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Dissecting The Interaction Between P53 And Trim24

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A

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It always seems impossible until it's done.
~Nelson Mandela

This thesis is dedicated to those who came before me, instilled in me the morals, curiosity, creativity and discipline to be an exceptional human being, and sacrificed to make my future limitless.
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Dissecting the Interaction of p53 and TRIM24

Aundrietta DeVan Duncan

Supervisory Professor, Michelle Barton, Ph.D.

p53, the “guardian of the genome”, plays an important role in multiple biological processes including cell cycle, angiogenesis, DNA repair and apoptosis. Because it is mutated in over 50% of cancers, p53 has been widely studied in established cancer cell lines. However, little is known about the function of p53 in a normal cell. We focused on characterizing p53 in normal cells and during differentiation. Our lab recently identified a novel binding partner of p53, Tripartite Motif 24 protein (TRIM24). TRIM24 is a member of the TRIM family of proteins, defined by their conserved RING, B-box, and coiled coil domains. Specifically, TRIM24 is a member of the TIF1 subfamily, which is characterized by PHD and Bromo domains in the C-terminus. Between the Coiled-coil and PHD domain is a linker region, 437 amino acids in length. This linker region houses important functions of TRIM24 including it’s site of interaction with nuclear receptors. TRIM24 is an E3-ubiquitin ligase, recently discovered to negatively regulate p53 by targeting it for degradation. Though it is known that Trim24 and p53 interact, it is not known if the interaction is direct and what effect this interaction has on the function of TRIM24 and p53. My study aims to elucidate the specific interaction domains of p53 and TRIM24. To determine the specific domains of p53 required for interaction with TRIM24, we performed co-immuoprecipitation (Co-IP) with recombinant full-length Flag-tagged TRIM24 protein and various deletion constructs of *in vitro* translated GST-p53, as well as the reverse. I found that TRIM24 binds both the carboxy terminus and DNA binding domain of p53. Furthermore, my results show that binding is altered when post-translational modifications of p53 are present, suggesting that the interaction between p53 and TRIM24 may be affected by these post-translational modifications. To determine the specific domains of TRIM24 required for p53 interaction, we performed GST pull-downs with *in vitro* translated, Flag-TRIM24 protein constructs and recombinant GST-p53 protein purified from *E. coli*. We found that the Linker region is sufficient for interaction of p53 and TRIM24. Taken together, these data indicate that the interaction between p53 and TRIM24 does occur *in vitro* and that interaction may be influenced by post-translational modifications of the proteins.

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Introduction:

Tumor suppressor *p53* is the most highly mutated gene in human cancer, and as “Guardian of the Genome” may be the most extensively studied (1,2). Remarkably, p53 was not always thought to be a tumor suppressor. First described in 1979, p53 was reported as an oncogene, because of its high expression levels in cells transformed by Simian Virus 40 (SV40) (3). Further investigation demonstrated its role as pro-apoptotic, and therefore a potential tumor-suppressor gene. Over the past 30 years, p53’s repertoire of functions expanded from a mere anti-cancer protein to a master regulator of gene expression, with involvement in cell cycle, DNA repair, aging, implantation, development, immunity, to even metabolism (3,4). It is striking that one molecule has the capability of regulating such a wide variety of biological functions. p53 can influence both positively and negatively the expression of hundreds of target genes by its function as a transcription factor, thereby affecting numerous downstream pathways (5).

Human p53 comprises 393 amino acids with five functional domains (Figure 1), including: the Transactivation domain (amino acids [aa] 1-42); the Proline Rich domain (aa 63-97), which is important for human p53 apoptotic function; the DNA binding domain (aa 98-292), which allows p53 to interact with its gene targets to effect transcription; the tetramerization domain (aa 342-355), essential for p53 to oligimerize, required for the function of p53; and the

Negative regulatory domain (aa 363-393) (6-11). Additionally, there are two Nuclear Export signals, one located

Figure 1: Schematic Representation of p53 Functional Domains

TA: Transactivation domain (amino acids [aa] 1-42); **PR:** Proline Rich domain (aa 63-97); **DNA Binding**: DNA binding domain (aa 98-292); **Tet:** tetramerization domain (aa 342-355); **Neg:** Negative regulatory domain (aa 363-393).

at the N-terminus (aa 11-27), the other at the C-terminus (aa 320-355) and one Nuclear Localization signal (aa 300-323) (12-14). p53 is mutated in over 50% of cancers and as a result may become non-functional. Many of the so-called “hot spots” of p53, codons where large numbers of tumor causing mutations are grouped, are located in the DNA binding domain (15,16). Moreover, these mutations occur at amino acids that are conserved throughout evolution, between trout, Xenopus, chicken, mouse, rat, monkey, and human, suggesting that these amino acids play an important role in p53’s structure and therefore its function (16). The high incidences of mutations of the DNA binding domain of p53 in cancer indicates the important function of p53 as a transcriptional regulator and thus why it is so important for p53 to always be functional and yet always be regulated.

Amongst numerous functions of p53, are its action to facilitate apoptosis and inhibit cell cycle progression. p53 is essential for cells to repair damaged DNA or to lead cells to programmed cell death when DNA damage can not be overcome, but if left unchecked p53 can have deleterious affects on the cell, therefore p53 protein levels must be finely regulated. The cell has an elegant system for controlling protein levels, by covalent addition of one or a chain of ubiquitin molecules (17). The number of ubiquitins determines the fate of the protein. For example, mono-ubiquitinated p53 is shuttled out of the nucleus into the cytoplasm, whereas poly-ubiquitinated p53 is directed to the 26S proteasome and degraded (17). Ubiquitination occurs at a specific Lysine reside on the target protein and linkages of additional ubituitin molecules at the

Lysine48 residue of the preceding ubiquitin molecule signals for degradation of the protein. Attaching ubiquitin to a protein requires the action of a cascade of enzymes which have specific functions: ubiquitin-activating (E1), ubiquitin-conjugating, (E2), and ubiquitin-ligating (E3) (17). The E3 ubiquitin ligase interacts directly with the E2, conjugated to ubiquitin and the target protein, or substrate and facilitates transfer of ubiquitin from the E2 to the protein, in a specific manner. One major class of E3 ubiquitin ligases is the really interesting new gene (RING) domain proteins. These RING domains have a consensus sequence of Cys-X2-Cys-X(9-39)-Cys-X(1-3)-His-X(2-3)-Cys-X2-Cys-X(4-48)-Cys-X2-Cys (X is any amino acid), whose three-dimensional structure can bind two zinc molecules and additional proteins. Ubiquitination is a stepwise process that begins with the E1 “charging” the E2 with ubiquitin; then the RING E3-ubiquitin ligases recruit and directly bind E2 ubiquitin-conjugating enzymes. Ubiquitin is transferred from E2 to the substrate. When forming polybiquitin chains, E2 dissociates from E3 and a new ubiquitin-ligated E2. Once polyubiquitin chains are formed, ubiquitin receptors bind and direct tagged protein to the proteasome where the protein is degraded, but the uniquitin molecules are recycled (17).

A widely studied RING E3-ubiquitin ligase is MDM2 (murine double minute 2), the predominant negative regulator of p53. It has four functional domains: the p53 binding domain (aa 1-110) through which MDM2 interacts with the transactivation domain of p53, the acidic domain (aa 210-300), the Zinc finger domain (aa 300-330) and finally the RING domain (aa 420-484), that has

the E3 ubiquitin ligase activity (18). MDM2 ubiquitinates p53, to target it for proteasomal degradation, and autoubiquitinates itself, through the E3 ligase activity of its RING finger (17-21). While this interaction is direct, post-translational modifications (PTMs) can affect the binding of p53 to MDM2(11, 22).

In response to cellular stress, p53 undergoes PTM including phosphorylation, acetylation, glycosylation, sumoylation, and ubiquitination (11). PTMs are added by an array of modifying enzymes and can influence p53's interaction with its regulator (23). Some enzymes can modify multiple residues, while others only modify one specific residue. Conversely, many residues are capable of undergoing multiple modifications by multiple enzymes. For example, in response to DNA damage, by IR or UV radiation, ATM and ATR protein kinases are activated. These kinases, in turn activate downstream checkpoint kinases (Chk1 and Chk2), which ultimately phosphrylate p53 at serines 6, 9, 15, 20 and 37, within the transactivation domain of p53. These phosphorylation events disrupt p53's interaction with MDM2; therefore, p53 protein can no longer be ubiquinated. This allows protein levels to accumulate and translocate into the nucleus to regulate the transcription of target genes (24, 25). Moreover, sumoylation of MDM2 inhibits autoubiquitination, which leads to stabilization of MDM2 protein and increased ubiquitination of p53, resulting in decreased p53 protein levels (26). The relationship of MDM2 and p53 is a well characterized interaction; however it has been primarily studied in established cancer cell lines.

Even with the numerous studies of p53, little is known about p53's role and regulation in a normal, non-cancerous system. To elucidate the role of p53 in normal cells, the Barton lab created a mouse embryonic cell line that expressed endogenously regulated p53 protein fused with a C-terminal Tandem Affinity Purification (TAP) tag (27). This powerful tool allowed them to purify endogenous p53, using TAP-chromatography. Mass spectrometry analysis not only confirmed the identity of p53 and two well established binding partners, MDM2 and 53BP1, but also identified a novel binding partner, Tripartite Motif Family Member 24 (TRIM24). Analysis of the novel interaction between p53 and TRIM24 further characterized TRIM24, which was previously identified as a gene target of p53 through a high throughput screen, as a negative regulator of p53 (27, 28).

TRIM24 was originally identified as a fusion partner of B-Raf in mouse hepatocellular carcinoma (HCC). Located on human chromosome 7q32, *TRIM24* encodes a 116kDa protein, which plays a role in cellular differentiation, development, and homeostasis (29, 30). The protein has five distinct functional domains (Figure 2). Beginning at the N-terminus, the RING, BBoxes and Coiled-coil domains make up the consensus tripartite motif, from which the TRIM family gets its name (28, 29). The RING domain acts as an E3 ubiquitin ligase, ubiquitinating p53, while the B-boxes are zinc finger domains and the coiled-coil domain is required for hetero-oligimerization with other Transcription Intermediary Factor (TIF) family members, including TRIM28 (32-34). Specific to the TIF subgroup to which TRIM24 (also known as TIF1α) belongs, are the C-

terminal PHD and Bromo domains (28,31). The PHD and Bromo domains are key to TRIM24's function as a chromatin reader, as they can interact with both unmodified lysine 4 on histone H3 (H3K4me0) and acetylated lysine 23 on histone H3 (H3K23ac) (35). Additionally, between the Coiled-coil and PHD domains is a 435 amino acid linker region which includes an LXXLL motif (aa 760-765), through which TRIM24 acts as a co-repressor of the retinoic acid (RA) receptor, thereby repressing RA signaling (30).

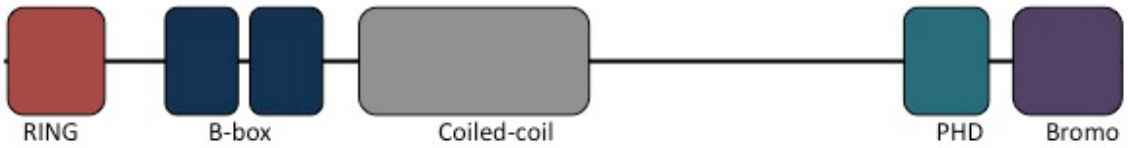

Figure 2: Schematic Representation of TRIM24 functional domains

Interestingly, TRIM24 and p53 have an opposing relationship, similar to that of MDM2 and p53 (27). At steady state, TRIM24 is present, keeping p53 protein levels down. When the cell is insulted with stress, such as DNA damage, p53 is stabilized and TRIM24 protein levels decrease. However, because TRIM24 is a target gene of p53, when p53 levels increase, it in turn activates transcription of *TRIM24*, which leads to more TRIM24 protein production which brings about the decrease in p53 protein (Figure 3). This observation suggests the existence of a negative regulatory loop between p53 and TRIM24. Understanding the way in which MDM2 and p53 physically interact has been pivotal in addressing the regulation of p53 and the role modifications and mutations can play in affecting the interaction and function of p53 and MDM2. Additionally, knowing where p53 interacts with its negative regulator(s), may be useful in creating drugs specifically targeting the interaction, resulting in re-activation of p53 (36).

Little is known about the nature of the interaction between p53 and TRIM24: where they interact and whether the interaction is direct; does the interaction require the presence of additional proteins or is affected by post-translational modifications. I hypothesize that the interaction between p53 and TRIM24 is direct and requires specific functional domain(s) of each protein. To test this hypothesis, I took advantage of the rabbit reticulocyte lysate system and recombinant proteins to perform a series of GST pull-downs or immunoprecipitations to elucidate the specific domain(s) of the interaction of p53 and TRIM24. The results of these experiments will be described here.

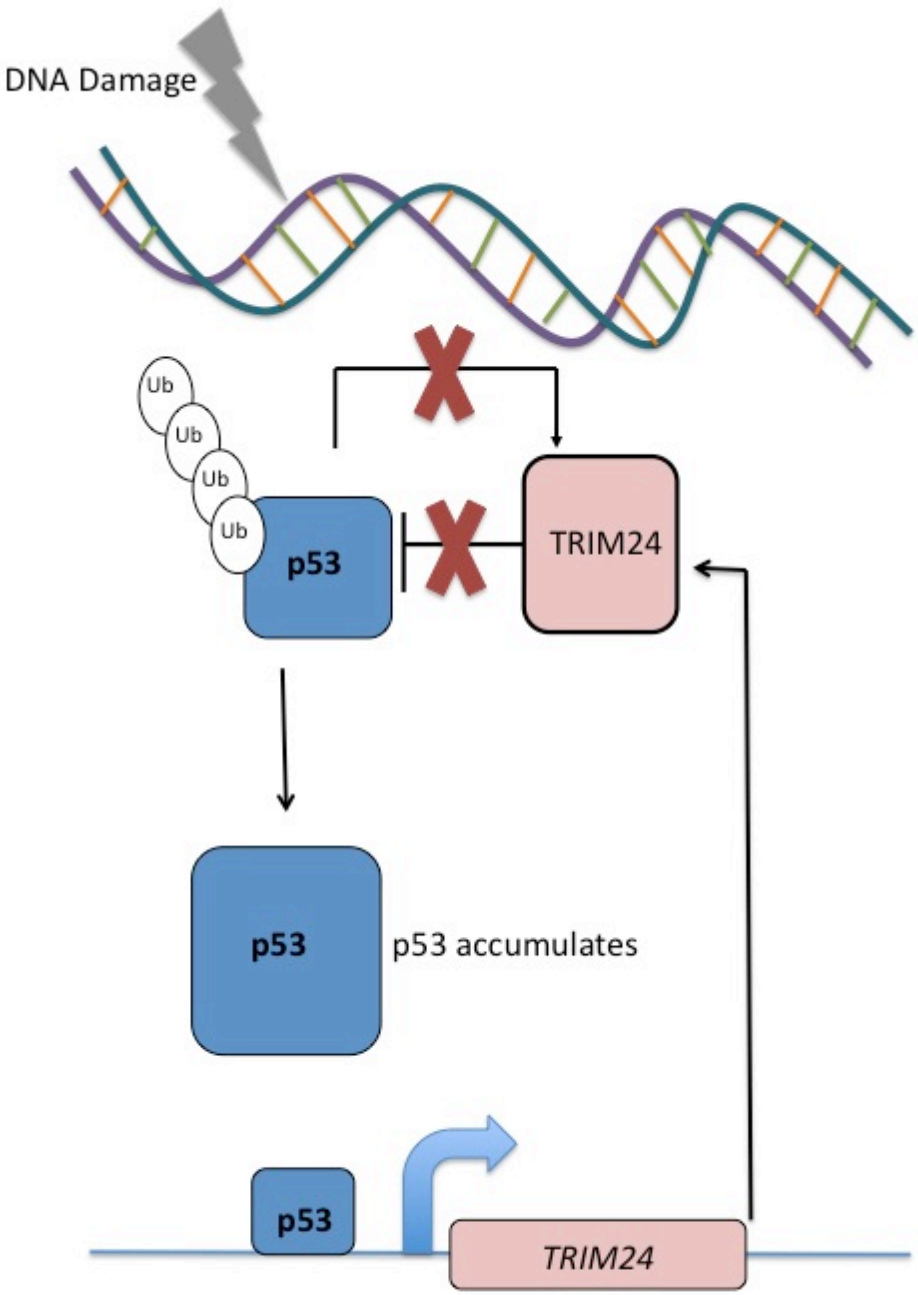

Figure 3: Diagram of the p53-TRIM24 negative regulatory loop.

During DNA damage,, the ubiquitination of p53 by TRIM24 is inhibited, allowing p53 to accumulate and bind the *TRIM24*, up-regulating expression, ultimately causing TRIM24 to again ubiquitinated p53.

Materials and Methods:

Solutions used in the following studies:

NTEP: 25mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 0.5% NP-40

NETN150: 50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40

NETN300: 50mM Tris-HCl pH 7.5, 300mM NaCl, 1mM EDTA, 0.5% NP-40

GST Purification Lysis Buffer: 1% Triton X, 0.1% β-merceptoethanol, 1mM PMSF, 1X Protease Inhibitors, PBS

GST Purification Lysis Buffer + NaCl: 1% Triton X, 0.1% β-merceptoethanol, 150mM NaCl,1 mM PMSF, 1X Protease inhibitors, PBS

Wash Buffer: 10mM Tris-HCl pH 8.0, 150mM NaCl, 2mM $MgCl_2$, 15% glycerol, 1mM DTT, 0.4mM PMSF, 1x Protease Inhibitor

Binding Buffer A: 10mM Tris-HCl pH 7.5, 150mM NaCl, 2mM $MgCl_2$, 15% glycerol, 4mM PMSF

2X Luria-Bertani Broth (2XLB): 20g/L tryptone, 10g/L yeast extract, 10g/L NaCl

List of Abreviations:

Flag-T24FL – Flag-TRIM24 Full Length

Flag-T24ΔR – Flag-TRIM24 ΔRING domain

Flag-T24ΔBBI – Flag-TRIM24 ΔRING and ΔBBox I domain

Flag-T24ΔBBII – Flag-TRIMM24 ΔRING, ΔBBoxes I and II

Flag-T24ΔBBC – Flag-TRIM24 ΔRING, ΔBBoxes I and II, and ΔCoiled-coil domains

Flag-T24PB - Flag-TRIM24 ΔRING, ΔBBoxes I and II, ΔCoiled-coil domains, and ΔLinker region

Flag-T24Bromo - Flag-TRIM24 ΔRING, ΔBBoxes I and II, ΔCoiled-coil domains, ΔLinker region, and ΔPHD domain

Flag-T24CC-L – Flag-TRIM24 coiled-coil domain and Linker region

Flag-T24Link – Flag-TRIM24 linker region only

GST-p53FL – GST-p53 Full Length

GST-p53ΔC – GST-p53 Δ30 aa from C-term (aa 1-363)

GST-p53C-term – GST-p53 C-terminus only (aa 286-393)

GST-p53N-term – GST-p53 N-terminus only (aa 1-110)

GST-p43DNAB – GST-p53 DNA Binding domain only (aa 91-295)

GST-p53DNAB/Tet – GST-p53 including end of DNA binding domain and half of the Tetramerization domain (aa 286-330)

GST -p53Tet/Neg – GST-p53 Tetramerization domain and half of the Negative regulatory domain (aa 328-380)

GST-p53Neg – GST-p53 Negative regulatory domain only (aa 361-393)

Plasmid construction:

The pCMX(flag)$_2$vector and pCMX-Flag-T24FL, pCMX-Flag-T24ΔR, pCMX-Flag-T24ΔBBI, pCMX-Flag-T24ΔBBII, pCMX-Flag-T24ΔBBC, pCMX-Flag-T24PB, and pCMX-Flag-T24Bromo were gifts from Dr. Abhinav Jain.

The T24CC-L construct was cloned into the pCMX(Flag)$_2$ vector using the following primers:

5'-GCG CTC TAG AAG ATC TGC TTT TCA GAA TCA GAA
3'- CGC GAT ATC CTC GAG TTA ATT GGG GTC ATC CTC

The Flag-T24Link construct was cloned into the pCMX(Flag)$_2$ vector using the following primers:

5'- GCG CTC TAG AAG ATC TAC CCA CCA CAC CAT CCA
3'- CGC GAT ATC CTC GAG TTA ATT GGG GTC ATC CTC

The pET19-GST-p53 plasmids were a generous gift from Dr. Mitchell Smith, previously published in Molecular and Cellular Biology, January 2008, Vol. 28, No.1, p.140-153.

GST protein purification.

BL-21AI (Invitrogen catalog# 44-0184) cells were transformed with GST-p53 constructs. Clones were selected and expanded in 2X LB and induced with 0.2% L-Arabinose and 100uM $ZnSO_4$, for 6 hours at 16°C. Cells were lysed in GST Purification Lysis Buffer. Non-specific proteins were removed by washing with GST purification lysis buffer with 150mM NaCl. Final proteins remained on Glutathione Sepharose bead (GE) in Lysis Buffer with 150mM NaCl.

Flag-TRIM24 protein purification.

Sf9 (Invitrogen catalog# 11496-015) cultures were infected with a baculovirus expressing Flag-TRIM24 at a 1:100 dilution for 72 hours at 27°C . Cells were collected and lysed with Baculovirus Purification Lysis Buffer F and bound to M2 (αFlag) beads (Sigma) in Baculovirus purification dilution buffer for 4 hours at 4°C. Non-specific protein were washed away with Baculovirus purification wash buffer. Purified proteins were visualized by both Coomassie and αFlag western blot.

In vitro Transcription/Translation

^{35}S-labled proteins were synthesized using the Promega TNT® Quick Coupled Transcription/Translation System. For a standard reaction, 500ng of DNA was added to the rabbit reticulocyte lysate Master Mix with ^{35}S-Methionine. Lysates were incubated at 30°C for 1.5 hours. Proteins were separated by SDS-PAGE

gels and exposed to an autoradiograph screen overnight. Sizes were confirmed and relative protein concentrations were determined by the Storm840 and Storm Scanner control Version 5.03 software (Amersham Biosciences).

Recombinant Co-Immunoprecipitation

500ng of recombinant GST-p53 or His-ERα protein, purified from *E.coil* was bound to 2ug recombinant Flag-TRIM24 (purified from baculovirus; on M2 bead) for 1.5 hours at 25°C, in 500uL NETN150. The M2 beads were washed 2X with NETN300, then washed 1X with NETN150. Proteins were removed from the beads by boiling, then separated by SDS-PAGE and bound proteins were visualized by immunoblotting for p53 (DO-1HRP Santa Cruz cat# sc-126 HRP 1:2,500 dilution), ERα (F-10 Santa Cruz cat# sc-8002 1:2,500 dilution), and Flag (αFlag M2-HRP Sigma-Aldrich cat# A8592).

GST-pull down

5μL lysate of *in vitro* translated TRIM24 protein was incubated with 2ug GST or GST-p53 (on Glutathione bead) for 1.5 hours at room temperature. Beads were washed 1x with NETN300 and 2X with NETN150. Beads were boiled in SDS loading dye, then proteins were separated by SDS-PAGE and the resulting gel was stained with Coomassie blue, de-stained and dried. The resulting, stained gel was then exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840 and Storm Scanner control Version 5.03 software (Amersham Biosciences).

Results:

The goal of this thesis was to define the interacting domains of p53 and negative regulator TRIM24. To address the directness of this interaction, these studies were carried out *in vitro*.

TRIM24 and p53 interact in vitro

The identification of TRIM24 as a binding partner of p53 was performed with mouse embryonic stem cells and confirmed in multiple cell lines including HEK293T, MCF7 and U2OS (23). However, the interaction between TRIM24 and p53 had yet to be demonstrated *in vitro* with recombinant proteins. To determine if TRIM24 and p53 directly interact *in vitro*, each of the proteins was expressed by *in vitro* translation or in either *E. coli* or Sf9 and further purified. The resulting proteins were then used for interaction studies by immunoprecipitation or GST pull-down assays.

Wild type GST-p53 was *in vitro* translated and labeled with ^{35}S-methionine. Flag-TRIM24, purified from Sf9 baculovirus was a gift from Kendra Allton. To assess the interaction between TRIM24 and p53 *in vitro*, ^{35}S-labeled GST-p53 was incubated with recombinant Flag-TRIM24 or flag beads only, as a negative control. As shown in Figure 3, GST-p53 specifically interacts with Flag-TRIM24. There is little or no non-specific interaction with the flag beads alone (lane 2), and the specific interaction of GST-p53 to Flag-TRIM24 is significantly

above that of flag beads only, which is taken as background (compare lanes 2 and 3).

To further confirm this interaction I did the reverse experiment. Flag-TRIM24 was *in vitro* translated and labeled with ^{35}S-Methionine and GST-p53 was purified from BL21-AI competent bacterial cells. ^{35}S-labeled Flag-TRIM24 was incubated with GST-p53 protein or GST alone, as a negative control. As shown in Figure 5, GST-p53 specifically interacts with Flag-TRIM24 (lane 3) *in vitro* while Flag-TRIM24 does not interact with GST protein alone (lane 2). This result further demonstrates that p53 and TRIM24 do interact *in vitro*. It is important to note that although equal amounts of protein were used for this experiment (Figure 5) and the former experiment (Figure 4), immunoprecipitating Flag-TRIM24 recovers more p53, than GST-p53 recovery of Flag-TRIM24. This result is seen repeatedly and could be an issue associated with the rabbit reticulocyte lysate, because it has other proteins that could interact with p53.

The interaction of p53 and TRIM24 is indirect

Although the previous results show the *in vitro* interaction of p53 and TRIM24, each experiment was performed in the presence of rabbit reticulocyte lysate, which may contain proteins that could contribute to the formation of a protein complex (37). To determine if the interaction between p53 and TRIM24 is direct and occurs without additional proteins, an *in vitro* binding assay was performed using recombinant purified proteins. Flag-TRIM24, purified from Sf9 cells, was incubated with GST-p53, purified from *E. coli*, or Estrogen Receptor α

(ERα), as a positive control. As shown in Figure 6, Flag-TRIM24 has a strong interaction with ERα, but it interacts with GST-p53 less than GST-p53 interacts with Flag beads only, used as background. This result was reproduced 4 times and did not change with varying amounts of Flag-TRIM24. From this we can conclude that while the interaction p53 and TRIM24 does occur *in vitro*, it does not occur without the assistance of additional binding partners, or may require post-translational modifications (PTMs) of one or more protein substrates.

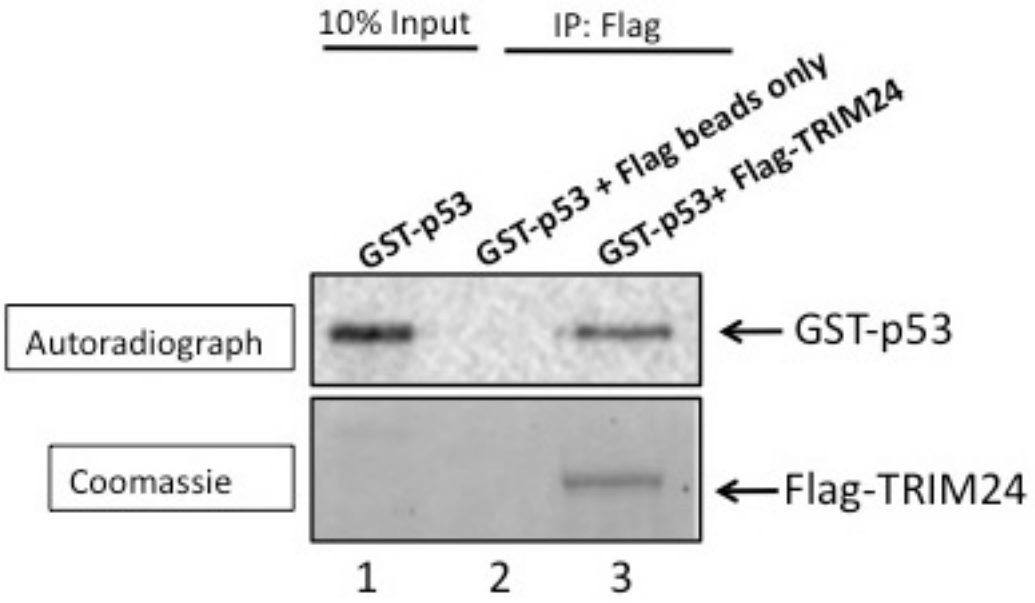

Figure 4: *In vitro* translated GST-p53 interacts with recombinant Flag-TRIM24 purified from baculovirus.
5µL lysate of *in vitro* translated GST-p53 protein was bound to 2ug Flag-TRIM24 or the equivalent volume of M2 flag beads for 1 hour at 25°C. Proteins were separated by SDS-PAGE, stained with Coomassie blue, then exposed to autoradiograph overnight and visualized using the Storm840.

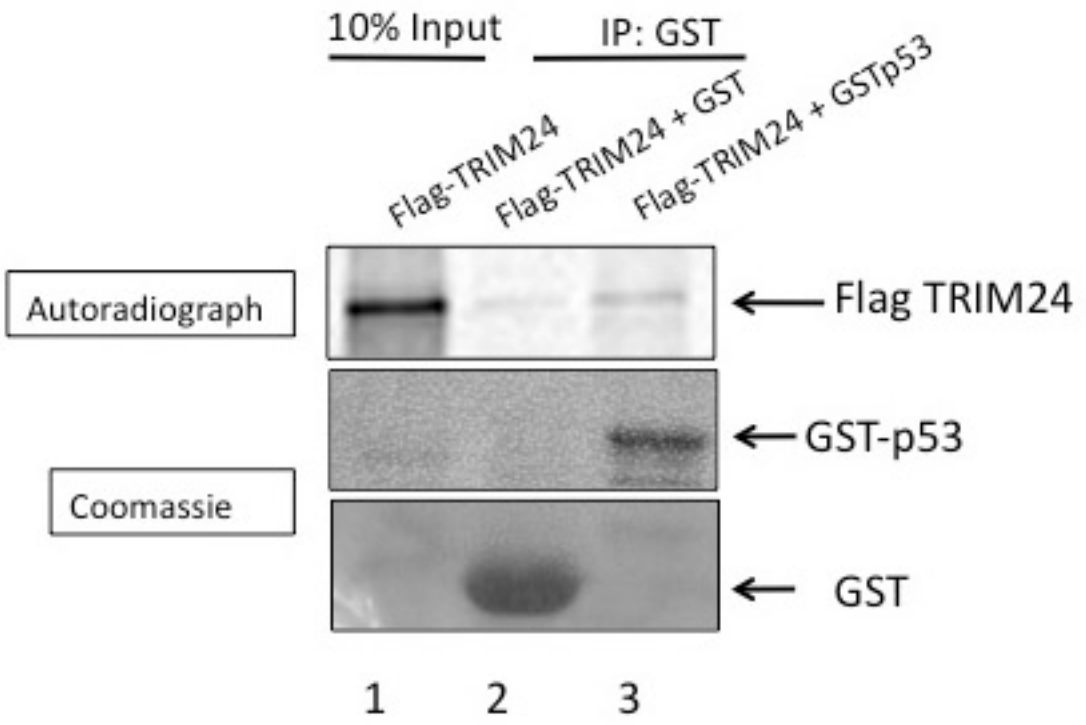

Figure 5: *In vitro* translated Flag-TRIM24 interacts with bacterial purified GST-p53.
10µL lysate of *in vitro* translated TRIM24 protein was bound to 1ug GST or GST-p53 (on bead) for 1.5 hours at 25°C. Proteins were separated by SDS-PAGE, stained with Coomassie blue, then exposed to autoradiograph overnight and visualized using the Storm840.

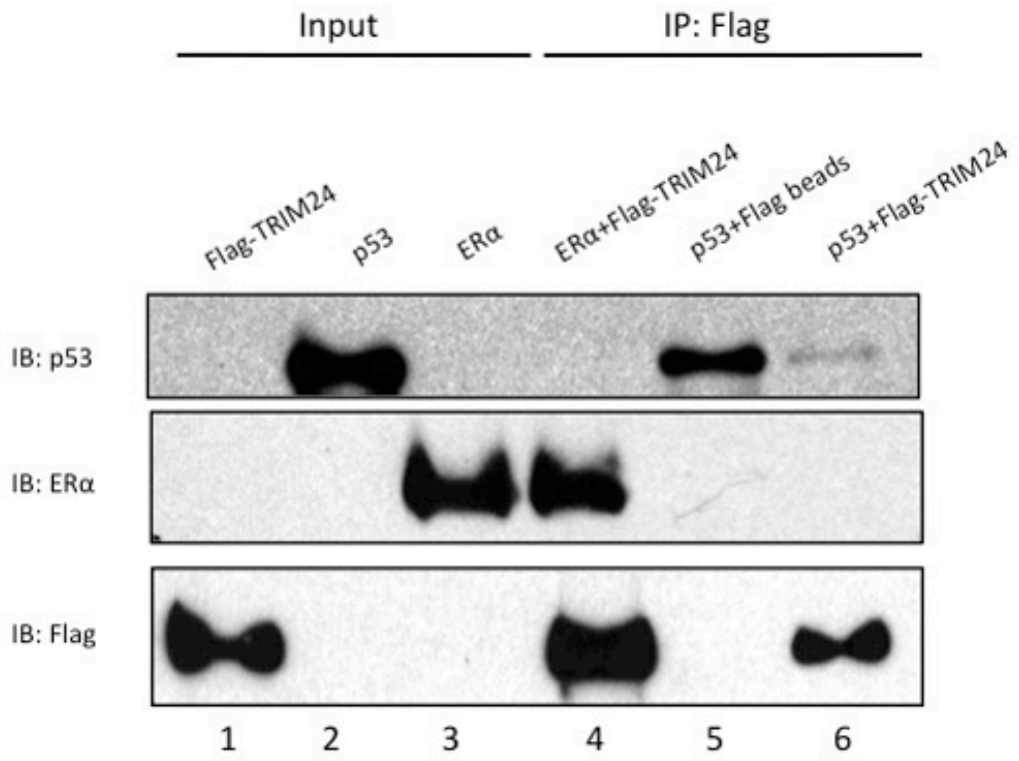

Figure 6: The interaction between p53 and TRIM24 is indirect.
500ng of recombinant GST-p53 or His-ERα protein was bound to 1ug recombinant Flag-TRIM24 (baculovirus; on bead) for 1.5 hours at room temperature, in 500uL NETN150. Proteins were separated by SDS-PAGE and bound proteins were visualized by immunoblotting for p53 (DO1-HRP), ERα (F10), and Flag (Flag-HRP).

p53 interacts with the Linker region of TRIM24

To determine which domain of TRIM24 interacts with p53, GST pull-downs were performed with GST-p53, purified from *E. coli* and ^{35}S-labeled Flag-TRIM24 deletion constructs. Deletion constructs of Flag-TRIM24 were *in vitro* translated using the Promega TNT® Quick Coupled Transcription/Translation kit. The Flag-TRIM24 constructs include: Flag-T24FL, Flag-T24ΔR, Flag-T24ΔBBI, Flag-T24ΔBBII, Flag-T24ΔBBC, Flag-T24PB, and Flag-T24Bromo (Deletion constructs illustrated in Figure 7; *in vitro* translated proteins shown in Figure 8). *In vitro* translated TRIM24 protein was incubated with GST or GST-p53 at 25°C for 1 hour. As shown in Figure 9, GST-p53 interacts with both Flag-TRIM24 Full Length and Flag-TRIM24ΔRING, but does not interact with the RING domain alone, indicating that the RING domain of TRIM24 is not involved in the interaction with p53. Additionally, the deletion of the BBoxes does not alter the binding of p53, while deletion of the Coiled-Coil domain drastically decreases the interaction (Figure 10, compare input in lane 3 to GST pull-down in lane 9). Removal of the LINKER region (Figure 7), between the Coiled-coil domain and PHD domain (aa 390-825) abolishes all interaction of the proteins (Figure 11). GST-p53 does not interact with either Flag-TRIM24PHDBromo or Flag-TRIM24Bromo (Figure 8 lanes 5 and 6). Taken together these results suggest that the Coiled-Coil domain and Linker region are essential for the binding between p53 and TRIM24.

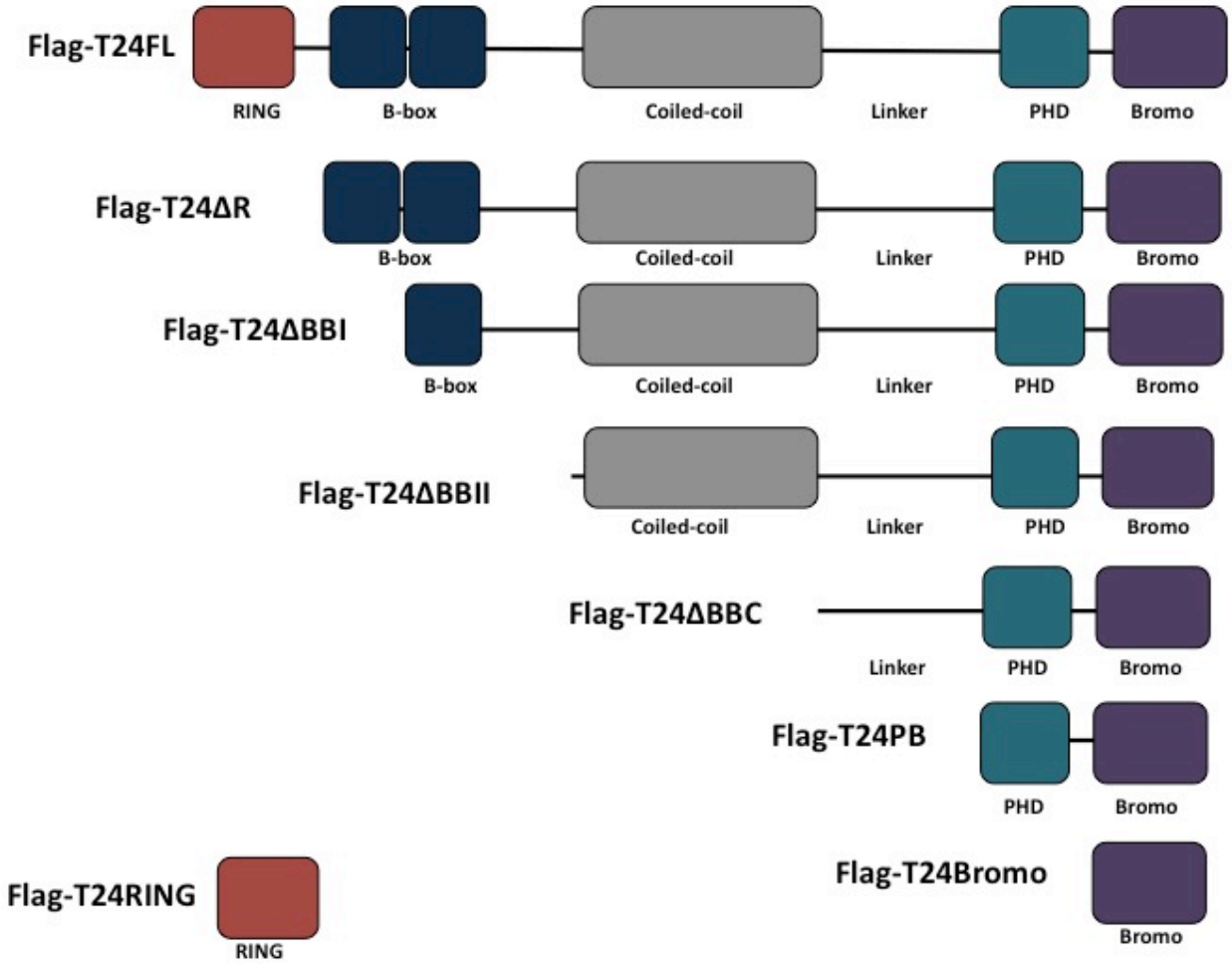

Figure 7: Schematic representation of Flag-TRIM24 Deletion constructs

A gift from Abhinav Jain, PhD, Flag-TRIM24 constructs are expressed in the pCMX vector .

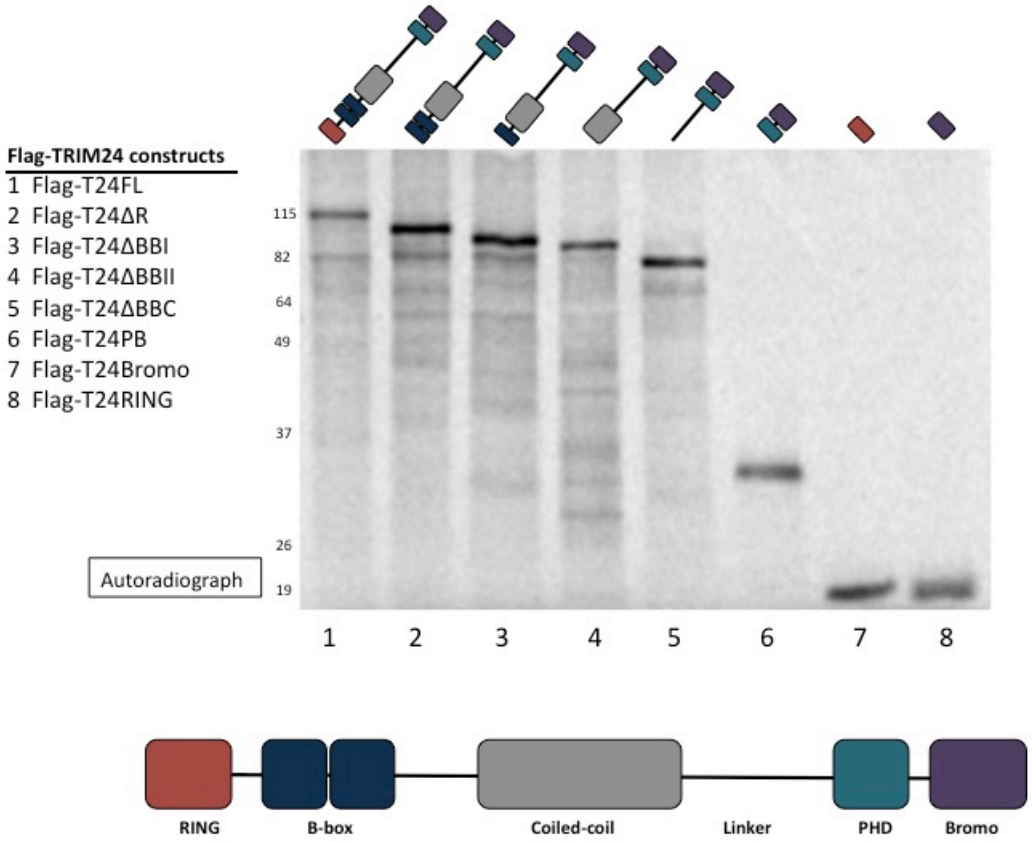

Figure 8: *In vitro* Translation of Flag-TRIM24 Deletion constructs
A)Upper panel shows autoradiograph of ^{35}S-labled TRIM24 proteins. ^{35}S-labled proteins were synthesized using the Promega TNT® Quick Coupled Transcription/Translation System. 500ng of DNA was added to the rabbit reticulocyte lysate Master Mix with ^{35}S-Methionine and at 30°C for 1.5 hours. Proteins were separated by SDS-PAGE; sizes and relative concentrations were confirmed using the Storm840. B) Lower panel is a schematic of Flag-TRIM24Full Length.

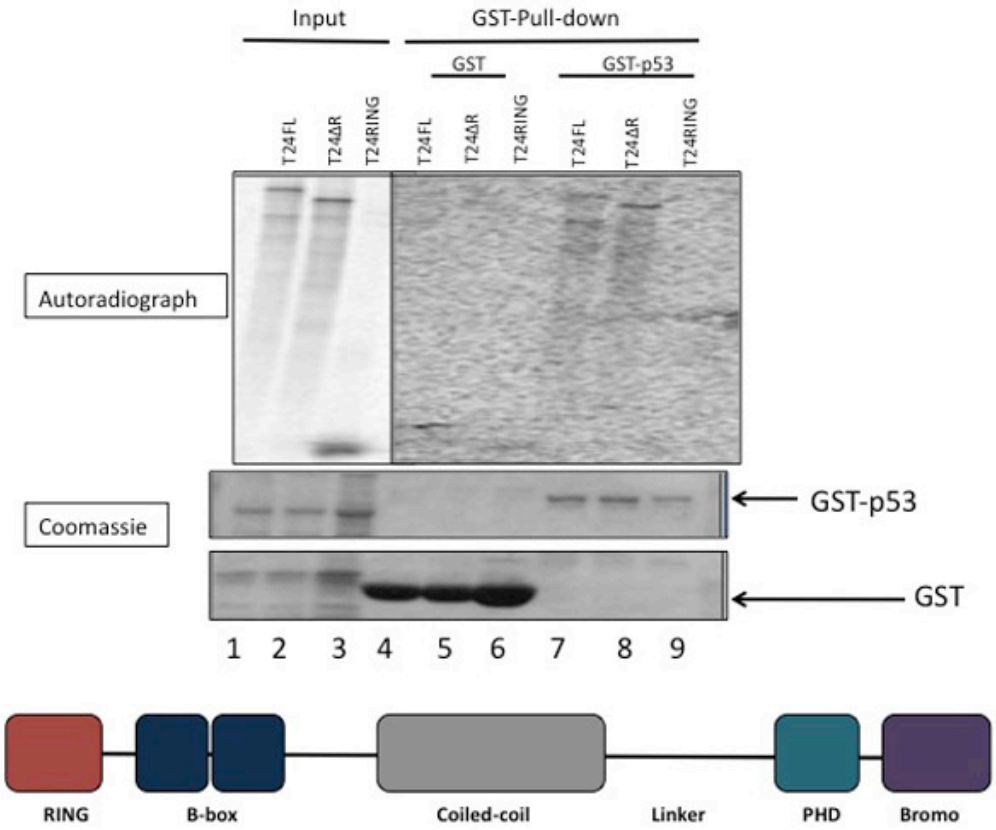

Figure 9: p53 does not interact with the RING domain of TRIM24
5µL lysate of *in vitro* translated TRIM24 protein was bound to 2ug GST or GST-p53 (on M2 bead) for 1.5 hours at room temperature. Proteins were separated by SDS-PAGE and the resulting gel was stained with Coomassie blue, then exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840. Upper panels are autoradiographs where the left panel is a short exposure, and the right panel is a long exposure. Bottom panel is a Coomassie.

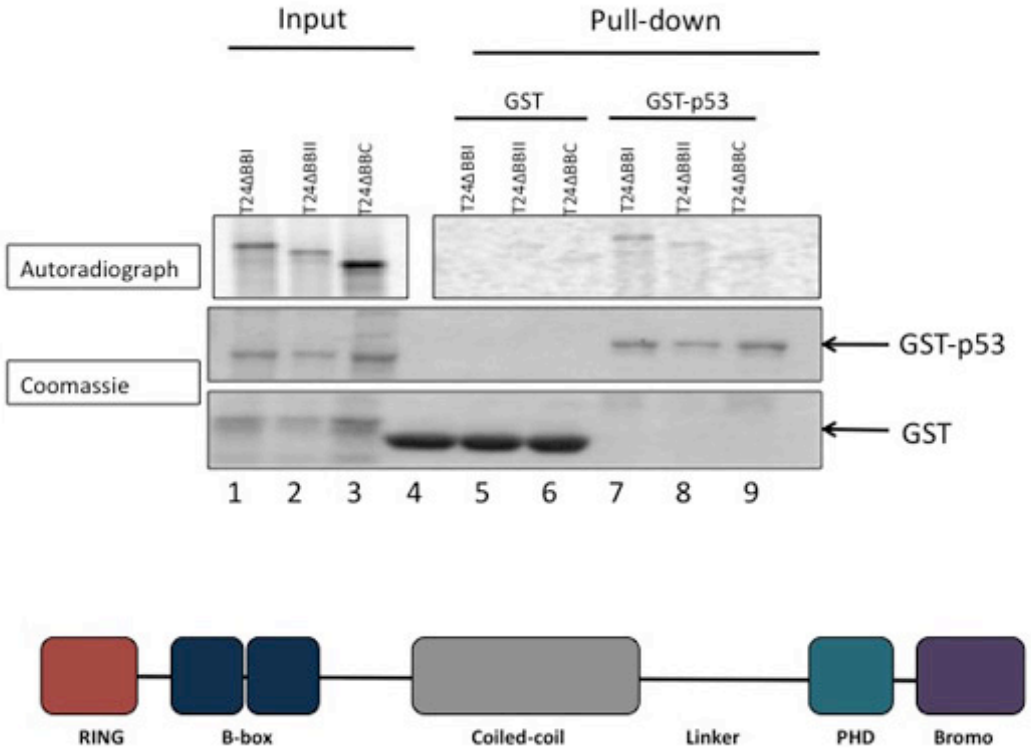

Figure 10: Deletion of the Coiled-Coil domain of TRIM24 decreases the binding of p53.

5µL lysate of *in vitro* translated TRIM24 protein was bound to 2ug GST or GST-p53 (on M2 bead) for 1.5 hours at room temperature. Proteins were separated by SDS-PAGE and the resulting gel was stained with Coomassie blue, then exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840. Upper panels are autoradiographs where the left panel is a short exposure, and the right panel is a long exposure. Bottom panel is a Coomassie.

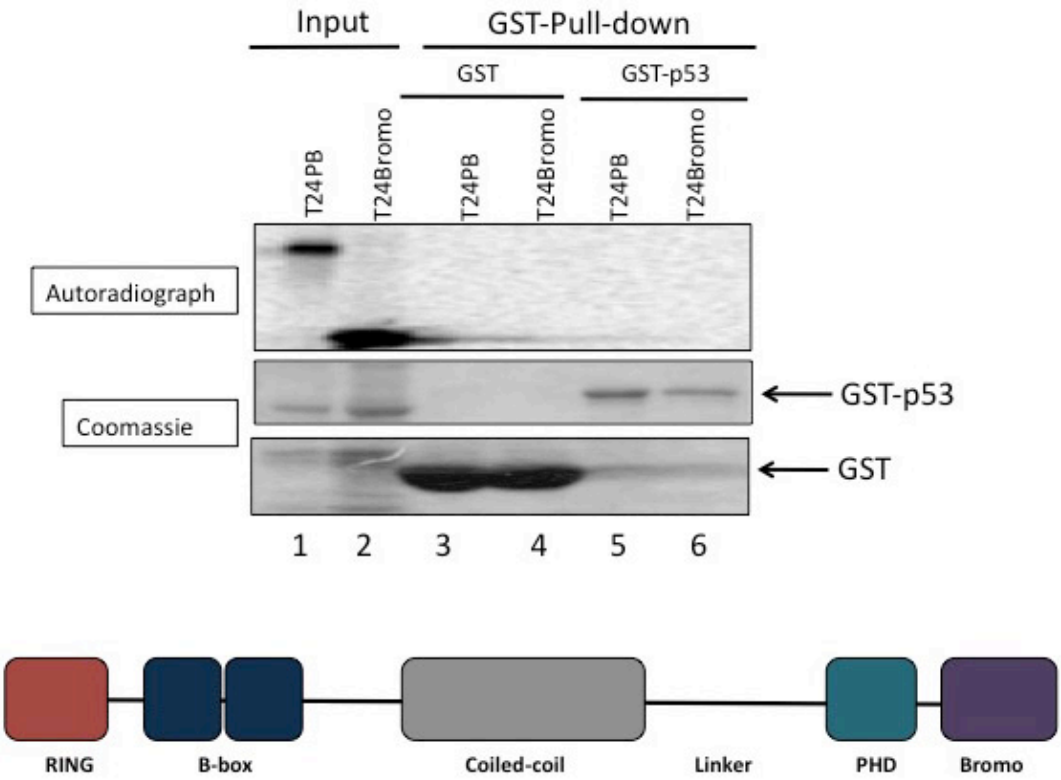

Figure 11: p53 does not interact with the C-terminus of TRIM24.
5µL lysate of *in vitro* translated TRIM24 protein was bound to 2ug GST or GST-p53 (on M2 bead) for 1.5 hours at room temperature. Proteins were separated by SDS-PAGE and the resulting gel was stained with Coomassie blue, then exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840. Upper panels are autoradiographs where the left panel is a short exposure, and the right panel is a long exposure. Bottom panel is a Coomassie.

To further investigate the role of the Linker region of TRIM24 in interactions with p53, a new construct was designed and cloned: pCMXFlag-TRIM24Linker (Flag-TRIM24Link) (Figure 12). This construct was *in vitro* translated and used in GST pull-down assays with GST-p53 as described above. As shown in Figure 13, GST-p53 interacts with Flag-TRIM24Link suggesting that the Linker region is sufficient for binding between p53 and TRIM24. However, it is important to note that Flag-TRIM24Link protein interacts with GST-p53 with less efficiency than Flag-TRIM24ΔBBC. The only difference between Flag-TRIM24Link and Flag-TRIM24ΔBBC is that the Flag-TRIM24Link protein lacks the PHD and Bromo domains. This suggests that while the PHD and Bromo domains may not be directly involved in the interaction of p53 and TRIM24 (Figure 12), their presence may contribute to a tertiary structure that is optimal for p53 binding. Taken together these results suggest that while p53 binding to TRIM24 is significantly deceased with the removal of the Coiled-Coil domain of TRIM24, the Linker region still maintains some ability to bind p53 (Quantification shown in Figure 14).

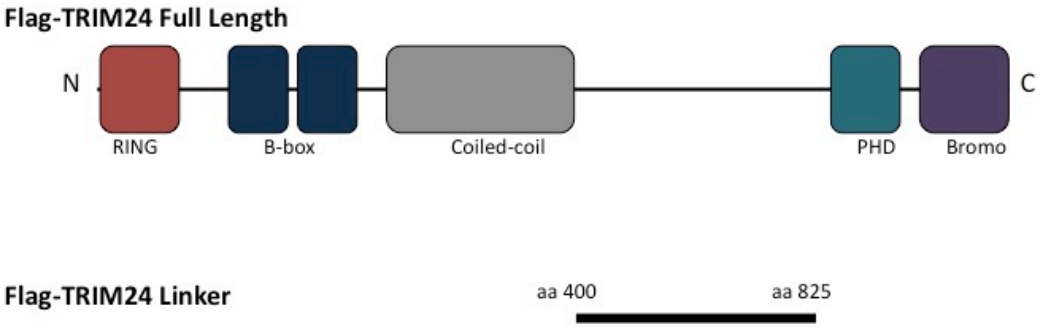

Figure 12: Schematic representation of Flag-TRIM24Linker.
Flag-TRIM24 Linker construct was cloned into the pCMX vector.

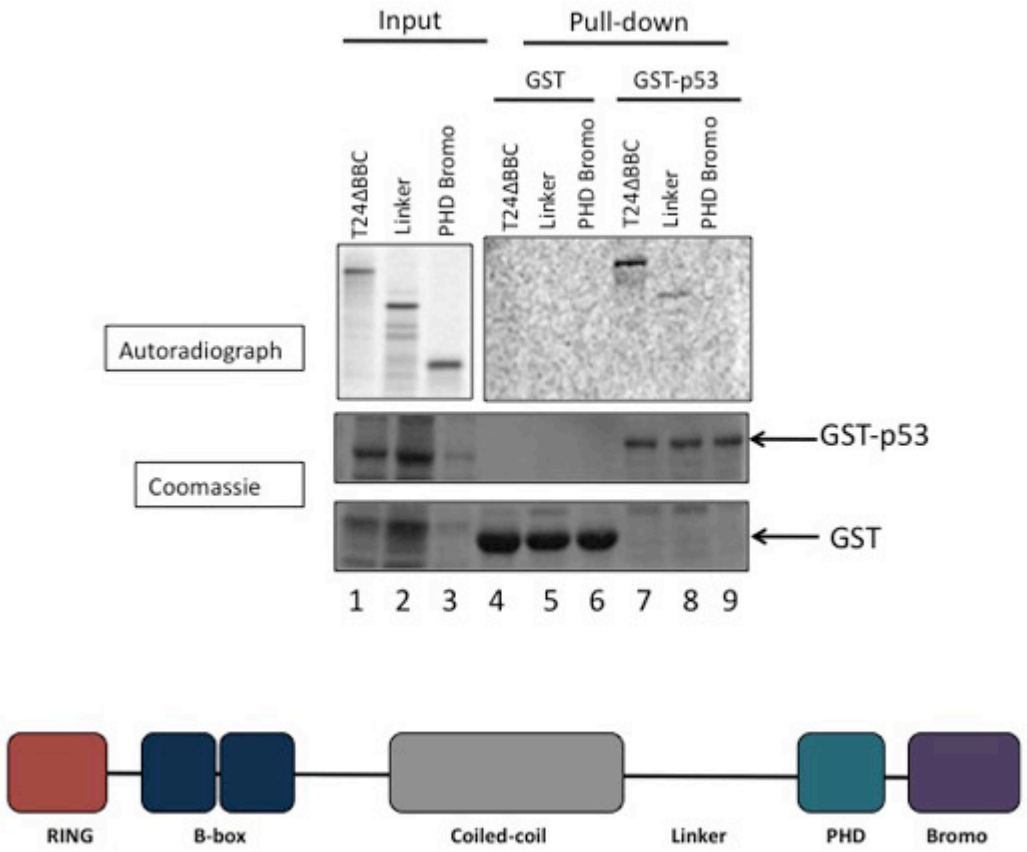

Figure 13: TRIM24 PHD and Bromo domains enhance interaction with p53. 5µL lysate of *in vitro* translated TRIM24 protein was bound to 2ug GST or GST-p53 (on M2 bead) for 1.5 hours at room temperature. Proteins were separated by SDS-PAGE and the resulting gel was stained with Coomassie blue, then exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840. Upper panels are autoradiographs where the left panel is a short exposure, and the right panel is a long exposure. Bottom panel is a Coomassie.

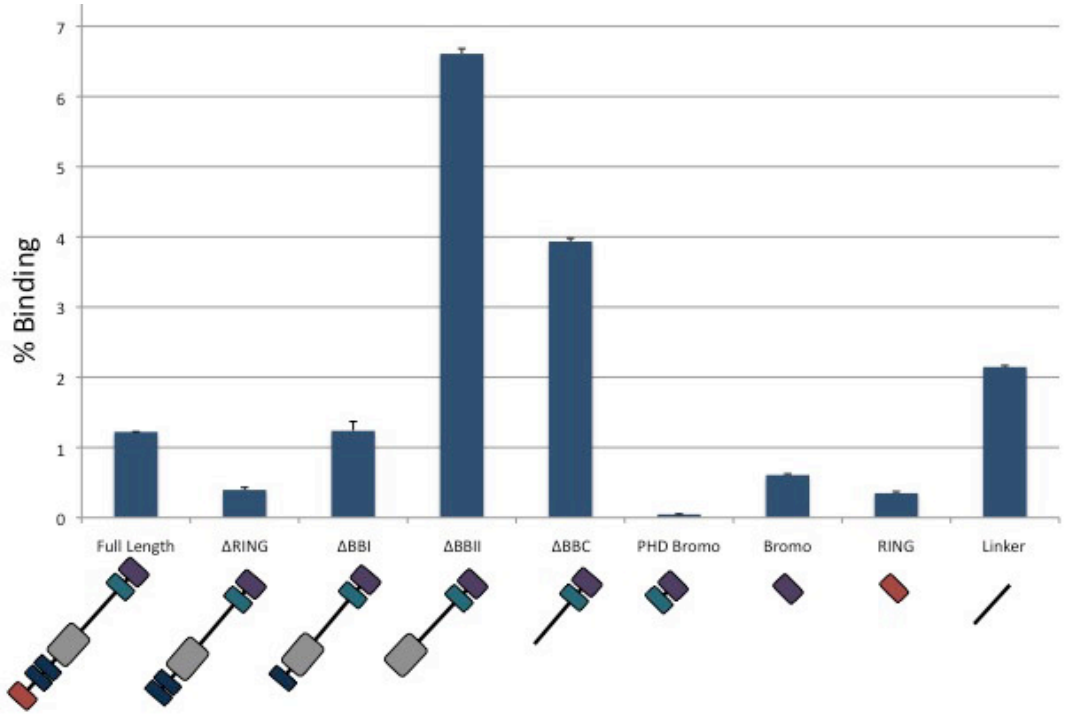

Figure 14: Relative binding of TRIM24 deletion constructs to p53
Percent binding was calculated by subtracting GST-only (background) from GST-p53-bound and diving by input: $\frac{GSTp53 - GST}{Input} \times 100$. Error bars represent standard deviation from 5 experiments.

TRIM24 interacts with the DNA Binding Domain and Carboxyl terminus of p53

To determine which domain of p53 interacts with TRIM24, a series of GST-p53 deletion constructs (Figure 15) were either *in vitro* translated (Figure 16) or purified from *E. coli* (Figure 17). *In vitro* translated GST-p53 proteins were incubated with recombinant Flag-TRIM24 for 1 hour. As shown in Figure 18, TRIM24 interacts with the Carboxy-terminus of p53. When the last 30 amino acids of p53 are removed, binding of TRIM24 is reduced to a level that is not significant over background (Figure 18 Compare lane 7 to lane 5). When only the last ~100 amino acids of p53 are present, TRIM24 binding is restored (Figure 18 lane 8), while the first 110 amino acids of p53 show no significant binding over background (Figure 18 lane 9). The core domain of p53, amino acids 91-295 has the greatest binding ability (Figure 19, lane 5). Interestingly, when the last 70 amino acids of p53 are intact, binding is weak; however dividing this region of p53 creates a robust interaction with TRIM24, almost equal to that of the full-length protein (Figure 19 Lanes 6-8; quantification in Figure 20).

To further investigate the role that PTMs might have in the interaction of p53 and TRIM24, the opposite experiment was performed: bacterial purified p53 was incubated with *in vitro* translated Flag-TRIM24. Conversely, when bacterial purified p53 is incubated with *in vitro* translated Flag-TRIM24, there is equal binding to GST-p53Full length and GST-p53ΔC (Figure 19, lanes 3 and 4; quantified In Figure 20). Notably, the ability for TRIM24 to interact withthe C-terminus of p53 is increased almost 2 fold, while the ability for TRIM24 to bind

the N-terminus, DNA binding domain and amino acids 286-330 is very weak. TRIM24 binds the final 70 amino acids of p53 with and almost equivalent ability as full-length p53, as in the previous experiment. These experiments are quantified and shown in figure 20.

The difference between the two previous experiments is the source of the proteins. In the first, GST-p53 was *in vitro* translated. My preliminary data suggest that p53 synthesized using the TNT-quick-coupled transcription/translation kit is modified by the lysate with known post translational modifications, including phosphorylation of serines 6 ,9, 15 and acetylation of lysine 373, while p53 purified from bacteria has no post translational modifications. Additionally, the post-translational modifications of TRIM24 differ between induced Sf9 expression and *in vitro* translation. Taken together, these data indicate that both the C-terminus and the DNA binding domain of p53 mediate and are sufficient for interaction with TRIM24 and the interactions may be dependant on PTMs.

In conclusion, this work demonstrates that the Linker region of TRIM24 is sufficient for p53 interaction and the DNA Binding domain and C-terminus of p53 are sufficient for TRIM24 interaction. It also suggests that post-translational modifications alter the binding affinity of the proteins. Determining the modifications is the next step in further characterizing this interaction.

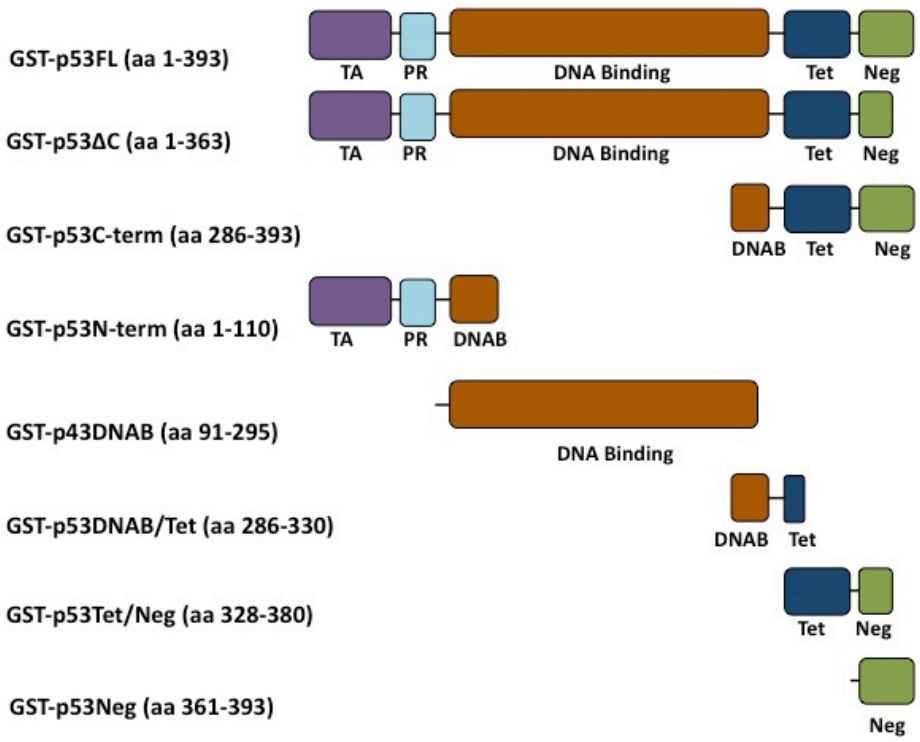

Figure 15: Schematic representation of GST-p53 deletion constructs
A gift from Dr. Mitchell Smith (University of Virginia), GST-p53 constructs are in the pET19b vector.

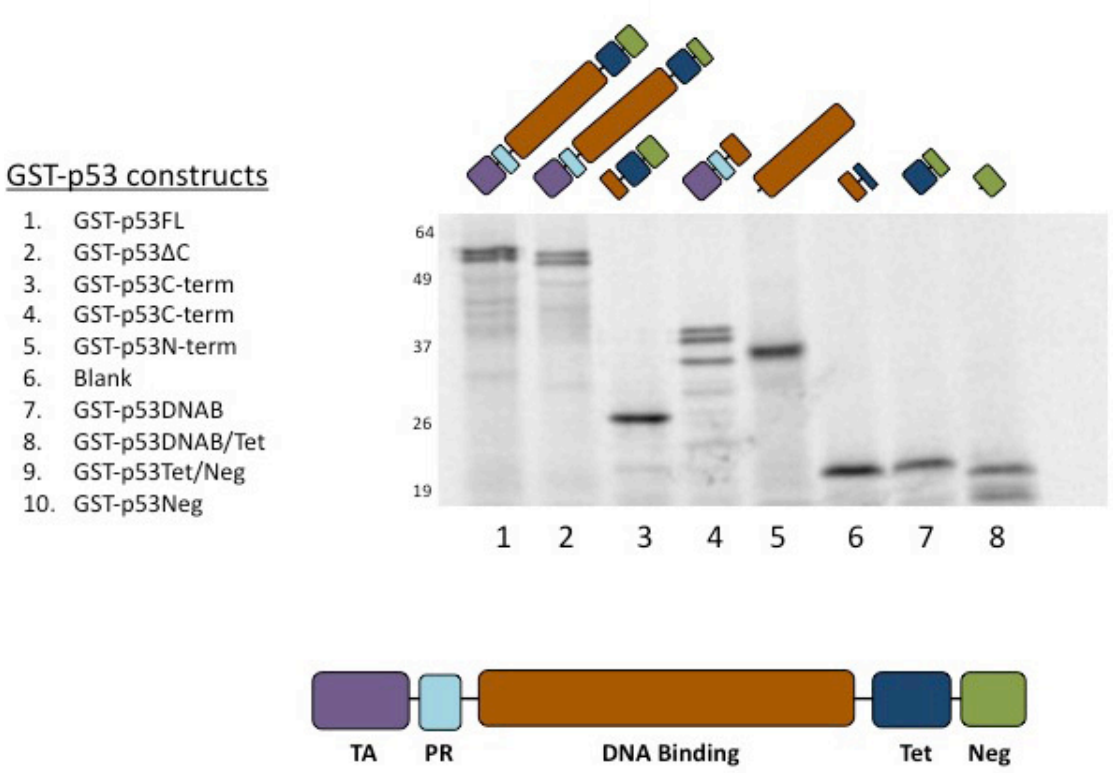

Figure 16: *In vitro* translation of GST-p53 constructs
Upper panel is an autoradiograph of ^{35}S-labeled GST-p53 protein. Proteins were synthesized using the Promega TNT® Quick Coupled Transcription/Translation System. 500ng of DNA was added to the rabbit reticulocyte lysate Master Mix along with ^{35}S-Methionine and incubated at 30°C for 1.5 hours. Proteins were separated by SDS-PAGE; sizes and relative concentrations were confirmed using the Storm840. Lower panel is a schematic representation of GST-p53 Full Length.

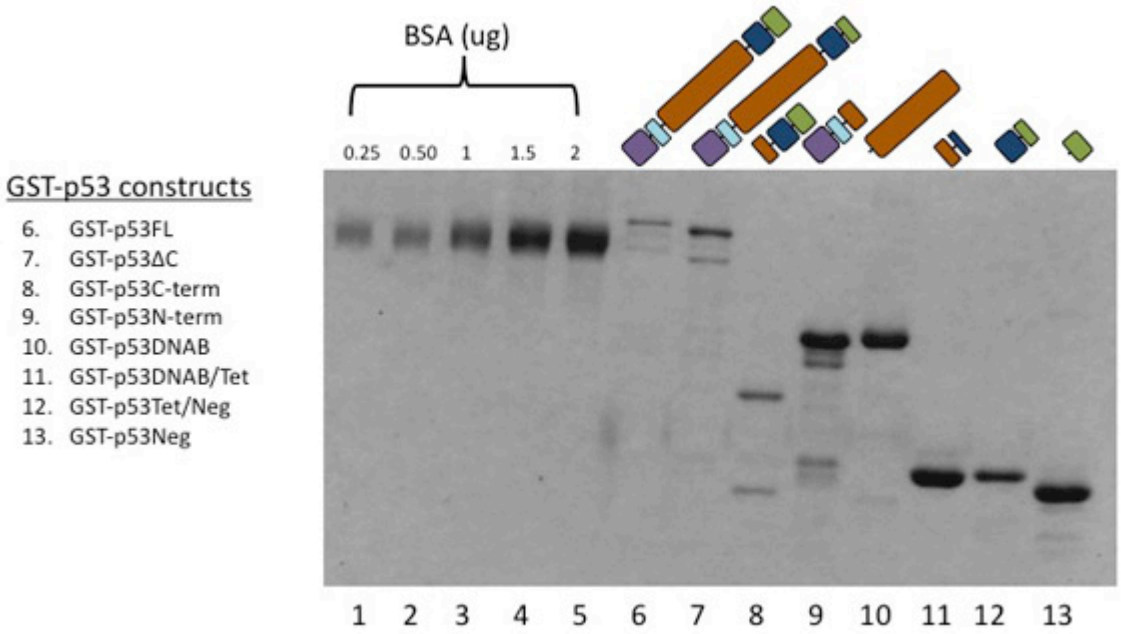

Figure 17: Purification of GST-p53 proteins from *E. coli*
Lanes 1-5 are BSA used to determine protein concentration. Sf9 cultures were induced with 0.2% L-Arabinose and 100uM $ZnSO_4$, and at 16°C. Here, 5 uL (50/50 slurry) of purified GST-p53 protein was loaded on an SDS-PAGE gel, and stained with Coomassie blue.

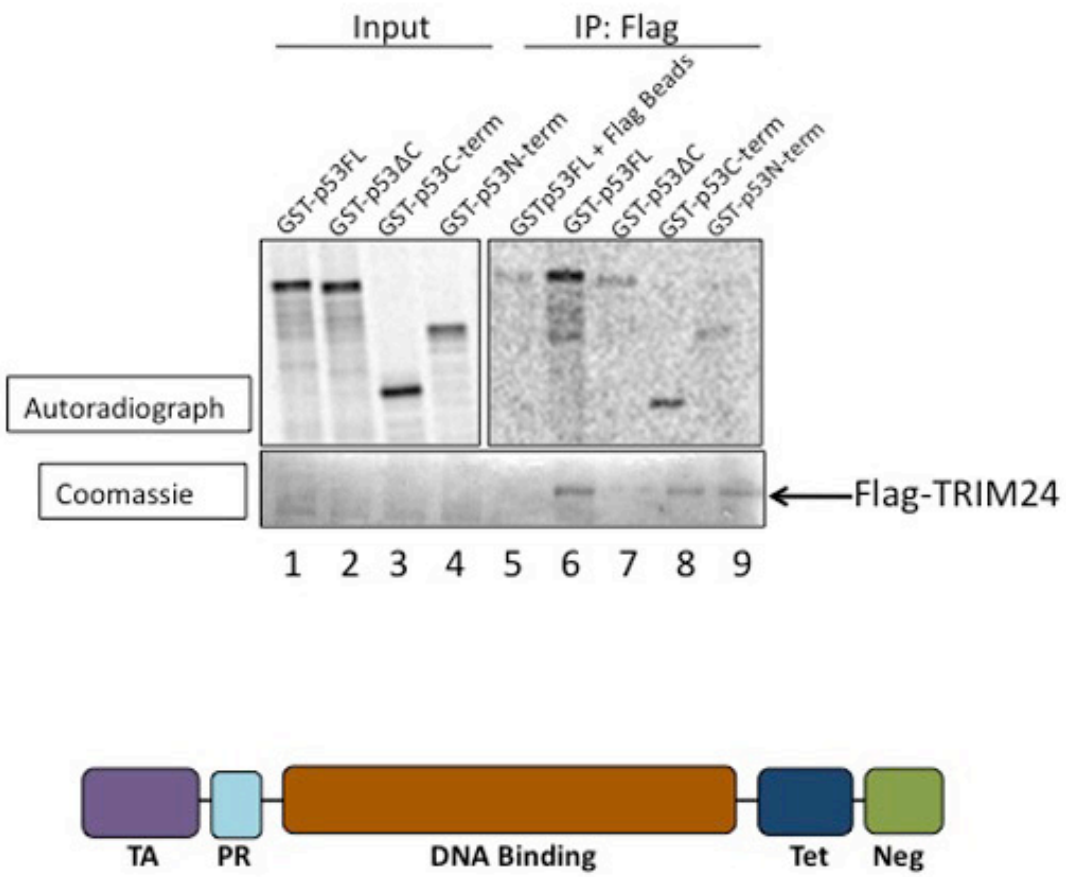

Figure 18: Flag-TRIM24 interacts with the C-terminus of *in vitro* translated GST-p53

A) Upper panel left panel shows short exposure of autoradiograph, right panel shows long exposure of the same autoradiograph. Coomassie stained gel is the same gel exposed to autoradiogram. B) Bottom panel is a schematic of the full-length p53 protein. 5µL lysate of *in vitro* translated GST-p53 protein was bound to 2ug Flag-TRIM24, purified from baculovirus or the equivalent volume of Flag beads alone for 1.5 hours at 25°C. Proteins were separated by SDS-PAGE and the resulting gel was stained with Coomassie blue, and exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840.

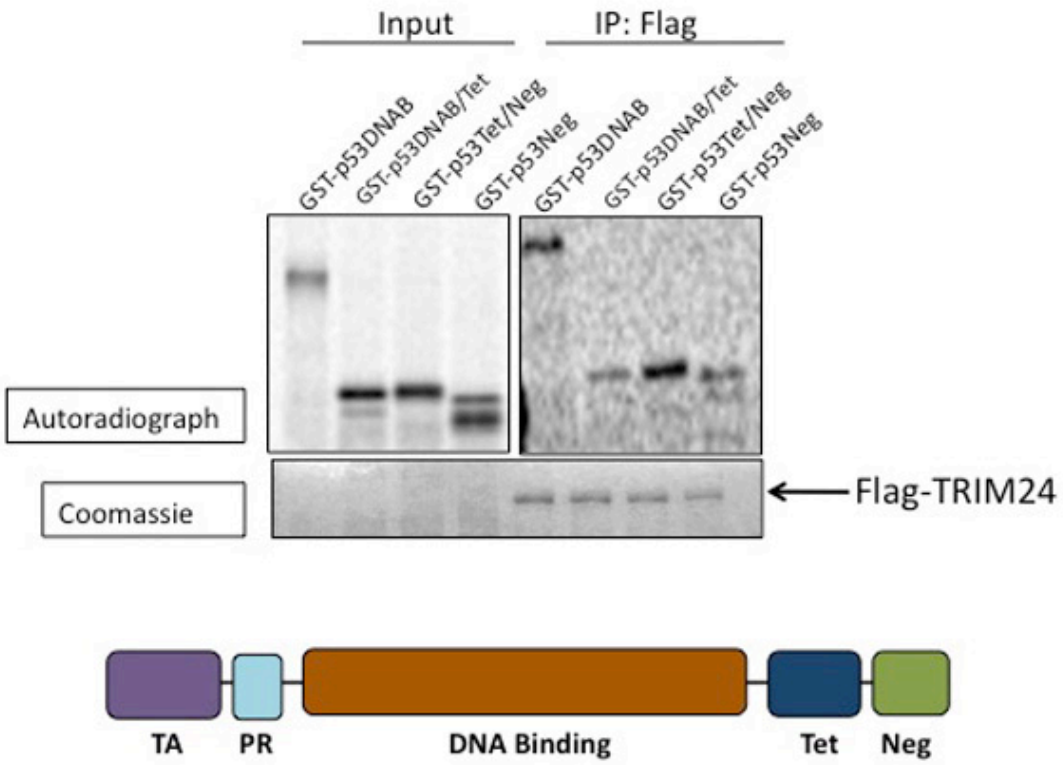

Figure 19: TRIM24 interacts with the DNA binding domain of p53
A) Upper panel left panel shows short exposure of autoradiograph, right panel shows long exposure of the same autoradiograph. Coomassie stained gel is the same gel exposed to autoradiogram. B) Bottom panel is a schematic of the full-length p53 protein. 5µL lysate of *in vitro* translated GST-p53 protein was bound to 2ug Flag-TRIM24, purified from baculovirus or the equivalent volume of Flag beads alone for 1.5 hours at 25°C. Proteins were separated by SDS-PAGE and the resulting gel was stained with Coomassie blue, then exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840.

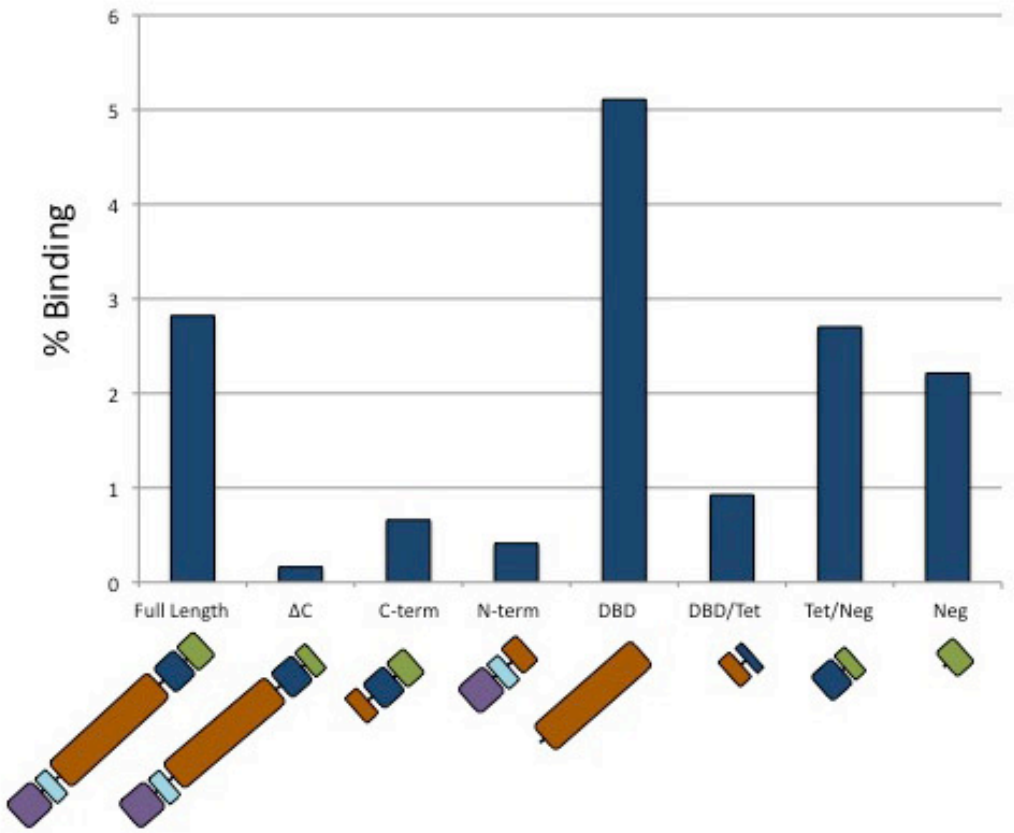

Figure 20: Quantification of the binding of Flag-TRIM24 to *in vitro* translated GST-p53

Percent binding was calculated by subtracting Flag-beads only (background) from Flag-TRIM24 bound and diving by input: $\frac{FlagTRIM24 - Flag\_beads}{Input} \times 100$.

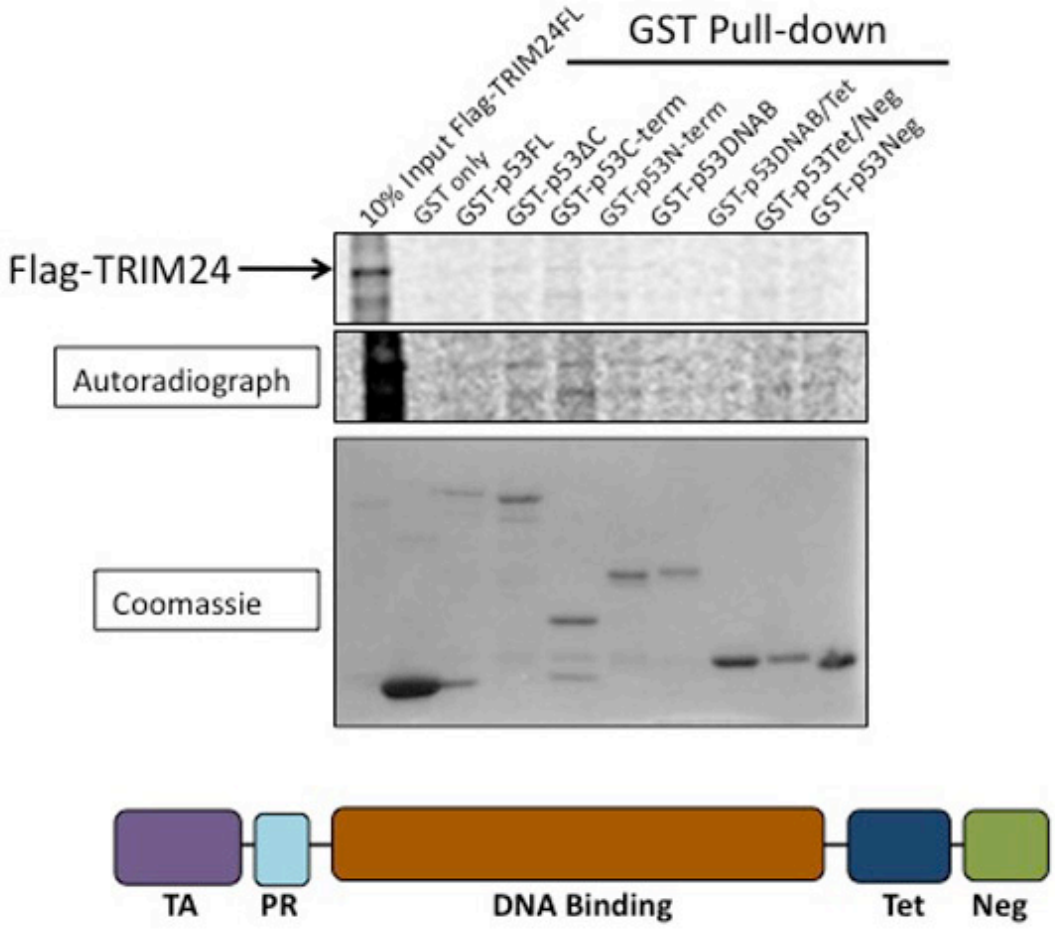

Figure 21: *In vitro* Translated TRIM24 interacts with the N-terminus of GST-p53.

Upper panel is a short exposure of an autoradiograph, middle panel is a long exposure of the same autoradigraph, bottom panel is the same gel, stained with Coomassie blue. *In vitro* translated TRIM24 protein was bound to iGST or GST-p53 (purified from *E. coli*, on Glutathione bead) for 1.5 hours at 25°C. Proteins were separated by SDS-PAGE, stained with Coomassie blue, then exposed to autoradiograph screen overnight. Bound proteins were visualized using the Storm840.

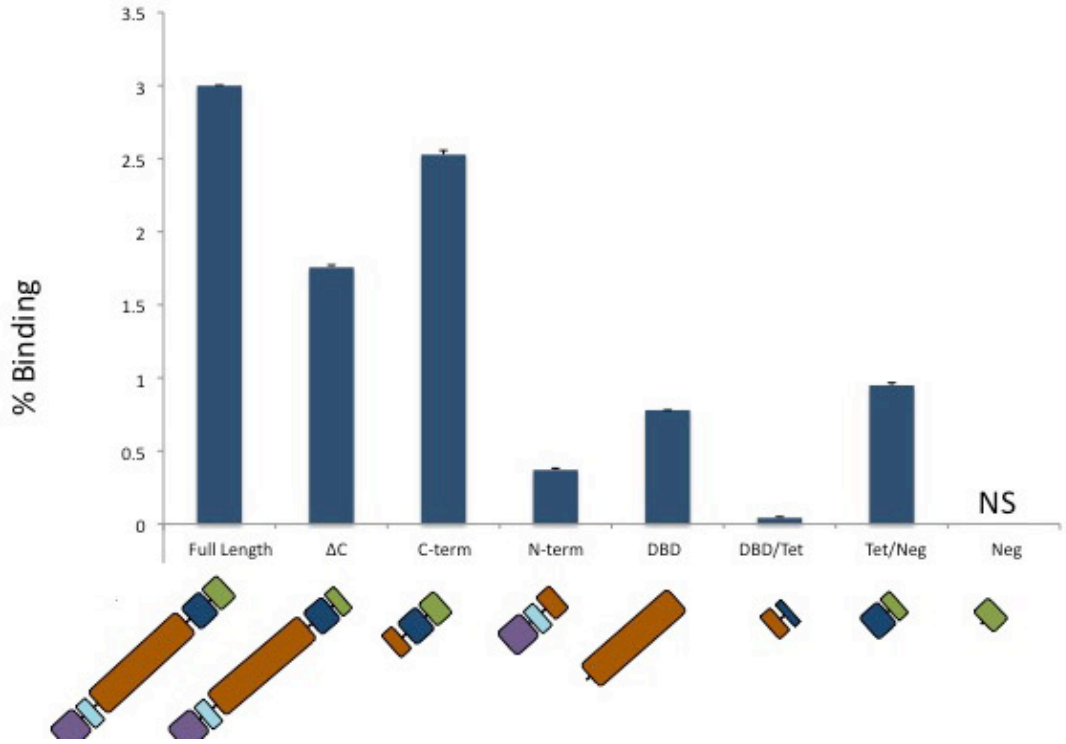

Figure 22: Quantification of GSTp53 binding to TRIM24.
Percent binding was calculated by subtracting GST-only (background) from GST-p53-bound and diving by input: $\frac{GSTp53 - GST}{Input} \times 100$.

Discussion:

p53 is involved in multiple regulatory networks, both as a target of regulation and as an effector of transcription regulation, as modulated by its numerous binding partners. With over 100 known binding partners, p53's influence on biological processes is vast and can have numerous outcomes, both positive and negative (38), therefore, understanding p53's relationship with its interacting and regulating proteins will better equip us to react when something goes awry with p53, specifically in disease and development. In this study, we have performed a series of GST pull-downs and immunoprecipitation assays to define the nature of the interaction of p53 and a recently discovered negative regulator, TRIM24. The results in this study confirmed that p53 and TRIM24 do interact *in vitro*, however this interaction is likely indirect and/or involves multiple protein partners. The linker region of TRIM24 is essential for interaction with p53. This is an intriguing finding as the linker region is highly conserved amongst family members and throughout evolution (39) and acts as a platform for other important TRIM24 functions: nuclear receptor interaction (40), and interaction with the heterochromatin binding protein HP1 (34, 39), and putative phosphorylation and caspase cleavage sites (A. Jain, Ph.D. unpublished data).

When p53 protein expressed in the rabbit reticulocyte lysate system is incubated with TRIM24 purified from baculovirus, the DNA binding domain and the C terminus of p53 are both sufficient for interaction. However, when *in vitro* translated TRIM24 is incubated with p53 purified from *E. coli, TRIM24* does not

interact with the DNA binding domain of p53. Together, these results suggest that there is a difference between the proteins when they are synthesized using the rabbit reticulocyte lysate system versus purification from bacteria and/or baculovirus. It is known that the rabbit reticulocyte lysate system may add PTMs to proteins (41). My preliminary data show those modifications may include, but are not necessarily be limited to, phosphorylation of serines 9, 15, 20, 37, 46, and 392 and acetylation of lysine 373. Each of these modifications correspond to p53's stress signal response and result in p53's disassociation from MDM2 (11). Conversely, *E. coli* do not have the ability to post-translationally modify proteins, thereby accounting for the initial difference in starting material of these experiments. Phosphorylation of specific residues in the MDM2-p53 interacting domain (p53's TA domain) inhibits the interaction. It is possible that a PTM present in the TRIM24-p53 interaction domain may interrupt this interaction. Interestingly, when PTMs that are associated with stress induced pathways of p53 regulation are present, TRIM24 interacts with the DNA binding domain, but when these marks are, presumably, absent (in the case of p53 purified from *E.coli* which is unable to post-translationally modify proteins), the interaction of TRIM24 with the DNA binding domain of p53 is lost. The DNA binding domain is essential for p53's function as a transcription factor, because in order to activate gene targets, p53 must first bind DNA. If TRIM24 is interacting with this domain, it could likely be masking p53's ability to bind DNA. Alternatively, *in vitro* translated TRIM24 may carry a modification that could be responsible for the loss of interaction with the DNA binding domain of p53. TRIM24 can be

phosphorylated, ubiquitinated and sumoylated (S. Appikonda and S. Jiang unpublished data), as with MDM2 (45).

In order to better characterize these interactions, further analysis will need to be conducted on p53 and TRIM24 before performing pull-down assays and probed for changes that occur during the assays. We are in the process of determining the PTMs on Flag-TRIM24 from baculovirus and rabbit reticulocyte lysate as well as p53 from rabbit reticulocyte lysate by immunoblotting and *in vitro* kinase assays. In the future we plan to send these proteins for mass spectrometry analysis, to verify putative modifications. This information will allow us to identify the modifications present on the proteins before interacting. It is possible that modifying enzymes present in the rabbit reticulocyte lysate could modify bacterial and baculoviral purified proteins during the incubation period, therefore affecting the binding. Mass spectrometry analysis repeated on the same p53 and TRIM24 after binding will allow us to assess if the modification status has been altered. Once we assess which residues are being differentially modified during binding, we can then begin to elucidate their role(s), by mutating the modified amino acid and repeated binding assays. Additionally, *in vitro* kinase assays or mimicking phosphorylation on bacterial or baculoviral purified protein, by mutating to Glutamate for example, would allow us to determine if post-translational modifications can enhance the binding *in vitro*, as opposed to the lack of binding shown in Figure 3. Most importantly, determining the modifying enzymes responsible for adding specific modifications and in response to what stresses, would give us invaluable knowledge about when and

why TRIM24 is regulating p53 and how that regulation is different from MDM2. The MDM2-/- mouse is embryonic lethal, which provides evidence that none of the other negative regulators of p53 are sufficient for keeping it at levels tolerable to the cell. However it is clear that other negative regulators have a crucial function in regulating the p53 pathway because, for example, aberrant expression of TRIM24 is directly correlated with both breast cancer and HCC (35, 32).

Determining if p53 and TRIM24 interact in a protein complex, with other binding partners could give further insight into the nature of the TRIM24-p53 regulatory loop. A caveat to the rabbit reticulocyte lysate system is that it contains a known p53 interacting protein, heatshock protein 70 (hsp70) (37,42). This could be the cause for poor recovery of TRIM24 when performing affinity purifications with GST-fused p53 as in Figure 5. Once we have a better understanding of PTM status of p53 and TRIM24 required for interaction, as determined by mass spectrometry, performing GST and/immunoprecipitation using recombinant proteins which we have modified accordingly will be a key experiment to truly define the interaction of p53 and TRIM24.

Bonus, the *Drosophilla melanogaster* homolouge of *TRIM24* is the only known negative regulator of p53 in fruit flies (27, 44). Yet, higher organisms evolved additional negative regulators. Understanding the differences between the relationship of p53 and TRIM24, and other negative regulators of p53 including Pirh2, compared to p53 and MDM2 may help us answer the question of the need for a seemingly redundant proteins.

Moreover, knowing exactly where p53 is interacting with TRIM24 can have clinical implications. Many researchers have suggested the idea of re-activating p53 in cancer cells as a promising treatment (36). However, that idea doesn't come without challenges. In cancers where TRIM24 is over-expressed, such as breast cancer, it correlates with poor prognosis of the patients (35, 45). Therefore, a more complete knowledge of how this negative regulator interacts with p53, in the normal cell and how that interaction is changed in the cancer cell, could lead to drug development of TRIM24-p53 interaction inhibitors, resulting in re-activation of p53. If, for example release of p53 from TRIM24 requires the presence of a phosphate group at a specific residue of TRIM24 or p53, but mutation of one of the genes inhibits this phosphorylation and therefore the downstream stabilization of p53, small molecules could be targeted to this direct site to release TRIM24's hold on p53, thereby re-activating downstream p53 pathways. Overall, the discovery of TRIM24 as a novel negative regulator of p53, that is associated with poor prognosis in cancer, is exciting finding because it further validates the necessity of p53 to maintain genomic stability. Additionally, this discovery gives us another avenue to address and possibly rectify mis-regulation of p53 in cancer.

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