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Peptide and protein chemistry approaches to study the tumor suppressor protein p53

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Abstract

The tumor suppressor and master gene regulator protein p53 has been the subject of intense investigation for several decades due to its mutation in about half of all human cancers. However, mechanistic studies of p53 in cells are complicated by its many dynamic binding partners and heterogeneous post-translational modifications. The design of therapeutics that rescue p53 functions in cells requires a mechanistic understanding of its protein-protein interactions in specific protein complexes and identifying changes in p53 activity by diverse post-translational modifications. This review highlights the important roles that peptide and protein chemistry have played in biophysical and biochemical studies aimed at elucidating p53 regulation by several key binding partners. The design of various peptide inhibitors that rescue p53 function in cells and new opportunities in targeting p53-protein interactions are discussed. In addition, the review highlights the importance of a protein semisynthesis approach to comprehend the role of site-specific PTMs in p53 regulation.

Introduction

Transcription factors (TFs) are a family of proteins that bind specific DNA sequences to either promote or repress the transcription of their target genes. Due to their critical roles in controlling gene function, TFs drive numerous cellular and physiological processes that are essential for the normal development of an organism. Significant alterations in either TF expression or function may lead to the deregulation of cellular pathways leading to disease development. Indeed, neurodegenerative disorders, diabetes, cardiac diseases and many human cancers are associated with TF malfunction. Not surprisingly, about 20% of human oncogenes identified encode TFs.[1]

The TF p53 is easily the best studied among the large family of human TFs, due to its critical role as a tumor suppressor protein. p53 suppresses abnormal cellular growth, which is a hallmark of cancer, by forcing cell-cycle arrest, cellular senescence, and in extreme instances cell death by apoptosis.[2, 3] The loss of functional p53 by various amino acid mutations is routinely detected in about 50% of all human cancers.[4] In many cases where p53 remains wild type it may still be functionally inactivated by binding to cellular oncoprotein inhibitors such as the Murine double minute 2 (MDM2) and Murine double minute X (MDMX) RING-domain containing proteins.[5, 6] Although p53 has been extensively investigated since its discovery in 1979,[7-9] for a significant period of time it was considered 'undruggable' or a 'difficult to drug' target. This was because the requisite mechanistic studies of p53 in cells were complicated by the vast number of p53-protein interactions in the nucleus, mitochondria and cytoplasm, as well as by numerous dynamic post-translational modifications (PTMs) found across the length of p53. Indeed, p53 is histone-like in the diversity and density of its modifications leading to the proposal of a p53 code for gene regulation.[10, 11] In the last two decades, however, substantial efforts have been made to

better understand its precise functions and the dynamic modes of p53 binding to its regulatory proteins and with its gene targets. These studies have identified new therapeutic opportunities to target p53 directly or indirectly in the treatment of cancer.[12-14] Based on our accumulated knowledge of the complexity of p53 functions in cells, it is even more important to first parse and then target specific p53-protein interactions implicated in human diseases. Furthermore, investigating the regulation of p53 by its various PTMs requires access to quantities of homogeneously site-specifically modified p53 that are currently inaccessible from cellular sources. Given the steep challenges in parsing the p53 code for gene regulation, we highlight recent applications of peptide and protein chemistry that have greatly increased our understanding of how p53 interacts with four key p53-binding proteins, MDM2/MDMX, the RNA polymerase II co-activator protein Positive Cofactor 4 (PC4), the breast cancer type two susceptibility protein (BRCA2), and the cell cycle regulator protein 14-3-3σ. Peptide-inhibitors of these regulatory p53-protein interactions are highlighted and discussed in the context of p53 function. Finally, we present the current state-of-the art in applying protein semisynthesis techniques to generate post-translationally modified full-length p53.

The multi-domain structure of p53

The p53 protein consists of several structural domains with unique functions.[15, 16] Its N-terminal region contains an intrinsically disordered trans-activation domain (TAD) which is subdivided into activation domain 1(TAD1, residues 1–42),[17, 18] activation domain 2 (TAD2, residues 43–63) [19] and a proline-rich domain (PRD, residues 64–91).[20] Both TAD1 and TAD2 are known to interact with the primary transcriptional machinery as well as with several regulatory proteins, like MDM2, PC4, BRCA2, and p300 (Figure 1).[21, 22] Mutations in the TADs lead to the

loss of p53 tumor suppressor function.[23-25] TADs also contain multiple phosphorylation sites that govern p53 activity,[26, 27] and direct the transactivation of numerous target genes involved in the induction of cell-cycle arrest and apoptosis.[28] Even though the PRD is not required for the transactivation of many promotors, it is essential for the induction of apoptosis, reactive oxygen species (ROS) production, and transcriptional repression.[29] The p53 core domain contains a structured DNA-binding domain (DBD, residues 100–312) that is required for sequence-specific DNA binding and this is the region where most point-mutations associated with human cancers are found.[30, 31] Several regulatory proteins that bind this domain also govern p53 functional outcomes (Figure1). The C-terminal region (residues 312-393) contains the key helical tetramerization domain (TD, residues 334–356), which forms a four-helix bundle that pre-organizes four p53 proteins into the tetrameric form that enhances its DNA-binding activity.[32, 33] The C-terminus also contains a lysine-rich C-terminal regulatory domain (CRD, residues 364 – 393) harboring sites for several PTMs such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation.[34-39]

The regulation of p53 activity

Several levels of p53 regulation are known and all are mediated by protein-protein interactions. These range from dictating the folded half-life of p53 inside cells to fine-tuning its DNA sequence specificity. Although p53 has been extensively studied since its discovery in 1979, mechanistic studies of p53 in cells are complicated by the vast number of p53-protein interactions and dynamic post-translational modifications found in p53. Indeed, p53 interacting proteins such as MDM2 and p300 play critical roles in negatively and positively regulating p53 stability and activity, respectively. Studying specific p53-protein interactions that are important for its unique

functions can also provide avenues to selectively control p53-mediated cellular outcomes. Indeed, a detailed investigation of numerous p53-protein interactions is imperative toward understanding normal cell development and the etiology of many cancers. The major challenges toward investigating crucial p53-protein interactions are the poor stability of full-length folded p53, its many post-translational modifications that lead to sample heterogeneity, and the challenges associated with purifying large multi-domain p53-interacting proteins in a stable and homogenous form. Even wild-type full-length p53 produced in *E. coli* is found in inclusion bodies, rendering it challenging to obtain the protein at reasonable concentrations required for biophysical techniques such as NMR, fluorescence anisotropy, fluorescence polarization (FP), surface plasmon resonance (SPR) or isothermal titration calorimetry (ITC).

To overcome these numerous challenges, peptide and protein chemistry provide powerful approaches to investigate specific domains of p53 and, more recently, the full-length wild-type and/or post-translationally modified protein. The use of short to medium-length peptides (10-35 amino acids) derived from either p53 or its binding partners is especially advantageous, as peptide are easily generated by solid-phase peptide synthesis (SPPS) and may be modified to include site-specific PTMs, fluorophores or NMR-active nuclei. Additionally, the relatively small surface area of peptides relative to full-length proteins has helped structural elucidation by X-ray crystallography and revealed interfacial residues critical to p53-protein interactions.

In this review, we highlight the enabling power of peptide chemistry, which has played a major role in elucidating multiple key p53-protein binding events including p53-MDM2, p53-PC4, p53-BRCA2, and p53-14-3-3σ interactions. Although a large number of p53 binding partners are known till date, the specific p53-protein interactions discussed herein are based on their crucial roles in controlling p53 function their association with human cancers, as well as the utility of

peptide chemistry in studying these interactions. Biophysical techniques such as X-ray crystallography, NMR, SPR, and ITC-based binding assays were employed toward understanding these interactions and the knowledge gained from these studies enabled researchers to design synthetic peptide and small molecule inhibitors or stabilizers that rescue p53 and enhance its transcriptional activity.

The p53-MDM2 Interaction

The proto-oncogene mouse double minute 2 homolog protein, MDM2, is an essential E3 ubiquitin ligase conserved from mice to humans that negatively regulates p53 activity and stability. As part of a crucial negative feedback loop that maintains low levels of p53 in healthy cells, MDM2 regulates p53 through the ubiquitin-dependent proteasomal degradation pathway.[40-42] MDM2 and its structural analogue MDMX contain a binding site for the N-terminal TAD1 domain of p53 and ubiquitylate several lysine (Lys) in the p53 CTD. MDM2 also directly inhibits p53 function in a subset of tumors by sterically disrupting its interaction with the general transcription machinery.[5] To counter negative regulation by MDM2 and increase its transcriptional activity, p53 is phosphorylated at Ser15 and Ser20 in TAD1 under conditions of cellular stress. This inhibits MDM2 binding and rescues p53 from ubiquitin-mediated degradation.[43, 44] In some tumors, however, the overproduction of MDM2 due to gene duplication events inhibits activation of the p53 pathway, leading to uncontrolled cell growth. Such an amplification of MDM2 activity tends to bypass the cellular response to either natural or chemotherapeutic signals to execute programmed cell death, i.e. apoptosis, and is associated with poor therapeutic efficacy of anti-cancer regimens.[45] Genetic and biochemical studies establishing the critical role of MDM2 in p53

regulation by proteasomal degradation led to the hypothesis that inhibiting the p53-MDM2 interaction may rescue p53 levels and restore p53-mediated apoptotic pathways in tumor cells.

Interestingly, several small molecules derived from the Nutlins, the first potent and selective MDM2 inhibitors, are currently in clinical trials.[46-49] However, one limitation of these molecules is that they have very poor activity against MDMX.[50-52] Although MDM2 and MDMX are structurally similar, their p53-binding regions differ sufficiently enough to pose a challenge for developing small-molecule *dual antagonists* of both proteins. This challenge led researchers to hunt for an effective dual inhibitor that can simultaneously inhibit MDM2 and MDMX. Due to their relatively large binding surface compared to small molecules, peptides were considered naturally privileged candidates for dual antagonism of the p53-MDM2/MDMX interaction.

Prior to designing a peptidic MDM2 inhibitor, a clear understanding and detailed characterization of the p53-MDM2 interaction was critical. Short peptides derived from the p53 TAD1 were employed to investigate this interaction and the first p53-MDM2 co-crystal structure was reported by Pavletich and co-workers in 1996.[53] This structure paved the way for subsequent studies leading to the discovery of several small-molecule and peptide-based MDM2 inhibitors.[54] The X-ray crystal structure of the N-terminal domain of MDM2 (residues 17-125) bound to a transactivation domain peptide of p53 (residues 15-29) revealed that MDM2 has a deep hydrophobic cleft in which three hydrophobic amino acid side-chains from the p53 peptide (Phe19, Trp23, and Leu26) bind as an amphipathic α-helix (Figure 2A).[53] The key hypothesis arising from this structural study was that a synthetic molecule displaying three hydrophobic groups in an orientation mimicking the alignment of Phe19/Trp23/Leu26 in p53 would occupy the MDM2 cleft and inhibit its interaction with p53. Soon thereafter, several reports employed traditional structure-

based design and combinatorial synthesis to develop numerous potent small-molecule and peptide-based antagonists of p53-MDM2 binding.[54, 55]

Several p53-derived peptides were shown to inhibit the p53-MDM2/X interaction,[54, 55] but their low potency mandated further optimization. By displaying short peptide libraries on the surface of bacteriophage, Chen and co-workers successfully identified a 12-residue peptide Dual Inhibitor (pDI) (Figure 2B) as a strong binder of the hydrophobic cleft in MDM2 and MDMX.[56] As pDI showed strong dual binding, it inhibited MDM2 and MDMX-p53 binding much more effectively (> 300-fold) than a short p53 peptide competitor.[56] The co-crystal structure analysis of MDM2 and MDMX complexes with pDI and another more potent p53-MDM2/MDMX inhibitor (PMI) (Figure 2B) revealed the structural basis for their inhibitory action.[56, 57] Further fine-tuning the sequence and structure of the inhibitory peptide pDI led to the design of a Quadruple mutant peptide (pDIQ) (Figure 2B), which remains one of the most potent MDM2 peptide inhibitors to date.[58]

Another promising approach was the design of α-helically *stapled* p53-based peptides that prevent the p53-MDM2/MDMX interaction. Based on the biologically active α-helical conformation found in the p53 TAD1, stable hydrocarbon-stapled α-helical peptides were designed.[59, 60] Stapled peptides typically display improved resistance towards cellular proteases and are found to promote cellular uptake.[61, 62] The stapled peptides designed by Chang et. al acted as dual antagonists against MDM2 and MDMX and their *in vitro* results showed a substantial reduction of MDM2 and MDMX bound p53, which would translate to higher p53 activity in cells. Indeed, the intravenous administration of a stapled peptide, ATSP-7041 (Figure 2C), resulted in significant tumor growth inhibition in human cancer xenograft models in mice and holds good promise for future therapies aimed at inhibiting the p53-MDM2/X interaction.[60]

The p53-PC4 interaction

The protein PC4 was first identified in nuclear extracts and found to stimulate activator-dependent DNA transcription through RNA polymerase II by means of its ability to bind TBP-TAFII.[63] It seems to be present in all stages of the cell cycle[64] and plays a key role in several cellular processes such as transcription, replication, DNA repair, and normal cellular growth.[63, 65] By binding single-stranded (ss) DNA and stimulating ligase-mediated DNA end-joining, PC4 can promote the repair of double-strand breaks by non-homologous end-joining.[66,67] The interaction between PC4 and p53 is critical for PC4-mediated activation of p53 function; moreover, PC4-assisted DNA bending also enhances p53-DNA binding and activates its downstream functions.[68] A variety of PTMs are known to modify PC4, further fine-tuning its interactions with DNA and other interacting proteins such as p53.[68] Its acetylation by the histone acetyltransferase p300, for example, improves double stranded (ds) DNA binding. Alternately, phosphorylation of an N-terminally serine-rich region in PC4 inhibits its acetylation and reduces dsDNA binding.[69] Several reports have suggested that p53-PC4 interactions play an important role in p53-mediated gene activation, but the precise modification state(s) of full-length PC4 may also make additional contributions to this interaction. In order to understand the mechanism of PC4 and p53 binding, a combination of biophysical and biochemical techniques were employed to identify the interaction sites in the two proteins.[69, 70] Through NMR and ITC experiments, Fersht and co-workers showed that TAD2 of p53 mediates a selective interaction with the C-terminal DNA-binding domain of PC4. Peptides derived from TAD2 were employed to show that binding of TAD2 and ssDNA to PC4 were mutually exclusive, suggesting that p53 and ssDNA share a common PC4 binding site.[70]

Molecular docking studies with the TAD2 peptide and PC4 also supported this NMR-based hypothesis.

Surprisingly, another p53 domain has also been reported to interact with PC4. A GST-pulldown study showed that the p53-CTD(364-393) selectively interacts with PC4, but no interaction was observed with the p53-TAD(1-93) region.[71] To further narrow down the p53 binding region, Roy and co-workers employed total correlation spectroscopy (TOCSY) NMR experiments and reported that p53(380-386) is primarily responsible for binding PC4.[70, 72] Studies with the p53(380-386) peptide revealed that acetylation at Lys381 and Lys382 in the p53 peptide is crucial for modulating its binding to PC4. Further, ^{1}H,^{15}N Heteronuclear Single Quantum Coherence (HSQC) studies with isotopically labeled PC4 and GST-pulldown experiments revealed that Ser73 of PC4 is key to the interaction, and intermolecular Nuclear Overhauser Effect (NOE) NMR experiments suggested that Asp76 in PC4 is adjacent to the p53 Lys-381 acetyl group. Although, these reports presented contradictory observations regarding the specific p53 domain that interacts with PC4, both studies supported their claims with a list of biophysical and biochemical experiments, such as ITC, NMR, molecular docking, GST pull down assays, Electrophoretic Mobility Shift Assays (EMSA), and fluorescence anisotropy. It remains unknown if either the CTD or TAD or indeed both p53 domains are important for PC4 binding in cells.

The p53-BRCA2 interaction

The breast cancer type two susceptibility protein, BRCA2, is a human tumor suppressor protein that is responsible for DNA damage repair in complex with the double-strand DNA break repair recombinase enzyme RAD51. Disease-associated mutations in BRCA2 that impair DNA damage repair lead to chromosomal instability and increase the risk of breast and ovarian cancers.[73]

BRCA2 binding to RAD51 and p53 also regulates their cellular functions in homologous recombination and the DNA damage repair pathway, respectively.[73, 74] Studies show that inactivation of both BRCA2 and p53 contributes to mammary tumorigenesis, and that impairment of the p53 pathway plays a pivotal role in BRCA2-associated breast cancers.[75] Indeed, in mutant BRCA2-associated breast cancers, ~70% of cases also present mutations in p53.[76]

Early immunoprecipitation studies from cell lysates indicated that p53 forms a complex with BRCA2 and suggested a potential direct physical interaction between the two proteins.[77, 78] However, the site and mechanism of this interaction remained unclear. Fersht and co-workers were the first to employ peptide-based biophysical techniques to investigate p53-BRCA2 interaction. The results from p53 pulldowns suggested oligonucleotide/oligosaccharide-binding (OB) domains, OB2 + OB3 of the C-terminal domain of BRCA2 ($BRCA2_{CTD}$) have a selective binding interaction with p53-(1-93). They confirmed pull-down results with the help of several biophysical experiments, including fluorescence anisotropy, ITC, and HSQC NMR studies with the ^{15}N-labeled TAD(1–93) of p53, and observed specific binding (K_d ~ 2-5 μM) with the OB2 and OB3 domains of the $BRCA2_{CTD}$.[78] Moreover, fluorescence anisotropy experiments established that binding of ssDNA and p53 TAD2 to the $BRCA2_{CTD}$ was mutually exclusive and that p53 TAD2 competes with ssDNA for binding to the same or proximal site. This study also revealed a secondary binding site. The eight BRC repeats in BRCA2 specifically bind the p53 DBD, which may lead to a head-to-tail complex between p53 and BRCA2. Their study also suggested that such specific interactions with BRCA2 may contribute to the suppression of p53 transcriptional activity.[78] Altogether, this study provided some critical biophysical and structural information of p53-BRCA2 interaction that is crucial in designing molecules to target the BRCA-p53 interaction. This awaits future testing with small molecule or peptide inhibitors.

The p53-14-3-3σ interaction

The protein 14-3-3σ belongs to a highly conserved family of adaptor proteins found in species ranging from parasites to humans. In humans, there are seven different isoforms of the 14-3-3 protein with sequence differences in discrete regions called β, γ, ε, η, σ, τ and ζ.[79] 14-3-3 proteins interact with over 200 different proteins and play an essential role in crucial cellular processes, including metabolism, apoptosis, cell cycle regulation, protein trafficking (nuclear transport), signal transduction, and differentiation.[80, 81] 14-3-3 proteins bind phosphorylated motifs rich in serine and threonine residues, and protein phosphorylation regulates their protein-protein interactions and oligomerization status.[80, 82] Although p53 does not contain any classical 14-3-3 binding motifs, evidence suggests that all isoforms of 14-3-3 are involved in regulating p53 activity through direct protein-protein interactions.[83, 84] Among all the 14-3-3 isoforms, the high frequency of 14-3-3σ inactivation during tumorigenesis indicates its important role in tumor development. Its downregulation has been directly linked with several cancer types, including breast, gastric, prostate, lung, and ovarian cancers.[85-87] In response to cellular stress caused by DNA damage, the p53-CTD is known to be phosphorylated at Ser376 and Thr387 and to interact with the binding groove of 14-3-3σ.[83, 84, 88, 89] As a result of this interaction, p53 is rescued from MDM2 mediated ubiquitylation in the CTD, and 14-3-3σ binding facilitates p53 tetramerization and DNA binding (Figure 3A, B).[83, 84] This leads to higher levels of p53 in cells, increased transcriptional activity, and cell cycle arrest. These findings suggest that targeting and stabilizing the 14-3-3σ-p53 interaction using small molecules or peptides has the potential to phenocopy the effect of p53-MDM2 binding inhibitors.

Prior to designing a molecule that could stabilize the p53-14-3-3σ interface, it was necessary to study the molecular mechanism of this interaction. Several biophysical and structural studies were undertaken to shed light on the p53-14-3-3σ interaction. Using a 9-mer peptide derived from the p53 CTD (385-393), Ottoman and co-workers were the first to solve the X-ray crystal structure of a 14-3-3σ complex with the p53-CTD phosphorylated at Thr387, which provided the first direct look at this interaction.[90] Their structural study revealed that the p53 peptide interacts with the 14-3-3σ binding groove by means of a unique turn conformation induced by Gly389 and Pro390 that allows the C-terminus of p53 to form a salt-bridge interaction with Arg60 of 14-3-3σ (Figure 3B). Importantly, this study also revealed that the p53 phosphopeptide occupies only around two-thirds of the 14-3-3σ peptide-binding groove. This creates a pocket at the binding interface that may in principle accommodate a small-molecule protein-protein interaction stabilizer, also called *molecular glue*.[90, 91] From this discovery a few small molecules such as Fusicoccin-A, AZ-008 and Amifostine were tested and observed to enhance the p53-14-3-3σ interaction and promote p53 activation.[92-94] However, FP, ITC, SPR, and transcriptional assay based studies revealed that these small molecule stabilizers had only 2-4 fold increase in p53-14-3-3σ binding, which nevertheless led to significant increases in p53 levels, transcriptional activity and nuclear localization of p53.[92-94] These results highlight the opportunity to identify new molecules, or potentially small rigidified cyclic peptides, that could bind and stabilize the p53-14-3-3 complex as a viable therapeutic strategy in human cancers.

Semisynthesis of p53

Although several studies focusing on the role of p53 PTMs in protein–protein interactions have been undertaken with short p53-derived peptides,[72, 78, 90] these are inherently limited in scope

due to the fact that p53 is a multidomain protein that is active in its homotetrameric form and may be post-translationally modified at numerous residues. In order to best understand p53 function, there is an urgent need to access the full-length tetrameric protein bearing site-specific PTMs. To address these challenges, Müller and co-workers developed a protein semisynthesis approach to generate site-specifically phosphorylated p53.[95] By combining synthetic p53 N-terminal phosphopeptides with heterologously expressed truncated p53 proteins using native chemical ligation, they accessed milligram quantities of homogenously site-specifically mono- and di-phosphorylated p53 tetramers.[95]

In order to access the full length phosphorylated p53, Müller and co-workers divided full-length p53(1-393) into two fragments, namely p53(1-39) and p53(40-393)Met40Cys. The N-terminal fragment p53(1-39) which covers most of TAD1, is also a site for several phosphorylation events at Ser15, Ser20, Ser33 and Ser37.[26] The p53 N-terminal peptide was synthesized by solid-phase peptide synthesis (SPPS) and two protected phosphoserine residues (pSer15 and pSer20) were incorporated in the growing polypeptide by HATU/DIEA mediated standard coupling conditions. The truncated p53(40-393) protein fragment was obtained from the recombinant expression of a His_6-SUMO-p53(40-393)Met40Cys mutant fusion protein and subsequent Ulp6-mediated deSUMOylation to release the p53(40-393)Met40Cys fragment. Finally, the phosphopeptide was converted into a C-terminal α-thioester and both fragments were ligated under native chemical ligation conditions (Figure 4). The authors utilized this semisynthetic phosphorylated p53 in biochemical experiments to demonstrate that p53 N-terminal phosphorylation enhances p300-mediated acetylation at C-terminal K373. Such biochemical crosstalk among p53 N-terminal phosphorylation and C-terminal acetylation may play an important role in rescuing p53 from MDM2-mediated degradation, and in further activating p53-

mediated transcription of genes that are critical for DNA-repair and cell survival.[95] This study highlighted the importance of the protein semisynthesis approach to understand p53 regulation by phosphorylation and may easily be extended to other modifications observed in p53 by a careful selection of disconnection points that do not interfere with p53 tetramerization and function. Our lab is pursuing similar studies aimed at investigating the CTD of full-length p53, where a single lysine is modified by both isoforms of SUMO, i.e. SUMO-1 and SUMO-3 with largely unknown outcomes. One caveat to the current state of the art in p53 semisynthesis, however, is the introduction of Cys mutation at ligation sites as neither the N- nor C-terminal regions of p53 contain native Cys residues.

Conclusion and future prospects

From the many pioneering studies described in this review, it was established that in addition to its key DNA-binding role, protein–protein interactions play a critical role in cellular p53 function. In order to elucidate the mechanisms underlying these interactions and their relevance to pathophysiological processes, numerous biophysical and biochemical techniques have been employed to characterize and quantify p53-protein interactions. Particular challenges toward investigating crucial p53-protein interactions include the poor thermal stability of full-length wild-type p53, numerous dynamic PTMs that lead to p53 heterogeneity, and the obvious challenges associated with purifying stable and homogenous forms of p53-interacting proteins and their complexes.

Synthetic peptides have played a crucial role in addressing these outstanding challenges. p53-derived peptides paved the way for the first X-ray crystal structure revealing the details of p53 binding in the MDM2 groove. This led to the development of peptides mimicking small molecules

such as Nutlin and its derivatives that are currently in clinical trials targeting different type of cancers such as acute myeloid leukemia,[46] metastatic melanoma,[48] advanced solid tumor,[49] neoplasm malignant.[47] Successes in inhibiting the p53-MDM2 interaction have inspired others to investigate various chemical and peptide scaffolds in deciphering key p53-protein interactions, such as with BRCA2 and PC4, that underlie DNA damage repair and transcription, respectively. Detailed studies of p53-protein interactions are imperative toward understanding their pathophysiological roles and in developing peptide and/or small molecule activators of p53 function. A major challenge for the rapidly growing field of peptide-based therapeutics remains the time required to convert a bioactive peptide that has desired inhibitory properties to a peptide-like reagent that also possesses the ideal properties of a small-molecule, such as easy cell penetration, longer bioavailability and resistance to cellular enzymes such as proteases and P450s. One promising approach in this regard is the synthesis of stapled peptides that retain key side-chain interactions found in natural peptide sequences but are more resistant of cellular proteases. Finally, recent exciting efforts in the semisynthesis of modified forms of p53 are rapidly leading to a more complete picture of p53 regulation by its various PTMs and will undoubtedly guide future peptide-based inhibitor designs that also account for the various modified forms of p53 and their proposed divergent roles in cells.

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Figure 1. Major functional domains of the transcription factor p53. These consist of the N-terminal trans-activation domain (TAD), the proline-rich domain (PRD), the DNA-binding domain (DBD), the tetramerization domain (TD) and the C-terminal regulatory domain (CRD). Examples of domain-specific p53-protein interactions and their associated functions that were investigated by peptide chemistry are shown.

Figure 2. The design of peptide inhibitors of the p53-MDM2 interaction. (A) p53-derived peptides employed in investigating p53-MDM2 binding. (B) Examples of peptide dual-inhibitors of the p53-MDM2/MDMX interaction selected by phage display. (C) Example of a stapled peptide dual inhibitor (Note, R8 and S5 is α-methylated and stapled linker consists of 11 carbon atoms). All peptide sequences are presented with one letter amino acid code. Amino acids whose side-chains bind to the hydrophobic cleft in MDM2 are colored yellow.

Figure 3. Role of the p53-14-3-3σ interaction in preventing MDM2-mediated degradation and rescuing p53 function. (A) Canonical MDM2-mediated p53 polyubiquitylation, followed by proteasomal degradation. (B) 14-3-3σ binding precludes MDM2 binding and promotes p53 tetramerization, DNA binding and transcriptional activation. (C) X-ray crystal structure showing key interactions between 14-3-3σ and the p53(385-393) peptide containing pT387 (PDB: 3LW1).

Figure 4. Semisynthesis of site-specifically phosphorylated full-length p53. A short p53(1-39)pS15pS20-thioester fragment was obtained by solid-phase peptide synthesis. Recombinant protein expression with a His6 (H6)-SUMO-solubilizing tag followed by proteolysis of SUMO led to the p53(40-393)M40C protein fragment with an N-terminal Cys. Native chemical ligation of

the two fragments followed by refolding of the full-length doubly phosphorylated p53 protein enabled biochemical studies of PTM crosstalk.

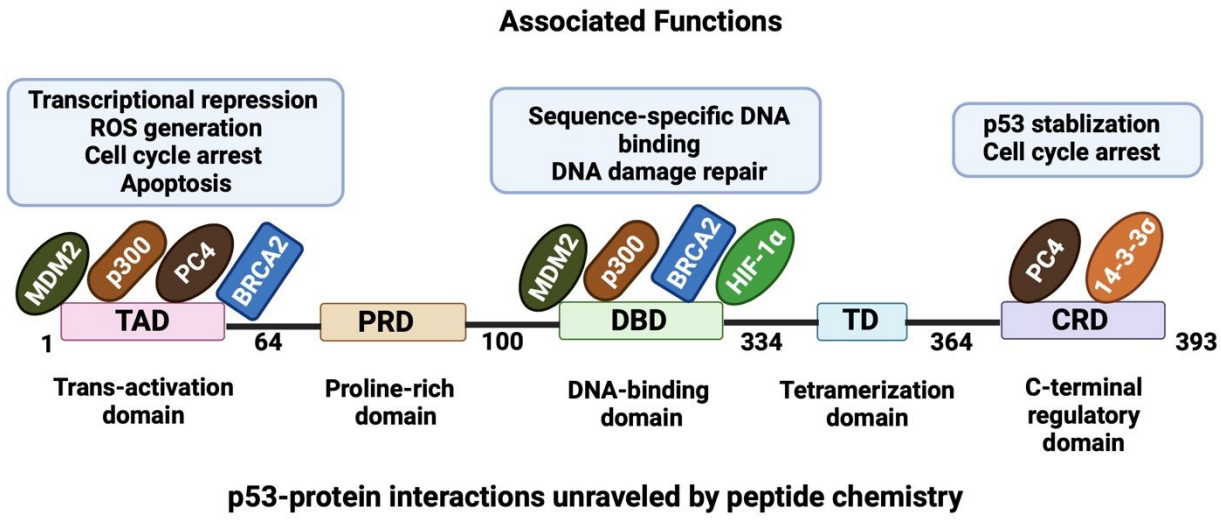

Figure 1

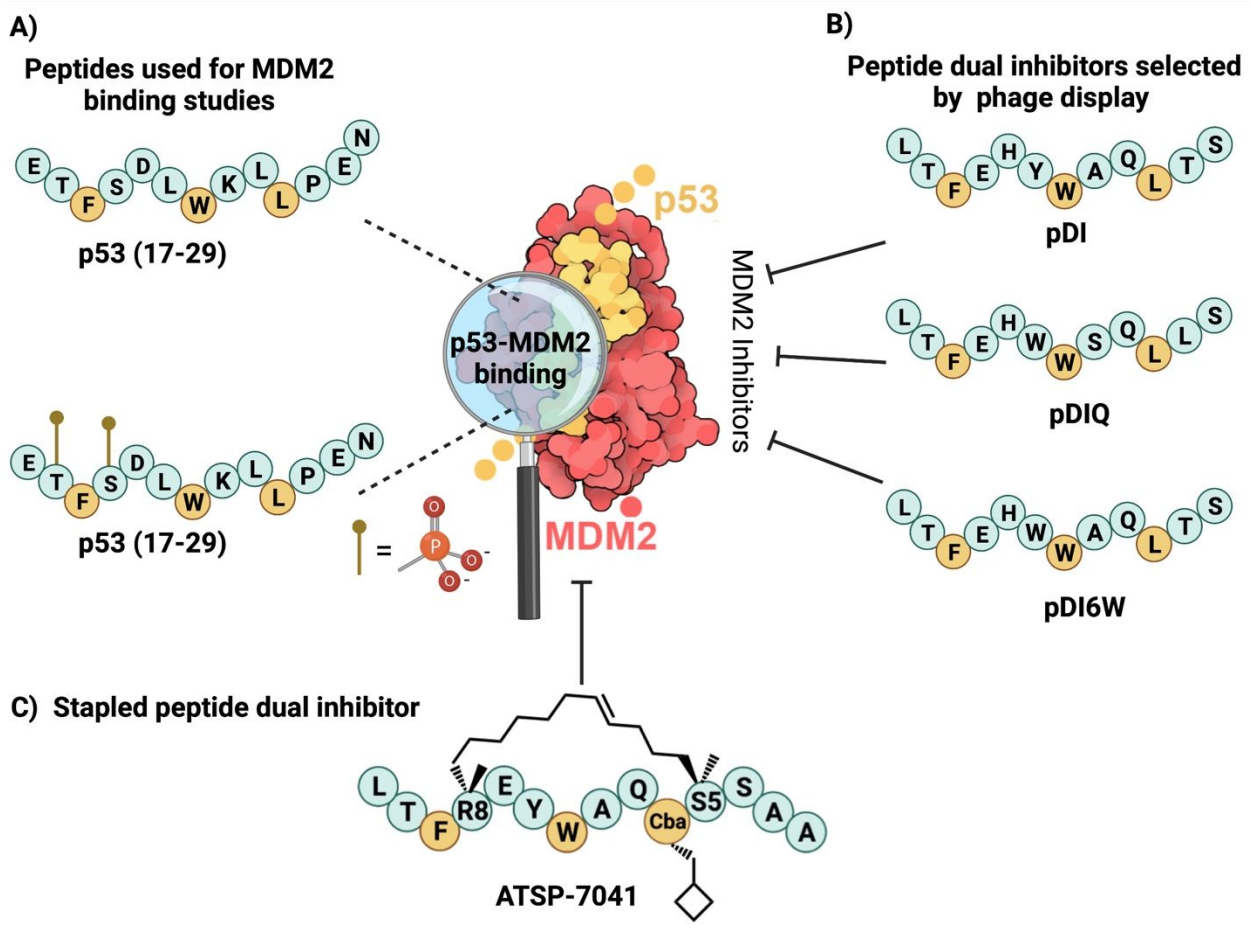
A)
Peptides used for MDM2 binding studies
p53 (17-29)
p53 (17-29)
p53
p53-MDM2 binding
MDM2
MDM2 Inhibitors
B)
Peptide dual inhibitors selected by phage display
pDI
pDIQ
pDI6W
C) Stapled peptide dual inhibitor
ATSP-7041

Figure 2

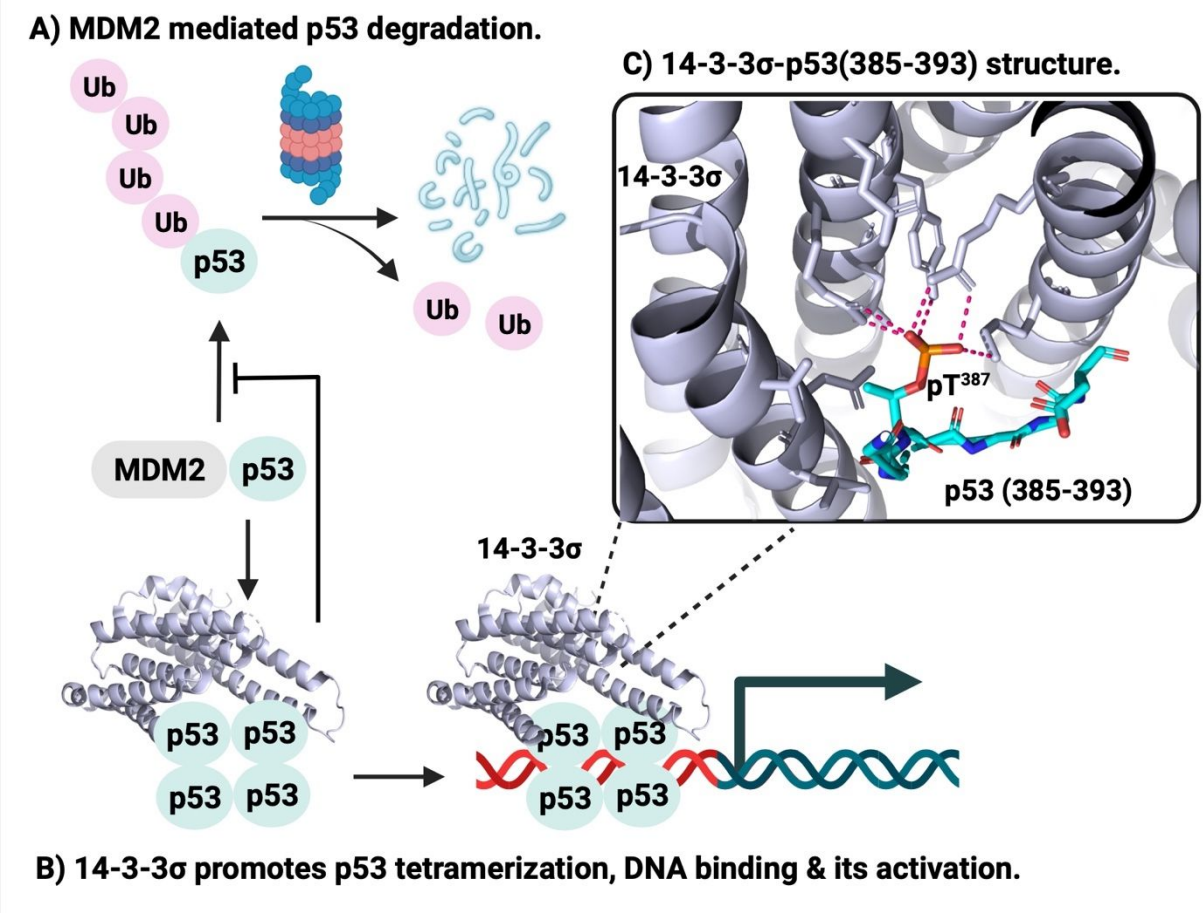
A) MDM2 mediated p53 degradation.
Ub
Ub
Ub
Ub
p53
Ub
Ub
MDM2
p53
p53
p53
p53
p53
C) 14-3-3σ-p53(385-393) structure.
14-3-3σ
pT387
p53 (385-393)
14-3-3σ
p53
p53
p53
p53
B) 14-3-3σ promotes p53 tetramerization, DNA binding & its activation.

Figure 3.

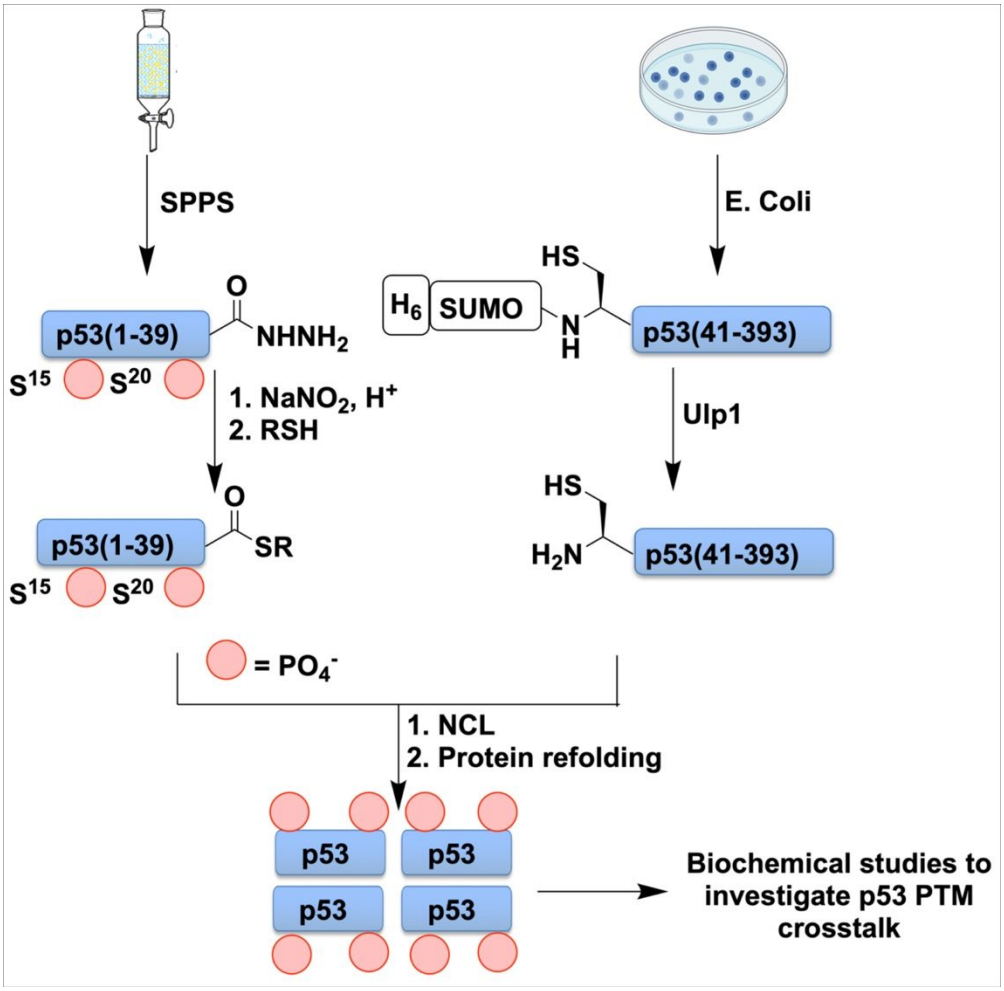
SPPS
E. Coli
p53(1-39)
NHNH2
S15
S20
1. NaNO2, H+
2. RSH
H6
SUMO
HS
N
H
p53(41-393)
Ulp1
HS
H2N
p53(41-393)
p53(1-39)
SR
S15
S20
= PO4-
1. NCL
2. Protein refolding
p53
p53
p53
p53
Biochemical studies to investigate p53 PTM crosstalk

Figure 4.