REGULATION OF HUMAN p53 TUMOR SUPPRESSOR GENE ACTIVITY BY THIOL-DEPENDENT OXIDOREDUCTASES

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ABSTRACT

REGULATION OF HUMAN p53 TUMOR SUPPRESSOR GENE ACTIVITY BY THIOL-DEPENDENT OXIDOREDUCTASES

Background: Occurrence of p53 mutations in more than half of human tumors indicates the importance of p53 gene in cancer prevention. Nevertheless, oxidation of cysteine –SH groups in p53 protein can inactivate the protein under oxidative conditions. The importance of p53 as a tumor suppressor and insufficient studies about redox regulation of p53 gene lead us to study redox regulation of p53 protein. **Methods**: In this study, yeast (Saccharomyces cerevisiae) was used as an *in vivo* model. All potential thiol-dependent antioxidant genes in yeast were identified based on specific characters in their structure, via REDOXCysSearch bioinformatics tool. To study human p53 gene activity in yeast cells, p53 gene and p53 RE Lac-Z reporter that is inducible by p53, were transformed into yeast. Antioxidant gene mutants were analyzed for LacZ reporter gene activity. **Results:** We identified several yeast mutants (Δ*sac1*, Δ*hnt3* and Δ*map1*) with lower p53 activity with respect to wild-type yeast. **Conclusion:** Due to conserved mechanisms of cell cycle regulation and oxidative stress tolerance between yeast and mammals, we believe that results from yeast studies may help us to understand the redox regulation of p53 in human cells. Thus, a new perspective may appear in the redox regulation of p53 gene.

ÖZET

İNSAN p53 TÜMÖR BASKILAMA GENİNİN TİYOL BAĞIMLI OKSİDOREDÜKTAZLAR İLE REGÜLASYONU

İnsan tümörlerinin yarısından çoğunda p53 mutasyonlarının görülmesi bu proteinin kanseri önlemede önemli bir rolünün olduğunu göstermektedir. Bununla birlikte, sistein aminoasitlerine bağlı –SH gruplarının oksidatif koşullar altında p53 proteinini inaktif hale getirebildiği bilinmektedir. p53'ün önemli bir tümör süpresör olması ve p53 proteininin redoks regülasyonu konusundaki çalışmaların yetersizliği bizi p53 proteininin redoks regülasyonunu çalışmaya yönlendirdi. Bu çalışmada maya (Saccharomyces cerevisiae) in vivo model olarak kullanılmıştır. Mayadaki bütün tiyol-bağımlı oksidoredüktazlar, yapılarındaki spesifik karakterlerin REDOXCysSearch biyoinformatik aracıyla taranmasıyla tespit edilmiştir. İnsana ait p53 geninin maya hücrelerinde çalışılabilmesi için, p53 geni ve p53 geni ile uyarılan p53 RE Lac-Z raportör sistemi mayalara transforme edilmiştir. Antioksidan gen mutantları da LacZ raportör gen aktivitesi yönünden incelenmiştir. Yabani tip mayaya göre daha düşük p53 aktivitesi gösteren bazı maya mutantları tespit edilmiştir. Hücre döngüsü regülasyonu ve oksidatif stress tolerans mekanizmalasının mayada ve memelilerde korunmuş olması nedeniyle, maya çalışmalarındaki sonuçların memeli hücrelerindeki p53'ün redoks regülasyonunu aydınlatmada yardımcı olacağı düşünülmektedir. Bu sayede, p53'ün redoks regülasyonu konusunda yeni bir görüş ortaya çıkabilecektir.

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CHAPTER 1

INTRODUCTION

1.1. TP53 Gene and p53 Protein

TP53 gene, which is located on 17p13.1 region of human genome, is one of the most important tumor suppressors found in human. It encodes for human p53 protein. p53 protein was firstly identified in a complex with large T-antigen in SV40 tumor virus transformed cells (Chang, Simmons et al. 1979; Kress, May et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979; Melero, Stitt et al. 1979). Then, it was characterized as a tumor antigen (Lane and Crawford 1979; Linzer and Levine 1979). p53 protein has been given that name because it runs as 53 kilodalton (kDa) protein on SDS page. However, actual mass of p53 protein is 43.7 kDa. Due to high amount of proline residues in p53 protein, it is observed heavier than it is when it is run on SDS page.

In a normal cell, p53 is found at low levels; however when a cellular stress condition like DNA damage, hypoxia, dNTP shortage or absence of telomerase activity is induced, p53 protein escapes from proteosome-mediated degradation and it is stabilized. Thus, p53 expression becomes much higher under cellular stress (Hainaut and Mann 2001; Vousden and Lu 2002). Stabilization of p53 protein initially leads to cell cycle arrest and activation of repair mechanisms. If, damage cannot be repaired then apoptosis is triggered and cell undergoes death.

p53 protein mainly functions as a transcription factor. When, p53 is triggered, its concentration in the nucleus increase, it specifically binds to DNA and positively or negatively affects the activity of many downstream genes. More than 100 genes were identified to be regulated by p53 and bioinformatic analysis revealed more than 4000 potential p53 binding sites in the genome (Lu 2005). p53 does not only fuction as a transcription factor but also it binds to several proteins involved in DNA replication, transcription and repair. Hence, it mediates certain antiproliferative responses (Ko and Prives 1996; Hainaut and Hollstein 2000) .

p53 has been one of the most extensively studied tumor suppressor proteins in literature. Approximately in 50% of all cancer types, p53 gene itself, is mutated which result in accumulation of dysfunctional p53 protein. In the remaining 50% of cancer cases, certain abnormalities can exist within the p53 pathway, which in turn attenuates its tumor suppressor function (Vogelstein, Lane et al. 2000). This is why p53 is important and why it takes the attention of many researchers in cancer biology.

Key role of p53 in many different cancers led to the development of various p53 based cancer therapies. However, only one of those therapies was approved for clinical usage. This method involves the administration of gene therapy by adenovirus vector and this therapy has been used in China since 2003 but not in other countries. On the other hand, small inhibitor molecules were developed to inhibit p53-Mdm2 interaction. Those molecules were detected to be functional in animal models and now being used in clinical trials for treatment of solid and hematologic malignancies. A new method called cyclotherapy which uses a p53 activator for protecting normal tissues from cytotoxic drugs and which is increasing therapeutic index of treating p53 mutant cancers, was shown to be effective in animal models (Cheok, Verma et al. 2011). Successful application of those therapies in clinical trials may open a new window in the treatment of p53 based cancers.

1.2. Structure of p53

There exist five main domains in p53 structure. They are transcriptional transactivation, proline rich, DNA-binding, tetramerization and negative regulation domains (Figure 1.1.). Each of those domains has distinct functions. Transactivation domain (activation domain I) is mainly responsible for transactivation of many proapoptotic genes. Also p53 interacts with many transcription factors like Hdm2 and acetyltransferases via its transactivation domain. Proline rich domain is important for stability and proapoptotic activity of p53. Between transactivation and proline-rich region, there exists activation domain (II) which is responsible for apoptotic activity. NLS is signal for nuclear localization of p53 whereas NES is signal for nuclear export of the protein. Plus, tetramerization domain plays important role for p53 activity in vivo whereas regulatory region is involved in the downregulation of DNA binding. DNA-

binding domain is the critical region for sequence specific DNA binding (Bai and Zhu 2006).

95% of p53 mutations occur in the DNA binding domain and causes loss in DNA binding ability of the protein (Vousden and Lu 2002). In addition, Pearson and Merill denoted the importance of redox status of cysteines that reside in the DNA binding domain. They found out that formation of intramolecular or intermolecular disulfide bridges at DNA binding domain could inhibit DNA binding of p53 to target region (Pearson and Merrill 1998).

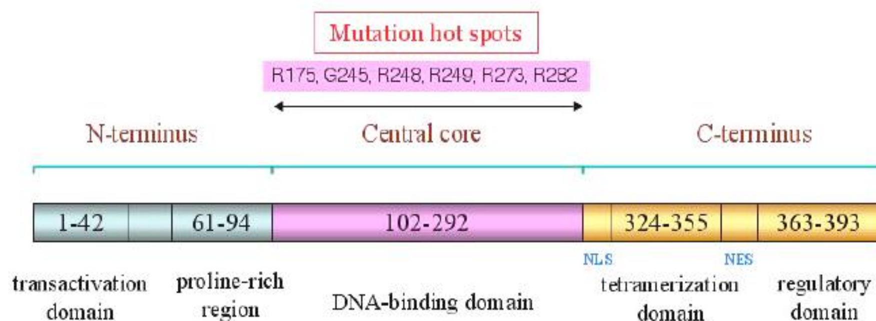

Figure 1.1. Domains in p53 protein
(Source: Bai and Zhu 2006)

1.3. Regulation of p53

Besides the effect of mutations on p53 protein structure, p53 can be regulated via many different pathways at transcriptional, post-transcriptional, translational, post-translational levels. Moreover, p53 function can be controlled by protein-protein interactions without modifying its expression status.

Transcriptional activation of p53 by interferon alpha, beta, gama was discovered by Takaoka, Hayakawa et al. Their study identified that interferon induced transcription of p53 increased both mRNA and protein levels of p53 and led to increase in apoptosis rate (Takaoka, Hayakawa et al. 2003). Moreover, post-transcriptional modifications of p53 mRNA has gained importance in p53 regulation. For, instance, U-rich sequence of 66 nucleotides at the 3’ end of human p53 mRNA was found to be required for translational repression of p53. On the other hand, murine p53 was identified to inhibit its own translation through binding the 5’ UTR of murine p53 mRNA (Zhang and Chen

2008). Furthermore, Mazan-Mamczarz, et.al and Galban et.al showed an enhanced rate in translation due to binding of HuR RNA-binding protein to p53 mRNA (Galban, Martindale et al. 2003; Mazan-Mamczarz, Galban et al. 2003). p53 can also be regulated independent from its expression. This type of regulation is mainly dependent on protein-protein interactions. For instance, mdmX proteins directly interact with N-terminal transactivation domain of p53 and supress its activity without causing degradation of p53. In addition, nucleoplasmin binds to N-terminal domain of p53 protein and represses p53 activity (Maiguel, Jones et al. 2004).

Advances in understanding post-translational modifications of p53 identified many different mechanisms regulating p53 protein at post-translational level. p53 was depicted to be subjected to many post-translational modifications like phosphorylation, acetylation, methylation, ubiquitination, sumoylation, neddylation, redox regulation, etc… Some of these modifications have stabilizing effect on p53 whereas others have destabilizing effect. Some modifications may have both stabilizing and destabilizing effect depending on the location of the modified residue and also the stimulant in the environment (Dai and Gu 2010).

An array of serine/threonine that reside in the C-terminal regulatory and N-terminal transactivation domains are subjected to phosphorylation. Phosphorylation can either have stabilizing or destablizing effect depending on the residue being phosphorylated. For instance, two of the widely studied phosphorylated sites of p53 protein are S15 and S20 residues in N-terminal transactivation domain. Phosphorylation of that site reduces the interaction between p53 and negative regulator of p53. Thus, S15/S20 phosphorylation has a positive influence on p53 activity (Toledo and Wahl 2006). In contrast, S392 hyperphosphorylation was found to be associated with poor prognosis, advanced tumor stage and tumor grade in cancer cases that are p53 positive (Matsumoto, Furihata et al. 2004; Matsumoto, Furihata et al. 2004; Bar, Slomska et al. 2009).

Acetylation is a strong way of activating function. Acetylation has been correlated with p53 activation via three different mechanisms. Initially, acetylation prevents ubiquitination at the same site and promotes p53 stabilization. Secondly, it inhibits HDM2/HDMX repressive complex formation on target promoters and finally acetylation is responsible for recruitment of certain cofactors to activate transcriptional activation of p53 (Dai and Gu 2010). Nine sites of p53 protein were identified to be

critical sites for acetylation. Tissue culture studies indicate the role of acetylation in activating, binding of p53 to DNA and transcriptional activity of p53. Thus, acetylation provides stability of p53 (Dai and Gu 2010). Acetylation level is balanced by deacetylases. For instance, SIRT1, an important protein with deacetylase activity was found to play a negative role on p53 activity via deacetylating p53 at K382. Therefore, p53 cannot induce its target genes for apoptosis (Luo, Su et al. 2000; Luo, Nikolaev et al. 2001).

There are number of lysine and arginine residues in p53 structure that are potential sites for methylation. Methylation may have either positive or negative effect on p53 function. For instance, monomethylation of K372 by SET7/9 was found to promote transactivation of p53 target genes whereas K382 monomethylation by SET8 was found to repress p53 transcriptional activity (Chuikov, Kurash et al. 2004; Huang, Perez-Burgos et al. 2006; Shi, Kachirskaia et al. 2007). Besides, there exists a correlation between repressive and inductive methylation modifications. For example, activating methylation of a certain lysine residue may repress repressive methylation of another lysine residue.

One of the most important post-translational modifications that have an effect of p53 regulation is ubiquitination. This modification involves the attachment of one or more (approximately) 8 kDa ubiquitin molecules to the protein. Monoubiquitination is responsible for cytoplamic translocation of p53 and initiates transcription independent roles of cytosolic p53 like triggering apoptosis or inhibiting autophagy (Marchenko and Moll 2007; Tasdemir, Maiuri et al. 2008; Green and Kroemer 2009). Nevertheless, polyubiqutination has a quite different effect on p53 activity. When cell is not under a stress condition, p53 levels are low due to Hdm2 activity. Hdm2 ubiquitinates p53 and directs it for proteosomal degradation. When there is a damage signal, it inhibits Hdm2 activity. Thus, p53 can escape from ubiquitination and degradation. By this way, p53 can be stabilized. Also, a deubiquitinase enzyme HAUSP was found to regulate p53 pathway by modifying p53, autoubiquitinated Mdm2 and ubiquitinated HDMX (Meulmeester, Maurice et al. 2005).

p53 is subjected to other ubiquitin-like post-translational modifications. Sumoylation and neddylation are structurally similar to ubiquitination and conserved in eukaryotes. However, they do not have any effect on stability or localization of p53. According to literature, sumoylation has been associated with increased transcriptional

activity of p53 and premature senescence (Melchior and Hengst 2002; Bischof, Schwamborn et al. 2006). In contrast, neddylation was found to inhibit transcriptional activity of p53 (Melchior and Hengst 2002; Fomenko 2005). On the other hand, low abundance of sumoylation and neddylation of p53 in vivo was associated with a challange in vivo. This indiates the importance of those modifications in p53 activity (Dai and Gu 2010).

In addition to all those post-translational modifications discussed above, there exist some other mechanisms that play role on p53 activity. Redox state of certain cysteine residues in p53 protein has been associated with regulation of p53 through disulfide bond formation. Because redox regulation of p53 is the main focus of my thesis, studies about this topic will be discussed under another subtitle.

1.4. Redox Regulation of p53

p53 can be regulated through the conserved cysteine residues which function as redox sensor. Those critical cysteine residues that reside especially in the DNA-binding domain can undergo covalent or oxidative modifications. Those modifications result in conformational changes in p53 and affect p53 function. An oxidizing environment can cause cysteines in p53 structure to be oxidized and lead p53 to undergo conformational changes. Thus, redox modifications influence p53 transcriptional activity and biological responses (Stommel, Marchenko et al. 1999).

Cysteine residues in the DNA binding region can participate in zinc coordination or can be regulated by redox regulation. Zinc coordination was shown to be essential in p53 function (Hofmann, Moller et al. 2002). Coordination of intrinsic zinc residues with cysteines keep p53 in the reduced state and keep its wild type function (Kim, Kundu et al. 2011). The rest of cysteines can be induced by oxidizing environment and can be modified via redox regulation which involves formation of disulfide bonds, S-Glutathiolation, nitrosylation, metal binding which alter p53 protein structure and lead to inhibition of p53 function.

There exist many different factors playing role in the redox regulation of p53 function (Figure 1.2.). Because redox regulation mainly occurs through critical cysteines in p53 structure and it is known that formation of intermolecular or

intramolecular disulfide bonds inhibit p53 activity, understanding formation of disulfide bonds from thiol groups can enlighten the redox regulation of p53.

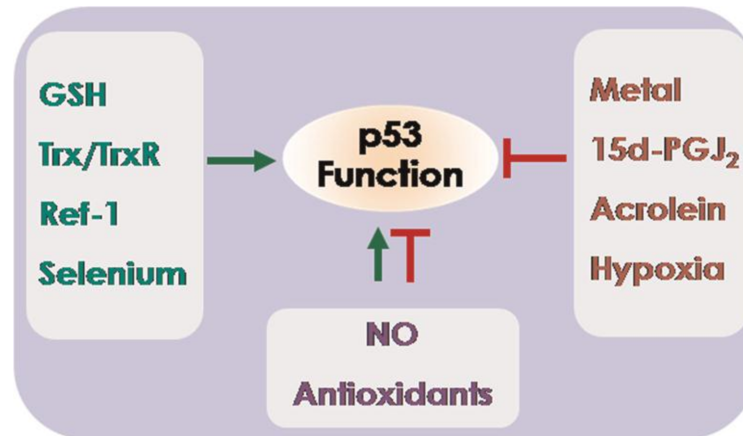

Figure 1.2. Factors that have role in redox regulation of p53
(Source: Kim, Kundu et al. 2011)

1.5. p53 Studies Using Yeast as a Model

Yeast has been an important organism for genetic studies. It has also been used in certain p53 studies. Despite not having an internal p53 gene, due to similarities in their cell cycle regulation mechanisms, yeast can be used to study human p53 gene. For example, the role of *PAK1* and *TRR1* genes on p53 activity has been determined via yeast studies. Thiagalingam, et al showed that *PAK1* overexpression in yeast resulted in increased DNA binding activity of p53. Due to its structural similarities of *PAK1* with Ser/Thr specific protein kinases, increase in p53 DNA binding activity was correlated with phosphorylation of p53 from its carboxy terminal by Pak1 (Thiagalingam, Kinzler et al. 1995). Nevertheless, in 1998, Pearson and Merrill found out the effect of thioredoxin reductase on p53 activity. Their system was based on the usage of a p53 binding site which was followed by a reporter gene. Their study with *trr1* gene knock out S.cerevisiae, exhibited very low reporter gene activity when compared to wild type yeast. The ectopic expression of *TRR1* gene complemented the activity loss of p53. The interesting thing was there was no change in the protein level of p53 in wild type and *trr1* knock out yeast. Therefore, they predicted that actity loss of p53 in *trr1* knock out yeast was sourced from post-translational modification of p53. Because *TRR1* functions

as a reducing agent for disulfide bridges, they thought that post-translational modification of p53 involved thiol oxidation of reduced cysteines (Pearson and Merrill 1998). Based on the literature, in this study, I would like to understand the effects of all known and potential yeast redox proteins, on p53 activity.

1.6. Antioxidant Genes in Yeast

Kinases, phosphatases and thiol dependent oxidoreductases play important roles in the regulation of protein activity. There exist two main redox mechanisms, thioredoxin and glutathione systems, in two of which NADPH electrons are transferred to the target protein. As mentioned in previous sections, cysteine residues in certain motifs were identified to be important in those redox mechanisms. Especially, CxxC motif has been extensively studied in those systems and detemined to reduce intermolecular and intramolecular disulfide bonds. CxxC motif contributes to redox regulation by applying the following mechanism. N-terminal cysteine in the motif has a decreased pK_a and functions as a nucleophilic attacking group in redox reactions. This event causes the oxidized cysteine in the substrate to form an intermolcular disulfide bond with the enzyme. Then, C-terminal cysteine in the CxxC motif, attacks the intermolecular disulfide bond. Thus, disulfide bond forms between two cysteines of the redox enzyme and the cyteine on the substrate is reduced (Holmgren 1989; Kortemme and Creighton 1995; Martin 1995). In addition to CxxC motif, different motifs like CxxS, TxxC, CxxT, SxxC were characterized to serve redox activity (Fomenko and Gladyshev 2003).

Antioxidant genes have significant roles in redox mechanisms. Therefore, genome wide identification of antioxidants is important to undestand those mechanisms. All oxido-reductive proteins in organisms whose genome is sequenced, can be identified based on searching certain redox motifs through bioinformatic analysis (Fomenko and Gladyshev 2002; Fomenko and Gladyshev 2003; Fomenko, Xing et al. 2007). The list of potential and known redox proteins in yeast, S. Cerevisiae, was generated via REDOXCysSearch bioinformatics tool with the help of Dr. Fomenko (Fomenko 2005). The algorithm of the bioinformatics tool initially searches the CxxS, CxxC, CxxT and SxxC motifs in the protein sequences of yeast genome in FASTA format. Then, metal binding proteins are eliminated from the list

because they can coordinate with metal ions. Remaining proteins in the list are analyzed for conservation through other species. Then, secondary structure of the protein is predicted to check the compatibility with the thioredoxin fold motif. Based on the structural and biochemical properties of the proteins, 88 proteins were classified as anti-oxidant. Some of them are known antioxidants and others have potential to function as an anti-oxidant (Fomenko and Gladyshev 2002; Fomenko and Gladyshev 2003; Fomenko, Xing et al. 2007). All yeast deletion mutants selected via REDOXCysSearch software were obtained commercially from EUROSCARF organization. All the deletion mutants in the list were analyzed for their role in p53 regulation previously by Beren Ataç. First analysis of those 88 mutants identified 7 critical genes with possible antioxidant activities (Atac 2008) . In my thesis, I aimed to further characterize those 7 genes for their effect on p53 activity.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plasmids

At the beginning of the study pRS315 RE-Z and pRS316 PGK p53 plasmids were used. Those vectors are shuttle vectors which can replicate both in yeast and E. coli. In addition, those plasmids are low copy plasmids.

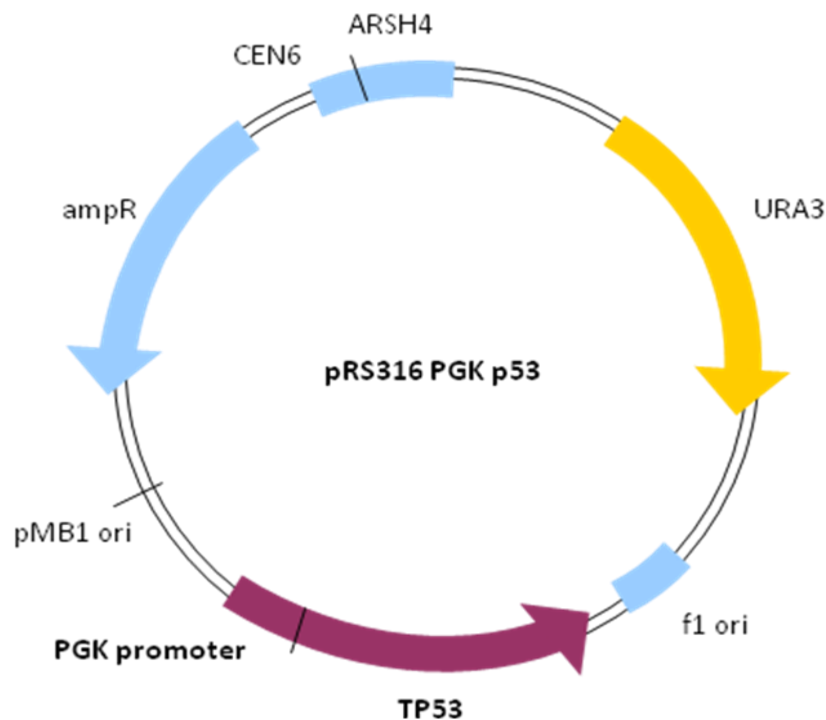

Figure 2.1. pRS316 PGK P53 plasmid
(Source: Atac 2008)

pRS316 PGK p53 plasmid is derived from pRS316 plasmid which includes a strong yeast promoter, PGK. This promoter binds to human TP53 gene terminating with a yeast terminator. *URA3* gene is the auxotrophic marker gene of the plasmid (Figure 2.1.).

pRS315 RE-Z plasmid is derived from pRS315 plasmid. The modified plasmid has a universal p53 binding site called “p53 Response Element” (RE) which is followed by Lac-Z reporter gene encoding for β-galactosidase enzyme. *LEU2* gene is the auxotrophic marker gene of the plasmid (Figure 2.2.).

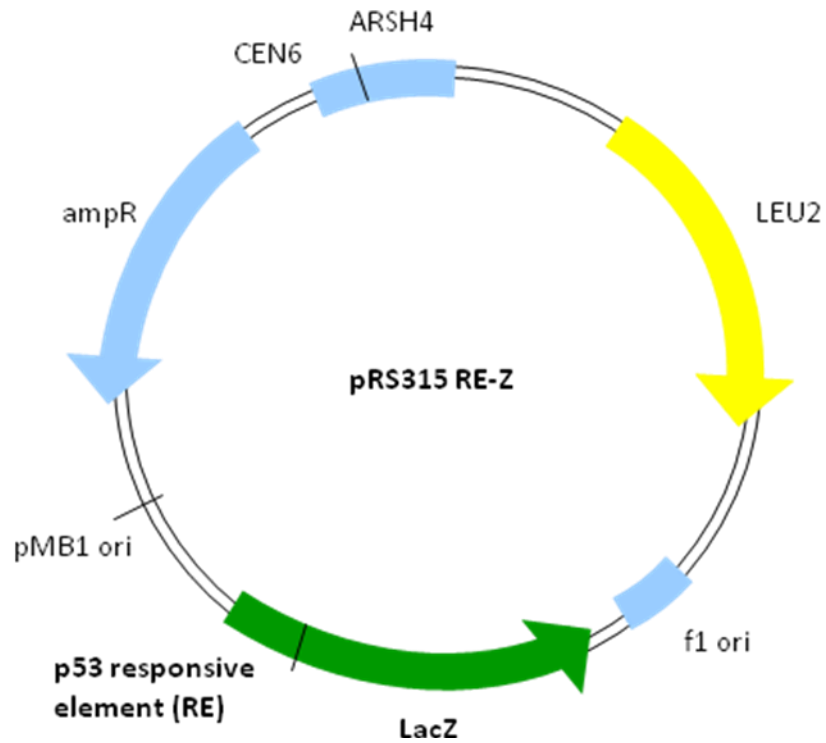

Figure 2.2. pRS315 RE_Z plasmid
(Source: Atac 2008)

When both, pRS315 RE_Z and pRS316 PGK p53, are transformed into yeast, if there exists functional p53 protein with DNA binding ability, p53 protein binds to p53 responsive element on the pRS315 RE_Z . When responsive element is induced via p53, β-galactosidase gene could be expressed and activity of reporter gene could be analyzed.

2.2. Transformation of Plasmids into Yeast via Li-Ac Method

BY4743 (MATα, Δ*his3,* Δ*ura3,* Δ*met15,* Δ*leu2*) was used as wild type strain to compare p53 activity with deletion mutants. All deletion mutants were derived from that strain.

Transformation of plasmids into yeast cells were carried out by lithium acetate method. One loop of yeast cells was transferred from YPD agar plates to 3ml of YPD liquid media. Cultures were grown overnight. In the morning, cultures were diluted (1/5). Then, cultures were grown at 30 ^{0}C shaking incubator for 3 more hours. Then cells were centrifuged at 5.000 rpm for 5 minutes, pellet was kept and resuspended in sterile dH_2O and centrifuged again to take the pellet. Then, cell pellet was resuspended in 0.1 M LiAc and pelleted by the same process two times. The pellet was resuspended in 240 ul 50% PEG, 36 ul 1M LiAc, 25ul salmon sperm single stranded carrier DNA(2 mg/ml), 5 ul (approximately 500 ng) plasmid and 20 ul 0.3 M DTT. Then, 25ul sterile water was added. Suspension was vortexed and incubated at 30 ^{0}C shaking incubator for 30 minutes, and then samples were subjected to heat shock at 42 ^{0}C for 20-25 minutes. Transformed cells were spreaded on YNB medium including all the essential amino acids required for the growth of transformants. Plates were kept at 30 ^{0}C for 2-3 days to give enough time for the colonies to grow. This transformation protocol was used for screening 7 redox gene mutants and their complementation assays.

2.3. β-Galactosidase Assay

After transformed colonies had grown, at least three colonies were selected and striken on YNB selection plates. One loop of grown cells was transferred into 5ml liquid selective YNB media and culture was grown overnight in 50 ml falcon tubes. In the morning, cultures were diluted in 10 ml YNB selective medium and given enough time to undergo 2 duplications. Then cells were collected between 0,4-0,6 OD values at 600 nm wavelength (OD 0,4-0,6 was selected as the optimized interval where the best reporter gene activity was observed). Then, 100 ul culture was taken into eppendorfs. For each sample, at least 3 replicates were collected. Then, cells were kept on ice to immediately begin the assay (Cells were not frozen because analysis with frozen samples exhibited decreased reporter gene activity).

Z-buffer which includes 60mM Na_2HPO_4, 2mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol and 0,2% L-Lauryl Sarcosine was prepared. Into all eppendorfs which have 100 ul cultures, 400 ul Z buffer and 100 ul YNB selective medium was added. Samples were vortexed vigorously. Then, samples were kept at 30 ^{0}C for 30 minutes via being vortexed every 10 minutes. 4 mg/ml ONPG was prepared by dissolving ONPG in Z buffer and 150 ul of that solution was added into each eppendorfs and mixtures were vortexed vigorously. Then, samples were incubated at 30 ^{0}C for 30 minutes. Reaction was stopped by adding 400 ul 1.5M Na_2CO_3. The mixture was pelleted at top speed for 1 minute and 200ul from the supernatant was transferred to 96-well plate for OD measurement at 420 nm wavelength (Kippert 1995).

Results were analyzed with respect to absorbance values of cultures at OD 600 nm and data was normalized according to absorbance values of the wild type sample.

2.4. Gene Cloning for Complementation

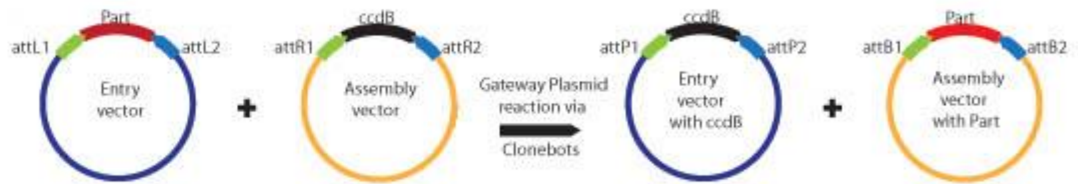

Figure 2.3. The logic behind gateway system
(Source: igem 2011)

Candidate genes were cloned into assembly vector, pAG413 GPD, via using Gateway Cloning system by Invitrogen. The system is based on two sequential reactions called BP and LR (Figure 2.3.). Everything in cloning steps was carried out according to manufacturer's protocol.

2.5. Fusion of Two Plasmids in One Plasmid

During complementation assays, three different plasmids were present together in yeast cells (pRS316 PGK p53, pRS315 RE-Z and pAG413 GPD). Because reporter system did not work in complementation analysis and yeast cells had great difficulty to grow in YNB selective media, it was determined to fuse two plasmids, pRS316 PGK

p53, pRS315 RE-Z, in one plasmid. Thus, decreasing the plasmid number in the yeast cells was aimed.

The cases in which three plasmids are present in yeast cells provide limiting conditions for cell growth because selective media is very restrictive for growth. Under such circumstances, expression of plasmids become problematic. For instance, expression of p53 gene is dependent on PGK promoter (promoter for phosphoglycerate kinase enzyme) activation. Cell has to be carrying out glycolysis reactions, for PGK to be active. Therefore, to examine reporter gene activity correctly, initially restrictive conditions should be removed. For this purpose, fusion of two plasmids to create a single plasmid was determined to be done. To do that, initially p53 gene under the control of PGK promoter in pRS316 PGK p53 plasmid was amplified via PCR using vector specific primers and then cloned into T/A vector (Fermentas) according to manufacturer's protocol. By using Xba1/HindIII sites on the T/A vector, p53 containing sequence was seperated from the rest of the plasmid and purified. Then, using the same enzymes, Xba1 and HindIII, pRS315 RE-Z plasmid was subjected to restriction. Then p53 carrying sequence was tried to be located between the restricted regions of the pRS315 RE-Z plasmid. This protocol was repeated many times due to problems in ligation of the fragments. At once, fusion of two plasmids in one plasmid was carried out successfully (restriction analysis of the newly generated plasmid and the sequence analysis of the same plasmid confirmed that plasmid fusion was successful however reporter system was not functional. It was thought that observing such a result might be normal because both p53 carrying and responsive element including sequences have their own promoter and terminator regions in the combined plasmid. In addition, there was only 50 bp between two promoter sequences. Therefore, those conditions might be inhibiting efficiently expression of the genes. For that reason, another strategy had to be developed.

2.6. Integration of Plasmids into the Yeast Genome

Due to expression problems in combined plasmid, a new strategy had to be developed. Chromosomal expression of genes was thought to be more stabile when compared to expression of genes from plasmids. Therefore, integration of p53 and p53 RE-Z containing casettes into the yeast genome by using genome integration vectors

was performed. Plasmid integration into yeast was based on strategy that was used by Sherman, F. (Sherman 1998) and Gietz transformation protocol (Gietz and Woods 2002) into yeast .

Initially, p53 and p53 RE-Z had to be integrated into chromosomal integration vectors. Because chromosomal integration vectors lack origin of replication, plasmid should integrate itself into the genome in order to replicate itself. Because 305 p53 RE-Z was kindly provided by Gary Merrill, this plasmid was not created by us. At first 305 p53 RE-Z plasmid sequence was analyzed bioinformatically to find out a rare cutter restriction enzyme which only cuts the *leu2* gene in the plasmid but does not recognize any other sequence in the plasmid. AflII enzyme was determined to be the most suitable enzyme for our purpose. Then our first integration vector, 305 p53 RE-Z, was subjected to restriction via AflII enzyme. The aim to do restriction was to linearize the plasmid because free ends are known to create recombination sites and increase the integration efficiency by this way. Basically, to integrate the integration vector to the right place in the genome, the vector should have homologous sites to the target region. Thus, homologous recombination occurs and integration plasmid can integrate itself into the genome. Then linearized plasmid was transformed into W303-1A wild type yeast. (Another strain had to be used because BY4741 and BY4743 strains are not compatible with genome integration strategy that was required to be used. Those sites lack the auxotrophic marker, *leu2* gene completely. Therefore, there is no homology site for integration to take place). Finally, transformants were seleced in the selective media.

Next step was the creation of 306-p53 plasmid. For this purpose, p53 gene was amplified from pRS316 PGK p53 plasmid via PCR with the help of site specific primers. pRS306 vector was subjected to restriction via KpnI and SacI enzymes. Then PCR product was ligated with pRS306 vector that was subjected to double digestion. Thus, pRS306-p53 plasmid was generated. Then, this plasmid was linearized via BstBI enzyme which cuts the *ura3* gene in the plasmid approximately from the middle point of the sequence. Linearized pRS306-p53 plasmid was transformed into the W303-1A transformant which had already been transformed with 305 p53 RE-Z plasmid. Colonies were selected on selective media.

2.7. Creation of Gene Knock Out in Yeast

After creation of pRS306-p53 and pRS305 p53 RE-Z containing W303-1A transformants, gene knock outs had to be created to continue with the project. To do this, a new strategy was developed by using corresponding gene mutants that are in BY4741 background. Those knock outs already has the kanmX casette inside. Therefore, by using specific primers for approximately 100 bp upsteam and downstream regions of the target genes, kanmX casettes and homology arms for the target gene to be deleted, were amplified via PCR from BY4741 strain of the corresponding knock out. For instance, to delete *HNT3* gene from W303-1A+ pRS306-p53+pRS305 p53 RE-Z strain, *hnt3* knock out of BY4741 was used. To amplify kanmX casette in that mutant, primers were designed from nearly 100 bp upstream and downstream regions of *HNT3* gene. Amplified region includes both *HNT3* specific homology regions in addition to kanmX casette. PCR fragments were transformed based on Gietz transformation protocol (Gietz and Woods 2002) and transformants were selected on G418(400 ug/ml) containing YPD plates. Due to problems in applied transformation protocol or inefficiency of homologous recombination frequency, still certain problems are encountered while performing knock out experiments.

2.8. Site Directed Mutagenesis

To understand the basis of redox regulation in mutants with lower LacZ activity, critical cysteines in the candidate genes were transformed into serine via site directed mutagenesis. For this purpose, Quick-Change II XL site directed mutation kit which is commercially available from Agilent Technologies, was used. Working principle of the kit is based on PCR reaction with mutation specific primers (Figure 2.4.). Nevertheless, primers specific for site directed mutagenesis were designed with the help of a bioinformatics tool on web provided by the manufacturer. Each step in the experiment was performed according to manufacturer’s protocol. The plasmids which involve site directed mutated versions of the critical cysteines in the p53 protein, were kindly provided by Gary Merrill.

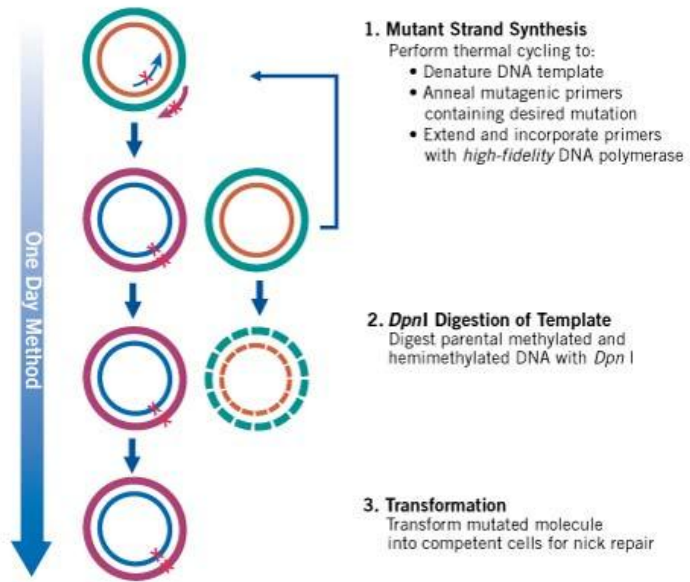

Figure 2.4. The basis of site directed mutagenesis.
(Source: agilent 2011)

CHAPTER 3

RESULTS

Initial screening of 88 different gene mutants indicated a list of 7 genes that exhibited significant decrease in reporter gene activity (Atac 2008). Those genes were further analyzed for decrease in Lac-Z reporter gene activity. Corresponding yeast gene mutants of those candidate genes and wild type control yeast were all transformed with pRS316 PGK p53 and pRS315 RE-Z plasmids again, grown for β-galactosidase assay and examined for reporter gene activity tests more than 3 times. Figure 3.1. indicates the mutants which exhibited the most significant reporter gene activity decrease when compared to wild type control. Those genes are *HNT3*, *MAP1* and *SAC1* genes which showed approximately 70%, 60% and 50 % decrease respectively in reporter gene activity.

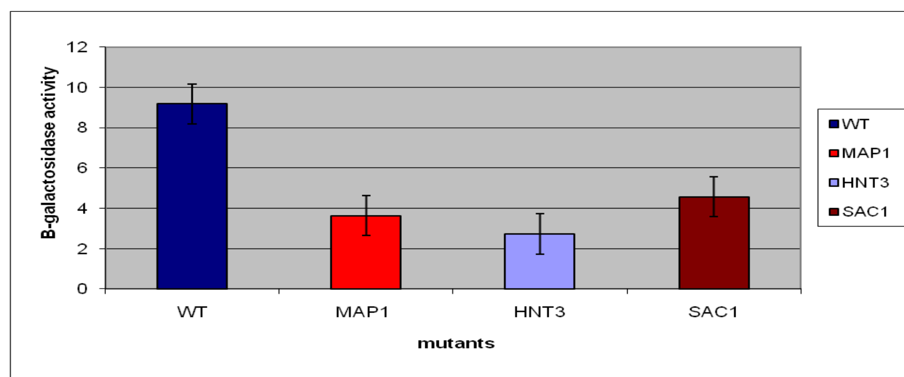

Figure 3.1. β-galactosidase assay results. Figure indicates the mutants that exhibited the most significant decrease in reporter gene activity.

After three genes had been selected as the most significant candidates, those genes were cloned into pAG413 vector via Gateway Cloning System. Then genes were sequenced to confirm whether the genes were cloned successfully. Sequence results confirmed that genes were cloned properly (data was not shown here).

After candidate genes had been cloned, pAG413 vectors carrying those genes and pAG413 empty vectors (ponly) were transformed into WT, Δ*hnt3,* Δ*map1* and Δ*sac1* yeast mutants one by one. Figure 3.2. indicates the complementation results.

There was no activity in any of the transformants. It was determined that in the presence of three plasmids in a yeast cell, reporter system was inhibited.

Transformants	B-gal activity
WT + ponly	0
WT + *HNT3*	0
WT + *MAP1*	0
WT + *SAC1*	0
Δ*hnt3* + ponly	0
Δ*hnt3* + *HNT3*	0
Δ*map1* + ponly	0
Δ*map1* + *MAP1*	0
Δ*sac1* + ponly	0
Δ*sac1* + *SAC1*	0

Figure 3.2. β-galactosidase assay results for complementations. ponly indicates empty pAG413 vector.

Problems that were encountered in handling three different plasmids in single yeast led the development of a new strategy to overcome the problems. To decrease the number of plasmids in the system, 2 plasmids carrying p53 and p53 RE-Z were tried to be combined in a single plasmid (see materials and methods section). Despite low efficiency in creating combined plasmid, sequence results indicated that combined plasmid was successfully created (data was not shown here). Via β-galactosidase assay analysis, reporter gene activity in WT yeast transformed with combined plasmid was analysed. Activity tests indicated wide variation when compared to the system with two plasmids. When the assays were repeated independently, results indicated that there was no or very low expression in reporter gene, when it was expressed from the combined plasmid (data was not shown here). Lack of reproducible results while using combined plasmid, resulted in the development of a new strategy to cope with the problem.

New strategy was the insertion of p53 and p53 RE-Z cassettes into the yeast genome. For this purpose, those cassettes were initially transferred into genome integration vectors and then transformed into W303-1A strain one by one (see materials and methods section). After both plasmids were inserted into the genome, the

transformant was analyzed for reporter gene activity to detect whether reporter system is functional when both plasmids are integrated into genome. Figure 3.3. indicates that the system is functional. This assay indicated that those integration plasmids integrated to the yeast genome properly. However, to confirm whether those plasmids did integrate into the correct location in the genome remains unknown until confirmation via PCR. For this purpose, site specific primers were designed. However, because those primers did not work properly, new primers were ordered and their arrival is expected. For that reason, site specific integration of those integration plasmids could not be confirmed via PCR. The next step was the creation of *hnt3*, *map1* and *sac1* gene knock outs on W3031A+305 RE-Z+306-P53 background (see materials and methods section). Creation of knock outs with homologous recombination based strategy did not work

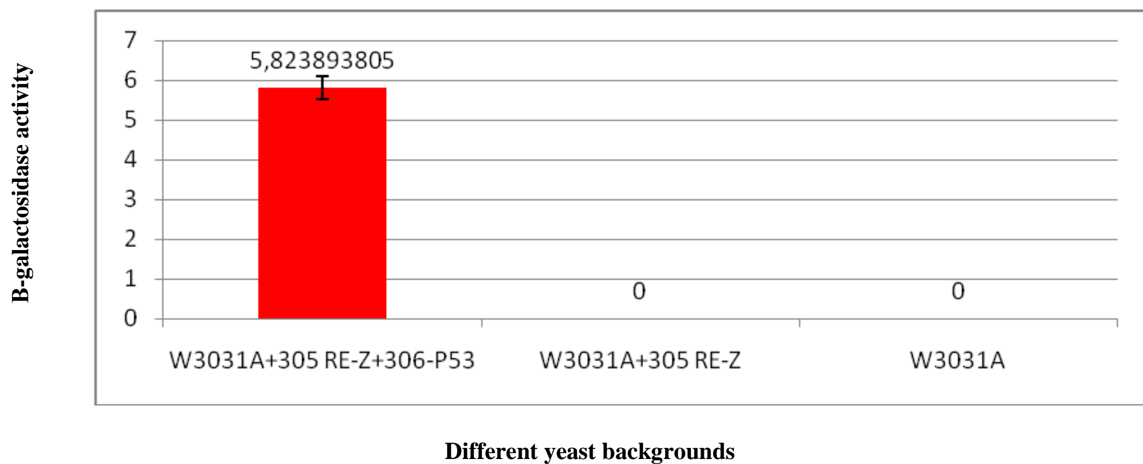

Figure 3.3. β-galactosidase results of W303-1A transformed with both of the integration vectors.

very efficiently. However, two of the knock outs (*hnt3* and *sac1*) were successfully created on W3031A+305 RE-Z+306-P53 background (Figure 3.4. , Figure 3.5.). After knock out transformants had been selected on G418 plates, genomic DNA isolation was performed from certain colonies and knock out positive colonies were confirmed via PCR. Figure 3.4. indicates that Δ*sac1* colony3 and Δ*sac1* colony4 exhibit the same band pattern with the PCR product used in the knock out transformation (band size suits expected 1922 bp band level), implies that Δ*sac1* colony3 and Δ*sac1* colony4 are positive for *SAC1* deletion.

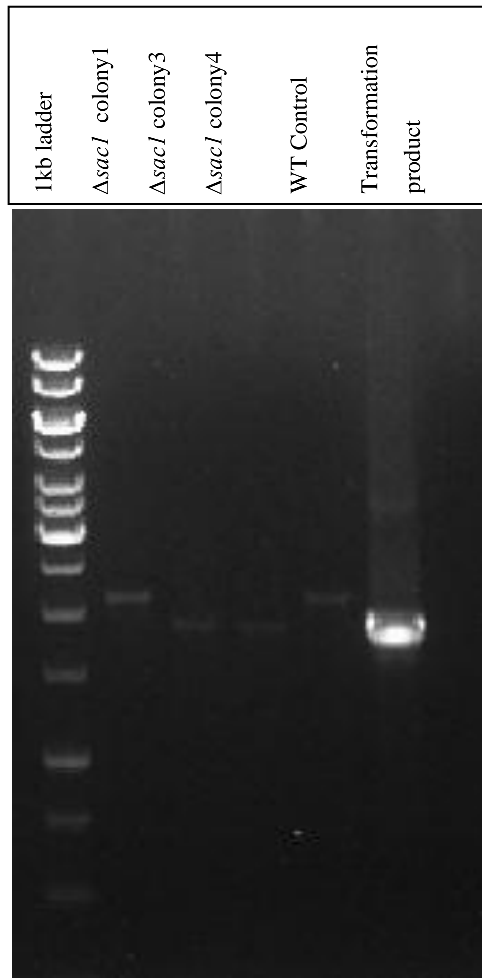

Figure 3.4. Creation of Δ*sac1*on W303-1A+305_REZ+306_p53 background

Figure 3.5 indicates that Δ*hnt3* colony1, Δ*hnt3* colony2, Δ*hnt3* colony4 and Δ*hnt3* colony5 exhibit the same band pattern with the PCR product used in the transformation (band size suits expected 1872 bp band level), implies that those colonies are positive for *HNT3* gene deletion. After creating knock outs, they were tested for reporter gene activity. Aim was to confirm whether in the absence of *SAC1* and *HNT3* genes in W3031A+305 RE-Z+306-P53 strain, there is decreased reporter gene activity compared to wild type control or not. Results showed that there was no

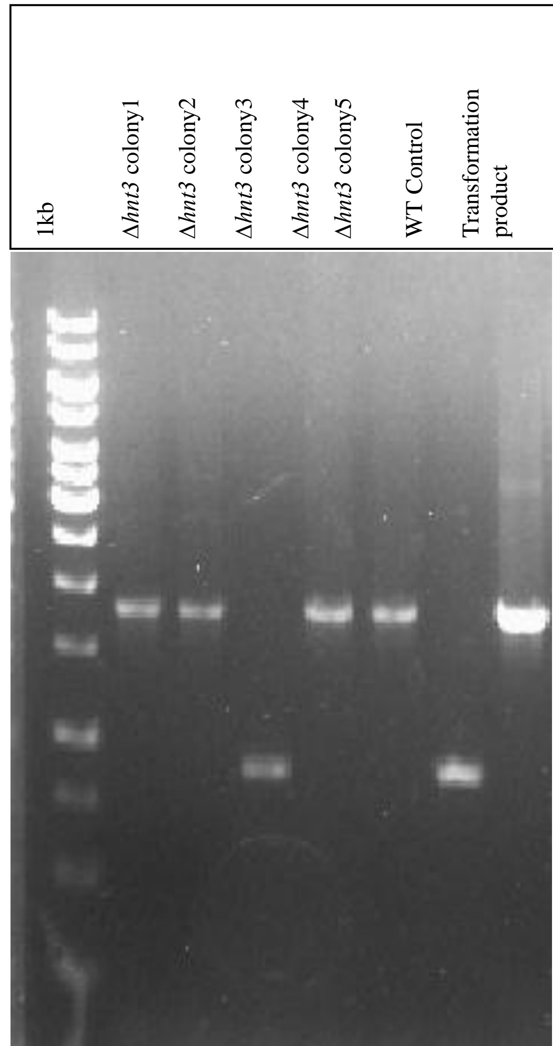

Figure 3.5. Creation of Δ*hnt3* on W303-1A+305_REZ+306_p53 background

reporter gene activity in knock out yeast whereas in wild type control, there was reporter gene activity (data was not shown here). Absence of reporter gene activity in knock out yeast could be explained in two ways. *i)* During creation of knock outs, homologous recombination might have affected the reporter sytem integrated into the genome and thus inhibit function of reporter system. *ii)* Removal of *SAC1* and *HNT3* genes from the genome may have such a significant effect on p53 activity that, reporter gene activity could not be observed. In order to test which explanation is valid, those yeast knock outs were complemented by transforming them with genes that they lack. Those transformants were subjected to reporter gene activity test. Results indicated that there was no reporter gene activity recovery after complementation (data was not shown

here). Thus, it was verified that, while creating knock outs, reporter system was damaged and became non functional.

Rarely, it might be possible that plasmids integrated into the genome can loop out and leave the genome. Therefore, knock out yeast on W3031A+305 RE-Z+306-P53 background, were grown in selective media one by one and their growth patterns were examined (Figure 3.6.). Here, only Δ*hnt3* colony2 results were shown as an example. In figure 3.5. , Δ*hnt3* colony2 was shown to be one of the successful knock outs. That sample was spreaded on different selective media and its growth pattern was examined overnight. Δ*hnt3* colony2 was able to grow in the absence of leucine but not in the absence of uracil. The result itself proposes that 306-p53 plasmid might have been removed while creating knock outs. Interesting thing is that those colonies can grow in the medium when both uracil and leucine are absent in the environment. This can be explained as in the following. Even if there is a few colonies that have both of the plasmids, it can trigger other yeast cells which lack 306-p53 to grow even in the selective media.

Figure 3.6. Growth pattern of Δ*hnt3* colony2 on different selective media

Results indicated that 306-p53 plasmid was less stabile in the genome. Therefore, p53 casette was determined to be introduced via pRS414-p53 plasmids into the cells which already has 305 RE-Z integrated to their genome. Those transformants were checked for reporter gene activity (Figure 3.7.). Results indicated that when 305 RE-Z plasmid is integrated into W303-1A genome, if p53 is expressed from a plasmid, reporter system is functional. Then, reporter system was tested to examine whether a second plasmid's (pAG413) transformation into the system block the reporter gene activity or not. Results indicated that addition of the second plasmid to the system did not inhibit reporter gene system (data was not shown here). Those results indicated that when there is single plasmid integration in the genome, transformation of two different plasmids into that yeast does not block the reporter system. Therefore, *hnt3*, *map1*, *sac1*

knock outs were determined to be created on W303-1A+305 RE-Z background. Up to now, *HNT3* gene was successfully removed from the genome (Figure 3.8.). Figure indicates that Δ*hnt3* colony1 and Δ*hnt3* colony2 are knock outs for *HNT3* gene.

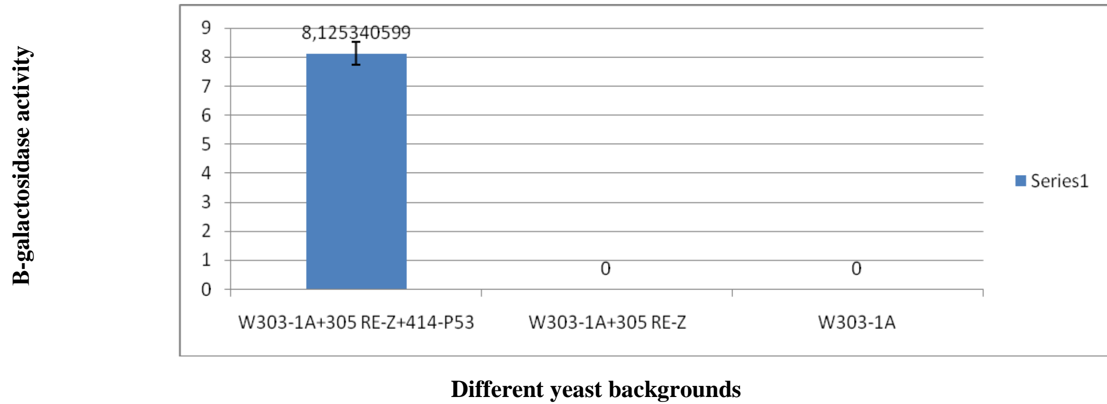

Figure 3.7. β-galactosidase activity in different transformants.

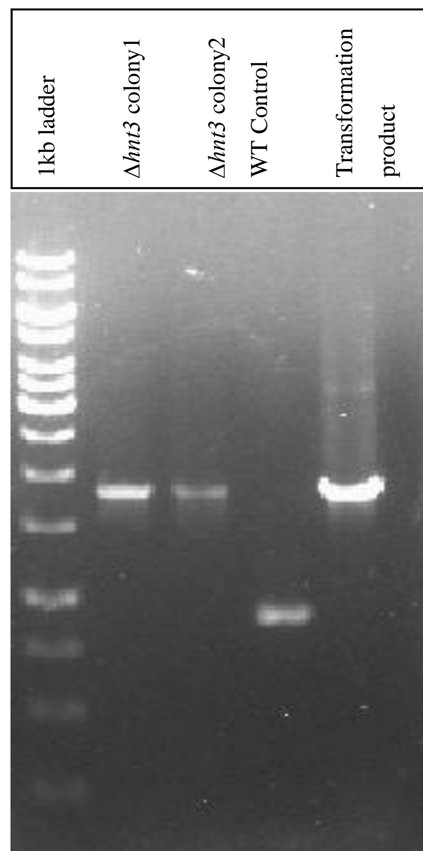

Figure 3.8. Creation of HNT3 k.outs on W303-1A+305 RE-Z background.

After creating *hnt3* knock out, it was tested for reporter gene activity. Aim was to confirm whether in the absence of *HNT3* gene in W3031A+305 RE-Z strain, there is decreased reporter gene activity compared to wild type control or not. Results indicated a significant decrease in the reporter gene activity in the absence of *HNT3* gene (Figure 3.9.). Further experiments should be done to examine the complementation sytem in *hnt3* knock out, to create other gene knock outs and to make their reporter system analysis.

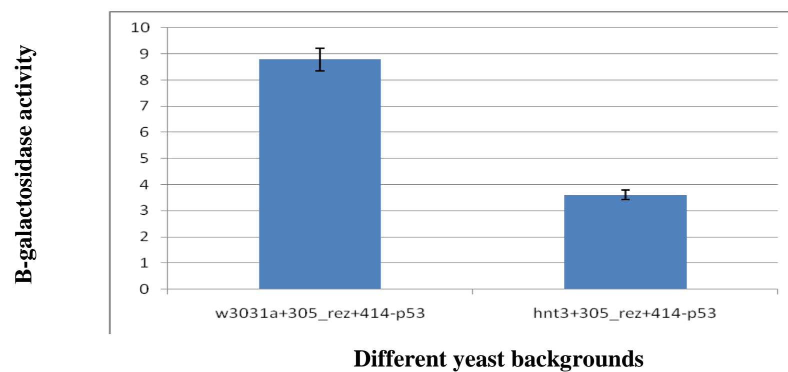

Figure 3.9. Reporter gene activity in Δ*hnt3* versus WT in W303-1A+305 RE-Z background.

CHAPTER 4

DISCUSSION

In this study, aim was to identify certain thiol-dependent oxidoreductases which may be regulating p53 protein activity post-translationally by reducing disulfide bonds in the target protein. Yeast, S.cerevisiae was used as a model organism to do the initial screening for examining those potential antioxidant genes which were classified so based on defined redox motifs in their structure. During the study, reporter gene system was used to detect critical antioxidant genes. The system was based on the binding of functional p53 protein to the DNA and then inducing the expression of reporter gene, LacZ.

Analysis of reporter gene activity in different potential antioxidant gene mutants indicated certain candidate genes which caused significant reduction in the reporter gene activity. Repetition of reporter gene activity analysis multiple times indicated the most significant potential antioxidant genes as *HNT3*, *MAP1* and *SAC1*. All of those three genes have their human homologs. One of those genes, *HNT3* is 5'AMP hydrolase which has been associated with DNA repair systems (Daley, Wilson et al. 2010). Yeast lacking *HNT3* was detected to be more sensitive to DNA damaging agents. In addition, *HNT3* was found out to be cooperating with Rad27-dependent LP-BER repair of oxidative and alkylating damage (Daley, Wilson et al. 2010). Its function in repairing oxidative damage may be associated with the antioxidant capability of Hnt3 protein. In addition, human homolog of *HNT3* gene, aprataxin was found out to have the same function with its yeast homolog. Recently, Sykora et al. identified that aprataxin played an important role in repair of mtDNA and defects in aprataxin led to mitochondrial dysfunction and oxidative stress (Sykora, Croteau et al. 2011). In the absence of aprataxin, mitochondrial dysfunction is initiated which is followed by ROS production and oxidative stress. Nevertheless, AOA1 patients with mutation in their aprataxin gene were identified to have elevated levels of oxidative DNA damage, lower levels of CoQ10 which is a potential antioxidant and also AOA1 patients have more genomic instability and tendency to develop cancer (Quinzii, Kattah et al. 2005; Le Ber, Dubourg et al. 2007). When all new findings are taken into account, aprataxin and its correlation

with oxidative stress makes *HNT3* gene as a critical gene for antioxidant like function. One of the other candidates in our analysis was *MAP1* gene which functions as a methionine aminopeptidase. *MAP1* human homolog is METAP1 gene that is structurally similar to METAP2 gene which is an important target of anticancer agents. It is known that removal of methionine is important for further amino terminal modifications like acetylation, myristoylation etc. In addition, addition of myristate residues to proteins predominantly occurs via thioester bond formation (Selvakumar, Lakshmikuttyamma et al. 2006). Due to their effects on modifying target proteins on their thiol groups, METAPs may also have oxidoreductase function revealing *MAP1* as a good candidate for antioxidant activity. The last candidate according to our analysis is *SAC1*, which is known to function as phosphoinositide phosphatase. Studies with mammalian *SAC1* revealed the importance of *SAC1* especially in golgi organization. In addition, *SAC1* was identified to work as a tumor suppressor (Liu, Boukhelifa et al. 2009). However, because it is known that *SAC1* is a phosphoinositide phosphatase, it is most likely that it can contribute post-translational modifications as a phosphatase instead of working like a thiol dependent oxidoreductease. Therefore, based on known function of *SAC1*, it does not seem to be a good candidate as an antioxidant. However, because some of the proteins may have multiple functions, *SAC1* gene also needs to be further analyzed.

Results presented here indicate the decrease in the p53 transactivation activity in the absence of certain potential antioxidant genes. However, those results do not indicate anything about the mechanism of the transactivation decrease or the inhibition mechanism is redox dependent or not. Therefore, based on the current data, it is not possible to propose whether p53 transactivation decrease is dependent on redox mechanism or an outcome of disruption in protein-protein interactions or any another type of regulation. In addition, absence of those genes may have an influence on one of the upstream regulators of p53 which causes a decrease in p53 transactivation capability. Also, a different post-translational mechanism rather than redox regulation may have an effect on decrease in p53 transactivation. For instance, in the case of *SAC1*, mechanism can be correlated with phosphorylation/dephosphorylation process of p53. On the other hand, using yeast as a model organism can be limiting the interactions between p53 and its upstream regulators. In addition, yeast is known to lack certain post-translational modifications found in higher eukaryotes, lack of those post-

translational modifications in upstream regulators of p53 may be inhibiting transactivation activity of p53.

It is possible enlighten the potential redox mechanism behind candidate genes through an appropriate experimental design. Initially, after creation of all the knock outs on W303-1A+305 RE-Z background, those gene mutants should be transformed with p53 including plasmid and reporter gene activity in them should be measured and compared with wild type control to confirm whether there is decrease in transactivation of p53 in gene mutants derived from W303-1A+305 RE-Z strain. If decreased reporter gene activity can be observed, those gene mutants should be complemented with the genes they lack to understand whether regain of the gene recovers decreased transactivation activity of p53. Then, the reporter gene activity should be tested by transforming yeast mutants with antioxidant genes and p53 whose cysteines were transformed into serines, the closest amino acid to cysteine. In the absence of cysteines in p53, stability of p53 protein is expected to be increased via eliminating oxidation sites. In contrast, in the absence of cysteines in potential antioxidant genes, p53 stability is expected to decrease due to inhibition in thiol-dependent oxidoreductase activity of the antioxidant gene on p53. In addition, to confirm that decrease in reporter gene activity is not dependent on change in the protein level of p53 but due to post-translational modification on p53, western blotting should be carried out. Finally, repetition of those experiments in reducing and non-reducing conditions at the same time, may give more idea about the redox regulation mechanism. Those experiments above can enlighten whether p53 activity decrease in this system is a redox-dependent mechanism or not.

CHAPTER 5

CONCLUSION

In conclusion, 7 antioxidant gene mutants of yeast were further analyzed for p53 transactivation activity and three of them *(Δhnt3, Δmap1, Δsac1)* were shown to exhibit the most significant decrease in p53 activity when compared to wild type yeast. Especially, *HNT3* gene which is homologous gene for human aprataxin is predicted to be a strong candidate for having a thiol-dependent oxidoreductase activity. Whether the p53 transactivation decrease was dependent on redox regulation of the protein or not could not be determined based on the results. However, complementation assays followed by analysis of Cys→Ser mutations may open a new perpective in the regulation of p53 protein by thiol-dependent oxidoreductases.

5.1. Future Perpectives

Using yeast, S.cerevisiae to study human p53 gene may be advantageous to perform an initial functional screening of potential antioxidant genes. However, data proposed here should be examined in different mammalian cell lines, to understand that mammalian homologs of those genes also have exactly the same function as their yeast homologs. Because, yeast cells lack certain post-translational modifications and some of the upstream regulators of p53, transactivation ability of p53 may change when the same reporter system of yeast is examined in the mammalian cell lines.

In clinical studies, DNA damaging agents have been widely used. Understanding of redox regulation of target genes in the cancer related pathways may lead the appearance of new perspectives in clinics. If importance of redox regulation of target proteins could be clearly identified, tissue specific application of certain thiol-dependent oxidoreductases may become a valuable therapy for treating many of the redox dependent cancers, in the future.

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APPENDIX A

PROTOCOLS

Plasmid Isolation from E. coli

For plasmid isolation from E.coli at low and large scale, PureLink™ Quick Plasmid Miniprep Kit and PureLink™ HiPure Plasmid Filter Midiprep Kit that are commercially available from Invitrogen were used respectively. All experimental steps were carried out based on manufacturer's recommendations.

Plasmid Transformation into E. coli

E.coli competent cells were defrosted on ice for 10 minutes. Then, approximately 200 ng plasmid was added into competent cells and mixture was kept on ice for 20 more minutes. Mixture was subjected to heat shock (42 ^{0}C) for 40 seconds and then kept on ice for 2 minutes. Then, 250 ul SOC medium is added to mixture and mixture is kept in a shaking incubator (37 ^{0}C) incubator for 1 hour. Mixture is spinned down and supernatant is removed then cell pellet is resuspended in 150 ul sterile water and spreaded on LB agar plate which includes selective antibiotic. Plate is kept at 37 ^{0}C overnight for colonies to grow.

PCR Reaction for Cloning Genes

1 ul BY4741 genomic DNA
5 ul HF buffer with $MgCl_2$
5 ul DNTP mix
0.5 ul forward primer (10 pmol)
0.5 ul reverse primer (10 pmol)
0.5 ul HF enzyme commercially available from Fermentas
37.5 ul UP water

PCR reactions were carried out by taking optimum temperature for primer annealing and gene product size into account. 35 PCR cycles are performed.

Agarose Gel Electrophoresis and Gel Extraction of PCR product

50 ul 1X TAE buffer
0.5 gr agarose
EtBr

All PCR products are loaded on gel and run at 100 volts for 1 hour. Then, PCR product at appropriate level is cut and subjected to gel extraction via Fermentas Silica Bead DNA Gel Extraction Kit according to manufacturer's recommendations.

Linearization of Genome Integration Plasmids, Plasmid Digestion

20 ul plasmid DNA (isolated via midiprep, approximately 40 ug)
15 ul UP water
15 ul appropriate enzyme buffer
3.5 ul appropriate restriction enzyme
0.2 ul DTT
Restriction reaction was kept at appropriate temperature for 3.5 hours and then reaction was stopped via heat inactivation.

APPENDIX B

PRIMERS

Primers for Gene Deletion

HNT3A:CTTACATCTTGCCCAAGGATGCAC
HNT3B:CGTGATCACATCAACCCTGATGA
MAP1A:TCATCCCAAGAACGCTATTGTGT
MAP1B:GCTTCTCATAATAACCAATTCGGAC
SAC1A:CTGCACTACTGCTTACCCACACTAC
SAC1B:ATAGCTTGTTCTTCCCTCTGGG

Primers for Site Directed Mutagenesis

primer name	primer sequence
SAC1 C392S	cattccgttgtaagaacaaacagtatggattgtttggacagaa
SAC1 C392SB	ttctgtccaaacaatccatactgtttgttcttacaacggaatg
SAC1 C395S	gtaagaacaaactgtatggatagtttggacagaacaaatgtcg
SAC1 C395SB	cgacatttgttctgtccaaactatccatacagtttgttcttac
MAP1C37S	gagaaacttcgtcccaaatgaaaagccctgtttgtc
MAP1C37SB	gacaaacagggcttttcatttgggacgaagtttctc
MAP1C40S	cccaaatgaaatgccctgttagtctaaaacaaggcatc
MAP1C40SB	gatgccttgttttagactaacagggcatttcatttggg
HNT3C188S	acctgaaagaacacgatttacttagctgctattgtcagc
HNT3C188SB	Gctgacaatagcagctaagtaaatcgtgttctttcaggt
HNT3C189S	gaaagaacacgatttactttgcagctattgtcagcgaaattttag
HNT3C189SB	ctaaaatttcgctgacaatagctgcaaagtaaatcgtgttctttc
HNT3C191S	gaacacgatttactttgctgctatagtcagcgaaattttagtaataaa
HNT3C191SB	tttattactaaaatttcgctgactatagcagcaaagtaaatcgtgttc