p53, a Novel Inhibitor of Apoptosis

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Declaration of Originality

The work presented in this thesis is planned and done by the author, however, with regular discussion and feedback from the supervisors, post-doctoral fellows and colleagues in the various laboratories. Works which are not done by the author are appropriately referenced.

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Abstract

p53 is a transcription factor known to induce apoptosis via transactivating the expression of pro-apoptotic proteins and by directly activating the mitochondria apoptotic pathway. p53 is also found to be mutated in 50% of human cancers with some of these tumours overexpressing both wild type (WT) and mutant p53. Overexpression of the p53 protein has been implicated with the more aggressive nature of these tumour cells, suggesting a possible gain-of-function of such mutants. In this study, the involvement of p53 in apoptosis via the caspase pathway was investigated. WT-p53 and three mutant p53 with mutations at the 1) N-terminus (D42Y), 2) central domain (R175H) and 3) C-terminus (R337H) were selected. The data collected demonstrated that p53 was able to inhibit the cleavage of caspases early in the apoptotic pathway. *In vitro* assays showed that addition of recombinant WT and the mutant p53 inhibited the cleavage of both caspase-9 and caspase-3 and subsequently PARP, while overexpression of p53 in mammalian cells yielded the same inhibition profile *in vivo*. Conversely, removal of p53 via siRNA and immunodepletetion showed accelerated caspase-9 activity and cleavage. Immunoprecipitation experiments and recombinant assay systems suggest that the inhibition by p53 is targeted at the active cleaved caspase-9. In addition, the presence of p53 (WT or mutant) in p53-null cells were able to confer a higher survival rate. These data therefore demonstrate that p53 may have an additional anti-apoptotic role via the inhibition of caspase-9, which is often masked by its pro-apoptotic functions. This anti-apoptotic role could manifest itself in a cancer background overexpressing mutant p53 which has lost its transcriptional activity and hence its ability to induce apoptosis via the induction of pro-apoptotic genes such as PUMA and NOXA. This newly discovered role of p53 could potentially explain the aggressive nature of cancers which overexpress p53.

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Chapter 1

Introduction

p53 is a transcription factor first discovered in 1979 as a mouse protein which interacts with the large T-antigen produced in simian virus 40 (SV40)-transformed cells [1]. The discovery came at a time when cancer research was focused on tumour-causing viruses.

In the 1970s, scientists were characterising proteins overexpressed in virus-transformed cells. Antibodies generated to detect these viral oncoproteins led to the discovery of the SV40 large and small T-antigen. Subsequent work by Lane and Crawford [1] found a non-viral protein of 53kDa that often forms a complex with the large T-antigen. This finding was concurrently reported by other laboratories around the world [2-5]. p53 was also later found in human cells, with a large quantity of this protein present in tumour cell lines that arose from spontaneous mutations or in SV40-transformed cells [6].

Due to its association with the SV40 large T-antigen protein, p53 was initially thought to function as an oncogene. In addition, p53 was also discovered to co-precipitate with the E1B tumour antigen, produced by another small DNA virus, the adenovirus [7]. Similar to other oncogenes, p53 was found to be overexpressed in many tumours [8]. It was not until almost a decade later that p53 was instead established to be a tumour suppressor.

As research on p53 progress, the *p53* gene was cloned in several laboratories. However, they soon realized that not all the cloned *p53* genes were able to result in cell transformation. Around the same time, p53 was also discovered to be inactivated by retroviral insertion in an Abelson murine leukaemia virus-transformed mouse cell line [9]. In another human derived leukaemic cell line, HL60, the *p53* coding sequence was found to be almost completely deleted [10]. Similarly, p53 knock-out mice displayed rapid malignant progression [11]. These findings pointed to the fact that *p53* might be an important gene in preventing the transformation of cells. As the researchers' findings converged, it was finally

revealed that the transformation abilities of p53 initially observed were due to the mutant forms of the *p53* gene [12,13], while the wild-type (WT) p53 actually functions as a tumour suppressor [14,15].

Since then, p53 has been shown to be implicated in most forms of cancer. Over the decades, 50% of human cancers were found to have mutations in the *p53* gene which directly compromise the functions of the p53 protein it encodes, while a large proportion of the rest have mutations in other genes which affect the functions of this important protein [16]. It is hence not surprising that current cancer research has focused on 1) p53 itself; 2) the numerous other proteins that either interacts directly with p53; 3) or on its transcriptional products; producing no less than 50,000 publications which are identified in a Pubmed search on "p53".

Besides being heavily implicated in cancer, p53 was shown to be involved in multiple cellular processes ranging from cell cycle arrest [17], senescence [18], stress [19], to apoptosis [20,21]. With its myriad roles, it is no wonder that mutations in this gene would result in considerable disturbance in cellular functions, affecting the survival of the cell, the organ, or the individual whose cells harbour the mutation(s).

1.1 Structure of p53

p53 is a polypeptide of 393 amino acids that is organized into five distinct domains (Figure 1.1). The amino-terminus encodes the transactivation domain (TAD), necessary for activating the target genes downstream of the promoter to which it binds. A Nuclear Export Signal (NES) is also within this domain. This is followed by the Proline-Rich Domain (PRD) which is critical for protein-protein interaction [22]. Next lies the DNA-binding domain (DBD), which is responsible for binding to the DNA consensus recognition element unique to *p53* [23]. Situated near the carboxyl terminus are several Nuclear Localisation Signal (NLS) as well as a NES. The NES is located within the Oligomerisation domain, also known as the

Tetramerisation Domain (TD). The last domain is the basic domain (BD), characterized by the highly basic amino acids which make up this segment. When p53 is activated, the protein forms a tetramer which allows the complex to remain in the nucleus and bind specifically to the promoters of its target genes [24,25].

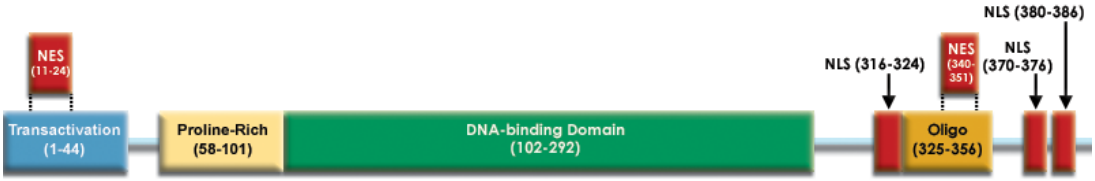


Figure 1.1. Structure of p53. Diagram showing the various domains of p53 and the amino acid residues that spans the domain [26].

1.1.1 The Transactivation and the Proline-Rich Domain

The TAD and the PRD (amino acid resides 1-101) make up the N-terminus of the p53 protein. Both domains are responsible for interacting with the transcriptional machinery of the cell to drive the expression of genes responsive to p53.

The TAD has a high content of aspartic and glutamic acid residues while the PRD, as the term suggest, consist of very high number of proline residues. Within the PRD region, five PXXP repeating sequences can be found, where P stands for Proline while X refers to any amino acid.

Based on the target genes that are activated, both domains can be reorganized into Activation Domain (AD) 1, which spans from amino acids 1-42, and AD2, which consists of amino acids 43-92. AD1 has been shown to drive the expression of genes involved in cell cycle arrest, while AD2 is responsible for transactivating genes involved in apoptosis [27]. Indeed, mutations to critical resides in both domains impairs the transactivation of the corresponding group of genes. The L22Q/W23S double mutation in AD1 resulted in apoptosis but not cell cycle arrest [28], while mutations to residues 53 and 54 (W53Q/F54S)

or deletion of residues 43–63 or 64–92 resulted in p53 proteins that are unable to induce apoptosis [29].

As the main function of the TAD and PRD is to transactivate genes, these domains have known interactions with several proteins involved directly in transcription. For example, the AD1 interacts with TATA box binding protein (TBP), TBP associated factors (TAF) TAFII31, TAFII70, p300/CBP and Pin1, while the AD2 interacts with p300/CBP, Pin1 and other transcriptional proteins. In addition, the PRD can interact with proteins with SH3 domains [30]. Pin1 positively regulates AD1 and AD2 through isomerisation of prolines within AD1 and AD2, resulting in conformational changes in p53. The conformational change stabilizes p53, which then promotes its DNA binding and transactivation functions [31].

An interesting fact to note within this region is that the PRP houses the polymorphic residue 72, which can either be a proline (P72) or arginine (R72), hence altering one of the five PXXP regions. The PXXP regions share similar properties to SH3-binding proteins, and thus were proposed to be involved in p53-mediated apoptosis [32] and for suppressing tumour growth [22]. The replacement of proline by arginine at residue 72 would cause a disturbance to the functions of p53, and has been shown to affect p53 activity. Interestingly, haplotype analysis has shown a strong emergence of the R72 allele which is clearly being actively selected for in humans living away from the equator [33].

1.1.2 The DNA-Binding Domain

The DBD occupies the central region of the p53 proteins and spans residues 93 to 292. The core function of this domain is to bind to the promoters of p53-responsive genes during the transactivation process. The promoters of these genes bear a canonical p53-responsive element (RE) consisting of one or two decamers of the sequence RRRCWWGYYY (where R=purine, C=cytosine, W=adenine or thymidine, G=guanine, and

Y=pyrimidine) with a spacing of 0 to 13 base pairs between each decameric recognition sequence [23]. Studies by Cho [34], showed that monomeric p53 binds to a pentameric sequence within the p53-RE, while an active tetrameric p53 binds to the full consensus sequence.

While the majority of p53-responsive genes carry the p53-RE within their promoters, research has shown that p53 can bind to other DNA sequences. For example, p53 can bind to the pentanucleotide polymorphicmicrosatellite $(TGYCC)_n$ sequence of the p53-induced gene 3 (PIG3) [35] and also the 12-bp palindromic sequence (CCCCACGTGAGG) of the phosphatase of activated cells 1 (PAC1) [36] gene. The diverse recognition sequences allow p53 to control and transactivate more genes in response to various cellular signals that converge on the activation of p53.

The binding of p53 to the REs is facilitated by the anti-parallel β-sheets that are formed by the DBD. Specific amino acid residues within the DBD form critical points of contact between the DBD and the DNA. For example, R273 associates with the DNA phosphate backbone while R280 interacts with the major groove [34]. As such, it is not surprising to find that almost 75% of p53 mutations in human tumours are in the central region of p53 [37]. One such mutation is the R273H. This mutation affects the binding of p53 to DNA [38], hence resulting in reduced transactivation of p53-responsive genes. A study by Bissonnette [39] showed that the R273H p53 mutant was unable to induce the expression of the pro-apoptotic, p53-responsive $p21^{WIP1/CIP1}$ gene.

Another hotspot mutation, R175H, disrupts the DBD structure by destabilizing the β-sandwich core domain and as a result the mutant lacks the appropriate scaffold for proper interaction with DNA. This seems to be a more potent mutation as it was able to transform mammalian epithelial cells when overexpressed, as compared to the R273H mutation, which

only induces prolonged cell survival [40]. Hence, it is clear that mutations in this domain can have undesirable effects on cells and their survival.

1.1.3 The Tetramerisation Domain

Tetramerisation of p53 is very important for its function as a transcription factor [41], as transactivation of p53-responsive genes can only take place when p53 tetramerises. The TD comprises of residues 326-356. A single TD folds to form the secondary structures of a β-sheet followed by an α-helix. p53 monomers dimerize through anti-parallel β-sheet and α-helix interactions. The dimers then tetramerise and are stabilized through hydrophobic forces generated by the helix–helix interactions [42].

Although mutations in this domain are less common than those in the DBD, the consequences of such mutations are still detrimental to p53's function, and the overall survival of the organism. A common mutation within the TD is R337H. This mutation has been implicated in the tumorigenesis of 36 children suffering from adrenocortical carcinoma (ACC) [43]. In this case study, the R337H mutation occurred in 35 out of the 36 children. The mutant R337H was found to be less stable and highly sensitive to pH, which could then contribute to the high incidence of ACC in these children. In a more recent study, a newly identified p53 mutant, K351N (found in cisplatin-resistant ovarian carcinoma cell line (A2780-cis) has been shown to have reduced stability and was further demonstrated to lack the ability to transactivate pro-apoptotic genes such as Bax [44] upon cisplatin challenge.

Overall, mutations in the TD disturb the tetramerisation of p53 and its ability to transactivate crucial genes involved in processes such as apoptosis, which could then possibly contribute to the tumorigenesis in cases such as the one mentioned above.

1.1.4 The Basic Domain

The BD spans the last 30 amino acids of the p53 protein. It is referred to as the BD as it largely consists of basic amino acids such as lysine and arginine. Unlike the DBD and the TD, this domain has no regular secondary structure. However, it does show some secondary structure upon association with specific proteins. For example, when complexed with S100B or phospho-CDK2-cyclin A, it folds to form a helical structure [45,46], while its binding to the deacetylase Sir2 or to the bromodomain of CBP results in an extended conformation [47,48].

However, the BD's contribution to the function of p53 lies not in the structure but the numerous post-translational modifications that could take place in the residues of this domain. These post-translational modifications include phosphorylation, methylation, acetylation, ubiquitination, neddylation, or sumoylation [49]. The regulation of p53 will be further discussed in Section *1.3*.

1.2 The Different Roles of p53

Since the discovery of p53 as a tumour suppressor, increasing evidence has been gathered to uncover novel functions of p53 that implicate the protein in enhancing cell survival. Generally, in cells damaged by cytotoxic insults, the role of p53 is to activate pathways which either repair this damage; or induce the damaged cells to enter apoptosis once these cells are deemed "beyond repair". The section here aims to bring attention to some of the more widely studied roles of p53.

1.2.1 In Cell Cycle

One of the roles of p53 is in controlling the progression of the cell cycle during cell division. $p21^{WAF1/CIP1}$, a transcriptional target of p53, is an inhibitor of cyclin-dependent kinases (CDKs). CDKs are key regulators of the cell cycle, allowing cells to enter S phase

from G1 phase. When DNA damage is detected, the induced expression of $p21^{WAF1/CIP1}$ by p53 halts cell cycle progression [17], enabling the damaged DNA to be repaired before the cell replication cycle is resumed.

In addition, p53 can also control cell cycle progression in the G2/M phase. A protein that functions in this is the 14-3-3σ. The induced expression of 14-3-3σ by p53 upon DNA damage helps to sequester the cyclin B1–CDK1 complexes outside the nucleus. This cytoplasmic mislocalisation of the complex maintains the cell in G2 arrest [50,51]. Another protein involved in this checkpoint is the Gadd45α protein. A p53-responsive protein, it binds to the proliferating cell nuclear antigen (PCNA) protein which is involved in DNA replication and repair. Thus, p53 functions to stimulate DNA repair through the expression of Gadd45α while at the same time inhibit the progression of cells into the mitotic phase [52].

Hence, the role of p53 here is to maintain genomic stability, minimize the replication of cells with DNA damage and mutation, and indirectly stimulate the repair of such damaged DNA.

1.2.2 In Senescence

As a more permanent control of aberrant cell proliferation, p53 is involved in inducing senescence in cells. Cells that are senescent cease to proliferate and accumulate G1 DNA content, indicating an arrest prior to the DNA replication stage in the cell cycle [53]. Plasminogen activator inhibitor-1 (PAI-1), a downstream target gene of p53, participates in this aspect to induce senescence in DNA damaged cells. Its knockdown in primary mouse embryo fibroblast through RNA interference resulted in continual cell replication, suggesting its importance in preventing oncogenic transformation in such damaged cells [54].

DNA damage such as that induced by dysfunctional telomeres is one of the triggers of senescence. In this case, upstream kinases such as ATM (ataxia-telangiectasia mutated) are

activated, which in turn phosphorylate factors such as checkpoint kinase (CHK)1, which then activate p53 [55].

Crosstalk between the senescence and the cell cycle arrest pathways is evident as activated p53 in senescence engages the same pathway as in cell cycle control involving $p21^{WAF1/CIP1}$ to stall cell proliferation.

1.2.3 In Oxidative Stress

Besides responding to cellular stress conditions such as DNA damage, hence activating cell cycle arrest or senescence, p53 also participates in activating pathways in response to conditions such as reactive oxygen species (ROS) and hypoxia. p53 reacts to the presence of ROS by inducing the expression of aldehyde dehydrogenase 4 (ALDH4)[19] and TIGAR (TP53-induced glycolysis and apoptosis regulator)[56] to reduce intracellular levels of ROS. p53 can also induce the expression of GLS2 (glutaminase 2), which increase the levels of glutathione, thereby also reducing the ROS levels [57]. Fast growing tumour cells often face hypoxic conditions where Hypoxia Inducible Factor 1-alpha (HIF 1-α) is activated, which in turns activates p53. p53 is then able to control cell proliferation in this oxygen-deprived environment by activating cell cycle arrest [58].

1.2.4 In Autophagy

In response to cellular stress such as nutrient deprivation [59] and serum withdrawal [60], the process of autophagy is induced. Autophagy (strictly termed macroautophagy, but often referred to as autophagy), is an evolutionary conserved membrane-trafficking process that operates at basal levels under normal conditions as a means of degrading cytosolic proteins and organelles [61]. Cytosolic proteins and damaged organelles such as the mitochondria are contained in double-membraned vesicles known as autophagosomes, which then fuse with lysosomes in the cytoplasm, to form the autolysosomes. The contents of the

autolysosomes are then degraded by the lysozymes, and released back into the cytoplasm [62]. This process allows for the recycling of cellular metabolites to counteract the effects of lowered levels of essential building blocks during nutrient deprivation.

The role of p53 in this aspect is to transactivate *DRAM* (damage-regulated autophagy modulator), which encodes a lysosomal protein that induces autophagy during cellular stress such as DNA damage [61].

Another set of genes that are transactivated by p53 are the *sestrins1* and *sestrins2* [63], which encodes proteins that inactivate mTOR (mammalian target of rapamycin), a kinase that inhibits autophagy [64].

However, studies by Tasdemir [65] demonstrated that cytoplasmically localised p53 actually inhibits autophagy. In fact, autophagy could only be induced if the function of the cytoplasmic p53 has been inactivated through the use of inhibitors such as pifithrin-α, the deletion of the *p53* gene or depletion of the protein. The implications of autophagy inhibition by cytoplasmic p53 in cancer will be discussed later.

1.2.5 p53 and Apoptosis

Another important function of p53 is in the induction of apoptosis. If cellular damage are beyond repair even after the activation of cell cycle arrest, p53 will then initiate the process of apoptosis to rid the body of such damaged cells. As a transcriptional factor and tumour suppressor, p53 has been shown to transactivate several genes whose products participate in the apoptotic pathway.

Apoptosis is a term coined by Kerr [66] to describe an intrinsic cell suicide program. Apoptosis is a regulated mode of cell death that occurs throughout development in all multicellular organisms. It is also involved in the death of cells during diseases [67,68] or when they are damaged [69,70], in tissue remodelling during embryogenesis [71,72], and in maintenance of homeostasis and immune functions [73]. Cells dying via apoptosis show

distinct morphological characteristics such as chromatin condensation, nuclear fragmentation, cell shrinkage, cytoplasmic condensation and apoptotic body formation [74]. The apoptotic bodies are then phagocytosed by macrophages, therefore preventing inflammation [75].

In order to better understand the mechanism in which a cell enters apoptosis, it is important to study in greater detail one of the main protein families involved in the apoptotic process, the caspases. The subject of this thesis, the interconnection between p53's functions and caspases will be highlighted.

1.2.5.1 The Caspase Family

The caspases are a distinct, highly conserved class of intracellular cysteine proteases. This family of proteins is characterized by their almost absolute specificity for aspartic residues in their substrate [76]. Caspases are constitutively expressed as inactive proenzymes [77]. They are only activated upon reception of death stimuli e.g. serum withdrawal. Activation of caspases requires the proteolytic cleavage at two sites within the protein [78]. The released subunits then assemble to form heterotetramers that are enzymatically active [79].

Caspases are divided into two subgroups based on their functions in the apoptotic pathways. The 'initiator' caspases consisting of caspases-1, -2, -4, -5, -8, -9, -10, -11, and -12 receive the initial death stimulus. The activation of this cascade of caspases usually converges upon a common set of the downstream 'effector' caspases. Effector caspases (consisting of caspases-3, -6, -7 and -14) are directly responsible for the cleavage of housekeeping proteins, causing death to the cells and also giving rise to the morphological characteristics observed during apoptosis. They can also inactivate proteins which inhibit apoptosis by cleaving them into fragments, for example the cleavage of Poly(ADP-ribose) polymerase (PARP), a nuclear protein involved in DNA repair [80].

In general, two different types of death stimuli exist which are able to activate distinct sets of caspases within the cells. These lead to the separate grouping of the caspases into two major apoptotic pathways. The two pathways are namely the death receptor (Fas-ligand)-mediated and the mitochondria-mediated pathways. The interaction of these two pathways and p53 are illustrated in Figure 1.2.

1.2.5.2 p53 and Fas-Ligand Pathway

The death receptor-mediated pathway is activated when ligands such as the tumour-necrosis factor (TNF) binds to the death receptor found on the surface of the cell. This leads to the recruitment of adaptor proteins such as FADD (Fas-associated death domain) and procaspase-8 to the receptor [81]. p53 is involved in this pathway through the transcriptional

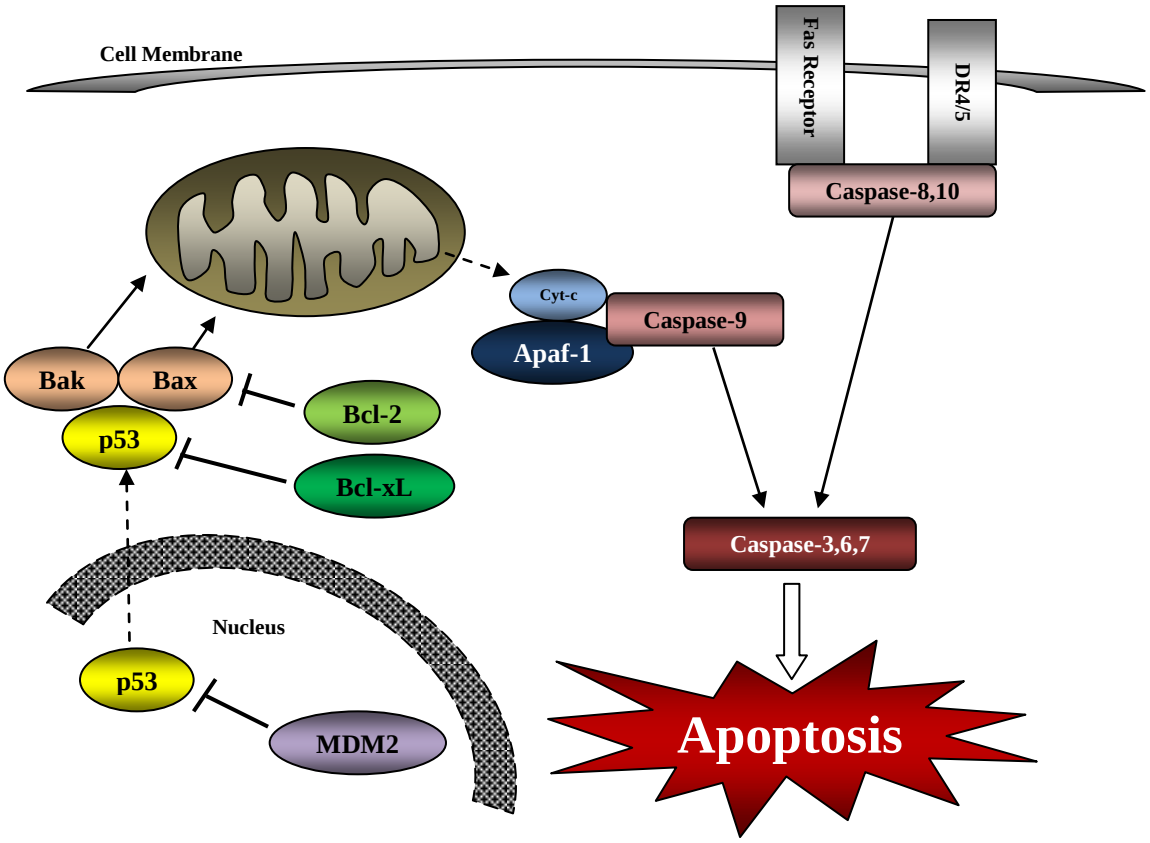


Figure 1.2. An illustration of the Role of p53 and Other Proteins Involved in the Various Apoptotic Pathways [82].

activation of genes encoding the death receptors located at the plasma membrane, including the Fas receptor [83] and DR4 [84]. This positive feedback system allows cells which are targeted for apoptosis to receive the death signal promptly for their ultimate destruction.

1.2.5.3 p53 and the Mitochondria Pathway

The mitochondrial apoptotic pathway is a complex network involving the caspases as well as the Bcl family of proteins. Cellular stress such as cytotoxins can damage the mitochondria, resulting in the disturbance of the membrane potential and the release of mitochondrial proteins such as cytochrome-*c*. Cytochrome-*c*, when present in the cytosol, triggers the formation of the complex 'apoptosome'. The apoptosome is composed of Apaf-1, cytochrome-*c* itself and caspase-9. This apoptosome complex can trigger the activation of both caspase-9 and caspase-3 within, thus leading to apoptosis [85].

Regulators of this pathway are provided by members of the Bcl family, which are either anti-apoptotic or pro-apoptotic. Anti-apoptotic members such as Bcl-2 and Bcl-xL have been shown to delay the activation of caspases by inhibiting the release of cytochrome-*c* [86,87]; while pro-apoptotic members such as Bax accelerates apoptosis by disturbing the mitochondrial potential via the formation of a complex within the mitochondrial membrane, thereby facilitating the release of cytochrome-*c* [88].

Bax is a p53-inducible protein. In normal cellular conditions, Bax is found associated with anti-apoptotic proteins such as Bcl-2 and Bcl-xL, while p53 is also kept in check through the association with these proteins [89]. PUMA is another transcriptional target of p53. In cellular stress, upregulation of PUMA by p53 allow the displacement of Bax [90] and p53 from Bcl-2 and Bcl-xL. This in turn allows Bax to induce apoptosis through the mitochondria, while p53 can further induce the upregulation of more pro-apoptotic genes to positively regulate this pathway. The importance of Bax in apoptosis was demonstrated in

report which showed that decreased levels of Bax correlated with lower apoptosis and increased tumour growth in mouse [91].

1.2.6 Transcription-Independent Role of p53 in Apoptosis

The majority of research on p53 is centred on its transcriptional role and its target genes. Judging from the extensive array of p53 target genes involved in the diverse cellular functions, the heavy focus on this area is more than justified. However, p53 does possess the ability to induce apoptosis that is independent of transcription.

One such clue came from the study of a temperature sensitive p53 mutant. At the permissive temperature, the upregulation of pro-apoptotic genes and apoptosis took place as expected. However, when drugs were added to inhibit transcription and translation, expression of the p53 target genes was abolished while apoptosis could still take place [92]. In other studies by Haupt [93,94], p53 proteins that did not possess the transactivation domain were able to induce apoptosis, although the known target genes of p53 in apoptosis were not found to be upregulated. Further evidence came from Marchenko [95], which demonstrated the localisation of p53 (a predominantly nuclear localised protein) to the mitochondria upon challenge by a cytotoxin e.g. camptothecin. This relocalisation of p53 was observed to be rapid and preceded changes in the outer mitochondrial membrane (OMM) potential, cytochrome-*c* release, and procaspase-3 activation.

In 2004, Chipuk [20] showed the activation of Bax directly by p53. The activated Bax induced the collapse of mitochondria potential, leading to release of cytochrome-*c* and caspase-9 and caspase-3 activation. This novel role of p53 was demonstrated convincingly using an *in vitro* system consisting of recombinant p53, recombinant Bax and isolated mitochondria. The combination resulted in the release of cytochrome-*c*, proving the transcription-independent role of p53 in apoptosis.

Another interacting partner of p53 in this transcription-independent role is Bak. Bak is a multidomain protein which is found integrated into the lipid layer of the outer mitochondria membrane. Similar to Bax, Bak is able to oligomerise to form a pore on the OMM, hence disrupting the membrane potential and inducing apoptosis via the release of cytochrome-*c* [96]. A small percentage of cellular p53, which is localised in the mitochondria, was shown to interact with Bak and induce its oligomerisation to result in the release of cytochrome-*c* upon cytotoxic challenge [97,98].

Interestingly, the transcriptional-independent induction of apoptosis by p53 is dependent on the different cellular location of p53. Upon cytotoxic stimulus, p53 can translocate from the nucleus into the cytoplasm [20,99] and the mitochondria [95,100]. The cytoplasmically localised p53 can then activate Bax while the mitochondria-located p53 activates Bak.

Apart from causing the release of cytochrome-*c* through inducing OMM permeabilization through Bak and Bax, p53 can also cause the same phenomenon by antagonizing the actions of anti-apoptotic proteins such as Bcl-xL and Bcl-2, allowing cytochrome-*c* release and apoptosis [21].

Hence, p53 performs its tumour suppressive functions not only by inducing the expression of gene in response to cellular stress and apoptosis, but also by directly interacting with proteins involved intimately in the apoptotic regulation. Its non-nuclear localisation also serves to ensure that destruction of the damaged cell can take place efficiently when the need arises.

1.3 Regulation of p53

From the diverse cellular situations that initiate the transactivation of the multitude of genes that are responsive to p53, it is no doubt that p53 functions as a node for organizing whether the cell responds to various types and levels of stress with cell cycle arrest and DNA

repair, senescence, cell metabolism, autophagy or apoptosis. Being termed the "guardian of the genome" [101] and the "cellular gatekeeper" [102], it is of utmost importance for the cell to regulate a protein such as p53.

1.3.1 Negative Regulation of p53 – MDM2 and Ubiquitination

Given that p53 is a tumour suppressor and that a constitutively active p53 could potentially result in dire outcomes, a normal healthy cell would want to keep this protein in an inactivated state and its cellular abundance in check. The stability and half-life of p53 is tightly regulated by the MDM2 (Murine Double Minute 2) [103], sometimes referred to as HDM2 in humans. By far, MDM2 is the most well-studied regulator of p53 stability.

p53 is constantly bound to MDM2 via the TAD [104] and DBD [105]. MDM2 is an E3 ligase responsible for targeting p53 for ubiquitin-dependent degradation [106]. It transfers ubiquitin moieties onto the lysine residues of p53 [104], which then exposes the NES in the C-terminus of p53, allowing it to dissociate from MDM2 and translocate from the nucleus into the cytoplasm [107]. In the cytoplasm, an ubiquitinated p53 is recognized by the 26S proteosome, which then targets it for degradation [108]. The importance of MDM2 in regulating p53 was highlighted in two studies. It was found that even when an extra copy of p53 were added to cells, a reduced expression of MDM2 were sufficient to regulate p53 levels and maintain homeostasis [109,110]. In addition, mice that were $MDM2^{-/-}$ were not viable and the embryonic lethality were rescued by a concurrent deletion of the *p53* gene, suggesting that the regulation of p53 by MDM2 is an essential cellular function [111]. It is therefore not surprising that over a third of human cancers with wild-type p53 contain multiple copies of MDM2 gene [112].

The ubiquitination of p53 by MDM2 is effective in regulating the stability and half-life of p53. The process of ubiquitination not only reduces the levels of p53, it also targets p53 into the cytoplasm, thereby reducing its levels in the nucleus, and hence its ability to

transactivate its target genes. At the same time, the binding of MDM2 to the TAD of p53 further prevents p53 from inducing the expression of its target genes [113].

In addition to MDM2, MDMX (sometimes referred to as MDM4), a MDM2 homolog, can interact and form a heterodimer with MDM2. This heterodimer of MDM2-MDMX was found to be a better ubiquitin ligase for p53, as compared to a MDM2 homodimer [114]. Similar to MDM2, $MDMX^{-/-}$ mice are embryonic lethal and can be rescued by the concurrent deletion of the p53 gene, hence demonstrating the importance of MDMX in p53 regulation [115].

1.3.2 Negative Regulation of p53 – Post-Translational Modifications of p53

Besides ubiquitination, p53 is extensively modified post-translationally through phosphorylation, acetylation, methylation, sumoylation and neddylation. It appears that most post-translational modifications serve to enhance p53 stability and activities, with a few as negative regulators of p53. To show the extent of p53 modification, a beautiful diagram by Boehme and Blattner [49] has been included as Figure 1.3.

Phosphorylation at the serine and threonine residues of p53 is common. Phosphorylations at serine 362 and 366, mediated by IκB kinase 2 (IKK2), targets p53 for degradation [116]. Also, COP9 signalosome kinase (CSN-K) has been found to promote p53 degradation by phosphorylating threonine 155 [117].

Methylation is another post-translational modification of p53, although not as extensive as phosphorylation. Huang [118] found that the methylation of lysine 370 by SMYD2 (a lysine methyltransferase) reduced the binding of p53 onto the promoters of genes such as *p21*, and that knockdown of *SMYD2* correlated with increased p21 expression and apoptosis.

Neddylation is a somewhat not so common form of modification. It involves the covalent addition of ubiquitin-like protein Nedd8 to the lysine residues of the modified

protein [119]. MDM2 has been found, among other proteins, to neddylate lysine residues K370, K372 and K373 in the C-terminus of p53 [120], thereby inhibiting its transcriptional activities.

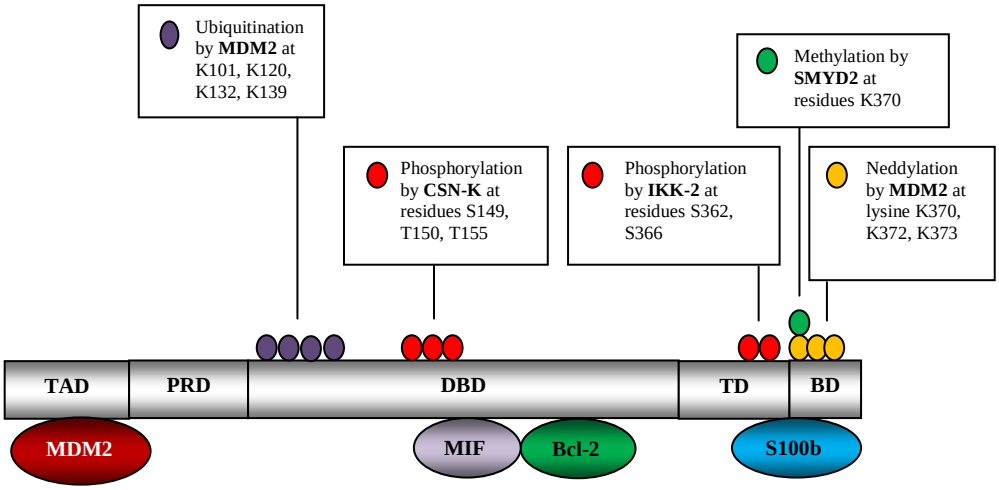


Figure 1.3. Negative Regulation of p53 through Post-Translational Modifications and other p53 Interacting Proteins. Illustration of the ubiquitination, phosphorylation, methylation and neddylation sites on p53 that increase its stability in the cell. All modifications are colour-coded with the corresponding protein responsible for the modifications identified in the same diagram.

1.3.3 Negative Regulation of p53 – The Role of its Interacting Partners

Through the years of research, interacting partners of p53 that regulate its activities were uncovered. Proteins that bind to the N-terminus and DBD of p53 generally suppress the transactivation properties of p53, for example MIF (macrophage migration inhibitory factor) [121]. Others associate with the TD of p53, thereby repressing p53 functions through preventing the formation of p53 tetramers. Examples of such proteins can be found in the S100 family, which prevent p53-mediated transactivation by hindering the oligomerisation and retaining p53 in the cytoplasm [122]. iASPP binds to the PRD of p53 and specifically inhibits the expression of Bax [123]. Apart from affecting the transactivation properties of p53, there are also proteins which can regulate its transcriptional-independent functions. Bcl-

2 interacts with p53 at the DBD to prevent the induction of apoptosis through p53-dependent activation of Bax and Bak proteins at the mitochondria [20,21].

1.3.4 Positive Regulation of p53 – Inhibiting the Inhibitor

In normal physiological conditions, p53 is constantly produced but the levels are maintained low by the recurring ubiquitin activities of MDM2 or by the other proteins mentioned above. However, in conditions where the functions of p53 are needed, the level of the protein has to be elevated. One of the major ways of increasing the levels of p53 is to relieve its inhibition from the negative regulation of MDM2.

To relieve this inhibition, phosphorylation of serine residues e.g. serine 20 within the TAD causes a conformation change, resulting in reduced affinity for MDM2 [124]. Tetramerisation of p53 then takes place, which hides the NES within the carboxyl terminus. Consequently, p53 is retained within the nucleus, allowing it to activate the expression of its target genes [25]. In addition, stability of p53 can also be enhanced by the degradation of its antagonizer MDM2, mediated by the alternate reading frame (ARF) protein, hence relieving p53 from the inhibitory actions of MDM2 [125].

1.3.5 Positive Regulation of p53 – Changing the Facet of p53

The stability and activity of p53 can also be mediated by post-translational modifications. Methylation at serine 372 by Set9 methyltransferase [126] and demethylation at lysine 382 by p53 binding protein-1 (53BP1) [127] increase the stability and thereby p53-mediated response to cellular stress.

Acetylation of p53 also plays a two-pronged role in stabilizing the protein. Acetylation of p53 can first disrupt its interaction with MDM2 [128] and MDMX [129], and subsequently increase the transactivation of pro-apoptotic genes such as Bax and p21 [130]. Acetylation of p53 is performed mainly through acetyltransferases such as p300/CBP [131].

Major sites of acetylation include lysine 164, 320, 373 and 382 [129,130,132]. In addition, acetylation of lysine 320 enhances the cytoplasmic localisation of p53. This could then enable p53 to perform its apoptotic roles in the cytoplasm [133].

The less common forms of post-translational modifications include neddylation and sumoylation. Studies on the process of neddylation seem to suggest that neddylated p53 are inactivated (as discussed in Section *1.3.2*), while there are conflicting roles of sumoylation on p53. In any case, for the enhancement of p53, studies have shown that sumoylation activates p53 and induces the transcription of pro-apoptotic genes such as *p21* [134,135].

1.3.6 Positive Regulation of p53 – The Roles of Other Proteins

While some proteins serve to prevent the binding of p53 to the promoters, others function as activators of p53. Such positive regulators of p53 mainly interact with p53 at the DBD and the TD. Apoptosis simulating p53 protein (ASPP)-1, ASPP-2 [136,137] and hematopoietic zinc finger (Hzf) [138] are examples of such proteins. These proteins function

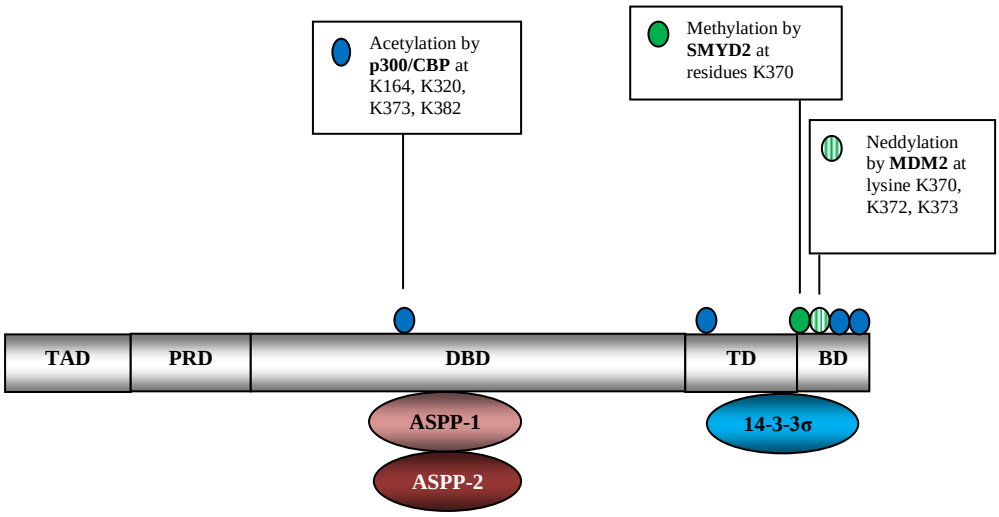


Figure 1.4. Positive Regulation of p53 through Post-Translational Modifications and other p53 Interacting Proteins. Illustration of the ubiquitination, phosphorylation, methylation and neddylation sites on p53 that increase its stability in the cell. All modifications are colour-coded with the corresponding protein responsible for the modifications identified in the same diagram.

to enhance the transactivation of p53 target genes by stimulating the binding of p53 to DNA. Positive regulators that interact with the TD of p53 enhance the tetramerisation of p53, and thereby induce the expression of its target genes. The Ref-1 (Redox/Repair-1) [139] and 14-3-3σ [140] belong to this group of proteins.

1.3.7 Regulation of p53 by p53

To add on to the complexity of the regulatory network of p53, p53 itself can be involved in the regulatory process, thereby forming feedback loops which can either positively or negatively regulate its own activities.

The most commonly discussed and studied feedback loops involve p53 and MDM2. MDM2 itself is a transcriptional target of p53 [141]. In circumstances where p53 is activated and free from the antagonistic actions of MDM2, the activated p53 can drive the expression of its targets genes in response to the stimulus, including MDM2. The consequential elevated expression of MDM2 would then inhibit p53 and reduce its levels, hence forming a negative feedback loop.

p53 can also positively regulate its stability and activities through feedback loops involving other proteins. For example, 14-3-3σ has been identified as a p53-inducible protein and a negative regulator of the cell cycle. Induced expression of this protein by p53 act to inhibit the ubiquitination of p53 by MDM2, and also promote the transactivation activities of p53 by enhancing its ability to oligomerise [140]. This feed forward loop may be necessary in times where the cell cycle needs to be halted, in order for p53-induced proteins involved in DNA repair to function, thereby reducing the propagation of DNA defective cells.

1.3.8 Regulation of p53 – A Delicate Balance

From the discussion on the regulation of p53, it is evident that several cellular stresses could result in the activation of p53; and that p53 can be activated through modifications of

itself, and also interactions with other proteins. The process that p53 elicit, could have conflicting outcomes for the cell. For example, DNA damage induces cell cycle arrest and DNA repair, while it could also cause cell death through apoptosis. Senescence and ultimately apoptosis could also result from genomic instability and cell cycle arrest. So how does a cell determine the cell fate in the face of the myriad of pathways elicited by p53?

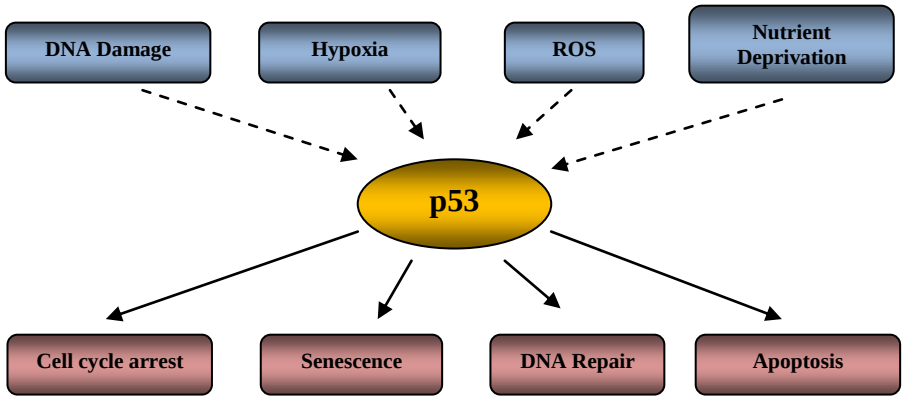


Figure 1.5. p53 as a Node for Integrating Cellular Stresses and its Possible Response. The diagram shows the various cellular process that could lead to the activation of p53 (box in blue), and also the responses that p53 can elicit (box in pink).

The answer lies in the controlling of the balance between the "activators" of each pathway. For example, while DNA damage could induce expression of proteins involved in DNA repair, cell cycle arrest, and even anti-apoptotic genes such as Bcl-xL[142], continual p53 activation would cause the expression of pro-apoptotic genes like Bax [143] and Noxa [144] at the same time. If the DNA damage is repaired, and modifications of p53 manage to inactivate p53 in time, the cell cycle resumes and apoptosis is halted i.e. the cell lives. However, should the damage be too severe, continual expression of pro-apoptotic proteins would have caused the balance to be tipped towards apoptosis. As a result, apoptosis ensues and the cell carrying the irreparable mutation is removed.

In summary, this delicate balance between the various pathways in a normal cell is held in place by its ability to regulate these pathways in response to various cellular

conditions. However, when certain genes in the pathways are mutated, the ability to maintain such a balance is lost, which could then result in oncogenic transformation and cancer.

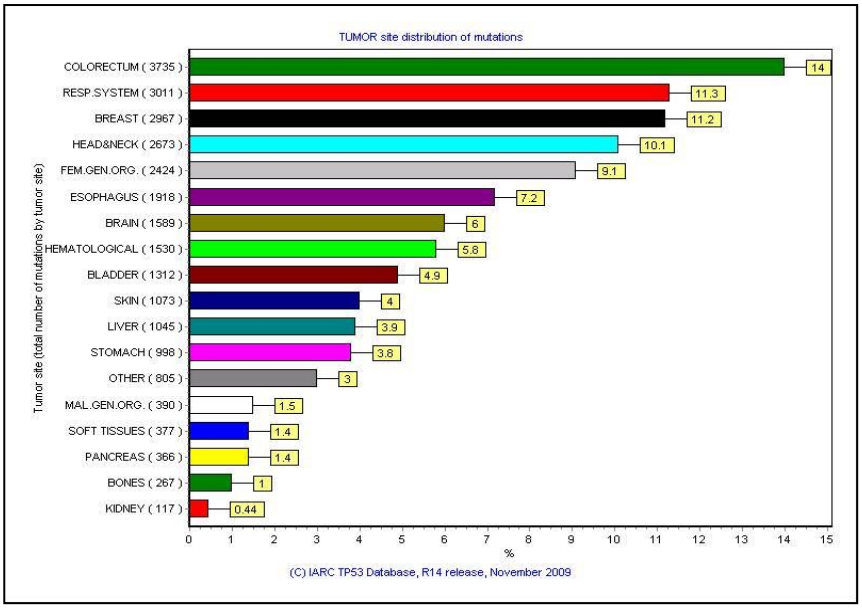
1.4 p53 in Cancer

With p53 playing such important roles in controlling cell survival and apoptosis, it is not surprising that mutation of such a gene would have disastrous consequences. In fact, throughout the 30 years of research on p53, it is now common knowledge that 50% of the human cancers contain mutations in the *p53* gene, while the rest of the cancers have mutations in genes that directly or indirectly affects the functions of p53.

1.4.1 The "Trends" of p53 Mutation in Cancer

Mutated p53 was found to correlate with the inability to induce apoptosis, with different p53 mutants conferring different degree of tumorigenesis [145,146]. From as early as 1985, the relationship between p53 mutation and cancer had been hypothesised. Le Beau's study [147] of acute promyelocytic leukaemia (APL) showed that the p53 gene located on chromosome 17 was translocated to chromosome 22 in patients suffering from APL. In 1989, Bodmer [148] found that mutations in p53 gene occurred in 50% or more of colorectal carcinomas. By 1991, a specific mutation has been linked to carcinogenesis. Hsu [149] and Bressac [150] found that a G to T substitution (causing an amino acid change from glycine to serine at codon 249) was prevalent in patients suffering from hepatocellular carcinoma (HCC) in China and South Africa. Hence, it was suggested by both groups that this mutation could be the factor that resulted in the carcinogenesis of HCC. Subsequently, increasing number of groups started focusing on a correlation between p53 mutation and diseases/cancer, with the first review connecting p53 mutation and cancer being published in 1991 [151].

A)



B)

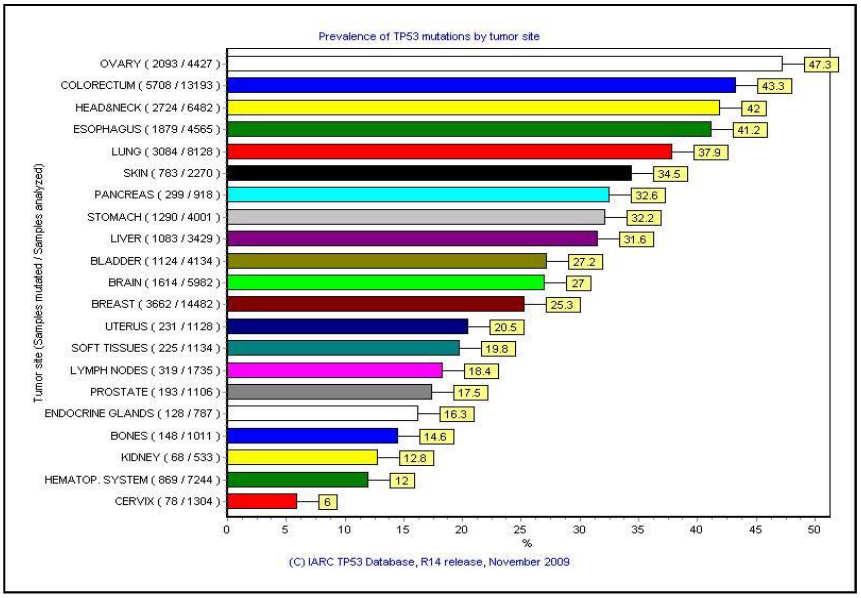
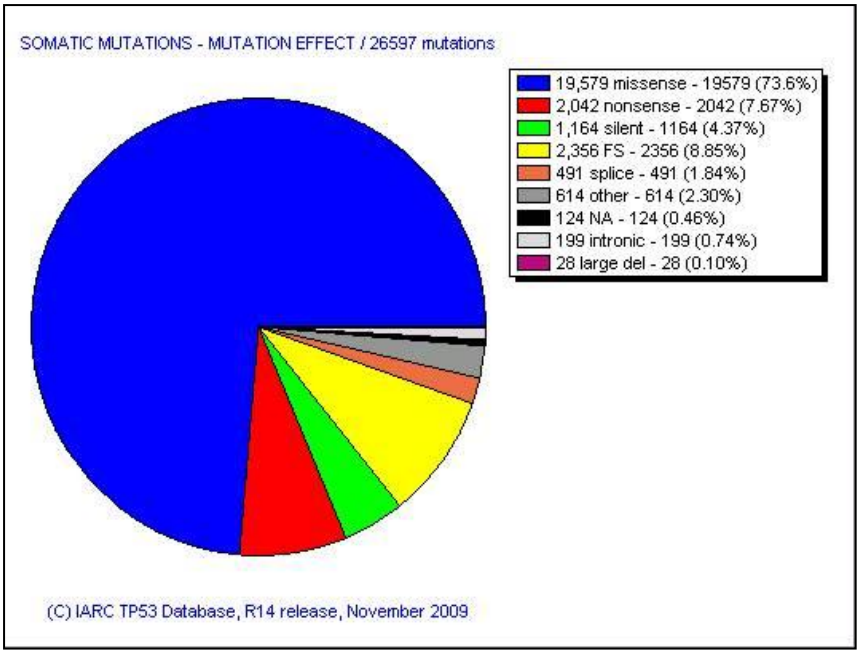


Figure 1.5. Database compilations showing the A) distribution of mutation and B) the prevalence of p53 mutation in tumours[152].

The International Agency for Research on Cancer (IARC) website on p53 is a database which consolidates and compiles the gene variations and mutations found in human and tumour samples [152]. The data are compiled from the peer-reviewed literature and from generalist databases. The database shows that to date, the cancer with the most number of different p53 mutations is the colorectal cancer, followed closely by the respiratory system

and breast cancer (Figure 1.5A). However, the cancer that contains tumours with the highest percentage of p53 mutation is ovarian cancer, with a prevalence level at 47.3% (Figure 1.5B).

A)



B)

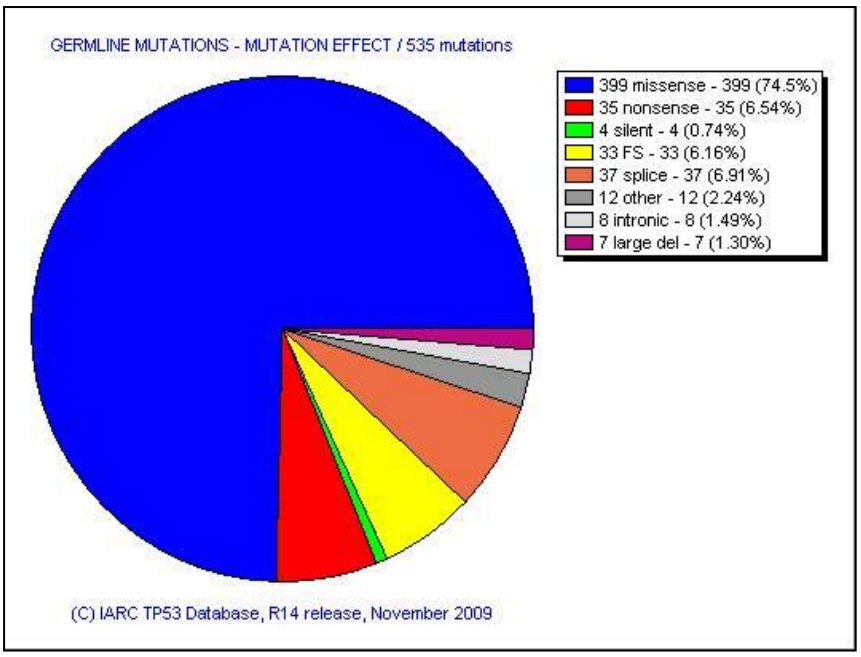


Figure 1.6. Diagram showing the distribution of the type of mutations found in p53 from A) somatic mutations and B) germline mutations[152]**.**

Many forms of mutation can be found in the *p53* gene. Deletion [153,154] or frameshift mutations [155,156] lead to the complete abrogation of p53 activities, while

translocation of the *p53* gene [147], and point/missense mutations [157-160], result in the production of full-length p53 but with altered functions. Interestingly, almost 74% of the mutations in p53 are missense mutations, that result in the synthesis of functionally defective p53 proteins [152,161] (see also Figure 1.6A).

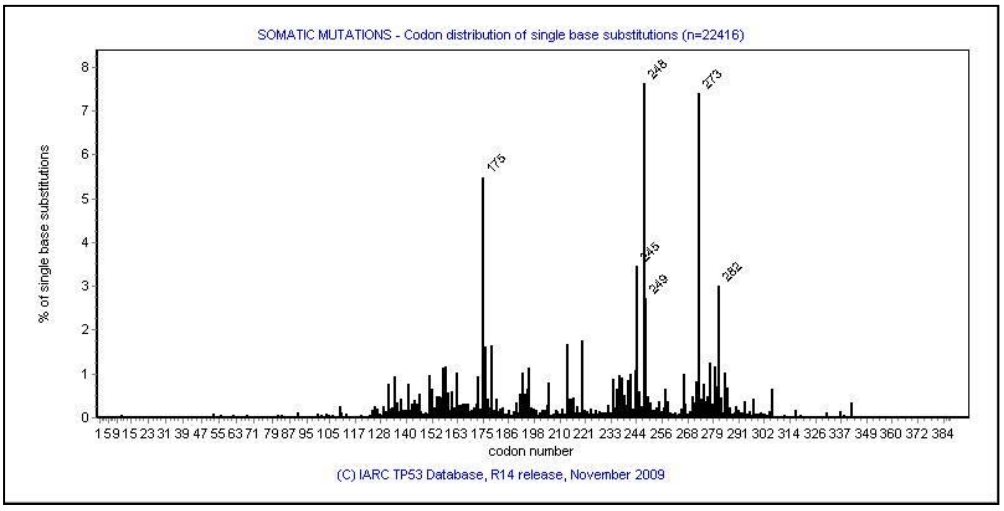
1.4.2 The Distribution of Mutation within p53

As 74% of the mutations in p53 are missense mutation, it had garnered a lot of attention from researchers in a bid to characterize and profile the missense mutations found in various types of cancer. Putting the data together, it is obvious that most of the mutations occur within the DBD (residues 93-292) [152,162] (Figure 1.7A). A closer look at the data shows that mutations at certain residues are more frequent than the others, especially at residues 175, 248 and 273. These residues have been termed "hot-spot" mutations [163,164].

Figure 1.7A depicts the distribution of the hot-spot residues found in cancers derived from somatic mutation of *p53*. However, not all cancers related to p53 are from somatic mutations. In fact, there is a condition known as the Li-Fraumeni Syndrome (LFS), in which individuals develop cancer as a result of a germline mutation in *p53*.

LFS was first described and named after the discoverer in 1988 who investigated a family of 151 blood relatives found to develop cancers such as bone cancers, brain tumours and leukaemia before the age of 45 years [165]. The development of these cancers was later attributed to inherited germline p53 mutation [166]. Like the profile found in somatic mutations, most mutations in LFS are missense mutations. However, LFS also carries a higher percentage of p53 gene deletion (1.3% - Figure 1.6B) as compared to diseases from somatic mutations (0.1%). Large p53 gene deletions were found in several families studied for their genetics in the p53 gene and the development of cancers [167]. In any case, missense mutations still stand at a majority of 74.5%, a figure that is comparable to those arising from somatic mutations.

A)



B)

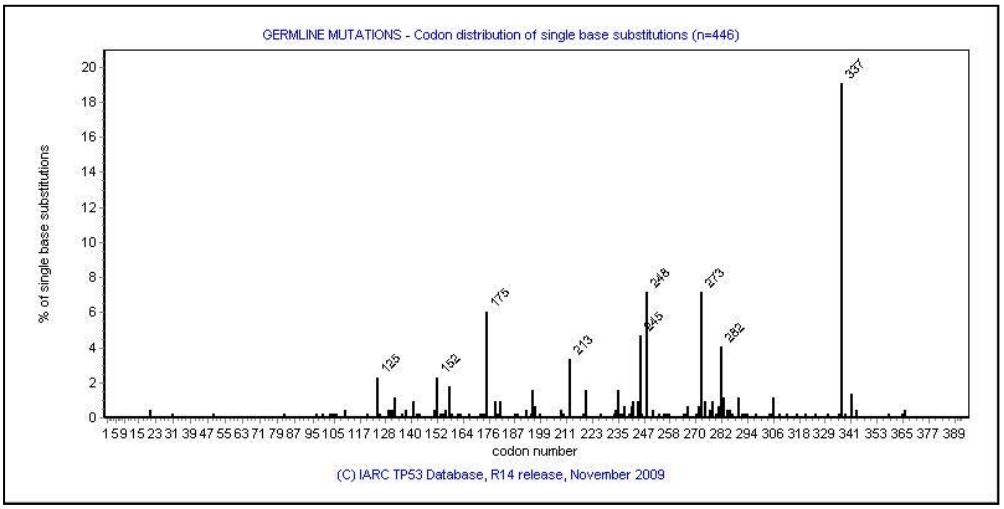


Figure 1.7. Profile of Codon Mutation Distribution in A) somatic mutation and B) germline mutation[152]**.**

The hot-spot mutations found in LFS are also relatively similar to those in somatic mutations, except for the arginine substitution at residue 337 (Figure 1.7B). As mentioned during the discussion on the TAD of p53, a study on the development of ACC in children in Africa showed that 47% of the p53 mutation were due to an amino acid change from arginine to histidine at residue 337 (R337H) [168]. This data hence contribute slightly different profile between those found in germline mutations and those in somatic mutations.

1.4.3 Hypothesis of Action of p53 Mutants

With such a high percentage of human cancers showing mutations in either p53 itself or in proteins that could affect its WT functions, studies were performed to explore the link between the mutations and the effect on prognosis. Indeed, tumours isolated from patients with p53 expression were usually associated with malignant progression, metastasis and poor prognosis across a wide range of cancers [169-172]. With such a high correlation between p53 mutation and poor survival rate, it is imperative to further study and understand the mechanism of mutant p53's impact on cellular functions, with the aim of developing therapeutic agents to reverse these adverse effects.

1.4.3.1 Inhibition of Transactivation Function in Mutant p53

With such a skewed distribution of mutation within the DBD of p53, a common hypothesis to explain the possible cause of these cancers is the inability of mutant p53 to bind to DNA, transactivate its target genes, and maintain homeostasis through the induction of cell cycle arrest and/or apoptosis. The profile of the hot-spot mutations seems to support this hypothesis.

As mentioned earlier, residues 175, 273 and 248 are critical for the binding of p53 to DNA [173]. Studies on these mutants showed their reduced ability to transduce expression of genes such as *p21* [173,174].

Another phenomenon observed in human tumours with p53 mutations is the aberrant cytoplasmic localisation of p53 in some breast cancer and neuroblastomas [175,176]. Since p53 functions as a transcription factor, a nuclear localisation of p53 is essential in its transactivation function. Sequestered in the cytoplasm, p53 would be unable to carrying out this function. Interestingly, constitutive cytoplasmic localisation of WTp53 has also been reported in breast carcinomas [177], colorectal carcinomas [178], hepatocellular carcinomas [179] and retinoblastomas [180]. Moreover, the phenomenon has been linked to tumour

metastasis and poor survival of these patients [181,182]. These studies underscored the importance of transactivation by p53 in controlling cell proliferation and tumour progression.

1.4.3.2 Gain-of-Function of Mutant p53

A common hypothesis is that oncogenesis in cancers with mutant p53 is caused by the reduced transactivation of p53 target genes. This hypothesis seemed logical in the presence of studies showing the relationship between reduced target gene expression levels and increased proliferation of tumour cells. Although it may seem adequate in explaining the phenomenon of tumours that habour mutant p53, it failed to explain the many concurrent findings of the overexpression of these mutant p53 proteins. So the question here is why is there a need for these tumour cells to overexpress mutant p53, when the missense mutations apparently are sufficient in impairing the functions of p53? All these seem to favour the hypothesis suggesting the possible gain-of-function (GOF) of p53 in eliciting oncogenic transformation.

One possible GOF mechanism involves the dominant negative effect that mutant p53 exerts over the WT counterpart. In a study by Brachmann [183], mutant p53 was found to form tetramers with wild-type p53. As mentioned above, the mutations in these mutants usually interfere with their DNA-binding and transactivation activity. The heteromers forms between the WT and mutant p53 hence impaired the transcriptional activities of WTp53 as well.

Supporting this hypothesis is evidence from several laboratories [184] which were investigating the post-translational modifications of mutant p53. The results showed that mutant p53 from tumour samples exhibited hyperphosphorylation at serine 392 [184-187]. Matsumoto [188] later found that hyperphosphorylation at serine 392 coincided with loss of DNA-binding and transactivation functions in these mutants. Since phosphorylation of WTp53 at serine 392 was found to stabilize and activate expression of genes [189], the hyperphosphorylation of serine 392 was proposed to produce a hyper-stable mutant p53,

which can hetero-oligomerise with its WT counterpart, thus preventing the proper functioning of WTp53. While it has yet to be proven that mutant p53 hyperphosphorylated at serine 392 indeed exerts a dominant-negative effect over WTp53, poor prognosis was found to correlate very well with this phenomenon [186]. In addition, oligomerisation of WT and serine 392 mutant p53 was found to exhibit a significantly reduced dissociation constant [190]. The formation of such stable oligomers could prevent functional WTp53 from performing its pro-apoptotic activities. Further evidence to support the dominant negative GOF hypothesis came from another study, where mutant p53 was able to induce WTp53 proteins to adopt a mutant conformation, thereby affecting the latter's functions [191].

Besides impairment of the inherent WT function, some studies showed that mutant p53 were able to transactivate new target genes, such as multidrug resistance gene (MDR1), found to be expressed in human cancer cells under the action of mutant p53. The overexpression of MDR1 confers resistance to cytotoxic drugs in these tumour cells [192]. C-myc, an oncogene found to induce immortalization through desensitization of contact inhibition of growth, is also another target gene of mutant p53 [193,194]. In addition, mutant p53 was also found to transactivate BAG-1, an anti-apoptotic protein that inhibits the action of proapoptotic proteins Bcl-2 and Bcl-xL [195]. The combination of target genes induced by mutant p53 thus act to prevent apoptosis while promoting cell division at the same time, contributing to the oncogenic malignancy.

The GOF of mutant p53 are not restricted to its transactivation properties alone. Studies have demonstrated that mutant p53 could inhibit process such as autophagy [196,197]. As discussed earlier, autophagy is a normal cellular function aimed at removing damaged proteins and organelles, thus facilitating the recycling of catabolites. Inhibition of autophagy by cytoplasmic p53 mutants could contribute to tumorigenesis by inhibiting the removal of damaged cellular organelles.

In summary, the mere presence of mutant p53 is no longer adequate in explaining the more aggressive nature of tumour cells that possess these mutant proteins. Many studies have demonstrated the "potency" of mutant p53 in oncogenesis by transfecting the genes for several mutant p53 into p53-null cells. Expression of mutant p53 in these cells have been shown to enhance "plating efficiency in agar cell culture", "tumourigenic potential in nude mice" and "lymphohaematopoietic metastatic potential and tissue invasiveness" [198] while silencing of endogenous mutant p53 in cancer cell lines led to decreased cell proliferation, tumourigenicity and drug resistance [199]. These studies suggest a strong correlation between p53 overexpression and tumour formation [200]. Hence, it is increasingly noted that these p53 mutants may actively enhance, rather than contribute passively, to the survival of tumour cells.

1.5 Therapies Targeting p53

Since p53 mutation is highly prevalent in cancer, numerous studies were undertaken to discover ways to reverse the effects of the mutation. The knowledge derived will thus bring us a step closer to helping the multitudes of people suffering from cancer caused by dysfunctional p53.

1.5.1 Reintroducing Functional p53

Considering the fact that the normal p53 functions are lost in most cancer, a way to reverse this effect is to introduce a functional *p53* gene into the genome. Encouraging results were shown in experiments involving the knock-in of functional *p53* gene into mouse genome. In both studies, restoring endogenous p53 expression led to increased apoptosis and reduced tumour development [201,202].

Even before these studies were published, clinical trials involving the introduction of a functional *p53* gene into human subjects were already ongoing. Adenovirus modified to

contain a *p53* gene was tested in patients suffering from recurrent glioma and non-small cell lung cancer among others. These trials yielded relatively promising results, whether used alone or in conjunction with chemo- or radiotherapy, with a slight majority of the patients showing partial to full regression of the disease [203-205]. As more of such studies are underway, progresses in this strategy is promising, with some trials already in Phase II [206,207]. Interestingly, p53 gene therapy is an approved medicine in China.

1.5.2 Activation of Endogenous p53

As mentioned earlier, most p53 related cancers possess p53 in the tumours, be it WT or mutant. Instead of trying to make these tumour cells express functional p53 through the introduction of the gene into the cell genome, another strategy is to try to reactivate the p53 proteins that already exist within the cells.

A method that was attempted involved the induction of p53 tetramerisation using pAb421, an antibody that recognizes an epitope that is found in some mutant p53. Use of this antibody allosterically activate sequence-specific DNA-binding by p53 [208,209]. No further progress has been reported for this potential treatment so far, possibly due to the difficulties in ensuring effective deliverance of large antibody protein into the cells.

With the elucidation of p53 protein structures, it became easier to study the interaction of p53 and other molecules using computer modelling. As such, small molecules can be designed to bind to mutant p53 and induce conformational change. Aided by the advances in molecular techniques and high throughput assays, large scale screening of small molecules capable of restoring WT conformation to the mutant p53 took flight, resulting in the discovery of PRIMA-1 [210]. PRIMA-1 was found to induce apoptosis in cells through the transactivation of Bax [211]. Currently, PRIMA-1 is in Phase II of clinical test [212].

Many similar studies are currently ongoing to identify other molecules which act like PRIMA-1. One advantage in using small molecules is that they can be designed for greater

cell permeability. Another advantage is that it holds the promise of being mutant specific through the optimisation of specific molecules, thus producing a whole spectrum of derivatives which can specifically bind and change the conformation of the various p53 mutants found in different human cancers.

1.5.3 Drugs Targeting the Negative Regulator of p53

Molecules that activate mutant p53 do so mainly by inducing a change in the conformation from mutant to WT, hence restoring its transactivation function. However, how would such molecules work on cancers habouring p53 with WT conformation? The answer lies in targeting the negative regulator of p53, MDM2. Currently, drugs that are either in clinical trials or already approved, work by disturbing the binding of WTp53 to MDM2, hence preventing degradation of the p53 proteins for pro-apoptotic functions.

A drug that is currently in Phase I testing is Nutlin [213]. The drug works by binding MDM2 at its p53 binding site. Nutlin was found to induce the accumulation and activation of p53 in both normal and cancer cells, and could lead to inhibition of tumour growth [214,215]. Another drug that works almost similarly is Actinomycin-D. Actinomycin-D inhibits p53-MDM2 binding indirectly by disturbing ribosomal biogenesis, resulting in the release of ribosomal protein L11. L11 then binds to MDM2, leading to the stabilization and activation of p53 [216]. Actinomycin-D, at low doses, act like nutlin to induce cell death [217,218].

While nutlin and Actinomycin-D holds promise for cancers which harbour WTp53, it may not be effective when used on cancers with cytoplasmic localised WTp53. For such cancer cells, inhibition of MDM2 and MDMX may not disrupt the possible cytoplasmic GOF of this subset of p53 protein. Hence, more work needs to be done to research on the mechanism of GOF in these cancers and determine how the cytoplasmic function can be inactivated.

1.6 Objective of this Study

The detection of mutant p53 being overexpressed in cancer is indeed an intriguing phenomenon. Despite the fact that most of the mutant protein remain localised in the nucleus, there are many observations that many tumours of diverse histological origins including the prostate cancer cell line, colorectal carcinoma and germ cell tumours [219,220] possess high levels cytoplasmically localised p53 (both WT and mutant). Several of these tumours with mislocalised p53 protein have been associated with greater survival and radio- and chemo-resistance. Clearly, there are reasons to suspect that p53, particularly those ectopically concentrated in the cytoplasm, may aid the evasion of apoptosis via means unknown thus far.

As discussed earlier, a commonly accepted hypothesis to explain the lack of apoptosis and chemotherapeutic resistance of such tumour cells include reduced transactivation, induction of new target genes that include resistance to drugs, and inhibition of autophagy. However, shouldn't the presence of WT or mutant p53 in the cytoplasm be able to induce apoptosis via direct activation of Bax and Bak directly? So why are these tumour cells not dying via apoptosis?

Not discounting the fact that all the proposed mechanism of GOF could contribute to tumorigenesis, the idea that p53 may possess novel roles in inhibiting cell death should also be explored. A strong motivation comes from the fact that elevated p53 in the cytoplasm and Bax expression was not able to induce apoptosis in cells. Studies have shown that the transactivation of Bax was not sufficient to control cellular growth [221] and on the contrary, overexpression of Bax was instead associated with poor prognosis in patients suffering from non-small-cell lung cancer [222]. Questions thus arise as to whether p53 functions to inhibit the apoptotic process downstream of Bax activation, and what the targets of p53's "inhibitory" action are likely to be.

To answer the above questions, this study aims to elucidate the possible effect of p53 on caspases, and overall cell survival. The hypothesis undertaken in this study is that p53 inhibits apoptosis via the interference of the caspase signalling pathways, thereby prevents the activation of downstream signalling process and consequently helps in averting death of these tumour cells.

Chapter 2

Materials and Methods

2.1 Construction of WTp53 Encoding Plasmids

WTp53 gene was cloned into the expression vector pET-32b (Novagen, EMD Chemicals, USA) and mammalian expression vector pX40-HA-GFP (a kind gift from Prof Low Boon Chuan, NUS [223]). Primers were designed to the N-terminus and C-terminus of WTp53 using gene sequence from IARC Tp53 Database [152].

WTp53 Forward primer: 5' – ATG GAG GAG CCG CAG TCA GA – 3'
WTp53 Reverse primer: 5' – TCA GTC TGA GTC AGG CCC TT – 3'

WTp53 were amplified using PCR in 1x AMV buffer, 1mM $MgSO_4$, 10mM dNTP, 0.7mM primers and 2.5 units of Tfl polymerase (Promega, USA) for 25 cycles. The PCR product was resolved in 1% agarose gel and the PCR product was purified using the Gel Extraction Kit from Qiagen, Germany. The purified product was then ligated to pGem-T-Easy (Promega, USA). The ligation mixture were transformed into MAX Efficiency® DH5α (Invitrogen, USA) and overnight cultures of the colonies were amplified overnight at 37^{o}C in Luria-Bertani (LB)-broth containing 50µg/ml ampicillin. Plasmids from the overnight cultures were extracted using Qiagen miniprep kit (Qiagen, USA). Positive clones were checked using restriction digestion with *NotI* enzyme (Promega, USA). The approximately 1.3kb product from the restriction digestion was resolved in 1% agarose gel and gel-extracted, followed by a final cloning into the pET-32b plasmid. The positive clones were sequenced using the Big dye (Applied Biosystems, USA), to determine the presence of the WTp53 gene as well as integrity of the sequence.

To clone the WTp53 gene into the pXJ40-HA-GFP plasmid, a new forward primer was designed so that the inserted gene can be cloned and expressed in frame. An additional adenine was placed in the 5' of the forward primer: -

WTp53 pXJ40-HA forward primer: 5' – AAT GGA GGA GCC GCA GTC AG – 3'

The cloning steps into pXJ40-HA were the same as cloning into the pET-32b plasmid. Both plasmids containing the WTp53 gene were amplified in large amounts using the Maxiprep kit (Qiagen, USA) according to manufacturer's instructions. The concentrations of the plasmids were quantified using the NanoDrop machine (Thermo Scientific, USA).

2.2 Construction of Mutant p53 Encoding Plasmids via Site-Directed Mutagenesis

From the pET32b-WTp53 and pXJ40-HA-GFP-WTp53 plasmids, various mutants of the p53 were generated using site-directed mutagenesis. Primers that contain the desired mutation were designed: -

D42Y Forward Primer: 5' – AGC AAT GGA T**(**T**)**A TTT GAT GCT – 3'
D42Y Reverse Primer: 3' – TCG TTA CCT A**(**A**)**T AAA CTA CGA – 5'

R175H Forward Primer: 5' – GTT GTG AGG C**(**A**)**C TGA CCC CAC– 3'
R175H Reverse Primer: 3' – CAA CAC TCC G**(**T**)**G ACG GGG GTG – 5'

R337H Forward Primer: 5' – GGG CGT GAG C(A)C TTC GAG ATG – 3'
R337H Reverse Primer: 5' – CCC GCA CTC G(T)G AAG CTC TAC – 3'

pET-His-WTp53 or pXJ40-HA-GFP-WTp53 were added as templates to a mixture of 1x *pfu* reaction buffer, 10mM dNTP mix, 0.7mM of the above overlapping primers and 0.8 units *pfu* turbo polymerase (Stratagene, USA).

The PCR reactions were transformed into MAX Efficiency® DH5α (Invitrogen, USA). Multiple colonies were picked, cultured overnight before plasmids were isolated. The

plasmids were sequenced to determine the presence of the point mutation, and also the integrity of the rest of the gene. Positive clones were amplified in large amounts using the Maxiprep kit (Qiagen, USA) according to manufacturer's instructions. The concentrations of the plasmids were quantified using nanodrop machine.

2.3 Preparation of Recombinant p53

Polyhistidine-tagged p53 (WT, D42Y, and R175H) in pET vector (obtained in section 2.4) and pET-32b-$p53^{R337H}$ (contributed by Phua [224]) was transformed into *Escherichia coli* BL21 Star^{TM} (DE3) pLysS bacteria (Novagen, USA). Cultures were grown in LB-broth containing 50μg/ml ampicillin at 37^{o}C till exponential phase and induced at 0.1 M isopropyl-1-thio-ß-D-galactopyranoside (IPTG) overnight at 30^{o}C.

Bacteria were harvested by centrifugation, resuspended in binding buffer (0.5M NaCl, 20mM Tris-HCl and 5mM imidazole, 1% protease inhibitor and 10 μl (per litre of bacteria culture) Benzonase, pH 7.9) followed by sonication at 40% amplitude for 4 min. Sonicated mixtures were centrifuged at 5500rpm for 15 min to collect a pellet containing inclusion bodies and cellular debris. This pellet was washed thrice by resuspension in binding buffer, sonication at 40% amplitude for 4 min and centrifugation at 5500rpm for 15 min. After the final sonication and centrifugation, the pellet was resuspended in 6M urea (dissolved in binding buffer) and incubated on ice for 1 hour to dissolve and denature the proteins in the inclusion bodies. Insoluble materials were then removed by centrifugation at 5500rpm for 30 min. Supernatant collected containing solubilised p53 as the dominant protein species was verified by 12% SDS-PAGE gel and Coomassie staining and were either stored at 4°C or proceeded with protein renaturation.

Recombinant p53 (wild-type and mutant) were renatured according to Bell [225]. In brief, solubilised p53 was diluted to 10μg/ml in refolding buffer (50mM tetrasodium diphosphate, 1M L-arginine, 2mM DTT, 0.2nM $ZnCl_2$, pH 8.0) and incubated overnight at

15°C. The solution was dialysed for 48 hours against dialysis buffer (50mM tetrasodium diphosphate, 4mM DTT, 5% (v/v) glycerol, pH 8) at 4°C. Dialysed samples were centrifuged at 5500rpm for 30min at 4°C to remove aggregates and concentrated using Vivaspin centrifugal concentrators (Sartorius, France) to a final protein concentration of 0.5µg/µl. The integrity of the recombinant p53 in the dialysed sample was reassessed by SDS-PAGE.

The 21kDa His-S-Thioredoxin tag (hereby referred to as HST-tag) expressed by the empty plasmid of pET-32b were also produced, refolded and renatured to perform as a negative control.

2.4 Glutaraldehyde Test for Tetramerisation in p53

10µg of the various refolded recombinant p53 were used for each test condition. 0.02% and 0.1% glutaraldehyde were used to induce cross-linking in the recombinant proteins for 1 minute at room temperature. 0% glutaraldehyde was used as controls. The reactions were stopped using equal volume of 1M Tris-HCl pH 7.4. The same conditions were used to induce cross-linking for 10µg of bovine serum albumin (BSA) as an additional negative control.

2.5 Cell Culture

Human embryonic kidney (HEK) 293T [226,227] and HEK293 cells [228] (both generous gifts from Dr Low Boon Chuan, NUS), A2780 (ECACC, United Kingdom), and PEO1 and PEO4 [229] were cultured in RPMI medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. Human large cell lung carcinoma H1299 $p53^{-/-}$ (ATCC, USA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Human colon tumour (HCT)-116 $p53^{-/-}$ [230] were cultured in McCoy 5A medium supplemented with 10% FBS and 1% penicillin-streptomycin.

2.6 Preparation of S100 Cytosolic Cell-Free Extracts

To isolate cytosolic fractions of cells, cells were harvested, washed twice in PBS and resuspended in ice-cold IDP buffer (20mM Hepes-KOH, pH 7.5, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1% protease inhibitor cocktail [AEBSF-Hydrochloride, Aprotinin, E-64-Protease Inhibitor, EDTA-Disodium, Leupeptin-Hemisulfate] and 3mM DTT). After incubation on ice for 10 min, the cells were disrupted by Dounce homogenizing for 25 times in a Wheaton Dounce homogenizer with a tight pestle. Cells extracts were directly centrifuged at 14,000rpm in a Beckam Optima TLX-100 ultracentrifuge for 20min at 4^{o}C. The resulting supernatants (S100 cell-free extracts) were collected.

2.7 Preparation of Dyna-beads® coupled to p53 Antibodies

1mg of M-280 Tosylactivated Dyna-beads® (Invitrogen, USA) were coupled to 10μg of anti-p53 antibodies (DO-1 (10 μg) and pAb240 (5 μg), both from Santa Cruz, USA; pAb421 (5 μg) from Calbiochem, USA) according to manufacturer's protocol. 20 μg of anti-HA antibodies (Santa Cruz, USA) were coupled to the beads as control.

2.8 Addition of recombinant proteins and in vitro Caspase Activation

S100 extracts of HEK293, HCT-116 $p53^{-/-}$ and A2780 cells were obtained as described in Section 2.5. Dialysis buffer, 0.1μM and 0.3μM of the various recombinant p53 produced in Section 2.3 were added separately into different portions of the S100 extracts. In addition, 0.3μM of recombinant GST (a kind gift from Baidah Bte Ahmad, NUS), GST-tagged WTp53 (Santa Cruz, USA) and S-tagged recombinant WT Nucleophosmin (NPM) (a kind gift from Tan Ban Xiong, NUS) were added to the S100 extracts from the various cell lines as additional controls. To per mg of protein in the HEK293 extract, 20nM dATP and 4μg of cytochrome-*c* were added to induce *in vitro* caspase activation, 20nM dATP and 12 μg of cytochrome-*c* for HCT-116 $p53^{-/-}$ lysates and 30nM dATP and 15μg of cytochrome-*c* for

A2780 lysates to do the same. Samples were incubated in the dark at room temperature for 1 hour before the reactions were stopped by freezing at -20^{o}C. Profile of p53, caspase-3 and -9, PARP, cytochrome-*c* and GAPDH in the samples was analyzed subsequently by Western blot (described in Section 2.12).

2.9 Immunodepletion and in vitro Caspase Activation

HEK293T and A2780 cells, harvested and lysed to obtain S100 cytosolic cell-free extracts, were incubated with the Dyna-beads® obtained from Section 2.6 for 2 hours on a rotator at 4°C. The lysates were removed from the magnetic beads and the appropriate amount of dATP and cytochrome-*c* were added to induce caspase activation. Samples were incubated in the dark for 15min and 1 hour at room temperate before storage at -20°C. Activation of various caspases was analyzed by Western blot subsequently.

2.10 Transfection and in vitro Caspase Activation

H1299 $p53^{-/-}$ cells, cultured to approximately 70% confluency in 10-*c*m plates, were transfected with various amounts of pXJ40-HA-GFP-WTp53/ pXJ40-HA-GFP-$p53^{D42Y}$/ pXJ40-HA-GFP-$p53^{R175H}$ / pXJ40-HA-GFP-$p53^{R337H}$ using Lipofectamine-2000 (Invitrogen, USA). Cells were harvested 6 hours post-transfection and lysed to obtain S100 cell-free extracts. Caspase cleavage was then induced by adding the appropriate dATP and cytochrome-*c*. Caspase-9 activity was measured using 200µM of LEHD-*pNA* substrate (BioVision, USA) and the yield of the coloured *pNA* product was measured at 405nm at 2 minute interval over a 12-hour period.. Efficiency of p53 transfection was analyzed by Western blot subsequently.

2.11 Knockdown of p53 and in vivo Caspase Activation

PEO1, PEO4 and A2780 were cultured to approximately 70% confluency in optical and clear 96-well plates. Transfection were performed using 50nM of control siRNA and

siRNA against p53 (Cell Signal, USA) with 0.125µl of DharmaFECT transfection reagent (Dharmacon, USA) per well. Cells were incubated overnight before the addition of 25µM of cisplatin for a further 24 hours. Control cells were treated with equivalent volume of PBS.

Cells cultured in clear 96-well plate were measured for cell density using the Sulphorhodamine B (SRB) assay. Briefly, cells were fixed in ice-*c*old 10% (wt/vol) trichloroacetic acid for 1 hour at 4^{o}C. Cells were then washed with water and air dried before staining with 0.057% (wt/vol) SRB solution for 30 minutes at room temperature. Stained cells were then washed four times with 1% (vol/vol) acetic acid. Finally, the stained cells were solubilised with 10mM Tris base solution (pH10.5) for 30 minutes before reading the plate at 510nm.

Cells cultured in optical plate were subjected to Caspase-Glow^{TM}-9 (Promega, USA) assay according to manufacturer's instructions. After 30 minutes incubation with Caspase-Glow$^{®}$-9 substrate, the activity of caspase-9 were measured with a fluorimeter at 535nm and normalized against cell density obtained from the SRB assay described above.

2.12 Knockdown of p53 and in vitro Caspase Activation

HEK293 and A2780 cells were cultured to approximately 70% confluency in 10-cm culture plates and p53 were knockdown were done as described in Section 2.9. For HEK293 cells, S100 lysates were obtained after 48 hours post-transfection, while for A2780, S100 lysates were obtained after 24 hours post-transfection. Caspase activation was then induced using the appropriate amount of dATP and cytochrome-*c* and analyzed using Western blot.

2.13 Detection of Interacting Partner of p53 using Immunoprecipitation

S100 lysates were obtained from HEK293 cells in IDP buffer containing 10mM DTT and caspase activation were induced using the appropriate amounts of dATP and cytochrome-*c*. The lysates containing the cleaved caspases were incubated with Dyna-beads® coupled to

either anti-HA or anti-p53 antibodies for 1 hour at room temperature. After the incubation, the lysates were removed and the beads were washed with the same IDP buffer. The bound p53 were eluted with Elution Buffer from Seize IP Kit (Pierce, USA). The eluent were analyzed for presence of p53 and caspase-9 using Western blot.

2.14 Detection of Interacting Partner of p53 using Immunoprecipitation and Cross-linking

S100 lysates were obtained from HEK293 cells in IDP buffer containing 10mM DTT and caspase activation were induced using the appropriate amounts of dATP and cytochrome-*c*. The lysates containing the cleaved caspases were incubated with 0.1µM of HST-tag or HST-tagged p53 proteins for 15 minutes at room temperature with agitation. 30µl of nickel beads (Qiagen, USA) were added to each fraction and incubated further 15 minutes with agitation. Nickel beads were pelleted at 1000rpm and lysates were removed. The nickel beads were washed once with PBS. 100µl of PBS were then added to the beads. 10mM of nickel acetate were added to the solution and incubated for 10 minutes at room temperature, followed by 30mM of magnesium monoperoxyphtaleic acid (MMPP) for a further 6 minutes. The beads were then washed thrice with PBS and analyzed for presence of p53 and caspase-9 using Western blot.

2.15 Electrophoresis and Coomassie Staining

Recombinant p53 from Section 2.3 were mixed with Laemmli's loading dye, boiled for 5 min at 95°C and subjected to 12% SDS-PAGE at 100V for 1.5h. The gels were stained by Coomassie brilliant blue.

Recombinant p53 from Section 2.4 were mixed with Laemmli's loading dye, boiled for 5 min at 95°C and subjected to 4-12% gradient SDS-PAGE at 100V for 1.5h. The gels were stained by Coomassie brilliant blue for analysis.

2.16 Electrophoresis and Western Blot Analysis

Equal amounts of proteins were mixed with Laemmli's loading dye, boiled for 5 min at 95°C and subjected to 12% SDS-PAGE at 100V for 1.5h. Separated proteins were electroblotted onto nitrocellulose membranes at 15V for 30 min followed by blocking with 3% skimmed milk for 15min. Specific proteins were probed with anti-p53 DO1 (Santa Cruz, USA, 1:3000), anti-caspases 3, 6, 8, 9 (Cell Signalling Technology, USA, 1:2000), anti-S-tag-HRP (Bethyl Laboratories, USA, 1:2000) anti-*c*ytochrome c (Pharmingen, USA, 1:2000), anti-GAPDH (Ambion, USA, 1:10000) or anti-PARP (Santa Cruz, USA, 1:2000) overnight. Membranes were rinsed with TBS-T and incubated with corresponding polyclonal anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:1000 to 1:3000) for 1h. Following 1h incubation, membranes were rinsed with TBS-T and last wash were done in TBS. Bound antibodies on membranes were detected using pico or femto enhanced chemiluminescence kit (Pierce, USA).

2.17 Electrophoresis and Far Western Blot Analysis

Recombinant caspase-9 was first expressed using the *in vitro* translation system (Pierce, USA) with 0.5µg of pET-32b-WT-caspase-9 (addgene, USA). 20µg of S100 lysates of H1299 $p53^{-/-}$ cells transfected with 2.5µg of pXJ40-HA-GFP-WTp53 from *Section 2.10* were used as a Western blot control. 0.5mg of HST-tag expressed by pET-32b plasmids were used as a negative control. 15ng of active caspase-9 (Calbiochem, USA) was used as the prey protein, while 1µl of the IVT reaction described above were used also as a negative control as it contains high concentration of the procaspase-9 protein. The proteins were mixed with Laemmli's loading dye, boiled for 5 min at 95°C and subjected to 12% SDS-PAGE at 100V for 1.5h. Separated proteins were electroblotted onto nitrocellulose membranes at 15V for 30 min. Based on the protocol by Wu [231], proteins on the membrane were refolded by

incubating the blots with AC buffer (10% glycerol, 0.1M NaCl, 20mM Tris-HCl-pH 7.4, 1mM EDTA, 0.1% Tween-20, 10mM DTT, 2% milk) containing 6M guanidine-HCl for 30 minutes, followed by AC buffer with 3M guanidine-HCl for 30 minutes, then AC buffer with 1M guanidine-HCl for 30 minutes. All of the above incubations were at room temperature. Subsequently, the blots were incubated at 4^{o}C in AC buffer with 0.1M guanidine-HCl for 30 minutes and finally in AC buffer containing no guanidine-HCl overnight at 4^{o}C. The blots were then blocked with TBS-T containing 5% milk for 1 hour at room temperature. 0.1µM of the HST-tag and the various recombinant HST-tagged-p53 were incubated with the blots separately in the interaction buffer (contents of the interaction buffer was similar to AC buffer except that 0.5mM EDTA were used) for 1 hour at room temperature. After incubation, the buffer containing the proteins was removed. The blots were washed and the bound p53 were detected using DO1-HRP (Santa Cruz, USA) and and S-tag-HRP antibodies (Bethyl Laboratories, USA) at 1:2000 dilution in TBS-T containing 1.5% milk. For blots that were subjected to cross-linking reactions, after the solutions containing the proteins were removed, 10ml of PBS were added to each blot. Cross-linking reactions were initiated with 10mM of nickel acetate for 10 minutes, followed by 30mM of MMPP for a further 6 minutes. The blots were finally washed and bound p53 were detected using DO1-HRP and S-tag-HRP antibodies (Santa Cruz, USA) at 1:1500 dilution in TBS-T containing 1.5% milk.

2.18 Kinetics of p53-Dependent Inhibition of Caspase Activity

The various recombinant HST-tagged-p53 were incubated with 2.8µM of active recombinant caspase-9, 20nM of active recombinant caspase-3, and 25nM of active recombinant caspase-6 (all recombinant proteins are from Calbiochem, USA) in 1x Reaction Buffer containing 10mM DTT (BioVision, USA). A positive control was set up with no addition of any recombinant protein while a negative control was set up with the addition of the highest concentration of the HST-tag in the particular set of experiment. 200µM of

LEHD-*pNA* substrate (for caspase-9), DEVD-*pNA* substrate (for caspase-3) and VEID-*pNA* substrate (for caspase-6) were added and the yields of the coloured *pNA* product were measured at 405nm at 2 minute interval over a 1-hour period.

2.19 Survival of Cells Transfected with various p53 in Cisplatin

HCT-116 $p53^{-/-}$ cells were first cultured in 6-well plates to approximately 60% confluency. Various dosages of cisplatin were then added to the medium and cells were exposed to the drug for 72 hours. PBS was added as the control. After 72 hours, dead cells were removed and the adherent cells were collected and lysed in Total Cell Lysis buffer (100mM HEPES pH 7.5, 5mM MgCl2, 150mM NaCl, 1mM EDTA, 1% Triton X-100 + 1% protease inhibitor cocktail [AEBSF-Hydrochloride, Aprotinin, E-64-Protease Inhibitor, EDTA-Disodium, Leupeptin-Hemisulfate]) for 10 minutes on ice, and centrifuged at 14000rpm to remove the cell debris. The supernatants were collected as the total cell lysate. Profile of caspase-9 and PARP were then analyzed using Western blot.

Subsequently, HCT-116 $p53^{-/-}$ cells were cultured in 24-well plates to approximately 40% confluency. 0.2µg of pXJ40-HA-GFP/pXJ40-HA-GFP-WTp53/ pXJ40-HA-GFP-$p53^{D42Y}$/ pXJ40-HA-GFP-$p53^{R175H}$/ pXJ40-HA-GFP-$p53^{R337H}$ were transfected into the cells using Lipofectamine 2000. At 4 hour post-transfection, cells were poisoned with 0.5mM cisplatin (Sigma-Aldrich, USA) with treatment with PBS as the control. This was considered as 0-hour. At 0-hour, cells were stained with 0.5µg/ml Hoechst-33342 (Sigma-Aldrich, USA) for 10 minutes. Cells were then washed twice with PBS to remove the dead cells. The cells were then viewed under Olympus inverted fluorescent microscope. Images of the GFP and Hoechst fluorescence were captured using the DP Controller software from Olympus. More images were captured subsequently at 24 hour interval after challenge with cisplatin. Finally, the fluorescence in the captured images were counted using ImageJ software. First, the percentage of the transfected cells in each well were calculated by expressing the amount of

transfected cells (GFP fluorescence) over the total number of cells in the well (Hoechst staining). The change in transfected cells between the PBS and cisplatin-challenged cells were next also expressed as a percentage. The percentages obtained at 0-hour for each p53 variant and the GFP control was used as a reference point. The change in percentages of differences in transfected cells for each p53 variant and the GFP control over the 72-hour period were obtained to illustrate the survivability of the cells transfected with the various p53 plasmids.

2.20 Cells Transfected with various p53 in Cisplatin and their Caspase-9 Activities

Similar to Section 2.15, HCT-116 $p53^{-/-}$ cells were cultured in 24-well plates to approximately 40% confluency. 0.2µg of pXJ40-HA-GFP/pXJ40-HA-GFP-WTp53/ pXJ40-HA-GFP-$p53^{D42Y}$/ pXJ40-HA-GFP-$p53^{R175H}$/ pXJ40-HA-GFP-$p53^{R337H}$ were transfected into the cells using Lipofectamine 2000. At 4 hour post-transfection, cells were poisoned with 0.5mM cisplatin (Sigma-Aldrich, USA) with treatment with PBS as the control. This was considered as 0-hour. At 72 hours post-transfection, 1µl of fluorescence caspase-9 substrate, Red-LEHD-FMK from the Caspase-9 Detection Kit (Calbiochem, Merck, Germany) were added to each well and incubated for 30 minutes. 10 minutes prior to end of assay, cells were stained with 0.5µg/ml Hoechst-33342 (Sigma-Aldrich, USA). At the end of the assay, cells were washed twice with Wash Solution to remove the dead cells and excess caspase-9 substrate. Fluorescent images were captured as per Section 2.15. ImageJ was used as an imaging and counting tool. Cells that had both caspase-9 and GFP fluorescence were scored as a percentage over the total number of transfected cells (GFP fluorescence) for each well. This percentage was next used to calculate the change in correlation between the caspase-9 and GFP fluorescence between the PBS and cisplatin-*c*hallenged cells for the GFP control and each p53 variant. Using GFP as the reference point, the change in correlation for each p53 variant was expressed as a percentage over GFP.

Chapter 3 – Results

p53 and its Mutants Directly Inhibits caspase-9's Activity

3.1 Introduction

The hypothesis of this study is that p53 inhibits apoptosis by interfering with the caspase signalling pathways, thereby preventing the activation of downstream caspase targets. This may in turn, increase the apoptotic threshold of cells, rendering them more susceptible to oncogenic transformation. Until recently, there has been no report of p53 as a direct inhibitor of any of the 14 known human caspases. Certain p53 mutants ($p53^{R175H}$ and $p53^{R273H}$) were associated with the downregulation of procaspase-3 and increased resistance to drug-induced apoptosis [232,233]. On the contrary, WTp53 was shown to lower the apoptotic threshold by inducing caspase-6 expression [234]. The apparently paradoxical situation suggests that while WTp53 may serve to facilitate apoptosis, mutant p53 may conversely increase the apoptotic threshold through suppression of caspase gene expression. A more direct link between p53 and caspases was demonstrated in another study in which knockdown of p53 in human osteoblast cells resulted in heightened caspase activities [235]. Although such reports are far and few between, it certainly does not reduce the motivation to explore p53's relationship with caspases in greater detail.

To fully understand how p53 and its various mutants may directly regulate caspase-9's activity in the cell death signalling process, four p53 isoforms were chosen for this current investigation: (1) WTp53 (which serves as a control for comparison); (2) $p53^{D42Y}$ (point mutation at the TAD region); (3) $p53^{R175H}$ (point mutation at the TAD region); (4) $p53^{R337H}$ (point mutation at the TD region). By selecting point mutations that span different domains of the p53 protein, this study aims to investigate how mutations at the different domains may influence the protein's effects on caspases. Of note here, $p53^{D42Y}$ was chosen partly because

the mutant was reported in ovarian cancer patients [236], a cancer which this study will attempt to focus on. A more detailed introduction to this cancer is covered in Chapter 5. Meanwhile, $p53^{R175H}$ mutant was chosen not only because the point mutation lies within the DBD, but also due to the fact that it is a structural mutant. This choice will further aid in determining a structural requirement for p53 in the regulation of caspase activities. Lastly, the fourth mutant $p53^{R337H}$ was selected as the point mutation at the TD affects protein tetramerisation, an important feature responsible for its transactivation property. At the same time, the study of this mutant could reveal clues to its probable pathological role(s) in LFS.

3.2 Results

3.2.1 Production of Highly Purified Recombinant p53 that is Capable of Tetramer Formation

The various forms of recombinant p53 were produced and refolded according to protocol reported by Bell [225]. In order to examine the purity of the recombinant proteins produced, equal amount of lysates from each round of sonication were saved, separated in a SDS-PAGE gel and Coomassie-stained. As seen in Figure 3.1 (A & B), a band of approximately 74kDa, which corresponds to the size of recombinant p53 fused with the His-S-Thioredoxin (HST) triple tags of approximately 21kDa, was isolated and purified.

To show that the recombinant p53 was refolded properly, the proteins were first assessed by Far-UV circular dichroism (CD). According to DiGiammarino [43], both WTp53 and $p53^{R337H}$ proteins exhibit the same CD spectrum. As expected, the CD data obtained for both proteins in this study were similar (Figure 3.1B), indicating that the proteins have been refolded properly.

Tegtmeyer [237] and Prives [238] reported that properly folded native murine and human p53 proteins were able to form tetramers. This forms the basis of the second test used to confirm if the recombinant p53 produced here was refolded correctly and was able to form tetramers. To do this, two concentrations of glutaraldehyde were used to induce cross-linking

between the monomers of the recombinant p53. As shown in Figure 3.1C, the various recombinant p53 forms were able to form tetramers (shown approximately at molecular weight of 300kDa). However, different degrees of tetramerisation were evident. WTp53 tetramers were apparent at 0.02% glutaraldehyde, seen from the shift in bands from 74kDa to approximately 300kDa. The mutant p53 proteins however, showed a reduced concentration of tetramers since at the same concentration of glutaraldehyde, the monomeric p53 form was present. Among the mutant p53 proteins, D42Y and R175H showed similar behaviour with the monomer species only absent at the 0.1% glutaraldehyde and some evidence of dimers not seen with the WT protein. Meanwhile, the tetrammerisation mutant $p53^{R337H}$ showed the lowest level of tetramers at all three concentration of gluteraldehyde used. BSA was used as a negative control. Although the band intensities of the protein shifted slightly upon exposure to glutaraldehyde, the changes were not significant (graph, Figure 3.1C). The magnitude of the changes in the intensities of the monomeric species of the various p53 proteins are also displayed in the same graph.

The cross-linking experiment here demonstrated the differential ability of the various p53 mutants to tetramerise, and this, in turn, correlates well to the known differential effects of the various point mutations on the structural integrity of the properly refolded protein. The purified and properly folded recombinant p53 were thus used for further tests in this study.

3.2.2 WT and Mutant Recombinant p53 Inhibit Caspase Activation

To investigate the hypothesis that p53 may inhibit caspase activity and activation, the recombinant p53 produced were added to S100 cytosolic fractions of HEK293 cells with endogenous WTp53. Apoptosis was initiated with the addition of cytochrome-*c*. In agreement with the hypothesis, inhibition of caspase cleavage was observed with the addition of the recombinant p53. Also, a dose-dependent inhibition of caspase cleavage was evident i.e. as

more recombinant p53 was added, the inhibition of caspase cleavage became more pronounced. The inhibition appeared to affect the caspase cascade upstream, with inhibition apparent on caspase-9, the most apical caspase to be activated upon cytochrome-*c* initiated apoptosis. As introduced in *Section 1.2.5.1*, caspases belong to a distinctive and highly conserved class of intracellular cysteine proteases. When activated by apoptotic signals, they are proteolytically cleaved at the aspartic acid residue into one large and one small subunit that assemble with each other to form enzymatically active heterotetramers [79]. The majority of the cleavage of caspase-9 occurs at the aspartic acid reside at position 315, generating two subunits of p35 and p12 [239]. Activation of caspase-3 occurs at the aspartic acid residue at position 175, producing two subunits of p20 and p11 [240]. The dimerization of p35 and p12 subunits forms the active unit of caspase-9 [241] while the dimerization of the p20 and p11 subunits forms the active unit of caspase-3 [242].

As seen from Figure 3.2, the addition of the various recombinant p53 led to a decrease in the p37 and p35 bands, the active subunits of caspase-9, with the concurrent restoration of the non-active p46 procaspase band. The inhibition of caspase-9 cleavage affected the activation and cleavage of direct downstream targets such as caspase-3 and PARP. As caspase-6 and caspase-8 are also reported to participate in the caspase amplification loop [243-245], these two caspases were probed for their cleavage patterns. Both caspases showed either reduced or no activation, as compared to caspase-9 and caspase-3, which are direct downstream targets of cytochrome-*c* induced apoptosis, indicating that these caspases were too far downstream and therefore were not affected by the caspase activation cascade.

To confirm this phenomenon, the experiment was repeated using S100 cytosolic fractions derived from HCT-116 $p53^{-/-}$ cells. This was done to eliminate the possible interference by endogenous p53 which is present in the cytosolic extracts of HEK293 cells. The same inhibitory effects on the caspases were observed (Figure 3.3), indicating that the

results from the experiments with both cell line lysates were solely due to the addition of recombinant p53 and not the endogenous p53.

In both experiments carried out with HEK293 and HCT-116 $p53^{-/-}$ cell lysates, the proteins affected by the cytochrome-*c*-induced apoptosis were caspase-9, caspase-3 and PARP. Meanwhile, caspase-6 and caspase-8 were demonstrated to be too far downstream to be affected by the inhibitory action of p53. For this reason, the profiles of both caspases were omitted from further experiments.

Although the results obtained using HEK293 and HCT-116 $p53^{-/-}$ cells were encouraging, these two cell lines were artificially generated from existing cell lines either through adenovirus-induced transformation (HEK293) [246], or homologous recombination to disrupt the endogenous WTp53 gene (HCT-116 $p53^{+}/^{+}$ cells) [230]. To eliminate the possibility that these additional manipulations used in creating these cell lines could have an effect on the results obtained, the experiments were repeated using A2780, an ovarian cell line established directly from the tumour tissues of the ovaries that expresses WTp53.

As shown in Figure 3.4, the addition of the various recombinant p53 proteins into the S100 lysates of A2780 were able to inhibit caspase-9, and the downstream proteins, similar to the effects found in both HEK293 and HCT-116 $p53^{-/-}$. Hence in this unmanipulated tumour cell line, the addition of exogenous recombinant p53 was still able to inhibit caspase-9 implying that the endogenous level of WTp53 in these cell extracts was not saturating for p53 dependant inhibition of caspase activity.

To further eliminate doubts about the inhibitory effect of the folded HST-p53 on caspase-9, a commercial source of active recombinant GST-tagged WTp53 was tested for its effect on caspase-9 in A2780 lysates induced to undergo caspase activation via the addition of recombinant cytochrome-*c*. Recombinant S-tagged nucleophosmin (NPM) was also included as a negative control as a recently published study by Leong [247] had demonstrated

its inhibitory effects on caspase-6 and caspase-8, but not on caspase-9 and caspase-3. As expected, only the tagged WTp53 were able to inhibit the cleavage of caspase-9 (Figure 3.5). One thing to note in this set of experiment is that, although the recombinant tagged p53 proteins were able to inhibit caspase-9 cleavage, the inhibition of the downstream proteins such as PARP was not as pronounced as those seen in Figures 3.2 to 3.4. Therefore, this would require another study to explore if selective inhibition could be seen at earlier time points.

3.2.3 In Vitro Depletion of Endogenous p53 Accelerates Caspase Activation

Since it was demonstrated that the presence of increased amount of p53 resulted in the inhibition of caspase-9, the next set of experiments were performed to determine if removal of p53 could accelerate caspase-9 activation. Immunodepletion of endogenous p53 from cells at normal physiological conditions served to minimize any disturbance to the normal cellular activities and functions. Endogenous p53 was immunodepleted from the cytosolic fraction of HEK293T cell lysates prior to treatment with cytochrome-*c*. In agreement with the hypothesis, the partial removal of p53 led to increased caspase-9, caspase-3 and PARP cleavage, compared to the non-immunodepleted cell lysates in which the endogenous p53 was still present (Figure 3.6).

As introduced earlier, WTp53 expressed in HEK293T cells were presumed to have been inactivated by the T-antigen of SV40, which is constitutively expressed in this cell line [228,248]. To eliminate the possibility of the inactivated p53 could have any bearing on the final outcome, the experiment was repeated using the cytosolic fractions derived from A2780 cells with functional WTp53. Indeed, the results obtained (Figure 3.7) were similar to those of HEK293T. Taken together, the results further supported the hypothesis that p53 may directly inhibit caspase activation.

3.2.4 Knockdown of p53 Accelerates Caspase Activation

To investigate if the inhibitory effect occurs in an *in vivo* environment, p53 was either removed through siRNA-mediated knockdown of p53, or by overexpression of these various p53 forms in p53-null cells. With the assumption that the inhibition is targeted at caspase-9, cells from both the knockdown and overexpression experiments were harvested for their cytosolic fraction and cytochrome-*c*-induced caspase-9/caspase-3 cleavage were investigated using immunoblotting.

In the knockdown experiments (Figures 3.8 and 3.9), reduction of endogenous p53 in both HEK293 cells and A2780 cells resulted in higher caspase-9, caspase-3 and PARP cleavage. Here, HEK293 cells were used instead of HEK293T cells. This is due to the fact that HEK293, which is isogenic to HEK293T cells, lack the expression of the T-antigen of SV40 [249]. A similar phenomenon was observed in both p53 knockdown experiment on HEK293 and p53 immunodepletion experiment on HEK293T cells. Therefore, the use of HEK293 cell line helped to substantiate that the results obtained from p53 immunodepletion experiment on HEK293T were indeed true. For A2780 cell line, an additional experiment was carried out which involved the measurement of caspase-9 activity *in vivo* after the cells was treated with siRNA and challenged with cisplatin. The fluorescent compound produced by activated caspase-9 was measured in the intact cells. Similar patterns of caspase-9 inhibition by p53 were observed both *in vivo* (Figure 3.9A) and *in vitro* (Figure 3.9C), showing lowered caspase-9 activities and cleavage respectively.

Both HEK293 and A2780 contain endogenous WTp53. It was therefore interesting to see if mutant p53 could likely inhibit caspases activation using cell lines containing endogenous mutant p53. For this purpose, a pair of ovarian cell lines PEO1 and PEO4 was used for comparison. PEO1 and 4 are isogenic cell lines derived from a patient with ovarian adenocarcinoma, whereby PEO1 is the original established cell line which is cisplatin-

sensitive while PEO4 was established when the same patient developed resistance to cisplatin [229]. Sequencing the p53 gene of these cell lines revealed a mutation at the p53 gene at codon 342, with a change of amino acid from arginine to proline (Figure 3.10A). Codon 342 lies within the TD.

Both cells lines were treated with 25μM cisplatin for 24 hours after knockdown of p53 to initiate apoptosis. The same assay used for A2780 (Figure 3.9A) was applied here to measure the caspase-9 activities. For the PEO1 cells (Figure 3.10B), no significant change in caspase-9 activity was observed with p53 knockdown. However, for the PEO4 cells (Figure 3.10D), caspase-9 activity was increased in the p53-knockdown cells, and the increase became more significantly with cisplatin treatment.

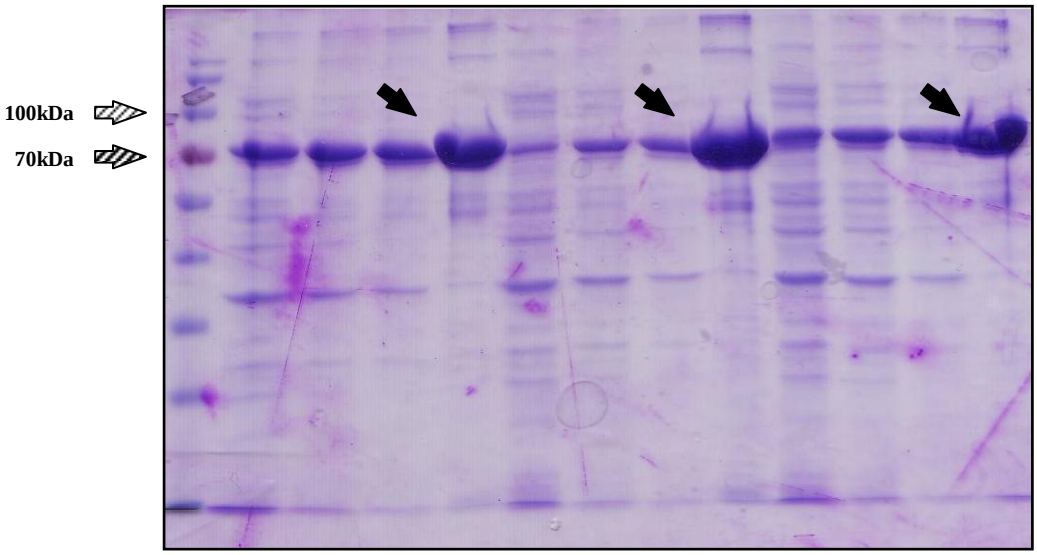
3.2.5 Overexpression of p53 Inhibits Caspase Activation

As results from the *in vivo* p53 knockdown experiments were consistent with the hypothesis, another set of experiment was carried to overexpress the various p53 proteins *in vivo* and to analyze if the increased in p53 in the cells could lead to inhibition of caspase activation. WT and mutant p53 were overexpressed in the lung carcinoma H1299 $p53^{-/-}$ cells. The genes encoding the various p53 proteins chosen for this study were cloned into the pXJ40-HA-GFP plasmid. The plasmid contains a cytomegalovirus enhancer at its promoter site which enables high levels of expression of the gene cloned downstream. Therefore, the cells transfected with the plasmids were harvested for caspase cleavage analysis as early as 6-hour post-transfection to minimize the effect of p53-transactivated pro-apoptotic genes brought about by the overexpression of p53. S100 cytosolic extracts were obtained from the cells after transfection followed by the initiation of caspase activation by the addition of cytochrome-*c*. Caspase-9 activities were then measured using a caspase-9 specific substrate, which when cleaved by active caspase-9, yields a quantifiable colorimetric product pNA.

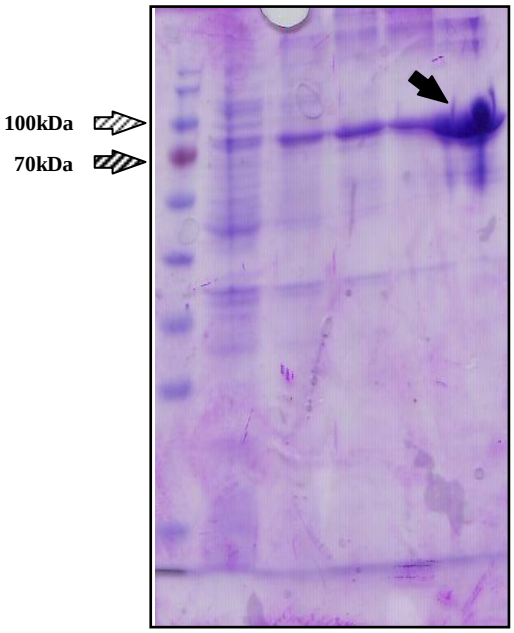
From Figures 3.11, it is apparent that increasing amount of the various mutant p53 in the cell lysates resulted in lower caspase-9 activities compared to the controls. These observations correlated with the results in Figures 3.1 and 3.2, where increasing amount of p53 caused decreased in caspase-9 activities. Interestingly, a different trend was observed in cells overexpressing WT-p53 (Figure 3.11A). The overexpression of low concentration of WT-p53 caused the slight inhibition of caspase-9 cleavage, while at higher concentration; there was a reverse in phenomenon, whereby the caspase-9 activities returned to the level observed in the control.

A)

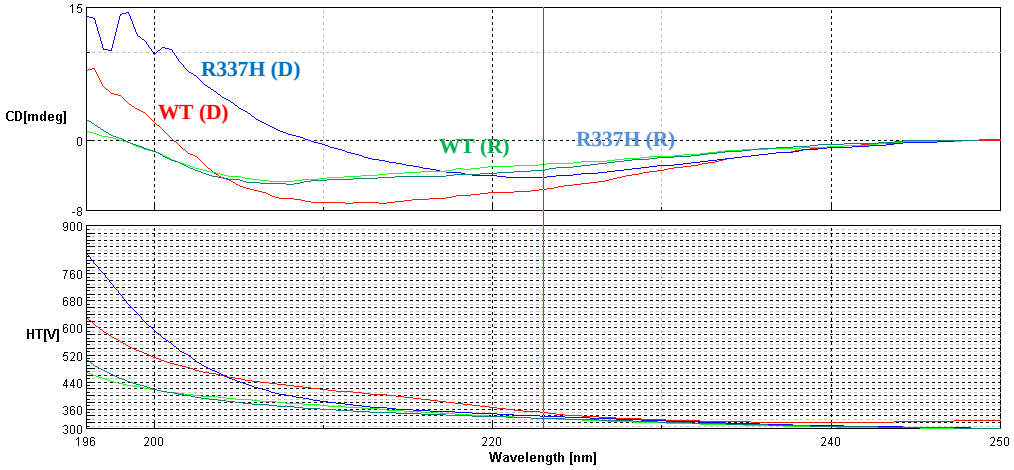
Recombinant p53	Marker	WT				D42Y				R175H			
Sonication Cycle		2nd	3rd	4th	6M Urea	2nd	3rd	4th	6M Urea	2nd	3rd	4th	6M Urea



Recombinant p53	Marker	R337H				
Sonication Cycle		1st	2nd	3rd	4th	6M Urea

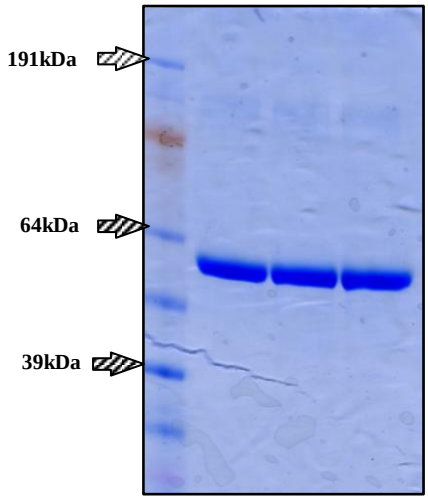


B)

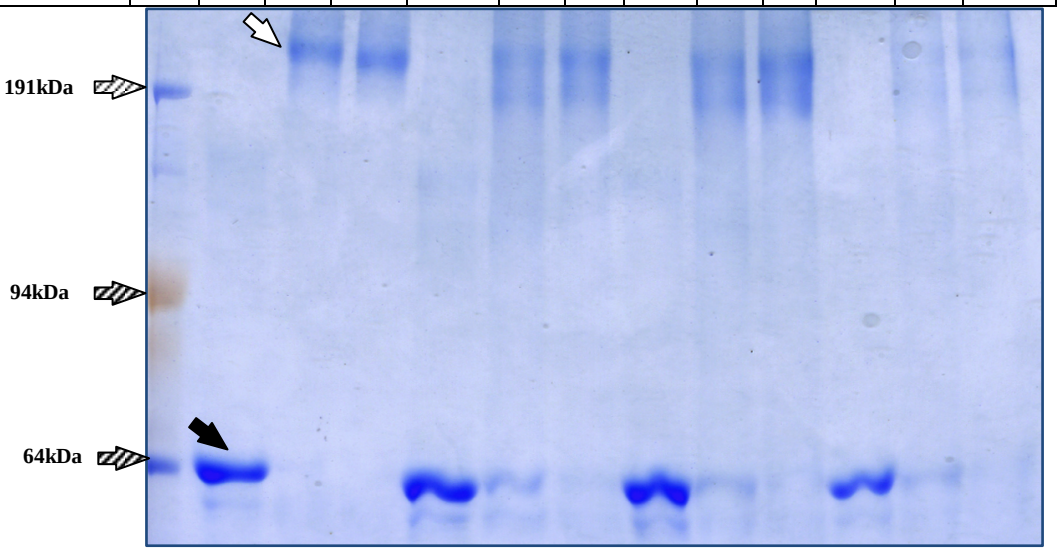


C)

Recombinant p53/BSA	Marker	BSA		
Glutaraldehyde (%)		0	0.02	0.1



Recombinant p53/BSA	Marker	WT			D42Y			R175H			R337H		
Glutaraldehyde (%)		0	0.02	0.1	0	0.02	0.1	0	0.02	0.1	0	0.02	0.1



D)

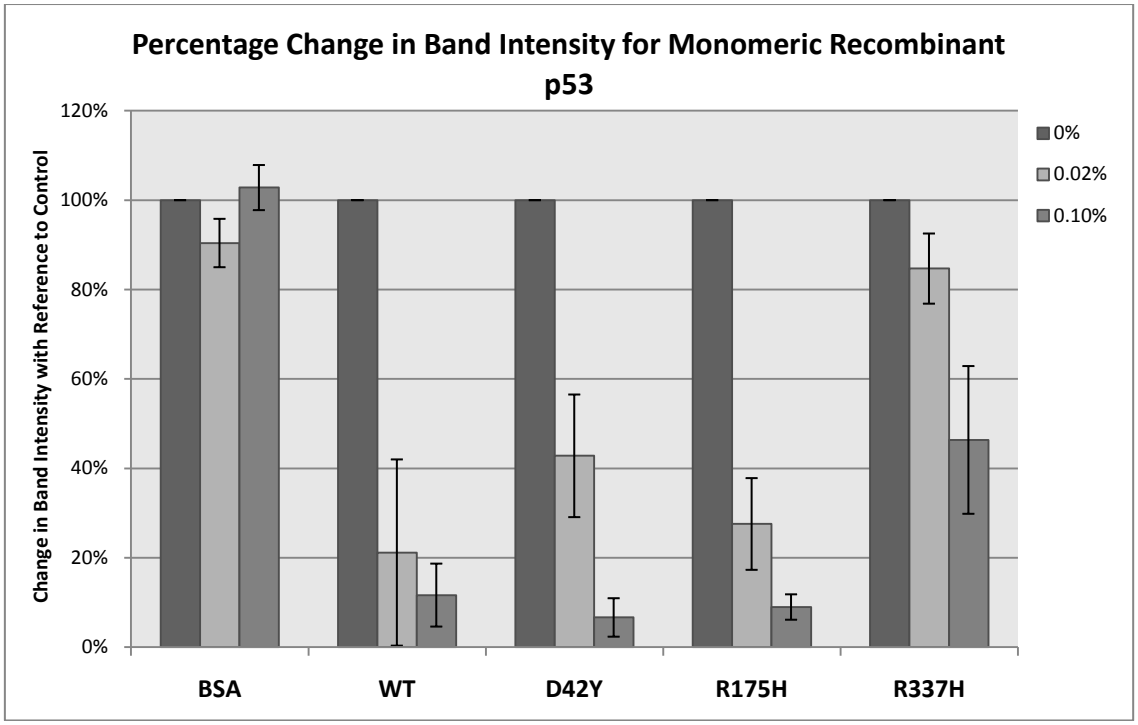
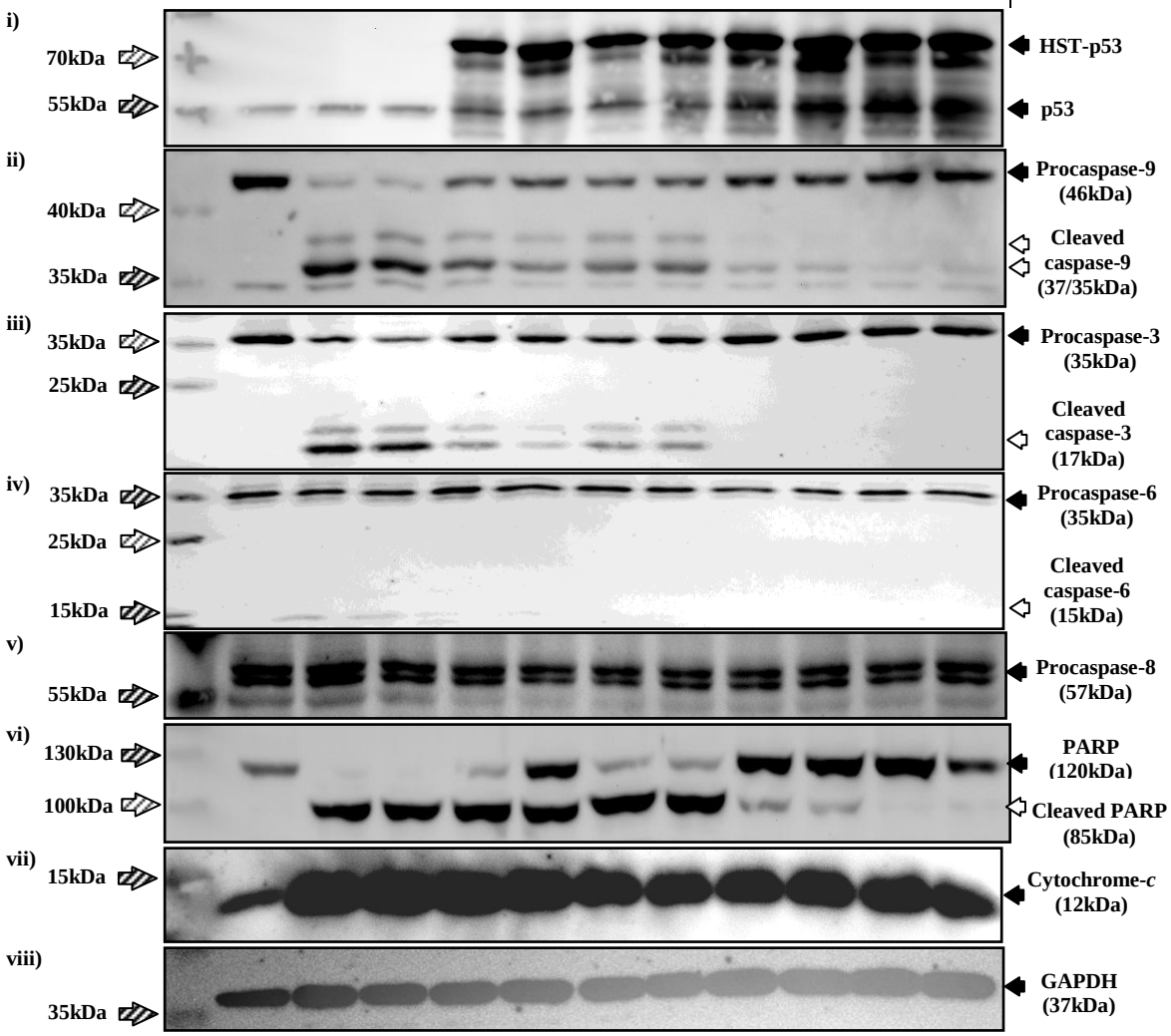


Figure 3.1. Verification of Purity and Structure of Recombinant p53. Recombinant p53 were overexpressed in *E.coli* BL21 cells by transfecting pET32b plasmids containing the various p53 genes. The shaded arrows ( ) marked the various molecular weight markers. The black arrow ( ) marked the monomeric recombinant p53 at approximately 74kDa. **A)** the recombinant p53 were extracted from the inclusion bodies in *E.coli* using sonication and the purity of the p53 protein extracted were shown in coomassie-stained SDS-PAGE gels. **B)** Far-UV circular dichoism data of recombinant WT and mutant $p53^{R337H}$ (extracted from Figure 6 Phua[224]). Data for WTp53 is in red, $p53^{R337H}$ is in blue, unfolded proteins of both species are in green and light blue. **C)** The structure of the recombinant p53 from (A) was tested using various concentrations of glutaldehyde to induce cross-linking between the p53 monomers and shown in Coomassie blue stained SDS-PAGE gel. The white arrow ( ) marked the tetrameric recombinant p53 at approximately 296kDa. **D)** Three sets of experiments presented in (C) were done and the band intensities for the various monomeric recombinant p53 at different glutaraldehyde concentrations were analyed and the change in band intensity with reference to the control (0% glutaraldehyde) were calculated (n=3).

A)

HST Tag (μM)	**Marker**	-	-	0.3	-	-	-	-	-	-	-	-
Recombinant p53 (0.1μM)		-	-	-	WT	D42Y	R175H	R337H	-	-	-	-
Recombinant p53 (0.3μM)		-	-	-	-	-	-	-	WT	D42Y	R175H	R337H
Cytochrome-*c*		-	+	+	+	+	+	+	+	+	+	+



B)

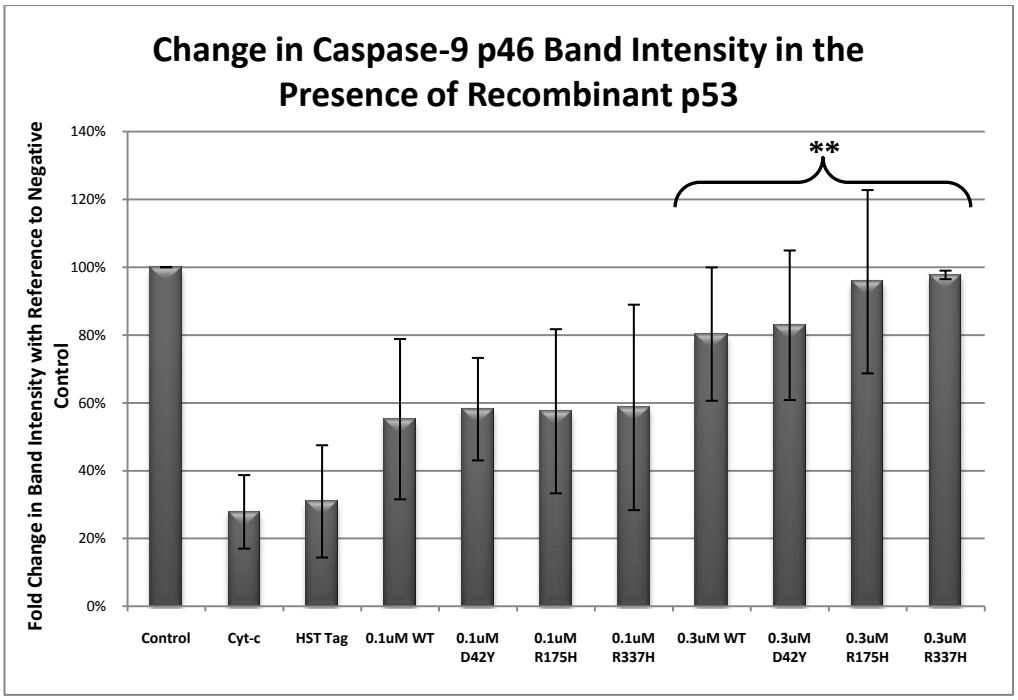
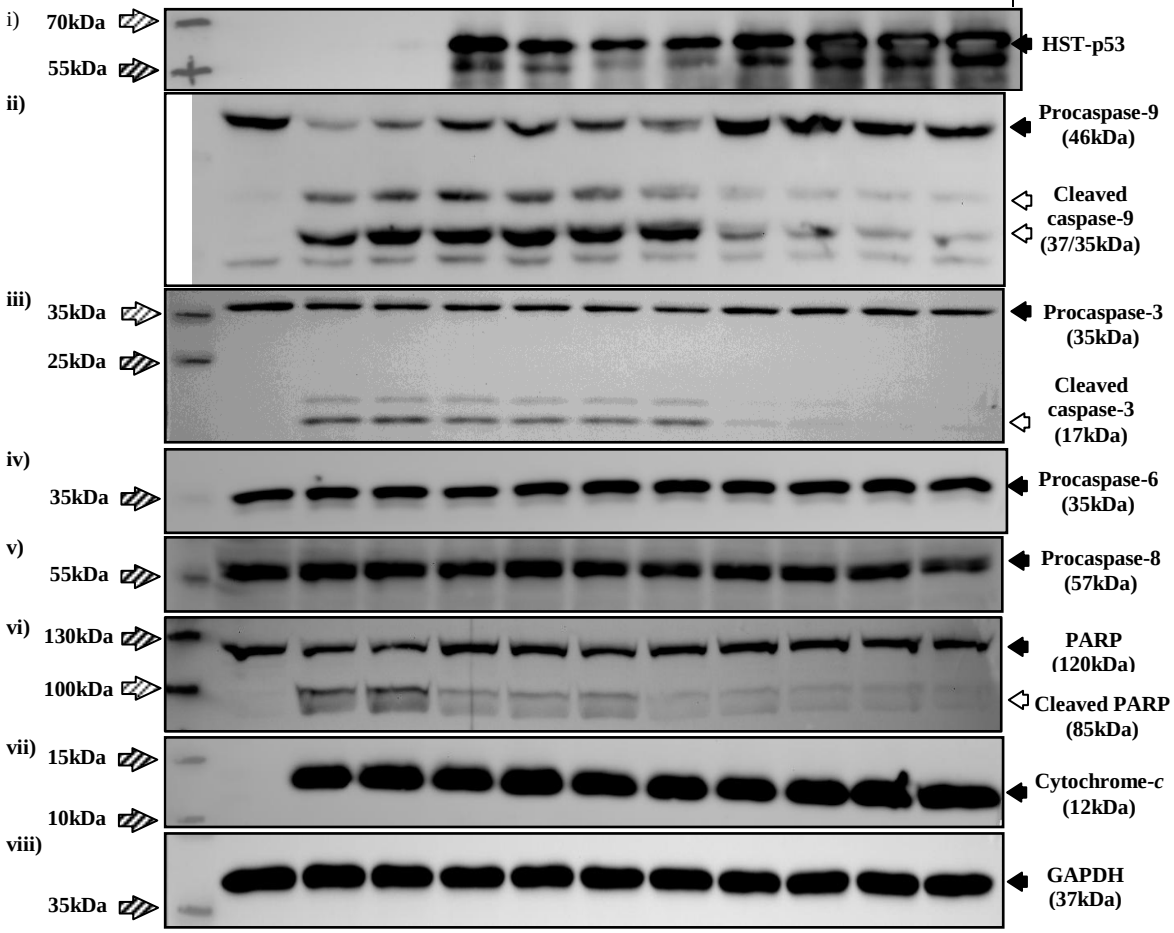


Figure 3.2. Addition of Recombinant p53 Inhibits Caspase Cleavage in HEK293 S100 Cell Lysate. A) S100 lysates from HEK293 cells were obtained and incubated with the various recombinant proteins for 1 hour after addition of equal amount of dATP and cytochrome-*c* to initiate caspase cleavage. Profile of p53 **(i)**, Caspase-9 **(ii)**, Caspase-3 **(iii)**, Caspase-6 **(iv)**, Caspase-8 **(v)**, PARP **(vi)**, cytochrome-*c* **(vii)** and GAPDH **(viii)**. Lane 1: lysate without cytochrome-*c* challenge; Lane 2: lysate with cytochrome-*c* challenge; Lane 3: lysate with cytochrome-*c* challenge and addition of 0.3µM HST-tag protein; Lanes 4-7: lysate with cytochrome-*c* challenge and addition of 0.1µM of the various recombinant p53; Lanes 8-11: lysate with cytochrome-*c* challenge and addition of 0.3µM of the various recombinant p53. **B)** Band intensities of the p46 fragment of caspase-9 in were measured using ImageJ and calculated with reference to the control lysate to obtain the percentage change in band intensities. ** denotes the fold change in the band intensity of the fractions with 0.3µM recombinant p53 vs. 0.3µM HST-tag was significantly different ($p<0.05$, $n=3$).

A)

HST Tag (μM)	**Marker**	-	-	0.3	-	-	-	-	-	-	-	-
Recombinant p53 (0.1μM)		-	-	-	WT	D42Y	R175H	R337H	-	-	-	-
Recombinant p53 (0.3μM)		-	-	-	-	-	-	-	WT	D42Y	R175H	R337H
Cytochrome-*c*		-	+	+	+	+	+	+	+	+	+	+



B)

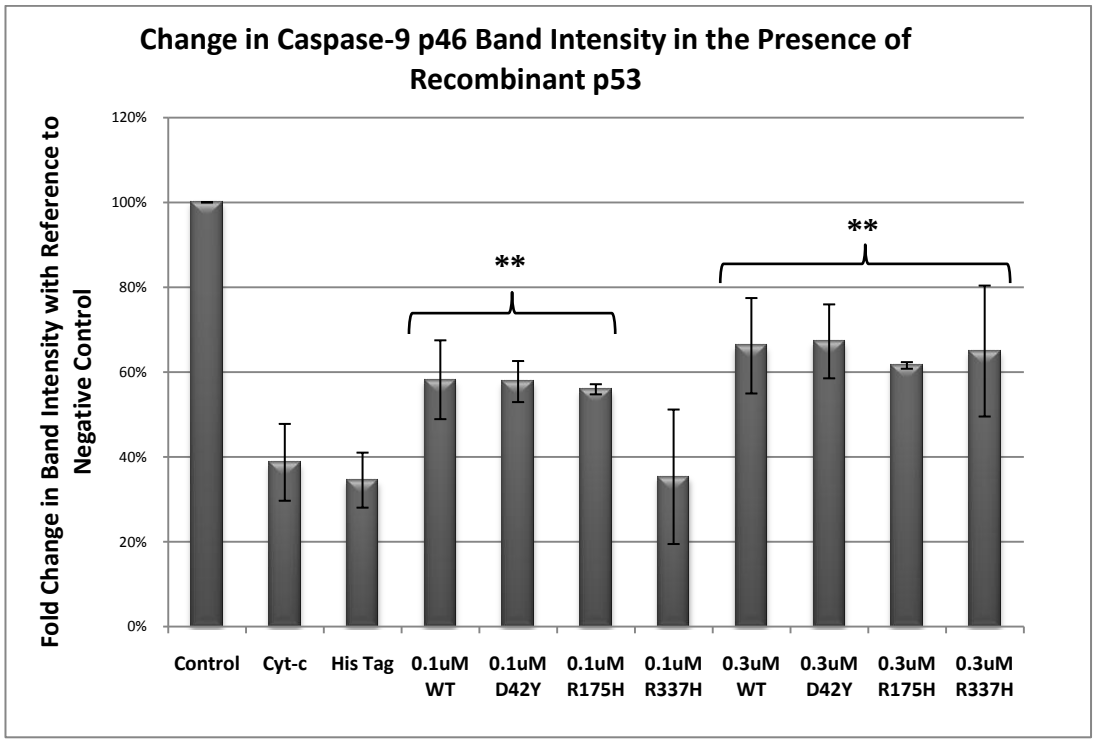
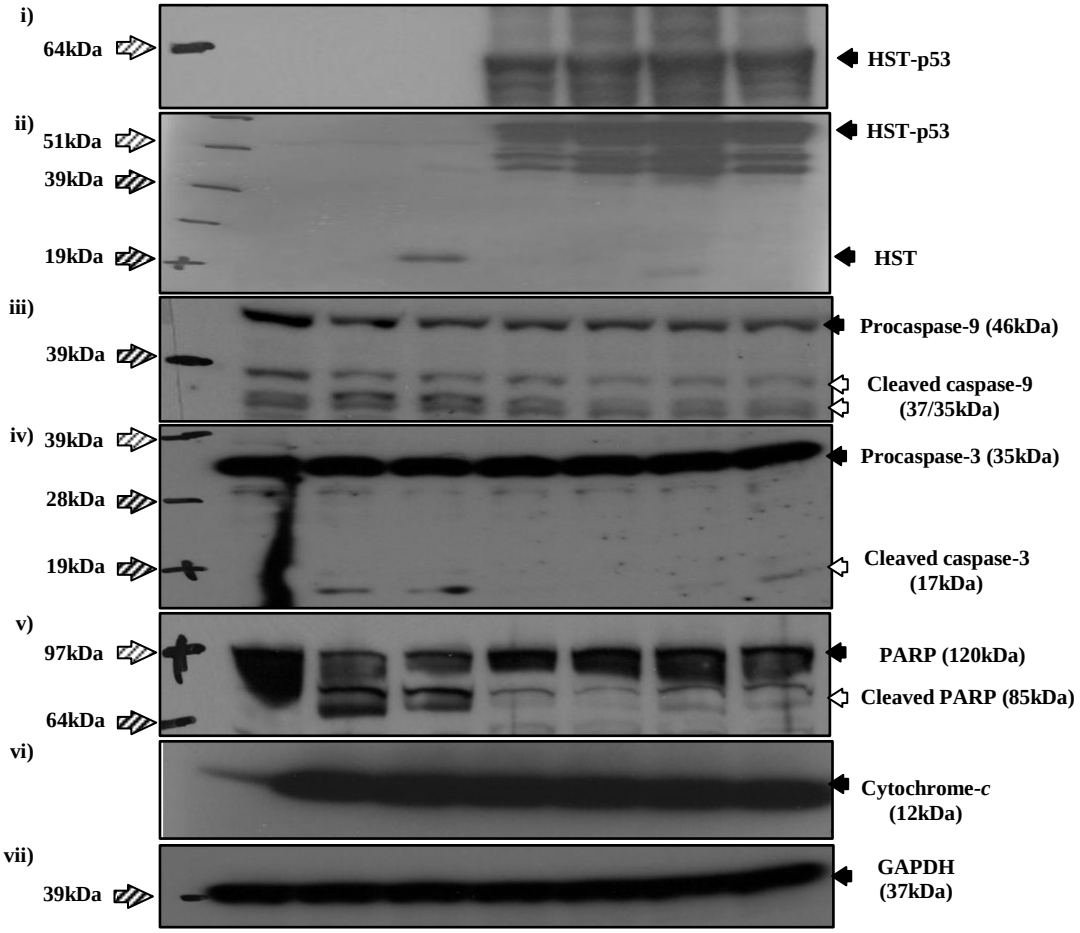


Figure 3.3. Addition of Recombinant p53 Inhibits Caspase Cleavage in HCT-116 p53$^{-/-}$ S100 Cell Lysate. S100 lysates from HCT-116 p53$^{-/-}$ cells were obtained and incubated with the various recombinant proteins for 1 hour after addition of equal amount of dATP and cytochrome-*c* to initiate caspase cleavage. Profile of p53 **(i)**, Caspase-9 **(ii)**, Caspase-3 **(iii)**, Caspase-6 **(iv)**, Caspase-8 **(v)**, PARP **(vi)**, cytochrome-*c* **(vii)** and GAPDH **(viii)**. Lane 1: lysate without cytochrome-*c* challenge; Lane 2: lysate with cytochrome-*c* challenge; Lane 3: lysate with cytochrome-*c* challenge and addition of 0.3µM HST-tag protein; Lanes 4-7: lysate with cytochrome-*c* challenge and addition of 0.1µM of the various recombinant p53; Lanes 8-11: lysate with cytochrome-*c* challenge and addition of 0.3µM of the various recombinant p53. **B)** Band intensities of the p46 fragment of caspase-9 in were measured using ImageJ and calculated with reference to the control lysate to obtain the percentage change in band intensities. ** denotes the fold change in the band intensity of the fractions with 0.3µM recombinant p53 vs. 0.3µM HST-tag was significantly different ($p<0.05$, $n=3$).

A)

HST Tag (μM)	**Marker**	-	-	0.3	-	-	-	-
Recombinant p53 (0.3μM)		-	-	-	WT	D42Y	R175H	R337H
Cytochrome-*c*		-	+	+	+	+	+	+



B)

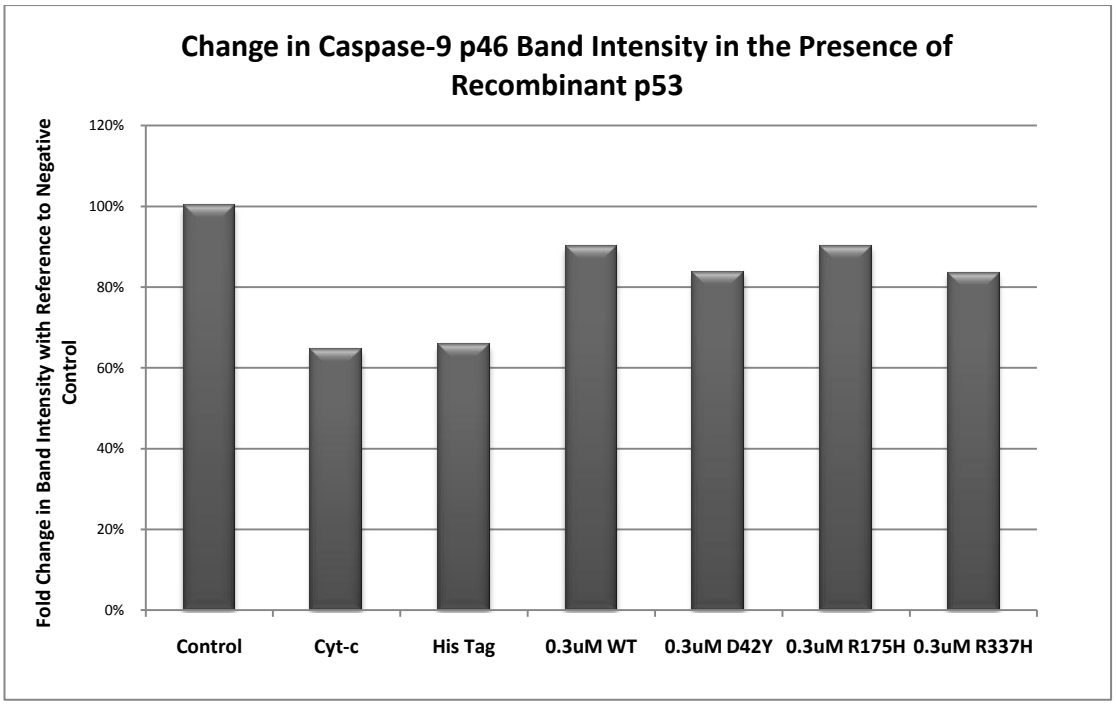
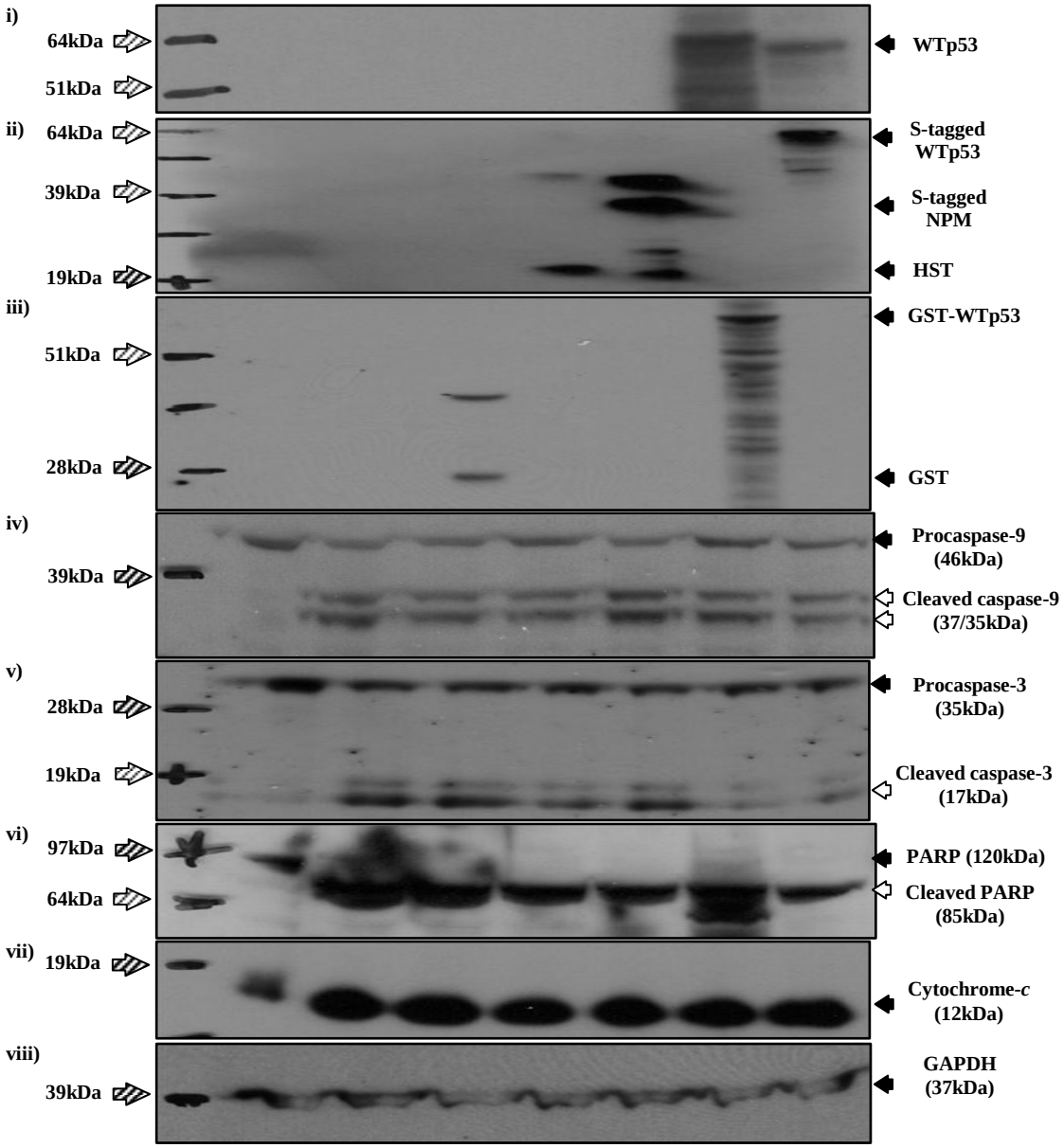


Figure 3.4. Addition of Recombinant p53 Inhibits Caspase Cleavage in A2780 S100 Cell Lysate. A) S100 lysates from A2780 cells were obtained and incubated with the various recombinant proteins for 1 hour after addition of equal amount of dATP and cytochrome-*c* to initiate caspase cleavage. Profile of p53 **(i)**, HST-tagged proteins **(ii)**, Caspase-9 **(iii)**, Caspase-3 **(iv)**, PARP **(v)**, cytochrome-*c* **(vi)** and GAPDH **(vii)**. Lane 1: lysate without cytochrome-*c* challenge; Lane 2: lysate with cytochrome-*c* challenge; Lane 3: lysate with cytochrome-*c* challenge and addition of 0.3µM HST-tag protein; Lanes 4-7: lysate with cytochrome-*c* challenge and addition of 0.3µM of the various recombinant p53. A light exposure of the p53 profile was used and endogenous p53 in A2780 is only visible when the blot is overexposed. **B)** Band intensities of the p46 fragment of caspase-9 in were measured using ImageJ and calculated with reference to the control lysate to obtain the percentage change in band intensities (n=2).

A)

GST Tag	**Marker**	-	-	+	-	-	-	-
HST Tag		-	-	-	+	-	-	-
NPM		-	-	-	-	+	-	-
Recombinant p53		-	-	-	-	-	C	WT
Cytochrome-*c*		-	+	+	+	+	+	+



B)

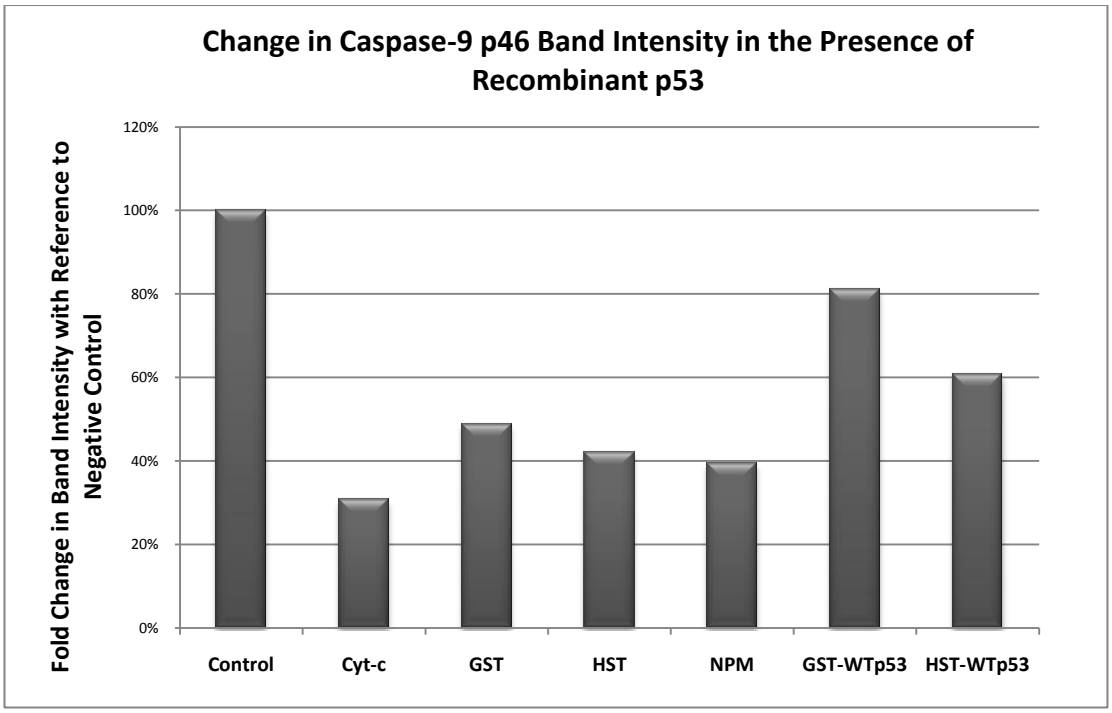


Figure 3.5. Only the Addition of Recombinant p53 Inhibits Caspase Cleavage in A2780 S100 Cell Lysate. S100 lysates from A2780 cells were obtained and incubated with the various recombinant proteins for 1 hour after addition of equal amount of dATP and cytochrome-*c* to initiate caspase cleavage. Profile of p53 (**i**), HST-tagged proteins **(ii)**, GST-tagged proteins **(iii)**, Caspase-9 **(iv)**, Caspase-3 **(v)**, PARP **(vi),** cytochrome-*c* **(vii)** and GAPDH **(viii)**. Lane 1: lysate without cytochrome-*c* challenge; Lane 2: lysate with cytochrome-*c* challenge; Lane 3: lysate with cytochrome-*c* challenge and addition of 0.3µM HST-tag protein; Lanes 4: lysate with cytochrome-*c* challenge and 0.3µM of GST-tag protein; Lane 5: lysate with cytochrome-*c* challenge and 0.3µM of recombinant nuclephosmin protein; Lane 6: lysate with cytochrome-*c* challenge and 0.3µM GST-WTp53 protein from commercial source; Lanes 7: lysate with cytochrome-*c* challenge and addition of 0.3µM of HST-tagged p53. A light exposure of the p53 profile was used and endogenous p53 in A2780 is only visible when the blot is overexposed. **B)** Band intensities of the p46 fragment of caspase-9 in were measured using ImageJ and calculated with reference to the control lysate to obtain the percentage change in band intensities (n=2).

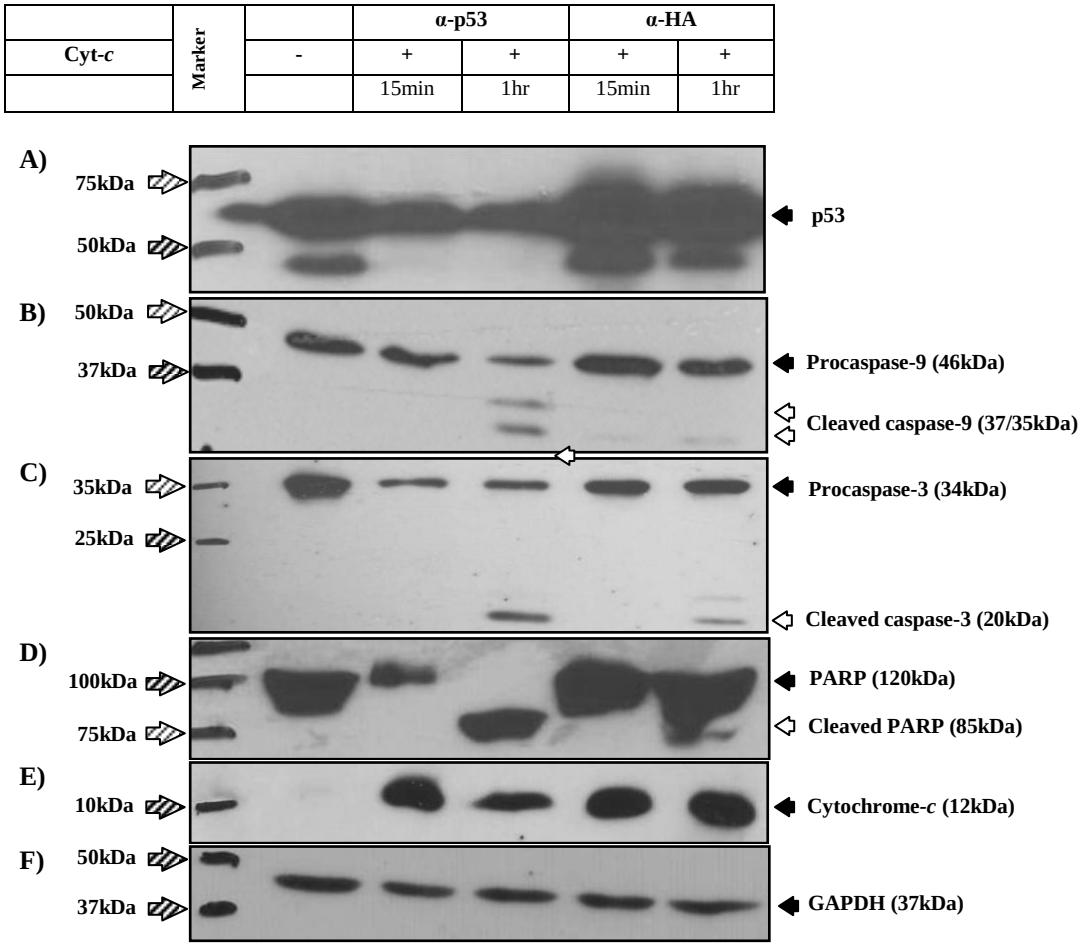


Figure 3.6. Immunodepletion of Endogenous p53 in HEK293 S100 Lysate Accelerates Caspase Cleavage. Profile of p53 **(A)**, Caspase-9 **(B)**, Caspase-3 **(C)**, PARP **(D)**, cytochrome-*c* **(E)** and GAPDH **(F)**. Cleavage Pattern upon addition of cytochrome-*c* to p53 immunodepleted HEK293T cell lysates. Lane 1: HEK293T S100 cytosolic lysate; Lane 2: HEK293T lysate immunodepleted with p53 antibody and challenged with cytochrome-*c* for 15min; Lane 3: lysate immunodepleted with p53 antibody and challenged with cytochrome-*c* for 1 hour; Lane 4: lysate immunodepleted with haemaglutinin (HA) antibody and challenged with cytochrome-*c* for 15min; Lane 5: lysate immunodepleted with HA antibody and challenged with cytochrome-*c* for 1 hour.

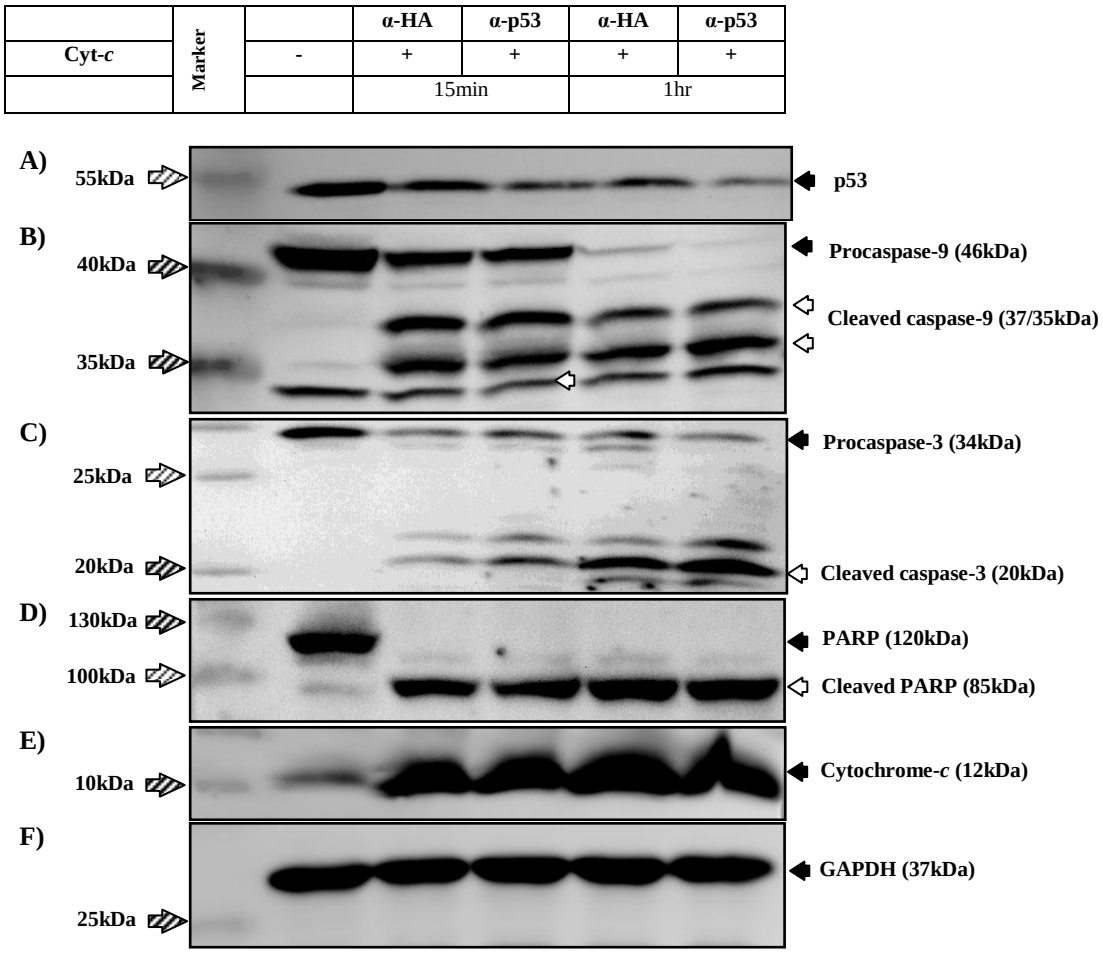


Figure 3.7. Immunodepletion of Endogenous p53 in A2780 cells Accelerates Caspase Cleavage. Profile of p53 **(A)**, Caspase-9 **(B)**, Caspase-3 **(C)**, PARP **(D)**, cytochrome-*c* **(E)** and GAPDH **(F)**. Cleavage Pattern upon addition of cytochrome-*c* to p53 immunodepleted A2780 cell lysates. Lane 1: A2780 S100 cytosolic lysate; Lane 2: A2780 lysate immunodepleted with HA antibody and challenged with cytochrome-*c* for 15min; Lane 3: lysate immunodepleted with p53 antibody and challenged with cytochrome-*c* for 15min; Lane 4: lysate immunodepleted with HA antibody and challenged with cytochrome-*c* for 1 hour; Lane 5: lysate immunodepleted with p53 antibody and challenged with cytochrome-*c* for 1 hour.

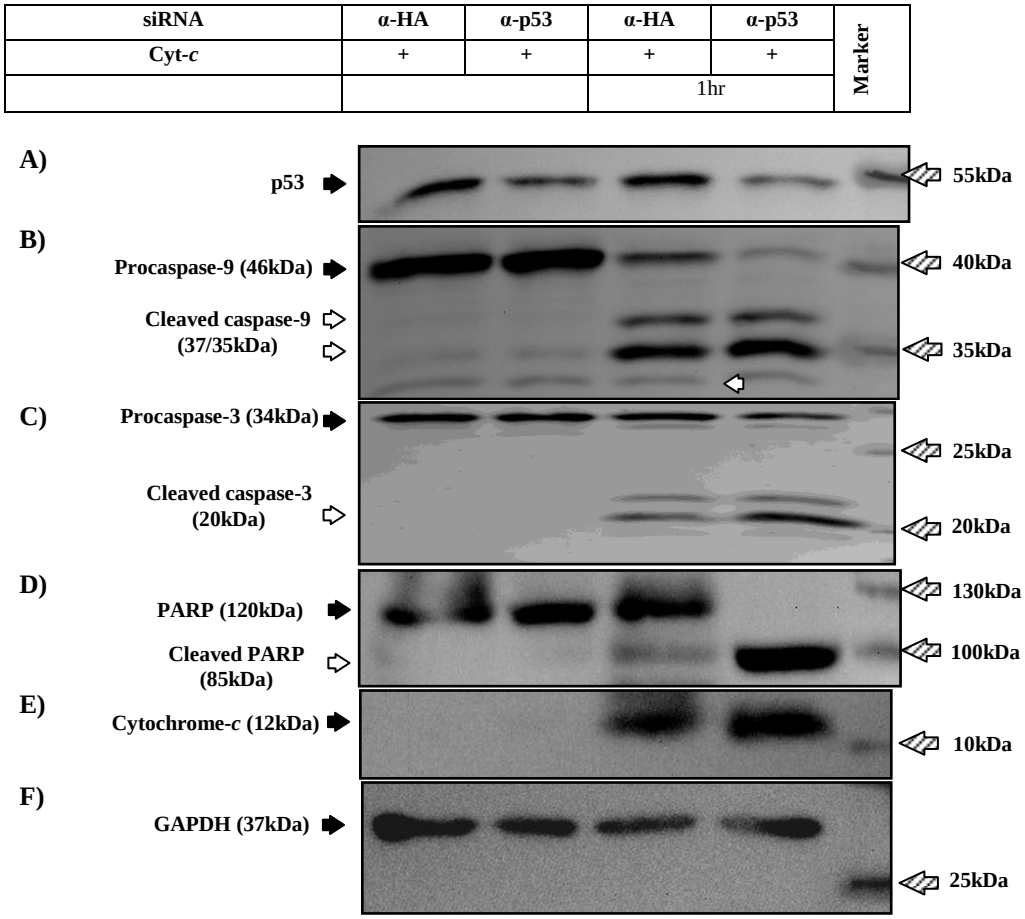
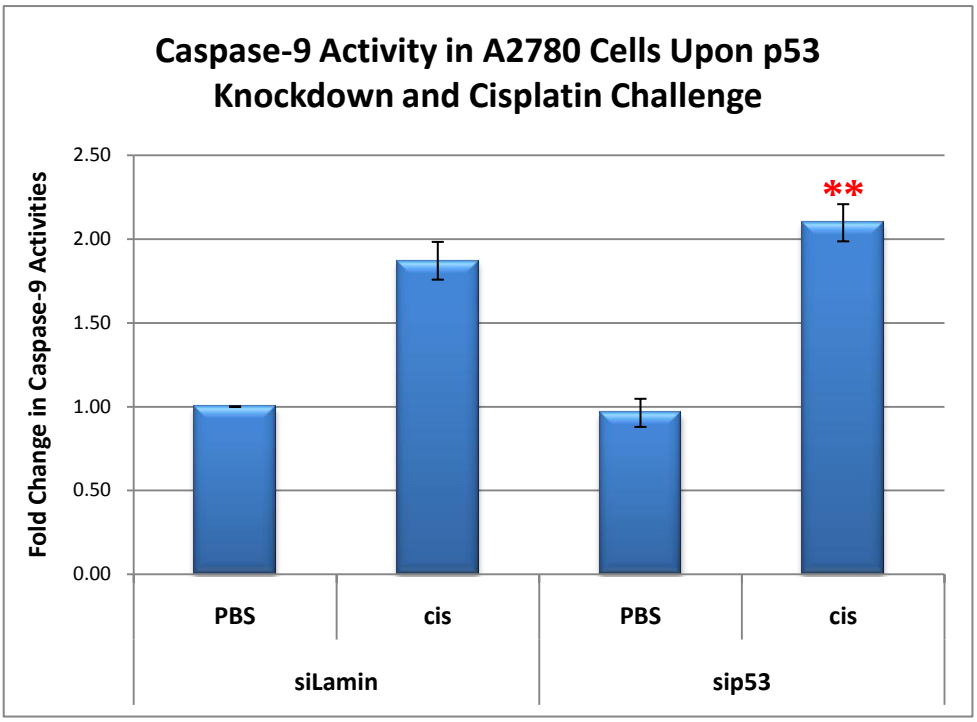


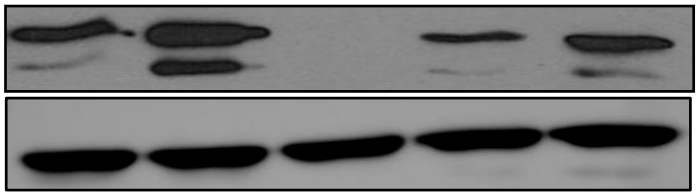
Figure 3.8. Knockdown of Endogenous p53 in HEK293 cells Accelerates Caspase Cleavage. Profile of p53 **(A)**, Caspase-9 **(B)**, Caspase-3 **(C)**, PARP **(D)**, cytochrome-*c* **(E)** and GAPDH **(F)**. Cleavage Pattern upon addition of cytochrome-*c* to HEK293 S100 cytosolic lysates which were subjected to knockdown of p53 over 48-hour period. NC represents negative control which contained siRNA with scrambled p53 sequences. Lane 1: lysate of HEK293 cells transfected with NC siRNA with no cytochrome-*c* challenge; Lane 2: lysate of HEK293 cells transfected with siRNA against p53 with no cytochrome-*c* challenge; Lane 3: lysate of HEK293 cells transfected with NC siRNA with cytochrome-*c* challenge for 15 minutes; Lane 4: lysate of HEK293 cells transfected with siRNA against p53 with cytochrome-*c* challenge for 15 minutes; Lane 5: lysate of HEK293 cells transfected with NC siRNA with cytochrome-*c* challenge for 1 hour; Lane 6: lysate of HEK293 cells transfected with siRNA against p53 with cytochrome-*c* challenge for 1 hour.

A)



B)

	A2780		H1299 $p53^{-/-}$	A2780	
siRNA	Lamin A/C		-	p53	
Cisplatin	-	+	-	-	+



C)

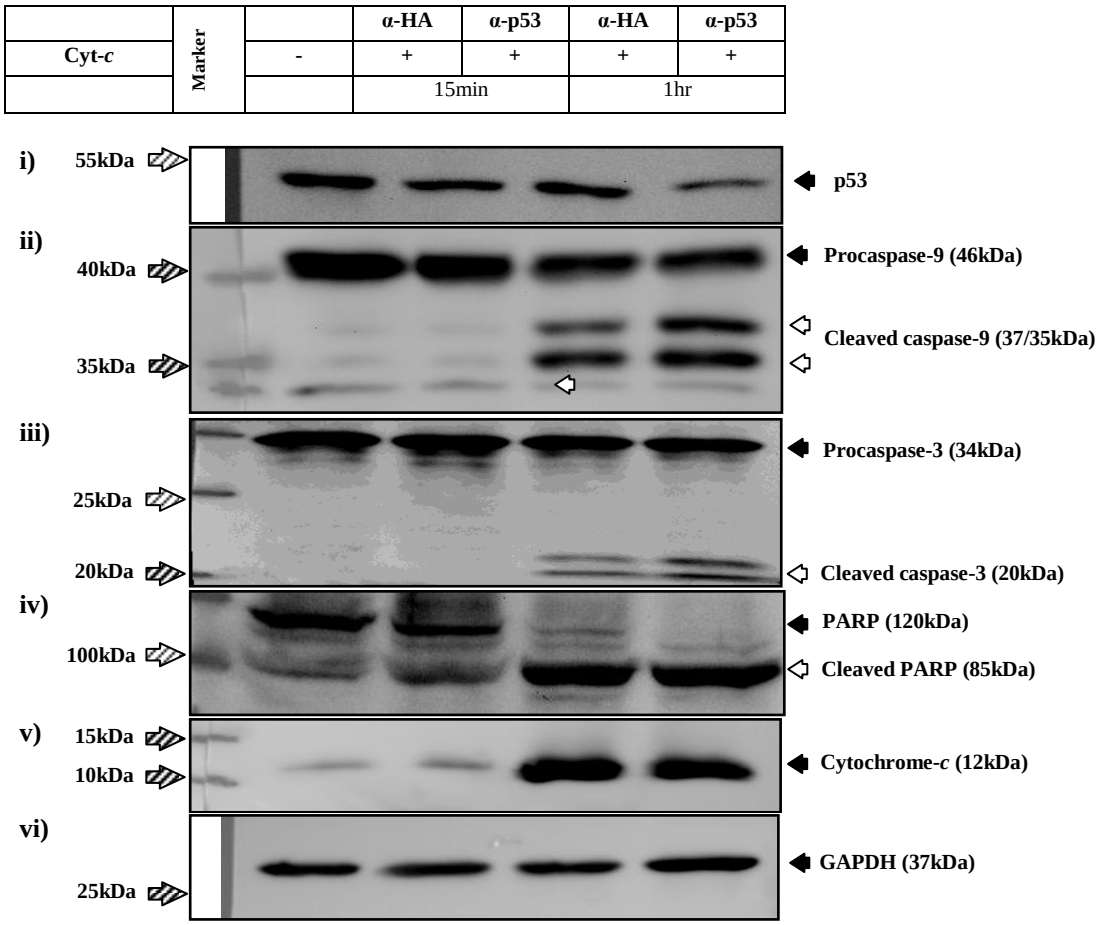
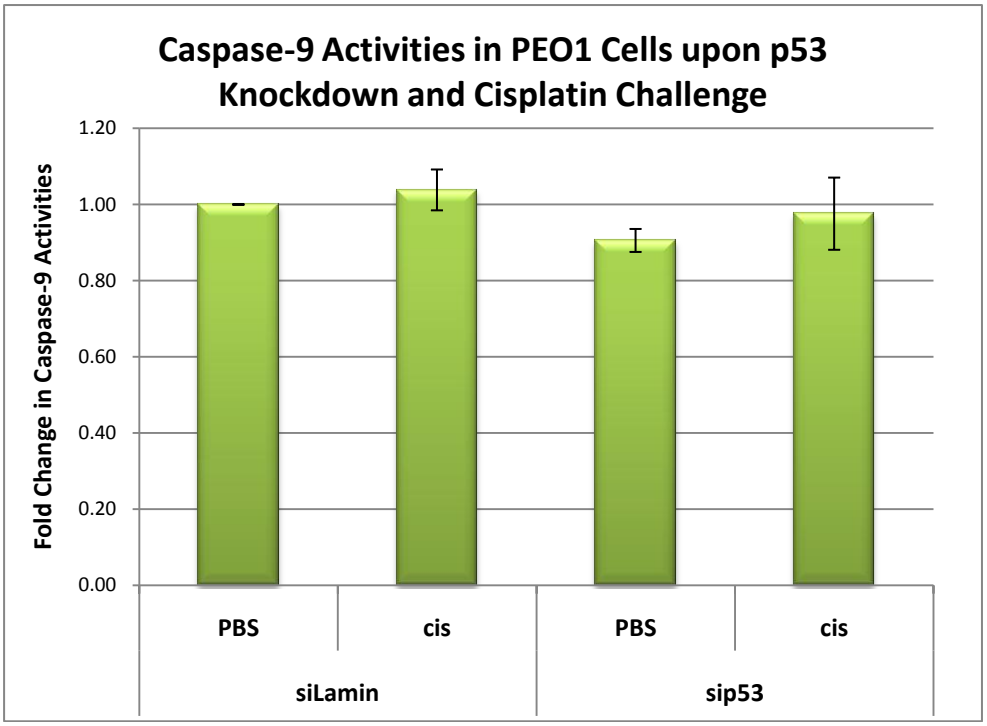


Figure 3.9. Knockdown of Endogenous p53 in A2780 cells Accelerates Caspase Activities. A) A2780 cells were subjected to knockdown of p53 for 24 hours and subsequent challenge with 25μM cisplatin for a further 24 hours. Endogenous caspase-9 activities of both cells lines were then measured using fluorescent substrate specific to caspase-9. ** denotes that the levels of caspase-9 activity between the siLamin and sip53 after cisplatin challenge are significantly different ($p<0.05$). **B)** Profile of p53 in A2780 total cell lysates. Lane 1: cells transfected with siRNA against Lamin A/C and challenged with PBS (control); Lane 2: cells transfected with siRNA against Lamin A/C and challenged with 25μM cisplatin; Lane 3: total cell lysate of H1299 $p53^{-/-}$ cells; Lane 4: cells transfected with siRNA against p53 and challenged with PBS; Lane 5: cells transfected with siRNA against p53 and challenged with 25μM cisplatin. **C)** Profile of p53 **(i)**, Caspase-9 **(ii)**, Caspase-3 **(iii)**, PARP **(iv)**, cytochrome-*c* **(v)** and GAPDH **(vi)**. Cleavage Pattern upon addition of cytochrome-*c* to A2780 S100 cytosolic lysates which were subjected to knockdown of p53 for 24 hours. NC represents negative control which contained siRNA with scrambled p53 sequences. Lane 1: lysate of A2780 cells transfected with NC siRNA with no cytochrome-*c* challenge; Lane 2: lysate of A2780 cells transfected with siRNA against p53 with no cytochrome-*c* challenge; Lane 3: lysate of A2780 cells transfected with NC siRNA with cytochrome-*c* challenge for 15 minutes; Lane 4: lysate of A2780 cells transfected with siRNA against p53 with cytochrome-*c* challenge for 15 minutes; Lane 5: lysate of A2780 cells transfected with NC siRNA with cytochrome-*c* challenge for 1 hour; Lane 6: lysate of A2780 cells transfected with siRNA against p53 with cytochrome-*c* challenge for 1 hour.

A)

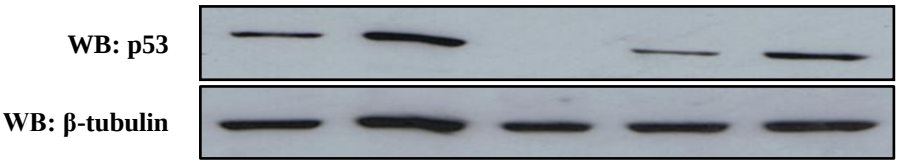
	Codon 339	340	341	**342**	343	344
WTp53	GAG	ATG	TTA	**CGA**	GAG	GTG
	E	M	F	R	E	L
PEO1 and PEO4	GAG	ATG	TTA	**CCA**	GAG	GTG
	E	M	F	P	E	L

B)

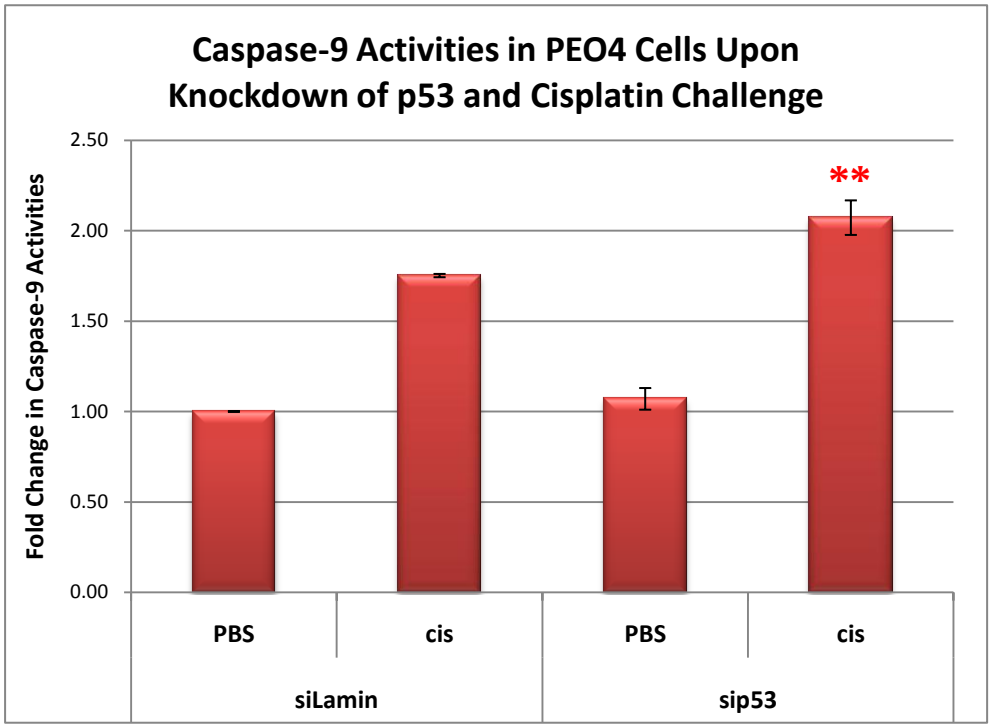


C)

	PEO1		H1299 $p53^{-/-}$	PEO1	
siRNA	**Lamin A/C**		-	**p53**	
Cisplatin	-	+	-	-	+



D)



E)

	PEO4		H1299 $p53^{-/-}$	PEO4	
siRNA	Lamin A/C		-	p53	
Cisplatin	-	+	-	-	+



F)

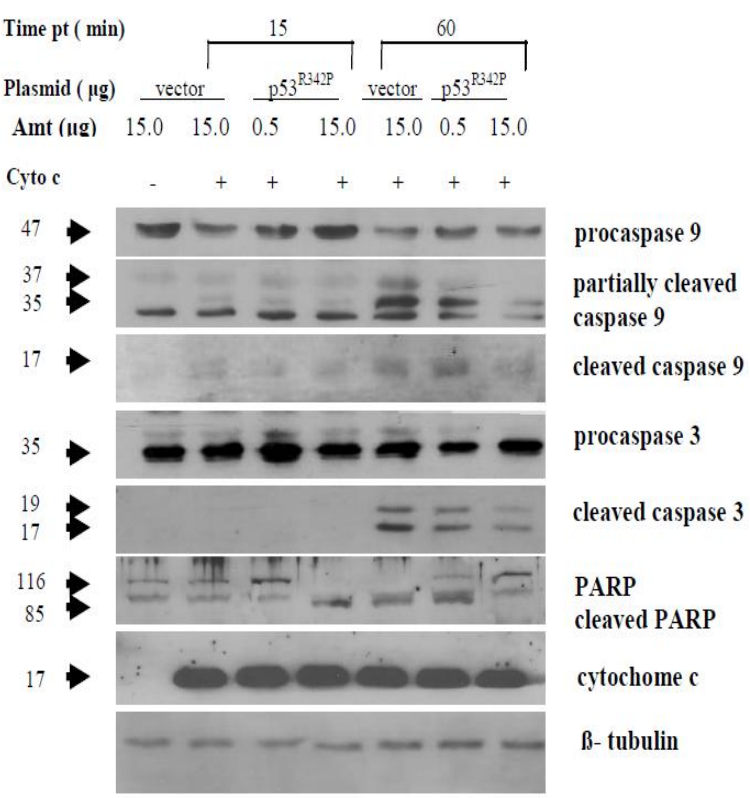
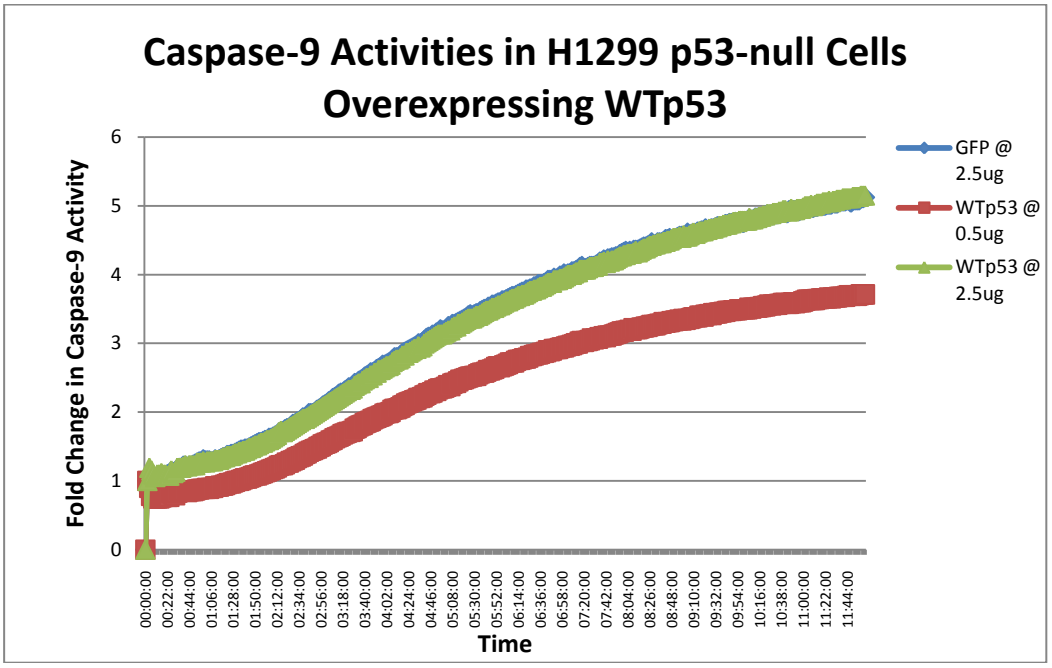


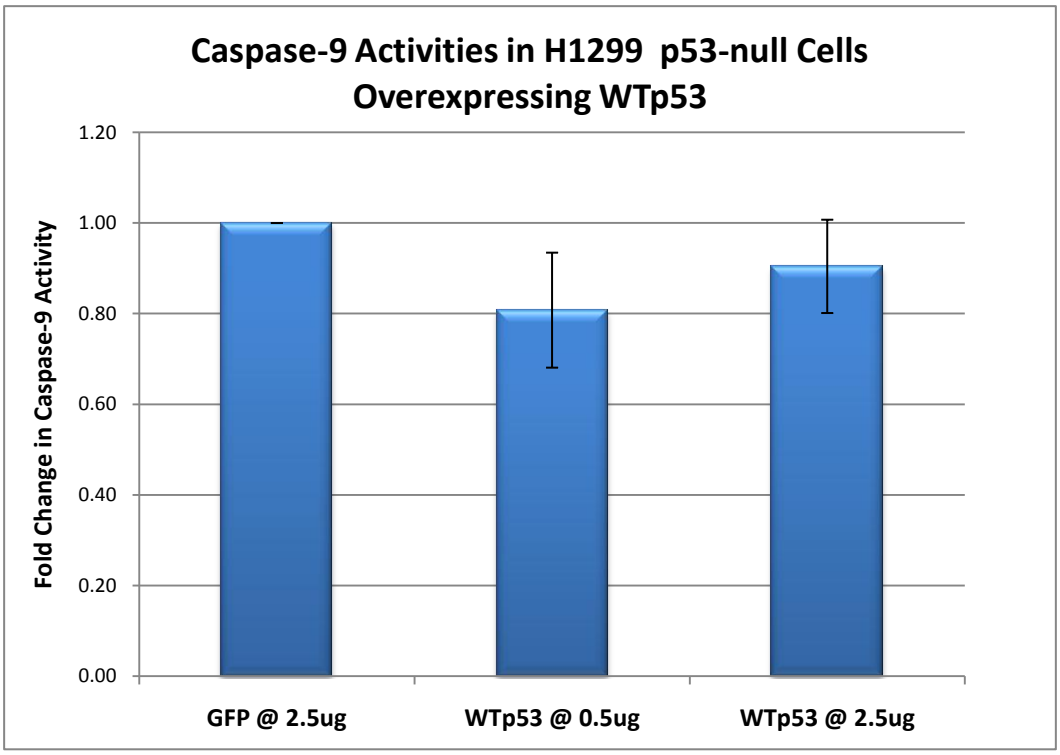
Figure 3.10. Knockdown of Endogenous p53 in PEO4 cells Accelerates Caspase Activities. **A)** Sequencing data of PEO1 and PEO4 DNA to show the mutation site at codon 342 of the p53 gene. **B & D)** Both cells lines were subjected to knockdown of p53 for 24 hours and subsequent challenge with 25µM cisplatin for a further 24 hours. Endogenous caspase-9 activities of both cells lines were then measured using fluorescent substrate specific to caspase-9. ** denotes that the levels of caspase-9 activity between the siLamin and sip53 after cisplatin challenge are significantly different ($p<0.001$). **C & E)** Profile of p53 of PEO1 (C) and PEO4 (E) total cell lysates. Lane 1: PEO1/4 cells transfected with siRNA against Lamin A/C and challenged with PBS (control); Lane 2: PEO1/4 cells transfected with siRNA against Lamin A/C and challenged with 25µM cisplatin; Lane 3: total cell lysate of H1299 $p53^{-/-}$ cells; Lane 4: PEO1/4 cells transfected with siRNA against p53 and challenged with PBS; Lane 5: PEO1/4 cells transfected with siRNA against p53 and challenged with 25µM cisplatin. **F)** Figure showing profile of the various caspases upon overexpression of $p53^{R342P}$ in H1299 cells (extracted from Figure 10 of Phua[224]).

A)

i)

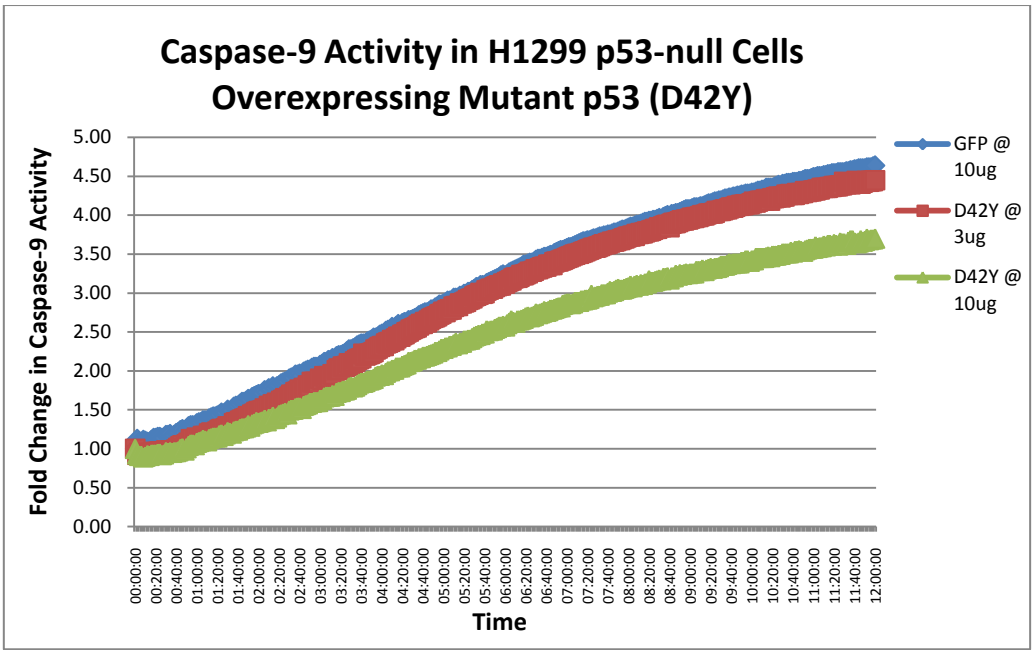

Caspase-9 Activities in H1299 p53-null Cells Overexpressing WTp53
Fold Change in Caspase-9 Activity
0
1
2
3
4
5
6
Time
00:00:00
00:22:00
00:44:00
01:06:00
01:28:00
01:50:00
02:12:00
02:34:00
02:56:00
03:18:00
03:40:00
04:02:00
04:24:00
04:46:00
05:08:00
05:30:00
05:52:00
06:14:00
06:36:00
06:58:00
07:20:00
07:42:00
08:04:00
08:26:00
08:48:00
09:10:00
09:32:00
09:54:00
10:16:00
10:38:00
11:00:00
11:22:00
11:44:00
GFP @ 2.5ug
WTp53 @ 0.5ug
WTp53 @ 2.5ug

ii)

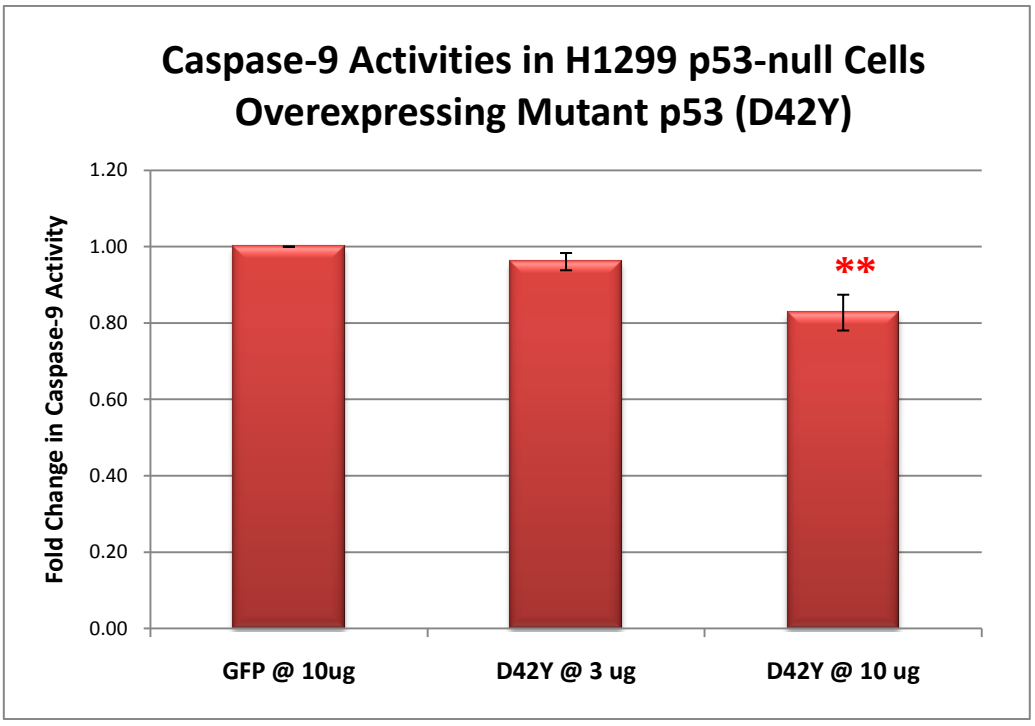

Caspase-9 Activities in H1299 p53-null Cells Overexpressing WTp53
Fold Change in Caspase-9 Activity
0.00
0.20
0.40
0.60
0.80
1.00
1.20
GFP @ 2.5ug
WTp53 @ 0.5ug
WTp53 @ 2.5ug

B)

i)

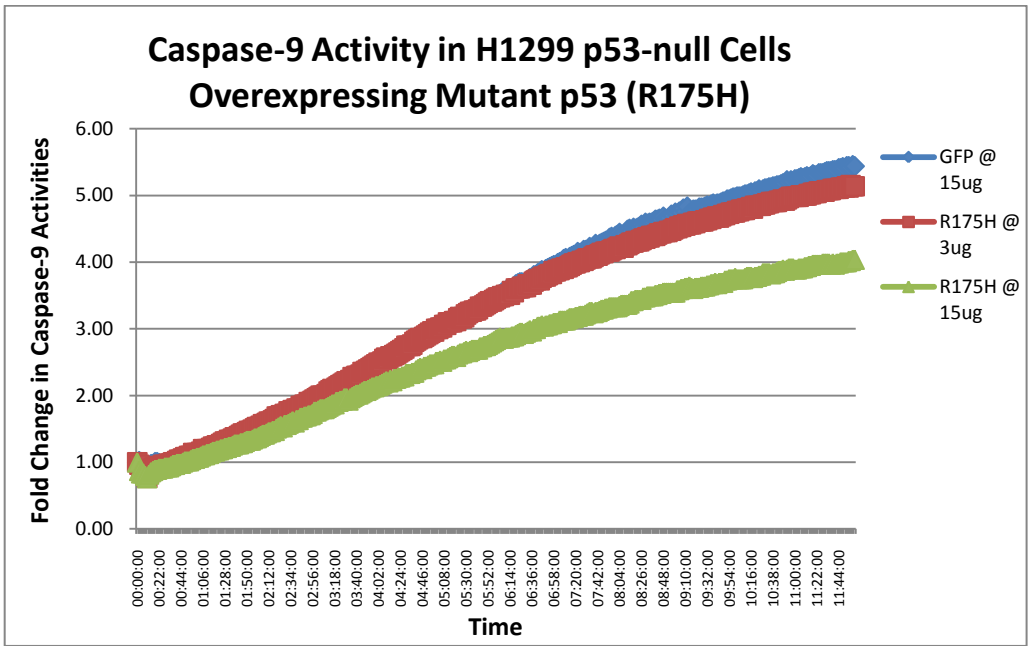

Caspase-9 Activity in H1299 p53-null Cells Overexpressing Mutant p53 (D42Y)
Fold Change in Caspase-9 Activity
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
00:00:00
00:20:00
00:40:00
01:00:00
01:20:00
01:40:00
02:00:00
02:20:00
02:40:00
03:00:00
03:20:00
03:40:00
04:00:00
04:20:00
04:40:00
05:00:00
05:20:00
05:40:00
06:00:00
06:20:00
06:40:00
07:00:00
07:20:00
07:40:00
08:00:00
08:20:00
08:40:00
09:00:00
09:20:00
09:40:00
10:00:00
10:20:00
10:40:00
11:00:00
11:20:00
11:40:00
12:00:00
Time
GFP @ 10ug
D42Y @ 3ug
D42Y @ 10ug

ii)

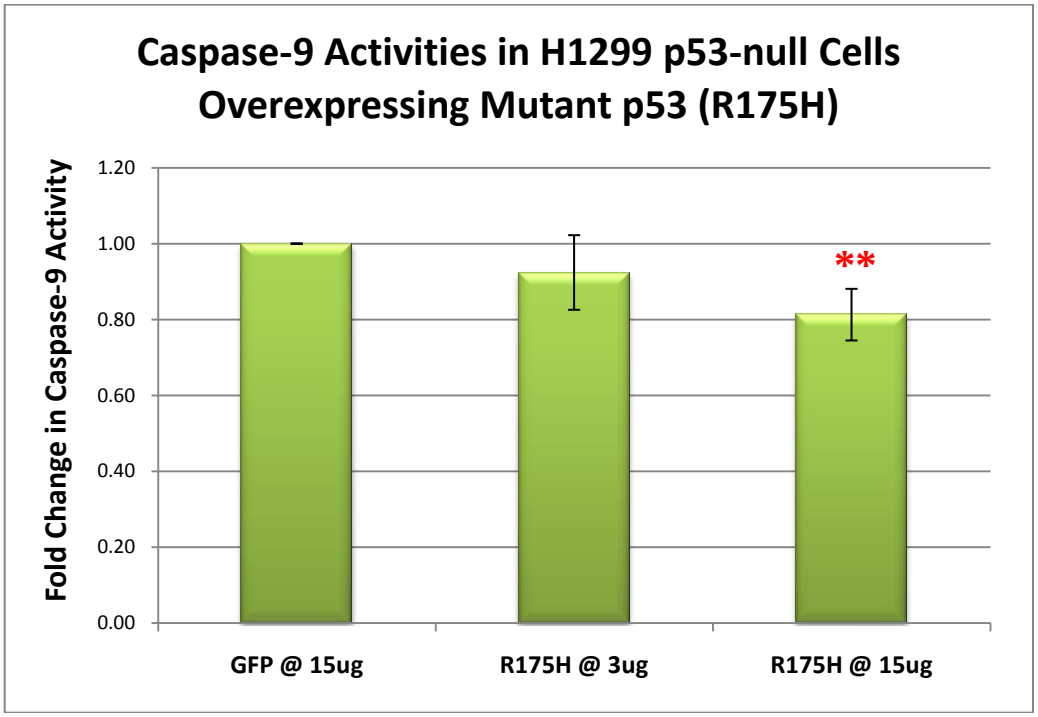

Caspase-9 Activities in H1299 p53-null Cells Overexpressing Mutant p53 (D42Y)
Fold Change in Caspase-9 Activity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
GFP @ 10ug
D42Y @ 3 ug
D42Y @ 10 ug

C)

i)

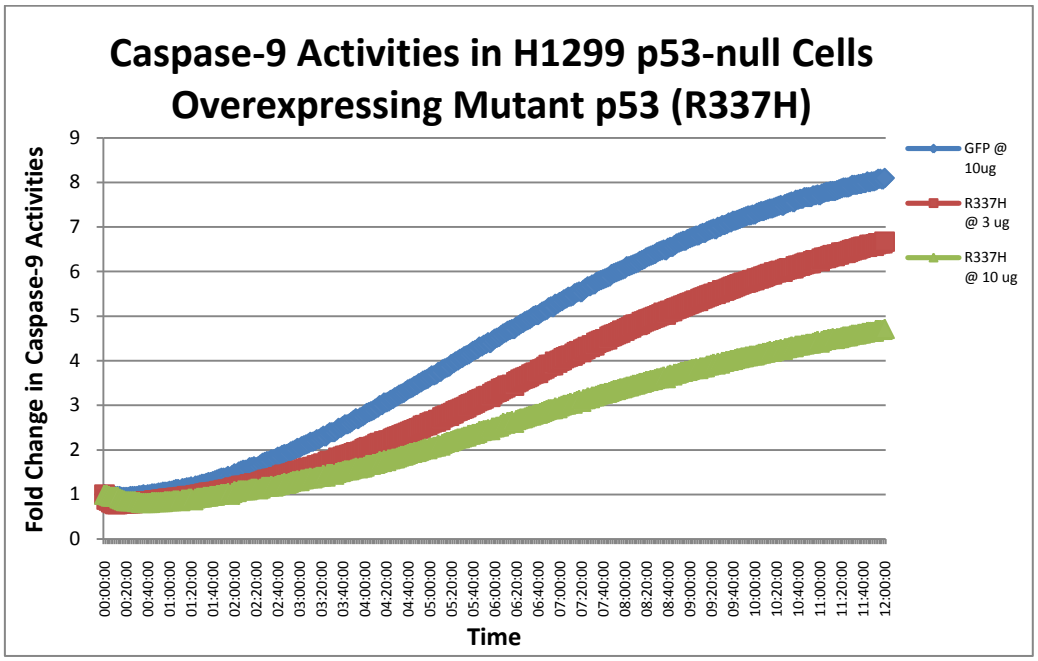

Caspase-9 Activity in H1299 p53-null Cells Overexpressing Mutant p53 (R175H)
Fold Change in Caspase-9 Activities
6.00
5.00
4.00
3.00
2.00
1.00
0.00
00:00:00
00:22:00
00:44:00
01:06:00
01:28:00
01:50:00
02:12:00
02:34:00
02:56:00
03:18:00
03:40:00
04:02:00
04:24:00
04:46:00
05:08:00
05:30:00
05:52:00
06:14:00
06:36:00
06:58:00
07:20:00
07:42:00
08:04:00
08:26:00
08:48:00
09:10:00
09:32:00
09:54:00
10:16:00
10:38:00
11:00:00
11:22:00
11:44:00
Time
GFP @ 15ug
R175H @ 3ug
R175H @ 15ug

ii)

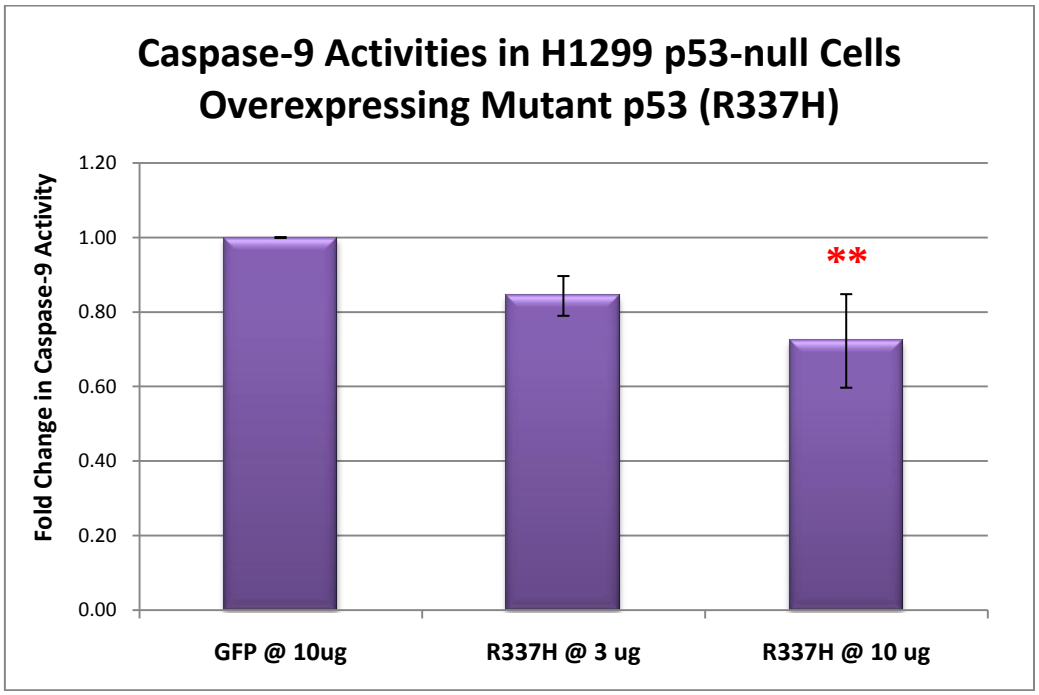

Caspase-9 Activities in H1299 p53-null Cells Overexpressing Mutant p53 (R175H)
Fold Change in Caspase-9 Activity
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
GFP @ 15ug
R175H @ 3ug
R175H @ 15ug

D)

i)


Caspase-9 Activities in H1299 p53-null Cells Overexpressing Mutant p53 (R337H)
Fold Change in Caspase-9 Activities
Time
GFP @ 10ug
R337H @ 3 ug
R337H @ 10 ug

ii)


Caspase-9 Activities in H1299 p53-null Cells Overexpressing Mutant p53 (R337H)
Fold Change in Caspase-9 Activity
**
GFP @ 10ug
R337H @ 3 ug
R337H @ 10 ug

E)

Marker	pXJ-HA40-GFP	pXJ-HA40-GFP-WTp53		pXJ-HA40-GFP-p53^{D42Y}		pXJ-HA40-GFP-p53^{R175H}		pXJ-HA40-GFP-p53^{R337H}	
	15µg	0.5 µg	2.5 µg	3 µg	10 µg	3 µg	15 µg	3 µg	10 µg

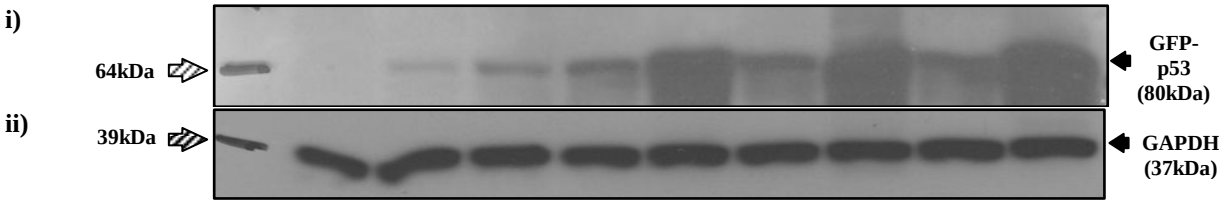


Figure 3.11. Overexpression of p53 in H1299 p53$^{-/-}$ Cells Inhibits Caspase-9 Activities. Profile of caspase-9 activities over a time course of 12 hours in S100 cytosolic lysates of H1299 p53$^{-/-}$ cells overexpressing various amounts of pXJ40-HA-GFP-WTp53 **(A)**, pXJ40-HA-GFP-p53^{D42Y} **(B)**, pXJ40-HA-GFP-p53^{R175H} **(C)** and pXJ40-HA-GFP-p53^{R337H} **(D)**; **A-D(i)** shows the kinetics of caspase-9 activity over a time course of 12-hours; **A-D(ii)** shows the end-point caspase-9 activities. ** denotes that the levels of caspase-9 activity between the overexpression of pXJ40-HA-GFP control and pXJ40-HA-GFP-p53 is significantly different ($p<0.05$); **E)** Western blot showing p53 expression **(i)** and GAPDH as the loading control **(ii)** in the same lysates used in (A) to (D).

3.3 Discussion

The results obtained in this study strongly indicate that p53 inhibits the cleavage of caspases. This was observed where removal of p53 accelerated caspase cleavage and activity, but the presence of p53 inhibited caspase cleavage. These results were initially surprising as it is a common knowledge that p53 (as reviewed in Introduction) should induce apoptosis. However, further in depth literature review reveals evidence suggesting that this novel function of p53 might indeed be true.

As mentioned earlier, Guillou [220], Jiang [219] and Ghavam-Nasiri [250] have observed a correlation between high levels of cytoplasmic mutant p53 and greater radio- and chemo-resistance of tumour cells. In accordance with this, ovarian cancer tumours are also found to exhibit overexpression of WTp53 [251]. In addition, there are many papers reporting the presence of p53 with missense mutations in tumour cells. These reports altogether seem to indicate that there are additional functions of p53 that are yet unknown. In this study, it appears that an unknown function is the inhibition of caspase activation by p53.

Since multiple cellular stress signalling pathways converge upon p53, it is logical and desirable that the master regulatory switch be able to initiate or inhibit apoptotic signalling. The additional control afforded by p53, through its inhibition of the important apical death signalling component caspase-9, will also help safe-guard against "accidental" death signalling e.g. caused by heightened amount of PUMA, BAX and Bak (which are all upstream of caspase signalling) as a result of mild cytotoxic provocation.

Given p53's importance in regulating cell fate, it is intriguing as to why this facet of p53 remained undiscovered until now. As reviewed in Chapter 1, the focus of research on p53 in oncology thus far has been on its inability to transactivate pro-apoptotic target genes such as *p21* and *Bax.* As caspases remained unknown as transcriptional target genes of p53 until very recently, p53's effect and direct interaction with caspases might have been

overlooked completely. Most importantly, for the current investigation, the use of a cell-free system derived primarily from cytosol and devoid of nuclear content, has allowed a focus on the transcription-independent influence of p53 in the caspase activation cascade. Also, the lack of mitochondria in the cell-free system, as well as the manual addition of cytochrome-*c* to the lysates limited the influence of p53 on Bax/Bak-mediated cytochrome-*c* release from the mitochondria, events which are upstream of caspase activation in an *in vivo* situation. All in all, the use of the cell-free system had been very crucial in uncovering the hitherto unknown oncogenic facet of p53 that is unrelated to its well-studied transactivation function.

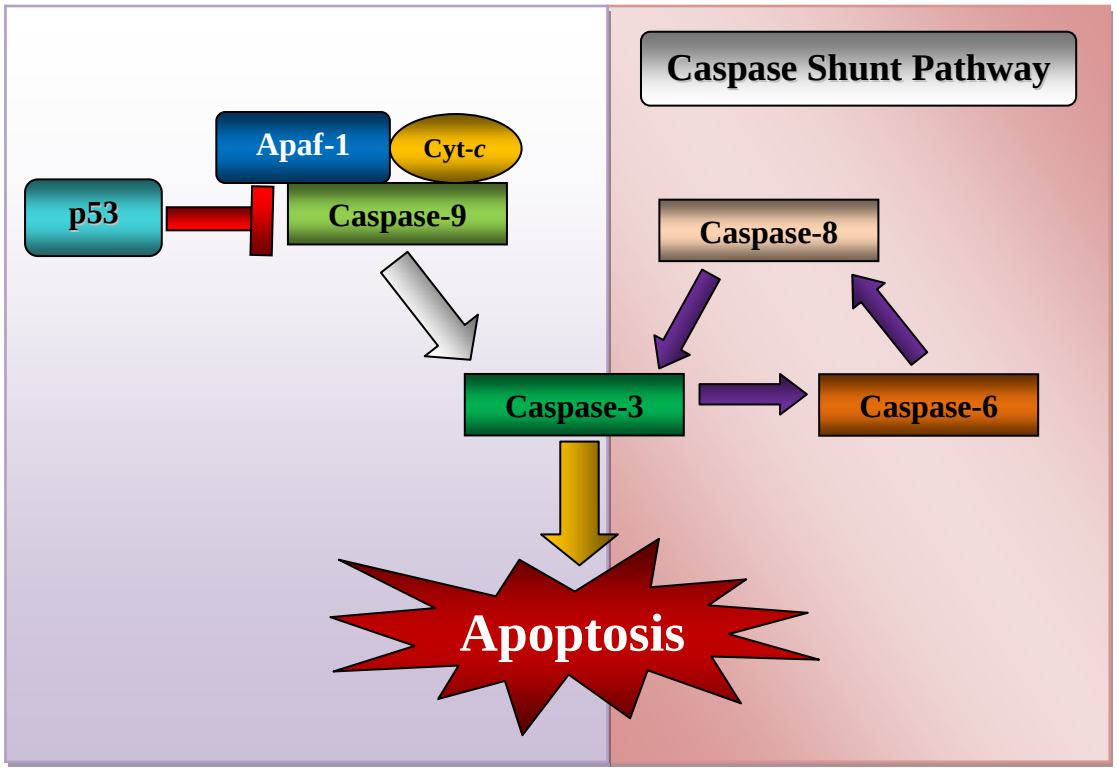


Figure 3.12. Diagram showing the relationship between caspases in the caspase shunt pathway. Inhibition of caspase-9 by p53 will arrest the progression of the normal as well as the caspase shunt pathway, thereby suppressing apoptosis in cells.

Caspase-9 is the most apical caspase activated in the mitochondrial apoptotic signalling pathway. When activated, downstream targets such as caspase-3 are also activated, which subsequently cleave and inactivate house-keeping genes like PARP to enable apoptosis to take place. In addition, a small amount of the caspase is usually shunted into the caspase

amplification loop for rapid signal amplification. It was previously demonstrated that caspase-9 activates caspase-3, which will in turn activate caspase-6 [244]. Caspase-6 then cleaves procaspase-8 [243], and activated caspase-8 back cleaves procaspase-3 [245], thereby completing the signal amplification loop (Figure 3.12). The caspase amplification loop is thus made up of various caspases cleaving one another in that sequential order, leading to an escalation of caspase activities enabling a swift death response. As such, inhibition of the apical caspase-9 by p53 not only serves to retard the mitochondrial death signalling pathway, but also effectively inhibits initiation and "snow-balling" of various caspase activations through the amplification loop. This will probably help protect against aberrant escalation of death signalling under very mild, non-threatening stress conditions.

The addition of the various recombinant p53 forms into HEK293, HCT-116 $p53^{-/-}$ and A2780 cell lysates demonstrated the inhibition of caspases activation and cleavage by p53, from the most upstream initiator caspase-9, to the downstream proteins such as caspase-3 and PARP (Figures 3.2 to 3.5). Since the inhibition was already evident starting from caspase-9, it was assumed that caspase-9 is the target of p53 inhibition in the caspase cascade. This assumption was supported by the fact that in this cell-free system, factors influencing caspase activation that are upstream of cytochrome-*c* induced apoptosome formation were absent. Also, if p53 had targeted other more downstream caspases such as caspase-6 and caspase-8, inhibition of cleavage of the most upstream caspase-9 would not have been so pronounced. In addition, this set of experiments showed that the inhibition of caspase-9 was specific to the addition of recombinant p53, regardless of the tag (GST or HST) and source (commercially available vs. those produced in the laboratory).

Using the same cell-free cytosolic system, the removal of endogenous p53 from both WTp53-expressing HEK293 and A2780 cells showed a reversed effect i.e. the acceleration of

caspase cleavage (Figures 3.6 and 3.7). This interesting observation could be the first evidence to show that endogenous WTp53 intrinsically inhibits caspase-9.

To further investigate if this phenomenon exists *in vivo*, p53 was removed from HEK293 and A2780 cells by using siRNA against p53. Various forms of p53 were also introduced into p53-null cells by overexpression. In line with the results obtained from the immunodepletion experiments, knockdown of p53 in HEK293 and A2780 cells showed accelerated caspase cleavage and activity (Figures 3.8 and 3.9). While the immunodepletion experiments eliminate potential p53-mediated transcriptional effects, the *in vivo* knockdown experiments took into account these transcriptional activities within the cellular milieu. The fact that p53's inhibitory effect on caspase-9 was recapitulated in the *in vivo* setting as well served to strengthen the hypothesis of this study.

p53's novel caspase inhibitory effect was further studied using ovarian cancer cell lines PEO1 and PEO4. The ability of p53 to inhibit caspase-9 was reproduced in the knockdown experiments in PEO4 cells (Figure 3.10D). Notably, the increase in caspase-9 activity due to p53-knockdown was only seen in cisplatin-resistant PEO4 cells and not in cisplatin-sensitive counterpart, PEO1. Although both cell lines harboured the R342P mutation, the different effects on caspases could be due to differential cytotoxin-induced p53 response in the two cell lines. Upregulation of p53 protein expression with cisplatin treatment appeared to be more pronounced in PEO4 than in PEO1 cells. This could thus explain the more obvious inhibitory effect exerted by p53 on caspase-9. The observation that overexpression of this mutation in H1299 $p53^{-/-}$ cells was able to inhibit caspase activation (Figure 3.10F) also helped to substantiate this interpretation. A more probable explanation is that the concentration of cisplatin used to induce apoptosis in PEO1 cells was at 25μM, which is five times the concentration of the IC_{50} for this cell line. Hence, most of the PEO1 cells would have been killed by the high dose of cisplatin. Thus, the caspase-9 activities

recorded would not an accurate reflection for this set of experiment. Taking all these into account, it is still very tempting to hypothesize that the mechanism of PEO4's cisplatin-resistance is due to p53's inhibitory action of caspases.

In the current investigation, both mutant and WT p53 were overexpressed in H1299 $p53^{-/-}$ cells in an attempt to substantiate the results obtained earlier on i.e. the addition of recombinant p53 into cell-free lysates. Expectedly, increased overexpression of the various mutants led to decreased caspase-9 activities measured using colorimetric assays (Figure 3.11). Although $p53^{R175H}$ was previously shown to be able to downregulate the expression of procaspase-3 [233], the activity of the more upstream caspase-9 was measured instead, and the trend registered was presumed not to be markedly affected by changes in the protein level of the downstream caspase-3.

It is of great interest to note that while the mutant p53 demonstrated a concentration-dependent inhibition of caspase-9 activities *in vivo*, WTp53 was only able to inhibit caspase-9 when overexpressed at very low amounts, although not at a level which is statistically significant (p=5.9%). At higher levels of WTp53 overexpression, caspase-9 activities increased to the control level. Bearing in mind that in the face of acute cytotoxic or genotoxic stress, dramatically heightened level of WTp53 can transactivate pro-apoptotic genes like *Bax* and *Bak*, expression of p53 at low level in this study might not be sufficient to tip the cell fate balance towards apoptosis. In this scenario, the slightly heightened amount of p53 may serve to instead inhibit nascent cell death signalling and perhaps allow for cell repair to occur. Conversely, with very high level of p53 overexpression, transactivation of the pro-apoptotic genes would have been rampant, and thus shifted the cell fate balance towards apoptosis. While the large amount of p53 could still inhibit active caspase-9, the p53-mediated expression of the array of pro-apoptotic genes such as *Bax* and *Bak* could have brought about massive cytochrome-*c* release from the mitochondria, wide-spread apoptosome formation and

consequently, run-away unbridled caspase-9 activation that became impossible to be quenched by the p53 expressed by the cells. However, it will be interesting to see if overexpression of an even lower concentration of WTp53 can result in a significant reduction in caspase-9 activity.

One thing to note in this set of experiments was that a much higher level of the mutant p53 had to be expressed for the inhibition of caspase-9 to be significant (Figure 3.11E). This is in contradiction to the earlier results where equal amount of recombinant p53 (WT or mutant) were able to inhibit caspase-9 activation. A possible explanation for this phenomenon is that in this *in vivo* experimental setup, the expressed p53 proteins may have undergone post-translational modification(s), which is most likely absent in the earlier experiments. Mutant forms of p53 have previously been reported to be hyperphosphorylated and acetylated as compared to its WT counterparts [184,185,252]. Among the sites which are phosphorylated in mutant p53, Ser392 was found to phosphorylated in high frequency in transitional cell carcinoma [185] and its phosphorylation correlated with lower patient survival in esophageal squamous cell carcinoma [187,188]. Phosphoryation of Ser392 was found to stablize p53 tetramers and increase its ability to bind DNA [253,254]. A possible mechanism which could have increase the demand for the mutant p53 protein in this experiment is that the mutant p53 proteins were sequestered at the various DNA binding sites due to its higher affinity for DNA in these Ser392-phosphorylated mutants. This leaves a lower level of free mutant p53 proteins which could then exert its inhibitory effects on caspase-9 and hence its activity.

In summary, the results have suggested a possible role of p53 in the inhibition of apoptosis, possibly through the inhibition of caspase-9. This was not only shown through the use of different *in vitro* and *in vivo* techniques, but also demonstrated in a variety of cell lines. Cell lines established from either viral-induced transformation - HEK293 and

HEK293T; or derived from tumours – HCT116 $p53^{-/-}$, H1299 $p53^{-/-}$ and A2780, were able to show similar phenomenon i.e. the inhibition of caspase activation when p53 is present. Although it can be argued that the p53 protein in HEK293 and HEK293T proteins are inactivated by E1B 55k [226,227] and SV40 large T-antigen [228] respectively, the fact that both immunodepletion and knock-down of p53 in the HEK cell lines as well as those derived from tumours (A2780 and PEO4) showed the same results suggested that the inhibitory function of p53 on caspases is independent of its interaction with the viral proteins. Moreover, the inhibition of p53 by both viral proteins (i.e. E1B 55k and SV40 large T-antigen) are targeted at its transactivation activities [227,255,256]. However, the technique used to initiate caspase activation in the above experiments was through the use of recombinant cytochrome-*c* in S100 lysates, which is transcription-independent. It can also be argued that the inhibition of caspases seen in the results obtained is due to E1B 19k which interacts and inhibits Bax-dependent apoptosis [257]. Again, the use of the S100 fractions and cytochrome-*c* inititated caspase activation is independent of the mitochondria and Bax-dependent perturbation of the mitochondria membrane. Hence, the participation of the viral proteins used in the immortalization of the HEK293 and HEK293T cells is minimal.

3.4 Concluding Remarks

The results obtained so far have been unexpected and could possibly open up a whole new dimension for p53 research in the study of oncology. The ability of p53 to induce apoptosis through transactivation and direct interaction with apoptotic proteins has been a major focal point for the past 30 years. Such dominant effects of p53 on apoptosis could have masked the hidden function of p53 in caspase inhibition, revealing itself only in the background of mutant p53 cells that are incapable of inducing transactivation-dependent apoptosis. Intriguingly, a study by Cao [40] showed that the abrogation of the transactivation activities of p53 was not sufficient to bring about transformation in epithelial cells. Apoptotic

resistance and aggressiveness in tumours harbouring such mutants appear to suggest that p53 does indeed possess some death-signalling related function that remained unknown until now.

Chapter 4 – Results

Direct Interaction between p53 and Caspase-9

4.1 Introduction

Protein-protein interactions form the basis of most cellular signalling processes. Such associations often lead to structural changes which inhibit or activate the target proteins. Some proteins even become permanently attached to their target to alter the latter's functions. A known interacting partner of p53 is MDM2, which binds to p53 directly for inhibition. More examples of negative regulators of p53 have been reviewed in Chapter 1, *Section 1.3*. p53 itself can also act as an inhibitor in other cellular processes. For example, Sommers [258] demonstrated that p53 regulated the WRN helicase activities through direct interaction between the two proteins. p53 can also function as an activator of the pro-apoptotic protein Bak through direct interaction as well [97].

In comparison to the deluge of reports focusing on the transactivation functions of p53, there are fewer reports on p53 as a modulator of cellular activities through direct interaction with the target proteins. This could be partly because such direct interactions are hard to detect. The discovery of p53's inhibitory effect on caspase-9 in this study could therefore be an addition to the few studies which report the transcription-independent roles of p53.

4.2 Results

4.2.1 p53 Inhibits the Activity of Caspase-9 Directly

To investigate if the inhibition of caspase-9 by p53 was direct, an assay monitoring the activity of active recombinant caspase-9 was used. Using a highly-purified recombinant system consisting of only recombinant p53 and active caspase-9, the direct effect that p53 exerts on the activity of caspase-9 was measured without interference of other proteins as

found in cell lysates or other *in vivo* systems. Hence, any significant changes in the activity of caspase-9 could be attributed purely to the action of the added recombinant p53. Figure 4.1 shows an at least 50% decrease in the activities of caspase-9 with addition of any of the four recombinant p53 (WT or mutants). The results showed that recombinant p53 is able to directly inhibit the activities of cleaved caspase-9.

Recombinant p53 was also added to caspase-3 and caspase-6 to investigate the former's effect on caspases other than caspase-9. From Figure 4.2, it can be seen that the recombinant p53 was unable to cause any significant reduction in the activities of both caspase-3 and caspase-6. It thus appears that p53 specifically inhibits caspase-9 and not other caspases.

In order to further affirm the specificity of the various recombinant p53, increasing concentrations of the recombinant p53 were titrated to determine if caspase-9 activities were correspondingly decreased. Indeed, all the recombinant p53 proteins tested were able to show an inverse correlation between the concentration of the p53 protein used and the caspase-9 activity, even though the difference were the least obvious in $p53^{R337H}$ (Figure 4.3).

4.2.2 Inhibition of Caspase-9 Activities Requires a Folded Recombinant p53

So far, the inhibition of caspase-9 activities in this Chapter was demonstrated using recombinant p53 proteins that were folded. The next question to ask was whether the inhibition of caspase-9 is dependent on the recombinant p53 acquiring the correctly folded structure, or on a specific amino sequence within the p53 protein. To answer this question, a comparison of the effects of the unfolded p53 (in denaturing 6M urea) and those that were refolded (dialysed with refolding buffer) on caspase-9 activities was performed. Both types of p53 were added to the S100 cytosolic lysates of HCT116 $p53^{-/-}$ cells to probe for the effects on the caspase cleavage patterns. To compensate for any effects which could have been caused by the 6M urea, an equivalent amount of 6M urea were added into the lysates where

folded recombinant p53 proteins were tested. Conversely, the equivalent amount of dialysis buffer was added into lysates where unfolded recombinant p53 proteins were used. In Figure 4.4, it was clear that only folded recombinant p53 was able to inhibit the cleavage of caspase-9 and the subsequent proteins in the pathway. Conversely, unfolded p53 were unable to show any inhibitory effects on active caspase-9. The results here indicate a structural requirement for p53's inhibitory activity on active caspase-9.

4.2.3 p53 Interacts with Active Caspase-9 in vivo

Since all the *in vitro* experiments were able to demonstrate clearly that p53 directly inhibits the active form of caspase-9, immunoprecipitation (IP) was performed to analyse if p53 interacts with caspase-9 *in vivo*. Cleavage of caspase-9 was initiated in S100 cytosolic extracts of HEK293 cells using cytochrome-*c*, and subsequently an antibody specific to p53 were used to immunoprecipate endogenous p53 from the lysates. From Figure 4.5, a band corresponding to the p35 band of caspase-9 co-precipitated with the endogenous p53. However, the result from this immunoprecipitation experiment was not always reproducible and thus a method for probing transient protein-protein interaction was utilised, as presented below.

4.2.4 Interaction between p53 and Caspase-9 is Transient

Despite numerous attempts to optimise the co-immunoprecipitation experiments, they were not successful in pulling down both caspase-9 and p53 together. Suspecting that the interaction between the two proteins is transient, attempts were made instead to detect transient protein-protein interaction using a cross-linking protocol reported by Melcher [259]. Based on this report, cross-linking between interacting proteins was induced with the addition of nickel acetate and MMPP. In this method, MMPP oxidizes the Ni(II) in the His-tag to highly electrophilic Ni(III), which in turn extracts an electron from a nearby aromatic side

chain, particularly a tyrosine. Finally, a covalent bond is formed between the His-tag and the tyrosine residues of the interacting proteins that are in close proximity. The nickel acetate helps to saturate the environment with free Ni^{2+} ions to aid the cross-linking reactions. All three components i.e. proteins, metal ions (Ni^{2+}) and oxidants (MMPP) must be present in order for cross-linking to take place [260]. HST-tagged recombinant p53 used in this study were added into the cytochrome-*c* challenged S100 lysates of HEK293 cells to allow for their interaction with the endogenous active caspase-9. Nickel beads specific for His-tagged proteins were added to the lysates to extract the HST-tag (control) and the HST-tagged p53 proteins. As the interaction was so transient, only one wash of the beads were performed before the cross-linking to avoid washing away the caspase-9 bound to the recombinant p53. Then, the three components used in Melcher's protocol were added to induce the formation of covalent bonds between the His-tag and the tyrosine residing in, presumably caspase-9. The beads were then washed extensively and analysed for the presence of p53 and caspase-9. Although lysates with HST-tagged p53 added showed higher pull-down of the p35 fragment of caspase-9, the control fraction were contaminated with small amount of both p53 and caspase-9 (Figure 4.6), which could arise from the initial not-so-stringent wash of the beads before the cross-linking reagents were introduced.

To further verify this result, experiments using the "far Western" technique were performed. The experimental protocol was based initially on the report by Srinivasula [239] which demonstrated the interaction between caspase-9 and XIAP using far Western, and subsequently the one published by Wu [231]. Initially, no positive result was observed. As results from the IP experiments provided strong evidences that the transient interaction between the two proteins could not be detected under normal circumstances, modifications were again made to include a cross-linking step into the far Western protocol. A detailed protocol of this set of experiments can be found in *Section 2.17*.

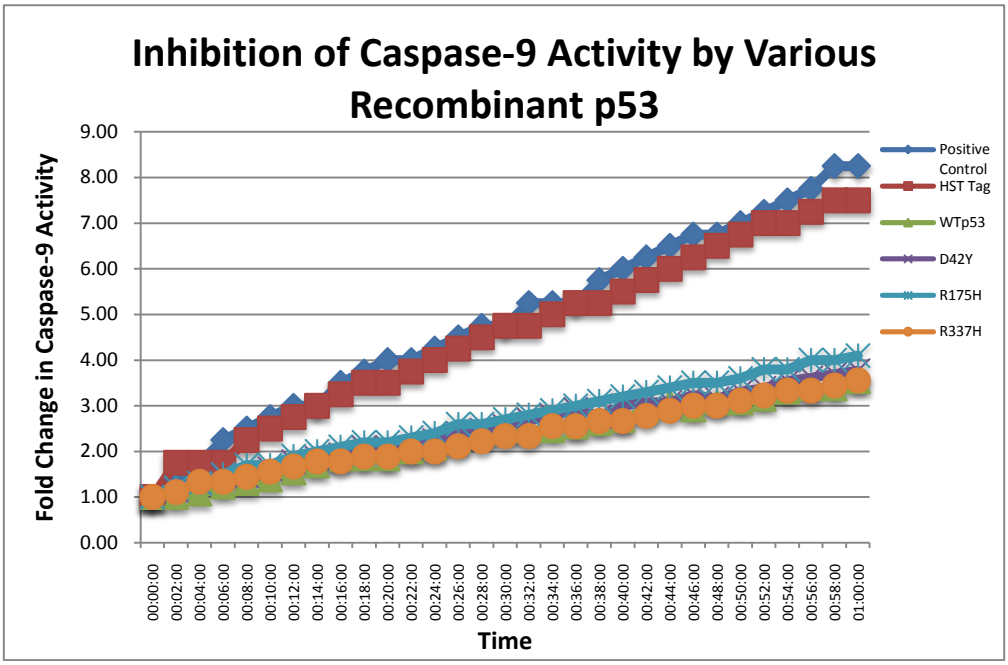
Nitrocellulose blots containing renatured proteins were exposed to equal amounts of either recombinant HST-tag or HST-tagged-p53, subjected to cross-linking reactions, and subsequently exposed to either anti-S-tag or anti-p53 antibodies to detect the bound HST-tagged p53. To visualise the profile of the caspase-9 proteins used for this experiment, a Western blot using antibodies against caspase-9 was performed. From Figure 4.7B, it can be seen that in Lane 1, both the p46 procaspase-9 and the p35 active caspase-9 bands were present at approximately equal amounts. In Lane 3, a thick band corresponding to the p35 fragment was present while small traces of the p12 band were also there. In Lane 4, the p46 procaspase-9 band and small traces of the p35 band were present. The HST-tag was loaded in Lane 2 to act as a control, and the bands showed up when the blots were exposed to anti-S-tag antibodies (Figure 4.7A, E & F).

Analyzing the profiles in greater detail, it can be seen that in Lane 1, multiple bands were observed when blots were exposed to recombinant p53 (Figure 4.7C & E) and not the HST-tag (Figure 4.7D & F). As Lane 1 contained proteins from the S100 cytosolic fraction from H1299 $p53^{-/-}$ cells, the multiple bands observed in Lane 1 were most probably contributed by the interaction of the recombinant p53 with the cytoplasmic components e.g. Bak. Moving on, there were no bands observed in Lane 2 when blots were exposed to recombinant p53 (Figure 4.7C & E). This hence showed that there were no unspecific interaction between the recombinant p53 or the HST-tag attached to the recombinant p53 and the HST-tag present on the blots. The presence of the HST-tag on the blots were clearly demonstrated when the blots were exposed to anti-S-tag antibodies (Figure 4.7D & F). In Lane 3, a band corresponding to the p35 fragment of caspase-9 was present in both blots exposed to recombinant p53 (Figure 4.7C & E) but not in blots exposed to HST-tag (Figure 4.7D & F). This hence further iterates that only the recombinant p53 was interacting with the caspase-9 and the reaction was not due to unspecific interactions with the HST-tag. Also, in

Figure 4.7D, there were no bands present except for one in Lane 1, which corresponded to the GFP-tagged-WTp53 expressed in the H1299 $p53^{-/-}$ cells. This result hence acts as an antibody control to verify that the results obtained for C) and E) were not false positives due to unspecific binding of the antibody. Finally in Lane 4, several bands were evident in all blots except the one exposed to HST-tag and anti-p53 antibodies (Figure 4.7D). This again showed that the HST-tag on the p53 proteins did not interact unspecifically with the caspase-9 present in this lane. Also, it is an important piece of evidence to prove that p53 does not interact with the p46 procaspase-9 fragment. The bands that showed up in Lanes 4 on the other blots could have been contributed by interaction between p53 and the IVT components (which were not removed as both the expressed caspase-9 and the IVT components were His-tagged).

In summary, blots that were exposed to recombinant WTp53 and anti-S-tag or anti-p53 antibodies showed a unique band in Lane 3 which corresponded to the 35kDa active caspase-9 fragment (indicated by the red arrows). No bands at 46kDa and 12kDa for lanes containing caspase-9 were detected, suggesting that the interaction occurs between p53 and specifically p35 fragment of caspase-9. This is in agreement with the results obtained for the IP experiments.

A)



B)

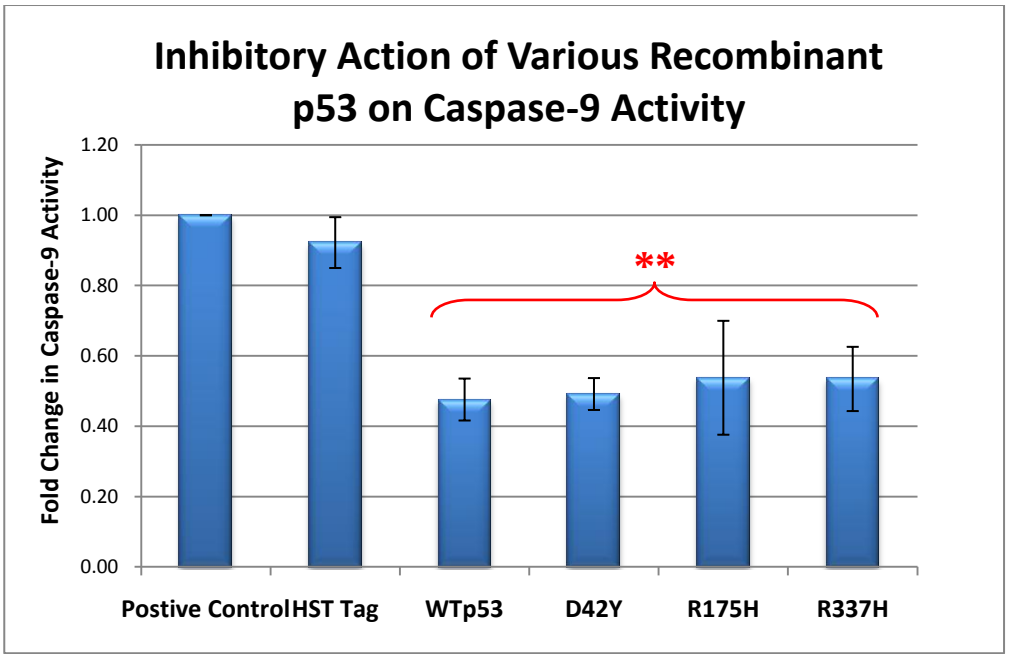
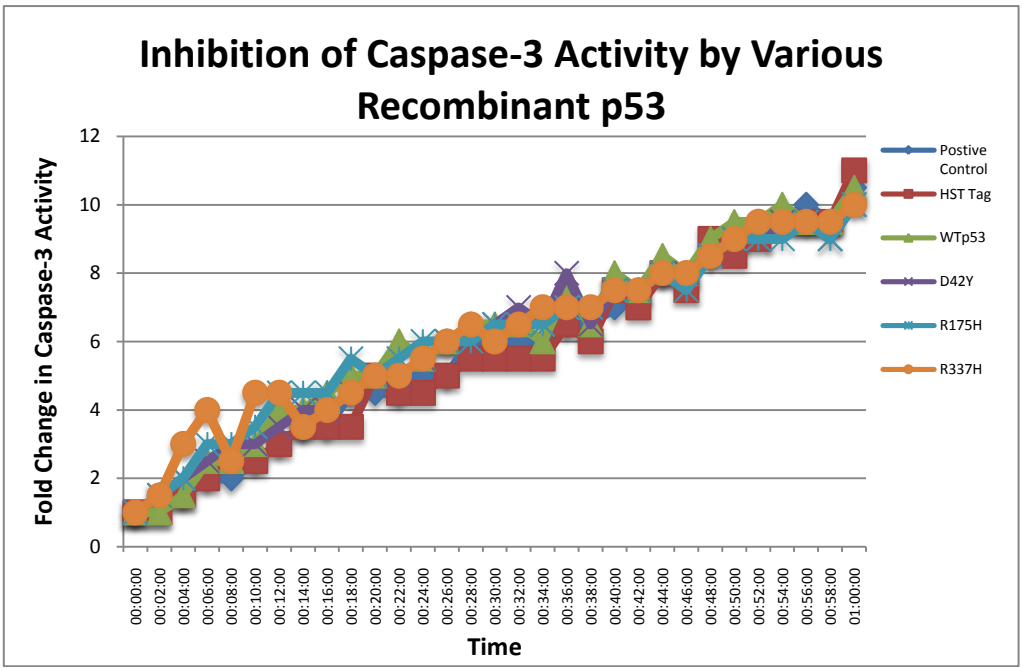
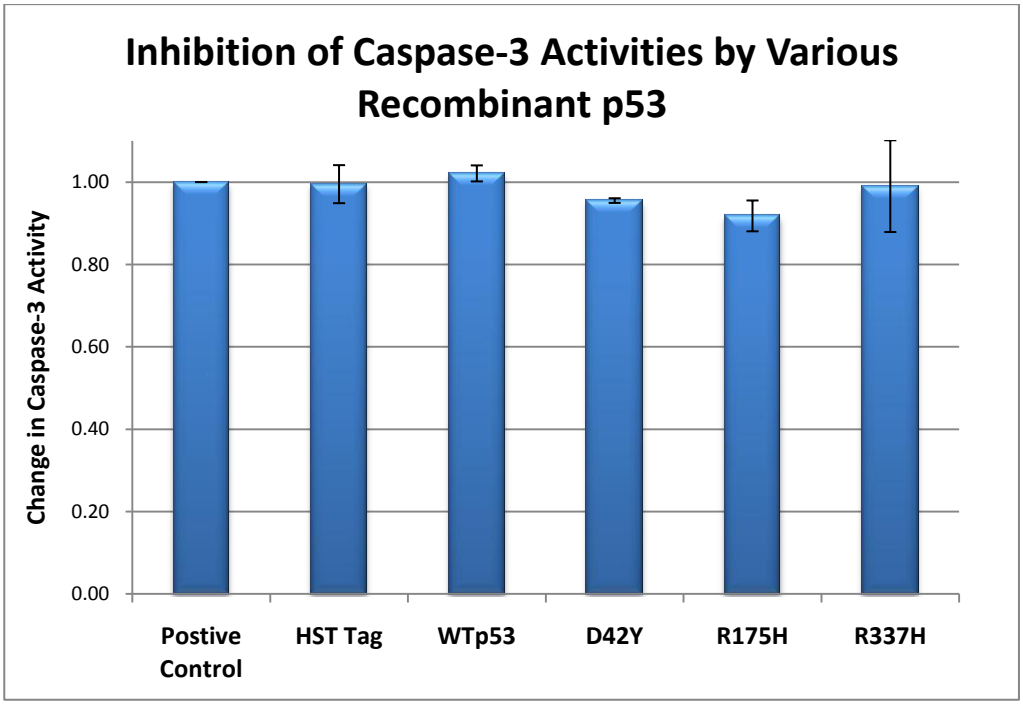


Figure 4.1. Recombinant WT-p53 and the Various Mutants inhibit the Activity of Recombinant Active Caspase-9 Directly. Activity of caspase-9 was measured through the release of *p*NA from the cleavage action of active caspase-9 on the LEHD-*p*NA substrate. 1µM of recombinant p53 was used with 2.8 µM of active recombinant caspase-9 and 200 µM of LEHD-*p*NA substrate. The data obtained were expressed as **A)** over a time course of 60 munites at 2-minute intervals and **B)** the final measured caspase-9 activities in the different conditions. ** denotes that the caspase-9 activity is significantly different from the positive control and the HST-tag (n=4, $p<0.001$).

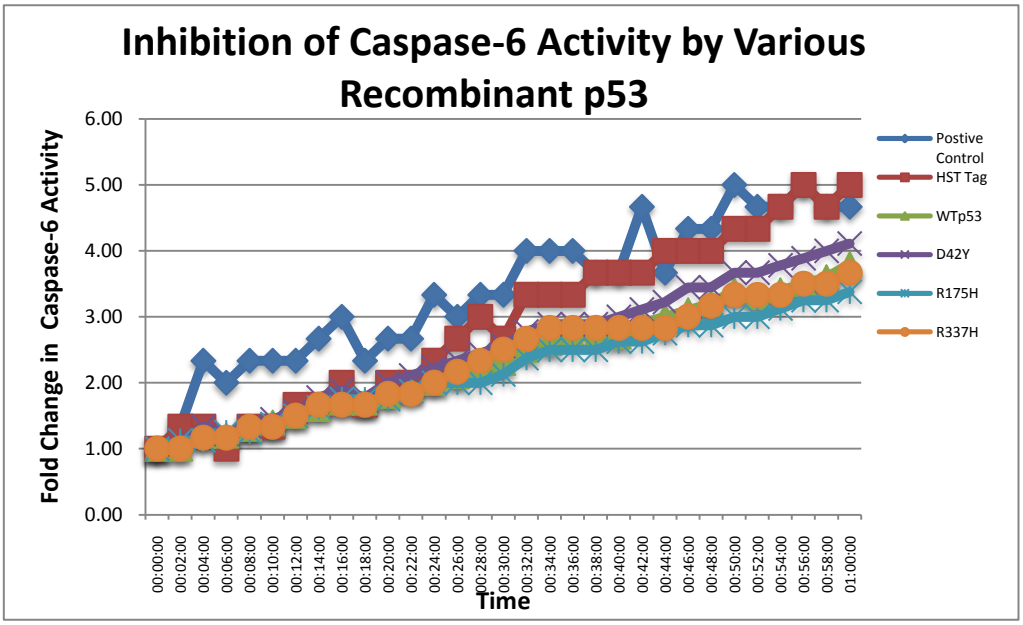
A)


Inhibition of Caspase-3 Activity by Various Recombinant p53
Fold Change in Caspase-3 Activity
12
10
8
6
4
2
0
00:00:00
00:02:00
00:04:00
00:06:00
00:08:00
00:10:00
00:12:00
00:14:00
00:16:00
00:18:00
00:20:00
00:22:00
00:24:00
00:26:00
00:28:00
00:30:00
00:32:00
00:34:00
00:36:00
00:38:00
00:40:00
00:42:00
00:44:00
00:46:00
00:48:00
00:50:00
00:52:00
00:54:00
00:56:00
00:58:00
01:00:00
Time
Postive Control
HST Tag
WTp53
D42Y
R175H
R337H

B)


Inhibition of Caspase-3 Activities by Various Recombinant p53
Change in Caspase-3 Activity
1.00
0.80
0.60
0.40
0.20
0.00
Postive Control
HST Tag
WTp53
D42Y
R175H
R337H

C)



D)

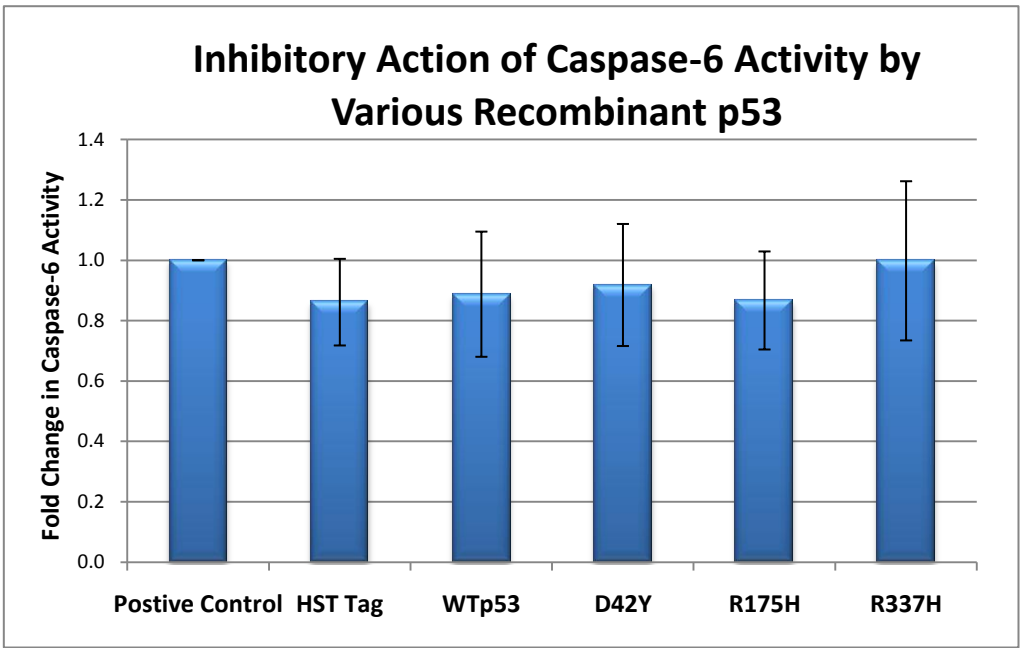
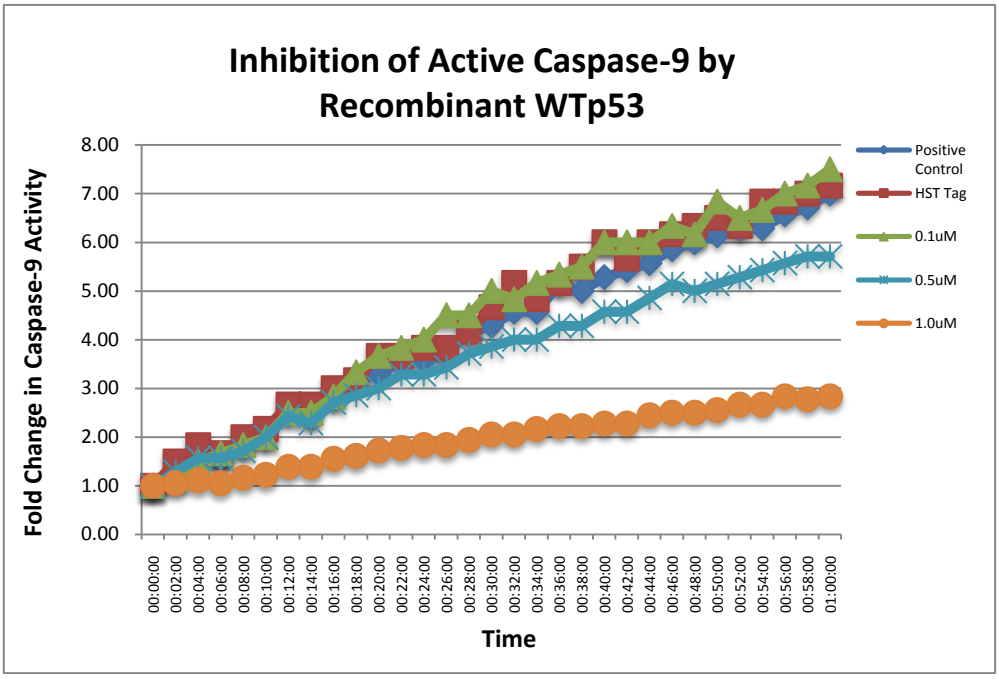
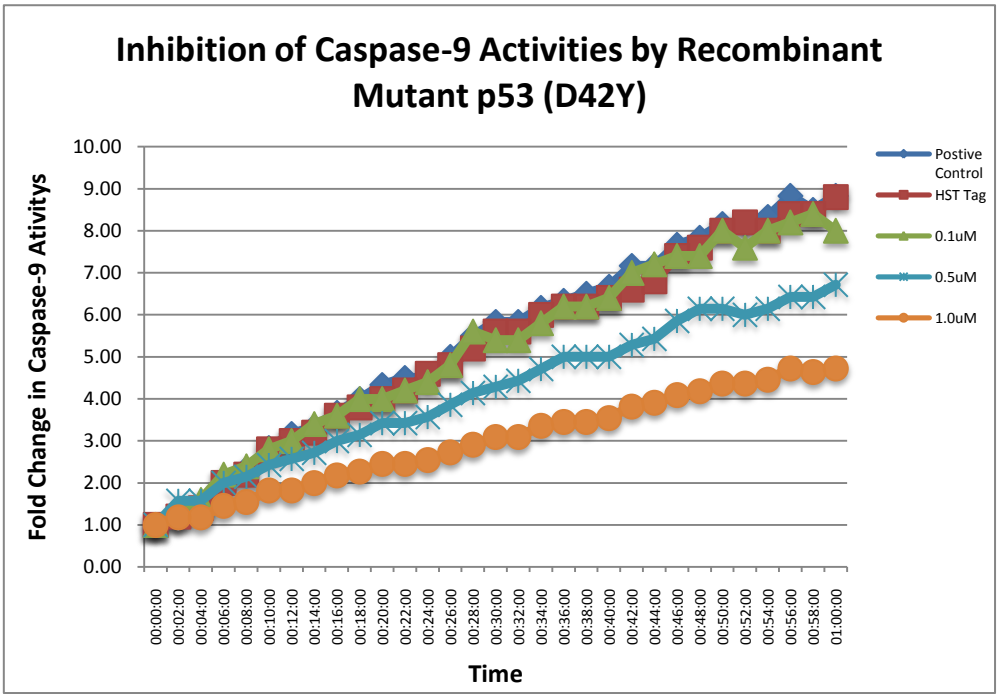


Figure 4.2. Inhibition of Caspase Activities is Specific to Caspase-9. Activities of the various caspases were measured through the release of *p*NA from the cleavage action of the active of the respective caspases on their substrate coupled to *p*NA. 1µM of the various recombinant p53 were used with **A & B)** 20nM of active recombinant caspase-3 were used with 200µM of Ac-DEVD-*p*NA substrate; **C & D)** 25nM of active recombinant caspase-6 were used with 200µM of Ac-VEID-*p*NA were used. All reactions were measured over a time-course of 60 minutes at 2-minute interval.

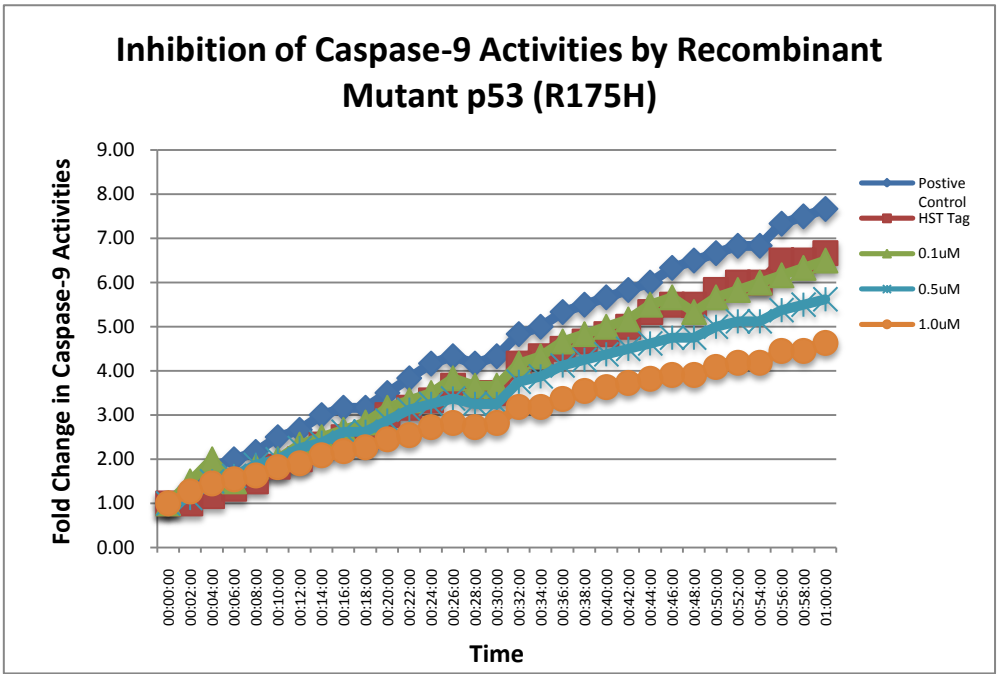
A)


Inhibition of Active Caspase-9 by Recombinant WTp53
Fold Change in Caspase-9 Activity
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
00:00:00
00:02:00
00:04:00
00:06:00
00:08:00
00:10:00
00:12:00
00:14:00
00:16:00
00:18:00
00:20:00
00:22:00
00:24:00
00:26:00
00:28:00
00:30:00
00:32:00
00:34:00
00:36:00
00:38:00
00:40:00
00:42:00
00:44:00
00:46:00
00:48:00
00:50:00
00:52:00
00:54:00
00:56:00
00:58:00
01:00:00
Time
Positive Control
HST Tag
0.1uM
0.5uM
1.0uM

B)


Inhibition of Caspase-9 Activities by Recombinant Mutant p53 (D42Y)
Fold Change in Caspase-9 Ativitys
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
00:00:00
00:02:00
00:04:00
00:06:00
00:08:00
00:10:00
00:12:00
00:14:00
00:16:00
00:18:00
00:20:00
00:22:00
00:24:00
00:26:00
00:28:00
00:30:00
00:32:00
00:34:00
00:36:00
00:38:00
00:40:00
00:42:00
00:44:00
00:46:00
00:48:00
00:50:00
00:52:00
00:54:00
00:56:00
00:58:00
01:00:00
Time
Postive Control
HST Tag
0.1uM
0.5uM
1.0uM

C)



D)

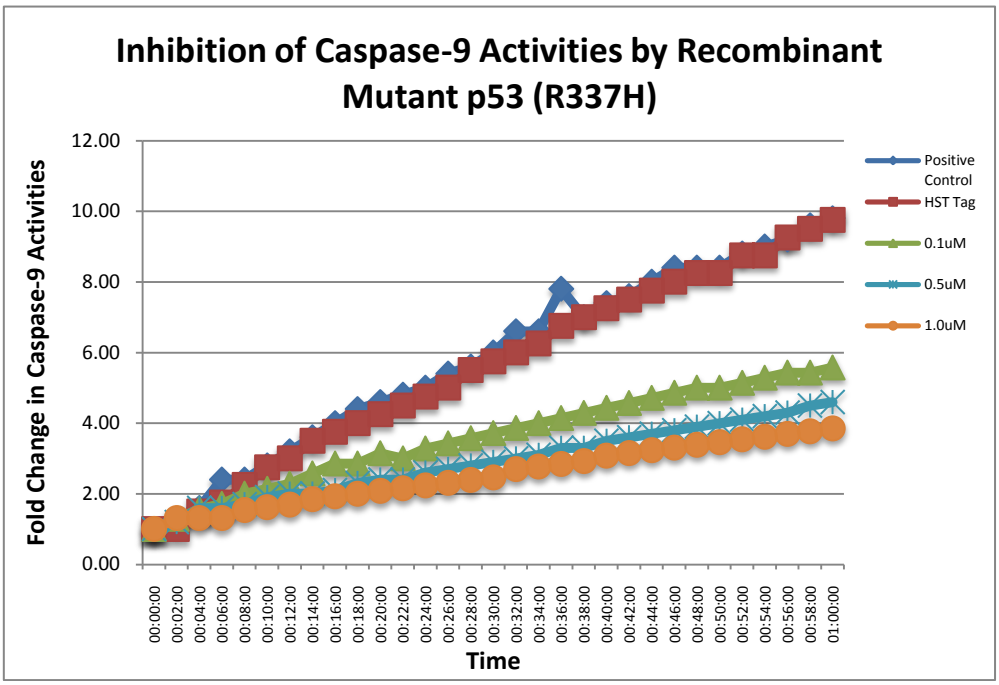


Figure 4.3. Dosage-Dependent Inhibition of Recombinant Active Caspase-9 Activity by Recombinant WT-p53 and the Various Mutants. Activity of caspase-9 was measured through the release of *p*NA from the cleavage action of active caspase-9 on the LEHD-*p*NA substrate. Various concentration of recombinant p53: WT **(A)**, D42Y **(B)**, R175H **(C)** and R337H **(D)** were used with 2.8 µM of active recombinant caspase-9 and 200 µM of LEHD-*p*NA substrate. Activties were measured over a period of 60 minutes at 2-minute interval.

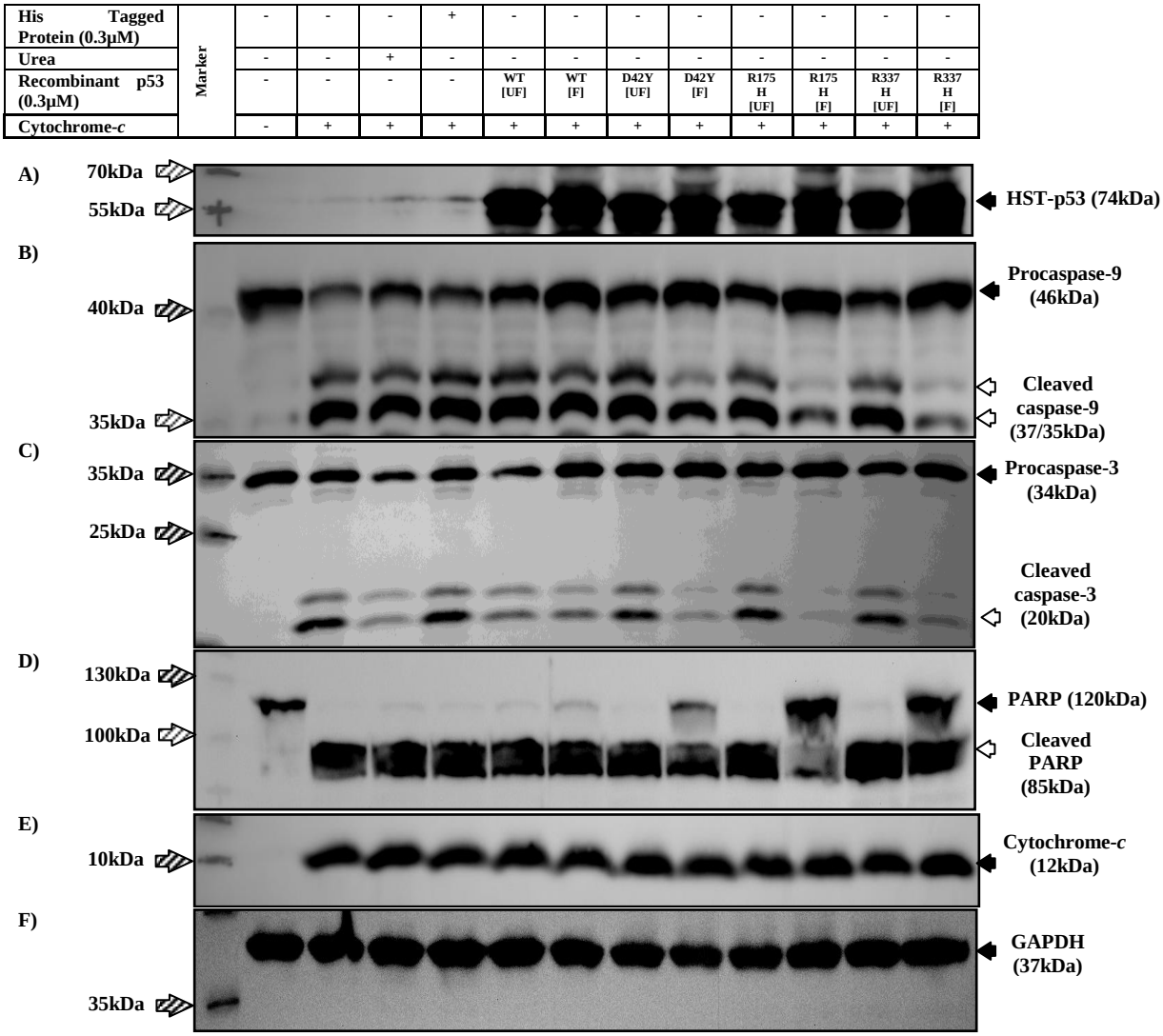


Figure 4.4. Inhibition of Caspase-9 by p53 Require a Folded Protein Structure. S100 lysates from HCT116 $p53^{-/-}$ cells were obtained and incubated with the various recombinant proteins for 1 hour after addition of equal amount of dATP and cytochrome-*c* to initiate caspase cleavage. [UF] depicts unfolded recombinant proteins whereby p53 still in 6M urea were added to refolding buffer (Section 2.3) without dialysis. [F] depicts recombinant p53 which has gone through the refolding and dialysis process. Profile of p53 **(A)**, Caspase-9 **(B)**, Caspase-3 **(C)**, PARP **(D)**, cytochrome-*c* **(E)** and GAPDH **(F)**. Lane 1: lysate without cytochrome-*c* challenge; Lane 2: lysate with cytochrome-*c* challenge; Lane 3: lysates with cytochrome-*c* challenge and addition of 0.13M of urea (this concentration was calculated based on the volume of 6M urea which would be present in the lysate after dilution with dialysis buffer); Lane 4: lysate with cytochrome-*c* challenge and addition of 0.3uM recombinant HST-tag protein; Lanes 5-12: lysate with cytochrome-*c* challenge and addition of 0.3μM of the various [UF] and [F] recombinant p53.

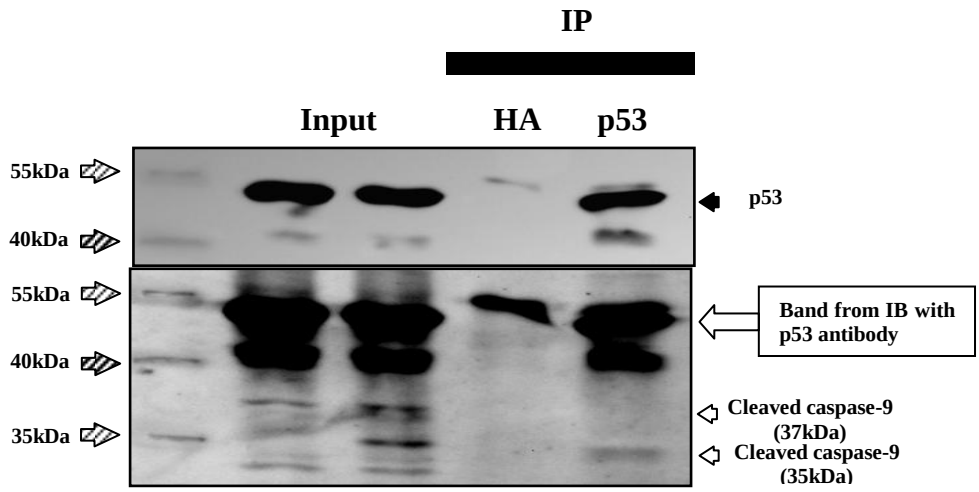


Figure 4.5. Immunoprecipitation of p53 in HEK293 Cells Shows Interaction of p53 and Active Caspase-9. S100 cell lysates were harvested from 293 cells. Lysates were challenged with cytochrome-*c* for 30 minutes before equal amounts of lysates were added to Dyna beads coupled with either anti-HA or anti-p53 antibodies. Western blot was then performed probing for p53 and caspase-9. Unchallenged lysates (Lane 1) and cytochrome-*c* challenged lysate (Lane 2) were loaded as positive control.

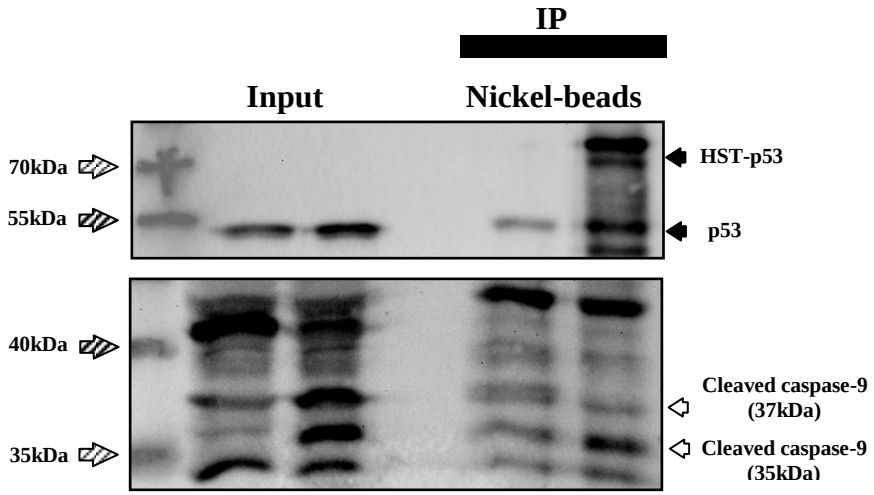
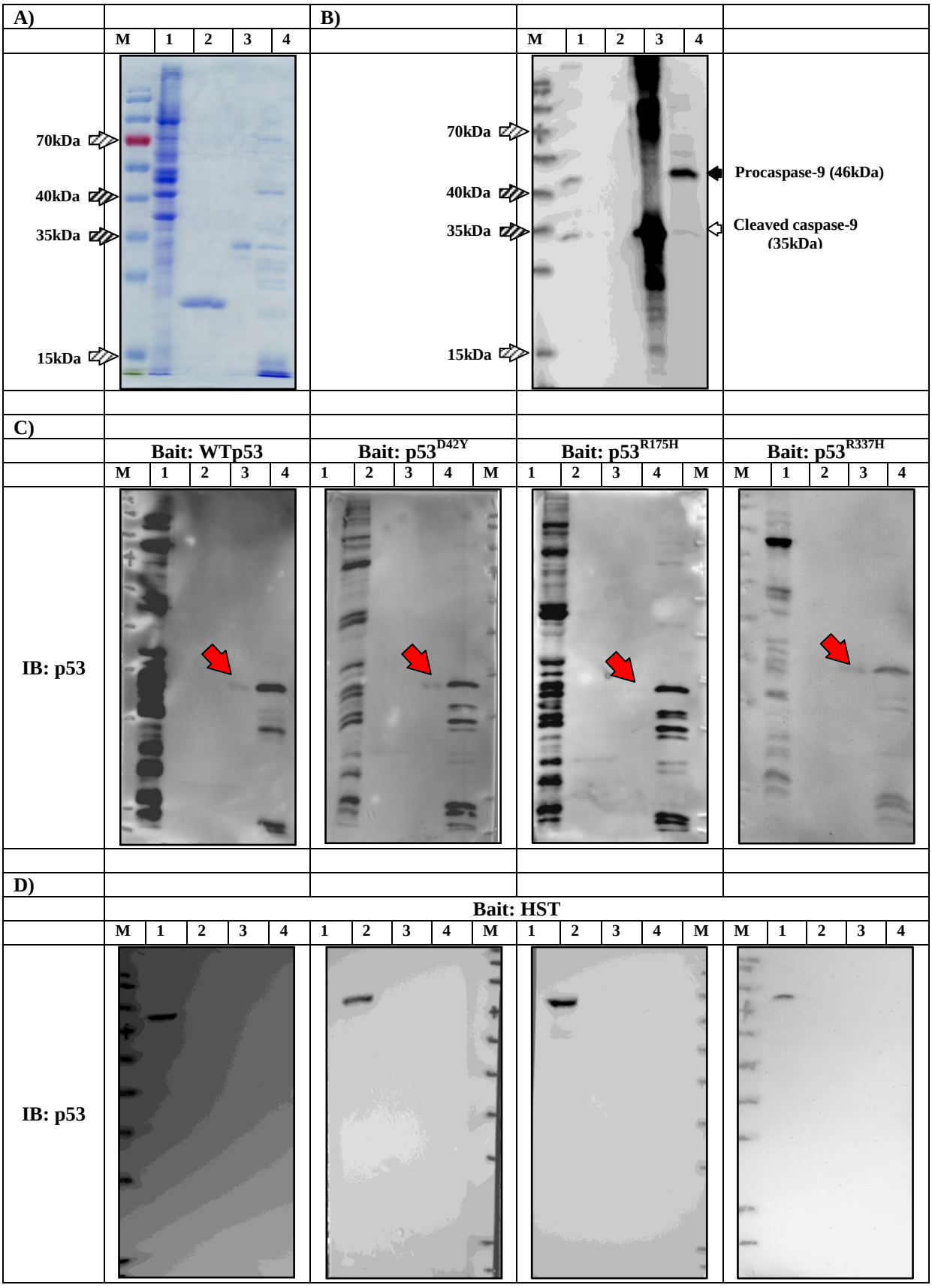


Figure 4.6. Immunoprecipitation of p53 in HEK293 Cells with the Addition of His-tagged Recombinant p53 showed more pull-down of Endogenous Active Caspase-9. S100 cell lysates were harvested from 293 cells. Lysates were challenged with cytochrome-*c* for 30 minutes before 0.1μM of His-tagged or His-tagged recombinant p53 were added to equal volumes of the cytochrome-*c* challenged lysate. Nickel beads specific for His-tagged proteins were used to extract the His-tagged proteins from both lysates. Western blot was then performed probing for p53 and caspase-9. Cytochrome-*c* challenged lysates (Lane 1) and unchallenged lysate (Lane 2) were loaded as positive control.


A)
M 1 2 3 4
70kDa
40kDa
35kDa
15kDa
B)
M 1 2 3 4
70kDa
40kDa
35kDa
15kDa
Procaspase-9 (46kDa)
Cleaved caspase-9 (35kDa)
C)
Bait: WTp53
Bait: p53D42Y
Bait: p53R175H
Bait: p53R337H
M 1 2 3 4
1 2 3 4 M
1 2 3 4 M
M 1 2 3 4
IB: p53
D)
Bait: HST
M 1 2 3 4
1 2 3 4 M
1 2 3 4 M
M 1 2 3 4
IB: p53

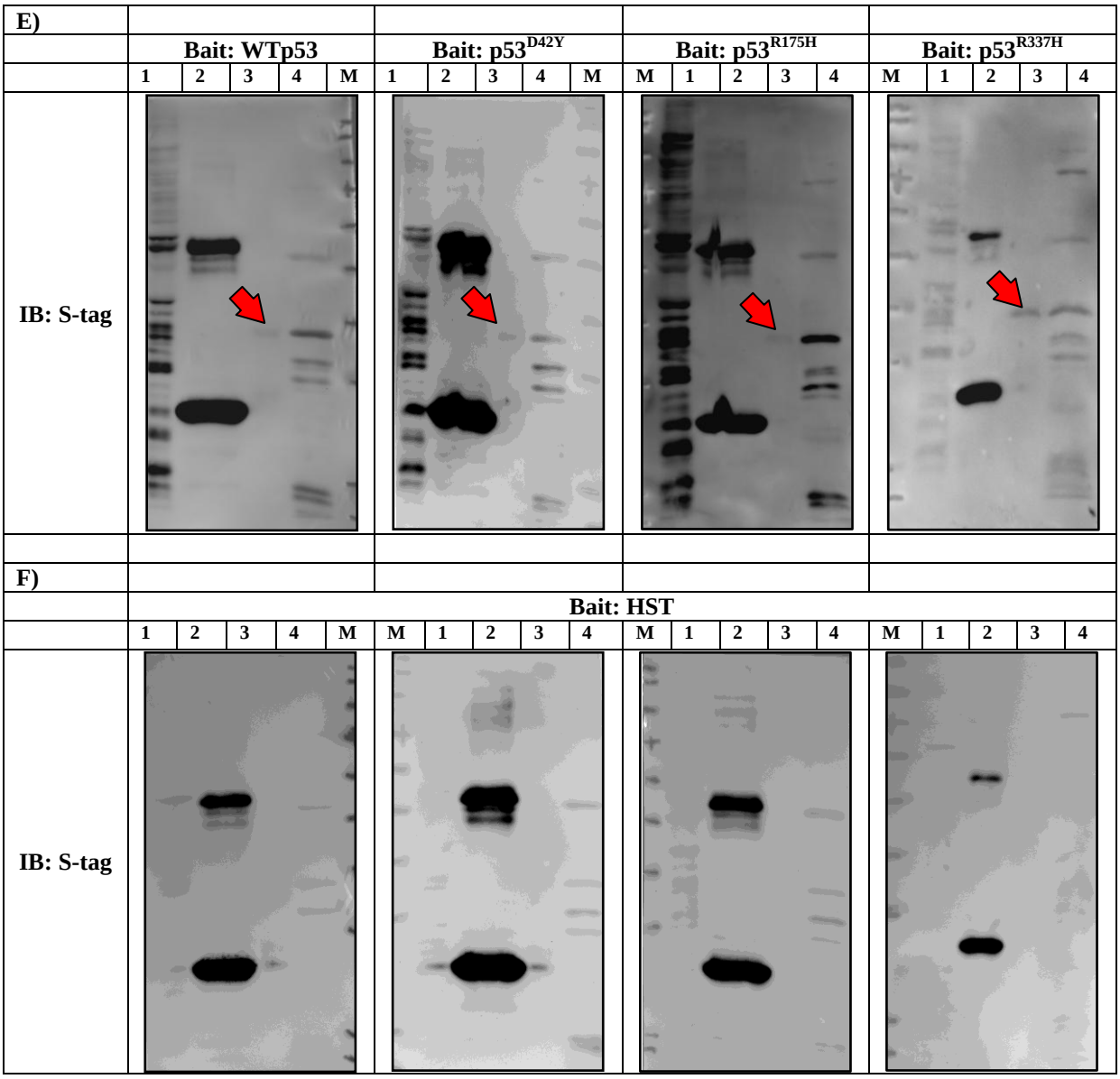


Figure 4.7. p53 Interacts with Active Caspase-9 via Far Western. The blots contained S100 lysate of HCT116 $p53^{-/-}$ overexpressing WTp53 (Lane 1); 0.5mg of HST-tag expressed from pET-32b plasmid (Lane 2); 15ng active caspase-9 (Lane 3); and 1µl of IVT mixture containing procaspase-9 (Lane 4). **A)** Protein profile shown using Coomassie staining. **B)** Caspase-9 profile detected using Western blot. **C&D)** Detection of interacting proteins using HRP-coupled-DO-1 p53 antibodies via far Western technique. **E&F)** Detection of interacting proteins using HRP-coupled-S-tag antibodies via far Western technique. The red arrows show the specific interaction between the recombinant WTp53 and the p35 fragment of caspase-9.

4.3 Discussion

So far, the results discussed in Chapter 3 revealed the phenomenon of caspase-9 inhibition by p53. In this Chapter, the results garnered support the hypothesis that p53 inhibits the action of caspase-9 through direct interaction.

Using pure recombinant systems, all four recombinant p53 were able to reduce the activity of active caspase-9 significantly (Figure 4.1). This inhibitory action was further shown to be specific to caspase-9 (Figure 4.2) and dependent on the concentration of the recombinant p53 proteins (Figure 4.3). The inhibitory effects of the four p53 forms were almost identical in this set of experiments except that $p53^{R337H}$ was able to inhibit caspase-9 activities more markedly at lower concentrations as compared to the other three p53 proteins. It could be that $p53^{R337H}$ possess the highest affinity for the active caspase-9 molecules and that a small amount the mutant protein was able to bring about a higher level of inhibition of caspase-9 as compared to the other p53 proteins. Notably, increasing $p53^{R337H}$ further to 0.5μM and 1.0μM did not bring about further marked decrease in caspase-9 activity, indicating that a saturation of the inhibitory effect might have been attained. Also, the inhibition on caspase-9 was shown to be structurally dependent (Figure 4.4). Most interestingly, differences in the inhibition of caspase-9 were obvious between the unfolded and folded structural mutant $p53^{R175H}$. This suggests that some protein structure might have been induced by the refolding procedure, and that this minimal structural conformation is sufficient in mediating the inhibition of caspase-9.

The idea of an inhibitor that targets the active form of caspases is not unprecedented in the field of apoptosis study. Proteins with similar mode of action have already been discovered, such as members of the IAP (inhibitor-of-apoptosis) family. The mammalian IAP proteins were discovered due to their homology to the baculoviral apoptotic inhibitor [261]. All family members possess a RING finger domain in their C-terminus and at least one BIR

(Baculovirus IAP Repeats) domain in the N-terminus [262]. To date, the better known members of this family include X-linked IAP (XIAP) [263], Survivin [264] and more recently, Livin [265]. Studies have revealed some very powerful properties of these proteins in coordinating cellular responses to apoptosis.

The IAP proteins have been demonstrated to regulate both initiator and effector caspases by binding to and inhibiting the actions of cleaved caspases such as caspase-9 and caspase-3 [265-267]. For example, XIAP was found to bind to monomeric caspase-9. As the activities of caspase-9 is amplified manifold when present as a heterodimer of the p35 and p12 subunits [239], the binding action of XIAP to monomeric caspase-9 retards the ability of caspase-9 to form the highly active heterodimer molecules [267]. Although there is no evidence so far in this study to determine the mode of inhibition of p53 on active caspase-9, it is plausible that p53 could act and inhibit caspase-9 in a fashion similar to XIAP.

Even though the inhibitory effects of p53 on caspase-9 can be easily observed as reported in Chapter 3, the direct interaction between p53 and caspase-9 was extremely difficult to prove using merely co-immunoprecipitation experiment. Out of every ten attempts (not including the numerous optimisation efforts) to pull down p53 and caspase-9 from cell lysates to show their interaction, only one succeeded (Figure 4.5).

Postulating that the interaction might only be possible at very low concentrations, modifications were performed to the IP protocol, e.g. overexpression of either WTp53 or caspase-9 in the cells to increase the concentration of both proteins. However, the results remained negative despite these efforts. This led to the next hypothesis that the interaction between p53 and caspase-9 might be transient. Going along this line of thought, attempts were made to crosslink the two proteins during the IP experiments. Results from Figure 4.6 appeared to show a pull-down of the p35 fragment of caspase-9 by the HST-tagged WTp53 in a background of lingering cytoplasmic proteins. As minimal washing steps were utilised in

the protocol after the nickel beads were extracted from the cytosolic lysates, small amounts of p53 and the p35 of caspase-9 were detected in both IP fractions (Figure 4.6). In addition, traces of p37 fragment of caspase-9 were also present in both fractions. However, as the intensity of this band was similar in both fractions, it thus could serve as an internal control to verify the greater pull-down of the p35 fragment of caspase-9 by p53. In spite of all the explanations, this could only be used as a supporting result and is not conclusive on its own to confirm the direct interaction between endogenous p53 and active caspase-9.

While showing the direct interaction between proteins using IP is an important step in determining whether the two proteins in questions work together, there are proteins which could influence the functions of their downstream targets without adhering to them. One such study was published by Chipuk [20]. In this report, p53 was shown to directly activate Bax and induce mitochondria membrane permeabilization. However, there were no evidence to demonstrate the direct interaction between p53 and Bax using traditional IP experiments. Instead, *in vitro* systems involving the pure components of the proteins under study i.e. p53, Bax, purified liver mitochondria, GST-Bcl-xL were reconstituted in different experimental conditions to study the effects of p53 on Bax and mitochondria permeabilization. Although no direct interactions between p53 and Bax were shown, the study proved undoubtedly that p53 could activate Bax directly. Hence, p53 could also influence the functions of caspase-9 that could not be detected under normal physiological conditions using IP.

In most experiments, interaction of proteins can be detected at ng or nM concentrations. For example, the report by Chipuk [20] as mentioned above was demonstrated with nM of recombinant Bax and p53, Li [85], showed the formation of the apoptosome at ng of the components, and Maiguel [268] proved the interaction between NPM and p53 using ng of GST-p53 protein. However, it does not exclude the possibility that interacting partners may physically associate only at much higher concentrations. Gannon

and Lane [269] demonstrated that interactions between T-antigen from SV-40 virus, p53 and DNA polymerase can only occur at concentrations of the components at µg levels. In another study, c-myc was shown to bind to DNA only at very high concentration [270]. In both cases, the high concentration needed to demonstrate the physical interactions did not undermine the physiological importance of such associations. In the current investigation, the reconstitution of the recombinant p53 and active caspase-9 at µg levels were able to show convincing trends of the inhibitory effect of p53 on caspase-9. Using conventional IP, µg level of endogenous p53 is already difficult to pull down. In addition, the detection of co-precipitated caspase-9 will already be difficult, should the p53-caspase-9 interaction occur only at high protein concentrations. As such, the task of detecting such interaction will be even trickier if the interaction is transient.

To circumvent the problems encountered using conventional IP protocols and the possibility of transient interaction between the two proteins, a modified far Western protocol was used. Srinivasula [239] employed this method to detect the interaction between XIAP and the p12 fragment of the active caspase-9 protein. After several optimisation and changes of protocols, the interaction between WTp53 and the p35 fragment of caspase-9 was finally detected (Figure 4.7C & E).

By adding the same cross-linking step into the far Western protocol, interaction between p53 (WT and mutant) and the p35 fragment of active caspase-9 was evident. One puzzling observation that emerged was that although the p35 caspase-9 fragment were in higher concentration to the ones generated using IVT (compare intensity of bands in Lane3 and 4 in Figure 4.7B), a stronger signal from the p35 band in Lane 4 (as compared to the one in Lane 3) was observed (Figure 4.7C & E). Even though it seems to suggest that the recombinant p53 binds better to the p35 fragment produced by the IVT system, a more plausible explanation would be that the stronger signal was due to the interaction between

p53 and one of the IVT components that bore similar molecular weight to the p35 fragment of caspase-9. The presence of other bands on Lane 4 though, could be attributed to unspecific interactions between the HST-tag of the recombinant p53 or the HST-tag itself and the components of the IVT systems.

In summary, the interaction between p53 and the p35 fragment of caspase-9 was demonstrated using recombinant systems i.e. activity assays and far Western, semi-recombinant systems (IP with HST-tagged recombinant p53) and endogenous proteins (conventional IP). The data obtained from all these experiments demonstrated the existence of the elusive p53-caspase-9 interaction, and appears to further point to a transient nature of such interaction.

4.4 Concluding Remarks

The results obtained so far help to substantiate the claim that the inhibitory action of p53 occurs via its direct action on active caspase-9. Furthermore, results from IP and far Western experiments confirmed the interaction of p53 and the p35 active subunit of caspase-9. This is in reminiscent to the interactions seen between caspase-9 and Survivin [271], Livin [265] and XIAP [239]. This interaction between p53 and caspase-9 appears to be transient and could only be detected when both proteins were present at very high concentrations. This could thus explain why the association between the two proteins had eluded detection by the scientific community for so long, even after the development of highly sensitive methods such as yeast-two-hybrid assay and Tandem affinity purification with mass spectrometry-based proteomics, to detect interacting partners.

Chapter 5 – Results

p53 Mediates Cell Survival Through Caspase-9 Inhibition

5.1 Introduction

Many of the known caspases are involved in death signalling, and thus inhibiting the activities of these caspases will perceivably impact on cell survival and oncogenic transformation. Given the findings reported in this thesis of p53 as a novel inhibitor of active caspase-9, the next logical step is to investigate how such inhibition would impact cell viability and possibly underlie the oncogenic processes.

As mentioned in Chapter 1, the proportion of ovarian cancer tumours with p53 mutations is the highest (approximately 50%), as compared to the other types of cancer tumours (Figure 1.5B). Indeed, in serous ovarian cancer, a recent report suggests that the mutation rate determined using clonal sequence may be over 96% [272]. Among the many studies attempting to correlate p53 mutation with p53 overexpression status and patient prognosis, the study by Rele [146] found that patient survival decreased when ovarian tumours had both p53 overexpression and mutation. Also, missense mutations in *p53* were found in the same study to be more prevalent in advanced ovarian cancer stage. In another report, patients who responded unfavourably to platinum-based chemotherapy had a higher occurrence of p53 mutation [273]. Taken together, these studies suggest that p53 mutation and overexpression in these tumours may indeed contribute to their more aggressive nature.

cis-diamminedichloroplatinum(II), or cisplatin, is a very common drug used in treating ovarian cancer. It was approved for clinical use in both ovarian and testicular cancer since 1978 [274]. It is also widely administered in bladder, cervical, head and neck, small-cell and non-small cell lung cancers [275]. Cisplatin belongs to a class of platinum-based drugs, which when aqueous, forms positively charged molecules that crosslink to DNA. Consequently, DNA/platinum adducts result from these cross-linkages [276]. The most

common adduct is an intrastrand crosslink between adjacent guanines [277]. The DNA damage caused by adduct formation then elicits a p53-dependent apoptotic response [278], thus leading to the demise of the actively dividing cancerous cells.

Studies have revealed that ovarian cancers with p53 mutation showed higher resistance to cisplatin treatment [146,279], thus suggesting that p53 may play a crucial role in enhancing the survival of these tumour cells. With this in mind, the hypothesis here is that the presence of both WT and p53 missense mutants can enhance cell survival in the face of cisplatin treatment, through the inhibition of apical caspase-9 and consequently, cell death signalling.

5.2 Results

5.2.1 Apoptosis was Elicited in HCT116 p53$^{-/-}$ cells when Challenged with Cisplatin

To ensure that the mitochondria pathway is activated in HCT116 p53$^{-/-}$ cells upon cisplatin administration, the cells were first exposed to different concentrations of cisplatin. From Figure 5.1B, the growing intensity of the cleaved PARP band with increasing cisplatin dosage used indicated that this apoptotic pathway was activated. However, at 1mM cisplatin, the procaspase-9 band was almost as intense as the one at 0.1mM dosage (Figure 5.1A), suggesting that this concentration of cisplatin was not efficient in activating the mitochondria pathway. Hence, a 0.5mM cisplatin concentration was selected to be used in the following experiments.

5.2.2 p53 Overexpression offered Protection Against Cisplatin-induced Cell Death

After establishing the concentration of cisplatin to use, HCT-116 p53$^{-/-}$ cells were transfected with pXJ40-HA-GFP plasmids containing the genes for WT or the various p53 mutants, followed by treatment with 0.5mM of cisplatin over 72 hours. In Figure 5.2A, the control cell population showed no significant difference in cell survival over the time course.

However, all forms of p53 (except $p53^{R175H}$) induced a greater extent of cell survival compared to the GFP control. The cytoprotection was most prominent at the end of the 72-hour period, with $p53^{D42Y}$ showing the highest percentage of cell survival, followed by WTp53 and lastly $p53^{R337H}$. For $p53^{R175H}$, there were no significant differences in the extent of cell death when compared to the GFP control, indicating that overexpression of this mutant in HCT-116 $p53^{-/-}$ cells might not confer marked cell death resistance against cisplatin.

5.2.3 Cytoprotection of Cells Harbouring p53 was Mediated through Reducing Caspase-9 Activity

After establishing that p53, WT or mutants alike, were able to afford protection against cell death in the presence of cisplatin, the next step was to determine if the enhanced resistance was due to the halted apoptotic signalling through the inhibition of caspase-9. HCT-116 $p53^{-/-}$ cells were again transfected with the pXJ40-HA-GFP plasmids containing the genes for the various p53 types, followed by treatment with 0.5mM cisplatin. After a 72-hour exposure to the cytotoxin, the caspase-9 activities in the cells were imaged. As can be seen from Figure 5.3A, cells overexpressing p53 had lower caspase-9 activity as compared to the GFP control. Also, there appears to be an inverse relationship between caspase-9 activity and the cytoprotection effect as shown in Figure 5.2A. Cells overexpressing WTp53 and $p53^{D42Y}$ (which displayed the most cytoprotection in Figure 5.2A) had the lowest caspase-9 activity. This is followed by cells overexpressing $p53^{R337H}$. As expected, $p53^{R175H}$ which showed no cytoprotective effect, did not exhibit any difference in caspase-9 activity when compared to the GFP control. Taken together, the results indicate the quenching of caspase-9 by p53 may mediate cell death resistance afforded by the latter in the face of potent cytotoxin treatment.

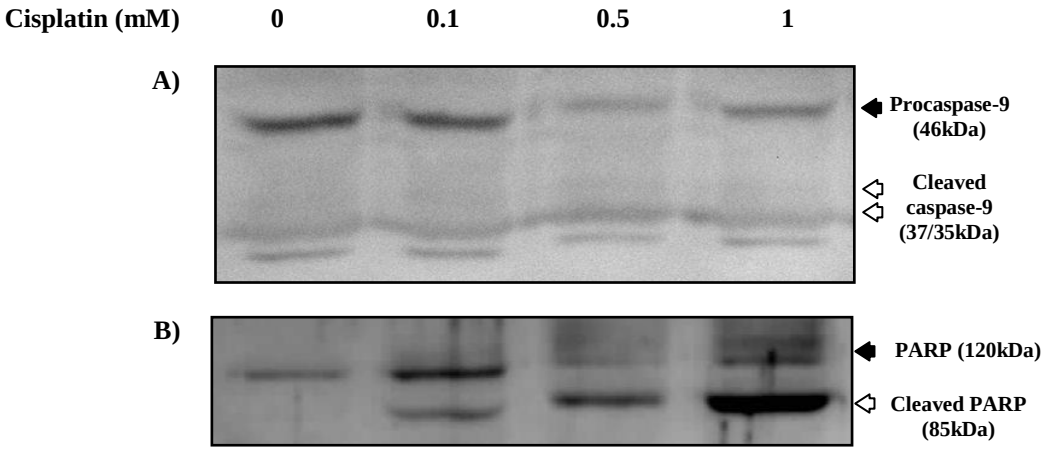
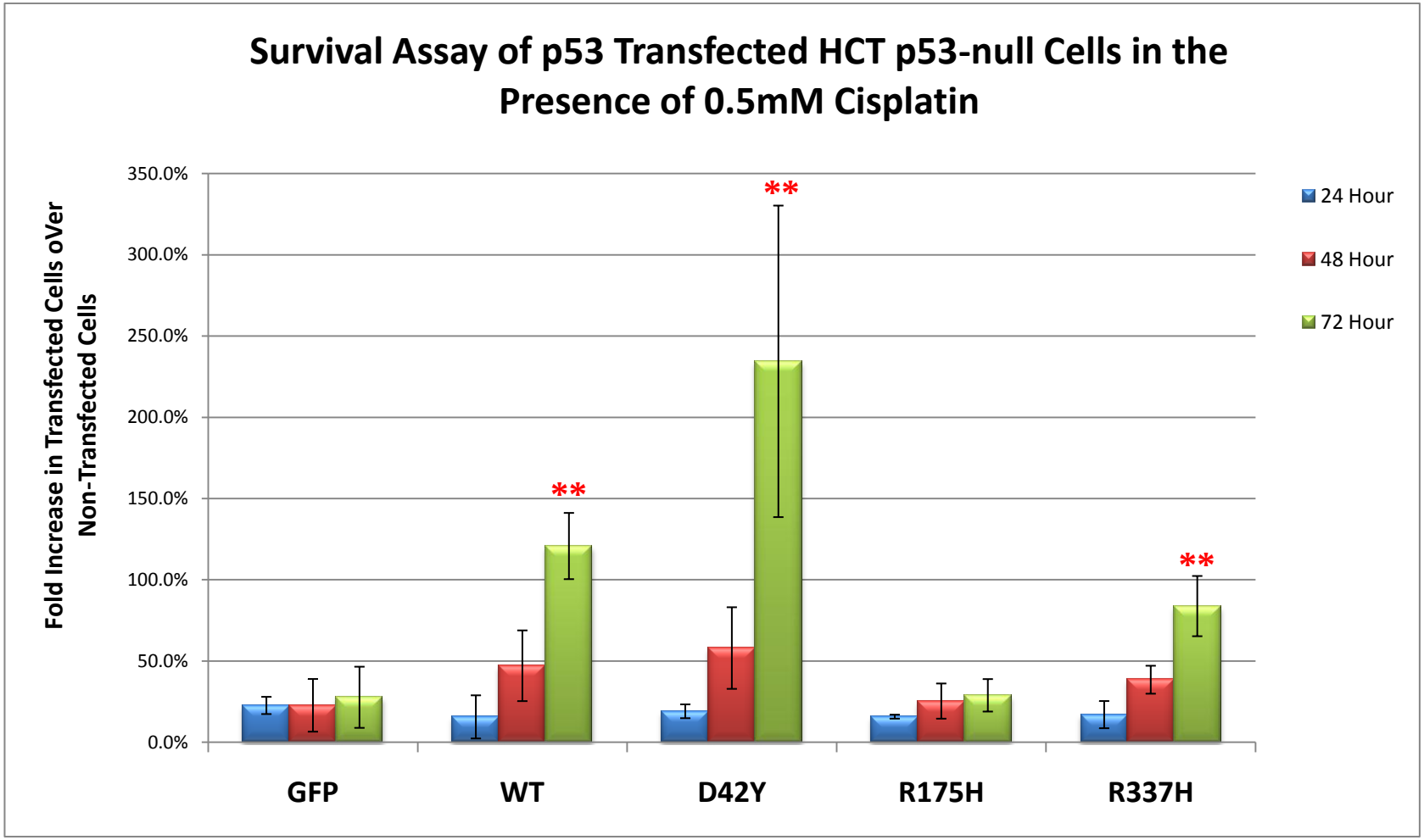


Figure 5.1. Apoptosis was elicited in HCT-116 p53$^{-/-}$ cells exposed to Cisplatin. HCT-116 p53$^{-/-}$ cells were exposed to increasing dosage of cisplatin for 72 hours and total cell lysate were obtained and probed for the profile of **A)** Caspase-9 and **B)** PARP protein.

A)


Survival Assay of p53 Transfected HCT p53-null Cells in the Presence of 0.5mM Cisplatin
Fold Increase in Transfected Cells oVer Non-Transfected Cells
350.0%
300.0%
250.0%
200.0%
150.0%
100.0%
50.0%
0.0%
**
**
**
24 Hour
48 Hour
72 Hour
GFP
WT
D42Y
R175H
R337H

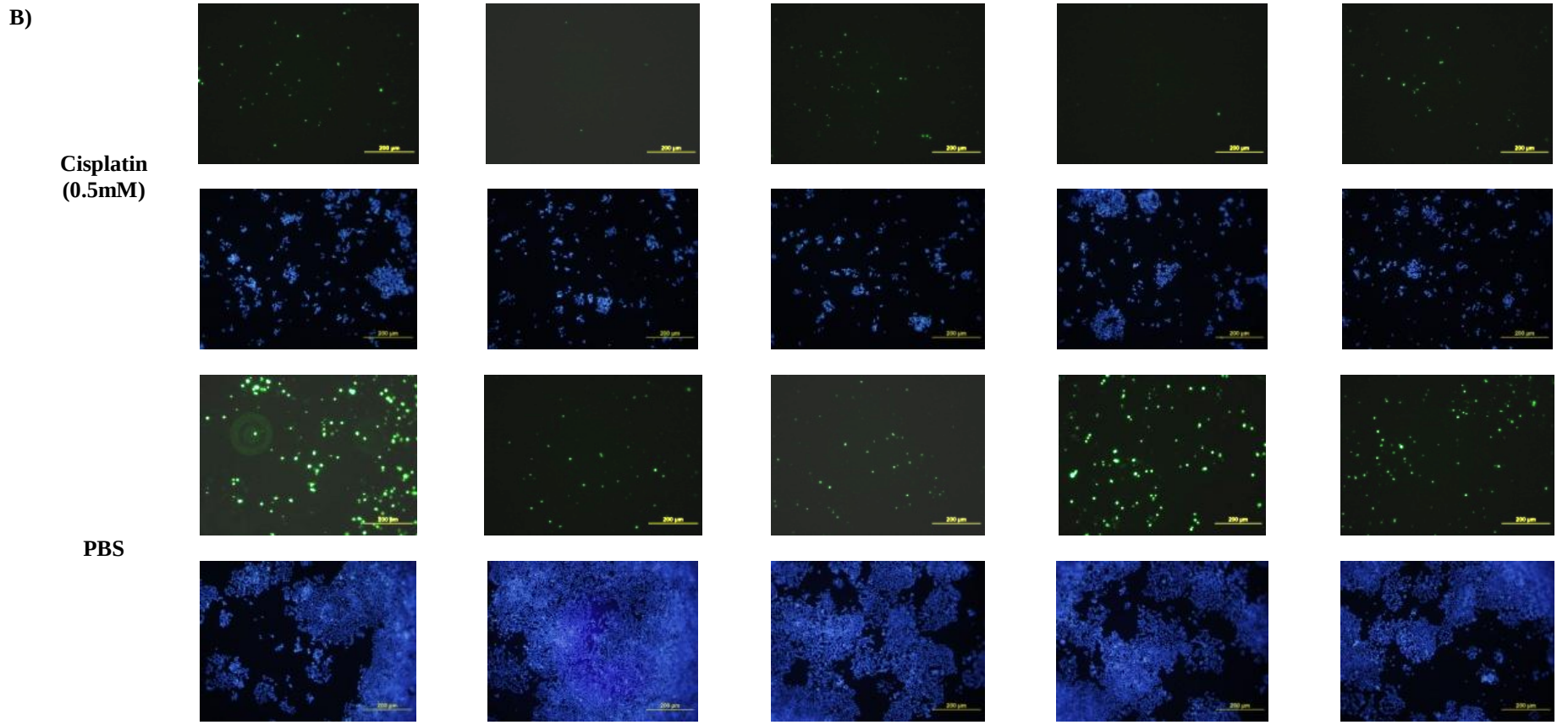
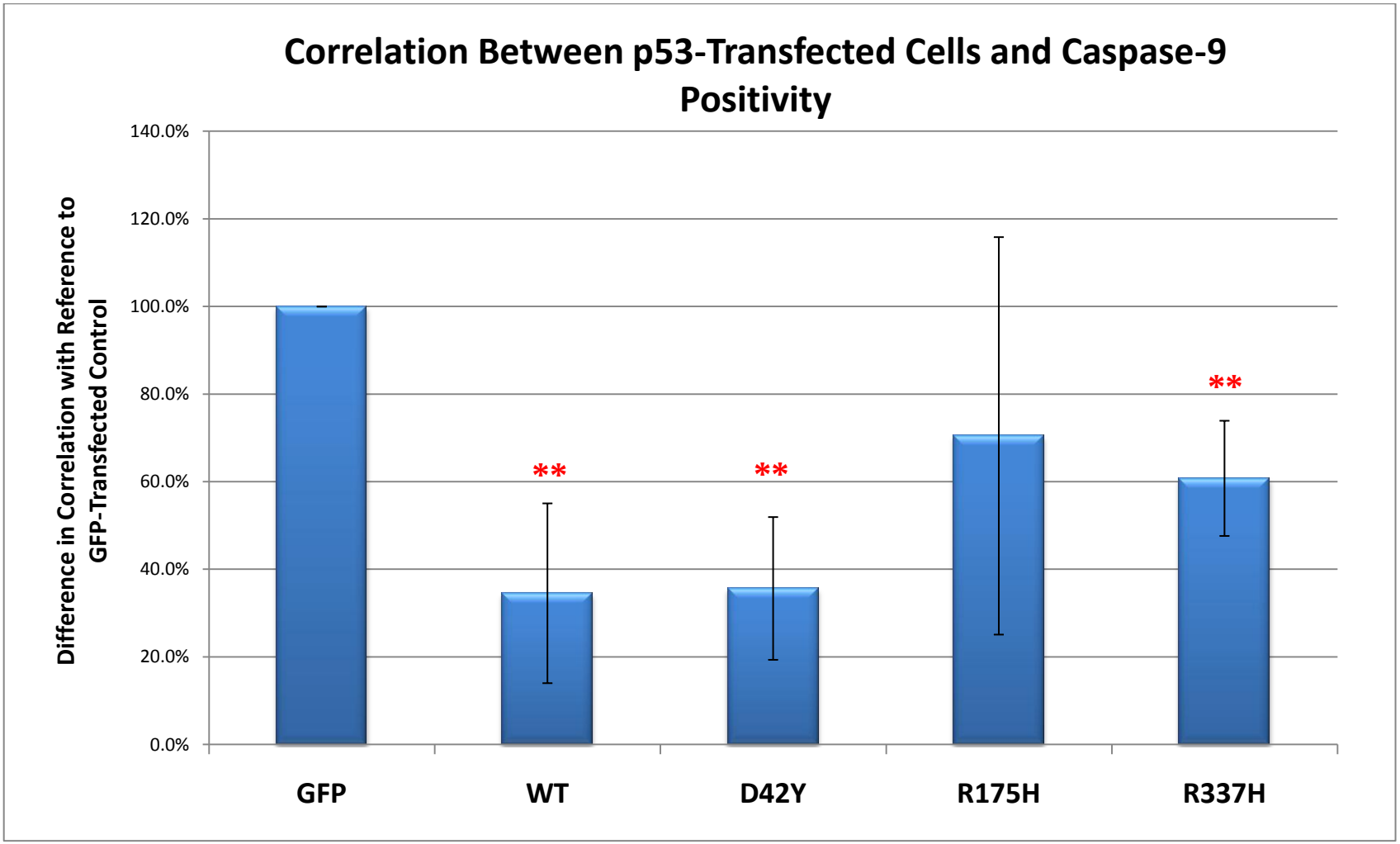


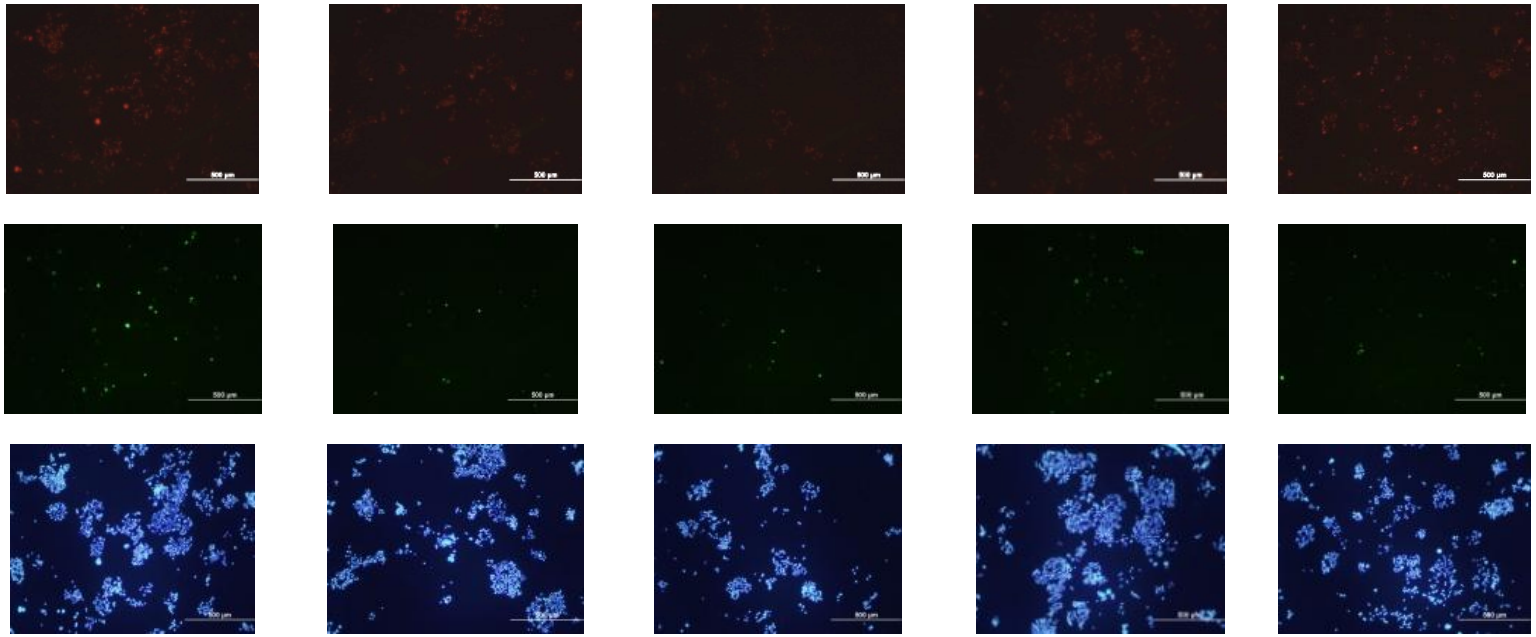
Figure 5.2. HCT-116 p53$^{-/-}$ cells transfected with p53 Survived Better Under Cisplatin Drug Challenge. HCT-116 p53$^{-/-}$ cells were transfected with 0.2µg of pXJ40-GFP-p53 for 4 hours before poisoning with 0.5mM of cisplatin. Cells were counted at every 24-hour period post-transfection. **A)** The percentages of transfected cells were calculated and the ratios of transfected cells in cisplatin to those in PBS were obtained. ** denotes that the cell survival at 72-hour of the cells overexpressing the various p53 is significantly different from the GFP control (n=3, $p<0.05$). **B)** shows the representative images used for the calculation of the ratios in (A).

A)


Correlation Between p53-Transfected Cells and Caspase-9 Positivity
Difference in Correlation with Reference to GFP-Transfected Control
140.0%
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
**
**
**
GFP
WT
D42Y
R175H
R337H

B)

Cisplatin (0.5mM)



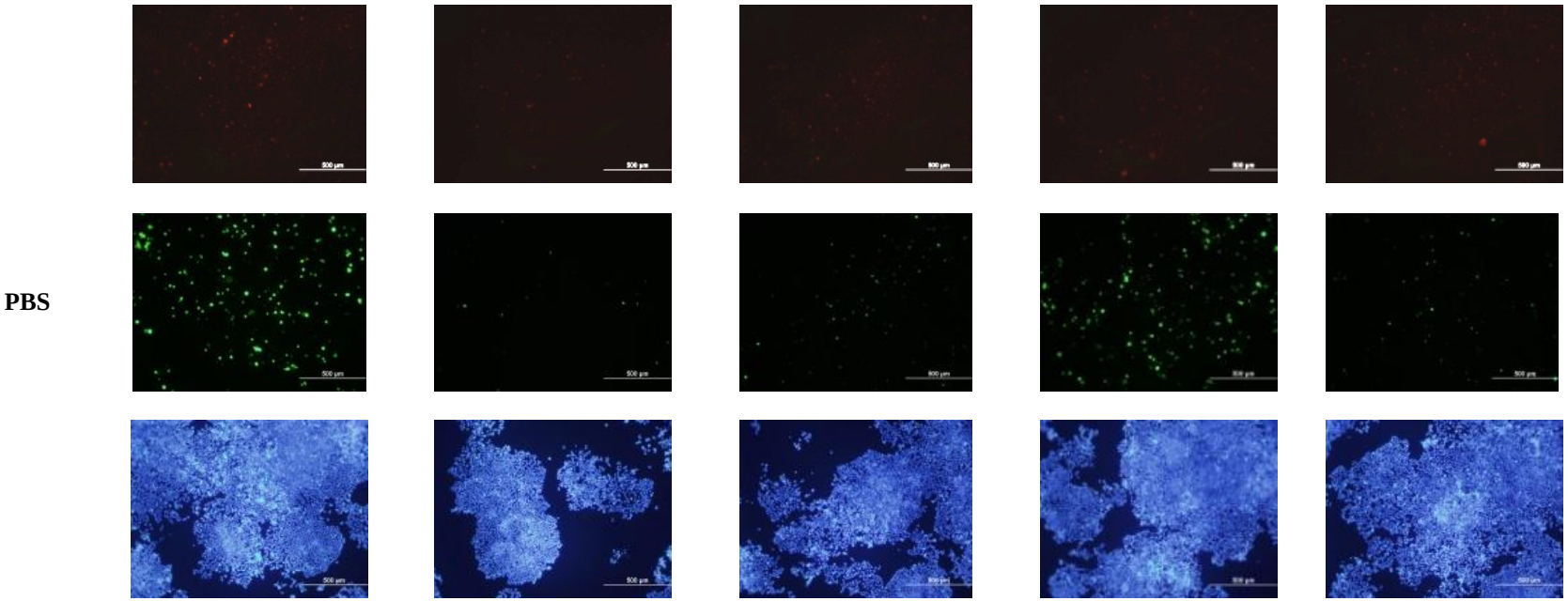


Figure 5.3. HCT-116 p53$^{-/-}$ cells transfected with p53 Survived Better Under Cisplatin Drug Challenge due to Inhibition of Caspase-9.

HCT-116 p53$^{-/-}$ cells were transfected with 0.2µg of pXJ40-GFP-p53 for 4 hours before poisoning with 0.5mM of cisplatin. Substrates that are specific to the action of active caspase-9 were added to the cells to yield a fluorescent product 1 hour prior to imaging. **A)** Cells positive for both GFP fluorescence and caspase-9 activity were counted and expressed as a ratio over the total number of GFP-fluorescence-positive cells. ** denotes that the caspase-9 activity of the cells overexpressing the various p53 were significantly different from the GFP control (n=3, $p<0.01$). **B)** shows the representative images used for the calculation of the ratios in (A).

5.3 Discussion

In line with the hypothesis stated at the beginning of this Chapter, the data demonstrated the enhanced survival of cisplatin-treated HCT-116 $p53^{-/-}$ cells through p53-mediated quenching of caspase-9 activity. Such cytoprotective effects of p53 (both WT and mutant) have already been reported in studies on ovarian cancer [146] and lung cancer [280]. With more reporting on the strong correlation between mutant p53 overexpression and enhanced cell survival, the long-standing hypothesis that the impairment of the transactivation property of mutant p53 is fully responsible for oncogenic transformation appears to be incomplete. This hypothesis cannot account for the phenomenon of tumours with overexpressed p53 showing enhanced invasion and metastasis in murine models [281,282]. In addition, the hypothesis also fails to explain the findings of tumours overexpressing WTp53 protein that has intact DNA binding ability, and hence the ability to induce apoptosis through transactivation of pro-apoptotic genes [161]. The findings that aggressive tumour cells usually exhibit both p53 mutation and overexpression are strong indications that the enhanced survival is not just dependent on the loss-of-function of WTp53, but also on a possible gain-of-function in mutant p53. The results here strongly indicate that the inhibition of caspase-9 activities by both WT and mutant p53 could contribute to the enhanced survival of tumour cells due to the impairment of cell death signalling.

In this study, overexpression of WTp53 in HCT-116 $p53^{-/-}$ cells resulted in greater extent of cell survival with cisplatin treatment, when compared to cells overexpressing $p53^{R175H}$ and $p53^{R337H}$ (Figure 5.2A). WTp53 possesses the ability to induce apoptosis through the transactivation and direct mitochondrial action. As such, the phenomenon observed here suggest that cells overexpressing the pro-apoptotic WTp53 were even more robust than its counterparts overexpressing the

mutants in the face of cisplatin treatment, is both intriguing yet fascinating. In addition, caspase-9 activity in these cells was lower than those in the GFP control (Figure 5.3A), suggesting a decrease in apoptotic signalling. Correlating this observation with other reports showing WTp53 overexpression in brain [283], ovarian [251], breast [182] colorectal [178], and head and neck cancer [284], the caspase inhibitory role of WTp53 uncovered in this study can potentially explain these seemingly paradoxical findings. In addition, the data also suggest that the inhibitory effect on caspase-9 is an inherent function of WTp53, which is often masked by its pro-apoptotic functions.

The p53 mutant that provided the most cell death-resistance to cisplatin is $p53^{D42Y}$. By the end of the 72-hour treatment duration, there was on average a ten-fold increase in cell survival as compared to the GFP control. As mentioned in *Section 3.1*, the choice of this mutation for this study is because of the location of the mutation (in the TAD region) and also because it is a mutation that was reported in ovarian cancer [236]. In addition, $p53^{D42Y}$ is characterised by the more drastic amino acid substitution from a positive electrically charged side group (aspartic acid – D) to one with a hydrophobic side group (tyrosine – Y), as compared to the other mutants chosen (all involving arginine to histidine substitution – with both amino acids possessing side chains that are positively charged), and this would provide a good contrast in this current investigation. As shown in the results, the more severe change in the side chain properties in the D42Y mutant seemed to accentuate cisplatin-induced cell death resistance and increase cell survival of cells overexpressing this mutant. The change in amino acid sequence and properties could have caused a structural change in the protein which retards p53's transactivation property, presumably by inhibiting the phosphorylation of serine 46. Phosphorylation at serine

46 was previously reported to be essential for the transactivation of the pro-apoptotic gene, *p53AIP1* [285]. Thus, coupled to the inhibition of caspase-9 activity by the mutant, cells with $p53^{D42Y}$ will be able to better resist the cytotoxic effects of cisplatin, as compared to the other p53 forms studied. Although the occurrence of this mutation in ovarian cancer is not very frequent [146,236], the potent effect of this mutant on cell death resistance and oncogenesis should not be dismissed. More work should be done to unravel the detailed mechanism underlying this mutant's influence on cell survival and drug resistance.

Compared to WTp53 and $p53^{D42Y}$, cells overexpressing $p53^{R337H}$ also afforded resistance to cisplatin treatment, though not as potently as the former two in terms of its cytoprotective effect. The mutation at amino acid residue 337 lowers the ability of p53 to tetramerise, which is an essential step for the transactivation of p53 target genes. Again, coupled to caspase-9 inhibition, the lack of transactivation by this mutant p53 could increase the threshold of cells to cytotoxin-induced apoptosis. Although the R337H mutation is not very commonly found in ovarian cancer, the tetramerisation mutants such as $p53^{K351N}$ mutation occurs naturally in the cisplatin-resistant ovarian cell line A2780-*c*is [44], while the $p53^{R342P}$ mutant was found in the PEO cell lines derived from ovarian cancer patients [229]. Similar to the R337H mutation, both the K351N and R342P lie within the TD region, which could destabilize the p53 tetramer. The reduction of its transactivation abilities could then ensue, thereby preventing the p53-mediated expression of pro-apoptotic proteins such as Bax. Hence, the significance of mutations in the TD region should not be discounted.

In contrast to the WT and the other two mutants, the structural mutant $p53^{R175H}$ offered no protection against the cytotoxic effect of cisplatin. Also, there

was no significant difference in the caspase-9 activity of this population of cells and the control. This is in sharp contrast to the inhibitory effects displayed when the recombinant protein of this mutant was added to S100 lysates of HEK293 (Figure 3.2) and HCT116 $p53^{-/-}$ cells (Figure 3.3) and when it was overexpressed in H1299 $p53^{-/-}$ cells (Figure 3.11). The only difference between these two sets of experiments is that the induction of caspase-9 activation in the two former experiments were done in cell-free systems, while the overexpression and cisplatin challenge were completely *in vivo*. Thus while $p53^{R175H}$ has the ability to inhibit caspase-9 *in vitro*, this structural mutant could no longer protect the cells from the effects of cisplatin in an *in vivo* setting. Cui [286] had reported previously that the neuroblast cell line SH-SY5Y overexpressing this mutant form of p53 was able to inhibit caspase-9 cleavage after doxorubicin administration. Coupled with the fact that R175H mutation is a rather prevalent mutation in ovarian cancer, it is rather surprising that this mutation did not afford protection against cisplatin treatment in the current investigation. Repeating the experiments using other ovarian cancer cell lines such as SKOV-3, will be required to determine if $p53^{R175H}$'s inability to protect against cell death is specific to just HCT-116 cells.

5.4 Concluding Remarks

The major focus of this segment of the study is to determine if p53 can augment cell survival in the presence of a cytotoxic drug, by reducing caspase-9 activity. From the results obtained, the hypothesis was proven true for WT and the various mutant p53 studied, with the exception of the R175H mutant. It hence appears that in an *in vivo* environment, the structure of p53 is important in the inhibition of caspase-9, since both mutants with slight ($p53^{R337H}$) or drastic structural changes ($p53^{R175H}$) showed compromised or no significant reduction in caspase-9

activity, and weakened cytoprotection against cisplatin. Intriguingly, the cytoprotection was also evident in cells overexpressing WTp53, suggesting the inhibition of caspase-9 as an inherent role of the WT protein and provides a mechanistic explanation to the phenomenon of WTp53 overexpression in various cancers.

Chapter 6

Conclusion

6.1 General Discussion

6.1.1 The Two Facets of p53

The results from this study demonstrated the inhibitory actions of p53 (both WT and mutant) on caspase-9 using several methods. This inhibition occurred through the direct interaction of p53 and the p35 active caspase-9 fragment, resulting in the suppression of caspase-9's activities both *in vivo* and *in vitro*. In addition, the enhanced resistance of cells to the cytotoxic effects of cisplatin in a p53-null background can be attributed to the reduction of caspase-9 activity. Taken together, the data obtained strongly suggest that p53 possesses an inherent anti-apoptotic function in the cell. This is in stark contrast to its very well-known tumour suppression functions. This unexpected activity of p53 uncovered by this study may well be the first report of such nature, even though the phenomenon that p53 can enhance survival of tumour cells has already been recorded for some time.

Although the anti-apoptotic function of p53 discovered in this study is novel, the idea that proteins can execute seemingly contradicting roles is not. In fact, there are already several examples of proteins with both facilitating and antagonising functions, switching to and fro between the two depending on circumstances. One known example of such is the c-myc protein.

6.1.1.1 c-myc, a Protein with "Contradicting" Roles

Like p53, c-myc is a transcriptional factor [287]. However, in contrast to p53, c-myc promotes cell cycle progression through the activation of transcriptional factors such as E2F1 [288] and E2F2 [289]. Also, it transcriptionally represses cell

cycle checkpoint genes like *GADD45* and *GADD153* [290]. In addition, it inhibits the function of cyclin-dependent kinase (CDK) inhibitors [291], either through direct repression of gene transcription or indirectly through degradation or sequestration. For cell differentiation to occur, downregulation of c-myc is required [292,293]. Apart from its functions to promote cell growth, c-myc can also initiate angiogenesis through the release of interleukin 1β [294].

The contrasting face of c-myc lies in its ability to induce apoptosis through direct interaction and activation of Bax [295] and indirectly inhibiting the functions of Bcl-2 [296] and Bcl-xL [297]. B-cells that were deficient in c-myc proteins were reported to be resistant to diverse apoptotic stimuli [298]. Interestingly, pathways involving c-myc and p53 crosstalk. In a study by Zindy [299], deregulated c-myc upregulated ARF, which in turn activate p53. The activated p53 then transactivate target genes involved in apoptosis and growth arrest.

6.1.1.2 Transforming Growth Factor-beta (TGFβ), Another Example

Another example of such proteins is TGFβ. TGFβ is a group of ligands consisting of TGFβ1, TGFβ2 and TGFβ3, acting through two receptors, namely TGFβ type 1 (TGFB1) and type 2 (TGFB2) receptors [300,301]. Upon activation, the phosphorylated receptors recruit and phosphorylate receptor-regulated Smads (R-Smads) [302] proteins. Activated R-Smads then form heteromeric complexes with SMAD4 and translocate into the nucleus [300]. The Smad complexes then regulate the expression of genes through the association with other transcription factors such as the forkhead, homeobox, zinc-finger, AP1, and basic helix–loop–helix (bHLH) families [303,304]. In addition, it can also repress transcription by associating with co-repressors such as retinoblastoma-like 1 (RBL1) [305]. Hence,

depending on which proteins partner with the Smad complexes in the nucleus downstream of TGFβ binding, the target genes can be either activated or repressed.

TGFβ can either promote or inhibit cell proliferation, although the two scenarios are dependent on cell type. TGFβ can inhibit cell cycle progression in the G1 phase through the induction of cyclin-dependent kinase inhibitors (CDKIs), InK4B and p21 [306,307]. TGFβ also represses the expression of c-myc [308]. In blood related cancers such as acute myeloid leukaemia, the human ecotropic viral integration site 1 (evI1) protein is transcriptionally activated by several recurrent chromosomal aberrations [309]. evI1 interacts with SMAD3 (an R-Smad protein) and antagonizes the growth inhibitory effects of TGFβ [310]. In another example, Tax, a transcriptional protein produced by the human T-*c*ell leukaemia virus type I (and implicated in various clinical manifestations in adult T cell leukaemia), disrupts the interaction of Smad complexes with the transcriptional co-activator p300 and contributes to resistance to growth inhibition by TGFβ [311,312]. In addition to the anti-proliferation activities, TGFβ can initiate the transcription of proapoptotic target genes such as *BIM* [313]. Bim promotes apoptosis by binding to Bcl-2 [314], thereby relieving its inhibitory effect on Bax, allowing Bax to induce the release of cytochrome-*c* [315].

In some other cell types, TGFβ appears to promote proliferation. For example, in certain mesenchymal cells (including smooth muscle cells), TGFβ enhances cell division through the induction of platelet-derived growth factor (PDGF) [316]. Overexpression of TGFβ was shown to be associated with a poor prognosis in patients with glioma [317]. Consistent with its pro-proliferation function, TGFβ can influence apoptosis through the transactivation of anti-apoptotic genes. *D*ifferentially *e*xpressed in *C*hondrocytes 1 (DeC1), a target of TGFβ [318], is

a bHLH transcription factor which could in turn activate the expression of Survivn [319], thereby inhibiting apoptosis. DeC1 has been found to be frequently overexpressed in certain cancers, including breast carcinomas [320].

6.1.2 A Balance is Needed

Due to the dual roles of p53 uncovered in this study, it appears now that cells are constantly balancing its pro-apoptotic and anti-apoptotic functions. As reviewed in Chapter 1, p53 is a node for the regulation of multiple cellular functions. Cells are not just sensitive to the activation state of p53, but also to the levels of the protein in the cellular environment. In cancer, the protein level of p53 is often affected, thereby contributing to the tumorigenesis and aggressiveness of the tumour cells.

Protein levels of a gene can be determined by the gene copy in a cell. Cells are extremely sensitive to the haploid/diploid state of the *p53* gene. Mice that are $p53^{+/-}$ showed enhanced tumour formation [321]. This suggests that one copy of the *p53* gene is inadequate in eliciting sufficient levels of apoptosis, which could be due to the reduced transactivation of pro-apoptotic genes and the ability to initiate apoptosis directly at the mitochondria. Further evidence of the importance of p53 protein level regulation for normal cell growth, cells with haploid MDM2/4 (the negative regulators of p53 level) showed increased cell death, and the phenomenon can be corrected by a concurrent deletion of one copy of the *p53* gene [322].

In cancer, this skewed p53 protein level is highly detrimental. A tumour cell with p53 mutation is analogous to a $p53^{+/-}$ environment. These tumour cells are usually heterozygous for p53 mutation, containing one allele carrying the mutation, while the other allele codes for the WT protein. As the mutant p53 can no longer carry out the functions of its WT counterpart, the decreased level of functional WT proteins probably account for the enhanced growth of these cells [323,324]. Not to

mention, there are additional mechanisms which have been suggested and reported that contribute to the aggressive nature of the tumour cells. This include the hypothesis that mutant p53 usually becomes dominant-negative by influencing the functions of the remaining WTp53 proteins through the formation of hetero-oligomers with WTp53. This interaction was found to change the conformation of the WTp53 proteins, hence inhibiting the normal functions of the WT protein [191]. A more detailed review has been presented in *Section 1.4.3.2*. In addition, there are many articles reporting the overexpression of mutant p53 in tumours. Hence, while the lowered levels of the WT protein could account for the reduced apoptosis, the elevated concentration of the mutant p53 could confer resistance to chemotherapeutic drugs. In this study, the uncovered function of p53's inhibition of caspase-9 can be a contributing factor to the enhanced resistance and aggressiveness of these tumour cells.

Not all tumour cells carry a p53 mutation. In fact, some tumours were found to overexpress the WTp53 protein instead. Accumulation of WTp53 in the cytoplasm has been reported in colorectal cancer [325], brain [326], breast and neuroblastoma [175,176]. Noon [327] reported that increased p53 expression contributed to reduced overall survival and more rapid disease progression in renal cell carcinoma. As cytoplasmically localised p53 was found to be resistant to ubiquitin-dependent degradation by MDM2 [328], the persistent presence of the WT protein in the cytoplasm could increase the threshold of the cells to apoptosis through the inhibition of caspase-9. The significance of this is that even in the face of its ability to induce the activation of Bax and the release of cytochrome-*c*, the ability of p53 to inhibit caspase-9 would still stem the progression of apoptosis, hence enhancing the capacity of these cells to survive.

6.1.3 Implications for Future Research and Therapies

With the discovery of p53's anti-apoptotic function as reported herein, the idea of activating endogenous p53 to the WT conformation would now need to include this new dimension of p53. As shown in several reports, the simple activation of p53 did not always yield the desired results of inducing more cell death. While studies by both Liu [329] and Song [330] demonstrated that viral-vector induced expression of WTp53 resulted in sensitisation of the transduced cells to the effects of irradiation and cisplatin, others have demonstrated that the induction of WTp53 did not increase apoptosis [331]. On the contrary, some work reported increased resistance after the introduction of WTp53 [332,333]. As such, more research has to be done to investigate if these transduced WTp53 are functionally similar to endogenous WTp53 in normal cells.

Apart from introducing WTp53 that are functionally active, the localisation of the protein should also be looked into. Reports mentioned earlier have demonstrated the higher chemoresistance in tumours with cytoplasmic accumulation of p53. Therefore, based on the results of this investigation, a cytoplasmic localisation would result in the inhibition of cytoplasmic caspase-9 activity, resulting in lowered sensitivity to apoptosis. At the current level of understanding, the introduction of a functional WTp53 that could enter the nucleus to perform its transactivation role to induce apoptosis would produce the best results, although the implications of activating other cellular processes such as cell cycle arrest and senescence should not be ignored. Another way, as discussed in *Section 1.5.3*, is to target MDM2/X, the negative regulators of p53. As both proteins are in the nucleus, the relief of the inhibition by MDM2/X would directly release WTp53 into the nuclear milieu, allowing it to perform its pro-apoptotic, transactivating functions. Also, the balance

between the levels of WT and mutant p53 proteins (in the case of tumours harbouring p53 mutation) must be analyzed before effective gene therapy can be administered.

The ideas suggested above are by no means exhaustive. Due to the dynamic nature of the cellular environment and the complex pathways involving p53, it will take more years of intensive research to completely understand the full functions of this intriguing protein. Only then, can p53 be manipulated successfully through the use of gene therapy, to obtain the desired outcome in treating cancer with dysfunctional p53. However, the challenge now will be to take into consideration this newly discovered role of p53 when designing therapies targeting cancers with p53 mutations and/or overexpression.

6.2 Critical Analysis and Future Works

Although the work performed for this study have provided evidence to prove the inhibitory effect of p53 on caspase-9 activity through direct interaction, more research work are needed to gain deeper insights into this novel p53 function.

6.2.1 Improvements to Experiments Done in this Study

Firstly, a few improvements can be done to experiments already performed in this study. A criticism of current work can be seen in Chapter 3, whereby the immunodepletion and knockdown of p53 in A2780 cells did not manage to show very significant differences in the enhancement of caspases and PARP protein cleavage (Figures 3.5 and 3.7). This was partly due to the difficulty in achieving substantial reduction in the p53 levels in both experiments. To better determine the impact of p53 reduction on caspase cleavage and activation on this cell line, further optimisation can be done in terms of increasing the knockdown duration, increasing

the concentration of the siRNA specific for p53 used, and increasing antibody concentration used for immunodepleting p53 from the lysates. The experiments can also be repeated in other ovarian cell lines with WTp53, such as OVCAR-4 or Sk-OV-3.

Similarly, the difference in the caspase-9 activities in PEO4 cells was not very obvious between the control and the one with p53 knockdown (Figure 3.8D). As seen from the Western blot for p53 levels (Figure 3.8E), there was no marked reduction of p53 protein level. Again, optimisation of the knockdown duration and dosage of siRNA should be performed for future works.

Another criticism is the observation that for cisplatin-sensitive PEO1 cell line, the measured caspase-9 activity of the control population upon cisplatin challenged (as compared to PBS) did not increase considerably (Figure 3.8B). It is possible that the number of times of passage for this cell line used during the experiment was too high. Also, the dosage of cisplatin used (25μM) may be overwhelming for this cisplatin-sensitive cell line, possibly resulting in massive necrosis which could not be detected in this experimental set up. In order to substantiate or verify that the cisplatin resistance in PEO4 cell lines were due to p53, PEO1 cells must first show sensitivity to cisplatin dosage in the control population, while displaying a lesser extent of increased caspase-9 activities, as compared to PEO4, upon p53 knockdown. Hence, a repeat of this set of experiments with lower cisplatin dosage is desirable.

The use of the PEO cell lines provided a p53 mutant background to study the effects of reducing endogenous mutant p53 on caspase-9 activities. However, only one such cell line was used on this study. To better show the inhibition of caspase-9 by mutant p53 *in vivo*, the use of other cell lines bearing mutations of p53 at other

sites could be incorporated. This may include ovarian cell lines such as 2274 and TOV-112D, both bearing hot-spot mutations R273H and R175H respectively [152]. In addition, cell lines of other backgrounds e.g. breast cancer cell line HCC1395 [334] and colon cancer cell line SW480 [335] can be used to reinforce the hypothesis of this study.

The general trend displayed by the results presented in Chapter 3 was that increasing p53 levels corresponded to reduced caspase-9 cleavage/activities. However, overexpression of WTp53 appears to not support this correlation. In Figure 3.9, the low levels of expression of WTp53 resulted in the inhibition of caspase-9, while much higher WTp53 levels resulted in the restoration of caspase-9 activity levels to that of the control. As discussed, this does not mean that WTp53 does not inhibit caspase-9. Rather, the pro-apoptotic effects observed with high p53 level, possibly due to the transactivation activities of WTp53, could contribute to the phenomenon observed. This actually provides an excellent case study to investigate the balance between the transactivation effects of p53 and the inhibition of caspase-9 activities by endogenous WTp53. To start, the concentration of plasmids used for overexpression can be further fine-tuned, to determine the tipping point in reversal of caspase-9 inhibition. In the meantime, the expression of pro-apoptotic target genes of p53 such as Bax or Bak can be probed using immunoblotting to determine their involvement in this phenomenon. Indeed, the use of an inducible cell line and careful measurement of p53 levels using a quantitative assay could give valuable information about the precise p53 level that controls this tipping point.

In Chapter 4, the specificity of the recombinant p53 refolded and used for many of the experiments in this study were shown by testing the inhibitory effect on other recombinant caspases such as caspase-3 and caspase-6 (Figure 4.2). The main

point of criticism here is that both caspases used are considered ‘effector’ caspases i.e. caspases which are downstream in the apoptotic pathways and their structural difference to ‘initiator’ caspases could have resulted in the lack of inhibition by the recombinant p53 [336]. As such, the inclusion of other ‘initiator’ caspases such as caspase-8 would further validate the specificity of the recombinant p53 used.

Also in Chapter 4, IP and cross-linking were used to demonstrate the interaction between the recombinant p53 and endogenous caspase-9 (Figure 4.6). However, the control beads showed ‘contamination’ of p53 and caspase-9. As discussed, the presence of both proteins could be due to the lack of stringency in the washing steps involved in the procedure before the cross-linking step. Further optimisation is hence needed to show a clear cut difference between the control (with the use of HST-tag), and the one with p53, so as to demonstrate unequivocally that the interaction is purely between p53 and caspase-9.

Lastly, in Chapter 5, the increase in cell survival through inhibition of caspase-9 was demonstrated using only one cell line and one type of drug. To show that this is a generic phenomenon, repeats of this experiment using other p53-null cell lines of other backgrounds (such as bone cell line, Saos-2 and lung carcinoma H1299) and other chemotherapeutic drugs (e.g. Actinomycin-D and etoposide) can be included. Also, other techniques such as cologenic assays and fluorescence activated cell sorting (FACS) analysis for Annexin V and propidium iodide can be used as additional methods to determine the changes in cell survival in response to cytotoxins in the presence of p53.

6.2.2 Domain Study to Investigate the Point of Interaction between p53 and Caspase-9

Since p53 has been shown to interact with caspase-9, it will be interesting to study the detailed mechanism of interaction. Firstly, domain studies can be performed to narrow down the point of interaction between these two proteins, and to investigate further the conditions in which such interaction/inhibition occurs. As it has been shown and discussed that the interaction between the two is transient and cannot be properly investigated using traditional IP experiments, other methods need to be employed. As demonstrated in Chapter 4, far Western coupled with cross-linking has been used successfully. By modifying this technique, the interaction site(s) can be potentially mapped out. This can be firstly done by producing recombinant p53 of various domains (e.g. the TAD only, TAD and DBD, DBD only etc), refolding it, and applying it as bait proteins to renatured active caspase-9 proteins on the nitrocellulose membranes, in order to elucidate which domain(s) is essential for the interaction. Next, site-directed mutagenesis of the identified domain(s) can be performed to pinpoint the site or amino acid(s) critical for this interaction. Similarly, this can be repeated for the p35 domain of caspase-9 to determine the crucial sites involved in the interaction.

A further extension of this study would be to overexpress the genes of both proteins bearing mutation to the critical sites to investigate if apoptosis can still be abrogated upon chemotherapeutic drug challenge. This would hence link the interaction of these proteins to enhanced cell survival, and possibly, the increased drug resistance in tumour cells.

6.2.3 Impact of Post-translational Modifications of p53 and Caspase-9

As this may be the first report to show the inhibitory effects of p53 on caspase-9, this study has concentrated on demonstrating the phenomenon of the inhibition as well as proving the interaction between these two proteins. However, it must be highlighted that both proteins may be subjected to post-translational modifications, which can influence the inhibitory effect observed so far.

As reviewed in *Section 1.3*, p53 can undergo extensive post-translational modifications to regulate its activities. In particular, phosphorylation of p53 at residue 46 has been shown to be involved in initiating apoptosis [285,337]. The enhanced resistance of p53-null cells overexpressing the D42Y mutant has been hypothesised in Chapter 5 to be partly due to the abrogation of this phosphorylation. Hence, it will be interesting to find out the phosphorylation status of p53 in the cell lines overexpressing the various mutant and WTp53.

Also reviewed in the same section, acetylation of p53 at various residues could bring about enhanced p53 activities either through the disruption of negative regulation of p53 by MDM2/MDM4 [128,129], or by increasing the cytoplasmic presence of p53, and thereby apoptosis [133]. Major sites of acetylation include lysine residues at position 164 and 320 [129,130,132], both of which are in close proximity to R175H and R337H mutations respectively. It will be interesting to see if acetylation was involved. It is possible that through the constitutive acetylation of p53, which brings forth an extended half-life of the p53 proteins, increased cytoplasmic localisation of p53 hence ensues, thereby resulting in the inhibition of caspase-9 and apoptosis.

Similar to p53, caspase-9 can also be regulated through post-translational modification. So far, phosphorylation of the protein has been identified to inhibit its

activity. Phosphorylation of serine 196 by Akt [338] and threonine 125 by ERK [339] and CDK1/cyclinB1 [340] inhibit caspase-9 activities and apoptosis. It will hence be worthwhile to study if the inhibition of p53 on caspase-9 involves the phosphorylation of caspase-9, both directly on the protein or indirectly through the influence of the other proteins responsible for the modifications.

To study the post-translational states of p53 and caspase-9, antibodies specific to these modifications are available commercially for detection through Western blot. By comparing the levels of such modification in the different experimental conditions, a better understanding of the relationship between this and the reduced level of apoptosis observed can be achieved.

6.2.4 Involvement of p53 in the Apoptosome

Caspase-9 forms part of the apoptosome with other proteins like Apaf-1 and cytochrome-*c* [341], leading to the activation of caspase-9 and caspase-3 [84]. Many studies focusing on the formation of the apoptosome as well as caspase activation have provided evidence to show that the activation of caspase-9 is dependent on dimerization [342] and association with the apoptosome complex [84][343].

In this study, p53 was shown to directly associate with caspase-9 and bringing about the inhibition of its activities. In an *in vitro* environment whereby caspase-9 is in an extremely high concentration, recombinant cleaved caspase-9 can easily dimerize to be active and hence the inhibition of its activities by recombinant p53 can be easily observed (Figures 4.1 and 4.3). In a cell-free and *in vivo* environment, due to the presence of endogenous Apaf-1, dATP and the added cytochrome-*c*, activation of caspase-9 can be achieved and similarly, inhibition of its cleavage can be detected (Figures 3.2 to 3.12). Combining these two findings, it seems to suggest that the mechanism of p53's inhibitory action on caspase-9 is to

firstly interact with the p35 active fragment, followed by the inhibition of its ability to cleave more procaspase-9 proteins, hence preventing the amplification of this apoptotic pathway. However, the results presented are inadequate in proving this hypothesis and it also could not show the relationship between the inhibition mechanism and the apoptosome. It will be interesting to see if p53 mount its 'attack' through sequestering active caspase-9 from the apoptosome, or by directly binding to the active caspase-9 fragment while it is part of the apoptosome complex, hence preventing the cleavage and activation of downstream proteins such as caspase-3 and PARP.

As the molecular weight of the apoptotsome complex (not including p53) is massive (>1MDa), the study of this structure had to be done using gel filtration techniques. Shiosaki [344] published his findings of the oligomerisation and activation of caspase-9 with the CARD domain of Apaf-1. Gel filtration, combined with Western blot, was used to elucidate the interaction of caspase-9 with Apaf-1 CARD, as interacting complexes will elute in different volumes due to the change in molecular weight. Similarly, this technique can be employed to study the involvement of p53 and the apoptosome.

6.2.5 Involvement of p53 Family of Proteins

The 'gain-of-function' of mutant p53 has already been long suspected by the scientific community although concrete evidences of its mechanism have not yet surfaced. Gaiddon [345] has demonstrated that mutant p53 could interact with p63 and p73 (both proteins related in sequence and structure to p53) and inhibit the transcriptional activities of these two proteins, resulting in reduced apoptosis and tumorigenesis.

In this study, the question whether the inhibitory role of p53 on caspase-9 is the sole mechanism in inhibiting apoptosis could not be addressed. It would be hence helpful to test if the involvement of the other two p53 family members affects apoptotic signalling as well. Mutations of the p53 gene, which can affect the interaction between the members of this family of proteins, can be generated to study if interaction of p53 and p63/p73 proteins will influence the p53-mediated cell death resistance already observed in this study.

In addition, elevated expression of p73 has been demonstrated in cells exposed to cisplatin [346]. Also, p73 was shown to increase the level of apoptosis [347] while the downregulation of its expression resulted in enhanced chemoresistance [348]. It would hence be beneficial to investigate if p73 protein levels are affected in cells overexpressing the various p53 and subjected to cisplatin challenge, and whether this contributed to the increased resistance to cisplatin.

6.2.6 Primary Cell Lines and Clinical Applications

The work done here involved the use of only immortalised cell lines. As this is a first effort to demonstrate the relationship between p53 and caspase-9, no research has been attempted on primary cell lines and animal models as yet. To prove that this inhibitory effect of p53 on caspase-9 is not just found in immortalised cancer cell lines, a repeat of the experiments using primary cell lines can be done. Also, the use of animal models with knock-in p53 mutation can also provide protein samples to verify the findings of this study. Similarly, clinical samples bearing p53 mutations are also valuable resources to give an added avenue to validate the results obtained.

In addition to the above, the results from this investigation have shown that the inhibition of caspase-9 could be possibly linked to cisplatin resistance (Figure

3.8D). Moreover, cells overexpressing the D42Y mutation (found in ovarian cancer tumours) displayed huge resistance to cisplatin and enhanced cell survival. As hypothesised earlier in this report, the abrupt change of amino acids properties with charged side chains to those bearing a benzene ring in the mutant could adversely affect the functions of normal p53, hence resulting in the augmented resistance to chemotherapeutic drugs observed. With this, it will be interesting to investigate the relationship between adverse change in side chain properties in tumours bearing p53 mutation and drug resistance. This can be achieved by sequencing the p53 genes of patient samples before and after cisplatin resistance, and looking out for changes in the p53 gene status (in particular for any side chain changes with vastly different properties), to correlate the two events. From here, the presence of such mutation could potentially be used as a biomarker for chemotherapeutic drug resistance, hence potentially allowing better design of treatment therapies for such cases.

6.3 Final Conclusion

The results obtained so far have provided evidence for a novel transcriptional-independent role of p53 in caspase inhibition. The direct interaction of p53 and active caspase-9 is the first report of such nature. In addition, the inhibition is evident not just in the mutant form, but also WTp53, which proves that this inhibitory role is an inherent p53 function. Its significance is such that firstly, the inhibition of an apical caspase bears consequential effects in preventing and/or substantially delaying a full-scale activation of the apoptotic pathway and thereby cell death. Secondly, this mechanism is downstream of p53's pro-apoptotic functions in terms of transactivation of Bax and Bak and the direct activation of Bax to result in cytochrome-*c* release. The ability of p53 to halt the progression of the very pathway it activates enable p53 to better regulate the whole progression of apoptosis,

allowing it to arrest cell death at the final moments before they are doomed to self destruct.

Although the inhibitory role of p53 has been proven in this study, more work has to be done, especially in the validation of the mechanism in primary cell lines, animal models, and clinical samples. Only then can it be safely concluded that such mechanism is an inherent function of p53. Upon the verification of this new role of p53, drug therapy design aimed at simply transducing cells to express WTp53 could no longer be sufficient. Localisation of the protein and the balance between WT and mutant p53 protein levels must also be optimised.

In conclusion, based on the knowledge of p53 functions so far, the delicate balance between the amounts of pro-apoptotic genes transactivated by p53, activated pro-apoptotic proteins, and the physical presence of p53 in the cytoplasm for caspase inhibition, is critical to the cell's survival. Any perturbation to this fragile balance, in particular in a diseased background, would affect cell fate and ultimately the overall survival of an individual.

Chapter 7 –

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