Charles University

Faculty of Science

Study programme: Cell biology

Bc. Lucie Vaníková

Functional analysis of selected variants of uncertain significance in cancer predisposing genes

Funkční charakterizace variant nejasného významu nádorových predispozičních genů

Diploma thesis

Supervisor: MUDr. Libor Macůrek, Ph.D.

Prague 2024

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 7. 8. 2024

Lucie Vaníková

Acknowledgements

First and foremost, I want to thank my supervisor MUDr. Libor Macůrek, Ph.D. His guidance, support and feedback have been invaluable throughout this journey. I couldn´t have done it without his precious advice and great leadership. I also want to express my gratitude to everyone at the Laboratory of Cancer Cell Biology at the Institute of Molecular Genetics of the Czech Academy of Sciences. The staff have been incredibly supportive, and I am especially grateful to Matouš Palek and Zuzana Chalupová for all their help in the lab. I´d also like to acknowledge the Institute of Molecular Genetics itself for allowing me to conduct the practical part of the thesis in its laboratories.

On a personal note, I want to thank my parents and grandparents, whose support have been a constant source of strength. And to my friends, my partner and my dogs for preserving my mental health during the tougher times.

Abstract

Inherited mutations in predisposing genes significantly contribute to the development of various cancers. Current genetic testing via panel sequencing efficiently identifies pathogenic germline mutations in the *TP53* gene, which can cause hereditary Li-Fraumeni syndrome (LFS) associated with greatly increased risk of tumor development. However, variants of uncertain significance (VUS) can complicate clinical interpretation. This thesis aims to evaluate the functional significance of selected variants in the *TP53* gene, identified by genetic screening particularly in the Czech population. *TP53* is crucial for cell cycle regulation, induction of apoptosis, DNA repair and other functions and its dysregulation is associated with cancer.

Using CRISPR/Cas9 technology, we generated a cellular model with inactivated *TP53* gene. Further work included preparing expression plasmids carrying coding sequences for fluorescent markers, cDNA of selected *TP53* variants, and selection cassettes. The function of the mutated versus the wild-type gene forms was compared after transfection and generation of stable cell lines. Eventually, the expression of *CDKN1A* and *MDM2* genes among the cell lines after nutlin-3a treatment was assessed. Additionally, the role of selected *TP53* variants in the p53 tetramer formation and colony formation assay was analysed.

This work contributes to a deeper understanding of the cellular functions of several *TP53* mutations. The findings provide insights into their roles in the cell cycle regulation, which will assist in evaluating cancer risk for mutation carriers in collaboration with clinical genetics. Overall, out of 35 analysed mutations we defined 14 as loss-of-function, 20 as benign and one of them (R267W) as functionally intermediate. Finally, we found that the E339del3 variant recently identified in a Czech cancer patient is a loss-of-function mutation and can promote LFS.

Keywords: *TP53*, variants of uncertain significance, cancer, *CDKN1A*, *MDM2*

Abstrakt

Dědičné mutace v predispozičních genech významně přispívají ke vzniku různých druhů rakoviny. Současné genetické testování pomocí panelového sekvenování účinně identifikuje patogenní zárodečné mutace v genu *TP53*, které mohou způsobit Li-Fraumeni syndrom (LFS) spojený s výrazně vyšším rizikem vzniku nádoru. Varianty nejasného významu (VUS) mohou však klinickou interpretaci komplikovat. Cílem této práce je zhodnotit funkční význam vybraných variant genu *TP53* identifikovaných genetickým screeningem zejména české populace. Gen *TP53* je klíčový pro regulaci buněčného cyklu, indukci apoptózy, opravu DNA a další funkce a jeho dysregulace je spojována s nádorovými onemocněními.

Pomocí technologie CRISPR/Cas9 jsme vytvořili buněčný model s inaktivovaným genem *TP53*. Další práce zahrnovala přípravu expresních plazmidů nesoucích kódující sekvence pro fluorescenční markery, cDNA vybraných *TP53* variant a selekční kazety. Po transfekci a vytvoření stabilních buněčných linií byla porovnána funkce mutovaných a wild-type forem genů. Nakonec byla napříč buněčnými liniemi vyhodnocena exprese genů *CDKN1A* a *MDM2* po použití nutlinu-3a. Kromě toho byla analyzována role vybraných variant *TP53* při tvorbě p53 tetrameru a testu tvorby kolonií.

Tato práce přispívá k hlubšímu pochopení buněčných funkcí některých mutací *TP53*. Výsledky přinášejí poznatky o jejich úloze v regulaci buněčného cyklu, což ve spolupráci s klinickými genetiky pomůže při hodnocení rizika vzniku rakoviny u nositelů mutací. Celkově jsme z 35 analyzovaných mutací určili 14 jako „loss-of-function“ (LOF), 20 jako benigní, a jedinou z nich (R267W) jako mutaci vykazující fenotypy mezi těmito dvěma kategoriemi. Dále jsme zjistili, že varianta E339del3, která byla nedávno identifikována u českého pacienta s rakovinou, je LOF mutací a může způsobit LFS.

Klíčová slova: *TP53*, varianty nejasného významu, rakovina, *CDKN1A*, *MDM2*

Table of contents

Abbreviations

3-meAde	3-methyladenine
A	adenine
AIP1	ASK1-interacting protein-1
ALOX15	arachidonate 15-lipoxygenase
AMPK	adenosine monophosphate-activated protein kinase
ARF	p14 alternative reading frame protein
ASK1	apoptosis signal-regulating kinase 1
ATM	ataxia-telangiectasia mutated kinase
ATP	adenosine trisphosphate
ATR	ataxia-telangiectasia and Rad3-related protein kinase
BAX	BCL2 associated X protein
BBC3	BCL2 binding component 3
BCL2	B-cell lymphoma 2
BCL-xL	BCL-extra large
BCL2L1	BCL2 like 1
BER	base excision repair
BH3	BCL-2 homology domain 3
C	cytosine
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CDC25	cell division cycle 25 phosphatase
CD95	cluster of differentiation 95
CDK	cyclin dependent kinase
CDKN1A/WAF1	CDK inhibitor 1A
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CK1	casein kinase 1
CREB	cAMP responsive element binding protein
CRISPR	clustered regularly interspaced short palindromic repeats
CTD/REG	carboxy-terminal domain
DBD	DNA-binding domain
DEN	diethylnitrosamine
DNA-PK	DNA-activated protein kinase
Dox	doxycycline
DPP4	dipeptidyl peptidase-4

DRAM	DNA damage regulated autophagy modulator
EI24	etoposide induced 2.4
G	guanine
G6PD	glucose-6-phosphate dehydrogenase
GADD45	growth arrest and DNA damage inducible protein
GLS2	glutaminase 2
GLUT1/GLUT4	glucose transporter 1/4
GOF	gain of function
GPX4	GSH peroxidase 4
GSH	glutathione
HAT	histone acetyltransferase
HR	homologous recombination
iASPP	inhibitor of apoptosis-stimulating protein p53
IF	immunofluorescence
IGFBP-3	insulin like growth factor binding protein 3
ISG20L1	interferon stimulated exonuclease gene 20kDa-like 1
KAT5	lysine acetyltransferase 5
KO	knockout
LFL	LFS-like syndrome
LFS	Li-Fraumeni syndrome
LOF	loss-of-function
LR	linker region
MAPK	mitogen-activated protein kinase
MDM2	mouse double minute 2
MDM4	double minute 4 human homolog
MGST3	microsomal glutathione S-transferase 3
miRNA	microRNA
MMR	mismatch repair
MOF	males absent on the first
Mono	monoclonal
MOZ	monocytic leukemia zinc finger
Ms	mouse
MSH2	MutS homolog 2
NADPH	nicotinamide adenine dinucleotide phosphate
NAT10	N-acetyltransferase 10
NES	nuclear export signal
NER	nucleotide excision repair

NHEJ	non-homologous end-joining
NOX1	nicotinamide adenine dinucleotide phosphate oxidase 1
NRF2	nuclear factor erythroid 2-related factor 2
Nut-3a	nutlin-3a
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PDK2	pyruvate dehydrogenase kinase isoform 2
PFK-1	phosphofructokinase-1
PIASγ	protein inhibitor of activated STAT
PMAIP1/NOXA	phorbol-12-myristate-13-acetate-induced protein 1
Poly	polyclonal
PPP	pentose phosphate pathway
pRb	retinoblastoma protein
PRD	proline-rich domain
PRDX6	peroxiredoxin 6
PUMA	p53 upregulated modulator of apoptosis
Rb	rabbit
RFP	red fluorescent protein
ROS	reactive oxygen species
RPE	retinal pigment epithelium
RRM2B	ribonucleoside-diphosphate reductase subunit M2 B
SAT1	spermidine/spermine N1-acetyltransferase 1
SCO2	synthesis of cytochrome c oxidase 2
SH3	Src homology 3
SLC7A11	solute carrier family 7 member 11
SREBP-1c	sterol regulatory element-binding protein 1
SSC	side scatter
STAT	signal transducer and activator of transcription
SV 40	simian virus 40
T	thymine
TAD	transactivation domain
TCA	tricarboxylic acid cycle
TD/TET	tetramerisation domain
TFIIH	transcription factor II H
TIGAR	TP53-induced glycolysis regulatory phosphatase
TNFR	tumor necrosis factor receptor
TNFRSF6	TNFR superfamily member 6

TP53	tumor protein p53
TRIAP1	TP53 regulated inhibitor of apoptosis 1
TRIML2	tripartite motif family-like 2
ULK1	unc-51 like autophagy activating kinase 1
UV	ultraviolet
VUS	variant of uncertain significance
WB	western blotting
XAF1	XIAP-associated factor 1
XIAP	X-linked inhibitor of apoptosis
XPB/XPC/XPD	xeroderma pigmentosum type B/type C/type D

1 Introduction

1.1 History of p53

Since it was discovered that protein p53 plays a major role in tumor suppression and in the cellular response to DNA damage, it has been intensively studied by the research community. However, this protein was first identified in 1979 as a 53 kDa large cell protein able to form complex with large T antigen of simian virus 40 (SV 40) (Lane & Lionel V. Crawford, 1979). Furthermore, it came to acknowledgement that transformed cells tended to possess more p53 than cells which did not undergo transformation (Kress et al., 1979). While p53 ordinarily functioned as a short-lived protein, transformed cells kept higher amount of this protein by its stabilization (Chandrasekaran et al., 1982). Later, scientists came up with a suggestion that p53 is involved in the cell cycle regulation. They believed that increased stability of this protein detected in G1 phase was responsible for the induction of DNA synthesis. Furthermore, constant steady-state level of p53 would prevent cells to entry G0 phase and therefore could contribute to their actively dividing state (Reich NC & Levine AJ, 1984).

Latter scientific experiments suggested that gene *TP53*, which encodes protein p53, might be an oncogene. They revealed that mutation of this gene enhanced its transformation efficiency (Jenkins JR et al., 1985). Other findings supported this oncogene theory by showing that expression of this gene seemed to be important for malignant transformation of cells. However, opposite to another oncogene c-myc, p53 was not detected in all malignant cells. On that account, c-myc and p53 were thought to be controlled by different cellular mechanism during tumor development (Stewart et al., 1986; Van Den Berg et al., 1989).

On the other hand, other scientific results were in contrary to the idea of *TP53* being an oncogene. Surprisingly, no detectable level of p53 protein was found in cell lines transformed by Friend leukaemia virus. Additionally, the authors proved that p53 was not essential for tumor formation *in vivo*. Hence, they believed that *TP53* gene inactivation could be important for malignant processes (Mowat M et al., 1985). Eventually, it was confirmed that p53 employed in the former experiments was not wild-type. In fact, there were dominant negative missense mutations located in the conserved region of p53 cDNAs from the studies. Those mutations were most likely responsible for alteration of the biological activity and

conformation of the protein (May & May, 1999). Further experiments showed that cells which were transfected with the wild-type p53 failed to grow or died. Nevertheless, *TP53* was thought of as a recessive oncogene on the grounds of that (Hinds et al., 1989).

In 1990, it was proved that transfection of the wild-type p53 into neoplastic cells with no detectable level of p53 expression led to suppression of their tumor phenotype. Even though cancer cells expressed mutated version of the protein, the wild-type p53 transfection caused identical results (P.-L. Chen et al., 1990). Other data suggested that in a two-allele configuration, the wild-type p53 has a dominant role over the mutant variant. As a result, the oncogenic effect could be induced by loss of the both wild-type p53 alleles or by deletion of the single wild-type allele when the other one is mutated (Nigro JM et al., 1989). During the following years, thousands of p53 mutations had been discovered in human tumors. At that time, the status of *TP53* changed from an oncogene to a tumor suppressor gene (Hollstein et al., 1996).

1.2 Structure of p53

In human karyotype, *TP53* gene is located on the short arm of chromosome 17, exactly in 17p13 (Mcbride et al., 1986). The gene covers 19.14 kB of genomic DNA in this region. Its coding sequence is composed of 10 exons, but overall, the gene possesses four more, the first one being non-coding and the others being alternatively spliced. As there are more than twelve p53 protein isoforms, these three exons give rise to the transcripts encoding them (Marcel et al., 2010; Hainaut & Pfeifer, 2016).

Since human p53 is a polypeptide containing 393 amino acids, its theoretical molecular weight is ~ 44 kDa. However, it was estimated that the major form of p53 is a bit more than 170 kDa. On behalf of that, protein p53 was identified to work as a tetramer. Eventually, a tetramer composed of p53 monomers or even multiples of tetramers together could generate a DNA-binding unit (Friedman et al., 1993). The full-length protein consists of multiple functional domains (Fig. 1). The N-terminus begins with two transactivation domains followed by a proline-rich region (Walker & Levine, 1996; Candau et al., 1997). Further in the protein length there is an important DNA-binding domain and a tetramerisation domain is to be found closer to the C-terminus. In addition, amino acids 357 – 393 comprise the carboxy terminal domain located at the very end of the C-terminal region (Pavletich et al., 1993).

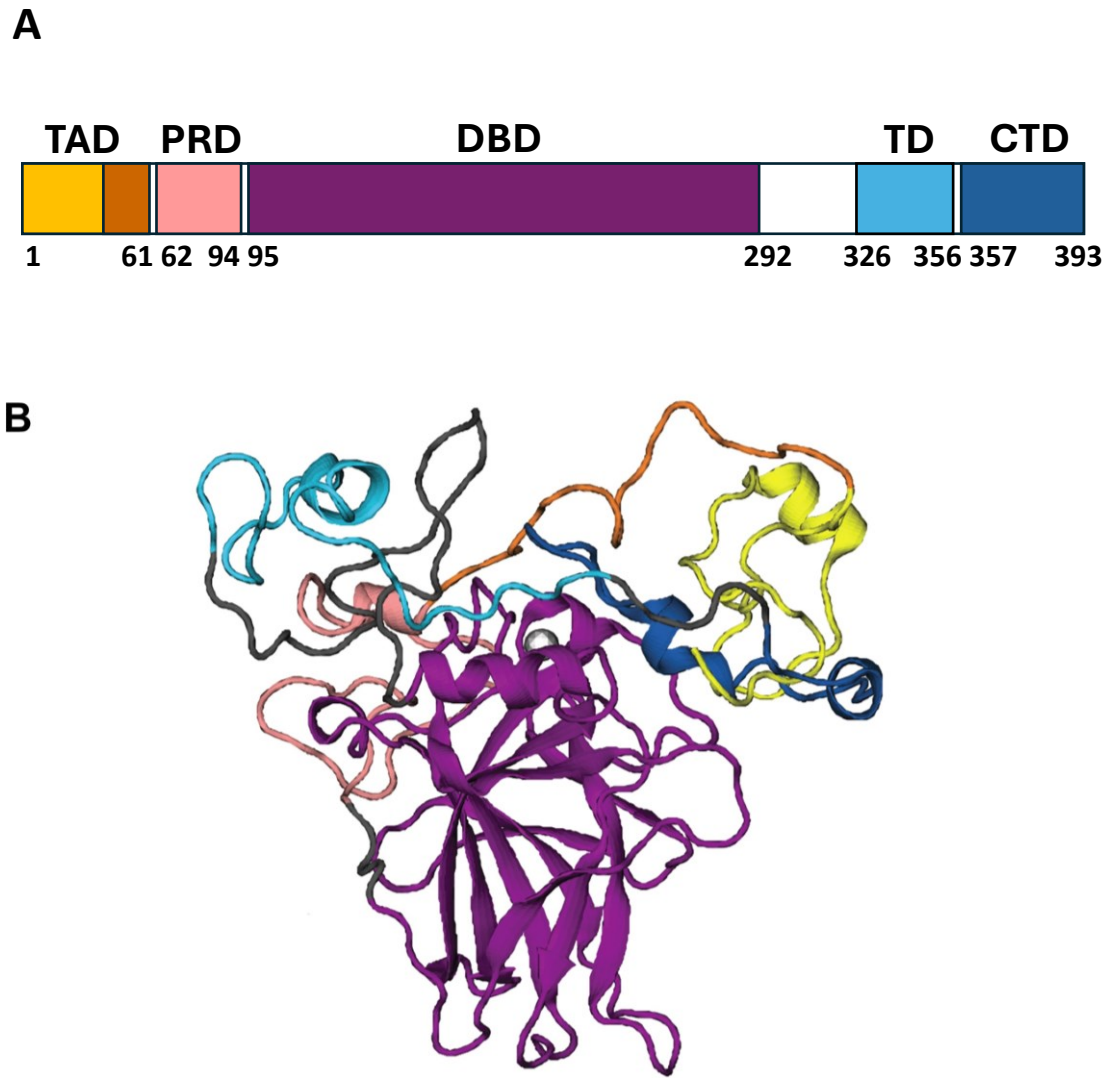


Figure 1. The p53 architecture. (A) Domain architecture of p53. TAD = transactivation domains; PRD = proline-rich domain; DBD = DNA-binding domain; TD = tetramerisation domain; CTD = carboxy-terminal domain. (B) Structure model of monomeric full-length p53 (adapted from Chillemi et al., 2013).

1.2.1 Transactivation domains

The first transactivation domain (TAD 1) is placed between amino acids 1-40 of protein p53. The second one (TAD 2) follows TAD 1 and comprises a region between residues 41-61 (Duffy et al., 2022). Together, TAD 1 and TAD 2 are capable of transcriptional regulation of several common genes, but each of them can as well provide transactivation of some genes on its own (Brady et al., 2011). Additionally, the domains bind p300/cAMP responsive element binding protein (CREB)-binding protein (CBP) that enhances the ability of p53 to activate expression of its targets (Scolnick et al., 1997). Hydrophobic amino acids leucine 22 and tryptophan 23

present in TAD 1 are important for the transcriptional activation function of the protein. Even though a double mutant in these two residues is still capable of binding p53-responsive DNA elements, it is unable to interact with the transcriptional machinery of the cell. Together with leucine 14 and phenylalanine 19, these four residues are essential for binding of the E3 ubiquitin ligase mouse double minute 2 (MDM2) (Lin et al., 1994). When this protein is bound to p53, it can inhibit eventual transcriptional activation (Momand et al., 1992). As the TADs not only bind MDM2, which is responsible for ubiquitination and subsequent degradation of p53, but also CBP, they may have impact on the stability of p53, depending on the binding partner (Scolnick et al., 1997; Fuchs et al., 1998). At the N-terminus, p53 can be phosphorylated at the residue serine 15. This posttranslational modification ought to activate and stabilize p53 upon a stress signal. Next, functional analysis showed that p53 phosphorylated at residue serine 46 binds several apoptotic proteins including B-cell lymphoma 2 (BCL2) binding component 3 (BBC3), BCL2 associated X protein (BAX), BCL2 like 1 (BCL2L1), tumor necrosis factor receptor superfamily member 6 (TNFRSF6) and TP53 regulated inhibitor of apoptosis 1 (TRIAP1) leading to apoptosis induction (Smeenk et al., 2011).

1.2.2 Proline-rich domain

The proline-rich domain is situated between residues 62 and 94. This domain is rich in PXXP motif repetitions, where P stands for proline and X for any amino acid (Duffy et al., 2022). Src homology 3 (SH3) protein domains can distinguish such motifs and bind to them (H. Yu et al., 1994). The indispensability of the proline-rich domain consists in its capability of suppressing cell growth. Overall, this domain is responsible for mediating tumor suppression by downstream antiproliferative signals (Walker & Levine, 1996).

1.2.3 DNA-binding domain

The largest and probably the most well-known domain expands from the amino acid residue 95 up to 292 (Duffy et al., 2022). The DNA-binding domain (DBD) forms a β sandwich comprised of two antiparallel β sheets, one with four β strands and the other with five. Besides the β sandwich, the domain also contains a loop-sheet-helix motif and two large loops, which are partially stabilized by a zinc atom (Fig. 2A). This atom is tetrahedrally coordinated by three cysteines (Cys176, Cys238 and Cys242) and histidine (His179). The structure accountable for DNA-binding in the major groove is the loop-sheet-helix motif. Especially arginine residues at

positions 248, 273 and 282 are known to make close contact with the DNA double-helix. The minor groove is occupied by arginine (Arg248) from one of the large loops. Both binding structures have residues that also contact DNA backbone between major and minor grooves (Cho et al., 1994). Within DNA, there are specific motifs recognizable by the p53 binding elements. These consensus sequences are two copies of a 10 bp long segment (Fig. 2B). Up to 13 base pairs divide the copies (El-Deiry et al., 1992). Several p53 amino acids, such as Arg175, Gly245, and Arg249, are essential for the stabilization of the whole protein structure, although they are not in the proximity to DNA. Apart from being key structural parts, the six mentioned amino acid residues were proved to be hot spots for mutations in cancer cells (Cho et al., 1994; Levine et al., 1994).

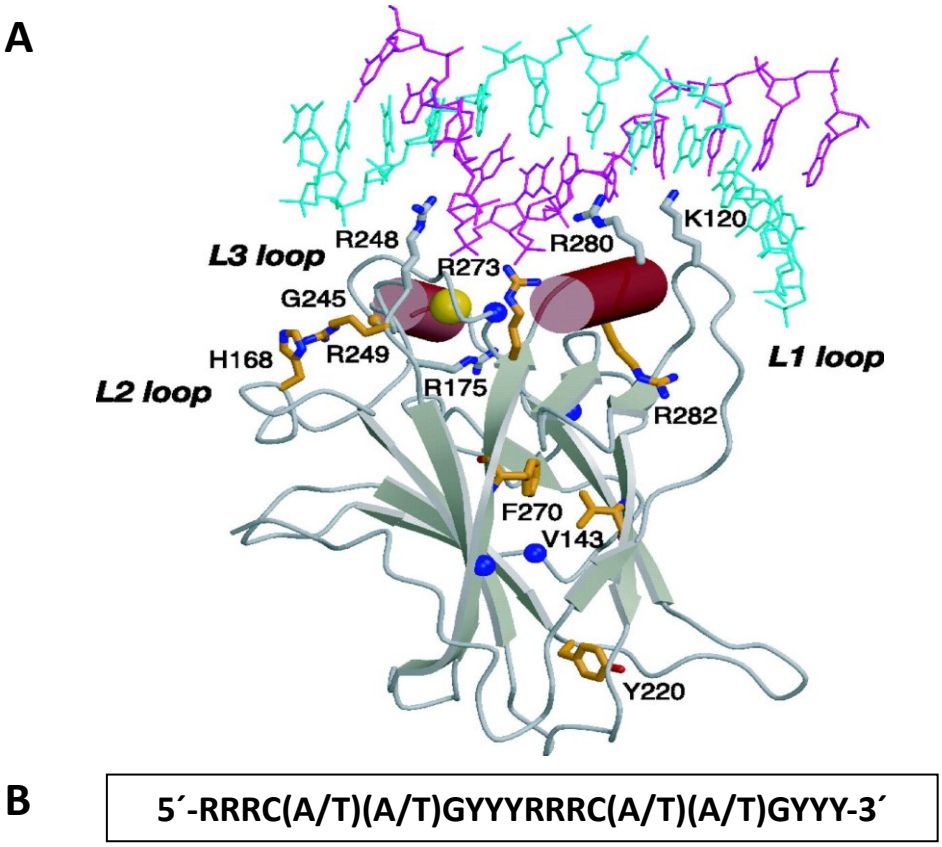


Figure 2. DNA-binding by p53. (A) Structure of the DNA-binding domain of p53 bound to consensus DNA. The two strands of bound consensus DNA are shown in blue and magenta. The bound zinc ion is displayed as a golden sphere (adapted from Joerger et al., 2006). (B) The consensus DNA sequence recognized by p53 (R = purine; C = cytosine; A = adenine; T = thymine; G = guanine; Y = pyrimidine).

1.2.4 Tetramerisation domain

Amino acids 293 – 325 encompass a linker region, which is followed by a tetramerisation domain (TD) situated between amino acids 326 – 356 (Duffy et al., 2022). The role of this domain is to mediate the correct conformation of protein p53. As p53 binds DNA in the form of tetramer, the TD also contributes to the DNA-binding function (Chène, 2001). Surprisingly though, even mutants with no TD are able to bind DNA (Bargonetti et al., 1993). The domain´s function most probably results in an increased affinity of binding and moreover, in bending and twisting DNA with significant differences (Balagurumoorthy et al., 1995; Nagaich et al., 1999). In addition, the oligomerization domain includes a highly conserved leucine-rich nuclear export signal (NES). When p53 monomers form a tetrameric structure, NES is no longer accessible. As a result, oligomerization state of p53 could determine the result of its shuttle between the nucleus and the cytoplasm (Stommel et al., 1999). Next, the TD can interact with several proteins, such as Ca^{2+}-dependent protein kinase C or adenovirus E4orf6 protein (Dobner T et al., 1996; Delphin et al., 1997). Additionally, this domain has influence on binding other proteins (for example MDM2), which do not interact with the TD directly, but bind p53 as a tetramer (Lomax et al., 1998).

1.2.5 Carboxy-terminal domain

The carboxy-terminal domain (CTD) stretches from the amino acid residue 357 to 393 (Duffy et al., 2022). This region contains a few lysine residues that can be post-translationally modified. In detail, when Lys370 and Lys382 become methylated, p53 transcriptional activity is repressed (Zhu et al., 2016). Greater stability and nuclear retention of p53 can be achieved by methylation of Lys372 (Chuikov et al., 2004). On the other hand, if Lys373 and Lys382 receive acetylation marks, the DNA-binding activity of p53 increases significantly (Gu & Roeder, 1997). Besides, acetylated Lys382, together with acetylated Lys320 from the tetramerisation domain, inhibit non-specific DNA-binding. Moreover, these acetylation modifications set by p300 and p300/CBP-associated factor (PCAF), respectively, are promoted by DNA damaged-induced phosphorylation of Ser33 and Ser37 (Sakaguchi et al., 1998).

1.3 Regulatory network of p53

Modifications of p53 are very extensive (Fig. 3) and their enumeration exceeds the purpose of this thesis, therefore only the most important ones will be described.

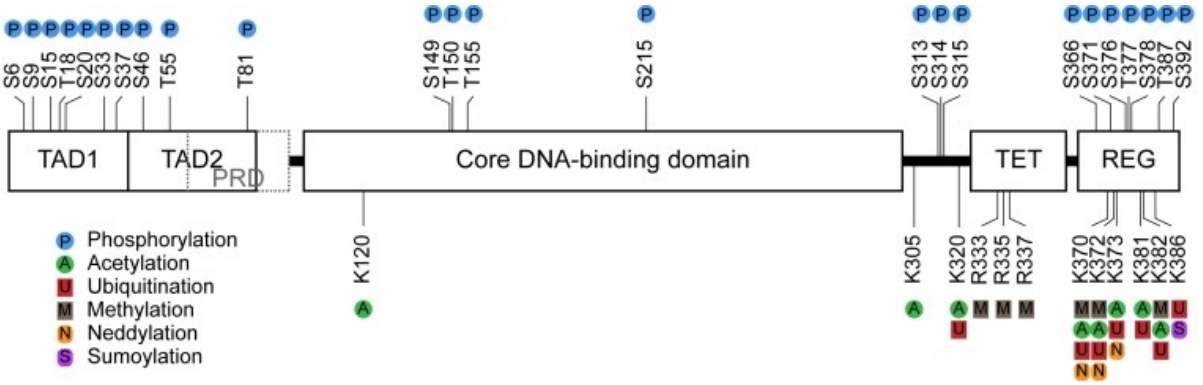


Figure 3. Sites of post-translational modifications on p53. Schematic representation of the 393 amino acid domain structure of human p53 showing the sites of post-translational modification including phosphorylation, acetylation, ubiquitination, methylation, neddylation, and sumoylation. Abbreviations: N-terminal transactivation domain (TAD); proline-rich domain (PRD); tetramerisation domain (TET); C-terminal regulatory domain (REG); arginine (R); lysine (K); serine (S); threonine (T) (adapted from Maclaine & Hupp, 2009).

Following genotoxic stress, p53 acquires multiple posttranslational modifications (Appella & Anderson, 2000). One of the most common modifications along p53 residues is phosphorylation, occurring at serine and threonine. As for the N-terminal part of the protein, phosphorylation marks mostly appear at Ser6, Ser9, Ser15, Ser20, Ser33, Ser37, Ser46 and at Thr18, Thr55 and Thr81. The phosphorylation process was identified to take place mainly after exposure to UV light or ionizing radiation. The DNA-binding domain gains significantly fewer phosphorylation modifications. They affect Ser149, Ser215, Thr150 and Thr155. Even residues situated within the linker region between the DBD and the TD get phosphorylated – Ser313, Ser314 and Ser315. Phosphorylation at the C-terminal part of p53 may arise after DNA damage. This modification then localizes to Ser392. Besides, Lys305 and Lys320 can get acetylated. Furthermore, acetyl groups can be found at Lys370, Lys372, Lys373, Lys381 and Lys382. In addition, sumoylation can also be found within the protein residues of p53, specifically at Lys386 of stressed cells (Appella & Anderson, 2001; Maclaine & Hupp, 2009). Transcription activation efficacy by p53 can be changed by both conformational change and DNA-binding affinity of the protein. Therefore, the numerous modifications affecting its conformation and binding regulate its function (Knights et al., 2006).

Phosphorylation mark at Ser15 can be induced by more than one kinase. This modification is often mediated by ataxia-telangiectasia mutated kinase (ATM), DNA-activated protein kinase (DNA-PK) or ataxia-telangiectasia and Rad3-related protein kinase (ATR) (Lees-Miller et al., 1992; Banin et al., 1998; Tibbetts et al., 1999). Depending on the distinct source of stress, also Ser20 might be phosphorylated by various kinases. The function can be again attributed to ATM, although its effect is indirect in this case. Additionally, other proteins that transfer phosphoryl group to this serine are casein kinase 1 (CK1), 5' adenosine monophosphate-activated protein kinase (AMPK), checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) (Craig et al., 2007; Maclaine & Hupp, 2009). Together, these two p53 modifications help stabilize the protein under stressful conditions, and hence, support its transactivation function (Chehab et al., 1999; Dumaz & Meek, 1999).

Acetylation of some p53 residues is generally performed by p300/CBP acetylase. Besides, some lysine residues are modified due to acetylases such as PCAF, lysine acetyltransferase 5 (KAT5, also known as Tip60), monocytic leukemia zinc finger protein (MOZ), males absent on the first (MOF) and N-acetyltransferase 10 (NAT10) (Gu & Roeder, 1997; Nagasaka et al., 2022). At the C-terminus of p53, acetylation stimulates greater interaction with its coactivators. One of them is histone acetyltransferase (HAT) that induces transcription through histone acetylation. In summary, acetylation of p53 is not necessary for increasing the DNA binding affinity, but for binding of specific transcription coactivators (Barlev et al., 2001). Especially acetylation of Lys320 and Lys373 is of great importance. Acetyl group at Lys320 contributes to the overall survival of the cell by transactivating numerous genes and hence, suppressing the apoptotic pathway. Acetylated Lys373 is important for p53 being able to bind low-affinity promoters of proapoptotic genes (Knights et al., 2006). Next, acetylated Lys382 enhances transactivation function of p53 by inhibiting non-specific DNA binding (Sakaguchi et al., 1998).

Apart from being phosphorylated and acetylated, p53 can acquire methylation, sumoylation, and neddylation at specific sites. Methyltransferase Set9 sets methylation mark at Lys372 in response to DNA damage. This modification leads to p53 nuclear retention and hence, supports transactivation of its target genes (Chuikov et al., 2004). Another methyltransferase is Set8, which is responsible for methylation of Lys382, thereby suppressing p53 functions and blocking acetylation of the residue (Shi et al., 2007). Methylation at Lys370 results in the same

process – downregulation of p53-responsive genes. An enzyme essential for this step is Smyd2 (J. Huang et al., 2006).

So far, not much information has been gathered about sumoylation and neddylation of p53. Tripartite motif family-like 2 (TRIML2) causes sumoylation of the protein resulting in enhanced transcription of apoptotic genes (Kung et al., 2015). On the contrary, sumoylation by protein inhibitor of activated signal transducer and activator of transcription (STAT) γ (PIASγ) contributes to the cytoplasmic localization of p53, making it impossible to transactivate its targets (Carter et al., 2007). In general, neddylation works against transactivation function of p53, although this modification is certainly worth of further examination (Abida et al., 2007).

Transcription factor p53 induces expression of MDM2, which, in turn, significantly reduces p53 levels. This regulatory feedback loop is maintained via direct p53-MDM2 association and eventual targeting of p53 for ubiquitin-mediated degradation (Haupt et al., 1997). Thus, the p53 protein levels are generally low due to this mechanism but rise after DNA damage events (Kastan et al., 1991). First, it was believed that phosphorylation of certain residues at the N-terminal of p53 might serve as an inhibition for MDM2 binding (Shieh et al., 1997). However, latter studies indicated that phosphorylation has little or no effect on disrupting interaction with MDM2 (Schon et al., 2002). Moreover, MDM2 can be negatively regulated, as well, by p14 alternative reading frame protein (ARF). ARF removes MDM2 from the nucleus to the nucleolus, providing that it can no longer bind nuclear p53 and block its activation (Weber et al., 1999). Double minute 4 human homolog (MDM4) also blocks the transactivation domain of p53, but, unlike MDM2, does not cause its degradation. MDM2 and MDM4 are homologs sharing similarities especially among the p53-binding domain (Shvarts et al., 1996). Besides promoting degradation of p53, ubiquitin E3 ligase MDM2 also promotes degradation of MDM4 (Pan & Chen, 2003).

In conclusion, the upstream regulatory network of p53 is extremely robust and p53 is able to acquire multiple posttranslational modifications that lead to various cellular events. Mainly, the protein can either be stabilized, degraded or its function can be inhibited. The specific outcome and subsequent p53-directed cellular process is generally given according to the type of cellular stress as signal stimulus. It has been shown that under various conditions, p53 levels result in diverse dynamics. Providing the cell suffered from transient damage, which could be presented by moderate doses of γ-irradiation, it generates repeated p53 pulses (Purvis et al.,

2012). This event allows cells to stop the cell cycle, repair the damage and further continue dividing. However, when the DNA damage is more severe, a sustained p53 pulse is created. Such situation might happen after exposure to UV radiation or after treatment with a small molecule nutlin-3, which disrupts p53-MDM2 interaction. Eventually, the sustained pulse promotes induction of genes associated with apoptosis or senescence (Vassilev et al., 2004; Purvis et al., 2012).

1.4 Functions of p53

Since *TP53* was classified as a tumor suppressor, plenty of scientific groups have started exploring the purpose of p53. Eventually, the collected data showed that protein p53 has a broad range of functions affecting multiple cellular mechanisms. Indeed, this protein controls processes that are directly related to tumor suppression as well as processes that may only contribute to this phenomenon. Overall, distinct stimuli tend to determine the exact p53-dependent signaling set off in a context-specific manner (Pitolli et al., 2019).

1.4.1 Canonical functions of p53

Protein p53 is a crucial component in maintaining cellular integrity (Fig. 4). Its canonical functions are essential for preventing the development of cancer by ensuring proper cellular responses to various stressors.

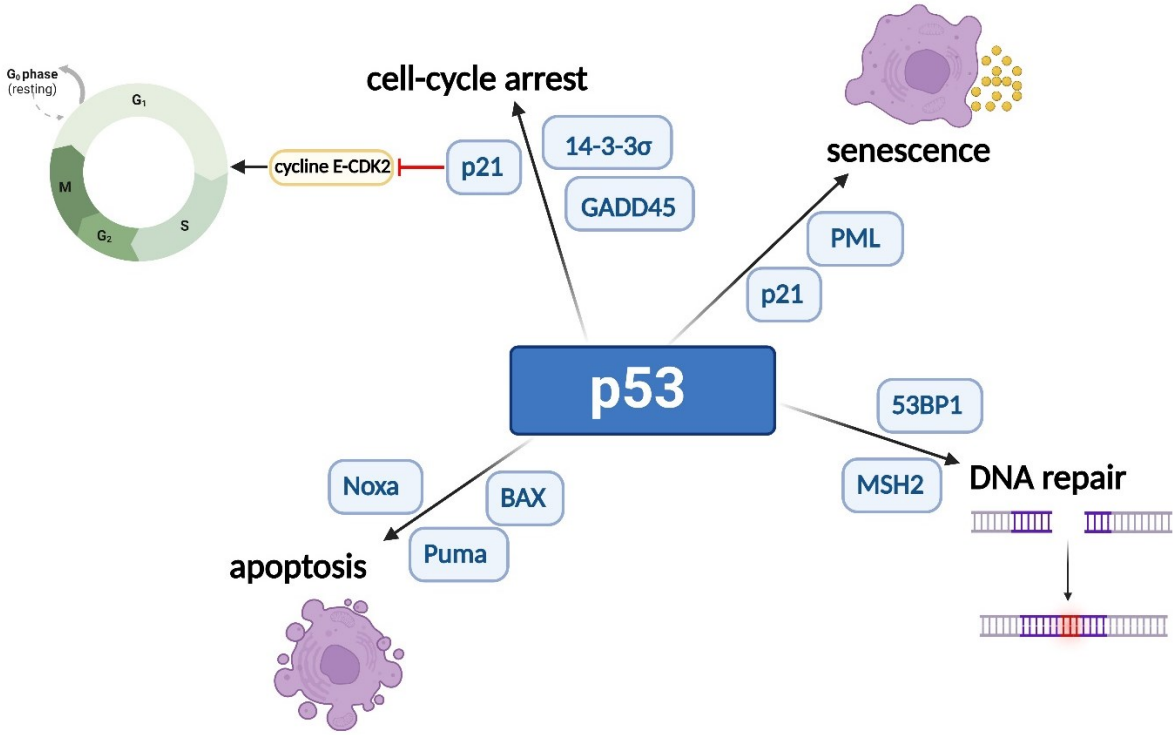


Figure 4. Selected canonical functions of p53. A few targets of p53 that promote cell-cycle arrest/apoptosis/senescence/DNA repair are presented in blue frames.

1.4.1.1 Role of p53 in cell cycle regulation

In 1991, it was discovered that the levels of this protein increase significantly after DNA damage. That happened possibly by a post transcriptional mechanism since the analysis showed that the levels of *p53* mRNA did not rise that much. In addition, p53 takes part in establishment of cell cycle arrest and thus, inhibition of DNA replication (Kastan et al., 1991). Eventually, cyclin-dependent kinase inhibitor 1A (*CDKN1A*/*WAF1*) gene was identified as a transcription target of p53 (El-Deiry et al., 1993).

Cell cycle is divided into four general phases, which are G1, S, G2 and mitosis. The transition from each phase to another one is controlled by proteins called cyclin dependent kinases (CDKs). Especially CDK1, CDK2, CDK4 and CDK6 have main impact on cell cycle progression regulation (Malumbres & Barbacid, 2005). Although the activity of these kinases remains stable during the cell cycle, their function is regulated by fluctuation of cyclin proteins (Tyson et al., 2002). Cyclins form regulatory subunits for their complementary CDKs. Cyclins D bind to CDK4/6 and therefore control the transition through G1 phase. In similar manner, cyclins E form complex with CDK2 and regulate passage from G1 to S phase. Eventually, cyclin A replaces

cyclin E binding CDK2 and ensures the whole process of cell successfully going from S phase to mitosis entry. At the end of G2 phase, cyclin B interacts with CDK1 to induce mitosis (Morgan, 1997).

The activity of CDKs is suppressed by CDK interacting proteins, among which p21 is to be found. This protein can interact with cyclin D1/CDK4, cyclin D2/CDK4, cyclin A/CDK2 and cyclin E/CDK2 complexes (Harper et al., 1993). CDK2 is important for retinoblastoma protein (pRb) hyperphosphorylation, a process important for progression from G1 to S phase (Mittnacht & Weinberg, 1991; Akiyama et al., 1992). As p21 binds to cyclin E/CDK2 complex, it can suppress its phosphorylation function and therefore prevent cell cycle progression from G1 phase. Upon DNA damage, p53 gets stabilized and, consequently, p21 is expressed. Hence, p53 (via p21 activation) plays a main role in ensuring that damaged DNA is not replicated, and cell does not proliferate under suboptimal conditions (Dimri et al., 1996). Moreover, p53 can also regulate cell division through sustaining G2 checkpoint. It has been shown that p21 inhibits activation phosphorylation of CDK1 and thus, blocks mitosis activation. However, the initial arrest in G2 phase is guaranteed by Chk1, which blocks cell division cycle 25 phosphatase (CDC25), therefore maintaining inhibitory phosphorylation of CDK1 (Smits et al., 2000).

Besides binding CDKs, p21 can also associate with proliferating cell nuclear antigen (PCNA) protein (Waga et al., 1994). PCNA is essential for DNA replication since it functions as an accessory protein for DNA polymerase δ and DNA polymerase ε. Additionally, the protein is also essential for DNA repair as well as DNA recombination (Kelman, 1997). During S phase, p21 is capable of suppressing elongation step of DNA replication by interacting with PCNA (Waga et al., 1994). By regulating DNA replication, p53 ensures that the risk of gene mutation is decreased, and oncogenes ought not to get activated (H. Wang et al., 2023).

Another transcriptional target of p53, 14-3-3σ, can also inhibit CDK1, and hence, contribute to G2 phase arrest. In fact, this protein anchors the CDK in the cytoplasm, where it loses its ability to promote mitosis. Moreover, growth arrest and DNA damage inducible protein (GADD45), also transcriptionally activated by p53, separates complex cyclin B1/CDK1 leading to the same result of blocking G2/M transition (Taylor & Stark, 2001).

1.4.1.2 Role of p53 in senescence

Cellular senescence is described as a process of irreversible loss of proliferative potential after cell cycle arrest. In this state, cells are no longer sensitive to proliferation or growth factors. Senescent cells differ from normal cells in their biochemical, morphological, and functional characteristics. In fact, they resemble aged cells that have stopped dividing (Hayflick & Moorhead, 1961; Bernadotte et al., 2016). Surprisingly, senescent cells keep high metabolic activity, therefore they tend to become enlarged. This fact suggests that cellular proliferation and growth of these cells are two disconnected processes (Salama et al., 2014). Moreover, senescence can be induced by a number of stimuli, that are both internal and external. Internal factors might include DNA damage or high levels of oxidative stress. External factors are sensed by receptors and further affect the cellular pathways (Passos et al., 2007; Kim et al., 2013; Salama et al., 2014).

Cells enter either senescence or apoptosis depending on the duration and the severity of a stress stimulus. Supposing the stress factor is more severe or longer, the cells undergo apoptosis (Spallarossa et al., 2009). Although not entirely, p53 regulates cell fate by driving it into senescence or apoptosis depending on p53 dynamics, as described before (Q. M. Chen et al., 2000).

1.4.1.3 Role of p53 in apoptosis

Other p53 function of great importance is the induction of apoptosis in cells under stress conditions. This type of cell death could be induced by two major pathways, as for mammalian cells (Strasser et al., 2000). The first pathway is called intrinsic or mitochondrial. It is activated by stress conditions and regulated by BCL2 (D. C. S. Huang & Strasser, 2000; Igney & Krammer, 2002). The second one, extrinsic pathway, is mediated via activation of tumor necrosis factor receptors (TNFRs) (S. Wang & El-Deiry, 2003).

It has been shown that protein p53 can activate promoter of *Noxa* gene (also known as phorbol-12-myristate-13-acetate-induced protein 1, *PMAIP1*), therefore promoting its further expression. Noxa is a BCL2 homology domain 3 (BH3)-only protein with ability to associate with anti-apoptotic mitochondrial proteins BCL-2 and BCL-extra large (BCL-xL) via its BH3 motif and inhibit them, thereby activating pro-apoptotic protein BAX. Afterwards, the protein is responsible for the release of cytochrome c from mitochondria to the cytosol, where this

molecule triggers caspase activation, and hence, apoptosis (Gross et al., 1999; Oda et al., 2000). Another p53-dependent proapoptotic gene is p53 upregulated modulator of apoptosis (*PUMA*). Again, this gene encodes a protein which contains a BH3 domain (BH3-only protein), therefore it also binds and functionally represses BCL-2 and BCL-xL, and eventually, leads to the equal result of cell death (J. Yu et al., 2001).

Furthermore, transcription-independent function of p53 might result in apoptosis as well. Cytosolic p53 can act in a similar way as a BH3-only protein. Following DNA damage, p53 directly activates BAX, and thus, initiates the intrinsic apoptosis pathway (Chipuk et al., 2004). However, it has also been shown that p53 binds promoter of *BAX* gene and further promotes its transcription. Surprisingly, the expression levels of BAX vary due to its tissue localization. Hence, the contribution of p53 to BAX basal expression is probably influenced by tissue-specific factors (Miyashita et al., 1994; Miyashita & Reed, 1995).

Besides regulating multiple components of the intrinsic apoptosis pathway, p53 can also affect the extrinsic pathway. Upon p53 upregulation, the protein induces expression of *FAS* gene encoding death-receptor cluster of differentiation 95 (CD95), which belongs to the TNF receptor superfamily (Müller et al., 1998; Magnusson & Vaux, 1999). After being triggered by the Fas ligand, the receptor mediates establishment of the death-inducing complex leading to the activation of cell-death effector protease caspase-8. Eventually, caspase-8 passes the apoptotic signal through downstream activation of caspase-3, accelerating cell death (Magnusson & Vaux, 1999).

Apart from regulating components of the apoptotic machinery, p53 also activates expression of some genes that are not directly involved in the apoptotic pathways, but still control the cell entry to apoptosis in a certain way. After being activated, p53 further induces transcription of genes encoding several microRNAs (miRNAs), including the family of miRNAs-34 (miR-34a, miR-34b and miR-34c). The members of this family can prevent cells from proliferation by inducing cell cycle arrest (He et al., 2007). Moreover, miR-34a has been proven to have a tumor suppressor function via activating caspase 3/7 apoptotic pathway (Welch et al., 2007). The upstream mechanism of this regulation might be the ability of miR-34 to decrease both the transcript and the protein levels of antiapoptotic factor BCL-2 (Bommer et al., 2007).

Altogether, p53 prevents the development of tumors by controlling multiple steps of apoptotic pathway. It can either function as a transcription factor of plenty of targets contributing to apoptosis or interact directly with apoptosis-related proteins.

1.4.1.4 Role of p53 in DNA repair

Apart from binding sequence-specific consensus transcription targets, p53 also binds non-sequence-specific DNA regions (Y. Liu & Kulesz-Martin, 2001). It has been shown that the C-terminus of p53 binds DNA damaged by enzymes or ionizing radiation with increased affinity. Therefore, p53 was thought to play a greater role in DNA damage pathways than just being a transcription factor of key genes involved in cell cycle arrest and repair processes. The protein ought to sense DNA damage and further promote the repair of DNA lesions (Reed et al., 1995). During the DNA-damage response, both transcription-dependent and transcription-independent functions of p53 are required for nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR). On the other hand, it seems that only transcription-independent processes guided by p53 are necessary for non-homologous end-joining (NHEJ) and homologous recombination (HR) (Sengupta & Harris, 2005).

Nucleotide excision repair is used to fix DNA damage caused by UV radiation (Ferguson & Oh, 2005). It has been proved that p53 is important for proper nucleotide excision and that this process is not controlled entirely by its transactivation activity. Indeed, p53 helps recruit transcription factor II H (TFIIH) helicase subunits, xeroderma pigmentosum type B and D (XPB and XPD), to the site of DNA damage (X. W. Wang et al., 1995; Chang et al., 2008). Additionally, suggesting p53 functions as a transcription factor, it induces expression of XPC and p48, which are both important for NER (Hwang et al., 1999; Adimoolam & Ford, 2002).

Base excision repair preserves cells from the damage initiated by spontaneous endogenous events or some exogenous agents (Seeberg et al., 1995). BER also consists of two distinct pathways. The first one, the short-patch pathway, leads to a single nucleotide repair. During the long-patch pathway, the repair aims for a few nucleotides, usually about seven (Frosina et al., 1996). Throughout BER, p53 can directly interact with DNA polymerase β and stabilize its association with abasic DNA. Surprisingly, the N-terminal part of the protein is necessary for the interaction, even though this process does not include transactivation function (Zhou et al., 2001). In fact, the transactivation function of p53 in BER remains a bit controversial.

Following γ-irradiation, cells tend to elevate the levels of 3-methyladenine DNA glycosylase (3-meAde DNA glycosylase), which is needed for the initial steps of BER, in a p53-dependent manner. However, under NO treatment, p53 downregulates the glycosylase activity of 3meAde DNA glycosylase by *trans* repression (Zurer et al., 2004).

Mismatch repair focuses on DNA damage occurring due to normal DNA metabolic processes. This repair system diminishes DNA biosynthetic errors, and therefore helps increase the fidelity of chromosome replication (Modrich, 1991). Mismatch repair gene MutS homolog 2 (*MSH2*) has been proved to rely on p53 for its transcriptional activation (Scherer et al., 2000). Furthermore, p53 associates with MSH2 protein at DNA damage sites, implying a possible transcription-independent function during DNA repair (Zink et al., 2002).

Non-homologous end-joining is the main pathway dealing with double-strand DNA break repair in mammalian cells. During NHEJ, two termini of the broken DNA molecule are directly ligated together without the need for extensive homology between them (Karran, 2000). Overall, the exact role of p53 in NHEJ is not yet well-established. Nevertheless, p53 can control this type of DNA repair either itself or by association with other proteins involved in NHEJ (Menon & Povirk, 2014). What is more, researchers showed that while p53 enhances error-free NHEJ by the recruitment of tumor suppressor 53 binding protein 1 (53BP1), it actually downregulates NHEJ of mismatched DNA termini (Akyüz et al., 2002; Y. H. Wang et al., 2022).

Homologous recombination is another pathway for double-strand break repair. Specifically, HR includes a process of synthetizing new DNA sequences guided by homologous template (Ray & Langer, 2002). Protein p53 directly modulates HR by interaction with HR factors RAD51 and RAD54. Eventually, through association with these proteins, p53 can in fact inhibit error-prone HR leading to genetic stability maintenance (Linke SP et al., 2003).

1.4.2 Non-canonical functions of p53

Previous studies stated that even though some p53 mutants are unable to facilitate the canonical p53 functions, they can still suppress cancer development through the non-canonical functions (Fig. 5) (Brady et al., 2011; T. Li et al., 2012). Since then, the interest in the likely vast field of these functions has increased.

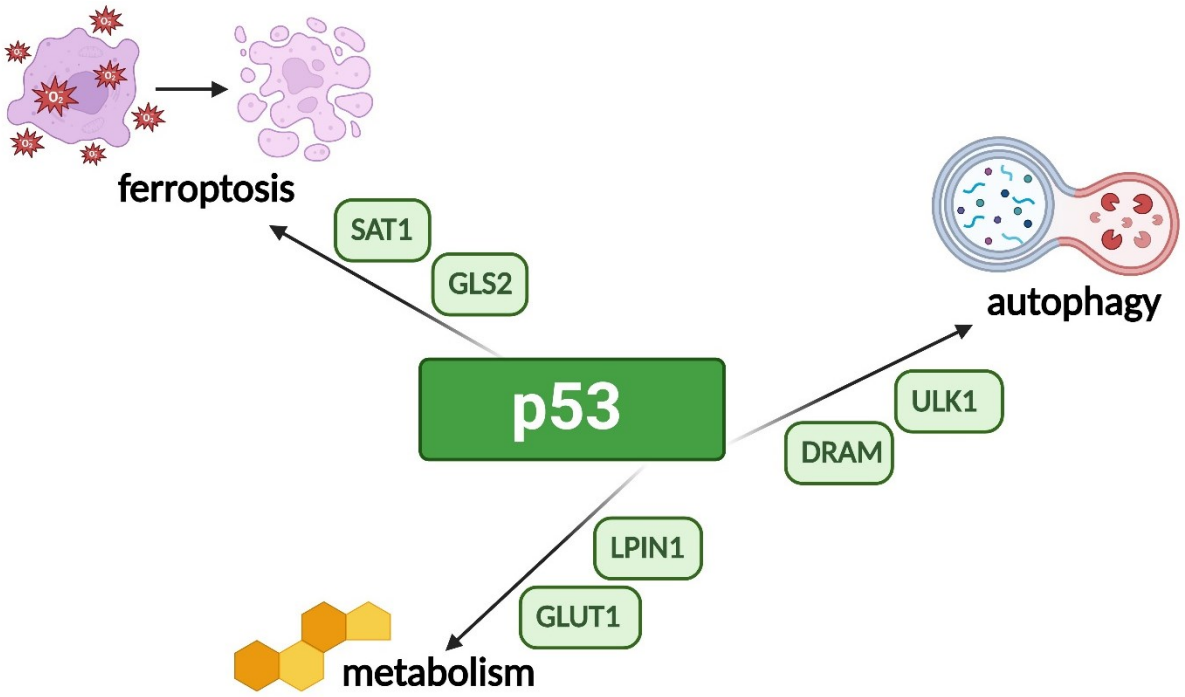


Figure 5. Selected non-canonical functions of p53. A few targets of p53 that control ferroptosis/autophagy/cellular metabolic pathways are displayed in green frames.

1.4.2.1 Role of p53 in ferroptosis

Ferroptosis is an iron-dependent type of cell death induced by increased levels of lipid reactive oxygen species (ROS) (Dixon et al., 2012). The mechanism leading to the accumulation of ROS could be an inhibition of the cystine/glutamate antiporter system x_c^- (Ogiwara et al., 2019). Following this repression, an important antioxidant glutathione (GSH) has no longer access to its substrate – cysteine (Bannai, 1986). Subsequently, GSH peroxidase 4 (GPX4) cannot oxidize GSH, thereby lipid peroxides do not get reduced and promote ferroptosis (Yang et al., 2014). This type of cell death act as an important tumor suppressing pathway. Even though some cells may be unable to commit cell cycle arrest, senescence, or apoptosis, they could still suppress cancer due in part to their ability to promote ferroptosis (L. Jiang et al., 2015).

Although the impact of p53 on ferroptosis has not been completely described yet, the protein certainly controls this cellular process. Surprisingly, it has been proved that p53 can both promote and inhibit ferroptosis, depending on the cellular context (Y. Liu & Gu, 2022). As for the positive effect of p53 on ferroptosis, it suppresses transcription of solute carrier family 7 member 11 (*SLC7A11*), which encodes a subunit of the cystine/glutamate antiporter.

Eventually, due to this effect the levels of lipid peroxides increase, and thus, ferroptosis is induced (L. Jiang et al., 2015; Lim & Donaldson, 2011).

Another ferroptosis-related gene is spermidine/spermine N1-acetyltransferase 1 (*SAT1*), whose transcription is positively regulated by p53. Overexpression of this protein may lead to increased levels of lipid peroxides, and therefore, ferroptosis. The molecular process responsible for this phenomenon is the downstream induction of arachidonate 15-lipoxygenase (ALOX15) by SAT1 (Ou et al., 2016). This enzyme has been previously implicated in generating lipid peroxides (Shintoku et al., 2017). Moreover, p53 stimulates transcription of phosphate-activated glutaminase 2 (*GLS2*) (Suzuki et al., 2010). *GLS2* encodes an enzyme involved in glutamine metabolism by hydrolysing glutamine to glutamate (Curthoys & Watford, 1995; Aledo et al., 2000; Márquez et al., 2016). Consequently, higher levels of extracellular glutamate ought to negatively affect the function of cystine/glutamate antiporter system x_c^- (Murphy et al., 1989). Besides, GLS2 protein can make cells less sensitive to apoptosis induced by ROS (Suzuki et al., 2010).

On the other hand, negative regulation of ferroptosis by p53 could be directed via transcription activation of p21. Recent study has discovered that upon p53 stabilization and consequent p21 expression, cells tend to stay more resistant to ferroptosis (Tarangelo et al., 2018). Another research has discovered the role of dipeptidyl peptidase-4 (DPP4) in ferroptosis. The enzyme interacts with nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1), thereby triggering lipid peroxidation. However, this step has shown to be successful only in *TP53*-deficient cells. Thus, p53 appears to block the DPP4-NOX1 interaction, leading to ferroptosis suppression (Xie et al., 2017).

Taken together, p53 does not always induce ferroptosis, nor does it only suppress the process. Although the impact of several p53-regulated genes affecting ferroptosis has been evaluated, the function of the protein in this type of cellular death is worth of further examination.

1.4.2.2 Role of p53 in autophagy

Autophagy is an important pathway in cellular homeostasis, it is described as degradation of cellular components by lysosome (Cuervo, 2004). This process can be triggered upon pathological stress, that may include nutrient deprivation, and bacterial or viral infection. Autophagy-dependent cell death shares several similarities with apoptosis – they both can be

activated by the same stimuli and end the same way. Nevertheless, cells usually use the autophagy pathway during development, so that they can be broken down internally. Supposing they are degraded this way, their death does not result in formation of large apoptotic bodies, that are not easy to get rid of (Kelekar, 2006).

Following DNA damage, p53 directly binds and further activates transcription of the autophagy-related gene DNA damage regulated autophagy modulator (*DRAM*). The gene encodes lysosomal protein DRAM, that has been proved to promote autophagy, although the specific mechanism behind the process remains unclear (Crighton et al., 2006). Next, chromatin immunoprecipitation sequencing and RNA sequencing revealed more p53 targets that eventually control autophagy. Among them was unc-51 like autophagy activating kinase 1 (*ULK1*) encoding a protein involved in autophagy initiation. Next discovered transcriptional target was etoposide induced 2.4 (*EI24*) (Kenzelmann Broz et al., 2013). Protein EI24 is important for controlling later steps during the autophagy pathway (Tian et al., 2010). Furthermore, interferon stimulated exonuclease gene 20kDa-like 1 (*ISG20L1*) is also transactivated by p53. Subsequent to genotoxic stress, protein ISG20L1 promotes cell death by autophagy pathway (Eby et al., 2010).

Finally, autophagy can also be managed by p53 through a transcription-independent mechanism. Indeed, cytoplasmic p53 has been shown to inhibit autophagy, so that its depletion leads cells autophagy-dependent cell death (Tasdemir et al., 2008).

1.4.2.3 Role of p53 in metabolism

Besides controlling more types of cell death, cell cycle, and DNA repair by both transcription dependent and independent pathways, p53 also affects number of aspects of cellular metabolism (Maddocks et al., 2011). Cancer cells possess a unique metabolic mark meaning that they prefer glycolysis to oxidative phosphorylation even in the presence of excess oxygen. Although this leads to production of smaller amount of adenosine trisphosphate (ATP), cancer cells utilize the intermediates of the glycolytic pathway for biosynthetic processes. These mainly include ribose synthesis for nucleotide production enabling the cells to grow and proliferate faster (Heiden et al., 2009; Liberti & Locasale, 2016).

Since p53 functions as a tumor suppressor, the protein ought to block the mechanisms cancer cells use for their high energy demands. Indeed, p53 inactivates gene transcription of glucose

transporter 1 (*GLUT1*) and glucose transporter 4 (*GLUT4*), making it harder for cancer cells to utilize the glucose supplies of the organism (Schwartzenberg-Bar-Yoseph et al., 2004). In addition, p53 also controls glycolysis by having impact on *TP53*-induced glycolysis regulatory phosphatase (*TIGAR*). This phosphatase decreases the levels of fructose-2,6-bisphosphate, which is a potent allosteric activator of glycolytic enzyme phosphofructokinase-1 (PFK-1). Thereupon, TIGAR protein serves as a negative regulator of glycolytic metabolic pathway (Bensaad et al., 2006).

In addition to glycolysis inhibition, p53 further regulates cellular metabolism by promoting the tricarboxylic acid cycle (TCA). The tumor suppressor protein downregulates the expression of pyruvate dehydrogenase kinase isoform 2 (*PDK2*), so that the protein PDK2 can no longer inactivate the pyruvate dehydrogenase complex. Hence, the complex allows conversion of pyruvate to acetyl-CoA, a substrate of the TCA (Harris et al., 2002; Contractor & Harris, 2012).

The pentose phosphate pathway (PPP) can be also controlled by p53. This metabolic pathway is essential for production of ribose 5-phosphate, important as a nucleotide precursor, and nicotinamide adenine dinucleotide phosphate (NADPH), an essential reductant for multiple cellular processes. Glucose-6-phosphate dehydrogenase (G6PD), a key protein in the PPP, was identified as a direct target of p53. Upon p53 activation, G6PD is unable to form a functional dimeric protein, thereby PPP is inhibited (P. Jiang et al., 2011).

Since cells utilize lipids as precursors for phospholipid membrane formation, cancer cells need to increase fatty acid synthesis in order to sustain high proliferation rate (Santos & Schulze, 2012). Multifunctional protein p53 has been shown to regulate lipid metabolism-associated processes, as well. Following glucose starvation, p53 promotes expression of *LPIN1* gene that encodes lipin-1, an enzyme involved in induction of fatty acid oxidation (Assaily et al., 2011). Furthermore, p53 represses lipid synthesis by downregulating the transcription of sterol regulatory element-binding protein 1 c (*SREBP-1c*) gene (Yahagi et al., 2003). SREBP-1c works as a transcription factor of genes promoting fatty acid synthesis (Foretz et al., 1999). Finally, p53 suppresses the promoter activity of genes specific for the mevalonate pathway, and hence, inhibits cholesterol synthesis (Moon et al., 2019).

1.5 Mutations of p53

In human cancer, *TP53* has been identified as the most frequently mutated gene (X. Chen et al., 2022). Germline mutations of the gene cause Li-Fraumeni syndrome (LFS) characterized by increased predisposition to cancer development. LFS patients usually develop tumors earlier in life, typically under the age of 45. Next, people with this genetic disorder ought to have a first-degree relative diagnosed with cancer before 45. Additionally, LFS carriers are also described by the fact that they have another first-degree or second-degree relative with developed any tumor before turning 45 or sarcoma anytime. Overall, most of the patients suffer from sarcomas, breast cancer and adrenocortical tumors. As assumed, older patients are more prone to malignancies formed in the digestive tract and the lung (F. P. Li et al., 1988; Olivier et al., 2003). Furthermore, providing that germline *TP53* mutation carriers do not obtain the features of LFS completely, they are defined as patients with LFS-like syndrome (LFL) (Birch et al., 1994). Even-though the germline mutations of *TP53* were previously considered as extremely rare, a study from 2009 found out that in fact, they might occur in 1 from 20,000 cases (Gonzalez et al., 2009). On the contrary, somatic *TP53* mutations are spontaneous and frequent in many types of human malignancies (Hollstein et al., 1991).

Most of *TP53* mutations are missense and occur in the DNA-binding domain, suggesting that this part of p53 is of great importance. The high number of mutations does not always lead to the loss-of-function phenotype of mutant *TP53* carriers, but instead, they can lead to multiple different phenotypic marks. Therefore, more specific classification of *TP53* mutations ought to be made in the future (Kastenhuber & Lowe, 2017). In general, there are eight types of *TP53* mutations – missense, nonsense, silent, frame-shift deletion, in-frame deletion, frame-shift insertion, in-frame insertion and splice-site (X. Wang & Sun, 2017).

Given the numerous mutations of this gene, they can lead to various outcomes. Structural mutations affect the DNA-binding activity of the protein by generating gaps and internal cavities in the p53 DNA-binding surface (Olivier et al., 2010). Mutational hotspots altering the structure were identified as R175H, G245S, R249S and R282W. Next, mutations within the same p53 domain, which directly influence DNA binding, are R248Q, R248W, R273C and R273H (Bullock et al., 2000). Moreover, since p53 transactivates a great number of downstream target genes, its mutations likely modify this activity. A comprehensive study from

2003 analysed the impact of *TP53* mutants on eight well-known p53-dependent genes, including *CDKN1A*, *MDM2*, *BAX*, *14-3-3σ*, apoptosis signal-regulating kinase 1 (ASK1)-interacting protein (*AIP1*), *GADD45*, *Noxa*, and ribonucleoside-diphosphate reductase subunit M2 B (*RRM2B*). The results revealed that about two thirds of the mutant variants kept the transactivation function at the similar level compared to the wild-type p53. The remaining third was divided into two subtypes, where the first one contained mutants with some residual activity, while the second subtype mutants exerted no activity at all (Kato et al., 2003). As assumed for the transactivation function of p53, plenty of the loss-of-function variants were localized in the DNA-binding domain. Conversely, variants outside the DBD were often classified as benign or with only reduced activity. However, some variants within the tetramerisation domain also proved to significantly alter the function (Kato et al., 2003).

Following DNA damage and the subsequent p53 stabilization, mutant p53 has the ability to inhibit the wild-type p53 activity within the tetramer, leading to a process called the dominant-negative effect (DNE) (Blagosklonny, 2000). Indeed, some mutants such as R175H or R248Q retain the capacity to bind the wild-type through their tetramerisation domain (Billant et al., 2016). Eventually, the mixed tetramers become less or completely inactive in terms of the transcription activation. Nevertheless, in some cases, the mutant p53 does not repress the wild-type p53 function entirely. The exact outcome of this regulation by mutant p53 probably depends on the posttranslational modifications of the protein (Gencel-Augusto & Lozano, 2020).

Additionally, mutations in the *TP53* gene may lead to gain-of-function (GOF) phenotype. The GOF p53 gains novel functions that are not present in cells with the wild-type protein. Furthermore, these functions may in fact increase cellular growth (Dittmer et al., 1993). For example, mutation R280K was proved to be less sensitive to apoptosis induction by curcumin (Bae et al., 2014). Moreover, in a recent study, the authors revealed that some GOF mutations of p53 may also result in increased resistance to anoikis, increased cell invasion and migration, as well as higher probability of growth factor-independent survival (Pal et al., 2023).

A large-scale study aimed at *TP53* mutations in 33 cancer types revealed that the mutation rate of the gene is higher than 50% in almost one third of them (X. Wang & Sun, 2017). On top of that, in more than 17 of the examined cancer types, the mutation rate is over 30%. Cancer types with the highest score of *TP53* mutation rate are as follows: uterine carcino-sarcoma and

ovarian serous cystadeno-carcinoma. Besides, significantly increased rate is also associated with lung cancer, gastro-intestinal cancers, head-and-neck squamous cell carcinoma and brain lower-grade glioma. On the contrary, mutations of *TP53* are not very common in kidney renal papillary-cell and clear-cell carcinoma, testicular germ-cell tumors, uveal melanoma, thyroid carcinoma, pheochromocytoma, paraganglioma, and thymoma (X. Wang & Sun, 2017).

Several dozens of polymorphisms in the *TP53* gene have been identified. Some of them have been already widely examined, but the impact of many of them on their carriers remains unknown. Therefore, it might be important to distinguish between different polymorphisms in order to determine, whether the subjects are likely to develop cancer or not. Additionally, supposing it would be enabled to have knowledge about the influence of each polymorphism on p53 function, treatment of the patients could become individualized, and thus more efficient (Hrstka et al., 2009).

Apart from conclusively pathogenic and benign mutations in the *TP53* gene, a large number of variants of uncertain significance (VUS) have also been identified. The effect of these variants discovered by genetic screenings of patients is not definite. Indeed, it remains unknown whether these mutations contribute to disease progression or not (Joynt et al., 2021). Since this fact complicates preventive care of the affected patients, more data about variants of uncertain significance should be collected. In 2019, a study examining 1844 patients for germline *TP53* mutations indicated that only twelve of them were identified as VUS carriers. Despite this small number, the authors highlighted that some of the VUS had been previously classified differently according to distinct classification systems. This matter of fact could result in a problem of divergent preventive care options of the mutation carriers (Bittar et al., 2019).

2 Aims

A significant portion of cancers is caused by inherited mutations in predisposition genes. In the framework of this project, we aimed to evaluate the functional significance of specific *TP53* gene variants found in cancer patients within the Czech population, together with analysis of data set including well-known loss-of-function variants, benign variants and VUS. The research involved creating cell models using CRISPR/Cas9 to inactivate the gene, followed by functional analyses to confirm defects in signaling pathways. Expression plasmids with fluorescent markers and selection cassettes were used to compare the functions of mutated and natural gene forms. The study focused on quantification of various cellular parameters and their statistical analysis to enhance the understanding of these genes at the cellular level. Insights gained are also intended for assessing cancer risks in mutation carriers.

The aims of this thesis were:

1. Generation of *TP53* knockout cells
2. Construction of plasmids containing selected *TP53* variants
3. Development of stable cell lines
4. Functional analysis of p21 and MDM2 expression among the cell lines
5. Tetramerisation assay of selected variants
6. Colony formation assay of selected variants

3 Materials and Methods

3.1 Materials

3.1.1 Primary antibodies

Table 1. List of used primary antibodies, their host and type (rb = rabbit; m = mouse; poly = polyclonal; mono = monoclonal), manufacturers, catalogue numbers, and dilutions for either immunofluorescence (IF) or western blotting (WB) experiments.

Molecular target	Host/type	Manufacturer	Catalogue #	Application	Dilution
p53	rb/poly	Santa Cruz	sc-6243	IF	1:100
p53	m/mono	Santa Cruz	sc-126	WB	1:750
p53	rb/poly	Cell Signaling	#9282S	WB (tetramerisation assay)	1:1000
p21	m/mono	Santa Cruz	sc-6246	IF	1:100
MDM2	m/mono	Calbiochem	OP46	IF	1:100

3.1.2 Secondary antibodies

Table 2. List of secondary antibodies, their manufacturers, catalogue numbers, and dilutions for either immunofluorescence or western blotting experiments.

Antigen	Manufacturer	Catalogue #	Application	Dilution
Alexa Fluor 488 GOAT anti rabbit	Life Technologies	A11034	IF	1:500
Alexa Fluor 647 GOAT anti mouse	Life Technologies	A21236	IF	1:500
Goat anti-Rabbit IgG (H+L)	Life Technologies	B2770	WB	1:5000
Goat anti-Mouse IgG (H+L)	Life Technologies	B2763	WB	1:5000

3.1.3 Buffers

Table 3. List of used solutions and their chemical composition.

Buffer	Chemical composition
2x Sample buffer	100 mM HEPES pH 7.5, 500 mM NaCl, 2% glycerol, 0.5% NP40
4x SDS loading buffer	250 mM Tris pH 6.8, 400 mM DTT, 40% glycerol, 8% SDS, few drops of bromphenol blue
Buffer 1	1.5 M Tris pH 8.8
Buffer 2	0.5 M Tris pH 6.8
Running buffer	2.5 mM Tris pH 8.3, 19.2 mM glycine, 0.01% SDS
Blotting buffer	2.5 mM Tris pH 8.3, 19.2 mM glycine, 20% methanol, 0.01% SDS
PBS	138 mM NaCl, 8 mM Na2HPO4, 2.7 mM KCl, 1.47 mM KH2PO4
PBS-T	0.1% Tween 20 in PBS
1x TAE buffer	1 mM EDTA, 40 mM Tris, 20 mM glacial acetic acid, pH 8.0
E1 buffer	50 mM Tris pH 8.0, 10 mM EDTA, 100 µg/mL RNase A
E2 buffer	200 mM NaOH, 1% (w/v) SDS
E3 buffer	3.1 M KAc pH 5.5
Lysis buffer	50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM imidazole, 1 mM TCEP, 1 µM ZnCl2
5x Isothermal buffer	25% PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM MgCl2, 50mM DTT, 1 mM each of the 4 dNTPs, 5 mM NAD
Tetramerisation lysis buffer	10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitors

3.1.4 Primers

Table 4. List of primers used for mutagenesis of selected sites and primer used for molecular cloning.

Target	Orientation	Sequence
E339G mutagenesis	forward	TGGGCGTGAGCGCTTCGGATGTTCCGAGAGCTGAATG
	reverse	CATTCAGCTCTCGGAACATCCGAAGCGCTCACGCCCA
E339del3 mutagenesis	forward	GCGTGAGCGCTTCGGAGAGCTGAATGAGGCCTT
	reverse	CTCATTCAGCTCTCCGAAGCGCTCACGCCCA
G262S mutagenesis	forward	CTGGAAGACTCCAGTAGTAATCTACTGGGA
	reverse	TCCCAGTAGATTACTACTGGAGTCTTCCAG
H168P mutagenesis	forward	CAAGCAGTCACAGCCCATGACGGAGGTTG
	reverse	CAACCTCCGTCATGGGCTGTGACTGCTTG
R158C mutagenesis	forward	CACCCGCGTCTGCGCCATGGCCATCTACA
	reverse	TGTAGATGGCCATGGCGCAGACGCGGGTG
R282L mutagenesis	forward	TGGGAGAGACCTGCGCACAGAGGAAGAGAA
	reverse	TTCTCTTCCTCTGTGCGCAGGTCTCTCCCA
V197E mutagenesis	forward	GCATCTTATCCGAGAGGAAGGAAATTTG
	reverse	CAAATTTCCTTCCTCTCGGATAAGATGC
Cloning of the variants into the vector	forward	GAAAACCCCGGTCCTAGGCTGCAGACGCGTGGTGGAATGGAGGAGCCGCAGTCAGAT
	reverse	TGTCTTCCCGGACTGAGGTCTGACTCCTAGGCCCCAACCCCAACGCGGAAAAGGTT

3.1.5 sgRNAs

Table 5. List of sgRNAs targeting *TP53* and their sequences.

	Sequence
sgRNA1	CAUUGCUUGGGACGGCAAGG
sgRNA2	AUCCAUUGCUUGGGACGGCA

3.2 Methods

3.2.1 Cell culture

The experiments were completed working with human nontransformed cell line RPE1-hTERT (hereafter referred to as RPE). The cells were cultured in high glucose DMEM (Sigma-Aldrich) supplemented with 6% fetal bovine serum (Sigma-Aldrich), 0.1mg/mL streptomycin (Sigma-

Aldrich) and 100 U/mL penicillin (Sigma-Aldrich). Cells were kept in incubator with 37°C and 5% CO_2 and regularly tested negative for mycoplasma contamination.

3.2.2 Generation of knockout cell line

One day prior transfection, RPE cells were seeded into 6-well-plate with approximate density 300,000 cells per well. First tube of CRISPR-Cas9 reagents included 125 µL Opti-MEM, 6 µL sgRNA (Synthego, 30 µM), 2 µL TrueCut Cas9 Protein (Thermo Scientific) and 5 µL PLUS Reagent (Thermo Scientific). The second tube consisted of 125 µL Opti-MEM and 7.5 µL Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Thermo Scientific). The tubes were mixed together and poured onto the cells. The mixture was left with the cells for 24 hours before it was replaced for fresh media. After 48 hours, cells were subjected to Nutlin-3a (Nut-3a, 9 µM) (MedChemExpress) treatment a few times within 18 days to enable selection of cells with inactivated *TP53*. Afterwards, the cells were plated in a single-cell manner into 96-well-plates by BD Influx (BD Biosciences), individual cell clones were expanded and tested for p53 expression by western blotting and immunofluorescence microscopy. Additionally, gDNA was isolated from the clones and was analysed by Sanger sequencing (SEQme).

3.2.3 Selection of tested variants and cloning

Our list of *TP53* mutations contained a set of well-known LOF variants, benign variants and VUS. Variants from this set were already purchased as constructed pCW57-RFP-P2A-TP53 vectors by Synbio Technologies, therefore no further cloning was needed. The variants of Czech patients were obtained from the Institute of Medical Biochemistry and Laboratory Diagnostics of the General University Hospital and the First Faculty of Medicine of Charles University. Plasmid pCW57-RFP-P2A-TP53 had been previously generated using pCW57-RFP-P2A-MCS as a backbone and pIRES-EGFP-TP53-WT (Addgene) as a template. The mutated *TP53* sequences were created by PCR amplification of the human *TP53* from pCW57-RFP-P2A-TP53 vector with designed overlapping primers with opposite orientation. The backbone for cloning of the *TP53* variants was made by opening pCW57-RFP-P2A vector with BamHI and MluI (Thermo Scientific). After that, the PCR products and the backbone were run on agarose gel and the DNA was isolated from the gel. Eventually, the mutated *TP53* sequences were cloned into the vector by Gibson assembly.

3.2.4 Restriction enzyme digestion

The reagents for restriction were 10 U BamHI, 10 U MluI, 6 µg plasmid DNA, 1x BamHI-Lsp1109I Buffer (Thermo Scientific) and 19 µL distilled water. After putting the components together, the solution was kept in 37°C overnight.

3.2.5 Polymerase chain reaction

The PCR reaction mixture consisted of 50 ng DNA template, 400 µM reverse primer, 400 µM forward primer, 1x Phusion HF Buffer (Thermo Scientific), 400 µM Deoxynucleotide Mix (Sigma-Aldrich), 2 U Phusion High-Fidelity DNA Polymerase (Thermo Scientific), and distilled water contributing to the total volume of 50 µL. All the reagents were mixed on ice. The reaction was composed of a 3-min initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds. When the cycles were done, the reaction finished by 5-min incubation at 72°C and eventual cooling down to 12°C.

3.2.6 DNA electrophoresis

Electrophoresis gels were prepared by mixing 1% Agarose (Sigma-Aldrich) with 1x Tris/Acetic acid/EDTA (TAE) buffer. Ethidium Bromide (BioChemica) was added to the solution in 0.5 µg/mL concentration for electrophoresis product visualization. Before loading on the gel, the DNA samples were mixed with 6x DNA Gel Loading Dye (Thermo Scientific). EnduroTM Horizontal Gel Boxes (Labnet International) were used to run DNA electrophoresis, together with PS 304 Minipec II (Apelex). The power supply was set on 100 V and the separation lasted 45 min. After that, DNA fragments of expected size were isolated from the gel with Agarose Gel Extraction Kit (Jena Bioscience). Total amount of DNA diluted in 20 µL of distilled water was measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific).

3.2.7 Gibson assembly

The Gibson reaction was composed of 165 ng linearized vector backbone and equimolar amount of PCR fragments, 15 µL 5x isothermal buffer, 0.033 U Phusion High-Fidelity DNA Polymerase (Thermo Scientific), 5.3 U Taq DNA Ligase (New England Biolabs), 0.0053 U T5 Exonuclease (New England Biolabs), and distilled water added to the total volume of 20 µL.

Then the mixture was incubated at 51°C for 25 min and eventually, 7 µL of the volume was used for bacteria transformation.

3.2.8 Bacteria transformation

Escherichia coli strain DH5α was incubated on ice with plasmid DNA for 15 min. After that, the competent cells were put into heat-shock at 42°C for 75 seconds. Following incubation on ice for 5 min, the mixture with cells was transferred to 1 µL LB media (25 g/L LB Broth (Miller), Sigma-Aldrich) and left at 37°C for an hour. The bacterial suspension from the tube with mixture was loaded onto LB Agar Ampicillin-100 Plates (Sigma-Aldrich) and incubated overnight at 37°C. Individual colonies were picked the following day and moved to new agar plates. After incubation at 37°C for 20 hours, the bacteria from the colonies were moved to tubes with 3 mL LB media and left at 37°C overnight to enable plasmid isolation the following day.

3.2.9 Plasmid isolation

The plasmids were isolated from overnight culture of transformed bacteria in LB media. First, the bacterial suspension was pelleted with a centrifugation speed of 2000 g for 5 min. After removing the supernatant, the bacterial pellet was resuspended in 200 µL E1 Buffer. Then, 200 µL E2 Lysis Buffer was added and the solution was mixed well. Subsequently, 350 µL E3 Neutralization Buffer was mixed into the solution. The mixture was incubated at room temperature for 3 min. After that, centrifugation speed of 2000 g for 10 min was used for sedimentation. The supernatant was loaded into new tubes, together with 500 µL isopropanol for plasmid DNA precipitation. The solution was mixed well and left at room temperature for 1 min. After centrifugation at 10,000 g and 4°C for 10 min, pelleted DNA was washed by cold 1000 µL 70% ethanol. The DNA was again spined down in centrifuge at 10,000 g and 4°C for 10 min. Air-dried DNA pellet was dissolved in 50 µL of distilled water. To make sure the cloning process was successful, the plasmid DNA was sequenced by SEQme.

As higher quality and purity of DNA was needed for cloning and transfection, the plasmids were also isolated by Plasmid Mini Kit (QIAGEN). After following the manufacturer´s protocol, the DNA was eluted in 100 µL distilled water and its concentration was measured using Nanodrop 2000.

3.2.10 Plasmid transfection

The cells were seeded into a 6-well-plate with density reaching about 300,000 cells per well. The next day, the cells were transfected with plasmid DNA using polyethylenimine PEI MAX® (MW 40,000) (Polyscience) as a transfection reagent. The exact amount of transfected DNA was 2 µg, which was diluted in 150 µL Opti-MEM I Reduced Serum Medium (Gibco), and then mixed with 12 µg PEI diluted in 150 µL Opti-MEM, as well. After that, the solution was added to the cells and the transfection process lasted approximately 4.5 hours. Next, the solution was changed for fresh media.

3.2.11 Generation of stable cell lines

The cells were transfected with the pCW57-RFP-P2A-TP53 plasmid. Two days after, G-418 Solution (Roche) was added to the media (7.5 mg G-418 per 10 mL media) to select the successfully transfected cells. Overall, the antibiotics was used three more times within three weeks of continuing selection. Cells positive for RFP after overnight doxycycline (Dox, 2 µg/mL) induction were selected by BD Influx into 6-well-plates as polyclonal cell lines.

3.2.12 SDS-PAGE

The cells were seeded into 6-well-plates to cover the area of the wells confluently within a day (approximate density was 500,000 cells per well). After that, the cells were washed with PBS, lysed with 2x sample buffer and the samples were incubated at 95°C for 5 min. Subsequently, the solutions were sonicated for 20 seconds at 25% amplitude with Q125 Sonicator (Qsonica). To load the samples onto gel in equal concentration, their concentration was measured by Pierce BCA Protein Assay Kit EnVision Multilabel Plate Reader. For this type of electrophoresis, resolving 4% to 20% gradient gel with 1.5 mm depth was prepared and left to solidify for 20 min. It was composed of distilled water, buffer 1, Acrylamide/BIS Solution 29:1 (30% w/v) 33% C (Serva), 10% Ammonium Persulfate (MP Biomedicals) and TEMED electrophoresis grade (MP Biomedicals). The exact amount of these chemicals is listed in the table below. The stacking gel was prepared from the same components, but instead of buffer 1, buffer 2 was used. After pouring the stacking gel onto the resolving one, it was let to harden well for 20 min. Before loading onto the gel, the samples were diluted with 4x SDS loading buffer. As a molecular weight marker, PageRuler Prestained Protein Ladder (Thermo Scientific) was used. The gels

were put in Mini-PROTEAN Tetra Cell (Bio-Rad) polyacrylamide gel electrophoresis system, together with running buffer. PowerPac Basic Power Supply (Bio-Rad) was connected to the system and switched on for one hour using current 50 mA per single gel.

Table 6. Chemical composition of gel used for SDS-PAGE.

Component	Stacking gel	Resolving gel 4%	Resolving gel 20%
Buffer 1/buffer 2 (mL)	0.63	1.25	1.25
Acrylamide/BIS (mL)	0.33	0.67	3.33
10% APS (µL)	24	30	30
TEMED (µL)	6	8	8
H_2O (mL)	1.54	3.08	0.42

3.2.13 Western blotting

After polyacrylamide gel electrophoresis, the gel was put into a blotting cassette, together with Amersham Protran 0.45 NC nitrocellulose Western Blotting membrane (Cytiva). These two components were covered with a sheet of filter paper and a foam from both sides. Next, the cassette was placed into Criterion Blotter with blotting buffer. The set-up was connected to PowerPac Basic Power Supply (Bio-Rad) and turned on for one hour and constant voltage of 100 V. Eventually, the membrane was dyed with Ponceau S (Serva) for 5 min. After that, it was incubated with 3% non-fat dry milk in PBS-T for 15 min on a shaker. Finally, incubation with primary antibodies diluted in 3% non-fat dry milk in PBS-T took place overnight at 4°C on a shaker. The next day, the membrane was washed three times in PBS-T. Following the washing, it was incubated with secondary antibody Goat anti-Mouse IgG (H+L)/Goat anti-Rabbit IgG (H+L) diluted in 3% non-fat dry milk in PBS-T for one hour at room temperature. After another triple membrane washing with PBS-T, reagents from Pierce ECL Western Blotting Substrate (Thermo Scientific) were mixed, poured on the membrane, and left there for 3 min. The membrane was carefully removed from the ECL reagents and put between layers of thin plastic foil. Last, the membrane was exposed on X-ray film (AGFA) in dark room and this film was further developed using OPTIMAX 2010 (PROTEC).

3.2.14 Immunofluorescence staining

The cells were seeded into 6-well-plates with 10 mm coverslips, so that they confluently covered the glass area (approximate density was 500,000 cells per well). After overnight

treatment with Nut-3a (9 µM) and Dox (2 µg/mL), the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS (VWR) for 15 min. Subsequently, the cells were washed with PBS three times and permeabilized with 0.2% Triton X-100 (Amresco) in PBS for 5 min. After another triple PBS washing, the blocking solution (1% BSA (Sigma), 0.3M glycine in PBS) was loaded onto the coverslips and left there for 30 min. Then, the cells were stained by primary antibodies diluted in blocking solution for two hours at room temperature. Staining with secondary antibodies diluted in blocking solution took place after triple washing with PBS. During this hour-long process, the coverslips were kept in dark at room temperature. Eventually, the coverslips were again washed in PBS three times and stained with 1 µg/mL DAPI (Sigma-Aldrich) for 3 min. The last washing was done with distilled water. Next, the coverslips were left to dry and further put into 2 µL drop of VECTASHIELD Antifade Mounting Medium (Vector Laboratories) onto microscope slide.

3.2.15 High-content microscopy

Automated microscope Olympus scanR was used for high-throughput screening of the stained cells. The UPLXAPO 40X, dry objective was used for cell imaging. After scanR acquisition, the scanR analysis software was utilized for results evaluation. The cell nuclei were determined by their DAPI signal. The examined cells were gated as p53-positive or p53-negative according to their maximal nuclear and mean nuclear p53 signal. The cells with outlier values (too low or too high p53 signal) were kept outside the p53-positive analysed gate to only work with cells with comparable p53 expression levels to control RPE parental cell line. For each experiment and variant, 300 cells within the gate were examined for their mean nuclear intensity of p21 or MDM2.

3.2.16 Widefield microscopy

The cell images were obtained using fluorescent motorized microscope Leica DM6000. HC PL APO 20x/0,75 CS2 was used as an objective.

3.2.17 Statistical analysis

The results were statistically analysed and graphically validated using GraphPad Prism 5.0. The specific tests utilized for the results evaluation are listed below the figures. Statistical

significance of obtained results was determined by P values (P). The figures contain either * representing P ≤ 0.05; ** representing P ≤ 0.01; or *** representing P ≤ 0.001.

3.2.18 Tetramerisation assay

The cells were seeded into 6-well-plates and treated with Dox (2 µg/mL) and Nut-3a (9 µM). The following day, the cells were lysed in 40 µL tetramerisation lysis buffer by incubating on ice for 30 min. Then, the extracts were centrifuged at 13,000 rpm for 15 min to remove cell debris. Afterwards, glutaraldehyde was added to the final concentration of 0.05 and the samples were incubated on ice for 15 min. Finally, the samples were diluted with 4x SDS loading buffer and analysed performing SDS-PAGE with 10% resolving gel and western blotting with polyclonal p53 antibody.

Table 7. Chemical composition of gel used for SDS-PAGE in tetramerisation assay.

Component	Stacking gel	Resolving gel 10%
Buffer 1/buffer 2 (mL)	0.63	2.5
Acrylamide/BIS (mL)	0.33	3.33
10% APS (µL)	24	70
TEMED (µL)	6	16
H_2O (mL)	1.54	4.17

3.2.19 Colony formation assay

To enable analysis of p53 positive cells only, 1000 RFP positive cells were sorted into 6-well-plates by BD Influx and treated with Dox (2 µg/mL) and Nut-3a (9 µM). After 11 days, cell colonies were washed with PBS and subsequently fixed and stained with 1% crystal violet (Sigma-Aldrich) in 20% ethanol for 30 min. The colonies were counted manually using ImageJ.

4 Results

4.1 Generation of *TP53* knockout cells

To address the selected aims of this thesis, we chose retinal pigment epithelium (RPE) cell line as a model for the experiments. These human diploid cells were ideal for our study because they are non-transformed, easy to manipulate, and possess a stable genome. We transfected RPE cells with CRISPR-Cas9 reagents containing two different sgRNA sequences (sgRNA1 and sgRNA2). To select cells with edited *TP53* gene, we treated the cells with Nut-3a, which disrupts p53-MDM2 interaction and stabilizes p53, so that only cells without p53 could proliferate. The polyclonal cell lines were tested for p53 expression by western blotting and immunofluorescence microscopy, which showed expected decrease in p53 levels. Subsequently, we seeded single cells into 96-well-plates and expanded individual clones.

When the clones made confluent cell populations, we treated them with Nut-3a to stabilize p53 expression. The following day, we made lysates from the cells and further used them for western blotting analysis with p53 antibody to confirm there was no p53 signal (Fig. 6A). Besides, we also fixed the cells on cover slips (after the same Nut-3a treatment), and subsequently stained them with p53 antibody. Next, we used immunofluorescence microscopy to distinguish if the newly generated cell lines had any p53 signal left (Fig. 6B). On top of that, we isolated DNA from the monoclonal cell lines and sent it to be sequenced by Sanger method (Fig. 7A). By *BLASTN* comparison of the mutated *TP53* sequenogram to the wild-type *TP53* sequenogram, we detected deletion in the *TP53* exon 4 sequence after using either sgRNA1 or sgRNA2 (Fig. 7B).

In summary, we prepared more individual clones of *TP53* knockout cells using two diverse sgRNAs. After the employed assays we could conclude that there was no significant difference in *TP53* levels among the clones, they all scored comparably. For our experiments, we chose the first derived clone generated using sgRNA1, which we refer to as RPE TP53-KO.

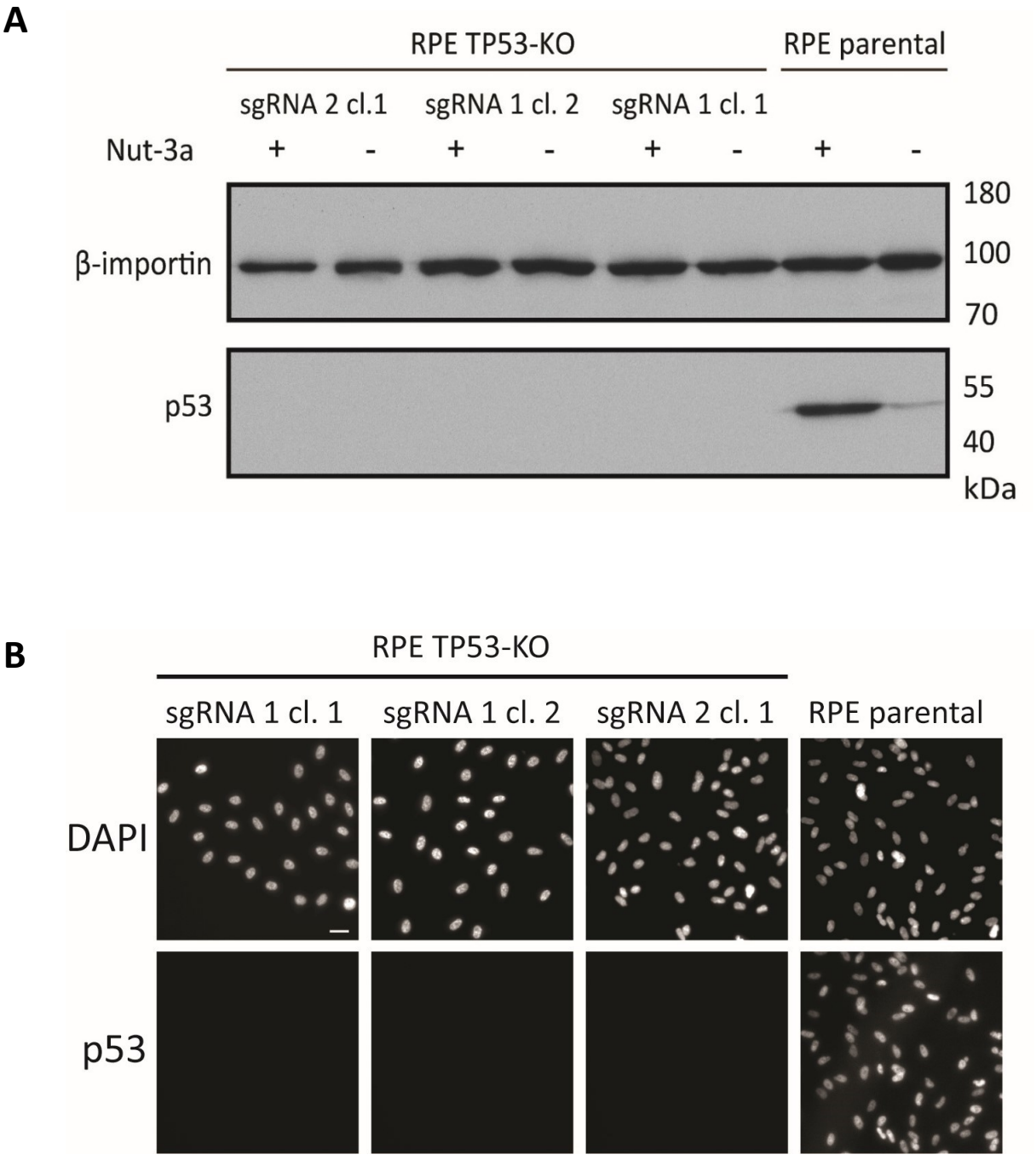
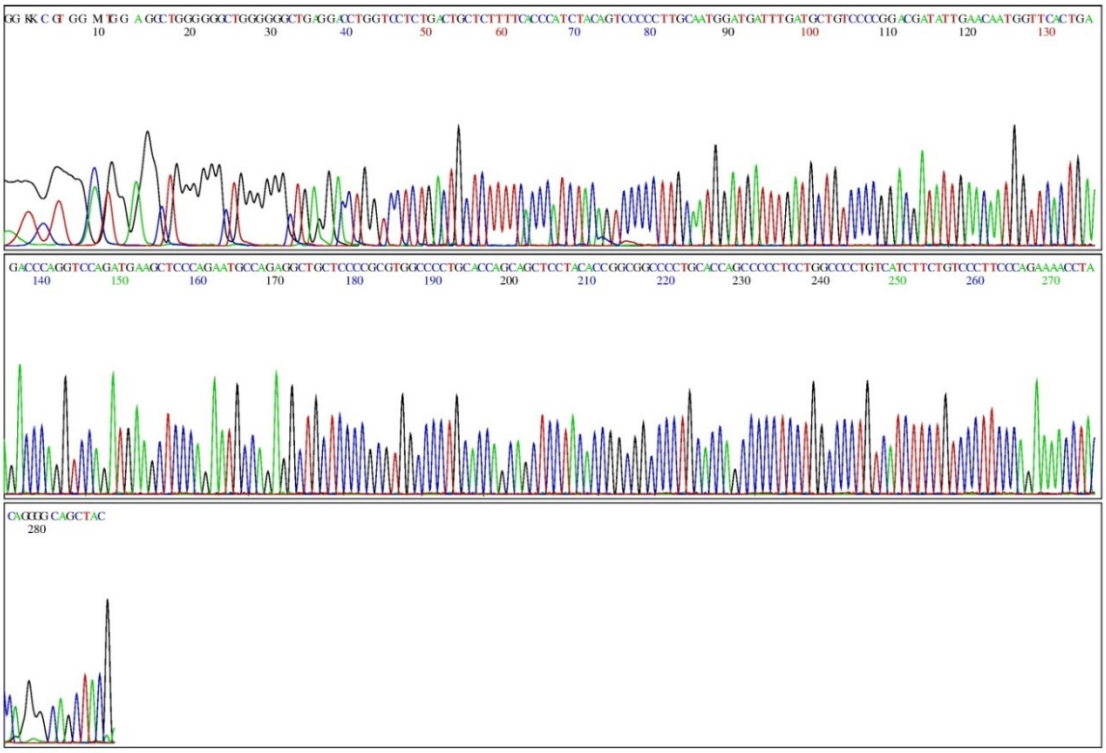


Figure 6. WB and IF analysis of *TP53* knockout cells. (A) For WB evaluation, cells were transfected with CRISPR/Cas9 transfection reagents, selected by multiple Nut-3a (9 μM) treatment within 18 days and individual clones were prepared by single-cell sorting. Following overnight treatment with Nut-3a (9 μM, 17 h), lysates were made from the cells and further used for WB with p53 and β-importin antibodies. **(B)** For IF evaluation, cells were treated with Nut-3a (9 μM) for 17 h, then fixed with paraformaldehyde and stained using p53 antibody. The scale bar represents 15 μm.

A



B

```
Query  6    CGTGGGCTGGAGGCTgggggctgggggCTGAGGACCTGGTCCTCTGACTGCTCTTTTC  65
            ||| || ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5    CGT-GGMTGGAGGCTGGGGGGCTGGGGGGCTGAGGACCTGGTCCTCTGACTGCTCTTTTC  63

Query  66   ACCCATCTACAGTCCCCCTTGCCGTCCCAAGCAATGGATGATTTGATGCTGTCCCCGGAC  125
            ||||||||||||||||||||          ||||||||||||||||||||||||||||||
Sbjct  64   ACCCATCTACAGTCCCCCTT----------GCAATGGATGATTTGATGCTGTCCCCGGAC  113

Query  126  GATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGATGAAGCTCCCAGAATGCCAGAG  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114  GATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGATGAAGCTCCCAGAATGCCAGAG  173

Query  186  GCTGCTCCCCGCGTGGCCCCTGCACCAGCAGCTCCTACACCGGCGGCCCCTGCACCAGCC  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  GCTGCTCCCCGCGTGGCCCCTGCACCAGCAGCTCCTACACCGGCGGCCCCTGCACCAGCC  233

Query  246  CCCTCCTGGCCCCTGTCATCTTCTGTCCCTTCCCAGAAAACCTACCAGGG-CAGCTAC  302
            |||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  234  CCCTCCTGGCCCCTGTCATCTTCTGTCCCTTCCCAGAAAACCTACCAGGGGCAGCTAC  291
```

Figure 7. Sequencing analysis of *TP53* knockout cells. (A) Chromatogram showing *TP53* exon 4 from RPE TP53 knockout cells prepared using sgRNA2 (clone 1). The sequencing was done by Sanger method by SEQme company. **(B)** Sequence alignments of *TP53* exon 4 of the wild-type (upper rows) and the *TP53* knockout (bottom rows), which was generated as the first derived individual clone using sgRNA2. The comparison was done using BLASTN, available from https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch.

4.2 Construction of plasmids containing selected *TP53* variants

In total, we selected 35 missense variants and one in frame deletion of the *TP53* gene to be cloned into pCW57 plasmid vector. Most of the variants (24) resided in the DNA-binding domain. Besides, there were a few variants in the transactivation domains (4), a couple in the linker region (2), and a small amount of them in the tetramerisation domain (4) and carboxy-terminal domain (2). Several of them have been classified as pathogenic (loss-of-function)/likely pathogenic or benign/likely benign, but to compare the variants of uncertain significance to the already examined variants, we worked with the whole list (Table 8). We were particularly intrigued by the E339del3 variant of uncertain significance, which was identified in a Czech patient diagnosed with breast cancer.

Table 8. List of the *TP53* variants including their location along the p53 domains (TAD = transactivation domains, DBD = DNA-binding domain, LR = linker region, TD = tetramerisation domain, CTD = carboxy-terminal domain) and classification (VUS = variant of uncertain significance) according to *ClinVar* database of genetic variants. Czech variants are highlighted by numbers in bold font.

	cDNA	protein	location	classification
1	91G>A	V31I	TAD	likely benign
2	139C>T	P47S	TAD	benign
3	206C>G	A69G	TAD	VUS
4	217G>A	V73M	TAD	likely benign
5	319T>C	Y107H	DBD	benign
6	404G>A	C135Y	DBD	VUS
7	404G>T	C135F	DBD	likely pathogenic
8	405C>G	C135W	DBD	VUS
9	467G>A	R156H	DBD	VUS
10	524G>A	R175H	DBD	pathogenic
11	541C>T	R181H	DBD	VUS
12	542G>A	R181C	DBD	VUS
13	733G>A	G245S	DBD	pathogenic
14	734G>A	G245D	DBD	pathogenic
15	742C>T	R248W	DBD	pathogenic
16	743G>A	R248Q	DBD	pathogenic
17	747G>T	R249S	DBD	pathogenic
18	799C>T	R267W	DBD	likely pathogenic
19	817C>T	R273C	DBD	pathogenic
20	818G>A	R273H	DBD	pathogenic
21	844C>T	R282W	DBD	pathogenic
22	848G>A	R283H	DBD	VUS
23	883C>T	P295S	LR	VUS
24	935C>G	T312S	LR	benign
25	1000G>C	G334R	TD	VUS
26	1010G>A	R337H	TD	likely pathogenic
27	1079G>C	G360A	CTD	likely benign
28	1096T>G	S366A	CTD	likely benign
29	760A>G	I254V	DBD	likely benign
30	503A>C	H168P	DBD	VUS
31	590T>A	V197E	DBD	VUS
32	1016A>G	E339G	TD	VUS
33	472C>T	R158C	DBD	VUS
34	784G>A	G262S	DBD	VUS
35	845G>T	R282L	DBD	VUS
36	1015-1023del	E339del3	TD	VUS

We prepared the Czech variants by site-directed mutagenesis of human *TP53* sequence using specially designed overlapping primers containing the mutant *TP53* sites of the variants. Then, we used BamHI and MluI endonucleases to prepare linearized pCW57-RFP-P2A backbone, which was suitable for Gibson assembly, together with the mutated *TP53* sequences. The rest of the variants were synthesised and inserted into pCW57 backbone by SynBio Technologies (Fig. 8).

After these steps, we transformed *Escherichia coli* competent cells with the constructed plasmids. To select transformed cells, we seeded the bacteria onto LB agar plates with ampicillin, therefore only the cells containing pCW57 plasmids with ampicillin resistance gene could survive. Subsequently, we selected five of the individual colonies and prepared liquid cultures from them. Furthermore, we isolated plasmids from the cultures, and validated them by bidirectional Sanger sequencing.

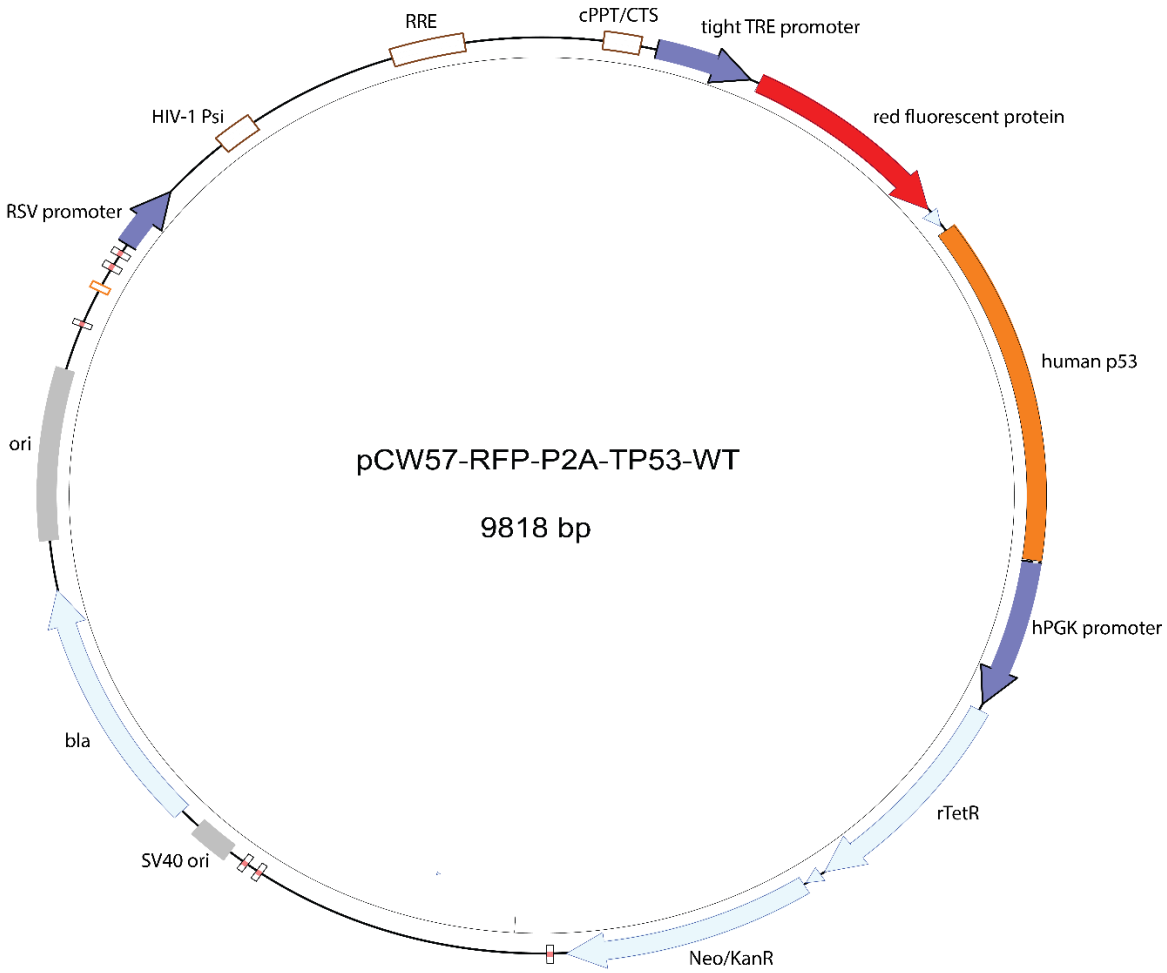


Figure 8. Graphical scheme of pCW57-RFP-P2A-TP53 plasmid used for the experiments. The vector contains the *TP53* variant, TurboRFP type of red fluorescent protein, and neomycin resistance gene (Neo/KanR). RFP gene and *TP53* are separated by self-cleaving P2A peptide, enabling co-expression from the same promoter. (tight TRE promoter = Tet-responsive promoter PTight; hPGK promoter = human phosphoglycerate kinase 1 promoter; rTetR = improved tetracycline-controlled transactivator; SV40 ori = SV40 origin of replication; bla = beta-lactamase; ori = high-copy-number ColE1/pMB1/pBR322/pUC origin of replication; RSV promoter = Rous sarcoma virus enhancer/promoter; HIV-1 Psi = packaging signal of human immunodeficiency virus type 1; RRE = REV response element of HIV-1; cPPT/CTS = central polypurine tract and central termination sequence of HIV-1)

4.3 Development of stable cell lines

After transfection of the cells with pCW57 plasmid containing gene for RFP and *TP53* variant, we performed selection of transfected cells by G-418 solution treatment, since there was a neomycin resistance gene in the plasmid. Then, using RFP as a marker, we sorted the successfully transfected cells into a polyclonal cell line (Fig. 9).

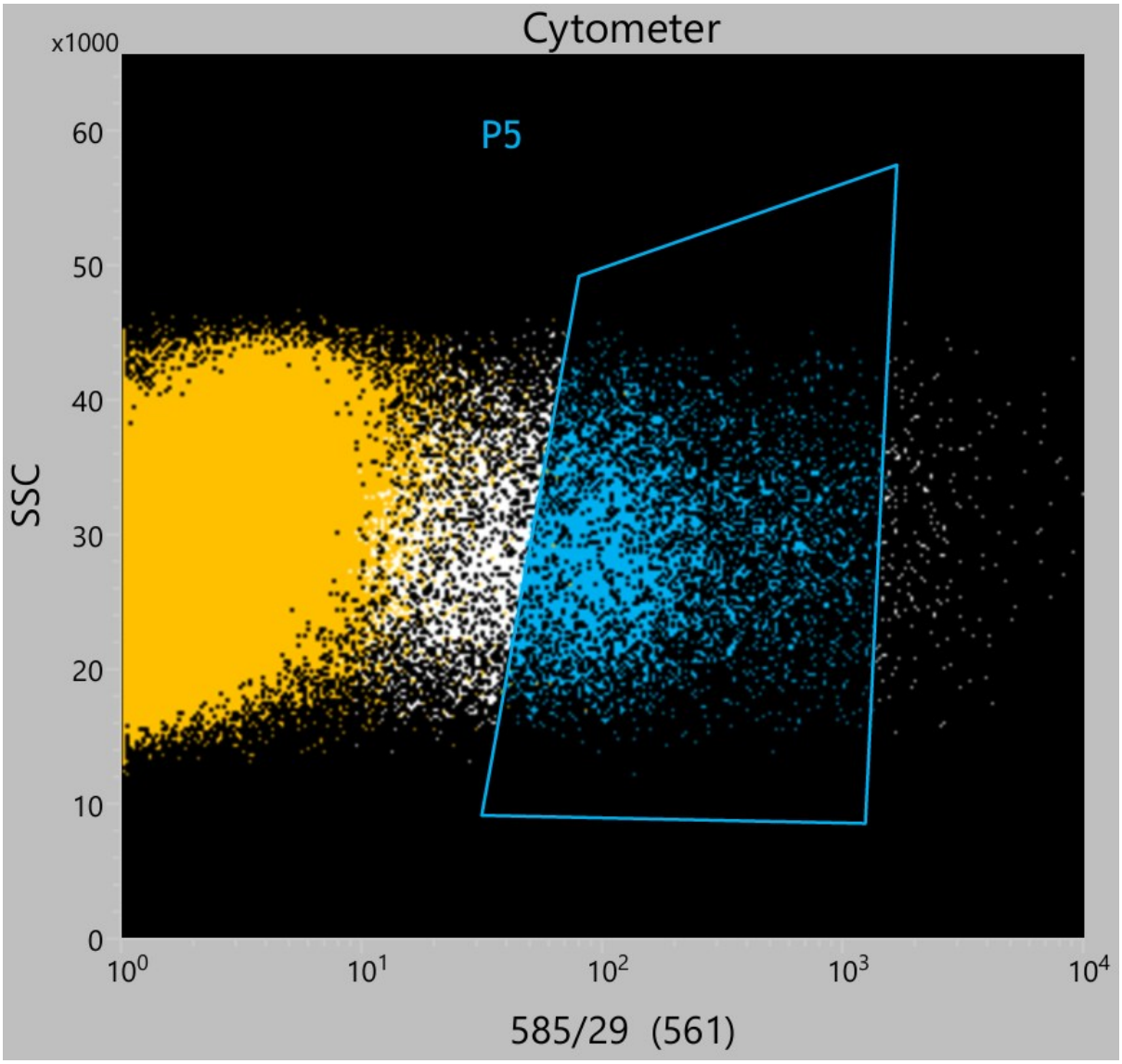
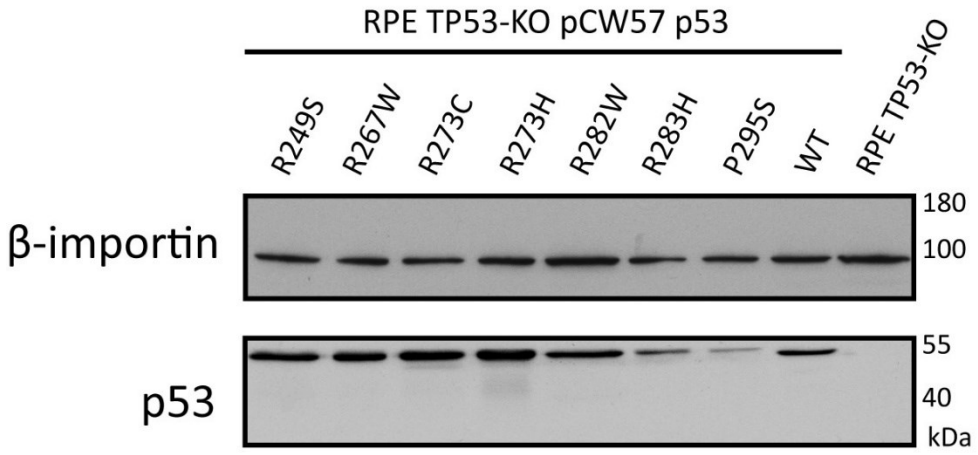


Figure 9. Flow-cytometry analysis of the polyclonal cell population after transfection with pCW57-RFP-P2A-TP53 plasmid and selection with G-418 solution. The cells were treated with Dox (2 μg/ml) a day prior cell sorting to induce RFP expression. RFP negative cells are shown in yellow, RFP positive cells are shown in white, the sorted cells are gated in the blue quadrangle. X axis represents RFP signal, Y axis represents side scatter (SSC).

To ensure the cell lines included the *TP53* variant, we treated the cells with Dox to induce p53 and RFP expression and further completed western blot analysis to detect p53 using specific antibody (Fig. 10A). Moreover, we seeded the cells onto cover slips, treated them with Dox and fixed them the following day. Then, we used immunofluorescence staining to confirm that the cells were both p53 and RFP positive (Fig. 10B).

A


RPE TP53-KO pCW57 p53
R249S
R267W
R273C
R273H
R282W
R283H
P295S
WT
RPE TP53-KO
β-importin
p53
180
100
55
40
kDa

B

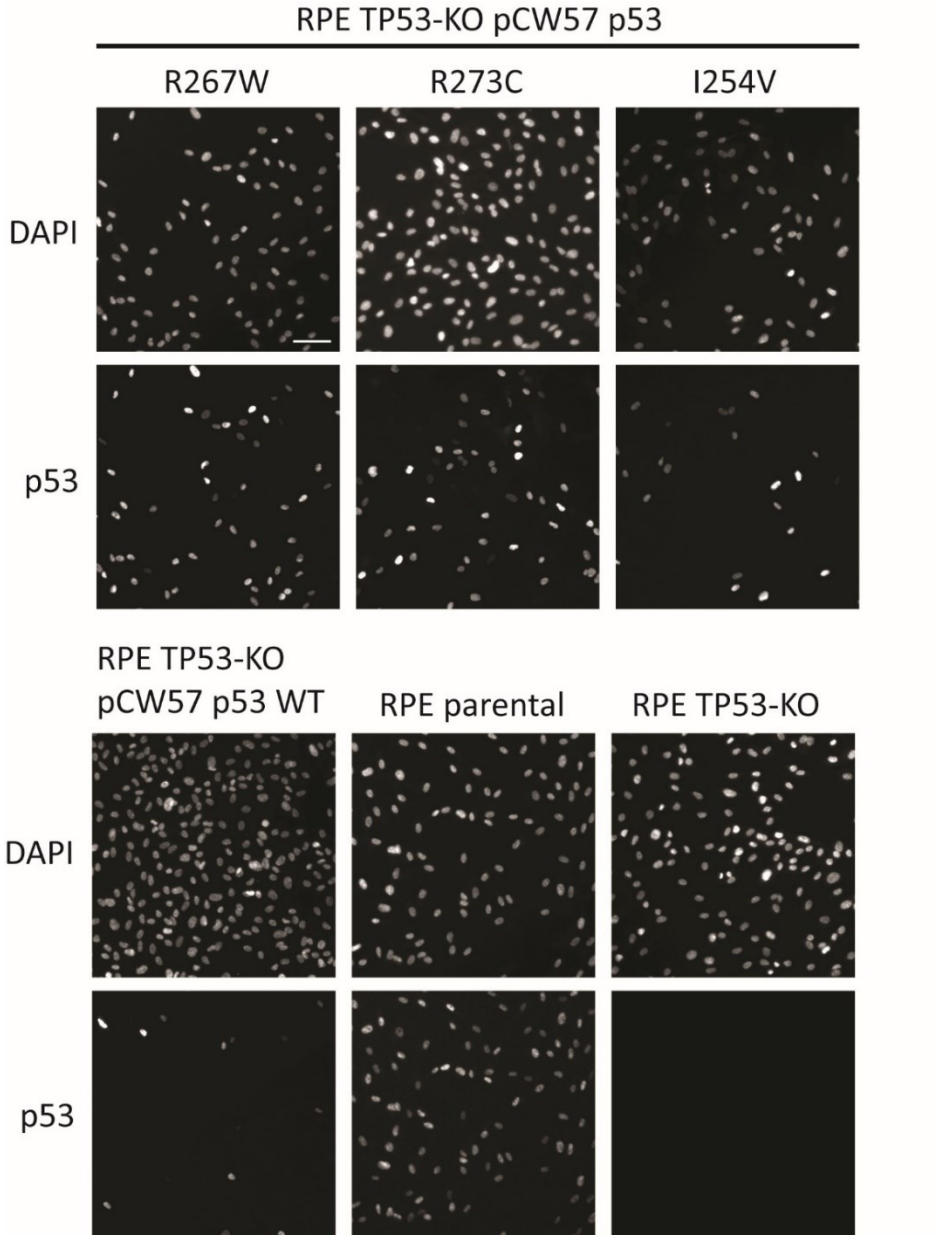

RPE TP53-KO pCW57 p53
R267W
R273C
I254V
DAPI
p53
RPE TP53-KO
pCW57 p53 WT
RPE parental
RPE TP53-KO
DAPI
p53

Figure 10. Detection of p53 expression among selected *TP53* variants. (A) Evaluation of p53 expression among 7 randomly selected *TP53* variants by western blotting analysis. After generation of stable cell lines, the cells were treated with Dox (2 µg/ml) for 17 h to induce p53 expression. Next, lysates were made from the cells and used for western blotting with p53 antibody. **(B)** Evaluation of p53 expression among three randomly selected *TP53* variants by immunofluorescence analysis. Three stable cell lines were randomly selected to present the results. The cells were seeded onto coverslips, transfected cells and *TP53* knockout cells were treated with Dox (2 µg/ml, 17 h), RPE parental cells were treated with Nut-3a (9 µM, 17 h). All the cells were fixed with 4% paraformaldehyde the following day. Afterwards, they were stained using p53 antibody. The scale bar represents 45 µm.

Unfortunately, it was not easy to distinguish between RFP positive and RFP negative cells in microscopy assays. It is possible that while this version of RFP (TurboRFP) works well in living cells, it does not survive paraformaldehyde fixation. Therefore, we decided to stain cells with RFP antibody to improve the detection ability. Nevertheless, although this antibody worked well in western blotting, it was not useful for immunofluorescence microscopy. Thus, we stained the cells with p53 antibody. Even though the p53 nuclear signal was easy to identify, it enabled us to find out that only a certain part of the cell population was indeed p53 positive. This fact may be due to a different level of p53 stability among the variants, incorrect gene incorporation of *TP53*, or to a certain extent due to the strict way of gating. Although it is highly unlikely since p53 and RFP are transcribed from a single promoter, there could also be a difference between p53 and RFP expression, so that when cells are sorted according to the RFP signal, it may not correspond to the p53 signal. Hence, we decided to employ single-cell assays which would not be influenced by that matter.

4.4 Functional analysis of p21 expression

Transcriptional activation of *CDKN1A* gene and subsequent cell cycle regulation is one of the most well-known and important functions of p53. Our aim was to find out the impact of several *TP53* variants on transactivation function of the protein. We treated the stable cell lines with Dox to induce expression of the *TP53* variants, and with Nut-3a to stabilize the level of p53. Eventually, after fixation with paraformaldehyde and subsequent immunofluorescence staining with p53 and p21 antibodies, we analysed the cell lines by high throughput scanR microscope. Since not all cells within the cell lines were p53 positive, we gated the analysed cell population for positive p53 nuclear signal. The p53 positive gate was set according to the p53 nuclear intensity of RPE parental cells, therefore we analysed cells with approximately normal levels of p53. We also left p53-low cells and p53-high cells out of the gate. These cells

with similar level of *TP53* expression from each variant were then examined for their *CDKN1A* expression level. We calculated a median of p21 mean nuclear intensity from 300 analysed cells for each cell line variant and normalised this value to the wild-type median generated the same way. Additionally, we performed the experiment two more times and calculated statistics for differences between cells expressing the wild-type p53 and individual variants (Fig. 11).

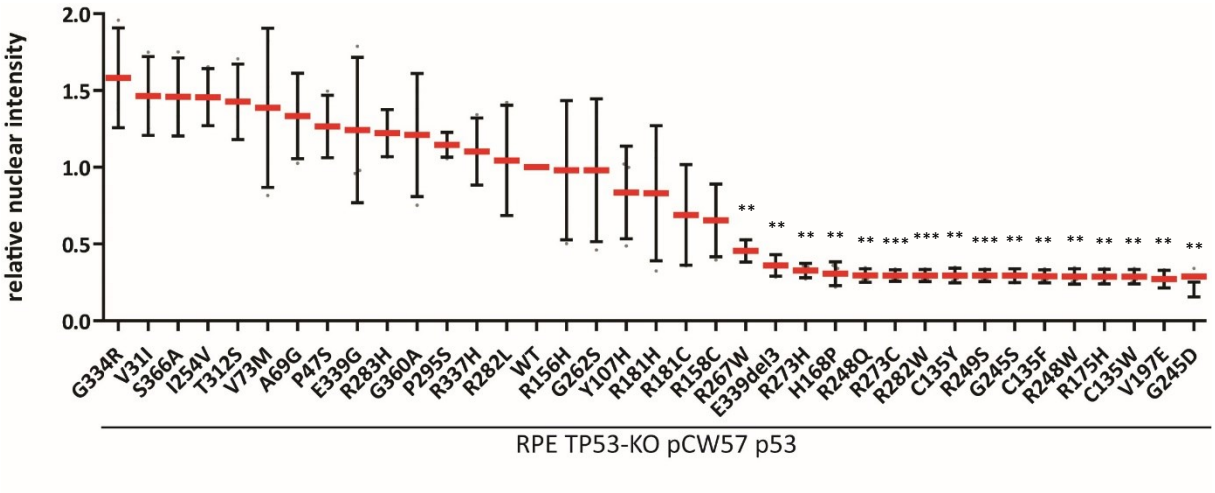


Figure 11. Quantification of p21 expression among the *TP53* variants, which are listed along the X axis. Stable cell lines containing the *TP53* variants were treated with Dox (2 µg/ml, 17 h) and Nut-3a (9 µM, 17 h), fixed with 4% paraformaldehyde and stained with p53 and p21 antibodies. The red lines show means of the wild-type normalised medians of p21 mean nuclear intensity from three independent experiments (medians were calculated out of 300 cells for each cell line), black lines show standard deviations. Significantly different results from the wild-type are highlighted. The significance was evaluated using single-sample two-tailed t-test, where n = 3.

As expected, we could not detect any p21 signal among the well-known pathogenic variants (R175H, G245S, G245D, R248W, R248Q, R249S, R273C, R273H, R282W) and the likely pathogenic variant C135F. However, the likely pathogenic R337H variant scored comparably to the wild-type in this assay. Next, we confirmed that the variants considered as benign/likely benign (V31I, P47S, V73M, Y107H, T312S, G360A, S366A, I254V) could activate expression of p21. Plus, we found out that 5 VUS showed LOF phenotype (C135Y, C135W, H168P, V197E, E339del3), while 11 others could express p21 (A69G, R156H, R181C, R181H, R283H, P295S, G334R, E339G, R158C, G262S, R282L). In addition to this, we identified a single variant (R267W) whose ability to activate transcription of *CDKN1A* was significantly lower than the ability of the wild-type, but at the same time significantly higher than the *TP53* knockout cells.

4.5 Functional analysis of MDM2 expression

As *MDM2* relies on p53 for its full expression, another goal of this project was to investigate how the variants influence this process (Fig. 12). The protocol remained the same as for the analysis of *CDKN1A* expression, but the combination of p53 and MDM2 antibodies was used.

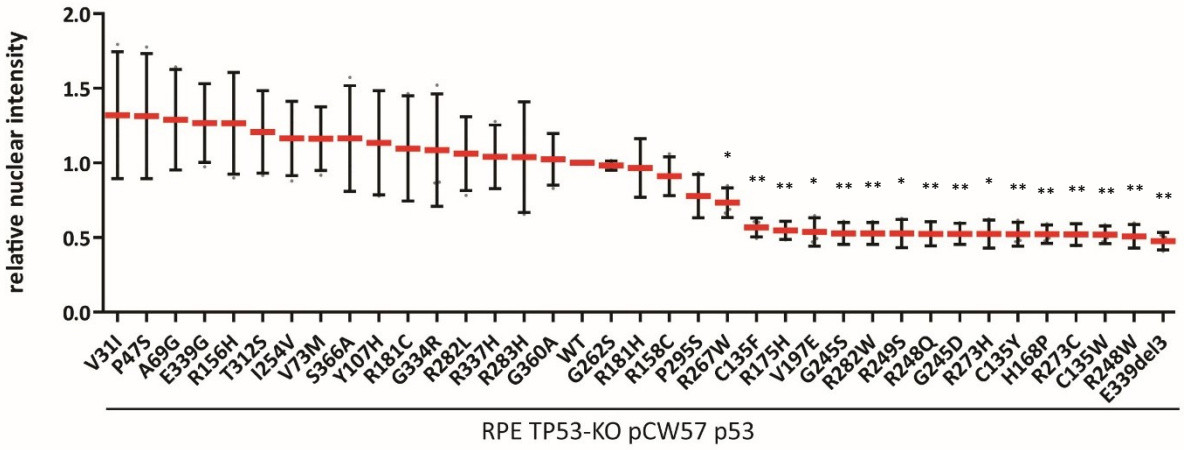


Figure 12. Quantification of MDM2 expression among the *TP53* variants, which are listed along the X axis. Stable cell lines containing the *TP53* variants were treated with Dox (2 µg/ml, 17 h) and Nut-3a (9 µM, 17 h), fixed with 4% paraformaldehyde and stained with p53 and MDM2 antibodies. The red lines show means of the wild-type normalised medians of MDM2 mean nuclear intensity from three independent experiments (medians were calculated out of 300 cells for each cell line), black lines show standard deviations. Significantly different results from the wild-type are highlighted. The significance was evaluated using single-sample two-tailed t-test, where n = 3.

According to this analysis we could summarize that the cell lines containing the *TP53* variants with the wild-type *CDKN1A* transactivation activity showed no significant defect in *MDM2* expression. Next, the cell lines with the LOF variants had no detectable level of *MDM2* expression. Again, the R267W variant scored significantly lower than the wild-type, but significantly higher than the *TP53* knockout in this assay. Overall, there were only a few minor differences in the level of expression of p21 and MDM2 within each cell line, including variants R181C (lower expression of p21 than MDM2) and P295S (lower expression of MDM2 than p21), although the dissimilarities were not found as significant. Thus, we suggest that the ability of inducing p21 expression among the variants coincided with the ability of inducing MDM2 expression.

Furthermore, we decided to compare the levels of p21 relative nuclear intensity to the levels of MDM2 relative nuclear intensity for each variant (Fig. 13). From the graph, we can clearly distinguish the LOF variants, the intermediate-scoring variant R267W and the variants that scored comparably to the wild-type.

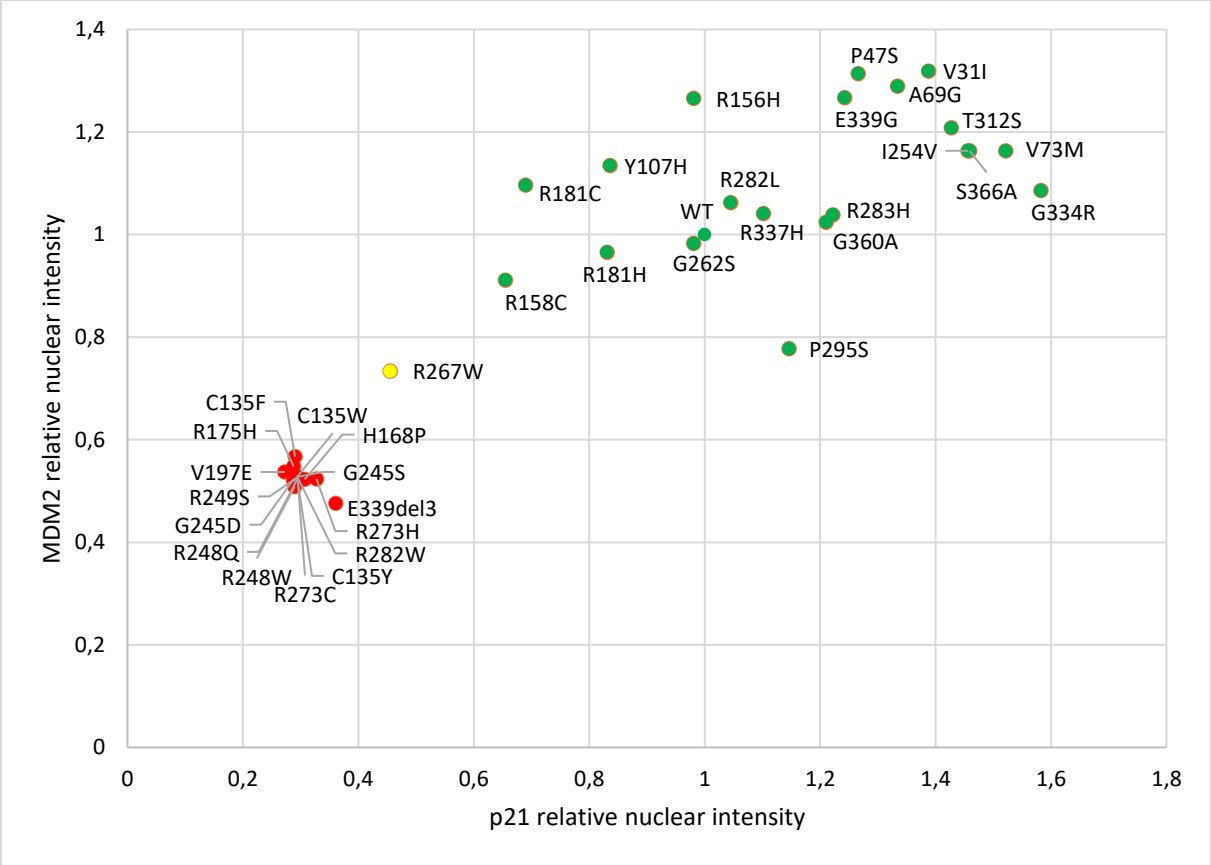


Figure 13. The comparison of p21 normalised nuclear intensity (X axis) to MDM2 normalised nuclear intensity (Y axis) among the *TP53* variants. Each dot represents a value of mean calculated from three medians normalised to the wild-type. The medians were derived from values of p21 or MDM2 mean nuclear intensities of 300 p53 positive cells within the analysed gate for each variant. LOF variants are presented in red, the intermediate variant is in yellow, and the benign variants are in green.

4.6 Tetramerisation assay

Within our list of *TP53* mutations, there were four variants located in the tetramerisation domain. Thus, we aimed to find out how these mutations affect the formation of the p53 tetramer using western blot analysis. Unfortunately, variants G334R, R337H, and E339G contained low percentage of p53-positive cells, so that the overall amount of the protein that could be detected was not comparable to the controls. Eventually, we completed this

experiment for the Czech deletion variant E339del3. For the assay, we seeded the cells into 6-well-plates and treated them overnight with Dox and Nut-3a. Next, we used the tetramerisation buffer to make lysates from them and further analysed the p53 tetramer formation by adding glutaraldehyde to the lysates and performing western blotting with p53 polyclonal antibody (Fig. 14).

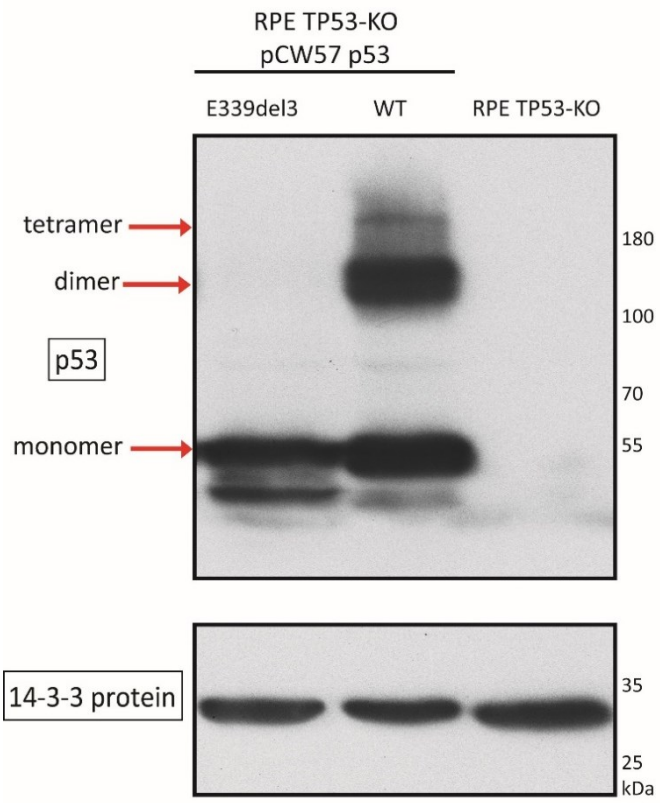


Figure 14. Western blotting analysis of impact of E339del3 on the p53 tetramer formation. The cells were seeded into 6-well-plates and treated with Dox (2 µg/ml) and Nut-3a (9 µM). The following day, lysates were made from them using tetramerisation lysis buffer. After adding glutaraldehyde (0,05%), the lysates were used for western blotting with p53 antibody.

We repeated this experiment two times and found out that variant E339del3 suppresses the tetramer formation. This result agrees with our previous conclusion that this variant is unable to transactivate both *CDKN1A* and *MDM2*.

4.7 Colony formation assay

We intended to perform the colony formation assay with the only intermediate scoring variant in the transactivation experiments (R267W) and with the newly identified Czech deletion variant (E339del3), as well. To analyse only p53 positive cells, we sorted 1000 RFP positive cells and subsequently, treated them with both Dox and Nut-3a. After 11 days, the cells were

washed with PBS, fixed and stained with crystal violet containing ethanol and the colonies were manually counted using ImageJ (Fig. 15).

Following three repetitions, we could conclude that the cells containing the intermediate variant were unable to form as many colonies as the *TP53* knockout cells, yet they made a lot more than cells with the wild-type p53. Hence, variant R267W proved to score as partial-LOF in both p21 and MDM2 transactivation assays and colony formation assay. Besides, the cells with the deletion variant E339del3 showed to be as efficient at colony formation as the knockout cells. Again, this result confirmed our previous findings that variant E339del3 resembles the phenotype of *TP53* knockout.

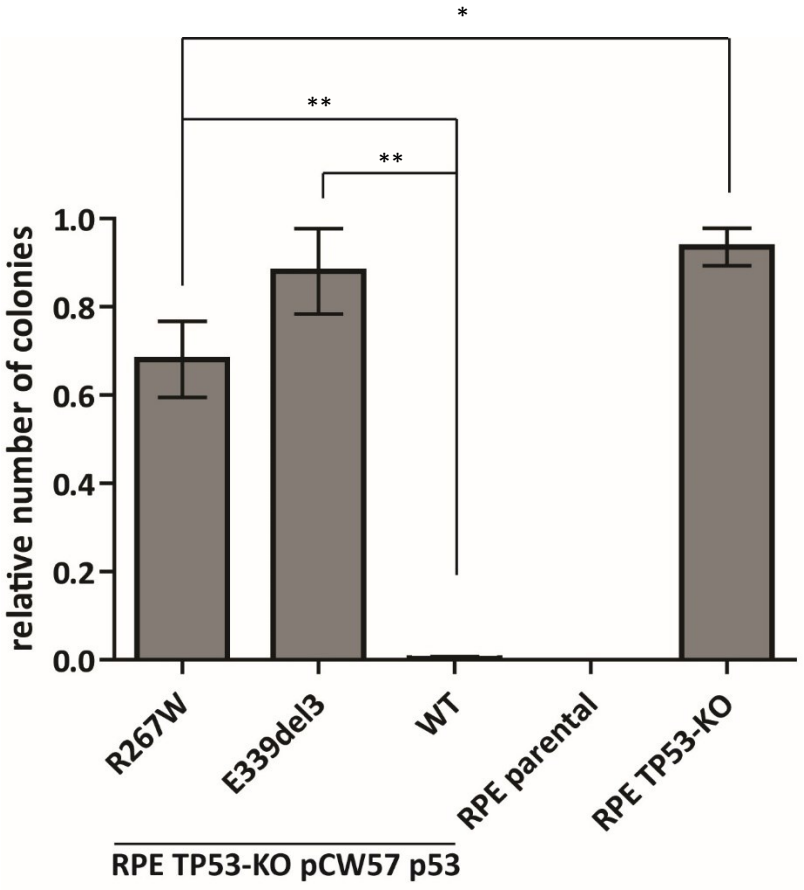


Figure 15. Graphical representation of colony formation assay results. For each cell line, 1000 RFP positive cells were sorted into 6-well-plates. The cell colonies were stained and fixed with 1% crystal violet in 20% ethanol after 11-day-long treatment with Dox (2 µg/ml) and Nut-3a (9 µM). Then, individual colonies were counted manually using ImageJ. Numbers of colonies formed by treated cells were normalised to non-treated cells. The graph shows means (presented as bars) from the normalised values from three independent experiments. Standard deviations are presented as black lines. Statistical significance was evaluated using two-tailed t-test, where n = 3.

5 Discussion

Functional analysis of 36 selected TP53 variants, of which 7 were obtained from Czech patients, confirmed that 10 of them are loss-of-function (C135F, R175H, G245S, G245D, R248W, R248Q, R249S, R273C, R273H, and R282W), as for the transactivation function of the protein, while 8 other variants remain (likely) benign (V31I, P47S, V73M, Y107H, I254V, T312S, G360A, and S366A). Besides, the experiments revealed useful information about 16 variants of uncertain significance. Overall, we could declare that most of the loss-of-function variants were located in the DNA-binding domain of p53, as assumed. On the other hand, benign variants resided mostly outside the DBD – at the N-terminal or C-terminal part of the protein.

However, when there is a substitution mutation in the tetramerisation domain of each p53 monomer, it leads to at least four mutated residues within the p53 tetramer. Hence, the TD is also sensitive to alterations in the DNA sequence. The overall mutation frequency of this domain among cancer patients is considered as low, possibly because the residues could be mutated in a manner that does not result in abolishing of p53 activity. Moreover, even if the mutation in the TD causes loss-of-function of the protein, it is unlikely it would show gain of function properties, in contrary to some of the DBD mutants (Mateu & Fersht, 1998).

Our list of *TP53* variants included four mutations in the TD, from which three are suggested to be VUS (G334R, E339G, E339del3) and the last one (R337H) is classified as likely pathogenic. The missense variants (G334R, R337H, E339G) scored not significantly differently from the wild-type p53 in the transcription activation experiments. Conversely, the fourth variant, the only deletion from the list (E339del3), was unable to induce transcription of both p21 and MDM2. Also, this newly identified variant among a Czech family, whose members were diagnosed with cancer, proved to be defective in the tetramer formation. Finally, cell line with *TP53*-E339del3 copied the results of cell line with *TP53* knockout in the colony formation assay.

Among 13 other variants of uncertain significance, we classified 4 of them as LOF (C135Y, C135W, H168P, and V197E). Next, 9 variants kept the wild-type transactivation function of p53 (A69G, R156H, R181C, R181H, R283H, P295S, R158C, G262S, and R282L). On top of that, variant R267W scored significantly lower than the wild-type p53, but significantly higher than the LOF variants for both p21 and MDM2 expression. We decided to test this variant using colony formation assay, in which the cells with *TP53*-R267W formed less colonies than *TP53*

knockout cells, but more colonies than cells with functional p53. This result suggests that R267W could be a partial-LOF mutant.

Previous experiments indicated that providing arginine at 267th position gets substituted for nonpolar tryptophane, p53 can no longer properly interact with DNA (Alvarez-Gonzalez et al., 2013). The interaction is interrupted even though Arg267 is not within the DNA-binding surface of p53. In fact, the partial loss of DNA-binding ability is due to the change of tertiary structure of the protein (Fulci et al., 2002).

Furthermore, in a *Saccharomyces cerevisiae*-based assay from 1998, this variant reached the same p21 expression level as the wild-type. Nevertheless, p53-R267W could not induce transcription (and hence, translation) of MDM2 (Di Como & Prives, 1998). However, another study claimed that p21 expression level was lowered in glioblastoma cell line cotransfected with p53-R267W expression vector and a reporter plasmid driven by p53 response element from the human *CDKN1A* (Fulci et al., 2002). These contradictory results could be explained by our finding that R267W exhibits an intermediate phenotype between LOF and benign variants.

Besides R267W, there were a few *TP53* variants which scored not significantly differently from the wild-type in p21 and MDM2 expression level assays, but still, the level of expression of the proteins seemed a bit lowered. The first was R158C, and although not much information has been gathered about it to date, it is generally considered as a functionally intermediate *TP53* mutant (Cardellino et al., 2007). Since in our assays this variant scored lower than the wild-type, but not significantly, it could be beneficial to find out its impact on other transactivation targets of p53 to classify this variant as LOF/partial-LOF/benign. Next variant of uncertain significance P295S was identified in genetic screening of cancer patients (Endris et al., 2013; Gabusi et al., 2019). However, there is no additional information about the influence of this mutation on cellular pathways controlled by p53, so our suggestion of P295S being a benign variant with a slight decrease in MDM2 expression might be important.

Another low-scoring variant, R181H, has been previously examined in osteosarcoma cell line transfected with *TP53*-R181H utilizing RNA sequencing. The results indicated that this mutation led to hypomorphic phenotype for both p21 and MDM2 expression. In this study, the variant was suggested being partial-LOF, but with apparent residual activity compared to a well-known LOF variant (R175H) (Klimovich et al., 2021). Our results partially supported this

statement, as R181H scored a bit lower than the wild-type in the transactivation assays, but not significantly. Plus, when Klimovich *et al.* expressed p53-R181H in colon cancer cell line, it showed no difference from the wild-type in ability to induce cell cycle arrest (Klimovich et al., 2021).

Interestingly, using *FoldX* energy functions, experiments from 2022 stated that variant R181H might lead to inhibition of p53 monomers binding, and therefore, destabilization of dimer formation. Moreover, by changing the conformation of the S6-S7 loop of p53, variant R181H enables the protein to interact with different molecules, and thus exert transcription-independent functions (Degn et al., 2022). Overall, this mutation has been assigned to conflicting interpretations. While it partially retains p53 canonical functions, it was identified among patients with various tumor types and could be the cause of adult-onset tumors (Fischer et al., 2023).

Variant R181C has also been studied more extensively. According to a study performed on *TP53*-R181C transfected osteosarcoma cell line, this mutation results in residual transcriptional activity, therefore it is claimed as partial-LOF. In fact, it resembles the results of the identically done analysis of p53-R181H but scores a bit lower in both p21 and MDM2 expression (Klimovich et al., 2021). Our results indicated lower expression of p21 in cells with p53-R181C, but surprisingly no decline in MDM2 expression. In addition, the study done by Klimovich *et al.* proved that substitution to cysteine at 181st position of p53 had no significantly different effect on the cell cycle regulation compared to the wild-type (Klimovich et al., 2021).

Although variant R181C is generally not considered as LOF, it is placed between the most prevalent germline mutations identified among cancer patients (Sidransky et al., 1992). Since arginine at this position is highly conserved among mammalian p53, it is probable that it plays an important role in the function of the protein (Soussi & May, 1996). On the other hand, there is no direct evidence that this variant could increase the risk of cancer predisposition (Sidransky et al., 1992). Interestingly, this mutation seems to be increased among Arab population, but this finding would require further validation (Lolas Hamameh et al., 2017; Zick et al., 2017).

Although variant R181C retains most of p53 transactivation activity of p21 and MDM2, it lost the ability to activate transcription of *BAX* and insulin like growth factor binding protein 3

(*IGFBP-3*) (Smith et al., 1999). Mutation at this position was also proved to result in higher amount of mitochondrial respiration. Therefore, increased levels of oxidative stress could cause DNA damage, genomic instability, and hence, tumor development (P.-Y. Wang et al., 2013). Thus, cancer might occur due to these phenomena, and not because of mild decrease in transactivation of *CDKN1A* and *MDM2*.

Surprisingly, it was discovered that the phenotype caused by *TP53*-R181C is reminiscent of the phenotype resulting from *TP53*-R337H (Lolas Hamameh et al., 2017). The latter substitution is commonly distributed among children with adrenocortical tumors in southern Brazil. Since pesticides and industrial chemicals are often used in this area with high agricultural productivity, they might be the cause of this mutation (Ribeiro et al., 2001). Overall, the substitution was identified among larger group of patients with various types of tumors. The other most frequent cancer types were soft tissue sarcomas, breast cancer and renal cancer (Achatz et al., 2007). Generally, the impact of p53-R337H on the increased risk of carcinogenesis likely depends on both the specific tissue and the patient´s age (Giacomazzi et al., 2014).

Arginine 337 is placed in the tetramerisation domain of p53. The residue forms a salt bridge with aspartate 352 from another p53 monomer. On the whole, the p53 tetramer is stabilized by four of these salt bridges (Jeffrey et al., 1995). When Arg337 is substituted for histidine, it does not result in block of the tetramer formation, although the structure of the protein is slightly changed (DiGiammarino et al., 2001). Compared to the wild-type dimer-dimer interaction, the mutation weakens the dimer hydrogen bonds by increasing the distance between the pairing residues. In addition, R337H disrupts the hydrophobic surface of the TD, which is important for the stability of the domain (Gordo et al., 2008).

However, it was found out that this mutation affects the thermal stability of the TD based on pH values. The higher the pH value (in the physiological range), the higher was the fraction of denatured TD molecules. Thus, the risk of cancer linked to p53-R337H may be affected by environmental pH changes. Furthermore, at all pH levels, the TD of p53-R337 begins to unfold at significantly lower temperatures compared to the wild-type (DiGiammarino et al., 2001). Also, a recent study stated that the stability of R337H is regulated by drastically distinct set of genes (including DnaJ homolog subfamily A member 1) than the stability of the wild-type (Lü et al., 2024).

Previous results indicated that this substitution variant resembles the wild-type activity. When overexpressed in both fibroblasts and osteosarcoma cells, the mutated protein kept the transactivation function of the wild-type. Moreover, p53-R337H, as well as the wild-type p53, was able to suppress colony growth of osteosarcoma cells without endogenous p53. In addition, when expressed at supraphysiological levels in non-small cell lung carcinoma, this variant showed no defect in inducing apoptosis (Ribeiro et al., 2001).

Other assays focused on knockin mouse model carrying homologous *TP53*-R337H mutation (R334H in mouse). Surprisingly, while median lifespan lowered and cancer incidence increased a bit, the results did not differ significantly from the wild-type animals. Upon genotoxic stress, using diethylnitrosamine (DEN) as a genotoxin, the tumors from mutant mice tended to be significantly bigger and heavier. Moreover, following DEN treatment, mutant mice expressed less p53 transactivation targets, such as p21, MDM2, BAX and PUMA. Finally, p53 isolated from mice liver was less efficient in both dimer and tetramer formation and less competent to bind *CDKN1A* response element (Park et al., 2018).

The downstream effects of *TP53*-R337H were proved to be influenced by X-linked inhibitor of apoptosis (XIAP)-associated factor 1 (XAF1). This factor probably acts as a tumor suppressor gene by controlling cell death (Liston et al., 2001). Besides, *TP53*-R337H resides in the same haplotype as nonsense variant *XAF1*-E134*. As for the transactivation function of p53, XAF1 presence leads to raise of this activity, but presence of XAF1-E134* does not. Since the extended haplotype with both *TP53*-R337H and *XAF1*-E134* has been frequently identified among cancer patients, it is assumed that it probably increases the risk of tumor development (Pinto et al., 2020). In our *TP53* project, the current aim is to find out the impact of *XAF1* knockout on cell line with *TP53*-R337H. First, we would like to investigate the possible fold change in the amount of expressed p53. Then, we might pay attention to comparison of the transactivation ability of cells with *TP53*-R337 and *XAF1*-KO cells with *TP53*-R337H.

Overall, the exact phenotypic outcome induced by *TP53-R337H* or other *TP53* variant may be also affected by different *TP53* polymorphisms (Figueiredo et al., 2006). Earlier studies found out that some polymorphisms may lead to changes in the activities of p53. For instance, the polymorphic variants at position 72 are considered as functionally varied. The polymorphic type *TP53*-P72 was identified to occur more frequently among African population. On the other hand, the higher the latitude, the lesser the incidence of P72 variant. In fact, it was

detected within 63 % of Nigerians, but only within 17 % of Swedish Saamis (Beckman et al., 1994). It was found out that cells with proline at the 72nd position of p53 are less prone to apoptosis. Providing there is arginine at the site, the protein localizes to mitochondria with higher probability, causing release of cytochrome c from the organelle and hence, apoptosis (Dumont et al., 2003). In addition, *TP53*-R72 is more efficient at inducing apoptosis by interacting less stably with inhibitor of apoptosis-stimulating protein p53 (iASPP) (Bergamaschi et al., 2006).

Apart from regulating the apoptotic pathway, this polymorphism has impact on many more cellular activities. First, the P72 variant was proved to induce the transactivation of p53-dependent DNA repair genes with greater efficiency (Siddique & Sabapathy, 2006). Next, *TP53*-R72 cancer cells show increased migration and invasion than cells with *TP53*-P72. The arginine variant also contributes to enhanced metastasis formation. Additionally, cancer cells containing *TP53*-R72 exhibit elevated oxidative phosphorylation and mitochondrial function in general. Eventually, polymorphic variant R72 decreases the survival of cancer patients with mutated p53. Together, these data suggest that R72 contributes to gain of function activity of mutated p53 (Basu et al., 2018). De Souza *et al.* confirmed that p53 mutants containing P72 show tumor suppressive behavior, whereas p53 mutants with R72 are more prone to be tumorigenic (De Souza et al., 2021).

Additionally, a less common polymorphism at position 47 was also proved to significantly alter the function of the protein (X. Li et al., 2005). Variant P47 is considered as wild-type, while variant S47 is found predominantly among a few percent of African-American population. However, it was found out that the transactivation function of p53 should not be influenced by different residues at the 47th position (Felley-Bosco et al., 1993). This statement agrees with our obtained results, at least for the activation of p21 and MDM2 transcription. Plus, when expressed in Calu-6 cancer cell line, p53-S47 retained the wild-type effect of suppressing cell proliferation (Felley-Bosco et al., 1993).

When serine resides at the 47th position p53, it affects the phosphorylation of the previous residue (serine 46), because p38 mitogen-activated protein kinase (p38 MAPK) recognizes its phosphorylation target due to the adjacent proline. Li *et al.* confirmed that variant S47 is indeed incapable of phosphorylation at S46 (X. Li et al., 2005). Since S46 phosphorylation is associated with increased apoptosis, impossibility of creating this posttranslational

modification might diminish the higher amount of cellular death (Smeenk et al., 2011). In fact, cells with *TP53*-P47 were shown to be more efficient at inducing caspase activation compared to cells with *TP53*-S47. Besides, the proline variant activates transcription of apoptotic gene *PUMA*. In contrast, the serine variant is partly defective in this function (X. Li et al., 2005).

Additionally, other experiments revealed that *TP53*-S47 cells tend to have a greater survival rate than *TP53*-P47 cells after treatment with DNA-damaging agent cisplatin. Following this idea, the S47 variant was also found to be less effective in transactivation of *NOXA* and synthesis of cytochrome c oxidase 2 (*SCO2*). Eventually, the same study claimed that mice with *TP53*-S47 are more prone to cancer development (Jennis et al., 2016).

In our experiments, we worked with *TP53* variants containing P47 and P72 polymorphic variants. Therefore, it might be interesting to investigate whether the other variants could influence the results of the study. Especially the R72 polymorphism combined with the mutation variants from our list is worth further testing, since it is quite frequently distributed among European population. Currently, we are working on comparison of the P72 and R72 variants of *TP53*, which are both considered as wild-type. First, we would like to focus on the possible differences in the transactivation function of the variants and furthermore, we might pay attention to some non-canonical functions of p53.

Altogether, the establishment of stable cell lines containing the *TP53* variants has brought great opportunities for further experiments. One of them may be the impact of the variants on ferroptosis induction, since it has been shown that the tumor suppression function of p53 can be also promoted via other pathways than the canonical ones. Recent studies proved that mice with mutated p53 acetylation sites, which are therefore impaired for inducing apoptosis, cell cycle arrest, and senescence, are still able to suppress cancer partly because they can regulate ferroptosis (L. Jiang et al., 2015).

Protein p53 controls ferroptosis in quite distinguished ways. Surprisingly, it has been proved that it can both promote and inhibit the process, and the outcome likely depends on the cellular context (Y. Liu & Gu, 2022). Therefore, it could be interesting to figure out how the *TP53* mutations affect this issue. It was found out that cells with the *TP53*-S47 polymorphism are resistant to erastin-induced ferroptosis. This mechanism of evading cellular death indicates a tumor-promoting activity of the variant (Jennis et al., 2016).

Additionally, mouse mutation hotspots R172H and R245W (R175H and R248W mutations in human) were examined for their role in ferroptosis. The experiments suggested that these LOF variants protect cancer cells from ferroptosis through their gain-of-function mechanism by interacting with nuclear factor erythroid 2-related factor 2 (NRF2) (Dibra et al., 2024). Transcription factor NRF2 is known for the regulation of antioxidant response (Chan et al., 2001). In this case, the protein activates transcription of glutathione-dependent peroxidases microsomal glutathione S-transferase 3 (*MGST3*) and peroxiredoxin 6 (*PRDX6*), which remove lipid peroxides (Dibra et al., 2024). Conversely, mutant p53 (R175H and R273H in this case) also interacts with NRF2 in an inhibiting manner, suppressing NRF2-mediated transcription of *SLC7A11*, thereby preventing ferroptosis induction (D. S. Liu et al., 2017).

Initially, we could focus on the effects of the *TP53* mutants from our list on SLC7A11 and GLS2 expression. Both proteins are linked to ferroptosis process, SLC7A11 blocks this cell death process, while GLS2 promotes it (Feng et al., 2021; Suzuki et al., 2022). Furthermore, it would be interesting to test which other variants are capable of interaction with NRF2, and consequently, how the possible interaction influences ferroptosis.

Next, it is crucial to conduct biological experiments not only *in vitro*, but also *in vivo* using mouse models. Although *in vitro* studies brought us valuable knowledge for better understanding of multiple *TP53* mutations, they unfortunately lack the complexity of a living system. To provide a more comprehensive insight into the examined biological processes, we would suggest utilization of mouse models, which share significant genetic and biological similarities with humans. Thus, by integrating both *in vitro* and *in vivo* approaches, we should get more robust results.

Taken together, our extensive analysis of *TP53* mutation variants from cancer patients has given us valuable information about distinct behavior and potential impacts of these mutations. Due to our obtained results, we can separate the variants into various groups of benign mutations, LOF mutations and partial-LOF mutations. These findings could help to understand how the variants contribute to cancer development and progression. Eventually, these efforts will guide the formulation of more effective management strategies for mutation carriers.

6 Conclusion

Overall, our investigation of 36 selected *TP53* variants has provided significant insights into their functional characteristics and potential implications for cancer biology. Through comprehensive analysis, we confirmed that 10 variants, including R175H, G245S, and R273H, exhibit loss-of-function effects on the transactivation function of p53. These variants predominantly reside within the DNA-binding domain (DBD), consistent with their expected impact on the ability of p53 to regulate transcriptional targets crucial for tumor suppression. Conversely, 8 other variants, including V31I and G334R, were classified as benign and are primarily located outside the DBD at the N-terminal or C-terminal regions of the protein. This segregation highlights the functional importance of specific domains within p53 and underscores the varied impact of mutations depending on their localization.

Moreover, variant R267W demonstrated partial LOF characteristics, influencing *CDKN1A* and *MDM2* expression levels in a manner distinct from both the wild-type p53 and well-known LOF variants. Of particular interest were the 16 variants categorized as variants of uncertain significance, for which our experiments provided valuable functional data. This study identified insights into less commonly studied variants, such as R158C and P295S, which displayed subtle changes in p53 function.

Looking ahead, the integration of our findings with ongoing research on *TP53* variants could lead to more targeted management of preventive care for mutation carriers, particularly those harboring VUS. Future studies using both *in vitro* and *in vivo* models will be crucial for validating our findings and exploring the broader biological consequences of these mutations, including their impact on pathways such as ferroptosis.

In summary, our comprehensive analysis of *TP53* variants provides a foundational framework for understanding their functional diversity. Most importantly, we identified a functionally intermediate *TP53* variant (R267W) and characterized the newly identified variant (E339del3) as a loss-of-function mutation. By elucidating the spectrum of effects associated with these variants, our work contributes to the ongoing efforts to personalize treatment and improve outcomes for *TP53* mutations carriers.

7 References

Abida, W. M., Nikolaev, A., Zhao, W., Zhang, W., & Gu, W. (2007). FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity. The Journal of Biological Chemistry, 282(3), 1797–1804. https://doi.org/10.1074/JBC.M609001200

Achatz, M. I. W., Olivier, M., Calvez, F. Le, Martel-Planche, G., Lopes, A., Rossi, B. M., Ashton-Prolla, P., Giugliani, R., Palmero, E. I., Vargas, F. R., Rocha, J. C. C. Da, Vettore, A. L., & Hainaut, P. (2007). The TP53 mutation, R337H, is associated with Li-Fraumeni and Li-Fraumeni-like syndromes in Brazilian families. Cancer Letters, 245(1–2), 96–102. https://doi.org/10.1016/J.CANLET.2005.12.039

Adimoolam, S., & Ford, J. M. (2002). p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene. Proceedings of the National Academy of Sciences of the United States of America, 99(20), 12985–12990. https://doi.org/10.1073/PNAS.202485699

Akiyama, T., Ohuchi, T., Sumida, S., Matsumoto, K., & Toyoshima, K. (1992). Phosphorylation of the retinoblastoma protein by cdk2. Proceedings of the National Academy of Sciences, 89(17), 7900–7904. https://doi.org/10.1073/PNAS.89.17.7900

Akyüz, N., Boehden, G. S., Süsse, S., Rimek, A., Preuss, U., Scheidtmann, K.-H., & Wiesmüller, L. (2002). DNA substrate dependence of p53-mediated regulation of double-strand break repair. Molecular and Cellular Biology, 22(17), 6306–6317. https://doi.org/10.1128/MCB.22.17.6306-6317.2002

Aledo, J. C., Gómez-Fabre, P. M., Olalla, L., & Márquez, J. (2000). Identification of two human glutaminase loci and tissue-specific expression of the two related genes. Mammalian Genome : Official Journal of the International Mammalian Genome Society, 11(12), 1107–1110. https://doi.org/10.1007/S003350010190

Alvarez-Gonzalez, R., Mendoza-Alvarez, H., Frey, M., & Zentgraf, H. (2013). Up-regulation of two distinct p53-DNA binding functions by covalent poly(ADP-ribosyl)ation: transactivating and single strand break sensing. Cancer Investigation, 31(9), 563–570. https://doi.org/10.3109/07357907.2013.845670

Appella, E., & Anderson, C. W. (2000). Signaling to p53: breaking the posttranslational modification code. Pathologie-Biologie, 48(3), 227–245. https://europepmc.org/article/med/10858956

Appella, E., & Anderson, C. W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. European Journal of Biochemistry, 268(10), 2764–2772. https://doi.org/10.1046/J.1432-1327.2001.02225.X

Assaily, W., Rubinger, D. A., Wheaton, K., Lin, Y., Ma, W., Xuan, W., Brown-Endres, L., Tsuchihara, K., Mak, T. W., & Benchimol, S. (2011). ROS-mediated p53 induction of Lpin1 regulates fatty acid oxidation in response to nutritional stress. Molecular Cell, 44(3), 491–501. https://doi.org/10.1016/J.MOLCEL.2011.08.038

Bae, Y. H., Shin, J. M., Park, H. J., Jang, H. O., Bae, M. K., & Bae, S. K. (2014). Gain-of-function mutant p53-R280K mediates survival of breast cancer cells. Genes and Genomics, 36(2), 171–178. https://doi.org/10.1007/S13258-013-0154-9/METRICS

Balagurumoorthy, P., Sakamoto, H., Lewis, M. S., Zambrano, N., Clore, G. M., Gronenborn, A. M., Appella, E., & Harrington, R. E. (1995). Four p53 DNA-binding domain peptides bind natural p53-response elements and bend the DNA. Proceedings of the National Academy of Sciences, 92(19), 8591–8595. https://doi.org/10.1073/PNAS.92.19.8591

Banin, S., Moyal, L., Shieh, S. Y., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., & Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science (New York, N.Y.), 281(5383), 1674–1677. https://doi.org/10.1126/SCIENCE.281.5383.1674

Bannai, S. (1986). Exchange of cystine and glutamate across plasma membrane of human fibroblasts. Journal of Biological Chemistry, 261(5), 2256–2263. https://doi.org/10.1016/S0021-9258(17)35926-4

Bargonetti, J., Manfredi, J. J., Chen, X., Marshak, D. R., & Prives, C. (1993). A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity wlien generated from wild-type but not from oncogenic mutant p53 protein. Genes Dev7, 7(12B), 2565–2574. https://doi.org/10.1101/gad.7.12b.2565

Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., & Berger, S. L. (2001). Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Molecular Cell, 8(6), 1243–1254. https://doi.org/10.1016/S1097-2765(01)00414-2

Basu, S., Gnanapradeepan, K., Barnoud, T., Kung, C. P., Tavecchio, M., Scott, J., Watters, A., Chen, Q., Kossenkov, A. V., & Murphy, M. E. (2018). Mutant p53 controls tumor metabolism and metastasis by regulating PGC-1α. Genes & Development, 32(3–4), 230–243. https://doi.org/10.1101/GAD.309062.117

Beckman, G., Birgander, R., Sjalander, A., Saha, N., Holmberg, P. A., Kivela, A., & Beckman, L. (1994). Is p53 Polymorphism Maintained by Natural Selection? Human Heredity, 44(5), 266–270. https://doi.org/10.1159/000154228

Bensaad, K., Tsuruta, A., Selak, M. A., Vidal, M. N. C., Nakano, K., Bartrons, R., Gottlieb, E., & Vousden, K. H. (2006). TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell, 126(1), 107–120. https://doi.org/10.1016/J.CELL.2006.05.036

Bergamaschi, D., Samuels, Y., Sullivan, A., Zvelebil, M., Breyssens, H., Bisso, A., Del Sal, G., Syed, N., Smith, P., Gasco, M., Crook, T., & Lu, X. (2006). iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72–polymorphic p53. Nature Genetics 2006 38:10, 38(10), 1133–1141. https://doi.org/10.1038/ng1879

Bernadotte, A., Mikhelson, V. M., & Spivak, I. M. (2016). Markers of cellular senescence. Telomere shortening as a marker of cellular senescence. Aging, 8(1), 3–11. https://doi.org/10.18632/AGING.100871

Billant, O., Léon, A., Le Guellec, S., Friocourt, G., Blondel, M., & Voisset, C. (2016). The dominant-negative interplay between p53, p63 and p73: A family affair. Oncotarget, 7(43), 69549–69564. https://doi.org/10.18632/ONCOTARGET.11774

Birch, J. M., Hartley, A. L., Tricker, K. J., Presser, J., Condie, A., Kelsey, A. M., Harris, M., Jones, P. H. M., Binchy, A., Crowther, D., Craft, A. W., Eden, O. B., Gareth, D., Evans, R., Thompson, E., Mann, J. R., Martin, J., Mitchell, E. L. D., & Santibã¡ã±ez-Koref, M. F. (1994). Prevalence and Diversity of Constitutional Mutations in the p53 Gene among 21 Li-Fraumeni Families.

Cancer Res, 54(5), 1298–1304. http://aacrjournals.org/cancerres/article-pdf/54/5/1298/2455940/cr0540051298.pdf

Bittar, C. M., Vieira, I. A., Sabato, C. S., Andreis, T. F., Alemar, B., Artigalás, O., Galvão, H. de C. R., Macedo, G. S., Palmero, E. I., & Ashton-Prolla, P. (2019). TP53 variants of uncertain significance: increasing challenges in variant interpretation and genetic counseling. Familial Cancer, 18(4), 451–456. https://doi.org/10.1007/S10689-019-00140-W

Blagosklonny, M. V. (2000). p53 from complexity to simplicity: mutant p53 stabilization, gain-of-function, and dominant-negative effect. The FASEB Journal, 14(13), 1901–1907. https://doi.org/10.1096/FJ.99-1078REV

Bommer, G. T., Gerin, I., Feng, Y., Kaczorowski, A. J., Kuick, R., Love, R. E., Zhai, Y., Giordano, T. J., Qin, Z. S., Moore, B. B., Macdougald, O. A., Cho, K. R., & Fearon, E. R. (2007). Article p53-Mediated Activation of miRNA34 Candidate Tumor-Suppressor Genes. Current Biology, 17(15), 1298–1307. https://doi.org/10.1016/j.cub.2007.06.068

Brady, C. A., Jiang, D., Mello, S. S., Johnson, T. M., Jarvis, L. A., Kozak, M. M., Kenzelmann Broz, D., Basak, S., Park, E. J., Mclaughlin, M. E., Karnezis, A. N., & Attardi, L. D. (2011). Distinct p53 Transcriptional Programs Dictate Acute DNA-Damage Responses and Tumor Suppression. Cell, 145(4), 571–583. https://doi.org/10.1016/j.cell.2011.03.035

Bullock, A. N., Henckel, J., & Fersht, A. R. (2000). Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. Oncogene, 19(10), 1245–1256. https://doi.org/10.1038/SJ.ONC.1203434

Candau, R., Scolnick, D. M., Darpino, P., Ying, C. Y., Halazonetis, T. D., & Berger, S. L. (1997). Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity. Oncogene, 15(7), 807–816. https://doi.org/10.1038/sj.onc.1201244

Cardellino, U., Ciribilli, Y., Andreotti, V., Modesto, P., Menichini, P., Fronza, G., Pellegrino, C., & Inga, A. (2007). Transcriptional properties of feline p53 and its tumour-associated mutants: a yeast-based approach. Mutagenesis, 22(6), 417–423. https://doi.org/10.1093/MUTAGE/GEM038

Carter, S., Bischof, O., Dejean, A., & Vousden, K. H. (2007). C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. Nature Cell Biology 2007 9:4, 9(4), 428–435. https://doi.org/10.1038/ncb1562

Chan, K., Han, X. D., & Kan, Y. W. (2001). An important function of Nrf2 in combating oxidative stress: Detoxification of acetaminophen. Proceedings of the National Academy of Sciences, 98(8), 4611–4616. https://doi.org/10.1073/PNAS.081082098

Chandrasekaran, K., Nagarajan, L., & of, L. P. (1982). The Amount of a Specific Cellular Protein (p53) I s a Correlate of Differentiation in Embryonal Carcinoma Cells. JOUHNAL OF CELLULAR PHYSIOLOGY, 113(1), 134–140. https://doi.org/10.1002/jcp.1041130122

Chang, Y. C., Jan, K. Y., Cheng, C. A., Liao, C. Bin, & Liu, Y. C. (2008). Direct involvement of the tumor suppressor p53 in nucleotide excision repair. DNA Repair, 7(5), 751–761. https://doi.org/10.1016/J.DNAREP.2008.01.019

Chehab, N. H., Malikzay, A., Stavridi, E. S., & Halazonetis, T. D. (1999). Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proceedings of the National Academy of Sciences of the United States of America, 96(24), 13777–13782. https://doi.org/10.1073/PNAS.96.24.13777

Chen, P.-L., Chen, Y., Booksmin, R., & Lee, W.-H. (1990). Genetic Mechanisms of Tumor Suppression by the Human p53 Gene. Science, 250(4987), 1576–1580. https://doi.org/10.1126/science.2274789

Chen, Q. M., Liu, J., & Merrett, J. B. (2000). Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H2O2 response of normal human fibroblasts. Biochemical Journal, 347(2), 543–551. https://doi.org/10.1042/BJ3470543

Chen, X., Zhang, T., Su, W., Dou, Z., Zhao, D., Jin, X., Lei, H., Wang, J., Xie, X., Cheng, B., Li, Q., Zhang, H., & Di, C. (2022). Mutant p53 in cancer: from molecular mechanism to therapeutic modulation. Cell Death & Disease, 13(11). https://doi.org/10.1038/S41419-022-05408-1

Chène, P. (2001). The role of tetramerisation in p53 function. Oncogene 2001, 20(21), 2611–2617. https://doi.org/10.1038/sj.onc.1204373

Chillemi, G., Davidovich, P., D'Abramo, M., Mametnabiev, T., Garabadzhiu, A. V., Desideri, A., & Melino, G. (2013). Molecular dynamics of the full-length p53 monomer. Cell Cycle, 12(18), 3098–3108. https://doi.org/10.4161/CC.26162

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., & Green, D. R. (2004). Direct Activation of Bax by p53 Mediates Mitochondrial Membrane Permeabilization and Apoptosis. Science, 303(5660), 1010–1014. https://doi.org/10.1126/SCIENCE.1092734

Cho, Y., Gorina, S., Jeffrey, P. D., & Pavletich, N. P. (1994). Crystal Structure of a p53 Tumor Suppressor-DNA Complex: Understanding Tumorigenic Mutations. Science, 265(5170), 346–355. https://doi.org/10.1126/SCIENCE.8023157

Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gamblin, S. J., Barlev, N. A., & Reinberg, D. (2004). Regulation of p53 activity through lysine methylation. Nature, 432(7015), 353–360. https://doi.org/10.1038/NATURE03117

Contractor, T., & Harris, C. R. (2012). p53 negatively regulates transcription of the pyruvate dehydrogenase kinase Pdk2. Cancer Research, 72(2), 560–567. https://doi.org/10.1158/0008-5472.CAN-11-1215.

Craig, A. L., Chrystal, J. A., Fraser, J. A., Sphyris, N., Lin, Y., Harrison, B. J., Scott, M. T., Dornreiter, I., & Hupp, T. R. (2007). The MDM2 ubiquitination signal in the DNA-binding domain of p53 forms a docking site for calcium calmodulin kinase superfamily members. Molecular and Cellular Biology, 27(9), 3542–3555. https://doi.org/10.1128/MCB.01595-06

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P. R., Gasco, M., Garrone, O., Crook, T., & Ryan, K. M. (2006). DRAM, a p53-Induced Modulator of Autophagy, Is Critical for Apoptosis. Cell, 126(1), 121–134. https://doi.org/10.1016/J.CELL.2006.05.034

Cuervo, A. M. (2004). Autophagy: Many paths to the same end. Molecular and Cellular Biochemistry, 263, 55–72. https://doi.org/10.1023/B:MCBI.0000041848.57020.57

Curthoys, N. P., & Watford, M. (1995). Regulation of glutaminase activity and glutamine metabolism. Annual Review of Nutrition, 15, 133–159. https://doi.org/10.1146/ANNUREV.NU.15.070195.001025

De Souza, C., Madden, J., Koestler, D. C., Minn, D., Montoya, D. J., Minn, K., Raetz, A. G., Zhu, Z., Xiao, W. W., Tahmassebi, N., Reddy, H., Nelson, N., Karnezis, A. N., & Chien, J. (2021). Effect of the p53 P72R Polymorphism on Mutant TP53 Allele Selection in Human Cancer. JNCI: Journal of the National Cancer Institute, 113(9), 1246–1257. https://doi.org/10.1093/JNCI/DJAB019

Degn, K., Beltrame, L., Dahl Hede, F., Sora, V., Nicolaci, V., Vabistsevits, M., Schmiegelow, K., Wadt, K., Tiberti, M., Lambrughi, M., & Papaleo, E. (2022). Cancer-related Mutations with Local or Long-range Effects on an Allosteric Loop of p53. Journal of Molecular Biology, 434(17), 167663. https://doi.org/10.1016/J.JMB.2022.167663

Delphin, C., Huang, K. P., Scotto, C., Chapel, A., Vincon, M., Chambaz, E., Garin, J., & Baudier, J. (1997). The in Vitro Phosphorylation of P53 by Calcium-Dependent Protein Kinase C. European Journal of Biochemistry, 245(3), 684–692. https://doi.org/10.1111/J.1432-1033.1997.T01-1-00684.X

Di Como, C. J., & Prives, C. (1998). Human tumor-derived p53 proteins exhibit binding site selectivity and temperature sensitivity for transactivation in a yeast-based assay. Oncogene, 16(19), 2527–2539. https://doi.org/10.1038/SJ.ONC.1202041

Dibra, D., Xiong, S., Moyer, S. M., El-Naggar, A. K., Qi, Y., Su, X., Kong, E. K., Korkut, A., & Lozano, G. (2024). Mutant p53 protects triple-negative breast adenocarcinomas from ferroptosis in vivo. Science Advances, 10(7). https://doi.org/10.1126/SCIADV.ADK1835

DiGiammarino, E. L., Lee, A. S., Cadwell, C., Zhang, W., Bothner, B., Ribeiro, R. C., Zambeti, G., & Kriwacki, R. W. (2001). A novel mechanism of tumorigenesis involving pH-dependent destabilization of a mutant p53 tetramer. Nature Structural Biology 2001 9:1, 9(1), 12–16. https://doi.org/10.1038/nsb730

Dimri, G. P., Nakanishi, M., Desprez, P.-Y., Smith, J. R., & Campisi, J. (1996). Inhibition of E2F Activity by the Cyclin-Dependent Protein Kinase Inhibitor p21 in Cells Expressing or Lacking a Functional Retinoblastoma Protein. Molecular and Cellular Biology, 16(6), 2987–2997. https://doi.org/10.1128/MCB.16.6.2987

Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A. K., Moore, M., Finlay, C., & Levine, A. J. (1993). Gain of function mutations in p53. Nature Genetics, 4(1), 42–46. https://doi.org/10.1038/NG0593-42

Dixon, S. J., Lemberg, K. M., Lamprecht, M. R., Skouta, R., Zaitsev, E. M., Gleason, C. E., Patel, D. N., Bauer, A. J., Cantley, A. M., Yang, W. S., Morrison, B., & Stockwell, B. R. (2012). Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell, 149(5), 1060–1072. https://doi.org/10.1016/J.CELL.2012.03.042

Dobner T, Horikoshi N, Rubenwolf S, & Shenk T. (1996). Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. Science, 272(5267). https://doi.org/10.1126/science.272.5267.1470

Duffy, M. J., Synnott, N. C., O'Grady, S., & Crown, J. (2022). Targeting p53 for the treatment of cancer. Seminars in Cancer Biology, 79, 58–67. https://doi.org/10.1016/J.SEMCANCER.2020.07.005

Dumaz, N., & Meek, D. W. (1999). Serine 15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. EMBO Journal, 18(24), 7002–7010. https://doi.org/10.1093/EMBOJ/18.24.7002

Dumont, P., Leu, J. I. J., Della Pietra, A. C., George, D. L., & Murphy, M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nature Genetics 2003 33:3, 33(3), 357–365. https://doi.org/10.1038/ng1093

Eby, K. G., Rosenbluth, J. M., Mays, D. J., Marshall, C. B., Barton, C. E., Sinha, S., Johnson, K. N., Tang, L., & Pietenpol, J. A. (2010). ISG20L1 is a p53 family target gene that modulates genotoxic stress-induced autophagy. Molecular Cancer, 9(1), 1–14. https://doi.org/10.1186/1476-4598-9-95/FIGURES/6

El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., & Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nature Genetics, 1(1), 45–49. https://doi.org/10.1038/ng0492-45

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., & Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell, 75(4), 817–825. https://doi.org/10.1016/0092-8674(93)90500-P

Endris, V., Penzel, R., Warth, A., Muckenhuber, A., Schirmacher, P., Stenzinger, A., & Weichert, W. (2013). Molecular Diagnostic Profiling of Lung Cancer Specimens with a Semiconductor-Based Massive Parallel Sequencing Approach: Feasibility, Costs, and Performance Compared with Conventional Sequencing. The Journal of Molecular Diagnostics, 15(6), 765–775. https://doi.org/10.1016/J.JMOLDX.2013.06.002

Felley-Bosco, E., Weston, A., Cawley, H. M., Bennett, W. P., & Harris, C. C. (1993). Functional Studies of a Germ-Line Polymorphism at Codon 47 within the p53 Gene. Am. J. Hum. Genet, 53, 752–759.

Feng, L., Zhao, K., Sun, L., Yin, X., Zhang, J., Liu, C., & Li, B. (2021). SLC7A11 regulated by NRF2 modulates esophageal squamous cell carcinoma radiosensitivity by inhibiting ferroptosis. Journal of Translational Medicine, 19(1), 1–16. https://doi.org/10.1186/S12967-021-03042-7/FIGURES/7

Ferguson, B. E., & Oh, D. H. (2005). Proficient Global Nucleotide Excision Repair in Human Keratinocytes but not in Fibroblasts Deficient in p53. Cancer Res, 65(19), 8723–8729. https://doi.org/10.1158/0008-5472.CAN-05-1457

Figueiredo, B. C., Sandrini, R., Zambetti, G. P., Pereira, R. M., Cheng, C., Liu, W., Lacerda, L., Pianovski, M. A., Michalkiewicz, E., Jenkins, J., Rodriguez-Galindo, C., Mastellaro, M. J., Vianna, S., Watanabe, F., Sandrini, F., Arram, S. B. I., & Boffetta, P. (2006). Penetrance of adrenocortical tumours associated with the germline TP53 R337H mutation. J Med Genet, 43, 91–96. https://doi.org/10.1136/jmg.2004.030551

Fischer, N. W., Vivian, Y. H., & Gariépy, J. (2023). Emerging insights into ethnic-specific TP53 germline variants. JNCI: Journal of the National Cancer Institute, 115(10), 1145–1156. https://doi.org/10.1093/JNCI/DJAD106

Foretz, M., Guichard, C., Ferré, P., & Foufelle, F. (1999). Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. Proceedings of the National Academy of Sciences of the United States of America, 96(22), 12737–12742. https://doi.org/10.1073/PNAS.96.22.12737

Friedman, P. N., Chen, X., Bargonetti, J., & Privest, C. (1993). The p53 protein is an unusually shaped tetramer that binds directly to DNA (tumor suppressor/chemical crosslinking/gel riltration/sucrose gradients/DNA-binding protein). Proc Natl Acad Sci U S A, 90(8), 3319–3323. https://doi.org/10.1073/pnas.90.8.3319

Frosina, G., Fortini, P., Rossi, O., Carrozzino, F., Raspaglio, G., Cox, L. S., Lane, D. P., Abbondandolo, A., & Dogliotti, E. (1996). Two Pathways for Base Excision Repair in Mammalian Cells. Journal of Biological Chemistry, 271, 9573–9578. https://doi.org/10.1074/jbc.271.16.9573

Fuchs, S. Y., Adler, V., Buschmann, T., Wu, X., & Ev Ronai, Z. ’. (1998). Mdm2 association with p53 targets its ubiquitination. Oncogene, 17(19), 2543–2547. https://doi.org/10.1038/sj.onc.1202200

Fulci, G., Ishii, N., Maurici, D., Gernert, K. M., Hainaut, P., Kaur, B., & Van Meir, E. G. (2002). Initiation of Human Astrocytoma by Clonal Evolution of Cells with Progressive Loss of p53 Functions in a Patient with a 283H TP53 Germ-line Mutation: Evidence for a Precursor Lesion 1. CANCER RESEARCH, 62, 2897–2905. www.rcsb.org/pdb/.

Gabusi, A., Gissi, D. B., Tarsitano, A., Asioli, S., Marchetti, C., Montebugnoli, L., Foschini, M. P., & Morandi, L. (2019). Intratumoral Heterogeneity in Recurrent Metastatic Squamous Cell Carcinoma of the Oral Cavity: New Perspectives Afforded by Multiregion DNA Sequencing and mtDNA Analysis. Journal of Oral and Maxillofacial Surgery, 77(2), 440–455. https://doi.org/10.1016/J.JOMS.2018.09.014

Gencel-Augusto, J., & Lozano, G. (2020). p53 tetramerisation: at the center of the dominant-negative effect of mutant p53. Genes & Development, 34(17–18), 1128–1146. https://doi.org/10.1101/GAD.340976.120

Giacomazzi, J., Graudenz, M. S., Osorio, C. A. B. T., Koehler-Santos, P., Palmero, E. I., Zagonel-Oliveira, M., Michelli, R. A. D., Neto, C. S., Fernandes, G. C., Achatz, M. I. W. S., Martel-Planche, G., Soares, F. A., Caleffi, M., Goldim, J. R., Hainaut, P., Camey, S. A., & Ashton-Prolla, P. (2014). Prevalence of the TP53 p.R337H Mutation in Breast Cancer Patients in Brazil. PLOS ONE, 9(6), e99893. https://doi.org/10.1371/JOURNAL.PONE.0099893

Gonzalez, K. D., Noltner, K. A., Buzin, C. H., Gu, D., Wen-Fong, C. Y., Nguyen, V. Q., Han, J. H., Lowstuter, K., Longmate, J., Sommer, S. S., & Weitzel, J. N. (2009). Beyond Li Fraumeni Syndrome: clinical characteristics of families with p53 germline mutations. Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology, 27(8), 1250–1256. https://doi.org/10.1200/JCO.2008.16.6959

Gordo, S., Martos, V., Santos, E., Menéndez, M., Bo, C., Giralt, E., & De Mendoza, J. (2008). Stability and structural recovery of the tetramerisation domain of p53-R337H mutant induced by a designed templating ligand. Proceedings of the National Academy of Sciences of the United States of America, 105(43), 16426–16431. https://doi.org/10.1073/PNAS.0805658105/SUPPL_FILE/0805658105SI.PDF

Gross, A., Mcdonnell, J. M., & Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev, 13(15), 1899–1911. https://doi.org/10.1101/gad.13.15.1899

Gu, W., & Roeder, R. G. (1997). Activation of p53 Sequence-Specific DNA Binding by Acetylation of the p53 C-Terminal Domain. Cell, 90(4), 595–606. https://doi.org/10.1016/s0092-8674(00)80521-8

Hainaut, P., & Pfeifer, G. P. (2016). Somatic TP53 mutations in the era of genome sequencing. Cold Spring Harbor Perspectives in Medicine, 6(11). https://doi.org/10.1101/cshperspect.a026179

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., & Elledge', S. J. (1993). The p21 Cdk-Interacting Protein Cipl Is a Potent Inhibitor of Gl Cyclin-Dependent Kinases. Cell, 75(4), 805–816. https://doi.org/10.1016/0092-8674(93)90499-g

Harris, R. A., Bowker-Kinley, M. M., Huang, B., & Wu, P. (2002). Regulation of the activity of the pyruvate dehydrogenase complex. Advances in Enzyme Regulation, 42, 249–259. https://doi.org/10.1016/S0065-2571(01)00061-9

Haupt, Y., Maya, R., Kazaz, A., & Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature, 387(6630), 296–299. https://doi.org/10.1038/387296A0

Hayflick, L., & Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. Experimental Cell Research, 25(3), 585–621. https://doi.org/10.1016/0014-4827(61)90192-6

He, L., He, X., Lim, L. P., De Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A. L., Linsley, P. S., Chen, C., Lowe, S. W., Cleary, M. A., & Hannon, G. J. (2007). A microRNA component of the p53 tumour suppressor network. Nature, 447(7148), 1130–1134. https://doi.org/10.1038/NATURE05939

Heiden, M. G. V., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. Science, 324(5930), 1029–1033. https://doi.org/10.1126/SCIENCE.1160809

Hinds, P., Finlay, C., & Levine, A. J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. Journal of Virology, 63(2), 739–746. https://doi.org/10.1128/JVI.63.2.739-746.1989

Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R., & Harris, C. C. (1996). Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic Acids Research, 24(1), 141–146. https://doi.org/10.1093/nar/24.1.141

Hollstein, M., Sidransky, D., Vogelstein, B., & Harris, C. C. (1991). p53 Mutations in Human Cancers. Science, 253(5015), 49–53. https://doi.org/10.1126/SCIENCE.1905840

Hrstka, R., Coates, P. J., & Vojtesek, B. (2009). Polymorphisms in p53 and the p53 pathway: Roles in cancer susceptibility and response to treatment. Journal of Cellular and Molecular Medicine, 13(3), 440–453. https://doi.org/10.1111/J.1582-4934.2008.00634.X

Huang, D. C. S., & Strasser, A. (2000). BH3-only proteins - Essential initiators of apoptotic cell death. Cell, 103(6), 839–842. https://doi.org/10.1016/S0092-8674(00)00187-2

Huang, J., Perez-Burgos, L., Placek, B. J., Sengupta, R., Richter, M., Dorsey, J. A., Kubicek, S., Opravil, S., Jenuwein, T., & Berger, S. L. (2006). Repression of p53 activity by Smyd2-mediated methylation. Nature, 444(7119), 629–632. https://doi.org/10.1038/NATURE05287

Hwang, B. J., Ford, J. M., Hanawalt, P. C., & Chu, G. (1999). Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. Proceedings of the National Academy of Sciences of the United States of America, 96(2), 424–428. https://doi.org/10.1073/PNAS.96.2.424

Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis. Nature Reviews Cancer, 2(4), 277–288. https://doi.org/10.1038/nrc776

Jeffrey, P. D., Gorina, S., & Pavletich, N. P. (1995). Crystal Structure of the Tetramerisation Domain of the p53 Tumor Suppressor at 1.7 Angstroms. Science, 267(5203), 1498–1502. https://doi.org/10.1126/SCIENCE.7878469

Jenkins JR, Rudge K, Chumakov P, & Currie GA. (1985). The cellular oncogene p53 can be activated by mutagenesis. Nature, 317(6040), 816–818. https://doi.org/10.1038/317816a0

Jennis, M., Kung, C. P., Basu, S., Budina-Kolomets, A., Leu, J. I. J., Khaku, S., Scott, J. P., Cai, K. Q., Campbell, M. R., Porter, D. K., Wang, X., Bell, D. A., Li, X., Garlick, D. S., Liu, Q., Hollstein, M., George, D. L., & Murphy, M. E. (2016). An African-specific polymorphism in the TP53 gene impairs p53 tumor suppressor function in a mouse model. Genes & Development, 30(8), 918–930. https://doi.org/10.1101/GAD.275891.115

Jiang, L., Kon, N., Li, T., Wang, S. J., Su, T., Hibshoosh, H., Baer, R., & Gu, W. (2015). Ferroptosis as a p53-mediated activity during tumour suppression. Nature 2015 520:7545, 520(7545), 57–62. https://doi.org/10.1038/nature14344

Jiang, P., Du, W., Wang, X., Mancuso, A., Gao, X., Wu, M., & Yang, X. (2011). p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. Nature Cell Biology 2011 13:3, 13(3), 310–316. https://doi.org/10.1038/ncb2172

Joerger, A. C., Ang, H. C., & Fersht, A. R. (2006). Structural basis for understanding oncogenic p53 mutations and designing rescue drugs. Proceedings of the National Academy of Sciences of the United States of America, 103(41), 15056–15061. https://doi.org/10.1073/PNAS.0607286103/SUPPL_FILE/07286TABLE3.PDF

Joynt, A. C. M., Axford, M. M., Chad, L., & Costain, G. (2021). Understanding genetic variants of uncertain significance. Paediatrics & Child Health, 27(1), 10–11. https://doi.org/10.1093/PCH/PXAB070

Karran, P. (2000). DNA double strand break repair in mammalian cells. Current Opinion in Genetics & Development, 10(2), 144–150. https://doi.org/10.1016/S0959-437X(00)00069-1

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., & Craig, R. W. (1991). Participation of p53 Protein in the Cellular Response to DNA Damage1. Cancer Res, 51(23 Pt 1), 6304–6311. http://aacrjournals.org/cancerres/article-pdf/51/23_Part_1/6304/2445789/cr05123p16304.pdf

Kastenhuber, E. R., & Lowe, S. W. (2017). Putting p53 in Context. Cell, 170(6), 1062–1078. https://doi.org/10.1016/J.CELL.2017.08.028

Kato, S., Han, S. Y., Liu, W., Otsuka, K., Shibata, H., Kanamaru, R., & Ishioka, C. (2003). Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. Proceedings of the National Academy of Sciences of the United States of America, 100(14), 8424–8429. https://doi.org/10.1073/PNAS.1431692100

Kelekar, A. (2006). Autophagy. Annals of the New York Academy of Sciences, 1066(1), 259–271. https://doi.org/10.1196/ANNALS.1363.015

Kelman, Z. (1997). PCNA: structure, functions and interactions. Oncogene, 14(6), 629–640. https://doi.org/10.1038/sj.onc.1200886

Kenzelmann Broz, D., Mello, S. S., Bieging, K. T., Jiang, D., Dusek, R. L., Brady, C. A., Sidow, A., & Attardi, L. D. (2013). Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. Genes & Development, 27(9), 1016–1031. https://doi.org/10.1101/GAD.212282.112

Kim, K.-H., Chen, C.-C., Monzon, R. I., & Lau, L. F. (2013). Matricellular protein CCN1 promotes regression of liver fibrosis through induction of cellular senescence in hepatic myofibroblasts. Molecular and Cellular Biology, 33(10), 2078–2090. https://doi.org/10.1128/MCB.00049-13

Klimovich, B., Merle, N., Neumann, M., Elmshäuser, S., Nist, A., Mernberger, M., Kazdal, D., Stenzinger, A., Timofeev, O., & Stiewe, T. (2021). p53 partial loss-of-function mutations sensitize to chemotherapy. Oncogene 2021, 41(7), 1011–1023. https://doi.org/10.1038/s41388-021-02141-5

Knights, C. D., Catania, J., Di Giovanni, S., Muratoglu, S., Perez, R., Swartzbeck, A., Quong, A. A., Zhang, X., Beerman, T., Pestell, R. G., & Avantaggiati, M. L. (2006). Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate. The Journal of Cell Biology, 173(4), 533–544. https://doi.org/10.1083/JCB.200512059

Kress, M., May, E., Cassingena, R., & May, P. (1979). Simian Virus 40-Transformed Cells Express New Species of Proteins Precipitable by Anti-Simian Virus 40 Tumor Serum. JOURNAL OF VIROLOGY, 31(2), 472–483. https://doi.org/10.1128/JVI.31.2.472-483.1979

Kung, C. P., Khaku, S., Jennis, M., Zhou, Y., & Murphy, M. E. (2015). Identification of TRIML2, a novel p53 target, that enhances p53 SUMOylation and regulates the transactivation of proapoptotic genes. Molecular Cancer Research, 13(2), 250–262. https://doi.org/10.1158/1541-7786.MCR-14-0385/80802/AM/IDENTIFICATION-OF-TRIML2-A-NOVEL-P53-TARGET-THAT

Lane, D. P., & Lionel V. Crawford. (1979). T antigen is bound to a host protein in SY40-transformed cells. Nature, 278(5701), 261–263. https://doi.org/10.1038/278261a0

Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E., & Anderson', C. W. (1992). Human DNA-Activated Protein Kinase Phosphorylates Serines 15 and 37 in the Amino-Terminal Transactivation Domain of Human p53. Moi.EcuLAR AND CELLULAR BIOLOGY, 5041–5049. https://doi.org/10.1128/mcb.12.11.5041-5049.1992

Levine, A. J., Perry, M. E., Chang, A., Silver, A., Dittmer, D., Wu, M., & Welsh, D. (1994). The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis. British Journal of Cancer, 69(3), 409–416. https://doi.org/10.1038/BJC.1994.76

Li, F. P., Fraumeni, J. F., Mulvihill, J. J., Blattner, W. A., Dreyfus, M. G., Tucker, M. A., & Miller, R. W. (1988). A Cancer Family Syndrome in Twenty-four Kindreds. CANCER RESEARCH,

48(18), 5358–5362. http://aacrjournals.org/cancerres/article-pdf/48/18/5358/2432690/cr0480185358.pdf

Li, T., Kon, N., Jiang, L., Tan, M., Ludwig, T., Zhao, Y., Baer, R., & Gu, W. (2012). Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. Cell, 149(6), 1269–1283. https://doi.org/10.1016/j.cell.2012.04.026

Li, X., Dumont, P., Della Pietra, A., Shetler, G., & Murphy, M. E. (2005). The codon 47 polymorphism in p53 is functionally significant. The Journal of Biological Chemistry, 280(25), 24245–24251. https://doi.org/10.1074/JBC.M414637200

Liberti, M. V., & Locasale, J. W. (2016). The Warburg Effect: How Does it Benefit Cancer Cells? Trends in Biochemical Sciences, 41(3), 211–218. https://doi.org/10.1016/J.TIBS.2015.12.001

Lim, J. C., & Donaldson, P. J. (2011). Focus on Molecules: The cystine/glutamate exchanger (System xc−). Experimental Eye Research, 92(3), 162–163. https://doi.org/10.1016/J.EXER.2010.05.007

Lin, J., Chen, J., Elenbaas, B., & Levine, A. J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are re.qmred for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev, 8(10), 1235–1246. https://doi.org/10.1101/gad.8.10.1235

Linke SP, Sengupta S, Khabie N, Jeffries BA, Buchhop S, Miska S, Henning W, Pedeux R, Wang XW, Hofseth LJ, Yang Q, Garfield SH, Stürzbecher HW, & Harris CC. (2003). p53 Interacts with hRAD51 and hRAD54, and Directly Modulates Homologous Recombination 1,2. CANCER RESEARCH, 63(10), 2596–2605. http://aacrjournals.org/cancerres/article-pdf/63/10/2596/2503167/ch1003002596.pdf

Liston, P., Fong, W. G., Kelly, N. L., Toji, S., Miyazaki, T., Conte, D., Tamai, K., Craig, C. G., McBurney, M. W., & Korneluk, R. G. (2001). Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. Nature Cell Biology 2001 3:2, 3(2), 128–133. https://doi.org/10.1038/35055027

Liu, D. S., Duong, C. P., Haupt, S., Montgomery, K. G., House, C. M., Azar, W. J., Pearson, H. B., Fisher, O. M., Read, M., Guerra, G. R., Haupt, Y., Cullinane, C., Wiman, K. G., Abrahmsen,

L., Phillips, W. A., & Clemons, N. J. (2017). Inhibiting the system xC−/glutathione axis selectively targets cancers with mutant-p53 accumulation. Nature Communications 2017 8:1, 8(1), 1–14. https://doi.org/10.1038/ncomms14844

Liu, Y., & Gu, W. (2022). p53 in ferroptosis regulation: the new weapon for the old guardian. Cell Death and Differentiation, 29(5), 895–910. https://doi.org/10.1038/S41418-022-00943-Y

Liu, Y., & Kulesz-Martin, M. (2001). p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis, 22(6), 851–860. https://doi.org/10.1093/CARCIN/22.6.851

Lolas Hamameh, S., Renbaum, P., Kamal, L., Dweik, D., Salahat, M., Jaraysa, T., Abu Rayyan, A., Casadei, S., Mandell, J. B., Gulsuner, S., Lee, M. K., Walsh, T., King, M. C., Levy-Lahad, E., & Kanaan, M. (2017). Genomic analysis of inherited breast cancer among Palestinian women: Genetic heterogeneity and a founder mutation in TP53. International Journal of Cancer, 141(4), 750–756. https://doi.org/10.1002/IJC.30771

Lomax, M. E., Barnes, D. M., Hupp, T. R., Picksley, S. M., & Camplejohn, R. S. (1998). Characterization of p53 oligomerization domain mutations isolated from Li-Fraumeni and Li-Fraumeni like family members. Oncogene, 17(15), 643–649. https://doi.org/10.1038/sj.onc.1201974

Lü, Y. Q., Cho, T., Mukherjee, S., Suarez, C. F., Gonzalez-Foutel, N. S., Malik, A., Martinez, S., Dervovic, D., Oh, R. H., Langille, E., Al-Zahrani, K. N., Hoeg, L., Lin, Z. Y., Tsai, R., Mbamalu, G., Rotter, V., Ashton-Prolla, P., Moffat, J., Chemes, L. B., … Schramek, D. (2024). Genome-wide CRISPR screens identify novel regulators of wild-type and mutant p53 stability. Molecular Systems Biology, 20(6), 719–740. https://doi.org/10.1038/S44320-024-00032-X

Maclaine, N. J., & Hupp, T. R. (2009). The regulation of p53 by phosphorylation: a model for how distinct signals integrate into the p53 pathway. Aging, 1(5), 490–502. https://doi.org/10.18632/AGING.100047

Maddocks, O. D. K., Vousden, K. H., Maddocks, O. D. K., & Vousden, K. H. (2011). Metabolic regulation by p53. J Mol Med, 89(5), 237–245. https://doi.org/10.1007/s00109-011-0735-5

Magnusson, C., & Vaux, D. L. (1999). Signalling by CD95 and TNF receptors: Not only life and death. Immunology and Cell Biology, 77(1), 41–46. https://doi.org/10.1046/J.1440-1711.1999.00800.X

Malumbres, M., & Barbacid, M. (2005). Mammalian cyclin-dependent kinases. Trends Biochem Sci, 30(11), 630–641. https://doi.org/10.1016/j.tibs.2005.09.005

Marcel, V., Perrier, S., Aoubala, M., Ageorges, S., Groves, M. J., Diot, A., Fernandes, K., Tauro, S., & Bourdon, J. C. (2010). Δ160p53 is a novel N-terminal p53 isoform encoded by Δ133p53 transcript. FEBS Letters, 584(21), 4463–4468. https://doi.org/10.1016/j.febslet.2010.10.005

Márquez, J., Matés, J. M., & Campos-Sandoval, J. A. (2016). Glutaminases. Advances in Neurobiology, 13, 133–171. https://doi.org/10.1007/978-3-319-45096-4_6/FIGURES/5

Mateu, M. G., & Fersht, A. R. (1998). Nine hydrophobic side chains are key determinants of the thermodynamic stability and oligomerization status of tumour suppressor p53 tetramerisation domain. EMBO Journal, 17(10), 2748–2758. https://doi.org/10.1093/EMBOJ/17.10.2748

May, P., & May, E. (1999). Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene, 18(53), 7621–7636. https://doi.org/10.1038/sj.onc.1203285

Mcbride, W., Merry, D., & Givolt, D. (1986). The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). Proc Natl Acad Sci U S A, 83(1), 130–134. https://doi.org/10.1073/pnas.83.1.130

Menon, V., & Povirk, L. (2014). Involvement of p53 in the repair of DNA double strand breaks: multifaceted Roles of p53 in homologous recombination repair (HRR) and non-homologous end joining (NHEJ). Sub-Cellular Biochemistry, 85, 321–336. https://doi.org/10.1007/978-94-017-9211-0_17

Mittnacht, S., & Weinberg, R. A. (1991). G1/S Phosphorylation of the Retinoblastoma Protein Is Associated with an Altered Affinity for the Nuclear Compartment. Cell, 65(3), 361–393. https://doi.org/10.1016/0092-8674(91)90456-9

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B., & Reed, J. C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene, 9(6), 1799–1805. https://europepmc.org/article/med/8183579

Miyashita, T., & Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell, 80(2), 293–299. https://doi.org/10.1016/0092-8674(95)90412-3

Modrich, P. (1991). Mechanisms and biological effects of mismatch repair. Annual Review of Genetics, 25, 229–253. https://doi.org/10.1146/ANNUREV.GE.25.120191.001305

Momand, J., Zambetti, G. P., Olson, D. C., George, D., & Levine, A. J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell, 69(7), 1237–1245. https://doi.org/10.1016/0092-8674(92)90644-R

Moon, S. H., Huang, C. H., Houlihan, S. L., Regunath, K., Freed-Pastor, W. A., Morris, J. P., Tschaharganeh, D. F., Kastenhuber, E. R., Barsotti, A. M., Culp-Hill, R., Xue, W., Ho, Y. J., Baslan, T., Li, X., Mayle, A., de Stanchina, E., Zender, L., Tong, D. R., D'Alessandro, A., … Prives, C. (2019). p53 Represses the Mevalonate Pathway to Mediate Tumor Suppression. Cell, 176(3), 564-580.e19. https://doi.org/10.1016/J.CELL.2018.11.011

Morgan, D. O. (1997). CYCLIN-DEPENDENT KINASES: Engines, Clocks, and Microprocessors. Annu Rev Cell Dev Biol, 13, 261–291. https://doi.org/10.1146/ANNUREV.CELLBIO.13.1.261

Mowat M, Cheng A, Kimura N, Bernstein A, & Benchimol S. (1985). Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. Nature, 314(6012), 633–636. https://doi.org/10.1038/314633a0

Müller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle, P. R., Stremmel, W., Oren, M., & Krammer, P. H. (1998). p53 Activates the CD95 (APO-

1/Fas) Gene in Response to DNA Damage by Anticancer Drugs. J. Exp. Med, 188(11), 2033–2045. https://doi.org/10.1084/jem.188.11.2033

Murphy, T. H., Miyamoto, M., Sastre, A., Schnaar, R. L., & Coyle, J. T. (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron, 2(6), 1547–1558. https://doi.org/10.1016/0896-6273(89)90043-3

Nagaich, A. K., Zhurkin, V. B., Durell, S. R., Jernigan, R. L., Appella, E., & Harrington, R. E. (1999). p53-induced DNA bending and twisting: p53 tetramer binds on the outer side of a DNA loop and increases DNA twisting. Proceedings of the National Academy of Sciences, 96(5), 1875–1880. https://doi.org/10.1073/PNAS.96.5.1875

Nagasaka, M., Miyajima, C., Aoki, H., Aoyama, M., Morishita, D., Inoue, Y., & Hayashi, H. (2022). Insights into Regulators of p53 Acetylation. Cells, 11(23), 3825. https://doi.org/10.3390/CELLS11233825

Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, & et al. (1989). Mutations in the p53 gene occur in diverse human tumour types. Nature, 342(6250), 705–708. https://doi.org/10.1038/342705a0

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., & Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science (New York, N.Y.), 288(5468), 1053–1058. https://doi.org/10.1126/SCIENCE.288.5468.1053

Ogiwara, H., Takahashi, K., Sasaki, M., Kuroda, T., Yoshida, H., Watanabe, R., Maruyama, A., Makinoshima, H., Chiwaki, F., Sasaki, H., Kato, T., Okamoto, A., & Kohno, T. (2019). Targeting the Vulnerability of Glutathione Metabolism in ARID1A-Deficient Cancers. Cancer Cell, 35(2), 177-190.e8. https://doi.org/10.1016/J.CCELL.2018.12.009

Olivier, M., Goldgar, D. E., Sodha, N., Ohgaki, H., Kleihues, P., Hainaut, P., & Eeles, R. A. (2003). Li-Fraumeni and Related Syndromes: Correlation between Tumor Type, Family Structure, and TP53 Genotype. CANCER RESEARCH, 63(20), 6643–6650. http://www.iarc.fr/P53/Germline.html.

Olivier, M., Hollstein, M., & Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harbor Perspectives in Biology, 2(1). https://doi.org/10.1101/CSHPERSPECT.A001008

Ou, Y., Wang, S. J., Li, D., Chu, B., & Gu, W. (2016). Activation of SAT1 engages polyamine metabolism with p53-mediated ferroptotic responses. Proceedings of the National Academy of Sciences of the United States of America, 113(44), E6806–E6812. https://doi.org/10.1073/PNAS.1607152113/-/DCSUPPLEMENTAL/PNAS.201607152SI.PDF

Pal, A., Gonzalez-Malerva, L., Eaton, S., Xu, C., Zhang, Y., Grief, D., Sakala, L., Nwekwo, L., Zeng, J., Christensen, G., Gupta, C., Streitwieser, E., Singharoy, A., Park, J. G., & LaBaer, J. (2023). Multidimensional quantitative phenotypic and molecular analysis reveals neomorphic behaviors of p53 missense mutants. NPJ Breast Cancer, 9(1), 78. https://doi.org/10.1038/S41523-023-00582-7

Pan, Y., & Chen, J. (2003). MDM2 promotes ubiquitination and degradation of MDMX. Molecular and Cellular Biology, 23(15), 5113–5121. https://doi.org/10.1128/MCB.23.15.5113-5121.2003

Park, J. H., Li, J., Starost, M. F., Liu, C., Zhuang, J., Chen, J., Achatz, M. I., Kang, J. G., Wang, P. yuan, Savage, S. A., & Hwang, P. M. (2018). Mouse homolog of the human TP53 R337H mutation reveals its role in tumorigenesis. Cancer Research, 78(18), 5375–5383. https://doi.org/10.1158/0008-5472.CAN-18-0016/653211/AM/MOUSE-HOMOLOG-OF-THE-HUMAN-TP53-R337H-MUTATION

Passos, J. F., Saretzki, G., Ahmed, S., Nelson, G., Richter, T., Peters, H., Wappler, I., Birket, M. J., Harold, G., Schaeuble, K., Birch-Machin, M. A., Kirkwood, T. B. L., & Von Zglinicki, T. (2007). Mitochondrial Dysfunction Accounts for the Stochastic Heterogeneity in Telomere-Dependent Senescence. PLOS Biology, 5(5), e110. https://doi.org/10.1371/JOURNAL.PBIO.0050110

Pavletich, N. P., Chambers, K. A., & Pabo, C. O. (1993). The DNA-binding domain of 53 contains the four conserved regions the major mutation hot spots. Genes Dev, 7(12B), 2556–2564. https://doi.org/10.1101/gad.7.12b.2556

Pinto, E. M., Figueiredo, B. C., Chen, W., Galvao, H. C. R., Formiga, M. N., Fragoso, M. C. B. V., Ashton-Prolla, P., Ribeiro, E. M. S. F., Felix, G., Costa, T. E. B., Savage, S. A., Yeager, M., Palmero, E. I., Volc, S., Salvador, H., Fuster-Soler, J. L., Lavarino, C., Chantada, G., Vaur, D., … Zambetti, G. P. (2020). XAF1 as a modifier of p53 function and cancer susceptibility. Science Advances, 6(26). https://doi.org/10.1126/SCIADV.ABA3231

Pitolli, C., Wang, Y., Candi, E., Shi, Y., Melino, G., & Amelio, I. (2019). p53-Mediated Tumor Suppression: DNA-Damage Response and Alternative Mechanisms. Cancers, 11(12). https://doi.org/10.3390/CANCERS11121983

Purvis, J. E., Karhohs, K. W., Mock, C., Batchelor, E., Loewer, A., & Lahav, G. (2012). p53 dynamics control cell fate. Science (New York, N.Y.), 336(6087), 1440–1444. https://doi.org/10.1126/SCIENCE.1218351

Ray, A., & Langer, M. (2002). Homologous recombination: ends as the means. Trends in Plant Science, 7(10), 435–440. https://doi.org/10.1016/S1360-1385(02)02327-0

Reed, M., Woelker, B., Wang, P., Wang, Y., Anderson, M. E., & Tegtmeyer, P. (1995). The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. Proceedings of the National Academy of Sciences of the United States of America, 92(21), 9455–9459. https://doi.org/10.1073/PNAS.92.21.9455

Reich NC, & Levine AJ. (1984). Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. Nature, 308(5955), 199–201. https://doi.org/10.1038/308199a0

Ribeiro, R. C., Sandrini, F., Figueiredo, B., Zambetti, G. P., Michalkiewicz, E., Lafferty, A. R., DeLacerda, L., Rabin, M., Cadwell, C., Sampaio, G., Cat, I., Stratakis, C. A., & Sandrini, R. (2001). An inherited p53 mutation that contributes in a tissue-specific manner to pediatric adrenal cortical carcinoma. Proceedings of the National Academy of Sciences of the United States of America, 98(16), 9330–9335. https://doi.org/10.1073/PNAS.161479898/SUPPL_FILE/4798FIG8LEGEND.HTML

Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., & Appella, E. (1998). DNA damage activates p53 through a phosphorylation–acetylation cascade. Genes & Development, 12(18), 2831. https://doi.org/10.1101/GAD.12.18.2831

Salama, R., Sadaie, M., Hoare, M., & Narita, M. (2014). Cellular senescence and its effector programs. Genes & Development, 28(2), 99–114. https://doi.org/10.1101/GAD.235184.113

Santos, C. R., & Schulze, A. (2012). Lipid metabolism in cancer. FEBS Journal, 279(15), 2610–2623. https://doi.org/10.1111/J.1742-4658.2012.08644.X

Scherer, S. J., Maier, S. M., Seifert, M., Hanselmann, R. G., Zang, K. D., Müller-Hermelinki, H. K., Angel, P., Welter, C., & Schartl, M. (2000). p53 and c-Jun functionally synergize in the regulation of the DNA repair gene hMSH2 in response to UV. The Journal of Biological Chemistry, 275(48), 37469–37473. https://doi.org/10.1074/JBC.M006990200

Schon, O., Friedler, A., Bycroft, M., Freund, S. M. V., & Fersht, A. R. (2002). Molecular Mechanism of the Interaction between MDM2 and p53. Journal of Molecular Biology, 323(3), 491–501. https://doi.org/10.1016/S0022-2836(02)00852-5

Schwartzenberg-Bar-Yoseph, F., Armoni, M., & Karnieli, E. (2004). The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Research, 64(7), 2627–2633. https://doi.org/10.1158/0008-5472.CAN-03-0846

Scolnick, D. M., Chehab, N. H., Stavridi, E. S., Lien, M. C., Caruso, L., Moran, E., Berger, S. L., & Halazonetis3, T. D. (1997). CREB-binding Protein and p300/CBP-associated Factor Are Transcriptional Coactivators of the p53 Tumor Suppressor Protein. Cancer Research, 57(17), 3693–3696. http://aacrjournals.org/cancerres/article-pdf/57/17/3693/2464680/cr0570173693.pdf

Seeberg, E., Eide, L., & Bjørås, M. (1995). The base excision repair pathway. Trends in Biochemical Sciences, 20(10), 391–397. https://doi.org/10.1016/S0968-0004(00)89086-6

Sengupta, S., & Harris, C. C. (2005). p53: traffic cop at the crossroads of DNA repair and recombination. Nature Reviews. Molecular Cell Biology, 6(1), 44–55. https://doi.org/10.1038/NRM1546

Shi, X., Kachirskaia, I., Yamaguchi, H., West, L. E., Wen, H., Wang, E. W., Dutta, S., Appella, E., & Gozani, O. (2007). Modulation of p53 function by SET8-mediated methylation at lysine 382. Molecular Cell, 27(4), 636–646. https://doi.org/10.1016/J.MOLCEL.2007.07.012

Shieh, S. Y., Ikeda, M., Taya, Y., & Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell, 91(3), 325–334. https://doi.org/10.1016/S0092-8674(00)80416-X

Shintoku, R., Takigawa, Y., Yamada, K., Kubota, C., Yoshimoto, Y., Takeuchi, T., Koshiishi, I., & Torii, S. (2017). Lipoxygenase-mediated generation of lipid peroxides enhances ferroptosis induced by erastin and RSL3. Cancer Science, 108(11), 2187–2194. https://doi.org/10.1111/CAS.13380

Shvarts, A., Steegenga, W. T., Riteco, N., Van Laar, T., Dekker, P., Bazuine, M., Van Ham, R. C. A., Van Der Houven Van Oordt, W., Hateboer, G., Van Der Eb, A. J., & Jochemsen, A. G. (1996). MDMX: A novel p53-binding protein with some functional properties of MDM2. EMBO Journal, 15(19), 5349–5357. https://doi.org/10.1002/J.1460-2075.1996.TB00919.X

Siddique, M., & Sabapathy, K. (2006). Trp53-dependent DNA-repair is affected by the codon 72 polymorphism. Oncogene, 25(25), 3489–3500. https://doi.org/10.1038/sj.onc.1209405

Sidransky, D., Tokino, T., Helzlsouer, K., Zehnbauer, B., Rausch, G., Shelton, B., Prestigiacomo, L., Vogelstein, B., & Davidson, N. (1992). Inherited p53 Gene Mutations in Breast Cancer. Cancer Res, 52(10), 2984–2986. http://aacrjournals.org/cancerres/article-pdf/52/10/2984/2446995/cr0520102984.pdf

Smeenk, L., van Heeringen, S. J., Koeppel, M., Gilbert, B., Janssen-Megens, E., Stunnenberg, H. G., & Lohrum, M. (2011). Role of p53 Serine 46 in p53 Target Gene Regulation. PLOS ONE, 6(3), e17574. https://doi.org/10.1371/JOURNAL.PONE.0017574

Smith, P. D., Crossland, S., Parker, G., Osin, P., Brooks, L., Waller, J., Philp, E., Crompton, M. R., Gusterson, B. A., Allday, M. J., & Crook, T. (1999). Novel p53 mutants selected in BRCA-associated tumours which dissociate transformation suppression from other wild-type p53 functions. Oncogene 1999 18:15, 18(15), 2451–2459. https://doi.org/10.1038/sj.onc.1202565

Smits, V. A. J., Klompmaker, R., Rijksen, G., Mä Kelä, T. P., & Medema, R. H. (2000). p21 Inhibits Thr 161 Phosphorylation of Cdc2 to Enforce the G 2 DNA Damage Checkpoint. Biol Chem, 275(39), 30638–30643. https://doi.org/10.1074/jbc.M005437200

Soussi, T., & May, P. (1996). Structural Aspects of the p53 Protein in Relation to Gene Evolution: A Second Look. Journal of Molecular Biology, 260(5), 623–637. https://doi.org/10.1006/JMBI.1996.0425

Spallarossa, P., Altieri, P., Aloi, C., Garibaldi, S., Barisione, C., Ghigliotti, G., Fugazza, G., Barsotti, A., & Brunelli, C. (2009). Doxorubicin induces senescence or apoptosis in rat neonatal cardiomyocytes by regulating the expression levels of the telomere binding factors 1 and 2. American Journal of Physiology - Heart and Circulatory Physiology, 297(6), 2169–2181. https://doi.org/10.1152/AJPHEART.00068.2009

Stewart, J., Evan, G., Watson, J., & Sikora, K. (1986). Detection of the c-myc oncogene product in colonic polyps and carcinomas. British Journal of Cancer 1986 53:1, 53(1), 1–6. https://doi.org/10.1038/bjc.1986.1

Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., & Wahl, G. M. (1999). A leucine-rich nuclear export signal in the p53 tetramerisation domain: regulation of subcellular localization and p53 activity by NES masking. The EMBO Journal, 18(6), 1660–1672. https://doi.org/10.1093/EMBOJ/18.6.1660

Strasser, A., O'connor, L., & Dixit, V. M. (2000). APOPTOSIS SIGNALING. Annu Rev Biochem., 69, 217–245. https://doi.org/10.1146/annurev.biochem.69.1.217

Suzuki, S., Tanaka, T., Poyurovsky, M. V., Nagano, H., Mayama, T., Ohkubo, S., Lokshin, M., Hosokawa, H., Nakayama, T., Suzuki, Y., Sugano, S., Sato, E., Nagao, T., Yokote, K., Tatsuno, I., & Prives, C. (2010). Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. Proceedings of the National Academy of Sciences of the United States of America, 107(16), 7461–7466. https://doi.org/10.1073/PNAS.1002459107

Suzuki, S., Venkatesh, D., Kanda, H., Nakayama, A., Hosokawa, H., Lee, E., Miki, T., Stockwell, B. R., Yokote, K., Tanaka, T., & Prives, C. (2022). GLS2 Is a Tumor Suppressor and a Regulator of Ferroptosis in Hepatocellular Carcinoma. Cancer Research, 82(18), 3209–3222. https://doi.org/10.1158/0008-5472.CAN-21-3914/707287/AM/GLS2-IS-A-TUMOR-SUPPRESSOR-AND-A-REGULATOR-OF

Tarangelo, A., Magtanong, L., Bieging-Rolett, K. T., Li, Y., Ye, J., Attardi, L. D., & Dixon, S. J. (2018). p53 Suppresses Metabolic Stress-Induced Ferroptosis in Cancer Cells. Cell Reports, 22(3), 569–575. https://doi.org/10.1016/J.CELREP.2017.12.077

Tasdemir, E., Maiuri, M. C., Galluzzi, L., Vitale, I., Djavaheri-Mergny, M., D'Amelio, M., Criollo, A., Morselli, E., Zhu, C., Harper, F., Nannmark, U., Samara, C., Pinton, P., Vicencio, J. M., Carnuccio, R., Moll, U. M., Madeo, F., Paterlini-Brechot, P., Rizzuto, R., … Kroemer, G. (2008). Regulation of autophagy by cytoplasmic p53. Nature Cell Biology 2008 10:6, 10(6), 676–687. https://doi.org/10.1038/ncb1730

Taylor, W. R., & Stark, G. R. (2001). Regulation of the G2/M transition by p53. Oncogene 2001 , 20(15), 1803–1815. https://doi.org/10.1038/sj.onc.1204252

Tian, Y., Li, Z., Hu, W., Ren, H., Tian, E., Zhao, Y., Lu, Q., Huang, X., Yang, P., Li, X., Wang, X., Kovács, A. L., Yu, L., & Zhang, H. (2010). C. elegans screen identifies autophagy genes specific to multicellular organisms. Cell, 141(6), 1042–1055. https://doi.org/10.1016/J.CELL.2010.04.034

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., & Abraham, R. T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes & Development, 13(2), 152–157. https://doi.org/10.1101/GAD.13.2.152

Tyson, J. J., Csikasz-Nagy, A., & Novak, B. (2002). The dynamics of cell cycle regulation. In BioEssays (Vol. 24, Issue 12, pp. 1095–1109). https://doi.org/10.1002/bies.10191

Van Den Berg, F. M., Tigges, A. J., Schipper, M. E. I., Den Hartog-Jager~, A., & Walboomers~, J. M. M. (1989). Expression of the nuclear oncogene p53 in colon tumours. JOURNAL OF PATHOLOGY, 157(3), 193–199. https://doi.org/10.1002/path.1711570304

Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., & Liu, E. A. (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science (New York, N.Y.), 303(5659), 844–848. https://doi.org/10.1126/SCIENCE.1092472

Waga, S., Hannon, G. J., Beach, D., & Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature , 369(6481), 574–578. https://doi.org/10.1038/369574a0

Walker, K. K., & Levine, A. J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. Proceedings of the National Academy of Sciences, 93(26), 15335–15340. https://doi.org/10.1073/PNAS.93.26.15335

Wang, H., Guo, M., Wei, H., & Chen, Y. (2023). Targeting p53 pathways: mechanisms, structures, and advances in therapy. Signal Transduction and Targeted Therapy 2023 8:1, 8(1), 1–35. https://doi.org/10.1038/s41392-023-01347-1

Wang, P.-Y., Ma, W., Park, J.-Y., Celi, F. S., Arena, R., Choi, J. W., Ali, Q. A., Tripodi, D. J., Zhuang, J., Lago, C. U., Strong, L. C., Talagala, S. L., Balaban, R. S., Kang, J.-G., & Hwang, P. M. (2013). Increased oxidative metabolism in the Li-Fraumeni syndrome. The New England Journal of Medicine, 368(11), 1027–1032. https://doi.org/10.1056/NEJMOA1214091

Wang, S., & El-Deiry, W. S. (2003). TRAIL and apoptosis induction by TNF-family death receptors. Oncogene, 22(53), 8628–8633. https://doi.org/10.1038/sj.onc.1207232

Wang, X., & Sun, Q. (2017). TP53 mutations, expression and interaction networks in human cancers. Oncotarget, 8(1), 624–643. https://doi.org/10.18632/ONCOTARGET.13483

Wang, X. W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J. M., Wang, Z., Friedberg, E. C., Evans, M. K., Taffe, B. G., Bohr, V. A., Weeda, G., Hoeijmakers, J. H. J., Forrester, K., & Harris, C. C. (1995). p53 modulation of TFIIH-associated nucleotide excision repair activity. Nature Genetics, 10(2), 188–195. https://doi.org/10.1038/NG0695-188

Wang, Y. H., Ho, T. L. F., Hariharan, A., Goh, H. C., Wong, Y. L., Verkaik, N. S., Lee, M. Y., Tam, W. L., van Gent, D. C., Venkitaraman, A. R., Sheetz, M. P., & Lane, D. P. (2022). Rapid recruitment of p53 to DNA damage sites directs DNA repair choice and integrity. Proceedings of the National Academy of Sciences of the United States of America, 119(10), e2113233119. https://doi.org/10.1073/pnas.2113233119

Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., & Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nature Cell Biology 1999 1:1, 1(1), 20–26. https://doi.org/10.1038/8991

Welch, C., Chen, Y., & Stallings, R. L. (2007). MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene, 26(34), 5017–5022. https://doi.org/10.1038/SJ.ONC.1210293

Xie, Y., Zhu, S., Song, X., Sun, X., Fan, Y., Liu, J., Zhong, M., Yuan, H., Zhang, L., Billiar, T. R., Lotze, M. T., Zeh, H. J., Kang, R., Kroemer, G., & Tang, D. (2017). The Tumor Suppressor p53 Limits Ferroptosis by Blocking DPP4 Activity. Cell Reports, 20(7), 1692–1704. https://doi.org/10.1016/J.CELREP.2017.07.055

Yahagi, N., Shimano, H., Matsuzaka, T., Najima, Y., Sekiya, M., Nakagawa, Y., Ide, T., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Gotoda, T., Nagai, R., Kimura, S., Ishibashi, S., Osuga, J. I., & Yamada, N. (2003). p53 Activation in adipocytes of obese mice. The Journal of Biological Chemistry, 278(28), 25395–25400. https://doi.org/10.1074/JBC.M302364200

Yang, W. S., Sriramaratnam, R., Welsch, M. E., Shimada, K., Skouta, R., Viswanathan, V. S., Cheah, J. H., Clemons, P. A., Shamji, A. F., Clish, C. B., Brown, L. M., Girotti, A. W., Cornish, V. W., Schreiber, S. L., & Stockwell, B. R. (2014). Regulation of ferroptotic cancer cell death by GPX4. Cell, 156(1–2), 317–331. https://doi.org/10.1016/J.CELL.2013.12.010

Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., & Schrelber, S. L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell, 76(5), 933–945. https://doi.org/10.1016/0092-8674(94)90367-0

Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., & Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. Molecular Cell, 7(3), 673–682. https://doi.org/10.1016/S1097-2765(01)00213-1

Zhou, J., Ahn, J., Wilson, S. H., & Prives, C. (2001). A role for p53 in base excision repair. EMBO Journal, 20(4), 914–923. https://doi.org/10.1093/EMBOJ/20.4.914

Zhu, J., Dou, Z., Sammons, M. A., Levine, A. J., & Berger, S. L. (2016). Lysine methylation represses P53 activity in teratocarcinoma cancer cells. Proceedings of the National Academy of Sciences of the United States of America, 113(35), 9822–9827. https://doi.org/10.1073/PNAS.1610387113/SUPPL_FILE/PNAS.201610387SI.PDF

Zick, A., Kadouri, L., Cohen, S., Frohlinger, M., Hamburger, T., Zvi, N., Plaser, M., Avital, E., Breuier, S., Elian, F., Salah, A., Goldberg, Y., & Peretz, T. (2017). Recurrent TP53 missense mutation in cancer patients of Arab descent. Familial Cancer, 16(2), 295–301. https://doi.org/10.1007/S10689-016-9951-Z/METRICS

Zink, D., Mayr, C., Janz, C., & Wiesmüller, L. (2002). Association of p53 and MSH2 with recombinative repair complexes during S phase. Oncogene 2002 21:31, 21(31), 4788–4800. https://doi.org/10.1038/sj.onc.1205614

Zurer, I., Hofseth, L. J., Cohen, Y., Xu-Welliver, M., Hussain, S. P., Harris, C. C., & Rotter, V. (2004). The role of p53 in base excision repair following genotoxic stress. Carcinogenesis, 25(1), 11–19. https://doi.org/10.1093/CARCIN/BGG186