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Review

Osmoregulation in *Saccharomyces cerevisiae* via mechanisms other than the high-osmolarity glycerol pathway

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The response of *Saccharomyces cerevisiae* to osmotic stress, whether arising from environmental conditions or physiological processes, has been intensively studied in the last two decades. The well-known high-osmolarity glycerol (HOG) signalling pathway that is induced in response to osmotic stress interacts with other signalling pathways such as the cell wall integrity and the target of rapamycin pathways. Osmotic balance is also maintained by the regulated opening and closing of channel proteins in both the cell membrane and intracellular organelles such as the vacuole. Additionally, environmental stresses, including osmotic shock, induce intracellular calcium signalling. Thus, adaptation to environmental stresses in general, and osmotic stress in particular, is dependent on the concerted action of components of multiple interacting pathways. In this review, we describe some of the major mechanisms and molecules involved in osmoregulation via pathways other than the high-osmolarity glycerol pathway and their known interactions with one another that have been discovered over the last two decades.

Introduction

All organisms experience a combination of biotic and abiotic stresses as a result of external environmental and internal physiological changes. The osmoadaptation response of *Saccharomyces cerevisiae* (baker's yeast, henceforth 'yeast') has contributed much to our understanding not only of the response of cells to changes in water activity but also of signalling mechanisms that couple stimuli to coordinated responses, thereby ensuring homoeostasis. The response to osmotic stress via the high-osmolarity glycerol (HOG) pathway, a signalling cascade that culminates in the activation of the Hog1p MAP (mitogen-activated protein) kinase, has been the subject of several reviews over the last two decades. In this review, we focus on a complementary facet of the osmoadaptation response, viz. non-HOG mechanisms, and highlight knowledge gained during the last 20 years. However, we have provided a brief overview of the HOG pathway as supplementary information to provide a conveniently accessible summary for the reader.

Abbreviations: CWI, cell wall integrity; ER, endoplasmic reticulum; ESR, environmental stress response; GAAC, general amino acid control; HACS, high-affinity Ca^{2+} influx system; HOG, high-osmolarity glycerol; LACS, low-affinity Ca^{2+} influx system; MIP, major intrinsic protein; PKA, protein kinase A; PKC, protein kinase C; TM, transmembrane; TOR, target of rapamycin; TORC1, TOR complex 1; TORC2, target of rapamycin complex 2; TRPC, transient receptor protein channel.

Three supplementary figures are available with the online Supplementary Material.

Changes in water activity and concentration of solutes within organisms and cells may occur as a result of environmental changes or even metabolic activity itself. For example, excessive glycerol synthesis in yeast acts as an endogenic stress (Hohmann, 2002a). From a physiological viewpoint, a critical ratio of free to bound water is required to maintain appropriate cell volume and provide a favourable milieu for biochemical reactions. External hyperosmotic stress results in plasmolysis due to water efflux. As a result, the cell shrinks, taking up ions and low molecular weight organic compounds (compatible solutes) in an attempt to achieve osmotic equilibrium. Bacteria such as *Escherichia coliSalmonella enterica* accumulate K^+ ions under hyperosmotic conditions (Moat, 2002). Halophilic bacteria accumulate glycine betaine, a novel amino acid (Varnam, 2000). Yeast is known to synthesize and accumulate glycerol as a compatible solute or osmolyte (Reed *et al.*, 1987). Adaptation to osmotic shock involves intricate response systems consisting of sensors and transducers that relay signals, as well as membrane channels that import and export a combination of water, compatible solutes and ions. The activities of these response elements must be spatiotemporally regulated in order to achieve satisfactory adaptation to osmotic shock.

We commence this review with an outline of the role of channel proteins in the cell and vacuolar membranes in the maintenance of osmotic balance. Subsequently, we discuss crosstalk and the sharing of effectors between the osmoadaptation response and other signalling cascades. Thereafter, the general response to environmental stress and its relation to

components of the osmoregulation pathway are briefly discussed, highlighting the utility of yeast as a model organism for studies of stress responses. This is followed by a brief description of osmolyte uptake in yeast. We conclude with remarks on the relative importance of adaptation at the levels of metabolism and gene regulation, highlight some impacts of the knowledge gained from studies of osmoregulation in yeast and present some perspectives for the future.

Regulation of water and glycerol transport by major intrinsic protein channels: form and function

The major intrinsic protein (MIP) family of protein channels is found in all three kingdoms of life (Hohmann, 2002b). These are known to transport water and glycerol, but their cargo can include other uncharged molecules and ions. The yeast MIP, the aquaglyceroporin Fps1p, is most closely related to the bacterial glycerol facilitators (Tamás *et al.*, 1999). MIP channels exhibit topological similarity, sharing six transmembrane (TM) helical segments per subunit (Hedfalk *et al.*, 2004). They assemble into homotetramers, with a pore being formed by each subunit (Hohmann, 2002b). One of the responses to osmotic stress in yeast is the closing of Fps1p, preventing the outflow of intracellular glycerol (Siderius *et al.*, 2000).

Fps1p is an unusual MIP channel in that its N- and C-terminal domains are much longer than those of other MIP channels and also because the conserved NPA motifs in loops B and E are replaced by the amino acid sequences NLA and NPS, respectively (Karlgren *et al.*, 2004; Fig. 1). Both loops B and E are located within the cell membrane, but loop B is located more toward the cytoplasmic side, whereas loop E is closer to the extracellular face (Hedfalk *et al.*, 2004; Karlgren *et al.*, 2005; Tamás *et al.*, 2003). The extended N- and C-terminal domains of Fps1p contain regulatory regions that penetrate the cell membrane as a consequence of their amphiphilicity, thereby interacting with NPS residues in the B-loop. Hedfalk *et al.* (2004) demonstrated that a conserved 12 amino acid stretch toward the end of the C-terminal (^{535}HESPVNWSLPVY^{546}) might be involved in the regulation of Fps1p by interacting with residues of loop B. Likewise, the sequence ^{219}MVKPK TLYQNPQTPTVLPSTYHPINKWSS^{247} present in the N-terminal domain interacts with residues in loop B. Thus, by acting as 'flaps' capable of restricting the channel orifice (see Fig. 1), the extended N- and C-terminal domains regulate glycerol transport (export or retention, as required) in response to osmotic shock (Hedfalk *et al.*, 2004; Tamás *et al.*, 2003). Certain amino acids in the N-terminal domain (K-223, Q-227, T-231, P-232 and P-236) and in loop B (G-348 and H-350) are important for this closure. Mutations in these residues (e.g. K223E, Q227R and T231A at the N-terminus and G348D, G348R and G348S in loop B) can result in a constitutively open pore (Karlgren *et al.*, 2004).

Recently, Geijer *et al.* (2012) have shown that the TM core of Fps1p is involved in regulating the transport of compatible osmolytes in response to osmotic stress. They found that expressing the Fps1p homologue from (an exclusively filamentous fungus) *Ashbya (Eremothecium) gossypii* (AgFps1p) in an *S. cerevisiae* strain harbouring a deletion of *FPS1* (*fps1*Δ) renders the cells osmosensitive relative to strains having a functional Fps1p channel. This phenotype was attributed to channel hyperactivity, i.e. inability to accumulate glycerol by channel closure under high-osmolarity conditions (0.8 M NaCl). Chimeras containing N- or C-terminal domains of *S. cerevisiae* Fps1p fusions to the TM core of AgFps1p (AgFps1-N_{Sc}, AgFps1-C_{Sc} and AgFps1-NC_{Sc}) conferred an osmosensitive phenotype to the *fps1*Δ strain, while chimeras having an *S. cerevisiae* TM core fused to N- or C-terminal domains of AgFps1p restored osmotolerance, indicating that this phenotype was attributable to the TM core. Subsequent screening for suppressors of the hyperactive N228A mutation (located within the NPS motif) revealed that a G519S mutation within the TM core domain 6 could restore osmotolerance. The Fps1p variants AgFps1p^{G519S} and ScFps1p$^{N228\text{-}A/G\text{-}519S}$ transported glycerol at rates comparable to those of WT ScFps1p when expressed in the *fps1*Δ strain, indicating that a narrower channel pore arising from steric crowding by the larger substituent (serine instead of glycine) results in the alleviation of the hyperactive phenotype.

Fps1p activity is regulated by Hog1p, the key MAP kinase of the HOG pathway, both directly and indirectly. Fps1p is directly phosphorylated on T-231 within the N-terminal domain by Hog1p (Thorsen *et al.*, 2006). Additionally, two paralogous protein kinases, Ypr115w (renamed Rgc1p for regulators of glycerol channel) and Ask10 (renamed Rgc2p) whose activity is partly dependent on their phosphorylation by Hog1p, probably act as positive regulators of Fps1p-mediated glycerol efflux (Beese *et al.*, 2009). Rgc2p binds close to the C-terminal regulatory domain of Fps1p and hinders the dipping action of the domain (Hedfalk *et al.*, 2004), thereby keeping the Fps1p channel pore open. Later studies (Lee *et al.*, 2013) demonstrated that phosphorylation of Rgc2p by Hog1p at three S/T-P sites – S-75, S-344 and S-944/948 – renders the protein inactive and unable to positively regulate Fps1p, resulting in channel closure. In strains having Rgc2p with mutated phosphorylation sites (*RGC2-3A* and *RGC2-7A*), Rgc2p remains bound to Fps1p resulting in a constitutively open channel. Hog1p also binds to the N-terminal domain of Fps1p and phosphorylates Rgc2p (and likely Rgc1p), which results in its displacement from the C-terminal domain of Fps1p (residues 544–581 and 611–617).

Adding further to the complexity of Fps1p regulation, Muir *et al.* (2015) have reported the presence of a Hog1p-independent regulatory pathway. This regulation of Fps1p is dependent on the target of rapamycin complex 2 (TORC2, discussed later) and its downstream effector Ypk1p (a Ser/Thr protein kinase). Under isotonic conditions, TORC2-mediated phosphorylation of Fps1p (on residues T-147, S-181, S-185 and S-570) by Ypk1p keeps the channel open. Hyperosmotic stress causes loss of this phosphorylation and

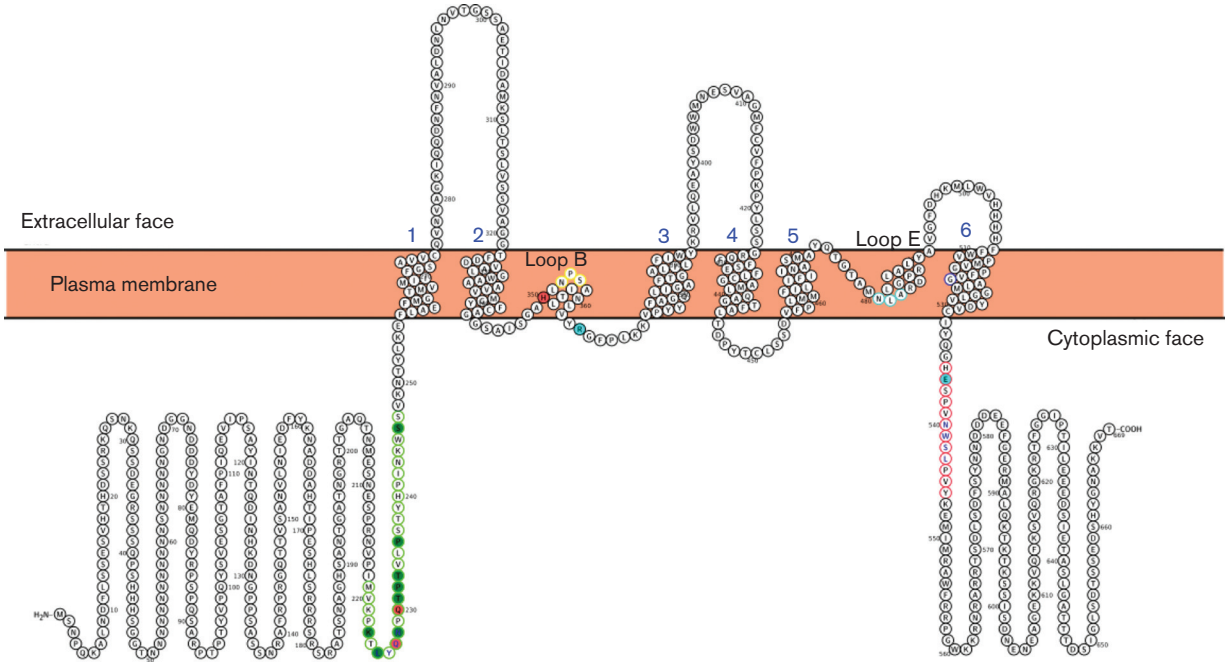

Fig. 1. Schematic representation of aquaglyceroporin Fps1p and its functionally critical domains. The amino acids are represented as circles. The six TM domains and loops B and E are marked. The brown bar depicts the cell membrane and the extracellular and cytoplasmic faces are indicated. Circles with green circumference depict the N-terminal regulatory domain. Likewise, circles with red circumference depict the C-terminal regulatory domain. The NPS motif of loop B and the NLA motif of loop E are highlighted by circles having yellow and cyan circumferences, respectively. Green-filled circles in the N-terminal regulatory domain represent the most important amino acids, i.e. L-225, N-228, T-231 and P-232, mutations in which cause osmosensitivity. Amino acids in the N-terminal regulatory domain, i.e. Q-227 or Q-230 (red-filled circles), may interact with H-350 of loop B. Similarly, E-536 in the C-terminal regulatory domain may interact with R-364 of loop B (cyan-filled circles). The amino acids in the blue font (LYQN in N-terminal domain and NWSL in C-terminal domain) depict similar but inverted regions of the form LXXN/NXXL and may help the two domains interact with each other. The residue G-519 of the TM core domain 6 has been shown to play an important role in regulating glycerol transport and has been depicted by circle having blue circumference {figure drawn using Protter [Omasits *et al.* (2014)] and based on Tamás *et al.* (2003); Karlgren *et al.* (2004); Hedfalk *et al.* (2004); Geijer *et al.* (2012)}.

the channel closes, thereby preventing glycerol loss. This closure is independent of both HOG and calcineurin pathways (discussed later) as mutants in pathway components, viz. *hog1p*Δ. *ssk1*Δ, *ssk2*Δ, *ssk22*Δ, *sho1*Δ, *pbs2*Δ (HOG pathway mutants) and *cna1*Δ*cna2*Δ (calcineurin mutants) exhibit marked reduction in the phosphorylation of Fps1p mediated by TORC2-Ypk1p.

MIP channels are known to exhibit marked substrate preferences. Channels transporting water do not transport other solutes such as glycine betaine, glycerol and so on, and vice versa (Hohmann, 2002b). In yeast, genome sequencing has led to the identification of four proteins encoding MIP channels. Two of them (Yfl054p and Fps1p) transport glycerol, and the other two – Aqy1p and Aqy2p – are aquaporins. Aqy1/2p are highly similar at the protein level (83 % amino acid similarity) and genes encoding both of them appear to be mutated in most laboratory strains (*AQY1* has two point mutations and *AQY2* has an 11 bp deletion), except for the *S. cerevisiae* WT strain Σ1278b, making the channels non-functional in all but this one strain. Aqy1p may play a role in spore maturation and spore germination, since it is heavily expressed in spores. Aqy2p may be involved in water efflux and maintenance of turgor pressure under hypoosmotic conditions, as its expression is diminished in an HOG-dependent manner under hyperosmotic conditions (Hohmann, 2002a). Strains in which *AQY1* and *AQY2* are deleted become more tolerant to hyperosmotic stress (Gonzalez-Hernandez, 2010). It seems that these mutated genes have persisted in laboratory strains due to unknown selection pressures.

Cation flows across cellular membranes

Ion channels

Several mechanisms contribute to ion homeostasis in yeast (Fig. 2). The transport of K^+ and Na^+ ions in yeast is mediated by ATP-driven active transport and H^+ antiport. Generally, yeast cells export Na^+ and accumulate K^+. Ena1p/Pmr2p

is a Na^+ exporter that belongs to the P-type ATPase family of autophosphorylable ion and lipid pumps. The transcription of *ENA1* is calcineurin dependent (Matsumoto *et al.*, 2002) and induced in response to high extracellular concentrations of both Na^+ and Li^+. Nha1p exports both Na^+ and K^+, while Trk1p/Trk2p contributes to K^+ uptake (Bañuelos *et al.*, 1998; Ko & Gaber, 1991). Nhx1p is a Na^+/K^+ exchanger located in the late endosome prevacuolar compartment that enables adaptation to high salt media by sequestering Na^+ in the vacuole, thereby reducing the cytosolic Na^+ concentration (Nass & Rao, 1999). Another K^+ channel in yeast is the outwardly rectifying Tok1p channel that preferentially causes K^+ efflux (Fairman *et al.*, 1999). It has been suggested that Tok1p is probably inhibited by Hog1p during Na^+ stress, which leads to depolarization of cell membrane and a lower influx of Na^+ (Ke *et al.*, 2013). (The roles of calcineurin and Ca^{2+} as a second messenger are detailed in the following section.)

Patch clamping experiments have been used to study channel protein activity in both prokaryotes and eukaryotes. In prokaryotes, stretch-activated channels MscL and MscS (membrane-sensitive channel of large/small conductance) are ubiquitous, and their homologues are present in bacteria, archea, fungi and plants (Edwards *et al.*, 2004). In such channels, membrane tension leads to progressive conformational change from closed to 'closed expanded' to opened, during which the central pore size increases from 1 to 16 Å (Hohmann, 2002b). One yeast homologue of MscL/S is Mid1p, a glycosylated plasma membrane channel protein that regulates Ca^{2+} influx (Iida *et al.*, 1994). It is a non-selective channel, allowing even anions to pass through, even though the exact conditions and mechanisms of such non-selective transport are unclear. The 36pS conductance of Mid1p during patch clamping persists in the plasma membrane of *mid1*Δ mutants, indicating that other, unidentified stretch-activated mechanosensitive channels exist in yeast (Martinac *et al.*, 2008). Muller *et al.* (2001) presented evidence in favour of two distinct Ca^{2+} influx systems, viz. a high-affinity Ca^{2+} influx system (HACS) and a low-affinity Ca^{2+} influx system (LACS). HACS is composed of Cch1p and Mid1p, functioning in low to moderate calcium environments. Co-localization of both the

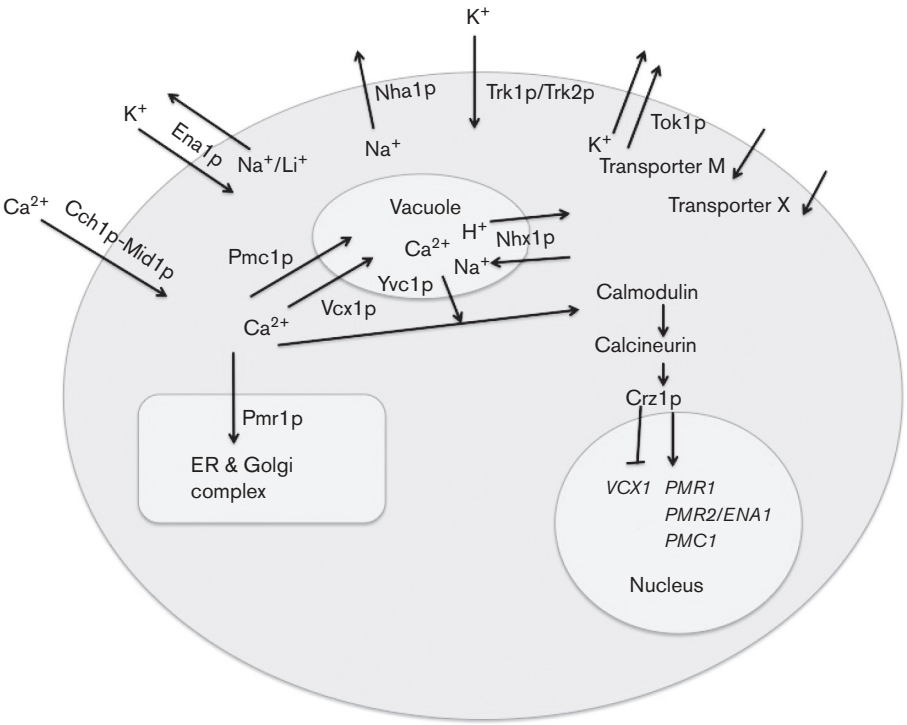

Fig. 2. Ion homeostasis and calcium signalling in yeast. In response to ionic stress, cytosolic calcium is transiently increased, attaining a higher steady-state concentration. Yvc1p releases vacuolar Ca^{2+} in the cytosol and Cch1p-Mid1p mediates Ca^{2+} influx from the extracellular environment in order to maintain $[Ca^{2+}]_{cyt}$. Meanwhile, Nhx1p, which is a vacuolar Na^+/H^+ exchanger, sequesters Na^+in the vacuole. Pmr1p helps restore the secretory Ca^{2+} in the ER and Golgi complex. Vacuolar transporters Pmc1p and Vcx1p import Ca^{2+} into the vacuole in response to high extracellular ionic concentrations. However, in response to high extracellular Ca^{2+}, Vcx1p activity, and perhaps Vcx1 expression, are downregulated in an unknown calcineurin-dependent manner. When Na^+/Li^+ is in excess in the cytosol, calcineurin also upregulates Pmr2p/Ena1p (which helps in Na^+ efflux and K^+ influx) and another Na^+ efflux pump, Nha1p. Other proteins that help in K^+ uptake are Trk1p/Trk2p and Tok1p. Transporters X and M are unidentified as yet [modified from Cui *et al.* (2009); also based on Cunningham & Fink (1994); Cunningham & Fink (1996)].

components of HACS on the plasma membrane and co-immunoprecipitation from soluble cell extracts indicates that they function as parts of the same system (Locke *et al.*, 2000). Thus, Ca^{2+} influx via HACS requires not only Mid1p but also Cch1p, an L-type voltage-gated Ca^{2+} channel (Iida *et al.* 2004). Interestingly, even a hyperactive mutant of Cch1p requires Mid1p for activity (Teng *et al.*, 2013).

Cch1p-Mid1p facilitates the entry of extracellular Ca^{2+} into the cell in response to various stimuli including high external ion concentration (Matsumoto *et al.*, 2002), endoplasmic reticulum (ER) stress (Bonilla & Cunningham, 2003), antifungals like euginol (Roberts *et al.*, 2012) and in the presence of mating pheromone (Muller *et al.*, 2001). The MAP kinase Mpk1p/Slt2p, the terminal MAP kinase of the cell wall integrity (CWI) pathway, is required for the activation of Cch1p-Mid1p during ER stress, and it counteracts the inhibition of Cch1p-Mid1p by calcineurin (Bonilla & Cunningham, 2003). The Cch1p-Mid1p channel plays an important role in regulating hyphal polarity, conidiation and the synthesis of cell wall components in *Aspergillus nidulans* (Wang *et al.*, 2012). HACS is stimulated by constitutive or overexpression of the Ste12p transcription factor [see Fig. 3, pheromone response (PR) pathway] in the presence of the calcineurin inhibitor FK506 in a mutant genetic background lacking the Ste12p inhibitors Dig1p and Dig2p (*dig1*Δ *dig2*Δ). Therefore, HACS activity likely involves calcineurin-dependent activation of Ste12p (Muller *et al.*, 2001). The work by Martin *et al.* (2011) indicates that, depending on the nature of the stimulus, HACS regulation may or may not involve calcineurin action. For example, calcium influx after high pH shock is calcineurin independent. However, PR involves the activation of the MAP kinase Fus3p (Fig. 3), which results in Cch1p activation, followed by dephosphorylation by calcineurin, which results in feedback inhibition (Muller *et al.*, 2003).

On the other hand, LACS, consisting of the polytopic plasma membrane protein Fig1p (Cavinder *et al.*, 2011), is not affected by calcineurin and can cause Ca^{2+} influx in *cch1*Δ *mid1*Δ double mutants (Muller *et al.*, 2001). Unlike HACS, it does not respond to multiple stimuli, but

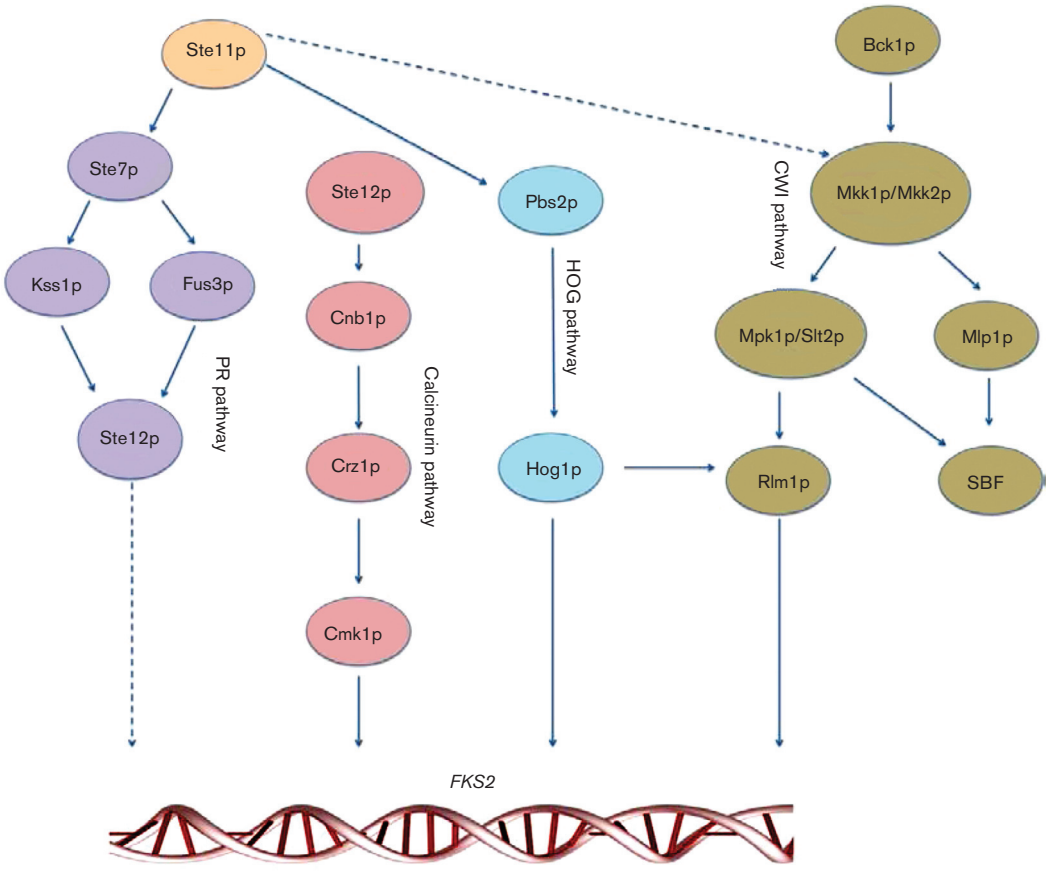

Fig. 3. Crosstalk among signalling pathways arising from the sharing of Ste11p (a sterile α-motif protein). The best understood role of Ste11p is as a MAPKKK in the SHO1 branch of the HOG pathway. However, it also functions in the PR pathway, thus activating transcription factor Ste12p that mediates calcineurin-dependent transcription of *FKS2* (glucan synthase), a target of the CWI pathway. Ste11p might itself play a role in CWI by directly activating the MAPKK Mkk1p/Mkk2p (dotted arrow), independently of MAPKKK Bck1p [from Wang *et al.* (2011), with permission].

it responds specifically to membrane stretching and morphogenesis during pheromone treatment. LACS has been demonstrated to be dependent on Bni1p (involved in formation of actin filaments, budding and mitotic spindle orientation), Far1p (an inhibitor of cyclin-dependent kinase Cdc28p mediates cell cycle arrest in response to pheromone) and Spa2p (a component of the polarisome assembly during polarized growth), all of these being involved in morphogenesis related to PR. Another example of a stretch-activated channel is Yvc1p [a member of the transient receptor protein channel (TRPC) family] that mediates Ca^{2+} efflux from vacuole to cytoplasm, in response to cytosolic Ca^{2+} depletion (Martinac *et al.*, 2008).

The role of vacuoles in osmoadaptation

As noted earlier, vacuoles tend to sequester Na^+ under high salt conditions using Nhx1p and help in countering high Na^+ stress. Nhx1p is a vacuolar membrane protein that functions as a $Na^+(K^+)/H^+$ antiporter. Deletion of *NHX1* leads to aberrant vacuolar morphology and defective vacuolar protein sorting. Vacuole fusion is important in ion homeostasis and Nhx1p has been shown to play a role in the fusion of vacuoles (Qiu & Fratti, 2010). In response to hyperosmotic conditions, cells lose water and vacuolar fragmentation occurs (Michaillat & Mayer, 2013). Hyperosmosis-induced fragmention of vacuoles is caused by Sty1p and Pmk1p in the fission yeast *Schizosaccharomyces pombe* (Bone *et al.*, 1998). Since Hog1p is the homologue of Sty1p and Slt2p/Mpk1p is the Pmk1p homologue in *S. cerevisiae*, this fragmentation process was initially thought to be HOG pathway dependent. However, Michaillat *et al.* (2012) have shown that it is the activity of the TOR complex 1 (TORC1) complex (discussed in the following section) during salt stress that leads to vacuolar fragmentation, indicating the absence of any direct involvement by HOG pathway members.

Crosstalk between the osmoadaptation response and other signalling pathways

Osmotic stress constitutes one of several, often simultaneous, environmental stimuli encountered by organisms, including yeast, and results in coordinated adaptive responses that depend on crosstalk between multiple signalling pathways. In this section, we will consider the effects of four important physiological signalling pathways on osmoadaptation: calcium mediated, CWI, target of rapamycin (TOR) and the general environmental stress response (ESR).

Calcium ions (Ca^{2+}) and the response to osmotic shock

Ca^{2+} plays a major role as a secondary messenger in eukaryotic organisms, including humans and plants. In yeast, cytosolic Ca^{2+} concentrations can increase during a shift to high temperature, hypoosmotic stress, sustained exposure to mating pheromone or an increase in the level of extracellular ions, such as Na^+, Ca^{2+}, Li^+ and so on. Calcium signalling is mediated by Ca^{2+} complexed with calmodulin (CaM), a highly conserved calcium-binding protein among eukaryotes. The Ca^{2+}/CaM complex in turn binds and activates a multisubunit serine/threonine phosphatase, calcineurin. In yeast, the catalytic domain of calcineurin is redundantly encoded by two homologous genes (*CNA1* and *CNA2*, while the regulatory domain is encoded by *CNB1*). Calcineurin enables the growth of yeast in media containing high levels of Na^+ and Li^+ (Cunningham & Fink, 1994) by dephosphorylating Ena1p/Pmr2p (a P-type ATPase located on the plasma membrane), thereby facilitating Na^+/Li^+ efflux. The calcium-dependent activation of calcineurin and its roles in the response to osmotic and ionic stresses are illustrated in Fig. 2. During hyperosmotic shock, secretory calcium stored in ER and Golgi apparatus is released into the cytoplasm through a Ca^{2+} efflux channel in the vacuolar membrane (Yvc1p), a homologue of the mammalian TRPC-type Ca^{2+} channel. The vacuolar Ca^{2+} ATPase Pmc1p and the H^+/Ca^{2+} exchanger Vcx1p sequester Ca^{2+} in the vacuole during hyperosmotic stress caused due to high extracellular Ca^{2+}, K^+ or Cl^-. This sequestering is controlled in a calcineurin-dependent manner and results from calcineurin-mediated deactivation of Vcx1p and the reduced expression of *Vcx1* by unknown mechanisms. Simultaneously expression of *PMC1* and *PMR1*, especially in response to high extracellular Ca^{2+} (Cunningham & Fink, 1996), is upregulated by the calcineurin via dephosphorylation of the transcription factor Crz1p (calcineurin-responsive zinc finger; Stathopoulos & Cyert, 1997; Matheos *et al.*, 1997). *PMR1* encodes a P-type ATPase similar to Pmc1p thaR130t is located in the Golgi complex. Cunningham & Fink (1994) demonstrated that double mutant yeast strains having a *pmc1*Δ *pmr1*Δ genotype are viable when calcineurin is inactivated by inhibitors such as cyclosporin A or FK506. Thus, calcineurin mediates accumulation of Ca^{2+} in the cytosol and its sequestration in intracellular organelles by Pmc1p and Pmr1p (Fig. 2).

Matsumoto *et al.* (2002) subjected *S. cerevisiae* to osmotic stress in two steps – an initial exposure to 0.5 M NaCl or 0.8 M sorbitol followed by 1 M NaCl or 2 M sorbitol, respectively. They found a rapid and sharp increase (within 1 min of stimulus) in cytoplasmic calcium levels, attaining a new higher steady-state concentration relative to the unstressed state. This may be due to a combination of the increased uptake of extracellular Ca^{2+}, release of Ca^{2+} from intracellular stores and limited vacuolar sequestration. Transient influx of extracellular Ca^{2+} is brought about by the Cch1p-Mid1p Ca^{2+} influx system alluded to in the earlier subsection on ion channels. Cch1p-Mid1p is activated by several stimuli such as membrane depolarization, depletion of secretory Ca^{2+} (Locke *et al.*, 2000), pheromone stimulation (Iida *et al.*, 1994; Muller *et al.*, 2001) and ionic stress (high extracellular Li^+ or Na^+) (Matsumoto *et al.*, 2002). Secretory calcium release is caused by Yvc1p, the vacuolar calcium channel, which is itself activated by Ca^{2+}

mediated by Cch1p-Mid1p uptake via an unknown mechanism (Fig. 2). However, the *yvc1*Δ mutant is unable to effect transient cytoplasmic calcium release in response to high extracellular ion concentrations for unknown reasons, even though Cch1p-Mid1p is functional, indicating that Yvc1p is involved in calcium uptake by Cch1p-Mid1p. Expression of *ENA1/PMR2* is transcriptionally induced by Hog1p or via Crz1p in a calcineurin-dependent manner in response to both NaCl and $CaCl_2$, indicating different pathways of *ENA1* induction during ionic/calcium stress. However, both pathways require a functional Cch1p-Mid1p channel (Matsumoto *et al.*, 2002). It must be borne in mind that all modes of entry for extracellular calcium in yeast are not known. For example, Cui *et al.* (2009) studied *cch1*Δ *yvc1*Δ double mutants and inferred the presence of at least two unknown transporters facilitating the entry of extracellular calcium into the cell.

The HOG and CWI pathways and calcineurin

A general observation of various research groups has been that the CWI [also termed the protein kinase C (PKC) pathway] pathway responds to hypoosmotic stress, heat stress, pheromone-induced morphogenesis, oxidative stress and so on (for a recent review, see Levin, 2011). Interestingly, it is also involved in cell cycle regulation. Similarly, the HOG pathway is also activated in response to multiple stresses, including citric acid (Lawrence *et al.*, 2004), heat (Winkler *et al.*, 2002), low temperature (Panadero *et al.*, 2006) and methylglyoxal (Aguilera *et al.*, 2005). Within the CWI pathway, GTPase-activating proteins and guanyl nucleotide exchange factors (GEFs) regulate the activation of the PKC MAP kinase cascade (see Fig. 3). When the CWI pathway is activated, the cell wall stress sensors Mid2p and Wsc1p bind to Rom2p, a GEF for Rho1p. Rho1p is a GTPase belonging the Rho subfamily of Ras-like proteins (Madaule & Axel, 1985). It effects compositional changes in the cell wall by acting as the regulatory subunit of the glucan synthase holoenzyme that synthesizes 1,3-β-D-glucan, a major cell wall component. Fks1p is the catalytic subunit of glucan synthase (Qadota *et al.*, 1996). The synthesis of 1,3-β-D-glucan is dependent on transcription factor Rlm1p that is itself activated by Slt2p/Mpk1p [a MAP kinase; (Dodou & Treisman, 1997)] and interestingly by Hog1p (Hahn & Thiele, 2002).

With regard to cell wall synthesis, a paralogue of *FKS1* (encoding Fks1p, above), *FKS2*, is transcribed only in the absence of *FKS1* expression and under conditions of high extracellular calcium (10 mM $CaCl_2$) and pheromone induction. Crz1p (described in the previous subsection) and the response regulator Skn7p, which also acts as a transcription factor, regulate *FKS1* (Stathopoulos & Cyert, 1997). Crz1p itself is activated by Rho1p-Skn7p via the CWI pathway (Williams & Cyert, 2001) or by the two-component osmosensing and signal relaying system Sln1p-Ypd1-Skn7p (Li *et al.*, 1998), which is part of the SLN1 branch of the HOG pathway (Fig. S1, available in the online Supplementary Material). [The turgor receptor Sln1p and the intermediate sensor Ypd1p make up the first part, and the response regulators Ssk1p and Skn7p constitute the second part of the signal relay system (Li *et al.*, 1998).] In a paper by Wang *et al.* (2011), the involvement of MAPKKK Ste11p was demonstrated across the three MAP kinase pathways in yeast, viz. HOG pathway, PR pathway and CWI pathway, eventually leading to transcription of *FKS2* (a glucan synthase gene) (Fig. 3). Interestingly, the calcineurin-Crz1p pathway has been found to have an antagonistic effect on the SLN1 branch of the HOG pathway during budding and in response to hyperosmotic stress via an unknown mechanism (Shitamukai *et al.*, 2004). This indicates that the same pathways may be regulated and interconnected differently in different contexts.

Rho1p also binds and activates Pkc1p (the only PKC in yeast) that initiates MAP kinase signalling by phosphorylation of the MAPKKK Bck1p. The cascade proceeds further as a result of phosphorylation of the MAPKK Mkk1p/Mkk2p, as well as subsequent phosphorylation and activation of the MAP kinase Slt2p/Mpk1p (Nonaka *et al.*, 1995, see Fig. 3). Mpk1p activates various transcription factors including Rlm1p (Watanabe *et al.*, 1995), Swi4p and Swi6p. Swi4p, a transcriptional activator, and Swi6p, a DNA-binding protein, form a heterodimer termed SBF (SCB binding factor) that activates transcription during the G1/S transition of the cell cycle (Madden *et al.*, 1997). Rlm1p and SBF are transcription factors activated on exposure to hypotonic solutions of NaCl, sorbitol or glucose (Davenport *et al.*, 1995). The transcription of *SLT2/MPK1* is also Rlm1p dependent (García *et al.*, 2016), which in turn is Hog1p dependent, and induced when the yeast cell experiences hyperosmotic stress (Hahn & Thiele, 2002). When the cell experiences hypoosmotic stress, the activation of Slt2p/Mpk1p by phosphorylation is carried out by the CWI pathway instead (Davenport *et al.*, 1995). The foregoing account illustrates that the HOG and CWI pathways regulate the osmotic homeostasis of the yeast by the sharing of effectors such as Slt2p/Mpk1p. In a recent paper by (Baltanás *et al.*, 2013), it was demonstrated that the PR pathway activates the HOG pathway in an Slt2p-dependent manner, dependent on changes in turgor pressure as a result of morphogenetic changes (shmooing) and opening of Fps1p channels (Fig. 4).

Interactions between the TOR and the CWI pathways during nutrient stress, and effects on osmotolerance

The TOR pathway is so named because it is inhibited by rapamycin and mediates the response to nutrient limitation, specifically to low nitrogen, glucose and amino acid availability (see Evans *et al.*, 2011 and Loewith & Hall, 2011 for reviews). Rapamycin is a macrolide produced by *Streptomyces hygroscopicus* that initially found use as an antifungal, but is currently repurposed as an immunosuppressant. It simulates conditions of nitrogen deprivation by forming a complex with Fpr1p, which is subsequently complexed to Tor1p, a serine/threonine (Ser/Thr) kinase, eventually inhibiting TOR signalling (Fuchs & Mylonakis, 2009).

(Fpr1p is the yeast orthologue of the human prolyl isomerase FK506-binding protein 12.) Interestingly, Tor1p performs two kinds of functions, one of which is rapamycin sensitive and the other one is rapamycin insensitive. This apparent dichotomy in function can be explained in terms of the enzyme being complexed with other proteins. Tor1p forms TORC1 consisting of Tor1p/Tor2p, Lst8p, Kog1p, Tco89 and Iml1p (Loewith, 2011). TORC1 performs rapamycin-sensitive functions involving both positive and negative regulations of its targets. For example, under favourable growth conditions, TORC1 (and TORC2) signalling results in the phosphorylation of Npr1p (a Ser/Thr kinase), rendering it inactive and preventing Npr1p-mediated degradation of the tryptophan permease Tat2p (Pagán-Mercado *et al.*, 2012; Schmidt *et al.*, 1998). Under the same conditions, Gap1p (a general aminoacid permease) is ubiquitinated by the ubiquitin protein ligase, Rsp5p, formerly designated Npi1p (Springael & André, 1998). The situation is reversed during the stationary phase or rapamycin treatment, when TOR signalling is lost and Npr1p is active, and this results in the degradation of Tat2p and the Npr1p-mediated protection of Gap1p from ubiquitination (Beck *et al.*, 1999). In the presence of rapamycin, TORC1 promotes sequestration of several nutrient-responsive transcription factors (Beck & Hall, 1999). TORC2 (consisting of Tor2p, Lst8p, Avo1p, Avo2p, Avo3p, Iml1p and Bit2p) performs rapamycin-insensitive functions, mainly during polarized growth of the cell via the control of the CWI

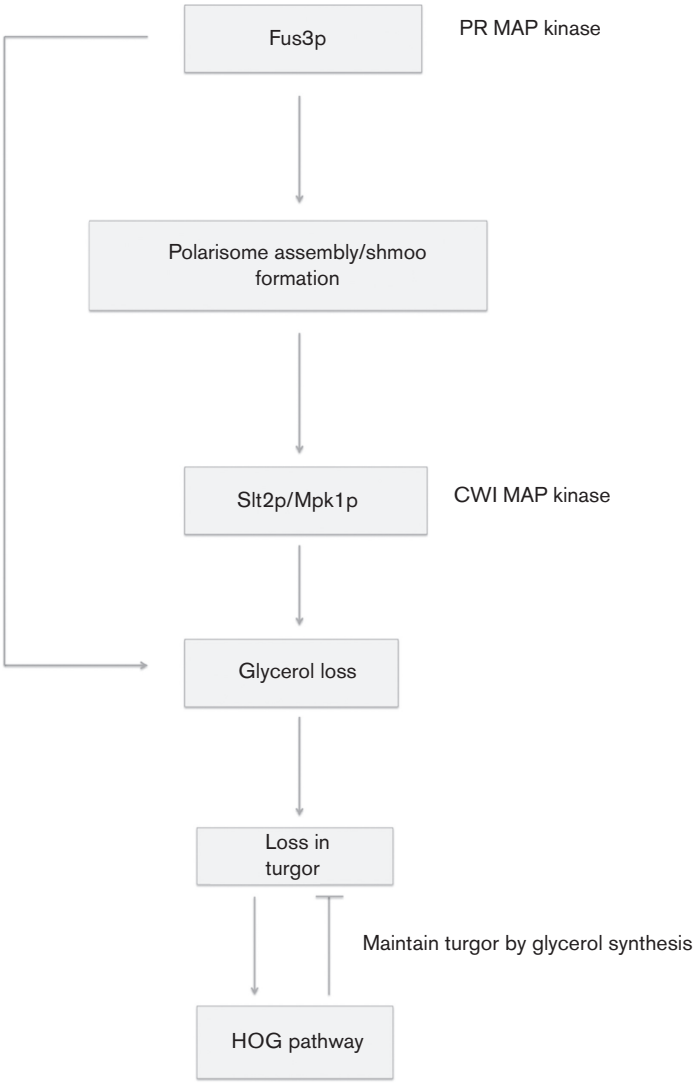

Fig. 4. Activation of the HOG pathway during PR. The PR pathway leads to activation of the HOG pathway in response to cell wall stress (loss of turgor) encountered during shmoo formation. Cell wall stress and polarisome assembly trigger the CWI pathway. The activated PR MAP kinase Fus3p and CWI MAP kinase Slt2p/Mpk2p increase glycerol loss, which in turn leads to loss of turgor. The loss of turgor leads to the activation of HOG pathway and the compensatory biosynthesis of glycerol [based on Baltanás *et al.* (2013)].

pathway (De Virgilio & Loewith, 2006; Loewith, 2011). It has been previously shown that, as a result of CWI pathway activation, the MAP kinase Slt2p/Mpk1p is phosphorylated by Mkk1/2 (García-Rodríguez *et al.*, 2005, see subsection 'The HOG and CWI pathways and calcineurin' and Fig. 3). Hence, it seems that the CWI pathway is also dependent to some extent on TOR activation.

Under conditions of nitrogen deficiency, shutdown of the TOR pathway results in the increased expression of *ENA1* (a Na^+/Li^+ efflux pump), incidentally resulting in increased salinity tolerance as well (Crespo *et al.*, 2001). Treatment with rapamycin induces *ENA1* expression in a manner dependent on Gat1p and Gln3p. Both Gat1p and Gln3p are TOR-dependent transcription factors that are localized in the nucleus when dephosphorylated by Sit4p (a TOR-controlled Ser/Thr protein phosphatase) in response to rapamycin or nitrogen deficiency. Treatment of *gln3*Δ *gat1*Δ mutants with rapamycin did not induce *ENA1* and they were found to be more sensitive to Na^+ and Li^+. *ENA1* expression was reduced in both standard rich medium (YPD) and under saline stress condition (YPD +0.4M NaCl) in the double mutant. Taken together, the results of the study (Crespo *et al.*, 2001) showed that *ENA1* is expressed in a TOR-dependent manner.

The TOR pathway is responsive to both osmotic and heat stress, and it is observed that osmotic stress diminishes amino acid uptake barring proline, probably due to its role as a compatible osmolyte (see the following section on osmolyte uptake). Amino acid deficiency or exposure to high salt (1M NaCl) is accompanied by the elevation of translation of Gcn4p, a transcription factor containing the basic leucine zipper DNA-binding domain and a master regulator of gene expression (Natarajan *et al.*, 2001). The elevated Gcn4p translation is caused by Gcn2p-dependent phosphorylation of eIF2α. Elevated levels of NaCl have been shown to reduce the uptake of many different amino acids, and this is one explanation for the activation of Gcn2p. However, *GCN4* expression can be induced in prototrophic strains in response to 1 M NaCl, arguing against a model in which limited amino acid uptake triggers activation of Gcn2p. Moreover, it has also been reported that high concentrations of sodium or potassium reduce amino acid uptake equally, without a noticeable induction of *GCN4* expression in the case of potassium. It has been proposed that dimerization of Gcn2p contributes to its activating *GCN4* and thus increases the expression of Gcn4p during NaCl-induced stress (Todeschini *et al.*, 2006). Recent work has indicated that the TOR and the general amino acid control (GAAC) pathways intersect, wherein TOR signalling (indicative of nutrient availability) represses the GAAC pathway (Staschke *et al.*, 2010). This also indicates that yeast strains that carry auxotrophic markers blocking amino acid biosynthetic pathways may have diminished osmotolerance compared to prototrophic strains and that such markers may account, in part, for the well-known strain differences in osmotolerance, as noted earlier.

Osmoregulation and the general response to environmental stress

The general ESR in yeast refers to the common changes observed in gene expression upon stress, regardless of the nature of the stress itself. The ESR involves the upregulation of ~300 genes and the downregulation of ~600 genes (Gasch *et al.*, 2000). The genes involved in ESR are activated by the binding of Msn2p and Msn4p transcription factors to the stress response element (STRE) within the promoter sequences. These transcription factors play non-redundant roles in the acquired resistance to multiple stress stimuli (NaCl, H_2O_2, heat stress, etc.) and not in the basal response to a single acute dose of one particular kind of stress (Berry & Gasch, 2008). Msn2p protein is present in the cytoplasm of unstressed cells, but it translocates to the nucleus when cells encounter stress. The nuclear exclusion of Msn2p in unstressed cells is attributed to phosphorylation mediated by cAMP-dependent protein kinase A (PKA; previously termed cAPK). Srb10p and Srb11p, constituents of the RNA polymerase II mediator complex, also downregulate the expression of STRE-containing genes by binding directly to Msn2p and inactivating it by phosphorylation (Bose *et al.*, 2005).

The genes that are upregulated in the ESR are involved in a wide variety of functions, including the overall feedback regulation of the ESR. In addition to PKA, TOR signalling also negatively regulates Msn2p and Msn4p by stimulating sequestration of these transcription factors in the cytoplasm (Beck & Hall, 1999). The ESR-induced genes encode both positive and negative regulators of the PKA and TOR pathways. Among the positive regulators are Tor1p and Tpk1/2p (PKA catalytic subunits) of the TOR and PKA pathways, respectively. The negative regulators are the regulatory subunit of PKA (Bcy1p), the phosphodiesterase Pde1p and the antagonist of both TOR and PKA – Yak1p. A major subset of genes downregulated during ESR are involved in protein synthesis, including those that encode ribosomal proteins; RNA processing and splicing factors; subunits of RNA polymerase I, II and III and other general transcription and translation factors. Additional genes involved in growth-related processes (such as cell cycle progression, secretion and metabolism) are repressed in both *S. cerevisiae* and *Schizosaccharomyces pombe* (Gasch, 2007).

Krantz *et al.* (2004), investigated whether osmotic stress leads to oxidative stress because hyperosmotic shock also activates genes involved in the oxidative stress response. However, they could not detect any major increase in intracellular reactive oxygen species under osmotic stress, regardless of whether the cultures were growing under aerobic or anaerobic conditions. They observed a more transient response to hyperosmotic stress in anaerobic cultures rather than the aerobically grown cultures of *S. cerevisiae*. This was attributed to increased glycerol production in anaerobic cultures, which in turn makes the cells more adapted to osmotic stress. This claim has been tested using transient expression of *STL1* (encoding a glycerol/H^+ symporter) as a

reporter of Hog1p activation. Similar results were obtained by comparing osmotolerant anaerobic strains to *GPD1* overexpressing strains. Interestingly, Hickman *et al.* (2011) also reported hypoxia-mediated activation of Hog1p and demonstrated that kinetics of Hog1p activation in response to hypoxia and osmotic stress are very different. Hog1p shows rapid transient activation in response to osmotic stress response as reported earlier, but it is activated slowly (over a period of 5 h) in response to hypoxia (Hickman *et al.*, 2011).

Thus, the HOG pathway interacts with, or is bypassed by, various other pathways at the level of signalling components or at the level of effector proteins and other molecules. It, along with the CWI pathway, mediates resistance to killer toxin HM-1, probably by an upregulation of glucan synthase (Miyamoto *et al.*, 2012). (Killer toxin HM-1 is a protein of 88 amino acids produced by the fungus *Williopsis saturnus* var. *mrakii* IFO 0895.) The CWI, HOG and calcineurin pathways mediate tolerance to low pH caused due to inorganic acids (de Lucena *et al.*, 2012). This repeated repurposing of a few 'core' pathways by either isolation or interaction in a context-dependent manner enables a wide range of complex physiological responses to environmental stimuli.

Osmolyte uptake in *S. cerevisiae*

Osmolytes such as glycerol can be taken up by yeast to in response to osmotic stress. Yeast can also synthesize glycerol in a Hog1p-dependent manner (Kaserer *et al.*, 2009). Gpd1p (glycerol-3-phosphate dehydrogenase), its isoform activated under anoxic conditions (Gpd2p) and Gpp1p/2p (glycerol-3 phosphatases) are enzymes involved in glycerol synthesis. The Fps1p channel is also closed during hyperosmotic stress to minimize the export of synthesized glycerol as discussed earlier. Initially, the active uptake of glycerol was studied by screening *gpd1*Δ*gpd2*Δ strains for glycerol uptake from the media under hyperosmotic conditions. Deletions in both paralogues encoding glycerol-3-phosphate dehydrogenase impair glycerol synthesis substantially, but not completely (see Oliveira & Lucas, 2004). Glycerol uptake in these doubly mutant strains was attributed to two proteins Gup1p (a putative membrane bound *O*-acetyltranferase) and its paralogue Gup2p (Holst *et al.*, 2000). Subsequently, Oliveira & Lucas, 2004 determined that while *GUP1* and *GUP2* are expressed constitutively, transient changes in their expression levels in response to osmotic stress before attaining equilibrium could not be ruled out. Ferreira *et al.* (2005), on the other hand, determined that active glycerol uptake was not attributable not either Gup1p or Gup2p but to the glycerol/H^+ symporter Stl1p (sugar transporter like). Recent studies have confirmed that the apparent osmosensitivity of *gup1*Δ mutants is due to the involvement of Gup1p in glycosylphophotidyl inositol lipid membrane remodelling, rather than in glycerol uptake (Yoko-o *et al.*, 2013).

Proline is a major osmolyte in plants that accumulates in response to saline and osmotic stress (Burg & Ferraris, 2008; Delauney & Verma, 1993; Szabados & Savouré, 2010). Therefore, it is likely that yeast may encounter abundant proline in their natural environment and utilize this osmolyte as well (Hohmann, 2002b). Indeed, overexpression of the proline dehydrogenase Put1p renders yeast cells hypersensitive to oxidative stress and heat (Chen *et al.*, 2006). *PUT1* is normally induced under nitrogen-limiting conditions, catalysing the conversion of proline into pyrroline-5-carboxylate, which is subsequently hydrolysed to γ-glutamic acid semialdehyde and that, in turn, is further reduced to glutamic acid by the action of Put2p. Thus, *PUT1* overexpression depletes proline stores. Global gene expression analysis has revealed that expression of *PUT4*, encoding a high-affinity proline and γ-aminobutyrate permease, is strongly stimulated under hyperosmotic and high salinity conditions (Rep *et al.*, 2000; Yale & Bohnert, 2001). *PUT4* induction is only partially Hog1p dependent and is induced by the Hot1p transcription factor; *HOT1* transcription is, in turn, activated by Hog1p (Rep *et al.*, 2000). *S. cerevisiae* encodes at least four permeases that are involved in proline transport – Put4p, Gap1p, Agp1p and Gnp1p (Andréasson *et al.*, 2004). The first two are nitrogen regulated while the latter two are regulated by the SPS (Ssy1p–Ptr3p–Ssy5) sensor of extracellular amino acids. The SPS sensor, when induced by extracellular amino acids, cleaves the N-terminal region of cytoplasmic Stp1p/2p (zinc finger transcription factors) that enables their nuclear entry and the transcriptional activation of amino acid permease genes (for a review, see Ljungdahl, 2009).

Osmolyte accumulation in the cell under hyperosmotic conditions, whether by synthesis or uptake, is essential for rapid Hog1p activation as well. Geijer *et al.* (2013) constructed a double mutant of *S. cerevisiae* with deletions in genes encoding both glycerol-3-phosphate dehydrogenase isoforms (*gpd1*Δ*gpd2*Δ) and expressing a rat aquaglyceroporin (rAQP9). Thus, osmoadaptation in this mutant has to rely not on glycerol synthesis but on the uptake of polyols (glycerol, erythrol xylitol and sorbitol in increasing order of molecular weight) from the medium via rAQP9. For this to occur, the initial recovery of the cells during the time course tested in these experiments should ideally be unaffected by glycerol import through the Stl1p glycerol/H^+ symporter, as *STL1* is a Hog1p-induced gene. The polyols function as osmotic stressors and compatible osmolytes upon uptake. When the mutant yeast cells were stressed with 1 M of any polyol, nuclear localization of activated Hog1p was delayed in direct proportion to the molecular weight of polyols available for uptake. Sorbitol stress maximally delayed Hog1p nuclear localization and the subsequent transcriptional response. However, Duskova *et al.* (2015) demonstrated, using the same yeast strain (BY4741), that Stl1p repression is not complete, indicating some polyol uptake via Stl1p, though this does not qualitatively affect the conclusions of Geijer *et al.* (2013).

Other studies (Babazadeh *et al.*, 2013; Miermont *et al.*, 2013) demonstrated that the delayed nuclear localization of Hog1p is not due to a lower rate of nuclear import, as the rate of import remains similar during different stress conditions (0.4 M and 0.8 M NaCl). Rather, the cytosolic diffusion of Hog1p is delayed due to molecular crowding in the cytosol upon volume loss under hyperosmotic conditions. It must be noted here that Babazadeh *et al.* (2013) studied *fps1*Δ, *ptp2*Δ and *ptp3*Δ single mutants in the context of Hog1p nuclear import. [*PTP2* and *PTP3* encode tyrosine phosphatases that inhibit Hog1p by dephosphorylation (see supplementary information). Ptp2p is present in the nucleus, while Ptp3p is present in the cytosol.] It was observed that cells having *ptp2*Δ and *fps1*Δ (but not *ptp3*Δ mutants) behaved similar to genetically 'pre-adapted' cells due to an increased basal Hog1p level and faster volume recovery, respectively. Thus, recovery of cell volume is required to restore 'normal' diffusion rates enabling Hog1p nuclear entry, and excessive molecular crowding due to cell shrinkage delays the signalling process. Babazadeh *et al.* (2013) also noted that the *gpd1*Δ*gpd2*Δ mutant displays 'delayed or absent initial responses' and attributed this to a lower initial cell volume (and hence greater crowding) that never quite attains 'normal' levels and that imposes a correspondingly longer delay on the response system.

Genetic regulation versus metabolic adaptation

While signalling pathways bringing about changes in gene expression are involved in responses to a variety of stimuli, research by Bouwman *et al.* (2011) sounded a cautionary note that metabolic adaptation, and not *de novo* changes in gene expression, quantitatively dominates the osmoadaptation response. Thus, the hyperosmotic shocking of yeast cultures results in an increase in the maximal velocity (V_{max}) of glycerol-3-phosphate dehydrogenase (Gpd1/2p) and glycerol-3-phosphatase (Gpp1p). Gpd1p converts dihydroxyacetone phosphate into glycerol-3-phosphate, which is dephosphorylated to glycerol by Gpp1p. Interestingly, the relative contribution of isozymes Gpd1p and Gpd2p to the glycerol flux indicated that Gpd1p was selectively upregulated in response to osmotic shock (also upregulated in response to cold stress, see Panadero *et al.*, 2006). Purely metabolic adaptation occurring at the post-translational level is missed in studies focusing a priorion gene activation and repression in response to stimuli (Bouwman *et al.*, 2011). Thus, under normal growth conditions and in the presence of sufficient glucose, TORC2-Ypk1p phosphorylation of Gpd1p reduces the catalytic activity of the enzyme. Under conditions of either glucose limitation or hyperosmotic stress, Gpd1p phosphorylation decreases, resulting in the increased production of glycerol. The downregulation of Ypk1p-mediated phosphorylation of Gpd1p is independent on Hog1p action as this dephosphorylation was found to occur in osmotically stressed *hog1*Δ cells as well (Lee *et al.*, 2012). In the work of Babazadeh *et al.* (2013), volume recovery and Hog1p nuclear entry are observed to be most rapid in the *fps1*Δ mutant, even at higher levels of osmostress (0.8 M NaCl) relative to both the WT and the 'pre-adapted' *ptp2*Δ mutant. Thus, this is indicative of the importance and effectiveness of an initally rapid adaptation to stress by glycerol retention (due to the deletion of *FPS1*) relative to changes in gene expression. In terms of adapting to fluctuating environmental conditions, Duskova *et al.* (2015) observed that the glycerol/H^+ symporter Stl1p was a critical component of osmoadaptation and that it was also involved in pH homeostasis. From their data, it would seem that the relative contribution of Stl1p function to survival under desiccation and subsequent rehydration and switching from hyperosmotic to hypoosmotic conditions is comparable to that of Hog1p.

Studies in which Hog1p signalling is abrogated by various means, preventing the usual activation of gene expression, have also highlighted the importance of metabolic adaptation. Westfall *et al.* (2008) constructed mutant yeast strains in which (a) the nuclear importin Ndm5p was deleted, preventing activated Hog1p translocation into the nucleus or (b) Hog1p was mislocalized by tethering to the plasma membrane. Both mutants were able to survive hyperosmotic conditions, indicating that transcriptional activation of target genes by Hog1p was not required. Westfall *et al.* further demonstrated that only mutations in genes involved in glycerol synthesis (i.e. *gpd1*Δ,.*tpi1*Δ and *gpp1*Δ *gpp2*Δ) resulted in non-viability (as opposed to partial viability) under hyperosmotic stress. Likewise, Babazadeh *et al.* (2014) report that a *hog1*Δ mutant can be rescued by ensuring higher glycerol synthesis under hyperosmotic conditions. Exploiting crosstalk between the PR and HOG pathways via sharing of Ste11p (see Figs 3 and S2), they demonstrated that driving the transcription of *GPD1* and *GPP2* by the Fus3p-dependent *FUS1* promoter could suppress the osmosensitive phenotype of *hog1*Δ mutant. Thus, in the context of osmoadaptation, glycerol synthesis and retention predominates and is crucial to the adaptation process.

Osmosensitivity and osmotolerance are contingent not only upon mechanisms that directly control intracellular osmolyte and ion levels. The composition, polarization and fluidity of the plasma membrane are important parameters in the stress resistance of yeast. For example, the synthesis of ergosterol in the fungal plasma membrane is repressed in a Hog1p-dependent manner via transcriptional repressors Mot1p and Rox1p during hyperosmotic stress, but it is repressed in a Hog1p-independent manner under oxidative stress (Montañés *et al.*, 2011). Recent work by Kodedová & Sychrová (2015) indicates that mutants with deletions in genes involved in the multiple steps of ergosterol synthesis are differentially susceptible/tolerant to various stresses, e.g. *erg5*Δ mutant has an osmotolerance equivalent to the WT (at 1.4 M NaCl), which is higher relative to *erg2*Δ, *erg3*Δ, *erg4*Δ or *erg6*Δ mutants. This should be seen in the light of earlier observations (Tanigawa *et al.*, 2012) that *erg2*Δ, *erg3*Δ and *erg6*Δ mutant exhibit elevated Hog1p

phosphorylation due to perturbation of the plasma membrane. However, the hyperosmotic sensitivity of these mutants observed by Kodedová & Sychrová, (2015) points to the essential contribution of plasma membrane functions for successful adaptation via HOG signalling. [Note that Kodedová and Sychrová used strain BY4741, while Tanigawa *et al.* used strain S288C. While BY4741 is also derived from S288C (Brachmann *et al.*, 1998), it is possible that these strains are not strictly comparable.]

Given these facts, what is the utility of *de novo* gene expression to osmoadaptation? Mettetal *et al.* (2008) studied recovery after osmotic shock in WT yeast cells that were exposed to cycloheximide to repeated pulses of NaCl. Recovery times from an initial pulse of osmotic shock were similar (~15 min) and glycerol accumulation attained similar levels. However, repetitive shocks in the presence of cycloheximide diminished glycerol accumulation when compared to cells not exposed to cycloheximide. The authors surmised that, while gene expression changes were not required for rapid recovery from a single osmotic shock, changes in gene expression enhanced recovery from repeated shocks of longer duration by an increase in glycerol production. Thus, the relative contributions of metabolic adaptation and changes in gene expression to osmoadaptation may be dependent on the intensity and duration of the stimuli encountered, with gene expression becoming a crucial component when undergoing prolonged and/or multiple simultaneous stresses. On the other hand, metabolic adaptation is sufficient to enable adaptation to a brief encounter with a single type of stress.

Future perspectives

The utility of studies of osmoregulation in *S. cerevisiae* goes well beyond providing an insight into information on adaptation mechanisms in a genetically tractable organism under well-defined conditions. Owing to the evolutionary conservation of several mechanisms of stress responses in a wide range of organisms, both unicellular and multicellular, these studies additionally enable us to make testable predictions and formulate experimental strategies in other organisms that are less well-characterized or otherwise experimentally less tractable owing to a variety of reasons. For example, molecular components mediating stress responses in pathogenic fungi can serve as potential targets for antifungals, as well as provide important information on the mode of antifungal action (recently reviewed by Brown *et al.*, 2014; Hayes *et al.*, 2014; Thewes, 2014; Liu *et al.*, 2015). Studies of TOR signalling in yeast in the context of lifespan extension by calorie restriction have uncovered previously unanticipated mechanisms, which are stress responsive and mediated by sirtuin-2, of suppressing genomic instability arising from repetitive ribosomal DNA (Medvedik *et al.*, 2007; see Schleit *et al.*, 2012, for a review). It has also been suggested that the study of yeast channel proteins having human homologues could offer insights into human channelopathies (Wolfe & Pearce, 2006). Insight into *S. cerevisiae* adaptive mechanisms to environmental stresses in general, and osmotic stress in particular, can also provide useful pointers to improving the performance of this workhorse of the fermentation industry by enhancing its tolerance to inhibitor build-up within bioreactors (reviewed by Caspeta *et al.*, 2015).

Our description of various osmoregulatory (and other) response systems in pure, clonal cultures of yeast implicitly assumes that the population being studied is essentially homogeneous in terms of its response to a defined stimulus. While this enables the dissection of adaptation mechanisms and the biomolecules involved, such analysis often misses intrinsic noise in gene expression and protein levels that can lead to bistability or multistability when networks of interacting components are considered (Viney & Reece, 2013). The pioneering study of protein abundance variation in individual yeast cells by Newman *et al.* (2006) found that proteins involved in responses to environmental conditions typically display a wide range of abundances, which could be advantageous in a fluctuating environment enabling the survival of at least some individuals within a population. Such knowledge provides an opportunity to examine phenotypic plasticity or the ability of a single genotype to express variable phenotypes depending on environmental conditions and is likely to be a fertile area for further enquiry.

While we have earlier commented on the evolutionary conservation of stress response elements, a comparative study of fungal stress responses by Nikolaou *et al.* (2009) has revealed a more nuanced situation. When stress response pathways are compared across several fungal species, it is found that there is extensive, niche-specific divergence of sensory and response elements, whereas core components of pathways (especially multifunctional components) are phylogenetically conserved. This serves to remind us that natural selection operates directly on phenotypes and only indirectly on genotypes. The availability of high-throughput data for a wide variety of species should not only enable comparisons and inferences but also inform and refine our efforts to model and manipulate regulatory systems of interest.

Though a deceptively simple-looking, unicellular eukaryote, *S. cerevisiae* has evolved mechanisms to cope with several types of environmental stresses, of which osmoregulation is a well-studied example. More often than not, response pathways interact to evoke a global and coordinated cellular response directed not only at mitigating the effects of the stress but also at ensuring that interlinked aspects of cell physiology such as growth rate and cell morphology are in consonance and commensurate with the response. Research on various aspects of *S. cerevisiae* biology over nearly a century, combined with more recent resources such as publicly available genome sequence, mutant libraries and large-scale expression data, can provide us unprecedented insights into organismal responses and adaptive strategies when confronted with both brief and sustained environmental stimuli in the proverbial struggle for existence.

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