Manzanares-Estreder, S.; Espí Bardisa, J.; Pascual-Ahuir Giner, MD.; Proft ., MH. (2016). Multi-layered control of peroxisomal activity upon salt stress in Saccharomyces cerevisiae. Molecular Microbiology. 104(5):851-868. doi:10.1111/mmi.13669

molecular microbiology

Multi-layered control of peroxisomal activity upon salt stress in Saccharomyces cerevisiae

Journal:	*Molecular Microbiology*
Manuscript ID	MMI-2016-16241
Manuscript Type:	Research Article
Date Submitted by the Author:	19-Oct-2016
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Key Words:	peroxisome, Saccharomyces cerevisiae, Hog1, Adr1, retrograde signalling

Multi-layered control of peroxisomal activity upon salt stress in *Saccharomyces cerevisiae*

Running title: Adaptation of peroxisomal activity and number upon stress

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Key words: peroxisome, Hog1, Adr1, retrograde pathway, stress adaptation, yeast

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Abstract

Peroxisomes are dynamic organelles and the sole location for fatty acid β-oxidation in yeast cells. Here we report that peroxisomal function is crucial for the adaptation to salt stress, especially upon sugar limitation. Multiple layers of control regulate both peroxisomal activity and number upon stress. Activated Hog1 MAP kinase triggers the induction of genes encoding enzymes for fatty acid activation, peroxisomal import and β-oxidation through the Adr1 transcriptional activator, which transiently associates with genes encoding fatty acid metabolic enzymes in a stress- and Hog1-dependent manner. Moreover, Na^+ and Li^+ stress induces an increase in peroxisomal number per cell in a Hog1-independent manner, which depends instead of the retrograde pathway and the dynamin related GTPases Dnm1 and Vps1. The strong activation of the Faa1 fatty acyl-CoA synthetase, which specifically localizes to lipid particles and peroxisomes, indicates that adaptation to salt stress requires the enhanced mobilization of fatty acids from internal lipid stores. Furthermore, the activation of mitochondrial respiration during stress depends on peroxisomes, mitochondrial acetyl-carnitine uptake is essential for salt resistance, and the percentage of peroxisomes attached to the mitochondrial network increases during salt adaptation, which altogether indicates that stress-induced peroxisomal β-oxidation triggers enhanced respiration upon salt shock.

Introduction

Peroxisomes are ubiquitous, DNA-free organelles present in most eukaryotic cells. They play important roles in different catabolic processes including the fatty acid β-oxidation, which in yeast cells exclusively occurs in peroxisomes while in mammalian cells it is

carried out mainly in mitochondria. Yeast peroxisomes readily adapt their number and physiological function in response to changes in the metabolic state of the cell. Low peroxisomal numbers and activity are typically found in yeast cells grown in the presence of sugar excess. However, following the shift to a less or non-fermentable carbon source such as fatty acids, ethanol or amino acids, peroxisomal proliferation is rapidly activated (Sibirny, 2016). This metabolic up-regulation of peroxisomal function is coordinately regulated by different specific transcription factors, Oaf1/Pip2 and Adr1 (Turcotte *et al.*, 2010). The Oaf1/Pip2 transcriptional activator seems to act in an analogous manner to the nuclear hormone receptors of higher eukaryotes (Phelps *et al.*, 2006, Thakur *et al.*, 2009). Oaf1 is activated by direct binding to fatty acids such as oleate (Phelps et al., 2006) and drives gene expression together with Pip2 from ORE (oleate response element) promoter sites present in many genes related to peroxisomal proliferation and β-oxidation (Karpichev *et al.*, 1997, Rottensteiner *et al.*, 1997). The Adr1 zinc cluster protein binds to a different consensus motif found in the same gene promoters and activates their expression in response to a shortage of intracellular energy stores mediated by the AMP-activated protein kinase Snf1 (Ratnakumar *et al.*, 2009, Ratnakumar & Young, 2010). Here we characterize an alternative mode of peroxisomal activation in yeast, which is modulated by salt stress. This finding indicates that peroxisomal dynamics are intimately linked to cell physiology beyond purely metabolic stimuli.

Salt and hyperosmotic stress triggers a complex adaptive response in yeast cells, which is coordinated by the HOG (high osmolarity glycerol) MAP kinase pathway (Brewster *et al.*, 1993). Once activated by stress, the Hog1 MAP kinase activates different physiological responses, which altogether contribute to survive and eventually resume growth on high osmolarity media (Hohmann, 2002, Saito & Posas, 2012). Activated Hog1

modulates transporter functions at the plasma membrane (Proft & Struhl, 2004), adapts translation efficiency (Warringer *et al.*, 2010) and cell cycle progression (Duch *et al.*, 2012) to the stress conditions and is responsible for the transient reprogramming of gene expression in the nucleus (de Nadal & Posas, 2010). Salt stress causes the transcriptional activation of many genes, which are involved in different physiological aspects of the stress response (Posas *et al.*, 2000, Rep *et al.*, 2000, Ni *et al.*, 2009). Among others, the up-regulated proteins are necessary for the enhanced biosynthesis of the osmolyte glycerol (Hohmann *et al.*, 2007), for intracellular cation balance (Marquez & Serrano, 1996), for the maintenance of the redox balance and detoxification of reactive oxygen species (Rep *et al.*, 2001, Schuller *et al.*, 1994). On the other hand, a regulated repression of metabolic pathways can be beneficial for cell survival upon salt stress as has been demonstrated for ergosterol biosynthesis (Montanes *et al.*, 2011).

Salt stress also activates mitochondrial functions in yeast cells, which is of special importance here (Martinez-Pastor *et al.*, 2010, Pastor *et al.*, 2009). Mutants with defects of mitochondrial respiration, Krebs cycle or organelle biogenesis fail to efficiently adapt to high salinity, and the expression of genes involved in mitochondrial respiration is coordinately activated via the Hog1 and Snf1 protein kinases (Pastor et al., 2009). As a consequence, mitochondrial respiration is reinforced during salt stress and in the absence of this regulation, an overproduction of reactive oxygen species (ROS) is observed (Pastor et al., 2009). Specific adaptation mechanisms of respiration to salt stress have also been described for the uptake of pyruvate via the mitochondrial Mpc carrier (Bricker *et al.*, 2012, Herzig *et al.*, 2012, Timon-Gomez *et al.*, 2013). Here, stress induces the formation of the more efficient Mpc1/Mpc3 complex in order to sustain higher respiration rates (Timon-Gomez et al., 2013, Bender *et al.*, 2015). In the present work, we find that upon salt stress,

the most important metabolic route to supply Acetyl-CoA for mitochondrial respiration is the peroxisomal β-oxidation.

The dynamic control of peroxisomal number and size is being studied intensively in yeast cells (Sibirny, 2016). More than 30 different *PEX* genes, which have specific functions in peroxisome biogenesis, have been identified in yeast (Purdue & Lazarow, 2001). Wild type cells with pre-existing peroxisomes multiply the organelle predominantly by fission upon non-fermentative growth conditions. Specific dynamin-like proteins such as Vps1 or Dnm1 or the fission protein Fis1 have been implied in this form of peroxisomal duplication (Hoepfner *et al.*, 2001, Motley *et al.*, 2008). Peroxisomes can also be formed de novo from the endoplasmic reticulum (ER), which has been discovered by re-induction of peroxisome biogenesis in peroxisome null mutants such as *pex3* or *pex19* (Hoepfner *et al.*, 2005, Tam *et al.*, 2005). In this process, Pex3 and Pex19 play essential roles in the early formation of peroxisomal pre-structures at the ER (Agrawal & Subramani, 2016).

Here we report that peroxisomal number and fatty acid β-oxidation are dynamically regulated upon salt stress, where peroxisomes serve to promote activated mitochondrial respiration. MAP kinase dependent activation of the expression of genes involved in oxidative degradation of fatty acids and the stimulation of peroxisomal fission and biogenesis are the principal mechanisms of peroxisomal adaptation, which is physiologically relevant for salt tolerance.

Results

Peroxisomal function is required for salt stress adaptation especially upon sugar limitation

We have previously shown that yeast cells respond to salt stress by a partial induction of mitochondrial respiration. Therefore we investigated whether the balance between fermentation and respiration had an effect on the adaptation to salt stress in yeast wild type cells. We quantified the growth efficiency of yeast cultures upon NaCl stress while continuously lowering the glucose content. As shown in Figure 1A, efficient growth on high salinity media was dramatically affected upon low sugar availability and the efficiency dropped from >80% on high glucose (4%) to approximately 20% on low glucose (0.5%). A possible explanation for this dramatic effect was that salt stress caused glucose starvation, which in turn would make necessary the use of alternative energy sources upon stress. One important energy resource are intracellular lipid stores, which can be mobilized during sugar starvation by peroxisomal β-oxidation in yeast cells. Therefore we directly tested whether peroxisomal function was important for salt stress tolerance. We employed two types of yeast deletion mutants, either completely lacking peroxisomes (*pex3*, *pex19*) or lacking individual enzymatic conversions of the β-oxidation (*pox1*, *fox2*, *eci1*). As shown in Figure 1B, loss of peroxisomal structures caused a mild sensitivity to NaCl upon high glucose, which was severely aggravated by lowering the glucose concentration. Individual interruptions of peroxisomal β-oxidation did not cause a detectable salt sensitivity upon high glucose. However, the same mutants became clearly hypersensitive to salt stress on low glucose media. Taken together, these results indicated that peroxisomal function was growth limiting upon salt stress especially upon low sugar availability.

Transcriptional activation of genes involved in fatty acid metabolism depends on the Hog1 MAP kinase and the Adr1 zinc cluster protein upon salt stress

We next tested whether the expression of genes involved in the peroxisomal mobilization of fatty acids was regulated upon salt stress. Representative gene functions were chosen to cover fatty acid activation (*FAA1*), fatty acyl-CoA import into peroxisomes (*PXA1*, *2*), β-oxidation (*POX1*, *ECI1*, *FOX2*) and acetyl-carnitine synthesis (*CAT2*) (Figure 2A). We found that, with the exception of the *FOX2* gene, the expression of all genes was rapidly and transiently induced upon NaCl shock, comparable to the osmostress inducible marker gene *GRE2* (Figure 2B). The transcriptional control occurred faster but less efficiently as compared to the induction during the shift from glucose to oleate metabolism (Figure 2B). The HOG MAP kinase pathway is the master regulator of gene expression upon osmostress in yeast cells. We confirmed that the Hog1 MAP kinase was indispensable for the transcriptional activation of genes involved in the mobilization of fatty acids (Figure 3A). Surprisingly we found that Hog1 was also responsible for a great part of the induction of the same genes upon oleate growth, indicating that Hog1 function was not restricted to salt stress at the genes related to fatty acid metabolism (Figure 3A).

We wanted to further characterize the salt inducible expression of genes related to fatty acid degradation and asked whether it occurred independently of the activation caused by non-fermentable growth. Therefore we compared the salt induced expression profiles of representative genes (*PXA2*, *POX1*, *ECI1*) in fermentative glucose repressed and non-fermentative glycerol/ethanol cultures. Expectedly, the steady state amount of mRNA for the respective genes is 20 to 200 fold higher in actively respiring yeast cells (Figure 3B). However, the expression of all three genes was readily activated upon NaCl shock, which

indicated that salt induction of genes related to peroxisomal metabolism occurs independently of the induction during the diauxic shift.

Next we wanted to shed light onto the molecular events underlying the transcriptional control of peroxisomal functions by salt stress. There are two known transcriptional activators acting on gene promoters of peroxisomal functions, Oaf1/Pip2 and Adr1. In order to discern their possible function in salt induction, we quantified the induction profile of representative peroxisomal genes in *oaf1* and *adr1* deletion mutants during salt shock. As shown in Figure 4A, with the exception of the *ECI1* gene, stress-induced activation was strongly diminished in the *adr1* mutant strain. We therefore concluded that the Adr1 zinc cluster protein is the responsible factor for salt inducible expression of peroxisomal genes. We finally followed Adr1 binding to selected peroxisomal gene promoters upon salt stress by in vivo ChIP. We observed a transient peak of Adr1 association to the *POX1*, *FAA1*, *PXA2* and *ECI1* promoters after NaCl treatment. Moreover, Adr1 recruitment was strongly reduced by deletion of Hog1. Therefore we conclude that Hog1 positively controls gene expression of peroxisomal functions by stimulating the binding of the Adr1 transcriptional activator.

Salt stress specifically induces the expression of Faa1, a fatty acyl-CoA synthetase localized to lipid particles and peroxisomes

The induction of genes related to the peroxisomal fatty acid metabolism suggested that salt stress adaptation implied the enhanced degradation of fatty acids. In order to clarify the origin of fatty acids which could be mobilized upon stress, we investigated the regulation of fatty acyl-CoA synthetases, which catalyze the first enzymatic step in the oxidative degradation of fatty acids. Budding yeast has five different fatty acyl-CoA synthetases,

Faa1-4 and Fat1. We quantified the expression of the five corresponding genes upon salt shock and found that *FAA1* was the most inducible gene under these conditions (Figure 5A). Interestingly, although still inducible, Faa1 is not the most activated fatty acyl-CoA synthetase upon oleate growth. Here, Faa2 is the far most inducible isoenzyme, which in turn is not induced upon salt stress. Taken together, salt stress and oleate growth induce different patterns of fatty acyl-CoA synthetase genes, with Faa1 being specific for salt stress.

We next determined the intracellular localization of Faa1. As shown in Figure 5B, Faa1 colocalized strictly with internal lipid particles and with most, but not all, peroxisomes. These data suggested that salt stress specifically induced the expression of the Faa1 isoenzyme to increase the mobilization of fatty acids from internal lipid storage particles.

Salt stress induces peroxisomal number in a Hog1 independent, but Rtg-, Dnm1- and Vps1-dependent manner

Having seen that salt stress rapidly induces the expression of genes encoding peroxisomal enzymes involved in β-oxidation, we wanted to assay possible stress-induced changes in peroxisomal abundance at the cellular level. We therefore visualized individual peroxisomes in yeast cells by the expression of GFP fused to the peroxisomal target sequence PTS1. As shown in Figure 6A, a clear increase in peroxisomal number during NaCl stress can be observed. This phenotype is observed upon Na^+ and Li^+ stress and is not seen upon general osmotic stress caused by sorbitol or by K^+ excess (Figure 6B). We could furthermore confirm that the increase of peroxisomal number upon salt stress occurred independently of Hog1 (Figure 6A). We next quantified the Na^+ and Li^+ induced

peroxisomal number in mutants affected in other signaling pathways related to peroxisomal function, namely the retrograde pathway (*rtg1*, *rtg2*), or in mutants lacking proteins involved in peroxisomal fission (*fis1*, *dnm1*, *vps1*). As shown in Figure 6C, the increase of peroxisome number upon Na^+ and Li^+ shock was not observed in yeast cells with an interrupted retrograde pathway. Additionally, Fis1 was dispensable for peroxisomal up-regulation, while the dynamin related GTPases Dnm1 and Vps1 were necessary to increase peroxisomal number upon stress. We conclude that salt stress increases the number of peroxisomes per yeast cell via signaling through the retrograde pathway and via specific GTPases necessary for peroxisomal fission.

We next explored whether the long term adaptation to salt stress could also involve the de novo synthesis of peroxisomes additionally to peroxisomal fission. We therefore measured the regulation of the *PEX3* gene, which is involved in the early events of peroxisomal biogenesis from the ER. We found that *PEX3* expression is several fold induced upon NaCl stress (Figure 6D). Furthermore, this up-regulation depends on the HOG and Rtg signaling routes since it is not observed in *hog1* and *rtg1* mutants. This regulation was different from the *ECI1* gene involved in β-oxidation, which was Hog1 dependent but Rtg1 independent (Figure 6D).

The function of peroxisomes in the salt stress induced activation of mitochondrial respiration

The above described results indicated that a genetic control of enzymes involved in the oxidative degradation of fatty acids together with the activation of peroxisomal fission and biogenesis reinforces β-oxidation in yeast cells as an important adaptation mechanism upon

salt stress. We have previously found that mitochondrial functions are partially activated under the same stress conditions. Therefore we wanted to clarify whether the up-regulation of peroxisomal function during stress serves to induce mitochondrial respiration. In yeast, there are two principal ways to introduce Acetyl-CoA, the primary substrate of the Krebs cycle, into mitochondria upon conditions, which require high respiration rates (Figure 7A). The first is the uptake of the glycolytic intermediate pyruvate via the Mpc1/Mpc3 carrier and the second is the uptake of Acetyl-carnitine derived from peroxisomal β-oxidation via the Crc1 carrier. The importance of both mitochondrial carriers for salt resistance and non fermentable growth was assayed using the *crc1* and *mpc1* deletion mutants. As shown in Figure 7B, cells lacking the mitochondrial carnitine carrier were hypersensitive to NaCl stress, especially upon glucose limitation. Mitochondrial pyruvate import was dispensable under the same stress conditions. In turn, the Mpc1 carrier was essential to sustain growth of yeast cells upon fully respiratory conditions, whereas Crc1 was not required (Figure 7B). These data indicated that Acetyl-carnitine import into mitochondria was essential for efficient salt stress adaptation, presumably by activating respiration. We tested this hypothesis by directly measuring the activity of mitochondrial electron transport through complex II, the succinate dehydrogenase (SDH) complex. SDH activity is activated upon salt stress and we tested whether this up-regulation was dependent on peroxisomal function. As shown in Figure 7C, SDH activation was impaired in the peroxisomal null mutants *pex3* and *pex19* as compared to the wild type. These results indicated that peroxisomal function was responsible for reinforced mitochondrial respiration upon salt stress. We finally investigated the physical association of peroxisomes with the mitochondrial network and asked whether any regulation occurred at this level. We used a yeast strain, which contained a peroxisomal GFP marker (GFP-PTS1) and a mitochondrial dsRed marker

(Om14-dsRed). As shown in Figure 7D, this fluorescent indicator strain was suitable to visualize both organelles in living yeast cells in order to determine the percentage of peroxisomes attached to the mitochondria. Flattened z-stack images of individual cells were analyzed for peroxisome-mitochondria colocalization. We found that peroxisomes tend to colocalize more with mitochondria in NaCl treated cells, so that during salt adaptation not only increases the absolute number of peroxisomes but also the percentage of peroxisomes, that are physically connected to mitochondria.

Discussion

Here we identify an environmental stress condition, which triggers a coordinated peroxisomal adaptation and we further show that this adaptation strategy contributes to the stress tolerance of yeast cells. This is important because it shows that peroxisomal dynamics are not exclusively regulated by nutritional signals and thus reveals insights into how environmental cues are connected to peroxisomal proliferation, which is still a poorly explored field of investigation (Smith & Aitchison, 2013). Interestingly, salt and other abiotic stresses lead to an increase in peroxisomal number and activity also in higher plants and a role of peroxisomes in ROS homeostasis has been postulated (Hu *et al.*, 2012, Mitsuya *et al.*, 2010, Sandalio & Romero-Puertas, 2015). However, the molecular mechanisms, which trigger peroxisomal dynamics in plants upon salt stress, are not known.

The dissection of signaling mechanisms which lead to the up-regulation of yeast peroxisomes upon high salinity has identified several layers of control as presented here. The stress-activated protein kinase Hog1 is the upstream regulator, which controls the expression of genes directly involved in fatty acid mobilization and peroxisomal β-oxidation. This adds a new and important physiological function for Hog1, which has been

already implied in many diverse cellular adaptations upon osmotic stress (Martinez-Montanes *et al.*, 2010). In the case of peroxisomal gene expression, Hog1 triggers transcriptional activation through the specific transcription factor Adr1, whose binding to peroxisomal gene promoters is stimulated by the MAP kinase. Our results indicate that the alternative peroxisomal activator Oaf1 is not involved in salt stress signaling. This might reflect a more specialized function of Oaf1 in situations when yeast cells have to metabolize fatty acids in the growth medium as the sole energy source. Indeed, Oaf1 is activated by direct binding of fatty acids such as oleate (Phelps et al., 2006).

Hog1 is known to directly regulate the activity of several transcription factors, such as Sko1, Hot1, Smp1, Rtg1 or Msn2 (Alepuz *et al.*, 2003, de Nadal *et al.*, 2003, Proft *et al.*, 2001, Ruiz-Roig *et al.*, 2012, Vendrell *et al.*, 2011). Whether Hog1 also regulates Adr1 by direct phosphorylation remains to be shown. Of note, Adr1 activity is additionally modulated by the Snf1 AMP activated protein kinase upon energy depletion, most likely by an indirect mechanism (Ratnakumar et al., 2009, Ratnakumar & Young, 2010). Therefore the HOG signal transduction pathway, which primarily responds to changes in the osmolarity of the cells environment, and glucose derepression, which activates Snf1 primarily upon ATP depletion, coincide at the Adr1 activator to stimulate fatty acid metabolism. Of note, both signaling pathways have been shown to interact functionally. Snf1 activity increases upon salt stress, although it is not clear whether this occurs directly or indirectly due to stress-induced energy starvation (Hong & Carlson, 2007). On the other hand, Hog1 is activated also upon glucose starvation, which seems to promote Hog1 phosphorylation indirectly through Snf1 (Piao *et al.*, 2012). Activation of the HOG pathway by sugar limitation could explain our finding that Hog1 plays an important role in

the transcriptional control of peroxisomal genes also upon the shift from glucose to oleate growth.

Another layer of peroxisomal control upon stress occurs at the level of the number of the organelle. Here, stimulated fission of the existing peroxisomes seems to roughly double the number of the organelle during the first hours of salt stress adaptation. The dynamin-related GTPases Dnm1 and Vps1 are critical in this response together with the retrograde signaling pathway. The fact that both upstream regulators (Rtg2) and downstream transcription factors (Rtg1/3) are required for stimulated peroxisomal bigenesis suggests that also in this case regulation is based on the control of gene expression. The retrograde signaling pathway is activated upon mitochondrial dysfunction (Liu & Butow, 2006), however it is functionally linked to osmostress signaling, since Hog1 directly controls the activity of the Rtg1 and Rtg3 transcriptional activators (Ruiz-Roig et al., 2012). Additionally, the retrograde pathway induces many peroxisomal genes upon mitochondrial damage (Epstein *et al.*, 2001). Our results further confirm this functional overlap and demonstrate that retrograde signaling activates peroxisomal fission upon salt stress.

Our results suggest that during the defense to salt stress, yeast cells switch from a fermentative metabolism to fatty acid oxidation to cover the energetic needs during the environmental challenge. Efficient biomass production upon salt stress depends very much on high sugar concentrations in the growth medium, and peroxisomal fatty acid oxidation becomes rate limiting upon sugar exhaustion. Therefore, salt stress might demand a high glycolytic flux for proper adaptation or might interfere with the glycolytic degradation of sugars. There are indications for both explications. High salinity induces glycerol as the major osmoprotectant of yeast cells (Hohmann, 2015), which deviates glycolytic

intermediates. Consequently, less efficiently fermentable carbon sources strongly interfere with glycerol biosynthesis and salt stress adaptation and resistance (Vanacloig-Pedros *et al.*, 2015). On the other hand, moderate NaCl concentrations significantly interfere with the sugar uptake in yeast cultures (Wei *et al.*, 1982), and sugar transporter genes are among the most highly induced genes upon salt stress (Posas et al., 2000, Rep et al., 2000). As a consequence, peroxisomal metabolism has to be rapidly reinforced, which starts with the mobilization of fatty acids from lipid droplets through the inducible Faa1 fatty acyl-coA synthetase. It is worth noting that apart from the here reported induction via Adr1, *FAA1* is additionally targeted by the Hog1-dependent Sko1 transcription factor (Proft *et al.*, 2005). Stimulated Acetyl-coA production in the peroxisomes is then destined to sustain high respiration rates at mitochondria. Introduction of Acetyl-carnitine is actually the rate limiting step during salt adaptation, which is further corroborated by the fact that expression of the mitochondrial carnitine carrier is highly inducible upon salt stress (Timon-Gomez et al., 2013). Peroxisomes can physically interact with mitochondria in yeast (Mattiazzi Usaj *et al.*, 2015, Shai *et al.*, 2016) and in fact the percentage of colocalization might reflect to what degree the peroxisomal metabolism is directly coupled to mitochondrial respiration. Here we show a tendency of peroxisomes to associate more efficiently with the mitochondrial network upon stress, which ultimately might indicate the need of a tighter coupling of peroxisomal β-oxidation with mitochondria during acute salt stress. Taking together, our study demonstrates that environmental stress adaptation involves the dynamic modulation of peroxisomal activity and biogenesis in order to reshape the cellular energy metabolism.

Experimental Procedures

Yeast strains

All strains used in this study are listed in table 1. Adr1 was tagged with 3xHA at the chromosomal locus according to (De Antoni & Gallwitz, 2000).

Growth conditions and sensitivity assays

Yeast cultures were grown in yeast extract-peptone containing 2% dextrose or oleate media. As indicated, NaCl, KCl, LiCl or sorbitol were added to the cultures from appropriate stock solutions. For salt tolerance assays upon glucose limitation, the indicated mixtures of dextrose and ethanol were applied in yeast extract-peptone media. Synthetic growth medium contained 0.67% yeast nitrogen base, 50mM succinic acid pH 5.5, and 2% dextrose. According to the auxotrophies of each strain, methionine (10 mg/l), histidine (10 mg/l), leucine (10 mg/l) or uracil (25 mg/l) were added. For sensitivity assays on solid media, fresh overnight cultures were adjusted to the same OD, and serial dilutions were spotted on the indicated agar media.

Plasmid constructions

For visualization of peroxisomes in yeast cells, a pTPI1-GFP-PTS1 fusion plasmid was employed (Motley et al., 2008). Constitutive expression of Faa1- and Om14-dsRed fusion proteins was achieved by cloning the respective ORF regions into the yeast Gateway plasmid pAG423-GPD-ccdB-dsRed (Alberti *et al.*, 2007).

Chromatin Immunoprecipitation

ChIP was performed as described previously (Kuras & Struhl, 1999). Quantitative PCR analyses at the *POX1* (-286/-211), *FAA1* (-320/-217), *PXA2* (-309/-207) and *ECI1* (-278/-195) promoter regions were performed in real time using an Applied Biosystems 7500 sequence detector with the *POL1* (+1796/+1996) coding sequence as an internal control.

Each immunoprecipitation was performed on three independent chromatin samples. All occupancy data are presented as fold IP efficiency over the *POL1* control sequence. All primer sequences are available upon request.

Continuous growth assays

For sensitivity assays in continuous growth fresh over night precultures of the indicated strains were diluted in triplicate in multiwell plates to the same initial OD. Growth was then constantly monitored in a Bioscreen C system (Thermo) for the indicated times. The growth curves were analyzed in Microsoft Excel and the maximal cell density determined. The growth efficiency was calculated as the percentage of the maximal cell density reached under the different stress conditions compared to the control (without NaCl). Three independent cultures were analyzed for each stress treatment.

RT-PCR analysis

Total RNA was isolated by acid phenol extraction from yeast cells grown in the indicated conditions. Total RNA samples were digested with DNaseI and purified with the RNeasy Mini kit (Qiagen). A total of 5 mg of RNA was converted into DNA using the Superscript III first strand cDNA synthesis kit (Invitrogen). The amount of DNA was determined with the indicated gene specific primers by quantitative PCR in real time using the EvaGreen qPCR Master Mix (Biotium) on an Applied Biosystems 7500 sequence detection system. The *ACT1* gene was used as a reference. Relative expression levels were calculated in triplicate from three independent cDNA samples.

Fluorescence Microscopy

Lipid droplets were stained with Bodipy 493/503 in living cells according to (Wolinski & Kohlwein, 2008). Cells were observed on a Leica confocal microscope TCS SP8 using the

following excitation and emission wavelengths: GFP (excitation 488nm; emission 509nm), Bodipy (excitation 493nm; emission 503nm), dsRed (excitation 545nm; emission 572nm).

Succinate Dehydrogenase (SDH) Assay

SDH assays were performed in yeast whole cell extracts with p-Iodonitrotetrazolium violet (INT) as an artificial electron acceptor for the SDH complex. Extracts were incubated in 300µl succinate buffer (10mM succinic acid hexahydrate in 50mM phosphate buffer pH 7.4) with 100µl INT solution (2.5mg INT in 50mM phosphate buffer pH 7.4). Reactions were stopped with 1ml Stop solution (10g Trichloroacetic acid in 100ml Ethylacetate/Ethanol (1vol:1vol)) and the absorbance was measured in the supernatant at 490 nm. Three independent cultures were analyzed in duplicate.

Acknowledgments

We thank E. Hettema for the kind gift of GFP-PTS1 expressing yeast strains. This work was supported only in the initial phase by a grant from Ministerio de Economía y Competitividad (BFU2011-23326). The authors declare no conflict of interest. MP and AP-A conceived and designed the study, SM-E, JE-B, MP and AP-A acquired, analyzed and interpreted data, MP wrote the manuscript.

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Figure legends

Fig. 1. Peroxisomal function is crucial for salt stress adaptation especially upon glucose limitation.

A. Yeast growth efficiency upon NaCl stress is highly dependent on glucose availability. The growth of yeast wild type cells (BY4741) was continuously monitored in YPD medium with the indicated concentration of glucose, either without NaCl or with the addition of 1M NaCl (upper panel). The growth efficiency (lower panel) was calculated as indicated in the *Experimental procedures.* Three independent cultures were analyzed for each growth condition. Mean values are depicted with the standard deviation.

B. Peroxisomal function is important for salt stress tolerance especially upon glucose limitation. The growth of wild type and the indicated deletion strains was assayed on YP agar plates containing the indicated concentrations of glucose and/or ethanol. Salt stress was induced by the addition of 1M NaCl.

Fig. 2. Transcriptional control of the peroxisomal β-oxidation pathway upon salt stress.

A. Representative genes involved in fatty acid activation, peroxisomal import and β-oxidation were analyzed.

B. Transcriptional induction of fatty acid metabolism genes upon NaCl shock. The mRNA levels of the indicated genes involved in fatty acid degradation were determined by RT-PCR together with the salt stress marker *GRE2* during the first 60min of adaptation to 0.4M NaCl in yeast wild type strain BY4741. Results shown are the mean values from three biological replicates including the standard deviation.

C. The mRNA levels of the same genes as in (B) were analyzed during the first 3 hours of transition from glucose to oleate medium.

Fig. 3. Transcriptional control of genes involved in peroxisomal function by the Hog1 MAP kinase.

A. NaCl and oleate induced transcription of peroxisomal genes depends on Hog1. The expression of the indicated genes upon salt shock (0.4M NaCl, upper panel) or upon oleate growth was quantified by RT-PCR analysis and normalized to the *ACT1* gene. Wild type expression levels are compared to the *hog1* mutant. Results shown are the mean values from three biological replicates including the standard deviation. The uninduced mRNA level at time point 0 was arbitrarily set to 1.

B. Salt induction of peroxisomal gene expression is independent of the induction during the diauxic shift. The expression levels of the indicated genes was determined as in (A) in

glucose repressed cells (upper panel) or glycerol grown cells (lower panel). The uninduced mRNA level at time point 0 of the glucose repressed cells was arbitrarily set to 1.

Fig. 4. Hog1 induces peroxisomal gene expression via the transcriptional activator Adr1.

A. The Adr1 zinc cluster protein is responsible for salt stress induction of genes involved in fatty acid metabolism. The mRNA levels of the indicated genes was determined by RT-PCR in the *adr1* and *oaf1* mutant strains upon salt shock (0.4M NaCl). Results shown are the mean values from three biological replicates including the standard deviation. The uninduced mRNA level at time point 0 was arbitrarily set to 1 for each gene.

B. Hog1 stimulates the association of Adr1 with genes involved in fatty acid activation and β-oxidation. Adr1-HA occupancy was determined by ChIP in vivo at the *POX1* (-486/-329), *FAA1* (-301/-121), *PXA2* (-492/-226), and *ECI1* (-251/-114) promoters in YPD or at the indicated times of salt stress (0.4M NaCl) in wild type or *hog1* mutant cells. The no-tag wild type strain is included as a control. The relative occupancy is given as the fold IP efficiency over the *POL1* control region. The results shown are mean values from three biological replicates including the standard deviation.

FIG. 5. Faa1 is a salt inducible fatty acyl-coA synthetase located at lipid particles and peroxisomes.

A. The expression of the five structural genes encoding fatty acyl-coA synthetases (*FAA1-FAA4* and *FAT1*) was quantified by RT-PCR in yeast wild type cells upon salt shock (0.4M NaCl) or oleate growth. Results shown are the mean values from three biological replicates including the standard deviation. The uninduced mRNA level at time point 0 was arbitrarily set to 1 for each gene.

B. Faa1 localizes to lipid particles and peroxisomes. Colocalization of Faa1-dsRed with peroxisomal GFP (GFP-PTS1) is shown in the upper panel. Colocalization of Faa1 with lipid particles in the lower panel was performed by Bodipy 493/503 staining of Faa1-dsRed expressing yeast cells.

FIG. 6. Peroxisomal number per cell is stimulated by salt stress dependent on the Retrograde pathway and specific dynamic related GTPases.

A. Na^+ and Li^+ increase the abundance of peroxisomal structures independently of Hog1. Peroxisomes were visualized by GFP-PTS1 expression in yeast wild type and *hog1* mutant cells under normal growth conditions or after a brief shock (2h and 4h) with 0.8M NaCl or 0.2M LiCl. Representative images are shown at the left. Flattened z-stack images were acquired for 50 cells under each growth conditions to determine the average number of peroxisomal structures per cell given in the graph at the right including the standard deviation.

B. Induction of peroxisomal number is cation specific. The average number of peroxisomes per cell was determined as in (A) upon normal growth and after 2h of exposure to 0.8M KCl, sorbitol and NaCl or 0.2M LiCl.

C. The retrograde pathway and the Dnm1 and Vps1 fission proteins are required for peroxisomal number increase upon salt stress. GFP-PTS1 was expressed in the indicated yeast deletion strains and the number of peroxisomal structures per cell was determined as in (A). Cells (n = 50 in each case) were grouped with respect to the number of peroxisomes. Deletion of Vps1 causes a general reduction in peroxisomal number and therefore the group distribution was changed in the last graph.

D. Hog1 and Rtg1 are necessary for *PEX3* induction upon salt stress. The expression of the *PEX3* and *ECI1* genes was quantified by RT-PCR in yeast wild type, *hog1* and *rtg1* mutant cells upon salt shock (0.4M NaCl). Results shown are the mean values from three biological replicates including the standard deviation. The uninduced mRNA level at time point 0 was arbitrarily set to 1 for each gene and strain.

Fig. 7. Enhanced supply of peroxisomal Acetyl-CoA is necessary to sustain mitochondrial respiration upon salt stress.

A. Schematic overview of the different sources of Acetyl-CoA for the mitochondrial Krebs cycle in yeast.

B. The mitochondrial carnitine carrier is important for salt stress adaptation, while the mitochondrial pyruvate carrier is essential for respiratory growth. Growth of the indicated yeast strains was assayed upon high glucose or low glucose media with or without addition of 1M NaCl (upper two panels) and on respiratory medium containing glycerol/ethanol.

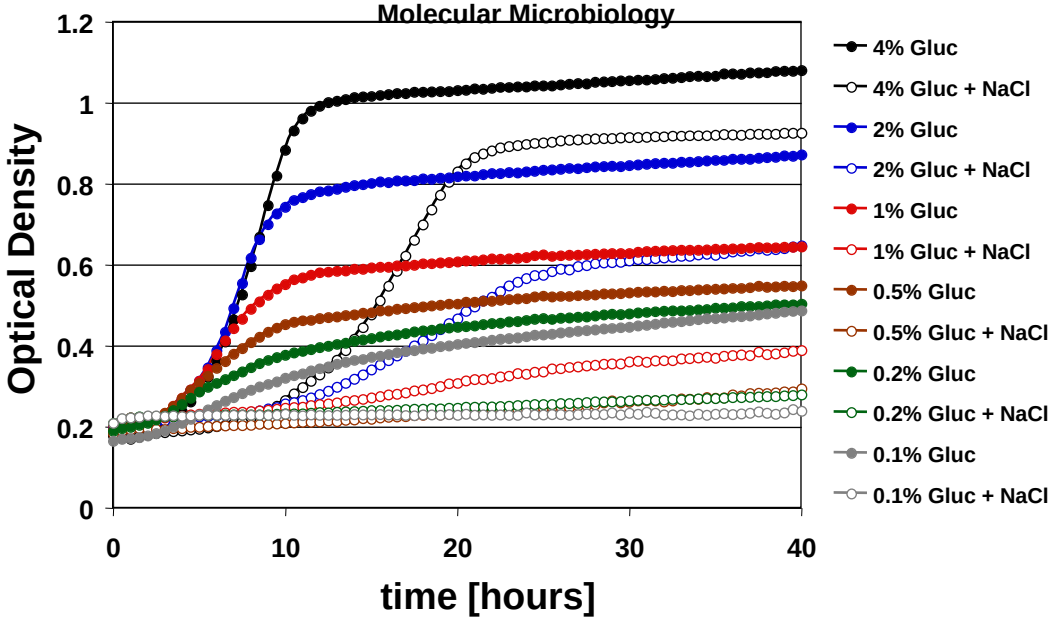
C. Salt induction of mitochondrial respiration depends on functional peroxisomes. The specific activity of succinate dehydrogenase (SDH) was determined in yeast wild type and the *pex3* and *pex19* deletion strains before and after the exposure to 0.4M NaCl for 2 hours. Enzyme activity was determined in duplicate in three independent cultures.

D. Association of peroxisomes with the mitochondrial network during salt stress adaptation. Yeast wild type cells expressing GFP-PTS1 (peroxisomal marker) and Om14-dsRed (mitochondrial marker) were analyzed by fluorescent microscopy upon normal growth or in high salinity medium (1M NaCl). Representative images are shown in the upper panel. Flattened z-stack images were acquired for 50 cells under each growth conditions to determine the degree of colocalization of both organelles.

Table 1. Strains used in this study.

Strain	Genotype	Source
BY4741	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	EUROSCARF
BY4741*pex3*	BY4741 *pex3::KanMX4*	EUROSCARF
BY4741*pex19*	BY4741 *pex19::KanMX4*	EUROSCARF
BY4741*pox1*	BY4741 *pox1::KanMX4*	EUROSCARF
BY4741*fox2*	BY4741 *fox2::KanMX4*	EUROSCARF
BY4741*eci1*	BY4741 *eci1::KanMX4*	EUROSCARF
BY4741*hog1*	BY4741 *hog1::KanMX4*	EUROSCARF
BY4741*oaf1*	BY4741 *oaf1::KanMX4*	EUROSCARF
BY4741*adr1*	BY4741 *adr1::KanMX4*	EUROSCARF
BY4741*rtg1*	BY4741 *rtg1::KanMX4*	EUROSCARF
BY4741*crc1*	BY4741 *crc1::KanMX4*	EUROSCARF
BY4741*mpc1*	BY4741 *mpc1::KanMX4*	EUROSCARF
Wt ADR1-HA	BY4741 *ADR1-3xHA::KAN MX*	This study
hog1 ADR1-HA	BY4741 *ADR1-3xHA::loxp hog1::KAN MX*	This study
Wt GFP$^+$-PTS1	BY4742 *can1::GFP$^+$-PTS1*	E. Hettema
Wt GFP$^+$-PTS1, Faa1-dsRed	BY4742 *can1::GFP$^+$-PTS1* with plasmid pAG423-GPD-FAA1-dsRed (HIS3)	This study
Wt GFP-PTS1	BY4741 with plasmid pTPI1-GFP-PTS1 (URA3)	This study
Wt Faa1-dsRed	BY4741 with plasmid pAG423-GPD-FAA1-dsRed (HIS3)	This study
hog1 GFP-PTS1	BY4741 *hog1::KAN* with plasmid pTPI1-GFP-PTS1 (URA3)	This study
rtg1 GFP-PTS1	BY4741 *rtg1::KAN* with plasmid pTPI1-GFP-PTS1 (URA3)	This study
rtg2 GFP-PTS1	BY4741 *rtg2::KAN* with plasmid pTPI1-GFP-PTS1 (URA3)	This study
fis1 GFP-PTS1	BY4741 *fis1::KAN* with plasmid pTPI1-GFP-PTS1 (URA3)	This study
dnm1 GFP-PTS1	BY4741 *dnm1::KAN* with plasmid pTPI1-GFP-PTS1 (URA3)	This study
vps1 GFP-PTS1	BY4741 *vps1::KAN* with plasmid pTPI1-GFP-PTS1 (URA3)	This study
BY GFP-PTS, Om14-dsRed	BY4741 with plasmids pTPI1-GFP-PTS1 (URA3) and pAG423-GPD-OM14-dsRed (HIS3)	This study

A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0.6
0.4
0.2
0
0
10
20
30
40
time [hours]
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4% Gluc + NaCl
2% Gluc
2% Gluc + NaCl
1% Gluc
1% Gluc + NaCl
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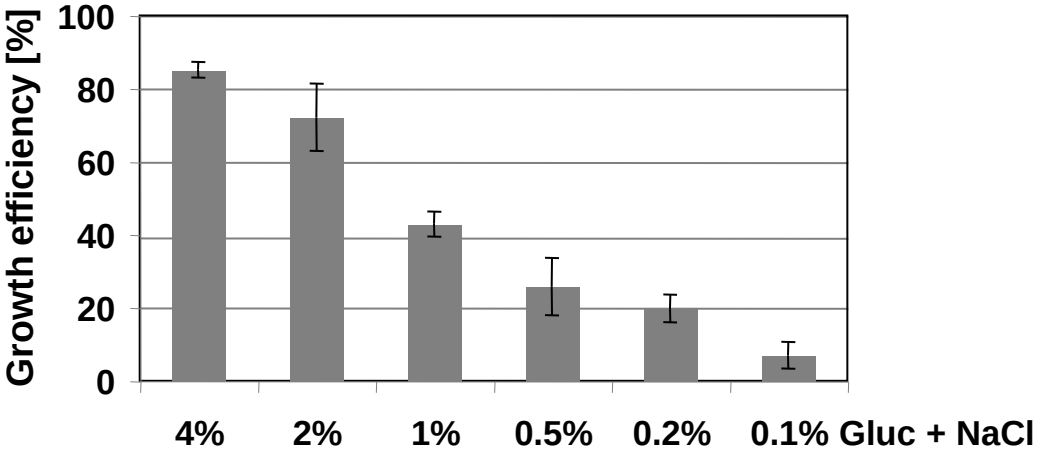
0.5% Gluc + NaCl
0.2% Gluc
0.2% Gluc + NaCl
0.1% Gluc
0.1% Gluc + NaCl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2%
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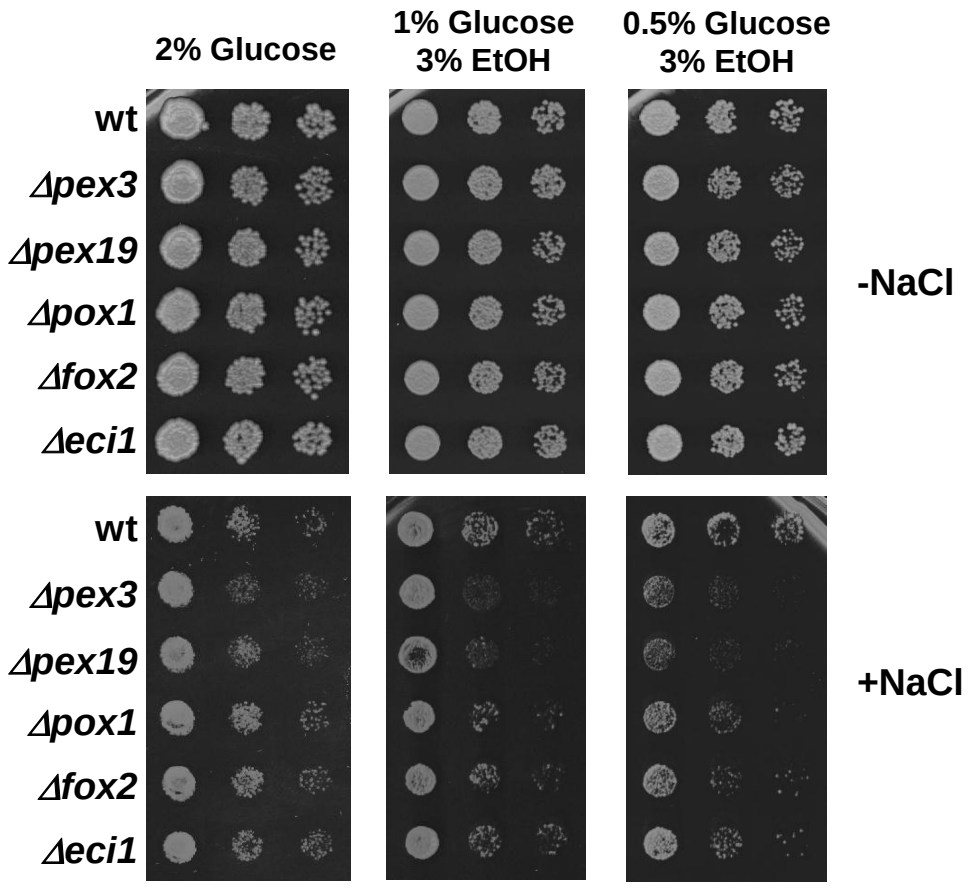
0.2%
0.1% Gluc + NaCl

B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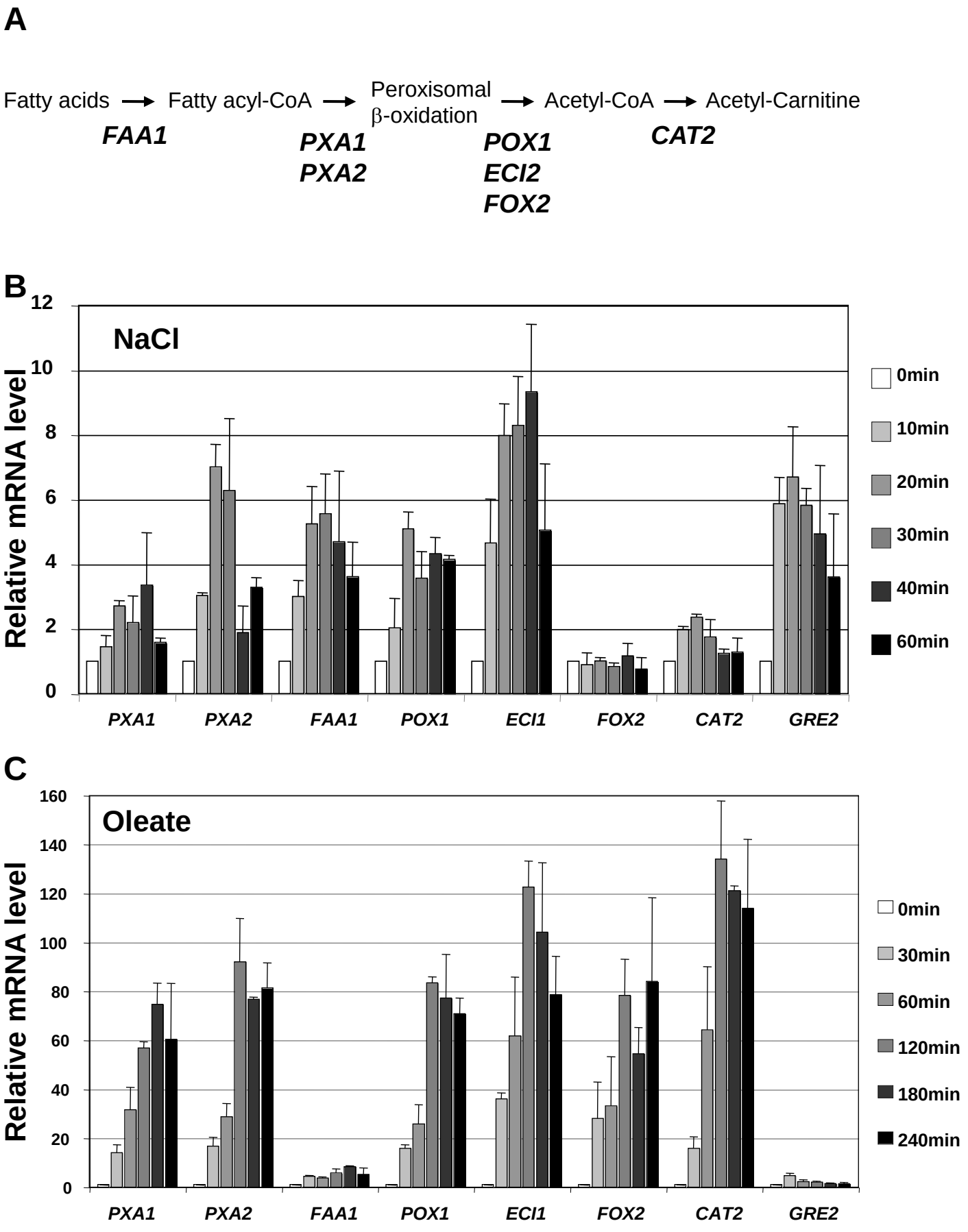
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Fatty acyl-CoA
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Acetyl-Carnitine
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PXA1
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POX1
ECI2
FOX2
CAT2
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POX1
ECI1
FOX2
CAT2
GRE2
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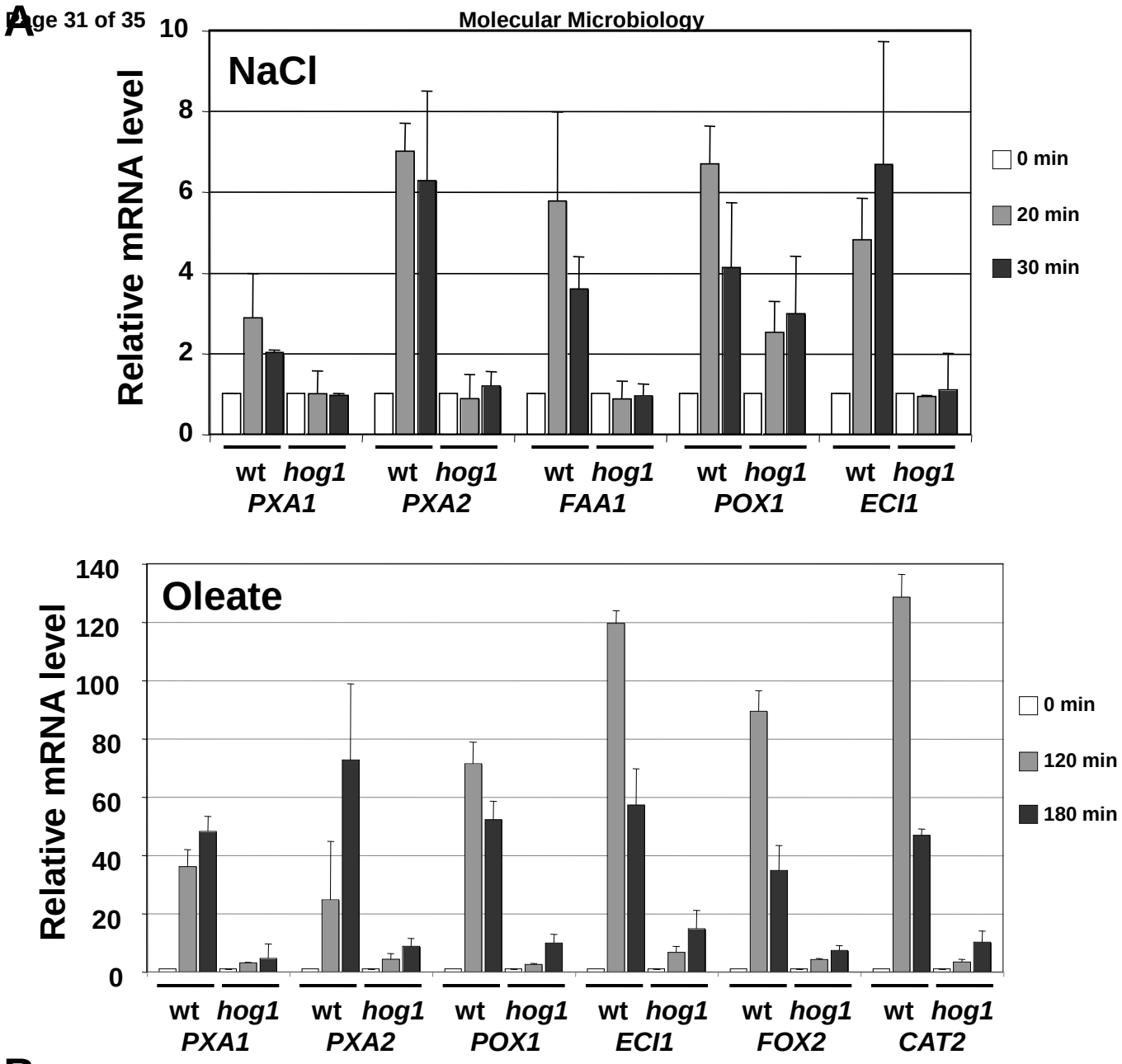
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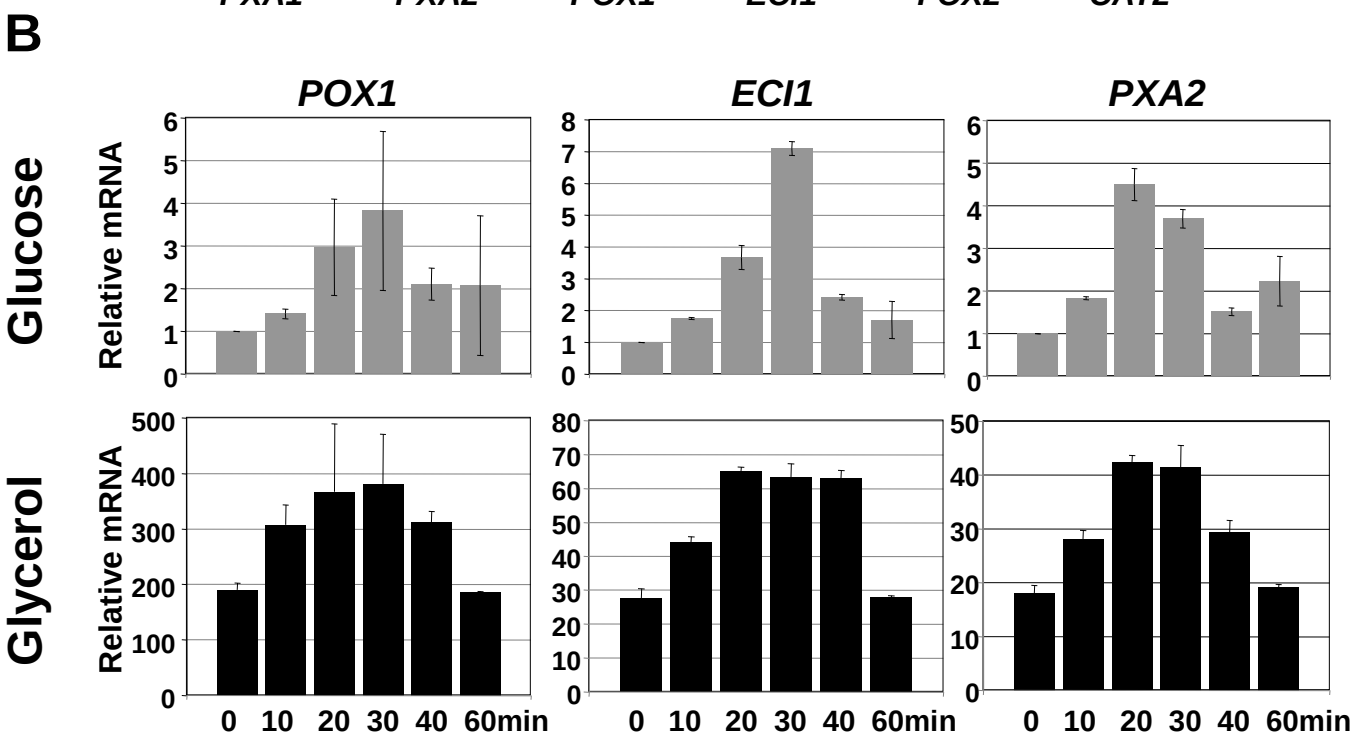
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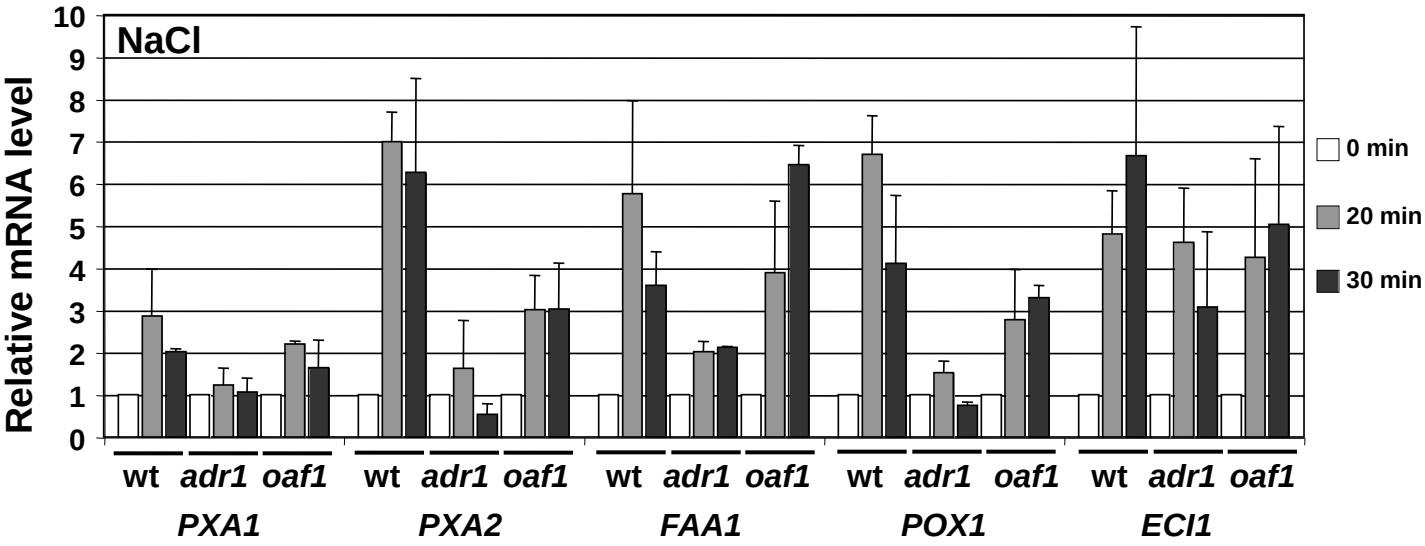
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POX1
ECI1

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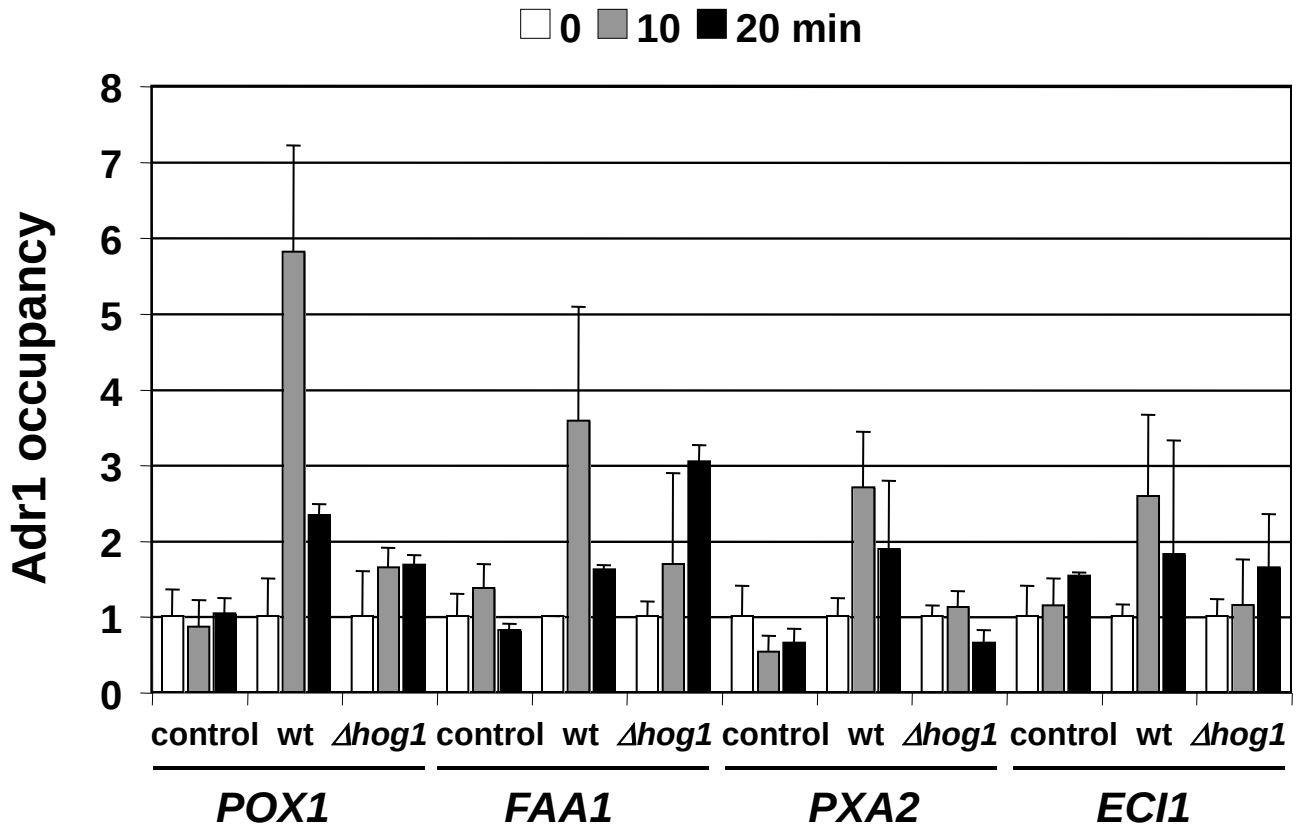
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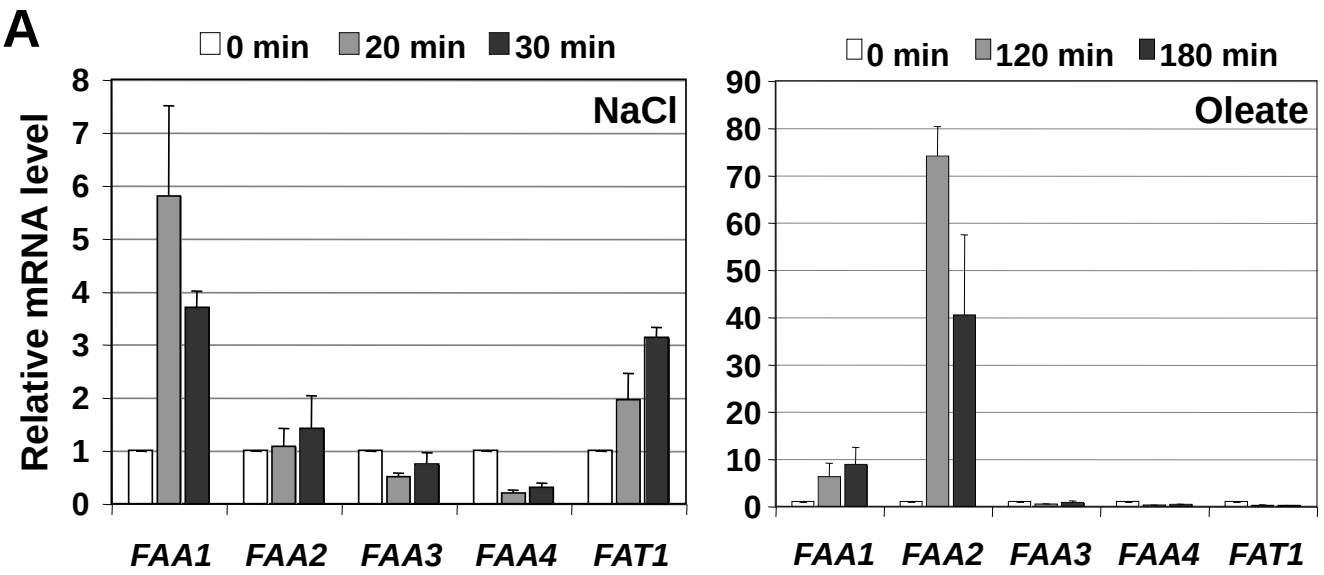
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FAA4
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180 min
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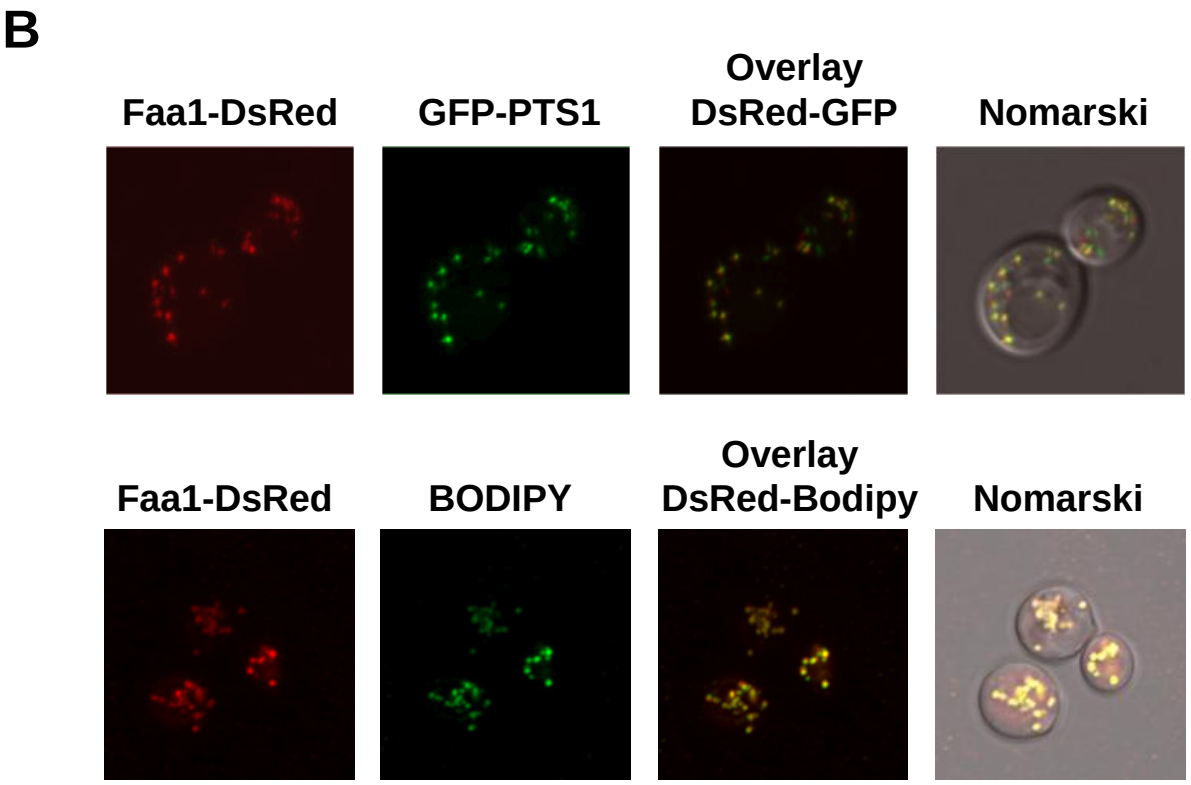
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Nomarski
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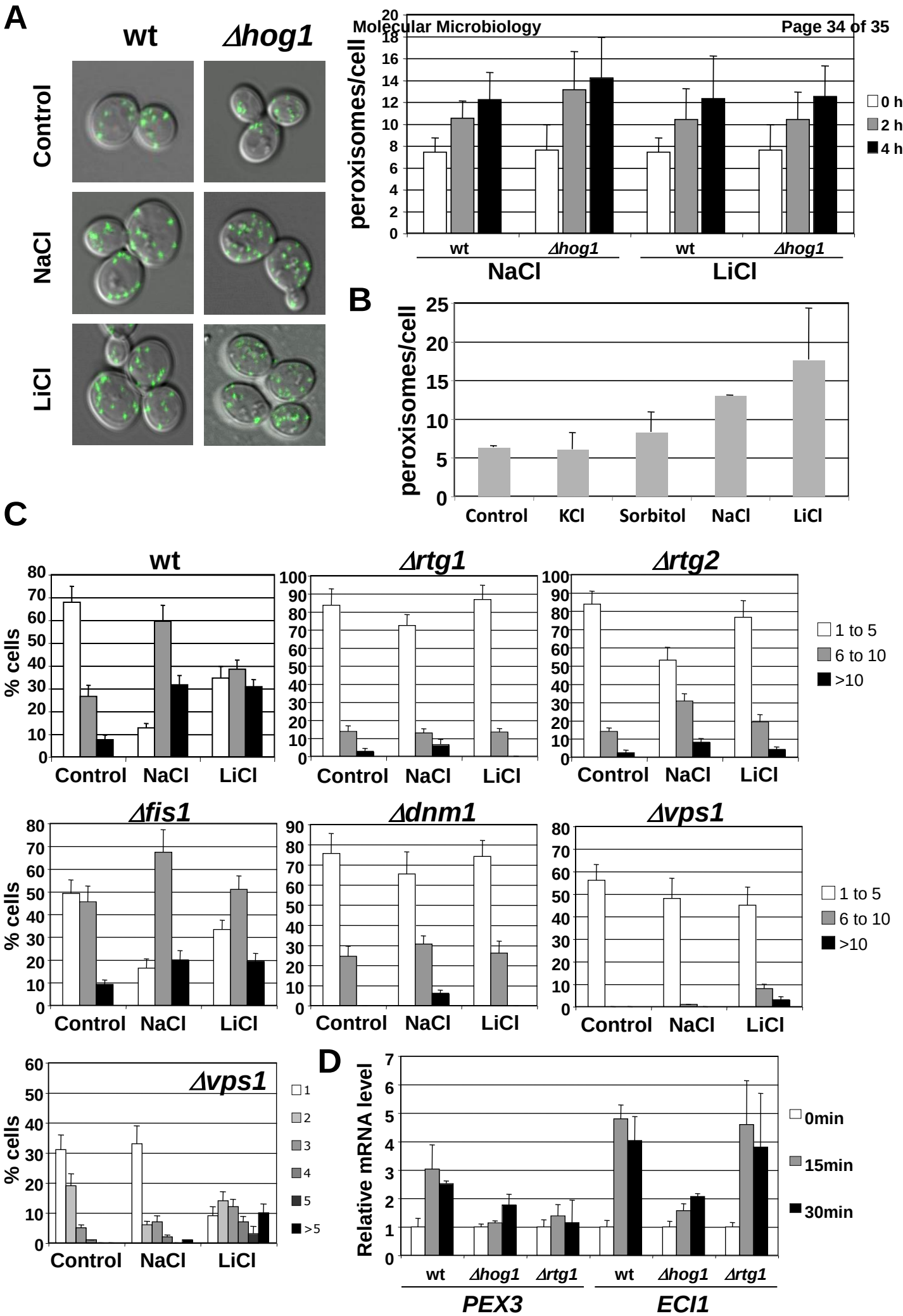
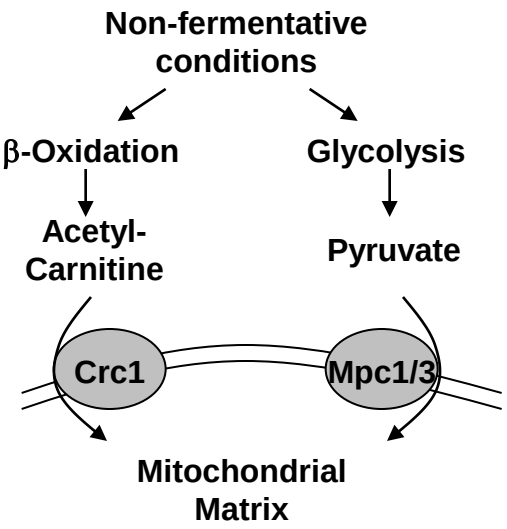
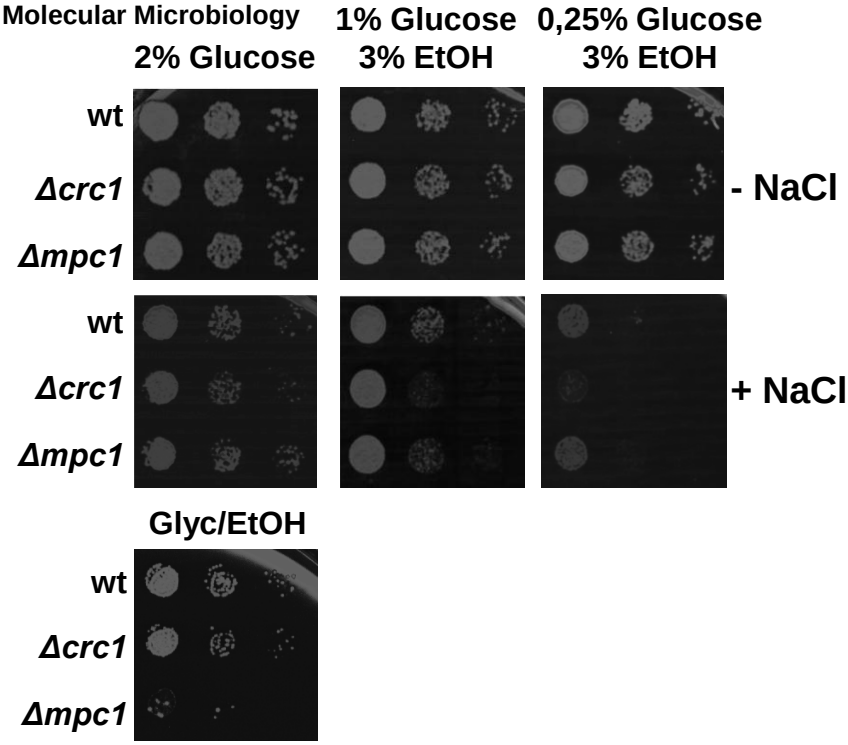
DsRed-Bodipy
Nomarski


A
wt
Δhog1
Control
NaCl
LiCl
peroxisomes/cell
0 h
2 h
4 h
wt
Δhog1
wt
Δhog1
NaCl
LiCl
B
peroxisomes/cell
Control
KCl
Sorbitol
NaCl
LiCl
C
wt
Δrtg1
Δrtg2
% cells
1 to 5
6 to 10
>10
Control
NaCl
LiCl
Δfis1
Δdnm1
Δvps1
Δvps1
1
2
3
4
5
>5
D
Relative mRNA level
0min
15min
30min
wt
Δhog1
Δrtg1
PEX3
ECI1

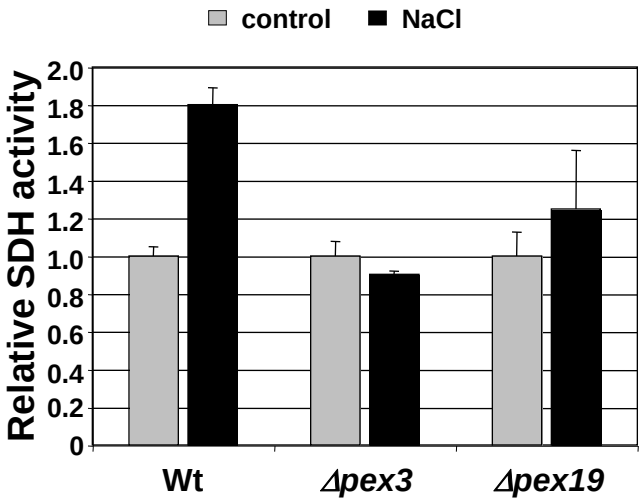
A


Non-fermentative conditions
β-Oxidation
Glycolysis
Acetyl-Carnitine
Pyruvate
Crc1
Mpc1/3
Mitochondrial Matrix

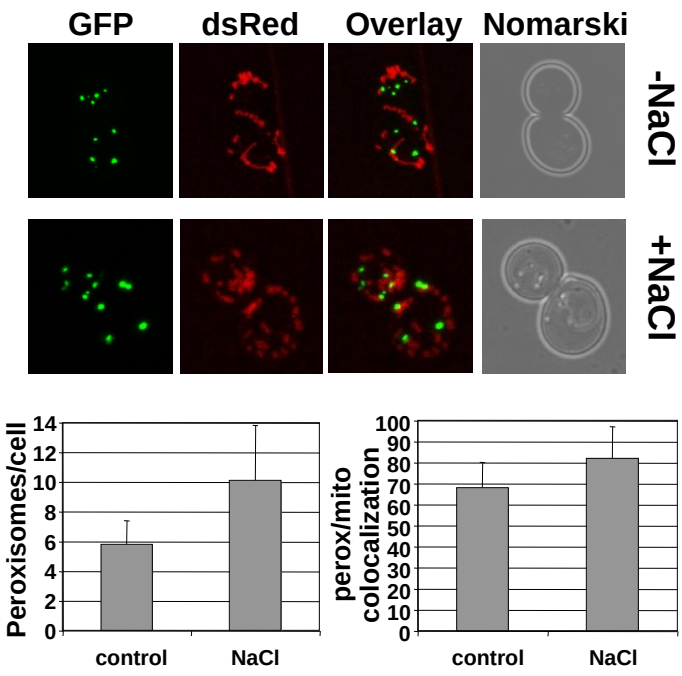
B


2% Glucose
1% Glucose 3% EtOH
0,25% Glucose 3% EtOH
wt
Δcrc1
Δmpc1
- NaCl
+ NaCl
Glyc/EtOH

C


control
NaCl
Relative SDH activity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Wt
Δpex3
Δpex19

D


GFP
dsRed
Overlay
Nomarski
-NaCl
+NaCl
Peroxisomes/cell
14
12
10
8
6
4
2
0
control
NaCl
perox/mito colocalization
100
90
80
70
60
50
40
30
20
10
0
control
NaCl