-dependent genes after heat shock (Düvel *et al*., 2003). Therefore, a functional TOR pathway would be involved in nuclear export of the transcription factors under non-stressing conditions, a fact that has already been demonstrated for Msn2p (Santhanam *et al*., 2004). Phosphorylated Tap42p competes with Cdc55p/Tpd3p for binding to the catalytic subunit Pph21/22p, increasing protein synthesis under these conditions (Jiang & Broach, 1999). Interestingly, disruption of either *TPD3* or *CDC55* causes cold sensitivity (Healy *et al*., 1991). These data strongly suggest that, like in plants, PP2A via Tap42p, Cdc55p and Tpd3p may play a role in cold response, and that the transcription factors Msn2/4p mediate the TOR signalling (Fig. 3, pathway 2). Hence, the cold-provoked induction of Msn2/4p-dependent genes would be signalled through TOR, as well as via the PKA pathway. In this respect, it has recently been proposed that the cAMP-PKA pathway is a TOR effector branch (Schmelzle *et al*., 2004) involved in Msn2p localisation and other readouts that are not controlled by Tap42p/Sit4p.

Further evidence that the TOR pathway is involved in cold response comes from the functional analysis of distinct TOR complexes (Loewith *et al*., 2002; Wullschleger *et al*., 2006). As well as its shared role with Tor1p in cell growth, Tor2p displays a unique function in the organization of cytoskeleton. Thus, *tor2* mutants exhibit abnormal polarized distribution of the actin cytoskeleton, a phenotype that is rescued by overexpression of the actin-specific TCP-1 chaperone (Schmidt *et al*., 1996). Interestingly, mutants in genes encoding different subunits of this chaperone (Stoldt *et al*., 1996), as *CCT2*, *CCT3* and *TCP1*, or in all the components of its co-chaperone, the GimC complex (Siegers *et al*., 1999), showed clear cold-sensitive phenotypes (Chen *et al*., 1994; Geissler *et al*., 1998). This suggests a Tor2p-mediated activation of protein complexes involved in maintaining the cytoskeleton under cold stress. Moreover, the abnormal actin polarization displayed by *tor2* mutants was reverted by enhanced expression of *PKC1*, *ROM2* and other effectors of the PKC pathway (Helliwell *et al*., 1998). Hence, it seems clear that maintenance of an adequate cell polarisation upon cold-shock requires interplay between TOR and PKC pathways.

However, we can not discard the possibility of an indirect effect of low temperatures in the activity of the TOR pathway. Changes in the physical state of the plasma membrane due to abrupt shifts in temperature could significantly alter the activity of membrane-associated enzymes and transporters, lowering nutrient uptake, and leading to TORC1 complex inactivation. For example, stress conditions, such as heat shock, have been reported to cause a severe decrease in amino acid import (Schmidt *et al*., 1998; Chung *et al*., 2001). This drop is a consequence of the vacuolar degradation of high affinity amino acid permeases such as Tat2p or Fur4p (Skrzypek *et al*., 1998; Bultynck *et al*., 2006), an event that is also stimulated by a downshift in environmental temperature (Abe & Horikoshi, 2000).

Role of the HOG pathway

As mentioned above, the histidine kinase Sln1p induces signalling through the HOG pathway in response to a downshift in temperature (Panadero *et al*., 2006; Hayashi & Maeda, 2006). The HOG pathway, one of the five MAP kinase pathways discovered in *S. cerevisiae* (Gustin *et al*., 1998; Schwartz & Madhani, 2004), has traditionally been thought to be involved in the genetic response to hyperosmotic stress alone (Hohmann, 2002; Westfall *et al*., 2004). However, recent findings have established novel roles for this pathway, such as adaptation to citric acid stress (Lawrence *et al*., 2004), heat-shock (Winkler *et al*., 2002), methylglyoxal resistance (Aguilera *et al.*, 2005), distribution of proteins within the Golgi (Reynolds *et al.*, 1998) or cell-wall maintenance (García-Rodriguez *et al.*, 2000). Recently, results obtained by our group (Panadero *et al.*, 2006) have revealed that downstream elements of the HOG-pathway are also involved in cold-signal transduction. The transcription factor Hog1p, which is the last step of the MAPK cascade, is phosphorylated after a downshift in temperature, a fact that is prevented in mutants lacking upstream elements of the pathway. Signalling through HOG causes the activation of *GPD1*, *GLO1* and other Hog1p-dependent osmotically activated genes in response to cold shock (Panadero *et al.*, 2006) (Fig. 4). These results are in good concordance with previously reported data regarding other organisms. The *Arabidopsis* MKK2 pathway mediates signal transduction upon both saline and cold stress (Teige *et al.*, 2004). In the fission yeast *Schizosaccharomyces pombe*, the homolog pathway to HOG (named SAPK) is also involved in the cold-shock response (Soto *et al.*, 2002). Similarly, activation by hypothermic stress of mammalian MAPK family members, including JNK (c-Jun N-terminal kinase) and p38, the functional homologues to Sty1p and Hog1p (de Nadal *et al.*, 2002), has been evidenced in human cells (Gon *et al.*, 1998; Ohsaka *et al.*, 2002; Roberts *et al.*, 2002). Both JNK and p38 are also activated during natural freezing and thawing of the wood frog (Greenway & Storey, 2000). This result strongly suggests that cold-stress responses by MAPKs, particularly p38 and relatives, may be adaptive in dealing with freeze stress, as recently reported (Panadero *et al.*, 2006). In fact, a yeast strain lacking *HOG1* showed impaired viability after freezing and frozen storage as compared with the wild-type (Panadero *et al.*, 2006).

Concluding remarks

Although *S. cerevisiae* cold-sensitive mutants were first described over 10 years ago, only very recent studies have started to reveal the existence of cold-shock and freezing-protective responses in this organism, giving some clues about their physiological significance. Many genes with up- or down-regulated expression at low temperatures are known to participate in cellular adaptation to other kinds of stressful situations, like thermal stress, high osmolarity, oxidation or presence of certain poisons. This phenomenon is very well known in yeast and has been called the General Stress Response. Hence, it is not surprising that exposure to low or sub-freezing temperatures are also included in the scope of this systemic mechanism. Similarly, known sensing molecules, signal transduction machinery and transcription factors that mediate the genetic response to other stressful conditions, appear to be involved in cold response. However, cold activation of several signalling pathways might be indirect. In this way, the loss in membrane fluidity, as a physical consequence of exposure to low temperatures, would diminish the protein mobility of integral membrane proteins, like amino acid transporters, and be interpreted by the cells as a situation of nutrient starvation.

Diversification of the functional roles of transcription pathways, which were initially believed to respond exclusively to one kind of stress, is an idea supported by an increasing number of new studies, especially in the case of the HOG pathway. Moreover, links between hyperosmotic stress, UFAs/SFAs ratio and membrane rigidification lead one to speculate about a scenario in which the level of membrane fluidity is the physical perturbation sensed by the cell that triggers the response to different kinds of stresses. However, not the whole cold-induced response is referred to multi-stress related genes. This is the case of the *TIP/TIR* genes, whose function and activation mechanism have yet to be clarified. Hence, the existence of an exclusive mechanism for cold response in yeast can not be discarded. Given the increasing interest shown by several groups in this field, one can expect that related works in the near future will shed light on the currently speculative aspects of this response.

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Figure legends

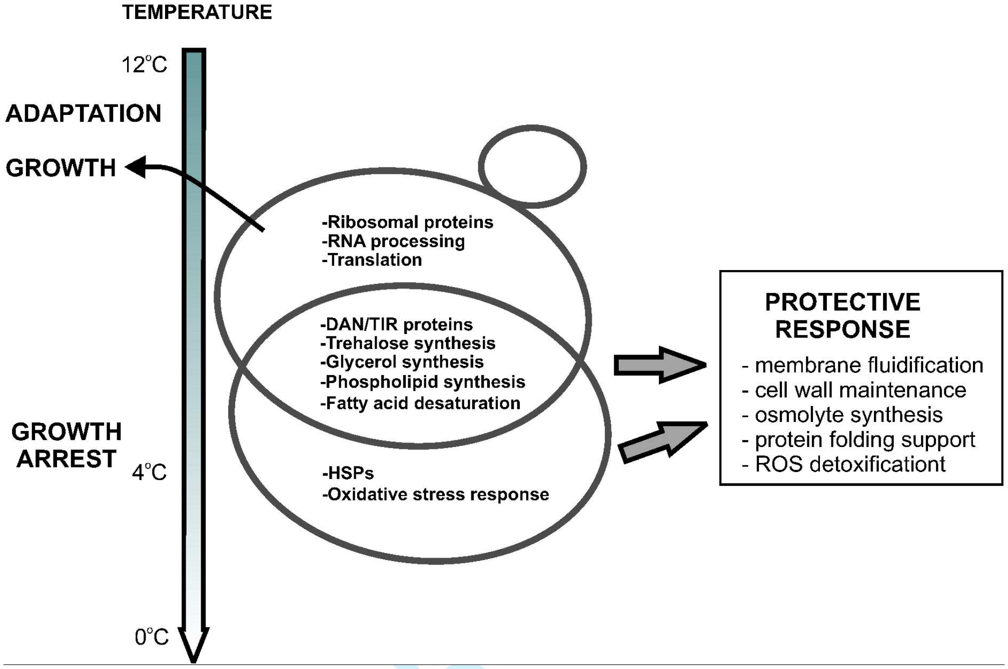
Fig. 1. Main responses of *Saccharomyces cerevisiae* to decreasing temperatures. At mid-low temperatures, specific transcriptional and translational machinery is up-regulated in order to enable cold-adapted growth. These mechanisms are switched off when the temperature is low enough to be growth-restrictive. On the other hand, freeze-protective mechanisms, like fatty acid desaturation and synthesis of osmolytes, are triggered even at permissive temperatures, and intensified at near-freezing conditions, a situation in which further protection systems, like HSPs, are also activated.

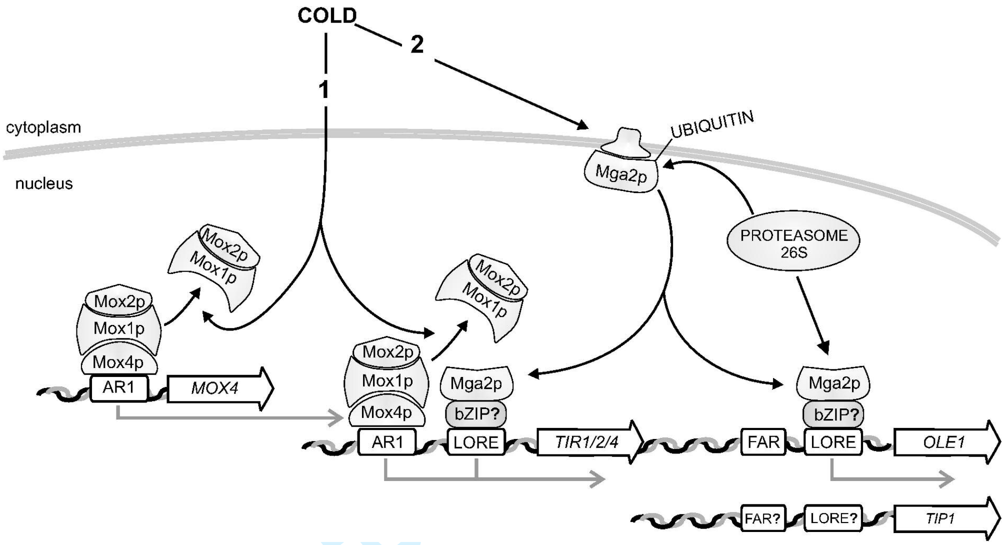
Fig. 2. Cold-instigated activation of the MOX factors and the RUP processing. 1, when the repressor complex Mox1p-Mox2p is released, the Mox4p factor promotes the transcription of *TIP*/*TIR* genes. The cold-provoked induction of these genes is eliminated in a *mox4* mutant, suggesting that low temperatures are able to release the repressor complex. 2, the transcriptional induction of *OLE1* under cold-shock is dependent on the transcription factor Mga2p, which is released from the nuclear envelope by proteasome-dependent deubiquitination provoked by the action of the Rsp5p factor. This action could therefore be triggered by cold stress (see text for details).

Fig. 3. cAMP-PKA and TOR pathways. 1, mutants in the Tpk1/2/3p (PKA) negative regulators Yvh1p and Mck1p show growth defects at low temperatures, indicating the involvement of the cAMP-PKA pathway in the cold response. This pathway starts with the formation of the GDP-binding complex Ras1/2p/Ira1/2p/Cdc25p, which activates Cyr1p for cAMP synthesis. Inhibition of this synthesis by cold, in addition to Yvh1p and Mck1p activation, would prevent Tpk1/2/3p-mediated phosphorylation of the transcription factors

Msn2/4p, avoiding their nuclear export. 2, disruption of either *TPD3* or *CDC55* causes cold sensitivity. Inhibition of Tap42p phosphorylation caused by cold would prevent the formation of the complex Tap42p/Php21/22p//Tpd3p/Cdc55p, also blocking the exportation of Msn2/4p from the nucleus, with the subsequent induction of genes encoding trehalose, glycerol and other STRE genes. On the other hand, localisation of Msn2/4p is regulated by PKA in a TORC1-dependent manner, whereas the TORC2 complex acts on Rom2p activation (see text for details).

Fig. 4. The HOG pathway. Sensitivity of the Sln1p/Ypd1p/Ssk1p histidine kinase complex to membrane rigidification caused by low temperature generates a signal that is transduced through the MAPK phosphorylation cascade. Hog1p is phosphorylated and migrates into the nucleus, promoting the transcription of genes for glycerol synthesis, among others (see text for details).


TEMPERATURE
12°C
ADAPTATION
GROWTH
-Ribosomal proteins
-RNA processing
-Translation
-DAN/TIR proteins
-Trehalose synthesis
-Glycerol synthesis
-Phospholipid synthesis
-Fatty acid desaturation
GROWTH ARREST
4°C
-HSPs
-Oxidative stress response
0°C
PROTECTIVE RESPONSE
- membrane fluidification
- cell wall maintenance
- osmolyte synthesis
- protein folding support
- ROS detoxificationt


COLD
1
2
cytoplasm
nucleus
UBIQUITIN
Mga2p
PROTEASOME 26S
Mox2p
Mox1p
Mox4p
AR1
MOX4
Mox2p
Mox1p
Mox2p
Mox1p
Mox4p
AR1
Mga2p
bZIP?
LORE
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OLE1
FAR?
LORE?
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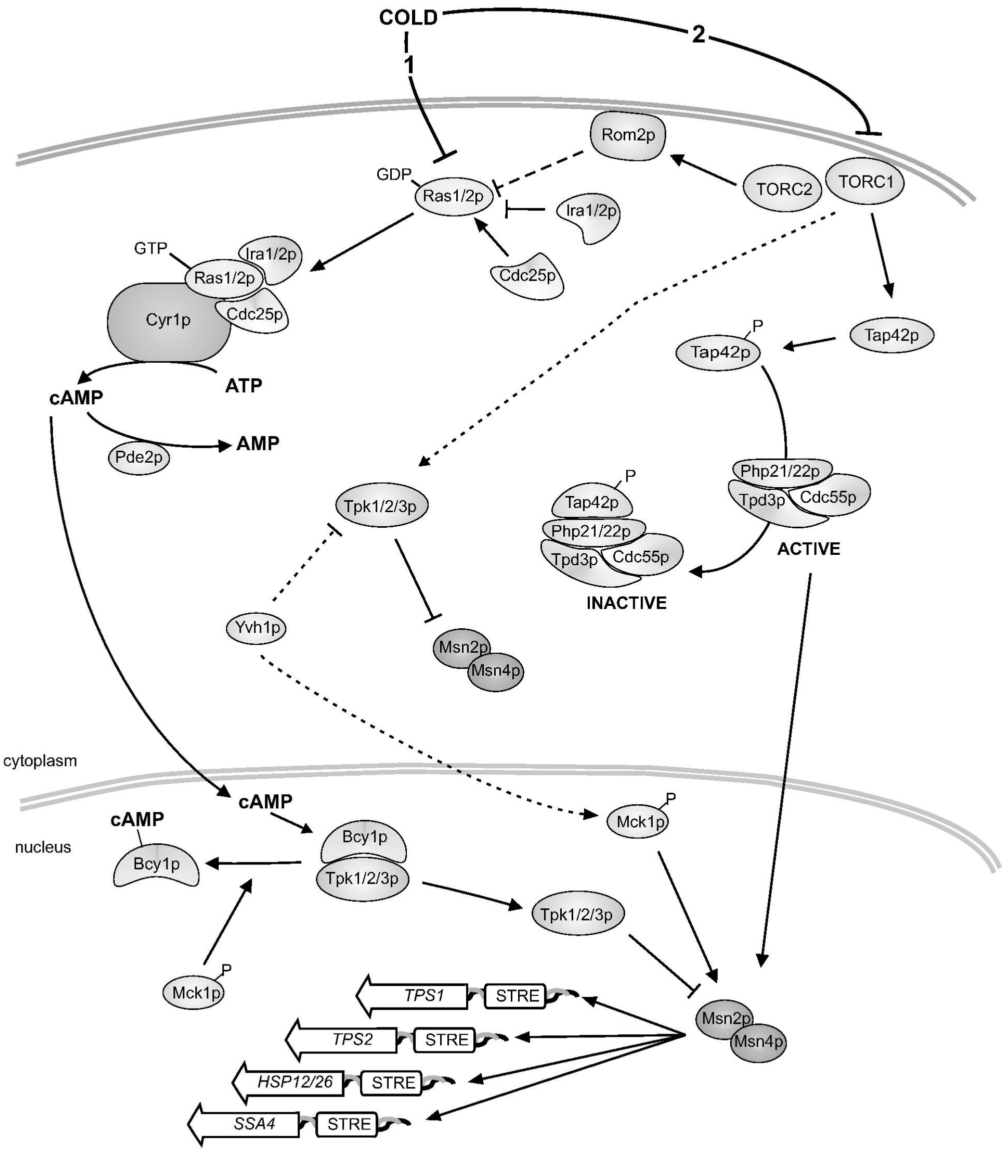
COLD
1
2
Rom2p
GDP
Ras1/2p
TORC2
TORC1
Ira1/2p
Cdc25p
GTP
Ras1/2p
Ira1/2p
Cyr1p
Cdc25p
P
Tap42p
Tap42p
cAMP
ATP
AMP
Pde2p
Php21/22p
Tpd3p
Cdc55p
ACTIVE
Tpk1/2/3p
P
Tap42p
Php21/22p
Tpd3p
Cdc55p
INACTIVE
Yvh1p
Msn2p
Msn4p
cytoplasm
cAMP
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P
Mck1p
nucleus
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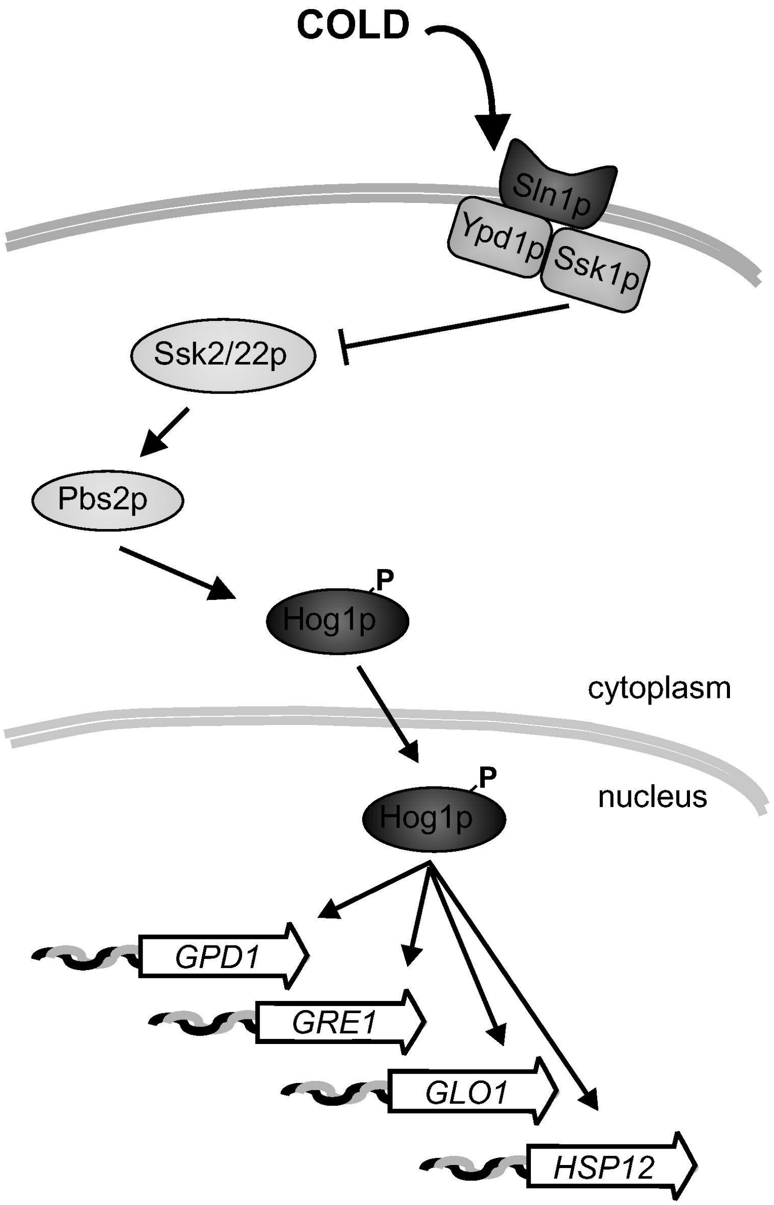
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Sln1p
Ypd1p
Ssk1p
Ssk2/22p
Pbs2p
P
Hog1p
cytoplasm
P
Hog1p
nucleus
GPD1
GRE1
GLO1
HSP12