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

TRANSLATIONAL READTHROUGH OF NONSENSE MUTANT TP53, RB1 AND PTEN TUMOR SUPPRESSOR GENES AS A STRATEGY FOR NOVEL CANCER THERAPY

Mireia Palomar Siles

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Translational Readthrough of Nonsense Mutant TP53, RB1 and PTEN Tumor Suppressor Genes as a Strategy for Novel Cancer Therapy

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By

Mireia Palomar Siles

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

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

Opponent:
Professor Alberto Inga
University of Trento
Department of Department of Cellular, Computational and Integrative Biology - CIBIO

Examination Board:
Professor Suparna Sanyal
Uppsala University
Department of Cell and Molecular Biology

Assoc. Prof. Ola Larsson
Karolinska Institutet
Department of Oncology-Pathology

Assoc. Prof. Olle Sangfelt
Karolinska Institutet
Department of Cell and Molecular Biology

To my family,

who made this journey possible

Popular science summary of the thesis

Cancer affects almost 20 million people around the world every year. It can be considered as several diseases with common characteristics. A key feature is the ability of cells to divide in an uncontrolled manner and escape normal cellular suicide programs. This will cause the formation of a tumor mass from which cells can eventually detach, and invade surrounding tissue and migrate to other organs in the body. This process is called metastasis. It is also known that cancer has a multifactorial origin that includes both intrinsic (not modifiable) and extrinsic factors (partially or totally modifiable). Some of the known causes of cancer are tobacco smoke, alcohol, some viruses and ultraviolet radiation. Cancer is a genetic disease in the sense that it arises due to mutations in specific genes in our cells.

Our genes are essentially DNA, which is made of the nucleotides adenine (A), guanine (G), thymine (T) and cytosine (C) arranged in a double helix in which A base-pairs with T and G base-pairs with C. A gene has a specific sequence of base-pairs and this is the code for making all the proteins that allow cells to perform all their various functions. Our genome contains around 20 000 different genes. Mutations are alterations in the DNA sequence. There are different classes of mutations, such as point mutations, insertions or deletions of nucleotides. This thesis is focused on the so-called *nonsense mutations*, which are base-pair substitutions in the DNA that result in a premature stop signal in a gene.

DNA is transcribed to messenger RNA (mRNA), which is translated into proteins. A gene produces an mRNA which has the code for synthesis of a protein with a specific function. Translation of mRNA to proteins is an important cellular process that deciphers the information in the DNA and mRNA to create the protein. Normally, the translation machinery reads the entire mRNA sequence from the beginning to the end, producing the complete and functional protein. However, if a nonsense mutation is present, it will act as a stop signal so that the translation machinery can only read the mRNA sequence up to that point. This will produce a truncated protein (shorter version), which will most likely not be functional.

Tumor suppressor genes are a class of genes that normally prevent uncontrolled cell proliferation or in other ways protect cells from becoming cancer cells. The most studied tumor suppressor gene is *TP53* that codes for the p53 protein, also known as the "Guardian of the genome" due to its important role in protecting the genome. p53 can block cell division or induce cell suicide by a process named apoptosis. The importance of *TP53* as tumor suppressor is highlighted by the fact that *TP53* is mutated in around half of all human cancers. Therefore, restoring normal function to mutated p53 is an interesting strategy for novel cancer treatment. The other tumor suppressor genes included in this thesis are *RB1* and *PTEN*. *RB1* codes for the Rb protein which has a critical role in the regulation of cell division. *PTEN* codes for the PTEN protein that is important for controlling cell growth and survival via other pathways. Nonsense mutations in any of these three genes cause the production of truncated and non-functional p53, Rb or PTEN proteins that can no longer act as brakes for cell proliferation, resulting in unbridled cell growth and development of cancer.

One strategy to restore the full-length protein from genes that carry a nonsense mutation is called *Translational readthrough*. This process tricks the translation machinery so that the ribosome, when it arrives at the nonsense mutation in the mRNA, continues translation until the correct end of the coding sequence instead of stopping at the premature stop signal. This results in the production of a full-length protein capable of regulating cell proliferation in a proper manner or even induce cell death. Translational readthrough can be pharmacologically induced by different compounds known as readthrough inducers. In the studies included in this thesis, the activity of already reported readthrough inducers by other researchers and of compounds found in our lab has been examined in the *TP53*, *RB1* or *PTEN* tumor suppressor genes.

In **Paper I**, the activity of two antibiotics (G418 and gentamicin), known to induce readthrough, was examined in combination with other compounds with the aim of reactivating nonsense mutant *TP53* in a more potent way. The idea was to find ways of reducing the concentration of these antibiotics, since they are quite toxic to patients, but to still maintain good levels of readthrough induction. Restoration of nonsense mutant *TP53* was also studied in **Paper II** in which we found that a commonly used chemotherapeutic drug (5-Fluorouracil) has the ability of inducing readthrough in *TP53*. We could show that this effect was mediated by one of its metabolites (5-Fluorouridine) and most likely through its ability to incorporate into the mRNA. Restored full-length p53 upon treatment with 5-Fluorouridine was functional as it induced p53-dependent cell death. Also, we could show that readthrough could be obtained *in vivo* in a mouse model where a tumor was formed with cells carrying nonsense mutated *TP53*. In **Paper III**, instead of *TP53* gene, we aimed to examine readthrough induction in nonsense mutant *RB1* by using the antibiotic G418 (also used in **Paper I**). We could prove for the first time that induction of readthrough in *RB1* is also possible. In addition, we used combination treatment of the antibiotic G418 and another already reported readthrough-inducing compound (CC-90009) and observed a marked enhancement in the full-length Rb protein production. Finally, in **Paper IV**, we discovered two potential novel readthrough inducers (C47 and C61) whose activity was enhanced by combination treatments with G418 in case of C47 and with CC-90009 (used in **Paper III**) and its related molecule CC-885 in case of C61.

In summary, we show clinical potential of pharmacologically waking up three crucial tumor suppressor genes from their inactive sleeping state using induction of translational readthrough.

Resumen divulgativo de la tesis

El cáncer afecta a casi 20 millones de personas cada año en el mundo. Se puede considerar como varias enfermedades con características comunes. Una característica clave es la capacidad de las células en dividirse de manera descontrolada y escapar de los programas normales de suicidio celular. Esto causará la formación de una masa tumoral de la cual algunas células pueden eventualmente desprenderse e invadir los tejidos adyacentes y migrar a otros órganos en el cuerpo. Este proceso se llama metástasis. También se sabe que el cáncer tiene un origen multifactorial que incluye tanto factores intrínsecos (no modificables) como factores extrínsecos (parcial o totalmente modificables). Algunas de las causas conocidas del cáncer son el tabaco, el alcohol, algunos virus y la radiación ultravioleta. El cáncer es una enfermedad genética ya que aparece debido a mutaciones en ciertos genes en nuestras células.

Nuestros genes son, esencialmente, ADN el cual está compuesto de los nucleótidos adenina (A), guanina (G), timina (T) y citosina (C) organizados en una doble hélice en la que A se empareja con T y G con C. A su vez, cada gen tiene una secuencia específica de pares de bases que es el código para crear todas las proteínas que permiten a las células hacer sus diferentes funciones. Nuestro genoma contiene alrededor de 20 000 genes diferentes. Las mutaciones son alteraciones en la secuencia de ADN. Existen diferentes clases de mutaciones como, por ejemplo, las *mutaciones puntuales*, inserciones o deleciones de nucleótidos. Esta tesis se centra en las llamadas *mutaciones sin sentido*, las cuales son sustituciones de pares de bases en el ADN que generan una señal de terminación prematura en un gen. El ADN se transcribe a ARN mensajero (ARNm) que a su vez se traduce a proteínas. Un gen produce un ARNm que tiene la información para sintetizar una proteína con una función específica. La traducción de ARNm a proteínas es un proceso celular importante que descifra la información en el ADN y el ARNm para crear la proteína. En condiciones normales, la maquinaria de traducción lee la secuencia entera de ARNm desde el principio hasta el final, produciendo así la proteína completa y funcional. Sin embargo, la presencia de una mutación sin sentido actuará como señal de terminación prematura haciendo que la maquinaria de traducción sólo pueda leer la información en el ARNm hasta ese punto y no la secuencia completa. Esto producirá una proteína truncada (una versión más corta de la proteína) la cual lo más probable no sea funcional.

Los *genes supresores de tumores* son un tipo de genes que normalmente evitan la proliferación celular descontrolada o que protegen a las células de convertirse en células cancerosas. El gen supresor tumoral más estudiado es *TP53* que codifica la proteína p53, también conocida como el *"Guardián del genoma"* debido a su importante rol en proteger el genoma. p53 puede bloquear la división celular o inducir suicidio celular a través de un proceso llamado apoptosis. La importancia de *TP53* como gen supresor tumoral es evidente debido al hecho de que *TP53* está mutado en aproximadamente la mitad de los cánceres humanos. Por este motivo, restaurar la función normal de p53 mutada es una estrategia interesante para el tratamiento del cáncer. Los otros genes supresores de tumores incluidos en esta tesis son los genes *RB1* y *PTEN*. *RB1* codifica la proteína Rb que está involucrada en la regulación de la división celular. *PTEN*

codifica la proteína PTEN que es importante para controlar el crecimiento y proliferación celular a través de otras vías. La presencia de mutaciones sin sentido en cualquiera de esto tres genes causa la producción de p53, Rb o PTEN truncadas y no funcionales. Estas proteínas truncadas no podrán actuar como frenos de la proliferación celular resultando en una división celular descontrolada y el desarrollo del cáncer.

Una estrategia para restaurar la proteína entera producida por genes que tienen una mutación sin sentido se llama *translational readthrough*. Este proceso es capaz de engañar a la maquinaria de traducción de manera que cuando llega a la mutación sin sentido en el ARNm, en vez de parar el proceso de traducción en ese punto prematuro de terminación, continúa hasta el punto de terminación normal. Esto producirá la proteína entera capaz de regular la proliferación celular de manera correcta o incluso inducir la muerte celular. El proceso *translational readthrough* se puede inducir farmacológicamente con diferentes tipos de compuestos conocidos como inductores de *readthrough*. En los estudios incluidos en esta tesis, la actividad de compuestos ya descritos por otros investigadores o de compuestos descubiertos en nuestro laboratorio han sido examinados en los genes *TP53*, *RB1* y *PTEN*.

En el **Estudio I**, la actividad de dos antibióticos (G418 y gentamicina), ya conocidos en inducir *translational readthrough*, fue examinada en combinación con otros compuestos con el fin de reactivar de manera más potente *TP53* mutado con una mutación sin sentido. La idea detrás de este estudio era encontrar maneras de reducir la concentración de antibióticos, ya que son bastante tóxicos para los pacientes, pero seguir manteniendo buenos niveles de *readthrough*. La restauración de *TP53* mutado también fue estudiada en el **Estudio II**, en el cual descubrimos que un quimioterapéutico usado comúnmente (5-Fluorouracilo) tiene la habilidad de inducir *readthrough* en *TP53*. Pudimos demostrar que este efecto es mediado por uno de sus metabolitos (5-Fluorouridina) y muy probablemente a través de su habilidad en incorporarse en el ARNm. La proteína completa p53 restaurada por el tratamiento con 5-Fluorouridina es funcional ya que es capaz de inducir muerte celular de manera dependiente de p53. Además, pudimos demostrar inducción de *readthrough in vivo* en un modelo de ratón en el que se creó un tumor con células portadoras de *TP53* mutado. En el **Estudio III**, en cambio de *TP53*, el objetivo era estudiar el proceso de *readthrough* en *RB1* mutado usando el antibiótico G418 (usado también en el **Estudio I**). En este estudio, pudimos demostrar por primera vez que la inducción de *readthrough* en *RB1* también es posible. Más allá, combinamos el antibiótico G418 con otro compuesto que también puede inducir *readthrough* (CC-90009) y observamos un marcado incremento en la producción de proteína Rb completa. Finalmente, en el **Estudio IV**, descubrimos dos potenciales nuevos inductores de *readthrough* (C47 y C61) cuya actividad es potenciada por la combinación con el antibiótico G418 en el caso del C47 y con CC-90009 (usado en el **Estudio III**) y su molécula relacionada CC-885 en el caso del C61.

En resumen, con estos resultados demostramos el potencial clínico de despertar farmacológicamente tres genes supresores de tumores de su estado dormido e inactivo usando la inducción de *translational readthrough*.

Abstract

A nonsense mutation causes a premature termination codon in the coding sequence of an mRNA. This leads to termination of translation and release of a truncated and in most cases non-functional protein. Tumor suppressor genes normally act to prevent tumorigenesis but inactivating mutations in these genes, for example nonsense mutations, can lead to cancer. *TP53*, *RB1* and *PTEN* are among the most well-known tumor suppressor genes. *TP53* codes for the p53 protein that regulates cellular processes such as cell cycle arrest, metabolism and cell death by apoptosis. Around 50% of all tumors carry *TP53* mutation and 11% of *TP53* mutations are nonsense mutations. The Rb protein encoded by the *RB1* tumor suppressor gene is a regulator of cell cycle progression. Around 25-34% of reported *RB1* mutations are nonsense mutations. The *PTEN* gene codes for the phosphatase PTEN that controls cell proliferation and cell survival via the PI3K-AKT pathway. *PTEN* nonsense mutations account for around 17.3% of all somatic mutations in this gene.

Pharmacological induction of translational readthrough has been applied as a strategy to rescue different types of nonsense mutant genes including tumor suppressor genes. A number of compounds have readthrough-inducing activity, including aminoglycoside antibiotics. In this thesis, readthrough-inducing agents have been examined as single treatments and in some cases in combination treatments to induce readthrough of nonsense mutant *TP53*, *RB1* or *PTEN*. In **Paper I**, we show that combination treatment with aminoglycosides G418 or gentamicin and Mdm2-p53 inhibitors Nutlin-3a or MI-773 or the proteasome inhibitor Bortezomib increased full-length p53 levels in a nonsense mutant *TP53* background. In **Paper II**, we found that the chemotherapeutic drug 5-Fluorouracil can induce functional full-length p53 in *TP53* R213X nonsense mutated cells via its metabolite 5-Fluorouridine that is incorporated into mRNA. We also showed induction of full-length p53 *in vivo* in a xenograft mouse model. In **Paper III**, we examined G418 as readthrough inducer of nonsense mutant *RB1*. We observed induction of full-length Rb by G418 and that this effect was markedly enhanced by combination treatment with the reported readthrough inducer CC-90009. Finally, in **Paper IV**, we performed chemical library screening and identified two novel candidate readthrough inducers, C47 and C61. We found that C47 synergizes with G418 and that C61 synergizes with CC-885/CC-90009 for induction of readthrough of R213X nonsense mutant *TP53*. C47 can also synergize with G418 for induction of readthrough of R130X, R233X and R335X nonsense mutant *PTEN*. The mechanisms of action of C47 and C61 remain unclear and require further studies.

In conclusion, this work shows that induction of translational readthrough of nonsense mutant *TP53*, *RB1* and *PTEN* tumor suppressor genes is feasible. We have studied compounds already known to induce readthrough, such as G418 and CC-885/CC-90009, as well as the 5-Fluorouracil metabolite 5-Fluorouridine and the novel candidate readthrough-inducing compounds C47 and C61. Combination treatments have been shown to enhance translational readthrough in the different genes. These results may facilitate the development of nonsense mutation-targeted cancer therapy in the future.

List of scientific papers

I. Meiqiongzi Zhang*, Angelos Heldin*, **Mireia Palomar-Siles**, Susanne Öhlin, Vladimir J.N. Bykov and Klas G. Wiman.
Synergistic Rescue of Nonsense Mutant Tumor Suppressor p53 by Combination Treatment with Aminoglycosides and Mdm2 Inhibitors.
Front Oncol (2018) 7:323.
*These authors contributed equally.

II. **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.
Translational readthrough of nonsense mutant TP53 by mRNA incorporation of 5-Fluorouridine.
Cell Death Dis (2022) 13:997.

III. **Mireia Palomar-Siles**, Viktor Yurevych, Vladimir J.N. Bykov and Klas G. Wiman.
Pharmacological induction of translational readthrough of nonsense mutations in the retinoblastoma (RB1) gene.
Manuscript

IV. 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.
Novel compounds that synergize with G418 or eRF3 degraders for translational readthrough of nonsense mutant *TP53* and *PTEN*.
Manuscript
*These authors contributed equally.

Contents

List of abbreviations

Abbreviation	Explanation
5-FU	5-Fluorouracil
A	Adenine
aa-tRNA	Aminoacyl-tRNA
APC	Adenomatous polyposis coli gene
Arg	Arginine
ATM	Ataxia telangiectasia mutated gene
ATR	Ataxia telangiectasia and Rad3-related gene
BUP-1	β-ureidopropionase
C	Cytosine
CDK	Cyclin-dependent kinase
CFTR	Cystic fibrosis transmembrane conductance regulator gene
COSMIC	Catalogue Of Somatic Mutations In Cancer
CTD	C-terminal domain of p53
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTNS	Cystinosin, lysosomal cystine transporter gene
Cys	Cysteine
DBD	DNA-binding domain of p53
DHFU	Dihydrofluorouracil
DHP	Dihydropyrimidinase
DMD	Dystrophin gene
DP1/2	Dimerization partners 1 and 2
DPD	Dihydropyrimidine dehydrogenase
dTMP	Deoxythymidinemonophosphate
dUMP	Deoxyuridinemonophosphate
$E2F^{TD}$	E2F transactivation domain of Rb
EGFP	Enhanced green fluorescent protein
EMA	European Medicines Agency
eRF	Eukaryotic release factor

F	Fluorine
FBAL	α-fluoro-β-alanine
FDA	U.S. Food and Drug Administration
FdUDP	5-Fluorodeoxyuridine diphosphate
FdUMP	5-Fluoro-2′-deoxyuridine-5′-monophosphate
FdUr	5-Fluoro-2'-deoxyuridine
FdUTP	5-Fluoro-2′-deoxyuridine-5′-triphosphate
FUDP	5-Fluorouridine diphosphate
FUMP	5-Fluorouridine monophosphate
FUPA	α-fluoro-β-ureidopropionic acid
FUr	5-Fluorouridine
FUTP	5-Fluorouridine triphosphate
G	Guanine
GI_{50}	50% growth-inhibitory concentrations
Gln	Glutamine
hCNT	Human concentrative nucleoside transporter
HDAC	Histone deacetylase
hENT	Human equilibrative nucleoside transporter
hNT	Human nucleoside transporter
hOAT2	Human organic anion transporter 2
LOH	Loss of heterozygosity
LOVD	Leiden Open Variation Database
LV	Leucovorin
Mdm2	Mouse double minute 2 protein
Mdm4/MdmX	Mouse double minute 4 protein
MRP	Multidrug resistance-associated protein
nc-tRNA	Near-cognate tRNA
NCI	National Cancer Institute
NMD	Nonsense-mediated decay
NTC	Normal termination codon
PABP	Poly(A)-binding protein

PBD	PIP_2 binding domain of PTEN
PD1	Programmed cell death protein 1
PDK	3-phosphoinositide-dependent kinase
PDZ-BD	PDZ-binding domain of PTEN
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B (AKT)
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PRD	Proline-rich domain of p53
PTC	Premature termination codon
PTEN	Phosphatase and tensin homolog gene
PTEN	Protein encoded by the *PTEN* gene
PTM	Post-translational modification
Rb	Retinoblastoma protein
RB1	Retinoblastoma gene
RBC	C-terminal domain of Rb
RBN	N-terminal domain of Rb
ROS	Reactive oxygen species
RPL26	Ribosomal protein L26
sfGFP	Superfolder green fluorescent protein
T	Thymine
TAD	Transactivation domain of p53
TD/OD	Tetramerization domain of p53
TK	Thymidine kinase
TP	Thymidine phosphorylase
TP53	Tumor protein p53 gene
TS	Thymidylate synthase gene
U	Uracil
UDPK	Uridine diphosphate kinase

UK	Uridine kinase
UMPK	Uridine monophosphate kinase
UP	Uridine phosphorylase
Ur	Uridine
WHO	World Health Organization
WT	Wild-type

1 INTRODUCTION

1.1 Cancer

Cancer is one of the main causes of death worldwide. However, cancer incidence has stabilized over the last few years, especially in males, and cancer-related mortality is decreasing in both males and females (Siegel *et al.*, 2022). This decrease is related to earlier detection and diagnosis, as well as more effective therapy. Cancer consists of several diseases that share the characteristics of a non-controlled cell proliferation and evasion of cell death. Cancer develops as a multistep process with characteristics of Darwinian evolution. Through the accumulation of genetic alterations cells may acquire the so-called "Hallmarks of Cancer", a set of characteristics including proliferation signal autonomy, unresponsiveness to anti-growth signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Reprogramming of cellular energetics and the evasion of immune cells destruction have subsequently been added as emerging hallmarks and genomic instability and inflammation that promotes tumor progression have been included as enabling characteristics (Hanahan and Weinberg, 2011). More recently, advances in cancer research have allowed the description and addition of the ability of unlocking phenotypic plasticity as a novel hallmark to the previously defined ones and the addition of epigenetic reprogramming as well as polymorphic microbiomes as enabling characteristics to further develop the described hallmarks of cancer. In addition, the importance of senescent cells in the tumor microenvironment has been highlighted (Hanahan, 2022). An updated overview of the characteristics shared among cancer cells that comprise the hallmarks is shown in Figure 1.

The cause of cancer is multifactorial and involves risk factors that can be classified as intrinsic and extrinsic. Intrinsic factors include errors that occur randomly during DNA replication and cannot be modified (Tomasetti and Vogelstein, 2015). Extrinsic factors, on the other hand, can be partially or totally modified. One group of extrinsic factors includes endogenous risk factors such as inflammation, presence of growth factors, status of the DNA repair machinery and biological aging. Another group among the extrinsic risk factors includes exposure to radiation, chemical carcinogens, viruses, as well as lifestyle behaviors such as smoking, exercise lacking and poor nutrition (Wu *et al.*, 2018).

1.2 Cancer treatment

For many years, the main options for treating cancer were surgery, radiotherapy (since the end of 19th century) and chemotherapy (since the 1940's) (Falzone *et al.*, 2018). Chemotherapy was developed after the discovery of the DNA alkylating agent *nitrogen mustard*, which caused bone marrow toxicity by inhibiting DNA replication and inducing cell death (Brookes, 1990). Around that time, another type of compounds was developed based on the structural modification of cellular metabolites to inhibit processes such as the purines synthesis that would in turn impair cell proliferation. These agents are called antimetabolites and are analogs

of folate, purines or pyrimidines, such as 5-Fluorouracil (Kaye, 1998), which will be discussed in the following section. Other chemotherapeutic drugs that are used today include the platinum derivatives cisplatin, carboplatin and oxaliplatin (Puyo *et al.*, 2014) as well as antimitotics of natural origin and antibiotics with cytotoxic effects (Falzone *et al.*, 2018).

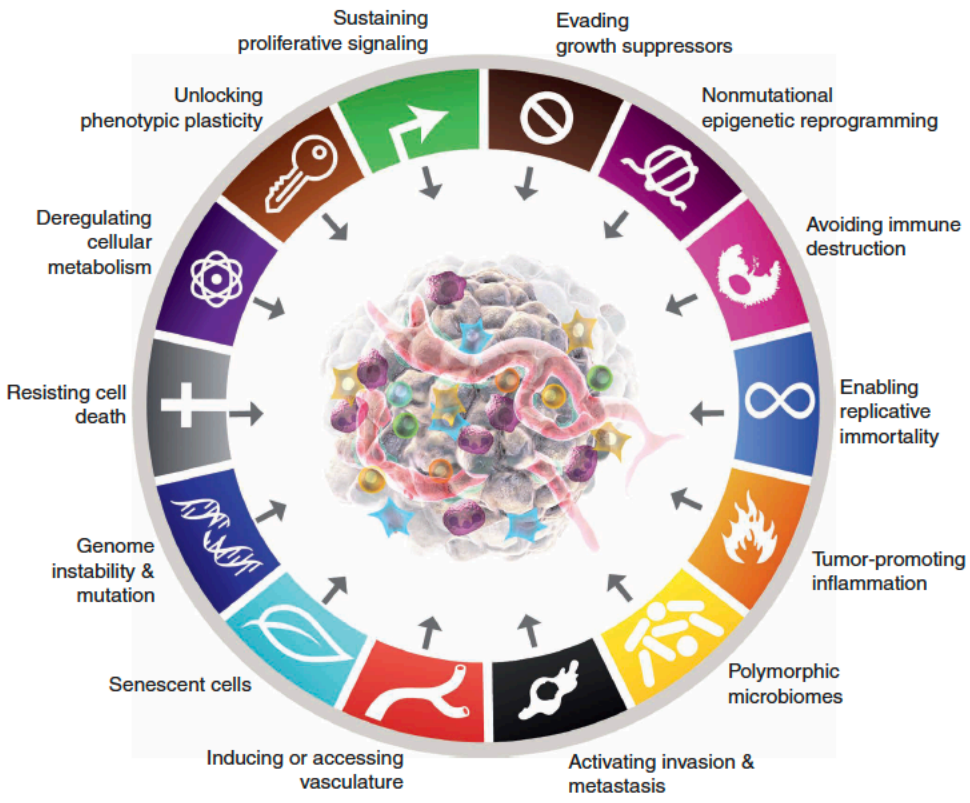

Figure 1. Hallmarks of Cancer. Current description of the common characteristics among different types of cancers. Reprinted from Cancer Discovery, ©2022, 12(1), 31-46, Hanahan D, *Hallmarks of Cancer: New Dimensions*, with permission from AACR.

Hormone therapy is used for specific types of cancer, mainly breast and prostate cancer. The receptor modulator of estrogen Tamoxifen and different aromatase inhibitors are among the most common hormonal treatments for breast cancers that are suitable for this type of treatment. For prostate cancer, the androgen deprivation therapy or androgen receptor inhibitors are used as hormone therapy (Abraham and Staffurth, 2016).

Over the last decades, major efforts have been made to develop novel cancer therapy that targets specific molecular defects in cancer cells. The hope is that such drugs will only hit the cancer cells and not cause major side effects.

One example of targeted therapy are tyrosine kinase inhibitors, which essentially compete with ATP at the tyrosine kinase catalytic binding site (Hartmann *et al.*, 2009). The first approved drug developed as a protein kinase inhibitor was Imatinib (Gleevec®). It inhibits the tyrosine

kinase Abelson (ABL) which is expressed as the fusion protein BCR-ABL deregulated in chronic myeloid leukaemia (CML) at high rates (Cohen *et al.*, 2021).

Another type of targeted therapy are monoclonal antibodies that act against overexpressed oncoproteins in tumor cells. The first tested monoclonal antibody in a clinical trial was Trastuzumab (Herceptin®) against HER-2 expressed in a subset of breast tumors in 1992 and was approved in 1998 and together with Rituximab (Rituxan® and MabThera®) against CD20 expressed on B lymphoma cells which got approval in 1997 marked an important advance in cancer therapy (Falzone *et al.*, 2018). Poly-ADP ribose polymerase (PARP) inhibitors are another type of targeted therapy which was developed for treatment of BRCA1/2-mutated or -deficient tumors. Interestingly, PARP inhibitors have a synthetic lethal interaction with tumors carrying mutant BRCA1/2 or lacking their expression due to their disrupted homologous recombination repair activity which is synthetically lethal when combined with the replication fork collapse after inhibition of PARP1 activity (Rose *et al.*, 2020).

The most recent type of cancer therapy is immunotherapy, which include the immune checkpoint inhibitors, they are monoclonal antibodies produced against antigens present in the T-cell membrane surface (anti-CTLA4) or in the cancer cells membrane (anti-PD1) (Seidel *et al.*, 2018). Inhibiting CTLA1 and PD1 pathways promote the antitumor immune responses, discovery of treatments targeting them was revolutionary in the treatment of cancer. In addition, the important discoveries on these two antigens led James Allison and Tasuku Honjo winning the Nobel Prize in Medicine in 2018 (Smyth and Teng, 2018).

Novel cancer therapeutics are approved yearly by the U. S. Food and Drug Administration (FDA) and European Medicines Agency (EMA). During the period from August 2021 to July 2022 8 new anticancer therapeutics were approved by the FDA and 10 already approved therapeutics were expanded for usage in other cancers (AACR, 2022).

1.2.1 5-Fluorouracil

1.2.1.1 Discovery of 5-Fluorouracil

5-Fluorouracil (5-FU) was one of the fluorinated pyrimidines presented in 1957 by Heidelberger and colleagues (Heidelberger *et al.*, 1957). These fluorinated pyrimidines were defined as a novel class of tumor-inhibitory molecules. 5-FU was designed based on the idea that malignant cells use more uracil compared to normal cells. Specifically, 5-FU contains a fluorine atom instead of a hydrogen at the carbon-5 position of the pyrimidine ring (Heidelberger *et al.*, 1957) (Figure 2). After its FDA approval in 1962 for colorectal cancer treatment (*See "Fluorouracil" at https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm*) 5-FU has been and it is still a commonly used chemotherapeutic drug for different solid tumors. The best results have been obtained in colorectal cancer (Longley *et al.*, 2003). The cancers that were most commonly treated with 5-FU worldwide in 2018 included colorectal and anal, esophageal, hepatocellular, cervical, breast and head and neck carcinomas, pancreatic and gastric adenocarcinomas and cholangiocarcinoma (Chalabi-Dchar *et al.*, 2021). Also, it is

considered as one of the essential medicines for cancer treatment by the World Health Organization (WHO) (Robertson *et al.*, 2016).

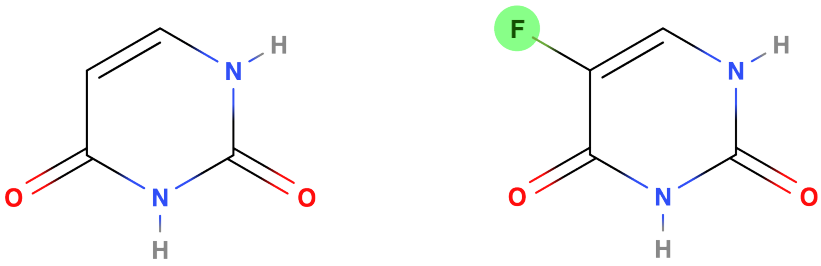

Figure 2. Chemical structures of uracil and 5-FU. The unique difference between uracil (left) and 5-FU (right) is the presence of a fluorine (F) atom in the carbon 5-position highlighted in green. Structures were exported using MolView software v2.4 (https://molview.org/).

1.2.1.2 Cellular uptake and efflux of 5-Fluorouracil

Nucleosides cellular intake is mediated by the membrane proteins human Nucleoside Transporters (hNT). These can be subcategorized into human Concentrative Nucleoside Transporters (hCNTs) comprised by three proteins: hCNT1-3, which are in charge of keeping a correct nucleoside homeostasis and human Equilibrative Nucleoside Transporters (hENT) comprised by four proteins (hENT1-4), which are related to sensing of nucleosides and signal transduction (Pastor-Anglada and Perez-Torras, 2018; Young *et al.*, 2008). Uridine is taken up by all the hNT except by the hENT4, which exclusively mediates the transport of adenosine and other organic cations (Young *et al.*, 2008). Although the mentioned transporters are able to transport uridine, the uracil analog 5-FU is only taken up by hENT1 and hENT2 (Boswell-Casteel and Hays, 2017), as well as by the organic anion transporter 2 (hOAT2) whose expression is markedly higher in liver (Kobayashi *et al.*, 2005) and kidney (Sun *et al.*, 2001). A part from entering cells via the aforementioned transporters, 5-FU has also been reported to enter cells by passive transport in Caco-2 cells, via the paracellular and transcellular routes (Imoto *et al.*, 2009). Regarding 5-FU efflux, it is studied that its monophosphate metabolites can be exported from cells via the Multidrug resistance-associated protein 5 (MRP5) (Pratt *et al.*, 2005) and MRP8 (Guo *et al.*, 2003; Oguri *et al.*, 2007) transporters.

1.2.1.3 5-Fluorouracil metabolism and mechanism of action

After administration of 5-FU, there are two routes by which the compound gets metabolized: the catabolic and the anabolic route (Grem, 2000) (Figure 3). The catabolic route inactivates around 80% of the administered 5-FU to dihydrofluorouracil (DHFU) by the enzyme dihydropyrimidine dehydrogenase (DPD). DHFU is then metabolized to α-fluoro-β-ureidopropionic acid (FUPA) by dihydropyrimidinase (DHP) and to α-fluoro-β-alanine (FBAL) by β-ureidopropionase (BUP-1) (Chalabi-Dchar *et al.*, 2021). FBAL has been related to neurotoxicity, cardiotoxicity and the hand-foot syndrome (Miura *et al.*, 2010). The DPD

enzyme is highly expressed in the liver, thus it is the organ which mainly catabolizes the drug (Diasio and Harris, 1989). The half-life of 5-FU ranges from 8-22 minutes (Diasio and Harris, 1989). In addition to the 80% of catabolized 5-FU to inactive metabolites in the liver, 5-20% of the administered drug is removed by urinary excretion and it is only around 1-3% that enters the anabolic route, which will produce the active metabolites of the drug (Chalabi-Dchar *et al.*, 2021). Regarding the anabolic route, upon entering into cells 5-FU gets metabolized to 5-Fluorouridine (FUr) by uridine phosphorylase and then to 5-Fluorouridine monophosphate (FUMP) by uridine kinase, or to FUMP directly by orotate phosphoribosyltransferase. FUMP gets further phosphorylated to the diphosphate (FUDP) and triphosphate (FUTP) forms. The metabolite FUTP, later on referred as FUr, is incorporated into RNA. At the same time, 5-FU also gets metabolized to 5-Fluoro-2'-deoxyuridine (FdUr) by thymidine phosphorylase and to 5-Fluoro-2'-deoxyuridine monophosphate (FdUMP) by thymidine kinase. FdUMP can be further phosphorylated to diphosphate (FdUDP) and triphosphate (FdUTP) forms by thymidine kinases. FdUTP incorporates into DNA causing DNA damage (Grem, 2000) (Figure 3).

Also, FUDP can be converted to FdUDP by ribonucleotide reductase and FdUMP can be converted to FdUr by 5'-Nucleotidases. In addition, deoxyuridine can convert to uracil and FdUr to 5-FU by thymidine phosphorylase, and at the same time, uridine can convert to uracil and FUr to 5-FU by uridine phosphorylase (Grem, 2000).

As briefly mentioned above, 5-FU has several mechanisms of action to induce cell death of cancer cells and are mediated by the metabolites FdUr and FUr. More specifically, FdUr in the monophosphate form inhibits thymidylate synthase (TS), which catalyzes the formation of thymidine 5'-monophosphate (dTMP) from 2-deoxyuridine 5'-monophosphate (dUMP), leading to impairment of DNA replication, and in the triphosphate form incorporates into the DNA creating DNA damage, these mechanisms of action were mostly studied for a long period of time as main mechanisms for 5-FU to induce cell death (Chalabi-Dchar *et al.*, 2021; Grem, 2000). In some early studies after the discovery of the compound, the FUr capability to incorporate in the RNA for its cytotoxic effects in both a human cell line (Kufe and Major, 1981) and in mice studies (Houghton *et al.*, 1979) were reported. In addition, FUr effects in RNA modification and processing pathways have recently been studied again (Liang *et al.*, 2022; Vodenkova *et al.*, 2020). Therefore, 5-FU induces cytotoxicity through interfering in several processes, which may well be one of the reasons for its efficacy and its long time of use in the clinics even up to the present time.

1.2.1.4 Use in the clinics

5-FU is given in the clinics for colorectal, gastric, pancreatic, breast and head and neck cancers, among others, via intravenous route as bolus, infusion or continuous infusion and it is usually combined with other compounds such as leucovorin (LV) as used for the treatment of colon cancer (Pharmacovigilance Risk Assessment Committee (PRAC) and EMA, 2020). There are also oral 5-FU prodrugs such as Tegafur used for rectal, colon, breast, gastric cancer and specific brain tumors. Tegafur can also be combined with gimeracil (also named CDHP), which is a DPD inhibitor, and oteracil (also named potassium oxonate or OXO), which is an orotate

phosphoribosyltransferase (OPRT) inhibitor. The combination of the three drugs is known as S-1 and it was developed to have increased activity and reduced gastrointestinal toxicity (Miura *et al.*, 2010).

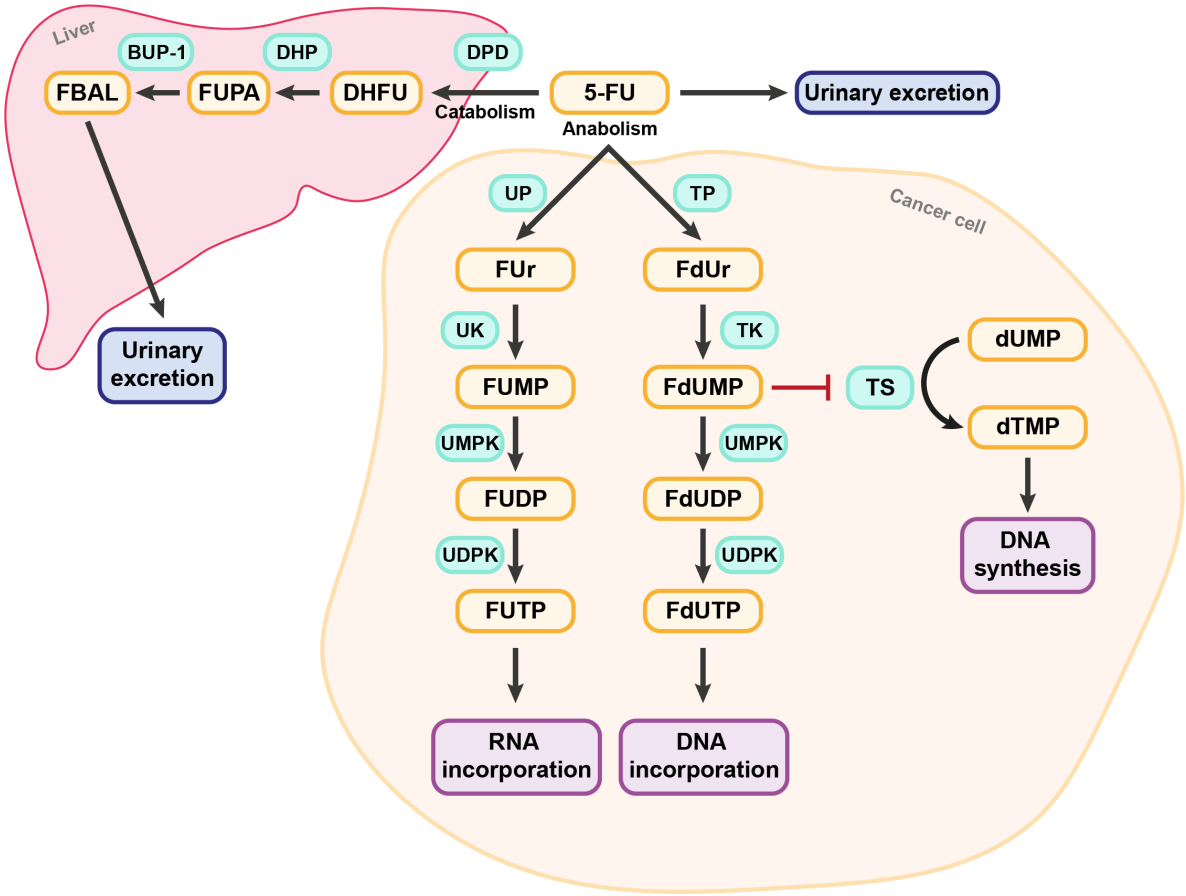

Figure 3. Metabolism of 5-FU. 5-FU catabolism in hepatic cells (top left), anabolism in cancer cells (center right) and their consequences are depicted. Compounds and metabolites are shown in yellow, enzymes in turquoise, cellular processes in purple and excretion pathway in dark blue. Abbreviations: 5-FU, 5-Fluorouracil; BUP-1, β-ureidopropionase; DHFU, Dihydrofluorouracil; DHP, Dihydropyrimidinase; DPD, Dihydropyrimidine dehydrogenase; dTMP, Deoxythymidinemonophosphate; dUMP, Deoxyuridinemonophosphate; FBAL, α-fluoro-β-alanine; FdUDP, 5-Fluorodeoxyuridine diphosphate; FdUMP, 5-Fluoro-2′-deoxyuridine-5′-monophosphate; FdUr, 5-Fluoro-2'-deoxyuridine; FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; FUDP, 5-Fluorouridine diphosphate; FUMP, 5-Fluorouridine monophosphate; FUPA, α-fluoro-β-ureidopropionic acid; FUr, 5-Fluorouridine; FUTP, 5-Fluorouridine triphosphate; TK, Thymidine kinase; TP, Thymidine phosphorylase; TS, Thymidylate synthase ; UDPK, Uridine diphosphate kinase; UK, Uridine kinase; UMPK, Uridine monophosphate kinase; UP, Uridine phosphorylase. Figure modified from Pharmacology & Therapeutics, 206, Vodenkova S, Buchler T, Cervena K, Veskrnova V, Vodicka P and Vymetalkova V, *5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future,* 107447, ©2020, with permission from Elsevier. Modifications to the figure have been authorized by Elsevier Ltd.

Another oral prodrug is capecitabine which was developed based on a previous compound called 5'-DFUR (Miura *et al.*, 2010). Capecitabine is used for colorectal, gastric and breast cancer. It can also be combined with docetaxel for breast cancer in an advanced stage or that has metastasized (Pharmacovigilance Risk Assessment Committee (PRAC) and EMA, 2020). UFT is another 5-FU prodrug that combines Tegafur with uracil for it to compete for the DPD enzyme and thus increase the 5-FU half-life (Miura *et al.*, 2010). Importantly, since 2020 EMA recommends the testing for DPD enzyme deficiency before starting the treatment with 5-Fluorouracil or with the same family of drugs as capecitabine and tegafur via injection or infusion (EMA, 2020). There are other combination treatments such as 5-FU plus LV and combined with oxaliplatin called FOLFOX or with irinotecan called FOLFIRI that are used in metastatic colorectal cancer with better survival results (Gustavsson *et al.*, 2015).

1.3 Tumor suppressor genes

Two types of genes have key roles in cancer. Oncogenes can drive tumor growth when illegitimately activated, e.g., by chromosomal translocation, gene amplification or point mutation. Tumor suppressor genes, on the other hand, contribute to tumor development when they are inactivated or lost, e.g. through truncating mutation or deletion (Weinberg, 2014). Thus, tumor suppressor genes are crucial for preventing cancer. In general, both alleles of a tumor suppressor gene must be inactivated in order to allow tumor development. Whole genome sequencing studies have provided a much more comprehensive and complete picture of genetic alterations in cancer (Kandoth *et al.*, 2013). Typically, cancer-associated mutations in oncogenes are missense and recurrent, resulting in an overactive gene product, whereas mutations in tumor suppressor genes are truncating and distributed over a larger region of the gene. Bert Vogelstein and colleagues have suggested that a cancer-driving gene with more than 20% recurrent missense mutations should be classified as an oncogene, and that a cancer-driving gene with more than 20% inactivating mutations should be classified as a tumor suppressor gene. Based on this simple 20/20 rule, it was estimated that the total number of cancer-driving genes is 125. If 10 oncogenes that are recurrently amplified and 3 tumor suppressor genes that show homozygous deletion are added, there are 138 cancer driver genes in total (Vogelstein *et al.*, 2013). This number may well increase as novel genes in either category are discovered.

1.3.1 The tumor suppressor *TP53*

1.3.1.1 TP53 gene and p53 protein

The tumor suppressor gene *TP53* codes for the p53 protein. The p53 protein was discovered in 1979 independently by several groups (DeLeo *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero *et al.*, 1979). *TP53* was first considered an oncogene due to the observed binding to the oncogenic large T-antigen of SV40, as described in the articles above, and due to the elevated p53 expression in various cancers and cancer cell lines (Lane and Benchimol, 1990). However, this classification rapidly changed upon the observation that wild-type (WT) p53 cDNA could inhibit cell transformation (Eliyahu *et al.*, 1989; Finlay *et al.*,

1989), and that both *TP53* alleles were altered in tumors either by deletion or mutation, a characteristic of tumor suppressor genes (Baker *et al.*, 1989; Nigro *et al.*, 1989). Also, inherited mutant *TP53* was associated with predisposition to cancer (Malkin *et al.*, 1990; Srivastava *et al.*, 1990) and a *TP53* knock-out mouse model showed a high rate of spontaneous tumors (Donehower *et al.*, 1992).

The human *TP53* gene is located on chromosome 17p13.1 (NIH, 2023b). The p53 protein consists on 393 amino acids and forms homotetramers that act as a transcription factor (Joerger and Fersht, 2010). The protein has two N-terminal transactivation domains (TAD1 and TAD2), followed by the proline-rich domain (PRD or PRR), the DNA-binding domain (DBD), the tetramerization domain (TD or OD) and the C-terminal domain (CTD) (Joerger and Fersht, 2010; Wen and Wang, 2022) (Figure 4). Only the DBD and the TD are folded regions while the rest are intrinsically disordered (Joerger and Fersht, 2010).

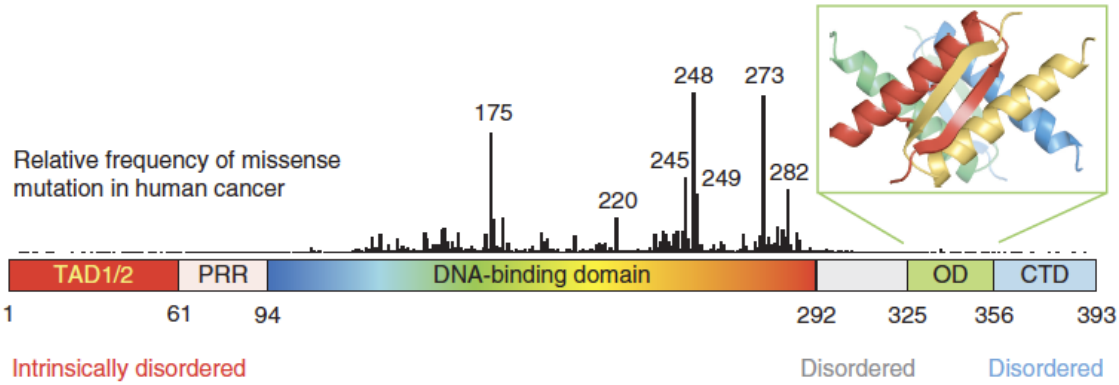

Figure 4. Domains of p53 protein and distribution of tumor-associated mutations. The different domains of p53 are shown: transactivation domains 1 and 2 (TAD1/2), proline-rich domain (PRR), DNA-binding domain, the tetramerization domain (OD) and the C-terminal domain (CTD). Common missense mutations are indicated. Figure reprinted from *The tumor suppressor p53: from structures to drug discovery*, Joerger AC and Fersht AR (2010) Cold Spring Harbor Perspectives in Biology, 2(6), a000919 with permission from copyright © 2010 Cold Spring Harbor Laboratory Press. Original figure was cropped.

The p53 family also includes p63 (Yang *et al.*, 1998) and p73 (Kaghad *et al.*, 1997). These two homologues share high sequence similarity with p53, especially in the DNA-binding domain, where the similarity reaches 60-63% (Levrero *et al.*, 2000). p63 and p73 have been reported to regulate p53 target genes, inducing cell cycle arrest and apoptosis (Khoury and Bourdon, 2010). In addition, DNA damage can induce both p53 and p73 (Levrero *et al.*, 2000). More specific functions for each member are the role in the development of the ectoderm and the limbs in case of p63 and in neural development, infections and inflammation in case of p73 (Van Nostrand *et al.*, 2017).

At least 12 isoforms of p53 have been identified that are generated due to alternative splicing of *TP53* mRNA, alternative initiation of translation, or differential promoter usage. Various p53 isoforms have been related to carcinogenesis as well as to a different response to cellular

stress. Some isoforms have antagonistic effects on p53 and others can have agonistic effects. For instance, apoptosis induction can be enhanced by the isoform p53β in complex with p53α (Khoury and Bourdon, 2011; Zhao and Sanyal, 2022).

1.3.1.2 TP53 mRNA and protein stabilization and regulation

TP53 translation was first reported to be upregulated upon DNA damage as increased levels of newly synthesized p53 were observed without mRNA levels increase (Kastan *et al.*, 1991) (Fu and Benchimol, 1997). Several factors have been reported to bind to either AU- or U-rich elements or cytoplasmic polyA signals at the 3'UTR of p53 mRNA with different consequences, from p53 mRNA stabilization or enhanced translation to destabilization and inhibition of translation (Haronikova *et al.*, 2019). One of these factors is the p53 target Zmat3 (Wig-1), which was suggested to form a positive feedback loop with p53 to sustain high levels of p53 (Vilborg *et al.*, 2009). The 5'UTR region has also been reported to control *TP53* mRNA translation, for example by enhancing it upon DNA damage mediated by Ribosomal protein L26 (RPL26) or by decreasing translation under normal conditions by nucleolin (Takagi *et al.*, 2005). In addition, the p53 protein itself was reported to regulate *TP53* mRNA by binding to its 5'UTR and inhibiting mRNA translation (Mosner *et al.*, 1995). Other factors that bind to the 5'UTR or the coding sequence of *TP53* mRNA have been shown to modulate either its stability or translation or both (Haronikova *et al.*, 2019).

At the protein level, one of the main regulators of p53 stability is the E3 ubiquitin ligase Mdm2 (mouse double minute 2) protein that binds p53 through a region in p53's N-terminal domain. Stress conditions such as DNA damage or metabolic changes can induce phosphorylation of p53 and/or Mdm2 at specific sites (see further below) that inhibit this binding and/or modulates the activity of Mdm2 as an E3 ligase for p53 (Levine, 2020). Also, the binding of Mdm2 to the p53 N-terminus inhibits the ability of p53 to activate transcription (Chen *et al.*, 1993a; Oliner *et al.*, 1993), but also targets it for proteasomal degradation via ubiquitination of the CTD (Giaccia and Kastan, 1998; Rodriguez *et al.*, 2000; Wen and Wang, 2022), one type of post-translational modifications (PTMs) mentioned below. The half-life of p53 is 5-20 minutes (Giaccia and Kastan, 1998), but increases after Mdm2 inhibition (Riley *et al.*, 2008). The resulting accumulation of p53 leads to transcriptional transactivation of p53 target genes that are involved in various processes, including cell cycle arrest, DNA repair and apoptosis (Levine, 2020).

The Mdm2 homolog MdmX (also known as Mdm4) also binds to the N-terminal part of p53 and suppresses its function as transcription factor (Shangary and Wang, 2009; Shvarts *et al.*, 1996). In addition, Mdm2 and MdmX can bind through their C-terminal domains and further bind to p53 and regulate its function (Shangary and Wang, 2009). Mdm2 and MdmX share a high degree of sequence homology, especially in the p53-binding domain and the zinc finger and the RING finger domain, located in the N- and the C-terminal part of both proteins, respectively (Shvarts *et al.*, 1997). In contrast to Mdm2, MdmX is not an E3 ligase but can nonetheless stabilize Mdm2 according to one report (Sharp *et al.*, 1999), thus enhancing Mdm2

function. As in the case of Mdm2, overexpression of MdmX can contribute to tumorigenesis and has been found in some types of cancer, such as retinoblastoma (Laurie *et al.*, 2006).

p53 activity can be modulated by PTMs at more than 36 residues located along the entire protein sequence. These PTMs include phosphorylation, acetylation, methylation, ubiquitination and hydroxylation, among others (Wen and Wang, 2022). As already alluded to above, p53 PTMs can be induced by different cellular stresses such as DNA damage or metabolic stress and are crucial for the p53 activity outcomes (Wen and Wang, 2022). p53 phosphorylation and acetylation are mainly related to p53 stabilization and nuclear accumulation, and its subsequent activation. Thus, they are mainly involved in activation of p53 as transcription factor (Bode and Dong, 2004). More specifically, the specific DNA-binding of p53 can be enhanced by phosphorylation (Hupp and Lane, 1994). Up to 17 residues in p53 have been reported to be modified by phosphorylation upon DNA damage (Bode and Dong, 2004). However, p53 dephosphorylation at, for example, Ser376 can also be important for p53 activation as it has been observed upon ionizing radiation (Waterman *et al.*, 1998). Some examples of kinases that phosphorylate p53 are ATM, Chk1/Chk2 and ATR, all of which can modify the p53 N-terminus upon DNA damage induced by e.g. UV light or ionizing radiation. These modifications can inhibit p53's interaction with Mdm2 and thereby stabilize p53. N-terminal sites phosphorylated are for example Ser6, Ser9, Ser15, Thr18 and Ser20 (Kruse and Gu, 2008; Olsson *et al.*, 2007). As mentioned above, generally, p53 phosphorylation and acetylation stabilize p53, although acetylation has also been associated with activation of transcription (Barlev *et al.*, 2001). On the other hand, p53 ubiquitination and further degradation has also been linked to deacetylation of p53 (Bode and Dong, 2004). Actually, the C-terminal residues in p53 that are acetylated can also be ubiquitinated by Mdm2. Thus, acetylation of C-terminal residues in p53 was proposed to lead to p53 stabilization through inhibition of ubiquitination by Mdm2 (Ito *et al.*, 2001; Rodriguez *et al.*, 2000).

1.3.1.3 p53 pathway

In the early nineties, p53 was dubbed "the Guardian of the genome" due to its ability to induce G1 phase cell cycle arrest upon DNA damage (Lane, 1992). This blockade allowed the damaged cell to induce repair before continuing division or in case of failure to repair, induce apoptosis as mentioned below (Lane, 1992). Different stresses other than DNA damage can also activate p53 such as hypoxia, oncogene activation and presence of short telomeres (Levine *et al.*, 2006). The first descriptions of p53 tumor suppression activity focused on p53's ability to reduce proliferation rate by inducing transitory or permanent cell cycle arrest and apoptosis. However, more recent evidence points towards important roles of other cellular processes such as metabolism and metastasis for the tumor suppression activity of p53 (Bieging *et al.*, 2014).

As a transcription factor, active p53 will upregulate expression of a number of target genes involved in cell cycle arrest, apoptosis, senescence, metabolism and DNA repair, allowing DNA repair before continued cell division or induction of cell death (Brown *et al.*, 2009; Kastenhuber and Lowe, 2017; Vousden and Prives, 2009) (Figure 5). More than 100 genes have been reported to be regulated by p53. These genes are related to different processes such

as apoptosis (e.g. *BAX*, *BBC3*, *FAS* and *PMAIP1*) and cell cycle arrest (e.g. *CDKN1A*) (Andrysik *et al.*, 2017; Fischer, 2017). p53 binds with high affinity and specificity to a DNA binding motif in p53 target genes. The consensus decamer sequence is 5′-RRRCWWGYYY-3′ in which G and C correspond to guanine and cytosine, respectively; R and Y to purine and pyrimidine, respectively; and W can be an adenine or a thymine (el-Deiry *et al.*, 1992; Funk *et al.*, 1992). The two halves (pentamers) of this sequence can be orientated in different ways and still create a consensus sequence for p53 in genomes of different organisms (Riley *et al.*, 2008).

In addition to the main mechanism by which p53 induces biological effects – transactivation of transcription – p53 can also act as an inhibitor of transcription and can enhance the permeabilization of the mitochondrial membrane to induce apoptosis (Bieging *et al.*, 2014). The transactivation of transcription function is exerted by direct DNA binding to a p53 binding motif in a target gene, as described above. After binding, p53 can recruit proteins that mediate transcription to the specific target gene promoter (Chen *et al.*, 1993b; Farmer *et al.*, 1996). The transcriptional repression mediated by p53 is thought to be due to both direct and indirect mechanisms. The direct mechanisms include p53 binding to the same DNA site that is also used by a more potent protein able to transactivate that gene; inactivation of other activators that are bound or unbound to DNA via protein-protein interactions; and lastly, recruiting histone deacetylases (HDACs) via binding to other proteins, as by deacetylating chromatin histones gene transcription is repressed (Riley *et al.*, 2008). The indirect mechanisms to repress transcription are firstly activation of *CDKN1A* expression that is able to bind cyclin D-CDK4 complexes (Löhr *et al.*, 2003). As discussed further below, this inhibition will result in the activation of Rb which will bind proteins in the E2F family. This in turn will prevent transcription of E2F target genes which will therefore be repressed. Secondly, p53 may bind to another transcription factor and thereby repress expression of a gene that does not have a p53 binding motif (Riley *et al.*, 2008).

Although important advances have been made in unravelling the role of p53 in tumor suppression, this process is still far from fully understood (Mello and Attardi, 2018). In addition, other factors apart from p53 are important to decide the cell fate under stress. This is indicative that activities exerted by other pathways can also be sensed by p53 and modulate its transcriptional activity (Riley *et al.*, 2008).

1.3.1.4 Mutations in TP53

TP53 is the most commonly mutated gene in human tumors. Around 50% of all cancers carry mutant *TP53* (Soussi and Wiman, 2007). Whole genome sequencing of 3281 tumors representing 12 major cancer types identified *TP53* mutations in 42% of the tumors (Kandoth *et al.*, 2013). Missense mutations leading to a single amino acid substitution in the p53 protein are the most common type of p53 mutations, accounting for around 63% of *TP53* alterations. The majority of these mutations are located in p53's DNA-binding domain and disrupt the DNA binding and transcription factor function (Joerger and Fersht, 2010) (Figure 4). However, around 11% are nonsense mutations, which will cause the insertion of a premature termination codon in the coding sequence and the production of a truncated and inactive p53 protein (Tate

et al., 2019). According to Catalogue Of Somatic Mutations In Cancer (COSMIC) database the most common *TP53* somatic nonsense mutation is R213X, followed by R196X, R342X, R306X and Q192X. Actually, *TP53* R213X nonsense mutation is one of the 10th most common *TP53* mutations overall (Tate *et al.*, 2019). Loss of p53 function by mutation allows evasion of normal cell cycle arrest and/or apoptosis, allowing accelerated tumor progression.

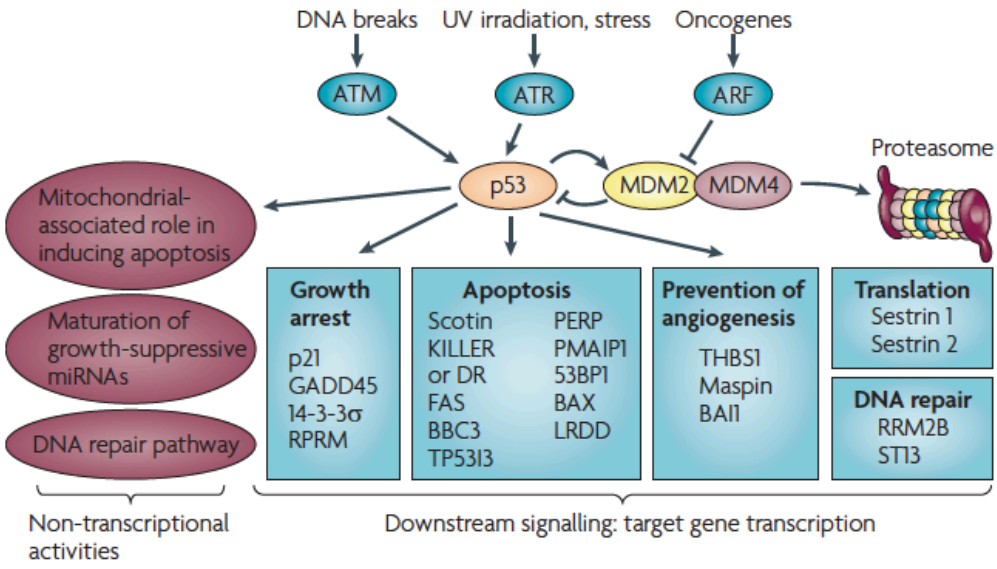

Figure 5. p53 pathway. Different cellular stresses cause the increase and activation of p53. Active p53 will activate transcription of target genes involved in processes such as growth arrest and apoptosis. In addition, p53 can also exert non-transcriptional activities upon activation. p53 levels are regulated by Mdm2 and Mdm4, which in complex are able to target p53 for proteasomal degradation. Material from: Brown CJ, Lain S, Verma CS, Fersht AR and Lane DP, *Awakening guardian angels: drugging the p53 pathway* (2009) Nature Reviews Cancer (Springer Nature) 9: 862-873 reproduced with permission of Springer Nature Customer Service Center GmbH (SNCSC).

1.3.1.5 Targeting p53 for cancer therapy

Mouse *in vivo* studies have demonstrated that restoration of WT p53 expression can lead to tumor regression (Martins *et al.*, 2006; Ventura *et al.*, 2007; Xue *et al.*, 2007). Moreover, the fact that p53 is mutated in such high percentage of human tumors and its important role in different pathways related to tumor suppression, makes p53 an interesting target for cancer therapy (Bykov *et al.*, 2018).

p53-targeted therapy can be approached from several angles. As mentioned above, the majority of *TP53* mutations are missense substitutions that disrupt p53 DNA-binding. Therefore, one strategy is to restore DNA binding to missense mutant p53. This idea is based on the fact that the DNA-binding domain of many missense mutant p53 proteins is improperly fold and therefore unable to bind DNA (Bykov *et al.*, 2003). Therapeutic strategies for targeting missense mutant p53 include small molecules that can promote refolding of mutant p53 to a WT conformation and thus reactivate the correct function of p53 and induce tumor cell death. The compound PRIMA-1 (Bykov *et al.*, 2002) and its structural analog APR-246 (PRIMA-

1^{MET} / Eprenetapopt) (Bykov *et al.*, 2005) were first identified as mutant p53-targeting compounds. Both are converted to MQ (methylene quinuclidinone), a Michael acceptor that binds to several cysteines in p53's core domain and enhance its thermostability (Degtjarik *et al.*, 2021; Lambert *et al.*, 2009; Zhang *et al.*, 2018). Further studies have shown that APR-246/MQ also targets Thioredoxin reductase (TrxR1), glutathione and other components of the cellular redox system which may induce p53-independent cell death (Birsen *et al.*, 2022; Ceder *et al.*, 2021; Liu *et al.*, 2017; Peng *et al.*, 2013; Tessoulin *et al.*, 2014). APR-246 has been tested in combination with azacitidine in phase II clinical trials in *TP53* mutant myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) (Cluzeau *et al.*, 2021; Sallman *et al.*, 2021) with promising results but results from a phase III trial did not reach statistical significance (*www.aprea.com*). The compound PC14586 that specifically targets Y220C missense mutant p53 is currently being tested in a phase I/II clinical trial (*www.pmvpharma.com*).

In many tumors carrying WT *TP53*, the p53 pathway can still be disrupted by other factors. For example, by overexpression of Mdm2 causing degradation of p53 or by the loss of the Mdm2 upstream regulator p14ARF (Asker *et al.*, 1999). Therefore, targeting the p53 pathway and reactivating it is interesting in this setting too. In fact, any treatment that causes DNA damage will activate and stabilize WT p53 (Wang and Sun, 2010), such as 5-Fluorouracil (Bunz *et al.*, 1999). However, inhibition of Mdm2-p53 binding with compounds that bind to the p53 binding pocket in Mdm2 or otherwise block this interaction will result in a more specific activation of WT p53 by preventing its degradation via the proteasome. This strategy appears feasible due to the small interaction surface between p53 and Mdm2, which comprises only three amino acids in p53 (Kussie *et al.*, 1996). There are several types of compounds that inhibit this binding, including Nutlins (Vassilev, 2004), benzodiazepinediones (Grasberger *et al.*, 2005) and the spiro-oxindoles derivatives (Ding *et al.*, 2006) from which different Mdm2 inhibitors (MI) were obtained (Wang and Sun, 2010), such as MI-773 (SAR405838) (Wang *et al.*, 2014).

Nutlins were identified as imidazoline compounds able to bind to the N-terminal part of Mdm2, where Mdm2 binds p53, essentially mimicking the three p53 amino acids mentioned above. Promising results were obtained with this type of compounds in terms of more potent effects in tumor cells compared to normal cells as well as nontoxic inhibition of tumor growth *in vivo* in a mouse xenograft model (Vassilev *et al.*, 2004). The second generation of Nutlin-class compounds include Idasanutlin (RG7388), which was shown to be a Nutlin analog with higher potency and selectivity (Ding *et al.*, 2013). In addition, it is the most advanced Mdm2 inhibitor as phase III clinical trials are on-going in relapsed or refractory AML patients with this compound in combination with cytarabine (clinical trial identifier: NCT01773408) (Duffy *et al.*, 2022). The clinical response to Idasanutlin seems to be related to the Mdm2 protein levels in the blasts of these patients. This relationship will be examined in future clinical trials as a potential biomarker to select patients likely to respond better to therapies that include Idasanutlin (Reis *et al.*, 2016). Apart from activating and stabilizing p53 by inhibiting the p53-

Mdm2 binding (Nutlins), inhibiting Mdm2 E3 ligase activity using compounds such as HLI98 has been reported to achieve the same outcome (Yang *et al.*, 2005).

Lastly, nonsense mutations in *TP53* also affect a large number of cancer patients. One strategy to reactivate this type of mutants is the pharmacological induction of translational readthrough. This therapeutic strategy will be reviewed in more detail in section 1.5.

1.3.2 The tumor suppressor *RB1*

1.3.2.1 The two-hit hypothesis and discovery of the RB1 gene

The first described tumor suppressor gene, *RB1*, was identified in retinoblastoma, a childhood eye tumor affecting 1 per 20 000 live births that can be either sporadic or inherited (Yun *et al.*, 2011). Based on the analysis of incidence curves of familial and sporadic retinoblastoma, Alfred Knudson formulated the classical two-hit hypothesis which says that two genetic hits are required for retinoblastoma to develop (Knudson, 1971). The responsible gene, *RB1*, was later identified and the two hits were shown to be mutation/inactivation of both alleles of the *RB1* gene. In the familial form, one mutant *RB1* allele is inherited and the second allele is subsequently inactivated by a somatic mutation, whereas both alleles are inactivated by somatic mutations in sporadic cases of the disease. These discoveries and the connection between retinoblastoma development and the *RB1* gene helped the cancer genetics community to understand what were tumor suppressor genes and what were their function at the molecular level (Berry *et al.*, 2019). Further studies of *RB1* and its encoded protein associated the lack of Rb function with other tumors such as osteosarcoma, cutaneous melanoma and soft tissue sarcomas (Abramson *et al.*, 2001).

1.3.2.2 Rb protein and pathway

The Rb protein is encoded by the *RB1* gene, which is located in chromosome 13 at position q14.2 (NIH, 2023a). Rb consists on 928 amino acids and it is generally known to have three domains, the structured N-terminal domain (RBN), the central pocket domain and the intrinsically disordered C-terminal domain (RBC) consisting of approximately the last 150 amino acids. The pocket domain can be considered one structural unit formed by the two interacting subdomains A and B (Dick and Rubin, 2013). It has a conserved region able to bind the E2F transactivation domain ($E2F^{TD}$) and a "L-X-C-X-E"-binding cleft that allows Rb to interact with many proteins (Figure 6).

Rb can be modified at several residues by PTMs, e.g. phosphorylation, acetylation and methylation (Dick and Rubin, 2013). Generally, Rb is inactivated by phosphorylation. Kinases that phosphorylate Rb include the cyclin-dependent kinases (CDKs) and the checkpoint kinase 2 (CHK2) (Munro *et al.*, 2012). More specifically, Rb phosphorylation that is regulated first by cyclin D in complex with CDK4 or CDK6 and then by cyclin E in complex with CDK2, is controlled by p16 and p21, respectively, upon stress signals (Ohtani *et al.*, 2004). Rb phosphorylation by CDKs causes the dissociation of the Rb-E2F complex, leading to de-repression of E2F which results in specific transcriptional activation and cell cycle progression

(Weinberg, 1995), as will be detailed below. On the contrary, Rb dephosphorylation results in its activation. This can occur during cell cycle progression under normal conditions but also upon cellular stress or conditions that inhibit cell proliferation. Dephosphorylation of Rb is mediated by protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). PP1 acts in late mitosis and early G1 phase and PP2A can act during the whole cell cycle and seems to be in a constant equilibrium with CDKs. When CDKs are less active, PP2A is active and vice versa (Kolupaeva and Janssens, 2013).

In contrast to phosphorylation, acetylation and methylation modifications are related to Rb activation upon different types of signals. Another characteristic of the Rb protein as cell cycle regulatory protein is that its inactivation does not usually cause its degradation (Dick and Rubin, 2013).

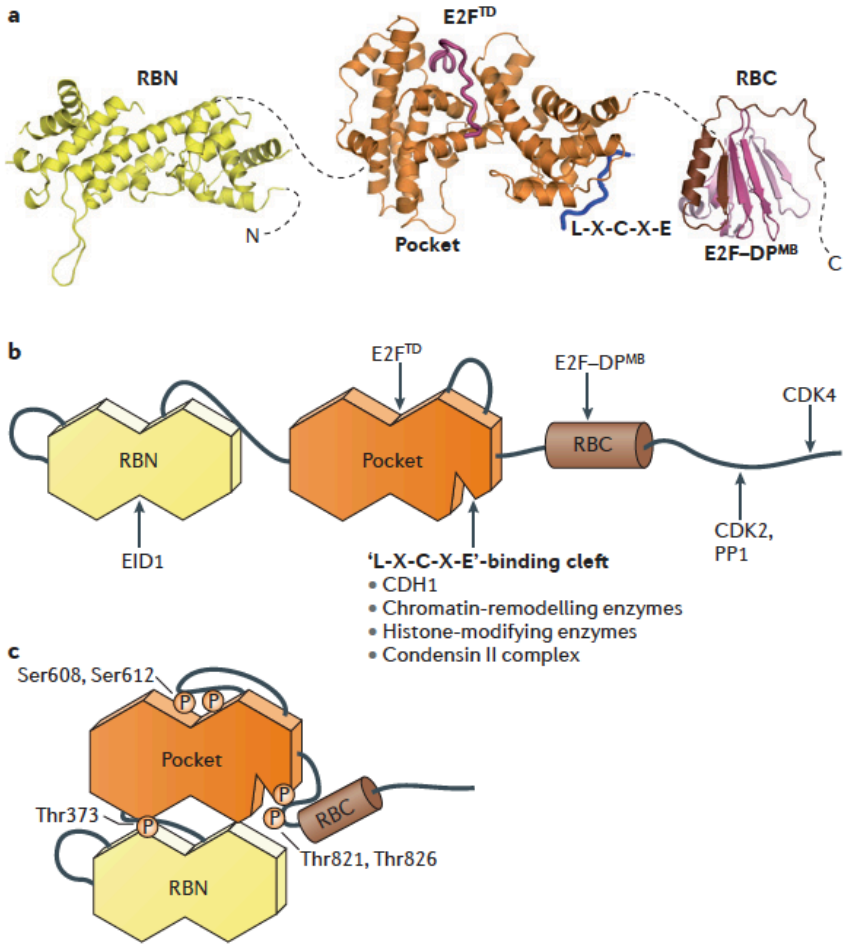

Figure 6. Domains and structure of Rb protein. A, Crystal structures of Rb protein domains, complexes with E2F and a L-X-C-X-E peptide. Structure codes from Protein Data Bank: 2QDJ, 1GUX, 1N4M and 2AZE. **B,** Diagram of Rb domains where binding sites for other proteins are indicated. **C,** Diagram of phosphorylated and inactive Rb. Abbreviations: CDK, cyclin-dependent kinase; DP^{MB}, differentiation-related polypeptide marked box; $E2F^{TD}$, E2F transactivation domain; PP1, protein phosphatase 1; RBC, Rb C-terminal domain; RBN, Rb N-terminal domain. Material from: Dick FA and Rubin SM, *Molecular mechanisms underlying RB protein function* (2013) Nature Reviews Molecular Cell Biology (Springer Nature) 14: 297-306 reproduced with permission of Springer Nature Customer Service Center GmbH (SNCSC).

Rb is a member of the *pocket protein family* of cell cycle regulators which includes the Rb-related proteins p107 and p130 that also bind E2Fs and dissociate from E2Fs upon phosphorylation (Cobrinik, 2005). One difference between the three pocket proteins is that Rb is expressed in both cells that are proliferating and those that are not, while p107 is more expressed in dividing cells and p130 in arrested cells (Classon and Dyson, 2001). The half-life of the Rb protein is longer than 10 hours and it is produced during all cell cycle phases (Mihara *et al.*, 1989).

Rb acts in the G1 phase of the cell cycle where it controls cell cycle progression by binding to the E2F family of proteins. E2F activates genes required for S phase progression. As mentioned above, phosphorylation of Rb by cyclin D-CDK4/6 disrupts binding to E2F, leading to the release of E2F and transcription of S phase genes (Henley and Dick, 2012; Knudsen *et al.*, 2019; Weinberg, 1995) (Figure 7). The phosphorylation sites that are crucial for Rb inactivation and that are dependent of CDKs are found in the linker sequences (Dick and Rubin, 2013). The family of E2F transcription factors can be divided into transcriptional *activators* and *repressors*. E2F1, E2F2 and E2F3a are activators preferentially regulated by Rb; whereas E2F3b, E2F4, E2F5, E2F6, E2F7 and E2F8 are repressors (Cobrinik, 2005; Rösner and Sørensen, 2019). E2F3b forms a complex with Rb to repress transcription and E2F4, and E2F5 forms complexes with p107 and p130, recruiting them to the nucleus and inhibiting transcription (Cobrinik, 2005). E2F6, E2F7 and E2F8 are also repressors but not through pocket proteins binding (Dimova and Dyson, 2005; Maiti *et al.*, 2005). In addition, E2F1-6 all bind to the transcription factors DP1 and DP2 (dimerization partner 1 and 2), forming heterodimers that are able to bind to DNA (Cobrinik, 2005). In addition to the cell cycle regulation function of Rb via E2F modulation, regulation of cell cycle independently from E2F as well as other roles have been ascribed to Rb, such as regulation of heterochromatin, genome stability and apoptosis (Dick and Rubin, 2013).

1.3.2.3 Mutations in RB1

RB1 is inactivated in all retinoblastomas and sarcomas in retinoblastoma families, and also in various other tumors although at lower frequency. The cancer genome sequencing study by Kandoth *et al.* showed an overall *RB1* mutation frequency in 12 common tumor types of 3.2% whereas 14.3% of bladder tumors were found to carry mutant *RB1* (Kandoth *et al.*, 2013). In this context, it is interesting to note that in a study that included 403 retinoblastoma patients, among the 399 analyzed mutations 40% of all inactivating *RB1* mutations were nonsense mutations (Price *et al.*, 2014). In addition, the most common *RB1* somatic mutations reported in COSMIC database correspond to nonsense substitutions which account for around 25.6% of all *RB1* mutations reported (Tate *et al.*, 2019). Among those, the positions that are more frequently mutated are R251X, R579X and R320X. Similarly, in the Leiden Open Variation Database (LOVD) (Lohmann and Novakovic, 2010) which collects information about both somatic and germline *RB1* mutations, 34.1% of the total number of *RB1* variants corresponded to nonsense mutations.

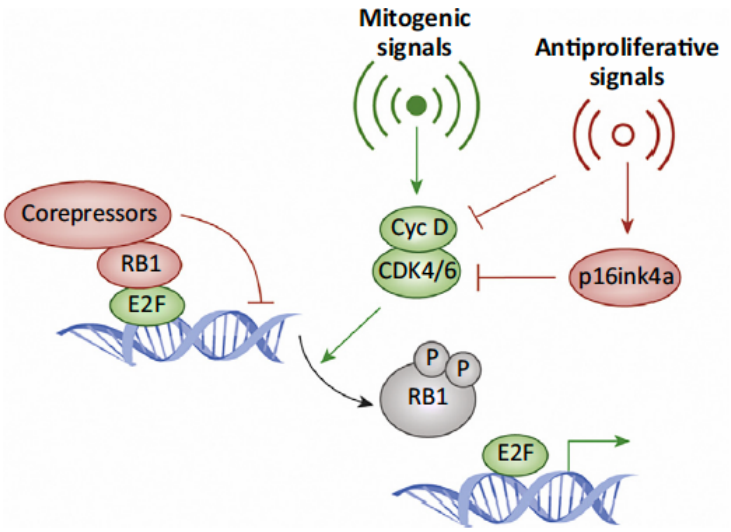

Figure 7. Canonical Rb pathway. Mitogenic signals activate cyclin-dependent kinases (CDK) 4 and 6 with cyclin D inactivating Rb by phosphorylation. Its inactivation causes the release of E2F bound to Rb and the consequent transcription activation of E2F target genes. On the other hand, antiproliferative signals and p16 can inhibit the phosphorylation and inactivation of Rb. Figure reprinted from Trends in Cancer, 5(5), Knudsen ES, Pruitt SC, Hershberger PA, Witkiewicz AK and Goodrich DW, *Cell cycle and beyond: Exploiting new RB1 controlled mechanisms for cancer therapy*, 308-324, ©2019, with permission from Elsevier.

1.3.2.4 RB1 status and response to cancer therapy

RB1 status has been shown to be important for the response to different types of therapies. *RB1* gene inactivation can be related to either good or bad prognosis depending on the type of treatment and cancer. Lack of functional Rb has been linked to resistance to hormone therapy in breast cancer and even recurrence of the tumor (Bosco *et al.*, 2007; Lehn *et al.*, 2011). In addition, resistance to kinase inhibitors that block cyclin D-CDK4/6 activity has been reported in a non-functional Rb background (Dick *et al.*, 2018). One example is the CDK4/CDK6 inhibitor Palbociclib which causes proliferation arrest in cancer cell lines carrying functional Rb but did not have any effect on tumor xenografts without a functional Rb (Sherr *et al.*, 2016). Another therapy which requires functional Rb is immunotherapy, most likely due to the importance of Rb in the immune function. Interestingly, a decrease in expression of factors related to the immune response have been reported from studies using models lacking *RB1* expression (Indovina *et al.*, 2019). Clinically, mutant *RB1* was correlated with poor response to two anti-PD-1 monoclonal antibodies – nivolumab and pembrolizumab in advanced non-small cell lung cancer patients (Bhateja *et al.*, 2019). On the other hand, better response and prognosis upon adjuvant and neoadjuvant chemotherapy have been reported in breast cancer patients with loss of *RB1* function or expression (Derenzini *et al.*, 2008; Herschkowitz *et al.*, 2008; Trere *et al.*, 2009; Witkiewicz *et al.*, 2012), and better response to radiation treatment has been associated with loss of Rb expression in bladder cancer (Agerbaek *et al.*, 2003).

1.3.3 The tumor suppressor *PTEN*

1.3.3.1 PTEN gene and PTEN protein

The phosphatase and tensin homolog *(PTEN)* gene was first identified as a candidate tumor suppressor gene on chromosome 10q23 (Li and Sun, 1997; Li *et al.*, 1997; Steck *et al.*, 1997). This chromosomal region is affected by loss of heterozygosity (LOH) in many cancers (Chen *et al.*, 2018).

PTEN gene encodes a protein consisting on 403 amino acids with phosphatase activity, which can act on both phosphopeptides and phospholipids (Chen *et al.*, 2018). The main substrate for dephosphorylation by PTEN is phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Downes *et al.*, 2007). The PTEN protein has five functional domains: 1) the N-terminal region consists of a phosphatidylinositol 2-phosphate binding domain (PBD), followed by 2) the catalytic phosphatase domain, 3) the C2 domain related to the lipid and membrane binding, 4) the C-terminal region which contains the PEST sequences, and 5) a binding motif defined as class I PDZ (PDZ-BD) (Lee *et al.*, 2018; Molinari and Frattini, 2014) (Figure 8). In order to dephosphorylate PIP_3, PTEN needs to translocate to the membrane where PIP_3 is located. This is mediated by the C2 domain and the PBD domain (Stiles, 2009). The C2 domain is also thought to be important for protein stability and supports the correct orientation of the phosphatase domain once located in the membrane (Georgescu *et al.*, 2000). PTEN has both cytoplasmic localization signals in the N-terminal part of the protein (Denning *et al.*, 2007) and nuclear localization signal-like motifs along the protein (Chung *et al.*, 2005). Its half-life is more than 2 hours (Georgescu *et al.*, 1999).

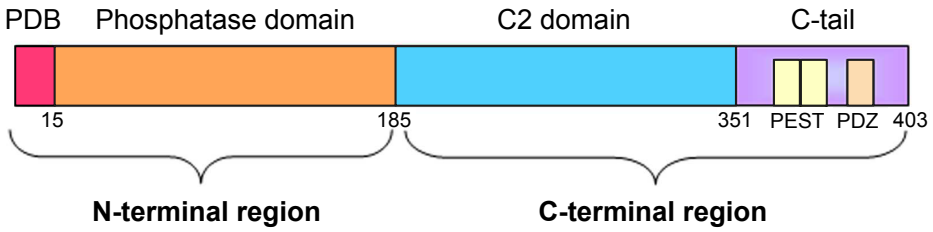

Figure 8. Domains of PTEN protein. The phosphatidylinositol 2-phosphate binding domain (PBD) and the phosphatase domain are part of the N-terminal region of the protein and the C2 domain and the C-tail containing the PEST (proline, glutamic acid, serine, threonine) sequences and the PDZ domain form the C-terminal region of the protein. Figure adapted from Molinari F and Frattini M (2014) *Functions and regulation of the PTEN gene in colorectal cancer*, Frontiers in Oncology, 3:326 under the terms of Creative Commons Attribution License Attribution 3.0 Unported (CC BY 3.0) (https://creativecommons.org/licenses/by/3.0/). The original colors of the figure were modified.

PTEN is transcriptionally regulated by p53 and several other factors (Stiles, 2009), as well as by microRNAs and long noncoding RNAs; post-transcriptional regulation of PTEN includes epigenetic silencing and repression of transcription (Lee *et al.*, 2018). At the post-translational level, PTEN is regulated by ubiquitylation, phosphorylation, oxidation and acetylation (Lee *et*

al., 2018). Ubiquinitation of PTEN takes place at the N-terminal and the C2 domain. The NEDD4-1 E3 ligase was reported to ubiquitinate it (Wang *et al.*, 2007). Monoubiquitination is related to nuclear import and polyubiquitination to protein degradation (Trotman *et al.*, 2007). In contrast, phosphorylation of PTEN occurs in the C-terminal region and is related to functions such as conformation stability and dimerization status. Many residues in PTEN have been shown to be phosphorylated, the most distinguished ones are Tyr46 in the phosphatase domain and several others in the C-terminal region (Lee *et al.*, 2018). High PTEN phosphorylation that promote its inactivation has been related to tumorigenesis in T cell acute lymphoblastic leukemia cells (Nakahata *et al.*, 2014; Silva *et al.*, 2008). In addition, phosphorylation of the C-terminal tail represses PTEN activity by promoting a change in its conformation that is closed, more stable and less prone to bind to phospholipids or other proteins (Odriozola *et al.*, 2007; Rahdar *et al.*, 2009; Vazquez *et al.*, 2001). In any case, it is worth noting that PTEN protein stability mediated by phosphorylation may vary between cell lines (Lee *et al.*, 2018). Cysteine 124 (Cys124) in the catalytic phosphatase domain can be oxidized which inhibits its phosphatase activity by the formation of a disulfide bond with another cysteine (Cys71) in the same domain (Lee *et al.*, 2018). Therefore, increased oxidative stress in cancer cells could inactivate PTEN via this mechanism. Acetylation modification occurs in the phosphatase domain, which inhibits its catalytic function, as well as in the C-terminal domain, which affects the ability of PTEN to bind to other proteins (Ikenoue *et al.*, 2008) and has been suggested to also regulate PTEN localization as it was observed to be excluded from the nucleus upon hyperacetylation (Chae and Broxmeyer, 2011).

Finally, it is worth mentioning the discovery of PTEN ability to form dimers with itself at the plasma membrane. This dimerization appears to be highly important for its full activation (Papa *et al.*, 2014). This study also showed that heterodimers of mutant PTEN with WT PTEN can be formed inhibiting its catalytic activity. Another study reported that the C-terminal tail of PTEN has a role in the stabilization of the dimer and that a closed conformation of PTEN occurs upon phosphorylation on C-terminal sites, inhibiting the formation of PTEN dimers (Heinrich *et al.*, 2015). PTEN dimerization is still poorly understood and further studies of the molecular mechanisms regulating this process are needed.

1.3.3.2 PTEN functions and pathways involved

PTEN regulates the so-called PI3K (phosphoinositide 3-kinase)-AKT signaling pathway (Downes *et al.*, 2007). This pathway includes phosphatidylinositol 4,5-biphosphate (PIP_2) and PIP_3 which is generated by phosphorylation of PIP_2 by PI3K in response to growth factor signaling (Stiles, 2009). As indicated above, dephosphorylation of PIP_3 to PIP_2 is mediated by PTEN (Figure 9). When PIP_3 accumulates in the membrane it activates the PI3K-AKT pathway by recruiting AKT to the membrane. This allows AKT phosphorylation and activation by the PDK1 kinase (3-phosphoinositide-dependent kinase 1). AKT is also known as protein kinase B (PKB) and is involved in cell survival, cell proliferation, growth, metabolism, migration and other pro-tumorigenic functions via phosphorylation of some of its substrates, resulting in inactivation of various Bcl-2 homology domain 3 (BH3)-only proteins or activation of mTOR

complex 1 (Manning and Cantley, 2007; Song *et al.*, 2012; Stambolic *et al.*, 1998). Therefore, the lack of functional PTEN promotes cell growth and survival by an indirect activation of the PI3K-AKT pathway through increased levels of PIP_3 in the membrane (Jamaspishvili *et al.*, 2018; Sun *et al.*, 1999).

PTEN has also been reported to have a role in cell cycle regulation by decreasing the transcription of cyclin D1 or by inhibiting its nuclear accumulation, as well as in cell cycle arrest by promoting p53 acetylation by forming a complex with p300, which in turn stabilizes and activates p53 (Li *et al.*, 2006). In addition, it has been proposed that nuclear PTEN can have pro-apoptotic functions (Gil *et al.*, 2006). Different studies have also attributed a scaffold function to PTEN and this role has also been connected to tumor suppression independently of the PI3K-AKT pathway (Lee *et al.*, 2018). Finally, later studies have also highlighted its importance in metabolic regulation (Worby and Dixon, 2014).

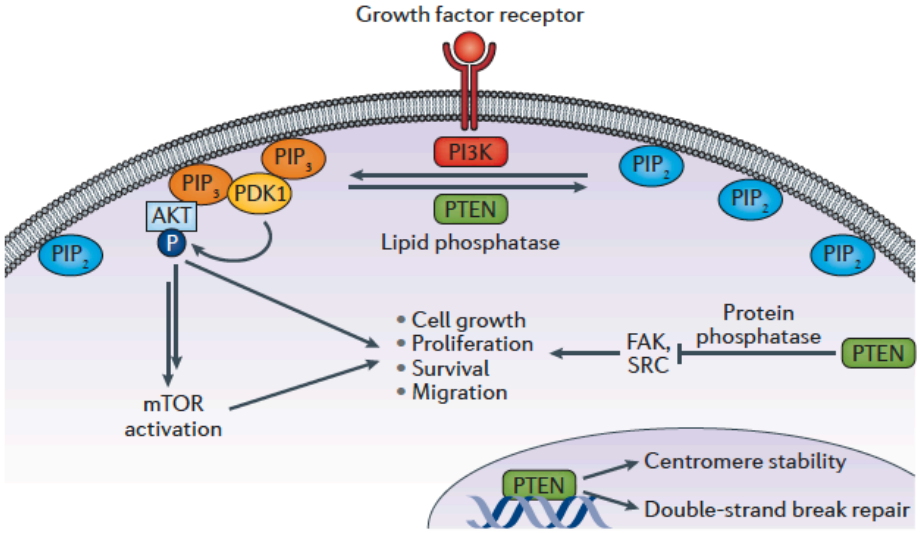

Figure 9. Different roles of PTEN. PTEN converts phosphatidylinositol 3,4,5-triphosphate (PIP_3) to phosphatidylinositol 4,5-biphosphate (PIP_2). Phosphoinositide 3-kinase (PI3K) phosphorylates PIP_2 to PIP_3 which recruits AKT to the plasma membrane. AKT is subsequently phosphorylated by 3-phosphoinositide-dependent kinase 1 (PDK1) and promotes processes such as cell growth and cell survival. Other functions are also associated to PTEN, such as promoting chromosome stability. Material from: Jamaspishvili T, Berman DM, Ross AE, Scher HI, De Marzo AM, Squire JA, and Lotan TL, *Clinical implications of PTEN loss in prostate cancer* (2018) Nature Reviews Urology (Springer Nature) 15: 222-234 reproduced with permission of Springer Nature Customer Service Center GmbH (SNCSC).

1.3.3.3 Mutations in PTEN

Already at the time of its discovery, *PTEN* was reported to be mutated at a high frequency in human tumors. The first studies showed mutations in glioblastoma, prostate cancer and breast cancer (Li *et al.*, 1997). Different types of mutations have been described, both as germline and somatic mutations. They include missense, nonsense, splice-site variants, deletions and insertions (Lee *et al.*, 2018). Interestingly, and similarly to p53, certain missense mutations in

PTEN have a dominant negative effect that inhibits the catalytic function of PTEN (Papa *et al.*, 2014).

According to the COSMIC database, 17.3% of all *PTEN* mutations in tumors are nonsense substitutions (Tate *et al.*, 2019), representing the second most common type of mutations after missense substitutions. The most common nonsense mutations are R233X, followed by R130X and R335X. Codon 130 is located in the phosphatase domain while the codons 233 and 335 are in the C2 domain. In addition, whole genome sequencing data from the 12 major cancer types revealed that *PTEN* is frequently mutated in uterine corpus endometrial carcinoma (63.5% of the cases) and in glioblastoma multiforme (30.7% of the cases) (Kandoth *et al.*, 2013). Overall, *PTEN* is mutated in 9.7% of the cases of these common tumor types, making *PTEN* the third most frequently mutated gene after *TP53* (42%) and *PIK3CA* (*PI3K*) (17.8%).

1.3.4 Nonsense mutations in tumor suppressor genes

There is strong association between nonsense mutations and cancer development (Prokofyeva *et al.*, 2013; Wei *et al.*, 2014). This type of mutations causes premature termination codons (PTCs) in the mRNA, which will therefore result in aberrant proteins unable to perform their normal function to prevent cancer onset and/or progression. When the ribosome encounters a PTC, no aminoacylated-tRNA can bind in that position. Release factors eRF1 and eRF3 are recruited, translation is finished and a truncated non-functional protein is released (Linde and Kerem, 2008). In addition, levels of these aberrant mRNAs are usually low due to degradation by nonsense-mediated decay (NMD), which can be activated by the presence of a PTC upstream of exon-exon junctions complexes (Lykke-Andersen and Jensen, 2015).

Interestingly, 18 codons out of the 61 sense codons can become stop codons (introducing a PTC) by the substitution of a single base. Two of the most common conversions that create nonsense mutations are CGA (Arg) to TGA and CAG (Gln) to TAG. This conversion is likely to be produced by frequent C>T substitutions which account for 27.4% of all possible single base substitutions (Lombardi *et al.*, 2022). Also, the majority of cytosines located at CpG islands in the human genome are modified by methylation which makes them more vulnerable to the C>T transition by spontaneous deamination of cytosine to thymine (Tomkova *et al.*, 2018).

1.4 Translation termination

Protein biosynthesis finishes with the well-described step translation termination. This process occurs when the ribosome encounters a stop codon, which can be UGA, UAG or UAA. The nascent polypeptide is released in the ribosomal P-site upon hydrolysis of the peptidyl-tRNA ester bond in the peptidyl transference center which is located in the large ribosomal subunit. In more detail, the tRNA-mimicking eukaryotic class-1 factor eRF1 recognizes any of the three stop codons in the ribosomal A-site which is located in the small subunit. eRF1 will then interact with eRF3 through the middle and C-terminal domain in eRF1. eRF3 will bind GTP through the C-terminal domain. This will allow GTP hydrolysis and the consequent release of

the polypeptide chain by a specific position of eRF1 in the peptidyl transferase center of the ribosome. In addition, interaction of poly(A)-binding proteins (PABPs) located in the 3'UTR of the mRNA with the complex is necessary for GTP hydrolysis (Dabrowski *et al.*, 2015).

The eRF1 protein has three domains: the N-terminal domain that recognizes the stop codons, the middle domain and the C-terminal domain, both of which can bind eRF3 (Hellen, 2018). Specifically in the middle domain, eRF1 has a conserved GGQ motif which is necessary for the hydrolysis of peptidyl-tRNA (Jackson *et al.*, 2012). In addition, there are studies showing that upon eRF1 binding to the ribosomal A-site, a modified mRNA configuration allows the fourth nucleotide to also be included in the A-site (Brown *et al.*, 2015). However, the peptide release process by eRF1 only is not sufficient and eRF3 is in charge of performing a fast and efficient peptidyl-tRNA hydrolysis (Alkalaeva *et al.*, 2006; Salas-Marco and Bedwell, 2004). eRF3 has a N-terminal domain that binds to PABP and UPF3b (a factor involved in nonsense-mediated decay), a GTP-binding domain and two β-barrel domains (Hellen, 2018). Different genes encode two different eRF3 isoforms, eRF3a and eRF3b, which have different N-terminal domains but both act as termination factors and are able to bind eRF1. The *a* isoform is expressed in all body tissues while the *b* isoform is mainly expressed in brain (Chauvin *et al.*, 2005). In addition, a study with human cell lines reported that only depletion of eRF3a but not eRF3b resulted in higher readthrough levels, but overexpression of both isoforms reverted the increase in readthrough by eRF3a depletion. Moreover, decreasing the levels of eRF3a affect eRF1 stability, causing a reduction in protein levels (Chauvin *et al.*, 2005).

Interestingly, it has been suggested that the complex process for translation termination in eukaryotes – involving eRF1, eRF3 and GTP – may be related to the fact that only eRF1 is needed to recognize the three termination codons, and so a more complicated system would help preventing induction of translation termination by recognition of the stop codon by eRF1 alone (Alkalaeva *et al.*, 2006).

1.5 Translational readthrough

Termination of translation is well regulated. However, it can sometimes fail, resulting in a process called translational readthrough (Bertram *et al.*, 2001). Readthrough can occur through the insertion of a near-cognate tRNA (nc-tRNA) by pairing 2 of the 3 nucleotides of the codon-anticodon sequence, instead of eRF1 (Dabrowski *et al.*, 2015). Consequently, the ribosome will continue translation until the next stop codon in frame. The rate of basal readthrough of normal stop codons is estimated to range from 0.001 to 0.1% (Keeling *et al.*, 2012), but higher readthrough efficiencies have been reported in specific cases (Loughran *et al.*, 2014). On the other hand, the basal readthrough rate of PTCs is significantly higher, ranging from 0.01 to 1% (Dabrowski *et al.*, 2018). This process can also be pharmacologically induced with readthrough-inducing compounds to rescue nonsense mutated genes (discussed in section 1.5.3 and 1.5.4) (Figure 10).

1.5.1 Readthrough efficiency factors

Several factors can influence readthrough efficiency. The amount of target mRNA present in cells, the efficiency of the readthrough *per se* as well as the characteristics of the newly synthesized proteins are factors to take into account for the clinical implementation of this strategy (Lombardi *et al.*, 2022).

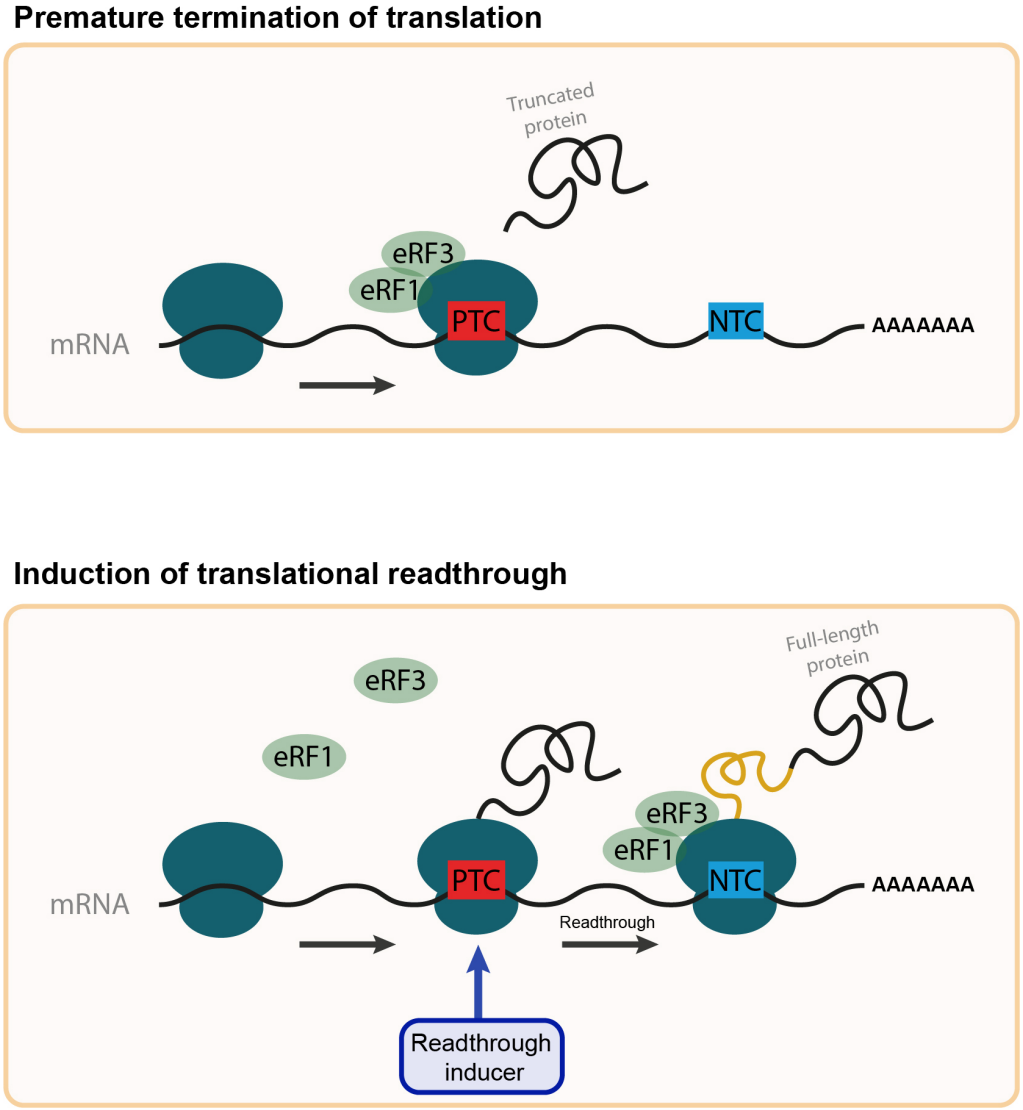

Figure 10. Premature termination of translation (top) and induction of translational readthrough (bottom). Upon encountering a premature termination codon (PTC), the ribosome stops translating the mRNA and the factors eRF1 and eRF3 promote the release of a truncated and non-functional protein (top). In the presence of a readthrough inducer, the ribosome continues translating past the PTC until the normal termination codon (NTC) producing the release of a full-length protein (bottom).

The main factor influencing translational readthrough induction is the PTC type and the surrounding sequences (Tate *et al.*, 2018). The three different stop codons show different efficiencies, with UGA being the most amenable to readthrough, followed by UAG and UAA (Manuvakhova *et al.*, 2000). In addition, the +4 nucleotide (if the first nucleotide in the PTC is

considered +1) has been confirmed to be highly important for termination of translation and readthrough efficiency. It appears to be so crucial that the termination signal for translation has been described as a tetranucleotide signal (McCaughan *et al.*, 1995). Essentially, the presence of a purine in the +4 position after the PTC or its presence in the +5 position when a pyrimidine is present in the +4 position results in a stronger termination of translation (McCaughan *et al.*, 1995). The fact that, as mentioned above, the fourth nucleotide after the stop codon is also included in the ribosomal A-site when eRF1 is interacting with the ribosome at a stop codon (Brown *et al.*, 2015) may explain the impact of the particular nucleotide present in that position. The mRNA compaction is mediated by stacking with a specific nucleotide of the 18S rRNA and this effect is weaker for pyrimidines. This could be the reason why cytosine and uracil located in the +4 nucleotide position are better for readthrough induction (Lombardi *et al.*, 2022). The four-nucleotide sequences that are more prone to readthrough induction are UGAC and UAGU, followed by UGA or UAG with any nucleotide in position +4 and finally UAA followed by any nucleotide at +4. The strongest four-nucleotide termination sequence is UAAA (Manuvakhova *et al.*, 2000).

In addition to the importance of the +4 nucleotide, the surrounding sequences of the PTC can affect readthrough efficacy. More specifically, higher readthrough levels have been reported with adenine in the -2 and -1 nucleotide positions (Tork *et al.*, 2004). Another study showed that the presence of uracil in the -1 position was favorable for gentamicin-induced readthrough (in addition to a cytosine in the +4 nucleotide) (Floquet *et al.*, 2012), and the +5 and +6 and +8 nucleotides were shown to be important for readthrough induction in a more complex manner together with the +4 nucleotide (Cridge *et al.*, 2018).

Moreover, and as mentioned in section 1.5.5 about differences between normal and premature termination codons, the levels of eRFs along the translated mRNA are also an important factor for readthrough induction, as eRFs are more concentrated at the normal stop codons compared to areas where the PTCs are located (Biziaev *et al.*, 2022). Finally, high mRNA levels are known to promote efficiency of readthrough, as more nonsense transcripts will be present for translation (Linde *et al.*, 2007).

1.5.2 Newly inserted amino acids in PTC after readthrough induction

The question as to which amino acids get incorporated at the PTC after readthrough induction is obviously very important since this may have a huge impact on the activity of the induced full-length protein. The binding of a nc-tRNA upon PTC readthrough is not random. The anticodons of the nc-tRNA have two matching nucleotides and one mismatch with the nonsense codon. Two studies have shown that the mispairing between the codon and the nc-tRNA occurs either in position 3 or 1 of the stop codon (Roy *et al.*, 2016; Roy *et al.*, 2015). Mispairing at position 1 was favored for readthrough of UAG, at position 3 for UGA and equally at position 1 or 3 for UAA (Roy *et al.*, 2015). Thus, theoretically, there are limited numbers of tRNAs that can bind to a given stop codon. Empirically, it appears that certain amino acids are more prone to incorporation than others. This was studied in human cells and

yeast which were transfected with nonsense mutation reporters and treated with PTC124 (Ataluren) or with aminoglycosides. The data demonstrated that the amino acids more prone to be incorporated after treatment were the same as the ones incorporated at endogenous readthrough. For UAA and UAG, Glutamine (Gln), Lysine (Lys) or Tyrosine (Tyr) were incorporated and for UGA Tryptophan (Trp), Arginine (Arg) or Cysteine (Cys) were incorporated. These results suggest that nonstandard base pairs such as U-G are preferred over other pairings at position 1 and that the mispairing A-C prevails over G-G followed by A-G at position 3 (Roy *et al.*, 2016).

Given the uncertainty regarding which amino acid will be inserted upon readthrough induction, a full functional restoration may or may not be achieved by readthrough induction as a therapeutic strategy. If restoration of expression of a full-length functional tumor suppressor protein is a therapeutic aim, the frequency of missense mutations at the nonsense mutant codon may provide a clue. If such mutations are frequent in tumors, this may indicate that the protein does not tolerate amino acid substitutions at that position in order to maintain its normal function. Appropriate functional assays should be performed after readthrough induction to validate restoration of WT function.

1.5.3 Readthrough-inducing compounds

So far, the most studied readthrough-inducing compounds are aminoglycosides, such as G418 (geneticin) and gentamicin. Already in 1996, the effect of G418 as a readthrough inducer was tested in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene carrying nonsense mutations. The full-length and functional protein observed after the treatment in a model of cystic fibrosis proved that G418 had readthrough-inducing activity (Howard *et al.*, 1996). Aminoglycosides have been shown to induce readthrough of several genes including *CFTR* (Bedwell *et al.*, 1997), *ATM* (Lai *et al.*, 2004), *DMD* (Bidou *et al.*, 2004) and *TP53* (Floquet *et al.*, 2011). However, their use in the clinics is limited due to their nephrotoxicity and ototoxicity (Guthrie, 2008; Huth *et al.*, 2011; Jiang *et al.*, 2017; Lopez-Novoa *et al.*, 2011; Wargo and Edwards, 2014). There are other non-aminoglycosides antibiotics that have been shown to have readthrough activity, including negamycin (Arakawa *et al.*, 2003) and macrolides such as tylosin, josamycin and spiramycin (Zilberberg *et al.*, 2010). Another non-aminoglycoside readthrough-inducing compound is PTC124 (Ataluren/Translarna) (Welch *et al.*, 2007), which got conditional authorization by the EMA for treatment of patients with Duchenne muscular dystrophy caused by a nonsense mutation in the dystrophin (*DMD*) gene (*see https://www.ema.europa.eu/*) (Haas *et al.*, 2015). However, in case of cystic fibrosis patients, no promising results have been reported in two clinical trials (Kerem *et al.*, 2014; Konstan *et al.*, 2020).

Other reported low-molecular weight readthrough-inducing compounds are GJ071 and GJ072 and their related compounds RTC204 and RTC219 (Du *et al.*, 2013). An adenosine nucleoside analog named clitocine was also found to induce readthrough of nonsense mutant *TP53* by incorporation in the mRNA instead of adenosine (Friesen *et al.*, 2017). Furthermore, the

synthetic eukaryotic ribosome-selective glycoside ELX-02, previously named NB124, has been shown to induce significant readthrough at PTCs while maintaining normal termination fidelity (Crawford *et al.*, 2020). NB124 was shown to restore up to 7% of the WT activity of CFTR in primary cystic fibrosis cells and rescue CFTR function in a mouse model expressing a nonsense mutated *CFTR* transgene (Xue *et al.*, 2014). It was also reported to induce readthrough of nonsense mutant *TP53* (Bidou *et al.*, 2017). ELX-02 has already completed phase I clinical studies (Leubitz *et al.*, 2019; Leubitz *et al.*, 2021) and is currently being tested in phase II trials in patients with nephropathic cystinosis with nonsense mutant cystinosin, lysosomal cystine transporter (*CTNS*) and in cystic fibrosis patients with nonsense mutations in the *CFTR* gene (Kerem, 2020). Another study examined the readthrough ability of the different components that form the aminoglycoside gentamicin and found that a minor component named gentamicin X2 was the most active in terms of readthrough induction (Friesen *et al.*, 2018). More recently, the purine derivative 2,6-diaminopurine (DAP) has also been shown to induce readthrough of UGA PTCs (Trzaska *et al.*, 2020). A recent drug screening led to the identification of the compound SRI-37240 and its more potent analog SRI-41315 as readthrough inducers of nonsense mutant *CFTR*, alone or in synergistic combination with G418 (Sharma *et al.*, 2021).

The compound CC-885 induces degradation of the release factor eRF3 (Matyskiela *et al.*, 2016). CC-885 belongs to the so-called immunomodulatory drugs that also include lenalidomide and pomalidomide (Ito *et al.*, 2010; Krönke *et al.*, 2014; Lu *et al.*, 2014). CC-90009 is another eRF3 degrader (Hansen *et al.*, 2021; Surka *et al.*, 2021). The observed activity of CC-885 and CC-90009 (explained in more detail in section 1.5.4) and the fact that decreased levels of release factors can promote readthrough, prompted studies of their effects in combination with G418. Indeed, combination treatment with CC-885 or CC-90009 and G418 induced synergistic readthrough of nonsense mutated *TP53* and was associated with decreased levels of eRF3a, eRF3b and eRF1 (Baradaran-Heravi *et al.*, 2021). A summary of selected readthrough-inducing compounds is shown in Table 1.

1.5.4 Mechanism of action of readthrough-inducing compounds

Aminoglycosides can inhibit bacterial protein synthesis by binding to the ribosomes (Fan-Minogue and Bedwell, 2008). Specifically, their binding in the decoding center of the ribosome produces a conformational modification as a consequence of the flipping out of two nucleotides (A1492 and A1493) from the helix 44 loop where they are located. This new conformation is similar to the one observed upon binding of a cognate aminoacyl-tRNA (aa-tRNA) and allows the incorporation of incorrect amino acids and inhibition of translation (Lombardi *et al.*, 2022; Ogle *et al.*, 2001). Aminoglycosides bind with lower efficiency to the eukaryotic ribosome as compared to the prokaryotic one (Fan-Minogue and Bedwell, 2008). However, this binding is strong enough to reduce the ability of the eukaryotic ribosome to distinguish between near-cognate aa-tRNAs and release factors at a PTC, and therefore promote readthrough by incorporation of amino acids at that position (Lombardi *et al.*, 2022). The aminoglycoside G418 is known to target the decoding center of the ribosome, specifically the internal loop of

the helix 44 of 18S rRNA. The decoding center contains the nucleotides A1755 and A1756, which are essential and universally conserved. The nucleotides G1645 and A1754 present in yeast and human ribosomes are also important for aminoglycoside-induced readthrough. The ring I of G418 interacts directly with G1645 and A1754. When G418 binds to the pocket, the nucleotides A1755 and A1756 flip out (Garreau de Loubresse *et al.*, 2014) and readthrough was proposed to occur due to miscoding and the efficient accommodation of a near- or non-cognate tRNA at the ribosomal A-site in the presence of a PTC (Prokhorova *et al.*, 2017). Gentamicin, on the other hand, was suggested to induce readthrough by other mechanisms, such as effects on intersubunit rotation that may obstruct the interaction between eRF1 and the ribosome (Prokhorova *et al.*, 2017). Non-aminoglycoside antibiotics act by a similar mechanism, impairing the ribosomal decoding (Arakawa *et al.*, 2003).

Table 1. Selected readthrough-inducing compounds. The putative mechanism of action of some of the reported readthrough inducers is shown, examples of genes tested for each compound are also annotated.

Compound	Mechanism of action	Genes studied	References
G418 (geneticin)	Binding to the decoding center or the ribosome, at helix 44 of 18S rRNA	*CFTR, ATM, DMD, TP53, CTNS*	(Bedwell *et al.*, 1997; Bidou *et al.*, 2004; Brasell *et al.*, 2019a; Floquet *et al.*, 2011; Lai *et al.*, 2004)
PTC124 (Ataluren/ Translarna)	Inhibition of the release factor complex termination activity	*DMD, CFTR, DYSF, HERG*	(Du *et al.*, 2008; Wang *et al.*, 2010; Welch *et al.*, 2007; Yu *et al.*, 2014)
Clitocine	mRNA incorporation instead of adenine	*TP53*	(Friesen *et al.*, 2017)
ELX-02 (NB124)	Binding to the decoding site in the ribosomal small subunit	*CFTR, TP53, CTNS*	(Brasell *et al.*, 2019b; Crawford *et al.*, 2020; Kerem, 2020; Xue *et al.*, 2014)
2,6-diaminopurine (DAP)	Inhibition of the FTSJ1 methyltransferase	*TP53, CFTR*	(Leroy *et al.*, 2023; Trzaska *et al.*, 2020)
SRI-41315	Reduction of eRF1 abundance	*CFTR*	(Sharma *et al.*, 2021)
CC-885/ CC-90009	Induction of eRF3 degradation	*TP53, IDUA, TPP1, DMD, COL17A1*	(Baradaran-Heravi *et al.*, 2021)

The mechanism of action of compounds other than aminoglycosides are even less clear, but some mechanisms have been proposed. Ataluren has recently been proposed to inhibit the

termination activity of the release factor complex (Huang *et al.*, 2022; Ng *et al.*, 2021). The adenosine nucleoside analog clitocine acts by incorporation into the mRNA by RNA polymerase instead of adenine, allowing the decoding of the PTC by a nc-tRNA. However, the exact molecular mechanism by which clitocine affects the ribosome to induce readthrough is not known. Clitocine is more likely to base-pair in the third codon position with G or A than with C, as Cysteine (UGU, UGC) was more prone to be incorporated than Tryptophan (UGG). Its presence in mRNA does not affect the normal decoding, meaning that it base-pairs with uracil during normal translation. Also, the codon UGcl (with clitocine in the third position) may be poorly recognized by eRF1, preventing proper termination and allowing nc-tRNA to bind (Friesen *et al.*, 2017). This different mechanism of action causes a more efficient induction of readthrough of UAA, followed by the UGA and UAG stop codons. The mechanism of action of other compounds, such as the RTC series, is unknown.

The mechanism of action of DAP is possibly related to inhibition of methylation of tryptophan tRNA which is exerted by the tRNA methyltransferase 1 (FTSJ1). Interestingly, the only amino acid reported to be incorporated at the UGA PTC is tryptophan (Trzaska *et al.*, 2020).

In the case of SRI-37240 and SRI-41315, readthrough was reported to occur by decreasing the levels of eRF1 and thus extending the pause at PTCs which allows the insertion of a nc-tRNA. Levels of eRF1 mRNA levels were almost unchanged and eRF3 protein levels were not affected by SRI-41315. These results suggest a post-transcriptional effect of the compound on eRF1, specifically by a pathway related to proteasomal degradation (Sharma *et al.*, 2021).

Both CC-885 and CC-90009 take advantage of the fact that specific proteins are recruited and ubiquitinated by the E3 ubiquitin ligase $CRL4^{CRBN}$, which leads to proteasomal degradation of the specific substrate. In case of CC-885, the protein eRF3a (GSPT1) was reported to be a new cereblon substrate dependent on the CC-885 effect. This was identified as the reason for the anti-proliferative activity of the compound (Matyskiela *et al.*, 2016). One drawback of CC-885 is the toxicity created by the degradation of several substrates apart from eRF3a. This leads undesired off-target effects that have hindered its clinical development. CC-90009, another compound that modulates the cereblon E3 ligase, is selective for eRF3a (Hansen *et al.*, 2021; Surka *et al.*, 2021). CC-90009 was discovered by structure-activity relationship (SAR) analysis based on the eRF3a depletion function exerted by CC-885. The differences between CC-885 and CC-90009 are small. The 2,2-difluoroacetamide part present in CC-90009 but not in CC-885 is important for a better selectivity for eRF3a (Dong *et al.*, 2021). CC-90009 decreased the levels of eRF3a also via ubiquitination and proteasomal degradation, but not of any other proteins that are degraded by CC-885 (Surka *et al.*, 2021).

1.5.5 Differences between normal and premature termination codons

One frequently raised concern regarding induction of readthrough of PTCs as a therapeutic strategy is the presumed readthrough effect on the normal termination codons, which might cause cellular toxicity. However, translation termination at normal stop codons differs from termination at PTCs. It is worth mentioning that during the translation process, the mRNA is

present in a closed conformation forming a loop, and this confers protection to the transcript ends from exonucleolytic degradation (Chen and Shyu, 2011) as well as increases the efficacy of the mRNA translation by recycling the components needed for this process (Keeling *et al.*, 2014). Three components are important to maintain the closed conformation of the mRNA: the cap-binding protein eIF4E at the 5'-cap, the PABP at the poly(A) tail and eIF4G that binds the two first mentioned components (Lombardi *et al.*, 2022). Due to this mRNA conformation, normal stop codons are located closer to the poly(A) tail of the 3'UTR in the mRNA. PABP interacts with eRF3 and increases termination efficiency; therefore, the efficiency of termination of translation would be reduced at longer distances between the PTC and the poly(A) tail (Hoshino *et al.*, 1999; Keeling *et al.*, 2012). This distance will also increase the retention time of the ribosome at PTCs (Amrani *et al.*, 2004), increasing the probability of readthrough. In addition, mRNAs often contain additional stop codons in tandem 3' of the normal stop codon, which ensures efficient termination after readthrough of the natural stop codon (Keeling *et al.*, 2012; Liang *et al.*, 2005). Moreover, concentrations of eRFs are higher at the canonical stop codon and are also recruited to the ribosome by factors such as PABP (Ivanov *et al.*, 2016). In contrast, concentrations of eRFs are low at PTCs due to the lack of factors present at the normal termination codons, i.e. the poly(A) tail. Thus, readthrough induction at the PTCs is more feasible due to the lower competition between eRFs and nc-tRNAs at this site (Biziaev *et al.*, 2022).

Recently, it has also been proposed that proteins with extended C-terminal regions might contain sequences that destabilize the protein (Arribere *et al.*, 2016). In another study, the authors examined the effect of extended proteins in the C-terminal domain and observed that they form aggregates that are eliminated by the lysosomes. The authors suggest that this may be a mechanism to control the presence of these C-terminally extended proteins that could have negative effects in cells (Kramarski and Arbely, 2020).

In summary, normal stop codons are much less likely to be affected by readthrough-inducing compounds, and even if they were, translation can be terminated by additional downstream stop codons. If a C-terminally extended protein is produced, it may be destabilized or eliminated.

2 RESEARCH AIMS

The overall aim of the thesis was to find novel compounds or repurpose compounds that are already used in the clinics for the induction of translational readthrough of nonsense mutations in the tumor suppressor genes *TP53*, *RB1* and *PTEN*.

In **Paper I** we aimed to study combination treatments with the known readthrough-inducing aminoglycosides G418 and gentamicin and Mdm2 inhibitors or a proteasome inhibitor to potentiate readthrough induction of nonsense mutant *TP53*.

The aim of **Paper II** was to find novel *TP53* readthrough-inducing compounds by an *in silico* screen of the National Cancer Institute database (NCI-60). This allowed us to find 5-Fluorouracil as a potential readthrough inducer and we therefore explored its capability to induce nonsense mutant *TP53* readthrough further.

In **Paper III** we examined the ability of the aminoglycoside G418 to induce readthrough of nonsense mutant *RB1* as well as combination treatments with another known readthrough inducer to potentiate the effect of G418.

Paper IV is focused on novel readthrough-inducing compounds for nonsense mutant *TP53* identified by chemical library screening. In addition, we examined different combination treatments with the selected compounds in the screening with already known readthrough inducer compounds and tested these single or combination treatments also in the nonsense mutant tumor suppressor gene *PTEN*.

3 METHODOLOGY

A detailed description of the materials and methods used in each study in this thesis can be found in the enclosed papers. Here, a brief summary of the most used methods is included.

3.1 Protein analysis

3.1.1 Western blotting

A classical method to analyze protein expression is Western blotting. It is a qualitative assay used to assess levels of proteins in cells and distinguish them by size. Proteins from a cell lysate are separated according to size by gel electrophoresis followed by the transfer of the proteins to a membrane that is then blotted with specific antibodies to visualize the protein of interest.

This was the main method used to study protein expression throughout the thesis and it was used in all the papers presented here. We used this assay to examine translational readthrough because it allowed us to study both expression of truncated and full-length proteins upon treatment. A variety of antibodies were used to detect readthrough induction in different cell lines and different genes. Collected cells after treatments *in vitro* or collected tumors from *in vivo* experiments were lysed and protein extracts were quantified using Bradford protein assay or DC™ Protein assay (both from Bio-rad, USA). Lysates were run using NuPAGE™ 10% Bis-Tris gels and MOPS SDS Running buffer (both from Thermo Fisher Scientific, USA). Protein transfer to membranes was performed either with iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific, USA) or with wet transfer. Membranes were blocked and blotted with different antibodies. Detection of proteins was performed using the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) and a FUJIFILM LAS-1000 Image Analyzer (Fujifilm, Japan) or an iBright FL1000 Imaging System (Thermo Fisher Scientific, USA).

It should be emphasized that Western blotting is a qualitative method not suitable for precise quantitation. Therefore, we also used two quantitative methods to analyze protein expression, Enzyme-Linked Immunosorbent Assay (ELISA) and flow cytometry, as described below.

3.1.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The Enzyme-Linked Immunosorbent Assay (ELISA) allowed us to detect the expression of proteins in a more quantitative manner. This technique, which was used in Paper II and Paper IV, is performed in a multi-well plate and relies on specific antibodies against the protein of interest. Wells in a plastic plate are coated with a specific antibody, a protein lysate is added and upon binding of the protein to the antibody immobilized on the well and a washing step, a second antibody conjugated to the HRP enzyme is added. The amount of immobilized protein can then be quantified.

We assessed amount of protein by determining the activity of HRP using 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, USA). The reaction was stopped with

1M HCl. Protein determination was performed by measuring absorbance at 450 nm with Varioskan™ LUX multimode microplate reader (Thermo Scientific, USA). In Paper II, we used ELISA to examine the ability of 5-FU/FUr/FdUr/G418 to induce readthrough of different nonsense mutations in *TP53*. In Paper IV, however, we used it to quantitatively determine the expression of full-length p53 upon treatment with C61 alone or in combination with CC-885 or CC-90009. This assay allowed us to perform synergy calculations of the ability of both compounds to induce readthrough.

3.1.3 Flow cytometry for EGFP detection

Another quantitative method to examine protein expression upon readthrough induction is flow cytometry. This technique has several applications, one of them is the detection of the reporter EGFP taking advantage of its intrinsic fluorescence. In Paper II and Paper IV, constructs with the sequence of interest carrying a premature termination codon and a C-terminal EGFP tag were used to detect readthrough by EGFP expression. Treated cells were harvested, washed and analyzed with NovoCyte flow cytometer (ACEA Biosciences, USA). After applying the necessary gatings to the data, cells were classified as positive or negative for EGFP to assess the number of cells that had undergone readthrough.

In Paper IV, taking advantage of the quantitative results obtained by this technique, EGFP expression measured by flow cytometry was further used to calculate synergy upon treatment with C47 alone or in combination with G418.

3.2 Functional studies

3.2.1 Cell viability

Cell viability and cell proliferation can be studied by a great variety of methods. For the studies included in this thesis, the WST-1 assay (Paper I, II and III) and the Incucyte S3® system (Paper II) were used.

The assay using the cell proliferation reagent WST-1 (Roche, Switzerland) is based on the ability of mitochondrial dehydrogenases to cleave tetrazolium salts (in this case WST-1 reagent) to formazan dye. Metabolically active and therefore viable cells will produce an increase in the activity of these enzymes and therefore the production of formazan. The absorbance of the substrate was determined at 450 nm with Varioskan™ LUX multimode microplate reader (Thermo Scientific, USA). Incucyte® S3 Live-Cell Analysis System (Essen BioScience, USA) allows the periodic imaging of living cells. We used it to measure cell confluence upon different treatments in Paper II. Collected data was analyzed using Incucyte® S3 Analysis Software.

3.2.2 Gene expression analysis (qRT-PCR)

Expression levels of several genes have been analyzed with different purposes throughout this thesis. Expression levels of *TP53* (Paper I and II) and of *RB1* (Paper III) upon readthrough

were studied in relationship to the literature reporting that induction of translational readthrough can promote an increase in the target mRNA. In addition, expression levels of a panel of p53 target genes were examined to study p53-dependent biological effects in terms of transcription activity (Paper I and II). Also, expression of an E2F target gene (Thymidylate synthase) was studied upon readthrough induction of Rb (Paper III).

RNA extraction was performed on collected cells at the end of the experiment using RNeasy mini kit (Qiagen, Germany). After RNA quantification using a NanoDrop Spectrophotometer (Thermo Scientific, USA), cDNA was synthesized using the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, USA). The system QuantStudio™ 7 Flex Real-Time PCR or the Applied Biosystems 7500 Real-Time PCR System (both from Applied Biosystems, USA) was used together with TaqMan Gene Expression Assays (Thermo Fisher Scientific, USA) and FastStart Universal Probe Master (Rox) (Roche, Switzerland) to perform quantitative Real-Time PCR (qRT-PCR). Analyses of qRT-PCR data were performed using the $2^{-\Delta\Delta Ct}$ method, with which the relative gene expression was calculated after normalization to GAPDH as endogenous control.

3.2.3 Cell death analysis

Cell death is a process that can be triggered by many different causes and can also occur in different ways. In this thesis, examination of cell death has been performed by Annexin V staining and by measuring the activity of caspase 3/7 in cells (both in paper II). In addition, assessment of cells in sub-G1 phase was used in Paper I to also examine cell death induction upon treatment.

Annexin V staining is a well-established method for the detection of apoptotic cells. It is based on the fact that the anionic phospholipid phosphatidylserine translocates from the inner part of the plasma membrane to the outer one during apoptosis. Annexin V is a calcium-dependent phospholipid-binding protein that has high affinity for phosphatidylserine, therefore it is used for its detection in the outer part of the membrane under apoptosis. Staining with Annexin V conjugated to fluorochromes was examined using NovoCyte flow cytometer (ACEA Biosciences, USA) and cells were classified as Annexin V positive or negative after the application of the needed gatings to determine percentage of dead or viable cells, respectively. In addition to the translocation of phosphatidylserine to the outer part of the membrane, apoptotic cells also present activated caspase-3 and caspase-7 enzymes. To detect their activation, the CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific, Sweden) was used in the Incucyte® S3 Live-Cell Analysis System (Essen BioScience, USA). Activated caspase 3/7 was measured by the green counts signal which was normalized to the cell confluence obtained also with Incucyte® S3 system. Finally, cell death can also be studied by assessing the sub-G1 cell fraction. This method is based on the fact that apoptosis involves DNA fragmentation. This results in a lower DNA content in apoptotic cells than in cells in G1-phase, which can be quantified by staining with propidium iodide and analysis by flow cytometry.

3.3 Ethical considerations

In **Paper II** experiments using a human tumor xenograft mouse model were performed. All animal studies were approved by the Stockholm Animal Experiments Ethical Committee, Sweden (Dnr 7054-2019; Dnr 15763-2020). In addition, animal care was in accordance with Karolinska Institutet guidelines.

In **Paper I**, **III** and **IV** commercially available cell lines and stably transfected cell lines created in our group using an original commercially available cell line were used.

No human patient material was used in any of the papers.

4 RESULTS AND DISCUSSION

4.1 Paper I

Synergistic Rescue of Nonsense Mutant Tumor Suppressor p53 by Combination Treatment with Aminoglycosides and Mdm2 Inhibitors

One strategy to restore nonsense mutated genes is the induction of translational readthrough. Aminoglycosides such as G418 and gentamicin are known to induce readthrough but they cause toxicity to patients. One possible approach for decreasing the doses of aminoglycosides given to patients is combination treatments with agents that potentiate the readthrough effect. In this paper, we combined either G418 or gentamicin with the p53-Mdm2 inhibitors Nutlin-3a or MI-773 or the proteasome inhibitor Bortezomib with the aim of achieving robust readthrough with lower aminoglycoside concentrations and therefore lower toxicity.

We first examined the ability of G418 or gentamicin to induce full-length p53 in the breast carcinoma cell line HDQ-P1 that carries endogenous R213X mutant *TP53*. We observed an increase in readthrough induction after treatment with both compounds, with higher potency by G418. In addition, an upregulation in p53 mRNA levels was observed upon both treatments but again stronger after G418. G418-induced full-length p53 was confirmed in the H1299 *TP53* null cells stably transfected with an R213X mutant *TP53* construct with or without an EGFP tag directly after the coding sequence.

In order to examine the functionality of the newly synthesized full-length p53 upon G418 or gentamicin treatment in HDQ-P1 cells, the expression of a panel of p53 target genes was examined. Wig-1 (Zmat3), p21, Fas, Mdm2 and Noxa were upregulated upon both G418 and gentamicin treatments and this effect was statistically significant. Bax was also significantly increased after gentamicin treatment. In general, treatment with G418 caused a more potent upregulation of p53 target genes. In addition, we examined p21 and Wig-1 protein levels in H1299-R213X and its control cell line H1299-EV and observed a stronger upregulation of both proteins in H1299-R213X cells than in H1299-EV cells. G418 was more potent than gentamicin and p21 showed the most pronounced upregulation.

We then proceeded to examine possible combination treatments of G418 or gentamicin with Bortezomib, Nutlin-3a or MI-773 in HDQ-P1 cells. No full-length p53 was detected in cells treated only with Bortezomib, Nutlin-3a or MI-773. However, upon combination with G418 or gentamicin, full-length p53 levels increased markedly. p53 target genes such as Wig-1, p21 Fas and Mdm2 were significantly upregulated upon combination treatment of G418 and Nutlin-3a. Wig-1, p21 and Mdm2 were also significantly higher after treatment with gentamicin and Nutlin-3a. p53 mRNA levels were increased by both combination treatments. In H1299-R213X cells, an increase in p21 protein was observed only after G418 alone or G418 combined with Nutlin-3a, but not after Nutlin-3a alone or after any treatment in H1299-EV cells. We finally studied the p53-dependent biological effects in terms of growth suppression and cell death upon G418 and Nutlin-3a single treatments or in combination. We found that the combination

treatment at 50 µM G418 caused a significantly increased growth suppression as compared to G418 treatment alone in H1299-R213X cells but not in the control cell line H1299-EV. We also observed a significant increase of the sub-G1 population in H1299-R213X cells but not in H1299-EV upon G418 and Nutlin-3a treatment compared to G418 alone. G418 alone also statistically increased the sub-G1 population as compared to vehicle control (DMSO).

This study allowed us to confirm the readthrough-inducing capacity of G418 and gentamicin on nonsense mutant *TP53* in cells with either endogenous or exogenous nonsense mutant *TP53* and to perform a functional assessment of the newly synthesized full-length p53. Furthermore, we could show that combining either G418 or gentamicin with a proteasome inhibitor or two p53-Mdm2 inhibitors resulted in enhanced full-length p53 expression and, in case of G418 and Nutlin-3a, a potentiated p53-dependent biological response (Figure 11).

Thus, we present an alternative strategy for the treatment of patients with tumors carrying nonsense mutant *TP53* by combination treatments that might allow decreased doses of aminoglycosides and therefore decreased toxicity, which is a concern when using these antibiotics.

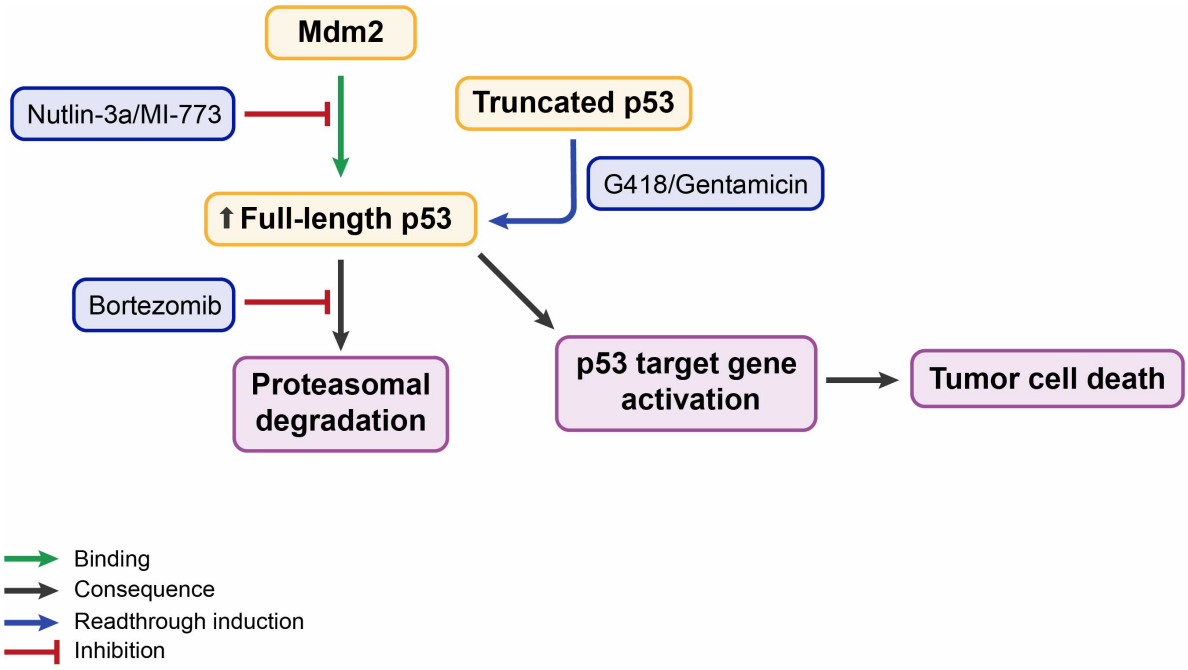

Figure 11. Model of the combination effects of G418 or gentamicin and Mdm2-p53 inhibitors or proteasome inhibitor for nonsense mutant *TP53* reactivation. Mdm2 targets p53 for proteasomal degradation. This process can be inhibited by Nutlin-3a or MI-773 via Mdm2-p53 protein binding inhibition or by Bortezomib via proteasome function inhibition. Also, full-length p53 can be obtained via readthrough with G418 or gentamicin treatment. These two processes promote the increase in full-length p53 which will activate transcription of its target genes that will lead to tumor cell death.

4.2 Paper II

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

In order to find compounds able to induce nonsense mutant *TP53* readthrough we analyzed 47 000 compounds in the NCI-60 database at the National Cancer Institute aiming to select compounds that were more efficient in inhibiting growth of tumor cell lines carrying nonsense mutant *TP53* compared to cell lines carrying WT *TP53* or other mutations. This analysis identified 5-Fluorouracil (5-FU) as a potential readthrough inducer. We first validated its ability to induce nonsense mutant *TP53* readthrough in both HDQ-P1 cells with endogenous R213X mutant *TP53* and originally *TP53* null H1299 cells stably transfected with an R213X mutant *TP53* construct. We also examined the expression levels of a panel of p53 target genes after treatment with 5-FU and observed a stronger upregulation of the p53 target genes p21, Zmat3 (Wig-1) and Fas in H1299-R213X compared to H1299-EV cells, which was statistically significant for Zmat3 and Fas.

We then proceeded to study the mechanism of action by which 5-FU induced readthrough and found that one of its metabolites, 5-Fluorouridine (FUr), was more active in inducing nonsense mutant *TP53* readthrough in both HDQ-P1 and H1299-R213X cells. It is known that WT p53 protein can be stabilized by 5-FU. Therefore, to rule out the possibility that the increased full-length p53 observed was only due to stabilization of p53 induced by basal readthrough, we included two p53-independent systems to study readthrough induction: 1) HCT116 cells stably transfected with superfolder GFP (sfGFP) carrying a PTC (UGA, UAG or UAA) at 150 position (HCT116 sfGFP150X); and 2) H1299 cells stably transfected with a construct carrying the EGFP reporter followed by a PTC (*TP53* R213X with flanking sequences) and a FLAG tag to allow detection of readthrough. In both systems, FUr showed induction of translational readthrough. In case of HCT116 sfGFP150X, readthrough was observed for the sublines carrying UGA and UAG but not UAA. The results in these p53-independent systems provide further support for induction of true translational readthrough by FUr readthrough rather than p53 stabilization only.

Ribosome profiling (Ribo-seq) allows precise quantification of ribosomes at each codon of the mRNA. It is therefore an attractive methodology to study readthrough levels in a specific gene as well as canonical stop codon readthrough in a genome-wide fashion. We used this technique to examine any increase in ribosomes after the R213X PTC compared to pre-PTC in *TP53* mRNA upon treatment with FUr, and also to examine possible readthrough at canonical stop codons genome-wide. Indeed, we observed an increase in *TP53* readthrough upon FUr treatment, reaching similar levels as that after G418 treatment used as positive control, although the differences were not statistically significant. Readthrough into 3'UTR regions – canonical stop codon readthrough – was increased over control but less potently than R213X readthrough induced by FUr and canonical stop codon readthrough induced by G418.

When performing Ribo-seq, RNA-seq needs to be performed in parallel, and so we leveraged the results from these two methodologies to further explore biological effects in terms of both translatome and transcriptome changes. We could observe increased transcription and translation of different p53 target genes upon FUr treatment in H1299-R213X cells. Gene expression levels of specific p53 target genes were further examined by qRT-PCR, showing overall an increased expression in H1299-R213X compared to H1299-EV cells upon FUr treatment. Since p53 activation can induce apoptosis, we tested the cell death response to FUr in different H1299 sublines. First, we observed an increased caspase 3/7 cleavage activity in H1299-R213X compared to H1299-EV cells after treatment with FUr. Second, more Annexin V-positive cells were observed in a H1299 subline carrying the full p53 cDNA sequence with the R213X codon followed by the EGFP reporter than in a similar subline with only the p53 cDNA up to the R213X mutation (not producing full-length p53 after readthrough) followed by EGFP. These two types of experiments confirmed p53-dependent cell death induction upon FUr treatment.

It has previously been shown that FUr gets incorporated into mRNA, and this was confirmed by LC-MS in our study. Thus, we postulate that readthrough induction by FUr is mediated by incorporation into the mRNA at the uracil position of the PTC. This idea is supported by studies in *E. coli* reporting that incorporation of 5-FU into the mRNA could act as a cytosine and pair with guanine (Champe and Benzer, 1962; Rosen *et al.*, 1969). Thus, FUr incorporation in UGA PTC would permit the base-pairing with Arg tRNA (CGA codon) and the restoration of a WT full-length p53 at codon 213.

We finally performed experiments *in vivo* in order to examine if full-length p53 could be induced in a tumor in a living organism upon systemic administration (intraperitoneal injection). For these experiments, we inoculated H1299 cells stably transfected with *TP53* sequence carrying the R213X mutation followed by a FLAG tag to track readthrough induction. Following 5 days treatment with 5-FU or FUr, we could observe an increase in full-length p53 levels by Western blotting and by immunohistochemical staining in case of FUr.

The fact that 5-FU is still one of the most widely used chemotherapeutic drugs makes the results in this study very relevant for patients with tumors that have nonsense mutated *TP53*. If 5-FU/FUr induces translational readthrough and expression of full-length functional p53 in patients with tumors that carry *TP53* R213X, one would expect that these patients would respond better to 5-FU than patients whose tumors carry *TP53* missense mutations or other types of *TP53* mutations. Ideally, data from patients that have undergone treatment with 5-FU should be analyzed with regard to *TP53* status to test this. In addition, if tumor samples are available, the expression of full-length p53 after treatment could be explored *ex vivo*. Such analysis should give us better insights into the consequences and the importance of the *TP53* status for the response to 5-FU treatment.

This study also emphasizes the importance of continuously study drugs that are already approved by the medical agencies and used in the clinics for possible new purposes and to dig deeper into their molecular mechanism of actions. In case of 5-FU, the main mechanism

responsible behind its anti-tumor effect is thought to be the inhibition of thymidylate synthase (TS) and the DNA damage produced by the metabolite 5-Fluoro-2'-deoxyuridine. However, it is clear that the cytotoxicity of 5-FU is also to a large extent mediated by the metabolite FUr and its incorporation into RNA.

To sum up, we present FUr as a readthrough-inducing compound capable of reactivating R213X nonsense mutant *TP53*, as well as another nonsense mutated gene. The full-length p53 induced by FUr is functional in terms of transcriptional activity and cell death induction. Therefore, we propose a dual mechanism model for 5-Fluorouridine which includes its translational readthrough activity on nonsense mutant *TP53* followed by the stabilization and activation of full-length p53. This will lead to transcription of p53 target genes and the consequent tumor cell death induction (Figure 12). Finally, we could show readthrough induction *in vivo* in a human tumor xenograft mouse model.

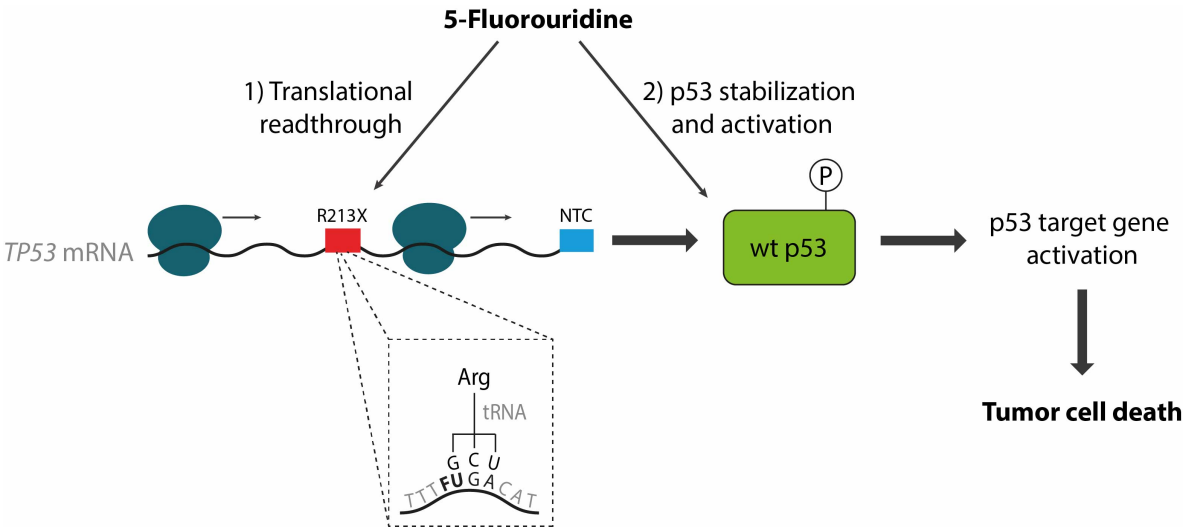

Figure 12. Proposed dual mechanism of 5-Fluorouridine to induce cell death. This model includes the induction of translational readthrough of nonsense mutant *TP53* due to incorporation of 5-Fluorouridine at the R213X PTC, allowing expression of full-length p53, which is then stabilized and activated in response to 5-Fluorouridine-induced cellular stress. This leads to transactivation of p53 target genes and tumor cell death.

4.3 Paper III

Pharmacological induction of translational readthrough of nonsense mutations in the retinoblastoma (RB1) gene

Translational readthrough induction has been studied for several tumor suppressor genes, such as *TP53*, *APC* and *PTEN*. However, there are other tumor suppressor genes for which the strategy of pharmacological induction of translational readthrough is also interesting when they carry nonsense mutations. The retinoblastoma (*RB1*) gene is mutated in not only retinoblastoma but also in for instance bladder cancer and lung cancer. As much as 25.6% of somatic mutations in *RB1* are nonsense substitutions. The *RB1* gene codes for the Rb protein that has a crucial role in regulation of G1 to S transition in the cell cycle by controlling E2F from binding to the promoters of its target genes. Its normal function is related to a correct control of the cell cycle as well as a correct chromosomal organization and preservation of the genomic stability. Dysfunctional Rb or complete lack of it allows uncontrolled cell division and proliferation and may ultimately lead to tumor development.

To our knowledge, reactivation of nonsense mutant *RB1* by induction of translational readthrough has not been reported. We therefore show for the first time that induction of this process is feasible for this gene. We demonstrate that G418 treatment in SW1783 cells carrying endogenous R579X nonsense mutant *RB1* or *RB1*-negative MDA-MB-436 cells transfected with R579X or Q702X nonsense mutant *RB1* constructs induces full-length Rb protein. As a control, we examined WT Rb protein levels upon G418 treatment in H1299 cells and observed no increase, providing further evidence that the effect observed in nonsense mutant *RB1* background is due to readthrough induction and not merely stabilization of full-length Rb that might have been produced by basal readthrough. Previous studies by others have indicated that translational readthrough inhibits the nonsense-mediated decay (NMD) pathway, which is in charge of degrading PTC-containing mRNAs, resulting in increased levels of the nonsense mutant mRNA *per se* upon readthrough induction. Therefore, the significant increase in *RB1* mRNA levels after G418 treatment in SW1783 cells provides further evidence for translational readthrough of nonsense mutant *RB1* in these cells.

Although it is difficult to examine Rb-dependent effects in SW1783 cells due to the lack of a suitable *RB1*-negative control cell line, we examined the levels of the E2F target gene thymidylate synthase (*TS*) upon G418 treatment. A significant decrease in *TS* mRNA levels was observed, which may be related to full-length Rb induction by readthrough.

Following a similar strategy as in Paper I, we tested combination treatments with G418 in order to potentiate nonsense mutant *RB1* readthrough. Based on a recently published study (Baradaran-Heravi *et al.*, 2021), we combined G418 with the cereblon E3 ligase modulator CC-90009 to examine readthrough of nonsense mutant R579X *RB1*. In both SW1783 and transiently transfected MDA-MB-436 cells with the R579X *RB1* mutant, a marked increase in full-length Rb could be observed upon combination treatment compared to single treatments.

The increase was more evident in the SW1783 cells that carry endogenous nonsense mutant *RB1*.

As a summary, this study shows as proof-of-concept that translational readthrough of the nonsense mutant tumor suppressor gene *RB1* can be achieved by treatment with the aminoglycoside G418, and that this effect is highly enhanced by combination treatment with CC-90009 (Figure 13). These results may have implications for future development of treatments for patients with nonsense mutant *RB1* tumors.

Finally, it is worth mentioning that functional Rb is not necessarily beneficial for the response to cancer therapy in general. The impact of Rb may vary depending on specific treatments and presumably also on other genetic alterations in the tumor. Therefore, it is very important to determine in which genetic background, and for which treatment, restoration of full-length functional Rb is beneficial in order to be able to select the most optimal treatment for each individual patient.

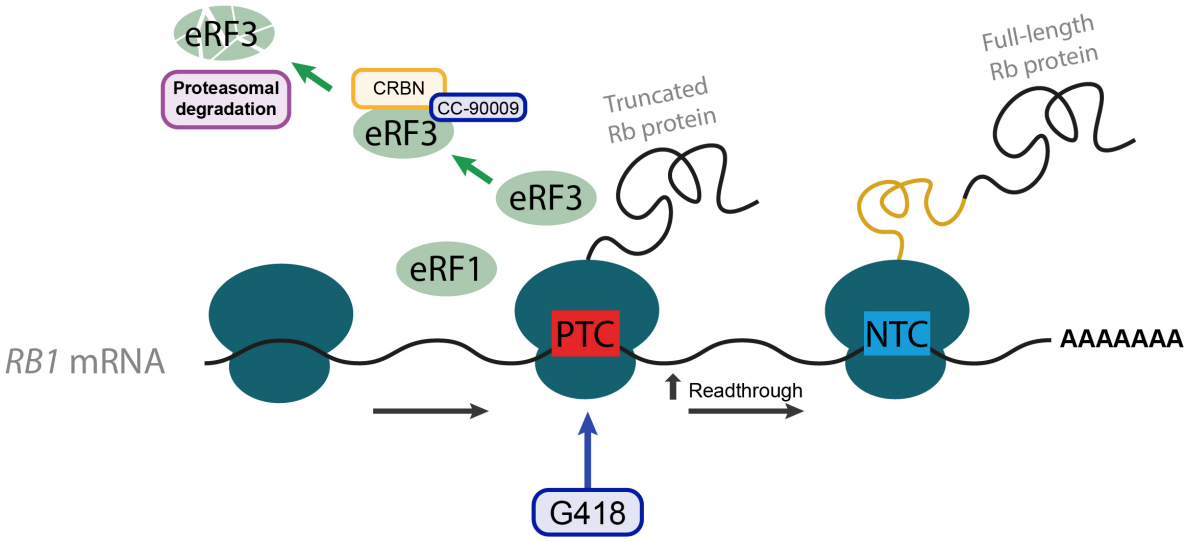

Figure 13. Model of the combined effect of G418 and CC-90009 treatment on induction of full-length Rb protein. G418 induces readthrough of nonsense mutated *RB1* acting at the decoding site of the ribosome. CC-90009 promotes the degradation of eRF3 by the E3 ubiquitin ligase $CRL4^{CRBN}$ (CRBN). These two processes together allow the efficient induction of full-length Rb protein.

4.4 Paper IV

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

In order to find novel potential readthrough inducers acting on nonsense mutant *TP53*, we performed a high-throughput chemical library screening based on the detection of p53 levels in HDQ-P1 cells carrying endogenous R213X *TP53* mutant. Starting with more than 30 000 compounds and after 3 rounds of screening, in HDQ-P1 cells plus validation by ELISA and Western blotting in the *TP53* null H1299 cells stably transfected with a *TP53* R213X construct containing the p53 coding sequence up to the R213X codon and followed by a FLAG tag (H1299p53-R213X-ΔC-FLAG) for detection of readthrough, we selected 2 compounds, C47 and C61, for further characterization as candidate readthrough inducers.

Both C47 and C61 induced full-length p53 in H1299 cells carrying the R213X mutation in the full *TP53* coding sequence (H1299p53-R213X-FLAG) and in H1299p53-R213X-ΔC-FLAG cells. As mentioned above, one important concern is that the expression of full-length protein after readthrough can be due to stabilization of the protein produced by low levels of basal readthrough. To test the ability of C47 and C61 to stabilize WT p53, HCT116 p53 WT cells were treated with these compounds at different concentrations. C61 did not stabilize WT p53 to any detectable extent at the concentrations tested and C47 had a weak stabilizing effect. In addition, the H1299 subline carrying a construct with the EGFP sequence followed by a PTC and a FLAG tag demonstrated clear readthrough induction capability of both compounds in a p53-independent model.

As the readthrough effect by C47 or C61 was relatively modest compared to that of G418, different combination treatment strategies were applied. We first combined either C47 or C61 with G418 and observed a marked increase in full-length protein upon combination of C47 and G418 but not with C61 and G418. This increase in readthrough induction was observed in H1299p53-R213X-FLAG cells, H1299p53-R213X-EGFP cells and HCT116 cells carrying a sfGFP protein with a PTC at codon 150, the PTC being UGA, UAG or UAA. In all tested systems the outcome of this combination treatment was similar. In contrast, when C47 or C61 were combined with the eRF degraders CC-885 or CC-90009, a marked increase in readthrough induction in R213X *TP53* was observed upon treatment with C61 combined with CC-885 or CC-90009 but not with C47 combined with the eRF degraders. Interestingly, while the combination of C61 and CC-885 induced higher readthrough levels in HCT116sfGFP150X cells, combination treatment with C61 and CC-90009 had no effect. Synergy upon both successful combination treatments was assessed with two different quantitative methods. For C47 and G418, flow cytometry was used to determine readthrough induction in the H1299-R213X cells carrying the EGFP reporter after the *TP53* sequence to detect readthrough induction. Synergy calculations confirmed the synergistic effect on readthrough induction with the combination of C47 and G418. A different quantitative method had to be applied to study synergistic effects of C61 and CC-885 or CC-90009, due to the autofluorescence of C61. Therefore, ELISA was used with the H1299p53-R213X-FLAG cells. As observed with the

previous treatments, combination of C61 with CC-885 or CC-90009 caused a synergistic increase in readthrough.

Finally, in order to test these compounds in another p53-unrelated system, their readthrough potential was examined in another tumor suppressor gene, *PTEN*. We used H1299 cells transfected with constructs representing three common *PTEN* nonsense mutations, R135X, R233X, and R335X, as well as U251 glioblastoma cells in which the same *PTEN* nonsense mutants had been introduced by a lentiviral vector. Since C61 did not promote any detectable increase in full-length PTEN, its further investigation was discontinued for this gene. However, promising results were obtained in all three different nonsense *PTEN* mutants upon treatment with C47 alone and even better induction was observed upon combination of C47 and G418. To elucidate the mechanism of action for C47 and C61 to induce readthrough, more experiments are required. However, we can speculate that the reason why C47 does not synergize with eRF inhibitors could be that it decreases eRF1 levels by itself. Regarding C61, we could not observe any decrease in eRF factors nor binding affinity for DNA or RNA. Therefore, this compound may induce readthrough by a different mechanism than that of aminoglycosides and eRF modulators.

As a conclusion, our chemical library screening allowed us to find two potential novel readthrough-inducing compounds, C47 and C61. Both compounds induced rather weak levels of readthrough which were enhanced by combination with G418 in case of C47 and with CC-885 or CC-90009 in case of C61 (Figure 14). Readthrough induction upon single and combination treatment was tested in several models including nonsense mutant *TP53*, sfGFP, EGFP reporter followed by a PTC and a FLAG tag and finally in *PTEN*. With some variation in the results, it can be concluded that in one system or another and with one combination or another, the combinations tested caused synergistic effects on readthrough induction.

Future experiments are required to further elucidate the mechanism of action of these compounds and the biological effects they induce in the different models used. Preliminary results showed no effect on p53 target gene levels, but different readouts may be chosen in the future to examine p53- or PTEN-dependent biological effects. This study contributes to the translational readthrough field by presenting two novel compounds to be further explored as potential readthrough inducers in different genes and models.

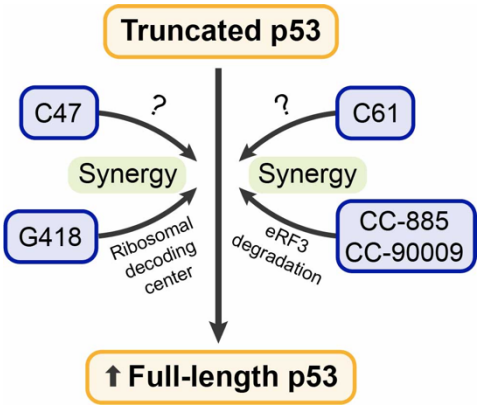

Figure 14. Model of different combination treatments to enhance readthrough of nonsense mutated *TP53*. C47 and C61 have, so far, unknown mechanisms of action for readthrough induction. C47 synergizes with G418 and C61 with CC-885/CC-90009 for *TP53* readthrough induction.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The studies presented in this thesis have several clinical implications that could be divided into more immediate implications (**Paper II**) and more long-term ones (**Paper I**, **Paper III** and **Paper IV**).

The main conclusion of **Paper I** was that the usage of combination treatments of aminoglycosides with other compounds can be an interesting strategy to potentiate the induction of translational readthrough. This is because the toxicity induced by aminoglycosides could be potentially decreased by the administration of lower concentrations of these compounds, but the readthrough-inducing effect could still be maintained and even potentiated when combined with either Mdm2-p53 inhibitors (Nutlin-3a and MI-773) or the proteasome inhibitor Bortezomib. These combinations should be explored further in *in vivo* experiments in order to validate that the p53 readthrough efficacy is maintained or even increased with lower aminoglycoside doses in combination with the indicated agents. In addition, the toxicity levels in mice should be explored upon combination treatments and should be compared to higher doses of aminoglycosides alone. If successful results *in vivo*, this study could have important clinical implications for treatment of patients with tumors carrying nonsense mutant *TP53*.

Paper II has more immediate impact since 5-Fluorouracil (5-FU) is already being used in the clinic. From this study, we could conclude that 5-FU is capable of inducing nonsense mutant *TP53* readthrough via its RNA-incorporating metabolite 5-Fluorouridine (FUr). In addition, we could observe p53-dependent biological effects upon treatment with FUr, which included upregulation of p53 target genes and induction of cell death. Since 5-FU is still used for treatment of various cancers, these results have several clinical implications:

1) Patients with *TP53* nonsense mutated tumor cells may respond better to 5-FU treatment.

2) Treatment with the metabolite FUr directly could be investigated further for patients with tumors carrying nonsense mutant *TP53*.

3) The readthrough-inducing ability of FUr could be tested in other nonsense mutated tumor suppressor genes.

Future directions of this study would be the analysis of clinical data to examine if the *TP53* status of the tumor correlates to better or worse prognosis when the patient is treated with 5-FU. This analysis should be feasible to perform as the parameters needed are the chemotherapeutic treatment given to the patient (in this case 5-FU), p53 status of the tumor cells (*TP53* sequencing of the tumor required) and outcome of the patient in terms of survival endpoints: 1) overall survival (length of patients' life), 2) progression-free survival (length of patients' life without progression of the tumor) or 3) relapse-free survival (length of patients' life without cancer relapse) or in terms of efficacy endpoints: 1) response rate (amount of patients in who the tumor has reduced), 2) complete response (complete disappearance of the tumor) or 3) duration of response (time during which the tumor responds to the treatment).

Paper III allowed us to conclude that nonsense mutations in another tumor suppressor gene, *RB1*, can also be targeted for translational readthrough by the aminoglycoside G418. This study is a proof-of-concept of *RB1* readthrough induction. Many nonsense mutant genes have been reported to undergo this process upon treatment with aminoglycosides or with other readthrough-inducing compounds. Therefore, we decided to go further and investigate different ways of potentiating *RB1* readthrough induction. Following a similar strategy as in **Paper I**, we combined G418 with a more recently reported readthrough-inducing compound, the eRF3 degrader CC-90009. Combination treatment of these two compounds resulted in a robust induction of full-length Rb in SW1783 cells carrying endogenous nonsense mutant *RB1*. The two compounds induce readthrough via different mechanisms – G418 most likely by binding to the ribosomal decoding center and CC-90009 by promoting degradation of eRF3 – and so they may presumably act in a complementary manner to induce strong readthrough. Future studies should include the validation of the readthrough-inducing effect of G418 alone and in combination with CC-90009 on other nonsense *RB1* mutations. In addition, it would be interesting to test if this combination treatment is less toxic *in vivo* than the combination treatment proposed in **Paper I**. Also, other possible combination treatments in the nonsense mutant *RB1* setting could be explored further, for instance combination of a readthrough inducer that generates full-length Rb and a compound that promotes Rb protein activity, such as the CDK4/6 inhibitor Palbociclib. Finally, reactivation of nonsense mutant *RB1* by translational readthrough in a context where this is expected to enhance sensitivity to already approved therapy, e.g. hormone therapy, immunotherapy or targeted therapy with a relevant kinase inhibitor is an interesting idea that should be explored further in the future.

Among the four studies presented in this thesis, **Paper IV** is the one that is the least advanced in terms of its possible clinical relevance. This study allowed us to identify two novel potential readthrough-inducing compounds, C47 and C61, by screening chemical libraries. The two compounds were able to induce readthrough of nonsense mutant *TP53* and in case of C47 also of nonsense mutant *PTEN*. One of the most interesting findings was the synergistic induction of readthrough observed when combining C47 with G418, or C61 with the eRF3 degraders CC-885/CC-90009. The mechanism of action of both compounds clearly requires further investigation. The downstream effects of full-length PTEN protein induced by C47 alone or in combination with G418 should also be explored, including its effect on the AKT pathway as well as the impact on cell proliferation and survival in relevant cellular *in vitro* and mouse *in vivo* models. Moreover, the ability of both compounds to induce readthrough of nonsense mutations in other tumor suppressor genes, for example *APC*, should be an interesting area of investigation in the future.

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