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Large-scale investigation of oxygen response mutants in *Saccharomyces cerevisiae*†

Bahram Samanfar,[ab] Katayoun Omidi,[ab] Mohsen Hooshyar,[ab] Ben Laliberte,[ab] MD Alamgir,[ab] Andrew J. Seal,[ab] Eman Ahmed-Muhsin,[ab] Duber Frey Viteri,[ab] Kamaleldin Said,[ac] Firoozeh Chalabian,[ad] Ardeshir Golshani,[e] Gabriel Wainer,[e] Daniel Burnside,[ab] Kristina Shostak,[ab] Magdalena Bugno,[ab] William G. Willmore,[ab] Myron L. Smith[ab] and Ashkan Golshani*[ab]

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A genome-wide screen of a yeast non-essential gene-deletion library was used to identify sick phenotypes due to oxygen deprivation. The screen provided a manageable list of 384 potentially novel as well as known oxygen responding (anoxia-survival) genes. The gene-deletion mutants were further assayed for sensitivity to ferrozine and cobalt to obtain a subset of 34 oxygen-responsive candidate genes including the known hypoxic gene activator, *MGA2*. With each mutant in this subset a plasmid based β-galactosidase assay was performed using the anoxic-inducible promoter from *OLE1* gene, and 17 gene deletions were identified that inhibit induction under anaerobic conditions. Genetic interaction analysis for one of these mutants, the RNase-encoding *POP2* gene, revealed synthetic sick interactions with a number of genes involved in oxygen sensing and response. Knockdown experiments for *CNOT8*, human homolog of *POP2*, reduced cell survival under low oxygen condition suggesting a similar function in human cells.

Introduction

Molecular oxygen is essential for a number of biological processes. It is used in many organisms as the terminal acceptor of electrons from the respiratory electron transport chain as well as being required in the biosynthesis of heme, sterols and fatty acids.[1–3] Toxic byproducts of oxygen metabolism include reactive oxygen species (ROS) such as oxygen ions and peroxides.[4,5] In general ROS have proposed roles in programmed cell death.[6–8] ROS production also has a positive role in host defense induction.[9,10] As oxygenation levels may fluctuate in the cellular environment, mechanisms must exist for detecting these changes and regulating the aforementioned oxygen-dependent biological processes.[11,12] Notable in this respect, studying cell growth in hypoxic conditions can add to our understanding of the biology of tumors.[13] Oxygen sensing mechanisms have been studied extensively and a number of important genes and pathways have been identified in humans and other model organisms.[14–18]

The budding yeast, *Saccharomyces cerevisiae*, is an interesting candidate for the study of oxygen response since it is a facultative anaerobe and can be grown under anoxic conditions. It demonstrates the Crabtree effect in the presence of high external glucose concentrations such that fermentation producing ethanol is favored over oxidative phosphorylation. The Crabtree effect produces a high-rate of low-cost ATP but at reduced efficiency.[19,20] Yeast has other advantages as a model eukaryote including a fast growth rate, it is easy to manipulate and its genome shares homology with the human genome including genes involved in human diseases. In budding yeast, some oxygen sensing processes have been identified but a great deal remains unknown.[21–23] In general, decreasing oxygen levels require cells to alter their gene expression profile by decreasing levels of aerobic- (or oxygen-) inducible genes and increasing levels of hypoxic-inducible genes. This is accomplished by regulation of genes involved in oxygen metabolism (including respiratory and biosynthetic genes for hemes, sterols and fatty acids) *via* control of transcription, mRNA stability and protein stability, and by management of ROS.[24–27]

[a] *Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, K1S 5B6, Canada. E-mail: ashkan_golshani@carleton.ca*
[b] *Ottawa Institute of Systems Biology, Carleton University, 1125 Colonel By Drive, Ottawa, K1S 5B6, Canada*
[c] *Department of Pharmaceutics, Qassim University, Buraidah, Saudi Arabia*
[d] *Department of Biology, Islamic Azad University, Tehran North Campus, Iran*
[e] *Department of Systems and Computer Engineering, Carleton University, 1125 Colonel By Drive, Ottawa, K1S 5B6, Canada*

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While some oxygen sensing processes have been identified in yeast, a great deal remains unknown.[21–23] In *S. cerevisiae*, two distinct pathways for the detection and subsequent regulation of genes involved in oxygen response have been found; one involves the transcription factor Hap1p (heme activated protein 1), which de-represses transcription factors Rox1p (repressor of hypoxic genes) and Mot3p (modifier of transcription),[28,29] while the other involves transcription activator Mga2p.[30] In the first pathway, Hap1p is activated by cytosolic heme and heme production which requires the presence of molecular oxygen.[28,31] At anoxic levels (0–0.2% oxygen) heme levels drop off, thus inactivating Hap1p.[32] Inactivation of Hap1 results in lower transcription of Rox1 and Mot3, which in turn leads to de-repression of hypoxic genes such as *ANB1*, *COX5A*, *ERG11*, and *HEM13*.[33] Rox1p and Mot3p work in combination to repress hypoxic genes since neither can accomplish this function alone at endogenous levels.[29,33,34] Additional factors such as Tup1p and Ssn6p have been identified that contribute to the repressor function of Rox1p, while others may not yet be identified.[35] It has also been proposed that Hap1p may repress Rox1p expression in the absence of heme.[28] The second pathway identified in yeast involves the mitochondrial electron transport chain and the activation of Mga2p.[3,30] While some of the steps involved in the activation of Mga2p have been elucidated, how its activity is regulated by oxygen tension has not been determined. However, once Mga2p is activated, it acts as an activator of transcription for genes possessing the *cis*-acting low oxygen response element (LORE) such as *OLE1*. While Mga2p does not possess a DNA binding motif, it is found in the transcription activating complex associated with LORE of Ole1p under hypoxic conditions.[36]

In order to identify novel candidates involved in oxygen responding pathways, two large-scale experiments were performed. The first identified gene deletions in yeast strains that resulted in sick phenotypes in anoxic conditions compared to normoxia. Those mutants that displayed a sick phenotype were further tested for sensitivity to each of ferrozine and cobalt, a ferrous ion chelator and a high-affinity competitor for protein binding to iron/copper, respectively. Since iron- and copper-bound proteins are critical to the function of the electron transport chain (ETC) and oxygen respiration,[37,38] disruption of these proteins should produce ETC-disruption-dependent induction of hypoxic response, including the proposed activation of Mga2p.[3] Together, these large-scale screens generated a list of interesting candidates. These candidates were then assessed for their involvement in activation of the hypoxia-induced promoter of OLE1, consisting of LORE response elements, conjugated with the *LacZ* gene.[39] Among several interesting genes identified in these experiments, *POP2* was selected for Synthetic Genetic Array (SGA) analysis. *POP2* encodes an RNase involved in 3′ to 5′ mRNA deadenylation, suggesting that processes such as regulation of mRNA stability may play an important role in the expression of hypoxic genes and in oxygen response. Down regulation of *CNOT8* gene which encodes for human homolog of Pop2p, reduced cell survival under low oxygen condition suggesting that the activity of Pop2p in response to oxygen appears to be conserved in human cells. All together this study identified novel candidates involved in oxygen response pathways and suggests that Pop2p has a role in responding to hypoxia and cell survival.

Material and methods

Strains and media

YPD (Yeast extract, Peptone, Dextrose) for yeast and LB (Luria-Bertani) for *Escherichia coli* were used as growth media. *S. cerevisiae* strains used included a library of gene-deletion mutants derived from the MATa strain BY4741,[40] and the MATα strain, BY7092. *E. coli* strain DH5α was used to replicate plasmids.[41] pAM6, a plasmid which has the hypoxia-induced promoter (LORE) from the OLE1 gene in front of the LacZ gene[39] was used to analyze β-galactosidase activity of mutants under anoxic and normoxic conditions. Plasmid extraction was performed using Pure Link Quick Plasmid Miniprep Kit (Invitrogen, Germany).

Large-scale gene-deletion array analysis and selection

Approximately 4500 yeast deletion strains (non-essential genes) were maintained on YPD + G418 (200 μg mL^{-1}) in a set of master plates, with each master plate containing an array of ~384 deletion strains.[42] Strains were inoculated onto replicate plates using a floating pin replicator[43] and grown for 2 days at 30 °C under normoxic (control) and anoxic conditions. Anoxic conditions were established using OXOID AnaeroGen Biopack (FLUKA) in an air-tight container system. Images of plates were captured and analyzed with some modifications.[44] Briefly, images were first converted to black and white representing media and colonies, respectively. Objects smaller than 0.00025 white pixels were considered artifacts and were eliminated. Colony locations were determined by colony isolation phase within the colony search area. The size of the colony search area includes target and adjacent colonies to compensate for missing or very small colonies. The colony size was determined as the number of pixels corresponding to a target colony. Average colony size (S_{ave}) was calculated by summing the size of all colonies on a plate and dividing by the total number of colonies on that plate (usually 384). Relative size of each colony was measured by subtracting S_{ave} from the colony size. Relative colony size between the control (normoxic) and experimental (hypoxic) conditions was used to assign sensitivity. Each large-scale experiment was repeated three times and those colonies that had a reduction of 20% or more in two of the three repeats or 15% or more in all three experiments were deemed "positive". Functional classification and enrichment analysis were performed using, Yeast Features (http://software.dumontierlab.com/yeastfeatures/), Yeast Genome Database (http://www.yeastgenome.org/) and GeneMANIA (http://www.genemania.org/). Sensitivity was calculated using the standard formula 100 × [TruePositives/(TruePositives + FalseNegatives)]; and specificity was calculated using 100 × [TrueNegatives/(TrueNegatives + FalsePositives)].

Gene knock-out and DNA transformation

Gene knock-outs in yeast were done by gene replacement with a selectable marker using homologous recombination.[40]

DNA transformation in yeast was by the Lithium Acetate (LiAc) method[45] and plasmid transformations in *E. coli* was carried out by chemi-competant method.[46]

Inhibitor sensitivity spot tests

Spot test analysis of serial dilutions of cell suspensions was carried out using two series of agar media, one containing 2.0 mM ferrozine and the other with 400 μM cobalt. The plates were incubated for 2–3 days at 30 °C in normoxic conditions. Sensitivity to the compounds was assessed by comparing the number and size of the colonies formed on each plate after 48 hours incubation at 30 °C in relation to the control.

β-galactosidase assay

A quantitative, plasmid-based (pAM6) β-galactosidase assay was carried out under anoxic and normoxic conditions using the substrate ONPG (*O*-nitrophenyl-beta-D-galactopyranoside) as described previously.[39,47–49] The deletion mutant for *MGA2* was used as a positive control in this assay since *MGA2*-deficient cells have lower induction from the LORE promoter under hypoxic conditions. Yeast strain BY4741 was used as a negative control in the assay since it should induce transcription through the LORE promoter.

SGA (Synthetic Genetic Array) analysis

SGA is a high-throughput screening method based on yeast mating followed by selection of haploid double mutants to identify synthetic growth defects that are indicative of genetic interactions.[40] For our query, we replaced the *POP2* gene with the nourseothricin-resistance (NAT) marker in the MATα strain, BY7092. This query strain was separately crossed to each deletion mutant in the MATa BY4741 library. After a few rounds of selection using G418 + NAT selective medium, haploid double mutant progeny were obtained and analyzed for growth defects using colony size measurements as above. The experiment was repeated three times and those interactions that were found in at least two experiments were considered for confirmation using random spore analysis.[40,50]

Cell culture, transfection and chemicals

Human embryonic kidney (HEK293, ATCC Number CRL-1573) cells were grown and transfected using standard laboratory methods.[51] Dubecco's modified Eagle's medium (DMEM), penicillin–streptomycin–antimycotic (P/S/A), horse serum (HS), Opti-MEM I Reduced-Serum Medium and Lipofectamine 2000 were purchased from Invitrogen (Invitrogen/Life Technologies, Carlesbad, CA). Restriction enzymes and buffers were from New England BioLabs (Ipswich, MA). Rabbit anti-human Cnot8p polyclonal antibody was purchased from Proteintech Group, Inc. (Chicago, IL). Goat anti-rabbit HRP antibody was purchased from Santa Cruz (DAKOCytomation, Mississauga, ON). 100 μM cobalt and 100 μM desferoxamine,[52] a mammalian specific chelator which works similar to ferrozine in yeast, were used for cell sensitivity analysis.

CNOT8 expression analysis

Ambion's p*Silencer*™ 4.1 CMV neo siRNA expression vector (Invitrogen/Life Technologies, Grand Island, NY) was used for expression of siRNA in HEK293 cells. Three oligonucleotides inserts were designed (using Ambion's siRNA Target Finder, Invitrogen/Life Technologies) to include two complementary 55-mer siRNA template and to encode a 19-mer hairpin sequence specific to the *CNOT8* mRNA target. Inserts were ligated into p*Silencer* using T4 DNA ligase. Inserts for GAPDH were ligated to p*Silencer* as a positive control.

DNA Insert 1:
5′-GATCA[ATCGTGCTCAGTTACAGTT]TTCAAGAGA[AACTGTAACTGAGCACGATTT]G-3′
3′-T[TAGCACGAGTCAATGTCAA]AAGTTCTCT[TTGACATTGACTCGTGCTAAA]CTCGA-5′

DNA Insert 2:
5′-GATCA[TGACTACAGTATGGACAAT]TTCAAGAGA[ATTGTCCATACTGTAGTCATT]G-3′
3′-T[ACTGATGTCATACCTGTTA]AAGTTCTCT[TAACAGGTATGACATCAGTAA]CTCGA-5′

DNA Insert 3:
5′-GATCA[TACTTGGCAGTTCAATTTC]TTCAAGAGA[GAAATTGAACTGCCAAGTATT]G-3′
3′-T[ATGAACCGTCAAGTTAAAG]AAGTTCTCT[CTTTAACTTGACGGTTCATAA]CTCGA-5′

PCR primers:
Forward: 5′-AGGCGATTAAGTTGGGTA-3′ and Reverse: 5′-CGGTAGGCGTGTACGGTG-3′

Semi quantitative real time RT PCR (Forward: 5′-AATTTCCAGGTGTTGTGGTGCGAC-3′ and Reverse: 5′-TGCAAAGTGCAGTGTGTCAATCCC-3′) was performed[53] using Bio-Rad iCycler (Hercules, CA). Western blot analysis was performed[51,54] using rabbit anti-human Cnot8p antibody (1 : 2000 dilution), and horseradish peroxidase-labelled goat anti-mouse IgG secondary antibody used at the dilutions of 1 : 2000 and 1 : 4000, respectively. As a loading control, membranes were probed with mouse anti-human β-tubulin (1 : 4000 dilution).

Cell sensitivity (MTT) and Annexin V assays

Cell sensitivity assay based on methylthiazolyldiphenyl-tetrazolium bromide (MTT) oxidation was used as before.[55,56] In brief, cells were seeded at a density of 50 000 cells mL^{-1} and grown for 48 h followed by target treatment for 24 h. MTT was then added (0.5 mg mL^{-1}) for 2 hours and the absorbance was read at 570 nm with correction at 630 nm. Annexin V assay was performed as in ref. 57.

Results and discussion

A large-scale non-essential gene-deletion array analysis identified candidate genes involved in anoxia response

The gene-deletion array analysis provided an easy and efficient approach to screen several thousands of yeast non-essential genes that are potentially involved in oxygen response. In this

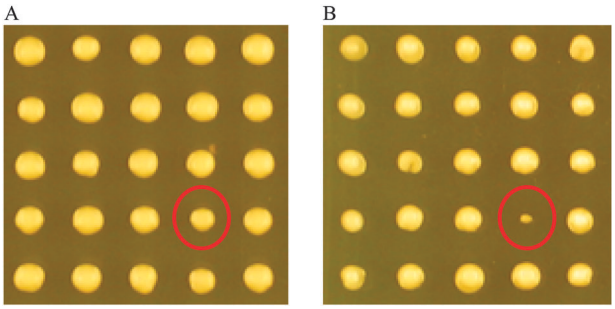

Fig. 1 Systematic growth of yeast colonies. Left panel (A) illustrates colonies grown in normoxic condition and right panel (B) those in anoxic condition. A representative colony with relative growth reduction in anoxic condition is highlighted by red circles.

experiment, which was repeated three times, approximately 4500 yeast deletion strains were exposed to normoxic and anoxic conditions. From this, a manageable set of 329 strains was selected that had significantly reduced colony growth rates under anoxic conditions relative to normoxic conditions (Fig. 1). This candidate gene set contains genes known to be involved in the oxygen response pathway (61 of the possible 72 genes or sensitivity of approximately 85%), in addition to genes that are involved in other pathways but not known to be involved in oxygen response, and functionally uncharacterized (unknown) genes (268 or specificity of approximately 95%). The deleted genes detected by our large-scale screening are hypothesized to be involved in oxygen response pathways or cell survival in hypoxic conditions. The set included known oxygen-responsive transcription factors such as Hap1p/3/4/5, Rox1p and Mot3p which add credibility to this large-scale investigation. In order to have a better representation of genes involved in the oxygen responding pathway, we selected additional mutants that have deletions in genes that are already thought to be involved in oxygen sensing but were not detected in our large-scale investigation, as well as some other interesting candidate genes. In total we selected 384 mutants to create an oxygen response nonessential gene-deletion array (ESI†).

34 yeast deletion strains show increased sensitivity to ferrozine and/or cobalt(II) chloride hexahydrate

To further characterize genes involved in oxygen response, we investigated the sensitivity of the selected 384 gene-deletion strains to different agents that induce oxidative stress such as ferrozine and cobalt(II) chloride hexahydrate by performing pinning analyses and spot tests. Ferrozine is an iron chelator that binds to free iron ions, effectively removing free iron from the growth medium, which results in the inhibition of oxygen uptake into the cell.[58] It was previously shown that transcription factors regulate known genes that respond to environmental changes in oxygen, do not sense oxygen directly, but sense a molecule, such as heme, whose biosynthesis is dependent on iron metabolism and oxygen.[59,60] Previous studies have suggested that overexposure to cobalt induces oxidative stress in yeast by increasing the production of reactive oxygen species and replacing essential metals such as iron, magnesium and calcium in various reaction pathways.[61] In particular, it has been shown that cobalt can substitute for iron in the porphyrin ring of heme, thereby decreasing its affinity for oxygen, creating a hypoxic environment.[62]

After pinning the selected 384 yeast deletion strains to media containing either sub-inhibitory concentrations of ferrozine (2.0 mM) or cobalt(II) chloride hexahydrate (400 μM) under normoxic conditions, it was observed that 34 of the selected strains showed decreased growth with one or both treatments. Sensitivity to the compounds was further tested for these 34 strains using spot test analyses in which the number and size of the colonies formed was compared to the control (data not shown). A representative example of spot test analysis is shown in Fig. 2. The degree of sensitivity to each compound was categorized as either sensitive (moderate or high) or not sensitive for each strain based on relative colony size and/or colony numbers (Table 1). Moderate sensitivity indicates a 40–70 percent reduction, while high sensitivity represents a 70–100 percent reduction in colony size and/or colony numbers. The relative sensitivity to these compounds by each of the 34 yeast strains could further indicate that the represented deleted genes are involved in an oxygen response pathway. In order

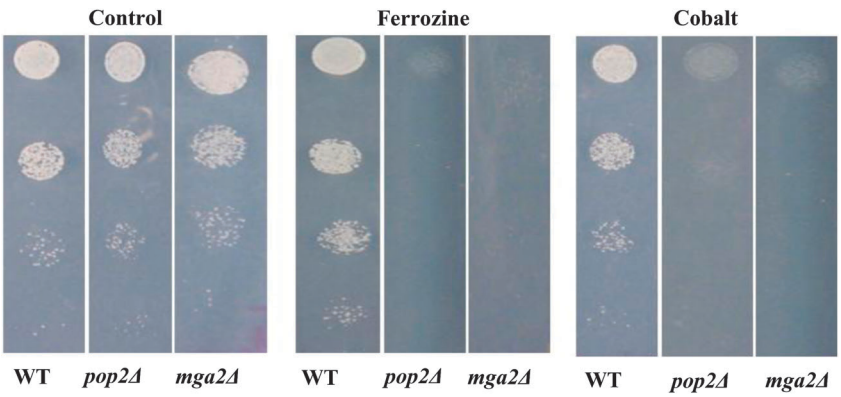

Fig. 2 Spot test analysis for wild-type and two representative yeast deletion strains for Pop2p and Mga2p from 34 anoxic response mutants grown under normoxic conditions on media with either 2.0 mM ferrozine or 400 μM cobalt(II) chloride hexahydrate.

Table 1 Candidate list of 34 deletion mutants show different levels of sensitivity to ferrozine (2.0 mM) and/or cobalt(II) chloride hexahydrate (400 μM) when grown under normoxic conditions. Moderate sensitivity indicates a 40–70% reduction, while high sensitivity represents a 70–100% reduction in colony size and/or colony numbers

	Level of sensitivity			Level of sensitivity	
Strain	Ferrozine	Cobalt	Strain	Ferrozine	Cobalt
aps2Δ	Moderate	NS	*qcr9Δ*[a,c,e]	Moderate	Moderate
cox5aΔ[a,c]	Moderate	NS	*rox1Δ*[d]	Moderate	High
cox7Δ[a,c]	Moderate	Moderate	*sac2Δ*	Moderate	High
cpa1Δ[b]	High	Moderate	*scd6Δ*	High	Moderate
dia2Δ[d]	Moderate	NS	*sec22Δ*	Moderate	NS
gal11Δ[d]	High	Moderate	*sel1Δ*[b]	High	High
gph1Δ[b]	Moderate	NS	*ser1Δ*	NS	Moderate
hmg2Δ[c]	High	High	*stv1Δ*[b]	Moderate	NS
hom6Δ[c]	Moderate	Moderate	*syt1Δ*	NS	High
idp2Δ[c]	High	Moderate	*vam3Δ*	High	Moderate
jlp1Δ[c]	High	Moderate	*vma4Δ*[b]	High	High
kha1Δ[e]	Moderate	Moderate	*vps41Δ*	High	High
mac1Δ[e]	High	High	*ycl014wΔ*	Moderate	Moderate
mga2Δ[d,f]	High	High	*ydr071cΔ*[d]	Moderate	Moderate
mlh1Δ[b]	Moderate	Moderate	*ygr161cΔ*	Moderate	NS
opi3Δ[b]	Moderate	Moderate	*ykl177wΔ*	NS	Moderate
pop2Δ[d]	High	High	*ynl134cΔ*[c]	Moderate	NS

NS = Not Sensitive (equal to wild-type sensitivity). Gene ontology annotation analysis: [a] Respiratory chain complex (*P*-value: 1.30×10^{-3}). [b] Phosphate and energy metabolism (*P*-value: 1.64×10^{-3}). [c] Oxidoreductase activity (*P*-value: 4.94×10^{-3}). [d] Transcription regulation (*P*-value: 1.11×10^{-3}). [e] Ion transportation (*P*-value: 4.79×10^{-3}). [f] Positive control.

to have a better understanding of the function of genes represented in the oxygen response set, we analysed the 34 candidate genes using gene ontology (GO) profiling software (GeneMANIA, g:profiler, ProfCom and Yeast Features). As illustrated in Table 1, GO annotation indicates that these candidate genes are significantly enriched ($P \leq 0.005$ in all cases) in pathways implicated in oxygen response, specifically involving respiratory chain complex, phosphate and energy metabolism, oxidoreductase activity, transcription regulation and ion transportation. 24 of these 34 yeast genes are conserved in human.

17 Yeast deletion strains show reduced induction of hypoxia induced LORE promoter

To further investigate the response of yeast deletion strains to anoxic conditions, the efficiency of a hypoxia inducible *LacZ* expression system was measured using a β-galactosidase assay under normoxic and anoxic conditions. The selected 34 yeast deletion strains from ferrozine and/or cobalt sensitivity analyses (Table 1) were each transformed with pAM6 plasmid, containing a *LacZ* expression cassette controlled by the hypoxia-induced promoter, LORE, from the OLE1 gene. Expression of β-galactosidase is accomplished through the induction of LORE under anoxic conditions, while colonies that have difficulties to induce this promoter have lower expression of β-galactosidase.[39] It was observed that 17 yeast deletion strains from the selected 34 strains had relatively low anoxic/normoxic ratios of β-galactosidase activity in comparison to the wild-type. This suggests that the deleted genes in these 17 strains play a regulatory role involving the LORE promoter under anoxic conditions, and are thus involved in oxygen response pathways (Table 2).

Table 2 Relative β-galactosidase activity for candidate gene deletion mutants

Gene names	Anoxia (MU)	Normoxia (MU)	Normalized ratio of β-galactosidase[a]
Control[b]	115.2	40.6	1
mga2Δ[c,g]	49.4	47.9	0.37
cox5aΔ[d,f]	77.5	43.7	0.63
cox7Δ[d,f]	29.5	23.9	0.44
cpa1Δ[h]	36.3	21.8	0.59
gal11Δ[g]	69.1	73.1	0.34
hom6Δ[h]	18.0	89.2	0.08
kha1Δ[e]	27.3	28.9	0.34
mac1Δ[g]	80.2	53.7	0.53
mlh1Δ[e]	9.6	35.2	0.10
opi3Δ[h]	26.7	20.7	0.46
pop2Δ[g]	53.2	61.9	0.31
qcr9Δ[d]	24.5	16.4	0.53
sec22Δ[h]	12.0	12.7	0.34
ubx2Δ[h]	7.4	10.3	0.25
vma4Δ[e]	61.2	30.2	0.72
vps41Δ[h]	86.0	84.9	0.36
ynl134cΔ[f]	1.1	13.8	0.03

MU stands for Miller Unit. [a] Normalized ratio of β-galactosidase activity in normoxic over anoxic conditions and related to control strain. [b] Negative control (BY4741). [c] Positive control. Gene ontology annotation analysis: [d] Mitochondrial respiratory chain (*P*-value: 3.37×10^{-2}). [e] Energy synthesis coupled electron transport (*P*-value: 3.77×10^{-2}). [f] Oxidative activity (*P*-value: 4.20×10^{-2}). [g] Transcription regulation (*P*-value: 4.33×10^{-2}). [h] Other gene ontology categories.

MGA2 was chosen as the positive control for β-galactosidase assay because it is a known mediator of hypoxic gene activation through the LORE promoter. The strain BY4741 (wild-type) is used as a negative control. Mga2p and Spt23p are paralogs and at least one of them is required for basal Ole1p expression and cell survival.[63] However, Mga2p but not Spt23p provides oxygen and fatty acid mediated regulation of Ole1p. Activation of Mga2p and Spt23p require the proteolytic cleavage of their C-terminal tails, which tether them to the endoplasmic reticulum (ER) membrane. This proteolytic cleavage is ubiquitin-dependent and requires Rsp5p mediated ubiquination.[64] Interestingly, Ubx2p, an ubiquitin-dependent proteolytic enzyme associated with the ER, was identified in our study as being involved in the anoxic induction of *OLE1* promoter. It is conceivable that this enzyme is involved in Mga2p processing. Additional novel oxygen response candidates were identified in our β-galactosidase assay. These included electron transport chain genes (*QCR9*, *COX5A*, and *COX7*), vesicle transport genes (*SEC22* and *VPS41*), transcription regulation (*POP2*, *GAL11* and *MAC1*), vacuolar ATPase-proton pump and K^+/H^+ antiporter (*VMA4* and *KHA1*) and *OPI3*. *OPI3* encodes for a protein involved in the biosynthetic pathway to phosphatidylcholine (PC), a phospholipid which is highly expressed in the membranes of mitochondria that is synthesized in the ER.[65] As illustrated in Table 2, gene ontology annotation analysis of the 17 gene deletions with relatively low hypoxia-induced β-galactosidase activity under anoxic growth conditions suggests the involvement of these candidate genes in oxygen response pathways.

Previous studies showed that full-length- and cleaved-Mga2p enhance *OLE1* mRNA stability in response to cytosolic saturated fatty acids, while cytosolic unsaturated fatty acids promote

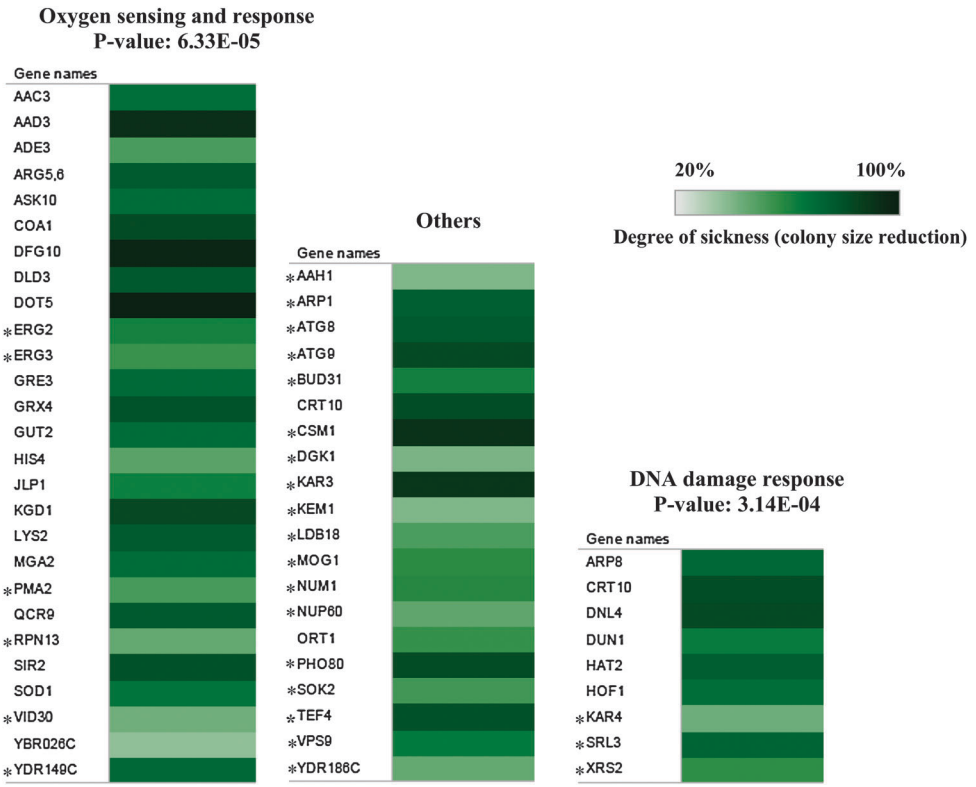

Fig. 3 Analysis of the union (from this study and literature) of synthetic sick interactions for *POP2*. The most dominant cluster is enriched for oxygen response and sensing (*P*-value: 6.33×10^{-5}). Relative growths are color coded as indicated. * Represents interactions included from literatures.

full-length-Mga2p-dependent *OLE1* mRNA degradation.[66] This mRNA degradation pathway does not involve Ccr4p-mediated deadenylation but does involve the 3′-to-5′ exonuclease activity of the SKI-complex.[66] Since it is generally accepted that 3′-to-5′ exosome-mediated degradation of functional mRNA requires deadenylation, it is likely that at least one of the yeast deadenylases encoded by *POP2*, *PAN2* or *PAN3* is involved.[67] Our data suggest that regulation of mRNA stability may also play an important role in the expression of hypoxic genes and may play a role in oxygen responding. Gene encoding Pop2p was identified as a potential member of an oxygen responding pathway in our *OLE1* promoter induction assay. Pop2p has been characterized as a member of the CCR4-NOT complex, a large multimeric transcription factor complex that can regulate transcription positively or negatively. It is also a regulator of mRNA stability through its 3′-to-5′ poly (A)-ribonuclease activity.[68,69] In previous studies of CCR4-NOT complex mutations, *POP2* deletion under certain conditions (exponential growth in glucose, diauxic shift, non-fermentative growth) differentially affected mRNA stability for genes involved in oxidative phosphorylation, stress response, hydrogen-transporting ATP syntheses activity, monosaccharide transport, ribonucleoside triphosphate biosynthesis and/or metabolism among others.[70]

Synthetic Genetic Array (SGA), analysis for *pop2Δ*

Synthetic Genetic Array (SGA) is a high-throughput genetic analysis method based on mating a reference mutant strain to each mutant in the yeast haploid gene deletion array and selecting for haploid progeny containing both mutations followed by scoring for genetic interactions.[40] Genetic interactions are recognized when double mutants have synergistic effects on growth. A common type of genetic interaction is an aggravating interaction called synthetic growth defect (synthetic sick) where double mutants have a lower growth rate than that expected from individual mutant growth phenotypes. These interactions often reveal genes that function in parallel pathways, where one gene/pathway often compensates the activity of the other. They may also expose cross-communication between different processes. Pop2p is a 3′-5′exoribonuclease known to be involved in mRNA degradation as well as involved in transcription regulation.[68,69] We selected Pop2p for genome-wide SGA analysis. We combined the interaction we observed for Pop2p encoding gene with those previously reported.[71] Fig. 3, represents functional categorization for the union of genes that formed synthetic growth defects with *POP2*. The most populated category is enriched by oxygen response and sensing genes (*P*-value: 6.33×10^{-5}) followed by DNA damage response (*P*-value: 3.14×10^{-4}). It is noteworthy that DNA damage response has a well documented association with oxygen availability especially through oxidative stress response.[72] Interestingly, *POP2* interacts with several genes that are directly involved in oxygen response pathway such as *COA1* (mitochondrial inner membrane protein required for assembly of the cytochrome *c* oxidase complex), *MGA2* (hypoxic transcription activator), *DOT5* (a peroxidase involved in oxidative stress), *QCR9* (a subunit of cytochrome *bc1* complex) and *AAC3* (Mitochondrial inner membrane ADP/ATP translocator). The observed genetic

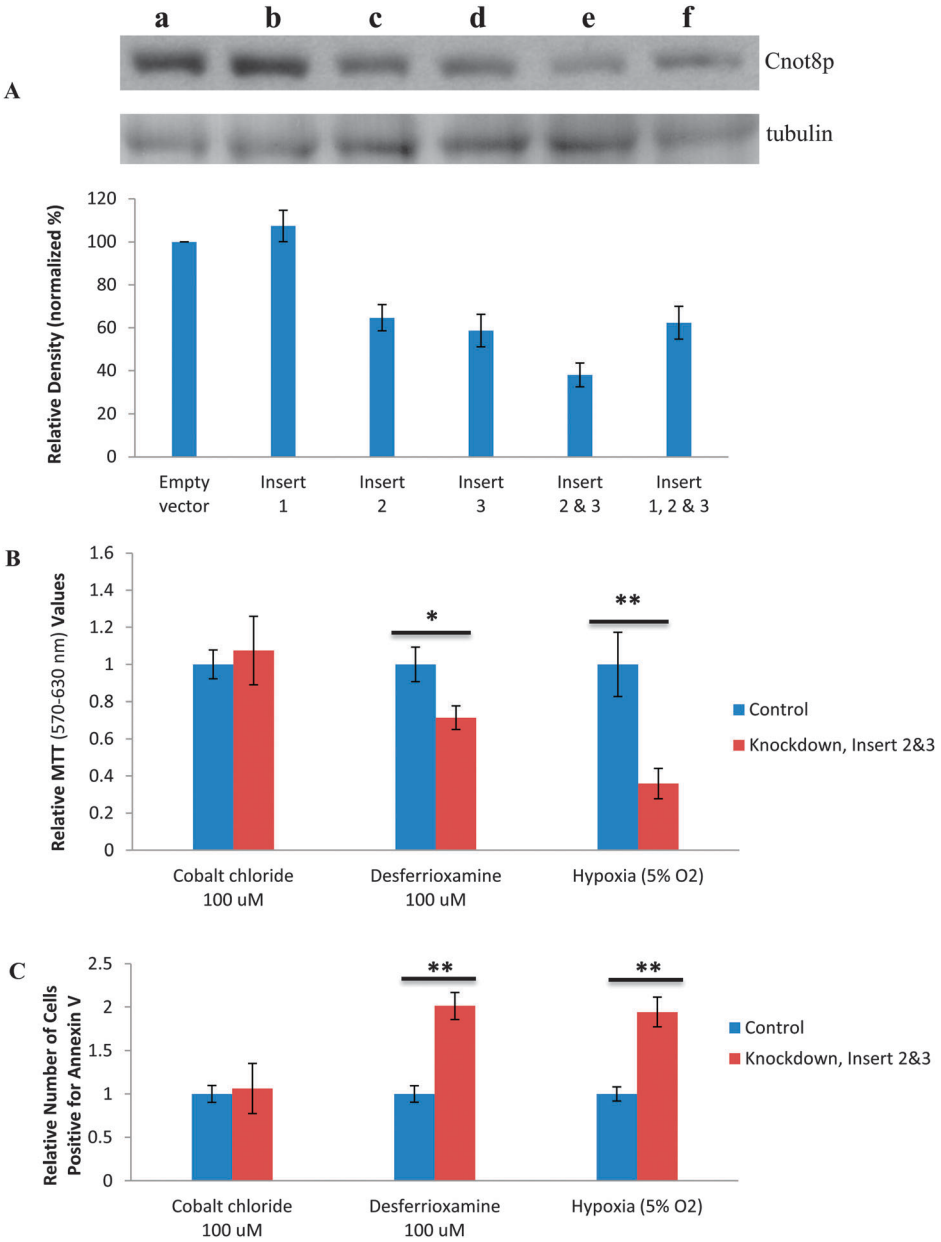

Fig. 4 Analysis of Cnot8p knockdown and cell survival in HEK293 cells. (A) Western blot analysis (top panel) of Cnot8p in lysates (30 μg total protein) from HEK293 cells transfected with siRNA constructs containing no insert (a), insert 1 (b), insert 2 (c), insert 3 (d), inserts 2 & 3 (e) and inserts 1, 2 & 3 (f) in p*Silencer* plasmid. Normalized density of Cnot8p band is quantified in the bar chart. Insert 2 & 3 reduced Cnot8p expression to approximately 40%. (B) Relative HEK293 cell viability normalized to normoxia. The ratio of knockdown cells to the control was approximately 0.74 under normoxia. Higher values represent greater cell survival. Insert 2 & 3 was used to obtain partial knockdown. (C) Ratio of Annexin V positive cells (apoptotic cells) for Cnot8p knockdown related to those in normoxia. Under hypoxia and desferrioxamine treatments the ratio of Annexin V positive cells approximately doubled. * (*P*-value $\leq$ 0.05) and ** (*P*-value $\leq$ 0.01) represent statistically significant results. Each experiment was repeated at least three times.

interaction profile for *POP2* resembles those for genes that encode Ccr4p and Kem1p, both of which are functionally related to Pop2p.[71,73,74] Ccr4p (like Pop2p) is a member of CCR4-NOT complex and Kem1p is a 5′-3′ exoribonuclease.

Knockdown of CNOT8, human homolog of POP2, reduces cell viability under hypoxia

To examine a possible conservation for Pop2p function in oxygen response, we studied its human homolog Cnot8p (37% identity; http://www.uniprot.org/uniprot/Q9UFF9) in HEK293 (Human Embryonic Kidney) cells. The expression of the gene encoding Cnot8p was knocked down by an siRNA-based approach followed by cell viability measurement under different treatment conditions. A reduction in the expression of Cnot8p was determined by semi-quantitative real time RT-PCR (data not shown) and confirmed by a Western blot analysis (Fig. 4A). A combination of DNA inserts 2 and 3 in p*Silencer* plasmid provided the best knockdown (by approximately 60%) for Cnot8p. It should be noted that this represents a partial knockdown and under our experimental conditions a complete knockdown was not

obtained. In agreement with our observation with deletion mutant strain for Pop2p in yeast, knockdown of Cnot8p reduced cell survival under hypoxic condition (Fig. 4B). Partial knockdown of Cnot8p in normoxia reduced cell proliferation by approximately 26%. The data presented in Fig. 4B is normalized for this. Reduction of cell proliferation by Cnot8p knockdown has been previously reported.[75] Similarly, reduction of Cnot8p expression made the cells more sensitive to desferoxamine (100 μM), a mammalian-specific chelator which works similar to ferrozine in yeast.[76] Unlike *pop2*Δ however, sensitivity to cobalt was not observed for concentrations as high as 100 μM at which human cell sensitivity is commonly measured. This discrepancy may stem from reported differences between oxygen sensing in yeast and mammalian cells.[77] A correlation between cell viability and apoptosis for Cnot8p knockdown was investigated using Annexin V assay which detects one of the earliest steps of apoptosis (Fig. 4C).[57] Under hypoxia and desferrioxamide treatments the ratio of Annexin V positive cells approximately doubled. Altogether these observations suggest a possible functional conservation for Pop2p and Cnot8p between yeast and human cells under hypoxia.

Conclusion

Our genome-scale investigation of oxygen sensing and response genes provided us with a list of 34 yeast gene deletion mutants (24 of which are conserved in human) that demonstrated growth sensitivity in the absence of oxygen, in addition to their sensitivity to cobalt and/or ferrozine. 17 of these gene deletion strains showed deficiencies in inducing gene expression *via* a previously reported anoxic-induced LORE promoter. Pop2p, a regulator of mRNA stability through its 3′-5′ ribonuclease activity, is one protein that was identified in this manner. Our genetic interaction analysis further supported an association for Pop2p in oxygen sensing and response. Our analysis using the human homolog of Pop2p, Cnot8p, indicated that the observed activity of Pop2p appears to be conserved between yeast and human cells. Further study of the additional candidates that we implicate in yeast oxygen sensing and response is highly recommended.

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