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UBIQUITINATION AND SUMOYLATION ANALYSIS OF THE P53 PROTEIN

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Dedication

This dissertation is dedicated to my mother, who passed on to become heavenly light from above. Even though we were separated by miles, your love, your strength and belief in me lifted me up through every challenge. I always aspired to make you proud and here is an academic achievement. To my family; although you weren't present in person, your prayers, sacrifices and faith in me had a way of leading and carrying me. Every time I experienced pride in my accomplishments, it was in a way that I knew all of you shared. This dissertation is a reflection of values you taught me, dreams you allowed me to dream, and love that would bridge any distance apart. I love you all

Abstract

The tumor suppressor protein p53 plays an essential role in the regulation of cell cycle control, DNA repair, and programmed cell death. These modifications regulate both p53 stabilization and localization. p53 levels and activity are regulated correctly by various post-translational modifications and disruption of these modifications can lead to the development of neurodegenerative diseases. In this study, ubiquitination, SUMOylation and acetylation sites on human p53 were predicted in silico, using GPS-Uber, GPS-SUMO/SUMOplot and ASEB, respectively. Two residues K386 and K164 are predicted to be sumoylated, ubiquitinated and acetylated, suggesting potential PTM crosstalk on these specific sites. The p53 SUMOylation at residue K386, located in the C-terminal regulatory domain, enhances retention in the nucleus and transcriptional activity. The modification of K164 located in the DNA-binding domain could allow degradation of P53 by ubiquitination or functional fine-tuning by acetylation and/or SUMOylation. Furthermore, the K351, present in the tetramerization domain, is predicted to be SUMOylated/Ubiquitinated. SUMOylation at K351 which could prevent MDM2-mediated ubiquitination, stabilize nuclear p53 retention in contrast to K351 ubiquitination which causes it degradation. Residues K292, K320 and K372 may host binary PTM overlaps, SUMOylationacetylation. In DNA-binding domain, this competition at residue K292 could alter DNA-binding efficiency, or transcriptional activity. However, in the C-terminal regulatory domain, this competition at K372, could potentially block ubiquitination at adjacent sites. Focusing on predictions of these three lysine modifications, not only allows us to identify candidate residues to consider for future experimental work, but it also clarifies our understanding of how the dynamic relationship with ubiquitination, SUMOylation and acetylation might regulate P53 activity.

Keywords:

p53, Ubiquitination sites, SUMOylation sites, Acetylation sites, Post-translational modifications, In silico analysis.

Résumé

La protéine suppresseur de tumeur p53 joue un rôle essentiel dans la régulation du contrôle du cycle cellulaire, la réparation de l'ADN et la mort cellulaire programmée. Les taux et l'activité de p53 sont étroitement régulés par diverses modifications post-traductionnelles (PTM) et la perturbation de ces dernières peut conduire au développement de maladies neurodégénératives. Dans cette étude, les sites d'ubiquitination, de SUMOylation et d'acétylation de la protéine p53 humaine ont été prédits in silico, en utilisant respectivement GPS-Uber, GPS-SUMO/SUMOplot et ASEB. Deux résidus K386 et K164 sont prédits comme étant sumoylés, ubiquitinés et acétylés, ce qui suggère une interaction potentielle des PTM sur ces sites spécifiques. La SUMOylation de p53 au niveau du résidu K386, situé dans le domaine régulateur C-terminal, augmente sa rétention dans le noyau et son activité transcriptionnelle. La modification du résidu K164, situé dans le domaine de liaison à l'ADN, pourrait permettre la dégradation de P53 par ubiquitination ou l'ajustement fonctionnel par acétylation et/ou SUMOylation. En outre, la protéine K351, présente dans le domaine de tétramérisation, est prédite pour être SUMOylée/Ubiquitinée. La SUMOylation au niveau de K351, qui pourrait empêcher l'ubiquitination médiée par MDM2, stabilise la rétention nucléaire de p53, contrairement à l'ubiquitination de K351 qui entraîne sa dégradation. Les résidus K292, K320 et K372 peuvent être le siège de deux MPT, SUMOylation-acétylation. Dans le domaine de liaison à l'ADN, cette compétition au niveau du résidu K292 pourrait altérer l'efficacité de la liaison à l'ADN ou l'activité transcriptionnelle. Cependant, dans le domaine régulateur C-terminal, cette compétition au niveau du résidu K372 pourrait potentiellement bloquer l'ubiquitination sur les sites adjacents. L'intérêt porté aux prédictions de ces trois modifications de lysine nous permet non seulement d'identifier des résidus candidats à considérer pour de futurs travaux expérimentaux, mais aussi de mieux comprendre comment la relation dynamique entre l'ubiquitination, la SUMOylation et l'acétylation pourrait réguler l'activité de la protéine P53.

Mots-clés :

p53, sites d'ubiquitination, sites de SUMOylation, sites d'acétylation, modifications post-traductionnelles, analyse in silico.

الملخّص

عناصر أساسية في تنظيم دورة الخلية، وإصلاح الحمض النووي، وآليات الموت p53تُعدّ بروتينات كابحة الأورام من نوع ونشاطه بدقّة عبر مجموعة من التعديلات ما بعد الترجمة، ويمكن لاختلال هذه p53يتم تنظيم مستويات .الخلوي المبرمج inationفي هذه الدراسة، تم التنبؤ بمواقع اليوبيكويت .التعديلات أن يسهم في تطوّر الأمراض التنكسية العصبية GPS-Uberالبشري باستخدام المحاكاة الحاسوبية عبر أدوات p53في بروتين Acetylationوالـ SUMOylationوالـ هما K164وK386أشارت النتائج إلى أن الحمضين الأمينيين .على التوالي ASEBو GPS-SUMO/SUMOplotو ، مما يدل على احتمال حدوث تفاعل Acetylationوالـ Ubiquitinationوالـ SUMOylationمرشحان للتعرض للـ متداخل للتعديلات في هذه المواقع.

، الواقع في المجال التنظيمي للطرف الكربوكسيلي، يعزز احتفاظ K386في الموقع SUMOylationتُظهر البيانات أن ، الموجود في مجال الارتباط بالحمض النووي، فقد K164أما التعديل عند الموقع .داخل النواة ويرفع نشاطه النسخي p53 كما .SUMOylationأو الـ/و Acetylationأو يضبط وظيفته عبر الـ inationعبر اليوبيكويت p53يسهّل عملية تحلّل قد .Ubiquitinationو SUMOylation، الواقع في مجال التتريمريّة، مرشح كذلك لتعديلي K351أُشير إلى أن الموقع ، مما يؤدي إلى تعزيز استقرار MDM2بوساطة inationعند هذا الموقع على منع اليوبيكويت SUMOylationتعمل الذي يوجّه البروتين نحو التحلّل K351عند Ubiquitinationداخل النواة، على عكس p53.

قد تكون عرضة لنوعين من التعديلات المتنافسة، وهي K372وK320وK292أظهرت النتائج أيضاً أن المواقع إلى التأثير K292في مجال الارتباط بالحمض النووي، قد يؤدي التنافس على الموقع .Acetylationو SUMOylation أما في المجال التنظيمي للطرف الكربوكسيلي، فقد يؤدي .بالحمض النووي أو نشاطه النسخي p53في كفاءة ارتباط في المواقع المجاورة Ubiquitinationإلى تعطيل حدوث K372التنافس على الموقع.

تساعد هذه التنبؤات المتعلقة بثلاثة أنواع من تعديلات الليزين في تحديد مواقع مرشحة للدراسة في الأبحاث المستقبلية، كما Ubiquitinationمن خلال التفاعل الديناميكي بين p53تساهم في فهم أعمق لكيفية تنظيم النشاط الوظيفي لبروتين وSUMOylation وAcetylation.

الكلمات المفتاحية

، التعديلات ما بعد الترجمة، تحليل Acetylation، مواقع الـSUMOylation، مواقع الـination، مواقع اليوبيكويتp53 in silicoحاسوبي

List of Abbreviation

List of abbreviations

ATM: Ataxia-Telangiectasia Mutated kinase

Bax: BCL-2-Associated X Protein

CRD: C-Terminal Regulatory Domain (of p53)

CTD: C-Terminal Domain (of p53)

DBD: DNA-Binding Domain (of p53)

DDR: DNA Damage Response

DUBs: Deubiquitinating Enzymes

E1,,: Ubiquitin-Activating Enzyme,

E2: Ubiquitin-Conjugating Enzyme,

E3: Ubiquitin Ligase

FBXO: F-Box Protein family (subset of E3 ligases)

FRDA: Friedreich's Ataxia

GO: Gene Ontology

GPS-SUMO: Group-Based Prediction System for SUMOylation

HD: Huntington's Disease

MOMP: Mitochondrial Outer Membrane Permeabilization

NLS: Nuclear Localization Signal(s)

NTD: N-Terminal Domain

OD: Oligomerization Domain (of p53)

PD: Parkinson's Disease

PIAS: Protein Inhibitor of Activated STAT (SUMO E3 ligase)

PRD: Proline-Rich Domain (of p53)

PTM: Post-Translational Modification

PSSM: Position-Specific Scoring Matrix

RD: Regulatory Domain (of p53)

ROS: Reactive Oxygen Species

SCF: Skp1-Cullin-F-box complex (type of E3 ligase)

SCA3: Spinocerebellar Ataxia Type 3

SENP: Sentrin/SUMO-Specific Protease

SUMO: Small Ubiquitin-like Modifier

TD: Tetramerization Domain

TAD: Transactivation Domain

TP53: Tumor Suppresor Protein 53

UBL: Ubiquitin-Like (modifier)

UPS: Ubiquitin-Proteasome System

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INTRODUCTION

introduction

The tumor suppressor protein p53 plays an essential role in the regulation of cell cycle control, DNA repair, and programmed cell death. It serves as a transcription factor that regulates transcriptional responses to a variety of cellular stress. Importantly, p53 levels and activity are regulated correctly by various post-translational modifications (PTM) (McLure & Trifunovic, 2023). Ubiquitination marks p53 for proteasomal degradation, ensuring tight control over its levels (Lee & Gu, 2021), while SUMOylation enhances its transcriptional activity by stabilizing nteractions with coactivators (Seeler & Dejean, 2023). These PTMs form a dynamic regulatory network that tailors p53's tumor-suppressive outputs (Zhang et al., 2022). Ubiquitination and SUMOylation dynamically compete for overlapping lysines of p53 with cellular conditions influencing which modification prevails. E3 ligases (e.g., MDM2) and SUMO-conjugating enzymes (e.g., UBC9), determine whether p53 will be degraded or stabilized and therefore transcriptionally active. This crosstalk incorporates stress severity and cellular context to modulate p53's functional fate (Jansson et al., 2021; Enserink et al., 2022). In as much as these two PTMs are vital to the regulation of the p53, other regulatory PTMs like acetylation may take place in the same sites as these two thus creating a dynamic regulatory PTM crosstalk, we therefore had a look into acetylation too and verified if it competes for lysine residues with ubiquitination and SUMOylation on the p53 how it could influence the activity of ubiquitination and SUMOylation.

This study seeks to find out the specific lysine residues which undergo ubiquitination and SUMoylation mainly, and then acetylation using computational predictive tools and then try to answer the essential question of how ubiquitination and SUMOylation work dynamically to govern the stability, localization and functional activity of p53 by finding out which lysine residues are mediating the regulatory crosstalk of the p53. Many diseases result from dysregulation of these PTMs notably neurodegenerative diseases. Our study aimed to predict the ubiquitination and SUMOylation sites using GPS-Uber and GPS-SUMO/SUMOplot, respectively. While this study remained on ubiquitination and SUMOylation with respect to PTM, acetylation site prediction was also provided for additional context using ASEB. Including acetylation prediction provides a more comprehensive analysis of potential crosstalk and competition for modification especially within functionally relevant regions of p53.

Focusing on predictions of these three lysine modifications not only allows us to identify candidate residues to consider for future experimental work, but it also clarifies our understanding of how the dynamic relationship with ubiquitination, SUMOylation and acetylation might regulate p53

Lastly, this manuscript is divided into two parts. Part 1 containing chapters 1–3. Chapter 1 reviews the structural and regulatory biology of p53, chapter 2 dives into the regulatory post translational modifications of p53 detailing Ubiquitination and SUMOylation, chapter 3 connects dysregulations of PTMs to neuronal pathology. Part 2 containing chapters 4 and 5, details the computational methodology that was used to generate the results, and discussion of the findings, climaxing it with a conclusion and perspectives.

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PART 1

THEMATIC SYNTHESIS

CHAPTER ONE

THE STRUCTURAL AND FUNCTIONAL BIOLOGY OF P53

1.1 Structure of p53

The p53 protein, commonly known as the "guardian of genome," is encoded by the TP53 gene and serves many roles in ensuring genomic stability. It is a globular protein of 393-amino acid residues, first identified in 1979 and subsequently named based on its molecular weight of 53 kDa (Levine & Oren, 2009; Soussi, 2010).

The human p53 protein contains three major domains: N-terminal domain (NTD), DNA-binding domain (DBD), and C-terminal domain (CTD). The NTD consists of the acidic N-terminus transactivation region (TAD) and a proline-rich region (PRD). CTD consists of a tetramerization domain (TD) also known as oligomerization domain (OD) and a regulatory domain (RD). The DBD is involved in binding to the DNA itself, along with the TD, which also plays a role in the formation of the tetramic quaternary structure of the p53 protein (Chene, 2001). The RD also known as the CRD (C-terminal regulatory domain) stabilizes the tetrameric structure and contributes to the regulation of other protein,

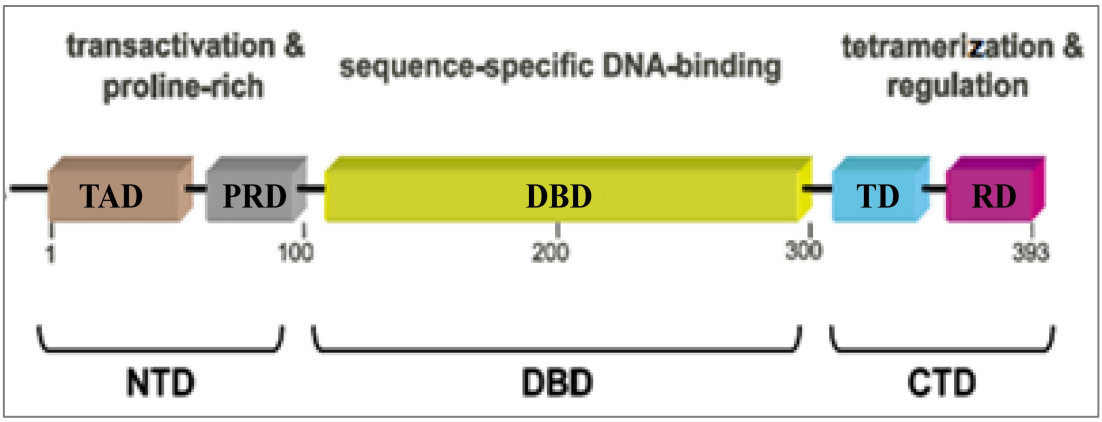


Figure 1.1 Structure of p53 (adapted from Sun Z, 2015)

1.1.1 N-Terminal Domain

The first 63 residues of p53 represent the N-terminal Transactivation Domain, which has been further defined as two distinct subdomains of the Transactivation Domain; TAD1 and TAD2 (residues 1-42 and 43-63 respectively). TAD1 induces the expression of pro-arrest genes, and binds transcriptional co-activators like p300/CBP. TAD2 stabilizes that interaction while activating pro-apoptotic targets like BAX. The NTD is also made up of the Proline-Rich Domain (residues 64-97) lies contiguous to TAD2 which utilizes PXXP motifs to recruit partners with an SH3/WW-domain to

direct stress-specific signals towards pro-apoptotic gene expression. The PRD also affects local phosphorylation and conformational changes that affect locally ubiquitination/SUMOylation to further adjust p53's stability, localization, and transcriptional specificity (Bode & Dong, 2004; Joerger & Fersht, 2010)

1.1.2 DNA-Binding Domain

The DBD is comprised of residues 102-292, and is characterized by a highly conserved β-sandwich scaffold together with a finger-like loop that coordinates zinc. More than 90% of mutations associated with p53 are clustered in DBD mostly influencing the integrity of binding to DNA or disrupted the DBD protein structure. The DBD is also a dynamic binding site for chaperones (like HSP70) that promote refolding, this enhances transcriptional activity (Joerger & Fersht, 2008)

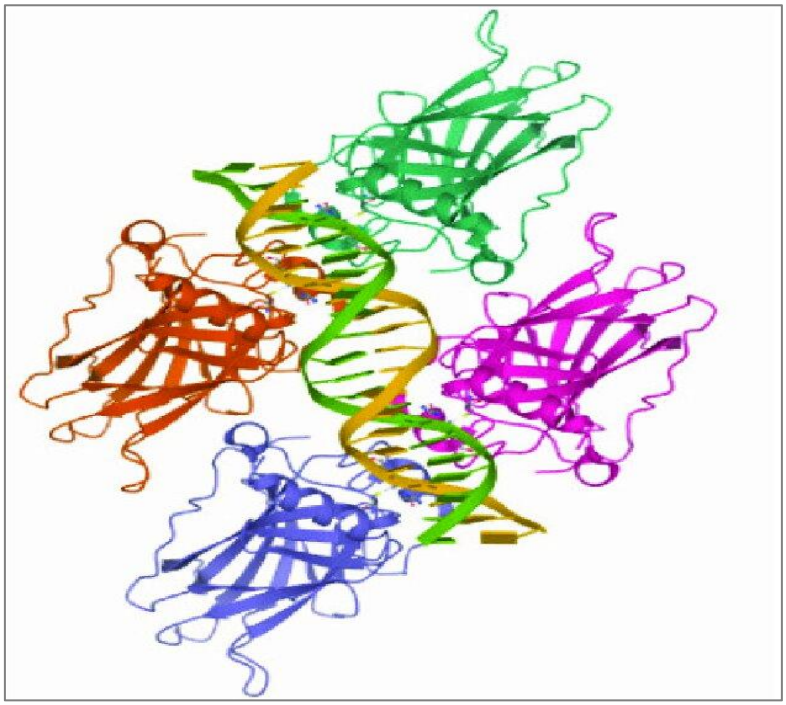


Figure 1.2: Crystal structure of p53 protein DBD

Referance: PDB ID: 4HJE.

1.1.3 Tetramerization Domain

The TD is made of residues 325 to 356, provide a dimer β-strand interface; two dimers come together through hydrophobic interactions to form a functional tetramer. Tetramerization is necessary for cooperativity in DNA binding, allowing p53 to regulate a wide variety of promoters (Joerger & Fersht, 2008)

1.1.4 C-Terminal Regulatory Domain

The CRD is found in the region of residues 363 to 393, This region contain nuclear localization signals (NLS) and phosphorylation sites. The CRD will autoinhibit DNA binding when cells are not stressed, however, post-translational modification (i.e., Ser392 phosphorylation) antagonizes repression and thus increase DBD activity. The CTD is the primary hub of ubiquitination and SUMOylation thus the domain responsible for regulating p53 stability and subcellular localization. (Kruse, J.-P.,& Gu, W., 2009 : Joerger & Fersht, 2008)

1.2 Tissular distribution of p53 protein

p53 is widely expressed in tissues across the human body, but it is often influenced by context, such as proliferation pressure or stress exposure. In rapidly dividing tissues such as the intestinal epithelium and skin, p53 is fully active at baseline and must cope with replication-related stresses and DNA damage to maintain genomic integrity (Kastenhuber & Lowe, 2017). Neuronal tissue maintains p53 activity in a tightly controlled manner to mitigate unneeded apoptosis. In the case of most neurons, p53 is typically retained within the cytoplasm at baseline, where it is prevented from unintended activation from basal activity via binding interactions with MDM2 or with chaperone proteins selected by the neuron (Tedeschi & Di Giovanni, 2019). Despite this, oxidative or genotoxic stress can initiate nuclear translocation to initiate the transcription of pro-apoptotic genes. Contractile skeletal muscle has p53-dependent checkpoints during regeneration (Vousden & Prives, 2018). The spatial regulation of p53 activity preserves local responses to p53 depending on tissue demands and highlights how p53 contributes to homeostasis and protects from tumorigenesis under a variety of physiological conditions (Bieging et al.,2014)

1.3 Physiological role of p53

p53 regulates the cell cycle and mediates the response to stress. It is kept inactive under normal conditions, but is activated when it is needed, usually under circumstances of DNA damage.

1.3.1 Cell Cycle Arrest and DNA Damage Repair

As a core component of the DNA damage response (DDR), p53 induces cell-cycle arrest, halting division until damage is repaired and preventing mutations from being passed to daughter cells. The DDR, in response to stress, can activate checkpoint kinases CHK1 and CHK2, that are able to inhibit cyclin-dependent kinases (CDKs) and their associated cyclins; they do this to establish checkpoints to effectively respond to lineages with DNA lesions.In response, p53 will then target and induce the CDK inhibitor, p21 (CDKN1A), which prevents any phosphorylation of the CDK4/6–cyclin D and the CDK2–cyclin E complexes. This inhibition prevents any phosphorylation of the retinoblastoma protein (Rb) and allows Rb to remain coupled to the E2F transcription factors (TF) to halt the progression from G_1 to S phase (Jones et al., 2022). Additionally, p53–p21 leads to the assembly of the DREAM complex (DP, Rb-like, E2F and MuvB) to repress additional cell-cycle genes.

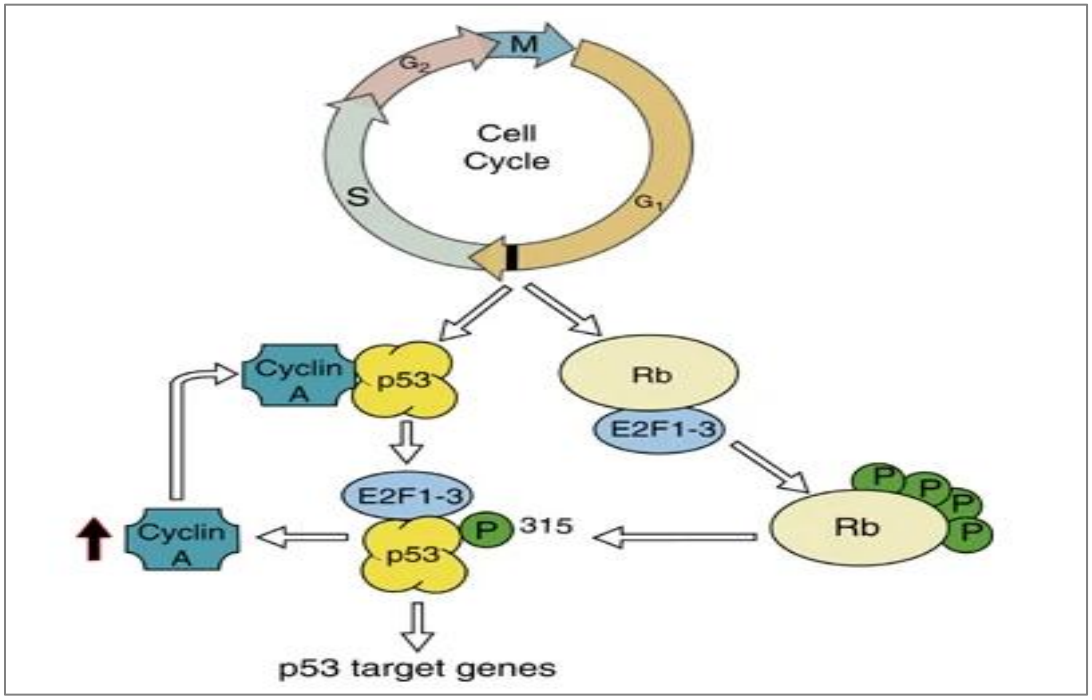


Figure1.4: An illustartion of how p53 regulates the cell cycle.

Once activated, p53 transcribes the target genes that inhibit Cyclin-CDK complexes, which inhibits phosphorylation of Rb and keeps Rb bound to E2F1-3 (which are the transcription factors required for entry into S-phase). If Rb remains unphosphorylated, it suppresses E2F1-3, inhibiting entry into S-phase. In normal conditions, Cyclin-CDKs will phosphorylate Rb and release E2F1-3 to activate genes that are required for DNA replication. p53 will pause damaged cells to repair them, or ultimately kill them via apoptosis, prioritizing genomic stability over division.

ATM/ATR kinases regulate p53 function by evaluating the traumatic impact of DNA damage, leading to cell repair in situations of low stress or, conversely, promote

apoptosis/senescence in situations of high stress (Vousden & Lane, 2007). p53 makes transcriptional decision mostly as a transcription factor, but it uses a non-transcriptional mechanism for some of its cell-fate decision making (Green & Kroemer, 2009).

1.3.2. Induction of Apoptosis and senescence

When damage cannot be resolved, p53 triggers programmed cell death (PCD) in order to eliminate defective cells. Nuclear p53 transactivates pro-apoptotic members of the BCL-2 family (B-Cell lymphoma2), including BAX (B-cell Associated X protein), PUMA (p53 upregulated Modulator of Apoptosis) and NOXA, to facilitate mitochondrial outer membrane permeabilization (MOMP), cytochrome c release and caspase activation (Vousden & Lu, 2020). In addition to transcriptional regulation, there is a cytosolic pool of p53 which can bind BCL-2/BCL-XL directly, thus displacing BAX to hasten MOMP. p53 is also an important regulator of both intrinsic and extrinsic death signalling. p53 can drive cells to become more sensitive to extrinsic signals of death.

Senescence is a growth arrest that is permanent and stops any duplication of damaged or pre-cancerous cells. The growth arrest that is senescence is brought about by p53, which drives it via the p21-Rb axis; here, p53-induced p21 inhibits cyclin-dependent kinases, leading to Rb-mediated E2F target repression like in apoptosis (Chang et al., 2023). How p53 operates can depend on biological context (cell type and type of stress); for example, acute stress can lead to the induction of arrest and repair, and chronic stress can enhance the signals leading to apoptosis or senescence this is illustrated in the pathway shown in figure 1.4 below.

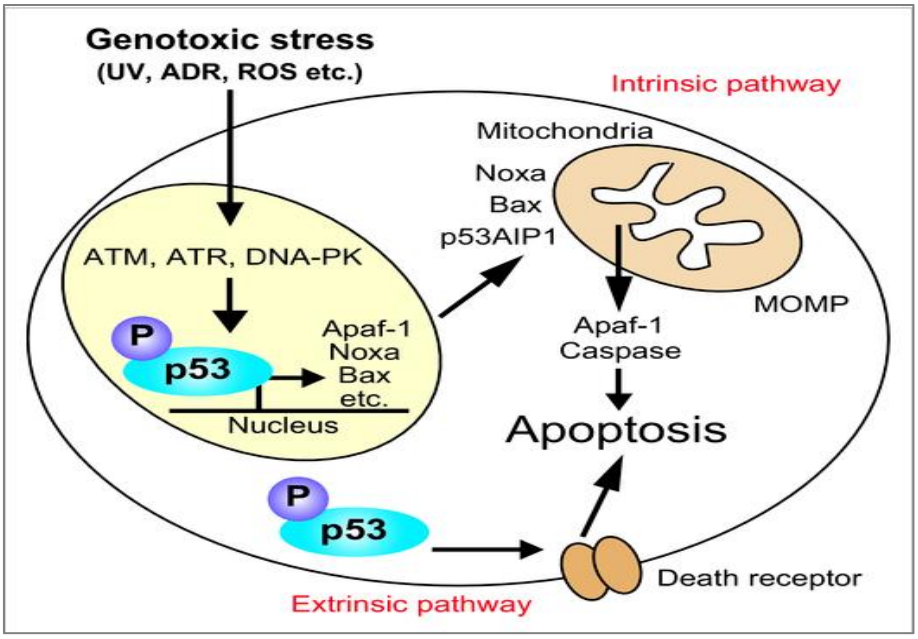


Figure 1.4: How p53 induces apoptosis. Genotoxic stress (UV, ADR, ROS) triggers ATM/ATR/DNA-PK kinases that phosphorylate and stabilize p53 in the nucleus which then drives transcription of pro-apoptotic genes (Bax, Noxa, p53AIP1, Apaf-1) that induce mitochondrial outer membrane permeabilization and caspase-9 activation (the intrinsic pathway), while also upregulating death receptors such as TNF-α receptor 1 to immediately activate caspase-8 (the extrinsic pathway) leading to apoptosis.

1.4 p53 in Neuronal Physiology

UnderUnder resting (basal) conditions in neurons, MDM2-mediated ubiquitin-dependent degradation of p53 occurs and p53 levels are kept low to stop cell death and cell malfunction (Tedeschi & Di Giovanni, 2019). Under mild metabolic or oxidative stress conditions (e.g. minor synaptic activity changes), p53 is stabilized and transcription is biased towards a cell-protective gene expression program. p53 can induce products that are antioxidants e.g. MnSOD and metabolic controls, as these can limit the presence of reactive oxygen species (ROS) and ensure that pathway proteins are induced to repair DNA and maintain genomic integrity (Lee & Ryu, 2020). Moreover, p53 behaviours extend beyond the immediate stress response, p53 can also impact mitochondrial health. When p53 coordinates these activities, it enables a neuron to withstand short-term and mild stresses, without demanding an apoptotic outcome.

In addition to its role in helping synapses adapt to mild changes in metabolic conditions, p53 is also important to neuronal synaptic homeostasis and synaptic

plasticity. p53 can cause differential expression of proteins that sit at the synapse (e.g. synaptophysin), and thereby, affect synaptic strength (Vogel et al., 2022). p53 also supports neuronal autophagy and promote mitochondrial clearance processes, by inducing proteins like Parkin . These properties help to remove damaged mitochondria and prevent ROS (Kim & Park, 2023).

Having established knowledge of the p53 domain architecture and the physiological relevance of p53, it becomes important to understand how this structure is altered through chemical modifications. Chapter 2 will consider the major post-translational modifications (ubiquitination, SUMOylation, acetylation, etc.) that fine-tune p53 stability, localization and activity.

CHAPTER TWO

REGULATORY POST TRANSLATION MODIFICATIONS OF P53

2.1 Overview and crosstalk of Post Translational Modifications

Post-translational modifications are covalent chemical modifications after the synthesis (translation) of the protein that can change its structure, function, location, stability, or its interactions. These are enzyme-mediated or enzyme regulated changes, e.g., phosphorylation, acetylation, ubiquitination, SUMOylation, and methylation, which provides functional diversity of the proteome so that dynamic and reversible control of cellular processes like signaling, gene expression, and stress response can take place.

PTM networks regulate several key post-translational modifications that act in concert to modulate p53's roles, linking environmental signals to specific transcriptional outputs. For instance, ubiquitination is linked to p53 degradation by the proteasome and targets p53 for degradation to maintain lower than basal levels of p53 in unstressed cells. Stress-induced PTM perturbs ubiquitination (i.e. phosphorylation, acetylation,) providing stabilization to p53 and allows the accumulation of p53 during DNA damage. After accumulation, SUMOylation stabilizes p53 in the nucleus, preventing nuclear export signals and increasing interactions with transcriptional co-activators. Taken collectively these PTMs stabilize p53 and allow increased spatial access to ther the many other secondary stress response gene promoters that p53 binds to, ultimately driving apoptosis or cell-cycle arrest.

Acetylation and phosphorylation have been shown to coincidentally block ubiquitin-dependent degradation while promoting transcriptional activation. In context of P53, even site-specific ubiquitination may provide transient suppression of DNA binding.

Variations between modification states create a "PTM code" that leads to differences in p53 stability, subcellular localization, or change in function to regulate p53 responses specifically to stimuli (Meek & Anderson, 2009). Although deciphering the p53 "code" is complicated, proper identification of the specific motifs for the PTM regulation is the key to designing precision therapies targeting p53 for a variety of diseases, including cancer and neurogenerative diseases, **figure 2.1** shows us all the different key regulatory PTMs of p53 and distinct p53 domains including specific amino acid residues from where they regulate p53 activity.

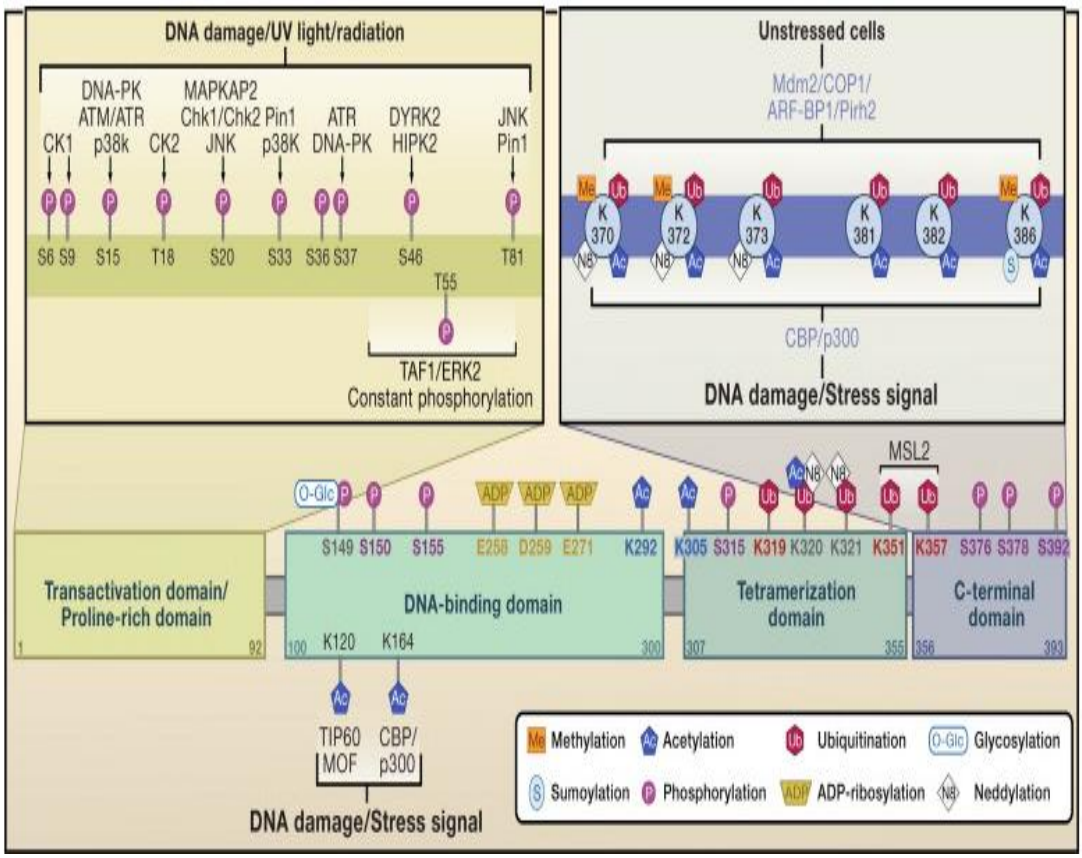


Figure 2.1: Distribution of PTMs of p53 per domain (adapted from Kruse, J.-P.,& Gu, W.,2009)

Ubiquitination, SUMOylation and the Ubiquitine-proteosome system

1.1) Ubiquitination

Ubiquitination, also known as ubiquitylation, is an enzymatic process that involves the bonding of an ubiquitin protein to a substrate protein.

Ubiquitin is conjugated to a substrate protein through an enzymatic catalytic process involving a number of steps. Ubiquitination is an important regulatory mechanism of protein turnover through the ubiquitin-proteasome system (UPS) as well as other roles related to DNA repair, cell signaling, and immune responses (Zheng & Shabek, 2017). Ubiquitination involves a series of reactions facilitated by three main enzyme species that have been well illustrated in **figure 2.2** below, E1 (the ubiquitin activating enzyme), E2 (the ubiquitin conjugating enzyme), and E3 (the ubiquitin ligase). The first enzyme, E1, activates the ubiquitin molecule in an ATP dependent biomolecular reaction, resulting in the generation of a high-energy thioester bond. Upon activation,

the ubiquitin molecule is transferred to the E2 enzyme, which is responsible for moving it. The last and most critical enzymatic step is performed by the E3 ubiquitin ligase. E3 ligases facilitate the transfer of ubiquitin and also confer substrate specificity in the ubiquitination process.

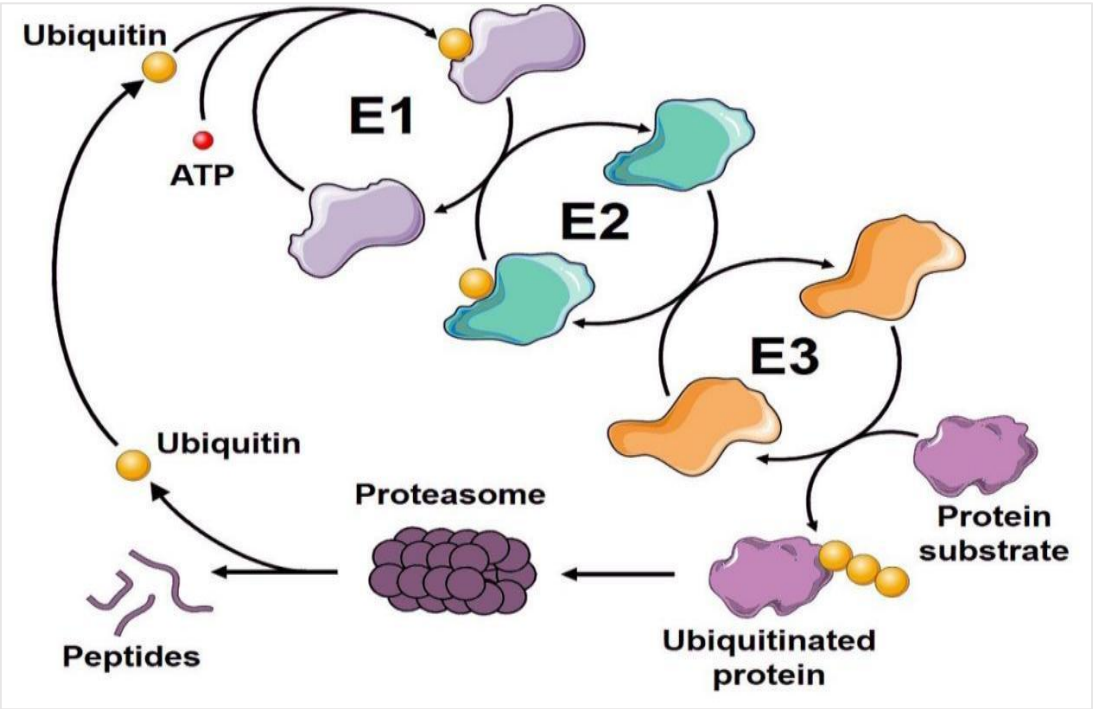


Figure 2.2: Ubiquitination enzymatic cascade

Ubiquitin protein

Ubiquitin is a highly conserved small, 76 amino acid compact globular protein. It consists of a central β-sheet that is presumed to be stable and surrounded by one α-helical structure and two flexible loops. Moreover, the single glycine residue at the C-terminus is the site of covalent linkage to lysines on target proteins. Ubiquitin's domains provide a variety of linkage types; thus, ubiquitin has numerous roles within the cell that are outside of protein degradation (Husnjak & Dikic, 2012).

1.2) SUMOylation

SUMOylation is a post-translational modification that modifies protein function, location, and stability. SUMOylation involves small ubiquitin-like modifier proteins—SUMO1, SUMO2, and SUMO3. The SUMO proteins require activation by a family of proteases known as sentrin-specific proteases (SENPs), which remove a

short tail at the C-terminus of the SUMO proteins to expose a glycine-glycine motif that is reactive (Hickey et al., 2012). After activation, SUMOylation involves the same enzymatic pathway described in **figure 2.2** that occurs in ubiquitination only that the the E1 activating enzyme which is a heterodimer of SAE1& SAE2 (Sumo Activating Enzymes).(Gareau & Lima, 2010). Moreso unlike in ubiquitination, the E2 conjugating enzyme in SUMOylation is specificically known as Ubc9 (Ubiquitin conjugating enzyme 9), which may transfer SUMO directly to the p53 or transfer it to E3 ligases, the precise description of the SUMOylation process has been ilustrated in **figure 2.3**. For the specific case of p53, E3 ligases such as PIAS1 and PIAS4 guide Ubc9 to the correct lysine residue. The E3 ligases are capable of identifying the target motifs on p53; Ψ-K-X-E/D, which are generally described as a hydrophobic residue (ψ), a lysine (K), a variable residue (X), and acidic residues (E or D) (Cappadocia & Lima, 2018).

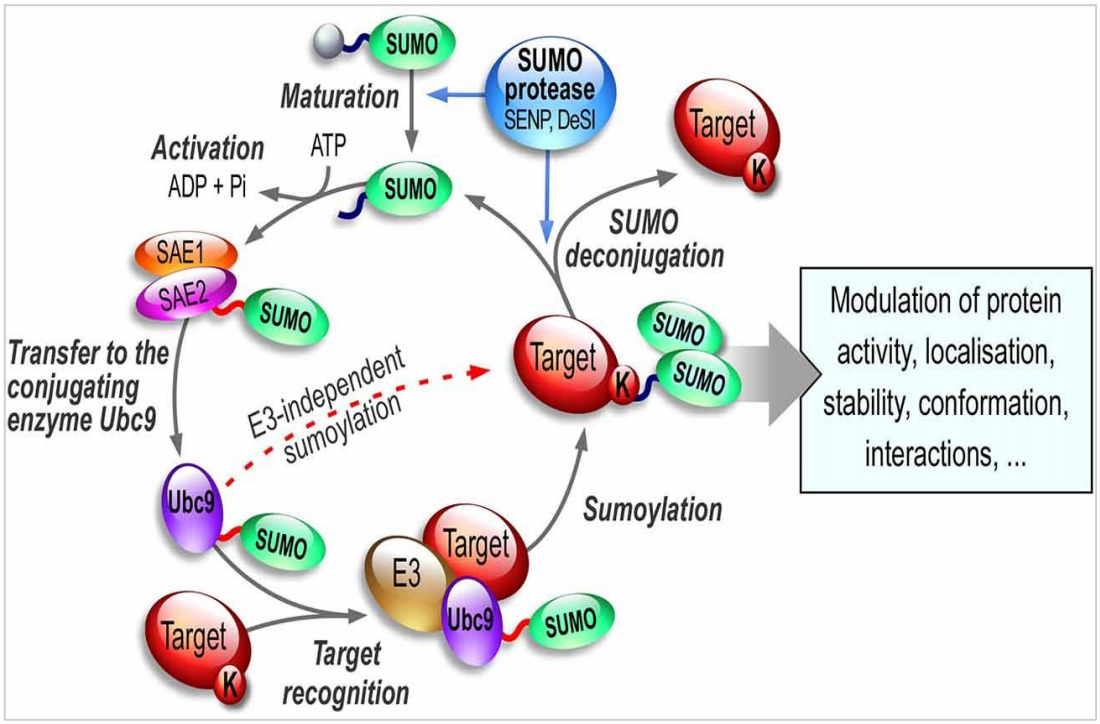


Figure 2.3: The SUMO enzymatic pathway (adapted from Schorova & Martin, 2016)

SUMO proteins

SUMO proteins are a group of small (approximately 10-12 kDa) ubiquitin-like (UBL) proteins that are evolutionarily conserved in eukaryotes. Structurally, SUMO exhibits a β-grasp fold, similar to ubiquitin, containing a central α-helix that is flanked by a β-sheet (β1-β5 strands) and a flexible c-terminal tail (Hay, 2001; Johnson, 2004).

Humans have five isoforms of SUMO proteins: SUMO1-SUMO5. SUMO isoforms have differentiated N-terminal extensions and surface charge distributions that dictate isoform-specific interactions (Wang & Dasso, 2009). SUMO conjugation (SUMOylation) occurs via a three-step enzymatic cascade similar to ubiquitination but with distinct enzyme specificity (Flotho & Melchior, 2013).

C) Ubiquitin-Proteosome System

The Ubiquitin-Proteasome System (UPS) is an important pathway for targeted protein degradation in eukaryotes. It plays a fundamental role in cellular quality control and regulation (Alberts et al., 2015). At the centre of the UPS is the 26S proteasome, an ATP-dependent protease. The 26S proteasome is made up of a 20S catalytic core and 19S regulatory particles (either one or two) (Finley, 2009). The 19S unit recognizes a polyubiquitinylated substrate protein through a specific series of E1, E2, and E3 enzyme same as that described in **figure 2.2**. The 19S particle utilizes ATP to hydrolysis remove the ubiquitin chains from the substrate protein, unfolds the substrate protein, and channels it into the 20S core for degradation. The UPS is selective for polyubiquitinated proteins, including mis-folded proteins, damaged proteins, and regulatory proteins (Min & Baek, 2013). The ATPase activity of the 19S cap is critical for substrate processing and recycling ubiquitin. Regulation of the UPS governs many fundamental cellular processes. The UPS also regulates the cell cycle, stress response, and protein homeostasis.

In the case of p53, the ubiquitin-proteasome system has two opposing functions. In unstressed cells, MDM2 ubiquitinates p53, leading to its degradation and low levels. However, during cellular stresses such as DNA damage, that process is inhibited. Stress signals can inhibit MDM2 activity, allowing p53 to escape degradation and bind to and activate genes that either inhibit cell cycle progression or promote cell death (Liu et al, 2019). This substrate regulation enables p53 to respond to damage appropriately.The pathway is also tightly regulated via other modifications, such as SUMOylation.

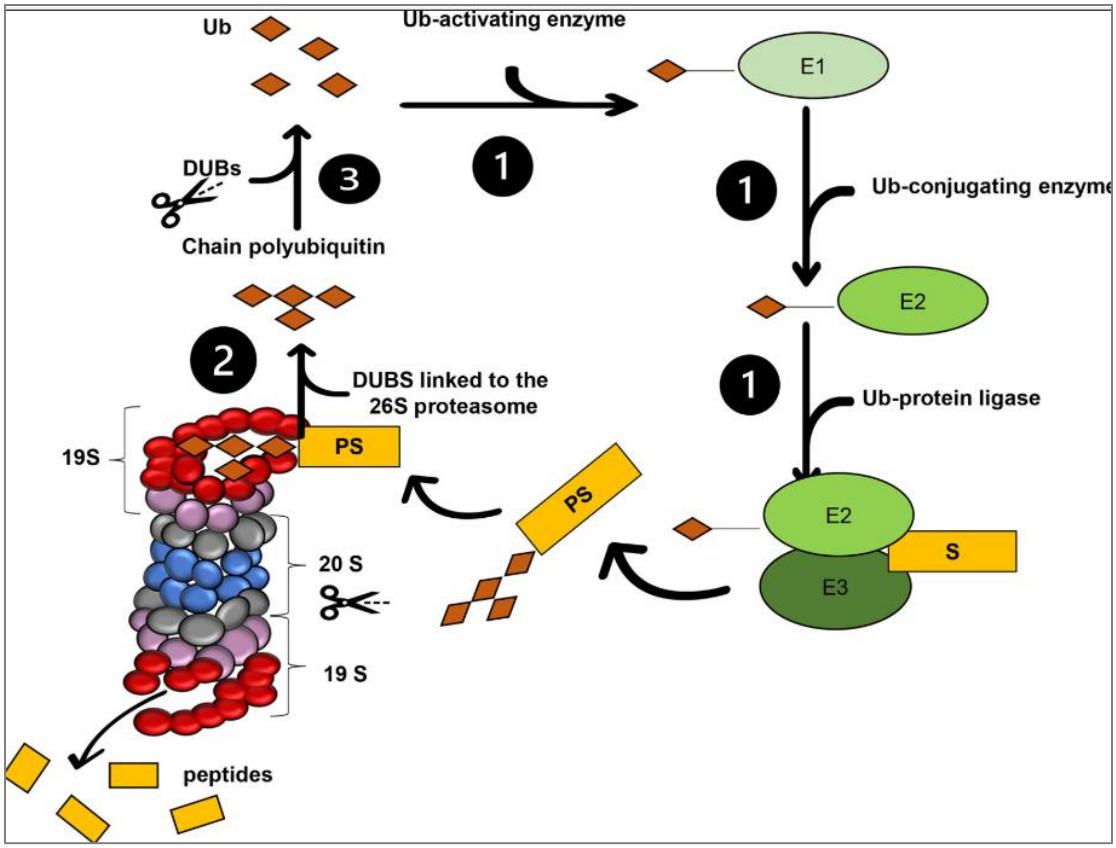


Figure 2.4: The Ubiquitine-Proteosome System. **(1)** E1 enzyme transfers ubiquitin to E2 enzyme, which, together with ubiquitin E3 ligase, transfers Ub to the target protein, forming the polyubiquitinated substrate(PS). **(2)** 19S Ub receptors recognize PS, deubiquitinating enzymes deubiquitinate PS and degrade it inside 20S **(3)** Polyubiquitin chain is cleaved by the deubiquinating enzymes (Patrocinio et al., 2022)

2.2 Ubiquitination and SUMOylation of p53

2.2.1 Ubiquitination of p53

p53 is tightly regulated by ubiquitination, which is mediated primarily through E3 ubiquitin ligases that regulate the cellular level of p53. The major E3 ligase that catalyzes ubiquitination of p53 is MDM2, which binds to the N-terminal transactivation domain of p53 and transfers ubiquitin, typically altering interactions to promote degradation by proteasomes or other cellular processes (Marine & Lozano, 2010). There is feedback in that p53 also stimulates its expression of MDM2. Other E3 ligases that mediate ubiquitination of p53 also exist including, PIRH2, COP1, and ARF-BP1 which may reside in separate, and are thought to be independent from

MDM2, polyubiquitination , and in some cases by targeting distinct lysine residues or by being activated by different types of stress signaling (Leng et al,. 2003; Dornan et al., 2004). Ubiquitination may involve mono- or polyubiquitin in the ubiquitination of proteins and the differences in chain type and linkage patterns can change downstream outcomes. The ubiquitination mechanisms may be affected by post-translational modifications on p53 itself or on E3 ligases in general, with effects on the efficiency and particularly the specificity as well as types of ubiquitination of p53 (Brooks & Gu, 2006).

2.2.1.1 Implications of ubiquitination to p53

Ubiquitination serves a large structural function in mediating the action of the p53. Ubiquitination can change the stability of the p53 protein, it's localization, and ultimately it's function. More generally, p53 ubiquitination targets specific lysines. Ubiquitin added at these sites induces a structural change in p53 that modifies its access to the proteasome and leads to its degradation. This is most commonly done through the MDM2. MDM2-mediated p53 ubiquitination at the CRD leads to failure of p53 DNA binding.

Structural alterations due to ubiquitination also impact p53's ability to associate with other proteins. Specifically, ubiquitinated p53 is much less likely to oligomerize into tetramers, which p53 has been suggested to be its active state. With the disruption of tetramerization, p53's ability to induce transcriptional control to initiate apoptosis, or halt progressing cells in the cell cycle from further proliferating is reduced. Liu et al.(2019) demonstrated that ubiquitination of the K386 site creates a loss of localization into the nucleus, effectively "sequestering" it in the cytoplasm preventing it from accessing DNA. This sequestration of p53 in the cytoplasm also diminishes its ability as a tumor suppressor. Furthermore, ubiquitination is a dynamic and reversible event, with the possibility of deubiquitinating enzymes (DUBs) completely removing ubiquitin from p53 restoring its functional stability. Zhang et al. (2020) are able to reveal that DUBs are important for the stabilization of p53 by reversing the ubiquitination. The dynamic regulation of ubiquitination and deubiquitination thereafter affords p53 the ability to respond to stress signals.

2.2.1.2 Regulation of Ubiquitination of p53

Ubiquitination of p53 is tightly regulated by MDM2 and specific deubiquitinases which can counteract MDM2 activity to keep protein levels in check. MDM2 mediates K48-linked polyubiquitination of p53 so it becomes degraded by the proteasome; by phosphorylating p53 at Ser15/20, DNA damage–responsive ATM/ATR kinases block MDM2 binding and thus, preventing ubiquitination (Maya et al., 2001). The ARF tumor suppressor can also sequester MDM2 in the nucleolus, and decrease p53 tagging (Kowalczyk et al., 2022). Under normal conditions, USP7 (Ubiquitin-Specific Peptidase 7) deubiquitinates MDM2 which indirectly continues to stabilize p53 by limiting its ubiquitination (Li et al., 2002). The type of ubiquitin linkage defines the fate of the protein: K48-linked chains are indicators for degradation, and K63-linked chains redirect p53 to non-degradative fates (Brooks & Gu, 2006). Ultimately the dynamic activity that safeguards p53- abundance allows p53 to be maintained in a poised state for rapid activation.

2.2.1.3 The MDM2 feedback loop

The MDM2 feedback loop represenst an essential brake-and-accelerator based system for p53. Under homeostatic, unstressed conditions, the low amounts of p53 present induce transcription of the MDM2 gene. Once MDM2 protein has built up sufficiently, it binds to p53, adds ubiquitin tags, and transports p53 to the proteasome for degradation. This creates a classic negative feedback loop where p53 induces its own inhibitor to avoid activation that would lead to unnecessary arrest, senescence or death of healthy cells (Yao et al., 2024).

Under conditions of genotoxic stresskinases, ATM and ATR recognize the stress and are able to phosphorylate specific serine residues on p53 and MDM2, resulting in a weakened interaction and preservation of p53 from degradation. Stabilized MDM2 is upregulated and accumulates in the nucleus leading to the transcription of genes responsible for halting the cell cycle (p21), repairing DNA, and initiating apoptosis. As stress is alleviated and damage is repaired, phosphatases reverse modifications to MDM2 and p53, MDM2 is able to reassociate with p53, and the feedback loop returns to original, low levels of p53.

Beyond the core circuit of regulation, other layers of regulatory processes can also provide more fine-tuning of the response. For instance, the ARF tumor suppressor can

sequester MDM2 to the nucleolus, further inhibiting MDM2's interaction and targeting of p53; MDMX (or MDM4) can heterodimerize with MDM2 that modulates its E3 ligase activity; and some members of the family of deubiquitinases can remove ubiquitin from p53 to help bias the balance towards stability and away from degradation. Gradually, these feedback loops and their interconnections enable the organism to regulate p53 throughout normal growth, yet be able to mount a strong defence when threatened with compromised genome integrity.

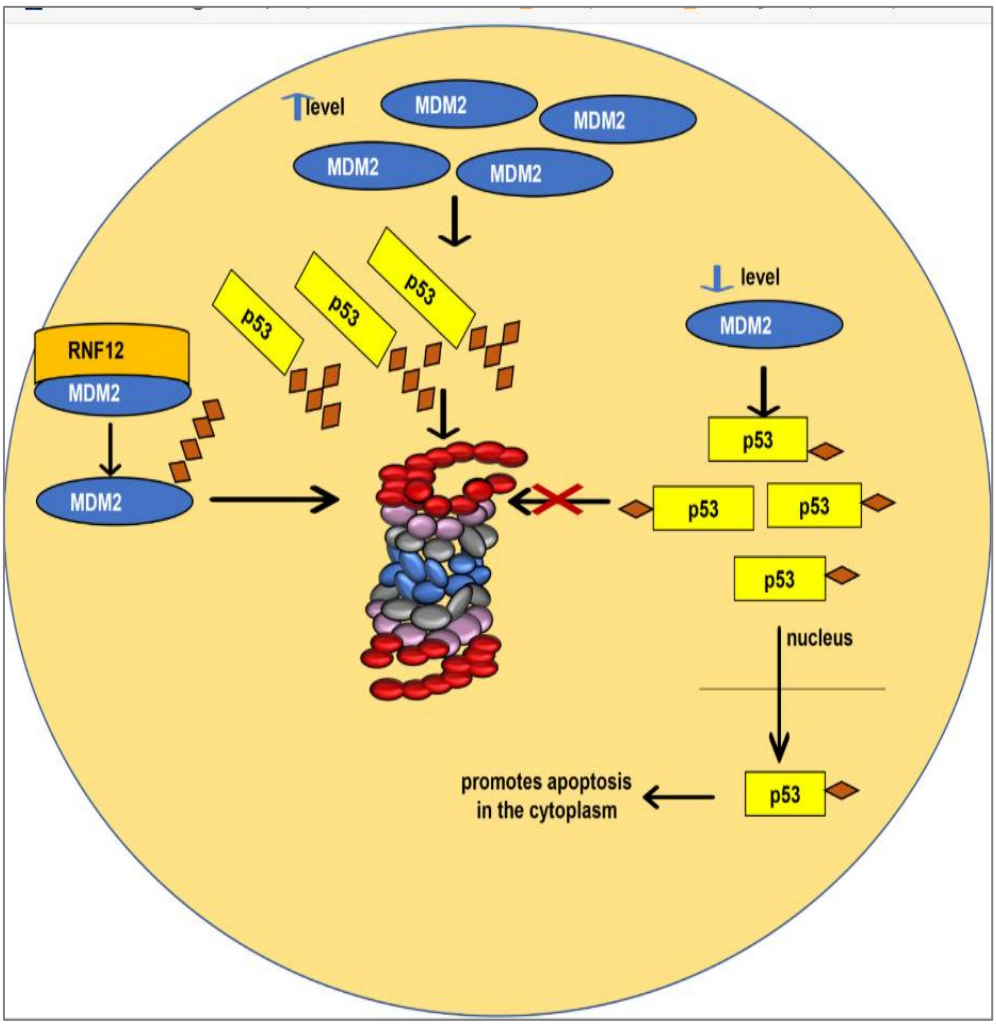


Figure 2.5: Illustration of the MDM2 feedback (adapted from Patrocinio et al., 2022)

2.2.2 SUMOylation of p53

p53 sumoylation results from the actions of the SUMO-activating enzyme (SAE1/SAE2), as well as the conjugating enzyme Ubc9, and E3 ligases primarily from the family of PIAS and RanBP2 have helped to direct SUMO-1 to a few of the

lysines on p53 (Gostissa et al., 1999; Tatham et al., 2001). SENP proteases, notably SENP1, SENP2 occupy the role of reversal, resulting in cleavage of SUMO from p53, changing the modifying state of p53 (Cheng et al., 2007). In this system the targets are comprised of multiple sites on p53 resulting in a more nuanced SUMO conjugation control at multiple sites without any preconceived destination for the one modification.

2.2.2.1 Implications of SUMOylation

SUMOylation alters the properties of the p53 in terms of the behavior of the cell while acting to protect the p53. One major mechanism of action through sumoylation is the stabilization of p53. Under normal conditions, p53 is highly regulated . Sumoylation serves to further inhibit degradation while also increasing the half-life of p53, helping to maintain activity when needed. Wang et al. (2021) show that sumoylation inhibits the interaction between p53 and MDM2, providing a possible mechanism by which p53 is stabilized during cellular stress.

The process of SUMOylation also plays a role in determining the cellular localization of p53. Following SUMOylation, p53 can accumulate in the nucleus and fully engage its DNA binding and gene regulatory functions necessary for cell cycle regulation and programmed cell death. Retention of p53 in the nucleus is crucial for a p53's role in maintaining genomic stability.

The structural changes that occur with sumoylation affect p53's interaction with many other regulatory proteins. SUMOylation can modulate p53's ability to bind to either co-repressors or co-activators depending on the context. Structural changes to p53 alter the ability of p53 to activate or repress target genes. During cellular stress responses, structural changes caused by sumoylation shift p53 activity to put p53 in a pro-apoptotic context that can eliminate damaged cells.

2.2.2.2 Regulation of SUMOylation

DeSUMOylating enzymes such as SENP1 and SENP2 can remove SUMO moieties from p53 in ways dynamic with cellular stress (Flotho, A. & Melchior, F., 2013). The coordination of SUMOylation with other PTMs, notably phosphorylation and ubiquitination, adds an additional layer of complexity in p53 regulation. Forinstance, via K386, SUMOylation stabilizes p53 by interfering with the ubiquitination mediated

by MDM2, and promotes its pro-apoptotic functions in the context of DNA damage (Jansson et al., 2020). SUMOylation could also biochemically repress p53 activity in specific contexts. In the absence of stress, p53 SUMOylation via PIAS1 hold p53 in nuclear bodies out of reach of target promoters (Bohren et al., 2020). This duality highlights SUMOylation's context dependent regulatory role, dependent on cell type determination, stress signals, and interacting partners.

Recent research has shown that oxidative stress plays a role in shaping p53 SUMOylation. The SUMOylation of p53 in response to ROS leads to the activation of its interaction with pro-survival genes, including SESN2 (sestrin 2) to stimulate antioxidant defenses (Enserink et al., 2018). On the other hand, excess SUMOylation associated with chronic stress leads to a correlation between p53 aggregation and loss of function, going back to its role in neurodegenerative models (Wang et al., 2022).

2.2.3 The Crosstalk Between Ubiquitination and SUMOylation of p53

Ubiquitination and SUMOylation act as opposing PTMs that dynamically regulate the stability, localization, and activity of p53. Ubiquitination and sumoylation often target the same lysine residues on p53, creating a feedback mechanism that consolidates the balance between p53 degradation or activation (Jansson et al., 2021; Wang & Chen, 2023) . Ubiquitination is important in maintaining low levels of p53 under basal states. In contrast, SUMOylation leads to stabilization by competing for the same lysine residues, inhibiting ubiquitination and thereby promoting the nuclear retention of p53 to enhance transcriptional activity. SUMOylation removes the ability for ubiquitination to occur, which functionally results in p53 becoming trapped in the nucleus to activate the expression of genes that mediate apoptosis or cell cycle arrest (Zhang et al., 2020).

Ubiquitination promotes the translocation of p53 to the cytoplasm for degradation whereas SUMOylation of p53 promotes mitochondrial spatial localization of p53, resulting in direct upregulation of apoptotic signaling. The action and relationship of these two PTMs with one another are dependent on the context; upon stress (i.e., DNA damage) the predominant modification is SUMOylation leading to stabilization of nuclear p53 to allow it to mediate cellular response to DNA damage. Wang et al.,

(2021) showed that SUMOylation prevents the interaction between p53 and MDM2, thereby protecting p53 from degradation.

We have now discussed how ubiquitin, SUMO, and other modifiers target specific lysines on p53 and affect its function in normal physiological context. In Chapter 3, we will discuss how dysregulation of ubiquitination/SUMOylation can drive p53 dysfunction in a variety of neurodegenerative disease.

CHAPTER THREE

DYSREGULATION OF UBIQUITINATION AND SUMOYLATION IN P53 AND ROLE IN NEURONAL PATHOLOGY

3.1 Background.

Dysregulation, the pathological disassembly of balanced PTM networks, arises when ubiquitination-SUMOylation crosstalk becomes uncoupled in p53 under specific stressors, or when these individual PTMs get impaired or disturbed which ends up causing a disruption in normal cellular processes and thus leading to diseases. Stressers like chronic oxidative stress that inhibits E3 ligase (Rufini et al., 2011); proteotoxic aggregates sequestering deubiquitinases (Schmidt et al., 2013); or mutations in ubiquitin-proteasome system (UPS)/SUMO machinery dysfunction (Zhou et al., 2021) may bring about this dysregulation. This perturbed state creates greater stabilization of pro-apoptotic states of p53, which drives neurodegeneration; the irreversible and progressive loss of neuronal structure and subsequently function, this follows our review in chapter 1 that discusses the role of p53 in neuronal homeostasis. This chapter seeks to extend the foundational PTM biology highlighted in Chapter 2 to the pathology of disease particularly neuronal pathology, by illustrating, for example, that dysregulated ubiquitination of p53 in Parkinson's and Friedreich's ataxia, and hyper-SUMOylation in Huntington's and spinocerebellar ataxia type 3, occur to underpin neuronal death. Identifying these mechanisms will be important to establish p53 PTM hotspots as therapeutic targets.

3.2 p53 ubiquitination and SUMOylation in Friedreich's Ataxia

Friedreich's Ataxia (FRDA), a disease caused by the absence of frataxin, shows experimentally verified disruption of p53 post-translational modification (PTM)-regulation of dynamics . Loss of frataxin leads to mitochondrial oxidative stress that, in turn, promotes UBC9-mediated p53 SUMOylation, especially on C-terminal lysines, while inhibiting MDM2-mediated p53 ubiquitination (Rufini et al., 2011 Hum. Mol. Genet.). The result of this PTM imbalance is stabilized nuclear p53 that regulates the transcriptional activation of pro-apoptotic targets like PUMA in neurons of the dorsal root ganglia (Shan et al., 2013 Cell Metab.). Most importantly, studies in vivo demonstrate that inhibition of p53 SUMOylation (i.e., with dominant-negative UBC9) restores mitochondrial function and neuron health in FRDA models (Puca et al., 2020). Thus, FRDA exemplifies that oxidative stress mediates PTM crosstalk-where ubiquitination outcompetes SUMOylation with pathological consequences

3.3 Ubiquitination of p53 in relation to Parkinson Disease

The dysregulation of p53 ubiquitination is an important pathogenic mechanism in Parkinson's Disease (PD). In normal conditions, p53 turnover is tightly regulated via ubiquitination by MDM2. In PD, this homeostasis is disrupted by two mechanisms. First, mutations found in PARKIN (a major E3 ubiquitin ligase) prevent ubiquitination of p53 and other substrates, leading to inappropriate p53 levels (Jiang et al., 2006). Second, chronic oxidative stress is a central feature of PD that maximizes p53 stability because it leads to post translational modification and inhibition of MDM2 activity (Kruse & Gu, 2009). The excess levels of p53 lead to degeneration of dopaminergic neurons through activation of pro-apoptotic genes (BAX, PUMA) that worsen mitochondrial dysfunction (Silva et al., 2005). Also, excess p53 interacts with α-synuclein in vulnerable neurons to promote the formation of toxic complexes that foster aggregate pathology and neuronal death (Alves da Costa et al., 2002). There is a dual role for p53, promoting both an apoptotic signalling pathway and the aggregation of proteins. Dysregulation of p53 ubiquitination can worsen PD progression.

3.4 Ubiquitination of p53 and Huntingtons' disease

In Huntington's disease (HD), mutant huntingtin (mHTT) profoundly alters the ubiquitin-mediated regulation of p53 which we have already discussed in chapter 2. However, mHTT aggregates inhibit nucleocytoplasmic transport by disrupting the nuclear pore complex (Grima et al., 2017), which prevents nuclear export of p53 to the cytoplasm, where ubiquitination by MDM2 and subsequent degradation occurs. As a result, p53 accumulates inappropriately in the nucleus.

The inhibited ubiquitination and degradation of p53 has dire consequences. When stable p53 accumulates in the nucleus, it will be activated so that it can act as a transcription factor. It hyperactivates pro-apoptotic targets genes such as PUMA and BAX (Bae et al., 2005). Not only does mHTT inhibit nucleocytoplasmic transport, but mHTT has also been shown to inhibit overall function of the proteasome thus lowering the capacity for degradation of ubiquitin tagged proteins like p53 (Jana et al., 2016). The rate of persistent p53 activity promotes transcriptional programs that are used to promote mitochondrial dysfunction and oxidative stress, which then

contributes to the selective neurodegeneration of striatal neurons, a well-characterized feature of the pathology of HD.

Table 3.1: Summary of some neurodegenerative disorders

Disease	**PTM Dysregulation**	**Affected PTM**	**Key Study**
Friedreich's Ataxia	Frataxin deficiency → ROS accumulation → UBC9-mediated hyper-SUMOylation of p53	SUMOylation	Rufini et al. (2011): *Puca et al. (2020)*
Parkinson's	FBXO7 mutation → impaired E3 ligase activity → defective p53 ubiquitination	Ubiquitination	Zhou et al. (2021);
Huntington's	mHTT aggregates → proteasome dysfunction → p53 accumulation + stress-induced SUMOylation	Ubiquitination	Bae et al. (2005)

3.5 The Interplay Between Ubiquitination and Sumoylation of p53 in Neurodegeneration

The functional duality of p53 is critically regulated by antagonistic post-translational modifications: ubiquitination and SUMOylation which have been broadly discussed in chapter 2. In neurodegenerative pathologies, this balance is catastrophically disrupted. Impairment of ubiquitin-mediated degradation, driven by disease-specific mechanisms such as proteasomal inhibition by amyloid-β in Alzheimer's disease (Myeku et al., 2016), PARKIN dysfunction in Parkinson's disease (Jiang et al., 2006), or mutant huntingtin-induced nucleocytoplasmic transport defects in Huntington's disease (Grima et al., 2017), leads to pathological p53 accumulation. Concurrently, chronic oxidative stress, a universal feature of neurodegeneration promotes p53 sumoylation through inhibition of SENP de-sumoylases (Yamaguchi et al., 2017).

Sumoylated p53 is translocated to the mitochondria and directly engages BAX/BAK dependent apoptosis independent of transcription (Zhang et al., 2021). These changes are critical because they create pathogenic synergy; for example, in Huntington disease, mutant huntington not only sequesters the SUMOylation components (Steffan et al., 2004), it also inhibits degradation via ubiquitination. The loss of degradation can create a circular reinforcing stabilization loop of p53 on top of other continuing

angles of stress causing pro-apoptotic activation. The disturbance of regulatory crosstalk results in a chronic signal of pro-apoptotic activity which results in synaptic breakdown and also leads to irreversible and unwanted neuronal death. This is a significant loss because post-mitotic neurons have no secondary opportunity for regeneration. Restoration of PTM balance may lead to a very effective neuroprotective intervention.

Based upon what we have seen regarding the structure and function of p53 in chapter 1, the regulation of p53 by ubiquitination and SUMOylation in chapter 2 and aberrant ubiquitination and SUMOylation of p53 in chapter 3 causing neuronal injury across numerous neurodegenerative diseases, we have our main question: what specific lysines on human p53 are the most likely to be modified? Which will be anwsered in the next chapters. Chapter 4 describes the computational tools and databases we utilized to perform the in silico prediction of both ubiquitination and SUMOylation sites, while also establishing the background for the results below.

PART 2

METHODLOGY, RESULTS AND DISCUSSION

CHAPTER FOUR

MATERIALS AND METHODS

4.1 Introduction to the chapter

4.1.1 Data collection

Using an in silico approach, we mapped post-translational modification sites on p53. Ubiquitination and SUMOylation sites were identified using GPS-Ub and GPS-SUMO, which provide high-sensitivity motif identification, in addition to SUMOplot for auxiliary SUMO consensus analysis. For assessing lysine acetylation, we used ASEB, which uses sequence conservation and residue physicochemical properties to assess predicted acetylation 'hotspots.' Predicted sites from each application were filtered according to the determined threshold for each tool to ensure a level of confidence. We then combined all of the high-confidence PTM sites into comprehensive tables, which allowed for cross-comparison of the ubiquitination, sumoylation, and acetylation layers. This resulting dataset provided the foundation for exploring PTM crosstalk and prioritizing targets for downstream experimental validation**.**

4.1.2 Why this approach?

Computational PTM screens are a relatively inexpensive way to screen modification sites before engaging in expensive wet-lab experiments.

Computational approaches offer a fast, high-throughput means to identify potential SUMOylation and ubiquitination sites across entire proteomes, their scalabilty and predictive accuracy help narrow experimental candidates saving time and resources . These approaches are very vital in biological research as they compliment experimental methods by guiding focused investigations into key PTM, as highlighted by He et al., (2021).

For customizability, all the tools we chose for our computational predictions allowed us to input species-specific sequence, and user defined parameters improving relevance to our research. More so, the computational predictions didnot need ethical regulations and restrictions, logistical complications or manual errors that would come as huge limitations to our study. The cross-validation across our use of all the tools indicates both reproducibility and a reasonable alignment with the referenced literature. By only keeping high-confidence sites we are limiting our workflow to true PTM crosstalk events and our downstream validation should be more targeted.

4.2 Application of Uniprot (Universal Protein Resource)

Protein sequences and functional annotations were obtained from Uniprot (Uniprot Consortium, 2023), accessed on 4th March, 2025 through this link: https://www.uniprot.org/blast. Uniprot is a durably curated protein database that facilitated the ability to analyze the same sequences in each of the subsequent analyses that were validated by experiment. Uniprot annotated information, including reported post-translational modifications and domain architectures, was reviewed with respect to predicted ubiquitination and sumoylation sites to help with biological relevance and experimentation prioritization.

Workflow: the protein of interest, p53, was searched for in Uniprot either with accession number, or gene name. Fasta format sequences of p53 were downloaded.

With the Uniprot ID of our protein being P04637, we obtained the FASTA sequence of p53 as show:

>sp|P04637|P53_HUMAN Cellular tumor antigen p53 OS=Homo sapiens OX=9606 GN=TP53 PE=1 SV=4

MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM DDLMLSPDDI EQWFTEDPGP

DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ KTYQGSYGFR LGFLHSGTAK

SVTCTYSPAL NKMFCQLAKT CPVQLWVDST PPPGTRVRAM AIYKQSQHMT EVVRRCPHHE

RCSDSDGLAP PQHLIRVEGN LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS

SCMGGMNRRP ILTIITLEDS SGNLLGRNSF EVRVCACPGR DRRTEEENLR KKGEPHHELP

PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FRELNEALEL KDAQAGKEPG

GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD

These fasta sequences data were then submitted as input for the computational tools that we used for lysin residue mapping; additionally, uniprot's annotated information helped identify biologically relevant sites to focus on in the validation work. Finally, Uniprot provided actively curated and non-redundant sequence information and annotation, and this helped provide a "gold-standard" to decrease errors that might

exist through fragment existences in analysis coming from the sequences publically available in other databases.

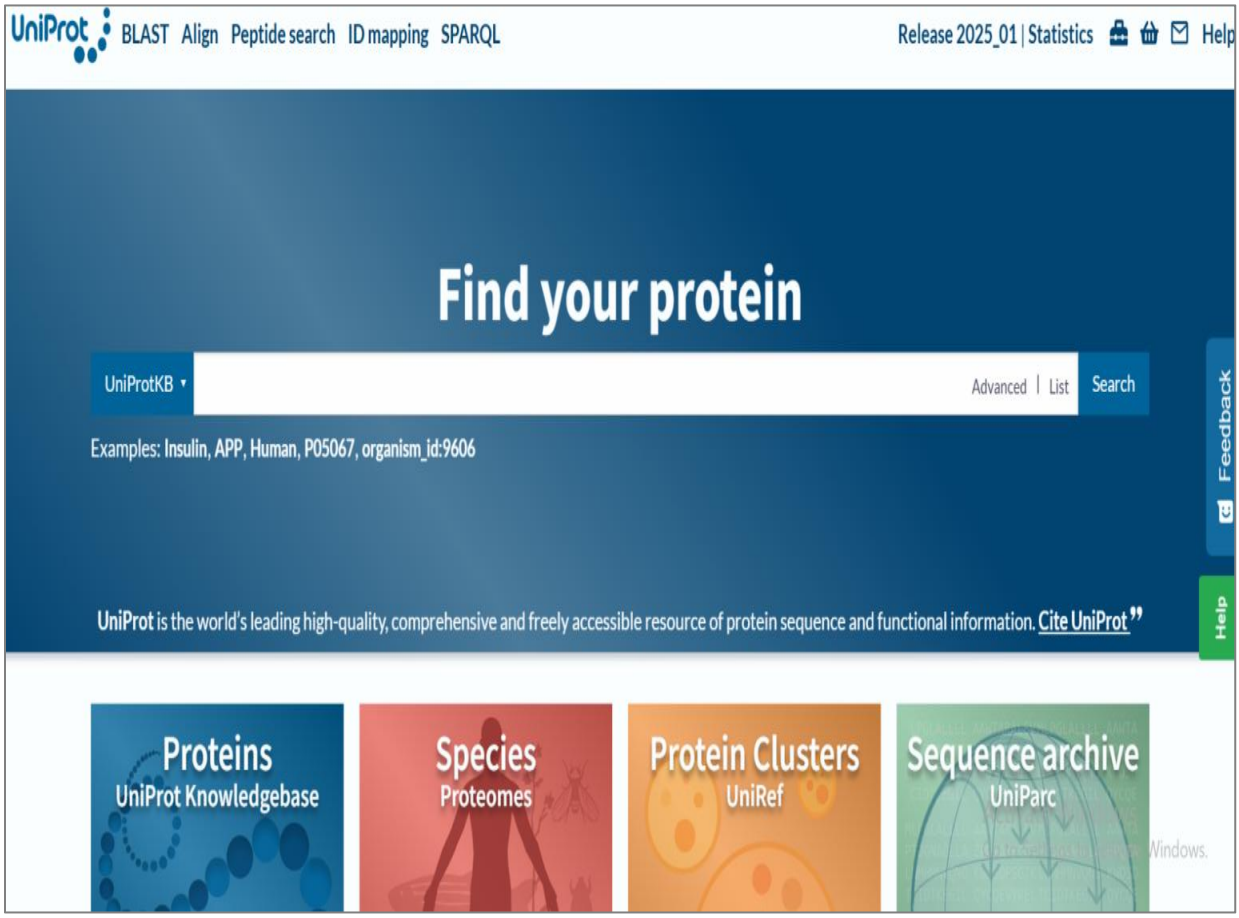


Figure 4.1: Uniprot webserver

4.3 Computational prediction tools

4.3.1 Ubiquitination Predictions using GPS-Uber

The use of GPS-Uber (Group-based Prediction System for Ubiquitination)(Wang et al., 2022) in our study created an important computational structure for identifying possible ubiquitination sites within the p53 protein. GPS-Uber was accessed through the link:GPS-Uber - Ubiquitin-protein ligase enzymes-substrate relationship prediction This tool used a group-based scoring algorithm trained on experimentally validated ubiquitination data to predict lysine residues to be ubiquitinated with high confidence. GPS-Uber located lysine residues in the p53 sequence that predispose to ubiquitination thus, providing finer-grained potential modification sites for ubiquitination and PTM hotspots. . We were able to identify lysine residues through GPS-Uber's peptide based outputs that are within important functional domains of p53.

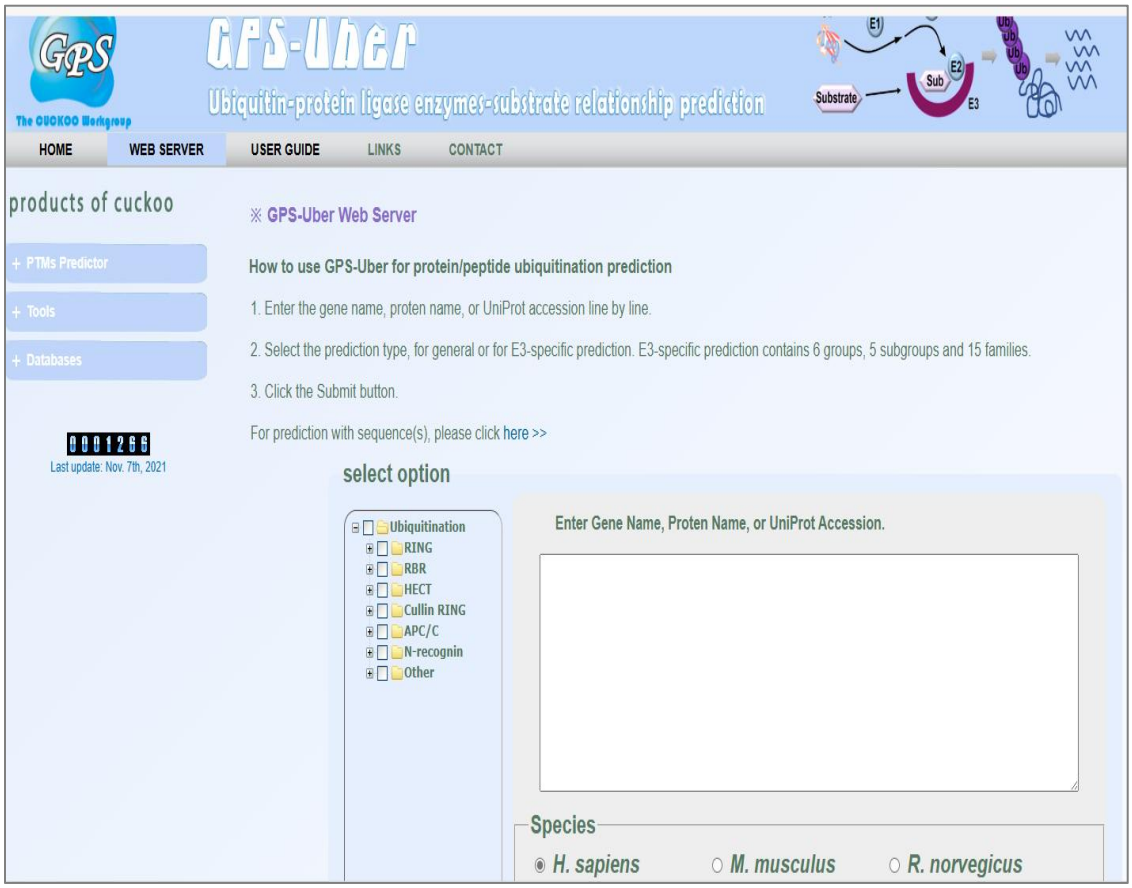


Figure 4.3: GPS-Uber webserver

4.3.2 Prediction of SUMOylation sites using GPS-SUMO

Accurate predictions of SUMOylation sites on p53, we utilized GPS-SUMO (group-based prediction system for sumoylation) (Gou et al., 2024) that we accessed through https://sumo.biocuckoo.cn/ It provided the best level of accuracy in comparison to other available tools such as SUMOsp, JAASSA, MusiteDeep, and ResSUMO. GPS-SUMO was judged to have better sensitivity and specificity overall. The GPS-SUMO tool functioned under a machine-learning system that included a position-specific Scoring Matrix (PSSM) score using a group-based prediction system. This combination has been evaluated based on large-scale datasets, including mass spectrometry output, and has performed reliably, especially for proteins that have been implicated in neurodegenerative disease states. Another benefit of using GPS-SUMO was the straightforward user interface and the ability to enter protein sequences quickly and generate results.

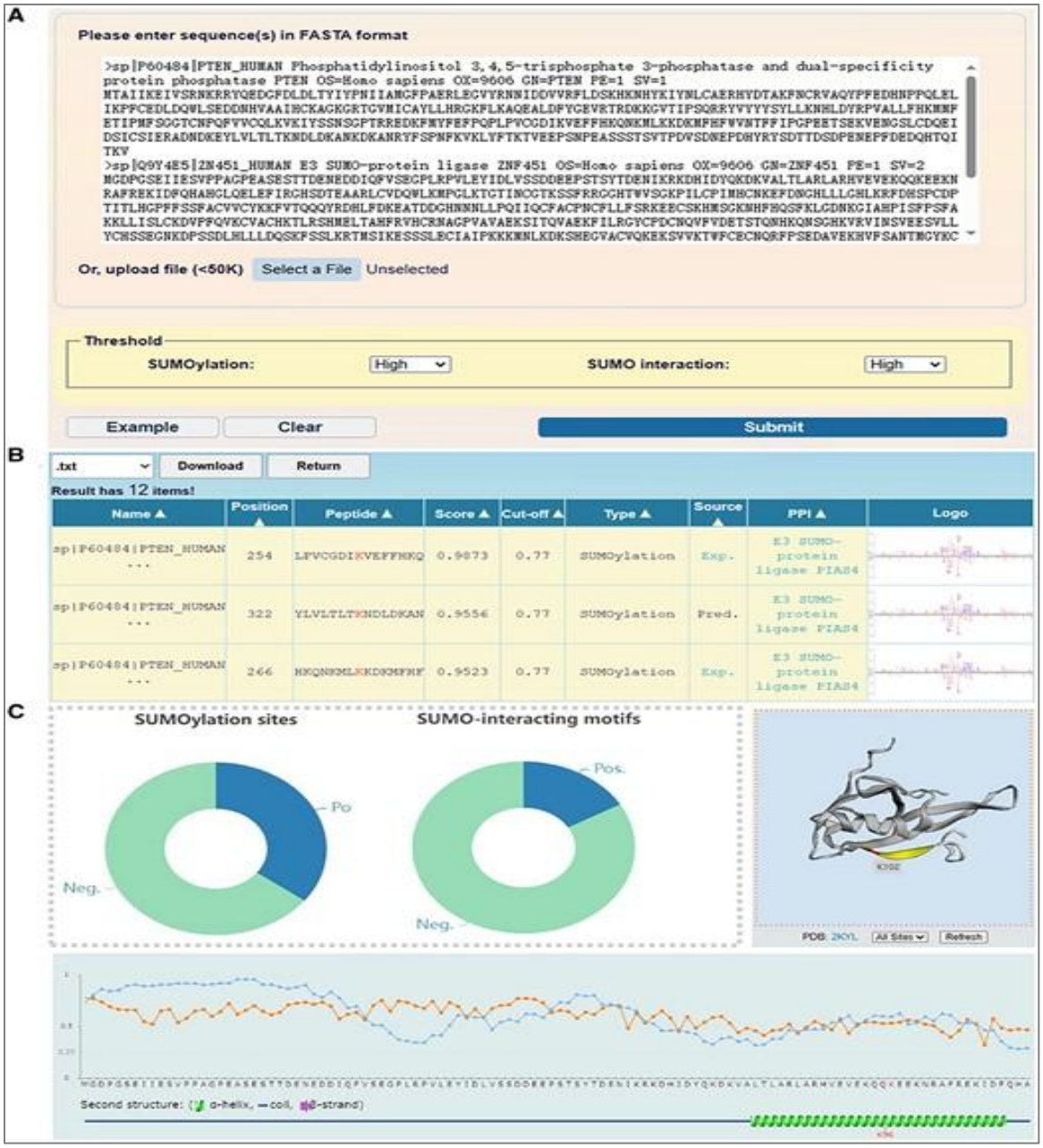


Figure 4.4: GPS-SUMO 2.0 webserve. **A**)The interface of sequence submission. The users can input the protein sequence in fasta format or enter uniprot accession number, and select 3 different thresholds for the prediction of modification sites. (**b**) the presentation of prediction results of the example. In tabular list, the predictive results contain the position of sumoylation site, the prediction score, cut-off value, identification by experimental or computational method, and ppi information. (**c**) the comprehensive annotations of the prediction results. The number of sumo modification sites predicted by GPS-SUMO 2.0 were presented, the location of sumoylation sites were illustrated in 3d structure derived from PDB database, the asa score and disorder score of sumoylation sites were calculated (Gou et al., 2024)

4.3.3 Prediction of SUMOylation using SUMOplot

In order to ensure solid and reliable predictions of the sumoylation sites, we supplemented the use of GPS-SUMO with the SUMOplot tool (Abgent. 2004), which we accessed via the following link: https://www.abcepta.com/sumoplot SUMOplot was chosen for its ability (1) to visualize prediction sites within protein sequences, so that we co-uld interpret positional context and how confident the scores we saw were, followed by (2) some experimentally validated SUMOylation data, obtained from a curated database to improve specificity of predicted sites.(Abgent. 2004). The tool analyses the lysine (K) residues in the input protein sequence or protein Uniprot ID by scanning for consensus motifs matching and substtitution of the consus acid residues that have similar hydrophobicity. For example, the canonical Ψ-K-x-E/D motif as describe by the tool webserver page, with ψ representing a hydrophobic residue and x representing any amino acid. (Yavuz & Sezerman, 2014; Zhou et al.,2005)

By cross-validating the results between GPS-SUMO and SUMOplot, we aimed to reduce the number of false positive predictions and thus bolster confidence in the identified sites. The algorithm of sumoplot integrated physicochemical properties and sequence context to predict SUMOylation sites based on SUMOylation predictions displayed in the original methodology.

Figure 4.5: SUMOplot webserver

4.3.4 Prediction of acetylation using ASEB

We incooperated the prediction of lysin acetylation sites into our work since this PTM occurs at specific lysin residues just like ubiquitination and SUMOylation, using this we would analyze if there is correlation between acetylation , Ubiquitination and SUMOylation.

We used the Acetylation Set Enrichment Based (ASEB)(Wang et al.,2012) http://bioinfo.ncu.edu.cn/ASEB/. which predicted Lysine Acetyl Transferases (KAT)-family-specific acetylation sites using a hierarchical GPS algorithm. First, potential acetylation sites were identified from our protein sequence that we had obtained from uniprot; second, cognate KAT families (e.g., p300, PCAF, MYST) were inferred via position-specific probability models trained on experimentally verified sites.

ASEB combined sequence-based features with an enrichment framework to score lysine sites for CBP/p300–mediated acetylation which is the only KAT we used in our prediction.

ASEB phases the query peptides against a curated acetylome then examine motif enrichment, yielding p-values that give site specific confidence .

Unlike solely machine-learning based predictors, ASEB's statistically based approach uses the assumption of the experimentally validated correlations to extract categories and reduce false positives. Its web interface was also easy to use and had rapid batch submissions without the need to adjust complex modes. The adjusted lens of accuracy and usability provided in ASEB makes it a strong candidate for our stud. (Wang et al., 2012).

Table 4.2: Summary of Computational Tools used for data collection

Tool	Acees link	Purpose	Citation
UniProt (Universal Protein Resource)	BLAST | UniProt	Retrieve human p53 sequence (FASTA, functional annotations)	UniProt Consortium
GPS-Uber (Group-based Prediction System for Ubiquitination)	GPS-Uber - Ubiquitin-protein ligase enzymes-substrate relationship prediction	Predict ubiquitination sites and highlight high-information lysine residues within functional domains	Wang et al. (2022)
GPS-SUMO (GPS-SUMO 2.0)	GPS-SUMO: Prediction of SUMOylation Sites & SUMO-interacting Motifs	Predict SUMOylation sites on p53	Gou et al.(2024)
SUMOplot	https://www.abcepta.com/sumoplot	Scan for consensus SUMOylation motifs and predict SUMOylation sites (sequence-based)	Yavuz and Sezerman.(2014)
ASEB (Acetylation Set Enrichment Based)	http://bioinfo.ncu.edu.cn/ASEB/	Predict lysine acetylation sites on p53 (to assess cross-talk with ubiquitination/SUMOylation)	Wang et al.,(2012)

With our in silico pipeline established (sequence retrieval, tool versions, criteria, access links and tool choice justification), we are prepared to see what actual predictions reveal. Chapter 5 will present the raw outputs of GPS-Uber, GPS-SUMO, SUMOplot, and ASEB, compare their overlaps, provide an interpretation of which lysine residues emerge as high confidence PTM candidates and then a discussion of results from each tool in relation to our research questions and objectives.

CHAPTER FIVE

RESULTS, INTERPRETATION AND DISCUSSION

5.1 Results from GPS-Uber

GPS-Uber (Wang et al., 2022) showed only predicted ubiquitination sites of the p53 protein and displayed the results showing the position for the lysine residues and a sequence of 21 amino acids in which this predicted lysine was centered . However for the purpose of our study, we used a strict 0.5 cut-off for our analysis of results which we have displayed in **table 5.1** below.

Ultimately, this tool provided an efficient means of identifying target lysines for our study.

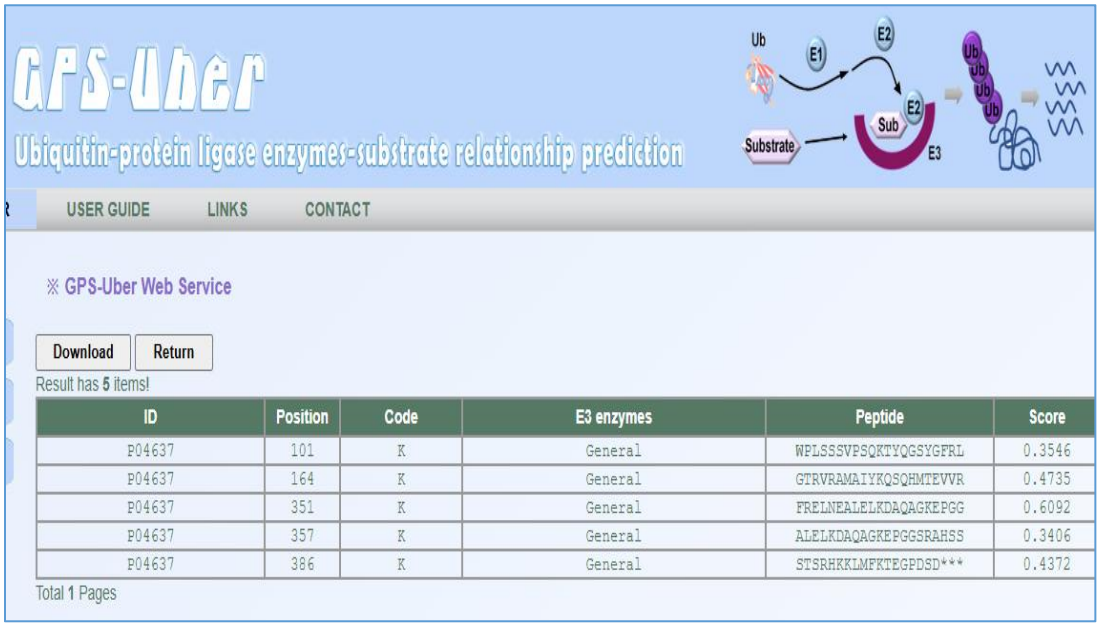


Figure 5.1: GPS-uber results

Table 5.1: Results from GPS-Uber

Position	Domain	Peptide	Score	Cutoff
351	TD	FRELNEALEL**K**DAQAGKEPGG	0.6092	0.5

The GPS-Uber analysis pinpointed 5 lysine residues on p53; K101 , K164, K351, K357, and K386 as displayed in the webserver results page in **figure 5.1.** Only one lysine (K351) had a score above our cutoff. K164 and K386 had scores that we would argue that have a biological relevance but their scores (0.4735, 0.4372 respectively) were slightly below our cutoff of 0.5 and thus we couldn't display them in our table of results.

5.2 Results from GPS-SUMO

The analysis identified 16 modification sites on the p53 protein as displayed by the results screenshot from the predictions tool below. This included 15 SUMOylation sites and one SUMO interaction motif. The SUMOylation sites had high confidence scores above the cut-off employed by the tool, all exceeded the 0.5 cut-off that we maintained for our analysis. The webserver as shown by the screnshot of the webserver in figure ,displayed results showing position of the lysine residues undergoing SUMOylation, a peptide section of about 15 aminoacids with the central one being the lysine residue that might undrgo SUMOylation and a colum of the source (predicted or experimentally validated)

.xls Download Return

Result has 19 items!

Name	Position	Peptide	Score	Cut-off	Type	Source	PPI	Logo
sp|P04637|P53_HUMAN C...	386	RHKKLMFKTEGPDSD	0.9253	0.67	SUMOylation	Exp.	Protein PML	
sp|P04637|P53_HUMAN C...	351	LNEALELKDAQAGKE	0.9113	0.67	SUMOylation	Pred.	Protein PML	
sp|P04637|P53_HUMAN C...	292	EEENLRKKGEPHHEL	0.8579	0.67	SUMOylation	Exp.	Protein PML	
sp|P04637|P53_HUMAN C...	251-255	GGMNRRPILTIITLEDSSG	0.8502	0.85	SUMO interaction	Pred.	SUMO1	
sp|P04637|P53_HUMAN C...	305	ELPPGSTKRALPNNT	0.8277	0.67	SUMOylation	Exp.	Protein PML	
sp|P04637|P53_HUMAN C...	382	QSTSRHKKLMFKTEG	0.8133	0.67	SUMOylation	Exp.	Protein PML	
sp|P04637|P53_HUMAN C...	370	RAHSSHLKSKKGQST	0.8126	0.67	SUMOylation	Exp.	Protein PML	

Figure 5.2: GPS-SUMO results

Table 5.2: Results from GPS-SUMO

Position	Domain	Peptide	Score	Source
K101	DBD	SSSVPSQ**K**TYQGSYG	0.7915	Pred.
K120	DBD	FLHSGTA**K**SVTCTYS	0.7245	Exp.
K139	DBD	KMFCQLA**K**TCPVQLW	0.7196	Exp.
K164	DBD	VRAMAIY**K**QSQHMTE	0.7294	Pred.
251-255	DBD	GGMNRR**PILTII**TLEDSSG	0.8502	Pred.
K291	DBD	TEEENLR**K**KGEPHHE	0.7773	Pred.
K292	DBD	EEENLRK**K**GEPHHEL	0.8579	Exp.
K305	-	ELPPGST**K**RALPNNT	0.8277	Exp.
K319	-	TSSSPQP**K**KKPLDGE	0.7719	Exp.
K321	-	SSPQPKK**K**PLDGEYF	0.7717	Exp.
K351	TD	LNEALEL**K**DAQAGKE	0.9113	Pred.
K357		LKDAQAG**K**EPGGSRA	0.7325	Exp.
K370	CRD	RAHSSHL**K**SKKGQST	0.8126	Exp.
K372	CRD	HSSHLKS**K**KGQSTSR	0.789	Pred.
K382	CRD	QSTSRHK**K**LMFKTEG	0.8133	Exp.
K386	CRD	RHKKLMF**K**TEGPDSD	0.9253	Exp.

In terms of retaining verified sites we only kept sites that had a confidence score greater than 0.5, but verification is only as reliable as the prediction we made. We also left out motifs that interacted with SUMO, only because we were not interested in any modifications beyond sumoylation.

Computational analysis resulted in the identification of ten sites were experimentally verified, while five were predicted. The strongest site was lysine 386 (K386) with a score of 0.9253. of five predicted SUMOylation sites on p53; K101 , K164, K291, K351, K372, and a motif at residues that passed a confidence score threshold of >0.50 thus identifying them as regulatory hotspots of p53. The highest confidence site, K351 located in the tetramization domain of the p53, lower confidence sites, such as K101 are also positioned in functional domain.

5.3 Results from SUMOplot

The program assigned a confidence score in the range of 0-1 to each lysine residue depending on similarity to lysine residues in previously published sequence motifs that support SUMOylation, as well as considering the physics/circuits of neighboring residues or the conservation/sequence of the motif itself. SUMOplot displayed the p53 sequence with motifs where SUMOylation could occur, marked according to likelihood of occurrance, those in red shed were flagged as motifs with high probability of occurance, those in blue with low probability and those in green were overlpping motifs, the screenshot from the webserver; figure is inserted below .

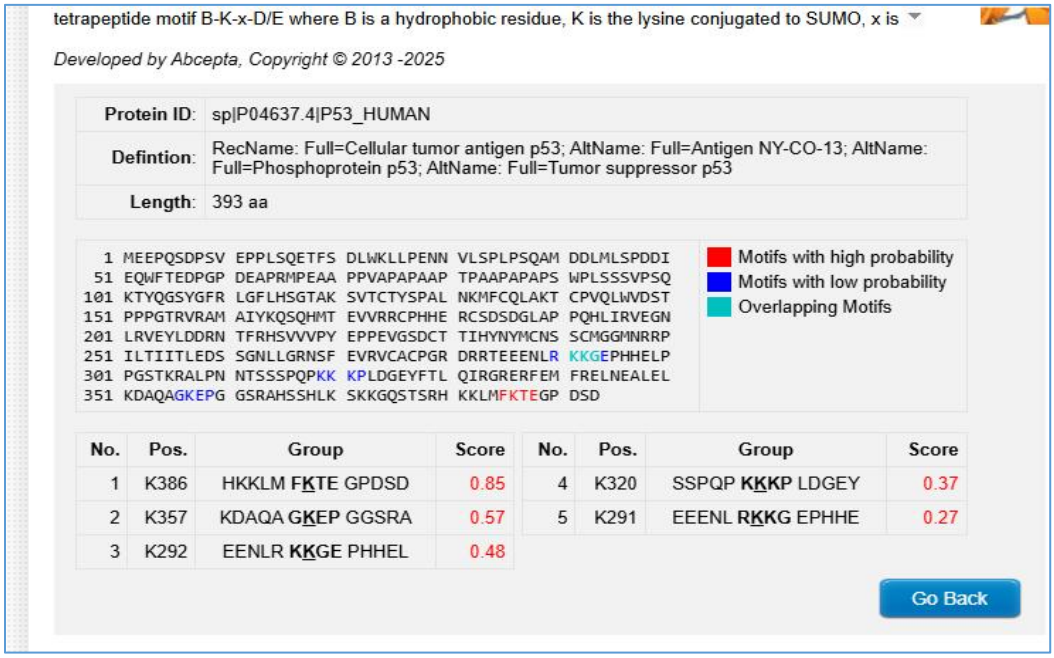


Figure 5.3: SUMOplot results

Table 5.3: Results from SUMOplot

Position	Domain	Group	Score
K386	CRD	HKKLM **FKTE** GPDSD	0.85
K357	TD	KDAQA **GKEP** GGSRA	0.57

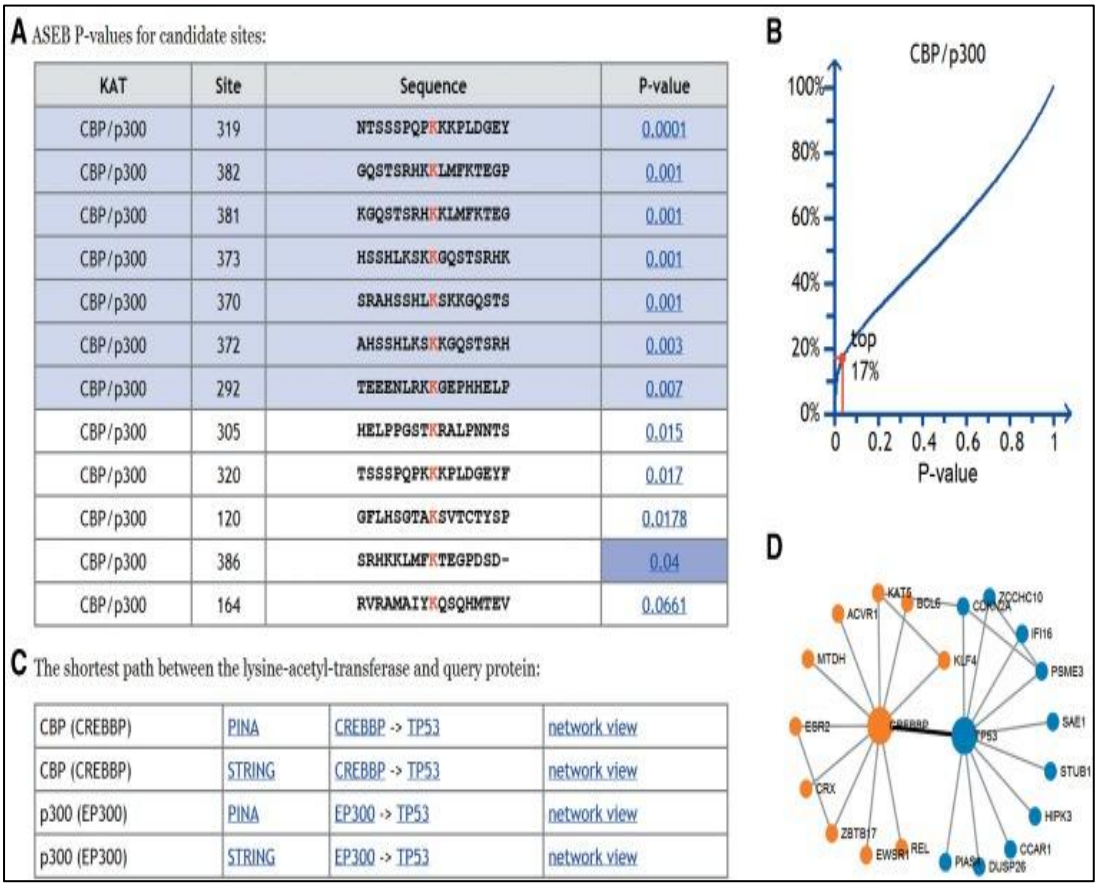
We maintained a cuttoff score of 0.5 for our 5 lysin resiues that were predicted as SUMOylation sites K386, K357, K292, K320, K291 as shown in figure 5.3 below. only those with scores above cutoff ; K386, K357 were displayed in the table.

The SUMOplot analysis identified two sites on p53 with high confidence as SUMOylation sites; K386 & K357 exceeded our 0.5 cutoff and deserved attention.

5.4 Results from ASEB

ASEB predicted lysine acetylation sites which it does by performing enrichment analysis against known KAT-specific substrate sets. It takes into account sequence motifs, evolutionary conservation, and protein–protein interaction information to produce enrichment scores and P-values. The tool gave us results specific to CBP/p300 KAT family, a speific site , a peptide segment in which this residue is located and respective p-values that we used to analyse the results.

For our data analysis, we formulated a table in which we displayed these results into 4 colums showing the position, the p53 domain in which this specific residue is located, the peptide segment in which the residue is located and the p-values. We organized our results using an ascending order of the positions of the lysine residues predicted, **Figure 5.4** shows the webserver display of the results from ASEB and then **table 5.4** displays our results that we used to analyze the ASEB tool results.



A ASEB P-values for candidate sites:

KAT	Site	Sequence	P-value
CBP/p300	319	NTSSSPQPKKKPLDGEY	0.0001
CBP/p300	382	GQSTSRHKKLMFKTEGP	0.001
CBP/p300	381	KGQSTSRHKKLMFKTEG	0.001
CBP/p300	373	HSSHLKSKKGQSTSRHK	0.001
CBP/p300	370	SRAHSSHLKSKKGQSTS	0.001
CBP/p300	372	AHSSHLKSKKGQSTSRH	0.003
CBP/p300	292	TEEENLRKKGEPHHELP	0.007
CBP/p300	305	HELPPGSTKRALPNNTS	0.015
CBP/p300	320	TSSSPQPKKKPLDGEYF	0.017
CBP/p300	120	GFLHSGTAKSVTCTYSP	0.0178
CBP/p300	386	SRHKKLMFKTEGPDSD-	0.04
CBP/p300	164	RVRAMAIYKQSQHMTEV	0.0661

B

C The shortest path between the lysine-acetyl-transferase and query protein:

CBP (CREBBP)	PINA	CREBBP -> TP53	network view
CBP (CREBBP)	STRING	CREBBP -> TP53	network view
p300 (EP300)	PINA	EP300 -> TP53	network view
p300 (EP300)	STRING	EP300 -> TP53	network view

D

Figure 5.4: ASEB results page

Table 5.4: Results from ASEB

Site	Domain	Sequence	P-value
120	DBD	GFLHSGTA**K**SVCYTCY	0.0178
164	DBD	AMALY**K**QSPGQSH	0.0661
292	DBD	EENLLRK**K**GEPHNEL	0.007
305	-	ELPRGST**K**RALPNNT	0.015
320	-	SSSPQPK**K**KPLDGEY	0.017
372	CTD	SSHLASKL**K**KFGQSTES	0.003
373	CTD	SHLASKLK**K**FGQSTESH	0.001
381	CTD	QSTSRH**K**KLMFKTEG	0.001
382	CTD	QSTSRHK**K**LMFKTEG	0.001
386	CRD	RHKKLMF**K**TEGPDSD	0.04

The ASEB analysis showed us 10 important acetylation hotspots, with specic sites; K372, K373, K381, and K382 standing out as the most likely ones with a high degree of confidence ($p \leq 0.003$). According to ASEB (Wang et al., 2012) the lower the p-values, the more likelihood the prediction made by the tool is. The high confidence acetylation sites are all in the CTD of the p53, This a strategic domain for p53 regulation because it's a hotspot for acetylation and other PTMs like ubiquitination and SUMOylation.

Lysine residues K120, K164, and K292 were located in the DBD where K292 had a higher likelihood of occurance due to its low p-value of 0.007. Analysing results from ASEB revels that the CTD is the region on p53 where acetylation is likely to occur and this could mean the CTD might be the region of p53 PTM crosstalk.

5.5 Discussion of predicted sites from all the prediction tools

Having done our predictions of Ubiquitination, SUMOylation and acetlylation using different predictive tools, we proceeded to evaluate the results from all these tools to

analyse lysine residues that could be shared by more than one PTM and this would help us establish an explanation about PTM crosstalk in p53 protein regulation as a result of competing PTMs.

Table 5.5: Results from all tools

Tool	PTM	Predicted Lysine Sites
ASEB	Acetylation	**K120**, K**164**, K**292**, K305, K**320**, K372, K373,K381, K382, K**386**
GPS-Uber	Ubiquitination	K101, K**164**, K**351**, K357, K**386**
GPS-SUMO/SUMOplot	SUMOylation	K101, K120, K139, K**164**, K291, **K292**, K319, K**320**, K321 , K**351**, K357, **K372,** K**386**
Shared	SUMOylation and Ubiquitination	**K164**, K**351**, K**386**
Shared	SUMOylation and Acetylation	**K164, K292, K320, K372, K386,**
Shared	Ubiquitination and Acetylation	**K164, K386**
shared	All the three	**K164, K386**

Integrated computational predictions suggest potential PTM crosstalk centered on specific lysine residues as illustrated in Table 5.5. Two residues K386 and K164 are predicted to be sumoylated, ubiquitinated and acetylated by all the four tools. The residue K386, located in the C-terminal regulatory domain, has been shown multiple times to become SUMOylated. p53 SUMOylation enhances retention in the nucleus and transcriptional activity of this protein when cells experience stress (Meulmeester et al., 2008). p53 SUMOylation could sterically affect ubiquitin attachment to preserve p53 in an active pool, potentially shifting p53 toward pro-apoptotic gene expression (Kim et al., 2019). The modification of K164 located in the DNA-binding domain could allow degradation of P53 by ubiquitination or functional fine-tuning by acetylation and/or SUMOylation.

Further more, residues K164, K351 and K386 are predicted to be target of both ubiquitination and SUMOylation tools. The residue K351, present in the tetramerization domain, could potentially modulate the tetramer-monomer ratio of p53. SUMOylation at K351 which could prevent MDM2-mediated ubiquitination and stabilize nuclear p53 in contrast to K351 ubiquitination which causes it degradation. In addition to being structurally important, K351 is placed near a nuclear export signal; amino acid residues 340 to 351 (Stommel et al., 1999), so PTMs at this site could also influence cellular localization of p53 within the nucleus or cytoplasm.

Residues K292, K320 and K372 may host binary PTM overlaps, SUMOylation-acetylation. In DNA-binding domain, this competition at residue K292 could alter DNA-binding efficiency, or transcriptional activity. However, in the C-terminal regulatory domain, the residue K372 which could also experience SUMOylation-acetylation competition, could potentially block ubiquitination at adjacent sites K373. Such spatial clustering might explain how stress-induced acetylation stabilizes p53.

In physiological and /or physiopathological context, competition at these residues could serve as aregulatory mechanism, tipping the balance between retaining p53 in the nucleus to carry out transcriptional responses or degradation within the cytoplasm. p53's fate will likely depend on the stress type and intensity, the upstream kinase signaling, and the relative activities of SUMO- or ubiquitin-conjugating enzymes. These modifications have been reported in cancer and not well documented in neurodegenerative diseases.

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5.6 Study limitations

Even with the extensive in silico pipeline that we have employed, there are several considerations for limitations. First of all, our in silico assessment relied entirely on predictive algorithms: GPS-Uber, GPS-SUMO, SUMOplot and ASEB. Thus, every site identified could be dependent on prior data made available in the algorithm and if we altered the threshold settings for the predictions, the returned results could have biases or overlook biologically relevant lysines without cuts offs, this could be a reason why different computational tools give different reults even when mapping for the same PTM hotspots. Secondly, without experimental validation (e.g., mass spectrometry or site-directed mutagenesis), we cannot ascertain if predicted

ubiquitination, SUMOylation, or acetylation events truly occur in neuronal cells when stressed. Thirdly, these tools do not determine how p53 undergoes dynamic, three-dimensional conformational changes in vivo, nor do they model how one PTM may impact other PTMs simultaneously. Lastly, since the majority of the underlying databases rely predominantly upon cancer, many of the predictions may not be fully representative of modifications that occur to p53 that would be specific to a neurodegenerative context.

Conclusively, while our computational predictions offered speed and cost effectiveness, these predictions were limited by biological complexity that couldnot be fully captured without experimental context.

CONCLUSION AND PERSPECTIVES

Conclusion

In this study, we employed a computational approach to map two post-translational modification sites, ubiquitination and SUMOylation on the human p53 protein, with additional insights into acetylation hotspots, to elucidate the regulatory crosstalk between these post-translational modifications. Our predictions identified key modification sites of residue lysine which might mediate the regulatory crosstalk of the p53. Notably two lysine residues K386 and K164 are predicted to be SUMOylated, ubiquitinated and acetylated, suggesting potential PTM crosstalk on these specific sites. The p53 SUMOylation at residue K386, located in the C-terminal regulatory domain, enhances retention in the nucleus and transcriptional activity. The modification of residue K164 located in the DNA-binding domain could allow degradation of P53 by ubiquitination or functional fine-tuning by acetylation and/or SUMOylation. Furthermore, the residue K351, present in the tetramerization domain, is predicted to be SUMOylated and Ubiquitinated. SUMOylation at residue lysine K351 which could prevent MDM2-mediated ubiquitination, stabilizes nuclear p53 retention in contrast to the ubiquitination of the residue K351 which causes it degradation. Residues K292, K320 and K372 may host binary PTM overlaps, SUMOylatio and acetylation. In DNA-binding domain, this competition at residue K292 could alter DNA-binding efficiency, or transcriptional activity. However, in the C-terminal regulatory domain, this competition at K372, could potentially
block ubiquitination at adjacent sites. These findings align with existing literature, reinforcing the notion that PTM interplay at specific residues fine-tunes p53's role in physiological context and in cellular stress responses, particularly in neurodegenerative diseases. This study advances our understanding of p53 regulation by mapping potential PTM crosstalk sites, offering a roadmap for further investigation into how ubiquitination, SUMOylation and acetylation orchestrate p53's activity.

By bridging computational predictions with biological relevance, we lay the groundwork for targeted interventions in neurodegenerative disorders where p53 dysregulation seems to play a pivotal role. In order to validate these predictions, the perspective is to experimentally corroborate each lysine residue modification site with its function using site-directed mutagenesis. Mass-spectrometry based proteomics in neuron-specific cell models, under basal and stress conditions, would allow to demonstrate the occurrence and stress-responsive nature of PTMs.

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