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Effects of Evolutionary Changes and Mutations on Tetramer Formation and Function in Tumor Suppressor Protein p53

癌抑制タンパク質 p53 における四量体化ドメインの
進化による変化および変異が四量体形成と機能に及ぼす影響

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Abbreviations

3Ala	monomer mutant p53 with 3 Ala at L330, I332, and L344
Aca	aminocaproic acid
BG values	background value
CD	circular dichroism
CH-p53TD	Chinese hamster p53 tetramerization domain
DAPI	4',6-diamidino-2-phenylindole
DTT	dithiothreitol
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
GP-p53TD	guinea pig p53 tetramerization domain
HU-p53TD	human p53 tetramerization domain
LFS	Li-Fraumeni syndrome
LOH	loss of heterozygosity
MALDI-TOF MS	Matrix assisted laser desorption/Ionization-time of flight mass spectrometry
NLS	nuclear localization signal
OP-p53TD	opossum p53 tetramerization domain
p53TD	tetramerization domain of p53 protein
PBS	phosphate-buffered saline
RE	response element
ROS	reactive oxygen species
SH-p53TD	sheep p53 tetramerization domain
TCEP	Tris(2-carboxyethyl)phosphine Hydrochloride
TD	tetramerization domain
TFA	trifluoroacetic acid
TS-p53TD	tree shrew p53 tetramerization domain
WT	wild type p53
ZF-p53TD	zebrafish p53 tetramerization domain.

1. General introduction

1.1. Tumor suppressor protein p53

The tumor suppressor protein p53 was originally identified as a 53 kDa SV40 large T antigen-binding protein [1,2]. Initially, p53, which is highly expressed in cancer cells, was classified as an oncogene, but it was later shown that there are many mutations in the p53 gene in these cancer cells. Subsequent studies established that the wild-type (WT) p53 protein encoded by the TP53 gene functions in the cell by inhibiting proliferation and tumorigenesis in cell culture [3], and that mutations in TP53 associated with increased expression levels of the resulting variant p53 proteins are common in a wide arrange of human tumors [4].

Consistent with its role as a tumor suppressor, mutations in TP53 are a hallmark of a hereditary cancer predisposition disease known as Li-Fraumeni syndrome [5,6], and TP53 knockout mice develop tumors at an unusually high rate [7]. To prevent tumorigenesis, the WT p53 protein responds to various genotoxic stresses, including UV light, radiation, oxidative stress, and starvation stress [8,9]. Once expressed at sufficient levels in cells, p53 adopts a tetrameric form, which is the active form that induces apoptosis and cell cycle arrest via transcription-independent, transcription-dependent, and miRNA processing. In transcription-dependent induction of apoptosis, the p53 protein binds as a tetramer to response elements present in its target genes and this leads to transcriptional activation of a number of pro-apoptotic genes, including Bax and Puma, as well as the pro-cell cycle arrest genes, CDKN1A (p21) and GADD45 [10]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis has also shown that p53 is activated by

numerous stress signals and increases in p53 protein levels is a major response to cell cycle arrest, cellular senescence, and apoptosis. It also activates transcription of negative feedback pathways such as MDM2 [<https://www.genome.jp/pathway/hsa04115>].

1.2. Mutations of p53 gene, *TP53*

The TP53 gene is one of the most frequently mutated genes identified in human tumors to date [11,12]. These mutations in TP53 lead to the formation of a wide range of variant p53 proteins including those that have been shown not only to impair the tumor suppressor function of p53, but also to acquire an inherent novel oncogenic function, often referred to as "gain-of-function". The presence of TP53 mutations is often associated with more aggressive tumors that are resistance to therapy, and thus have poor prognosis for the patient. The majority of mutations in TP53 that have been found in tumors are missense mutations. These missense mutations are often located within the region of TP53 encoding for the DNA-binding domain in the p53 protein and they often lead to amino acid substitutions that decrease the protein's binding affinity for its target DNA sequences in genes it regulates, thereby impairing its function as a transcription factor . As a result, p53 may fail to activate or suppress the expression of target genes involved in cell cycle arrest and apoptosis.

In addition to mutations within the region coding for the DNA-binding domain, mutations within the region coding for the tetramerization domain (TD) of p53 (residues 325-358 in the Human p53 sequence) can lead to variant p53 proteins with altered functions associated with various cancers. The effect of tumor-derived missense

mutations on the stability and status of the oligomeric structure of the resulting p53 protein has been reported. In a comprehensive study, 40 unique peptides containing amino acid substitutions in the TD of human p53 associated with germline mutations in TP53 associated with different cancers were quantitatively analyzed to determine the effect of these substitutions on the thermal stability of the TD and its ability to form tetramers. [13]. Interestingly, these amino acid substitution had a wide range of effects both on the ability of the peptide to form a tetrameric structure varied greatly as well as on their thermal stability. These results suggest that even a very slight destabilization of the tetrameric structure of the p53 protein in cells may lead to a decrease in its ability to function as a tumor suppressor, since formation of the tetrameric structure is critical for protein-protein interactions, DNA binding, and post-translational modifications of p53. It also suggests that the threshold for loss of tumor suppressor activity that result from mutations associated with the TD region of TP53 may be very low in terms of destabilization of the tetrameric form of the p53 protein. As a result, small molecules, such as calixarene [14,15], that have the capacity to bind and stabilize the tetrameric form of the p53 protein are actively being considered as a potential therapeutic strategy as anticancer agents.

1.3. p53 family

The p63 [16] and p73 [17,18] proteins are considered to be members of the p53-family family of proteins due to the fact that they are structural and functional homologs of p53. These three proteins share a similar domain architecture and sequence homology with including the ability to form tetramers, bind to specific DNA sequences, activate

p53-responsive genes as well as mediate cell cycle arrest, cellular senescence, and apoptosis in response to DNA damage. Although p63 and p73 share many functional properties with p53 and cooperate with p53 to regulate tumorigenesis in the absence of stress, the most important role of these p53-family members is their ability to regulate cellular differentiation and development. In particular, the p63 protein plays an important role in controlling epidermal development[19,20], whereas the p73 protein has an essential role in regulating neuronal differentiation and it is essential for the development of the nervous and olfactory systems [21,22]. This is demonstrated by the fact that p63-deficient mice are characterized with severe limb amputation, lack of skin, hair, teeth, mammary, lacrimal, and salivary glands [23,24], whereas p73-mice display complex defects in neuronal development [21,25].

1.4. p63

The p63 protein is actually the oldest member of the p53 family in terms of evolution. It is highly expressed in the ectoderm of embryos and in the nuclei of basal regenerative cells of many adult epithelial tissues, including skin, breast myoepithelium, oral epithelium, prostate and urinary tract skin. In contrast to the tumor suppressive functions associated with p53, overexpression of select splice variants of p63 have been observed in many squamous cell carcinomas, suggesting that p63 may in fact act as an oncogene. Furthermore, it is known that the TP63 gene has two promoters that result in the production of different proteins, one with transcriptional activation (TA) domain at the N-terminal region of the protein (TAp63) and a second one that is missing the TA domain

(Δ Np63) [26,27]. Importantly, basal cells of normal human epithelia, including epidermis, strongly express the p63 protein. However, they primarily express the Δ Np63 isoform (ratio of Δ Np63 to TAp63 is approximately 100:1), which disappears as soon as these cells leave the stem cell compartment. Zebrafish embryos require the Δ Np63 protein to suppress the activity of p53 and this allows epidermal proliferation and limb development [28,29]. This study demonstrates an essential and ancient role of Δ Np63 in skin development.

1.5. p73

Like p63, p73 is also a member of an evolutionary more ancient member of the p53-family proteins. As seen for p63, selective splicing at the 3'-end of the p73 gene results in seven different splice variants that contain different C-termini and they are referred to as α to η [30,31]. Although a variety of functions and activities have been reported for the p73 proteins collectively, the individual roles for the specific variants are currently not clearly established. The p73 gene *Trp73* is rarely mutated in tumors. The p73 mutants differ slightly in their N-terminal sequences, but all lack the transcriptionally active domain and are collectively referred to as Δ N isoforms. Thus, based on the N-terminus, we can distinguish between TA isoforms that contain the TA domain and Δ N isoforms that do not. In general, the Δ N isoforms act as dominant negatives of the p73 TA isoform as well as the p53 and p63 TA isoforms.

1.6. Structure of p53 family

In terms of the domain architecture of p53-family proteins, they all possess an N-terminal transcription activation domain (TAD), which is followed sequentially by a proline-rich domain (PRD), a DNA-binding domain (DBD), and a tetramerization domain (TD). However, the proteins vary at the C-terminal ends of the three proteins. The p53 protein contains a disordered basic domain (OD), whereas the p63 and p73 proteins have a SAM domain [32,33]. The N-terminal TAD is a highly negatively charged region that is required for transcriptional activity. Despite being intrinsically disordered, this region has been shown to interact with a variety of transcriptional regulatory factors, including several histone acetyltransferases (HATS) and the E3-ubiquitin ligase MDM2. The PRD region has also been shown to play a role in regulating the stability of p53, and deletion of this region renders the p53 protein more susceptible to degradation by MDM2. The DBD is considered to be the core region of the p53 protein and it is essential for sequence-specific DNA binding capacity of the protein and thus its transcriptional activity. The C-terminus of the p53 protein also functions as a negative regulatory domain and is involved in the induction of cell death. Of all of the domains of p53, the DBD is the most highly conserved region when comparing the sequences of p53 and this includes not only when comparing the mammalian p53-family proteins, but also when including homologs from *Drosophila melanogaster* and *Caenorhabditis elegans*. The high degree of conservation within the DBD of p53 family proteins is consistent with the fact that the majority of mutations in *TP53* associated with cancer are missense mutations within the region coding for amino acids within the DBD.

1.7. Evolution of p53 family

The common ancestor proteins of the p53 family are expressed in invertebrate collar flagellates and anemones, and the amino acid sequence and structure of the DBD are very similar and evolutionarily conserved in higher organisms. Surprisingly, the specific DNA sequences to which p53 family proteins bind in invertebrates and vertebrates are also virtually identical. Subsequently, three clear p53 homologs were identified in the genome of early vertebrate Lamprey, followed by clear presence of genes for expressing p53, p63, and p73 in the genomes in the ray-finned fish, a subsequently evolved bony fish [34–36]. The function of this ancestral p53 family gene in sea anemones is to prevent DNA damage in the gametes of germ cells. Subsequently this p53 gene has evolved to where it now functions as a tumor suppressor in vertebrates as well as in somatic cells. However, it has not been clear until now as to what changes caused p53 to function differently from the other family proteins, p63 and p73 during the course of evolution.

1.8. Aims of this study

Tetramer formation of the p53-family proteins is essential for most if not all of their physiologically critical functions and there is strong correlation between the stability of the tetrameric structure formed by p53-family proteins and their activities, and in particular their ability to activate transcription. Mutations in *TP53* in germline cells are often localized in the region coding for residues within the tetramerization domains. However, the mechanism by which these mutations that lead to amino acid substitutions in the tetramerization domains are involved in cellular oncogenesis is unknown. Moreover,

despite the importance of the p53-family proteins in vertebrate evolution, changes in the stability of these tetrameric structures during the evolutionary process and their effects on function are unknown. Therefore, it is important to elucidate the structures of these different tetramerization domains of the p53-family members from different species to better understand how their functional roles have evolved during the course of evolution. To this end, I have explored the effects of mutations in tumors and evolutionary substitutions in the tetramerization domain on the structural stability and heterotetramer formation, and the role of the tetramer formation in the specific functions of the p53 family.

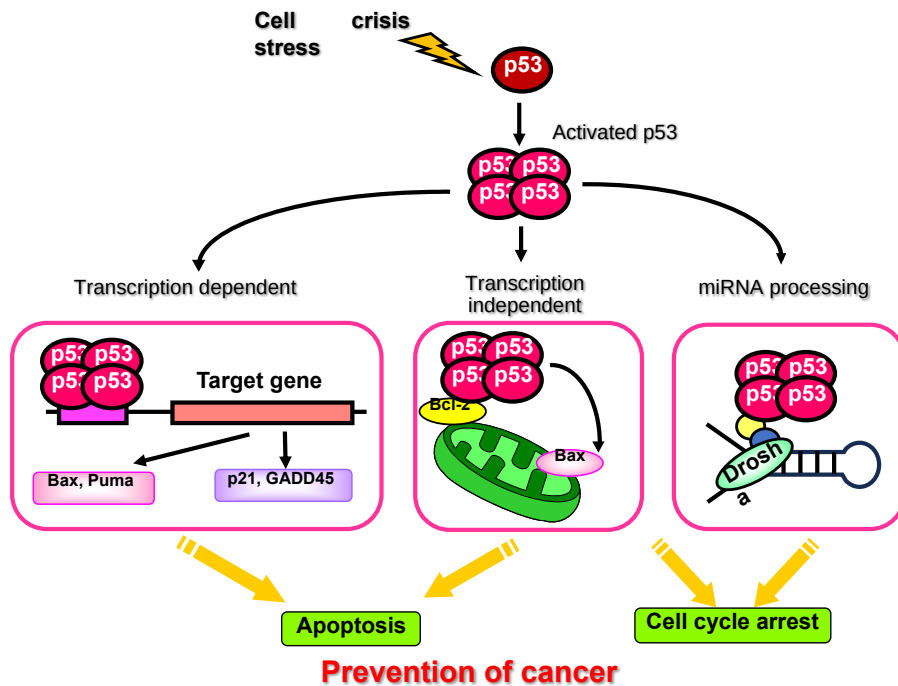


Figure 1-1. Function of Tumor Suppressor Protein p53

The tumor suppressor protein p53 is tetramerized and activated in response to cellular crisis stresses such as oxidative stress and UV radiation. Activated p53 induces apoptosis and cell cycle arrest through transcription-dependent, transcription-independent, and miRNA processing. The transcription-dependent pathway promotes transcription of genes involved in apoptosis (Bax, PUMA) and cell cycle arrest (p21, GADD45). The transcription-independent pathway disrupts the Bcl2/Bax complex, an anti-apoptotic protein, in mitochondria and induces Bax-mediated apoptosis. p53 interacts with the miRNA-processing Drosha complex to promote post-transcriptional maturation of miRNAs and induce cell cycle arrest. Induces cell cycle arrest by promoting post-transcriptional maturation of miRNAs.

1	61	101	300	324	358	393
TAD	PRD	DBD		TD	BD	
10	20	30	40	50		
MEEPQSDPSV	EPPLSQETFS	DLWKLLPENN	VLSPLPSQAM	DDLMLSPDDI		
60	70	80	90	100		
EQWFTEDPGP	DEAPRMPEAA	PPVAPAPAAP	TPAAPAPAPS	WPLSSSVPSQ		
110	120	130	140	150		
KTYQGSYGFR	LGFLHSGTAK	SVTCTYSPAL	NKMFCQLAKT	CPVQLWVDST		
160	170	180	190	200		
PPPGTRVRAM	AIYKQSQHMT	EVVRRCPHHE	RCSDSDGLAP	PQHLIRVEGN		
210	220	230	240	250		
LRVEYLDDRN	TFRHSVVVPY	EPPEVGSDCT	TIHYNMCNS	SCMGMNRRP		
260	270	280	290	300		
ILTIITLEDSE	SGNLLGRNSF	EVRVCACPGR	DRRTEENLR	KKGEPHHELP		
310	320	330	340	350		
PGSTKRALPN	NTSSSPQPKK	KPLDGEYFTL	QIRGRERFEM	FRELNEALEL		
360	370	380	390	393		
KDAQAGKEPG	GSRAHSSHLK	SKKGQSTSRH	KKLMFKTEGP	DSD		

Figure 1-2. Schematic structure and amino acid sequence of p53

TAD: transactivation domain (green), PRD: proline-rich domain (yellow), DBD: DNA binding domain (cyan), TD: tetramerization domain (magenta), BD: basic domain (blue). The TD targeted in this paper is a domain consisting of 35 amino acid residues located on the C-terminal side of p53.

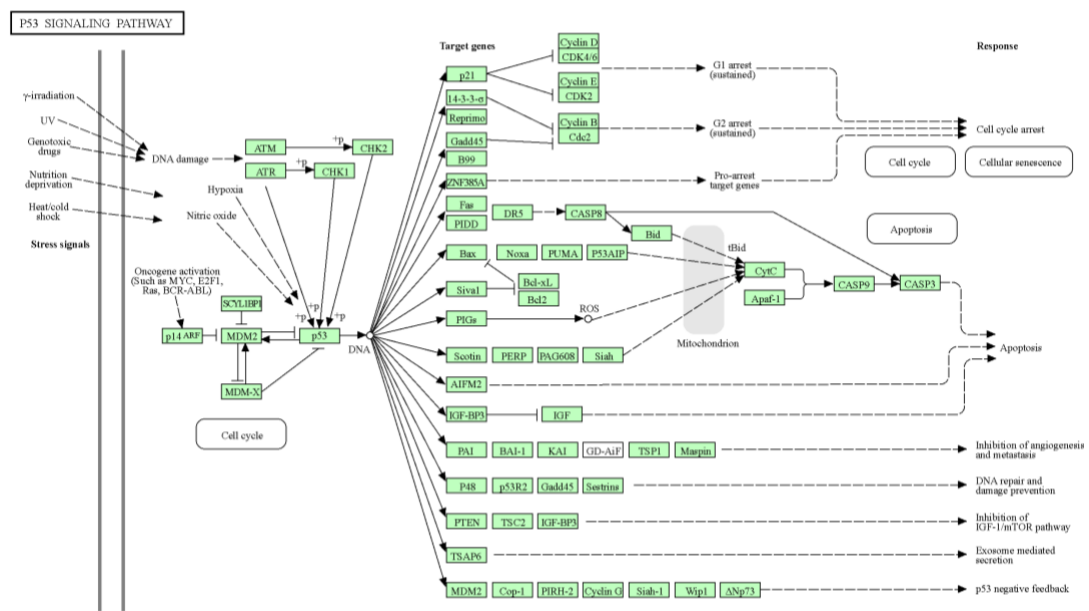


Figure 1-3. p53 signaling pathway

The results of KEGG pathway analysis are shown. p53 is activated by various stress signals such as DNA damage and oxidative stress. Transcription of p53-regulated genes is activated by activated p53, inducing major responses such as cell cycle arrest, cellular senescence, and apoptosis. It is also involved in genes involved in DNA damage recovery. Transcription of genes such as MDM2, which is involved in negative feedback of p53, is also activated. [<https://www.genome.jp/pathway/hsa04115>]

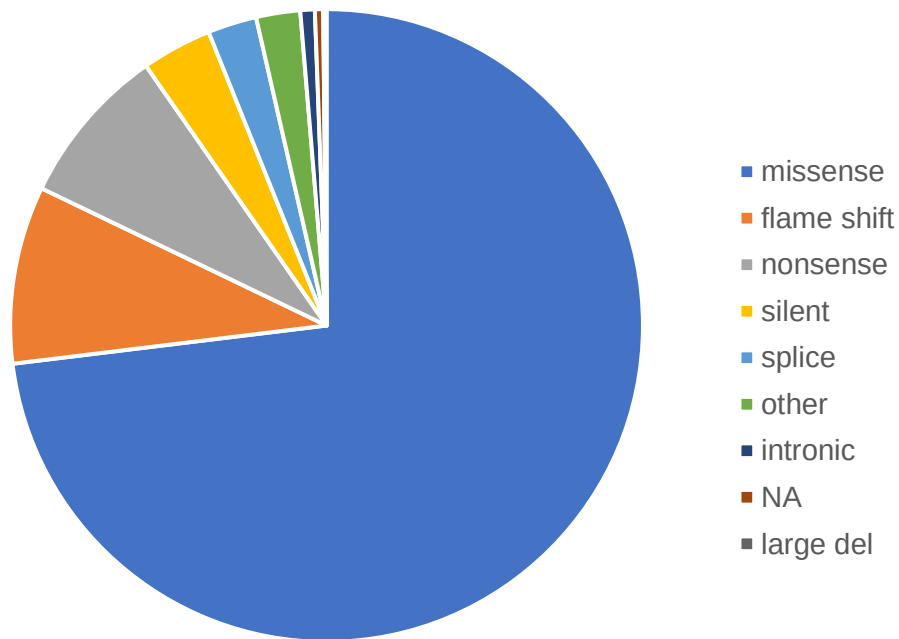
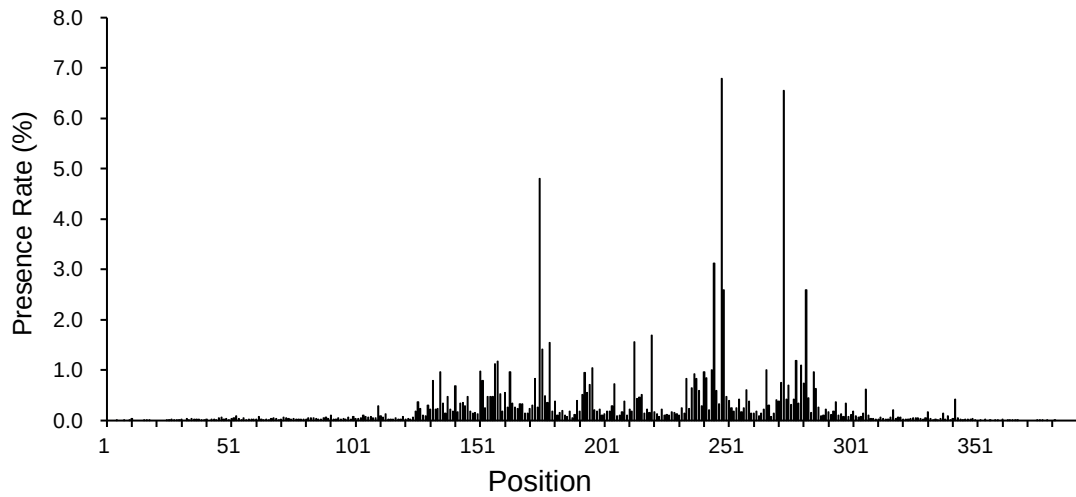


Figure 1-4. Types of p53 mutant variants in tumors.

Missense mutations account for about 3/4 of p53 mutations (N=28,855) in tumor, followed by frameshift (FS) and nonsense mutations as the most prevalent types. These mutations may have reduced or lost the effectiveness of p53, leading to tumorigenesis. The numbers are based on data in the version R20 of "The *TP53* Database" [<https://tp53.isb-cgc.org>], [12].

A Somatic mutations



B Germline mutations

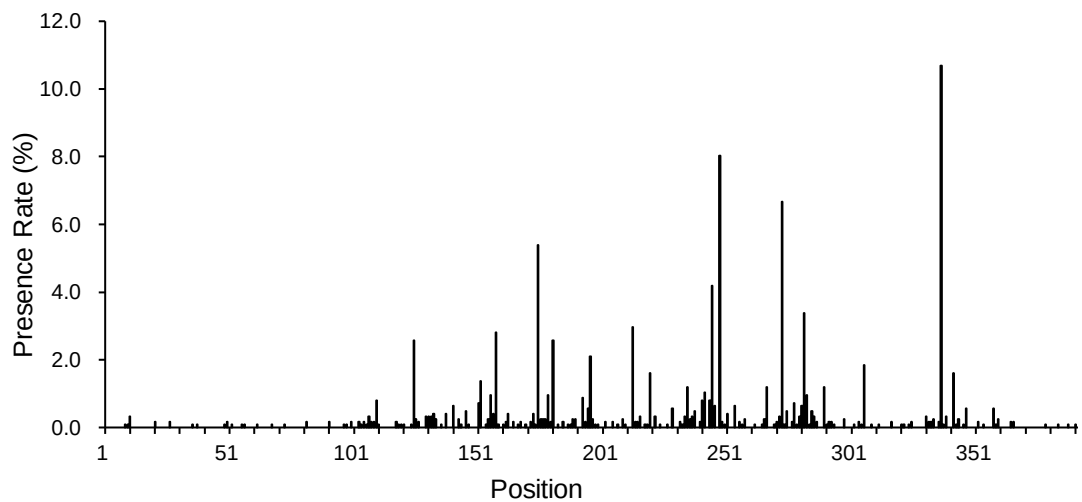


Figure 1-5. Codon distribution of p53 mutants in tumors.

(A) The distribution of *TP53* mutations in human somatic tumors (N=27,847) was shown to be concentrated in the DNA-binding domain. (B) Many mutations were also found in the tetramerization domain for germline cells (N=1247). The numbers are based on data in the version R20 of "The *TP53* Database" [<https://tp53.isb-cgc.org>], [12].

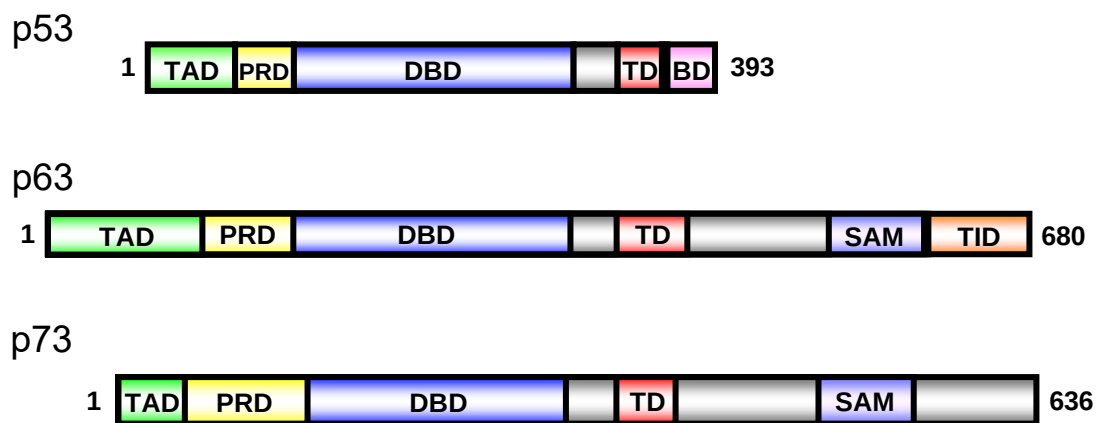


Figure 1-6. Schematic structure of the human p53 family proteins.

TAD: transactivation domain, PRD: proline-rich domain, DBD: DNA binding domain, TD: tetramerization domain, BD: basic domain, SAM: Sterile α Motif domain, TID: transactivation inhibitory domain

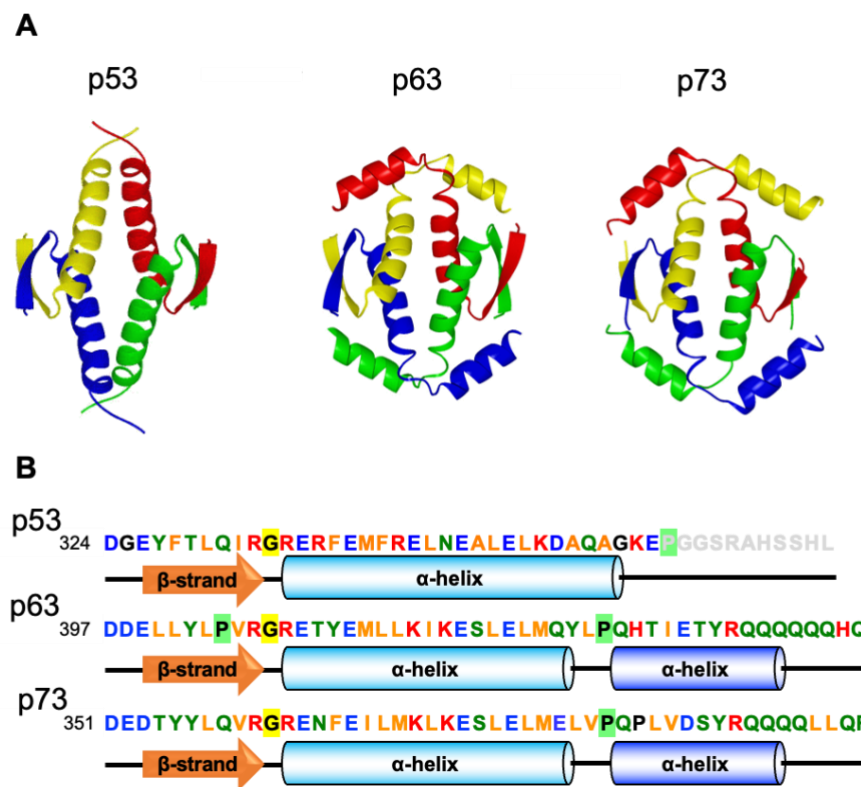


Figure 1-7. Structures of the tetramerization domain of the Human p53 family

The tetramerization domain of the Human p53 family consists of a beta strand, a tight turn, and an alpha helix. p63 and p73 have a slightly shorter first helix followed by a second helix that is not present in p53. A: Crystal structure of the Human p53(PDB: 1AIE), p63(PDB: 3ZY1), and p73(PDB: 2WQI) tetramerization domain. B: Amino acid sequence of Human p53, p63, and p73 and corresponding secondary structure.

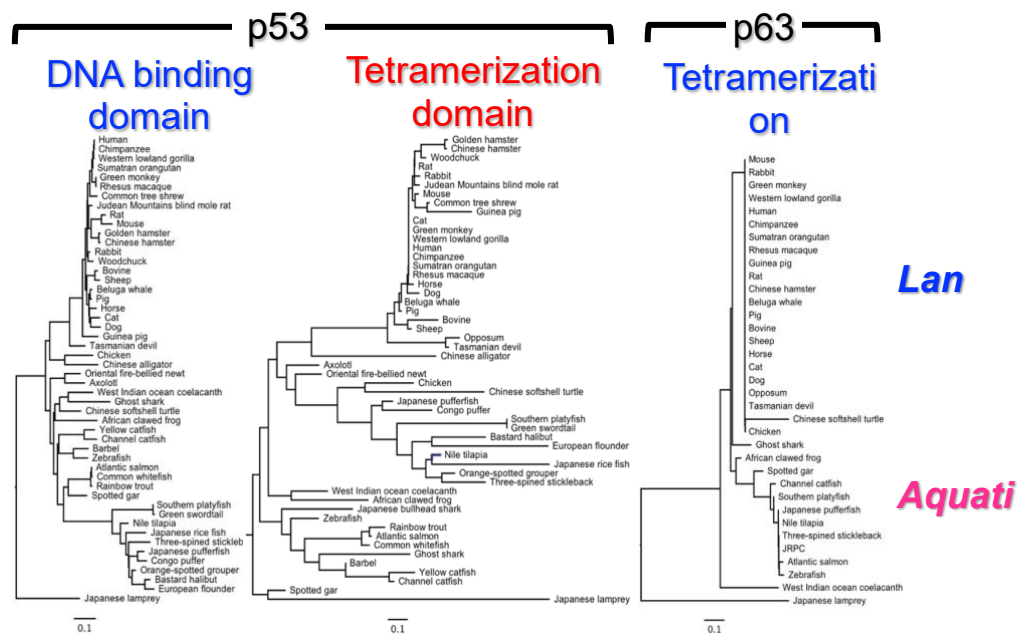


Figure 1-9. comparison evolution rate

The results of phylogenetic tree analysis of the respective domains of p53 and p63 in vertebrates are shown. Terrestrial and aquatic organisms are shown at the top and bottom, respectively. p53's tetramerization domain has longer branch lengths across species, indicating evolutionary diversification compared to p53's DNA binding domain and p63's tetramerization domain.

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2. Tetramerization of mutant p53 found in cells of Li-Fraumeni syndrome

2.1. Abstract

Li-Fraumeni syndrome (LFS) is inherited genetic disorders caused by heterozygous mutations in the *TP53* gene. Among the *TP53* mutations associated with Li-Fraumeni syndrome, missense mutations of Arg337, namely R337C and R337H, are particularly frequently found. Although there are many reports about individual mutant in Li-Fraumeni syndrome, the interaction of TD mutants and wild-type p53 in Li-Fraumeni syndrome cells remains unclear.

In this study, a novel assay system was developed to analyze the ability of the p53 TD mutants expressed with the wild-type protein to form heterotetramers as well as the overall transcriptional activity and employed to LFS-associated p53 TD mutants.

The results showed that whereas the heteromeric activity of the R337C p53 mutant with WT p53 was only slightly reduced compared to WT p53, its transcriptional activity was reduced by more than 50% of the activity of the wild-type protein. In addition, even though the heterotetramer formation ability of the R337H mutant was more-or-less normal, its transcriptional activity was markedly reduced. Taken together, these results indicate that these mutants produce a previously unknown dominant-negative-like effect. They also suggest that the decrease in activity observed with these mutations is not merely due to a decrease in their ability to form a tetrameric structure but also to the stability of the tetramer structure. Interestingly, these LFS-associated p53TD mutants showed a more pronounced decrease in activity against the low-affinity apoptosis-inducing *bax*

responsive element than the high-affinity *CDKN1A* responsive element involved in cell cycle arrest. This study shows for the first time that mutations in the tetramerization domain of p53 can have a dominant-negative effect and these findings have the potential to greatly advance our understanding of the role of multimerization in the mechanism of LFS pathogenesis.

2.2. Introduction

Li-Fraumeni syndrome (LFS) is inherited genetic disorders [1–3], and patients with these disorders frequently develop juvenile cancers. The syndrome was first reported by Frederick Li and Joseph Fraumeni in 1969 [1]. The syndrome is mainly caused by heterozygous mutations in the *TP53* gene [4–6]. Among the *TP53* mutations associated with Li-Fraumeni syndrome, missense mutations in Arg at the position 337 which include R337C and R337H are particularly frequently found [7–10]. The *TP53* gene encodes for the tumor suppressor protein p53, which is a 393-residue transcription factor that is activated in response to genotoxic stress and plays a central role in tumor suppression through transcriptional regulation of multiple target genes [11–13]. To activate transcription, the p53 protein must adopt a homotetrameric structure and tetramerization occurs through a short tetramerization domain (TD) located between residues 326–356 in the sequence [14]. The position of Arg337 of the LFS mutation located in the TD and the salt bridge between Arg337 and Asp354 plays an important role in stability of the tetramer formation.

Dominant negative effects occur when variant proteins out compete the wild-type proteins and inhibit the normal functions of the protein [15,16]. These dominant negative effects are generally attributed to mutations that lead to amino acid substitutions in function domains and they appear to be more common in proteins who function in multimeric forms. Many of the *TP53* mutations found in tumor cells are missense point mutations, which are frequently located in the DNA binding domain [17–20]. Many tumors have *TP53* mutations in heterozygotes and thus express WT and mutant p53

simultaneously. These DBD mutants of p53 form a heterotetramer with the wild-type p53 and inactivate it by the dominant-negative effects. Although there are many reports about individual mutant in Li-Fraumeni syndrome, the interaction of TD mutants and wild-type p53 in Li-Fraumeni syndrome cells remains unclear. Therefore, the analysis of the hetero-oligomer formation is important for the mechanism of cellular oncogenesis via p53 inactivation in those cells.

This study aims to elucidate the dominant-negative effect of LFS-associated p53 TD mutants by analyzing their ability to form heterotetramers with wild-type p53 protein as well as what effect they have on the ability of p53 target genes to be transcriptionally activated. To accomplish this, a novel assay system was developed for analyzing hetero-oligomerization of the WT p53 and the p53 TD mutants as well as their transcriptional activity in cell simultaneously. This assay system was applied to analyze LFS associated p53 TD mutants.

2.3. Materials and methods

2.3.1. Synthesis of p53TD peptides

The peptides corresponding to the human WT p53TD (residues 319–358 of human p53), the R337H, R337C, R337P p53TD mutants and the WT p63TD (residues 397–444 of human p63) were synthesized as previously described [35]. The peptide sequences are shown in Figure 4-2. The synthesized peptides were deprotected and cleaved from the resin by using Reagent K (TFA:H₂O:thioanisole:ethanedithiol:phenol = 82.5:5:5:2.5:5). All peptides were purified to homogeneity using reverse-phase HPLC over a C8 column (Vydac, Hesperia, CA, USA) and the correct molecular weight of each peptide was verified by MALDI-TOF-MS analysis. Peptide concentrations were determined using an extinction coefficient, $\epsilon_{280} = 1,490 \text{ M}^{-1} \text{ cm}^{-1}$, which corresponds to a single Tyr residue present in all of the p53TD and p63TD.

2.3.2. Avidin Biotin pulldown assay for in vitro heterotetramer formation

For the in vitro avidin-biotin pulldown experiments to identify formation of heterotetramers, a biotin labeled WT p53TD was mixed with the non-biotinylated mutant p53TD peptides. To prepare the biotinylated p53TD and p63TD peptides, biotin was conjugated on resin at the N-terminus of the peptide with two residues of aminocaproic acid (Aca) residues as a linker by regular solid-phase peptide synthesis. The Biotin-(Aca)₂-p53TD and Biotin-(Aca)₂-p63 peptides were then dialyzed into a binding buffer consisting of 50 mM Na phosphate buffer (pH 7.5) 100 mM NaCl. For the binding

reaction, 40 μ L of NeutrAvidinTM UltraLinkTM Resin (Thermo SCIENTIFIC) beads (50% slurry) was placed in a tube and centrifuged to remove the supernatant. Then, 40 μ L of the binding buffer was added and centrifuged to remove the supernatant. This procedure was repeated twice to buffer exchange the beads for the binding reaction. For the binding reaction, the Bio-(Aca)₂-p53TD peptide was added at a concentration of 10 μ M to 10 μ M of the mutant p53TD. The mixed solution was boiled at 100°C for 5 min and first cooled at room temperature for 1 h and then at 4°C for an additional 1 h. Then, 40 μ L of buffer-exchanged beads and 60 μ L of buffer were added and incubated at 4°C for 2 h to bind Bio-(Aca)₂-p53Tet to the Avidin beads. The supernatant was removed by centrifugation, washed with 200 μ L of binding buffer, and then washed a second time with H₂O. Next, 200 μ L of 80% acetic acid was added and incubated at 4°C for 2 h to elute the bound peptides from the avidin beads. After centrifugation, 125 μ L of the supernatant was mixed with buffer A (0.05% TFA/H₂O) to make a final solution of 525 μ L. If the mutant p53TD sequence contained a Cys, the reducing agent TCEP was added to a final concentration of 1 mM. The solution was then analyzed by reverse-phase HPLC to determine the amount of each peptide that bound to the resin. A SHISEIDO Proteonavi column (Cat No. 80205) was used for the HPLC analysis of the WT and p63TD experiments and a GL Sciences Inter Sustain C8 column (Cat No. 5020-16021) was used for the HPLC analysis of the R337C, R337H, and R337P experiments. The results were expressed as a ratio of either the mutant p53TD or p63TD pulled down with respect to the amount of biotinylated p53TD.

2.3.3. Cells used in the experiments

p53-null lung cancer cell line H1299 (American Type Culture Collection) derived from human lung cancer were used for the analysis. Cells were cultured in RPMI-1640 (SIGMA) medium with 10 % Fetal Bovine Serum (FBS; Thermo Scientific) and Penicillin-Streptomycin (Gibco) (penicillin (100 units/mL) streptomycin (100 µg/mL)). Culture conditions were 37°C 5 % CO₂.

2.3.4. Plasmid construction

Plasmids for expressing p53 protein fused with fluorescent proteins for FRET assay (Figure 2-1) used in this study were as follows:

phCMV-p53(WT)-Venus-2A-p53(WT)-Cerulean (**WT/WT**);
phCMV-p53(3Ala)-Venus-2A-p53(3Ala)-Cerulean (**3Ala/3Ala**);
phCMV-p53(3Ala)-2A-p53(WT)-Cerulean (**3Ala/WT**);
phCMV-Venus-NLS-2A-p53(WT)-Cerulean (**del/WT**);
phCMV-p53(R273H)-Venus-2A-p53(R273H)-Cerulean (**R273H/R273H**);
phCMV-p53(R273H)-Venus-2A-p53(WT)-Cerulean (**R273H/WT**);
phCMV-p53(R337H)-Venus-2A-p53(R337H)-Cerulean (**R337H/R337H**);
phCMV-p53(R337H)-Venus-2A-p53(WT)-Cerulean (**R337H/WT**);
phCMV-p53(R337C)-Venus-2A-p53(R337C)-Cerulean (**R337C/R337C**);
phCMV-p53(R337C)-Venus-2A-p53(WT)-Cerulean (**R337C/WT**);
phCMV-p53(R337P)-Venus-2A-p53(R337P)-Cerulean (**R337P/R337P**);
phCMV-p53(R337P)-Venus-2A-p53(WT)-Cerulean (**R337P/WT**).

If p53-Venus and p53-Cerulean form hetero-tetramer, FRET signal between Venus and Cerulean can be observed in this system.

For the p53 reporter plasmids, pp53RE(*bax*)-mCherry-NLS-AU2 and pp53RE(*CDKN1A*)-mCherry-NLS-AU2 were used in this study (Figure 2-2). There are two different types of p53 responsive elements used for the reporter site. The p53RE(*bax*), a responsive element present in the apoptosis-inducing *bax* gene (Figure 2-2); and the p53RE(*CDKN1A*), a responsive element from the *CDKN1A* gene (Figure 2-2). In Figure 2-3 and Figure 2-4, the underlined bases indicate the consensus p53-binding sites, whereas the capital letters indicate palindromic consensus sequences that bind to p53.

2.3.5. Measurement of intracellular p53 tetramerization and transcriptional activity

H1299 cells were seeded at a 3.0×10^5 cells/dish density in a 35 mm diameter tissue culture dish. 24h later, 1.2 μ g of each type of plasmid DNA (p53 expression plasmid and reporter plasmid) was added to 100 μ L of Opti-MEM, mixed with 6 μ L of lipofectamine 2000 (Thermo Fisher Scientific), and left at room temperature for 10 min. After washing twice with 1.5 mL of PBS per dish, the mixture was combined with 700 μ L of RPMI-1640 (without 10 % FBS P/S), added to the dish, and incubated at 37 °C, 5 % CO₂ for 1h. After the incubation, the mixture of RPMI-1640 (without 10 % FBS P/S) and lipofectamine 2000 was removed, and 1.5 mL of RPMI-1640 (without 10 % FBS P/S) was added, followed by incubation at 37 °C and 5 % CO₂. After 11h, the medium was removed, 700 μ L of 10 % formalin PBS solution was added, and the cells were shaken in

an invitroshaker (TAITEC) for 10 min. Then, the 10 % formalin solution was removed, and 700 μ L of 0.2 % Triton-X-100 PBS solution was added, followed by shaking at room temperature for 10 min. Next, 0.2 % Triton-X-100 PBS solution was removed, washed three times with PBS, and 1 mL of 1000-fold diluted DAPI PBS solution was added to the dish and shaken for 10 min. Then, another 1 mL of PBS was added.

A BIOREVO BZ-9000 fluorescence microscope (Keyence) was used for observation. The fluorescence mirror units used were YFP-BP for Venus, CFP-2342C for Cerulean, DAPI-BP for DAPI, TRITC for mCherry, and FRET for FRET observation (Table 2-2). A Nikon Plan Fluor D 10 $\times$ / NA0.30 ph 1 objective lens was used, and the exposure times were FRET 1/2 s, Cerulean 1/2 s, DAPI 1/4 s, Venus 1/2 s, and mCherry 1/2 s. The images were acquired at 8 bits. Images were acquired at 8 bits, and 5 $\times$ 6 fields of view, shifted by one field of view from the center, were acquired in the order of upper right, lower right, lower left, and upper left, centered on the (0, 0) point. After the DAPI, Venus, FRET, and Cerulean fluorescence images were acquired in this order, the FRET mirror unit was changed to TRITC, and only mCherry fluorescence images were acquired in 4 planes of 30 consecutive fields of view.

2.3.6. Image analysis methods

Images were analyzed using ImageAnalysis_3 FRET and DataAnalysis_4, programs for image analysis created in our laboratory.

In this analysis software, the fluorescence intensity is first averaged over 144 pixels, of which 12 pixels $\times$ 12 pixels is one square. Then, the average value of the lower half

(72 pixels) is used as one square's provisional Background value (BG value). Next, the 25th intensity value, the median of the tentative BG values in 49 squares (7×7) with that square as the center, is used as the BG value for that square. 144 pixels in a square are uniformly subtracted from this BG value. After subtracting the BG value of the DAPI fluorescence image, one nucleus is detected as a contiguous 50-pixel or larger mass showing an intensity greater than or equal to the BG value $\times 0.5 + 20$. Next, Venus, Cerulean, FRET, and mCherry fluorescence intensity analysis is performed for each nucleus for which DAPI fluorescence intensity is detected. For Venus, Cerulean, and mCherry, the average of the raw data without subtracting the BG value for each pixel in the cell nucleus detected by DAPI is taken; for FRET, the FRET fluorescence intensity for each pixel in the nucleus is calculated from the FRET fluorescence intensity of the raw data. The FRET signal value was calculated by subtracting Venus $\times 0.15$ and Cerulean $\times 0.33$ [22], which are contributions from the fluorescence of Venus and Cerulean alone, from the FRET fluorescence intensity of the raw data per pixel per nucleus, and the average of these values per nucleus was taken as the FRET signal value.

DataAnalysis_4 was used for data integration and sorting. Since previous studies have shown that the fluorescence intensity ratio of p53-Venus and p53-Cerulean is 1:1 [22], it is necessary to analyze only cells with a Venus: Cerulean fluorescence intensity ratio of 1:1. In this program, cells whose Venus and Cerulean fluorescence intensities both fall within a range of 2, such as 0 to 2 and 2 to 4, were sorted as cells whose Venus and Cerulean fluorescence intensities are of their average values. This is used to calculate the average FRET signal to Cerulean Intensity, the average mCherry Intensity, and the

mCherry Intensity to FRET signal for a group of cells with a Venus: Cerulean fluorescence intensity ratio of 1:1. FRET signal to Cerulean Intensity and mCherry Intensity to FRET signal were calculated.

2.4. Results

2.4.1. Analysis of hetero-tetramerization by avidin-biotin pull down assay

Avidin-Biotin pull down assays were performed to analyze the potential of the p53TD and TD mutants (R337C, R337H and R337P) to form hetero-tetramers in vitro (Figure 2-2). Biotinylated p53TD and untagged p53TD mutant peptides were used to analyze the ratio of p53TD mutant that is pulled down with respect to the amount of biotinylated p53TD. The results indicate that each p53TD mutant can form heterotetramers with the WT p53TD in vitro. For the binding assay, the ratio of WT-to-WT was 0.81. The R273H mutation within the DBD, which had been shown to possess dominant-negative activity, contains no additional mutations in the TD region and it is pulled-down at the same ratio as the WT p53TD. Furthermore, the ratio of mutant-to-WT were 0.33 for the R337C mutation, 0.53 for the R337H mutation, and 0.15 for the R337P mutation when the biotinylated Bio(Aca)₂-p53TD and the untagged p53TD mutants peptides were used at equal concentrations (10 μ M). On the other hand, the pull-down ratio for the p63TD, which has not been reported to form a heterotetramer with WT p53TD, was only 0.02. The results demonstrate that the mutations tested in this study (R337C, R337H and R337P) can form heterotetramers with WT p53TD in vitro. These results strongly suggest that the R337 mutants (R337H, R337C) found in LFS form a heterotetramer with WT when present as heterozygous mutations.

2.4.2. Construction of dual-assay system for hetero-tetramer formation and transcriptional activity by FRET

In order to analyze hetero-oligomerization between the WT p53 and LFS-associated p53 TD mutants, a novel construct was created. In this construct, two p53 proteins fused to the blue fluorescent protein Cerulean and the yellow fluorescent protein Venus, respectively, are arranged in tandem with a 2A sequence in between (Figure 2-3). By using the construct, the WT-p53 and the p53 TD mutants (R337C and R337H) were expressed in cells and the amount of heteromeric p53 (WT with mutants) was quantified by intensity of FRET. Another construct was also created to determine the transcriptional activity. The construct contains the red fluorescence protein mCherry with nuclear localization signal which is controlled by a p53-responsive element.

First, the tetramer formation and transcriptional activity of the wild-type p53 protein was analyzed by using the pp53RE(*bax*)-mCherry-NLS-AU2 and phCMV-p53(WT)-Venus-2A-p53(WT)-Cerulean (**WT/WT**). p53-null H1299 cells were co-transfected with these plasmids, and FRET signal and mCherry signal were observed. Using this system, the FRET signal and mCherry signal can be observed and they were directly correlated with the p53 expression level, indicating that formation of the tetramer and transcriptional activity can be detected in a p53 dependent manner by this system (Figure 2-7).

Next, the monomeric mutant p53-3Ala was analyzed by using the same FRET assay. In this protein, the hydrophobic residues L330, L344, and I332 of p53 are substituted with an Ala residue, which prevents the p53 protein forming a multimeric structure (dimers or tetramers). No FRET signal was not detected for the monomeric mutant 3Ala following

either homo (hCMV-p53(3Ala)-Venus-2A-p53(3Ala)-Cerulean; **3Ala/3Ala**) and hetero (phCMV-p53(3Ala)-2A-p53(WT)-Cerulean; **3Ala/WT**) expression, which indicates that there is no tetramer formation. In addition, the transcriptional activity of homo- and hetero-expression of 3Ala were compared with the transfection of hCMV-Venus-NLS-2A-p53(WT)-Cerulean plasmid as a model of hetero-deletion of *TP53*. The results showed that **3Ala/3Ala** was little to no transcriptional activity, indicating that 3Ala is transcriptionally inactive and is incapable of forming a multimer. In addition, the transcriptional activity of hetero-expression of 3Ala and WT (**3Ala/WT**) was the same as that of the heterozygous deletion model **del/WT**. These data indicated that 3Ala does not form a heterotetramer with the wild-type protein, therefore does not affect to transcriptional activity of wild-type protein (Figure 2-8).

Next the R273H mutant of p53 was analyzed using the cellular FRET assay. The R273H mutation is located in the DNA-binding domain of the p53 protein. This mutant has been previously shown to have dominant-negative effects. With this mutant, the levels of tetramer formation were similar to (**WT/WT**) p53 with either homo (phCMV-p53(R273H)-Venus-2A-p53(R273H)-Cerulean; **R273H/R273H**) or hetero (phCMV-p53(R273H)-Venus-2A-p53(WT)-Cerulean; **R273H/WT**), indicating that this mutation did not alter the ability of p53 to form tetramers. However, the (**R273H/R273H**) p53 protein showed no transcriptional activity. Furthermore, the hetero-expression (**R273H/WT**) p53 protein was also determined to have very low transcriptional activity which was lower than the heterozygous deletion (**del/WT**). This indicates that a

dominant-negative effect was observed with this mutant, as heterologous expression greatly reduced the transcriptional activity of the WT p53 protein (Figure 2-9). These results demonstrated that this system can be used to quantify both the amount of hetero-tetramerization and the transcriptional activity of p53 mutants and determine whether they have a dominant-negative effect.

2.4.3. p53 tetramerization and transcriptional activity for different responsive elements

Using our verified FRET-based analysis system, I analyzed the transcriptional activity of mutants at R337 of p53 that have been associated to either the Li-Fraumeni syndrome or a Li-Fraumeni-like syndrome. More specifically I determined the transcriptional activity of an R337C, R337H and R337P p53 variant protein with the p53RE(*bax*) gene using the FRET assay system.

The amount of homotetramerization of R337C (**R337C/ R337C**) was 0.7 FRET signal at Cerulean Intensity 15 (Figure 2-10). Since the FRET signal of **WT/WT** at this point was 2.3, the amount of homotetramerization of R337C was about 30% of that of WT homotetramerization. This result indicates that the amount of homotetramerization of R337C is lower than that of WT. The homotetramer of R337C showed no transcriptional activity from *bax* promoter, as did 3Ala of the monomeric mutant. However, the FRET signal of the heterotetramer with WT (**R337C/WT**) was 1.6, about 70% of that of the WT homotetramer. Despite R337C mutant could form heterotetramer with WT, the transcriptional activity of **R337C/WT** was lower than that of the

heterozygous deletion model **del/WT**. This indicates that R337C, when heterologously expressed, has a dominant-negative effect that reduces the activity of WT.

R337H formed a homotetramer (**R337H/R337H**) with the FRET signal of 1.7 which was about 80% of **WT/WT** (Figure 2-11). This value was greater than that of R337C. The transcriptional activity of **R337H/R337H** from *bax* promoter at this time was as low as that of **del/WT**. The amount of heterotetramer (**R337H/WT**) was slightly higher than that of WT homotetramer. However, its transcriptional activity was slightly higher than **R337H/R337H**, but was reduced to the same level as **del/WT**.

Under the experimental conditions, there was no FRET signal observed following expression of the **R337P/R337P** p53, which indicate that there is no interaction between the R337P p53 monomers (Figure 2-12). In contrast, the FRET signal of **R337P/WT** was about 30% of that of **WT/WT**, which demonstrates that the R337P p53 could form heterotetramers with the WT p53 protein. In addition, the **R337P/WT** was not transcriptionally active when expressed alone but showed the same activity as **del/WT** when co-expressed with the WT p53 protein, indicating that co-expression of R337P did not affect the transcriptional activity of wild-type p53.

To determine whether changes in protein affinity for DNA target sequences lead to changes in transcriptional activity, the transcriptional activity of R337 p53 mutants associated with Li-Fraumeni syndrome or Li-Fraumeni-like syndrome was analyzed using a reporter containing a *CDKN1A* response element. The WT p53 proteins exhibits a six-fold higher affinity for the *CDKN1A* responsive element in comparison with the *bax*

responsive element. As seen with the *bax* promoter, the **R273H/R273H** was also not transcriptionally active with the *CDKN1A* response element under the experimental conditions. However, the transcriptional activity of the heterogously expressed **R273H/WT** was only slightly lower than that of the **del/WT**, and no significant dominant-negative effect was observed for this promoter as was seen with the *bax* promoter (Figure 2-13).

Unlike with its activity for the *bax* promoter, the **R337C/R337C** p53 homotetramer protein was slightly transcriptionally active against *CDKN1A* promoter, and when heterogously expressed (**R337C/WT**), the transcriptional activity from the *CDKN1A* promoter was comparable to that of **del/WT** (Figure 2-14).

R337H showed transcriptional activity from *CDKN1A* promoter when homo-expressed (R337H/R337H) and its activity was higher activity than **del/WT** and lower activity than **WT/WT** when hetero-expressed (**R337H/WT**) (Figure 2-15).

Similar to the results for the *bax* promoter, R337P was not transcriptionally active when expressed homologously (**R337P/R337P**) and was as active when heterologously expressed (**R337P/WT**) as **del/WT** (Figure 2-16).

2.4.4. Comparison of transcriptional activity for *bax* and *CDKN1A* response elements

Since the tetramer-formation abilities of the tetramerization domain mutants differed from each other, their normalized transcriptional activity was analyzed by WT homotetramerization levels. The results showed that only **R337H/WT** was active against

the *bax* promoter, while **R337C/WT** and **R337P/WT** had little activity (Figure 2-17). Furthermore, analysis of transcriptional activity against *CDKN1A* showed that **R337H/WT** was active, as was the case with the *bax* promoter. More interestingly, unlike the DNA binding domain mutant **R273H/WT**, **R337C/WT** and **R337P/WT** were found to be slightly active (Figure 2-18).

To compare the transcriptional activity of each mutant against the *bax* promoter with that against *CDKN1A*, the transcriptional activity of each mutant at heterotetramerization was normalized with **WT/WT** at Cerulean fluorescence intensities of 10-20 (Figure 2-19).

The **WT/WT** homo-tetramer with the *CDKN1A* promoter was 410% more active than that with the *bax* promoter, and **del/WT**, the hetero deletion model, was 4.1 times more active with the *CDKN1A* promoter than that with the *bax* promoter. The heterozygous deletion model **del/WT** was 50% active with the *bax* promoter and 230% active with *CDKN1A*, while **R337H/WT** was 50% active with the *bax* promoter, comparable to **del/WT**, and slightly active with the *CDKN1A* promoter, 300% active. **R337P/WT** and **R337C/WT** had similar transcriptional activity to **del/WT** at 45% and 250% and 40% and 240%.

The transcriptional activity of each mutant at the time of hetero-tetramerization was normalized and analyzed with that of **WT/WT** for the *bax* promoter and *CDKN1A* promoter (Figure 2-20). Differences in affinity between p53 and DNA were not taken into account. The DNA binding domain mutant **R273H** showed 25% transcriptional activity against *bax* and 45% transcriptional activity against *CDKN1A*, both lower than **del/WT**,

while R337C and R337P mutants showed 40% and 45%, respectively, for the *bax* promoter and 50% and 55% for the *CDKN1A* promoter, respectively, these activities were comparable to **del/WT**, hetero deletion model of *TP53* gene. On the other hand, R337H showed a value of 55% for the *bax* promoter, similar to **del/WT**, and a slightly higher activity of 65% for the *CDKN1A* promoter.

Mutant Name	p53 Sequences																								
WT	K	K	K	P	L	D	G	E	Y	F	T	L	Q	I	R	G	R	E	R	F	E	M	F	R	E
R337H	K	K	K	P	L	D	G	E	Y	F	T	L	Q	I	R	G	R	E	H	F	E	M	F	R	E
R337C	K	K	K	P	L	D	G	E	Y	F	T	L	Q	I	R	G	R	E	C	F	E	M	F	R	E
R337P	K	K	K	P	L	D	G	E	Y	F	T	L	Q	I	R	G	R	E	P	F	E	M	F	R	E
p63 Sequences																									
p63	D	D	E	L	L	Y	L	P	V	R	G	R	E	T	Y	E	M	L	L	K	I	K	E	S	L

Figure 2-1. The sequences of p53TDs with mutations at R337 used in the pull-down assay Conserved residues in terms of human p53TD are gray, substituted residues are hydrophobic, orange; acidic, red; basic, blue; others, black. Residues forming a hydrophobic core are shadowed in light blue.

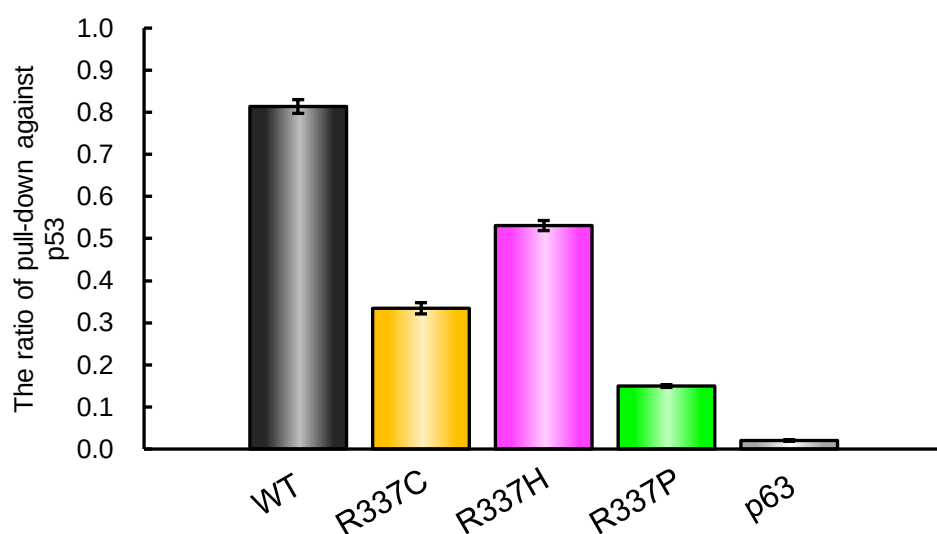


Figure 2-2. Ratio of mutant p53TD pulled-down versus WT p53TD.

Experiments were performed in 50 mM Na phosphate buffer (pH 7.5), 100 mM NaCl, and a peptide solutions containing the Bio-(Aca)₂-p53TD and p53TD mutants at equimolar concentrations (10 μ M). The results demonstrate that the p53TD was pulled-down in WT(0.81) R337C (0.33), R337H (0.53) and R337P(0.15). No substantial pull-down of p63TD was observed(0.02).

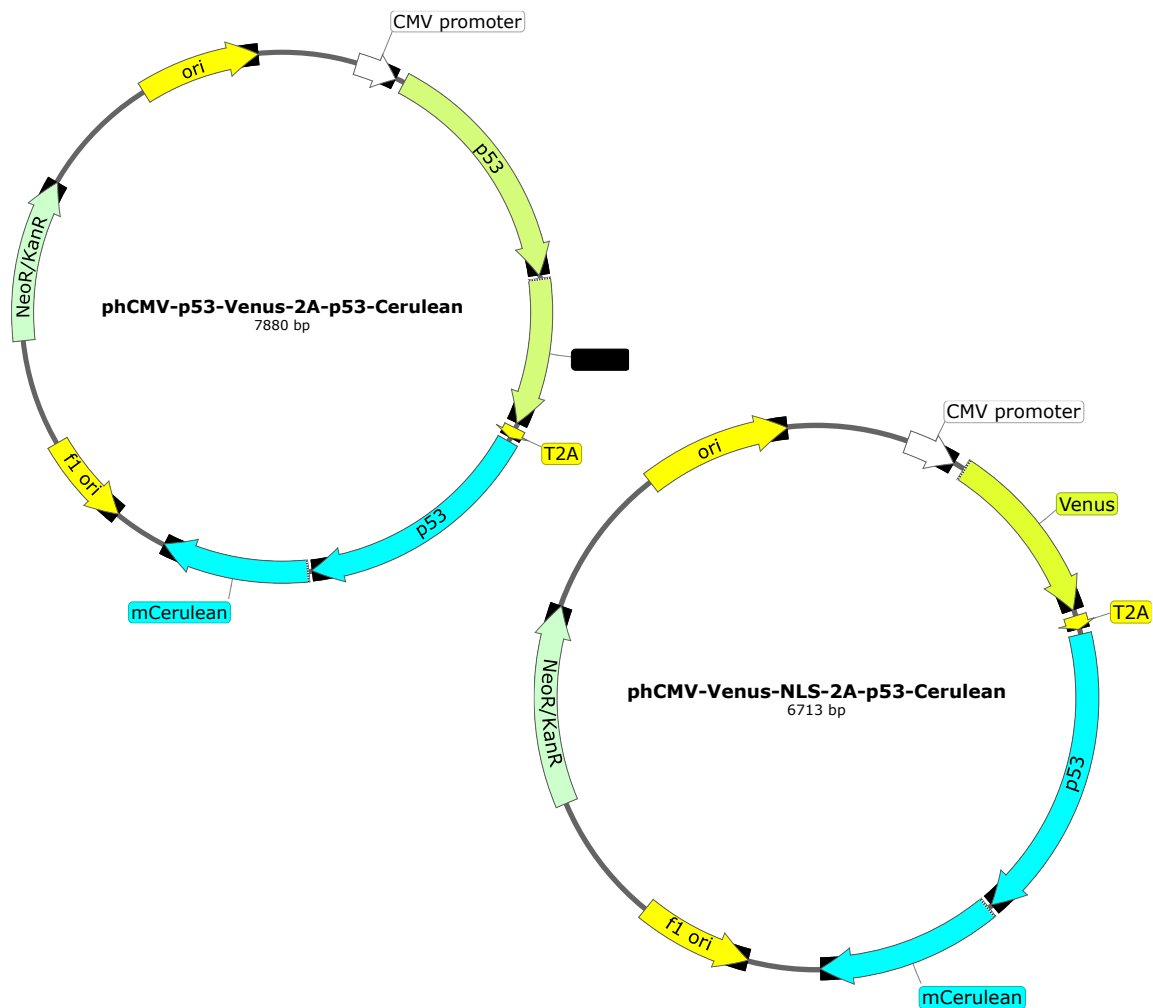


Figure 2-3. Expression vectors of phCMV-p53-Venus-2A-p53-Cerulean and phCMV-Venus-NLS-2A-p53-Cerulean

Plasmids expressing Venus- and Cerulean-tagged p53 were created to analyze p53 tetramerization in cells. 2A sequences were designed to be inserted between the two fluorescent-tagged p53 sequences so that two types of p53 could be expressed intracellularly in cells. The Venus tag NLS was used as a control.

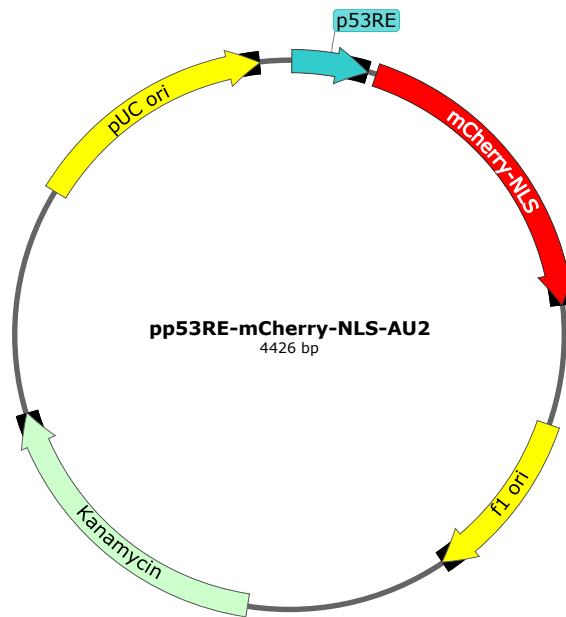
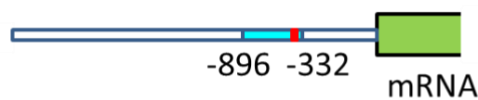


Figure 2-4. Preparation of pp53RE(*bax/CDKN1A*)-mCherry-NLS-AU2

pp53RE(*bax/CDKN1A*)-Venus-NLS and pp53RE-mCherry-NLS-AU2 were cleaved by restriction enzymes AgeI and MluI, the mCherry-NLS-AU2 portion was then connected to pp53RE(*bax/CDKN1A*)-Venus NLS to obtain the pp53RE(*bax/CDKN1A*)-mCherry-NLS-AU2.

bax (565 bp)



(-896)

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ccctgctgat ctatcagcac agattagttt ctgccacttt ttaaacttca
tattcctttt ctttttacac aaacacaaac attcgagtca tgactgggtg
gggtggctca agcctgtaat ctcagcactt tgggaggcca aggtgcgagg
atcgcttgag tctgggagtt cagagaccag cctgggcaac atagagagac
ctcatctcca cataaaaagt tttaaaaatt aaccaggggc ggtgtagtcc
cagctactca ggaggctgag gtgggaggct tcagcccggg aattccagac
tgcagtgagc catgattggg ccactgcact ccagcctggg caacacagtg
agaccctgtc tcaaaaaaaaa aaaaaaaaaa aacaggaaaa aacaaacaaa
cagaaaagca ggcctggcgc ggtagctcat gcctgtaatc ccagcgcttt
ggaaggctga gacggggtta tctcttgggc tcACAAGTIa gAGACAAGCC
IGGGCgIggg CTAtaIIgct agatccaggt ctctgcaaaa aacaaaacca
ctcagttttt agtca

```

(-332)

Figure 2-5. *bax* response element sequence

The DNA sequence of the response element from the *bax* gene is shown. Red letters indicate the p53-binding region, and the underlined capital letters indicate the palindromic consensus p53-binding sequence. The number of bases from the transcription start point are indicated in parentheses.

CDKN1A(1014 bp)

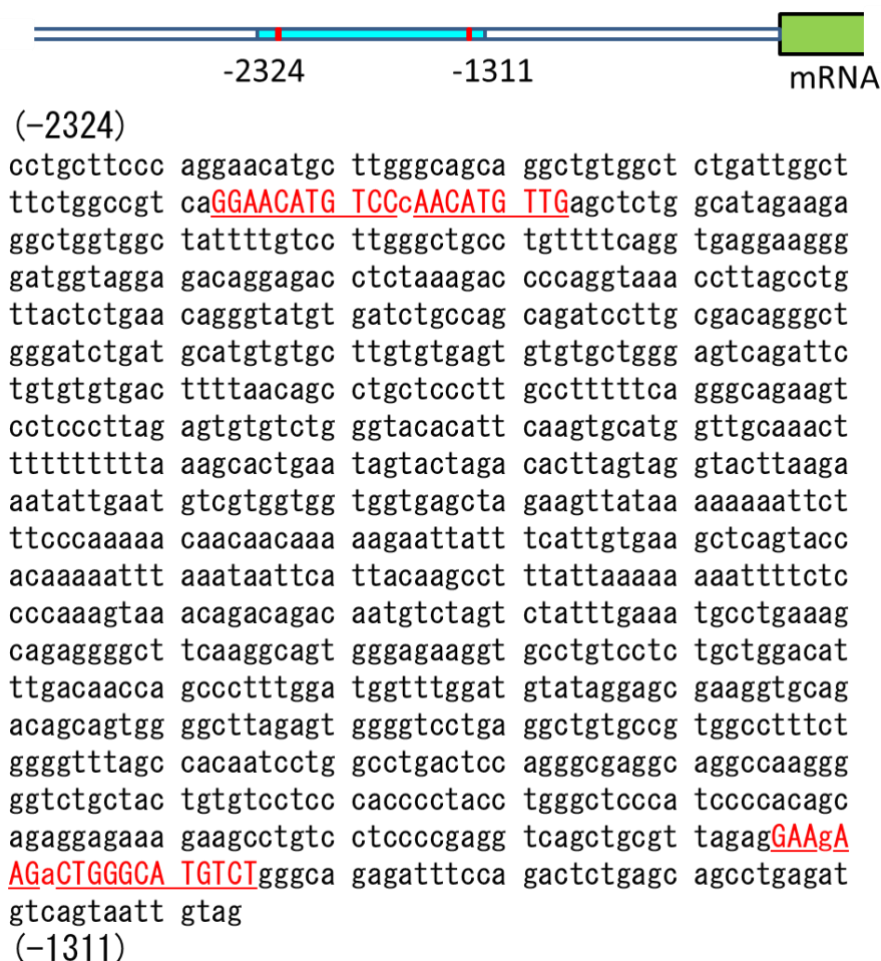


Figure 2-6. *CDKN1A* response sequence

The DNA sequence of the response element from the *CDKN1A* gene is shown. Red letters indicate the p53-binding region, and the underlined capital letters indicate the palindromic consensus p53-binding sequence. The number of bases from the transcription start point are indicated in parentheses.

Table 2-1 Characteristics of fluorescent mirror units used for fluorescence microscopy

	Ex. (nm)	DIC (nm)	Em. (nm)
YFP-BP	500 / 25	520	542 / 27
CFP-2342C	438 / 24	458	483 / 32
DAPI-BP	360 / 40	400	460 / 50
TRITC	540 / 25	565	605 / 55
FRET	438 / 24	458	542 / 27

YFP-BP, CFP-2432C, DAPI-BP, TRITC, and FRET were used for Venus, Cerulean, DAPI-BP, mCherry, and FRET observation, respectively, as fluorescent mirror units.

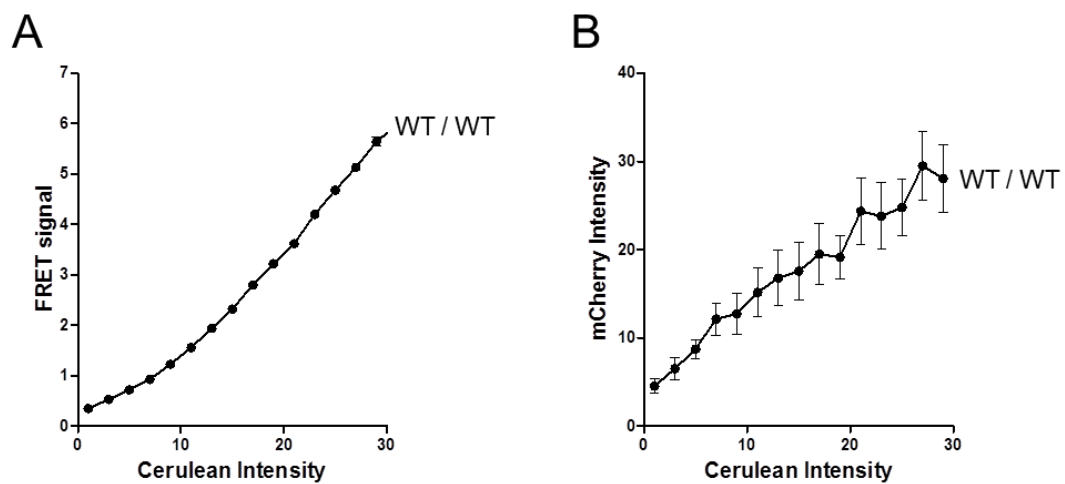


Figure 2-7. Analysis of p53 wild-type protein using the FRET-based Assay

Using the FRET-based assay, I measured the relative amount of tetramer formed (A) as well as the transcriptional activity for the *bax* promoter (B). For this assay, p53 tetramerization in response to pp53RE(*bax*)-mCherry-NLS-AU2 were analyzed when phCMV-p53(WT)-Venus-2A-p53(WT)-Cerulean (**WT/WT**) was transfected. The levels of FRET signal and mCherry signal correlated with p53 expression levels, which indicated that this system can be used to evaluate p53 tetramerization and p53 transcriptional activity.

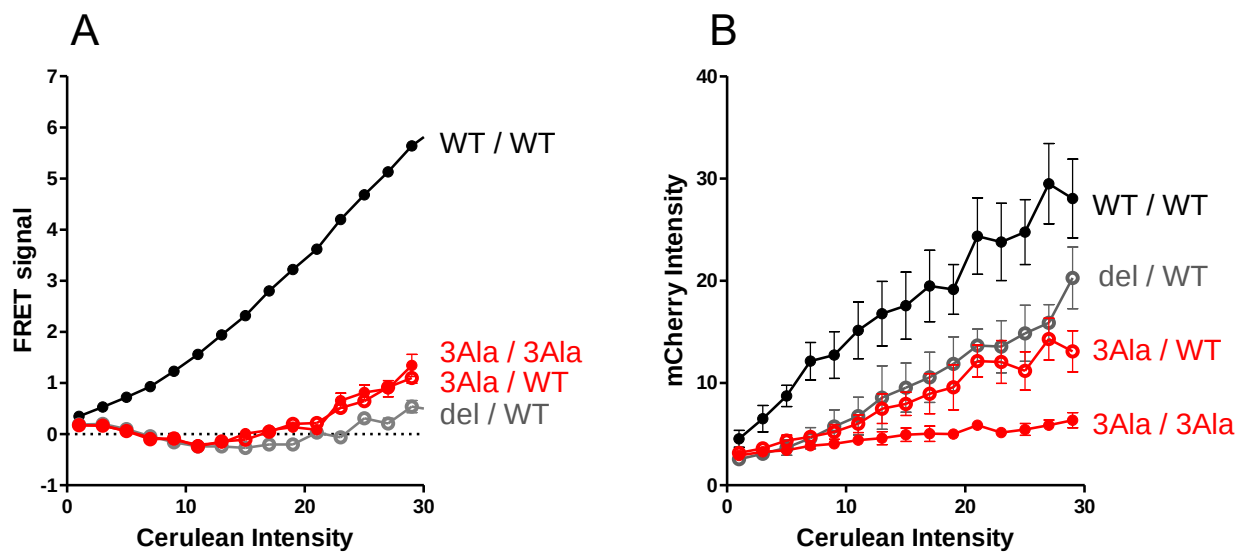


Figure 2-8. Analysis of p53-3Ala mutant protein using the FRET-based Assay

Cells were transfected with phCMV-p53(3Ala)-Venus-2A-p53(WT)-Cerulean (3Ala/WT) and phCMV-p53(3Ala)-Venus-2A-p53(3Ala)-Cerulean (3Ala/3Ala) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(bax)-mCherry-NLS-AU2 (B). Little or no FRET signal was not detected with the monomeric p53-3Ala and there was also little to no transcriptional activity. Combined the results support the hypothesis that transcriptional activity is correlated to the amount of tetramer formation.

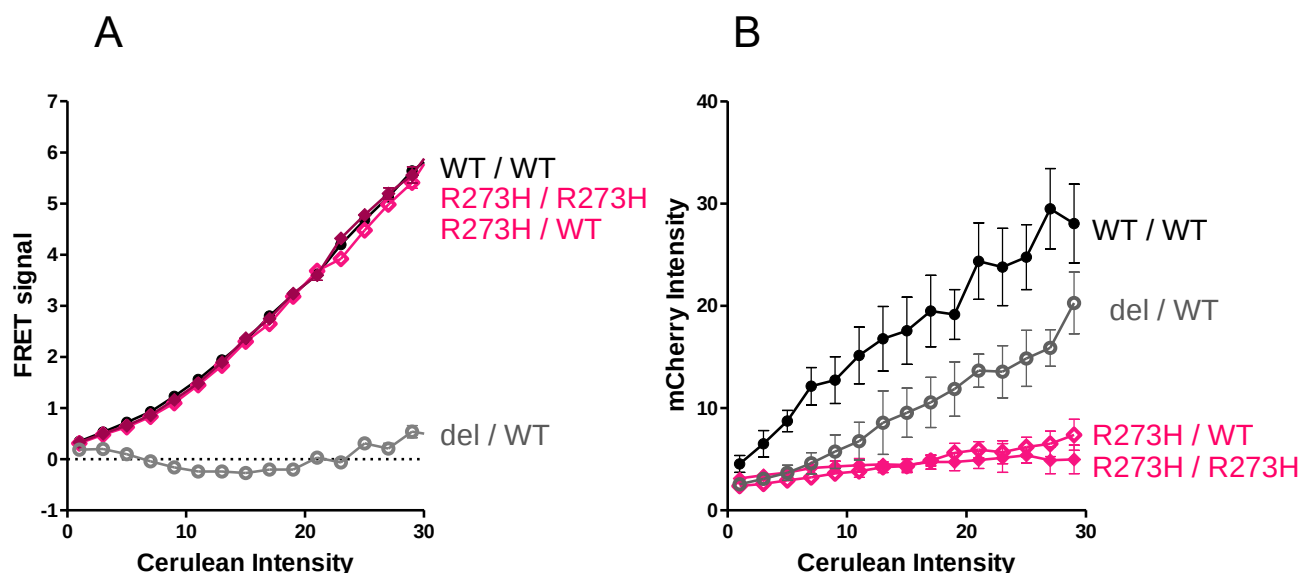


Figure 2-9. Analysis of p53(R273H) mutant protein using the FRET-based Assay

Cells were transfected with phCMV-p53(R273H)-Venus-2A-p53(WT)-Cerulean (R273H/WT) and phCMV-p53(R273H)-Venus-2A-p53(R273H)-Cerulean (R273H/R273H) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(bax)-mCherry-NLS-AU2 (B). Both the hetero-tetramer of R273H with WT and the homo-tetramer of R273H showed the same signal as the homo-tetramer of WT, indicating that R273H forms homo- and hetero-tetramers. However, transcriptional activity was lower than that of the negative control del/WT. These results indicate that R273H has a dominant-negative effect as reported.

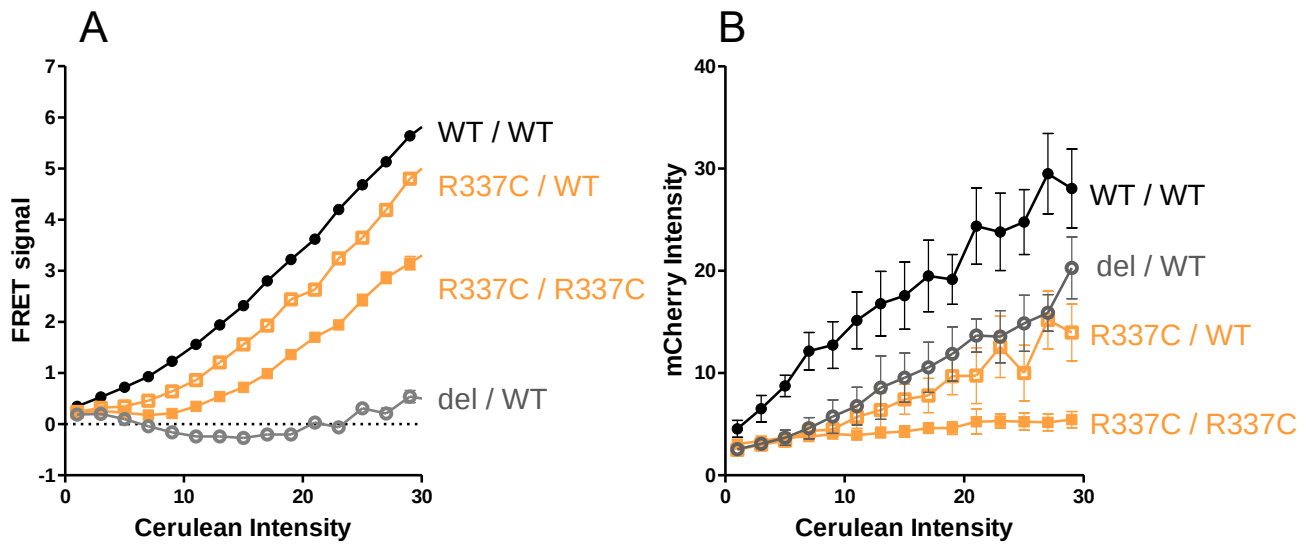


Figure 2-10. Analysis of p53(R337C) mutant protein using the FRET-based Assay

Cells were transfected with phCMV-p53(R337C)-Venus-2A-p53(WT)-Cerulean (R337C/WT) and phCMV-p53(R337C)-Venus-2A-p53(R337C)-Cerulean (R337C/R337C) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(bax)-mCherry-NLS-AU2 (B). The homo-tetramer of R337C showed low tetramerization signals, whereas the hetero-tetramer with WT showed mostly tetramerization signals, indicating that R273H forms homo- and hetero-tetramers. However, transcriptional activity was lower in the homo-tetramer than in the negative control **del/WT**, while the hetero-tetramer showed activity similar to that of **del/WT**. These results indicate that R337C has a dominant-negative-like effect.

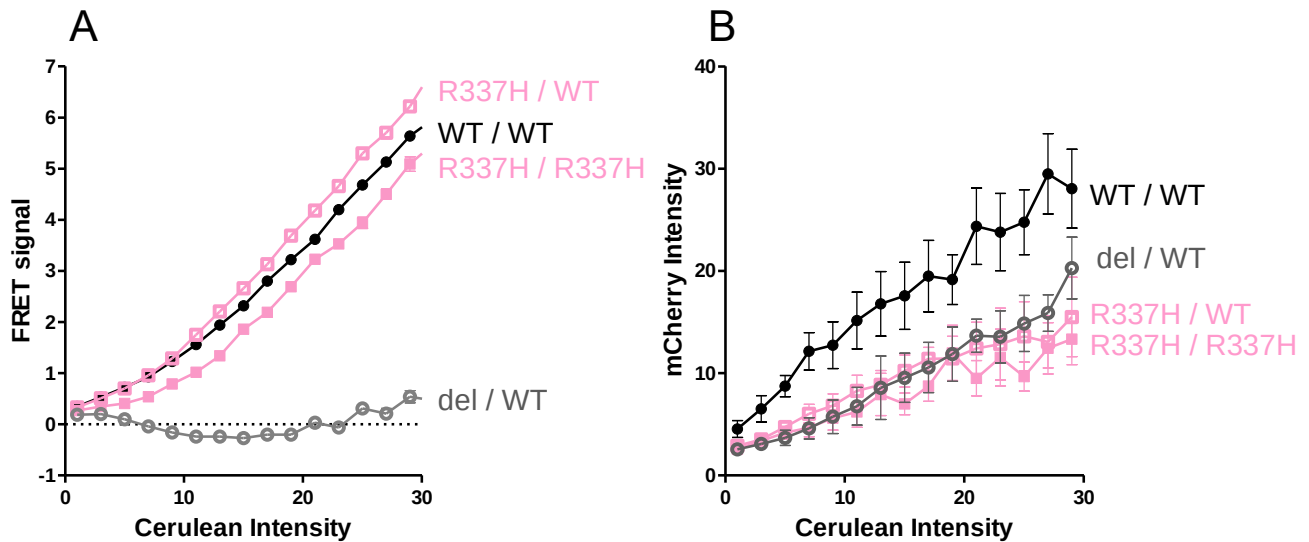


Figure 2-11. Analysis of p53(R337H) mutant protein using the FRET-based Assay

Cells were transfected with phCMV-p53(R337H)-Venus-2A-p53(WT)-Cerulean (R337H/WT) and phCMV-p53(R337H)-Venus-2A-p53(R337H)-Cerulean (R337H/R337H) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(bax)-mCherry-NLS-AU2 (B). Both the hetero-tetramer of R337H with WT and the homo-tetramer of R337H showed the same signal as the homo-tetramer of WT, indicating that R337H forms homo- and hetero-tetramers. However, transcriptional activity showed a similar signal to the negative control **del/WT**. These results indicate that R337H has a dominant-negative effect as reported.

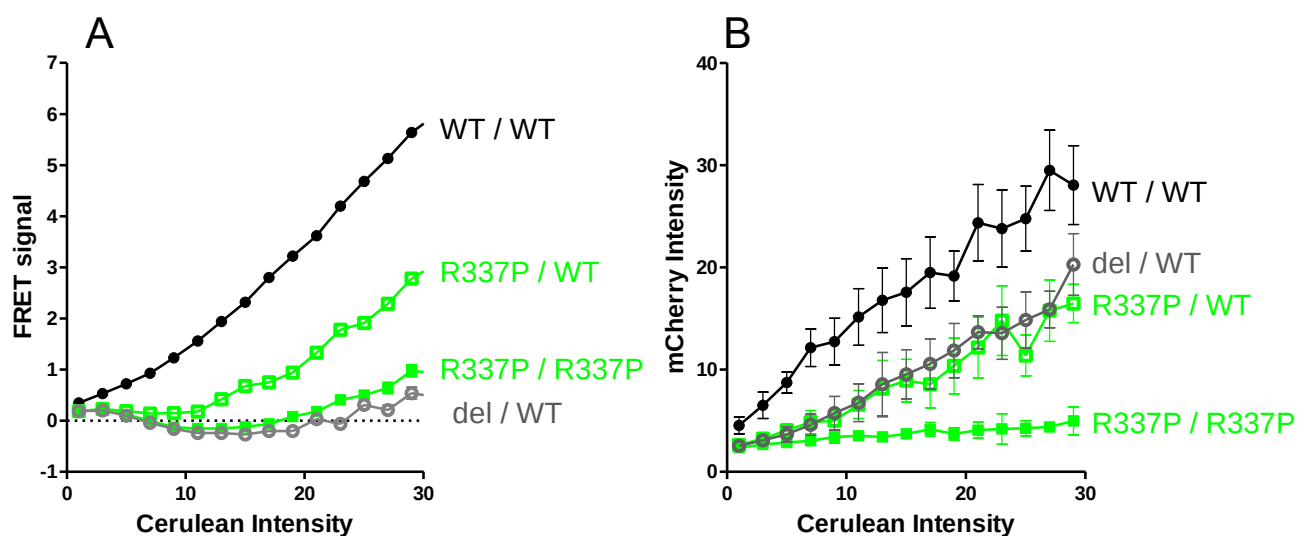


Figure 2-12. Analysis of p53(R337P) mutant protein using the FRET-based Assay
Cells were transfected with phCMV-p53(R337P)-Venus-2A-p53(WT)-Cerulean (R337P/WT) and phCMV-p53(R337P)-Venus-2A-p53(R337P)-Cerulean (R337P/R337P) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(bax)-mCherry-NLS-AU2 (B). The homo-tetramerization of R337P had the same signal as Venus/WT, indicating that it was not formed. On the other hand, R337P/WT had a low signal but showed hetero-tetramerization. However, transcriptional activity showed a similar signal to the negative control del/WT. These results indicate that R337P has a dominant-negative effect as reported.

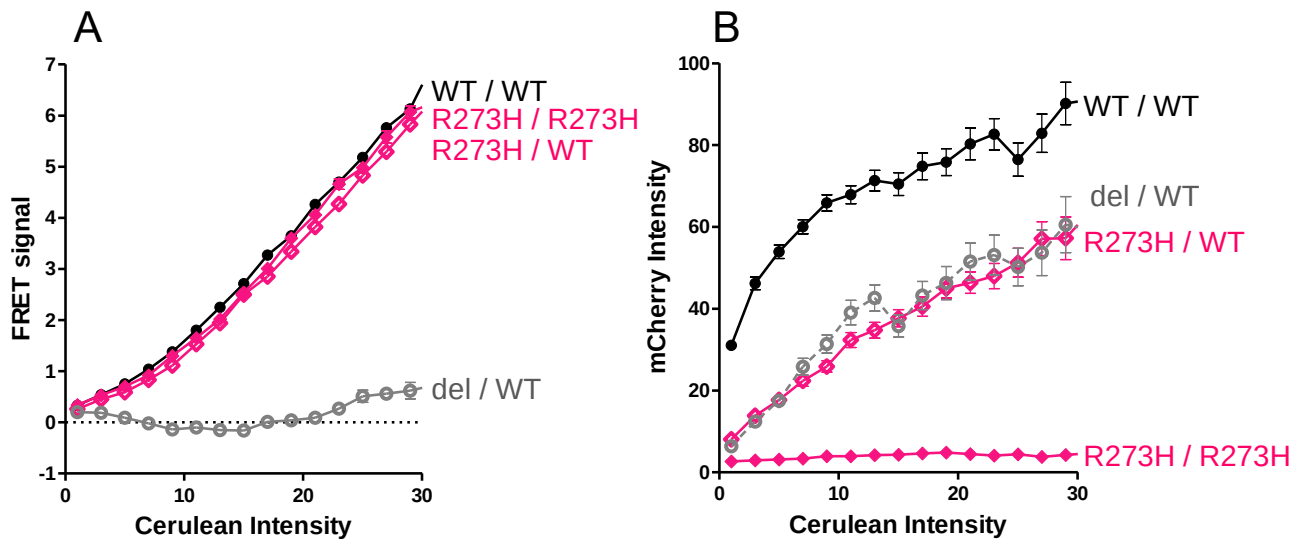


Figure 2-13. Analysis of p53(R273H) mutant protein using the p53RE(*CDKN1A*)

Cells were transfected with phCMV-p53(R273H)-Venus-2A-p53(WT)-Cerulean (R273H/WT) and phCMV-p53(R273H)-Venus-2A-p53(R273H)-Cerulean (R273H/R273H) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(*CDKN1A*)-mCherry-NLS-AU2 (B). The homo-tetramer showed no transcriptional activity at all, and that of the hetero-tetramer was only slightly lower than that of **del/WT**. These results indicate that no significant dominant-negative effect was observed for this promoter as was seen with the *bax* promoter.

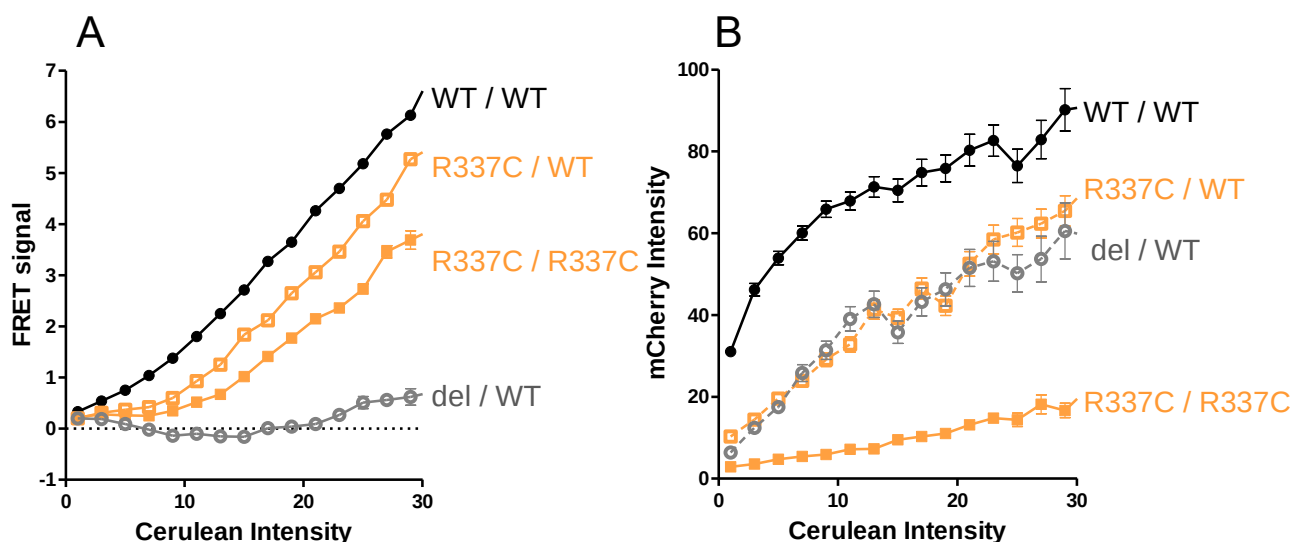


Figure 2-14. Analysis of p53(R337C) mutant protein using the p53RE(*CDKN1A*)

Cells were transfected with phCMV-p53(R337C)-Venus-2A-p53(WT)-Cerulean (R337C/WT) and phCMV-p53(R337C)-Venus-2A-p53(R337C)-Cerulean (R337C/R337C) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(*CDKN1A*)-mCherry-NLS-AU2 (B). The homotetramer was slightly transcriptionally active against the *CDKN1A* promoter, while the heterotetramer was equivalent to **del/WT** p53 protein in transcriptional activity from the *CDKN1A* promoter.

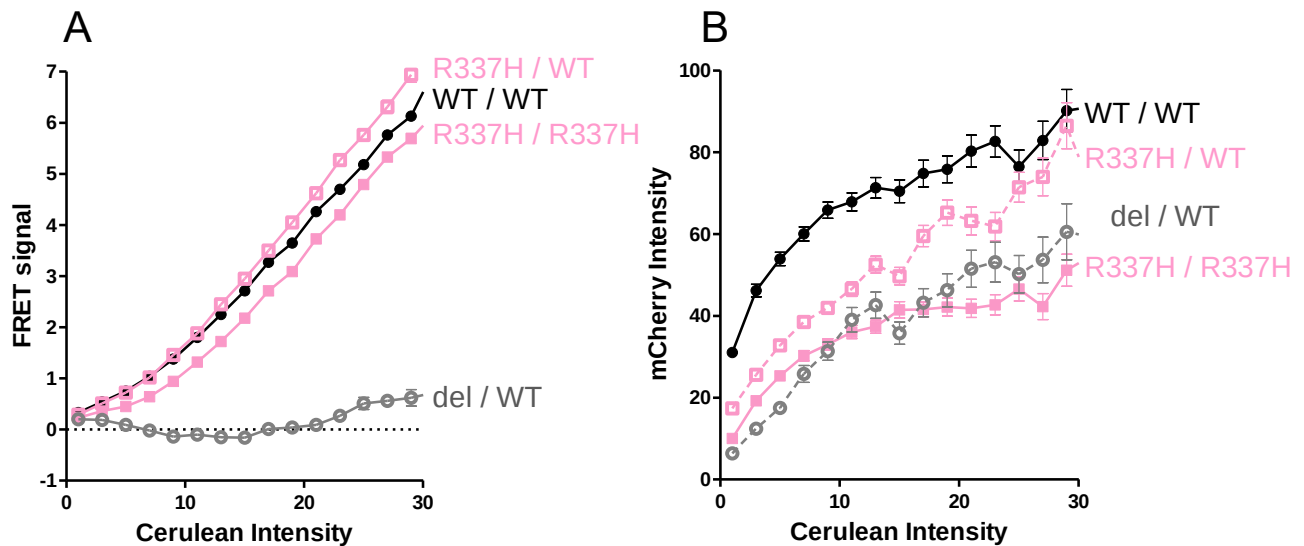


Figure 2-15. Analysis of p53(R337H) mutant protein using the p53RE(*CDKN1A*)

Cells were transfected with phCMV-p53(R337H)-Venus-2A-p53(WT)-Cerulean (R337H/WT) and phCMV-p53(R337H)-Venus-2A-p53(R337H)-Cerulean (R337H/R337H) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(*CDKN1A*)-mCherry-NLS-AU2 (B). Homo-tetramers showed higher transcriptional activity than **del/WT** and hetero-tetramers showed lower transcriptional activity than **WT/WT**.

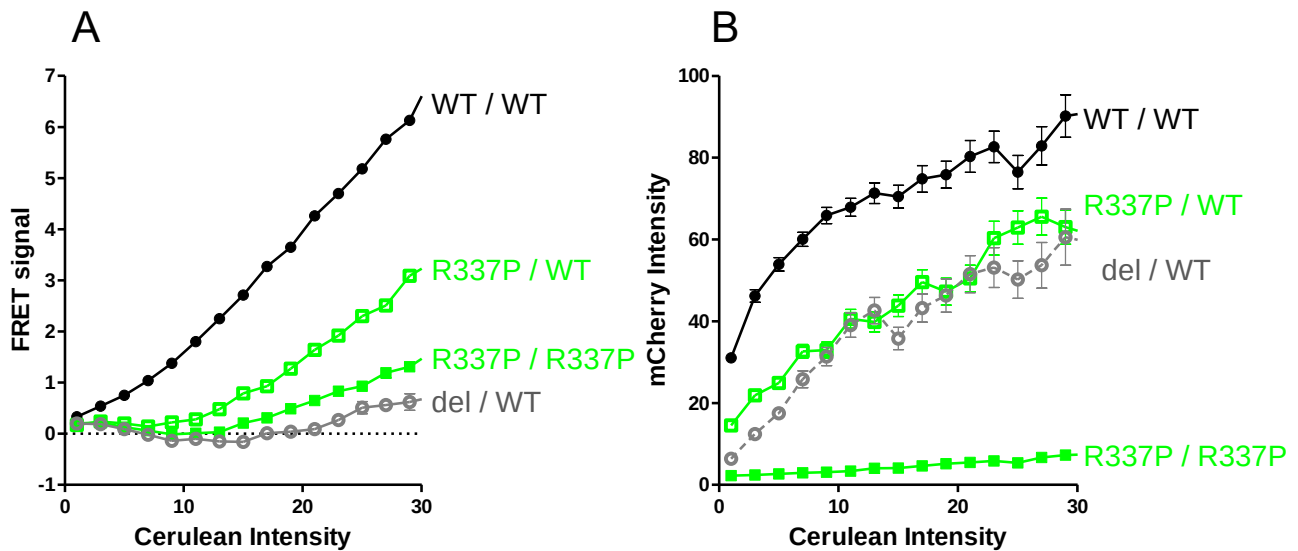


Figure 2-16. Analysis of p53(R337P) mutant protein using the p53RE(*CDKN1A*)

Cells were transfected with phCMV-p53(R337P)-Venus-2A-p53(WT)-Cerulean (R337P/WT) and phCMV-p53(R337P)-Venus-2A-p53(R337P)-Cerulean (R337P/R337P) and analyzed for formation of p53 either homo- or hetero-tetramers (A) as well as transcriptional activity for the pp53RE(*CDKN1A*)-mCherry-NLS-AU2 (B). Similar to the results for the bax promoter, the homo-tetramer was not transcriptionally active, while the hetero-tetramer was active as in **del/WT**.

Table 2-2 Summary of tetramer formation analysis and transcriptional activity analysis in each mutant

		3Ala	R273H	R337C	R337H	R337P
Tetramerization	Mut/Mut	-	++	+	++	-
	Mut/WT	-	++	++	++	+
Transcriptional activity(bax)	Mut/Mut	--	--	--	-	--
	Mut/WT	-	--	-	-	-
Transcriptional activity(p21)	Mut/Mut	N.D.	--	--	-	--
	Mut/WT	N.D.	-	-	-	-

The effect of homotetramer Mut/Mut and heterotetramer Mut/WT formation on transcriptional activity with the bax and p21 promoters are summarized.

If the activity of a particular tetramer was similar or identical to the activity of the del/WT tetramer, the result was categorized as "-". If the activity of a particular tetramer formation was similar to the activity of the WT/WT tetramer, the result was categorized as "++". The activity of a tetramer was categorized as "+" if it was lower than that of the WT/WT tetramer, but displayed tetramer formation. If no transcriptional activity was observed, the result was categorized as "--". "N.D." indicates that No Data was obtained.

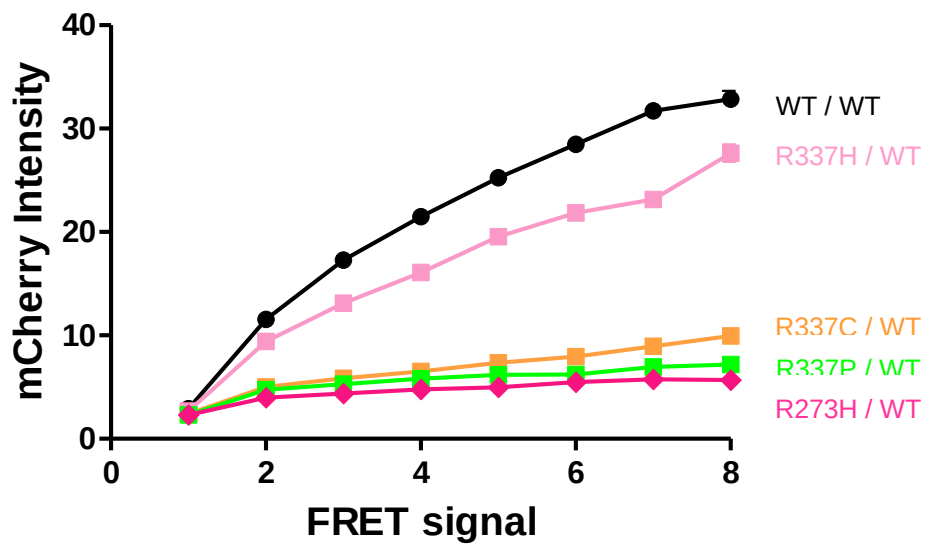


Figure 2-17. Transcriptional activity against bax response sequence per amount of p53 tetramerization

Comparison of transcriptional activity against pp53RE(bax)-mCherry-NLS-AU2 per homo-tetramerization of each mutant revealed that R337C, R337P, and R273H are mostly tetramers without transcriptional activity, whereas R337H forms a slightly less active tetramer compared to WT. R337H forms a slightly less active tetramer compared to WT.

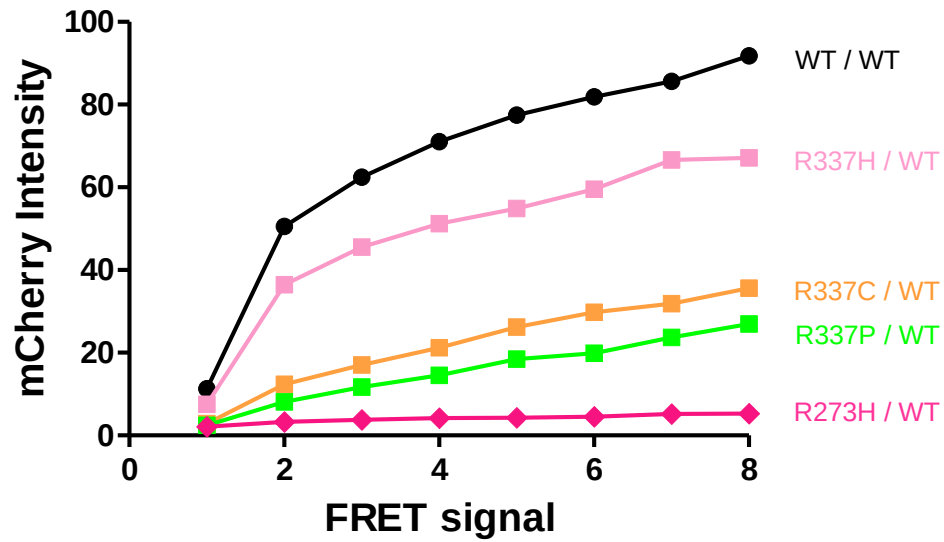


Figure 2-18. Transcriptional activity against the CDKN1A response sequence per amount of p53 tetramerization

The transcriptional activity per amount of each mutant homotetramer against pp53RE(CDKN1A)-mCherry-NLS-AU2 was compared. The results showed that R273H formed a tetramerization with no activity, while R337C and R337P formed a tetramerization with slight activity.

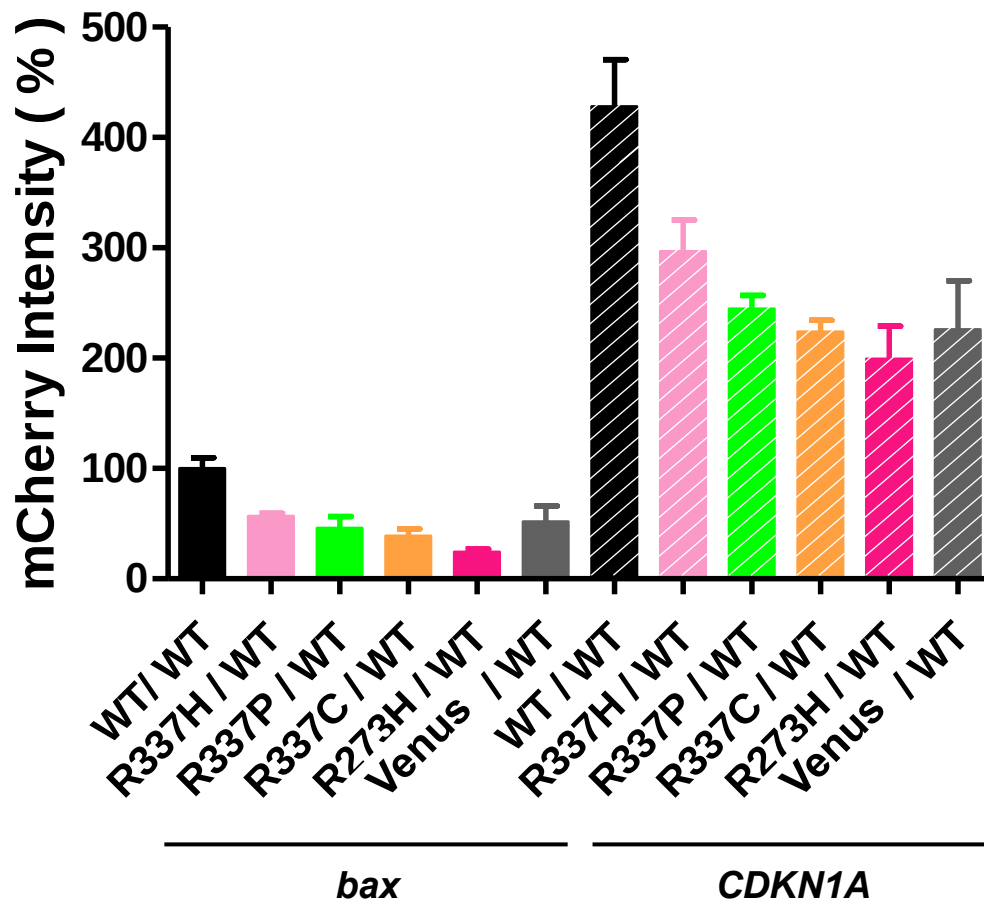


Figure 2-19. Transcriptional activity against each promoter for each mutant when normalized by WT / WT transcriptional activity against the *bax* response sequence
The WT/WT homo-tetramer with the *CDKN1A* promoter was 410% more active than that with the *bax* promoter, and **del**/WT was 4.1 times more active.

The hetero deletion model **del**/WT was 50% active with the *bax* promoter and 230% active with *CDKN1A*, while R337H/WT was 50% active with the *bax* promoter, comparable to **del**/WT, and slightly active with the *CDKN1A* promoter, 300% active. **R337P**/WT and **R337C**/WT had similar transcriptional activity to **del**/WT at 45% and 250% and 40% and 240%.

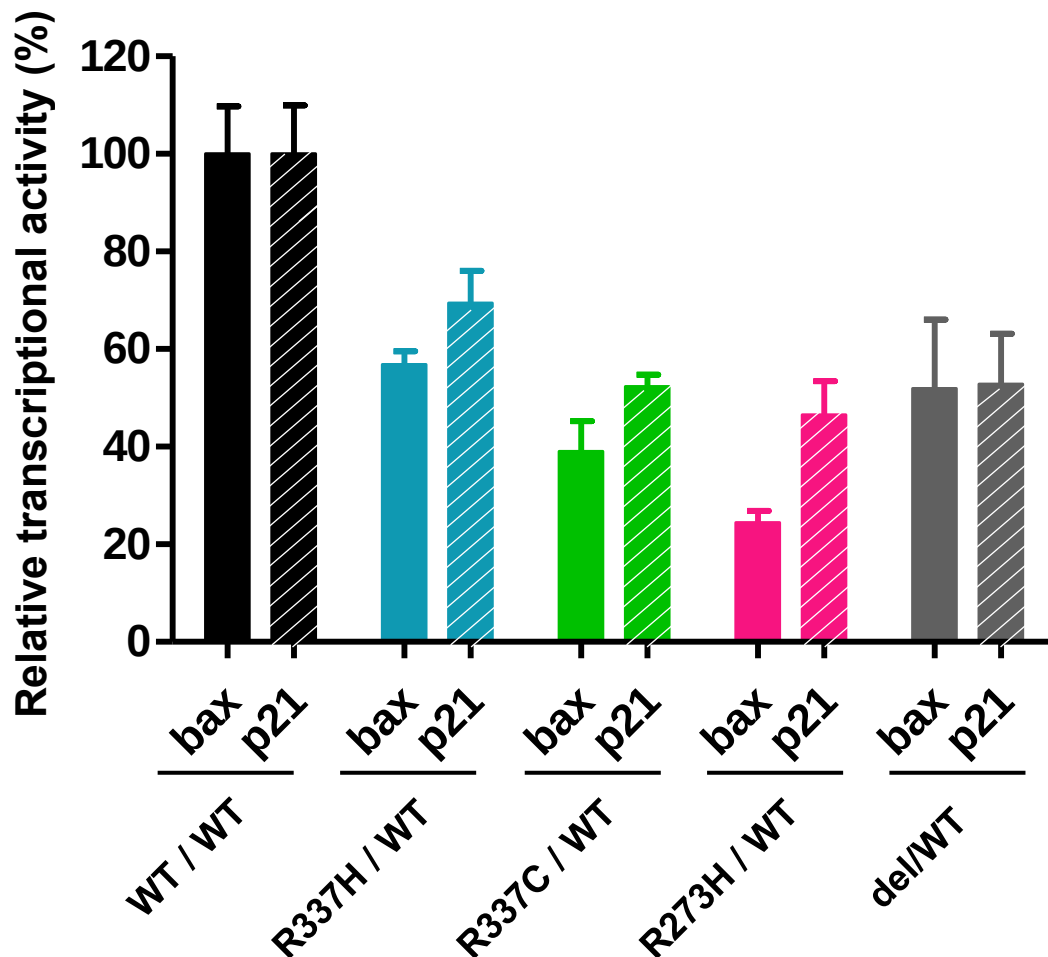


Figure 2-20. Transcriptional activity against each promoter for each mutant when normalized by the respective WT / WT transcriptional activity against the bax and CDKN1A (p21) response sequences

R273H showed 25% transcriptional activity against bax and 45% transcriptional activity against *CDKN1A*, both lower than **del/WT**, while R337C and R337P mutants showed 40% and 45%, respectively, for the *bax* promoter and 50% and 55% for the *CDKN1A* promoter, respectively, these activities were comparable to **del/WT**. On the other hand, R337H showed a value of 55% for the *bax* promoter, similar to the **del/WT**, and a slightly higher activity of 65% for the *CDKN1A* promoter.

2.5. Discussion

In this study, a system that enables simultaneous analysis of p53 expression level-dependent tetramerization and transcriptional activation capacity was successfully established. The high correlation between the amount of p53 tetramerization and transcriptional activity in this system makes it possible to evaluate dominant-negative effects of p53 mutants. The analysis of p53 (Arg337) mutants from Li-Fraumeni syndrome and Li-Fraumeni-like syndrome revealed for the first time that the dominant negative effect of R337C, the tetramerization domain mutant, and the dominant negative-like effect of R337H and R337P, which had long been questioned. I define a dominant-negative-like effect as a reduction in function compared to hetero-deficiency, as observed in the present study, even though the amount of multimer formation is higher than in hetero-deficiency.

R337C is a tetramerization domain mutant found in patients with Li-Fraumeni syndrome [21,22]. FRET analysis of the tetramer formation ability of R337C showed that the amount of heterotetramer between R337C and WT was slightly less than the amount of WT homotetramer. The amount of homotetramer of R337C was even less than the hetero-tetramer of R337C and WT, suggesting that R337C is more stable in hetero-tetramer with WT than in homo-tetramer of R337C. Arg at position 337 stabilizes the tetramerization structure by forming a salt bridge with Asp at position 352 of the other p53 molecule that forms a dimer [23,24]. This suggests that the R337C homotetramer cannot form a salt bridge, which greatly destabilizes the tetramer structure. However, the

presence of p53(WT) during heterotetramerization allows salt bridges to form between Asp352 of p53(R337C) and Arg337 of p53(WT). This is thought to stabilize the heterotetramer rather than the homotetramer.

The R337C homotetramer showed little transcriptional activity, while the transcriptional activity of hetero-tetramer of **R337C/WT**, in which R337C and WT are co-expressed, was lower than that of **del/WT**, a heterozygous deletion model. Thus, it is clear that R337C has a dominant negative effect on the transcriptional activity of wild-type p53 when heterologously expressed. Evaluation of transcriptional activity against homotetrameric levels (Figs. 22 and 23) indicated that the R337C homotetramer has little transcriptional activity.

R337H is a variant derived from Li-Fraumeni-like syndrome, and patients with this mutation are at high risk for adrenocortical carcinoma [7–9]. R337H mutation induced strong destabilization of the homo-tetrameric structure of p53 [27, 36]. FRET analysis also showed that homo-tetramer of R337H showed slightly lower amount compared with homo-tetramer of WT, because of the destabilization effect by R337H mutation. Due to the stabilization effect by formation of hetero-tetramer with WT, **R337H/WT** hetero-expression showed slightly increase in hetero-tetramer formation.

The transcriptional activity of R337H was reduced to the same level as that of **del/WT** in both homo **R337H/R337H** and hetero **R337H/WT**. This indicates that R337H has a dominant-negative-like effect, in which its activity is only as high as that of **del/WT**, even when hetero-expressed in which the expression level of total p53 protein is double than **del/WT**. The fact that the transcriptional activity was reduced to the same level in

the **R337H/R337H** and **R337H/WT** cases in the present results suggests that the **R337H/WT** hetero-tetramer has same transcriptional activity as the **R337H/R337H** homo-tetramer, indicating that the activity is greatly reduced when even a single molecule of R337H is included in the tetramer of p53WT.

R337P is a mutation that has been reported in one case of Li-Fraumeni syndrome [25]. Kamada et al. had shown that R337P alone did not form oligomer and existed as monomer [26], and the FRET analysis in this study revealed that it does not form a homotetramer but does form a hetero-tetramer with WT to some extent. In addition, no transcriptional activity was observed when only R337P was expressed, consistent with the absence of tetramerization, but when R337P was hetero-expressed with WT, despite forming a heterotetramer, its transcriptional activity was reduced to the same level as that of Venus/WT-Cerulean, indicating that, R337P also showed dominant-negative like effect as well as R337H.

The three tetramerization domain mutants analyzed in this study, R337C, R337H, and R337P, when heterologously expressed with WT, had their transcriptional activity reduced to the same level or lower than that of the heterozygous deletion model. All of the mutants were capable of heterotetramerization with WT, suggesting that the total amount of p53 tetramer is greater than **del/WT**, the heterozygous deletion model when each mutant is heterologously expressed with WT. This analysis also suggests that the decrease in transcriptional activity is not only due to decreased tetramerization but also to the formation of unstable tetramers.

The relative transcriptional activity from *CDKN1A* promoter of homo/hetero-expression of p53 mutants normalized by WT/WT transcriptional activity for the *bax* response sequence were increased roughly 4-fold compared with the activity from *bax* promoter (Fig. 24). The KD values of the *CDKN1A* response sequence with p53 are 4.9 nM at the 5' site and 12.0 nM at the 3' site, whereas the *Kd* value of the *bax* response sequence is 73 nM [27]. Interestingly, the effects of hetero-expression of p53 mutants on the transcriptional activity of the wild-type p53 protein were different between the *bax* promoter and the *CDKN1A* promoter.

In order to induce transcription from the p53RE promoter, p53 required to bind and bend the DNA [28–30]. For this DNA bending, it is important to form and maintain stable tetramer through its tetramerization domain during DNA binding and bending. Since the tetramerization domain mutant analyzed in this study is known to have an unstable tetrameric structure due to the R337 mutation, we propose a model in which the tetramer structure is broken because it is not able to withstand the load during bending, making it difficult to regulate transcription. It was shown that p53RE for apoptosis-related genes, with weak DNA binding affinity required stable tetramer formation.

The R337 mutant analyzed in this study showed a more pronounced dominant-negative-like effect on the apoptosis-inducing *bax* response element than on the cell cycle arrest-inducing *CDKN1A* response sequence. In actual cancers, there are many reports that the ability to induce cell cycle arrest is maintained, but the ability to induce apoptosis is lost. This suggests that the loss of apoptosis-inducing ability is involved in cellular

oncogenesis. In general, target genes of p53 involved in cell cycle arrest have higher binding affinity to p53 than the genes involved in apoptosis (cell death). The dual reporter assay in this study demonstrated that p53 mutation had severe effects on the transcription from the apoptosis-related gene with lower affinity against p53. These data suggests that p53 ability to induce apoptosis has easy to be affected by p53 mutation than that to induce cell cycle arrest.

The germline mutations in TP53 that cause LFS show a different distribution than the somatic mutations. In particular, Arg337 is significantly more abundant in the germline, while it is less abundant in somatic cells. The present results indicate that the R337 mutant does not cause as marked a decrease in activity as the dominant-negative effect shown by the functional domain mutant R273H, but it does have a dominant-negative-like effect that reduces activity to the same level as heterozygosity. This suggests that the dominant-negative-like effect, which causes a milder reduction in activity, rather than the dominant-negative effect of R273H, which is common in somatic mutations, is a contributing factor in the onset of LFS. Therefore, although patients develop cancer at a younger than typical age, they are expected to survive to reproductive age and thereby inherit the TP53 mutation.

It has also been reported that loss of heterozygosity (LOH), in which the allele of the normal *TP53* gene carrier is lost in tumor tissue from LFS patients and in successive passages of cells derived from patients, has occurred [31,32]. From this, it is expected that p53 function would be almost completely lost when only mutant p53 is expressed by LOH, given the congenital occurrence of TP53 mutations in the patient's cells. Indeed,

even though dominant-negative-like effects were observed in the present results when the p53 mutants were heterologously expressed, homotetramers of all mutants except R337H had little or no transcriptional activity. Therefore, the complete loss of p53 function due to the occurrence of LOH in the cells of LFS patients may be related to carcinogenesis. In addition, R337H alone was slightly active even in the homotetrameric form, suggesting that the degree of loss of p53 function may be related to the carcinogenicity rate and age of onset of cancer in patients.

In summary, this study demonstrates that R337C, R337H, and R337P, p53 tetramerization domain mutants, have dominant-negative effects or dominant-negative-like effects that reduce activity to the same degree as heterozygous deletions. The dominant-negative effect of mutations in the multimeric domain of multimeric proteins has been a "long-standing question," and these findings are the first to clarify the dominant-negative effect of mutations in the multimeric domain of a multimeric protein. This finding is the first clarification of the dominant negative effect of mutations in the multimeric domain of multimeric proteins.

Furthermore, as a mechanism for the dominant-negative effect caused by the p53 tetramerization domain mutation, we proposed a model in which the tetramer structure cannot be maintained until transcriptional activation via DNA bending due to tetramer structure destabilization of p53, in addition to a decrease in the amount of tetramerization. Further studies on the mechanism of dominant-negative-like effects caused by tetramer structural instability are expected to greatly advance our understanding of the mechanism of LFS pathogenesis.

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3. p53 Protein Tetramerization Domains from Different Mammalian Species

3.1. Abstract

The p53 protein is a transcriptional regulatory factor and many of its functions require that it forms a tetrameric structure. Although the tetramerization domain of mammalian p53 proteins (p53TD) share significant sequence similarities, it was recently shown that the tree shrew p53TD is considerably more thermostable than the human p53TD. Biophysical, functional, and structural studies to compare the properties of the p53TDs from six mammalian model organisms (human, tree shrew, guinea pig, Chinese hamster, sheep, and opossum). were used to determine whether other mammalian species display differences in this domain. The results indicate that the p53TD from the opossum and tree shrew are significantly more stable than the human p53TD, and there is a correlation between the thermostability of the p53TDs and their ability to activate transcription. Structural analysis of the tree shrew and opossum p53TDs indicated that amino acid substitutions within two distinct regions of their p53TDs can dramatically alter hydrophobic packing of the tetramer, and in particular substitutions at positions corresponding to F341 and Q354 of the human p53TD. Together, the results suggest that subtle changes in the sequence of the p53TD can dramatically alter the stability, and potentially lead to important changes in the functional activity, of the p53 protein.

3.2. Introduction

The p53 protein is the founding member of a family of transcriptional regulatory proteins that also includes the related factors p63 and p73 [1–4]. In humans, the p53 protein functions as an important tumor suppressor through its role in controlling key cellular processes associated with DNA repair and genome maintenance [5–7]. Significantly, mutations in the p53 gene have been shown to be directly linked to approximately 50% of all known human cancers [8–10]. The human p53 protein contains 393 amino acids and it can be roughly divided into four key functional domains. The four domains include a central core DNA-binding domain [11], an N-terminal transcriptional activation domain [12], a tetramerization domain (TD) [8] located just C-terminal to the DNA-binding domain, and a C-terminal regulatory domain that is controlled through numerous post-translational modifications [13–16].

To bind to a specific DNA target site and function as a transcriptional regulatory factor, the human p53 protein must first form a homo-tetrameric structure [17]. Formation of the tetrameric structure occurs in response to increases in cellular levels of p53 following the activation of its expression, and the activation of p53 expression occurs in response to a variety of external stimuli including oxidative stress and DNA damage [18–20]. Importantly, the stability of the tetrameric form of p53 appears to correlate with its transcriptional activity [21,22]. The formation of the p53 tetrameric structure requires a short TD region, which is located between residues 324 and 356 in the sequence of human p53 (HU-p53TD). The structure of HU-p53TD has been determined by both NMR spectroscopy and X-ray crystallography, and it consists of four monomers that each

contain a single β -strand (residues 326–333) followed by a single α -helix (335–356), which are separated by a tight turn of Gly334 [23,24]. To generate the tetrameric form of p53, two monomers form an initial dimeric structure through interactions between their β -strands that results in a two-stranded β -sheet as well as hydrophobic packing between the helices from each monomer. Next, two dimers come together to form the tetramer, which is stabilized by a helical bundle that forms through a series of hydrophobic and electrostatic contacts between the side chains of the amino acids located in the four α -helices from each monomer.

Although all members of the p53 family of proteins bind to their target DNA sequences as tetramers, the structure of HU-p53TD is distinct from the structure of both HU-p63TD and HU-p73TD. The TDs of human p63 and p73 both contain an additional helical segment, which is located at the C-terminal end of the TD, creating a $\beta\alpha\alpha$ motif in comparison to the more simplistic $\beta\alpha$ motif found in HU-p53TD [25,26]. In the case of p73, the second helical domain has been shown to not only increase the stability of the homo-tetramer, but also to play a crucial role in the formation of hetero-tetramers between the human p63 and p73 proteins. In contrast, the formation of hetero-tetramers between the human p53 protein and either p63 or p73 has not been observed, and this inability of p53 to form hetero-tetramers has been attributed to the absence of the second helical segment in HU-p53TD [27]. Interestingly, this second helical region has been shown to be present in the TD of the Zebrafish p53 (ZF-p53TD), which is from a subgroup of bony fishes; however, to date, there is no evidence that ZF-p53TD forms hetero-tetramers with either its p63 or its p73 [28]. The presence of these structural variations in p53TD between

different species suggests that the TD of p53 may have evolved so that it could perform different functions in the transition from aquatic species to mammalian terrestrial-based species [29–31].

The common tree shrew is a small rodent-like animal that has drawn considerable scientific interest since it possesses numerous characteristics in common with primitive primate species. Although the tree shrew is estimated to have diverged from larger primates about 90 million years ago, it is still regarded as a potential model for examining early primate evolution since it represents an early precursor in the evolution pathway leading to apes and humans [32–34]. Surprisingly, it is reported that the TD of the tree shrew p53 protein (TS-p53TD) is dramatically more thermostable than HU-p53TD despite the fact that the two TDs share very a high sequence identity percentage [35]. It was postulated that this difference was important in helping the tree shrew adapt to its unusual diet that includes the daily consumption of large quantities of alcohol, which poses an enhanced risk of DNA damage due to exposure to high levels of the genotoxic agent acetaldehyde. This increase in the thermostability of the TS-p53TD also suggests that, within different mammalian species, the p53 proteins may have evolved to serve different functional roles by altering the biophysical properties of their TD, which can be produced through minimal alterations in the sequence.

The secondary structure, the thermostability, the transcriptional activity, and several of the structures of the p53TDs from six different mammalian species (human, tree shrew, guinea pig, Chinese hamster, sheep, and opossum) were examined to determine whether the p53TDs from different mammalian species have distinct biophysical properties

despite sharing high sequence identity percentages. These mammalian species were selected because they represent important model systems in scientific research. Our results demonstrate that, despite sharing very high sequence identity percentages and similar overall three-dimensional structures, there are significant differences in the thermostability of the different p53TDs, with the opossum and tree shrew showing the largest enhanced thermostability relative to the HU-p53TD. This enhanced thermostability appears to correlate with an increase in transcriptional activity of the proteins, and, in the case of the tree shrew p53TD, with slower rates of oxidation of a methionine residue within its hydrophobic core. Structural characterization of the p53TD from tree shrew and opossum highlights how even subtle changes in the sequence can result in a more compact organization of the hydrophobic core formed between the four monomers within the tetrameric structure to enhance the thermostability. Together, these results further demonstrate how substitutions within the sequence of mammalian p53TDs can alter their properties, and suggest that simple changes may be associated to important functional differences in the p53 protein in the different species as well as to cancer-associated mutations in humans.

3.3. Materials and Methods

3.3.1. Synthesis of p53TD peptides

The peptides corresponding to the human p53 TD (HU-p53TD; residues 324–358 of human p53), the tree shrew p53 tetramerization domain (TS-p53TD; residues 324–358 of tree shrew p53), the guinea pig p53 tetramerization domain (GP-p53TD; residues 321–355 of guinea pig p53), the Chinese hamster p53 tetramerization domain (CH-p53TD; residues 324–358 of Chinese hamster p53), the sheep p53 tetramerization domain (SH-p53TD; residues 313–347 of sheep p53), and the opossum p53 tetramerization domain (OP-p53TD; residues 296–330 of opossum p53) were synthesized as previously described [35]. The peptide sequences are shown in Figure 3-1. The synthesized peptides were deprotected and cleaved from the resin by using Reagent K (TFA:H₂O:thioanisole:ethanedithiol:phenol = 82.5:5:5:2.5:5). The peptides were all purified to homogeneity using reverse-phase HPLC over a C8 column (Vydac, Hsperia, CA, USA) and the correct molecular weight of each peptide was verified by MALDI-TOF-MS analysis. Peptide concentrations were determined using an extinction coefficient, $\epsilon_{280} = 1,490 \text{ M}^{-1} \text{ cm}^{-1}$, which corresponds to a single tyrosine present in all of the p53 proteins.

3.3.2. Determination of oligomeric state, secondary structure, and thermal stability

To verify that the p53TD peptides from the different mammalian species form tetrameric structures, the peptides were analyzed by gel filtration chromatography on a Superdex 75 (GE Healthcare, Chicago, IL, USA) column. For this analysis, 20 μ L of each peptide at 100 μ M concentration in 50 mM phosphate buffer, pH 7.5 with 100 mM NaCl, was injected onto the column and eluted at room temperature using a flow rate of 0.1 mL/min with UV-monitoring at 214 nm.

To determine the secondary structure content of the different p53TD peptides, circular dichroism (CD) spectra at a peptide concentration of 10 μ M in a buffer consisting of 50 mM sodium phosphate, pH 7.5 with 100 mM NaCl, were recorded at 4 °C on a Jasco J-805s spectropolarimeter in a 1 mm quartz cell. Scans were recorded continuously over the region between 195 and 260 nm at a rate of 50 nm/min and a bandwidth of 0.2 nm. The percentages of secondary structural elements were calculated using the commercially supplied JASCO JWSSE-480 software 1.53.07 package and fit according to previously published methods for analysis [36]. For the thermal denaturation assay, CD spectra were recorded between 4 °C and 96 °C and the temperature was increased at a fixed rate of 1.0 °C/min. Ellipticity was measured at 222 nm for monitoring the α -helical content of the peptides. The thermodynamic parameters of the peptides were determined as described previously [21]. The theoretical values were calculated using the PDB:3SAK structure [23].

3.3.3. Transcriptional Activity Assay

A series of reporter plasmids, p(p53RE(CDKN1A))-mCherry-NLS-hCMV-Venus-chimeric p53, were constructed to test the transcriptional activity of the different mammalian p53TDs. The plasmid carried the p53RE derived from the CDKN1A gene. For each of the species, a chimeric p53 expression plasmid was prepared, in which the TD of the human p53 protein was replaced by the TD from the other mammalian species (tree shrew, guinea pig, Chinese hamster, sheep, and opossum). The cDNA sequences encoding the TD from the different species were inserted into the human p53 sequence in the vector.

The transcriptional activities of chimeric p53 proteins containing TD sequences from each of the mammalian species were determined as described previously with minor modifications [37]. H1299 p53 null cells (American Type Culture Collection, Manassas, VA, USA) were seeded in a 35 mm-diameter plastic dish (Falcon, Corning, NY, USA) and grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in 5% CO₂. After 12 h, cells were washed three times with 1.5 mL PBS. Next, 100 µL Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA), 2.4 µg plasmid, and 6 µL Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) were mixed and incubated for 20 min at room temperature. Then, 700 µL Opti-MEM was added to the mixture (transfection mixture). The transfection mixture was added to the dish containing the cells and incubated for 1 h at 37 °C. Afterward, the transfection mixture was removed, and the cells were grown in 1.5 mL of normal culture medium at 37 °C. After 11 h, the

cells were washed three times with 900 μ L PBS and fixed with 900 μ L of a 10% neutral formalin buffer solution (Wako, Osaka, Japan) for 10 min at room temperature with agitation. The formalin solution was removed, and the cells were incubated in 900 μ L 0.2% Triton-X-100/PBS for 5 min at room temperature. The cells were washed three times with 900 μ L PBS and incubated for 15 min in 1 mL 0.1% DAPI/PBS at room temperature to stain the nuclei. The cells were then washed twice with 900 μ L PBS and 1 mL of PBS was added to the dish. The cells were observed by fluorescence microscope (BIOREVO BZ-9000, Keyence, Osaka, Japan) with the TRITC filter (excitation filter: 540/25 nm; emission filter: 605/55 nm; dichroic mirror: 565 nm) for mCherry; the YFP filter (excitation filter: 500/24 nm; emission filter: 542/27 nm; dichroic mirror: 520 nm) for Venus; the CFP filter (excitation filter: 448/20 nm; emission filter: 482/25 nm; dichroic mirror: 458 nm) for Cerulean; the DAPI filter (excitation filter: 360/40 nm; emission filter: 460/50 nm; dichroic mirror: 400 nm) for DAPI. The objective lens was a Nikon Plan Fluor 10 $\times$ /0.30 and the image and data quantitative analyses of fluorescence intensity were performed as previously described [37].

3.3.4. Peptide expression and purification in *E. coli* cells

The DNAs corresponding to the HU-p53TD, the TS-p53TD, the OP-p53TD, and the GP-p53TD were synthesized as oligonucleotides (Integrated DNA Technologies) with flanking BamHI- and EcoRI-restriction enzyme sites and cloned into a modified pGEX-2T vector (Amersham, Merck, Darmstadt, Germany) with a Tobacco Etch Virus (TEV)

protease cleavage site replacing the original thrombin cut site. The corresponding plasmids were transformed into *E. coli* Topp2 cells for expression.

For the expression, the cells were first grown overnight (16 h) in 2 L of LB with ampicillin (100 mg/L) at 37 °C, and this culture was diluted the following morning with 6 L of LB with ampicillin and induced for 4 h at 30 °C by the addition of IPTG (125 mg/L). After 4 h of induction with IPTG, the cells were harvested by centrifugation and re-suspended in lysis buffer. The cells were lysed using a French press and centrifuged at 105,000× *g* for 30 min at 4 °C. The resulting supernatant was incubated in the presence of glutathione resin (GE Healthcare) for 1 min at 4 °C. Afterwards, the supernatant was removed by centrifugation at 3000× *g* for 5 min and washed three times with TEV buffer (25 mM Na phosphate, 125 mM NaCl, 5 mM DTT). The GST-tag was removed by the overnight incubation at room temperature in the presence of 100 units of TEV protease. The solution was then centrifuged at 3000× *g* for 5 min and the supernatant was applied to an ion exchange column (buffer A: 20 mM Tris-HCl, 1 mM DTT, pH 7.2; buffer B: 20 mM Tris-HCl, 1 mM DTT, 1 M NaCl, pH 7.2). SP Sepharose or Q Sepharose column (GE Healthcare) were used. The fractions from the ion exchange column containing tag-free p53 Tet proteins were pooled and dialyzed into 20 mM Tris-HCl, 1 mM DTT, pH 7.5 for subsequent purification by gel filtration on a Superose™ 12 (GE Healthcare). The tetramer structures and their stability were measured by CD analysis as well as synthetic p53TD peptide (Figure 3-2).

3.3.5. Crystallization and structure determination

Crystals of the HU-p53TD, TS-p53TD, and OP-p53TD were grown by the vapor diffusion method at 20 °C using either 1:1 or 2:1 mixtures of peptide solution (4.0 mg/mL) and precipitant buffer. The precipitant buffer was 20% PEG 3350 with 0.2 M NH_4NO_3 . Before the samples were flash-frozen, 24% ethylene glycol was added as a cryoprotectant. Diffraction data were collected from single crystals using the ID7B2 beamline at MacCHESS (Cornell University, Ithaca, NY, USA) or at the Canadian Light Source (CLS) (Saskatoon, SK, Canada). All data sets were processed with HKL2000. The initial phases for determining the structures were obtained by molecular replacement using the structure of the HU-p53TD as a search template. Phases were improved by iterative cycles of model building with Coot 0.9.8.93, and refinement was performed with PHENIX 1.19.2-4158. The figures were visualized using PyMOL 2.5.

3.3.6. Determination of oxidation status of methionine residues

The HU-p53TD and TS-p53TD peptides (100 μM) were first preincubated for 1h in 50 mM phosphate buffer (pH 7.5) containing 100 mM NaCl. After preincubation, hydrogen peroxide (H_2O_2) was added to the peptide mixture at a concentration of 100 mM to initiate the oxidation reaction. Samples were taken and analyzed by HPLC on a C8 column (4.6 $\times$ 250 mm, CAPCELL PAK C8 100, 5 mm, SHISEIDO, Tokyo, Japan) at set time intervals (0–24 h). The samples were eluted on the HPLC at a flow rate of 0.7 mL/min using buffer A (0.05% TFA in water) and buffer B (0.04% TFA in acetonitrile), using a linear gradient from 15% to 35% buffer B over 20 min to elute the peptides. Following separation on the HPLC, the different peaks were collected and digested with

trypsin (0.92 mg/L, 0.16 µg) for 12 h at 35 °C. The trypsin reaction mixture was then desalted using a Macro SpinColumn™ (Harvard Apparatus, Holliston, MA, USA) and analyzed using an Ultraflex extreme MALDI TOF/TOF mass spectrometer from Bruker Daltonics (Billerica, MA, USA) to verify the precise Met residues that were oxidized.

3.3.7. Statistical analysis

Statistical analyses were performed using the Kruskal–Wallis test with Dunn’s multiple comparison post-hoc tests for transcriptional activity using GraphPad Prism (Version 9.5.1, GraphPad Software Inc., San Diego, CA, USA). For the comparisons, a *p* value of <0.05 was considered statistically significant. *** *p* < 0.0005, **** *p* < 0.0001.

3.4. Results

3.4.1. Selection and preparations of TDs from mammalian p53 protein

Six mammalian species (human, tree shrew, guinea pig, Chinese hamster, sheep, and opossum) were chosen for testing of the biophysical and functional properties of their p53TD, because they each represent an important mammalian model species [38–44]. The amino acid sequence selected as the TD for each species was chosen based on sequence alignment to the well-characterized HU-p53TD, which corresponds to residues 324–358 of the human p53 protein (Figure 3-1).

Thus, the TDs from each of the six species contain 35 amino acids. Peptides containing the sequences of the six different p53TDs were initially prepared by solid-phase peptide synthesis using Fmoc-chemistry. Comparing the sequences of the p53TDs from the six species shows (Figure 3-1) that the percentages of sequence identity of the five other mammalian TD sequences relative to the HU-p53TD range from a high of 91.4% for the Sheep p53TD (SH-p53TD) to a low of 71.4% for both the Opossum p53TD (OP-p53TD) and the Guinea pig p53TD (GP-p53TD). In addition, the five sequences shared more than 90% similarity with the HU-p53TD, with a high of 97.1 for the SH-p53TD and OP-p53TD, whereas the low was 91.4% for the GP-p53TD. Overall, the sequences for the p53TDs from the different mammalian species are very similar.

3.4.2. Secondary structure composition of the six p53TD based on CD analysis

To initially characterize their structural characteristics, the secondary structure compositions of the six different mammalian p53TDs were determined using circular dichroism (CD) spectroscopy. Based on the previous structures of the HU-p53TD, the peptides should theoretically contain 63% helix, 23% beta, 3% turn, and 11% random structural content (Table 3-1). Analysis of the six p53TDs by CD spectroscopy in solution indicated that the OP-p53TD and TS-p53TD give the closest matches in terms of helical structure to the theoretical value of 63%, with predicted helical contents of 62% and 64%, respectively, based on CD analysis (Figure 3-3 and Table 3-1).

In terms of beta content, the GP-p53TD and the TS-p53TD were the closest matches to the theoretical value of 23%, with both containing 24% beta content as estimated by CD analysis (Table 3-1). Overall, the secondary structure content of the TS-p53TD in solution based on the CD analysis was the closest to the theoretical values based on the crystal structure of the HU-p53TD. Although NMR measurements have demonstrated that the HU-p53TD is dynamically stable in solution between residues 325 and 347, the differences between the theoretical values based on the reported structure [23] and the measured values for the HU-p53TD determined by CD analysis could represent the relative association and disassociation of the HU-p53TD that occurs in solution.

3.4.3. Verification of tetrameric structure of the mammalian p53TDs by gel filtration chromatography

To verify that they form tetrameric structures, the six mammalian p53TDs were analyzed by gel filtration chromatography. Using this methodology, we previously demonstrated that the HU-p53TD and the TS-p53TD exist predominantly in the tetrameric form at a concentration of 100 mM in 50 mM phosphate buffer with a pH of 7.5 and with 100 mM NaCl. Using identical experimental conditions, the SH-p53TD, GP-p53TD, OP-p53TD, and CH-p53TD are also all predominantly in their tetrameric form (Figure 3-4).

The differences between the monomeric and tetrameric forms can be distinguished in this experiment by comparing them with the retention time by gel filtration chromatography for a triple-alanine substituted variant of the HU-p53TD (3Ala), which has been shown to only exist in a monomeric form (Figure 3; grey line). All six of the mammalian p53TDs are predominantly in their tetrameric form under the experimental conditions used.

3.4.4. Determination of the thermostability of mammalian p53TDs by CD spectroscopy

Given that the TS-p53TD was previously shown to have much higher thermostability than the HU-p53TD despite the two sharing a very high sequence identity percentage, we measured the thermostability of the other four mammalian p53TDs based on CD measurements at variable temperatures. In these experiments, the ellipticity of each p53TD at 222 nm was measured between 4 °C and 96 °C, and these measurements were

used to generate denaturation curves for each peptide (Figure 3-5). The different denaturation curves were then used to calculate the T_m , the ΔT_m , and the $\Delta G_u^{37^\circ\text{C}}$ for each of the six mammalian p53TDs (Table 3-2). As seen with the TS-p53TD, all of the other mammalian p53TDs are significantly more stable than the HU-p53TD, with the exception of the CH-p53TD. Of the six mammalian p53TDs tested, the OP-p53TD is the most thermostable. In the case of the OP-p53TD, the calculated T_m was 78.0 °C and the $\Delta G_u^{37^\circ\text{C}}$ was 36.6 kcal/mol, which is very similar to the TS-p53TD, which has a T_m of 77.1 °C and a $\Delta G_u^{37^\circ\text{C}}$ of 36.4 kcal/mol, but significantly higher than the HU-p53TD's calculated T_m of 67.8 °C and $\Delta G_u^{37^\circ\text{C}}$ of 32.4 kcal/mol. In addition to the dramatic increase in thermostability observed for the OP-p53TD and TS-p53TD, the SH-p53TD and the GP-p53TD showed an intermediate increase in thermostability relative to the HU-p53TD and CH-p53TD. For the SH-p53TD, the calculated T_m was 75.1 °C and the $\Delta G_u^{37^\circ\text{C}}$ was 34.0 kcal/mol, and for the GP-p53TD, the calculated T_m was 74.7 °C and the $\Delta G_u^{37^\circ\text{C}}$ was 35.4 kcal/mol. Taken together, these results suggest that there are considerable differences in the thermostabilities of the p53TDs from the six different mammalian species despite the fact that their sequences are often quite similar.

3.4.5. Transcriptional activity of chimeric p53 protein with TD of each species

It has been suggested that the transcriptional activity of the p53 protein is correlated with the stability of its tetrameric structure. In order to test whether changes in the thermostability of the different mammalian p53TDs alter their relative transcriptional

activity, expression vectors to produce chimeric p53 proteins were generated. In the vector, the TD from each of the five other mammalian species was substituted into the corresponding position in the human p53 with p53TD, and the transcriptional activity of each chimeric p53 protein was measured using a reporter assay (Figures 3-6 and 3-7). The transcriptional activities of several of the chimeric p53 proteins were slightly but significantly increased for several species in comparison with the wild-type human p53 protein. Among the different mammalian species, the chimeric p53 containing either the OP-p53TD or the TS-p53TD showed the highest transcriptional activity towards the p21 promoter used in this assay, suggesting that enhanced thermostability of the p53TD increases the transcriptional activity under these experimental conditions. Overall, these results were consistent with our previous data which indicated that the transcriptional activity of p53 was associated with the thermostability of p53TD [21,22].

3.4.6. Crystal structures of HU-p53TD, TS-p53TD and OP-p53TD

To help define the differences between the HU-p53TD and the mammalian p53TDs with significantly enhanced thermostability at the atomic level, crystals of the OP-p53TD and the TS-p53TD were generated, as well as the HU-p53TD using the identical sequences employed in the other assays. After screening a variety of different conditions, crystals were obtained for the HU-p53TD that diffracted to a resolution of 1.1 Å, for the TS-p53TD that diffracted to a resolution of 1.17 Å, and for the OP-p53TD that diffracted to a resolution of 1.35 Å. these crystals yielded the first high-resolution structures for the

TS-p53TD and the OP-p53TD as well as a very high-resolution structure for the HU-p53TD. As expected, the structures of the three p53TDs are very similar, in that they consist of a tetramer that is composed of two dimers and each monomer contains a $\beta\alpha$ fold (Figure 3-8)

In comparing the TS-p53TD with the HU-p53TD structure, there are two major regions where structural differences are observed, and these differences occur in areas that contain the three amino acid differences in the two sequences within the structured region of these p53TDs. The first difference occurs in the region around L350 and Q354 in the HU-p53TD, which corresponds to L350 and M354 in the TS-p53TD (Figures 3-9 and 3-10). In the structure of the TS-p53TD (Figure 3-9A), the side chains of L350 and M354 from the adjacent monomers are in proximity to each other and in position to make hydrophobic contacts with the same residues of the other monomer within the dimer (Figure 3-9A, left panel and 3-10, upper panel). This results in the formation of two hydrophobic clusters, one at the C-terminal end of the helices in each dimer. In contrast, in the HU-p53TD (Figure 3-9B), the side chain of Q354 from one monomer forms a hydrogen bond with the second Q354 of the adjacent monomer in the dimers in the HU-p53TD (Figure 3-9B, left panel and 3-10, upper panel); however, the two L350 residues from the adjacent monomers remain more distant from each other than the corresponding residues in the TS-p53TD (4.5 versus 8.2 Å). The second key difference between the TS-p53TD and HU-p53TD structures occurs in the hydrophobic core, formed between the four helices, that stabilizes the tetrameric structure. In this region, the aromatic side chain

of F341 in the HU-p53TD is substituted by the aliphatic L341 in the TS-p53TD and L344 in the HU-p53TD is substituted by I344 in the TS-p53TD. This substitution at position 341 leads to a series of subtle difference in the packing of the side chains that form the hydrophobic core between the four helices. It is noteworthy that the distances between the sulfur atoms of the M340 residues from the different dimers are 1.1 Å closer (3.2 versus 4.3 Å) to each other in the TS-p53TD structure (Figure 3-9A, right panel and 3-10, lower panel) than in the HU-p53TD structure (Figure 3-9B, right panel and 3-10, lower panel). In addition, the sulfur atom of M340 from one dimer is 0.5 Å closer (4.0 versus 4.5 Å) to the nearest methyl group of I344 of the other dimer in the TS-p53TD (Figure 3-9A, right panel) than the sulfur atom of M340 is to the nearest methyl group of L344 of the other dimer in the HU-p53TD (Figure 3-9B, left panel). Together, these two differences help explain the enhanced thermostability of the TS-p53TD in comparison to the HU-p53TD.

As seen with the TS-p53TD, there are also two major structural differences observed in comparing the structure of the OP-p53TD (Figures 3-10C and 3-11) with the structure of the HU-p53TD (Figures 3-10B and 3-11), and these differences occur in the same two regions. In the first region, L350 and Q354 in the HU-p53TD correspond to L322 and H326 in the OP-p53TD, and in the structure of the OP-p53TD (Figure 3-9C, left panel and 3-11, upper panel), the imidazole rings from the side chains of the H326 residues from the adjacent monomers are in proximity (2.9 Å) to form a hydrogen bond between each other. As seen with the TS-p53TD (Figure 3-9A, left panel and 3-11, upper panel), this helps stabilize a hydrophobic interaction by bringing the side chains of the L322

residues from the adjacent monomers in proximity to each other (4.5 Å in the OP-p53TD versus 8.2 Å in the HU-p53TD) [45]. The second key difference between the OP-p53TD and HU-p53TD structures occurs in the hydrophobic core formed between the four helices. In this region, as seen with the TS-p53TD, the aromatic side chain of F341 in the HU-p53TD is substituted by the aliphatic L313 in the OP-p53TD and L344 in the HU-p53TD is substituted by I316 in the OP-p53TD. In addition, M340 of the HU-p53TD is replaced by L312 in the OP-p53TD (Figure 3-9C, right panel and 3-11, lower panel). As seen with the TS-p53TD (Figure 3-9A, right panel and 3-11, lower panel), this substitution at position 341 leads to a series of subtle difference in the packing of the side chains that form the hydrophobic core between the four helices in comparison to what is observed for the HU-p53TD (Figure 3-9B; right panel and 3-11; lower panel). Together, these two differences not only help explain the enhanced thermostability of the OP-p53TD in comparison to the HU-p53TD, but also explain why its thermostability is similar to what is observed for the TS-p53TD.

3.4.7. Oxidation of methionine residues in HU-p53TD and TS-p53TD

The HU-p53TD contains one Met residue (M340) in its hydrophobic core, whereas the TS-p53TD contains two Met residues (M340 and M354), one in the hydrophobic core and one near the C-terminal interface of the helices. Given that the M354 of the TS-p53TD is the key determinant of its enhanced stability relative to the HU-p53TD and that M340 in the HU-p53TD appears to be more surface-exposed than the M340 of the TS-

p53TD, the relative accessibility of these three Met residues to oxidation by hydrogen peroxide (H_2O_2) was evaluated. The rate of oxidation of the Met residues was assessed using an HPLC assay in combination with mass spectral (MS) analysis (Figure 3-12 and Table 3-3).

In the presence of H_2O_2 , the M340 of the HU-p53TD oxidized in a time-dependent manner and one additional peak (oxi) appeared in the HPLC chromatogram due to oxidation of the M340. After 2 h, more than half of the M340 was in the oxidized form, and after 8 h, more than 95% was oxidized. By 14 h, the HU-p53TD was completely in an oxidized form. In contrast, three new peaks appeared in the HPLC chromatogram following the addition of H_2O_2 to a solution containing the TS-p53TD. The MS analysis demonstrated that two of these peaks were the peptide in which either M354 (mono-oxi1) or M340 (mono-oxi2) was oxidized, and the other peak corresponded to the peptide in which both Met residues were oxidized (di oxi). The level of the non-oxidized TS-p53TD was less than 5% of the total amount after 2 h. The M354 oxidized peak was present only at 30 min and 2 h after the initiation of the oxidation reaction, whereas the M340 oxidized peak increased over the first 2 h and then began to decrease. The di-oxidized peak gradually increased in a time-dependent manner and exceeded 95% at 16 h after the initiation of the oxidation reaction.

To identify the oxidized products, each peak from the HPLC separation of the two reactions was collected and treated with trypsin (Table 3). MS analysis of peptide fragments from the different peaks following the trypsin treatment revealed that the mono-oxi1 peak corresponds to the oxidation of M340 in both the HU-p53TD and TS-

p53TD samples. This result indicates that the mono-oxi² peak from the TS-p53TD sample corresponded to the oxidation of M354, and this indicates that M354 is more easily oxidized than M340 in the TS-p53TD. In addition, the results demonstrate that M340 in the TS-p53TD oxidizes at a slower rate than the M340 of the HU-p53TD under these conditions, which is presumably due to the increased stability of the TS-p53TD relative to the HU-p53TD.

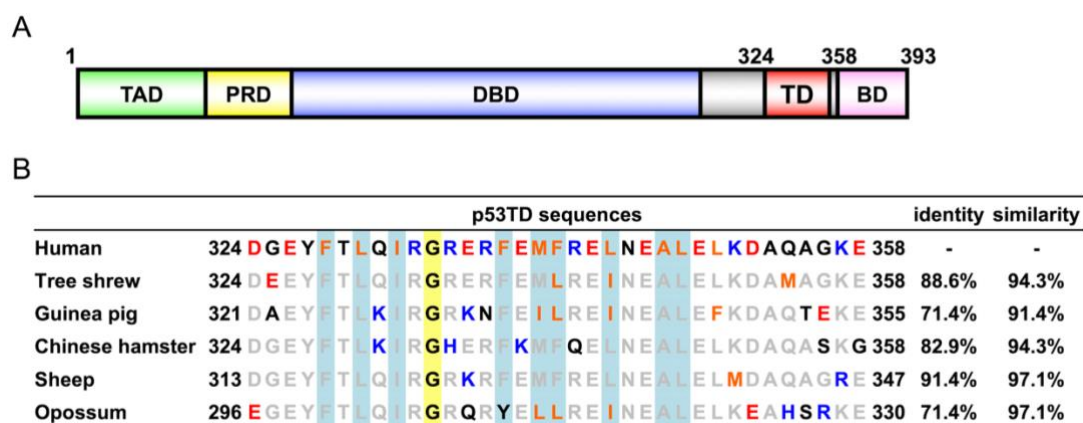


Figure 3-1. Mammalian p53TDs.

(A) Schematic structure of the human p53 protein. TAD: transactivation domain, PRD: proline-rich domain, DBD: DNA binding domain, TD: tetramerization domain, BD: basic domain. (B) Sequences of the mammalian p53TDs and % of identity/similarity relative to the HU-p53TD. Conserved residues in terms of human are gray, substituted residues are hydrophobic, orange; acidic, red; basic, blue; others, black. Residues forming a hydrophobic core are shadowed in light blue.

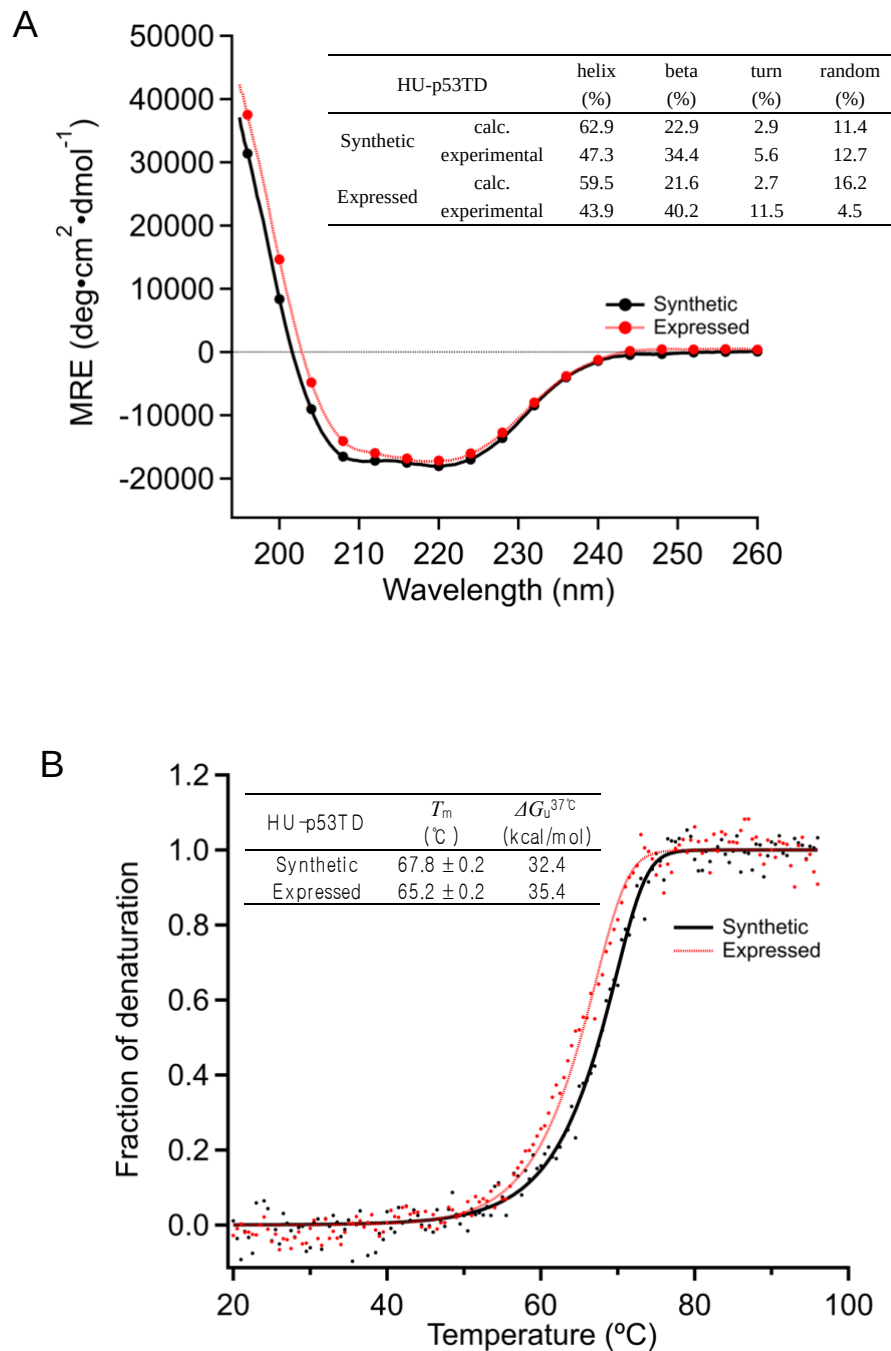


Figure 3-2. CD spectra and thermal denaturation curves of the synthetic and the expressed HU-p53TD peptides.

(A) The secondary structure of the HU-p53TD peptides were measured by CD spectrometry at 4°C. The percentage of secondary structural elements were calculated. (B) The signals from the HU-p53TDs (synthetic and expressed in *E.coli*) were monitored by CD spectrometry at 222 nm between 4°C and 96°C. The fraction of denaturation at each temperature was plotted to generate the denaturation curves.

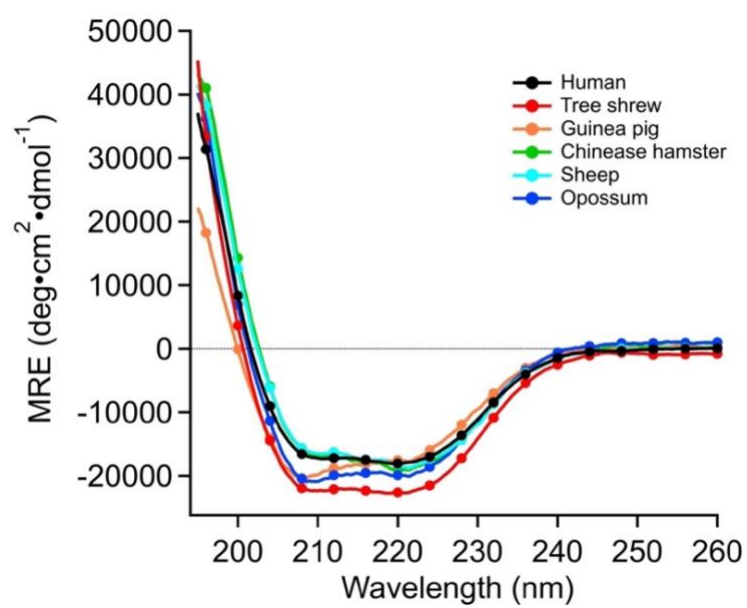


Figure 3-3. CD spectra of the mammalian p53TDs.

The secondary structure of the mammals p53TD were measured by CD spectrometry at 4°C.

Table 3-1 Secondary structural elements present in peptides from CD analysis.

Peptides	helix (%)	beta (%)	turn (%)	random (%)
Human (calc.)*	62.9	22.9	2.9	11.4
Human	47.3	34.4	5.6	12.7
Tree shrew	64.2	23.7	0.0	12.1
Guinea pig	47.9	23.8	0.0	28.3
Chinese hamster	50.5	33.8	13.5	2.2
Sheep	47.0	38.8	8.8	5.3
Opossum	62.0	12.7	14.7	10.7

*The contents were calculated based on the reported structure [23].

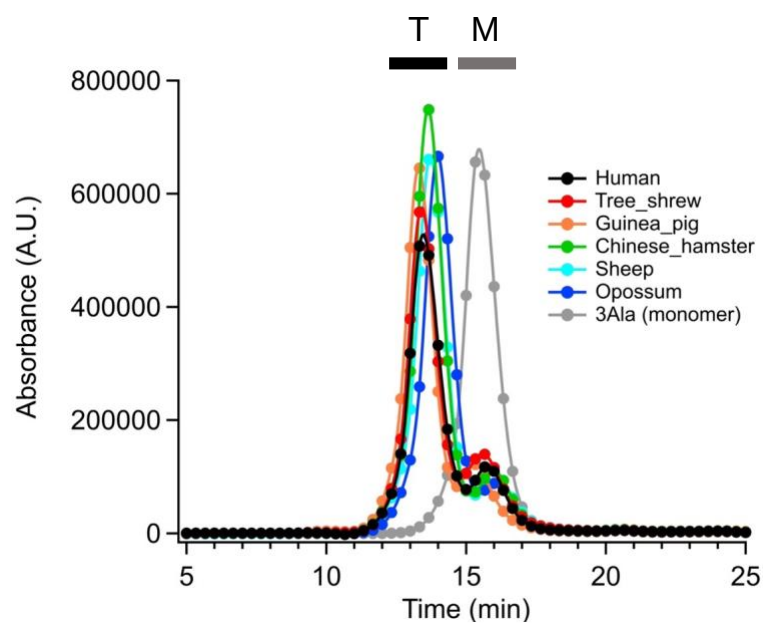


Figure 3-4. Gel filtration of mammals p53TD.

The oligomeric states of the mammalian p53TDs and 3Ala (monomer mutant) were measured by gel filtration chromatography. The black and gray bar represented the tetramer (T) and monomer (M) fraction, respectively [21]. The UV absorbance was monitored at 214 nm.

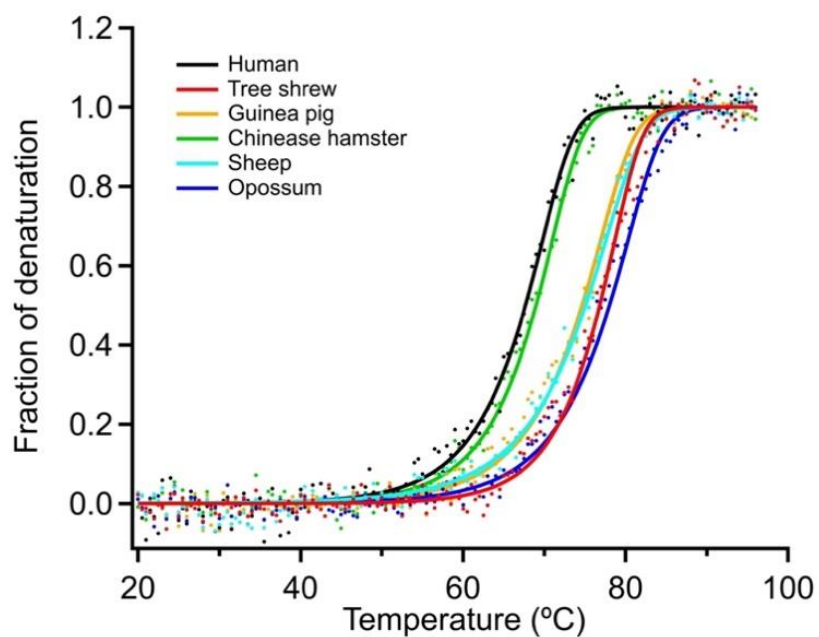


Figure 3-5. Thermal denaturation curves of the mammalian p53TDs.

The signals from the mammalian p53TDs were monitored by CD spectrometry at 222 nm between 4°C and 96°C. The fraction of denaturation at each temperature was plotted to generate the denaturation curves.

Table 3-2. Thermodynamic parameters of tetramerization domain peptides.

Peptides	T_m (°C)	ΔT_m (°C)	$\Delta G_u^{37^\circ\text{C}}$ (kcal/mol)	$\Delta\Delta G_u^{37^\circ\text{C}}$ (kcal/mol)
Human	67.8 ± 0.2	-	32.4	-
Tree shrew	77.1 ± 0.2	9.3	36.4	4.0
Guinea pig	74.7 ± 0.5	6.9	35.4	3.0
Chinese hamster	69.0 ± 0.2	1.2	35.4	3.0
Sheep	75.1 ± 0.4	7.3	34.0	1.6
Opossum	78.0 ± 0.2	10.2	36.6	4.2

T_m (transition temperature) and $\Delta G_u^{37^\circ\text{C}}$ (variation in the free energy of unfolding at 37°C) were calculated by denaturation analysis. The standard errors of fittings were indicated. $\Delta\Delta G_u^{37^\circ\text{C}} = (\Delta G_u^{37^\circ\text{C}} \text{ of each species}) - (\Delta G_u^{37^\circ\text{C}} \text{ of Human})$

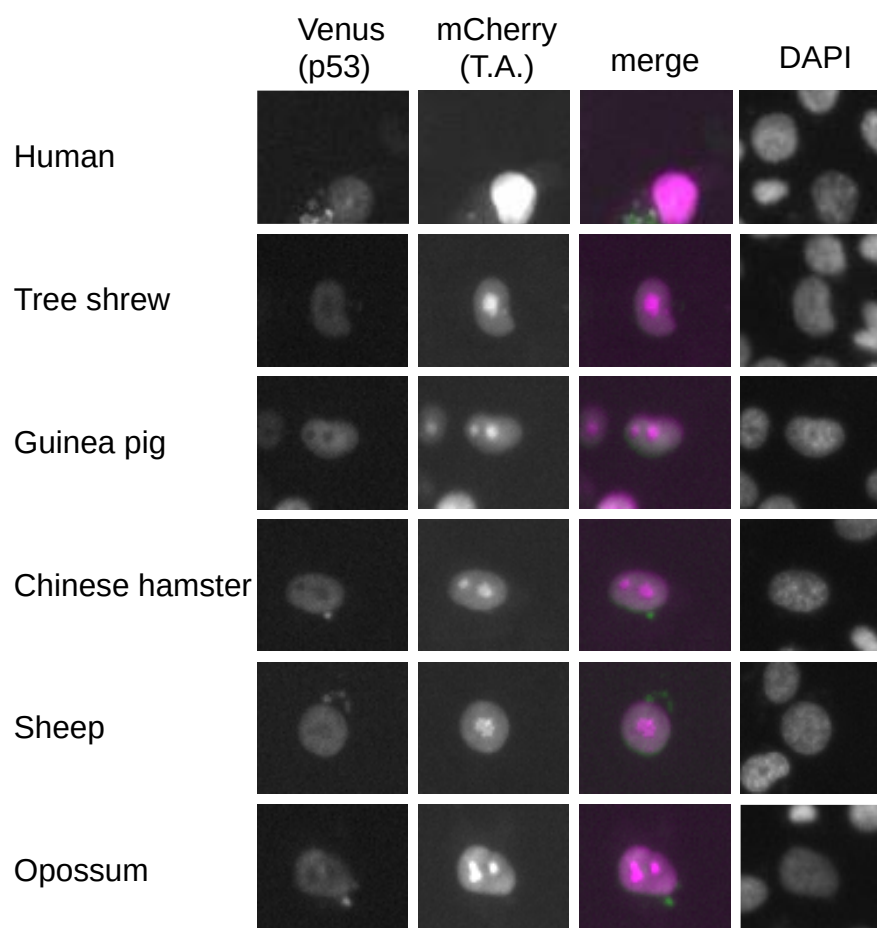


Figure 3-6. Fluorescence images of the reporter assay.

Chimeric p53 protein (Venus), p53-dependent transcriptional activity (mCherry), merged images of Venus (green) and mCherry (magenta), and DAPI staining were shown.

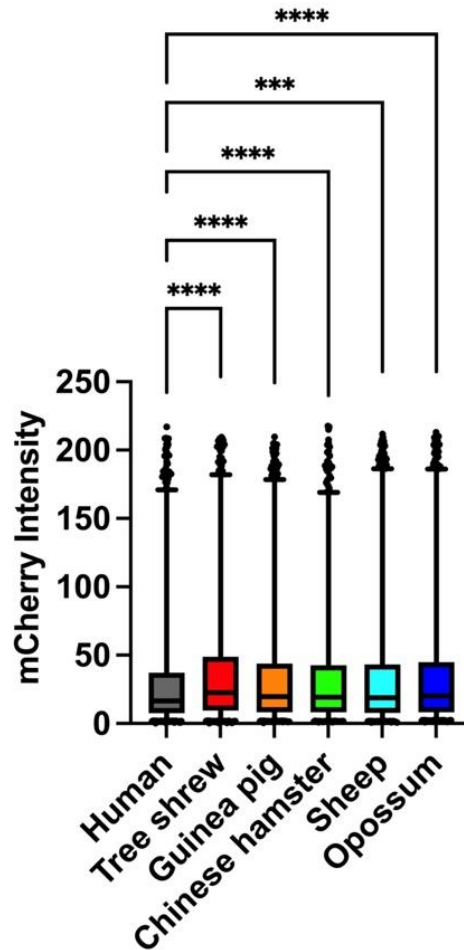


Figure 3-7. Transcriptional activity of the chimeric human p53 protein substituted with the TDs from the other mammalian p53 proteins in single cells.

The proteins were expressed at the physiological protein level corresponding to levels of the human p53 in cells. The mCherry (p53-dependent transcription) fluorescence signals in each single cell were quantified. Boxes and whiskers include the values between the 25th and 75th and 1st and 99th percentiles, respectively. More than 3,000 cells were analyzed under each condition from three independent experiments. Significance was analyzed by using Kruskal-Wallis test. *** $p < 0.0005$, **** $p < 0.0001$.

Table 3-3 Data collection and refinement statistics for p53TDs

Dataset	HU-p53TD	OP-p53TD	TS-p53TD
Data Collection			
Beamline	ID7B2, CHESS	ID7B2, CHESS	ID7B2, CHESS
Wavelength (Å)	0.9686	1.0000	1.0000
Space group	P 21 21 21	P 21 21 21	C 2 2 2
Unit cell parameters			
a, b, c (Å)	30.973, 56.437, 61.656	38.275, 50.887, 60.240	62.871, 65.806, 32.511
α, β, γ (°)	90, 90, 90	90 90 90	90 90 90
Resolution range (Å)	41.63 – 1.22 (1.264 – 1.22)	32.31 – 1.35 (1.398 – 1.35)	45.46 - 1.16 (1.201 - 1.16)
No. of unique reflections	30235 (1953)	24100 (1080)	21822 (1027)
Multiplicity	5.5 (2.4)	5.6 (1.9)	9.9 (2.8)
Completeness (%)	91.77 (60.35)	90.51 (39.14)	91.70 (43.72)
R _{merge}	0.04487 (0.2466)	0.08437(.9251)	0.04206 (0.3908)
CC _{merge}	1.0 (0.959)	0.999 (0.662)	1.0 (0.84)
I/σ(I)	19.68 (2.06)	12.46 (0.51)	28.35 (1.62)
Refinement Statistics			
Resolution (Å)	41.63 – 1.22	32.31 – 1.35	45.46 - 1.16
Reflections (total/test) ^a	30234 (1992)	24002 (1992)	21817 (2001)
R _{work} /R _{free} (%)	0.1464/0.1764	0.1711/0.2025	0.1736/0.1915
CC _{work}	0.967 (0.937)	0.964 (0.474)	0.947 (0.891)
CC _{free}	0.967 (0.804)	0.956 (0.395)	0.942 (0.861)
No. of atoms (excluding hydrogens)			
Protein	1155	1107	536
Water	147	133	99
B factors			
Protein	16.87	21.59	16.55
Water	29.03	32.73	28.90
Root-mean-square deviation			
Bond length (Å)	0.022	0.011	0.009
Bond angle (°)	1.74	1.11	1.09
Ramachandran (%)^b			
Favored	98.39	100.00	100.00
Outliers	0.00	0.00	0.00

Values in parentheses are for highest-resolution shell. $R_{\text{sym}} = \frac{\sum_{\mathbf{hkl}} \sum_i |I_{\mathbf{hkl},i} - \langle I_{\mathbf{hkl}} \rangle|}{\sum_{\mathbf{hkl}} I_{\mathbf{hkl}}}$, where $I_{\mathbf{hkl},i}$ is the intensity of an individual measurement of the reflection with Miller indices $\mathbf{hkl}$ and $I_{\mathbf{hkl}}$ is the mean intensity of the reflection. $R_{\text{work}} = \frac{\sum_{\mathbf{hkl}} ||F_o| - |F_c||}{\sum_{\mathbf{hkl}} |F_o|}$, where $|F_o|$ is the observed structure-factor amplitude and $|F_c|$ is the calculated structure-factor amplitude. R_{free} is the [R factor](#) based on at least 500 test reflections that were excluded from the refinements. CLS (Canadian Light Source) and CHESS (Cornell High Energy Synchrotron Source).

^a Reflection for $F_o > 0$. ^b MolProbity analysis.

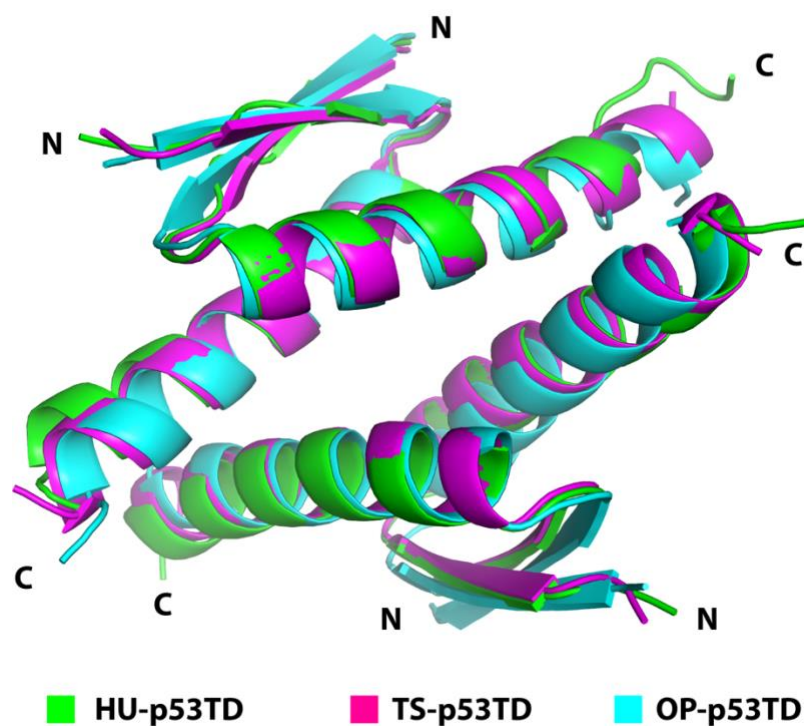


Figure 3-8. Overlay of the crystal structures of HU-p53TD (Green), TS-p53TD (Magenta) and OP-p53TD (Aqua).

The structures are shown as cartoon representations and are overlaid based on the Ca positions of the peptide backbone. The N-terminal (N) and the C-terminal (C) of each monomer is indicated for clarity. The root mean square deviation (rmsd) for the Ca positions of the peptide backbone are 0.39 Å between the HU-p53TD and the TS-p53TD, 0.95 Å between the HU-p53TD and the OP-p53TD and 0.75 Å between the TS-p53TD and the OP-p53TD.

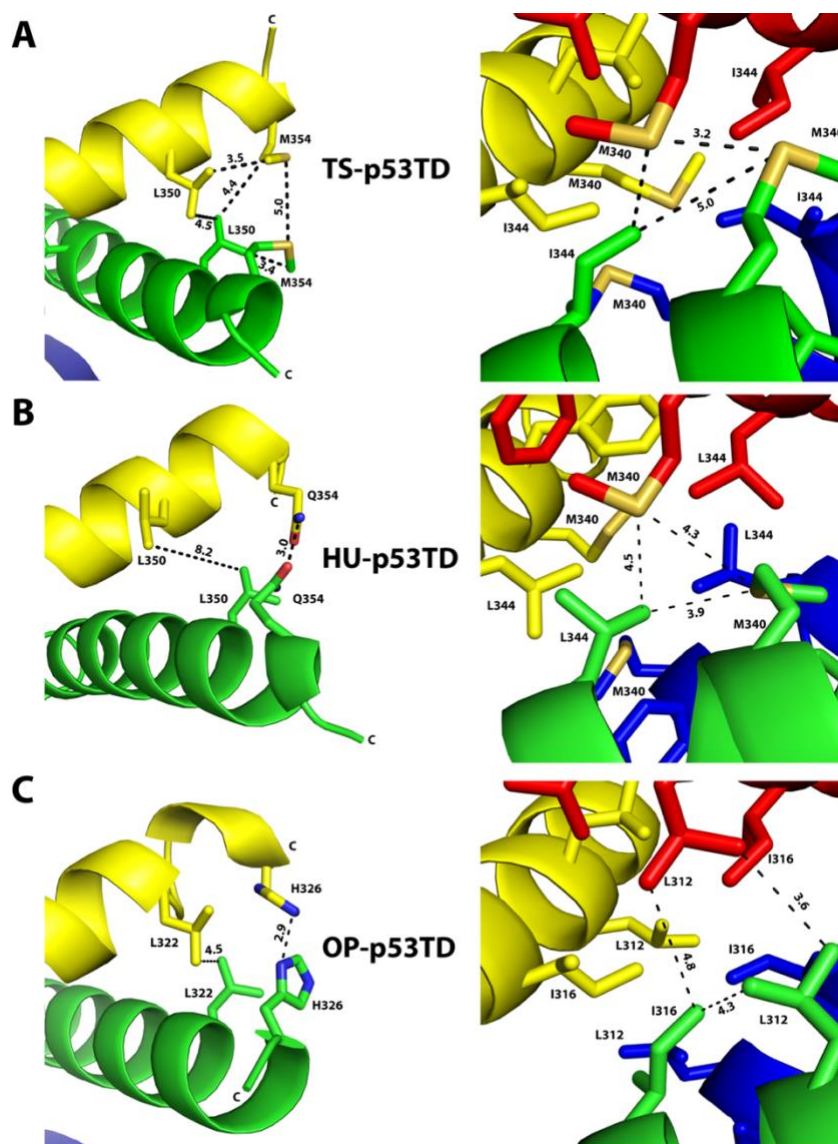


Figure 3-9. Key differences in the dimeric and tetrameric interfaces of the TS-p53TD and OP-p53TD that enhance their thermostability in comparison to the HU-p53TD.

Close up and metrics (dashed distance lines between residues in Å) of the TS-p53TD (A), HU-p53TD (B) and OP-p53TD (C) highlighting the differences in the C-terminal regions of dimers (left panel) and the hydrophobic core of the tetrameric interfaces (right panel). The three structures are shown as cartoons with each monomer in a unique color (red, blue, yellow and green) and the interacting amino acids associated with the key differences as stick conformations. The N-terminal (N) and the C-terminal (C) of each monomer is indicated for clarity when possible, and the side chains are color coded based on the atom type (S-yellow, O-red and N-blue).

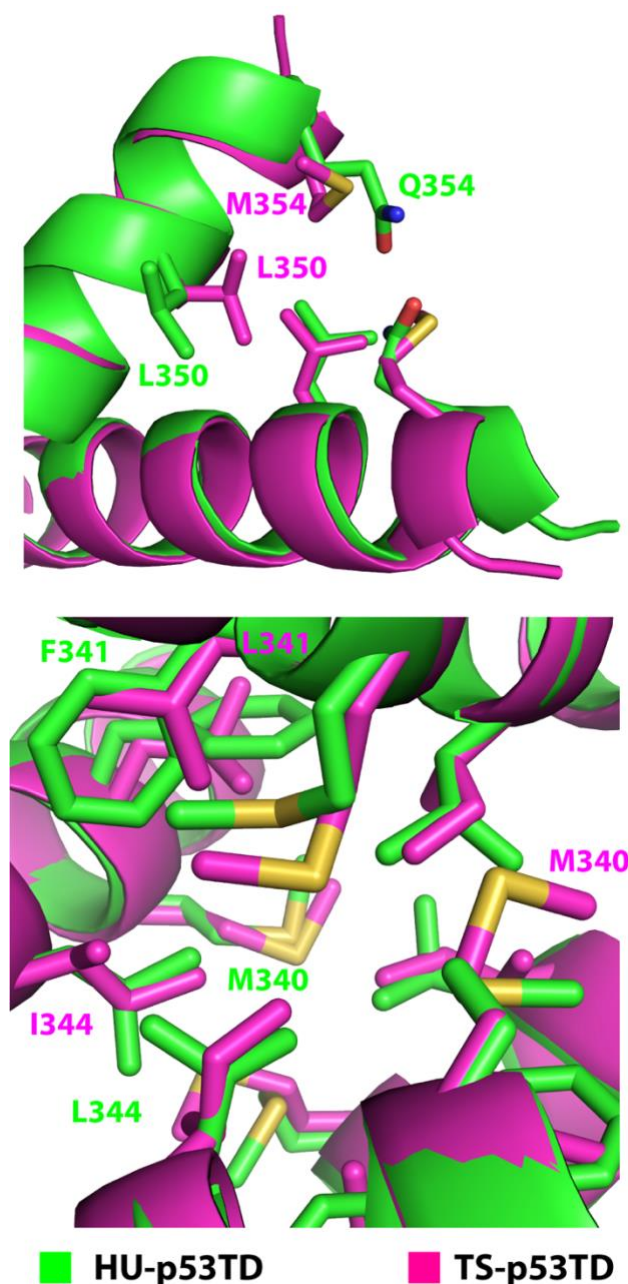
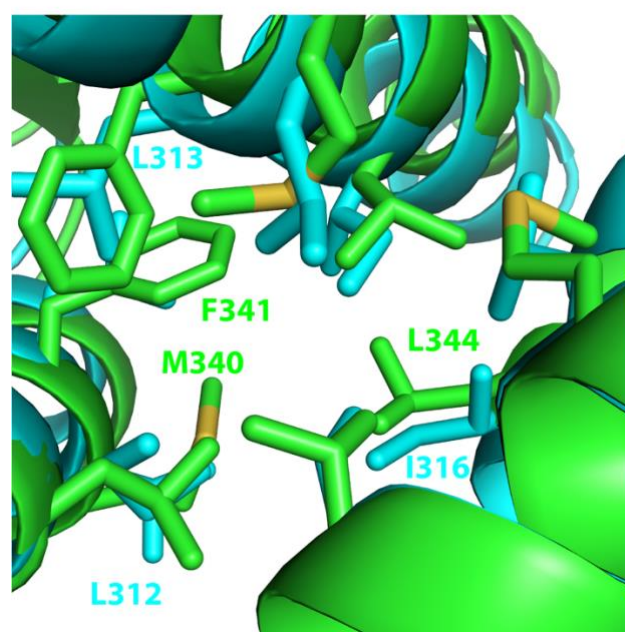
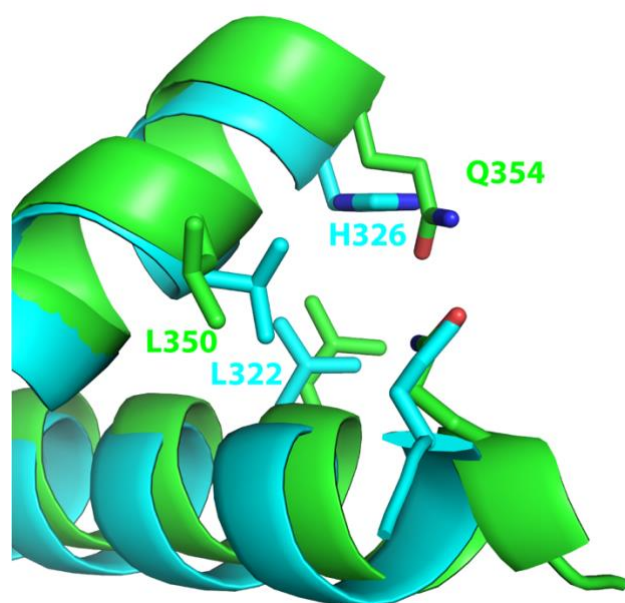


Figure 3-10. Overlay of the regions containing the key structural differences between the HU-p53TD (green) and the TS-p53TD (Magenta).
 The upper panel shows a zoom of the C-terminal end of the α-helix highlighting the side chains of residues L350 and Q354 of HU-p53TD (green) and the corresponding L350 and M354 of TS-p53TD (magenta). The lower panel shows the zoom of the central region of the α-helix highlighting the side chains of residues M340, F341 and L344 of HU-p53TD (green) and the corresponding M340, L341 and I344 of TS-p53TD (magenta).



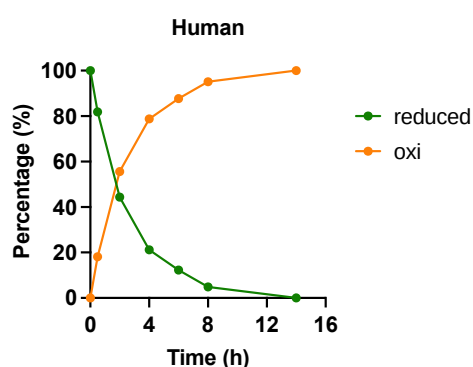
■ HU-p53TD

■ OP-p53TD

Figure 3-11. Overlay of the regions containing the key structural differences between the HU-p53TD (Green) and the OP-p53TD (Aqua).

The upper panel shows a zoom of the C-terminal end of the α -helix highlighting the side chains of residues L350 and Q354 of HU-p53TD (Green) and the corresponding L322 and H326 of OP-p53TD (Aqua). The lower panel shows the zoom of the central region of the α -helix highlighting the side chains of residues M340, F341 and L344 of HU-p53TD (Green) and the corresponding L312, L313 and I316 of OP-p53TD (Aqua)

A



B

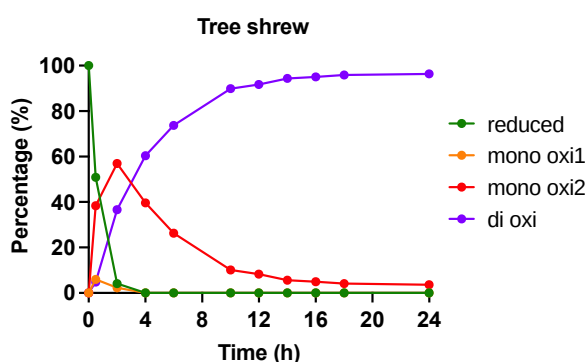


Figure 3-12. Oxidation of the mammalian p53TDs (A: HU-p53TD, B: TS-p53TD) by hydrogen peroxide.

The peptide solution was incubated in the presence of hydrogen peroxide. The reaction mixture of each indicated time point was analyzed by HPLC and observed peak areas corresponding to each oxidation status, which were determined by MALDI-TOF MS (Table 3-3), were plotted.

Table 3-3. Molecular wight of the p53TD peptides after oxidation reaction.

	MS+1(324-358)*	MS+1(336-342)**
reduced	4231.16	980.84
mono oxi1	4247.01	996.82
mono oxi2	4247.24	980.84
di oxi	4262.91	996.82

TS-p53TD peptide was incubated in the hydrogen peroxide. *Molecular weight of the TS-p53TD peptide after oxidation reaction. **Molecular weight of the fragment of p53TD, (residue from 336 to 342) was analyzed using MALDI-TOF MS from the fraction eluted from reverse-phase HPLC of the oxidation reaction.

3.4. Discussion

Amongst the different mammalian species analyzed in this study, the opossum, as a member of the marsupial family, is the most evolutionarily ancient species, whereas the tree shrew, as a member of the Scandentia family, is evolutionarily the closest to humans. Despite these two species being the farthest and nearest to humans in terms of evolution, the TDs from these two species displayed the most significant enhancements in thermostability and ability to activate transcription relative to that of the HU-p53TD. Based on the crystal structures of the OP-p53TD and TS-p53TD, the structural changes that lead to their enhanced thermostability appear to be centered in the same two regions of their sequence. The first region is centered around the C-terminal of the α -helix and this leads to an enhanced stabilization of the dimers, and the second region is centered around residues in the central area of the α -helix that form the hydrophobic core to stabilize the tetrameric structure. These two major differences are consistent with the fact that, in the amino acid sequences of the different mammalian p53TDs, the sequences of the α -helical region are considerably more diversified than the sequences of the more highly conserved β -sheet region.

Our analysis of the crystal structures of the TS-p53TD and the OP-p53TD indicates that the key residues in the C-terminal region that help to enhance the stability of the dimers are M354 in the TS-p53TD and H326 in the OP-p53TD, which corresponds to Q354 in the HU-p53TD. In both the TS-p53TD and OP-p53TD, the side chains of M354 or H326 form key interactions with themselves as well as with a neighboring Leu residue (L350 and L332 in tree shrew and opossum, respectively) located four residues in front

of them in their sequences. This enables that Leu residue from one monomer to form hydrophobic contacts with the same Leu residue on the other monomer of the dimer. In the HU-p53TD, Q354 is not able to stabilize the hydrophobic contacts between the two Leu350 residues of the dimer and this results in a decrease in the thermostability of the HU-p53TD relative to the TS-p53TD and OP-p53TD. In the central region of the α -helix, the hydrophobic core is formed by the side chains of the residues F328, L330, I332, F338, M340, F341, L344, A347, and L348 [46,47]. Within these residues that make up the hydrophobic core, M340, F341, and L344 in the HU-p53TD appear to be more commonly substituted for other amino acids in the sequences of other mammalian p53TDs. These three residues in the HU-p53TD correspond to L312, L313, and I317 in the OP-p53TD, and M340, L341, and I344 in the TS-p53TD, respectively. In comparing the three sequences, the biggest difference appears to be the presence of the aromatic F341 in the HU-p53TD relative to the aliphatic Leu residue in the OP-p53TD (L313) and the TS-p53TD (L341). Our analysis of the three crystal structures showed that the OP-p53TD and TS-p53TD form a more compact hydrophobic core relative to what is observed for the HU-p53TD. In the TS-p53TD, this results in the sulfur atoms of M340 being closer to each other as well as to the nearest methyl group of I344 than they are in the HU-p53TD. This more compact hydrophobic core is also supported by the fact that the oxidation rate of M340 was slower for the TS-p53TD in comparison with the HU-p53TD. Overall, this suggests that replacing the aromatic F341 residue in the hydrophobic core with a hydrophobic aliphatic amino acid increases the stability of the TS-p53TD and OP-p53TD.

The role of aromatic F341 in destabilizing the core of the HU-p53TD relative to the OP-p53TD and TS-p53TD is interesting in light of previous studies examining the role of the three different Phe residues in the stability of the HU-p53TD. In this study, the aliphatic cyclohexylalanine group was substituted in place of F328, F338, and F341 in the HU-p53TD. When this hydrophobic aliphatic group was substituted for either F328 or F338, a decrease in the thermostability of the HU-p53TD was observed, suggesting that it was important to have the aromatic residue in these positions. In contrast, substituting the aliphatic cyclohexylalanine group for F341 of the HU-p53TD lead to a significant enhancement in the thermostability from a T_m of 67.8 °C to a T_m of more than 90 °C [48]. These results suggest that the substitution of an aliphatic group at the positions corresponding to the aromatic F328 and F338 of the HU-p53TD significantly decreases the thermostability of the p53TD, but the substitution of the aliphatic hydrophobic group in place of an aromatic F341 of the HU-p53TD leads to a significant enhancement. These results support the conclusion that substitution of the aromatic F341 by an aliphatic hydrophobic amino acid in the TS-p53TD and OP-p53TD is in part responsible for their enhanced thermostability relative to the HU-p53TD.

Based on the conclusions that differences in two regions make important contributions to the overall stability of the p53TD, these regions were examined for the three remaining species evaluated as part of this study. The CH-p53TD sequence contains the same amino acid residues in the positions corresponding to Q354 at the C-terminal as well as M340, F341, and L344 in the center of the α -helix of the HU-p53TD. This is consistent with our results showing that the CH-p53TD has nearly the same

thermostability as the HU-p53TD. In contrast, the GP-p53TD has three aliphatic residues (Ile, Leu, and Leu) in the central region of the α -helix, like the TS-p53TD and the OP-p53TD, but a Gln residue in the position of Q354 in the C-terminal helical region, like the HU-p53TD. Thus, there should be enhanced stability in the core region of the tetramer due to the replacement of the aromatic F341 residue, but no enhanced stability in the C-terminal region of the helix relative to the HU-p53TD. Again, this is consistent with our results which demonstrated that the GP-p53TD has an intermediate thermostability between what is observed for the HU-p53TD and what is observed for the TS-p53TD and OP-p53TD. The last sequence to compare is that of the SH-p53TD, which is the same as the HU-p53TD and CH-p53TD in both regions. However, the SH-p53TD possesses an intermediate thermostability relative to the HU-p53TD and the TS-p53TD/OP-p53TD. Surprisingly, the HU-p53TD and SH-p53TD share 91.4% of their sequence identities and the only two differences occur at E336 and K357 of the HU-p53TD, which correspond to K325 and R346, respectively, in the SH-p53TD. Given that the K357/R346 residue is outside of the structured region of the p53TD, this indicates that E-to-K substitution at position 336 in the HU-p53TD (position 325 in SH-p53TD) must be responsible for the intermediate thermostability observed for the SH-p53TD.

In conclusion, the results demonstrate that the biophysical properties of the different mammalian p53TDs can be altered fairly dramatically by rather subtle changes in the amino acid sequence, and this could lead to important functional differences for their respective p53 proteins. In tree shrew, the average body temperature is approximately the same as for humans, but the circadian range of body temperature differs by two-fold [49].

Mitochondrial UCP1-mediated thermogenesis is important for regulating body temperature, and radical oxygen species (ROSs) are produced during this process[50]. This suggests that tree shrews are more likely to be exposed to ROS-mediated oxidative stress than humans due to heat production associated with circadian fluctuations. To increase its resistance to ROS-induced oxidative stress, the TS-p53TD appears to have undergone a structural evolution to become more thermostable, which may be advantageous to its survival through changes in the activity of its p53 proteins. In the case of opossums, they are marsupial, and unlike humans and other placentals, they have their offspring immature, which grow in their pouches by feeding on their mother's milk. Since these immature progenies are exposed to various stresses early in life, including UV radiation [44,51], an enhanced stability of the p53 protein may have been required for a more optimal tumor suppressor function. In humans, a number of tumors have been reported that contain single point missense mutations within the p53TD. In many cases, these point mutations lead to the substitution of an amino acid residue in the hydrophobic core of the tetramer, which results in the destabilization of the tetrameric structure and, in many cases, the complete loss of the proteins' functions [21,44]. However, our results indicate that mutations that lead to subtle amino acid substitutions in the p53TD could also lead to an enhancement in the overall stability and transcriptional activity of the p53 protein. This could result in an increase in function that could either be beneficial or detrimental to the organism. Future studies will be needed to identify mutations within the p53TD that may have resulted in enhanced activity of the p53 protein as opposed to mutations that result in the loss of activity.

3.5. References

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4. Homo- and hetero-oligomerization of vertebrate p53 and p63

4.1. Abstract

The primary function of the p53 family proteins, p63 and p73, are in development and differentiation, which is distinct from p53, which functions as a tumor suppressor. In all cases, homotetramerization is essential for the functional properties of these p53 family proteins and in all cases this requires a tetramerization domain (TD) that is present just C-terminal to the DNA-binding domain. In human p53, the TD consists of a bba fold whereas the TDs of p63 and p73 contain a second helix in a bbaa fold. Interestingly, it has been previously reported that the p53TD of Zebrafish, a ray-finned fish, has a second helix and a bbaa fold similar to TDs of Human p63 and p73, which indicates that the p53TD has undergone key structural changes during evolution. However, the details of the effect of this structural change on the function of p53TD are not clear. In this study, I have analyzed the thermostability, structures and the ability to form heteromultimers of the TDs of p53 and p63 from different species that appeared at different times along the evolution scale. The results showed that sharing high sequence homology, the thermal stability of the p63TDs from vertebrates varied greatly between the different species. X-ray crystallography revealed that there is a unique disulfide bond present in the structure of Zebrafish p63TD that forms across the monomers that form the two dimers of the tetramer and this is most likely present in the Gar and Lamprey p63TD. Furthermore, we analyzed the hetero-multimeric formation ability of p53TD and p63TD, and found that the hetero-multimeric formation ability of the early vertebrate Lamprey was lost during

both the evolution to fish and the evolution to terrestrial organisms. Based on these results, we propose a novel model in which the p53 family proteins lost their capacity to form heteromultimers by increasing the stability of their TDs through structural changes and in this process the p53 protein acquired its tumor suppressor specific functions.

4.2. Introduction

The tumor suppressor protein p53 is a key transcriptional regulatory protein and protein interactome studies have demonstrated that it forms interactions with multiple different partner proteins making it one of the cells key “hub” proteins [1–3]. The p53 protein is the founding member of a family of related transcription factors that also includes the related protein p63 and p73 [4,5]. The p53-family proteins are grouped as a family since they share many common structural features such as a transcription activation domain (TAD) at the N-terminal of their proteins, followed by a proline-rich domain (PRD), a central core DNA-binding domain (DBD), and a tetramerization domain. However, the three proteins differ from each other in their C-terminal regions of the proteins. In the case of p53, the C-terminal region is a disordered transcriptional regulator region with multiple sites of posttranslational modification, whereas p63 and p73 contain a sterile-alpha motif (SAM) as well as a transcription inhibition domain.

To carry out many of its tumor suppressor functions, p53 must adopt a homo-tetrameric structure which occurs when its cellular levels increase in response to diverse cellular stresses such as UV light and oxidative stress. In the tetrameric form, p53 binds to consensus DNA-binding sites in the promoter regions of genes, which enables it to carry out key functions, including inducing apoptosis and cell cycle arrest via proteins such as p21 and Bax, and maintaining genome integrity. Like p53, p63 and p73 also form homo-tetramers and as is the case with p53 their tetrameric form is required to carry out most of their essential functions. In the case of p53, the TD monomer is composed of a

β -sheet, a turn, and an α -helix forming a dimer, and two dimers forming a tetramer via a central 4-helix bundle. Like p53, the TD of p63 and p73 are also composed of a β -sheet, a turn, and an α -helix, but they also contain a second short α -helix at the C-terminal end of the domain. Interestingly, p63 and p73 are also able to form hetero-tetramers with each other and these p63/p73 hetero-tetramers can also play key functional roles associated with these proteins [6–8]. The heterotetramers of Human p63/p73 appear to consist of dimers of dimers so that a homodimer of Human p63 and a homodimer of Human p73, come together to form the heterotetramer. However, there is still some debate as to whether other combinations of the monomers of p63 and p73 in the heterotetramer are possible and whether or not the ratios of p63 and p73 in the heterotetramer can vary in different species. In contrast, there is currently no evidence that p53 can form heterotetramers with either p63 or p73 in either Humans or any other species. Therefore, it is thought that the presence of this second helix of p63 and p73 is the reason why they have the ability to form heterotetramers, but the precise mechanistic details as to why this is the case are not yet clear.

The common ancestor of the p53-family proteins is thought to have been first expressed in the invertebrate sea anemone, which occurred roughly 550 million years ago[9]. The Cambrian explosion that led to the evolution to vertebrates occurred about 540 million years ago and in the genome of Lamprey, the first vertebrate, there are three different paralogs of p53. Subsequently, through the acquisition of new functions to adapt to environmental changes via genetic mutations, vertebrates evolved into eight different orders: agnatha, chondrichthyans, ray-finned fish, lobe-finned fish, amphibians, reptiles,

ornithopods, and mammals. The appearance of the three distinct p53-family proteins found in Humans (p53, p63, and p73) are first found in the genome of different species of ray-finned fish and bony fish. The separation into the three distinct proteins with varying functions is thought to be due to the evolution of vertebrates from terrestrial animals to aquatic species, which occurred in two evolutionary stages.

Protein evolution proceeds through mutations in the bases of key genes within an organism and the accompanying changes in amino acid composition that occur in the proteins that are expressed from these genes [10]. Currently, the neutral theory is the dominant theory in the molecular evolution of organisms [11,12]. This theory is predicated on the theory that most genetic changes linked to the evolution of organisms are neutral, which means that they are neither advantageous nor disadvantageous for survival of the organism, but they accumulate in a population due to genetic flotation, which is an accidental factor and is considered to be the main factor in evolution. Recently, our laboratory conducted a phylogenetic tree analysis for each of the domains found in the p53 and p63 proteins and determined that the DNA-binding domain (DBD) of p53 and the tetramerization domain (TD) of p63 have matured in the course of evolution, whereas the TD of p53 has diversified with time. This suggests that mutations in the coding region for the TD of p63 are likely to be detrimental to survival of the organism, whereas mutations in the coding region for the TD of p53 will be more neutral in terms of organism survival. This analysis is consistent with the fact that the TD of Zebrafish p53 protein, a member of the ray-finned fish class commonly used as a model organism for vertebrate embryology and genetics, contains two helices [13]. The presence of the

two helices in the Zebrafish p53TD is more similar to what is found in the TDs from the Human p63 and p73 proteins as opposed to the one helix found in the Human p53TD. Taken together, this suggests that the structure of the p53 TD has undergone major changes through the course of its evolution. However, the interactions of the p53 protein with the other family proteins and the evolution of p63 due to structural changes in p53 are not clear. In this study, I have analyzed the thermostability and performed structural analysis on peptides corresponding to the TDs from p53 and p63 protein from nine different species along the evolutionary process. In addition, I have examined the ability of these p53 and p63 TDs from different species to form a hetero-tetramers, and based on our results we propose a novel model by which p63 has acquired a p53-specific tumor suppressive function.

4.3. Materials and Methods

4.3.1. Synthesis of p53TD and p63TD peptides

The peptides corresponding to the human p63TD (residues 397–444 of human p63, Figure 4-1), the Softshell turtle p63TD (residues 288–335 of Softshell turtle p63), the Frog p63TD (residues 298–346 of Frog p63), the Coelacanth p63TD (residues 296–343 of Coelacanth p63), the Catfish p63TD (residues 300–347 of Catfish p63), the Zebrafish p63TD (residues 298–345 of Zebrafish p63), the Gar p63TD (residues 397–444 of Gar p63), the Ghost shark p63TD (residues 421–468 of Ghost shark p63) and the Lamprey p53TD (residues 474–523 of Lamprey p63) were synthesized as previously described. The peptide sequences are shown in Figure 4-2. The synthesized peptides were deprotected and cleaved from the resin by using Reagent K (TFA:H₂O:thioanisole:ethanedithiol:phenol = 82.5:5:5:2.5:5). The peptides were all purified to homogeneity using reverse-phase HPLC over a C8 column (Vydac, Hesperia, CA, USA) and the correct molecular weight of each peptide was verified by MALDI-TOF-MS analysis. Peptide concentrations were determined using an extinction coefficient, $\epsilon_{280} = 1,490 \text{ M}^{-1} \text{ cm}^{-1}$, which corresponds to a single Tyr residue present in all of the p53 proteins.

4.3.2. Determination of oligomeric state, secondary structure, and thermal stability

To verify that the p53TD peptides from the different mammalian species form tetrameric structures, the peptides were analyzed by gel filtration chromatography on a

Superdex 75 (GE Healthcare, Chicago, IL, USA) column. For this analysis, 20 μ L of each peptide at 100 μ M concentration in 50 mM phosphate buffer, pH 7.5 with 100 mM NaCl, was injected onto the column and eluted at room temperature using a flow rate of 0.1 mL/min with UV-monitoring at 214 nm.

To determine the secondary structure content of the different p53TD peptides, circular dichroism (CD) spectra at a peptide concentration of 10 μ M in a buffer consisting of 50 mM sodium phosphate, pH 7.5 with 100 mM NaCl, were recorded at 4 °C on a Jasco J-805s spectropolarimeter in a 1 mm quartz cell. Scans were recorded continuously over the region between 195 and 260 nm at a rate of 50 nm/min and a bandwidth of 0.2 nm. For the thermal denaturation assay, CD spectra were recorded between 4 °C and 96 °C and the temperature was increased at a fixed rate of 1.0 °C/min. Ellipticity was measured at 222 nm for monitoring the α -helical content of the peptides. The thermodynamic parameters of the peptides were determined as described previously [14].

4.3.3. Avidin Biotin pulldown assay for in vitro heterotetramer formation

For the in vitro avidin-biotin pulldown experiments to identify heterotetramer formation, biotin labeled versions of the p53TD from the tested species (Lamprey, Gar, Zebrafish, Coelacanth, and Human) were mixed with their corresponding non-biotinylated p63TD peptides. To prepare the biotinylated p53TD peptides, biotin was conjugated on resin at the N-terminus of the peptide with two residues of aminocaproic acid (Aca) residues as a linker by regular solid-phase peptide synthesis. The Biotin-(Aca)₂-p53TD and p63 peptides were then dialyzed into a binding buffer of 50 mM Na

phosphate buffer (pH 7.5) 100 mM NaCl. For those peptides containing a Cys residue in their sequence 1 mM TCEP was also added to the buffer. For the binding reaction, 40 μ L of NeutrAvidinTM UltraLinkTM Resin (Thermo SCIENTIFIC) beads (50% slurry) were placed in a microcentrifuge tube and centrifuged to remove the supernatant. Then, 40 μ L of the binding buffer was added and centrifuged to remove the supernatant. This procedure was repeated twice to buffer exchange the beads for the binding reaction. For the binding reaction, the Bio-(Aca)₂-p53TD peptides from Lamprey, Gar, Zebrafish, Coelacanth, and Human peptides were added at a fixed concentration of 10 μ M to varying concentrations (10 μ M, 20 μ M, and 40 μ M). of the corresponding p63TD from the same species. The mixed solution was boiled at 100°C for 5 min and first cooled at room temperature for 1 h and then at 4°C for an additional 1 h. Then, 40 μ L of buffer-exchanged beads and 60 μ L of buffer were added and incubated at 4°C for 2 h to bind Bio-(Aca)₂-p53Tet to the Avidin beads. The supernatant was removed by centrifugation, washed with 200 μ L of binding buffer, and then washed a second time with H₂O. Next, 200 μ L of acetic acid was added and incubated at 4°C for 2 h to elute the bound peptides from the avidin beads. After centrifugation, 125 μ L of the supernatant was mixed with buffer A (0.05% TFA/H₂O) to make a 525 μ L solution. If the sequence contained Cys, TCEP was added to a final concentration of 1 mM. The solution was then analyzed by reverse-phase HPLC (SHISEIDO Proteonavi Cat No. 80205) to determine the amount of each peptide that bound to the resin. The results were expressed as a ratio of p63TD pulled down with respect to the amount of biotinylated p53TD.

4.3.4. Peptide Expression and Purification in *E. coli* cells

For the expression of peptides in *E. coli*, the cells were first grown for overnight (16 h) in 2 L of LB with ampicillin (100 mg/L) at 37°C, and this culture was diluted the following morning with 6 L of LB with ampicillin and induced for 4 h at 30°C following the addition of IPTG (125 mg/L). After 4 h of induction, the cells were harvested by centrifugation and re-suspended in lysis buffer. The cells were lysed using a French press and centrifuged at 105,000g for 30 min at 4°C. The resulting supernatant was incubated in the presence of glutathione resin (GE Healthcare) for 1 min at 4°C. Afterwards, the supernatant was removed by centrifugation 3000g for 5 minutes and washed three times with TEV buffer (25 mM Na phosphate, 125 mM NaCl, 5 mM DTT). The GST-tag was removed by the overnight incubation at room temperature in the presence of 100 units of TEV protease. The solution was then centrifuged at 3000g for 5 min and the supernatant was applied to an ion exchange column (buffer A: 20 mM Tris, 1 mM DTT, pH7.2; buffer B: 20 mM Tris, 1 mM DTT, 1 M NaCl, pH7.2). SP Sepharose or Q Sepharose column (GE Healthcare) were used. The fractions from the ion exchange column containing p53TD and p63TD proteins were pooled and dialyzed into 20 mM Tris, pH 7.5, 1 mM DTT for subsequent purification by gel filtration on a SuperoseTM 12 (GE Healthcare).

4.3.5. Crystallization and Structure Determination

Crystals of the p53TD and p63TD were grown by the vapor diffusion method at 20°C using either 1:1 or 2:1 mixtures of peptide solution (4.0 – 8.0 mg/mL) and precipitant buffer. The crystals of the peptides were generated using the hanging drop vapor diffusion

method. The precipitant buffer was 20% PEG 3350 with 0.2 M NH_4NO_3 . Before the samples were flash frozen, 24% ethylene glycol was added as a cryoprotectant. Diffraction data collected using Pilatus3S 6M at beamline 08-ID of the Canadian Light Source (CLS) or either a Pilatus3S 6M detector or at the beamline IDB7 of the Macromolecular Cornell High Energy Synchrotron Source (MacCHESS). Datasets were indexed, integrated and scaled using HKL2000 (HKL Research, Inc.).

For calculation of the structures from the crystal data, the initial phases were generated by molecular replacement using the crystal structure of p53TD (PDB: 1C26) or p63TD (PDB:1GNU) as a search template. The final phases were determined following iterative cycles of model building with Coot and refinement using PHENIX [33]. Test data sets were selected randomly from the observed reflections prior to refinement. Statistics for the final models were obtained with PHENIX and Molprobability, and the figures were prepared with PyMOL [34].

4.3.6. NMR experiments

All NMR experiments were carried out at 300 K on a 4-channel Bruker BioSpin 700-MHz spectrometer in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl and 90% H_2O , 10% D_2O . For the ^1H - ^{15}N heteronuclear single quantum coherence (^1H - ^{15}N HSQC) spectra experiments, unlabeled p63TD peptides (Gar or Human) were added to 0.5 mM (monomer concentration) of ^{15}N -labeled-p53TD peptide (Gar or Human) at a ratio of 1:1. The NMR data were processed with NMRPipe/NMRDraw and analyzed with CcpNMR.

4.4. Results

4.4.1. Thermal stability for oligomerization of vertebrate p63

Based on the phylogenetic tree analysis, nine species were selected for analysis and the species selected to investigate how evolution has altered the structure of the p63 TD included at least one member of each class of vertebrates. For the p63TD peptides, 48 residue peptides (corresponding to residues 397-444 of human p63Tet) were prepared by solid-phase peptide synthesis for each species. To initially compare these p63TD peptides, the T_m values of each peptide was determined by CD spectroscopy (Figure 4-3, Table 4-1). The T_m values of the p63TD from the ghost shark, the frog and the three ray-finned fish species (gar, catfish and zebrafish) were much higher in comparison to the 6 other species. One possible explanation for the higher T_m values for the p63TDs of the three ray-finned species is that they each contain a Cys residue near the N-terminal of their peptide sequence, which have the potential to oxidize and form intramolecular disulfide bonds within the homotetrameric structure. Therefore, I also determined the T_m values for these peptides in the presence of 1 mM TCEP to prevent the possibility of disulfide bond formation via oxidation (Figure 4-4, Table 4-2). In the presence of TCEP, the T_m values decreased by 11.7°C for Catfish, 3.1°C for Zebrafish, and 13.8°C for Gar in comparison to the T_m values obtained in the absence of TCEP. These results suggests that the p63TD peptides of the three ray-finned fish species form disulfides bond during the process of tetramerization and these disulfide bond contribute significantly to the overall stability of their homotetrameric structure. Interestingly, the T_m values of p63TD peptides that share

the highest sequence homology, also differed among species, as we have observed with the p53TD peptides. This suggest that like the TD of p53, the TD of p63 has evolved to increase stability throughout the course of evolution.

4.4.2. Crystal Structures of vertebrate p53TD and p63TD

In order to identify the factors responsible for the differences in thermal stability of the p63TD among the different species at the atomic level, X-ray crystallographic analysis was attempted with several of the different p63TD peptides. From these efforts, I was able to obtain a crystal structure of Zebrafish p63TD for the first time at 1.8 Å resolution (Figure 4-5A). Similar to the structure of the Human p63TD (Figure 4-5C), the Zebrafish p63TD is composed of a two stranded β -sheet on the N-terminal side of the protein, followed by two alpha helices. Interestingly, there is also a unique disulfide bond present in the structure (Figure 4-6). This disulfide bond is formed through two Cys303 residues from two monomers and the disulfide bond is between the two monomers in each of the homodimers. This disulfide bond appears to be one of the key factors responsible for the remarkably high T_m value of Zebrafish p63TD obtained in the thermal stability experiments.

I was also able to obtain a crystal structure for the Coelacanth p63TD for the first time at 2.5 Å resolution (Figure 4-5B). Overall, the structure of the Coelacanth p63TD is not significantly different from that of the Human p63TD. However, the β -sheet in the Coelacanth p63TD is two residues shorter in comparison to β -sheet in the Human p63TD, and the second helix in the Coelacanth p63TD is 3 or 4 residues shorter in some of the

monomers due to lack of electron density (Figure 4-7). This would suggest that the C-terminal end of the helix in the Coelacanth p63TD is more dynamic (less stable) than the helix in the Human p63TD. Thus, there seems to be an overall decrease in the proportion of residues present in defined secondary structural elements when compared to what is observed in the structure of the Human p63TD. These differences would help to explain the lower thermal stability of Coelacanth p63TD relative to the Human p63TD. Structural alignment of Coelacanth and Zebrafish and Human p63TD with β -sheets revealed that the N-terminal side of the first helix and the orientation of the second helix are very different from those of Human and Zebrafish. It is suggested that this conformational change is responsible for the reduced thermal stability.

I also attempted to obtain a crystal structure of the Gar p63TD and we did obtain crystals that diffracted to 2.67 Å resolution. Unfortunately, I was not able to refine the structure with these crystals due to twinning of the crystals. Therefore, I am attempting to obtain additional crystals using different conditions for the crystallization to help resolve these difficulties.

The crystal structure of Gar p53TD was also obtained at 1.97Å (Figure 4-8). As expected from its sequence homology with Zebrafish p53TD, the structure of the Gar p53TD was very similar to the previously reported structure of the Zebrafish p53TD. More specifically, it was found to have a β -sheet on the N-terminal side and a slightly shorter first helix than Human p53TD, but it contained a second short helix C-terminal to the first helix. This result indicates that the p53TD had a second helix in organisms present at the time of divergence into ray-finned fish and that the evolution of p53TD in

ray-finned fish continued to possess a second helix that is not present in the p53TD of mammals and other higher organisms.

4.4.3. Analysis of hetero-tetramerization by avidin-biotin pull down assay

Avidin-Biotin pull down assays were performed to analyze the potential of p53 and p63 from each species (Lamprey, Ghost shark, Gar, Zebrafish, Catfish, Coelacanth, Frog, Softshell turtle, and Human) to form hetero-tetramers in vitro (Figure 4-9). Biotinylated p53TD and untagged p63TD peptides were used to analyze the ratio of p63TD pulled down with respect to the amount of biotinylated p53TD. The results indicate that p63TD from the early vertebrate Lamprey, the early divergent ray-finned fish Gar and the lobe-finned fish Coelacanth can form heterotetramers with their corresponding p53TD in vitro. For the binding assay, the ratio of p63-to-p53 were 0.60 in Lamprey, 0.39 in Gar, and 0.23 in Coelacanth when Bio(Aca)₂-p53TD and p63TD peptides were used at equal concentrations (10 μ M). On the other hand, in Catfish and Zebrafish, which are also ray-finned fish but diverged after Gar, the pull-down ratio of p63TD was only 0.04 and 0.05, respectively. This indicates that the p53-p63 pairs from these species do not form a heterooligomers as observed with Gar. Ghost shark (0.06), a chondrichthyan, and the terrestrial animals Frog (0.03), an amphibian, Softshell turtle (0.04), a reptile, and Human (0.02), a mammalian, also failed to show the ability to form heterotetramers. The results revealed that the ratio of pulled-down p63TD, which indicates the amount of p63 in the hetero-oligomer, increased in a concentration-dependent manner in Lamprey (1.14 and 2.93) slightly increased in Gar (0.42 and 0.59) and decreased in Coelacanth (0.05 and

0.05). These results indicate that the percentage of p63TD in the hetero-oligomer increased as the percentage of p63TD increased in Lamprey. Furthermore, when p63TD was 4 equivalents of p53TD, the ratio was 2.93, suggesting that almost all hetero-oligomers were at the theoretical maximum occupancy of p53:p63 = 1:3 (Figure 4-10). This suggests that in Lamprey, hetero-oligomers of p53TD and p63TD are formed by stoichiometric equilibrium, with a mixture of 1:3, 2:2, and 3:1 types of p53:p63 combinations when the heterotetramer was formed. While increasing p63TD in Gar did result in the formation of hetero-multimers, the percentage of p63TD in the hetero-oligomer did not increase dramatically as in Lamprey. This indicates that in Gar, heterotetramerization is not due to stoichiometric equilibrium as in Lamprey. Furthermore, Coelacanth, which formed a slight hetero-oligomer when p53TD and p63TD were 10 μ M each, showed no hetero-oligomer formation when the amount of p63TD was increased. This suggests that the hetero-oligomerization ability of Coelacanth, unlike that of Lamprey and Gar, is very weak and that homo-oligomerization of p53TD and p63TD is preferentially formed.

4.4.4. Analysis of hetero-tetramerization by intracellular FRET assay

To verify the results of our in vitro pull down assays examining heterotetramer formation with the TDs of p53 and p63, we examined the ability of the full-length p53 and p63 proteins to form hetero-tetramers in cells (Figure 4-11). The formation of the heterotetramers was analyzed by expressing Venus-tagged p53 proteins and Cerulean-tagged p63 proteins from different species in human cells that were p53-deficient. This

system allows for the identification of interactions between the p53 proteins with the p63 proteins from the different species to be observed by FRET analysis in the context of a cellular setting. The p53 and p63 proteins from the early vertebrates Lamprey and Coelacanth which were observed to form hetero-oligomers in an in vitro pull-down assay, were selected for the testing of heterotetramer formation and the p53 and p63 proteins from Human served as controls for the assay. Cells expressing the Human (p53/p53) protein positive control exhibited an increase in FRET signal in a Cerulean intensity-dependent manner, with a FRET signal of 4.1 at a Cerulean intensity of 15, whereas no increase in the FRET signal was observed in cells expressing the negative control Venus-tagged p53(p53/-) protein as the Cerulean intensity increased, and the FRET signal was 1.9 when the Cerulean intensity was 15. Likewise, expression of the Human (p53/p63) proteins, which have been reported to not form hetero-oligomer, showed a similar FRET signal pattern as cells expressing the negative control Human (p53/-) protein. With the Lamprey p53 and p63 proteins, where in vitro pull down assays showed strong heterotetramer formation between the TD peptides, cells expressing the Lamprey (p53/p53) showed a slightly lower FRET signal when compared to cells expressing the Human (p53/p53) protein at low Cerulean intensities. However, the FRET signal was stronger than that of cells expressing the Human(p53/p53) protein at Cerulean intensities above 9. This indicates that Lamprey's p53 is more like to form homotetramers at high intracellular concentrations. In cells expressing Lamprey (p53/p63) proteins, a lower FRET signal was observed than in cells expressing than the Human (p53/p63) proteins when the Cerulean intensity was low (between 1-7). More interestingly, a higher FRET

signal was observed with cells expressing Lamprey (p53/p63) proteins than the Human (p53/p53) protein when the Cerulean intensity was 9 or higher, and when the Cerulean intensity was 15, the FRET signal was 3.7. The results indicate that hetero-oligomers are formed when the intracellular concentrations of Lamprey's p53 and p63 proteins are raised. In Coelacanth, which showed slight heteromultimeric activity in the in vitro pull-down assay, expression of the Coelacanth(p53/p53) protein displayed a Cerulean intensity-dependent increase in FRET signal, but at all Cerulean intensities the FRET signal was less than that obtained in cells expression the Human(p53/p53) protein. This is consistent with a decrease in the ability to form stable homotetramers due to the lower thermal stability of the Coelacanth p53TD relative to that of the Human p53TD. Expression of the Coelacanth(p53/p63) proteins showed almost the same value for their FRET signal as the FRET signal of cells expressing the Human(p53/p63) proteins at Cerulean intensity values between 1-13, and a slightly larger value (2.3) than Human(p53/p63) at Cerulean intensity of 15. The results showed that Coelacanth p53 and p63 proteins rarely form heterotetramers in the cell. In summary, it is clear that in the early vertebrate Lamprey, the p53 and p63 proteins are able to form heterotetramer even in cellular conditions with the full-length proteins, while the more evolved species of Coelacanth and Human do not.

4.4.5. NMR-based analysis of the heterotetramer formation between the Gar and Human p53TD/p63TD pairs

In an attempt to analyze the proportion of hetero-tetramers that form in aqueous solution, I performed nuclear magnetic resonance (NMR) spectroscopy chemical shift perturbation experiments. ^1H - ^{15}N heteronuclear single quantum coherence (^1H - ^{15}N HSQC) spectra were recorded with ^{15}N -labeled Gar-p53TD (Figure 4-12) in both the absence and presence of unlabeled Gar-p63TD. As a negative control, an identical set of spectra were recorded with ^{15}N -labeled Human-p53TD (Figure 4-13) in both the absence and presence of unlabeled Human-p63TD. In the first set of experiments, the samples were heated at 95°C for 5 min after mixing the two peptides to promote oligomer formation, and then allowed to stand at room temperature for 1h and at 4°C for 1h prior to recording the NMR spectra. With the Gar p53TD/p63TD pair, a number of new signals associated with the ^{15}N -labeled Gar-p53TD (roughly 15 signals) appeared in the spectrum, and these new signals appear to be a doublet signal of one of the existing signals from the homo-tetrameric Gar-p53TD that appeared at a ratio of approximately 45:55. In contrast, no new signals were observed when the human peptides were mixed and no chemical shift changes were observed for the ^{15}N -labeled Human-p53TD in the presence and absence of the Human-p63TD. These results suggested that the Gar p53TD/p63TD peptides were forming hetero-tetramers to a significant extent under these conditions, in contrast to the absence of any significant quantity of hetero-tetramer with the human p53TD/p63TD peptides. In the second set up experiments, the samples were left at room temperature for 24h after mixing the two peptides prior to recording the NMR spectra (figure 4-14). The results in this set of experiments were identical to what was observed in the first set of experiments with heating. Again, we saw additional signals with the Gar

p53TD/p63TD pair associated with the ^{15}N -labeled Gar-p53TD and no changes in the spectra with the Human p53TD/p63TD pair. Again the results from the NMR studies suggest the formation of hetero-tetramers and the ratio of the doublet signals was virtually identical to what was seen in the first set of experiments with heating. Taken together, the NMR studies suggest that the Gar p53TD and p63TD peptides readily form hetero-tetramers in solutions when mixed. Although it is impossible to measure the exact ratio, it appears that the amount is most likely greater than 50% since the ratio was 45% for the signals associated with the hetero-tetramer and 55% for the signals associated with the homo-tetramer. However, the signal associated with the hetero-tetramer contains half the amount of monomers that are ^{15}N -labeled in comparison to the signals associated with the homo-tetramer. If corrected for the amount of label, the ration of hetero-tetramer to homo-tetramer would be 90:50 or roughly 65% hetero-tetramer and 35% homo-tetramer.

1	67	170	362	397	444	541	610
TAD	PRD	DBD		TD		SAM	TID
MNFETSRCAT	LQYCPDPYIQ	RFVETPAHFS	WKESYYRSTM	SQSTQTNEFL			
SPEVFQHIWD	FLEQPICSVQ	PIDLNFVDEP	SEDGATNKIE	ISMDCIRMQD			
SDLSDPMWPQ	YTNLGLLSNM	DQQIQNGSSS	TSPYNTDHAQ	NSVTAPSPYA			
QPSSTFDALS	PSPAIPSNTD	YPGPHSFDVS	FQQSSTAKSA	TWTYSTELKK			
LYCQIAKTCP	IQIKVMTPPP	QGAVIRAMPV	YKKAEHVTEV	VKRCPNHEL			
REFNEGQIAP	PSHLIRVEGN	SHAQYVEDPI	TGRQSVLVPY	EPPQVGTEFT			
TVLYNFMCS	SCVGGMNRRP	ILIIIVTLETR	DGQVLGRRCF	EARICACPGR			
DRKADEDSIR	KQQVSDSTKN	GDGTRKPFQR	NTHGIQMTSI	KKRRSPDDEL			
LYLPVRGRET	YEMLLKIKES	LELMQYLPQH	TIETYRQQQQ	QQHQHLLQKQ			
TSIQSPSSYG	NSSPPLNKMN	SMNKLPSVSQ	LINPQQRNAL	TPTTIPDGMG			
ANIPMMGTHM	PMAGDMNGLS	PTQALPPPLS	MPSTSHCTPP	PPYPTDCSIV			
SFLARLGCS	CLDYFTTQGL	TTIYQIEHYS	MDDLASLKIP	EQFRHAIWKG			
ILDHRQLHEF	SSPSHLLRTP	SSASTVSVGS	SETRGERVID	AVRFTLRQTI			
SFPPRDEWND	FNFDMDARRN	KQQRKEEGE					

Figure 4-1. Schematic structure and amino acid sequence of p63

TAD: transactivation domain (green), PRD: proline-rich domain (yellow), DBD: DNA binding domain (cyan), TD: tetramerization domain (magenta), SAM: sterile alpha motif domain (purple), TID: transcription inhibition domain (orange).

Common Name		p53 Sequences																									
Human	HU	D	G	E	Y	F	T	L	Q	I	R	G	R	E	R	F	E	M	F	R	E	L	N	E	A	L	E
Softshell turtle	SO	D	K	E	V	F	C	L	E	V	H	G	R	E	N	Y	E	M	L	K	K	I	N	N	A	L	E
Frog	FR	D	E	E	I	F	T	L	R	I	K	G	R	S	R	Y	E	M	I	K	K	L	N	D	A	L	E
Coelacanth	CO	E	E	E	L	Y	S	L	Q	V	R	G	R	E	R	Y	E	M	F	K	K	L	N	N	A	L	E
Catfish	CA	D	E	E	I	Y	T	L	Q	V	R	G	K	E	R	Y	E	F	L	K	K	I	N	D	G	L	E
Zebrafish	ZE	D	E	E	I	F	T	L	Q	V	R	G	R	E	R	Y	E	I	L	K	K	L	N	D	S	L	E
Gar	GA	E	E	E	I	F	T	L	Q	V	R	G	R	E	R	Y	E	M	L	K	K	I	N	E	S	L	E
Ghost shark	GH	D	T	E	V	F	T	L	Q	V	T	G	R	E	R	Y	E	T	L	K	Q	I	N	E	S	L	E
Lamprey	LA	K	E	E	L	F	L	I	P	V	R	G	R	E	N	Y	E	L	L	H	L	K	E	S	L	E	M

Common Name		p63 Sequences																									
Human	HU	D	D	E	L	L	Y	L	P	V	R	G	R	E	T	Y	E	M	L	L	K	I	K	E	S	L	E
Softshell turtle	SO	D	D	E	L	L	Y	L	P	V	R	G	R	E	T	Y	E	M	L	L	K	I	K	E	S	L	E
Frog	FR	D	D	E	V	L	Y	L	P	V	K	G	R	E	I	Y	E	M	L	L	K	I	K	E	S	L	E
Coelacanth	CO	D	D	E	V	L	Y	I	A	V	R	G	R	E	N	Y	E	V	L	Q	K	V	R	E	S	L	E
Catfish	CA	D	E	E	V	F	C	L	P	I	K	G	R	E	I	Y	E	I	L	V	K	I	K	E	S	L	E
Zebrafish	ZE	D	E	E	V	F	C	L	P	I	K	G	R	E	I	Y	E	I	L	V	K	I	K	E	S	L	E
Gar	GA	D	D	E	L	F	C	L	P	I	K	G	R	E	I	Y	E	I	L	K	I	K	E	S	L	E	
Ghost shark	GH	E	E	E	L	F	Y	L	P	V	R	G	R	E	T	Y	E	V	L	L	K	I	K	E	S	L	E
Lamprey	LA	Q	E	D	I	F	P	L	L	Q	G	R	E	N	F	E	I	L	K	I	K	E	S	L	E	M	

Figure 4-2. Alignment of p53 and p63 sequences for the species used

Alignment of p53TD and p63TD of the organism species used (agnatha, chondrichthyans, ray-finned fish, lobe-finned fish, amphibians, reptiles, and mammals). Acidic amino acid residues (red), basic amino acid residues (blue), hydrophobic amino acid residues (orange). The p53TD of ray-finned fish is very similar to p63TD in the C-terminal Pro residue and YRQ sequence.

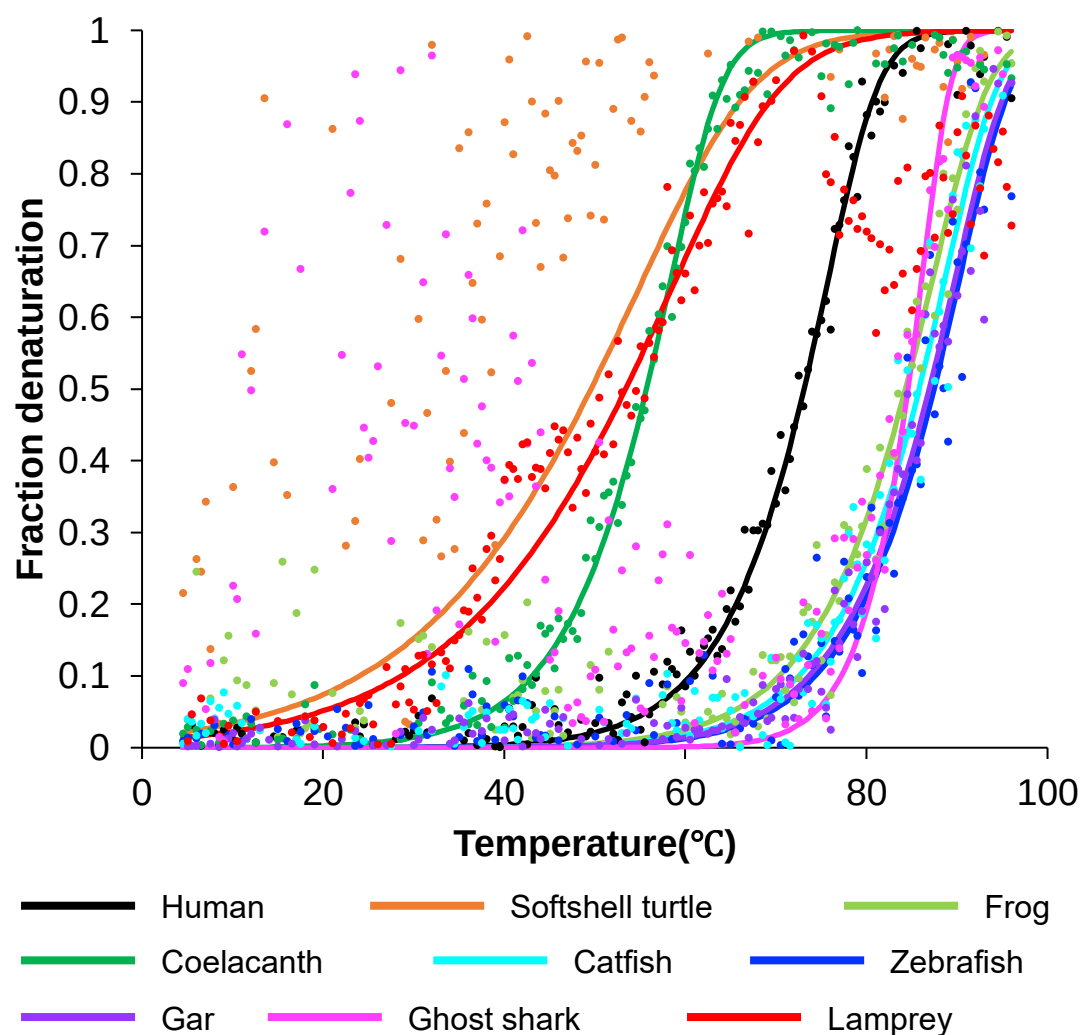


Figure 4-3. Thermal denaturation curves of the vertebrate p63TDs.

The signals from the vertebrate p63TDs were monitored by CD spectrometry at 222 nm between 4 °C and 96 °C. The fraction of denaturation at each temperature was plotted to generate the denaturation.

Table 4-1 Thermodynamic parameters of p63TDs based on CD analysis

Classification	Species	T_m (°C)	$\Delta G^{37^\circ\text{C}}$ (kcal/mol)
Mammalia	Human	71.4	29.8
Reptilia	Softshell turtle	59.0	25.3
Amphibian	Frog	76.5	26.2
Lobe-finned fish	Coelacanth	53.8	25.0
Ray-finned fish	Catfish	85.8	31.1
	Zebrafish	81.3	27.6
	Gar	86.9	25.9
Cartilaginous fishes	Ghost shark	85.5	26.4
Agnatha	Lamprey	69.0	33.3

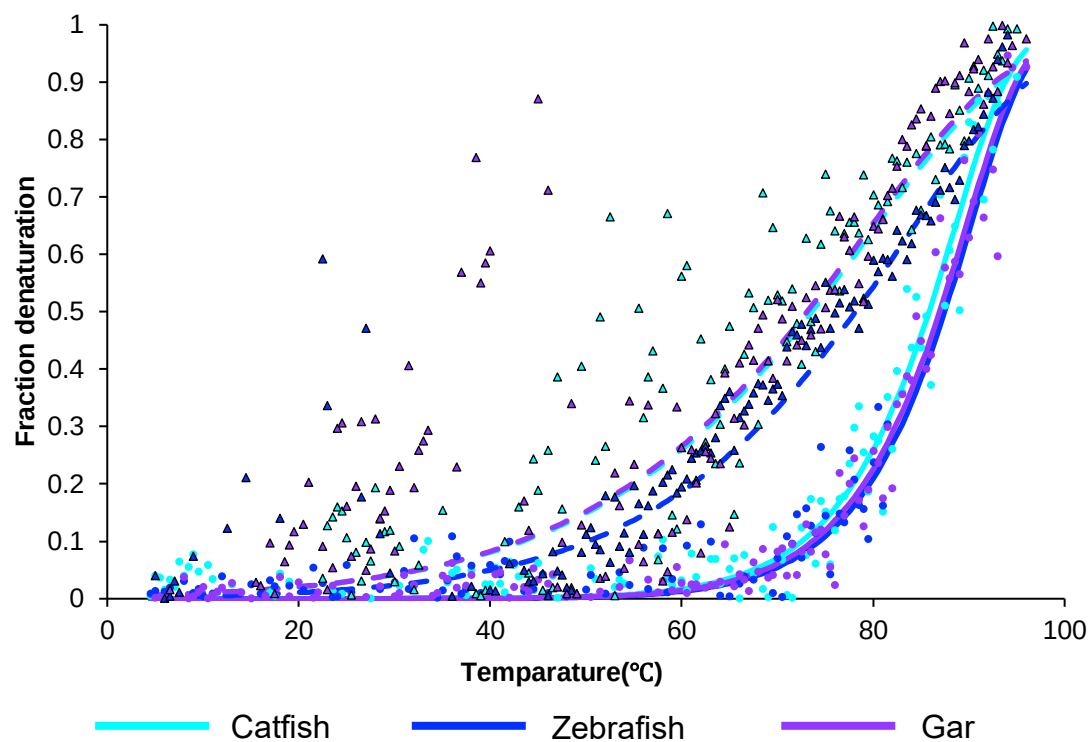


Figure 4-4. Comparison of the thermal denaturation curve of ray-finned fish p63TDs with the condition of adding the reducing agent TCEP.

When TCEP was added, T_m decreased and destabilized by 11.7°C for Catfish, 3.1°C for Zebrafish, and 13.8°C for Gar. This suggests that these three p63TDs form disulfide bonds under oxidative conditions.

Table 4-2. Thermodynamic parameters of p63TD of the above school based on CD analysis under oxidation and reduction conditions

Condition	Species	T_m (°C)	$\Delta G^{37^\circ\text{C}}$ (kcal/mol)
Oxidative	Catfish	85.8	31.1
	Zebrafish	81.3	27.6
	Gar	86.9	25.9
Reductive	Catfish	73.6	23.7
	Zebrafish	78.2	24.1
	Gar	73.1	23.7

Table 4-3. Data collection and refinement statistics for p53TD and p63TDs

Dataset	Zebrafish p63TD	Coelacanth p63TD	Gar p53TD
Data Collection			
Space group	C 1 2 1	C 2 2 21	R 3 2 :H
Unit cell parameters			
a, b, c(Å)	79.932, 81.461, 54.037	50.492, 77.449, 189.442	169.895, 169.895, 196.363
$\alpha, \beta, \gamma(^{\circ})$	90 132.545 90	90 90 90	90 90 120
Resolution range(Å)	29.44 - 1.964 (2.035 - 1.964)	47.36 - 2.25 (2.331 - 2.25)	48.39 - 2.743 (2.841 - 2.743)
No. of unique reflections	17801 (1472)	17892 (1613)	25968 (1217)
Multiplicity	5.7 (3.0)	6.1 (3.9)	12.1 (1.5)
Completeness(%)	91.02 (62.74)	96.82 (71.02)	89.90 (41.31)
R _{merge}	0.08336 (1.009)	0.2077 (2.597)	0.1976 (1.092)
CC _{merge}	0.999 (0.779)	0.998 (0.577)	0.999 (0.197)
I/ σ (I)	12.08 (0.73)	5.03 (0.39)	9.84 (0.46)
Refinement Statistics			
Resolution(Å)	29.44 - 1.964	47.36 - 2.25	48.39 - 2.743
Reflections(total/test) ^a	16591 (1133)	17543 (1282)	25790 (1158)
R _{work} /R _{free} (%)	0.2197/0.2521	0.2460/0.3008	0.2260/0.2940
CC _{work}	0.977 (0.570)	0.952 (0.420)	0.931 (0.122)
CC _{free}	0.964 (0.323)	0.940 (0.433)	0.881 (-0.028)
No. of atoms(excluding hydrogens)			
Protein	1623	2826	5261
water	65	54	59
B factors			
Protein	61.52	63.90	68.92
Water	56.67	54.25	60.57
Root-mean-square deviation			
Bond length(Å)	0.009	0.007	0.01
Bond angle(°)	1.49	1.1	1.19
Ramachandran(%)^b			
Favored	92	97.76	96.59
Outliers	2.86	0.00	0.51

Values in parentheses are for highest-resolution shell. $R_{\text{sym}} = \frac{\sum_{\mathbf{hkl}} \sum_i |I_{\mathbf{hkl},i} - \langle I_{\mathbf{hkl}} \rangle|}{\sum_{\mathbf{hkl}} I_{\mathbf{hkl}}}$, where $I_{\mathbf{hkl},i}$ is the intensity of an individual measurement of the reflection with Miller indices $\mathbf{hkl}$ and $I_{\mathbf{hkl}}$ is the mean intensity of the reflection. $R_{\text{work}} = \frac{\sum_{\mathbf{hkl}} ||F_o| - |F_c||}{\sum_{\mathbf{hkl}} |F_o|}$, where $|F_o|$ is the observed structure-factor amplitude and $|F_c|$ is the calculated structure-factor amplitude. R_{free} is the [R factor](#) based on at least 500 test reflections that were excluded from the refinements. CLS (Canadian Light Source) and CHESS (Cornell High Energy Synchrotron Source).

^a Reflection for $F_o > 0$. ^b MolProbity analysis.

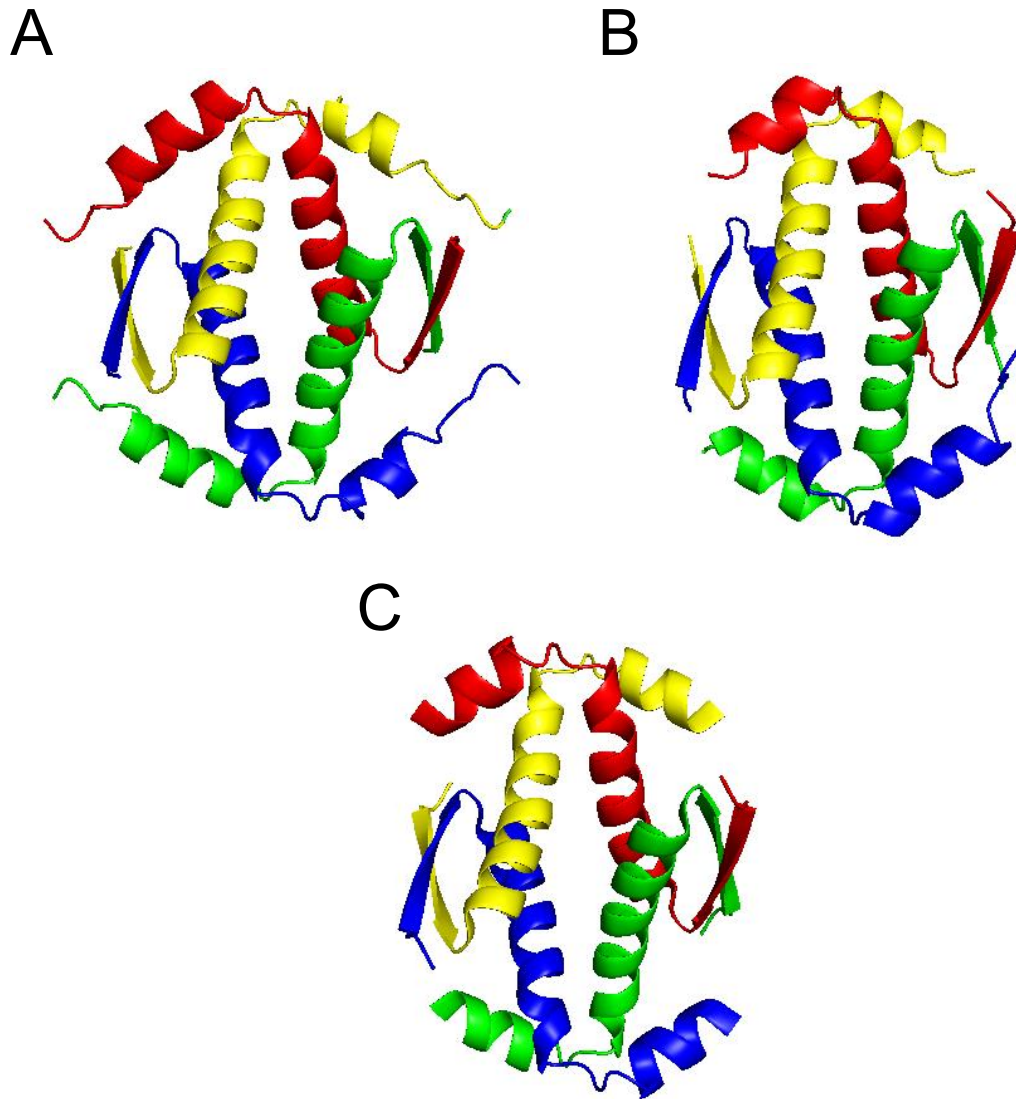


Figure 4-5. Crystal structure of p63TD of Zebrafish, Coelacanth, Human
 (A)Zebrafish, (B)Coelacanth, (C)Human(PDB:3ZY1). Both Zebrafish p63TD and Coelacanth p63TD had a β -sheet and two helix structures as did Human. However, there was a monomer of Coelacanth with a shorter β -sheet and second helix length than that of Human.

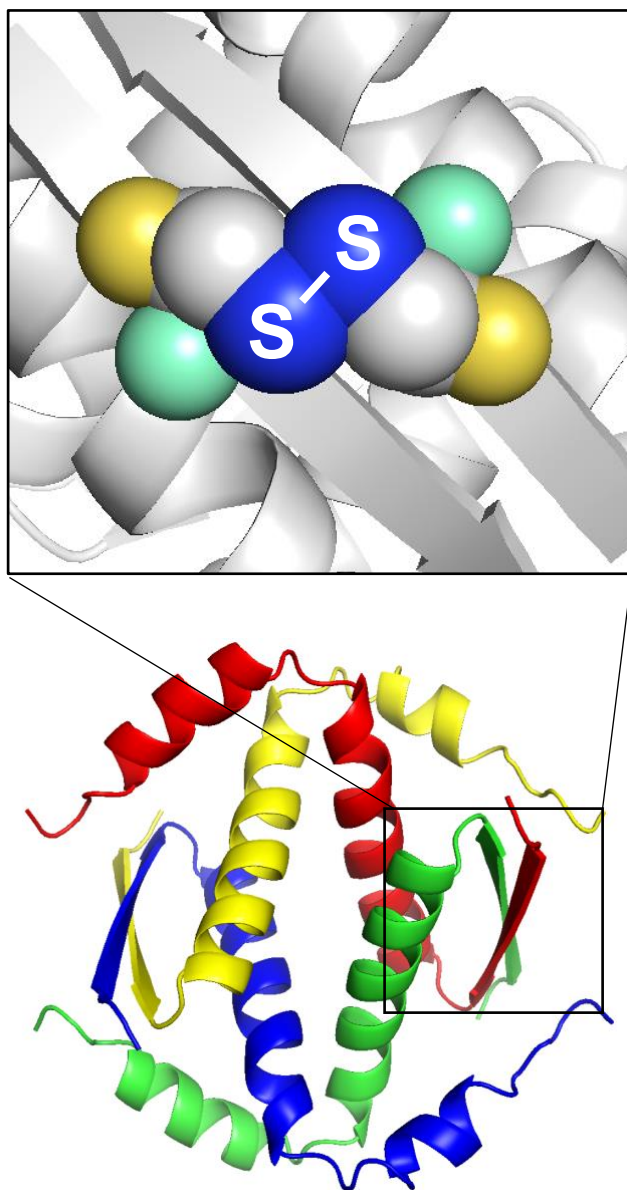


Figure 4-6. Crystal structure of Zebrafish p63TD

The structure is similar to that of Human p63TD, Zebrafish p63TD is composed of a two stranded β -sheet on the N-terminal side of the protein, followed by two alpha helices. Interestingly, there is also a unique disulfide bond present in the structure. This disulfide bond is formed through two Cys303 residues from two monomers and the disulfide bond is between the two monomers in each of the homodimers.

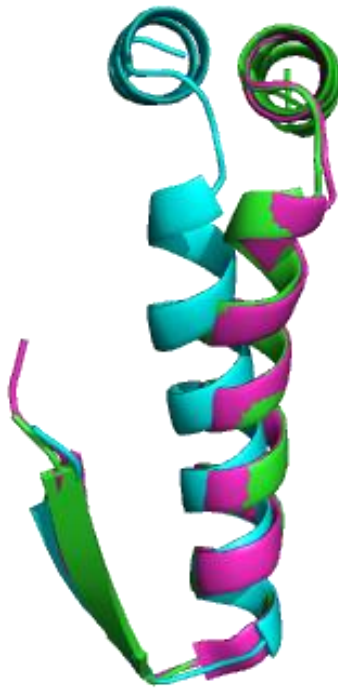


Figure 4-7. Comparison of crystal structures of monomers of p63TD in Zebrafish, Coelacanth, and Human

(green)Zebrafish, (cyan)Coelacanth, (magenta)Human. Alignment of the three crystal structures with the β -sheet structure revealed that from the middle of the first helix to the second helix, the orientation of Coelacanth p63TD is different from that of Human and Zebrafish. It is suggested that this structural difference may be one of the reasons for the low stability of Coelacanth p63TD.

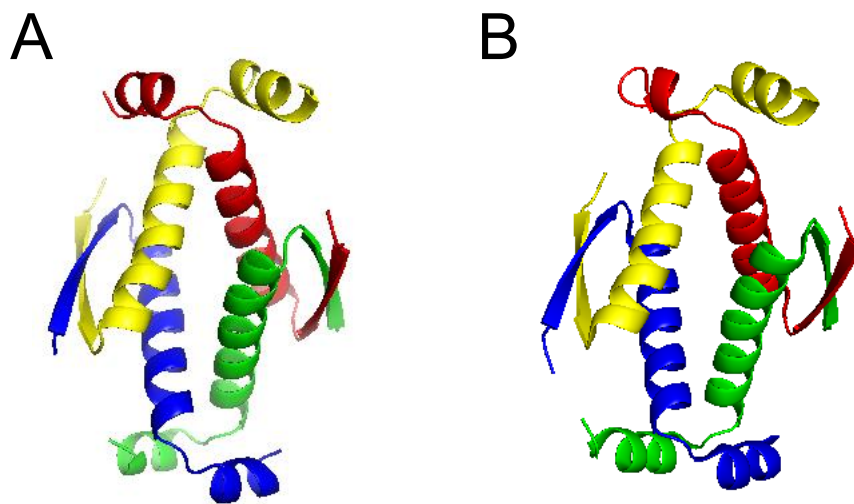


Figure 4-8. Crystal structure of p53TD in Gar and Zebrafish

(A) Gar p53TD, (B) Zebrafish p53TD (4D1M)

The structure of Gar p53TD, which belongs to ray-finned fish and diverged earlier than Zebrafish, was similar to that of Zebrafish p53TD, unlike Human p53TD. This suggested that at the time of evolution to ray-finned fish, p53TD evolved retaining the second helix.

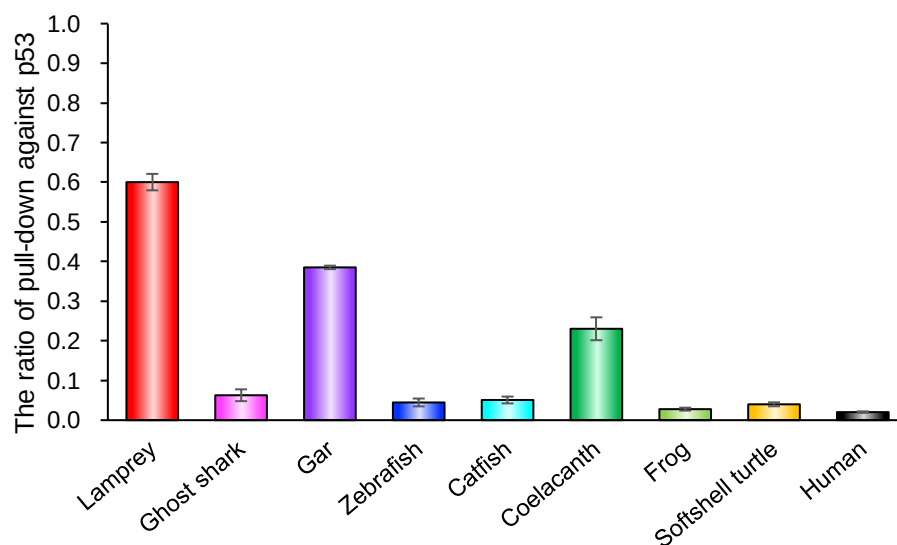


Figure 4-9. Ratio of pulled-down p63TD to p53TD for each species

Experiments were performed with 50 mM Na phosphate buffer (pH 7.5), 100 mM NaCl, and peptide solutions mixed with Bio-(Aca)2-p53Tet and p63Tet at a concentration of 10 μ M. The results showed that p63Tet was pulled-down in the early vertebrates Lamprey (0.60), Gar (0.39), which is an early branch of the ray-finned fish, and Coelacanth (0.23), a lobe-finned fish. No substantial pull-down of p63Tet was observed in other species such as Ghost shark (0.06), Zebrafish (0.04), Catfish (0.05), Frog (0.03), Softshell turtle (0.04) and Human (0.02).

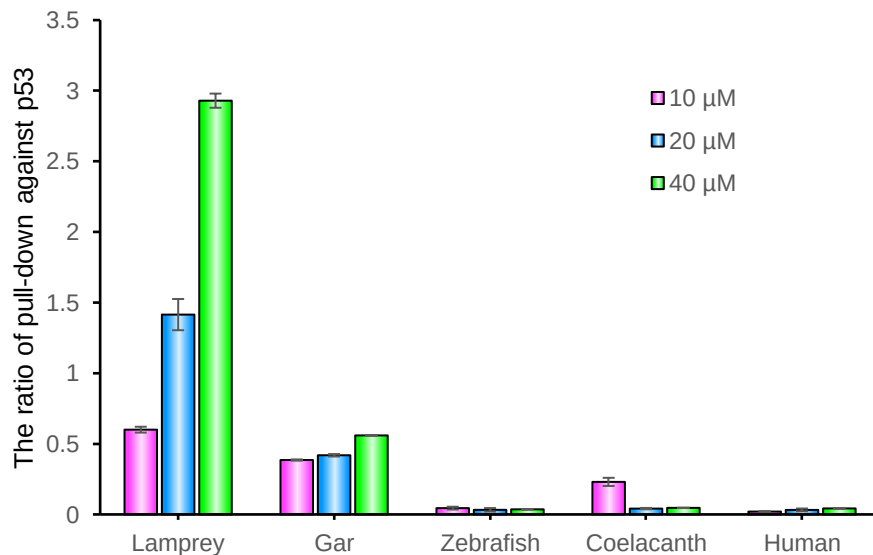


Figure 4-10. Ratio of pulled-down p63TD to p53TD at increasing amounts of p63TD in each species

Experiments were performed using 50 mM Na phosphate buffer (pH 7.5), 100 mM NaCl, and peptide solutions mixed at concentrations of 10 μ M Bio-(Aca)-p53Tet and 10 μ M, 20 μ M and 40 μ M p63Tet. The results showed a dose-dependent increase in the percentage of p63TD pulled-down in early vertebrates Lamprey to 0.60, 1.41, and 2.93. Gar, on the other hand, showed only slight dose-dependent increases in p63TD (0.39, 0.42, and 0.56). Furthermore, Coelacanth showed no substantial pull-down with increasing amounts of p63TD (0.04, 0.05); Zebrafish and Human, which showed no pull-down at 10 μ M, showed no pull-down with increasing amounts of p63TD (0.03, 0.04 in Zebrafish and 0.03, 0.04 in Human).

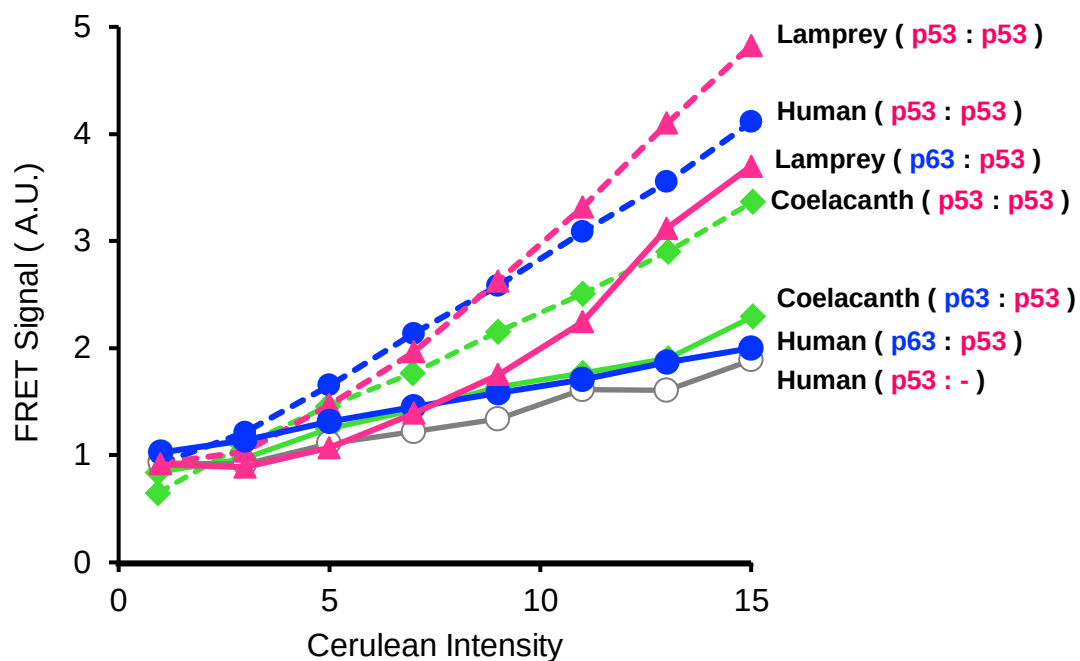


Figure 4-11. Analysis of p53 and p63 hetero-oligomer formation in cells using FRET

Intracellular FRET results using Human and Lamprey and Coelacanth p53 family proteins in H1299 cells are shown. Coelacanth p53 / p63 showed a similar signal to the negative control, but a small amount of FRET was observed at a Cerulean intensity of 15. On the other hand, Lamprey p53 / p63 showed a significant increase in signal, similar to that observed when p53 was co-expressed with human p53, the positive control.

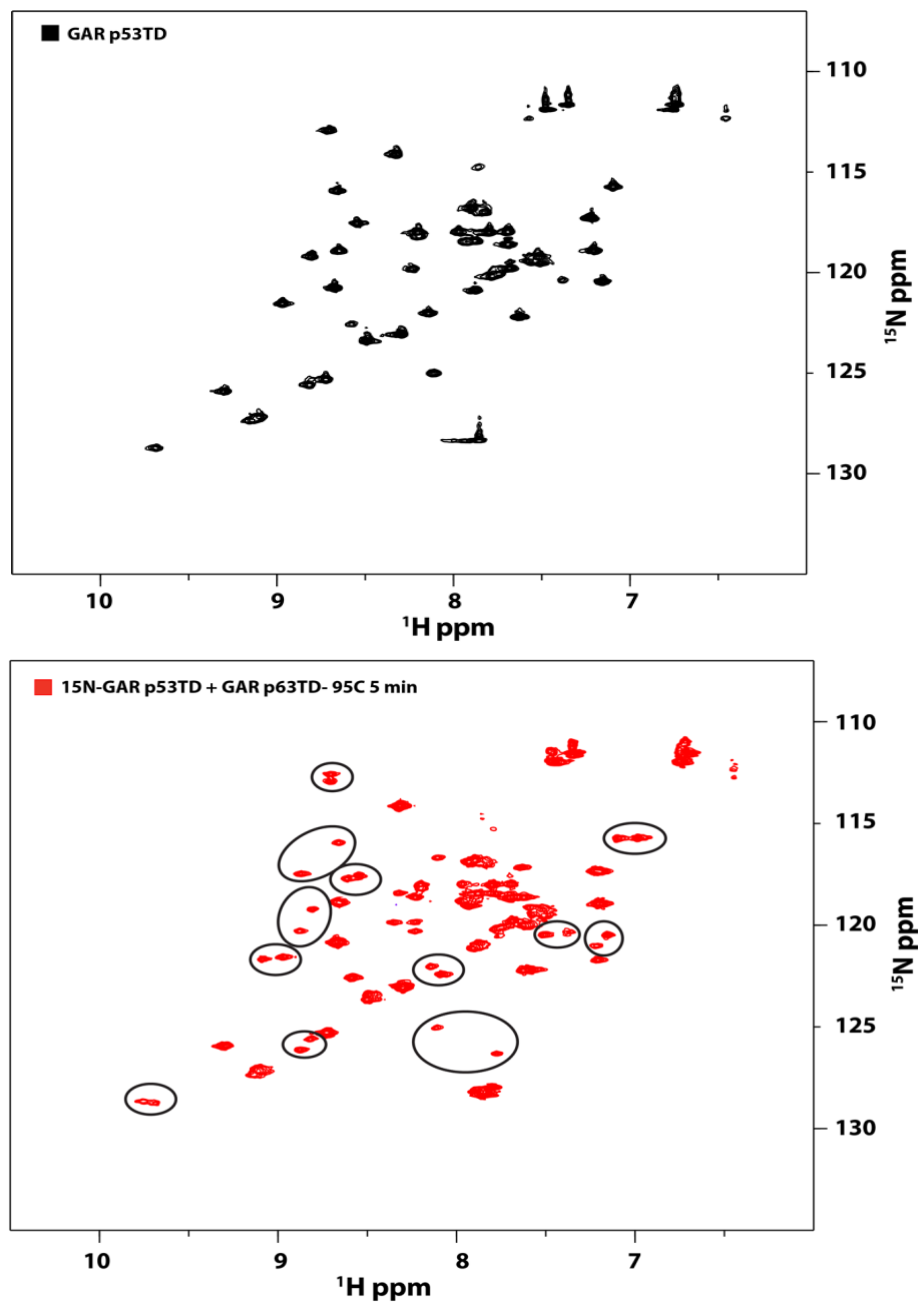


Figure 4-12. ^1H - ^{15}N heteronuclear single quantum coherence (^1H - ^{15}N HSQC) spectra using Gar p53TD and p63TD.

Spectra measured with ^{15}N -labeled p53TD alone (top); spectra measured with samples mixed with ^{15}N -labeled p53TD and unlabeled p63TD and allowed to stand at 95°C for 5 min, room temperature for 1 h, and 4°C for 1 h (bottom). Compared to the spectrum with p53TD alone, roughly 15 new signals were observed in the condition with the addition of p63TD, which is considered to be a doublet signal. The intensity ratio of the original signal to the doublet signal was 45:55.

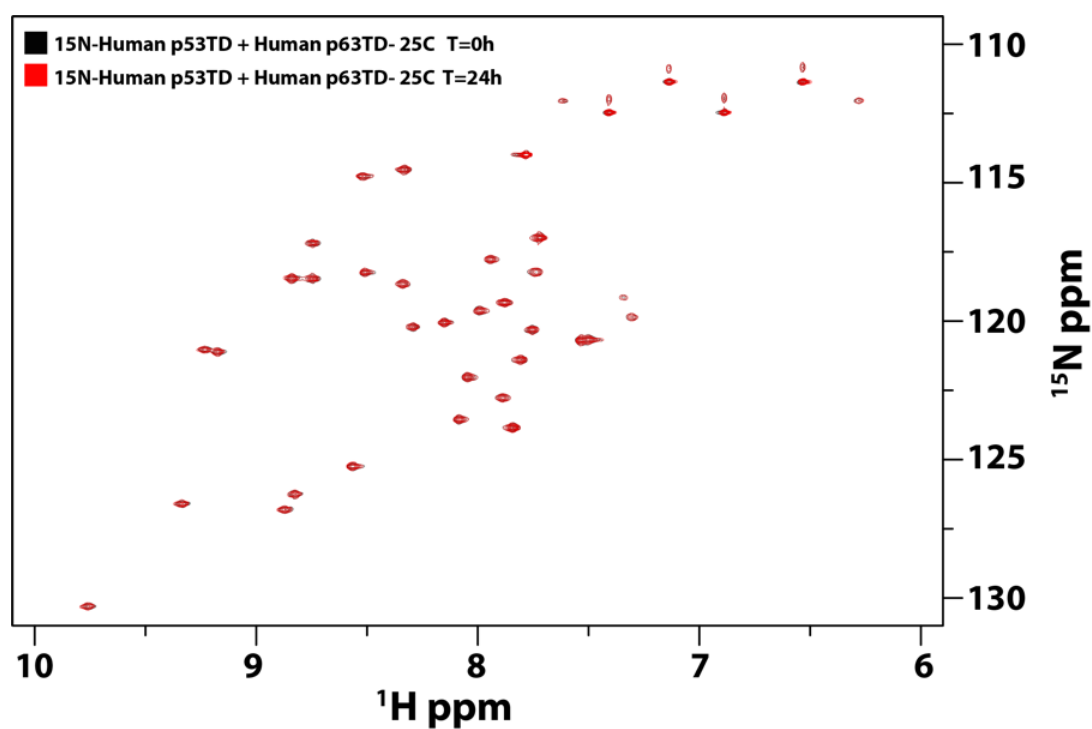


Figure 4-13. ^1H - ^{15}N heteronuclear single quantum coherence (^1H - ^{15}N HSQC) spectra using Human p53TD and p63TD.

No new signals were observed when comparing Human's ^{15}N -labeled p53-only signal (black) with the signal in the p63-added condition (red). This result indicates that Human p53 does not form a hetero-tetramerization with p63 as reported.

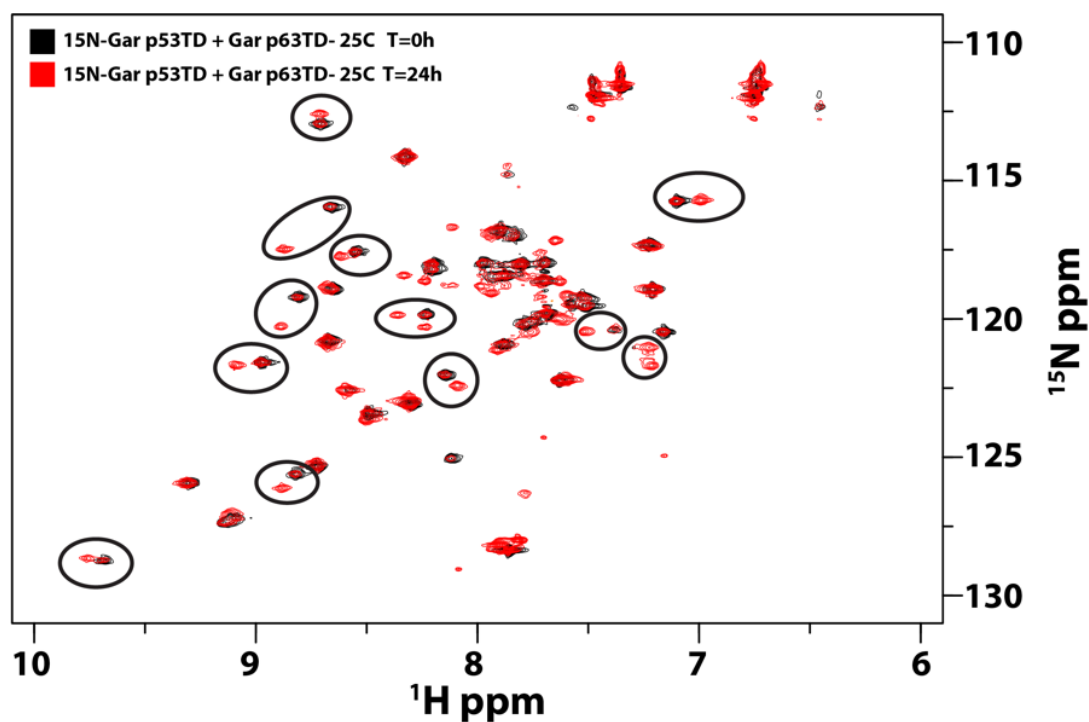


Figure 4-14. ^1H - ^{15}N heteronuclear single quantum coherence (^1H - ^{15}N HSQC) spectra using Gar p53TD and p63TD in different condition.

Spectra measured on a sample immediately after mixing ^{15}N -labeled p53TD and unlabeled p63TD (black) and on a sample left at 25°C for 24h (red). Similar to the first condition, a new doublet signal was observed when p63TD was added compared to p53TD alone. This indicates that Gar's p53 forms a hetero-tetramerization with p63 even under mild conditions.

4.5. Discussion

The average sequence homology of the p53TDs among the eight other species used in this study (excluding Human) is relatively low (62%), whereas the average sequence homolog of their p63TD is much high (88%). However, there is considerable differences in the thermal stability of p63TD among the various species, with the Zebrafish possessing the highest T_m of 87.5°C and the Softshell turtle having the lowest T_m of 37.9°C. In particular, the T_m of Gar (86.9°C), Zebrafish (87.5°C), Catfish (85.8°C), Frog (84.3°C), and Ghost shark (84.2°C), which belong to the ray-finned fish, amphipods, and cartilaginous fish, respectively, have significantly higher T_m values than the other four species. Among these, it appears that the stability of the three ray-finned fish species occurs via formation of a disulfide bond between Cys residues on the β -sheet of different monomers in the tetrameric structure, as seen in our crystal structure of the Zebrafish p63TD (Figure 4-8). Therefore, we suspect the structure of the Gar p63TD will be very similar to what we obtained for the Zebrafish p63TD as it also contains the Cys residues in its sequence in the same position.

The role of these disulfide bond in the stabilization of these tetrameric forms is supported by our results demonstrating a decrease in T_m of 11.7°C for Gar, 3.1°C for Zebrafish, and 13.8°C for Catfish when the CD analysis is performed under reducing conditions in the presence TCEP. Although the normal intracellular state is somewhat reducing, the stability of p63 may be altered by transient oxidative conditions or oxidative conditions inside the endoplasmic reticulum, and this could be a mechanism for regulating the associated transcriptional activity[15–17]. The increased stability observed

for the Frog and Ghost shark p63TDs, which are not members of the ray-finned fish family, may be due to compact packing of the hydrophobic core and amino acid substitutions in the vicinity of the core of the helical bundle that stabilizes the tetrameric form. On the other hand, the overall stability of the Lamprey, Softshell turtle and Coelacanth p63TDs are very low in comparison. The presence of a of Pro residue in the sequence of the p63TD of Lamprey that aligns with the region is not present in the other species, and the presence of this Pro residue should result in the formation of a shorter lengthened β -sheet and thus lower thermal stability of the domain. Likewise, the crystal structure of Coelacanth p63TD reveals that it also possesses a shorter β -sheet as well as a shorter second helix, and both of these changes may contribute to its relatively low thermal stability of its p63TD in comparison the other species. In the case of Softshell turtles, they differ only in having a shorter second helical region, which again suggests that changes in this region have resulted in producing TDs with decreased thermal stability.

Looking at these changes in thermal stability of the different p63TDs from the perspective of the evolutionary processes, aquatic species up to ray-finned fish and terrestrial species up to mammals have evolved to enhance the thermal stability of this important structural domain within the p63 protein. As far as their ability to form hetero-oligomers between their p53 and p63, it appears to be strong in the early vertebrate Lamprey. However, later evolution showed that only the early fish Gar and the lobe-finned fish Coelacanth have the ability to form hetero-polymers, which was completely absent in the more evolved species. When the ratio of p63TD to p53TD was increased

from 1, 2, to 4 equivalents, the amount of p63TD pulled-down by the Lamprey p53TD increased in a concentration-dependent manner from 0.53 to 1.14, and then to 2.93 equivalents of p63. This result alone does not clarify the ratio of p53 to p63 in the hetero-oligomer formation. However, the value of 2.93 when four equivalents of the p63TD are added suggests that the p63TD ratio is the highest in the p53-p63 hetero-tetramerization, with p63TD forming at a ratio of one p53TD to three p63TDs. This suggests that Lamprey's p63TD readily incorporates its p53TD to form a hetero-oligomer. Interestingly, among the ray-finned fish species that evolved while retaining the second helix, Gar, an early branching species, formed hetero-oligomers, whereas Zebrafish and Catfish, later branching species, do not. This results suggests that species belonging to the ray-finned fish family have different characteristics. Gar showed only a slight increase in the percentage of p63TD that was pulled-down (0.39, 0.42, and 0.59) when the ratio of p63TD to p53TD was increased; this smaller increase compared to what was observed with the Lamprey p53TD/p63TD pair indicates that the ability of p63TD and p53TD to form hetero-tetramers is weaker for Gar.

The results from the NMR experiments using the Gar p53TD and p63TD strongly suggests that there is only one type of heterotetramer of p53TD to p63TD formed. Furthermore, the p63TD from ray-finned fish all have the Cys residue in the same position in their sequences that is part of the β -sheet, and this Cys forms a disulfide bond within the dimers in the Zebrafish p63TD. Given that there is high sequence homology between the Zebrafish and Gar p63TDs, this suggests that the Gar p63TD will also form very stable homodimers and that the hetero-oligomer formed by Gar would most likely consists of a

homodimers of p53 and p63, respectively, forming the hetero-tetramer. This would be similar to what is observed for the heterotetramers formed between the TDs of Human p63 and p73 [6].

Despite having high sequence homology including having a Cys in the p53 domain, Zebrafish and Catfish had pull-downs of 0.04 and 0.05, respectively. The reason for this large difference between the two species, even though they belong to the same ray-finned fish, can be attributed to the whole-genome duplication [18,19]. Whole genome duplication is a phenomenon in which the amount of DNA in a normally diploid cell doubles to become tetraploid, and vertebrates have experienced whole genome duplication at least twice before humans. This phenomenon temporarily doubles the number of genes, but most of the genes are eliminated by selection or become pseudogenes, and the total number of genes decreases. However, since genes from one parent or the other remain in the doubled gene number, amino acid substitutions are more likely to occur, and a small percentage of genes are known to either change part of their original function or change to a completely different function. The neopinnae, to which Gar belongs, experienced this whole-genome duplication twice, whereas eukaryotes, to which Zebrafish and Catfish belong, have been reported to have experienced a third whole-genome duplication during their evolution [20,21]. This third whole-genome duplication is predicted to have resulted in substitutions in p53 that resulted in the loss of p53-p63 hetero-oligomer formation in Zebrafish and Catfish.

Coelacanth, a lobe-finned fish that is believed to have evolved just before vertebrates expanded onto land [22,23], forms only a small percentage of heterooligomers and this

occurred only when the concentration of p63TD was low (0.20). Interestingly, the Coelacanth p53TD does not have a second helix as observed with the Gar p53TD and this strongly suggests that this may be the reason why Coelacanth lost its ability to form hetero-oligomers. In our laboratory, we conducted a transcriptional activity analysis using chimeric p53, in which the TD of Human p53 was changed to the TD of each of the different vertebrates. The results showed that those p53TDs that have high thermal stability in the CD assay also had high transcriptional activity in the context of the full-length Human p53 protein. In the present results, the high thermal stability of p63TD in striated fins, suggests that there is a correlation between transcriptional activity and thermal stability, and that the evolution of p63 has been favorable for the development of fins, which are very important for the survival of fish.

Based on the changes in the thermal stability of the TDs of these p53 family members as well as their ability to form homooligomers or hetero-oligomers, I propose the following novel model. In the early vertebrate such as Lamprey, the stability of p53 and p63 is very low and they are able to readily form hetero-oligomers, which means that the specificity of their respective functions is lower. However, changes in amino acid side-chain interactions and increased stability during evolution have led to the loss of functions associated with the formation of heteromultimeric proteins and increased the specificity of the individual functions of the p53 and p63 proteins. This novel model is expected to be relevant not only to the p53 family of proteins but also to a wide variety of other proteins that form oligomers, and this highlights the evolutionary significance of the loss of hetero-oligomer formation. In terms of biological evolution, the function of

maintaining genome integrity is expected to be strong in ray-finned fish, especially eukaryotes, which are species with very stable p53 proteins and a corresponding high level of transcriptional activity, but it is clear that the species are very diverse. This is expected to be related to the loss of the ability to form hetero-oligomers with p63, since species diversification is not so strong in neopinnae such as Gar.

4.6. References

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5. Conclusions

The tumor suppressor protein p53 is a key factor in protecting genomic integrity in response to various cellular stresses, including DNA damage, hypoxia, oxidative stress, virus infection and starvation stress. The p53 gene, *TP53*, is the most frequently mutated gene in human tumors and mutations in *TP53* are predominantly missense mutation. Although mutations in somatic cell cancers occur most frequently in the region coding for the DBD of p53, mutations in the region coding for the TD are important in germline cells, including in Li-Fraumeni syndrome. Understanding the function of p53 and the p53 pathway in various diseases and abnormalities is crucial for cancer therapy and prevention. Therefore, a wide range of research on p53 has been conducted and more than 40,000 papers with "p53" in the title have been published to date.

The formation of a tetrameric structure by the p53 protein is essential for most of the functions it carries out as a tumor suppressor protein and the stability of the tetrameric form correlates strongly with the transcriptional activity of the p53 protein. Dominant negative effect between the WT p53 and variant p53 proteins that arise due to mutations that occur within the sequence coding for the DNA-binding domain, such as R273H, have been known for several decades now. However, it is still not clear how p53 variants that arise from mutations within the sequence coding for the TD can affect the functional properties of the WT p53 protein. Up to our work, it was also unclear whether p53 proteins from different species in the evolutionary process could alter the functions of the other p53-family members via heterotetramer formation. In this study, I addressed the question of heterotetramer formation by examining the p53 proteins from two

different species using *in vitro* biochemical, crystallographic, NMR, and intracellular molecular biological methods. In many tumor cells including the Li-Fraumeni syndrome, one allele expresses the WT p53 protein whereas the other allele expresses a variant p53 protein that arises as a result of a missense mutation in the TP53 gene. However, the interaction that results from this heterologous expression of a variant p53 protein containing an amino acid substitution in the TD and the WT p53 protein, as well as the effects of these substitutions on overall p53 function, were unclear.

In chapter 2, I demonstrated that TD variants of the p53 protein formed heterotetramers with the WT p53 protein and decreased total p53 regulated transcriptional activities. The results suggested a novel concept of the dominant negative-like effect by the mutations that alter the amino acid composition of the TD of p53. This effect may be a key part of the mechanism of tumorigenesis by the mutation associated with the Li-Fraumeni syndrome. A detailed investigation of the binding of the Li-Fraumeni associated variant p53 proteins to each p53 regulated response element in target genes is required to better understand the mechanism underlying this dominant negative-like effect. To start transcription of the genes, recruitment of the transcriptional machinery to the response element of the target gene require DNA bending. Therefore, it would be valuable to be perform experiments to examine how mutations that lead to proteins with substitutions in the TD change the ability of the resulting p53 proteins to bend DNA.

In Chapter 3, we performed heat stability analysis, transcriptional activity analysis, and crystal structure analysis of p53 TDs from several different mammalian species (human, tree shrew, guinea pig, Chinese hamster, sheep, and opossum). The results

showed that, despite sharing high sequence homology, the thermal stability of tree shrew and opossum p53TDs was significantly higher than that of other mammals tested. The high stability was also accompanied by high transcriptional activity. Crystal structure analysis also revealed that the packing of the hydrophobic core that stabilized the tetrameric structure is important for the high stability of these two p53TDs. The high stability of these two p53TDs suggests that they have evolved in line with mammalian ecology, such as to counter circadian changes in body temperature in the tree shrew and cell tumorigenesis in the opossum fetus. These results also suggest that subtle changes in the sequence of p53TD can dramatically alter the stability of the p53 protein and cause important changes in its functional activity.

In chapter 4, I perform a comprehensive analysis of the evolution of the TDs of p53-family proteins in vertebrates. Analysis of the p63TDs from different species revealed surprising differences in their thermal stability, which is in sharp contrast to the high sequence similarity between them. Furthermore, structural analysis by X-ray crystallography revealed that the Zebrafish p63TD, which belongs to ray-finned fish, forms a disulfide bond between the two monomers that form the dimer, which is not found in other species and this helps to explain the reason for the high thermal stability observed for the Zebrafish p63TD. Furthermore, analysis of the hetero-polymorphism involving p53TD and p63TD in vertebrates revealed that this occurs in the early vertebrate Lamprey, but appears to have been lost during the subsequent evolution to ray-finned fish as well as to terrestrial organisms including humans. Based on these results, we propose a model in which p53 has acquired its cancer-suppressing function by evolving to a homotertramer

only protein which occurs as a result of changes in the thermal stability of its p53-family proteins. I think that the findings on the structures and interaction of p53 and its homolog p63 in this study provide insight into the specific function and significance of the tetrameric formation of p53 family proteins.

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