From Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

PHARMACOLOGICAL TARGETING OF NONSENSE MUTANT TP53 AND PTEN IN CANCER

Angelos Heldin

Karolinska
Institutet

Stockholm 2023

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2023
© Angelos Heldin, 2023
ISBN 978-91-8017-088-8
Cover art: *Magnum opus* by Angelos Heldin

Pharmacological targeting of nonsense mutant *TP53* and *PTEN* in cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Angelos Heldin

The thesis will be publicly defended in lecture hall R8:00, Cancer center Karolinska, Karolinska University Hospital, Solna, Sweden.

Friday, 20th October 2023 at 09:30 am

Principal Supervisor:
Professor Klas Wiman
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor:
Assoc. Prof. Vladimir J.N. Bykov
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Kanaga Sabapathy
Nanyang Technological University
School of Biological Sciences

Examination Board:
Professor Per Lindahl
Göteborgs Universitet
Department of Medical Biochemistry & Cell Biology

Docent Susanne Schlisio
Karolinska Institutet
Department of Oncology-Pathology

Docent Fredrik Wermeling
Karolinska Institutet
Department of Medicine

To my family, friends and colleagues,

thanks for all the support throughout this endeavor.

Popular science summary of the thesis

A cell is the basic building block of life. Just as bricks come together to form a house, cells come together to form organisms, be it plants, animals or humans. Inside almost every cell is a nucleus where the crucial code called DNA is stored. DNA is like the instruction manual of life. It's a long molecule made up of units called nucleotides, arranged in a unique sequence for every individual. This sequence determines our characteristics including the color of our eyes to our susceptibility to certain diseases. DNA is the ultimate instruction manual for building and operating an organism. In addition, there is an intermediate carrier of genetic information called mRNA that acts as a messenger that takes the information from the DNA and transcribes it into a language that the cellular machinery responsible for making proteins can work with. The cellular protein factory is called the ribosome and translates the code from the mRNA and assembles different building blocks called amino acids according to the instructions in the mRNA. When a chain of amino acids has been linked together by the ribosome, the amino acids start interacting with each other, forming complex 3D structures more commonly referred to as proteins. These proteins carry out most functions in our cells.

Sometimes, the genetic code in the DNA gets damaged or changed due to UV radiation, certain chemicals or other factors. This error, if it is not corrected, is passed on to the mRNA and finally the resulting protein. This is commonly referred to as a mutation and can be as small as a single nucleotide change in the genetic code or as a large missing section. These errors can lead to proteins that are either nonfunctional or have gotten an alternative function, possibly resulting in disease. All our cells have safety mechanisms called tumor suppressor genes like *TP53* and *PTEN*. They function by detecting DNA damage and activate quality control mechanisms that either repairs the damage or force the affected cell to die in a process called apoptosis. But if the DNA damage occurs in one of these tumor suppressor genes, the safety mechanism will not work as intended and the ability of the cell to detect damaged DNA will be inactivated. This will in the long run lead to additional mutations and eventually cancer which is characterized by uncontrolled cell growth. There are different categories of mutations, one of them is called nonsense mutation which creates an early stoppage in the code, causing the ribosome to stop protein production before reaching the end. This results in a shorter version of the affected protein which often is nonfunctional and that cannot perform its intended function. These shortened or truncated proteins can disturb normal cellular function and lead to various diseases, such as cancer. Certain drugs like the antibiotic Geneticin (G418) can trick the ribosome to ignore the early stop signal in a process called translational readthrough. The ribosome

can then continue protein production from the mRNA resulting in production of the full-length protein.

Mouse models are commonly used in research to help researchers understand a disease or to test new treatments. They are created by introducing a specific mutation in the mouse DNA that is also found in humans. This allows us to observe how a disease develops and test new drugs to see if they work as intended. Mouse models are therefore extremely important tools for the development of new drugs.

In summary, the human body is a fine-tuned machine where DNA, RNA, and proteins work in harmony to maintain the balance of life. When this balance is disrupted by mutations or other factors, diseases like cancer may result. However, new techniques, such as translational readthrough and new mice models, offer a glimpse into future therapies that could restore this balance.

Abstract

The *TP53* tumor suppressor gene encodes p53 and is inactivated by mutations in around half of all human tumors. Approximately 11% of *TP53* mutations are nonsense mutations, resulting in the premature termination of translation and the production of truncated and non-functional p53 proteins. Aminoglycosides such as G418 are known to induce translational readthrough, a process in which the ribosome overcomes the stop signal introduced by a nonsense mutation and translates full-length protein. However, the clinical use of aminoglycosides is restricted due to severe side effects. We have demonstrated that combination treatments with proteasome inhibitors or compounds that disrupt the binding of p53 to the ubiquitin ligase MDM2 can synergistically enhance the levels of full-length p53, improving the efficacy of readthrough compared to aminoglycosides alone. These combinations were proven to produce at least partially active full-length p53, as shown by the suppression of cell growth and the induction of cell death. In parallel, chemical library screenings led to the discovery of two novel compounds, C47 and C61, showing readthrough activity and synergizing with G418 and eRF3 degraders CC-885 and CC-90009, respectively. Remarkably, C47 also exhibit readthrough activity for nonsense mutant phosphatase and tensin homolog (*PTEN*), expanding the scope for targeted cancer therapies. Furthermore, we have identified the 5-fluorouracil (5-FU) metabolite 5-Fluorouridine (FUr) as a potent readthrough-inducing compounds capable restoring full-length p53 expression in cells harboring nonsense mutant *TP53*. In vivo studies further substantiated the capability of FUr to reinstate full-length p53 expression in human tumor xenografts with TP53 R213X nonsense mutations. Finally, the first *Trp53* R210X nonsense mutant knock-in mouse model has been generated. R210X corresponds to human *TP53* R213X. Observations on tumor development, lifespan and other phenotypic traits in these mice provide valuable insights into the impact of *TP53* nonsense mutation in a multi-organ system. These results also provide a platform for the preclinical evaluation of novel therapeutic strategies for targeting nonsense mutant *TP53*.

In summary, these findings offer a multi-faceted approach towards understanding *TP53* nonsense mutations and advancing targeted cancer therapy through pharmacological induction of translational readthrough. The discovery of novel readthrough inducing compounds, the application of combination therapy in translational readthrough, the discovery of a novel therapeutic application for 5-FU and its metabolite FUr, as well as the generation of a novel animal model collectively set the stage for the further development of personalized treatments for patients with tumors harboring nonsense mutant *TP53*.

List of scientific papers

I. Synergistic rescue of nonsense mutant tumor suppressor p53 by combination treatment with aminoglycosides and Mdm2 inhibitors.
Frontiers in Oncology (2018), 7:323
Meiqiongzi Zhang*, **Angelos Heldin***, Mireia Palomar-Siles, Susanne Öhlin, Vladimir J. N. Bykov and Klas G. Wiman.
*Authors contributed equally

II. Novel compounds that synergize with aminoglycoside G418 or eRF3 degraders for translational readthrough of nonsense mutant *TP53* and *PTEN*.
RNA Biology (2023), 20:368
Angelos Heldin, Matko Cancer*, Mireia Palomar-Siles*, Meiqiongzi Zhang, Susanne Öhlin, Anna Mariani, Alexander Sun-Zhang, Jianping Liu, Vladimir J.N. Bykov and Klas G. Wiman
*Authors contributed equally

III. Translational readthrough of nonsense mutant *TP53* by mRNA incorporation of 5-Fluorouridine.
Cell Death & Disease (2022), 13:997
Mireia Palomar-Siles, **Angelos Heldin**, Meiqiongzi Zhang, Charlotte Strandgren, Viktor Yurevych, Jip T. van Dinter, Sem A. G. Engels, Damon A. Hofman, Susanne Öhlin, Birthe Meineke, Vladimir J. N. Bykov, Sebastiaan van Heesch and Klas G. Wiman.

IV. A novel tumor-prone mouse model harboring the Trp53R210X nonsense mutation.
Manuscript
Charlotte Strandgren, **Angelos Heldin**, Susanne Öhlin and Klas G. Wiman

Contents

1 INTRODUCTION 1
1.1 Cancer 1
1.1.1 Cancer genes 3
1.1.2 Cancer therapeutic approaches 4
1.1.3 Conventional cancer therapy 5
1.1.4 Targeted therapy 6
1.1.5 Combination treatment 8
1.2 The *TP53* gene 8
1.2.1 TP53 function 9
1.2.2 TP53 regulation 11
1.2.3 TP53 mutations 11
1.2.4 Trp53 deficient mouse models 12
1.2.5 p53 targeted therapies 13
1.3 The PTEN gene 15
1.3.1 PTEN function 15
1.3.2 PTEN regulation 16
1.3.3 PTEN mutations 17
1.4 Protein translation 17
1.4.1 The Ribosome 18
1.4.2 Initiation 18
1.4.3 Elongation 19
1.4.4 Termination 19
1.5 Nonsense-mediated decay 20
1.6 Translational Readthrough 21
1.6.1 Factors affecting readthrough efficiency 22
1.6.2 Readthrough-inducing compounds 23
1.6.3 Nonsense mutant mouse models 25
1.7 Synergy 26
2 MATERIALS AND METHODS 28
2.1 Western Blotting 28
2.2 Enzyme-Linked Immunosorbent Assay (ELISA) 28
2.3 Flow Cytometry EGFP readthrough 29
2.4 Immunofluorescence staining 30
2.5 Quantitative Real-Time PCR (qRT-PCR) 30
2.6 Cell Cycle analysis 29

2.7 WST-1 30
2.8 Annexin V 29
3 ETHICAL CONSIDERATIONS 31
4 RESEARCH AIMS, RESULTS AND DISCUSSION 32
4.1 Paper I 32
4.2 Paper II 33
4.3 Paper III 35
4.4 Paper IV 36
5 CONCLUSIONS AND FUTURE PERSPECTIVES 38
6 ACKNOWLEDGEMENTS 40
7 REFERENCES 42

ABBREVIATIONS

5-FU	5-fluorouracil
ALK	Anaplastic lymphoma kinase
AKT	Protein kinase B (PKB)
APC	Adenomatous polyposis coli
APC^{min}	Adenomatous polyposis coli $^{\text{multiple intestinal neoplasia}}$
A-T	Ataxia-telangiectasia
Atm	A-T mutated
Aptx	Aprataxin
A-site	Aminoacyl-site
BAX	Bcl-2-like protein 4
BCL-2	B-cell lymphoma 2
CML	Chronic myeloid leukemia
CRC	Colorectal cancer
CRBN	Cereblon
CTLA-4	Cytotoxic T lymphocyte–associated antigen 4
DAP	2,6-diaminopurine
DBD	DNA binding domain
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
EGR1	Early Growth Response 1
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
FAP	Familial adenomatous polyposis
E-site	Exit-site

eEF	Elongation factor
eIF	Eukaryotic initiation factor
EJC	Exon-junction complex
eRF	Eukaryotic release factors
FUr	5-Fluorouridine
FdUr	5-Fluoro-2′-deoxyuridine
GOF	Gain-of-function
GSH	Reduced glutathione
HER2	Human epidermal growth factor receptor 2
IMRT	Intensity-modulated radiation therapy
ICI	Immune checkpoint inhibitor
LFS	Li-Fraumeni syndrome
LNP	Lipid nanoparticle
LOH	Loss of heterozygosity
MDM2	Mouse double minute 2
NMD	Nonsense mediated decay
MQ	Methylene quinuclidinone
mRNA	Messenger RNA
MuLV	Moloney murine leukemia virus
NLS	Nuclear localization signal
NSCLC	Non-small cell lung cancer
P-site	Peptidyl-site
PABP	Poly(A) binding protein
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death-ligand 1

PHTS	PTEN Hamartoma Tumor Syndrome
PI3K	Phosphatidylinositol-3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B (AKT)
PPARγ	Peroxisome Proliferator-Activated Receptor γ
PRD	Proline-rich domain
PTC	Premature termination codon
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	Quantitative Real-Time PCR
RB	Retinoblastoma
Ribo-seq	Ribosome sequencing
rRNA	Ribosomal RNA
SBRT	Stereotactic body radiation therapy
SURF	**S**MG-1/**U**pf1/e**RF**1/eRF3 complex
SV40	Simian virus 40
SMG	Suppressor of morphogenesis of genitalia
TAD 1/2	Transactivation domain 1/2
TD	Tetramerization domain
TMV	Tobacco mosaic virus
TKI	Tyrosine kinase inhibitor
tRNA	Transfer-RNA
TrxR1	Thioredoxin reductase
TS	Thymidylate synthase
TP53	Tumor protein 53

UPF	Up-frameshift
VEGF	Vascular endothelial growth factor
WST-1	Water-soluble tetrazolium-1
WT	Wild-type
WHO	World Health Organization
ZIP	Zero Interaction Potency

1 INTRODUCTION

1.1 Cancer

Cancer is a word used to describe a diverse group of disorders that share certain traits, the most fundamental being abnormal or disrupted regulation of cell proliferation and survival (Hanahan and Weinberg, 2011, Hanahan and Weinberg, 2000). Cancer development is caused by dysregulation of one or more of these regulatory mechanisms and can occur due to activating mutations in genes that drive cell growth (oncogenes) or inactivating mutations in genes that prevent cancer development (tumor suppressor genes). Mutations occur regularly because of exposure to external and internal mutagenic sources, such as ultraviolet light and ionizing radiation, oxidation and other metabolic reactions, chemical carcinogens and biological carcinogens, such as infections by viruses, bacteria and parasites. However, mutations are not only the result of external and internal mutagenic sources but may occur due to deoxyribonucleic acid (DNA) replication errors during cell proliferation (Stratton et al., 2009, Vogelstein and Kinzler, 2004, Hanahan and Weinberg, 2011).

An important contribution to our understanding of cancer originated from the work of Theodor Boveri in the early 20th century. Boveri proposed the chromosomal theory of cancer, suggesting that chromosomal abnormalities within cells could lead to cancer development. Boveri's hypothesis was founded on his observations in sea urchin eggs, where irregularities in chromosome distribution during cell division resulted in abnormal development (Boveri, 1914, Manchester, 1995). Boveri's work significantly influenced the present understanding of cancer as a genetic disease and formed a founding concept for the widely accepted genetic model of cancer today (Manchester, 1995, Hanahan, 2022).

Over the past century, significant advancement has been made in elucidating the fundamental principles of cancer development and progression, summarized in three seminal papers by Douglas Hanahan and Robert A. Weinberg. In their initial paper, "The Hallmarks of Cancer," Hanahan and Weinberg proposed six key hallmarks that collectively characterize cancer. One of the hallmarks is "self-sufficiency in growth signals." Unlike normal cells, which require external signals for proliferation, cancer cells exhibit the ability to proliferate autonomously. This is achieved through the production of their own growth factors or the presence of overactive or hypersensitive signaling pathways. Another hallmark is "insensitivity to anti-growth signals," where cancer cells can evade inhibitory signals from tumor

suppressors that typically regulate cell proliferation, thereby avoiding the control mechanisms that prevent uncontrolled cell division. The hallmark "evading apoptosis" reveals that while most normal cells possess a mechanism for programmed cell death, or apoptosis, cancer cells have evolved strategies to resist this process, contributing to their uncontrolled proliferation. Another hallmark, "limitless replicative potential", highlights that cancer cells, unlike their normal counterparts, can replicate indefinitely. This capability is attributed to the overactivation of the telomerase enzyme, enabling cancer cells to continuously divide and replicate by lengthening their telomers. The fifth hallmark, "sustained angiogenesis," underscores that tumors require nutrients and oxygen for growth and metastasis. To meet these demands, they stimulate the formation of new blood vessels, a process known as angiogenesis. Finally, the hallmark "tissue invasion and metastasis" is the most destructive feature of cancer, accounting for 90% of all cancer-related deaths. This hallmark characterizes how cancer cells reorganize their cytoskeleton and interact with the extracellular matrix, facilitating invasion of adjacent tissue and metastasis to distant sites (Hanahan and Weinberg, 2000).

In their subsequent paper, "Hallmarks of Cancer: The Next Generation," Hanahan and Weinberg expanded upon the original six hallmarks to incorporate two enabling characteristics and two emerging hallmarks. The first enabling characteristic, "genome instability and mutation," indicates that cancer cells are predisposed to acquiring and accumulating genetic alterations that facilitate the development of the other hallmarks. The tumor suppressor gene *TP53* is instrumental in maintaining genomic integrity by inducing senescence or apoptosis in aberrant cells. The second enabling characteristic, "tumor-promoting inflammation," describes how inflammation contributes to multiple hallmarks by providing tumor promoting factors to the tumor microenvironment. These molecules encompass growth and survival factors, proangiogenic factors, and extracellular matrix molecules that mediate angiogenesis, invasion, and metastasis. The emerging hallmark, "reprogramming energy metabolism," emphasizes that while normal resting cells primarily generate energy through oxidative phosphorylation, cancer cells predominantly utilize aerobic glycolysis. This hallmark indicates that cancer cells undergo metabolic reprogramming to support growth, survival, proliferation, and long-term maintenance. The emerging hallmark, "avoiding immune destruction," asserts that under normal conditions, the immune system identifies and eliminates aberrant cells. However, cancer cells often manage to evade the immune system by suppressing immune responses and establishing an immune-privileged status (Hanahan and Weinberg, 2011).

Recently, a new iteration of the Hallmarks of Cancer series was published wherein additional emerging hallmarks and enabling characteristics were proposed. The author propose that disrupted cellular differentiation, nonmutational epigenetic reprogramming, the microbiome as well as senescent cells of the tumor micro-environment, all play important roles in tumor development and progression (Hanahan, 2022).

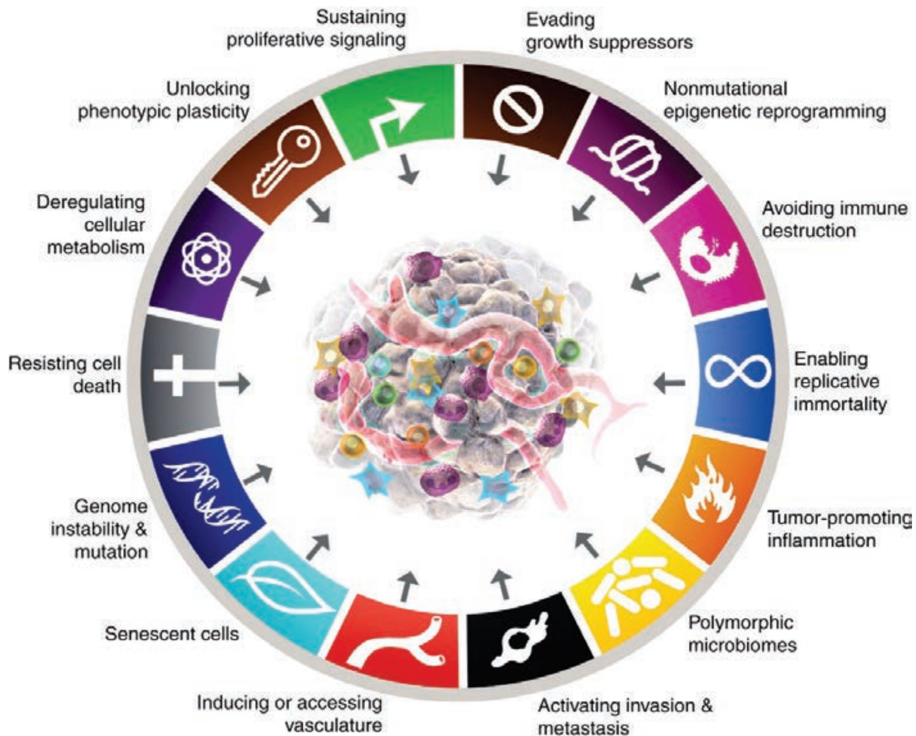

Figure 1. Hallmarks of cancer. An illustration summarizing all suggested cancer hallmarks throughout the Hallmarks of Cancer series. Illustration from: Hanahan D, *Hallmarks of cancer: new dimensions*, American association for cancer research

1.1.1 Cancer genes

As mentioned previously, cancer arise when key genes become mutated and lose their function or gain new functions. These genes can be classified into two main categories based on their canonical functions to either promote cell proliferation or inhibit it. Proto-oncogenes are genes that, under normal conditions, play a role in promoting cell growth and division. However, when proto-oncogenes are mutated, they convert to oncogenes that are constitutively active causing normal cells to become malignant (Vogelstein et al., 2013). Some notable oncogenes are the *MYC* gene coding for the transcription factor Myc who functions by regulating

cell growth and proliferation. However, if *MYC* becomes overexpressed or mutated it can cause uncontrolled cellular proliferation and tumorigenesis (Dang, 2012). The *RAS* family of genes (*KRAS*, *NRAS* and *HRAS*) are the most mutated oncogenes in human cancers and play a pivotal role in intracellular signal transduction. Mutated *RAS* can cause persistent signaling that induces uncontrolled cell growth via activation of MAPK and PI3K signaling pathways (Moore et al., 2020). The *ABL* gene encodes a non-tyrosine kinase essential for cell differentiation and proliferation. Aberrant activation of *ABL*, as a consequence of chromosomal translocation leading to formation of the fusion protein Bcr-Abl, is associated with the development of chronic myeloid leukemia (CML) (Sawyers, 1999).

Tumor suppressor genes on the other hand, carry out inhibitory effects by regulating cell division and ensuring cells do not grow uncontrollably. Mutations that inactivate these genes promote tumorigenesis. The retinoblastoma susceptibility gene (*RB1*) and its gene product pRB is one notable example. pRB functions by limiting transcription of genes that regulate cell cycle progression primarily through regulation of the transcription factor E2F. Loss of *RB1* through mutation or increased phosphorylation of pRB lead to a pro-oncogenic phenotype due to compromised cell-cycle regulation (Dyson, 2016). The adenomatous polyposis coli (*APC*) tumor suppressor gene is involved in cell cycle regulation and adhesion. Around 80% of all colorectal cancers (CRC) harbors mutations in *APC* causing dysfunction in the cytoskeletal regulation, promoting cell migration, reduced cell adhesion and adenoma development (Aghabozorgi et al., 2019). *TP53* and *PTEN* are two other important tumor suppressor genes which will be discussed in detail in upcoming sections.

1.1.2 Cancer therapeutic approaches

Historically, three primary therapeutic modalities have been utilized in oncology: surgery, radiation, and chemotherapy. Each plays a crucial role in cancer management and continues to be a significant part of the treatment repertoire.

Surgery can be used both curative by completely removing a localized tumor or to alleviate symptoms by excising as much tumor mass as possible, paving the way for subsequent treatments (Siamof et al., 2020).

Radiation therapy is another key therapeutic approach for cancer. It involves the use of high-energy radiation to induce DNA damage in cancer cells, leading to cell death. Radiation therapy is commonly employed in conjunction with surgery or chemotherapy to enhance the efficacy of these treatments. Innovative techniques, such as intensity-modulated radiation therapy (IMRT) and

stereotactic body radiation therapy (SBRT), have allowed for more precise targeting of tumors, while sparing adjacent normal tissues (Corradini et al., 2019).

Chemotherapy represents a systemic approach that employs cytotoxic agents aimed at exterminating rapidly dividing neoplastic cells. The mechanism of action of chemotherapeutic agents involves perturbation of cellular division processes, indiscriminately affecting cancerous and healthy cells alike, leading to potential adverse effects, including nausea, alopecia, and immunosuppression. Noteworthy examples of chemotherapeutic drugs include cisplatin and 5-fluorouracil (Longley et al., 2003, Rosenberg et al., 1965).

While these three pillars remain fundamental in cancer treatment, it is essential to recognize the increasing role of targeted therapies, immunotherapies, and hormone therapies in cancer management. These newer approaches, often used in combination with the traditional methods, represent a more personalized and tailored approach to cancer treatment (Wargo et al., 2015).

1.1.3 Conventional cancer therapy

Conventional chemotherapy plays a pivotal role in management of numerous malignancies. Traditional cytotoxic drugs, such as 5-fluorouracil (5-FU), cisplatin, doxorubicin, methotrexate and cyclophosphamide, have formed the backbone of oncology therapeutics for several decades. 5-FU, a pyrimidine analog, was discovered in 1957 and continues to be a crucial part of treatment regimens for colorectal, breast, and head and neck cancers (Heidelberger et al., 1957). The mechanism of action of 5-FU is mediated by its metabolites 5-fluorouridine (FUr) and 5-fluoro-2'-deoxyuridine (FdUr). The DNA-incorporating metabolite FdUr inhibits thymidylate synthase (TS) in its monophosphate form causing impaired DNA replication. The triphosphate form of FdUr incorporates into DNA causing DNA damage and subsequent p53 activation. FUr incorporates into RNA and affects RNA processing causing cytotoxicity (Grem, 2000, Chalabi-Dchar et al., 2021). Additionally, the clinical efficacy of 5-FU is occasionally tempered by issues of toxicity and the development of resistance (Longley et al., 2003).

Cisplatin is a platinum-based compound that was discovered by Rosenberg et al., in the middle of the 1960s (Rosenberg et al., 1965). It has served as a cornerstone in the treatment of various malignancies, including testicular, ovarian, bladder, head and neck and lung cancers. The mechanism of action for cisplatin is complex involving several signaling pathways. Cisplatin is initially inert and is activated in the cytoplasm by aquation reactions forming highly reactive mono- and bi-aquated cisplatin. These active forms of cisplatin interact with many cytoplasmic

substrates, for example reduced glutathione (GSH) causing a redox shift, ultimately leading to oxidative stress. Additionally, aquated cisplatin binds DNA leading to DNA damage, ultimately triggering DNA repair pathways and induction of apoptosis. Cisplatin treatment is often associated with serious side effects, such as nephrotoxicity, and the emergence of resistance (Galluzzi et al., 2012).

Doxorubicin, a non-selective class I anthracycline, has been used extensively for the treatment of a range of malignancies, including solid tumors, leukemia and lymphomas. It exerts its anti-tumor effects by DNA helix intercalation, causing DNA damage and activation of apoptosis pathways. Doxorubicin also inhibits the topoisomerase enzymes I and II, causing inhibition of DNA replication and transcription. A significant drawback of doxorubicin therapy is the risk of cardiotoxicity, limiting its long-term use (Tacar et al., 2013).

Methotrexate is a folate analog that inhibits the enzyme dihydrofolate reductase, which is involved in *de novo* synthesis of purines and pyrimidines, thus starving highly proliferative cells of their DNA and RNA building blocks, thereby disrupting DNA replication (Cronstein, 1997).

Cyclophosphamide is a prodrug that requires metabolic activation resulting in the active product phosphoramide mustard which is responsible for its cytotoxic effects. Phosphoramide mustard causes interstrand and intrastrand DNA crosslinks, disrupting DNA replication and inducing cell death (Emadi et al., 2009).

These conventional chemotherapeutic agents have significantly improved the survival rates for many cancers, but challenges including drug resistance and adverse effects persist. The evolution of targeted chemotherapeutic agents and the stratification of patients based on genetic markers have improved the specificity and efficacy of chemotherapy. Overcoming drug resistance, often the reason for therapeutic failure, remains a major challenge in oncology. Current research is directed towards understanding the mechanisms underlying drug resistance to develop more efficacious chemotherapeutic regimens (Holohan et al., 2013).

1.1.4 Targeted therapy

In the last two decades, the landscape of cancer treatment has been dramatically revolutionized by the development of targeted therapies. These therapeutic strategies aim at specifically target molecular alterations associated with tumor progression while minimizing toxicity to normal cells. Imatinib (Glivec) is a tyrosine kinase inhibitor (TKI) and targets the constitutively active tyrosine kinase BCR-

ABL fusion protein commonly found in CML, thus blocking oncogenic signals and inducing apoptosis. The introduction of Glivec has completely changed the outcome for CML patients from a fatal disease into a manageable chronic condition, demonstrating the potential of targeted therapies in cancer treatment (Druker et al., 2001). Another tyrosine kinase inhibitor, crizotinib, targets the anaplastic lymphoma kinase (ALK) and is used to treat tumors harboring ALK rearrangements in for example non-small cell lung cancer (NSCLC), substantially improving patient outcomes (Shaw et al., 2013). Approximately 40-60 % of malignant cutaneous melanomas have a mutation in the BRAF gene causing constitutive activation of the BRAF kinase, driving cancer growth. Vemurafenib is a BRAF inhibitor that selectively targets mutated BRAF and has shown significant improvements in overall survival in melanoma patients (Chapman et al., 2011). The human epidermal growth factor receptor 2 (HER2) is overexpressed in about 25-30% of all breast cancers. Tumors with overactivation of HER2 can be targeted by the monoclonal antibody trastuzumab (Herceptin) alone or in combination canonical chemotherapeutic drugs. These treatments have shown significant benefits in both early and metastatic HER2-positive breast cancer, prolonging survival and improving quality of life (Slamon et al., 2001). Another monoclonal antibody, Bevacizumab (Avastin) inhibits vascular endothelial growth factor (VEGF), that has an important role for formation of new blood vessels. The prevention of angiogenesis in tumors lead to inhibition of tumor growth (Ferrara et al., 2004).

Immune checkpoint inhibitors (ICIs) have emerged as a revolutionary treatment in the field of oncology, significantly impacting the management of a broad spectrum of malignancies. The antitumor effect is achieved by enhancing the immune system´s response to cancer cells. ICIs have demonstrated remarkable efficacy in producing durable tumor responses, sometimes even in advanced, previously untreatable cases. The first immune checkpoint receptor to be investigated was the cytotoxic T lymphocyte–associated antigen 4 (CTLA-4). The CTLA-4 immune checkpoint receptor is upregulated on the plasma membrane of activated T-cells where it transmits inhibitory signals to T-cells. The realization that CTLA-4 is a negative regulator of immunity prompted efforts to investigate if antibody blockade of CTLA-4 could elicit an anti-tumor response. Another immune checkpoint targeted by monoclonal antibodies is programmed cell death protein 1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1). PD-1 functions by negatively regulating T-cell activity following binding to its ligands PD-L1 and PD-L2. Monoclonal antibodies against both PD-1 and PD-L1 have been developed and have demonstrated highly durable responses. Targeting immune checkpoint proteins, such as CTLA-4 and PD-1/PD-L1, have proven remarkable efficacy in

certain types of cancer. By blocking these pathways, ICIs enhance the ability of immune cells to recognize and attack cancer cells (Postow et al., 2015).

1.1.5 Combination treatment

As previously mentioned, cancer is a collective term describing complex diseases characterized by multiple genetic and epigenetic alterations, heterogeneity and adaptability. Consequently, single-agent therapy often results in treatment resistance, disease progression or recurrence. To overcome these obstacles, combination therapies have emerged as a powerful strategy in the oncology field. Combination therapy involves the concurrent use of two or more therapeutic agents or modalities. This approach provides several potential benefits. For example, tumor cells can develop resistance to single-agent therapies through various mechanisms, such as gene mutations, activation of compensatory pathways, and alterations in drug targets. By simultaneously targeting different signaling pathways or cancer hallmarks, it is less likely that resistance emerges (Holohan et al., 2013). Additionally, simultaneous targeting of multiple tumor-associated pathways can lead to synergistic effects, enhancing therapeutic efficacy beyond what can be achieved with single-agent therapy. For instance, the combination of BRAF and MEK inhibitors in BRAF-mutant melanoma has shown improved survival rates compared to BRAF inhibitor monotherapy (Larkin et al., 2014). Combination therapy can also often allow therapeutic efficacy at lower dosages of each individual agent, which may mitigate treatment-associated toxicities (Al-Lazikani et al., 2012). Tumors often consist of a heterogenous population of cells. The subclones residing in a tumor can have different molecular alterations affecting different signaling pathways. The combination therapy approach can target multiple aberrations, thus accounting for the intratumor heterogeneity more effectively than single-agent therapies (McGranahan and Swanton, 2017).

1.2 The *TP53* gene

The *tumor protein 53* (*TP53*) tumor suppressor gene codes for a transcription factor (p53) that is involved in several processes striving to maintain cellular integrity, including DNA repair, cell cycle arrest, metabolism, apoptosis and senescence (Vousden and Prives, 2009). The p53 protein is divided into several distinct domains, namely two N-terminal transactivation domains (TAD 1/2), a proline-rich domain (PRD), a central DNA-binding domain (DBD) and a C-terminal part containing several elements including a tetramerization domain (TD) and a

nuclear localization signal (Kastenhuber and Lowe, 2017). Since its discovery in 1979 by several independent research groups (Linzer and Levine, 1979, Lane and Crawford, 1979, DeLeo et al., 1979, Melero et al., 1979), the perception of *TP53* in carcinogenesis has dramatically evolved, shifting from a presumed oncogene function to being a genuine tumor suppressor gene. Initially, p53 was identified as a protein that binds to the viral large T-antigen in cells transformed by simian virus 40 (SV40), leading to the belief that p53 was an oncogene (Lane and Benchimol, 1990). The p53 discovered in these early studies was later recognized as a mutated form, which explains its association with transformed cells. The view of p53 as an oncogene was challenged by several subsequent studies. A key experiment was performed by Finlay et al., who introduced wild-type (WT) p53 into cancer cell lines and observed that these cells underwent growth arrest and apoptosis, indicating that p53 acts as a tumor suppressor (Finlay et al., 1989). The breakthrough in understanding the role of p53 in cancer came when a series of studies showed that p53 was frequently mutated in various human tumors (Nigro et al., 1989). The majority of *TP53* mutations occur in its DNA-binding domain, impairing its function as a transcription factor and therefore its tumor-suppressive activity (Sabapathy and Lane, 2018). These findings have led to the recognition of p53 as "the guardian of the genome," a molecule that plays a critical role in maintaining genomic stability and preventing malignancy (Donehower et al., 2019, Lane, 1992).

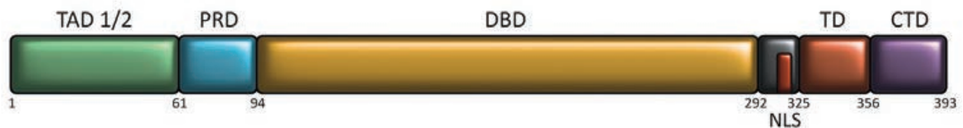

Figure 2. Domains of p53. Schematic illustration showing the various domains of the p53 protein. Featured are the transactivation domains 1 and 2 (TAD 1/2), the proline-rich domain (PRD), the DNA binding domain (DBD), the tetramerization domain (TD), and the C-terminal domain (CTD). Additionally, the nuclear localization signal (NLS) is highlighted.

1.2.1 p53 function

Activation of p53 occurs as a response to a multitude of malignant stress factors including DNA damage, oncogene activation and hypoxia. Activation of p53 can evoke several responses aimed at preserving cellular integrity by induction of cell cycle arrest, senescence, apoptosis and repair of genotoxic damage (Vousden and Lu, 2002). The cellular response of p53 is achieved by activation of p53 target genes, such as *CDKN1A* , *BAX, PUMA, FAS, NOXA, WIG-1* and many more (Vousden and Prives, 2009). One of the most classical target genes of p53 is *CDKN1A*,

encoding p21, which is activated following DNA damage, resulting in cell cycle arrest at the G1 check point, thus allowing the cell to activate DNA repair mechanisms before further cell division occurs. If the damage is irreparable, p53 can induce apoptosis or senescence via activation of cell cycle inhibitors such as p16 and the retinoblastoma (RB) protein. The pro-apoptotic property of p53 comes from its ability to transactivate members of the B-cell lymphoma 2 (BCL-2) gene family, such as bcl-2-like protein 4 (BAX) and p53 upregulated modulator of apoptosis (PUMA) that act at mitochondria and trigger caspase activation and apoptosis (Shaw et al., 1992, Kastenhuber and Lowe, 2017). It has also been shown that p53 has a role in regulating cellular metabolism. It modifies metabolic pathways, such as glycolysis and oxidative phosphorylation, to adapt to metabolic stresses and prevent oxidative DNA damage (Berkers et al., 2013).

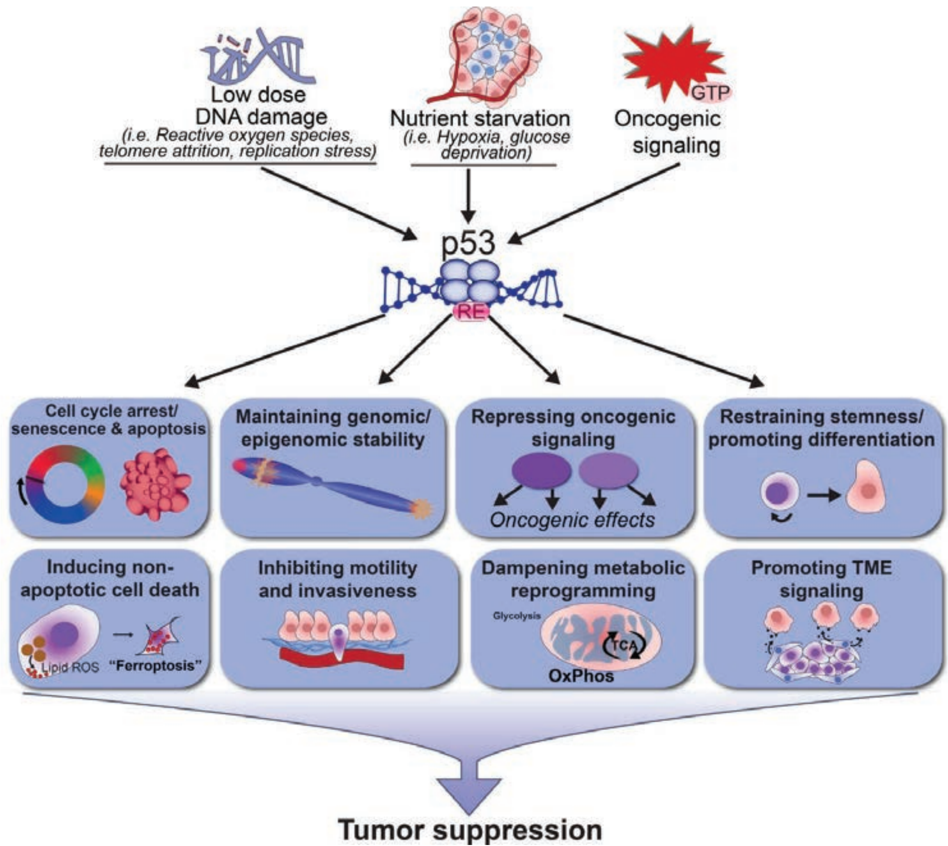

Figure 3. Activation and function of p53. Schematic representation of p53 activation pathways as well as the intracellular responses elicited by its upregulation. Illustration from Mello SS and Attardi LD, *Deciphering p53 signaling in tumor suppression (2018)*, Current Opinion in Cell Biology

1.2.2 p53 regulation

Under normal conditions, p53 levels are kept low due to stringent control by an E3 ligase, encoded by the p53 target gene mouse double minute 2 (*MDM2*) (Brooks and Gu, 2006). The inhibitory effect of *MDM2* is achieved by binding of its protein product Mdm2 to the N-terminal transactivation domain of p53 and ubiquitination of the C-terminal regions of p53. By binding the transactivation domain of p53, Mdm2 effectively inhibits p53's ability to interact with the transcriptional machinery. The C-terminal ubiquitination of p53 causes transport into the cytoplasm where p53 is degraded by the proteasome (Moll and Petrenko, 2003). However, in response to various cellular stress factors such as oncogenic signaling or DNA damage, p53 is rapidly stabilized through a series of post-translational modifications causing p53 levels to rise. The post-translational modifications of p53 mainly occur in the transactivation domain located in the N-terminal, disrupting p53-mdm2 binding, and in the C-terminal regulatory domain (Lavin and Gueven, 2006). The manner in which p53 is stabilized differs depending on the stress stimuli the cell encounters. DNA damage promotes p53 phosphorylation via activation of kinases, such as ATM and CHK2, while oncogenic signaling promotes induction of the ARF tumor suppressor that inhibits MDM2 (Kastenhuber and Lowe, 2017). Oncogenic signaling can also induce DNA replication stress that activates p53 via phosphorylation by the above mentioned kinases (Halazonetis et al., 2008).

1.2.3 *TP53* mutations

According to the World Health Organization (WHO), there are over 19 million new cancer cases reported globally every year. (Sung et al., 2021). *TP53* is commonly inactivated in human cancers; in fact, around 50 % of all human tumors carry inactivating *TP53* mutations (Vogelstein et al., 2000). Most *TP53* mutations (around 63%) are missense mutations resulting in a single nucleotide change that alters the amino acid sequence. This results in altered binding capacity or misfolding and subsequent inactivation of the p53 protein (Soussi and Wiman, 2007). The most common missense mutations are R175H, R248Q and R273H. Another type of mutation commonly seen in *TP53* is called nonsense mutation, in which a single nucleotide substitution results in formation of a premature termination codon (PTC) causing translation of truncated and often non-functional p53 (Tate et al., 2019). Around 11 % of all *TP53* mutations are nonsense mutations, translating into approximately 1 000 000 new cancer cases harboring nonsense mutant *TP53* annually (Sung et al., 2021, Tate et al., 2019). Loss of p53 function due to mutations is associated with cancer development and

progression (Vousden and Prives, 2009). Inactivation of p53 is also associated with genomic instability that can lead to accumulation of other driver mutations that potentially act to accelerate transformation, metastasis and drug resistance. As a result, mutations in *TP53* are linked to poor prognosis in many cancers (Kastenhuber and Lowe, 2017).

1.2.4 Trp53 deficient mouse models

Human p53 and mouse p53 share a high degree of similarity, making mouse models an invaluable tool for in vivo evaluation of the biological functions and mechanisms of both mutated and WT p53 in tumorigenesis and treatment. In the early 1990s, the first germline *Trp53* (human *TP53* homologue) null (*Trp53*$^{-/-}$) mice were generated by deleting one or more *Trp53* exons, leading to knockout alleles with no WT p53 activity (Donehower et al., 1992, Jacks et al., 1994). A decade later, researchers reported the first mouse models with germline missense *Trp53* knock-in mutations, encoding the structural mutants Trp53 R172H and Trp53 R172P, which altered the p53 DNA-binding domain conformation, and the contact mutant Trp53 R270H, affecting p53 residues that interact with DNA (collectively referred to as *Trp53*^{Mis}) (Lang et al., 2004, Liu et al., 2004, Olive et al., 2004).

Trp53$^{-/-}$ and *Trp53*^{Mis} mice exhibit a pronounced cancer phenotype, characterized by a high incidence of spontaneous tumors in various tissues and reduced survival compared to WT mice (Donehower et al., 1992, Jacks et al., 1994, Lang et al., 2004, Olive et al., 2004), highlighting p53's critical role as a tumor suppressor. Both *Trp53*$^{-/-}$ and *Trp53*$^{Mis/Mis}$ mice start developing tumors from 3 to 6 months of age, while tumors appear around 9 to 10 months in heterozygot *Trp53*$^{+/-}$ and *Trp53*$^{Mis/+}$ mice, usually associated with loss of the WT allele. The tumor spectrum in both *Trp53*$^{-/-}$ and *Trp53*$^{Mis/Mis}$ mice primarily includes lymphomas and sarcomas, while heterozygotes also develop carcinomas. Interestingly, *Trp53*$^{Mis/+}$ models exhibit a higher carcinoma rate than *Trp53*$^{+/-}$ mice. *Trp53*$^{R172H/+}$ and *Trp53*$^{R270H/+}$ mice tend to develop more invasive and metastatic tumors compared to *Trp53*$^{+/-}$ mice (Lang et al., 2004, Olive et al., 2004).

Recently, three novel germline missense knock-in mouse models for hotspot p53 mutations were published: the contact mutant *Trp53*^{R245W} (human *TP53*^{R248W}) (Xiong et al., 2022), and tetramerization domain mutations *Trp53*^{R339P} and *Trp53*^{A344D} (human *TP53*^{R342P} and *TP53*^{A347D}) (Gencel-Augusto et al., 2023). When compared to *Trp53*^{R172H}, *Trp53*^{R270H}, and *Trp53*$^{-/-}$ the *Trp53*^{R245W} mice showed similar loss of heterozygosity (LOH) in tumors and inhibitory effects on WT *Trp53*

transcription. However, the two contact mutants demonstrated stronger gain-of-function (GOF) activities (Xiong et al., 2022).

1.2.5 p53 targeted therapies

In cancer cells, the function of p53 is often compromised through its interaction with the negative regulator, MDM2, which is often overexpressed in human cancers. Therapeutic strategies have been developed to reactivate or stabilize the function of WT p53, primarily by inhibiting the interaction between p53 and MDM2. Nutlin-3, a cis-imidazoline analog, was one of the first compounds identified as an inhibitor of the MDM2-p53 interaction. Preclinical studies have demonstrated that Nutlin-3 can induce levels of p53, resulting in cell cycle arrest and apoptosis in cancer cells (Vassilev et al., 2004). Subsequent research has led to the development of more potent and selective MDM2 antagonists, such as RG7112 and RG7388 (idasanutlin). In particular, idasanutlin has shown enhanced potency and selectivity for MDM2 relative to Nutlin-3 (Tovar et al., 2013, Andreeff et al., 2016, Ding et al., 2013). Another notable MDM2 antagonist is AMG-232 (KRT-232), a small molecule that has shown significant antitumor activity in preclinical studies. AMG-232 has been assessed in clinical trials for the treatment of various cancers, and the results have provided valuable insights into its potential usefulness (Canon et al., 2015, Gluck et al., 2020). In addition to targeting MDM2, there has been interest in inhibiting the interaction between p53 and MDMX, an MDM2 homolog that also binds to and inhibits p53. ALRN-6924, a stapled peptide, has been developed to inhibit both MDM2 and MDMX, thereby activating p53. Preclinical studies have shown that ALRN-6924 has antitumor activity, and it is currently undergoing clinical evaluation for various cancers (Carvajal et al., 2018, Saleh et al., 2021).

Overall, therapeutic targeting of WT p53 holds promise for the treatment of cancer. However, challenges remain, such as toxicity and the development of resistance to these agents. Further research is needed to optimize the efficacy and safety of these therapies, identify appropriate patient populations, and explore combination strategies.

As mentioned earlier, *TP53* mutations are present in approximately 50% of all human cancers, prompting efforts to find compounds that target the mutated p53. Both PRIMA-1 and APR-246 (PRIMA-1Met) has emerged as promising strategies for the reactivation of mutant p53. These compounds are prodrugs that are spontaneously converted to the active product methylene quinuclidinone (MQ). They function by covalently binding to several cysteines in the p53 core domain thereby promoting WT conformation of the mutant protein. This process

re-establishes the DNA binding and transcriptional activity of p53, resulting in apoptosis and cell cycle arrest in cancer cells (Bykov et al., 2002, Bykov et al., 2018). In clinical trials, APR-246, in combination with other chemotherapy agents, has demonstrated promising antitumor effects in various malignancies harboring p53 mutations (Sallman et al., 2021). APR-246/MQ can also target glutathione, thioredoxin reductase (TrxR1) and other cellular redox regulators (Bykov et al., 2016).

COTI-2 is a third-generation thiosemicarbazone that has been found to reactivate the normal p53 pathway in tumors harboring mutated p53. By inducing a conformational change in mutant p53, COTI-2 can restore the protein's normal function, leading to apoptosis and inhibition of tumor growth. Preclinical studies have demonstrated the potential effectiveness of COTI-2 against various cancer types (Lindemann et al., 2019, Synnott et al., 2020).

ATO is a trivalent arsenic compound that binds covalently to cysteine residues, inducing a refolding of the mutant p53 protein to a WT conformation. Recent studies have highlighted the role of ATO in inducing cell cycle arrest and apoptosis in cancer cells harboring mutant p53 (Liu et al., 2003). ATO's ability to restore p53's tumor suppressor function makes it a promising candidate for targeted cancer therapy, especially in tumors driven by p53 mutations (Chen et al., 2021). Moreover, the application of ATO for p53 reactivation complements its other known anti-tumor mechanisms. In acute promyelocytic leukemia, ATO promotes degradation of the oncogenic PML-RARα fusion protein and induces differentiation and apoptosis in leukemic cells (Zhang et al., 2010).

Moreover, compounds that specifically target the p53 missense mutation Y220C have been identified. The small molecule PK7088 acts by binding to misfolded Y220C p53, thus increasing its melting point and promoting re-folding into WT conformation. The resulting p53 product was shown to be functionally active by upregulation of p53 target genes (Liu et al., 2013). PC14586 is a more recently developed small molecule that has a similar mechanism of action as PK7088 by binding to misfolded Y220C p53 and promoting WT conformation. PC14586 is currently being tested in a First-in-human clinical trial in patients with advanced solid tumors harboring the *TP53* Y220C mutation (Dumbrava et al., 2022). Lastly, KG13 targets Y220C mutant p53 by covalently binding the mutant cysteine, restoring thermal stability and WT conformation (Guiley and Shokat, 2023).

1.3 The *PTEN* gene

The tumor suppressor gene *phosphatase and tensin homolog* (*PTEN*) was identified in 1997 following genetic mapping of chromosome 10q23, a locus frequently mutated in human cancers. *PTEN* plays an essential role in regulating several cellular processes including regulating cell cycle progression, cellular growth, survival and proliferation (Li et al., 1997, Chen et al., 2018). The *PTEN* gene produces a bifunctional phosphatase protein with the capacity for both lipid and protein dephosphorylation. It modulates the Phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathway by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP_3) converting it to phosphatidylinositol-4,5-bisphosphate (PIP_2) (Song et al., 2012). The protein is structured into several domains that mediate its complex functions. The PIP_2-binding domain is located at the N-terminal and facilitates binding to PIP_2. The phosphatase domain is central to the enzymatic activity of PTEN, enabling the dephosphorylation of its substrates. The function of the C2 domain is to facilitate PTEN binding to the cell membrane, positioning the phosphatase domain near its lipid substrate (Lee et al., 1999). Finally, two PEST sequences and a C-terminal PDZ-binding domain, interacting with PDZ domain-containing proteins, potentially influence PTEN's stability and activity (Valiente et al., 2005).

Figure 3. PTEN protein domains. A schematic representation of the PTEN domains including the PIP_2-binding domain (PBD), the phosphatase domain, the C2 domain and the C-tail. The two PEST sequences and the PDZ binding motif are located in the C-terminal tail.

1.3.1 PTEN function

One of the most prominent functions of PTEN is its activity as a lipid phosphatase, particularly its role in inhibiting the PI3K/AKT signaling pathway. This pathway is central for the regulation of cell proliferation, survival, and growth (Maehama and Dixon, 1998). PTEN acts primarily by dephosphorylating PIP_3, a lipid second messenger involved in the transmission of signals for cell growth and survival; the reduction of PIP_3 levels effectively downregulates the PI3K/AKT signaling pathway, thus promoting apoptosis and inhibiting cell proliferation (Lee et al., 2018). Loss of function mutations or deletions in the *PTEN* gene have been identified in several types of human cancers, reinforcing its critical role as a tumor suppressor. Mutations can result in uncontrolled activation of the PI3K/AKT pathway, thereby

contributing to unregulated cell proliferation and tumor development (Li et al., 1997). In addition to its function as a tumor suppressor, PTEN also plays an essential role in other biological processes. For example, it is involved in cellular adhesion and migration, thus influencing how cells interact with each other and their environment (Milella et al., 2015). Furthermore, PTEN has been shown to be involved in neuronal survival and plasticity, suggesting a potential role in neurologic disorders (Endersby and Baker, 2008). Given the essential role of PTEN in maintaining cellular homeostasis, understanding its function and regulation is of high significance for the development of therapeutic strategies against diseases associated with its dysfunction, including cancer.

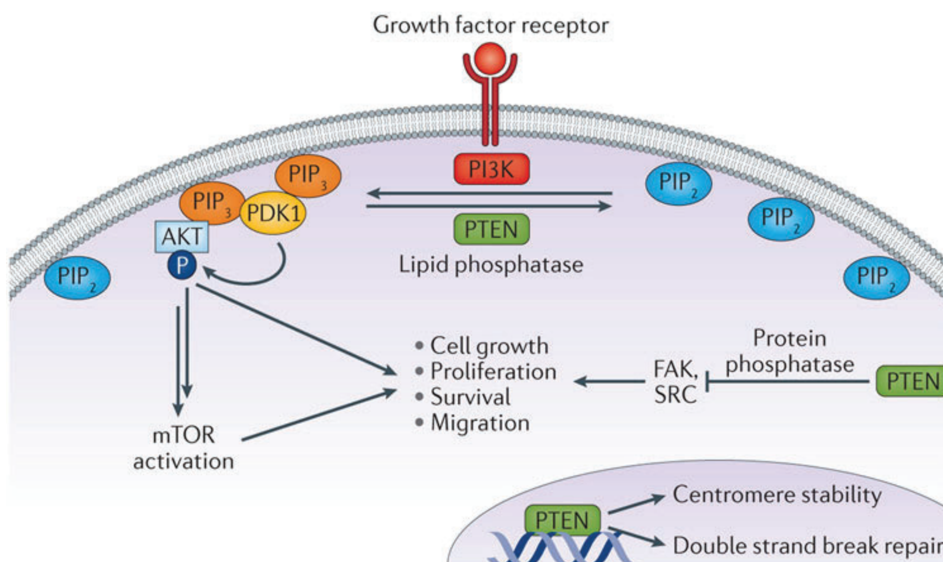

Figure 4. PTEN function. Schematic representation showing how PTEN dephosphorylates PIP_3 to PIP_2, thus inhibiting PI3K signalling and subsequent inhibition of proliferative signals. illustration from Jamaspishvili T, et al. *Clinical implications of PTEN loss in prostate cancer*, Nature reviews Urology.

1.3.2 PTEN regulation

Transcriptionally, PTEN expression is regulated by various transcription factors such as Peroxisome Proliferator-Activated Receptor γ (PPARγ), p53 and Early Growth Response 1 (EGR1). The p53 transcription factor upregulates PTEN expression by binding to the *PTEN* promoter, providing a link between these two critical anticancer pathways (Freeman et al., 2003, Chen et al., 2018). EGR1 is another transcription factor that binds to the *PTEN* promoter to stimulate its expression, providing a mechanism of PTEN regulation in response to cellular

stress (Virolle et al., 2001). Similarly, PPARγ stimulates PTEN transcription and inhibits AKT phosphorylation and thereby cell proliferation (Patel et al., 2001). PTEN is also subject to regulation via post-translational modifications, including phosphorylation and ubiquitination which affect its stability, cellular localization, and enzymatic activity (Vazquez et al., 2000, Leslie and Foti, 2011).

1.3.3 *PTEN* mutations

Mutations in the *PTEN* gene are frequently observed in several types of cancer, including breast cancer, glioblastoma and prostate cancer (Li et al., 1997). These mutations can range from deletions, insertions, missense and nonsense mutations, and can lead to the loss of protein function (Hollander et al., 2011). A significant proportion of PTEN mutations are located within the phosphatase domain of the protein, which is critical for its enzymatic activity, hence disrupting its ability to downregulate the PI3K/AKT pathway (Lee et al., 2018, Waite and Eng, 2002). *PTEN* mutations can also result in less severe functional defects or changes in protein stability or subcellular localization, leading to a partial loss of function phenotype. For instance, certain *PTEN* mutations affect its nuclear-cytoplasmic shuttling, impairing its cellular functions, including its role in maintaining genomic stability (Planchon et al., 2008). Germline *PTEN* mutations are associated with a group of disorders commonly referred to as *PTEN* Hamartoma Tumor Syndrome (PHTS), which includes Bannayan-Riley-Ruvalcaba syndrome, Cowden syndrome, Proteus syndrome and Proteus-like syndrome. Individuals with PHTS have an increased lifetime risk of developing certain types of cancer, including breast, thyroid, endometrial and renal cancers (Eng, 2003).

1.4 Protein translation

In 1953, James Watson and Francis Crick made a discovery that would forever change the understanding of life at the molecular level. They unveiled the double-helix structure of DNA, marking a significant turning point in molecular biology and genomics (Watson and Crick, 1953). While the discovery of DNA's structure was indeed a monumental breakthrough, understanding how genetic information stored in DNA was converted into functional proteins remained a mystery. The missing link was provided by Sydney Brenner and François Jacob's work in 1961, which introduced the concept of messenger RNA (mRNA) (Brenner et al., 1961). This molecular intermediary transcribes information from DNA and translates it into proteins, significantly advancing our understanding of gene expression and regulation. The discovery of mRNA brought clarity to the flow of genetic

information from DNA to RNA to proteins, cementing what is now known as the central dogma of molecular biology (Crick, 1970). The discovery of the triplet nature of codons was a significant milestone in molecular biology, pieced together by several pioneering researchers. In the early 1960s, it was demonstrated that the genetic code was read in triplets, as deletions or insertions of a single nucleotide caused a frameshift mutation, while those of three nucleotides did not (Crick et al., 1961).

1.4.1 The Ribosome

The ribosome makes up the foundation of the translational machinery. There are three sites within the ribosome with different functions during translation. The aminoacyl-site (A-site) is the site to which incoming cognate aminoacyl-transfer-RNAs (tRNA)s binds. The P-site, or peptidyl-site, is the second tRNA position and is responsible for carrying the growing nascent polypeptide until proper base pairing of an aminoacyl-tRNA has occurred at the A-site. The E-site, or exit-site, is the third and final site in which a tRNA resides within the ribosome before being released. The two main functions of the ribosome are to decode the information contained within the mRNA and to form nascent polypeptides. These functions are carried out by two ribonucleoprotein particles called the small 40S subunit and the large 60S subunit. The small subunit is responsible for decoding the mRNA template, a process which involves base pairing the tRNA anticodon with the mRNA template. The large subunit contains the peptidyl transferase activity which is responsible for transferring the nascent polypeptide chain carried by the tRNA in the P-site to the newly bound aminoacyl-tRNA in the A-site (Lafontaine and Tollervey, 2001). The nucleotides within a codon are named according to the position they have in the codon. The first nucleotide is called +1, the second +2 and the third +3. The nucleotides surrounding a particular codon also follow this nomenclature using the codon in question as reference, meaning that the nucleotide directly downstream of the codon will be called +4 and the nucleotide directly upstream -1 etcetera (Cridge et al., 2018).

1.4.2 Initiation

Prior to initiation of protein translation, assembly of key components is required to form the initiation complex. This process is mediated by eukaryotic initiation factors (eIFs) functioning in aiding the initiator tRNA, the mRNA, and the two ribosomal subunits to come together and form the initiation complex. This process can be divided into four stages: 1) Recruitment of the initiator tRNA to the

small 40S ribosomal subunit mediated by eIF2. Together these proteins form the 43S preinitiation complex. 2) Binding of the mRNA template to the 43S complex which involves recognition of the 5´-cap of the mRNA by eIF4F. 3) The 43S then moves along the 5´nontranslated region of the mRNA until it reaches the initiation codon where it base pairs the initiation codon to the initiator tRNA forming a 48S initiation complex. 4) eIFs and other proteins are removed from the 48S complex to accommodate binding of the large 60S ribosomal subunit forming the 80S ribosome. During this process, the initiator tRNA has been moved into the P-site of the ribosome (Pestova et al., 2001).

1.4.3 Elongation

Following formation of the mature 80S ribosome, with the initiation tRNA and initiation codon paired and positioned in the P-site, elongation of the amino acid sequence can commence. At this stage, the second codon of the open reading frame is in the A-site, ready to accept binding of a cognate aminoacyl-tRNA. The eukaryotic elongation factor (eEF) 1A mediates this interaction by binding to and directing cognate aminoacyl-tRNAs via a GTP-dependent process to the A-site. Upon codon recognition at the A-site, GTP hydrolysis causes eEF1A release, thus enabling accommodation of the cognate aminoacyl-tRNA into the A-site. Following this step, peptide bond formation with the P-site peptidyl-tRNA occurs rapidly and the nascent polypeptide chain is transferred onto the A-site tRNA. Binding of the GTPase eEF2 in complex with GTP causes the ribosome to move one codon downstream in the open reading frame. This movement places the peptidyl-tRNA from the A-site into the P-site and frees up the A-site for binding of a new cognate aminoacyl-tRNA, while also moving the deacylated tRNA previously residing in the P-site to the E-site. The deacylated tRNA is later expelled from the ribosome (Dever and Green, 2012).

1.4.4 Termination

When the stop codon (UGA, UAG or UAA) at the end of the coding sequence enters the A site, termination of translation is initiated. Translational termination is catalyzed by the binding of the eukaryotic release factors (eRF) 1 and eRF3 to the decoding center of the ribosome. The tRNA-shaped eRF1 protein contains three domains; 1) an amino-terminal domain recognizing the stop codon via a highly conserved NIKS motif; 2) a middle domain, similar to the tRNA acceptor stem and extends into the peptidyl transferase center to cause release of the peptide through the conserved GGQ motif. 3) A carboxyl terminus involved in interactions

with eRF3. The GTPase eRF3 acts to accelerate peptide release and increase termination efficiency through GTP-hydrolysis, resulting in release of the protein (Dever and Green, 2012).

The different stop codons have different termination efficiencies: UAA>UAG>UGA, where UAA is the most potent termination codon and UGA being the leakiest stop codon. The level of translational termination efficiency is also dependent on the neighboring nucleotides, both upstream and downstream of the stop codon. The +4 nucleotide downstream of the stop codon has proven to be most influential in determining termination efficiency (C>U>A>G, C being most leaky and G most stringent) (Bidou et al., 2012). The reason why the +4 nucleotide is so influential in determining termination efficiency might be due to a conformal change of the ribosomal RNA (rRNA) upon binding of eRF1 to the A-site. The conformal change causes the rRNA residue A1825 to adapt a flipped-out conformation enabling stacking of the +2 and +3 nucleotides of the stop codon allowing accommodation of the +4 nucleotide into the A-site (Brown et al., 2015).

1.5 Nonsense-mediated decay

Nonsense-mediated decay (NMD) is an evolutionary conserved quality control mechanism. NMD functions by selectively targeting mRNA transcripts containing PTCs for degradation to reduce translation of potentially deleterious truncated proteins. There are several proteins involved in the NMD response of which the up-frameshift (UPF) 1, 2 and 3, as well as the suppressor of morphogenesis of genitalia (SMG) kinase family of proteins 1 to 7, are of particular importance. UPF1 has helicase and ATPase activity which are both essential for NMD activity by mediating assembly of other NMD factors (Karousis and Muhlemann, 2019). UPF2 serves as a scaffolding protein, linking UPF1 and UPF3 together and mediating UPF3 interaction with exon-junction complex (EJC) core factors. The SMG proteins have many important NMD functions including phosphorylation of UPF1, endonucleolytic activity and recruitment of other degradation factors needed for mRNA transcript degradation (Karousis et al., 2016).

The first and most important step in the NMD process is recognition of PTC-containing mRNA transcripts. Four different PTC recognition models have been proposed: (1) The faux 3´UTR model proposes that normal stop codon recognition is aided by the interaction between eRF3 on the ribosome and the poly(A) binding protein (PABP) on the poly(A)-tail. But in the case of premature termination, the longer distance between eRF3 on the ribosome at the PTC and PABP on the poly(A)-tail prevents their interaction, instead promoting UPF1 binding to eRF3

leading to activation of the NMD pathway (Singh et al., 2008). (2) The exon-junction complex dependent model suggests that during splicing, the multiprotein EJCs that are deposited onto mRNA 20-40 nucleotides upstream of exon-exon junctions and act as NMD activators. During normal translation the EJCs are displaced by the ribosome as it moves along the mRNA template until translation is completed. At this stage all EJCs have been removed. For mRNAs containing a PTC, the translational machinery will not be able to remove the EJCs downstream of the PTC, thus triggering recruitment of NMD effectors UPF2 and UPF3 as well as the SURF protein complex (SMG1, UPF1 and eRF1 and eRF3) subsequently leading to mRNA degradation. (3) The unified model implements both previously described models acknowledging the importance of UPF1 and PABP competition for binding to eRF3 bound to the ribosome while proposing that the remaining EJCs act to augment NMD signaling by positioning UPF2 and UPF3 close to the terminating ribosome. (4) The ribosome release model suggests that NMD is triggered due to early ribosome release which exposes the mRNA normally covered by the translating ribosome. This makes the mRNA susceptible for nucleases which degrade the mRNA template (Fang et al., 2013). After identification of PTC-containing mRNAs, NMD effectors effectively cause degradation of aberrant mRNA transcripts. The NMD pathway degrades mRNA through interactions between UPF1 bound to the targeted mRNA and the endonucleolytic SMG6 protein or a heterodimer consisting of SMG5 and SMG7 which recruits other factors that degrade the transcript (Karousis et al., 2016).

1.6 Translational Readthrough

As previously described, translational termination occurs when the ribosome is confronted with one of the three stop codons (UGA, UAG, UAA). However, translation termination is not 100% efficient and is dependent on several factors, one being competition between stop codon recognition by eRF1 and binding of near-cognate tRNAs in the A-site. If decoding of the stop codon by a near-cognate tRNA occurs instead of binding of eRF1, translation will continue through the stop codon, an event called translational readthrough. Translational readthrough occurs at extremely low rates (< 0.1%) during normal conditions. As previously described, the rate varies depending on several factors including which of the three types of stop codons is encountered and the surrounding mRNA nucleotide context (Bidou et al., 2012, Keeling et al., 2012). It has been shown that certain viruses like the Moloney murine leukemia virus (MuLV) and the tobacco mosaic virus (TMV) use translational readthrough to increase their coding capacity and diversify protein output (Skuzeski et al., 1991, Wills et al., 1991).

However, for humans, unwanted C-terminal protein extensions may cause dysfunction of normal cellular processes and toxicity. This has been the main concern when translational readthrough is discussed as a possible strategy for treatment of diseases caused by nonsense mutant genes (Dabrowski et al., 2015). This flaw in the translational machinery can be taken advantage of to treat genetic diseases caused by nonsense mutations. By using compounds that induce translational readthrough it is possible to promote translation of full-length protein, thus alleviating the source of the disease and restoring WT function of the affected gene. Worth noting is that the insertion of near-cognate tRNAs at the PTC following translational readthrough is semi-random meaning that several variants of the full-length protein will be produced. For example, the UGA stop codon is most often decoded as tryptophan but can also be misread as cysteine or arginine. UAG and UAA can be decoded as glutamine, tyrosine or lysine (Dabrowski et al., 2015).

1.6.1 Factors affecting readthrough efficiency

1.6.1.1 Type of premature termination codon

The type of stop codon at a PTC influences the translational readthrough efficiency since the three stop codons, i.e., UAA, UAG, and UGA have varying susceptibilities to readthrough. UGA codons have the highest readthrough propensity, followed by UAG and UAA (Beznoskova et al., 2016). Differences in readthrough efficiency stem from stop codon recognition by translation termination machinery. All three different stop codons are recognized by eRF1 but with different affinities, affecting readthrough rates (Frolova et al., 2000, Brown et al., 2015).

1.6.1.2 Nucleotide context

The surrounding nucleotide context adjacent to the PTC significantly influences the amenability to translational readthrough. The +4 nucleotide (following the stop codon) has been shown to play a crucial role in determining readthrough efficiency (Cassan and Rousset, 2001). One plausible reason why the +4 nucleotide is of particular importance is that it enters the ribosomal A-site upon eRF1 binding during initiation of translation termination, effectively resulting in a tetranucleotide termination signal. This is possibly due to a conformal change leading to stacking of the +2 and +3 nucleotides of the PTC, thus allowing for the +4 nucleotide to enter the A-site (Brown et al., 2015). Additionally, the nucleotide

context downstream and upstream of the PTC has also been shown to impact readthrough efficiency (Loughran et al., 2014). It has also been shown that the secondary structure of mRNA surrounding the PTC can influence readthrough. Open mRNA structures are typically associated with higher readthrough efficiencies. Furthermore, specific RNA elements, such as stem-loop structures and pseudoknots, can affect the interaction between ribosomes and release factors, impacting readthrough efficiency (Belcourt and Farabaugh, 1990, Jungreis et al., 2011).

1.6.1.3 Normal vs premature termination codons

There are several distinct differences between PTC and normal stop codons protecting the cells from systematic readthrough of all stop codons resulting in translational turmoil. The first and probably most important feature of normal stop codons residing at the end of the open reading frame is the existence of tandem stop codons (Keeling et al., 2012). These stop codons exist downstream of the primary stop codon in the open reading frame providing protection against unwanted errors in translational termination. These multiple stop codons are more abundant in highly expressed genes in which translation termination occurs at a higher rate. A second difference is that the ribosome pauses longer at PTC compared to normal stop codons before translational termination. This reduced efficiency of termination has been suggested to be part of the reason why PTC are more susceptible to pharmacological induction of translational readthrough than normal stop codons (Amrani et al., 2004). A third difference between the normal and PTCs is the interaction between eRF3 in the termination complex and PABP at the Poly(A) tail (Cosson et al., 2002). The proximity of normal stop codons to the poly(A) tail makes this interaction easy, while the random positioning of PTC in the open reading frame can result in a significant distance between PABP and the stop codon, thus preventing interaction between the two and reducing termination efficiency (Keeling et al., 2012).

1.6.2 Readthrough-inducing compounds

The aminoglycoside family of antibiotics are well known to induce translational readthrough and amongst them G418, gentamicin and paromomycin are the most potent and well-studied, but their clinical use is limited by nephrotoxicity and ototoxicity. The way in which aminoglycosides are thought to induce translational readthrough is by binding the ribosomal decoding center, causing less stringent tRNA-anticodon base paring at the PTC, and thus allowing near-cognate tRNA

insertion and subsequent translational readthrough. Additionally, it is thought that ribosome-aminoglycoside binding causes conformal changes that sterically impairs binding of release factors which are necessary for successful translational termination (Bidou et al., 2012). Due to the toxicity observed by the existing aminoglycosides, several analogues have been synthesized to reduce toxicity while maintaining or enhancing the readthrough potential. The most prominent aminoglycoside-like compound is ELX-02 (NB124) which has proven effective at inducing translational readthrough in several nonsense mutant genes (Xue et al., 2014, Crawford et al., 2020).

There are also several non-aminoglycoside readthrough-inducing compounds that have been identified by high throughput screenings, amongst which PTC124 (or Ataluren) is the only compound to reach phase III clinical trials and receiving provisional authorization by the European medicines agency (EMA) for treatment of Duchenne muscular dystrophy (DMD) caused by nonsense mutations (Haas et al., 2015, Bidou et al., 2012). However, the results from the clinical trials have been inconsistent resulting in termination of several trials. Also, there has been some controversy regarding the mechanism of action for Ataluren since the compound was evaluated in a firefly luciferase reporter system and as it turns out, Ataluren stabilizes firefly luciferase quite effectively, resulting in bias when analyzing the results (Auld et al., 2009).

The small molecule compound Clitocine is a nucleoside analog capable of inducing readthrough. Clitocine is thought to exert its readthrough effect by incorporating into mRNA instead of adenine thus allowing for near-cognate tRNA insertion by the translational machinery at a PTC (Friesen et al., 2017). Another notable readthrough inducing compound is 2,6-diaminopurine (DAP) which exclusively acts on the UGA stop codon. DAP´s mechanism of action is thought to be by inhibiting the methyltransferase FTSJ1, thus leading to tRNA alterations allowing for tryptophan to be inserted at UGA PTCs (Trzaska et al., 2020).

CC-885 and CC-90009 are two eRF3a inhibitors and have been identified as potent readthrough inducing compounds (Surka et al., 2021). They function by facilitating the binding of the E3 ubiquitin ligase cereblon (CRBN) and eRF3a leading to ubiquitination of eRF3a and subsequent proteasomal degradation. This causes a decrease in intracellular levels of translational release factors, thus inhibiting translational termination and promoting translational readthrough (Baradaran-Heravi et al., 2021).

Recently, a novel approach to induce translational readthrough was reported. Human tRNAs were engineered altering various functional segments, such as the anticodon stem or loop (controlling codon decoding) or the TΨC-stem

(responsible for binding elongation factors). These modified tRNAs, or sup-tRNAs, were generated through *in vitro* transcription and were modified to enable pairing to the UGA PTCs, thus preventing translation termination and promoting translational readthrough. To enable intravenous injections of the sup-tRNAs in mice a lipid nanoparticle (LNP) encapsulation approach was employed. Potential *in vivo* off-target readthrough of canonical stop codons was assessed by ribosome profiling of tissue from lung and liver. The results revealed no increased readthrough of canonical stop codons compared to untreated control mice (Albers et al., 2023).

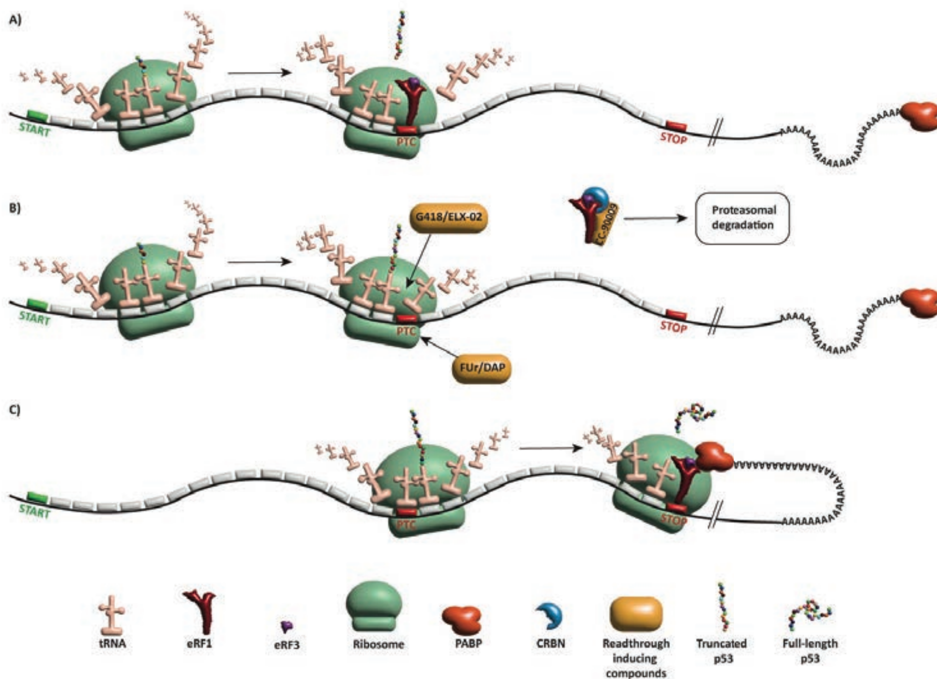

Figure 5. Different translational outcomes. (**A**) Translational termination occurring at a PTC facilitated by binding of the eRF1 and eRF3 complex at the A-site causing release of truncated protein. **(B)** Pharmacological induction of translational readthrough promoting binding of a near cognate tRNA at the A-site, thus allowing elongation of the nascent polypeptide chain passed the PTC. Different known readthrough inducers are indicated. **(C)** A complete translational readthrough event showing the ribosomes journey beyond the PTC, all the way to the canonical stop codon where PABP interacts with the termination complex to facilitate proper termination of translation and release of full-length protein.

1.6.3 Nonsense mutant mouse models

Nonsense mutant mouse models have become an invaluable resource in the study of human diseases caused by nonsense mutations. By studying mice with these genetic alterations, researchers can gain insights into the role of specific

genes in the development of various diseases and test potential therapeutic approaches.

One of the most well-known nonsense mutant mouse models is the APC^{min} (adenomatous polyposis coli multiple intestinal neoplasia) mouse model which was generated by Moser et al. in 1990 and harbors a nonsense mutation in the *Apc* gene at codon 850 leading to protein truncation and inactivation resulting in subsequent formation of intestinal tumors. The APC^{min} mouse model has been a valuable tool in studying the pathogenesis of familial adenomatous polyposis (FAP), which is characterized by the development of numerous polyps in the colon and rectum (Moser et al., 1990, Su et al., 1992).

The mdx mouse model was generated in the 1980's and has been instrumental in the study the cellular and molecular mechanisms of DMD, a severe neuromuscular disorder caused by nonsense mutations in the dystrophin gene (Bulfield et al., 1984). The mdx mouse has a single nucleotide substitution (C to T transition) at position 3185 in exon 23 resulting in insertion of a PTC. This model has been essential for studying the pathophysiology of DMD and testing potential therapeutic approaches (Gaina and Popa Gruianu, 2021).

More recently, another mouse model was established, harboring nonsense mutant *A-T mutated* (*Atm*) and knocked out *Aprataxin* (*Aptx*) gene. These mutations were targeted to accurately mimic the genetic landscape found in ataxia-telangiectasia (A-T). The specific mutation introduced was a C to T transition at codon 103 in the *Atm* gene. This mutation results in a PTC in the *Atm* gene, leading to loss of Atm expression. In these mice, heterozygous PTC mutations resulted in about half the normal Atm expression, while homozygous mutations led to complete loss of Atm expression (Perez et al., 2021).

1.7 Synergy

The phrase "the whole is greater than the sum of its parts" encapsulates the essence of synergy, but when it comes to therapeutic application and drug discovery, a more precise understanding is needed. The interaction between drugs can be complicated, with the outcomes being influenced by numerous factors, such as dosage, timing and the specific mechanisms of action of the drugs (Earp et al., 2004). Synergy models offer a mathematical approach that quantitatively measures the interactions between drugs. They enable precise numerical values to be assigned to describe the extent and type of interaction between drugs (Chou and Talalay, 1984, Tallarida, 2006). Several synergy models have been created attempting to accurately evaluate synergistic interactions

between drugs including the Loewe additivity model, Bliss independence model and the Zero interaction potency (ZIP) model. All these models must make assumption regarding the drugs to build the mathematical framework that governs them.

The Loewe additivity model is based on the principle of dose equivalence meaning that for any given effect produced by drug A, there is a corresponding dose in drug B that can produce the same effect, effectively making the drugs interchangeable (Vlot et al., 2019). Additionally, Loewe's additivity is defined by the principle of a "sham experiment" stipulating that a compound cannot synergize with itself. An agent combined with itself must result in an additive effect. Therefore, two different drugs are considered to be synergistic if they have an effect that exceeds their individual effects at doses with equal efficacy. The model is simple and widely accepted for drugs with similar mechanisms of action and provides a clear distinction between synergistic, additive, and antagonistic interactions. However, this model struggles when the dose-response curve is not linear, which is not the case for many drugs (Greco et al., 1995).

The Bliss Independence model assumes that drugs act independently, and calculates the expected combined effect of the individual drug effects (Slinker, 1998). Synergy is defined when the observed combined effect is greater than the expected effect. The model is applicable to drugs with different mechanisms of action, as it does not rely on dose equivalence. It is a simple and intuitive method for calculating synergy. A noteworthy limitation is its propensity to overestimate synergy given its inherent assumption of absolute independence between drug actions. Moreover, it might not always be applicable for drugs that have similar mechanisms of action (Greco et al., 1995).

Both previously mentioned models for synergy have been heavily criticized due to their limitations in accuracy. Several attempts have been made to generate model that more accurately predicts synergy. One such example is the ZIP model that encompass the strengths of both the aforementioned models while overcoming many of their shortcomings. The ZIP model makes the assumption that two drugs cause minimal change to their respective dose-response curves if they are non-interacting. It provides a single delta score that detects any deviation of the observed effect compared the expected effect given zero interaction between the drugs (Yadav et al., 2015).

2 MATERIALS AND METHODS

A detailed description of the methods used throughout this thesis can be found in the included papers. A brief general summary of the most common methods is presented below.

2.1 Western blotting

Western blotting was used as the principal method to detect protein expression. This method was mainly used as a qualitative measure of protein expression and determination of protein size in cellular extracts. Western blot relies on gel electrophoresis for size-dependent protein separation and subsequent transfer to a nitrocellulose membrane which can then be probed with antigen-specific antibodies. Western blotting was utilized in all included studies, specifically to investigate translational readthrough, allowing for analysis of both truncated and full-length proteins post-treatment. Different antibodies were used for readthrough detection across various cell lines and genes. Following treatment, cells were lysed, and protein concentration was quantified employing Bradford (Bio-Rad, USA) or DC™ Protein assays (Bio-Rad, USA). Gel electrophoresis was conducted using NuPAGE™ 10% Bis-Tris polyacrylamide gels (Thermo Fisher Scientific, USA) and MOPS sodiumdodecyl sulfate (SDS) Running buffer (Thermo Fisher Scientific, USA). Membrane transfer was performed using iBlot™ 2 Gel Transfer Device. Membranes were then blocked with milk and probed with antibodies. Protein visualization was accomplished using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA) and either a LAS-1000 Image Analyzer (Fujifilm, Japan) or an iBright FL1000 Imaging System (Thermo Fisher Scientific, USA).

2.2 Enzyme-linked immunosorbent assay (ELISA)

96-well plates (Thermo Fisher Scientific, USA) were coated with anti-FLAG antibody FLAG M2 (Sigma-Aldrich/Merck, Germany) and incubated overnight at 4°C. The wells were then blocked for 2h using 5% milk in PBS-T and then washed in PBS-T. Each well was loaded with 100 µg protein in 50 µl of blocking buffer and 50 µl lysis buffer before incubation overnight at 4°C. All wells were washed with PBS-T, after which a HRP-conjugated DO-1 antibody (Santa Cruz, USA) diluted 1:500 in blocking buffer was added to each well and incubated for another 2 hours at 4°C. Another wash with PBS-T was then conducted before 100 µl of the 1-Step™

Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) was added to each well. The reaction was conducted at room temperature until a satisfactory signal strength was achieved. The reaction was stopped by adding 10 µl of 1 M HCL. Absorbance was then determined at 450nm wavelength using a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, USA).

2.3 Flow cytometry (EGFP readthrough)

Cells were seeded onto 12-well plates and left to settle for 24-hour before being treated at specified dosages of compound. After a subsequent incubation period of 72 hours, the cells were collected via trypsinization and subjected to analysis for EGFP expression. This was accomplished using a NovoCyte flow cytometer (ACEA Biosciences, USA). As a control measure, cells without EGFP were evaluated following identical treatment to determine any background fluorescence attributed to the compounds. Any detected background fluorescence was subtracted from the corresponding measurements from the cells with EGFP.

2.4 Flow cytometry (cell cycle analysis)

Indicated cells were cultured in six-well plates at an appropriate density. 24h following seeding they were treated with G418 and/or nutlin-3a at indicated concentrations and incubated for 72 h. Cells were washed with PBS and fixed overnight using ice-cold 100% ethanol. The cells were subsequently washed before incubation with a mixture of propidium iodide at 0.025 mg/ml (Sigma-Aldrich/Merck, Germany) and RNase A at 0.125 mg/ml (Sigma-Aldrich/Merck, Germany), and left in the dark at 37°C for 30 minutes. 50 µl PBS was added to each sample before analysis using the NovoCyte® flow cytometer (ACEA Biosciences, USA).

2.5 Flow cytometry (Annexin V)

Indicated cells lines were seeded at an appropriate density in 6-well plates. After 24h, the cells were treated with FUr, FdUr, or G418 at the specified concentrations. Following 72h incubation, both adherent and floating cells were collected by Trypsinization (Sigma-Aldrich/Merck, Germany). The cells were resuspended in PBS supplemented with $CaCl_2$ and $MgCl_2$ (DPBS; Gibco, USA). Cells were incubated in 1X Annexin V binding buffer. They were then incubated with either BD Horizon™ V450 Annexin V antibody (BD Biosciences, USA) or Annexin V Alexa Fluor™ 647

conjugate (Thermo Fisher Scientific, USA). Following 15 minute incubation in the dark, cells were washed with chilled DPBS and then analyzed using the NovoCyte flow cytometer (ACEA Biosciences, USA). Events were gated using SSC-H/FSC-H and single cells were gated using FSC-H/FSC-A gates. The cells were categorized as either Annexin V positive or negative.

2.6 Immunofluorescence staining

Cells were seeded onto coverslips and subjected to indicated treatments prior to fixation utilizing 4% PFA after which permeabilization was achieved using 0.2% Triton in a PBS solution. The cells were then blocked for 1 h using 2% BSA in PBS. Following this, the cells were incubated with the indicated antibody for 1-2h. Finally, the cells were mounted using FluoroshieldTM incorporated with DAPI (Sigma-Aldrich/Merck, Germany). Detection of the immunofluorescent staining was conducted using a Zeiss AxioImager M2 microscope.

2.7 Quantitative real-time PCR (qRT-PCR)

Cells were seeded and left to settle for 24 h before treatment with designated compounds for 72 h. All cells were harvested using Trypsin-EDTA solution (Sigma-Aldrich/Merck, Germany). RNA extraction was done using RNeasy mini kit (Qiagen, Germany) according to the manufacturer's specifications. A NanoDrop Spectrophotometer (Thermo Scientific, USA) was used to quantify the extracted RNA. The RNA was reverse transcribed into cDNA utilizing SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, USA). Quantitative real-time PCR (qRT-PCR) assays were performed using a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, USA), TaqMan Gene Expression Assays, and FastStart Universal Probe Master (Rox) (Roche, Switzerland). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression levels, using GAPDH as the internal normalization control.

2.8 WST-1

Cells were seeded onto 96-well plates and treated with C47 and G418 at the specified concentrations. Following 72 h incubation, the water-soluble Tetrazolium-1 dye (WST-1; Roche, Switzerland) was added as per the guidelines provided by the manufacturer. The reaction mixture was incubated 30–60 minutes until reaching satisfactory colorimetric change. Absorbance

measurements were then taken at 440 nm using the Varioskan™ LUX multimode microplate reader (Thermo Scientific, USA).

3 ETHICAL CONSIDERATIONS

Papers I and II: Both commercially procured cell lines and cell lines engineered within our research team was used as experimental models.

Paper III: Human tumor xenografts in mice were used (ethical approval numbers Dnr 7054-2019 and Dnr 15763-2020).

Paper IV: A novel mouse model was generated (ethical approval number Dnr 14188-2019).

All in vivo experiments were conducted under the authorization of the Stockholm Animal Experiments Ethical Committee, Sweden, and conformed to the animal welfare guidelines set forth by Karolinska Institutet. Importantly, no human patient-derived materials were utilized in any of the aforementioned studies.

4 RESEARCH AIMS, RESULTS AND DISCUSSION

4.1 Paper I

Synergistic rescue of nonsense mutant tumor suppressor p53 by combination treatment with aminoglycosides and Mdm2 inhibitors

In this study, we investigated potential benefits of a combination based therapeutic approaches for the restoration of nonsense mutant p53. Aminoglycosides, specifically G418 and gentamicin, have previously been extensively studied for their capacity to induce translational readthrough. However, the associated toxicity has been a significant limitation rendering them unsuitable for long term treatment. To overcome this, we explored combination therapies with agents that potentially could potentiate the readthrough output, ultimately aiming at reducing the concentrations of aminoglycosides needed and consequently the toxicity.

We combined either G418 or gentamicin with Nutlin-3a, MI-773 or bortezomib. The rationale behind this strategy was that G418 would induce translational readthrough of nonsense mutant p53 while the p53-Mdm2 interaction inhibitors Nutlin-3a and MI-773 or the proteasome inhibitor bortezomib would prevent degradation of the readthrough product. This would increase total levels of intracellular p53, hopefully leading to p53-dependant effects. Our initial experiments focused on the induction potential of G418 or gentamicin alone in the HDQ-P1 breast carcinoma cells, which harbors an endogenous homozygous R213X nonsense mutation. Both compounds were found to induce translational readthrough, with G418 demonstrating superior potency in both full-length p53 expression and in p53 mRNA induction. We further confirmed these results in H1299 *TP53* null cells, stably transfected with an R213X nonsense mutant *TP53* construct (H1299-R213X) showing a robust induction of full-length protein.

We then assessed the functional capacity of the newly synthesized full-length p53 in HDQ-P1 cells following G418 or gentamicin treatment. There was a robust upregulation in p53 target gene mRNA, such as Wig-1 (Zmat3), p21, Fas, Mdm2, and Bax following treatment with both compounds, with G418 again showing a more prominent effect. Additionally, the functionality of the readthrough product was confirmed at protein level in H1299-R213X cells showing upregulation of p21 and Wig-1 following treatment.

To investigate the synergy potential, HDQ-P1 cells were treated with G418 or gentamicin together with either bortezomib, Nutlin-3a or MI-773. While no substantial full-length p53 was detected with Bortezomib, Nutlin-3a, or MI-773

alone, a marked increase was observed in the combination treatments with the aminoglycosides. Additionally, combination treatment with G418 or gentamicin together with Nutlin-3a resulted in an upregulation of p53 target genes detected by RT-PCR analysis. Substantial upregulation of p21 protein could also be detected in H1299-R213X cells following combination treatment with G418 and Nultin-3a. Importantly, this effect was not seen in H1299-EV cells, indicating at p53-dependent upregulation of p21 protein. The biological consequence of these treatments was subsequently examined, focusing on growth inhibition and cell viability by WST-1 assay and cell cycle analysis. Combination treatment with G418 and Nutlin-3a resulted in increased growth suppression/cell death in H1299-R213X cells compared to the single treatments and compared to the same treatment in the empty vector control cell line.

In summary, this research shows the potential of G418 and gentamicin in inducing readthrough in cells carrying either endogenous or exogenous nonsense mutant p53. The results demonstrate that the readthrough product is at least partially functional and that the combination treatments successfully increased p53 dependent apoptosis or growth inhibition. Consequently, this study offers a promising avenue for treating patients with tumors harboring nonsense mutant p53, possibly allowing for reduced aminoglycoside dosages and therefore reduced toxicity.

4.2 Paper II

Novel compounds that synergize with aminoglycoside G418 or eRF3 degraders for translational readthrough of nonsense mutant *TP53* and *PTEN*

In this paper, our objective was to discover new molecules that could promote translational readthrough of nonsense mutant *TP53*. We conducted a high-throughput microscopy screening with HDQ-P1 cells that carry homozygous R213X nonsense mutation in the *TP53* gene. Immunofluorescence staining was used as readout for p53 protein expression. Over 33 000 compounds were screened, and promising compounds were validated by both ELISA and Western blot using H1299 cells transfected with a *TP53* R213X construct with a truncation following the PTC and a C-terminal FLAG-tag (H1299-R213X-ΔC-FLAG). Following validation of all hit compounds, the two compounds C47 and C61 were identified as the most promising candidates.

Both C47 and C61 induced levels of full-length protein in several nonsense mutant p53 cell line variants, indicative of readthrough induction. One critical aspect that needed investigation was whether the compounds themselves stabilized p53,

which could be misinterpreted as translational readthrough. To evaluate any p53 stabilization by C47 and C61, we treated HCT116 p53 WT cells and did not detect any significant increase in p53 levels. Moreover, a parallel experiment using a p53-independent nonsense mutant EGFP cell line, we confirmed the readthrough-inducing capabilities of both compounds.

Considering the relatively modest readthrough induction observed by C47 or C61, we investigated whether we could potentiate the readthrough potential of these compounds by combination treatments. Several known readthrough inducing compounds were assessed for synergistic interactions with C47 and C61. The combinations that yielded the best results were C47 + G418 and C61 + the two eRF3a degraders CC-885 or CC-90009. A robust increase of full length p53 was detected by Western blot following combination treatment compared to the single treatments alone for these combinations. The readthrough potential was also validated in a p53-independent reporter cell line harboring a nonsense mutation in sfGFP (HCT116sfGFP), further strengthening previous results.

To determine if the induction seen following combination treatment was synergistic or additive quantitative methods were employed. Analysis by flow cytometry using HCT116sfGFP cells was used for the C47 and G418 combination, while ELISA was chosen for the combinations of C61 with CC-885 or CC-90009, due to the autofluorescence exhibited by C61. Both C47 and C61 were shown to exhibit synergistic interactions in their corresponding combinations, dramatically increasing the output of full-length protein.

To investigate if our novel readthrough-inducing compounds were able to induce translational readthrough of additional nonsense mutant tumor suppressor genes, we extend our study to include nonsense mutant *PTEN*. The readthrough potential of C47 was of particular interest, especially in combination with G418 which generated a strong and synergistic response in nonsense mutant *PTEN* cells.

The precise mechanism of action for C47 and C61 in promoting readthrough remains to be determined. Preliminary results suggest mechanisms distinctly different from that of G418 and CC-885/CC-90009, but further studies are needed to confirm this.

In summary, our high-throughput screening of chemical libraries yielded two promising readthrough-inducing compounds, C47 and C61. Despite their modest effects individually, combination treatments exhibited synergistic induction of translational readthrough across several tumor suppressor genes and cellular models, underscoring their potential. Future investigations will be needed to define the underlying mechanisms and the broader biological implications of these compounds. This research could pave the way for the advancement of

targeted cancer therapies through pharmacological induction of translational readthrough.

4.3 Paper III

Translational readthrough of nonsense mutant *TP53* by mRNA incorporation of 5-Fluorouridine

In this paper we set out to identify compounds capable of inducing readthrough in nonsense mutant *TP53*, similar to that described for paper II. However, we employed a completely different screening approach for this current study. We systematically analyzed 47 000 compounds from the National Cancer Institute-60 database. The aim of the study was to select compounds that demonstrate greater potency in impeding proliferation of tumor cell lines containing nonsense mutant *TP53* compared to those with WT *TP53* or alternative *TP53* mutations. Following analysis of the mean 50% growth-inhibitory concentrations (GI50) of the compounds found in the data base, 5-FU was selected for further investigation. We validated the readthrough-inducing capability of 5-FU in several cell lines including HDQ-P1, H1299-R213X and HCT116sfGFP cells. Additionally, we assessed the expression patterns of specific p53 target genes following 5-FU treatment and detected upregulation of p53 target genes such as *CDKN1A* (p21), *ZMAT3* (Wig-1), and *FAS*.

To elucidate the mechanism by which 5-FU exerts its readthrough effect, we tested the readthrough capabilities of two of its metabolites, specifically the RNA-incorporating FUr and the DNA incorporating FdUr. It was shown that FUr exhibited prominent readthrough activity while FdUr induced negligible levels of readthrough, suggesting that mRNA incorporation could be the primary mechanism of action. This was tested by a competitive binding assay where increasing concentrations of Uridine resulted in diminished translational readthrough activity of FUr.

Both 5-FU, FUr and FdUr stabilizes WT p53 which could result in a false positive conclusion. To test whether the observed induction of translational readthrough was due to p53 stabilization or in fact a result of translational readthrough, several methods were used. First, we utilized reporter cell lines such as HCT116sfGFP and H1299-EGFP-X-FLAG, the latter was generated from H1299 cells stably transfected with EGFP succeeded by a stop codon and a C-terminal FLAG-tag. Translation of full-length protein was observed following treatment with FUr in both reporter cell lines, indicative of translational readthrough. We also used ribosome profiling (Ribo-seq), a technique enabling quantification of ribosome

position on each mRNA codon. We observed increased *TP53* readthrough upon FUr treatment further strengthening our hypothesis that FUr induces genuine translational readthrough.

RNA-sequencing analysis displayed increased expression of p53 downstream target genes following FUr treatment in H1299-R213X cells. Due to the increase in p53 downstream target activity, we tested whether FUr is capable of inducing p53-dependent apoptosis in H1299-R213X and the empty vector control cell line. We observed increased caspase 3/7 cleavage as well as increased Annexin-V expression in the nonsense mutant cell lines compared to the empty vector control. We also performed *in vivo* studies where we assessed if systemic administration could evoke full-length p53 expression in a human xenograft tumor in mice. An increased level of full-length p53 following FUr treatment could be detected from excised tumors by Western blot analysis and immunohistochemical staining.

In conclusion, this research underscores the importance of continuous evaluation of approved drugs to reveal potential new applications and deepen our understanding of their molecular mechanisms. We have identified FUr as a readthrough inducing agent that can reinstate full-length expression from R213X nonsense mutant *TP53* and potentially other nonsense mutated genes. The full-length p53 readthrough product exhibits functional transcriptional activity.

4.4 Paper IV

A novel tumor-prone mouse model harboring the Trp53^{R210X} nonsense mutation

Here we present a novel mouse model harboring the Trp53 R210X nonsense mutation corresponding to the *TP53* R213X nonsense mutation observed in humans. This was achieved using the CRISPR/Cas9 genome editing system to introduce a PTC at codon 210 in the *Trp53* gene. Approximately 35% of the initial offspring (F0) derived from the edited zygotes harbored the R210X mutation. Subsequent breeding confirmed germline transmission of the Trp53 R210X mutation to the F1 generation.

Upon studying the gender distribution of the offspring, it was noted that the intercrosses of Trp53R210X/R210X mice produced significantly smaller litters, with a notable reduction in female pups. Observations related to body weight trends revealed that homozygous female mice were considerably smaller than their

heterozygote littermates from as early as four weeks of age, while homozygous male mice exhibited reduced weight only post 34 weeks of age.

The lifespan of the Trp53R210X mice was notably reduced, with homozygous mice exhibiting the shortest lifespan in comparison to heterozygous and WT mice. Survival comparisons between Trp53R210X and Trp53R172H strains showed no significant differences. Regarding tumorigenesis, 87.8% of homozygous Trp53R210X mice developed tumors, predominantly lymphomas (64.7%), sarcoma (19.61%) and carcinomas (15.89%) by the end of their lifespan.

Furthermore, to determine the potential for translational readthrough of the R210X mutation, T-cell lymphoma lines, designated X405 and X481, were derived from Trp53R210X/R210X mice. Following treatment with G418, a dose-dependent translational readthrough of full-length p53 was observed in both T-cell lines. Additionally, experiments using FUr displayed a weak yet dose-dependent induction of the full-length p53 protein in the X405 cells which correlates with recent observations in human cancer cells in paper III.

In conclusion, the Trp53R210X mouse model will serve as a valuable tool for comprehensive understanding of the biological implications of the *TP53* R213X mutation in humans. The significant impact of the mutation on mouse phenotype, breeding capacity and lifespan, highlights its importance in the context of a multiorgan system and tumor development. Moreover, the potential use of translational readthrough as a therapeutic intervention for nonsense mutations is underscored by the successful induction of full-length protein following treatment with G418 and 5-fluorouridine in cells derived from this mouse model. Additionally, evaluating the long-term impact of prophylactic translational readthrough therapy might pave the way for a novel therapeutic intervention for humans with Li-Fraumeni syndrome (LFS).

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis and its content serve to expand the understanding of translational readthrough of nonsense mutant tumor suppressors. This was achieved by demonstrating the impact of a combinational therapeutic approach, presenting novel readthrough inducing compounds, revealing a novel therapeutic target for the chemotherapeutic agent 5-FU and its metabolite FUr and finally by developing of a novel mouse model that will be instrumental in development of future therapeutics targeting nonsense mutant p53. The presented scientific discoveries have the potential to be of great scientific and clinical importance.

In **Paper I** we provide evidence that a combination treatment approach using aminoglycosides and other agents can potentiate the effects of translational readthrough. These combinations allow a reduction in aminoglycoside concentrations, consequently lowering their toxicity. In a cellular setting, G418 in combination with the Nutlin-3a, MI-773 or bortezomib induces a distinct p53-centric response. Future studies should examine if the biological effect seen *in vitro* effect is transferable into an *in vivo* model, like the one presented in paper IV. Additionally, newer aminoglycoside derivates that are designed to exhibit less toxicity, such as ELX-02, should be investigated in combination with Nutlin-like compounds to determine if the results presented in this paper can be optimized even further.

In **Paper II**, we set out to tackle one of the most important problems within the research field of pharmacological induction of translational readthrough, i.e. the lack of potent and non-toxic readthrough inducing compounds. Even though C47 and C61 demonstrated a relatively modest readthrough activity on their own, their combination treatments showed a robust induction of full-length protein. Furthermore, preliminary results suggest that C47 and C61 have a mechanism of action distinctly different from that of G418 and the eRF3a degraders. Further studies should focus on systematically investigating analogue compounds to elucidate the precise mechanism of these compounds as well as improving the readthrough potency. Additionally, more combination treatments should be tested to identify even more potent synergistic interactions.

Paper III holds immediate significance due to the clinical application of 5-FU. The study demonstrates that 5-FU induce readthrough of nonsense mutations in *TP53*, an effect predominantly linked to the RNA-integrating metabolite FUr. Additionally, FUr treatment elicited p53 dependent biological effects such as apoptosis and the upregulation of p53 downstream target genes. The revelation that 5-FU and FUr are readthrough inducing agents should warrant further investigation into the *TP53* status of patients treated with 5-FU and if there is any

correlation between overall survival and *TP53* mutational status. Additionally, FUr should be investigated in other nonsense mutant genes to see if the readthrough effect observed in nonsense mutant *TP53* is transferrable to other genes.

Lastly, in **Paper IV** we present a novel nonsense mutant Trp53 mouse model. Due to the lack of prior nonsense mutant p53 mouse models, this has the potential to have an important clinical and scientific impact. The *Trp53*^{R210X} mouse model will allow the study of therapeutic effects of existing and future readthrough inducing compounds in a clinically relevant multiorgan system. This will aid in the development of nonsense mutant p53 targeted therapeutics. Additionally, all the findings of papers I, II and III can be validated and further explored in this relevant in vivo model.

In summary, the collective research presented herein not only elucidates critical molecular mechanisms in nonsense suppression, but also presents the possibility for innovative and more effective therapeutic modalities in patients afflicted with nonsense mutant tumors in tumor suppressor genes. It is important to remember that nonsense mutations are not exclusive to tumor suppressor genes, implying that compounds capable of inducing translational readthrough of nonsense mutant p53 might also be of use for other nonsense mutant genes.

6 ACKNOWLEDGEMENTS

Completing this thesis would not have been possible without the guidance, support, and encouragement from numerous individuals. I wish to express my profound gratitude to them as well as **Karolinska Institutet** and all the **funders**.

First and foremost, I would like to express my deepest appreciation to my main supervisor **Klas Wiman** whose expertise, understanding, kindness and patience have truly made my doctoral studies a fun and rewarding experience. Your guidance and support throughout this journey have been invaluable. I would also like to extend my deepest gratitude to my co-supervisor **Vladimir Bykov** who always have taken the time to talk about science and life. Our discussions throughout the years have ranged from silly scientific ideas to future perspectives on life. Thank you so much for all the help and support!

I would first like to thank the present members of the Wiman group: **Amos**, **Lotta**, **Alessandra**, thank you for all the laughs and scientific input these last couple of years. It has been a pleasure working with you guys! A special thanks goes out to **Susanne** who has helped me so much with everything from technical advice to performing whole experiments for my projects. Secondly, I would like to thank the past members of the Wiman crew: **Sophia**, we have had so many good times both in the lab and outside! Everything from late night beers in the office to traveling around the world. It has been amazing getting to know such a fun, smart and inspiring person! **Mireia**, you have been like mi hermana pequeña throughout this whole journey. You have always spread so much positive energy and laughter in and outside work and have always been available for scientific input and/or discussions. **Matko**, we have known each other for many years, starting already in Uppsala. You spread your lively energy with singing, happy dancing and gossip sessions. **Julie**, thank you for all the help and your advice throughout my PhD regarding both science and life, inside and outside of the lab. **Sofi**, you are always so kind and knowledgeable and a true joy to work with. **Emarn**, thank you for many great memories and for being such a kind person! **Alexander**, Sandy, Xander, Sandman, kärt barn har många namn. I could not have asked for a better student to supervise! We have laughed, talked about life and much more. Btw, it's time for a beer soon! **Mei** & **Qiang**, thank you for many fond memories, I especially remember the Chinese-style crayfish!

CCK people: **Christos** and **Hanif**, thank you for all the fantastic moments laughing, gossiping and traveling. You guys always spread joy wherever you go, and it has

been a true privilege getting to know both of you. **Fredrik**, even though our paths never crossed at work, you have contributed significantly to my PhD journey as my mentor. I want to thank you for invaluable advice during our regular mentor lunches and for being a fantastic and inspiring person.

The after work crew, aka the Thai food channel: **Aarren**, **Solrún & Steffi**: I only ate Thai once, but we drank many beers. You guys have made the last couple of years so much fun! Thank you for listening to all my weird YouTube knowledge and indulging me when bringing up weird topics to discuss during lunch. You are all amazing people!

Bioclinicum/KI people: **Nathan, Martin, Jessica, Muyi & Huaitao**, I have had a lot of fun with you guys! We have shared many laughs during some intellectual, and some not-so-intellectual discussions/debates during lunch. **Katja**, you are so kind, funny and caring, it is always a pleasure to talk with you. **Andreas**, thank you for all the guidance during the early years of my PhD. It is always a pleasure to talk to you regardless of the topic! **Mirco**, you are an amazingly friendly and happy person. We had a lot of fun at the Nobel night cap! Thank you **Pedro**, **Ilias**, **Sonia**, **Mireia**, **Kristina**, **Tom**, **Chen**, **Lucas**, **Lena**, **Hao**, **Anderson**, **Sean**, **Francesca**, **Soniya** and all the rest of the people that made coming to work so much fun!

To my Uppsala friends: **Andreas**, **Ben**, **David**, **Henry**, **Isak**, **Jesper**, **Joakim**, **Kristoffer**, **Lukas**, **Marcus**, **Mattias**, **Oscar**, **Pontus** & **Viktor**, we grew up together and have shared countless (usually beer-soaked) adventures throughout the years. I feel truly fortunate to have had you as friends for all these years. You guys are the best!

Aris, thank you for being such a kind and fantastic person and for letting me work in your lab during the first couple of years of my academic career and thank you **Jon** for supervising me and teaching me how to run western blots, a skill that has been heavily utilized throughout my academic journey.

I also want to thank my family: **Mamma & Pappa**, **Johan & Lena**, **Erik & Eleni** as well as the little ones **Astrid & Alfred, Christos & Angelos** for supporting me in all aspects of life leading up to this moment. You have made me the person I am today, and I am very thankful for having you as my family. **Tess**, my fantastic partner who has been by my side through the arguably most hectic period of my PhD. Your love has given me so much happiness and strength during demanding periods. Thank you for being you <3

7 REFERENCES

AGHABOZORGI, A. S., BAHREYNI, A., SOLEIMANI, A., BAHRAMI, A., KHAZAEI, M., FERNS, G. A., AVAN, A. & HASSANIAN, S. M. 2019. Role of adenomatous polyposis coli (APC) gene mutations in the pathogenesis of colorectal cancer; current status and perspectives. *Biochimie,* 157**,** 64-71.

AL-LAZIKANI, B., BANERJI, U. & WORKMAN, P. 2012. Combinatorial drug therapy for cancer in the post-genomic era. *Nat Biotechnol,* 30**,** 679-92.

ALBERS, S., ALLEN, E. C., BHARTI, N., DAVYT, M., JOSHI, D., PEREZ-GARCIA, C. G., SANTOS, L., MUKTHAVARAM, R., DELGADO-TOSCANO, M. A., MOLINA, B., KUAKINI, K., ALAYYOUBI, M., PARK, K. J., ACHARYA, G., GONZALEZ, J. A., SAGI, A., BIRKET, S. E., TEARNEY, G. J., ROWE, S. M., MANFREDI, C., HONG, J. S., TACHIKAWA, K., KARMALI, P., MATSUDA, D., SORSCHER, E. J., CHIVUKULA, P. & IGNATOVA, Z. 2023. Engineered tRNAs suppress nonsense mutations in cells and in vivo. *Nature,* 618**,** 842-848.

AMRANI, N., GANESAN, R., KERVESTIN, S., MANGUS, D. A., GHOSH, S. & JACOBSON, A. 2004. A faux 3'-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. *Nature,* 432**,** 112-8.

ANDREEFF, M., KELLY, K. R., YEE, K., ASSOULINE, S., STRAIR, R., POPPLEWELL, L., BOWEN, D., MARTINELLI, G., DRUMMOND, M. W., VYAS, P., KIRSCHBAUM, M., IYER, S. P., RUVOLO, V., GONZALEZ, G. M., HUANG, X., CHEN, G., GRAVES, B., BLOTNER, S., BRIDGE, P., JUKOFSKY, L., MIDDLETON, S., RECKNER, M., RUEGER, R., ZHI, J., NICHOLS, G. & KOJIMA, K. 2016. Results of the Phase I Trial of RG7112, a Small-Molecule MDM2 Antagonist in Leukemia. *Clin Cancer Res,* 22**,** 868-76.

AULD, D. S., THORNE, N., MAGUIRE, W. F. & INGLESE, J. 2009. Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression. *Proc Natl Acad Sci U S A,* 106**,** 3585-90.

BARADARAN-HERAVI, A., BALGI, A. D., HOSSEINI-FARAHABADI, S., CHOI, K., HAS, C. & ROBERGE, M. 2021. Effect of small molecule eRF3 degraders on premature termination codon readthrough. *Nucleic Acids Res,* 49**,** 3692-3708.

BELCOURT, M. F. & FARABAUGH, P. J. 1990. Ribosomal frameshifting in the yeast retrotransposon Ty: tRNAs induce slippage on a 7 nucleotide minimal site. *Cell,* 62**,** 339-52.

BERKERS, C. R., MADDOCKS, O. D., CHEUNG, E. C., MOR, I. & VOUSDEN, K. H. 2013. Metabolic regulation by p53 family members. *Cell Metab,* 18**,** 617-33.

BEZNOSKOVA, P., GUNISOVA, S. & VALASEK, L. S. 2016. Rules of UGA-N decoding by near-cognate tRNAs and analysis of readthrough on short uORFs in yeast. *Rna,* 22**,** 456-466.

BIDOU, L., ALLAMAND, V., ROUSSET, J. P. & NAMY, O. 2012. Sense from nonsense: therapies for premature stop codon diseases. *Trends Mol Med,* 18**,** 679-88.

BOVERI, T. 1914. *Zur frage der entstehung maligner tumoren,* Jena,, G. Fischer.

BRENNER, S., MESELSON, M. & JACOB, F. 1961. An Unstable Intermediate Carrying Information from Genes to Ribosomes for Protein Synthesis. *Nature,* 190**,** 576-+.

BROOKS, C. L. & GU, W. 2006. p53 ubiquitination: Mdm2 and beyond. *Mol Cell,* 21**,** 307-15.

BROWN, A., SHAO, S., MURRAY, J., HEGDE, R. S. & RAMAKRISHNAN, V. 2015. Structural basis for stop codon recognition in eukaryotes. *Nature,* 524**,** 493-496.

BULFIELD, G., SILLER, W. G., WIGHT, P. A. L. & MOORE, K. J. 1984. X-Chromosome-Linked Muscular-Dystrophy (Mdx) in the Mouse. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences,* 81**,** 1189-1192.

BYKOV, V. J., ISSAEVA, N., SHILOV, A., HULTCRANTZ, M., PUGACHEVA, E., CHUMAKOV, P., BERGMAN, J., WIMAN, K. G. & SELIVANOVA, G. 2002. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med,* 8**,** 282-8.

BYKOV, V. J. N., ERIKSSON, S. E., BIANCHI, J. & WIMAN, K. G. 2018. Targeting mutant p53 for efficient cancer therapy. *Nat Rev Cancer,* 18**,** 89-102.

BYKOV, V. J. N., ZHANG, Q., ZHANG, M. Q. Z., CEDER, S., ABRAHMSEN, L. & WIMAN, K. G. 2016. Targeting of Mutant p53 and the Cellular Redox Balance by APR-246 as a Strategy for Efficient Cancer Therapy. *Frontiers in Oncology,* 6.

CANON, J., OSGOOD, T., OLSON, S. H., SAIKI, A. Y., ROBERTSON, R., YU, D., EKSTEROWICZ, J., YE, Q., JIN, L., CHEN, A., ZHOU, J., CORDOVER, D., KAUFMAN, S., KENDALL, R., OLINER, J. D., COXON, A. & RADINSKY, R. 2015. The MDM2 Inhibitor AMG 232 Demonstrates Robust Antitumor Efficacy and Potentiates the Activity of p53-Inducing Cytotoxic Agents. *Mol Cancer Ther,* 14**,** 649-58.

CARVAJAL, L. A., BEN NERIAH, D., SENECAL, A., BENARD, L., THIRUTHUVANATHAN, V., YATSENKO, T., NARAYANAGARI, S. R., WHEAT, J. C., TODOROVA, T. I., MITCHELL, K., KENWORTHY, C., GUERLAVAIS, V., ANNIS, D. A., BARTHOLDY, B., WILL, B., ANAMPA, J. D., MANTZARIS, I., AIVADO, M., SINGER, R. H., COLEMAN, R. A., VERMA, A. & STEIDL, U. 2018. Dual inhibition of MDMX and MDM2 as a therapeutic strategy in leukemia. *Science Translational Medicine,* 10.

CASSAN, M. & ROUSSET, J. P. 2001. UAG readthrough in mammalian cells: effect of upstream and downstream stop codon contexts reveal different signals. *BMC Mol Biol,* 2**,** 3.

CHALABI-DCHAR, M., FENOUIL, T., MACHON, C., VINCENT, A., CATEZ, F., MARCEL, V., MERTANI, H. C., SAURIN, J. C., BOUVET, P., GUITTON, J., VENEZIA, N. D. & DIAZ, J. J. 2021. A novel view on an old drug, 5-fluorouracil: an unexpected RNA modifier with intriguing impact on cancer cell fate. *NAR Cancer,* 3**,** zcab032.

CHAPMAN, P. B., HAUSCHILD, A., ROBERT, C., HAANEN, J. B., ASCIERTO, P., LARKIN, J., DUMMER, R., GARBE, C., TESTORI, A., MAIO, M., HOGG, D., LORIGAN, P., LEBBE, C., JOUARY, T., SCHADENDORF, D., RIBAS, A., O'DAY, S. J., SOSMAN, J. A., KIRKWOOD, J. M., EGGERMONT, A. M., DRENO, B., NOLOP, K., LI, J., NELSON, B., HOU, J., LEE, R. J., FLAHERTY, K. T., MCARTHUR, G. A. & GROUP, B.-S. 2011. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med,* 364**,** 2507-16.

CHEN, C. Y., CHEN, J., HE, L. & STILES, B. L. 2018. PTEN: Tumor Suppressor and Metabolic Regulator. *Front Endocrinol (Lausanne),* 9**,** 338.

CHEN, S., WU, J. L., LIANG, Y., TANG, Y. G., SONG, H. X., WU, L. L., XING, Y. F., YAN, N., LI, Y. T., WANG, Z. Y., XIAO, S. J., LU, X., CHEN, S. J. & LU, M. 2021. Arsenic Trioxide Rescues Structural p53 Mutations through a Cryptic Allosteric Site. *Cancer Cell,* 39**,** 225-+.

CHOU, T. C. & TALALAY, P. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul,* 22**,** 27-55.

CORRADINI, S., ALONGI, F., ANDRATSCHKE, N., BELKA, C., BOLDRINI, L., CELLINI, F., DEBUS, J., GUCKENBERGER, M., HORNER-RIEBER, J., LAGERWAARD, F. J., MAZZOLA, R., PALACIOS, M. A., PHILIPPENS, M. E. P., RAAIJMAKERS, C. P. J., TERHAARD, C. H. J., VALENTINI, V. & NIYAZI, M. 2019. MR-guidance in clinical reality: current treatment challenges and future perspectives. *Radiation Oncology,* 14.

COSSON, B., COUTURIER, A., CHABELSKAYA, S., KIKTEV, D., INGE-VECHTOMOV, S., PHILIPPE, M. & ZHOURAVLEVA, G. 2002. Poly(A)-binding protein acts in translation termination via eukaryotic release factor 3 interaction and does not influence [PSI+] propagation. *Molecular and Cellular Biology,* 22**,** 3301-3315.

CRAWFORD, D. K., ALROY, I., SHARPE, N., GODDEERIS, M. M. & WILLIAMS, G. 2020. ELX-02 Generates Protein via Premature Stop Codon Read-Through without Inducing Native Stop Codon Read-Through Proteins. *J Pharmacol Exp Ther,* 374**,** 264-272.

CRICK, F. 1970. Central dogma of molecular biology. *Nature,* 227**,** 561-3.

CRICK, F. H., BARNETT, L., BRENNER, S. & WATTS-TOBIN, R. J. 1961. General nature of the genetic code for proteins. *Nature,* 192**,** 1227-32.

CRIDGE, A. G., CROWE-MCAULIFFE, C., MATHEW, S. F. & TATE, W. P. 2018. Eukaryotic translational termination efficiency is influenced by the 3' nucleotides within the ribosomal mRNA channel. *Nucleic Acids Res,* 46**,** 1927-1944.

CRONSTEIN, B. N. 1997. The mechanism of action of methotrexate. *Rheum Dis Clin North Am,* 23**,** 739-55.

DABROWSKI, M., BUKOWY-BIERYLLO, Z. & ZIETKIEWICZ, E. 2015. Translational readthrough potential of natural termination codons in eucaryotes--The impact of RNA sequence. *RNA Biol,* 12**,** 950-8.

DANG, C. V. 2012. MYC on the path to cancer. *Cell,* 149**,** 22-35.

DELEO, A. B., JAY, G., APPELLA, E., DUBOIS, G. C., LAW, L. W. & OLD, L. J. 1979. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc Natl Acad Sci U S A,* 76**,** 2420-4.

DEVER, T. E. & GREEN, R. 2012. The elongation, termination, and recycling phases of translation in eukaryotes. *Cold Spring Harb Perspect Biol,* 4**,** a013706.

DING, Q., ZHANG, Z., LIU, J. J., JIANG, N., ZHANG, J., ROSS, T. M., CHU, X. J., BARTKOVITZ, D., PODLASKI, F., JANSON, C., TOVAR, C., FILIPOVIC, Z. M., HIGGINS, B., GLENN, K., PACKMAN, K., VASSILEV, L. T. & GRAVES, B. 2013. Discovery of RG7388, a potent and selective p53-MDM2 inhibitor in clinical development. *J Med Chem,* 56**,** 5979-83.

DONEHOWER, L. A., HARVEY, M., SLAGLE, B. L., MCARTHUR, M. J., MONTGOMERY, C. A., JR., BUTEL, J. S. & BRADLEY, A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature,* 356**,** 215-21.

DONEHOWER, L. A., SOUSSI, T., KORKUT, A., LIU, Y. X., SCHULTZ, A., CARDENAS, M., LI, X. B., BABUR, O., HSU, T. K., LICHTARGE, O., WEINSTEIN, J. N., AKBANI, R. & WHEELER, D. A. 2019. Integrated Analysis of TP53 Gene and Pathway Alterations in The Cancer Genome Atlas (vol 28, pg 1370, 2019). *Cell Reports,* 28**,** 3010-3010.

DRUKER, B. J., TALPAZ, M., RESTA, D. J., PENG, B., BUCHDUNGER, E., FORD, J. M., LYDON, N. B., KANTARJIAN, H., CAPDEVILLE, R., OHNO-JONES, S. & SAWYERS, C. L. 2001. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med,* 344**,** 1031-7.

DUMBRAVA, E. E., JOHNSON, M. L., TOLCHER, A. W., SHAPIRO, G., THOMPSON, J. A., EL-KHOUEIRY, A. B., VANDROSS, A. L., KUMMAR, S., PARIKH, A. R., MUNSTER, P. N., DALY, E., DE LEON, L., KHADDAR, M., LEDUKE, K., ROBELL, K., SHEEHAN, L. I., ST LOUIS, M., WIEBESIEK, A., ALLAND, L. & SCHRAM, A. M. 2022. First-in-human study of PC14586, a small molecule structural corrector of Y220C mutant p53, in patients with advanced solid tumors harboring a TP53 Y220C mutation. *Journal of Clinical Oncology,* 40.

DYSON, N. J. 2016. RB1: a prototype tumor suppressor and an enigma. *Genes Dev,* 30**,** 1492-502.

EARP, J., KRZYZANSKI, W., CHAKRABORTY, A., ZAMACONA, M. K. & JUSKO, W. J. 2004. Assessment of drug interactions relevant to pharmacodynamic indirect response models. *J Pharmacokinet Pharmacodyn,* 31**,** 345-80.

EMADI, A., JONES, R. J. & BRODSKY, R. A. 2009. Cyclophosphamide and cancer: golden anniversary. *Nat Rev Clin Oncol,* 6**,** 638-47.

ENDERSBY, R. & BAKER, S. J. 2008. PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene,* 27**,** 5416-30.

ENG, C. 2003. PTEN: one gene, many syndromes. *Hum Mutat,* 22**,** 183-98.

FANG, Y., BATEMAN, J. F., MERCER, J. F. & LAMANDE, S. R. 2013. Nonsense-mediated mRNA decay of collagen -emerging complexity in RNA surveillance mechanisms. *J Cell Sci,* 126**,** 2551-60.

FERRARA, N., HILLAN, K. J., GERBER, H. P. & NOVOTNY, W. 2004. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov,* 3**,** 391-400.

FINLAY, C. A., HINDS, P. W. & LEVINE, A. J. 1989. The P53 Proto-Oncogene Can Act as a Suppressor of Transformation. *Cell,* 57**,** 1083-1093.

FREEMAN, D. J., LI, A. G., WEI, G., LI, H. H., KERTESZ, N., LESCHE, R., WHALE, A. D., MARTINEZ-DIAZ, H., ROZENGURT, N., CARDIFF, R. D., LIU, X. & WU, H. 2003. PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell,* 3**,** 117-30.

FRIESEN, W. J., TROTTA, C. R., TOMIZAWA, Y., ZHUO, J., JOHNSON, B., SIERRA, J., ROY, B., WEETALL, M., HEDRICK, J., SHEEDY, J., TAKASUGI, J., MOON, Y. C., BABU, S., BAIAZITOV, R., LESZYK, J. D., DAVIS, T. W., COLACINO, J. M., PELTZ, S. W. & WELCH, E. M. 2017. The nucleoside analog clitocine is a potent and efficacious readthrough agent. *RNA,* 23**,** 567-577.

FROLOVA, L. Y., MERKULOVA, T. I. & KISSELEV, L. L. 2000. Translation termination in eukaryotes: Polypeptide release factor eRF1 is composed of functionally and structurally distinct domains. *Rna,* 6**,** 381-390.

GAINA, G. & POPA GRUIANU, A. 2021. Muscular dystrophy: Experimental animal models and therapeutic approaches (Review). *Exp Ther Med,* 21**,** 610.

GALLUZZI, L., SENOVILLA, L., VITALE, I., MICHELS, J., MARTINS, I., KEPP, O., CASTEDO, M. & KROEMER, G. 2012. Molecular mechanisms of cisplatin resistance. *Oncogene,* 31**,** 1869-83.

GENCEL-AUGUSTO, J., SU, X., QI, Y., WHITLEY, E. M., PANT, V., XIONG, S., SHAH, V., LIN, J., PEREZ, E., FIOROTTO, M. L., MAHMUD, I., JAIN, A. K., LORENZI, P. L., NAVIN, N. E., RICHIE, E. R. & LOZANO, G. 2023. Dimeric p53 Mutant Elicits Unique Tumor-Suppressive Activities through an Altered Metabolic Program. *Cancer Discov,* 13**,** 1230-1249.

GLUCK, W. L., GOUNDER, M. M., FRANK, R., ESKENS, F., BLAY, J. Y., CASSIER, P. A., SORIA, J. C., CHAWLA, S., DE WEGER, V., WAGNER, A. J., SIEGEL, D., DE VOS, F., RASMUSSEN, E. & HENARY, H. A. 2020. Phase 1 study of the MDM2 inhibitor AMG 232 in patients with advanced P53 wild-type solid tumors or multiple myeloma. *Invest New Drugs,* 38**,** 831-843.

GRECO, W. R., BRAVO, G. & PARSONS, J. C. 1995. The search for synergy: a critical review from a response surface perspective. *Pharmacol Rev,* 47**,** 331-85.

GREM, J. L. 2000. 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. *Invest New Drugs,* 18**,** 299-313.

GUILEY, K. Z. & SHOKAT, K. M. 2023. A Small Molecule Reacts with the p53 Somatic Mutant Y220C to Rescue Wild-type Thermal Stability. *Cancer Discov,* 13**,** 56-69.

HAAS, M., VLCEK, V., BALABANOV, P., SALMONSON, T., BAKCHINE, S., MARKEY, G., WEISE, M., SCHLOSSER-WEBER, G., BROHMANN, H., YERRO, C. P., MENDIZABAL, M. R., STOYANOVA-BENINSKA, V. & HILLEGE, H. L. 2015. European Medicines Agency review of ataluren for the treatment of ambulant patients aged 5 years and older with Duchenne muscular dystrophy resulting from a nonsense mutation in the dystrophin gene. *Neuromuscul Disord,* 25**,** 5-13.

HALAZONETIS, T. D., GORGOULIS, V. G. & BARTEK, J. 2008. An oncogene-induced DNA damage model for cancer development. *Science,* 319**,** 1352-5.

HANAHAN, D. 2022. Hallmarks of Cancer: New Dimensions. *Cancer Discov,* 12**,** 31-46.

HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HEIDELBERGER, C., CHAUDHURI, N. K., DANNEBERG, P., MOOREN, D., GRIESBACH, L., DUSCHINSKY, R., SCHNITZER, R. J., PLEVEN, E. & SCHEINER, J. 1957. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature,* 179**,** 663-6.

HOLLANDER, M. C., BLUMENTHAL, G. M. & DENNIS, P. A. 2011. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. *Nat Rev Cancer,* 11**,** 289-301.

HOLOHAN, C., VAN SCHAEYBROECK, S., LONGLEY, D. B. & JOHNSTON, P. G. 2013. Cancer drug resistance: an evolving paradigm. *Nature Reviews Cancer,* 13**,** 714-726.

JACKS, T., REMINGTON, L., WILLIAMS, B. O., SCHMITT, E. M., HALACHMI, S., BRONSON, R. T. & WEINBERG, R. A. 1994. Tumor spectrum analysis in p53-mutant mice. *Curr Biol,* 4**,** 1-7.

JUNGREIS, I., LIN, M. F., SPOKONY, R., CHAN, C. S., NEGRE, N., VICTORSEN, A., WHITE, K. P. & KELLIS, M. 2011. Evidence of abundant stop codon readthrough in Drosophila and other metazoa. *Genome Research,* 21**,** 2096-2113.

KAROUSIS, E. D. & MUHLEMANN, O. 2019. Nonsense-Mediated mRNA Decay Begins Where Translation Ends. *Cold Spring Harb Perspect Biol,* 11.

KAROUSIS, E. D., NASIF, S. & MUHLEMANN, O. 2016. Nonsense-mediated mRNA decay: novel mechanistic insights and biological impact. *Wiley Interdiscip Rev RNA,* 7**,** 661-82.

KASTENHUBER, E. R. & LOWE, S. W. 2017. Putting p53 in Context. *Cell,* 170**,** 1062-1078.

KEELING, K. M., WANG, D., CONARD, S. E. & BEDWELL, D. M. 2012. Suppression of premature termination codons as a therapeutic approach. *Crit Rev Biochem Mol Biol,* 47**,** 444-63.

LAFONTAINE, D. L. & TOLLERVEY, D. 2001. The function and synthesis of ribosomes. *Nat Rev Mol Cell Biol,* 2**,** 514-20.

LANE, D. P. 1992. Cancer. p53, guardian of the genome. *Nature,* 358**,** 15-6.

LANE, D. P. & BENCHIMOL, S. 1990. p53: oncogene or anti-oncogene? *Genes Dev,* 4**,** 1-8.

LANE, D. P. & CRAWFORD, L. V. 1979. T-Antigen Is Bound to a Host Protein in Sv40-Transformed Cells. *Nature,* 278**,** 261-263.

LANG, G. A., IWAKUMA, T., SUH, Y. A., LIU, G., RAO, V. A., PARANT, J. M., VALENTIN-VEGA, Y. A., TERZIAN, T., CALDWELL, L. C., STRONG, L. C., EL-NAGGAR, A. K. & LOZANO, G. 2004. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell,* 119**,** 861-72.

LARKIN, J., ASCIERTO, P. A., DRENO, B., ATKINSON, V., LISZKAY, G., MAIO, M., MANDALA, M., DEMIDOV, L., STROYAKOVSKIY, D., THOMAS, L., DE LA CRUZ-MERINO, L., DUTRIAUX, C., GARBE, C., SOVAK, M. A., CHANG, I., CHOONG, N., HACK, S. P., MCARTHUR, G. A. & RIBAS, A. 2014. Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N Engl J Med,* 371**,** 1867-76.

LAVIN, M. F. & GUEVEN, N. 2006. The complexity of p53 stabilization and activation. *Cell Death Differ,* 13**,** 941-50.

LEE, J. O., YANG, H., GEORGESCU, M. M., DI CRISTOFANO, A., MAEHAMA, T., SHI, Y., DIXON, J. E., PANDOLFI, P. & PAVLETICH, N. P. 1999. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell,* 99**,** 323-34.

LEE, Y. R., CHEN, M. & PANDOLFI, P. P. 2018. The functions and regulation of the PTEN tumour suppressor: new modes and prospects. *Nat Rev Mol Cell Biol,* 19**,** 547-562.

LESLIE, N. R. & FOTI, M. 2011. Non-genomic loss of PTEN function in cancer: not in my genes. *Trends Pharmacol Sci,* 32**,** 131-40.

LI, J., YEN, C., LIAW, D., PODSYPANINA, K., BOSE, S., WANG, S. I., PUC, J., MILIARESIS, C., RODGERS, L., MCCOMBIE, R., BIGNER, S. H., GIOVANELLA, B. C., ITTMANN, M., TYCKO, B., HIBSHOOSH, H., WIGLER, M. H. & PARSONS, R. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science,* 275**,** 1943-1947.

LINDEMANN, A., PATEL, A. A., SILVER, N. L., TANG, L., LIU, Z. Y., WANG, L., TANAKA, N., RAO, X. Y., TAKAHASHI, H., MADUKA, N. K., ZHAO, M., CHEN, T. C., LIU, W. W., GAO, M., WANG, J., FRANK, S. J., HITTELMAN, W. N., MILLS, G. B., MYERS, J. N. & OSMAN, A. A. 2019. COTI-2, A Novel Thiosemicarbazone Derivative, Exhibits Antitumor Activity in HNSCC through p53-dependent and -independent Mechanisms. *Clinical Cancer Research,* 25**,** 5650-5662.

LINZER, D. I. & LEVINE, A. J. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell,* 17**,** 43-52.

LIU, G., PARANT, J. M., LANG, G., CHAU, P., CHAVEZ-REYES, A., EL-NAGGAR, A. K., MULTANI, A., CHANG, S. & LOZANO, G. 2004. Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. *Nat Genet,* 36**,** 63-8.

LIU, X., WILCKEN, R., JOERGER, A. C., CHUCKOWREE, I. S., AMIN, J., SPENCER, J. & FERSHT, A. R. 2013. Small molecule induced reactivation of mutant p53 in cancer cells. *Nucleic Acids Res,* 41**,** 6034-44.

LONGLEY, D. B., HARKIN, D. P. & JOHNSTON, P. G. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer,* 3**,** 330-8.

LOUGHRAN, G., CHOU, M. Y., IVANOV, I. P., JUNGREIS, I., KELLIS, M., KIRAN, A. M., BARANOV, P. V. & ATKINS, J. F. 2014. Evidence of efficient stop codon readthrough in four mammalian genes. *Nucleic Acids Res,* 42**,** 8928-38.

MAEHAMA, T. & DIXON, J. E. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem,* 273**,** 13375-8.

MANCHESTER, K. L. 1995. Theodor Boveri and the Origin of Malignant Tumors. *Trends in Cell Biology,* 5**,** 384-387.

MCGRANAHAN, N. & SWANTON, C. 2017. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell,* 168**,** 613-628.

MELERO, J. A., STITT, D. T., MANGEL, W. F. & CARROLL, R. B. 1979. Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T antigen and present in SV40-infected and -transformed cells. *Virology,* 93**,** 466-80.

MILELLA, M., FALCONE, I., CONCIATORI, F., CESTA INCANI, U., DEL CURATOLO, A., INZERILLI, N., NUZZO, C. M., VACCARO, V., VARI, S., COGNETTI, F. & CIUFFREDA, L. 2015. PTEN: Multiple Functions in Human Malignant Tumors. *Front Oncol,* 5**,** 24.

MOLL, U. M. & PETRENKO, O. 2003. The MDM2-p53 interaction. *Mol Cancer Res,* 1**,** 1001-8.

MOORE, A. R., ROSENBERG, S. C., MCCORMICK, F. & MALEK, S. 2020. RAS-targeted therapies: is the undruggable drugged? *Nature Reviews Drug Discovery,* 19**,** 533-552.

MOSER, A. R., PITOT, H. C. & DOVE, W. F. 1990. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science,* 247**,** 322-4.

NIGRO, J. M., BAKER, S. J., PREISINGER, A. C., JESSUP, J. M., HOSTETTER, R., CLEARY, K., BIGNER, S. H., DAVIDSON, N., BAYLIN, S., DEVILEE, P. & ET AL. 1989. Mutations in the p53 gene occur in diverse human tumour types. *Nature,* 342**,** 705-8.

OLIVE, K. P., TUVESON, D. A., RUHE, Z. C., YIN, B., WILLIS, N. A., BRONSON, R. T., CROWLEY, D. & JACKS, T. 2004. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell,* 119**,** 847-60.

PATEL, L., PASS, I., COXON, P., DOWNES, C. P., SMITH, S. A. & MACPHEE, C. H. 2001. Tumor suppressor and anti-inflammatory actions of PPAR gamma agonists are mediated via upregulation of PTEN. *Current Biology,* 11**,** 764-768.

PEREZ, H., ABDALLAH, M. F., CHAVIRA, J. I., NORRIS, A. S., EGELAND, M. T., VO, K. L., BUECHSENSCHUETZ, C. L., SANGHEZ, V., KIM, J. L., PIND, M., NAKAMURA, K., HICKS, G. G., GATTI, R. A., MADRENAS, J., IACOVINO, M., MCKINNON, P. J. & MATHEWS, P. J. 2021. A novel, ataxic mouse model of ataxia telangiectasia caused by a clinically relevant nonsense mutation. *Elife,* 10.

PESTOVA, T. V., KOLUPAEVA, V. G., LOMAKIN, I. B., PILIPENKO, E. V., SHATSKY, I. N., AGOL, V. I. & HELLEN, C. U. 2001. Molecular mechanisms of translation initiation in eukaryotes. *Proc Natl Acad Sci U S A,* 98**,** 7029-36.

PLANCHON, S. M., WAITE, K. A. & ENG, C. 2008. The nuclear affairs of PTEN. *J Cell Sci,* 121**,** 249-53.

POSTOW, M. A., CALLAHAN, M. K. & WOLCHOK, J. D. 2015. Immune Checkpoint Blockade in Cancer Therapy. *J Clin Oncol,* 33**,** 1974-82.

ROSENBERG, B., VANCAMP, L. & KRIGAS, T. 1965. Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode. *Nature,* 205**,** 698-+.

SABAPATHY, K. & LANE, D. P. 2018. Therapeutic targeting of p53: all mutants are equal, but some mutants are more equal than others. *Nat Rev Clin Oncol,* 15**,** 13-30.

SALEH, M. N., PATEL, M. R., BAUER, T. M., GOEL, S., FALCHOOK, G. S., SHAPIRO, G. I., CHUNG, K. Y., INFANTE, J. R., CONRY, R. M., RABINOWITS, G., HONG, D. S., WANG, J. S., STEIDL, U., NAIK, G., GUERLAVAIS, V., VUKOVIC, V., ANNIS, D. A., AIVADO, M. & MERIC-BERNSTAM, F. 2021. Phase 1 Trial of ALRN-6924, a Dual Inhibitor of MDMX and MDM2, in Patients with Solid Tumors and Lymphomas Bearing Wild-type TP53. *Clin Cancer Res,* 27**,** 5236-5247.

SALLMAN, D. A., DEZERN, A. E., GARCIA-MANERO, G., STEENSMA, D. P., ROBOZ, G. J., SEKERES, M. A., CLUZEAU, T., SWEET, K. L., MCLEMORE, A., MCGRAW, K. L., PUSKAS, J., ZHANG, L., YAO, J., MO, Q., NARDELLI, L., AL ALI, N. H., PADRON, E., KORBEL, G., ATTAR, E. C., KANTARJIAN, H. M., LANCET, J. E., FENAUX, P., LIST, A. F. & KOMROKJI, R. S. 2021. Eprenetapopt (APR-246) and Azacitidine in TP53-Mutant Myelodysplastic Syndromes. *J Clin Oncol,* 39**,** 1584-1594.

SAWYERS, C. L. 1999. Chronic myeloid leukemia. *N Engl J Med,* 340**,** 1330-40.

SHAW, A. T., KIM, D. W., NAKAGAWA, K., SETO, T., CRINO, L., AHN, M. J., DE PAS, T., BESSE, B., SOLOMON, B. J., BLACKHALL, F., WU, Y. L., THOMAS, M., O'BYRNE, K. J., MORO-SIBILOT, D., CAMIDGE, D. R., MOK, T., HIRSH, V., RIELY, G. J., IYER, S., TASSELL, V., POLLI, A., WILNER, K. D. & JANNE, P. A. 2013. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med,* 368**,** 2385-94.

SHAW, P., BOVEY, R., TARDY, S., SAHLI, R., SORDAT, B. & COSTA, J. 1992. Induction of Apoptosis by Wild-Type P53 in a Human Colon Tumor-Derived Cell-Line. *Proceedings of the National Academy of Sciences of the United States of America,* 89**,** 4495-4499.

SIAMOF, C. M., GOEL, S. & CAI, W. 2020. Moving Beyond the Pillars of Cancer Treatment: Perspectives From Nanotechnology. *Front Chem,* 8**,** 598100.

SINGH, G., REBBAPRAGADA, I. & LYKKE-ANDERSEN, J. 2008. A competition between stimulators and antagonists of Upf complex recruitment governs human nonsense-mediated mRNA decay. *Plos Biology,* 6**,** 860-871.

SKUZESKI, J. M., NICHOLS, L. M., GESTELAND, R. F. & ATKINS, J. F. 1991. The signal for a leaky UAG stop codon in several plant viruses includes the two downstream codons. *J Mol Biol,* 218**,** 365-73.

SLAMON, D. J., LEYLAND-JONES, B., SHAK, S., FUCHS, H., PATON, V., BAJAMONDE, A., FLEMING, T., EIERMANN, W., WOLTER, J., PEGRAM, M., BASELGA, J. & NORTON, L. 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England Journal of Medicine,* 344**,** 783-792.

SLINKER, B. K. 1998. The statistics of synergism. *Journal of Molecular and Cellular Cardiology,* 30**,** 723-731.

SONG, M. S., SALMENA, L. & PANDOLFI, P. P. 2012. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol,* 13**,** 283-96.

SOUSSI, T. & WIMAN, K. G. 2007. Shaping genetic alterations in human cancer: the p53 mutation paradigm. *Cancer Cell,* 12**,** 303-12.

STRATTON, M. R., CAMPBELL, P. J. & FUTREAL, P. A. 2009. The cancer genome. *Nature,* 458**,** 719-24.

SU, L. K., KINZLER, K. W., VOGELSTEIN, B., PREISINGER, A. C., MOSER, A. R., LUONGO, C., GOULD, K. A. & DOVE, W. F. 1992. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science,* 256**,** 668-70.

SUNG, H., FERLAY, J., SIEGEL, R. L., LAVERSANNE, M., SOERJOMATARAM, I., JEMAL, A. & BRAY, F. 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin,* 71**,** 209-249.

SURKA, C., JIN, L., MBONG, N., LU, C. C., JANG, I. S., RYCHAK, E., MENDY, D., CLAYTON, T., TINDALL, E., HSU, C., FONTANILLO, C., TRAN, E., CONTRERAS, A., NG, S. W. K., MATYSKIELA, M., WANG, K., CHAMBERLAIN, P., CATHERS, B., CARMICHAEL, J., HANSEN, J., WANG, J. C. Y., MINDEN, M. D., FAN, J., PIERCE, D. W., POURDEHNAD, M., ROLFE, M., LOPEZ-GIRONA, A., DICK, J. E. & LU, G. 2021. CC-90009, a novel cereblon E3 ligase modulator, targets acute myeloid leukemia blasts and leukemia stem cells. *Blood,* 137**,** 661-677.

SYNNOTT, N. C., O'CONNELL, D., CROWN, J. & DUFFY, M. J. 2020. COTI-2 reactivates mutant p53 and inhibits growth of triple-negative breast cancer cells. *Breast Cancer Research and Treatment,* 179**,** 47-56.

TACAR, O., SRIAMORNSAK, P. & DASS, C. R. 2013. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *Journal of Pharmacy and Pharmacology,* 65**,** 157-170.

TALLARIDA, R. J. 2006. An overview of drug combination analysis with isobolograms. *J Pharmacol Exp Ther,* 319**,** 1-7.

TATE, J. G., BAMFORD, S., JUBB, H. C., SONDKA, Z., BEARE, D. M., BINDAL, N., BOUTSELAKIS, H., COLE, C. G., CREATORE, C., DAWSON, E., FISH, P., HARSHA, B., HATHAWAY, C., JUPE, S. C., KOK, C. Y., NOBLE, K., PONTING, L., RAMSHAW, C. C., RYE, C. E., SPEEDY, H. E., STEFANCSIK, R., THOMPSON, S. L., WANG, S., WARD, S., CAMPBELL, P. J. & FORBES, S. A. 2019. COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res,* 47**,** D941-D947.

TOVAR, C., GRAVES, B., PACKMAN, K., FILIPOVIC, Z., HIGGINS, B., XIA, M., TARDELL, C., GARRIDO, R., LEE, E., KOLINSKY, K., TO, K. H., LINN, M., PODLASKI, F., WOVKULICH, P., VU, B. & VASSILEV, L. T. 2013. MDM2 small-molecule antagonist RG7112 activates p53 signaling and regresses human tumors in preclinical cancer models. *Cancer Res,* 73**,** 2587-97.

TRZASKA, C., AMAND, S., BAILLY, C., LEROY, C., MARCHAND, V., DUVERNOIS-BERTHET, E., SALIOU, J. M., BENHABILES, H., WERKMEISTER, E., CHASSAT, T., GUILBERT, R., HANNEBIQUE, D., MOURAY, A., COPIN, M. C., MOREAU, P. A., ADRIAENSSENS, E., KULOZIK, A., WESTHOF, E., TULASNE, D., MOTORIN, Y., REBUFFAT, S. & LEJEUNE, F. 2020. 2,6-Diaminopurine as a highly potent corrector of UGA nonsense mutations. *Nat Commun,* 11**,** 1509.

VALIENTE, M., ANDRES-PONS, A., GOMAR, B., TORRES, J., GIL, A., TAPPAREL, C., ANTONARAKIS, S. E. & PULIDO, R. 2005. Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. *J Biol Chem,* 280**,** 28936-43.

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,* 303**,** 844-8.

VAZQUEZ, F., RAMASWAMY, S., NAKAMURA, N. & SELLERS, W. R. 2000. Phosphorylation of the PTEN tail regulates protein stability and function. *Molecular and Cellular Biology,* 20**,** 5010-5018.

VIROLLE, T., ADAMSON, E. D., BARON, V., BIRLE, D., MERCOLA, D., MUSTELIN, T. & DE BELLE, I. 2001. The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling. *Nat Cell Biol,* 3**,** 1124-8.

VLOT, A. H. C., ANICETO, N., MENDEN, M. P., ULRICH-MERZENICH, G. & BENDER, A. 2019. Applying synergy metrics to combination screening data: agreements, disagreements and pitfalls. *Drug Discov Today,* 24**,** 2286-2298.

VOGELSTEIN, B. & KINZLER, K. W. 2004. Cancer genes and the pathways they control. *Nat Med,* 10**,** 789-99.

VOGELSTEIN, B., LANE, D. & LEVINE, A. J. 2000. Surfing the p53 network. *Nature,* 408**,** 307-10.

VOGELSTEIN, B., PAPADOPOULOS, N., VELCULESCU, V. E., ZHOU, S., DIAZ, L. A., JR. & KINZLER, K. W. 2013. Cancer genome landscapes. *Science,* 339**,** 1546-58.

VOUSDEN, K. H. & LU, X. 2002. Live or let die: the cell's response to p53. *Nat Rev Cancer,* 2**,** 594-604.

VOUSDEN, K. H. & PRIVES, C. 2009. Blinded by the Light: The Growing Complexity of p53. *Cell,* 137**,** 413-31.

WAITE, K. A. & ENG, C. 2002. Protean PTEN: form and function. *Am J Hum Genet,* 70**,** 829-44.

WARGO, J. A., REUBEN, A., COOPER, Z. A., OH, K. S. & SULLIVAN, R. J. 2015. Immune Effects of Chemotherapy, Radiation, and Targeted Therapy and Opportunities for Combination With Immunotherapy. *Seminars in Oncology,* 42**,** 601-616.

WATSON, J. D. & CRICK, F. H. C. 1953. Molecular Structure of Nucleic Acids - a Structure for Deoxyribose Nucleic Acid. *Nature,* 171**,** 737-738.

WILLS, N. M., GESTELAND, R. F. & ATKINS, J. F. 1991. Evidence that a downstream pseudoknot is required for translational read-through of the Moloney murine leukemia virus gag stop codon. *Proc Natl Acad Sci U S A,* 88**,** 6991-5.

XIONG, S., CHACHAD, D., ZHANG, Y., GENCEL-AUGUSTO, J., SIRITO, M., PANT, V., YANG, P., SUN, C., CHAU, G., QI, Y., SU, X., WHITLEY, E. M., EL-NAGGAR, A. K. & LOZANO, G. 2022. Differential Gain-of-Function Activity of Three p53 Hotspot Mutants In Vivo. *Cancer Res,* 82**,** 1926-1936.

XUE, X. P., MUTYAM, V., TANG, L. P., BISWAS, S., DU, M., JACKSON, L. A., DAI, Y. Y., BELAKHOV, V., SHALEV, M., CHEN, F. Q., SCHACHT, J., BRIDGES, R. J., BAASOV, T., HONG, J., BEDWELL, D. M. & ROWE, S. M. 2014. Synthetic Aminoglycosides Efficiently Suppress Cystic Fibrosis Transmembrane Conductance Regulator Nonsense Mutations and Are Enhanced by Ivacaftor. *American Journal of Respiratory Cell and Molecular Biology,* 50**,** 805-816.

YADAV, B., WENNERBERG, K., AITTOKALLIO, T. & TANG, J. 2015. Searching for Drug Synergy in Complex Dose-Response Landscapes Using an Interaction Potency Model. *Comput Struct Biotechnol J,* 13**,** 504-13.

ZHANG, X. W., YAN, X. J., ZHOU, Z. R., YANG, F. F., WU, Z. Y., SUN, H. B., LIANG, W. X., SONG, A. X., LALLEMAND-BREITENBACH, V., JEANNE, M., ZHANG, Q. Y., YANG, H. Y., HUANG, Q. H., ZHOU, G. B., TONG, J. H., ZHANG, Y., WU, J. H., HU, H. Y., DE THE, H., CHEN, S. J. & CHEN, Z. 2010. Arsenic trioxide controls the fate of the PML-RARalpha oncoprotein by directly binding PML. *Science,* 328**,** 240-3.