p90 and UHRF1, Two Novel Regulators of the p53 Signaling Pathway

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ABSTRACT

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To ensure proper and differentiated regulation of stress response pathways, the p53 tumor suppressor calls for an intricate network of control of activation and fine tuning of transcription activity, which is offered largely through post-translational modifications. Accumulating evidence supports the indispensability of acetylation in the activation of p53 function and indicates modulation of cell fate decision; however the underlying molecular mechanisms are not well understood and identification of the regulatory mechanisms controlling p53 acetylation remains an important step in furthering the understanding of p53 regulation *in vivo*. In this study we identify p90 and UHRF1 as two novel members of the p53 regulatory network upstream of TIP60-mediated p53 acetylation.

Through biochemical purification, p90 was identified as a unique regulator for p53. p90 (also called CCDC8, coiled-coil domain containing 8) interacts with p53 both *in vitro* and *in vivo*. Depletion of p90 by RNAi has no obvious effect on p53 stability or p53-mediated activation of p21, but specifically abrogates PUMA activation. Moreover, p90 also interacts with the TIP60 acetyltransferase and stimulates TIP60-dependent Lys120 acetylation of p53, therefore enhancing the

apoptotic response of p53. These data reveal p90 as an upstream regulator of the Tip60-p53 interaction and demonstrate that p90 is specifically required for p53-mediated apoptosis upon DNA damage.

We also report that the epigenetic regulator UHRF1 (ubiquitin-like with PHD and RING finger domains 1) interacts with TIP60 and induces degradation-independent ubiquitination of TIP60. Moreover, UHRF1 markedly suppresses the ability of TIP60 to acetylate p53. In contrast, RNAi-mediated inactivation of UHRF1 increases endogenous p53 acetylation and significantly augments p53-mediated apoptosis. To elucidate the mechanisms of this regulation, we found that the interaction between TIP60 and p53 is severely inhibited in the presence of UHRF1, suggesting that UHRF1 modulates TIP60-mediated functions in both K120 acetylation-dependent and -independent manners. Consistent with this notion, UHRF1 knockdown promotes activation of p21 and PUMA but not HDM2. These findings demonstrate that UHRF1 is a critical negative regulator of TIP60 and suggest that UHRF1-mediated effects on p53 may contribute, at least in part, to its role in tumorigenesis.

This study provides insight for understanding the regulation of p53 acetylation and cell fate decision. Both p90 and UHRF1 are previously unidentified members of the p53 regulatory network. Although both function upstream of the TIP60-p53

interplay, they act through distinct and opposing mechanisms to dynamically regulate TIP60-mediated effects on p53 *in vivo*.

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DEDICATION

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CHAPTER 1

INTRODUCTION

1.1 p53 is a tumor suppressor

p53, encoded by the *TP53* gene, is often regarded as "guardian of the genome" because of its pivotal role in tumor suppression [1]. p53 was initially discovered independently by David Lane and Arnold Levine in 1979 as a simian virus 40 (SV40) large T antigen interacting partner that migrates at 53 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [2,3]. Early work revealing excessive p53 production in transformed and cancer cells and work demonstrating that p53 cooperates with the Ras oncoprotein to transform or immortalize cells led to the classification of p53 as an oncoprotein [4-7]. It was later found that the *TP53* cDNA initially cloned from tumor cell mRNA was a dominant negative allele containing a valine (V) to alanine (A) mutation at codon 135 that activates transforming properties. In 1989, work by the Oren group and Levine group showing that wild type p53 could suppress oncogene driven transformation and work by the Vogelstein group demonstrating frequent mutations of the *TP53* gene in human colorectal carcinomas collectively characterized p53 as a tumor suppressor [8-10].

Following these initial observations, *TP53* mutations were reported in a wide spectrum of human cancers, with mutation rates ranging from ~10% in hematopoietic malignancies to ~50%-70% in ovarian, colorectal, lung, and head and neck malignancies [11]. *Trp53* (encoding mouse p53) deficient mice are

susceptible to early onset spontaneous tumorigenesis [12], and germline mutation of p53 in humans which leaves only one functional allele of the *TP53* gene is associated with the Li-Fraumeni syndrome characterized by a 25-fold increase in cancer susceptibility and early onset of a wide range of malignancies such as breast cancer, brain tumors, and soft tissue sarcomas [13,14]. It is now known that p53 mutations or perturbation of the p53 regulatory network exist in over half of all human cancer cases [15-17].

1.2 p53 functions as a sequence-specific transcription factor

The p53 protein comprises several domains: an amino (N-) terminal transactivation domain (TAD; consisting of two transactivation subdomains, TAD-I, residues 1-42, and TAD-II, residues 43-62) [18-20], a proline rich domain (PRD; residues 63-97), a central DNA binding core domain (DBD; residues 100-300) [21,22], a tetramerization domain (TD; residues 307-355) [23,24], and a carboxyl (C-) terminal regulatory domain (CTD; residues 356-393) [25].

Soon after its characterization as a *bona fide* tumor suppressor, p53 was identified to possess binding affinity, through its central domain, to specific DNA sequences termed “the p53 consensus binding site” or “the p53 response element” [26]. The consensus sequence consists of two 5’-PuPuPuC(A/T)(T/A)GPyPyPy-3’

decameric palindromes called “p53 binding half-sites” separated by 0-13 basepairs [26].

The presence of p53 response elements in the regulatory regions (promoters, introns, and upstream sequences) of genes predicts transcription regulation by p53. A combination of gene expression microarrays, chromatin-immunoprecipitation-based microarrays (ChIP-chip) and ChIP sequencing analysis have to date identified at least 500 p53 binding loci throughout the human genome [27-30]. At least 100 genes have been identified to possess p53 response elements and are experimentally validated as p53 target genes [31,32].

DNA sequencing of tumor samples bearing mutant p53 revealed that the vast majority of p53 mutations are missense mutations within the DNA binding domain, resulting in mutant p53 proteins with altered conformation and attenuated sequence-specific binding to DNA [33]. The significance of p53 mutations in tumorigenesis is 3-fold: (i) they abolish wild type p53 function, (ii) they create dominant negative activity through tetramer formation with wild type p53, and (iii) they convey “oncogenic” function through the selective growth advantages of cells with the mutations, the transactivation of new target genes or via inappropriate interaction with other cellular proteins [34].

1.3 p53 centrally coordinates cellular responses to a wide range of stresses

p53 exerts tumor suppressive capacities by centrally coordinating a regulatory circuit that monitors and responds to a variety of stress signals. Under homeostasis, both p53 abundance and p53 transcription activity is kept low by its primary negative regulators Human Double Minute 2 (HDM2, mouse ortholog is Mdm2) and Human Double Minute X (HDMX, mouse ortholog is MdmX). In the event of genotoxic stresses such as DNA damage, abnormal oncogene activation, telomere erosion, hypoxia etc, p53 is rapidly stabilized and activated to transcribe target genes that mediate cell cycle arrest, apoptosis, DNA repair, senescence, energy metabolism, or autophagy. Through executing and balancing these cellular responses, p53 ultimately protects cellular and genomic stability, preventing the propagation of genetic lesions and tumor formation.

A multitude of chemo-reagents converge onto the activation of p53. The cytotoxic agent etoposide forms a ternary complex with DNA and the topoisomerase II enzyme, thus preventing re-ligation of the DNA strand and causing DNA strand breaks in cancerous cells that undergo rapid DNA replication and cell division [35]. The anthracyclines (doxorubicin, daunorubicin, and their derivatives) work by intercalating DNA as well as undergoing redox reactions that generate reactive oxygen species (ROS) [36]. DNA strand breaks are recognized by the MRN complex (consisting of three proteins Mre11, RAD50,

and NBS1) which in turn activates ATM-CHK1 or ATM-CHK2 kinase cascades that transmit this information to p53 through phosphorylating both p53 and HDM2, ultimately inhibiting their association and stabilizing p53 [37]. Reagents that disrupt rRNA biogenesis, such as actinomycin D, increase ribosomal stress and release ribosomal proteins from the nucleoli, which in turn bind to HDM2 and result in p53 stabilization [38]. The uracil analogue 5-fluorouracil (5-FU) antimetabolite functions through misincorporation into nascent RNA and irreversibly blocking thymidylate synthase, causing dTMP depletion in rapidly dividing cells [39], as well as triggering a ribosomal stress response that releases ribosomal proteins to activate p53 by ablating the HDM2-p53 feedback loop [40].

Although the p53 effects are predominantly exerted through its transcription activation of target genes, our knowledge of p53 functions have been expanded into transcription repression [41], regulation of translation [42] and homologous recombination [43], and the induction of a transcription-independent apoptotic response [44].

1.4 p53 and the "big three": growth arrest, apoptosis and senescence

Growth arrest, apoptosis, and senescence are the most well characterized cellular responses following p53 activation and thought of as major mediators of

the tumor suppressive function of p53. The ability of p53 to remove damaged cells through apoptosis is a more evolutionarily conserved function: in lower eukaryotes, including *D. melanogaster* and *C. elegans*, p53 is critical for eliminating damaged cells to preserve germline and tissue integrity [45]. In higher eukaryotes, genotoxic stresses activate p53, leading to cell cycle pauses allowing time for damage repair or the irreversible cellular senescence or apoptosis in the event of prolonged damage as safeguards against neoplasia [31].

Here I will briefly revisit the means by which p53 regulates each of these pathways, and discuss their roles in tumor suppression.

1.4.1 Growth arrest

Cell cycle checkpoint is a common theme of regulation in eukaryotes to ensure fidelity of DNA replication and mitosis, thus protecting from propagation of genetic lesions and progressive accumulation of genomic changes that eventually leads to neoplastic transformation. Halting the cell cycle at checkpoints presumably permits repair of damage before the cell reinitiates DNA replication (G1 arrest) or enters mitosis (G2 arrest).

The first line of evidence suggesting p53 control of cell cycle progression comes from the work from Kastan and colleagues demonstrating that ataxia telangiectasia mutated (ATM), p53 and GADD45 comprise a signal transduction

pathway that controls the mitotic checkpoint upon DNA damage [46]. Soon afterwards, p53 was shown to be required for G1 checkpoint arrest following DNA damage, primarily through transcription activation of one of the best characterized p53 target genes *CDKN1A* encoding $p21^{CIP1/WAF1}$, a cyclin-dependent kinase (CDK) inhibitor [47,48]. Elevated p21, through binding to and inactivating cyclin/CDK complexes required for the G1/S transition, arrests cells in the G1 phase to allow time for DNA damage repair.

Mouse embryonic fibroblasts (MEFs) derived from mice lacking p21 are almost entirely deficient in G1 arrest following DNA damage, underlying the significance of p21 in the G1 checkpoint arrest [49]. However, unlike $Trp53^{-/-}$ mice, $p21^{-/-}$ mice are not susceptible to early onset of spontaneous tumor development [49]. Nevertheless, loss of p21 promotes tumor initiation, progression or metastasis in some mouse tumor models driven by carcinogens, activated oncogene or γ-irradiation [50,51], suggesting that p21 deficiency promotes tumorigenesis in certain settings.

1.4.2 Apoptosis

The finding of p53 regulation of apoptosis comes from work by Oren and colleagues utilizing a temperature-sensitive p53 mutant that behaved like wild type p53 at the permissive temperature. Re-introduction of p53 into p53-deficient

myeloid leukemia cells potently induced apoptosis that could be counteracted by a pro-survival cytokine [52].

It is now known that at least three apoptotic pathways exist (the mitochondrial pathway, the death receptor pathway, and the endoplasmic reticulum pathway) and they cross-communicate with each other and converge to a common downstream caspase activation that eventually leads to programmed cell death [53].

p53 can transactivate a wide array of downstream death effectors including the pro-apoptotic Bcl-2 family members Bax [54], Bid [55], PUMA [56] and NOXA [57] involved in the mitochondrial apoptotic pathway, Killer/Dr5 and Fas (also called CD95 and Apo-1) of the death-receptor pathway [58-61], and Scotin of the endoplasmic reticulum pathway [62]. In addition to transactivating death effectors, p53 can mediate transcription repression of anti-apoptotic proteins (Bcl-2, Bcl-XL, and survivin) [63-65], or cytoplasmic p53 can translocate to the mitochondria and directly interact with pro- and anti-apoptotic Bcl-2 family members to induce mitochondrial outer membrane permeabilization (MOMP) [66-71].

Despite the many p53-activated death effectors and the transcription-independent apoptotic function of cytoplasmic p53, p53-induced apoptosis *in vivo* is mediated predominantly by PUMA and to a lesser extent by NOXA [57,72-75],

because *Bbc3 (puma)* knockout mice recapitulates nearly all apoptotic deficiencies in *Trp53* knockout mice, although in a tissue specific manner further loss of *Pmaip1 (noxa)* was required for the complete abolishment of apoptosis following whole body gamma-irradiation [75].

p53 mediated apoptosis undoubtedly plays an important role in suppressing tumor growth and progression in response to oncogenic events or DNA damage. Using a brain cancer mouse model in which the pRB tumor suppressor is perturbed, Dyke and colleagues showed the first evidence that apoptosis contributes to p53 tumor suppression function *in vivo*: tumors develop aggressively in the absence of p53 but grow slowly in the presence of p53, and that this is attributed to high levels of p53-dependent apoptosis [76]. In addition, in Eμ-Myc transgenic mice, a model for B-cell lymphoma, disruption of apoptosis downstream of p53 through Bcl-2 or dominant negative caspase 9 expression, recapitulates the tumor growth advantage observed for loss of p53 [77]. Furthermore, lymphoma development driven by c-Myc or low dose γ-irradiation is significantly accelerated by loss of *puma* and/or *noxa* [78-80].

1.4.3 Senescence

Cellular senescence is the process of irreversible cell-cycle arrest in spite of mitogenic signals, and was first described almost fifty years ago by Hayflick and colleagues when they showed that normal cells had a finite proliferative capacity

in culture [81]. Senescent cells manifest phenotypic changes including a flattened/enlarged morphology, increased adherence, and the expression of senescence-associated β-galactosidase (SA-β-GAL), the staining of which is a common and reliable method for detection of senescence [82,83]. Senescent cells also acquire an altered gene expression profile, including upregulation of inflammatory cytokines and other immune modulators [84].

Although replicative senescence is triggered by telomere erosion, premature senescence can also be acutely achieved through oncogene activation, oxidative stress, DNA damage and treatment with anticancer drugs [85-87], all of which seemingly converge upon the activation of the DNA damage response (DDR).

Both telomere- and damage-initiated cellular senescence depend strongly on p53 mediated induction of the pleiotropic CDK inhibitor p21. In many cases, this is followed by a delayed stable activation of $p16^{Ink4A}$ (encoded by *CDKN2a*) CDK inhibitor [88], which itself is a tumor suppressor frequently mutated in cancer [89,90]. It is believed that p53 acts to initiate senescence through the induction of p21, while the subsequent increase in p16 level then acts to maintain senescence.

The fact that cancer cells are immortal and can proliferate indefinitely suggests that cellular senescence needs to be bypassed at some point prior to malignant transformation. One such example would be the common benign human tumor melanocytic naevi (moles), which frequently possess oncogenic

mutations but typically remain in a growth arrested state for decades and only rarely progress to malignant melanomas [91]. Indeed, senescence markers are expressed by nevi *in vivo* [91], demonstrating that cellular senescence efficiently suppresses malignant transformation of benign tumors.

Studies with oncogene driven tumor mouse models support the role of p53-mediated cellular senescence in suppressing tumor *in vivo*. Expression of oncogenic Eμ-N-Ras in p53 knockout mice readily drives aggressive T cell lymphomas, whereas in the presence of wild type p53 Eμ-N-Ras transgenic mice developed nonlymphoid neoplasia with prevalent signs of senescence [92]. An oncogenic K-Ras^{G12V} transgenic mouse model also demonstrates senescence in the early stages of lung and pancreatic tumors [93].

Importantly, reactivation of p53 in p53-deficient liver carcinoma induced the cellular senescence program, in turn triggering tumor clearance *in vivo* through the innate immune system [94], highlighting the potential of tailored pro-senescence therapies in cancer treatment.

1.4.4 Tumor suppression: the "big three" and beyond

Over the past 30 years of p53 research, the tumor suppressive capacity of p53 cell-cycle arrest, apoptosis, and senescence targets have been rigorously tested using target gene knockout mouse models, and numerous studies have

demonstrated the importance of several key targets in suppressing tumor in the context of oncogene activation, tumor suppressor deficiency, irradiation, and DNA damage. However, increasing evidence suggests that p53-mediated tumor suppression is more complex than just the "big three".

A number of knockout mice lacking single p53 target genes have been generated, and none of these could recapitulate the dramatic and completely penetrant phenotype of spontaneous tumor predisposition observed in *Trp53* null mice [95]. This then led to generation of compound mouse knockouts such as *p21*$^{-/-}$*puma*$^{-/-}$, *puma*$^{-/-}$*noxa*$^{-/-}$ and *p21*$^{-/-}$*puma*$^{-/-}$*noxa*$^{-/-}$ mice, which again were not prone to spontaneous early onset of tumorigenesis [78,96,97].

A recent p53$^{3KR/3KR}$ knockin mouse generated by the Gu team, in which three lysine (K) acetylation sites (K117, K161, and K162; human counterparts are K120 and K164) were collectively mutated to the non-acetylable arginine (R) residue, was still resistant to spontaneous tumorigenesis despite loss of DNA-damage induced growth arrest, apoptosis, and senescence [98], suggesting that loss of all three functions is insufficient for abrogating p53 tumor suppression *in vivo*. Strikingly, p53$^{3KR/3KR}$ retains regulation of non-conventional target genes involved in energy metabolism (upregulation of *GLS2*, encoding a mitochondrial glutaminase that modulates mitochondrial respiration and ATP generation; downregulation of *GLUT3*, encoding a glucose transporter) and reactive oxygen

species production (upregulation of *TIGAR*, encoding a a fructose bisphosphatase that downregulates glycolysis by reducing cellular levels of fructose-2, 6,-bisphosphate), implying that these non-canonical p53 functions may be more relevant to suppression of early onset tumorigenesis *in vivo*.

Interestingly, loss of growth arrest, apoptosis, and senescence seems to confer a certain degree of genome instability. Indeed, in the $p53^{3KR/3KR}$ background, *de novo* mutation of $Trp53^{3KR}$ gene is observed in several animals, contributing to latent spontaneous tumorigenesis [98]. Similarly, taking advantage of the hypomorphic $p53^{R172P}$ mutant that delays spontaneous tumor onset due to complete loss of apoptosis and partial loss of cell cycle arrest, the Lozano team showed that $Trp53^{R172P/R172P}p21^{-/-}$ mice display accelerated tumor onset compared to $Trp53^{R172P/R172P}$ mice due to genome instability as demonstrated by aneuploidy and chromosomal aberrations that were absent in $Trp53^{R172P/R172P}$ malignancies [99].

1.4.5 Summary

It is probable that different p53-dependent response pathways are differentially required for tumor suppression under different biological settings or tumor types, and the composite loss of several effector pathways (coordination of cell cycle arrest, apoptosis, senescence, DNA repair, energy metabolism, etc.)

collectively accounts for the high penetrance and early onset of tumors when p53 is mutated in mice [12] and in humans (Li-Fraumeni syndrome) [14].

For instance, in an unchallenged state, cell cycle arrest, apoptosis and senescence may keep damaged cells in check; however these may not be the rate limiting step in protecting from tumor formation. Instead, loss of apoptosis and temporary or permanent growth arrest allows proliferation of damaged cells and accumulation of genome instability, eventually leading to surpassing certain thresholds in energy metabolism, allowing for selective growth advantage of cancer cells.

Importantly, unlike laboratory animals, humans are frequently challenged by environmental insults, increasing chances for acquiring carcinogen or oncogene-driven mutation. Under these stress conditions, cell cycle, apoptosis, and senescence may be of particular importance for p53 tumor suppressor function.

1.5 Regulation of p53 function

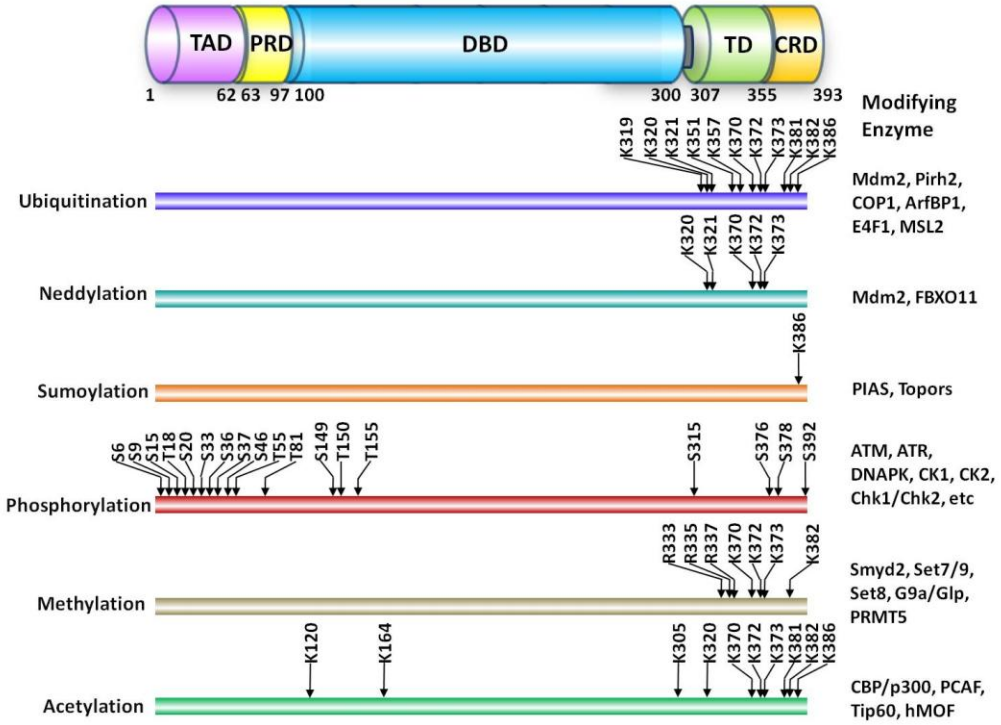
In order to coordinate a wide variety of cellular processes, p53 demands a refined and complicated regulatory network consisting of many positive and negative regulators. At homeostasis, the steady state level of p53 is kept low and p53 function is repressed mainly by the negative regulators HDM2 and HDMX. Under stress conditions, however, p53 is stabilized, translocated to the nucleus, released from repression, and its transcription activity is further activated in a promoter-specific manner.

Significantly, covalent post-translational modifications play a pivotal role in the regulation of p53 under homeostasis and every aspect of the stress induced p53 response. p53 harbors many conserved amino acid residues that can be regulated by a multitude of post-translational modifications, including ubiquitination, phosphorylation, acetylation, methylation, sumoylation and neddylation (Fig. 1). Interestingly, a single enzyme may target several p53 sites for modification, and a single site may be targeted for multiple modifications. Many modifications display dramatic regulatory effects on p53 function as demonstrated in various *in vitro* and cell culture based studies, however transgenic mice expressing mutant p53 deficient in a single residue modification often show modest phenotypes, supporting a certain degree of regulatory redundancy.

Here I will revisit some of the most important modes of regulation of p53 stability, localization, DNA binding, cofactor recruitment and promoter-specific transcription activity, highlighting recent advances in our understanding of post-translational modifications with key roles in modulating these aspects of p53 regulation, their regulatory effects *in vivo*, and how deregulated p53 modifications contribute to tumorigenesis.

Figure 1. Overview of p53 domain structure and post-translational modifications

The major sites for p53 phosphorylation, ubiquitination, neddylation, sumoylation, acetylation and methylation are plotted. The enzymes responsible for each type of modification are shown on the right. Abbreviations: TAD, transactivation domain; PRD, proline rich domain; DBD, DNA-binding domain; TD, tetramerization domain; CRD, C-terminal regulatory domain.



1.5.1 Regulation of p53 stability

The cellular protein stability of p53 is tightly controlled: p53 has a very short half-life in normal unstressed cells, ranging from 5-30 min [100], and the rapid stabilization of p53 following stress stimuli allows for exertion of diverse response pathways, such as the halter of cell cycle, the activation of the DNA damage repair response, and the induction of the apoptotic response, to combat theses stresses and protect cellular and genomic stability. The tight control of p53 stability is made possible by the ubiquitin-dependent proteasome degradation pathway, with HDM2 being the chief mediator of p53 ubiquitination and degradation.

1.5.1.1 Ubiquitination overview

Ubiquitination refers to the covalent conjugation of one or more ~8 kDa ubiquitin molecules to a protein substrate, and requires the consecutive function of three enzymes. The E1 ubiquitin-activating enzyme links the C-terminal glycine of the ubiquitin molecule to its own active site cysteine through the formation of a thioester bond; the ubiquitin molecule is then transferred to the active site cysteine of the E2 ubiquitin-conjugating enzyme; and finally an E3 ubiquitin-ligating enzyme transfers the ubiquitin molecule to the protein susbstrate and directing it to rapid degradation by the 26S proteasome [101].

E1 and E2 enzymes have low substrate specificity: a single E1 can bind to dozens of E2s, and a single E2 can bind to hundreds of E3s in a hierarchical way. Unlike E1 and E2, the E3 ubiquitin ligase displays high target specificity, usually through a specific substrate recognition domain or through other cofactors in the case of multi-subunit E3 ubiquitin complexes.

E3 ligases can be divided into two types: those that harbor a Really Interesting New Gene (RING) domain and those with a Homologous to the E6-AP Carboxyl Terminus (HECT) domain [102].

1.5.1.2 p53 ubiquitination by HDM2

HDM2 is the pivotal E3 ubiquitin ligase and negative regulator of p53 [103,104]. HDM2 targets six p53 lysine (K) residues within the C-terminal regulatory domain (K370, K372, K373, K381, K382, and K386; Fig. 2), promotes p53 degradation by its E3 ubiquitin ligase function and ultimately inhibits p53 transcription activity. p53 is poly-ubiquitinated by high levels of HDM2 and mono-ubiquitinated by low levels of HDM2 [105]. HDM2-mediated suppression of p53 is 2-fold: (i) as an E3 ubiquitin ligase it targets p53 for ubiquitin-dependent proteasomal degradation and (ii) it inhibits p53 transcriptional activation by directly binding to and repressing p53 [106] (discussed in more detail in Chapter 1.5.3).

Importantly, the gene encoding HDM2 is a p53 transcription target, therefore the stress-induced increase in p53 levels leads to the expression of its own negative regulator HDM2, which in turn downregulates p53, creating an autoregulatory feedback loop [107]. The stabilization and activation of p53 go hand in hand with the inhibition of HDM2 E3 ubiquitin ligase function [108,109]. The p53/HDM2 feedback loop is regulated by multiple factors including the Alternate Reading Frame of the INK4a/ARF locus (ARF) tumor suppressor [110], the E3-ligase activity-lacking HDM2 homolog HDMX (also known as HDM4) [106] , the deubiquitinating enzyme ubiquitin specific protease 7 (USP7, also known as herpesvirus associated ubiquitin specific protease (HAUSP)) [111], and post-translational modifications of HDM2 such as phosphorylation and acetylation [112-114].

The critical role for HDM2 suppression of p53 is best illustrated by the overactivation of p53 in *mdm2* null mice leading to embryonic lethality, which can be rescued by the loss of p53 [115]. Furthermore, mice expressing a cysteine (C) 462A mutated version of mdm2 (equivalent to C464A in HDM2), which loses its E3 ligase activity but retains p53 binding capacity, die during embryogenesis but can be rescued by the loss of p53 [116], demonstrating that the E3 ligase activity of mdm2 is indispensable for the repression of p53.

1.5.1.3 p53 ubiquitination by HDM2-independent E3 ubiquitin ligases

Despite the elevated p53 level and the spontaneous activation of p53 function in mdm2 null mice [117], supporting that HDM2 is the principal endogenous E3 ubiquitin ligase targeting p53 with high specificity [118-120], p53 still undergoes degradation in the absence of mdm2 [117], suggesting the existence of HDM2-independent degradation pathways.

Indeed, several other E3 ligases have been shown to regulate p53 degradation and localization independent of HDM2. In cell culture, the RING domain containing p53-Induced protein with a RING-H2 domain (PIRH2) [121], Constitutively Photomorphogenic 1 (COP1) [122], Carboxy terminus of Hsp70p-Interacting Protein (CHIP) [123], Caspase 8/10-Associated RING Proteins (CARPs) and SYNOVIOLIN [124,125], the HECT domain containing ARF-Binding Protein 1 (ARF-BP1) [126] as well as Ubiquitin-Conjugating enzyme 13 (UBC13) (containing neither domain) [127] poly-ubiquitinate p53 and target it for proteolysis. Whether these E3 ligases regulate p53 stability *in vivo* needs further genetic validation.

Recent studies also support the existence of E4 ubiquitin ligases that specifically target mono-ubiquitinated p53 in the cytosol for homeostatic proteolytic degradation [128], possibly antagonizing the transcription-independent

apoptotic functions of cytosolic p53, which requires mono-ubiquitinated p53 in the mitochondria.

The presence of multiple ubiquitin ligases that control p53 stability suggests a "fail-proof" redundancy in negative regulation. The capacity of these ligases to repress p53 function predicts that these p53-specific E3 ubiquitin ligases could be oncogenes. Indeed *PIRH2*, *COP1* and *WWP1* are amplified or overexpressed in certain cancers [129-131].

1.5.1.4 p53 deubiquitination by USP7

The ubiquitination of p53 is counteracted mainly by the USP7 deubiquitinating enzyme. USP7 deubiquitinates p53, auto-ubiquitinated HDM2 and ubiquitinated HDMX [132], and changes in USP7 levels produce non-linear effects on the p53-HDM2/HDMX pathway, therefore USP7 plays a dynamic role in tumorigenesis.

Moderate down regulation of USP7 preferably stabilizes HDM2, therefore leading to p53 destabilization and favors cell proliferation [133]. These data lend support to the finding in a study of patient samples of Non-small Cell Lung Cancer (NSCLC) that nearly 50% of NSCLC samples with wild-type p53 display reduced USP7 mRNA expression [134]. In contrast, complete loss of USP7 function through a robust small interfering (si)RNA knockdown or knockout of

the *usp7* gene destabilizes HDM2 and HDMX, therefore stabilizing p53 and would inhibit tumor growth [132]. This is consistent with the observation that no *USP7* mutation was identified in the $TP53^{+/+}$ NSCLC samples [134].

Inhibition of USP7, therefore, presents a promising therapeutic approach for treating cancers that retain wild-type p53. Indeed, a small molecule inhibitor HBX41108 identified for USP7 by high-throughput screening stabilizes p53 in tissue culture and inhibits tumor cell growth [135], warranting further studies to confirm the anti-tumor effect *in vivo*.

1.5.2 Regulation of p53 localization

In normal cells under homeostasis, p53 is shuttled between the nucleus and the cytoplasm [136]. In response to stress, however, p53 is rapidly translocated to the nucleus to exert its biological function as a transcription factor. The recent discovery of transcription-independent functions of p53 in the cytoplasm, including direct activation of apoptosis at the mitochondria and inhibition of autophagy, further underscore the importance of regulation of p53 localization [137-139]. Indeed, interference with p53 localization has detrimental effects *in vivo*: constitutive cytoplasmic localization of p53 has been linked to poor response to chemotherapy, tumor metastasis and poor prognosis [140-142].

1.5.2.1 Cytoplasmic targeting of p53 by ubiquitination

p53 ubiquitination not only targets it for proteasomal degradation, but also plays a key role in regulating the cellular localization of p53 (Fig. 2). p53 is polyubiquitinated when HDM2 levels are high and monoubiquitinated when HDM2 levels are low [105]. Poly-ubiquitination primarily targets p53 for proteasomal degradation, while mono-ubiquitination facilitates p53 cytoplasmic translocation through exposing a C-terminal nuclear export signal and promoting dissociation from HDM2 [103,143,144].

Several other E3 ubiquitin ligases also preferentially target p53 for nuclear export independent of HDM2. WW domain-containing Protein 1 (WWP1) mediates p53 ubiquitination and, unlike HDM2, stabilizes p53 at the protein level and causes cytoplasimc accumulation of p53 [145]. Male-Specific Lethal-2 (MSL2) also promotes p53 ubiquitination but does not regulate p53 protein level; instead it preferentially targets p53 for nuclear export [146].

1.5.2.2 Nuclear import of p53 through deubiquitination by USP10

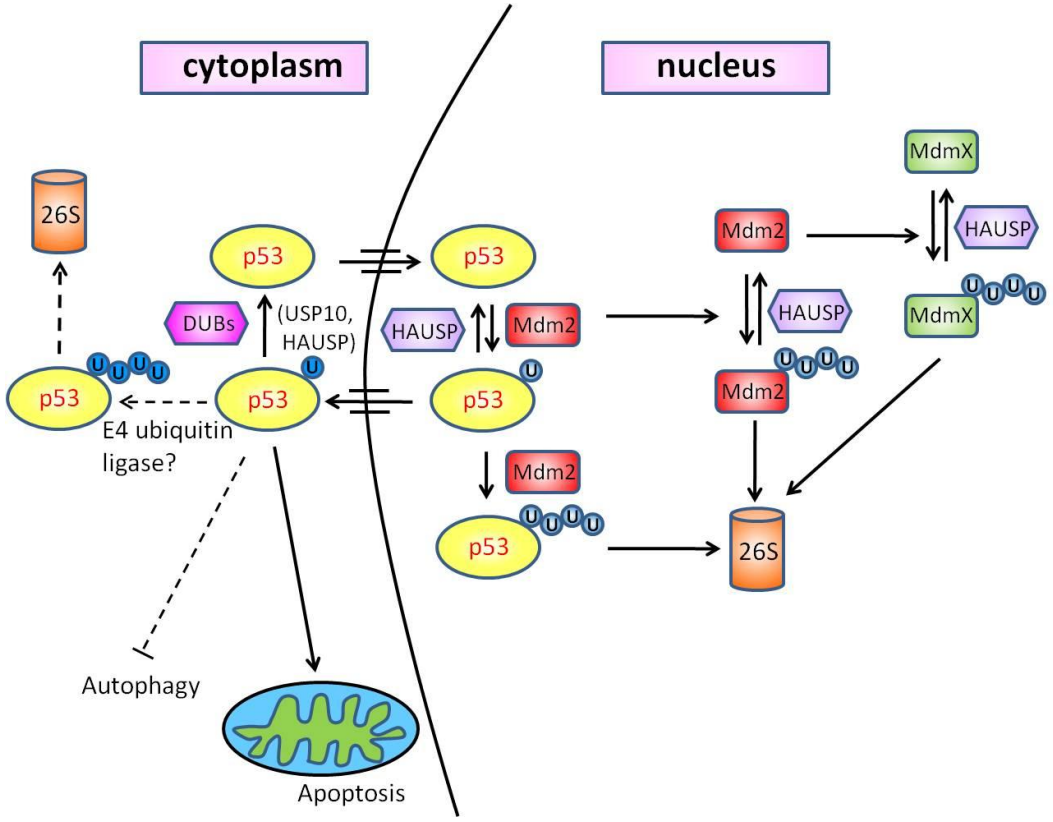
Another member of the large deubiquitinase (DUB) family [147], USP10, has been shown to remove ubiquitin chains from p53. However, unlike USP7, USP10 does not deubiquitinate HDM2 or HDMX. Rather, USP10 reverses HDM2-induced p53 nuclear export, thereby recycling cytoplasmic p53 back to the nucleus [148]. Thus, although both USP7 and USP10 target p53 for deubiquitination, they function in different compartments: USP7 deubiquitinates

and stabilizes p53 primarily in the nucleus [111], whereas USP10 largely deubiquitinates cytoplasmic p53 during homeostasis, although it retains deubiquitinase activity upon translocation to the nucleus following DNA damage [148].

Using human Renal Cell Carcinoma (RCC) cell lines, Yuan and colleagues showed that USP10 is capable of stabilizing both wild-type and mutant p53; therefore USP10 might have different roles in tumorigenesis depending on the p53 status [148]. In RCC cell lines that retain wild-type p53, USP10 behaves like a tumor suppressor and upregulation of USP10 is favorable for repression of cancer growth. In RCC cell lines that have mutant p53, USP10 promotes cancer cell proliferation, and downregulation of USP10 would be beneficial for the inhibition of cancer growth. *usp10* knockout mice studies would facilitate our understanding of the physiological role of USP10 in tumorigenesis. It is perceivable that discovery of USP10-activating or -inhibiting drugs would offer promising treatments for cancers with wild-type or mutant p53.

Figure 2. Regulation of p53 stability and localization by ubiquitination

Nuclear p53 is targeted by HDM2 for monoubiquitination promoting cytoplasmic translocalization or polyubiquitination promoting proteosomal degradation. The abundance of HDM2 and HDMX are also regulated by ubiquitination and deubiquitination. USP7 stabilizes p53, HDM2, and HDMX through deubiquitination. In the cytoplasm, USP10 deubiquitinates monoubiquitinated p53, reversing nuclear export and recycling p53 into the nucleus. Monoubiquitinated p53 in the cytoplasm can possibly be further ubiquitinated by E4 ubiquitin ligases and targeted for degradation. Cytoplasmic p53 also has transcription-independent roles in activating apoptosis through permeabilization of the mitochondrial outer membrane and the inhibition of autophagy through mechanisms yet to be discovered. Abbreviations: U, Ubiquitination.



1.5.3 p53 repression on promoters by HDM2 and HDMX

It was originally believed that p53 exists in a DNA-free form until cells encounter stress stimuli, which in turn stabilizes and activates sequence-specific DNA binding. However, increasing evidence now supports p53 basal binding to DNA in a non sequence-specific manner, and the presence of p53 repressors at target gene promoters prevent transcription activation until a stress stimuli occurs.

1.5.3.1 p53 is bound to DNA at homeostasis

Early studies focusing on the sequence specific DNA binding capacity of p53, often utilizing *in vitro* assays such as Electrophoretic Mobility Shift Assay (EMSA), led to the presumption that p53 exists in a DNA-free form under homeostasis and that only stress-activated p53 could bind to DNA. DNA binding was also thought to be mediated exclusively by the p53 central core domain and requires stringent conformity to the consensus p53 response element. However global Chromatin immunoprecipitation (ChIP) and microarray analysis of p53 binding to genomic DNA reveal considerable divergence from the consensus p53 binding response element [29,149]. Instead, the majority of p53-binding events *in vivo* were found at non sequence-specific regions.

It is now understood that both the p53 central core domain and the C-terminal regulatory domain possess DNA binding capacities [150,151], with the former

providing primarily sequence-specific DNA binding and the latter recognizing DNA structure and topology [150,152], thereby enabling DNA binding within the vicinity of canonical p53 binding sites and providing a basis for sliding and searching for specific sequences.

Additionally, although promoter-binding of p53 is increased in response to genotoxic stress, quantitative ChIP assays reveal disproportionality to the fold induction of target gene mRNA; instead a portion of p53 is bound to target gene promoters in the absence of stress, and genotoxic stresses further enhances promoter binding [153]. These studies support a model in which p53 is bound to DNA but under constant repression.

1.5.3.2 Repression of p53 at promoters by HDM2/HDMX

Both HDM2 and HDMX interact directly with p53 and are recruited to p53 response elements in a p53-dependent manner. HDM2, HDMX and p53 form a protein complex on target gene promoters and repress p53 function by preventing access to the general transcriptional machinery [154,155].

The repression of p53 by HDM2 and HDMX is non-overlapping, because neither regulator can compensate for the embryonic lethality caused by the loss of the other [33]. The importance of HDM2 and HDMX in repressing p53 tumor suppressor function is further supported by the prevalence (around 1/3 of human

tumors) of HDM2 or HDMX gene amplification or overexpression in human tumors retaining wild-type p53 [33,156].

1.5.3.3 De-repression of p53 is required for transcription activation

While DNA binding alone may be sufficient for p53 to maintain basal level transcription of p53 negative regulators such as HDM2 and Pirh2 [106], in order to induce a stress response through transactivating distinct subsets of target genes p53 must first be released from HDM2/HDMX mediated repression. The necessity for disrupting HDM2 mediated repression is highlighted by the Nutlin-3A small molecule HDM2 antagonist, currently in phase I clinical trial, that is sufficient to restore p53 transcription activity in cells with a wild type *TP53* gene [157].

De-repression of p53 from HDM2 and HDMX can be achieved through several mechanisms. Post translational modifications on certain p53 residues facilitate the dissociation of p53 from HDM2 (discussed in detail in Chapter 1.5.4). In response to DNA damage, HDM2 and HDMX also undergo a number of post-translational modifications that either decrease protein stability or disrupt interaction with p53 [112-114,158-160]. One such example is the ATM-dependent phosphorylation of HDM2 and HDMX, which reduces their affinity for the USP7 deubiquitinase and therefore accelerates HDM2 and HDMX degradation [112]. Furthermore, in response to oncogenic activation, $p14^{ARF}$

directly interacts with the central region of HDM2, thereby antagonizing its activity toward p53 [110]. Several nucleolar or ribosomal proteins also interact directly with HDM2 and prevent its negative regulation of p53 [38,161,162].

1.5.4 Regulation of p53 transcription activity by post-translational modifications

p53 is subject to a diverse and complex array of post-translational modifications that influence its transcription activity at specific target gene promoters. The most commonly reported post-translational modifications affecting p53 transcription activity include phosphosphorylation of serines and/or threonines and acetylation, sumoylation, neddylation and methylation of lysine residues (Fig. 1). The presence of multiple p53 residues targeted by a single enzyme and multiple modification possibilities on C-terminal lysines allows for a multitude of combinations of post translational modifications that can be conferred on the p53 protein. These serve as a "histone-like" code to dictate correct and differentiated activation of certain sets of downstream targets involving different cellular responses.

1.5.4.1 Phosphorylation

Human p53 harbors an array of serine (S)/threonine (T) phosphorylation sites that span the entire protein but are concentrated in the N-terminal transactivation

domain and the C-terminal regulatory domain (Fig. 1). The majority of these sites are rapidly phosphorylated following cellular stress, although a few (e.g. T55 and S376) are constitutively phosphorylated in unstressed cells and dephosphorylated following stress [163,164]. p53 phosphorylation at the N terminus shows significant redundancy; a single site can be phosphorylated by multiple kinases and a single kinase can phosphorylate multiple sites [106].

1.5.4.1a Phorphorylation at Ser15/Ser20

The most extensively studied N-terminal p53 phosphorylation sites are S15 and S20 (S18 and S23 in mice). S15/S20 phosphorylation reduces p53 affinity for its primary negative regulator HDM2, and promotes the recruitment of transcriptional co-activators p300 and CBP on p53 target gene promoters [33]. Studies with mice containing single and double S to alanine (A) mutations reveal a certain level of redundancy in the physiological importance of these two phosphorylation sites. Although the individual mutations in gene knock-in experiments in mice only marginally change p53 stability and transactivation activity, the mice bearing p53 with both S15A and S20A mutations display a more severe phenotype including tissue-specific deficiency in pro-apoptotic capacity, mildly compromised replicative senescence and a latent development of a spectrum of tumors [165].

1.5.4.1b Phosphorylation at Ser46

S46 phosphorylation has recently attracted much attention. Phosphorylation of S46 is critical for p53-mediated induction of pro-apoptotic genes such as p53-regulated Apoptosis-Inducing Protein 1 (p53AIP1) but is not required for the induction of cell cycle arrest targets [166,167]. Indeed, the resistance of a human oral squamous cell carcinoma cell line HSC-3 to p53 is attributed to deficiency in S46 phosphorylation, and the introduction of the exogenous phospho-mimic p53S46D (aspartic acid) mutant enhanced transcription of the pro-apoptotic target *Noxa* and restored apoptosis in HSC-3 cells [168]. A study with knock-in mice expressing the human *TP53* gene with the S46A mutation partially supports the idea that S46 has a physiological role in differentially regulating cell cycle arrest and apoptosis. The mutant mice, compared to knock-in mice expressing the wild-type human *TP53* gene, displayed modestly reduced p53 transcription of some pro-apoptotic targets and compromised apoptosis but not cell cycle arrest, although the effects were tissue-specific [169].

1.5.4.1c Phosphorylation at Ser392

Phosphorylation of C-terminal S392 in response to Ultra-Violet (UV) light activates specific DNA binding through the stabilization of the p53 tetramer [34]. Knock-in mice with a S389A (human S392A) mutation displayed normal p53 stability but an increased predisposition to UV-induced skin cancer as well as altered expression of p53 target genes compared to wild-type mice [170-172],

supporting a physiological role for S392 phosphorylation in the tumor suppressive responses of p53 to UV. However, some studies report a correlation between S392 hyper-phosphorylation and poor prognosis, advanced tumor stage and tumor grade in p53-positive cancers [173,174]. How does a tumor-suppressive modification acquire tumor-promoting functions? Perhaps S392 phosphorylation enhances the tetramer formation of certain gain-of-function p53 mutants, turning these mutants into more potent oncoproteins. Further investigation is needed to determine whether S392 phosphorylation is common to both wild-type and mutant p53, and if so, how it might contribute to tumor progression.

1.5.4.2 Ubiquitin-like modifications

p53 is targeted by two ubiquitin-like proteins, Small Ubiquitin-like Modifier (SUMO) and Neural precursor cell Expressed Developmentally Down-regulated protein 8 (NEDD8), both of which are evolutionarily conserved in eukaryotes and resemble ubiquitin in both their three-dimensional structure and their mechanism of conjugation through lysines [175-177]. p53 is sumoylated at a single site K386 by members of the Protein Inhibitor of Activated Stat (PIAS) family and Topors [178,179]. Neddylation of p53 is mediated by HDM2 and F-box protein 11 (FBXO11): HDM2 catalyzes the neddylation of three C-terminal lysines (K370, K372 and K373) that are also targeted for ubiquitination [180], FBXO11 neddylates two lysines (K320 and K321) [181]. Unlike ubiquitination,

neddylation and sumoylation have not been demonstrated to affect p53 stability or localization. Neddylation inhibits p53 transcriptional activation activity [180,181], whereas the functional consequences of K386 sumoylation is interesting, albeit not well-defined; some reports have linked it to increased p53 transcriptional activity and premature senescence [178,182-184].

It is noteworthy, that the low abundance of SUMO- or NEDD-8 modified p53 *in vivo*, normally less than 5% of total cellular p53, poses a challenge for defining the cellular roles of these modifications. Reconstituted systems allow robust testing of the roles of these ubiquitin-like modifications *in vitro*, but are unlikely to recapitulate the physiological conditions in which these modifications occur. It remains to be determined under what circumstances sumoylation and neddlyation might affect p53 function.

1.5.4.3 Methylation

The large number of lysine and arginine residues in p53 presents the potential for regulation by methylation (Figure 1). Arginine methylation has only been shown for one methyltransferase, Protein Arginine N-Methyl Transferase 5 (PRMT5) [185,186], which targets R333, R335 and R337 in the tetramerization domain, and methylation of these residues differentially affect the target gene specificity of p53 [186]. p53 lysine methylation is better understood: p53 is monomethylated by three different Lysine Methyl Transferases (KMTs) and

dimethylated by at least two KMTs [187]. The functional consequences of p53 lysine methylation can be either activating or repressive, depending on the location of the modification and the number of methyl groups attached.

Monomethylation at K372 is mediated by SET7/9 (also known as KMT7) and this modification promotes the transactivation of target genes [188]. SET8 (also known as KMT5A)-mediated K382 monomethylation and SMYD2 (also known as KMT3C)-mediated K370 monomethylation repress p53 transcriptional activity [189,190]. G9A (also known as KMT1C) and G9A-like Protein (GLP, also known as KMT1D) dimethylate p53 at K373, thereby negatively regulating p53-mediated apoptosis [191]. Interestingly, however, conjugation of a second methyl group to K370 (K370me2), by a currently unknown enzyme, leads to a distinct functional consequence from monomethylation. K370me2 increases in response to DNA damage and promotes p53 function by facilitating the association of p53 with the coactivator p53 Binding Protein 1 (53BP1) [192]. Lysine Specific Demethylase 1 (LSD1, also known as KDM1) preferentially removes this positive-acting second methyl group thereby repressing p53 function by inhibiting the association of p53 with 53BP1 [192]. These findings suggest that p53 methylation and demethylation dynamically regulate p53 function, at least in part by allowing or disallowing p53 binding to coactivators.

Interestingly, there appears to be crosstalk between p53 methylation at different sites and between p53 methylation and acetylation. Activating methylation of K372 inhibits the repressive methylation of K370 by preventing SMYD2 binding to p53 [189]. Moreover, the repressive methylation of K382 normally prevents acetylation at this same site by CBP/p300 [190]. Upon DNA damage, the level of methylation at K382 decreases, reversing its inhibitory effect and allowing CBP/p300 acetylation of K382 and thereby promoting p53 activity. Together, the interplay between p53 methylation sites as well as between p53 methylation and acetylation provide mechanisms for triggering a rapid increase in p53 transcriptional activity in response to stress.

The presence of negatively acting lysine methylation sites and KMTs that normally maintain p53 in an inactive state suggests the possibility that abnormally high levels of KMTs could be oncogenic. Indeed, the SET domain containing methyltransferase G9A is upregulated in many cancer cell types and its homolog GLP is also overexpressed in brain tumors and multiple myeloma [191].

1.5.4.4 Acetylation

The acetylation of p53 is a powerful mechanism for activating function. The significance of p53 acetylation is three-fold: (i) it promotes p53 stabilization by excluding ubiquitination on the same site; (ii) it inhibits the formation of

HDM2/HDMX repressive complexes on target gene promoters; and (iii) it recruits cofactors for the promoter specific activation of p53 transcriptional activity.

Ten acetylation sites have been identified for p53, and the Histone Acetyl Transferases (HATs) responsible for these modifications include the structurally related p300 (also known as K(lysine) acetyltransferase 3B (KAT3B)) and CREB-Binding Protein (CBP, also known as KAT3A), P300/CBP-Associated Factor (PCAF, also known as KAT2B) and the MYST (named for members MOZ, Ybf2/Sas3, Sas2 and Tip60) family HATs, Tat-Interactive Protein of 60 kDa (TIP60, also known as KAT5) and human Males absent On the First (hMOF, also known as MYST1/KAT8) [25,193-195] (Fig. 1).

1.5.4.4a Acetylation at the C-terminus

Six lysine residues (K370, K372, K373, K381, K382 and K386) in the C-terminal regulatory domain are acetylated by CBP/p300 and ubiquitinated by HDM2 [193] (Fig. 1). Acetylation in tissue culture systems activates sequence-specific binding of p53 to DNA and its transcriptional activation activity and enhances the stability of p53, owing to the mutual exclusion of acetylation and ubiquitination. Nevertheless, despite some cell type-specific differences in transcriptional profiles, mice expressing C-terminal acetylation-deficient p53 ($p53^{6KR}$ and $p53^{7KR}$ knock-in mice) generally exhibited no major difference in cell cycle control, apoptosis or tumor suppression [196,197], which is in line with the

fact that mutation in the p53 C-terminal regulatory domain is rarely found in human cancers (UMD_TP53 Mutation database http://p53.free.fr/).

1.5.4.4b Acetylation at Lys320

K320 in the tetramerization domain is acetylated by PCAF [198]. It has been reported that the competition between the mutually exclusive ubiquitination and acetylation of K320 tips the cell fate balance. The atypical E3 ubiquitin ligase E4F1 mediates non-degraded K48-linked oligo-ubiquitination of p53 on K320, and competes with PCAF mediated acetylation [199]. High levels of K320 ubiquitination resulting from E4F1 overexpression specifically favors cell survival by promoting p53-mediated induction of p21 [199]. This is supported by studies using K317R (equivalent to human K320R) knock-in mice, showing increased expression of pro-apoptotic target genes and enhanced p53-dependent apoptosis upon irradiation [200], suggesting apoptotic repression by K320 acetylation.

1.5.4.4c Acetylation in the DNA binding domain

Two additional acetylation sites, K120 (K117 in mice, acetylation mediated by TIP60/hMOF) [194,201] and K164 (K161 and K162 in mice, acetylation by CBP/p300) [155] were discovered in the DNA binding domain. Importantly, both K120 and K164 are recurrently mutated in cancer (UMD_TP53 Mutation

database http://p53.free.fr/), implying that these two modifications might have profound and nonredundant effects on p53 function.

K120 acetylation is indispensable for the activation of target genes involved in apoptosis but not cell cycle arrest [194,201], suggesting a means for controlling promoter specificity and hence cell fate. Indeed, in $p53^{K117R}$ knock-in mice p53-dependent cell cycle arrest and senescence remain intact but apoptotic induction following ionizing radiation is completely abrogated [98], confirming the indispensability of K120 acetylation to p53-mediated apoptosis. Additionally, K120 acetylation might be required for p53 to effectively displace the proapoptotic protein BCL2-Antagonist/Killer 1 (BAK) from the oncoprotein Myeloid Cell Leukemia sequence 1 (MCL-1) at the mitochondria [202]. Therefore, it is probable that K120 acetylation by TIP60 contributes to both transcription-dependent and transcription-independent apoptotic functions of p53.

In cell culture based assays using human p53, individual K to R mutation can be compensated for by acetylation at other sites; however the collective mutation of eight acetylation sites ($p53^{8KR}$: mutation at K120, K164, and six CBP/p300-targeted C-terminal sites) completely abolishes p53-mediated cell cycle arrest and apoptosis [155], demonstrating that acetylation is indispensible for the canonical p53 functions. Mechanistically, acetylation allows p53 to evade HDM2 and

HDMX repression by blocking recruitment of HDM2 and HDMX to target gene promoters [155].

In mice, however, the collective loss of acetylation at K117 (human K120) and K161/K162 (human K164) seems sufficient to recapitulate the phenotypes seen with human $p53^{8KR}$. $p53^{3KR}$ knock-in mice are completely deficient in eliciting growth arrest, apoptosis, or senescence *in vivo* [98], confirming the physiological importance of acetylation in the transcription activation of canonical p53 targets.

1.5.4.4d Deacetylation by HDACs and SIRT1

Equilibrium in the acetylation of p53 is maintained by the Histone Deacetylases (HDACs), HDAC1 and Sirtuin 1 (SIRT1) [203,204]. SIRT1 preferentially deactylates p53 at K382 and has a profound negative impact on the capacity of p53 to induce the expression of target genes involved in apoptosis, such as *PUMA* and *BAX*. Thymocytes of Sirt1-deficient mice exhibit p53 hyperacetylation and increased radiation-induced apoptosis compared to wild-type thymocytes [205]. *SIRT1* is negatively regulated at the transcriptional level by Hypermethylated In Cancer 1 (HIC1) and at the translational level by the microRNA (miR)-34a [206,207], both of which are targets of p53 [208-212]. SIRT1 expression is elevated in leukemia [213], prostate cancer [214] and skin cancer [215], and it is negatively regulated by Deleted in Breast Cancer 1 (DBC1)

[216,217], supporting a role for SIRT1 in tumorigenesis. However, the suppression of intestinal tumorigenesis and colon cancer growth in a β-catenin-driven mouse model of colon cancer by ectopic induction of Sirt1 [218] suggests that it also has tumor-suppressive properties.

The evidence that SIRT1 harbors both tumor-promoting and tumor-suppressing functions generates interest in developing SIRT1-targeted drug therapies for cancer treatment [219]. The most promising SIRT1 inhibitors discovered to date are tenovin-1 and its more water-soluble derivative, tenovin-6 [220]. At low micromolar concentrations, tenovins potently inhibit the deacetylase activities of SIRT1 and SIRT2, significantly increase the level of p53 K382 acetylation in tissue culture and decrease tumor growth in xenograft mouse tumor models. Studies on activators of SIRT1 focus on resveratrol, which is abundant in grapes. Although dietary intake of resveratrol delays aging in mice [221], more studies are needed to assure that resveratrol activation of Sirt1 does not impose cancer susceptibility.

1.5.4.5 Concluding remarks

Although biochemical and cell culture based studies have highlighted the crucial role of a number of post-translational modifications in the activation of p53 transcription activity, the relatively mild and tissue/cell type-specific phenotypes of many knock-in mice with a single point mutation that abolishes a

certain modification suggest functional redundancy, perhaps important for the "fail-proof" regulation of p53 considering its central role in tumor suppression. Although each site/modification might only fine-tune p53 function, the numerous possible combinations of different modifications could dictate p53 activity in a promoter-specific manner, allowing p53 to exert a spectrum of functions.

The striking phenotype of the $p53^{3KR}$ mice, however, undeniably underscores the absolute requirement for p53 acetylation in activating the transcription of canonical targets involved in the classic growth arrest, apoptosis and senescence response pathways.

1.6 Summary

Although accumulating evidence supports the indispensability of acetylation in the activation of p53 function and indicates cell fate modulation, the underlying mechanisms are not completely understood. The experiments in this study were designed to identify novel regulators of p53 acetylation and to study the mechanisms modulating p53-mediated cell fate decision.

In this study we identify p90 and UHRF1 as two novel members of the p53 regulatory network. Although both function upstream of the TIP60-p53 interplay,

they act through distinct and opposing mechanisms to dynamically modulate TIP60-mediated effects on p53 *in vivo*.

CHAPTER 2

DIFFERENTIAL EFFECTS ON p53-MEDIATED CELL CYCLE ARREST VS. APOPTOSIS BY p90

2.1 Introduction

p53 was the first nonhistone protein known to be regulated by acetylation and deacetylation [25,203]. There is accumulating evidence indicating that acetylation of p53 plays a major role in activating p53 function during stress responses [222,223]. Following early findings of C terminus p53 acetylation [25], the Gu team and others recently showed that p53 is also acetylated by TIP60 (also known as KAT5)/MOF (human ortholog of males absent on the first) at residue Lys120 (K120) within the DNA-binding domain [194,201,224]. K120 acetylation is crucial for p53-mediated apoptosis but has no obvious effect on p21 expression, an essential target of p53-mediated growth arrest [98]. Notably, although TIP60 is required for K120 acetylation of p53 *in vivo*, the levels of K120 acetylation are dynamically regulated *in vivo* and the interaction between p53 and TIP60 is not very stable, indicating that additional regulators may play a role in controlling K120 acetylation and subsequent p53-mediated apoptotic response [225-227].

Through biochemical purification, we identified p90 as a unique regulator for p53. p90, also called CCDC8 (coiled-coil domain containing 8), which was previously found down-regulated in human cancer cells [228,229], interacts with p53 both *in vitro* and *in vivo*. Knockdown of p90 has no obvious effect on p53-mediated activation of p21 but specifically abrogates its effect on p53 upregulated modulator of apoptosis, also known as Bbc3 (PUMA) activation. Moreover, p90

also interacts with TIP60 and promotes TIP60-dependent Lys120 acetylation of p53, therefore enhancing the apoptotic response of p53. These data reveal p90 as an upstream regulator of the TIP60-p53 interaction and demonstrate that p90 is specifically required for p53-mediated apoptosis upon DNA damage.

2.2 Results

2.2.1 Identification of p90 as a unique component of p53-associated complexes

To further elucidate the mechanisms of p53-mediated promoter-specific activation *in vivo*, p53-associated protein complexes were isolated from human cells. Attempts to purify p53-containing protein complexes were hindered in the past because cells cannot tolerate expressing even low levels of wild-type p53. Interestingly, recent studies by the Gu team indicate that $p53^{8KR}$, in which all eight p53 acetylation sites are mutated to arginine, is inactive in inducing cell cycle arrest or apoptosis [155]. Moreover, $p53^{8KR}$ retains the capacity to bind target gene promoters as well as to activate the p53-HDM2 feedback loop, suggesting that $p53^{8KR}$, unlike the hot spot tumor mutant $p53^{H175R}$, may retain a similar conformation as wild-type p53 in human cells. Therefore we have utilized an H1299 p53-null lung carcinoma cell line that stably expresses a double tagged human $p53^{8KR}$ mutant protein with N-terminal FLAG and C-terminal HA epitopes (FLAG-$p53^{8KR}$-HA) (Fig. 3A).

To ensure physiological interactions, H1299 derivatives were selected such that the expression level of the ectopic $p53^{8KR}$ protein is not much higher than endogenous p53 in HCT116 colon cancer cells upon DNA damage treatment. As

expected, HDM2 is activated in the $p53^{8KR}$ stable line to a similar level compared to that induced by DNA damage in HCT116 cells. Consistent with previous findings, pro-apoptotic and growth arrest targets such as PUMA and p21 are not activated in the $p53^{8KR}$ stable line (Fig. 3B).

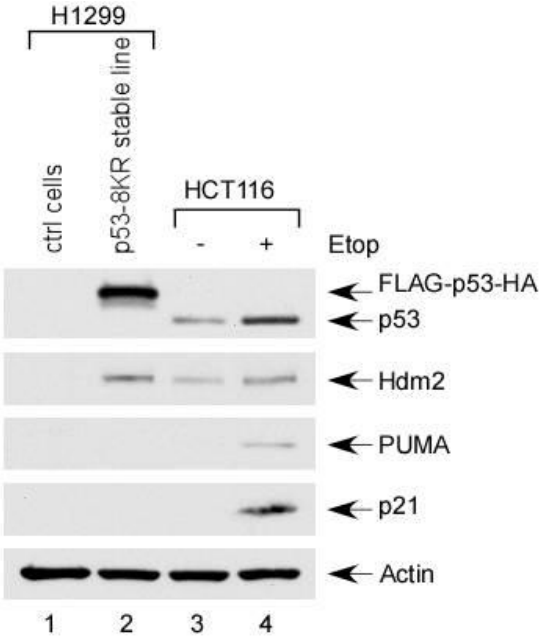
Figure 3. Construction of p53^{8KR} stable line in H1299

(A) Schematic representation of the p53^{8KR} protein used for protein complex purification. Mutations of acetylation sites are indicated. TAD, transcription activation domain; PRD, proline rich domain; DBD, DNA-binding domain; TD, tetramerization domain; CTD, C-terminal regulatory domain.

(B) Characterization of H1299 cells stably expressing p53^{8KR}. Total cell extracts from FLAG-p53^{8KR}HA/H1299 stable cell line and HCT116 cells with or without 8hr treatment with 20 μM etoposide were assayed by Western blot analysis using antibodies against p53, β-actin, Mdm2, PUMA, and p21.

A

B



To isolate $p53^{8KR}$-containing complexes, cell extracts from the stable line were subjected to a two-step affinity chromatography previously described [126]. The tandem affinity-purified p53-associated proteins were analyzed by liquid chromatography (LC) MS/MS. As expected, known p53 binding proteins such as HDM2, tumor protein 53 binding protein 1 (p53BP1), USP7, and the CREB binding protein (CBP) were identified as specific components of the p53 complex (Fig. 4A). In addition, MS analysis of a protein band p90 (with the apparent size at approximately 90 kDa molecular mass) revealed six peptide sequences matched with a signal cDNA sequence in the database, which is also named CCDC8 (Fig. 4B). Because none of the peptide sequences of p90 were identified from the control complexes purified in parental H1299 cells, p90 is likely a unique binding partner of p53.

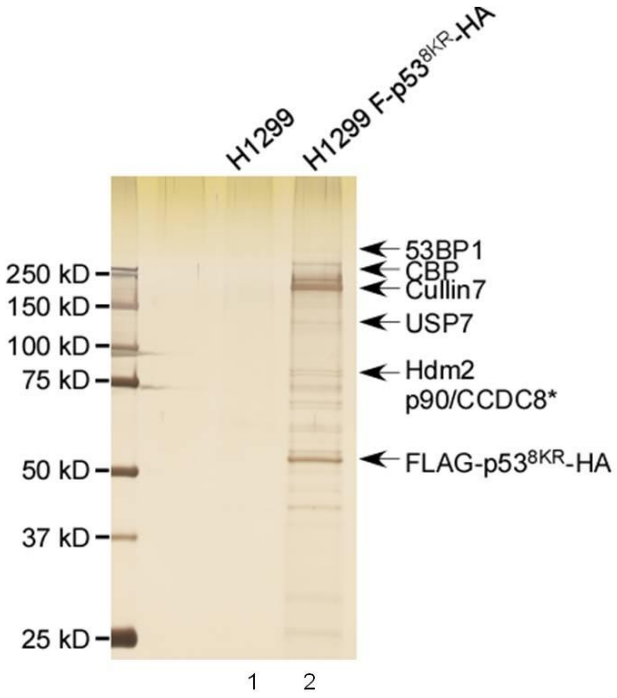
The cDNA of p90/CCDC8 encodes a 538 amino acid protein possessing no known functional domains other than two small coiled-coil regions that are likely to mediate protein–protein interactions (Fig. 4B). Although p90/CCDC8 has been reported as a candidate tumor suppressor gene in renal cell carcinoma (RCC), the molecular function of this protein is unclear [230].

Figure 4. Identification of p90 as a component of a p53-containing protein complex

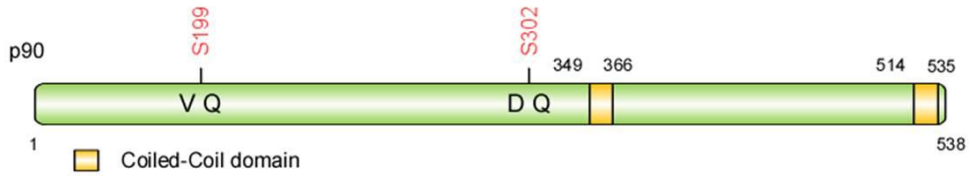
(A) Silver staining of purified $p53^{8KR}$ containing protein complex. Peptide sequences identified from the mass spectrometric analysis are presented.

(B) Schematic representation of the p90 protein. p90 contains two coiled-coil regions and two potential ATM/ATR phosphorylation sites.

A



B



peptides identified:
VQQMVTPPPR
LASTAVESAGVSSAPEGTSPGDR
AGAPAEEGAEAADNQREEAADNQR
APAVQGADNQR
AGAPGIQEAEVSAAQGTTGTAPGAR
AEAEQGEQEDQL

2.2.2 p90 is a *bona fide* p53 interacting protein

To investigate a role for p90 in regulating p53 function *in vivo*, the interaction between p90 and p53 was first tested. Thus, H1299 cells were transfected with expression vectors for FLAG-tagged p53 and HA-tagged p90, and Western blot analysis revealed that p90 is readily detected in p53-associated immunoprecipitates (Fig. 5A).

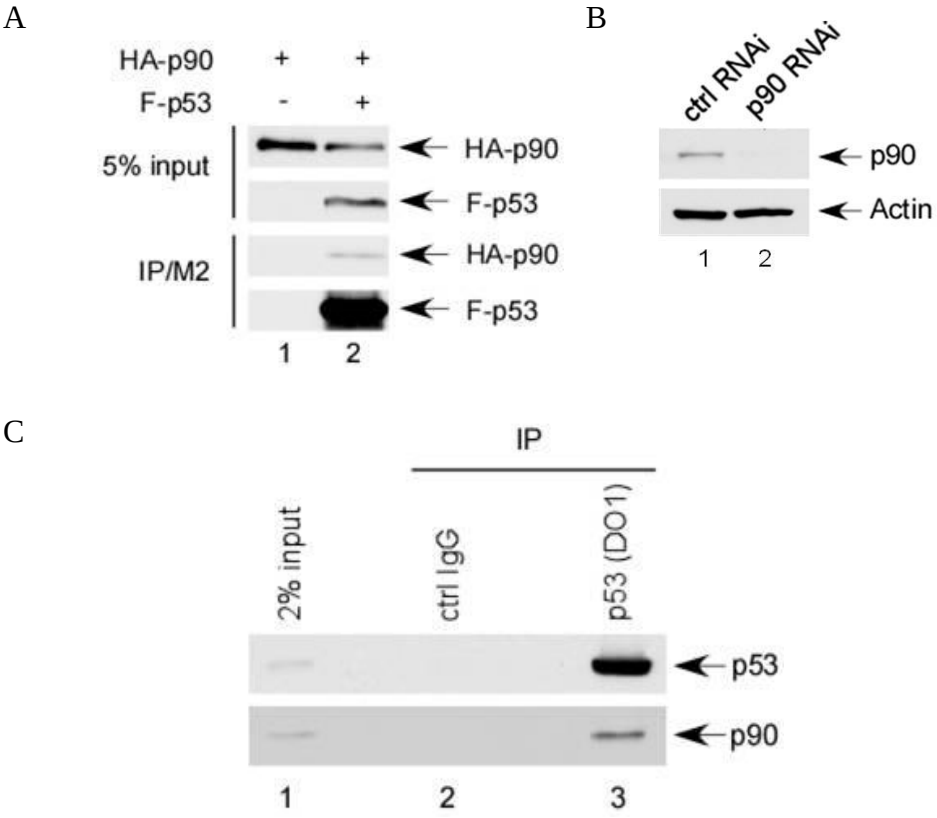
To further elucidate this interaction under physiological settings, a polyclonal antiserum was then raised against the full-length p90 protein. Upon Western blot analysis, the affinity-purified antibody specifically detected in human cells an approximately 90 kDa polypeptide, the level of which decreases significantly after treatment with p90-specific siRNA oligos (Fig. 5B). To investigate the interaction between endogenous p90 and p53 proteins, extracts from U2OS osteosarcoma cells were immunoprecipitated with α-p53 antibody or with the control IgG. As expected, the α-p53 antibody immunoprecipitated endogenous p53; more importantly, p90 is easily detected in the immunoprecipitates obtained with the α-p53 antibody but not the control IgG (Fig. 5C, lanes 2 and 3), confirming that p90 and p53 interact endogenously.

Figure 5. p90 interacts with p53 *in vivo*

(A) p90 coimmunoprecipitates with p53 in an overexpression system. H1299 cells were transiently transfected with plasmid DNA expressing HA-p90 or/and FLAG-p53. The cell extracts and the M2 immunoprecipitates (IP) were analyzed by Western blot analysis using α-HA and α-p53 antibodies.

(B) Purified p90 antisera specifically recognizes p90 protein. U2OS cells were transiently transfected with either control siRNA or p90-specific siRNA. Whole cell lysates were analyzed with α-90 antisera.

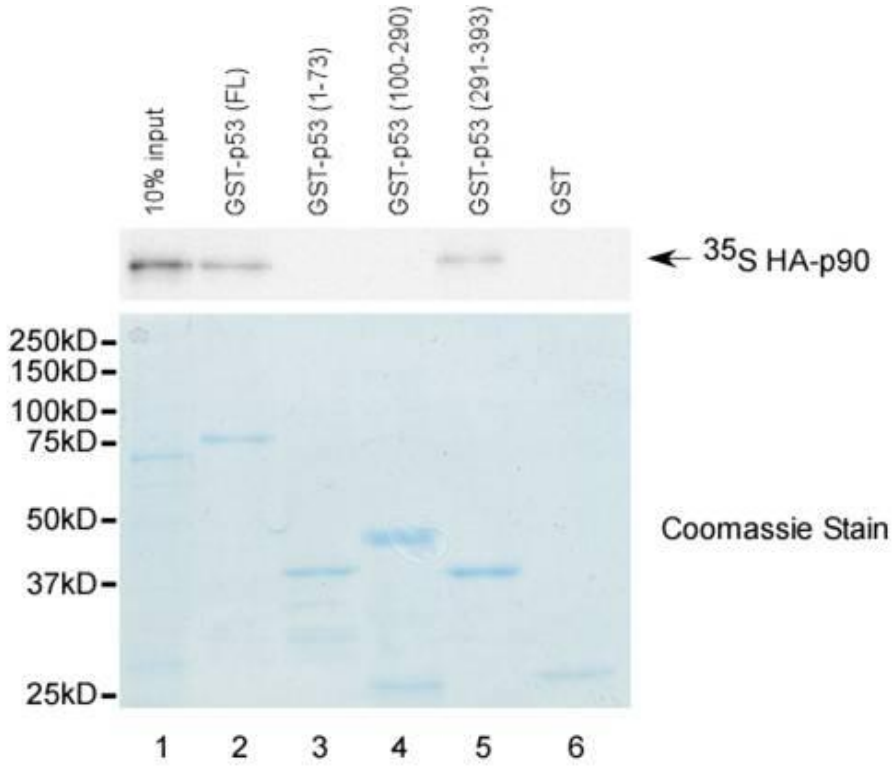
(C) p90 interacts with p53 endogenously. Total cells extracts from U2OS cells were immunoprecipitated with α-p53 (DO-1) antibody or a control mouse IgG . Extracts and immunoprecipitates were assayed by Western blot analysis using α-p90 and α-p53 antibodies.



An *in vitro* GST-pulldown assay was performed to further assess direct interaction. p53 can be divided into an N-terminal (NT) fragment containing the transactivation domain, a middle fragment (M) containing the DNA-binding domain, and a C-terminal (CT) fragment containing the tetramerization domain as well as the regulatory domain. Purified recombinant GST-tagged p53 full-length and fragment proteins were incubated with *in vitro* translated ^{35}S-methione-labeled HA-p90. Following immobilization with GST resins and recovery of captured complexes using reduced glutathione, the eluted complexes were resolved by SDS-PAGE and analyzed by autoradiography. ^{35}S- methionine-labeled HA-p90 strongly bound immobilized GST-tagged full length and CT fragment of p53 (Fig. 6, lanes 2 and 5), but not the NT and middle fragments of p53 or GST alone (Fig. 6, lanes 3, 4, and 6). These data demonstrate that p90 interacts with p53 *in vitro* through binding directly to the C-terminal portion of p53.

Figure 6. p90 interacts with the C-terminal domain of p53 *in vitro*

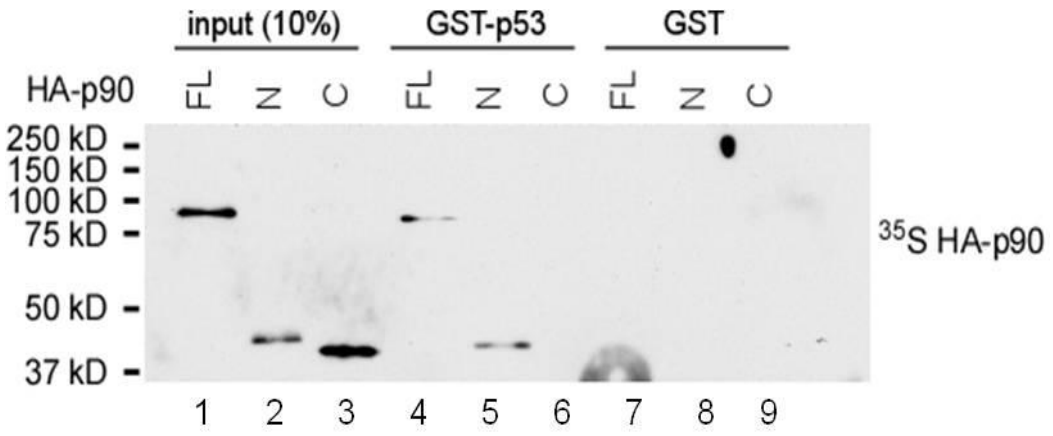
The bacteria purified full-length and fragmented GST-p53 fusion proteins or GST were used in the GST-pulldown assay with *in vitro* translated ^{35}S-methionine labeled HA-p90 protein. The complexes were captured with GST sepharose beads and eluted with reduced glutathione. The eluted complexes were resolved by SDS-PAGE and analyzed by autoradiography. The levels of the GST fusion proteins are shown in the bottom panel stained by Coomassie blue.



We further mapped the binding fragment on p90. The N-terminal and C-terminal fragments of p90 were subcloned into HA expression vector, and *in vitro* translated 35S-methionine labeled HA-p90 fragments were incubated with GST-p53 or GST. Following capture and elution, the complexes were resolved by SDS-PAGE and analyzed by autoradiography. The full length p90 protein and the N-terminal fragment, but not the C-terminal fragment of p90 bound GST-p53 (Fig. 7). These data demonstrate that the N-terminal fragment of p90 protein and the C-terminal portion of p53 interact directly *in vitro*.

Figure 7. p90 interacts with p53 through its N-terminal fragment

The N-terminal and the C-terminal fragments of p90 are subcloned into HA-tagged expression vector. The bacteria purified GST-p53 or GST were used in the GST-pulldown assay with *in vitro* translated ^{35}S-methionine labeled HA-p90 fragments, essentially as in Fig. 7. The eluted complexes were resolved by SDS-PAGE and analyzed by autoradiography.



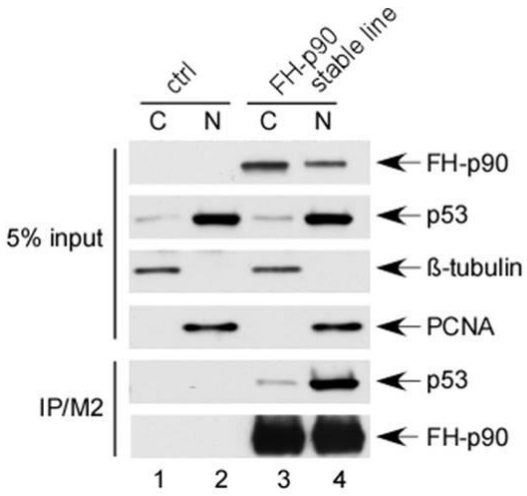
Because p90 was identified in the p53 complex purified from whole cell extracts, we assessed the cellular localization of the p90–p53 interaction. To this end, U2OS cell line stably expressing FLAG and HA double tagged p90 was established. Using parental U2OS cells as control, cell extracts from the p90 stable line were fractionated, and both nuclear and cytoplasmic fractions were immunoprecipitated with M2/FLAG agarose beads. Western blot analysis showed that p53, as expected, localizes mainly in the nuclear fraction, whereas p90 is present in both fractions but more so in the cytoplasmic fraction (Fig. 8A). Furthermore, in the M2 immunoprecipitate, p53 is primarily found in the nuclear fraction (Fig. 8A). The difference in p90/p53 ratio in the cytoplasmic and nuclear fractions as well as the p53 abundance in the nuclear M2 immunoprecipitate indicate that, although p90 is present in both the cytoplasm and the nucleus, it interacts with p53 predominantly in the nucleus. In agreement with the fractionation experiment, immunostaining of HA-p90 transfected U2OS cells revealed that p90 is localized to both the nucleus and the cytoplasm, regardless of etoposide challenge. p53 was stabilized following etoposide treatment and co-localized with p90 in the nucleus. (Fig. 8B). These data demonstrate that p90 interacts with p53 primarily in the nucleus and probably regulates p53 nuclear activities.

Figure 8. p90 interacts with p53 in the nucleus

(A) p90 localizes in both the cytoplasm and nucleus but interacts with p53 predominantly in the nucleus. Parental U2OS or FLAG-HA-p90/U2OS stable cell line were fractionated. Extracts and M2 immunoprecipitates from the cytoplasmic and nuclear fractions were assayed by Western blot analysis using α-HA and α-p53 antibodies. β-tubulin and proliferating cell nuclear antigen (PCNA) were used as cytoplasmic and nuclear protein markers, respectively.

(B) p90 localization remains unchanged regardless of DNA damage and p90 colocalizes with activated p53. U2OS transiently transfected with expression plasmid for HA-p90 was subjected to 8hr of etoposide treatment and immunostained with α-HA and α-p53 (DO-1) antibody.

A



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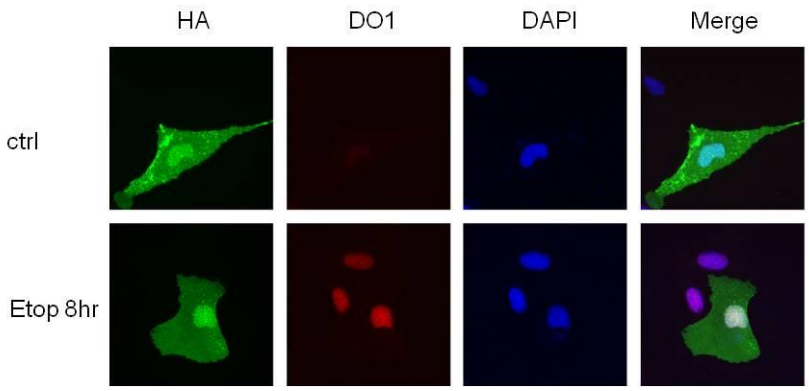


2.2.3 Inactivation of p90 attenuates p53-mediated activation of PUMA but not p21

To understand the physiological role of p90, we examined whether inactivation of endogenous p90 has any effect on the stability and functions of p53. To this end, U2OS cells were transfected with a p90-specific (p90-RNAi#1) siRNA oligo or a control (control-RNAi) siRNA oligo. As shown in Fig. 9A, lanes 1 and 2, the level of endogenous p90 polypeptides was severely re- duced after transfection with p90-RNAi. p53 protein level was unaffected by p90 ablation, suggesting that p90 does not regulate p53 stability. We then assessed the effect of p90 inactivation on the level of two important p53 downstream targets: the growth arrest target p21 and the apoptotic target PUMA. Surprisingly, p90 ablation displayed differential effects on the two different endogenous targets: the level of PUMA was significantly reduced, whereas p21 expression remained unchanged. To exclude off-target effects, cells were treated with three additional p90 siRNAs (p90-RNAi#2, p90-RNAi#3; p90-RNAi#4) that target different regions of the *p90* mRNA. Again, the levels of PUMA were decreased by p90 knockdown, although there was no significant change for the levels of p53 and p21 (Fig. 9A, lanes 3–5).

Because p53 is strongly activated upon DNA damage and regulates downstream targets, we wanted to assess whether p90 affects p53 and

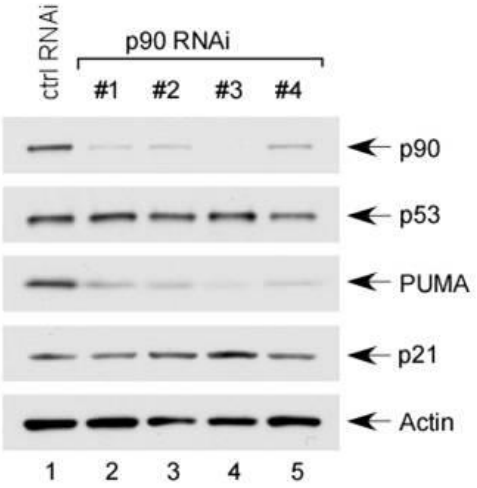
downstream target activation upon DNA damage. U2OS cells were transfected with p90-specific siRNA oligos followed by treatment with the DNA damage reagent etoposide. As expected, p53 levels increased drastically upon DNA damage (Fig. 9B, lane 2 vs. lane 1), and notably, RNAi-mediated ablation of p90 displays no effect on p53 accumulation following etoposide treatment (Fig. 9B, lane 4, 6, 8, and 10 vs. lane 2). p21 was strongly induced upon treatment, however, damage-induced PUMA expression was severely attenuated in the cells treated with p90-RNAi (Fig. 9B, lanes 4, 6, 8, 10 vs. lane 2).

Figure 9. p90 inactivation reduces basal PUMA level and differentially affects PUMA and p21 induction upon DNA damage

(A) p90 RNAi does not affect p53 stability or p21 basal level but reduces basal PUMA expression. U2OS cells were transiently transfected with either control siRNA or four different p90-specific siRNA oligos. Cell extracts were assayed by Western blot analysis using the indicated antibodies.

(B) p90 RNAi attenuates PUMA but not p21 activation upon DNA damage. U2OS cells transfected with the indicated siRNAs were treated with or without 20 μM etoposide for 8hr. Total cell extracts were assayed by Western blot analysis using the indicated antibodies.

A



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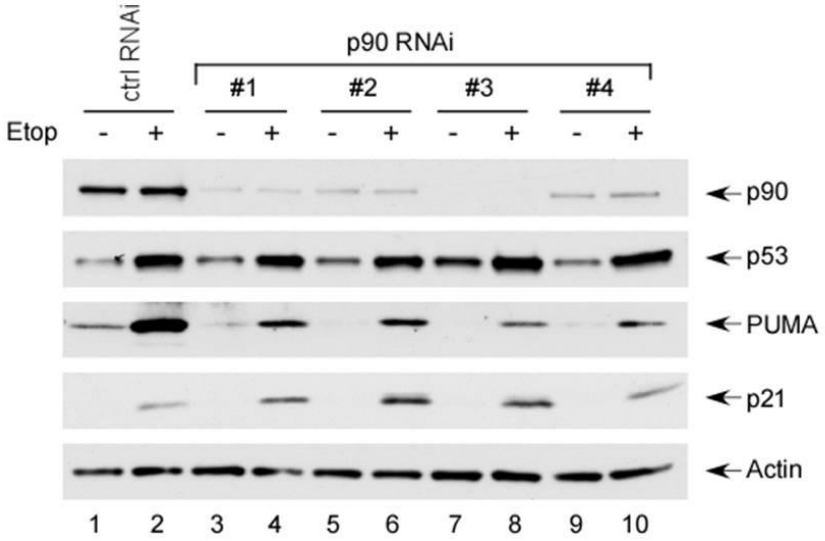
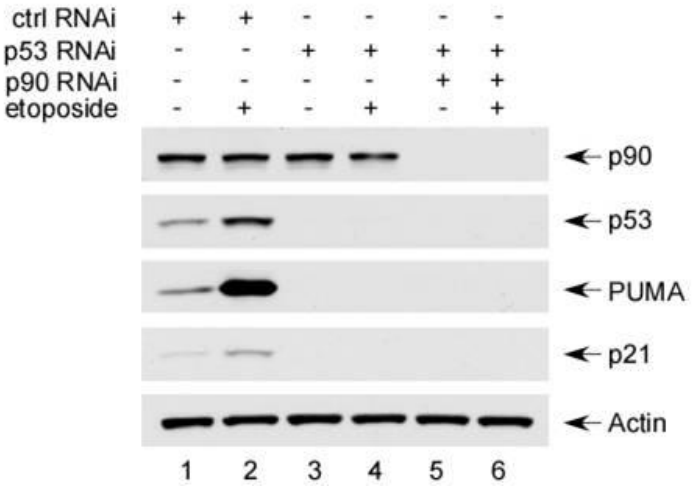


To validate that the differential effect of p90 on PUMA and p21 is p53 dependent, we inactivated both p53 and p90 in U2OS using RNAi prior to etoposide treatment. In cells transfected with the control siRNA, p53 accumulates and both PUMA and p21 are activated significantly upon treatment (Fig. 10, lanes 1 and 2). In a p53-deficient background, DNA damage fails to activate PUMA and p21 (lanes 3 and 4), and more importantly p90 ablation displayed no effect on PUMA and p21 in the absence of p53 (lanes 5 and 6). Taken together, these data demonstrate that p90 inactivation differentially affects PUMA and p21 induction in a p53-dependent manner.

Figure 10. Differential regulation of PUMA and p21 activation by p90 is dependent on p53

p53 alone, or both p53 and p90 were inactivated in U2OS cells using RNAi. Subsequently, cells were treated with or without 20 μM etoposide for 8 hr before extraction and Western blot analysis using the indicated antibodies.



2.2.4 p90 is required for p53-mediated apoptosis upon DNA damage

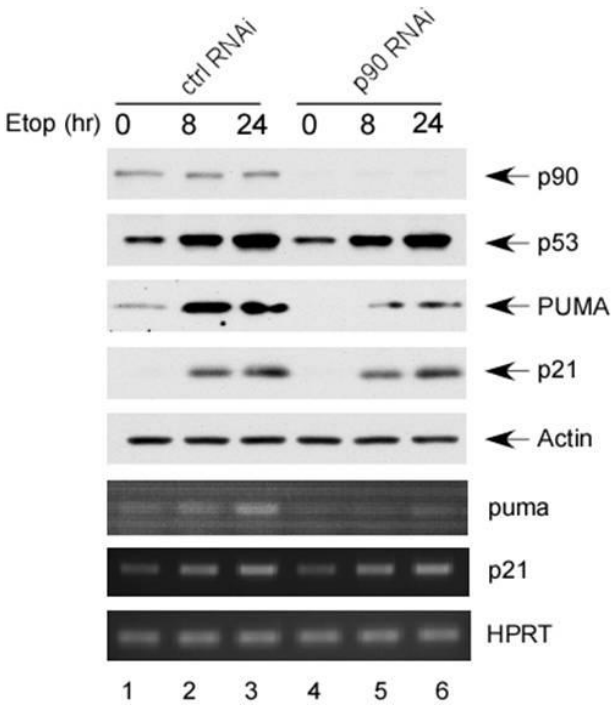
To further confirm the differential effects on p53-mediated activation of p21 versus PUMA, we collected the cells at different time points following treatment with etoposide. At all time points, p53 accumulation and p21 activation were unaffected by p90 ablation but PUMA induction was severely attenuated (Fig. 11A, lanes 5 and 6 vs. lanes 2 and 3).We further confirmed that p90 ablation affected p53-dependent activation of p21 and PUMA at the transcription level by examining the mRNA levels of these targets. Indeed, basal PUMA mRNA was reduced in samples treated with p90-RNAi, consistent with our finding that p90 ablation reduces basal PUMA protein level (Fig. 11A). PUMA activation was attenuated at the mRNA level following p90 ablation, whereas p21 mRNA level increased upon etoposide treatment at all time points and remained unaffected in samples treated with p90-RNAi.

To further confirm these differential effects of p90 in p53 responses, we repeated these experiments in the cells treated with another DNA damage reagent doxirubicin. Again, we observed the differential effects of p90 on p53-dependent p21 and PUMA activation upon doxirubicin treatment (Fig. 11B, lanes 6–8 vs. lanes 2–4). These results suggest that p90 is crucial for p53-dependent activation of PUMA, which is a very important mediator of p53-mediated apoptosis [56,57,231].

Figure 11. Inactivation of p90 attenuates p53-dependent PUMA activation in time point experiments

(A and B) p90 RNAi reduces PUMA but not p21 activation upon DNA damage. U2OS cells transiently transfected with either control siRNA or p90-specific siRNA were treated with 20 μM etoposide or 0.34 μM doxirubicin for the indicated time. Cell extracts were analyzed by Western blot analysis using the indicated antibodies. Total RNA were isolated from the same experiment. Followng reverse transcription, PCR was performed to detect the *PUMA* and *p21* transcripts.

A



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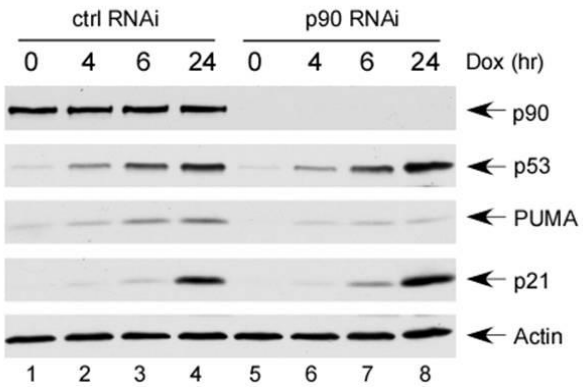


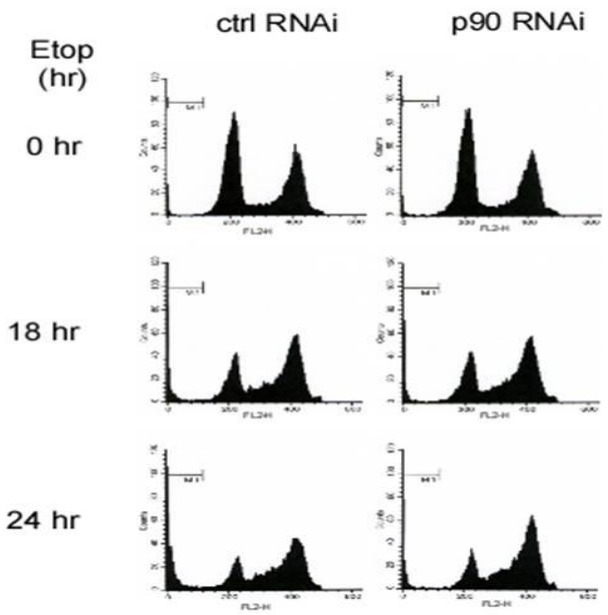
We therefore speculated whether the attenuation of PUMA activation by loss of p90 can be translated into a phenotypic effect on apoptosis. To this end, we transfected U2OS cells with either control siRNA or p90 siRNA prior to etoposide treatment. Cells were collected at different timepoints, stained with propidium iodide (PI), and analyzed by flow cytometry for apoptotic cells according to DNA content. As shown in Fig. 12A, basal level sub-G1 content is minimally affected by inactivation of p90. However, following 18hr or 24hr of etoposide treatment, an average of 18.67% or 26.84% of cells transfected with control siRNA were apoptotic, whereas only 8.04% or 11.49% of cells transfected with p90 siRNA were apoptotic (Fig. 12B). These data demonstrate that p90 is crucial for p53-mediated apoptosis.

Figure 12. Inactivation of p90 impairs p53-mediated apoptosis upon damage

(A) FACS analysis of p90-inactivated U2OS cells treated with etoposide. U2OS cellstransiently transfected with either control siRNA or p90 siRNA were treated with 20 μM etoposide for the indicated time. Cells were fixed in cold methanol, stained with propidium iodide and subjected to DNA content analysis by flow cytometry.

(B) p90 RNAi attenuates apoptosis. Percentages of apoptotic cells from (A) are presented. Values are an average of three independent experiments. Error bars, 1 standard deviation.

A



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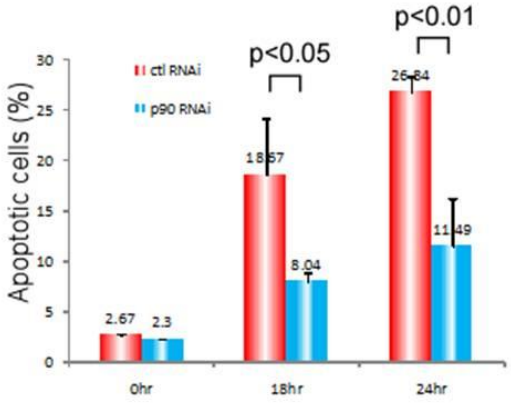


2.2.5 Mechanistic insights into p90-mediated effect on p53-dependent apoptotic responses

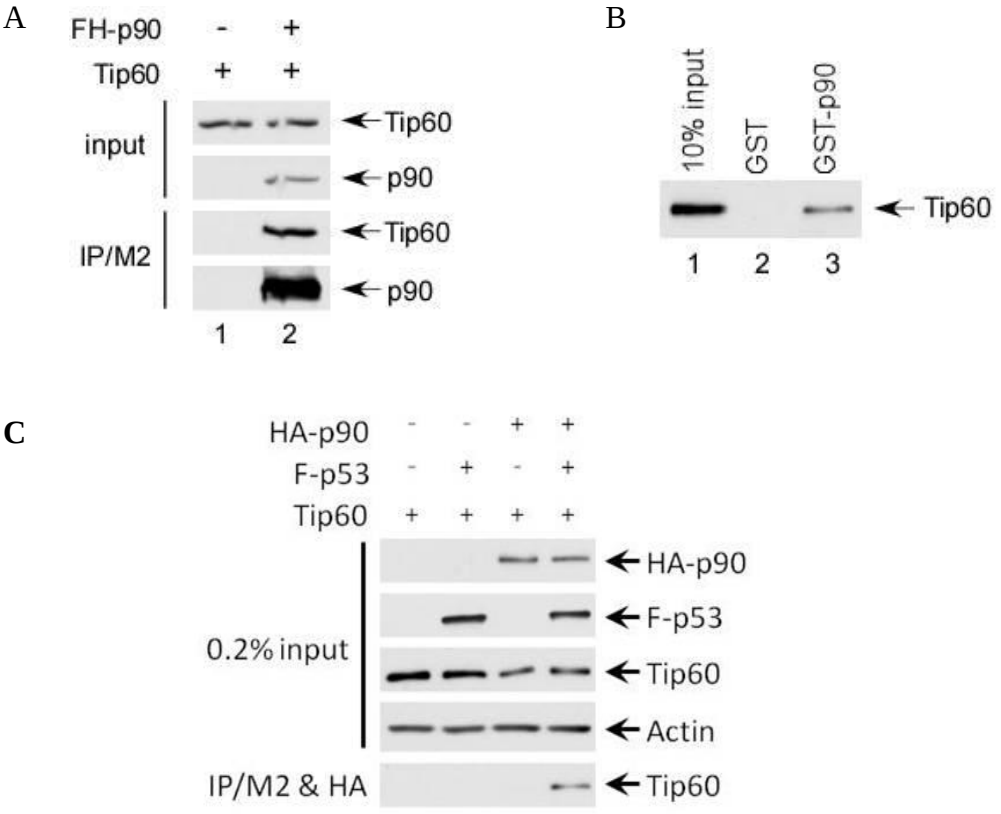
Previous studies demonstrated that p53 acetylation at Lys120 (p53 AcK120) by TIP60 is indispensable for apoptosis but not required for growth arrest [194,201], leading to our speculation that p90 may regulate p53-mediated PUMA activation through promoting TIP60-dependent acetylation of p53 at K120. To investigate the mechanism underlying the effect of p90 on the apoptotic target PUMA, we first assessed the interaction between p90 and TIP60. To this end, H1299 cells were transfected with expression vectors for TIP60 and FLAG/HA double-tagged p90. Western blot analysis revealed that TIP60 is readily detected in p90 associated immunoprecipitates (Fig. 13A). Using a GST-pulldown assay, we further tested the *in vitro* interaction of TIP60 and p90. As shown in Fig. 13B, TIP60 bound to immobilized GST-tagged p90 but not GST alone, demonstrating that p90 and TIP60 interacts directly. Further, p90, TIP60 and p53 form a ternary complex (Fig. 13C).

Figure 13. p90 interacts with TIP60 and forms a ternery complex with TIP60 and p53

(A) TIP60 coimmunoprecipitates with p90 in an overexpression system. H1299 cells were transiently transfected with the plasmid DNA expressing TIP60 or/and FLAG-HA-p90. Cell extracts and M2 immunoprecipitates were assayed by Western blot analysis using α-TIP60 and α-HA antibodies.

(B) p90 interacts with TIP60 *in vitro*. The GST-p90 fusion protein or GST alone was used in the GST-pulldown assay with in vitro translated ^{35}S-methione labeled FLAG-HA-TIP60 protein. The immobilized complexes were resolved by SDS-PAGE and analyzed by autoradiography.

(C) Total cell extracts from H1299 cells transfected with HA-p0, F-p53 and Tip60 are subjected to a 2-step immunoprecipitation using M2/Flag and HA agarose beads. Extracts and eluates are assayed by Western Blot using antibodies against HA, p53, and TIP60.



To investigate the role of p90 in p53 K120 acetylation by Tip60, we examined whether TIP60-mediated p53 acetylation is modulated by p90 status. As expected, in a cotransfection system, p53 was readily acetylated by TIP60. Notably, although p90 itself does not acetylate p53, p53 acetylation by TIP60 was significantly enhanced upon p90 expression (Fig 14A). These data demonstrate that p90 promotes the aceylation of p53.

In order to confirm the effect of p90 on Tip60-mediated p53 K120 acetylation under physiological settings, we inactivated p90 in U2OS cells via RNAi and assessed endogenous acetylation of p53 at K120. Because the steady-state levels of K120 acetylation are dynamically regulated by both acetylases and deacetylases, in order to exclude the potential effect on p53 acetylation levels by deacetylases, cells were treated with deacetylase inhibitors trichostatin A (for inhibiting histone deacetylase 1/histone deacetylase 2-mediated deacetylation of p53) and nicotinamide (for inhibiting Sirt1-mediated deacetylation of p53) prior to harvesting [201,225,227]. Cell extracts were immunoprecipitated with α-Ac-p53K120 or control IgG. As shown in Fig. 14B, p53 acetylation at K120 was easily detected in the cells with the deacetylase inhibitor treatment; however, the levels of p53 acetylation at K120 were significantly reduced upon p90 knockdown (Fig. 14B, lane 4 vs. lane 2). Taken together, these data indicate that

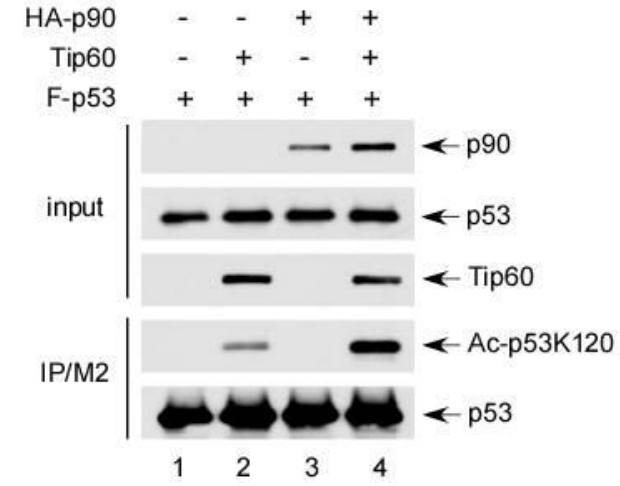
p90 is a critical cofactor for p53-mediated apoptosis through promoting K120 acetylation of p53.

Figure 14. p90 promotes TIP60-mediated p53 acetylaiton at K120

(A) p90 promotes p53 acetylation by TIP60 at K120. H1299 cells were transiently transfected with plasmid DNA expressing FLAG-p53, Tip60, and/or HA-p90. Cell extracts and M2 immunoprecipitates were assayed by Western blot analysis using antibodies against HA, p53, TIP60, and p53-AcK120.

(B) Inactivation of p90 significantly reduces p53 acetylation at K120. U2OS cells were transiently transfected with either control siRNA or p90 siRNA, and treated with 1 μM trichostatin A (TSA) and 5 mM nicotinamide (NTA) 6 h prior to harvesting. Cell extracts and immunoprecipitates obtained with α-Acp53K120 or control IgG were analyzed by Western blot analysis using antibodies against p90, p53, and β-actin.

A



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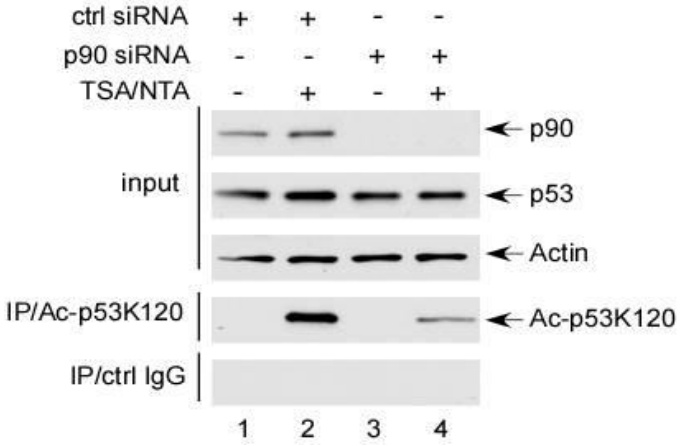


2.3 Discussion

Our findings reveal that p90 is a p53 interacting protein with differential effects on p53-mediated activation of target genes. Here, we have demonstrated that p90 is a *bona fide* p53 interacting protein and that this interaction primarily occurs in the nucleus. Inactivation of p90 attenuates apoptosis due to downregulation of p53-mediated PUMA activation upon DNA damage. However, p90 does not appear to affect growth-arrest targets such as p21. To dissect the molecular mechanism underlying this differential regulation, we found that p90 interacts with the TIP60 acetyltransferase and promotes TIP60-mediated acetylation of p53 at K120, a posttranslational modification that has previously been reported to modulate the decision between cell cycle arrest and apoptosis [194,201,224]. Thus, p90 likely serves as an upstream regulator of the p53-TIP60 interplay that is required for apoptotic signaling and allows for transcription induction of PUMA in cells at risk of DNA damage.

K120 is located within the p53 DNA-binding domain and is recurrently mutated in cancer (UMD_TP53 mutation database http://p53.free.fr/). Acetylation at K120 is indispensable for activation of pro-apoptotic targets but is not required for activation of growth-arrest targets [194,201]. Although the mechanism underlying this target specificity remains to be elucidated, it is possible that acetylation at K120 may impose specificity through altering the p53 quaternary

structure and thus endowing p53 binding to low-affinity response elements that are found on pro-apoptotic promoters [232,233].

We also noticed a small amount of cytoplasmic p53–p90 interaction. Cytoplasmic localization of p53 was originally thought to passively block transactivation in the nucleus. However increasing evidence suggests cytoplasmic p53 has important roles in regulating apoptosis and autophagy. Cytoplasmic p53 promotes apoptosis through increasing mitochondrial outer-membrane permeabilization and release of cytochrome c [66,69,70]. Basal levels of wild-type p53 in the cytoplasm also inhibits autophagy, although the exact mechanism remains to be understood [138]. It will be interesting to explore the possibilities of p90 regulating transcription-independent functions of p53 in the cytoplasm.

It is noteworthy that p90 itself is underexpressed in human tumors, including kidney cancer and myeloma, based on the cancer gene expression profile database from Oncomine Research [228,229]. In this regard, p90 has also been identified as a candidate tumor suppressor gene as hypermethylation and transcriptional silencing of the p90 promoter was found in 35% of primary RCC tumor samples [230]. It will be interesting to test whether p53-mediated apoptosis is abrogated in the human tumors lacking p90 expression and whether reactivation of silenced p90 promotes apoptosis thereby contributing to tumor suppression.

Finally, protein modifications of the components in the p53 pathway are well accepted as the key mechanisms for controlling p53 function during stress responses [146,177](2, 35). Interestingly, p90 contains two potential ataxia telangiectasia mutated/ataxia telangiectasia and Rad3 related (ATM/ATR) phosphorylation sites at Ser-199 and Ser-302 (Fig. 4B). Indeed, in a screen assay performed by the Elledge Group [234], a phosphorylated peptide derived from p90 was identified as an ATM/ATR substrate. Future investigations are required to validate if p90 undergoes damage-induced phosphorylation by ATM/ATR and dissect whether p90 phosphorylation modulates its interaction with p53 and Tip60 as well as p53-mediated apoptotic responses. It is possible that p90 is functionally regulated by ATM/ATR mediated phosphorylation during the DNA damage response to control the decision between cell cycle arrest and apoptosis mediated by p53.

2.4 Materials and methods

Plasmids

The full-length p90 cDNA was PCR-amplified from Human MGC Verified FL cDNA (Open Biosystems) and subcloned into pcDNA3.1/V5-His-Topo vector (Invitrogen), pCIN4-FLAG-HA, or pCIN4-HA expression vector [235]. To construct the GST-p90 plasmid, cDNA sequences corresponding to the full-length p90 were amplified by PCR from other expression vectors and subcloned into pGEX-2T (GE Healthcare) vector for expression in bacteria.

Cell culture

H1299 and U2OS cells were maintained in DMEM (Cellgro) and HCT116 cells in McCoy's 5A medium (Cellgro). All media were supplemented with 10% fetal bovine serum (Gibco), 100 I.U./mL penicillin and 100 ug/mL streptomycin (Cellgro). The stable cell lines were established by transfecting H1299 or U2OS cells with the plasmids pCIN4-FLAG-p538KR-HA and pCIN4-FLAG-HA-p90, respectively, followed by selection with 1 mg/mL or 0.5 mg/mL G418 (EMD Biosciences). Independent clones were selected and evaluated for expression by immunoblot. Transfections with plasmid DNA were performed using the calcium phosphate method and siRNA transfections by Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol.

Antibodies

The rabbit polyclonal antibody specific for p90 was generated by Covance. Rabbits were immunized with purified full-length GST-p90 protein. Antisera from the immunized rabbits were first depleted with a GST-affinity column, then affinity purified by use of a GST-p90 affinity column using the Aminolink Plus Immobilization kit (Thermo Scientific). Antibodies used for immunoprecipitation are p53 (DO-1) from Santa Cruz, α-FLAG M2 affinity gel from Sigma, and α-HA affinity gel from Roche. Antibodies used for Western blot analysis are p53 (DO-1), p53 (FL-393), β-tubulin (D-10), p21 (C-19 and SX118), and PCNA (PC10) from Santa Cruz, β-actin (AC-15), PUMA (NT), and FLAG M2 from Sigma, Mdm2 (Ab-5) from EMD Biosciences, HA (3F10) from Roche Applied Science. α-Acp53K120 antibody has been described [201]. α-TIP60 (CLHF) was a gift from Chiara Gorrini and Bruno Amati (European Institute of Oncology, Milan, Italy).

Protein complex purification from H1299/FLAG-p538KR-HA stable cell line

To purify p53 containing protein complexes, a large scale two-step affinity purification was performed using H1299/FLAG-p53^{8KR}-HA stable cell line. Cells were lysed for 2 hr in cold BC300 buffer [20 mM Tris, (pH 7.9), 300mM NaCl, 10% glycerol, 0.2 mM EDTA, 0.4% Triton X-100, and freshly supplemented

protease inhibitor], then for another 2 hr following addition of equal volume of BC0 buffer [20 mM Tris, (pH 7.9), 10% glycerol, 0.2 mM EDTA, and freshly supplemented protease inhibitor]. Following high-speed centrifugation (21,885 xg for 15 min), the cleared extract was then subjected to overnight immunoprecipitation with α-FLAG M2 affinity gel at 4 °C. After five washes with BC150 buffer [20 mM Tris, (pH 7.9), 150mM NaCl, 10% glycerol, 0.2 mM EDTA, and freshly supplemented protease inhibitor], the bound proteins were eluted twice using FLAG-peptide (Sigma) in BC150 buffer for 2 hr each at 4 °C. The eluted material was subjected to a second round of immunoprecipitation with α-HA affinity gel (Roche). After five washes with BC150 buffer, the bound proteins were eluted with 0.1% trifluoroacetic acid in 50% acetonitrile. The eluted complexes were then lyophilized using a freeze dryer (FreeZone 2.5Plus, Labconco), resuspended in SDS sample loading buffer and assayed by SDS-PAGE.

Western blot analysis and immunoprecipitation

For Western blot analysis, immunoprecipitation of ectopically expressed FLAG-tagged proteins, or from the U2OS FH-p90 stable line, cells were lysed in cold FLAG lysis buffer [50 mM Tris-HCl (pH 7.9), 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 10% glycerol, and freshly supplemented protease inhibitor cocktail]. For immunoprecipitation, extracts were

incubated with the α-FLAG M2 affinity gel (Sigma) at 4 °C overnight. After five washes with the lysis buffer, the bound proteins were eluted using FLAG-peptide (Sigma) in BC100 for 2 h at 4 °C. The eluted material was resolved by SDS-PAGE and immunoblotted with antibodies as indicated. To immunoprecipitate endogenous p53, cells were lysed in BC100 buffer and cell lysates were pre-cleared by incubating with 20 uL protein A/G agarose beads (Santa Cruz) for 2 hours with gentle rotation. The cleared supernatants were incubated with α-p53 (DO-1) antibody at 4 °C overnight before addition of 20ul of protein A/G agarose beads for 4 hours. After five washes with the lysis buffer, the immunoprecipitated materials were eluted with the SDS sample buffer with boiling, resolved by SDS-PAGE and detected with antibodies as indicated.

Preparation of cytoplasmic and nuclear fractions

Cytoplasmic extracts were prepared by resuspension of pelleted cells in hypotonic buffer [10 mM Tris-HCl (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, supplemented with fresh protease inhibitor] followed by Dounce homogenization (six strokes with Type A pestle) and subsequent low-speed pelleting of nuclei (600 xg for 10 min). The supernatant was removed for use as cytoplasmic extract. The pellet from the low-speed spin was washed once with hypotonic buffer containing 0.1% Nonidet P-40, and further extracted with BC200 [20 mM Tris (pH 7.9), 200 mM NaCl, 10% glycerol, 0.2 mM EDTA, 0.4% Triton X-100,

supplemented with fresh protease inhibitor]. The nuclear extract was then clarified by high-speed centrifugation (21,885 xg for 15 min). For subsequent immunoprecipitation, both fractions were adjusted to a final concentration of 150 mM NaCl and 0.2% Triton X-100.

GST-pulldown assay

GST and GST-tagged protein fragments were purified as described previously [236]. ^{35}S-methione labeled proteins were prepared by *in vitro* translation using the TNT Coupled Reticulocyte Lysate System (Promega). GST or GST-tagged fusion proteins were incubated with *in vitro* translated ^{35}S-methione-labeled proteins overnight at 4 °C in BC100 buffer containing 0.2% Triton X-100 and 0.2% BSA. GST resins (Novagen) were then added, and the solution was incubated at 4 °C for 3 h. After five washes, the bound proteins were eluted for 1.5 h at 4 °C in BC100 buffer containing 0.2% Triton X-100 and 20mM reduced glutathione (Sigma), and resolved by SDS-PAGE. The presence of ^{35}S-labeled protein was detected by autoradiography.

siRNA-mediated ablation of p90 and p53

Ablation of p90 was performed by transfection of U2OS cells with siRNA duplex oligonucleotides synthesized by Dharmacon:

p90-RNAi-1(5'-GGACUUGACAACUGACGAA-3');

p90-RNAi-2(5’-GGCAAGAAGGUGCGCAAAA-3’);

p90-RNAi-3(5’-CAGAUAAUCAGAGGGCGG-3’);

p90-RNAi-4(5’-ACACAAUGGGGUUGCGUCA-3’).

Ablation of p53 was performed by transfection of U2OS cells with siRNA duplex oligoset (On-Target-Plus Smartpool L00332900, Dharmacon). Control RNAi (On-Target-Plus siControl nontargeting pool D00181010, Dharmacon) was also used for transfection. RNAi transfections were performed two times with Lipofectamine 2000 at a final concentration of 100 pM according to the manufacturer’s protocol (Invitrogen). 72hr after the first transfection, cells were either harvested for Western blotting or subjected to drug treatment.

Immunofluorescent staining

Cells were washed with lukewarm phosphate buffered saline solution (PBS), and fixed with 4% paraformaldehyde in PBS at 37 °C for 30 min, rehydrated for 5 min in serum-free DMEM, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were incubated in blocking buffer (1% bovine serum albumin (BSA), 5% goat serum in PBS) for 45 min. Primary antibodies (as indicated) were added in blocking buffer for 1 hr at room temperature. After three washes with 1% BSA/PBS, Alexa Fluor conjugated secondary antibodies were added and incubated for 30 min at room temperature. Finally, cells were counterstained with DAPI to visualize the nuclei.

CHAPTER 3

NEGATIVE REGULATION OF THE TIP60-P53 INTERPLAY BY UHRF1

3.1 Introduction

TIP60 is a ubiquitously expressed and evolutionarily conserved founding member of the MYST family of lysine acetyltranferases. It was originally isolated in a yeast-2-hybrid screen as a protein interacting with and augmenting the function of the potent HIV gene transactivator HIV-1-Tat [237]. Tip60 possesses acetyltranferase activity, through acting in multiprotein complexes that are structurally and functionally conserved from yeast to human, with specificity for histones (core histones H2A, H3 and H4) [238-241] and non histone proteins [241-244].

Histone acetylation occurs at the N-terminal tail and on the surface of the nucleosome core. Acetylation removes the positive charge on histones, thereby decreasing the affinity of histones to the negatively charged phosphate groups of DNA. As a consequence, the local chromatin structure becomes more relaxed, often leading to greater levels of gene transcription. Apart from acetylating histones, TIP60 can also acetylate transcription factors and directly affect DNA binding, or recruit other coactivators/corepressors. TIP60 can positively or negatively regulate transcription activation depending on the specific transcription factor with which it is complexed. For example, TIP60 promotes the transcription activity of HIV-1-Tat [237] and nuclear hormone receptors such as the androgen receptor, the estrogen receptor and progesterone receptor [245,246], but promotes

transcription repression when complexed with Signal transducer and activator of transcription 3 (STAT3) [247] or TEL [248], which frequently translocates and fuses to Acute Myeloid Leukemia protein (AML) in pediatric leukemia.

In addition to its function in transcriptional regulation, TIP60 plays important roles in the DNA damage response pathway: activation of the ataxia telangiectasia mutated (ATM) DNA damage sensor is dependent on the acetyltransferase activity of TIP60, and TIP60 is recruited to sites of DNA lesions in Drosophila to facilitate DNA repair [249,250]. A key study of large-scale inhibitory RNA (RNAi) screening identified TIP60 to be essential for p53-dependent cell growth arrest, thereby suggesting TIP60 as a component of the p53 pathway [251]. This was soon confirmed by studies demonstrating TIP60 interaction with p53 and TIP60-mediated p53 acetylation at K120, which specifically favors expression of p53-dependent apoptotic targets [194,201]. Although TIP60 is a potent positive regulator of p53 activation, the dynamically regulated levels of TIP60-mediated K120 acetylation and the unstable interaction between TIP60 and p53 suggests additional players in regulating the TIP60-p53 interplay.

Recent studies have identified Tip60 to be a new member in the macromolecular epigenetic regulating protein complex that contains UHRF1 (Ubituitin-like containing PHD Ring Finger 1, also known as ICBP90 in humans and Np95 in mice), the maintenance DNA methyltransferase DNMT1, the de

novo DNMT3a/3b, the histone deacetylase 1 (HDAC1), ubiquitin specific protease 7 (USP7, also known as HAUSP), proliferating cell nuclear antigen (PCNA), and the euchromatic histone lysine N methyltransferase 2 (EHMT2, also called G9a) [252-255].

UHRF1 was originally isolated in a yeast-1-hybrid screen to enhance expression of topoisomerase IIα by binding to the CCAAT box of its promoter region [256,257]. UHRF1 is a key component and critical coordinator of the epigenetic regulating complex. This is made possible by the multiple protein modalities of UHRF1 (Fig. 15) that facilitate the coordination of other epigenetic regulators through linking DNA methylation and histone modifications. UHRF1 interacts with PCNA at the replication fork and, through its Set and Ring associated (SRA) domain that is distinctive to the UHRF family, recognizes hemimethylated DNA [258-260] and tethers DNMT1 to methylate the newly-synthesized DNA strand [261], thereby maintaining genomic DNA methylation patterns. In addition, UHRF1 also recruits G9a to methylate Histone H3K9. Trimethylated histone H3K9 is then read by UHRF1 through its tudor domain [262], and HDAC1 is recruited through the SRA domain [263]. HDAC1 in turn deacetylates histones, causing them to become positively charged and tightly bound to the negatively charged DNA, causing heterochromatin formation to perpetuate transcription repression of certain tumor suppressor genes.

The implication of UHRF1 involvement in tumorigenesis stems from studies reporting high UHRF1 expression level in actively proliferating tissues and low expression level in quiescent cells and highly-differentiated tissues [256,264]. Indeed, UHRF1 was found upregulated in numerous cancers, including breast cancer [263,265], pancreatic cancer [266], brain tumor [267], lung cancer [268,269], bladder and kidney cancer [270], cervical cancer [271], and colon cancer [263,265-267,269-272].

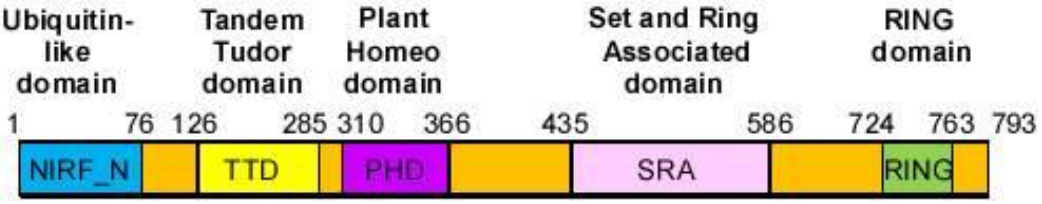
The carboxyl (C-) terminal RING domain of UHRF1 confers intrinsic E3 ligase activity towards histones and non-histone proteins [262,269,273,274]. It has been shown that UHRF1 is capable of mediating ubiquitination-dependent degradation of the promyelocytic leukemia protein (PML) [274], a tumor suppressor protein capable of promoting apoptosis, inhibiting cell proliferation, inducing senescence, and suppressing cell migration [275]. Further, a recent study reported that UHRF1 ubiquitinates and targets DNMT1 for proteasomal degradation through coordinating other DNMT1-associated proteins including TIP60 [276].

Although coexistence of TIP60 and UHRF1 in the same macro-molecular protein complex is indicated, direct interaction has not been reported [254,276]. Here we have identified UHRF1 as a direct interacting partner of TIP60 and a unique negative regulator of the TIP60-p53 interplay. UHRF1 expression induces

TIP60 ubiquitination, which does not trigger proteolysis but partially contributes to marked suppression of p53 K120 acetylation mediated by TIP60. Ablation of UHRF1 promotes K120 acetylation and p53-mediated apoptosis. Through its SRA and RING domains UHRF1 binds to TIP60 and severely inhibits TIP60-p53 interaction, thereby modulating transcription of K120 acetylation-dependent and -independent p53 targets PUMA and p21. These data reveal that UHRF1 negatively regulates TIP60 and modulates TIP60 function in the p53 response pathway both dependent and independent of K120 acetylation.

Figure 15. Schematic representation of UHRF1 domain structure

UHRF1 possesses multiple protein modalities. NIRF_N: ubiquitin-like domain; TTD: tandem tudor domain; PHD: plant homeo domain; SRA: set and ring associated domain; RING: really interesting new gene domain.



3.2 Results

3.2.1. UHRF1 interacts with TIP60 both *in vitro* and *in vivo*

Recent studies demonstrated correlation of UHRF1 overexpression with tumor growth and aggressiveness and poor prognosis in prostate cancer and colorectal cancer [277,278]. The oncogenic role of UHRF1 has long been implicated through epigenetic regulation, however recent indication of coexistence of UHRF1 in the same multi-protein complex with the TIP60 acetyltransferase [254,276] that modulates p53- dependent growth arrest and apoptosis leads to the attractive hypothesis that UHRF1 may be linked to the TIP60-p53 interplay and modulate the p53-dependent damage response pathway.

We first sought to confirm interaction between UHRF1 and TIP60 by performing coimmunoprecipitation experiments in H1299 p53 null lung carcinoma cells transfected with FLAG-Tip60 and HA-UHRF1 expression vectors. Cell extracts were subjected to immunoprecipitation with M2/FLAG antibody. Western blot analysis of M2 eluates revealed that TIP60 was effectively precipitated and that UHRF1 is readily detected in TIP60-associated immunoprecipitates (Fig. 16A). To further elucidate this interaction under physiological settings, we made use of a 3xFLAG-tagged TIP60 knock-in HCT116 colon carcinoma cell line. As expected, these cells express endogenous

3xFLAG-TIP60 protein that can be specifically recognized by α-FLAG antibody, and the level of UHRF1 protein expressed is comparable to that in control HCT116 cells (Fig. 16B, lanes 1 and 2). When we subjected extracts from TIP60 knock-in cells or control HCT116 cells to immunoprecipitation with M2/FLAG antibody, TIP60 was specifically immunoprecipitated from the knock-in cells; more importantly UHRF1 is easily detected in the immunoprecipitates obtained from the knock-in cells but not the control cells (Fig. 16B, lane 2 vs. lane 1), suggesting that UHRF1 interacts with TIP60 endogenously. Inversely, extracts from U2OS osteosarcoma cells were immunoprecipitated with the α-UHRF1 antibody or with the control IgG. As expected, endogenous UHRF1 of ~95 kD was specifically immunoprecipitated with the α-UHRF1 antibody; more importantly, TIP60 is readily detected in the immunoprecipitates obtained with the α-UHRF1 antibody but not the control IgG (Fig. 16C, lanes 2 and 3). Thus, reciprocal immunoprecipitation confirms that UHRF1 and TIP60 interact endogenously.

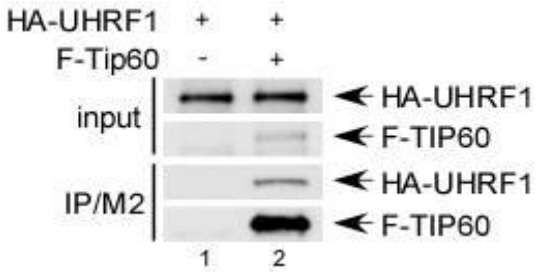
Figure 16. UHRF1 coimmunoprecipitates with TIP60 exogenously and endogenously

(A) UHRF1 coimmunoprecipitates with TIP60 in an overexpression system. Whole cell extracts or immunoprecipitates with M2/FLAG antibody from H1299 cells transiently transfected with plasmid DNA expressing HA-UHRF1 or/and FLAG Tip60 were subjected to Western blot with α-FLAG and α-HA antibodies.

(B) UHRF1 interacts with TIP60 endogenously in 3xFLAG-TIP60 knock-in cells. Whole cell extracts or M2/FLAG immunoprecipitates from control HCT116 cells or HCT116 3xFLAG-Tip60 knock-in cells were subjected to Western blot with α-UHRF1 and α-FLAG antibodies.

(C) TIP60 interacts with UHRF1 endogenously in U2OS cells. U2OS-derived nuclear extracts or immunoprecipitates with a control IgG or α-UHRF1 antibody were subjected to Western blot with α-TIP60 and α-UHRF1 antibodies.

A



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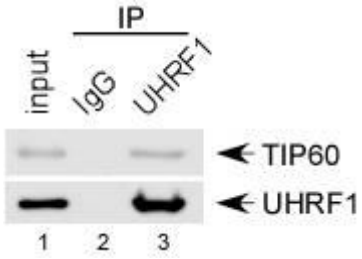

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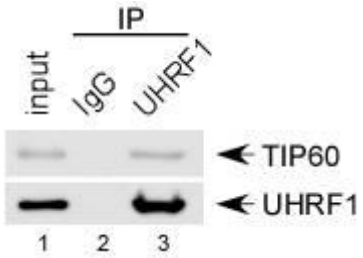
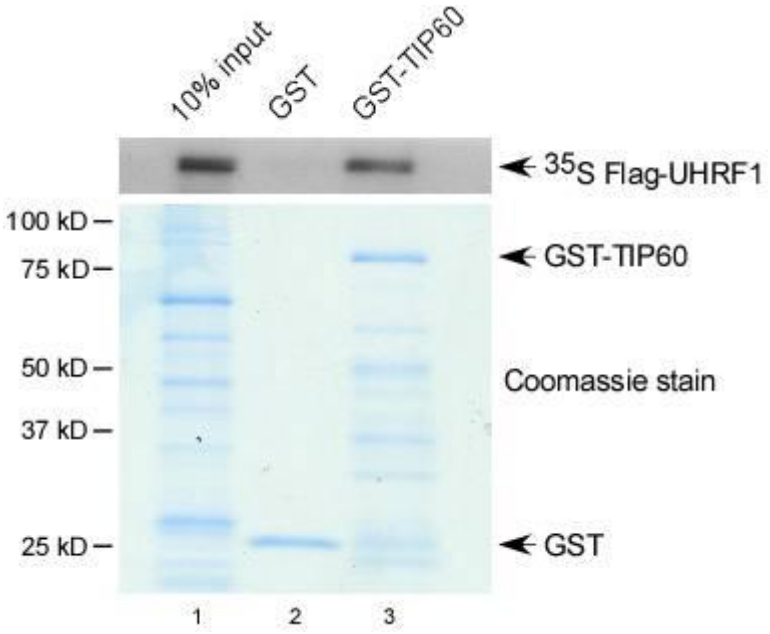


An *in vitro* GST-pulldown assay was performed to further assess direct interaction. Purified GST or GST-tagged TIP60 protein was incubated with *in vitro* translated ^{35}S-methione labeled FLAG-UHRF1 protein. Following capture with GST resins and recovery of immobilized complexes, the eluted complexes were resolved by SDS-PAGE and analyzed by autoradiography. ^{35}S-UHRF1 strongly bound immobilized GST-TIP60, but not GST alone (Fig. 17), demonstrating direct UHRF1-TIP60 binding *in vitro*. Taken together, these data confirm that UHRF1 is a *bone fide* interacting partner of TIP60.

Figure 17. UHRF1 interacts with TIP60 directly *in vitro*

In vitro translated ^{35}S-methione labelled 3xFLAG-UHRF1 protein was incubated with purified GST-TIP60 or GST alone. Complexes immobilized with GST resins and recovered using reduced glutathione were subjected to SDS-PAGE and analyzed by autoradiography. The levels of purified GST- TIP60 and GST are shown in the bottom panel stained by Coomassie blue.



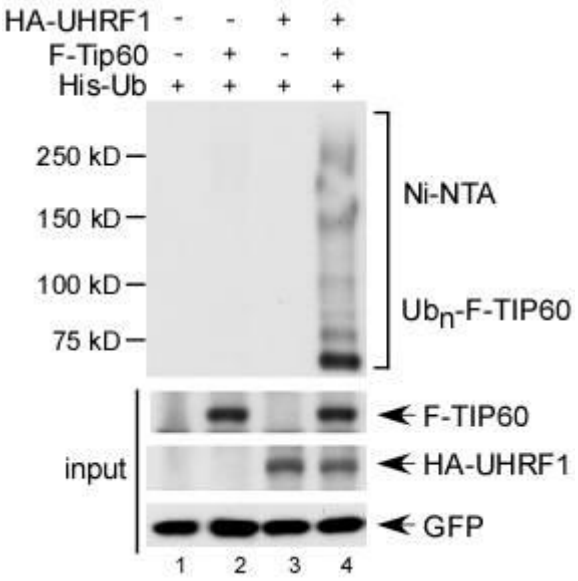
3.2.2 UHRF1 induces degradation-independent ubiquitination of TIP60

The C-terminal RING domain endows UHRF1 with intrinsic E3 ubiquitin ligase activity. Recent studies have identified PML (promyelocytic leukemia protein) and DNMT1 to be substrates for UHRF1-mediated ubiquitin-dependent proteolysis [274,276]. We therefore first tested the possibility of UHRF1-mediated Tip60 ubiquitination.

To test this hypothesis, a cell-based ubiquitination assay was performed where H1299 cells were transfected with expression vectors for FLAG-Tip60 and His-ubiquitin alone or in combination with HA-UHRF1. Ubiquitinated proteins were captured with nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and analyzed by Western blot. Immunoblotting with α-FLAG antibody revealed that coexpression of UHRF1 and TIP60 produced significant levels of ubiquitinated TIP60 (Fig. 18A, lane 4).

Figure 18. UHRF1 promotes ubiquitination of TIP60

UHRF1 induces ubiquitination of TIP60 *in vivo*. H1299 cells were cotransfected with expression vectors encoding FLAG-Tip60 or/and HA-UHRF1 in combination with His-ubiquitin. Whole cell extracts and Ni-NTA affinity-purified fractions were analyzed by Western blot with α-FLAG and α-HA antibodies. GFP was used as a control to confirm equal transfection.

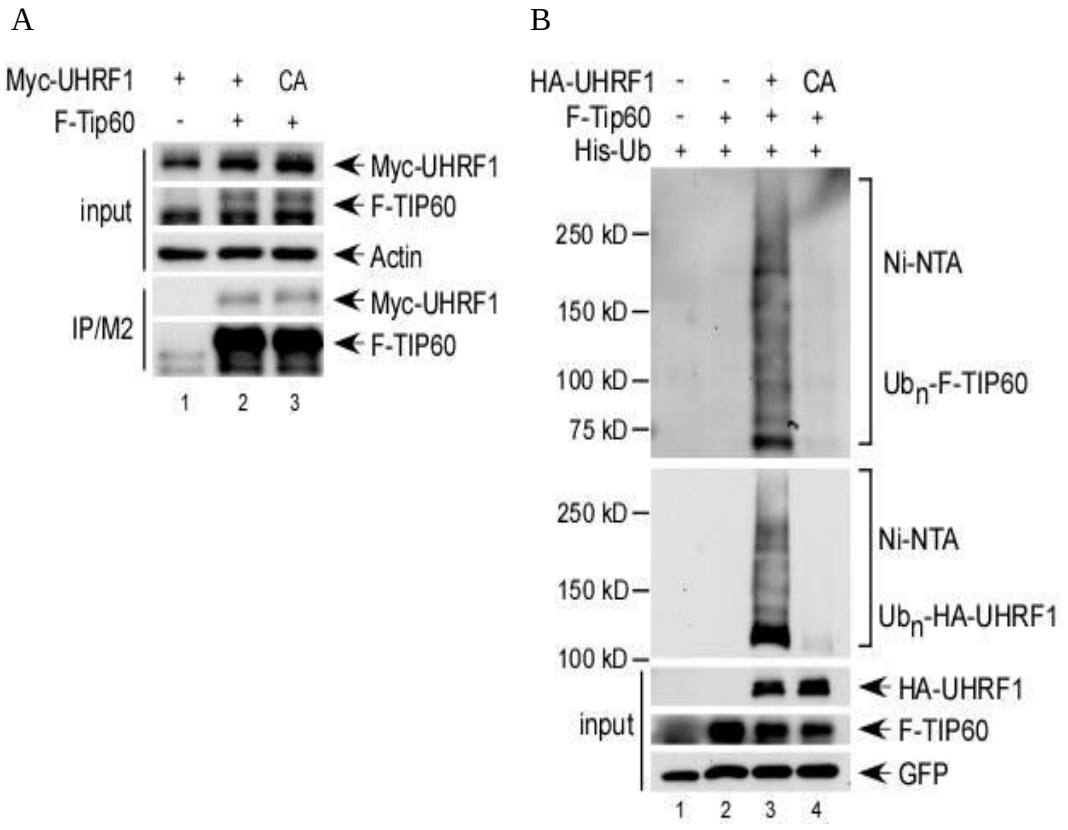


To confirm that UHRF1 induces TIP60 ubiquitination through its E3 ubiquitin ligase activity, we further made use of C724A UHRF1, a RING finger point mutant that retains TIP60 binding affinity (Fig. 19A) but was previously reported deficient in *in vitro* autoubiquitination [269]. In a cell-based ubiquitination assay, wild-type UHRF1 displays robust autoubiquitination while the C724A UHRF1 mutant lack E3 ligase activity (Fig. 19B, middle panel, lane 3 vs. lane 4). More importantly, coexpression of wild-type UHRF1 strongly induced TIP60 ubiquitination, while in the absence of UHRF1 E3 ligase activity TIP60 ubiquitination was undetectable (Fig. 19B, upper panel, lane 3 vs. lane 4). Together these data demonstrate that UHRF1 induces TIP60 ubiquitination directly via its E3 ligase activity conferred by the RING domain.

Figure 19. UHRF1 induces TIP60 ubiquitination directly through its E3 ligase activity

(A) C724A UHRF1 mutant retains interaction with TIP60. H1299 were transiently transfected with FLAG-Tip60 in combination with Myc-UHRF1 or Myc-C724A UHRF1. Whole cell extracts or immunoprecipitates with M2/FLAG antibody were analyzed by Western blot with α-MYC and α-FLAG antibodies.

(B) UHRF1 directly ubiquitinates TIP60 through its E3 ubiquitin ligase activity. H1299 cells were cotransfected with FLAG-Tip60 and His-ubiquitin alone or together with either HA-UHRF1 or HA-C724A UHRF1 expression vectors. Whole cell extracts and Ni-NTA affinity purified fractions were analyzed by Western blot with α-FLAG and α-HA antibodies. GFP was used as control to confirm equal transfection.



Because ubiquitination is most frequently associated with proteosomal degradation, we sought to test whether UHRF1 promotes Tip60 degradation. To this end, H1299 cells were transfected with FLAG-HA-Tip60 in combination of increasing amounts of HA-UHRF1 expression vector. Western blot analysis of total cell extracts revealed that exogenous TIP60 protein levels remain unchanged in the presence of increasing levels of UHRF1 (Fig. 20A). To further confirm that UHRF1 is incapable of regulating Tip60 stability, we inactivated endogenous UHRF1 in 3xFLAG-TIP60 knock-in HCT116 cells. Following two rounds of transfection with either the control siRNA or UHRF1-specific siRNA, total cell extracts were analyzed by Western blot and untreated parental HCT116 was used as a control to confirm expression of 3xFLAG tagged TIP60. As expected, the level of endogenous UHRF1 protein was severely ablated after transfection with UHRF1-RNAi (Fig. 20B, lane 2 vs. lane 1). More importantly, TIP60 protein level was unaffected by UHRF1 ablation (Fig. 20B, lanes 1 and 2 vs. lane 3), suggesting that UHRF1 does not regulate TIP60 protein stability. Furthermore, we assessed *Tip60* mRNA level in HCT116 cells that were transfected with UHRF1-specific siRNA oligos or a control siRNA oligo. Quantitative real time PCR analysis reveals that the level of *Tip60* mRNA remains unaffected by UHRF1 ablation (Fig. 20C). Taken together, these data suggest that UHRF1 does not promote degradation of Tip60 or regulate *Tip60* at the transcription level.

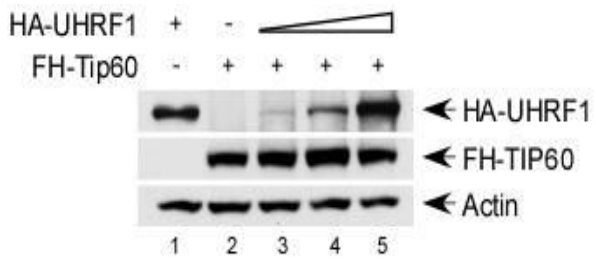
Figure 20. UHRF1 does not promote TIP60 degradation or affect *Tip60* at the transcription level

(A) UHRF1 expression does not induce TIP60 degradation. H1299 cells were transfected with FLAG-HA-Tip60 in combination with increasing levels of HA-UHRF1. Whole cell extracts were analyzed by Western blot with α-HA antibody.

(B) UHRF1 depletion does not affect TIP60 protein level. HCT116 cells were treated with 2 rounds of knock-down with either control RNAi, UHRF1 RNAi, or TIP60 RNAi. Whole cell extracts were analyzed by Western blot with α-UHRF1 and α- TIP60 antibodies.

(C) UHRF1 depletion does not affect Tip60 mRNA level. Total RNA was extracted from control RNAi or UHRF1 RNAi treated HCT116. Following reverse transcription, the abundance of Tip60 mRNA was assessed using quatitative real time PCR.

A



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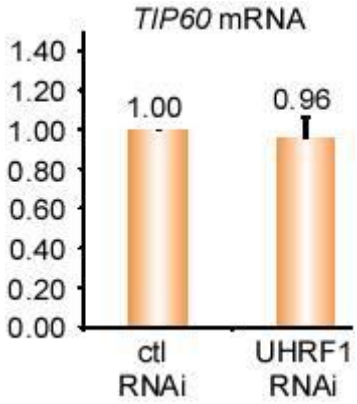
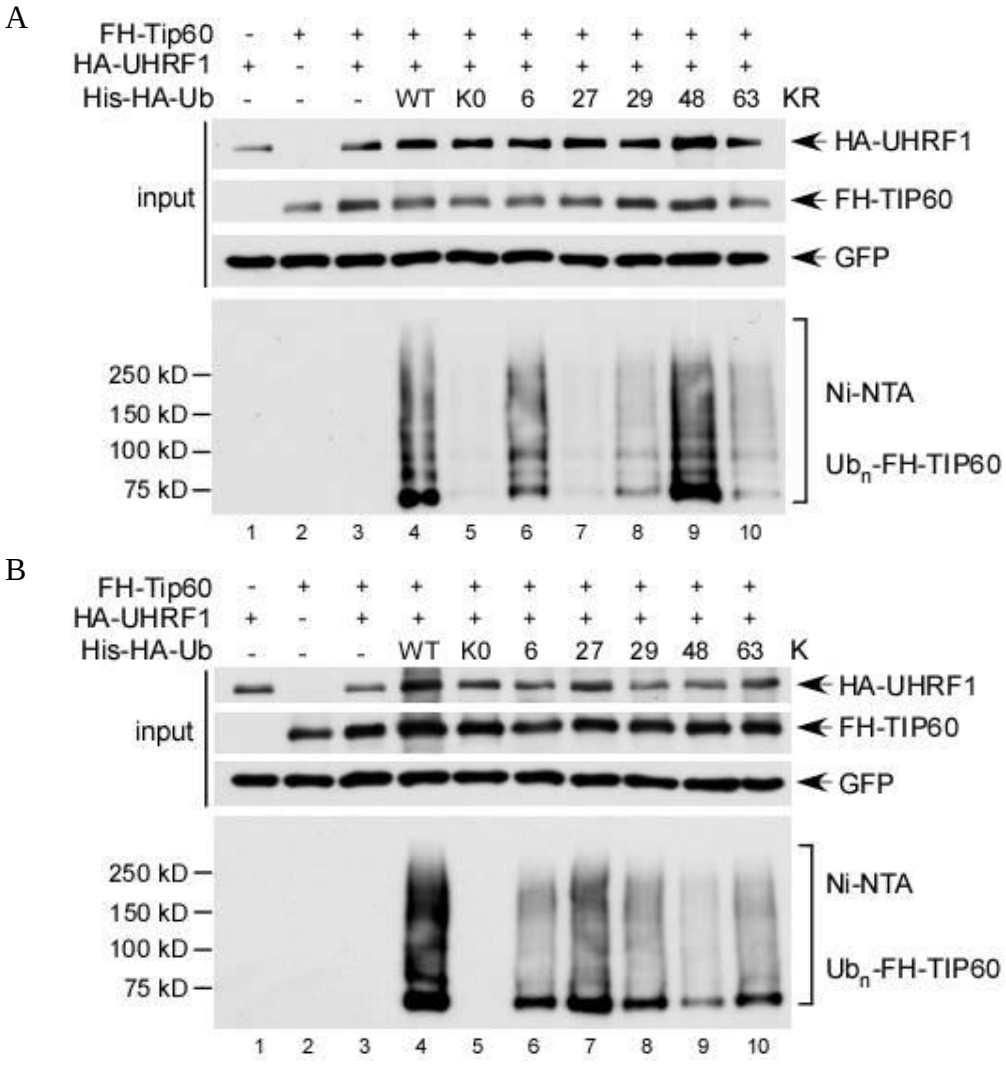


Our finding that the TIP60-conjugated polyubiquitin chains generated by UHRF1 do not serve as a targeting signal for proteolysis predicts that they may not be of the typical K48-linked ubiquitin chains. Chains that conjugate through K63 of ubiquitin, for example, have been reported to involve protein trafficking or DNA repair [279,280]. In a cell-based ubiquitination assay performed with either wild-type ubiquitin (WT-Ub), mutant ubiquitin with arginine (R) replacing specific lysine sites (KR-Ub), or a mutant ubiquitin with all lysines replaced (K0-Ub) (Fig. 21A), TIP60 ubiquitination was retained in the presence of K48R-Ub. In contrast, K27R-Ub diminished TIP60 ubiquitination level to almost that obtained with K0-Ub; K6R-, K29R- or K63R-Ub also attenuated TIP60 ubiquitination to different extents (Fig. 21A). Conversely, by utilizing mutant ubiquitin containing only one unaltered lysine (K-Ub) in the same assay, K27-Ub was most readily incorporated by UHRF1, and K6-, K29-, and K63 ubiquitin could all be incorporated to a lesser extent (Fig. 21B). Together these data confirm that K48-dependent linkage, which is characteristic of ubiquitin-mediated proteolysis, is excluded and suggest that TIP60 ubiquitination by UHRF1 relies on K27 as a major site for conjugation but likely involves a combination of K6, K29 and K63 linkages as well.

Figure 21. UHRF1 ubiquitinates TIP60 through atypical ubiquitin lysine linkages

(A and B) UHRF1 mediates K48-independent polyubiquitin chain conjugation on TIP60. H1299 cells were cotransfected with expression vectors for FLAG-HA-Tip60 and HA-UHRF1, in combination with His/HA double tagged ubiquitin mutants. Ni-NTA purified fractions were immunoblotted with α-FLAG antibody; whole cell lysates were immunoblotted with α-HA and α-FLAG antibodies. GFP was used as a control for equal transfection.



3.2.3 UHRF1 depletion increases TIP60-mediated p53 acetylation at K120 and enhances apoptosis

Because TIP60 is a known regulator of p53 acetylation at the K120 residue, we first assessed whether UHRF1 modulates TIP60-dependent acetylation of p53. M2/FLAG immunoprecipitation of extracts from H1299 cells transfected with FLAG-p53, Tip60, and HA-UHRF1, revealed that p53 is easily acetylated by TIP60 and acetylation was markedly attenuated upon UHRF1 expression (Fig. 22A).

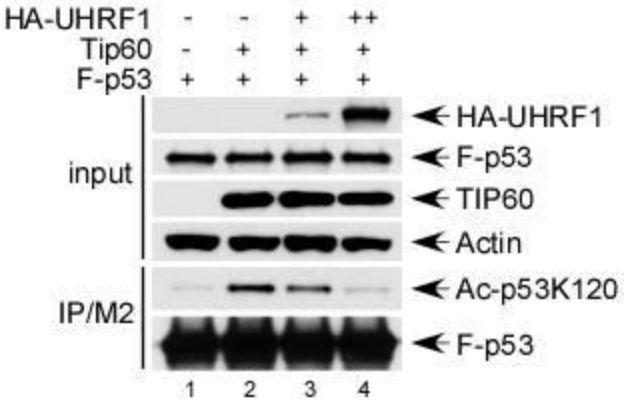
UHRF1 was further ablated in U2OS cells via RNAi and endogenous p53 acetylation was assessed by immunoprecipitating cell extracts with the α-Ac-p53-K120 antibody. Prior to harvesting, cells were subjected to trichostatin A (for inhibiting HDAC1/HDAC2-mediated p53 deacetylation) and nicotinamide (for inhibiting SIRT1-mediated p53 deacetylation) treatment to enrich acetylated endogenous p53. As shown in Fig. 22B, p53 K120 acetylation was readily detected with treatment of deacetylase inhibitors, and significantly enhanced upon UHRF1 ablation, suggesting that UHRF1 is a potent suppressor of TIP60-mediated acetylation of p53 at K120.

Figure 22. UHRF1 suppresses TIP60-mediated p53 acetylation at K120

(A) UHRF1 expression inhibits p53 acetylation by TIP60 at K120. H1299 cells were transiently transfected with plasmid DNA expressing FLAG-p53, Tip60 and HA-UHRF1. Total cell extracts and M2 immunoprecipitates were assayed by Western blot using antibodies against HA, p53 and p53-AcK120.

(B) UHRF1 inactivation significantly increases p53 acetylation at K120. U2OS cells were transiently transfected with either control siRNA or UHRF1 siRNA, and treated for 6 hr with 1μM trichostatin A (TSA) and 5mM nicotinamde (NTA) prior to harvesting. Cell extracts and immunoprecipitates obtained with α-Ac-p53K120 or control IgG were analyzed with Western blot using α-UHRF1, α-p53 and α-Ac-p53K120 antibodies.

A



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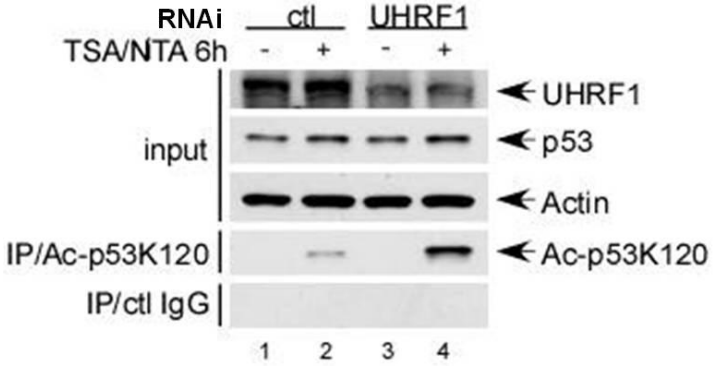


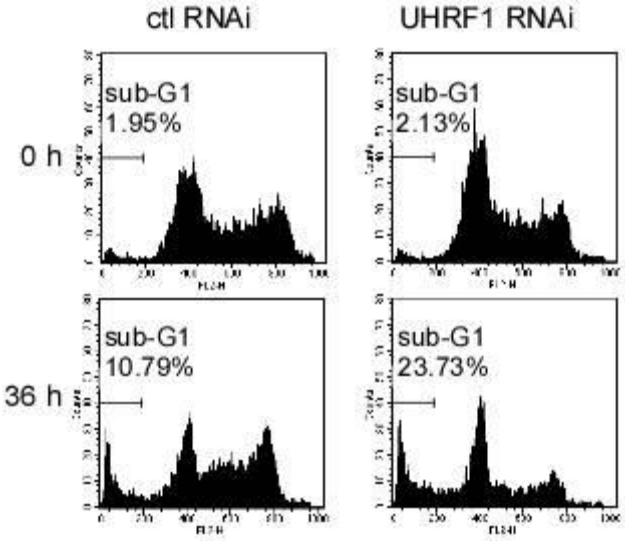
The Gu team and others previously demonstrated that p53 K120 acetylation is indispensable for apoptosis, leading to our speculation that UHRF1 might modulate apoptosis in cells at risk of DNA damage. Apoptosis was therefore assessed by flow cytometric analysis of DNA fragmentation in UHRF1-ablated U2OS cells treated with etoposide and stained with propidium iodide (PI). As shown in Fig. 23A, UHRF1 inactivation minimally affected basal level sub-G1 content but markedly increased apoptosis following etoposide challenge. Quantitative analysis revealed that following 36 or 44 hr of etoposide treatment, an average of 10.21% and 21.58% of control RNAi-treated cells were apoptotic, whereas a dramatically elevated 26.91% and 34.96% of UHRF1 RNAi-treated cells underwent apoptosis (Fig. 23B). Collectively, these data demonstrate that UHRF1 negatively regulates damage-induced apoptosis through attenuating TIP60-mediated p53 K120 acetylation.

Figure 23. UHRF1 depletion augments damage-induced apoptosis

(A) FACS analysis of UHRF1-inactivated U2OS cells treated with etoposide. U2OS cells transiently transfected with either control siRNA or UHRF1 siRNA were treated with 20uM etoposide for the indicated time. Cells were subsequently fixed in 80% cold methanol, stained with propidium iodide and subjected to DNA content analysis by flow cytometry.

(B) UHRF1 RNAi increases apoptosis. Apoptosis was assessed as in (A) and percentages of apoptotic cells are presented as average values of three independent experiments. Error bars, ±1 standard deviation.

A



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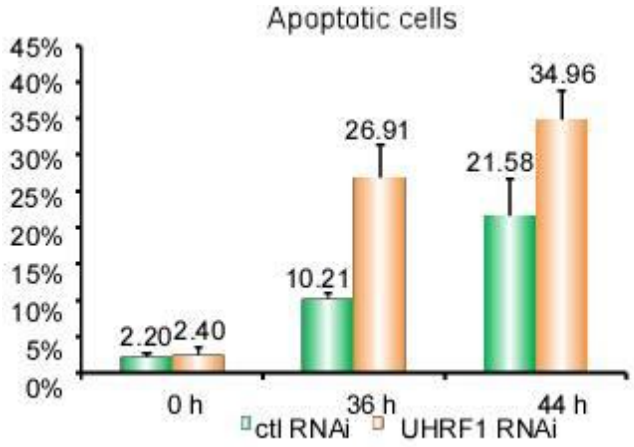


3.2.4 UHRF1 inhibits TIP60-p53 interaction

We further assessed UHRF1 regulation of TIP60-p53 interaction by transfecting H1299 with increasing amounts of Myc-UHRF1 in the presence of p53 and FLAG-Tip60 and subjecting cell extracts to M2/FLAG immunoprecipitation. p53 was readily detected in TIP60-associated immunoprecipitates; however, upon UHRF1 expression the amount of TIP60-bound p53 decreased drastically (Fig. 24A), indicating that UHRF1 inhibits TIP60 interaction with p53.

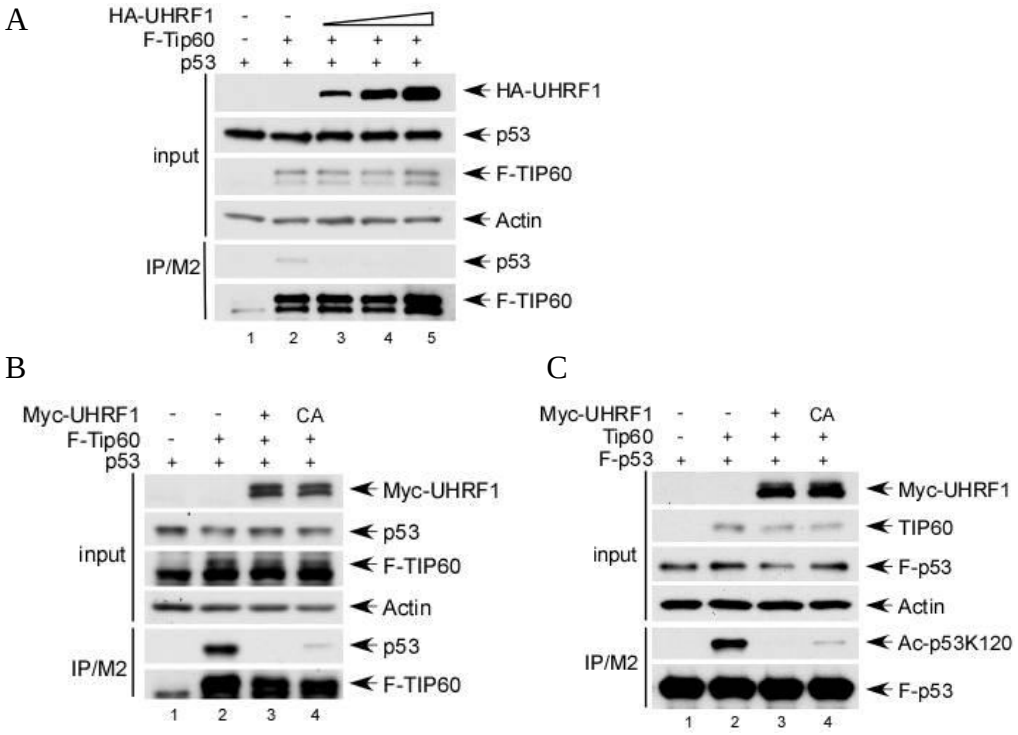
Having established that UHRF1 is capable of attenuating the TIP60-p53 interplay, we next sought to test whether this is achieved through TIP60 ubiquitination. Coimmunoprecipitation of p53 and TIP60 was performed in extracts of H1299 cells transfected with p53, FLAG-Tip60 and Myc-tagged UHRF1 or C724A UHRF1. As shown in Fig. 24B, expression of wild-type UHRF1 diminished the amount of p53 detected in TIP60-associated immunoprecipitates, whereas the ligase activity deficient C724A mutant only partially inhibited TIP60 interaction with p53. Furthermore, while wild-type UHRF1 completely abolished p53 K120 acetylation by TIP60, the C724A mutant mildly diminished p53 acetylation (Fig. 24C). Taken together, these results demonstrate that UHRF1 inhibits TIP60-p53 interaction and TIP60-mediated p53 acetylation, and that UHRF1-induced TIP60 ubiquitination may contribute partially to the suppression of the TIP60-p53 interplay.

Figure 24. UHRF1 suppresses TIP60-p53 interaction partially through promoting TIP60 ubiquitination

(A) UHRF1 expression inhibits TIP60 interaction with p53. H1299 cells were transiently transfected with FLAG-Tip60, p53 and HA-UHRF1 expression vectors. Cell extracts and M2 immunoprecipitates were assayed by Western blot using α-HA, α-FLAG and α-p53 antibodies.

(B) Loss of UHRF1-mediated TIP60 ubiquitination partially suppresses TIP60 interaction with p53. Total cell extracts and M2 immunoprecipitates from H1299 transiently transfected with FLAG-Tip60, p53 and Myc-tagged UHRF1 or C724A UHRF1 expression vectors were assayed by Western blot using antibodies against MYC, FLAG, and p53.

(C) Loss of UHRF1-mediated TIP60 ubiquitination partially inhibits p53 acetylation by TIP60 at K120. Total cell extracts and M2 immunoprecipitates from H1299 transiently transfected with FLAG-p53, Tip60 and Myc-tagged UHRF1 or C724A UHRF1 expression vectors were assayed by Western blot using antibodies against MYC, TIP60, p53 and p53-AcK120.



3.2.5 SRA and RING domains of UHRF1 are indispensable for UHRF1 suppression of TIP60-p53 interaction

The partial suppression of the TIP60-p53 interplay by the ligase activity deficient UHRF1 mutant suggests that UHRF1 may elicit inhibitory effects through mechanisms including but not limited to TIP60 ubiquitination. It would therefore be interesting to elucidate what other functional domains might be responsible for exerting the inhibition. To this end, we generated a series of Myc-tagged UHRF1 truncation mutants as diagrammed in Fig. 25A with deletion of one or more functional domains.

UHRF1-TIP60 interaction was determined by transfecting H1299 with F-Tip60 and Myc-tagged mutant UHRF1, followed by Western blot analysis of M2/FLAG immunoprecipitates. Mutant UHRF1 lacking the N-terminal ubiquitin-like domain (ΔNIRF), the Plant Homeo domain (ΔPHD), or the truncation mutant possessing only the SRA (Set and Ring Associated) and RING domains (S+R) bound strongly to TIP60 (Fig. 25B, lanes 3, 6, and 7); mutant UHRF1 that lacked either the SRA domain (ΔSRA) or the RING domain (ΔRING) showed weaker interaction with TIP60 (Fig. 25B, lanes 4 and 8); whereas loss of both SRA and RING domains (ΔS+R) completely abolished TIP60 interaction (Fig. 25B, lane 5), indicating that the SRA and RING domains are indispensable for UHRF1 interaction with TIP60.

Figure 25. The SRA and RING domains of UHRF1 confer interaction with TIP60

(A) Schematic representation of UHRF1 deletion mutants used in interaction domain mapping. Full-length UHRF1 and all deletion mutants were subcloned into pCMV-Myc expression vector.

(B) UHRF1 interacts with TIP60 through its SRA and RING domains. H1299 cells were transiently transfected with expression vectors for FLAG-Tip60 and MYC-tagged UHRF1 deletion contructs. Total cell extracts and M2 immunoprecipitates were assayed by Western blot using antibodies against MYC and FLAG.

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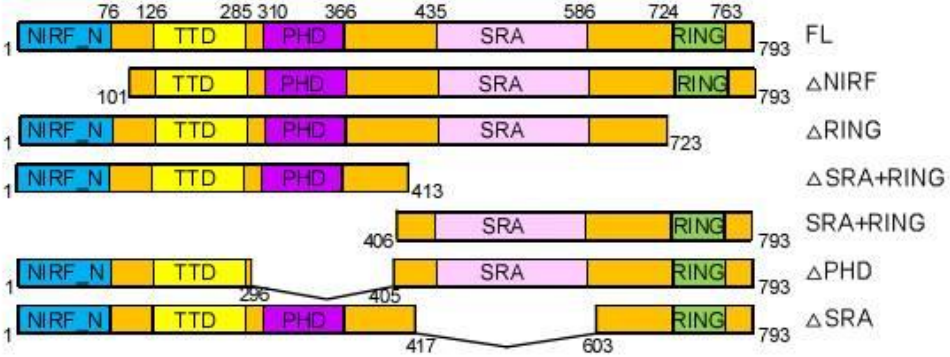

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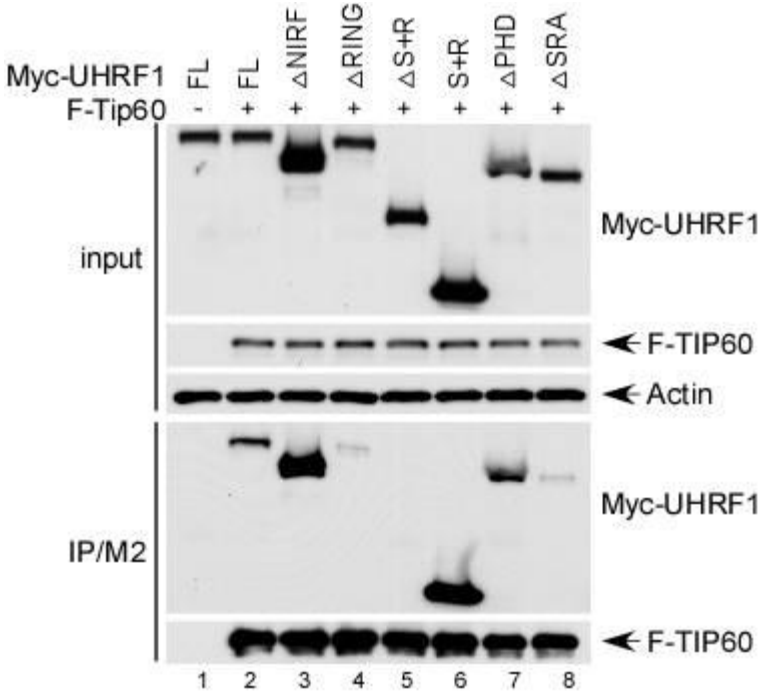


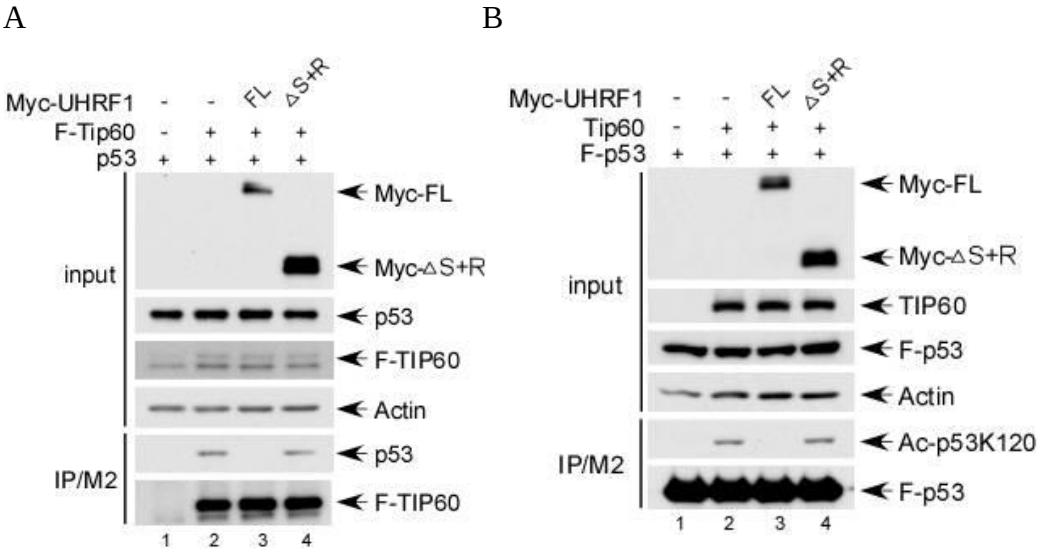
To test whether the inhibition of TIP60-p53 interaction requires UHRF1 affinity for TIP60, coimmunoprecipitation of p53 with TIP60 was performed in extracts of H1299 cells transfected with p53, FLAG-Tip60, and Myc-UHRF1 or Myc-ΔS+R UHRF1. As expected, Western blot analysis of M2/FLAG eluates revealed that the level of TIP60-associated p53 was severely ablated upon UHRF1 expression (Fig. 26A, lane 3 vs. lane 2). Strikingly, the ΔS+R mutant, which lacks binding affinity or TIP60, was incapable of suppressing TIP60-p53 interaction (Fig. 26A, lane 4 vs. lane 2).

In an effort to test whether p53 K120 acetylation is affected by loss of UHRF1-TIP60 interaction, the level of K120 acetylation was assessed by M2/FLAG immunoprecipitation of total p53 from H1299 cells transfected with FLAG-p53, Tip60, and Myc-UHRF1 or Myc-ΔS+R UHRF1 expression vectors and immunoblotting with α-Ac-p53K120 antibodies. Consistent with Fig. 24C, wild-type UHRF1 significantly diminished p53 acetylation by TIP60 (Fig. 26B, lane 2 and lane 3). However the ΔS+R mutant completely lost inhibition of p53 K120 acetylation (Fig. 26B, lane 4).

Figure 26. The SRA and RING domains of UHRF1 are indispensable for inhibition of TIP60-p53 interaction and p53 acetylation

(A) The SRA and RING domains of UHRF1 are indispensable for inhibition of TIP60-p53 interaction. Total cell extracts and M2 immunoprecipitate from H1299 transiently transfected with FLAG-Tip60, p53 and Myc-tagged full-length UHRF1 or ΔS+R UHRF1 expression vectors were analyzed by Western blot using antibodies against MYC, FLAG, and p53.

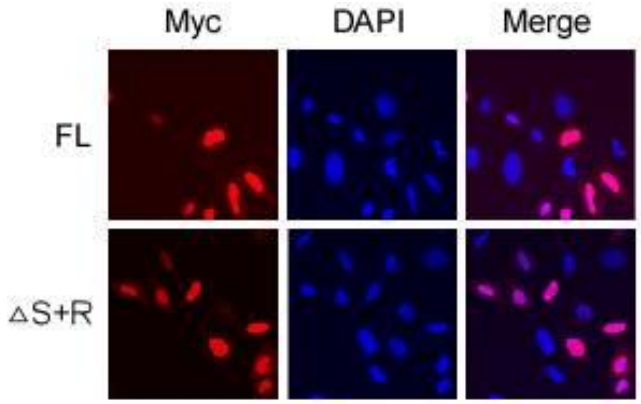
(B) The UHRF1ΔSRA+RING mutant loses inhibition of TIP60-mediated p53 acetylation at K120. Total cell extracts and M2 immunoprecipitates from H1299 transiently transfected with FLAG-p53, Tip60 and MYC-tagged full-length UHRF1 or ΔS+R UHRF1 expression vectors were analyzed by Western blot using α-Myc, α- TIP60, α-p53 and α-Acp53K120 antibodies.



We then sought to validate that the loss of TIP60 affinity is conferred through the deletion of SRA and RING domains rather than altered protein localization. Therefore cellular localization of full-length and mutant UHRF1 was assessed by immunostaining Myc-UHRF1 or Myc-ΔS+R UHRF1 transfected U2OS cells. Co-staining with the α-Myc antibody and the nuclei labeling reagent 4,6-diamidino-2-phenylindole (DAPI) demonstrated that both the full-length UHRF1 protein and the ΔS+R mutant localize to the nucleus (Fig. 27). These data collectively suggest that UHRF1 inhibits TIP60-p53 interaction through competitively binding to TIP60 via its SRA and RING domains, and ablation of UHRF1-TIP60 binding completely loses inhibition of the TIP60-p53 interplay.

Figure 27. The UHRF1ΔSRA+RING mutant retains nuclear localization

The UHRF1ΔSRA+RING mutant retains nuclear localization. U2OS cells were transiently transfected with expression vectors for Myc-tagged full-length UHRF1 or ΔS+R UHRF1. 24h posttransfection, cells were fixed with paraformaldehyde, immunostained with α-Myc antibody and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Subcellular localization was assessed by fluorescent microscopy.



3.2.6 UHRF1 depletion upregulates activation of PUMA and p21 but not HDM2

Previous studies demonstrate that apart from promoting p53 acetylation at the K120 site, which is specifically required for the activation of apoptotic targets, TIP60 also regulates p53 transcription of *p21* in the absence of K120 acetylation, through p53-dependent recruitment to the *p21* promoter and modulation of histone H4 acetylation [201]. In contrast, TIP60 is not recruited to the *HDM2* promoter and activation of the p53 feedback target HDM2 is not dependent on TIP60 [201]. Therefore our finding that UHRF1 inhibits not only p53 acetylation but also TIP60-p53 interaction predicts that UHRF1 ablation should result in increased activation of both PUMA and p21 in response to DNA damage because an increased amount of TIP60 now becomes available for promoter co-recruitment and/or co-activation of p53. Conversely, HDM2 induction by p53, which is independent of TIP60, should remain unaffected regardless of UHRF1 status.

To test this hypothesis, we performed RNAi-mediated inactivation of UHRF1 in HCT116 cells followed by treatment with the 5-fluorouracil (5-FU) antimetabolite that strongly activates p53 and induces p53-dependent growth arrest and apoptosis in HCT116 [281]. As expected, upon 5-FU treatment p53 levels increased drastically; and notably UHRF1 inactivation affected neither

basal p53 level (Fig. 28A, lane 6 vs. lane 1) nor the accumulation of p53 protein. Furthermore, at all time points assessed, HDM2 induction was unaffected by UHRF1 depletion but PUMA and p21 activation was significantly increased in the UHRF1-depleted group (Fig. 28A, lanes 7-10 vs. lanes 2-5).

To exclude off-target effects of RNAi and validate that our finding was not specific to HCT116 cells or the 5-FU drug, we ablated UHRF1 in U2OS using three siRNA oligos targeting different regions of the *UHRF1* mRNA, and further treated these cells with another DNA damage reagent doxorubicin. UHRF1 was effectively ablated by all three siRNA oligos and doxorubicin-induced HDM2 induction remain unaffected by UHRF1 depletion, however PUMA and p21 activation was significantly increased by UHRF1 ablation using all three oligos (Fig. 28B).

Figure 28. UHRF1 depletion upregulates activation of PUMA and p21 but not HDM2 following DNA damage

(A) UHRF1 RNAi in HCT116 cells upregulates 5-FU induced PUMA and p21 activation but not HDM2 activation. HCT1116 cells were treated with 2 rounds of knock-down with either control RNAi or UHRF1 RNAi. Following treatment with 400μM 5-FU for the indicated time, whole cell extracts were analyzed by Western blot with the indicated antibodies.

(B) UHRF1 RNAi upregulates doxorubicin induced PUMA and p21 but not MDM2 activation in U2OS cells. U2OS cells were transiently transfected with control siRNA or three different U2OS-specific siRNA oligos and treated with or without 0.5μM doxorubicin for 16 hr. Total cell extracts were analyzed by Western blot using the indicated antibodies.

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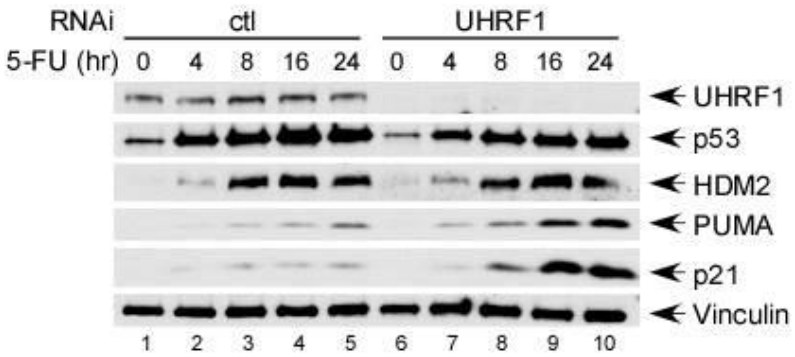

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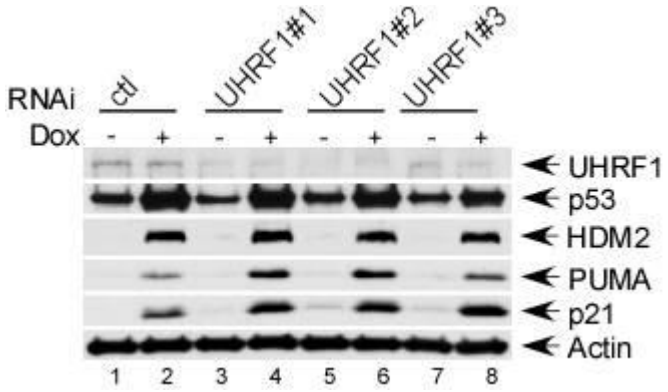
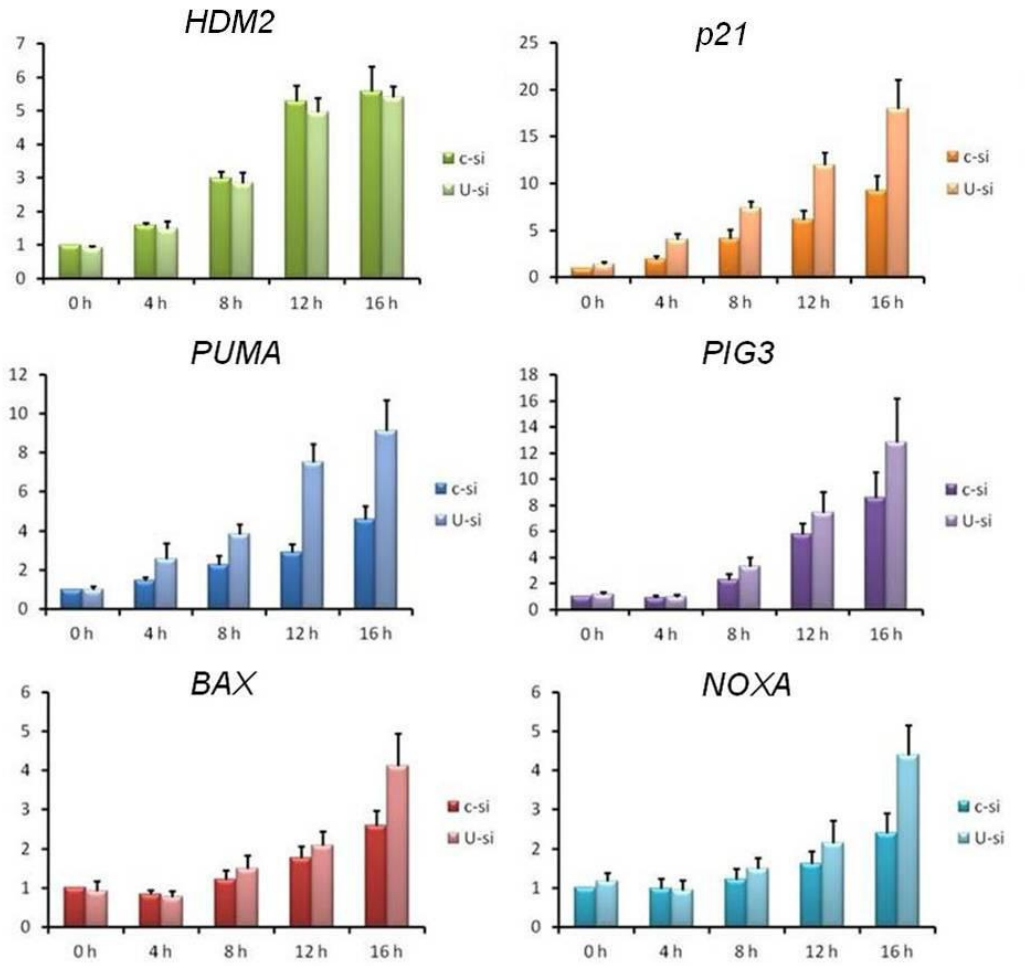


We further confirmed that UHRF1 ablation affected p53-dependent transcription of *PUMA* and *p21* but not *HDM2* by examining the mRNA levels of these targets using qRT-PCR (Fig. 29). Indeed, basal *HDM2*, *PUMA* and *p21* mRNA levels were unaffected by UHRF1 depletion. Upon 5-FU treatment, *PUMA* and *p21* transcription was considerably augmented following UHRF1-RNAi, whereas *HDM2* mRNA level increased upon 5-FU treatment and remained unaffected in the UHRF1-RNAi treated group (Fig. 29). mRNA levels of other p53 apoptotic targets such as *BAX*, *PIG3*, and *NOXA* were also assessed; and UHRF1 ablation augmented the mRNA induction of these targets, especially at later time points (Fig. 29). These data confirm that UHRF1 antagonizes p53-dependent transcription of growth arrest and apoptotic targets, but not the HDM2 feedback target.

Together, these data validate UHRF1 ablation does not affect p53-mediated transcription of HDM2, but increases transcription of apoptotic and growth arrest targets.

Figure 29. UHRF1 RNAi upregulates 5-FU induced growth arrest and apoptotic target transcription but not HDM2 transcription

HCT116 were treated with 400μM 5-FU for the indicated time following control RNAi or UHRF1 RNAi. Total RNA was extracted and cDNA was prepared by reverse transcription. mRNA abundance for the indicated genes assessed using quantitative real time PCR.



To verify that the effect of UHRF1 on PUMA and p21 activation is p53-dependent, we ablated UHRF1 in the HCT116 $p53^{+/+}$ and $p53^{-/-}$ pair prior to 5-FU treatment. Following damage, p53 accumulated in $p53^{+/+}$ HCT116 and PUMA and p21 were activated, the extent to which were significantly increased by UHRF1 depletion (Fig. 30A, lanes 1-4). In a p53-deficient background 5-FU failed to activate PUMA and p21; and UHRF1 ablation did not increase PUMA and p21 level upon 5-FU treatment in the absence of p53 (Fig. 30A, lanes 5-8). 5-FU induced HDM2 only in the presence of p53, and no difference in HDM2 activation was observed upon UHRF1 RNAi.

Alternatively, p53-dependency was confirmed in U2OS by double inactivation of UHRF1 and p53 followed by doxorubicin treatment (Fig. 30B). Inactivating UHRF1 alone strongly increased doxorubicin-induced PUMA and p21 activation, when combined with p53 depletion UHRF1 ablation could not affect PUMA and p21 levels before and after damage, suggesting that the effect of UHRF1 on PUMA and p21 activation upon damage treatment is p53-dependent.

Figure 30. UHRF1 modulation of damage-induced PUMA and p21 activation in a p53-dependent manner

(A) p53 dependency in HCT pair. UHRF1 is inactivated by RNAi in either p53+/+ or p53-/- HCT116 cells. Subsequently, cells were treated with or without 400μM 5-FU for 8 hr before extraction and Western blot analysis using the indicated antibodies.

(B) p53 dependency using double knocking down in U2OS. UHRF1 alone, or both p53 and UHRF1 were ablated in U2OS cells using RNAi. Subsequently, cells were treated with or without 1μM doxorubicin for 16 hr before extraction and Western blot analysis with the indicated antibodies.

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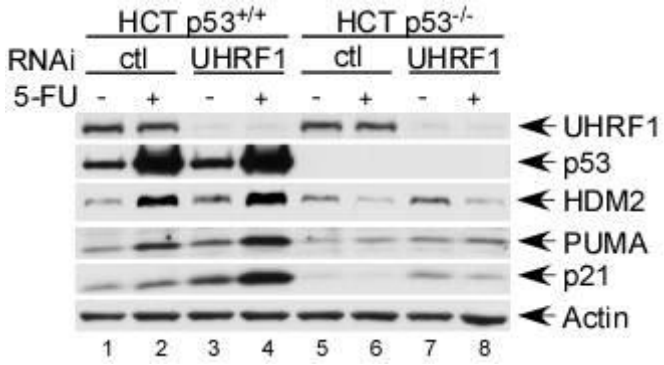

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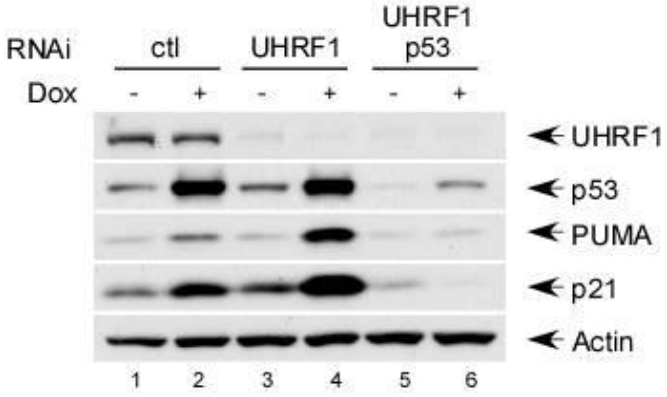
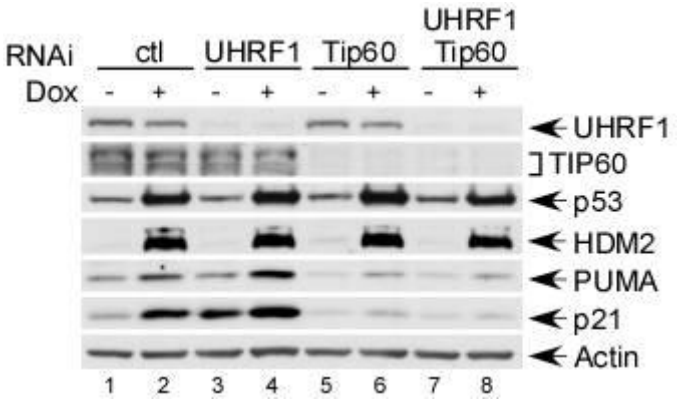


To further validate that these effects are also dependent on TIP60, we inactivated UHRF1 or TIP60 alone, or in combination in U2OS cells using RNAi. p53 levels accumulated and HDM2 was activated normally following doxorubicin treatment in samples that were depleted of either UHRF1, TIP60 or both. However the induction of PUMA and p21 expression was severely diminished by TIP60 ablation (Fig. 31, lanes 5 and 6); and more importantly, in the TIP60-deficient background UHRF1 depletion displayed no effect on PUMA and p21 levels before and after doxorubicin treatment (Fig. 31, lanes 5-8).

Figure 31. UHRF1 modulates damage-induced PUMA and p21 activation in a TIP60-dependent manner

UHRF1 or TIP60 alone, or both UHRF1 and TIP60 were inactivated in U2OS using RNAi. Subsequently, cells were subjected to 16 hr 0.5μM doxorubicin treatment before extraction and Western blot analysis using the indicated antibodies.



Because UHRF1 ablation increases p53-mediated damage-induced p21 expression, the physiological role of UHRF1 in regulating p53-mediated cell growth arrest was also tested. To this end, the effect of UHRF1 ablation on cell growth was examined by monitoring BrdU incorporation, a marker for proliferating cells, following etoposide treatment. U2OS cells were transfected with either control siRNA or UHRF1 siRNA prior to treatment with etoposide. Following 16 or 24 hr of etoposide treatment, these cells were then labeled for 1 hr with BrdU, fixed in paraformaldehyde and double stained with the α-BrdU antibody and 4,6-diamidino-2-phenylindole (DAPI). As shown in Fig. 32, at 0 timepoint, U2OS cells transfected with UHRF1 siRNA showed a similar level of BrdU incorporation (~50%) compared to cells transfected with control siRNA. In contrast, following 16 or 24 hr of etoposide treatment, only 24.37% and 16.5% of cells transfected with UHRF1 siRNA were detected BrdU-positive, whereas 44.74% and 31.23% of cells transfected with control siRNA were BrdU-positive. These data validate that UHRF1 negatively regulates p53-mediated growth arrest.

Together our data suggest that UHRF1 modulates p53 activity through negatively regulating TIP60-mediated functions in both K120 acetylation-dependent and -independent manners.

Figure 32. UHRF1 depletion upregulates damage-induced growth arrest

(A) BrdU incorporation of UHRF1-inactivated U2OS cells treated with etoposide. U2OS cells were transiently transfected with either control siRNA or UHRF1 siRNA. 72 hr post-transfection, cells were treated 1 hr with 10μM BrdU and immunostained with the α-BrdU antibody. The nuclei are in blue (DAPI), and BrdU-positive nuclei are shown in red.

(B) UHRF1 RNAi decreases cel proliferation. BrdU positive cells in (A) were counted in 6-8 microscopic fields and percentages of BrdU positive cells are presented as average values from 3 independent experiments. Error bars, ±1 standard deviation.

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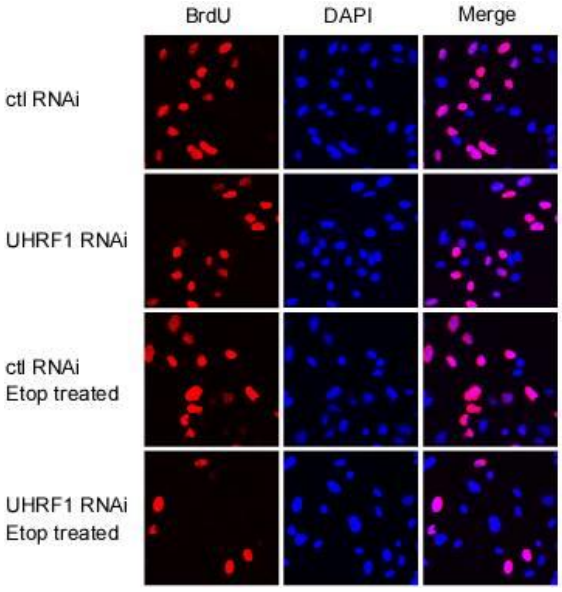

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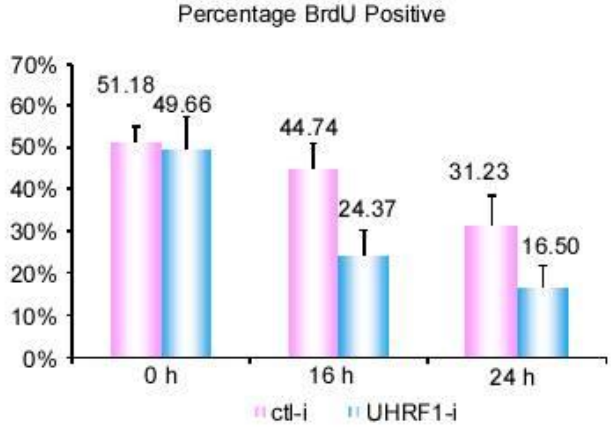

3.3 Discussion

Our findings identify that UHRF1 is a direct interacting partner of TIP60 and a potent negative regulator of the TIP60-p53 interplay. UHRF1 expression induces degradation-independent ubiquitination of TIP60, which partially contributes to the marked suppression of TIP60-mediated p53 acetylation at K120. In contrast, UHRF1 ablation significantly increases p53 K120 acetylation, upon which p53-mediated apoptosis is dependent. Further elucidating the underlying mechanism, we found that UHRF1 severely inhibits TIP60 interaction with p53, leading to UHRF1 modulation of TIP60 function both dependent and independent of its ability to acetylate p53 at K120. Upon DNA damage, UHRF1 inactivation augments PUMA and p21 transcription, both of which rely on TIP60 but are differentially dependent on p53 K120 acetylation; in contrast, UHRF1 depletion does not affect stressed induced HDM2 transcription by p53, which is independent of TIP60 status. Therefore, our findings suggest that UHRF1 acts as a critical negative regulator of TIP60 upstream of the p53 pathway, thereby negatively regulating TIP60-dependent transcription of key targets involved in growth arrest and apoptosis in cells at risk of DNA damage.

Based on our observation, we propose a model of tumorigenesis and/or tumor progression in the presence of high levels of cellular UHRF1 (Fig. 33). In normal cells, p53 is stabilized and activated upon DNA damage and induces transcription of the HDM2 feedback regulator in the absence of TIP60 recruitment and histone

H4 acetylation in the vicinity of the *HDM2* promoter. TIP60-p53 interaction is required for TIP60 recruitment to p53 target promoters and the induction of histone H4 acetylation, leading to p21 transactivation and growth arrest. Finally in cells that have undergone excessive damage, TIP60 acetylates p53 at K120, resulting in induction of PUMA and activation of the irreversible apoptotic pathway, thereby maintaining cellular and genomic stability and suppressing tumorigenesis (Fig. 33A). In contrast, UHRF1 overexpression leads to excessive UHRF1-TIP60 interaction and sequestration of TIP60 from associating with p53. This suppresses stress-induced TIP60 recruitment to p53 target promoters, acetylation of histone H4, and p53 actylation at K120. As a result, p53-dependent p21 and PUMA transactivation are abolished, and proprogation of the damaged genome leads to tumorigenesis and/or tumor progression (Fig. 33B).

Figure 33. A model for tumorigenesis/tumor progression in cells with UHRF1 overexpression

See text for details.

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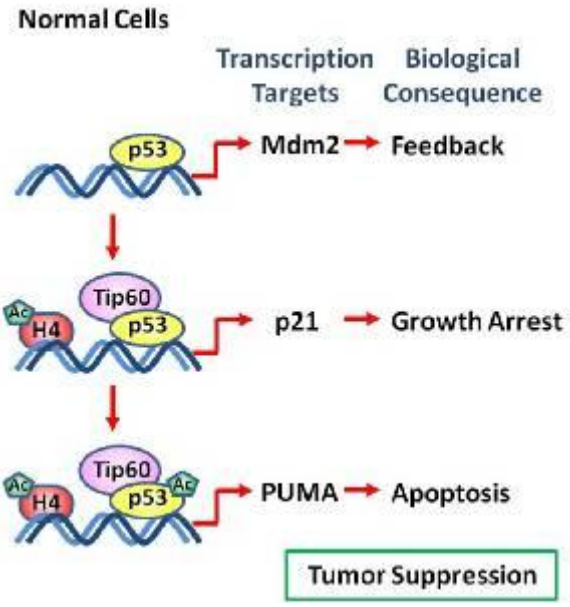

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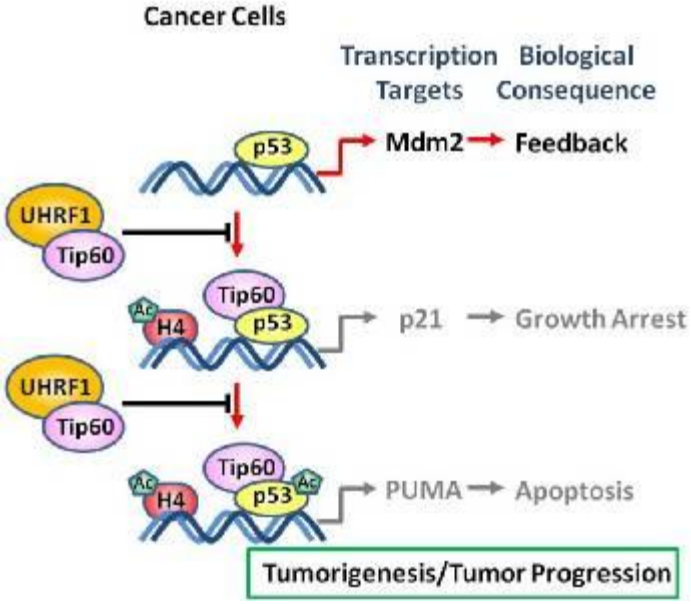


TIP60 is a haplo-insufficient tumor suppressor with well-documented functions in regulating transcription, DNA damage repair, and p53-mediated growth arrest and apoptosis [282]. Therefore TIP60 function should require tight regulation, which to date has been shown achievable through post-translational modification or protein-protein interaction. Post-translationally, phosphorylation and autoacetylation of TIP60 upon DNA damage are required for TIP60 HAT activity and p53-mediated apoptosis [227,283,284]; whereas the E3 ligases HDM2 and CUL3 have been reported to target TIP60 directly for ubiquitin-dependent proteolysis [285,286]. With regards to protein-protein interaction, a few studies have implicated that interaction with viral transforming proteins attenuates TIP60 HAT activity, de-stabilizes TIP60, or abrogates p53- dependent apoptosis [226,287]. Recently, we have identified the p90 protein (also known as CCDC8) to specifically enhance p53-dependent apoptotic response through binding to TIP60 and promoting TIP60-mediated p53 acetylation [288]. Our current study identifies UHRF1 to be another upstream regulator but functions to repress p53-dependent damage response through binding to TIP60, inhibiting TIP60-p53 interaction and TIP60-mediated p53 acetylation. Thus p90 and UHRF1, while both regulating upstream of TIP60 through protein-protein interaction, controls p53 function via distinct and opposing mechanisms.

At present, it is unclear how UHRF1 binding to TIP60 releases p53 from TIP60 interaction and renders TIP60 inactive in acetylating p53. TIP60 comprises

an N-terminal chromodomain and a catalytic MYST domain. It is possible that UHRF1 and p53 compete for the same binding site within TIP60, or that UHRF1 binding induces a conformation change that makes TIP60 inaccessible for p53 binding. Further mapping of UHRF1-TIP60 and TIP60-p53 interaction domains and structural analysis of binding pockets may shed light in this respect. In addition to directly inhibiting TIP60-p53 binding, UHRF1 may change TIP60 conformation to compromise its HAT activity towards p53 and histone H4, the acetylation of which is required on p21 and PUMA promoters but not HDM2 promoter for transcription activation of respective gene targets [201].

Our results also reveal a previously unrecognized mechanism of TIP60 regulation through degradation-independent ubiquitination. Two previous studies report regulation of TIP60 stability by E3 ubiquitin ligases [285,286], which likely contribute to maintenance of low TIP60 protein level in the absence of damage. Here we show that UHRF1 mediates TIP60 ubiquitination, which does not affect protein stability but rather negatively regulates TIP60-p53 interaction and TIP60 acetyltransferase activity. Although ectopic expression of E3 ligase-deficient mutant UHRF1 mildly suppresses the TIP60-p53 interplay, this could be owing to limited amount of cellular TIP60 being ubiquitinated by UHRF1. While physiological functions of degradation independent ubiquitination are not entirely understood, there have been a few studies implicating signal transduction, recruitment of interacting partners and regulation of enzymatic activities [289].

That ubiquitinated TIP60 loses acetyltransferase activity as suggested by our data is an interesting hypothesis and needs to be investigated further. It is also possible that UHRF1-mediated ubiquitination of TIP60 decreases its affinity for p53 or compromises recruitment to chromatin.

Overexpression of UHRF1 is found in a wide array of human tumors, including breast cancer, pancreatic cancer, brain tumor, lung cancer, bladder and kidney cancer, cervical cancer, and colon cancer [263,265-267,269-271]. Recently UHRF1 overexpression has been linked to tumor progression and poor prognosis in prostate cancer and colorectal cancer [277,278]. UHRF1 function in heterochromatin formation and inheritance of genomic DNA methylation patterns has long been implicated as its major oncogenic role. A number of anticancer drugs have been developed to target UHRF1 complex members such as HDAC1 and DNMT1 [270,290], both of which are upregulated in tumors [255,291-296]. However, the presence of multiple members of the HDAC family and the ubiquitous basal expression of HDACs and DNMT1 in normal cells create significant challenge for high specificity and low side effect [270,296]. In contrast to HDAC1 and DNMT1, the basal expression of UHRF1 in normal tissues is significantly lower and almost non-detectable in differentiated tissues [255,297], making UHRF1 a very attractive therapeutic target and suggests that UHRF1 inhibitors, if available, could have fewer side effects than current drugs.

Our study suggests that apart from epigenetic regulation, the oncogenic functions of UHRF1 may also be conferred through inhibition of the TIP60-p53 interplay and p53-dependent damage-induced apoptosis and growth arrest. It will be interesting to test whether UHRF1 overexpression and p53 mutation are mutually exclusive in human tumors, and in tumors with wild-type p53 whether downregulation of UHRF1 or treatment with small molecule inhibitors targeting UHRF1-TIP60 interaction would de-repress the TIP60-p53 interplay and reactivate p53-dependent growth arrest and apoptosis, thereby inhibiting tumor growth.

3.4 Materials and methods

Plasmids

All UHRF1 expression vectors were constructed by PCR amplification from a pET28a-UHRF1 expression vector that was generously gifted from Dr. Zhenghe Wang at Case Western Reserve University. UHRF1 was subcloned into either the pCMV-Myc-N or pCMV-HA expression vectors (Clontech). The 3xFLAG-UHRF1 construct used for *in vitro* translation is also a gift from Dr. Zhenghe Wang. Deletion mutants of UHRF1 were further constructed by PCR amplification from the full length expression plasmids and subcloning into respective vectors. Point mutants of UHRF1 were introduced using the Quikchange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol.

Cell culture

H1299 and U2OS cells were maintained in DMEM (Cellgro) and HCT116 cells in McCoy's 5A medium (Cellgro). All media were supplemented with 10% fetal bovine serum (Gibco), 100 I.U./mL penicillin and 100 ug/mL streptomycin (Cellgro). Transfections with plasmid DNA and siRNA were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol.

Antibodies

Antibodies used in this study include UHRF1 (H-65 and H-8), p53 (DO-1), p21 (SX118), PUMA (H-136), TIGAR (E-2) and Myc (9E10) from Santa Cruz, β-actin (AC-15) and Flag (M2) from Sigma, MDM2 (Ab-5) from EMD Biosciences, HA (3F10) from Roche Applied Science, GFP (JL-8) from Clontech, and α-Ac-p53K120 antibody [201]. α-TIP60 (CLHF) antibody was a generous gift from Dr. Bruno Amati at European Institute of Oncology.

Western blot and immunoprecipitation

For Western blot analysis, cells were lysed in cold RIPA buffer (20mM Tris-HCl, pH8.0, 150mM NaCl, 1% Triton-X100, 1% DOC, 1mM EDTA, 0.05% SDS and freshly supplemented protease inhibitor cocktail).

Co-immunoprecipitation assays were performed as decribed previously (Dai et al., 2011). In brief, cells were lysed in cold BC100 bufffer (20mM Tris-HCl, pH7.9, 100mM NaCl, 10% glycerol, 0.2mM EDTA, 0.2% Triton X-100 and freshly supplemented protease inhibitor) with mild sonication. 5% of cell extracts were saved for input, and the rest was first pre-cleared with A/G PLUS agarose beads (Santa Cruz Biotechnology) at 4ºC with rotation, then incubated with the antibody or control IgG at 4ºC overnight. A/G PLUS agarose beads were then

added for 3 hr incubation at 4ºC. After five washes with the lysis buffer, the bound proteins were eluted by boiling with SDS sample buffer.

For immunoprecipitation of ectopically expressed FLAG-tagged proteins, cells were lysed in Flag lysis buffer (50mM Tris-HCl, pH7.9, 137mM NaCl, 10mM NaF, 1mM EDTA, 1% Triton X-100, 0.2% sarkosyl, 10% glycerol and freshly supplemented protease inhibitor). When detection of acetylated proteins was desired, lysis buffer was freshly supplemented with 2μM trichostatin A and 10mM nicotinamide. Cell extracts were incubated with the monoclonal M2/FLAG agarose beads (Sigma) at 4ºC overnight. After five washes with the lysis buffer, the bound proteins were eluted with FLAG-peptide (Sigma) in BC100 for 2 hr at 4ºC.

siRNA-mediated Ablation of UHRF1, TIP60 and p53

Ablation of UHRF1 was performed by transfection of HCT116 cells or U2OS cells with siRNA duplex oligonucleotides (Silencer Select S26553, S26554, S26555) synthesized by Ambion. Ablation of TIP60 was performed by transfection with a siRNA duplex: 5'-ACGGAAGGUGGAGGUGGUUdTdT-3' and 5'-AACCACCUCCACCUUCCGUdTdT-3' synthesized by Dharmacon. Ablation of p53 was performed by transfection with siRNA duplex oligoset (On-Target-Plus Smartpool L00332900, Dharmacon). Control siRNA (On-Target-Plus

siControl nontargeting pool D00181010, Dharmacon) was also used for transfection.

RNAi transfections were performed 2 times in HCT116 cells and 3 times in U2OS cells with Lipofectamine2000 according to the manufacturer's protocol (Invitrogen).

Apoptosis

Apoptosis was measured by FACs analysis of DNA content. After treatment with DNA damage reagents for the indicated time, dead and live cells were collected by mild trypsinization, washed with PBS, fixed in cold 80% methanol, and stored at -20ºC until stained. After fixation, cells were washed twice with cold PBS and incubated 20 min at room temperature with 50µg/ml of RNaseA in PBS and stained 5min with 25µg/ml of propidium iodide. Flow cytometry was performed with a FACScalibur flow cytometer (BD Biosciences). Analysis was performed using the CellQuest software.

BrdU incorporation

To examine growth arrest, cells were treated with 10 µM BrdU for 1 hr. Cells were then fixed in 4% paraformaldehyde, and costained with the anti-BrdU antibody and DAPI. Cells were then visualized with a fluorescent microscope.

Cell-based Ubiquitination Assay

Cell-based ubiquitination assays were performed essentially as described [105] with some modifitions. H1299 cells were transfected with FLAG-Tip60, HA-UHRF1 and His-ubiquitin. 24 hr post transfection, 10% of the cells were lysed with FLAG lysis buffer and extracts were saved as input. The rest of the cells were lysed with phosphate/guanidine buffer (6M guanidin-HCl, 0.1M Na2HPO4, 6.8mM NaH2PO4, 10mM TrisHCl, pH8.0, 0.2% Triton-X100, freshly supplemented with 10mM β-mercaptoethanol and 5mM imidazole) with mild sonication and subjected to Ni-NTA (Qiagen) pulldown overnight. Ni-NTA captured fractions were then washed with phosphate/guanidine buffer and urea wash buffer (8M urea, 0.1M Na2HPO4, 6.8mM NaH2PO4, 10mM Tris-HCl, pH8.0, 0.2% Triton-X100, freshly supplemented with 10mM β-mercaptoethanol and 5mM imidazole) once each, and further washed 3 times with buffer (8M urea, 18mM Na2HPO4, 80mM NaH2PO4, 10mM TrisHCl, pH6.3, 0.2% Triton-X100, freshly supplemented with 10mM β-mercaptoethanol and 5mM imidazole). Precipitates were eluted by 30 min incubation with Elution buffer (0.5M imidazole, 0.125M DTT) and resolved by SDS-PAGE.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from cells using TRIzol (Invitrogen) and treated with DNase I (Ambion). 2µg of total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis Supermix (Invitrogen) and random primers following the manufacturer's protocol. PCR was performed in triplicates using SYBR green mix (Applied Biosystems) with a 7500 Fast Real Time PCR System (Applied Biosystems). The relative amount of specific mRNA was first normalized to β-actin and then to control sample (ctl RNAi, 0 hr).

For the qRT-PCR analysis of human transcripts the following primers were used: *HDM2* forward 5'-CGATGAATCTACAGGGACGCCATCG-3',

HDM2 reverse 5'-TCCTGATCCAACCAATCACCTG-3';

p21 forward 5'-CCATGTGGACCTGTCACTGTCTT-3',

p21 reverse 5'-CGGCCTCTTGGAGAAGATCAGCCG-3';

PUMA forward 5'-GGTCCTCAGCCCTCGCTCTC-3',

PUMA reverse 5'-GTACGACTTGTCTCCGCCGCTCGTAC-3'.

GST-Pulldown Assay

GST and GST-tagged protein fragments were purified as described previously [236]. ^{35}S-methione labeled proteins were prepared by *in vitro* translation using

the TNT Coupled Reticulocyte Lysate System (Promega). GST or GST-tagged fusion proteins were incubated with *in vitro* translated ^{35}S-methione-labeled proteins overnight at 4 °C in BC100 buffer containing 0.2% Triton X-100 and 0.2% BSA. GST resins (Novagen) were then added, and the solution was incubated at 4 °C for 3 h. After five washes, the bound proteins were eluted for 1.5 hr at 4 °C in BC100 buffer containing 0.2% Triton X-100 and 20mM reduced glutathione (Sigma), and resolved by SDS-PAGE. The presence of ^{35}S-labeled protein was detected by autoradiography.

Immunofluorescent Staining

Cells were washed with lukewarm phosphate buffered saline solution (PBS), and fixed with 4% paraformaldehyde in PBS at 37 °C for 30 min, rehydrated for 5 min in serum-free DMEM, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were incubated in blocking buffer (1% bovine serum albumin (BSA), 5% goat serum in PBS) for 45 min. Primary antibodies (as indicated) were added in blocking buffer for 1 hr at room temperature. After three washes with 1% BSA/PBS, Alexa Fluor conjugated secondary antibodies were added and incubated for 30 min at room temperature. Finally, cells were counterstained with DAPI to visualize the nuclei.

CHAPTER 4

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The critical role of p53 in tumor suppression is underscored by the high mutation rate of the *Tp53* gene or the inactivation of the p53 regulatory pathway in human cancers. As a central coordinator of cellular responses to various stress types, p53 represses the propagation of damaged cells by transcription activation of specific target gene sets that transmit to the appropriate responses, including cell cycle arrest, apoptosis, cellular senescence, DNA repair, energy metabolism, and autophagy. The mechanisms defining which specific target gene sets are activated and which cellular outcomes are adopted upon p53 activation are not fully understood. This ignorance hampers the development of therapies that could employ the apoptotic potential of p53 for the selective elimination of cancer cells.

Interestingly, the recently identified evolutionarily conserved acetylation site within the central DNA binding domain of p53 plays a key role in the selective activation of pro-apoptotic target genes and the apoptotic cell fate. Following acute DNA damage, as is often the case with chemotherapy and radiotherapy, Lys120 undergoes rapid acetylation mediated by the TIP60 acetyltransferase. Abrogation of this modification alone, as seen in the tumor-derived Arginine (R) mutant, completely abolishes the p53-dependent apoptotic response but retains the capactity to induce transient or permanent growth arrest. Although K120 acetylation is indispensable for p53-dependnet apoptosis *in vivo*, the levels of K120 acetylation are dynamically regulated and the interaction between p53 and

TIP60 is quite unstable, indicating that additional regulators may play a role in controlling K120 acetylation and subsequent p53-mediated apoptotic response [225-227].

The identification of p90 and UHRF1 in this study as novel regulators of the TIP60-p53 interplay and TIP60-mediated p53 acetylation shed light on our understanding of how p53 acetylation at the K120 key site is achieved, and also provide insight on the regulation of the p53-mediated apoptotic response.

4.1 p90 and renal cell carcinoma

The differential effect of p90 on p53-mediated cell cycle arrest and apoptosis is of particular interest because it suggests that the level of cellular p90 could dictate the successful elimination of cancer cells through chemo- and/or radiotherapy induced apoptosis in wild type p53 retaining cancers. Importantly, *p90* is one of several candidate tumor suppressor genes identified to be epigenetically inactivated in more than 30% of primary renal cell carcinomas (RCC) using the technique of methylated DNA immunoprecipitation (MeDIP) combined with high-density whole-genome microarray analysis [230]. Inactivation of p90 through RNAi also conferred anchorage-independent growth advantage in the kidney cancer derived HEK293 cells, dubbing p90 as a potential tumor suppressor whose inactivation may play in a role in kidney tumorigenesis. Interestingly, RCC is one of several tumor types that is highly resistant to chemo-

and radiotherapy, probably because RCC commonly retains wild type yet functionally inactive p53 [298]. In fact, in response to genotoxic stress, p53 showed intact protein stabilization, nuclear translocation, and specific DNA binding in RCC [299], but fail to activate transcription. Functional and expression analysis of well known and commonly inactivated p53 regulators reveal that the observed defects of p53 function in RCC are not attributed to HDM2, HDMX or ARF mutation and/or inactivation. This suggests that the repression of p53 transcription activation and the lack of apoptotic response to therapies in RCC are probably conferred through a less well-characterized p53 regulator.

Future investigations are needed to elucidate if inactivation of p90 in RCC contributes, at least in part, to the repression of therapy induced p53-dependent apoptosis. Efforts should also be directed at reactivating p90 in RCC and determining if this may sensitize wild type p53-bearing tumors to chemo- and/or radiotherapies, although the selective reactivation of specific genes without impacting the global chromatin structure and epigenetic profile remains to be a major challenge.

4.2 p90 and post-translational modifications

p90 contains two sites (Ser 199 and Ser 302) (Fig. 5B) that can be potentially modified by the ATM/ATR DNA damage checkpoint protein kinases. ATM/ATR

substrates often contain serine or thereonine residues with glutamine (Q) at the +1 position (the SQ/TQ motif) and immediately preceded by a hydrophobic or negatively charged amino acid [300]. In a large-scale proteomic screen assay performed by the Elledge Group utilizing SQ/TQ peptide immunoprecipitation, phosphorylated S302 peptide derived from p90 was identified as an ATM/ATR substrate [234]. Future efforts are needed to develop phospho-specific antibodies to p90 and validate if p90 undergoes DNA damage induced phosphorylation mediated by the ATRM/ATR kinases. Further investigations are also required to dissect whether p90 phosphorylation plays a modulating role on the p90-p53 interaction, the p90-TIP60-p53 ternary complex formation, or the p53-mediated apoptotic response. It is possible that following DNA damage ATM/ATR mediated phosphorylation functionally regulates p90 to control the decision between cell cycle arrest and apoptosis mediated by p53.

Bioinformatic analysis of p90 reveals a number of additional sites that are potentially modified through amidation, glycosylation, phosphorylation, and myristalation [301]. The specific enzymes catalyzing these modifications and their potential functional roles in regulating the p90-p53 interplay remain to be understood.

4.3 p90 as a potential promoter specific cofactor for p53

Given that p90, TIP60 and p53 can form a ternary protein complex, whether this complex exists on chromatin is an interesting question. In the absence of TIP60 recruitment and histone H4 acetylation, transcription activation is limited to the HDM2 feedback target. When TIP60 is recruited to p53 responsive gene promoters and acetylates histone H4 in the vicinity of the promoter, transcription activation of growth arrest targets such as p21 is enabled; however the full activation of p53 transcription activity and the induction of apoptotic targets such as PUMA require not only histone H4 acetylation but also p53 K120 acetylation mediated by TIP60. It is possible that p90 coexists with TIP60 and p53 at apoptotic target promoters, facilitates K120 acetylation, and serve as a transcription coactivator. Although owing to antibody limitation, we did not detect co-recruitment of p90 at the PUMA promoter, this is an interesting hypothesis that should be further tested should a ChIP quality antibody for p90 becomes available.

4.4 The regulation of TIP60

Downregulation of TIP60 expression has been observed in colon and lung cancers [302], and the *HTATIP* gene encoding TIP60 is a frequent target of mono-allelic loss in human carcinomas, including lymphomas, head-and-neck carcinomas and mammary carcinomas [282]. Because TIP60 can broadly regulate transcription, DNA damage repair, growth arrest and apoptosis, TIP60

acetyltransferase activity should require tight regulation, since even a 2-fold reduction of its activity would result in accelerated lymphomagenesis driven by Myc activation [282] and excessive activation of TIP60 would result in apoptosis [303].

Several studies have demonstrated that TIP60 activity is regulated by acetylation and phosphorylation. The autoacetylation of TIP60, which is augmented by UV radiation and negatively regulated by SIRT1, activates TIP60 HAT activity through facilitating the dissociation of TIP60 oligomers and enhancing substrate binding [227]. The glycogen synthase kinase-3 (GSK-3) phosphorylates TIP60 at S86, and S86 phosphorylation of Tip60 is required for TIP60-mediated acetylation of p53 at K120, histone H4 acetylation at the PUMA promoter, and the induction of PUMA but not p21 transcription [283]. A recent study identified that TIP60 is phosphorlated at Y44 by the c-Abl proto-oncogene in response to damage, and Y44 phosphorylation of TIP60 triggers its acetyltransferase activity towards ATM [304]. c-Abl was previously shown to modulate p53 functions through phosphorylating HDM2 and impairing the inhibition of p53 by HDM2 [305,306]. It would be interesting to test if p53 acetylation by TIP60 is also dependent on Y44 phosphorylation by c-Abl, as this would add another layer of regulation to p53 activation by c-Abl.

The identification of UHRF1 as a p53 regulator upstream of TIP60 also broadens our understanding of TIP60 regulation through ubiquitination. UHRF1 targets TIP60 ubiquitination through atypical ubiquitin lysine linkages and TIP60 ubiquitination mediated by UHRF1 does not promote protein turnover; nevertheless, the ubiquitinated form of TIP60 partially loses its acetyltransferase activity. Interestingly, two recent studies implicate that the USP7 deubiquitinase targets both UHRF1 and TIP60 for removal of ubiquitin chains [252,307]. Given that UHRF1 inhibits TIP60 function through both ubiquitination and direct impairment of the TIP60-p53 interaction, future investigations are required to determine the conditions that favor UHRF1 or TIP60 deubiquitination by USP7. It is likely that the modulation of the UHRF1-TIP60 interplay by USP7 is complex and non-linear, as in the case with USP7 modulation of HDM2/HDMX and p53.

4.5 UHRF1 and cancer therapy

UHRF1 expression is virtually non-detectable in terminally differentiated tissues, but detected exclusively in actively proliferating and/or undifferentiated cells and tissues, such as hematopoietic stem cells, germinal center B cells, and endometrial lining of the uterus [256,297,308,309]. UHRF1 expression is downregulated accompanied by differentiation [256,264,308,310]. Overexpression of UHRF1 is found in a wide array of human tumors, including breast cancer, pancreatic cancer, brain tumor, lung cancer, bladder and kidney

cancer, cervical cancer, and colon cancer [263,265-267,269-271], and the extent of UHRF1 overexpression has been linked to tumor progression and poor prognosis [270,277,278].

The oncogenic role of UHRF1 has traditionally been attributed solely to its function in causing heterochromatin formation and transcription repression at promoters of certain tumor suppressor genes, including *CDKN2A* (encoding $p16^{INK4A}$) [311], *Human Mutant L homologue 1* (*hMLH1*) [312], *Breast Cancer Eearly Onset* (*BRCA1*) [313] and *Retinoblastoma 1* (*RB1* encoding pRB) [314]. The current study adds another layer to the oncogenic role of UHRF1: UHRF1 promotes tumorigenesis and/or tumor progression through inhibition of the TIP60-p53 interplay and p53-dependent damage-induced apoptosis and growth arrest. Future efforts are needed to investigate whether UHRF1 overexpression and p53 mutation are mutually exclusive in human tumors, identify wild type p53 retaining tumors in which UHRF1 is upregulated, and test in these tumors whether downregulation of UHRF1 or treatment with small molecule inhibitors targeting UHRF1-TIP60 interaction would de-repress the TIP60-p53 interplay and reactivate p53-dependent growth arrest and apoptosis, thereby inhibiting tumor growth.

Unlike genetic mutations which are irreversible, epigenetic alterations are reversible and sensitive to environmental conditions including nutritional changes

[315,316], making them interesting therapeutic targets. To date two large families of specific inhibitors have been developed to target DNMT1 and HDAC1 [290], key partners of UHRF1 and components of the epigenetic regulating complex that are also upregulated in cancer [255,291-293,295]. For instance, the HDAC inhibitor Vorinostat (Zolinza™, Merck, NJ) received approval by the US Food and Drug Administration (FDA) in 2006 and have shown efficacy for treatment of cutaneous T-cell lymphoma (CTCL) [290]; several other HDAC inhibitors are in clinical trial and may follow soon. However the presence of 11 classical members of the HDAC family and the basal expression of HDACs in normal cells and tissues create significant challenge for high specificity: the currently available HDAC inhibitors target all or at least several of the 11 classical HDAC family members [291,296]. Cytosine analogues are another type of chemotherapeutic drugs targeting the UHRF1 complex. They work through occupying the cytosine recognition pocket of DNMT1, thereby inhibiting DNMT1 activity and promoting proteasome degradation [255]. Two such commercially available DNMT1 inhibitors are azacitidine (Vidaza™, Phamion, CO) and decitabine (Dacogen™, SuperGen, CA, and MGI Pharma, MN). However because DNMT1 is ubiquitously expressed, including in vital organs such as the heart and kidney, cytosine analogues causes various adverse reactions and impose risks for causing genome-wide hypomethylation that contributes to further aggravation of cancer [255].

Considering that UHRF1 is required for DNMT1 and HDAC1 to fully exert their effects, and that UHRF1 has the additional function of suppressing the TIP60-p53 interplay, inhibition of UHRF1 would theoretically not only mimic the cumulative effects of HDAC1 and DNMT1 inhibitors, but also reactivate the p53 pathway to achieve killing of cancer cells. Furthermore, in contrast to HDAC1 and DNMT1, the basal expression of UHRF1 is almost non-detectable in normal tissues [255,297] and no expression of UHRF1 is observed at the protein level in vital organs including heart, lungs, liver, kidneys, and bladder [270]. This offers a foreseeable advantage that UHRF1 inhibitors may display a higher selectivity for tumor cells and have fewer side effects than currently available drugs targeting HDAC1 or DNMT1. One of the potential strategies for interfering with UHRF1-TIP60 binding would be the utilization of a permeable dominant negative peptide that is a partial region of UHRF1 or TIP60 in their respective binding motifs. Similar approaches have proven successful in the treatment of breast cancer: a peptide derived from AMAP1 specifically blocked AMAP1-cortactin binding and effectively inhibited breast cancer invasion and metastasis [317].

4.6 Acetylation is required for all major steps of p53 activation

Based on current knowledge of p53 research reviewed in this thesis and the results in this study, we propose that the key steps in p53 transcription activity are

sequence specific DNA binding, de-repression from its key inhibitor HDM2, and promoter specific recruitment of corepressors/coactivators.

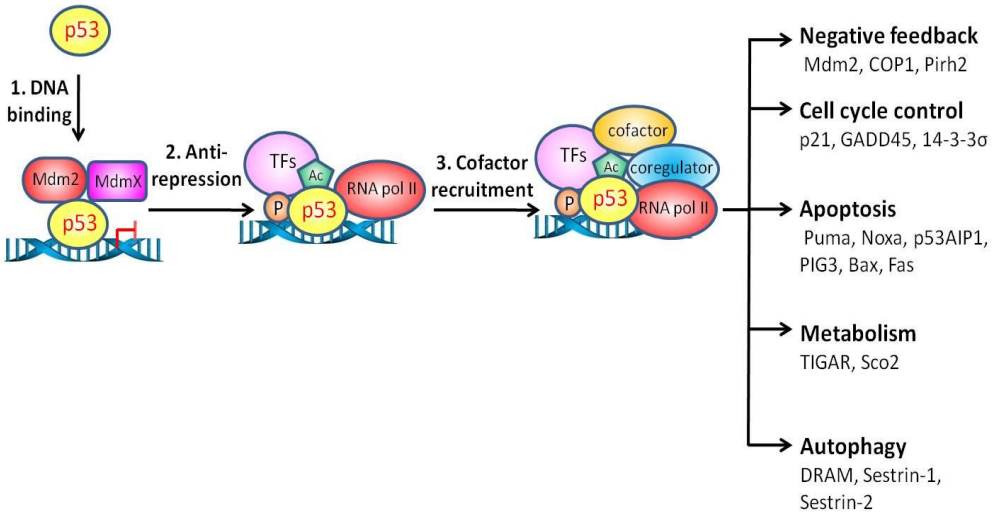
Importantly acetylation is required for all the major steps of p53 activation: (i) p53 acetylation at the C-terminus enhances sequence-specific binding to DNA at target gene promoters [318]; (ii) C-terminal and K164 acetylation disrupts the repressive HDM2-p53-DNA complex on chromatin; and (iii) K120 acetylation is required for the full induction of transcription activity of p53 on pro-apoptotic targets and acetylated p53 recruits additional cofactors that are required for promoter specific transcription activation.

The importance of p53 cofactors in regulating target specificity has been implicated by several studies. For example, the ankyrin-repeat, SH3-domain- and proline-rich-region-containing proteins (ASPP) family, consisting of two pro-apoptotic mediators (ASPP1 and ASPP2) and one anti-apoptotic mediator iASPP, specifically regulates the p53-mediated apoptotic response. ASPP1 and ASPP2 selectively stimulates the apoptotic function of p53 by enhancing p53 binding to *BAX*, *PUMA* and *PIG3* promoters [319], whereas iASPP counteracts the effects of ASPP1 and ASPP2 and inhibits p53-mediated apoptosis [320]. Interestingly the expression ratio of ASPP1/2 to iASPP correlates with the cellular sensitivity to apoptosis-inducing drugs [320]. In contrast, the hematopoietic zinc finger (HZF) protein, through direct interaction with the p53 DNA binding domain, enhances

p53 binding to *p21* and *14-3-3σ* promoters and attenuates PUMA and BAX expression, thereby favoring the pro-survival cell fate [321]. This current study identifying p90 to be another cofactor of p53 that stimulates TIP60-dependent acetylation at K120 and is specifically required for p53-mediated apoptosis broadens our knowledge of p53 cofactors and supports the importance of cofactor-determined promoter specificity.

Figure 34. Three-step activation of p53 transcriptional activity

p53 transcriptional activity is activated through three sequential steps: (i) sequence-specific DNA binding; (ii) anti-repression; and (iii) cofactor recruitment. Under homeostasis, p53 is bound to target gene promoter DNA but is repressed by Hdm2 and HdmX. Cellular stress triggers phosphorylation and acetylation at key p53 residues and facilitates the release of p53 from Hdm2 and HdmX mediated repression. The exact combinations of cofactors and post-translational modifications present on p53 provide promoter specificity. Anti-repression alone is sufficient for the induction of the p53 negative feedback loop. Cell cycle control requires partial activation of p53 through further modifications. Apoptotic activation requires the full activation of p53 activity via specific cofactors and an array of modifications. The control of p53 transcriptional regulation of metabolism and autophagy remains to be understood. Important p53 target genes for each cellular outcome are listed on the right. Abbreviations: TFs, transcription factors; P, phosphorylation; Ac, acetylation.



4.7 Other modifications/cofactors for p53 regulation of metabolism, antioxidant defense and autophagy

It is noteworthy that although the best studied function of p53 is its control of temporary and permanent growth arrest and apoptotic cell death, ever-increasing evidence demonstrates that p53 regulates cellular metabolism, antioxidant defense, and autophagy [322-326]. A complete review of p53 regulation of target genes involved in these processes is beyond the scope of this thesis; instead, a few examples would be visited briefly.

Cancer cells adopt a fundamentally different metabolic profile to sustain their rapid growth and survival in the stressful and dynamic microenvironment of the solid tumor [327]. One of the best characterized metabolic phenotypes of cancer cells is the "Warburg effect", which describes the shift of energy generation from oxidative phosphorylation to aerobic glycolysis [328]. In normal cells, incoming glucose is converted through glycolysis to pyruvate, which then enters the mitochondrial tricarboxylic acid (TCA) cycle for maximal ATP generation through oxidative phosphorylation. Cancer cells, however, convert most of the incoming glucose to lactate even when oxygen is plentiful (thus termed "aerobic glycolysis"); this provides a high flux of biosynthetic substrates for macromolecule building, therefore offering a biosynthetic advantage to cancer cells [327]. Because ATP generation from aerobic glycolysis is less efficient in

terms of molecule of ATP generated per unit of glucose consumed, this must be compensated by a higher rate of glycolysis. Indeed cancer cells demand a significantly higher rate of glucose uptake and proliferating cancer cells typically have glycolytic rates up to 200 times higher than cells derived from their normal tissue of origin.

p53 is known to downregulate glucose metabolism via transcription of the TP53-Induced Glycolysis and Apoptosis Regulator (TIGAR) [324]. TIGAR is an inhibitor of fructose-2,6-bisphosphate (Fru-2,6-P2), which strongly stimulates glycolysis at the third step of glucose breakdown through allosteric modulation of phosphofructokinase 1 (PFK-1) [329]. TIGAR directs glucose metabolism away from glycolysis and towards the pentose phosphate shunt, generating maximal levels of NADPH thus causing an increase in cellular glutathione (GSH), which acts as Reactive Oxygen Species (ROS) scavengers. ROS quenching protects the cell from p53-mediated apoptosis as a result of genotoxic stress [324]. Furthermore, increased NADPH inhibits apoptotic effector caspases, thus also contributing to the neutralization of the p53 apoptotic response [329].

Glutaminase 2 (GLS2) is a p53 transcription target that plays important roles in the antioxidant defense mechanism. As a key enzyme in the conversion of glutamine to glutamate [326,330], GLS2 facilitates glutamine metabolism and synthesis of the glutathione antioxidant, thus lowering the levels of intracellular

ROS and decreasing overall DNA oxidation. By transcriptionally activating GLS2, p53 fights intracellular ROS, and prevents the accumulation of genomic damage, allowing cell survival after mild and repairable genotoxic stress [326].

Autophagy allows recycling of intracellular constituents as an alternative energy source during periods of metabolic stress, thereby enabling homeostasis and viability [331]. Nuclear p53 induces autophagy following genotoxic stress by transcriptionally upregulating the mammalian Target Of Rapamycin (mTOR) inhibitors, Phosphatase and Tensin homolog (PTEN) and Tuberous Sclerosis 1 (TSC1), or the p53-regulated autophagy and cell death gene Damage-Regulated Autophagy Modulator (DRAM) [323,325], whereas basal levels of cytoplasmic p53 inhibit autophagy through transcription-independent mechanisms such as AMP-Activated 10 Protein Kinase (AMPK) activation and mTOR inhibition [138,325,332].

Interestingly, although loss of acetylation abolished p53-mediated transcription of canonical target genes involved in cell cycle arrest, apoptosis and senescence, the activation of metabolic and antioxidant defense targets such as TIGAR and GLS2 is retained by the $p53^{3KR}$ mice [98], likely contributing to the suppression of early onset spontaneous tumorigenesis. Because the role of p53 in regulating metabolism, antioxidant defense and autophagy has only recently begun to be appreciated, *in vitro* and *in vivo* studies of p53 post-translational

modifications and their mediators have generally overlooked these aspects of p53 function. Future efforts are required to further elucidate the exact combinations of post-translational modifications and the cofactors recruited for the transcription activation of such targets. Furthermore, previously generated p53 mutant mice and any future mouse models should also be carefully investigated for p53 transcription dependent and -independent functions on metabolism, antioxidant defense and autophagy.

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