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Isabel Sá-Correia *Editor*

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Physiological Genomic Approaches

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Isabel Sá-Correia
Editor

Yeasts in Biotechnology and Human Health

Physiological Genomic Approaches

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Chapter 1
Physiological Genomics of Multistress Resistance in the Yeast Cell Model and Factory: Focus on MDR/MXR Transporters

Cláudia P. Godinho and Isabel Sá-Correia

Abstract The contemporary approach of physiological genomics is vital in providing the indispensable holistic understanding of the complexity of the molecular targets, signalling pathways and molecular mechanisms underlying the responses and tolerance to stress, a topic of paramount importance in biology and biotechnology. This chapter focuses on the toxicity and tolerance to relevant stresses in the cell factory and eukaryotic model yeast *Saccharomyces cerevisiae*. Emphasis is given to the function and regulation of multidrug/multixenobiotic resistance (MDR/MXR) transporters. Although these transporters have been considered drug/xenobiotic efflux pumps, the exact mechanism of their involvement in multistress resistance is still open to debate, as highlighted in this chapter. Given the conservation of transport mechanisms from *S. cerevisiae* to less accessible eukaryotes such as plants, this chapter also provides a proof of concept that validates the relevance of the exploitation of the experimental yeast model to uncover the function of novel MDR/MXR transporters in the plant model *Arabidopsis thaliana*. This knowledge can be explored for guiding the rational design of more robust yeast strains with improved performance for industrial biotechnology, for overcoming and controlling the deleterious activities of spoiling yeasts in the food industry, for developing efficient strategies to improve crop productivity in agricultural biotechnology.

Keywords *Saccharomyces cerevisiae* · Response and resistance to stress · Physiological genomics · MDR/MXR transporters · Eukaryotic model · Cell factory

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1.1 Multidrug/Multixenobiotic Resistance (MDR/MXR) and MDR/MXR Transporters in *Saccharomyces cerevisiae*

Understanding the complexity of cellular responses to environmental insults is a major challenge in Biology given that the survival of living cells depends on their ability to sense alterations in the environment and to appropriately respond to the new stressing situations by remodelling genomic expression. In the particular case of the yeast *Saccharomyces cerevisiae*, its role as cell model and factory in biotechnology implicates exposure to stress factors that may not be present in its natural environment. The highly useful eukaryotic model *S. cerevisiae* shares with more complex and less accessible eukaryotes, from the experimental point of view, molecular mechanisms that are conserved in the eukaryotic cell. Therefore, studies in this experimental model can provide clues on multistress resistance in other eukaryotes, as it is, for example, the case of resistance to pesticides and other stresses of agricultural relevance in plants (Teixeira et al. 2007; Cabrito et al. 2011). Many studies focusing on more complex eukaryotes rely on the heterologous expression in yeast of genes from those eukaryotes to unveil their function and impact in stress response and resistance (Remy et al. 2012, 2015, 2017; Cabrito et al. 2009). Also in biomedical, clinical and medicinal research, the model yeast *S. cerevisiae* has been highly useful to examine the toxicological response to get a genome-wide view and to understand adaptation and resistance to drugs relevant to fight pathogenic fungi or cancer cells (dos Santos et al. 2012). Concerning yeast as a cell factory, alcoholic fermentation and other biotechnological processes also impose multiple chemical and physical stresses to the yeast cell as detailed later in the chapter.

Among the mechanisms used by yeast to overcome the deleterious effects imposed by chemical and other environmental stresses is the action of plasma membrane transporters belonging to the Major Facilitator Superfamily (MFS) (Sá-Correia et al. 2009; dos Santos et al. 2014) or to the ATP-binding cassette (ABC) superfamily (Gulshan and Moye-Rowley 2007; Higgins 2007; Jungwirth and Kuchler 2006; Piecuch and Obłak 2014). Although these transporters involved in multidrug/multixenobiotic resistance (MDR/MXR) have been considered as drug/xenobiotic efflux pumps, thus contributing to the decrease of the intracellular concentration of a given toxic compound by actively pumping it out of the cell, this simplistic concept is currently under scrutiny (dos Santos et al. 2014; Godinho et al. 2018; Prasad and Panwar 2004; Cabrito et al. 2011). In fact, it is puzzling that a wide range of structurally and functionally unrelated substrates might be exported from the cell by a specific transporter, especially when the organism is not expected to be in contact with them in the natural environment. The physiological function of a number of these MDR/MXR transporters is being revealed in yeast and in other organisms and it is now clear that they might have natural substrates (dos Santos et al. 2014; Prasad et al. 2016; Tarling et al. 2013). It is hypothesized that their eventual role in drug/xenobiotic transport might occur only fortuitously or opportunistically or that their action might affect the partition of cytotoxic compounds indirectly by influencing drug/xenobiotic

accumulation in the cell interior by promoting physical or chemical changes in the plasma membrane (Prasad et al. 2016; Sherlach and Roepe 2014). The large number of homologous MDR/MXR transporters from the MFS and the ABC superfamily that are encoded in the genomes of *S. cerevisiae* and other eukaryotes strongly suggests that they may play important physiological roles even in the absence of cytotoxic compounds. Accumulating evidence has shown that ABC and MFS transporters required for MDR/MXR perform endogenous activities extending beyond their accepted role as drug/xenobiotic exporters (dos Santos et al. 2014; Godinho et al. 2018; Prasad and Panwar 2004; Prasad et al. 2016). The understanding of the physiological function of the multiple MDR/MXR transporter homologues can also provide clues into the mechanisms behind their action in multistress resistance.

Plasma membrane composition and function is essential for the ability of yeast cells to overcome chemical and environmental stresses, given that plasma membrane is an active interface between the cell interior and the surrounding medium. Plasma membrane lipid composition and organization greatly impact yeast resistance to stress as these parameters influence plasma membrane role as a selective barrier to the entrance of compounds by passive diffusion (Peetla et al. 2013; Bosmann 1971; Rank et al. 1978; Mukhopadhyay et al. 2002). Also, yeast plasma membrane is a lipid matrix for several embedded proteins, and thus its composition and structure can influence the kinetics of solute transport, the maintenance of the transmembrane electrochemical potential and the activity of the MDR/MXR transporters (Peetla et al. 2013; Rank et al. 1978; Mukhopadhyay et al. 2002; Shahi and Moye-Rowley 2009; Kodedová and Sychrová 2015). Remarkably, several ABC required for MDR/MXR are involved in lipid trafficking impacting cell membrane lipid composition and properties (Jungwirth and Kuchler 2006; Prasad et al. 2016).

1.2 The *S. cerevisiae* Transporters of the Major Facilitator Superfamily

Transporters belonging to the Major Facilitator Superfamily (MFS) are secondary carriers of small solutes, involved in uniport, symport and antiport transport processes in response to a chemiosmotic gradient (dos Santos et al. 2014; Pao et al. 1998; Sá-Correia and Tenreiro 2002). It is proposed that some MFS transporters play a role in multidrug/multixenobiotic resistance (MDR/MXR) by performing the antiport of drugs/xenobiotics with protons (dos Santos et al. 2014).

Structurally, the MFS-MDR/MXR transporters are composed of 500–600 amino acids in a single-polypeptide chain, with two structural units of six or seven transmembrane-spanning α-helical domains (TMD), connected by a cytoplasmatic loop (Fig. 1.1) (Marger and Saier 1993; Nelissen et al. 1997). MFS-MDR/MXR transporters were first classified into two subfamilies, depending on whether the protein contains 12 or 14 transmembrane segments: the 12-spanner drug:H^+ antiporter family 1 (DHA1) and the 14-spanner drug:H^+ antiporter family 2 (DHA2) (Nelissen

et al. 1995, 1997). More recently, a combined phylogeny and gene neighbourhood analysis revealed that the DHA2 family of transporters shares a common root with two other MFS transporter families, the siderophore transporters (ARN) and the glutathione exchangers (GEX) (Dias and Sá-Correia 2013). Consequently, these three families of MFS transporters were proposed to be included in the new MFS gene family, designated DAG (DHA2/ARN/GEX) family (Dias and Sá-Correia 2013) (Fig. 1.1).

The DHA1 family of transporters in *S. cerevisiae* is composed of 12 proteins, encoded by *AQR1*, *DTR1*, *FLR1*, *QDR1*, *QDR2*, *QDR3*, *TPO1*, *TPO2*, *TPO3*, *TPO4*, *YHK8* and *HOL1* genes (Sá-Correia et al. 2009; dos Santos et al. 2014; Dias et al. 2010). The DAG family includes 16 transporters, encoded by *ATR1, AZR1, SGE1, VBA1, VBA2, VBA3, VBA4, VBA5, ATR2, AMF1, ARN1, ARN2, ARN3, ARN4, GEX1* and *GEX2* (Sá-Correia et al. 2009; dos Santos et al. 2014).

A summary of the subcellular localization, susceptibility profile to drugs/xenobiotics and physiological roles identified so far for several MFS-MDR/MXR transporters, from DHA1 and DAG families, is provided in Tables 1.1 and 1.2. Studies dedicated to examine the physiological role of these transporters in the cell, even in the absence of any stress, have been scarce but for a few MFS-MDR/MXR transporters a biological function was already unveiled.

The MFS transporters Yhk8 and Hol1 have no reported role in MDR/MXR. However, *YHK8* transcription was found to be up-regulated in cells exhibiting reduced susceptibility to azole drugs (Barker et al. 2003) and mutations in *HOL1* have been shown to enhance the ability of yeast cells to import histidinol (a precursor of histidine) as well as mono- and divalent cations (Wright et al. 1996).

1.3 Transcriptional Regulation of *S. cerevisiae* MFS-MDR/MXR Transporter-Encoding Genes in Response to Stress

A very large amount of information regarding the transcriptional regulation of *S. cerevisiae* MFS-MDR/MXR transporter-encoding genes, as well as of the other yeast genes, is gathered in the YEASTRACT database (www.yeastract.com). A very large part of this information is based on the use of genome-wide transcriptomic analysis (Teixeira et al. 2018). This precious and user-friendly information system is essential to guide further analysis to unveil the complex regulatory networks acting on the transcriptional regulation of MDR/MXR transporters in yeast cells challenged with different environmental stress conditions. To exemplify this approach, the YEASTRACT database was used to prepare Fig. 1.2 by extracting the information behind the regulatory networks underlying the activation of MFS transporters in the presence of different stresses. Both DNA binding and expression evidences were considered as the source of information, but this study was restricted to MFS transporters with a documented role in MDR/MXR, as shown in Tables 1.1 and 1.2.

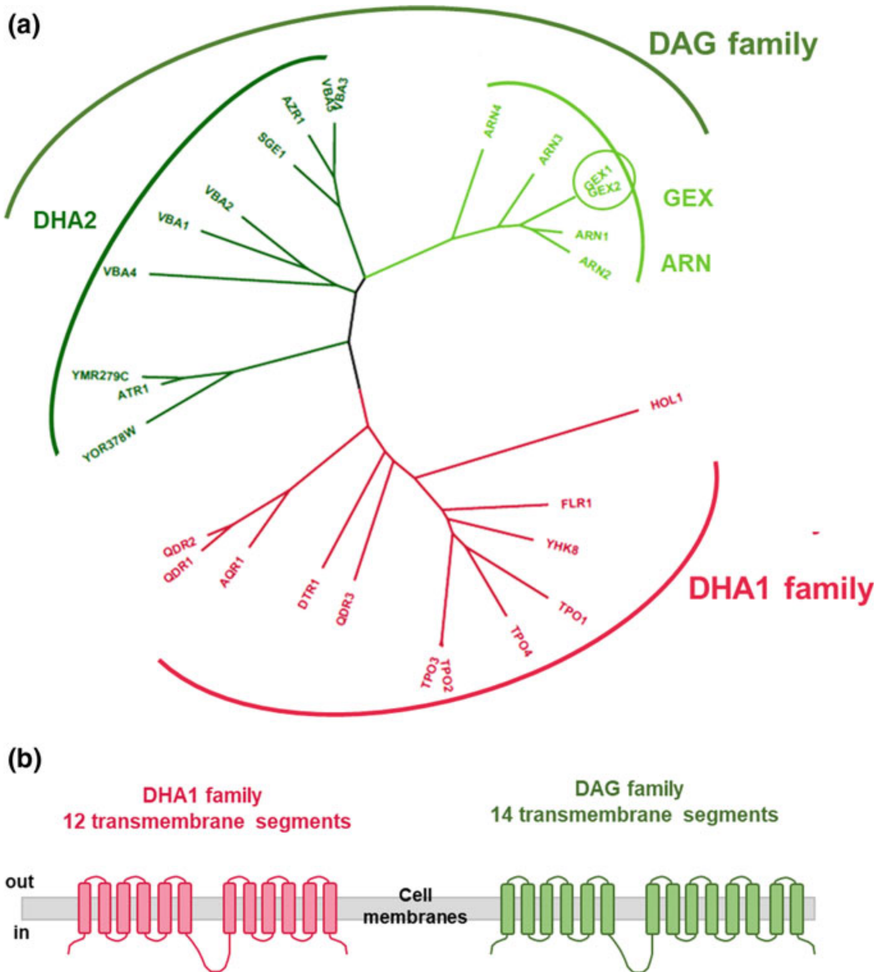

Fig. 1.1 Phylogenetic tree and predicted topology of yeast MFS-MDR/MXR transporters. **a** Maximum likelihood phylogenetic tree constructed using the protein sequences of the MFS transporters from the DHA1 and DAG families of *S. cerevisiae* S288c, retrieved from SGD. Alignment and phylogenetic reconstruction were performed using the function 'build' of ETE3 v3.0.0b32 (Huerta-Cepas et al. 2016) as implemented on the GenomeNet (http://www.genome.jp/tools/ete/). Alignment was performed with MUSCLE v3.8.31 with the default options (Edgar 2004). ML tree was inferred using PhyML v20160115 ran with model JTT and parameters: -f m –pinv e -o tlr –alpha e –nclasses 4 –bootstrap -2 (Guindon et al. 2010). Branch supports are the Chi^2-based parametric values return by the approximate likelihood ratio test. Tree output visual formatting was performed using FigTree v1.4.3. **b** Predicted MFS transporters topology for each family, adapted from Sá-Correia et al. (2009)

Table 1.1 MFS-MDR/MXR transporters from the DHA1 family and the corresponding subcellular localization and described roles in MDR/MXR and yeast cell physiology. MFS-DHA1 transporters for which a physiological role in the cell was already proposed are highlighted in green. Information concerning subcellular localization is based on experimental evidence

Systematic name	Standard name	Subcellular localization	MDR/MXR phenotype (classes of compounds)	Physiological role	References
YNL065W	*AQR1*	Plasma membrane; internal membranes	Anti-arrhythmic and antimalarial drugs; azole fungicides; cationic dyes; weak acid food preservatives	Excretion of excess amino acids; role in DNA replication during stress response	Tenreiro et al. (2002), Velasco et al. (2004), Tkach et al. (2012)
YBR180W	*DTR1*	Plasma membrane	Anti-arrhythmic and antimalarial drugs; weak acid food preservatives	Translocation of bisformyl dityrosine during spore wall maturation	Felder et al. (2002)
YBR008C	*FLR1*	Plasma membrane	Agriculture fungicides; aniline analgesics; immunosuppressants; mutagens; oxidizing and alkylating agents; antibiotics	Unknown	Alarco et al. (1997), Brôco et al. (1999), Oskouian and Saba (1999), Jungwirth et al. (2000), Nguyên et al. (2001), Srikanth et al. (2005), Teixeira et al. (2008)
YIL120W	*QDR1*	Plasma membrane	Anti-arrhythmic drugs; azole fungicides; herbicides	Unknown	Nunes et al. (2001), Vargas et al. (2004), Tenreiro et al. (2005)
YIL121W	*QDR2*	Plasma membrane	Anti-arrhythmic drugs; azole fungicides; chemotherapy agents; herbicides	Potassium and copper homeostasis	Vargas et al. (2004, 2007), Tenreiro et al. (2005), Ríos et al. (2013)
YBR043C	*QDR3*	Plasma membrane	Anti-arrhythmic drugs; herbicides and chemotherapy agents; cations	Involved in spore wall assembly; Polyamine homeostasis	Tenreiro et al. (2005), Teixeira et al. (2011), Lin et al. (2013)

(continued)

Table 1.1 (continued)

Systematic name	Standard name	Subcellular localization	MDR/MXR phenotype (classes of compounds)	Physiological role	References
YLL028W	*TPO1*	Plasma membrane	Metal ions; antimalarial drugs; immunosuppressants; herbicides; azole and agricultural fungicides; weak acids; chemotherapy agents; nonsteroidal anti-inflammatory drugs	Hypothesized role in lipid homeostasis; Polyamine homeostasis	Cabrito et al. (2009), Dias et al. (2010), Mira et al. (2009), Uemura et al. (2005), Kennedy and Bard (2001), Desmoucelles et al. (2002), Mima et al. (2007), Borrull et al. (2015), Legras et al. (2010), Tomitori et al. (1999, 2001), Albertsen et al. (2003), Alenquer et al. (2006), Do Valle Matta et al. (2001), Teixeira and Sá-Correia (2002), Berra et al. (2014), Hillenmeyer et al. (2008)
YGR138C	*TPO2*	Plasma membrane	Weak acid food preservatives	Polyamine homeostasis	Tomitori et al. (2001), Albertsen et al. (2003), Fernandes et al. (2005)
YPR156C	*TPO3*	Plasma membrane	Weak acid food preservatives	Polyamine homeostasis	Tomitori et al. (2001), Albertsen et al. (2003), Fernandes et al. (2005)
YOR273C	*TPO4*	Plasma membrane; bud	Anti-arrhythmic drugs; antibiotics	Polyamine homeostasis	Tomitori et al. (2001), Albertsen et al. (2003), Do Valle Matta et al. (2001), Delling et al. (1998), Huh et al. (2003)
YHR048W	*YHK8*	Unknown	Unknown	Unknown	Barker et al. (2003)
YNR055C	*HOL1*	Plasma membrane	Unknown	Uptake of histidinol and cations	Huh et al. (2003), Wright et al. (1996)

Table 1.2 MFS-MDR/MXR transporters from the DAG family and the corresponding subcellular localization and described roles in MDR/MXR and yeast cell physiology. MFS-DAG transporters for which a physiological role in the cell was already proposed are highlighted in green. Information concerning subcellular localization is based on experimental evidence

Systematic name	Standard name	Subcellular localization	MDR/MXR phenotype (classes of compounds)	Physiological role	References
YML116W	*ATR1*	Plasma membrane; vacuole	Cations; herbicides; mutagens; precursors in chemical industry; purine and pyrimidine analogues; weak acids	Unknown	Gömpel-Klein and Brendel (1990), Kanazawa et al. (1988), Kaya et al. (2009), Mack et al. (1988)
YGR224W	*AZR1*	Plasma membrane	Azole drugs; cationic dyes; fungicides; weak acid food preservatives	Unknown	Tenreiro et al. (2000), Venturi et al. (2012)
YPR198W	*SGE1*	Plasma membrane	Cationic dyes; guanidinium compounds; imidazolium ionic liquids; mutagens	Unknown	Ehrenhofer-Murray et al. (1994, 1998), Jacquot et al. (1997), Bowie et al. (2013), Higgins et al. (2018)
YMR088C	*VBA1*	Vacuolar membrane	Anti-arrhythmic drugs; azole fungicides	Vacuolar transporter for basic amino acids	Kawano-Kawada et al. (2016), Shimazu et al. (2012)
YBR293W	*VBA2*	Vacuolar membrane	Anti-arrhythmic drugs; azole fungicides	Vacuolar transporter for basic amino acids	Kawano-Kawada et al. (2016), Shimazu et al. (2012)
YCL069W	*VBA3*	Vacuolar membrane	Unknown	Vacuolar transporter for basic amino acids	Kawano-Kawada et al. (2016), Shimazu et al. (2012)
YDR119W	*VBA4*	Vacuolar membrane	Anti-arrhythmic drugs; azole fungicides	Vacuolar transporter for basic amino acids; vacuolar morphology	Kawano-Kawada et al. (2016), Shimazu et al. (2012)
YKR105C	*VBA5*	Plasma membrane	Unknown	Unknown	Shimazu et al. (2012)
YMR279C	*ATR2*	Unknown	Weak acids	Unknown	Kaya et al. (2009), Bozdag et al. (2011)
YOR378W	*AMF1*	Plasma membrane	Unknown	Ammonium transport to the intracellular medium	Chiasson et al. (2014)

(continued)

Table 1.2 (continued)

Systematic name	Standard name	Subcellular localization	MDR/MXR phenotype (classes of compounds)	Physiological role	References
YHL040C	*ARN1*	Endosome; plasma membrane (dependent on ferrichrome concentrations)	Unknown	Siderophore-iron chelates transport to the intracellular medium; possible role in DNA damage response	Tkach et al. (2012), Heymann et al. (2000)
YHL047C	*ARN2/TAF1*	Unknown	Unknown	Siderophore-iron chelates transport to the intracellular medium	Heymann et al. (1999)
YEL065W	*ARN3/SIT1*	Vacuolar membrane	Unknown	Siderophore-iron chelates transport to the intracellular medium	Huh et al. (2003), Lesuisse et al. (1998)
YOL158C	*ARN4/ENB1*	Unknown	Unknown	Siderophore-iron chelates transport to the intracellular medium	Heymann et al. (2000)
YCL073C	*GEX1*	Vacuolar membrane; plasma membrane	Metal cations; oxidative stress agents	Excretion of glutathione to the vacuole lumen and extracellular media	Dhaoui et al. (2011), Thorsen et al. (2012)
YKR106W	*GEX2*	Vacuolar membrane; plasma membrane	Metal cations	Excretion of glutathione to the vacuole lumen and extracellular media	Dhaoui et al. (2011), Thorsen et al. (2012)

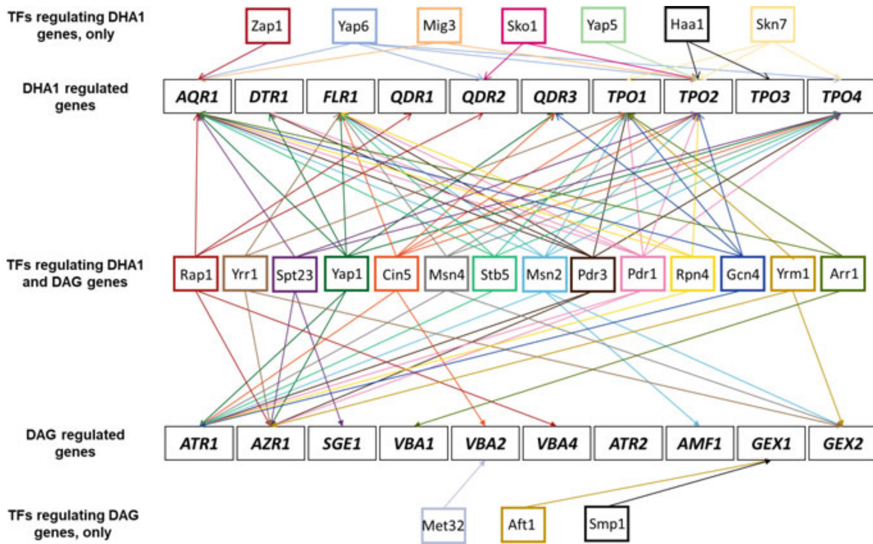

Fig. 1.2 Transcriptional regulatory networks controlling the expression under stress of *S. cerevisiae* MFS-MDR/MXR transporter genes from the DHA1 and DAG families, currently documented as MDR/MXR. The regulatory network was built based on information gathered in the YEASTRACT database (August 2018). Only documented regulations (DNA binding or expression evidence) were considered, and the environmental condition 'stress' was selected. TF—transcription factor

The transcription factors Pdr1 and Pdr3, considered the main regulators of the MDR/MXR phenomenon in yeast, were found to activate the transcription of eight or seven genes, respectively, out of the 20 MFS genes under study. These TFs form homo- or heterodimers (Mamnun et al. 2004) and recognize the same nucleotide sequence in the promoter region of target genes, the so-called Pdr1/Pdr3 pleiotropic drug responsive element (PDRE) (Mahé et al. 1996; Katzmann et al. 1996; Wolfger et al. 1997). The transcription factors Msn2 and Msn4, considered the key regulators of the general stress response, were found to activate the transcription of eight or five genes, respectively, encoding MFS-MDR/MXR transporters. Yap1, a zinc-finger transcription factor that is considered the master regulator of the oxidative stress response in *S. cerevisiae* (Rodrigues-Pousada et al. 2010), was found to regulate seven genes of the dataset, as well as another Yap family member, Cin5/Yap4, active upon osmotic stress.

The role played by Gcn4, a transcription factor involved in yeast response to amino acid and nitrogen limitation, in the transcriptional regulation of genes encoding MDR/MXR transporters from the MFS was also described in several studies. Gcn4 was found to activate the transcription of the DHA1 transporter-encoding gene *QDR3*, when yeast cells are challenged by toxic concentrations of spermine or spermidine, although the expression of *GCN4* itself seems not to be required for increased tolerance towards polyamine-induced stress (Teixeira et al. 2010). Given

the involvement of Gcn4 in yeast response to amino acid and nitrogen limitation, the transcript levels of *QDR3* in these conditions was investigated and found to increase, in the dependence of Gcn4 (Teixeira et al. 2010), similarly to what was observed for the DHA1 gene *QDR2* (Vargas et al. 2007). The activation of Gcn4 and the Gcn4-dependent activation of *TPO1* was described under stress induced by the food preservative benzoic acid that leads to the reduction of the intracellular pools of amino acids and polyamines (Godinho et al. 2017). This activation was related with a more rapid adaptation to the food preservative benzoic acid-induced stress (Godinho et al. 2017)

Although no demonstrated role for MFS-MDR/MXR transporters in lipid transport has been reported so far, contrary to what has been demonstrated for a number of ABC transporters (Jungwirth and Kuchler 2006; Prasad et al. 2016), there are transcription factors involved in the regulation of lipid metabolism that also regulate the expression of MFS transporters. This is the case of the transcription factor Spt23, involved in the regulation of the Δ9 desaturase *OLE1* gene (Zhang et al. 1999), and reported to regulate 6 members of the MFS-MDR/MXR dataset (Auld et al. 2006). It is likely that this fact may have a meaning.

The *TPO1* gene was chosen as an example of the complexity of the regulatory networks acting on the transcriptional activation of MFS-MDR/MXR genes upon different stress conditions (Fig. 1.3). *TPO1* gene transcription was found to be activated by the pleiotropic drug resistance (PDR) network regulators Pdr1 and Pdr3 in stress imposed by a wide range of drugs/xenobiotics such as artesunate (Alenquer et al. 2006), fluphenazine (Fardeau et al. 2007), benomyl (Lucau-Danila et al. 2005), and the chlorinated phenoxyacetic acid herbicides MCPA and 2,4-D (Teixeira and Sá-Correia 2002). The transcriptional activation of *TPO1* was also found to be mediated by the oxidative stress response transcription factor Yap1 in the presence of the anticancer drug bleomycin (Berra et al. 2014). Besides Gcn4-dependent activation of *TPO1*, *TPO1* expression was found to be regulated by the transcription factor paralogues Yrr1 and Yrm1 in the presence of hop iso-α-acids, which are secondary metabolites of the hop plant *Humulus lupulus* L., with antiseptic properties and responsible for the typical bitter flavour of beer (Hazelwood et al. 2010). Also, Arr1 was found to regulate *TPO1* transcriptional activation in the presence of arsenic compounds, although the mechanisms and triggers were not reported so far (Haugen et al. 2004).

1.4 The *S. cerevisiae* ATP-Binding Cassette (ABC) Superfamily of Transporters

ABC transporters constitute one of the largest classes of transporters and are widespread among prokaryotes and eukaryotes (Higgins 2001; Holland and Blight 1999). These transporters are responsible for catalysing the transport of solutes across cell membranes against a concentration gradient, directly depending on ATP

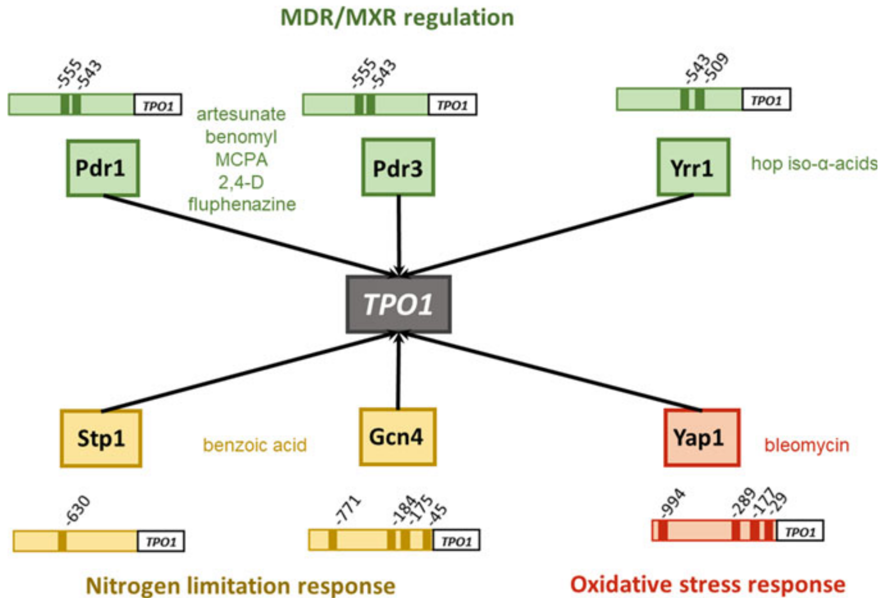

Fig. 1.3 Regulatory network behind the activation of *TPO1* transcription under stress. The representation of the regulatory network and TFs binding sites on *TPO1* promoter were built based on information gathered in the YEASTRACT database (August 2018), and manually curated to include more recent research (Godinho et al. 2017). Only documented regulations (DNA binding or expression evidence) and TFs with a documented binding site in *TPO1* promoter were considered, and the environmental condition 'stress' of YEASTRACT tools was selected

hydrolysis. ABC transporters share a common architecture of two hydrophobic regions each comprising six transmembrane domains (TMD), and two hydrophilic domains including a conserved cassette with ATP-binding motifs (nucleotide binding domain—NBD) (Linton 2007; Locher 2009; Paumi et al. 2009), although some variations can occur (Fig. 1.4). For example, some yeast, mammalian and plant ABC transporters that confer MDR/MXR are known to possess the reverse topology $[NBD\text{-}TMD]^2$ (Lamping et al. 2010; Van Den Brûle and Smart 2002; Kerr et al. 2011). The functioning of ABC transporters is considered to obey the ATP switch model that proposes that the binding of a given substrate to the TMD open to the cytosol triggers a conformational change in the NBDs that lead to the opening of the substrate binding pocket to release the substrate to the extracellular media (Higgins and Linton 2004). According to this model, the conformation of the ABC transporter is recovered in an energy-dependent manner, provided by the hydrolysis of the ATP molecule (Higgins and Linton 2004).

Complete inventories of the ABC transporters in *S. cerevisiae* identified 30 members of this superfamily (Fig. 1.4), of which only 22 have predicted transmembrane spans and are therefore considered true ABC transporters (Paumi et al. 2009). The true ABC transporters were found to cluster into 6 phylogenetic subfamilies (Paumi et al.

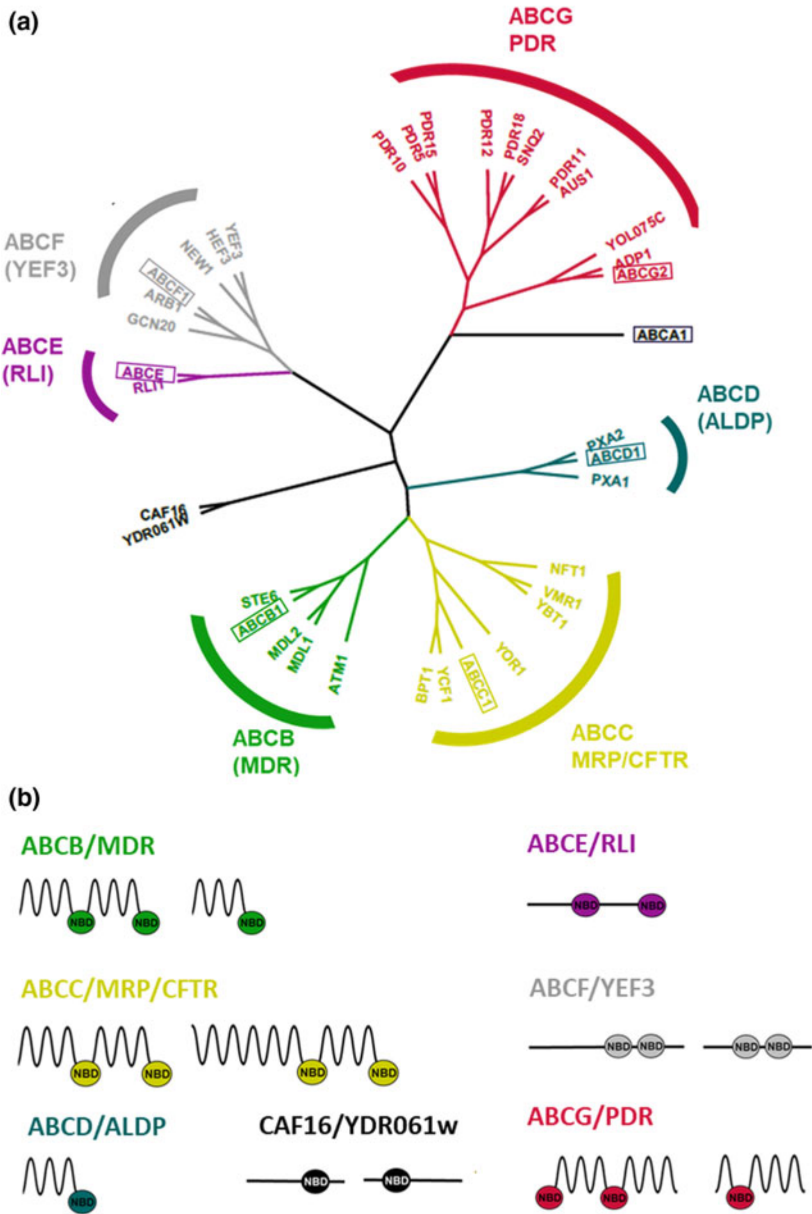

Fig. 1.4 Phylogenetic tree and predicted topology of yeast ABC transporters. **a** Maximum likelihood phylogenetic tree constructed using the protein sequences retrieved from SGD of the complete set of 30 ABC transporters identified in *S. cerevisiae*. An element of each family of ABC transporters from *Homo sapiens* was retrieved from UniProt and included in the phylogenetic analysis (marked in the tree with a square). Alignment and phylogenetic analysis were performed as described for Fig. 1.2. Each cluster is identified by the name of the corresponding family used in the two nomenclatures currently in use. **b** Predicted ABC transporters topology for each family, adapted from Paumi et al. (2009). Abbreviations: NBD nucleotide binding domain

2009; Decottignies and Goffeau 1997). For these subfamilies, it has been recently adopted the nomenclature proposed by HUman Genome Organization (HUGO) classification, from ABCB to ABCG. However, two transporters (*CAF16* and *YDR061w*) cannot be classified into any of the HUGO subfamilies (Paumi et al. 2009). Transporters from the ABCG subfamily are one of the most thoroughly studied in yeast. They exhibit a characteristic reverse topology structure $[NBD\text{-}TMD]^2$ and are commonly associated with Pleiotropic Drug Resistance, therefore known as PDR transporters (Jungwirth and Kuchler 2006; Balzi and Goffeau 1995).

The inventory of all PDR transporters in *S. cerevisiae* considers six PDR sensu stricto transporters (Pdr5, Snq2, Pdr12, Pdr15, Pdr10 and Pdr18) based on the fact that these transporters possess the $[NBF\text{-}TMD]^2$ topology, the presence of a cysteine residue instead of the lysine residue in N-terminal Walker A motifs, a specific NVEQ motif in the C-terminal ABC signature and a documented phenotype of multidrug resistance (Seret et al. 2009). The Pdr11, Aus1 and YOL075c are considered members of the PDR family as they share the characteristic $[NBF\text{-}TMD]^2$ topology, but since no role in MDR/MXR was reported until now and they do not present the K/C substitution in the Walker A motif, they were considered as Pdrp sensu lato (Seret et al. 2009). The subcellular localization, role in MDR/MXR and physiological role in *S. cerevisiae* for the PDR transporters are summarized in Table 1.3.

Several members of the PDR family of transporters have been found to play physiological roles in yeast cells related to plasma membrane lipid homeostasis (Prasad et al. 2016). For example, Pdr5 is implicated in the translocation of phospholipids between the two plasma membrane monolayers, thus contributing to plasma membrane asymmetry (Decottignies et al. 1998; Kaur and Bachhawat 1999; Ruetz et al. 1997). Although no clear physiological role was described for Snq2, it was shown that Snq2 is involved in alleviation of estradiol toxicity in *S. cerevisiae* (Mahé et al. 1996), which led to some speculation on a possible role in lipid translocation (Mahé et al. 1996; Kuchler et al. 1997). Pdr10 contributes to the normal expression, and/or sorting and trafficking, and/or catalytic capacity of Chs3 and Pdr12 (Rockwell et al. 2009). Although the mechanism by which Pdr10 controls the microdomain localization of Pdr12 is not completely understood. By analogy to its close homologue Pdr5, one possibility is that Pdr10 catalyses the outward translocation of a yet unidentified lipid substrate (Rockwell et al. 2009). Pdr18 is a paralog of Snq2, only encoded in the genome of *Saccharomyces* genus species, consistent with the hypothesis that it arose from a recent duplication event in the common ancestor and *PDR18* translocation to the subtelomeric region of chromosome XIV (Seret et al. 2009; Akache and Turcotte 2002). Although the evolutionary processes acting on the duplicated copies need further investigation, Snq2 and Pdr18 show little functional overlap regarding their role in MDR/MXR (Godinho et al. 2018). While no biological role in the cell was attributed to Snq2 so far, its role in the alleviation of estradiol toxicity in *S. cerevisiae* led to the hypothesis of an affinity for lipid transport, especially for the estradiol structurally related molecule ergosterol (Mahé et al. 1996; Kuchler et al. 1997). On the other hand, Pdr18 was proposed to be responsible for the active transport of ergosterol at the plasma membrane level, allowing the high physiological ergosterol concentration be present in this membrane, especially under acetic acid

Table 1.3 ABC transporters from the PDR family and the corresponding subcellular localization and described roles in MDR/MXR and yeast cell physiology. ABC-PDR transporters for which a physiological role in the cell was already described are highlighted in green. Information concerning subcellular localization is based on experimental evidence

Systematic name	Standard name	Subcellular localization	MDR/MXR phenotype (classes of compounds)	Physiological role	References
YOR153W	*PDR5*	Plasma membrane	Agricultural and clinical fungicides; antibiotics; cations; chemotherapy drugs; herbicides; human steroid hormones	Phospholipid translocation; quorum sensing	Mamnun et al. (2004), Balzi and Goffeau (1994), Bissinger and Kuchler (1994), Emter et al. (2002), Kolaczkowski et al. (1998), Mahé et al. (1996), Rogers et al. (2001), Miyahara et al. (1996), De Thozée et al. (2007), Decottignies et al. (1998)
YDR011W	*SNQ2*	Plasma membrane	Antibiotics; cations; chemotherapy agents; detergents; ionophores; mutagens; steroid hormones	Possible role in lipid translocation	Huh et al. (2003), Mahé et al. (1996a, b), Kolaczkowski et al. (1998), Wehrschütz-Sigl et al. (2004), Servos et al. (1993), Ling et al. (2013), Miyahara et al. (1996), Nishida et al. (2013), Tsujimoto et al. (2015), Ververidis et al. (2001)
YOR328W	*PDR10*	Plasma membrane	Anionic dyes; organic solvents	Plasma membrane microdomain formation	Nishida et al. (2013), Rockwell et al. (2009)
YDR406W	*PDR15*	Plasma membrane	Antibiotics; detergents	Unknown	Wolfgert et al. (2004), Snider et al. (2013)
YPL058C	*PDR12*	Plasma membrane	Moderately lipophilic weak acids (benzoic, propionic and sorbic acids); short-chain alkanols (n-butanol, n-propanol and n-pentanol)	Unknown	Piper et al. (1998, 2001), Hatzixanthis et al. (2003)
YNR070C	*PDR18*	Plasma membrane	Alcohols; Anti-arrhythmic and antimalarial drugs; cations; clinical and agricultural fungicides; herbicides; polyamines; weak acids	Ergosterol transport at the plasma membrane	Cabrito et al. (2009), Godinho et al. (2018a, b), Snider et al. (2013), Teixeira et al. (2012)
YIL013C	*PDR11*	Plasma membrane	Unknown	Sterol uptake	Gulati et al. (2015), Li and Prinz (2004), Wilcox et al. (2002)
YOR011W	*AUS1*	Plasma membrane	Unknown	Sterol uptake	Gulati et al. (2015), Li and Prinz (2004), Wilcox et al. (2002)
YOL075C	–	Plasma membrane	Unknown	Unknown	Snider et al. (2013)
YCR011C	*ADP1*	Endoplasmic reticulum	Unknown	Unknown	Huh et al. (2003)

stress (Godinho et al. 2018; Cabrito et al. 2011). This role correlates with the maintenance of adequate plasma membrane physical properties, essential for its function as a selective barrier and matrix for embedded proteins involved in the import of nutrients and excretion of toxic metabolites (Godinho et al. 2018). Consistently, Pdr18 has a documented role in yeast tolerance to a very wide range of chemical compounds (Tarling et al. 2013; Wolfgert et al. 2004; Li and Prinz 2004; Akache and Turcotte 2002). Pdr11 and Aus1 are responsible for the uptake of exogenously supplied ergosterol in anaerobic conditions when ergosterol biosynthesis is impaired, and are considered to play a role in the transport of exogenously supplied ergosterol to the Golgi apparatus, where esterification occurs (Gulati et al. 2015; Li and Prinz 2004; Wilcox et al. 2002). However, neither Pdr11 nor Aus1 was found to confer yeast with improved tolerance to any chemical compound.

1.5 Transcriptional Regulation of PDR Transporters Encoding Genes in Response to Stress

Regulation of PDR transporters entails a regulatory network of transcription factors, centred in the homologous $Zn(II)_2Cys_6$ zinc cluster paralogue transcription factors Pdr1 and Pdr3 (Akache and Turcotte 2002; Balzi et al. 1987; Delaveau et al. 1994; Kolaczkowska and Goffeau 1999), which were found to activate the expression of 4 and 6, respectively, out of the 6 PDR transporters involved in MDR/MXR in our dataset (Fig. 1.5). There are additional transcription regulators that modulate Pdr1/Pdr3 activity, such as Stb5 that forms heterodimers with Pdr1 or Pdr3 competing with the Pdr1/Pdr3 heterodimers for the binding in PDRE locations (Akache and Turcotte 2002). Stb5 was found to regulate the transcription of two genes of our PDR transporters dataset (Fig. 1.5). Other transcription factors are involved in the activation of PDR genes' expression: Yap1 was found to activate three genes of our dataset (Fig. 1.5). The general stress response transcription activators Msn2 and Msn4 also play a role in the regulation of the PDR network, as they were found to be behind the activation of 4 and 2 PDR genes, respectively. MDR/MXR transcription factor paralogues Yrr1/Yrm1 were also found to play a role in the activation of the genes under study, 3 or 2 out of the dataset of the six genes, respectively. The role of Pdr12 in yeast tolerance to moderately lipophilic weak acid stress was found to depend on the transcription factor War1, that do not apparently play a role in the activation of any of the remaining PDR transporter-encoding genes (Piper et al. 1998, 2001; Hatzixanthis et al. 2003).

Another relevant fact that raises the importance of the PDR network in the lipid composition of cell membranes is that some enzymes involved in sphingolipid synthesis such as Ipt1, Lcb2, Sur2 and Lac1 contain a pleiotropic drug responsive element (PDRE) within the promoter regions of the encoding genes, having their expression induced by Pdr1 and Pdr3 upon environmental stimuli (Han et al. 2010). These facts

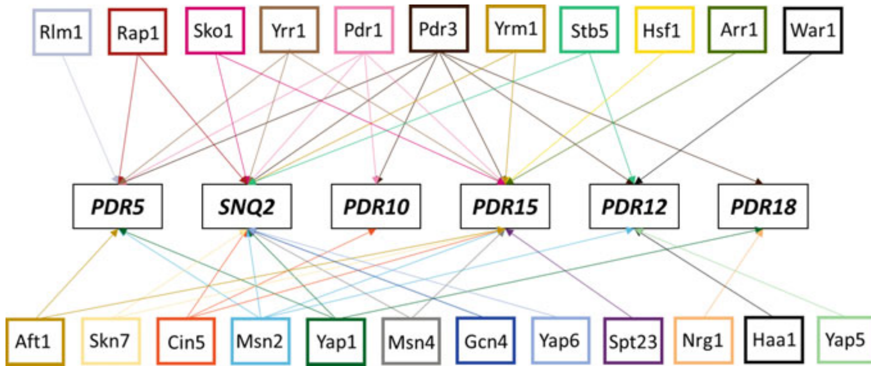

Fig. 1.5 Transcriptional regulatory networks controlling the expression under stress of six *S. cerevisiae* PDR transporters encoding genes currently documented as MDR/MXR. The regulatory network was built based on information gathered in the YEASTRACT database (August 2018). Only documented regulations (either DNA binding or expression evidence) are considered, and the environmental condition 'stress' of YEASTRACT tools was selected

are further indications of the relevance of the PDR network in the regulation of plasma membrane composition and asymmetry (Gulshan and Moye-Rowley 2007).

As an example of the complexity of the network behind the activation of expression of PDR transporters in response to a given stress is the activation of *PDR18* transcription in response to 2,4-D-imposed stress which was found to be dependent on Nrg1, Yap1, and Pdr3 (Cabrito et al. 2011). Although the deletion of *PDR3* or *YAP1* genes that are key players in broad stress defence responses lead to a reduction of Pdr18 activation under 2,4-D stress, *NRG1* deletion abolished completely the transcriptional activation registered for *PDR18*, a role that was proven to be indirect (Cabrito et al. 2011). Also, the up-regulation of *PDR18* transcription in the presence of acetic acid was found to be coordinated with an up-regulation of genes encoding enzymes of the ergosterol biosynthetic pathway (Godinho et al. 2018). Remarkably, other transcriptional co-regulations of genes involved in yeast metabolism and in membrane transport have been described (Prasad et al. 2016; Coste et al. 2004; DeRisi et al. 2000; Khakhina et al. 2015; Kihara and Igarashi 2004).

1.6 Interplay Between MDR/MXR Transporters Activity and Plasma Membrane Composition and Organization in the Response and Resistance to Multiple Stresses

Yeast plasma membrane plays crucial biological roles in the yeast cell such as physical protection and control of osmotic stability, control of the entrance and exit of soluble compounds, the anchoring of the cytoskeleton, the functioning of endocytosis and exocytosis processes, signal transduction, cell-to-cell recognition, and adhesion

(van der Rest et al. 1995). Membrane-embedded proteins confer a patchwork organization to the plasma membrane (Spira et al. 2012), and the lipid environment greatly influences the trafficking, localization and activity of plasma membrane transporters, as it is the case of the raft-associated H^+-ATPase Pma1 and the non-raft-associated Pdr12 (Kodedová and Sychrová 2015; Rockwell et al. 2009; Aguilera et al. 2006; Bagnat et al. 2001). On the other hand, the maintenance of plasma membrane lipid homeostasis and asymmetries are influenced by the activity of plasma membrane transporters. This is the case of the ABC transporters Pdr5 and Yor1 that play a role in maintaining the asymmetry between the two plasma membrane leaflets, by performing the outward movement of phosphatidylethanolamine (Decottignies et al. 1998). Pdr10 was proposed to act as a detergent, thus inhibiting the formation of microdomains in the plasma membrane, by a mechanism still unclear (Rockwell et al. 2009). Pdr11 and Aus1 are responsible for the uptake of exogenously supplied ergosterol when ergosterol biosynthesis is impaired, and are considered to play a role in the transport of exogenously supplied ergosterol to the Golgi apparatus, where esterification occurs (Gulati et al. 2015; Li and Prinz 2004; Wilcox et al. 2002). Pdr18 mediates ergosterol transport at the plasma membrane and is found to be essential to reduce toxicity induced by the herbicide 2,4-D and the food preservative and toxicants of biotechnological relevance such as acetic acid and ethanol, which negatively impacts ergosterol plasma membrane levels and are membrane-permeabilizing agents (Godinho et al. 2018; Cabrito et al. 2011; Teixeira et al. 2012). Pdr18 biological role was found to deeply affect plasma membrane composition and organization, especially under membrane-disturbing stresses, as cells not expressing Pdr18 exhibit a more disorganized plasma membrane (Godinho et al. 2018). Another fact that demonstrates the interplay between plasma membrane transporters activity and plasma membrane lipid homeostasis is the coordinate control of expression of lipid metabolism genes and MDR/MXR transporters (Gulshan and Moye-Rowley 2007; Godinho et al. 2018; Shahi and Moye-Rowley 2009; Hallstrom et al. 2001; Kolaczkowski et al. 2004). The interplay described above is essential for yeast tolerance to industrially associated stresses of major relevance in the cell factory *S. cerevisiae*, which include the accumulation of fermentation products such as ethanol and weak acids, and the operation temperatures that, even when suboptimal, together with the cytotoxic metabolites accumulated in the fermentation medium may become supra-optimal for the fermentation process (Cheng and Kao 2014; van Uden and da Cruz 1981).

High ethanol concentrations and supra-optimal temperatures both impact yeast cells' plasma membrane and have synergistic effects on growth and death kinetics, depending on the stress level. Exposure of yeast cells to these stress factors leads to increased plasma membrane fluidity, decreased plasma membrane thickness and the consequent decrease of membrane integrity (Adachi et al. 1995; Barry and Gawrisch 1994; Kranenburg and Smit 2004; Feller et al. 2002; Verghese et al. 2012; Piper 1995). These deleterious effects at the plasma membrane structure and lipid organization levels are responsible for the increase of plasma membrane permeability and loss of function as a selective barrier to the influx of solutes by passive diffusion, as well as the dissipation of the proton motive force that drives the secondary transport of solutes

(Aguilera et al. 2006; Alexandre et al. 1994). Changes in membrane thickness can also result in the exposure of hydrophobic amino acid residues of integral membrane proteins, leading to membrane protein aggregation that minimizes the exposure of hydrophobic portions (Lee 2004; Killian 1998). Growth in the presence of increasing concentrations of ethanol correlates with the increase in ergosterol levels in yeast cells and ergosterol incorporation was proposed to maintain yeast plasma membrane fluidity (del Castillo 1992). Further studies confirmed that increased sterol incorporation, alone or combined with increased content of unsaturated lipids, counteracts ethanol-induced formation of the interdigitation of plasma membrane (Vanegas et al. 2010, 2012; You et al. 2003). Consistently, based on a toxicogenomics study, it was suggested that yeast plasma membrane lipid composition suffers remodelling in response to ethanol stress, since several genes involved in ergosterol (*ERG2* and *ERG24*) and glycerophospholipid (*KCS1*, *LIP5*, *OPI3*, *PDX3*, and *IDI1*) biosynthesis were found to be determinants of yeast tolerance to high ethanol concentrations (Teixeira et al. 2009). A study based on adaptive laboratory evolution selecting for thermotolerant mutants led to the conclusion that all isolates exhibiting improved tolerance contained nonsense mutations in the *ERG3* gene, accumulating the intermediate bended sterol fecosterol (Caspeta et al. 2014). Previous reports already have related the incorporation of branched sterols, such as sitosterol and bended sterol-like lipids such as bacteriohopanetetrol, in protection of *Archaea* and plant cells' membranes from high temperatures (Dufourc 2008). This observation calls attention to the fact that both improved sterol content and sterol structure can impact plasma membrane organization, providing improved tolerance to membrane-disturbing agents. The activity of the plasma membrane H^+-ATPase that contributes to counteract the dissipation of plasma membrane electrochemical potential was found to be strongly dependent on plasma membrane lipid environment (Aguilera et al. 2006; Serrano 1988; Wach et al. 1990). The ABC transporter Pdr18, a determinant of yeast tolerance to ethanol, was found to contribute to decreased intracellular accumulation of ethanol (Teixeira et al. 2012). Further analysis revealed that the involvement of Pdr18 in ethanol tolerance is related to its physiological role in ergosterol transport contributing to higher ergosterol content in the plasma membrane and, consequently, lower ethanol-induced permeabilization (Godinho et al. 2018; Cabrito et al. 2011; Teixeira et al. 2012). The overexpression of *PDR18* gene was found to enable a better performance of yeast cells in high gravity fermentation, by greatly restricting non-specific permeabilization induced by the accumulation of ethanol in the fermentation medium, thus allowing higher ethanol production (Teixeira et al. 2012).

Yeast ability to overcome stress induced by weak acids, and acetic acid in particular, is crucial since acetic acid is an important by-product of alcoholic fermentation that together with the increasing concentration of ethanol can lead to stuck or sluggish fermentations. Also, bioethanol production from lignocellulosic materials requires substrate pretreatment for the release of fermentable sugars. This pretreatment leads to the release of many inhibitory compounds, a mixture in which acetic acid prevails (Palma et al. 2017). On the other hand, the preservation of several acidic foods and beverages relies on the use of weak acid food preservatives such as sorbic, benzoic, propionic and acetic acids. Therefore, the understanding of the mechanisms behind

S. cerevisiae tolerance to weak acid stress, in particular those involved in the intracellular reduction of the weak acid counter-ion, presumably dependent on the action of specific efflux pumps, as well as in the remodelling of the membrane lipid composition, may provide clues to guide new food preservation techniques to control spoilage yeast or to improve the robustness of industrial strains (Palma et al. 2017).

A complete lipidomic profiling of acetic acid adapted and unadapted cells of *S. cerevisiae* has implicated the incorporation of complex sphingolipid in yeast membranes, in response to acetic acid stress (Lindberg et al. 2013). Also, the higher fraction of sphingolipids incorporated in the highly acetic acid resistant yeast *Zygosaccharomyces bailii,* compared to *S. cerevisiae,* reinforce the correlation between sphingolipid content and acetic acid tolerance (Lindberg et al. 2013). Studies on model membranes confirmed that the higher incorporation of sphingolipids results in thicker and more dense membranes, with lower permeability to acetic acid passive diffusion (Lindahl et al. 2016). The remodelling of plasma membrane lipid composition in response to weak acid stress was very recently reported for other weak acids, such as the less lipophilic formic and levulinic acids and the more lipophilic cinnamic acid (Guo et al. 2018). The changes observed in glycerophospholipid classes and fatty acyl chain length between control and acid stress conditions were found to depend greatly on the lipophilicity of the acid and higher ergosterol levels were confirmed to improve yeast viability under weak acid-induced stress (Guo et al. 2018). Several genes encoding ergosterol biosynthetic enzymes are determinants of acetic acid resistance (Mira et al. 2010) and the coordinated transcriptional activation of these genes and of the ergosterol transporter Pdr18 encoding gene in yeast adaptation to acetic acid stress demonstrated and related with reduced plasma membrane permeabilization and transmembrane electrochemical potential dissipation (Godinho et al. 2018). Noticeably, Pdr18 expression was recently reported to improve yeast tolerance to several other weak acids (Godinho et al. 2018).

1.7 Response and Resistance to Agriculturally Relevant Stresses: From the Yeast Model and Expression Host to the Plant Model

The plant model *Arabidopsis thaliana* has important advantages for laboratory cultivation and manipulation, justifying its extensive use in genetic, biochemical and physiological studies (Provart et al. 2016). This small flowering plant of no agronomic relevance, with a rapid life cycle, self-pollination and prolific seed production (Provart et al. 2016; Sivasubramanian et al. 2015) has the relatively small genome sequence of around 135 Mb, available since 2000 (The Arabidopsis Genome Initiative 2000). Moreover, transformation protocols and large collections of mutant lines are currently available, and genetic tools such as RNAi and CRISPR/Cas9 have been improved in the plant model (Provart et al. 2016). However, in spite of the increased ability to conduct research in this plant model and other more complex

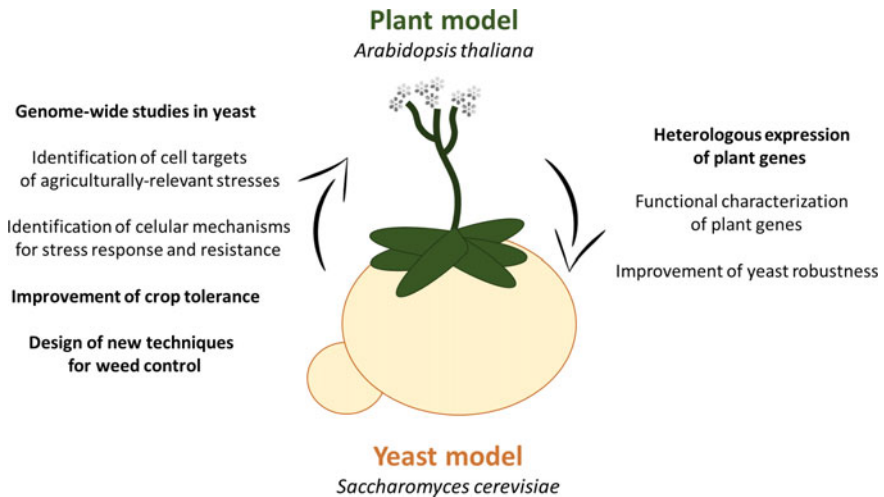

Fig. 1.6 The yeast model *S. cerevisiae* is used to unveil cellular mechanisms in the plant model *A. thaliana*. *S. cerevisiae* is a pivotal tool both as expression host and experimental model for cell biology studies in *A. thaliana*. This knowledge may ultimately lead to crop improvement in the presence of stresses of agricultural interest or to the endurance of yeast cells for the biotechnological industry

eukaryotes in the recent years, the budding yeast is still a powerful model eukaryote. It is essential both as a single cell experimental model organism and as a host for the expression and functional analysis of heterologous proteins from more complex and, experimentally, less accessible eukaryotes. In particular, the exploitation of yeast to uncover the mechanisms of resistance to stresses relevant in Plant Biology and Biotechnology and to characterize the role of plant membrane transporters involved in MDR/MXR is considered in this chapter section, as a proof of concept (Fig. 1.6). Moreover, combined with the plant model, the simpler model yeast *S. cerevisiae* can also be explored to unveil cellular mechanisms that can be extrapolated to plants of economic interest (Fig. 1.6).

Herbicides are extensively used agrochemicals to improve crop productivity by controlling the growth of undesired weeds. However, weeds can acquire resistance and tolerate the presence of increasing concentrations of the herbicide. This phenomenon can jeopardize crop productivity and prompt to the use of higher herbicide doses at higher rates, which ultimately will impact environmental and public health. MDR/MXR in plants can, however, be beneficial when the mechanisms behind this phenomenon are explored in agriculturally relevant crops to improve biotic and abiotic stress resistance and consequently crop productivity. Among the herbicide families more prone to induce herbicide resistance in plants are synthetic auxins, the auxin-like herbicide 2,4-dichlorophenoxyacetic acid 2,4-D among the most used members of the family. This lipophilic weak acid herbicide enters the cell by passive diffusion of its undissociated form, present in acidic soils. Mechanistic insights into

2,4-D toxicity and the corresponding adaptive responses based on studies carried out using *S. cerevisiae* and *A. thaliana* as model organisms, at a genome-wide scale, were reviewed (dos Santos et al. 2012). This is a paradigmatic example that showed that, in general, mechanisms of toxicity, response and adaptation to 2,4-D, are highly conserved between yeast and plants (Cabrito et al. 2011; Teixeira et al. 2005, 2006, 2007). Among the expected advantages of using the yeast model is the capacity to get the necessary knowledge to guide the design and development of safe and effective weed control strategies (Fig. 1.6). Yeast is also useful for the development of rapid and inexpensive assays that can be used to screen a large number of agrochemicals for toxicity, since the majority of the chemicals in commercial use have not been comprehensively tested for human toxicity. Complementary studies in *A. thaliana* have provided important insights into the specific action of 2,4-D in target weeds and development of herbicide resistance (Teixeira et al. 2007; Cabrito et al. 2011; Remy et al. 2013) (Fig. 1.6).

Several MDR/MXR transporters from the ABC superfamily and the MFS are activated upon a sudden exposure of unadapted yeast cells to 2,4-D (Cabrito et al. 2011; Teixeira and Sá-Correia 2002; Teixeira et al. 2006). However, among them, only the ABC transporters Pdr5 and Pdr18 and the MFS transporter Tpo1 were confirmed determinants of resistance to this weak acid herbicide (Cabrito et al. 2011; Teixeira and Sá-Correia 2002). Transcription levels from yeast genes encoding Pdr5 and Tpo1 suffer a rapid and transient up-regulation in yeast, dependent on Pdr1 and Pdr3 transcription factors (Teixeira and Sá-Correia 2002) and *TPO1* gene expression leads to the decrease of intracellular accumulation of this herbicide (Cabrito et al. 2009). Remarkably, the heterologous expression of ScTpo1 and ScPdr5 transporters at *A. thaliana* plasma membrane was demonstrated to confer resistance to 2,4-D in planta, among other chemical compounds of agricultural interest (Remy et al. 2017).

Although the *A. thaliana* genome encodes 15 PDR transporters, only a few have a characterized functional role in MDR/MXR (Van Den Brûle and Smart 2002). The Pdr5-homolog AtPdr8 was found to exhibit a plasma membrane localization and to be a heavy metal extrusion pump, detoxifying the plant cells in the presence of inhibitory concentrations of cadmium and lead (Kobae et al. 2006; Kim et al. 2007; Strader and Bartel 2009). Also, mutations that inactivate AtPdr8 were shown to cause arrested development of root hair and cotyledon expansion due to a defect in the efflux of the auxinic hormone Indole-3-butyric acid (IBA), putatively performed by this ScPdr5 homolog (Strader and Bartel 2009). Another ScPdr5 homolog, AtPdr9, localizes to the plasma membrane and catalyse the efflux of 2,4-D, as well as other auxinic compounds (Ito and Gray 2006; Strader et al. 2008). AtPdr12 was first reported to alleviate toxicity induced by lead (Pb^{2+}) and lead-derived compounds, by extruding them out of plant cells (Lee et al. 2005). More recent studies describe AtPdr12 as responsible for the uptake of the hormone abscisic acid (ABA), therefore contributing to stomatal closure and regulation of water loss, in response to drought stress conditions (Lee et al. 2005; Kang et al. 2010). The physiological role described for AtPdr12 was found to be behind its relevance in plant tolerance to lead-derived compounds, since stomatal closure reduces lead translocation to the shoot through the transpirational stream, and the presence of ABA in the cell induces the activation

of the expression of genes required for lead tolerance (Kang et al. 2010; Chatthai et al. 1997; Perfus-Barbeoch et al. 2002; Talanova et al. 2000).

The heterologous expression of novel putative *A. thaliana* MFS transporters conferring MDR/MXR in *S. cerevisiae* is successfully allowing the elucidation of the functional role of plant transporters. The homologue of ScTpo1 in *A. thaliana*, At5g13750 (named ZIFL1—zinc-induced facilitator-like 1), when expressed in yeast was found to complement the absence of the *ScTPO1* gene and improve parental strain resistance to 2,4-D. In the other way, the expression of either *ScTPO1* or *AtZIFL1* in yeast leads to the decrease of the intracellular accumulation of 2,4-D in yeast cells. Increased expression of *At5ZFL1* also renders yeast cells more tolerance to the auxinic hormone indole-3-acetic acid (IAA), Al^{3+} and Tl^{3+} (Cabrito et al. 2009). In *A. thaliana*, the functional splicing variants of the *ZIFL1* transporter were found to have different tissue and subcellular localization (Remy et al. 2013; Duque 2013). The full-length *ZIFL1.1* protein localizes to the tonoplast of root cells and participates in auxin efflux during shootward transport, whereas the truncated isoform ZIFL1.3 localizes to the plasma membrane of leaf stomatal guard cells, improving plant drought tolerance (Remy et al. 2013). Heterologous expression of both *ZIFL1.1* and *ZIFL1.3* in yeast contributed to further detail the role played by these transporters in MDR/MXR, as they increase yeast tolerance to weak acids and ions (Remy et al. 2013). Complementation experiments in yeast showed that both splice variants complement the absence of *QDR2* expression, and the protein was found to exhibit high-affinity K^+ transport activity (Remy et al. 2013). The finding of K^+ being the physiological substrate for *ZIFL1* is consistent with the roles attributed to the splice isoforms given that K^+ fluxes favour auxin efflux and are regulators of guard cell turgor in response to stressing conditions (Remy et al. 2013).

The function of *ZIFL2* (zinc-induced facilitator-like 2), another member of the Major Facilitator Superfamily (MFS) of membrane transporters and the second closest homolog of the *ZIFL1* transporter in *A. thaliana*, was associated to K^+ and Cs^+ homeostasis. This conclusion was reached also by combining functional analysis studies in *A. thaliana* and heterologous expression of the plant gene in *S. cerevisiae* in which this transporter was found to mediate K^+ and Cs^+ influx (Remy et al. 2015). These are important phenotypes because K^+ is an essential mineral nutrient for plant growth and development, with numerous membrane transporters and channels being implicated in the maintenance and regulation of its homeostasis. The cation Cs+ is toxic for plants but shares similar chemical properties with K^+, competing with K+ for membrane transporters. Following detailed studies in the plant model, it was proposed that the activity of AtZifl2 promotes cellular K^+ efflux in the root, thereby restricting Cs^+/K^+ xylem loading and subsequent root to shoot translocation under conditions of Cs^+ or high K^+ external supply (Remy et al. 2015).

1.8 Concluding Remarks

The elucidation of the mechanisms underlying *S. cerevisiae*'s ability to tolerate and thrive in the presence of multiple toxic compounds is of paramount importance, given the dual role of this yeast as a model organism and cell factory. Multidrug/multixenobiotic resistance (MDR/MXR) transporters belonging to the ABC and MF Superfamilies have been traditionally considered efflux pumps involved in the active expulsion of a wide spectrum of unrelated cytotoxic compounds. However, evidence supporting the concept that their role in MDR/MXR may result from their specific physiological function is being obtained in recent years. In particular, several ABC transporters of the PDR family were implicated in plasma membrane lipid homeostasis, and their role in controlling plasma membrane lipid composition is very likely behind the corresponding MDR/MXR phenotype.

The functional characterization of MDR/MXR transporters and of the impact of their physiological functions in yeast tolerance to multiple stresses is expected to advance our understanding of the MDR/MXR phenomenon in yeast and in more complex eukaryotes such as plants. The heterologous expression of yeast MDR/MXR transporters in the plant model *A. thaliana* and vice versa has proven to be a promising strategy to improve the robustness of both yeast and plants (Remy et al. 2017; Cabrito et al. 2009). Moreover, the complex regulatory networks behind the activation of MDR/MXR transporters involve not only drug/xenobiotic or stress response specific transcription factors but also transcription factors responsible for the regulation of yeast metabolism. MDR/MXR transporters and their regulators are potential candidates as targets for the improvement of yeast strains with higher performance during bioprocesses and for design of more effective food preservation practices and for crop improvement.

Physiological genomic approaches are proving to be vital to unveil the global mechanisms underlying toxicity, response and adaptation to multiple stresses involving MDR/MXR transporters in *S. cerevisiae*, an invaluable cell factory and experimental model in industrial and agro-food biotechnology.

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Chapter 2
Mechanisms of Yeast Adaptation to Wine Fermentations

Estéfani García-Ríos and José Manuel Guillamón

Abstract Cells face genetic and/or environmental changes in order to outlast and proliferate. Characterization of changes after stress at different "omics" levels is crucial to understand the adaptation of yeast to changing conditions. Wine fermentation is a stressful situation which yeast cells have to cope with. Genome-wide analyses extend our cellular physiology knowledge by pointing out the mechanisms that contribute to sense the stress caused by these perturbations (temperature, ethanol, sulfites, nitrogen, etc.) and related signaling pathways. The model organism, *Saccharomyces cerevisiae,* was studied in response to industrial stresses and changes at different cellular levels (transcriptomic, proteomic, and metabolomics), which were followed statically and/or dynamically in the short and long terms. This chapter focuses on the response of yeast cells to the diverse stress situations that occur during wine fermentations, which induce perturbations, including nutritional changes, ethanol stress, temperature stress, oxidative stress, etc.

Keywords Yeast · Wine · Temperature · Ethanol · Sulfites · Nitrogen

2.1 Introduction

The grapes were domesticated between the Black Sea and Iran during the 7000–4000 BC period. The first evidence for winemaking lies in the presence of tartaric acid found in an ancient jar dated from 5400 to 5000 BC at the Neolithic site of Tepe in Mesopotamia, and also from grape juice remains dating back to 5000 BC at the Neolithic site of Dikili Tash, Greece (Sicard and Legras 2011). The colonization

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process by de Romans spread the wine around the Mediterranean Sea. In 500 BC, wine was being produced in France, Italy, Spain, Portugal, and North Africa.

The cultivation of grapes was also spread to Balkan States, and the Romans took it into Germany and other parts of northern Europe, and it eventually reached parts as far as Britain. In the sixteenth century, European explorers introduced the vine into the New World. In 1655, Dutch settlers planted French vine cuttings on the lower slopes of the Cape of Good Hope's majestic Table Mountain in South Africa. Planting in California followed soon after, and also in Australia and New Zealand more than a century later, in 1813 (Pretorius 2000).

Antonie van Leeuwenhoek developed an optic device which permitted him to observe yeast for the first time in seventeenth century. In the following centuries, scientist worked hard to understand the nature of alcoholic fermentation through analytical chemistry. Our knowledge of the fermentation process arose from the work of chemist Louis Pasteur. Pasteur was the first person to experimentally demonstrate that fermented beverages result from the action of living yeast transforming glucose into ethanol. He also demonstrated that only microorganisms were capable of converting sugars into alcohol from grape must in the absence of oxygen (Barnett 2000).

Later in 1890, Müller-Thurgau introduced the concept of inoculation with yeast culture (Pretorius 2000). Nowadays, most wine production relies on the use of selected pure yeast culture as part of the enological practice followed to produce wine with desirable characteristics and to guarantee the homogeneity of successive vintages.

2.2 Alcoholic Fermentation

Alcoholic fermentation is the principal metabolic process in winemaking and consists in transforming grape sugars (glucose and fructose) into ethanol and CO_2 (Pretorius 2000). Late in the 1850s, Louis Pasteur established that yeasts were the organisms responsible for fermentation, the process was nitrogen-dependent, and ethanol and CO_2 were not the sole products of fermentation as yeast synthesized the cell biomass. Yeasts are strongly predisposed to carry out alcoholic fermentation under both anaerobic and aerobic conditions (van Dijken et al. 1993). Fermentation is usually carried out anaerobically and generates energy in the form of adenosine triphosphate (ATP). Anaerobic metabolism generates only two ATPs per glucose molecule, compared to 36–38 ATPs during aerobic oxidation. At sugar concentrations above 20 g L^{-1} or even less, *Saccharomyces cerevisiae* uses the fermentative pathway for sugar metabolism and aerobic respiration is blocked, even in the presence of oxygen (O_2). This is known as the Crabtree effect (van Dijken et al. 1993). During fermentation, yeast metabolizes the sugars in grape musts to pyruvate via glycolysis. Pyruvate is decarboxylated to acetaldehyde, which is reduced to ethanol. One glucose molecule yields approximately two molecules of ethanol and CO_2, and approximately 95% of sugars are transformed into these two major metabolites. Only a small percent-

age of sugars are converted into secondary minor metabolites and in biomass (yeast growth). However, the majority of sugars are fermented in the nongrowing stationary phase (Roustan and Sablayrolles 2002).

2.3 Wine Ecology and Population Dynamics

Microbiologically, fermentation is carried out by different yeast species, but *S. cerevisiae* is the most abundant yeast species because it better resists to a wide variety of stresses. Standard wine fermentation is integrated by three different steps that are influenced by various stresses: lag phase, exponential phase, and stationary phase. The lag phase shows the time that yeast cells need to adapt to their new environment by synthesizing the ribosomes and enzymes required in the next step (López et al. 2004). The duration of this step depends on the initial population size and the environmental conditions. Once cells start active metabolism, they begin DNA replication, and cells divide shortly after. This process gives rise to the second growth phase called the exponential phase. This is the period during which cells duplicate at a maximum specific growth rate (μ_{max}). The time needed to double a population is known as the generation time. The generation time is influenced by different factors, such as yeast strain, growth medium, and temperature. The objective of industrial fermentations is to extend this phase in order to maximize the production of the biomass and metabolites (López et al. 2004). The third phase in yeast growth is the stationary phase; a period of no growth when metabolism slows down and cell division stops.

Wine fermentation-related natural microbiota are extremely complex and change depending on rainfall, grape variety, temperature, and on other influences, such as soil, fertilization, viticultural practices, and irrigation (Jolly et al. 2014). Genus *Hanseniaspora* species predominate on surfaces of grape berries and explain 50–75% of the whole yeast population. Species of *Candida* (e.g., *C. zemplinina*, *C. stellata*, *C. pulcherrima*), *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Metschnikowia* and *Pichia*, and *Rhodotorula* (Querol and Fleet 2006) are not as prevalent as these apiculate yeasts in numerical terms. Despite popular beliefs, the fermentative species of the genus *Saccharomyces*, particularly *S. cerevisiae*, appear on healthy undamaged grapes in very low populations, and are very rarely isolated from vineyards soils and intact berries (<0.1%). Damaged grapes are believed an important source by supplying inocula of 10^2–10^3 cells/mL of grape must (Fleet 2008). During the fermentation process, the antimicrobial activity of added sulfur dioxide, anaerobic conditions, depletion of nutrients, and rising ethanol levels extend the selectivity of the medium. The above-described non-*Saccharomyces* yeasts in grape juice, e.g., *Metschnikowia*, *Candida*, *Hanseniaspora* (*Kloeckera*), *Kluyveromyces*, and *Pichia,* could proliferate to final populations of some 10^6–10^7 cfu/mL, and start declining when the ethanol production by *S. cerevisiae* goes over 5–7%. *S. cerevisiae*-based ethanol production is a major factor which affects the growth of non-*Saccharomyces* yeasts (Fleet 2008;

Fleet 1998), along with increasing temperatures caused by *Saccharomyces* during fermentation (Salvadó et al. 2011).

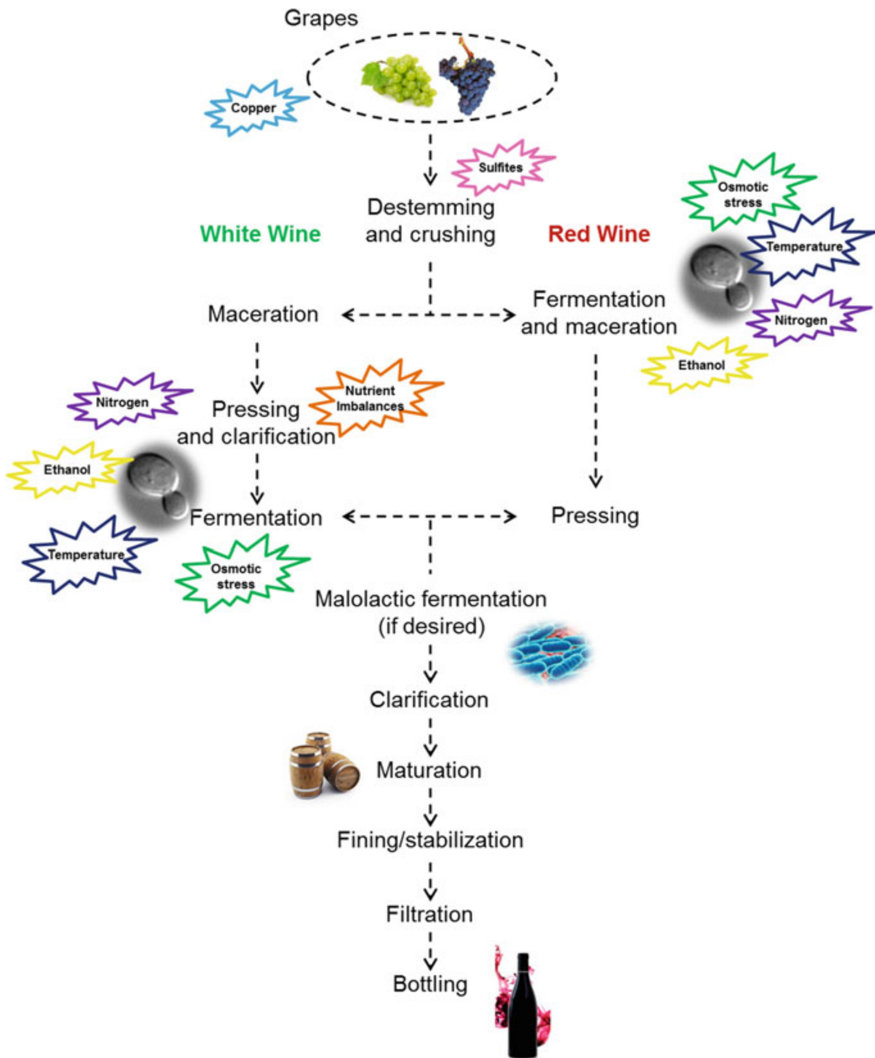

Fig. 2.1 Schematic representation of winemaking production and the stresses encountered during the process

2.4 Wine Fermentation: A Harsh Environment

Fermentation exposes yeasts to multiple environmental stresses (Fig. 2.1), including high osmotic pressure, low pH, low O_2, high sugar, elevated ethanol, nutrient starvation, addition of sulfites, and temperature fluctuations (Bauer and Pretorius 2000; Marks et al. 2008; Marsit and Dequin 2015; de Nadal et al. 2011; Gasch et al. 2000; Matallana and Aranda 2017). Nitrogen is the most limited nutrient in grape musts, and its availability correlates directly with biomass production, which controls the production of aroma compounds and the fermentation rate. Consequently, nitrogen deficiency is one of the most important causes of stuck and sluggish fermentations (Bisson 1999; Bell and Henschke 2005). Ethanol is produced during alcoholic fermentation and influences yeast metabolism, growth and the cell membrane composition, which is the primary target for its action. Other stresses in this phase are also noteworthy: nutrient starvation, which could take place at the mid or end of fermentation; high temperature (not very usual with the control systems presently used in wineries); low temperature (14–18 °C) in the current fermentation procedures used to improve wine's organoleptic properties (Torija et al. 2003; Beltran et al. 2002). Extreme conditions in the aforementioned factors may reduce the growth speed and survival rate and, therefore, tend to diminish fermentation efficiency, which can finish in stuck fermentations.

2.5 Wine Yeast Adaptation to Anthropic Niches

S. cerevisiae wine strains are very specialized organisms. They have evolved to employ various anthropic niches or environments during a process during the so-called "unaware domestication" process, which is responsible for these yeast's specific genetic characteristics (Fay and Benavides 2005; Liti et al. 2009; Almeida et al. 2015; Legras et al. 2018; Peter et al. 2018). The yeast genomes exposed to dynamic mechanisms produce genetic polymorphisms that have various evolutionary effects (Sicard and Legras 2011; Bisson 2012). The classifications of these mechanisms include short sequence insertions or deletions, single nucleotide polymorphisms (SNPs), short tandem duplications, recombination gross chromosomal rearrangements (GCRs), and gene conversion, gene and segmental duplications, ploidy changes, and interspecific hybridization, all of which help wine yeast genomes to adapt (Pretorius 2000; Blondin et al. 2009; Dequin and Casaregola 2011; Guillamón and Barrio 2017; Escalera-Fanjul et al. 2018).

2.6 Adaptation of Wine Yeast to Nutritional Deficiencies

Yeast cells have to adapt to the shortage of essential nutrients during wine fermentation. Some of these nutrients are rapidly depleted in the first stages of fermentation, and they have developed approaches to quickly and effectively deal with new conditions. As in other stresses, different yeast cell pathways are activated as response to nutrient depletion, which coordinate general responses, such as cellular proliferation and stress resistance. The cells of *Saccharomyces cerevisiae* stop mitotic division and arrest in the G1 phase when an essential nutrient is drained, entering into a non-proliferative state or stationary phase of growth (Conrad et al. 2014). Recent studies have shown that the capability of yeasts to survive depends on not only how they get in the starvation state, but also on the type of exhausted nutrient (Boer et al. 2008). The main signaling pathways activated by nutritional deficiencies are TOR (Rohde et al. 2008; Smets et al. 2010) or PKA (Roosen et al. 2005), together with other signaling pathways that work at the plasma membrane level (Shin et al. 2009).

Nitrogen is the main limiting nutrient during wine fermentation, and is highly correlated with fermentation kinetics, especially the maximum CO_2 production rate and the final yeast population (Bisson 1999; Bell and Henschke 2005). However, not all the nitrogen sources support the same growth rate and reach the same population size (Gutiérrez et al. 2013a). In complex mixtures of nitrogen compounds, *S. cerevisiae* have developed a mechanism, known as nitrogen catabolite repression (NCR), to select first the preferred nitrogen sources and to uptake later the least interesting ones. The presence in the grape must of good nitrogen sources (glutamine, asparagine, and ammonium) downregulates the transcription of the genes required to use poor nitrogen sources, lowering the levels of the enzymes and permeases encoded by these genes (Magasanik and Kaiser 2002). During wine fermentation, yeast cells change from a nitrogen-repressed condition to a nitrogen-de-repressed condition as nitrogen is consumed (Beltran et al. 2005). Thus, the transcriptional activity of these NCR genes is a good marker for sensing nitrogen limitation during grape must fermentation (Gutiérrez et al. 2013b). Some studies have proved that the profile of amino acid uptake can be changed by altering NCR regulation. Salmon and Barre (Salmon and Barre 1998) reported the increase in the consumption of proline during wine fermentation by deleting *URE2*, the main repressor of NCR genes. Proline is one of the most abundant amino acids in grape musts but repressed when preferred nitrogen sources are available. The Δ*ure2* strain produced more biomass and exhibited a higher maximum CO_2 production rate than the wild type under enological conditions. Beltran et al. (Beltran et al. 2006) also reported a relaxed NCR when fermentations were carried out at low temperature, as deduced from the gene expression of ammonium and amino acid permeases (*MEP2* and *GAP1*) and the uptake of some amino acids subjected to NCR (arginine and glutamine).

However, nitrogen requirements are strain-dependent and it is not well-known the molecular mechanisms determining this nitrogen demand during wine fermentation. Recently, some authors have identified allele variants in *S. cerevisiae* involved in mechanisms responding to nitrogen availability that affect the fermentation rate.

Brice et al. (Brice et al. 2014) showed how the presence of specific variants of the genes *MDS3* and *GCN1* had a different effect on the fermentation rate in the strains harboring these alleles. Both genes are involved in sensing and signaling nitrogen, highlighting the role of nitrogen signaling in controlling the glycolytic flux in nitrogen starvation, and support the hypothesis that the TOR pathway plays a key role in controlling fermentation capacity in nitrogen-starved cells (Brice et al. 2014). Horizontal gene transfers (HGT) are also adaptive solutions for nutrient limitations (Marsit et al. 2015; Galeote et al. 2010). Novo et al. (Novo et al. 2009) described the presence of a 65-kb region in the genome of *S. cerevisiae* wine yeasts that came from *Torulaspora microellipsoides*. The further study of this region revealed the presence of *FOT* genes, which enhanced the uptake capacity of oligopeptides from grape must and improved its fermentation capacity in a nitrogen-limiting medium (Marsit et al. 2015). Thus, HGT events can be also considered as an adaptive advantage of wine yeasts to improve their fitness in a poor nitrogen medium. In this HGT region, these authors have also described the presence of *FSY1*, the first active fructose transporter carrier described in *S. cerevisiae*, which also provides better fitness to the wine yeast strains harboring this gene (Galeote et al. 2010).

Although nitrogen is the main limiting nutrient in wine fermentation, lipids are also essential nutrients for a correct growth and fermentation performance of *S. cerevisiae*. Some enological practices, such as excessive must clarification and a short contact time between grape solids and juice, can significantly lower the concentration of sterols and unsaturated fatty acids in grape must, which can affect the fermentation rate and cell viability (Zara et al. 2009; Ochando et al. 2017; Luparia et al. 2004; Fornairon-Bonnefond et al. 2002; Varela et al. 2012; Tesnière et al. 2015; Tesnière et al. 2013). However, a lethal combination for wine yeasts is a strong unbalance among nitrogen and lipids (Duc et al. 2017). Excess of nitrogen in a lipid-limited grape must enhances yeast cell death. Conversely, lowering nitrogen levels restores yeast cell viability (Zara et al. 2009; Ochando et al. 2017). Thus, restriction of lipids, growth factors, and vitamins (ergosterol, oleic acid, pantothenic acid) trigger yeast cell death in a nitrogen-dependent manner (nitrogen excess) during wine alcoholic fermentation (Zara et al. 2009; Ochando et al. 2017).

Oxygen is not required for *S. cerevisiae* metabolism during wine fermentation because all the energy demands came from the glycolytic ATP. However, oxygen is needed for the synthesis of sterols and unsaturated fatty acids, and complete anaerobic environments lead to unbalance in the membrane lipid compositions. Thus, strong restrictions of oxygen during wine fermentation should be considered as a limiting situation for a proper yeast growth and fermentation performance. Wine yeasts have also developed strategies that favor survival under oxygen limitation conditions, such as flor yeast strains (Luparia et al. 2004). These strains aggregate or form a biofilm on the surface of fortified wines to get access to oxygen, where oxidative metabolism is possible (Fidalgo et al. 2006; Soares 2011). This process is typical of the sherry-type wine production. This capacity to form biofilm on the surface of wines mainly depends on a couple of mutations in the gene *FLO11*. A deletion in the promoter region and a rearrangement in the central tandem repeat domain of the coding region induce an upregulation in the transcription of this gene and a more hydrophobic

protein, respectively. The increase in this hydrophobic Flo11 variant explains the capability of the flor strains to form velum on the surface of wines. More recently, the whole-genome sequencing of three flor strains from different geographic regions corroborated how the expansion of the coding region of *FLO11*, which alter the balance between other *FLO* genes family members, underlies as the main molecular mechanism for velum formation (Eldarov et al. 2018).

2.7 Adaptation of Wine Yeasts to Osmotic Stress

High sugar concentrations at the beginning of wine fermentation also induce a strong osmotic stress to yeast cells. This high osmolarity in the grape must turns out in a reduction of both growth and viability as a consequence of a loss of the osmotic gradient across the plasma membrane and changes in cellular water activity. Osmotic stress is rapidly perceived by *S. cerevisiae* through multiple signaling pathways that allow cells to quickly respond to altered osmolarity (Saito and Posas 2012). The most well-known response is mediated by the MAPK HOG (high osmolarity glycerol) pathway, in which Hog1p is phosphorylated and rapidly accumulated in the nucleus (Posas et al. 2000; Gasch et al. 2017). The presence of Hog1p in the nucleus activates the expression of genes involved in osmotic stress defense (Rep et al. 1999). Among these genes, it is worth mentioning *GPD1* and *GPP2,* which encode glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, respectively. Both are responsible for the production of glycerol, the main metabolite synthesized by yeasts cells to protect from osmolarity (Blomberg and Adler 1989). Moreover, this rise in the intracellular glycerol is coordinated with a faster closure of the glycerol exporter Fps1, which prevents glycerol outflow. Thus, among the genes upregulated by the phosphorylated Hog1p are the genes responsible for glycerol import (*STL1*) and glycerol production (*GPD1* and *GPP2*) (Brewster et al. 1993). Hence, the intracellular accumulation of glycerol allows yeast cells adapting to high concentrations of sugar, NaCl, or other solutes in their medium by increasing internal osmolarity (Blomberg and Adler 1989). Other physiological alterations in the presence of high solute concentrations are changes in the physicochemical structure of the cell wall and plasma membrane and cell volume (de Nadal et al. 2011; Saito and Posas 2012; Posas et al. 2000).

2.8 Adaptation of Wine Yeasts to the Compounds Used in Vineyards

Sulfur dioxide has been used as a common preservative in wine since at least the nineteenth century. Its use is essential in winemaking not only because it is antioxidant, but also thanks to its antiseptic properties (Divol et al. 2012). One example of the

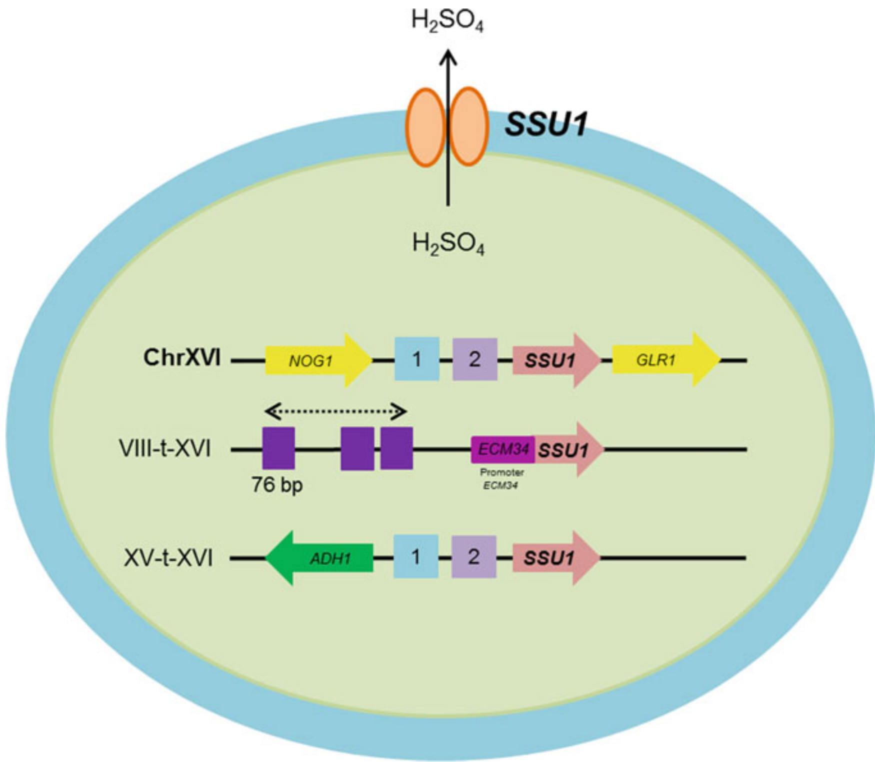

Fig. 2.2 Ectopic translocations described to be involved in sulfite resistance. VIII-t-XVI was mediated by the crossing-over mediated by the homology in the promoters of *ECM34* and *SSU1*. Several 76-bp (in purple) repetitions were found in the promoters, along with a direct relationship between the number of 76-bp repeats and sulfite tolerance. XV-t-XVI involves the Adr1 (1) and Fzf1 (2) binding regions of the promoter of *ADH1* and *SSU1*, respectively

adaptive advantage is the reciprocal translocation that occurs between chromosomes VIII and XVI, which is widespread among wine yeasts (Fig. 2.2). This translocation generates an allele of the sulfite pump, *SSU1*-R1, with higher expression levels than *SSU1*, and confers greater resistance to sulfites (Pérez-Ortín et al. 2002; Yuasa et al. 2004; Goto-Yamamoto et al. 1998). Recently, another translocation between chromosomes XV and XVI has been identified in relation to quantitative trait loci (QTL) of the lag phase duration during wine fermentation. This translocation also increased the expression of gene *SSU1* (Zimmer et al. 2014). Both translocations have been observed only in wine yeasts, the VIII-t-XVI translocation is the more frequent and the XV-t-XVI form has been found only in commercially selected wine strains, which suggests a more recent event. Both translocations confer resistance in a medium containing SO_2. Therefore, the widespread use of sulfites in winemaking likely causes a convergent evolutionary rearrangement that confers a growth advantage to the strains carrying the *SSU1* recombinant forms.

Another potential domestication-related trait is the acquisition of resistance to copper sulfate. Copper is an oxidizing agent needed for many single electron transfer reactions in the cell and is toxic at high concentrations. The sensitivity to copper sulfate of natural isolates of *S. cerevisiae* has been reported to vary, and resistance to copper sulfate may be a recently acquired adaptation as a result of applying copper sulfate as a fungicide to treat powdery mildew and other vine diseases in vineyards (Fogel and Welch 1982; Sun et al. 2018; Besnard et al. 2001; Fay et al. 2004; Warringer et al. 2011).

Good copper tolerance in the European and Sake yeast lineages has been associated with the wide copy number variation (CNV) of *CUP1,* which encodes copper-binding metallothionein (Warringer et al. 2011). *CUP1* is commonly duplicated among wine yeast strains, but not among yeasts in a closely related natural oak lineage (Almeida et al. 2015; Strope et al. 2015; Steenwyk and Rokas 2017). Recently, a promoter variant of *CUP1* with increased expression variability has been identified in wine yeast strain EC1118, which is an advantage when facing stress and suggests that, together with the increase in the copy number of genes, gene expression modulation is another potential adaptation mechanism in yeast (Liu et al. 2015).

2.9 Adaptation of Wine Yeast to Oxidative Stress

In general, all eukaryotic organisms have oxygen-dependent metabolism because throughout evolution, this molecule has been selected as a final electron acceptor during the respiration process. The consequence is that all aerobic organisms are subjected to the so-called "oxygen paradox" (Davies 1995) because they depend on it for their survival during cellular respiration but, at the same time, the result of oxidizing power is toxic compounds called reactive oxygen species (ROS). Oxidative stress is said to occur when ROS overwhelm antioxidant defenses, which results in genetic degeneration and physiological dysfunction, and eventually leads to cell death (Halliwell and Gutteridge 1986).

Oxidative damage activates synthesis in enzymes capable of detoxifying ROS. Superoxide dismutases transform the superoxide anion. Then, catalases degrade H_2O_2 and several peroxiredoxins eliminate a range of peroxides (Herrero et al. 2008; Ayer et al. 2014). The redox status of cells is controlled by oxidative stress response systems to avoid and repair damage to oxidation-prone molecules; e.g., the thiol residues of proteins. Two basic redox-controlling systems exist: one based on tripeptide glutathione and a second on small proteins known as thioredoxins (Ayer et al. 2014).

Oxidative stress is the main stress that operates against the yeast cells that grow in fermenters during biomass production. A respiratory metabolism is then imposed, so more biomass is produced, which results in higher oxidative stress (Pérez-Torrado et al. 2005). Cells have developed different strategies to deal with this situation. The overexpression of *TRX2,* which codes for a thioredoxin, leads to a wine strain with increased biomass production (Gómez-Pastor et al. 2010; Gómez-Pastor et al. 2012).

The drying process also causes internal oxidative stress as markers of oxidative damage increase. Induction of genes with antioxidant function (*TRR1*), overexpression of hydrophilines *SIP18* and *STF2*, accumulation of intracellular trehalose, and strong catalase and glutathione reductase activities (Gómez-Pastor et al. 2010; Gamero-Sandemetrio et al. 2014; López-Martínez et al. 2013; López-Martínez et al. 2015; Rodríguez-Porrata et al. 2012; López-Martínez et al. 2012) are strategies to overcome this stressful situation.

2.10 Adaptation of Wine Yeast to Temperature Fluctuations

Wine yeasts are also exposed to a wide range of temperatures. Red wine fermentation is carried out mainly at high temperatures (25–30 °C), whereas white and rosé wine fermentations typically take place at lower temperatures (15–20 °C) to preserve wine aroma.

Low temperatures strongly affect the physiology and metabolism of yeast, and cells need to rapidly respond and adapt. This response is accompanied by major changes in gene expression and enzyme activity, which allow yeast to maintain growth and survival at low temperatures (Sahara et al. 2002; Schade et al. 2004; Tai et al. 2007; García-Ríos et al. 2014; Aguilera et al. 2007). There are differences between *S. cerevisiae* strains in terms of their physiological and transcriptional response to cold temperatures, but yeasts are generally prone to maintain metabolic functions during cold temperature stress. A study by Tai et al. 2007demonstrated that, although the activity of glycolytic enzymes was ~7.5-fold lower at 12 °C than at 30 °C, yeasts were able to maintain the same glycolytic flux level in chemostat cultures ($D = 0.03\ h^{-1}$). Wine yeasts are typically better at adapting to cold temperatures than laboratory strains (Pizarro et al. 2008).

Cold temperature has a massive effect on cell physiology (Fig. 2.3). These effects include decreased membrane fluidity, increased stabilization of DNA and RNA secondary structure, reduced protein translation and protein folding efficiency, increased protein denaturation, clustering of integral membrane proteins, and decreased enzyme activity (Sahara et al. 2002; Al-Fageeh and Smales 2006). The cell membrane, which contains a large proportion of lipid molecules, is considered the primary target of low temperature trauma (López-Malo et al. 2013; López-Malo et al. 2014; Redón et al. 2011; Tronchoni et al. 2012; García-Ríos et al. 2017). Temperature directly affects the organization of these membrane lipids by causing them to solidify, decreasing membrane fluidity, and reducing intra- and extracellular transport and diffusion rates of compounds and ions (Inouye and Phadtare 2004). The cell counteracts this effect by increasing the synthesis of unsaturated fatty acids and short-chain fatty acids which, in turn, implies enhanced fluidity in the plasma membrane (homeoviscous adaptation) (Al-Fageeh and Smales 2006; López-Malo et al. 2013; López-Malo et al. 2014).

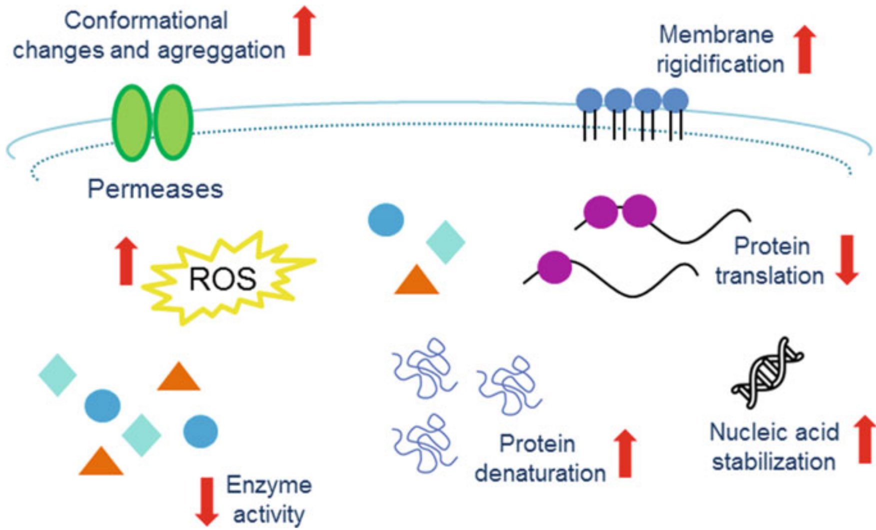

Fig. 2.3 Physiological effects on yeast cells during low temperature stress

Nucleic acid secondary structure stabilization is considered a unique consequence of low temperatures and it lowers mRNA elongation rates during transcription and mRNA movement in ribosome during translation (Inouye and Phadtare 2004). The mRNA 5' untranslated region (5'UTR) tends to form stable secondary structures at low temperatures by becoming less accessible to the ribosome (Fortner et al. 1994; Hilliker et al. 2007; Li and Brow 1996; Perriman and Ares 2007; Staley and Guthrie 1999; Zavanelli et al. 1994). The upregulation of the genes and proteins involved in translation is a compensatory mechanism to overcome the blockage of this process at low temperature (Tronchoni et al. 2014; García-Ríos et al. 2016a). Low temperature also produces a higher stabilization of proteins and folding problems. To counteract any problems associated with protein misfolding and denaturation at low temperature, yeast synthesizes HSPs, which act as protein chaperones and help restore enzyme activity at low temperatures (Murata et al. 2006; Pacheco et al. 2009).

During cold stress, some authors have reported that yeast cells produce large amounts of protectant compounds, such as reserve carbohydrates trehalose and glycerol, to protect internal cellular components (Sahara et al. 2002). The disaccharide trehalose, which acts as a chaperone for protein and membrane stabilization, accumulates after cells are incubated for approximately 12 h at 10 °C, 4 °C or 0 °C. Glycogen production is also induced after this period, but its role in the cold response has not yet been elucidated (Aguilera et al. 2007). Glycerol, an osmoprotective solute, accumulates at higher levels at low temperature and the accumulation rate depends on the Stl1 glycerol active transporter (Aguilera et al. 2007). Glycerol protects the cell by counteracting osmotic shrinkage effects caused by freeze and thaw cycles (Panadero et al. 2006). Recent studies (Oliveira et al. 2014; Pérez-Torrado et al. 2018) have observed

major differences in the regulation of glycerol synthesis in cryotolerant species *S. uvarum* and *S. kudriavzevii* compared to *S. cerevisiae*. *S. kudriavzevii* exhibits a higher *GPD1* expression, enhanced enzymatic parameters, and increased activity for the enzyme (Oliveira et al. 2014). *S. uvarum* also exhibits a different regulatory mechanism: a higher *GPD1* expression as in *S. kudriavzevii*, and a higher expression of *STL1* by encoding a glycerol proton symporter of the plasma membrane (Pérez-Torrado et al. 2018). All these differences show that *S. kudriavzevii* and *S.uvarum* have remodeled their metabolism to promote the glycolysis involved in glycerol production in order to adapt to cold niches and to maintain the NAD^+/NADH ratio in alcoholic fermentations.

Naturally, cold-tolerant strains like *S. kudriavzevii*, *S. uvarum* or *S. eubayanus* can be potentially used for low temperature fermentations, but tend to have higher ethanol sensitivity than *S. cerevisiae* and may, therefore, be less suitable for alcoholic fermentation (Arroyo-López et al. 2010). The hybridization process between *Saccharomyces* species has been proposed as an adaptation mechanism to different stresses, especially to low temperature (Sipiczki 2008). The hybrids described in wine have physiological properties from both parentals. Hybrids might have inherited the ability to grow at low temperatures (10–16 °C) from their *S. kudriavzevii*, *S. uvarum* and *S. eubayanus* parental, as well as the ability to grow at high temperatures (30–37 °C), with enhanced ethanol tolerance from their *S. cerevisiae* parental (Alonso del Real et al. 2017; Origone et al. 2018; Belloch et al. 2008; Gamero et al. 2013; Magalhães et al. 2017a, b).

The antioxidant response is also elicited at low temperatures to protect cells from ROS and free radicals, which are formed under environmental stress (Ballester-Tomás et al. 2015; Paget et al. 2014; Salvadó et al. 2008; Zhang et al. 2003; García-Ríos et al. 2016b). Antioxidant compounds and enzymes, including glutathione, catalase, and superoxide dismutase, are all induced at low temperatures by detoxifying ROS to maintain viability (Murata et al. 2006; Ballester-Tomás et al. 2015; Paget et al. 2014; Zhang et al. 2003; García-Ríos et al. 2016b). A recent study has demonstrated that the coordinated upregulation of the genes involved in the sulfur and glutathione pathways may lead to higher intracellular glutathione concentrations, whose protective effect improves the fermentation process at low temperature (García-Ríos et al. 2014, 2016).

In the tumultuous phase of red wine fermentation, temperatures can reach values of around 37–40 °C. At these temperatures, yeast cells activate the heat shock response (HSR) and alter some other components of their physiology, including membrane composition and carbohydrate flux (Morano et al. 2012; Lindquist 1986; Richter et al. 2010; Pereira et al. 2018). The nature of the response depends on not only but also on the increase in temperature and the proximity of temperature to produce growth inhibition (Gasch et al. 2000; Gasch 2003). At high temperatures, the genes related with rescuing unfolded proteins from degradation, trehalose synthesis, restoring unfolded proteins, restoring the DNA structure, recovering the microfluidic state of membranes, protecting splicing from disruption, and protection against excessive energy consumption are usually overexpressed (Piper 1995; Castells-Roca et al. 2011; Yost and Lindquist 1991). Conversely, the genes related with ribosome and

protein synthesis, and those involved in cell cycle progression, are usually expressed at lower concentrations. Sterols are essential for the formation of lipid "rafts" and the regulation of membrane dynamics to maintain the microfluidic state and to perform essential biological processes, including cellular sorting, cytoskeleton organization, and asymmetric growth and signal transduction (Caspeta et al. 2013; Caspeta et al. 2016; Caspeta and Nielsen 2015; Lingwood and Simons 2012).

2.11 Adaptation of Wine Yeast to Ethanol

Ethanol is the main stress factor that the yeast cell faces during fermentation. The continuous increase in ethanol throughout fermentations inhibits the viability and growth of yeasts (Stanley et al. 2010; Teixeira et al. 2011). In physiologically terms, ethanol is an inhibitor of yeast growth at relatively low concentrations, and it decreases cell volume, slows down the maximum specific growth rate, inhibits cell division, and increases cell acidification (Birch and Walker 2000). Ethanol also influences cell metabolism and macromolecular biosynthesis by inducing the production of heat shock-like proteins, lowering the RNA and protein accumulation rate, enhancing the frequency of "petite" mutations, altering metabolism, denaturing intracellular proteins and glycolytic enzymes, and reducing their activity (Hu et al. 2007). Moreover, the plasma membrane is also a key target of ethanol toxicity. Ethanol increases the fluidity of the plasma membrane. In response to this altered fluidity, yeast cells may change membrane composition by increasing the levels of unsaturated fatty acids (UFAs) and ergosterol (Jimenez and Benitez 1987; Navarro-Tapia et al. 2016). Furthermore, the addition of some amino acids (proline, tryptophan, and arginine) (Cheng et al. 2016; Du and Takagi 2007; Takagi et al. 2005) and inositol (Ohta et al. 2016; Kelley et al. 1988) can enhance ethanol tolerance, probably through enhanced membrane stability.

Genome-wide analyses have been used to identify the genes involved in yeast response and resistance to ethanol stress (Alexandre et al. 2001; Fujita et al. 2006; Teixeira et al. 2009; Dinh et al. 2008; Kubota et al. 2004; Voordeckers et al. 2015; Ma and Liu 2010; Lewis et al. 2014; Auesukaree 2017). Adaptation to high ethanol levels involves copy number variation, changes in ploidy, and the appearance of different phenotypes (Voordeckers et al. 2015). The expression of the factors that stabilize and/or repair protein denaturation, such as trehalose (*TPS2* and *NHT1*), glycogen (*GSY1* and *GSY2*) and heat shock proteins (*HSP12*, *HSP26*, *HSP30*, *HSP78*, *HSP82*, *HSP104*, *SSA3*, and *SSA4*), directly correlates with high ethanol tolerance (Fujita et al. 2006; Wang et al. 2014; Trevisol et al. 2011; Alexandre et al. 2001). After ethanol stress, activation of the genes related with the unfolded protein response and its transcription factor Hac1 has been observed (UPR) (Navarro-Tapia et al. 2016; Navarro-Tapia et al. 2018). Mutants lacking mitochondrial manganese-superoxide dismutase (MnSOD) are sensitive to ethanol, which indicates that *SOD2* is essential for ethanol tolerance (Costa et al. 1997). The peroxisomal function also appears to be associated with ethanol tolerance as deletions of the genes encoding proteins of

both the peroxisome transport machinery and peroxisomal membrane protein import machinery are more sensitive to ethanol (Teixeira et al. 2009; Yoshikawa et al. 2009).

A recent study (Voordeckers et al. 2015) used experimental evolution in the presence of ethanol to show that evolved clones carry an extra copy of chromosome III and/or ChrXII; or clones even have a smaller region (introgression) of chromosome XII. The GO enrichment of this region belonging to chromosome XII showed cell wall formation as one of the key processes to be affected by these amplifications. Some genes involved in stress response, intracellular signal transduction, the cell cycle, and the genes related to membrane composition and organization, have also been found to arise in the evolved populations.

2.12 Concluding Remarks and Perspectives

The vast amount of data produced by the global analysis of the transcriptome, proteome, and metabolome of wine yeasts under industrial conditions, together with the wide availability of genome sequences, will provide a complete view of the behavior of these organisms in forthcoming years. Hence, the first step is to assess the relationship between genotype and phenotype in a population to investigate the patterns of polymorphisms in a large sample of individuals (Liti and Schacherer 2011). With the rise in new long-reads sequencing technologies, we will unveil the variability hidden in subtelomeric regions. Resolving the structure of chromosome ends is essential as they contain many genes involved in secondary metabolisms and, therefore, play key roles in individual variation. The availability of complete genome assemblies will also provide a complete picture for other polymorphisms by resolving their impact on evolution and fitness (Liti and Louis 2012).

Multiple adaptation strategies to fermentative conditions have been discussed in this chapter. Knowledge of these adaptive mechanisms will provide us with the tools to generate better adapted yeast to wine fermentations. The solutions could be the rational selection of new yeasts with improved enological characteristics, engineering evolution-based experiments that take advantage of acquired knowledge, or even the application of so-called GMO techniques, such as directed mutagenesis.

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Chapter 3
Development of Robust Yeast Strains for Lignocellulosic Biorefineries Based on Genome-Wide Studies

Ming-Ming Zhang, Hong-Qi Chen, Pei-Liang Ye, Songsak Wattanachaisaereekul, Feng-Wu Bai and Xin-Qing Zhao

Abstract Lignocellulosic biomass has been widely studied as the renewable feedstock for the production of biofuels and biochemicals. Budding yeast *Saccharomyces cerevisiae* is commonly used as a cell factory for bioconversion of lignocellulosic biomass. However, economic bioproduction using fermentable sugars released from lignocellulosic feedstocks is still challenging. Due to impaired cell viability and fermentation performance by various inhibitors that are present in the cellulosic hydrolysates, robust yeast strains resistant to various stress environments are highly desired. Here, we summarize recent progress on yeast strain development for the production of biofuels and biochemical using lignocellulosic biomass. Genome-wide studies which have contributed to the elucidation of mechanisms of yeast stress tolerance are reviewed. Key gene targets recently identified based on multiomics analysis such as transcriptomic, proteomic, and metabolomics studies are summarized. Physiological genomic studies based on zinc sulfate supplementation are highlighted, and novel zinc-responsive genes involved in yeast stress tolerance are focused. The dependence of host genetic background of yeast stress tolerance and roles of histones and their modifications are emphasized. The development of robust yeast strains based on multiomics analysis benefits economic bioconversion of lignocellulosic biomass.

Keywords Lignocellulosic biomass · Biorefinery · *Saccharomyces cerevisiae* · Stress tolerance · Metabolic engineering · Multiomics analysis

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3.1 Introduction

With the gradual depletion of fossil fuels, the aggravation of environmental pollution and the increasing demand for energy, development, and utilization of alternative sources for the production of biofuels and biochemicals are urgently desired. Microbial metabolic engineering has contributed to bioproduction in a sustainable and environmentally friendly way, and has received considerable attention (Liao et al. 2016).

Lignocellulosic biomass, such as agricultural residues, energy crops, as well as forest and municipal wastes, is the richest renewable resource on Earth. It is estimated that as much as 170 million tons of lignocellulosic biomass is generated annually (Ho et al. 2014), and extensive studies have been carried out on the production of biofuels and biochemicals from lignocellulosic feedstocks using microbial cell factories (Chandel et al. 2018). However, economic biorefinery of lignocellulosic feedstocks is restricted by various factors, among which low cell viability and poor fermentation performance due to various inhibitors generated during the pretreatment process are common problems (Jönsson and Martín 2016). Inhibitors in cellulosic hydrolysates can reduce the growth and fermentation ability of microbial strains, and lead to low concentration and productivity of biofuels, thereby resulting in high energy consumption and low economic profits.

The budding yeast *Saccharomyces cerevisiae* is widely used in the production of biofuels and bio-based chemicals, and great efforts have been made to develop robust yeast strains for lignocellulosic biofuels production (Ko and Lee 2017). To satisfy efficient lignocellulosic biorefinery, robust *S. cerevisiae* strains that are tolerant to various inhibitors present in the lignocellulosic hydrolysates, as well as other environmental stress factors, including high temperature, low pH, and inhibitory compounds and products, are highly desired (Deparis et al. 2017; Zhao et al. 2016). Excellent stress tolerance of yeast strains can maintain suitable fermentation activity and promote cell recycle. Therefore, it is of profound significance to study the tolerance mechanisms of *S. cerevisiae* to various environmental stresses and to develop stress-resistant strains for biorefinery applications.

Since the past decade, significant advances have been achieved in the elucidation of the underlying mechanisms of yeast stress response and tolerance. In addition to the fact that expression of multiple genes can be changed by stresses, genome-wide studies also revealed that mechanisms of stress tolerance can be host-dependent in different yeast strains (Sardi et al. 2016, 2018). Multiomics analysis has been employed to identify key genes involved in stress tolerance for further genetic engineering, and the results have contributed greatly to yeast strain development for efficient bioconversion of lignocellulosic biomass (Zhao et al. 2016; Caspeta et al. 2015).

In this chapter, genome-wide studies on stress tolerance of *S. cerevisiae* are summarized, focusing on industrial strains and their applications. Successful examples on the development of stress-tolerant yeast based on multiomics analysis are provided, and future prospect in development of robust yeast strains is presented.

3.2 Inhibitory Conditions and Yeast Stress Response

Lignocellulosic materials are mainly composed of cellulose, hemicellulose, and lignin. These three components are entangled in complex structures, which hinder the effective release of cellulose, and finally affect the efficiency of enzymatic hydrolysis. Therefore, pretreatment is critical in the process of cellulosic biofuel production, which can increase the surface area of cellulase and hemicellulase, and reduce the relative molecular weight and the crystallinity of cellulose, thus effectively release monosaccharides which can be used by microbes (Mood et al. 2013). In the process of pretreatment, sugar release is usually accompanied by the generation of inhibitors, such as weak acids, furans, and phenolic compounds (Jönsson and Martín 2016).

3.2.1 Inhibitors in Cellulosic Hydrolysates

3.2.1.1 Weak Acids

The most common weak acids in lignocellulosic hydrolysates are acetic acid, formic acid, and levulinic acid. The toxicity of weak acids varies under different pH conditions. Weak acids in their molecular state enter into cells and then dissociate to protons and anion ions, which result in intracellular acidification and cause toxicity to yeast cells. The underlying mechanisms of weak acid inhibition on *S. cerevisiae* include uncoupling mechanism and intracellular anion accumulation (Palmqvist and Hahn-Hägerdal 2000). Through these two suppression mechanisms, weak acids inhibit yeast growth, prolong the lag phase, and reduce the productivity and yield. Genome-wide studies have revealed the molecular mechanisms of acetic acid tolerance, which can be found in the recent review (Palma et al. 2018).

As a primary part of weak acids, acetic acid is mainly derived from hydrolysis of hemicellulose (Jönsson and Martín 2016). Meanwhile, acetic acid is also produced as a byproduct of ethanol fermentation. Previous study has shown that acetic acid at a concentration of 5 g/L severely suppressed the growth and fermentation phenotype of yeast cells (Sousa et al. 2012). Undissociated acetic acid enters yeast cells by passive diffusion and through aquaglyceroporin Fps1p, and then dissociates into protons and acetate ions (Mollapour and Piper 2007). Accumulated protons induce acidification of intracellular environment, leading to an imbalance of intracellular pH state. To defense this imbalance, cellular protectional systems, such as H^+-ATPase and other ATP-dependent transmembrane proteins, can be activated by acetic acid stress (Piecuch and Obłąk 2014). Protons can be pumped out of the cell or be stored in vacuoles by consuming ATP. Therefore, reduced growth rate occurs due to ATP consumption for proton export, not only in case of acetic acid stress, but also under other acid stress conditions (Guo and Olsson 2014). In case that ATP is depleted, lack of intracellular energy then affects cell metabolism and even leads to programmed cell death (PCD) (Guo and Olsson 2014; Sousa et al. 2013). Meanwhile, acetate ions

could activate Hog1p, a high osmolality glycerol response protein, and further induce phosphorylation and degradation of channel protein Fps1p, leading to a reduction in the absorption of nutrient substances (Mollapour and Piper 2007; Mollapour and Piper 2006). In addition, protons and acetate ions would influence the process of electron transfer chain, causing electronic leakage, leading to the accumulation of reactive oxygen species (ROS), which results in oxidative stress (Giannattasio et al. 2008, 2013). DNA damage was also observed in the yeast cells treated with acetic acid (Ribeiro et al. 2006). The toxic effects of acetic acid for lignocellulosic biorefinery depend on the pH and the concentration of acetic acid, which should be considered based on specific process conditions.

3.2.1.2 Furans

Furans, mainly referring to furfural and 5-hydroxymethylfurfural (5-HMF), are released from the degradation of glucose and xylose under high-temperature and high-pressure condition. Furans affect the physiological activity of yeast cells in many ways such as causing damage to DNA, RNA, and protein and reducing intracellular ATP content (Ask et al. 2013). For example, a 53% decrease of the maximal specific growth rate, 25% reduction of the maximal biomass, and 10% decrease of the maximum ethanol concentration were observed when yeast strain was cultured in the presence of 4.0 g/L furfural. Meanwhile, increased permeability of membrane was also detected when 4.0 g/L furfural was added in the medium (Lopes da Silva et al. 2017). In addition, accumulation of nontranslating mRNAs and an attenuation of bulk translation activity could be caused by furfural and 5-HMF in *S. cerevisiae* (Iwaki et al. 2013). Fortunately, microorganisms have the ability to degrade furans to less toxic compounds through NAD(P)H-dependent reductive pathways. Furfural could be reduced to less toxic furfural alcohol by *S. cerevisiae* under anaerobic condition (Liu et al. 2008). Furfural induces accumulation of ROS inside the yeast cells (Allen et al. 2010), which caused damages to mitochondria and vacuole membranes, the actin cytoskeleton, and nuclear chromatin (Liu et al. 2008).

3.2.1.3 Phenolic Compounds

Phenolic compounds, which are released during the pretreatment of lignocellulosic biomass, are potential inhibitors that seriously suppress growth and ethanol production rate of yeast cells. The concentration of phenolic compounds in wheat straw hydrolysate treated by wet alkali oxidation is 0.27 g/L, however, the concentration could reach to 2–3 g/L when bagasse was hydrolyzed via dilute sulfuric acid (Martinez et al. 2001). Although in a relatively low concentration, phenolic compounds still suppress yeast growth and fermentation due to their lower water solubility and the higher hydrophobicity than other inhibitors, making it difficult to be removed via washing or over-liming (Gu et al. 2014). In general, phenolic compounds mainly are composed of syringyl, guaiacyl, and para hydroxyl (Wang et al. 2017a). The lower the

molecular mass of phenolic compounds, the higher the toxicity. It was reported that when yeast cells were treated with 0.5 g/L phenol, about 50 metabolites with changed levels were detected. These changed metabolites were mainly involved in amino acid metabolism, such as tyrosine, tryptophan, valine, and leucine, etc. Moreover, phenol could cause disruption of cell wall integrity and defects in cell growth through inhibiting the regulation of Pkc1p-MAPK pathway (Ding et al. 2011). Vanillin is a lignin-derived inhibitor, which could induce processing bodies and stress granules accumulation through repressing the translation process and blocking ribosomes assembly (Iwaki et al. 2013). Meanwhile, mitochondrial dysfunction and ROS accumulation were found when yeast cells were treated with vanillin, which then triggered oxidative stress (Nguyen et al. 2014). It was suggested that ergosterol content was related to vanillin tolerance (Zheng et al. 2017).

3.2.2 Inhibitory Conditions Related to Fermentation

3.2.2.1 Osmotic Stress

Excessive salts in cellulose hydrolysate may cause osmotic stress on *S. cerevisiae* cells during the process of biofuels fermentation. In general, osmotic stress is caused by an imbalance of osmolytes between extracellular and intracellular situation, which will lead to an alteration in cellular physiology. In response to osmotic stress, the intracellular water and the expansion pressure of cells will be lost, and then cells shrinkage, which at last leads to mitochondrial dysfunction, and may also result in apoptotic cell death (Martínez-Montañés et al. 2010). Previous results showed that with 10% sorbitol addition, the redox signal intensity of *S. cerevisiae* BY4741 decreased about 15%. Similar results were also detected when 15% glucose or 1.5 M glycerol was added, respectively (Kumar et al. 2015). Decreased mitochondrial membrane potential and increased ROS production were determined when yeast cells were treated with 0.6 M NaCl for 30 min, suggesting the dysfunction of mitochondrial induced by osmotic stress (Liu et al. 2009). The general mechanisms of maintaining osmotic balance in and out of the environment is the accumulation of compatible substances, for example, glycerol, which is modulated by mitogen-activated protein kinases (MAPKs). The high-osmolarity glycerol (HOG) MAPK cascade is well-studied MAPKs pathway related to response to hyperosmotic stress (Martínez-Montañés et al. 2010). Knockout of genes encoding glycerol synthetase gene and HOG pathway revealed that mutant strains were highly sensitive to osmotic stress (Albertyn et al. 1994). Global reprogramming of gene expression by the HOGMAPK cascade and the protein kinase A pathway was reviewed previously (Martínez-Montañés et al. 2010).

3.2.2.2 Ethanol Stress

In addition to inhibitors in cellulosic hydrolysates, accumulation of ethanol to a certain level during fermentation also causes stress on cells. It was found that ethanol toxicity is a major limiting factor for high-solid fermentation of cellulosic ethanol (Jin et al. 2017; Nguyen et al. 2017). Ethanol passes through the cell membrane through free diffusion, which generates proteins denaturation and reduces the fluidity of the cell membrane (Navarro-Tapia et al. 2018). At the same time, the previous study showed accumulation of proton and decreased intracellular pH due to the increased membrane permeabilization that was induced by highly lipophilic alcohols (Charoenbhakdi et al. 2016). In addition, ethanol inhibits the growth and metabolic activity of yeast cells, affects the transport system of substances such as amino acids and glucose, and inhibits enzymes in glycolysis and other important carbon metabolic pathways (Kim et al. 2016). By comparing the difference of the transcriptional results between sake wine yeast and the laboratory yeast strains under the stress of ethanol, the authors found that the tryptophan synthesis-related genes played an important role in ethanol tolerance of the wine yeast (Hirasawa et al. 2007). Overrepresentation of genes related to amino acid metabolism can improve cell membrane stability, reduce the influence of ethanol on cell membrane fluidity, and decrease the toxicity of ethanol to cells (Yoshikawa et al. 2009). After knockout of genes related to the synthesis of tryptophan, it was found that the ethanol tolerance of the strain decreased significantly, and the addition of tryptophan could also effectively improve ethanol tolerance (Teixeira et al. 2009). It was found that ethanol-tolerant yeast strains exhibit activated unfolded protein response (UPR) (Navarro-Tapia et al. 2016), suggesting that UPR may be a target for improving ethanol tolerance.

3.2.2.3 Thermal Stress

Cooling water systems with intensive capital investment and energy consumption are needed during the cellulosic ethanol fermentation. Fuel ethanol production by *S. cerevisiae* is generally performed at about 30 °C. Simultaneous saccharification and fermentation (SSF) is highly preferred for producing cellulosic ethanol from lignocellulosic biomass due to significant benefits with such a process in preventing glucose repression on cellulases, avoiding microbial contamination, and savings in production cost. However, the preferred temperature for enzymatic hydrolysis, which is 50 °C, is a lethal temperature for yeast cells (Zhao et al. 2012). Therefore, the improvement of yeast thermal tolerance is highly desired. Yeast cellular responses to heat shock and/or heat stress have been investigated extensively. The response of yeast cells to heat stress and the tolerance mechanisms were reviewed elsewhere (Auesukaree 2017). On the other hand, high-temperature fermentation also elicits accumulation of acetic acid, which further aggravates toxicity (Woo et al. 2014).

Integration of different omics analysis was performed on studies of thermal tolerance. Whole genome transcriptional profiling revealed that several single-nucleotide variations (SNVs), which could affect membrane composition and structure were

detected in the mutant obtained from laboratory adaptive evolution (LAE), among which changes in the *ERG3* gene, encoding a C-5 sterol desaturase was overlapped in all of the adaptive strains. Subsequently, metabolomics analysis revealed the significant increase of fecosterol content, accompanied by the reduction of ergosterol content, aroused by the mutation of *ERG3* (Caspeta et al. 2014).

It should be noted that although both heat shock stress and prolonged heat stress (thermal stress) belong to high-temperature stress, they have obviously different outcomes in *S. cerevisiae*. Proteomics analysis has shown that under heat shock, most differentially expressed proteins were unrelated. In contrast, under long-term heat stress (LTHS), about 70% proteins with changed levels were downregulated. Proteins involved in central carbon metabolism, oxidative stress response, as well as protein folding and degradation, were mainly upregulated by heat shock, which could provide maximal energy and reductive power to defense stress. In contrast, the changed proteins under LTHS could be categorized into central carbon metabolism, energy metabolism, amino acid metabolism and vesicle organization (Shui et al. 2015). Hence, it is very important to focus on thermal stress response instead of heat shock for industrial strain development for biorefinery.

3.2.2.4 Oxidative Stress

Oxidative stress is also a major stressor encountered during a variety of fermentation processes (Auesukaree 2017). As discussed in the previous section, the toxicity of ethanol and inhibitors in cellulosic hydrolysate (e.g., acetic acid, furfural, etc.) is all related to ROS accumulation. Oxidative stress is caused by the imbalance of intracellular oxidants and antioxidants, resulting in the increase of oxidants. The most abundant oxidants in the cell are ROS, derived from oxygen, including one or more unpaired electrons. Under aerobic conditions, intracellular ROS mainly come from the process of electron transport chain in mitochondria, especially when redox reaction is incomplete. Besides in mitochondria, ROS can also be generated in endoplasmic reticulum and peroxisome (Bin-Umer et al. 2014). The intracellular ROS mainly includes superoxide anion, hydroxyl group, and hydrogen peroxide (H_2O_2). ROS are reactive with many macromolecules such as lipids, proteins, DNA, and RNA, causing their oxidation and loss of normal functions (Schieber and Chandel 2014). High ROS levels lead to a process that is called "oxidative stress". ROS-dependent oxidation of DNA can generate several different DNA damages, including base modifications, single-strand breaks and intra/interstrand DNA crosslinks (Schieber and Chandel 2014). DNA lesions can block the progression of replication, causing double-strand breaks. Oxidative damages and the resulting genomic instability are major contributing factors for carcinogenesis. Several categories of enzymes directly perform the function as ROS detoxification. Superoxide anions could be converted to H_2O_2 by cytoplasmic and mitochondrial superoxide dismutases (SODs). Enzymes catalases (CATs), glutathione peroxidases (GPXs), and thioredoxin-dependent peroxidases (TSAs) could decompose H_2O_2 to H_2O. The mutants with deletion of *SOD1*, which encodes a cytosolic copper–zinc superoxide dismutase, is sensitive to a variety of

environmental stresses including oxidative stress, desiccation, alkaline pH, and the presence of various chemicals (Rattanawong et al. 2015).

3.3 Improvement of Stress Tolerance for Efficient Bioconversion Based on Genome-Wide Studies

Despite the complicated network of gene transcription and translation related to various stress factors, significant progress has been made in the development of yeast stress tolerance for efficient production of cellulosic biofuels and biochemicals. Genome-wide studies have provided molecular targets for metabolic engineering of stress tolerance of different yeast strains, which is the main topic of this chapter. Due to the multiple genes related to yeast stress tolerance, random methods, including random mutagenesis, genome shuffling, and adaptive evolution are still required. Genome-wide studies can be employed both under different physiological conditions and using the mutants obtained from these random processes. Furthermore, it is of great importance to integrate process conditions in strain design and development, because the regulatory network of gene expression depends heavily on dynamic process conditions.

In this chapter, the applications of multiomics analysis in the improvement of yeast stress tolerance are reviewed, and strain development for enhancement of cellulosic ethanol production by budding yeast *S. cerevisiae* is focused.

3.3.1 Laboratory Adaptive Evolution (LAE) and Genome-Wide Studies

LAE has been proved useful for selection of yeast strains both for tolerances to inhibitors and other stress conditions (Caspeta et al. 2014; Shui et al. 2015; Cakar et al. 2012; Thompson et al. 2016). LAE was used to enhance yeast tolerance to vanillin. After 180 batches of adaptive evolution, a high-tolerance strain EMV-8 was obtained, which maintained a specific growth rate of 0.104/h in 2 g/L vanillin, whereas the reference strain could not grow. Physiological studies revealed that the vanillin reduction rate of EMV-8 is 1.92-fold higher than its parent strain (Shen et al. 2014). In another report, after 100 transfers by cultures of yeast to synthetic media containing increasing concentrations of inhibitors, *S. cerevisiae* 307-12H60 and 307-12H120 that showed enhanced ability to reduce furfural or HMF were obtained (Wang et al. 2017a). Along with long-term evolution engineering development, the short-term adaption cultivating yeast under conditions that resembled the subsequent fermentation or visualizing evolution in real time (VERT) was established. By increasing amounts of hydrolysate in the propagation in the pre-cultivation, adaptation improved cell viability by >10% and increased vitality by >20% in SSF. Subsequent growth

kinetic analyses of the mutants that obtained through VERT in individual and combinations of common inhibitors present in hydrolysates (acetic acid, furfural, and 5-HMF) showed differential levels of resistance to different inhibitors, with enhanced growth rates up to 57%, 12%, 22%, and 24% in hydrolysates, acetic acid, 5-HMF, and furfural, respectively (Almario et al. 2013).

Genome-wide studies, such as genome sequencing, transcriptomic analysis, and their combinations, have been performed in the evolved strains, and molecular mechanisms for the improved growth rate under various stress conditions were revealed. For example, the transcription factor Yrr1p was revealed to suppress vanillin resistance by combined genome sequencing and transcriptomic analysis of a vanillin-resistant strain EMV-8 which was obtained by adaptive evolution (Wang et al. 2017b). In another report, genome sequencing identified cell periphery-related proteins performed positive effect on thermal tolerance and inhibitor tolerance of the evolved strain *S. cerevisiae* strain ISO12 (Wallace-Salinas et al. 2015). Whole genome sequencing and RNA-seq analysis were performed to study yeast resistance to low pH exerted by HCl and L-lactic acid. It was found that evolution outcomes are different with the different property of the acids, and also carbon sources used for LAE are an important determinant to influence evolutionary (Fletcher et al. 2017). The above-mentioned studies demonstrated the effectiveness of genome-wide studies integrated with LAE for the elucidation of the underlying mechanisms of yeast stress tolerance. Based on the understanding of important molecular functions and metabolic processes, rational design can be further employed to construct stress-tolerant yeast. For example, genome sequencing of an evolved thermal tolerant strain derived from *S. cerevisiae* MT8-1 revealed a critical point mutation in the gene *CDC25*, and thermotolerant yeast was reconstructed by introducing the point mutation (Satomura et al. 2016). It can be expected that more critical targets can be identified by further studying the evolved yeast strains with improved stress tolerance phenotypes.

3.3.2 Genome-Wide Studies Related to Medium Optimization

Nutrients in the medium exert significant influences on cell metabolism and gene regulation, and yeast stress tolerance is also modulated by medium compositions. Here, we highlight the recent studies on metal ions and amino acids.

3.3.2.1 Metal Ions

Metal ions play important roles in the maintenance of the conformation of intracellular bio-macromolecules, and can assist the enzyme to function normally in the form of coenzymes. Metal ions can also participate in the regulation of intracellular redox potential and osmotic pressure. Studies have shown that in the presence of various inhibitory conditions, the addition of appropriate amounts of metal ions,

such as potassium (Lam et al. 2014), calcium, copper, and zinc (Ismail et al. 2014), can effectively improve the tolerance of yeast cells.

Zinc ion not only acts as the cofactor of various enzymes, but also serves as the critical structural component of many important proteins, including zinc finger proteins which exert control in gene regulation (Zhao and Bai 2012). However, the effects of zinc ion on yeast stress tolerance have been not fully understood. In our previous studies, increased cell viability in response to 20% (v/v) ethanol shock of the self-flocculating yeast SPSC01 was achieved through supplementation of proper concentration of zinc sulfate (Xue et al. 2008). The protective effect of zinc ion on yeast thermal tolerance was also discovered (Zhao et al. 2009). During very high gravity (VHG) fermentation, yeast cells with zinc supplementation exhibited improved glucose uptake rate and ethanol production as well as decreased glycerol production, when compared with that without zinc supplementation (Xue et al. 2010). In the follow-up studies, enhanced ethanol fermentation efficiency of the flocculating yeast SPSC01 was observed with zinc addition under acetic acid stress condition (Wan et al. 2015). We, therefore, explored the underlying mechanisms by comparative transcriptomic analysis and metabolic profiling analysis. Compared with that of the control group without zinc supplementation, transcription of key genes involved in nucleotide metabolism (*ADE1*, *ADE17*, and *RNR3*), carboxylic acid transporters (*ADY2*, *JEN1*, and *ATO2*) and zinc finger proteins (*SET5*, *ZAP1*, and *PPR1*) were significantly affected by zinc addition. We further demonstrated that deletion of *ADY2* improved yeast acetic acid tolerance (Zhang et al. 2017). Zinc can act as an antioxidant (Eide 2011), which may contribute to protecting yeast cells against long-term acetic acid stress treatment. In our metabolic profiling results, the addition of zinc under acetic acid stress significantly decreased intracellular ROS content, and dynamic changes in the content of amino acid and intermediates of central carbon metabolism were also detected (Wan et al. 2015). The important roles and possible mechanisms of zinc in yeast stress response and tolerance have been summarized (Zhao and Bai 2012).

In addition to zinc, other metal ions also are involved in oxidative stress. Copper is an important component of Cu/Zn superoxide dismutase (Sod1p), metallochaperone Atx1p, and metallothioneins Cup1p and Cup2p, and also exerts antioxidant properties. Copper supplementation increased intracellular SOD activity and reduced ROS content of *S. cerevisiae* BY4741, which contributed to enhanced freeze–thaw stress resistance. Meanwhile, deletion of genes (*MAC1* and *CTR1*) involved in copper ion homeostasis exhibited freeze–thaw sensitivity (Takahashi et al. 2009). Overexpression of *FRE1*, which encodes a plasma membrane high-affinity copper transporter, increased intracellular copper content and led to enhanced oxidative stress tolerance (Berterame et al. 2018). Besides, iron and manganese also act as antioxidants, which exert function in the regulation of both SOD and CAT activities (Ribeiro et al. 2015). Iron supplementation or increased expression of *CTH2*, which is involved in iron homeostasis, enhanced yeast growth under oxidative stress (Matsuo et al. 2017). Acetic acid stress could be alleviated by manganese supplementation during xylose fermentation through an isomerase-based xylose-utilizing strain, which resulted in a

52% increased ethanol (Ko et al. 2016). Hence, the regulation of metal ions homeostasis is important for yeast stress tolerance.

The effects of different metal ions on yeast stress tolerance have also been compared in the case of acetic acid stress using a recombinant yeast strain-utilizing xylose (Ismail et al. 2014). Ethanol production from xylose under acetic stress was enhanced by supplementation of three metal ions (Zn^{2+}, Mg^{2+}, and Ca^{2+}), and comparative transcriptomics was performed. Distinct changes of gene transcription were observed with different metal ions, but upregulation of *FIT2*, which encodes the facilitator of iron transport, was observed in common. In a follow-up report, upregulation of genes involved in transition metal ion homeostasis (iron and zinc, respectively) was found in the heat and acid-resistant yeast mutant obtained by genome shuffling (Inokuma et al. 2017). Further studies will unveil how metal ions affect global gene transcription and translation, which will deepen our knowledge on the important roles of metals in yeast stress tolerance.

3.3.2.2 Amino Acids

Amino acids are essential nutrients for yeast cells, and their involvement in stress tolerance has also been extensively studied. As mentioned in the previous section, manipulation of tryptophan biosynthesis genes enhanced stress tolerance to ethanol. Supplementation of tryptophan in the culture medium showed similar enhancing effects (Hirasawa et al. 2007).

Metabolomics studies revealed that intracellular contents of some amino acids (such as alanine, γ–aminobutyric acid (GABA), valine, proline, and serine, etc.) were obviously higher in the superior tolerant yeast strains (Ding et al. 2011). According to the "omics" results, exogenous supplementation and endogenous accumulation of amino acids have been performed exerting a beneficial effect on ethanol fermentation in response to inhibitory conditions. Intracellular levels of proline have been correlated with stress resistance in yeast. Improved viability to freezing, desiccation, or acetic acid has been demonstrated with proline addition previously (Takagi et al. 1997; Liang et al. 2014). In another report, yeast strains with 1.5 g/L proline supplementation displayed shortened lag phase and fermentation time in the presence of inhibitor mixtures [furfural, acetic acid, and phenol (FAP)]. Meanwhile, with *PRO1* overexpression, which could increase approximately twofold higher intracellular proline in BY4742, nearly 50 h shortened fermentation time was achieved when the FAP inhibitors were present (Wang et al. 2015). Other amino acids (such as methionine and arginine) have also been investigated, and their protective effects on oxidative stress and ethanol stress were uncovered (Campbell et al. 2016; Cheng et al. 2016a).

In our recent studies, metabolic profiling revealed the increased accumulation of alanine under acetic acid stress when zinc sulfate was supplemented. Subsequently, we proved that the addition of alanine decreased ROS production and improved yeast cell growth as well as ethanol fermentation under acetic acid stress (Wan et al. 2015). These results implied that metal ion homeostasis and amino acid metabolism are interconnected, both of which exert control in yeast stress tolerance.

3.3.2.3 Other Protectants Related to Yeast Stress Tolerance

In addition to metal ions and amino acids that are discussed above, trehalose is also a well-known protectant for yeast stress tolerance [reviewed in Auesukaree (2017)]. For example, *S. cerevisiae* ZS*p*T*ΔA* containing higher intracellular trehalose content exhibited improved growth ability and fermentation performance under osmotic stress and ethanol stress conditions (Wang et al. 2014). Other yeast stress protectants were also reported. Increased yeast growth under FAP or ethanol stress was achieved through supplementation of 500 mg/L myo-inositol, as well as by the overexpression of *INO1*, which encodes inositol-3-phosphate synthase (Wang et al. 2015). In addition, quercetin, glutathione, and melatonin, also exerted a protective effect against oxidative stress, acetic acid stress, and furan stress in *S. cerevisiae* (Ask et al. 2013; Alugoju et al. 2018; Vázquez et al. 2018).

Polyamines, including spermine, putrescine, and spermidine (SPD), are closely associated with a defense to diverse environmental stresses, which have been demonstrated by spermine addition or *SPE1*, *SPE2*, and *SPE3* overexpression in *S. cerevisiae* in response to furfural and acetic acid stress (Kim et al. 2015).

Studies involving key molecules in yeast stress tolerance are providing increasing evidence on the dependence of nutrient conditions of yeast cell viability in the presence of inhibitory conditions. Genome-wide studies have aided the exploration of nutrient effects, which provide key genes for metabolic engineering of yeast stress tolerance.

3.3.3 Metabolic Engineering of Yeast Stress Tolerance Based on Genome-Wide Studies

Genome-wide studies using LAE and nutrient modulation have provided abundant data on the possible gene targets for further development of robust yeast strains for biorefinery. Here, we review the recent progress on metabolic engineering of yeast stress tolerance based on genome-wide studies.

3.3.3.1 Key Genes Involved in Stress Tolerance

With the advancement of high-throughput sequencing technology, comprehensive multiomics analysis, including genomics, transcriptomics, proteomics, and metabolomics has become convenient. On the other hand, multiomics analysis provides a basis for the study of the interaction of different cellular components and the analysis of the dynamic process of biological systems at specific process conditions (for example, temperature, different concentrations of inhibitors, etc.). Besides the target genes discussed in the previous section, other representative genes involved in stress tolerance to different inhibitory conditions that were recently identified, which

Table 3.1 Selected genes responsible for stress tolerance in *S. cerevisiae*

Gene name	Function of the protein product from SGD	Inhibitor	Reference
ACE2	Transcription factor required for septum destruction after cytokinesis	Acetic acid, furfural	Chen et al. (2016)
PMA1	Plasma membrane P2-type H^+-ATPase	Weak acids, ethanol, etc.	Lee et al. (2016)
PPR1	Transcription factor positively regulates transcription of URA genes that are involved in de novo pyrimidine biosynthesis	Acetic acid to H_2O_2	Zhang et al. (2015)
PRS3	Phosphoribiosyl pyrophosphate synthetase gene synthesizes PRPP that is required for nucleotide, histidine, and tryptophan biosynthesis	Acetic acid	Cunha et al. (2018)
RPB7	RNA polymerase II subunit B16 regulates cellular lifespan via mRNA decay process	Ethanol	Qiu and Jiang (2017)
RTT109	Histone H3 acetyltransferase; critical for cell survival in presence of DNA damage during S phase	Acetic acid, heat	Cheng et al. (2016b)
SET5	Histone methyltransferase involved in methylation of histone H4 Lys5, -8, -12	Acetic acid, H_2O_2	Zhang et al. (2015)
SFP1	Predicted RNA polymerase II transcription factor involved in the activation of transcription of ribosomal protein genes and in the regulation of cell size	Acetic acid, furfural	Chen et al. (2016)

include genes encoding transcription factors, histone modification enzymes, RNA polymerase II subunits, among others, were highlighted in Table 3.1.

3.3.3.2 Condition-Specific Stress Tolerance

It is important to know that the same gene may play different roles in the case of different stress environments. For example, the plasma membrane is important for yeast stress tolerance. Transcriptional level of genes encoding plasma membrane transporters was upregulated in response to inhibitors (Zhang et al. 2017; Dong et al. 2017), which implied the importance of membrane transporters in stress resistance of yeast. The deletion of *TRK1* gene, which encodes a membrane transporter responsible for potassium import lead to reduced tolerance of yeast cells to acetic acid (Mira et al. 2010). However, in response to formic acid toxicity, improved growth ability of yeast cells was observed when *TRK1* was deleted. Further analysis revealed that Trk1p deletion resulted in decreased accumulation of intracellular formic acid and therefore contributed to improved cell growth, leading to the hypothesis that Trk1p may catalyze the uptake of formic acid/formate (Henriques et al. 2017). Therefore,

for strain development, key genes should be carefully selected based on the specific conditions.

As discussed above, the mechanisms of heat shock are very different from that of thermal tolerance. Therefore, it is important to consider specific conditions related to the fermentation process. In our previous studies, key genes involved in acetic acid tolerance and thermal tolerance of industrial yeast strains were identified using an artificial zinc finger library (Khatun et al. 2017; Ma et al. 2015). We found that *QDR3* is involved in long-term acetic acid stress, whereas *IKS1* only shows effects upon acetic acid shock treatment (Khatun et al. 2017). Therefore, genome-wide studies should focus on more customized conditions closely related to real fermentation.

3.3.3.3 Roles of Transcription Factors and Histones in Yeast Stress Tolerance

Transcription factors (TFs) are important targets for the development of stress-tolerant yeast strains. For example, genome-wide transcriptional analysis revealed that Haa1p is the main regulator in controlling acetic acid tolerance of yeast as it may regulate the transcription of approximately 80% of the acetic acid-activated genes (Mira et al. 2011). *HAA1* overexpression conferred yeast superior growth and higher sugar consumption capacities of *S. cerevisiae* acetic acid condition (Cunha et al. 2018; Swinnen et al. 2017). Ace2p and Sfp1p were top two transcription factors regulating genes with varied transcription in the presence of acetic acid and furfural. Nearly four times improved specific ethanol productivity for *SFP1* overexpression and three times enhanced fermentation rate for *ACE2* overexpression were observed in *S. cerevisiae* SR8, respectively, under the stress of acetic acid and furfural (Chen et al. 2016). Other TFs, such as Ppr1p and Yrr1p were also identified through omics analysis, which was involved in the regulation of yeast stress response (Wang et al. 2017a; Zhang et al. 2015).

On the other hand, regulation of biosynthesis of histones and their modifications, which can cause chromatin remodeling and alter gene expression, is a different level of regulation that is different from traditional transcriptional regulation by transcription factors. In our previous studies, the transcriptomic analysis revealed enhanced transcription level of *SET5,* which encodes a methyltransferase for methylation of histone H4, in the zinc sulfate-supplemented cells comparing with that of the non-addition control. Overexpression of *SET5* resulted in improved growth ability under acetic acid and H_2O_2 stress condition, and also improved ethanol yield in the presence of toxic level acetic acid as well as in the corn stover hydrolysate (Zhang et al. 2015). In addition to histone methylation and demethylation, histone acetylation and deacetylation are also well known to affect gene expression. We found a variation of *RTT109*, which encodes a histone acetyltransferase for the acetylation of histone H3, in our transcriptomic analysis with zinc sulfate addition when yeast cells were employed in continuous ethanol fermentation. Subsequently, improved acetic acid stress tolerance and tolerance to oxidative stress was observed through deletion of *RTT109* (Cheng et al. 2016b). Previously, it was reported that mutations in

H4K8, which is the target site of 2-hydroxyisobutyrylation, and also a mutation in H3T11, the target site of phosphorylation, led to reduced growth ability of yeast strain under 4 mM H_2O_2 stress condition (Huang et al. 2017; Li et al. 2015). On the other hand, mutations in histone also affect yeast stress tolerance. From the comprehensive screening of a histone H3/H4 mutant library, 24 histone H3/H4 mutants with varied robustness were identified, which include 6 acetic acid resistant and 18 sensitive mutants (Liu et al. 2014). Further studies on histone and histone modification enzymes will unveil a novel regulatory network and provide efficient strategies for yeast strain development.

3.3.3.4 Host Genetic Backgrounds and Stress Tolerance

It is of great importance to point out that yeast stress tolerance is significantly dependent on host genetic background (Sardi et al. 2016, 2018). It is not surprising that key genes identified in one strain work poorly in another strain. Therefore, it is essential to screen natural isolates that are tolerant to stressful conditions employed in industrial applications. For example, to obtain furfural-resistance yeast, over 70 environmental and industrial isolated strains were analyzed, and furfural-resistant strain was isolated (Field et al. 2015). A robust *S. cerevisiae* strain LC 269108 displayed acid-tolerant properties. This strain has great potential for fermentation of acid-pretreated substrate (Nwuche et al. 2018). Strain with high thermotolerance properties is an important criterion for strain screening for avoiding contamination and reducing economic consumption. Therefore, isolation of thermal tolerant yeast strains is necessary. Highly thermotolerant yeast strains that were isolated from the natural source were summarized in Table 3.2.

Genome-wide studies have been used to unveil the mechanisms of host genetic backgrounds of industrial strains. For example, industrial yeast *S. cerevisiae* CAT-1 and PE-2 were isolated from Brazilian distilleries, and are still being used now (Basso et al. 2008). Comparative proteomics was performed to compare these two strains. It was found that CAT-1 strain with improved fermentation performance contains more proteins related to oxidative stress (Sod1p and Trx1p) and trehalose synthesis

Table 3.2 Isolation of thermotolerant yeasts strains as potential hosts for cellulosic biorefinery

Samples and localities	Yeast isolate/upper limit temperature	Reference
Fruits and vegetables, Thailand	*S. cerevisiae* TR2/42 °C	Koedrith et al. (2008)
Ripe banana peels, Nigeria	*S. cerevisiae* R-8/42 °C	Brooks (2008)
Soils and fruits, India	*S. cerevisiae* MTCC 170/40 °C	Ali and Khan (2014)
Daqu from Chinese liquor factory, China	*S. cerevisiae* G13105/43 °C	Gong et al. (2014)
Foods, milk, yogurt, flowers, Sudan	*S. cerevisiae* A/49°C	Ali et al. (2017)

(Tps3p) than that in PE-2 in batch fermentation, which is consistent with improved oxidative stress and trehalose accumulation in CAT-1 (Santos et al. 2017). These results indicate the correlation of oxidative stress tolerance with efficient fermentation ability of industrial yeasts.

3.3.3.5 Roles of Yeast Stress Tolerance in Heterologous Expression: Case Study in Xylose Utilization

Strains with both superior stress tolerance and better xylose utilization properties are beneficial for mixed-sugar utilization during bioconversion of lignocellulosic hydrolysates. In our recent studies, global transcription of *S. cerevisiae* YB-2625 which was isolated from bagasse was compared with that of the model yeast strain *S. cerevisiae* S288c during glucose and xylose fermentation. We found that enhancing oxidative stress tolerance benefits xylose utilization (Cheng et al. 2018). These results implied the important roles of oxidative stress in the bioconversion of xylose, which is abundant in the cellulosic hydrolysate. We assume that metabolic engineering of stress tolerance is not only important for cell defense from external stress factors (e.g., inhibitors and other fermentation-related stresses), but can be also relevant for combating endogenous stress factors, for example, toxic intermediates, metabolic burden aroused from overexpression of proteins (van Rensburg et al. 2012). It is still not clear how heterologous genes or pathway, such as xylose utilization pathway, cellulase genes, among others, introduced into yeast cells induce stress response, but it is worthwhile to investigate such internal stress responses for improved biorefinery efficiency.

3.3.3.6 Yeast Strain Development for Efficient Biorefinery

Strategies for the development of robust yeast strains through process optimization and metabolic engineering are summarized in Fig. 3.1. Although CRISPR-Cas9-based genome editing is not focused in this chapter, this method has been popular for yeast genome engineering, and novel methods have been developed for rapid engineering of yeast stress tolerance (Bao et al. 2018; Garst et al. 2017). CRISPR-Cas9-based genome editing is of great importance to develop highly efficient methods to improve yeast robustness, which is controlled by multiple genes. However, so far most studies employ laboratory yeast strains, it is vital to establish related methods for development of industrial yeast strains, and genome-wide studies can also facilitate the identification of customized gene targets. We emphasize that it is vital to perform precise strain engineering to improve robustness by combining with process condition and specific host genetic background.

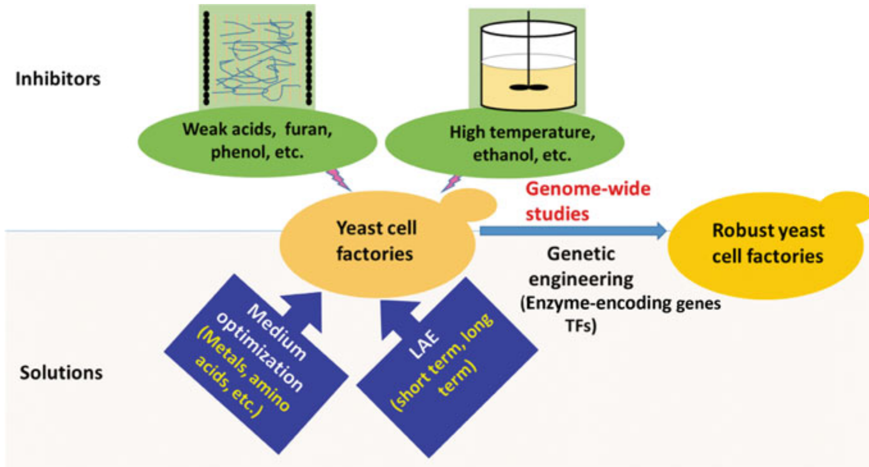

Fig. 3.1 Development of robust yeast cell factories with the aid of genome-wide studies Abbreviations: TF, transcription factor: LAE, laboratory adaptive evolution

3.4 Conclusion

Economic lignocellulosic biorefinery requires robust yeast strains with high cell viability and excellent fermentation performance in the presence of various toxic inhibitors present in the cellulosic hydrolysates. On the other hand, resistance to other environmental stress factors such as high temperature and low pH also benefits product yields under various process conditions. Genome-wide studies not only provide information on the genetic basis for robust phenotypes, but also reveal changes in global gene transcription and protein expression under different process conditions and host genetic backgrounds. This information will continually provide evidence for the identification of key genes for the improvement of stress tolerance. An efficient lignocellulosic biorefinery is expected to be achieved by the combination of both strain breeding and process optimization based on genome-wide studies.

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Chapter 4
Physiological Genomics of the Highly Weak-Acid-Tolerant Food Spoilage Yeasts of *Zygosaccharomyces bailii* sensu lato

Margarida Palma and Isabel Sá-Correia

Abstract *Zygosaccharomyces bailii* and two closely related species, *Z. parabailii* and *Z. pseudobailii* ("*Z. bailii* species complex", "*Z. bailii* sensu lato" or simply "*Z. bailii* (s.l.)"), are frequently implicated in the spoilage of acidified preserved foods and beverages due to their tolerance to very high concentrations of weak acids used as food preservatives. The recent sequencing and annotation of these species' genomes have clarified their genomic organization and phylogenetic relationship, which includes events of interspecies hybridization. Mechanistic insights into their adaptation and tolerance to weak acids (e.g., acetic and lactic acids) are also being revealed. Moreover, the potential of *Z. bailii* (*s.l.*) to be used in industrial biotechnological processes as interesting cell factories for the production of organic acids, reduction of the ethanol content, increase of alcoholic beverages aroma complexity, as well as of genetic source for increasing weak acid resistance in yeast, is currently being considered. This chapter includes taxonomical, ecological, physiological, and biochemical aspects of *Z. bailii* (*s.l.*). The focus is on the exploitation of physiological genomics approaches that are providing the indispensable holistic knowledge to guide the effective design of strategies to overcome food spoilage or the rational exploitation of these yeasts as promising cell factories.

Keywords *Zygosaccharomyces* · Physiological genomics · Food spoilage · Weak acid tolerance · Nonconventional yeasts · Microbial cell factories · Weak acids production

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4.1 Introduction

Zygosaccharomyces bailii and the two other species in the *Z. bailii* sensu lato clade, *Z. parabailii* and *Z. pseudobailii*, have long been associated with spoilage of food products, in particular, acidified preserved foods and beverages containing fermentable sugars (James and Stratford 2011; Thomas and Davenport 1985; Loureiro and Malfeito-Ferreira 2003; Suh et al. 2013). Spoilage associated with growth and metabolic activity of these yeast species can lead to significant economic losses in the food and beverage industry (James and Stratford 2003). The remarkable spoilage capacity of these species results from the combination of a number of physiological characteristics: (i) high tolerance to the weak acids frequently used as food preservatives, (ii) ability to ferment fructose and glucose, (iii) ability to cause the spoilage of foods and beverages even at a low cell concentration, and (iv) osmotolerance (although moderate, when compared to *Z. rouxii*) (James and Stratford 2003). These traits also make *Z. bailii* sensu lato species relevant biological platforms for particular biotechnological processes. For example, these species have been suggested to be potentially interesting cell factories for the production of organic acids (Sauer et al. 2004; Dato et al. 2010), or based on their genomic patrimony, as a source of genes for expression in *S. cerevisiae* envisaging the increase of tolerance to weak acids of this biotechnologically established species (Palma et al. 2015, 2017). In the food industry, *Z. bailii* (sensu lato) was also suggested to have potential for the reduction of wine ethanol content (Contreras et al. 2015; Gobbi et al. 2014), and increase of the aroma complexity of alcoholic beverages (Domizio et al. 2011; Garavaglia et al. 2015; Xu et al. 2017).

4.2 Taxonomy of *Zygosaccharomyces bailii* sensu lato Clade

The genus *Zygosaccharomyces* was initially described by Barker in 1901 to accommodate *Saccharomyces*-like yeasts that exhibited conjugation prior to ascus formation (James and Stratford 2011). However, the number of species included in this genus has been altered through the years, not only because new species were isolated and identified, but also because of improvements in the methodologies used for microbial identification that are currently mostly centered on DNA-based methods, such as ribosomal RNA (rRNA) gene sequencing. At the time of publication of the fifth edition of *The Yeasts: A Taxonomic Study* (Kurtzman et al. 2011), six species were allocated to the genus *Zygosaccharomyces*: *Z. kombuchaensis*, *Z. lentus*, *Z. mellis*, *Z. bailii*, *Z. bisporous*, and *Z. rouxii* (James and Stratford 2011). However, the genus has been greatly expanded with the identification of the new species *Z. machadoi* (Rosa and Lachance 2005), *Z. gambellarensis* (Torriani et al. 2011), *Z. favi* (Čadež et al. 2015), *Z. sapae* (Solieri et al. 2013), *Z. siamensis* (Saksinchai et al. 2012), *Z. pseudobailii*, and *Z. parabailii* (Suh et al. 2013) (Fig. 4.1).

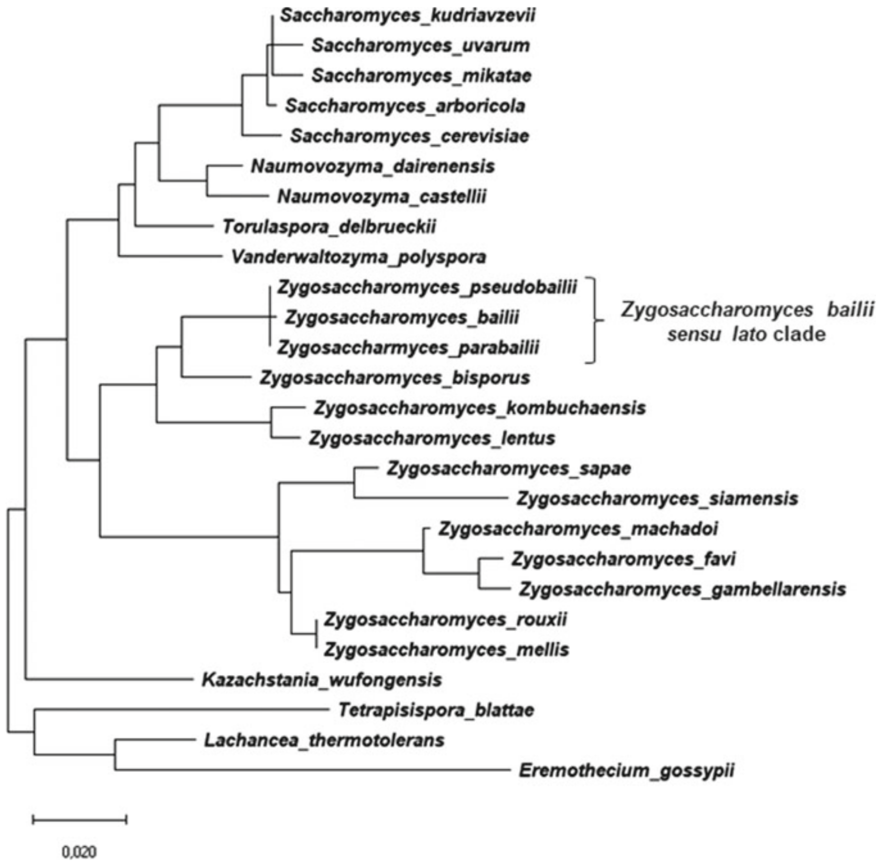

Fig. 4.1 Molecular phylogenetic analysis of *Zygosaccharomyces* species. The partial sequence of the large subunit (LSU) ribosomal RNA gene from 26 yeast species belonging to the *Zygosaccharomyces* genus and to other genus of Saccharomycetaceae family were used. LSU sequences from *Z. bailii* sensu lato clade species are those corresponding to the type strains described in Suh et al. (2013). Phylogenetic analysis was inferred by using the Maximum Likelihood method. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018)

Z. pseudobailii and *Z. parabailii* are names that were given to strains that were formerly considered to be *Z. bailii*, but which were more recently recognized to be species separate from *Z. bailii*, based on phylogenetic comparison of their nucleotide sequences in the internal transcribed spacer (ITS) and D1/D2 regions of rRNA (Suh et al. 2013). Furthermore, both *Z. parabailii* and *Z. pseudobailii* are hybrid species, having genomes twice as large as the genome of *Z. bailii* (Ortiz-Merino et al. 2017; Braun-Galleani et al. 2018). *Z. parabailii* ATCC 60483 was shown to be an interspecies hybrid strain that resulted from the mating of two parents (one of them being *Z. bailii*) that differed by 7% in their genome sequences (Ortiz-Merino et al. 2017). A similar process of interspecies mating produced *Z. pseudobailii* MT15, which also

has *Z. bailii* as one parent but its second parent is different from the second parent of *Z. parabailii*. Interspecies hybrids are in general asexual, replicating only mitotically. Remarkably, these interspecies *Zygosaccharomyces* hybrids were initially sterile, but in both cases, they regained fertility when one copy of their mating-type locus became damaged. Due to this phenomenon, *Z. parabailii* and *Z. pseudobailii* now behave as haploids, being able to go through mating-type switching and autodiploidization, followed by sporulation (Ortiz-Merino et al. 2017; Braun-Galleani et al. 2018). The existence of natural interspecific hybrids within the *Zygosaccharomyces* genus (Xu et al. 2017; Ortiz-Merino et al. 2017; Braun-Galleani et al. 2018; James et al. 2005; Mira et al. 2014) suggests that other species taxonomically related to *Z. bailii* may remain to be identified—in particular, pure strains representing the second parents of *Z. parabailii* and *Z. pseudobailii* have not yet been found. In summary, the *Z. bailii* sensu lato clade, which is also called the *Z. bailii* species complex, consists of three species (Fig. 4.1): *Z. bailii*, whose genome is approximately 10 Mb (Palma et al. 2017; Galeote et al. 2013), and two hybrid species *Z. parabailii* and *Z. pseudobailii* whose genomes are approximately 20 Mb (Ortiz-Merino et al. 2017; Braun-Galleani et al. 2018; Mira et al. 2014).

Prior to the recognition that the *Z. bailii* species complex contains three distinct species (Suh et al. 2013), and the more recent discovery that two of these species are hybrids, many publications reporting physiological, biochemical, and genome-wide studies used the name “*Z. bailii*” to refer to strains that are now known to be in fact *Z. parabailii* or *Z. pseudobailii*. The literature can therefore be confusing. For example, strain ISA1307 was referred to as *Z. bailii* in the phenomics (Rodrigues et al. 2001; Pina et al. 2004), quantitative proteomics (Guerreiro et al. 2012, 2016), and metabolomics (Rodrigues et al. 2012) studies summarized in Table 4.1. However, in 2014 when its genome was sequenced, strain ISA1307 was discovered to be an interspecies hybrid between *Z. bailii* and a closely related species (Mira et al. 2014) and following this publication, ISA1307 was called an interspecies hybrid in subsequent studies (Palma et al. 2015; Guerreiro et al. 2016). In 2017, strain ISA1307 was identified as *Z. parabailii* (Ortiz-Merino et al. 2017). In order to update and clarify the information available in the literature, in this chapter, we will consider the most recent taxonomic classification of the strains examined whenever we have that information. Also for these reasons, we have chosen to state systematically the strain associated to each species and published study. The terms “*Z. bailii* sensu lato” (meaning “in a broad sense” and abbreviated *s. l.*) and “the *Z. bailii* species complex” are synonyms, and they both refer to the group of three species (*Z. bailii*, *Z. parabailii*, and *Z. pseudobailii*).

4.3 Ecology of *Z. bailii* sensu lato Species

Yeasts of the genus *Zygosaccharomyces* are widely distributed through different ecological niches ranging from high osmotic pressure environments to raw, preserved or fermented foods and beverages (James and Stratford 2011; Sá-Correia et al. 2014).

Table 4.1 Physiological genomics studies performed in *Z. bailii* sensu lato clade species

Z. bailii clade species	Strain	Description of the study	Reference
Z. bailii (Type strain: ATCC 58445^T = CBS 680^T = NCYC 1416^T = NRRL Y-2227^T = CLIB213^T)	NCYC 1427	Construction of a genomic library of strain NCYC 1427 and its transformation in *S. cerevisiae (MAT*a *ura3-52, his3-Δ200, leu2-Δ1, trp1-Δ63, pdr12-ΔhisG)* allowed the identification of *ZbURA3* and *ZbTRP1*	Mollapour and Piper (2001a)
	CBS 7555	Lipidomic profiling of the major lipid species found in the plasma membrane in *S. cerevisiae* (CEN.PK 113_7D) and *Z. bailii* (CBS 7555) cultured with acetic acid	Lindberg et al. (2013)
	IST302	Study of the transcriptional alterations occurring during early response to acetic acid or copper mediated by ZbHaa1 by mRNA-seq	Antunes et al. (2018)
Z. parabailii (Type strain: ATCC 56075^T = NBRC 1047^T = NCYC 128^T = CBS 12809^T)	ISA1307	Construction of a genomic library of strain ISA1307 and its transformation in *S. cerevisiae* W303-1A (*MAT*a *ade2*-1 *his3*-11, 15 *ura3*-1 *leu2*-3, 112*trp1*) allowed the identification of *ZbLEU2*	Rodrigues et al. (2001)
		Transformation of the genomic library of strain ISA1307 for functional complementation of an *S. cerevisiae* hxt-null strain (EBY.VW4000) led to the identification of *Z. bailii* fructose transporter Ffz1	Pina et al. (2004)
		Transformation of the genomic library of strain ISA1307 in the highly acetic acid susceptible mutant *S. cerevisiae* BY4741 *haa1*Δ allowed the identification of several determinants of tolerance to acetic acid	Palma et al. (2015)
		The pathways of acetic acid metabolism in cells of strain ISA1307 were identified in vivo by carbon-13 (^{13}C) nuclear magnetic resonance spectroscopy and ^{14}C incorporation experiments	Rodrigues et al. (2012)
		The alterations in the protein content in response to sudden exposure or during exponential growth in the presence of an inhibitory sublethal concentration of acetic acid were examined by quantitative two-dimensional electrophoresis (2DE)	Guerreiro et al. (2012)
		The alterations in the expression of mitochondrial proteins in cells exposed to lethal concentrations of acetic acid were assessed by quantitative two-dimensional electrophoresis	Guerreiro et al. (2016)
	ATCC 36947	Construction of a genomic library of strain ATCC 36947 and its transformation in *S. cerevisiae* GRF18U (*MAT*α, leu2-3,112, his3-11, 15, ura3) allowed the identification of *ZbHIS3*	Branduardi (2002)
	ATCC 60483	Investigation of the transcriptional alterations occurring in response to lactic acid by mRNA-seq	Ortiz-Merino et al. (2018)
Z. pseudobailii (Type strain: ATCC 56074^T = CBS 2856^T = NBRC 0488^T)	MT15	Study of the transcriptomic features of flavor metabolism in strain MT15 under heat stress at 30 and 37 °C by mRNA-seq	Xu et al. (2017)

Z. bailii, *Z. parabailii*, and *Z. pseudobailii* share the same natural habitat and exhibit similar physiological traits, this being one of the reasons why the three species are hard to distinguish using conventional physiological tests (Suh et al. 2013). *Z. bailii* (*s.l.*) species are often found in acidified, preserved foods, containing or not high concentrations of fermentable sugars, such as fruit juices, fruit syrups, jams, honey, wine and wine must, vinegar, pickles, soft drinks, and salad dressings (James and Stratford 2011; Thomas and Davenport 1985; Loureiro and Malfeito-Ferreira 2003; Suh et al. 2013; Sá-Correia et al. 2014). Nevertheless, apart from its presence in food and beverage industrial settings, the natural habitats of the species in *Z. bailii* complex are dried fruits, gummy exudates from fruit trees, vineyards and orchards (Thomas and Davenport 1985; Sá-Correia et al. 2014).

The distribution of *Z. bailii* (*s.l.*) species in nature and their remarkable spoilage capacity result from a combination of several physiological and biochemical traits that characterize these species. *Z. bailii* (*s.l.*) species can tolerate ethanol concentrations above 15% (v/v) (Thomas and Davenport 1985), pH in the range of 2.0–7.0 and a_w in the range of 0.80–0.99 (James and Stratford 2003; Martorell et al. 2007), high concentrations of glucose (72% (w/v)) and to vigorously ferment fructose and glucose (Martorell et al. 2007). Nevertheless, one of the physiological hallmarks of *Z. bailii* (*s.l.*) is its remarkable tolerance to the weak acids that are used as food preservatives, as it is the case of acetic, benzoic, sorbic, and lactic acids, even at values above those allowed by some food legislations (Stratford et al. 2013; European Comission 2011). For example, the limit concentrations approved for sorbic and benzoic acids utilization as food preservatives range, in general, from 0.5 to 2 g/L, depending on the food product (European Comission 2011) while the average minimum inhibitory concentration (MIC) determined for several *Z. bailii* strains is approximately 8 and 10 g/L (at pH 4.0) for sorbic and benzoic acids, respectively (Stratford et al. 2013). Concerning the use of acetic and lactic acids as food preservatives, the concentration approved for both acids is in general *quantum satis* (European Comission 2011), which means that these acids should be used in food products under conditions that do not result in consumer's deception. Remarkably, *Z. bailii* and *Z. parabailii* tolerate concentrations of acetic acid and lactic acid which are, in average, 1.5- to 3-folds higher than those determined for *S. cerevisiae* (Mira et al. 2014; Guerreiro et al. 2012; Stratford et al. 2013).

4.4 *Z. bailii* sensu lato are Fructophilic Yeasts

Species of *Z. bailii* complex have a fructophilic metabolism, that is, have a preference for fructose over glucose when both sugars are available in the environment (Sousa-Dias et al. 1996; Cabral et al. 2015). The fructophilic behavior of *Z. parabailii* ISA1307 was demonstrated by comparing fructose and glucose uptake kinetics (Pina et al. 2004; Sousa-Dias et al. 1996). Although the two hexose sugars are consumed simultaneously by *Z. parabailii* ISA1307, the overall hexose transport shows a higher capacity (higher Vmax) for fructose than for glucose (Sousa-Dias et al. 1996). This

fact was explained by the identification of two plasma membrane hexose transport mechanisms. The first is a low-capacity and high-affinity glucose uptake system that is also able to transport fructose; when the two sugars are available, fructose promotes the inactivation of the glucose transporter, thereby preventing the utilization of glucose (Sousa-Dias et al. 1996). The second is the high-capacity and low-affinity fructose facilitator Ffz1, specific for fructose (Pina et al. 2004; Sousa-Dias et al. 1996). The access to the genome sequences of *Z. bailii (s.l.)* species allowed the identification of several genes putatively involved in the uptake of hexoses (Ortiz-Merino et al. 2017; Mira et al. 2014; Palma et al. 2017; Galeote et al. 2013). Five genes homologous to *S. cerevisiae* hexose transporters (HXTs) (Wieczorke et al. 1999) and one gene homologous to the fructose facilitator *FFZ1* were identified in the genome sequence of *Z. bailii* strains IST302 and CLIB213^{T} (Palma et al. 2017; Galeote et al. 2013). In *Z. parabailii* strains ISA1307 and ATCC 60483, the number of hexose transporter encoding genes is higher due to their hybrid nature (Ortiz-Merino et al. 2017; Mira et al. 2014). Interestingly, Ffz1 transporter was found to be phylogenetically distant from the yeast H^+-fructose symporters Fsy1 of *S. carlsbergensis* (Gonçalves et al. 2000) and Frt1 of *Kluyveromyces lactis* (Diezemann and Boles 2003), and from *S. cerevisiae* Hxt-like hexose facilitators (Wieczorke et al. 1999), all belonging to the sugar porter family (Palma et al. 2007). Ffz1 is phylogenetically close to transporters belonging to the drug/H^+-antiporter family 1 (DHA1) (Leandro et al. 2011), although it holds conserved sequence motifs not present in this family, neither in the sugar porter family (Leandro et al. 2011).

The existence of two energy-independent fructose uptake systems confers to *Z. bailii (s.l.)* species a physiological and adaptive advantage over other yeast species in fructose-rich food products (Sousa-Dias et al. 1996). When containing sucrose, food products are also susceptible to contamination by species of the *Z. bailii* complex because some strains are able to produce an extracellular invertase that hydrolyses sucrose into glucose and fructose (James and Stratford 2011; Arez et al. 2014) and sucrose hydrolysis is also possible at the low pH environments where *Z. bailii* (*s.l.*) species thrive.

4.5 Physiological Genomics of *Z. bailii* sensu lato

Despite the recognized interest in understanding the mechanisms underlying *Z. bailii* (*s.l.*) spoilage capacity, the lack of appropriate genetic engineering tools and genomic information has delayed the investigation of the molecular players associated with their food spoilage capacity until the last decade. This contrasted with the situation for *S. cerevisiae* whose complete genome annotated sequence was released in 1996 (Goffeau et al. 1996). This knowledge paved the way to the development and exploitation of genome-wide analyses that made *S. cerevisiae* an instrumental eukaryotic model in the field of toxicogenomics, as well as cell factory for the production of added-value chemicals and biofuels (dos Santos and Sá-Correia 2015). The function of a large part of *S. cerevisiae* genes has been thoroughly characterized

over the years and Omics approaches were exploited and combined with metabolic engineering strategies. Therefore, the model yeast has been at the forefront of several genome-wide approaches aiming at understanding yeast adaptation and tolerance to several toxicants and other environmental stresses (dos Santos and Sá-Correia 2015).

In the case of the food spoilage yeasts of the *Z. bailii* species complex, genome-wide studies were hindered for years due to the lack of sequenced genomes. However, prior to the release of *Z. bailii* and *Z. parabailii* annotated genome sequences, genomic libraries from these two species were constructed and used for the isolation, identification, and functional analysis of a number of genes (Table 4.1) (Palma et al. 2015; Rodrigues et al. 2001, 2004; Pina et al. 2004; Mollapour and Piper 2001a; Branduardi 2002). For instance, the fructose transporter Ffz1 was discovered by functional complementation of an *S. cerevisiae* strain whose hexose transport is fully impaired with a *Z. parabailii* ISA1307 genomic library (Pina et al. 2004). The identification of *Z. bailii* essential genes involved in the biosynthesis of histidine (Branduardi 2002), uracyl and tryptophan (Mollapour and Piper 2001a), and leucine (Rodrigues et al. 2001) was also performed by the functional complementation of *S. cerevisiae* auxotrophic mutants for these compounds using *Z. bailii* NCYC 1427 and *Z. parabailii* strains ISA1307 and ATCC 36947 cDNA libraries. The identification of these amino acid biosynthetic genes was an essential step for the construction of *Z. bailii* or *Z. parabailii* auxotrophic strains (Dato et al. 2010).

The release of the genome sequences of *Z. bailii* strains CLIB213^T (Galeote et al. 2013) and IST302 (Palma et al. 2017), of *Z. parabailii* strains ISA1307 (Mira et al. 2014) and ATCC 60483 (Ortiz-Merino et al. 2017), and of *Z. pseudobailii* MT15 (Xu et al. 2017; Braun-Galleani et al. 2018) led to the recent emergence of physiological genomics studies, in particular envisaging the investigation of the global mechanisms underlying the adaptation and tolerance to weak acids in *Z. bailii* sensu lato species (Table 4.1).

The *Z. bailii* strain IST302 proved to be amenable to genetic engineering and physiological studies because it does not form cell aggregates and is easy to transform, contrary to the type strain CLIB213^T (Palma et al. 2017). Therefore, the sequencing and annotation of *Z. bailii* IST302 haploid genome were considered an important step forward to allow physiological genomics studies in this species (Palma et al. 2017). *Z. bailii* IST302 was recently used to identify genome-wide transcriptional alterations occurring in this yeast species during early response to acetic acid or copper (Antunes et al. 2018), as detailed in the next section. Transcriptomics studies were also recently performed in the other two species of the *Z. bailii* complex. The transcriptional alterations occurring in *Z. parabailii* ATCC 60483 cells cultured in a medium with glucose plus inhibitory concentrations of lactic acid highlighted the importance of genes related to respiratory functions and oxidative stress defense in the response to that weak acid (Ortiz-Merino et al. 2018). The comparative transcriptomic analysis of *Z. pseudobailii* MT15 fermentation of Chinese *Maotai*-flavor liquor at different temperatures was also useful to reveal the genes potentially involved in the production of specific flavor compounds (Xu et al. 2017).

Based on the functional complementation of the *S. cerevisiae* acetic acid susceptible mutant *haa1*Δ (*HAA1* codes for the major transcription factor for adaptation and

tolerance to acetic acid) with a *Z. parabailii* ISA1307 genomic library, it was possible to identify genes that are determinants of tolerance to acetic acid (Palma et al. 2015). Among them are genes related to cellular transport and transport routes, protein fate, protein synthesis, amino acid metabolism and transcription (Palma et al. 2015). A quantitative proteomics analysis based on two-dimensional gel electrophoresis (2-DE) also contributed to the identification of the alterations occurring in the protein content of *Z. parabailii* ISA1307 in response to sudden exposure to acetic acid or during exponential adapted growth in the presence of acetic acid and glucose (Guerreiro et al. 2012). The increased content of proteins involved in carbohydrate metabolism (Mdh1, Aco1, Cit1, Idh2, and Lpd1) and energy generation (Atp1 and Atp2) indicates that, when glucose is also present, acetate is channeled into the tricarboxylic acid cycle, being co-consumed with the sugar. These results were corroborated by in vivo ^{13}C-NMR spectroscopy studies of acetic acid fate during *Z. parabailii* ISA1307 growth in single (acetic acid) or mixed (glucose and acetic acid) substrates (Rodrigues et al. 2012). A quantitative proteomic analysis focused on mitochondrial proteins of *Z. parabailii* ISA1307 cells exposed to lethal concentrations of acetic acid highlighted the importance of mitochondrial energetic metabolism in acetic acid-induced regulated cell death response (Guerreiro et al. 2016).

The profiling of the major lipid species found in the plasma membrane of exponentially growing cells of *Z. bailii* CBS 7555 and *S. cerevisiae* CEN.PK 113_7D, under control and acetic acid stress conditions, highlighted the correlation established between the higher basal levels of complex sphingolipids in *Z. bailii* when compared to *S. cerevisiae*, and the consequent reduced plasma membrane permeability to acetic acid in the highly tolerant *Z. bailii* species (Lindberg et al. 2013).

The mechanisms underlying adaptation and tolerance to weak acids in species of *Z. bailii* sensu lato clade, recently revealed by Omics-based analyses in combination with more classical physiological and biochemical studies, will be detailed in the next section.

4.6 Mechanistic Insights into *Z. bailii* sensu lato Adaptation and Tolerance to Weak Acids

4.6.1 Plasma Membrane Composition and Weak Acid Uptake

The antimicrobial potential of weak acids is determined by their hydrophobicity, volatility, and pK_a (Mira et al. 2010a). At an external pH below the weak acid pK_a value, the undissociated form of the acid prevails (RCOOH) and may passively diffuse across the cell membrane lipid bilayer (Fig. 4.2a) (Piper et al. 2001). At an external pH above the weak acid pK_a, two types of carriers have been described in *Z. parabailii* ISA1307: (i) a constitutive carrier specific for acetate and (ii) a glucose-repressible acetate proton symporter that is also able to uptake propionate and formate, but not lactate or pyruvate (Sousa et al. 1996, 1998) (Fig. 4.2a).

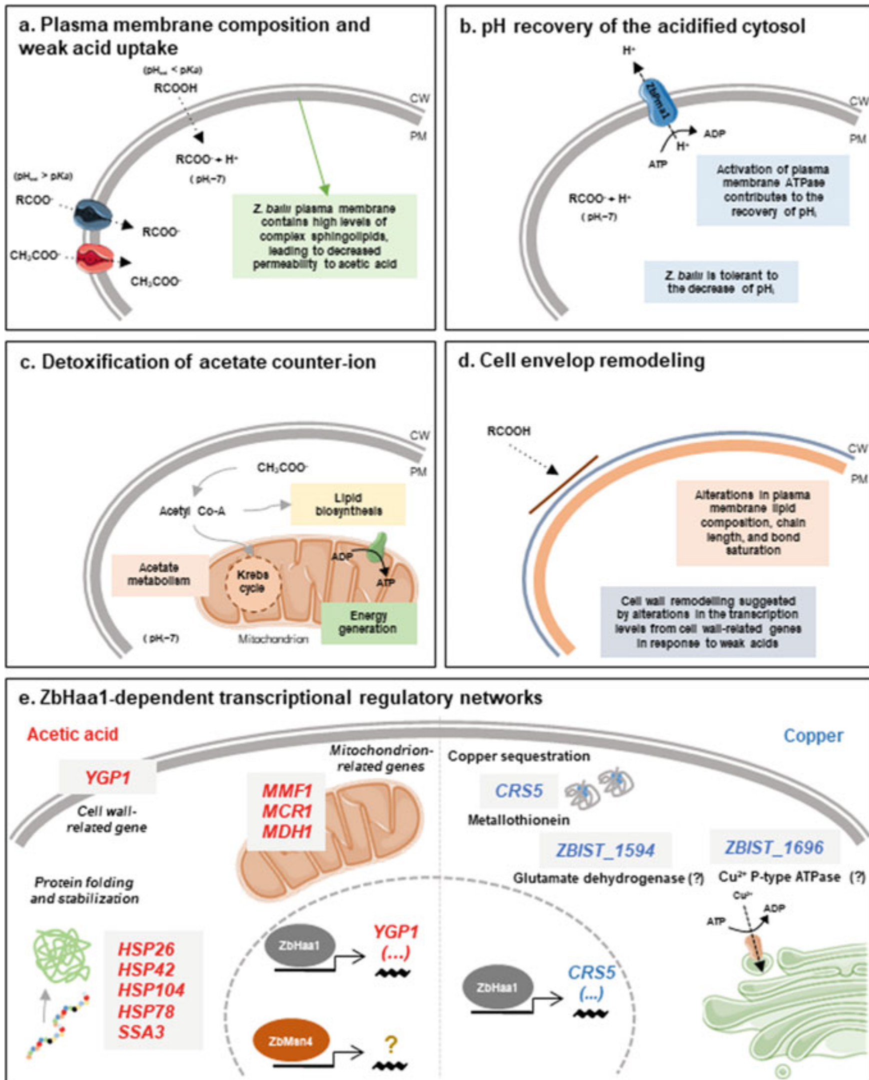

Fig. 4.2 **Schematic model for the mechanisms underlying adaptation and tolerance to weak acids in *Z. bailii*** sensu lato. **a** Plasma membrane composition and weak acid uptake; **b** pH recovery of the acidified cytosol; **c** detoxification of acetate counterion; **d** cell envelope remodeling; **e** ZbHaa1-dependent transcriptional regulatory networks. Details are provided in the text. pH_{ext}, external pH; pH_i, intracellular pH; PM, plasma membrane; CW, cell wall

It has been demonstrated that plasma membrane lipid composition has an important role in the diffusion of acetic acid across the plasma membrane. A lipidomic profiling of the major lipid species found in the plasma membrane revealed significant differences in their cellular lipid content in *Z. bailii* CBS 7555 cells compared with *S. cerevisiae* (Lindberg et al. 2013). Although the levels of total glycerophospholipids were similar in both species, a higher phosphatidylinositol content and a slightly lower phosphatidylethanolamine content was registered in *Z. bailii* (Lindberg et al. 2013). Moreover, the glycerophospholipid chain length was, in general, two carbons longer in *Z. bailii*, and the fatty acid chains contained double unsaturations that were not found in *S. cerevisiae* (Lindberg et al. 2013; Pilkington and Rose 1989). However, the most remarkable difference between *Z. bailii* and *S. cerevisiae* plasma membrane lipids was the higher basal level of complex sphingolipids found in *Z. bailii*. These results lead to the suggestion of a link between high sphingolipid levels in plasma membrane and the intrinsic remarkable tolerance of *Z. bailii* to acetic acid (Lindberg et al. 2013). The correlation between the sphingolipids content and membrane permeability to acetic acid was further investigated and confirmed based on in silico simulations of model membranes (Lindahl et al. 2016). The plasma membrane lipid composition is altered during adaptation to acetic acid stress, as described in Sect. 4.6.4. Nevertheless, *Z. bailii* extraordinary ability to tolerate high concentrations of weak acids does not only rely on the highly effective plasma membrane barrier to the entry of weak acids into the cell, but other metabolic strategies contribute to its remarkable resilience to weak acids, as detailed below.

4.6.2 pH Recovery of the Acidified Cytosol

Once inside the cell, weak acids dissociate at the near-neutral pH of the cytosol leading to the accumulation of the corresponding counter-anions ($RCOO^-$) and protons (H^+), the accumulation of protons leading to intracellular acidification (Fig. 4.2b). Yeast plasma membrane H^+-ATPase, an ATP-driven proton efflux pump, is responsible for the extrusion of protons caused by weak acid dissociation, being activated in *S. cerevisiae* upon exposure to weak acid stress (Piper et al. 2001; Holyoak et al. 1996; Viegas and Sá-Correia 1991; Carmelo et al. 1997). The activation of the plasma membrane H^+-ATPase activity was demonstrated also in *Z. bailii* NCYC 563 under benzoic acid stress (Macpherson et al. 2005), although the abundance of this plasma membrane protein is maintained under weak acid stress (Piper et al. 2001). *Z. bailii* was suggested to better tolerate a short-term decrease of intracellular pH (pH_i) compared with *S. cerevisiae* (Arneborg et al. 2000). A significant pH_i drop was observed during *Z. bailii* exponential phase of growth in the presence of acetic acid, glucose and fructose, being later restored at the stationary phase (Dang et al. 2012). Although this response is representative of the global cell population, individual *Z. bailii* cells exposed to weak acids were found to exhibit variable tolerance to a number of weak acids, specifically to acetic, sorbic and benzoic acids (Stratford et al. 2013; Steels et al. 2000). The most tolerant cell subpopulation exhibits the lower pH_i values

(Stratford et al. 2013; Steels et al. 2000), which implicates a reduced intracellular dissociation of the weak acid and consequently, a reduced accumulation of the counterion in the cytosol, thus implicating a decreased susceptibility to any weak acid (Stratford et al. 2013). Therefore, the high tolerance of *Z. bailii* to weak acids is not mainly dependent on the specific structure of the acid, but apparently relies on a general mechanism related to decreased uptake and/or accumulation of the weak acid in the cell (Stratford et al. 2013).

4.6.3 Detoxification of the Weak Acid Counterion

There are apparently important differences between *Z. bailii* (*s.l.*) species and *S. cerevisiae* with regard to the mechanisms used by each species to avoid the accumulation of weak acid counterions. In *S. cerevisiae*, the intracellular accumulation of inhibitory concentrations of weak acids causes an increase in turgor pressure, oxidative stress, protein aggregation, lipid peroxidation, inhibition of membrane trafficking processes, and alterations of the spatial organization of membranes (Reviewed by Mira et al. (2010a), Piper et al. (2001), Palma et al. (2018)). *S. cerevisiae* strategy to reduce the intracellular pool of weak acid counterions relies on the expression of several specific transporters, involved in multidrug/multixenobiotic (MD/MX) resistance (Piper et al. 1998; Holyoak et al. 1999; Fernandes et al. 2005; Kawahata et al. 2006; Mira et al. 2010b; Tenreiro et al. 2002). To date, no active transporter was implicated in the expulsion of weak acid counterions in species of the *Z. bailii* complex and the single homologue of *S. cerevisiae* putative acetate exporters *TPO2* and *TPO3* was found to be downregulated under acetic or lactic acid stresses in *Z. bailii* and in *Z. parabailii*, respectively (Antunes et al. 2018; Ortiz-Merino et al. 2018). Instead of actively exporting the weak acid counterion for detoxification, results gathered so far suggest that *Z. bailii* and *Z. parabailii* have the ability to oxidatively degrade several weak acids, using these compounds as carbon sources in the mitochondria (Mollapour and Piper 2001a, b; Sousa et al. 1998). For example, the expression of ZbYme2, the homologue of *S. cerevisiae* mitochondrial inner membrane protein Yme2, was implicated in the degradation of sorbate and benzoate (Mollapour and Piper 2001a, b). Moreover, the utilization of acetate by *Z. parabailii* as a carbon source was also demonstrated when glucose is also present in the growth medium (Guerreiro et al. 2012; Rodrigues et al. 2012). This trait is consistent with the threat posed by *Z. bailii* (*s.l.*) species in the spoilage of sugar-rich foods and beverages preserved using weak acids. Moreover, from the biological perspective, the poor susceptibility to the Crabtree effect distinguishes species of the *Z. bailii* complex from *S. cerevisiae* and other yeast species that, by virtue of catabolite repression, are unable to use alternative carbon sources when glucose is available (Gancedo and Gancedo 1986). The utilization and fate of acetate in the presence of glucose involves the expression of a non-glucose repressible acetic acid transporter controlled by the internal concentration of acetate and the activity of a partly glucose-repressed acetyl-CoA synthetase (Guerreiro et al. 2012; Rodrigues et al. 2012, 2004; Sousa et al. 1998). This regulatory control allows

the maintenance of a metabolic flux compatible with relatively low levels of intracellular acetate and, while glucose is catabolized through the glycolytic pathway, acetic acid is used as an additional source of acetyl-CoA both for lipid synthesis and Krebs cycle (Fig. 4.2c) (Rodrigues et al. 2012, 2004). Due to the co-metabolization of glucose and acetic acid, *Z. bailii* (*s.l.*) species benefit from an additional carbon source for respiratory metabolism, even under glucose-fermentative conditions (Rodrigues et al. 2012). An expression proteomics approach based on quantitative 2-DE revealed that several proteins involved in the Krebs cycle (Mdh1, Aco1, Cit1, Lpd1, and Idh2) have an increased content in *Z. parabailii* ISA1307 cells adapted and exponentially growing in the presence of acetic acid and glucose (Guerreiro et al. 2012). Interestingly, the genome-wide transcriptional profiling of *Z. bailii* IST302 early response to acetic acid showed that mRNA levels from *Z. bailii* homologues of *MDH1*, *ACO1* and *CIT1* increased after one hour of exposure of unadapted *Z. bailii* cells to acetic acid, and the active functioning of the Krebs cycle replenishes the oxidative phosphorylation process with reduced cofactors (Guerreiro et al. 2012; Antunes et al. 2018). Consistent with this idea, in *Z. parabailii* ISA1307 cells exponentially growing in glucose and acetic acid, an increase in the content of the mitochondrial ATP synthase subunits Atp1 and Atp2 involved in oxidative phosphorylation was found to occur (Guerreiro et al. 2012). Moreover, the registered increase of the mRNA levels from *Z. bailii* IST302 homologues of *ATP16* (subunit of the central stalk of mitochondrial F1F0 ATP synthase), *CYC1* (cytochrome c isoform 1), and *COR1* (core subunit of the ubiquinol-cytochrome c reductase complex) corroborates the hypothesis that acetate is channeled into energy generation processes (Antunes et al. 2018).

4.6.4 Cell Envelope Remodeling in Response to Weak Acids

The remodeling of the cell wall and plasma membrane composition and structure has been proposed as one of the most important mechanisms of adaptation and tolerance to weak acids in *Z. bailii* (*s.l.*) species, as reported for *S. cerevisiae* (Lindberg et al. 2013; Palma et al. 2018; Simões et al. 2006; Mollapour et al. 2009; Guerreiro et al. 2016; Godinho et al. 2018). The alteration of the cell wall and plasma membrane chemical structure and properties promotes the reduction of cellular envelope permeability, thereby decreasing the passive diffusion of weak acids. This strategy counteracts the futile cycle resulting from the active expulsion of the counterion through specific active transporters and the continuous uptake of the lipophilic form of the weak acid by passive diffusion occurring in *S. cerevisiae* (Piper et al. 2001).

Along with the high basal level of complex sphingolipids related with the intrinsic resistance of *Z. bailii* to acetic acid uptake, this species also has the ability to undergo major rearrangements in the composition, chain length, and bond saturation of plasma membrane lipids in response to acetic acid (Lindberg et al. 2013) (Fig. 4.2d). The levels of complex sphingolipids (Inositol phosphate-ceramide IPC, Mannosyl-inositol phosphate-ceramide MIPC, Mannosyl-di-inositol phosphate-ceramide $M(IP)_2C$) increase in both *Z. bailii* CBS 7555 and *S. cerevisiae*

CEN.PK 113_7D cells under acetic acid stress. The content of glycerophospholipids was found to be slightly lower in *Z. bailii* than in *S. cerevisiae*, but the degree of saturation of these molecules increased in *Z. bailii* cells adapted to acetic acid (Lindberg et al. 2013). On the other hand, ergosterol levels were not significantly altered in acetic acid-challenged *Z. bailii* cells (Lindberg et al. 2013), despite the recognized role that this lipid species has in *S. cerevisiae* response and tolerance to acetic acid-induced stress (Godinho et al. 2018). The higher saturation of long chain bases and the longer fatty acyl chains in sphingolipids were proposed as the alterations that make the plasma membrane thicker and less permeable (Lindberg et al. 2013; Pilkington and Rose 1989) and, therefore, more tolerant to acetic acid (Lindahl et al. 2016). Moreover, a reduction in the levels of phosphatidylcholine, one of the most abundant phospholipids in the plasma membrane, was observed during late exponential phase of *Z. parabailii* growth in the presence of lactic acid, leading to the suggestion that this reduction could be the cause for a more compact and less permeable plasma membrane (Kuanyshev et al. 2016).

Several genes involved in cell wall structure assembly and remodeling, or in the synthesis of cell wall polysaccharides are demonstrated determinants of tolerance, or transcriptionally responsive to weak acids, in *S. cerevisiae, Z. bailii* and *Z. parabailii* species, as indicated by several genome-wide studies (Palma et al. 2015, 2017; Antunes et al. 2018; Ortiz-Merino et al. 2018; Kawahata et al. 2006; Mira et al. 2010b, c; Kuanyshev et al. 2016; Desmoucelles et al. 2002; Simões et al. 2003; Abbott et al. 2007; Schüller et al. 2004). For instance, the *Z. parabailii* ISA1307 genes homologous to *S. cerevisiae PMT1* and *KTR7* were considered strong candidate determinants of acetic acid tolerance in this species (Palma et al. 2015). In *S. cerevisiae*, both *PMT1* and *KTR7* code for mannosyltransferases that have been described as key enzymes for protein glycosylation known to be essential for cell wall rigidity (Gentzsch and Tanner 1996; Lussier et al. 1997). The *Z. bailii* IST302 *YGP1* homologue that in *S. cerevisiae* codes for a cell-wall-related secretory glycoprotein (Destruelle et al. 1994) was also found to be upregulated in *Z. bailii* early response to acetic acid stress (Palma et al. 2017; Antunes et al. 2018). However, only for *S. cerevisiae* this gene was so far demonstrated to be a determinant of weak acid tolerance (Kawahata et al. 2006; Mira et al. 2010c; Abbott et al. 2007). Moreover, the mRNA levels from *Z. bailii* IST302 genes homologous to *S. cerevisiae* genes involved in cell wall modulation, in particular, *ANP1*, *ECM33*, and *HSP150* were also found to increase upon sudden exposure to acetic acid (Antunes et al. 2018). Nevertheless, several genes involved in cell wall remodeling were downregulated during *Z. parabailii* ATCC 60483 response to lactic acid (Ortiz-Merino et al. 2018). This effect was associated with the reported decrease in the levels of cell wall mannans, β-1 → 3 and β-1 → 6 glucans during *Z. parabailii* growth in the presence of lactic acid (Kuanyshev et al. 2016). Interestingly, *Z. bailii* homologues of *KRE6* and *KNH1* that in *S. cerevisiae* are involved in β-1 → 6 glucan synthesis were also found to be downregulated during the early response to acetic acid (Antunes et al. 2018). These results confirm that extensive alterations occur in the cell wall of *Z. bailii* and *Z. parabailii* upon exposure to acetic and lactic acid stress.

4.6.5 Transcriptional Regulatory Networks Governing the Response and Tolerance to Weak Acids

Weak acids, or any external insult, can trigger several cellular responses orchestrated by transcription factor-associated networks. Differently from *S. cerevisiae*, in *Z. bailii* (*s.l.*) species the regulatory mechanisms involved in the response and tolerance to weak acids are still poorly characterized (Piper et al. 2001; Palma et al. 2018; Teixeira et al. 2011). To date, only two transcription factors were demonstrated as being involved in the response and tolerance to acetic acid in *Z. bailii* and *Z. parabailii*: ZbMsn4 (Palma et al. 2015), the single homologue of *S. cerevisiae* Msn4 and Msn2 general stress response activators (Gasch et al. 2000); and ZbHaa1 (Palma et al. 2017), the homologue of *S. cerevisiae* transcription factor Haa1 required for the direct or indirect activation of 80% of the acetic acid-responsive genes (Mira et al. 2010b, c). The transcription factor ZbMsn4 from *Z. parabailii* ISA1307 was identified among the genes whose expression was able to suppress the high acetic acid susceptibility phenotype of *S. cerevisiae haa1*Δ mutant; the transformation of *ZbMSN4* from *Z. bailii* IST302 in *S. cerevisiae* single and double deletion mutants *msn4*Δ and *msn*2Δ*msn4*Δ was also able to suppress the acetic acid susceptibility phenotype in these mutants (Palma et al. 2015). However, the genes regulated by ZbMsn4 in *Z. bailii* response to weak acids are still unidentified. Likewise, ZbHaa1 was able to rescue the acetic acid susceptibility phenotype of *S. cerevisiae haa1*Δ and the disruption of *ZbHAA1* gene or the expression of an extra *ZbHAA1* copy in *Z. bailii* confirmed *ZbHAA1* as a determinant of acetic, benzoic and sorbic acids tolerance in this yeast species (Palma et al. 2017). In addition, ZbHaa1 (the single orthologue of *S. cerevisiae* Haa1 and Cup2) was demonstrated to have a role in metalloregulation, being involved in copper tolerance and copper-induced transcriptional regulation, a role associated to *S. cerevisiae* Cup2, but not to *S. cerevisiae* Haa1 (Palma et al. 2017). This evidence, together with the results from phylogenetic and gene neighborhood analyses have suggested that, after the whole-genome duplication event, the subfunctionalization of *Z. bailii* ancestral bifunctional protein Haa1/Cup2 originated *S. cerevisiae* Haa1 and Cup2 paralogues (Palma et al. 2017). The transcriptional alterations of *Z. bailii* IST302 early response to acetic acid or copper stresses mediated by ZbHaa1 were recently characterized by RNA sequencing (Antunes et al. 2018). This transcription factor was found to regulate approximately 26% of the genes activated upon sudden exposure to acetic acid stress in *Z. bailii* (Antunes et al. 2018). Among these genes, ZbHaa1 was found to activate the transcription of several genes homologous to *S. cerevisiae* genes involved in protein folding and stabilization (*HSP26*, *HSP42*, *HSP104*, *HSP78*, *SSA3*), and of *YGP1*, during the early response to acetic acid stress (Palma et al. 2017; Antunes et al. 2018) (Fig. 4.2e). Interestingly, *YGP1* and *HSP26* are also activated by Haa1 in acetic acid-challenged *S. cerevisiae* cells (Mira et al. 2010c). ZbHaa1 also activates mitochondrial gene homologues involved in different functions: *MMF1* (mtDNA maintenance); *MCR1* (mitochondrial NADH cytochrome b5 reductase); and *MDH1* (mitochondrial malate dehydrogenase).

Among the genes activated by ZbHaa1 in the early response to copper stress is the copper-binding metallothionein encoding gene *CRS5* (ORF ZBIST_3713) and the ORFs ZBIST_1696 and ZBIST_1594 putatively encoding a cation transport ATPase and a glutamate dehydrogenase Gdh3, respectively (Fig. 4.2e) (Palma et al. 2017; Antunes et al. 2018). ZbHaa1-dependent activation of, for example, the *CRS5* homologue (activated by Cup2 in *S. cerevisiae* upon copper stress) and of the *YGP1* homologue (activated by Haa1 in *S. cerevisiae* upon acetic acid stress) reinforces the concept of ZbHaa1 bifunctionality (Palma et al. 2017; Antunes et al. 2018).

4.7 Biotechnological Potential of *Z. bailii* sensu lato

Although species of the *Z. bailii* complex have been for years mainly regarded as a threat in the food industry, the biotechnological potential of these species is currently being considered due to the advantages associated with *Z. bailii* (*s.l.*) physiological traits. In fact, several studies have been conducted in order to (i) use their genetic patrimony for expression in *S. cerevisiae* in order to increase its tolerance to weak acids, (ii) examine their potential as cell factories for the production of organic acids, and (iii) explore them to reduce the ethanol content and increase the aroma complexity of alcoholic beverages.

The increase of *S. cerevisiae* tolerance to acetic acid has been in the spotlight of several biotechnological processes to increase their productivity and sustainability. In fact, acetic acid is a byproduct of alcoholic fermentation carried out by *S. cerevisiae* (Garay-Arroyo et al. 2004; Graves et al. 2006) that can achieve levels that when combined with high concentrations of ethanol and other toxic metabolites may lead to fermentation arrest or reduced ethanol productivity (Garay-Arroyo et al. 2004; Graves et al. 2006; Rasmussen et al. 1995). This tolerance phenotype is currently highly desired also because acetic acid is one of the most important inhibitory compounds present in lignocellulosic hydrolysates used for bioethanol production in the context of biorefineries (Jönsson et al. 2013). For all these reasons, genetic, metabolic, and evolutionary engineering of *S. cerevisiae* strains has been explored to increase the robustness of yeast industrial strains against acetic acid-induced stress in Industrial Biotechnology (reviewed by Palma et al. (2018)). The heterologous expression of *Z. bailii* IST302 genes *ZbMSN4*, *ZbTIF3*, or *ZbHAA1* (Palma et al. 2015, 2017) was found to increase *S. cerevisiae* tolerance to acetic acid. However, the relevance of this first attempt to improve acetic acid tolerance in *S. cerevisiae* still needs to be confirmed in industrial settings, as well as other *Z. bailii* and *Z. parabailii* candidate genes (Palma et al. 2015, 2017).

The remarkable tolerance to weak acids of *Z. bailii* (*s.l.*) species has brought to light the potential of these nonconventional yeast species as alternative cell factories for high-level production of weak acids, in particular, lactic acid (Sauer et al. 2004; Dato et al. 2010). L-lactic acid is a precursor of the bioplastic polymer polylactide (poly lactic acid, PLA), which is a bio-based and biodegradable polymer considered a valuable alternative to petroleum-derived polymers (Chen and Nielsen

2016). Lactic acid bacteria have been the main choice microorganisms for lactic acid production, but they require complex media and controlled pH to avoid bacterial metabolism inhibition thereby increasing the costs of lactic acid production and recovery (Vaidya et al. 2005). Due to yeast tolerance to low pH and ability to grow in synthetic media, they are promising alternatives for the production of pure lactic acid (Liu and Lievense 2005). *S. cerevisiae* does not hold the native pathway to produce lactic acid, but the deletion of pyruvate decarboxylase encoding genes and the heterologous expression of a lactate dehydrogenase allowed its engineering toward lactic acid production (Liu and Lievense 2005). Since such production demands highly weak acid-tolerant producing strains, *Z. parabailii* ATCC 60483 (the first *Z. parabailii* isogenic auxotrophic strain) was used to heterologously express the L-lactate dehydrogenase gene (LDH) from *Lactobacillus plantarum* (Dato et al. 2010). However, the concentration of lactic acid produced was similar to that produced by *S. cerevisiae* (Branduardi et al. 2006). The production and secretion of L-ascorbic acid were also attempted in *S. cerevisiae* and in *Z. parabailii* ATCC 60483 cells expressing simultaneously *S. cerevisiae ALO1* gene (D-Arabinono-1,4-lactone oxidase) and *Arabidopsis thaliana LGDH* gene (L-galactose dehydrogenase) (Sauer et al. 2004). Nevertheless, engineered *S. cerevisiae* produced higher concentrations of L-ascorbic acid when compared to recombinant *Z. parabailii* (Sauer et al. 2004). If increased levels of organic acids can be produced, species of the *Z. bailii* complex may become competitive microbial cell factories.

There is a recent growing interest in reducing the alcohol content of wines (reviewed in Varela et al. 2015; Querol et al. 2018). The sequential inoculation or the co-inoculation of non-*Saccharomyces* and *S. cerevisiae* yeasts have proven to be an efficient strategy (Contreras et al. 2014, 2015; Gobbi et al. 2014; Morales et al. 2015; Canonico et al. 2016; Englezos et al. 2016). Among the non-*Saccharomyces* yeast species used to reduce the alcohol content of wines, *Z. bailii* is considered a promising species to be used with *S. cerevisiae* in sequential fermentations (Contreras et al. 2015). Using chemically defined grape juice medium with aeration, the fermentation carried out by *Z. bailii* AWRI1578 and *S. cerevisiae* AWRI1631 led to the reduction of 2.0% (v/v) of the final ethanol content when compared to a fermentation where *S. cerevisiae* was used as a single starter (Contreras et al. 2015). When used as a single starter, *Z. bailii* anaerobic fermentation of two different grape juice media also led to a significant reduction in the ethanol yield by 4.1% when compared to *S. cerevisiae*; however, the organoleptic characteristics of the wine and the concentration of residual sugars were not considered (Gobbi et al. 2014).

It is widely accepted that non-*Saccharomyces* yeasts can positively influence the sensorial complexity of alcoholic beverages, having an impact on the primary (determined by the grape variety) and secondary (determined by the fermentation process) aroma through the production of enzymes and metabolites (Padilla et al. 2016; Ciani et al. 2010). *Z. bailii* BCV 08 was shown to produce high levels of ethyl esters (Garavaglia et al. 2014), which are the volatile compounds responsible for fruity and floral aromatic notes (Rojas et al. 2001), and selected as a potential candidate for use as a co-starter in wine fermentation (Garavaglia et al. 2015). Higher amounts of ethyl esters were produced during *Z. bailii* BCV 08 and *S. cerevisiae* co-fermentation (1:1

proportion of each species) under static conditions and at 28 °C (Garavaglia et al. 2015). The use of these two species as co-starters also produced higher amounts of polysaccharides than pure *S. cerevisiae* cultures (Domizio et al. 2011). The beneficial effect of polysaccharides in wine taste and body, aroma persistence, and protein and tartrate stability suggest that this co-fermentation may increase wine quality (Domizio et al. 2011).

Z. pseudobailii and *S. cerevisiae* are also found among the dominant species responsible for the fermentation of Chinese *Maotai*-flavor liquor, *Z. pseudobailii* being responsible for the production of various flavor compounds, including alcohols, acids, esters, aldehydes, and ketones during liquor fermentation (Xu et al. 2017; Wu et al. 2013). Given that *Maotai*-flavor liquor is produced at temperatures up to 37 °C, the comparison of *Z. pseudobailii* M15 genome-wide transcriptional alterations occurring at 30 and 37 °C and relation to flavor compounds produced at different temperatures suggested that the upregulation of genes involved in amino acid transport and metabolism at 37 °C may be responsible for increased flavor production at this temperature (Xu et al. 2017).

The contribution of *Z. bailii* and of other non-*Saccharomyces* species to the sensorial characteristics of alcoholic beverages may not be directly related with the production of a particular compound during fermentation, but with the production of specific enzymes. Research on the ability of different non-*Saccharomyces* yeasts to produce extracellular enzymes of enological relevance (β-glucosidases, pectinases, proteases, amylases, and xylanases) during fermentation has revealed that enzymatic activities are strain-dependent, with some *Z. bailii* strains showing β-glucosidase, pectinase and also cellulase activity (Escribano et al. 2017; Merín and Morata de Ambrosini 2015; Ganga and Martínez 2004).

4.8 Final Remarks

The characterization of *Z. bailii* (*s.l.*) species from the taxonomic, physiological, and genomic point of view and the elucidation of mechanisms of adaptation and tolerance to weak acids were possible during the last decade by exploring physiological genomics approaches. The development of molecular tools to genetically manipulate *Z. bailii* (*s.l.*) species, the identification of a haploid *Z. bailii* strain and the sequencing and annotation of *Z. bailii*, *Z. parabailii*, and *Z. pseudobailii* genomes are placing these nonconventional yeast species in a prominent position in Biotechnology research. The first studies envisaging the understanding of the transcriptional regulatory mechanisms associated with the response and adaptation to weak acids in *Z. bailii* (*s.l.*) species are emerging. It is anticipated that the availability of new strains of the *Z. bailii* complex more prone to genetic and laboratory manipulations and the development of new molecular genetic tools will boost the development of mutant strains and accelerate the characterization of transcriptional regulatory networks under environmental challenges. Efficient genome editing is also essential to explore the potential of these species in Industrial and Food Biotechnology. These

are essential directions to be taken in the future envisaging the understanding of physiological genomics aspects of species of *Z. bailii* complex in order to guide the effective control of these food spoilage yeasts or their sustainable use as microbial hosts for synthetic biology strategies.

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Chapter 5
Yeast Genome-Scale Metabolic Models for Simulating Genotype–Phenotype Relations

Sandra Castillo, Kiran Raosaheb Patil and Paula Jouhten

Abstract Understanding genotype–phenotype dependency is a universal aim for all life sciences. While the complete genotype–phenotype relations remain challenging to resolve, metabolic phenotypes are moving within the reach through genome-scale metabolic model simulations. Genome-scale metabolic models are available for commonly investigated yeasts, such as model eukaryote and domesticated fermentation species *Saccharomyces cerevisiae*, and automatic reconstruction methods facilitate obtaining models for any sequenced species. The models allow for investigating genotype–phenotype relations through simulations simultaneously considering the effects of nutrient availability, and redox and energy homeostasis in cells. Genome-scale models also offer frameworks for omics data integration to help to uncover how the translation of genotypes to the apparent phenotypes is regulated at different levels. In this chapter, we provide an overview of the yeast genome-scale metabolic models and the simulation approaches for using these models to interrogate genotype–phenotype relations. We review the methodological approaches according to the underlying biological reasoning in order to inspire formulating novel questions and applications that the genome-scale metabolic models could contribute to. Finally, we discuss current challenges and opportunities in the genome-scale metabolic model simulations.

Keywords Genome-scale metabolic model · Genotype–phenotype dependency · Yeast metabolism · Metabolic flux · Strain design

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5.1 Introduction to Genome-Scale Metabolic Models

Since the early distinction of genotypes from phenotypes (Johannsen 1911) life science research has sought for understanding their dependency. The dependency is inherently complex and dynamic. Single genotype may manifest several phenotypes (i.e., clonal heterogeneity) and different genotypes may translate to indistinguishable observable phenotypes. While the complete genotype–phenotype dependencies are challenging to resolve, metabolic phenotypes are moving within the reach through genome-scale metabolic model simulations. A genome-scale metabolic model is a description of the complete biochemical conversion potential encoded in an organism's genome as a network of reactions (Fig. 5.1). The stoichiometries of these reactions form mass conservation constraints of cellular metabolism. When a biological optimality principle (e.g., fast cell growth) is additionally introduced, a steady-state metabolic phenotype can be simulated using powerful linear programming solvers. Such simulations holistically consider cellular resource, energy, and redox requirements for biochemical synthesis. A myriad of applications has been derived from the original undecorated phenotype simulation. The applications vary from simulating metabolic genotype–phenotype dependencies for finding cancer drug targets to designing genotype manipulations for achieving desired phenotypes in microbial hosts for industrial biotechnology needs.

Yeasts, unicellular eukaryotes, are suitable hosts for industrial biotechnology owing to their robustness against harsh growth environments, established genetic engineering tools for several species, and eukaryotic protein modification. They have scientific relevance also as simpler model system for higher cells and some yeasts are pathogenic causing difficult infections. Furthermore, yeasts,

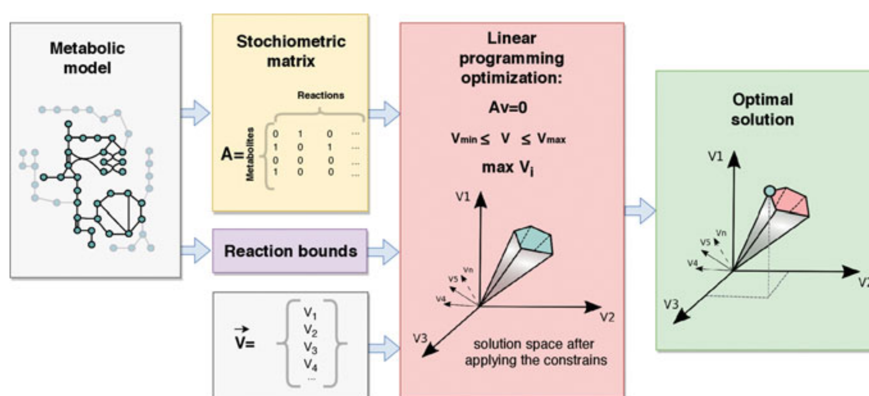

Fig. 5.1 Metabolic capacity of cells represented as a network of reactions or further as stoichiometric matrix allows simulations of metabolic phenotypes using linear programming. Metabolic steady-state assumption renders the system of metabolite mass balances linear. Reaction capacity and thermodynamic constraints can be included and limit the space of feasible metabolic phenotypes (i.e., metabolic fluxes)

Saccharomyces cerevisiae, in particular, have been domesticated for food and beverage fermentations and baking already since ancient times. While *S. cerevisiae* is by far the most well studied and broadly used yeast in applications, several other species attract considerable interest as well. For instance, *Pichia pastoris* is a widely used protein production host, *Kluyveromyces lactis* is known for beta-galactosidase synthesis, *Yarrowia lipolytica* is an oleaginous yeast attractive for lipid production, *Scheffersomyces stipitis* is a naturally xylose-utilizing yeast, and pathogenic yeasts *Candida tropicalis* and *Candida glabrata* cause difficult infections urging for more efficient treatments to be developed. The variety of yeast species of scientific and application interest can be expected to broaden following the rise of CRISPR/Cas9 and other generally applicable genetic engineering tools such as synthetic expression system universal for fungi (Rantasalo et al. 2018). Genome sequences are already available for a large variety of yeasts. Reference genomes for 98 yeast species are available from NCBI (www.ncbi.nlm.nih.gov/genome).

5.1.1 Genome-Scale Metabolic Model Reconstruction

Genome sequence is the starting point for reconstructing a genome-scale metabolic model. Semi-automatic reconstruction methods are available for building the first drafts of genome-scale metabolic models from the genome sequences (Swainston et al. 2011; Agren et al. 2013; Pitkänen et al. 2014; Castillo et al. 2016; Dias et al. 2015). The quality of draft reconstructions after the semi-automatic processes is strongly dependent on the comprehensiveness and quality of the source reaction database used. The reaction database has to contain links from the reactions to corresponding gene/protein sequences either within the database or by proving adequate identifiers such as EC numbers for external mapping. Reactions need to essentially be atom balanced for mass conservation in the reconstructed model. Popular reaction databases for genome-scale metabolic model reconstruction include Kegg (Kanehisa et al. 2017), Rhea (Morgat et al. 2017), MetaCyc (Caspi et al. 2014), BiGG (Schellenberger et al. 2010), and Reactome (Fabregat et al. 2018). A confidence score for the presence of a reaction from the reaction database in the metabolic repertoire of the species is derived by most of the semi-automatic reconstruction methods. Then, the high scoring reactions are pulled to the model after which gap filling algorithms are used for introducing lower scoring reactions that are essential for the *in silico* synthesis of biomass. Gap filling benefits greatly from experimental data on the growth of the species under different nutrient environments (Tramontano et al. 2018). Alternatively, to the two-phase process of introducing high scoring reactions followed by gap filling for a functional model, a single step process of carving out the organism-specific metabolic network from a universal gapless model (CarveMe) has recently been proposed (Machado et al. 2018). When the universal model is well curated, simulatable species-specific models are fast to reconstruct using CarveMe (Machado et al. 2018). Further, using a universal model standardizes the quality of input reaction data for reconstructing different species models.

However, there are also other sources of uncertainty in the model reconstruction such as the quality of the genome and the annotations, and the availability of similar annotated sequences in databases. Given the data, several models of a species could score equally well in the automatic reconstruction. Therefore, an approach has been suggested for simulating an ensemble of equally likely models simultaneously instead of a single reconstruction (Biggs and Papin 2017). Yet, evaluating the quality of models reconstructed for less well-studied non-model species is challenging. The reconstruction algorithms themselves can be evaluated against manually curated models and experimental data on model organisms such as metabolic gene knockout phenotypes. Metabolic gene knockout phenotypes can be simulated using the gene annotations of the models. The genes are annotated to the reactions whose catalyzing enzymes they encode. Preferably, the gene annotations include also Boolean rules describing whether the genes annotated to the reaction encode isoenzymes (i.e., OR rule) or whether they form a complex whose all components are required for activity (i.e., AND rule). Thereby, the Boolean rules allow propagating the genetic state into reaction activity state for performing mutant phenotype simulations. Simulated mutant phenotypes can be compared against experimental deletion mutant phenotypes for validating models. Though many metrics have been proposed for assessing the quality of reconstructed models (Sanchez and Nielsen 2015; Lopes and Rocha 2017), experimental growth and phenotype data are necessary for true evaluation (Tramontano et al. 2018).

5.2 Yeast Genome-Scale Models

Several genome-scale metabolic models have been reconstructed for *S. cerevisiae* during the last 15 years. The first *S. cerevisiae* model was created in 2003 by Föster et al. 2003 and was named iFF708 after the main developers and the number of genes supporting the reactions in the model. Slightly different and variable numbers of genes were annotated to metabolic reactions in the three following *S. cerevisiae* models (iND750, iLL672, and iIN800) derived directly from iFF708. Creating the first consensus model for *S. cerevisiae* was a collaborative effort. It was built on the iLL672 and iMM904 models (derived from iND750 model) and published in 2008 (Herrgård et al. 2008). After several updates of, in particular, lipid metabolism and transport reactions, the consensus model version 7 was published in 2013 by Aung et al. (2018). Since then the consensus yeast model has gone through several smaller updates (https://github.com/SysBioChalmers/yeast-GEM). Heavner and Price (2015) compared the 12 (*S. cerevisiae*) metabolic models created from 2003 until 2015. Though the coverage (i.e., number of genes annotated) and predictive power (i.e., in terms of gene essentiality predictions) had increased over time, the coverage of the models does not always correlate with the predictive ability. Extensive models annotating higher number of genes do not necessarily have better essentiality prediction capabilities than simpler ones. Introducing additional minor activity encoding genes may decrease the predictive capacity if the encoded enzymes cannot

alone sustain the corresponding reactions (Pereira et al. 2016). However, in addition to using the models for predictive simulations of genotype–phenotype translation, the genome-scale metabolic models can also be seen as knowledge bases containing all known biochemical conversion potential of the organism. Including the minor activity encoding genes and the corresponding reactions in a model are valuable for a knowledge base or a biochemical interaction network use. In conclusion, the several genome-scale metabolic models of *S. cerevisiae* have been developed and evolved independently for different purposes and none of them is generally the best.

Genome-scale metabolic models have been reconstructed, and manually curated, also for other yeasts than *S. cerevisiae* (Fig. 5.2). The models have commonly been reconstructed in a comparative manner using an *S. cerevisiae* model as a template. The reconstruction tool RAVEN especially supports the comparative reconstruction using an *S. cerevisiae* and CoReCo exploits species relatedness in scoring the reactions (Pitkänen et al. 2014). The models for industrially relevant species *K. lactis*, *P. pastoris*, *S. stipitis*, and *Y. lipolytica*, and for pathogenic *C. glabrata* have been derived using *S. cerevisiae* models as templates. For pathogenic *C. tropicalis* and for

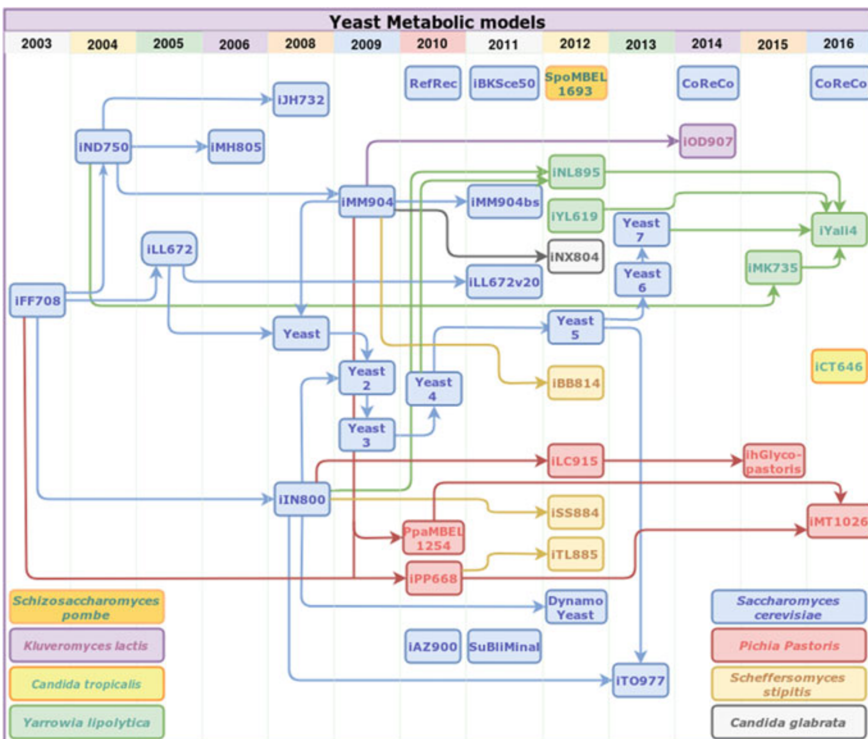

Fig. 5.2 Time line of genome-scale metabolic models for yeasts. Information partially extracted from (Sanchez and Nielsen 2015; Lopes and Rocha 2017). The arrows start from the template models used in reconstruction

scientifically relevant *S. pombe* model reconstructions no *S. cerevisiae* framework has been reported. In addition, a large set of draft fungal models, including yeast models, reconstructed using CoReCo (Pitkänen et al. 2014; Castillo et al. 2016) are available in the BioModels database (Chelliah et al. 2015). In addition to the BioModels database and the developer's specific sites, genome-scale metabolic models for various species can be downloaded from other public databases such as BiGG database (http://bigg.ucsd.edu/) (King et al. 2016).

5.3 Methods for Metabolic Phenotype Simulations Derived from Flux Balance Analysis (FBA)

A myriad of methods for performing phenotype simulations using genome-scale metabolic models derived from Flux Balance Analysis (FBA) (Varma and Palsson 1994). FBA solves a linear programming problem of optimizing biologically relevant objective function (typically growth) under metabolic steady-state mass conservation, enzyme capacity, and thermodynamic constraints. Steady-state assumption implies that the intracellular metabolite concentrations are constant (i.e., their time derivatives are zero). Thus, the steady-state assumption renders the problem linear (Fig. 5.2) and eliminates the need to describe the reaction kinetics that are functions of reactant abundances often with several unknown parameters. The steady-state assumption linearizing the problem is well justified for many metabolic states. Particularly well the steady-state assumption holds when microbial cells divide unlimited by the external conditions or grow in continuous cultivations under constant conditions. Under these conditions, FBA-optimized growth yields have been found to closely match experimental observation in microbial species (Edwards et al. 2001). Yet, other optimality principles than growth such as maximization of energy generation in terms of ATP have been suggested and evaluated (Schuetz et al. 2007). Model simulations of optimizing defined objective functions take globally into account cellular energy and redox balancing requirements when fulfilling mass balance, enzyme capacity, and thermodynamic constraints in the whole metabolic network. Enzyme capacity and thermodynamic constraints are introduced into the FBA problem as flux upper and lower bounds. Commonly, the sign of flux value describes the net flux direction of the reaction but alternatively forward and backward reactions can be separately represented in the model. When thermodynamics do not allow for a particular reaction direction under cellular conditions (Flamholz et al. 2012), the flux bounds can be assigned accordingly for simulations.

Phenotype simulations with FBA and derived tools and genome-scale metabolic model manipulations are facilitated with frameworks supporting method development and/or tools with higher level interfaces for analysis (Table 5.1). While Python-based frameworks, relying on COBRApy (Ebrahim et al. 2013), are currently the primary choice of developers, there are R (R Development Core Team 2018) (Sybil (Gelius-Dietrich 2013)) and MATLAB (www.mathworks.com) (COBRA toolbox,

Table 5.1 Development frameworks and higher level tools for genome-scale metabolic model manipulations and simulations

Tool name	Description	Ref.
COBRApy	Python package containing model manipulation, analysis, and design methods, for developers and end users	Ebrahim et al. (2013)
COBRA Toolbox	Software suite based on Matlab containing model manipulation, analysis, and design functions, for developers and end users	Heirendt et al. (2017)
Sybil	R-package containing algorithms for model manipulation and analysis, for developers and end users	Gelius-Dietrich (2013)
BIOMET Toolbox	Web based collection of tools for reconstruction and analysis of metabolic models, for end users	Cvijovic et al. (2010), Garcia-Albornoz et al. (2014)
CAMEO	Python based COBRApy compatible tool with several *in silico* metabolic engineering tools implemented, for end users	Cardoso et al. (2018)
CellNetAnalyzer	Graphical user interface for metabolic modeling using elementary flux modes approach, Matlab based, for end users	Klamt et al. (2007), Klamt and von Kamp (2011), von Kamp et al. (2017)
MetaFlux	Graphical user interface or lisp API for metabolic model reconstruction and flux balance analysis, for end users	Latendresse et al. (2012)
OptFlux	Java based tool for *in silico* metabolic engineering, for end users	Rocha et al. (2010)

(Schellenberger et al. 2011; Heirendt et al. 2017)) based frameworks available as well. The frameworks and tools commonly offer interfaces to external LP (and commonly also Mixed-Integer Linear Programming (MILP) and Quadratic Programming (QP)) solvers (e.g., glpk (www.gnu.org/software/glpk/), cplex (www.ibm.com/analytics/cplex-optimizer), gurobi (www.gurobi.com)) to be recruited for different applications. External libraries may also be engaged by the tools, in particular, for manipulating models in common Systems Biology Markup Language (SBML) format (Hucka et al. 2003) (SBML toolbox (Keating et al. 2006), libSBML (Bornstein et al. 2008)). Tools with higher level interfaces allow also experimental scientists analyzing metabolism with genome-scale models and designing genotype manipulations, as will be reviewed below.

Genome-scale metabolic model simulations using FBA with alternative, other than biological design principle mimicking, objectives can be used to explore an organism's metabolic potential, possible metabolic states it may have. For instance, under the given mass balance, enzyme capacity, and thermodynamic constraints, the optimal theoretical yields of biotechnologically relevant molecules can be solved with simulations. The simulations can be done by assigning alternative nutritional conditions mimicking different growth media or bioconversion substrates. In case substrate utilization rates are available, they can be introduced to the models as exchange fluxes between cells and the environment, and FBA can be used to predict optimal steady-state growth (1/h) and specific production rates (mmol/(g cell dry weight * h)) instead of yields. While the optimal value solved for the chosen objective by FBA (i.e., yield or rate) is global and unique, the other fluxes (i.e., variables of the optimization problem) may adopt different values under optimality. Thus, there may be several, alternative, yet equally optimal metabolic phenotypes in terms of the defined objective function.

5.3.1 Parsimonious Flux Balance Analysis (pFBA)

Parsimonious Flux Balance Analysis (pFBA) aims at reducing the set of alternative equally optimal flux states in a biologically relevant way (Lewis et al. 2010). pFBA derives from FBA and includes a bi-level optimization where first the biological design objective (e.g., growth) is optimized after which, under the optimality condition, another linear programming problem is solved to minimize the sum of the fluxes. The flux-sum minimization in pFBA can be seen biologically relevant in optimizing the enzyme usage, and thereby the cellular resource utilization. Flux-sum minimization efficiently omits futile flux cycle artifacts from the returned flux vector. Yet, fluxes may adopt alternative values also under pFBA optimality.

5.3.2 Flux Variability Analysis (FVA)

The ranges of possible values fluxes may adopt under particular optimality can be assessed with Flux Variability Analysis (FVA) (Burgard and Maranas 2001; Mahadevan and Schilling 2003). FVA can be performed under the optimality of the assigned objective (i.e., commonly growth) or different levels of it. The computation involves solving two subsequent linear programming problems, minimization and maximization, for each of the fluxes. The fluxes whose ranges do not pass zero are coupled to the objective and can thus be considered essential for the particular objective. General analysis of flux coupling in a metabolic network is derived from FVA (Burgard et al. 2004).

5.3.3 Simulating Mutant Cell Phenotypes

The above FBA-derived simulation approaches assume optimal distribution of flux in the metabolic network. In case of FBA simulation with an objective function mimicking biological optimality principle, the premise is justified by evolutionary optimization of organism's metabolism (Ibarra et al. 2002). However, mutant strains engineered in laboratory cannot be assumed to function optimally. Minimization of Metabolic Adjustment (MoMA) approach was developed to simulate the metabolic state of such engineered mutant strains (Segrè et al. 2002). MoMA solves a quadratic optimization problem of minimizing the flux differences to a reference flux state (i.e., wild-type flux state) given the constraints arising from the engineered modifications to the strain (e.g., gene deletions). There is also a linearized version, linear Minimization of Metabolic Adjustment (lMoMA) of the algorithm (Burgard et al. 2003; Becker et al. 2007). In biological sense MoMA and lMoMA assume that the wild-type regulation is still driving the distribution of metabolic fluxes in engineered but not evolutionarily streamlined strains. Wild-type regulation-driven flux distribution in engineered cells is also simulated with Minimization of Metabolites Balance (MiMBl) algorithm (Brochado et al. 2012). In contrast to MoMA and lMoMA, MiMBl is independent of the stoichiometric representation of the reactions. While multiplicating the stoichiometric coefficients of particular reaction(s) (which does not affect the reaction stoichiometry or elemental balance) would alter the output of MoMA computation, MiMBl solution would be unaffected. MiMBl computation minimizes the flux distribution difference to the wild-type state in terms of metabolite turnovers instead of fluxes. Yet another approach for simulating the metabolic state of engineered, but not evolved organisms is Regulatory On/Off Minimization (ROOM) algorithm (Shlomi et al. 2005). ROOM minimizes the number of fluxes that are changed in mutant cells compared to wild-type cells. The underlying premise in ROOM is the same as in MoMA, lMoMA, and MiMBl in assuming that the wild-type regulation drives the distribution of fluxes in a non-evolved mutant strain. In ROOM simulations, it is further assumed that the mutant metabolic state is reached through

only the necessary transient metabolic changes mediated by the regulatory network. The necessary changes are simulated with ROOM by solving a Mixed-Integer Linear Programming (MILP) problem.

5.4 Examples of Genotype–Phenotype Simulations: Single and Double Gene KOs

The above-introduced simulation tools using genome-scale metabolic models allow predicting phenotype effects following from gene deletions (Förster et al. 2003). *In silico* metabolic gene deletions are propagated through the Boolean gene-reaction rules into reaction activities. If a regulatory model is integrated as in rFBA approach (Covert et al. 2001; Herrgård et al. 2006), the regulatory gene deletions can be first propagated to the status of metabolic genes through the regulatory Boolean rules, and then through the metabolic model's gene-reaction rules into reaction activity states. The phenotype simulation is then performed with updated reaction activity states. FBA or another simulation algorithm, not assuming the metabolism in mutant could necessarily become optimized, can be used. In case the simulated growth is negligible, the deleted gene is predicted essential. Double gene deletion simulations predict *in silico* synthetic lethal gene pairs (Suthers et al. 2009). Since experimental screens of gene deletion mutants in model organisms are available in genome-scale, comparison to *in silico* model predicted essentialities and synthetic lethalities can be used for validating metabolic model reconstruction algorithms.

5.5 *In Silico* Metabolic Engineering—Strain Design

Since the genome-scale metabolic models allow predicting translation of genotype to phenotype, they can be used to design genotype manipulations leading to desired phenotypes. Overproducer phenotypes are especially sought for industrial biotechnology applications. While native strains are evolved to distribute the available resources for growth and survival, feasible industrial production using a microbial fermentation process requires cells to divert substantial resources to product synthesis. Diverting cellular resources toward production is the aim of metabolic engineering of the industrial biotechnology host organisms, like yeasts, in addition to introducing the production pathways in case of heterologous products. Strategies to achieve the desired metabolic flux re-regulation diverting resources efficiently to the production pathway can be computationally designed using genome-scale metabolic models. An elegant solution for the inherent competition of growth and product synthesis for resources is to align those objectives through metabolic network modifications. Aligning the growth and production objectives in cells can be achieved with specific metabolic gene deletions resulting in growth-coupled production. The specific

metabolic gene deletions reduce the metabolic network in such a way that the cells cannot grow (optimally or at all) unless they simultaneously synthesize the product. In other words, some growth essential pathway produces the desired product as an unavoidable side stream. OptKnock was the pioneering method for finding growth–product coupling creating deletion targets using metabolic models (Burgard et al. 2003). It was implemented as a bi-level MILP. An alternative implementation of *in silico* growth–product coupling design is OptGene in which the phenotype simulation is embedded in a genetic algorithm allowing for nonlinear design objectives and searching larger target gene sets (Patil et al. 2005; Asadollahi et al. 2009). OptGene has been used successfully to design, for example, succinate and terpenoid overproducing *S. cerevisiae* strains (Otero et al. 2013; Asadollahi et al. 2009). For vanillin production in *S. cerevisiae* (in form of vanillin glycoside to reduce toxicity), OptGene was used to identify deletion targets out of which *GDH1* (glutamate dehydrogenase encoding) and *PDC1* (pyruvate decarboxylase encoding) deletions were experimentally implemented and evaluated (Brochado et al. 2010). Single deletion mutants, a double deletion mutant, and a double deletion mutant with *GDH2* overexpression to improve nitrogen assimilation defect in *gdh1*Δ were constructed. The mutant strains except single *gdh1*Δ mutant showed 1.5 fold increase in vanillin glucoside yield in batch cultures compared to the non-host metabolism optimized strain. Furthermore, optimizing the synthetic, four-step, production pathway of vanillin glucoside in *S. cerevisiae* did not improve the production, before the OptGene identified targets to optimize the host metabolism were implemented (Brochado et al. 2010; Brochado and Patil 2013). Later, Tepper and Shlomi (2010) released their RobustKnock version for extracting such growth–product coupling creating deletions that force product synthesis with an additional optimization step (Tepper and Shlomi 2010). Growth–product coupling creating manipulations to genome fix the relative yields of biomass and target product. However, the rates are amenable for improvement through Adaptive Laboratory Evolution (ALE) of the mutant strains. While faster growing cells are selected for, the coupled production rate is improved on the side (Otero et al. 2013). If the growth–product coupling relies on a carbon–carbon bond cleaving reaction splitting a precursor for growth and production, the coupling is likely to be very robust in ALE. An *Anchor* reaction producing an essential precursor for growth and another product convertible to the target product is biochemically essential for a growth–product coupled reduced metabolic network (Jouhten et al. 2017). Carbon–carbon bond cleaving *Anchor* reactions are a subset of all possible *Anchors*. Growth-coupled succinate production in *S. cerevisiae* relies on carbon–carbon bond cleaving isocitrate lyase as an *Anchor* reaction (Otero et al. 2013). The initial production rate after the metabolic network reduction for growth–product coupling was substantially improved with ALE along with relieving glycine auxotrophy (Table 5.2).

Metabolic network manipulations for achieving growth–product coupling are identifiable also with elementary-mode analysis methods (Schuster and Hilgetag 1994; Schuster et al. 2000; Trinh and Srienc 2009; Unrean et al. 2010; Hädicke and Klamt 2011). Elementary modes are minimal sets of reactions allowing a steady-state operation (Heinrich and Schuster 1998). Engineering strategies are

Table 5.2 Examples of reported overproducer yeast strains whose development has been involved using genome-scale metabolic model simulation tools

Product	Species	Tools	Year	Ref.
Ethanol	*S. cerevisiae*	in house script (FBA)	2006	Bro et al. (2006)
Sesquiterpene	*S. cerevisiae*	MOMA, OptGene	2009	Asadollahi et al. (2009)
Vanillin	*S. cerevisiae*	MOMA, OptGene, OptKnock	2010	Brochado et al. (2010)
2,3-butanediol	*S. cerevisiae*	OptKnock	2012	Ng et al. (2012)
Fummaric acid	*S. cerevisiae*	FBA	2012	Xu et al. (2012)
Succinic acid	*S. cerevisiae*	OptGene	2013	Otero et al. (2013)
Tyrosine	*S. cerevisiae*	OptKnock	2013	Cautha et al. (2013)
Dihydroartemisinic acid	*S. cerevisiae*	MOMA, OptStrain, OptForce, OptKnock	2013	Misra et al. (2013)
Muconic acid	*S. cerevisiae*	FBA	2013	Curran et al. (2013)
Malate	*C. glabrata*	FBA	2013	Chen et al. (2013)
Triacetic acid lactone	*S. cerevisiae*	OptKnock	2014	Cardenas and Da Silva (2014)
Human recombinant protein	*P. pastoris*	FSEOF, MOMA	2014	Nocon et al. (2014)
Ethanol	*S. cerevisiae*	FBA, EMA	2014	Toro et al. (2014)
Acetoin	*C. glabrata*	FBA	2014	Li et al. (2014)
Amorphadiene	*S. cerevisiae*	MOMA, FBA	2014	Sun et al. (2014)
Succinate	*S. cerevisiae*	FBA	2014	Rosdi and Abdullah (2014)
3-hydroxypropionic acid	*S. cerevisiae*	FBA	2015	Borodina et al. (2015)
Patchoulol	*S. cerevisiae*	EMA	2015	Gruchattka and Kayser (2015)
Lipid	*Y. lipopytica*	FBA	2015	Kavscek et al. (2015)
Tyrosine	*S. cerevisiae*	OptKnock	2015	Gold et al. (2015)
β-Farnesene	*S. cerevisiae*	pFBA	2016	Meadows et al. (2016)
3-hydroxypropionic acid	*S. cerevisiae*	pFBA	2016	Kildegaard et al. (2016)
Muconic acid	*S. cerevisiae*	FBA	2016	Suastegui et al. (2016)
Biomass	*S. stipitis*	FBA	2016	Unrean et al. (2016)
Growth on Methanol or glycerol	*P. pastoris*	FBA	2017	Tomas-Gamisans et al. (2018)
Polymalic acid	*A. pullulans*	FBA	2017	Feng et al. (2017)
Ethanol	*S. stipitis*	FBA	2017	Acevedo et al. (2017)
Triacylglycerol	*Y. lipopytica*	FBA	2018	Koivuranta et al. (2018)
Lipid	*R. toruloides*	FBA	2018	Castañeda et al. (2018)

designed for disabling undesired elementary modes while retaining the desired ones (Hädicke and Klamt 2011). Introducing flux capacity constraints to the elementary-mode framework, as in FBA-derived methods, is enabled using Elementary Flux Vectors (EFVs) allowing also designing growth–product coupling strategies (Urbanczik 2007; Klamt and Mahadevan 2015). The scalability of searching metabolic engineering strategies *in silico* using elementary-modes-based approaches has been limited but is improving through algorithmic developments (von Kamp and Klamt 2014). Currently, minimum sets of genetic engineering targets can be exhaustively identified enabling evaluations also in yeast hosts. Beyond identifying growth–product coupling strategies, genome-scale metabolic models allow designing also other kinds of engineering strategies for improving production. While the methods for designing strategies to optimize the cellular fluxes for production are broadly reviewed elsewhere (e.g., Maia et al. (2016)) many of them are yet to be evaluated for yeasts. Among the variety of approaches, there are methods for identifying not only knockouts but also up- and downregulation targets for improving production. OptReg identifies combined strategies of deletions, overexpressions, and downregulations for host optimization as bi-level MILP solutions (Pharkya and Maranas 2006). Similarly, OptForce identifies combined strategies in a comparative manner against the wild-type flux status by classifying reactions based on the type of manipulation they require for optimizing production (Ranganathan et al. 2010). Flux Scanning based on Enforced Objective Flux (FSEOF) considers the wild-type flux status by identifying upregulation engineering targets as genes annotated to reactions whose flux is increased *in silico* when the production objective is enforced while biological objective (i.e., growth) prevails (Choi et al. 2010). FSEOF-identified targets have successfully been implemented in *P. pastoris* yeast for improving protein production (Nocon et al. 2014). The strain improvement strategies may also benefit from augmenting metabolic models with additional information on metabolic enzymes or wild-type phenotype. For instance, k-OptForce integrates available enzyme kinetic information to improve predictions by considering metabolite concentration effects on the distribution of fluxes (Chowdhury et al. 2014). OptFlux allows using gene expression data for using a comparative approach against the wild type for identifying overexpression and downregulation targets in a metaheuristic optimization framework (Gonçalves et al. 2012). Importantly, considering the wild-type gene expression data allows relieving the optimality assumption from the native operation of cells allowing a comparative strain design also in secondary metabolic pathways (Kim et al. 2016). Accordingly, transcriptomics-based Strain Optimization Tool (tSOT) identifies the metabolic engineering targets by considering the wild-type flux regulatory status inferred from gene expression data (Kim et al. 2016). However, a word of caution though, the gene expression status of central metabolic enzymes may not very well reflect the actual flux status in yeast cells as (Machado and Herrgård 2014) observed when integrating gene expression data to genome-scale metabolic models.

5.6 Integrating Omics Data into Models

Genome-scale metabolic models offer frameworks for integrating omics data since they connect metabolic genes/proteins to reaction fluxes through which biochemical conversion of metabolites occurs. Fluxes together with metabolite abundances are the metabolic phenotype determined by and reciprocally regulating the underlying transcriptional and translational states in a cell. Evolutionarily shaped cellular regulation can vary the metabolic phenotypes within the ultimate limits of the laws of mass conservation and chemical thermodynamics. Therefore, transcriptomics, proteomics, or metabolomics data have been integrated to the models for shrinking the space of feasible metabolic states to improve flux estimation outcomes. Indeed, flux predictions would often benefit from specific constraints representing the regulation of the metabolic network utilization under particular conditions (e.g., repression of respiration in *S. cerevisiae* on high glucose). Several methods have been developed for inferring the flux states from gene expression data, the most abundantly available omics data type. iMAT (Shlomi et al. 2008), GiMME (Becker and Palsson 2008), GIM3E (Schmidt et al. 2013), RELATCH (Kim and Reed 2012), and INIT (Agren et al. 2012) methods derive expected or allowable flux states from the gene expression data. However, flux estimation could also be misled by gene expression data (Machado and Herrgård 2014) as post-transcriptional regulation of metabolic phenotypes is prevalent. Consequently, additional constraints derived from proteomics data integrated with enzyme-specific turnover numbers (kcat) (Sanchez et al. 2017; Vazquez and Oltvai 2016) have allowed reproducing, using model simulations, metabolic phenotypes (e.g., overflow metabolism) that are not well captured with plain FBA or apparent in gene expression data. Further, time derivatives of extracellular metabolites in a cell culture (i.e., rates of consumption and production) can readily be integrated into the models as bounds on exchange fluxes between cells and environment, allowing simulations of consistent intracellular flux states (Mo et al. 2009). However, while the exchange flux, gene expression, and proteomics data derived constraints can directly be assigned to the fluxes in models, integration of intracellular metabolite abundance data to steady-state simulations is less straightforward. Metabolite concentrations can be used to refine reaction thermodynamics for resolving feasible reaction directions (Henry et al. 2007; Kümmel et al. 2006). Further, constraints for flux changes have been derived from relative metabolomics data through the connectivity of metabolites with several reactions in the metabolic network (Sajitz-Hermstein et al. 2016). Vice versa, metabolite concentration changes can be predicted using gene expression data and the network neighborhood (Zelezniak et al. 2014). When the metabolite concentration change prediction from gene expression data and network connectivity fails, the particular metabolite is likely to be connected to a post-transcriptionally regulated enzyme (Zelezniak et al. 2014). Likely post-transcriptionally regulated enzymes can similarly be identified in disagreements of gene expression data and flux estimates (Shlomi et al. 2008). Thus, omics data integration with model simulations allows also uncovering how the cells have achieved the observed metabolic phenotypes. Recently, (Strucko et al. 2018) uncovered in molecular detail how *S. cerevisiae*

achieved an efficiently glycerol-utilizing phenotype through Adaptive Laboratory Evolution (ALE). Classical genetic crossing, genome-scale metabolic model simulations, whole genome sequencing, and omics analyses revealed involvement of all levels of cellular regulation, in a pathway-dependent manner, in achieving the glycerol utilization trait. The ALE for glycerol utilization was performed for a laboratory strain of *S. cerevisiae*, commonly lacking the ability to grow on glycerol in absence of amino acid supplementation. Interestingly, some wild *S. cerevisiae* strains can grow on glycerol as the sole carbon source, and the metabolic network structure of *S. cerevisiae* does not object the conversion of glycerol to biomass even without amino acids being provided. By gradually decreasing the amino acid supplementation, evolved lineages growing on glycerol as the sole carbon source were obtained (Strucko et al. 2018). Whole genome sequencing of evolved lineages revealed mutations that arose during the ALE. Few metabolic genes and genes involving osmoregulation controlling glycerol accumulation in cells had been repeatedly hit by mutations. A lineage not having loss-of-function mutations in osmoregulation involved genes was characterized in controlled bioreactors and analyzed on different omics levels (i.e., RNA sequencing, proteomics, and metabolomics). Further, genome-scale metabolic model simulations were run for identifying the necessary but minimum re-regulation of wild-type metabolic fluxes for achieving an optimally glycerol-utilizing phenotype. The identified necessary flux changes were overlaid with the mutated genes and the omics data on the metabolic network. The model simulations had revealed a necessary downregulation of TCA cycle activity while maintaining respiratory function for glycerol utilization which was in perfect concordance with the otherwise obscure *KGD1* (encoding alpha-ketoglutarate dehydrogenase in the TCA cycle) loss-of-function mutation gained repeatedly in ALE. Further, the model simulations predicted also an activation of GABA shunt bypass of the TCA cycle for optimizing glycerol utilization. Indeed, reactant ratios from metabolomics data were in agreement with the GABA shunt activation. In addition, gene/protein expression changes were in agreement with the model simulated prediction of decreased TCA cycle flux. In conclusion, the flux change predictions with model simulations effectively reconciliated the separate observations in omics data and the genes repeatedly mutated in ALE.

5.7 Regulation of Yeast Metabolism: Key Nodes and Their Impact on Flux Distribution—Future Directions of Reincorporating These into Models

While metabolic models have greatly improved our ability to systematically map genotype–phenotype relations, they have also brought forward key gaps in the understanding of the complex interactions between different metabolic pathways and between metabolic and regulatory processes. This becomes evident when considering the dramatically reduced performance of genome-scale metabolic models from well

predicting the essentiality of single genes to the low accuracy in predicting genetic interactions (Brochado et al. 2012). A major limitation of the models, especially when tackling higher order complex interactions, is the large degrees of freedom, i.e., multiple ways that the resource (carbon and other elemental) fluxes can be distributed in the cell. Without considering additional constraints imposed by protein abundance and activity status (e.g., phosphorylation), metabolite concentrations, and allosteric regulations, the models will not be able to narrow down the predictions on the actual routes operating in cells. Different approaches have been proposed toward constraining the solution space of metabolic models for improving the accuracy of predictions in a biologically sound manner. These include knowledge-based heuristics imposing constraints on flux distribution at key branch points (Pereira et al. 2016), constraining the fraction of protein resources allocated to metabolic processes (Sanchez et al. 2017), imposing a constraint on maximum Gibbs energy dissipation from cells (Niebel et al. 2019), and large-scale kinetic models that include metabolite concentrations and enzyme kinetic parameters (Chakrabarti et al. 2013; Stanford et al. 2013; Smallbone et al. 2010). The last mentioned would be an ideal approach encompassing various complexities in their mechanistic detail. Yet, the lack of reliable in vivo data on enzyme kinetics, metabolite concentrations, and enzyme/metabolite distributions within a cell limit the use of kinetic modeling to well-studied conditions and relatively small perturbations. Further, introducing a constraint on Gibbs energy dissipation to the metabolic models is computationally demanding as it results into nonlinear and non-convex model. Thus, the first two approaches are likely to be the most fruitful in the near future. Indeed, the distribution of major metabolic fluxes in yeast cells are tied to the redox and energy cofactor balance, which, in turn, are closely coupled with the flux distribution in pentose phosphate pathway and pyruvate nodes. The former largely determines the NADPH production and the latter affects NADH and ATP turnover. Indeed, a recent study (Yu et al. 2018) elegantly demonstrates this by replacing ethanol production by fatty acid production. Given that ethanol accumulation is a hallmark of yeast metabolism, this is a remarkable feat and yet can be understood in terms of redox balance rewiring. Along similar lines, an approach considering protein allocation constraint has suggested that lower protein requirement of ATP generation through fermentation is the trade-off factor underlying the switch from respirative to fermentative metabolism at higher glucose utilization rates in yeast (Nilsson and Nielsen 2016). The ongoing efforts in expanding the models to incorporate transcriptional and translational processes (Yang et al. 2018) are likely to complement the abovementioned approaches in expanding the scope of metabolic models as well as in improving their accuracy which is capturing complex metabolic traits.

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Chapter 6
Emerging Mechanisms of Drug Resistance in *Candida albicans*

Rajendra Prasad, Remya Nair and Atanu Banerjee

Abstract Drug resistance mechanisms in the commensal human pathogen *Candida albicans* are continually evolving. Over time, *Candida* species have implemented diverse strategies to vanquish the effects of various classes of drugs, thereby emanating as a serious life threat. Apart from the repertoire of well-established strategies, which predominantly comprise permeability constraints, increased drug efflux or compromised drug import, alteration, overexpression of drug targets, and chromosome duplication, *C. albicans* has evolved novel regulatory mechanisms of drug resistance. For instance, recent evidences point to newer circuitry involving different mediators of the stress-responsive machinery of oxidative, osmotic, thermal, nitrosative, and nutrient limitation, which contribute to the emergence of drug resistance. Contemporary advances in genome-wide studies of transcription factors, for instance, the Zn_2Cys_6 transcription factors, *TAC1* (transcriptional activator of CDR) in *Candida albicans,* or *YRR1* in yeast have made it feasible to dissect their involvement for the elucidation of unexplored regulatory network of drug resistance. The coordination of implementers of the conventional and nonconventional drug resistance strategies provides robustness to this commensal human pathogen. In this review, we shed light not only on the established strategies of antifungal resistance but also discuss emerging cellular circuitry governing drug resistance of this human pathogen.

Keywords Antifungal resistance · *Candida albicans* · Efflux pumps · Stress response pathway · Hsp90 · Hsf1

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6.1 Introduction

Antifungal resistance is currently, the paramount concern in the field of medical mycology. The predominant human fungal pathogens, *Candida albicans*, *Aspergillus fumigatus,* and *Cryptococcus neoformans* can thrive in various anatomically distinct sites within the host and are, therefore, capable of fostering deep-seated infections in susceptible patients (Cornet and Gaillardin 2014). Additionally, the challenge associated with eukaryotic pathogens is predominantly due to their close evolutionary relationship with human hosts, and hence limiting the range of drug targets, exclusively found in fungal pathogens (Shapiro et al. 2011). The continual appearance of novel resistant profiles of these fungal pathogens in recent decades highlights their capacity to adapt to disparate antifungal selective pressure. According to phylogenetic analysis, the pathogenic fungi are not evidently clustered away from the other nonpathogenic fungal species; hence, much of the pathogenic traits can be attributed to multiple evolutionary transitions that develop over the time (Cowen and Steinbach 2008). The evolution of drug resistance in the microbial communities, especially in fungal pathogens, is accompanied by an incessant surge in mortality rates in humans (Cowen 2008). Broadly, resistance elicited by a particular pathogen may either be found inherently without prior exposure to the drug, i.e., intrinsic or primary resistance, for instance, fluconazole resistance in *Candida krusei* or echinocandin resistance in the basidiomycete *Cryptococcus neoformans*. The other category, i.e., acquired or secondary resistance, comprises resistance acquired by previously susceptible species owing majorly to altered gene expression on continual exposure to certain drugs (Kanafani and Perfect 2008).

Traditionally, drug resistance has been associated with cellular determinants that prevent a drug from entering a cell, promote drug extrusion, inactivate the drug, or prevent it from inhibiting its target. However, recently, noncanonical mechanisms of drug resistance that include mechanisms that allow the cell to cope with and adapt to the drug-induced stress have gained remarkable attention. As the ever-increasing instances of antifungal drug resistance dramatically outpaces the development of new antifungal compounds, the importance of the understanding of the novel or unconventional evolutionary mechanisms that command the development of resistance has emerged.

6.2 Conventional Mechanisms of Drug Resistance in *Candida albicans*

Even though numerous novel mechanisms of resistance have been revealed over the years, the classical mechanisms of antifungal resistance still hold central importance as far as various *Candida* species are concerned. The conventional mechanisms of drug resistance are as follows.

6.2.1 Alterations in Drug Target

The predominant mechanism by which *C. albicans* acquires resistance toward antifungals is through mutations in the gene encoding the drug target. Most of the target mutations compromise drug-binding abilities resulting in reduced drug efficacy. *ERG11*, encoding a lanosterol demethylase, serves as the main target of azoles, and thus is one hotspot for mutations (Marichal et al. 1999; Prasad et al. 2017; Robbins et al. 2017). Since *ERG11* mutations are often accompanied by loss of heterozygosity (LOH) events, azole resistance is further strengthened under such conditions (Robbins et al. 2017). Azole resistance owing to *ERG11* mutations has been observed in other fungal pathogens as well, for instance, *C. neoformans* and *A. fumigatus* (Rodero et al. 2003; Garcia-Effron et al. 2008). Apart from azoles, resistance to echinocandins in *C. albicans* is also mediated through mutations in the target gene, which is, in this case, the β-1,3 glucan synthase encoding gene *FKS1*(Prasad et al. 2017; Garcia-Effron et al. 2009). Mutations in the *FKS1* gene have been found to be clustered in two different regions, namely, Hot spot 1 (HS1) and Hot spot 2 (HS2) in drug-resistant clinical isolates of *C. albicans* (Sanglard 2017). As is often the case with azoles, mutations in *FKS1* too are followed by LOH in *C. albicans* (Robbins et al. 2017). Interestingly, certain mutations in *FKS1* result in decreased processivity rather than affecting affinity of the drug for its target (Garcia-Effron et al. 2009).

In addition to point mutations, overexpression of the drug target is also an effective mechanism to achieve drug resistance. With an increase in the number of target proteins, higher and infeasible concentration of drug is required to inactivate them, thus leading to drug resistance. Gain of function (GOF) mutation in a zinc cluster finger transcription factor (TF), *UPC2* has been found to be responsible for upregulation of *ERG11* and fluconazole resistance in *C. albicans* clinical isolates (Dunkel et al. 2008; Hoot et al. 2011).

6.2.2 Permeability Constraints

6.2.2.1 Active Efflux

One principal mechanism by which fungal cells evade toxicity of xenobiotics is through activation of efflux pump proteins. These proteins include members of ATP-binding cassette (ABC) superfamily and major facilitator superfamily (MFS) which are involved in rapid drug extrusion (Prasad et al. 2017). Among 26 ABC superfamily members encoded by the *C. albicans* genome, *Candida* drug resistance 1 (CaCdr1) and *Candida* drug resistance 2 (CaCdr2) are the two major efflux pump proteins whose overexpression hold key to clinical azole resistance and represents one of the major mechanisms of MDR (Prasad et al. 2015). Even though CaCdr1 protein seems to be more important than CaCdr2 in azole resistance, as established by Holmes

and coworkers, transcriptional activation *CaCDR2* is also seen in several clinical isolates (Holmes et al. 2008; White et al. 2002; Chen et al. 2010). GOF mutations in *TAC1,* which is a key regulator of *CaCDR1/CaCDR2* expression, are associated with a number of azole-resistant *C. albicans* isolates (Coste et al. 2006).

Recently, one transporter Cdr6 has been found to efflux xenobiotics such as berberine; however, its role in clinical drug resistance is not established (Khandelwal et al. 2017). Besides *C. albicans,* other *Candida* species and fungal pathogens such as *A. fumigatus* and *C. neoformans* also overexpress ABC transporters encoding genes which are responsible for antifungal resistance (Moran et al. 1998; Barchiesi et al. 2000; Posteraro et al. 2003; Sanguinetti et al. 2006; Slaven et al. 2002). Among 95 MFS proteins which *C. albicans* genome possesses, only CaMdr1 protein is implicated in clinical drug resistance where its overexpression in azole resistance is widely reported (Prasad et al. 2017; White 1997). The transcriptional activation of *CaMDR1* in response to drugs is regulated by a zinc cluster TF, multidrug resistance regulator 1 (*MRR1*) (Morschhäuser et al. 2007). Invariably, most of the fluconazole-resistant clinical isolates exhibiting *CaMDR1* upregulation display GOF mutations in *MRR1*. Some other MFS transporters such as Cg*TPO1_2* and Ca*FLU1* have also been implicated in contributing to resistance to antifungals and peptides; however, their clinical importance remains unidentified (Pais et al. 2016; Li et al. 2013).

The promiscuity of these drug efflux proteins of ABC and MFS superfamilies is intriguing. These proteins can export a wide range of structurally unrelated compounds such as antifungal drugs, herbicides, steroids, lipids, fluorescent dyes, etc., which poses a major challenge in designing inhibitors/modulators for these proteins (Prasad et al. 2015; Gaur et al. 2008). The predicted topology diagrams of Cdr1/Cdr2 in Fig. 6.1 illustrate that both proteins are made up of two transmembrane domains (TMDs) connected with each other by means of intracellular and extracellular loops (ICLs and ECLs, respectively) and two nucleotide-binding domains (NBDs). Each TMD is made up of six alpha helices. While the TMDs form the substrate-binding site, the NBDs function as powerhouse and provide energy via ATP hydrolysis for drug extrusion. On the other hand, CaMdr1p is made up of two TMDs, each with six alpha helices connected by ECLs and ICLs (Fig. 6.1). Further, there is also a long N-terminal extension. Mdr1 utilizes electrochemical gradient of protons to fuel uphill transport of xenobiotics (Redhu et al. 2016). A number of structural and functional studies have been undertaken to reveal the nature of substrate-binding pocket of Cdr1 and Mdr1. Two notable studies which dealt with the same are by (Rawal et al. 2013) and (Redhu et al. 2018), respectively. Both these studies involved mutagenesis of the entire primary structure of TMDs followed by extensive biochemical analysis along with deduced 3D molecular homology modeling to identify the important helices and residues which contribute to substrate binding and transport. One interesting finding made with respect to Mdr1 is that polyspecificity is conferred in this pump protein by residues situated at the periphery of the binding pocket (Redhu et al. 2018). Another study by Redhu and coworkers revealed an important arginine residue R215 of Mdr1p which is important for proton transport (Redhu et al. 2016). Interestingly, mutagenesis of ICLs also revealed a number of residues to be important for transport functions of Cdr1 and Mdr1 (Mandal et al. 2012; Shah et al. 2015).

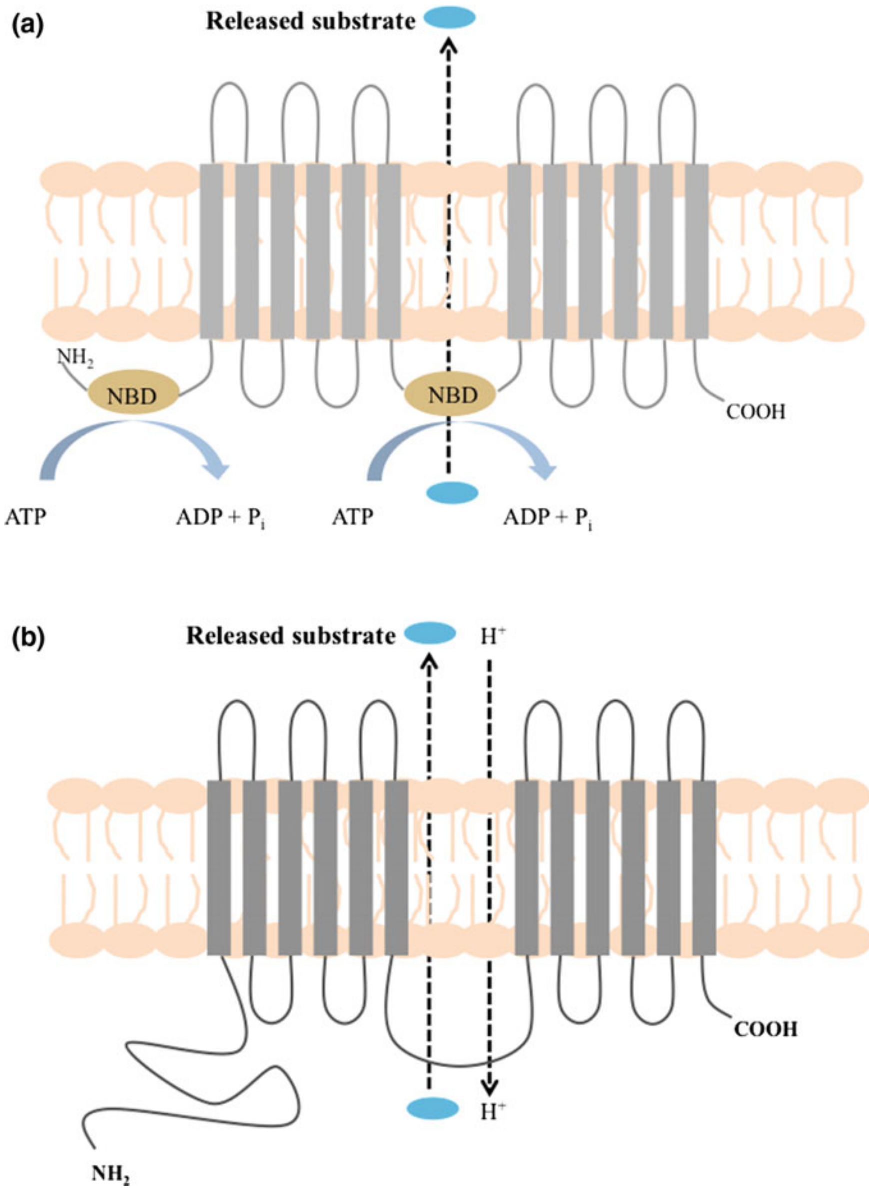

Fig. 6.1 Schematic representation of **a** Cdr1/Cdr2 (ABC transporter) and **b** Mdr1 (MFS transporter) of *C. albicans*. Cdr1 and Cdr2 harness the energy from ATP hydrolysis to power substrate efflux across the bilayer. Mdr1 utilizes proton-motive force to efflux drugs

Recently, suppressor genetics has been exploited as a tool to dissect out residues involved in inter-domain cross talk in Cdr1p. In particular, residues within or around highly conserved motifs of NBD2 such as Q-loop and D-loop have been identified to be functioning as essential entities involved in the mechanism (Shah et al. 2015; Banerjee et al. 2018).

6.2.2.2 Reduced Drug Import

Interestingly, reduced drug import has also been implicated as a mechanism of azole resistance in *C. albicans*. The first study which highlighted such a phenomenon was by Mansfield and colleagues which reported that fluconazole import proceeds by facilitated and nonpassive diffusion via some membrane-bound transporter (Mansfield et al. 2010). Concordantly, the authors also observed azole import to vary among resistant isolates of *C. albicans* implying a plausible mechanism of azole resistance. Recent study by Khandelwal et al. showed that the homozygous deletion of PM-localized *CDR6/ROA1* results in azole resistance by affecting membrane microviscosity and TOR signaling (Khandelwal et al. 2017). Despite the fact that this particular transporter has been found to be upregulated in a number of clinical isolates of *C. albicans*, no direct correlation could be deduced between its overexpression and azole resistance in the isolates.

Sequestration of drugs in intracellular and extracellular compartments can also lead to increased drug resistance. For instance, *C. albicans* possesses the remarkable feature of forming biofilms on various biotic/abiotic surfaces. Biofilms are extracellular structures made up of glycoproteins, carbohydrates, polysaccharides, etc. Owing to its dense and complex architecture, several antifungals such as azoles, polyenes fail to find a way to the cell's interior rendering the drugs ineffective (Taff et al. 2013). As far as intracellular sequestration is concerned, there exists a single report of fluconazole sequestration in intracellular structures of *C. albicans* (Maebashi et al. 2002). The authors described the organelles as vesicular vacuoles.

6.2.3 Metabolic Bypass

This particular mechanism remains rather insignificant and of compensatory nature in comparison to the previously described mechanisms. Nonetheless, it holds sufficient relevance in clinical context. Loss of function mutations in *ERG3* which encodes for $\Delta^{5,6}$ desaturase present the best example for such a mechanism. The enzyme converts 14α-methylated sterols which result from azole exposure into a toxic derivative (Kelly et al. 1995). Thus, mutations which inactivate this enzyme lead to azole resistance because of the absence of toxic derivative (Martel et al. 2010; Morio et al. 2012). Since, under such conditions, ergosterol is replaced by 14α-methylated sterols, the organism also evades the toxic effect of Amphotericin B (AmB) which relies on the physical extraction of ergosterol from the fungal cell membranes (Prasad et al.

2017; Anderson et al. 2014). Mutations in some other ergosterol pathway genes, viz., *ERG2, ERG6,* and *ERG24* also display similar compensatory effects (Sanglard 2016).

6.3 Multidrug Resistance: Adding on to the Misery

Multidrug resistance or MDR is defined as resistance to at least two different classes of drugs. In addition to efflux pumps, which can contribute to MDR owing to their promiscuous nature in terms of their substrates, fungal pathogens can display MDR via mutations in single or multiple genes. As discussed in the previous section, loss of function mutations in *ERG3* can lead to resistance to both azoles as well as Amphotericin B. Simultaneous mutations in multiple genes can also result in MDR. In one clinical isolate of *C. albicans*, mutations in *ERG11* and *ERG5* led to resistance to azoles as well as a polyene, i.e., AmB (Martel et al. 2010).

One frightening evidence of evolution of resistance toward three classes of antifungal drugs via gradual accumulation of mutations came up recently (Jensen et al. 2015). Herein, gene sequencing of sequential isolates collected from different sites of a single patient revealed that resistance emerged closely following the course of treatment which began with fluconazole followed by caspofungin and AmB. With exposure to fluconazole, GOF mutation in *TAC1* resulted in resistance toward it. Similarly, caspofungin treatment led to a mutation in *FKS1* which later countered the treatment. Finally, AmB exposure was countered by a loss of function mutation in *ERG2* which led to AmB resistance. The final strain harbored all the three mutations and displayed resistance to the three classes of antifungals. Thus, combination therapy represents the only effective option in cases where resistance encompasses multiple classes of drugs

6.4 Novel Strategies of Drug Resistance

The most common contributors of drug resistance as discussed above comprise minimizing the impact of the drug on the fungus, such as the overexpression of multidrug transporters or alterations of the drug target that prohibits the drug from inhibiting its target. However, in recent times, myriad unconventional strategies are being employed by microorganisms to thwart the impact of routinely used antifungal drugs. Survival of the pathogen within the host relies on stringent coordinated circuitry of initiating the befitting cellular response in accordance to the environmental stimuli encountered (Lafayette et al. 2010). Consequently, microorganisms have evolved robust mechanisms to sense and respond to diverse environmental stresses, encompassing thermal stress, oxidative stress, osmotic stress, changes in pH, and nutrient limitation (Brown et al. 2017). Recent studies have manifested that novel resistance mechanisms that curtail drug toxicities are often dependent on stress responses that

assist in cell survival (Robbins et al. 2017). For instance, targeting core regulators of cell wall stress response may be employed as a strategy to enhance the efficacy of antifungal drugs that target the cell wall, as with the echinocandins. Wherein, in many models of infection, it has been observed that the inhibition of Hsp90 enhances the efficacy of echinocandins against diverse fungal pathogens by modulating the levels of its client protein, calcineurin (Singh et al. 2009). By developing drugs capable of differentiating the cells of pathogens from the host, conserved eukaryotic cellular regulators can be exploited as potential antifungal targets. How the regulators of major stress-responsive pathways are contributing to drug resistance is discussed below.[1]

6.4.1 Oxidative Stress Response Regulator

In a cell, reactive oxygen species (ROS) are conventionally generated endogenously as a consequence of varied metabolic processes. It causes severe damage to cellular DNA, lipids, and proteins, thereby inducing oxidative stress (Jamieson 1998). Multitude of observations indicates that *C. albicans* cells encounter oxidative stress

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[1]Three stresses described in Fig. 6.2:

(1) **Oxidative Stress**
Oxidative stress regulator, Cap1, induces the overexpression of *MDR1* by recruiting Ada2, a subunit of the SAGA/ADA coactivator complex on *MDR1* promoter, thereby leading to an activation of drug resistance genes. Cap1 and Mrr1, both bind to the *MDR1* promoter, and cooperate to promote *MDR1* upregulation in response to inducing chemicals.

(2) **Metal Stress**

(i) **Iron depletion** leads to downregulation of *ERG11* gene, along with a parallel upregulation of *ERG3* gene. The downregulation of *ERG11* increases the membrane fluidity leading to a rise in passive drug diffusion, and hence increased drug susceptibility. The increase in Erg3 levels leads to accumulation of toxic sterols on the cell membrane resulting in cell death.
(ii) **Magnesium depletion** influences drug resistance majorly toward echinocandins. Its deficiency influences mutations in the histidine kinase gene, *NIK1*, thereby blocks the activation of Hog1 in response to the drug, enhancing caspofungin activity.
(iii) **Calcium depletion** either by chelating extracellular calcium with ethylene diamine tetraacetic acid (EDTA) or the inhibition of calcium importers with benidipine and nifedipine, leading to enhanced azole activity against *C. albicans.*

(3) **Heat Stress**

(i) By binding to and chaperoning calcineurin, the thermal stress regulator, Hsp90, regulates calcineurin-dependent stress responses, thereby enabling the cell to survive the membrane stress induced by azoles. Several downstream effectors of calcineurin mediate cellular responses to azoles, including the transcription factor Crz1.
(ii) Heat shock factor 1, Hsf1, has been observed to be crucial for the survival of *C. albicans* in the presence of various classes of drugs. The susceptibility shown toward certain classes of drugs was seen to be iron dependent.

during infection and that adaptation to it is essential for pathogenicity (Dantas et al. 2015). All aerobic organisms have, therefore, evolved anti-oxidant defense mechanisms to survive and protect the cells from such unfavorable stress conditions. In *S. cerevisiae,* the basic leucine zipper (bZip) transcription factor, Yap1 translocates to the nucleus to activate oxidative stress response. In the case of *C. albicans* and *C. glabrata*, which are comparatively more tolerant to oxidative stress, the Yap1 homologues, Cap1 (*Candida* AP-1 protein) and Cgap1 (*C. glabrata* AP-1), govern the oxidative stress response. Interestingly, *C. glabrata* could survive higher concentrations of H_2O_2 than both *S. cerevisiae* and *C. albicans* (Cuéllar-Cruz et al. 2008). Cap1 target includes genes involved in the detoxification of oxidative stress (e.g., catalase and superoxide dismutase: *CAT1* and *SOD1*), glutathione synthesis (e.g., gamma-glutamylcysteine synthetase: *GCS1*), redox homeostasis, and oxidative damage repair (e.g., glutathione reductase and thioredoxin: *GLR1* and *TRX1*). Together, these enzymes sequester ROS and mediate cellular adaptation to stress. Catalase, Cta1, and superoxide dismutases, Sod1 and Sod5, are major detoxifiers of ROS and are implicated to have role in virulence in mouse models of systemic candidiasis (Chauhan et al. 2006). Expectedly, the inactivation of Cap1 attenuates the induction of these stress genes, rendering *C. albicans* cells sensitive to oxidative stress. Further, robust oxidative stress response of *C. albicans* apart from maintaining

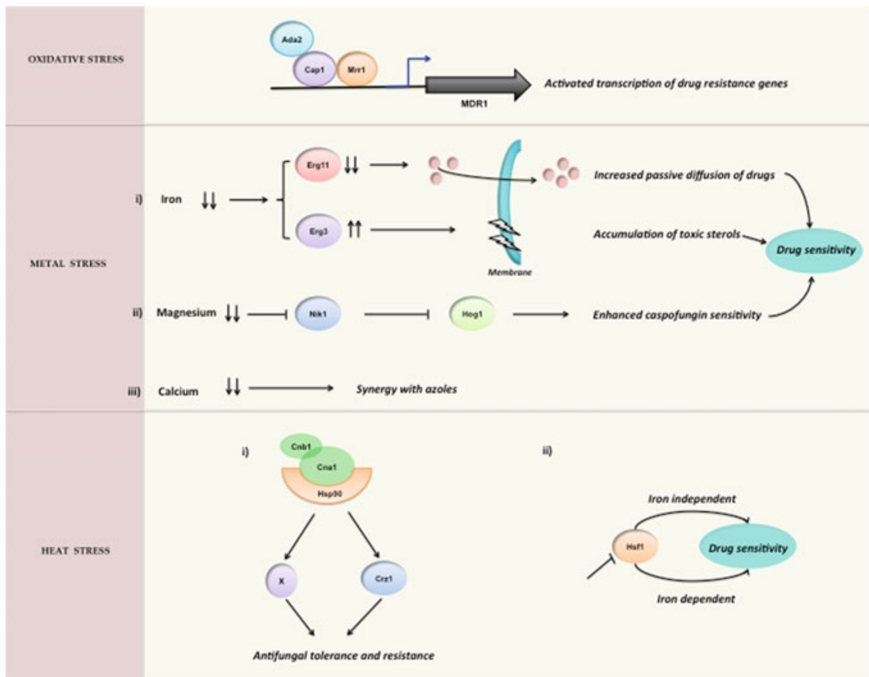

Fig. 6.2 Non-conventional regulators of drug resistance: (1) oxidative stress; (2) metal stress; and (3) heat stress (see footnote 1 for detailed description)

cellular homeostasis is also a major contributor in virulence attributes (Dantas et al. 2015).

The underpinnings of the involvement of this major oxidative stress regulator in regulation of *MDR1*, one of the major drug efflux pumps in *C. albicans* has been previously established. Upregulation of the *MDR1* gene is perceived in several clinical isolates of *C. albicans* as a resistance mechanism to some of the commonly used antifungal agents (Alarco et al. 1999). As discussed previously, its expression is mediated chiefly by gain of function mutations in the zinc cluster transcription factor, Mrr1, making it constitutively active while mutants lacking Mrr1 display little or no induction of *MDR1*. However, recent evidences also suggest the participation of the major oxidative stress response regulator, Cap1 in governing the expression of *MDR1*, wherein both Mrr1 and Cap1 bind to the promoter of *MDR1* to induce its expression in response to particular chemicals (Rognon et al. 2006). Cap1, is not only required for the induction of *MDR1* transcription on exposure to H_2O_2 but also contributes to benomyl-induced *MDR1* expression (Fig. 6.2). Recent studies have revealed that Cap1 and not Mrr1 induces the overexpression of this drug efflux pump by recruiting Ada2, a subunit of the SAGA/ADA coactivator complex on *MDR1* promoter, thereby leading to an activation of drug resistance genes (Ramírez-Zavala et al. 2014). Additionally, the *C. glabrata* Yap1p ortholog is also functionally involved not only in the oxidative stress response, but also in resistance to various drugs by activating the *MDR1* homologue, *FLR1* (Chen et al. 2007).

6.4.2 Metal Deficiency and Drug Tolerance

Transition metals such as iron, zinc, manganese, and copper are the structural and catalytic cofactors for several enzymes and proteins that influence standard metabolic processes in a cell and are, hence, vital for life (Kehl-Fie and Skaar 2010). Metal homeostasis is a key nexus in both the host as well as pathogen, influencing virulence factors. While the pathogen continually evolves diverse strategies to acquire the required nutrient metal ions from the host, in parallel sequesters these elements in order to restrict their availability to the invading pathogens, a concept termed as "*nutritional immunity*" (Citiulo et al. 2012). Perturbing metal homeostasis may be employed as a major strategy to cope up with myriad infectious diseases. For instance, iron chelators have been used effectively for superficial mycoses, while broad-spectrum chelators are shown to enhance the efficacy of certain classes of drugs (Butts et al. 2017).

The mechanisms of iron, an essential metal of several pathogens, its homeostasis, and impact on host–pathogen interactions are extensively studied. It is the most abundant metal in humans, its sequestration, especially during infection, represents a potent tool employed by the mammalian innate immune system (Chen et al. 2011). The underpinnings that iron impacts antifungal drug resistance has been widely established. Various iron chelators like doxycycline and other tetracycline antibiotics, deferasirox, and lactoferrin have been shown to possess antifungal activity (Butts et al. 2017). Generally, when confronted with azole antifungals, fungi upreg-

ulate several of the ergosterol biosynthesis genes to compensate for the inhibition of lanosterol demethylase. Many of these enzymes require heme cofactors to function and their upregulation increases the overall cellular demand for iron. Thus, restricting the availability of iron inhibits an efficient azole stress response leading to enhanced antifungal activity (Butts et al. 2017). The role of iron is recently extended to include its impact on clinical drug resistance in *C. albicans*. It is now well established that the deprivation of iron from the media by external chemical chelators results in highly drug-susceptible *C. albicans* cells. Notably, other Candida species such as *C. kefyr, C. glabrata, C. tropicalis, C. krusei, C. dubliniensis,* and *C. parapsilosis* also manifest enhanced drug susceptibility upon iron deprivation. Expectedly, the extent of iron-dependent drug susceptibility is variable among the different species of Candida (Prasad et al. 2006). Apart from metal chelators, iron acquisition transporter mutants Δ*FTR1,* Δ*FTR2,* Δ*FTR11* Δ*FTR2,* and Δ*CCC2* of *C. albicans* also display iron-deprivation-mediated drug susceptibility. The deregulation of iron homeostasis is also associated with the enhanced drug susceptibility of *C. albicans* cells. For instance, the zinc sulfur cluster protein, Upc2, implicated in ergosterol biosynthesis or Hsf1, the major heat stress response regulator, both display iron-mediated drug sensitivity (Vasicek et al. 2014; Nair et al. 2017).

Notably, the drug efflux transporters implicated in clinically observed azole resistance in *C. albicans* do not seem to play a tangible role in iron-induced drug susceptibility. For instance, the null mutants of efflux pump-encoding genes such as Δ*CDR1,* Δ*CDR2,* Δ*CaMDR1,* Δ*FLU1,* and Δ*CDR1*Δ*CDR2* do not display any further impression on drug susceptibility in iron-deprived *C. albicans* cells (Prasad et al. 2006). Moreover, iron deprivation has no impact on the expression of genes encoding these major drug efflux pumps. However, in the case of the parasitic protozoan, *Leishmania enriettii*, the multidrug resistance protein 1 (*LeMDR1*) mediates iron-dependent drug resistance. LeMdr1, located intracellularly, was proposed to mediate drug resistance by sequestering drugs into intracellular organelles rather than by active efflux (Wong and Chow 2006). However, additional molecular insights into iron-mediated drug resistance reveal that two important genes of the ergosterol biosynthetic pathway, *ERG11* and *ERG3,* display a pattern of reciprocal regulation upon iron deprivation, wherein *ERG11* is considerably downregulated with a parallel upregulation of *ERG3*. The downregulation of *ERG11* could be further correlated with the lowering of ergosterol content of iron-deprived cells leading to elevated passive drug diffusion. While the upregulation of *ERG3* results in increased accumulation of toxic sterols, which acts synergistically with the drugs administered under iron-limited conditions, thus leading to enhanced drug susceptibilities (Fig. 6.2) (Hameed et al. 2011).

Apart from exploiting a pathogen's iron requirements as a defense strategy, vertebrates also sequester other metals expanding the scope of nutritional immunity beyond iron. For instance, upon endothelial invasion, *C. albicans* relies on the zinc scavenger Pra1 for the acquisition of zinc from the host. Deletion of this protein not only impaired fungal sequestration and utilization of host zinc but also specifically blocked host cell damage in the absence of exogenous zinc (Citiulo et al. 2012). Additionally, zinc depletion by a broad-spectrum chelator of di- and trivalent

cations, diethylenetriaminepentaacetic acid (DTPA), also induced filamentation in a temperature-independent manner (Polvi et al. 2016).

While the chelation of magnesium was observed to influence echinocandin drug resistance, it turned out that DTPA synergizes with caspofungin, as the depletion of magnesium was sufficient to impair growth of the echinocandin-resistant clinical isolate in the presence of caspofungin. Notably, DTPA-mediated mutations in the histidine kinase gene, *NIK1,* block the activation of Hog1 in response to the drug, thereby enhancing caspofungin activity. Notably, the mode in which this chelator potentiates echinocandin antifungal activity is distinct from the conventional mode of resistance to echinocandins, i.e., mutations in the target gene, *FKS1* (Polvi et al. 2016).

Owing to the fact that calcium acts as a second messenger, its signaling has been associated with regulation of myriad cellular stress responses including response to drug-induced stress. Perturbation of the intricate balance between calcium import and storage has been seen to result in inability to respond appropriately to antifungals (Liu et al. 2016). Additionally, its involvement in response to drug became more evident with the depletion of calcium levels, either by chelating extracellular calcium with ethylene diamine tetra-acetic acid (EDTA) or the inhibition of calcium importers with benidipine and nifedipine, leading to enhanced azole activity against *C. albicans* (Butts et al. 2017).

Together, this implies that the bioavailability of certain metals in distinct niches within the host may also have a profound impact on drug susceptibility. Hence, the impact of metal depletion provides a proficient strategy to exploit cellular pathways governing morphogenesis, virulence traits, and drug resistance.

6.4.3 Thermal Stress Response Regulators

Fungi occupy diverse environmental niches, and therefore encounter heterogeneous stress conditions. Interestingly, adaptive responses to commonly encountered cellular stresses have diverged considerably across the fungal kingdom. Surprisingly, the response to thermal fluctuations or the heat shock response has been evolutionarily conserved to a great extent in *Candida* species. In *C. albicans*, the heat shock element (Hsf1–HSE) regulon is crucial for the maintenance of thermal homeostasis, wherein, Hsf1, the master regulator and premier transcription factor mediates heat shock response via multiple signaling cascades (Leach et al. 2012). Hsp90 plays a major role in the regulation of the Hsf1–HSE regulon by functioning in an autoregulatory circuit comprising Hsf1 and Hsp90 modulating both short- and long-term thermal adaptations. Notably, both Hsf1 and Hsp90 are essential for the growth and viability of evolutionarily divergent yeasts, for example, *S. cerevisiae, Schizosaccharomyces pombe,* and *C. albicans*, even under normal growth conditions. Apart from mentioned conventional heat shock responsive roles, these thermal stress regulators also drive considerable attention, owing to their propensity to orchestrate cellular sig-

naling that governs drug resistance and morphogenesis in diverse fungal pathogens (Nair et al. 2017; Shapiro et al. 2012; Nicholls et al. 2009).

6.4.3.1 Hsp90-Mediated Drug Resistance

In eukaryotes, Hsp90 is amply present in the cell even at normal temperatures and is chiefly induced under stress conditions (Cowen and Lindquist 2005). Recently, it has been shown that depletion of Hsp90 interferes with the development of resistance to azoles and echinocandins. It is well established that Hsp90 signaling mediates azole resistance in *C. albicans* and *S. cerevisiae* by employing its client protein, calcineurin (Cowen et al. 2006). This regulatory circuitry essential for response to drug-induced stress, coordinated by Hsp90 relies on its post-translational modifications like phosphorylation that enables it to stabilize and interact with its client proteins like calcineurin. By binding to and chaperoning calcineurin, Hsp90 regulates calcineurin-dependent stress responses, thereby enabling the cell to survive the membrane stress induced by azoles (Fig. 6.2). Additionally, clinical evidences also reveal that patients receiving calcineurin-inhibitor-based immunosuppressive therapy develop lesser invasive fungal infections as compared to those on other regimens. Hence, the pharmacological inhibition of either Hsp90 or calcineurin not only blocks the emergence of azole resistance in *C. albicans*, but also abrogates resistance of laboratory mutants and clinical isolates that evolved resistance in human host (Cowen and Lindquist 2005).

More recently, the implications of Hsp90-mediated resistance was further extended in the case of an azole resistant, ABC transporter mutant, Δ*CDR6*. The resistance of the mutant to azoles was attributed to Tor1 (target of rapamycin) hyperactivation that resulted in Hsp90-dependent calcineurin stabilization, and thereby contributing to increased azole resistance in the mutant (Khandelwal et al. 2017). Parallelly, some other studies have also shown that inhibition of Tor1 leads to inhibition of Hsp90 activity, resulting in hypersensitivity to azoles in *S. cerevisiae* and *C. albicans* (Shekhar-Guturja et al. 2016).

The commonly used echinocandins inhibit the synthesis of β-1,3-D-glucan, a prime component of the fungal CW. Interestingly, both Hsp90 and calcineurin regulate the maintenance of CW integrity in response to echinocandin-mediated cell wall stress, by upregulating CW components and CW integrity signaling pathways (Singh et al. 2009). This is observed not only in *C. albicans* but also in *A. fumigatus*. While the tolerance to CW targeting antifungal drugs is mostly associated with the protein kinase C (PKC) pathway, the role of PKC signaling in mediating responses to azoles and other drugs targeting the ergosterol biosynthesis pathway, via downstream effectors linking Hsp90 is recently demonstrated. Not only was the activation of calcineurin on exposure to drugs dependent on PKC signaling, but also compromising PKC function, phenocopies inhibition of Hsp90, or its client protein, calcineurin (Lafayette et al. 2010).

It is, therefore, proposed that by inhibiting Hsp90, not only can the efficacy of the currently used antifungals be exalted, but also the emergence of drug resistance

can be curtailed (Cowen 2009), thus making this thermal stress response regulator a promising target with therapeutic potential for myriad fungal diseases.

6.4.3.2 Hsf1-Mediated Drug Resistance

Human Hsf1 induces a multidrug resistance phenotype toward the commonly used anti-cancerous drug, doxorubicin, in a non-heat-shock-dependent manner that is mediated partly through the constitutive activation of the multidrug resistance gene 1 (*MDR1*). While in *S. cerevisiae*, besides its traditional roles, Hsf1 is linked to oxidative and heavy metal stress response, *C. albicans* Hsf1 is indispensable for its growth and virulence (Nicholls et al. 2011). That similar to Hsp90, Hsf1 could also be correlated to drug resistance in *C. albicans* which became evident from recent studies by Dhamgaye and coworkers (Dhamgaye et al. 2014) where for the first time it was demonstrated that Hsf1 mutant is not only sensitive to the herbal alkaloid, berberine, but also exhibited collateral susceptibility toward drugs targeting cell wall (CW) and ergosterol biosynthesis. The drug susceptibility perceived was, however, independent of known attributes of MDR (Tchenio et al. 2006). For instance, the expression of major coordinators of conventional drug resistance, *CDR1, CDR2, MDR1, ERG11, TAC1, and UPC2,* remains unaltered in the Hsf1 conditional mutant both in presence and absence of berberine (Dhamgaye et al. 2014). Additionally, more recently an intricate relationship between cellular iron and Hsf1-mediated drug susceptibility of *C. albicans* is disclosed, wherein iron deprivation further enhanced Hsf1-mediated drug susceptibility (Fig. 6.2). At normal temperatures (30 °C), the Hsf1 mutant showed susceptibility not only to azoles like fluconazole but also to oxidative stress conditions. This susceptibility could be attributed to low intracellular levels of iron in the Hsf1 mutant at basal conditions as these non-heat shock phenotypes observed could be rescued upon exogenously supplementing iron (Nair et al. 2017). Additionally, it has also been observed from genome-wide ChIP-on-Chip studies that Hsf1 associates to the promoters of genes involved in a myriad of functions under both basal- and iron-deprived conditions, highlighting its ubiquitous roles in *C. albicans* (Nair et al. 2018). Taken together, these observations bring together the importance of nonconventional contributors to drug resistance—metal homeostasis and thermal stress response.

6.5 Perspectives

Conventionally, drug resistance has been typically perceived from a mechanistic perspective, as in, recognizing the cellular determinants that prevent a drug from entering a cell, diverse modes, or cellular proteins involved in drug extrusion, inactivating a drug or impeding a drug from inhibiting its target. However, studies over the past decade present a different outlook toward antifungal resistance, mainly considering the revelation of cellular pathways which integrate with antifungal resistance mech-

anisms. Therefore, it is of much importance to consider antifungal drug resistance as an evolving network and not treat each mechanism as a separate entity. Further, clinical components of drug resistance should not be ignored. Finally, emphasis should be laid on the search for novel agents, which can augment existing therapies. In this regard, inhibitors for components of cellular pathways such as stress response pathways hold promise for usage in combination therapy alongside known antifungal drugs.

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Chapter 7
Genome-Wide Response to Drugs and Stress in the Pathogenic Yeast *Candida glabrata*

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Abstract *Candida glabrata* is the second most common cause of candidemia worldwide and its prevalence has continuously increased over the last decades. *C. glabrata* infections are especially worrisome in immunocompromised patients, resulting in serious systemic infections, associated to high mortality rates. Intrinsic resistance to azole antifungals, widely used drugs in the clinical setting, and the ability to efficiently colonize the human host and medical devices, withstanding stress imposed by the immune system, are thought to underlie the emergence of *C. glabrata*. There is a clear clinical need to understand drug and stress resistance in *C. glabrata*. The increasing prevalence of multidrug resistant isolates needs to be addressed in order to overcome the decrease of viable therapeutic strategies and find new therapeutic targets. Likewise, the understanding of the mechanisms underlying its impressive ability thrive under oxidative, nitrosative, acidic and metabolic stresses, is crucial to design drugs that target these pathogenesis features. The study of the underlying mechanisms that translate *C. glabrata* plasticity and its competence to evade the immune system, as well as survive host stresses to establish infection, will benefit from extensive scrutiny. This chapter provides a review on the contribution of genome-wide studies to uncover clinically relevant drug resistance and stress response mechanisms in the human pathogenic yeast *C. glabrata*.

Keywords *Candida glabrata* · Drug resistance · Host stress resistance · Stress response · Genome-wide analyses

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7.1 Introduction

Candida spp. are commensals that asymptomatically colonize healthy humans but act as opportunistic pathogens in immunocompromised patients. The incidence of infections caused by *Candida* spp. has increased considerably over the past three decades, mainly due to the rise of the AIDS epidemic, an increasingly aged population, higher numbers of immunocompromised patients and the widespread use of indwelling medical devices (Silva et al. 2012). Especially concerning is the occurrence of mortality rates up to 40% associated with systemic candidiasis (Pfaller and Diekema 2007).

Although *Candida albicans* remains the predominant causative agent of all forms of candidiasis, candidaemia epidemiology has changed in the past few decades. This changing is characterized by a shift in species distribution, from a preponderance of *C. albicans* to more frequent isolation of less drug-susceptible *Candida* species such as *Candida glabrata*. *C. glabrata* is an important human fungal pathogen, being currently the second-most frequent cause of candidiasis, causing 15–30% of these infections in humans (Schwarzmüller et al. 2014). The number of invasive candidiasis reports caused by *C. glabrata* has continuously increased over the last decades, a phenomenon that has been associated with its ability to resist antifungal therapy (Le et al. 2017; Singh-Babak et al. 2012). Indeed, *C. glabrata* has an unusual ability to rapidly acquire resistance to azole antifungals, which limits their clinical effectiveness (Whaley and Rogers 2016). Other features that underlie *C. glabrata* pathogenesis include its ability to adapt to host stresses and evade the immune system (Miramón et al. 2013).

C. glabrata is phylogenetically closer to the non-pathogenic yeast *S. cerevisiae* than to *C. albicans* and other *Candida* spp. from the CUG clade. This is reflected in the distinct strategies that *C. glabrata* applies to adapt to host niches and survive within the host, when compared to *C. albicans*. *C. glabrata* does not form true hyphae (it only produces pseudo-hyphae under nitrogen limitation (Csank and Haynes 2000), therefore it does not actively invade host tissues or causes damage during colonization, as observed for *C. albicans* (Brunke and Hube 2013; Kasper et al. 2015). Similarly, it does not exhibit relevant levels of secreted protease activity, another virulence mechanism displayed by *C. albicans* (Naglik et al. 2004; Kaur et al. 2005). Rather, it is hypothesized that *C. glabrata* may penetrate host tissues through induction of endocytosis by epithelial cells (Li et al. 2007).

Upon phagocytosis, *C. albicans* actively escapes from phagocytic cells by pyroptosis or by producing hyphae and bursting the phagocyte membrane (Wellington et al. 2014; Uwamahoro et al. 2014). *C. glabrata*, due to lack of hyphae production, does not actively escape from immune cells. Alternatively, it survives phagocyte-mediated stress and continues to replicate within phagocytes, eventually lysing these cells due to overwhelming intracellular fungal load (Seider et al. 2011; Dementhon et al. 2012). In general, *C. glabrata* adopts an infection strategy based on persistence, rather than active invasion. The referred traits of *C. glabrata* infection strategies and immune interaction result in a low inflammatory response, verified by low cytokine

production (Li et al. 2007; Schaller et al. 2002; Jacobsen et al. 2010), which could be an important attribute in the establishment and persistence of infections.

In general, the molecular mechanisms underlying virulence features and response to host stress are poorly understood in *C. glabrata*. In order to shed some light on the molecular basis of its pathogenicity, this chapter analyzes *C. glabrata* genome-wide responses to antifungal drugs and stresses encountered inside the human host.

7.2 Response and Resistance to Drugs in *C. glabrata*: A Genome-Wide View

Drug resistance poses a significant challenge in antifungal therapy since resistance has been found in *C. glabrata* clinical isolates for all known classes of antifungal drugs. Currently available antifungals belong to four major drug classes: polyenes (e.g. amphotericin B), azoles (e.g. fluconazole), echinocandins (e.g. caspofungin), and pyrimidine analogs (e.g. 5-fluorocytosine). These antifungals are able to disrupt membrane integrity, ergosterol biosynthesis, cell wall function, and DNA/RNA/protein synthesis, respectively. Although they are extensively used, the drugs within each class hold various limitations besides the development of resistance, including toxicity (polyenes), narrow spectrum of activity (echinocandins), and lack of efficacy as a single agent (5-fluorocytosine). Considering this, the development of new drugs and/or the sighting of new drug targets is urgent in order to fight increasing *Candida*-associated infections.

7.2.1 Azoles

Azoles are by far the most common antifungal drugs in clinical use to treat or prevent fungal infections (Sheehan et al. 1999). They exert their function through the impairment of ergosterol biosynthesis, leading to fungal growth inhibition. Azoles bind to and inhibit the activity of the cytochrome P450 lanosterol 14-α-sterol-demethylase, which is a key enzyme in the ergosterol biosynthesis pathway encoded by the *ERG11* gene in yeasts. Ergosterol is the main sterol component in fungal cell membranes and its depletion damages the cell membrane resulting in defective structural properties, loss of fluidity and altered functions such as signaling, transport, exocytosis and endocytosis. Moreover, inhibition of Erg11 activity leads to the accumulation of alternate and toxic sterol precursors, for instance lanosterol, 4,14α-dimethyl zymosterol and 14α-methyl ergosta 8,24(28)-dien-3β,6α-diol, leading to growth inhibition (Whaley et al. 2014).

Azoles are five-membered heterocyclic synthetic compounds containing a nitrogen atom and at least one other non-carbon atom (e.g. nitrogen, sulphur, or oxygen) as part of the ring (Eicher et al. 2012). They can be divided into imidazole

(clotrimazole, miconazole, thioconazole, econazole and ketoconazole) and triazole (fluconazole, itraconazole, voriconazole and posaconazole) derivatives, as they have two or three nitrogen atoms in their azole ring, respectively. The imidazole class (particularly ketoconazole) was a tremendous breakthrough and quickly became the drug of choice for many fungal infections for nearly a decade. However, because of their limited spectrum of activity, high toxicity, severe side effects and numerous interactions with other drugs, they were replaced by the triazoles and are currently limited to the treatment of superficial mycosis (Yan et al. 2013; Pais et al. 2016a). The first-generation triazoles (fluconazole and itraconazole) were groundbreaking, exhibiting a broader antifungal activity spectrum as compared to imidazoles and having a significantly improved safety profile. However, both have clinical limitations such as their fungistatic nature instead of fungicidal, leading to increased probability of resistance outbreaks. To solve this problem, second-generation triazoles (voriconazole and posaconazole) were developed. They are considered fungicidal and have a broad spectrum of activity (Petrikkos and Skiada 2007). Nevertheless, although being inactive against infections caused by filamentous fungi and having a narrower spectrum activity, the first-generation triazole fluconazole is still the most widely used drug in the treatment of *Candida* infections due to its favorable bioavailability and safety profile (Sun et al. 2018).

C. glabrata infections have risen dramatically in frequency since the introduction of azole drugs in the 1980s, in part due to its rapid acquisition of resistance to these antifungal drugs, which are very effective in eradicating infections caused by other *Candida* species (Wiederhold 2017). In fact, the increasing number of *C. glabrata*-associated infections has been accompanied by an increase in the prophylactic use of azoles for high-risk individuals (Shapiro et al. 2011; Anderson 2005), which undoubtedly contributes to the development of resistance. Additionally, *C. glabrata* presents higher levels of intrinsic resistance to azoles than *C. albicans* and develops further resistance during prolonged azole therapy. Previous studies reported that the average fluconazole minimum inhibitory concentration (MIC) for *C. glabrata* is 32-fold higher than for *C. albicans* (Borst et al. 2005).

The major described mechanism of acquired azole resistance in *C. glabrata* clinical isolates is the increased drug efflux due to the upregulation of drug efflux pumps (vanden Bossche et al. 1992; Sanglard et al. 1999). This is caused by Gain-Of-Function (GOF) mutations within the gene encoding the key transcriptional regulator of drug resistance, *CgPDR1* (Vermitsky and Edlind 2004), which increases the expression of ATP-binding cassette (ABC) transporter-encoding genes, *CgCDR1* and *CgCDR2,* that catalyze the extrusion of azole drugs (Sanglard et al. 2001).

In *C. albicans*, mutations in the gene encoding azoles target, *ERG11,* are reported to be one of the main mechanisms underlying azole resistance. Interestingly, despite the potential for *ERG11* point mutations to have a greater impact in haploid organisms, as is the case of *C. glabrata*, several studies suggest that mutations in *ERG11* are not involved in clinical azole resistance in this pathogen (Vermitsky and Edlind 2004; Caudle et al. 2011; Sanguinetti et al. 2005).

Otherwise, a high frequency of acquired azole resistance in vitro in *C. glabrata* populations has been linked to loss of mitochondrial function (Defontaine et al. 1999).

It was proposed that this pathogen can switch between states of mitochondrial competence (azole-susceptible) and incompetence (azole-resistant) in response to azole exposure, probably through chromatin epigenetic modifications (Kaur et al. 2004). In fact, this human pathogen can live without mitochondrial DNA (mtDNA), and the drug-resistant mutants most commonly lost their mitochondrial genome (Sanglard et al. 2001; Kaur et al. 2004), (Hallstrom and Moye-Rowley 2000). This mitochondrial deficiency is called *petite* phenotype and corresponds to the absence of growth on non-fermentable carbon sources, deficient growth in media supplemented with glucose, reduced oxygen consumption and partial or total mtDNA deletion (Brun et al. 2004). It was first proposed that the respiratory deficiency observed in the *petite* mutants could promote the exhibited azole resistance, since the biosynthesis of P-450-dependent 14α-sterol demethylase is stimulated by anaerobic conditions (Defontaine et al. 1999). Nonetheless, loss of mitochondrial activity has been linked as well to increased drug efflux through constitutive activation of *CgPDR1* (Sanglard et al. 2001). In dysfunctional mitochondria, changes in the membrane association characteristics of the mitochondrial inner membrane protein Psd1 create a signal for activation of the transcription factor Pdr1, leading to upregulation of genes encoding efflux pumps which mediate drug resistance and have additional roles in phospholipid homeostasis, as well as genes required for sphingolipid metabolism (Shingu-Vazquez and Traven 2011). Although loss of mtDNA and associated drug resistance is relatively common in *C. glabrata* in vitro cultures, very few reports of clinical azole resistance are linked to mitochondrial dysfunction, and thus, the relevance of this mechanism in the clinic remains to be studied.

Although *C. glabrata* azole resistant isolates often exhibit *CgPDR1* GOF mutations, different mutations have been found to lead to various degrees of impact on CgPdr1 target genes, even if the mutations occur in the same domain/region of the gene (Tsai et al. 2010). For instance, strains with higher expression of *CgPDR1* seemed to always lead to higher expression levels of *CgCDR1*. On the other hand, the impact of *CgPDR1* GOF mutations on *CgCDR2* appeared to be much weaker when compared to *CgCDR1*. Moreover, not all *CgPDR1* GOF mutations lead to increased expression of *SQN2*, which is also an ABC transporter involved in *C. glabrata* drug resistance.

Interestingly, Ferrari and co-workers (Ferrari et al. 2009) have demonstrated that GOF mutations in *CgPDR1* also result in increased fitness and virulence. Additionally, they found that the only two genes coordinately upregulated by all *CgPDR1* GOF mutations present in their set of resistant isolates were *CgCDR1*—ABC transporter—and *CgPUP1*—mitochondrial protein with unknown function. Since the increased virulence phenotype was seen with all *CgPDR1* GOF mutations, these genes are suspected to be involved. In fact, deletion of *CDR1* and *PUP1* in an azole-resistant clinical isolate revealed that both genes were required for increased virulence.

7.2.2 Amphotericin B

Polyenes are macrocyclic organic molecules composed, most of them, by a 20–40 carbon macrolactone ring conjugated with a d-mycosimine group and they were the first antifungal drugs applied to clinical use (Mayers et al. 2017). These compounds are fungicidal and have the broadest spectrum of activity compared to any other antifungal molecules. Nystatin, natamycin and amphotericin B, natural products isolated from the cultivation broths of *Streptomyces noursei*, *S. natalensis* and *S. nodosum*, respectively, are the only three polyenes in clinical use (De Pauw 2000).

Due to their amphipathic structure, these antifungals act by binding to the lipid layer and form a complex with ergosterol, producing pores on the cell membrane, which increases cell permeability, leakage of cytoplasmic contents and oxidative damage resulting in fungal cell death (Rodrigues et al. 2014; Andes 2003). Nonetheless, polyenes have a lower but non-negligible affinity for cholesterol. This slight affinity for cholesterol explains the high toxicity associated with these antifungals and is responsible for numerous side effects (Lemke et al. 2005).

Nystatin and natamycin are only used as topical agents due to their low absorption in the gut and their high toxicity, while amphotericin B is the most used polyene for the treatment of systemic infections. Nonetheless, due to its hydrophobicity and poor absorption through the gastrointestinal tract, amphotericin B is administered intravenously which might cause adverse effects in kidneys and liver (Lemke et al. 2005; Odds et al. 2003).

For over 50 years, amphotericin B, as lipid formulations, has remained the powerful but toxic last line of defense in treating life-threatening fungal infections in humans with minimal development of microbial resistance (Anderson et al. 2014). However, resistance toward this antifungal has been found in many clinical isolates, including *C. glabrata* (Cho et al. 2014; Krogh-Madsen et al. 2006). The molecular mechanisms that lead to polyene resistance are still poorly documented, especially in non-*albicans* yeasts. However, mutations in the *ERG6* gene, encoding an enzyme involved in late steps of ergosterol biosynthesis, have been found in polyene resistant clinical isolates, apparently leading to decreased ergosterol concentration in the plasma membrane and accumulation of late sterol (Vandeputte et al. 2008; Vandeputte et al. 2007). Lower ergosterol concentration leads to decreased binding of amphotericin B, thus decreasing its toxic effect. It seems that *ERG6* mutants of *C. glabrata*, which are obtained more easily in this species than in diploid *Candida* species, may be selected by the prophylactic or therapeutic use of amphotericin B (Vandeputte et al. 2008).

In 2014, Schwarzmüller and colleagues (Schwarzmüller et al. 2014) generated a large-scale collection encompassing 619 bar-coded *C. glabrata* mutants, each lacking a single gene, and determined the susceptibility of the collection to major antifungal compounds, including amphotericin B. Among the 13 amphotericin B sensitive strains, the five mutants displaying the most pronounced susceptibilities lack genes that play diverse roles in phospho- and sphingolipid signaling, including *YPK1*, *CKA2*, *DEP1*, *SNF6* and *VPS15*. Additional determinants of resistance

include *KRE1* and *SAC7*, which encode proteins implicated in glucan homeostasis, as well as *KTR6*, *KTR2*, *CWH41*, whose products affect surface protein glycosylation. Further studies are needed to understand the roles of cell wall and phospho- and sphingolipid metabolism as resistance mechanisms against amphotericin B in *C. glabrata*. Besides, to the best of our knowledge, *C. glabrata* response to amphotericin B at the transcriptomic or proteomic level has never been addressed.

7.2.3 Flucytosine

Flucytosine (5-FC; 5-fluorocytosine) is one of the oldest synthetic antimycotic compounds, first synthesized in 1957 (Duschinsky et al. 1957). It is a fluorinated pyrimidine analog with fungistatic activity that interferes with pyrimidine metabolism, as well as RNA/DNA and protein synthesis. 5-FC itself has no antifungal activity. Its antimycotic activity results from the rapid conversion of 5-FC into 5-fluorouracil (5-FU) within fungal cells (Benson and Nahata 1988). 5-FC is taken up by a cytosine permease, which is also the transport system for adenine, hypoxanthine and cytosine. These compounds competitively antagonize the uptake of 5-FC (Polak and Grenson 1973). Once inside the cells it is converted by cytosine deaminase to 5-fluorouracil (5-FU) which is transformed by UMP pyrophosphorylase into 5-fluorouridine monophosphate (5-FUMP). This compound is then phosphorylated and incorporated into RNA, instead of UTP, resulting in inhibition of protein synthesis. 5-FU also undergoes conversion into 5-FdUMP (5-fluorodeoxyuridine monophosphate), a potent inhibitor of thymidylate synthase, that inhibits fungal DNA synthesis and nuclear division (Dowell et al. 2004). Flucytosine has the advantage of being selectively toxic to fungi as there is little or no cytosine deaminase activity in mammalian cells (Zhao et al. 2010). However, it's use is limited as it displays significant adverse effect, myelotoxicity and hepatotoxicity in particular, and should be used in combination with other antifungals as resistance rapidly develops with monotherapy (Roger et al. 2018).

Whether it is innate in some fungal species or acquired in others, resistance to 5-FC is a frequent phenomenon in pathogenic fungi. However, due to its complex mode of action, the molecular mechanisms leading to 5-FC resistance have been poorly investigated, particularly in the yeast *C. glabrata*. Although the most common mechanism of resistance to pyrimidine analogs is a point mutation in the *FUR1* gene, coding an uracil phosphoribosyltransferase (Chapeland-Leclerc et al. 2010), other mechanisms may contribute to 5-FC resistance which may be easily acquired in *C. glabrata*, probably due to its haploid genome. It was reported that point mutations in *FCY1*, coding the cytosine deaminase, and *FCY2*, coding a purine-cytosine transporter, genes and overexpression of the thymidylate synthase coded by *CDC21*, key enzyme of DNA synthesis, may provide 5-FC-resistant phenotype in *C. glabrata* (Vandeputte et al. 2011).

To our knowledge, a membrane proteomics analysis regarding *C. glabrata* response to 5-FC (Pais et al. 2016b), is the only study reporting the response of

this pathogen to flucytosine in a global perspective. The largest functional group identified in the 5-FC membrane proteome response, including a third of the proteins with altered content, is related to RNA metabolism. The expression of seven proteins involved in ribosome biogenesis and translation was found to increase in flucytosine stressed cells, which may be related to the specific mechanism of action of 5-FC. It is thus possible to assume that the RNA- and protein-metabolism-related genes identified in our study as responding to 5-FC challenge may be involved in counteracting its primary toxic action. Additionally, a group of five multidrug transporters was found to exhibit altered levels of expression in flucytosine stressed cells. Four of them, previously implicated in azole drug resistance (Sanglard et al. 1999; Vermitsky et al. 2006; Torelli et al. 2008), were actually found to be down-regulated, while the fifth, *CgFLR1*, was found to be more than 2-fold up-regulated upon *C. glabrata* exposure to flucytosine.

7.2.4 Echinocandins

Echinocandins are the only novel class of antifungal drugs approved for clinical use in decades (Shapiro et al. 2011). Their development has helped to fulfill the need for more efficacious and safer antifungal drugs, since the fungicidal effect exerted is accomplished by disrupting cell wall synthesis, an ideal target because no comparable structure is present in human cells (Rosenwald et al. 2016). Specifically, echinocandins (e.g., caspofungin, micafungin and anidulafungin) act as noncompetitive inhibitors of β-(1,3)-D-glucan synthase enzyme complex, specifically targeting the Fks1 subunit, which catalyzes the production of glucan the major component in *Candida* cell walls (Shapiro et al. 2011; Denning 2003). The disruption of (1,3)-β-D-glucans impairs the structure of growing cell walls, resulting in loss of structural integrity, osmotic instability and cell death.

Echinocandins present good safety profiles and their toxicity is very low due to their unique target, that is absent in mammalian cells. Additionally, interactions with other drugs are minimal (Petrikkos and Skiada 2007). However, despite these advantages, the pharmacokinetic and stability properties of the currently approved echinocandins impose limitations on their use. Because of short half-lives and poor oral absorption, they were each developed for once-daily administration by intravenous infusion (Krishnan et al. 2017). A novel echinocandin, rezafungin (CD101), is presently being developed as a once-weekly intravenous formulation for the treatment of candidemia and invasive candidiasis. Rezafungin has potent in vitro activity against *C. albicans* and *C. glabrata*, including azole- and echinocandin-resistant isolates (Lakota et al. 2018). The stability and solubility features of CD101 not only provide advantages for manufacturing and storage, but also enable expansion of echinocandin use to include weekly intravenous infusions and topical and subcutaneous forms (Krishnan et al. 2017).

Generally, MICs of echinocandins are much lower than for amphotericin B and fluconazole against all *Candida spp*. (Matsumoto et al. 2014), since most *Candida*

species have a low rate of echinocandin resistance (Pfaller and Diekema 2007). Nevertheless, recent studies reported that echinocandin resistance is more common in *C. glabrata* compared to other species and that this rate can be attributed to the high potential of *C. glabrata* for developing resistance mutations (Matsumoto et al. 2014; Dannaoui et al. 2012). It has been reported that the reduced susceptibility to echinocandins is due to mutations in "hot-spot" regions of the *CgFKS1* and *CgFKS2* genes, which encode subunits of the glucan synthase enzyme (Katiyar et al. 2006; Costa-de-Oliveira et al. 2011). Such mutations alter the kinetics of the target glucan synthase resulting in significantly higher MIC levels and inhibition constant (Garcia-Effron et al. 2009a).

Although echinocandin resistance is systematically associated with point mutations in either *CgFKS1* or *CgFKS2* genes (Singh-Babak et al. 2012; Sanglard 2016; Garcia-Effron et al. 2009b; Cowen et al. 2002), cell wall integrity signaling mediated via protein kinase C (PKC), the protein phosphatase calcineurin, and the molecular chaperone Hsp90 are vital in enabling echinocandin drug tolerance and compensatory mechanisms such as upregulation of chitin synthesis (Singh-Babak et al. 2012; Singh et al. 2009; Stevens et al. 2006). Interestingly, it was seen that Hsp90 and calcineurin regulate echinocandin resistance by controlling expression of the resistance determinant *CgFKS2*, providing a novel mechanism via which Hsp90 and calcineurin contribute to echinocandin resistance in *C. glabrata* (Singh-Babak et al. 2012).

Recently, Schwarzmüller and colleagues (Schwarzmüller et al. 2014) identified 48 *C. glabrata* mutants with altered caspofungin susceptibilities, among the 619 single deletion strains tested, 38 of which were strongly hypersensitive while another 10 were mildly sensitive. Remarkably, the group of genes found to affect caspofungin sensitivity included several genes playing a role in the PKC cell integrity signaling pathway, calcium/calcineurin signaling, general cell wall homeostasis, including mannosylation and glycosylation, as well as transcriptional regulators. Deletion of several genes encoding components of the chromatin and histone modification machinery also modulated caspofungin susceptibility, suggesting an important regulatory role for chromatin in controlling surface homeostasis and caspofungin susceptibility. Hyperresistance to echinocandins can result from mutations in glucan synthase genes as well as through PKC pathway that mediates caspofungin tolerance in the phylogenetically close yeast *S. cerevisiae* and in *C. albicans* (Markovich et al. 2004; Walker et al. 2008). Accordingly, *C. glabrata Δwsc1*, *Δslt2*, *Δmkk1*, *Δbck1*, *Δrlm1* and *Δfks1* mutants, all lacking key genes of this central pathway, were found to be hypersensitive to caspofungin.

Rosenwald and co-workers (Rosenwald et al. 2016) also applied a global approach to identify genes important for cellular resistance to caspofungin in *C. glabrata*. They screened a collection of ∼27,000 *C. glabrata* insertion mutants, covering about 75% of the nonessential genes in the genome, for altered sensitivity to caspofungin. 48 genes were identified as determinants of caspofungin resistance. However, the list of caspofungin resistance genes identified in this study, compared to that performed by Schwarzmüller et al. (2014) only overlap in a total of 8 genes (*CgCNA1*, *CgSTL2*, *CgMKK1*, *CgPMT2*, *CgMNN10*, *CgCCH1*, *CgMID1*, *CgSIN3*). These include some controlling the cell-wall integrity (CWI) pathway, *CgMKK1*, *CgCNA1* and *CgSLT2*

(Chen et al. 2012; Cota et al. 2008), reinforcing the idea that chemical modulators of the CWI pathway may increase the efficacy of caspofungin. Interestingly, it has been reported that *S. cerevisiae* strains with deletions of CWI pathway genes *ScBCK1* and *ScSLT2* are sensitive to the anti-malarial drug chloroquine, and that chloroquine and caspofungin show synergy in *S. cerevisiae*, *C. albicans* and in *C. glabrata* (Islahudin et al. 2013). Moreover inhibition of Ca^{2+} influx via high affinity Ca^{2+} uptake system (HACS) complex may also increase the efficacy of echinocandins. In both screens, the disruption of the HACS members *CgCCH1* and *CgMID1* was found to confer increased caspofungin susceptibility. Nevertheless, none of these 8 genes are currently associated with clinical resistance, rather resistance in the clinic is associated primarily with "hot-spot" mutations in *CgFKS1* and *CgFKS2* (Singh-Babak et al. 2012; Krogh-Madsen et al. 2006; Vallabhaneni et al. 2015).

To the best of our knowledge, there is no transcriptomics or proteomics analysis of the changes in *C. glabrata* cells exposed to echinocandins or even between clinical resistant and susceptible isolate pairs. Therefore, it is likely that there are important resistance players still to be unveiled.

7.3 Drug Resistance in *C. glabrata*: A Genome-Wide Integrated Perspective

The last decade has seen a burst in the amount of biological data generated by an ever-increasing number of techniques enabling the simultaneous detection of a large number of alterations in molecular components (Schneider and Orchard 2011). As expected, the use of such genome-wide approaches has been applied to the sudy of drug resistance in *C. glabrata* as well, although with a limited extent when compared to *C. albicans* or *S. cerevisiae*.

In 2006, Vermitsky and co-workers (2006) reported the first application of microarrays-based transcriptomics analysis to *C. glabrata*, which enabled the identification of multiple genes that are co-regulated with *CgCDR1* and *CgCDR2* in fluconazole-resistant strains, when compared to susceptible strains, and are likely to impact *C. glabrata* resistance toward azoles. The major upregulated functional groups found were involved in transport, lipid, fatty acid or sterol metabolism, stress response, DNA metabolism, transcription and cell wall function, similarly to those reported to be upregulated by Tsai and colleagues (2010) in their azole-resistant isolates.

Collecting all available genome-wide expression data from the comparison of azole resistance vs azole susceptible isolates with azole exposed vs control cells in *C. glabrata* (Pais et al. 2016a; Caudle et al. 2011; Tsai et al. 2010; Vermitsky et al. 2006; Shen et al. 2015; Rogers et al. 2006; Salazar et al. 2018; Loureiro y Penha et al. 2010), only *CgCDR1*, *CgSNQ2*, *CgQDR2*, *CgHFD1*, *CgPGK1* and *CAGL0L01485g*, were found to be both upregulated in *C. glabrata* cells exposed to azole drugs and upregulated in azole-resistant strains (Fig. 7.1). However, it is important to point

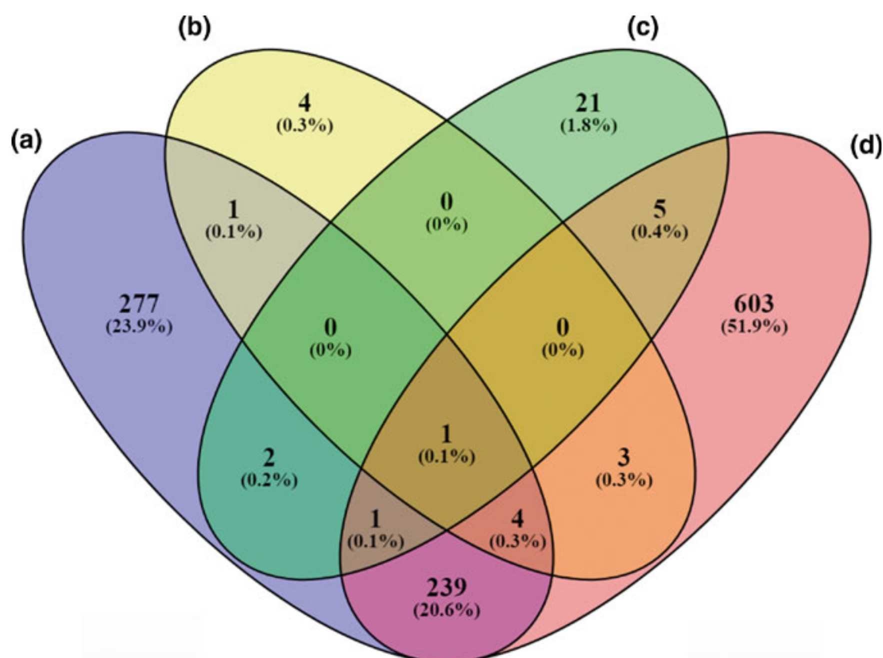

Fig. 7.1 Response and resistance to azoles in *C. glabrata*: the Influence of *CgPDR1*. **a** Genes found to be upregulated in azole-resistant versus azole-susceptible strains (Caudle et al. 2011; Tsai et al. 2010; Vermitsky et al. 2006; Shen et al. 2015; Rogers et al. 2006; Salazar et al. 2018; Loureiro y Penha et al. 2010). **b** Genes found to be upregulated in wild-type susceptible cells exposed to azole antifungals (Pais et al. 2016a). **c** Genes found to confer azole resistance based on knockout libraries screening (Schwarzmüller et al. 2014; Kaur et al. 2004). **d** Documented CgPdr1 targets (http://pathoyeastract.org (Monteiro et al. 2017))

out that, to the extent of our knowledge, there is only one study that compares the transcriptome or proteome of *C. glabrata* wild-type azole-susceptible strain in control conditions versus drug exposure (Pais et al. 2016a).

CgCdr1, CgCdr2, CgSnq2 and CgQdr2 are multidrug transporters known to be involved in *C. glabrata* azole drug resistance (Sanglard et al. 1999; Torelli et al. 2008; Miyazaki et al. 1998; Costa et al. 2013a), which is compatible with the predominant role of drug extrusion as a primary mechanism of defense against azoles used by *C. glabrata*. CgHfd1 is a putative mitochondrial fatty aldehyde dehydrogenase known to be induced in menadione-associated oxidative stress response (Roetzer et al. 2011). Its role in azole response and resistance may come from counteracting the apparent pro-oxidant effect of azoles (Mahl et al. 2015; Kobayashi et al. 2002). The roles played by CgPgk1, a putative 3-phosphoglycerate kinase, and *CAGL0L01485g*, encoding a putative endoplasmic reticulum (ER) protein involved in hexose transporters' targeting (Sherwood and Carlson 1999), in azole adaptive response, may

derive from their role in energy generation, much needed to fight back azole accumulation in *C. glabrata* cells (Kobayashi et al. 2002).

Since azole drug resistance in *C. glabrata* is highly associated, at the transcriptional level, with CgPdr1, we used the data gathered in the PathoYeastract database (Monteiro et al. 2017) to investigate which of genes found to be involved in azole response/resistance through different approaches are regulated by this transcription factor (Fig. 7.1). From all documented CgPdr1 targets (856), only about 30% appear to be involved in resistance/response to azoles. Since *CgPDR1* is considered the master regulator in this phenomenon and in its absence *C. glabrata* cells display severely decreased growth ability in the presence of azole antifungals, these numbers suggest that there may be still unidentified players controlling azole response.

Interestingly, the only azole response/resistance modulator found to be commonly identified through all genome-wide approaches considered in Fig. 7.1 is the multidrug transporter CgCdr1, highlighting the notion that it plays an incomparable role in azole resistance.

In the quest for potential genes involved in *C. glabrata* resistance against multiple antifungal drugs, large-scale *C. glabrata* deletion libraries were created. All information regarding *C. glabrata* knockout collection studies on drug resistance (Schwarzmüller et al. 2014; Kaur et al. 2004; Rosenwald et al. 2016) was collected.

In 2004, Kaur et al. (2004) generated a collection of 9,216 random insertion mutants, whose screening enabled the identification of 17 fluconazole resistance determinants. Later, Schwarzmüller and co-workers (2014) generated a large-scale collection of 619 bar-coded *C. glabrata* knockout mutants, and determined their susceptibility to azoles (fluconazole and voriconazole), amphotericin B and caspofungin, finding 38, 14 and 13 determinants of resistance to caspofungin, azoles and amphotericin B, respectively. Rosenwald and colleagues (2016) carried out the same type of study, but focusing in caspofungin resistance. To the best of our knowledge no genome-wide screening for *C. glabrata* determinants of resistance to flucytosine was performed.

In the pursuit for potential genes involved in *C. glabrata* resistance against multiple antifungal drugs, a comparison of genes involved in either the response or the resistance to azoles, amphotericin B, echinocandins and flucytosine was carried out (Fig. 7.2). The number of overlapping genes is quite reduced, or even absent in some cases, which is consistent with the distinct mechanisms of action of the different antifungal drug families. Nevertheless, it should be noted that there are very few genome-wide studies regarding *C. glabrata* response/resistance to drugs other than azoles. Therefore, it is likely that the overlap between the genes related to azole response/resistance and the other considered antifungals is underestimated.

There are 3 genes reported to be important for resistance to both azoles and amphotericin B: *CAGL0M05841g* (ortholog to *S. cerevisiae KTR2*), *CgCKA2* and *CgCWH41*. Despite azoles specifically target Erg11 and amphotericin B directly bind to ergosterol, both antifungals have the cell membrane as target, which might explain this overlap. CgCka2, for instance, is a catalytic subunit of casein kinase 2, involved in regulation of sphingolipid biosynthesis being essential for membrane homeostasis (Healey et al. 2012).

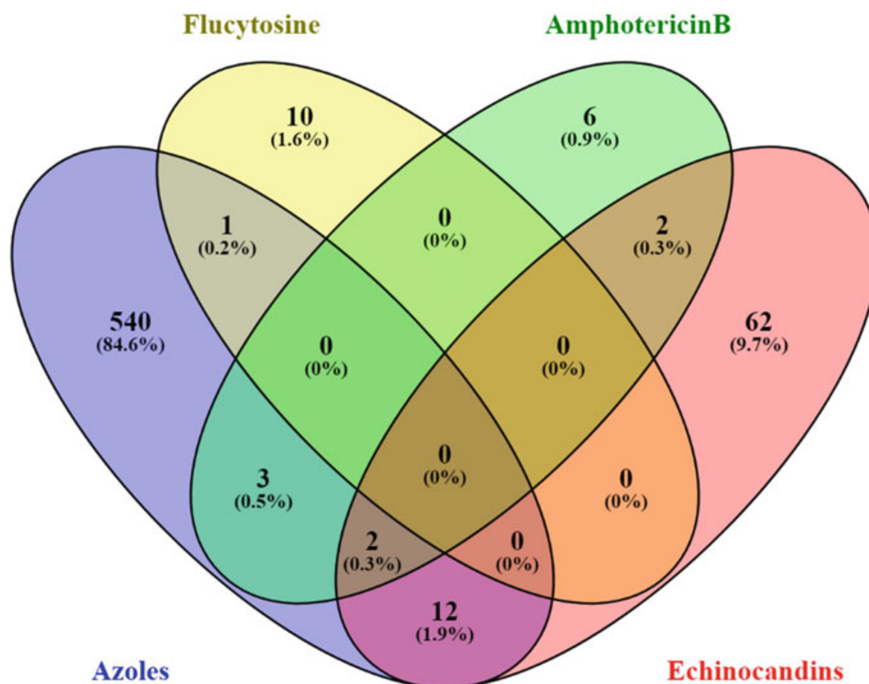

Fig. 7.2 Genes involved in resistance and/or response to multiple drugs in *C. glabrata*. Data gathered from transcriptomics, proteomics or knockout library screening studies focused on the resistance or response to azoles (blue) (Schwarzmüller et al. 2014; Pais et al. 2016a; Caudle et al. 2011; Defontaine et al. 1999; Tsai et al. 2010; Vermitsky et al. 2006; Shen et al. 2015; Rogers et al. 2006; Salazar et al. 2018; Loureiro y Penha et al. 2010), flucytosine (yellow) (Pais et al. 2016b), amphotericin B (green) (Schwarzmüller et al. 2014) and echinocandins (red) (Schwarzmüller et al. 2014; Rosenwald et al. 2016) in *C. glabrata*

Notably, even though they have different targets, there are, at least, 12 genes involved in the response/resistance to both echinocandins and azoles: *CgPDR16*, *CgCCH1, CgMID1, CgSTV1, CgECM7, CgCNB1, CgCNA1, CgSLG1, CgSLT2, CgPST1, CgSWI4* and *CAGL0K03377g*. CgCnb1 and CgCna1 are involved in cell wall integrity through calcineurin signaling pathway (Chen et al. 2012), while CgCch1, CgMid1 and CgEcm7 are membrane proteins responsible for Ca^{2+} uptake. These observations suggest that calcium signaling and cell wall integrity are required for the adaptation of *C. glabrata* cells to both stresses.

The same happens with amphotericin B and echinocandins, although having different cell targets, there are 2 genes involved in the response/resistance to both drugs: *CgDEP1* and *CAGL0E02783g*. *CgDEP1* is an ortholog of *S. cerevisiae DEP1* which is a component of the Rpd3L histone deacetylase complex and transcriptionally modulates the regulation of structural phospholipid biosynthesis genes, thus playing a role in membrane homeostasis (Lamping et al. 1994). *CAGL0E02783g* is ortholog

to *S. cerevisiae SLA1* which is a cytoskeletal protein binding protein required for assembly of the cortical actin cytoskeleton (Holtzman et al. 1993).

Interestingly, genes involved in *C. glabrata* response to flucytosine seem to be very exclusive, having only 1 overlap with azole induced genes (*CAGL0E03201g*, a putative enzyme that catalyzes the first step of phosphatidylcholine biosynthesis). This might be due to the fact that the mechanism through which 5-FC exerts its antifungal activity is completely different from the other antifungals. Whereas amphotericin B and azoles target cell membrane and echinocandins target the cell wall, 5-FC interferes with pyrimidine metabolism, as well as RNA/DNA and protein synthesis. Nevertheless, it should be noted that there is very few data regarding *C. glabrata* genome-wide response to this antifungal, thus it is likely that there are many other important players yet to be identified.

Altogether, these findings highlight the need for genome-wide studies addressing *C. glabrata* resistance/response toward antifungals, especially those that are not azoles. The information provided by these studies would be important to improve our understanding concerning *C. glabrata* response that enables its rapid acquisition of resistance. The ultimate goal would be finding new targets in order to develop suitable therapeutic strategies that could target multiple drug resistance pathways.

7.4 Response and Resistance to Pathogenesis-Associated Stresses in *C. glabrata*: A Genome-Wide View

One of the main features that makes *C. glabrata* a successful pathogen is its ability to survive confrontation with the host immune system. As such, the study of the interactions of *C. glabrata* cells with macrophages and neutrophils is a promising research topic to better understand the pathogenic nature of this yeast. Once internalized by phagocytes, *C. glabrata* has to cope with a panoply of antimicrobial strategies applied by host immune cells: nutrient limitation, oxidative and nitrosative stress, phagosomal pH acidification and production of antimicrobial peptides (Otto and Howard 1976; Kaur et al. 2007).

A study probing a library of *C. glabrata* mutants led to the identification of 56 genes required for survival within human macrophages (Rai et al. 2012). It was observed that genes involved in DNA repair (*DNA2*, *MRE11*, *RAD5*, *RTT107* and *SGS1*) and chromatin organization (*ARP7*, *CHZ1*, *FPR4*, *HFI1*, *RSC3* and *RTT109*) contribute to *C. glabrata* survival/replication in macrophages. Moreover, differential chromatin modification was seen to take place in macrophage-internalized *C. glabrata* cells, as different chromatin architectures were detected over the course of macrophage internalization. Chromatin remodeling during survival and/or replication in macrophages was postulated to be important for survival to Reactive Oxygen Species (ROS)-induced DNA damage, rewiring of transcriptional networks to adapt to poor-nutrient conditions, activation of stress signaling pathways and cell wall integrity (Rai et al. 2012).

Macrophage-ingested cells also show altered epigenetic signature, with elevated histone methylation and diminished acetylation marks. Other than histone modification, reduced protein acetylation levels can be used to modulate the activity of metabolic enzymes or generate an acetate pool to the generation of energy through acetyl-CoA. Although genes involved in acetyl-CoA production (*ACS1* and *ACS2*) are upregulated in response to exposure to macrophage environment, it is unclear if the acetate resulting from protein deacetylation enters metabolic pathways (Rai et al. 2012). Reduced histone acetylation modifies the chromatin to an inactive state to suppress transcription, regulate cell cycle and protect against DNA damage. Together with the activation of DNA damage signaling upon macrophage internalization, these observations indicate that a concerted action between chromatin remodeling and/or DNA damage response may contribute to survivability of *C. glabrata* cells in macrophages (Rai et al. 2012).

The fungal cell wall establishes the interface between fungal and host cells, mediating the interaction between the two. This comprises mutual recognition and activation of the correspondent signal transduction pathways. Concurrently, cell wall integrity and remodeling are correlated with *C. glabrata* survival within macrophages, as well as virulence in a murine model of disseminated candidiasis (Kaur et al. 2007; Seider et al. 2014). Cell wall metabolism upon macrophage internalization is associated with the putative GPI-linked aspartyl proteases of the *YPS* gene family. Several genes from this family were reported to be upregulated upon phagocytosis by both macrophages and neutrophils (Kaur et al. 2007; Fukuda et al. 2013). The *YPS* gene family in the phylogenetically close *S. cerevisiae* is associated with cell integrity. Phenotypic characterization showed that *C. glabrata YPS1* and *YPS7* are critical for cell wall integrity, although the expression of either gene is unaltered during macrophage contact (Kaur et al. 2007). On the other hand, the *YPS* genes that are upregulated upon macrophage internalization (*YPS2/4/5/8/9/10/11*) do not display an apparent role in cell wall integrity, but are required for the modulation of macrophage activation (NO• production) and have a role in *C. glabrata* survival inside the host (Kaur et al. 2007). The induction of *YPS* genes is also reported to take place in neutrophil phagocyted *C. glabrata* (Fukuda et al. 2013). The role of the Yps proteases in cell wall remodeling has been proposed to be related with the processing (namely degradation/removal) of cell wall proteins, such as the adhesin Epa1 (Kaur et al. 2007). Remodeling of the cell surface through removal of cell wall proteins was proposed to lead to the incorporation of more suited cell wall proteins according to different niches. Moreover, removal of such proteins could be an important mechanism to avoid immune recognition and immune attack, as, for example, Epa1 induces immune cell adhesion, phagocytosis and cytokine production (Swanson 2008).

Accessibility of β-glucan and/or chitin in the *C. glabrata* cell wall also modulates the inflammatory response of macrophages by altering TNF-α proinflammatory cytokine production, meaning that cell wall alterations influence the recognition of *C. glabrata* cells and that these factors ultimately influence the ability of *C. glabrata* cells to survive macrophage attack (Seider et al. 2014). Additionally, β-glucan exposure and chitin content were also found to influence ROS production (Seider et al. 2014), thus interconnecting cell wall integrity and remodeling with activation of the

host immune system. Moreover, changes in cell surface may also influence pathogen uptake by phagocytic cells, possibly by contributing to evade immune recognition (Gow et al. 2007; Netea et al. 2006). A relevant role of cell wall in macrophage-mediated stress resistance is further emphasized by the fact that there is a significant enrichment of caspofungin sensitivity phenotypes in macrophage-attenuated survival phenotypes, indicating that maintenance of a robust cell wall is crucial for surviving the adverse environment of the phagosome (Seider et al. 2014).

Illustrating the number of factors that may influence survival of *C. glabrata*, a mutant library screening for reduced viability of *C. glabrata* within human monocyte-derived macrophages identified several genes involved in biological processes that affect yeast survivability. These comprise cell membrane and cell wall biogenesis (*LRG1, GNT1, ERG5, SLG1*), nutritional response (*ARG81, GPR1, GPA2, FRE8, CAGL0M12496g*), response to stress (*CCH1, SLM1, SHO1*), protein glycosylation (*OST6, MNN4, MNS1, PMT2, PMT4*) and genes with other functions (*BAR1, CDC12, CKA2, CAGL0G07887g*) (Seider et al. 2014). The majority of these genes was found to affect *C. glabrata* replication rate inside the macrophages, which can be correlated with the observed reduced survival phenotype of the correspondent mutants (Seider et al. 2014).

7.4.1 Nutrient Limitation Response

Upon phagocytosis, *C. glabrata* is challenged with a nutrient-poor medium inside macrophages and neutrophils, especially suffering from carbohydrate and amino acid deprivation. As such, yeast cells must adapt their metabolic program to adjust to the new environmental conditions, which involves the utilization of alternative carbon sources and the ability to overcome nitrogen limitation.

A transcriptomics analysis of genome-wide *C. glabrata* response to murine macrophages upon phagocytosis shows that this response closely resembles the one described for *C. albicans* (Lorenz et al. 2004; Fradin et al. 2005). Phagocyted *C. glabrata* cells upregulate genes involved in gluconeogenesis (e.g. *FBP1* and *PCK1*), β-oxidation of fatty acids (e.g. *FAA2*, *FOX2*, *POT1*, *POX1*), glyoxylate cycle (e.g. *ICL1*, *ACO1*, *MLS1*) and methylcitrate cycle (*PDH1*, *CIT4*, *ICL2*). Additionally, the acetate transporter encoding gene *ADY2* is also upregulated. Similarly to what is observed in *C. albicans*, there is downregulation of translation (ribosomal protein genes, tRNA synthetases, translation initiation and elongation factors) after phagocytosis (Kaur et al. 2007).

Genome-wide transcriptional profiling of human macrophage-internalized *C. glabrata* cells revealed that internalized yeast cells utilize fatty acids as the main carbon source and can use acetyl-CoA from fatty acid oxidation via glyoxylate cycle to generate energy (Rai et al. 2012). As described previously concerning murine macrophages (Kaur et al. 2007), *C. glabrata* shuts down translational machinery, glycolysis and upregulates glyoxylate and citrate cycles (Rai et al. 2012). This is consistent with a known hallmark of macrophage-internalized fungal pathogens: repro-

grammed carbon metabolism based on increased nucleogenesis, glyoxylate cycle and fatty acid degradation (Lorenz and Fink 2001).

Notably, *RSC3-A* encoding a DNA binding protein and *RTT109* encoding a histone acetyltransferase were found to affect proliferation and epigenetic modifications in internalized *C. glabrata*. Transcriptional profiling revealed that these genes are required for the expression of respiratory metabolism and energy-related genes upon phagocytosis, thus implying a role for chromatin organization in cellular energy homeostasis (Rai et al. 2012). Plus, *C. glabrata* displays a similar epigenetic response post-macrophage internalization and under glucose-limiting conditions, strengthening the notion of a carbohydrate poor environment in the phagosome (Rai et al. 2012).

Transcriptomics analysis of human neutrophil-phagocyted *C. glabrata* cells shows a similar carbon and energy metabolism response compared to the one reported in murine macrophages. Namely, upon neutrophil contact, upregulated carbohydrate processes include gluconeogenesis (*FBP1*, *PCK1*, *TDH3*, *PYC1*), glyoxylate cycle (*ICL1*, *MLS1*) and utilization of extracellular trehalose (*NTH1*) (Fukuda et al. 2013). Trehalose has a protective role against environmental stresses (Fetter and Kwon-Chung 1996), and in vitro glucose starvation transcription profiling results in upregulation of trehalose biosynthesis genes (*TPS1/2*) and other central carbon metabolism genes (*GPH1, UGP1, GSY1, GLK1*) (Roetzer et al. 2008). Other than additional processes such as actin cytoskeleton organization (*HUA1*) and multidrug resistance (*PDR1*); autophagy and pexophagy pathways were also upregulated in neutrophil engulfed *C. glabrata*, possibly providing a way to sequester resources in a nutrient-poor environment (Fukuda et al. 2013; Roetzer et al. 2010; Tsai et al. 2004). It is noteworthy to point out that the observed *C. glabrata* transcriptional response to neutrophil phagocytosis closely resembles that of glucose deprivation, reinforcing that carbon starvation is a stress to be dealt with in neutrophils, as is during macrophage internalization (Fukuda et al. 2013). Likewise, such a correlation is also verified in downregulated processes, including protein synthesis, ribosomes and membrane sterols (*ERG1, ERG2, ERG3, ERG11, ERG13, ERG25*) are repressed during in vitro glucose starvation (Roetzer et al. 2008). Genes controlling mannan biosynthesis (*PSA1* and *CIS3*) and beta-glucan metabolism (*GSC2*, *EXG1* and *UTR2*) were also significantly repressed. These observations suggest that *C. glabrata* shuts down processes of protein synthesis, cell membrane and cell wall biosynthesis due to carbohydrate depletion in neutrophils (Fukuda et al. 2013).

Another mechanism employed by *C. glabrata* cells to cope with phagocytosis-induced nutrient starvation is autophagy (Roetzer et al. 2010). It contributes for mobilization of intracellular resources in a nutrient-poor environment and is beneficial for *C. glabrata* survival in macrophages (Roetzer et al. 2010). In particular, the autophagy pathway genes *ATG11* and *ATG17* were found to be required for *C. glabrata* survival in macrophages (Roetzer et al. 2011; Roetzer et al. 2010). Likewise, 7 genes from the autophagy pathway are upregulated upon phagocytosis by human neutrophils, including *ATG11* and *ATG17* (Fukuda et al. 2013). Interestingly, *ATG11* is required for selective autophagy of peroxisomes, indicating that not only non-selective autophagy takes place upon *C. glabrata* phagocytosis, but also that

pexophagy is required for *C. glabrata* survival (Roetzer et al. 2010). The autophagy and pexophagy pathways appear to be activated in latter stages of internalization. As stated previously, nutrient limitation response includes utilization of fatty acids as energy source, which is consistent with the expression of peroxisomal genes (Fukuda et al. 2013). However, induction of peroxisomes was found to occur in a transient manner, being observed at an early stage post-internalization (5 h), followed by a decrease in the number of peroxisomes at 24 h, consistent with pexophagy (Roetzer et al. 2010). The activation of autophagy and pexophagy in phagocyted *C. glabrata* has been recognized as a nutrient recycling mechanism during nutrient starvation in the phagosome (reviewed in Oku and Sakai 2010; Till et al. 2012).

Consistent with the notion that *C. glabrata* is subjected to nitrogen limitation inside macrophages, induction of aminoacid transporters (*GAP1*, *CAN1*) and the upregulation of arginine and lysine biosynthetic pathways was observed in macrophage internalized cells (Kaur et al. 2007). A similar response was observed in neutrophil engulfed cells, where the transport of ammonium (*ADY2*), aminoacids (*GAP1*) and methionine (*MUP1*) is activated (Fukuda et al. 2013). Furthermore, lysine and methionine (*STRE3*) metabolic processes are also induced (Fukuda et al. 2013). Plus, the observed response in these processes closely resembled the transcriptional response of *C. glabrata* during nitrogen deprivation growth, demonstrating the occurrence of nitrogen limitation in the neutrophil environment (Fukuda et al. 2013).

7.4.2 Iron Limitation Response

From the 23 genes identified by a screening of deletion mutants found to influence the ability of *C. glabrata* to survive inside macrophages, 11 are required for growth under iron limiting conditions (Seider et al. 2014). Despite only 1 gene (*FRE8*) being currently known to participate in iron homeostasis, the correlation between macrophage phagosome survival and iron limitation growth suggests that iron acquisition is a relevant factor for *C. glabrata* inside the phagosome (Seider et al. 2014). Indeed, within the phagosome, iron is sequestered from pathogens by the action of scavengers (e.g. lactoferrin, ferritin, transferrin) and transporters (e.g. NRAMP1) (Almeida et al. 2009; Masson et al. 1969; Cellier et al. 2007; Forbes and Gros 2001). The ability of pathogens to acquire iron inside the human host is considered a main virulence trait.

A phenotypic screening of a deletion mutant library identified 36 mutants with growth defects under iron-limiting conditions (Gerwien et al. 2016). Among them are genes involved in chromatin remodeling (*CYC8*, *PHO23*), iron acquisition (*FTR1*, *FET3*) and transcription regulation of iron acquisition (*AFT1*, *SEF1*). The absence of either *AFT1* of *SEF1* decreases intracellular iron content in *C. glabrata* (Gerwien et al. 2016). As expected, expression profiling upon confrontation with iron deprivation reveals that *C. glabrata* activates iron uptake (*FTR1*, *FET3*, *SIT1*) and iron recycling (*FTH1*, *HMX1*, *SMF3*); whereas it downregulates iron consuming processes (*CYC1*, *COX6*, *CCP1*, *CCC1*, *HEM15*). The regulator Aft1 plays a dual role in this response, activating genes involved in iron uptake and recycling while

repressing iron consumption. In turn, Sef1 seems to regulate specific processes such as the TCA and iron sulfur cluster-dependent functions (*ACO1, IDH1, IDH2, ISA1*) during iron starvation; and is also involved in the regulation of iron consuming processes in the presence of iron (*CCP1, CYT1, HEM15, CCC1, ACO1*) (Gerwien et al. 2016). Additionally, Aft1 induces the expression of *CTH2*, a mediator of mRNA degradation of iron acquisition genes during iron limitation. Both regulators (Aft1 and Sef1) are required for *C. glabrata* survival in an ex vivo human blood model, in agreement with the iron limiting conditions during the initial immune defense against systemic fungal infections (Gerwien et al. 2016).

Through transcriptomics analysis of *C. glabrata* cells exposed to a low-iron environment, Srivastava et al. uncovered, other than the upregulation of iron uptake genes (*FTR1, FET3*), activation of mitosis, RNA processing, DNA repair, autophagy, chromosome segregation and protein transport (Srivastava et al. 2015). On the other hand, downregulated genes included nucleotide and amino acid biosynthesis, ion transport, translational elongation and cellular respiration (Srivastava et al. 2015). Interestingly, upregulation of the respiratory electron transport chain during iron-surplus conditions indicates a reciprocal regulation of the electron transport chain by iron. In agreement with the low-iron conditions, *C. glabrata* halts the synthesis of iron-containing enzymes, translated by the downregulation of *ACO2, GLT1, LEU1, LYS4, ILV3, MET5, HEM1, HEM2, HEM4, HEM5*. Interestingly, the upregulation of DNA repair and autophagy is not verified in *S. cerevisiae* (Srivastava et al. 2015) and could represent a pathogenic trait of *C. glabrata* upon iron limiting conditions in the host. Similarly, iron-responsive differential expression of the protease Yps5 and the adhesin Epa1 is also specific for *C. glabrata*, establishing a link between iron availability and the regulation of virulence factors (Srivastava et al. 2015). Moreover, the kinase Hog1 participates *C. glabrata* iron response, as a *Δhog1* mutant accumulates more intracellular iron and presents deregulation of iron-associated genes: *AFT2, ATM1, FTR1, FRE6, FRE8, SMF3* and several target genes regulated by the iron response regulators Aft1 and Aft2 (Srivastava et al. 2015).

7.4.3 Oxidative Stress Response

The production of ROS is one of the mechanisms used by host cells to kill the phagocyted pathogen (Vieira et al. 2002; Haas 2007). Nonetheless, *C. glabrata* is able to detoxify ROS or inhibit their production (Seider et al. 2014). Maintenance of the redox homeostasis within the cell, resistance to oxidative stress and survival within the host is mediated by the activity of catalase (Cta1), superoxide dismutases (Sod1/2) and the glutathione and thioredoxin pathways (Roetzer et al. 2011; Fukuda et al. 2013; Cuéllar-Cruz et al. 2008; Briones-Martin-Del-Campo et al. 2014). The action of oxidative burst in the context of infection and the role of ROS detoxification systems in *C. glabrata* virulence is therefore a relevant subject.

Interestingly, 9 genes out of a set of 23 found to influence *C. glabrata* survivability in macrophages were seen to contribute to oxidative stress resistance in vitro (*CCH1,*

SHO1, CAGL0M12496g, *LRG1, ERG5, OST6, CDC12, HEK2, MPS3*). Some of these genes have functions related with cell wall composition and cytokine production, demonstrating the interplay between *C. glabrata* strategies to survive phagocytosis (Seider et al. 2014). Another crucial feature in *C. glabrata* response to ROS is the capability to modulate ROS production by phagocytes. In fact, from the 23 genes found to contribute to *C. glabrata* survival within macrophages, 15 were found to modulate ROS production (*CCH1, SLM1, GPR1, GPA2, LRG1, GNT1, ERG5, SLG1, MNN4, MSN1, PMT2, PMT4, CDC12, CKA2, HEK2*) (Seider et al. 2014). Interestingly, *C. glabrata* response to neutrophils includes the up-regulation of genes related to oxidative stress, including genes encoding catalase Cta1, the superoxide dismutase Sod2, the putative glutamate decarboxylase Gad1 and the regulator Ask10 (Fukuda et al. 2013).

A transcriptomics analyses of *C. glabrata* response to the oxidative stress inducers hydrogen peroxide (H_2O_2) and menadione allowed to clarify important aspects of this response (Roetzer et al. 2008, 2011). A core response to hydrogen peroxide comprises generic oxidative stress response genes, including *TRR1/2, TRX1/2, TSA1/2, GPX1/2, CTA1, SOD1* and most of these genes are also dependent on the main oxidative stress regulators Yap1 and Skn7 (Roetzer et al. 2008, 2011). Yap1 also regulates other genes involved in redox processes (*ADH6, GRE2, SCS7, OYE2*). Additional up-regulated genes involved in oxidative stress response also included mitochondrial processes (*ACP1, OPI3, HSP10, MRP10*) (Roetzer et al. 2011). Interestingly, 26 genes were found to be induced by both oxidative stress and glucose starvation (including *CTA1, GPX2, TRX2*) (Roetzer et al. 2011). Moreover, the PathoYeastract database (Monteiro et al. 2017) predicts that genes activated during glucose starvation (Roetzer et al. 2008, 2011) are regulated by the oxidative stress regulators Yap1 and Skn7. This gene set could represent relevant targets to study in the context of *C. glabrata* infection of phagocytic cells, where both oxidative and glucose starvation stresses are exerted. Interestingly, comparing the genome-wide response to hydrogen peroxide to that of menadione, there was limited overlap between both activated regulons (Fig. 7.3). Moreover, only 11 genes were dependent on Yap1/Skn7 on both stresses, pointing out to specific oxidative stress regulons in *C. glabrata* (Roetzer et al. 2011). However, the survival of *C. glabrata* cells engulfed by mouse macrophages is dependent on both Yap1 and Sod1, but not on Yap1 and Sod1 alone, and Yap1 is not required for virulence on a murine model of infection (Roetzer et al. 2011; Chen et al. 2007). On the other hand, Skn7 is involved in virulence in a murine model of infection (Saijo et al. 2010), despite not being required for survival in macrophages (Roetzer et al. 2011). This indicates a more complex role of oxidative stress response genes and regulators in *C. glabrata* survival and virulence.

A phenotypic screening of a collection of mutant strains in zinc cluster genes aimed at identifying transcriptional regulators required for response to multiple stresses (Klimova et al. 2014). Among them, the transcription factor (TF) Stb5 was found to be required for hydrogen peroxide resistance and to be involved in oxidative stress response (Klimova et al. 2014). Stb5 is also an activator of genes from NADPH production, a cofactor involved in oxidative stress resistance (Larochelle et al. 2006).

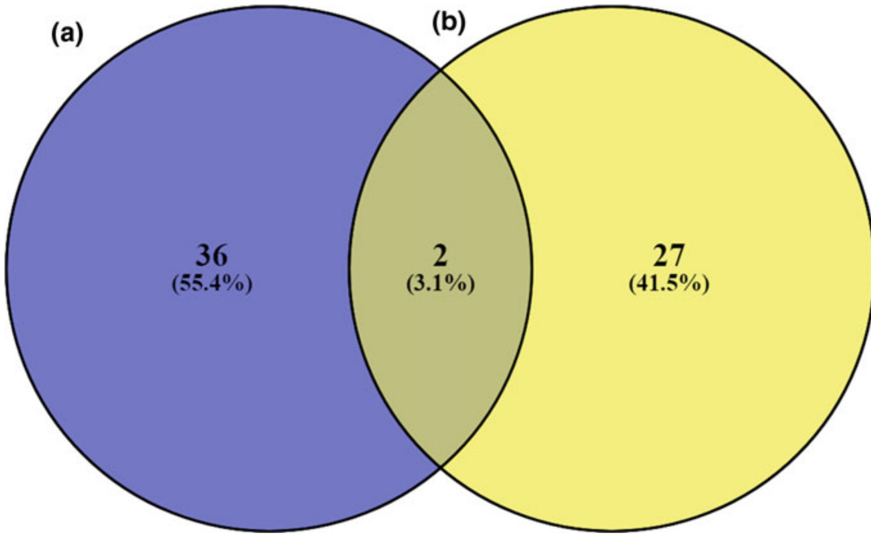

Fig. 7.3 Oxidative stress activated genes in *C. glabrata*. **a** Genes found to be upregulated in response to H_2O_2 (Roetzer et al. 2011). **b** Genes found to be upregulated in response to menadione (Roetzer et al. 2011)

As stated previously, macrophage-ingested cells show altered epigenetic signature, with altered histone acetylation marks that can be used to modulate energy metabolism (Rai et al. 2012). A role for differential histone acetylation levels, mediated by the histone deacetylase Hst1, was also observed to regulate *C. glabrata* oxidative stress response (Orta-Zavalza et al. 2013). The Hst1 sirtuin mediates local silencing to suppress oxidative stress response through control of the expression of *CTA1* and the general stress regulator *MSN4*. This epigenetic regulation was also described to regulate specific adhesins and resistance to xenobiotics through control over the major antifungal resistance determinants *PDR1* and *CDR1* (Orta-Zavalza et al. 2013).

7.4.4 Nitrosative Stress Response

The production of reactive nitrogen species (RNS), such as nitric oxide (NO•), in phagocytic cells causes nitrosative stress in engulfed pathogens (Brown et al. 2009).

C. glabrata transcriptional profiling during nitrosative stress induced by S-nitrosoglutathione revealed that a significant portion of its genome (48%) is differentially expressed (Linde et al. 2015). A significant part of the responsive genes are linked to RNA polymerase III activity. Concurrently, genes relative to RNA processing steps and translation as well as those involved with oxidation-reduction processes are also enriched in this context, indicating that the cells are under stress. Compar-

ing the nitrosative stress response of *C. glabrata* with that of the closely related non-pathogenic yeast *S. cerevisiae*, a much larger number of genes participates in this response (Linde et al. 2015). This could reflect the importance of nitrosative stress response during the interaction with the host. Commonly upregulated genes between *C. glabrata*, *C. albicans* and *S. cerevisiae* include the flavohemoglobin *YHB1*, involved in nitric oxide detoxification (Linde et al. 2015; Merhej et al. 2015) and the inositol 1-phosphate synthase *INO1*; while the genes *NOP13, NOP58, CBF5, NSA2* and *ENP2* are specifically activated in *C. glabrata* (Linde et al. 2015). These genes are involved in RNA interaction (Linde et al. 2015). Interestingly, a subset of the genes differentially expressed under nitrosative stress were predicted by the PathoYeastract database (Monteiro et al. 2017) to be regulated by the TFs Asg1 and Hal9, a duo of pH responsive TFs that will be addressed further on this chapter.

7.4.5 pH Stress Response

C. glabrata is able to colonize diverse host niches with varying pH conditions. Oral and blood infections are established in environments of neutral to slightly basic pH, whereas the commonly colonized vaginal tract, stomach and duodenum constitute acidic environments and the intestine and kidneys alkaline ones (Bairwa and Kaur 2011; Schmidt et al. 2008). As such, *C. glabrata* must have factors required to survive and thrive in diverse host niches, making pH adaptation a critical factor for host adaptation (Brunke and Hube 2013; Bairwa and Kaur 2011; Penalva and Arst 2002).

Transcriptional profiling analysis of *C. glabrata* response to low pH shows that a wide variety of cellular processes are differentially regulated in response to acidic conditions (Bairwa and Kaur 2011; Lin et al. 2017; Wu et al. 2015). Namely, *C. glabrata* induces the expression of genes involved in stress response, carbohydrate and amino acid metabolism, gluconeogenesis, phospholipid metabolism, nucleic acid binding, trehalose catabolic process, protein modification and transport; while genes involved in signal transduction, cofactors and vitamins metabolism, nucleotide metabolism, cell division, chromosome segregation, stress response and glucose metabolism are repressed (Bairwa and Kaur 2011; Lin et al. 2017; Wu et al. 2015). Additionally, genes coding for tRNA synthetases and cell wall proteins are also downregulated (Bairwa and Kaur 2011).

Notably, several genes from the *YPS* gene family were found to be activated in response to low external pH (Bairwa and Kaur 2011). As stated previously in this chapter, various *YPS* genes are also upregulated upon *C. glabrata* internalization by phagocytes (Kaur et al. 2007; Fukuda et al. 2013). *YPS* were proposed to be involved in cell wall metabolism by participating in the processing of cell wall proteins (Kaur et al. 2007). In fact, *YPS1* is required for survival of *C. glabrata* in a low-pH environment and this was associated with a role of Yps1 in cell wall restructuring under acidic environmental conditions and intracellular pH homeostasis (Bairwa and Kaur 2011). Intracellular pH acidification is associated with oxidative damage by induc-

ing endogenous ROS production (Lagadic-Gossmann et al. 2004). Accordingly, the lack of *YPS1* results in intracellular pH reduction leading to increased ROS production and consequent decreased viability under acidic environmental conditions. Moreover, a role in pH homeostasis mediated by *YPS1* was associated with plasma membrane proton pump activity via post-translational regulation of the H^+-ATPase Pma1 in low pH conditions (Bairwa and Kaur 2011).

Tolerance to low pH also comprises changes in sterol membrane composition, found to be mediated by the Mediator complex subunit 3 (Med3) and the Mediator tail subunit Med15B (Lin et al. 2017; Qi et al. 2017). Both Med3 subunits, encoded by *MED3A* and *MED3B*, and Med15B regulate the expression of a subset of genes in lipid biosynthesis and metabolism pathways. The sterol content of *C. glabrata* plasma membrane (including lanosterol and ergosterol, among others) is affected in a *Δmed3AB* double deletion strain (Lin et al. 2017); while ergosterol and phospholipid content is altered in a *Δmed15B* strain (Qi et al. 2017). In both strains, these alterations result in defective membrane integrity, rigidity and H^+-ATPase activity (Lin et al. 2017; Qi et al. 2017). The *C. glabrata* Med3 complex was proposed to regulate membrane composition, while Med15B exerts transcriptional regulation over acid stress response genes and lipid composition, enabling tolerance to low pH stress (Lin et al. 2017; Qi et al. 2017).

Acidic pH tolerance in *C. glabrata* is mediated by the transcription factors *ASG1* and *HAL9* (Wu et al. 2015). The absence of either regulator decreases growth in a low pH environment due to reduction of intracellular pH, partly due to diminished activity of the proton pump Pma1, and two-fold higher ROS levels. This is consistent with the knowledge that intracellular ROS production is closely related to intracellular pH (Wu et al. 2015). Transcriptomics analysis revealed that both TFs influence MAPK signaling pathways, which could explain the acid stress phenotypes. Moreover, absence of either regulator induced the expression of *BTN1* (involved in vacuole pH homeostasis), while the expression of the pH response transcription factor *RIM101* decreased (Wu et al. 2015). It appears that *C. glabrata* responds to acid stress through multiple pathways, at least partly regulated by Asg1 and Hal9.

Acidification of pH is also an antimicrobial feature applied by host immune cells. After pathogen phagocytosis, the acidification of phagosomes is crucial for the activation of hydrolytic enzymes and constitutes a harsh environment for the pathogen (Vieira et al. 2002). Maturing phagosomes go through a decrease in pH ranging from 6.0 in early endosomes to up 4.5 in phagolysosomes (Seider et al. 2011). It is interesting to note that *C. glabrata* appears to prevent phagosome maturation, thus preventing the acidification of its environment (Seider et al. 2011). This hypothesis was emphasized by the comparison of genome-wide transcriptional profiles of *C. glabrata* cells exposed to pH 4.5 in vitro (as found in phagolysosomes) and of *C. glabrata* cells recovered from human macrophages (Seider et al. 2011). Indeed, only 5.5% of the genes upregulated at pH 4.5 were also induced in macrophages, leading to the likely conclusion that *C. glabrata* is not exposed to an acidic environment within macrophages (Seider et al. 2011).

During the course of infection and contact with the host, pathogens must adapt to dynamic environments such as changing pH. Therefore, the shift from an acidic

environment to an alkaline environment is also valuable to investigate (Linde et al. 2015; Schmidt et al. 2008). During the shift from acidic (pH 4.0) to alkaline pH (pH 8.0), *C. glabrata* differentially regulates 834 genes (409 upregulated and 426 downregulated) (Linde et al. 2015). Heme-binding protein encoding genes are differentially expressed, indicating that *C. glabrata* adjusts iron homeostasis in response to pH changes. Moreover, genes involved in cytochrome-c reductase activity and oxidation-reduction processes are also differentially expressed, showing that the redox state of the cells is changed. According to this observation, the ranking of TFs that could regulate this response predicted that a subset of the genes can be regulated by the Yap1 and Skn7 oxidative stress response TFs (Monteiro et al. 2017). Furthermore, genes involved in cell wall assembly are also differentially expressed, implying that cell wall remodeling is taking place during pH shift (Linde et al. 2015). Interestingly, 15 differentially expressed genes in *C. glabrata* have homologues that are not differentially regulated under pH stress in the phylogenetically related *S. cerevisiae*. These genes may be related to host-specific adaptation of *C. glabrata* which is not present in the non-pathogenic *S. cerevisiae* (Linde et al. 2015).

The proteome response during shift from an acidic to an alkaline environment (pH 4.0-8.0) revealed that *C. glabrata* shows decreased expression of proteins involved in energy generation through glycolysis/gluconeogenesis and TCA cycle (Aco1, Tdh3, Krs1, Mdh1, Lsc1, Tkl1, Fba1, Cdc19, Pgi1) and fermentation (Adh3, Dld1, Pdc1); cytochrome-c reductase activity (Rip1); oxidation-reduction processes (Trr1, Ahp1, Tsa1); protein folding (Sse1, Hsp82) and protein complex assembly (Hsp82, Cox12); cytoskeleton and cell polarization (Act2); chromosomal segregation and endocytosis (Nuf2) (Schmidt et al. 2008). On the other hand, proteins involved in cell signaling (Bcy1), endocytosis (Lsp1, Pil1), cytoskeleton organization (Aip1, Pph21), intracellular transport (Sec28, Vma2, Ssa3) and protein catabolism (Lap4, Rpt3) showed increased expression upon acidic to alkaline shift. Interestingly, *C. glabrata* also displays differential regulation of protein expression between neutral and alkaline pH (pH 7.4 and pH 8.0). Translation factors (Yef3), regulators (Gcn3), proteins involved in oxidative stress response (Cta1), stress response (Rhr2), protein folding (Cpr1), amino acid (Ilv5) and glucose metabolism (Eno1, Pgk1, Rhr2, Tpi1) were found to be upregulated at pH 7.4 when comparing to pH 4.0, but to be downregulated at pH 8.0 (Schmidt et al. 2008). Overall, proteomic response to different pH environments appears to be based on the expression of proteins from a core set of functional groups, with distinct proteins being differentially regulated at each pH: proteins implicated in microtubule cytoskeleton organization, amine metabolism and protein synthesis, folding and complex assembly were specifically downregulated at pH 8.0, whereas actin cytoskeleton organization and biogenesis, protein catabolism and response to stress proteins were upregulated. As stated previously, energy-related proteins were significantly enriched upon pH shift. Enzymes involved in glucose metabolism, TCA cycle and respiration were specifically downregulated in the alkaline environment; while the subunits of a glycolytic enzyme (Pfk1/2) were upregulated (Schmidt et al.

2008). Overall, this data suggests that *C. glabrata* perceives an alkaline environment as more stressful than the acidic one (Schmidt et al. 2008).

7.4.6 Stress Imposed by Weak Acids

Other than the pH stress exerted over *C. glabrata* during phagosome acidification and in host niches, *C. glabrata* has to cope with high concentrations of weak acids in low pH environments, such as acetic and lactic acids in the vaginal tract. To assess *C. glabrata* mechanisms responsible for acetic acid tolerance, transcriptomics analysis revealed that the expression of 538 genes is activated. These genes are enriched for central carbon metabolism, amino acid metabolism, ion transport, response to low pH and oxidative stress response. Previously, acetic acid was described to induce oxidative stress in *S. cerevisiae* (Semchyshyn et al. 2011). Additionally, several genes related with cell wall metabolism are also overexpressed, including β-glucan synthesis (*FKS2, KNH1, KRE6, GAS5*), mannoproteins (*TIR1, TIR3, YEH2*) and cell wall structure (*CWP1/2*). On the other hand, *C. glabrata* downregulates 80 genes during acetic acid stress, which are involved in protein synthesis, ribosomal biogenesis and ER-Golgi transport (Bernardo et al. 2017). As part of *C. glabrata* acetic acid response, the TF Haa1 was found to regulate 75% of the acid-responsive genes, making it a major regulator of acidic tolerance. This is consistent with the increased sensitivity of the *Δhaa1* strain to acetic acid and the increased intracellular accumulation of acetic acid in this mutant strain. This response was partly associated with the proton pump Pma1, responsible for intracellular pH homeostasis (Bairwa and Kaur 2011; Bernardo et al. 2017). As previously referred in this chapter, Pma1 is post-translationally regulated by the protease Yps1; and *YPS1* is required for *C. glabrata* survival to acetic acid stress (Bairwa and Kaur 2011), indicating a central role for proton pump activity in response to low pH and acidic stress, mediated by the Haa1 TF and the protease Yps1. Consistent with the synergistic effect of acidic pH and weak acid stress, *C. glabrata* transcriptional response to acetic acid was predicted to be, in part, mediated by the pH response regulators Asg1 and Hal9 (Monteiro et al. 2017). The role of Haa1 in acetic acid intracellular accumulation was associated with its export by the plasma membrane transporter Tpo3 (Bernardo et al. 2017). Interestingly, the plasma membrane transporter Aqr1 was previously identified as a determinant of acetic acid resistance, although it was not related directly with its transport (Costa et al. 2013b). Equally relevant, Haa1 plays a role in the adhesion and colonization of a vaginal epithelium model in the presence of acetic acid (Bernardo et al. 2017), unveiling a possible mechanism applied by *C. glabrata* to surpass stress and colonize the host in one of its most typical niches.

7.5 Multiple Host-Associated Stress Response

Inside the host, *C. glabrata* has to cope with a number of host related stresses. In most instances, the pathogen is challenged with concurrent stresses at one given time. Therefore, multi stress responses are probably crucial for survival of *C. glabrata* in host niches.

Phagocyted pathogens have to cope with oxidative stress in order to survive the phagosome. Hydrogen peroxide and menadione are among the most commonly found oxidative stress agents and it is interesting to note that *C. glabrata* responds differently to each agent (Fig. 7.3). The common antioxidant response is based on the activation of the catalase Cta1 and the thioredoxin peroxidase Tsa2, indicating the core of oxidative stress response. Most genes known to be associated with antioxidant resistance are upregulated in response to H_2O_2; including *TRR1/2*, *TSA1* and *GPX2*; but not to menadione. In turn, response to menadione comprises the specific upregulation of the cystathionine beta-lyase Str3. This highlights that *C. glabrata* adopts a specific response to different types of oxidative stress agents, albeit backed by a core response based on the hallmark OSR gene *CTA1*.

Upon phagocytosis, pathogens are subject to multiple stresses, such as oxidative stress and glucose starvation. As observed in Fig. 7.4, the transcriptional profile of phagocyted *C. glabrata* comprises genes found to be activated in response to glucose starvation and oxidative stress resistance. Interestingly, there are 7 (1.5%) genes commonly activated in all instances, which could represent the core players in *C. glabrata* genome-wide response to the stressful environment of the macrophage. In fact, these genes belong to distinct functional categories, highlighting the multifactorial response in this environment: OSR (*CTA1*), transmembrane transport (*AQY1*), heme biosynthesis (*HEM15*), carbonic anhydrase activity (*NCE103*), NADPH regeneration (*ALD4*), mitochondrial function (*RIP1*) and cell damage response (*TOS8*). Due to the activation of OSR genes in macrophage-engulfed cells, it is widely accepted that *C. glabrata* suffers from oxidative stress upon phagocytosis. Indeed, comparing with genome-wide OSR response, 26% of the genes are concurrently activated in phagocyted cells; including *CTA1*, *STR3*, *TRR1* and *TRX2*. However, such response only represents 5% of the genes activated in phagocyted cells. These observations implicate catalase and thioredoxin activity as the main strategy underlying *C. glabrata* response to oxidative burst during immune attack and that this response is only a fraction of the genome-wide changes upon host confrontation. By looking at the glucose starvation response, a similar conclusion can be drawn. Transcriptomics comparison shows that 27% of the activated genes during glucose starvation are concurrently activated by *C. glabrata* inside macrophages, although this response makes up 6% of the global gene activation in phagocyted cells (Fig. 7.4). As widely described, these genes are involved in several pathways of carbohydrate metabolism, including glycogen and fatty acid metabolism. It is interesting to note some overlap between activated genes in both glucose starvation and OSR responses (e.g. *GPX2*, *GRE3*, *GDB1*, *OPI3*). This comes in agreement with the observation by Roetzer et al. of commonly activated genes between these two conditions

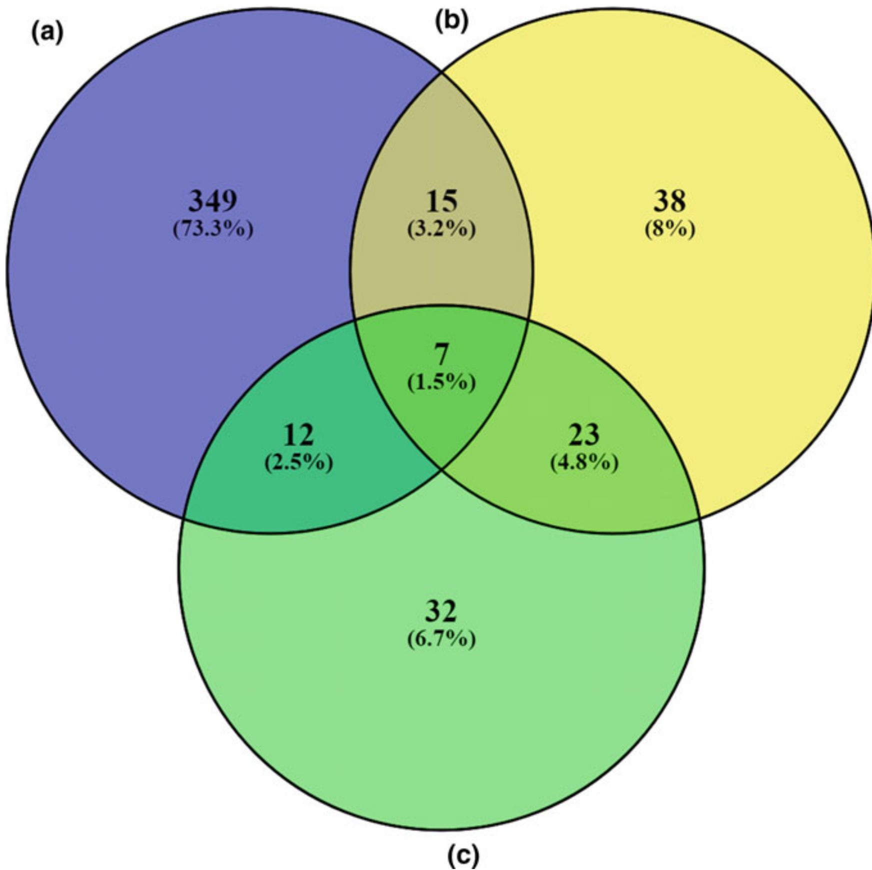

Fig. 7.4 Macrophage and macrophage-imposed stress activated genes in*C. glabrata*. **a** Genes found to be upregulated upon internalization by human and murine macrophages (Seider et al. 2011; Kaur et al. 2007). **b** Genes found to be upregulated in response to glucose starvation (Roetzer et al. 2011; Roetzer et al. 2008). **c** Genes found to be upregulated in response to oxidative stress (Roetzer et al. 2011)

(Roetzer et al. 2011) and can be related with the increase of reductive metabolism to generate NADPH in response to glucose (energy) starvation, as noted by *HBN1, GCY1, GRE3*. Moreover, the activation of the same genes in both conditions and upon *C. glabrata* phagocytosis reaffirms the importance of multi stress resistance for *C. glabrata* prevalence within the host.

The maturation of the phagolysosomes to become an acidic environment (pH 4.5) is yet another stress pathogens have to cope during immune attack (Seider et al. 2011). Moreover, *C. glabrata* is also exposed to an acidic environment in host niches, such as the vaginal tract, were the presence of weak acids (e.g. acetic acid) may act synergistically with low pH. As such, the ability of *C. glabrata* to cope with low

pH and weak acid conditions can be an important feature that mediates its interaction with the host. This fact is supported by the concurrent activation of genes in both acidic pH and presence of acetic acid (Fig. 7.5), including the polyamine and acetate transporter *TPO3* (Bernardo et al. 2017; Costa et al. 2014) and the pH homeostasis required proton pump *PMA1* (Bairwa and Kaur 2011; Bernardo et al. 2017). Given these observations, it is apparent that transport activity (possibly to extrude acetate from the intracellular medium) works in conjunction with proton pump activity to maintain intracellular pH homeostasis in face of external stress. Nevertheless, their relevance in surviving macrophage attack needs to be elucidated, given that their expression was not found to be activated in such instance. However, the putative weak acids transporter coding ORF *CAGL0M07293g* (*PDR12*) is upregulated during both low pH and acetic acid stress, but also upon macrophage internalization (Fig. 7.5). Upon phagocytosis, *C. glabrata* may activate a low pH response, as 19% of the activated genes under low pH conditions are also upregulated in phagocyted cells (Fig. 7.5), although representing 5% of the global response and apparently not being directly involved in processes typically associated with pH homeostasis. Interestingly, there is significant overlap between upregulated genes in acetic acid exposure and macrophage exposed cells. Stress response to weak acids is, at a first glance, predominantly associated with colonization of niches such as the vaginal environment. However, the activation of acetic acid responsive genes upon macrophage confrontation makes one wonder about a possible role played by weak acids after phagocytosis and raises the question if pH acidification of the phagosome and acetic acid may act synergistically as an antimicrobial strategy.

Once there is limited overlap between *C. glabrata* response to macrophage engulfment and each separate stress condition, it appears that the response to this event is more extensive than what can be probed in vitro. Is this response based on additional stress responses, or is it associated with the activation of additional genes from the same functional groups? Among all the datasets under consideration, there are 253 genes exclusively upregulated inside macrophages. These genes were clustered into functional groups according to their associated GO terms. Other than known biological processes, such as carboxylic acid metabolism, fatty acid catabolism and oxidation-reduction processes, this dataset is enriched for aminoacid biosynthetic pathways; especially arginine, glutamine and lysine. Related processes as ribosome biogenesis and rRNA processing are also overrepresented, possibly indicating a reprogramming of protein synthesis underlying the stress response. ncRNA processing is also enriched, possibly fine-tuning gene expression reprogramming.

In order to understand which determinants could underlie a cellular response that is translated in phenotypic effects, Fig. 7.6 crosses genes activated upon macrophage internalization with those observed to be required for macrophage survival. Surprisingly, only 1.1% of these datasets overlap. They comprise pathways of aminoacid biosynthesis (*MET15, LYS1*), tRNA methylation (*GCD14*), oligosaccharide metabolism (*CAGL0L10582g*) and weak acid tolerance (*PDR12*). These could represent good candidates to extend the knowledge on *C. glabrata* survival from the host immune system.

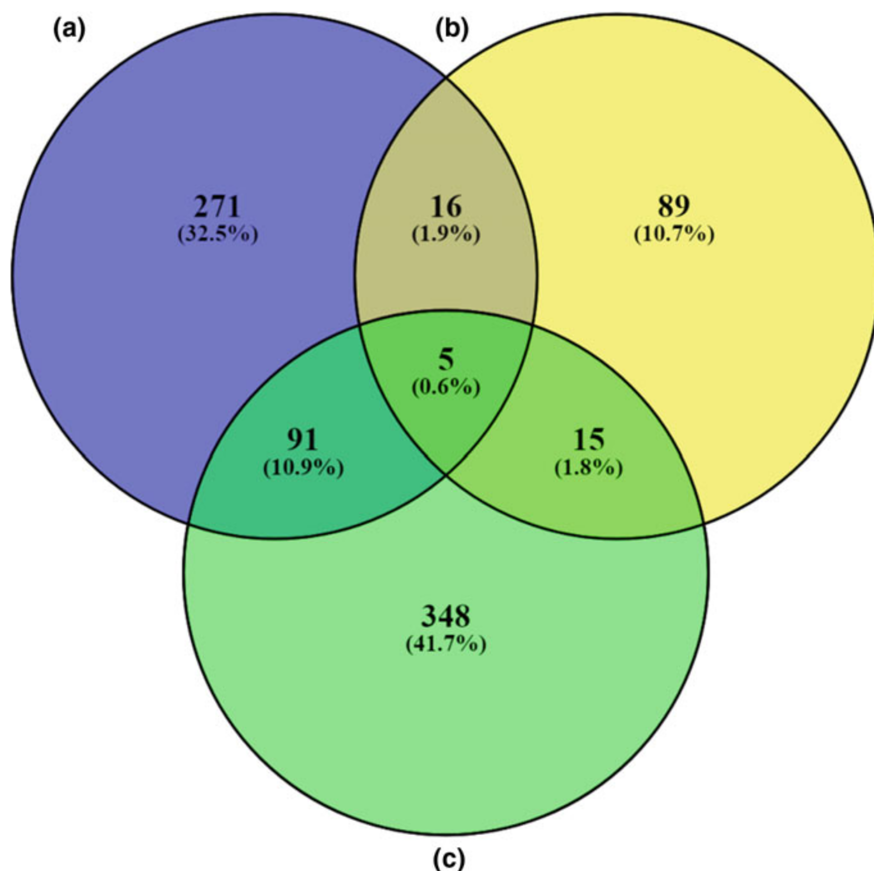

Fig. 7.5 Macrophage and host-associated stress activated genes in *C. glabrata*. a Genes found to be upregulated upon internalization by human and murine macrophages (Seider et al. 2011; Kaur et al. 2007). **b** Genes found to be upregulated in response to low pH (Seider et al. 2011; Wu et al. 2015). **c** Genes found to be upregulated in response to acetic acid stress (Bernardo et al. 2017)

7.6 Conclusions

Genome-wide studies have been key to identify major cellular responses to various stresses in pathogens. The application of these methodologies have provided a sneak peek into the features that make *C. glabrata* a successful pathogen.

A comparison between transcriptomics, proteomics and phenotypic studies probing antifungal resistance response/phenotypes can prove beneficial to identify core players in multidrug resistance. The participation of the master regulator of azole resistance Pdr1 and of multidrug resistance transporters is highlighted by all large-scale surveys. However, multiple studies hint to additional processes outside drug efflux that can be relevant as drug resistance mechanisms, such as calcium signal-

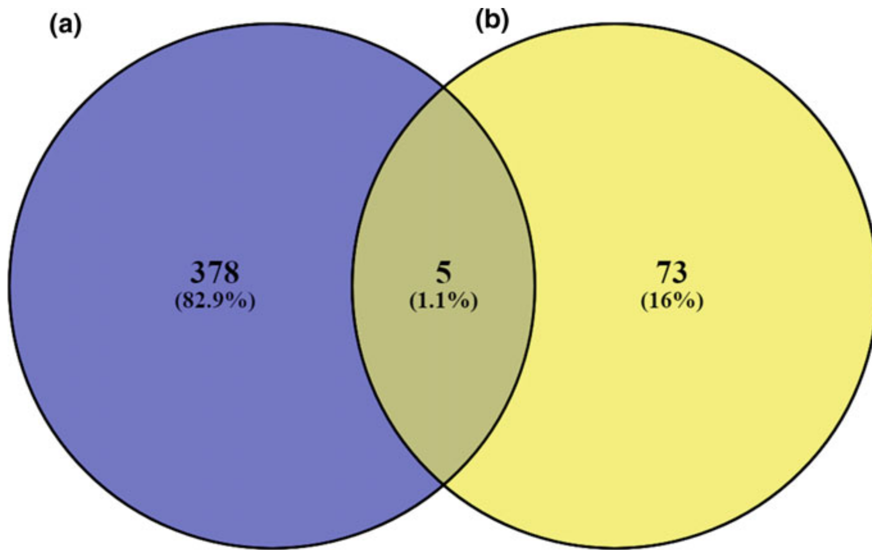

Fig. 7.6 Macrophage activated and macrophage survival genes in *C. glabrata*. a Genes found to be upregulated upon internalization by human and murine macrophages (Seider et al. 2011; Kaur et al. 2007). **b** Genes found to be required for *C. glabrata* survival upon internalization by macrophages (Seider et al. 2014)

ing, energy metabolism or cell wall dynamics. Interestingly, despite distinct modes of action, there is a small number of genes mediating response/resistance to both azoles and amphotericin B, such as *CKA2*, a catalytic subunit of casein kinase 2, involved in regulation of sphingolipid biosynthesis being essential for membrane homeostasis (Healey et al. 2012). Additionally, 12 genes were found to be involved in the response/resistance to both echinocandins and azoles, including *CNB1* and *CNA1*, involved in cell wall integrity through calcineurin signaling pathway (Chen et al. 2012). The occurrence of cell wall related genes is expectable in response to echinocandin antifungals, given their mode of action and cellular target, but a role for cell wall in azole response has also been observed (Pais et al. 2016a). More than just common antifungal resistance genes, albeit limited, it is striking to see concerted biological processes playing a role in diverse antifungal responses. This analysis could provide a better understanding of complementary resistance mechanisms applied by *C. glabrata*, beyond traditional pathways, possibly pointing out to the existence of multiple antifungal stress resistance genes.

The comparison of *C. glabrata* response to multiple stress conditions can also be key in identifying relevant determinants of resistance to the host, as a whole, which could ultimately be used as therapeutic targets. We observed that the catalase gene *CTA1* is concurrently activated not only during OSR, but also during glucose starvation and upon engulfment by macrophages. This is concordant with the observation that oxidative stress and glucose starvation activate a set of identical genes (Roetzer

et al. 2008, 2011). Moreover, its activation inside macrophages, where such stress conditions occur, makes it a central piece in host stress response of *C. glabrata*. Another interesting possible target is the cytochrome C peroxidase Ccp1, upregulated in OSR, low pH and inside macrophages. Although it lacks characterization in *C. glabrata*, its *S. cerevisiae* homolog is involved in oxidative stress signaling (Charizanis et al. 1999); while *C. albicans* Ccp1 is also induced by macrophage interaction (Lorenz et al. 2004). From these findings, it appears that determinants of oxidative stress resistance are a key feature in *C. glabrata* response to host stresses. Another concurrently activated gene upon OSR, glucose starvation, low pH and internalization by macrophages is *HEM15*, encoding a ferrochelatase involved in heme biosynthesis. Interestingly, its *C. albicans* homolog is not regulated by iron levels (Santos et al. 2004), but the fact that it is activated upon internalization by macrophages, although iron consuming processes are believed to be shutdown due to iron limitation, makes this gene an appealing candidate for further study. In the same situation is *RIP1*, encoding a putative ubiquinol cytochrome c reductase repressed by low levels of iron and by nitric oxide in *C. albicans* (Hromatka 2005; Singh et al. 2011); despite being seemingly required in *C. glabrata* for multiple host stresses. The only gene found to be activated in all conditions (oxidative stress, glucose starvation, low pH, acetic acid stress and internalization by macrophages) encodes the putative transcription factor Tos8. Tos8 contains a homeodomain typically involved in the transcriptional regulation of key eukaryotic developmental processes. In *S. cerevisiae* it is involved in the regulation of cell cycle and response to cell damage conditions (e.g. alkylating agents) (Horak et al. 2002; Jelinsky et al. 2000). It would be interesting to determine if *TOS8* acts as a general stress regulator and if it constitutes a virulence determinant. Plus, its role as a regulator of possible multiple stress responses makes it a promising therapeutic target.

Understanding the broad spectrum of stress responses by fungal pathogens, both during antifungal treatment and inside the host, is of paramount importance to overcome the increasing impact of fungal infections. The study and thorough analysis of genome-wide stress responses seems to be required for the full understanding of microbial stress in fungal pathogens.

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Chapter 8
Lipidomics Approaches: Applied to the Study of Pathogenesis in *Candida* Species

Ashutosh Singh, Nitesh Kumar Khandelwal and Rajendra Prasad

Abstract High rate of reported cases of infections in humans caused by fungal pathogens pose serious concern. Potentially these commensal fungi remain harmless to the healthy individuals but can cause severe systemic infection in patients with compromised immune system. Effective drug remedies against these infections are rather limited. Moreover, frequently encountered multidrug resistance poses an additional challenge to search for alternate and novel targets. Notably, imbalances in lipid homeostasis which impact drug susceptibility of *Candida albicans* cells do provide clues of novel therapeutic strategies. Sphingolipids (SPHs) are unique components of *Candida* cells, hence are actively exploited as potential drug targets. In addition, recent research has uncovered that several SPH intermediates and of other lipids as well, govern cell signaling and virulence of *C. albicans*. In this chapter, we highlight the role of lipids in the physiology of *Candida*, particularly focusing on their roles in the development of drug resistance. Considering the importance of lipids, the article also highlights recent high-throughput analytical tools and methodologies, which are being employed in our understanding of structures, biosynthesis, and roles of lipids in fungal pathogens.

Keywords Lipids · Pathogenic fungi · Functions · Mass spectrometry

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Abbreviations

MDR	Multidrug resistance
CW	Cell wall
CRS-MIS	Caspofungin reduced susceptibility—micafungin increased susceptibility
LCB	Long-chain sphingoid bases
PM	Plasma membrane
ABC	ATP binding cassette
MFS	Major facilitator superfamily
PC	Phospahtidylcholine
FD	Facilitated diffusion
QDR	Quinidine drug resistance
PHS	Phytosphingosine
PG	Phosphatidylglycerol
PS	Phosphatidylserine
PSD	Phosphatidylserine decarboxylase
PE	Phosphatidylethanolamine
BMDM	Bone marrow-derived macrophage
PI	Phosphatidylinositol
MS/MS	Triple quadrupole mass spectrometry
MSn	Tandem quadrupole-linear ion trap mass spectrometry
TOF	Time-of-flight mass spectrometry
ESI	Electrospray ionization
MALDI	Matrix-assisted laser desorption ionization
SPE	Solid phase extraction
HPLC	High-performance liquid chromatography
m/z	Mass-to-charge ratio
MPIS	Multiple precursor ion scanning
Pre	Precursor
NL	Neutral loss
PGL	Phosphoglyceride
SPH	Sphingolipids
PA	Phosphatidic acid
IPC	Inositolphosphorylceramide
MRM	Multiple reaction monitoring
SRM	Single reaction monitoring
LC-ESI/MSMS	Liquid chromatography electrospray ionization tandem mass spectrometry
MIPC	Mannosylinositolphosphoryceramide
$M(IP)_2C$	Mannosyldiinositolphosphoryceramide
GCMS	Gas chromatography mass spectrometry

8.1 Introduction

One of the most dominant fungal species causing secondary infections in humans belongs to genus *Candida*. *Candida* species contribute to more than 60% of all reported hospital cases. These include *Candida albicans, Candida glabrata, Candida tropicals, Candida paropsilosis, Candida dubliniensis, Candida guilliermondii, Candida krusei, Candida lusitaniae,* and *Candida auris,* etc. (Colombo et al. 2017; Pappas et al. 2018; Spivak and Hanson 2018; Webb et al. 2018). The resistance to antifungals like azoles, allylamines, polyenes, and echinocandins in majority of *Candida* infection cases has become a common occurrence in hospitals across the globe (Cowen et al. 2014; Morschhäuser 2016). The available arsenals of drugs against these *Candida* infections are rather limited. The frequently encountered Multidrug Resistance (MDR) poses an additional challenge to therapy and prompts for a serious search for new alternate and novel targets.

8.2 Mechanisms of Antifungal Drug Resistance

MDR is a multifaceted phenomenon since it is manifested with multiple mechanisms, which are well characterized over the years. In *Candida*, a decrease in intracellular accumulation of drugs, especially of azoles represents one of the major mechanisms of azole resistance. *Candida* genomes harbor a large number of such drug transporters belonging to either ABC (ATP Binding Cassette) or MFS (Major Facilitator Superfamily) proteins. Notwithstanding, among the large numbers of existing members of ABC or MFS superfamilies, only few have been implicated in manifestation of clinical drug resistance. Recent reviews highlight the structure and function of these ABC and MFS proteins (Prasad et al. 2015; Redhu et al. 2016). High expressions of genes encoding these efflux pumps namely Cdr1 or Cdr2 (ABC transporter superfamily) and/or Mdr1 (MFS transporter superfamily) are hallmark for azole tolerant isolates of *C. albicans*. These tolerant strains also showed a significantly lower intracellular accumulation of azoles, suggesting that azole tolerance is tightly linked to an overexpression of efflux pump proteins (Prasad et al. 1995). Mutational studies in Cdr1 and Mdr1 proteins have highlighted the key amino acids within and outside the active site that are involved in drug extrusion (Rawal et al. 2013; Shah et al. 2015; Redhu et al. 2018).

Drug diffusion also appears to direct the drug susceptibility of *Candida* cells. Recently, homozygous nulls of a PM localized ABC transporter Cdr6 (previously known as Roa1) in *C. albicans* were shown to display high PM rigidity due to ensuing lipid imbalances. The decrease PM viscosity hindered the drug diffusion process; which in turn resulted in decreased intracellular accumulation of azoles and enhanced resistance. This study represents an instance where membrane permeability compulsions imposed by *C. albicans* cells contribute to the development of azole resistance (Khandelwal et al. 2018). Few earlier reports have also implied that azoles

could be imported by facilitated diffusion (FD) rather than by passive diffusion in *C. albicans* and an altered FD could result in the development of MDR (Mansfield et al. 2010).

Notably, not all ABC or MFS members of superfamilies are drug transporters or even PM localized. For instance, ABC transporter Mlt1 of *C. albicans* is localized in vacuolar membrane and transports phosphatidylcholine (PC) into the vacuolar lumen (Khandelwal et al. 2016). In another instance, disruptants of Quinidine Drug Resistance (QDR) protein family members Qdr1, Qdr2, and Qdr3 (belonging to MFS superfamily), although localized on PM, do not recognize azoles, polyenes, echinocandins, polyamines, or quinolines as substrates (Shah et al. 2014). Together, there are many instances to support that several members of MFS and ABC superfamilies are not drug transporters.

MDR is also encountered by echinocandins which target β1,3-glucan synthase of cell wall (CW) (Perlin 2015). The resistance to echinocandins has been attributed to mutations in the catalytic subunit of β1,3-glucan synthase, Fks1 (and its paralog Fks2) (Martí-Carrizosa et al. 2015). In *C. glabrata*, resistance to echinocandins is strongly linked to mutations in Fks1/Fks2 (Katiyar et al. 2012). Notably, the Caspofungin Reduced Susceptibility—Micafungin Increased Susceptibility (CRS-MIS), phenotype of *C. glabrata*, is Fks-independent (Healey et al. 2011, 2012). Disruptants of *C. glabrata* Fen1 and Cka2 confer CRS-MIS and also show accumulation of long-chain sphingoid bases (LCB). On the other hand CRS-MIS phenotype is accompanied by mutations in Fen1, Sur4, Ifa38, and Sur2, all proteins involved in the SPHs biosynthetic pathway of *C. glabrata*. This evidence suggests that SPHs modulate Fks-echinocandin interaction in CRS-MIS in *C. glabrata* (Healey et al. 2015).

8.3 Lipid Imbalances Impact MDR

Imbalances in lipid homeostasis have been revealed to impact drug susceptibility of *C. albicans*. It is observed that variations in membrane lipids impact membrane rigidity and drug susceptibility of yeast cells (Kohli et al. 2002; Prasad and Singh 2013). Disruption of genes particularly those involved in either ergosterol or SPH biosynthesis, renders *C. albicans* cells hyper-susceptible to drugs. The imbalances in membrane ergosterol or SPHs not only results in increased drug susceptibility but also impact protein trafficking (Mukhopadhyay et al. 2004; Pasrija et al. 2008; Branco et al. 2017).

ABC drug transporter Cdr1 of *C. albicans* is preferentially localized in lipid rafts (membrane micro-domain, (Rella et al. 2016)), in contrast, MFS drug transporter Mdr1, does not show such a preference. Supporting this conclusion, the fluctuations in the levels of both ergosterol and SPH biosynthetic pathway intermediates selectively led to mislocalization of ABC transporter protein (Pasrija et al. 2008). It has been established that any disproportionate distribution of raft specific lipids not only affect membrane fluidity, but also impact functions of membrane localized ABC transporters. The ability of Cdr1 protein to translocate phospholipid between the

two monolayers of PM to maintain lipid asymmetry is an additional function which further highlights the relevance of lipids (Prasad et al. 1995; Smriti et al. 2002).

More recently, the deletion of orthologs of Fen1 and Sur4 in *C. albicans* and *C. glabrata* has been linked to increased susceptibility to amphotericin B. This observation is well supported by the fact that wild-type *C. glabrata* strains became susceptible to amphotericin B, if pretreated with myriocin (an inhibitor of serine palmitoyl transferase), and this phenotype could be reversed by the addition of phytosphingosine (PHS) in the growth medium (Healey et al. 2015, Kumar and Shahi et al. unpublished data). Likewise, imbalances in ergosterol contents also affect drug susceptibilities of *C. albicans* cells. For instance, the accumulation 14α-methyl-3,6-diol in *ERG11* (target of azoles) mutant, disrupts the membranes, results in intracellular toxicity and cell death in *C. albicans* (Martel et al. 2010; Flowers et al. 2012; Feng et al. 2017). More recently, ergosterol is also implicated in vacuolar ATPase function. The disruptants of ergosterol biosynthesis (Erg24) and vacuolar ATPase (Vma2) of *C. albicans* cells fail to acidify vacuoles. A similar phenotype is also observed when cells are treated with fluconazole, pointing towards a strong correlation between ergosterol content and vacuolar acidification in *C. albicans*. Additionally, disruption of either *ERG24* or *VMA2* results in drastic defects in mitochondrial respiration, cell wall biogenesis and virulence in *C. albicans* (Zhang et al. 2010).

Apart from raft lipid components namely ergosterol and SPHs, fluctuations in phosphoglycerides are also critical for drug susceptibility of *C. albicans* cells. Our group has shown that phosphatidylglycerol (PG) is linked to azole resistance, mitochondrial function and cell wall (CW) integrity (Singh et al. 2012). In another study, homozygous null mutants of *PSD1/PSD2* (phosphatidylserine decarboxylases) and *CHO1* (a PS synthase) lacking PE and PS, are linked to compromised CW integrity (Chen et al. 2010). The defect in *Psd1/Psd2* impacts membrane viscosity, which in turn affects azole tolerance (Khandelwal et al. 2018b). Apart from the fact that fluctuations in lipid affects drug tolerance, the levels of Psd1, Psd2, Cho1, Cdr6, and vacuolar transporter Mlt1, also affect virulence characteristics of *C. albicans* and point to a strong correlation between phospholipid homeostasis, azole resistance, and virulence in *C. albicans* (discussed below).

8.4 Lipids Impact on Hyphae and Biofilm Development

Biofilm formation is one of the crucial properties in various pathogenic fungi. The biofilm of *C. albicans* composed of a mixture of yeast and hyphae cells along with complex biofilm matrix. The biofilm matrix consist 55% protein, 25% carbohydrate, 15% lipid, and 5% nucleic acid as macromolecular classes (Zarnowski et al. 2014). Among lipid species, glycerolipids contribute major portion of 99.5% (neutral glycerolipids 89.1%, polar glycerolipids 10.4%), where SPHs (0.5%) exist as minor component. Between the glycerolipids present in the biofilm matrix, phosphatidylethanolamine (PE) is the most abundant polar glycerolipids (Zarnowski et al. 2014).

Ghannoum group has shown that lipid emulsion induces the hyphae germination and biofilm formation in *C. albicans* (Swindell et al. 2009). A comparative lipid analysis between yeast and hyphae form show that glycerophospholipid and SPH composition changes between the two morphological forms (Lattif et al. 2011). Notably, SPHs only contributes a small portion in biofilm matrix; however, they are crucial for its formation. Inhibition of SPH biosynthesis in *C. albicans* either by chemicals (myriocin or aureobasidin A) or by genetic intervention (deletion of *IPT1* gene) prevents biofilm formation (Lattif et al. 2011). Similarly, the deletion of other SPH biosynthesis pathway genes like *FEN1* and *FEN12* also lead to defective hyphae and biofilm formation (Alfatah et al. 2017). Along with SPH biosynthesis genes, the genes involved in sterol biosynthesis also seem to have a role in hyphae development (Pasrija et al. 2005; Prasad et al. 2005). Interestingly, in the biofilm matrix, the only detectable sterol is ergosterol which remains at a very low level (Zarnowski et al. 2014). This suggests that azoles may not be very effective against biofilm and one should explore alternate drugs, which target other lipids or a combinational therapy as a better option to combat the development of biofilms.

8.5 Lipids Impact Virulence

A screening of homozygous null mutant library of *C. albicans* revealed the involvement of several genes of glucosylceramide biosynthesis in its pathogenicity (Noble et al. 2010). The screen revealed that homozygous null mutants for glucosylceramide biosynthesis pathway genes, *HSX11* (glucosyltransferase), *SLD1* (SPH desaturase), and *MTS1* (SPH C9-methyltransferase) show attenuated virulence. Interestingly, glucosylceramide is absent in nonpathogenic yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* However, its presence in pathogenic fungi such as *Cryptococcus neoformans* and *C. albicans* impacting virulence capacity of the pathogens is an interesting observation (Noble et al. 2010; Raj et al. 2017). Additionally, several other lipid metabolism genes (*CYB1*, *HET1, OAR1*, and orf19.6411) are recognized as probable virulence factors (Noble et al. 2010). The ceramide synthase (CerS3) deficient mice showed that it lacked all SPHs with ultra long chain fatty acids resulting in water permeability barrier disruption. Interestingly, CerS3-deficient mice skin became prone to *C. albicans* infection, highlighting the role of SPHs in permeability barrier of mice and in promoting seeding of fungal skin infection (Jennemann et al. 2012).

Interestingly, mutants of *CHO1* and *PSD1*/*PSD2* of *C. albicans* which display attenuated virulence in a mouse model of systemic candidiasis (Chen et al. 2010), also affect the immune-stimulatory phenotypes. In a specific example, the extracellular vesicles from the *CHO1* deletion mutant fail to activate the NF-kB activation in macrophage cells from bone marrow-derived macrophage (BMDM) and the murine macrophage-like J774.14 cell line (Wolf et al. 2015). Since fungal Cho1 is conserved among fungal species and mammalian phosphatidylserine synthase enzymes are not orthologous of fungal Cho1, it is also considered as a potential drug target.

Additionally, the phosphoinositide 3-kinase (PI3K; CgVps34) signaling is central to intracellular survival and pathogenesis of *C. glabrata*. The enzyme PI3K phosphorylates phosphatidylinositol (PI) into phosphatidyl inositol-3-phosphate—an important class of phospholipid implicated in protein trafficking processes (Sharma et al. 2016).

The levels of oleic acid in cellular lipids are reported as critical components for morphogenetic competence of *C. albicans*, where *OLE1* (encoding fatty acid desaturase) is shown to be essential for viability (Nguyen et al. 2011). The study reveals that a modest reduction in oleic acid does not affect growth in the yeast form, but prevents hyphal development. Apparently, the impact of oleic acid levels on membrane fluidity is not directly responsible for the morphogenetic potential of *C. albicans*, but probably suggestive of a role in activating specific morphogenetic pathways (Krishnamurthy et al. 2004). Earlier studies have pointed out the role of other fatty acids in virulence of *C. albicans* cells. For instance, *NMT* of *C. albicans* encoding myristoylCoA:protein-N-myristoyltransferase is essential for its vegetative growth. A point mutant strain displaying reduced activity of this enzyme shows attenuated virulence in a mouse model (Weinberg et al. 1995). Similarly, a mutant lacking the *FAS2* gene, which encodes a fatty acid biosynthesis enzyme subunit, shows attenuated virulence in a mouse model of systemic and rat model of oropharyngeal candidiasis (Zhao et al. 1996, 1997). These emerging examples collectively point that lipids in general are important factors, which affect morphogenesis and virulence capacity of *C. albicans* cells, and positions itself as having great potential of becoming novel drug targets.

8.6 Mass Spectrometry Based Lipidomics

The overwhelming evidence of lipids in the development of drug tolerance, signaling and virulence in *Candida* discussed above warrant a detailed understanding of the composition and regulation of lipid biosynthetic pathways. Considering the importance of lipids, the following section discusses high-throughput systems-level approach being increasingly employed in determining the absolute abundance of molecular lipid species under a variety of biological conditions in yeast.

Earlier the lipid analysis was limited to techniques like thin layer chromatography, radioactive labeling, gas–liquid chromatography, and high-performance liquid chromatography. But more recently, high-throughput mass spectrometry based lipid analysis has evolved as a new field of “lipidomics” (Wenk 2005). Researchers have employed high-throughput lipidomics to map the lipid dynamics in a variety of systems including in fungi. By using *S. cerevesiae* as an example, the first automated shotgun lipidomics analysis of yeast was conducted, which enabled lipidome-wide absolute quantification and identification of individual molecular lipid species (Ejsing et al. 2009). The study provided a platform and an example of molecular characterization of eukaryotic lipidomes and established shotgun lipidomics as a powerful platform for systems-level analysis (Ejsing et al. 2009). The study was followed by a report from our group where high-throughput approach was employed to ana-

lyze the phospholipidome of eight *Candida* species (Singh et al. 2010). Our study detected no large phosphoglyceride (PGL) compositional differences among *Candida* species; however, fatty acyl chain composition greatly varied and pointed to a typical molecular species specific imprint of each *Candida* species. This followed several high-throughput lipidomic analysis of yeast, in understanding the structure, biosynthesis and role of lipids in fungal pathogenesis, virulence and MDR (Khandelwal et al. 2018; Singh et al. 2012, 2013; Hameed et al. 2011; Singh and Prasad 2011; Sharma et al. 2012; Mahto et al. 2014).

Over the years a wide variety of platforms have been developed for lipidomic studies. Some common examples include triple quadrupole (MS/MS), tandem quadrupole-linear ion trap (MSn), time-of-flight (TOF), etc. These techniques require prior ionization of analytes using ionizers like electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI), etc. The complete ionization of the analytes into the gaseous phase is an extremely crucial step for their detection on mass analyzer. Various aspects regarding the choice of analyzer and ionizers depending upon the required analysis have been reviewed in detail (Köfeler et al. 2012). In the following, we focus on some examples of mass spectrometry based methods employed in *Candida* and discuss the implications of these lipidomic analyses on understanding the observed phenotypes.

8.6.1 Sample Preparation for High-Throughput Analysis

For lipidomics study employing high-throughput mass spectrometry platform, the sample preparation is a key step leading to robust and reproducible datasets. Extraction of lipids requires pre-breaking of fungal cells in solvents with glass beads using alternate sonication and vortexing; or by a homogenizer. The use of solvent depends upon the lipid of interest. While chloroform:methanol (2:1, v/v) extraction procedure has been a gold standard for isolating lipids (Bligh and Dyer 1959), lately a two-step extraction method involving chloroform:methanol (17:1, v/v) followed by chloroform:methanol (2:1, v/v) extraction has also been used (Ejsing et al. 2009). Mild alkaline hydrolysis of lipid extracts is recommended for sterol and SPH analysis (Singh et al. 2017). For SPHs, Mandala extraction has shown good efficiency (Mandala et al. 1995). Purification of more specific lipid classes like glycosphingolipids requires solid phase extraction (SPE), which is not a common practice in routine lipid analysis (Singh and Del Poeta 2016).

Mass spectrometry is a sensitive technique and does not require large quantities of samples; however high-quality samples are a prerequisite. In addition, before the samples are subjected to mass spectrometry, lipids are further separated on a High-Performance Liquid Chromatography (HPLC) column. Both normal and reverse phase HPLC columns are used depending on the class of lipids to be separated. While SPHs can be easily resolved on a reverse phase C8 column, PGLs a normal phase C18 column is preferred (Singh and Del Poeta 2016). The idea is to resolve any lipid species that has similar mass-to-charge ratios (*m/z*), as these are difficult

to distinguish on a mass spectrometer. Further, pre-separation of lipids species on an HPLC significantly improves the sensitivity of detection and their ionization efficiencies. For yeast samples, where the PGLs represent an abundant class of lipids, separation of individual lipids on an HPLC is often not required; and therefore, are detectable using the direct infusion of the sample onto the mass spectrometer. An exception would be if one wants to focus on differences in *cis-trans* lipid species, which requires both mass spectrometry and HPLC to accomplish (Bird et al. 2012). Notably, a differential mobility spectrometry approach, which could distinguish *cis-trans* lipid species without the HPLC separation, has recently been described (Baba et al. 2017). Nonetheless, separation of analytes on a HPLC is recommended prior to their analysis on a mass spectrometer.

8.6.2 Analysis of PGLs

PGLs are one of the most abundant groups of lipids present in fungi. There are over 20 classes of PGLs among various biological samples so far identified [LIPID MAPS® Lipidomics Gateway]. Each PGL species has a glycerol backbone, ester linked to fatty acyl at the *sn*-1 and *sn*-2, with a polar head group at the *sn*-3 position. Subclasses of PGLs are based on the polar head groups, which are esterified with –OH of glycerol at *sn*-3 position. Multiple Precursor Ion Scanning (MPIS) is the most commonly employed approach for the detection of PGLs species. Sequential precursor (Pre) and neutral loss (NL) scans of the lipid extracts (Bligh and Dyer extract) produce a series of spectra revealing a set of lipid species containing a common head group fragment on an electrospray ionization tandem mass spectrometer (ESI/MSMS) (Singh et al. 2010). Lipid species are detected with the following scans: PC and LysoPC, $[M + H]^+$ ions in positive ion mode with Pre 184.1; PE and LysoPE, $[M + H]^+$ ions in positive ion mode with NL 141.0; PA, $[M + NH_4]^+$ in positive ion mode with NL 115.0; PG, $[M + NH_4]^+$ in positive ion mode with NL 189.0; PI, $[M + NH_4]^+$ in positive ion mode with NL 277.0; PS, $[M + H]^+$ in positive ion mode with NL 185.0; LysoPG, $[M - H]^-$ in negative mode with Pre 152.9 (Fig. 8.1).

Mass spectrometry approaches have revealed that PGLs composition in *Candida* comprises PC, PS, PE, PI, PG, and phosphatidic acid (PA) (Singh et al. 2010). Also, the compositions of individual molecular species for each class, including their lyso forms, have been qualitatively and quantitatively determined. In a typical PGL analysis, over 200 lipid species from nine different classes of PGLs are detectable. Structures of six major PGL classes are shown in Fig. 8.2. In *Candida* species, the abundances of PGLs are in the order: PC>PE>PI>PS>PA>PG; and those of lysoPGLs are in the order: LysoPC>LysoPE>LysoPG. In *C. albicans*, the most abundant molecular lipid species for different PGL classes include: LysoPG 18:0 detected as *m/z* 511.4 (Fig. 8.1a); PG 34:2 and PG 34:1 detected as *m/z* 764.7 and 766.7, respectively (Fig. 8.1b); PA 34:2 and PA 34:1 detected as *m/z* 690.7 and 692.7, respectively (Fig. 8.1c); PS 34:2 and PS 34:1 detected as *m/z* 760.7 and 762.8, respectively (Fig. 8.1d); PI 34:2 and PI 34:1 detected as *m/z* 852.7 and 854.7, respectively

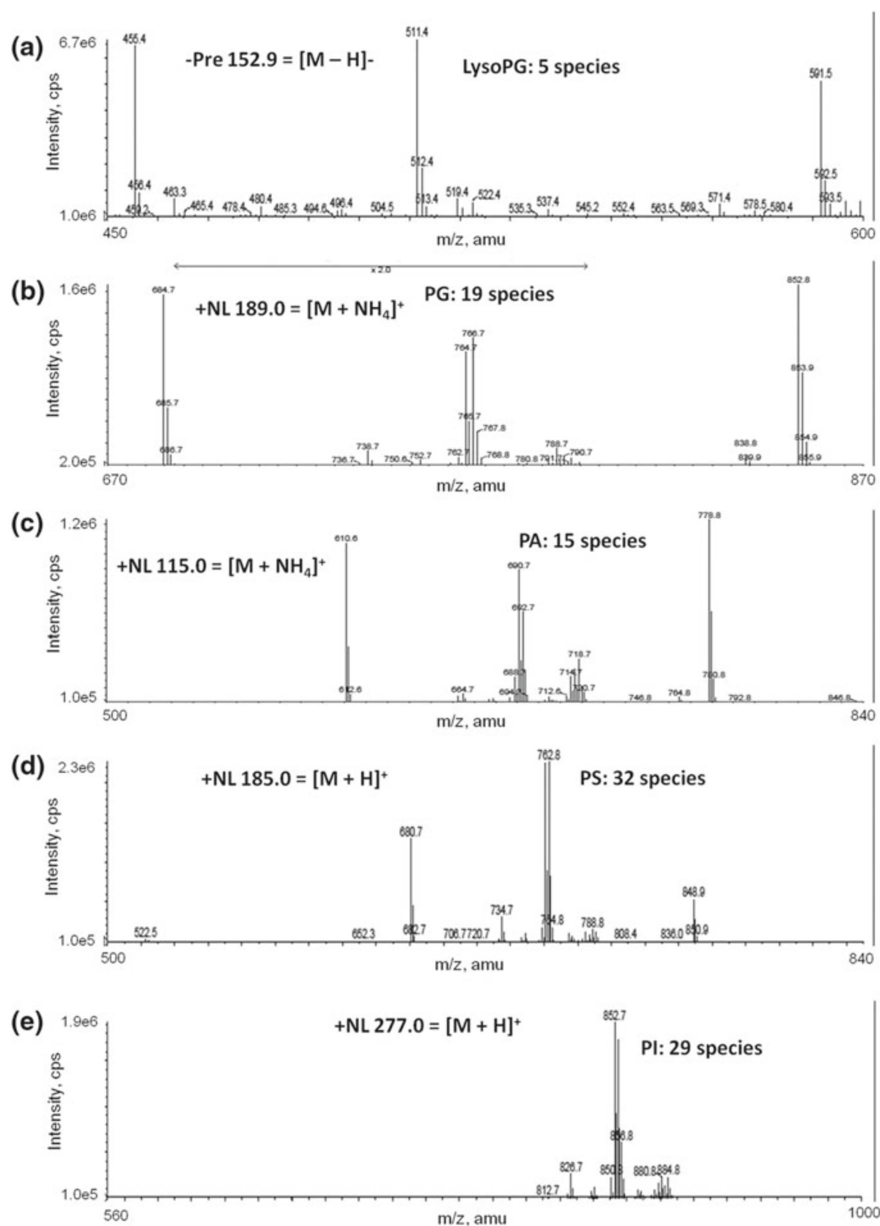

Fig. 8.1 Analysis of PGLs using positive Pre and NL scanning using QTRAP-ESI/MSMS in *C. albicans*. **a** LysoPG species detection using negative Pre 152.9 as $[M - H]^-$. **b** PG species detection using positive NL 189.0 as $[M + NH_4]^+$. **c** PA species detection using positive NL 115.0 as $[M + NH_4]^+$. **d** PA species detection using positive NL 185.0 as $[M + H]^+$. **e** PI species detection using positive NL 277.0 as $[M + H]^+$. **f** LysoPE and PE species detection using positive NL 141.0 as $[M + H]^+$. **g** LysoPC and PC species detection using positive Pre 184.1 as $[M + H]^+$. Various scans are represented as *m/z*. Numbers of species detected in each scan are also shown

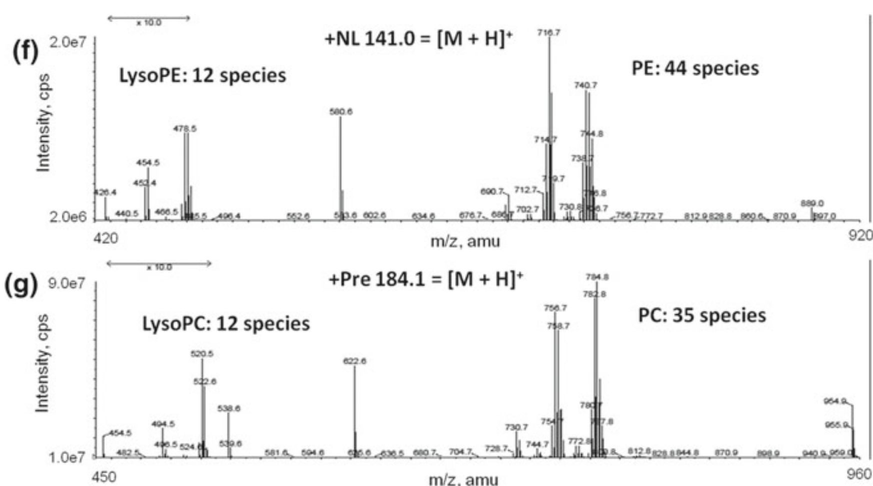

Fig. 8.1 (continued)

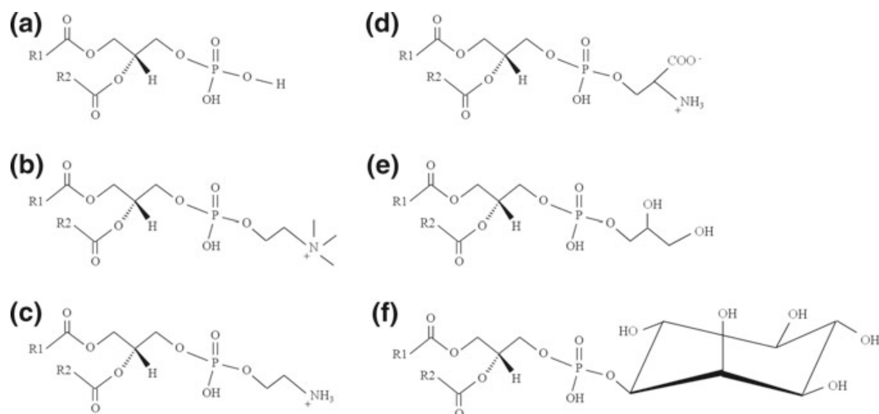

Fig. 8.2 Structures of major PGL classes detected in *Candida*. **a** PA. **b** PC. **c** PE. **d** PS. **e** PE and **f** PI. 'R1' and 'R2' represent the fatty acyls of different chain lengths

(Fig. 8.1e); LysoPE 18:2 and LysoPE 18:1 detected as *m/z* 478.5 and 480.5, respectively (Fig. 8.1e); PE 34:2, PE 34:1, PE 36:4, and PE 36:3 detected as *m/z* 716.7, 718.7, 740.7, and 742.8, respectively (Fig. 8.1e); LysoPC 18:2 and LysoPC 18:1 detected as *m/z* 520.5 and 522.6, respectively (Fig. 8.1f); PC 34:3, PC 34:2, PC 36:4 and PC 36:3 detected as *m/z* 756.7, 758.7, 782.8, and 784.8, respectively (Fig. 8.1f). The compositions of these PGL species vary significantly among different *Candida* species (Singh et al. 2010). The odd-chain fatty acyl containing PGL species are also detectable in *Candida* species (Singh et al. 2010, 2012). While it is known that the odd-chain fatty acyls are incorporated into the PGL pool via the PHS degradation pathway (Kondo et al. 2014); the functional significance of these lipids is not well

understood and further studies are required in this area. Our ability to detect these changes at molecular level by tools like mass spectrometry has enabled us to better understand the importance of lipids in molecular basis of pathogenicity not only in *Candida* but in other yeasts and fungi as well.

8.6.3 Analysis of SPHs

SPHs represent a diverse and important group of structures found in biological samples and are divided into about 11 different classes (LIPID MAPS® Lipidomics Gateway). In most *Candida* species, the range of these structures varies from low molecular weight simple SPHs like LCBs to high molecular weight complex SPHs like inositolphosphorylceramide (IPC) derivatives and glucosylceramides (Prasad and Singh 2013; Singh and Del Poeta 2016). Two common approaches that are used to analyze these lipids (base hydrolyzed lipid extracts, see above) in *Candida* are discussed below.

First is the MPIS approach, which has been extensively used to detect complex SPH structures (Fig. 8.3). For example, the IPC structures consist of a phytoceramide backbone with a phosphoryl inositol moiety linked at the C1-hydroxy position (Guan and Wenk 2008). Various IPC derivative structures are detectable using the specific head group fragments: IPC, $[M - H]^-$ in negative mode with Pre 259 (corresponding to $[IP]^-$) or Pre 241 (corresponding to $[IP - H_2O]^-$); mannosylinositolphosphoryceramide (MIPC), $[M - H]^-$ in negative mode with Pre 421 (corresponding to $[MIP]^-$); mannosyldiinositolphosphoryceramide ($M(IP)_2C$), $[M - H]^-$ in negative mode with Pre 663 (corresponding to $[M(IP)_2]^-$). Fragments m/z 241 and 259 are detected in all IPC derivatives (Singh et al. 2010; Angelini et al. 2012). In *Candida*, 11 species each for IPC, MIPC, and $M(IP)_2C$ are detectable (Fig. 8.3). In *Candida*, the most abundant IPC derivative species include: MIPC (42:0-3), MIPC (42:0-4), MIPC (44:0-3), and MIPC (44:0-4) detected as *m/z* 1087, 1103, 1115, and 1131, respectively (Fig. 8.3a); IPC (42:0-3), IPC (42:0-4), IPC (44:0-3), and IPC (44:0-4) detected as *m/z* 924.9, 940.8, 952.9, and 968.9, respectively (Fig. 8.3b); $M(IP)_2C$ (42:0-3), $M(IP)_2C$ (42:0-4), $M(IP)_2C$ (44:0-3) and $M(IP)_2C$ (44:0-4) detected as *m/z* 1329, 1345, 1357, and 1373, respectively (Singh et al. 2010). Notably, detection in negative Pre ion scanning results in poor ionization efficiency of $M(IP)_2C$, in turn resulting in lower sensitivity limits.

Glucosylceramides represent another important class of complex SPH structures in *Candida* (Noble et al. 2010). In a glucosylceramide structure, a glucose moiety is linked at C1 position to the ceramide backbone by a β-glycosidic bond (Del Poeta et al. 2014). Glucosylceramide structures with 3 different backbones are reported in *Candida*. These are 4-sphingenine (d18:1), 4,8-Sphingadiene (d18:2) and 9-methyl-4,8-sphingadiene (d19:2) (Noble et al. 2010). In the study by Noble et al., the glucosylceramide structures containing these backbones, identified $[M + Na]^+$ with exact masses of 766.8 for d18:1-glucosylceramide, 764.7 for d18:2-glucosylceramide and 778.9 for d19:2-glucosylceramide (Noble et al. 2010). These structures can also be

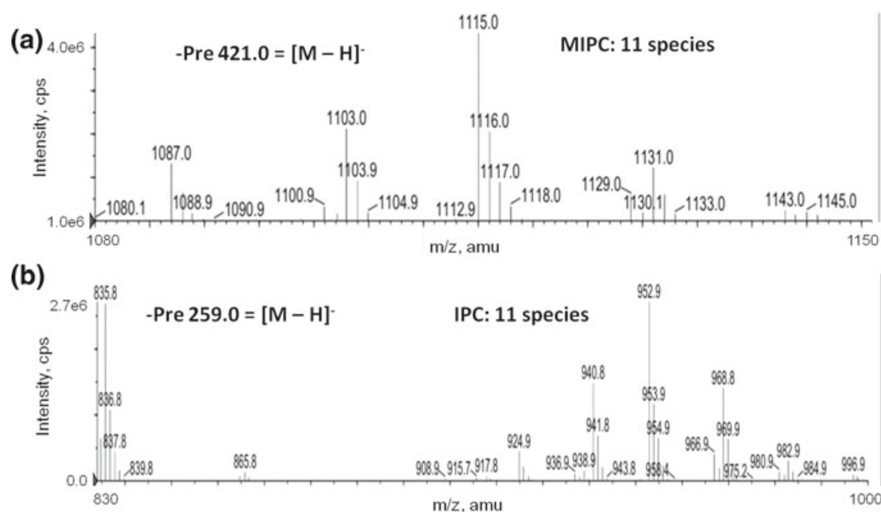

Fig. 8.3 Analysis of IPC derivatives using negative Pre ion scanning using QTRAP-ESI/MSMS in *C. albicans*. a MIPC species detection using negative Pre 421.0 as $[M - H]^-$. **b** IPC species detection using negative Pre 259.0 as $[M - H]^-$. Scans are represented as *m/z* and 11 species were detected for MIPC and IPC each

validated by using the positive Pre ions of *m/z* 264.4 for d18:1-glucosylceramide (parent ion fragment of *m/z* 744.4), 262.4 for d18:2-glucosylceramide (parent ion fragment of *m/z* 742.4) and 276.3 for d19:2-glucosylceramide (parent ion fragment of *m/z* 756.4) structures, as $[M + H]^+$ (Singh and Del Poeta 2016).

A second more targeted approach to analyze SPHs is the multiple reaction monitoring (MRM) using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MSMS) platform. The approach utilizes the monitoring *m/z* of a select parent or Pre ion (in the first quadrupole) and *m/z* of a select daughter ion (in the third quadrupole) which results in collision-induced dissociation in the collision cell (second quadrupole) at specific collision energy. Such single reactions are termed as “single reaction monitoring” (SRM). Based on the scanning efficiency of the instrument several SRM reactions can be monitored simultaneously as the MRM, allowing us to detect multiple SPH species in a single sample run (Singh and Del Poeta 2016). An example of MRM approach being used to analyze a SPH mixture is depicted in Fig. 8.4. The following “parent ion → daughter ion” SRM reactions are used in a single MRM to detect the SPH species composition of the sample: (i) *m/z* 286.4 → 268.3 for sphingosine (C17 base); (ii) *m/z* 288.5 → 252.3 for sphinganine (C17 base); (iii) *m/z* 366.5 → 250.3 for sphingosine-1-P (C17 base); (iv) *m/z* 368.5 → 252.3 for sphinganine-1-P (C17 base); (v) *m/z* 806.6 → 264.3 for lactosyl(β)C12-ceramide; (vi) *m/z* 644.3 → 264.3 for glucosyl(β)C12-ceramide; (vii) *m/z* 482.5 → 464.4 for C12-ceramide; (viii) *m/z* 647.7 → 184.1 for C12-sphingomyelin; (ix) *m/z* 561.8 → 264.3 for C12-ceramide-1P; (x) *m/z* 664.7 → 646.6 for C25-ceramide. These scans are performed in positive ion mode and all ions are detected as $[M + H]^+$. Use of C8 column based HPLC prior to mass spectrometry allows a descent sep-

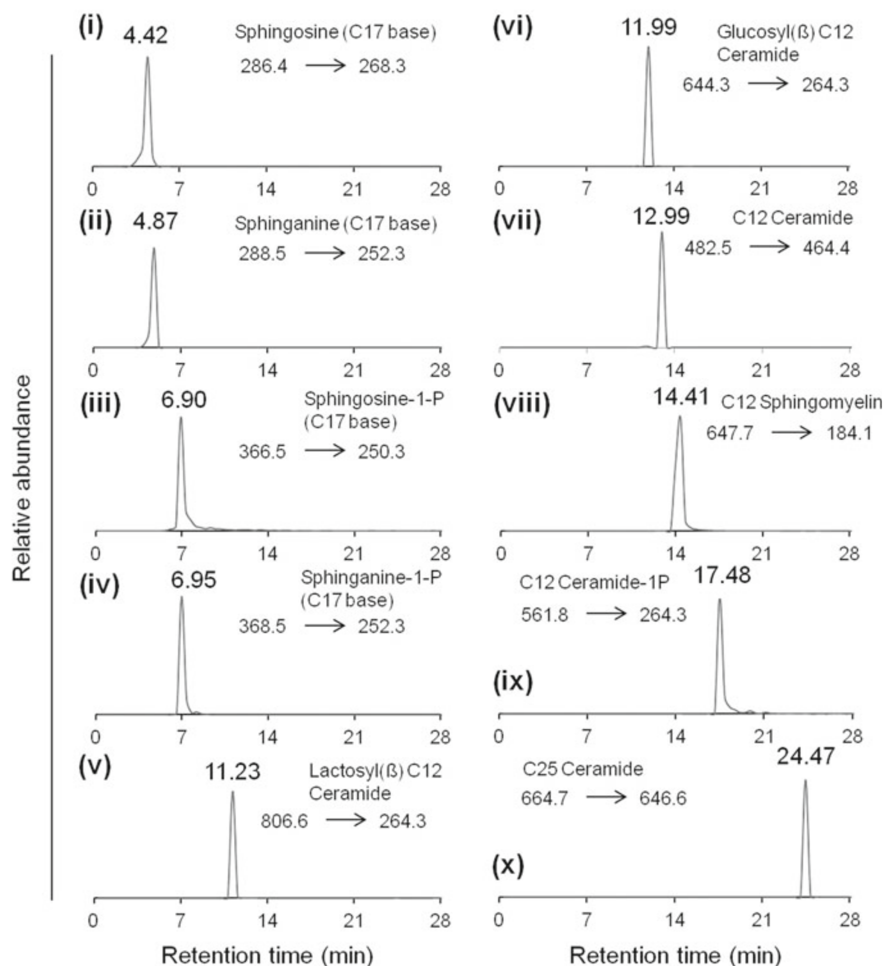

Fig. 8.4 Analysis of various sphingolipid species using MRM approach on LC-ESI/MSMS. The figure shows single reactions for 10 sphingolipid species (**i**–**x**) that were detected in a single MRM scanning. Transitions are represented as *m/z* of parent ion to daughter ion. This data was acquired using a standard sphingolipid mixture I (Avanti Polar Lipids, Inc.)

aration of analytes as evident by their retention times (Fig. 8.4). The MRM approach has been extensively used in targeted sphingolipidomics in recent years, discussed in detail by Singh and Del Poeta (Singh and Del Poeta 2016). Ability to accurately determine the compositions of SPH pool has allowed us to better attribute the functional importance of various SPH structures in pathological mechanisms of *Candida* as well other human pathogenic fungi (Prasad and Singh 2013; Lattif et al. 2011; Beckmann et al. 2003; Oura and Kajiwara 2008; Cheon et al. 2012; Tafesse et al. 2015; Rollin-Pinheiro et al. 2016).

8.6.4 Analysis of Sterols

Functionally, three different sterol classes are implicated in growth and pathogenesis of *Candida* which include: free sterols, sterol esters, and steryl glucosides (Singh et al. 2013; Bailey and Parks 1975; Warnecke et al. 1999; Lv et al. 2016; Xu et al. 2017). These sterol structures can be detected using a wide variety of mass spectrometry platforms. For example, sterol esters are detectable in positive ion scanning on an ESI/MSMS instrument. Specifically, NL scans of *m/z* 271.2, 273.2, 295.2, 297.2, 299.2, and 301.2 can be used to detect 16:1, 16:0, 18:3, 18:2, 18:1, and 18:0—containing sterol ester species as $[M + NH_4]^+$ (Singh and Prasad 2011). Similar scans are used to detect the diacylglycerol and triacylglycerol species compositions. However, this approach of detecting sterol ester is not very quantitative because of overlapping masses of other lipid species (Singh and Prasad 2011); therefore, prior separation on HPLC is recommended.

Sterols show poor ionization efficiency on an ESI source. Hence, Gas Chromatography Mass Spectrometry (GCMS) is a more reliable technique to analyze sterol compositions. One major limitation of sterol analysis by GCMS is that sterols are not readily ionized into the gaseous phase; therefore a chemical derivatization step is mandatory prior to analysis. Base hydrolyzed lipid extracts are derivatized using BSTFA/TMCS (N,O-bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane) reagent (Sigma Aldrich, US) resulting in trimethylsilyl-derivatives (TMS-derivatives) of sterols (Singh et al. 2013). Analysis of sterols on GCMS results in sterol species specific fragmentation patterns that are used to identify the species (Nes et al. 2009; Chang et al. 2014). A few common sterol species and their specific fragments are: *m/z* 117.1, squalene; *m/z* 366.2, zymosterol; *m/z* 376.3, dehydroergosterol; *m/z* 363.4, ergosterol (Fig. 8.5); *m/z* 343.4, fecosterol; *m/z* 365.4 episterol; *m/z* 367.4, fungisterol; *m/z* 349.3, zymosterone; *m/z* 393.3, obtusifoliol; *m/z* 484.4, 4α-methyl fecosterol; *m/z* 407.4, eburicol; *m/z* 393.3, lanosterol (Singh et al. 2017). An example of TMS-derivatized ergosterol is shown in Fig. 8.5. A mass spectrum of TMS-ergosterol reveals characteristic fragments: *m/z* 468.4, 378.3 (Fig. 8.5 inset), 363.3 (major fragment), 337.3, 253.2, 131.1, 73.1 representing M^+, $M–(CH_3)_3SiOH$, $M–(CH_3)_3SiOH–CH_3$, $M–(CH_3)_3SiO{=}^+CH–CH_2–CH_3$, $M–(CH_3)_3SiOH$-side chain, $(CH_3)_3SiO{=}^+CH–CH_2–CH_3$ and $(CH_3)_3Si$ structural fragments (Honda et al. 1996). Similar GCMS based approaches have been used to detect the steryl glucoside structures (Warnecke et al. 1999; Rella et al. 2015) and the free fatty acyl contents in pathogenic fungi (Singh et al. 2017).

These mass spectrometry based analysis very well compliment the genetic approaches, which allow researchers to map the biosynthetic pathways as well as assign biological functions to lipids (Noble et al. 2010; Cheon et al. 2012).

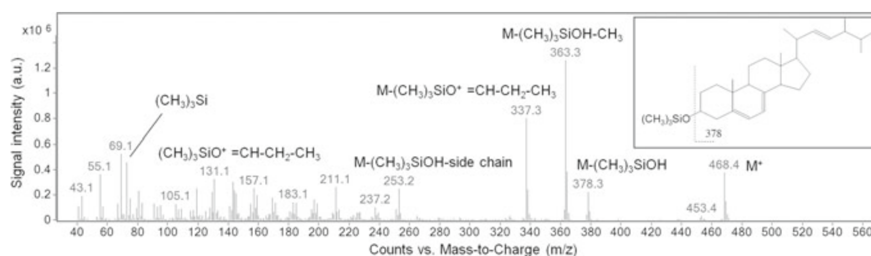

Fig. 8.5 Fragmentation pattern of TMS-derivatized ergosterol using EI-MS. Major fragments observed at 70 eV collision energy are labeled. Inset figure shows the structure of TMS-derivatized ergosterol

8.7 Concluding Remarks

In this chapter, we have summarized some key functions of lipids in human pathogenic *Candida*. Apart from the fact that lipids are a key structural component of cells, their optimal levels are necessary to maintain an environment of homeostasis in cellular membranes. Disruption in lipid biogenesis significantly alters the growth and phenotypic properties of *Candida*. Role of lipids in drug tolerance and virulence are beginning to be realized which may lead to improve therapeutic strategies. Therefore, it becomes critical to determine the structure and levels of lipids in these emerging pathogens. Considering the emerging significance of lipids in *Candida*, we have discussed some examples of recent mass spectrometry based methodological approaches that have been used to analyze lipid compositions of *Candida*.

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Chapter 9
Yeast at the Forefront of Research on Ageing and Age-Related Diseases

Belém Sampaio-Marques, William C. Burhans and Paula Ludovico

Abstract Ageing is a complex and multifactorial process driven by genetic, environmental and stochastic factors that lead to the progressive decline of biological systems. Mechanisms of ageing have been extensively investigated in various model organisms and systems generating fundamental advances. Notably, studies on yeast ageing models have made numerous and relevant contributions to the progress in the field. Different longevity factors and pathways identified in yeast have then been shown to regulate molecular ageing in invertebrate and mammalian models. Currently the best candidates for anti-ageing drugs such as spermidine and resveratrol or anti-ageing interventions such as caloric restriction were first identified and explored in yeast. Yeasts have also been instrumental as models to study the cellular and molecular effects of proteins associated with age-related diseases such as Parkinson's, Huntington's or Alzheimer's diseases. In this chapter, a review of the advances on ageing and age-related diseases research in yeast models will be made. Particular focus will be placed on key longevity factors, ageing hallmarks and interventions that slow ageing, both yeast-specific and those that seem to be conserved in multicellular organisms. Their impact on the pathogenesis of age-related diseases will be also discussed.

Keywords Ageing · Nutrient-sensing pathways · Autophagy · Chronological life span · Replicative life span · Yeast · Proteostasis

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Abbreviations

aSyn	Alpha-synuclein
AD	Alzheimer's disease
Aβ	Amyloid-β
AMPK	AMP-activated protein kinase
ATG	Autophagy gene
CLS	Chronological life span
CORE	Cross-organelle stress response
CR	Caloric restriction
DDR	DNA damage responses
ERCs	Extrachromosomal rDNA circles
HD	Huntington's disease
Htt	Huntingtin
IPOD	Insoluble protein deposit
INQ	Intranuclear quality control compartment
GTA	Genotoxin-induced targeted autophagy
JUNQ	Juxta nuclear quality control site
NQ	Non-quiescent
OXPHOS	Oxidative phosphorylation
PD	Parkinson's disease
PAS	Phagophore assembly site
PKA	Protein kinase A
Pho85	Phosphate metabolism protein 85
PolyQ	Polyglutamine
Q	Quiescent
ROS	Reactive oxygen species
RLS	Replicative life span
RNR	Ribonucleotide reductase
Snf1	Sucrose non-fermenting protein 1
TOR	Target of rapamycin
TORC1	Target of rapamycin complex 1
UPS	Ubiquitin proteasome system
VPS	Vacuolar protein sorting

9.1 Introduction

Ageing is a complex and multifactorial process driven by genetic, environmental and stochastic factors that lead to the cumulative incorporation of imbalances at the genomic and proteomic level in a multidimensional process resulting in the progressive decline of biological systems and decreased cellular fitness over time. In spite the fact that eukaryotic species have their own set of age-related diseases, the hallmarks

of cellular ageing are surprisingly conserved. These include primary events that trigger the progressively accumulation of cellular damage with ageing such as genomic instability, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing and mitochondrial dysfunction (reviewed in Lopez-Otin et al. (2013)). Due to the well-conserved hallmarks of cellular ageing, the budding yeast *Saccharomyces cerevisiae* has been widely used as a model of cellular and organismal ageing (Kaeberlein 2010; Longo et al. 2012; Sampaio-Marques et al. 2014a). The first study of yeast ageing, published more than 60 years ago, showed that yeasts have a finite replicative capacity (Mortimer and Johnston 1959). Mortimer and Johnston plotted their data and made the remarkable observation that the mortality curve for a yeast population resembles the mortality curves for many other organisms, including humans (Mortimer and Johnston 1959). Based on this observation, Replicative Life Span (RLS) was defined as the number of daughter cells produced by a single mother cell before dying. This definition underlies what has become a valuable model for studying ageing of mitotic cells. A second yeast model of ageing—the Chronological Life Span (CLS) model—was first proposed for budding yeast in 1980 (Muller et al. 1980). CLS is defined as the time that yeast cells can survive in a non-dividing state after exhaustion of the carbon source (Fabrizio and Longo 2003). Therefore, this single-celled organism provides a unique opportunity to study the ageing of both mitotic and post-mitotic cells (Kaeberlein et al. 2007). Not surprisingly, much of the advances on ageing research can be traced back to yeast that facilitates discovery of the evolutionarily conserved molecular and cellular mechanisms through which genetic and environmental interventions promote longevity. Yeasts were especially pivotal in the discovery of sirtuins and the TOR signalling pathway linking environmental nutrients to longevity. Different studies using these two yeast models of ageing have found that reducing glucose in the media can increase both RLS and CLS (Fabrizio and Longo 2003; Jiang et al. 2000). This paradigm linking environmental nutrients to longevity has been referred to as calorie restriction (CR) and is a major focus on ageing research.

This chapter intends to present some of the leading evidence and relevant advances on ageing and age-related diseases research in yeast models, with particular focus on longevity-promoting effects and proteostasis control.

9.2 Genes and Pathways Modulating Yeast Ageing: Replicative Versus Chronological Life Span

The two yeast models of ageing constitute important paradigms for the progressive accumulation of damage during ageing. While in the Replicative Life Span (RLS) damage is accumulated in mother cells due to the asymmetrical inheritance of damage, in the Chronological Life Span (CLS), the non-dividing cells cannot dilute the damage accumulated during ageing. Although both ageing models have different molecular and genetic determinants, they are not entirely independent. Indeed, there

is evidence that chronological aged cells have reduced RLS once they re-enter into the cell cycle (Ashrafi et al. 1999; Murakami et al. 2012; Piper 2006). Furthermore, some longevity-promoting interventions extend both CLS and RLS, while defects in protein quality control contribute to decreased survival in both ageing models (reviewed in Sampaio-Marques et al. (2014a)). This evidence gives strong support to damage-based theories of ageing but also recognizes that some molecular and genetic factors play a key role in ageing.

The key finding that defines the RLS is the fact that individual cells have a finite number of divisions (around 20–30), which is followed by cell death. Nearly, 100 yeast genes have been identified as involved in ageing and whose deletion enhances RLS (Kaeberlein et al. 2005; Longo et al. 2012). One of the best understood replicative ageing pathways involves the gene *SIR2* encoding a member of the sirtuin family of NAD^+-dependent deacetylases. Overexpression of *SIR2* was shown to extend yeast RLS (Kaeberlein et al. 1999) among other mechanisms, by suppressing homologous recombination of rDNA that leads to the formation of extrachromosomal rDNA circles (ERCs) (Fig. 9.1). These ERCs were thought to limit the mother's cells RLS due to their self-replicating capacity and asymmetrically segregation to the mother cells (Sinclair and Guarente 1997). Recently, this idea is being challenged by the suggestion that rDNA instability, rather than ERCs, is the primary cause of mother cells' senescence and death (Lindstrom et al. 2011). Importantly, mutations that suppress the rDNA instability such as deletion of *FOB1*, an rDNA replication fork block protein (Kaeberlein et al. 1999), are able to overcome the decreased RLS of *sir2Δ* mother cells (Longo et al. 2012). Several groups have later demonstrated that overexpression of Sir2 homologs, Sir2.1 in *Caenorhabditis elegans* and dSir2 in *Drosophila melanogaster*, extends life span (Rogina and Helfand 2004; Tissenbaum and Guarente 2001). Therefore, the first highly conserved determinant of ageing, Sir2, was discovered in yeast.

The role of Sir2 in the regulation of the RLS is not restricted to the formation of ERCs and rDNA instability. Deletion of *SIR2* results in a defect in the asymmetric retention of damage, particularly of oxidatively damaged cytoplasmic proteins in the mother cell leading to a shorter RLS of daughter cells (Aguilaniu et al. 2003). This defective asymmetric retention of damage promoted by deletion of *SIR2* can be overcome by the overexpression of *HSP104* (Erjavec et al. 2007) (Fig. 9.1).

Sir2 has also been shown to be fundamental to the regulation of the epigenetic modifications to histones. The best example is the increase in the H4K16 acetylation concurrently with a decline in Sir2 levels during ageing (Dang et al. 2009). Furthermore, Sir2 can also control RLS in an ERC-independent manner, by controlling cytoskeleton function and polarity (Liu et al. 2010). Importantly, overexpression of *SIR2* orthologs in worms and flies promotes longevity and activation of the mammalian Sir2-ortholog, SIRT1, can enhance health span in mice (reviewed in Finkel et al. (2009)). Although these results have been questioned by many, there appears to be a general consensus that SIRT1 interacts with important ageing-related pathways in mammals.

Mitochondrial function also plays a critical role in RLS determination, as mitochondrial oxidative phosphorylation (OXPHOS) deteriorates and mitochondrial ROS

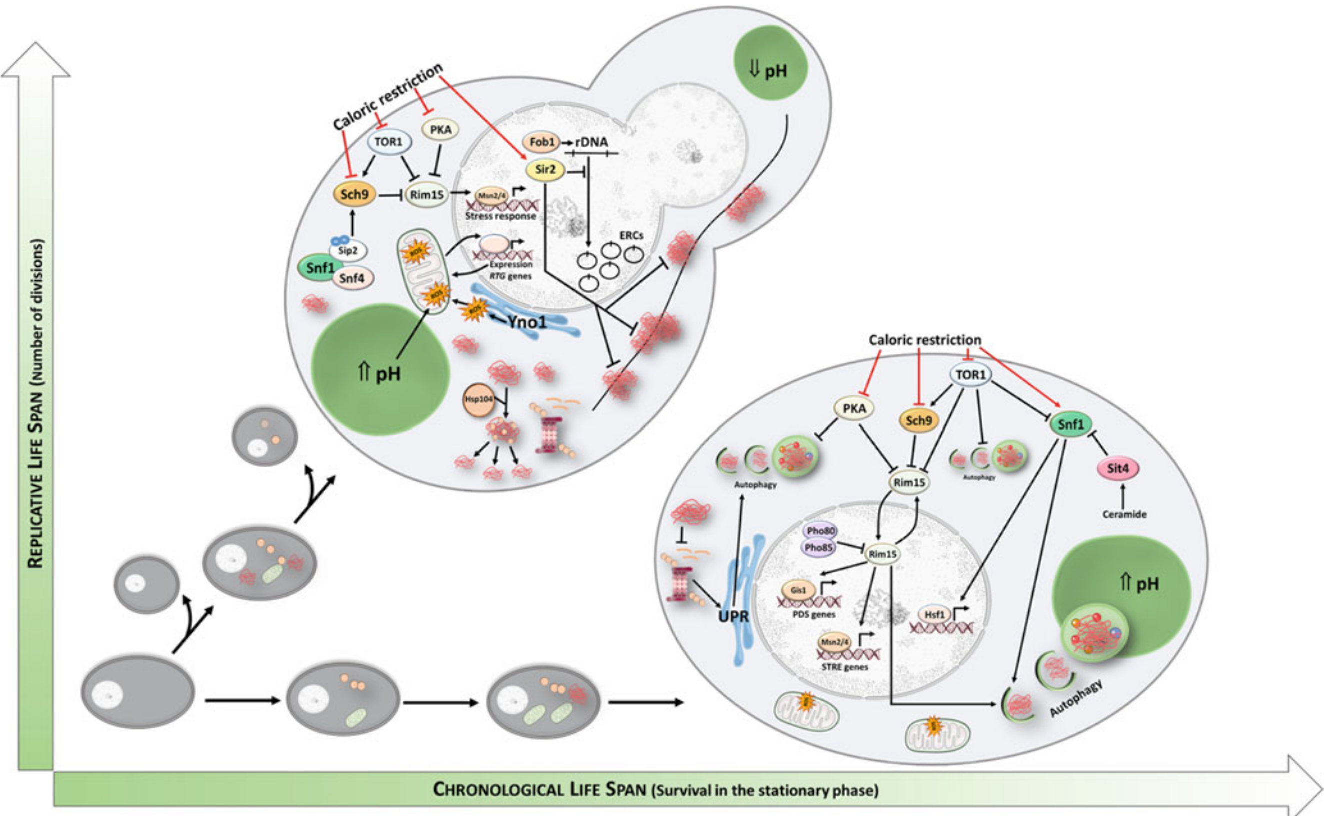

Fig. 9.1 Genes and pathways modulating replicative and chronological life spans. Shown are illustrated the main players and pathways that modulate ageing in the yeast *S. cerevisiae*. See text for details

generation increases with age. Jazwinski and colleagues have demonstrated that induction of the retrograde response pathway, which transmits signals of mitochondrial stress to the nucleus, extends RLS in certain genetic backgrounds (Kirchman et al. 1999). Therefore, RLS is extended by enhancement of mitochondrial biogenesis, correct mitochondrial segregation and inheritance, prevention of mitochondrial proteotoxic stress, and maintenance of proper nuclear–mitochondrial communication through activation of mitochondrial retrograde signalling pathways (Fig. 9.1; reviewed in Ruetenik and Barrientos (2015)). Recently, it was shown that the shortening of RLS due to mitochondrial dysfunction was not related to the accumulation of ERCs, but to increased ROS generation of the ER-localized NADPH oxidase Yno1 (Yi et al. 2018). Furthermore, mitochondrial function is also impaired due to the decline of vacuolar acidity that occurs with age. The age-dependent increase of pH promotes the storage of amino acids in the mitochondria that consequently affects membrane potential and mitochondrial function (Fig. 9.1) (Hughes and Gottschling 2012).

Recently, studies indicate that as cells age, damaged protein aggregates and oxidatively damaged organelles, as mitochondria, are predominantly sequestered in mother cells contributing as ageing factors (Aguilaniu et al. 2003; Denoth Lippuner et al. 2014; Erjavec and Nystrom 2007; Lam et al. 2011; Liu et al. 2010; McFaline-Figueroa et al. 2011). In contrast, disruption of endoplasmic reticulum diffusion barriers can result in the segregation of misfolded protein aggregates into daughter cells (Higuchi-Sanabria et al. 2014), see for review Smith and Schneider (2018).

The abovementioned detrimental age-dependent changes can be mitigated by anti-ageing intervention such as caloric restriction (CR). Mounting evidences demonstrated that CR extends life span and health span in several model organisms (Fontana et al. 2010). In yeast, CR is achieved by reducing glucose concentration from 2 to 0.5% or below (Lin et al. 2000). The *SIR2* gene and functional NAD^+ salvage genes were reported to be required for CR-mediated RLS extension (Anderson et al. 2002, 2003; Lin et al. 2000). Although it is still debated the role of Sir2 on the CR-mediated RLS extension, it has been accurately established that CR can also extend life span via Sir2-independent mechanisms and that Sir2 and CR work in parallel pathways (Kaeberlein et al. 2004). Additional discussions of the role of Sir2 and the other yeast sirtuins in RLS extension promoted by CR can be found in recent reviews of this topic Kaeberlein (2010), Kaeberlein and Powers (2007). Curiously, enhanced proteasome activity is able to increase RLS by a mechanism that is genetically distinct from both CR and Sir2 (Kruegel et al. 2011).

Accumulated evidence indicates that CR-mediated RLS extension is largely dependent on reduced Ras-PKA and TOR/Sch9 pathways signalling that play a concerted role in regulating growth, metabolism and stress resistance in response to nutrient availability (Kaeberlein et al. 2005). Importantly, Sch9 activity can be regulated independently of CR to influence RLS through acetylation of Sip2, a component of the yeast AMP-activated protein kinase complex, Snf1 (Lu et al. 2011). These nutrient and energy sensing pathways play a similar role in modulating yeast CLS, as well as longevity in worms, flies and mice, providing strong evidence for their conserved effects on ageing throughout eukaryotes (Fontana et al. 2010).

CLS defines the survival of stationary-phase cells after depletion of nutrients, and glucose is commonly the first limiting nutrient. Importantly, two main cell populations could be defined when glucose is exhausted at the diauxic shift. One of these populations corresponds to quiescent (Q) cells that are in G0, a non-proliferative state. Q cell population is mainly composed of unbudded daughter cells (Aragon et al. 2008; Li et al. 2013; Miles et al. 2013; Werner-Washburne et al. 2012) that are highly resistant to stress and present rigid cell walls and high accumulation of glycogen and trehalose and low accumulation of reactive oxygen species (ROS). These cells are able to re-enter mitosis when nutrients become available (Aragon et al. 2008; Leonov et al. 2017; Miles et al. 2013). Another cell population is composed of non-quiescent (NQ) cells, most or all of which are first-generation and higher generation mother cells (Aragon et al. 2008; Li et al. 2013; Miles et al. 2013; Werner-Washburne et al. 2012). These NQ cells can be metabolically active, with or without clonogenic capacity, or may exhibit hallmarks of apoptosis or necrosis (Aragon et al. 2008; Li et al. 2013; Miles et al. 2013; Werner-Washburne et al. 2012).

CLS is controlled by a complex signalling network including TORC1 (target of rapamycin complex 1), a highly conserved serine/threonine protein kinase complex that is the major regulator of the signalling network controlling cell growth; PKA (protein kinase A), a major regulator of metabolism, proliferation and stress resistance; and the protein kinase Sch9, a serine/threonine protein kinase that plays a central role in nutrient-mediated signalling (Smets et al. 2010). These three kinase complexes are the so-called ménage-à-trois that integrates inputs from several nutrient-sensing systems to regulate metabolism, intracellular trafficking, proteome integrity, autophagy, stress resistance, cell size, progression, growth and sporulation (reviewed in Deprez et al. (2018)). In addition to these regulators, other energy and sensing pathways play important roles in regulating longevity. This is the case for Snf1 (sucrose non-fermenting, protein 1), a member of the conserved AMP-activated protein kinase (AMPK) family that is a major sensor of cellular energy levels, and the Pho85 (phosphate metabolism, protein 85), which together with Pho80 forms a kinase complex with a major role on the cellular response to changes in extracellular and/or intracellular phosphate levels.

These nutrient-sensing pathways may overlap and create redundancy in the modulation of many downstream effector proteins including (among others) Rim15, a serine/threonine protein kinase that is essential for cell cycle arrest at G1 and entry of cells into quiescence. Rim15 is regulated by the TORC1, Sch9, PKA and Pho85 (reviewed in Leonov et al. (2017, Sampaio-Marques et al. (2014a), Smets et al. (2010)). Yak1 is another serine/threonine protein kinase under the control of PKA, and it is required for cell cycle arrest at G1 (reviewed in Leonov et al. (2017), Sampaio-Marques et al. (2014a), Smets et al. (2010)). TORC1 and PKA also control Mck1, a dual-specificity serine/threonine and tyrosine protein kinase. Msn2/4 and Gis1 are also downstream effectors of these two kinases. These transcription factors are controlled by TORC1 and PKA, as well as by Snf1—they activate the expression of genes involved in stress response and diauxic transition (reviewed in Leonov et al. (2017), Smets et al. (2010)). Another essential transcription factor is Hsf1, which is controlled by Snf1 and is involved in the expression of many genes, particularly those

related to control of proteostasis and energy generation. Snf1 and TORC1 regulate the transcriptional factor Gln3, which regulates genes involved in the metabolism of nitrogen, and eIF2α, a factor involved in the initiation of protein synthesis. It is also important to highlight the regulation of the Atg1–Atg13 complex, controlled by TORC1, PKA, Snf1 and Pho85, which initiates autophagy by enabling phagophore assembly site (PAS) formation (reviewed in Leonov et al. (2017)). Globally, these downstream effectors enhance several protective systems including glycogen and, glycerol and antioxidant enzymes and mechanisms related to the maintenance of proteostasis, such as HSPs and autophagy (Sampaio-Marques et al. 2014b).

Despite the different mechanisms of life span extension promoted by the inactivation of these signalling pathways, autophagy seems to be a common denominator. Reduced TOR signalling induces autophagy (Noda and Ohsumi 1998), while deletion of *SCH9*, a TOR effector that can function independently, has a minor impact on autophagy. Nevertheless, the role of Sch9 on vacuole acidification has to be considered, as deletion of *SCH9* could extend both RLS and CLS by contributing to the maintenance of vacuole acidification in aged cells (reviewed in Ruckenstuhl et al. (2014), Tyler and Johnson (2018a)). These nutrient-sensing pathways together with PKA are crucial for the regulation of pH homeostasis through their influence on the proton pumping activity of the V-ATPase, and possibly also on Pma1, from the plasma membrane ATPase (Deprez et al. 2018). pH homeostasis is a crucial regulator of autophagy, as the final step of autophagy is linked to vacuolar membrane integrity and acidification of the vacuolar lumen (reviewed in Deprez et al. (2018)). Besides PKA's role in pH homeostasis, inactivation of PKA also induces autophagy associated with RLS and CLS extension, albeit less efficiently than autophagy induction promoted by TOR inactivation (Budovskaya et al. 2004) or by deletion of both *SCH9* and PKA (Yorimitsu et al. 2007). In contrast, the positive regulator of autophagy, Snf1, promotes a reduction of CLS when deleted (Wang et al. 2001).

Regarding Sir2, its role on CLS is far more complex. Depending on the strain background and growth media, deletion of *SIR2* either has no effect or induces a moderate increase of CLS (reviewed in Wierman and Smith (2014)). Consequently, Sir2 has been mainly assigned a pro-ageing role in CLS (reviewed in Sampaio-Marques et al. (2014a)). Although Sir2 might antagonize CLS extension promoted by CR (Fabrizio et al. 2005), it was also shown that CR extends CLS independently of the sirtuins including Sir2 (Smith et al. 2007). We have shown that autophagy maintenance at homeostatic levels promoted by CR or *TOR1* deletion is achieved by decreasing Sir2 levels and activity (Guedes et al. 2017). Although *SIR2* deletion does not have a major effect on CLS, it does compromise the extension of CLS observed in *SCH9* deleted cells and in cells treated with the life span-promoting agent resveratrol (Fabrizio et al. 2005; Howitz et al. 2003). Furthermore, Sir2 plays an important role in autophagy regulation during CLS in certain scenarios. We have previously shown that Sir2, similar to mammalian SIRT1, activates autophagy and mitophagy through the transcriptional regulation of *ATG8* and *ATG32* under proteotoxic conditions (Sampaio-Marques et al. 2012). Therefore, it is tempting to speculate that Sir2 supports life span extension of *SCH9* deleted cells by maintaining autophagy.

Besides the activation of a general stress response, the pro-longevity effects linked to reduced activity of nutrient-sensing pathways appears to be also associated with ROS signalling and increased mitochondria function. Our studies have shown that abrogation of catalase activity or of nutrient-sensing pathways by CR extends CLS by producing hydrogen peroxide, which leads to the activation of superoxide dismutases that inhibit the accumulation of superoxide anions (Mesquita et al. 2010). These findings established a role for hormesis effects of hydrogen peroxide in promoting longevity. Later, it was reported that during exponential-growing phase, *TOR1* or *SCH9* deleted cells generate mitochondrial ROS, as an adaptive hormetic signal, which results in the reduction of ROS levels at stationary phase and extension of CLS (Pan et al. 2011). A few years ago, the beneficial effects of hormetic mitochondrial ROS on longevity signal that extends yeast CLS were shown to involve epigenetic alterations and the DNA damage responses (DDR) kinases, Tel1 and Rad53 (Schroeder et al. 2013). This hormetic pathway is independent and distinct from the nuclear DDR and involves histone modifications (Schroeder et al. 2013).

The existence of a nuclear pathway that senses mitochondrial ROS generation/accumulation points to the crucial role of mitochondrial function on ageing. In fact, the longevity-promoting effects of the global activation of general stress response by decreasing the nutrient-sensing pathways' activity appear to be associated with an increase in mitochondria function. In line with this, the lack of mitochondrial respiration severely impacts the longevity of stationary-phase cells (reviewed in Sampaio-Marques and Ludovico (2018)). In contrast, long-lived cells deleted on *TOR1* (Bonawitz et al. 2007; Ocampo et al. 2012; Pan et al. 2011) or *SCH9* (Lavoie and Whiteway 2008) presented an increased respiratory capacity. The promotion of longevity encompasses the activation of the Msn2/4 and Gis1 stress response (Fig. 9.1) (Ewald et al. 2016) and the Rph1-dependent epigenetic silencing by triggering a non-canonical activation of the DDR pathways (Schroeder et al. 2013).

More recently, novel ageing determinants were identified, as proteins involved in chromatin remodelling (Swr1, Arp6 and Swc3), Arv1, a lipid homeostasis factor that modulates autophagy, Tep1, the homologue of the human tumour suppressor PTEN, and proteins associated with phosphatidylinositol phosphate metabolism (Garay et al. 2014). A connection between sphingolipids and cell signalling through TOR, Sch9 and the ceramide-activated protein phosphatase Sit4 was recently shown to impact on mitochondria function, autophagy and CLS (Vilaca et al. 2018) (Fig. 9.1). Details on the link between sphingolipids signalling and CLS can be found in (Oliveira et al. 2017).

Numerous studies point to distinct determinants of yeast RLS and CLS. For example, deletion of *SIR2* or *RAS2* has dissimilar effects on RLS and CLS (reviewed in Smith and Schneider (2018)). In contrast, both RLS and CLS are extended in response to CR and other interventions that abrogate nutrient-sensing pathways. Nevertheless, it remains unclear whether similar downstream molecular events are common to both yeast ageing paradigms (Sampaio-Marques et al. 2014a). Importantly, both yeast ageing paradigms are connected. As briefly mentioned above, chronologically aged yeast cells show a proportional reduction in RLS (Ashrafi et al. 1999; Murakami et al. 2012; Piper et al. 2006) that is prevented by CR, suggesting that the metabolic

state and mitochondrial function of stationary-phase cells determine their replicative potential upon transfer to growth conditions (Delaney et al. 2013).

9.3 Proteostasis and Yeast Ageing

Ageing is driven by accumulation of damage in highly conserved cell-intrinsic processes such as chromosome structure/organization, transcriptional regulation, nuclear export/import, protein translation and quality control, recycling of damage/unnecessary organelles, maintenance of cytoskeletal structure and extracellular signalling (DiLoreto and Murphy 2015). These processes have the ability to communicate with each other resulting in an intricate interplay that governs cells' ageing. Therefore, the knowledge of the specific cellular and molecular mechanisms underlying ageing represents one of the most complex issues that have yet to overcome.

Several studies focused on molecular alterations occurring during yeast ageing revealed a series of progressive events that collectively contribute to ageing phenotypes. These events integrate damage and dysfunction with stress pathways, including oxidative stress associated with mitochondrial dysfunction and accumulation of ROS, genomic instability associated with nuclear DNA damage, mutagenesis and replication stress, metabolic alterations and loss of proteostasis. Importantly, DNA damage and error-prone DNA repair systems have been assumed as key for the mechanisms behind age-dependent genomic instability observed during ageing. The results of several studies consistently point to a role for oxidative damage that induces senescence and cell death as an important determinant of life span (reviewed in Weinberger et al. (2013)). However, the relationships between ROS, ageing and age-related diseases suggest increased complexity in this scenario (Ludovico and Burhans 2014; Weinberger et al. 2013).

Protein quality control systems as autophagy play a key role in the DDR by controlling the levels of proteins involved in cell cycle checkpoints and DNA synthesis/repair mechanisms. For example, in *S. cerevisiae*, DNA damage induces the autophagic degradation of ribonucleotide reductase 1 (Rnr1) (Dyavaiah et al. 2011), the large subunit of ribonucleotide reductase (RNR), which is a highly conserved enzymatic complex catalysing the formation of deoxyribonucleotides required for both DNA replication and repair. This DDR-dependent autophagic pathway in yeast was called genotoxin-induced targeted autophagy (GTA) and requires the involvement of the DDR kinases, Mec1 and Rad53, as well as a central component of the selective autophagy machinery, Atg11 (Eapen et al. 2017). Recently, it was reported that the kinase Mec1 plays a fundamental role in protein homeostasis (Corcoles-Saez et al. 2018). In agreement, it is becoming well recognized that one of the major determinants of ageing is proteostasis and that the other ageing hallmarks are intimately related to it. It is the example of the nutrient-sensing pathways that when inactivated mainly contribute to the maintenance of the proteome during ageing (Sampaio-Marques et al. 2014a). In this sense, it is proposed that early changes on protein homeostasis network that result in the cellular loss of proteostasis could

be one of the earliest events dictating ageing progression, affecting a multitude of downstream processes (Labbadia and Morimoto 2014). In fact, cells have multiple stress-responsive mechanisms to combat loss of proteostasis associated with cellular ageing as described below.

The ability of cells to maintain protein homeostasis, or proteostasis, in response to intrinsic cellular and environmental insults, which accumulate over time, is one of the main determinants of life span (Morimoto and Cuervo 2014). Proteostasis, referred as the healthy maintenance of the cellular proteome, comprises highly complex and interconnected pathways that govern the fate of proteins. Proteostasis is controlled by a multi-compartmental system that has the ability to coordinate protein synthesis, processing, trafficking, folding, localization, assembly/disassembly and degradation (Sampaio-Marques and Ludovico 2018). A major determinant of loss of proteostasis and protein aggregation is the overproduction and accumulation of unstable proteins (Lopez-Otin et al. 2013). For example, it was demonstrated that inhibition of protein translational machinery with cycloheximide blocks the formation of protein aggregates indicating that active protein translation is required for stress-induced protein aggregation in yeast (Zhou et al. 2014). Although the mechanisms underlying this observation remain unclear, a reduction in handling the burden of newly translated unfolded proteins and an increase in free molecular chaperones as well as in the activity of the degradation pathways could be simple explanations (Medicherla and Goldberg 2008).

Molecular chaperones assist in the folding/refolding of proteins (Fig. 9.2). Chaperones can be found in the cytoplasm but also in the ER and mitochondria. A recent study revealed that decline in chaperone activity in each cellular compartment triggers a response in other compartments that result in loss of respiration capacity, demonstrating the dependence of mitochondrial activity on cell-wide proteostasis. This phenomenon has been called cross-organelle stress response (CORE) and has a protective role by extending both CLS and RLS (Peric et al. 2016). Although several physical organelle contact sites exist in yeast, their involvement in CORE and the cell-wide proteostasis system is yet to be elucidated. Importantly, it was demonstrated that ER-formed protein aggregates are frequently associated with or are later captured by mitochondria (Zhou et al. 2014). In line with this concept, aged replicative cells exhibit a gradual decline of aggregate–mitochondria association decreasing mobility and leakage of aggregates from mother into the bud contributing to the decreased life span of daughter cells (Zhou et al. 2014).

The activity of ATP-dependent chaperones is greatly affected by the age-dependent reduction of cellular energy, due to reduced mitochondrial functionality and deregulation of lipid and glucose metabolism (Ma and Li 2015; Ritz and Berrut 2005). The activity of molecular chaperones could also be affected by their availability, which does not meet the needs of aged cells. These phenomena might be aggravated by protein modifications that are enhanced during ageing, such as accumulation of advanced glycation end products through non-enzymatic modifications that interfere with the chaperone's ability to recognize the target (Vanhooren et al. 2015), resulting in the accumulation and aggregation of the defective proteins (Kumar et al. 2007). Consistent with this possibility, it was shown that downregulation of

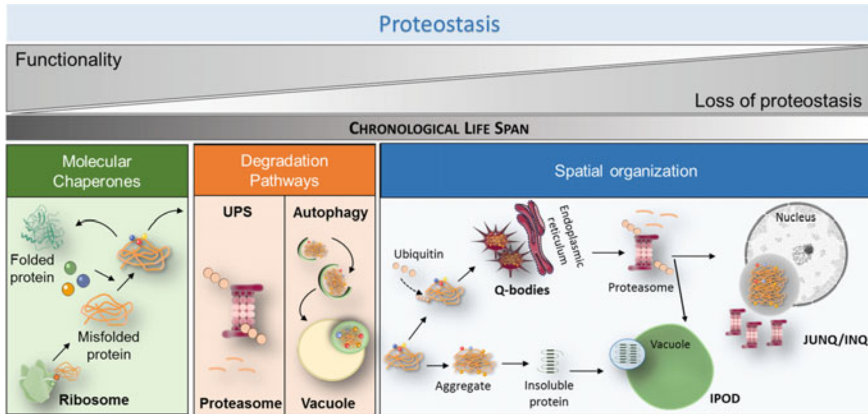

Fig. 9.2 Proteostasis and yeast ageing. Proteostasis is mainly maintained by the action of molecular chaperones and the two degradation pathways, the ubiquitin–proteasome system (UPS) and autophagy. In addition, yeast cells have developed a spatial quality control system where misfolded and aggregated proteins are sequestered into larger protective inclusions: the juxtanuclear quality control site (JUNQ)/the intranuclear quality control compartment (INQ) and the peripheral vacuole-associated, insoluble protein deposit (IPOD). See text for more details

yeast Hsp90 activity results in an increase in heat shock protein synthesis due to the inability to efficiently repress Hsf1 (Duina et al. 1998; Harris et al. 2001), which is correlated with increased viability over time. Thus, an enhancement of chaperone activity is associated with increased longevity. However, age-mediated alterations in proteostasis are due not only to decreased chaperones activity, but also to alterations in degradation pathways, the ubiquitin–proteasome system (UPS) and autophagy (Fig. 9.2). In yeast chronological ageing, proteasome dysfunction occurring over time induces the accumulation of protein aggregates and formation of inclusions that further obstruct proteasome function in a vicious cycle (Andersson et al. 2013). Our experimental results showed an accumulation of ubiquitinated proteins associated with an increase in levels of *RPN4* along chronological ageing, reflecting an impairment of proteasome activity during yeast ageing (Sampaio-Marques and Ludovico 2018).

Proteasome dysfunction might also be potentiated by the sequestration in protein aggregates of factors required for proteasome activity, such as ubiquitin ligases/proteases or proteasome activators (Andersson et al. 2013). For example, the production and accumulation of ROS lead to proteasome activity reduction and subsequent accumulation of carbonylated proteins and specific modifications in certain proteins, such as E1 and/or E2 enzymes, which results in the impairment of the ubiquitin-binding (da Cunha et al. 2011). In contrast, overexpression of key molecules, such as the proteasome chaperone Ump1, results in the proteasome-mediated protein degradation enhancement with the consequent longevity extension (Chen et al. 2006).

Accumulating evidence also suggests that damaged proteins are not randomly distributed in the cell during ageing. Yeast cells have developed a spatial quality control system where misfolded and aggregated proteins are sequestered into larger protective inclusions (Alvers et al. 2009a) (Fig. 9.2). This spatial compartmentalization of protein aggregates is a complementary protein quality control strategy that acts in parallel with temporal quality control. The presence of these inclusions is not essential for their degradation, but it may facilitate refolding/degradation by increasing the proximity of chaperones and their substrates limiting the toxic interactions of misfolded proteins. Several studies suggest that immediately upon misfolding, an active chaperone-dependent transport of damaged proteins to dynamic compartments called Q-bodies, which are attached to the ER, takes place (Escusa-Toret et al. 2013). The damaged proteins inside of these Q-bodies are rapidly cleared through the UPS—however, if clearance is impaired, these misfolded proteins concentrate in one of the two major protein quality control compartments, the juxta nuclear quality control site (JUNQ) and the peripheral vacuole-associated, insoluble protein deposit (IPOD) (Kaganovich et al. 2008) (Fig. 9.2). More recently, JUNQ was found inside the nucleus, where it serves as a new intranuclear quality control compartment (INQ) for the deposition of both nuclear and cytosolic misfolded proteins, irrespective of ubiquitination (Miller et al. 2015). Proteins are targeted to the JUNQ by a ubiquitin-based sorting mechanism, while they are sent non-ubiquitinated to IPOD (Kaganovich et al. 2008) (Fig. 9.2). Recently, it was found that the small Hsp42 can assemble into versatile dynamic oligomers—the Hsp42-containing stationary-phase granules (Hsp42-SPGs), which contain protein components including molecular chaperones, metabolic enzymes and regulatory proteins (Lee et al. 2018). These Hsp42-SPGs are enriched in long-lived quiescent cell populations, suggesting that these granules may help quiescent cells to combat various stresses during stationary phase, by mechanisms that remain unclear (Lee et al. 2016). Furthermore, Hsp42-SPGs may work as centres that control both protein quality and quantity in stationary-phase cells (Lee et al. 2018).

Like the other proteostasis pathways, spatial quality control also declines with age, and cells that lack this ability show accelerated ageing (Escusa-Toret et al. 2013). During ageing, the increase in the load of aggregated proteins and the inactivation of cellular chaperones could provide one explanation for the age-associated loss of spatial protein quality control (Hill et al. 2017). Furthermore, the reduction on efficiency of this spatial protein quality control system might be also related with the failure of organelles function. For example, vacuolar pH alterations could cause a breakdown in vesicle trafficking and fusion to the vacuole, an important process for spatial sequestration of aggregated proteins. Furthermore, disruption of a functional actin cytoskeleton might also affect vesicle trafficking and fusion, mitochondrial inheritance, and increase ROS accumulation—this could explain the inefficient inclusion formation observed in ageing cells (reviewed in Hill et al. (2017)).

Surveillance of proteostasis is mainly played by the chaperones and the main protein degradation pathways, the UPS and the autophagy. Although UPS is the primary cellular route for protein degradation, it does not allow for the degradation of unfolded or large protein complexes. Therefore, larger substrates, such as

large protein inclusions, can be directly degraded by autophagy. In the budding yeast *S. cerevisiae*, macro(autophagy) occurs through the formation of a double-membrane vesicle—autophagosome—that sequesters cytosol and organelles and fuses with the vacuole releasing the content to be degraded and recycled. Besides bulk unspecific degradation, autophagy can occur by selective mechanisms encompassing the degradation of specific cargos such as organelles (reviewed in Galluzzi et al. (2017)). Selective autophagy requires functional actin cytoskeleton for specific degradation of mitochondria, peroxisomes, mature ribosomes, and cytosolic proteins such as acetaldehyde dehydrogenase Ald6 (reviewed in Smith and Schneider (2018)). The functioning of autophagy is supported by two main groups of genes, autophagy-related genes (ATG) and vacuolar protein sorting (VPS) genes (Reggiori and Klionsky 2013; Tyler and Johnson 2018b). Collectively, the processes underlying autophagy are highly complex and beyond the scope of this chapter and have been extensively reviewed elsewhere Yin et al. (2016).

Similar to UPS, autophagy activity decreases during ageing, as reported in different model systems (reviewed in Rubinsztein et al. (2011)). However, due to the complexity of the autophagy process and the stochastic nature of ageing, the mechanisms underlying decreased autophagy remain largely unclear. The decline of autophagy activity promoted by ageing enhances the accumulation of aberrant proteins/aggregates, causing additional molecular and cellular damage, as a vicious cycle. Furthermore, an age-associated increase of vacuolar pH (Fig. 9.1), which limits the activities of vacuolar proteases and results in the loss of vacuolar homeostasis, may contribute to autophagy impairment during ageing (Nakamura et al. 1997). Autophagy deregulation in aged cells can also be a consequence of persistent activity stimulation. Although increased autophagy might initially have a favourable outcome, if maintained at a high rate, it can promote the depletion of functional organelles/proteins and essential autophagic molecules, contributing to cell death and thus shortening of life span (Meijer and Codogno 2007; Sampaio-Marques et al. 2012). In agreement, we showed that heterologous expression of human alpha-synuclein (aSyn) in yeast cells results in aberrantly high activation of autophagy associated with shortening of CLS (Sampaio-Marques et al. 2012). Furthermore, all the genetic and environmental manipulations reducing aSyn toxicity resulted in decreased autophagy activity (Guedes et al. 2017; Sampaio-Marques et al. 2012) indicating that autophagy should be maintained under homeostatic levels. It is, nonetheless, well recognized that autophagy is required for maximal CLS and has been implicated in almost all the CLS promoting interventions, as discussed herein and reviewed in Sampaio-Marques et al. (2014a), Tyler and Johnson (2018a). Different genetic studies have demonstrated that genes encoding proteins involved in autophagy machinery as *ATG1*, *ATG2*, *ATG7*, *ATG8*, *ATG16* or *VPS21* are required for life span extension (Alvers et al. 2009a; Aris et al. 2013; Fabrizio et al. 2010; Matecic et al. 2010). Dietary interventions such as caloric or methionine restriction were also shown to be dependent on autophagy (Aris et al. 2013; Ruckenstuhl et al. 2014). As we have discussed in the previous section, nutrient-sensing pathways also have profound effects on autophagy and longevity, as well as other relevant players linking metabolism, autophagy and longevity. Notably, accumulation of acetyl-

CoA, a critically important molecule in metabolism, has been shown to result in the hyperacetylation of histones that transcriptionally repress autophagy genes and negatively impact ageing (Eisenberg et al. 2014). The Esa1 and Rpd3 antagonistic acetyltransferase–deacetylase pair has also been shown to transcriptionally regulate autophagy—implicating epigenetic regulation of autophagy also in RLS (Yi et al. 2012). Together, these studies clearly demonstrate a bona fide role for epigenetics in the regulation of autophagy and yeast life span.

Lipid metabolism can also engage the autophagy machinery to positively regulate longevity, as referred above. The Arv1, a protein involved in sterol and sphingolipids metabolism, was identified as involved in the regulation of CLS through autophagy (Garay et al. 2014). An additional study demonstrated that supplementation of nutrient medium with phosphatidylethanolamine (PE) or genetic interventions that result in increased PE levels activate autophagy and extend CLS (Rockenfeller et al. 2015).

Interestingly, due to the large number of conserved ageing-related genes and ageing mechanisms in yeast and humans, testing candidate anti-ageing molecules in yeast has proven highly successful in the search for potential anti-ageing therapies. Yeast-based studies have helped to understand the mode of action of anti-ageing molecules such as rapamycin, spermidine or resveratrol. Rapamycin is a macrolide antibiotic with antifungal and immunosuppressive properties, which inhibits the TOR signalling pathway. Autophagy activation was shown to be essential for rapamycin-mediated life span extension in yeast (Alvers et al. 2009b). The polyphenol resveratrol and the natural polyamine spermidine are currently the most promising potential anti-ageing agents that were discovered in yeast and shown to activate autophagy and extend both RLS and CLS (Eisenberg et al. 2009; Morselli et al. 2011). Collectively, these observations are consistent with results from the large number of studies that point to general anti-ageing properties of autophagy. In spite of the role of autophagy in CLS, autophagy does not appear to contribute to RLS under normal growth conditions. Indeed, deletion of most of the *ATG* genes has negligible effects on RLS, and in some cases even results in extension of RLS (Ghavidel et al. 2015; McCormick et al. 2015).

Altogether, these studies demonstrate that yeast is an invaluable tool for the identification and characterization of conserved mechanisms that promote cellular longevity, and that autophagy plays an important role in nearly all known longevity-promoting interventions, as reviewed elsewhere Tyler and Johnson (2018a).

9.4 Modelling Age-Related Diseases in Yeast

Deregulation of protein network functionality is correlated with ageing and is a major risk factor for the development of a wide spectrum of age-related protein diseases (Morimoto and Cuervo 2014). The budding yeast *S. cerevisiae* is a simple unicellular eukaryotic organism that shares well-conserved molecular and cellular mechanisms with higher eukaryotes and has been particularly useful as a biological model for ageing and age-related diseases (Tenreiro et al. 2013). Thus, *S. cerevisiae*

has played an extremely important role in the discovery of key molecular events associated with neurodegenerative diseases, including Parkinson's (PD), Alzheimer's (AD) and Huntington's (HD) diseases. These protein misfolding disorders are age-related degenerative diseases in which misfolded proteins are prone to form intra- or extracellular aggregates with specific composition and localization for each disease. While intracellular aSyn and huntingtin (Htt) aggregates are hallmarks of PD and HD, respectively, extracellular aggregates of tau protein and amyloid-β (Aβ) peptide are characteristic of AD. Depending on the disease, the resulting aggregates might result in the loss of protein function and/or in the gain of a cytotoxic function. Mitochondrial dysfunction, transcriptional dysregulation, trafficking defects and loss of proteostasis are some of the molecular and cellular mechanisms conserved from yeast to human that underlie the pathogenesis of these diseases (reviewed in Tenreiro and Outeiro (2010)).

It is estimated that around 25–30% of the genes linked to human diseases have yeast orthologues (Bassett et al. 1996). Thus, if a gene related to a human disease has a yeast homologue, its role can be investigated by simply deleting or overexpressing this gene in yeast. For example, yeast-based studies on the *SOD1* and *YHF1* genes, homologs of the human genes involved in Friedreich's ataxia and amyotrophic lateral sclerosis, respectively, contributed greatly to our understanding of these disorders. Furthermore, even if a human gene is absent from the yeast genome, its role in disease can be modelled by the heterologous expression of the human gene in yeast cells. Yeast models for PD, HD and AD, which are examples of this strategy, have been extensively exploited and have greatly contributed to the elucidation of the molecular and cellular aspects of these disorders, as detailed below (Miller-Fleming et al. 2008; Sampaio-Marques and Ludovico 2015; Sampaio-Marques and Ludovico 2018).

A yeast HD model can be generated by the heterologous expression of mutant human Htt exon 1 with different polyglutamine (polyQ) expansions (more than 35 glutamine residues) in yeast cells, reproducing many of the cellular and molecular features of HD pathology. For example, expression of mutant fragments of Htt resulted in polyQ length-dependent aggregation and toxicity, endocytosis impairment, transcriptional dysregulation, mitochondrial dysfunction, oxidative stress and apoptosis (reviewed in Tenreiro and Outeiro (2010)). Furthermore, genetic screens using yeast HD models have identified different modulators of mutant Htt aggregation and toxicity. Notably, chaperones members of Hsp40 and Hsp70 families have been identified as potential therapeutic targets (reviewed in Tenreiro and Outeiro (2010)). Yeast HD models have also been useful in screening drugs.

Studies on AD primarily make use of human cell lines and transgenic mouse models. However, yeast AD models are becoming increasingly important to unravel fundamental molecular aspects of AD. Pathological hallmarks of this disease include the presence of extracellular plaques of Aβ and intracellular neurofibrillary tangles of phosphorylated tau protein. Tau and Aβ have no functional yeast orthologues and thus, different yeast models have assessed the cellular consequences of expressing Aβ peptides or Tau. Different yeast models have been used, and some of them fuse Aβ40 or Aβ42 to C-terminal part of Sup35, a translation termination factor without the prion domain, to create an oligomerization assay to find specific point mutations able to

inhibit Aβ oligomerization (Bagriantsev and Liebman 2006). These models have been used in high-throughput screens resulting in the identification and validation of two compounds with anti-oligomeric effects (Park et al. 2011). In other yeast AD models, the Aβ42 peptide is directed to the secretory pathway (D'Angelo et al. 2013; Treusch et al. 2011).

Although only a few studies have employed yeast to study the biology of Tau, the data obtained revealed that yeast cells have an enormous potential to disclose key aspects of Tau pathophysiology, since these models recapitulate central features of the AD, including Tau hyperphosphorylation at pathological residues, conformational changes and aggregation (reviewed in Verduyckt et al. (2016)). For example, in yeast, Tau phosphorylation is regulated by the kinases Mds1p and Pho85p, the orthologues of human GSK3b and CDK5, respectively, at the same residues that Tau is hyperphosphorylated in neurons (Vandebroek et al. 2005). Downregulation of Pho85 increases Tau phosphorylation and aggregation, while deletion of *MDS1* is associated with reduced Tau phosphorylation (Vandebroek et al. 2005). Furthermore, oxidative stress and dysfunctional mitochondria exacerbate Tau aggregation, although Tau is less phosphorylated under those conditions, suggesting that other mechanisms are involved in Tau aggregation (Vanhelmont et al. 2010) (for review see Seynnaeve et al. (2018)).

Insoluble aggregates of aSyn are found in synucleinopathies including idiopathic and familial forms of PD. Duplication or triplications of *SNCA* gene, which encodes aSyn, are linked to sporadic PD, while aSyn point mutations (A30P, E46K, H50Q, G51D, A53T and A53E) are associated with familial PD forms, with early onset (reviewed in Sampaio-Marques and Ludovico (2015)). Cell-based models for PD include yeast models, immortalized cell lines, primary neuronal cultures, stem cells and patient-derived cell models. These cellular models have been widely explored to dissect molecular mechanisms behind pathology using unbiased genetic screens, as well as multi-omic approaches to identify relevant genes and proteins. In addition, they can be easily manipulated genetically and pharmacologically at a reduced cost and in the absence of ethical issues. Nevertheless, these cellular models cannot reproduce several features of disease related to multicellularity and require validation in animal models.

The yeast *S. cerevisiae* is one of the best characterized eukaryotic organisms that provides a relevant biological context for the study cellular pathologies associated with PD (reviewed in Tenreiro et al. (2017)). Several molecular aspects of PD have been modelled in yeast, even though yeast lacks orthologs for aSyn. The first PD yeast model was reported in 2003 (Outeiro and Lindquist 2003), and since then a number of different humanized yeast PD models have been developed and employed to investigate PD. Similar to observations in other PD models, aSyn heterologous expression in yeast inhibits cell growth and promotes cell death in a concentration-dependent manner (Outeiro and Lindquist 2003). Independent studies identified mitochondrial dysfunction associated with oxidative stress (Buttner et al. 2008; Sampaio-Marques et al. 2012; Sharma et al. 2006), proteasome impairment (Chen et al. 2005; Sharma et al. 2006), autophagy and mitophagy dysfunction (Petroi et al. 2012; Sampaio-Marques et al. 2012), vesicular trafficking defects (Outeiro and Lindquist 2003) and

ER-to-Golgi trafficking impairment (Cooper et al. 2006) as relevant features of PD (Fig. 9.3). Furthermore, several post-translational modifications such as phosphorylation, ubiquitination, sumoylation and acetylation appear to influence aSyn toxicity and inclusion formation (reviewed in Tenreiro et al. (2017)). Our group was the first to develop a yeast model to study aSyn toxicity during ageing. Most yeast PD models are based on the heterologous expression of human aSyn under the control of a strong *GAL* promoter. Nonetheless, to avoid metabolic manipulations during chronological ageing, the *GAL* promoter was replaced by the *TPI1* promoter, which results in aSyn expression at lower levels when compared to *GAL* promoter, but allows for constitutive expression of aSyn during growth and ageing (Fig. 9.3).

Ageing constitutes a major risk factor for neurodegenerative diseases including PD and other synucleinopathies. By exploring the pathobiology of aSyn during yeast ageing, we observed that aSyn-expressing cells display a dramatic increase of autophagy and particularly of mitophagy that is deleterious for cells and shortens life span (Sampaio-Marques et al. 2012). Although increased autophagy can help aSyn clearance in functionally competent cells, it might also affect autophagy efficiency and selectivity in aged cells that have lost proteostasis. Studies in other cellular models have associated aSyn toxicity with aberrantly high activation of autophagy (Choubey et al. 2011; Stefanis et al. 2001; Xilouri et al. 2009). In addition, our experimental results showed that impairment of mitophagy by deletion of the yeast mitophagy-specific genes, *ATG11* and *ATG32*, resulted in CLS extension, further implicating mitophagy in aSyn toxicity. When exploring the pathways underlying autophagy and mitophagy after they have been aberrantly stimulated, we found that deletion of the *SIR2* gene alleviated aSyn toxicity as evidenced by CLS extension, and this phenomenon is linked to a drastic inhibition of autophagy and mitophagy (Sampaio-Marques et al. 2012) (Fig. 9.3). Notably, Sir2 was determined to be essential for the transcriptional regulation of *ATG8* and *ATG32* in stationary-phase cells expressing aSyn toxic variants (Sampaio-Marques et al. 2012). Our work emphasizes the fact that increased autophagy/mitophagy activity mediated by Sir2-mediated transcriptional regulation of *ATG* genes is an important phenomenon linked to aSyn toxicity during ageing.

In support of an association between exacerbated autophagy and aSyn toxicity, we have also shown that interventions that extend longevity and are associated with autophagy regulation, such as CR and inactivation of the TOR signalling pathway, are able to abolish aSyn toxicity and restore normal chronological longevity by maintaining autophagy at homeostatic levels (Guedes et al. 2017). In general, our results strongly suggest that it is important for life span extension to maintain autophagy under homeostatic levels, as has been reported in other biological systems. Furthermore, together with other findings, our data clearly indicate the relevance of proteostasis control in this age-related disease and confirm the utility of yeast as a model system for investigating different aspects of aSyn toxicity.

In conclusion, yeast cell-based models for neurodegenerative diseases provide valuable tools for deciphering the biological mechanisms of pathogenesis of these diseases as well as the discovery of novel therapeutic targets for treating them. Notably, yeast is also a well-recognized cellular ageing model that makes it possible to inves-

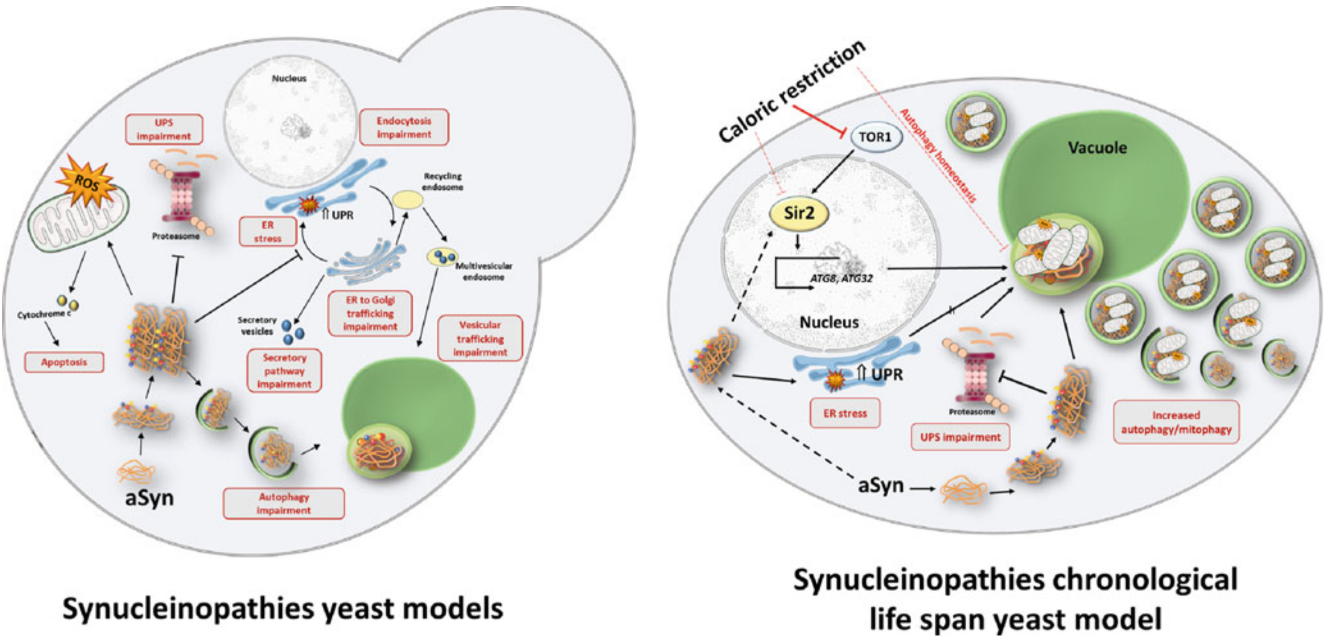

Fig. 9.3 Yeast models for synucleinopathies including Parkinson's disease. Shown are the pivotal pathways associated with aSyn-mediated toxicity in yeast. See text for additional details

tigate ageing as a component of neurodegenerative and age-related diseases in a manner that may not be possible in other cellular and animal models. To date, yeast ageing models have not been used to assess the toxicity of different factors in either HD or AD. This should provide fruitful avenues of investigation of these diseases in the future, as has been the case for Parkinson's and other synucleinopathies.

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