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REVIEW

Oxidative Stress in Yeast

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Abstract—The mechanisms of production and elimination of reactive oxygen species in the cells of the budding yeast *Saccharomyces cerevisiae* are analyzed. Coordinative role of special regulatory proteins including Yap1p, Msn2/4p, and Skn7p (Pos9p) in regulation of defense mechanisms in *S. cerevisiae* is described. A special section is devoted to two other well-studied species from the point of view of oxidative stress – *Schizosaccharomyces pombe* and *Candida albicans*. Some examples demonstrating the use of yeast for investigation of apoptosis, aging, and some human diseases are given in the conclusion part.

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Key words: yeast, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, oxidative stress response, regulatory proteins

Interest in free radicals has increased continuously since the 1950s when their existence in biological systems was discovered and involvement in development of many pathologies as well as aging was suggested [1]. Logically, in the first stage of free radical research they were seen only as dangerous oxidants when the enzyme detoxifying superoxide anion radical – superoxide dismutase (SOD) – was described [2]. It became clear that living systems possess not only systems for generation of free radicals, but also specific systems of their elimination. In the end of 1970s a factor inducing relaxation of endothelial smooth muscle was described – the free radical nitric oxide [3]. Therefore, it was concluded that free radicals might serve as regulators of certain processes. Superoxide anion, hydrogen peroxide, etc. might be signaling molecules along with nitric oxide. Finally, in the 1960s, leucocytes were found to produce free radicals for defense against infections [4]. Therefore, it is now well known that free radicals can damage cells, but at the same time they are involved in intra- and intercellular communication and can be used as well for protection against infections.

How are all the above listed functions of free radicals realized simultaneously? Free radicals are produced either as side products of aerobic metabolism or by specific systems. Their elimination is also provided by complex multilevel systems. Therefore, a fine balance between generation and degradation of free radicals exists in the cell. This balance under normal conditions is provided by many systems of feedback mechanisms. This means that intensification of free radical production can enhance the capacity of elimination systems. The operation of defense systems protecting organisms against free radicals was not clear for a long time. However, in the 1990s molecular sensors for superoxide anion and hydrogen peroxide were described in bacteria [5]. At least some sensor proteins "recognize" oxidant molecules because of reversible oxidation of cysteine residues. The activation of protein sensors provides their ability to stimulate transcription of genes, some of which encodes enzymes directly involved in antioxidant defense. They are, for example, different isoforms of SOD, catalase, etc. Sensors of free radicals in eukaryotes were described in the 1990s, and the yeast *Saccharomyces cerevisiae* was one of the first in this aspect [6, 7]. Since that time it has become possible to analyze principal similarities and difference between corresponding systems of pro- and eukaryotes. The reversible oxidation of cysteine plays a key role here also. However, the transduction of the signal from cytosol to nucleus is necessary, because eukaryotic cells possess a nucleus, but sensors are localized mainly in cytosol. Therefore, the reception of the signal in cytosol results in migration of a sensor (or mediator molecule)

Abbreviations: AMT, aminotriazole; cAMP-PKA, cAMP-dependent protein kinase A; DDC, diethyldithiocarbamate; G6PDH, glucose-6-phosphate dehydrogenase; ROS, reactive oxygen species; SOD, superoxide dismutase.

into the nucleus and its accumulation there with subsequent activation of specific genes [6, 7].

The cellular responses of eukaryotes toward oxidants have been described for many cell types. However, it seems the molecular mechanisms of signal transduction and adaptive response to oxidative stress are best studied in yeasts among all eukaryotes.

Here we will focus mainly on baker's yeast *S. cerevisiae* because most available information on cellular response to oxidative stress was obtained with this organism. That will be analyzed how yeast cells "feel" the signal and transform it into response, increasing antioxidant potential, which allow the cells to survive under unfavorable conditions. Certain attention will be paid to other yeast species, particularly *Schizosaccharomyces pombe* and *Candida albicans*.

INDUCTION OF OXIDATIVE STRESS IN YEAST

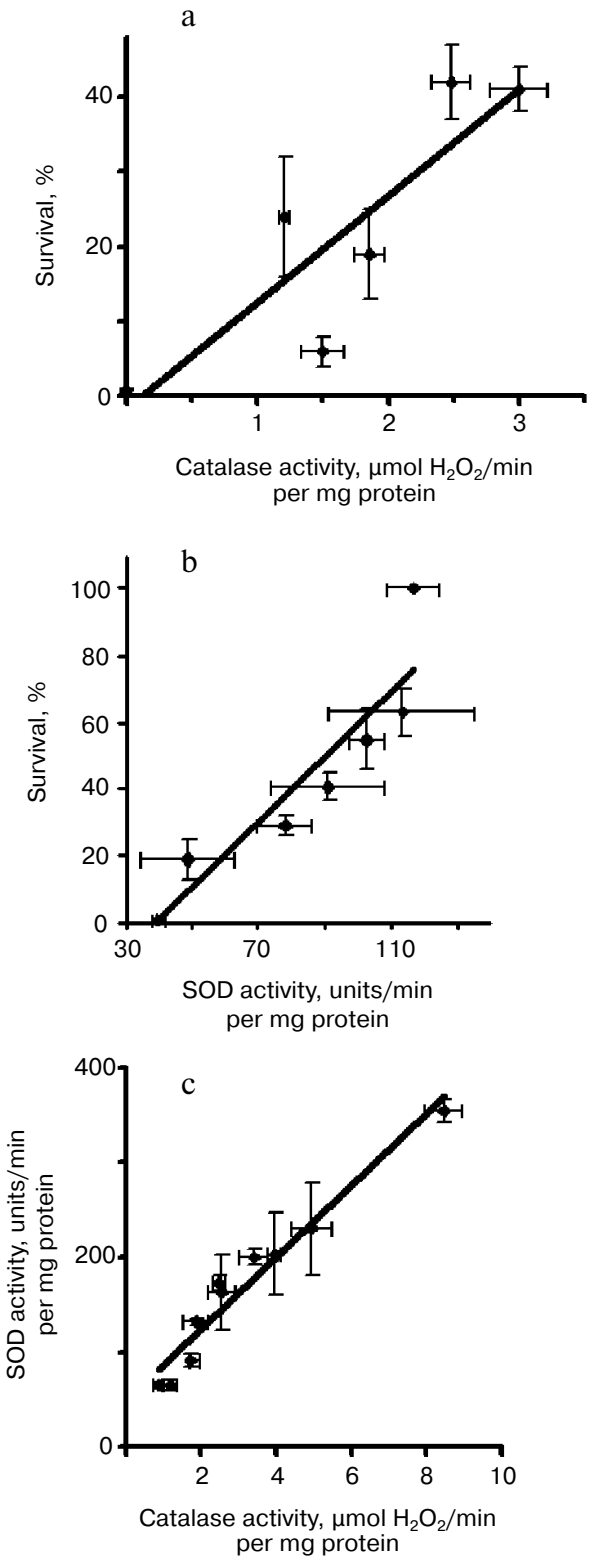
There are many ways to induce oxidative stress in these unicellular fungi. The sensitivity to stress depends on functional state of organisms. In experiments with stationary cultures of microorganisms several stages are known: lag phase (cells actively adapt to cultivation conditions and prepare to actively divide), logarithmic or exponential phase (cells actively divide), stationary or steady-state phase (the number of new cells corresponds to those that have died), and death culture phase (the number of dying cells is higher than those being newly created). In most cases, cultures in exponential and stationary phases are used in studies. When glucose is used as the energy and carbon source, the metabolism of *S. cerevisiae* in exponential phase is mainly anaerobic, i.e. most energy is produced by glycolysis or fermentation. The involvement of mitochondria is negligible, their number is small, and oxygen consumption is minimal. This results in low capacity for reactive oxygen species (ROS) production. For these reasons, the activity of antioxidant enzymes in the exponential phase of yeast culture is low [8, 9]. The transition of *S. cerevisiae* culture into stationary phase is characterized by increased utilization of ethanol, formed in the previous phase, as a source of carbon and energy. Complete oxidation of ethanol, which requires functioning of mitochondria, is provided by increase in their number, and as a consequence, generation of ROS is intensified. The latter is followed by an increase in activity of antioxidant enzymes [8-10] and causes enhanced capability to maintain ROS level within secure limits. Therefore, the transition of yeast culture from exponential to stationary phase is accompanied by enhanced ROS generation, i.e. can potentially cause oxidative stress. However, in counterbalance, the activity of the antioxidant system is increased and the system either returns to the initial state or reaches a new steady-state (or quasi-stationary) level. Anyway, the transition of yeast culture, as well as other cellular cultures with similar cultivation properties, from exponential to stationary phase can result in development of oxidative stress.

Since the ROS level is a dynamic parameter, it can be enhanced by decreasing the rate of ROS elimination. For example, it was demonstrated that deletion of genes encoding isoforms of catalase induced oxidative stress in yeast [11]. That was evidenced by enhanced content of protein carbonyl groups – a measure of free radical-induced oxidation of proteins. That was accompanied also by decreased activity glucose-6-phosphate dehydrogenase (G6PDH), an enzyme sensitive to free radical oxidation [11]. Consequently, the inactivation of genes encoding catalases can result in oxidative stress.

The use of inhibitors of antioxidant enzymes is the second experimental approach to reveal the role of enzymes in protection during oxidative insults. It should be noted that serious attention should be given here also, because the question of specificity of inhibitor effects cannot be ignored. Thus, in our works we used two inhibitors, which are active components of certain herbicides: aminotriazole (AMT) and diethyldithiocarbamate (DDC). The first creates the stable complex with active site of catalases [12], while the second extracts copper ions from active sites of enzymes, particularly SOD isoform containing in active center ions of copper and zinc – Cu,Zn-SOD [13]. This extraction inactivates the enzymes. The incubation of yeast cells with AMT and DDC suppressed catalase [12] and SOD [13] activities, respectively. Therefore, we concluded that inhibition of ROS degradation might induce oxidative stress in yeasts. Both deletion of corresponding genes and inhibition of superoxide dismutase by DDC in yeast cells allow us to demonstrate that *in vivo* SOD can be either anti- or prooxidant [13, 14]. This issue was discussed by us earlier [15].

In experiments, oxidative stress is most commonly induced by certain oxidants. Hydrogen peroxide, menadione, and paraquat are the most popular in yeast oxidative stress research. Since the situation with hydrogen peroxide is rather clear, only the use of two other compounds needs some explanation. Menadione it a synthetic analog of vitamin K, and paraquat is an efficient component of some herbicides. Their action is realized after entrance into the cell where redox systems can reversibly oxidize them [16]. Receiving electrons from cellular reducers, they can donate them to molecular oxygen, resulting in generation of superoxide anion with a following chain of events. Since the effects of hydrogen peroxide are most predictable an interpretable, it is, therefore, the most commonly used inducer of oxidative stress in yeasts and other organisms as well.

The supplementation of yeast cell suspension with hydrogen peroxide kills cells in a concentration-dependent manner, and catalase, by dismutating hydrogen per-



Correlation between activity of catalase and survival of yeast under treatment by hydrogen peroxide (a), activity of SOD and yeast survival under hydrogen peroxide exposure (b), and relationship between activities of SOD and catalase in YPH250 *S. cerevisiae* strain under treatment by different hydrogen peroxide concentrations during 30 min (c). Modified from [18]

oxide, protects yeast cells against this toxicant [17]. Interestingly, when we plotted at graph the survival of yeast cells under treatment with hydrogen peroxide against catalase activity, a clear linear relationship was found (figure, panel (a)) [18]. Although a similar relationship of protective role of catalase was postulated earlier based on single experiments, the correlation found by us substantially strengthens the general ideas on protective role of catalases. It should be added that in comparing yeast survival under hydrogen peroxide challenges and SOD activities, we also found clearly linear dependence (figure, panel (b)). Although these data are more complicated than in the case of catalase, it can be concluded that superoxide dismutase can also play a certain role in protection of yeast cells against hydrogen peroxide-induced oxidative stress. The application of correlative analysis allowed us to demonstrate that under the conditions used a linear positive correlation between the activities of SOD and catalase exists (figure, panel (c)). Therefore, it can be concluded that antioxidant enzymes play a substantial role in protection of yeast cells under hydrogen peroxide-induced oxidative stress, and, moreover, they operate in a coordinated manner.

YEAST CELL RESPONSE TO OXIDATIVE STRESS

Increase in steady-state ROS concentration. In many studies with fluorescent dye dichlorofluorescein, it was shown that introduction in the media with yeast of menadione, paraquat, and other compounds entering reversible redox cycles enhanced the fluorescence intensity of the dye [19, 20]. That was supposed to reflect the increase in steady-state ROS level.

Free radical-induced lipid oxidation. Two approaches were used earlier to evaluate this process in yeast. The first was measurement of products interacting with thiobarbituric acid (TBARS) [11]. Oxidative stress induced by inactivation of genes encoding catalase did not affect TBARS level. On the induction of mild oxidative stress by nitric oxide donor, the measurement of lipid peroxide level with xylenol orange did not show any change [21]. It seems that low content of unsaturated fatty acids and/or the absence of polyunsaturated fatty acids prevented us from using this marker as a reliable index of oxidative stress development in yeast grown without externally added polyunsaturated fatty acids.

Glutathione status. Despite certain complications connected with the use of glutathione as a marker of oxidative stress in yeast, it has been used for this purpose many times [22, 23]. Under oxidative stress one can register decrease in total glutathione level and increase in its oxidized form, which frequently is reflected by enhanced [GSSG]/[GSH] ratio. Interestingly, this ratio is substantially higher in strains with deleted catalase genes [24].

This parameter seems is a good marker of oxidative stress in yeast and, therefore, can be recommended for this use.

Activities of enzymes sensitive to oxidation. Aconitase seems to be the most sensitive to activated forms of oxygen and nitrogen among studied yeast enzymes [21]. At least this specific enzyme is most frequently used for this purpose. Inactivation of aconitase was found during induction of mild oxidative stress by low concentrations of nitric oxide donor sodium nitroprusside, which had virtually no effect on any other of markers of oxidative stress [21].

Other enzymes that are inactivated by ROS *in vitro* can also be used as markers of oxidative stress. Their sensitivity as markers of oxidative stress can be ordered in the series: G6PDH > glutathione reductase > isocitrate dehydrogenase > malate dehydrogenase > lactate dehydrogenase [13, 14].

Carbonyl groups of proteins. This index is among the most frequently used markers of oxidative stress in yeast. The methods of modern protein chemistry let to identify which specific proteins are oxidized during induction of oxidative stress in yeast [25, 26]. In some works even the character of protein modification was disclosed. Free radical oxidation of proteins, particularly in yeasts, was analyzed by us recently [15].

Change in permeability of plasma membrane for oxidants. In 2001 during study of effects of hydrogen peroxide on the enterobacterium *Escherichia coli*, we concluded that it freely penetrates their plasma membrane [27]. However, in works carried out with other *E. coli* strains and under different conditions exogenous hydrogen peroxide was supposed not always to freely enter the cells [28]. In 2004 data were published that demonstrated that hydrogen peroxide not only did not freely penetrate the plasma membrane of *S. cerevisiae* yeast cells, but that this penetration might be regulated in response to exogenously added oxidant [29]. In stationary growth phase, yeast membranes demonstrated 5-fold lower permeability for hydrogen peroxide than in exponential phase. It was suggested that the change in ergosterol biosynthesis could be the reason for modified permeability of yeast membranes for hydrogen peroxide. From the works described above [27-29] one can conclude that the active regulation of permeability of cellular membranes for oxidants, and for hydrogen peroxide particularly, can also be one of the mechanisms of yeast protection under oxidative insults. The concept of regulated hydrogen peroxide fluxes in the cells of bacteria, yeasts, plants and animals was systematized in review [30], where the possible involvement of the transmembrane protein aquaporin and its functional homologs in transport of oxidants was discussed.

Nucleic acids. The question the effect of oxidative stress on the genetic apparatus of yeasts is very important. Since yeasts, similarly to other organisms, possess a powerful system of DNA reparation in order to avoid mutations, it is rather complicated to register damage to DNA [31]. However, the use of strains defective in reparation disclosed different types of damage. Thus, the works [31, 32] described in details the molecular basis of yeast protection against injuring effects of oxidants, particularly hydrogen peroxide. Since this direction is not in the field of the author's interests, here only the system involved will be mentioned. These ways involve base excision repair (BER), nucleotide excision repair (NER), recombination (REC), and translesion synthesis (TLS).

COORDINATING ROLE OF Yap1p

This protein belongs to AP-1 type family of transcription factors. These are bZip DNA-binding proteins, which bind homo- or heterodimers with AP-1-binding sequences of DNA [33, 34]. The AP-1 protein family is highly conservative among eukaryotes and is a member of the nonspecific group of transcription activators involved particularly in gene response toward different signals. In mammalian cells AP-1 proteins are involved in regulation of proliferation, differentiation, apoptosis, and response to different kinds of stress.

The yeast AP-1 form, called Yap1p (other name is Gcn4p) in order to indicate its yeast specificity, binds the same DNA sites as the mammalian Jun and Fos factors and activates transcription of many genes in response to amino acid starvation [35]. At least eight proteins of the Yap family, called Yap1p-Yap8p, respectively, have been found in yeast cells. All of them have different although similar specificity for DNA binding and can be involved in regulation of cellular response to oxidative stress and cytotoxic agents, including drugs and ions of heavy metals, but their functions can differ [35-37].

Saccharomyces cerevisiae Yap1p plays a key role in cellular response toward oxidative stress and many drugs (multidrug resistance (MDR)) [38, 39]. As mentioned above, it belongs to the bZip family (basic domain/leucine zipper) transcription factors, which include yeast Gcn4p and mammalian AP-1 proteins – Fos and Jun [36]. Yap1p activates transcription via binding with specific DNA sequences localized in promoters of regulated genes [40]. Many genes involved in response to oxidative stress are known. They include *TRX2* (thioredoxin) and *GSH1* (γ-glutamylcysteine synthase) [6], *GSH2* (glutathione synthase) [41], *TRR1* (thioredoxin reductase) [42], *GPX2* (glutathione peroxidase) [43], *TSA1* (thioredoxin peroxidase) [44, 45], *AHP1* (alkylhydroperoxide reductase) [46, 47], etc. Yap1p also regulates the transcription of genes encoding membrane transporters, such as *YCF1* encoding a transporter with so-called ATP-binding cassette (ABC) operating as a glutathione-S-conjugating protein class [48], and *ATR1* and *FLR1* encoding MDR transporters of a family of big proteins facilitating membrane transport of many com-

pounds [49, 50]. The important role of Yap1p is strengthened by its direct or indirect involvement in regulation of many genes not listed above [45, 51]. Especial attention should be given to regulation of expression of genes encoding antioxidant enzymes.

Yap1p was initially identified basing on its capability to recognize and bind an element of activator protein 1 (AP-1) in SV-40 enhancer (5′-TGACTAA-3′) [36]. This sequence was found for the first time in promoter of the *GSH1* gene as critically important for regulation of its expression [41]. However, it was shown later that Yap1p preferably interacts with sequence 5′-TTAC/GTAA-3′ [35]. This palindromic sequence contains two identical hemi-sites TAA and operates in orientation-independent manner [49]. It was found in promoters of genes *TRX2*, *YCF1*, *GLR1*, and *ATR1* and was involved in gene transcription activation by Yap1 protein [6, 44, 49]. Therefore, it is accepted that the sequence 5′-TT/GAC/GTAA-3′ is a consensus element for response to the effect of Yap1p (named YRE, Yap1p response element).

The activity of Yap1p is mainly regulated at the level of export from the nucleus (Scheme 1). Under normal conditions Yap1p is freely distributed between the cytoplasm and nucleus, but mainly it is located in the former. Yap1p is actively exported from the nucleus by nuclear export protein Crm1, which interacts with C-end domain (cysteine rich domain (CRD)) of Yap1 protein containing many cysteine residues [52]. This domain also contains amino acid sequence enriched in leucine residues and three cysteine residues. This sequence is necessary for export of certain proteins from the nucleus and is highly conservative among proteins homologous to Yap1p [52, 53]. Mutant proteins with deleted CRD fragment or with certain amino acids replaced in the nuclear export sequence, especially the cysteine residues, or yeast cell treatment by oxidative agents, for example, hydrogen peroxide, diamide, or diethyl malate results is the impossibility to form Yap1p–Crm1p complex. Therefore, Yap1p is accumulated in the nucleus and induces Yap1p-dependent activation of expression of target genes [52, 53]. Due to this it is suggested that CRD is a specialized site responsible for the export of Yap1p from the nucleus and sensitive toward cellular redox state. Oxidation of certain Yap1p cysteine residues prevents its interaction with Crm1p, providing in this manner its accumulation in the nucleus and enhancing the expression of target genes [53].

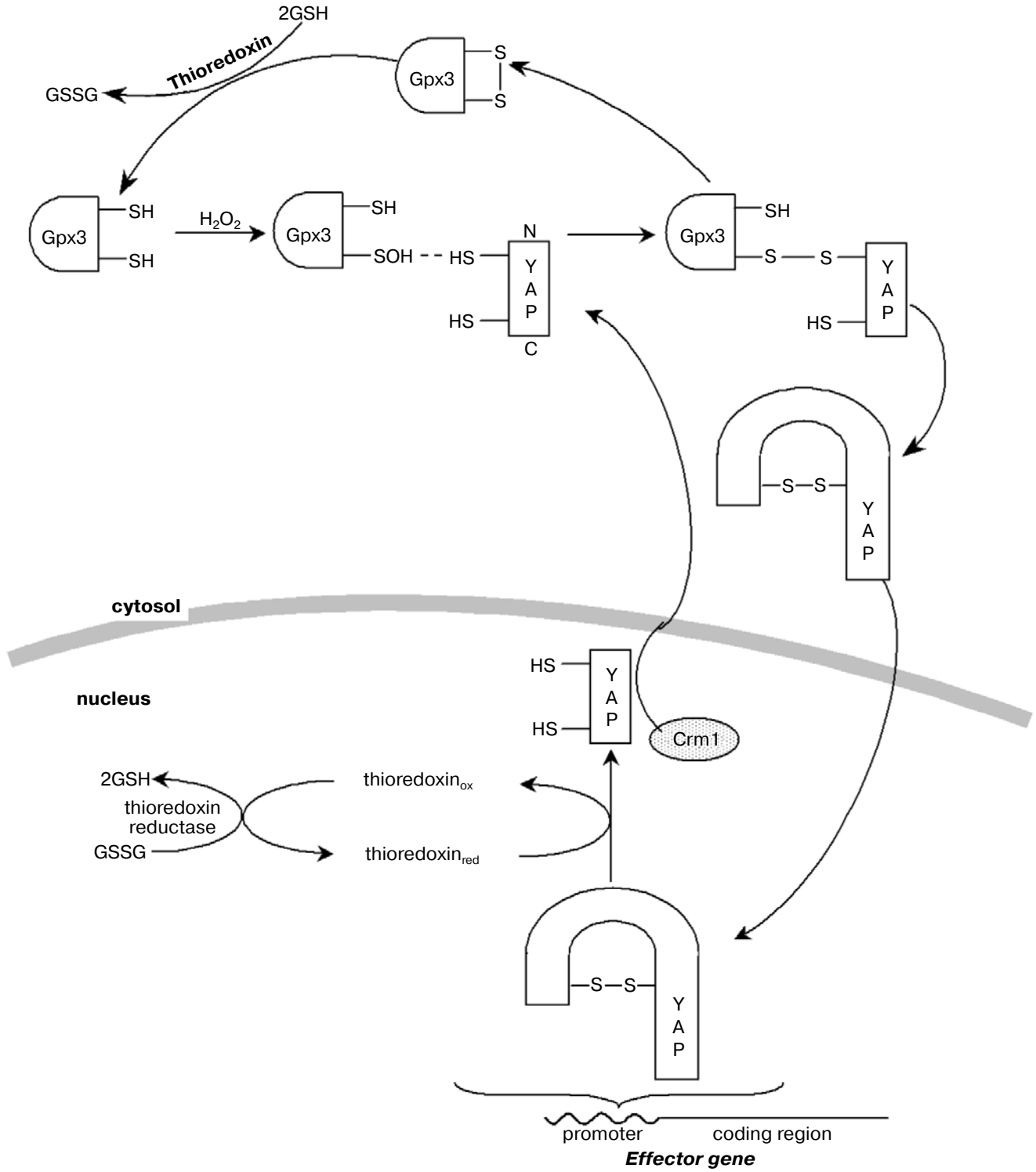
General principles of operation of Yap1 regulatory protein are given in Scheme 1. Its reduced form is relatively freely distributed between cytoplasm and nucleus. But due to the presence of Crm1p in the nucleus, the binding of the latter with reduced Yap1p is followed by export from the nucleus to the cytoplasm. Oxidation of Yap1p changes the site responsible for binding with Crm1p resulting in nuclear accumulation of Yap1p and enhances the expression of target genes. Reduction of Yap1p provides the availability of Crm1p to its nuclear export sequence leading to extrusion from the nucleus and decrease in expression of target genes.

Molecular mechanisms of Yap1p activation have been established (Scheme 1). The question of response to stress induced by hydrogen peroxide has been studied most detail [7, 48]. In fact, Yap1p is not the H_2O_2 sensor. The protein Gpx3, a glutathione-dependent peroxidase, is a sensor in this case. At the first stage, one of the cysteine residues of Gpx3 is oxidized to sulfoxide, which further interacts with a cysteine residue of Yap1p leading to the formation of an intermolecular disulfide bond. Breakage of this bond results in the formation of an intramolecular disulfide bond in Yap1p, i.e. it becomes oxidized. As described above, the oxidized Yap1p cannot bind Crm1p, and this results in Yap1p accumulation in the nucleus and activation of target genes. Oxidized Yap1p can be reduced by thioredoxin, which restores its capability to sense hydrogen peroxide. Thioredoxin is reduced by thioredoxin reductase at the expense of glutathione, which is reduced by glutathione reductase, respectively. The latter uses NADPH provided, for example, by G6PDH. Therefore, it became clear how the level of reduced Yap1p is connected with cellular redox status and particularly with concentrations of reduced and oxidized glutathione and NADP, and also with carbohydrate metabolism.

It should be noted, that oxidants other that hydrogen peroxide can activate Yap1p in a different manner. For example, Gpx3p is not involved in Yap1p activation by diamide [7, 48, 54].

The interaction of Yap1p with a protein called Ybp1 (Yap1p-binding protein) is necessary for response toward hydrogen peroxide, but not diamide [55]. Although its role has not been established, yeast mutants defective in Ybp1p do not form oxidized Yap1p. Therefore, Yap1p is not accumulated in the nucleus and consequently the expression of genes of the Yap1 stimulon is not activated. However, deletion of Ybp1 gene does not affect yeast response toward the stress induced by diamide. A homolog of Ybp1p, first named Ybp2p and later renamed Ybh1p, was found in yeasts [56]. It also affects yeast sensitivity toward hydrogen peroxide, but it seems these two homologs act via different mechanisms. Therefore, it was concluded that although proteins Ybp1 and Ybh1 are involved in coordination of yeast response toward exogenous oxidant, they operate in parallel manner as positive regulators of cell sensitivity to hydrogen peroxide.

The protein Yap1 is also involved in regulation of yeast sensitivity toward cadmium ions by activating the *YCF1* gene, encoding membrane transporter of ABC type [50, 57, 58]. In addition, it was found that this regulator is closely connected with yeast adaptation toward such toxicants as cycloheximide and 4-nitroquinoline-N-oxide [50].

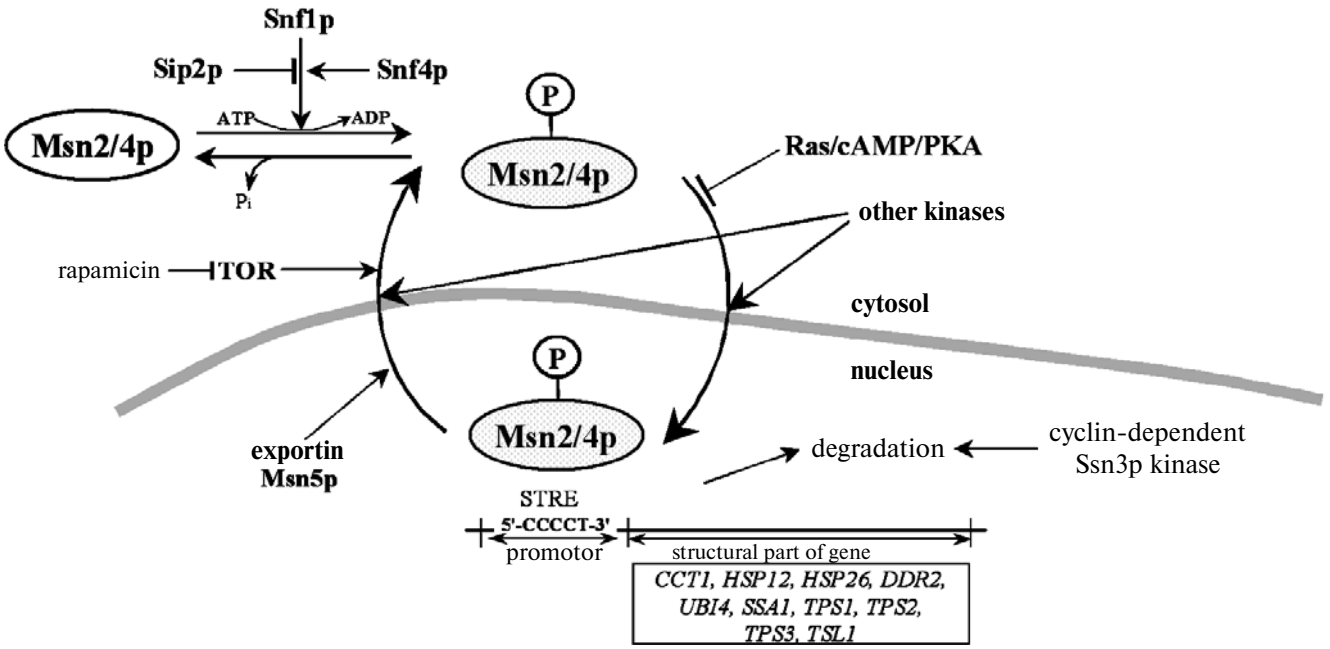


Operation of Yap1 regulatory protein during treatment of *S. cerevisiae* with hydrogen peroxide

Scheme 1

REGULATION BY TRANSCRIPTION FACTORS Msn2/4p

The transcription factors Msn2/4p bind DNA and activate genes contained in promoter regions called stress response element (STRE) – CCCCT [59, 60]. The latter is involved in yeast transition to diauxic growth and in response to heat-, osmotic-, acid-, and H_2O_2-induced stresses. The Msn2/4 proteins contain Zn-type DNA-binding domains, Cys2-His2, which bind STRE sequence.



Operation of regulatory proteins Msn2/4p in yeast cell response to stress factors

Scheme 2

Although their functions seem to be similar and individually they look not important for adaptation to oxidants, double Msn2/4 mutants are highly sensitive to stress. The full-scale stress response depends on the presence of two proteins – Msn2 and Msn4 [59]. In response to stress Msn2p and Msn4p are reversibly translocated into the nucleus, and this translocation is suppressed by cAMP, which provides the basis for an idea on control of cellular response via protein kinase A (PKA) [61]. In particular, it was shown that phosphorylation of the Ser620 residue in Msn2p by PKA provided its cytoplasmic localization and, therefore, inhibits its involvement in response toward stress (Scheme 2) [61]. During cell adaptation to stress Msn2p quickly exits the nucleus [62]. The diauxic transition, when the activity of PKA is decreased, provides conditions for Msn2/4p accumulation in the nucleus, might be partially responsible for enhanced yeast tolerance to environmental stresses in stationary growth phase. Although being slow, the degradation of Msn2/4p is another factor regulating its operation [62].

A French team [60] evaluated the role of Msn2/4p in the response of *S. cerevisiae* toward hydrogen peroxide at the level of protein expression in details. It was shown that Δ*msn2*Δ*msn4* strain was more sensitive only to high H_2O_2 concentrations, while triple mutant Δ*msn2*Δ*msn4*Δ*yap1* was only slightly more sensitive to the oxidant than Δ*yap1*. Moreover, each of the deletions Δ*msn2*Δ*msn4* or Δ*yap1* decreased the capability of the yeast to adapt to low H_2O_2 concentrations, and the abovementioned triple mutant did not adapt at all. These results demonstrate that although Msn2/4p and Yap1p determine yeast sensitivity toward H_2O_2, their functions can differ. About 30 proteins were induced by hydrogen peroxide in Msn2/4p-dependent manner. High yeast sensitivity toward H_2O_2 in the strain defective in phosphodiesterase encoded by the *PDE2* gene, and, therefore, possessing increased intracellular cAMP concentration was found. On the other hand, overexpression of Pde2p enhanced yeast tolerance to the oxidant. Deletion of the *BCY1* gene, encoding the regulatory subunit of PKA and enhancing the activity of the enzyme, increased yeast sensitivity toward H_2O_2 also [60].

Two stimulons, Yap1p and Msn2/4p, being involved in response to oxidants, are regulated by oxidative stress differently. Anyway, since both of them regulate the expression of the same genes, the question on their specificity arises. The Yap1p stimulon mainly includes enzymes some of which take part in metabolism of glutathione and thioredoxin. They operate mainly in defense against oxidative stress via ROS degradation and binding. The Msn2/4p stimulon consists of genes encoding only two antioxidant enzymes – cytosolic catalase Ctt1 and thiol transferase Grx1, while the other genes encode certain proteases, some enzymes of ubiquinone and proteasome pathways, chaperons, heat shock proteins, and some metabolic enzymes [60]. The maximal induction of target genes regulated by Msn2/4p is reached at higher H_2O_2 concentrations than those activated by Yap1p. Therefore, it is assumed that if Yap1p is important for prevention of oxidative stress and immediate adaptation to it, Msn2/4p is more important for recovery from the stress and providing of reparation and/or degradation of damaged molecules and/or restoration of metabolic

routes. Modification of the Ras-cAMP-PKA-Msn2/4p pathway demonstrated the role of PKA in functioning of both Msn2/4p and Yap1p. The latter does not possess consensus phosphorylation sequence (RRXS/TB, RXRXXS/T) and seems not to be a substrate for PKA [60]. However, PKA modifies yeast response to H_2O_2-induced stress via Yap1p in an unknown manner. Due to that, it can be concluded that PKA substantially affects yeast response to oxidative stress, but the mechanisms involved need to be elucidated.

The data obtained with microchips on H_2O_2-induced Msn2/4p dependent gene expression [62, 63] mainly correspond to those described above at proteome level, but they seem to less accurately reflect the adaptive response of the cell.

REGULATION BY Skn7 (Pos9) FACTOR

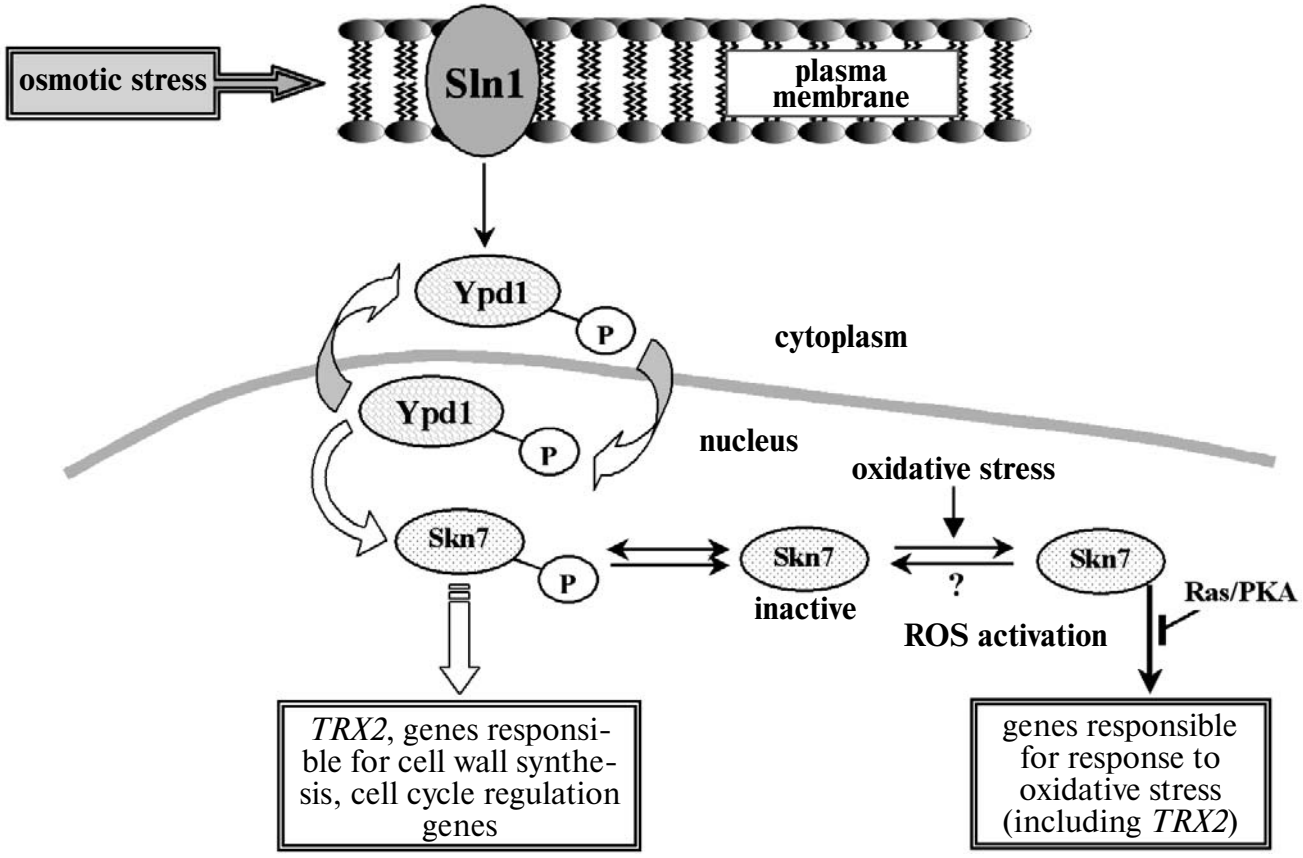
In 1996 Krems and colleagues reported that Pos9 (peroxide sensitivity) protein is involved in regulation of *S. cerevisiae* sensitivity to hydrogen peroxide, methyl viologen, and enhanced oxygen pressure, but did not affect yeast growth under normal conditions [64]. Isolation and sequencing of the Pos9 gene demonstrated its identity with Skn7 protein. The latter possesses a domain with high homology to prokaryotic proteins involved in the so-called "two-component" system of signal transmission where phosphate from autophosphorylated sensor histidine kinase is transferred to a conservative aspartate residue of the regulatory protein. Replacement of the corresponding aspartate residue in Skn7 (Pos9) protein confirmed its possible involvement in cellular response toward hydrogen peroxide. Two-hybrid screen experiments suggest the possible oligomerization of Skn7 protein. It was concluded that Skn7p might be an element of the two-component system involved in yeast response on oxidative stress [64].

Skn7 protein is a transcription factor involved in regulation of biosynthesis of cell wall and cell cycle [43, 65, 66]. Skn7p was demonstrated to regulate the induction of two genes – *TRX2* encoding thioredoxin and *TRX1* encoding thioredoxin reductase [66]. Skn7p in concert with Yap1p increased the expression of *TRX2* gene via binding its promoter. J. Lee and colleagues studying protein expression level found a rather large set of genes the expression of which was enhanced in response to hydrogen peroxide and depended on both Skn7 and Yap1 proteins [47]. The two regulatory proteins were shown to be involved in upregulation of cytochrome-*c*-peroxidase, catalase T, Cu,Zn-SOD, Mn-SOD, thioredoxin 2, thioredoxin reductase 2, thiol-specific antioxidant, alkylhydroperoxide reductase, heat shock proteins, and some others. Most proteins controlled by Yap1p were not regulated by Skn7p, and several proteins the expression of which was induced in Skn7p-dependent manner were not regulated by Yap1p. Therefore, it was concluded that although both Skn7p and Yap1p coordinate yeast response to hydrogen peroxide and there are many genes under control of both these proteins, the full set of target genes is substantially different. Interestingly, global changes in expression of proteins/enzymes led to the conclusion that there is a switch of metabolic fluxes from energy providing to maintaining of reductive potential, the concentrations of glutathione and NADPH [47].

Scheme 3 demonstrates current concepts on the operation of Skn7 protein in regulation of cellular processes. The regulation of cellular response to osmotic stress is given on the left side of the scheme. Under hypo-osmotic stress, membrane protein kinase Sln1 is autophosphorylated at residue His576. The phosphate is transferred to the conservative Asp1144 residue of acceptor domain of the same protein [67, 68]. Further, the phosphate is transferred to a conservative residue of Ypd1 protein, resulting in diffusion of the latter into the nucleus. Finally, in the nucleus where Skn7p is permanently localized [68], phosphorylated Ypd1p donates phosphate to a conservative aspartate residue of the acceptor domain of Skn7 protein. This is so-called two-component route of phosphate transfer. It participates in regulation of genes involved in cellular response to hypo-osmotic shock. The question here is: how is the above described mechanism related to this review? It seems to be really connected, although not directly. First, Skn7p can enhance the expression of one of the genes, *TRX2*, encoding the antioxidant enzyme thioredoxin [67]. Second, as seen from Scheme 3, the phosphorylation of Skn7p can prevent its involvement in oxidative stress-induced activation of antioxidant and other genes. It should be noted that the mechanism of dephosphorylation of Skn7p has not been established.

The induction of oxidative stress activates Skn7 proteins via an unknown mechanism. However, it was found that Skn7p activation lead to its binding with promoters of effector genes [43, 64, 69, 70]. Interestingly, non-phosphorylated Skn7p in some way provides basal transcription of the *TRX2* gene [67]. Up to now neither the oxidative stress-induced Skn7p activation mechanism nor its inactivation has been deciphered. It cannot be ruled out that thioredoxin peroxidase-1 (Tsa1p) can regulate its functional state [71].

Skn7 protein in cooperation with heat shock factor Hsf1p regulates the induction of heat shock proteins [72]. It was found that Skn7p can form homo-oligomer(s), but the formation of hetero-oligomers with Hsf1p coordinating yeast response to heat shock cannot be excluded. Two more proteins involved in regulation of calcium-dependent processes, namely the Ca^{2+}-activated protein phosphatase calcineurin and transcription factor Crz1/Tcn1p, function together with Skn7p also [73]. The formation of this complex stabilizes Crz1p resulting in increased expression of genes involved in calcium metabolism. It



Involvement of Skn7 protein in gene expression in yeast *S. cerevisiae*. Modified from [67, 68]

Scheme 3

seems that the interaction with other proteins, phosphorylation, and formation of homo-oligomers can substantially affect the ability of Skn7p to regulate yeast adaptation to oxidative stress. The possible involvement of phosphorylation in response to oxidative stress via Skn7p cannot be excluded because Ras/PKA (via cAMP-dependent PKA) negatively regulates the involvement of Skn7p in cell response to stress [74].

Therefore, it can be concluded that Skn7 protein in cooperation with Yap1p regulates yeast cell response to oxidative stress. Since it is also involved in other regulatory pathways, particularly in response to hyper- and hypoosmotic and heat shock stresses, regulation of calcium homeostasis, and possibly in other interactions, it can serve as a coordinator of cellular response to effects of external factors and adaptation to unfavorable conditions.

RESPONSE OF *Sch. pombe* AND *C. albicans* TO OXIDATIVE STRESS

Similarly to *S. cerevisiae*, *Sch. pombe* is also a rather well studied yeast species that is grown vegetatively. Modern genetic approaches are also well developed for it. Therefore, it is also a convenient eukaryotic model to study the effects of external factors on yeast, particularly oxidative stress. *Candida albicans* is the second species the response of which to oxidative challenge will be described in this section. It is a component of normal human and animal microflora and is present in two forms: individual cells (yeasts), which is dominant, and in the form of mycelium, a parasitic form. This yeast is a facultative pathogen that induces systemic infections in individuals with suppressed immunity. The transition from noninfective to infective form is induced by changed conditions, for example, temperature or pH [75]. The immune system of humans and animals is used to fight *C. albicans*. Thus, it is known that macrophages are able to absorb *C. albicans* cells and kill them in an oxygen-dependent manner, probably via ROS generation [76]. Therefore, for successful colonization of the host's body the *C. albicans* cell has to "feel" ROS and be able to adapt to their increased level. In fact, it was shown that inactivation of regulation of the antioxidant system decreased pathogenicity of *C. albicans* [76].

Schizosaccharomyces pombe yeasts possess Pap1 protein homologous to transcription factors AP-1 of mammals and Yap1p of *S. cerevisiae*. Similarly to Yap1p, Pap1p

is concentrated in the nucleus in response to oxidative stress [77], where it induces the transcription of many genes, particularly those encoding antioxidant enzymes: *ETT1* (cytosolic catalase), *TRX2* (thioredoxin), *TRR1* (thioredoxin reductase), and *PGR1* (glutathione reductase) [78]. It was found that similarly to *S. cerevisiae*, *Sch. pombe* mutants defective in *PAP1* are highly sensitive to many cytotoxic agents, heavy metal ions, and oxidants, particularly to hydrogen peroxide and diamide [79, 80].

The mechanisms of regulatory effects of Pap1p in *Sch. pombe* and Yap1p in *S. cerevisiae* are very similar. The genetic approaches demonstrated that nuclear protein Crm1 is also necessary for Pap1p concentration in the nucleus in response to oxidative stress [79, 80]. Pap1p in the C-end region contains the sequence responsible for its export from the nucleus – nuclear export signal (NES). Similarly to Yap1p, excision of the NES fragment or inactivation of exportin Crm1p results in Pap1p accumulation in the nucleus leading to expression of target genes without oxidative stress [52, 80]. This shows that the localization of Pap1p in the nucleus is enough for induction of transcription response. Similarly to Yap1p, Pap1p activation requires the formation of an intracellular disulfide bond [81]. The oxidant diethyl maleate activates Pap1p, directly modifying one of the cysteine residues of the NES sequence [52, 81]. Probably, oxidative modification of Pap1p leads to impossibility to interact with Crm1p, which provides its accumulation in the nucleus and increases the expression of target genes.

In 1998 W. Toon and colleagues reported that the MAP-kinase pathway might be involved in regulation of *Sch. pombe* response via Pap1p [79]. On the other hand, later work led to the opposite conclusion [82]. It is possible that MAP-kinase has an indirect effect. Since the regulation of several genes was studied, it is possible that not cooperation at regulatory pathway level, but rather interaction at level of target genes, particularly catalase *CTT1* and thioredoxin reductase *TRR1*, might take place. In particular, regulatory regions of these genes contain binding sites for transcription regulators – sites of different pathways of signal transduction [83].

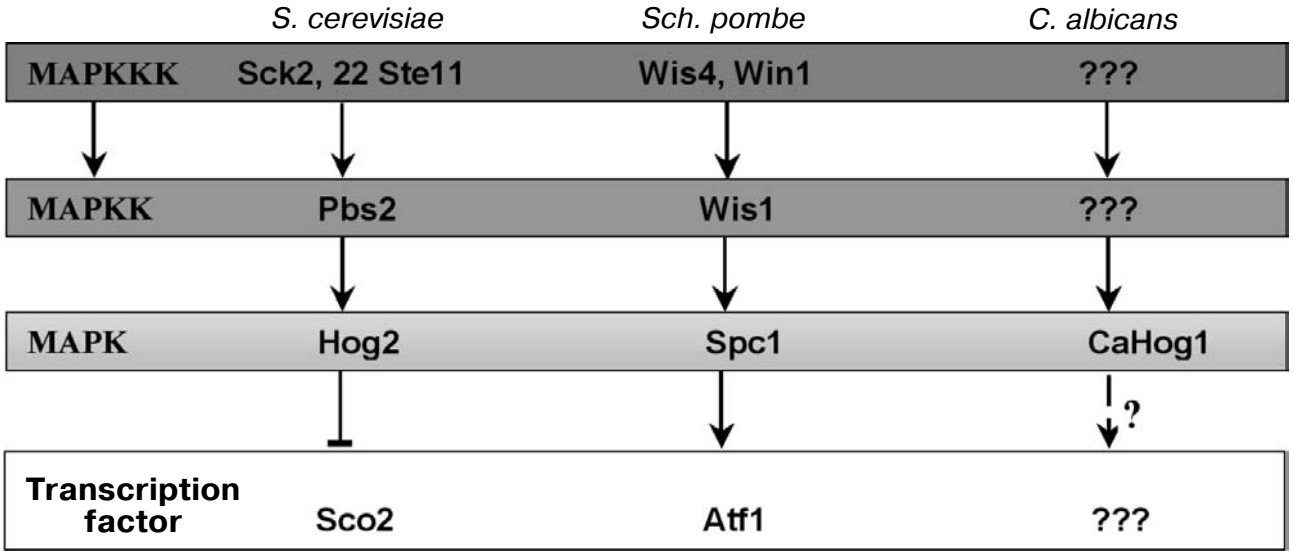
In *C. albicans*, the homolog of Yap1p called Cap1p was found using approaches developed for *S. cerevisiae*. The functional complementation of *yap1*$^-$ (Δ*YAP1*) was applied in this case [84]. The protein Cap1 bound the same DNA sequences as Yap1p [85]. Mutants defective in Cap1p were hypersensitive to hydrogen peroxide and cadmium ions and did not respond to oxidative stress by increased expression of genes encoding antioxidant enzymes, particularly glutathione reductase [84, 85]. This shows that Cap1p is necessary for protection of *C. albicans* against oxidative stress. Similarly to Yap1p and Pap1p, Cap1p activity as transcription activator was regulated via its accumulation in the nucleus, and the oxidation of a cysteine residue in the C-end part of the protein might also take place [85].

It seems that the multistep phosphorelay pathway plays a substantial role in sensing of oxidative stress signaling and coordination of response in cells of *Sch. pombe* and *C. albicans*. In *S. cerevisiae*, phosphate transfer probably participates in operation of Skn7p via the kinase system Sln1–Ypd1 induced by osmotic shock. Probably, Skn7p in this way is removed from regulation of response to oxidative stress. This pathway was recently analyzed in detail in review [86], and here it will be discussed briefly.

In *Sch. pombe* the phosphotransfer regulatory pathway was discovered as an alternative to the regulatory one to stress-activated protein kinases (SAPK). Three orthologs of the *SLN1* gene of *S. cerevisiae* called *MAK1*, *MAK2*, and *MAK3* were found in this yeast [87]. In contrast to the budding yeast *S. cerevisiae*, in the conventional yeast *Sch. pombe* the Mak1-Mpr1-Mcs4 pathway receives and transduces oxidative stress signal to the SAPK cascade (Scheme 4). In contrast to Sln1p, Mak2 and Mak3 possess GAF domains similar to the redox-sensitive domain of transcription factors of plants [87]. This additional domain might provide the sensitivity of Mak-kinases to oxidative signals. There are several evidences for the involvement of Mak2 and Mak3 in the transduction of oxidative stress signal in the MAPK cascade via response regulators Mpr1p and Mcs4p. First, the deletion of *MAK2* and *MAK3* genes leads to weaker MAPK activation [88]. Second, the deletion of the *MPR1* gene or substitution in the protein of a histidine residue involved in transfer of phosphate residue also weakens the system response to oxidative stress [89]. Third, mutation of the *MCS4* gene with substitution of an aspartate residue involved in phosphate transfer by a non-phosphorylatable asparagine also weakens signal transduction of oxidative stress to the MAPK cascade [90]. Fourth, Mpr1 protein binds with Mcs4p in response to oxidative stress [82]. Fifth, phosphate transfer between Mpr1p and Mcs4p was found *in vitro* [89]. It should be added that none of the mutants in *MAK2* and *MAK3*, *MPR1* or *MCS4* demonstrated defects in activation of the MAPK cascade in response to osmotic stress or heat shock. That can be interpreted as evidence for existence of a Mak2p/Mak3p-Mpr1p-Mcs4p phosphate transfer pathway that is specific for transduction of oxidative stress signal. Finally, it was shown that Mcs4 protein binds with MAPKKK kinases and initiates the activation of the cascade of MAP-activated protein kinases (Schemes 4 and 5) [89, 91].

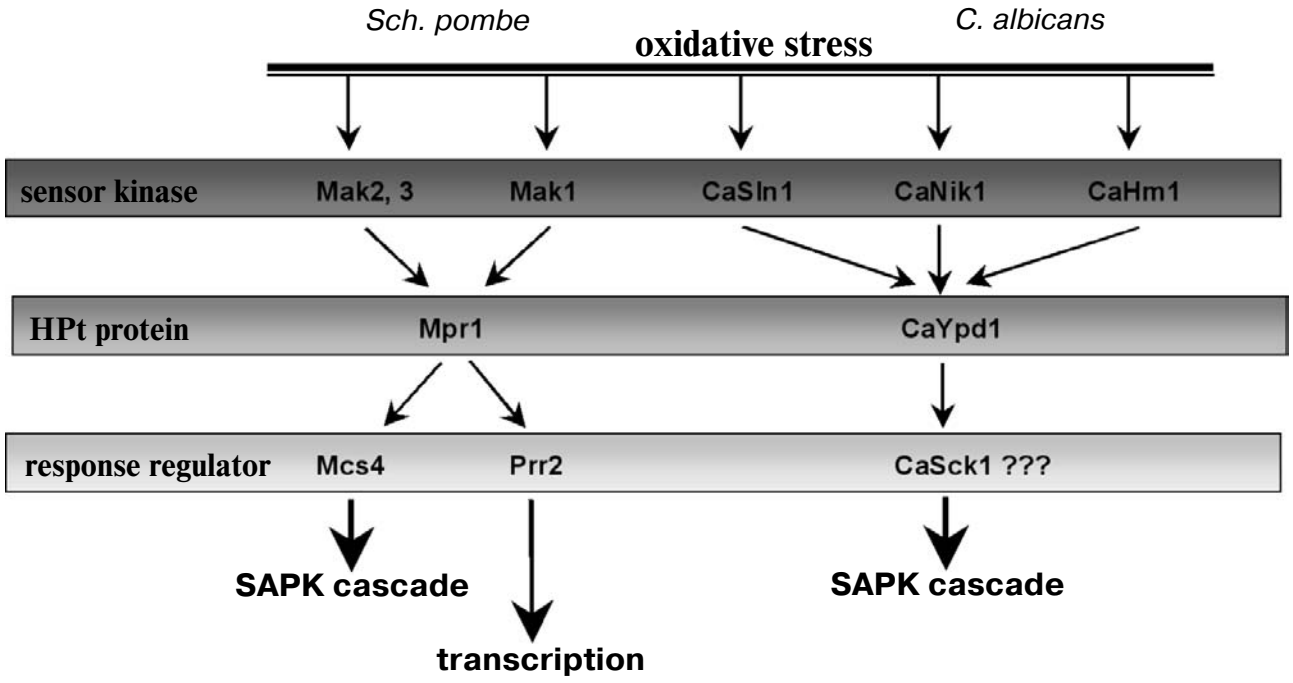
Although the sensor kinase Mak1p is not involved in activation of the MAP-kinase pathway of response to oxidative stress, deletion of the gene encoding this protein leads to partial defects in hydrogen peroxide-induced expression of genes of catalase (*CTT1*) and thioredoxin reductase (*TRR1*) [82, 92].

The gene encoding Prr1 protein in *Sch. pombe* is the ortholog of the *SKN7* gene of *S. cerevisiae*. In *Sch. pombe* Prr1 protein operates as a transcription regulator containing a DNA-binding domain [92]. The inactivation of the



Stress responsive MAP-kinase (SAPK) cascades of three yeast species. Modified from [86]

Scheme 4



Phosphorelay pathways in *Sch. pombe* and *C. albicans*. Modified from [86]

Scheme 5

PRR1 gene does not affect activation of the MAP-kinase cascade [82]. Therefore, the involvement of the phosphorelay pathway Mak1p-Mpr1p-Prr1p in regulation of response to oxidative signal by the enhanced expression of antioxidant genes is suggested [82]. Moreover, this pathway operates independently from the MAP-kinase pathway (Scheme 4).

Three genes of histidine sensor kinases, *CaSLN1*, *CaNJK1/COS1*, and *CaNK1* [93, 94], as well as *CaYPD1* gene encoding HPt protein [95], have been found in *C. albicans*. It was found that the regulatory gene *CaSSK1* of *C. albicans*, an ortholog of genes *SSK1* of *S. cerevisiae* and *MCS4* of *Sch. pombe*, might be involved in a phosphorelay pathway of oxidative stress signaling to the SAPK cascade

[96, 97]. A *C. albicans* strain defective in the *CaSSK1* gene was highly sensitive to hydrogen peroxide and menadione. In addition, the activation of the SAPK cascade in response to oxidative stress was not found, but SAPK was activated by osmotic stress similarly to the "wild" type strain. Therefore, despite very few works being carried out in this direction in *C. albicans*, the phosphorelay pathway from sensor histidine kinase via proteins HPt and CaSsk1 to the MAP-kinase cascade is supposed to be involved in coordination of response to oxidative stress similarly to *Sch. pombe* (Scheme 4).

The involvement of a phosphorelay pathway in MAP-kinase inactivation in response to oxidative stress was discussed above. To emphasize that these kinases are activated by stresses, they were called as stress responsive MAPK (SAPK). This pathway of signal transduction is evolutionarily conservative in eukaryotes and is responsible for accepting of different environmental signals [86]. It regulates multiple cellular processes including proliferation, differentiation, cell death, and homeostasis. The "typical" MAPK cascade consists of three protein kinases that are phosphorylated and activated in response to effects of stimulators (Scheme 5). MAPK are activated via the phosphorylation of tyrosine and threonine residues of their activation loop. This process is provided by kinase of MAP-kinase (MAPKK, also called MEK). MAPKK is also activated by phosphorylation of other kinase called "MAPKK kinase" (MAPKKK or MEKK). Activated MAP-kinase phosphorylates different substrates such as cytosolic proteins and/or transcription factors involved in cellular response to different environmental stimuli [86].

Involvement of MAP kinases in cell response to stress was first described for the budding yeast *S. cerevisiae* where MAP kinases participate in coordination of response to osmotic stress. This pathway is named HOG (high osmolarity glycerol response). It increases glycerol in response to increase in osmolarity of the external medium [98, 99]. Subsequently, similar regulatory cascades were found in other species in response to osmotic stress, oxidative stress, heat shock, starving, etc. Therefore, this particular group of kinases got the name SAPK – stress-activated kinases [86].

SAPK seems the best studied in *S. cerevisiae* [88]. This issue was briefly discussed during analysis of its involvement in operation of Skn7p. However, since SAPK are not involved in the response of *S. cerevisiae* to oxidative stress [87], further we will describe the system in two other yeast species where its involvement has been demonstrated to some extent.

In *Sch. pombe* a SAPK cascade is involved in response to osmotic stress, heat shock, starvation, and oxidative stress, particularly to effects of such oxidants as hydrogen peroxide, paraquat, UV-radiation, alkylating agents, and arsenic [87]. In this conventional yeast, genetic inactivation of SAPK components enhanced sensitivity to the above listed environmental factors. Proteins Wis4 and Wis1 phosphorylate Ser469 and Thr473 residues of MAPKK (Wis1p) leading to inactivation of the latter. Since Wis1p inactivation cancelled Spc1p activation in response to oxidative stress, it is assumed that MAPKK are involved in signal transduction to Spc1p [100]. The stress-activated Spc1p is translocated from the cytoplasm into the nucleus where via phosphorylation it activates a bZip-type transcription factor [101, 102]. The inactivation of the *SPC1* and *ATF1* genes of *Sch. pombe* allows identification with microchips of more than 200 genes induced by oxidative stress via this pathway [103, 104]. Cytosolic catalase (Ctt1), glutathione peroxidase (Gpx1), thioredoxin reductase (Trr1), and glutathione-S-transferases (Gst1, Gst2, and Gst3) were among these induced genes [86].

An interesting regulation pathway in response to oxidative stress is described for *Sch. pombe*. Under oxidative stress Spc1p phosphorylates cytosolic protein Csx1, which can bind to mRNA [104]. Csx1 binds and stabilizes mRNA encoding protein Atp1p. Mutants deficient in *CSX1* gene are particularly sensitive to hydrogen peroxide and respond with lowered expression of genes regulated by Atp1p and some other stress-induced genes. Discovery of Csx1 and elucidation of its involvement in yeast cell response to oxidative stress revealed that this expression is regulated both at the level of transcription and the level of mRNA stabilization. Another substrate for MAPK Spc1p is protein kinase Cmk2 [86, 105]. *Schizosaccharomyces pombe* mutants deficient in this protein have increased sensitivity to hydrogen peroxide and arsenite. MAPK Spc1p binds to Cmk2p and phosphorylates it. Unfortunately, the molecular mechanisms of the action of Cmk2p protein kinase are not yet established [86].

To investigate the role of SAPK in *C. albicans* adaptation to oxidative stress, the knowledge gained with *S. cerevisiae* and *Sch. pombe* was used. By genetic complementation of budding yeast mutants defective in Hog1 and using the gene library of *C. albicans*, the *CaHOG1* gene was identified [99]. Amino acid sequence of CaHog1p was highly homologous to Hog1p of the budding yeasts and Spc1p of the conventional yeast. Although upstream components of the *C. albicans* Hog1 pathway are not described, it is possible that they possess this highly conservative pathway (Scheme 5). Genetic studies demonstrated that a defect in the CaHOG1 pathway resulted in enhanced sensitivity to such oxidants as hydrogen peroxide and menadione, as well as to UV-radiation [106]. In addition, CaHog1p is subjected to activating phosphorylation during oxidative stress. It was also shown that the mutant *C. albicans* strain was a less virulent inducer of fungal infections, which suggests a key role of CaHog1p in pathogenesis of candidoses [107].

Recently, an extensive review of N. Chauhan and colleagues was published [108] where the authors summarized data on transduction of signals and response to

oxidative stress in *C. albicans* and *Aspergillus fumigatus*. Since these processes in the first species were analyzed above in detail, and the second is not an yeast, although it is a fungus, we suggest that it would be enough to call attention of readers to this work. Interestingly, the response of *A. fumigatus* to oxidative stress is rather similar to that of yeast.

CONCLUSIONS AND PERSPECTIVES

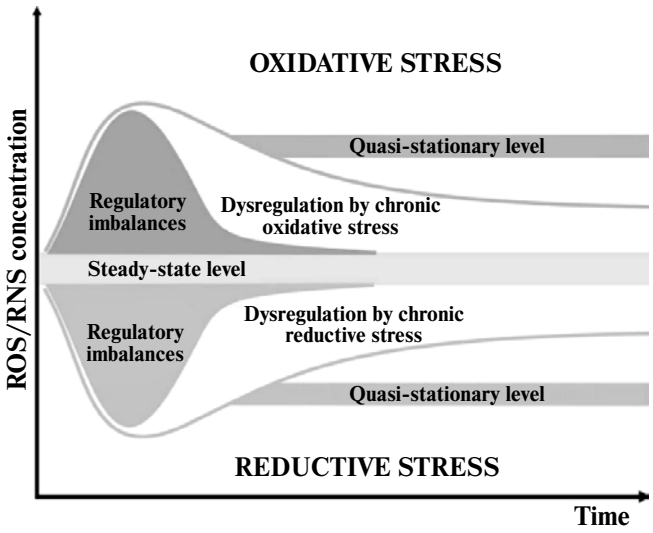
Since the generation of reactive species of oxygen, nitrogen, and other elements is an inevitable part of living in an oxygen environment, organisms developed many specific defense mechanisms. It should be emphasized that we are not talking about elimination of all ROS, but about maintaining them at a certain level that is necessary for normal functions. The elimination of ROS can have negative consequences because, as mentioned above, they play signal and protective roles and additionally some more vital functions. The danger of fighting against ROS was described in book of A. Hailey "Strong Medicine". The main hero synthesized a powerful antioxidant that was introduced as a pharmacological form. After some time information on side effects started to appear, and they were related with weakening of the immune system. It is clear why! The immune system fights infections using activated forms of oxygen, nitrogen, etc. [16]. This example clearly demonstrates the importance of taking into account all factors when evaluating affects in biological systems. But here first of all we are interested in ROS metabolism in general, in particular in yeasts.

The concentration of ROS is maintained within a certain range and fluctuates similarly to other parameters in the organism in according to homeostasis theory (Scheme 6). However, under some circumstances the concentration may leave this range due to increase/decrease of production or change of efficiency of the catabolism system. The state when ROS level is transiently increased is referred to oxidative stress, and when decreased it is reductive stress. The problem of oxidative stress is investigated rather well, while reductive stress studies are only in the infant state. In the latter even methodological approaches have not been developed. Large changes in ROS level, out of a certain range of "norm", stimulate a system of feedback relationships. They are abundant and multilevel, which provides fine regulation in ROS level in a certain range of concentrations. There are two principally different scenarios. In the first case, after induction of oxidative/reductive stress the ROS level returns into the initial range. In the second case, the system reaches a new steady-state range, and this is a new "normal" range of concentrations. The new steady-state range or quasi-steady state range appears.

Above we have analyzed the mechanisms of the development of oxidative stress, defense against ROS, and regulation of protective mechanisms in yeasts. Interestingly, many regulatory mechanisms are rather similar in all organisms, especially in eukaryotes. We will not discuss the core vital processes, propagation, homeostasis, etc., or biochemical principles of metabolism of compounds of main classes, energy generation, maintaining of transmembrane gradients of different compounds, etc. In order to demonstrate that, several examples will be given here using the yeast *S. cerevisiae* as a model subject to reveal molecular mechanisms of certain processes. Here we focus only on the processes closely associated with modification of free radical reactions.

Yeasts and particularly *S. cerevisiae* are efficiently used to investigate basic biological processes. For example, they are a convenient model to study apoptosis and aging. The first problem was analyzed in detail in review article of Russian researchers [109]. Interestingly, the main features of apoptosis in yeasts are rather similar to those in animals. Information on the use of yeast to study the aging process can be found in review articles [110, 111]. Since apoptosis and aging processes in yeast are well highlighted in the literature, here we will not discuss them.

Most key vital processes are similar in yeasts and humans. Therefore, it is very attractive to use the former to study the molecular mechanisms of certain pathologies in humans and animals [112, 113]. This approach provides an excellent tool for development of prophylactics and treatment of certain diseases and for screening of potential drugs [114, 115]. The use of yeasts to study cancer provides a good example [115, 116]. That might seem



Schematic representation of modern ideas on metabolism of free radicals in biological systems. The steady-state ROS level either can enhance inducing oxidative stress, or decrease causing reductive stress. Further the system can return to the initial state or stabilize at a new (quasi-stationary) level. The disturbance of response of a biological system toward any stress can result in certain pathologies. RNS, reactive nitrogen species

Scheme 6

strange, but yeast can serve as a good model even for investigation of neurodegenerative diseases. The above-mentioned diseases frequently are related with induction of oxidative stress and incorrectly folded protein molecules [117-119]. Genetically modified yeast strains are more and more frequently used to study different pathologies.

One more problem where yeasts are used as model should be highlighted. The ROS-induced oxidation of proteins and nucleic acids leads to many consequences. It is interesting that oxidized forms of these components can also produce ROS. For example, so-called advanced glycation end products can be ROS generators [120, 121]. Similarly ROS-oxidized nucleic acids also can be ROS sources [31]. Due to the processes described above a closed circle is formed – free radicals modify proteins and nucleic acids (similarly other compounds), and further modified compounds enhance ROS production. A similar situation takes place in Alzheimer's disease in which neurodegenerative changes are supposed to be related with accumulation of a myeloid β-peptide via precursor oxidation and resulting in formation of assembles which become ROS sources [122]. Recently transformed yeasts were successfully used to investigate molecular mechanisms of this pathology [123, 124]. In conclusion, it is clear that yeasts are a convenient object to study molecular mechanisms of human pathologies and develop prophylactic and therapeutic approaches.

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