ANALYSIS OF THE P53 REGULATOR MDM2 AND THE IDENTIFICATION OF THE NOVEL P53 TARGET GENE LRP1

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ABSTRACT

Patrick Lee Leslie: Analysis of the p53 regulator MDM2 and identification of the p53 target gene LRP1
(Under the direction of Yanping Zhang)

The transcription factor p53 responds to many stresses and regulates many different pathways. The earliest characterized functions of p53 include the induction of cell cycle arrest, apoptosis, and senescence; however, more recent studies have shown that p53 regulates other pathways, including lipid and glucose metabolism, DNA damage repair, and autophagy. While the activation of many of these pathways likely overlaps in many contexts, a current model proposes that p53 plays a critical role in deciding cell fate in response to stress. Indeed, depending on the type and severity of stress, p53 can induce genes that promote the resolution of cellular damage thereby allowing the cell to continue to proliferate, or p53 can induce genes that promote apoptosis to prevent the cell from propagating deleterious mutations. The chief negative regulators of p53, MDM2 and its homologous binding partner MDMX, are overexpressed in many cancers, especially those with wild-type p53. Although MDM2 and MDMX have been intensely studied, basic aspects regarding their interaction, such as how MDM2 preferentially heterooligomerizes with MDMX over homooligomerizing with MDM2, remain unknown. In my research, I generated multiple MDM2 mutant constructs to test their ability to homooligomerize with MDM2 and heterooligomerize with MDMX. Surprisingly, despite many studies suggesting that the C-terminal Really Interesting New Gene (RING) domain is critical for both MDM2

homooligomerization and MDM2-MDMX heterooligomerization, my results show that MDM2 RING structural mutations that prevent MDM2 enzymatic function and MDMX binding retain the ability to homooligomerize. Interestingly, deletion of the regulatory central acidic domain of MDM2 inhibits the ability of MDM2 to homooligomerize but does not impede its ability to heterooligomerize with MDMX, suggesting that MDM2-MDM2 homooligomerization and MDM2-MDMX heterooligomerization occur through different mechanisms. In another study, I identified the gene *low-density lipoprotein receptor related protein 1* (*LRP1*) as a novel p53 target gene. Further analysis revealed that LRP1 protein induction occurs in response to sub-lethal but not lethal p53-activating stresses. Interestingly, although lethal p53-activating stress can induce LRP1 transcription, protein expression is impeded at the translational level. Collectively, these studies contribute to our knowledge of p53 regulation as well as the p53 regulome.

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5FU: 5-fluorouracil

AD: acidic domain

APAF1: apoptotic peptidase activating factor 1

ApoE: apolipoprotein E

ARF: alternative reading frame

ATM: ataxia telangiectasia mutated

ATR: ATM-related

Aβ: amyloid-beta

BAX: Bcl-2-associated X protein

Bcl-2: B-cell CLL/lymphoma 2

CDKN1A: cyclin-dependent kinase inhibitor 1A

ChIP: chromatin IP

CK1/2: casein kinase 1/2

CQ: chloroquine

CRISPR: clustered regularly interspersed palindromic repeats

DBD: DNA binding domain

DNA: deoxyribonucleic acid

Dox: doxorubicin

DRAM: damage-regulated autophagy modulator

FAO: fatty acid oxidation

GADD45: growth arrest and DNA damage-inducible 45-alpha

GLS2: glutaminase 2

GOF: gain-of-function

GPX1: glutathione peroxidase 1

HIPK2: homeodomain-interacting protein kinase 2

IP: immunoprecipitation

IR: ionizing radiation

LDL: low-density lipoprotein

LDLR: LDL receptor

LRP1: LDLR-related protein 1

MCD: malonyl coA dehydrogenase

MDM2: mouse double minute 2

MDMX: mouse double minute 4

MGMT: O-6-methylguanine DNA methyltransferase

miRNA: microRNA

NES: nuclear exportation signal

NLS: nuclear localization signal

NOXA: phorbol-12-myristate-13-acetate-induced protein 1

p53BD: p53 binding domain

p53ER: p53-estrogen receptor

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PIG3: p53-induced gene 3

PML: promyelocytic leukemia

PTM: post-translational modification

PUMA: p53-upregulated modulator of apoptosis

RE: response element

RING: really interesting new gene

RISC: RNA-induced silencing complex

RNA: ribonucleic acid

RP: ribosomal protein

RRM2B: ribonucleotide reductase subunit M2B

SCO2: cytochrome C oxidase assembly protein

SILAC: stable isotope labeling by amino acids in cells

Sp1: specificity protein 1

SV40: simian virus 40

TGF-beta: transforming growth factor-beta

TIGAR: tp53-inducible glycolysis and apoptosis regulator

tPA: tissue-type plasminogen activator

Ub: ubiquitin

ULK1: UNC51-like autophagy activating kinase

UTR: untranslated region

UV: ultraviolet

WT: wild-type

XPC: xeroderma pigmentosum complementation group C

CHAPTER 1: INTRODUCTION[1]

Known to many as the "guardian of the genome," the transcription factor p53, which interestingly enough was originally characterized as an oncogene, has gradually gained widespread notoriety as a general stress response coordinator. Indeed, p53 can be activated by several types of stress, including DNA damage, oncogene activation, ribosomal stress, hypoxia, nucleotide deficiency, oxidative stress, and chromosomal aberrations, among others (Figure 1-1). Likewise, p53 controls the expression of numerous genes involved in many different cellular processes corresponding to the nature of the activating stress (Figure 1-1). Early reports on p53 function discovered a prominent role for p53 in the induction of cell cycle arrest, apoptosis, and senescence, so-called canonical p53 functions. However, more recent studies have also shed light on various non-canonical functions of p53, including DNA damage repair, glucose and lipid metabolism, and autophagy. These advancements in our knowledge of p53 have helped shape and change our perception of the role of p53 in the cellular and organismal context. Many novel p53 targets are being discovered as more and more high throughput screens are conducted. In Chapter 3, the identification and characterization of the novel p53 target gene *low-density lipoprotein receptor-related 1* (*LRP1*) will be discussed.

[1] The sections of this chapter describing MDM2 and MDMX were adapted from a review article currently in press at *Oncogene.* I wrote and edited the entire review with guidance from my mentor. The advance online publication citation is as follows: Leslie PL, Zhang Y. MDM2 oligomers: antagonizers of the guardian of the genome. *Oncogene* advance online publication, 4 April 2016 (DOI 10.1038/onc.2016.88).

In addition to understanding p53 function, another important research subject regarding p53 is how it is regulated. Commonly accepted as the most important negative regulator of p53, the gene product mouse double minute 2 (MDM2, sometimes referred to as H(uman)DM2) potently inhibits p53 function in two ways: MDM2 binds directly to the p53 activation domain thereby masking p53 function as a transcription factor, and MDM2 catalyzes the transfer of ubiquitin to p53 to mark p53 for proteasomal degradation. Studies on MDM2 have shown that it is as important in the context of p53 functions as p53 itself. Interestingly, as will be discussed in more detail below, the manipulation of MDM2 alone can produce phenotypes that are identical to the deletion or manipulation of p53, suggesting that MDM2 is an important consideration for most, if not all, p53 studies. In Chapter 2, a mutational analysis of MDM2 will be described in detail. Some of the novel findings of this study include evidence that mutations in MDM2 that were previously thought to inhibit its ability to homooligomerize actually do not affect homooligomerization. Importantly, I show evidence that homooligomer formation involves domains of MDM2 that are only beginning to be acknowledged for their role oligomer formation.

In this Introduction chapter, I will provide an overview of p53 activation, function, and regulation as well as a discussion of current p53 regulation models supported by the body of our knowledge of p53. Moreover, as a significant part of the study detailed in Chapter 3, I will provide background information on LRP1 structure and function, which could provide clues as to its function in the context of the p53-dependent response. I will also introduce and discuss in detail the functions of the p53 regulator MDM2. First, I begin

with a brief introduction describing the initial discovery and the surprisingly tortuous path towards the characterization of p53.

Discovery of p53

The initial discovery of p53 was reported by multiple labs that found that p53 interacts with the simian virus 40 (SV40) large T antigen. These labs, which included those of David Lane, Arnold Levine, Pierre May, Robert Carroll, and Alan Smith, found that upon immunoprecipitation of the SV40 large T antigen, a cellular protein of approximately 53 kDa in size could be co-precipitated.[152, 163, 187, 212, 282] Further investigation into this 53-kDa protein, eventually named p53, found that not only could p53 be stabilized by SV40 large T antigen but p53 was generally stabilized in transformed tissues compared with normal tissues. These studies collectively showed that higher levels of p53 correlate with tumor formation and progression, which led to the initially false assumption that p53 functions as an oncoprotein. However, the characterization of p53 as an oncoprotein came under scrutiny when completely opposite phenotypes were observed in cell lines transfected with cloned p53 constructs from different labs.[174] These discrepancies were explained when sequencing of these clones revealed that wild-type p53 produced a cell suppression effect, whereas mutated versions of p53 acted as an oncogene and promoted cellular transformation. Part of the problem originated from the fact that p53 was most often cloned from cDNA obtained from tumor cell lines, which typically express much higher levels of p53 than normal cells and thus were presumed to offer an advantage for cloning purposes. Because tumors typically express mutated versions of p53, many labs reported on the gain-of-function (GOF) effects of mutant p53 well before it was known that the

normally tumor suppressive p53 could be transformed into a tumor-promoting factor through the mutation of single residues. Studies thereafter (by our own Dr. Dirk Dittmer studying in the Levine lab) confirmed that gain-of-function effects are associated with p53 mutations, which manifests in a pro-tumor phenotype.[70]

Function of p53

The function of p53 as tumor suppressor was further confirmed by studies analyzing the effects of p53 mutation. In patients who present with mutations in a single p53 allele, acquired either through germline transmission or through somatic mutation early in development, show a particularly high penetrance of tumor development of many types at a young age. This disorder, termed Li-Fraumeni syndrome, displays many of the characteristics observed upon deletion of p53 in mice, suggesting the autosomal dominant nature of the disease. Deletion of p53 in mice shows a similarly high rate of tumor formation in early adulthood.[74] Consistently, mutation of p53 has been observed in approximately half of all cancers, consistent with its function as a tumor suppressor.[53, 108]

After being confirmed as a tumor suppressor, focus on determining the mechanism of action of p53 intensified. Evidence for a role as a transcription factor emerged in the early 1990s, when transcriptionally active regions in p53 were discovered.[87, 255] Other studies revealed that p53 could form higher molecular weight complexes in a manner that was consistent with oligomerization.[150] Closer analysis revealed that p53 oligomers were predominantly tetrameric in nature, especially when bound to DNA, suggesting that the tetramer could be the functional configuration of p53 as a transcription factor.[91] As the field progressed, p53 was further confirmed through direct means as a tetrameric DNA

binding transcription factor composed of two dimers.[198] Subsequent studies identified sequence-specific binding sites for p53 that are consistent with its symmetric oligomeric nature.[80, 140] El-deiry *et al*. identified the p53 consensus binding site as consisting of two 5'-RRRCWWGYYY-3' half sites separated by 0-13 base pairs (R=purine, Y=pyrimidine, W=adenine or thymine). The identification of the consensus p53 binding site was an important discovery for the p53 field, as it allowed for the prediction of putative p53 target genes once genomic sequence data became available. Subsequent high-throughput analyses further refined the strongest p53 candidate response elements as consisting of the two decamer half sites separated by either zero or one base pair.[32] Based on the identified p53 consensus binding sequence, multiple high-throughput studies have been conducted, identifying multiple verified and putative p53 response genes.[32, 226, 281] Nonetheless, even before the development of high throughput techniques, shortly after the identification of the p53 consensus sequence, the first p53 target, p21 (also known as CDKN1A), was reported.[81]

Since the discovery of *p21* as a p53-regulated gene, multiple other p53 target genes have been identified, including the apoptotic gene products PUMA, NOXA, BAX, AIP1, and APAF1, the autophagy gene products DRAM1, ULK1, and sestrin 1, the senescence gene products GADD45 and PML, the DNA damage repair gene products ribonucleotide reductase subunit M2B, XPC and MGMT, the metabolism gene products TIGAR, SCO2, MCD, and GLS2, antioxidant gene products such as GPX1, and other gene products, including MDM2 and various miRNAs (Figure 1-1). Thus, while p53 was characterized early on as a tumor suppressor, decades of research thereafter discovered that p53 mediates its tumor suppressive effect by regulating many different pathways. It should be noted that, while

some of the mechanistic details remain obscure, many p53 target genes and pathways can have overlapping functions and can be activated in different combinations depending on context, which will be discussed below.

Another milestone in p53 research was achieved when the first successful crystal structure of p53 bound to its response element was reported. This crystal structure offered insight into which p53 residues interact directly with DNA.[51] Details provided by the crystal structure provided a physical explanation why common p53 mutations prevent p53 from binding to its response element.[131] Interestingly, the vast majority of tumor-associated p53 mutations are point mutations and not deletions, which can be explained by the selective pressure for clones that harbor the oncogenic GOF effects associated with p53 point mutations. Comparing mice deficient for p53 with mice harboring a single point mutation in p53 (R175H in human p53) revealed a relative survival advantage for the p53-null mice, suggesting that p53 point mutations indeed promote cancer progression.[164] *In vitro* experiments using cells derived from the p53 R175H mice revealed that these cells possessed several characteristics that are beneficial to rapid tumor growth, including increased transformation potential, increased clonogenicity, and more rapid proliferation, when compared with p53-null cells. Moreover, p53 point mutation-bearing mice display a different spectrum of tumors, compared with p53-null mice.[235]

Although p53 point mutations have been found throughout the entire length of the gene, most of these mutations occur within the DNA binding domain (DBD), which spans exons 4-9. Moreover, of the p53 DBD mutations, approximately 30% occur among six residues (R175, G245, R248, R249, R273, and R282) referred to as mutational "hot spots." Some of these mutations are considered less severe than others, and p53 mutations have

been broadly classified into "structural mutants," which induce the unfolding of p53, and "contact mutants," which describe mutations that specifically affect that ability of p53 to bind DNA but do not significantly induce protein unfolding.[51, 277] Although all p53 mutations likely unfold p53 to some extent, the unfolded nature of mutant p53 may play a role in its associated gain-of-function phenotypes. Interestingly, a recent study showed that several common p53 mutations reconfigure the folded p53 protein structure to allow binding to and redirection of the chromatin remodeling machinery, including the methyltransferases MLL1 and MLL2 as well as the acetyltransferase MOZ. This results in the expression of a distinct set of genes that promotes tumor cell growth and metastasis.[340] Other studies have also shown that mutant p53 could produce an opposite outcome compared with WT p53 with respect to miRNA-mediated gene regulation.[151] Because of the importance of p53 GOF mutations in cancers, this area of research remains popular and continues to evolve.

Regulation of p53

While genes such as p21 are regulated by p53, p53 itself is also subject to tight regulation, as too little p53 can result in tumor development, and too much p53 can result in widespread apoptosis. Therefore, p53 is subject to strict regulation through many different cellular components. Under normal conditions, p53 protein is maintained at low levels to prevent the aberrant activation of cell cycle arrest or apoptotic genes. Rapidly dividing cells are particularly susceptible to p53 overactivation, which includes tumor cells but also includes hematopoietic and intestinal cells. The increased sensitivity of rapidly dividing cells to p53-mediated apoptosis forms part of the basis for many cancer therapies,

as rapidly proliferating cancer cells that lack the basic cell cycle regulation infrastructure are more prone than almost all normal cells to catastrophic outcomes from unrestrained proliferation in the presence of DNA damage or other defects in cellular structures (e.g., taxol-induced microtubule stabilization). Cancer treatment modalities, such as radiotherapy and DNA damaging chemotherapies, exploit the rapid proliferative capacity of cancer cells and offer therapeutic value, as normal cells generally respond by inducing cell cycle arrest and/or DNA damage repair pathways through tumor suppressor genes such as p53. Interestingly, the absence of one or both alleles of p53 increases the sensitivity of mice to the development of tumors after a single dose of sub-lethal irradiation, which highlights the importance of p53 in preventing tumorigenic transformation.[139] On the other hand, the absence or inhibition of p53 is sufficient to protect mice from otherwise lethal doses of irradiation.[144, 322] In response to a lethal dose of irradiation, p53 WT mice show extensive apoptosis in many tissues and ultimately succumb to hematopoietic failure. These results suggest that p53 overactivation in normal tissue is just as important a consideration for the treatment of tumors as p53 underactivation in tumor tissue. To keep p53 inactive under normal conditions and active under stress, p53 is regulated at the transcriptional and translational levels;[223] however, the main modality of p53 regulation occurs at the post-translational level. The post-translational regulation of p53 is mediated predominantly in terms of p53 stability involving various post-translational marks (PTMs). p53 protein can be modified by phosphorylation, acetylation, ubiquitination, SUMOylation, methylation, and neddylation, among others. Some of the better characterized PTMs on p53 are described below and are shown in Figure 1-2.

p53 phosphorylation

Many p53 residues can be phosphorylated by various kinases under various conditions, contributing to the regulation of p53. Indeed, based on the rapid kinetics, p53 phosphorylation is thought to be one of the most important first steps for p53 stabilization in response to stress. One of the best characterized phosphorylation-inducing stresses is DNA damage, which results in p53 phosphorylation on several residues. Most prominent among these residues is serine 15, which is phosphorylated by ATM, ATR, and DNA-PK.[26, 154, 209, 274] $p53^{Ser15}$ phosphorylation results in p53 stabilization in part by inhibiting its ability to interact with its negative regulator MDM2.[273] Interestingly, $p53^{Ser15}$ phosphorylation also enhances the affinity of p53 for its response elements.[192] Moreover, $p53^{Ser15}$ phosphorylation behaves as a sort of gateway phosphorylation mark, whereby $p53^{Ser15}$ phosphorylation precedes and acts as a prerequisite to the subsequent phosphorylation of other p53 residues, including serines 6, 9, 20, and 46 as well as Thr18, by other kinases such as casein kinase 1, casein kinase 2, JNK, and HIPK2 (Figure 1-2).[76, 262, 263] Importantly, mutation of $p53^{Ser15}$ to alanine is sufficient to prevent the p53-dependent transactivation of target genes in response to DNA damage.[192] $p53^{Ser15}$ is often used as a DNA damage marker in response to various stresses.

Interestingly, mutation of any of these p53 phosphorylation sites, including Ser15, in various mouse models has produced an intermediate phenotype in terms of the effect on the p53 response upon challenge with p53-activating stress when compared with WT or p53-null mice.[35, 195, 280, 328] This suggests that *in vivo*, these phosphorylation sites only partially contribute to p53 activation and that other mechanisms of activation are involved. Indeed, p53 as well as MDM2 is post-translationally modified in many different manners,

some of which could be more important physiologically in the p53 response than even $p53^{Ser15}$ phosphorylation. For example, mutation of one phosphorylation mark on MDM2 Ser395 (normally phosphorylated by ATM) to alanine prevents death in mice exposed to a lethal dose of ionizing radiation.[92]

p53 residue Ser46 is another well characterized phosphorylation site that is preferentially phosphorylated in response to apoptotic stress.[229] Ser46 is phosphorylated by the kinase homeodomain-interacting protein kinase 2 (HIPK2) and has been used to differentiate between the apoptotic and cell cycle arrest programs of p53 (discussed more below).[28, 29, 58, 69, 168] Similarly to the function of Ser15 phosphorylation as a gateway mark to additional phosphorylation marks, Ser46 phosphorylation facilitates the acetylation of Lys382,[107] as p53 acetylation serves as another important mark for p53 regulation.

p53 is also subject to dephosphorylation by various phosphatases. The most prominent p53 phosphatase is Wip1, which has been shown to dephosphorylate $p53^{Ser15}$ but not $p53^{Ser46}$.[193] Interestingly, Wip1 is expressed in a p53-dependent manner, suggesting the existence of negative feedback loop involving Wip1 phosphatase.[88] As one would expect, p53 dephosphorylation of these residues by Wip1 has the opposite effect of p53 phosphorylation, namely inhibition of p53 transcriptional activity. Another p53 phosphatase that has been reported to inhibit p53 activity is protein phosphatase-1 (PP-1), which was shown to dephosphorylate Ser15 and Ser37.[177] The protein phosphatase 2A (PP2A) subunit B56γ has also been reported as a p53 phosphatase that dephosphorylates residue Thr55.[179] Interestingly, the authors report that B56γ-mediated dephosphorylation of Thr55 is necessary to stabilize p53 and induce apoptosis, suggesting that phosphorylation of certain p53 residues could also inhibit its activity.

p53 acetylation

As alluded to above, another important PTM on p53 is acetylation. The acetyltransferases CBP/p300 (hereafter referred to as p300), PCAF, and Tip60 have been shown to directly acetylate various p53 Lys residues (p300: lysines 164, 370, 372, 373, 381, 382, and 386, PCAF: lysine 320, Tip60: lysine 120) thereby resulting in p53 activation.[154, 188] Some of the acetylation sites in p53, especially near the C-terminus, overlap with ubiquitination sites within p53 that are crucial for its degradation. Therefore, it appears as though p53 acetylation functions in part by blocking ubiquitination and proteasomal degradation. p53 lysine acetylation has also been shown to affect p53 function in other respects. In one study, mutant p53 that was unable to be acetylated on Lys120 showed increased binding affinity between p53 and the response elements for cell cycle arrest genes p21 and MDM2,[294] suggesting that p53 acetylation may play a role in differential gene transcription (discussed in detail below). Lys382 acetylation appears to play a similar role in specifying p53 transcriptional activity. Moreover, some p53 acetylation marks appear to interfere with or promote the acetylation or phosphorylation of other p53 residues, which could affect which genes are transcribed by p53 in response to a given stress;[142] an example of this was provided in the preceding section whereby Ser46 phosphorylation enhances Lys382 acetylation.

p53 is also subjected to the activity of deacetylases, the most prominent of which is SIRT1, which plays a key role in p53 stability and function.[309] SIRT1 overexpression inhibits the ability of p53 to transcribe its target gene p21, highlighting its importance in p53 regulation. Interestingly, SIRT1 shows specificity for the deacetylation of the pro-apoptotic mark on Lys382, which could imply a role for SIRT1 in the selection of the

activation of the various p53 programs.

p53 ubiquitination

A major avenue through which p53 is regulated on the post-translational level occurs through ubiquitin attachment. In fact, p53 degradation occurs primarily through the ubiquitin-proteasome pathway. The importance of ubiquitination on p53 degradation can be illustrated by MG132-mediated proteasome inhibition, which prolongs the half-life of p53 from less than 30 minutes to several hours.[197] Ubiquitination generally occurs through a cascade of reactions involving the activation and transfer of individual ubiquitin (Ub) molecules to the substrate. Ub activation occurs when an E1 Ub-activating enzyme catalyzes the conjugation of an adenylate moiety to Ub. Once activated, an E2 Ub-conjugating enzyme catalyzes the transfer of the activated Ub to itself thereby forming an E2-Ub intermediate. The final step of the Ub cascade is catalyzed by an E3 ligase, which binds to its specific substrate and the E2-Ub to facilitate the transfer and covalent attachment of Ub to the substrate. This reaction can occur in iterations to produce a polyubiquitin tail that can mark the targeted protein for degradation. E3 ligases constitute the largest and most diverse class of the ubiquitination cascade enzymes, which allows the ubiquitination system to target only a handful of proteins at a time. Interestingly, p53 can be ubiquitinated through multiple E3 ligases, including COP1,[75] PirH2,[171] and Topors,[253] any of which can enhance p53 proteasomal degradation. However, the best characterized E3 ligase for p53 is MDM2 and its homolog MDMX. The ubiquitination mechanism of p53 remains unclear, as the extent of p53 ubiquitination could vary depending on the system.[109, 160, 319] One of the particular details of p53 ubiquitin-mediated degradation that

remains unclear is how nuclear p53 is ubiquitinated and then transported to the cytoplasm where the proteasome resides. It is thought that a signal is required for p53 to be expelled to the cytoplasm, and studies have suggested that the signal is ubiquitin attachment.[20, 96] One model proposes that p53 is monoubiquitinated in the nucleus by MDM2, which exposes the p53 nuclear export sequence (NES) and promotes p53 expulsion from the nucleus.[160, 180] Interestingly, although MDM2 also possesses an NES, only the p53 NES and MDM2 ubiquitin ligase activity are necessary for p53 export. Once in the cytoplasm, p53 could be further polyubiquitinated and degraded by the proteasome.

Studies have also shown that once p53 becomes expelled from the nucleus (possibly through monoubiquitination), cytoplasmic p53 can exert transcription-independent activity by directly interacting with proteins such as Bax, Bcl-2, and Bcl-xL.[49, 50, 216] This leads to another unresolved question in the field, which is if p53 is monoubiquitinated and expelled into the cytoplasm, then what determines whether mono-Ub-p53 is immediately degraded or allowed to exert extra-transcriptional effects. Part of the answer appears in the fact that p53 can also be deubiquitinated through the action of the cytoplasmic deubiquitinase USP10.[335] Though the regulatory mechanism remains unclear, these results suggest that mono-Ub-p53 could be subject to deubiquitination under certain circumstances after being expelled from the nucleus.

p53 methylation

Another intriguing area of p53 regulation is methylation. Although this area remains less well researched compared with p53 phosphorylation, acetylation, and ubiquitination, p53 can be methylated on Lys372 by the methyltransferase Set9.[52] Set9 methylation of

Lys372 results in p53 stabilization and increased transcription of p53 target genes. Interestingly, p53 can also be methylated on Lys370 by Smyd2.[114] In contrast to Lys372 methylation, Lys370 methylation was reported to suppress p53 activity. Moreover, methylation at Lys370 or Lys372 (by Set9) appeared to counter-regulate each other, suggesting that p53 methylation marks cross-talk with one another and produce different outcomes depending on the modified residues.

p53 differential gene activation

As discussed above, p53 can transactivate genes that are involved in many different types of pathways, including cell cycle arrest, senescence, apoptosis, metabolism, and autophagy, among others (Figure 1-1). Despite the diverse array of p53-regulated genes, stresses that activate p53 do not always result in the same outcome. For example, although different doses of a single DNA damaging agent, such as doxorubicin, can activate p53, the outcomes can be completely different. Whereas low dose doxorubicin-activated p53 results in cell survival, higher doses produce more widespread cell death.[258] Likewise, different p53-activating stresses can also result in not only variable outcomes but also different p53-regulated gene expression patterns. These differences suggest that p53 functions as a sort of decision node tasked with choosing which of the different gene programs to activate as an appropriate response to the context and severity of a given stress.[153] Indeed, this idea that p53 can promote either cell survival and adaptation or cell death and senescence is thought to depend on whether the damage resulting from a given stress is repairable or not. Intuitively, if a stress induces DNA damage in a cell to an extent that overwhelms the machinery and resources required for repair, then in the context of the organism, apoptosis

is the most beneficial outcome. On the other hand, because the generation of new cells requires significant investment of energy and resources, if DNA damage is resolvable, then the most beneficial outcome would be repair and adaptation. This dual function of p53 fits well with its role as a preserver of genomic fidelity and organismal fitness, as severe DNA damage can result in mutations that lead to transformation. An interesting question regarding the p53-regulated gene programs concerns the antagonistic effects of some programs towards others. One of the more obvious examples is that during the p53-dependent induction of apoptosis, the simultaneous p53-dependent activation of DNA repair or antioxidant pathways could act counter to the intended apoptotic outcome. Indeed, p21 has long been known to exert anti-apoptotic effects, which suggests that suppressing p21 could be beneficial for a rapid apoptotic response.[196, 316] Therefore, pleiotropic transcription factors such as p53 likely require a mechanism to selectively transcribe some genes but not others depending on the context. As one of the major mysteries that persists regarding p53 regulation, several models have been proposed to explain mechanistically how p53 decides which programs to activate, many of which have focused on differences in p53 itself (overall level, PTM marks, expression patterns, etc.). Notably, these proposed models are not necessarily mutually exclusive, and some of the models overlap considerably (e.g., PTMs on p53 affect the affinity between p53 and various response elements).

p53 threshold model

Multiple studies have suggested that the outcome of a cell depends on the level of p53.[149, 252] Moreover, a recent study using a p53-inducible cell model that allows for the

precise control of p53 levels in the absence of genotoxic stress showed a strong correlation between the level of p53 and the expression of both cell cycle arrest and apoptosis genes.[149] In this study, the authors provided additional evidence suggesting that a physiological threshold dictates whether a cell responds to stress in an apoptotic manner. In one experiment, the authors showed that the activation of apoptosis requires a relatively long duration of high p53 levels, as low p53 induction for an extended duration failed to induce appreciable apoptosis. In another experiment, the authors showed that the theoretical p53 apoptotic threshold could be lowered by inhibiting anti-apoptotic Bcl-2 family member proteins. Interestingly, inhibition of Bcl-2 family proteins resulted in increased apoptosis in the presence of an otherwise sub-lethal level of p53. These results support a model whereby only high levels of p53 are able to induce apoptosis, whereas, while low levels of p53 can activate some apoptotic gene expression, the abundance of apoptotic gene expression does not surpass the threshold required for cell death. This model fits well with several studies that have shown that to trigger an apoptotic response, a certain threshold of pro-apoptotic gene expression (relative to anti-apoptotic gene expression) must occur.[14, 286]

Differences in p53 response elements

Another popular model for p53 differential gene regulation is based on the binding affinity between p53 and cell cycle arrest or apoptotic p53 response elements. Through the analysis and confirmation of several dozen authentic p53 REs, one observes that many of these REs deviate, sometimes considerably, from the consensus RE $(RRRCWWGYYY)_2$. Additionally, multiple studies have analyzed p53 binding affinity to various cell cycle arrest

and apoptotic response elements in an attempt to identify a pattern. In one study comparing the p53 REs of the cell cycle arrest gene *p21* and the apoptotic gene *TP53I3* (also called *PIG3*), the p53 RE for *p21* was bound more rapidly than that of *TP53I3* in response to DNA damage, suggesting that various p53 REs bind p53 at different rates and affinities.[295] In another study, p53 was shown to bind preferentially to the REs for *p21* and the senescence target gene *GADD45* in senescent cells. This binding pattern was not observed in cells treated with an acute dose of doxorubicin, which show increased accumulation of p53 at the promoters of apoptotic target genes.[124] Using a biophysical approach, the Fersht group determined the *in vitro* dissociation constants for 20 known p53 response elements for the p53 tetramer and found that all of the low-affinity response elements corresponded to apoptotic genes, which could also be consistent with the p53 threshold model.[321] Although these studies provide evidence that at least some of the p53 REs display different affinities for p53 binding, some of these studies have also hinted at the possibility that certain p53 PTMs could be required to enhance its binding to the low-affinity promoters.

p53 post-translational modifications

Many studies have revealed that p53 is subject to a large and diverse array of post-translational modifications (PTMs). More detailed analyses of these PTMs have found that the modification of certain residues, particularly by phosphorylation or acetylation, could contribute to the ability of p53 to activate cell cycle arrest or apoptosis genes. Much like the "histone code," p53 PTMs are thought to provide at least part of the instructive information from which p53 target genes are selectively expressed in response to various stresses. As described above, the phosphorylation of Ser-46 in p53 correlates strongly with the

induction of apoptotic genes and an apoptotic outcome.[229] The mutation of Ser-46 to Ala results in the attenuation of apoptosis induction by p53. p53 acetylation at residue Lys 382 occurs subsequent to Ser46 phosphorylation, and this mark is required for p53 apoptotic gene expression.[107] Other PTMs include those induced by the E3 ubiquitin ligase E4F1, which monoubiquitinates p53 at Lys320 and results in the upregulation of the p53 cell cycle arrest program.[166] Interestingly, Lys320 is not ubiquitinated by MDM2, suggesting an MDM2-independent mechanism. In addition to ubiquitination at Lys320, p53 can also be functionally acetylated at this residue. One study showed that p53 acetylation at Lys320 and Lys373 results in the expression of cell cycle arrest and apoptotic genes, respectively.[142] In this study, the authors show that acetylation of either residue prevents the other from being acetylated, implying antagonism between these two marks. Moreover, acetylation at Lys320 prevents the phosphorylation of N-terminal p53 residues thereby limiting p53 to high-affinity promoters such as those of cell cycle arrest gene *p21*. In contrast, acetylation of Lys373 promotes the phosphorylation of N-terminal p53 residues and allows p53 to bind to low-affinity promoters such as those of the apoptotic target genes. Another acetyl PTM mentioned above, acetylation of Lys120 also appears to dictate the affinity between p53 and cell cycle arrest and apoptosis gene response elements.[294] Lys120 acetylation is necessary for the induction of p53 apoptotic genes, as the mutation of this residue inhibits the ability of p53 to upregulate apoptotic gene expression. Other PTMs could also play roles in the ability of p53 to bind to certain promoters, and this remains a popular area of p53 research.

p53 dynamic pattern

Most studies investigating p53 activation have analyzed lysates obtained from populations of cells. Using this method, the average p53 response across many cells can be determined; however, the effect of a given stress on the dynamic behavior of individual cells cannot be resolved. With advances in time-lapse microscopy and single cell tracking, the effects of stress on the p53 expression pattern in single cells could be determined. Analyzing single cells offers an opportunity unparalleled by other methods to determine how expression levels of certain genes change over time. In conjunction with fluorescently labeled p53 constructs, this method was used to precisely determine the dynamics of p53 expression in response to ionizing radiation (IR).[159, 252] Interestingly, these studies showed that p53 expression in response to IR shows a pulsed pattern, whereas ultraviolet (UV) irradiation induced a sustained pattern of p53 activation. Interestingly, the pulsed patterns of p53 induction tend to result in non-lethal, reversible outcomes for the cell, whereas sustained activation of p53 results in cell death or senescence. The pulses of p53 in response to IR show similar amplitudes but vary in frequency depending on the magnitude of the initial stress.[159, 252] Importantly, the manipulation of the pulsed p53 induction pattern associated with IR into a sustained response using the p53-activating molecule nutlin-3a can convert the cell survival outcome associated with the pulsed pattern into a senescence outcome. Thus, these studies collectively suggest that the pattern of p53 induction plays a role in whether a cell commits apoptosis in response to a given stress. Interestingly, p53 induction patterns also appear to be associated with the phase of the cell cycle, whereby cells synchronized and released from mitosis show a peak of p53 induction soon after release that subsides within a few hours.[44] This pattern can be exploited by

timing DNA damage to occur when p53 levels are highest, which predisposes the cell to a cell death outcome as opposed to a cell cycle arrest and survival outcome. Of note, the comparison in the Purvis study only analyzed the activation of p53 without addressing other potential differences related to the DNA damage stress itself. Because the authors compared the effects of two entirely different types of DNA damage (UV induces mainly thymidine dimers, whereas IR induces mainly double strand breaks) that activate different repair pathways, it is possible that this dichotomy of pulsed *versus* sustained p53 expression is unique to this stress comparison. Notably, the pulsed pattern of p53 induction has not been reported for other types of DNA damage. Moreover, our personal experience with p53 induction over time in response to a sub-lethal, brief (1 h) dose of the double strand break-inducing chemotherapeutic doxorubicin showed a pattern that most closely resembles the sustained pattern of UV-induced DNA damage. Constant doxorubicin treatment at the same concentration causes a similar albeit gradually increasing expression pattern for p53 (see Chapter 3). Thus, investigation and comparison of the p53 induction pattern in response to other types of stress may offer additional insight into the p53 dynamic pattern model of p53 induction and the nature of the pulsed pattern in response to IR.

p53 co-regulation model

Another plausible model that could explain the differential regulation of p53 programs is based on the binding of co-regulating transcription factors for the expression of certain genes. An example of p53 co-regulation involves the SMAD transcription factors. Reports have suggested that p53 target genes, including *p21*, are co-regulated by SMAD2/3

through the concomitant binding of the promoter region by p53 and SMAD2/3.[56] The nature of this co-regulatory mechanism appears to require individual binding sites for SMAD2/3 and p53, although these transcription factors are also able to bind directly to each other. Moreover, reports have suggested that the p53 and SMAD transcriptomes overlap substantially, particularly concerning cell cycle arrest genes,[77] which is consistent with the roles of p53 and SMAD transcription factors in tumor suppression. The SMAD transcription factors have been implicated in cell cycle inhibition as well as cell cycle progression in a context-dependent manner, much like p53. In addition to the SMADs, p53 has been shown to bind directly to the transcription factor Sp1 (specificity protein 1).[147, 148] p53-Sp1 co-regulation is required for the efficient upregulation of several p53 target genes, including *p21* and *PUMA*.[148] A recent high-throughput analysis further suggested that Sp1 co-regulation is a key determinant for p53-dependent apoptotic gene expression but not for cell cycle arrest gene expression.[178] Collectively, these studies support a co-regulation mechanism that dictates whether p53 transactivates the expression of cell cycle arrest genes or apoptotic genes.

p53 miRNA model

Finally, another model that has recently been proposed regarding p53 differential gene regulation involves p53-dependent miRNA upregulation. miRNAs are small non-coding RNA species approximately 22-23 nucleotides in length that bind to the 3' untranslated region (UTR) of their target genes to induce the degradation or translational inhibition of their target gene(s). Any given miRNA can simultaneously regulate several genes, and any given mRNA transcript can be regulated by several miRNAs. Several miRNA

genes are regulated by p53 as direct transcriptional targets.[86] Interestingly, some p53-regulated miRNAs have been reported to target known p53-regulated genes. In one such example, the p53-regulated miR-23a is expressed in response to p53-activating stresses. Upon upregulation, miR-23a can down-regulate the expression of the apoptotic p53 target gene *APAF1*. Through this mechanism, miR-23a expression can promote apoptotic resistance in some cancer cells.[43, 270, 333] Although these studies were performed in cancer cells, it is likely that p53-regulated miRNAs contribute to the regulation of other p53 target genes in normal cells as well. Of interest to my studies on p53 regulation of LRP1, the doses and stresses used in the studies showing p53-dependent miR-23a targeting of *APAF1* were sub-lethal (~37 μM 5-fluorouracil in HCT116 cells), suggesting that miRNAs could play a role in the manipulation of p53 responses. These studies investigating p53-miRNA feedback loops represent a relatively unknown aspect of p53 differential gene expression, which I address in part in Chapter 3 of this dissertation.

What p53 programs are important for tumor suppression?

At this point, one of the major issues that remains to be discussed is which p53 programs are most important for its tumor suppressor activity. Based on the earliest findings of p53 function, the transactivation of genes involved in cell cycle arrest, apoptosis, and senescence were thought to play major roles in tumor suppression. One can intuitively grasp how these functions would antagonize tumor initiation and development. However, multiple recent studies have hinted that p53-dependent cell cycle arrest, apoptosis, and senescence might not be as important as once thought for the prevention of tumor formation. A study from the Attardi lab reported that mice expressing a p53 knock-in

variant ($p53^{L25Q,W26S}$, so-called $p53^{25,26}$), which shows deficiencies in activating cell cycle arrest and apoptosis, retain the ability to suppress KRAS-induced tumors.[21] However, this mouse model retains the ability to induce senescence, which could account for the tumor suppressive effects of $p53^{25,26}$. This possibility was addressed in a study by the Gu lab, where the authors analyze *Tp53*$^{3KR/3KR}$ mice, which harbor three point mutations (K117R, K161R, and K162R) that inhibit the acetylation of p53 and thus effectively abrogate the induction of p53-dependent cell cycle arrest, apoptosis, and senescence programs.[181] In this paradigm-shifting study, the *Tp53*$^{3KR/3KR}$ mouse remains resistant to spontaneous tumor development compared with *Tp53*-null mice, which develop early onset lymphomas. These results suggest that these canonical functions of p53, which have long been thought to be absolutely necessary for tumor suppression, are actually dispensable for p53-mediated tumor prevention. In another mouse model that supports this conclusion, triple deletion of the p53 target genes *p21*, *Puma*, and *Noxa*, which are involved in cell cycle arrest, apoptosis, and senescence, reveals no difference in susceptibility to spontaneous tumor development.[306] Collectively, these studies suggest that p53-dependent cell cycle arrest, apoptosis, and senescence are not necessary for effective tumor suppression, which in turn suggests that other non-canonical p53-regulated programs are sufficient for tumor suppression. Although p53 has been reported to exert extra-transcriptional activities on various cellular components (glucose-6-phosphate dehydrogenase[130] and autophagy[300]), these functions of p53 also appear to be insufficient for tumor suppression.[129] Therefore, p53 transcriptional activity appears to be key to its function in tumor suppression. Which pathways are most important for p53-mediated tumor suppression remain to be determined, although intriguing candidates include p53-regulated metabolism and DNA

damage repair pathways. A better understanding of how p53 decides between the transactivation of its various target genes could facilitate the development of the appropriate models to dissect this complex question.

Low-density lipoprotein receptor-related protein 1

In chapter 3 of this dissertation, a detailed discussion of the discovery and characterization of the novel p53 target gene *low-density lipoprotein receptor-related protein 1* (*LRP1*) is included. Through this study, I offer insight into a gene product that was initially characterized as an endocytic lipoprotein receptor involved in the importation of lipids by the cell. Being highly expressed in liver tissue, at first glance, LRP1 appears to play a role in p53-mediated lipid metabolism regulation. Interestingly, subsequent studies have ascribed additional functions to LRP1, including the involvement in the endocytosis of various other substrates as well as in the transduction of signaling events to cytoplasmic kinases to mediate cellular responses to various factors. Based on the known functions of LRP1, our identification of the multi-functional LRP1 as a p53 target gene contributes to our understanding of the complement of p53-regulated genes that contribute to the non-canonical p53 programs. The identification of novel p53 target genes involved in the non-canonical p53 programs contributes to our knowledge of the breadth of p53 functions and possibly to the tumor suppressive mechanism of p53.

LRP1 is a member of the low-density lipoprotein receptor (LDLR) family, and presents as a large, bipartite membrane protein that harbors multiple motifs associated with clathrin-dependent endocytosis. LRP1 is transcribed and translated through the secretory pathway as a single 600-kDa protein that is cleaved by furin protease within the

Golgi apparatus to generate 515-kDa and 85-kDa peptides (alpha and beta subunits, respectively) that remain attached via non-covalent interactions.[325] After cleavage, LRP1 is transported to the plasma membrane where it functions in its mature form. The alpha subunit is entirely extracellular, whereas the beta subunit harbors a single transmembrane segment and is partially cytoplasmic, containing an ~100-amino acid tail. LRP1 is unique among the LDLR family members in that it displays the most rapid rate of endocytosis due in large part to the presence of two NPXY motifs and two dileucine motifs within the cytoplasmic tail of its beta subunit.[143] The alpha and beta subunits have been shown to interact with a wide array of different proteins (reviewed by [183]). Being entirely extracellular and constituting over 80% of the receptor, the ligands bound by the LRP1 alpha subunit offer insight into LRP1 function. One of the first LRP1 ligands identified was apolipoprotein E (ApoE), which is associated with certain types of lipoprotein particles.[13] ApoE contributes to the solubility of lipids in circulation and encodes the ligand by which receptors such as LRP1 can bind and endocytose lipoprotein particles. The function of ApoE in the clearance of serum lipids is exemplified by patients who lack both copies of *APOE*.[266] In *APOE*-deficient patients, very low-density lipoprotein and intermediate-density lipoprotein particles accumulate in the serum, suggesting that these lipoproteins require ApoE for proper clearance. Although *APOE* deficiency in humans and mice results in normal embryonic development, *LRP1* deficiency is more severe, as whole-body *LRP1* knockout mice results in embryonic lethality before day E13.5.[104] Thus, to investigate the role of LRP1 *in vivo*, multiple tissue-specific knockout mouse models have been developed.

The tissue-specific deletion of *Lrp1* in smooth muscle cells (*smLrp1*$^{-}$) in an atherogenic genetic background (LDL receptor (*LDLR*)-null) results in increased

atherosclerotic lesions compared with *Lrp1* WT mice, which the authors show is due to the role of LRP1 in PDGF-bb-dependent cell signaling.[19] Importantly, atherosclerosis in the *smLrp1*$^{-}$ mice can be reversed by treating the mice with the kinase inhibitor Gleevec (imatinib), which inhibits the PDGFR signaling pathway. Interestingly, tissue-specific knockout of *Lrp1* in the liver[83] and macrophages[112, 237] also point to a role for LRP1 in the prevention of atherosclerosis. Hepatocyte-specific *Lrp1* knockout mice (in an *Ldlr*- and *ApoE*-null background) show decreased serum triglyceride and cholesterol levels compared with the corresponding *Lrp1* WT mice. Despite the decrease in serum triglyceride and cholesterol levels, liver-specific *Lrp1* knockout mice show a dramatic increase in atherosclerotic lesions, which coincides with increases in the serum levels of several potentially atherogenic LRP1 ligands, including tissue-type plasminogen activator (tPA), von Willebrand factor, coagulation factor VIII, and lipoprotein lipase. Thus, this study highlights the importance of hepatic LRP1 in the binding and regulation of various atherogenic ligands independent of ApoE and shows that these functions constitute an important part of the LRP1 atheroprotective repertoire. In macrophage-specific *Lrp1* deletion mice, a similar increase in atherosclerotic lesions is observed in an *Ldlr*- and *ApoE*-null background compared with corresponding WT mice.[112]

Interestingly, deletion of *Lrp1* in adipocytes results in generally healthier mice. These mice display impaired clearance of post-prandial serum lipids as well as reduced body weight, smaller fat stores, improved glucose tolerance, and increased energy expenditure. These findings are consistent with a role for LRP1 in the importation of serum lipids by the adipocytes. Moreover, considering the multitude of ligands bound to LRP1, if adipocytes could be specifically targeted, adipocyte-expressed LRP1 could be an interesting

candidate for anti-obesity drug development.[106]

As mentioned above, LRP1 binds to many ligands. One interesting LRP1 alpha subunit ligand is amyloid beta (Aβ, isoforms 40 and 42),[146] which has been shown to play a role in the development of amyloid plaques and presumably in the pathogenesis of Alzheimer's disease.[267] Direct binding of Aβ by LRP1 results in Aβ endocytosis and degradation, defects of which could presumably result in the accumulation of Aβ plaques in neural tissue and the pathological sequelae of Alzheimer's disease. Interestingly, Aβ also binds to ApoE, which is thought to enhance its clearance and degradation in part by LRP1.[290, 291] One of the few known aspects regarding the pathogenesis of familial Alzheimer's disease is a strong predisposition for the disease based on *APOE* polymorphisms. In a study analyzing 42 families with familial Alzheimer's disease, it was found that the risk of developing the disease increased with the number of *APOE* E4 alleles present in an individual.[57] Three major alleles of *APOE* exist based on polymorphisms at amino acid residues 112 and 158, whereby *APOE* allele 2 is Cys112/Cys158, *APOE* allele 3 is Cys112/Arg158, and *APOE* allele 4 is Arg112/Arg158. Interestingly, individuals who are homozygous for the *APOE* 4 allele display an approximately 50% chance of developing Alzheimer's disease, compared with a 20-30% chance for *APOE* 3/4 heterozygotes.[95] It is thought that *APOE* allele 4 binds amyloid beta with more avidity and prevents Aβ clearance, at least in part by LRP1.[27, 290]

Other LRP1 ligands include growth factors, lipases, and protease-protease inhibitor complexes, including TGF-beta, platelet-derived growth factor (PDGF), hepatic lipase, lipoprotein lipase, tPA and tPA-associated complexes, and α2-macroglobulin. Although it is beyond the scope of this introduction to discuss all of the ligands that bind to LRP1, this list

is intended to illustrate a fraction of the array of diverse ligands engaged by LRP1, which hints at the importance of our results identifying *LRP1* as a p53-regulated gene.

Conclusions related to p53 function and regulation

Despite the wealth of knowledge produced on many aspects of p53, we lack p53-related clinical applications with respect to p53-specific diagnostic or therapeutic modalities. Nonetheless, significant efforts are underway to develop the first clinically approved p53-activating drug.[141] Understanding how p53 activation affects the gene expression landscape under controlled conditions is important to comprehend how p53 activation in tumors by targeted therapies or traditional chemotherapeutics can be improved. As discussed above, many factors contribute to p53 regulation, chief among which is post-translational stability principally through ubiquitination. Because the principal E3 ligase for p53 is MDM2, understanding how p53 is ubiquitinated by MDM2 is also critically important to understand all aspects of p53, particularly for the development of p53-targeting therapeutics. In the following sections, our understanding of MDM2 will be discussed in detail. This section will begin with a discussion of the history of MDM2 and its homologous partner MDMX.

Discovery of MDM2 and MDMX

MDM2 was initially identified as one of three genes (MDM1 and MDM3 being the others) that were highly overexpressed in spontaneously transformed 3T3-DM mouse cells. The MDM genes were present in the form of amplified minute chromosomes, and these minute chromosomes turned out to be amplified arms of mouse chromosome 10.

Subsequent analyses determined that only the overexpression of MDM2 resulted in the transformation of these cells, suggesting its oncogenic potential and its connection to cellular transformation. Research interest in MDM2 increased dramatically alongside p53 once MDM2 was recognized as an important E3 ligase tasked with targeting p53 for degradation. The MDM2 homolog MDMX (also known as MDM4) was discovered by the Jochemsen lab shortly thereafter based on a cDNA library screen using purified p53.[276] Upon further analysis, MDMX showed considerable homology to MDM2 and has since been characterized as a p53-regulating protein that synergizes with MDM2.

Function of MDM2 and MDMX

As mentioned above, one of the best known regulators of p53 is MDM2, which is an E3 ubiquitin ligase capable of ubiquitinating p53, thereby marking p53 for proteasomal degradation.[102, 109, 155] MDM2 has also been shown to inhibit p53 transcriptional activity directly through binding to the transactivation domain of p53.[220, 234] MDM2 remains the most experimentally consistent ubiquitously expressed ubiquitin ligase that can effect the degradation of p53 *in vitro* and *in vivo*. Therefore, while p53 has been referred to as the "guardian of the genome," MDM2 can be thought of as the guardian of the "guardian of the genome." MDM2 is frequently overexpressed in several types of tumors (many of which harbor WT p53),[22, 199, 233, 256, 278] which has led to increased interest in developing drugs that inhibit MDM2 activity to stabilize and activate p53.[314] However, many drugs that have shown promise in preclinical models have failed to translate into therapeutically effective drugs. One factor that could contribute to the eventual success of p53-activating drugs is a more comprehensive understanding of MDM2 function. For example, although we know

that MDM2 requires the ability to oligomerize to efficiently ubiquitinate p53,[46, 120, 299] understanding the mechanics, function, and regulation of MDM2 oligomerization could be useful to optimize the potency, specificity, and synergy of p53-activating drugs. In the following sections, I discuss our current understanding of MDM2 function with an emphasis on evidence of MDM2 oligomerization and function in p53 regulation. I discuss the importance of the MDM2-MDMX (also known as MDM4) heterooligomers and evidence of MDM2 homooligomer function. Although MDMX shares many structural features with MDM2, it is less understood and less studied than MDM2 (12-fold more MDM2 publications than MDMX accessible on PubMed). I further speculate on potential models that could help explain *in vivo* MDM2 behavior. Finally, I end with a discussion of efforts to target MDM2 oligomers to restore p53 activity in tumors.

Background of MDM2 and MDMX

MDM2 is a member of the RING (Really Interesting New Gene) domain-containing E3 ligase family and contains at least three distinct regions that are highly conserved and critical to its function as an E3 ligase for p53. These three domains include an N-terminal p53 binding domain, a central acidic/zinc finger domain, and a C-terminal RING domain (Figure 1-3). The p53 binding domain, which resides within the first ~100 amino acids, is necessary for substrate recognition and transcriptional inactivation of p53.[158, 170, 234] The isolated MDM2 p53 binding pocket, which minimally involves amino acids 25-108,[219] appears to be sufficient to bind p53, at least *in vitro*.[158] The central acidic domain (AD), which can be considered to include the central zinc finger domain (residues 300-326), has been largely characterized as a regulatory domain; the AD binds to several small proteins

that can inhibit MDM2 and stabilize p53. The AD and adjacent intervening sequence can also be post-translationally modified by various kinases, including ATM (ataxia telangiectasia mutated),[207] ATR (ATM-related),[275] c-Abl,[99] Akt,[208] casein kinase 1δ,[326] and casein kinase 2,[105] which serves as another way (or a prerequisite) to achieve MDM2 enzymatic regulation (Figure 1-3). Although less well studied, some of these kinases also modify residues on the MDM2 homolog MDMX, which could contribute to the regulation of MDM2 function and p53 stability.[39, 40, 245, 343] A summary of the basic ways in which MDM2 can be inhibited through the AD is shown in Figures 1-3 and 1-4. Finally, the RING domain is responsible for the heterooligomerization of MDM2 with its homologous partner MDMX as well as for homooligomerization with other MDM2 molecules. The RING domain also contains the enzymatic activity of MDM2 and catalyzes the transfer of ubiquitin from the E2 to p53. The deletion of any of these three domains inhibits MDM2 function and stabilizes p53, suggesting that all three are required for MDM2-mediated p53 degradation.[110, 137, 156, 214] Other aspects of MDM2 worth noting include a nuclear localization sequence (residues 181-185) and a nuclear export sequence (residues 190-200), which are responsible for shuttling MDM2 into and out of the nucleus. Moreover, a cryptic nucleolar localization sequence has been identified near the C-terminus of MDM2.[190]

In its functional configuration as an E3 ligase, MDM2 forms homooligomers and heterooligomers with MDMX.[271, 299] MDMX is structurally similar to MDM2 (Figure 1-3), which reflects its evolution through a gene duplication event involving *MDM2* approximately 440 million years ago.[221] Despite extensive homology between these two genes (especially in the RING and p53 binding domains), MDMX itself does not possess appreciable intrinsic E3 ligase activity towards p53.[202, 276] Recent mutational analyses

offered insight into the basis for this difference in catalytic proficiency. Interestingly, only two point mutations (N448C and K478R) are sufficient to restore E3 ligase activity to the MDMX RING domain *in vitro*.[79] However, it appears as though additional regions of MDMX that deviate from MDM2, including the AD and the analogous NoLS region of MDMX, may be equally necessary to convert MDMX into a functional p53 E3 ligase in cells.[123] MDMX also does not contain a nuclear localization sequence (NLS), which accounts for its predominantly cytoplasmic localization. Despite not possessing an NLS, MDMX can be transported to the nucleus by piggybacking onto MDM2 in a RING domain-dependent manner.[176] Purified MDMX RING domains have recently been shown to form oligomers suggesting that MDMX may also form homooligomers in cells.[17] Interestingly, MDM2 transcription is upregulated by p53, forming an autoregulatory inhibitory feedback loop.[10, 327] For many years following its discovery, MDMX expression was thought to occur independently of p53 control;[276, 302] however, recent reports have identified a functionally active p53 response element within intron 1 of the *MDMX* locus.[175, 247] This response element can be induced in a p53-dependent manner in response to at least some stresses. Thus, MDM2 and MDMX appear to participate in negative feedback loops to control the p53 response, which could play a role in the rapid attenuation of the p53 response when an apoptotic outcome is not warranted. MDM2 and MDMX also share the ability to inhibit p53 transcriptional activity through direct binding and masking of the p53 transactivation domain.[220, 233] Another common thread between MDM2 and MDMX is their ability to be ubiquitinated by MDM2. At least under overexpressed conditions, MDM2 can auto-ubiquitinate itself, and MDM2 can ubiquitinate MDMX in *trans* resulting in their respective degradation. While MDM2 may be subject to degradation through other E3 ligases under

physiological conditions,[120] the ubiquitination of MDM2/MDMX by MDM2 offers an autoregulatory mechanism through which MDM2 can reduce its activity. One of the major questions that remains to be determined is how MDM2 directs its ligase activity towards p53, MDMX, or itself, such as in the context of the MDM2-MDMX-p53 ternary complex.[62, 136, 239] Recent studies have suggested that binding of small proteins such as p14ARF to the MDM2 AD could direct the substrate specificity of MDM2 toward MDMX.[182, 232] Under non-stressed conditions, MDMX is thought to stabilize MDM2, which could contribute to the increased potency of MDM2-MDMX heterooligomers for p53 ubiquitination relative to MDM2 homooligomers.[9, 138, 185, 319] On the other hand, the absence of MDMX effectively stabilizes p53, as the deletion of the *MdmX* gene in mice (like the deletion of the *Mdm2* gene in mice) results in an embryonic lethal phenotype that can be rescued by the concomitant deletion of p53.[241] Nonetheless, several studies have shown that MDM2 alone is capable of ubiquitinating and degrading p53.[299] Moreover, MDM2 may be sufficient for p53 degradation, as the conditional deletion of *MdmX* in adult tissue does not cause extensive p53 stabilization and apoptosis when compared with *Mdm2* deletion.[89]

Genetic mouse models have shown that the reactivation of p53 signaling is sufficient for the ablation of tumors.[204, 310, 332] Moreover, the activation of p53 signaling appears to be specifically damaging enough to cancer cells to spare normal tissue from severe side effects from therapeutics that stabilize or restore the activity of p53. To fully capitalize on the potential of drugs that activate p53, such as MDM2 inhibitors, understanding how p53 is controlled by MDM2 oligomers is important. Moreover, to understand how to manipulate MDM2 oligomers to stabilize p53, we must understand MDM2 oligomers in detail. In the following few sections, I discuss our current knowledge on MDM2 oligomer formation.

MDM2/MDMX oligomerization *in vitro* studies

The initial pieces of evidence for MDM2-MDMX synergy have come from *in vitro* studies. In 1999, Tanimura *et al*.,[299] and subsequently Sharp *et al*.,[271] revealed that MDM2 binds to MDMX through their respective RING domains. By 2003, Linares *et al*. showed that MDMX enhances MDM2 E3 ligase activity.[185] Subsequent studies revealed additional mechanistic details regarding the method of binding between MDM2 and MDMX. For example, although binding between the RING domains of MDM2 and MDMX was known, multiple studies showed that the extreme C-terminal tails (479-491 and 478-490 in human MDM2 and MDMX, respectively) of both proteins must be present for MDM2 oligomerization and E3 ubiquitin ligase activity.[249, 304] Uldrijan *et al*. also showed that point mutations within the MDM2 extreme C-terminus can be introduced that do not disrupt MDM2 oligomerization but inhibit p53 ubiquitination, suggesting that the extreme C-terminus may participate directly in the ubiquitin transfer reaction. Moreover, the lengths of the extreme C-terminal tails of MDM2 and MDMX are highly conserved, and the addition of residues that extend the length of either C-terminal tail inhibits MDM2 E3 function but not homo- or heterooligomerization.[72]

Another milestone in understanding MDM2-MDMX heterooligomerization is the publication of an x-ray crystal structure of a heterooligomer between the MDM2 and MDMX RING domains.[186] This structure was preceded by the NMR-based resolution of the MDM2 RING domain homooligomer and the MDM2-MDMX RING domain heterooligomer in solution.[145] These studies were largely consistent in describing the structure of the MDM2-MDMX RING heterooligomer; however, due to unstructured regions in MDM2, we lack full-length MDM2 protein oligomer structures. Without a full-length crystal structure of MDM2,

determining whether other domains participate in homo- and heterooligomerization must be accomplished by other techniques. Participation of other residues in addition to the RING domain and extreme C-terminus of MDM2 in oligomer formation seems likely, especially concerning MDM2 homooligomers. In describing the purification of the MDM2 RING homooligomer, Linke *et al*. declared that the relative instability of MDM2*s* (residues 432-491) homooligomers could be improved by including additional N-terminal residues (MDM2*l*, residues 417-491). Moreover, a recent study performed by Dolezelova *et al*. suggested that MDM2 homooligomers and MDM2-MDMX heterooligomers likely form through different mechanisms.[72] Another recent study (Chapter 2 of this dissertation) using overexpression co-immunoprecipitation experiments provided evidence supporting this theory, suggesting that MDM2 homooligomers and MDM2-MDMX heterooligomers require different domains of MDM2.[172] Thus, despite extensive similarities in the reported oligomeric RING structures of MDM2 RING homooligomers and MDM2-MDMX RING heterooligomers, MDM2 appears to bind to MDMX and other MDM2 molecules through different mechanisms, which could imply differences in MDM2 oligomer function or efficiency. Whether these differences in binding have implications on the cellular level and whether these differences have implications for the development of effective MDM2 inhibitors remains to be determined.

Further complicating the issue of MDM2 oligomerization, MDM2 and MDMX can form intramolecular interactions (Figure 1-5). The MDM2 RING domain can fold back and bind the AD, thereby generating additional tertiary structure that could play a role in oligomerization and MDM2 activity.[47] Similarly, in MDMX, the p53 binding domain and the RING domain can each fold back and interact intramolecularly with the AD, although

whether the AD can interact simultaneously with both the p53 binding domain and the RING finger remains unknown.[17, 41] Although these interdomain interactions may have direct implications for previously described intermolecular oligomerization (e.g., RING domain of one MDM2 molecule interacts with the AD of another MDM2 molecule[61]), intramolecular domain interactions likely play a more significant role based on their kinetic favorability due to covalent attachment. Interestingly, these intramolecular interactions have been implicated in MDM2 E3 ligase activity[47] as well as in MDMX nuclear localization.[41] Disruption of the MDMX RING-AD interaction increases nuclear localization, suggesting that its ability to bind MDM2 for nuclear import is impeded when present in the RING-AD intramolecular configuration. These types of studies offer hints to the supramolecular assembly of the MDM2 oligomers for which we do not have direct structural data. Collectively, these studies suggest that MDM2 ternary complexes involving p53 require more than simple RING-RING and p53-p53 binding domain interactions.

Regulation of MDM2(/MDMX) oligomers

Based on the importance of MDM2 oligomerization for regulating p53, understanding how oligomer formation is regulated is of crucial importance. MDM2 and MDMX levels and activities are regulated in many different ways, including at the transcriptional level (p53-MDM2/MDMX autoregulatory loops), direct binding by other proteins (ARF,[336] RPL5,[59, 201] RPL11,[191, 337, 339] CK1α,[40] CK1δ,[84, 326]), subcellular localization,[15] and post-translational modifications (phosphorylation, ubiquitination, neddylation, reviewed by[314]). The respective ADs of MDM2 and MDMX appear to feature prominently in the regulation of intermolecular interactions, including oligomerization. In

addition to the newly discovered intramolecular interactions involving the MDM2 and MDMX ADs, binding of the AD by small proteins and phosphorylation of the AD likely also contribute to MDM2 oligomer regulation. The AD-RING intervening sequence also appears to be a key regulatory point for MDM2 oligomerization. A study by Cheng *et al*. showed that phosphorylation of sequence upstream of the RING domain of MDM2 results in the inhibition of MDM2 RING domain oligomerization, which correlates with reduced E3 ligase activity toward p53.[46] Moreover, ATM-mediated phosphorylation of these residues in MDM2 inhibits the ability of MDM2 to oligomerize.[45] Interestingly, enforced oligomerization using a cross-linkable FKBP domain fused to the N-terminus of the MDM2 RING domain significantly enhances MDM2 E3 activity, which provides direct evidence that MDM2 oligomerization is a critical point of regulation for MDM2 activity. As one might expect based on homology, MDMX phosphorylation at a similar region could result in decreased MDM2-MDMX heterooligomer formation and stabilized p53 levels as well.

MDM2 E3 ligase activity may also be regulated indirectly by manipulating MDMX. Because MDM2-MDMX heterooligomers are more efficient E3 ligases, it is thought that directing MDM2 E3 ligase activity towards MDMX could effectively reduce MDM2 E3 activity and thus stabilize p53. In a recent study, overexpression of the small protein ARF could decrease MDMX levels and stabilize p53 by directing MDM2 E3 ligase activity toward MDMX instead of p53.[182] Consistently, in the absence of ARF (as in many tumors that express WT p53), MDM2 E3 ligase activity appears to be less readily diverted towards MDMX degradation, which could explain why tumors that retain WT p53 are pressured for the mutation of the ARF locus. Because ARF binds to the MDM2 AD and possibly not the MDMX AD,[318] it is tempting to speculate that the positively charged ARF is required to

stabilize the quaternary structure between the two MDM acidic domains. Similar observations were reported for RPL11.[97] In another study analyzing RPS27l knockout mouse cells, the induction of ribosomal stress by the absence of RPS27l resulted in MDM2-mediated MDMX degradation, which reduced MDM2-MDMX heterooligomer formation and stabilized p53.[331] The effect of RP binding on MDM2 oligomer formation has not been tested directly; however, based on the reported effects of ARF-MDM2 binding,[182] I suspect that RP-MDM2 binding may behave in a similar manner in terms of reducing MDM2-MDMX heterooligomer formation. Based on these studies, it would be interesting to determine whether ARF- or RP-mediated stabilization of MDM2 AD-MDMX AD binding could offer a viable method through which to generate x-ray crystallographs using the full-length proteins. Notably, Linke *et al*. reported that the presence of L-arginine, a positively charged amino acid, was necessary to obtain crystals of MDM2-MDMX RING domains.[186] If the conditions for full-length crystal structures of MDM2 can be resolved, then it would be interesting to determine how the binding of small proteins, such as ARF or RPL11, affects the overall structure and substrate selection of MDM2.

MDM2-MDMX heterooligomers are required *in vivo*

In addition to the abundance of *in vitro* evidence dissecting MDM2 oligomerization mechanics, several mouse models have been developed that offer complementary *in vivo* platforms to investigate MDM2 oligomerization. When the results of these studies are analyzed in aggregate, they offer mechanistic insight as well as a clearer picture of the importance of MDM2 oligomerization in p53 regulation. The earliest mouse models based on the knockout of *Mdm2* or *MdmX* have revealed p53-dependent embryonic lethal

phenotypes for either gene.[132, 222, 241] These studies were critical to show the importance of each of these two genes in p53 regulation. Moreover, these models show that neither MDM2 nor MDMX can completely compensate for the deficiency of the other. Of note, overexpressing an *Mdm2* transgene in mice can compensate for MDMX deficiency.[289] Nonetheless, although MDM2 and MDMX have been attributed with the ability to physically mask the p53 transactivation domain, it appears as though this activity is insufficient for the control of p53 during mouse development. This idea was supported by a study that showed that mice homozygous for the MDM2 RING domain structural mutation C462A, which abrogates MDM2 E3 activity and MDM2-MDMX heterooligomerization, die *in utero* in a p53-dependent manner.[120] As mentioned in the preceding section, the corresponding human MDM2 RING mutant C464A retains the ability to form homooligomers under overexpressed conditions,[172] which may contribute to residual p53 inhibitory activity, as MDM2 likely requires some type of oligomerization to function (discussed further below).

Subsequent knock-in mouse studies have hinted at the importance of MDM2-MDMX heterooligomer formation for the control of p53 activity during embryonic development. Two similar studies by Pant *et al*. and Huang *et al*. showed that mice expressing mutant versions of MDMX lacking the RING domain or lacking proper RING domain structure display an embryonic lethal phenotype.[115, 240] In these mice, although the p53 binding domain of MDMX remains intact and MDM2 is wild-type, the mice die *in utero* in a p53-dependent manner (approximately day E9.5). These studies suggest that heterooligomerization is the key to p53 control, at least during the embryonic stages of development. Interestingly, heterooligomerization is dispensable for p53 regulation during adulthood.[240] Consistently, the death of these mice, as well as all embryonic lethal

Mdm2/*MdmX* knock-in mice, approximately coincides with the stage in which p53 expression is dramatically and ubiquitously upregulated, suggesting the crucial need for effective p53 regulation during this stage of embryonic development (~E8.5).[268] Expounding on the C462A and RING *MdmX* knock-in mouse studies, Tollini *et al*. showed that mice expressing an *Mdm2* knock-in mutant (Y487A) that is E3-dead but retains the ability to heterooligomerize with MDMX are viable with no phenotypic differences under unstressed conditions.[303] Because the *Mdm2*$^{Y487A/Y487A}$ mouse lacks the ability to ubiquitinate p53, in the context of the *MdmX* knock-in models, it appears as though MDM2-MDMX heterooligomerization is sufficient to control p53 during embryonic development and is dispensable during adulthood, whereas MDM2 E3 ligase activity is sufficient (and necessary) to control p53 in adult tissue, especially in the presence of stress, and is dispensable during embryonic development.

Another thing that is apparent from analyzing the various *Mdm2*/*MdmX* mouse models is that MDM2 is a more effective regulator of p53 *in vivo* than MDMX.[312] Indeed, the loss of MDM2 is consistently more detrimental to mice than the equivalent loss of MDMX (reviewed in [312]). For example, in a study of mice expressing a brain-specific Cre-inducible p53 allele, Francoz *et al*. showed that *p53*$^{LSL/-}$*;MdmX*$^{-/-}$*;Nes-Cre* mice survived birth, whereas *p53*$^{LSL/-}$*;Mdm2*$^{-/-}$*;Nes-Cre* mice died during development.[89] In this study, assuming Cre-mediated gene excision occurred at similar efficiencies in the two mice, the presence of MDM2 alone resulted in a better outcome than the presence of MDMX alone. This trend is noticeable in other mouse models as well. Knockout mice homozygous for *Mdm2* deletion die at a slightly yet consistently earlier embryonic stage when compared with homozygous *MdmX* knockout mice (E5.5 for *Mdm2*-null vs. E7.5 for *MdmX*-null mice).[132, 222, 241]

Consistent with a more severe phenotype, *Mdm2* knockout mice display extensive apoptosis, whereas *MdmX* knockout mice display extensive cell cycle arrest.[37] In another study, brain-specific loss of MDM2 in mice resulted in death at E12.5 compared with brain-specific loss of MDMX, which resulted in death at E17.5.[329] Moreover, using a tamoxifen-inducible p53 fusion protein (p53ER) expressed in mice, the Evan lab showed in separate studies that the activation of p53ER in adult *Mdm2*$^{-/-}$ mice results in lethality around 5 days, whereas p53ER activation in adult *MdmX*$^{-/-}$ mice results in lethality in approximately 29 days.[93, 259] Interestingly, mice harboring an E3-dead, heterooligomerization-deficient *Mdm2*$^{C462A/C462A}$ mutant die around E7.5, which is closer to the *MdmX*-null mutant mouse, suggesting that MDM2^{C462A} might possess residual ability to inhibit p53 that is not present in the *Mdm2*-null mice.[120] What could explain the ability of the MDM2-C462A mutant to inhibit p53 *in vivo*? One possibility is the p53 binding domain, which can still bind to p53 *in vivo*.[120] The MDM2 p53 binding domain actually displays a 3- to 4-fold greater affinity for p53 than that of MDMX, which suggests that the presence of the MDM2 p53 binding domain in the *Mdm2*$^{C462A/C462A}$ mice offers an advantage in transcriptional suppression over that of the MDMX p53 binding domain, which is also present in these mice. Physical MDM2-C462A-p53 interaction could contribute to the delay in death of mice that express the MDM2 p53 binding domain compared with mice that do not (e.g., *Mdm2*$^{C462A/C462A}$ mice vs. *Mdm2* knockout mice),[162, 227] although further experiments will be required for confirmation. Moreover, although it is possible that MDM2^{C462A} protein retains the ability to suppress p53 through direct binding of the p53 transactivation domain, whether the homooligomerization capacity of MDM2 is required remains to be determined *in vivo*.

The MDM2-MDMX heterooligomer is a clearly more efficient E3 ligase platform than the MDM2 homooligomer.[110, 138] However, the secret behind the increased ligase efficiency of the heterooligomer (as well as the basic requirement for MDMX, which lacks E3 activity) remains a mystery. Structural data imply that MDMX binding to MDM2 creates a unique platform capable of more efficiently transferring the ubiquitin molecule from the E2 to p53.[145, 186] However, other models cannot be excluded based on current data. For example, MDM2 is commonly thought to bind directly to the E2. However, because direct evidence for E2 binding to MDM2 remains elusive, it is conceivable that MDMX could bind to and transport the cognate E2 to MDM2, which in turn catalyzes the transfer of ubiquitin to p53. Circumstantial evidence in support of this possibility includes the currently inexplicable observation that overexpression of MDMX in the presence of many different types of E3-dead MDM2 constructs can rescue MDM2 E3 ligase activity.[279, 303, 304] If MDMX is able or necessary to transport the E2-Ub to MDM2, then overexpression of MDMX could increase the intracellular concentration of MDM2-MDMX-Ub complexes in the cell, forcing the transfer of Ub to p53 and resulting in E3 ligase rescue. Observations in our lab suggest that MDMX shows specific binding to the E2 UbcH5 when compared with MDM2 (unpublished observations). Another consideration is that MDMX could bind to a different E2 than MDM2 does resulting in the targeting of different lysine residues or in the conjugation of differently linked Ub chains. The endogenous E2 associated with MDM2-dependent ubiquitination of p53 has not yet been confirmed, thus speculation persists regarding MDM2-MDMX-mediated ubiquitin transfer to p53. Perhaps the greatest advances in predicting and understanding MDMX enhancement of MDM2 E3 ligase activity could be

achieved through the analysis of complete full-length crystal structures of the heterooligomer compared with the homooligomer.

MDM2-MDMX heterooligomerization is important, what about MDM2 homooligomerization?

A uniting factor in all of the *Mdm2*/*MdmX* mutant mouse models is that the presence of MDM2 (even structural mutant MDM2^{C462A}) is sufficient to delay lethality. Notably, prior to the evolution of *MDMX*, MDM2 was tasked with the regulation of p53 for organismal survival. Thus, although MDM2 likely retains the ability to inhibit p53 by itself, the predominant mechanism *in vivo* remains to be determined. Early evidence has suggested that the direct binding of MDM2 to p53 is sufficient to suppress p53 activity.[220, 234] Although direct binding and inhibition of p53 could account for this delay in lethality, whether MDM2 homooligomerization also contributes to p53 degradation/inhibition *in vivo* is unknown. Studies confirming the *in vivo* existence and the role of MDM2 homooligomers need to be conducted; however, inherent difficulties are associated with analyzing MDM2 homooligomers *in vivo* because of the identical nature of the alleles of *MDM2*. With the increasing popularity and standardization of modern-era genome editing tools, a useful mouse or genome-edited cell model that could address these questions is one that expresses different epitope tags on each of the two *MDM2* alleles. Cells expressing endogenous levels of multiple epitope-tagged *MDM2* alleles could be used to confirm the existence of MDM2 homooligomers under physiological conditions. Another *Mdm2* knock-in model of interest is an MDM2 mutant that selectively prevents homooligomer formation while leaving heterooligomer formation intact. This model could be used to determine how

the abrogation of MDM2 homooligomers under physiological conditions affects MDM2 function and organismal fitness. Although point mutations may not be sufficient to achieve the selective inhibition of MDM2 homooligomers, the deletion of acidic domain residues appears to confer a selective defect in MDM2 homooligomer formation while leaving MDM2-MDMX heterooligomerization and E3 ligase activity largely intact, at least under overexpressed conditions.[172] Because E3 ligase activity appears to remain intact in the presence of MDM2 AD deletion constructs,[4, 341] one would expect viable offspring. However, the construction of a conditional knock-in mutant mouse harboring an inducible deletion of the MDM2 acidic domain could be a worthwhile endeavor to determine the effect of MDM2 AD deletion on survival and p53 stability during the adult stages. A better understanding of how MDM2 oligomers form and function *in vivo* could provide valuable insight into the design of MDM2-targeting drugs.

Targeting the MDM2(/MDMX) oligomer

Considering their crucial role in p53 regulation, particularly in the context of DNA damage, targeting MDM2/MDMX is a promising avenue of pharmacological research. MDM2/MDMX dual inhibitors could be particularly effective in tumors that overexpress these targets and harbor WT p53 (Figure 1-6). Nonetheless, most compounds developed to date offer relatively specific MDM2 inhibition by targeting the p53 binding domain on MDM2.[308, 338] These inhibitors have been carefully optimized, and some have shown strong efficacy in preclinical studies; however, their efficacy in patients has been underwhelming. The lack of clinical success with MDM2 inhibitors can be attributed to multiple factors, the most prominent of which is on-target toxicity.[254] Thus, although highly potent MDM2

inhibitors are currently available (RG7112, K_d=11 nM; MI-888, K_d=0.44 nM; RO-5963, IC_{50}=33 nM), better targeting mechanisms or dosing optimization, perhaps by combining MDM2 inhibitors with other therapies, may be necessary to achieve the best patient outcomes. However, even if side effects could be minimized, it is possible that MDM2 inhibition alone could be ineffective for several types of cancers.[313] For example, inhibitors that target only MDM2 and not MDMX, such as nutlin-3a (400-fold less effective against MDMX than against MDM2), are ineffective against cancer cells that overexpress MDMX presumably through MDMX-mediated inhibition of p53.[111, 242, 311] Moreover, long-term treatment with MDM2 inhibitors introduces a selective pressure that could promote the development of p53 mutations and multi-drug resistance.[8, 215] Another issue unique to MDM2-specific inhibitors is the requirement for high doses of the drug to counter the p53-regulated increase in MDM2 expression as a result of inhibiting MDM2 in the first place. Although some of these issues also apply to MDM2/MDMX dual inhibitors, current efforts in MDM2 inhibition have paid more attention to the effects of MDMX as well.

Differences in the structures of the p53 binding pockets of MDM2 and MDMX present a challenge to the development of drugs that effectively target both molecules.[257] Nonetheless, recent drug candidates have emerged that simultaneously target MDM2 and MDMX ([34, 100, 167] see [141] and [23] for detailed reviews of MDM2 drugs). In a study by Graves *et al*., the authors show that RO-5963, an indolyl hydantoin derivative, binds the p53 binding pocket of MDM2 and MDMX with similar affinities *in vitro*. Moreover, RO-5963 binding simultaneously engages MDM2 and MDMX, resulting in the enforced oligomerization of the two molecules. This results in p53 stabilization and cell death in several cancer cell lines, especially in cancer cells expressing high levels of MDMX, which is a promising proof-of-

principle aspect of dual MDM2-MDMX inhibition. Although the clinical utility of this compound as a single agent may not be likely possibly due to the compensatory induction of MDM2, the use of compounds such as RO-5963 in conjunction with other p53-activating compounds may prove effective for certain types of tumors.

In addition to preventing interaction with p53, other methods of inhibiting MDM2 and MDMX have involved the inhibition of E3 ligase activity and MDM2/MDMX heterooligomerization. Early studies suggested that the MDM2-MDMX RING-RING oligomer was not an attractive drug target due to the lack of a defined catalytic site and a predominantly hydrophobic interaction-based binding mechanism.[145, 186] Nonetheless, experimental evidence has suggested that the inhibition of MDM2-MDMX heterooligomerization could be effective, as MDMX RING domain overexpression competes for MDM2 binding, inhibits endogenous MDM2-MDMX oligomer formation, and results in the stabilization and activation of p53.[138, 287] Small molecules that inhibit MDM2 enzymatic activity have been reported, including HLI98 and MDM2 E3 Ligase (MEL) inhibitors. While HLI98 showed specificity for MDM2 inhibition *in vitro*, it also displayed non-specific effects and p53-independent effects at higher concentrations in cells as well as poor pharmacological characteristics overall.[334] A more promising candidate of the MDM2 ligase inhibitor class, the MEL series of inhibitors (MEL-23 and MEL-24) showed specific inhibition of MDM2-MDMX E3 ligase activity, stabilization of p53, and the induction of apoptosis in a p53-dependent manner.[103] Though more work using *in vivo* models is required to validate the MEL inhibitors, targeting MDM2-MDMX enzymatic activity may not be as difficult as once thought. In an even more recent study using computational and rational methods, a peptide-based inhibitor of MDM2-MDMX oligomerization was

reported.[243] This inhibitor, named Peptide3, showed a p53-dependent apoptotic response; however, its reported mechanism of action is peculiar in that only *nuclear* MDM2-MDMX heterooligomerization is affected. Perhaps using a more thorough and unbiased screening method, such as phage display, coupled with emerging peptide technologies, such as peptide stapling, could yield even more effective peptide-based MDM2-MDMX oligomerization inhibitors.

Another area of intense research includes the development of p53 mimetic stapled peptides. Stapled peptides are short polypeptide sequences that are optimized to bind to specific clefts within proteins and are stabilized with a hydrocarbon "staple." p53 appears to be an ideal candidate for the development of stapled peptide mimetics because binding between the transactivation domain of p53 and its inhibitor MDM2 requires a p53 alpha-helix that makes three critical contacts with the MDM2 binding cleft.[158] This alpha-helix of the stapled peptide can form spontaneously in solution; however, the inclusion of a hydrocarbon staple not only locks the alpha-helix in the proper configuration but also enhances the non-polar nature of the overall molecule thereby possibly enabling diffusion through the membranes of cells. In a study by Chang *et al*., the authors describe a stapled α-helical peptide that binds to the p53 binding domains of MDM2 and MDMX with low-nanomolar affinities.[34] Though the mechanism of delivery remains controversial, stapled peptide technology offers the unique opportunity to specifically mimic the p53 binding site in MDM2 and/or MDMX with strong affinity. It will be interesting to see how far p53 stapled peptide mimetic drug candidates, such as ATSP-7041, can progress through clinical trials.

Expanding the tumor spectrum of MDM2/MDMX inhibitors

What is even more exciting is how the spectrum of tumors that respond to drugs that inhibit MDM2 oligomerization and function (wild-type p53 and/or overexpressing MDM2/MDMX) could be increased by combining them with complementary drugs that also target the MDM2/MDMX-p53 pathway. For example, the compound APR-246 (also known as PRIMA-1^{MET}), a mutant p53 reactivator, has shown some promise in patients harboring mutant p53 tumors in an early-stage clinical trial.[169] The combination of APR-246 with various standard chemotherapeutics has also shown synergistic effects in the treatment of cancer cells and warrants further investigation.[24, 224, 296] Moreover, considering the reduced efficacy of MDM2 inhibitors in many p53-mutant tumors as well as the selective pressure that MDM2 inhibitors, such as nutlin-3a, exert on tumors to develop p53 mutations,[8, 215] p53 mutant reactivators could be effective adjuvants to increase the scope of MDM2/MDMX inhibitors. In fact, because many p53 mutations result in gain-of-function phenotypes,[70] the treatment of p53 mutant tumors with MDM2 inhibitors may actually require p53 reactivation.

Another therapeutic modality being investigated is the use of aptamers, short single-stranded DNA or RNA constructs that can be engineered to bind specific proteins. Though delivery of nucleic acids remains an obstacle, aptamers offer the ability to specifically target deleterious point mutations characteristic of tumors, such as the p53 mutation R175H.[42] Moreover, delivery of p53R175H aptamers via nanoparticles showed impressive efficacy in a mouse xenograft tumor model. Similarly to the potential for PRIMA-1^{MET} synergism with MDM2 inhibitors, aptamers could also be useful adjuvants to expand the effective tumor profile of MDM2 inhibitors.

Because cancer is a heterogenous disease with multiple subclonal populations, future curative cancer treatment attempts may focus on targeting all of the tumor clone subsets. Because aberrant p53 signaling is an early event in many types of tumors, combining direct p53-targeting treatments with drugs that target upstream factors like MDM2 inhibitors could result in synergistic effects that could be effective in many types of tumors. Though no p53-activating drugs have been approved for clinical use, the future of p53-targeting drugs offers a promising, relatively unexplored avenue through which a broad array of tumors may be treated.

Concluding remarks on MDM2/MDMX

Though we have made great progress in understanding MDM2 oligomerization and how MDM2 can affect tumor development, basic concepts remain to be determined. Some crucial aspects about MDM2 oligomerization that remain to be discovered include the following: How does MDMX enhance MDM2 E3 ligase activity toward p53? What role do MDM2 homooligomers play *in vivo*? What mechanisms control the direction of MDM2 E3 activity toward p53 vs. other substrates (e.g., MDMX)? What residues of MDM2 are necessary for homo- vs. heterooligomerization? Are other domains of MDM2 worthy targets for the development of small molecules that can activate p53? As we ponder new methods to address these questions, we eagerly anticipate new insights from the brilliant minds of the MDM2/p53 field.

Dissertation questions and goals

How do different mutations in MDM2 affect its oligomerization capacity and its ability to degrade p53?

Considering the importance of MDM2 oligomers in the regulation of p53, understanding how MDM2 functions, especially in terms of its most active oligomeric form, is important. In Chapter 2, I present my original mutational analysis study on MDM2 homo- and heterooligomer formation. In the design of this study, I sought to investigate how mutations in key residues and domains of MDM2 affect its ability to interact with MDM2 or MDMX as well as its ability to regulate p53. In this study, I show evidence that MDM2 homooligomerizes in a manner that depends on the C-terminal residues (but not the structure) as well as the acidic domain of MDM2. Moreover, MDM2-MDMX heterooligomerizes in a manner that depends on the structure of the RING domain but does not require the acidic domain of MDM2. This study offers novel insight suggesting that MDM2 homooligomers and MDM2-MDMX heterooligomers form in uniquely different manners. Moreover, through this study, I identified an MDM2 mutation (Δ245-295) that can selectively inhibit MDM2 homooligomer formation but not MDM2-MDMX heterooligomer formation or p53 ubiquitination. In Chapter 4, I speculate on additional questions that could be investigated using the findings of this study.

Identify novel p53 targets and determine how it fits into the p53 regulome

As introduced in the above sections, Chapter 3 describes my original study identifying and characterizing LRP1 as a novel p53 target gene that is selectively induced in response to sub-lethal stress. Based on the widespread functions of p53, identifying novel

target genes contributes to our understanding of how p53 might coordinate its tumor suppressive effect. The identification of p53 target genes outside of the canonical p53 gene programs (cell cycle arrest, apoptosis, and senescence) is especially useful, considering the studies described above that found these programs dispensable for the tumor suppressive effect of p53.[21, 181, 306] I show that LRP1, a multi-ligand-binding surface receptor that is associated with many different diseases, is upregulated at the transcriptional level in response to sub-lethal and lethal doses of doxorubicin but is only effectively expressed at the protein level in response to sub-lethal stress. I provide evidence suggesting that LRP1 *de novo* translation is impaired in response to lethal doses of doxorubicin, possibly through a p53-regulated miRNA-dependent mechanism. I also observe evidence of a possible p53 threshold type of mechanism, as only high levels of doxorubicin are able to induce certain p53 target genes, including some miRNAs. Although the study presented is not complete, our results thus far suggest the existence of a new layer of self-regulation for p53 target genes that involves the selective expression of miRNAs and possibly the repression of certain p53 target genes. In Chapter 4, I discuss in detail future experiments that would be interesting to test our observations *in vivo* and *in vitro* as well as clinical implications for our findings.

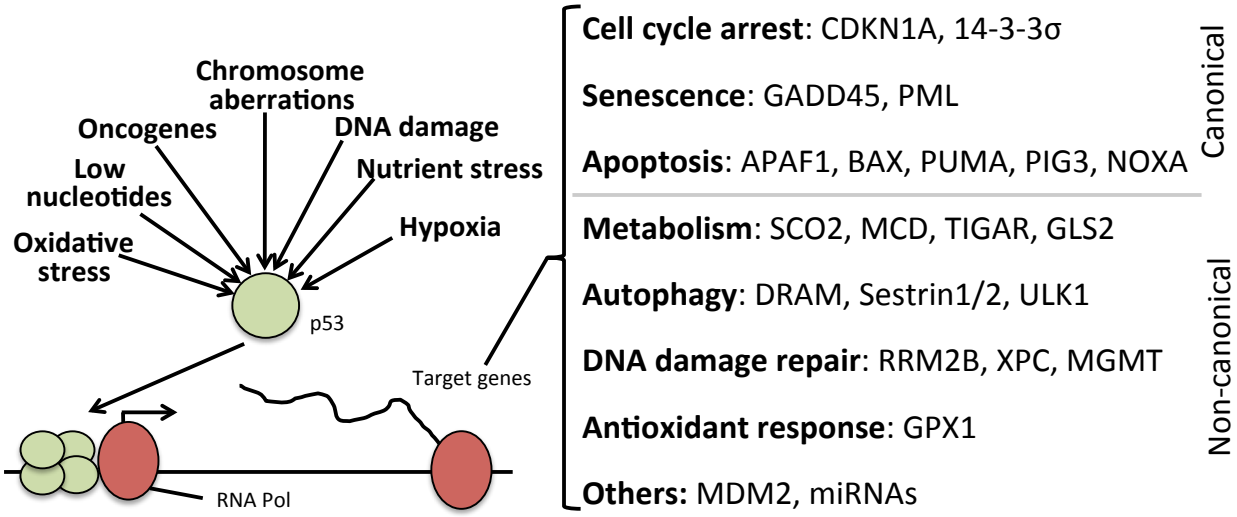

Figure 1-1. p53 contributes to the regulation of several genes and pathways.
p53 is activated by numerous stresses and functions as a transcription factor for a diverse array of genes involved in a diverse array of pathways. p53 binds to a canonical well-defined response element near target genes in a tetrameric form. A non-exhaustive list of activating stresses and target genes/pathways is presented above. RNA pol: RNA polymerase, CDKN1A: cyclin-dependent kinase inhibitor 1A, GADD45: growth arrest and DNA damage inducible 45-alpha, PML: promyelocytic leukemia, APAF1: apoptotic peptidase activating factor 1 , BAX: Bcl-2-associated X protein, PUMA: p53 upregulated modulator of apoptosis, PIG3: p53-induced gene 3, NOXA: phorbol-12-myristate-13-acetate-induced protein 1, SCO2: cytochrome C oxidase assembly protein, MCD: malonyl coA dehydrogenase, TIGAR: tp53-inducible glycolysis and apoptosis regulator, GLS2: glutaminase 2, DRAM: damage-regulated autophagy modulator, ULK1: UNC-51 like autophagy activating kinase 1, RRM2B: ribonucleotide reductase subunit M2B, XPC: xeroderma pigmentosum complementation group C, MGMT: O-6-methylguanine DNA methyltransferase, GPX1: glutathione peroxidase 1, MDM2: mouse double minute 2, miRNA: microRNA.

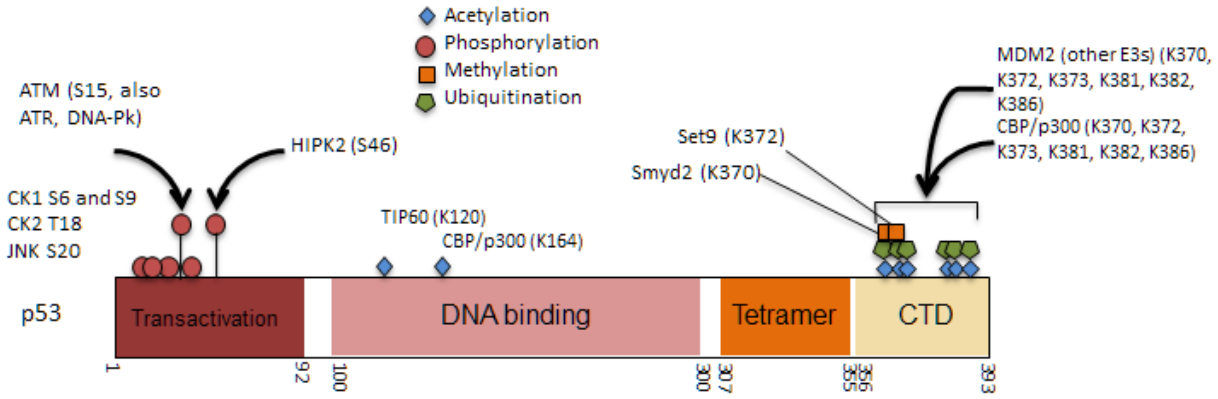

Figure 1-2. Diagram of p53 protein domains and modifications.
p53 consists of four domains: the N-terminal (AA1-92) transactivation domain, a central DNA binding domain (AA100-300), and C-terminal tetramerization (AA307-365) and C-terminal regulatory (AA366-393) domains. p53 is subject to several post-translational modifications (PTMs) that affect its activity and stability, including acetylation, phosphorylation, methylation, and ubiquitination, among others. Some of the major PTMs are indicated above along with the corresponding protein that introduces these PTMs.

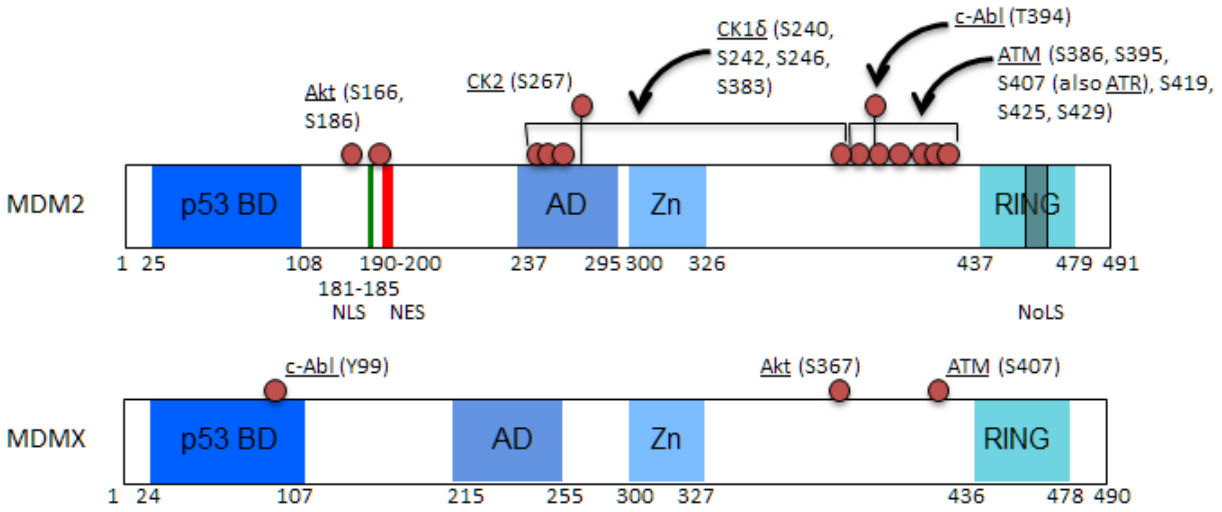

Figure 1-3. MDM2 and MDMX share significant homology.
MDM2 and MDMX harbor similar structural domains, including an N-terminal p53 binding domain, a central acidic domain and C4 zinc finger domain, and a C-terminal RING domain. The central AD and Zn regions serve as binding sites for several small proteins, including ribosomal proteins and ARF, any of which results in the inhibition of MDM2 E3 ligase function. MDM2 can bind to other MDM2 molecules of to MDMX through their respective C-terminal RING domains. In contrast to MDMX, MDM2 possesses a nuclear localization sequence (NLS), a nuclear export sequence (NES), and a cryptic nucleolar localization sequence (NoLS). Moreover, whereas MDM2 exerts E3 ligase activity towards p53, MDMX does not possess appreciable E3 ligase activity. Red circles indicate phosphorylation sites. p53 BD: p53 binding domain, AD: acidic domain, Zn: C4 zinc finger domain, RING: really interesting new gene domain, NLS: nuclear localization sequence, NES: nuclear export sequence, NoLS: nucleolar localization sequence, CK: casein kinase.

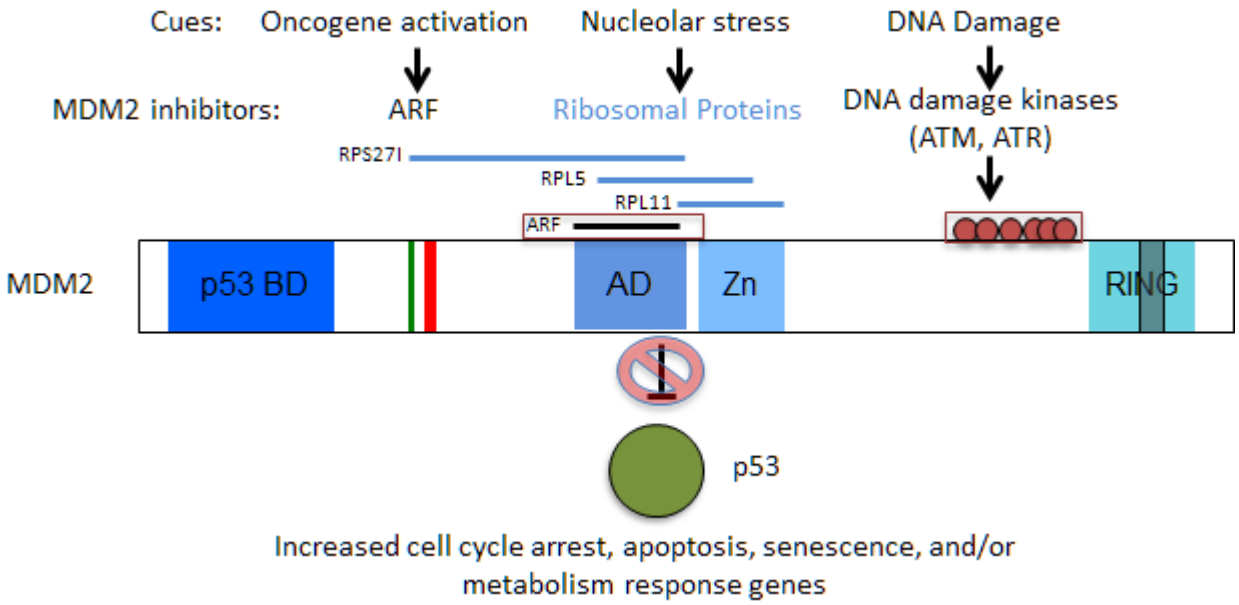

Figure 1-4. Many different p53-activating stresses are channeled through MDM2. MDM2 is modified post-translationally (phosphorylation by DNA damage kinases) or by direct protein binding (RPs and ARF) in response to various stresses (cues). When affected by any of the MDM2 inhibitors, MDM2 loses its ability to bind to and regulate p53, resulting in stabilized p53 levels and increased p53 transcriptional activity. Ablation of any of the three arms of MDM2 regulation severely impairs the activation of p53 in response to the corresponding stress cue. Red circles denote phosphorylation sites, the blue lines denote the binding sites of the indicated RP, and the black line represents the binding site of ARF. The inhibitors boxed in red have been shown to affect MDM2 homo- or heterooligomer formation. ARF: alternative reading frame.

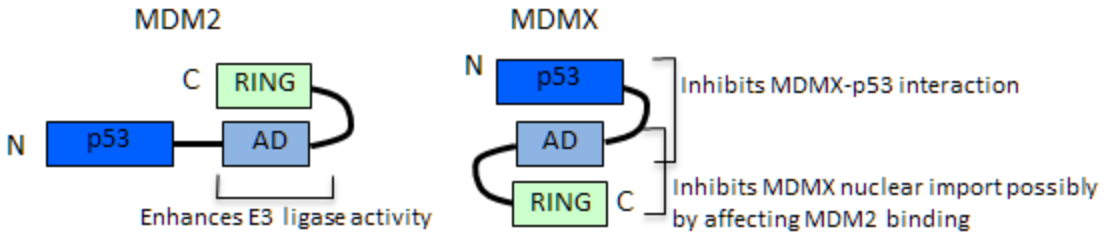

Figure 1-5. MDM2 and MDMX form intramolecular interactions.
Recent studies have shown that MDM2 forms intramolecular interactions that involve RING-AD interactions. MDMX is also capable of RING-AD and p53BD-AD intramolecular interactions. The implications of these intramolecular interactions remain unclear particularly in terms of function and oligomerization. However, these intramolecular interactions have clear effects on p53 regulation and likely effects on MDM2-MDMX oligomerization. p53: p53 binding domain, AD: acidic domain, RING: really interesting new gene domain.

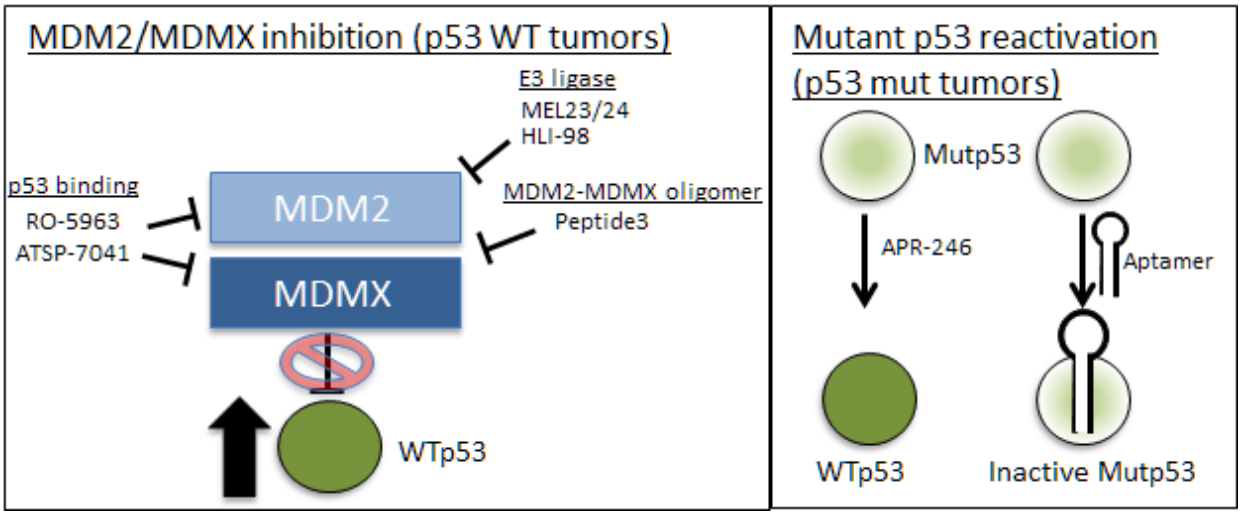

Figure 1-6. The clinical efficacy of reactivation, inactivation, and stabilization of p53 through direct means or through MDM2/MDMX inhibition are being evaluated.
The MDM2/MDMX-p53 pathway is currently being targeted through two broad mechanisms: MDM2/MDMX inhibition through small molecules (RO-5963, MEL23/24, HLI-98) and stapled peptides (ATSP-7041) and mutant p53 reactivation through compounds such as APR-246 and inactivation through compounds such as aptamers. MDM2/MDMX inhibition could be particularly useful for p53 WT tumors, whereas mutant p53 reactivation/inactivation could be useful for mutant p53 tumors and for the expansion of the tumor spectrum for which MDM2 inhibitors may be used. Mutp53: mutant p53, WTp53: wild-type p53.

CHAPTER 2: THE MDM2 ACIDIC DOMAIN AND RING DOMAIN PLAY DISTINCT ROLES IN MDM2-MDM2 HOMODIMERIZATION AND MDM2-MDMX HETERODIMERIZATION[2]

INTRODUCTION

True to its title of "guardian of the genome," the tumor suppressor p53 is a transcription factor that promotes the transcription of an array of genes responsible for preventing cells from proliferating in the presence of DNA damage. Various stresses such as DNA damage activate p53, which in turn upregulates several genes involved in cell cycle arrest and apoptosis.[68, 81, 225, 228] As a major factor involved in the inhibition of cell growth and division, the *TP53* gene is mutated in many different types of cancer.[246] Interestingly, many cancers that do not harbor a mutation directly affecting p53 often harbor mutations in genes that regulate p53, resulting in p53 inactivation.[121, 161] The oncoprotein murine double minute 2 (MDM2) is a particularly important p53 regulator that is overexpressed in nearly one-third of sarcomas that harbor wild-type p53, consistent with the idea that the overexpression of MDM2 could substitute for p53 mutation.[233]

MDM2 is best known for its role as an E3 ubiquitin ligase that ubiquitinates p53, thereby targeting p53 for proteasomal degradation.[102, 109, 155] Knockout of *Mdm2* results in

[2] This chapter is adapted from an original research article published in the *Journal of Biological Chemistry*. The conceptual design of the study was developed by me and Yanping Zhang. Assistance with pyMOL molecular modeling was provided by Henming Ke. All experiments were performed by me. The manuscript was written and edited by me under the mentorship of Yanping Zhang. The original citation is as follows: Leslie PL, Ke H, Zhang Y. (2015) The MDM2 RING Domain and Central Acidic Domain Play Distinct Roles in MDM2 Protein Homodimerization and MDM2-MDMX Protein Heterodimerization. *Journal of Biological Chemistry*. 290(20): 12941-50.

embryonic lethality in mice that can be rescued through the concomitant deletion of p53, emphasizing the importance of MDM2 in p53 regulation.[132] In its active form, MDM2 forms homooligomers and/or heterooligomers with its homologous partner MDMX presumably through their C-terminal Really Interesting New Gene (RING) domains.[299] In addition to its role in oligomerization, the RING domain of MDM2 is also necessary for catalyzing the transfer of ubiquitin to p53.[85] Although MDMX contains a RING domain that is very similar to that of MDM2, MDMX lacks appreciable E3 ligase activity towards p53. Nonetheless, the MDM2-MDMX heterooligomer appears to be required for p53 degradation at least during embryonic development, as mice harboring MDMX deletion or MDMX mutations that prevent heterooligomerization with MDM2 show an embryonic lethal phenotype that can be rescued by concomitant p53 deletion.[115, 240]

Many of the studies that have contributed to our understanding of MDM2 oligomerization have used isolated domains of MDM2 such as the RING domain (human MDM2 residues 384-491).[72, 304] Although the study of isolated protein domains can offer insight into the function of individual protein domains, these studies fail to account for the behavior of these domains in the context of the full-length protein. Therefore, a comprehensive understanding of MDM2 behavior and function can only be obtained by studying the full-length protein. In this study, I use several known and novel MDM2 mutant constructs in the context of the full-length protein to show that MDM2-MDM2 interaction requires the central acidic domain and the extreme C-terminal residues of MDM2, whereas MDM2-MDMX interaction requires the proper RING domain structure and the extreme C-terminal residues of MDM2.

RESULTS

MDM2 residue N447 is important for p53 degradation

Kostic *et al.* performed a detailed investigation of MDM2-MDMX RING domain binding by NMR.[145] In this study, radiolabeled purified MDM2 RING domains were titrated with unlabeled purified MDMX RING domains, and differences in the heteronuclear single quantum coherence (HSQC) spectra were obtained for each MDM2 residue within the RING domain upon MDMX binding. The greatest chemical shift occurred at asparagine residue 447 in MDM2 suggesting that N447 may be important for MDM2-MDMX binding. Initial analysis of the MDM2 sequence indicated that many of the residues within the C-terminal MDM2 RING domain show a high degree of identity among different species. N447 is conserved in many species from humans to zebrafish, consistent with the idea that this residue serves an important role in MDM2 function (Figure 2-2A). Additionally, upon analyzing the MDM2-MDMX RING domain heterodimer crystal structure,[186] N447 appears to form an intramolecular hydrogen bond with the backbone amino group of Phe residue 462 (Figures 2-1A and B). To determine whether the N447 side chain is indeed important for MDM2 function, I used site-directed mutagenesis to convert the N447 residue to aspartic acid or alanine (Figure 2-2B). I predicted aspartic acid, which contains a side chain of comparable size and polarity to asparagine, should maintain the hydrogen bond and the proper three-dimensional structure and function of MDM2 (Figure 2-1C). However, the alanine substitution mutant, which lacks a side chain oxygen residue and is considerably smaller than the asparagine side chain, should lose the ability to bond with F462 thereby preventing proper structure and function of MDM2 (Figure 2-1D).

To test the importance of N447 in MDM2 function, MDM2 point mutation constructs were assembled in untagged and FLAG-tagged expression plasmids. Consistent with our modeling predictions, endogenous p53 levels decreased in cells transfected with either WT MDM2 or the N447D mutant when compared with the empty vector control. Likewise, p53 levels increased in cells transfected with the E3-dead C464A mutant or the N447A mutant (Figure 2-2C). These results suggest that whereas the N447A mutation inhibits the ability of MDM2 to degrade p53, the N447D mutation does not appreciably affect MDM2-mediated p53 degradation. I observed similar results when I tested these mutants on exogenously over-expressed p53 in H1299 cells (p53-null, Figure 2-2D). Mutation of N447 to either Val or Glu also inhibits the ability of MDM2 to degrade p53, further highlighting that small perturbations in the N447 residue are sufficient to inhibit MDM2 activity (Figure 2-3). Although cells transfected with the N447D mutant showed significant p53 degradation, a noticeable increase in p53 levels was observed with the N447D mutant when compared with WT MDM2, which reflects decreased E3 ligase activity likely due to differences between the Asp side chain and the naturally occurring Asn side chain. Treatment of the transfected cells with MG132 prevented degradation of p53 and MDM2 by WT MDM2 and the N447D mutant, suggesting that degradation occurs predominantly through the proteasome (Figure 2-2E).

N447A half-life increases due to lack of ubiquitination activity

To further determine the effect of the N447 mutations on p53 and MDM2 stability, half-life assays were conducted for the N447A and N447D mutants in U2OS cells. Expression of the E3-active WT or N447D mutant constructs shortened the half-life of p53

(58 min and 69 min, respectively, compared with 116 min for empty vector), which is consistent with intact MDM2 E3 activity (Figure 2-4A and B). Conversely, the p53 half-life was dramatically extended in the presence of the C464A or N447A MDM2 mutants (over 210 min for both compared with 116 min for empty vector), consistent with diminished MDM2 E3 activity (Figure 2-4C and D). Analysis of the half-life of the overexpressed MDM2 revealed a similar trend whereby WT and N447D mutants showed relatively short half-lives (69 mins and 77 min, respectively) when compared with C464A and N447A mutants (>210 min for both), consistent with the ability of ectopically overexpressed WT and N447D MDM2 proteins to promote their own degradation (Figure 2-4A-D).

Next, I conducted *in vivo* ubiquitination assays to determine whether the MDM2 mutants show differences in p53 and/or MDM2 ubiquitination. To assess p53 ubiquitination, H1299 cells (p53 negative) were transfected with FLAG-p53, HA-ubiquitin (HA-Ub), and untagged MDM2 constructs for 24 hours. Consistent with our previous results (Figure 2-4), WT MDM2 and the N447D mutant polyubiquitinated p53, whereas the C464A and N447A mutants were unable to ubiquitinate p53 (Figure 2-5A). To assess MDM2 autoubiquitination, the FLAG-MDM2 constructs were individually transfected into H1299 cells along with HA-ubiquitin. Consistent with the p53 ubiquitination assay results, WT and the N447D mutant showed autoubiquitination laddering, whereas C464A and N447A mutants displayed significantly less laddering (Figure 2-5B).

RING-disruptive mutations inhibit MDM2 binding to MDMX but not to MDM2

Because MDM2 is thought to require homo- and/or heterooligomerization with MDMX to function as an effective E3 ligase,[109, 299] I sought to determine how these

mutations affect MDM2 homo- and heterooligomerization. To this end, U2OS cells were co-transfected with myc-tagged WT MDMX and each of the FLAG-MDM2 mutant constructs and were then subjected to co-immunoprecipitation (co-IP). As expected, the MDM2 constructs capable of degrading p53 (WT and N447D) formed robust heterooligomers, whereas MDM2 constructs incapable of degrading p53 (C464A and N447A) failed to heterooligomerize (Figure 2-6A). I noticed a consistent decrease in heterooligomerization between MDMX and the N447D mutant relative to WT MDM2, suggesting that the N447D mutation marginally affects MDM2-MDMX binding.

To determine whether the MDM2 mutant constructs could interact with MDM2, I conducted a similar co-IP experiment with WT myc-MDM2 instead of myc-MDMX. Surprisingly, all of the constructs were able to pull down WT MDM2 to similar extents (Figure 2-6B). Because the RING domain of MDM2 has been thought to be responsible for MDM2 homooligomerization, I expected that the C464A and N447A mutants, which were unable to heterooligomerize with MDMX, would also fail to interact with MDM2. However, despite harboring point mutations that prevent binding with MDMX, C464A and N447A mutant MDM2 maintained robust binding with WT MDM2 to an extent similar to WT MDM2 itself, suggesting that MDM2-MDMX and MDM2-MDM2 interactions require different sets of residues for binding.

Deletion of the extreme C-terminal residues prevents MDM2 interaction with MDM2 and MDMX

Previous studies have shown that MDM2 sequence at the extreme C-terminus is necessary for homo- and heterooligomer formation.[249, 304] Because our co-IP results

suggested that MDM2 homo- and heterooligomers may require different residues, I sought to determine the effect of an extreme C-terminus deletion of MDM2 on its ability to interact with full-length MDM2. To this end, I constructed MDM2 C-terminal deletion mutants lacking the RING domain (amino acids 437-479, ΔRING), the extreme C-terminal 12 residues (480-491, ΔC-term), or both the RING domain and the C-terminal residues (437-491, ΔRING/ΔC-term) (Figure 2-7A). Consistent with our expectations, all of these constructs lacked the ability to degrade endogenous p53 (Figure 2-7B). Furthermore, co-IP experiments conducted to determine their ability to bind MDMX or MDM2 revealed that all three deletion constructs failed to interact with either MDMX or MDM2 full-length constructs (Figures 2-8A and B). These data suggest that although the proper structure of the RING domain may not be required for MDM2-MDM2 interaction, the presence of the RING domain and the C-terminal residues that lie downstream of the RING domain of MDM2 are necessary for interaction with both MDMX and MDM2.

MDM2 acidic domain is necessary for interaction with MDM2 but not with MDMX

MDM2 has been reported to oligomerize through RING-acidic domain (AD) interactions.[61] However, a more recent study has suggested that AD-RING interactions occur intramolecularly, which may actually be more physiologically relevant than potential intermolecular AD-RING domain interactions. Consistent with the idea that intermolecular RING-AD interactions may not significantly affect MDM2-MDM2 binding in the cellular milieu, the ΔRING MDM2 mutant, which harbors an intact AD, is severely impaired in its ability to bind to WT MDM2, which contains both the AD and the RING domain (Figure 2-8B). Nonetheless, to investigate the possible effects of the AD on the observed interactions

of our mutant constructs, I deleted a portion of the AD (Δ245-295) in the WT, C464A, N447A, and N447D constructs to determine the effect of AD deletion on MDM2 oligomerization and p53 degradation (Figure 2-9A). Consistent with previous reports, AD deletion inhibited the degradation of endogenous p53 (Figure 2-9B) and ectopically expressed p53 (Figure 2-9C).

Co-IP analysis for oligomerization with MDMX revealed that AD deletion does not affect heterooligomer formation of WT or N447D MDM2, consistent with the idea that heterooligomerization is necessary for ubiquitination activity (Figure 2-10A, also Figure 2-6A). Furthermore, these results suggest that the AD is dispensable for heterooligomerization, which likely occurs exclusively through RING domain/C-terminal interactions. However, when analyzed for their ability to interact with WT myc-MDM2, the AD deletion mutants displayed an inverse trend. When the AD was deleted in the E3-dead C464A or N447A constructs, the ability to interact with WT MDM2 was largely unaffected. However, when the AD was deleted in the E3-active WT or N447D constructs, their ability to bind WT MDM2 was abrogated, suggesting that MDM2-MDM2 binding depends on the AD (Figure 2-10B).

To further investigate the E3-dead (C464A and N447A) AD deletion mutants, the extreme C-terminal residues were deleted to determine whether these oligomers require the C-terminus. When the extreme C-terminus was deleted, the C464A and N447A AD deletion mutants failed to interact with WT MDM2 (Figure 2-10C), suggesting that the C-terminal tail is absolutely required for MDM2 oligomerization.

When subjected to p53 and MDM2 *in vivo* ubiquitination assays, AD deletion did not affect ubiquitination of p53 or MDM2 (Figures 2-11A and B). These results are consistent

with some early reports analyzing various AD deletions and may suggest that the AD is necessary for p53 and MDM2 degradation downstream of ubiquitination.[4, 341]

To address whether co-expression of an AD-binding protein might affect MDM2 oligomer formation, I co-transfected U2OS cells with constructs encoding FLAG-MDM2, myc-p14ARF (0 μg, 0.1 μg, or 0.5 μg), and either myc-MDMX or myc-MDM2. After protein extraction, the lysates were probed with anti-FLAG beads and then examined by western blot to determine how much myc-MDMX or myc-MDM2 was pulled down. Our results show that p14ARF co-expression decreased MDMX levels resulting in less MDMX co-IP while increasing MDM2 levels and correspondingly increasing the amount of MDM2 pulled down by co-IP (Figures 2-12A and B, respectively). While it is difficult to determine whether there is a change in binding affinity between FLAG-MDM2 and myc-MDMX or myc-MDM2 using this method, it appears that p14ARF at least increases the overall amount of MDM2 homooligomers and decreases the overall amount of MDM2-MDMX heterooligomers, which is consistent with previous findings.[45, 46, 119, 182]

DISCUSSION

MDM2 has long been known to form homooligomers and heterooligomers with the structurally related MDMX protein. Although data have convincingly shown the importance and necessity of MDM2-MDMX binding for p53 regulation, the physiological significance of MDM2 homooligomers remains unknown. *In vitro* studies have shown that purified MDM2 in the absence of MDMX is sufficient to ubiquitinate and presumably elicit the degradation of p53.[109, 249] Furthermore, several independent mouse studies have shown that when MDM2 is present but unable to bind MDMX, embryonic development progresses to a later

stage than *Mdm2* knockout mice (~E7.5 or greater vs. E5.5), suggesting that MDM2 alone may offer a primitive mechanism of p53 control.[115, 120, 132, 222, 240, 241] MDM2 homooligomers are indeed stable enough to be detected when overexpressed *in vitro* and in cell-based assays, suggesting that MDM2 homooligomers could also exist *in vivo*.[46, 145]

To better understand MDM2 homooligomers and MDM2-MDMX heterooligomers, detailed crystal structures of the two oligomers could be particularly valuable; however, complete crystal structures of MDM2 oligomers remain elusive. Thus, many structure- and mechanism-based MDM2 studies have focused on isolated MDM2 domains such as the RING domain. Although these studies have provided valuable insight into the binding mechanisms of the individual domains of MDM2, they are unable to assess MDM2 binding in the context of the complete protein. Although protein binding studies, such as ours, require verification using purified proteins in an *in vitro* binding assay, our experience using purified recombinant full-length MDM2 and MDMX protein suggests that they behave differently under *in vitro* conditions when compared with their behavior in the cytoplasmic milieu (data not shown). This could indicate that other factors, such as small proteins that bind MDM2 or the intracellular environment itself, are required for proper function. However, using full-length proteins overexpressed in cells, our study provides mechanistic insight with respect to the domains of MDM2 that are necessary for homo- and heterooligomerization within the cell.

Using point mutations in the highly conserved C464 and N447 residues, which reside within the RING domain but interact with different residues, I show that the proper MDM2 RING domain structure may not be necessary for interaction with WT MDM2 but is critical for heterooligomerization with MDMX. Previous studies investigating the conserved

zinc-coordinating residues within the RING domain have shown the importance of these residues for heterooligomerization and p53 degradation.[110, 120] Our study expounds on these observations by showing that RING domain mutants (C464A and N447A) retain the capacity to form stable homooligomers with WT MDM2 comparable to WT MDM2 itself (Figure 2-6B). This observation was unexpected because previous studies using isolated MDM2 and MDMX RING domains have suggested that while some residues may differ, MDM2 homooligomers and heterooligomers form in a similar fashion.[145, 186]

The extreme C-terminal residues have been implicated in MDM2 RING domain homooligomer formation.[249, 304] By deleting a small portion of the C-terminus of full-length MDM2, our data confirm that the C-terminal residues are required for MDM2 binding to WT MDM2 and MDMX. Furthermore, deletion of the C-terminal 12 residues (ΔC-term) abrogated MDM2 interaction in all constructs tested (WTΔAD, C464AΔAD, and N447AΔAD, Figures 2-8 and 2-10C). Although the presence of the C-terminus appears to be necessary for MDM2 homo- and heterooligomerization, the mechanisms that dictate the formation of homo- and/or heterooligomers appear to involve the MDM2 AD.

Previous studies have suggested that the AD plays a role in MDM2 regulation and function.[4, 137, 214, 341] The AD could also play a role in regulating MDM2 protein binding such as oligomerization preference. Deletion of the MDM2 AD can affect binding between MDM2 and other proteins such as p300 and various proteasome components.[18, 157] It is possible that our AD deletion mutants lack a motif required for their interaction with MDM2, as AD deletion from WT MDM2 selectively inhibits binding with WT MDM2. Furthermore, I observe an increase in MDM2-MDMX heterooligomerization upon AD deletion, suggesting that the AD may also be inhibitory towards MDM2-MDMX heterooligomerization (Figure 2-

10A compare lanes 1 and 3). These data collectively suggest that the AD may control the balance between MDM2 homo- and heterooligomerization. The Chen lab and others have shown that manipulation of the MDM2 AD in response to various p53-activating stimuli can affect MDM2 oligomerization thereby stabilizing p53.[45, 46, 119, 182] I suspect that modification of the MDM2 AD or binding by p14ARF and/or ribosomal proteins in response to stress could also affect MDM2 homo- and heterooligomerization. Consistent with this idea, our data show that overexpression of the MDM2-binding protein p14ARF decreases the overall amount of MDM2-MDMX heterooligomers while increasing the amount of MDM2-MDM2 homooligomers in the cell, likely by affecting MDM2 and MDMX protein stability (Figures 2-12A and B).

Interestingly, when the AD is deleted from E3-inactive MDM2 constructs (C464A, N447A), the ability of these mutants to interact with WT MDM2 persists (Figure 2-10B). Although I find this observation intriguing, I am currently unable to fully explain this E3-associated correlation. One possible explanation that will require further investigation is that the AD functions as a trigger that, when activated, allows homooligomerization to occur through the C-terminal residues. The unstructured state of the C464A and N447A RING domains could constitutively expose the C-terminus for homooligomerization, whereas the WT and N447D RING domains maintain the C-terminus in a state that allows homo- and heterooligomer formation depending on the state of the AD. When the AD is removed from WT MDM2, then only heterooligomers can form. Future studies investigating possible RING structure-dependent binding mechanisms could shed light on the binding mechanics of MDM2 and other oligomeric E3 ligases.

Altogether, our results are the first to definitively show evidence for differences in the mechanism of oligomerization for MDM2 homooligomers and heterooligomers. Our data show that whereas MDM2-MDMX heterooligomers form through RING domain and extreme C-terminal contacts, MDM2 homooligomers form in an AD- and extreme C-terminal residue-dependent manner (Figure 2-13). To our knowledge, this AD deletion mutant represents the first reported MDM2 mutation that can selectively inhibit MDM2-MDM2 interaction while leaving heterooligomerization and E3 activity intact. I believe that further investigation into the MDM2 AD will prove critical for a more comprehensive understanding of MDM2 regulation.

EXPERIMENTAL PROCEDURES

Cell culture and reagents

U2OS and H1299 cells were obtained from ATCC and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Sigma or Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Cells were grown at 37°C in a humidified incubator in the presence of 5% CO_2. MG132 was purchased from Calbiochem (cat. no. 474790). Cycloheximide was purchased from Sigma (cat. no. C7698). Phusion polymerase (cat. no. M0530S) and all restriction enzymes were purchased from NEB. XtremeGene HP transfection reagent was purchased from Roche through the UNC Tissue Culture Facility and transient transfections were performed according to the manufacturer's instructions. Mouse anti-actin (MAB1501, Chemicon), mouse anti-p53 (DO-1, Labvision AB-6 cat. no. MS-187P), mouse anti-FLAG (M2, Sigma cat. no. F3165), mouse anti-HA (16B12, Covance cat. no. MMS101P) antibodies were purchased. Mouse anti-MDM2

(4B11 and 2A10) and rabbit anti-myc (9E10) antibody supernatants were obtained from hybridoma cell lines.

Cloning and plasmids

MDM2 mutants were generated by using the QuikChange II XL site-directed mutagenesis protocol (Agilent Technologies cat. no. 200521). Briefly, wild-type human MDM2 encoded in pCMV mammalian expression vector was used as a template for all site-directed mutagenesis reactions. PCR reactions (50 µl total volume) contained 100 µg template plasmid DNA, 20 pmol forward primer, 20 pmol reverse primer (primer sequences in Supplementary Table 1), 1x Phusion polymerase buffer, 50 µM dNTPs (ThermoScientific cat. no. R0182), 2 U Phusion polymerase, and distilled water. Primers used were as follows: N447A (Tm 55°C) F-GTGATTTGTCAAGGTCGACCTAAAGCCGGCTGCATTGTCCATGGCAAAAC R-GTTTGCCATGGACAATGCAGCCGGCTTTAGGTCGACCTTGACAAATCAC; N447D (Tm 55°C) F-GTGATTTGTCAAGGTCGACCTAAAGACGGCTGCATTGTCCATGGCAAAAC R-GTTTTGCCATGGACAATGCAGCCGTCTTTAGGTCGACCTTGACAAATCAC; C-terminal deletion (Tm 55°C) F- CCCTGCCCAGTATGTAGATAGTTGACCTGTCTATAAGAGAATT R-AATTCTCTTATAGACAGGTCAACTATCTACATACTGGGCAGGG; RING deletion (Tm 55°C) F-CCCTTAATGCCATTGAACAACCAATTCAAATGATTG R-CAATCATTTGAATTGGTTGTTCAATGGCATTAAGGG; RING and C-terminal deletion (Tm 55°C) F- CCCTTAATGCCATTGAATAGTTGACCTGTCTATAAGAGAATT R-AATTCTCTTATAGACAGGTCAACTATTCAATGGCATTAAGGG; acidic domain deletion (Tm 60°C) F- ATCAGGATTCAGTTTCAGATCAGGAAATTTCCTTAGCTGACTATTGG R-

CCAATAGTCAGCTAAGGAAATTTCCTGATCTGAAACTGAATCCTGAT. Mutagenesis primers were designed to harbor a novel restriction endonuclease site that did not alter the protein coding sequence and were used to amplify the intended product using a thermocycler (Applied Biosystems model 2720) with the following program: 98°C for 4 mins, then 30 cycles of 98°C for 30 s, 60°C for 30 s, and 72°C for 5 mins. A final extension step at 72°C for 10 mins was performed after the final cycle. Reactions were digested with DpnI (NEB) for 2 hours and then 10 µl of each reaction was transformed into chemically competent XL-1 blue *E. coli* cells. All clones were submitted to the University of North Carolina Genome Analysis Facility for sequence verification.

Transfections

Cells were plated in 6-well plates overnight and transfections were performed using XtremeGene HP transfection reagent (Roche) according to the manufacturer's instructions. Briefly, cells were transfected at approximately 90% confluence with a mixture of 2 µg total DNA and 6 µl transfection reagent. All transfections included GFP plasmid to visually confirm transfection efficiency (in all transfections, at least 50% of cells were GFP-positive). Twenty-four hours after the transfection, the indicated treatment was applied or the cells were collected and lysed for western blotting.

Immunoprecipitation

Cells were transfected with the appropriate plasmids for 24 h, after which the cells were lysed in 0.1% NP-40 buffer containing 1x protease inhibitor cocktail (leupeptin L2884, aprotinin A1155, benzamidine B6506, trypsin inhibitor T9003 all Sigma), 1 mM

phenylmethylsulfonyl fluoride (PMSF, Sigma P7626), 1 mM $NaVO_3$ (Fisher Scientific S454-50), and 1 mM dithiothreitol (DTT, Roche 03117014001) for 2 h. Lysates (500 µg each) were probed with 5 µl EZ-view anti-FLAG M2 affinity gel (Sigma F2426) by rotating the samples at 4°C for 2 h to overnight. Beads were washed three times in 0.1% NP-40 lysis buffer, after which the beads and inputs were analyzed by western blotting.

Half-life assay

U2OS cells were transfected with the indicated vectors for 24 h, after which cycloheximide (CHX, 100 µg/ml) was added to all samples. Cells were collected in 0.5% NP-40 lysis buffer containing 1x protease inhibitor cocktail, 1 mM PMSF, 1 mM $NaVO_3$, and 1 mM DTT at the indicated time point after CHX addition. Cells were lysed for 2 h by rotation at 4°C, after which the samples were centrifuged, and the supernatants collected. Lysates were separated by SDS-PAGE and subjected to western blotting for the indicated proteins. Bands were quantified after normalization to actin, and then were plotted as the amount of protein remaining relative to time 0. Bands were compared quantitatively by using ImageJ software version 1.48.

Western blotting

Cell lysates (100 µg per sample) were separated on 10% or 12.5% SDS-PAGE gels, after which the proteins were transferred to nitrocellulose membranes (BioRad). Transfers were assessed by staining the membranes with Ponceau S (Sigma P3504) for 5 min followed by several brief washes with ddH_2O. Membranes were blocked for at least 1 h in phosphate-buffered saline containing 5% non-fat milk and 0.1% Tween-20. Membranes

were then incubated with the appropriate primary antibody diluted in blocking buffer for 2 h to overnight. Membranes were washed three times in PBS-T and then incubated with the appropriate HRP-conjugated secondary antibody diluted in blocking buffer for 1 h. Membranes were washed four times in PBS-T and then developed with Supersignal West Pico chemiluminescent substrate according to the manufacturer's instructions (Pierce cat. no. 34080).

In vivo *Ub assay*

Equal amounts of U2OS or H1299 cells were transfected with the indicated plasmid constructs for 24 h after which the cells were trypsinized and collected. One-fifth of each sample was lysed with 0.1% NP-40 lysis buffer as inputs. The remaining cells were boiled in SDS lysis buffer containing 1x protease inhibitor, 1 mM PMSF, 1 mM $NaVO_4$, and 1 mM DTT for 10 min. Then, the lysates were diluted into 0.1% NP-40 lysis buffer containing protease inhibitors and subjected to immunoprecipitation using anti-FLAG beads. Western blotting was performed to probe for the indicated proteins.

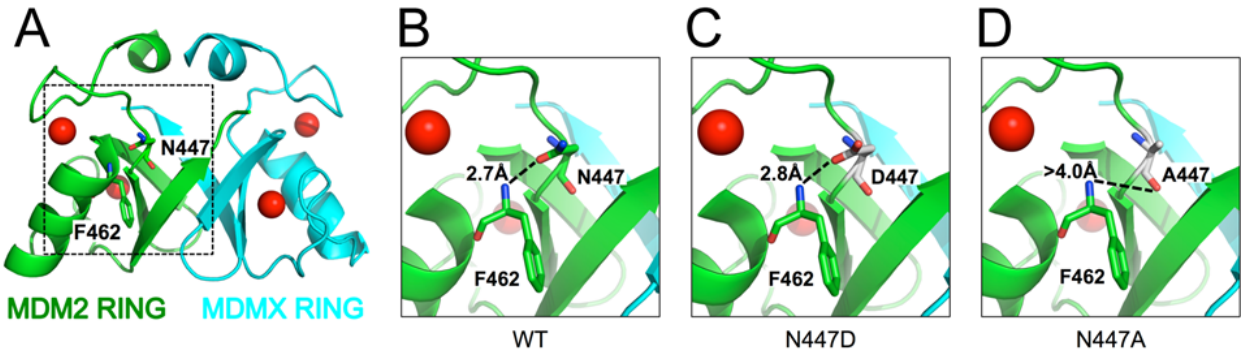

Figure 2-1. Simulations of the different N447 mutations.
A) PyMOL image based on the published crystal structure of the human MDM2 (green)-MDMX (blue) RING domain heterooligomer. Residues F462 and N447 are highlighted as stick figures.
B) Close-up image of (A) depicting the intramolecular bond between the N447 side chain and the amino group of F462.
C) PyMOL image simulating the conservative N447D mutation.
D) PyMOL image simulating the non-conservative N447A mutation.

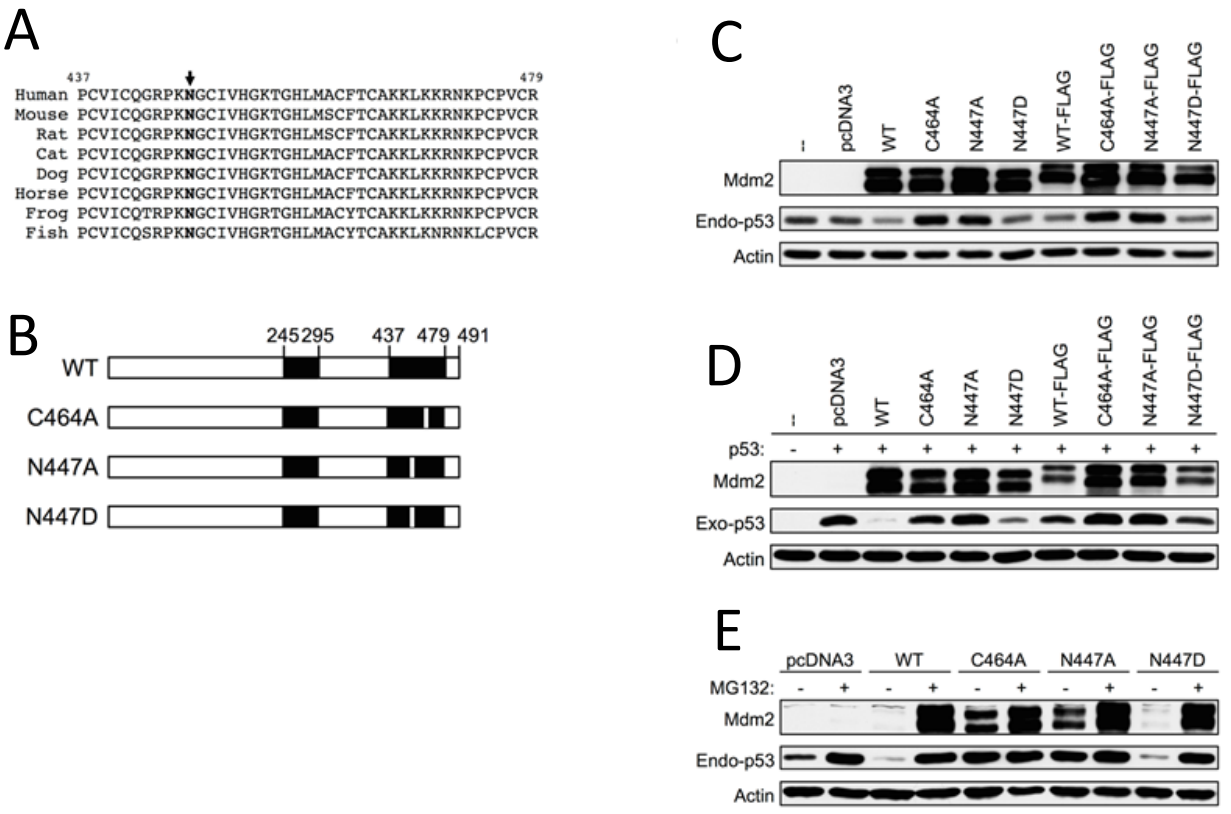

Figure 2-2. MDM2 N447D but not N447A is able to degrade p53.
A) Protein sequence alignment for the MDM2 RING domain (human residues 437-479) in several species.
B) Diagram of MDM2 with amino acid positions of AD (245-295), RING (437-479), and extreme C-terminus (479-491) indicated.
C) U2OS cells (WT p53) were transfected with empty vector or untagged or FLAG-tagged MDM2 constructs and then blotted for MDM2 (4B11) and p53 (DO-1).
D) H1299 cells (p53-null) were co-transfected with WT p53 and empty vector or untagged or FLAG-tagged MDM2 constructs and then blotted for MDM2 (4B11) and p53 (DO-1).
E) U2OS cells were transfected with empty vector or FLAG-MDM2 constructs for 24 h and then treated for 2.5 h with 10 μM MG132 and 100 μg/ml cycloheximide (CHX). Membranes were blotted for MDM2 (2A10) and p53 (DO-1).

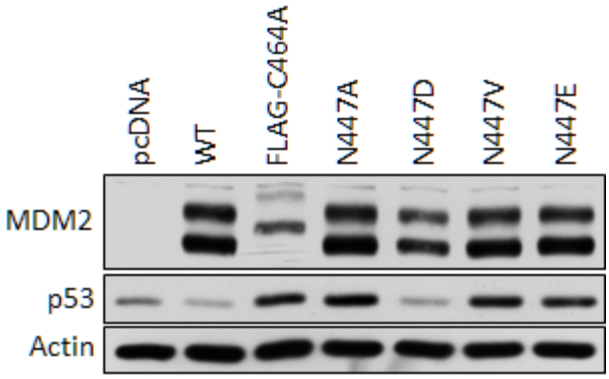

Figure 2-3. N447V and E mutants are unable to degrade p53.
U2OS cells were transfected with the indicated MDM2 constructs for 24 h, after which lysates were collected and probed for p53 levels.

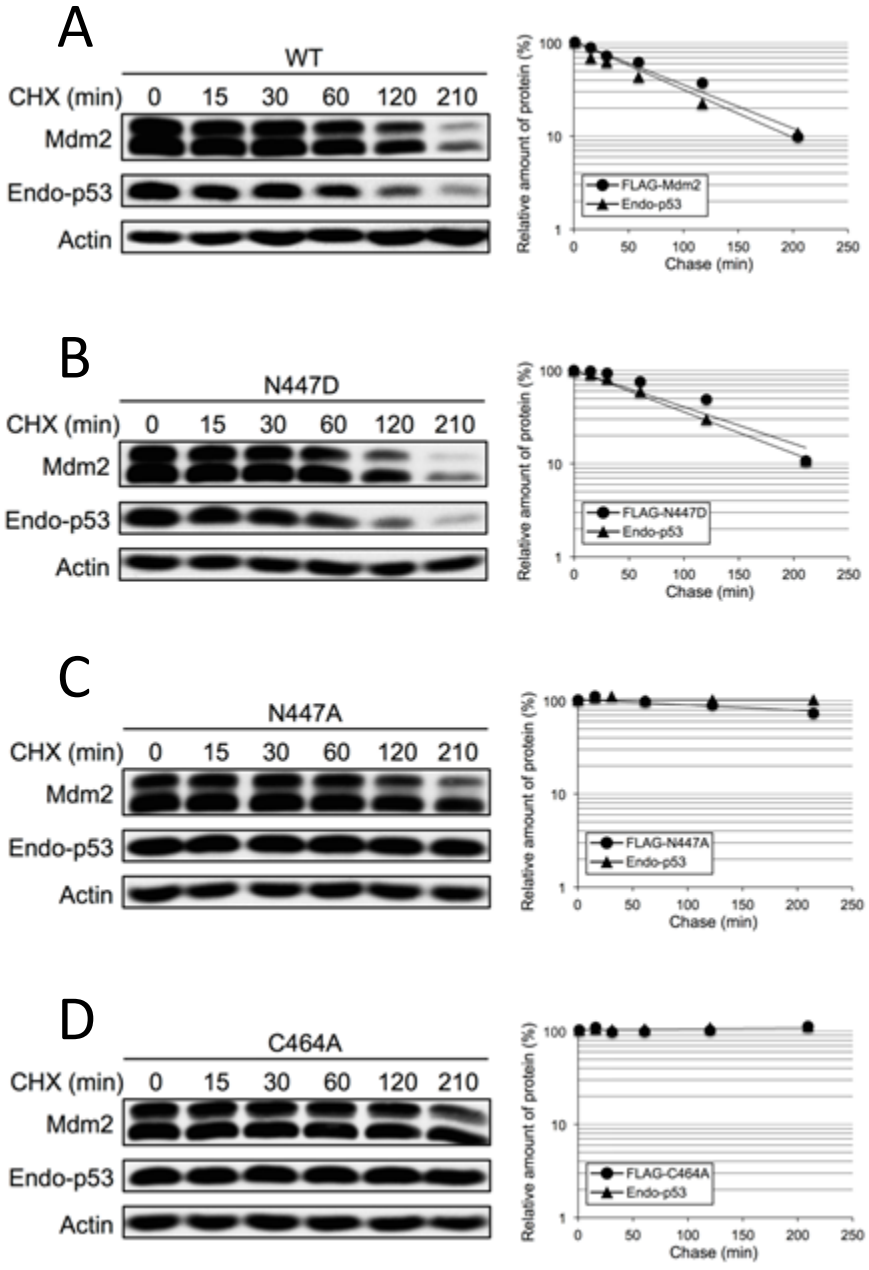

Figure 2-4. Half-life of p53 and MDM2 are extended in the presence of MDM2 N447A but not N447D.

U2OS cells were transfected with the constructs encoding MDM2 WT (A), N447D (B), N447A (C), or C464A (D) for 24 h, and then chased with 100 μg/ml CHX for various amounts of time. Lysates were resolved by SDS-PAGE and then probed by western blot for p53 (DO-1) and MDM2 (4B11).

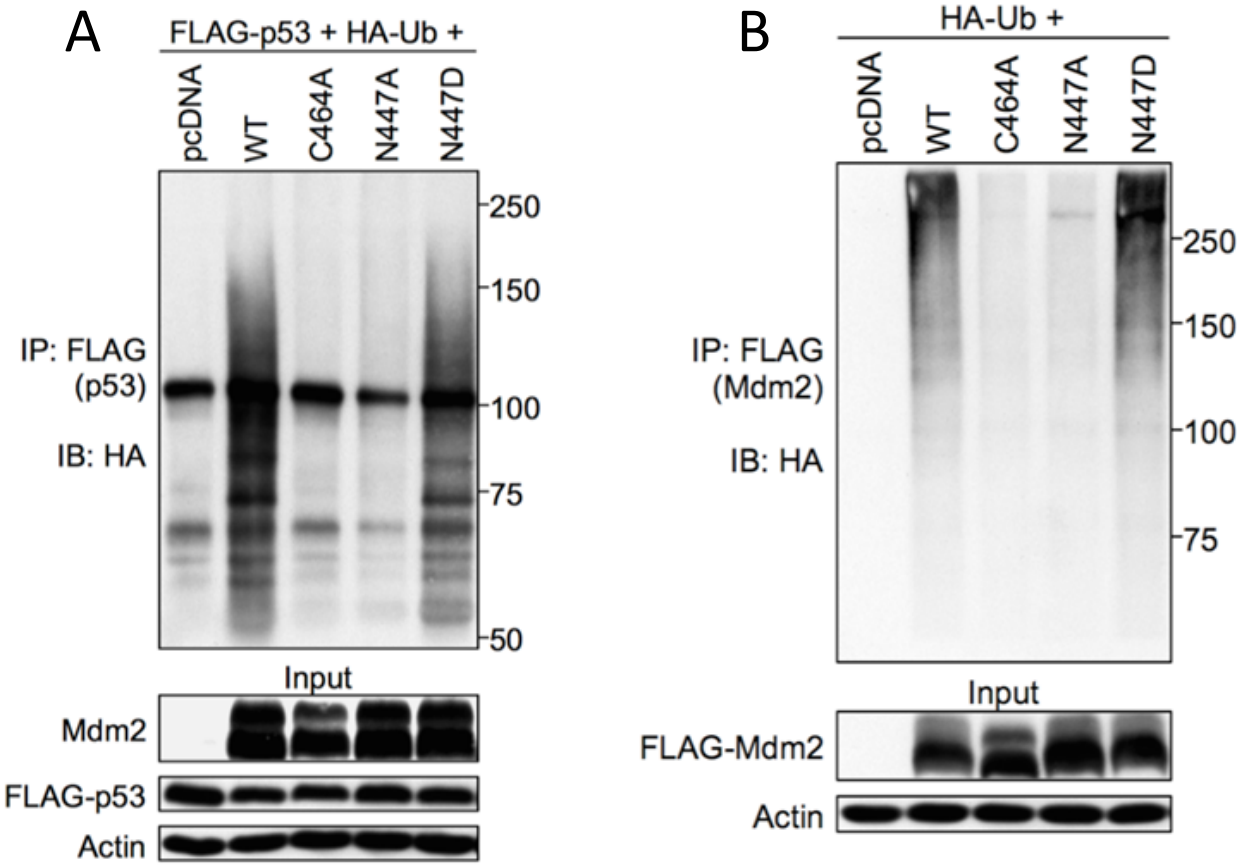

Figure 2-5. N447D but not N447A retains the ability to ubiquitinate itself and p53. U2OS cells were transfected with HA-ubiquitin, FLAG-p53, and one of the untagged constructs as indicated (A) or with HA-ubiquitin and one of the FLAG-tagged constructs as indicated (B) for 24 h. Cell lysates were immunoprecipitated with anti-FLAG beads and then subjected to western blot analysis probing with anti-HA antibody. Inputs representing 20% of the total protein immunoprecipitated are included.

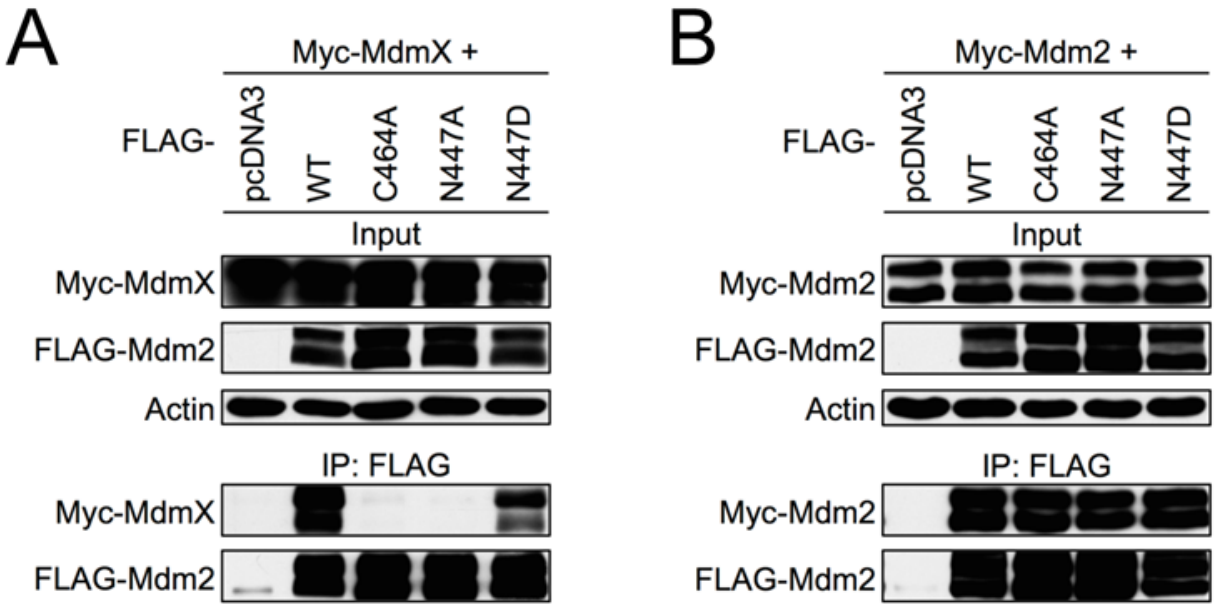

Figure 2-6. MDM2 heterooligomerization but not homooligomerization requires the proper structure of the RING domain.
A and B) U2OS cells were co-transfected with constructs encoding myc-MDMX (A) or myc-MDM2 (B) and each of the FLAG-MDM2 constructs for 24 h. Cell lysates were subjected to immunoprecipitation, resolved by SDS-PAGE, and then probed with anti-myc antibody and then reprobed with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included.

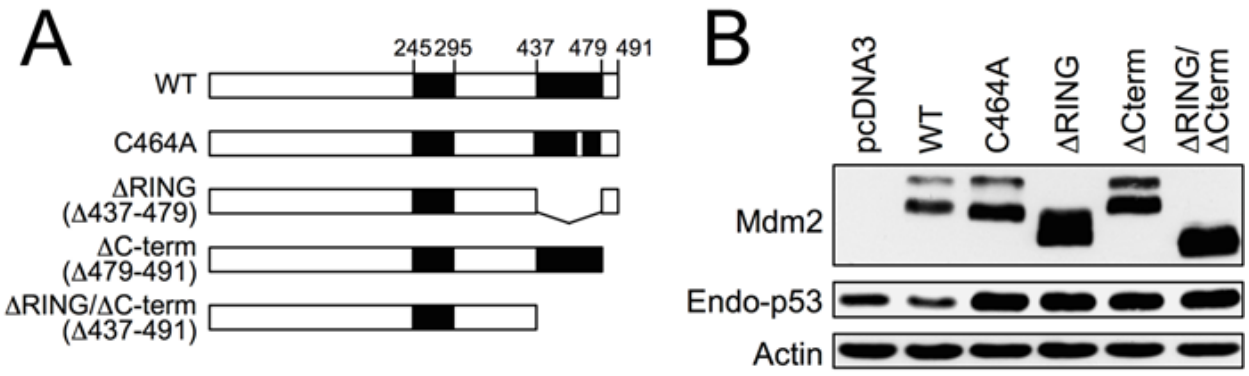

Figure 2-7. Deletion of the MDM2 RING domain or the extreme C-terminus inhibits p53 degradation.

A) Diagram of WT and mutant MDM2 constructs.

B) U2OS cells were transfected with empty vector or FLAG-MDM2 constructs for 24 h, after which the cell lysates were resolved by SDS-PAGE and subjected to western blotting probing for FLAG and p53.

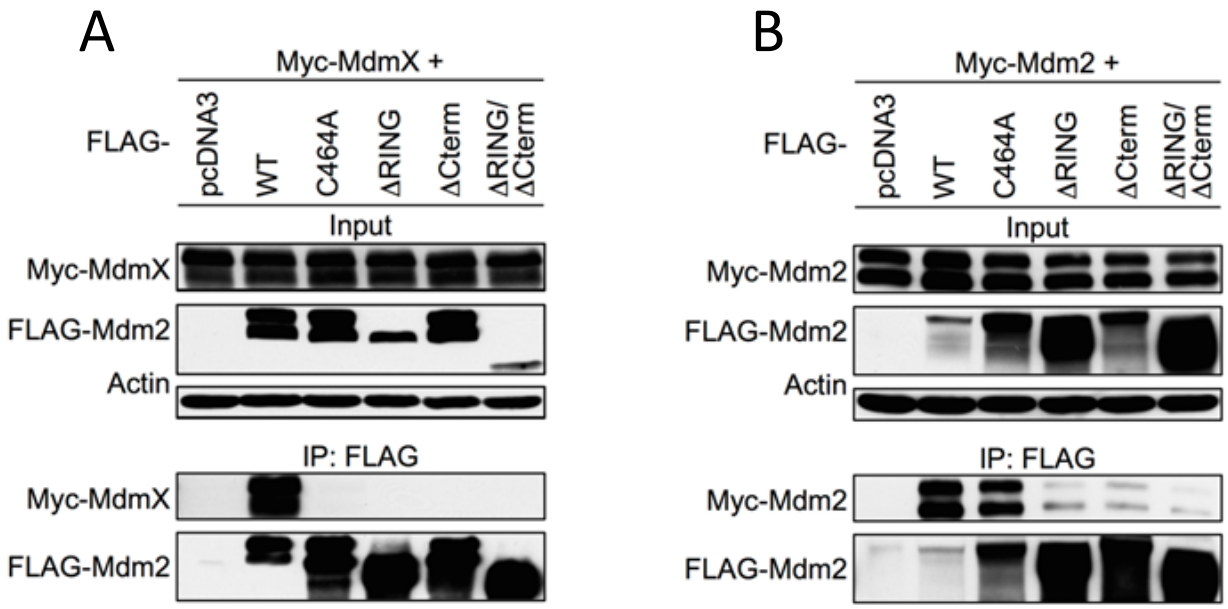

Figure 2-8. MDM2 RING or extreme C-terminal deletion prevents MDM2-MDM2 homooligomerization and MDM2-MDMX heterooligomerization.
A and B) U2OS cells were co-transfected with constructs encoding myc-MDMX (A) or myc-MDM2 (B) and each of the indicated FLAG-MDM2 constructs for 24 h. Cell lysates were immunoprecipitated with anti-FLAG beads, resolved by SDS-PAGE, and then subjected to western blotting probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included.

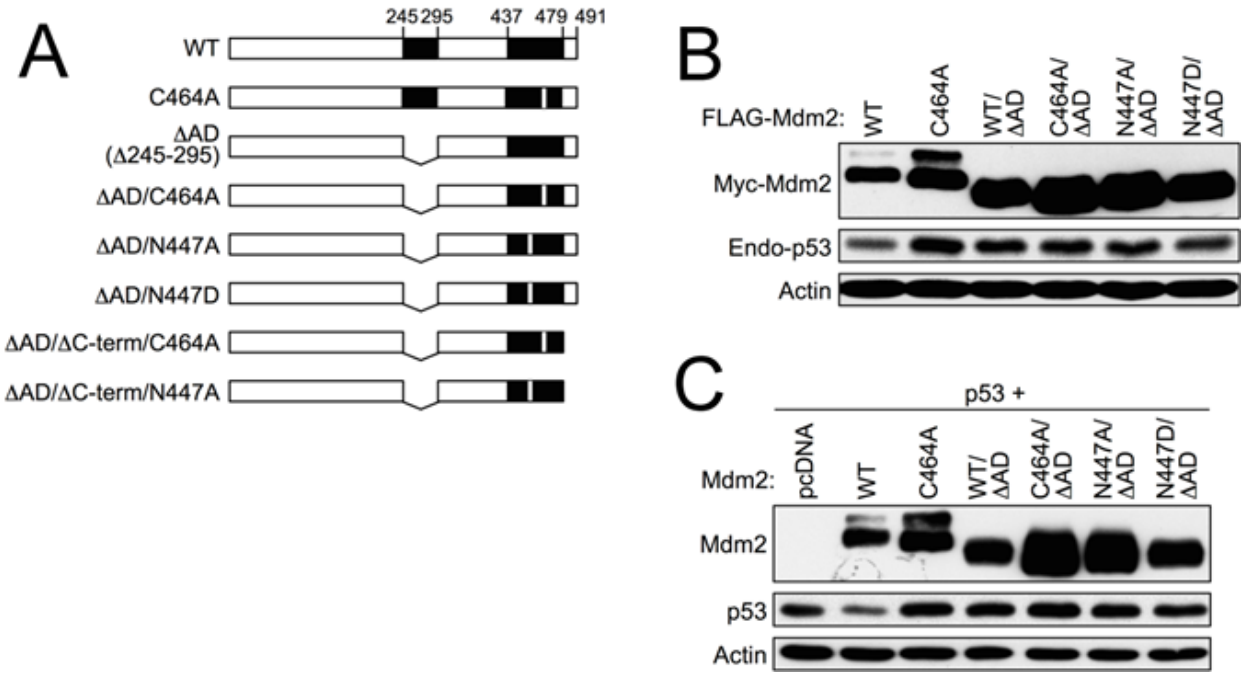

Figure 2-9. AD deletion inhibits the ability of MDM2 to degrade p53.
A) Diagram of WT and mutant MDM2 constructs.
B) U2OS cells were transfected with empty vector or FLAG-MDM2 constructs for 24 h, after which cell lysates were subjected to western blotting probing for FLAG and p53.
C) H1299 cells were co-transfected with WT p53 and empty vector or FLAG-MDM2 constructs for 24 h, after which cell lysates were subjected to western blotting for FLAG and p53.

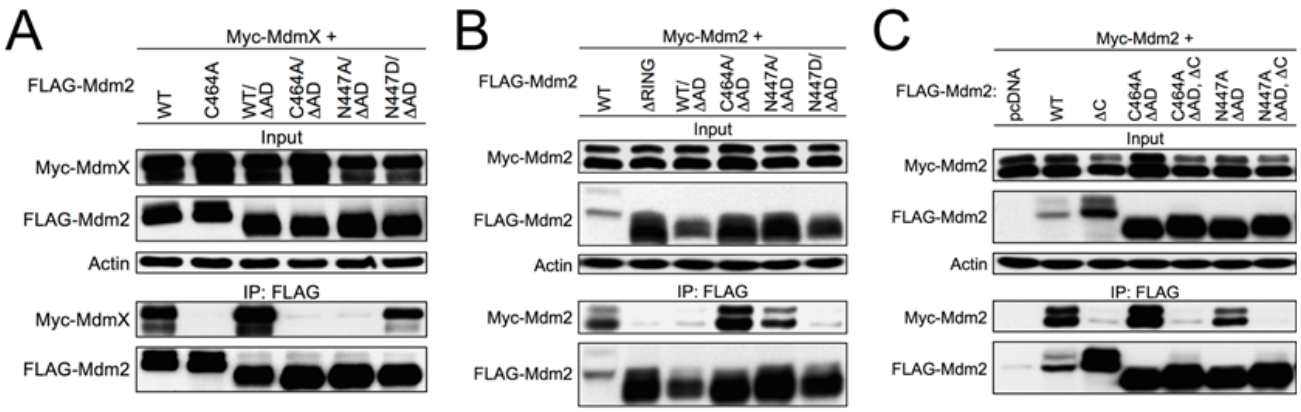

Figure 2-10. AD deletion inhibits homooligomer but not heterooligomer formation in E3-competent MDM2 constructs.

A and B) U2OS cells were co-transfected with constructs encoding myc-MDMX (A) or myc-MDM2 (B) and each of the indicated FLAG-MDM2 constructs for 24 h. For (A), cells were treated for 3 h with 20 μM MG132 prior to lysis. Cell lysates were precipitated with anti-FLAG beads overnight and then subjected to western blotting probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included. C. U2OS cells were co-transfected with myc-MDM2 and each of the indicated FLAG-MDM2 constructs for 24 h. Cell lysates were immunoprecipitated with anti-FLAG beads and then subjected to western blotting probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included.

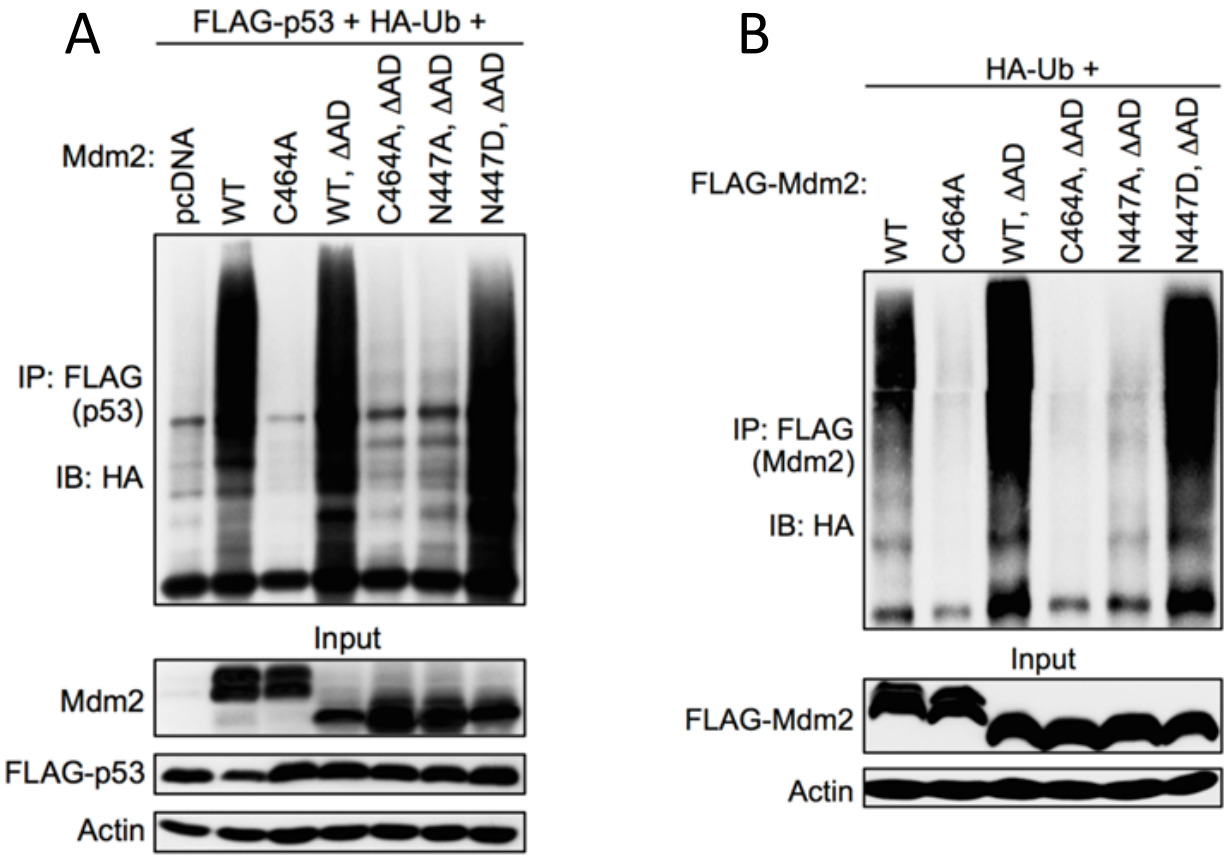

Figure 2-11. AD deletion does not affect the ubiquitin ligase activity of E3-competent MDM2 constructs.
U2OS cells were transfected with HA-ubiquitin, FLAG-p53, and one of the untagged constructs as indicated (A) or with HA-ubiquitin and one of the FLAG-tagged constructs as indicated (B) for 24 h. Cell lysates were immunoprecipitated with anti-FLAG beads and then subjected to western blotting probing with anti-HA antibody. Inputs representing 20% of the total protein immunoprecipitated are included.

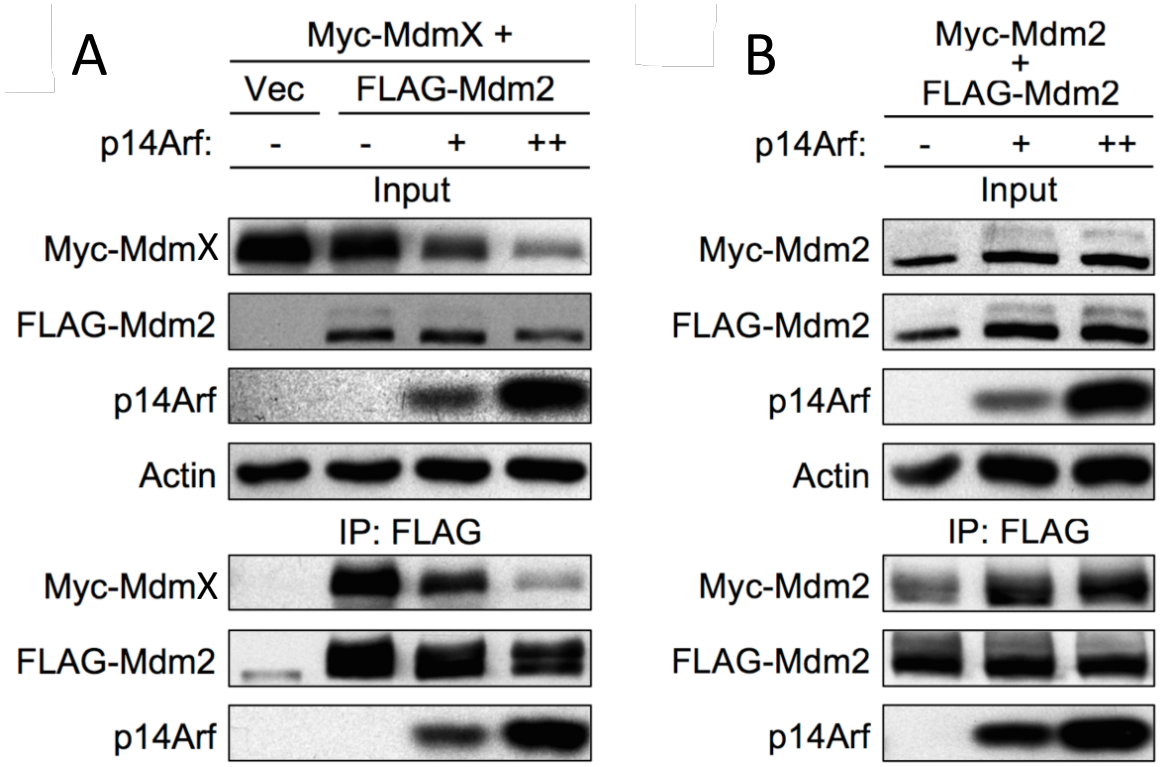

Figure 2-12. ARF affects the formation of MDM2-MDM2 homooligomers and MDM2-MDMX heterooligomers.
U2OS cells were co-transfected with constructs encoding myc-MDMX (A) or myc-MDM2 (B) and each of the indicated FLAG-MDM2 and p14ARF constructs for 24 h. Cell lysates were precipitated with anti-FLAG beads overnight and then subjected to western blotting probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included.

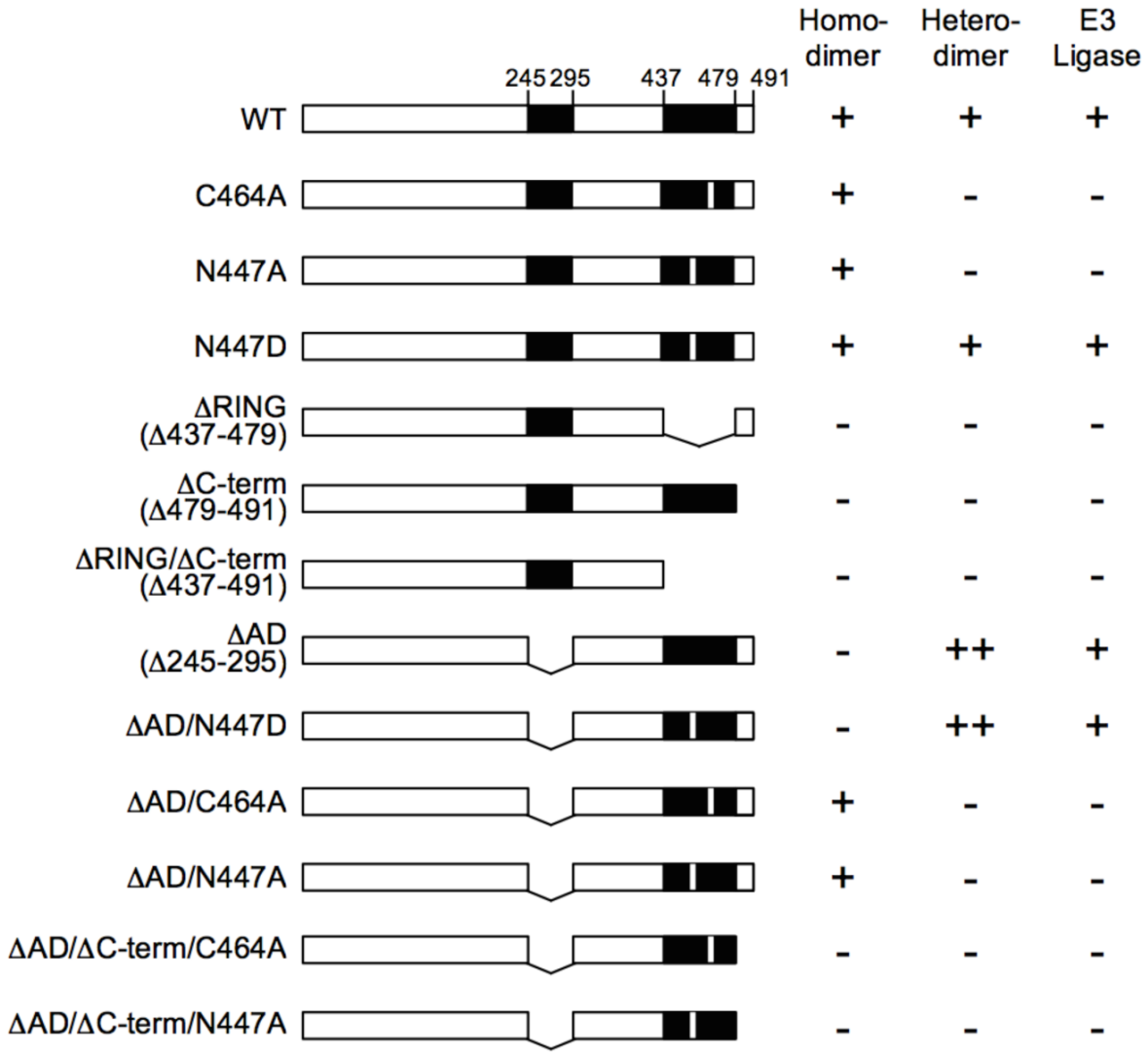

Figure 2-13. Summary of results.
MDM2 requires the AD and C-terminal residues to interact with MDM2, whereas MDM2 requires the properly structured RING domain and C-terminal residues to interact with MDMX. AD, acidic domain.

CHAPTER 3: LRP1 IS A NOVEL P53 TARGET GENE WHOSE PROTEIN PRODUCT IS INDUCED IN RESPONSE TO SUB-LETHAL STRESS[3]

INTRODUCTION

The tumor suppressor p53 is a diverse transcription factor that contributes to the regulation of numerous pathways. Some of the classical functions of p53 include the induction of cell cycle arrest and apoptosis, whereas more recently characterized functions of p53 include the induction of metabolic alterations, DNA damage repair pathways, and antioxidant factors. As the regulatory functions of p53 increase in scope, it has become increasingly apparent that the functions of p53 can be broadly categorized into a pro-death and a pro-survival dichotomy that is highly context-dependent. Thus, whereas previous studies have explored p53 in either a pro-survival or a pro-apoptotic context, the current body of knowledge on p53 suggests that its role depends on multiple factors, including cell type and the stressors to which the cell is exposed. For example, multiple studies have shown that p53 activates predominantly pro-survival genes in response to a low concentration of DNA damaging agents but activates predominantly pro-apoptotic genes in response to high concentrations of DNA damaging agents.[258] These observations suggest that p53 might serve as a decision node for the cell under stress that must weigh various inputs, such as the extent of DNA damage and oxidation, and commit the cell to repair the

[3] This chapter presents an ongoing project. The study design and almost all experiments were performed by me under the guidance of my mentor. Dr. Yong Liu performed the radioactive labeling experiment and provided assistance with the data analysis. This manuscript draft of the study was written and edited by me.

damage or apoptose if the damage is irreparable.

The importance of p53 in the decision between life and death of the cell helps explain why p53 signaling is almost universally perturbed in cancers by direct mutations or by mutations to proteins that regulate p53. p53 is the most commonly mutated gene in cancers, which has contributed to its popularity as a research topic. However, despite the tens of thousands of publications on p53 and its frequent mutation in cancers, we have yet to generate a clinically useful tool for the imaging, prognostication, or treatment of patient cancers based on p53. Though several p53-targeting drugs are in the clinical trial stage of development, the possibility exists that these drugs will be either ineffective or counter-productive. While reactivation of p53 has been shown to be sufficiently selective to target tumor cells while sparing normal cells in mice, whether this holds true for humans remains uncertain. Moreover, multiple lines of evidence have shown that p53 activation in tumors could generate a pro-tumor effect in response to chemotherapies by allowing the cell a chance to repair DNA damage.[31, 125] The pro-tumor role of WT p53 could also be true in human tumors.[16]

One of the barriers preventing the advancement of the p53 field in terms of developing medically useful products is the incomplete state of our knowledge on the p53 regulome. Understanding not only which genes are regulated by p53 but also in which contexts they are regulated is important to grasp a comprehensive picture of the effects of p53 activation by various methods. In this study, I identify and confirm the novel p53 target gene *low-density lipoprotein receptor-related protein 1* (*LRP1*), a large, multi-functional, transmembrane protein involved in endocytosis and signal transduction. I show that *LRP1* is a late p53 target gene that is induced in response to several p53-activating stimuli in a

p53-dependent manner. Interestingly, I found differences in the upregulation of LRP1 protein depending on whether the p53-activating stress is lethal or sub-lethal. Whereas LRP1 transcript expression is upregulated in response to both lethal and sub-lethal concentrations of doxorubicin, LRP1 protein is selectively increased in response to sub-lethal doses of doxorubicin. I further show that the translation of LRP1 could be suppressed through a post-transcriptional mechanism involving miR-103 and miR-107.

RESULTS

p53 induces LRP1 expression

In an effort to identify potential novel p53 target genes that could be involved in the non-canonical p53 gene regulation programs, I mined a microarray screen previously reported by our lab. In this microarray screen, I compared the expression profiles of three different mouse embryonic fibroblast (MEF) genotypes that express p53 at a low ($MDM2^{WT/WT}$), medium ($Mdm2^{-/-}$), or high ($Mdm2^{462/462}$) level according to the competency of MDM2 as an E3 ligase.[66] Correspondingly, p53 activity in these cells mirrors the level of p53 and allows for the identification of potential p53 target genes based on their adherence to the low-medium-high pattern. One of the target genes identified through this screen was *low-density lipoprotein receptor-related protein 1* (*LRP1*), which closely mirrored the expression pattern of the p53 target gene *p21* (Figure 3-1A). Importantly, p53-dependent expression is not observed for the related genes *low-density lipoprotein receptor* (*LDLR*) or *LRP2* (Figure 3-1A). To confirm that LRP1 is expressed in a p53-dependent manner, I compared p53 isogenic colon cancer HCT116 cells (WT and $p53^{-/-}$) and found that both transcript and protein induction occurs only in HCT116 WT cells but not in $p53^{-/-}$ HCT116

cells upon treatment with the MDM2 inhibitor nutlin-3a (Figures 3-1B and C). To show that p53 is sufficient to upregulate LRP1 expression, I transfected p53-null H1299 cells with WT or mutant p53 (R273H), treated with nutlin-3a, and then analyzed LRP1 protein expression levels. Consistent with a role in LRP1 induction, WT p53 transfection induced LRP1 protein expression and showed a nutlin-3a-dependent further increase in expression (Figure 3-2A). LRP1 expression was unchanged in the empty vector-transfected cells regardless of nutlin-3a treatment. Interestingly, the p53R273H mutant was able to induce LRP1 protein expression above background levels and showed a nutlin-3a response, suggesting that this mutant derivative of p53 might retain some transcriptional activity when overexpressed. Consistent with the p53 rescue data, CRISPR-Cas9-mediated knockout of p53 in HCT116 cells using guide RNAs targeting p53 exons 3 (p53KO-3) or 5 (p53KO-5) not only reduced basal LRP1 protein expression levels to barely detectable levels but also abrogated the effect of nutlin-3a treatment on LRP1 induction (Figure 3-2B). Adenoviral infection of the p53KO-5 HCT116 line with WT p53 rescued LRP1 induction (Figure 3-2C).

Next, I investigated whether LRP1 is a direct p53 target. Analyzing the upstream promoter region of the LRP1 genomic locus revealed one putative p53 response element that displayed extensive similarity with the consensus sequence $(RRRCYYGWWW)_2$ (Figure 3-3A). Thus, I tested whether this putative p53 RE could be bound by p53 by chromatin immunoprecipitation (ChIP)-qPCR. The putative p53 response element could be immunoprecipitated in a p53-dependent manner. Moreover, this binding was enhanced by nutlin-3a treatment (Figure 3-3B). Cloning the putative LRP1 p53 RE upstream of a luciferase reporter construct was also sufficient to drive luciferase expression in a p53-

dependent manner, suggesting that the LRP1 promoter contains a *bona fide* p53 RE (Figure 3-3C).

LRP1 is induced by several p53-activating stresses

Next, I tested a panel of p53-activating stimuli to determine which stresses most strongly upregulate LRP1 expression. For this experiment, I tested physical DNA damage inducers ionizing radiation and UV-C irradiation, topoisomerase inhibitors doxorubicin and etoposide, ribosomal stresses low-concentration actinomycin D (ActD) and 5-fluorouracil (5FU), and the DNA adduct-inducing agent cisplatin. Nutlin-3a treatment was used as a positive control. Although all of these stresses were able to induce p53, LRP1 protein induction varied considerably (Figure 3-4A). I noted that the highest levels of LRP1 induction occurred in response to the double strand break-inducing stresses (IR, doxorubicin, and etoposide). Moreover, p53 protein induction was not necessarily indicative of LRP1 induction, as strong p53 induction was observed in response to ActD and 5FU, but only modest LRP1 induction was observed. LRP1 expression is heavily dependent on p53 in HCT116 cells in response to acute stress, as p53 CRISPR knockout HCT116 cells (p53KO-5) show no detectable induction of LRP1 expression (Figure 3-4A). LRP1 transcript expression levels were consistent with protein expression levels (Figure 3-4B). These results are not cell type-specific, as they could be reproduced in the osteosarcoma cell line U2OS (Figure 3-5). Because some of the strongest LRP1-inducing stresses were double strand break inducers, which activate p53 at least in part through ATM and ATR kinases, I pre-treated cells with the pleiotropic PI3K inhibitor caffeine and then subjected cells to various DNA damage stresses. Caffeine treatment reduced the

protein expression levels of LRP1, p53, and p21, which is consistent with a dependence on the DNA damage surveillance machinery to activate p53 (Figure 3-6).

Sub-lethal stresses induce LRP1 expression

Based on our panel of p53-activating stresses, I could observe a rough correlation within each stress whereby stresses that induce extensive cell death fail to induce LRP1. I also observed that not only could 200 nM doxorubicin induce LRP1, but also 1 hour of a (otherwise non-LRP1-inducing) 1 μM doxorubicin treatment (dox pulse) could induce LRP1 expression to high levels (Figure 3-7). Moreover, the induction of LRP1 in response to all of the treatments that I tested correlated with the cellular outcome, whereby treatments that resulted in cell survival resulted in LRP1 induction, and treatments that resulted in cell death resulted in no LRP1 expression. Therefore, I further investigated whether this correlation was true on the molecular level as well. To this end, I compared the expression of LRP1 and various cell death markers in two sub-lethal-lethal pairs of stresses (IR vs. UV and dox pulse vs. high dox). Propidium iodide-based cell cycle analysis of these treatment pairs revealed the expected large percentage of cells in sub-G1 for UV and high doxorubicin at 24 and 48 h time points that was noticeably reduced in the sub-lethal stresses (Figure 3-8A). Analysis of protein lysates taken from the same experiment showed that each stress showed a strong inverse correlation between the induction of LRP1 protein and the expression of apoptotic markers cleaved caspase 3 and cleaved PARP at 24 and 48 hours (Figure 3-8B). This correlation could be observed for sub-lethal and lethal doses of etoposide as well (Figure 3-9A and B). One possibility that I initially considered was that because LRP1 is expressed several hours after exposure to stress, the lethal stresses might

kill the cell too quickly for LRP1 transcription to occur. Therefore, I attempted a rescue experiment using the potent, general caspase inhibitor QVD-OPh (QVD). Although QVD treatment effectively prevented cell death as determined by microscopy and by the inhibition of caspase 3 cleavage, QVD treatment was insufficient to allow cells to express LRP1 in the presence of lethal stress (Figure 3-10). Next, I analyzed the transcript levels of various p53 target genes associated with the apoptotic response or with other p53 responses in the presence of sub-lethal and lethal doxorubicin treatment. I found that lethal doxorubicin but not sub-lethal doxorubicin was able to induce the transcript expression of p53 apoptotic target genes *BAX*, *PUMA*, *APAF1*, and *NOXA* (Figure 3-8C). Interestingly, although I observed low expression for not only LRP1 but also various non-apoptotic p53 target genes at the protein level in response to high doxorubicin compared with low doxorubicin, I saw higher transcript expression of LRP1 and other non-apoptotic genes in the presence of high doxorubicin relative to low doxorubicin (Figure 3-8C). Moreover, I observed strong APAF1 protein expression at 24 and 48 h in the presence of high doxorubicin but not low doxorubicin, suggesting that non-apoptotic genes but not pro-apoptotic genes may be actively suppressed at the translational level in response to high doxorubicin (Figure 3-8B). Because I used the same stress while only varying the concentration or length of time, I hypothesized that the intensity of a given p53-inducing stress could have a threshold beyond which the expression of certain proteins must be repressed to effectively induce apoptosis.

Sub-lethal but not lethal doxorubicin induces LRP1 protein expression

To further investigate my hypothesis, I analyzed the effects of sub-lethal and lethal doxorubicin treatment on LRP1 expression. Because I saw higher LRP1 transcript in cells treated with high dox, I conducted a detailed time course to determine whether LRP1 is induced at any time between 1 and 24 hours. Interestingly, although p53 is induced with similar kinetics but a higher ceiling in cells treated with high doxorubicin compared with low doxorubicin, only the low doxorubicin treatment is able to induce LRP1 protein, and induction occurs sometime between 12 and 24 hours after treatment (Figures 3-11A-C). This pattern was also observed when comparing ionizing radiation and UV-C treatment (Figure 3-12A-C). These observations are not cell type-specific, as similar trends were observed in the breast cancer cell line MCF-7 for low and high doxorubicin (Figure 3-13A-C) as well as IR and UV irradiation (Figure 3-14A-C). More detailed analysis of the induction of LRP1 protein in HCT116 cells in response to low doxorubicin shows that LRP1 protein occurs in a gradual manner beginning approximately 13 hours after treatment and continuing through hour 24 (Figure 3-15). To determine the range of doses that correspond to LRP1 induction, I tested the ability of several different doses of doxorubicin to induce LRP1 after a 24-h treatment period. I found that doses from as low as 50 nM to as high as 900 nM showed a clear increase in LRP1 expression at 24 h. The highest concentration of doxorubicin (1000 nM), which is also the high dose used in all previous experiments, did not induce LRP1 protein expression appreciably (Figure 3-16). Interestingly, I found a dose-dependent increase, peak, and decrease in not only LRP1 expression but also in p21 and MDM2 expression (Figure 3-16). Although in these experiments, apoptotic/floating cells were washed from the dish prior to lysate collection,

collection of floating cells prior to lysis does not affect the doxorubicin dose-dependent pattern of LRP1 expression (Figure 3-17). Moreover, I was unable to detect any LRP1 cleavage products by western blot, suggesting that other mechanisms of LRP1 repression are responsible for our observation (Figure 3-18). Testing the effect of another lethal stress on LRP1 protein expression, I noticed dose-dependent (24 h post-treatment collection) and time-dependent (25 J/m^2 constant dose) decreases in LRP1 protein expression in response to UV-C treatment (Figure 3-19A and B). Analysis of LRP1 transcript induction in response to 50, 200, or 1000 nM constant doxorubicin treatment revealed that both LRP1 and p21 showed dose-dependent increases in transcript expression (Figure 3-20A). p53 ChIP-qPCR analysis in an experiment using the same doses of doxorubicin also revealed increased p53 binding at the LRP1 promoter for all doxorubicin treatments (Figure 3-20B). These results collectively suggest that p53-mediated LRP1 transcript expression remains increased in the presence of all concentrations of doxorubicin despite the observed low levels of LRP1 protein.

Based on the observed decrease in LRP1 protein, I tested whether there might be a difference in LRP1 protein stability. The half-life of LRP1 under unstressed conditions has been reported at approximately 3 h in cancer cells and 24 h in smooth muscle cells.[25, 213] To investigate whether a change in the half-life of LRP1 could account for the difference in protein expression between the high and low doxorubicin treatments, I treated HCT116 cells with a lethal or sub-lethal dose of doxorubicin for 24 h, after which I added the general translation inhibitor cycloheximide (CHX) for various amounts of time to observe the rate of LRP1 protein degradation. Because high doxorubicin treatment is extremely stressful on cells, especially in the presence of CHX, I added QVD to all of the samples to prevent cell

death. Interestingly, I observed a 4-fold decrease in the LRP1 half-life in the presence of high doxorubicin compared with low doxorubicin (4.5 h vs. 18 h), suggesting that LRP1 protein stability is indeed affected by lethal concentrations of doxorubicin (Figure 3-21A and B). Interestingly, I observed no difference in the half-lives of either p53 or p21 when comparing the two treatments (Figure 3-21B and C).

Next, I attempted to determine which protein degradation pathway might account for the difference in LRP1 stability. LRP1 has been reported to undergo proteasome-mediated degradation;[30] therefore, I determined whether MG132-mediated proteasome inhibition could rescue LRP1 expression in response to lethal stress. Contrary to previous reports, I observed no MG132-dependent rescue of LRP1 protein expression in response to lethal or sub-lethal stress. Unexpectedly, I observed a paradoxical decrease in LRP1 protein upon MG132 treatment for the sub-lethal stresses (Figure 3-22). Because LRP1 plays a major role in the endocytosis-lysosome pathway, I also attempted to rescue LRP1 protein expression using the lysosome inhibitor chloroquine (CQ), but CQ was unable to rescue LRP1 expression (Figure 3-22). I considered the possibility that the decreased half-life of LRP1 protein might not account for the entire difference in LRP1 protein levels between the two treatments, particularly because LRP1 transcript levels remain elevated in the presence of high doxorubicin treatment, which implies a potential futile cycle between the synthesis and degradation of LRP1 protein. Therefore, I hypothesized that other mechanisms could contribute to the lethal doxorubicin-dependent repression of LRP1 protein expression. Consistent with my hypothesis, the results of a ^{35}Cys/^{35}Met pulse labeling experiment showed that *de novo* translation of LRP1 is indeed reduced in the presence of lethal doxorubicin compared with sub-lethal doxorubicin, suggesting that

doxorubicin dose-dependent regulation of LRP1 protein levels occurs at least in part through translation inhibition (Figure 3-23).

LRP1 protein could be suppressed in a miRNA-dependent manner in response to lethal doxorubicin

Next, I investigated the mechanism through which LRP1 protein translation is suppressed in response to high- but not low-concentration doxorubicin. To this end, I used TargetScan to identify potential miRNA binding sites in the 3'UTR of LRP1. My search revealed three candidate seed regions for miR-205, miR-200b/c, and miR-103/107 (Figure 3-24). Interestingly, all of these miRNA species can be regulated by one or more of the p53 family of transcription factors (p53, p63, and p73).[33, 248] To follow up on these candidates, I used TaqMan miRNA assays to determine whether I could observe a difference in the expression levels of the mature miRNAs in the presence of high or low doxorubicin. Although I saw no doxorubicin dose-dependent difference in the levels of miR-205, miR-200b, or miR-200c, I found significant differences in the expression levels of both miR-107 and miR-103 between sub-lethal and lethal doxorubicin (Figure 3-25A-E). I also observed a doxorubicin dose-dependent increase in the expression of both miR-103 and miR-107, suggesting that these miRNAs could contribute to LRP1 suppression (Figure 3-26A and B). MiR-103 and miR-107 bind to identical seed regions; therefore, I pursued the predicted seed region in the LRP1 3'UTR as the binding site for both miRNAs using multiple test and genomic DNA control primer sets (Figure 3-27). First, I confirmed that the LRP1 3'UTR binding site for miR-103/107 is present in HCT116 cells (Figure 3-28). In the next steps, I

plan to use luciferase reporter assays to determine whether the endogenous miR-103/107 seed region is sufficient to suppress signal upon miR-103 or miR-107 mimic transfection.

LRP1 deletion confers a growth and survival advantage to established colon cancer cell line HCT116 in response to IR stress

To analyze the effect of deleting LRP1 on HCT116 cell survival and growth after an IR challenge, I used the CRISPR-Cas9 system to target *LRP1* exons 1, 2, and 3 at the endogenous locus. I hypothesized that because LRP1 is induced in response to sub-lethal doses of stress, LRP1 could play a role in cell growth and adaptation, particularly in response to DNA damage. To test my hypothesis, I treated several HCT116 LRP1 CRISPR-KO clones with 5 Gy of IR and then obtained cell counts at multiple time points after treatment to determine whether LRP1 might play a role in cell survival and proliferation. Interestingly, compared with the control knockout cells, LRP1 knockout resulted in consistently higher cell counts at all post-IR time points analyzed (Figure 3-29A). Consistent with a role in cell survival, all LRP1 KO lines tested showed fewer trypan blue-positive cells compared with control KO cells, suggesting that the advantage in cell numbers shown by the LRP1 KO lines was due at least in part to reduced cell death (Figure 3-29B). Next, I analyzed the same cell lines using a colony formation assay and found a clear increase in the number and size of the colonies present after an IR challenge (Figure 3-30). Collectively, these results suggest that LRP1 deletion confers a survival advantage to HCT116 cells compared with LRP1-expressing HCT116 cells in response to IR stress.

DISCUSSION

Although the regulome of p53 continues to expand, basic mechanisms by which p53 regulates gene expression remain unresolved. In this study, we contribute to the knowledge of both aspects of p53. I identified LRP1 as a novel p53 target gene that is selectively expressed at the protein level in the presence of sub-lethal stress but is actively suppressed in response to lethal stress. Interestingly, while LRP1 transcript is induced by p53 in response to sub-lethal and lethal stress, LRP1 protein expression is suppressed in response to lethal stress possibly by miR-103 and miR-107, two p53-regulated miRNAs. Thus, our results provide the basis for the further investigation of a possible p53-dependent miRNA regulatory feedback mechanism whereby stresses that are irreparable invoke the p53-dependent suppression of potentially antagonistic cell survival genes.

Interestingly, I observed similar expression patterns at the protein and transcript levels of other p53 target genes, such as *p21* and *MDM2* (Figures 3-8C and 3-16), whereby although transcript levels are elevated in response to lethal and sub-lethal doxorubicin, protein levels show a tendency toward increased expression in response to sub-lethal doxorubicin. Because the panoply of p53 target genes includes those that could be counter-productive to the rapid induction of apoptosis, these results could suggest that p53 has a built-in mechanism that allows the active suppression of cell survival-type p53 target genes under acutely lethal conditions. Various p53 target genes have been reported to exert anti-apoptotic effects. For example, p21 deletion predisposes cells to initiate an apoptotic p53 response, suggesting that p21 could play a role in preventing apoptosis.[196, 315, 316] Several mechanisms have been proposed to explain how p21 prevents apoptosis, including procaspase-3 inhibition,[293] inhibition of pro-apoptotic cyclin-dependent kinases (CDKs),[194,

[283] and inhibition of apoptosis signal-regulating kinase 1.[116] Thus, in response to acute apoptotic stress, it seems likely that inhibition of cell survival genes, such as *p21*, would be necessary to rapidly induce apoptosis. Other p53-regulated cell survival and adaptation pathways, including lipid and glucose metabolism, antioxidant pathways, and DNA damage repair, are also likely counterproductive in the presence of acute apoptotic stresses.

Based on the reported roles of LRP1 in endocytosis and cell signaling, processes that promote cell maintenance, growth, and survival, I reasoned that *LRP1* would fall within the category of cell survival-type genes. Interestingly, in contrast to the reported apoptosis-sensitizing effect of *p21* deletion, I observed that *LRP1* deletion in HCT116 cells protects cells from IR-induced cell death, and thus offers a growth advantage to cancer cells. This is consistent with the down-regulated nature of LRP1 in many types of cancers[65, 134] but is counterintuitive to the sub-lethal-specific induction of LRP1 protein. A possible explanation is that LRP1 contributes to the cell cycle arrest/DNA damage repair process, and deletion of LRP1 allows more cells to continue to divide before completely repairing the DNA damage. In future studies, it would be interesting to determine whether LRP1-deficient cells display more extensive DNA damage due to defects in damage repair than their LRP1-proficient counterparts. Another possible explanation is that clonal selection of the LRP1 CRISPR deletion cells could have promoted compensatory mutations/adjustments that result in more rapid proliferation in the presence of DNA damage. Future experiments analyzing transient LRP1 knockdown could help determine the validity of this possibility.

Our results have interesting clinical implications for the use of various dsDNA break-inducing chemotherapies. Doxorubicin has become a staple for the treatment of several solid tumors, including cancers of the uterus, cervix, prostate, pancreas, liver, and

various sarcomas. Therefore, our finding that the dose of doxorubicin could actually promote a p53-dependent cell survival/adaptation response suggests that sub-optimal doses of doxorubicin could be a substantial problem in the three-dimensional tumor. Because tumors are often poorly perfused in sometimes large areas, it is conceivable that intravenous drugs such as doxorubicin might not accumulate at a high enough concentration in hypoperfused portions of the tumor, which could result in the induction of a distinct set of genes that play a role in cell survival and tumor cell longevity.[217] In future studies, it would be interesting to determine the actual concentration of drugs like doxorubicin at poorly perfused tumor areas to determine whether different p53 target genes show an inverse expression pattern dependent on doxorubicin concentration. The naturally fluorescent nature of doxorubicin offers a convenient method to detect the spatial distribution of doxorubicin perfusion in cryosections of tumors.[54]

Our results also show the importance of the effective dose of a given drug in a tumor because as little as a two-fold dose change can have a dramatic effect on protein expression and cell outcome (Figure 3-16). Moreover, because these differences may not be manifested at the transcriptome level, the use of transcriptomic analysis to determine the effects of drugs on cells should ideally be coupled with proteomic analysis to offer a clearer, more comprehensive picture of gene expression. Future experiments analyzing the effects of different doses of chemotherapeutic agents such as doxorubicin could yield valuable insight into the heterogeneous effects that likely characterize the treatment of tumors. Moreover, our findings highlight the importance of identifying methods that increase the local concentration of chemotherapeutics throughout the tumor without requiring dose

increases, especially because drugs such as doxorubicin can cause secondary cancers such as leukemia or cardiotoxicity when administered in high doses.

In conclusion, in addition to the identification of the heavily researched endocytic receptor LRP1 as a novel p53 target, I show evidence for a novel context-dependent p53 post-transcriptional regulatory mechanism that depends on the expression of miRNAs. I offer novel insight into how p53 can actively suppress its own cell survival program without requiring changes in its transcriptome. I anticipate that future studies into p53-regulated genes may yield many more p53 targets that are regulated in a similar manner.

EXPERIMENTAL PROCEDURES

Cell lines and reagents

HCT116 WT and $p53^{-/-}$ cells, as well as all HCT116 derivatives, and MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS at 37°C in the presence of 5% CO_2. MG132 was purchased from Calbiochem (cat. no. 474790). Cycloheximide and doxorubicin were purchased from Sigma (cat. nos. C7698 and D1515, respectively). QVD-OPh was purchased from ThermoFisher. Phusion polymerase (cat. no. M0530S) and all restriction enzymes were purchased from NEB. Effectene transfection reagent was purchased from Qiagen through the UNC Tissue Culture Facility. Anti-LRP1 antibody recognizing the beta-subunit of LRP1 was purchased from Abcam (ab92544 Cambridge, UK). Mouse anti-actin (MAB1501, Chemicon), mouse anti-p53 (DO-1, Labvision AB-6 cat. no. MS-187P), goat anti-p21 (C19, SC-397G Santa Cruz), mouse anti-PARP (clone C2-10, #556362, BD Biosciences), rabbit anti-cleaved caspase 3 (D175, #9661S, Cell Signaling Technology) antibodies were purchased. miRNA assays, miRNA reverse

transcription kit, and TaqMan miRNA Universal PCR Master mix were ordered from ThermoFisher. Oligonucleotides for cloning, PCR or reverse transcription were ordered through the UNC Nucleic Acid Core Facility. For doxorubicin treatments, pulsed doxorubicin (a sub-lethal dose of doxorubicin) refers to the treatment of cells for 1 h with 1 μM doxorubicin followed by a PBS wash and fresh DMEM. High doxorubicin refers to treating cells with a constant 1 μM dose of doxorubicin for 24 h unless stated otherwise.

Polymerase chain reaction (PCR) and cloning

PCR primers for cloning or amplification were designed with melting temperatures near 60°C. All PCR reactions were conducted using the following reaction conditions: 98°C for 2 mins followed by 30 cycles of 98°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec/kb and a final elongation step of 72°C for 7 mins. The following PCR primer sets were used: .

Plasmid transfection

Cells plated at approximately 80% confluency were transfected using Effectene reagent (Qiagen) according to the protocol recommended by the manufacturer. Briefly, 0.4 μg of plasmid DNA was mixed with 3.2 μl of enhancer reagent, and 8 μl of Effectene reagent. The DNA-effectene mixture was incubated at room temperature for 15 min, after which the mixture was added directly to the cells in culture. Transfections were allowed to proceed for 24 h, after which cells were collected and processed or the medium was replaced.

Viral transduction

Cells plated in 6-cm dishes at approximately 50% confluency were incubated with 500 µl of DMEM containing adenoviral particles encoding wild-type p53 or GFP. Infection was allowed to proceed for 3 hours, after which the virus was removed, and the cells were incubated in DMEM for an additional 24 hours. Cells were collected and processed for the indicated analysis.

Western blotting

Cells were lysed in 0.5% or 0.1% Nonidet P-40 or 2% SDS, as indicated. Immediately before lysis, 1x protease inhibitor cocktail (leupeptin L2884, aprotinin A1155, benzamidine B6506, trypsin inhibitor T9003, all from Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma P7626), 1 mM $NaVO_3$ (Fisher Scientific S454-50), and 1 mM dithiothreitol (DTT, Roche 03117014001) was added to the lysis buffer, and the cells were scraped in lysis buffer. For NP-40 lysis, the cells were rotated at 4°C for up to 2 h to lyse the cells, after which the cell debris was pelleted, and the supernatants were collected. For SDS lysis, the cells were scraped and transferred to microfuge tubes, after which the lysate was passed 15 times through a 25-gauge needle attached to a 1-ml tuberculin syringe (BD Biosciences). Protein concentrations for NP-40 lysates were determined using a Bradford assay, and protein concentrations for SDS lysates were determined using a BCA assay based on a standard curve constructed from bovine serum albumin. An equal amount of total protein for each sample was loaded onto 12.5% or 10% SDS-PAGE gels. After separation, proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were blocked in 5% non-fat milk in PBS with 0.1% Tween 20 (PBS-T) for one

hour at room temperature, after which primary antibody was added to the appropriate membrane and incubated at room temperature for 2 hours or overnight at 4°C with shaking. Membranes were washed three times in PBS-T, after which the membranes were incubated with the appropriate secondary antibody at RT for 1 h with shaking. Membranes were washed four times with PBS-T, and then bands were developed using ECL reagent (Advansta), Pico reagent, or Dura reagent (Pierce). Images were acquired using a BioRad digital imager (BioRad Hercules, CA).

Reverse transcription-PCR

After treatment, RNA was purified from cells using the Zymo Quick RNA mini-prep kit according to the protocol recommended by the manufacturer (Zymo Research R1057). Then, cDNA was synthesized using a BioRad iScript cDNA Synthesis kit (BioRad 1708891) for total RNA or a TaqMan microRNA Reverse Transcription kit for miRNA (ThermoFisher 4366596) according to the protocol recommended by the manufacturer. cDNA was diluted 1:5 (total RNA analysis) or undiluted (miRNA), and then analyzed using SYBR Green reagent (BioRad 1525271) or the appropriate TaqMan assays (ThermoFisher #4427975 (all human) miR-205-5p: Assay ID000509, miR-200b-3p: 002251, miR-200c-3p: 002300, miR-103-3p: 000439, miR-107: 000443, U6: 001973) according to the protocol recommended by the manufacturer. Data were analyzed using the $\Delta\Delta C_t$ method, and experiments were normalized to actin, GAPDH, or U6 miRNA. Primer sequences included the following: LRP1: Fwd CAACAGATCAACGACGATGG, Rev GGGTGGCGTCAGAGAAGTAG; PUMA: Fwd ACGACCTCAACGCACAGTACG, Rev GTAAGGGCAGGAGTCCCATGATG; Bax: Fwd CATGTTTTCTGACGGCAACTTC, Rev CAGCCCATGATGGTTCTGAT; Apaf1: Fwd

GGAGGACCCTCAAGAGGATATG, Rev GGATTTCTCCCAATAGGCCACT; Noxa: Fwd AGAAGGCGCGCAAGAAC, Rev GCACCTTCACATTCCTCTCAG; MDM2: Fwd GGTGGGAGTGATCAAAAGGA, Rev CCTGATCCAACCAATCACCT; CDKN1A: Fwd GTCAGAACCCATGCGGCAGCAAG, Rev CAGGTCCACATGGTCTTCCTCTG; Actin Fwd AGAAAATCTGGCACCACACC, Rev CTCCTTAATGTCACGCACGA; GAPDH Fwd CCTGACCTGCCGTCTAGAAAAACCT, Rev CCATGAGGTCCACCACCCTGTT.

Luciferase assay

The WT or a mutant version of the putative response element for LRP1 was cloned into the pGL3 basic vector. The resultant pGL3 construct was co-transfected into p53-null H1299 cells in 6-well plates along with a constitutive renilla luciferase expression construct and either empty vector or a p53-expressing construct. A positive control construct harboring the native p21 p53 response element was included for comparison. Transfection was conducted for 24 h, after which the cells were analyzed for luciferase signal using a Promega DualGlo Luciferase kit (Promega) according to the protocol recommended by the manufacturer. Briefly, after transfection, cells were trypsinized and transferred to 96-well plates. Cells were lysed with equal volume of DualGlo reagent, and then firefly luciferase signal was determined using a SpectraMax plate reader. Next, one volume of Stop & Glo reagent was added to each well, and the wells were read for renilla luciferase signal. For analysis, firefly luciferase signal was normalized to the corresponding renilla luciferase signal. After normalization, the luciferase signal in the presence of WT p53 was normalized to the luciferase signal in the presence of empty vector. Data represent the averaged and associated standard deviation of two independent experiments

performed in triplicate. The following oligonucleotides were ordered and annealed for cloning to construct the luciferase vectors to test LRP1 promoter activity: LRP1 Fwd TCGAGGAGCCCCACGCGGGC GGACAAGCTCCGGCGTGTCCCCTCGGGTGTCCCTGA, Rev AGCTTCAGGGACACCCGAGG GGACACGCCGGAGCTTGTCCGCCCGCGTGGGGCTCC; LRP1 mutant Fwd TCGAGGAGCCCCACGCGGGCGGATAATCTCCGGAGTATCCCCTCGGGTGTCCCTGA, Rev AGCTTCAGGGACACCCGAGGGGATACTCCGGAGATTATCCGCCCGCGTGGGGCTCC; p21 Fwd CAGGGTACCGCTTTCTGGCCGTCAGGAACATGTCCCAACATGTTGAGCTCTGGCAAGCTTGAC, Rev GTCAAGCTTGCCAGAGCTCAACATGTTGGGACATGTTCCTGACGGCCAGAAAGCGGTACCCTG. The p53 R273H mutant construct was made using site-directed mutagenesis based on a pCMV-WT p53 vector using the following primers: Fwd GGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGG, Rev CCAGGACAGGCACAAACATGCACCTCAAAGCTGTTCC.

CRISPR/Cas9 deletion

CRISPR/Cas9 constructs were designed and assembled based on previous reports.[55] Briefly, high-scoring guide RNAs (gRNAs) were identified using the CRISPR design tool developed by the Feng Zhang lab. LRP1 exons 1, 2 and 3 and p53 exons 3 and 5 were used as input to identify gRNAs, and the gRNAs were synthesized, annealed and cloned into the PX260 plasmid. gRNA annealed oligonucleotides included the following: LRP1 exon1: Fwd CACCGGCTCTCAGCTCTGGTCGCGG, Rev AAACCCGCGACCAGAGCTGAGAGCC; LRP1 exon2: Fwd CACCGTCAAAGGGCTGGCGGTGCGA, Rev AAACTCGCACCGCCAGCCCTTTGAC; LRP1

exon3: Fwd CACCGGCTCGTTTGGCTGGCATCGC, Rev AAACGCGATGCCAGCCAAACGAGCC; p53 exon3 Fwd CACCGTCCTCAGCATCTTATCCGAG, Rev AAACCTCGGATAAGATGCTGAGGAC; p53 exon5 Fwd CACCGCCATTGTTCAATATCGTCCG, Rev AAACCGGACGATATTGAACAATGGC. The CRISPR constructs were transiently transfected into cells for 24 h, after which puromycin was added for 72 h to select CRISPR transfectants. After selection, individual clones were isolated using a limiting dilution method. After two weeks, colonies were screened for the absence of LRP1 or p53, and knockout clones were used for subsequent analyses.

Colony formation assay

Equal amounts of cells (1 x 10^4 cells per sample) were plated in 6-well plates in triplicate. The next day, cells were treated with 5 Gy of IR using an x-irradiator (RS-2000). Cells were allowed to recover for 7 days, during which 1 ml of fresh medium was added every other day so as not to excessively disrupt the remaining cells. After day 7, medium was replaced every other day until visible colonies were formed (approximately 3 weeks). Cells were then fixed in 10% formalin overnight and then stained for 20 mins with 0.05% crystal violet stain in a solution composed of 1% methanol and 10% PBS. Colonies were destained with several 1-ml washes of distilled water. Images were scanned using an Epson office scanner.

Cell growth curve

Cells (1 x 10^5 cells per sample) were counted and plated in duplicate in 6-cm dishes. The next day after plating, cells were treated with 5 Gy of ionizing radiation using an x-

irradiator (RS-2000). The cells were incubated for an additional 7 days with fresh medium added every 2 days to allow for recovery. On days 7, 9, and 12 after treatment, cells were counted using an automated cell counter (BioRad model TC20). Trypan blue stain was included in the counted samples to assess the extent of membrane integrity. Each sample was counted twice and averaged to obtain the count for each plate. Two plates were counted for each cell line for each time point, and the data were compiled in a growth curve based on the average and standard deviation of the two cell counts.

Half-life assay

Cells were pre-treated with 10 μM QVD-OPh for 30 mins, and then 1 μM doxorubicin was added to the cells as indicated. Doxorubicin treatment was conducted for 22 h, after which cycloheximide (CHX, 100 μg/ml) was added to the indicated samples. At several time points, cells were collected in 0.5% NP-40 lysis buffer containing 1x protease inhibitor cocktail, 1 mM PMSF, 1 mM $NaVO_3$, and 1 mM DTT and then frozen at -80°C until all time points were collected. Cells were then lysed for 2 h by rotation at 4°C, after which the samples were centrifuged, and the supernatants collected. Lysates were separated by SDS-PAGE and subjected to western blotting for the indicated proteins. Bands were quantified after normalization to actin, and then were plotted as the amount of protein remaining relative to time 0. Bands were compared quantitatively by using ImageJ software version 1.48.

Chromatin immunoprecipitation

ChIP assays were conducted using the QuickChIP kit (Novus Biologicals) according to the protcol recommended by the manufacturer with a few adaptations for p53 immunoprecipitation. All of the reagents were included in the kit except for p53 antibody (FL-393 rabbit anti-human p53). HCT116 cells were grown and treated with the indicated drug in 10-cm dishes, after which p53 was cross-linked to the DNA by incubating with 1% formalin solution at 37°C for 10 mins. Formalin was neutralized with glycine, and then the cells were washed twice with cold PBS. Cells were scraped in cold PBS, pelleted, and then resuspended in 1 ml of SDS lysis buffer. DNA was sonicated and then precleared with protein-A/G agarose beads. Precleared DNA was subjected to IP with 0.6 µg of p53 antibody by rotating at 4°C overnight. Antibody-p53/DNA complexes were pulled down by incubating with protein A/G beads at 4°C for 1 h. Beads were washed with 1 ml of buffers of differing salt concentrations. Then, p53-DNA complexes were eluted from the beads by incubating with two 250-ul aliquots of elution buffer at RT for 15 mins. Cross-links were reversed by adding 20 µl of 5 M NaCl and incubating at 65°C overnight. Samples were RNase-treated and then incubated with 10 mM EDTA, 25 mM Tris-HCl (pH 6.5), and 20 µg/ul proteinase K for 1 h at 45°C. DNA was purified using a Qiagen PCR purification kit, and then DNA was quantified using qPCR. Primers for ChIP qPCR included the following: LRP1 p53RE: Fwd AATGAGCCCCGACTTCTTG, Rev TCGGAGTAAACAGGGACACC; p21 p53RE: Fwd CTGGACTGGGCACTCTTGTC, Rev CTCCTACCATCCCCTTCCTC.

^{35}Cys/^{35}Met labeling assay

Cells were treated in 6-cm dishes with the indicated treatment for 24 h. After treatment, cells were starved with Cys/Met-free DMEM for 30 mins, after which 170 uCi of ^{35}Cys/^{35}Met was added to each dish. Cycloheximide was included in one sample to control for *de novo* translation-mediated incorporation of ^{35}S label. Label was applied for 30 mins followed by a 30 min chase period with complete DMEM. Then, protein lysates were obtained by lysing the cells in 0.1% NP-40 lysis buffer containing inhibitors. Lysates were subjected to IP using an anti-LRP1 antibody (Abcam ab92544). After separation on 10% SDS-PAGE gels, the gels were dried for 4 h on a vacuum gel drying apparatus, and then the gel was exposed to autoradiography film for several days.

Statistical analysis

All statistical analysis was conducted using GraphPad software version 5.0 (La Jolla, CA). Comparisons were conducted using Student's unpaired t-tests. P-values < 0.05 were considered statistically significant. *, **, and *** correspond to p-values < 0.05, 0.01, and 0.001, respectively.

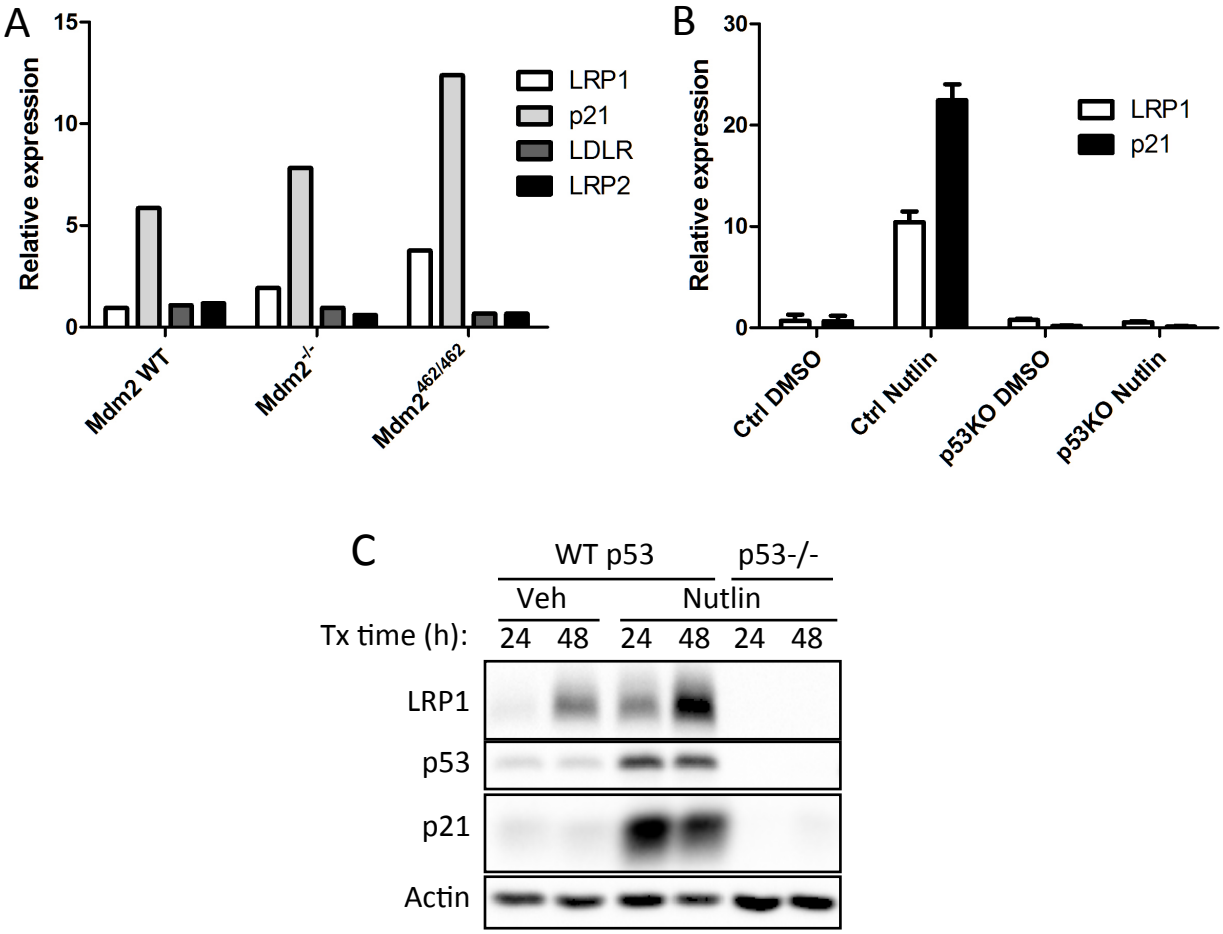

Figure 3-1. LRP1 transcript and protein is upregulated in response to nutlin-3a treatment.

A) Microarray screen of $Mdm2^{WT/WT}$, $Mdm2^{-/-}$, and $Mdm2^{462/462}$ MEF cells reveals a similar expression pattern between LRP1 and p21. Control genes LDLR and LRP2 do not show increasing expression patterns.

B) LRP1 transcript shows a p53-dependent increase in expression. Cells were treated with 10 μM nutlin-3a.

C) LRP1 protein shows a p53-dependent increase in expression. Cells were treated as in (B).

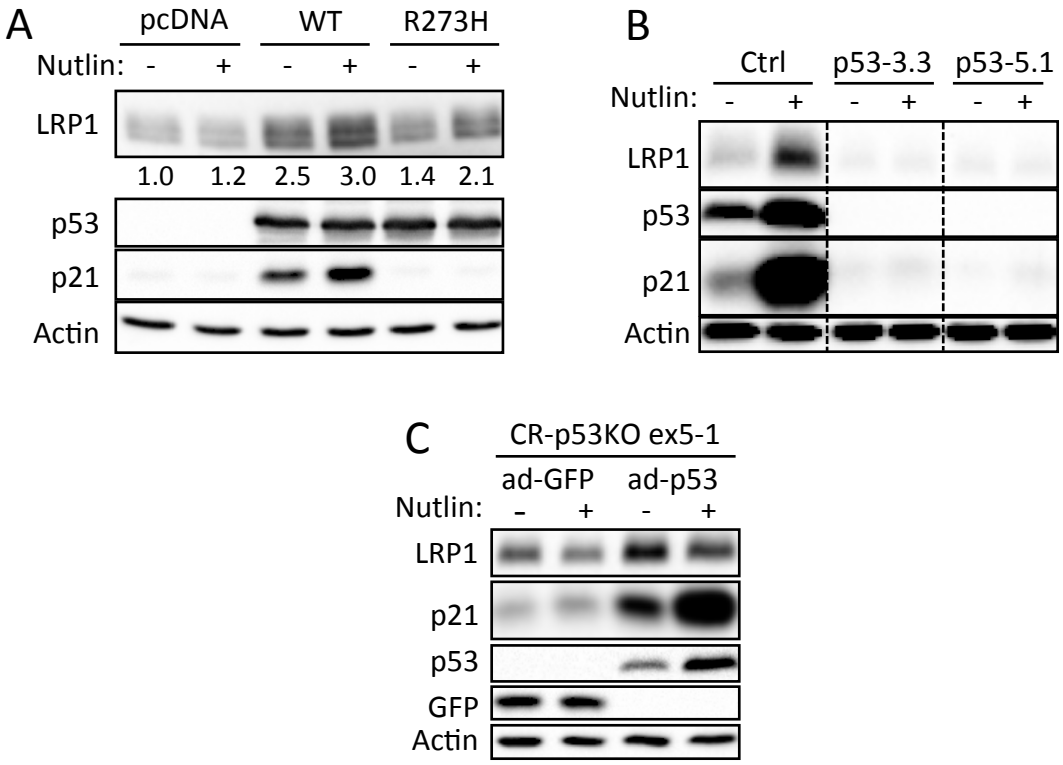

Figure 3-2. LRP1 expression is p53-dependent.
A) WT p53 rescues LRP1 expression. H1299 cells were transfected with WT or mutant (R273H) p53 for 24 h, after which the cells were treated with vehicle or 10 μM nutlin-3a for 24 h.
B) p53 knockout inhibits LRP1 expression. HCT116 cells were subjected to CRISPR-mediated p53 knockout. After clonal selection, cells were treated with vehicle or 10 μM nutlin-3a for 24 h.
C) LRP1 expression could be rescued in HCT116 CRISPR p53 knockout cells. p53 CRISPR knockout cells were infected with WT p53- or GFP-expressing adenovirus for 24 h, after which cells were treated with 10 μM nutlin-3a for an additional 24 h.

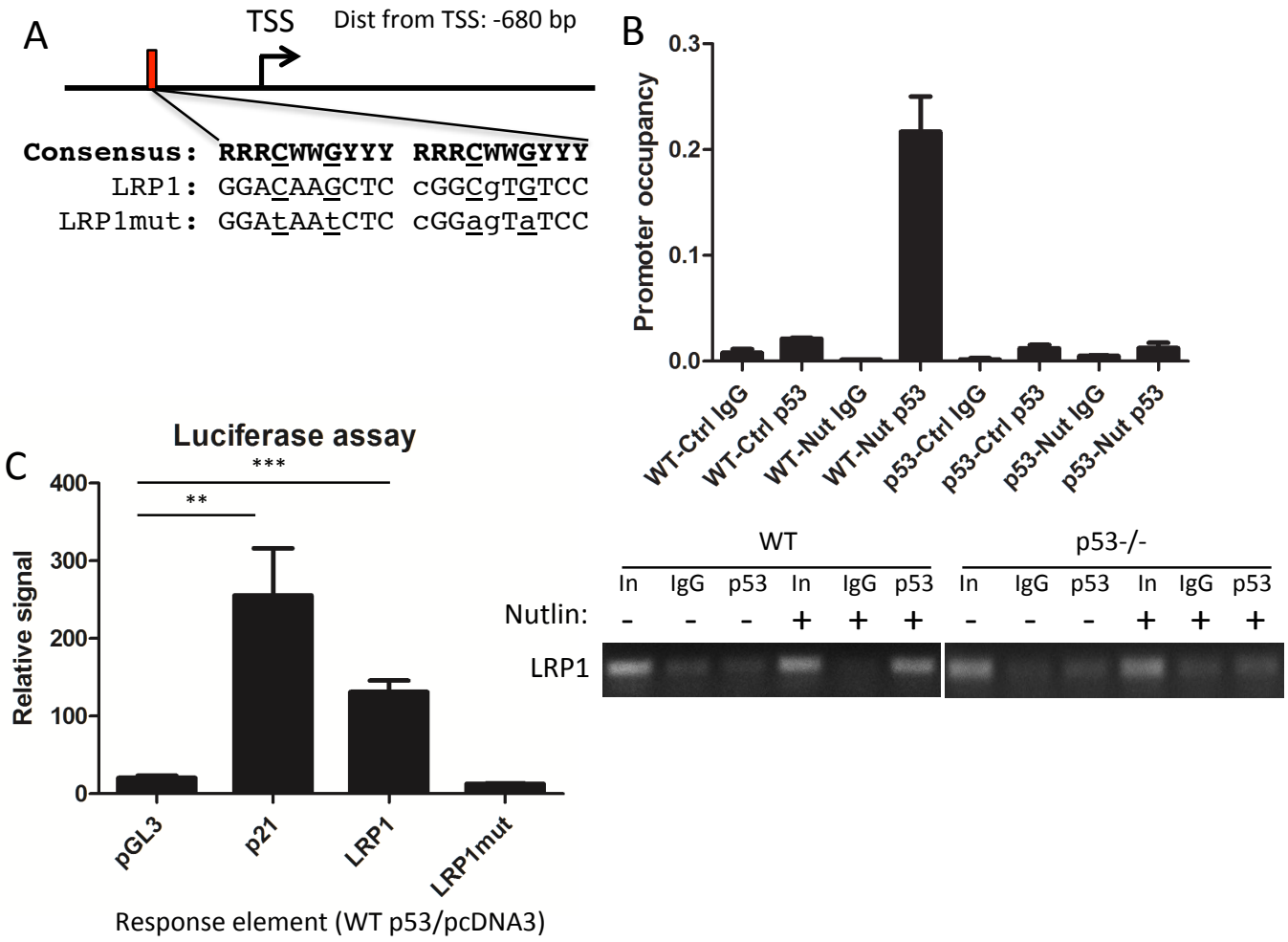

Figure 3-3. LRP1 is a direct p53 transcription target.
A) A putative p53 response element was identified in close proximity to the LRP1 transcription start site.
B) p53 binds directly to the putative p53 response element in the LRP1 promoter. HCT116 WT and p53-null cells were treated with vehicle or 10 μM nutlin-3a for 24 h, after which the cells were subjected to chromatin immunoprecipitation with p53 antibody or non-specific IgG. The PCR products were analyzed by gel electrophoresis and are shown below the graph.
C) LRP1 p53 response element is sufficient to drive reporter gene expression. H1299 cells were transiently transfected with luciferase plasmids containing the endogenous WT LRP1 p53 RE or a mutated derivative of the LRP1 p53 RE. The p21 WT p53 RE was included as a positive control. Data were normalized to renilla luciferase signal, and the data are presented as the luciferase signal in the presence of WT p53 to the signal in the absence of p53.

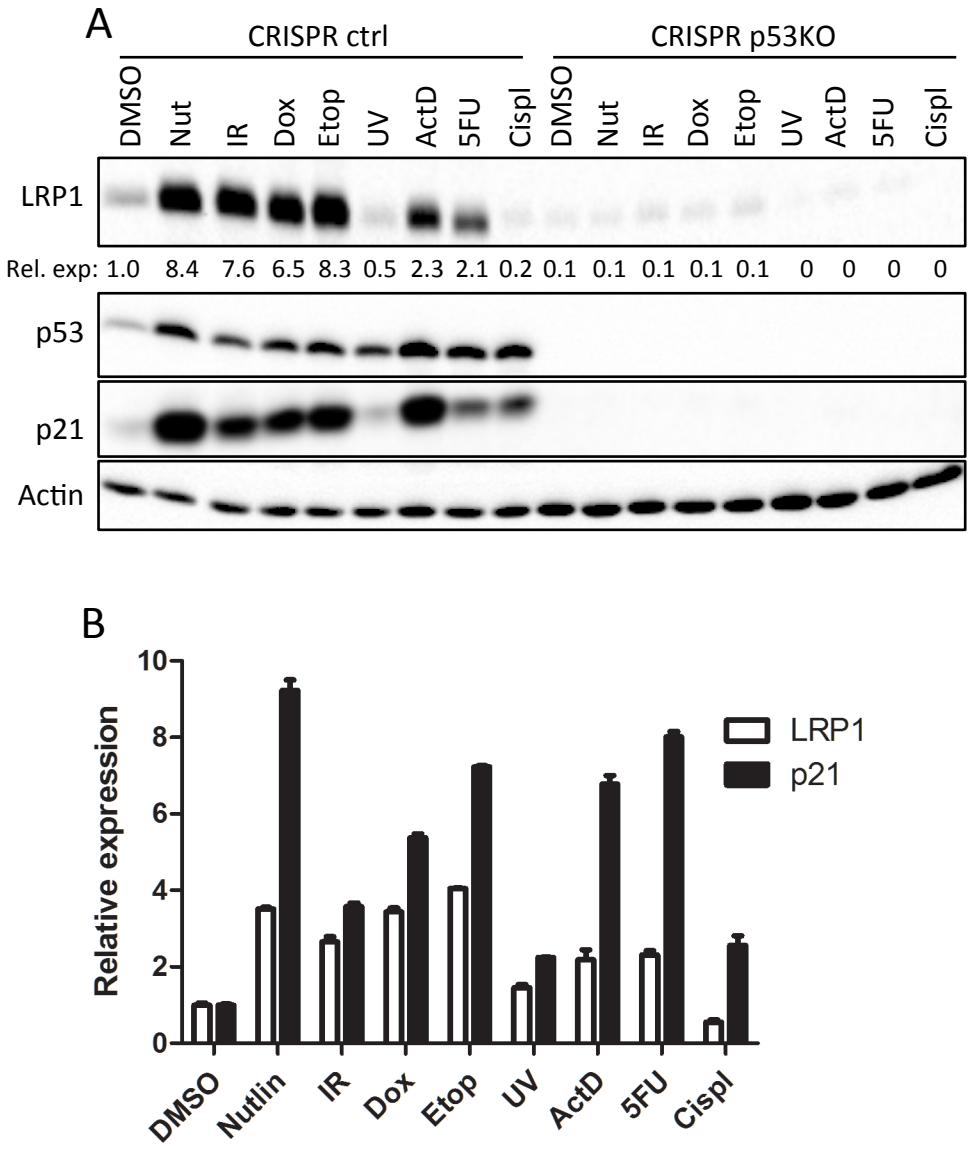

Figure 3-4. LRP1 is induced in response to canonical p53-activating stresses.
A) LRP1 protein is expressed in response to several types of stress. HCT116 CRISPR ctrl or p53 knockout cells were treated with the indicated stresses for 24 h and then probed for the indicated proteins.
B) HCT116 cells were treated with the indicated stresses for 24 h, after which transcript levels were evaluated. Treatment abbreviations: Nut, 10 μM nutlin-3a; IR, 10 Gy ionizing radiation; Dox, 200 nM doxorubicin; Etop, 10 μM etoposide; UV, 25 J/m^2 UV-C; ActD, 5 nM actinomycin D; 5FU, 100 μM 5-fluorouracil; Cispl, 10 μM cisplatin.

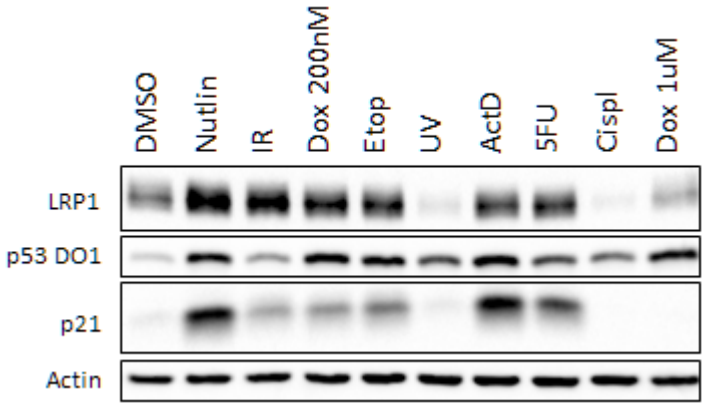

Figure 3-5. U2OS cells show a similar trend in LRP1 expression.
U2OS cells were treated with the indicated treatment for 24 h, after which lysates were collected and subjected to western blot. Treatment abbreviations: Nut, 10 μM nutlin-3a; IR, 10 Gy ionizing radiation; Dox, 200 nM doxorubicin; Etop, 10 μM etoposide; UV, 25 J/m^2 UV-C; ActD, 5 nM actinomycin D; 5FU, 100 μM 5-fluorouracil; Cispl, 10 μM cisplatin.

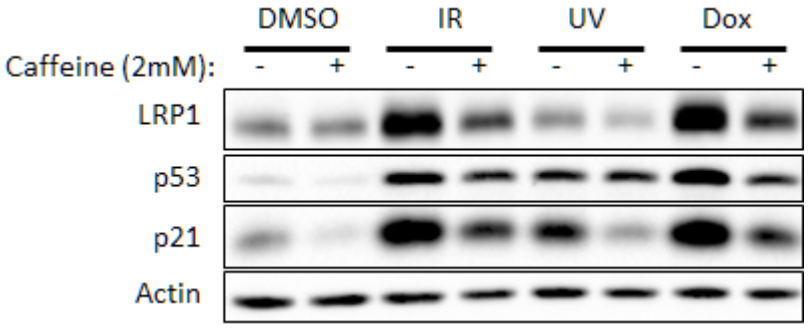

Figure 3-6. LRP1 induction is attenuated in the presence of the pleiotropic inhibitor caffeine.
HCT116 WT cells were treated with the indicated stresses for 24 h in the presence or absence of caffeine. Treatment abbreviations: IR, 10 Gy ionizing radiation; UV, 25J/m^2 UV-C; Dox, 200 nM doxorubicin.

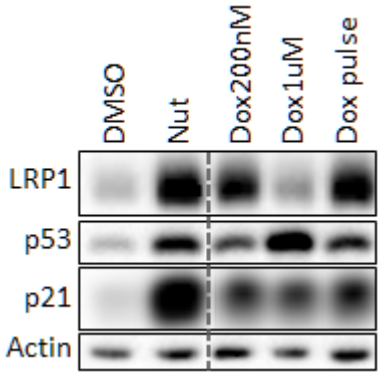

Figure 3-7. Sub-lethal doxorubicin can induce LRP1 expression.
HCT116 cells were treated with the indicated dose of doxorubicin for 24 h, after which lysates were collected and subjected to western blot. Vehicle and 10 μM nutlin-3a treatments were included as negative and positive controls, respectively. Dox pulse refers to a 1-h treatment with 1 μM doxorubicin followed by drug withdrawal and culture in regular medium for an additional 23 h.

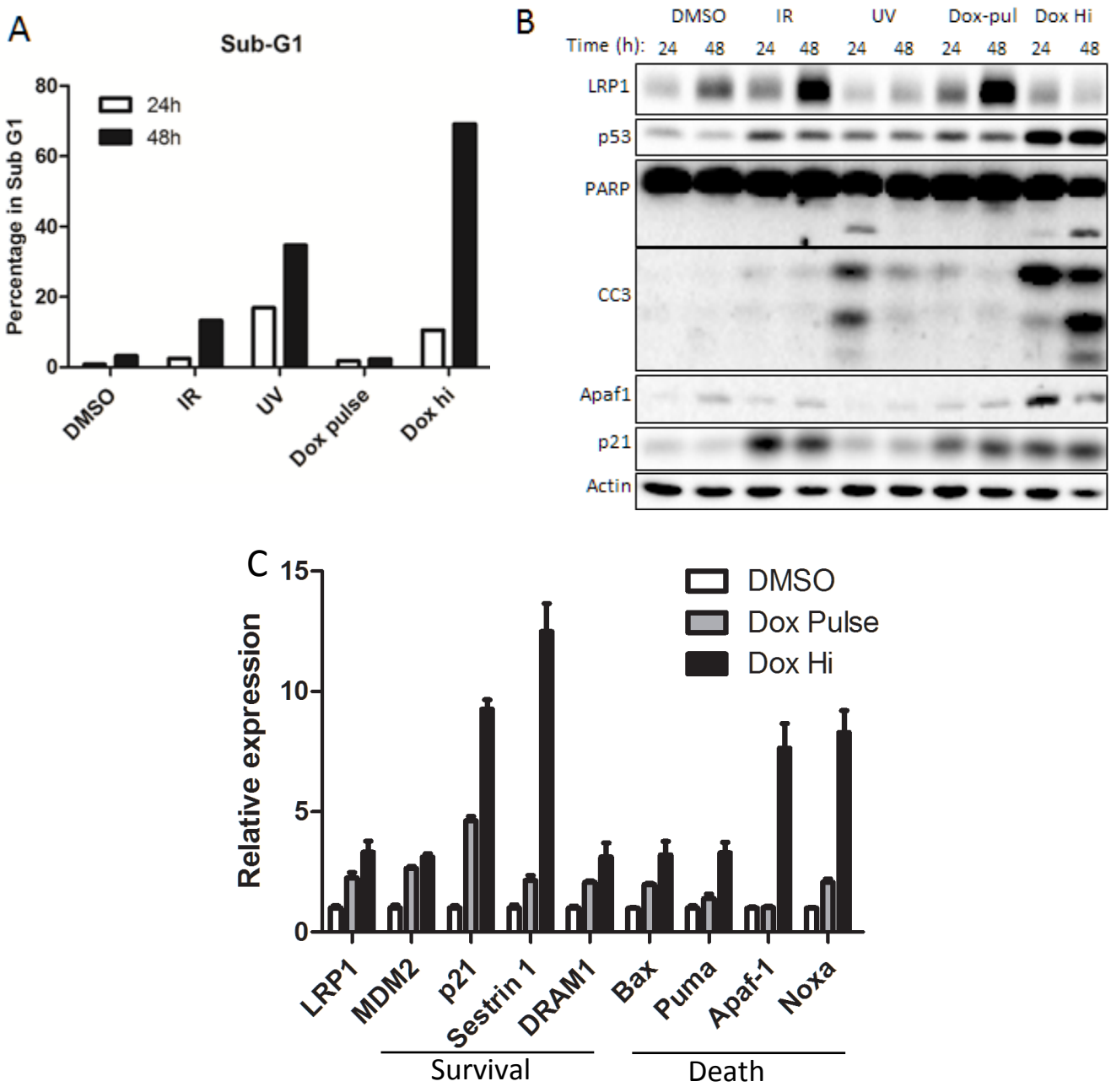

Figure 3-8. LRP1 protein is expressed in response to sub-lethal stresses.
A) HCT116 cells were treated with the indicated stress for 24 or 48 h, after which the cells were subjected to PI staining and flow cytometry analysis. The percentages of sub-G1 cells are quantified in the graph. Treatment abbreviations: IR, 10 Gy ionizing radiation; UV, 25J/m^2 UV-C; Dox pulse and Dox Hi, see Experimental Procedures.
B) HCT116 cells were treated with the indicated stress for 24 or 48 h, after which lysates were subjected to western blot.
C) Lethal but not sub-lethal doxorubicin strongly induces pro-apoptotic p53 target gene transcription.

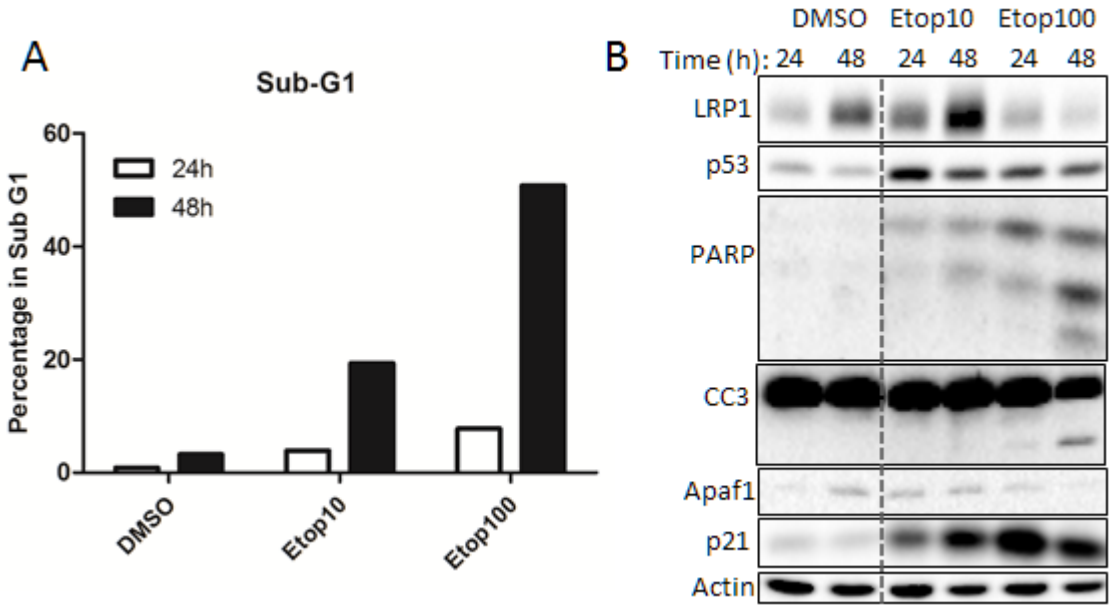

Figure 3-9. HCT116 cells show dose-dependent induction of LRP1 in response to etoposide.
A) HCT116 cells were treated with 10 M or 100 M etoposide for 24 or 48 h, after which cells were stained with propidium iodide and analyzed by flow cytometry. The sub-G1 population of cells is quantified in the graph for each treatment.
B) Protein lysates taken from a sample of the cells analyzed in (A) were analyzed by western blot for cell death markers and LRP1 expression.

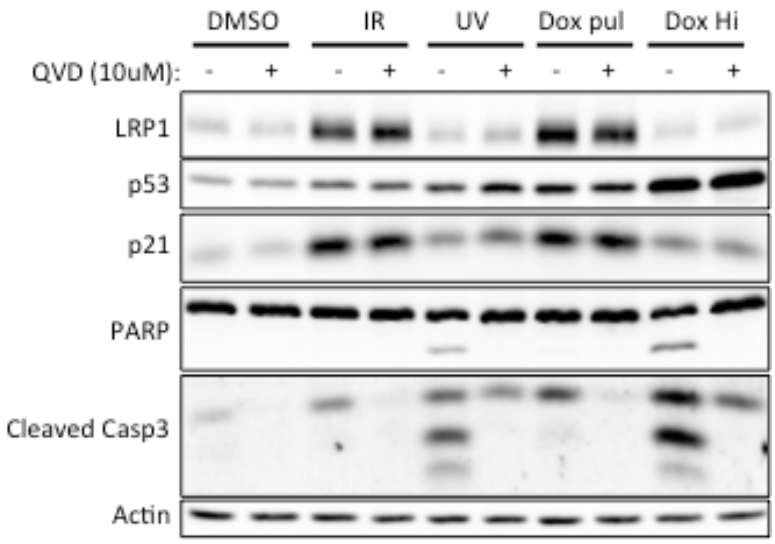

Figure 3-10. Caspase inhibition does not rescue LRP1 expression.
HCT116 cells were treated with the indicated stresses in the presence or absence of the caspase inhibitor QVD-OPh, 24 h after which lysates were subjected to western blot. Treatment abbreviations: IR, 10 Gy ionizing radiation; UV, 25J/m2 UV-C; Dox pulse and Dox Hi, see Experimental Procedures.

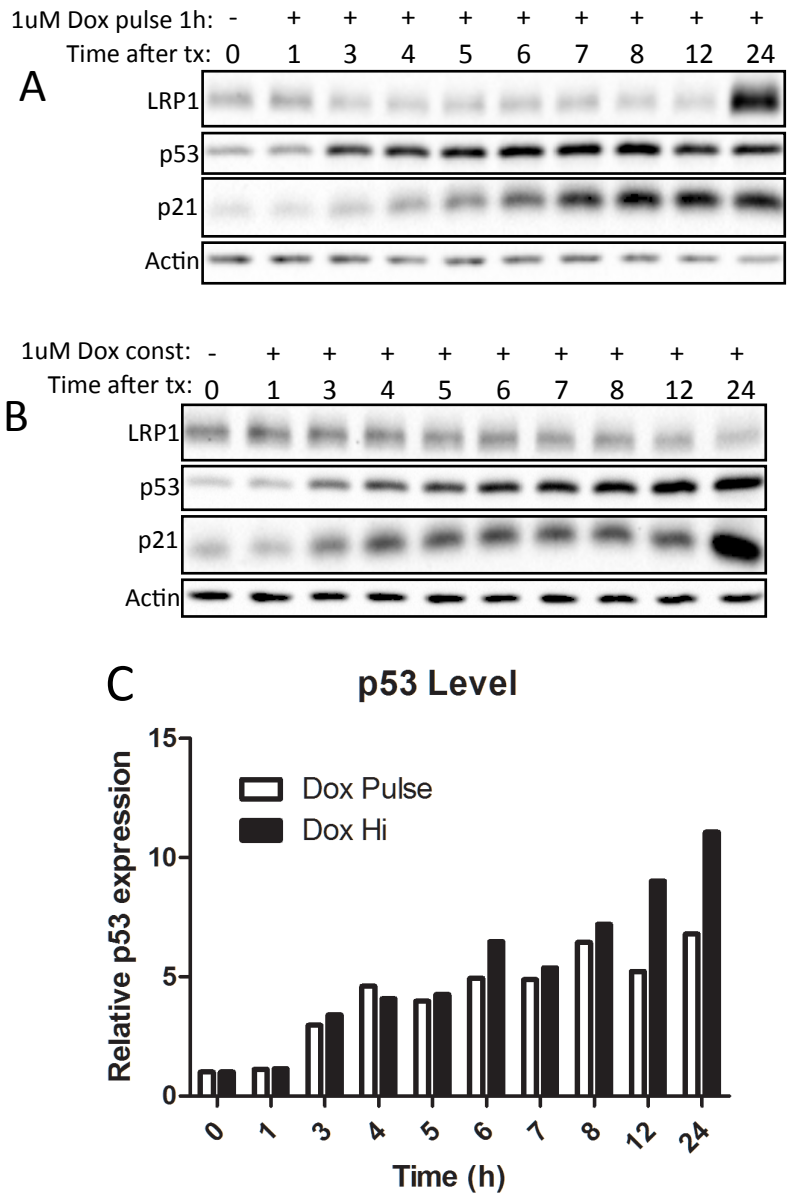

Figure 3-11. LRP1 is a late-expressed gene that occurs in response to sub-lethal doses of doxorubicin.

A) HCT116 cells were treated with 1 μM doxorubicin for 1 h, after which the drug was withdrawn and the cells were cultured for an additional amount of time as indicated.

B) HCT116 cells were treated with 1 μM doxorubicin for the indicated amount of time, at which point lysates were collected and analyzed by western blotting.

C) p53 levels were quantified in the graph to the right after normalization to actin.

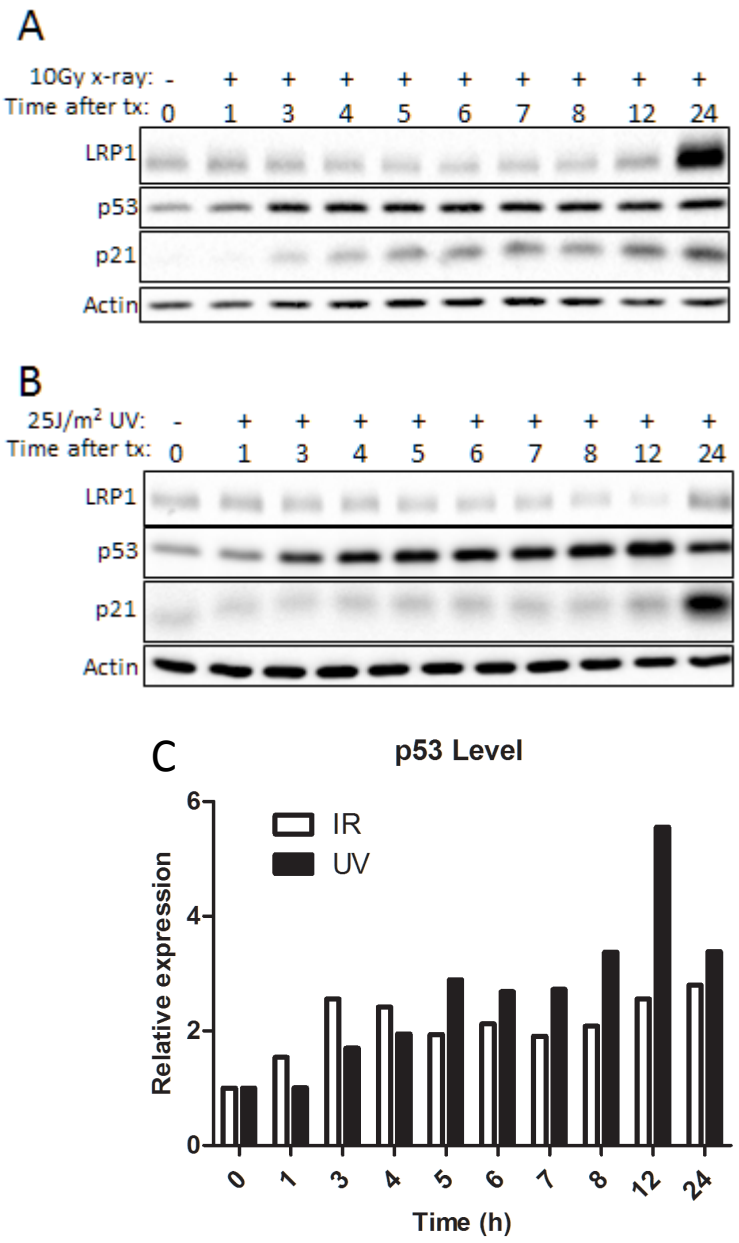

Figure 3-12. X-irradiation and UV irradiation produce distinct patterns of p53 induction and different ability to induce LRP1.
A) HCT116 cells were treated with 10 Gy of x-irradiation, and lysates were collected at the indicated time points and subjected to western blot.
B) HCT116 cells were treated with 25 J/m^2 of UV irradiation, and lysates were collected at the indicated time points and subjected to western blot.
C) p53 protein levels were normalized to actin and quantified.

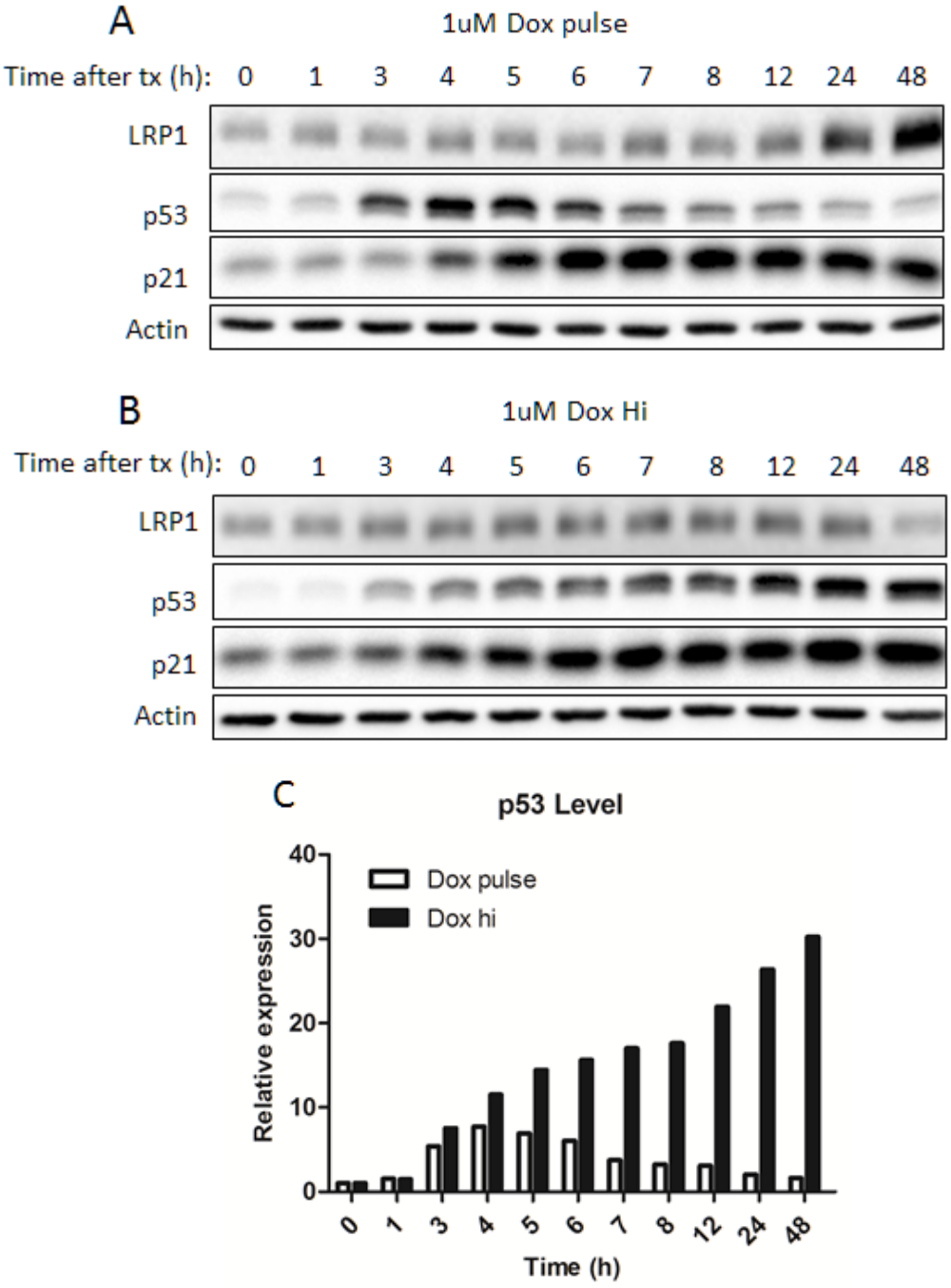

Figure 3-13. MCF7 cells show a similar pattern of p53 induction to HCT116 cells in response to doxorubicin.
A) MCF7 cells were treated with 1 μM doxorubicin for 1 h followed by drug withdrawal, and lysates were collected at the indicated time points and subjected to western blot.
B) MCF7 cells were treated with 1 μM doxorubicin in a constant manner, and lysates were collected at the indicated time points and subjected to western blot.
C) p53 protein levels were normalized to actin and quantified.

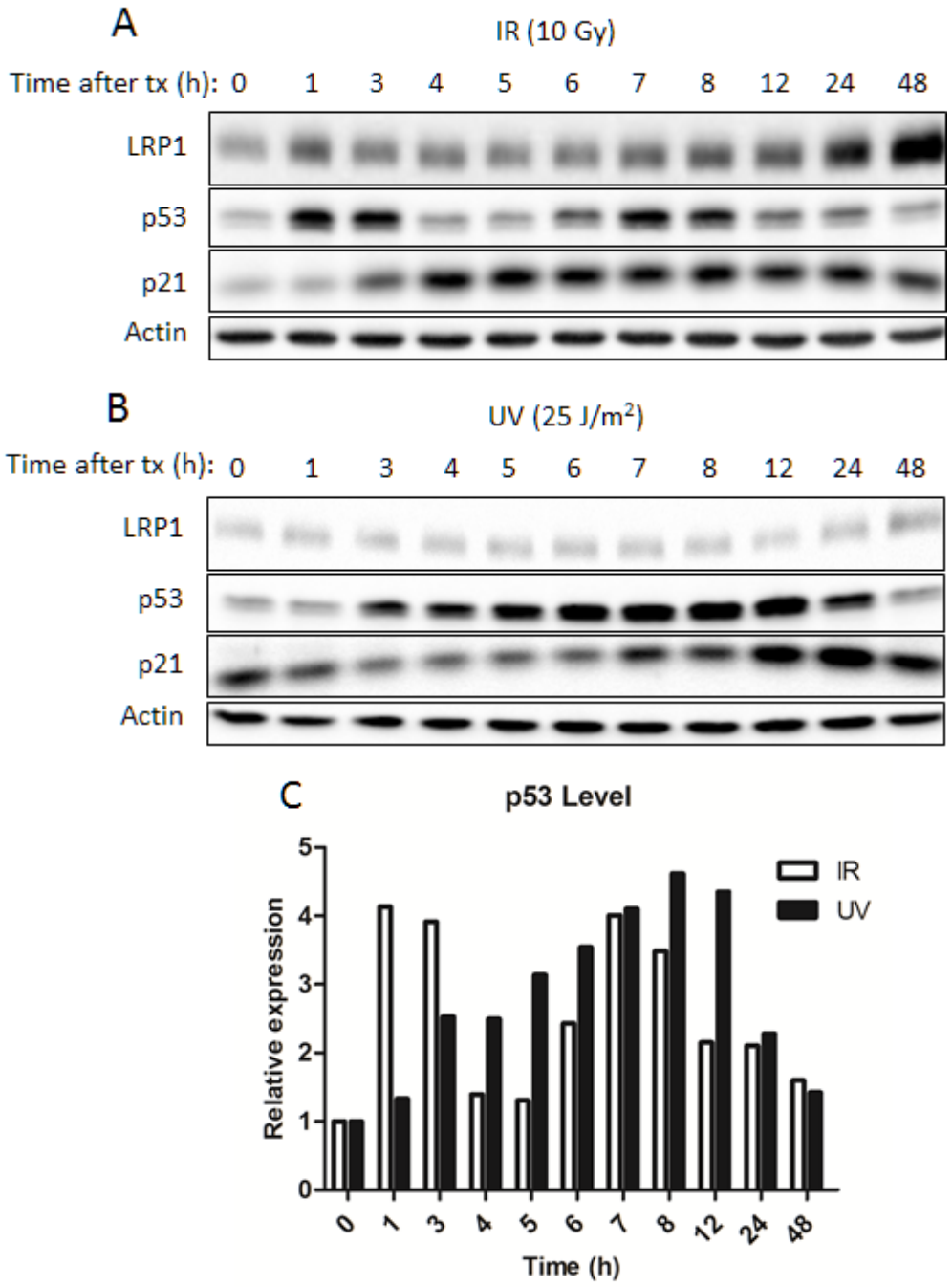

Figure 3-14. MCF7 cells show a similar pattern of p53 induction to HCT116 cells in response to IR and UV.
A) MCF7 cells were treated with 10 Gy of IR, and lysates were collected at the indicated time points and subjected to western blot.
B) MCF7 cells were treated with 25 J/m^2 of UV, and lysates were collected at the indicated time points and subjected to western blot.
C) p53 protein levels were normalized to actin and quantified.

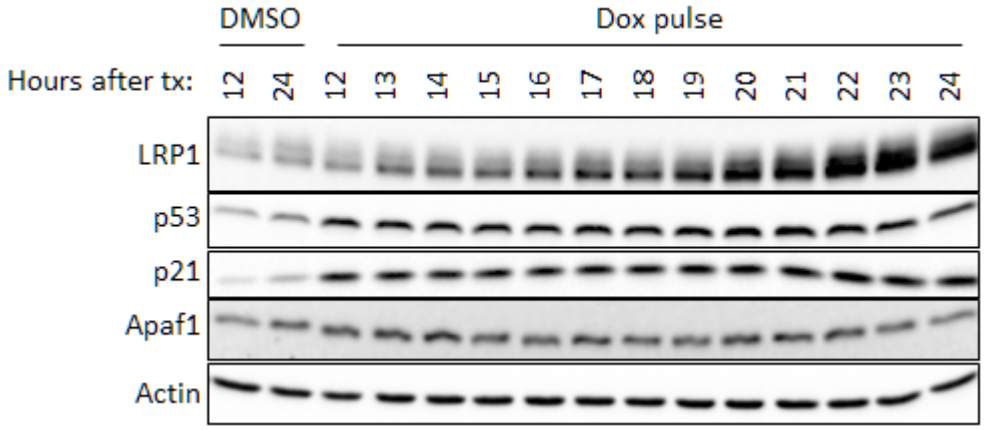

Figure 3-15. LRP1 protein is induced gradually between 12 and 24 h after sub-lethal doxorubicin treatment.
HCT116 cells were treated with a 1-h pulse of doxorubicin and then were collected at the indicated time point after treatment, lysed, and analyzed by western blot.

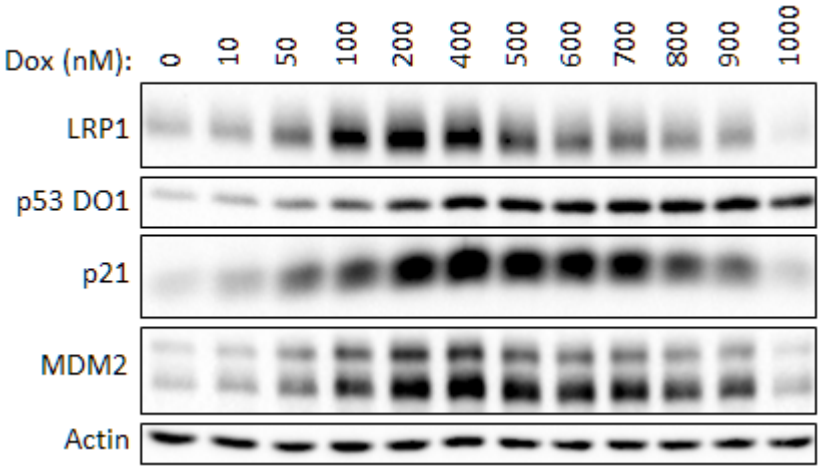

Figure 3-16. Protein expression of LRP1 and other p53-regulated proteins are sensitive to the concentration of doxorubicin
HCT116 cells were treated with the indicated dose of doxorubicin for 24 h, after which lysates were collected and analyzed by western blot.

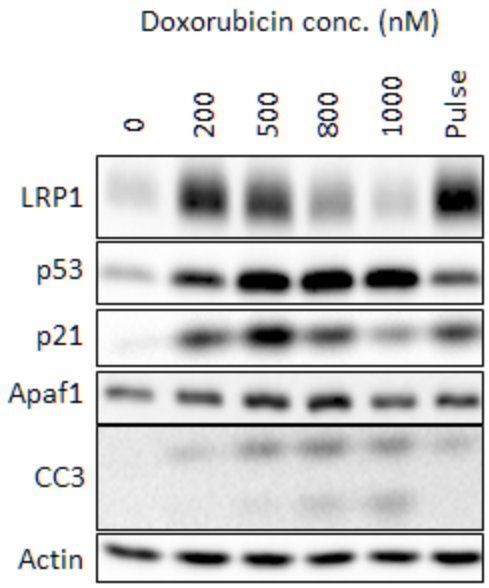

Figure 3-17. Collection of floating cells does not affect protein expression levels. HCT116 cells were treated with the indicated dose of doxorubicin for 24 h, after which lysates were collected and analyzed by western blot.

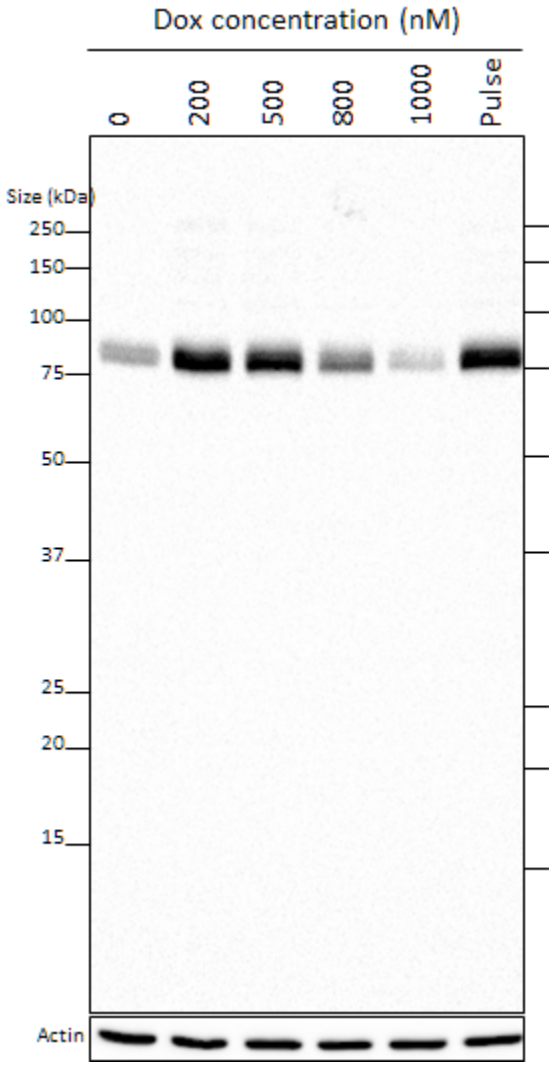

Figure 3-18. Doxorubicin dose-dependent decrease in LRP1 protein level does not produce any detectable cleavage products.
HCT116 cells were treated with the indicated dose of doxorubicin for 24 h, after which lysates were collected and analyzed by western blot.

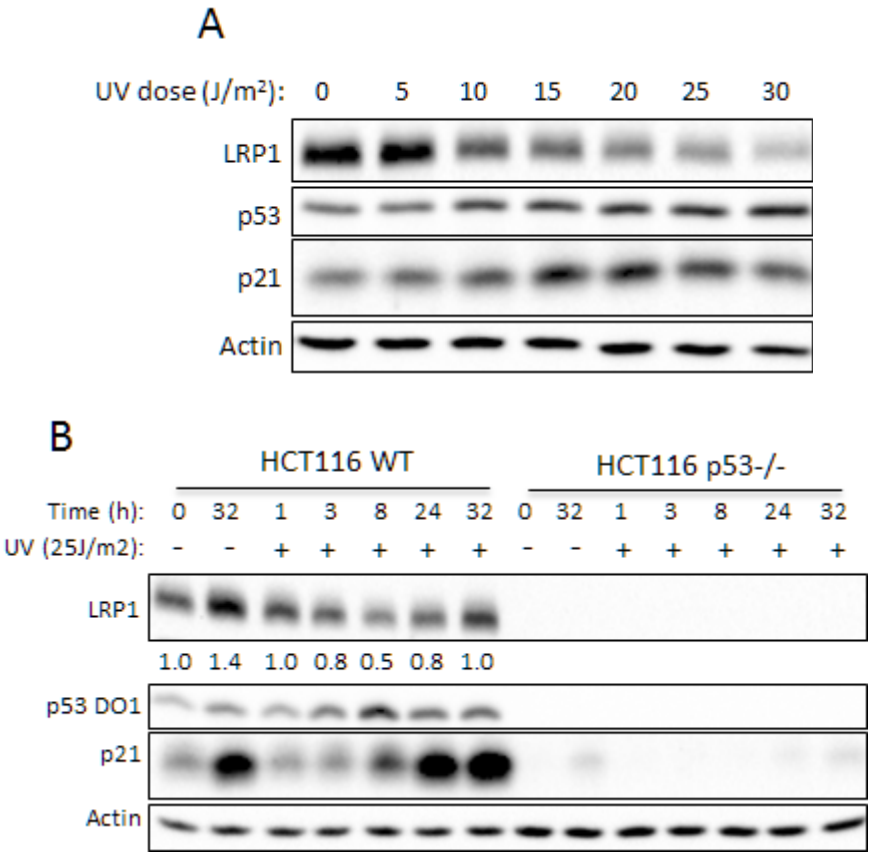

Figure 3-19. LRP1 protein also shows a dose-dependent decrease in response to UV irradiation.
A) HCT116 WT cells were treated with the indicated dose of UV-C for 24 h, after which lysates were collected and analyzed by western blot.
B) HCT116 WT or $p53^{-/-}$ cells were treated with 25 J/m^2 of UV-C, after which lysates were collected at the indicated time points.

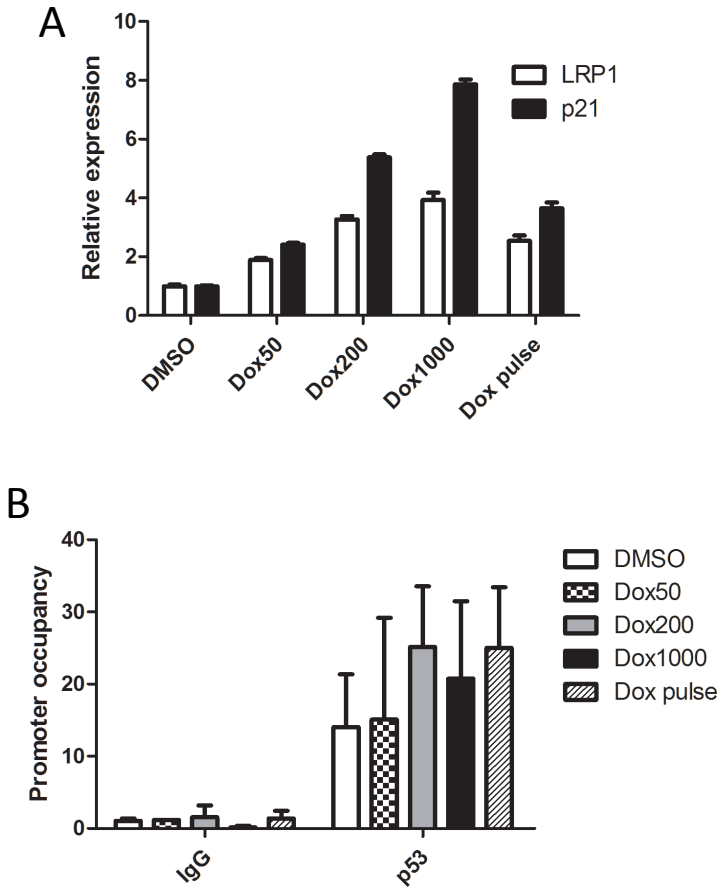

Figure 3-20. LRP1 transcript expression is elevated in response to lethal doxorubicin.

A) HCT116 cells were treated with the indicated dose of doxorubicin (in nM) for 24 h, after which RNA was collected and subjected to RT-qPCR analysis.

B) HCT116 cells were treated with the indicated dose of doxorubicin for 24 h, after which cells were subjected to chromatin immunoprecipitation analysis using anti-p53 antibody and non-specific IgG.

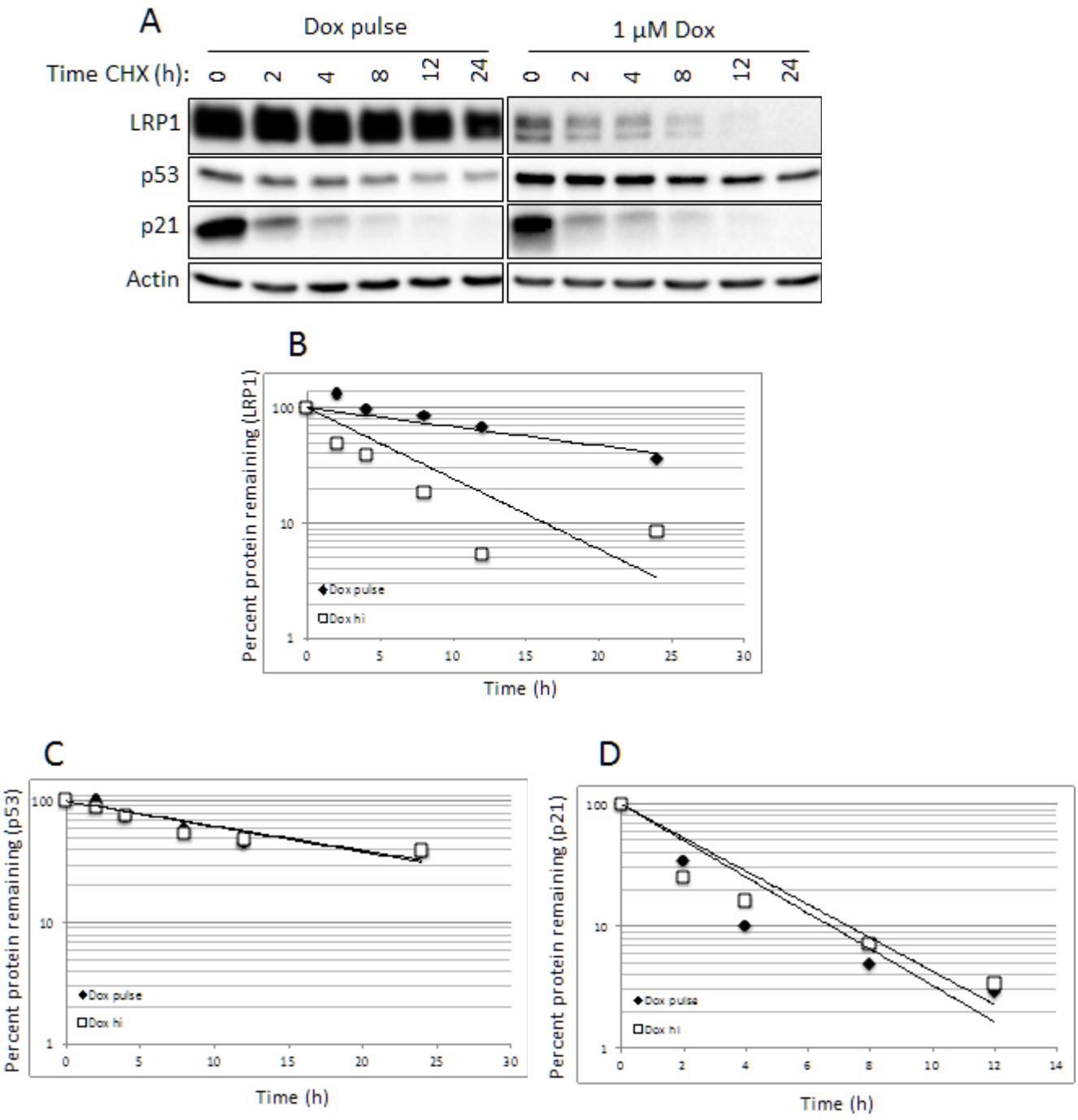

Figure 3-21. LRP1 half-life is decreased upon treatment with lethal doxorubicin.
A) HCT116 cells were treated with lethal or sub-lethal doxorubicin for 24 h, after which the cells were treated with cycloheximide (CHX) for the indicated amount of time.
B) Protein degradation graph for LRP1 protein.
C) Protein degradation graph for p53 protein.
D) Protein degradation graph for p21 protein.

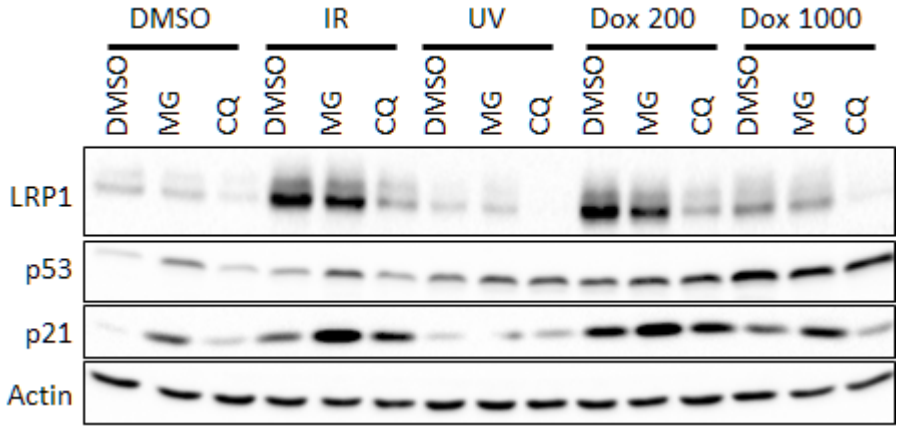

Figure 3-22. Neither MG132 nor chloroquine can rescue LRP1 expression.
HCT116 cells were treated with the indicated stress for 24 h, after which vehicle, MG132 (MG), or chloroquine (CQ) was added to the cells for an additional 8 hours. After MG or CQ treatment, lysates were collected and subjected to western blot.

Figure 3-23. *De novo* LRP1 translation is reduced in response to lethal doxorubicin compared with sub-lethal doxorubicin.
HCT116 cells were treated with the indicated course of doxorubicin for 24 h, after which cells were labeled with ^{35}S-Met/Cys for 30 mins, chased with complete DMEM for another 30 mins, and then subjected to LRP1 immunoprecipitation.

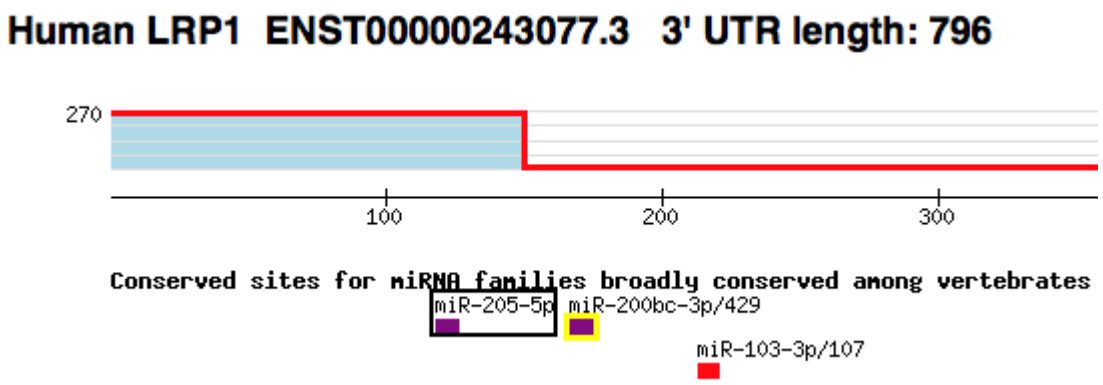

Figure 3-24. TargetScan results for LRP1 3'UTR.
TargetScan analysis of the 3'UTR of human LRP1 revealed high scoring conserved seed regions for miR-205, miR-200b/c, and miR-103/107.

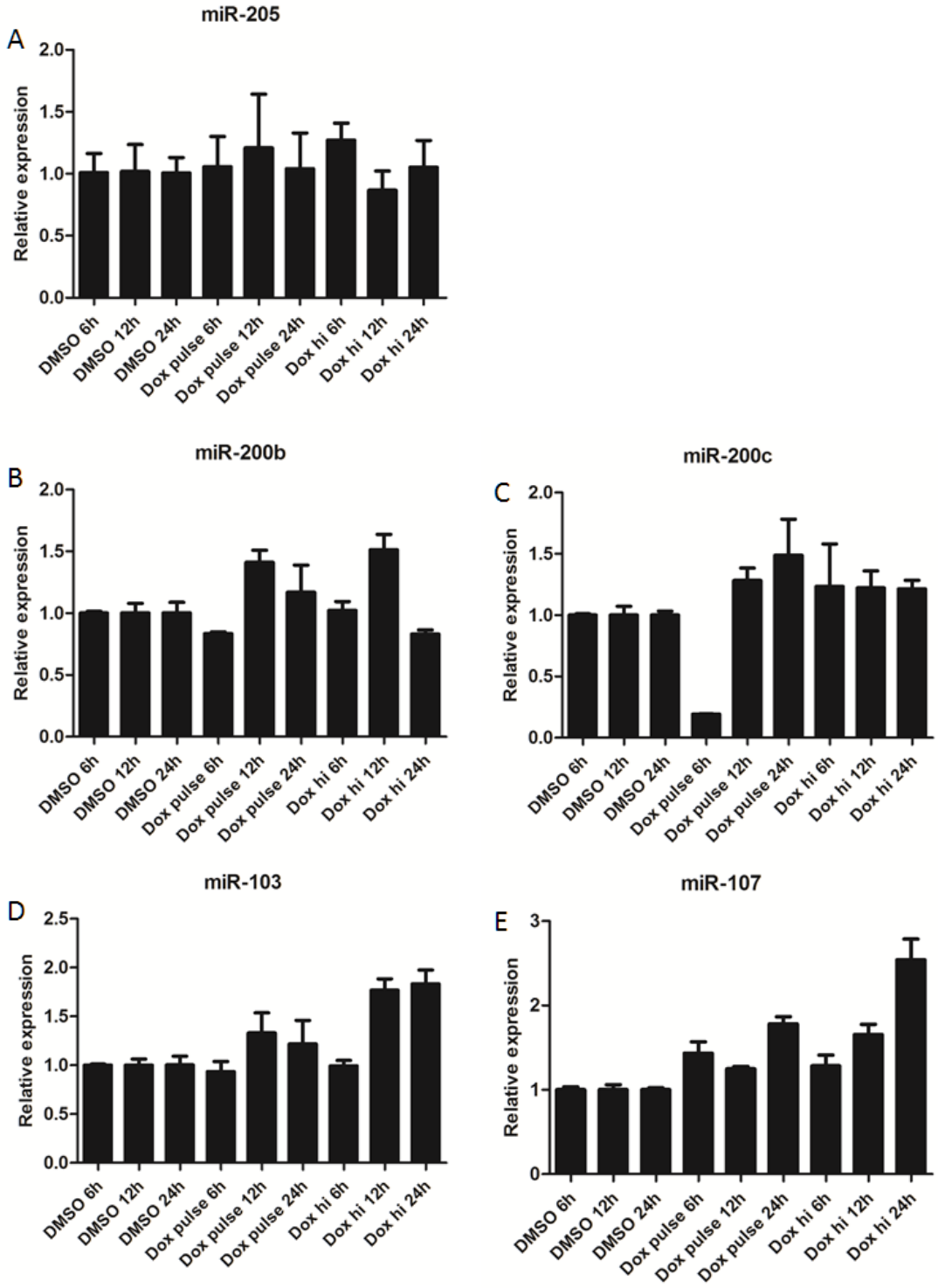

Figure 3-25. miR-103 and miR-107 show significantly higher induction in response to lethal doxorubicin compared with sub-lethal doxorubicin.
HCT116 cells were treated with vehicle or sub-lethal or lethal doxorubicin for the indicated amount of time, after which total RNA was harvested and tested for the induction of various putative LRP1-targeting miRNAs, including miR-205 (A), miR-200b (B), miR-200c (C), miR-103 (D), and miR-107 (E).

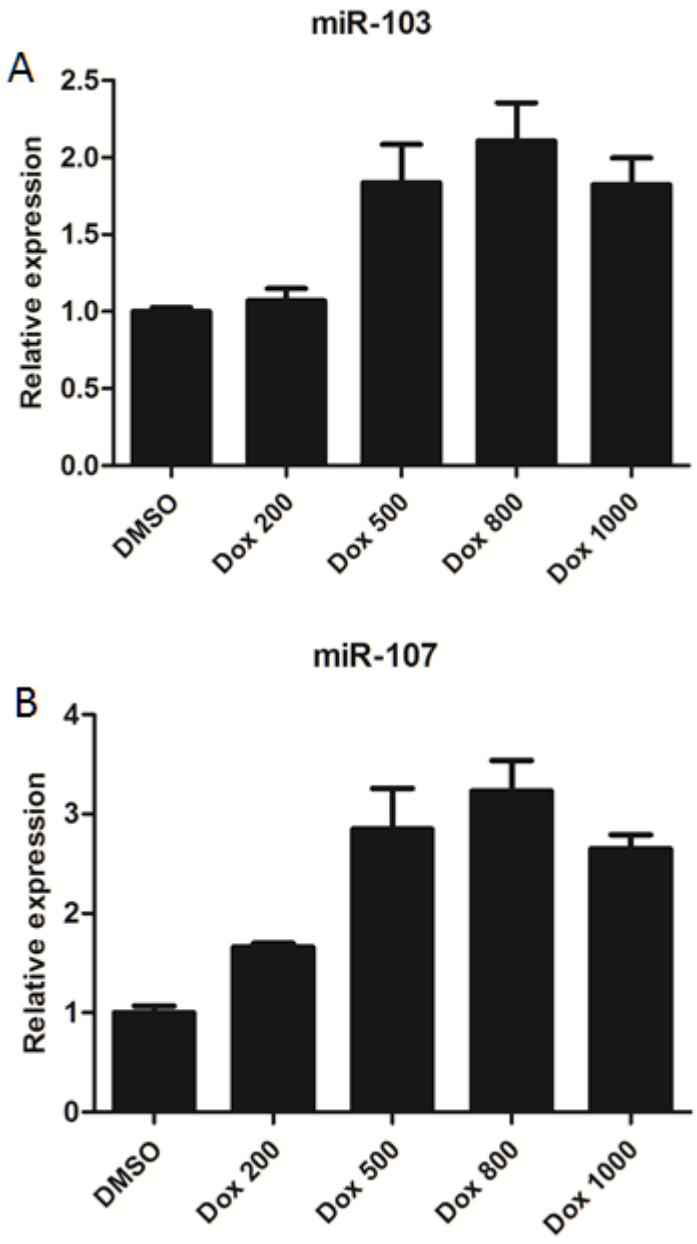

Figure 3-26. miR-103 and miR-107 are induced in a doxorubicin dose-dependent manner.
A) HCT116 cells were treated with the indicated dose of doxorubicin for 24 h, after which total RNA was isolated and probed for miR-103 expression.
B) HCT116 cells were treated with the indicated dose of doxorubicin for 24 h, after which total RNA was isolated and probed for miR-107 expression.

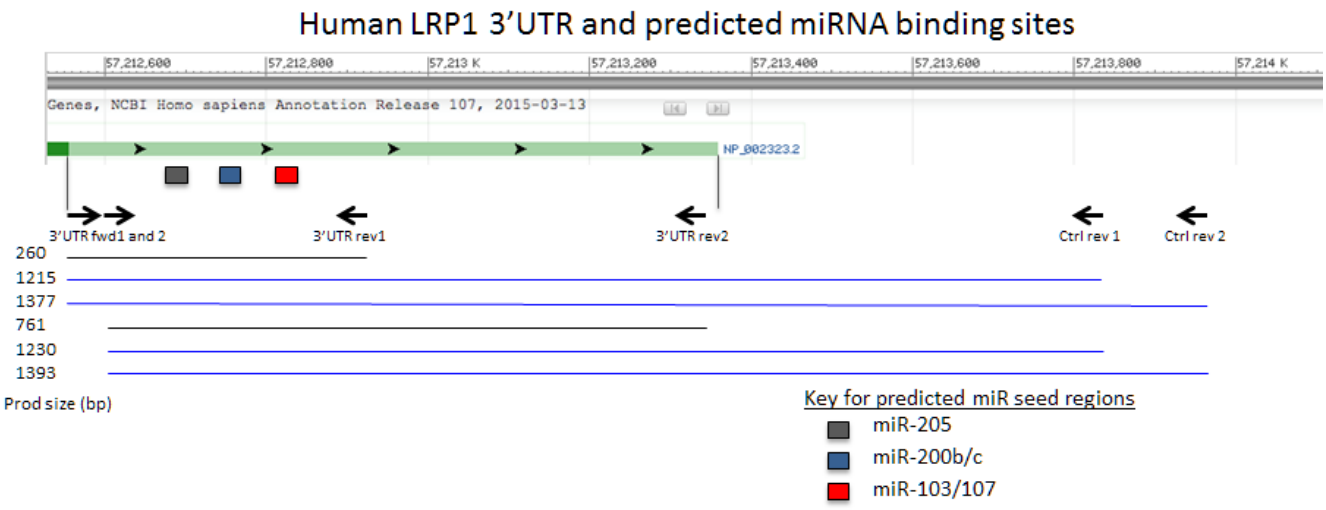

Figure 3-27. Primer design to confirm the presence of the miR-103/107 seed region in the LRP1 3'UTR in HCT116 cells.
The 3'UTR of LRP1 is depicted in light green bar along with the terminal coding sequence of LRP1 (dark green bar) and genomic DNA (downstream of the light green bar). miRNA seed regions were predicted using TargetScan algorithm and are indicated along with the approximate locus with the short gray (miR-205), blue (miR-200b/c), and red (miR-103/107) bars. Arrows are included to indicate the binding sites of primers used to amplify HCT116 cDNA to confirm the presence of the LRP1 3'UTR as well as control primers designed to bind to genomic regions beyond the 3'UTR. The product sizes for each primer set used is indicated in black (correct product size amplified) or blue (no correct products amplified) lines.

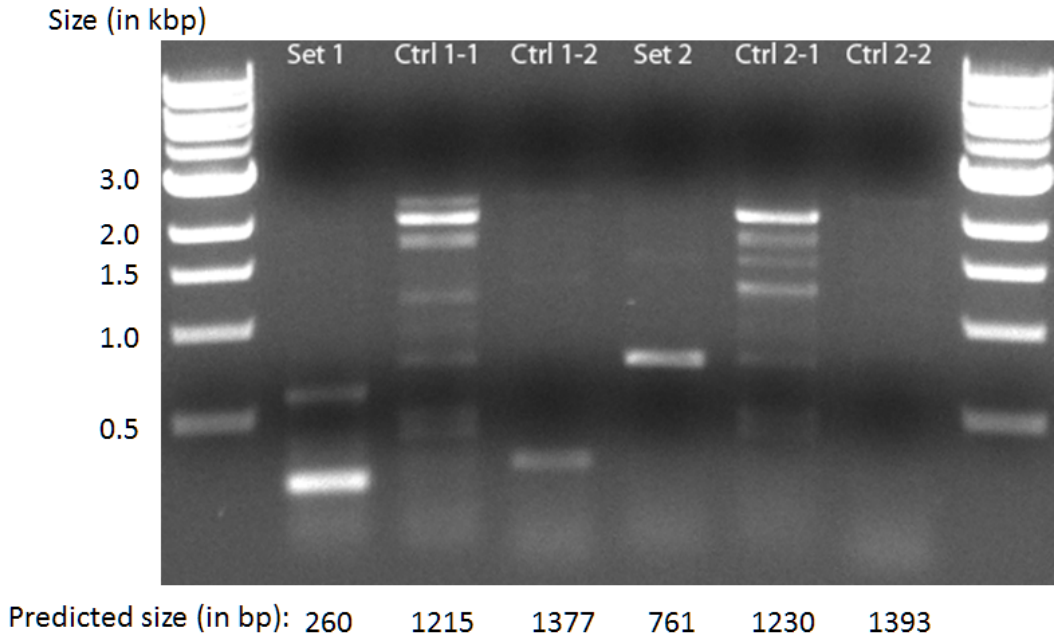

Figure 3-28. Predicted miR-103/107 binding site is present in HCT116 cells. RNA was isolated from untreated HCT116 cells and converted to cDNA. Then, the cDNA was probed using primer sets designed to amplify segments of the 3'UTR (260 bp and 761 bp in size, lanes 1 and 4, respectively). Additional primer sets were included to control for the presence of genomic DNA contamination.

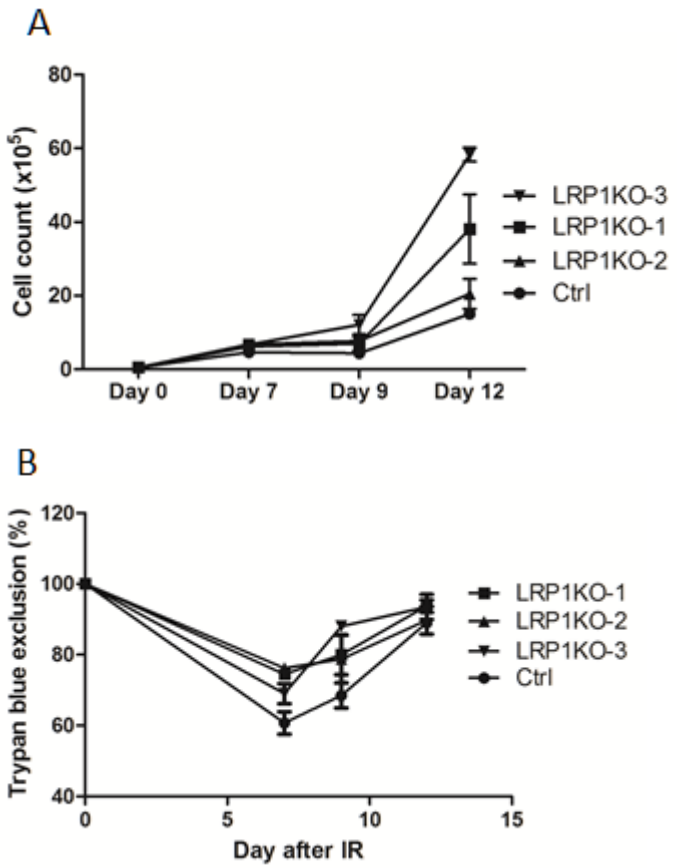

Figure 3-29. HCT116 LRP1 knockout lines show a survival advantage.
A) HCT116 LRP1 knockout cell lines were treated with 5 Gy of IR and were evaluated for cell proliferation by cell counting on days 7, 9, and 12 after treatment. LRP1 KO cells show an advantage in cell number compared with control cells.
B) Trypan blue staining at each of the time points in (A) revealed resistance in all LRP1 knockout lines to cell death characteristics.

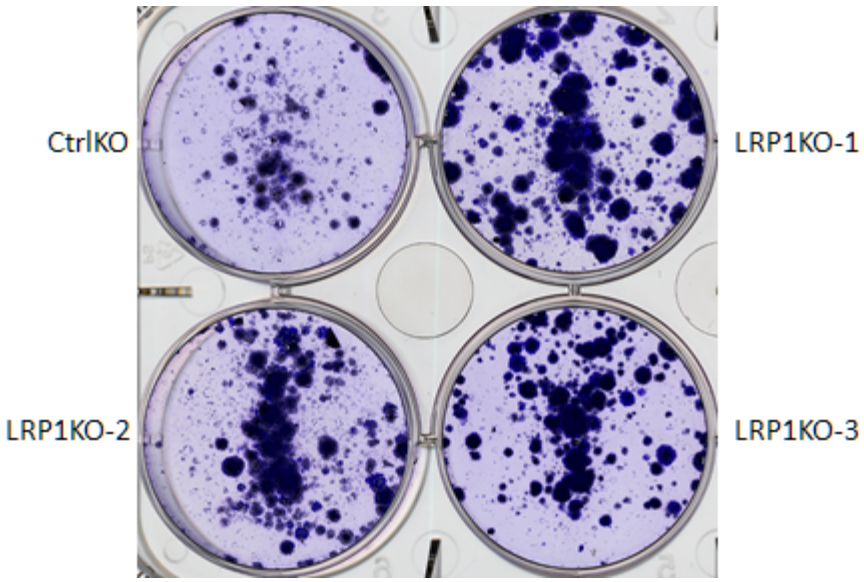

Figure 3-30. HCT116 LRP1 knockout lines show enhanced colony formation ability compared with control cells.
Colony formation assay was conducted comparing three LRP1 knockout clones with a control knockout line. Crystal violet staining results show a clear growth advantage in the absence of LRP1.

CHAPTER 4: REMAINING QUESTIONS AND FUTURE DIRECTIONS

In these studies, we offer insight into multiple aspects of the p53 signaling pathway. In one study, I provide evidence suggesting that the p53 negative regulator MDM2 forms homooligomers and heterooligomers through distinct mechanisms. In another study, I showed that LRP1 is a novel p53 target gene whose protein product is selectively expressed depending on the severity of p53 activating stresses. Thus, this dissertation advances the field with respect to our understanding of how p53 upstream factors function to regulate p53 and how p53 regulates its target genes. In the following pages, I discuss in more detail the implications of our findings with an emphasis on remaining questions and future studies that I would very much like to pursue.

What could explain the difference in MDM2 homo- and heterooligomerization mechanisms?

Because MDM2 homo- and heterooligomerization has long been thought to occur through very similar mechanisms and involve similar domains, the identification of the involvement of the AD in MDM2 homooligomerization but not heterooligomerization is the first direct piece of evidence that suggests otherwise. Interestingly, deletion of the AD in MDM2 prevents the ability of MDM2 to interact with WT MDM2, which could suggest that both ADs are required for MDM2-MDM2 binding. The role of the AD in dictating the MDM2 oligomerization partner requires further investigation. I have developed several

hypotheses based on the available data that would be interesting to test in future studies.

Some of the most important pieces of data regarding MDM2 binding mechanisms are the reports of various MDM2 structures. As mentioned above, MDM2-MDM2 and MDM2-MDMX RING-RING structures have been reported.[145, 186] Structures have also been reported for other domains of MDM2, including portions of the AD[113, 339] and the p53 binding domain.[2, 158] Because we lack full-length MDM2 structures, in part because of inherently disordered regions of MDM2, we must employ other techniques to determine how MDM2 behaves in cells. Recent *in vitro* studies have shown that MDM2 and MDMX form stable intramolecular interactions involving their respective ADs, which hints at additional complexity involved in not only the configuration of MDM2 monomers but also in MDM2 oligomers. In the context of the MDM2 homooligomer, the presence of identical ADs at potentially similar locations could present considerable charge repulsion that might contribute to the relative instability of the MDM2 homooligomer. Our data suggest that the presence of small basic MDM2 AD-binding proteins such as ARF contributes to MDM2 homooligomer stability while simultaneously contributing to MDMX destabilization. One of my hypotheses is that small proteins such as ARF could neutralize the charge repulsion between the two MDM2 ADs in the homooligomer. Moreover, the offset nature of the MDMX AD relative to the MDM2 AD presents a way to avoid this charge repulsion without requiring a neutralizing basic protein. The differences in the MDM2 and MDMX AD positions within their respective proteins could also help explain why MDM2-MDMX heterooligomers are inherently more stable than MDM2-MDM2 homooligomers under purified protein conditions.[271, 299] To test whether the AD position plays a role in the relative stabilities of MDM2 homo- and heterooligomers, it would be interesting to

genetically engineer the AD to positions upstream or downstream of its natural position. If the position of the MDM2 AD interferes with MDM2 homooligomer formation, I expect that altering the position of the AD in one MDM2 molecule would enhance its binding affinity for WT MDM2. A potentially interesting follow up experiment would involve the co-expression of two tagged versions of MDM2 (e.g., FLAG-MDM2 and myc-MDM2) where one version harbors a variably positioned AD to mimic the positioning of the AD of MDMX to determine the effect on MDM2 oligomer stability.

Do MDM2-MDM2 homooligomers and MDM2-MDMX heterooligomers perform different functions *in vivo*?

Another intriguing idea regarding the selective role of the MDM2 AD in homo- and heterooligomerization pertains to the respective roles of the oligomers *in vivo*. I identified the first MDM2 manipulation that could selectively affect MDM2 homooligomerization while leaving MDM2-MDMX heterooligomerization intact. Moreover, the MDM2ΔAD mutant shows the ability to ubiquitinate but not degrade p53. Therefore, it would be interesting to test the MDM2ΔAD mutation under endogenous conditions in a mouse model to determine whether a mouse expressing an MDM2 mutant that possesses these characteristics develops normally. Because the MDM2ΔAD mutation used in our study involves the deletion of a large portion of the protein (50 amino acids), one of the first steps would be to define the minimal sequence to delete/mutate that prevents MDM2 homooligomerization but does not affect MDM2-MDMX heterooligomerization to minimize the possibility of off-target effects. Then, the generation of mice bearing this mutation could be investigated to determine whether the abrogation of MDM2 homooligomer formation

affects p53 regulation and survival in an *in vivo* setting.

Additional questions that could be addressed with this model involve the nature of the failure of MDM2ΔAD to degrade p53 despite no difference in p53 ubiquitination. Interestingly, whether p53 can be ubiquitinated depends on the section of the AD deleted. Two studies have shown that MDM2 AD deletion constructs retain the ability to ubiquitinate p53, which is similar to our findings (ΔAD:222-272 (mouse MDM2),[4] ΔAD: 217-246,[341] ΔAD: 245-295[172]). Interestingly, larger MDM2 AD deletions can inhibit its ability to ubiquitinate p53, suggesting that certain residues within the AD are necessary for E3 ligase function (ΔAD: 222-303[137] and ΔAD: 202-303[214]). A more recent study showed that multiple small deletions (~5-6 amino acids) within the MDM2 AD are sufficient to prevent p53 degradation without affecting p53 ubiquitination.[73]. Although these mutations were not tested for their effects on homo- and heterooligomerization, some of these mutants may offer a starting point by which to identify the minimal deletion necessary to inhibit homooligomerization without affecting heterooligomerization or p53 ubiquitination. Some preliminary data of ours suggest that small AD deletions could indeed selectively affect MDM2 homooligomerization, as the deletion of a 5-residue span within the AD (ΔDEDDE), which prevents MDM2-mediated degradation of p53,[73] shows a modest decrease in MDM2 binding (Figure 4-1). The effect of DEDDE deletion suggests that additional small deletions could produce selective homooligomerization inhibition possibly without dramatically affecting the overall structure or other characteristics of the protein. It would be interesting to screen additional small MDM2 AD mutations or deletions compounded with the DEDDE deletion to determine the minimal mutation that accomplishes selective homooligomerization deficiency.

As mentioned above in Chapter 1, confirmation that MDM2 homooligomers form under physiological conditions is an important preliminary step required before further investigating the role of MDM2 homooligomers *in vivo*. This could be accomplished using recently standardized genomic engineering tools such as the CRISPR/Cas9 system. The integration of an N-terminal epitope tag in one or both of the alleles of MDM2 could provide a valuable tool with which to determine whether MDM2 homooligomers can be detected under endogenous expression conditions. CRISPR/Cas9-mediated genomic tagging has already been successfully used to tag various endogenous proteins.[60, 264, 265] To identify MDM2 homooligomers using a CRISPR/Cas9 system, either single allele tagging or unique tagging of both alleles would be required to allow for the differentiation of two otherwise identical proteins. Single allele tagging is inherently more difficult using currently published protocols, and optimization of CRISPR for this purpose would likely be required. One possible modification to current CRISPR/Cas9-mediated epitope tagging protocols could involve the use of two different selection markers to permit the simultaneous identification of the integration of both epitopes in a single cell. Otherwise, screening a large amount of clones for single allele tag integration would be necessary. Nonetheless, the high efficiency of the CRISPR/Cas9 system relative to standard homologous recombination techniques renders single allele tagging a reasonable possibility.

How are p53-independent functions of MDM2 related to MDM2-MDM2 homooligomers and MDM2-MDMX heterooligomers?

Another area of MDM2 research that has not yet been discussed in this dissertation

is the p53-independent functions of MDM2. MDM2 can ubiquitinate and degrade several other proteins in addition to p53, including TERT,[231] Slug,[317] RUNX3,[48] IGF-1R,[98] HIPK2,[258] ribosomal proteins S27L,[330] S7,[342] and L26,[230] activating transcription factor 3 (ATF3),[218] and androgen receptor[184] among others. MDM2 has also been reported to activate or inhibit several other proteins in a p53-independent manner, including DNA polymerase ε,[7] E2F1,[203] p65,[101] the polycomb repressor complex subunit EZH2,[323] and the DNA damage repair protein Nbs1.[1] These interactions have been shown to affect crucial cellular processes and attributes such as stem-like character,[231, 323] cancer cell invasion,[317] DNA damage repair and recombination,[1, 7] protein synthesis,[230, 330, 342] and cell cycle progression.[203] Thus, although the vast majority of literature on MDM2 regulation entails p53-dependent functions and effects, a potentially significant proportion of the MDM2 regulome involves proteins outside of the p53-MDM2 loop, which is an important consideration to understand MDM2 function in a cancer context. Interestingly, several splice variants of MDM2 that lack the ability to bind p53 have been identified in patient tumors, suggesting that MDM2 functions outside of p53 regulation could offer an additional benefit to cancer cells.[11, 90] Because the regulome of MDM2 remains incompletely characterized, it would be interesting to determine whether MDM2 homo- and/or heterooligomers play unique roles in these respects. Moreover, using MDM2 mutants that selectively inhibit homo- or heterooligomerization offers a manner through which to specifically address this question. Thus, other potential future experiments might entail a comparison of the ability of homooligomerization-deficient and heterooligomerization-deficient MDM2 constructs to bind to and regulate any or all of the above-mentioned MDM2-regulated proteins.

LRP1 is a novel p53 target gene that shows context-dependent protein induction involving a translation regulatory mechanism

The identification of LRP1 as a p53-regulated gene is particularly interesting considering its multi-faceted nature. LRP1 has been tied to several diseases for which the etiology remains incompletely understood, including atherosclerosis, Alzheimer's disease, and cancer, which likely relates to the wide array of ligands bound by LRP1 and the multitude of pathways influenced by LRP1 function. Moreover, LRP1 is ubiquitously expressed, which suggests that p53-mediated LRP1 expression, which occurs in response to several types of stress, could be an important response in several tissues to counteract the effects of these stresses. Because LRP1 appears to play unique roles depending on the tissue under consideration, it would be interesting to determine how p53-mediated LRP1 expression in response to whole-body irradiation, for example, could induce distinct responses in different tissues with or without LRP1. The potential diversity of responses of different cell types to p53-mediated LRP1 induction presents an interesting question regarding the context-dependent physiological effects of LRP1. Our results suggest that LRP1 protein is selectively induced in response to sub-lethal stresses, which could imply that LRP1 protein could confer a survival advantage to cells. As discussed in the Introduction chapter, p53 target genes can be differentially expressed depending on the type of stress and in correlation with the ultimate outcome of the cell (i.e., cell cycle arrest vs. apoptosis). I was able to recapitulate this relationship regarding LRP1 expression, and interestingly, we show evidence of a potential miRNA-mediated mechanism through which p53 can differentially regulate target gene expression. I present evidence of a potential regulatory circuit for LRP1 in which sub-lethal p53-activating stress induces the

transcriptional and translational activation of LRP1, whereas a corresponding lethal p53-activating stress induces the transcriptional but not the translational activation of LRP1. Our results further show that although the half-life of LRP1 is reduced four-fold in response to lethal doxorubicin, enhanced LRP1 degradation only partially contributes to the difference in LRP1 protein levels between the two treatments, as *de novo* LRP1 protein synthesis is also impaired (Figure 3-23). Interestingly, I found that MG132-mediated proteasome inhibition, chloroquine-mediated lysosome inhibition, and QVD-OPh-mediated caspase inhibition were each unable to rescue LRP1 protein expression in response to lethal stress despite reports suggesting that LRP1 is degraded via the proteasome.[25, 213] In addition to the decreased half-life of LRP1 by an as yet unknown mechanism, I found that *de novo* LRP1 protein translation is inhibited in the presence of lethal stress but not sub-lethal stress, suggesting that lethal stress invokes the translational inhibition of LRP1 transcript (Figure 4-2).

The translational suppression of LRP1 appears to occur through the specific targeting of LRP1, as the expression of other proteins, such as p53, remain unaffected. Moreover, I observed a measurable and consistent LRP1 induction in response to 5 nM actinomycin D (ActD) treatment, which selectively inhibits RNA polymerase I activity and produces ribosomal stress on the cell. ActD treatment at this concentration results in the inhibition of ribosomal RNA synthesis and ribosome activity.[244, 260] The increase in LRP1 protein expression in response to ActD treatment further suggests that general translational inhibition is not a major factor in the difference in protein expression levels of LRP1. Collectively, our data support a miRNA-dependent suppression mechanism of LRP1 translation.

Are miRNAs responsible for LRP1 dose-dependent expression?

miRNAs are small non-coding RNAs that inhibit the expression of various target genes at the post-transcriptional level through direct complementary interaction between the miRNA and the target transcript. Upon binding to the transcript, the miRNA can either trigger the degradation of the transcript, or it can suppress translation, leaving the transcript levels unchanged.[122] Although the mechanistic details regarding how miRNA binding results in translational suppression instead of transcript degradation remain unknown, a considerable contingent of miRNA-mRNA interactions can result in the inhibition of translation without extensive mRNA decay.[205, 211, 324] Moreover, several studies have proposed a two-step mechanism whereby translational suppression is a required first step that precedes transcript degradation.[12, 71, 210] In an effort to identify which miRNAs might contribute to the translational suppression of LRP1 in response to lethal stress, I performed a TargetScan search to identify candidates and then analyzed the levels of these miRNAs in the presence of lethal and sub-lethal stress. Of the three candidate miRNA seed regions I found in LRP1 (miR-205, miR-200b/c, and miR-103/107), only miR-103 and miR-107 showed significant upregulation in the presence of lethal doxorubicin compared with sub-lethal doxorubicin. Interestingly, not only are miR-103 and miR-107 p53-regulated miRNAs, but so are miR-205 and miR-200b/c. Therefore, I considered a model whereby in response to sub-lethal or lethal stress, p53 upregulates LRP1 transcription, the translation of which depends on whether LRP1-regulating miRNAs are concomitantly upregulated. In response to lethal stress, p53 levels are induced to much higher levels, and consistent with the p53 threshold model, the p53 level may cross a threshold necessary to induce the expression of apoptotic genes (Figure 4-2). As more miRNA targets become identified as

p53-regulated genes, it would be interesting to determine whether the promoters of different miRNAs show different affinities for p53, as was shown for p53 binding to apoptotic promoters.[321] One would expect that the affinity of p53 for the promoters of miRNAs that target cell survival genes would be lower than promoters of miRNAs that target apoptosis genes. Consistent with this idea, the initial study reporting that p53 induces miR-205 expression indicated that the miR-205 promoter could be a low-affinity binding site for p53.[248]

Although I only observed an increase in the levels of miR-103 and miR-107, I initially considered miR-205 as a likely candidate for LRP1 translational suppression, as miR-205 can selectively inhibit LRP1 translation without appreciably affecting LRP1 transcript levels.[284] Coupled with its reported p53-dependent regulation, I cannot rule out the possibility that miR-205 affects LRP1 expression in a p53-dependent manner. A very recent study showed that p53 could affect the proportion of certain miRNAs that are loaded onto the Ago2 argonaute protein.[151] The argonaute proteins, of which four are known in humans (Ago1-4), bind to mature miRNAs to form the minimal RISC (RNA-induced silencing complex) complex, which is necessary to guide miRNA-mRNA binding. Interestingly, p53 binds directly to Ago2 and increases the loading of some miRNAs, such as the let-7 (lethal-7) miRNAs, which in turn affects the post-transcriptional landscape. The authors showed that in HCT116 p53 WT cells but not HCT116 p53-null cells although the levels of some miRNAs do not change, the Ago2-associated miRNA fraction could be significantly and functionally different. Moreover, mutant p53 maintains the ability to bind to Ago2 but instead reduces Ago2 association with the let-7 miRNAs. Thus, no changes in the overall levels of some miRNA species does not necessarily preclude a difference in the

regulatory effect of that miRNA, especially considering the ability of p53 to regulate the loading of miRNAs onto the RISC complex. These results imply that Ago2-associated miRNA levels could be worth analyzing in the context of doxorubicin dose-dependent regulation of LRP1. A more global analysis of the Ago2-associated miRNAs could offer insight into how p53-dependent post-transcriptional regulation through miRNAs changes in response to stress of different intensities.

As mentioned above, although the other two miRNA species identified through TargetScan analysis, miR-200b and c, did not show upregulation in response to doxorubicin, these miRNAs are also considered p53-regulated genes.[151, 297] Interestingly, analysis of the Ago2-bound fraction of miRNA revealed that although p53 does not upregulate overall levels of miR-200b (as found in our study), p53 increases the fraction of miR-200b that is loaded onto Ago2, suggesting that these miRNAs could also contribute to LRP1 post-transcriptional regulation.[151] Thus, future analyses investigating the ability of miR-200b to suppress LRP1 expression could be worth pursuing.

Interestingly, p53-regulated miRNAs have been reported to regulate apoptotic p53 targets, including *APAF1*.[270] In this study, the authors show that the apoptotic efficacy of a dose of 5-fluorouracil (5FU) equivalent to the IC50 of colorectal cancer cells can be enhanced by inhibiting the expression of the *APAF1*-targeting miRNA miR-23a. In my experience, the dose of 5FU used in this study for HCT116 cells (37 μM) is unable to induce considerable cell death within 24 h, suggesting that the dose used is sub-lethal. Importantly, miR-23a is a p53-regulated miRNA.[33, 333] The trend of miR-23a-mediated APAF1 suppression has been shown in other models as well, collectively suggesting that p53-regulated miRNAs could also play a role in suppressing pro-apoptotic p53 genes in

response to sub-lethal stresses.[43] This likely plays an important role in maintaining low levels of pro-apoptotic proteins when a pro-survival p53 response is warranted. Although this has not been shown directly, the regulation of various pro- and anti-apoptotic p53-regulated miRNAs and their corresponding target genes could be an interesting future study that could offer insight into p53 differential gene regulation.

Are lethal stress-induced translation/protein suppression mechanisms applicable to other p53 targets?

The lethal stress-specific translational suppression of LRP1 is likely not unique to LRP1, as I observe similar trends for p21 expression, whereby lethal doses of doxorubicin strongly induce p21 transcript levels but not p21 protein levels. Although we did not conduct translational analyses for p21, I anticipate that p21 translation is also inhibited in the presence of lethal stresses in a manner similar to LRP1. I also detect no difference in the half-life of p21 (Figure 3-21D). The possibility that many p53 target genes could be regulated in a stress-dependent manner on the post-transcriptional level suggests that proteomic studies analyzing differences in p53 target gene expression could be particularly interesting. Previous studies characterizing the p53 proteome and transcriptome in parallel have been conducted;[117, 133] however, to the best of our knowledge, comparisons of the p53 proteomes in the presence of lethal and sub-lethal doses of drugs such as doxorubicin have not been conducted. I anticipate that these sorts of analyses could yield important insight into other p53 targets that are regulated in a manner similar to LRP1. Moreover, miRNA sequencing of the same treatment comparison could be used to help identify potential miRNA genes that contribute to the proteomic differences observed

between lethal and sub-lethal stress.

Most high-throughput studies conducted to date investigating the p53 regulome have focused on transcript levels through either microarray or RNA-seq methods. Although these studies have expanded our knowledge of p53-regulated genes, they lack the ability to identify differences in protein expression. Thus, based on our analyses of LRP1, the transcript profile of lethal doxorubicin-treated cells is probably not consistent with the corresponding protein profile. One study that could be very interesting is a stable isotope labeling of amino acids in cells (SILAC) experiment designed to make pair-wise comparisons between untreated and lethal doxorubicin-treated cells as well as between sub-lethal and lethal doxorubicin-treated cells. SILAC involves the labeling of two cell populations with different amino acid isotopic variants, such as Arg/Lys labeled with ^{13}C or ^{12}C, such that proteins are close but detectably different in mass upon analysis by mass spectrometry. The difference in mass is quantifiable, such that relative amounts of protein can be determined between two different cell populations. Thus, in conjunction with high-throughput transcript analysis techniques such as microarray and RNA-seq, SILAC offers the ability to comparatively analyze the proteome as well as the transcriptome in samples treated in parallel with sub-lethal and lethal doxorubicin.

What are the intratumoral expression patterns of p53 genes?

As discussed in Chapter 3, the clinical implications of the gene expression differences depending on the dose of doxorubicin are apparent. Moreover, the relatively higher protein expression of multiple p53 target genes (*LRP1*, *p21*, *MDM2*) in response to several types of sub-lethal stress suggests that the local dose of chemotherapeutics to

which p53-competent cancer cells are exposed could activate completely different p53 gene programs. This is important because in response to low level stress, p53 can invoke an adaptive/cell survival type of response, which could theoretically condition some cancer cells to adopt a more resistant phenotype.[153] In the three-dimensional environment of the tumor, all cells are not exposed to the same dose of a systemically administered drug. Part of the complication associated with homogeneous drug distribution throughout the tumor is the irregular vascular network and biomechanical stresses associated with rapidly growing tumors.[128, 292] Indeed, increases in intratumoral pressure by increases in tumor cell number and mass, stromal tissue constriction, and lymphedema can not only affect blood and lymph flow by physically compressing tumor-associated vessels but it can also prevent the efflux of drugs carried through the blood vessels by dissipating the natural pressure gradient favoring drug delivery to the tumor.[126, 173, 238] The increase in intratumoral pressure contributes to reduced blood flow to large parts of the tumor, which can contribute to hypoxia-driven alterations in gene expression, as well as reduced exposure to systemically administered chemotherapeutics such as doxorubicin (Figures 4-3 and 4-4, see [217] for an excellent review). Another major vascular-associated issue inherent in many rapidly growing tumors is the increased distance between many tumor cells and the closest blood vessel, which has been estimated for some cells at over 100 μm.[301] The increased distance from blood vessels means that a considerable proportion of tumor cells rely on cell-to-cell diffusion of drugs and other components in the blood, which correlates with reduced nutrient and oxygen availability to distant cells as well as attenuated proliferation.[217] Moreover, because many anti-cancer strategies are based on the rapid proliferation rate of tumor cells relative to normal cells, this reduced proliferative

state of distant tumor cells presents a physically more resistant cell population that could contribute to the regrowth and resistance of tumors to standard treatment regimens. In the case of doxorubicin, which shows poor tissue diffusion characteristics due to its high affinity for DNA binding,[250] cancer cells distant from the tumor vasculature are also exposed to relatively low concentrations doxorubicin, which, based on our study, suggests that p53 survival pathways could also be activated in these cancer cells (Figure 4-3).[165, 250]

Intracellular and intratumoral doxorubicin distribution has been determined based on its natural fluorescent properties.[54, 63] Because doxorubicin absorbs and emits characteristic wavelengths of light, we could analyze cross-sections of established tumor xenografts treated intravenously with doxorubicin by microscopy to obtain a tumor-wide view of the distribution of doxorubicin in tumors. This technique has been demonstrated in tumor sections obtained from patients and mice treated with doxorubicin, and indeed the distribution shows a gradient type of pattern soon after administration.[165, 250, 320] In the context of our findings regarding the doxorubicin dose-dependence on gene expression, it would be interesting to determine whether the gradient of doxorubicin in tumors correlates with the expression levels of p53 genes such as *p21* and *LRP1*. Using co-staining methods, we could stain for several p53 target genes involved in the pro-survival and pro-apoptotic type of signature to determine whether a trend exists with respect to the concentration of doxorubicin. I would predict that cells that are less well-perfused by doxorubicin would not only show increased expression of p53 pro-survival-type gene products but would also reside within the interface of normoxic and hypoxic tumor regions. Another interesting marker to include in these analyses is an indicator of tissue hypoxia, such as pimonidazole or EF5, which would allow us to define which regions of the

tumor show hypoxic characteristics.[307] The combination of doxorubicin auto-fluorescence and hypoxia marker staining in tumors has been used to show that doxorubicin fails to penetrate hypoxic regions of tumors;[250] however, whether the sub-optimally dosed cells might actually display molecular evidence of a p53-dependent survival advantage remains to be determined. Moreover, the investigation of the gene expression patterns in p53 mutant tumors could also provide insight into other ways in which the p53 regulome could be altered in response to sub-optimal doses of drugs. The activation of p53 survival pathways in a subset of tumor cells could actively promote the regrowth of the tumor once the drug is withdrawn (Figure 4-5). Thus, if our observations of p53 survival program gene expression are true in the tumor context, then this corroborates the reported benefits of more constant/frequent dosing regimens to provide pro-apoptotic pressure on the tumor as continuously as possible.[64]

Because a considerable aspect of the difficulty in treating solid tumors with chemotherapeutics involves the irregular vasculature, which results in poor perfusion, efforts have been made to increase tumor perfusion to enhance drug delivery and efficacy. There are generally two methods to improve tumor perfusion, including the reduction of biomechanical stresses (extracellular matrix, other stromal components, etc.) that induce the intratumoral pressure that physically blocks the tumor vascular system and restoring the integrity of tumor-associated blood vessels through anti-angiogenic therapy. Anti-angiogenic therapy is thought to temporarily increase vessel integrity by targeting the removal of immature blood vessels and by promoting pericyte coverage of the blood vessels, which restores the pressure gradient between the blood vessels and the surrounding tissue, thereby allowing smaller drugs to enter the tumor tissue with more

efficiency.[127] Interestingly, both strategies improve tumor perfusion, which enhances drug delivery to otherwise inaccessible portions of the tumor.[36] Moreover, these strategies have resulted in improved outcomes when used as adjuvants in both mice and humans.[82, 236, 251, 285] Based on our study characterizing the p53 gene program as highly dependent on drug concentration, it would be interesting to determine whether the rescue of tumor perfusion through one of these methods could enhance doxorubicin perfusion to the extent that all of the tumor cells are exposed to high concentrations of drug and express pro-apoptotic p53 markers.

Other methods of enhancing drug penetration in tumor tissue that are currently under investigation include nanoparticle-mediated delivery and drug modifications that enhance perfusion. One of the major advantages of nanoparticle delivery is the ability to be relatively selective in terms of payload delivery, as many nanoparticles are designed to exit leaky blood vessels, which is a characteristic of many tumors.[5, 305] Moreover, nanoparticles can be engineered to contain surface components that enhance their specificity for tumor tissue. In theory, the tumor-targeting characteristics of nanoparticle delivery suggest that very high doses of otherwise toxic or non-soluble drugs can be administered with a relatively low threat of normal tissue toxicity because the drug remains nanoparticle-bound until it is delivered to the tumor. However, as discussed above, a major drawback to this method is that many rapidly growing tumors possess hypoxic regions and display intratumoral pressure that can compress blood vessels, which severely limits nanoparticle delivery to tumor tissue. Thus, nanoparticles may also benefit from the combination with agents that increase tumor perfusion. Another method is the modification of drugs to enhance perfusion. These modifications vary from introducing amphipathic moieties that

allow a drug to better permeate tumor tissue to designing pro-drugs that are converted to cytotoxic agents only under conditions that are unique to tumors (e.g., hypoxia, acidity). Several of these methods are in clinical use or are under investigation.[200]

What accounts for stability change in response to high dose doxorubicin?

I also observed the destabilization of LRP1 protein in the presence of high doxorubicin, which likely accounts for part of the difference in LRP1 protein levels between low and high doxorubicin. However, I was unable to identify which component of the cellular protein degradation machinery was responsible for the increased degradation rate. I speculate that a degradation mechanism other than proteasome-, lysosome-, and caspase-dependent degradation is responsible for LRP1 degradation. One possible explanation is the action of gamma-secretase, which is a membrane-embedded protease that can induce the cleavage of LRP1.[189, 206] Another possibility that cannot be entirely ruled out as a protein degradation mechanism is protein oxidation through mechanisms such as carbonylation. Carbonylation describes the non-enzymatic, irreversible addition of carbonyl-containing adducts to the side chains of certain proteins.[288] The carbonylation of proteins can result in unfolding, inactivation, aggregation, and degradation of the target protein. Although the reason is unknown, certain proteins are strikingly more susceptible to carbonylation than others. Moreover, because doxorubicin induces a large amount of reactive oxygen species (ROS), high doses of doxorubicin could induce more carbonylation than low doses. Indeed, a recent study showed that the cardiotoxicity observed in patients treated with doxorubicin could be due at least in part to the increased carbonylation of cardiac myosin binding protein (MyBPC), which enhances its degradation.[6] Interestingly,

this study showed that high doxorubicin (>500 nM) has a significant carbonylation and pro-degradation effect on MyBPC that is not as apparent at lower doses. It is unknown whether LRP1 protein is carbonylated significantly in response to doxorubicin or other treatments; however, carbonylation could conceivably induce the degradation of LRP1 protein thereby accounting for the decreased half-life in the presence of high doxorubicin. Arguing against a considerable carbonylation effect in LRP1 protein levels, I did not observe a similar dose-dependent threshold in response to ionizing radiation, another known inducer of ROS (Figure 4-6). Nonetheless, it would be interesting to determine whether LRP1 (or other p53 target gene products) are carbonylated to any extent, and whether carbonylation could contribute to LRP1 stability. Carbonylation analysis tools and methods have been standardized, including carbonylation-specific antibodies that could be used to determine whether the proportion of LRP1 protein is significantly more carbonylated in the presence of high doxorubicin than in the presence of low doxorubicin.

What does p53-induced LRP1 accomplish for the cell?

Another question related to the p53-dependent regulation of LRP1 worth pursuing is the mechanistic implications of LRP1 expression in response to p53-activating stresses. The multi-functional, multi-ligand-binding role of LRP1 presents difficulty with respect to narrowing our focus to certain pathways. Moreover, as mentioned above, LRP1 likely functions in a context-dependent manner depending on the tissue and other potential mutations present (in a tumor context). Based on the correlation between cell death-inducing stresses and LRP1 translational suppression, one of my initial hypotheses regarding the function of p53-dependent LRP1 expression was based on the DNA and

cellular structural repair aspects of the p53 transcriptome. A recent study using radiolabeled substrates showed that knockdown of CPT1A, a fatty acyl-carnitine transporter required for fatty acid oxidation (FAO), greatly reduces the incorporation of radioactive signal into nucleic acids, suggesting that lipids contribute to *de novo* nucleotide production and cell proliferation in endothelial cells.[269] Because regulation of the nucleotide pool is a significant aspect of the p53 DNA damage response,[38, 118, 298] it seems reasonable to hypothesize that p53-mediated LRP1 induction could be necessary to increase the uptake of exogenous lipids to facilitate nucleotide production and DNA damage repair as well as the replacement of oxidized lipids within membranous cellular structures. However, analyzing U2OS cells in which LRP1 was deleted by CRISPR revealed no apparent difference in the resolution of p-H2AX signal, suggesting that DNA damage repair occurs normally in response to ionizing radiation even in the absence of LRP1 (Figure 4-7).

Another consideration was that LRP1 could play a role in tumor suppressive signaling pathways such as TGF-beta. As discussed in the Introduction section, TGF-beta co-regulates the expression of several genes with p53, many of which are involved with the cell cycle arrest and adaptation program of p53. LRP1 was identified as a TGF-beta receptor, which invoked the hypothesis that p53-mediated LRP1 expression could function as a means of concomitantly upregulating TGF-beta signaling under sub-lethal conditions that induce p53. The bimodal expression of LRP1 in response to sub-lethal or lethal stress is consistent with the bimodal function of TGF-beta signaling in the induction of cell cycle arrest and apoptosis.[67] Thus, I hypothesized that p53 upregulates LRP1 as a cross-talk mechanism to upregulate the TGF-beta signaling pathway and enhance cell cycle arrest.

Although our initial analyses revealed no difference in TGF-beta responsiveness in U2OS LRP1 isogenic clones, it is possible that TGF-beta signaling could occur predominantly through other mechanisms in these cells (Figure 4-8). The context dependence of both TGF-beta and p53 signaling pathways require the use of the appropriate model system to ensure that the results are reproducible and accurately portray physiological phenomena. Thus, in future studies, I would like to generate LRP1 CRISPR knockout cell lines or use LRP1-targeting siRNA constructs in non-transformed cells to investigate the effects of LRP1 signaling in a more normal genetic background. Moreover, overlap of other TGF-beta receptors expressed in the cell could have masked a possible LRP1-dependent effect. Therefore, the manipulation of the expression of other TGF-beta receptors could be necessary to observe an LRP1-dependent effect. One of the phenomena that has been commonly accepted for a while is cross-talk between the TGF-beta and p53 signaling pathways; however, although the co-regulation of genes by p53 and the SMAD transcription factors is known, we lack basic mechanistic insight into how p53 and TGF-beta pathways are coordinated with one another. It would be interesting to determine whether LRP1 might contribute to p53-TGF-beta pathway cross-talk.

The context-dependent nature of the role of LRP1 in different tissues raises interesting questions with respect to the analysis of different model systems to identify LRP1 effects in the context of the p53 response. LRP1 is directly involved in the phagocytosis of apoptotic cells by macrophages through the binding of surface-expressed calretculin on the target cell.[94] Considered in the context of our results, the upregulation of LRP1 by p53 in macrophages makes intuitive sense, as stresses such as ionizing radiation often result in the death of cells that require clearance, typically by macrophages. Thus, it

would be interesting to determine whether p53-dependent LRP1 expression in macrophages could facilitate the clearance of apoptotic cells. This could serve as a general mechanism through which p53 can prime phagocytic cells such as macrophages to increase their surveillance for apoptotic debris when stress is detected.

Counter-intuitively, I observed a clear survival advantage in HCT116 clones in which LRP1 was deleted, which is inconsistent with a role of LRP1 in a pro-survival function. This could be partially explained by the method used to evaluate cells in the absence of LRP1. Because I used stable clones, I cannot rule out that the cells experienced compensatory alterations in other pathways that produced a survival advantage in the presence of IR. Therefore, validation experiments using transient siRNA knockdown of LRP1 could be necessary to account for this possibility.

Could LRP1 be a suitable drug target?

Despite strong ties to major diseases, including Alzheimer's and atherosclerosis, we lack candidate drugs that target LRP1 activity and/or expression. One of the challenges in targeting LRP1 is that like p53, both too much LRP1 and too little LRP1 in a given tissue can promote a diseased state. In Alzheimer's disease, increased LRP1 activity in the brain and liver would presumably be beneficial for the clearance of amyloid-beta and amyloid plaques.[135, 261, 272] LRP1 also binds directly to an amyloid precursor protein variant, which enhances its internalization and degradation.[146] However, in the presence of increased lipids, as in the case of post-prandial serum lipid increase, increased LRP1 activity in adipose tissue increases susceptibility to lipid accumulation and obesity based on its ability to enhance the uptake of lipoproteins, exemplifying the complex nature of LRP1 as a

potential drug target.[106] Nonetheless, with LRP1 implicated in deadly diseases for which treatments are limited or non-existent, the investigation of LRP1 as a therapeutic target for conditions like Alzheimer's disease makes sense. Our study implies that the activation of p53 could increase the expression of LRP1, which in the context of Alzheimer's disease and amyloid-beta removal could be beneficial therapeutically. Although many of the anti-amyloid-beta biologicals under clinical investigation have produced underwhelming results to date, at least one study reported improvements in Alzheimer's patient outcomes and has been advanced to Phase III trials, suggesting that the anti-amyloid-beta strategy could prove effective (studies NCT02484547 and NCT02477800, see [267] for review). Moreover, multiple Alzheimer's disease mouse models have been developed, which offers a platform for the *in vivo* investigation of potential anti-amyloid-beta therapies.[78] As a follow up study, it would be interesting to determine whether the p53-mediated expression of LRP1 could be exploited using current p53 activators such as nutlin-3a or RITA to counter amyloid-beta accumulation. I expect that based on the ability of LRP1 to internalize and degrade amyloid-beta, p53 activation could increase the rate of amyloid-beta degradation and delay Alzheimer's disease progression. Moreover, targeted p53 upregulation in the mouse in relevant tissues such as the brain and liver could offer a more controlled model through which to determine whether p53 is involved in Alzheimer's disease progression. Because most Alzheimer's mouse models are based on mutations in genes involved in the amyloid-beta degradation pathway, perhaps a more appropriate model would be the *Octodon degus* rodent, which has been reported to spontaneously develop Alzheimer's-like symptoms with age.[3] One of the obvious gaps in our knowledge of Alzheimer's disease, and particularly the sporadic form of the disease (as opposed to the familial form), is the

etiology. Thus, although a more in-depth understanding of how Alzheimer's disease occurs and progresses would be very useful for the development of effective therapeutics, the established link between LRP1 and Alzheimer's disease offers an early potential target that could be exploited.

Conclusions

The identification of a potential p53 self-regulating mechanism involving the induction of miRNAs that target pro-survival p53 target genes is an interesting concept that deserves more in-depth investigation. I present evidence of a translation deficiency in LRP1 expression in the presence of lethal doses of stress, and I present preliminary evidence suggesting that miRNAs could be involved. I find the dose-dependent difference in the protein expression levels of various p53 target genes worth follow-up using an *in vivo* model. I am especially interested in determining whether the gradient pattern of doxorubicin distribution in tumors that is dependent on proximity of tumor cells to blood vessels could actually contribute to the p53-dependent survival of tumor cells that receive low doses of doxorubicin. If so, then the adjuvant use of agents that normalize the perfusion of drugs throughout tumor tissue could gain recognition as a necessary measure for effective treatment. Moreover, considering the anti-apoptotic functions of some p53 target genes such as p21, I am also interested in pursuing the potential for the use of certain pro-survival p53 target genes as biomarkers for the distribution of drugs to p53 WT tumors. The work presented in this dissertation provides novel insight into multiple aspects of the p53 pathway but also in turn raises several questions that would be interesting to pursue further.

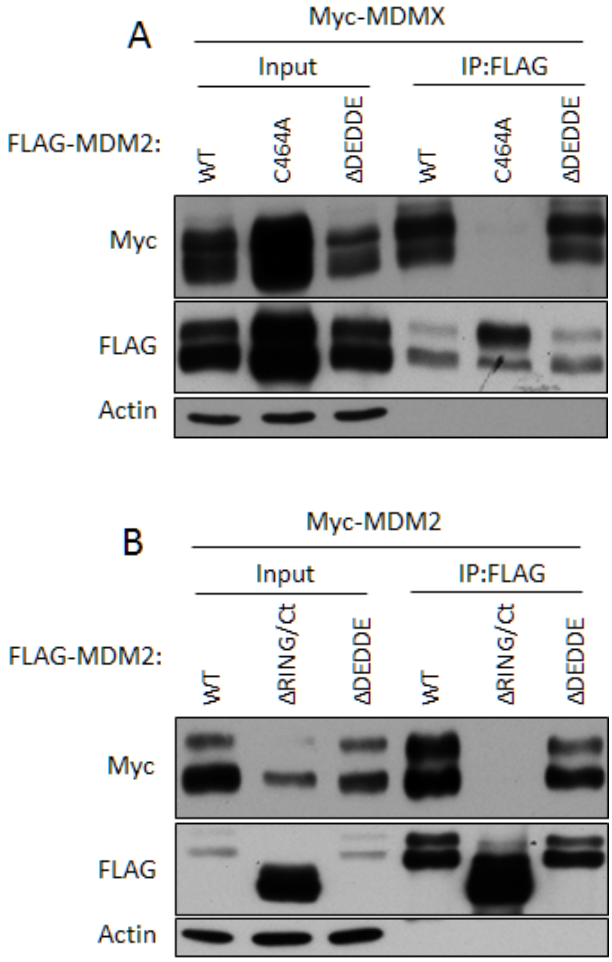

Figure 4-1. Small deletions of the MDM2 acidic domain may selectively impair MDM2-MDM2 homooligomerization.
A) U2OS cells were co-transfected with the indicated constructs for 24 h and then were subjected to IP for the FLAG-MDM2 constructs. Membranes were first probed for myc-MDMX co-IP and then reblotted for FLAG-MDM2 constructs.
B) U2OS cells were co-transfected and subjected to IP as in (A) with the substitution of myc-MDM2 for myc-MDMX.

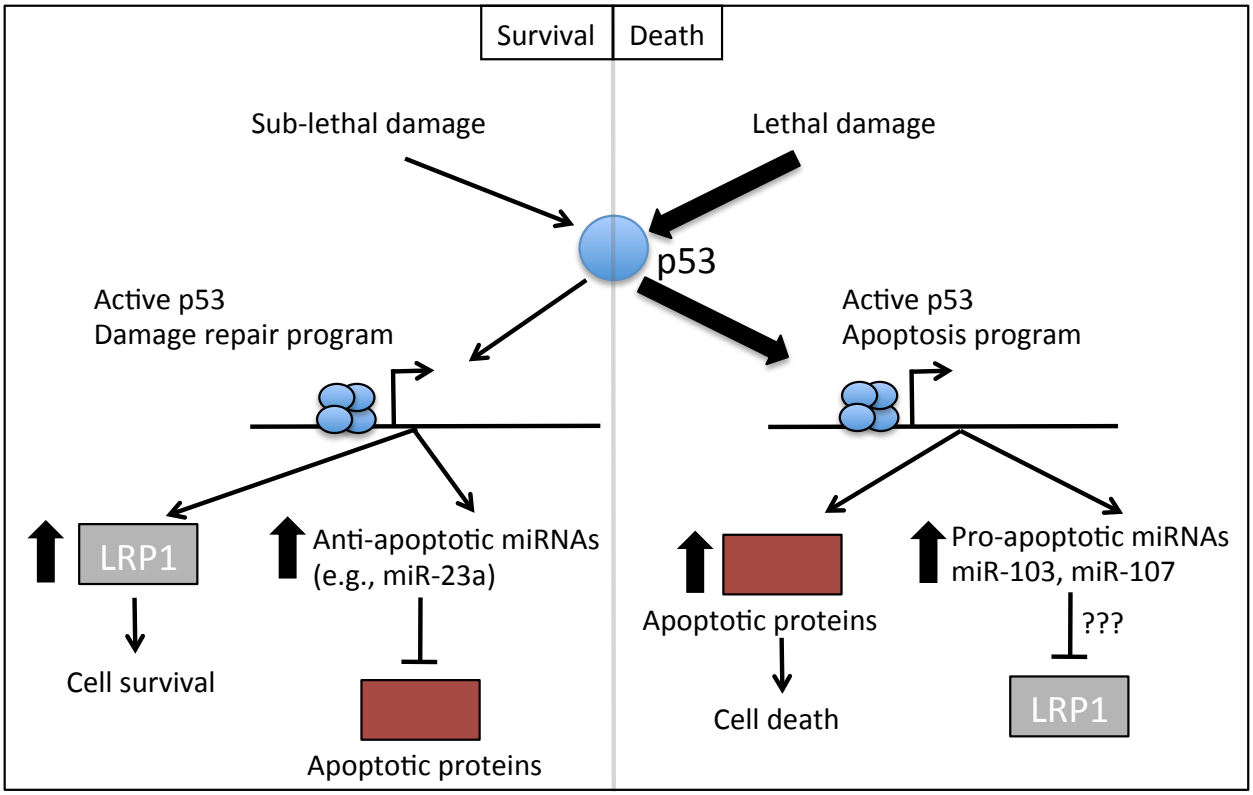

Figure 4-2. A model based on the integration of the current study on p53 regulation of LRP1 and other studies investigating miRNA regulation.
This model reflects our data, which support a dichotomy involving p53 that depends on the nature of a given stress. Stresses that result in cell survival likely activate the p53 cell survival/adaptation pathway, which appears to involve LRP1 protein expression. Moreover, p53-regulated miRNAs (e.g., miR-23a) have been reported to suppress the expression of pro-apoptotic p53 target genes, such as Apaf-1, in response to sub-lethal stresses (see discussion). Stresses that result in cell death appear to activate the expression of p53 pro-apoptotic target proteins, such as Apaf-1. Moreover, because the expression of pro-survival p53 target genes could interfere with the induction of apoptosis, it is likely that the p53 cell survival program needs to be suppressed. Our data show that p53-regulated miRNAs could play a role in the suppression of p53 target genes, such as LRP1, which implies the existence of a natural feedback loop allowing p53 to rapidly induce apoptosis.

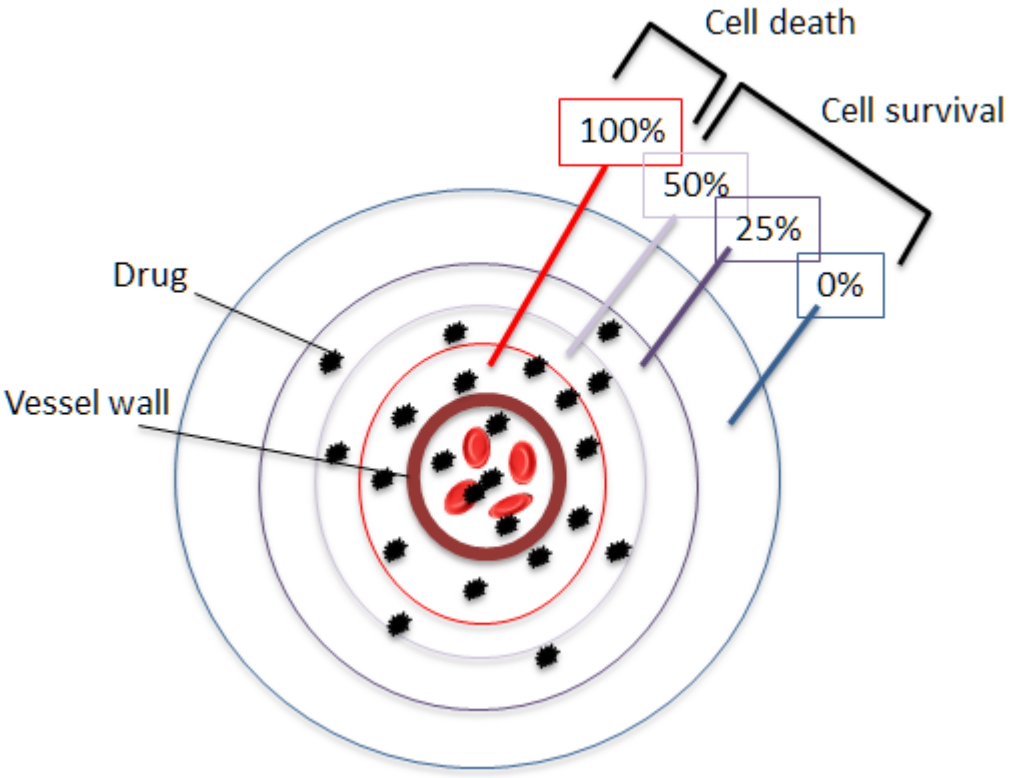

Figure 4-3. Intravenous drug delivery often delivers therapeutic doses of drug to only the most proximal tumor cells.
Many drugs that are delivered intravenously rely on the vascular system for delivery to tumor cells. However, in rapidly growing tumors, a considerable portion of the tumor resides to distal (>100 μm) from blood vessels to be exposed to cell death-inducing concentrations of the drug. Doxorubicin is one drug that displays poor tumor diffusion characteristics, as the vast majority of drug that exits the tumor vasculature into the tumor tissue binds tightly to the most proximal cell, which results in a gradient of doxorubicin concentration. It is possible that p53 WT tumor cells exposed to sub-optimal concentrations of doxorubicin (as shown in the figure) respond in a p53-dependent manner by upregulating pro-survival p53 programs, which could also facilitate the development of resistance.

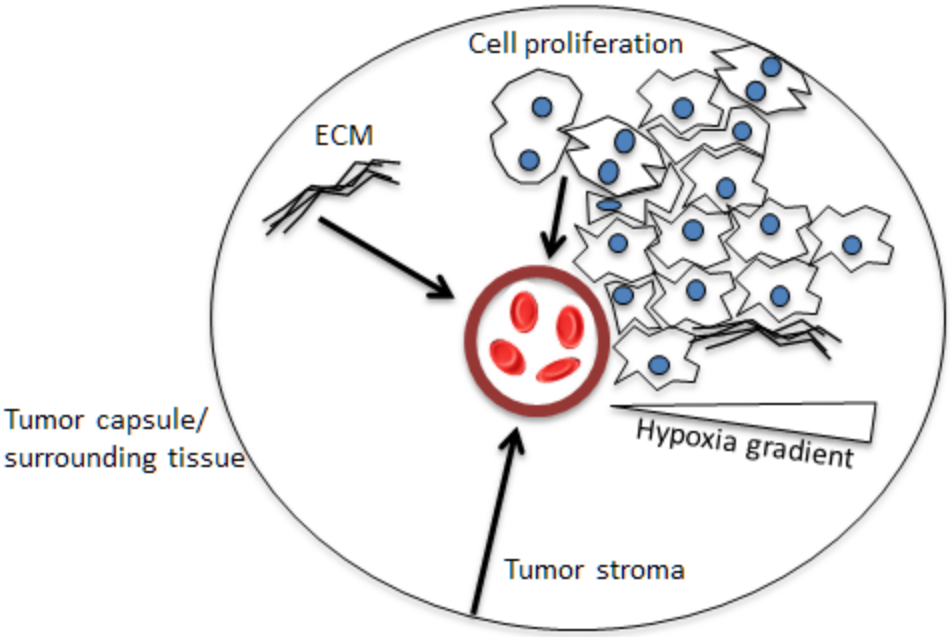

Figure 4-4. Rapidly growing tumors display high levels of intratumoral pressure. Intratumoral pressure contributes to the poor diffusion of chemotherapeutics. Rapidly growing tumors exert physical pressure (indicated by the arrows) on intratumoral vessels, such as blood vessels and lymphatics from several sources, including cell proliferation, extracellular matrix (ECM), and tumor stroma (connective tissue, non-tumor tissue). The increase in pressure can physically occlude blood vessels, which can result in the development of hypoxic regions within the tumor, especially in regions that are distant from blood vessels. Hypoxic regions are not only inherently less perfused by components of the blood (oxygen, IV drugs, nutrients) but these regions also contain fewer actively dividing cells, which reduces the efficacy of many cancer drugs that rely on the rapid proliferation characteristics of tumor cells to offer a therapeutic benefit.

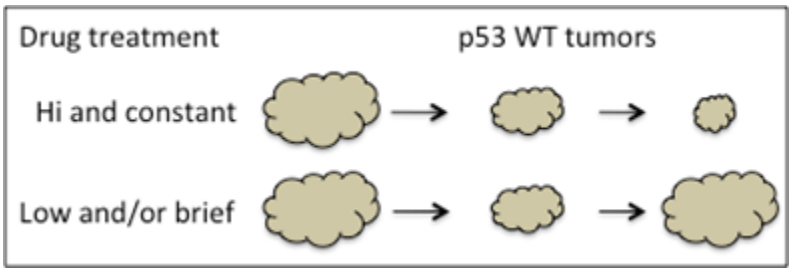

Figure 4-5. p53 WT tumors exposed to sub-optimal dosing regimens of some chemotherapeutic drugs may employ p53 cell survival programs to increase their resistance.
In p53 WT tumors, physical tumor characteristics that prevent the delivery of clinically therapeutic doses of a drug to the entire tumor may activate p53 survival pathways in certain tumor cells, which could promote the development of resistance. A major limiting factor for several chemotherapeutics is the susceptibility of normal tissue to the effects of the drug, which limits the duration and dose that can be administered in any round of chemotherapy.

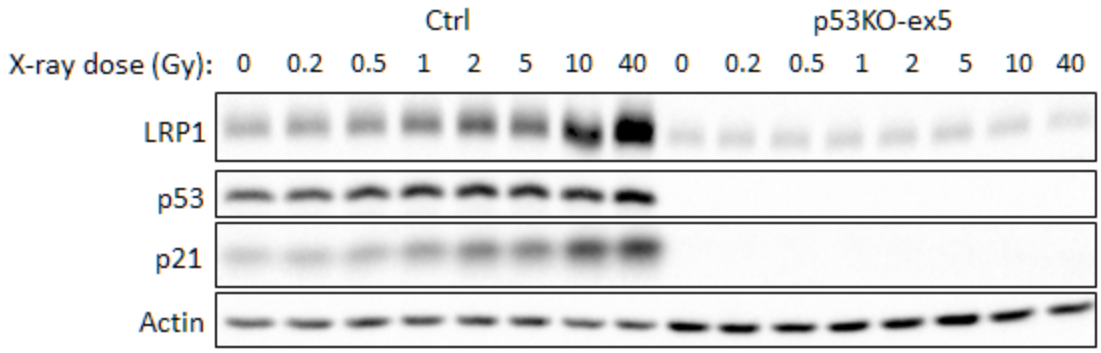

Figure 4-6. IR-dependent LRP1 induction does not show protein suppression at high doses.
HCT116 CRISPR ctrl or p53 knockout cells were treated with the indicated dose of IR (x-rays) and harvested 24 h later. Lysates were obtained and analyzed by western blot.

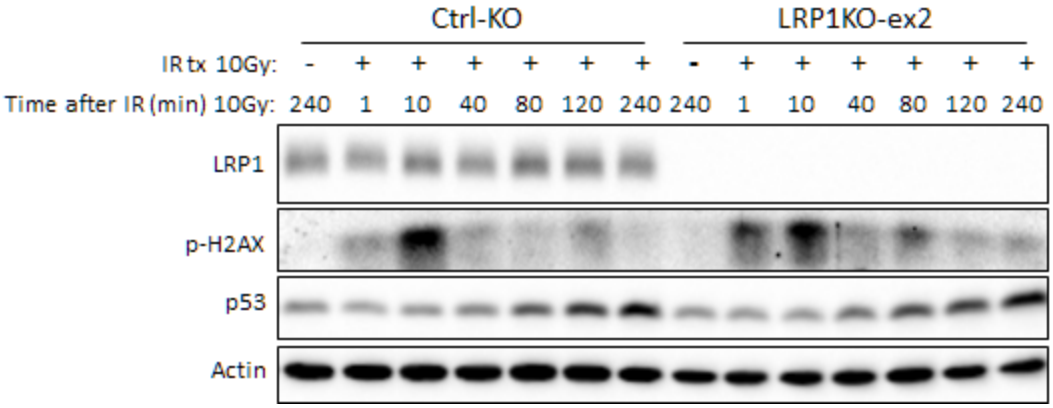

Figure 4-7. LRP1 deletion does not affect p-H2AX resolution in cancer cells.
A U2OS LRP1 knockout cell line was treated with 10 Gy of IR, 24 h after which samples were collected at different time points to determine p-H2AX signal resolution.

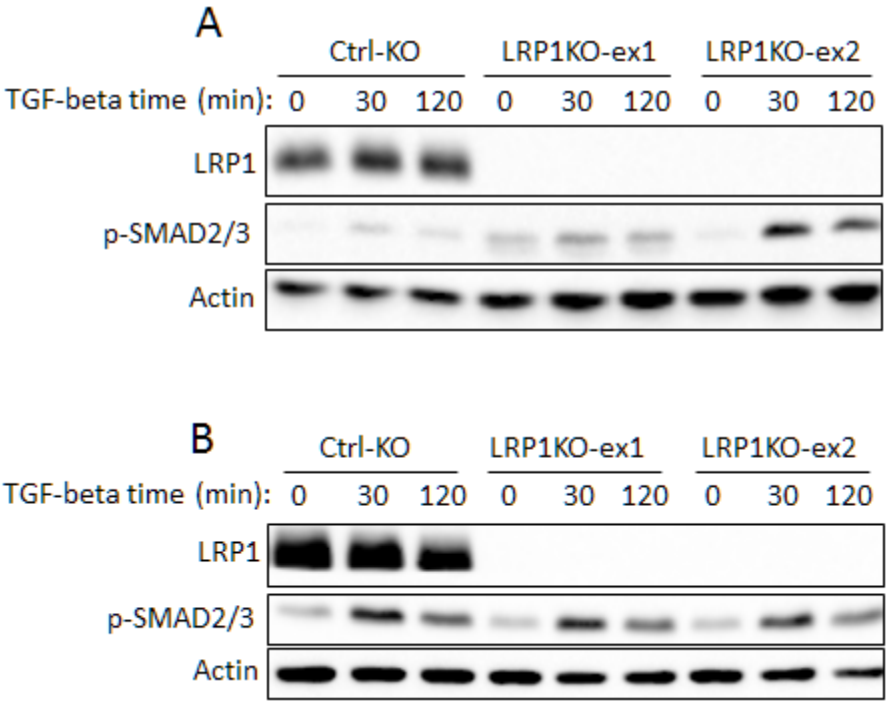

Figure 4-8. TGF-beta signaling is not affected by LRP1 deletion in cancer cells.
A) U2OS CRISPR lines were treated with 2.5 ng/ml TGF-beta for the indicated amount of time and then were analyzed for p-SMAD2/3 expression.
B) HCT116 cells were treated with 2.5 ng/ml TGF-beta for the indicated amount of time and then were probed for p-SMAD2/3

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