Investigating radiation therapy treatments with cell cycle and DNA repair inhibitors in triple-negative breast cancer

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Abstract

Triple-negative breast cancers (TNBCs) are the most aggressive and the deadliest of all breast cancer molecular subtypes. Patients with TNBCs have limited treatment options and their life-threatening condition is most often clinically unfavorable. To date, there are no approved treatments nor effective or approved targeted therapy against TNBC, highlighting a clear medical gap and unmet clinical need for these deadly tumors. Our lab previously showed that inhibition of cyclin-dependent kinase 4/6 (CDK4/6) gene expression or activity, using a pharmacological inhibitor (palbociclib) led to decreased expression of DNA repair genes, such as Rad51 and BRCA1.

In addition, palbociclib was previously shown to sensitize ER+/HER2- breast cancer cell lines to radiation therapy (RT) and hormone receptor-positive (HR+) breast cancer can respond well to RT followed by palbociclib *in vitro* and *in vivo*. Palbociclib may thus act synergistically with RT, according to these preclinical evidence. RT has been a standard locoregional treatment option for breast cancer. Postmastectomy radiation (PMRT) is applied when women have locally advanced breast cancer, and local or regional recurrence (LRR) after radiation treatment has been shown to cause resistance and reduce overall survival. However, the potential use of CDK4/6 inhibition (CDK4/6i)/RT combination therapy in TNBC has not been investigated yet and will be the focus of this thesis.

High RAD51 expression is observed in basal breast cell lines, such as TNBC and is associated with resistance to RT. As CDK4/6 regulates RAD51 expression, we investigated the combination treatment effect of CDK4/6 knockout (KO) cell lines with RT. For this we generated single KO TNBC cell lines for CDK4, CDK6, and Rad51, using CRISPR/Cas9 gene editing technology. We then assess the effects of CDK4/6i combined with RT *in vitro* as well as *in vivo*, using preclinical models of TNBC.

Our results show that CDK4/6 KO can effectively restore TNBC sensitivity to RT, as evidenced by reduced cell viability *in vitro* and inhibition of tumor growth *in vivo*. We also found that RAD51 KO can sensitize TNBC cells to DNA-PK inhibitor. This study thus highlights potential treatments combining with RT that sensitizes targeted therapies and provides a rationale for developing novel therapies for TNBC.

Résumé

Les cancers du sein triple négatifs (CSTN) sont les plus agressifs et les plus mortels parmi tous les sous-types moléculaires du cancer du sein. Les patientes atteintes de CSTN disposent de peu d'options de traitement et leur état de santé est le plus souvent cliniquement défavorable. À ce jour, il n'existe ni traitements approuvés ni thérapies ciblées efficaces contre le CSTN, soulignant un besoin médical clairement identifié et non satisfait pour ces tumeurs possiblement léthales. Notre laboratoire a précédemment montré que l'inhibition de l'expression ou de l'activité du gène de la kinase dépendante des cyclines 4/6 (CDK4/6), en utilisant un inhibiteur pharmacologique (palbociclib), entraînait une diminution de l'expression des gènes de réparation de l'ADN, tels que Rad51 et BRCA1.

De plus, il a été démontré que le palbociclib sensibilise les lignées cellulaires du cancer du sein ER+/HER2- à la radiothérapie (RT) et que les cancers du sein positifs aux récepteurs hormonaux (HR+) pouvaient bien répondre à la RT suivie du palbociclib *in vitro* et *in vivo*. Selon ces preuves précliniques, le palbociclib pourrait donc agir de manière synergique avec la RT. La RT est une option de traitement locorégional standard pour le cancer du sein. La radiothérapie post-mastectomie (PMRT) est appliquée lorsque les femmes ont un cancer du sein localement avancé, et la récidive locale ou régionale (LRR) après le traitement par radiothérapie a été montrée comme causant une résistance et réduisant la survie globale. Cependant, l'utilisation potentielle d'une thérapie combinée d'inhibition de CDK4/6 (CDK4/6i) et de RT dans le CSTN n'a pas encore été étudiée et sera l'objet de cette thèse.

Mais encore, comme pour les CSTN, une expression élevée de RAD51 est observée dans les lignées cellulaires basales du sein et est associée à une résistance à la RT. Comme CDK4/6 régule l'expression de RAD51, nous avons étudié l'effet de traitement combiné des

lignées cellulaires knock-out (KO) CDK4/6 avec RT. Pour cela, nous avons généré des lignées cellulaires KO TNBC uniques pour CDK4, CDK6 et Rad51, en utilisant la technologie d'édition génomique CRISPR/Cas9. Nous avons ensuite évalué les effets de la combinaison CDK4/6i et de la RT *in vitro* ainsi qu'in vivo, en utilisant des modèles précliniques de CSTN.

Pour finir, nos résultats montrent que CDK4/6 KO peut restaurer efficacement la sensibilité des CSTN à la RT, comme en témoignent la viabilité cellulaire réduite *in vitro* et l'inhibition de la croissance tumorale *in vivo*. Nous avons également constaté que le KO de RAD51 pouvait sensibiliser les cellules TNBC à un inhibiteur de DNA-PK. Cette étude met donc en évidence des traitements potentiels combinés avec la RT qui sensibilisent les thérapies ciblées et fournit une justification pour le développement de nouvelles thérapies pour le CSTN.

Acknowledgements

First, I would like to thank my supervisor, Prof. Jean-Jacques Lebrun, for giving me the opportunity to do research in his laboratory and providing me with knowledge and strength to pursue this study. I must say that fate is amazing to lead me to meet such a great teacher, and his teaching has been the best gift in my life. Under his guidance, I have learned how to think independently and design a scientific research project, which is exactly what I had hoped to gain from pursuing graduate school. I am also very grateful to you for being my anchor when things were difficult for me. I can always share various things with you, which makes me feel once again that you are the best teacher I have ever met!

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I am also thankful to my committee member, Dr. Torban Elena, who provided me with the direction that I need to explore in my study, , and to staff members of the School of Medicine and Health Sciences for their ongoing support during my studies in the Experimental Medicine program.

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Preface (Contribution of the Authors)

Throughout the writing process of my thesis, my supervisor, Dr. Lebrun, reviewed and provided suggestions, while I was responsible for writing the entire thesis. I contributed to data analysis and conducted the experiments under Dr. Lebrun's supervision. Dr. Meiou Dai assisted in designing the experiments and supervised my preparation for the animal experiments.

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AZD7648 treatments

List of Abbreviations

AIs	aromatase inhibitors
AURKB	Aurora Kinase B
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia telangiectasia and Rad3 related
BRCA2	breast cancer 2
BCT	breast conservation therapy
BRIP	BRCA1 Interacting Protein
BLBC	basal-like breast cancer
BMPR1B	Bone Morphogenetic Protein Receptor Type 1B
Bub	Budding uninhibited by benzimidazole
Cas	CRISPR-associated proteins
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CKI	CDK inhibitors
CHEK2	Checkpoint kinase 2
CRISPR	clustered regularly interspaced short palindromic repeat
CSC	cancer stem cells
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DDR	DNA damage response
DSB	double-strand break
EMI1	early mitotic inhibitor 1
EMT	epithelial–mesenchymal transition
ER	Estrogen receptors

ET	endocrine therapy
HF	hypofractionated
HDR	homology directed repair
HFRT	hypofractionated radiation therapy
HER2	human epidermal growth factor receptor 2
HR	homologous recombination
HRD	homologous recombination deficiency
INK4	Inhibitors of CDK4
LRR	locoregional recurrence
Mad	Mitotic arrest-deficient
MDM2	murine double minute 2
MEC1	Mitosis Entry Checkpoint 1
MYT1	Myelin Transcription Factor 1
NBS1	Nijmegen breakage syndrome
NACT	neoadjuvant chemotherapy
NHEJ	non-homologous end joining
NSCLC	non-small-cell lung cancer
NST	neoadjuvant systemic therapy
OS	overall survival
PR	progesterone
PAM	protospacer-adjacent motif
PAM50	Prediction Analysis of Microarray 50
PARP	Poly ADP-ribose Polymerase
pCR	pathologic complete response
PFS	progression free survival
PI3K	Phosphatidylinositol 3-kinase

PIKK	phosphatidylinositol 3-kinase-related kinase
PALB2	Partner and localizer of BRCA2
PMRT	post-mastectomy radiation therapy
RB	retinoblastoma
SAC	spindle assembly checkpoint
SSBs	Single-strand breaks
Tyr15	Tyrosine 15
TNBC	Triple-negative breast cancer
WBRT	whole breast radiation therapy

Definitions

Breast cancer originates in the breast's lobules or milk ducts and accounts for about 10.4% of cancer cases in women globally. It is the second most common cancer in women after lung cancer and also the second most common cause of cancer death among Canadian women. [1] (Canadian Cancer Society) Breast cancer is classified into three main subtypes: hormone receptor-positive/ERBB2-negative (70%), ERBB2-positive (15-20%), and triple-negative (15%), which lacks all three molecular markers. These subtypes influence treatment options and prognosis. [2] In 2004, the global death toll from breast cancer was estimated at 519,000. [3]

Cell cycle, a four-stage event which includes G1, S, G2, and M, is the process of cells division. By this process, a single cell makes 2 new cells (termed daughter cells) that will divide again. G1 is the time that cells prepare to divide, followed by S phase which is DNA synthesis. In S phase, 46 chromosomes are duplicated. After DNA is double copied, cells enter the G2 stage where it prepares for division. M means mitosis and it is the phase that cells really divide into two, with each cell having identical genetic material. The primary underlying cause of cancer is the persistent, uncontrolled proliferation of cancer cells. [1]

The checkpoint is intended as a quality control check: you are not allowed to move on to the subsequent process or event until the one you are now engaged in is finished. [4] Cell cycle checkpoints are important for cells to monitor the order of cell cycle and ensure its fidelity. Proteins that drive cell cycle to the next stage are called cyclin-dependent kinases (CDKs). [5]

Cyclin-dependent kinases (CDKs) are regulatory serine/threonine protein kinases which phosphorylate essential selected proteins for further events like DNA synthesis. In cell cycle, it is impossible to return, and stage transition is controlled strictly by CDKs. Cyclin protein can activate CDKs, and different cyclins associate with specific CDKs. We have known that D type

cyclins bind to CDK4 and CDK6, and that this complex will further phosphorylate the protein pRB and promote the progression of the cell cycle into the G1 phase. [6]

Cancer cells use these checkpoints to escape from the immune system. Genomic integrity is protected by several mechanisms like cell cycle checkpoints and DNA damage repair pathway, and it maintains both somatic and germ cells. [7] Loss of checkpoint functions are common in cancer. In the case of p21, which is an inhibitor of CDK, will phosphorylate pRB and initiate E2F transcription factor for entering into S phase. It is a tumor suppressor gene and loss of p21 expression is common in human breast cancer. [8] TNBCs commonly contain mutations or deletions in the RB1 gene, impairing the integrity of cell cycle through the Rb/E2F/CDK4/6 pathway. [9]

Radiation therapy in cancer is a treatment that uses high doses of radiation to kill cancer cells, and it is usually combined with other treatments like chemotherapy. For breast cancer, following breast conserving surgery and in individuals with locally advanced breast cancer following mastectomy, radiotherapy is administered. [10] In addition, a combination of lumpectomy and radiation therapy is also used for early-stage breast cancer. [3] As the molecular classification of breast cancer progresses, treatments for different breast cancer subtypes become more specific.

Genetic screening is a tool used to find specific genetic components linked to a desired trait. It is a type of phenotypic screen to identify a subgroup of people who have the disease. There are two types of genetic modulation used in screenings: gain-of-function and loss-of-function. In particular, the advent of CRISPR (clustered regularly interspaced short palindromic repeats) has offered a powerful method for assessing gene function in a high-throughput format. [11]

RAD51 recombinase, a key protein in the repair of DNA double-strand breaks, has been linked to cancer patient outcomes. It functions by locating and invading homologous DNA sequences, playing a crucial role in maintaining genomic stability. [12] RAD51 has been found to be

upregulated in various malignant solid tumors. [13] In invasive breast cancer, patients with absent RAD51 nuclear expression have shown poorer prognostic indicators and shorter survival times. [14]

Since TNBC is a deadly and non-treatable breast cancer, we aim to find genes which confer radio resistance and sensitivity to this type of cancer. The following section discusses the overview of the CDK4/6 inhibitors, which pause the cell cycle in G1, and radiation therapy, which plays an important role in cancer treatments. [15] This project aims to discover a new treatment combination using radiation therapy with CDK4/6 inhibitors and RAD51 KO in TNBC cell lines and mice, with the intention of curing TNBC patients.

CHAPTER I: Review of the literature

1.1 Molecular subtypes of breast cancer and characteristics of TNBC

Due to the development of molecular technology, TNBC can now be classified into basal-like breast cancer (BLBC) and non-basal-like breast cancer which includes Claudin-low breast cancer, HER2-enriched breast cancer, Luminal A breast cancer, and Normal-like breast cancer. It has reached an agreement that TNBC is a heterogenous disease with different molecular subtypes these years. [16] Despite the fact that basal-like and triple-negative are sometimes used interchangeably, there is a 25–30% discrepancy between molecular profiling and the standard immunohistochemistry classification using estrogen (ER), progesterone (PR), and HER2 (EGFR2) as markers. [17]

All breast cancers develop from a genetically normal precursor that has accumulated oncogenic hits. Breast cancers might vary depending on the type of genetic alterations present or the origin cell's characteristics. [18] Prediction Analysis of Microarray 50 (PAM50), which divides tumors into five intrinsic subtypes (luminal A, luminal B, HER2-enriched, basal, and normal-like) based on mRNA expression of a panel of 50 genes, has increased the understanding of breast cancer. [19] There are two ways to determine subtype: gene-based assays and IHC-based markers. [20]

Luminal A: The 13th St Gallen International Breast Cancer Conference (2013) determined that this subtype is estrogen receptor positive (ER+), progesterone receptor (PR) ≥ 20%, human epidermal growth factor receptor 2 (HER2) negative, and expresses a cellular marker for proliferation Ki67 < 14% with low recurrence rate based on gene-based assays. [20] These cancer cells have the differentiated goblet-like/enterocyte subtypes, and also surprisingly have the poorly differentiated stem-like heterocellular subtype including stem-like tumors (45%), stem-

like tumors (17%), enterocyte (15%), inflammatory (12%), and transit-amplifying subtypes (11%). [16,21]

Luminal B: The luminal B subtype is the most prevalent, making up about 40% of all breast cancers. [22] This subtype can be divided into: HER2- and HER2+. The HER2- subtype is defined as ER+, HER2-, and at least one of the following: Ki67 ≥ 20% or PR negative, while the HER2+ is defined as ER+, HER2+, and any levels of Ki67 and PR. [20] Luminal B has more aggressive clinical behavior and higher percentages of lymph node involvement, which also shows higher Ki-67 level, compared with luminal A. [23,24]

HER2+: About 15–20% of breast cancers are classified as HER2 positive, which harbors overexpression of HER2 protein as determined by immunohistochemistry status (IHC3+) or by fluorescence in situ hybridization (FISH) detection methods measuring HER2 copy number (≥6.0) or a ratio of HER2/CEP17 (≥2.0). [25-27] The prevalence of brain metastases can occur in up to 50% of patients with HER2+ metastatic breast cancer. [25]

Basal-like: The basal-like subtype lacks expression of ER, PR, and HER2, which is so called triple-negative disease. Because of this, despite the frequent interchange of the terms basal-like and triple-negative, there is a 25–30% discrepancy between gene expression profile and protein expression of immunohistochemistry. [17,28] In one study, a basal-like gene profile was present in 71% of triple-negative breast cancers, but only in 29% of cases. [28] The basal-like subtype also expresses high level of cytokeratins CK5/6, CK14, and CK17, and has been demonstrated to show overexpression of p16 and p53 immunoreactivity, as well as lack of pRB, in roughly 30% of basal-like breast cancers. [29]

Normal breast-like: This subtype makes up between 5% and 10% of all breast cancers. [30] Normal-like and luminal A tumors show the same condition of basic IHC markers, which are ER or PR+, HER2-, Ki67-, although their expression patterns differ as the normal-like tumors are

similar to the normal breast profiling but with poor prognosis. [31] Since they are deficient in the expression of ER, PR and HER2, they can be classified as *triple-negative* and typically do not react to neoadjuvant chemotherapy. [30] Adipose tissue and other non-epithelial tissue genes are highly expressed in this group. [30,32] However, the existence of the normal breast-like subtype is debatable, and some scientists think it may be an artifactual mistake brought on by the sample's high proportion of normal breast tissue compared to its low tumor concentration. [16]

Claudin-low: The claudin-low subtype is a subset of TNBC with reduced claudin gene expression and has characteristics of stem cell- and epithelial–mesenchymal transition (EMT)-like features. [17,33] Claudin-low tumors are the least prevalent subtype overall (prevalence ~12–14%) [34] and have been associated with poor prognosis. It is also believed to be triple-negative, yet it is distinguished from basal-like tumors. [17,35] Claudin-low tumors have higher Ki-67 than luminal A and normal-like tumors, but lower than in basal-like tumors. [33]

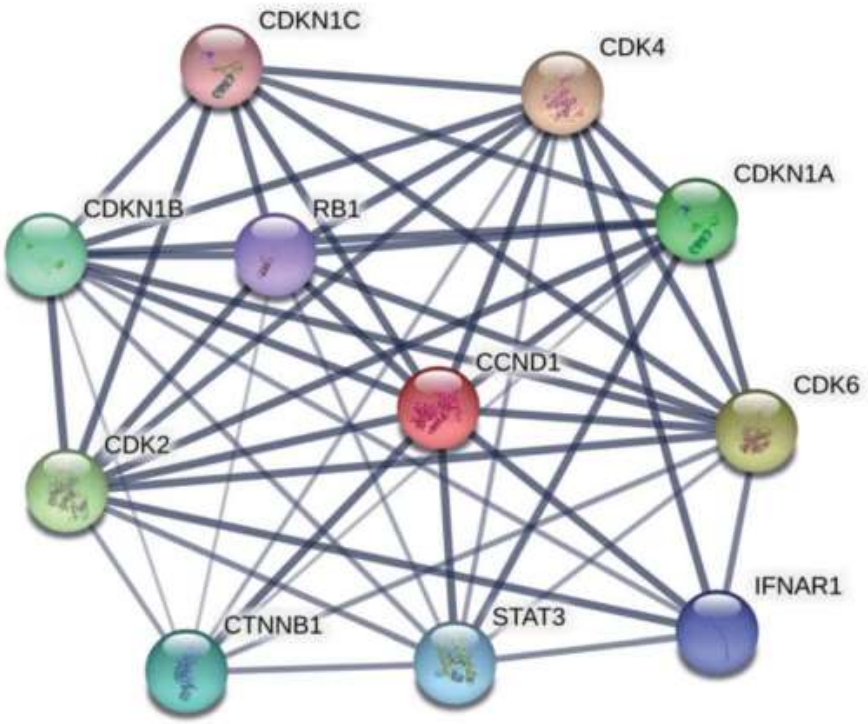

Figure 1: The protein–protein interaction network of cyclin D1 with retinoblastoma and other families of cyclin. A dark line of connection in the network indicates the strong binding interaction vice versa. [36]

Overview of CDKs

1.2 The discovery of Cyclin-dependent Kinase (CDKs)

CDKs were discovered a long time ago. Genetic and biochemical investigations in model organisms like yeasts and frogs led to the initial discovery of CDKs. In genetic searches for *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* mutants with flaws in the cell division cycle, the original member of the Cdk family (currently called Cdk1) was found. It was discovered that this protein, known as Cdc2 in S. pombe and Cdc28 in S. cerevisiae, is necessary for cell-cycle progression. [37] Human CDKs were then cloned soon after by several independent groups. Currently, there are 20 CDKs and 29 cyclins identified in human cells [38]

1.2.1 Cyclin-dependent kinase (CDK) regulation

During G1 phase (CDK4, CDK6 and CDK2), S (CDK2), G2 and M (CDK1), CDKs phosphorylate their downstream target proteins. It is necessary for the three D type cyclins (cyclin D1, cyclin D2, and cyclin D3) to bind to CDK4 and CDK6 for entry into G1. [6] Cyclin E, another G1 phase cyclin, collaborates with CDK2 to control the transition from G1 to S phase. [39] Cyclin A binds with CDK2, which is necessary during S phase. [40] Cyclin A/CDK2 specifically phosphorylates mammalian CDC6 and regulates CDC6 function. [41] More than 20 cyclins or cyclin-like proteins have been discovered, many of which are unknown in terms of their function. The cyclins have conserved “cyclin box” regions which have around 150 amino acid

residues and can be recognized structurally. The residues are organized into 5 helical regions and are crucial for binding partner proteins like CDKs. [42]

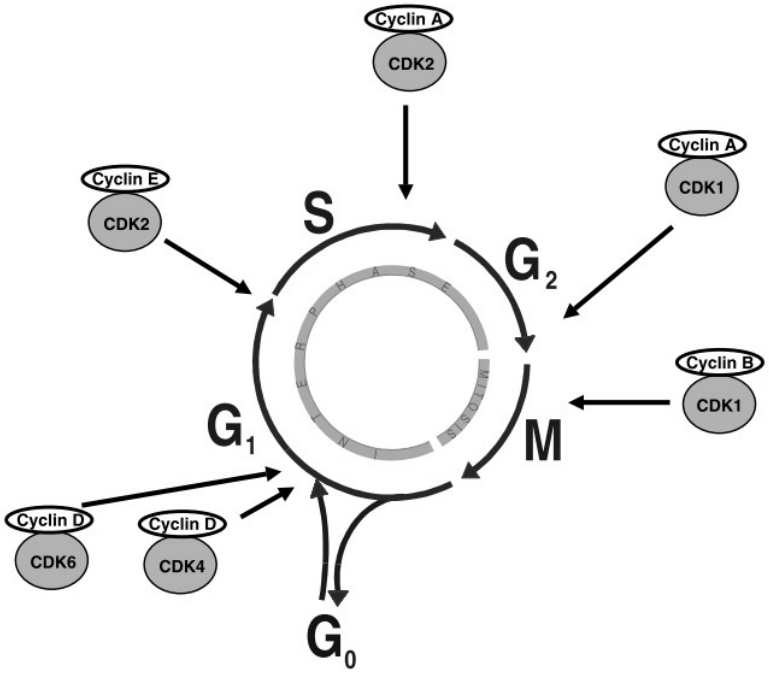

Figure 2: The stages of the cell cycle. The site of activity of regulatory CDK/cyclin complexes is also indicated. [6]

Along with CDK binding, CDK needs phosphorylation to be activated. During the S and G2 phases, CDK2 is phosphorylated on threonine-14 and tyrosine-15 due to the MYT1 and WEE1 kinases, while Cdc25 dephosphorylates it. [43] [44] [45] In contrast, it is widely known that inhibition of CDK phosphorylation plays a crucial role in causing cell cycle arrest in response to checkpoint activation. Tyr-15 phosphorylation plays a key role in the regulation of the unperturbed cell cycle by directly regulating the entry into mitosis and inhibiting CDK1 activity. [46]

Cell cycle inhibitory proteins, known as CDK inhibitors (CKI), can decrease CDK

activity by binding to CDK alone or to the CDK-cyclin complex. CDK inhibitors have been researched since the 1990s. Flavopiridol, roscovitine, and other pan-CDK inhibitors are part of the first generation of CDK inhibitors. Most of the first generation pan-CDK inhibitors were not successful in clinical trials as they were highly toxic. [47] Two families of CDK inhibitors, Ink4 and Cip/Kip, are involved in tumor suppression and cellular senescence. The INK4 family includes p16INK4a, p15INK4b, and p18INK4c, which restrains G1/S progression by binding CDK4 and CDK6. [48] Three CKIs make up the Cip/Kip family: $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. The Cip/Kip family consists of highly homologous individuals who share about 50% of their sequences. [49] It has also been shown that $p57^{Kip2}$ participates in the stability of CDK4-cyclin D complexes *in vivo*. [50]

The cyclin-dependent kinase inhibitor p21 (Cip1/Waf1/CDKN1A) is encoded by the *CDKN1A* gene, which was initially identified as p53's transcriptional target. [51] It has been demonstrated that p21 mediates p53-induced G1 cell cycle arrest. [52] As a result of p53 activation, $p21^{Cip1}$ levels are increased in response to DNA damage and genotoxic stress. [49] There is a p53-binding site on the p21 gene promoter, which enables p53 to activate the p21 gene transcriptionally. [6] By affecting either Rb phosphorylation or degradation, the p21 protein has been proven to be both a positive and negative regulator of Rb. P21 expression is increased after p53 is activated, followed by RB-E2F complex formation and downregulation of several cell cycle genes. RB and p53 proteins are frequently found to be inactive in different types of tumors. [53]

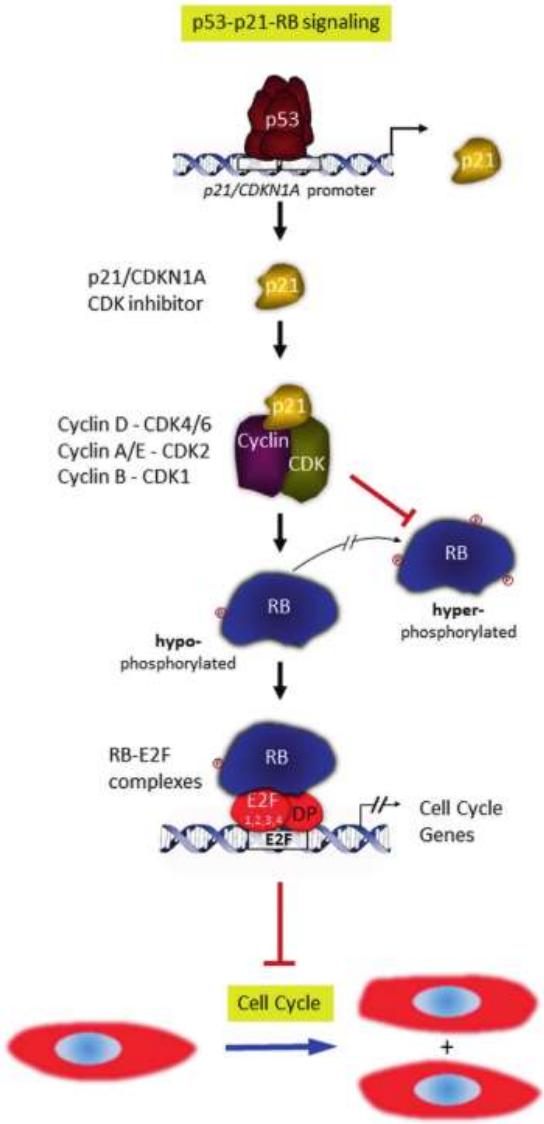

Figure 3: p53-p21-RB signaling. Following p53 activation, transcription of p21/CDKN1A is strongly induced as a direct target of p53. The cyclin dependent kinase inhibitor p21 then blocks activity of several cyclin-CDK complexes. This results in hypophosphorylation of RB, which fosters RB-E2F complex formation and their binding to E2F sites in target promoters. Many target genes are downregulated as a consequence of this mechanism of indirect p53-dependent transcriptional repression. As most repressed genes are involved in cell cycle progression, their downregulation causes cell cycle arrest. [53]

Different cell cycle-regulating proteins' intracellular localization also aid proper cell cycle progression. Interesting studies have shown that cytosolic localization and p27 down-regulation are directly linked to poor prognosis for several cancer types. [54] Additionally, p27 phosphorylation is markedly increased in many proliferative diseases, which has historically been associated with its cytoplasmic localization. [55]

1.2.2 Cell cycle quality control: checkpoints and restriction points

The restriction point, first mentioned by Arthur Pardee in 1974, is a term used to describe the time during G1 when a normal cell decides it is going to enter S phase and stops needing growth factors. The RB, p53, and CDK inhibitors' restraining effects significantly contribute to the establishment of the restriction point and set it apart from both G0 and the G1/S deoxyribonucleic acid (DNA) damage checkpoint. [56] Cell cycle checkpoints protect transformed cells from both internal and external stress, such as genotoxic stress and replicative stress, preserving the genome's integrity. In cancer patients, these checkpoints are usually dysregulated. [57]

DNA damage is one of the causes of cell cycle arrest; for instance, the DNA damage response proteins ATM and ATR can cause G1 and S/G2 arrest through the ATM-CHK2 and ATR-CHK1 pathways, respectively. Since their capacity to repair DNA damage is already impaired, cells that have homologous recombination deficiency (HRD) may be especially susceptible to checkpoint inhibitors that prevent the cell cycle checkpoints from appropriately initiating cell arrest. [58] Compounds like ATM/ATR/CHK1/CHK2/WEE1 inhibitors have been developed for treating p53 mutated tumors due to their impaired G1/S checkpoint. [59] The G1 checkpoint is a crucial component of the cell cycle, and p53 is a key regulator of this process. [58] Cancer cells are known to be dependent on a functional G2-M arrest when the tumor suppressor p53 is lost, as it eliminates the G1/S cell cycle checkpoint. [60] Mdm2 is important in regulating p53: the p53 protein is degraded by the MDM2 oncoprotein through ubiquitination, which in turn negatively affects p53 activity. [6,61,62]

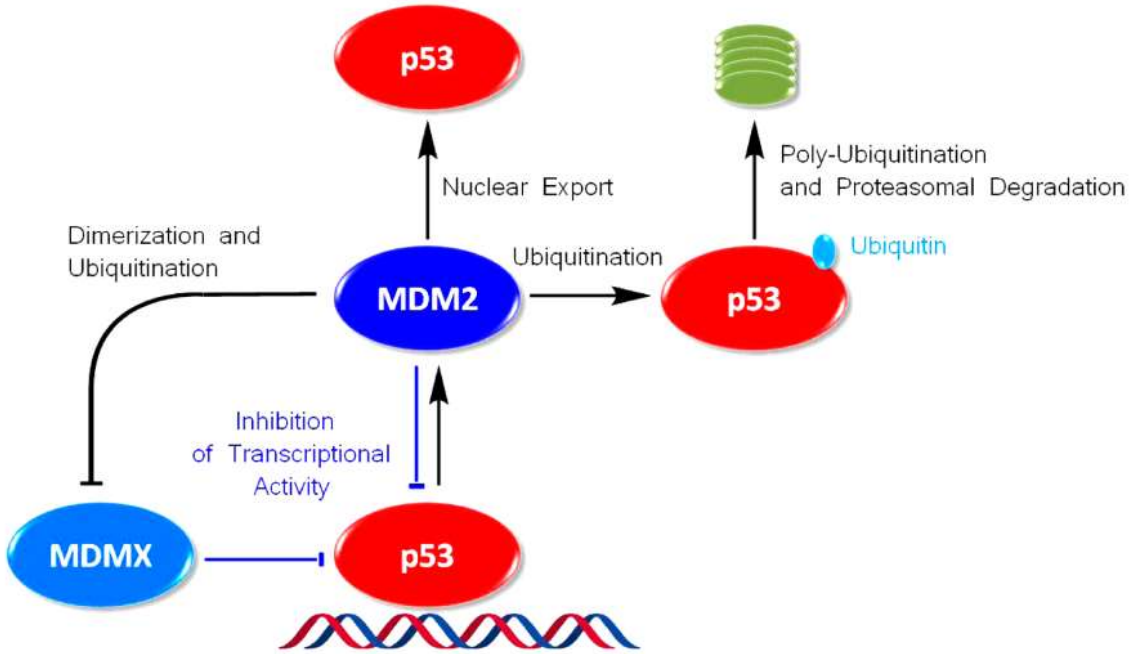

Figure 4: Inhibition of p53 by MDM2 and MDMX. [62]

In budding yeast, it has been shown that S phase checkpoint can regulate SUMOylation. [63] The S phase checkpoint is essential for the entire S-M control. Cells lacking Mec1/ATR, the DNA damage checkpoint initiator, can still keep damaged chromosomes segregated. [64,65]

CDK1 plays an essential role in G2/M phase transition and activation of homologous recombination (HR) DNA repair pathway. [66] When mitosis begins, one of the crucial processes that greatly raises CDK1's kinase activity is its attachment to cyclin B1. [67] The following step is dephosphorylating the site of Thr14 and Tyr15 through cdc25, which is a phosphatase. These actions are crucial for mitotic entry. [66] A report has confirmed that Menadione, used to treat hypoprothrombinemia and as a dietary supplement, can cause G2/M cell cycle arrest in gastric cancer cells by decreasing CDK1, cyclin B1 and CDC25C protein levels in a dose-dependent manner. [68]

The spindle assembly checkpoint (SAC) is a monitoring mechanism that operates the mitotic phase of the cell cycle and ensures faithful chromosome segregation. [69,70] The 'SAC'

pathway in yeast monitors cell cycle before it enters anaphase. Some SAC proteins, like Budding uninhibited by benzimidizole (Bub) proteins (e.g.,Bub1), have been identified to be in conjunction with kinetochore proteins including the Mitotic arrest-deficient (Mad) proteins, Mad1, Mad2 and Mad3. [71,72]

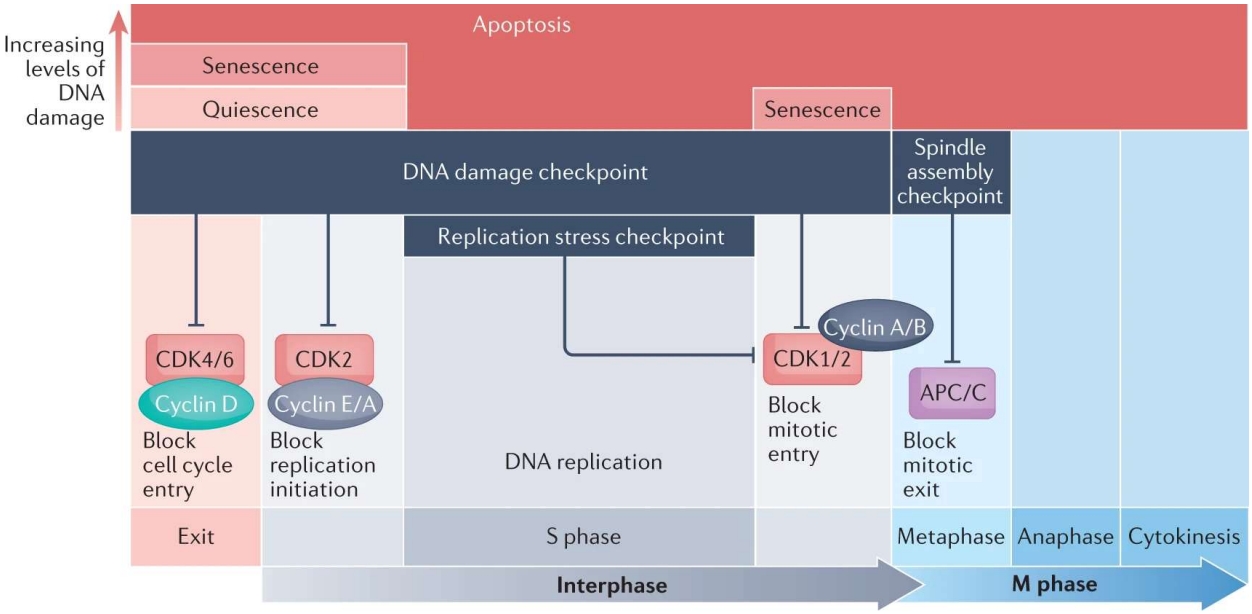

Figure 5: Checkpoint-dependent cell cycle arrest and exit. [73]

1.3 Cell cycle and cancer

Many cell cycle proteins are overexpressed or overactive in human cancers. [74] Deregulation of the cell cycle results in uncontrolled cell proliferation and hence lead to the development of cancer. Genetic alterations are common in human cancers. Recent research has shown that rather than causing uncontrolled cell cycle progression, the ongoing cell division is caused by mutations that prevent apoptosis and compromise cell cycle exit. [73] Mutations can occur in two types of genes: 1) proto-oncogenes and 2) tumor suppressor genes. [6] The observed cancer heterogeneity can be explained by the finding that only a small number of genes are

commonly mutated in breast cancer, compared to a large number that are infrequently mutated. [75]

1.3.1 Deregulation of the cell cycle in breast cancer

In hormone-receptor positive (ER+)/HER2-negative "luminal" breast cancers, malignant cells rely on estrogen for its mitogenic activities. Breast cancer frequently exhibits dysregulation of the oncogenes ErbB2, PI3KCA, MYC, and CCND1 (Cyclin D1). [75] Amplification of Cyclin D1 DNA occurs frequently in luminal tumors as Cyclin D1 is an ER target gene that drives cell cycle progression through the restriction point. [9] Activating mutations in PIK3CA which encodes the catalytic subunit p110α of PI3K, exist in 30–50% of advanced ER+/HER2− breast cancers and contribute to cell cycle progression. [9,76,77]

Breast cancer risk is considerably increased by germ-line mutations in ATM, CHK2, NBS1, RAD50, PALB2, and BRIP. [75] Radiation and genotoxins potently activate ATM and Chk2, which causes DSBs. [78] The RAD50 gene is essential for repairing DNA DSBs and is a breast cancer susceptibility gene associated with genomic instability. [79,80] PALB2 encodes a protein that interacts with BRCA2. [81] Several pathways involving key lipid and protein kinases are frequently deregulated in breast cancer as a result of mutations in these genes. [75]

On the contrary, TNBCs display RB1 mutations or deletions that jeopardize the integrity of the Rb/E2F/CDK4/6 pathway's control over the cell cycle. [9] *BRCA* gene mutations result in dysfunctional DNA repair pathways and BRCA1 mutation carriers are more likely to develop TNBC, according to several research. [82] Numerous research showed that TNBC tumors depend on the SAC and have high expression levels of DNA repair proteins and mitotic checkpoint genes, such as TTK, BUB1, MAD2, and AURKB, likely because of their high levels of genomic

instability. [9]

1.3.2 Overview of CDK4/6 inhibitor

HR+ breast cancer accounts for 70% of breast cancer cases in women and is typically treated with endocrine therapy (ET) including selective estrogen receptor inhibitors and aromatase inhibitors (AIs). However, resistance exists in up to 50% of patients with advanced breast cancer after ET as a standard treatment for HR+ breast cancer. Targeted treatments for HR+ breast cancer have been approved to overcome resistance. [83] Since scientists have learned more about CDKs' function in maintaining the oncogenic state in cells and controlling transcription, CDK inhibitors have become ground-breaking pharmacological targets and new therapeutic approaches in the fight against cancer. Evidently, this led to the approval of abemaciclib, palbociclib, and ribociclib for the treatment of patients with stage IV or metastatic breast cancer expressing HER2- and HR+. [36]

Palbociclib

Palbociclib, also known as PD-0332991 (Pfizer, Inc., New York, NY, USA), is an oral CDK4/6 inhibitor. PD-0332991 effectively and equally inhibits the activity of CDK4 and CDK6-cyclin D1 kinase. [84] Reversible neutropenia, which is not typically linked to significant infections, stands out in the well-described toxicity profile of palbociclib. [85] Previous research using ER+ breast cancer models and low doses of CDK4/6i with RT show clinically significant radiosensitization. In addition, a report has shown that palbociclib radiosensitizes TNBC cell lines with wildtype (WT) RB protein, including MDA-MB-231 and SUM159. RAD51, which is a recombinase mediating the repair of DNA DSBs, decreased after palbociclib treatment with radiation compared with radiation alone. [86] However, palbociclib does not radiosensitize all cell

lines. For instance, radiosensitivity is promoted in A549 and HCT116 cell lines but not in HT-29 and H1299 cell lines. (78) In line with other studies, the current data suggests that the ATM signaling pathway is a key factor in radiosensitivity that is mediated by palbociclib. [87] Notably, palbociclib therapy clearly caused senescence in healthy fibroblasts as well as in breast cancer and certain melanoma cells. [88]

1.4 Radiation therapy in triple negative breast cancer

Radiation therapy is administered to around half of all cancer patients during their illness. Cancer cells are destroyed by high doses of radiation, a physical substance used in radiation therapy, as a treatment for cancer to eradicate cancer cells and reduce tumor size. Radiation can be used as a palliative treatment to ease patients' cancer-related suffering and with the goal of curing the disease. [89] Radiotherapy plays an cricial role in treating breast cancer nowadays [90], but radiotherapy based on molecular subtyping of breast cancer in clinical practice is rare. [16]

The major objectives of therapy for nonmetastatic breast cancer include eliminating the tumour from the breast and regional lymph nodes; the treatment targets for metastatic breast cancer are life extension and symptom relief. [2] Radiotherapy also has an important role in local control after surgery. [91]

TNBC has been shown to be a deadly breast cancer. Patients with TNBC normally receive breast conservation therapy (BCT) or post-mastectomy radiation therapy (PMRT). Patients with TNBC have worse locoregional relapse for either breast conservation therapy or PMRT compared to luminal subtypes. BCT is still used as part of a treatment plan for breast cancer. According to research done in 2016, BCT showed similar 5-year local relapse (12-17%)

for TNBC and non-TNBC patients. [17]

Distant relapse usually occurs in the brain and the lungs 2-3 years after diagnosis. Regional nodal recurrences are usually found in TNBC patients in the axilla and the supraclavicular region. As such, use of locoregional irradiation should be considered in the treatment of TNBC. [92] Data from Danish Trials discovered that while PMRT reduced locoregional recurrence in TNBC patients, it did not improve overall survival. [93]

Types of cancer that are treated with radiation therapy:

External beam radiation therapy: This type of radiation therapy uses one or more beams from outside the body to deliver high-energy X-rays to malignant tumors.

Brachytherapy: It is a radiation therapy commonly used in women's reproductive system cancers such as cervical cancer and endometrial cancer. Brachytherapy has been the longest researched technique for administering partial breast irradiation (PBI), however, it is not often used due to the shortage of services and lack of clinician expertise. [94]

Systemic radiation therapy: Radioactive iodine treatment (I-131) is used to treat selected thyroid cancer patients after surgery. Thyroid tissue is the only organ that can take up iodine in human cells. I-131 can release β radiation with weaker penetrating power, which can destroy cell tissue in a short distance and be used as targeted radiation therapy.

1.4.1 Locoregional management

There are two primary locoregional treatment options for aggressive, early-stage breast cancer: 1) breast conservation therapy or 2) mastectomy. Numerous randomized studies, as well

as a meta-analysis, have shown that both mastectomy and BCT have a long-term equally high rate of survival, and that additional whole breast radiation therapy (WBRT) contributes to locoregional and survival outcomes of patients who receive lumpectomy. [17,95,96] Breast conservation therapy refers to lumpectomy, which is frequently followed with WBRT to minimize any microscopic residual disease. [17] WBRT includes typical fractionation with the dose fractionated into 30–35 fractions over 6–7 weeks. [96,97] However, some randomized trials have found that hypofractionated radiation therapy in early breast cancer is equivalent or superior to typical fractionation in terms of disease control and toxicity. Wang et al. conducted a phase 3 randomized trial testing the difference between hypofractionated radiation therapy and typical fractionation in patients with T1/2N0-3 breast cancer. The results showed a 5-year cumulative local recurrence rate of 1.2% in the hypofractionated group compared to 2.0% in the conventional group. [98] Classifying patients by breast cancer subtype can aid in deciding locoregional treatment. [17]

Breast conservation for TNBC has raised some concern due to the aggressiveness of TNBC and its high risks of recurrence. Regarding this matter, a meta-analysis by Wang et al. showed that BCS+RT was less likely to cause locoregional recurrence than mastectomy, although stage-specific comparisons weren't made. [99] Some studies showed the results of breast conservation with adjuvant radiotherapy in TNBC and non-TNBC patients: overall, locoregional recurrence was lower than 10% for most of the studies except for Haffty et al. which showed 17% at 5 years for both TNBC and non-TNBC. Most of the studies showed the same overall survival (OS) at 5 years. [92]

Study	Year	Patients (N)	TNBC patients (N)	Stage	Follow-up (months)	Locoregional recurrence* (%)		P value	Overall survival*		P value
						TNBC	Non-TNBC		TNBC	Non-TNBC	
Haffty *et al.* (11)	2006	482	117	I–III	95	17.0	17.0	NS	80%	89%	NS
Nguyen *et al.* (14)	2008	793	89	I–III	70	7.1	2.0	–	–	–	–
Solin *et al.* (15)	2009	519	90	I–III	47	8.0#	4.0	0.041	84%	88%	0.780
Freedman *et al.* (16)	2009	753	98	I–III	44	3.2	–	0.360	90%	–	0.150

*, values reported at 5 years; #, values reported at 8 years.

Figure 6: Reported locoregional recurrence (LRR) and overall survival (OS) after breast conserving therapy (BCT) in triple negative breast cancer patients (TNBC) and non-TNBC. [92]

1.4.2 Historical advancements in hypofractionated radiation therapy

Clinical radiation therapy is thought to have begun around 1922. [100] A French radiologist named Henry Coutard observed that prolonged and hyper-fractionated teletherapy administered at high total doses might be clinically advantageous. Experience with the application of hypofractionation was later achieved in Germany and Austria. [97] The radiologist Paterson began employing radical small field x-ray treatment in Manchester around the early 1930s. With the advance of megavoltage radiotherapy, patients received a small increase of single and total doses each time, provided in a fixed 20 fractions per month at The Hammersmith Hospital like Robert Morrison (1975). [97]

For many breast cancer patients, 15–16 fractions are the preferred standard treatment. [101] Since 2008, a number of national and international guidelines have advocated whole breast irradiation (WBI) with hypofractionated (HF) radiation as the new standard. Hypofractionated radiation therapy (HFRT) was primarily recommended for certain BC patients, and then was expanded to include all patients needing WBI. [102] Reducing the number of fractions has advantages. A phase II trial of once-weekly hypofractionated breast irradiation was described by

Dragun et al., in which each patient received 30 Gy over the course of five weekly fractions, with or without a boost. Although worries concerning recurrence and overall survival persisted, acute toxicity was well tolerated. [103]

1.4.3 Radiation therapy equipment

Radiation therapy that is administered from outside the body is known as external radiation. It is typically transmitted using protons, electrons, or photons. Radiation oncologists use ^{60}Co units or linear accelerators to deliver external-beam radiation, with the linear accelerator being the most commonly used device. Based on the photon's energy, external beam radiation therapy includes orthovoltage and megavoltage radiation therapy. [104] Orthovoltage X-rays are produced at ranges from 150 to 500 kV; megavoltage radiation emits photons with levels equal to or higher than 1 million electron volts (1 MeV) or one MeV. Megavoltage radiation is becoming more popular since it has better tissue penetrating capabilities than orthovoltage X-rays which have low energy. [105]

The principle of plaque brachytherapy is to place a radioactive radiation source in the tumor area, so that the tumor receives a high dose while the dose to the surrounding normal tissue is rapidly reduced. [106] Brachytherapy has now become another treatment option for early-stage prostate cancer. [107]

1.4.4 Neoadjuvant systemic therapy

The systemic treatment of breast cancer prior to surgical therapy is referred to as neoadjuvant therapy. It is usually indicated as neoadjuvant chemotherapy (NACT), and later, neoadjuvant endocrine therapy and targeted therapy. Systemic therapy is given to reduce any risks of distant recurrence and to decrease the severity of breast and lymph node diseases. A group from Milan showed that preoperative chemotherapy for stage II breast cancer allowed for women who would have needed a mastectomy having BCT with downstaging efficacy. [108] Several trials confirmed preoperative systemic therapy could increase rates of breast conservation therapy. [109]

1.4.5 Breast conservation therapy versus mastectomy

There are few results from phase 3 studies comparing mastectomy with breast conservation therapy for TNBC. Several studies have shown that breast conservation therapy provides better locoregional outcome compared to mastectomy for early-stage TNBC. It has long been assumed that the HER2-positive subtype and TNBC have similar regional recurrence. However, how to reduce local recurrence rates still remain a challenge for TNBC patients after breast conservation therapy. [16] According to Abdulkarim's cohort study, recurrence of breast-conserving surgery plus radiotherapy, modified radical mastectomy without adjuvant radiotherapy, and modified radical mastectomy with adjuvant radiotherapy were respectively 94%, 85%, and 87% for patients with early-stage TNBC after 7.2 years follow-up. [16,110] The majority of patients who are candidates of neoadjuvant chemotherapy have a chance of having breast-conserving surgery in roughly 70% of cases. Following neoadjuvant chemotherapy, an examination of 5477 breast cancer patients revealed that the tumour biology, not the type of surgery, was what affected patients' locoregional recurrence (LRR) free survival. [111]

In an analysis, there were no statistically significant differences in disease-free survival between patients who underwent mastectomy and those who underwent breast conservative surgery (BCS), even after adjusting for age and histological features. [112] In light of the data from research, regional lymphatic radiation therapy may be effective in the treatment of breast cancer. [113]

1.4.6 Association between BRCA status and TNBC

The basal-like subtype of TNBC is present in 75% or more of breast cancers linked to BRCA1, despite the fact that only 10–20% of sporadic TNBCs have germ-line BRCA1 mutations. [17] Individuals with breast cancer who were BRCA1 mutation carriers exhibited high expression of nuclear grade more frequently than patients with BRCA2 mutation. [82] Increased radiosensitivity has also been attributed to the BRCA mutant propensity in TNBC patients, which renders the tumour DNA repair-deficient. [114] Additionally, sporadic TNBC and BRCA1-associated cancers share some clinicopathological similarities. Determining the germ-line BRCA mutation status is thus essential for establishing locoregional treatment choices. [17]

1.5 Combination treatments with radiation for TNBC

TNBC patients do not benefit from hormonal or trastuzumab-based therapy due to the absence of target receptors like ER, PR, and HER2. Therefore, it appears that surgery and chemotherapy are the only treatment options, either separately or together. Certain receptors have, however, been named as potential targets for novel therapeutic medications in some studies. [115] Over the past few years, there have been more and more applications of neoadjuvant

systemic therapy (NST) in breast cancer. [92] The primary goal of NST is to reach pathologic complete response (pCR), which has been found to be related with disease-free and overall survival benefit. A study has shown that only one-third of the basal-like subtype can achieve a complete response compared to the luminal A subtype, which demonstrates excellent prognosis. [116]

There are some molecularly targeted methods in TNBC, which include poly ADP-ribose polymerase (PARP) inhibition, immune checkpoint inhibition, inhibition of signaling kinases, angiogenesis, epigenetic modifications, and cell cycle. [117] Clinical trials have demonstrated a synergistic effect of radiotherapy and immunotherapy for increased anti-tumor activity and better prognostic results. [118]

1.5.1 PARP inhibitor and radiation therapy

Single-strand breaks (SSBs) are recognized by PARP, and through its parylation activity, PARP recruits proteins that facilitate DNA repair. [119] Numerous cancers have increased PARP activity. [120] Due to the PARP inhibitor (PARPi)-induced inhibition of the DNA homologous recombination repair system and the radiation-induced production of DNA single- and double-strand breaks, which are currently being researched in the preclinical context, this combination offers a powerful rationale. [121]

Lynparza® (Olaparib) has been approved and used to treat adult patients with germline BRCA1/2 mutations who have locally progressed or metastatic breast cancer and are HER2-negative since February 2019. [121] Preclinical research has shown that PARP inhibitors are effective against TNBC cell lines. [122] Olaparib (AZD-2281) is an oral PARP inhibitor that has been studied in the treatment of breast, pancreatic, ovarian, and prostatic cancer. A phase I trial

RADIOPARP, which seeks to assess the tolerability of the combination of radiation therapy and olaparib for the treatment of TNBC, advises utilizing olaparib 200 mg twice per day and examining its conjunction with breast radiation therapy for TNBC. [122] Another PARP inhibitor, Veliparib (ABT-888) was also shown to radiosensitize breast cancer cells. [123] Veliparib has been coupled with paclitaxel and carboplatin in a phase III trial (NCT02032277) to treat TNBC with standard neoadjuvant chemotherapy. [117]

1.5.2 CDK4/6 inhibitors and radiation therapy

There are several CDK4/6 inhibitors analogues which have different effects on cytotoxic behaviors. Pyrimidine based analogues, 4-thiazol-N-(pyridin-2-yl) pyrimidin-2-amine derivatives for example, are potent bioavailable CDK4/6 inhibitors that suppress pRb phosphorylation at a specific amino acid residue, Ser780, at 12 and 24 hours. [36] The G1 phase of the cell cycle and Rb/E2F transcription are primarily regulated by cyclin dependent kinases 4 and 6, together with cyclin D. There are several medicines that are approved and only a few that are being used as CDK4/6 inhibitors in clinical studies. There are some clinical trials combining CDK4/6 inhibitors with existing drugs like Gemcitabine which serves as the exclusive first-line therapy for pancreatic cancer and carboplatin which has also been used to treat TNBC. [36]

Palbociclib has demonstrated impressive efficacy in breast cancer patients. According to previous research, this CDK4/6 inhibitor sensitizes ER+/HER2- breast cancer cell lines to RT regardless of the presence or absence of p53. Radiotherapy followed by palbociclib showed superior cytostatic effects in cell lines and mouse models. [124] The presence of wild type p53 is essential for radiosensitizing palbociclib [87]. Hence, TNBC cell lines carrying TP53 mutations, such as MDA-MB-231, are supposed to have limited effect of palbociclib in combination with

RT. [87,124] HR+ breast cancer can benefit from RT followed by palbociclib *in vitro* and *in vivo*. [124] However, research about radiation therapy for TNBC patients with combined palbociclib is rare, so is the TNBC cell lines. For studies of non breast cancer, palbociclib with RT enhanced tumour cell apoptosis in two trials using glioblastoma cell lines in comparison to monotherapy. [125,126] Palbociclib with radiation therapy may have a synergistic impact, according to preclinical research, hence clinicians remain concerned that radiation therapy could exacerbate the palbociclib 's toxicity, especially neutropenia and leukopenia. Clinicians hardly ever use this combination. [127]

1.5.3 DNA-PK inhibitors and radiation therapy

The DNA-dependent protein kinase (DNA-PK) is a nuclear protein serine/threonine kinase that detects DNA double-strand breaks (DSBs). [128,129] DNA-PK belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family and plays an essential role in the regulation of non-homologous end joining (NHEJ). [130] In combination with genotoxic chemotherapy or ionizing radiation, inhibition of DNA-PK has become a promising cancer therapy. [128]

AZD7648 is a new and potent inhibitor of DNA-PK. [129] When combined with RT, AZD7648 was found to induce tumor shrinkage in a large percentage of mice bearing murine colon adenocarcinoma or colorectal carcinoma tumors. [131] A prolonged G2/M cell cycle arrest was also observed in non-small-cell lung cancer (NSCLC) cell lines when receiving combination of AZD7648 with IR. [132] AZD7648 is also an efficient sensitizer of doxorubicin, which is an inducer of DSBs, and shows improved effectiveness when combined with olaparib. [129,132,133]

The resulting repair is more prone to mistake since NHEJ does not rely on the presence of template DNA. As a result, the accumulation of unresolved dsDNA breaks makes breast cancer cell lines more susceptible to the toxic effects of RT. This is how small-molecule DNA-PK inhibitors like NU7761 and AZD7648 work. [134] However, no articles have been reported to apply this combination in breast cancers.

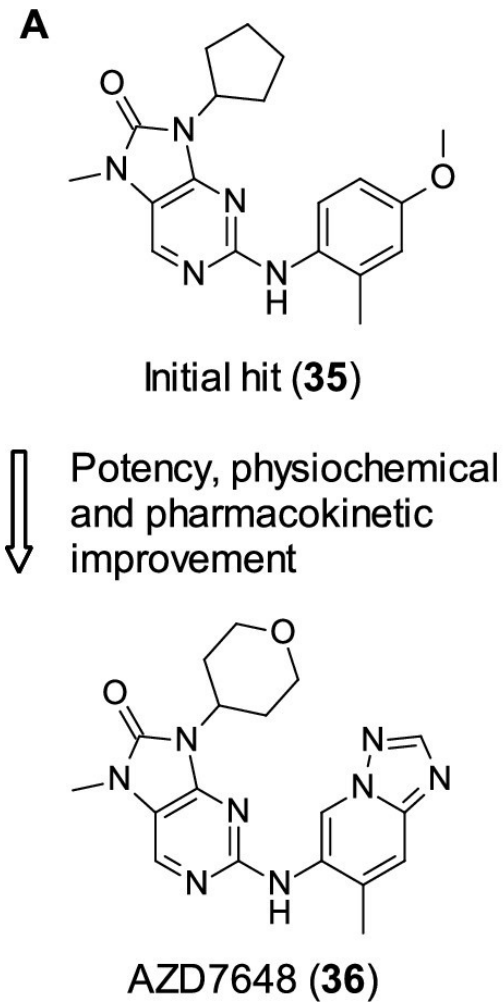

Figure 7: Optimization of DNA-PK inhibitor 35 to AZD7648 (36) by AZ. [135]

1.5.4 RAD51 KO and radiation therapy

In lung cancer, prostate cancer, and ER+ breast cancer, higher RAD51 expression has been linked to poor clinical outcome. As a surrogate marker for chemo and radioresistance, RAD51 has received substantial research as a marker of DNA repair ability. [136] A study has shown that RAD51 is involved in SOS responses to DNA damage by γ -irradiation and alkylating reagents [137], which suggests that targeting RAD51 and radiation can be used as a combination therapy. Loss of heterozygosity in the RAD51 gene has been observed in breast tumors and *BRCA* mediates recruitment of RAD51 after DNA damage. [138,139] However, there are no studies discussing about RAD51 inhibition and radiation therapy in breast cancer.

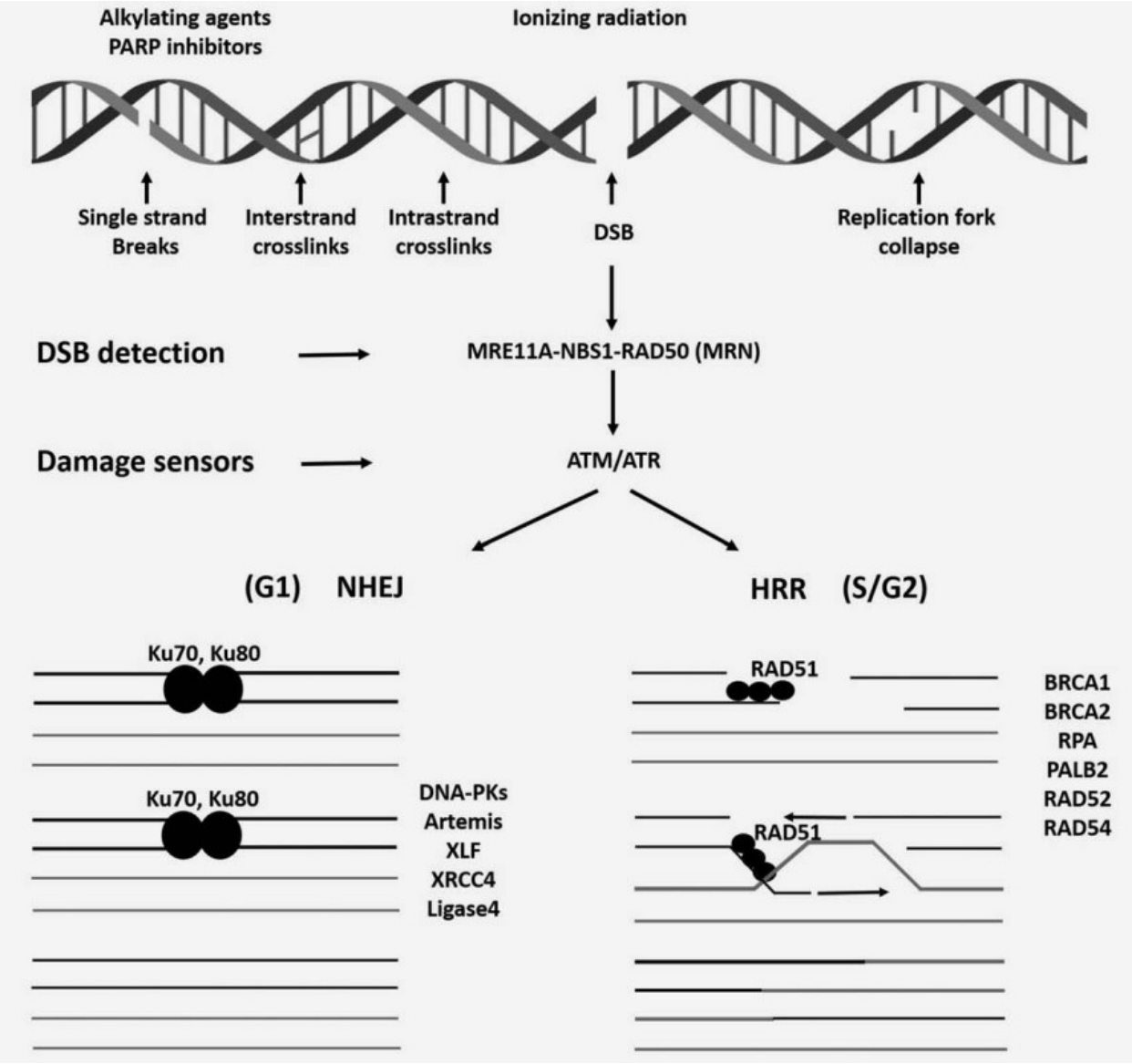

Figure 8: Schematic representation of DNA double strand break (DSB) repair. [139]

1.6 Overview of Rad51

RAD51 plays a key role in DNA double-strand break repair. It is overexpressed in multiple tumor types and correlates with high-grade metastatic breast tumors, including TNBC, and poor prognosis. [136,140] Rad51 is identified as a potential mediator of resistance to PARPi in cancer stem cells (CSC). [141] BRCA1 and BRCA2 associate with RAD51 at DNA replication and damage sites, activating RAD51-mediated homologous recombination repair. [142] A study found that RAD51 was highly expressed and had a substantial correlation with both homologous recombination deficiency (HRD) and intratumor heterogeneity, comparing cancer to normal tissues. [143]

The expression of RAD51 in the cytoplasm ($RAD51C^{+}$) and absence of nuclear expression ($RAD51\ N^{-}$) were linked to aggressive behavior characteristics and aberrant expression of BRCA1. A report from the MD Anderson Cancer Center showed that in non-small-cell lung cancer (NSCLC) patients undergoing neoadjuvant chemotherapy, increased levels of cytoplasmic RAD51 were linked to shorter overall survival (OS) times. [144] In addition to RAD51-mediated HR, RAD51 can also be involved in the NHEJ pathway by interacting with BRCA2, PALB2 and RAD52, which are single-strand DNA-binding proteins. [14]

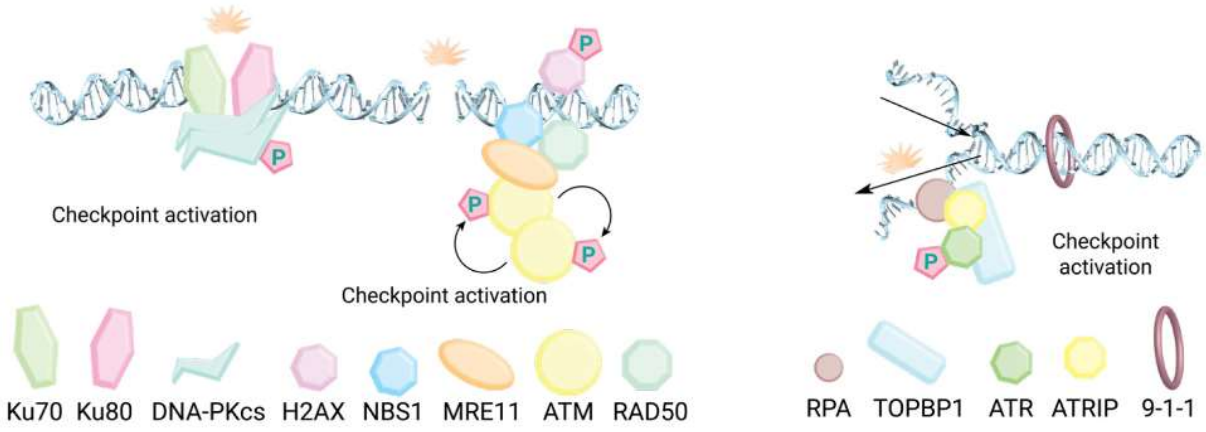

Figure 9: Recognition of DNA DSBs (left) and SSBs (including stalled replication forks, right) and activation of the DNA damage checkpoints kinases ATM, DNA-PKcs, and ATR. [144]

1.6.1 Rad51 and DNA damage response

RAD51 is important in DNA homologous recombination (HR) during double-strand break (DSB) repair. DSB is acknowledged as the most dangerous sort of DNA damage that may occur inside a mammalian cell. Genomic instability, which is related to DSB, occurs in many types of cancers and is caused by defects in the life cycle of cells. [14] RAD51 expression is highly upregulated in several types of cancers including breast, which over-activates HR and causes uncontrolled DNA DSB repairs and cancer cell survival. [143]

1.6.2 Rad51 pathway

BRCA1 and BRCA2 both co-localize with RAD51 at DNA replication and damage sites, activating RAD51-mediated HR repair of DSBs. [142] A previous study has shown that Rad51

could be phosphorylated on tyrosine. Phosphorylation of two tyrosines on Rad51, which are Tyrosine 315 (Y315) and Tyrosine 54 (Y54), have been published. The c-Abl family, which consists of the two members: c-Abl and Arg, contains the kinases that phosphorylate Rad51. Rad51 can also be phosphorylated by BCR/Abl, the oncogenic fusion tyrosine kinase. [145] Particularly, c-ABL phosphorylates RAD51 at Y54, perhaps through forming a tripartite complex with ATM, which prevents RAD51 from binding to DNA. In an ATM-dependent way, IR-triggered c-Abl tyrosine phosphorylates Rad51 at Tyr315 to promote interaction with Rad52 and chromatin in the recombination complex. [146] Phosphorylation of RAD51 at Y315 is required for chromatin association of oligomerization-defective RAD51 mutants. [144]

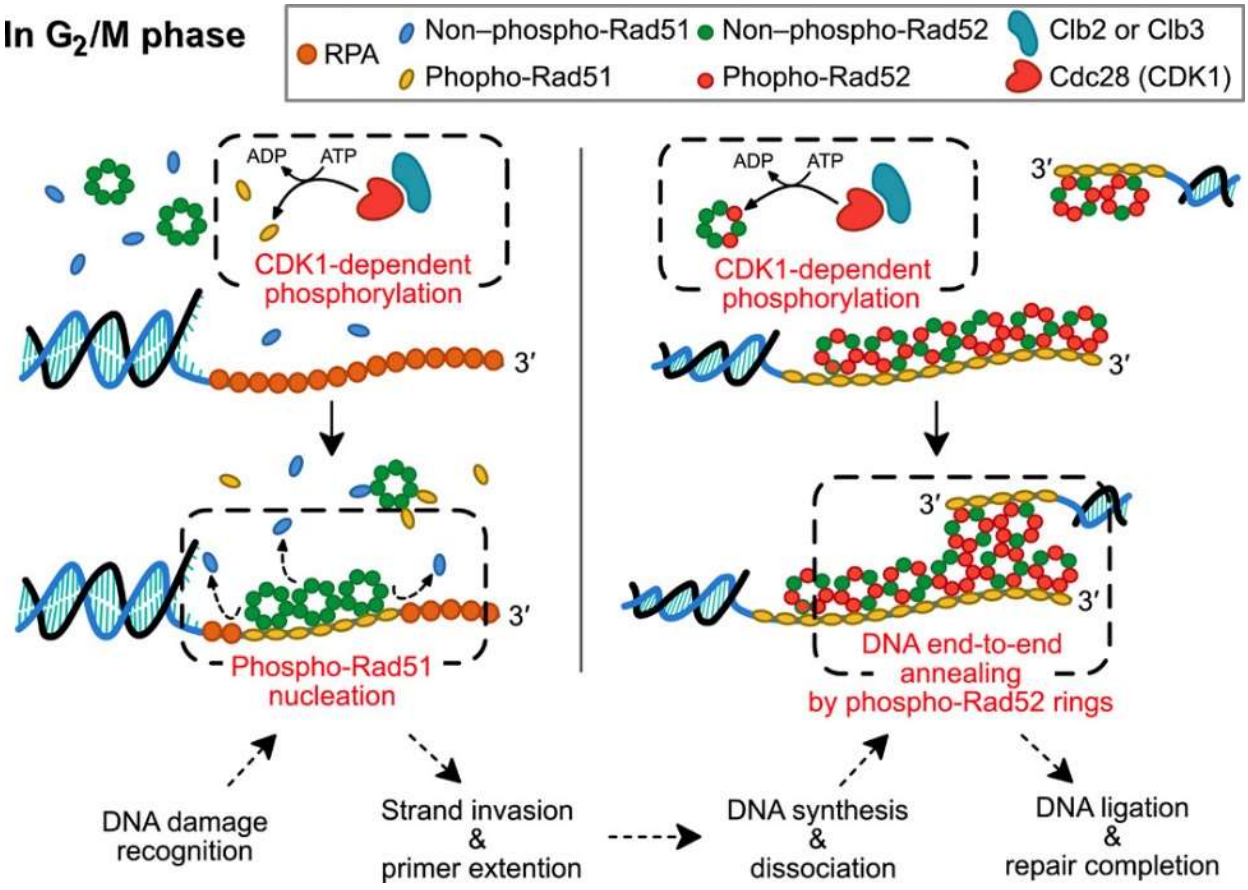

Figure 10: A study demonstrated that Rad51 and Rad52 are both G2/M-phase CDK1 substrates and that phosphorylation of both proteins is necessary for the proper activation of HR. Rad51 is phosphorylated to increase its DNA binding affinity. As a result, phosphorylated Rad51 can

attach to ssDNA that has been coated by Rad52 and the RPA complex. [147]

1.6.3 Rad51 and cancer

In several tumour types, including ovarian cancer, breast cancer, lung tumours, pancreatic adenocarcinomas, and malignant gliomas, increased expression of RAD51 is linked to tumour aggressiveness. [140] Numerous cancer cell lines have been identified to overexpress RAD51, and this has been linked to treatment resistance that causes DNA damage. The RAD51 gene has polymorphisms that may have clinical implications, according to several research. In refractory breast cancer cell lines, Marsden et al. discovered a novel RAD51 polymorphic variation 151 G/D that was connected to resistance to ionising radiation and DNA-damaging agents. [148] When RAD51 was downregulated, metformin's impact was strengthened, whereas RAD51 overexpression prevented the metformin-mediated inhibition of migration and invasion. [140] In METABRIC, RAD51 expression was considerably higher in BRCA mutant cancers than in BRCA wild-type tumors, however the TCGA cohort did not support this finding. RAD51 was most highly expressed in TNBC and high expression of RAD51 was correlated with poor survival outcome in ER+ patients. [149] Breast cancers with high levels of RAD51 were more immunogenic due to their higher mutational burden and increased quantity of neoantigens. [143]

In recent years, the potential marker RAD51 for predicting breast cancer therapy response has been emphasized. It is known that breast and ovarian tumors with HRD that lacked BRCA exhibited sensitivity to PARP inhibitors and DNA-damaging drugs like platinum. RAD51 was found to increase in samples collected right away following radiation-induced DNA damage. Direct inhibition of RAD51 with miR-155 or RAD51 siRNA can radiosensitize breast cancer cell lines with RT, which inhibits the HR of cells. [134] In addition, TNBC primary tumors

did not achieve pCR after NACT when RAD51 expression was high. [143] Research has shown that resistance to PARP inhibitor is positively linked with the frequency of RAD51 nuclear foci. Only RAD51 and BRCA1 mutant bulk tumor cells are efficiently targeted by the PARPi. [150,151] Conjugates of olaparib-RAD51 inhibitor may thus disrupt TNBC cell resistance to olaparib treatment and sensitize breast cancer cells independent of BRCA status. [152]

Adult T-cell leukemia-lymphoma cells were shown to become more chemosensitive when RAD51 was targeted by decreasing DNA DSB repair. [153] A mitotic regulator implicated in RAD51 degradation, EMI1, generates an increase in the protein level of RAD51 in these cells in addition to BRCA1 mutations that restore BRCA1-RAD51 interactions and make it easier for RAD51 to load on ssDNA. [154]

1.6.4 Blocking the Rad51 Pathway

As of current, some research groups are investigating treatments that potentiate the inhibition of RAD51 through combinations with cytotoxic agents. The homology directed repair (HDR) factors BRCA1 and RAD51 are suppressed by both hypoxia and PARP inhibition, which causes E2F4/p130 to bind to the BRCA1 and RAD51 promoters. [155] A study showed that Brca1 loss stimulates Akt1 to inhibit Chk1-Rad51 signaling, resulting in faulty HR, genetic instability, and tumor development. [156] Cancer and a FA-like syndrome can both result from inactivating RAD51, which can have significant impacts on genomic stability. However, cells that overexpress RAD51 are resistant to DNA damaging agents such as radiation and cisplatin. RAD51 overexpression also promotes genome instability and is seen in many cancers. Targeting and changing RAD51 activity using small molecules such as DIDS and B02 are hence being developed as novel cancer treatments. [157] TNBC cells might also become re-sensitive to a dual

PARP and RAD51 inhibitor. [152]

1.7 CRISPR screening

Recent advances have made RNA-guided CRISPR (clustered regularly interspaced short palindromic repeat)-associated Cas proteins useful tools for studying and engineering the genome. [158] Direct gene knockout (CRISPRko) or knockdown (CRISPRi) explains how genes function based on interference with gene expression. [159] Protein functions can be changed if the genome sequence is altered since proteins are generated based on the genome sequence. [160] Through gene mutations, a protein can lose its function (loss-of-function, LOF) or confer new activity (gain-of-function, GOF). [159,160] Genetic screens are often used to find out the relationship between the genotype and phenotype by introducing perturbations into genes at a large scale. [161] Loss-of-function screens can be used to determine essential genes by their decreased capacity to produce a desired phenotype or overall viability. [159] In CRISPRko, a frameshift or stop codon results in the DNA sequence, which targets protein-coding sequences or noncoding regions. CRISPRko pooled screens benefit from DNA cleavage mechanisms carried out by sgRNA-guided Cas9 that are afterwards repaired by NHEJ. [159] Cas9 is an RNA-dependent DNA endonuclease that creates double-stranded DNA breaks using a sgRNA. sgRNAs can be directed to target any sequence in the genome that comes before a "NGG" protospacer-adjacent motif (PAM) in mammalian cells. [162] The PAM exists in prokaryotic immune systems to allow them to distinguish the foreign DNA sequence (nonself) and the same sequence in CRISPR arrays (self). [163,164] The utilization of a library sgRNA molecules that targets numerous genes is necessary for pooled screens which depend on physically dividing cells into subpopulations that are either enriched or deficient for the desired phenotype. [159]

1.7.1 Screens for drug resistance/sensitization

Resistance/sensitization screens are an extension of fitness screens and allow for the evaluation of cell growth and viability in response to drug treatment. [165] Oncogene-dependent cancers are subjected to intense evolutionary pressure when they are treated with a therapy that targets an oncogene, or a gene that promotes carcinogenesis. [166] To identify possible therapeutic targets used for patient selection in clinical studies, a compilation of integrative information encompassing 941 genome-wide CRISPR screens, performed in a human cancer cell panel from 30 cancer types, was created. [167] When drug exposure is involved, CRISPR and shRNA screening are particularly helpful for identifying biological pathways in cells that affect sensitivity or resistance. [168] Oncology is increasingly favoring the use of numerous FDA-approved medicines in combination for the best possible therapeutic response. [169] Since a patient's mutational burden, which is the number of mutations in a tumor cell, may affect sensitivity to drug treatment, personalized treatment based on a patient's mutations is important. [169,170]

1.8 Objectives and Hypothesis

The CDK4/6 – RB pathway plays a central role in regulating cell proliferation and deregulation of this pathway often causes cells to grow and divide uncontrollably, eventually leading to tumorigenesis. CDK4/6 inhibitors have been shown to improve progression free survival (PFS) in advanced HR+ breast cancer when combined with hormonal therapy. [171] However, the effects of CDK4/6 inhibitor treatment on TNBC have not been fully investigated yet. I hypothesize that interfering with CDK4/6 expression/activity or their downstream DNA repair target genes may potentiate TNBC tumor response to RT treatments. My project's specific goal is to elucidate the potential benefits and effects of specific drugs affecting these pathways in combination with radiation therapy on aggressive TNBC cells.

CHAPTER II: Materials and methods

2.1 Cell Culture

SUM159 cells were used to generate cell lines expressing CDK4, CDK6, and Rad51 KO using lentiviral system designated as SUM159/lentiCRISPR v2 according to Feng Zhang's lab. [172] Mesenchymal triple-negative breast cancer cell line SUM159 (invasive phenotype, basal-B/claudin-low) was cultured in Ham's F12 (GIBCO) nutrient mixture supplemented with 5% Fetal Bovine Serum (FBS, GIBCO), 1 µg/mL hydrocortisone (Sigma-Aldrich), and 5 µg/mL insulin (Sigma-Aldrich). MDA-MB-231 (highly aggressive, epithelial, poorly differentiated triple-negative breast cancer) was cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 10 % FBS. All cells were kept in culture for fewer than 30 passages. Cells were maintained in a 37 ºC incubator with 5% CO2. Cells were detached from cell culture plates using trypsin (0.25%, Wisent), followed by washing with fresh completed Ham's F12 or DMEM to neutralize the trypsin and cultivate further in the incubator.

2.2 Primers (for sgRNAs)

The primers were designed using CHOPCHOP v3 which is a web tool for designing CRISPR-Cas single guide RNA (sgRNA) targets. [173] All primers were designed using this program and specificity was confirmed by UCSC Genome Browser on Human (GRCh38/hg38). We selected the top 3 predictive sequence. They were all purchased from Invitrogen.

Rad51 sgRNA g1

Forward primer: CACCGCATACCTCTAACCGTGAAAT

Reverse primer: AAACATTTCACGGTTAGAGGTATGC

Rad51 sgRNA g2

Forward primer: CACCGTAGCTTCCCATTGACCGGGG

Reverse primer: AAACCCCCGGTCAATGGGAAGCTAC

Rad51 sgRNA g3

Forward primer: CACCGTAGCTCCTTCTTTGGCGCAT

Reverse primer: AAACATGCGCCAAAGAAGGAGCTAC

2.3 Establishment of stable cells lines of the single knockout of the Rad51 gene using the CRSIPR/Cas9 one-vector lentivirus (virus' production using polyethylenimine method)

LentiCRISPR v2 (from Feng Zhang Addgene plasmid #52961) was used to generate Rad51 (human) *in vitro* genome editing. Three sgRNA (single guide RNAs) sequences for Rad51 were shown above and were predicted by CHOPCHOP v3. To construct the lentiCRISPR v2-RAD51-KO plasmid, lentiCRISPR v2 was digested by FastDigest BsmBI enzyme and ligated with annealed sgRNAs according to methods provided in relevant publications using golden gate reaction. [172,174] To generate the knockout cell lines, lentiCRISPR v2-RAD51-KO plasmid, together with the packing plasmids pMD2.G (Addgene plasmid #12259) and psPAX2 (Addgene plasmid #12260), were transfected into HEK293(F)T cells. DNA can be introduced into a host cell by transfection with polyethylenimine (PEI), a stable cationic polymer. [175] Cell supernatants containing the lentivirus were collected 48 h later and used to infect SUM159 and MDA-MB-231 cells. The CDK4/6-KO SUM159 cell lines were from my fellow lab member Dr. Meiou Dai who created and confirmed this cell lines.

After adding 8 µg/mL polybrene, viruses were then added to cell culture media. 3 days post-infection, infected cells were selected with 2 µg/mL puromycin for 7 days. Surviving pools

were collected on day 7 for immunoblotting. [176]

2.4 Clonogenic assay setup

Plating efficiency

Different cell lines have different plating efficiencies. Since we expect to obtain well-distributed colonies without overlapping after collecting results, first we must define growth properties of cells in culture which is plating efficiency (PE). When untreated cells are plated as a single-cell suspension at low densities of 2–50 cells cm^{-12}, they will grow to colonies. The colony is defined to consist of at least 50 cells. [177]

PE is the ratio of the number of colonies to the number of cells seeded:

$$\textbf{PE} = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100\%$$

The number of colonies that arise after treatment of cells, expressed in terms of PE, is called the Surviving fraction (SF):

$$\textbf{SF} = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} \times \text{PE}}$$

2.5 *In vitro* irradiation and clonogenic assays

For experiments with confluent populations, cells were seeded in 6-well plates at various densities to determine the appropriate density for confluence. The optimal density was found to be 300 cells/well (SUM159) or 250 cells/well (MDA-MB-231), and cells were seeded and grown for 72 hours, at which point they reached confluence. Cells were then pre-treated with olaparib, palbociclib, or AZD7648 (or DMSO vehicle control) for 4 hours followed by ionizing radiation treatment.

Four hours before irradiation, cells were treated with 2 or 4 nM AZD7648; 100 or 200 nM palbociclib; 2.5 or 5 nM olaparib and irradiated with the mentioned doses of IR using the X-Ray Irradiation System (Faxitron MultiRad 225). Culture medium was replaced 24 h after IR and refreshed every 6 days until the end of the experiment. Medium was removed in plates, cells were rinsed with 1X PBS. Cell viability was evaluated 13 days post-plating incubating with 10% formalin for fixation for overnight, staining was done with a solution containing 0.5% crystal violet in water for overnight at room temperature in mild rocking on the following day. Counting of clones was done on the following day.

Irradiation was done using Copper 0.30 mm with 225.0 kV and 13.0 mA. The irradiation dose varied between 0 and 10 Gy. The plates were photographed, and the colonies were counted with the naked eye under the stereomicroscope (from Dr. Bassam Abdulkarim's lab). The colonies with less than 50 cells were discarded.

Figure 11: Faxitron MultiRad 225 X-ray irradiation system.

Picture from: Utilizing the Faxitron MultiRad 225 X-ray irradiation system for the construction of mouse chronic whole brain radiation model. (We used this machine for *in vitro* experiments)

2.6 Animal experiments and irradiation

Mice were irradiated by me and Dr. Ni Wang at the Animal Resources Division (ARD) at the Research Institute of the McGill University Health Centre (RI-MUHC).

A total of 1 x 10^6 CDK4 or CDK6 KO SUM159 cells (15 tumors per group) were subcutaneously implanted into female mice sourced from the ARD. Mice surgery was operated by Dr. Ni Wang. Subcutaneous injection is used for creating solid tumor models by injecting cancer cells just under the skin, which makes it easier to observe tumor growth and response to radiation. Tumors were allowed to grow to approximately 100 mm^3. To ensure that four treatment groups' tumor sizes were distributed equally, randomization was done. Radiation was given one time (single 10 Gy or 20 Gy dose). Irradiation was done using Copper 0.30 mm with

225.0 kV and 13.0 mA. Tumor diameters were measured using calipers roughly three times per week. All animal procedures were approved by the ARD at the RI-MUHC. [178] [179]

2.7 Western blotting

Sample preparation

Place the cell culture dish on ice and wash the cells with cold 1X PBS pH7.4. Cells were then collected in lysis buffer (1 M Tris-HCL, 5 M NaCl, 10% Triton, 100 mM EDTA, 100 mM Na_3VO_4, H_2O). 100X protease (100 µM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin) and phosphatase inhibitors (Roche) were added prior to lysis. After centrifugation and aspirate the supernatant to a new fresh tube, the supernatant was mixed with 5X SDS Sample Buffer (10% SDS, 50% Glycerol, 250nM Tris-HCL, and 0.5% bromophenol blue dye, PH6.8). Samples were heated at 95 °C for 5 mins to break the interactions and denature the proteins.

Protein quantification

Protein quantification was performed by using the BCA Protein Assay Kit (ThermoFisher) following the manufacturer's instructions. Protein lysates were diluted with water (1:5), then a serial dilution was done by adding BCA reagents, followed by 37 ºC incubation for 30 minutes. The optical density (OD) was determined by using Epoch Microplate spectrometer to measure the absorbance at 570nm. Protein concentration will be calculated based on the standard curve.

SDS-PAGE and transfer

Aliquots of lysates equivalent to 80 µg of protein were electrophoresed through polyacrylamide gels and separated by SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), using electrophoresis apparatus. Once separated in the gel, the proteins are transferred onto a 0.45 µm PVDF membranes for further detection of specific proteins through immunoblotting. A wet transfer apparatus was used.

Immunoblotting

The PVDF membrane was blocked by soaking in 5% (w/v) milk in TBST (25 mM Tris, 0.2 M NaCl, HCl, pH 7.4) + 0.1 % Tween 20 for 1 hour and incubated with primary antibody solutions prepared in 5% (w/v) milk/TBST overnight at 4°C. Antibodies were used for the detection of: RAD51, RB1, and CDK4.

The membrane was washed with washing buffer and was incubated in blocking buffer containing HRP-conjugated goat anti-mouse or anti-rabbit antibodies at room temperature for 1 hour. After three 5-minute washes with washing buffer, the membranes were detected using ECL and chemiluminescence imaged using the ChemiDoc Imaging System (Bio-Rad).

2.8 Statistical analysis

GraphPad Prism 8.0 was used for statistical calculations. Statistical significance was evaluated using two-sided unpaired t-tests.

CHAPTER III: Results

3.1 SUM159 cells are radioresistant

Cell-cycle entry in early G1 phase relies on the activation of CDK4 and CDK6 by binding of cyclin D. Multiple mechanisms, including a RB1 mutation or decreased levels of the CIP/KIP proteins, p21, and p27, contribute to resistance to CDK4/6 inhibitors. [180] Loss of both alleles or loss of a single copy, gene deletions, promoter methylation, or small-range mutations within the RB1 gene or its promoter are among the genetic abnormalities for RB1 that have been reported. [181] CDK4/6 inhibitor resistance has been found in BC patients with acquired RB1 mutations. [182] Our lab has previously proved CDK6 played the role of the downstream DNA replication and regulating HR DNA repair proteins BRCA1 and Rad51. Patients with BC improve significantly when palbociclib is combined with anti-hormonal therapy. [183] However, the majority of patients with TNBC and one-third of those with ER+ BC did not react to CDK4/6 inhibition when combined with aromatase inhibitors. [184] To investigate whether using some combination therapy will decrease cell viability of TNBC cell lines, a highly metastatic basal-like TNBC cell line SUM159 was used.

To investigate survival of irradiated TNBC cells through clonogenic assay, SUM159 cell was used first. Cells were seeded at low densities and cultured for 13 days for colony formation. The result revealed that SUM159 is a radio-resistant cell line determined by surviving fraction at 2 Gy (SF2), as shown in figure 13.

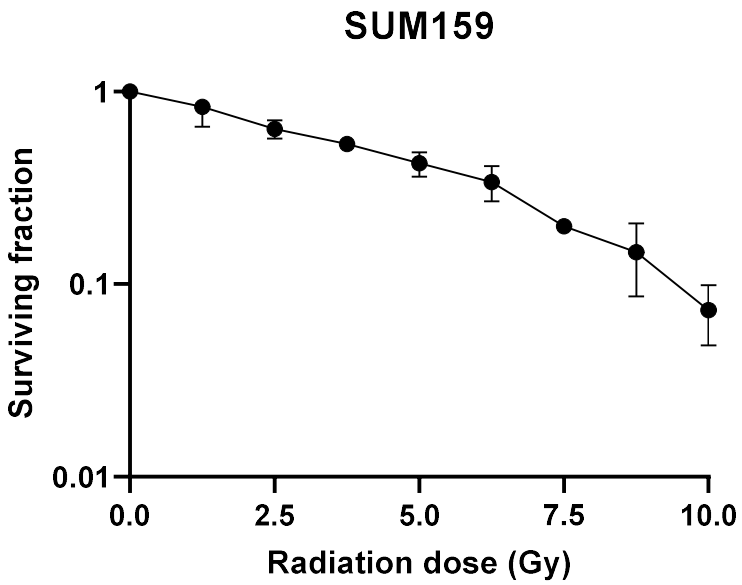

Figure 12: Sensitivity of SUM159 cells to radiation therapy.

Survival curve of SUM159 cells received different doses of radiation indicated. Survival fractions represent the ratio of the plating efficacy of the treated cells to the non-treated cells. The colonies were counted at 13 days after the IR. The SF2 was used to quantify radiosensitivity.

3.2 CDK4 or CDK6 single knockout didn't enhance the radiosensitization on SUM159

To further explore the effect of radiation on CDK4/6 KO SUM159, I examined the CDK4/6 CRISPR KO SUM159 cell lines generated in the Lebrun laboratory. Our lab has previously demonstrated that CDK4 specifically affects the regulation of cancer stem cell (CSC) self-renewal, while CDK6 regulates HR DNA repair by regulating downstream DNA replication and repair gene expressions. [184] It has been documented that CDK4/6 activation resulted in TSC2 inactivation which led to mTORC1 activation. [185] Given our preclinical data, we

speculated that CDK4 and CDK6 single KO could radiosensitize SUM159 cells.

The survival curves of the clonogenic cell survival assays are shown in figure 14. Clonogenic survival assay was used to measure radioresistance after exposure to increasing radiation doses. However, as shown in the figure 14, little difference was observed between the surviving colony numbers in the scramble SUM159 and CDK4/6 KO SUM159 cells. This suggests that a double CDK4 and CDK6 KO may be required to modify the radiosensitivity response in SUM159.

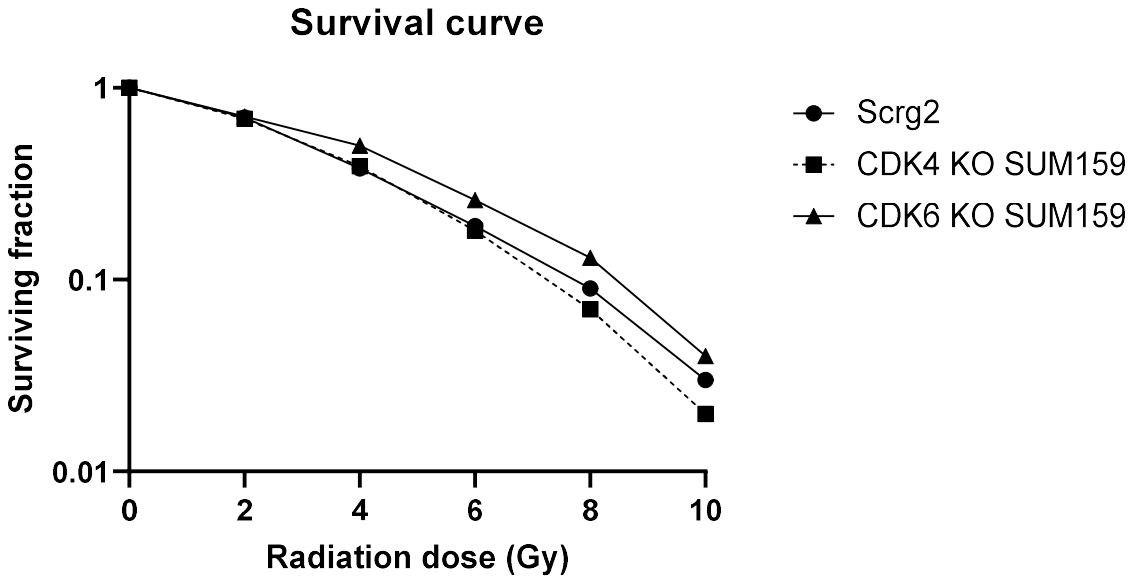

Figure 13: Radioresistance measurement by clonogenic survival assay.

CDK4/6 KO SUM159 and parental SUM159 cells irradiated with different doses (0, 2, 4, 6, 8, and 10 Gy) were graphed at 15 days after irradiation. Colonies of more than 50 cells were counted.

To further test if inhibiting both CDK4 and CDK6 can sensitize RT, we examined the effect of palbociclib which is a CDK4/6 inhibitor. We also used olaparib and AZD7648 in combination with palbociclib to see the effect on RT. Our lab has previously determined the IC50 concentration of palbociclib, olaparib, and AZD7648 used on SUM159 and therefore we applied the same condition to the following experiments.

3.3 Palbociclib, AZD7648 and olaparib enhance the IR activity in SUM159 cells

Numerous mechanisms, such as changes in the efficiency of the DNA damage response, cell cycle reassortment, changes in oxygenation, enhancement of apoptosis, and induction of the cellular senescence phenotype can lead to radiosensitization. [186,187] CDK4/6 inhibitors combined with IR increase DSB and cause HR deficiency via reducing Rad51 and ataxia telangiectasia mutated (ATM) kinase expression. [186] Palbociclib has been shown to radiosensitize TNBC cell lines with WT RB1. Our lab has shown that deletion of CDK6 effectively prevented tumor growth and progression in SUM159 cells (a RB WT cell line), offering a potential new targeted treatment for breast cancer. [184] About 50% of cancer patients have radiotherapy as part of their treatment. In January 2018, the initial study about the radiotherapy/palbociclib combination was published. [188] CDK4/6i may enhance the anticancer effects of radiation by first regulating cell progression from G1 phase to the more radioresistant S phase and secondly by suppressing DNA DSB repair pathways. [188] Preliminary results from several small sample clinical studies showed that CDK4/6 inhibitors combined with radiation displayed a synergistic effect, however, most clinical trials for combination therapy are still in progress. [186] Our goal is to make radiation therapy better and hence to see if palbociclib affected the sensitivity of SUM159 to irradiation. I performed clonogenic assay using palbociclib treated for 4 hours with concentrations at the IC_{50}

value followed by radiation.

DNA-PK is a key player in repairing DNA DSBs. AZD7648, a powerful and highly selective DNA-PK inhibitor, is an effective sensitizer of radiation-induced DNA damage. [132] In addition, a previous study has suggested that inhibiting DNA-PK with AZD7648 could cause delay in DNA repair and radiosensitize SUM159. [189] Hence, DSBs introduced by external sources like IR, make sense to combine DNA-PK inhibitors. AZD7648 was also shown to enhance olaparib activity. [132] I used SUM159 parental cell as a model to assess these effects. (Figure 15)

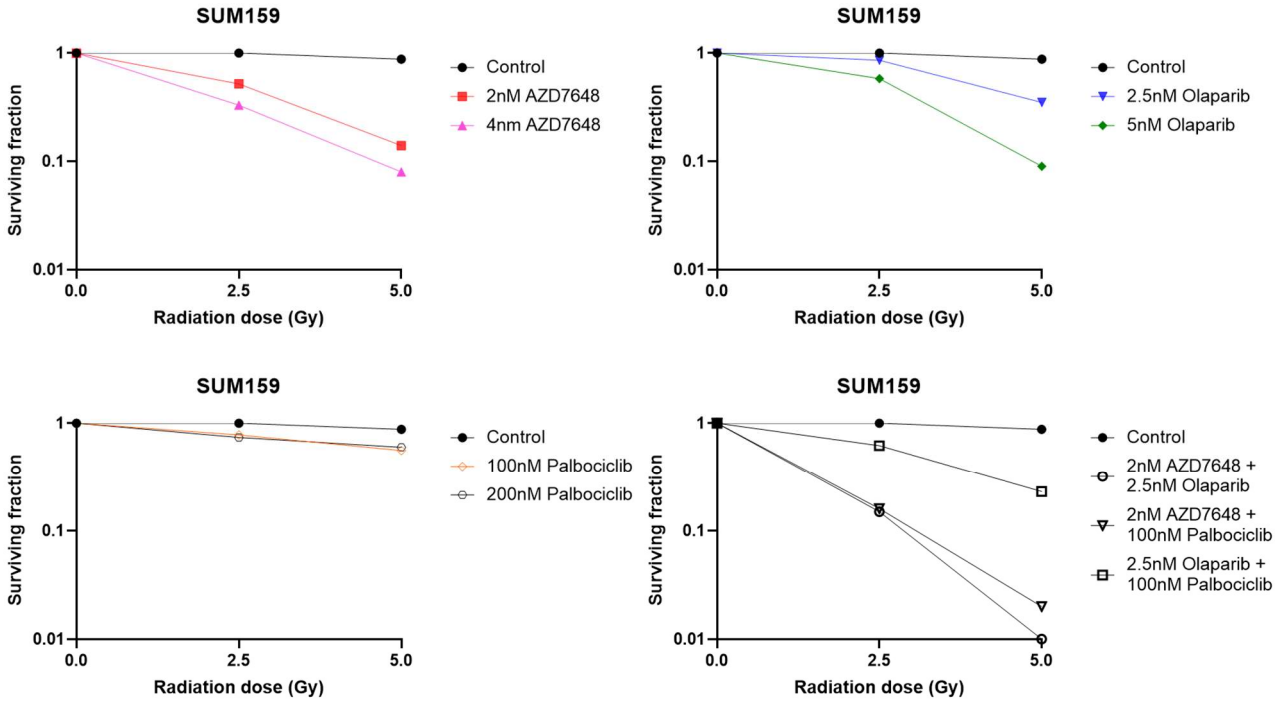

Figure 14: Sensitivity of SUM159PT cells to DNA damaging agents upon drugs treatments. Clonogenic assay performed in SUM159 after treatment with different doses of AZD7648, palbociclib, or olaparib for 4h before irradiation with 2.5 to 5 Gy irradiation and allowed to grow for 13 days. AZD7648 combined with olaparib or palbociclib can sensitize RT in a

radiation dose-dependent manner.

Figure 15 shows that AZD7648 affects the clonogenic survival after radiation treatment in a concentration dependent manner. The combination of AZD7648 with olaparib led to a concentration-dependent decrease in clonogenic survival of SUM159 cells after IR. Clonogenic survival curves showed the surviving colony numbers of SUM159 cells were significantly lower at 5 Gy (Figure 15D). The effect of 5nM olaparib on SUM159 at 5 Gy was higher than at 2.5 Gy (Figure 15B). Both 100nm and 200nm palbociclib alone didn't show any effect with IR. However, palbociclib combined with AZD7648 radiosensitize SUM159 significantly compared to AZD7648 treatment alone (Figure 15D). We speculated that there might be other unknown mechanisms that affect the radiosensitization of CDK4/6 inhibition. Next, we investigated if CDK4/6 knockout with palbociclib or in combination with other drugs could enhance the effects of RT.

3.4 Palbociclib and AZD7648 treatments have no synergistic effects with CDK4/6 gene knockout in SUM159

We next examined the radiosensitization under CDK4/6 KO SUM159. CDK4/6 acts as a key point in tumorigenesis pathways since it intersects with numerous oncogenes in cancer cells by activating the CDK4/6-Rb-E2F pathway. In addition, overexpressed and hyperactivated CDK4/6 are common in diverse cancers including breast cancer. CDK4/6 is important in tumor formation, which has been confirmed by gene knockout studies. [190] As shown in the figure 16,

there is no radiosensitization after palbociclib treatment nor with AZD7648 in CDK4/6 KO SUM159 cells. Knocking out CDK4/6 expression didn't change the effects of AZD7648 on RT.

One possible issue to explain the lack of response following inhibition of CDK4/6 could be due to other mechanisms affecting the CDK4/6-Rb-E2F pathway. Indeed, previous reports showed that in the absence of CDK4/6 proteins, cyclin D binds to CDK2 which may function as CDK4/6 and promote G1/S conversion. [190,191] In addition to the compensative CDK2, proteins related to cyclin D-CDK4/6-Rb like other cyclins and transcription factors may also play an essential role in the control of cell proliferation. [190,192]

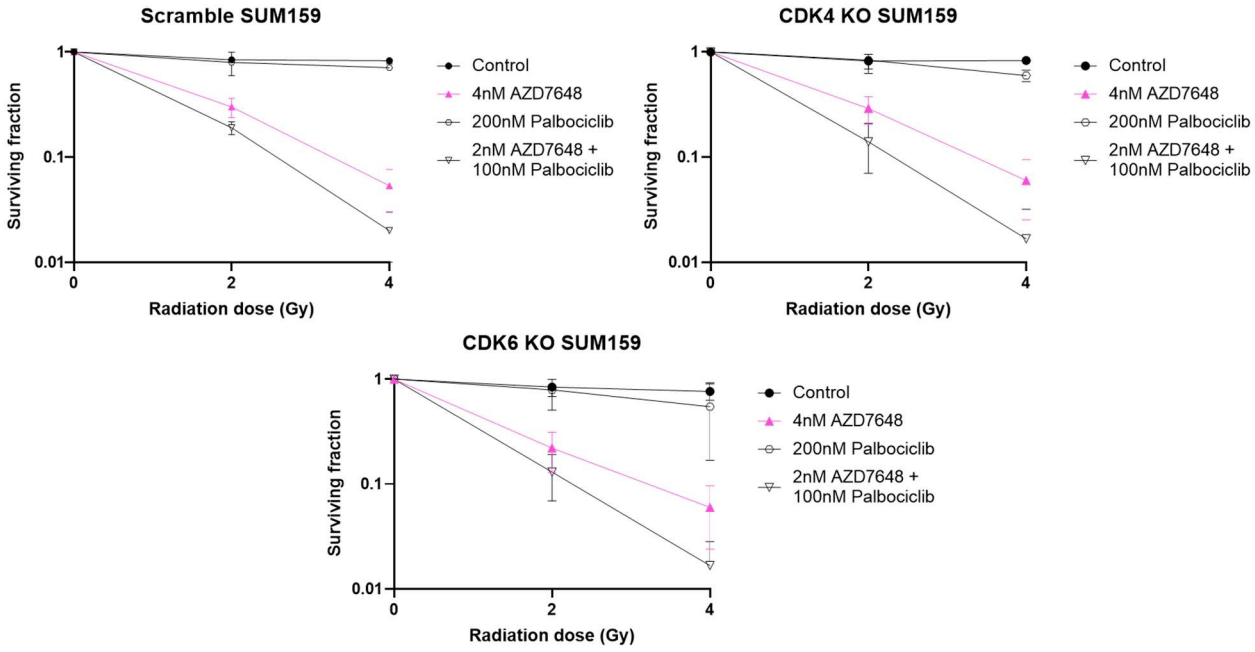

Figure 15: Clonogenic assay for AZD7648, Palbociclib, and IR treatment in KO SUM159 cells.

CDK4 KO SUM159 and CDK6 KO SUM159 cells were treated with 4nM AZD7648 or 100nM palbociclib 4h prior to IR (0, 2, 4 Gy) and the number of clones were counted 13 days after IR.

All experiments were repeated three times. The error bar represents standard error of the mean (SEM).

3.5 Knockout of CDK6 shows SUM159 sensitivity to radiation *in vivo*

In order to be closer to clinal settings, we next investigated the radiation response *in vivo* using a breast cancer xenograft model. Based on a previous study, radiation has minimal impact on SUM159 tumor growth. [193] The estimated TCD_{50} value for SUM159 was 44.9 Gy in nude mice, which demonstrated that SUM159 tumors were resistant to radiation. [193] Our lab's previous data showed that either CDK4/6 knockout or inhibition by palbociclib significantly reduced the tumor size of SUM159 transplanted orthotopically into the mammary fat pads of NSG mice. [184] We then further evaluated whether targeting CDK4/6 with the RT would represent better effects on regulating tumor development using SUM159 xenografts. In addition, research showed that RB WT TNBC could be radiosensitized by CDK4/6i but not RB-null TNBC. [86] SUM159 cells are RB proficient and as such should respond, at least partly, to CDK4/6i treatments. [194] Up to now, there are no studies doing research on radiation on CDK4/6 KO SUM159 *in vivo*. For this, we injected SUM159 CRISPR/Cas9 scramble2 and SUM159 knockout of CDK4/6 gene (1×10^6 cells for each mouse) cells subcutaneously into the NSG mice to artificially induce the formation of TNBC tumor. Once the tumor volume reached 100-200 mm^3, mice were randomized and received a single dose of 10 Gy or 20 Gy. Since mice can die from high radiation doses, we set the highest single-fraction dose at 20 Gy. We used animal irradiator (X-RAD SmART) consisting of a heavy-duty X-ray tube equipped with precision collimators to do radiation on mice. [195] Tumors were locally targeted by X-ray irradiation using Cobalt-60 filter.

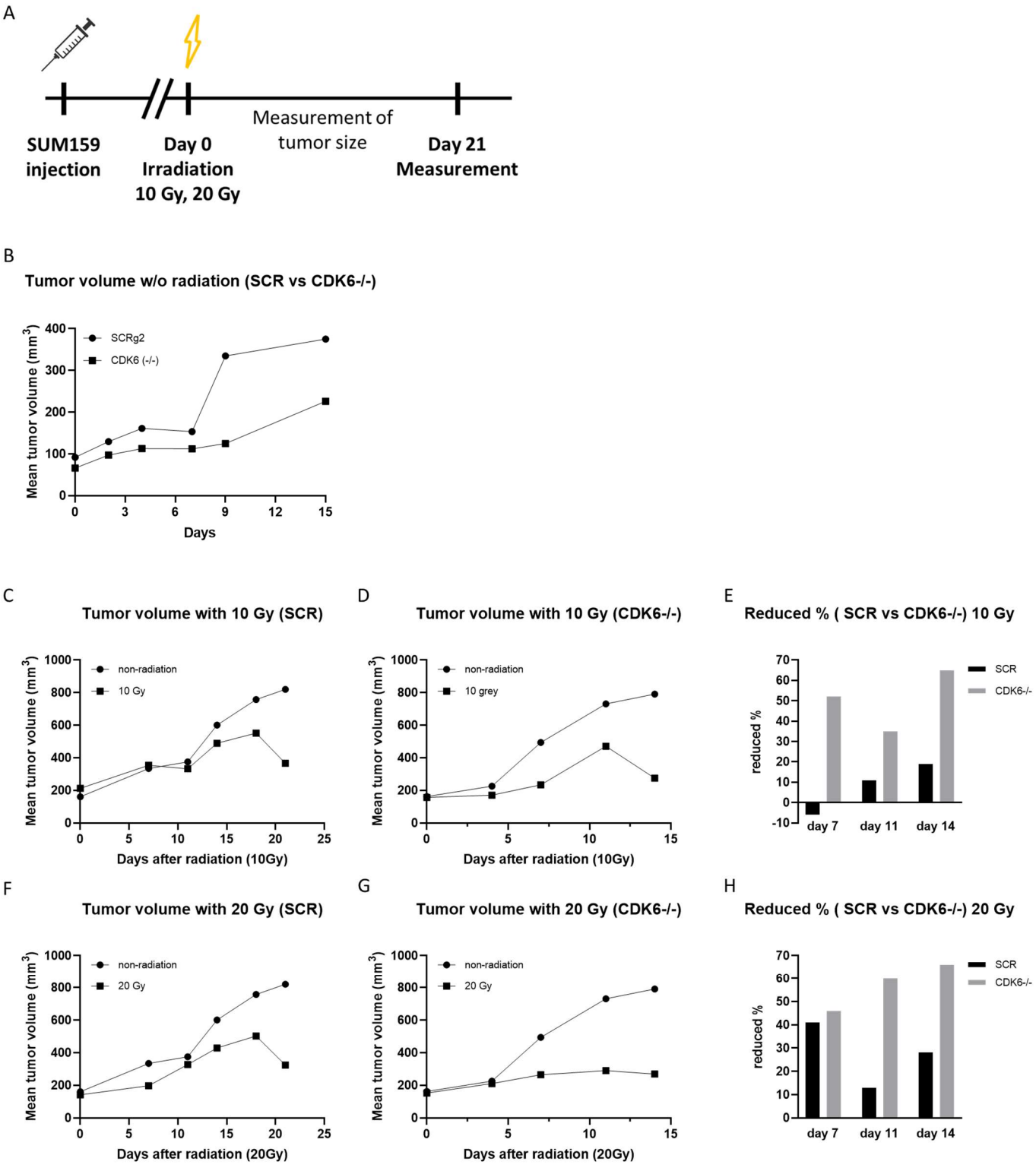

Figure 16: Radiation response in CDK6 KO SUM159 xenografts.

This figure shows tumors that received radiation therapy at the same time, and their

measurement times are also consistent. (A) Experimental scheme. CDK4 or CDK6 KO SUM159 cells were subcutaneously implanted into mice and the animals were treated one time with radiation (single 10 Gy or 20 Gy dose) or controls. (B) Mice without radiation. (SCR and CDK6-/-) CDK6 knockout significantly reduced the tumor size of SUM159 transplanted subcutaneously into the NSG mice. (C,F) Mice treated with 10 Gy or 20 Gy (SCR) showed a reduction in tumor growth when compared with the non-radiation group. (D,G) The growth curves of CDK6 KO tumors receiving 10 Gy or 20 Gy. (E,H) Bar chart represents the percentage of tumor reduction with 10 Gy or 20 Gy on 3 different days.

As shown in figure 17B, the tumor volumes of CDK6 KO without radiation were smaller than the group of SCR significantly on day 7, day 9, and day 15. In general, the CDK6 KO SUM159 tumors are more sensitive to radiation than the SCR group. When the tumor volume of non-radiation versus 10 Gy or 20 Gy radiation, both the group of SCR and CDK6 KO have 2 or 3 measurements of significant reduction. I observed that CDK6 KO tumor suppression effect begins after day 4 for both the 10 Gy and 20 Gy radiation with a maximum of 65% and 66% reduction on day 14 at 10 Gy and 20 Gy, respectively. (Figure 17E, H) In addition, the percentage tumor reduction volume in the CDK6 KO group was much higher than the one in the control SCR group at days 7, 11, and 14 following exposures to 10 Gy or 20 Gy radiation.

3.6 Knockout of CDK4 shows SUM159 sensitivity to radiation with higher dose *in vivo*

We next investigated radiation response in CDK4 KO SUM159 tumors *in vivo*. The data indicated that there was no significant difference in the tumor volumes when comparing the CDK4 KO with SCR with no radiation (Figure 18A). Interestingly, while no significant differences were observed with low radiation doses (10 Gy), the radiation effects were potentiated in the CDK4 KO model, when using 20 Gy radiation level (Figures 18B-18G). These findings indicate that CDK4 plays an essential role in radiation resistance in SUM159, and knocking out its expression could effectively restore SUM159 sensitivity to radiation *in vivo*.

Taken together, the efficacy of combined treatments essentially relates to the doses of radiation cobalt 60 in TNBC cells. The better combined effect with IR was observed to be related to AZD7648 in CDK4/6 KO cells. (Figure 16) These results provide an initial concept to support the development of radiation therapy combinations in TNBC patients, although again, some results only have one biological replicate (n = 1), and hence larger experiments are needed.

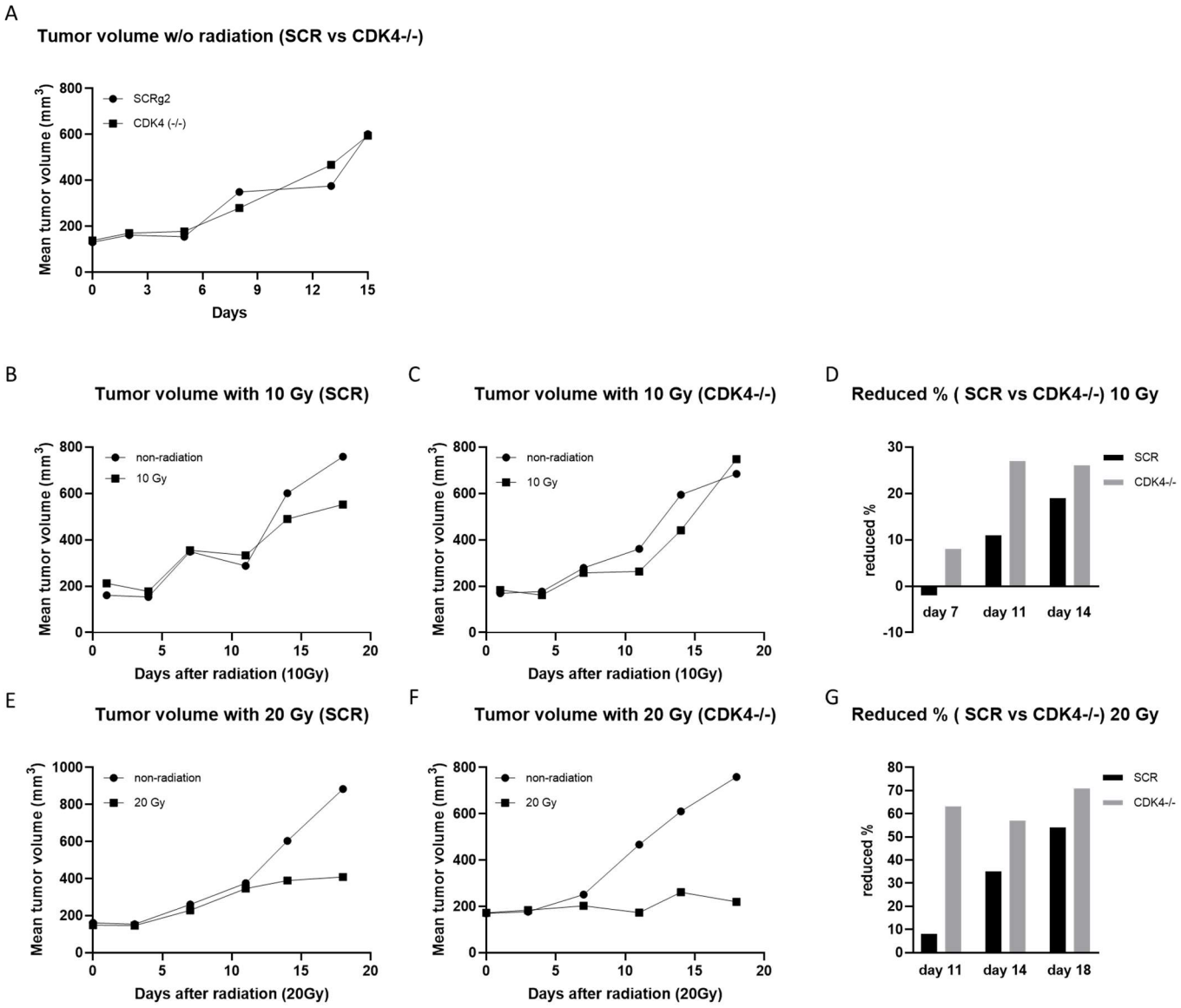

Figure 17: Radiation response in CDK4 KO SUM159 xenografts.

This figure shows tumors that received radiation therapy at the same time, and their measurement times are also consistent. (A) Mice without radiation. (SCR and CDK4-/-) There was no significant decrease in tumor size observed in CDK4 KO. (B, E) Mice treated with 10 Gy or 20 Gy (SCR) showed a reduction in tumor growth when compared with the non-radiation group. (C, F) The growth curves of CDK4 KO tumors receiving 10 Gy or 20 Gy. (D, G) Bar chart represents the percentage of tumor reduction with 10 Gy or 20 Gy on 3 different days.

Next, we would like to discover more potential genes which can radiosensitize SUM159 cells, so that we had a wider range of options when we started our research in earnest. It is currently unknown if deficiencies in DNA DSB repair genes contribute to the onset and development of sporadic breast cancer. One study showed that RAD51 is a potential biomarker and RAD51 expression is increased during breast cancer progression. [136] No one has done a study combining cobalt 60 and RAD51 in TNBC. We hypothesised that aberrant DSB repair with additional ionizing radiation may contribute to better cell survival of TNBC. In addition, since AZD7648 is a potent radiosensitizer *in vitro*, we want to see if a better effect occurs between RAD51 KO and AZD7648 in SUM159 cells.

3.7 Depletion of RAD51 leads to possible sensitization to RT in SUM159 cells after AZD7648 treatment

It has been established that RAD51 is an alternative synthetic lethal target in tumors with BRCA1 mutations and a target in synthetic lethal screening for PARP inhibition in breast cancer. [196] Depletion of RAD51 leads to possible sensitization to radiation treatment for pancreatic cancer and multiple myeloma. [136] Moreover, it has shown that inhibition of DNA-PK combined with RT could increase the time for DSBs and was accompanied by the increase of Rad51 foci. [197] However, there are few research studying the impact of RAD51 on RT on TNBC cells, I evaluated the efficacy of RAD51 KO SUM159 cells in combination with radiation and DNA-PK inhibitor on cell growth. Research of RT with DNA-PK inhibition has not been studied before. I generated individual CRISPR/Cas9 knockouts of RAD51 in SUM159, and the condition set up for RAD51 KO SUM159 cells was also applied on the other KO cell lines we used above.

First, three specific gRNAs targeting RAD51 gene were generated. SUM159 cells were transfected with RAD51 gRNA to determine the gRNA with best efficiency, which was then subsequently used in the experiments that followed. We evaluated the RAD51 knockout efficiency at the protein level using western blot, which showed. The best gRNA was RAD51 gRNA 2 as shown in the figure 19.

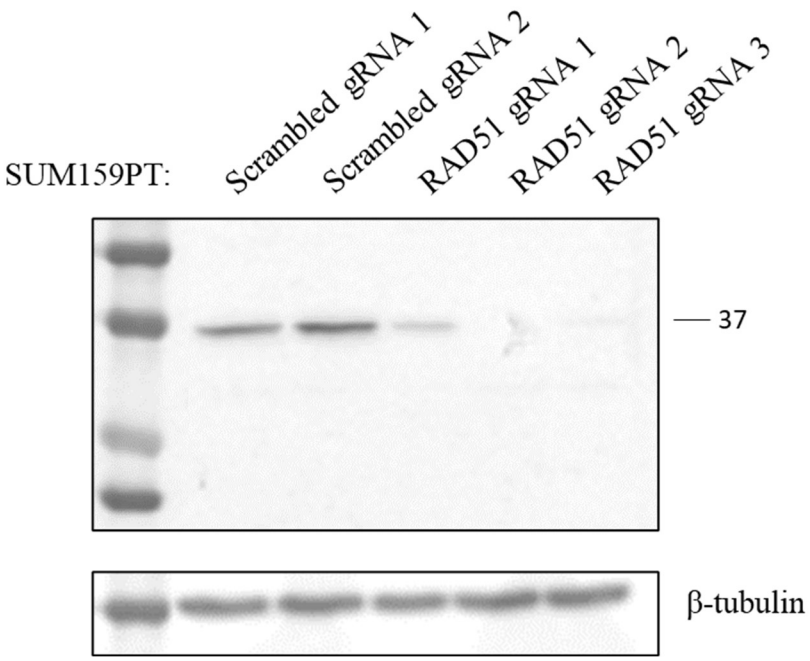

Figure 18: CRISPR knock out of RAD51 in TNBC cells.

SUM159 cells were transfected with specific sgRNA targeted towards RAD51 using Polyethylenimine. Cells were seeded onto 6 well plates for 7 days, lysed and samples analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-RAD51 antibody. β-tubulin was used as a loading control.

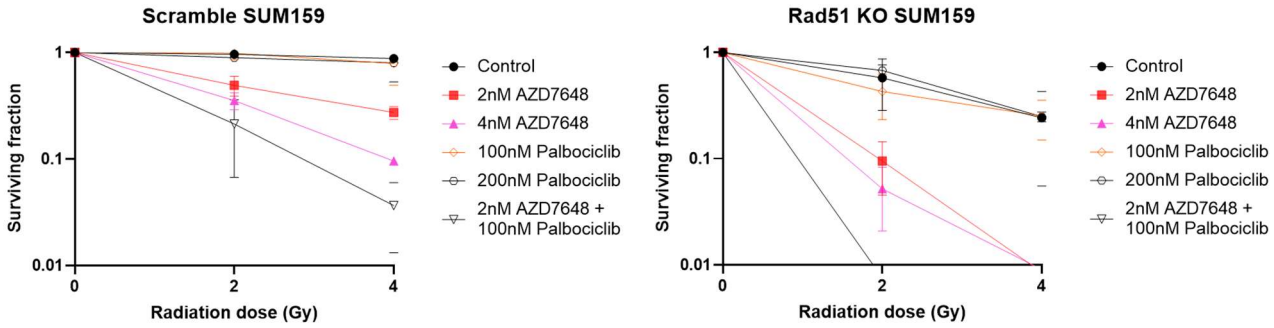

Figure 19: Knockout of Rad51 increases sensitivity of SUM159 cells to radiation and AZD7648 treatments.

Knockout of Rad51 increases sensitivity of SUM159 cells to radiation and AZD7648. Knockout and Rad51 control cells were exposed to the indicated doses of radiation 4h before drugs treatments. After 13 days, the formed colonies were fixed, stained, and counted. The fractional survival curves were calculated from the number of colonies in each dose of radiation. The results are presented as means ± SEM (n=2).

Deficiencies in DNA repair genes are one cause of many cancers, and DNA damage is a critical factor in cancer development. As shown in figure 20B, knock-out of RAD51 results in radiosensitization of SUM159 cells. Compared to the scramble SUM159, RAD51 KO SUM159 treated with 2nM AZD7648 resulted in 70% decrease in cell viability and 64% decrease with 4nM AZD7648 at 2Gy. (Figure 20) 2nM AZD7648 combined with 100nM palbociclib showed 50% decrease in RAD51 KO SUM159 compared to the scramble SUM159 at 2 Gy. When compared to the control in RAD51 KO SUM159 cells (Figure 20B), 2nM AZD7648 combined with 100nM palbociclib at 2Gy showed over 80% decrease in cell viability.

We confirmed the activity of AZD7648 as a potent inhibition of RAD51 KO SUM159 after irradiation in a dose-dependent manner, which has not been studied before. We did not

observe growth inhibition of palbociclib alone treated with radiation in RAD51 KO SUM159. However, palbociclib combined with AZD7648 radiosensitized RAD51 KO SUM159 significantly compared to AZD7648 treatment alone. Consistent with our results, preclinical data suggested that palbociclib combined with RT may have a synergistic effect. [127] Moreover, palbociclib increased the anticancer effect of radiation by delaying DNA DSB repair. [198] A study showed that in SUM159 cells, palbociclib combined with RT reduced the formation of IR-induced RAD51 foci as a functional biomarker of HRR. [86,199]

CHAPTER IV: Discussion and Conclusion

4.1 Discussion

Patients with early TNBC are frequently treated with neoadjuvant chemotherapy, which aims to target DNA repair and cell growth processes. TNBC is made more challenging by 1) the dearth of therapeutic alternatives outside of cytotoxic chemotherapy, and 2) the lack of predictive and prognostic biomarkers to personalized therapy. [200] We concentrated on TNBC in particular because of its aggressiveness and limited therapy choices. Many cancer cells require the phosphorylation of retinoblastoma-associated protein 1 (RB1) by cyclin-dependent kinases (CDK) 4/6 to move from G1 to S phase. The CDK4/6 is known to control the G1 to S phase cell cycle transition. [201] Proliferation is increased as a result of dysregulation of the cyclin D-CDK4/6-INK4-Rb pathway, which is frequently seen in many cancer types. [202] Therefore, the significance of CDK4/6 activity in cancer cells has led to the development of CDK4/6 inhibitors as viable cancer therapeutic options. Although preclinical studies suggest that inhibition of CDK4/6 by palbociclib, prolongs progression-free survival of patients with hormone receptor positive BC [203], studies have shown that BC cells have developed resistance to palbociclib. In addition, basal-like tumors are more likely to be resistant to palbociclib because this subtype expresses the missing RB protein more frequently. [204] It has been shown that palbociclib could reduce cell growth in SUM159 [205], and led to a significant decrease of the stem cell [206]. Since cell cycle arrest can cause cell apoptosis, we hypothesize that CDK4/6 inhibition combined with DNA damage reagent can maximize inhibition of DNA damage repair. [207] Thus, our first goal in designing this study was to assess the effects of radiation on inhibiting CDK4/6 in SUM159 cells. According to previous research, CDK4/6i may be used to suppress the DNA damage response in RB-dependent cancers. [86] However, palbociclib and radiation therapy have not been shown to have clinically significant effects. [208] Second, to clarify the role of CDK4 and CDK6

individually in SUM159 cells and how they respond to radiation treatment, we created CDK4 and CDK6 single KO in SUM159.

Radiation therapy is a crucial part of the multimodal approach to treating breast cancer in women. However, little is known about radiation response among TNBC. Recent clinical evidence leads to the possibility that each phenotype may be accompanied by specific patterns. Data from the Early Breast Cancer Trialists' Collaborative Group suggests that the degree of clinical benefit from radiation differs across ER-positive and ER-negative breast tumor subtypes. [209] SUM159 (basal subtype) is a mesenchymal TNBC cell line which retains RB expression. A study showed that SUM159 cell lines were resistant to radiation therapy compared to other breast cancer cells with different phenotypes. HCC1954 and SUM159 cells underwent G2/M arrest in response to radiation and they held mutant p53, yet MCF7 cells expressing wild-type p53 had G1 and G2/M arrests after radiation. [209] Radiosensitivity can be calculated as the percentage of cells that survive a single 2 Gy dose of radiation (survival fraction at 2 Gy: SF2). Although there are alternative ways to assess the radiation sensitivity of cell lines, the gold standard method is SF2, which has clinical support. [210,211]

We first used radiation to observe the reactivity of SUM159 cells to radiotherapy. We found that SUM159 displays high resistance to radiation therapy, as indicated by a surviving fraction of over 50% at 2 Gy. There hasn’t been extensive research on radiotherapy’s effects on SUM159 cells, despite its known specific lethality to tumor cells. Since SUM159 cells is resistant to radiotherapy, other treatments are needed. Based on our earlier research, inhibiting CDK4/6 could lead to a reduction in cell viability. Therefore, we hypothesize that palbociclib combined with radiotherapy could produce synergistic antitumor effects. Our results suggest that no significant decrease in survival fraction after palbociclib treatment was found. This is consistent with the data published by Pesch et al. [86] Apart from inhibiting CDK4/6, we also used other drugs targeting DNA repair pathway to investigate if there are better results in SUM159

cells.

PARP, poly(ADP-ribose) polymerase, uses nicotinamide adenine dinucleotide (NAD+) as a substrate to form a repair complex, transferring ADP- ribose to target proteins to facilitate the repair of single-strand DNA breaks [212], and PARP inhibitor is known for the treatment of breast cancer having BRCA1/2 inherited gene mutation, which leads to synthetic lethality. Olaparib, which has been demonstrated to enhance the effects of DNA-damaging agents, is a potent inhibitor of PARP1 and PARP2. We therefore used olaparib as a radiosensitizer in combination with radiotherapy for SUM159 cells treatment. Our results suggest that combination treatment of targeting PARP and radiation may reduce cell growth of SUM159 *in vitro*, and it may be a promising therapeutic strategy for TNBC patients.

DNA-PKcs, combines with the Ku80/Ku70 heterodimer to create the active DNA-PK holoenzyme, playing essential roles in DNA damage response (DDR) [213] which is a cellular signaling pathway involved in development of resistance [214] of radiotherapy and chemotherapy of breast cancer. [214,215] The DDR pathway finally converges on the control of CDK activity because CDKs regulate cell-cycle progression. [216] Many DDR-related factors are recruited to DNA damage sites, resulting in the formation of unique DNA damage-induced nuclear foci. [132] A study showed that SUM159 treated with AZD7648 for 1h before radiation caused prolonged DSBs compared to control. [189] Given our previous data, we hypothesize that AZD7648 could radiosensitize SUM159 cells. As shown in our data, AZD7648 combined with radiation caused an obvious decrease in SUM159 cell viability. Moreover, we also found synergistic effects in SUM159 cells when combining AZD7648 with palbociclib and olaparib. Although palbociclib alone with radiotherapy didn't show effects, we found that when palbociclib was combined with AZD7648, it substantially enhanced the effects of radiotherapy. There is currently a lack of literature studying about the combined use of DNA-PK inhibitor and CDK4/6 inhibitor in conjunction with radiotherapy, which is worthy of further discussion. In addition, we showed the

significantly enhanced efficacy of AZD7648 combining radiation and olaparib, which can be a future combination strategy to treat TNBC tumors. Apart from the application of drug inhibitions on SUM159 cells, we hypothesize that inhibition of CDK4/6 and DNA-PK cojoining with knockout of CDK4 or CDK6 could reach better treatment outcome with radiation.

Our investigation into the radiation response of SUM159 breast cancer xenografts has provided valuable insights into the potential therapeutic benefits of combining CDK4/6 inhibition with radiation therapy. Despite the inherent resistance of SUM159 tumors to radiation, as demonstrated by a high TCD50 value [193], our findings indicate that CDK6 or CDK4 knockout significantly enhances the tumor's sensitivity to radiation. Specifically, CDK6 knockout tumors exhibited notable reductions in volume. These results suggest that the disruption of the CDK4/6-RB pathway plays a critical role in radiosensitizing SUM159.

A next step in our research will be to validate the use of RAD51 as a radiosensitizer. RAD51 plays an important role in homologous recombination and RAD51 has been reported to maintain metastasis in TNBC. [217] When compared to SUM149 cells which have BRCA1 mutation, DNA damage significantly increased the number of cells with RAD51 foci in the BRCA1-wild-type SUM159 cell line. Targeting RAD51 may be used as a strategy to increase the sensitivity of PARPi, according to a study demonstrating that RAD51 KO could sensitize SUM159 cells to olaparib *in vitro* and *in vivo*. [141] Furthermore, previous studies have shown that inhibiting DNA-PK sensitizes cells to radiation. [132] Given the role of RAD51 in initiating HR in DSB situations, we hypothesize that inhibiting RAD51 could synergize with DNA-PK inhibitors. In addition, ionizing radiation causes DSB, followed by DDR including ATM/ATR which initiates HR. [139] We first confirmed that the RAD51 KO SUM159 cell lines did not express RAD51 by western blot. Our result suggests that SUM159 cells harboring sgRAD51 treated with radiation showed a decreased number of cells. We found that inhibiting RAD51 could radiosensitize AZD7648 in SUM159 cells. In addition, a recent clinical trial has shown

that palbociclib reduced the induction of RAD51, resulting in decreased HR in locally advanced head and neck squamous cell carcinoma. [218] We hypothesize that palbociclib could also enhance the sensitivity of RAD51 KO SUM159 cells to radiation. However, our study indicates that targeting RAD51 could further enhance the sensitivity of SUM159 cells to radiation and palbociclib but not palbociclib alone. Yet it is still necessary to understand the molecular mechanisms of RAD51 activity. In conclusion, radiation therapy combined with AZD7648 could synergize with RAD51 KO cells. These highlight the potential usage of RAD51 as a target to enhance the effectiveness of radiation.

4.2 Conclusion

Cyclin D-CDK4/6 complexes phosphorylate the RB protein and the CDK4 protein is overexpressed in a large portion of patients with breast cancer. [219] RB1 activity is lost in most basal-like TNBC and hence basal-like BC cell lines are resistant to CDK4/6 inhibitors. [201] However, CDK4/6i exhibits favorable effects when combined with targeted therapies and radiotherapy. Most combined therapy clinical trials are still in progress. [186] Thus, we found that radiotherapy could be a promising combination therapy with CDK4/6i in TNBC *in vitro* and *in vivo*. To attain the best clinical effects, better patient stratification and additional clinical trials will be needed to target the TNBC with the most suitable molecular subtypes.

Our study aimed to explore the effects of combining radiation therapy with CDK4/6 inhibition in SUM159 TNBC cells, which are known to be radiation-resistant. Our findings indicate that while palbociclib alone did not significantly decrease cell viability, combining it with radiation did not produce a clinically significant effect. However, targeting DNA repair pathways, particularly with PARP inhibitors like olaparib and DNA-PK inhibitors such as AZD7648, in combination with radiation showed promising results in reducing SUM159 cell growth. Then, we further investigated the role of RAD51, a key player in HR, as a potential radiosensitizer. Our results suggest that inhibiting RAD51 can enhance the sensitivity of SUM159 cells to radiation, particularly when combined with AZD7648.

Overall, our study provides valuable insights into the potential benefits of combining radiation therapy with CDK4/6 and DNA repair pathway inhibitors in TNBC. These findings suggest that such combination therapies could be a promising strategy to overcome the inherent resistance of TNBC to radiation, thereby improving patient outcomes.

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