Section 1 - Introduction

Important Roles of p53 & MDM2 in Cancer

p53 was first detected in rodent cells transformed by SV40 in a complex with the SV40 large T-antigen [1, 2] and mutant forms were found to be associated with inherited cancer-susceptibility in patients with Li-Fraumeni syndrome [3-5]. An early study using homologous recombination in mouse ES cells to derive chimeric progeny with a Tp53 null allele found that mice homozygous for the mutant Tp53 gene appeared normal, but were susceptible to various types of tumors prior to reaching 6 months of age. The most frequently observed tumors being lymphomas occurring in a number of various organs including the thymus, heart, liver, spleen, lung, kidney, and brain. Sarcomas occurred at a relatively high frequency within the population as well [3].

Amplification of the mdm2 gene mapped to chromosome 12q13-14, along with its respective mRNA and protein occurred in sarcoma cell lines with gene amplification [6]. Moreover, mdm2 gene amplification is believed to be associated with tumor progression and metastasis in osteosarcomas [7]. Altered patterns of Tp53 and mdm2 expression are good indicators of survival times in patients with soft tissue sarcomas, with their overexpression a predictor of poor prognosis [8]. Although gene amplification did not occur in leukemias, mdm2 mRNA overexpression levels were comparable to those observed in sarcomas in some cases. Furthermore, this overexpression appears to be associated with unfavorable chromosomal abnormalities (translocations, reciprocal translocations, etc.) suggesting MDM2 may play a significant role in leukemogenesis [9].
Structure and Function Domains of p53

The tumor suppressor p53 is a transcription factor that, upon tetramerization and subsequent binding to a p53 response element (RE) [10], plays a critical role in monitoring genetic stability and circumventing tumorigenesis by up regulating transcriptional activation of numerous proteins responsible for cellular senescence, cell cycle arrest, apoptosis, and DNA repair [11, 12]. Located at the amino-terminus of p53, the transactivation domain (TAD) is responsible for the recruitment of both activating and repressing co-factors to p53 REs. The most important protein interacting directly with p53 is MDM2 (also known as HDM2 in humans, but will remain MDM2 for the purpose of this dissertation) which binds to and inhibits the TAD’s function as a transactivation domain by masking this region of p53 [13]. The two transactivation domains (TAD I aa1-42, TAD II aa43-63) act both together and independently of each other. For example, TAD II is necessary for apoptosis when TAD I is mutated or deleted, and mutation of TAD II at residues 53-54 abrogates apoptosis altogether [14]. Just adjacent to TAD II, the proline rich domain (PRD) comprising residues 60-94 (Figure 1.1) consists of five repeating PXXP motifs (where P = Proline and X = any amino acid), which are important for apoptotic functioning of p53 [15]. The PRD may also play a critical role in the transmission of antiproliferative signal transduction by its interaction with SH3 domains of other proteins [16]. Interestingly, this domain retains partial apoptotic activity with only one of its PXXP motifs intact while mutants devoid of all PXXP motifs are inactive [17].

The DNA sequence-specific binding domain of p53 resides in the central region of the protein (amino acids 98-289) and consists of residues 99-289.
The DNA Binding Domain or DBD, along with the C-terminal region under conditions inhibiting the C-terminal domains (CTD) non-specific binding function, is considered to play a role in DNA binding specificity [19] by recognizing the p53 consensus sequence Pu-Pu-Pu-C(A/T)|(A/T)G-Py-Py-Py-Py (where Pu denotes a Purine nucleotide and Py represents a Pyrimidine) [20]. This sequence can be separated by up to 20 base pairs, with the icosamer (0 nucleotide separation) found most commonly *in vivo* [21]. In 1992, Hupp discovered that monoclonal antibody PAb421 enhanced sequence specific DNA binding by p53’s DBD. Moreover, deletion of the last 30 C-terminal amino acids constitutively activated p53 [22]. The DBD is where the vast majority of tumor-derived substitution mutations are found in mutant p53. The four “hot spot” mutations found to overlap with the most highly conserved regions in the DBD include amino acids 114-139, 168-178, 231-255, 267-283 of the protein [19]. More recent research identified the dimerization interface as another hot spot for mutations upon oligomerization that includes residues associated with DNA contact and protein stabilization [23]. The basic residues K117 (K120 human), R280 (283) and, R245 (248), which are often mutated in human cancers, are thought to be essential for DNA recognition [18].

Regulation of p53 DBD sequence specific binding involves the basic C-terminal domain or (CTD) spanning residues 364 to 390 in the murine p53 (367 to 393 in the human ortholog) [24]. For example, a study using murine NSp53 (Normally Spliced) with a C-terminal highly homologous with human p53 and murine ASp53 (Alternatively Spliced) replacing the mostly basic C-terminal 26 amino acids of NSp53 with a different 17 amino acid sequence, found that
NSp53 retains non-specific DNA binding while the alternatively spliced version remains constitutively active [24-26] suggesting a possible dominant negative role for the AS mutants and further evidence for CTD non-specific binding to DNA. Various models to explain the p53 ssDNA/RNA binding and renaturing functions exist from prior investigations. One study suggests a model in which the p53 CTD recognizes DNA structures resulting from DNA damage and this interaction regulates the sequence specific binding of p53 via conformational changes [27]. Another study reported that p53 has a C-terminal regulated affinity for Insertion/Deletion (IDL) mutations [28]. Recent studies find that p53-RNA interactions require the CTD in vitro and in vivo in a sequence-nonspecific fashion. Nevertheless, p53 with a post translationally modified CTD, the major in vivo form of the protein, does not appear to bind RNA at all [29]. Perhaps most importantly, the binding of non-specific DNA sequences to the CTD indirectly regulates p53s DBD-DNA specific interaction by sequestering p53 in locations separate from p53 REs, again confirming its role as a negative regulator of specific p53-DNA binding [30].

The tetramerization domain (TET) of residues 326-355 [31] is important for p53’s ability to stably bind DNA. Conformational switching between the active and latent forms occurs when p53 exists as a homotetramer [32]. Recent NMR and X-Ray Crystallographic structures depicted the homotetramer binding to a p53 consensus sequence [18] finding a glycine residue at position 334 resulting in the TET forming a V shape with a β-strand and an α-helix forming each side of the structure [33, 34]. Experimental evidence indicates that the TET is a dimer of dimers with two monomers associating via their β-strands to form an anti-parallel
double stranded sheet and double helical bundle [35]. This interaction leads to the formation of eight backbone hydrogen bonds as well as a hydrophobic core. The residues located within the hydrophobic core are the major stabilizing force in this conformation [36]. One model for explaining p53-DNA interaction involves cooperative binding between the dimers engaging the consensus sequence and forming a homotetramer [18, 23]. Therefore, the TET influences both affinity and the interaction strength of the entire p53-DNA complex [37, 38]. Furthermore, certain kinases have been shown to bind and phosphorylate the TET and CTD; including Casein Kinase II [39] and PKC which phosphorylates residues S360, T365, S370, and T377 resulting in the loss of sequence specific DNA binding within the C-terminal region [40]. ChK1 phosphorylation (see Regulators Section: ATM/ATR) is abolished when the TET is deleted from p53 [41] and the ubiquitylation of p53 by E3 ligases requires the oligomerization of p53 [42], thus the degradation of p53 via the binding of MDM2 is inhibited without its quaternary structure in the homotetrameric conformation [43]. Moreover, p53s tetramerization at higher concentrations buries the Nuclear Export Signal (NES) located within the TET [44] effecting p53’s subcellular location. Interestingly, missense mutations related to Li-Fraumeni syndrome, R337C [45] and L344P [46] suggest that TET mutations can exist in the germline as well as sporadic somatic cellular tumorigenesis (Figure 1.1).

The main mechanism for the cellular localization of p53 is via three nuclear localization signals (NLS) and 2 nuclear export signals (NES) [44, 47-49]. Of the 3 NLS’, 2 are mono-partite consisting of a single stretch of several basic residues located in the far C-terminal regions (369-375), (379-384) and referred to as NLS
III / NLS III, respectively [50]. NLS I is responsible for nucleocytoplasmic shuttling of p53, is bi-partite and encompasses residues 305-322 in the following configuration – K^{305}-R^{306} - 9 residues – PQPKKKP [47]. As the functioning NLS of p53, its role is essential in ensuring p53 nuclear accumulation upon various stress signals [51]. p53 is subject to both nuclear import and export via an active pathway that relies on the expenditure of energy [52]. Specifically, nuclear import involves the binding of the NLS I to an importin α/β complex followed by docking at the nuclear pore where the small G protein RAN and its GTPase activating protein RanGAP1 assist in the transport of p53 into the nucleus [53]. p53’s C-terminal NES is located between residues 340-351 and consists of a sequence rich in leucine [44]. Due to the location of this NES, the tetramerization of p53 can mask its nuclear export signal, sequestering p53 in the nucleus due to the inability of this NES to bind to its receptor CRM1 upon reaching a threshold level [44, 51]. Under normal conditions CRM1 translocates p53 to the nuclear pore where another Ran GTPase along with its regulator, a guanine exchange factor (GEF) called RCC1, assists p53’s entrance into the cytoplasm [53]. p53’s other NES resides at the N-terminal and comprises residues 11-27 [49]. It is proposed to work in collaboration with the C-terminal NES and contains 2 serine residues, 18 and 23 in the human ortholog, that are phosphorylated after DNA damage by either ATM/ATR or ChK1/ChK2 depending on the source of the stress and tissue involved, preventing the nuclear export of p53 (Figure 1.2) [49].

In light of these comprehensive studies, it is evident that p53’s relative binding affinity to DNA and/or proteins is regulated through each of its functional
domains via multiple mechanisms as detailed in the following text in order to activate its transcriptional activity.

**p53 Target Genes and the Functions of Their Encoded Proteins**

As mentioned in the previous section, the major function of p53 is to regulate the transcription of numerous genes involved in cellular activities such as senescence, cell cycle arrest, apoptosis, and DNA repair [10-12]. Also, some p53 target genes encode proteins that are important for regulating angiogenesis, re-entry into the cell cycle, response to oxidative stress, and cell fate [54], as well as longevity, ageing, and the glycolytic pathway [55]. It would be hard to detail all of these p53 target genes here, and thus I will discuss some representative and well-studied ones as follows.

**p21**

p21\textsuperscript{WAF1/CIP1} (p21) is a universal inhibitor of CDKs or cyclin dependent kinases [56]. The gene encoding p21 is mapped to chromosomal region 6p21.2 and contains a cis-acting 20bp p53 response element located approximately 2.4kb upstream of the p21 gene. It was not surprising then, when studies found p53 to activate the transcription of p21 [57]. Soon after cyclins and cyclin dependent kinases were found to regulate the cell cycle between each transitional phase (G0-G1-S-G2-M), a group of KIPs (Kinase Inhibitor Proteins) including p21 and p27\textsuperscript{KIP1} were also discovered. p21 plays a crucial role in the G1-S checkpoint and probably also an inhibitory role in the G2-M checkpoint [58].

*Under normal cellular conditions*, mitogen signaling induces the expression of D-type cyclins [59]. However, in early to mid G1, the p21 gene is induced if cell cycle arrest is required and incorporated into the formation of cyclin D-CDK 4/6
complexes [60] to inhibit their kinase activity. The active (proliferative cell nuclear antigen) PCNA-cyclin D-CDK 4/6 complexes phosphorylate retinoblastoma (Rb) protein and release it from the Rb-E2F-complex, consequently allowing E2F to function as a transcription factor important for the expression of various genes encoding proteins for DNA synthesis or S phase transition. In situations, such as DNA damage, requiring cell cycle arrest, p21 acts as a G1-S checkpoint regulator to inactivate the activity of the next round of CDKs (CDK2/4-cyclin D/E) complexes, thus resulting in G1 or Restriction point arrest upon p21 overexpression in response to active p53 after stress.

During the G2-M checkpoint p21 is phosphorylated on Thr-57 and CDK2 is the most likely candidate for p21s extensively phosphorylated state. Studies have shown p21 to enter the nucleus during S phase, become hyperphosphorylated at the S-G2 boundary and interact with Ser126 of cyclin B1 prior to Cdc2 kinase activation by Cdc25C (i.e. dephosphorylation of Cdc2 at Y15) and by CAK (i.e. phosphorylation of Cdc2 at Tyr161). Although some studies have found the association between p21 and cyclin B1-Cdc2 appears to promote Cdc2-B1 kinase activity at the G2-M checkpoint [61], when cellular conditions dictate, DNA damage or oncogenic signaling for instance, can result in the overexpression and universal binding of p21 to each of the cyclin-CDKs [56], resulting in p53 initiated cell cycle arrest.

14-3-3σ

The 14-3-3σ protein is one of the seven human and highly conserved 14-3-3 family members [62-64], which was originally discovered and characterized as a human mammary epithelium-specific marker 1 (HME1) downregulated in
mammary carcinoma cells [63]. The 14-3-3σ gene was found to be a direct transcriptional target of p53 [65]. 14-3-3σ performs a number of functions within a cell upon DNA damage. For example, other isoforms sequester Cdc25C phosphatase in the cytoplasm, preventing its dephosphorylation of the Cdc2-B1 complex at Tyr 15 and Thr 14 in order to keep cyclin-B1:Cdc2 in an inactive state [66, 67]. 14-3-3σ also sequesters Cdc2 in the cytoplasm to prevent the induction of mitosis [66], arresting cycling cells at the G2/M transitional checkpoint in response to active p53.

One study, exposing colorectal cancer (CRC) cells to various DNA damaging agents in combination with gene expression profiles, found that 14-3-3σ expression increases dramatically following gamma-irradiation; moreover, exogenous introduction of 14-3-3σ into proliferating cells produced G2 arrest [65]. Also, 14-3-3σ appears to interact directly with endogenous p53 and to enhance its activity via a positive feedback-loop upon DNA damage [68]. This interaction leads to stabilization of p53 by blocking MDM2-mediated ubiquitylation of p53. It appears that 14-3-3σ overexpression enhances MDM2 turnover and sequesters MDM2 in the cytoplasm blocking MDM2-mediated p53 nuclear export [68]. On the other hand, 14-3-3σ contains a Leucine rich Nuclear Export Sequence between residues 202 and 212 that might play a role in the nuclear export of MDM2 interfering with its export, E3 ligase ubiquitylation and 26S proteosomal degradation of p53 [68].

14-3-3σ shares many highly conserved regions with its family members while retaining some that differ from the other 14-3-3 isoforms. These distinct regions are apparently responsible for its unique interactions with specific
effector proteins within the cell. For example, although all 14-3-3 proteins bind to common pSer/pThr containing peptide motifs corresponding to Mode-1 (RSXpSXP) or Mode-2 (RXXXpSXP) sequences [69, 70], it is not the critical determinant in providing 14-3-3σ its unique biological properties. Instead, 14-3-3σ appears to contain a second ligand binding site allowing 14-3-3σ the ability to bind unique ligands. An x-ray crystal structure along with an alignment of all known 14-3-3 isoforms revealed a unique 3 amino acid substitution in the αH-αI linker region of 14-3-3σ that apparently plays a role in this second ligand-binding region [71]. Finally, 14-3-3σ is the only isoform that functions as a homo-, but not hetero-, dimer in the 14-3-3 family.

**GADD45**

The GADD45 family consists of GADD45a (GADD45), GADD45b (MyD118), GADD45g (CR6) [72], which are ~18Kda, highly acidic, and evolutionary conserved proteins with 55-57% overall residue identity. They respond to various physiological and environmental stressors [73]. For example, GADD45b, induced by terminal differentiation, shares 57% identity with GADD45a, which is encoded by a target gene of p53 [74, 75]. One study showed p53 transcriptional induction of *gadd45a* by immunoprecipitating both mutant and WT p53 proteins, the third intron RE fragment co-precipitated in the presence of WT, but not mutant p53 [76], thus validating the involvement of p53 in the transcriptional activation of *gadd45a*.

Previous studies have found that GADD45a plays a crucial role in cell cycle arrest at the G2/M boundary in response to certain genotoxic agents [66, 73]. Microinjection of GADD45a into primary human fibroblasts resulted in arresting
them with partially condensed chromatin, an intact nuclear membrane, and
centrosome segregation in 15% of the cells which tested positive for mitosis
specific antigens [66, 77]. This arrest does not occur in Li-Fraumeni fibroblasts
which are p53-null, linking p53 to the G2/M arrest. Furthermore, murine
lymphocytes exposed to UV had a 75% reduction in mitosis, but no effect on
p53−/− lymphocytes. Microinjection of cyclin B1 and cdc25c rescued the GADD45a
induced G2/M arrest, indicating that GADD45a acts via inhibition of these
molecules [66, 77]. Indeed, in vitro studies revealed that GADD45a directly
inhibits the cdc2-B1 complex by physical interaction with cdc2 via its central
domain [66, 78]. In line with these studies, another in vivo study found that
GADD45a deficient mice display genomic instability as depicted by anuploidy,
chromosome aberrations, gene amplifications (as evidenced by double minutes),
and centosome disturbances (example, fusions probably resulting from the
anuploidy). These phenotypic results usually accompany abnormalities in
mitosis, cytokinesis, and growth regulation [66, 79], many of which also result
from p53-null experiments [79]. Given that GADD45a interacts with cdc2, PCNA,
p21, MTK1, and core histones [80, 81], GADD45a may act as a monitor for
genomic instability, scanning for perturbations in G2-M cell cycle progression [73,
79] and/or DNA repair from genotoxic agents such as UV exposure.

As mentioned previously, GADD45a binds to PCNA in vitro [79, 81] and
can stimulate excision repair in vitro [80]. According to another study using
Tp53−/−, gadd45−/−, and p21−/− MEFs, GADD45a is also involved in global genomic
repair (GGR), a subpathway of nucleotide excision repair (NER) [81] responsible
for the repair of incorrectly incorporated nucleotides into the genome during DNA
replication. A strong evidence linking GADD45a to NER is that gadd45a<sup>−/−</sup> MEFs displayed deficient NER ability, as specifically 50% of 6-4pps [Pyrimidine-(6-4)-Pyrimidone photoproduct(s)] lesions were removed from genomic DNA in WT MEFs while only 15% were repaired in gadd45a<sup>+/−</sup> cells [81]. Also, GADD45a protects against other UV-induced skin lesions and promotes apoptosis and stress signaling via the p38/JNK/MAPK pathway resulting in inadequate p53 activation and the loss of G1 and G2 checkpoints [82] leading to a lack of growth inhibition.

In summary, GADD45a as one of the p53 downstream effectors plays an important role in regulating DNA repair, cell growth and death [83].

<sup>rrm2b</sup> & <sup>p53R2</sup>

<sup>rrm2b</sup> encodes a 351 amino acid polypeptide [54] that shares 83.5% identity with R2 [84] and is also transcriptionally activated by p53 with the p53 response element located in the 1<sup>st</sup> intron of the gene sequence. The resulting protein from <sup>rrm2b</sup> is the small subunit of p53 controlled ribonucleotide reductase or p53R2. The heterotetrameric enzyme, ribonucleotide reductase, consists of one homodimeric R1 subunit and one homodimeric R2 subunit and is responsible for the <em>de novo</em> conversion of ribonucleoside 5′-diphosphates into deoxyribonucleoside 5′-diphosphates. This conversion is an essential step in the synthesis of DNA during the S phase of the cell cycle [84, 85], p53R2 is an R2 homolog supplying dNTPs for DNA repair [86].

Previous studies discovered p53R2 expression upon gamma/UV irradiation, oxidative stress, or adriamycin exposure [54, 84]. Using knockout mice, it was found that <sup>rrm2b</sup> plays a role in DNA repair. <sup>rrm2b</sup> null and
heterozygous MEF cells were immortalized much easier than wild type MEFs. Also, \textit{rrm2b}−/− MEFs, upon H$_2$O$_2$ exposure, succumbed to apoptosis at a significantly higher rate than WT MEFs, due to severe attenuation of the dNTP pool [54, 87]. Given that dNTP pools in cells are regulated via allosteric controls through ribonucleotide reductase specificity, unbalanced dNTP pools can result in misincorporation of dNTPs into DNA, resulting in potential errors in the genetic code. Finally, the lack of p53R2 in kidney cells after oxidative stress resulted in an increase in the mutation rate and activation of p53 dependent apoptotic pathways \textit{in vivo}, causing renal failure, growth retardation, and early mortality in all mice after 14 weeks [54, 87].

The \textit{rrm2b} gene is also believed to be involved in an inheritable disease known as mitochondrial DNA Depletion Syndrome (MDS). MDS is a prevalent cause of oxidative phosphorylation disorders characterized by a reduction in mtDNA copy number [84]. Among 7 cases of extreme mtDNA depletion (1-2% residual mtDNA in muscle) from 4 unrelated families, germline mutations were identified in the form of nonsense, missense, splice-site, and in-frame deletions in the \textit{rrm2b} gene [84]. These results imply that p53R2 plays a critical role in supplying dNTPs for mtDNA synthesis.

In summary, p53R2 plays p53-dependent and p53-independent roles in DNA synthesis by maintaining the pool of dNTPs. Mutations brought on by environmental stressors such as gamma/UV irradiation or oxidative stress can result in cell cycle aberrations and activation of p53 apoptotic pathways resulting in cell death, organ failure, and early mortality. The following section will discuss p53 target genes responsible for both intrinsic and extrinsic apoptosis as well as
several mitochondrial proteins that interact with p53 directly at the mitochondrial outer membrane.

**Transcription Dependent Regulation of Apoptosis by p53**

Apoptosis is an evolutionarily conserved process by which organisms remove unwanted cells and/or damaged cells. For example, p53 dependent regulation of KILLER/DR5-mediated apoptosis is conserved from humans to mice [88]. Two major apoptotic pathways exist in mammalian cells, both of which involve p53 along with a number of various p53 target genes differentially expressed depending on the tissue and environment both within and outside of the cell. Here I will discuss the transcription dependent regulation of apoptosis by p53 in both the extrinsic (signaling via cytokines outside the cell, transduced through death receptors, and ultimately culminating in the activation of Caspase 8 or 10 [89]) and intrinsic (mitochondrial release of cytochrome c and other pro-apoptotic proteins followed by activation of Apaf-1 and Caspase 9 [90]) pathways followed by transcription independent regulation of apoptosis by p53.

p53’s role in the regulation of the extrinsic pathway began with the discovery of the fas/apo-1 receptor gene as a target gene transactivated by p53 in response to DNA damage caused by cisplatin, mitamycin, methoterate, mitoxantrane, doxorubicin, and bleomycin at therapeutic levels [91]. This gene contains p53 REs at both the promotor (-1837, -1445, -615bp from the start codon) and intronic (779bp from start codon) regions [91, 92]. The FAS/APO-1 receptor contains a cytoplasmic death domain (DD) located between amino acids 228-293, a transmembrane domain, and three extracellular Cys rich domains.
FAS/APO-1R shares 19% identity with another p53 regulated tumor necrosis factor or TNF death receptor called TRAIL-R2 (KILLER/DR5) [94] [92].

Also included within the TNF family of proteins, the TRAIL death receptors are found in close proximity to one another on human chromosome 8p21-22 and bind the ligand TRAIL or APO-L2 [93, 95, 96]. DNA damaging agents and irradiation transactivate the TRAIL (TNF-Related-Apoptosis-Inducing Ligand) death receptors R1 and R2 in a p53 dependent manner [94, 96]. TRAIL R1 and R2 both contain a cytoplasmic death domain (DD), a transmembrane region, and two extracellular Cys rich domains responsible for binding their shared ligand APO-L2 [93]. The R1 protein is 454 amino acids long while R2 is only 411 residues in length [93, 94]; nevertheless the two receptors bind similar “adaptor” proteins that also contain death domains. Upon APO-L2 binding the death receptors homotrimerize in preparation for activation via interaction with intracellular adaptor proteins [97-99]. FADD/MORT1 is an adaptor protein containing a death domain near its C-terminal between amino acids 153-215 which binds to almost identical Fas/Death Receptor domains upon APO-L1/L2 activation [98, 99]. Once endogenous FADD/MORT1 proteins (within BL60, BJAB, CEM, and JURKAT cells) are bound to the trimerized death receptors via DD-to-DD they recruit procaspase-8 via their Death Effector Domains (DED).

This process occurs within seconds of APO-L2 binding resulting in the Death Initiating Signaling Complex (DISC) [98, 100]. Procaspsase-8 transforms into its active conformation via proteolytic activation and DNA fragmentation is seen soon thereafter [100]. In the extrinsic pathway, receptor trimerization followed by the entrance of adaptor proteins and procaspase enzymes marks the
convergence of signals from the various p53 induced death receptors, resulting in a caspase enzyme proteolytic cascade and ending with apoptosis. Clearly this pathway is responsive to active p53.

Increasing evidence has also demonstrated that the intrinsic pathway is also regulated through p53's transcriptional activity. The proteins involved in the intrinsic pathway are mostly related to the BH3-only family and usually reside at or near the outer mitochondrial membrane in order to inactivate Bcl-2 family members, such as Bcl-2 and Bcl-XL, or activate BH123 or multi-domain Bcl family members, such as Bak and/or Bax [101-104]. The ultimate outcome is the activation of apoptosis in cells where the p53 dependent transactivation of BH3-only family members takes place through either direct activation or derepression from other anti-apoptotic Bcl-2 family members [102, 104].

Bax is one protein within the intrinsic pathway that p53 not only interacts with directly at the outer mitochondrial membrane, but also acts as a key regulator of transcription for this BH123 family member [101, 105]. Additional BH3-only members of the Bcl-2 family whose expression is induced by p53 are Noxa, Puma, and BID [106-108].

Noxa is a pro-apoptotic protein that interacts with anti-apoptotic Bcl-2 proteins at the outer mitochondrial membrane, resulting in the activation of caspase-9 leading to the Apaf-1/caspase-9 proteolytic cascade and apoptosis [106]. The gene encoding the BH3-only containing protein PUMA (p53 upregulated modulator of apoptosis) resides on chromosome 19. The genomic structure of puma allows the cells posttranscriptional splicing machinery to create four alternative splice variants, two of which contain the BH3 domain responsible
for protein-protein interaction at the outer mitochondrial membrane. The full-length transcript contains four exons; puma-α contains exons 2, 3, 4 and puma-β contains exons 1, 3, 4 while both contain the BH3 domain. Both variants bind to Bcl-2 in co-precipitation experiments resulting in the release of cyt c as well as caspase 9 and 3 activation in vitro. Mutating their BH3 domains abolishes Bcl-2 interaction in vitro; however, whether PUMA produces apoptosis by inhibiting anti-apoptotic proteins or activating proapoptotic proteins (i.e. Bak, Bax) in vivo still remains unclear. PUMA-(α/β) has a p53 binding site within the first intron of the puma gene and there is a rapid increase in the level of puma mRNA upon increased p53 levels, strongly suggesting p53 activates the transcription of puma. Moreover, endogenous p53 induction using chemotherapeutic drugs or p14ARF activation results in increased PUMA expression in both normal and tumor cells [107].

The next BH3-only protein involved in the intrinsic pathway and transactivated by p53, Bid, stands out among its relatives in that a post-translational modification is required for its functions at the outer mitochondrial membrane. Specifically, in response to etoposide, Bid was cleaved to its truncated form (tBid) by executioner caspases 3 and/or 7. It is the truncated C-terminal with a now exposed and myristoylated glycine residue at its new N-terminal that translocates to the mitochondrial outer membrane and interacts with other proteins of the BH123 family (presumably Bak), resulting in the release of cytochrome c, Smac/DIABLO, and Omi that converge to activate caspase 9 within the apoptosome complex. The cleavage of Bid to tBid by the executioner
caspases creates a feed forward amplification of the original signal via tBids activation of Bak [108, 109].

Pidd (p53-induced protein with a death domain) is a more recently discovered p53 inducible protein, presumably involved in apoptosis, which differs from the BH3-only members of the Bcl-2 family of proteins. This protein contains two functional domains: the N-terminal has a region with seven tandem leucine rich repeats (LRR), and the C-terminal contains a Death Domain between residues 797-877. The gene is highly expressed in mouse spleen, kidney, and lung with low levels in the muscle and brain. Pidd inhibits cell growth in a p53-like manner, resulting in the induction of apoptosis [110]. Hence, Pidd is also likely in the p53 dependent apoptotic pathway.

In conclusion, p53 regulates the expression of a number of proapoptotic genes that encode membrane, cytosolic and mitochondrial proteins important for apoptosis.

**Transcription Independent Regulation of Apoptosis by p53**

For a number of years research has shown that p53 plays a role in apoptosis via activation of gene transcription, resulting in various effects on apoptosis. However, recent studies have documented that p53 also acts directly on both pro and anti-apoptotic proteins residing at the outer mitochondrial membrane [101, 102, 111, 112].

First, Bax has been shown to permeabilize the mitochondria and trigger the apoptotic process upon direct interaction with p53 [101]. Moreover, the increase in cytosolic p53 results in the discharge of the aforementioned transcription dependent pro-apoptotic BH123 and BH3-only proteins, releasing them from their
Bcl-2/Bcl-X₇ sequestration [101]. p53 not only regulates the transcription of Bax, but also frees it from Bcl-2/X₇ sequestration at the outer mitochondrial membrane, activating the release of cytochrome c. These results suggest the p53-Bax interaction is part of a feed-forward loop resulting in the initiation of apoptosis.

Then, how is p53 transported from the nucleus to the cytoplasm and then still onto the mitochondria? Recent studies reveal that MDM2 mediated mono-ubiquitylation of p53 along its six C-terminal Lysines located near p53’s NES result in nuclear export of the partially assembled p53 proteins [113]. These Ks are usually concealed due to tetramerization of p53, but accessible to the nuclear export machinery when mono-ubiquitylated keeping p53 in monomer or dimer configurations (see section 1.2 - p53 TET domain) [44]. It was shown that high levels of MDM2 promote p53 poly-ubiquitylation while low levels of MDM2 result in the mono-ubiquitylation of p53. Thus, the ratio between the two proteins may determine whether or not the cell would undergo apoptosis [114] (see the following text for detailed description of MDM2).

Alternatively, a new E3 ubiquitin ligase called MSL2 (male-specific lethal-2) has been recently identified to transport p53 out of the nucleus independently of MDM2 interaction. This ligase contributes to establishing cytoplasmic pools of p53 in resting cells by ubiquitylating Lysines in p53, specifically K351 and K357 [115] different from those by MDM2. Moreover, stress induced increases in cytoplasmic p53 have been attributed to the stabilization of these p53 pools residing in the cytoplasm as opposed to the shuttling of nuclear p53 into the cytoplasm upon apoptotic signaling [111, 116]. Fractionation experiments
suggest that both nuclear and cytoplasmic pools are stabilized independently, allowing accumulation of mono-ubiquitylated p53 in the cytoplasm [115]. Upon cellular stress requiring programmed cell death, these pools are available to engage the pro/anti-apoptotic proteins residing at the outer mitochondrial membrane much more rapidly than the energy dependent p53 export from the nucleus prior to arriving at mitochondria [103].

Once at the outer mitochondrial membrane, p53 undergoes rapid deubiquitylation by mitochondrial HAUSP (a deubiquitylase) via a stress induced mitochondrial p53-HAUSP complex. This generates an apoptotically active non-ubiquitylated p53 [111]. Upon activation, p53 is able to interact with multiple proteins from the pro-apoptotic BH3-only and BH123 as well as the pro-survival Bcl-2 and Bcl-XL proteins [103]. For example, activated p53 interacts with the pro-apoptotic BH123 mitochondrial membrane protein Bak. The interaction of p53 with Bak produces the oligomerization of Bak and the release of cytochrome c as well as other pro-apoptotic proteins and biomolecules from the mitochondria [103]. The central region of p53 encompassing amino acids 92-318 contains the Bak binding domain as well as the DBD or DNA Binding Domain (See section 1.2, p53-DBD-aa99-298) [18, 103, 117]. Bak, as a member of the Bax family, contains three BH domains and in vitro binding analysis suggests that p53 interacts with Bak via a pocket structure formed by the BH1, BH2, and BH3 domains [103]. Bak is complexed with the anti-apoptotic mitochondrial protein Mcl1 during periods of cellular rest from stressors via its BH3 domain as determined by in vitro mapping experiments. p53 activation at the outer mitochondrial membrane results, either directly or indirectly, in the disruption of
the Bak-Mcl1 complex upon cellular stress and this hindrance in the Bak-Mcl1 complex correlates with the formation of the p53-Bak heterodimer, Bak oligomerization and the release of cytochrome c from mitochondria [103].

Additional evidence uncovers the direct interaction between p53 and anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL and this interaction results in the release of cytochrome c as well as other pro-apoptotic proteins and biomolecules [102]. As mentioned previously, the anti-apoptotic members, specifically Bcl-2 and Bcl-XL, constitutively reside at the outer mitochondrial membrane and mediate their pro-survival functions by sequestering pro-apoptotic BH123 and BH3-only members and/or preventing the release of numerous other factors involved in the apoptotic program [102, 104].

Hence, in addition to its transcription-dependent regulation of apoptosis, p53 can also trigger apoptosis via direct interaction with a plethora of mitochondrial apoptotic proteins independently of its transcriptional activity.

MDM2 (HDM2)

The *mdm2* (murine double minute 2) gene, originally identified during an investigation of amplified DNA sequences present in a tumorigenic mouse cell line designated 3T3DM, was one of the three genes (*mdm1, 2, and 3*) overexpressed greater than 50-fold due to amplification. These genes were located on extrachromosomal, acentromeric, short pieces of normal chromosomes retained in cells only if providing a growth advantage to those cells [118]. Perhaps of the utmost importance though was that sequences homologous to *mdm-1* and *2* resided in the genomes of several species, including *Homo sapiens* [119]. MDM2 is an E3 ubiquitin ligase whose main function is the
regulation of p53 steady state levels in unstressed cells by binding to and
ubiquitylating the transcription factor in a ubiquitin ligase RING domain dependent
manner [120-122]. The \textit{mdm2} gene appears to play a role in tumor formation via
amplification in approximately 1/3 of human soft tissue sarcomas retaining wt
p53, suggesting that overexpression of MDM2 may possibly inactivate p53 via
sequestration as well [123, 124]. Numerous studies have suggested a multitude
of mechanisms for these functions. Although the exact mechanisms underlying
certain MDM2 functions remain elusive at the present time, the domains that
allow the oncoprotein to perform various functions within the cell have been well
characterized [125] (Figure 1.3).

The N-terminal p53 Binding Domain of MDM2 consists of residues 26-108
and forms a deep hydrophobic pocket responsible for binding p53’s
transactivation domain (TAD), inhibiting p53-co-activator and/or co-repressor
interactions and blocking transcription of p53 target genes [126, 127]. The central
acidic domain (CAD) consisting of residues 221-274 within MDM2 is an essential
region for the ubiquitylation and degradation of p53. Multiple studies have
generated MDM2/MDMX chimeric proteins and revealed that a complete MDM2
RING finger E3 ligase domain (aa437-491) is necessary for the mono and/or
polyubiquitylation and degradation of p53. Interestingly, a chimeric MDM2/MDMX
protein containing only the CAD of MDMX (aa202-302) loses its ability to
ubiquitylate p53 even though it still self-ubiquitylated. Furthermore, when
chimeric MDMX contains only the RING finger domain of MDM2, it becomes
unstable due to self-ubiquitylation; however, it is unable to ubiquitylate p53 in
vivo until the addition of the MDM2 central acidic domain [128, 129]. These
results not only show the necessity of the CAD for the ubiquitylation of p53, but also suggest different mechanisms for MDM2 self-ubiquitylation and the MDM2-mediated ubiquitylation of p53. Just downstream of the CAD between residues 305-322, MDM2 contains a Zinc finger domain that is involved in its regulation, nuclear export and ribosomal protein inhibition as well as its ability in promoting 26S proteosomal p53 degradation [130]. Adjacent to the p53-binding domain are two nucleocytoplasmic localization signals, an NLS between aa178-192 and an NES comprising residues 197-211 providing MDM2 nucleocytoplasmic shuttling ability as determined by studies using Leptomycin B (LMB) which blocks nuclear export of certain proteins [125, 131]. Also, mutation of these signals abolishes detectible nucleocytoplasmic shuttling [132]. In addition to these domains, MDM2 has a NoLS (nucleolar localization signal) within the ring finger domain comprising residues 464 to 471 [125].

Because the *mdm2* gene is regulated at the level of transcription via a p53 responsive element located in the second intron, MDM2 serves as a negative feedback regulator of p53 via protein-protein interaction [125, 133]. Under normal physiological conditions within the cell, this loop keeps the two proteins at a low steady state level consistent with their half-lives of approximately 20-30 minutes each. As described above, MDM2 regulates the stability and activation of p53 mainly by two mechanisms: ubiquitylation-dependent 26S proteosomal degradation of p53 and binding-dependent suppression of its transcriptional activity [113, 134, 135] [13, 122, 123, 126]. Hence, to activate p53, cellular stress signaling must activate or employ cellular proteins, peptides, and/or biomolecules.
that either modify or interact with MDM2 and p53, leading to suppression of MDM2 functions.

**Regulators of the Mdm2-p53 feedback loop**

It is well established that the MDM2-p53 feedback loop is subject to regulation by multiple biomolecules and proteins in response to various signaling pathways. A detailed discussion involving all possible regulators of this negative feedback loop is beyond the scope of this dissertation. Therefore, I will discuss in some detail four well studied proteins and enzymes followed by a table (Table 1.1) summarizing a number of other enzymes and their modifications to the MDM2-p53 pathway. These regulatory proteins envelope a broad range of structures and functions from structured and unstructured (highly disordered) proteins that simply interact with their targets, such as MDMX and ARF, to enzymes, such as kinases (ATM/ATR) and deubiquitylases (HAUSP) that chemically modify their respective substrates.

**ARF**

\(p14^{\text{ARF}}\) shares the genomic sequence with \(p16^{\text{INK4a}}\), which was the first inhibitor of cyclin dependent kinases (CDKs) of the INK family of kinase inhibitors discovered to bind and inhibit the CDK4-Cyclin D complex. The \(p16^{\text{INK4a}}\) protein is encoded by the INK4a (\(cdkn2a\)) gene located on chromosome 9 in humans (4 in mice) and blocks CDK4/6 from phosphorylating the Rb protein inducing G1 cell cycle arrest [136, 137]. Soon thereafter, a second INK4 gene (\(cdkn2b\)) was found at the INK4 locus encoding the \(p15^{\text{INK4b}}\) protein also found to block passage through the G1-S restriction point via CDK inhibition. An additional exon designated \(1\beta\), located between the \(INK4a\) and \(INK4b\) genes, is responsible for
an alternatively spliced mRNA that also incorporates sequences encoded by exons 2 and 3 of \textit{INK4a}. The alternate reading frame-encoded p14\textsuperscript{ARF} protein suppresses abnormal cell proliferation by indirectly stabilizing and activating p53 upon receiving signals of oncogenic stress [138, 139]. However, whereas the standard INK4 proteins (p15\textsuperscript{INK4B}, p16\textsuperscript{INK4A}, p18\textsuperscript{INK4C}, p19\textsuperscript{INK4D}) assist in regulating the cell cycle by interfering with CDK-Cyclin complexes directly, p14\textsuperscript{ARF} responds to inappropriate proliferative cellular signals and assists in tumor suppression mediated by the exon 1\textbeta encoded N terminal domain of ARF binding to and inhibiting MDM2 thus stabilizing and activating p53 [138-140].

Investigators have proposed a number of different models in attempting to explain how ARF inhibits MDM2. For example, a recent study found a highly conserved NoLS within the first 22 residues of the ARF proteins N-terminal and that this region alone can relocalize MDM2 to the nucleolus, inhibiting its ability to degrade p53 through sequestration [141]. Another study found that certain forms of ARF inhibit MDM2 in the nucleus, but the subcellular localization depends on the environment of the cell [142]. Finally, it has also been suggested that ARF can interact with both MDM2 and p53. In this scenario, ARF binds p53-DNA complexes via the first 62 amino acids of its N-terminal domain [143].

\textbf{ATM/ATR}

The ataxia telangiectasia mutated (ATM) protein and the (ATM and Rad3-related) ATR protein kinases exhibit substrate specificity in response to different genotoxic agents. ATM responds to DNA damage in the form of IR induced double strand breaks (DSBs) by phosphorylating Ser15 on p53 [144, 145]. Meanwhile, UV induced DNA damage, such as pyrimidine dimers, activates ATR
to phosphorylate p53 at Ser 15 [146]. In fact, phosphorylation on Ser15 of p53 initiates a series of sequential post-translational modifications resulting in an activated and stabilized p53 transcription factor. Two of these sites include Y18 and S20, both of which lie in close proximity of the MDM2 binding region on p53 and most likely sterically interfere, independently of each other, with MDM2 binding and inhibition of p53 as well as bock p53s N-terminal NES (aa11-27 – see “p53 domains” section) sequestering p53 in the nucleus [147]. The covalent modification of p53 can also occur via downstream effectors of ATM/ATR. For example, oxidative damage by IR irradiation induces both ATM and ATR to phosphorylate the checkpoint kinases, ChK 1 (S345) and ChK 2 (T68) as well as p53 (S15) [146]. This phosphorylation activates the checkpoint kinases (which both phosphorylate similar locations on p53, MDM2, and other proteins) to phosphorylate S20 on tetrameric p53 [41]. An example of MDM2 phosphorylation occurs at residues S395 by ATM and T394 by c-Abl (another downstream effector of ATM). Each covalent modification is thought to act independently and effect MDM2’s E3 ligase and 26S proteosomal degradation of p53 along with MDM2 mediated p53 nuclear export [147]. The ATM/ATR activation and subsequent phosphorylations of certain specific substrates following different stress signals is a nice example of a kinase cascade protecting the genome from genotoxic agents.

**MDMX**

MDMX appears to have a paradoxical relationship with its homolog MDM2 that depends on the stress signal(s) involved in its activation. Specifically, under normal conditions MDMX can bind MDM2 via RING domain interaction forming a
heterodimer that allows MDM2 to shuttle MDMX into the nucleus and synergistically act to extend the half-life of MDM2 resulting in p53 degradation via MDM2s E3 ligase activity [148]. Moreover, studies have shown that MDMX knockout mice die in utero despite having endogenous MDM2 suggesting that both are necessary for normal cellular functioning [149]. However, under certain circumstances, MDMX acts as a stimulator of MDM2s E3 ligase ability for not only p53, but also MDM2 itself [150]. The homologous regions between MDM2 and MDMX are in their N and C-terminals and although MDMX does not have E3 ligase capabilities, the homology with MDM2 allows it to interact independently with many of the same substrates as MDM2, but not with others [151]. For example, during periods of ribosomal stress, RPL11 as well as other ribosomal proteins will not bind to MDMX even when MDMX is overexpressed, allowing MDMX to form complexes with p53 monomers independently of MDM2, sequestering p53, blocking tetramer formation and target gene transactivation [152]. However, during periods of DNA damage, the ATM/ATR- ChK1/ChK2 kinase cascade post translationally modifies MDMX allowing it to enhance p53 activity. After UV exposure, ChK1 phosphorylates MDMX at Ser367 enhancing 14-3-3γ binding, sequestering MDMX in the cytoplasm, and thus stabilizing p53 due to its subcellular localization [62].

**HAUSP**

The deubiquitylase HAUSP (herpesvirus-associated ubiquitin specific protease), first identified via MS of affinity purified p53 associated factors, strongly stabilizes p53 through deubiquitylation both *in vitro* and in vivo [153]. However, HAUSP also deubiquitylates and stabilizes MDM2 in a p53
independent manner. HAUSP and the death domain-associated protein (DAXX) mediating the stabilizing effect of HAUSP on MDM2 results in the destabilization of p53 under certain circumstances due to inhibition of MDM2 self-ubiquitylation and subsequent 26S proteosomal degradation [154, 155]. On the other hand, the tumor suppressor protein RASSF1A regulates restriction point progression (G1-S) in a p53 dependent manner via promoting MDM2 self-ubiquitylation and preventing p53 degradation by disrupting the interaction between MDM2, DAXX, and HAUSP [156].
In addition to the aforementioned regulators of the p53-MDM2 feedback loop, there are a number of other molecules that have been reported, though not detailed here, to regulate this loop, as summarized in Table 1.1 below.

**Table 1.1 – Regulators of the MDM2-p53 Feedback Loop**

<table>
<thead>
<tr>
<th>Ref #</th>
<th>Protein</th>
<th>Function</th>
<th>Target</th>
<th>Effect on Mdm2-p53 Negative Feedback Loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>[157, 158]</td>
<td>AMPK</td>
<td>Phosphorylation</td>
<td>p53</td>
<td>Induces apoptosis in energetic stress (AMP/ATP is high)</td>
</tr>
<tr>
<td>[159]</td>
<td>HIPK2</td>
<td>Phosphorylation</td>
<td>p53</td>
<td>Phosphorylates S46 upon DNA damage mediating apoptosis</td>
</tr>
<tr>
<td>[160]</td>
<td>Pirh2</td>
<td>Ubiquitylation</td>
<td>p53</td>
<td>Possibly another neg feedback loop since it has p53 RE, Ubiquitylates p53</td>
</tr>
<tr>
<td>[161]</td>
<td>COP1</td>
<td>Ubiquitylation</td>
<td>p53</td>
<td>Negative regulation of p53</td>
</tr>
<tr>
<td>[162]</td>
<td>ARF-BP</td>
<td>Ubiquitylation</td>
<td>ARF</td>
<td>Negative mediator of ARF tumor suppressor</td>
</tr>
<tr>
<td>[163]</td>
<td>YY1</td>
<td>Trans Factor</td>
<td>p53</td>
<td>Strengthens interaction between p53 and Mdm2 promoting Ub of p53</td>
</tr>
<tr>
<td>[164]</td>
<td>CK1</td>
<td>Phosphorylation</td>
<td>Mdm2</td>
<td>Phos-Mdm2 in the central acidic domain under normal conditions</td>
</tr>
<tr>
<td>[165]</td>
<td>Wip1</td>
<td>De-Phos</td>
<td>Mdm2</td>
<td>Activates Mdm2</td>
</tr>
<tr>
<td>[166, 167]</td>
<td>CBP/p300</td>
<td>Acetylation</td>
<td>p53</td>
<td>Ac of k120, K164; C-terminal K Acetylation = promotor specific binding</td>
</tr>
<tr>
<td>[168]</td>
<td>CBP/p300</td>
<td>Acetylation</td>
<td>Mdm2</td>
<td>Inhibits Mdm2 degradation</td>
</tr>
<tr>
<td>[169, 170]</td>
<td>PCAF</td>
<td>Acetylation</td>
<td>p53</td>
<td>Acetylation at residue K320</td>
</tr>
<tr>
<td>[171-173]</td>
<td>Tip60</td>
<td>Acetylation</td>
<td>p53</td>
<td>Assists in decision between cell cycle arrest and apoptosis</td>
</tr>
<tr>
<td>[174]</td>
<td>hSIR2</td>
<td>De-Acetylation</td>
<td>p53</td>
<td>Affects cell growth and apoptosis</td>
</tr>
<tr>
<td>[175]</td>
<td>Set7/9</td>
<td>Methylation</td>
<td>p53</td>
<td>Methylates c-term Ks (K372) leads to increase in p21</td>
</tr>
<tr>
<td>[176]</td>
<td>Smyd2</td>
<td>Methylation</td>
<td>p53</td>
<td>Methylation K370 and represses p53 activity</td>
</tr>
<tr>
<td>[177]</td>
<td>Set 8</td>
<td>Methylation</td>
<td>p53</td>
<td>Methylates K382 and represses p53 activity</td>
</tr>
<tr>
<td>[178]</td>
<td>SUMO-1</td>
<td>Sumoylation</td>
<td>p53</td>
<td>Nuclear export of p53</td>
</tr>
</tbody>
</table>
Nucleolar Stress and the Stabilization and Activation of p53

As discussed above, there are various ways to stabilize and activate p53. These involve circumventing the interaction between MDM2 and p53, whether through direct disruption of the MDM2-p53 interaction or interruption of MDM2 mediated p53 ubiquitylation, subsequent nuclear export, and 26S proteosomal degradation [181]. Both of these involve post transcriptional and/or translational modifications to either one or both proteins in many cases. Also certain stressors, such as oncogenic stress, produce their effects by derepression of p53 via direct protein-protein interaction with MDM2 and/or MDMX. Lately, another type of stress called nucleolar or ribosomal stress has been found to activate p53 and induce cell growth arrest [182]. In this case, upon ribosomal stress excess ribosomal proteins are released to the nucleoplasm where they interact with MDM2 via its central acidic domain, preventing its ability to ubiquitylate and degrade p53, consequently stabilizing and activating p53 [12, 183, 184].

Nucleolar stress may result from various reagents or mutations in nucleolar proteins and/or ribosomal RNAs resulting in the perturbation ribosomal biogenesis in the nucleolus. In general, these stress factors can be broken down into two fundamental categories, maintaining balance among ribosomal constituents or defects in ribosomal proteins and/or their processing factors [185]. Accumulating evidence suggests that the balance among constituents making up the ribosome and stresses that accompany disturbances in nucleolar homeostasis plays a critical role in p53 activation and cell growth control. For example, small doses of the chemotherapeutic agent actinomycin-D (5.0nM) result in the stalling of RNA Polymerase I perturbing ribosomal biogenesis by
decreasing the levels of available rRNAs, which reduces the rate of ribosomal assembly and increases ribosome-free forms of ribosomal proteins, consequently resulting in increased interaction between MDM2 and ribosomal protein L11 (RPL11) [183]. Other stressful conditions, such as serum starvation, also result in RPL11-MDM2 binding and suppressing MDM2 inhibition of p53 [186]. In addition, mycophenolic acid (MPA) inhibits inosine monophosphate dehydrogenase (IMPDH) and results in the depletion of guanine nucleotides. As a result, it disrupts nucleolar homeostasis and consequently induces activation of p53 via the inhibition of MDM2 by RPL5 and RPL11, leading to cell cycle arrest and apoptosis [184]. Another example involving the balance among ribosomal constituents involves chemotherapeutic agent 5-flurouracil, which converts into 5-flurouridine triphosphate and incorporates into rRNA molecules ultimately leading to the inhibition of rRNA processing. This triggers a ribosomal stress response resulting in the release of ribosomal RPL5, RPL11, and RPL23, which in turn activate p53 by inhibiting MDM2 activity via interaction with its central domain [187] (Figure 1.3).

The second fundamental category for nucleolar stress is defects in nucleolar proteins and/or processing factors [185]. Bop1 and nucleostemin (NS), are two nucleolar proteins with the ability to bring about nucleolar stress, when mutated, inhibited, or overexpressed in some fashion these insults effect cellular functioning. Bop1 contains WD40 repeats, is upregulated during mid-G1, and involved in rRNA processing and ribosome assembly [182, 188]. Expression of a Bop1 mutant lacking the first 231 residues from the amino-terminal results in decreased activity of Bop1 [182, 189] in a dominant negative fashion, resulting in
inhibition of the 36S precursor conversion to 32S pre-rRNA and a complete inhibition of mature 28S and 5.8S rRNA synthesis from the 32S pre-RNA, thus abolishing new 60S ribosomal subunit formation [189]. The deficiency of the 60S large ribosomal subunit results in a reversible cell cycle arrest in 3T3 fibroblasts. Specifically, CDKs 2 and 4 are downregulated and expression of CKIs p21 and p27 increase resulting in an expected lack of Rb hyperphosphorylation and the inability to traverse the G1-S restriction point. Most importantly, these studies reveal the p53-MDM2 pathway as an essential link for signaling between the cell cycle and ribosomal biogenesis, thus bringing about the possibility of irregular ribosomal biogenesis causing nucleolar stress and resulting in p53 dependent cell cycle arrest [182, 188, 189]. It is of note to mention that MDM2-p53-RPL5-5SrRNA ribonucleoprotein complexes were found during p53 dependent cell cycle arrest [190].

NS is another nucleolar protein essential for cell proliferation and early embryogenesis whose aberrant expression activates p53 and induces cell cycle arrest via inhibition of MDM2 by RPL5 and RPL11 [11]. The seeming paradox with NS is that its depletion and overexpression both inhibit cell proliferation. Earlier studies have suggested that the oncogenic signaling protein ARF is an upstream regulator of NS with the two proteins locked in an inversely proportional relationship [191]. Later investigations reveal that NS regulates p53 activity by binding to the central acidic domain of MDM2 directly upon its overexpression. However, depletion of NS results in increased interaction between MDM2 and RPL5 and RPL11 again via the MDM2 central acidic domain. Knockdown of either RPL5 or RPL11 abrogated this NS depletion-induced p53 activation and
resulting cell cycle arrest [11]. Once again, both scenarios place p53 as a cell cycle checkpoint monitoring NS levels through the impediment of MDM2, further advancing the proposal of p53 as the “guardian of the genome” [11, 191].

There are many questions on the topic of nucleolar stress and the subsequent release of RPs from the nucleolus. For example, how do the proteins cross over into the nucleoplasm from the nucleolus and from the nucleus into the cytoplasm? How do the proteins enter the nucleus or nucleolus, active (importan-α, transporten) transport or passive diffusion? Are there other methods employed in order to carry out this essential function? What are the various triggers that result in release of RPs from the nucleolus? How much difference does the stage of development and the cellular environment make when considering their release? Upon their arrival at the proposed MDM2 target, what is the exact mechanism behind the interaction of the ribosomal proteins and their target? Do all RPs bind MDM2 as a complex, interact with MDM2 individually, or is the interaction a combination of these depending on various factors within the cell? Apparently, there is still a long way to go in order to uncover all the mysteries behind the RP-MDM2-p53 pathway.

Previous studies have mapped out the ribosomal protein binding domains of MDM2 with similar but varying outcomes (see Figure1.3). For example, while several studies have found that RPs bind to the central acidic domain; at least one study has found that RPL5 and RPL11 bind the MDM2 Zinc finger domain as well as the central acidic domain [12, 130, 192]. Studies have also revealed the MDM2 binding regions of RPL11 and RPL23. The RPL11 central MDM2 binding domain is located between residues 51-108 and RPL23 binds MDM2 via its
middle domain (~aa35-105) [12, 193]. It remains to map the MDM2 binding region(s) of RPL5, as this information would be critical for further understanding the role of RPL5 in regulating the MDM2-p53 feedback loop.

In this dissertation research, I have dissected the MDM2 binding regions of RPL5 using a set of in vitro GST-fusion protein-protein association assays. As a result, I found that MDM2 appears to bind both the amino (aa1-39) and C-terminals of RPL5. I will discuss how this result compares with a number of different studies regarding interaction between RPL5 and 5SrRNA; specifically, the binding and shuttling of 5SrRNA between the cytoplasm and the nucleolus of cells and the involvement of NoLS/NES RPL5 domains in this process. Finally, I will discuss proposed models describing the interaction between RPs and MDM2, resulting in the stabilization and activation of p53 and their possible implications for future studies.
Figure 1.1 – p53 Functional Domains

Figure 1.1 – depicts the tumor suppressor p53’s functional domains. Beginning at the amino-terminus, p53 has 2 TransActivation Domains that work both together and independently of one another in the recruitment of co-repressors or activators to locations of p53-DNA interaction at p53REs. The oncogene MDM2 binds this area of p53, inhibiting the protein. Just downstream is the Proline Rich Domain responsible for initiating apoptotic signals. The DNA Binding Domain is responsible for locating and binding to DNA consensus sequences when p53 is in the homodimer/tetramer quaternary structure. “L” stands for the linker region separating the DBD and TET domain. The TET or Tetramerization Domain induces p53 monomers and/or dimers to form the active homotetramer conformation and begin the transactivation of p53 target genes. The CTD or C-Terminal Domain negatively regulates p53 by binding to nonspecific DNA sequences, sequestering p53 from binding to specific p53 REs. Of course, all of these domains are subject to post-translational modifications that modify their effectiveness and sometimes their function.

Figure 1.2 – p53 Nuclear Localization & Export Signals

Figure 1.2 – p53 contains 3 Nuclear Localization Signals (NLS) and 2 Nuclear Export Signals (NES) that allow it to traverse the boundary separating the nucleus and cytoplasm using the RAN GTPase pathway, thus expending energy in the form of GTP→GDP to enter the nucleus and GDP→GTP to enter the cytoplasm. Where the NESs work together in transporting p53 out of the nucleus, only NLS I located in the Linker region is responsible for importing p53 into the nucleus.
Figure 1.3 – A depiction of MDM2 showing all of its functional domains including the Nuclear Localization and Export Signals along with the Nucleolar Localization Signal near the RING finger domain. Directly below the MDM2 structure are the Ribosomal Proteins along with the regions of MDM2 to which they bind. Of note are the two different positions for RPL5 as this binding location has yet to be determined with complete confidence.
Section 2 - Materials and Methods

Cell Lines and Plasmids

The plasmids utilized in this study to transform BL21(DE3) cells included pGEX4T-1 for generating GST-RPL5- deletion mutant fusion proteins and pET24a containing a 3’ His tag for producing soluble MDM2 proteins.

Generation of pGEX4T-1 Expression Vector

The pGEX4T-1 vector was amplified via inoculating 4ml of LB medium plus 4 µl stock ampicillin [80mg/ml] with a small fraction of pGEX4T-1-RPL5 from glycerol stock stored in -80°C from prior experiments. The culture was placed in 37°C overnight with 250rpm agitation followed by performing a DNA mini-prep (QIAGen) on 2ml of culture to isolate purified pGEX4T-1-RPL5 expression vector. This vector was digested separately from the RPL5 deletion mutants in the following step; however, purification of the digested pGEX4T-1 vector was ran alongside the non-digested RPL5 deletion mutant insert PCR products on Gel#021408.

RPL5 Deletion Mutant Insert Generation

RPL5 wild type (wt) and deletion mutant inserts were amplified from pcDNA3-2FLAG-RPL5-WT template using primers specific for each RPL5 PCR product. The primers were engineered with 3’ EcoRI and 5’ BamHI restriction sites for later digest, purification, and directional ligation into a pGEX4T-1 expression vector. The following lists the primers used and their corresponding insert products:
1) L5-WT – Forward P1 (5'CGC GGA TCC ATG GGG TTT GTT AAA GTT G-3') and Reverse P2 (5'CCG GAA TTC TTA GCT CTC AGC AGC CCG CTC-3')
2) L5-(aa1-251) – Forward P1 and Reverse L5-A (5' CCG GAA TTC CTA TGG ATT CTC TCG TAT AGC AGC CCG CTC-3') and Reverse L5-A
3) L5-(aa39-251) Forward L5-B (5' CGC GGA TCC CAA GAT AAA AAT AAA TAC ACA CCC CCG-3') and Reverse L5-A
4) L5-(aa112-297) Forward L5-D (5' CGC GGA TCC AGG AAA GGC ATG GAC AAG ATC GAC-3') and Reverse P2
5) L5-(aa1-50) Forward P1 and Reverse L5-E (5'CCG GAA TTC CTA CCT GTA TTT GGG TGT GTT G-3')

PCR reactions for insert amplification were set up as follows:

a. Total reaction volume = 50.0µl
   i. ddH20 42.0
   ii. 10x buffer 5.0
   iii. 10mM dNTPs 1.0
   iv. Taq Pol 0.5
   v. Fwd Primer [100pmols/µl] 0.5
   vi. Rev Primer [100pmols/µl] 0.5
   vii. Template [50ng/µl] 0.5

b. Thermocycler Program
   i. 94 Deg – 3min
   ii. 94 Deg – 30sec
   iii. 60 Deg – 30sec
   iv. 72 Deg – 2min
   v. GoTo Step 2 – 29x
   vi. 72 Deg – 10min
   vii. 4 Deg – 24hrs

The PCR amplified RPL5 inserts were purified via 1% agarose gel, bands were cut out and placed into separate 1.5ml Eppendorf tubes prior to extracting them using the QIAgen gel extraction spin kit. In the case of the pGEX4T-1 vector, only the pGEX4T-1 vector was cut from Gel#021408, leaving the original RPL5 insert in order to insure accuracy that all RPL5 inserts were products of the same template (pcDNA3-2FLAG-RPL5-WT). Therefore, it was ran beside the PCR amplified RPL5 protein inserts even though it’s amplification and restriction digest was performed separate from the deletion mutant PCR. The purified
inserts and pGEX4T-1 vector were quantified using a combination of absorption and relative 1% agarose gel band intensities (Figure 2.1 - GEL#021408, Figure 2.2 - GEL#073008-3).

Restriction Digest and purification of RPL5 inserts and pGEX4T-1 vector

A double restriction digest of the now purified expression vector pGEX4T-1 and PCR amplified RPL5 inserts was performed using NEB products and following NEB protocol regarding quantities used and total reaction volume where possible. Each digest requires ~ 2-5µg insert and vector DNA.

Inserts – insert concentrations were ~120ng/µl X 21µl = 2.5µg of insert/reaction. Vector – vector concentration was ~253.2ng/µl X 20µl = 5.0µg of vector/reaction. Total Reaction volume for digests = ~30µl:

-Insert DNA 21.0µl  -vector DNA 20.0µl  
-NEB2 10x Buffer 3.0µl  -NEB2 10x buffer 3.0µl  
-BamHI 2.5µl  -BamHI 3.0µl  
-EcoRI 2.5µl  -EcoRI 3.0µl  
-BSA .3µl  -BSA .2µl

Each digest was incubated in a 37°C water bath for 45min.

Restriction digest products were purified via 1% agarose gel, bands removed and placed into separate Eppendorf tubes and then extracted via the spin gel extraction protocol (QIAgen). There are double bands on GEL#021508 due to a side effect from the restriction enzymes; therefore, the bottom bands matching the molecular weight of the inserts were removed from the gel, sequenced for accuracy, and utilized in the next step (Figure 2.3 - GEL#021508; Figure 2.4 - GEL#080408).
Ligation of RPL5 deletion mutant inserts with pGEX4T-1 vector

An example of Promega’s equation for determining the amount of insert to add for each ligation is provided for reference. In my work the molar ratio of insert to vector was between 1:1 and 3:1 depending on the concentration(s) of inserts following purification from the previous step; nevertheless, the total reaction volume never differed from 10µl suggested in the protocol. The Promega equation for calculating ng of insert per reaction is as follows:

\[
\frac{\text{ng of vector} \times \text{Kb of insert}}{\text{Kb of vector}} \times \frac{3}{1} \text{ (insert)} = \text{ng of insert}
\]

**EXAMPLE:**

Promega: 10X buffer 1µl  Control: 10X buffer 1µl
T4 Ligase 1µl  T4 Ligase 1µl
Inserts 2µl  Inserts ----
pGEX 1µl  pGEX 1µl
H2O-nuc free 5µl  H2O-nuc free 7µl
Total reaction vol = 10µl  Total reaction vol = 10µl

Ligation reactions for RPL5 (aa1-297) (aa1-251), (aa39-251), (aa112-297) were incubated overnight (~8-10hrs) at 16°C. The RPL5 (aa1-50) was incubated at room temperature for 3hrs.

Transformation of *E. coli* BL21(DE3) cells with pGEX4T-1-rpl5 recombinant plasmids

The chemical procedure was used to transform BL21(DE3) cells. The entire 10µl of ligation mix was added to a tube previously stored in -80°C containing 50µl of competent BL21(DE3) cells and mixed well followed by an incubation period of 30min on ice. The cells were then placed in a 42°C heat block for 45sec and immediately placed on ice for 1.5min. 1ml of LB (without ampicillin) was added and the newly transformed cells were incubated at 37°C
for 1hr with shaking at ~230rpm. In order to select for those cells transformed with recombinant plasmids, the culture was centrifuged and the top 800µl of LB supernatant removed to increase the cell concentration. The remaining 200µl of concentrated culture was resuspended, plated (100µl /plate) and incubated @ 37°C overnight (16hrs) on agar plates with [80µg/ml] ampicillin.

Mushui Dai, PhD provided the pET24a-mdm2-wt insert expression vector from in lab Glycerol stock.

Inoculation, induction and expression of recombinant plasmid pGEX4T-1-rpl5 mutant and pET24a-mdm2-wt transformed cells

5 colonies were picked per plate for each recombinant expression plasmid containing RPL5 wt and deletion mutant inserts and used to inoculate five 15ml vials each containing 3-5ml of LB and 1:1000 dilution of stock [80mg/ml] ampicillin. These were grown overnight (16hrs) at 37°C with 250rpm shaking. The next day 500ml flasks of 250ml LB medium + 250µl ampicillin were inoculated with a 1:100 dilution from one of the five overnight cultures. Each 250ml newly inoculated culture was incubated at 37°C with 250rpm shaking until reaching an OD₆₀₀ reading between .5 and .7 Abs (~3hrs 15min). 250µl of .5M IPTG was added and incubation continued for another 4hrs under the same conditions. Once the induced culture was centrifuged at 4°C and 5K rpm for 15min the supernatant was decanted and the pellet stored in -80°C for future purification. However, first samples were taken and restriction digests, SDS-PAGE protein expression gels and sequencing were employed to verify the accuracy of the clones (Figure 2.5 - GEL-022708-1 SDS PAGE GST-RPL5-aa1-
The procedure for BL21(DE3) *E. coli* transformed with pET24a-mdm2-wt expression vector was the same with the following exceptions.

1. 5µl of Kanamycin was added to the 5ml quantity of LB resulting in a 1:1000 dilution
2. 500ml of LB was inoculated with 500µl Kan stock [34mg/ml] and 5ml of overnight culture.
3. Resulting inoculated and incubated culture was centrifuged for 20min.

**Sequencing purified GST-rpl5 fusion and 6x-His tagged mdm2 constructs**

All GST-rpl5-deletion mutant and His-mdm2-wt constructs were sequenced to insure accuracy.

**Buffers and Reagents**

Glutathione-sepharose beads were incubated and washed with 1x Phosphate Buffered Saline (PBS) twice prior to adding a final 1:1 bead bed volume of PBS to GSH creating a 50% slurry. Cold GST lysis buffer (1x PBS, 10% Glycerol, .1% NP40, ddH₂O, pH7.4) and GST lysis buffer plus 150mM NaCl were used for all GST-RPL5-fusion protein purifications. A Tris based lysis buffer (50mM Tris, .5% NP40, 1mM EDTA, 150mM NaCl, ddH₂O pH8.0) was used to wash all GST-RPL5 fusion protein - MDM2-His association products. PMSF and DTT with final concentrations at [.2mM] and [1mM], respectively, were added to each buffer just prior to use in each experiment.

6x-His tagged MDM2 proteins were purified via affinity chromatography using cold Phosphate Buffered Saline (50mM NaH₂PO₄·H₂O, 300mM NaCl,
pH 8.0) with buffers A (flow through), B (wash), and C (elution) containing 10mM, 20mM and 250mM Imidazole, respectively. PMSF and Glycerol were added to final concentrations 0.2mM and 5%, respectively and just prior to their use in the assay. The affinity chromatography elution products were then dialyzed using cold BC100 buffer pH 8.0 (20mM Tris, 0.1mM EDTA, 10% glycerol, 100mM KCl and 4mM MgCl2) and PMSF [0.2mM], DTT [1mM] and [10mM] BME (final concentrations) were added to the BC100 buffer just prior to its use in dialysis.

**Purification of GST-RPL5 fusion proteins**

All GST-RPL5 fusion protein pellets were washed with cold 1x PBS and transferred to 50ml centrifuge tubes and centrifuged 10 min at 10K rpm and 4°C. The pellets were resuspended in cold PBS lysis buffer (described above) and then lysed, while on ice, via sonication (6x at 15sec/x with 30sec-1min cool down between sonications). After centrifuging the lysed cells 10 min at 8K rpm and 4°C the supernatant was decanted into a 15ml conical vial and 100-150µl of GSH 50% slurry was added followed by rotating each vial for 20min in 4°C to allow the GSH beads to interact with and bind the GST-RPL5 fusion proteins. The now glutathione bead bound GST-RPL5 fusion proteins were collected by centrifugation at 2K rpm and 4°C and the supernatant again decanted into a new 15ml conical vial and stored in -80°C for future use. The bead bound GST-RPL5-fusion proteins were washed 5x (3x in PBS lysis / 2x in PBS lysis-150mM NaCl) and each wash consisted of addition of ~500µl of buffer, rotation in 4°C for 5 min, centrifuging at 2K rpm for 1min in 4°C followed by suctioning off of supernatant leaving the now bead bound GST-RPL5 deletion mutant fusion proteins. Finally,
PBS lysis buffer with PMSF [.2mM] and DTT [1mM] was added at a 1:1 ratio (buffer to bead bed volume) and stored in 4°C.

**Purification of 6x-His-tagged MDM2 soluble protein**

As discussed earlier, Ni-NTA affinity chromatography purified the His-MDM2-wt proteins for use in association reactions (see buffers and reagents for specifics on Buffers A, B and C). The induced His-MDM2-wt expression pellet was resuspended in 10ml of lysis buffer A, lysed via sonication (6x at ~12 seconds/x with 30sec-1min cool down between sonications) and then centrifuged at 10K for 20min and 4°C. The resulting supernatant was decanted into a 50ml conical vial and 400µl of Ni-NTA (50% slurry) agarose beads were added followed by a 4hr rotating incubation in 4°C. After incubation, a (Polyprep) chromatography column was prepared in a 4°C cold room and the resultant mixture of lysis buffer A and Ni-NTA bound His-MDM2 was poured into the column and the flow through saved in -80°C. The His-MDM2 bound Ni beads remaining in the column were washed by adding lysis buffer B two times successively at 500µl/time. Buffer C was then added in 200µl aliquots (1:1 bead bed volume) to elute His-Mdm2 proteins, collected in separate 1.5ml Eppendorf tubes and stored in -80°C. SDS-PAGE (1.5mm, 10% acrylamide) was employed to confirm and quantify the presence of MDM2 protein (Figure 2.9 - GEL-091008-1 CS, GEL-091008-2 WB).

Dialysis was performed on elution His-MDM2 volumes using BC100 buffer described earlier in the buffers and reagents section. An 8kDa filter tube was prepared by cutting it to ~3inches in length, rinsed with milli-Q ddH₂O, and clipped off at one end using a small dialysis clip prior to adding all 200µl of each
elution (E1-E5) separately from the affinity purification described above. A second clip was used to seal off the other end of the dialysis bag leaving a small air pocket. The dialysis bag was then submerged in 500ml of cold BC100 in 4°C and incubated 2hrs with stirring via a magnetic stir bar and plate. Upon removal of dialysis bag, the 200µl of His-Mdm2 solution was removed from the bag via tipped pipette and divided into 50µl aliquots prior to storing in -80°C for future use.

Quantifying purified proteins

Samples were purified via running 1.5mm, 10% SDS-PAGE and measuring band intensities against those of known quantity BSA samples. Samples were prepared by using defined volumes and adding 4X SDS loading buffer plus 1x PBS to bring total volume to approximately 24µl. Samples were vortexed, centrifuged 1min at 2K rpm, and boiled in a heating block at 97°C for 8min. This procedure was repeated one more time prior to loading samples into SDS Gel wells along with 5µl of 11kDa – 170kDa reference ladder (Fisher Scientific) in the far left lane. Gels were run in a Bio-Rad electrophoresis apparatus at 150V for 20min followed by 160V for 45min. The gel(s) were soaked in .1% Coomassie Blue stain immediately after completing electrophoresis (4-8hrs) and then incubated in destain buffer (60% H₂O, 30% MetOH, 10% glacial acetic acid) overnight with shaking (Figure 2.8 - GEL-081908-1 SDS PAGE GST-RPL5-aa1-50 - Quantitative).

The corresponding band intensities for each GST-RPL5-fusion protein were calculated against a linear plot of BSA VS Band Intensity. Each gel was
scanned using an HP-photoscanner, cropped and converted into 8 bit black and white format. Adobe Photoshop 3 (CS3) was used to measure band intensities by first correcting the gel background, setting it to an input level equivalent to that of the darkest spot on the gel. The BSA bands were outlined and intensities recorded using the formula (Mean X Count = Intensity) and were graphed along the Y-axis against the known quantities of BSA along the X-axis. A trendline was created using a scatter plot and the R^2 values analyzed to insure accuracy. Once sample band intensities were found using the procedure previously described, their quantities were calculated in µg using the linear equation y(I)=aX(µg) and solving for X: X(µg)= y(I)/a; where (a) equals the slope of the Intensity VS µg BSA trendline. Dividing the resulting number by the volume of sample per well gave the concentration for each sample in µg/µl.

Figure 2.10 – 2009GEL-0126 (RPL5 quantification derived from Band Intensity VS µg of BSA)

**In vitro GST-RPL5 fusion protein-His-MDM2 association assays**

Each reaction tube contained 200ng of His-MDM2-wt and 4µg of GST-RPL5 deletion mutant or wt fusion proteins suspended in PBS lysis buffer (see buffers and reagents for detail) for a total reaction volume of 40µl. Each vial was incubated at room temperature for 55min while mixing (Vortex Genie 2) on setting 3. Upon the completion of the association reaction, the proteins were centrifuged for 30sec at room temperature and 5K rpm. 20µl of supernatant was withdrawn from the GST-0 reaction vial (negative control) after this step to be used as input (50%) during the SDS-PAGE step of the experiment. From this point forward the reaction vials were kept on ice between washing steps, each of
which was carried out at 4°C. The first wash differed from the remaining three in
that only 150µl of Tris GST lysis buffer was added and tube was inverted 4-5x,
centrifuged 1min at 3K rpm and the supernatant suctioned of leaving 25-30µl of
Mdm2 bound GST-RPL5 fusion proteins. The other three washes consisted of
adding 500µl of Tris GST lysis buffer followed by rotation for 5min and
centrifugation at 5K rpm for 1min. Again, the supernatant was suctioned off
leaving 25-30µl in the bottom of each reaction tube.

The washed samples were prepared for SDS-PAGE using the exact same
procedure described under the protein purification section and the entire sample
loaded into each 1.5mm, 10% SDS polyacrylamide gel well. 5µl of 11kDa –
170kDa reference ladder (Fisher Scientific) was loaded into the far left lane well.
Gels were run at 150V for 20min and 160V between 32-40min.
Upon completion of electrophoresis, each gel was cut at the 72kDa marker and
the portion of the gel <72kDa was incubated in Coomassie Blue stain with
shaking at room temperature followed by submersion in destaining buffer (see
above) for approximately 12-16hrs prior to scanning and measuring band
intensities for the GST-RPL5 fusion proteins. Standard gel-to-membrane transfer
followed by immunoblotting was performed on the portion of the gel >72kDa. The
1° antibody (MC 2A10 α-Mdm2) in 1x TTBS (1:50 dilution) was incubated with
the nitrocellulose membrane for 1hr after which the membrane was washed in 1x
TTBS three times (10min/time) before adding the secondary antibody (GAM) in
1x TTBS (1:10K dilution) for 25min followed by washing in 1xTTBS as described
above.
Chemiluminescence was measured using a detector (Ultra-Lum) and ECL Supersignal West Pico Substrate (Thermo Scientific) with two-minute exposure times. Relative binding intensities were calculated for each reaction by dividing the WB band intensity by the CS band intensity using GST-0 as the negative control and GST-RPL5-wt as the positive control. Within each relative binding assay, the positive control was normalized to one and the relative binding intensities along with standard deviations calculated.
Figure 2.1 – Amplified RPL5 Inserts & pGEX4T-1-RPL5 Restriction Digest

Figure 2.2 – PCR Amplified RPL5-aa1-50 Insert
Figure 2.3 – Restriction Digest of RPL5 Deletion Mutant Inserts

<table>
<thead>
<tr>
<th>NEB 1K</th>
<th>RPL5 1-251</th>
<th>RPL5 39-297</th>
<th>RPL5 39-251</th>
<th>RPL5 112-297</th>
<th>RPL5 1-100</th>
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<tbody>
<tr>
<td>.5-10Kb</td>
<td>753bp</td>
<td>774bp</td>
<td>636bp</td>
<td>555bp</td>
<td>300bp</td>
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GEL-021508

Figure 2.4 – Restriction Digest Quantitative Gel of RPL5-aa1-50

Restriction Digest of RPL5-aa1-50 – Quantitative Gel

<table>
<thead>
<tr>
<th>NEB 1K</th>
<th>.5-10K</th>
<th>2.0ul</th>
</tr>
</thead>
</table>

150bp RPL5-aa1-50

GEL-080408
**Figure 2.5 – GEL-022708-1 SDS PAGE GST-RPL5-aa1-251**

SDS PAGE – GST-RPL5-aa1-251

75kDa

50kDa

37kDa

GST-RPL5-aa1-251 M + +

**Figure 2.6 – GEL-022708-1 SDS PAGE GST-RPL5-aa39-251**

SDS PAGE – GST-RPL5-aa39-251

75kDa

50kDa

37kDa

GST-RPL5-aa39-251 M + +
Figure 2.7 – GEL-022708-2 SDS PAGE GST-RPL5-aa112-297

SDS GEL PAGE GST-RPL5-aa112-297

50kDa

37kDa

GST-RPL5-aa112-297 - + - +

Figure 2.8 – GEL-081908-1 Quantitative for GST-RPL5-aa1-50

GEL-081908-1 Quantitative for GST-RPL5-aa1-50

CS

Marker
GST-L5-(1-50)
BSA [.5ug/ul]

+ 10 15 .5 1 2 6 10
Figure 2.9 – GEL-091008-1 CS & GEL-091008-2 WB

2A10-αMDM2
Blot from lanes 2-4 of the same gel as below

WB

M E1 E2 E3 E4 NA .5 2 4

MDM2

Lanes 2-4 MDM2 Elutions from Ni Affinity Assay
Lanes 6-8 contain BSA samples in ug/lane
Marker is Fisher Scientific 11kDa – 170kDa
Figure 2.10 - RPL5 Quantification Based on Band Intensity VS µg of BSA

### 2009GEL-0126 - Quantification of 1-25-09 Purifications of L5-WT, L5(39-259), L5(112-297) - all original purifications

<table>
<thead>
<tr>
<th>µg BSA</th>
<th>Intensity</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>6.8</td>
</tr>
<tr>
<td>1</td>
<td>15.1</td>
</tr>
<tr>
<td>2</td>
<td>28.9</td>
</tr>
<tr>
<td>3</td>
<td>40.4</td>
</tr>
<tr>
<td>4</td>
<td>58.4</td>
</tr>
</tbody>
</table>

Intensity \( \frac{I}{14.251} = \text{µg} \)

<table>
<thead>
<tr>
<th>Intensity</th>
<th>µg/µl</th>
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<tbody>
<tr>
<td>12.7</td>
<td>0.111</td>
</tr>
<tr>
<td>121.3</td>
<td>1.064</td>
</tr>
<tr>
<td>122.7</td>
<td>1.076</td>
</tr>
</tbody>
</table>

1-25-09 Orig. Purifications

- L5-WT(1-297) (1/25/09) 8µl 0.111
- L5(39-251) (1/25/09) 8µl 1.064
- L5(112-297) (1/25/09) 8µl 1.076
Section 3 - Results

**In Vitro GST-Fusion Protein-Protein Association Assays**

As mentioned previously, the main objective of this dissertation is to characterize the Mdm2 binding domains of RPL5. However, in order to first determine that Glutathione-S-Transferase protein expressed without an RPL5 insert (GST-0) does not bind to the soluble protein MDM2, I performed a set GST-fusion protein-protein assays titrating increasing amounts of GST-0, GSH, GST-RPL5 and holding His-MDM2 constant. The reading for GST-0 was not visible until 500ng and did not show any signs of binding MDM2 while the GST-RPL5 showed intense association with MDM2 (Figure 3.1). Once I confirmed non-binding, the decision was made to use the pGEX4T-1 vector as the negative control and GST-RPL5-wt for the positive control, which was normalized to 1.00 when calculating the final relative binding intensities between the two proteins. GST-0 had a relative binding intensity of only 2% and displayed binding in only 3 out of 12 protein-protein association assays (Figures 3.3 and 3.4).

**In Vitro Mapping of GST-RPL5 Fusion Protein - 6x tagged MDM2 Associations**

In order to determine the RPL5 domains responsible for binding MDM2, I engineered (glutathione-s-transferase) GST-RPL5-fusion protein deletion mutants (Figure 3.2). These were used in 12 sets of GST-fusion protein-protein association assays conducted using GST-RPL5-deletion mutant fusion proteins and His-MDM2 purified from transformed BL21(DE3) bacterial cultures. The relative binding intensities between MDM2 and each of the RPL5 deletion mutants were calculated upon completion of the reactions. Each sample was run on a 10% SDS gel followed by Coomassie Blue staining to identify GST-RPL5
fusion proteins (Figure 3.3) and immunoblotting with mono-clonal α-MDM2 (2A10) to identify MDM2-RPL5 deletion mutant association (Figure 3.4).

Prior research has shown RPL5 to bind to MDM2’s central acidic domain [194]. However, RPL5’s exact mechanism of interaction with MDM2 is left to further investigation (Figure 3.5) [130]. My research found that the hydrophobic middle region (39-251) which includes the Nuclear Export Sequence (NES) rich in leucine and spanning amino acids 101-111 displayed little affinity for MDM2 with a binding intensity of 12% when compared to the GST-RPL5-WT protein. Meanwhile, the N-terminal of GST-RPL5 consistently showed a relative binding intensity equivalent to 150% to that of the full-length RPL5 for the region containing aa1-251 and 71% for the aa1-50 region. Combined, these findings provide a picture of GST-RPL5-MDM2-His interaction involving the 1st 39 amino acids of RPL5 including the extremely basic (47%) 17 amino acid Nucleolar/Nuclear Localization Sequence (NoLS) from amino acids 21 to 37. The C-terminal of GST-RPL5 displayed binding to His-MDM2 as depicted by the mutant containing aa112-297. This region contains the C-terminal 11 amino acid NoLS that is 72% basic with mostly lysines providing its basic characteristics. Previous research has found this NLS to mediate nuclear import as well as nucleolar accumulation, but with a much slower translocation kinetic when compared with a classical NLS [195]. This research found these findings reflected in the C-terminal’s relative binding intensity to MDM2-His, which was only 43% compared to the GST-RPL5 positive control.

Taken together, these findings appear to depict the MDM2 binding regions to be at both the N and C-terminals of RPL5 within the amino acid sequences 1-
39 and 252-297 with the N-terminal of RPL5 having a higher relative binding intensity than the C-terminal (Figure 3.6 – RBI bar chart).
Figure 3.1 – MDM2 Titration against GSH, GST-0, & GST-RPL5-WT

Figure 3.2 – GST-RPL5 Deletion Mutant Binding to MDM2
Figure 3.3 – Coomassie Stain of GST-RPL5 Fusion Proteins

Figure 3.4 – Western of MDM2 with GST-RPL5 Deletion Mutants
Figure 3.5 - MDM2 Functional Domains with Proposed RPL5 Regions

Figure 3.6 - RBI Summary Chart RPL5-WT Normalized to 1.00
Section 4 - Discussion

Previous research has found that the 297 amino acid RPL5 contains nuclear import and nucleolar localization signals mapping to amino acids 21-37 (NLS/NoLS-1) at the N-terminal and C-terminal residues 255-265 (NLS/NoLS-2). RPL5 also contains a leucine rich nuclear export signal residing within its central region between residues 101-111 that displays efficient nuclear export, especially upon removal of the NoLS ends from the protein [195]. Moreover, RPL5s 5SrRNA binding sites overlap with the basic localization signals. Indeed, entering the nucleolus without bound RNA appears to pose greater difficulty for RPL5 than when RPL5 attempts to enter alone [195]. Given this information, I engineered GST-RPL5 deletion mutants based around RPL5’s localization and export signals (Figure 3.2) and performed a series of GST-fusion protein-protein association assays. My study found that MDM2’s binding regions overlap with the 5SrRNA region and both NLS domains at RPL5s N and C-terminals, specifically residues 1-39 and 252-297 (Figure 4.1).

One of the first studies regarding interaction between RPL5 and MDM2 incorporated a method similar to the one described below in that it was based on the retention of 5SrRNA; however, it also included 5.8SrRNA and p53 in the formation of a ternary complex [190]. Several other studies have found that RPL11, when overexpressed, is also necessary to stabilize and activate p53 via the inhibition of MDM2, although the specifics of each model differ in their scope and implementation [183, 186, 192, 193].

A recent article in Cancer Cell identified hnRNP K as a co-activator of p53 dependent p21 transcriptional activation. The article discusses hnRNP K in the
context of DNA damage and nutlin3a, MDM2 undergoes inhibition and degradation upon the addition of nutlin3a, which blocks MDM2’s binding and subsequent degradation of p53 leaving it open to interact with hnRNP K and transactivate p21 induced growth arrest as opposed to apoptosis [196].

Based upon these findings, I propose an alternate hypothesis connecting hnRNP K with nucleolar stress. In this scenario, upon ribosomal perturbation RPL5 along with other ribosomal proteins (RPL11 and RPL23) exit the nucleolus, bind to, and sequester hnRNP K. Given RPL5’s proclivity for binding RNA molecules (5SrRNA) via its extremely basic N and C-terminals, nucleolar RPL5 seems an excellent candidate for interacting with the RNA associated with hnRNP K. This interaction blocks p53 from transcribing p21 and prevents the cell from entering growth arrest. At the same time RPL11, sensing a disturbance in the nucleus, binds free MDM2 in the nucleus while reserve pools of RPL5-5SrRNA RNPs currently residing in the cytoplasm [197] enter the nucleus via RPL5’s NLSs and bind MDM2, using RPL5’s basic N and/or C-terminals to form a complete complex with RPL11 via a 5SrRNA bridge [198]. Blocking p53 transactivation of p21 via sequestering hnRNP K frees p53 to transactivate apoptotic genes instead, resulting in cell death as opposed to p21 induced cell cycle arrest.

In another event similar to the one just discussed above, excess nuclear MDM2 moves out into the cytoplasm using its NES where the protein interacts with cytoplasmic RPL5-5SrRNA ribonucleoprotein particles. Again, the NLSs on RPL5 direct the MDM2-RPL5-5SrRNA heterotrimer into the nucleus where excess RPL11 binds the complex inhibiting MDM2 and resulting in p53 stabilization and activation.
Both scenarios are dynamic, allowing the cell to decide rapidly if p53 should begin transactivation of apoptotic genes and thus induce apoptosis or continue the cell cycle arrest based on not only the availability of hnRNP K but also derepression from MDM2.

Yet another possible RPL5-MDM2 interaction again involves the basic terminals of RPL5, only this time RPL5 is overexpressed and one RPL5 terminal binds to the central acidic domain of MDM2 while the other binds the MDM2 Zinc finger domain and possibly a portion of the small acidic domain just downstream from the Zinc finger. This scenario would explain why cancer-associated mutations in MDM2’s Zinc finger domain appear to disrupt RPL5-MDM2 interactions and increase MDM2-induced p53 degradation [130]. However, a previous study by HF Horn and KH Vousden in 2008 consistently depicted physiological levels of RPL5 alone as insufficient to effectively inhibit MDM2 and stabilize p53 in comparison to p14ARF. Nevertheless, overexpression of RPL5 in the cell does come very close to p14ARF induced p53 stabilization. When RPL5 is co-expressed with RPL11, the two ribosomal proteins act in a synergistic fashion to stabilize and activate p53 via inhibition of MDM2 at intensities in the vicinity of those resulting from p14ARF [198].

Taken together and in relation to the findings of RPL5’s MDM2 binding regions, it appears that the stronger N-terminal of RPL5 interacts with MDM2 when other ribosomal proteins are present in the nucleus. For example, when forming a quadruple complex with RPL11 and RPL23 resulting in MDM2 inhibition and p53 stabilization and transactivation [12, 194]. However, there exists the possibility that RPL5 overexpression results in the use of both RPL5’s
N and weaker C-terminals to bind MDM2’s acidic domain(s), resulting in the stabilization of p53.

So far, this dissertation has discussed protein-protein and/or protein-RNA interactions that fall under noncompetitive or uncompetitive binding to RPL5, but what about competitive binding? That is to say, of 5SrRNA and MDM2 competing for the same regions of RPL5 and the effects this scenario would have on ribosomal assembly.

The free RPL5s may possibly, due to leaving the nucleolus because of nucleolar stress and upon reaching certain threshold levels in the nucleus, complex with excess free MDM2 induced from the increase in p53 activity and sequester RPL5 from engaging 5SrRNA as under normal cellular conditions. The result might possibly be even less 5SrRNAs in the nucleolus as a direct result of MDM2 competition for RPL5-5SrRNA binding regions, causing further nucleolar stress from perturbation of ribosomal biogenesis. Therefore, a partial inhibition of MDM2 as found by Horn and Vousden [198] could actually exacerbate the instability within the nucleolus due to MDM2 replacing 5SrRNA on RPL5s basic domains, preventing not only 5SrRNA but also RPL5 from entering the nucleolus.

The next step in characterizing RPL5-MDM2 interaction is to mutate certain residues within the full-length RPL5 N and C-terminals followed by running GST-fusion protein-protein association assays similar to the assays performed in this study. These experiments will provide greater specificity in determining which amino acids are involved in binding MDM2. Once the in vitro information is confirmed, RPL5-MDM2 binding will be analyzed using co-immunoprecipitation experiments. Another interesting area to investigate is the possible interactions
between hnRNP K and RPL5, RPL11, and RPL23 using protein-protein association assays in order to identify any potential interactions between these proteins in vitro.

In the future, chemotherapeutic agents might possibly take the form of small molecular inhibitors (SMIs) or peptides resembling an integral part of the MDM2 inhibitors discussed in this paper. Specifically, SMIs or peptides might contain the reactive portion of RPL5 and inhibit MDM2 via binding its central acidic domain or smaller acidic domain just downstream of MDM2’s Zn finger or both, preventing MDM2’s interaction with p53, thus stabilizing and activating p53 in cells exhibiting high proliferation with tumor cell specificity.
Figure 4.1 – Depicting the Nuclear Localization and Nucleolar Localization Signals at both the N & C-terminals of the full-length (aa-1-297) ribosomal protein L5. The N & C-terminal NLS/NoLS domains consisting of residues 21-37 and 255-265, respectively and are depicted in red. The Nuclear Export Signal (NES) is in green and covers residues 101-111. MDM2 binding regions are blue and cover residues 1-39 and 252-297.


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Curriculum Vitae

Kevin D. Plummer

EDUCATION

08/2006 – 05/2010 Indiana University Indianapolis, IN
Department of Biochemistry and Molecular Biology – MS Program
➤ GPA 3.33
  My research was performed in the lab of Dr. Hua Lu and involved characterizing protein-protein and protein-nucleic acid associations.

08/89 – 05/90 & 08/92 – 12/94 Indiana University Bloomington, IN
Degree Conferred: December 1994
BA - double major - Chemistry & Biology
➤ Studies outside of majors included a concentration of Psychology courses.
➤ Worked for The Institute of Molecular and Cellular Biology, performing various laboratory procedures including cell transformations, PAGE, Sequencing, PCR and mini/maxi DNA preparations.

THESIS LABORATORY EXPERIENCE

10/2007 – 03/2009 IU- School of Medicine Indianapolis, IN
Degree Conferred: May 2010
Master of Science in Biochemistry
➤ Designed and engineered deletion mutant constructs.
➤ Sequenced purified GST-rpl5 fusion and 6X-His-mdm2 constructs to insure template accuracy prior to proceeding.
➤ Performed transformations of _E.coli_ BL21(DE3) cells with pGEX4T-1-RPL5 recombinant plasmid constructs.
➤ Inoculated, induced, and expressed recombinant plasmid pGEX4T-1-RPL5 deletion mutants and pET24a-mdm2-wt newly transformed cells.
➤ Purified GST-RPL5 fusion proteins via GSH bead and GST-RPL5 Fusion Protein interaction.
➤ Performed purification of 6x-His-tagged MDM2 soluble proteins via Ni-NTA affinity chromatography.
➤ Performed dialysis on affinity chromatography elution volumes.
➤ Quantified purified proteins by comparing to BSA trendline.
➤ Performed In Vitro GST-RPL5 fusion protein: His-MDM2 association assays
➤ Washed, immunoblotted, and stained reactants to analyze Relative Binding Intensities between MDM2 and RPL5 deletion mutants
INDUSTRY LABORATORY EXPERIENCE

07/1995 – 06/1996  Lark Sequencing Technologies  Houston, TX  
Project Manager  
Responsible for managing the flow of DNA Sequencing projects through the laboratory. These responsibilities included: receiving and reviewing projects, ordering initial incoming quality controls, designing primers for difficult nucleotide sequences and ordering reactions necessary to clear these ambiguities, analyzing final data, compiling, drafting and submitting final reports before project due dates.

Worked closely with the laboratory director, technologists and sales/marketing departments to effectively communicate potential issues, provide status updates and information necessary to insure accurate and on-time completion of project deliverables.

02/1995 – 07/1995  Identigene  Houston, TX  
Molecular Technologist  
Used STRs to solve paternity cases. Daily activities included performing DNA Extraction and Isolation, Polymerase Chain Reaction, PAGE, Silver Staining, allele resolution and data analysis.

OTHER EXPERIENCE

07/2004 – 04/2006  Kelly Scientific Resources  Indianapolis, IN  
Scientific Recruiter  
Recruited science professionals to fill technician, scientist and laboratory management positions for companies located in Indiana. Daily activities included sourcing, screening, and interviewing potential candidates, working with clients to determine the best possible candidates, presenting candidates to clients, developing job orders, writing and posting job descriptions.

03/2002 – 04/2004  I.T. Staffing  Houston, TX  
Account Manager / Sr. Account Manager  
Responsible for marketing full service Information Technology staffing solutions to executive management of fortune 1000-500 companies headquartered in Houston. Daily activities included successfully penetrating accounts via cold calling and referrals, giving presentations, assessing the client’s needs and recruiting/placing various skill levels from Desktop Technicians and Network Engineers to IT Directors.
10/2001 – 02/2002 Crescent Communications Inc. Houston, TX
03/1999 – 08/2000 Crescent Technology Account Executive / Sr. Account Executive

Marketed Information Technology solutions to executive management of mid-sized companies headquartered in Houston. Daily duties included new business development, managing current accounts, drafting proposals, presenting proposed solutions and effectively establishing and maintaining strategic alliances with key vendors. As the single point of contact between internal technical resources, vendors and clients, I played a crucial role in communicating and coordinating their combined efforts to insure smooth transitions during the implementation phase of projects.

09/1996 – 02/1999 Allstar Systems Houston, TX
Service Account Manager

Responsible for marketing Information Technology outsourcing solutions to executive management of fortune 500 companies headquartered in the Houston area. Daily activities included developing new business, managing current accounts, interfacing with technicians, engineers and management to communicate client needs and effectively identify the best possible solutions, negotiating service contracts, drafting technical proposals and presenting proposed solutions.

MILITARY SERVICE

1990-92 U.S. Army Ft. Sam Houston/San Antonio, TX
Infantry / Laboratory Technician

· Honorable discharged in 1992.
· Distinguished Honor Graduate – Academy of Health Sciences, Ft. Sam Houston
· Wartime Service Ribbon and Good Conduct Medal