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ORIGINAL ARTICLE

Over-Expression of *YLR162W* in *Saccharomyces cerevisiae* Inhibits Cell Proliferation and Renders Cells Susceptible to the Hypoxic Conditions Induced by Cobalt Chloride

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Abstract *YLR162W* is an uncharacterized *Saccharomyces cerevisiae* ORF whose transcript level is elevated in cells under environmental stress, during α-factor response and in stationary phase. We obtained a partial cDNA clone of *YLR162W* by subtractive hybridization cloning of genes that were not expressed in a $CoCl_2$ resistant DNA synthesis mutant but expressed in its wild type counterpart. Our studies demonstrated that *YLR162W* transcript level was reduced in BY4741 cells upon exposure to the hypoxia mimetic agent $CoCl_2$, and continuous expression of full length *YLR162W* from a plasmid borne copy of the gene rendered BY4741 cells extremely susceptible to the hypoxic conditions induced by $CoCl_2$. At initial time points following the induction of *YLR162W* expression, cell cycle progression was inhibited with the emergence of a distinct sub-G1 peak indicative of apoptotic cells, mitochondrial membrane potential was also decreased along with an increase in the fraction of cells permeable to propidium iodide; none of the above was further affected by $CoCl_2$. The up-regulation of Ylr162wp in cells exposed to environmental stress and in non-replicating cells appears to be related to its growth inhibitory properties presented in this report.

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Introduction

YLR162W is an uncharacterized *S. cerevisiae* ORF that resides ~20 kb upstream of the chromosomal rDNA repeat in chromosome XII and is supposed to have arisen from a duplication event. It's third half and attached flanking region (a total of 1 kb) is more than 99% similar to the reverse complement of the 25S rRNA coding region. *YLR162W* encodes a hypothetical membrane protein of a molecular mass of 13,055 Da (NCBI accession no. 568478) containing 118 amino acids with one putative transmembrane domain coded by residues 37–53. Ylr162wp has been classified as a type-2 membrane protein [1] that does not have a cleavable signal sequence and displays an *N*-terminal extracellular and *C*- terminal cytoplasmic orientation [2].

Very little experimental data is available on the *YLR162W* gene product. Its over-expression provides resistance against an anti-microbial peptide (MiAMP1) in a strain of *S. cerevisiae* that is susceptible to MiAMP1 [3]. Expression studies have shown that *YLR162W* transcript level was increased by 4.3 folds in cells over-expressing *MLH1* [4] i.e., under conditions of elevated spontaneous mutation rate and genomic instability. *YLR162W* expression was also significantly elevated during high-pressure stress [5], upon Mg^{2+} starvation [6], during response to α-factor (50 folds) [7] and in stationary phase [8]. Its expression is reduced under both oxidative and reductive stress [9] and during hypoxia followed by reoxygenation in both glucose and galactose [9]. In spite of the available expression data, a specific function is yet to be assigned to

Ylr162wp. Available data indicate that *YLR162W* mRNA level increased in cells under various stress conditions and in non-replicating cells.

Cobalt chloride is a well-established hypoxia mimetic agent in *S. cerevisiae* [10] and in mammalian cell in culture [11]. It has hence been widely used to study the effects of hypoxia on cellular activities.

In the present communication, we report functional characterization of the *YLR162W* gene product.

Materials and Methods

Chemicals, Growth Media and Growth Condition

Media components were purchased from Becton Dikinson and Co. and all reagents used were of molecular biology grade. *S. cerevisiae* cells were grown at 30°C either in SD (synthetic dextrose) or in SG (synthetic galactose) media containing appropriate supplements. Wherever indicated $CoCl_2$ was added to a final concentration of 0.75 mM to the media to induce hypoxia specific gene expression. Hypoxia specific repression of *ROX1* and induction of *OLE1* was confirmed in control experiments in cells exposed to $CoCl_2$ in parallel with cells exposed to hypobaric hypoxia (Table 1).

Cloning Techniques, DNA Sequencing and Identification of *YLR162W* by Sequence Similarity Search

All cloning experiments were performed according to Sambrook et al. [12]. $CoCl_2$ resistant DNA synthesis mutants were isolated from BJ5416 cells based on their ability to incorporate α-^{35}SdATP into their chromosomal DNA in the presence of $CoCl_2$. Subtractive hybridization [13] was performed with the PCR-select subtractive hybridization kit (BD Biosciences) according to the manual supplied by the manufacturer. Equal amounts of Poly-A^+ RNA (1 μg) isolated from the mutant and BJ5416 cells either following exposure to 0.75 mM $CoCl_2$ for 3 h or without any exposure were mixed before cDNA synthesis to ensure equal representation of RNAs expressed under stressed and non-stressed conditions in the two strains. Subtractive hybridization was carried out with cDNA synthesized from Poly-A^+ RNA isolated from the mutant as driver and that from BJ5416 cells as tester. The subtracted library was amplified twice by PCR using primers (specific for the tester) supplied in the kit, digested with *Rsa* I and ligated into *Eco*R I digested, end-filled Bluescript. The library was transformed into *E. coli* XL1-Blue cells and transformants containing inserts were identified by blue-white screening. Recombinant plasmids isolated

Table 1 Microbial strains and vectors used in this study

Strains	Genotype	Source
BJ5416 (ABC709)	*MATa ura3-52 lys2-801leu2-D1his3-D200 GAL*	Dr. S. Biswas JNU, Delhi, India.
BY4741 (ABC733)	*MATa his3*Δ*0 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	Dr. A. Bachhawat. IMTECH, Chandigarh, India.
BY4741pBG1805*YLR162W-3xHA*	BY4741pBG1805*YLR162W-3xHA*	This work.
Y800*OLE1-3xHA*	*MATaleu2* − Δ98*cry1*^{R}*ade2* − 101*HIS3* *Ura3* − 52*can1*^{R}lys2 − 801 *CYH2rrp1* − 1 *OLE1* :: *OLE1* − 3*xHA*	Open Biosystems.
	*MAT*α*leu2* − Δ98*CRY1*^{R}*ade2* − 101*his3* − Δ200*Ura3* − 52*CAN* *1lys2* − 801 *cyh2*^{R}*TRP1Cir*^{0}*OLE1* :: *OLE1* − 3*xHA*	
E. coli XL1-Blue	[12]	Bangalore Genei
Bluescript	[12]	Dr. S. Biswas JNU, Delhi, India.

from the transformants were digested with *Bam*H I and *Hind* III to confirm clones containing inserts. DNA sequencing was performed commercially (Lab India Pvt. Ltd) using both T3 and T7 universal primers and the region between restriction sites *Bam*HI and *Hin*dIII was used as a query for similarity search in the Saccharomyces ORF database (SGD).

Cell Survival Assay

Cell survival assays were performed by spotting ten fold serial dilutions (10^3, 10^2, 10^1, 10^0 cells) of log phase cells of the appropriate strain onto plates containing media and additives as indicated in the respective figures. Plates were incubated at 30°C for three days before being photographed.

Northern Blotting

Northern blotting was performed as per standard procedure [12]. ^{32}P-labeled single stranded probes were prepared by primer extension with the Klenow fragment of *E. coli* DNA polymerase I using anti-sense primer (*YLR162W*AS: 5′ GGATCCGAATTCCTA GCAACGGGTGCTCTTGGCG GAAAGGCC 3′) and PCR amplified *YLR162W* as template in the presence α-^{32}PdATP (Primers *YLR162W*S: 5′ ATC GATAAGCTT ATATGCAGCACGCTTACCCGGACCG CCTCT 3′ and *YLR162W* AS).

Protein Isolation and Western Blotting:

Cell lysis was performed as described in [14] and proteins of interest were detected by immuno-blotting using appropriate Antibodies.

Cell Cycle Progression by Flow Cytometry:

Cell cycle progression during growth under optimal conditions and in the presence of 0.75 mM $CoCl_2$ was determined by flow cytometry essentially as described in [15] except that cells were synchronized by overnight incubation in minimal media containing 5 μg/ml α1-mating factor (Sigma). 10^4 events were acquired per sample and counts versus FL2-A histograms were obtained in a Becton–Dickinson flow cytometer (488 nm argon laser) and further analyzed using Cell Quest software.

Estimation of Cell Death by Propidium Iodide Staining

Log phase cells were shifted to galactose containing media to induce *YLR162W* expression and either exposed to $CoCl_2$ (0.75 mM) for 3 h or kept at 30°C without $CoCl_2$ as the untreated control. 2.5×10^6 cells from each aliquot were withdrawn, washed with PBS and suspended in 500 μl PBS followed by the addition of 15 μl of 1 mg/ml propidium iodide (PI) solution. After incubation at 30°C for 30 min, aliquots of 10^4 cells were analyzed by flow cytometry (Becton–Dickinson, 488 nm argon laser) for fluorescence. An acquisition protocol was defined to measure Forward scatter (FSC), Side scatter (SSC) and fluorescence (FL2-H) on a four decades logarithmic scale. Further analysis was done by Cell Quest software.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured essentially as described in [16]. Briefly, log phase cells in a total volume of 3 ml were shifted to galactose containing media to induce *YLR162W* expression and were harvested at different intervals of time. NaN_3 was added to a final concentration of 20 mM to a separate culture to be used as a control. 10^6 cells were harvested for each reading, washed twice with ice–cold water and suspended in water at 10^6 cells/ml, Rhodamine123 (Rh123; Sigma) was then added to a final concentration of 25 nM and incubated for 10 min at 30°C in dark. 2×10^4 cells were analyzed using an acquisition protocol to measure Forward scatter (FSC), Side scatter (SSC) and Green Fluorescence (FL1-H) on a four decade logarithmic scale. Further analysis was done by cell Quest software.

Results and Discussion

Cloning of *YLR162W*

We performed subtractive hybridization cloning of genes that were not expressed in a $CoCl_2$ resistant DNA synthesis mutant in comparison to its wild type counterpart (BJ5416) to identify factors responsible for efficient DNA synthesis in a hypoxic environment. Positive clones were confirmed by restriction digestion and all the inserts obtained were between 200 and 300 bp in length (Fig. 1a). Following sequencing of the inserts and similarity search in the *Saccharomyces* ORF Database one of the clones was found to be identical to a part of *YLR162W*–an uncharacterized *S. cerevisiae* ORF (Fig. 1b and a, Lane 3). Northern blotting experiments indicated that *YLR162W* transcript level was reduced in BJ5416 cells during exposure to $CoCl_2$ in comparison to growth under optimal conditions. It was undetectable in the mutant both in the presence and absence of $CoCl_2$ (Fig. 1c). Since we had set out to clone genes that were not expressed in the $CoCl_2$ resistant DNA synthesis mutant, the northern blotting data vindicated our subtractive hybridization experiment.

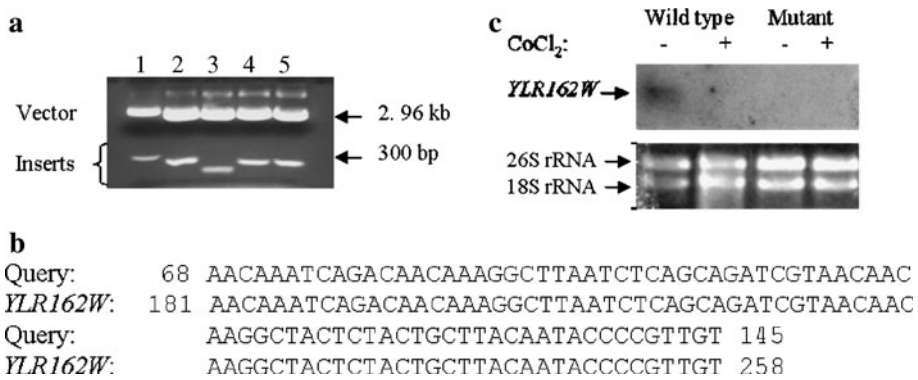

Fig. 1 *YLR162W* cloning and expression under $CoCl_2$ induced hypoxia. **a** Restriction digestion of clones obtained following subtractive hybridization: recombinant plasmid isolated from the clones were digested by *Bam*H I and *Hind* III and analyzed by agarose gel electrophoresis. **b** Sequence similarity between the cloned gene (query) and *S. cerevisiae* ORF *YLR162W*. **c** *YLR162W* transcript level during exposure to $CoCl_2$: 20 μg total RNA isolated from BJ5416 cells and $CoCl_2$ resistant DNA synthesis mutant either following exposure to 0.75 mM $CoCl_2$ for 30 min or without any exposure was analyzed for *YLR162W* expression by Northern blotting and hybridization with full length *YLR162W* anti-sense probe. The experiment was carried out with two different populations of total RNA and representative results are shown. **c** Lower panel: 2 μg of the total RNA used in the above blot was analyzed by agarose gel electrophoresis to determine if the RNA was intact

YLR162W Over-Expression Rendered BY4741 Cells Susceptible to $CoCl_2$

For functional characterization of the *YLR162W* gene product, we studied the phenotypic effects of its over-expression in BY4741 cells. Continuous expression of *YLR162W* had a slight inhibitory effect on cell proliferation but rendered cells extremely sensitive to chronic $CoCl_2$ exposure (Fig. 2a). Neither acute exposure to $CoCl_2$ for 2 h during *YLR162W* expression nor chronic exposure to $CoCl_2$ alone had any significant effect on cell viability (Fig. 2a). Figure 2b and c are controls demonstrating the induction of hypoxia specific Ole1p expression during exposure to $CoCl_2$ (Fig. 2b) and expression of Ylr162wp in media containing galactose (Fig. 2c), respectively.

Cell Cycle Progression was Inhibited in Cells Expressing *YLR162W*

Further characterization of the growth inhibitory properties of Ylr162wp especially in the presence of $CoCl_2$ was carried out by cell cycle progression studies of cells expressing *YLR162W* in the presence and absence of $CoCl_2$. Results demonstrated that following removal of the α-factor block a fraction of the cells expressing *YLR162W* entered the S phase (15–30 min, Fig. 3) but did not enter the G2 phase of the cell cycle, instead, the sub-G1 and G1 peaks re-emerged (45 min onwards, Fig. 3). Since a sub-G1 peak is indicative of apoptotic cells [17 and 18] it appears that *YLR162W* expression induced apoptosis in BY4741 cells. The presence of $CoCl_2$ in the growth media did not have any significant additional effect. Inhibition of cell cycle progression is known to require the activation of checkpoint pathways [19]; we hence determined the response of checkpoint pathway mutants to $CoCl_2$ during *YLR162W* expression. The response of the *chk1*Δ, *rad9*Δ and *dun1*Δ cells towards exposure to $CoCl_2$ during *YLR162W* expression was essentially identical to that of BY4741 cells (data not shown; similar to Fig. 2a). Our results suggested that the combined effect of *YLR162W* and $CoCl_2$ on cell viability was irrespective of checkpoint functions since the absence of key checkpoint proteins could not overcome the above inhibitory effect. Some apoptotic pathways however are known to be independent of checkpoint functions in yeast [20].

Cell Death and Decreased Mitochondrial Membrane Potential in *YLR162W* Expressing Cells

Apoptosis induced by *YLR162W* over-expression (Fig. 3) was further investigated by determining the percentage of cells that were permeable to propidium iodide (dead cells) at early time points following the induction of *YLR162W* expression. Results (Fig. 4a) showed that *YLR162W* overexpression caused an increase in the number of cells permeable to propidium iodide as indicated by the flow cytometry data (Fig. 3). $CoCl_2$ did not have any additional effect over that observed during *YLR162W* expression alone (as also observed in the flow cytometry data, Fig. 3). Since mitochondrial membrane potential (ψ_m) is decreased upon initiation of apoptosis [18], we measured ψ_m in *YLR162W* expressing cells. Mitochondrial membrane potential began decreasing within 30 min of induction of *YLR162W* expression and continued to decrease further for the next

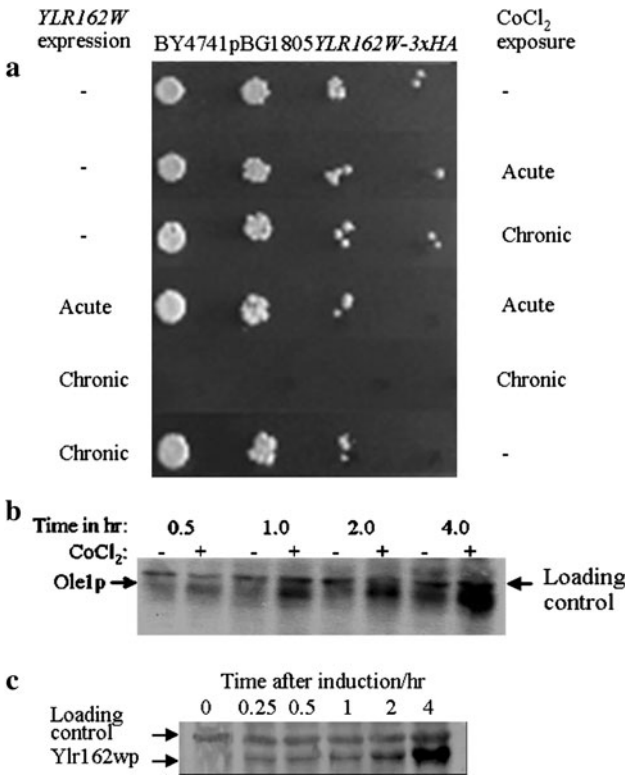

Fig. 2 Effect of *YLR162W* expression on cell viability in the presence and absence of $CoCl_2$: **a** for acute exposure to $CoCl_2$ in the presence of *YLR162W*, cells were grown in SG broth containing 0.75 mM $CoCl_2$ for 2 h, exposure to $CoCl_2$ alone was carried out in SD broth. Chronic exposure to $CoCl_2$ was carried out in plates containing 0.75 mM $CoCl_2$, SG plates were used for concurrent expression of *YLR162W* and SD plates for exposure to $CoCl_2$ alone. Cells were spotted in tenfold serial dilutions and SG plates were incubated for four days at 30°C to compensate for the slower growth rate in galactose containing media. **b** Induction of *OLE1* expression by $CoCl_2$: Ole1p levels were detected by immunoblotting, 40 µg total protein isolated fromY800*OLE1-3xHA* that were either untreated or treated with 0.75 mM $CoCl_2$ for periods of time as indicated in the figure was electro-blotted onto PVDF membranes and probed with anti-HA Ab and developed with HRP conjugated secondary Ab and chemiluminescent HRP substrate. **c** *YLR162W* expression in media containing galactose: 40 µg of total cellular protein isolated at different time points from a log phase culture of BY4741pBG1805*YLR162W-3xHA* cells following transfer to galactose containing media was analyzed for Ylr162wp levels as described in (**b**) with anti-HA Ab and alkaline phosphatase conjugated secondary Ab, blots were developed with BCIP and NBT

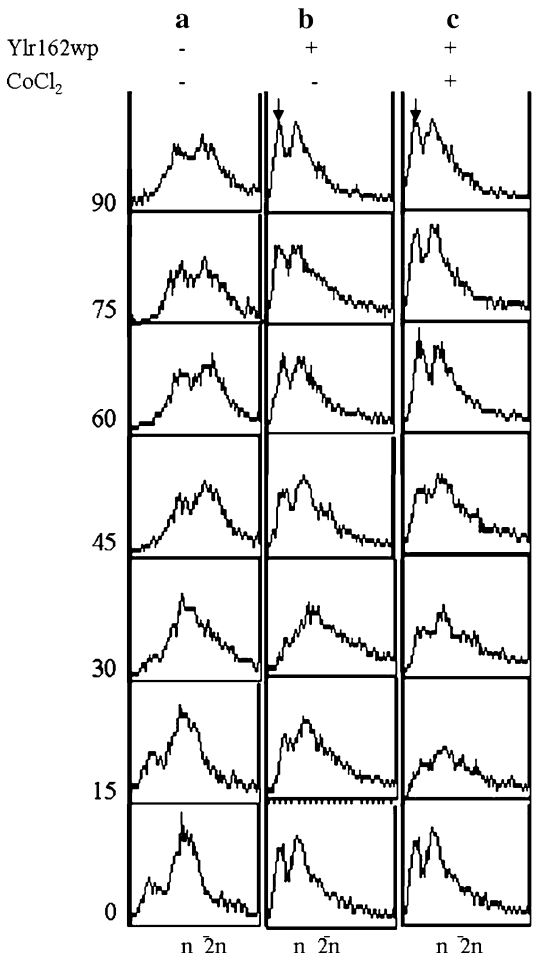

Fig. 3 Cell cycle progression of *YLR162W* expressing cells. Cell cycle progression of **a** BY4741 in SD media (Control) **b** BY4741pBG1805 *YLR162W-3xHA* in SG media and **c** BY4741pBG1805*YLR162W-3xHA* in SG media containing 0.75 mM $CoCl_2$ was determined by flow cytometry of aliquots taken out at regular intervals of time following removal of the α-factor block. The sub-G1 peaks indicative of apoptotic cells have been indicated by *arrows* (↓)

30 min (Fig. 4b). The data (Fig. 4a, b) demonstrated that over-expression of *YLR162W* induced cell death and caused a substantial reduction in ψ_m.

Functional analysis of Ylr162wp based upon phenotypic studies of cells expressing the protein demonstrated that, at early time points following the induction of *YLR162W* expression in BY4741 cells, cell cycle progression was inhibited (Fig. 3), mitochondrial membrane potential was decreased (Fig. 4b) and cell death was increased (Fig. 4a). Cells expressing *YLR162W* were rendered extremely susceptible to the hypoxic conditions induced by $CoCl_2$ (Fig. 2a).

The growth inhibitory property of Ylr162wp seems to explain its up-regulation under stress conditions and when cells do not actively proliferate; Ylr162wp may hence be part of the cellular stress response mechanism that inhibits cell proliferation during exposure to stressful conditions and thereby conserves cellular ATP reserves that is required during adaptation to adverse environmental conditions. In addition, the hitherto uncharacterized *S. cerevisiae* ORF *YLR162W* or peptides synthesized based upon Ylr162wp may be important pharmaceutical agents for its growth inhibitory properties especially during exposure to hypoxic conditions.

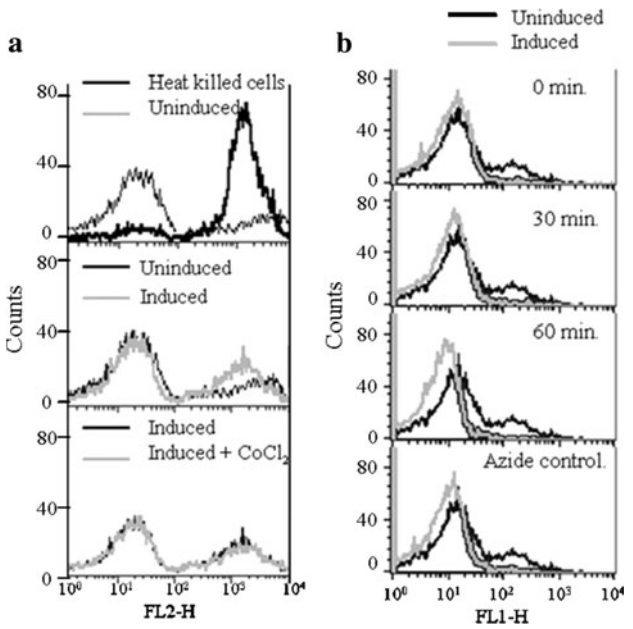

Fig. 4 Effect of *YLR162W* expression on **a** Cell death and **b** Mitochondrial membrane potential. Experiments were performed essentially as described in materials and methods

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