REDOX REGULATION OF HUMAN P53 TUMOR SUPPRESSOR GENE ACTIVITY: IDENTIFICATION OF REDOX GENES THAT PLAY ROLE IN HUMAN P53 REPORTER GENE ACTIVITY

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ABSTRACT

REDOX REGULATION OF HUMAN P53 TUMOR SUPPRESSOR GENE ACTIVITY: IDENTIFICATION OF REDOX GENES THAT PLAY ROLE IN HUMAN P53 REPORTER GENE ACTIVITY

The occurence of *p53* gene mutations in many human tumors shows that p53 protein plays an important role in preventing cancers. One of the most important function of p53 protein is its ability to stimulate transcription of other genes that inhibit cell cycle progression and DNA repair mechanisms or apoptosis pathways. p53 gene activity is controlled by a series of mechanisms among which redox regulation has taken little attention. Because of its importance as a tumor suppressor, its role as a cell cycle control protein and transcription factor, we decided to focus on p53 transcriptional activity. Human p53 can be studied in yeast where genetic tools can be used to identify proteins that affect its ability to stimulate transcription of reporter genes. Several studies have shown that the p53 protein is prone to oxidative inactivation. Although yeast does not contain a *p53* gene, the similarity of cell cycle control mechanisms and oxidative stress response pathways prompted us to ask whether human p53 was active in yeast cells lacking individual antioxidant genes. In this study, using yeast deletion mutants, p53 reporter gene activity was assayed in different antioxidant mutants that were identified by REDOX-Cys-Search bioinformatic tool by previous studies. Seven antioxidant genes were found to be important in regulating p53 activity. These genes played role in phosphatidylinositol pathway, protein dephosphorylation, cellular iron metabolism, DNA mismatch repair, and three other unknown biochemical pathways.

Identification of these new proteins that regulate p53 activity may have broad implications in understanding the complex behaviour of p53 and tumor formation in humans.

ÖZ

İNSAN P53 TÜMÖR BASKILAYICI GEN AKTİVİTESİNİN REDOX REGÜLASYONU: İNSAN P53 RAPORTÖR GEN AKTİVİTESİNDE ROL OYNAYAN REDOKS GENLERİNİN BELİRLENMESİ

İnsan tümörlerinin çoğunda p53 mutasyonlarının görülmesi bu proteinin kanser önlemede önemli rol oynadığını gösterir. p53 proteinin bir transkripsiyon faktörü olarak en önemli fonksiyonu, başka proteinleri aktive ederek hücre döngüsünün durmasını ve DNA tamiri veya kontrollü hücre ölümü yolaklarının aktive edilmesini sağlamasıdır. p53 aktivitesini kontrol eden birçok mekanizma bilinmesine rağmen redoks kontrol mekanizması fazla çalışılmamıştır. Tümör süpressör olarak büyük öneme sahip olması ve hücre döngüsünün kontrolünde önemli roller üstlenmesi nedeni ile bu projede p53 proteinin redoks regülasyonunu çalışmak istedik. İnsan p53'ü, mayada raportör genlerin transkripsiyonunu uyaran proteinleri belirleyen genetik araçların kullanılmasıyla çalışılabilir. Bazı çalışmalar p53 proteininin oksidatif inaktivasyona yatkın olduğunu göstermiştir. Mayada p53 geni bulunmamasına rağmen benzer hücre döngüsü kontrol mekanizmaları ve oksidatif stres yanıt yolakları bizi insan p53'ünü ayrı antioksidan genleri eksik olan maya hücrelerinde aktif olup olmadığını sorgulamaya yöneltti. Yaptığımız ön çalışmalar ile maya hücrelerinde bulunan bütün antioksidan genler, yapılarında bulunan bazı ayırt edici karakterler sayesinde REDOXCysSearch olarak bilinen biyoinformatik programının yardımı ile tespit edildi. Bu çalışmada antioksidan genlerin delesyon mutantları kullanılarak insan p53 raportör gen aktivitesini etkileyen antioksidant genler tespit edildi. Bu genler biyokimyasal olarak fosfotidil inositol, protein defosforilasyonu, hücresel demir metabolizması, DNA yanlış eşleşme tamiri (mismatch repair) yolaklarında ve üç ayrı bilinmeyen yolakta daha rol oynamaktadır. p53 aktivitesini düzenleyen yeni proteinlerin belirlenmesinin geniş uygulama alanları olacaktır. p53'ün redoks regülasyonu transkripsiyon faktörü olarak aktivitesinin kontrolü açısından yeni bir örnek teşkil edecektir.

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CHAPTER 1

INTRODUCTION

1.1. p53 Protein

p53 is one of the most important tumor suppressor proteins. It is shown to be mutated in more than 50% of the tumor cells. p53 protein is discovered firstly in SV40 tumor virus containing tissues in high levels and defined as a tumor antigen in 1979 (Lane and Crawford 1979, Linzer and Levne 1979). p53 is found in low levels in a normal cell; however under cellular stress conditions like DNA damage, hypoxia, dNTP shortage and absence of telomerase activity, it is shown to be one of the most induced proteins (Vousden and Lu 2002). These stress conditions stabilizes and activates p53 that causes cause cell cycle arrest and activates repair mechanisms. However, if the damage can not be repaired, apoptosis is initiated. There are more than 100 genes determined as activated or inhibited by p53 as a transcription factor. According to bioinformatic studies there are more than 4000 potential p53 binding sites in genome (Vousden and Lu 2002). These studies enlighten the importance of p53.

1.2. p53 Structure

p53 protein has five domains including transactivation, proline rich, DNA-binding, tetramerization and negative regulation domains (Figure 1.1). This protein performs as a tetramer by binding to DNA sequence specifically (Figure 1.2). 95% of the p53 mutations shown to be in the DNA binding domain resulted with the loss of DNA binding ability of the protein (Vousden and Lu 2002). DNA binding ability of p53 can also be affected by the redox state of cystein residues in the protein especially in its DNA binding domain. Intermolecular or intramolecular extra disulfate bridges could prevent the molecule from binding to DNA (Pearson and Merill 1997).

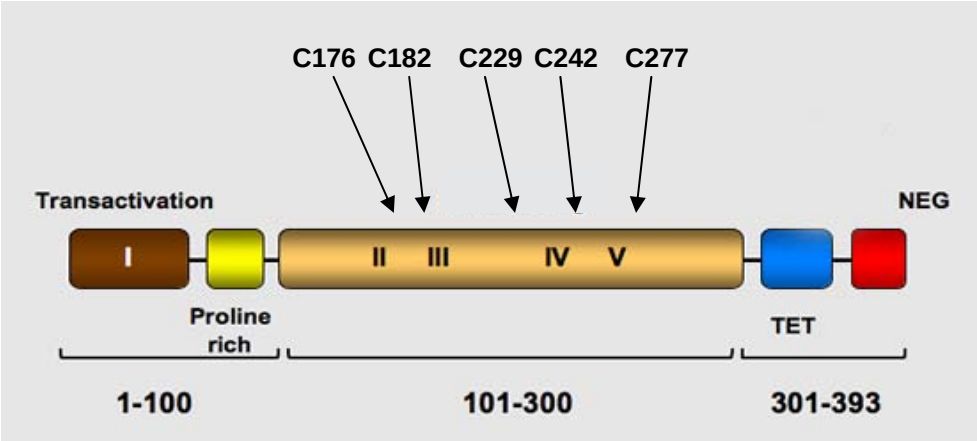

Figure 1.1. Domains of p53 protein and location of the cyctein residues in DNA-binding domain (Source: p53.free.fr)

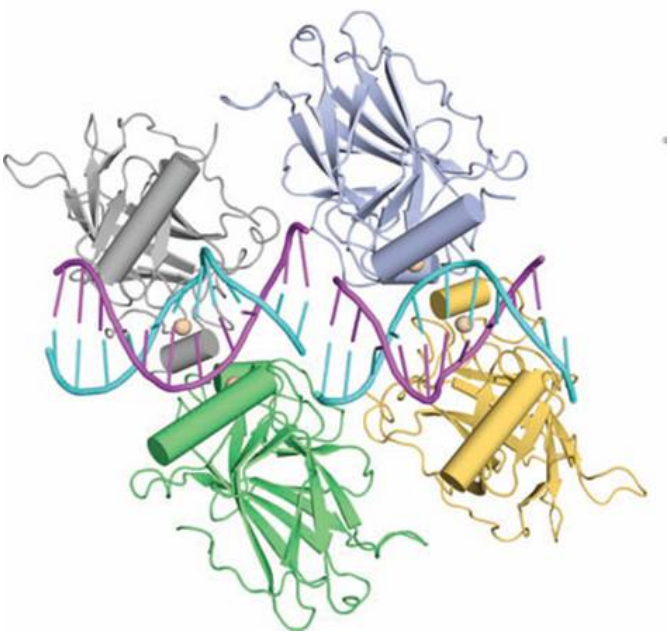

Figure 1.2. p53 tetramere bound to DNA (Source: Joerger and Fersht 2008)

1.3. Regulation of p53

In addition to known effects of mutations to p53 structure and activity can be altered transcriptionally, translationally, post-translationally or by controlling its function independent from its expression.

Regulation of p53 at transcriptional level was shown by inductive effect of interferon α/β in 2003 (Takaoka, et al. 2003). At translational level, RNA binding HuR protein is shown to increase the p53 expression (Mazan-Mamczarz, et al. 2003, Galban, et al. 2003).

Post-transcriptionally p53 level is hold in low levels mainly by Mdm2 under normal conditions. Mdm2 has E3 ubiquitin ligase activity. It has ability to bind to p53 and to mark it for proteozomal degradation (Honda, et al. 1998). Mdm2 is transcriptionally induced by p53, thus p53 controls the cellular level of itself by negative feed back (Momand, et al. 2000). DNA damage and stress signals inhibit the interaction between Mdm2 and p53 then increase the stability and cellular level of p53. In this way p53 can bind to specific sequences to activate specific genes of interest (Kaustov, et al. 2006). In some tumors, the increased level of proto-onco gene Mdm2 inactivates p53 without any mutation. (Momand et al. 2000). While the transcription factor YY1 increrases Mdm2 originated p53 degradation (Gronroos, et al. 2004, Sui, et al. 2004), AbI, $p14^{ARF}$, L11, PML and Rb proteins decrease (Hsieh, et al. 1999, Lohrum, et al. 2003, Bhat, et al. 2004, Zhang, et al. 2003). Except that Mdm2, other E3 ubiquitin ligases like Pirh2, COP1, CHIP and ARF-BP1 proteins take part in regulation of p53 levels by protein-protein interaction. (Leng, et al. 2003, Dornan, et al. 2004, Esser, et al. 2005, Chen, et al. 2005).

There are numerous post-transcriptional covalent modifications that p53 can encounter. Phosphorylation, acetylation, methylation, ubiquitination, sumoylation, glycosylation and neddylation are those identified while some of them stabilizing, destabilizing or both (Chuikov, et al. 2004, Xirodimas, et al. 2004) (Figure 1.3). Other than covalent modifications, there are some molecules which regulate the activity of p53 by non-covalent interactions.

Phosphorylation sites of p53 are shown to be serine and threonine residues found in 17 different positions. (Xu 2003, Bode and Dong 2004). There are different effects of p53 phosphorylation. The effect of modification differs as stabilizing or destabilizing depend on the residue that was phosphorylated. (Katayama, et al. 2004, Li, et al. 2004). Different kinases are determined to phosphorylate different residues and more than one site could be phosphorylated at the same time. This situation is thought to be related with different responses to different stimulants. (Xu 2003; Bode and Dong 2004). However, phosphorylation of p53 does not known to increase the DNA binding ability *in vivo*. (Feng, et al. 2005).

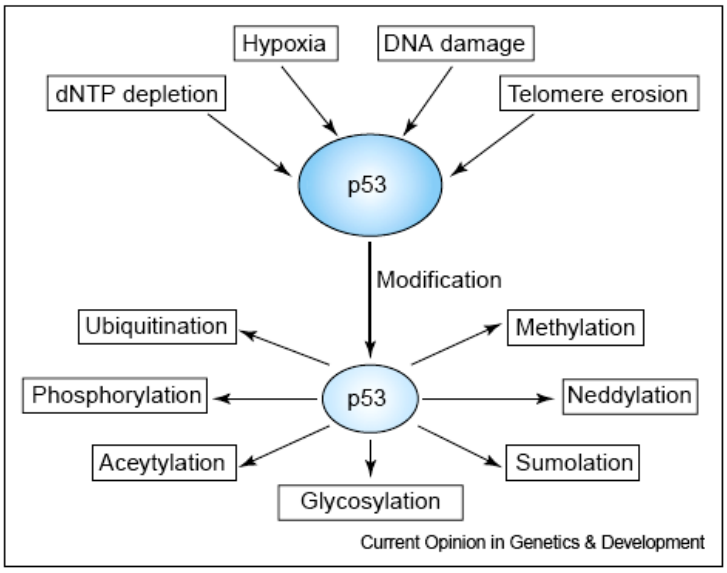

Figure 1.3. p53 modifications in response to cellular stres

(Source: Lu 2005)

The methylation, acetylation and sumoylation of p53 is shown to make the molecule stable and increase its transactivation and DNA binding ability (Chuikov, et al. 2004, Luo, et al. 2004; Lu 2005). Methylation of p53 ensures the molecule to stay in the nucleus and increase its transcriptional ability. In case of DNA damage, p53 is shown to be methylated at the 372th lysine residue by a methyl transferase Set9 both *in vitro* and *in vivo*. (Chuikov, et al. 2004). p53 activating enzyme p300/CBP acts as an co-activator by histon acetylation. (Liu, et al. 2003). Other studies show that p300/CBP also acetylates p53 at the carboxy terminal and could affect its DNA binding ability (Luo, et al. 2004). SUMO-1 binds to transcription factors like p53 and increases its transcriptional activity. Mechanism of the increased activity as a result of the sumoylation at 386th lysine residue is not known. The cross-interaction between acetylation and sumoylation which occurs both at the carboxy terminal does not identified (Liu and Chen 2006).

Deacetylation, ubiquitination and neddylation are known to decrease the stability and transcriptional activity of p53. By deacetylating the acetylated p53, histone deacetilase (HDAC) 1, 2 and 3 are shown to decrease its transcriptional activity (Juan, et al. 2000; Insinga, et al. 2004). HDAC does not interact directly with p53. Metastasis associated protein 2 (MTA2)/PID, Sir2α and PML-retionic acid receptor (RAR) are found to take part to repress p53 by histon deasetylases. (Luo, et al. 2000, Luo, et al. 2001, Insinga, et al. 2004).

Ubiquitination is a pathway used for selectively degrading cellular proteins. Addition of ubiquitin molecules covalently to proteins marks them for proteozomal degradation. (Hershko and Ciechanover 1998). While mono-ubiquitination of p53 is the signal to exit the nucleus, poly-ubiqutination is the signal for its proteozomal degradation by 26S proteosome. For normal progress of cell cycle p53 levels should be regulated thightly which mainly controlled by ubiquitination of p53 by Mdm2. (Li, et al. 2003, Yang, et al. 2004). Mdm2 can both mono ubiqutinate and by the help of p300, poly-ubiquitinate p53 (Grossman, et al. 2003). The ubiquitination of p53 by both Mdm2 and other E3 ubiquitin ligases is shown to be recycling. Herpes virus associated ubiquitin specific protease (HAUSP) is shown to stabilize p53 both *in vitro* and *in vivo*. However the effect of HAUSP is not understood completely. (Li, et al. 2002).

NEDD8 is an ubiquitin like molecule is also named as Rub1 or Apg12. Neddylation of p53 is promoted by Mdm2 similar to its ubiquitination and the process is resulted with the loss of transcriptional activity (Xirodimas, et al. 2004).

There are some other molecules regulating p53 activity like Ref-1. (Liu and Chen 2006). Regulating the redox state of some proteins and acting as a DNA repair endonuclease are its two distinct functions of Ref-1. It is shown to induce the DNA binding ability of p53 independent of its carboxy terminal when oxidized or dependent when reduced (Gaiddon, et al. 1999). Moreover, selenomethyonine which has anti-cancer properties requires Ref-1 in redox mechanism to activate p53 (Seo, et al. 2002). In contrast to other known modifications, redox regulation of p53 is not quite studied. Consequently, I want to study redox regulation of p53 to understand the influence of anti-oxidant genes on one of the most important tumor supressor protein.

1.4. Previous p53 Studies with Yeast

Yeast is a preferable model organism to take advantage of genetic studies. It is also used in some p53 studies. Although yeast does not have p53 gene there are similar cell cycle control mechanisims with human. The role of *PAK1* and *TRR1* genes are identified by this way. p53 activating kinase (PAK1) over-expressing *Saccharomyces cerevisiae* strains are showed increased DNA binding capasity. Increased transactivation of p53 is thought to be originated from phosphorylation by *PAK1* from its carboxy terminal. (Thiagalingam, et al. 1995). In 1998, it is found that in the absence *S.*

cerevisiae thioredoxin reductase coding gene, the DNA-binding activity of p53 is diminished (Pearson and Merrill 1998). This effect is understood by the reduced expression of the reporter gene coded after multiple p53 binding sites. This binding site is determined as RRRC(A/T)GYYY by oligonucleotide selection experiments (Funk, et al. 1992). The ectopic expression of disulphate bridge reducing *TRR1* gene in *TRR1* mutant yeast strain recovered p53 activity (Pearson and Merrill 1998). *TRR1* studies suggest that the inhibition of p53 activity is caused by the reduced cycteins of directly p53 or indirectly other related proteins as a result of thiol oxidation (Merrill, et al. 1999, Merwin, et al. 2002). In the light of the literature, I want to explore the effects of the known and potential redox proteins of *S. cerevisiae* on p53 by deletion mutants of those genes in this study.

1.5. Yeast Anti-oxidant Genes

Main mechanisms that regulate protein activity are kinase/phosphotases and thiol dependent oxido-reductases. There are mainly two redox mechanisims which are thioredoxin and glutathione systems. Both systems transfer the electrons of NADPH to their target molecule (Holmgren 2000). These type of proteins include a beta-sheet at the center surrounded by alfa-helices with one or two cycteins at the active site which is called thioredoxin-fold motif (Martin 1995). Cysteins have important redox functions in protein activity by controlling reversible oxidation and reduction reactions. Most studied redox motif is CXXC as two cycteins are separated by two other residues. Recent studies suggest that CXXS, TXXC, CXXT, SXXC motifs also provide redox activity (Fomenko and Gladyshev, 2003). Other amino acids located on both sites of these motifs and secondary structures determines the function of these proteins (Woycechowsky and Raines, 2000).

Bioinformatic studies enable us to study these antioxidant genes genome-wide instead of searching in single gene level. All oxido-reductive proteins can be determined in organisms those gemome was sequenced by using bioinformatic algorithms (Fomenko and Gladyshev, 2002, Fomenko and Gladyshev, 2003, Fomenko, et al. 2007). The list of potential and known redox proteins in *S. cerevisiae* was composed by the help of Dr. Fomenko previously (Table-1). The software used for this study can be found in its web site (Genomics, 2008). The algorithm that REDOXCysSearch software

followed is given in Figure 1.4. The obtained protein sequences of yeast in FASTA format are searched for containing the specific motifs which are CXXS, TXXC, CXXT and SXXC. Metal binding proteins are eliminated from the selected ones as these motifs are also capable of binding metal ions. The remaining proteins are analysed for conservation through other species. If the candidate protein is conserved then secondary structure prediction is made to determine the compatibility of the motif with thioredoxin-fold motif. The selected proteins are analysed individually for their structural and biochemical properties and as a result 88 known and potential yeast proteins are selected as anti-oxidant (Fomenko and Gladyshev 2002, 2003, Fomenko et al, 2007).

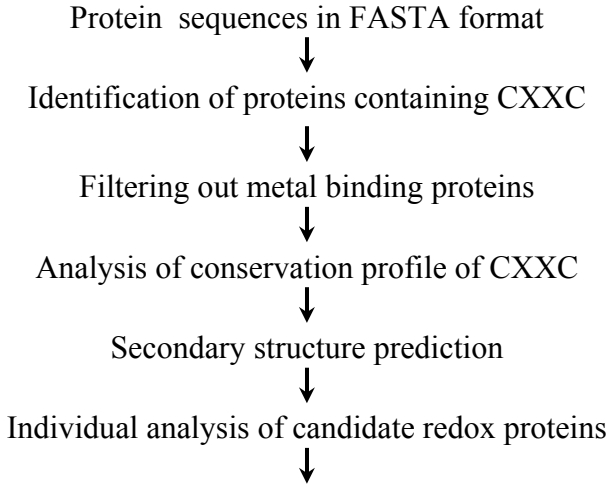

Figure 1.4. Schematic representation of the algorithm for identification of thiol/disulfide oxidoreductases (Fomenko and Gladyshev 2002, 2003, Fomenko et al, 2007)

The yeast deletion mutants selected according to the REDOXCysSearch results are listed in Table-1 and they were obtained from EUROSCARF organization commertially (Euroscarf, 2000). All the deletion mutants in this table will be analysed for p53 gene activity to enlighten the roles of antioxidant genes in the regulation of p53.

Table 1.1. Redox active yeast genes

gi number	Protein length	CxxC position	Secondary structure	Description
6319806	***522***	***60*** ***405***	***b-CGHC-a*** ***b-CGHC-a***	protein disulfide isomerase; Pdi1p
6319814	***110***	***26***	***b-CPYC-a***	Glutaredoxin; Grx1p

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Table 1.1 (cont.)

6319925	***127***	***54***	***b-CGPC-a***	Mitochondrial thioredoxin; Trx3p
6320492	***114***	***30***	***b-CGLC-a***	thioredoxin (COG)
6320720	***143***	***60***	***b-CPYC-a***	Glutaredoxin
6681846	***189***	***29*** ***129***	***CRSC-a*** ***a-CNWC-a***	Protein essential for mitochondrial biogenesis and cell viability; Erv1p
6321648	***104***	***30***	***b-CGPC-a***	thioredoxin; Trx2p
6322186	***701***	***59*** ***199***	***b-CPHC-a*** ***b-CDKC-a***	Thiol-disulfide isomerase
6322811	***256***	***195***	***b-CTSC-a***	Nifu-like protein; Nfu1p
6323072	***103***	***29***	***b-CGPC-a***	thioredoxin;
6323396	***109***	***24***	***b-CPDC-a***	Glutaredoxin (COG- thioredoxin)
6323505	***561***	***348*** ***351***	***a-CVQC-a*** ***a-CDRC-a***	involved in protein disulfide bond formation in the ER; Ero1p
6324484	***277***	***55***	***b-CQHC-a***	Thiol-disulfide isomerase (thioredoxin?????)
6324862	***318***	***58***	***b-CGHC-a***	Thiol-disulfide isomerase (thioredoxin?????)
12621477	**82**	**46**	**CPSC-a**	Similar to Hemiascomycetous yeast protein
6320163	**562**	**371**	**b-CVTC-a**	regulator of silencing at HML, HMR, telomeres, and rDNA; Sir2p
6320169	**114**	**38**	**a-CNSC-**	Hypothetical ORF; Ydl034wp
6320293	**382**	**121**	**a-CRNC-a**	splice site choices and 2nd step of splicing; Slu7p
6321227	**193**	**21**	**a-CELC-a**	Predicted ATPase of the PP-loop superfamily implicated in cell cycle control (COG)
6322432	**111**	**52**	**a-CFAC-a**	peroxisome proliferator-activated receptor
6322474	**198**	**7**	**-CGIC-a**	Translation initiation factor (SUI1) (COG)
6322548	**954**	**71** **207**	**b-CCYC-a** **-CIYC-**	inhibitor of apoptosis protein
6322637	**623**	**391**	**b-CMDC-a**	integral membrane protein localizing to the ER and Golgi
6323301	**274**	**247**	**b-CFFC-a**	Hypothetical protein Ylr271wp
6323600	**280**	**243** **255**	**b-CSIC-a** **-CVNC-a**	Hypothetical protein
6323622	**332**	**77**	**b-CQLC-a**	Putative new 37kDa subunit of N-oligosaccharyltransferase complex; Ost6p
6323747	**620**	**529**	**a-CRRC-a**	Homolog of samB gene of Aspergillus nidulans (deletion of samB results in mislocalization of septa
6324005	**879**	**4666**	**b-CIDC-a**	FIG4 expression is induced by mating factor.;
6324209	**493**	**2** **365**	**CQRC-a** **b-CQIC-a**	Predicted ATPase of the PP-loop superfamily implicated in cell cycle control (COG)
6324224	**1183**	**445**	**b-CLDC-a**	Synaptojanin-like protein
6324311	**112**	**23**	**a-CEKC-a**	Hypothetical protein
6324504	**503**	**317**	**b-CVTC-a**	Homolog of SIR2
6324659	**350**	**72**	**b-CSLC-a**	Catalyzes the transfer of oligosaccharide from dolichol-oligosaccharide donor to consensusglycosylation acceptor sites (asparagines) in newly synth. proteins - ER lumen; may enhance

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Table 1.1 (cont.)

				oligosacch. transfer to subset of acceptor substrates
6324683	**1107**	**420**	**b-CLDC-a**	Synaptojanin-like protein
6324770	**414**	**184**	**b-CRFC-a**	Lipoate synthase (COG)
6324832	**217**	**187**	**CCYC-a**	histidine triad superfamily, third branch;
6324848	**428**	**374**	**a-CNVC-a**	RNA isopentenyl transferase
6325150	**248**	**90**	**-CVNC-a**	Hypothetical protein
6325296	**196**	**120**	**a-CGEC-a**	homologous to ERV1; Erv2p
6319647	**580**	**144** **215**	**-CEYC-** **-CSKC-**	Nuclear pore or nuclear pore-associated protein required for nuclear membrane integrity and nuclear transport
6320126	**1049**	**984**	**-CPIC-**	Required for the vacuolar morphogenesis in yeast
6320667	**374**	**130**	**-CSEC-**	Hypothetical protein
6324347	**270**	**130**	**-CDYC-**	Hypothetical protein
6321419	**278**	**183**	**-CPSC-**	beta (38kDa) subunit of casein kinase II (CKII)
6321421	**503**	**19**	**-CGYC-**	arginyl-tRNA-protein transferase
6321725	**375**	**102**	**-CKYC-**	Biotin synthase
6321824	**630**	**47**	**-CLFC-**	Hypothetical ORF
6321882	**282**	**264**	**-CPEC-**	Yeast homolog of mammalian Ing1
6322217	**364**	**306**	**-CPGC-**	nitrogen starvation-induced protein phosphatase
6323273	**387**	**36**	**-CPVC-**	methionine aminopeptidase
6323933	**732**	**125**	**-CTQC-**	TFIIF interacting Component of CTD Phosphatase
6324552	**1001**	**51**	**-CTKC-**	Possible component of RCC1-Ran pathway
6324613	**258**	**166**	**-CPSC-**	Casein kinase II, beta' subunit
6324911	**958**	**187**	**-CGPC-**	Cytoplasmic alanyl-tRNA synthetase
6325171	**557**	**117**	**-CVYC-**	elongator protein; histone and other protein acetyltransferase; has sequence homology to known HATs and NATs
6325439	**768**	**79**	**-CPIC-**	cytoplasmic GTPase-activating protein
6226540	**251**	**221**	**-SELC-**	subunit II of cytochrome c oxidase
SxxC motif including proteins				
6323138	***176***	***58***	***b-SPTC-a***	Ahp1 alkyl hydroperoxide reductase
6320661	***196***	***44***	***b- SFVC-a***	Tsa2
6324090	**483**	**93**	**SGRC-a**	aminopeptidase of cysteine protease family
6321488	**107**	**64**	**SAPC-a**	Hypothetical ORF
14318479	**375**	**13**	**-SGMC-**	Involved in cell polarization, endocytosis and other cytoskeletal functions
CxxS motif including proteins				
6319488	***203***	***108***	***-CPYS-***	Glutaredoxin
6320193	***231***	***136***	***-CSYS-***	Glutaredoxin
6320303	***285***	***211***	***-CGFS-***	Glutaredoxin
6321022	***244***	***171***	***-CGFS-***	Glutaredoxin
6325198	***150***	***60***	***-CGFS-***	Glutaredoxin
6320726	***517***	***62*** ***405***	***-CLHS-*** ***-CIHS-***	Protein thiol-disulfide isomerase, ER-resident protein
6321642	***148***	***90***	***-CMLS-***	Required for arsenate resistance
6325458	***130***	***76***	***-CTGS-***	Required for arsenate resistance

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Table 1.1 (cont.)

6320882	**449**	**198**	**CRES-□**	S-adenosyl-L-homocysteine hydrolase
6324041	**373**	**181**	**□-CVAS-□**	Hypothetical ORF; Caf40p
6324588	**757**	**583**	**□-CISS-□**	B-type regulatory subunit of protein phosphatase 2A
6320878	**72**	**24**	**□-CASS**	Hypothetical ORF; Yer039c-ap
6321792	**472**	**3**	**CTVS-□**	Component of pheromone response pathway
6319497	**1165**	**448**	**CIAS-□**	Required for chitin synthesis
6321005	**801**	**565**	**□-CIVS-□**	Secretion (golgi retention) deficient
6322745	**717**	**405**	**□-CGTS-□**	Required for biosynthesis of cell wall
6322811	***256***	***199***	***-CSSS-***	NifU-like protein
6324247	**904**	**848**	**□-CRSS-□**	Required for mismatch repair in mitosis and meiosis
6325025	**1887**	**1305**	**CATS-□**	Trifunctional enzyme
6319816	***168***	***157***	***-CVNS-***	Methionine-Rsulfoxide reductase
14318476	**674**	**239**	**-CFNS-**	Hypothetical ORF
6323001	***250***	***244***	***-CGES-***	HesB-like protein
6324521	**396**	**284**	**-CGYS-**	S-adenosylmethionine decarboxylase
6325324	**185**	**177**	**-CGSS-**	HesB-like protein
CxxT motif including proteins				
6319721	***162***	***36***	***b- CGFT-a***	Glutathione peroxidase paralogue; Gpx2p
6321593	***356***	***30***	***b- CPFT-a***	glutathione S-transferase
6321876	**688**	**143**	**a- CLKT-a**	Involved in pheromone and pseudohyphal growth signal transduction
6322228	***163***	***35***	***b- CGFT-a***	Hydroperoxide resistance conferring gene; Hyr1p
6322826	***167***	***35***	***b- CAFT-a***	Glutathione peroxidase paralogue; Gpx1p
6323871	**524**	**253**	**b- CSGT-a**	Glycolipid anchored surface protein
6324049	**438**	**381**	**a- CLAT-a**	sterol C-14 reductase; Erg24p
6324082	**767**	**62**	**b- CGPT-a**	Protein required for cell viability; Ynl247wp
TxxC motif including proteins				
6319407	***261***	***87***	***b- TPVC-a***	mPrx Peroxiredoxin
6322180	***215***	***103***	***b- TPGC-a***	Derepression Of Telomeric silencing; Dot5p
6319726	**370**	**340**	**b- TDAC-a**	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase
6323067	**83**	**23**	**- TKHC-a**	essential during assembly for full cytochrome c oxidase activity
6323613	***196***	***44***	***b- TFVC-a***	antioxidant enzyme that provides protection against oxidation systems capable of generating reactive oxygen and sulfur species; Tsa1p
6324800	***156***	***84***	***b- TFGC-a***	Iron-sulfur cluster nifU-like protein; Isu2p
6325122	***165***	***92***	***b- TFGC-a***	Iron-sulfur cluster nifU-like protein; Isu1p

CHAPTER 2

MATERIALS AND METHODS

2.1. Plasmids

pRS315 and pRS316 plasmids used in this study are shuttle vectors which can replicate both in bacteria and yeast. *Escherichia coli* was used to replicate these plasmids in high amount. These plasmids are centromeric and found in 1-3 copy numbers in yeast cells.

pRS316 plasmid carries highly strong yeast promoter PGK followed by human *TP53* gene coding for p53 which is terminated with a yeast terminator. *URA3* gene is the selection marker gene of the plasmid (Figure 2.1).

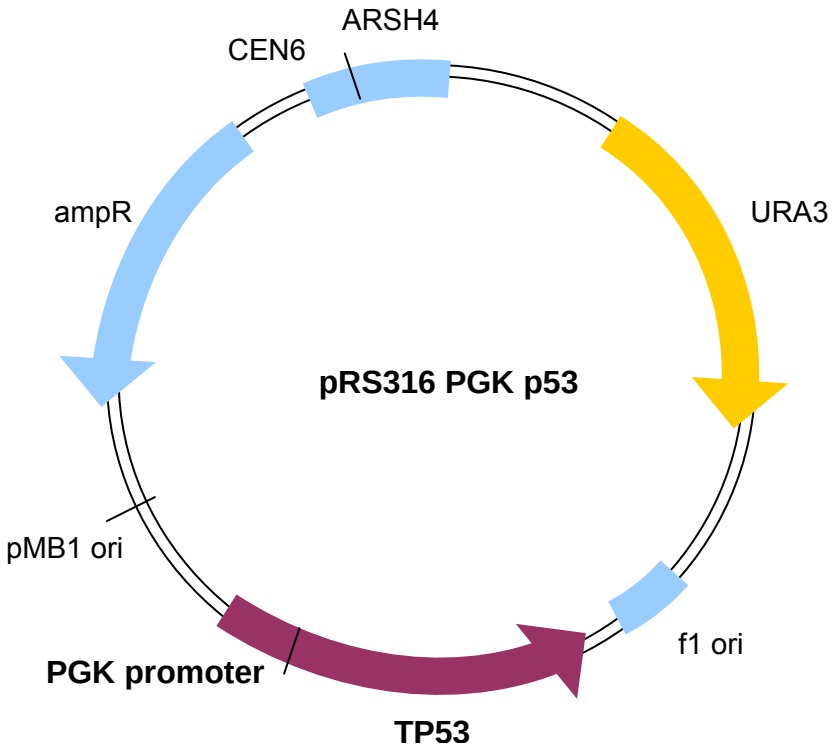

Figure 2.1. pRS316 PGK p53 plasmid

pRS315 plasmid carries p53 universal DNA binding site "Response Element" (RE) followed by Lac-Z gene coding for β-galactosidase enzyme. *LEU2* gene is the selection marker gene of the plasmid (Figure 2.2).

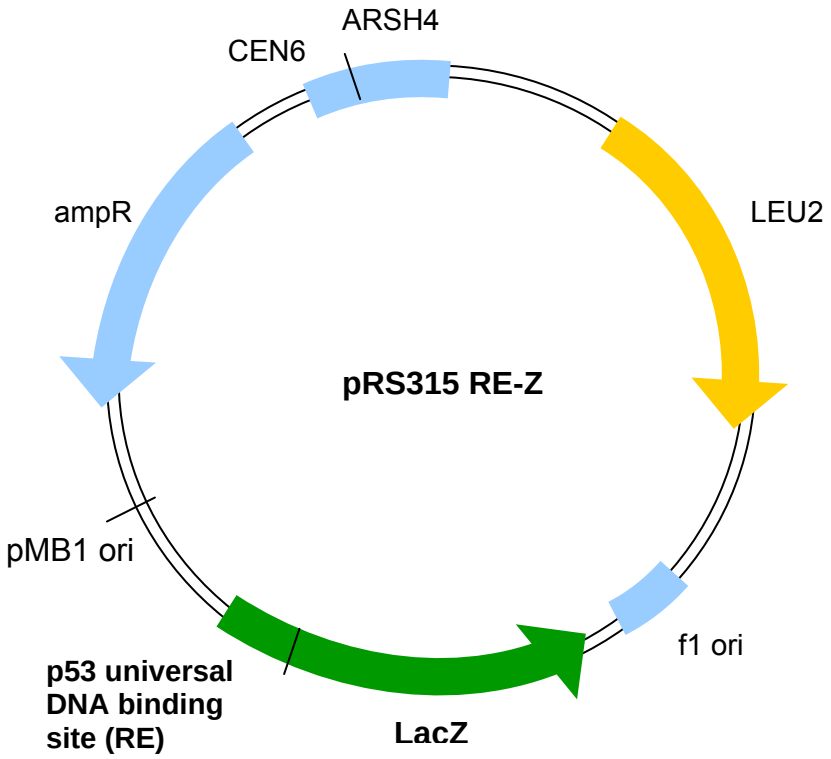

Figure 2.2. pRS315 RE-Z plasmid

In case of transformation of both plasmids to yeast, we expect that the expressed p53 protein bind to RE and induce the expression of β-galactosidase enzyme which is the reporter protein, if the p53 protein is functional and has the DNA binding ability.

The system was tested initially with X-gal which is a substrate of β-galactosidase. Wild type strain is transformed with both plasmids, pRS315 RE-Z only and pRS316 PGKp53 only. Cells were grown and simply disturbed with glass beads. The β-galactosidase activity of the double transformed cells was compared with non-transformed and single transformed cells.

2.2. Yeast transformation with Li-Ac method

BY4741 (MATα, Δ*his3*, Δ*ura3*, Δ*met15*, Δ*leu2*) is the wild type strain used as control to compare the p53 activity with deletion mutants. All deletion mutants were derived from this strain.

Transformation of yeast strains was achieved by lithium acetate method (Amberg, et al. 2005). Strains steaked on YPD agar plate overnight. Grown cultures harvested by

a loop and washed with sterile dH_2O. Cells centrifuged in top speed for 5sec to pellet the cells. Pellet is resuspended in 0,1M Li-Ac and pelleted by the same process. The pellet then resuspended in 240 µl 50% PEG, 36 µl 1M Li-Ac, 5 µl salmon sperm single stranded carrier DNA (10mg/ml) 5 µl pRS316 PGK p53 plasmid, 5 µl pRS315RE-Z plasmid and 69 µl sterile dH_2O respectively. Suspention was vortexed vigorously and incubated for 30min at 30°C then heat shocked at 42°C for 20-25min. Transformed cells were spreaded on synthetic agar media (YNB) including methionine and histidine but without uracil and leucine (YNB-leu-ura). Colonies were allowed to grow for 2-3 days. The transformation protocol was applied to 88 redox mutants.

2.3. B-galactosidaside assay

At least three transformed colonies taken by a loop and spreaded on a YNB -leu-ura agar plate to allow them to grow. Grown cells were inoculated in 5ml YNB -leu-ura liquid media for 24 hours. Optical density (OD) of cultures were diluted to 0,1 in 10ml YNB -leu-ura media according to absorbance value at 600nm. Cells were allowed to grow for at least 4 hours to ensure that they are in logarithmic growth phase. Between 0,2 and 0,5 OD values at 600nm 100 µl of culture is taken and freezed in -80 °C.

Frozen cultures were defrost in 100 µl YNB -leu-ura media and 400 µl Z-buffer which includes 60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol and 0,2% L-Lauryl Sarcosine. After 30min preincubation at 30°C, 150 µl Z-buffer with 4mg/ml ONPG was added to suspensions and the mixtures were incubated in 30 °C for 30min. The reaction is stopped by adding 400 µl 1.5M Na_2CO_3. The mixture was pelleted at top speed for 1 min and the supernatant is used for the measurement at 420nm (Kippert 1995).

The absorbance values were normalized according to their OD values and compared to the absorbance values of the wild type strain.

2.4. Determination of oxidative stress tolerance

Selected yeast mutants are tested for their oxidative stress tolerance. Two different agents are used to induce oxidative stress on cells which are H_2O_2 and diamide. H_2O_2 is a natural by-product of oxygen metabolism and substrate of peroxidases which is

decomposed to H_2O and oxygen. Diamide is a sulfhydryl-oxidizing agent, which can interact with the cystein residues of proteins and as a result cause denaturation (Eaton 2005).

Halo assay was used to test its effect on mutant strains. Cells were grown overnight and diluted to 0.2 at OD_{600}. Approximately 50.10^6 were spreaded on YPD plate 30 min before 5µl 8.8M (%30) H_2O_2 was pipetted at the center. The plates were incubated for 2 days and the diameter of the hole at the center of the plate without growth was measured to indicate the H_2O_2 sensitivity of the strains in compared to wild type.

Strains were grown overnight and diluted to 0.2 at OD_{600}. A set of dilution series were prepared: 2.10^{-1}, 2.10^{-2}, 2.10^{-3} and 2.10^{-4}. 5µl from each dilution is dropped on YPD plates containing no diamide, 0.5mM, 1mM, 1.5mM and 2mM diamide. Plates were incubated for 2 days to observe the sensitivity difference among each other.

CHAPTER 3

RESULTS

The initial testing of the plasmid system gave positive result to X-gal in double transformed cells wild type cells while negative in non-transformed and single transformed cells as expected (Figure 3.1). Thus we proceed to the transformation of mutant strains.

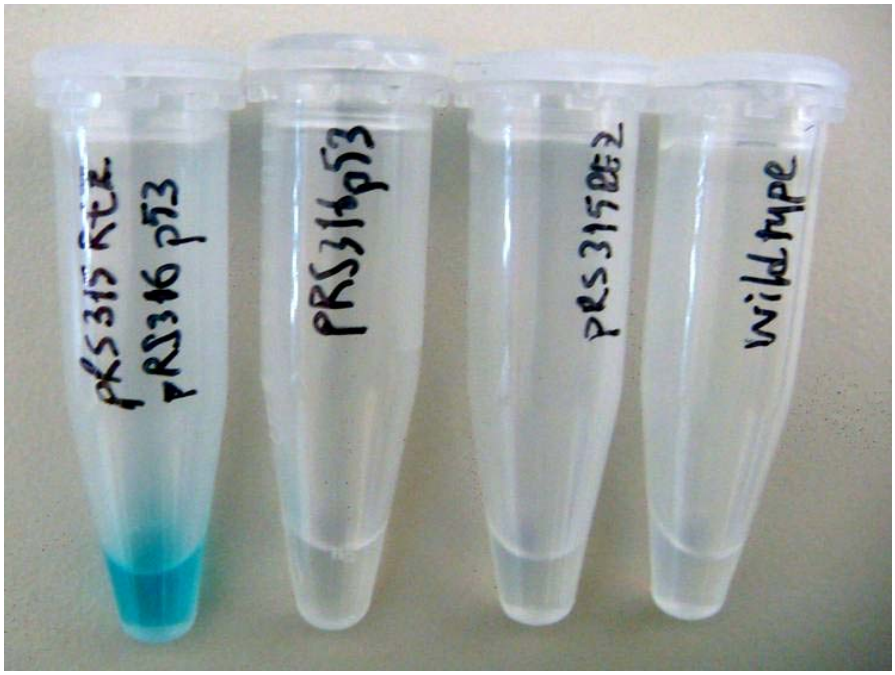

Figure 3.1. Testing of plasmids and experimental design by X-gal

3.1. B-galactosidaside assay

After transformation and β-galactosidase assay of 88 mutant strains in three replicates, 19 of them were selected for the second and third repeats of the experiment. They were transformed all over again and grown for β-galactosidase assay. As a result, 7 of the deletion mutants were selected for decreased p53 activity 2 to 13 fold according to the reporter gene activity of the wild type (Figure 3.2). These are SAC1, PMS1, RTS1, NFU1, HNT3, YDR286C and YHR035W.

YDR286C and YHR035W are uncharacterized open reading frames (Giaever, et al. 2002). SAC1 is known as lipid phosphoinositide phosphatase of the ER and Golgi (Guo, et al. 1999). PMS1 is determined as an ATP-binding protein required for DNA mismatch repair in mitosis and meiosis (Prolla, et al. 1994). RTS1 is the B-type regulatory subunit of protein phosphatase 2A (PP2A) and also homolog of the mammalian B' subunit of PP2A (Giaever, et al. 2002). NFU1 is a protein involved in iron metabolism in mitochondria (Giaever, et al. 2002). HNT3 is the member of the third branch of the histidine triad (HIT) superfamily of nucleotide-binding proteins however its molecular function is not determined yet (Huttenhower and Troyanskaya 2008).

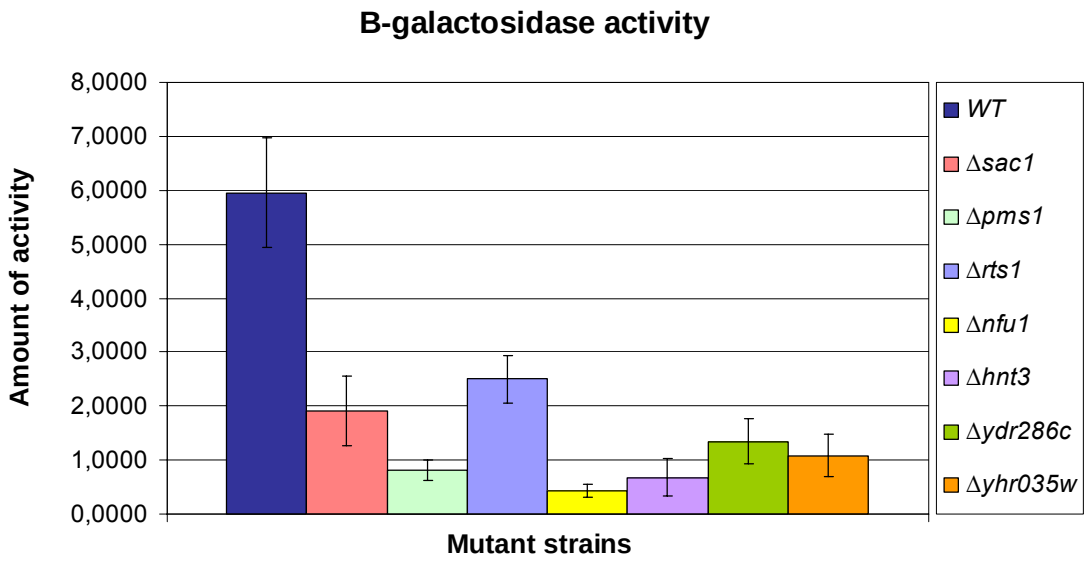

Figure 3.2. β-galactosidase assay results

3.2. Oxidative stress tolerance

The mutants showing decreased p53 activity were analysed for further characterization. Their oxidative stress tolerance were measured compared to wild type. Two different stress agents were used to understand whether they are more sensitive or not than wild type. Halo assay results showed that all mutants were 15% to 50% more sensitive to H_2O_2 than wild type (Figure 3.3).

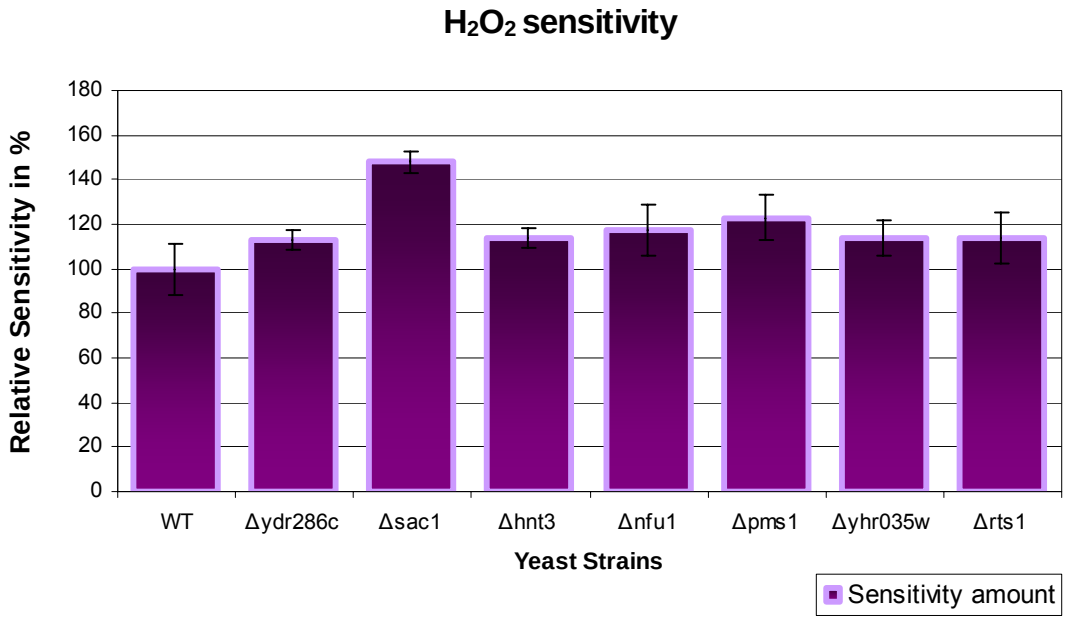

Figure 3.3. Halo assay results

Spotting assay results for diamide showed different sensitivity levels of mutants in compared to wild type (Figure 3.4). 0.5mM and 1mM diamide concentrations did not show any effect on mutants. 1.5mM diamide showed that the most sensitive mutant for diamide effect is SAC1 mutant. The other mutants and wild type showed almost no sensitivity. However, on 2mM diamide, almost all strains showed growth defect. According to the results, YDR286C and NFU1 mutants were less sensitive to the effect of diamide than the wild type.

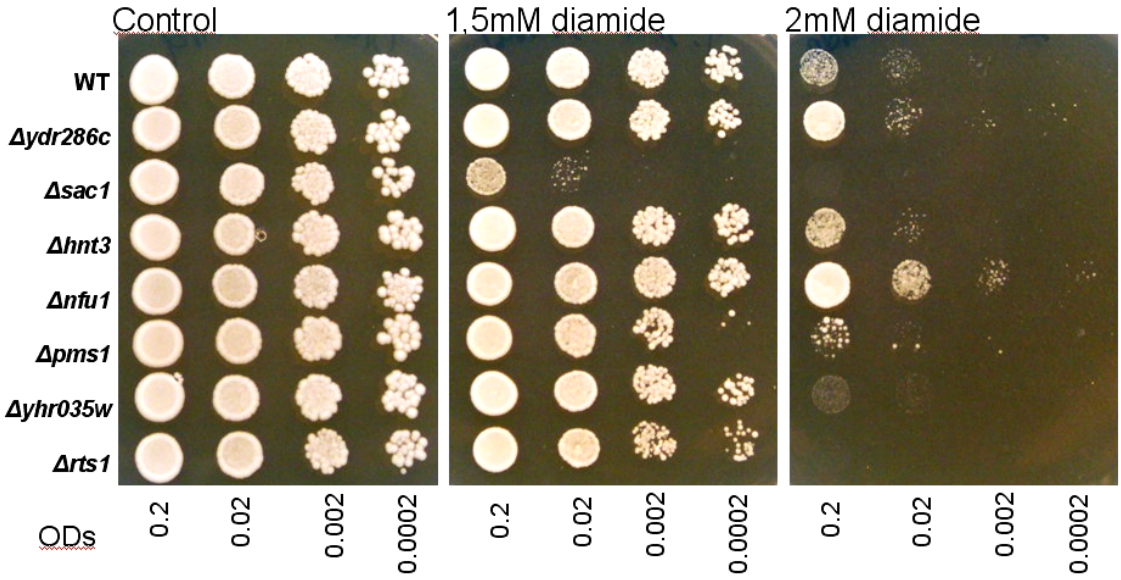

Figure 3.4. Spotting assay results

CHAPTER 4

DISCUSSION

B-galactosidase assay results showed that absence of some of the anti-oxidant proteins can decrease p53 transactivation activity. These results neither reflect the mechanism of the inactivation nor the inactivation is redox dependent.

Five of the seven genes express an identified protein. Two of them are open reading frames of unknown function. All of the seven genes have human homologs. Identified five proteins have similar function in humans. Unidentified YDR286C gene homolog in human codes for a glutaredoxin-like protein, while YHR035W gene homolog in human codes for a transport protein. One of the seven genes is denoted as anti-oxidant gene (YDR286C) while others have known or predicted distinct functions. Many proteins have more than one function. Therefore, these proteins might also have oxido-reductive effects on other proteins. However, what we do not know is, if their effect on p53 transactivation activity is redox dependent or not, or a result of distruption of the protein-protein interactions or any other type of regulations. The absence of these genes might interrupt the interaction of an upstream protein in a pathway which might resulted with decreased p53 activity. On the other hand, by using yeast as the model organism instead of a human cell, we minimize the interactions of regulatory proteins with the expressed p53. The other possibility is the interaction of the proteins of interest with p53 might be resulted with a known post-translational modification other than redox regulation. As mentioned before, two of the seven genes were known as phosphatases. Therefore the activity loss of p53 might be corelated with a phosphorylation/dephosphorylation process of p53 or the related proteins.

Moreover, the oxidative stress tolerance tests cleared that stress agents show their effect in different ways as their results are not correlated. Especially, effect of diamide on mutants is interesting because instead of being more sensitive two of them (YDR286C and NFU1) were appearently less sensitive than wild type to sulfhydryl-oxidization. While we expect oxidation of cystein residues during redox regulation of p53, these two mutants are less prone to redox dependent activity loss of p53.

In contrast to these speculations, there is also the possibility of direct interaction and redox regulation p53 by the subjected proteins. So, we need to do further investigation to understand the reason of decreased transactivation activity.

Complementation of the mutant genes should be the first step to observe whether p53 activity is restored or not, to confirm the results. Then Cys → Ser point mutations can be introduced to both anti-oxidant genes and p53. Serine is the most similar amino acid to cysteine, it has an –OH group instead of a –SH group in its side chain. Introducing serine instead of cysteine in key points like DNA binding domain of the p53 would eliminate an oxidation side on the protein and the protein as a result could store its 3D structure or its active side. These modifications on necessary genes will enable us to enlighten the relationship between p53 and the selected anti-oxidant genes so we can understand whether p53 activity decrease in mutants is purely redox dependent or not.

CHAPTER 5

CONCLUSION

In conclusion, 88 anti-oxidant gene mutants of yeast were analyzed for the p53 transactivation activity and seven mutants were shown to have 2- to 13-fold decrease in p53 activity compared to the wild type. These mutants were analyzed for oxidative stress tolerance using diamide and H_2O_2. They were shown to be all sensitive to H_2O_2, however two of them were less sensitive to diamide than wild type. Mutants were experiencing intrinsic oxidative stress which may have resulted in oxidation and inhibition of p53 or the related proteins. The less affected two mutants from diamide are less prone to affect p53 activity in a redox dependent manner.

To understand the reason of decreased transactivation activity of p53, complementation of deletion mutants will be achieved. As a result of complementation, we expect the mutant strains to restore their p53 activity. For further investigations, Cys → Ser point mutations can be made by site-directed mutagenesis to understand the inactivation of p53 is whether related with oxidation or not.

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