

From DEPARTMENT OF ONCOLOGY-PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

**Finding synergies for cancer treatment —
New ways to modulate DNA damage
repair by CX3CR1 and PFKFB3 inhibition**

Jemina Lehto



**Karolinska
Institutet**

Stockholm 2021

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

Printed by Universitetservice US-AB, 2021

© Jemina Lehto, 2021

ISBN 978-91-8016-238-8

Cover illustration: A nucleus (blue) of an osteosarcoma cell treated with Mitomycin C, displaying FANCD2 nuclear foci (violet).

Finding synergies for cancer treatment — New ways to modulate DNA damage repair by CX3CR1 and PFKFB3 inhibition

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Jemina Lehto

The thesis will be defended in public at Air & Fire, SciLifeLab, Tomtebodavägen 23a, Solna
Wednesday, 23rd of June, 2021 at 14:00

Principal Supervisor:

Assistant Professor Nina Gustafsson
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):

Professor Thomas Helleday
Karolinska Institutet
Department of Oncology-Pathology

Dr Ali Moshfegh
Karolinska Institutet
Department of Oncology-Pathology /
Linköping University
LiU Innovation

Dr Li-Sophie Rathje
Karolinska Institutet
Department of Medical Epidemiology and
Biostatistics, Biobank

Opponent:

Assistant Professor Sylvie Noordermeer
Leiden University Medical Center
Department of Human Genetics

Examination Board:

Professor Andrzej Wojcik
University of Stockholm
Centre for Radiation Protection Research,
Molecular Biosciences, The Wenner-Gren Institute

Associate Professor Malin Wickström
Karolinska Institutet
Department of Women's and Children's Health
Division of Paediatric Oncology and Paediatric
Surgery

Associate Professor Leonard Girmata
Karolinska Institutet
Department of Oncology-Pathology

We should always have three friends in our lives: one who walks ahead, who we look up to and follow; one who walks beside us, who is with us every step of our journey; and then, one who we reach back for and bring along after we've cleared the way.

Michelle Obama

POPULAR SCIENCE SUMMARY OF THE THESIS

Despite major advances in cancer treatment during the last century, cancer remains the leading cause of death. Together with surgery, radiotherapy and chemotherapeutics are cornerstones of cancer therapy and nearly all cancer patients receive some of these treatments. Radiotherapy (ionizing radiation) and chemotherapeutics kill cancer cells mostly by damaging the genetic material, the DNA. Cell death following DNA damage is a protective mechanism for the entire organism to prevent the generation of tumors as cancer can arise from mutations caused by DNA damage. Since normal cells are also vulnerable to DNA damage, these cancer therapies can damage healthy cells in the body. Thus, the treatment doses must be kept at a low enough concentration for the normal cells not to be harmed, which often leads to survival of some cancer cells. As a result, these therapies initially kill most of the cancer cells, leading to tumor shrinkage or disappearance, but some more resistant cancer cells may remain in the body and initiate new tumors, which results in relapse. Since the relapsed cancer has developed from the cancer cells that survived the treatment, the new tumor is often resistant to this treatment, and the patient can no longer be treated with the same therapy at safe doses.

Cancer cells can become resistant to DNA damaging treatments by enhancing their DNA repair capacity. Therefore, targeted therapies are being developed which block the drivers of this enhanced DNA repair activity of cancer cells, and thus sensitize them to DNA damaging treatments while sparing normal cells. This thesis work focused on investigating the role of two proteins, CX3CR1 and PFKFB3, in DNA repair in cancer cells. The aim was to determine their molecular function and whether blocking of these cancer targets would impair DNA repair and sensitize them to DNA damaging chemo- (*i.e.* platinum drugs) and radiotherapy. Due to the lack of drugs blocking PFKFB3, we developed KAN0438757, a drug targeting PFKFB3. Along with KAN0438757, we evaluated if KAND567, a drug blocking CX3CR1, could be combined with platinum drugs and ionizing radiation to kill cancer cells.

We reveal that PFKFB3 has a role in a certain type of DNA repair mechanism called homologous recombination repair and that inhibition of PFKFB3 by KAN0438757 blocks this repair and sensitizes cancer cells to ionizing radiation. Furthermore, we discovered that blocking CX3CR1 with KAND567 reduces survival of ovarian cancer cells, impairs their DNA replication, and has potential to sensitize cancer cells to ionizing radiation and platinum drugs. We continued this investigation by showing that CX3CR1 blockage leads to sensitization of especially platinum resistant cancer cells to platinum, without affecting normal cells. We discover that CX3CR1 regulates a specific DNA repair pathway, the Fanconi Anemia (FA) pathway, which repairs DNA damage caused by platinum drugs. Finally, we reveal that platinum resistant cancer cells are re-sensitized to platinum upon PFKFB3 inhibition. We further identify that PFKFB3 interacts with DNA repair proteins in the FA pathway and is key to establish a functional FA repair pathway. In summary, these studies reveal novel functions for CX3CR1 and PFKFB3 in DNA repair pathways in cancer cells and demonstrate that blocking their function by using targeted drugs results in cancer-specific sensitization to DNA damaging anti-cancer treatments, even in treatment-resistant cancer cells.

ABSTRACT

The goal of targeted cancer therapy is to selectively kill cancer cells based on their molecular survival mechanisms. DNA repair is as a promising cancer target as many cancers have chronic replication stress and deficiencies in the DNA damage response. Moreover, combining DNA damaging chemo- and radiotherapy with inhibitors of DNA repair can lead to improved treatment responses, reduced resistance to treatments, as well as lowering of effective doses and thereby reduced toxicity to healthy tissues. In this thesis, two cancer targets, CX3CR1 and PFKFB3, were investigated for their emerging roles in DNA repair. Furthermore, small molecule inhibitors KAN0438757, developed in **Paper I** to target PFKFB3, and KAND567 targeting CX3CR1, were evaluated in combination treatments with ionizing radiation (IR) and platinum drugs *in vitro*.

In **Paper II** and **III** we characterize the role of CX3CR1 in the DNA damage response. We reveal that CX3CR1 inhibition by KAND567 reduces cancer cell survival and impairs DNA replication, reducing RPA and ATR activation (**Paper II**). CX3CR1 inhibition increases DNA damage levels and S phase arrest when combined with platinum drugs, resulting in reduced cancer cell survival at doses not affecting non-transformed cells (**Paper II and III**). Mechanistically, we reveal that upon DNA damage induction CX3CR1 is relocated to the nucleus and regulates interstrand crosslink (ICL) repair by facilitating the recruitment of the key repair proteins in the Fanconi Anemia (FA) repair pathway, FANCD2 and FANCI, to the chromatin (**Paper III**). Notably, CX3CR1 inhibition sensitizes cancer cells to platinum treatment and especially platinum resistant cancer cell lines demonstrate good synergy for this combination treatment (**Paper III**).

In **Paper I** and **IV**, we identify novel roles for PFKFB3 in regulating DNA repair. We show that PFKFB3 locates to DNA damage sites upon IR and PFKFB3 inhibition results in impairment of DNA double-strand break repair by homologous recombination (HR). Mechanistically, PFKFB3 triggers recruitment of RRM2, responsible of local nucleotide supply, and the HR factors, RPA and RAD51, to DNA damage sites, to allow for DNA repair (**Paper I**). Moreover, we develop a selective small molecule inhibitor, KAN0438757, that targets PFKFB3 and selectively radiosensitizes transformed cells (**Paper I**). In **Paper IV**, we discover a role for PFKFB3 in FA repair upon ICL induction in cancer cells. We demonstrate that PFKFB3 associates to the chromatin following treatment with ICL-inducing agents and regulates establishment of the FA repair pathway, needed for initiation of ICL repair. Importantly, we demonstrate that PFKFB3 inhibition synergizes with platinum treatments in blocking proliferation of transformed cells.

In summary, our work identifies novel roles of CX3CR1 and PFKFB3 in DNA repair processes critical for cancer cell survival following treatment with DNA damaging agents.

LIST OF SCIENTIFIC PAPERS

I. **Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination.**

Nina M.S. Gustafsson, Katarina Färnegårdh, Nadilly Bonagas, Anna Huguet Ninou, Petra Groth, Elisee Wiita, Mattias Jönsson, Kenth Hallberg, **Jemina Lehto**, Rosa Pennisi, Jessica Martinsson, Carina Norström, Jessica Hollers, Johan Schultz, Martin Andersson, Natalia Markova, Petra Marttila, Baek Kim, Martin Norin, Thomas Olin, Thomas Helleday.
Nature Communications 2018, 9(1):3872.

II. **Blocking the fractalkine receptor disrupts replication and ovarian cancer cell proliferation.**

Jemina Lehto, Anna Huguet Ninou, Petra Marttila, Emma Åkerlund, Greta Gudoityte, Ulrika Joneborg, Joseph Carlson, Brinton Seashore-Ludlow, Nina M.S. Gustafsson.
Manuscript.

III. **Targeting CX3CR1 suppresses the Fanconi Anemia DNA repair pathway and synergizes with platinum.**

Jemina Lehto, Anna Huguet Ninou, Dimitrios Chioureas, Jos Jonkers, Nina M.S. Gustafsson.
Cancers 2021, 13(6):1442.

IV. **PFKFB3 regulates repair of DNA interstrand crosslinks via modulation of the Fanconi Anemia repair pathway.**

Anna Huguet Ninou, **Jemina Lehto**, Dimitrios Chioureas, Hannah Stigsdotter, Korbinian Schelzig, Emma Åkerlund, Greta Gudoityte, Ulrika Joneborg, Joseph Carlson, Jos Jonkers, Brinton Seashore-Ludlow, Nina M.S. Gustafsson.
Manuscript.

CONTENTS

1	INTRODUCTION.....	1
1.1	Therapeutic approaches in cancer treatment.....	1
1.1.1	DNA crosslinkers.....	3
1.1.2	Ionizing radiation.....	3
1.1.3	Synthetic lethality.....	4
1.1.4	Drug synergism.....	6
1.1.5	Case: Ovarian cancer.....	6
1.2	The DNA damage response.....	7
1.2.1	Recognition and signaling of DNA damage.....	7
1.2.2	Homologous recombination.....	10
1.2.3	The Fanconi Anemia pathway.....	11
1.2.4	Replication stress.....	13
1.3	CX3CR1: Emerging roles for a chemokine receptor.....	15
1.3.1	CX3CR1 in cancer.....	16
1.3.2	CX3CR1 in DNA repair.....	18
1.3.3	CX3CR1 as a therapeutic target.....	18
1.3.4	KAND567.....	19
1.4	PFKFB3: A bifunctional enzyme at the crossroads of metabolism and DNA repair.....	20
1.4.1	PFKFB3 in cancer.....	21
1.4.2	PFKFB3 as a therapeutic target.....	22
2	RESEARCH AIMS.....	23
3	METHODOLOGICAL APPROACHES.....	25
3.1	Drug synergy studies.....	25
3.2	Colony-formation assay.....	25
3.3	Subcellular protein fractionation.....	26
3.4	Detection of nuclear repair foci by confocal microscopy.....	26
3.5	Generation of knockdown cell lines by lentiviral transduction.....	27
3.6	DNA fiber assay.....	27
3.7	Flow cytometry analysis of cell cycle progression of replicating cells.....	28
3.8	Ethical considerations.....	28
4	RESULTS.....	29
4.1	Paper I.....	29
4.2	Paper II.....	31
4.3	Paper III.....	32
4.4	Paper IV.....	33
5	DISCUSSION.....	35
5.1	CX3CR1.....	35
5.1.1	The nuclear role of CX3CR1.....	36
5.1.2	The MAPK/ERK pathway.....	37
5.1.3	The PI3K/Akt pathway.....	38
5.1.4	β -arrestins.....	39
5.1.5	CX3CR1 and replication.....	39
5.2	PFKFB3.....	40
5.2.1	KAN0438757 as a selective PFKFB3 inhibitor.....	40
5.2.2	Nuclear and cytoplasmic roles of PFKFB3 in DNA repair and replication.....	41
6	CONCLUSIONS AND FUTURE PERSPECTIVES.....	45
7	ACKNOWLEDGEMENTS.....	47
8	REFERENCES.....	51

LIST OF ABBREVIATIONS

53BP1	Tumor suppressor p53-binding protein 1
Akt	The Ser/Thr kinase AKT
alt-NHEJ	Alternative non-homologous end-joining
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
BLM	Bloom helicase
BER	Base-excision repair
BRCA1/2	Breast and ovarian cancer susceptibility protein 1/2
CBP	<i>Cis</i> -diammine(1,1-cyclobutanecarboxylato)platinum(II); carboplatin
CDDP	<i>Cis</i> -diamminedichloroplatinum(II); cisplatin
CDK	Cyclin dependent kinase
CHK1/2	Checkpoint kinase 1/2
CtIP	C-terminal binding protein 1 interacting protein
CX3CL1	C-X3-C motif chemokine ligand 1 / fractalkine
CX3CR1	C-X3-C motif chemokine receptor 1 / fractalkine receptor
DDR	DNA damage response
DMR	Dynamic mass redistribution
DNA-PK(cs)	The DNA-dependent protein kinase (catalytic subunit)
dNTP	Deoxynucleoside triphosphate
DSB	DNA double-strand break
dsDNA	Double-strand DNA
EdU	5-ethynyl-2'-deoxyuridine
EGFR	Epidermal growth factor receptor
EOC	Epithelial ovarian carcinoma
ERK	Extracellular signal-regulated kinase
F2,6BP	Fructose-2,6-bisphosphate
FA	Fanconi Anemia
FAAP	Fanconi Anemia core complex associated protein
FANCD2	Fanconi anemia complementation group D2
FANCI	Fanconi anemia complementation group I
FANCM	Fanconi anemia complementation group M
FDA	Food and Drug Administration
GPCR	G protein-coupled receptor
HGSOC	High-grade serous ovarian cancer
HIF-1 α	Hypoxia-inducible factor
HR	Homologous recombination
HU	Hydroxyurea
ICL	Interstrand crosslink

ID2	FANCI-FANCD2 complex
IR	Ionizing radiation
MAPK	Mitogen-activated protein kinase
MDC1	Mediator of DNA damage checkpoint protein 1
MMC	Mitomycin C
MRN	MRE11-RAD50-NBS1 complex
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor NF-kappa-B
NHEJ	Non-homologous end-joining
NER	Nucleotide-excision repair
PARP	Poly (ADP-ribose) polymerase 1
PCNA	Proliferative nuclear antigen
PFK-1	Phosphofructokinase 1
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
PI3K	Phosphoinositide 3-kinase
PIKK	PI3-kinase-related kinase
p53	Tumor Protein P53
pRb	Retinoblastoma protein
RAD50	RAD50 double-strand break repair protein
RAD51	DNA repair protein RAD51 homolog 1
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
RPA	Replication protein A
RRM1	Ribonucleotide Reductase Catalytic Subunit M1
RRM2	Ribonucleotide Reductase Regulatory Subunit M2
UV	Ultraviolet
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
SSB	DNA single-strand break
ssDNA	Single-strand DNA
TLS	Translesion synthesis
γH2AX	Histone H2AX phosphorylated at serine 139

1 INTRODUCTION

The start of modern cancer research begun in the early 1900s and grew rapidly towards to the end of the century, resulting in expansion of treatment options and the discovery of cancer-causing genes¹. The 21st century marks the beginning of the era of targeted therapies with an increasing amount of cancer drugs brought into the clinic¹. Cancer survival rates have dramatically increased over the decades due to advanced treatment options and screening programs allowing early detection of malignant lesions but, although some cancers are now curable, we still lack effective treatment options for many cancers. Today, personalized medicine approaches and analyses of whole cancer genomes by international research consortiums, along with improved methods to study cancer vulnerabilities, has led the way to a better understanding of this complex group of diseases with the aim to improve treatment responses and quality of lives².

1.1 THERAPEUTIC APPROACHES IN CANCER TREATMENT

Cancer is often characterized by genome instability which is one of the enabling characteristics of cancer, as reviewed by Hanahan and Weinberg (2011)³. Along the progression of cancer, mutations occurring in the genome maintenance and surveillance systems endanger the integrity of the genome. This genome instability results in new mutations and genomic rearrangements driving cancer progression via selection of favorable phenotypes. However, genomic instable cancer cells are more vulnerable to DNA damage than non-malignant cells since their DNA repair machinery often is deficient⁴. This cancer cell vulnerability, along with the high proliferation rate of cancer cells, enables the usage of DNA damaging cancer treatments (Figure 1) such as ionizing radiation (IR) and chemotherapy, which have been used in cancer treatment for nearly a century^{4,5}. However, genome instability in cancer is more elegantly harnessed by synthetic lethality approaches, described in Chapter 1.1.3, which target cancer cells specifically by directly interfering with drivers of cancer cell survival⁶.

Chemotherapeutics are a diverse group of cytotoxic drugs with different, and usually with multiple, mechanisms of action⁷. This thesis work focused on studying the DNA crosslinkers platinum (cisplatin and carboplatin) and mitomycin C (MMC) chemotherapeutics as well as IR in synergistic treatments with small molecule inhibitors that target DNA repair. These cytotoxic agents distort the cells' DNA in multiple ways, resulting in DNA damage which activates several DNA repair pathways (Table 1)⁸⁻¹². The main aspects of DNA crosslinkers and IR in relation to DNA damage will be reviewed in this chapter, followed by introduction to the principles of synthetic lethality and drug synergy. Finally, ovarian cancer will be used as an example, highlighting the clinical need for innovative treatment approaches.

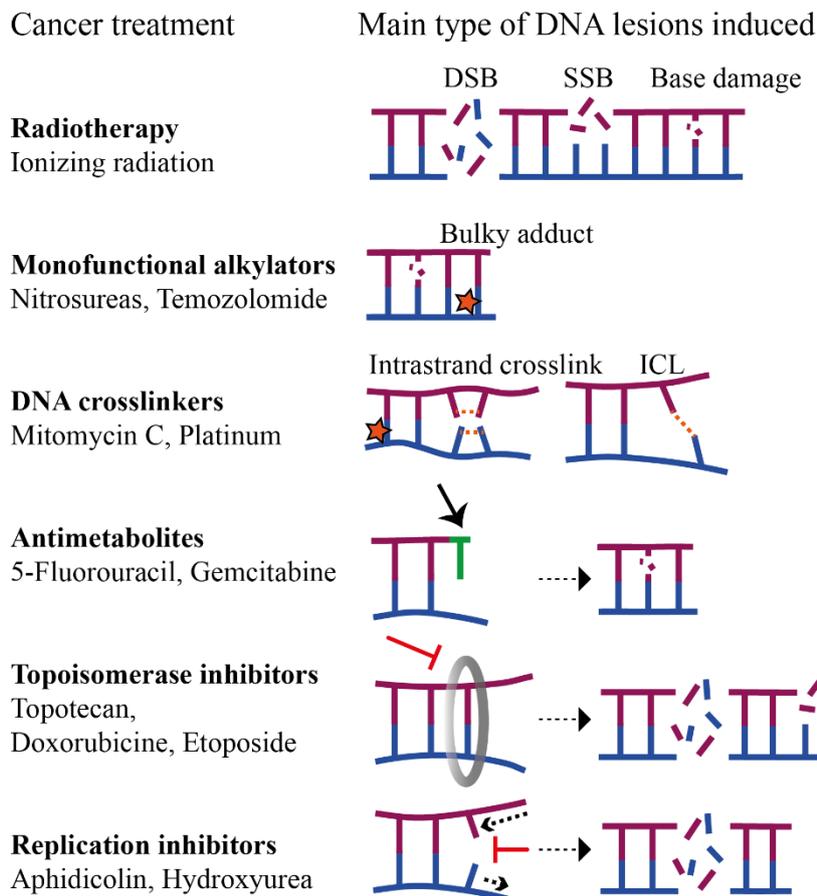


Figure 1. Examples of DNA-damaging anti-cancer treatments and the main types of DNA lesions they induce. In addition, all the chemotherapeutics interfere with DNA replication. This thesis focuses on radiotherapy and DNA crosslinkers (platinum drugs and Mitomycin C). The DNA damage caused by these agents is described in more detail in Table 1. DSB=DNA double-strand break; SSB=DNA single-strand break; ICL=Interstrand crosslink. Figure created based on the reviews by Helleday et al. (2008) and Jackson&Bartek (2009)^{5,13}.

Agent	DNA Damage	Main repair pathways activated
DNA crosslinkers:	ICLs (P: 2-5 %, M: 5-13 %)	Fanconi Anemia pathway (ICLs)
Platinum	Intrastrand crosslinks	<i>Nucleotide excision repair</i>
Mitomycin C	Monoadducts	<i>Translesion synthesis</i>
	DNA-protein crosslinks	<i>Homologous recombination</i>
	Base damage	Non-homologous end-joining
		Mis-match repair
		Replicative bypass
Ionizing radiation	Double-strand breaks (40) Single-strand breaks (500-1000) Base damage (1000-2000) DNA crosslinks (30) DNA-protein (150) Sugar damage (800-1600)	Homologous recombination (DSBs) Non-homologous end-joining (DSBs) alt-NHEJ Single-strand annealing Base excision repair Nucleotide excision repair

Table 1. Various types of DNA damage caused by DNA crosslinkers (platinum and Mitomycin C) and ionizing radiation. The most relevant DNA damage responsible of cytotoxic effects and main repair pathways are highlighted in **bold**. *Cursive* displays different repair pathways involved in Fanconi Anemia repair of ICLs. The brackets show the amount of break/damage caused by one Gy of ionizing radiation and the % of ICLs caused by DNA crosslinkers of all crosslinks. Gy=Grey, ICL=interstrand crosslink, DSB=DNA double-strand break, SSB=DNA single-strand break, alt-NHEJ=alternative non-homologous end-joining, P=platinum, M=Mitomycin C. Table created based on references:⁸⁻¹².

1.1.1 DNA crosslinkers

Platinum drugs are some of the most commonly used anticancer drugs¹⁴. They react with the bases of DNA, resulting in DNA lesions and generation of DNA adducts which distort the structure of DNA, inhibiting replication and transcription, and activating multiple DNA repair mechanisms (Table 1)^{8,9,15}. Intrastrand crosslinks comprise 90 % of the crosslinks and occur most frequently between adjacent purine residues on the same DNA strand¹⁶. In addition, platinum treatment results in the formation of DNA monoadducts, interstrand crosslinks (ICL) and DNA-protein crosslinks⁹. ICLs link two opposite DNA strands together, preventing their separation with irreversible covalent bonds. They constitute up to 5 % of the platinum damage but are the major cytotoxic lesion^{17,18}.

ICLs are considered the most severe type of lesion as they cause the replication machinery to stall while an intrastrand crosslinks can be bypassed by DNA polymerases¹⁵. ICLs are detected and removed via the Fanconi Anemia (FA) pathway in order to allow for replication to resume^{15,18}. FA repair of ICLs involves coordination of multiple DNA repair pathways described in detail in Chapter 1.2.3¹⁵. The cellular effects of platinum compounds are not limited to direct DNA damage—they also induce reactive oxygen species (ROS), including free radicals, which can cause damage to components of the cell by peroxidation of lipids and to the DNA through nucleic acid damage⁸. Moreover, platinum compounds can modulate apoptosis and survival signaling pathways and alter gene expression, some of which can contribute to development of platinum resistance in cancer cells⁸.

Cisplatin (*cis*-diamminedichloroplatinum(II)) and its newer derivative carboplatin (*cis*-diammine(1,1-cyclobutanecarboxylato)platinum(II)) generate intra- and interstrand crosslinks in similar ratio, but cisplatin is more reactive and has faster DNA binding kinetics than carboplatin⁸. In addition, carboplatin is retained longer in the body than cisplatin and has generally less side effects, which makes it more suitable for high-dose chemotherapy than cisplatin^{8,19}. Carboplatin is preferred over cisplatin in the treatment of ovarian cancer, but due to lower efficacy in many other cancers, cisplatin is still widely used in anticancer treatment¹⁹.

Mitomycin C (MMC) belongs to a group of cancer drugs called anti-cancer antibiotics due to the fact that it is naturally produced by the bacterium *Streptomyces caespitosus*. Following an enzymatic reduction of MMC, it is a potent crosslinker of DNA and causes ICLs by *N*-alkylation of nucleosides²⁰. It is estimated that the ICLs consists about 5-13 % of all lesions induced by MMC, which is about twice the amount of ICLs caused by platinum¹⁸. In addition to ICLs, MMC can also form DNA monoadducts²¹ and generate highly reactive free radicals^{20,22} (Table 1). MMC is used in the treatment of esophageal and bladder cancer but bone marrow toxicity (leukopenia and thrombocytopenia) limits its use^{7,15}.

1.1.2 Ionizing radiation

Radiation therapy is the most common cancer treatment after surgery. About 50 % of cancer patients receive radiation therapy and it is estimated to constitute 40 % of the curative cancer modalities²³. Radiation therapy can be used as neo-adjuvant therapy (before surgery) or as

adjuvant therapy to kill remaining cancer cells and is administered as external beam radiation or as brachytherapy (internal radiation)²³.

Radiation therapy uses ionizing radiation to deliver electrically charged particles (electrons or ions) to the tumor site¹¹. This high energy radiation results in various types of DNA lesions (Table 1), either via direct ionization of DNA molecules or via formation of free radicals. Of these DNA lesions, DNA double-strand breaks (DSBs) are considered the most lethal¹². DNA DSBs are mainly repaired via the HR or non-homologous end-joining (NHEJ) DNA repair pathways, which are described in more detail in Chapter 1.2. However, if the damage level is beyond repair, cells undergo apoptosis or senescence¹¹. Radiosensitivity of a tumor depends on underlying mutations in DNA repair genes, activity of survival pathways and tumor suppressors, as well as the tumor microenvironment¹¹. Due to radiation resistance and toxicity to normal cells, combination treatments sensitizing cancer cells to ionizing radiation or protecting normal tissue hold potential to improve clinical outcomes²⁴.

1.1.3 Synthetic lethality

A focus of current cancer research is to find specific molecular mechanisms that cancer cells uniquely depend on for survival. The idea behind targeted therapies is that these anti-cancer targets are either not expressed in most non-malignant cells, or that they are especially vital for cancer cells but not for healthy cells⁴. Thus, an optimal targeted therapy gives clinicians a large therapeutic window to treat cancer. One of the most effective targeted therapy approaches is the synthetic lethal therapy (Figure 2)⁶.

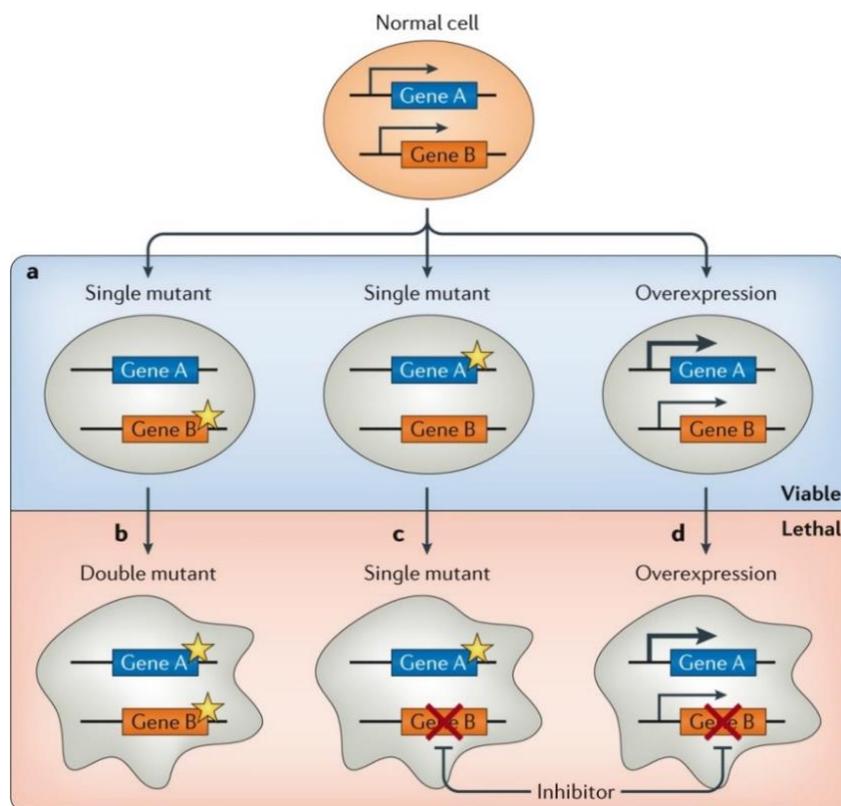


Figure 2. The basic principle of synthetic lethality in cancer. Synthetic lethality can be achieved by mutation of two genes (b) or by pharmaceutical inhibition of “Gene B” in a single mutant cell (c). Also overexpression of “Gene A” can lead to synthetic lethality with inhibition of “Gene B” (d)⁶. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Genetics, Synthetic lethality and cancer, Nigel J. O’Neil, Melanie L. Bailey and Philip Hieter, Copyright © 2017.

The term synthetic lethality was first presented in the context of genetics for two genes that, when both mutated, are lethal for the organism although mutation of one is harmless^{25,26}. Today, the synthetic lethality principle in cancer comes from the realization that cancers are often dependent on certain process or pathway due to disturbance of another parallel process, for example resulting from a mutation of a gene^{6,27}. Therefore, inhibiting the remaining functional pathway will result in cancer cell death (Figure 2). Furthermore, synthetic lethality can be expanded to entail conditional synthetic lethality, which means that certain conditions such as hypoxia or metabolic changes enable synthetic lethal interactions. On the other hand, synthetic cytotoxicity specifies a situation where a targeted therapy results in cell death in cells with certain genetic alterations in combination with a DNA damaging agent⁶.

The synthetic lethality approach in cancer treatment is best exemplified by PARP inhibitors (Figure 3). The breast/ovarian cancer susceptibility genes 1 and 2 (*BRCA1* and *BRCA2*), which are commonly mutated in hereditary breast and ovarian cancer, play a key role in HR repair^{28,29}. PARP proteins bind DNA single-strand breaks (SSB) and recruit repair factors in several repair pathways such as base-excision repair (BER) and nucleotide excision repair (NER)³⁰. Consequently, inhibition of PARP impairs the repair of SSBs which will be converted to DSBs. However, *BRCA1*- or *BRCA2*-mutated cells are unable to repair these DSBs via HR, which renders cells deficient in both HR and SSB repair leading to cancer cell death due to unresolved DSB damage (Figure 3)⁶. Moreover, PARP modulates the replication fork progression, and inhibition of PARP results in increased speed of the replication fork elongation, causing replication stress³¹. PARP inhibitors also trap PARP to the DNA, making PARP unable to dissociate which interferes with replication and potentiates the cytotoxic effects of PARP inhibitors enabling the use of PARP inhibitors beyond *BRCA*-mutated cancers^{32,33}. Repair of PARP-DNA complexes requires repair pathways such as FA and HR and factors required for removal of DNA adducts³⁴.

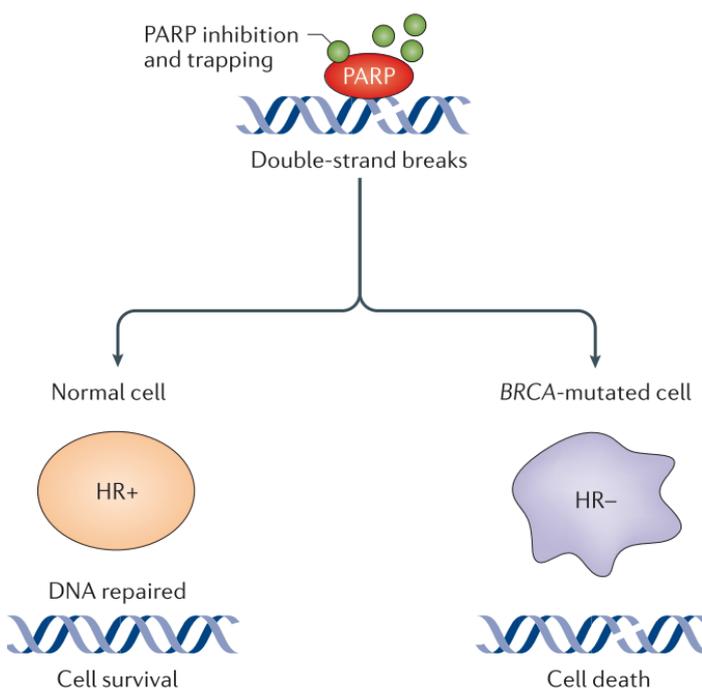


Figure 3. Principle of the use of PARP inhibitors in *BRCA*-mutated cancers. PARP inhibition leads to ineffective repair of DNA single-strand breaks which are then converted to DNA double-strand breaks and repaired via HR. *BRCA*-mutated cells cannot use HR which results in cell death. In addition, PARP trapping leads to improved efficacy of PARP inhibitors⁶. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Genetics, Synthetic lethality and cancer, Nigel J. O’Neil, Melanie L. Bailey and Philip Hieter, Copyright © 2017.

1.1.4 Drug synergism

Drug combinations are widely used in cancer treatment to increase the therapeutic responses and to combat the development of cancer drug resistance. Since new drug combinations can be tested only in limited amount of clinical trials and *in vitro* screens, rational design of drug combinations is needed³⁵. Drug synergy in cancer treatment can be achieved by classical synthetic lethal interactions by targeting complementary pathways or processes of cancer cells with deficiency in a parallel mechanism, as summarized in the previous chapter³⁶. However, also targeting the same pathway can result in more complete response than either drug alone leading to synergistic effects³⁷. Drug synergy is more than the additive effect of two drugs and several mathematical methods have been developed that aim to determine whether a combination effect on cancer cell phenotype is truly synergistic and not a mere additive effect of two drugs when drug combinations are tested *in vitro*³⁸. Chapter 3.1 summarizes the method used in this thesis to measure drug synergy in cell viability assays.

In addition to pure *in vitro* screens, semi-computational methods to predict drug synergies have been developed. Example of this is Cancer Drug Atlas, that was successfully used to predict drug synergies based on single-compound drug response data matched with corresponding molecular mechanism of drug sensitivity³⁷. The benefit of this predictive approach is especially highlighted in the ability to detect multi-drug synergies, that are impossible to test with non-computational methods due to the large number of possible combinations.

1.1.5 Case: Ovarian cancer

Ovarian cancer is an example of a highly lethal cancer that lacks effective treatment options. More than 50 % of ovarian cancers are detected at a late stage, which partly explains the poor overall survival rate of 40 %³⁹⁻⁴¹. Although 80 to 90 % of ovarian cancer patients respond to first line therapy, 75 % of them relapse within 18 months with insensitivity to the same chemotherapy⁴². A majority ovarian cancers are defined as epithelial ovarian carcinoma (EOC) which can be further divided in histotypes⁴³. High-grade serous ovarian cancer (HGSOC) is a histotype that accounts for about 70 % of all ovarian cancer cases and has the worst prognosis⁴⁴. Approximately 15 % of EOC arise from hereditary preposition⁴⁰. Of these, 75 % are caused by mutations in *BRCA1* and *BRCA2*⁴⁵⁻⁴⁷. In HGSOC the *BRCA1/2* mutation rate can be as high as 22 %⁴⁸. In addition, all HGSOC patients are evaluated to have a deficiency at least in one main DNA repair pathway⁴⁹. Due to deficiency in DNA repair genes and mutations in the tumor suppressor gene *TP53*, HGSOC is characterized by high genomic instability, which increases the aggressiveness of tumors and development of drug resistance⁵⁰. On the other hand, in the presence of DNA repair deficiencies, the repair capacity of another, complementary, DNA repair pathway can be enhanced by overexpression of repair proteins, which contributes to treatment resistance⁴⁹. Mutated *TP53* and activation of phosphoinositide 3-kinase (PI3K) pathway are common early events in HGSOC⁵¹.

Despite various histological and molecular differences between histotypes, ovarian cancer is mostly treated as a single disease. Standard treatment after surgery involves chemotherapy with

platinum drugs (cisplatin, carboplatin) and the microtubule-binding agent paclitaxel⁵². In addition, the anti-angiogenic monoclonal antibody bevacizumab is used during, and after, first line chemotherapy⁵³. As a second-line therapy, after relapse and platinum resistance, topotecan, doxorubicin, etoposide, and gemcitabine (Figure 1) can be used³⁴. Due to resistance and toxic side-effects of the current therapies, targeted and personalized therapies alongside with early detection, are urgently needed. A successful example of targeted therapies based on molecular characteristics of ovarian cancer is the approval of the PARP inhibitor olaparib for the treatment of *BRCA1/2* germline mutated ovarian cancers by Food and Drug Administration (FDA) in 2014^{54,55}. To date, two additional PARP-inhibitors, rucaparib and niraparib, are approved for the treatment of ovarian cancer^{56,57}. Other PARP inhibitors, veliparib and talazoparib, have not been approved for ovarian cancer treatment yet, but their use is under clinical investigation^{58,59}.

1.2 THE DNA DAMAGE RESPONSE

The genome is constantly facing endogenous and exogenous insults for example through intrinsic DNA replication errors, ROS, ultraviolet (UV) radiation and environmental toxins. The DNA damage response (DDR) is an evolutionary conserved signaling cascade that detects, signals, and repairs DNA damage in cells while cell cycle progression is halted⁵. If the damage is beyond repair, cell initiates apoptosis in order to avoid chromosomal aberrations, which could for example lead to the onset of cancer⁶⁰. This chapter describes the DDR process, highlighting aspects related to the recognition and repair of DNA damage caused by platinum and IR treatments, connecting these processes into understanding of synergistic treatment opportunities and treatment resistance in cancer discussed in last chapters of this thesis.

1.2.1 Recognition and signaling of DNA damage

The components of the DDR can be divided into DNA damage sensors, adaptors / mediators and downstream transducers and effectors⁵. PI3 kinase-related kinases (PIKKs), Ataxia telangiectasia and Rad3 related (ATR), ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK), are key transducers of DNA damage signaling orchestrating the control of DNA repair and cell cycle progression (Figure 4)⁶¹. Different PIKKs are recruited to the DNA damage sites depending on the cell cycle phase and the nature of the DNA damage. ATR responds to ssDNA, which is often generated upon replication stress deriving from replication impairments⁶¹. On the other hand, ATM and DNA-PK are activated by DSB formation⁶².

In the canonical mode of action, ATR is recruited to replication protein A (RPA) at the ssDNA via the ATR-interacting protein (ATRIP)⁶³. RPA is important factor in the protection of ssDNA and upon ssDNA formation, RPA readily coats it and protects the ssDNA from degradation and from the formation of secondary structures⁶³. ATR is subsequently activated by DNA topoisomerase 2-binding protein 1 (TopBP1) or Ewing Tumor Associated Antigen 1 (ETAA1), and further phosphorylates the adaptor claspin which mediates the phosphorylation of the effectors such as checkpoint kinase 1 (CHK1) which elicits cell cycle arrest at different cell cycle checkpoints to allow time for DNA repair⁶⁴. A complex called 9-1-1, which consists of

RAD9, RAD1 and HUS1 proteins, is involved in the TOPBP1-mediated activation of ATR⁶⁵⁻⁶⁷.

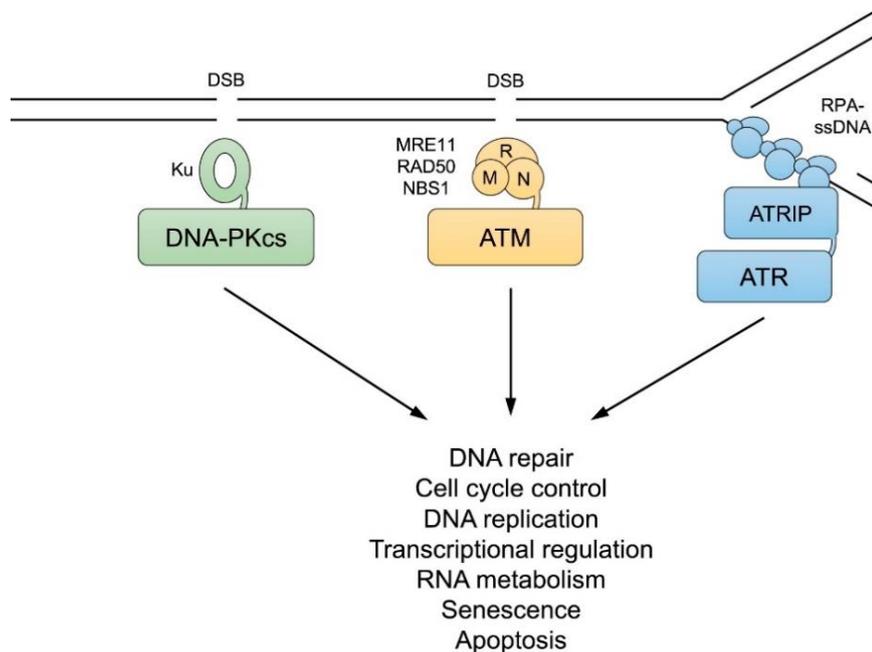


Figure 4. DNA-PK, ATM and ATR are the main kinases responsible of sensing DNA double-strand breaks and single-stranded DNA. They control DNA damage signaling cascades leading to DNA repair, cell cycle control, apoptosis, and other cellular responses aiming to maintain the integrity of the genome. Details are described in the text. DSB=double-strand break; ssDNA=single-strand DNA. Reprinted from Molecular Cell, 66 (6), Andrew N. Blackford, Stephen P. Jackson, ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response, 801-817, Copyright © 2017, with permission from Elsevier.

Upon DSB formation, ATM is recruited and activated by the MRN sensor complex which consists of double-strand break repair protein MRE11 (MRE11), RAD50 double-strand break repair protein (RAD50), and nibrin (NBS1)⁶⁸. When activated, ATM further phosphorylates the histone H2AX at serine 139, forming γ H2AX⁶⁹, and the mediator of DNA damage checkpoint 1 (MDC1) which is recruited to γ H2AX⁶¹. MCD1 further recruits MRN to the chromatin, promoting HR, creating a positive feedback loop that amplifies ATM recruitment and activation⁶¹. A linker histone H1 is ubiquitinated by E3 ubiquitin ligase ring finger protein 8 (RNF8)⁷⁰. This results in the recruitment of another ubiquitin ligase, RNF168, which ubiquitinates H2AX, leading in the recruitment of tumor suppressor p53-binding protein 1 (53BP1) which protects DNA ends from resection via the shieldin complex⁷¹⁻⁷⁴. ATM-mediated threonine 68 phosphorylation of CHK2 is a marker for CHK2 activation, which is the canonical event downstream of ATM signaling and catalyzes further phosphorylation events to elicit cellular responses for DSB damage⁶⁴.

The second PIKK involved in DSB repair, DNA-PK, consists of a catalytic subunit (DNA-PKcs) and a regulatory heterodimer of Ku proteins (Ku70 and Ku80)⁷⁵. In contrast to ATM, DNA-PK is essential in the NHEJ repair of DSBs⁶². As reviewed by Blackford and Jackson (2017), DNA-PKcs is recruited to the DSB and activated at the DNA ends by Ku proteins⁶¹. Ku proteins and activated DNA-PKcs further recruit and activate other NHEJ core factors needed for the ligation process. The interplay between BRCA1 and 53BP1 plays an important

role in the regulation of DSB repair pathway choice between HR (Chapter 1.2.2) and NHEJ⁷⁶ via mechanisms that are out of the scope of this thesis. Briefly, in G1 phase HR is suppressed by 53BP1 via its effector RIF1 which inhibits BRCA1-mediated DNA end-resection required for HR⁷⁷ and by inhibition of BRCA2 recruitment via suppression of BRCA1–Partner and localizer of BRCA2 (PALB2) interaction⁷⁸. On the other hand, in the S/G2 phases BRCA1, together with C-terminal binding protein 1 interacting protein (CtIP), antagonizes 53BP1 promoting HR⁶¹.

The PIKKs have overlapping functions; they can activate multiple repair factors and regulate several repair pathways⁶¹. Table 2 summarizes DNA repair pathways and what type of damage they can repair. HR and NHEJ can both repair double-strand breaks but HR is active only in the S and G2 phases since it needs sister chromatid as a template for repair⁵. On the contrary, NHEJ merely ligates resected DSBs together and can therefore repair DNA in any phase of the cell cycle⁵, whereas the FA pathway is a pivotal process in the initiation of ICL repair and it is mainly active in S phase⁷⁹. HR and FA repair pathways, which are key pathways in the repair of DNA damage caused by platinum and IR in replicating cells, will be described in more detail in the following chapters.

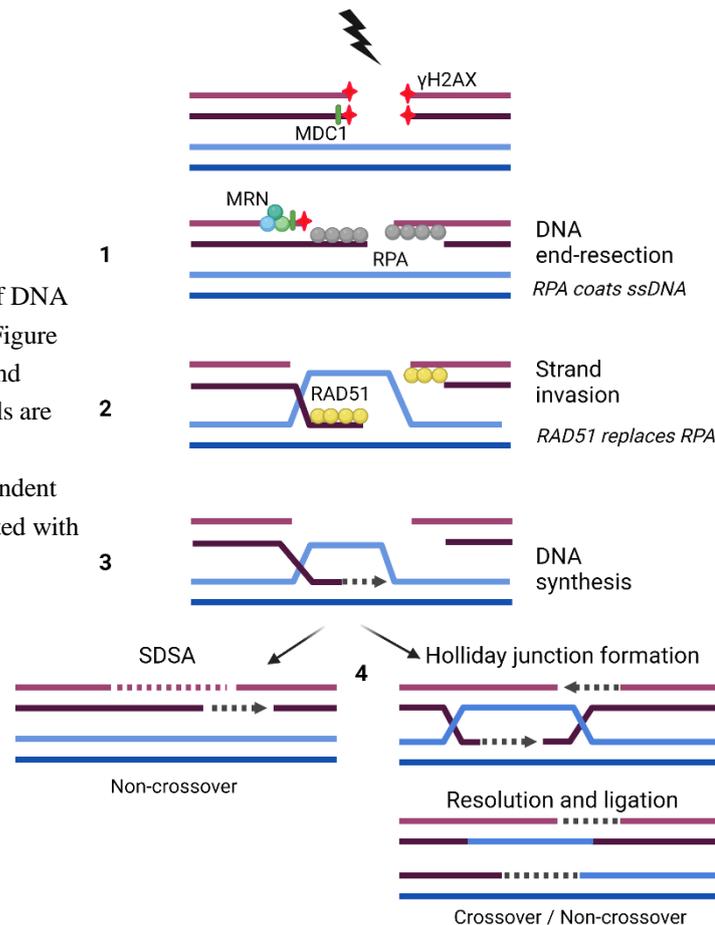
DDR mechanism	Prime DNA damage lesions acted upon
Direct DNA-lesion reversal	UV photo-products, O ⁶ alkylguanine
Mismatch repair (MMR)	DNA mismatches and insertion/deletion loops arising from DNA replication
Base excision repair (BER) and single-strand break repair	Abnormal DNA bases, simple base-adducts, SSBs generated as BER intermediates, by oxidative damage or by abortive topoisomerase I activity
Nucleotide excision repair (NER)	Lesions that disrupt the DNA double-helix, such as bulky base adducts and UV photo-products
Translesion synthesis (TLS)	Base damage blocking replication-fork progression
Non-homologous end-joining (NHEJ)	Radiation- or chemically-induced DSBs
Alternative NHEJ (alt-NHEJ)	Repair of DSBs based on microhomology-mediated end-joining
Homologous recombination (HR)	DSBs, stalled replication forks, interstrand DNA crosslinks and abortive Topoisomerase II action
Fanconi anemia pathway	Interstrand DNA crosslinks
ATM-mediated DDR signaling	DSBs
ATR-mediated DDR signaling	ssDNA, resected DSBs

Table 2. Summary of the main DNA repair pathways and DDR signaling mechanisms and the primary lesions they recognize. Homologous recombination and Fanconi Anemia pathway are described in the text in more detail. DDR=DNA damage response; DSB=double-strand break; ssDNA=single-strand DNA; UV=ultraviolet; SSB=DNA single-strand break. Table adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature, The DNA-damage response in human biology and disease, The DNA-damage response in human biology and disease, Stephen P. Jackson¹ and Jiri Bartek, Copyright © 2009.

1.2.2 Homologous recombination

HR is regarded as an error-free DSB repair mechanism compared to NHEJ, as it uses the sister chromatid as a template for repair⁶¹. This is the preferred repair pathway for DSBs that occur at active replication forks⁸⁰. HR is a multistep process involving a number of factors but it can be separated to following main steps: 1) DNA resection, 2) strand invasion, 3) DNA synthesis and 4) resolution (Figure 5)⁸¹. HR repair requires both ATM and ATR-mediated signaling as both DSB and generation of ssDNA occur during the repair process⁸².

Figure 5. Homologous recombination repair of DNA double-strand breaks. Figure illustrates key events and factors involved. Details are described in the text. SDSA=Synthesis-dependent strand-annealing. Created with BioRender.com.



MRN complex localizes to DSBs via γ H2AX and MDC1 and initiates the DNA resection by its own endolytic cleavage activity, promoted by CtIP⁸³, and by recruitment of endonucleases such as Exonuclease I (ExoI) and Bloom helicase (BLM) for 5'-3' resection of DNA (step 1)⁸². The resection step leads to generation of ssDNA which is coated by RPA. BRCA1 is phosphorylated by ATM at DSB sites and works as a scaffolding factor to recruit for example the BRCA2-RAD51 complex to the ssDNA, which enables the homology search between DNA templates and catalyzes the strand invasion (step 2)⁸⁴. In this step, RAD51 forms a RAD51-nucleoprotein filament with the ssDNA, displacing RPA from the ssDNA⁸⁵ and mediates the connection between the invading DNA and the template DNA, which generates a D-loop structure⁸⁶. Subsequently, the invading DNA is extended by DNA polymerases (step 3), followed by resolution of possible Holliday junctions and final ligation step (step 4) (Figure 5).

Upon DNA damage induction in S phase, dNTPs levels increase about four-fold compared to already elevated levels during S phase to support DNA repair synthesis⁸⁷. As reviewed by Niida et al. (2010), this higher demand is reached by increasing the transcription of Ribonucleotide reductase (RNR) genes, by subcellular localization of RNR to the nucleus and by concentrating dNTPs at the DNA damage sites⁸⁷. RNR is responsible for catalyzing the generation of deoxyribonucleotides from ribonucleotides and consists of the Ribonucleotide Reductase Catalytic Subunit M1 (RRM1) and Ribonucleotide Reductase Regulatory Subunit M2 (RRM2) or its isoform, RRM2B, which is p53 inducible⁸⁷.

1.2.3 The Fanconi Anemia pathway

The FA pathway recognizes and coordinates the repair of ICLs. It derives its name from the Fanconi Anemia disorder, which is a genetic disease caused by biallelic germline mutations in the FA complementation group proteins (FANCA-FANCW)⁷⁹ and presents as bone marrow failure during childhood due to vast genomic instability⁸⁸. The FA proteins and FA-related proteins (FAAPs) initiate the ICL repair which requires the coordination of NER, TLS and HR repair pathways^{79,89}. It consists of; lesion recognition and fork convergence by FA proteins, nucleolytic incision and unhooking by NER, lesion bypass by TLS and DSB repair by HR, illustrated in Figure 6.

In the first step of the FA pathway, (a) BRCA1 functions to evict the CMG replicative helicase complex, consisting of the Cell division control protein 45 homolog (Cdc45), the Minichromosome maintenance proteins 2-7 (Mcm2-7) and the DNA replication complex GINS (GINS)⁹⁰, from the stalled forks allowing one replication fork to approach the ICL (fork convergence)⁹¹. The ICL is recognized (b) by Ubiquitin like with PHD and ring finger domains 1 (UHRF1) protein⁹² and a complex consisting of FANCM, FAAP24 and histone-fold-containing FANCM-associated proteins MHF1 and MHF2⁹³. The BLM helicase promotes the recruitment of FANCM to stalled replication forks by interacting with FAAP24⁹⁴, whereas (c) FANCM promotes the checkpoint response via ATR⁹⁵. Simultaneously, ATR phosphorylates the FANCI protein, which acts as on-switch for the FA pathway⁹⁶. Then, the FANCM-FAAP24-MHF1-MHF2 complex recruits the FA core complex to the ICL, which in its turn (d) ubiquitinates a heterodimer consisting of FANCD2 and FANCI (ID2 complex)⁷⁹. The monoubiquitination of the ID2 complex results in a conformational change in the complex which clamps the FANCI-FANCD2 heterodimer to the dsDNA, stabilizing it⁹⁷. The ATR-mediated FANCI phosphorylation maintains FANCD2 ubiquitination, protecting it from deubiquitination⁹⁸. Subsequently, the ID2 complex recruits other FA and HR proteins to the damage site⁹⁹. Next, ubiquitinated FANCD2 together with SLX4 structure-specific endonuclease subunit, recruit structure-specific nucleases (SSEs) to (e,f) unhook the ICL, which generates a DSB in the opposite strand from the ICL¹⁰⁰⁻¹⁰². Consequently, one DNA strand is still tethered to a nucleotide and in this strand the ICL is (g) bypassed by TLS, which involves the translesion polymerases REV1 or Pol ζ ^{79,103}.

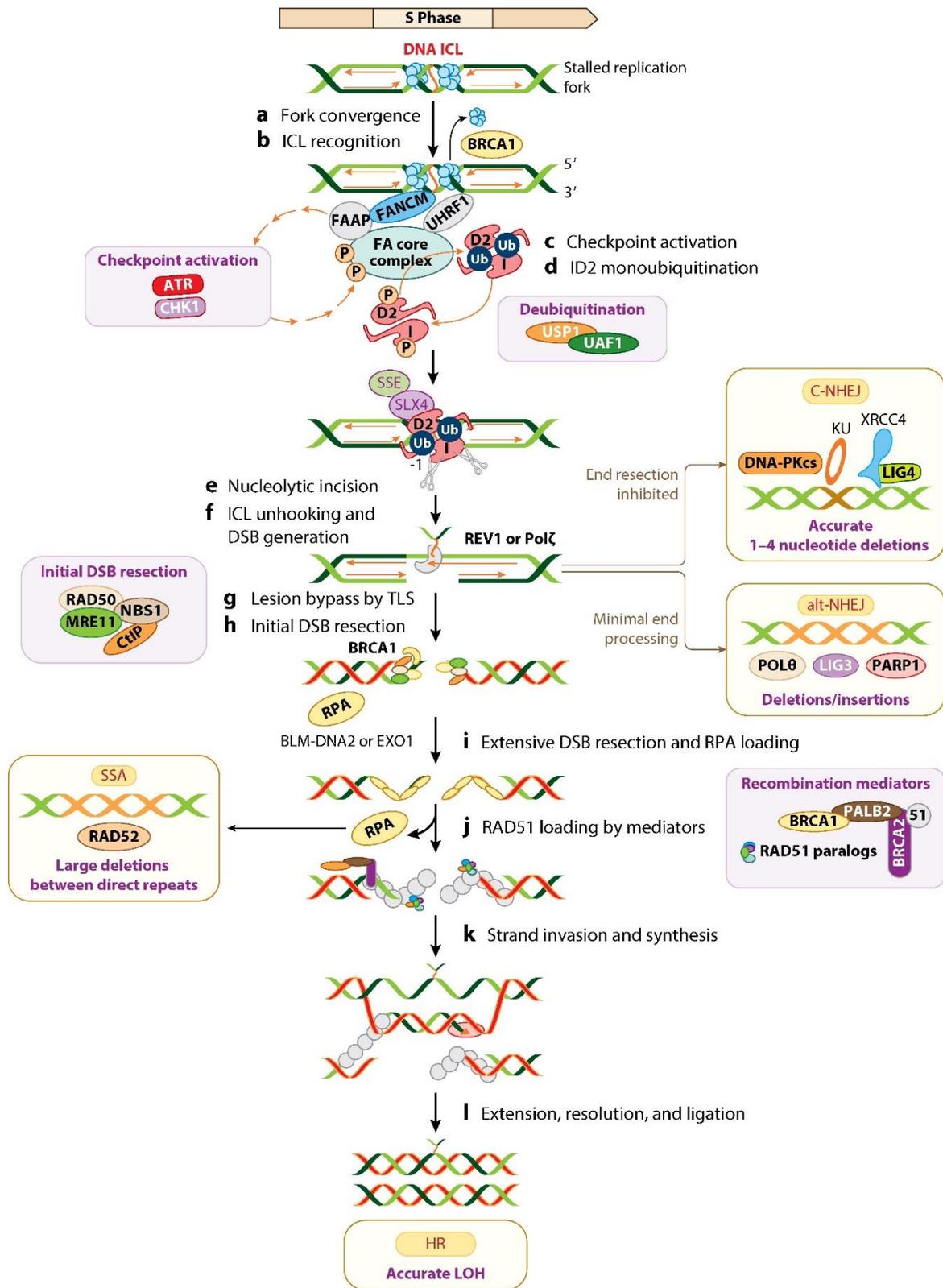


Figure 6. Overview of the repair of ICL damage by the Fanconi Anemia pathway. Details are described in the text. Republished with permission of Annual Reviews, Inc., from The fanconi anemia pathway in cancer, Niraj, Joshi; Färkkilä, Anniina; D'Andrea, Alan D, Volume 3, Issue 1, Copyright © 2019; permission conveyed through Copyright Clearance Center, Inc.

The DSB generated during ICL unhooking can be repaired via HR, NHEJ (also referred as canonical NHEJ; C-NHEJ), alternative NHEJ (alt-NHEJ) or single-strand annealing (SSA)⁷⁹. In S phase, DSB repair is initiated by the (h) resection of DSB ends with the DSB resection machinery¹⁰⁴. If the resection is minimal, the break can be repaired via alternative NHEJ (alt-NHEJ) utilizing polymerase θ (POL θ), which is error-prone⁷⁹. However, (i) extensive resection mediated by BLM, Exonuclease 1 (EXO1), DNA replication ATP-dependent helicase/nuclease DNA2 and CtIP leads to longer ssDNA stretches coated by RPA⁷⁹. The RPA coating is subsequently replaced either by RAD52 to mediate repair via SSA or (j) RAD51 to promote (k,l) HR⁷⁹. Briefly, in SSA homologous repeats that flank the DSB are annealed together which forms an intermediate synapsis¹⁰⁵. Following this, ssDNA tails are processed by endonucleolytic cleavage and remaining gaps are filled by polymerases and ligated. In contrast to HR, SSA causes deletions and rearrangements of the DNA, and increases genomic instability, however SSA can be preferred over HR in case of dysfunctional HR, or if the DSB occurs in early of mid-S phase far prior replication fork when sister chromatid is not present¹⁰⁵.

1.2.4 Replication stress

The high fidelity of DNA replication ensures that the genome is duplicated correctly from one cell division to another. Replication stress is defined as transient replication blockage or slowdown in response to DNA lesions, aberrant replication fork structures and other replication fork obstacles as well as and oncogene activation in cancer^{106,107}. Genomic instability is a cause and consequence of replication stress and, consequently, cancer cells are often characterized by increased levels of replication stress¹⁰⁸. Likewise, cancer treatments such as platinum and IR induce replication stress by DNA damage induction and by interfering with DNA replication.

Replication stress leads to the activation of the replication stress response to stabilize and restart the replication fork and to maintain genomic stability¹⁰⁶. The replication stress response is activated by ssDNA, which is generated upon replication fork stalling by the uncoupling of the replicative helicase from DNA polymerases¹⁰⁹. Phosphorylation of ATR by RPA elicits the ATR-CHK signaling cascade which leads to cell cycle arrest and other cell-protective events described in Chapter 1.2.1. However, recent findings suggest that already replication fork stalling is able to activate ATR-CHK1 pathway in 9-1-1/TOPBP1-dependent manner before generation of excess ssDNA and RPA coating, which functions only after the fork stalling to amplify the ATR-CHK1 activation¹¹⁰. Importantly, activated ATR reciprocally phosphorylates the chromatin-bound RPA at serine 33, which promotes RPA hyperphosphorylation by CDKs and limits the release of extensive ssDNA¹¹¹. Notably, in response to replication stress H2AX is phosphorylated at serine 139 in ATR dependent but not ATM independent manner, in contrast to DSBs, leading to recruitment of other DDR factors such as proliferative nuclear antigen (PCNA), BRCA1 and 53BP1 at stalled replication forks¹¹².

High levels of replication stress can lead to fork collapse and breakage generating DSBs, *i.e.* replication catastrophe, which is a lethal event for a cell¹¹³. Even in physiologically normal levels of initial replication stress, replication catastrophe can occur if the replication stress

response fails to protect the stalled fork, for example in the absence of RPA¹¹⁴. RPA excess in the cell attenuates the replication catastrophe and acts as a buffer against lethal threshold of replication stress by increasing tolerance to ssDNA (Figure 7). Interestingly, ATR protects cells against the exhaustion of RPA during replication stress by suppressing global origin firing. Conversely, depletion of ATR increases ssDNA formation depleting the available RPA pool and increasing fork breakage¹¹⁴.

In addition, studies using hydroxyurea treatment have demonstrated that the FA pathway is also activated in high levels of replication stress without ICL damage⁷⁹. On the other hand, in response to low levels of replication stress, FANCD2 and FANCI seem to have FA pathway-independent roles mediated by ATR, resulting in suppression of firing of dormant and new origins, which highlights the diverse roles of the FA pathway proteins in the maintenance of genomic stability⁷⁹.

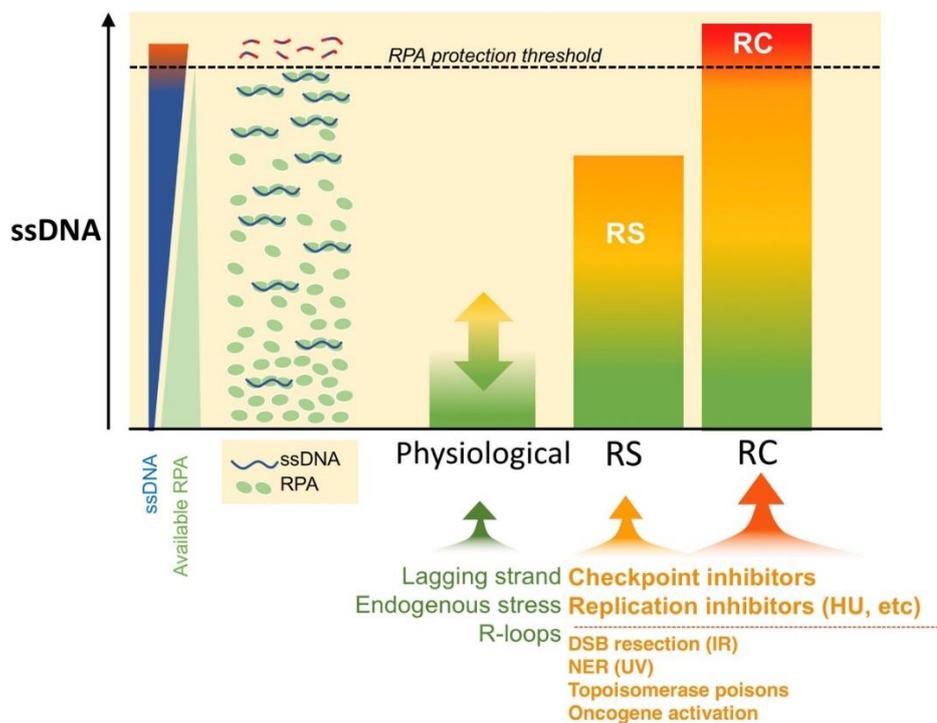


Figure 7. RPA protects ssDNA from breakage and degradation increasing the replication stress tolerance¹¹³. Physiological levels of ssDNA are generated by replicative events such as the lagging DNA strand and R-loops as well as endogenous replication stress (green). Checkpoint inhibitors and replication inhibitors such as hydroxyurea (HU) lead to large quantities of ssDNA generation and can cause replication catastrophe by RPA exhaustion. Ionizing radiation (IR), ultraviolet (UV) radiation, topoisomerase poisons and oncogene activation can further increase ssDNA formation and replication stress (RS). DSB=double strand break; NER=nucleotide excision repair. Reprinted from Molecular Cell, 66 (6), Luis Toledo, Kai John Neelsen and Jiri Lukas, Replication Catastrophe: When a Checkpoint Fails because of Exhaustion, Pages 735-749, Copyright © 2017, with permission from Elsevier.

1.3 CX3CR1: EMERGING ROLES FOR A CHEMOKINE RECEPTOR

C-X3-C motif chemokine receptor 1 (CX3CR1/fractalkine receptor) is heptahelical receptor belonging to the G protein-coupled receptor (GPCR) superfamily¹¹⁵. The binding of the natural ligand for the receptor, C-X3-C motif chemokine ligand 1 (CX3CL1/fractalkine)^{116,117}, leads to the activation of the G_i protein which triggers several major intracellular signaling events such as PI3K/Protein kinase B (Akt) and Mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinase (ERK) pathways, depending on the cellular context (Figure 8)^{118–123}. Moreover, CX3CR1 can transactivate the epidermal growth factor receptor (EGFR/ErbB-1) and human epidermal growth factor receptor 2 (HER2/ErbB-2)^{123–125}. Fractalkine is the only chemokine in the structural C-X3-C group of chemokines and exists in both membrane-bound and soluble forms¹¹⁶. Unlike other chemokines, fractalkine selectively binds to CX3CR1¹²⁶. CX3CR1 signaling promotes cell adhesion via the membrane-bound ligand and migration, proliferation, anti-apoptosis, angiogenesis and epithelial-mesenchymal transition via its soluble ligand¹²⁷.

Owing to the canonical role of chemokines, fractalkine and CX3CR1 were discovered due to their role in inflammation. Fractalkine was first reported to be expressed by inflammatory endothelial cells, in response to inflammatory cytokines, to promote migration and invasion of leukocytes that, subsequently, were demonstrated to express CX3CR1^{115,116,128}. In its membrane-bound form, fractalkine promotes adhesion of CX3CR1 positive leukocytes to the inflamed endothelium¹¹⁶. In addition, CX3CR1 signaling has been connected to survival of monocytes¹²⁹. Furthermore, the CX3CL1/CX3CR1 axis has a pivotal role in the brain in the elimination of damaged CX3CL1-expressing neurons by CX3CR1-expressing microglia, in neuronal protection and plasticity as well as neurogenesis¹³⁰. CX3CL1/CX3CR1 signaling is involved in the pathogenesis of several cancers and other conditions such as multiple sclerosis, neuronal pain, and reperfusion injury after myocardial infarction^{130–132}.

In addition to leukocytes and the central nervous system (microglia, astrocytes, and hippocampal neurons)¹³³, CX3CR1 is also expressed in osteoclasts¹³⁴ and in cancer cells¹³⁰, and its expression is induced by fractalkine, interleukin 10, interferon γ and hypoxic conditions^{120,135,136}. Besides the brain, inflammatory endothelium and fibroblasts, CX3CL1 mRNA is found in wide range of tissues such as heart, lung, kidney, intestines, skeletal muscles and some epithelial cells¹¹⁶. Importantly, both CX3CR1 and CX3CL1 can be expressed by the same cell, and CX3CL1 can induce its own expression and that of CX3CR1¹²¹. Thus, cells can activate CX3CR1 signaling in an autocrine manner.

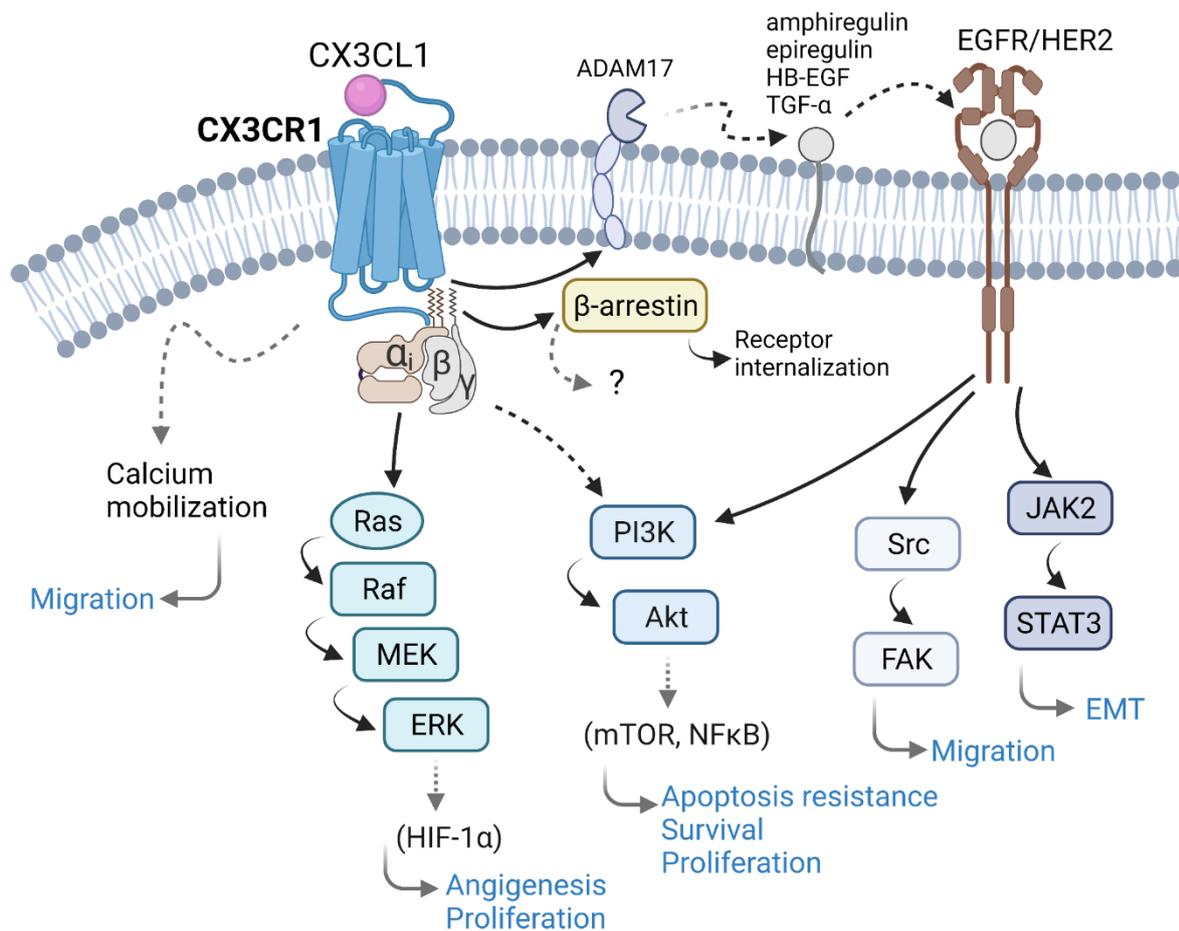


Figure 8. CX3CR1 activation by CX3CL1 can lead to the activation of several signaling pathways depending on the cellular context via G_i protein or by transactivation of the epidermal growth factor receptor (EGFR) by matrix metalloproteinases, such as ADAM17, that release EGFR-activating ligands^{126,127,130}. Examples of proteins activated by these pathways are written in brackets and potential cellular outcomes, many of which are related to pro-tumorigenic phenotypes, are written in blue. After G protein activation, β -arrestin is recruited, internalizing the receptor into early endosomes which can result in the termination of the CX3CR1-induced signals. However, β -arrestins can elicit further signaling events and G proteins can continue to be active in intracellular compartments^{137–139}. Activation of CX3CR1 induces also calcium mobilization, but there is evidence that this cannot happen without active an G_{α_q} protein¹⁴⁰. ADAM17=A disintegrin and metalloproteinase 17; HB-EGF=heparin-binding epidermal growth factor; TGF- α =Transforming growth factor α ; EGFR=Epidermal growth factor receptor; HER2= human epidermal growth factor receptor 2; RAS=Ras GTPase; ERK=Extracellular signal-regulated kinase; FAK=Focal adhesion kinase; JAK2=Janus kinase 2; STAT3=Signal Transducer And Activator Of Transcription 3; EMT=Epithelial-mesenchymal transition. Figure based on reviews by Korbecki et al. 2020, White et al. 2012 and Rivas-Fuentes et al. 2020^{126,127,130}. Created with BioRender.com.

1.3.1 CX3CR1 in cancer

CX3CR1 is involved in cancer invasion and metastasis^{125,141}, proliferation and survival^{142,143} as well as modulation of the tumor microenvironment in several cancers^{144–148}. The CX3CL1/CX3CR1 axis contributes to the pro-cancer phenotype of for example ovarian¹⁴¹, breast¹²⁴, prostate¹²⁵, colorectal¹⁴⁹, testicular¹⁴⁵, pancreatic¹⁵⁰, lung¹⁵¹, gastric cancer¹⁵², B cell malignancies¹⁵³ and glioblastoma¹⁵⁴. CX3CR1 expression has been associated with poor patient outcomes in ovarian cancer¹⁴¹ and clear cell renal cell carcinoma¹⁵⁵. CX3CR1 is upregulated in primary and metastatic EOC, but it is almost absent in normal ovarian surface

epithelium¹⁵⁶. Furthermore, CX3CR1 expression increases over the course of ovarian cancer progression¹⁴¹ and is expressed early on during pancreatic carcinogenesis potentially promoting invasiveness¹⁵⁷, indicating that CX3CR1 might be involved in the early steps of tumorigenesis and promote aggressiveness of cancers.

However, CX3CR1 expression is also associated with better prognosis in some cancers, such as colorectal cancer¹⁴⁹, hepatocellular carcinoma¹⁵⁸ as well as pancreatic ductal adenocarcinoma¹⁵⁷. Interestingly, fractalkine signaling can have a dual role in the spread of cancer; if CX3CL1 is expressed distant from the tumor site it promotes metastasis of cancer cells that express CX3CR1^{125,141}, but if CX3CL1 is expressed locally at tumor site, it anchors cancer cells and prevents metastasis^{149,158}.

Regarding the involvement of CX3CL1/CX3CR1 in the tumor microenvironment, CX3CL1 has been found to promote recruitment of CX3CR1-expressing tumor infiltrating macrophages (TAMs) in testicular germ cell cancer¹⁴⁵, endometrial carcinoma¹⁴⁶, skin cancer¹⁴⁸ and pancreatic ductal adenocarcinoma¹⁴⁷, thereby contributing to the tumorigenic cancer microenvironment. On the other hand, CX3CL1 can attract CD8+ T cells and NK cells resulting in a better prognosis for gastric adenocarcinoma¹⁵⁹. Furthermore, the CX3CL1/CX3CR1 axis can modulate the cancer microenvironment by promoting angiogenesis, as seen in multiple myeloma¹⁴⁴.

CX3CR1 activation can mediate proliferation of cancer cells via several mechanisms (Figure 8). For example in pancreatic cancer, CX3CL1/CX3CR1 induces the upregulation of anti-apoptotic molecules and downregulation of pro-apoptotic molecules via activation of Akt¹⁶⁰. In addition, CX3CR1 signaling can promote cancer cell proliferation and cell cycle progression possibly via activation of the Akt pathway in ovarian cancer¹⁶¹ and via the Akt/Nuclear factor NF-kappa-B (NFκB) pathway in pancreatic cancer¹⁶⁰. Moreover, CX3CR1 can transactivate the EGFR pathway in breast cancer¹²⁴. Interestingly, activation of CX3CR1 in pancreatic carcinoma can increase glucose uptake and lactate secretion via induction of Hypoxia-Inducible Factor-1α (HIF-1α) in a PI3K/MAPK-dependent manner and thus favors anaerobic glycolysis for cell proliferation¹⁶². Finally, CX3CL1/CX3CR1 has been seen to promote epithelial-to-mesenchymal (EMT) transition in ovarian cancer and prostate cancer^{136,163}.

In summary, the CX3CL1/CX3CR1 pathway can either have pro- or anti-tumor effects depending on the tissue origin of the cancer and the pathways activated, co-expression status of both ligand and receptor, and the effect of tumor microenvironment. When dissecting the involvement of the CX3CL1/CX3CR1 axis in cancer, it is important to separate the immunomodulatory functions of CX3CL1 in the tumor microenvironment and the role of CX3CR1 in cancer metastasis and cell survival.

1.3.2 CX3CR1 in DNA repair

In 2018, a study suggested that CX3CR1 increases resistance to DNA damaging treatments in cancer cells¹⁶⁴. Xie et al. reported that CX3CR1 knockdown by small interfering RNA (siRNA) treatment sensitized OC cell lines to ionizing radiation, cisplatin, and carboplatin in long term proliferation assays¹⁶⁴. Furthermore, knockdown of CX3CR1 inhibited phosphorylation of ATM, DNA-PKcs, CHEK1 and CHEK2, and delayed IR-induced γ H2AX foci formation, which resulted in high amount of DSBs¹⁶⁴. The regulatory effect of CX3CR1 on these DDR factors was attributed to reduced protein levels of RAD50 and disturbance of MRN complex¹⁶⁴. *In vivo*, CX3CR1 knockdown and ionizing radiation revealed an organ-specific synergy in HGSOC tumor reduction. Notably, in this xenograft model using SKOV3 cell line, CX3CR1 knockdown alone significantly reduced omental metastasis¹⁶⁴, unlike in a syngeneic ovarian carcinoma mouse model published earlier¹⁴¹. Reduced omental metastasis was partially explained by reduced uptake of fatty acids from omental adipocytes by cancer cells upon CX3CR1 knockdown¹⁶⁴. Importantly, high CX3CR1 mRNA expression significantly correlated with worse overall survival of ovarian cancer patients that were treated with platinum drugs and other DNA damaging agents¹⁶⁴. Taken together, CX3CR1 has an emerging role in the regulation of the DDR response in cancer cells and CX3CR1-mediated enhanced DNA repair capacity could confer proliferative advantage in cancers that display genomic instability and resistance to DNA damaging agents.

1.3.3 CX3CR1 as a therapeutic target

About half of all FDA approved drugs currently in the market target GPCRs due to their overall druggability and involvement in a myriad of diseases^{137,165}. Chemokine receptors are attractive therapeutic targets due to their role in inflammation and immunity and efforts have been made to pharmaceutically target various chemokine receptors in different diseases^{166,167}. For example, the CCR5 antagonist Maraviroc is used in the clinic in a combination treatment of HIV and its use in cancer is also under evaluation^{168,169}. There are currently several inhibitors or antibodies targeting chemokine receptors in clinical cancer trials¹⁷⁰ (<https://www.clinicaltrials.gov/>) and two in use (the monoclonal CCR4 antibody Mogamulizumab and the CXCR4 inhibitor AMD3100) for hematological cancers^{166,170}.

Since CX3CL1/CX3CR1 signaling is involved in pathogenesis of several diseases, but supports few normal processes in adults, several drugs targeting CX3CR1 or CX3CL1 are under development. An antibody targeting CX3CL1 (E6011; Eisai Pharmaceuticals)¹⁷¹ has shown efficacy in a Phase 2 clinical trial of rheumatoid arthritis¹⁷². KAND567 is the first selective small molecule inhibitor targeting CX3CR1 (Kancera AB)^{173,174} and it has successfully passed clinical phase 1 trial with healthy volunteers¹⁷⁵. Furthermore, an anti-CX3CR1 nanobody (BI 655088; Boehringer/Ablynx)¹⁷⁶ has recently been evaluated in Phase 1 trial (NCT02696616) and it inhibits atherosclerotic plaque formation in mice¹⁷⁶. Due to the selectivity and clinically proven safety of these antagonists in late development, CX3CR1 inhibitors show potential for clinical use.

1.3.4 KAND567

The small molecule CX3CR1 inhibitor KAND567 (previously AZD8797)^{173,174} was used to inhibit CX3CR1 in the studies included in this thesis. KAND567 was the first potent and selective inhibitor of CX3CR1 to be published^{173,174}. KAND567 was functionally characterized by Cederblad et al. (2016) who showed that KAND567 is a negative allosteric modulator of CX3CR1 but the exact binding site is not known¹⁷⁴. KAND567 binds CX3CR1 in a non-competitive manner and increases the dissociation of CX3CL1 from CX3CR1, eventually displacing CX3CL1. This displacement was hypothesized to be conferred via uncoupling of the G protein from CX3CR1 upon KAND567 binding. KAND567 antagonized CX3CL1-induced G protein signalling in isolated Chinese hamster ovary (CHO) cell membranes stably expressing human CX3CR1 with an IC₅₀ value of 340 nM, when 2 nM CX3CL1 was used, and prevented the CX3CL1/CX3CR1-mediated capture of human blood leukocytes to endothelial cells with a similar IC₅₀¹⁷⁴.

Interestingly, Cederblad and colleagues showed that KAND567 potentiates CX3CL1-mediated β -arrestin recruitment at low concentrations, therefore acting as positive allosteric modulator in this assay¹⁷⁴. In contrast, high concentrations of KAND567 almost totally abolished the CX3CL1-induced β -arrestin recruitment. This duality was attributed to the fact that lower concentration of KAND567 is needed to bind CX3CR1 than to displace CX3CL1 from the receptor. In this context, the KAND567 concentration conveying agonistic function in the β -arrestin assay was comparable to the concentration of KAND567 receptor-binding, and the concentration eliciting antagonist function was similar to the concentration of KAND567-mediated CX3CL1 displacement. It was therefore suggested that when both KAND567 and CX3CL1 are bound to CX3CR1, β -arrestin recruitment is potentiated. Furthermore, KAND567 alone did not induce β -arrestin recruitment. However, KAND567 alone at high concentrations induced a dynamic mass redistribution (DMR) response which was reduced with the addition of pertussis toxin. DMR assay is a label-free technique to study GPCRs based on an optical biosensor that detect changes in cellular morphology and adhesion as well as cytoskeletal rearrangement¹⁷⁷. Change in DMR upon KAND567 indicates that binding of KAND567 to CX3CR1 induces cellular responses that result in mass relocation. Therefore, KAND567 has partial agonist functions which were suggested to be mediated via CX3CR1. However, CHO cells without CX3CR1 expression elicited a mild increase in DMR response and a 10-fold higher concentration of KAND567 was needed for this agonist effect on DMR than for KAND567 to abolish CX3CL1 binding¹⁷⁴.

KAND567 has a good oral availability¹⁷³ and further *in vivo* studies have showed efficacy of KAND567 in attenuating multiple sclerosis in mice with calculated effective IC₅₀ mean concentration of 2 μ M¹⁷⁸ and suppressing inflammation^{179,180}. In addition, KAND567 shows cardioprotective effects in rodent disease models¹⁸¹ and after successfully passing Phase 1, is now under phase II development in myocardial infarction¹⁷⁵, and in a phase II study for Covid-19-related hyperinflammation indications (EudraCT: 2020-002322-85).

1.4 PFKFB3: A BIFUNCTIONAL ENZYME AT THE CROSSROADS OF METABOLISM AND DNA REPAIR

Altered metabolism is a hallmark of cancer³ and high rate of glycolysis, the conversion of glucose to pyruvate to produce high energy products ATP and NADP, is characteristic to many tumors¹⁸². Although oxidative phosphorylation is a more efficient way to produce energy, tumor cells often prefer the glycolytic pathway even in the presence of oxygen—this phenomenon is referred to as the “Warburg effect”¹⁸³. The reason for cancer cells to prefer glycolysis over mitochondrial ATP production, although around 18-fold lower in efficiency, is attributed to the production of glycolytic intermediates that can be used in the biosynthesis of macromolecules and organelles required for tumor growth¹⁸⁴. Another reason for favoring glycolysis is hypothesized to be an establishment of low pH environment via lactate production, that could cause apoptosis in surrounding non-malignant cells expressing functional p53 protein¹⁸⁵. This metabolic switch in cancer cells is achieved by activation of oncogenes and inactivation of tumor suppressors^{186,187}.

In the glycolysis (Figure 9) the bifunctional metabolic enzymes, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFBs), play a key role by synthesizing and degrading fructose-2,6-bisphosphate (F2,6BP), which is an allosteric activator of Phosphofructokinase 1 (PFK-1)¹⁸⁸. PFK-1 is a rate-limiting enzyme in glycolysis and can be negatively regulated by ATP when high level of energy is no longer needed¹⁸⁹. However, abundant F2,6BP can surpass this negative feedback loop, enhancing glycolysis and consumption of glucose. Thus, positive regulation of F2,6BP, for example by oncogenes, is connected to pro-cancer phenotype of cells¹⁹⁰. PFKFB3, one of the PFKFB enzymes regulating the production of F2,6BP, possesses a dominant kinase function over its phosphatase activity, differing from the other isoforms PFKFB1, 2 and 4¹⁸⁸. Unlike other PFKFBs, PFKFB3 is an inducible isoform promoted by mitogenic stimuli, hypoxia, inflammation and oncogenic transcription factors^{191–193}, indicative of its oncogenic function and involvement in the metabolic reprogramming of cancers.

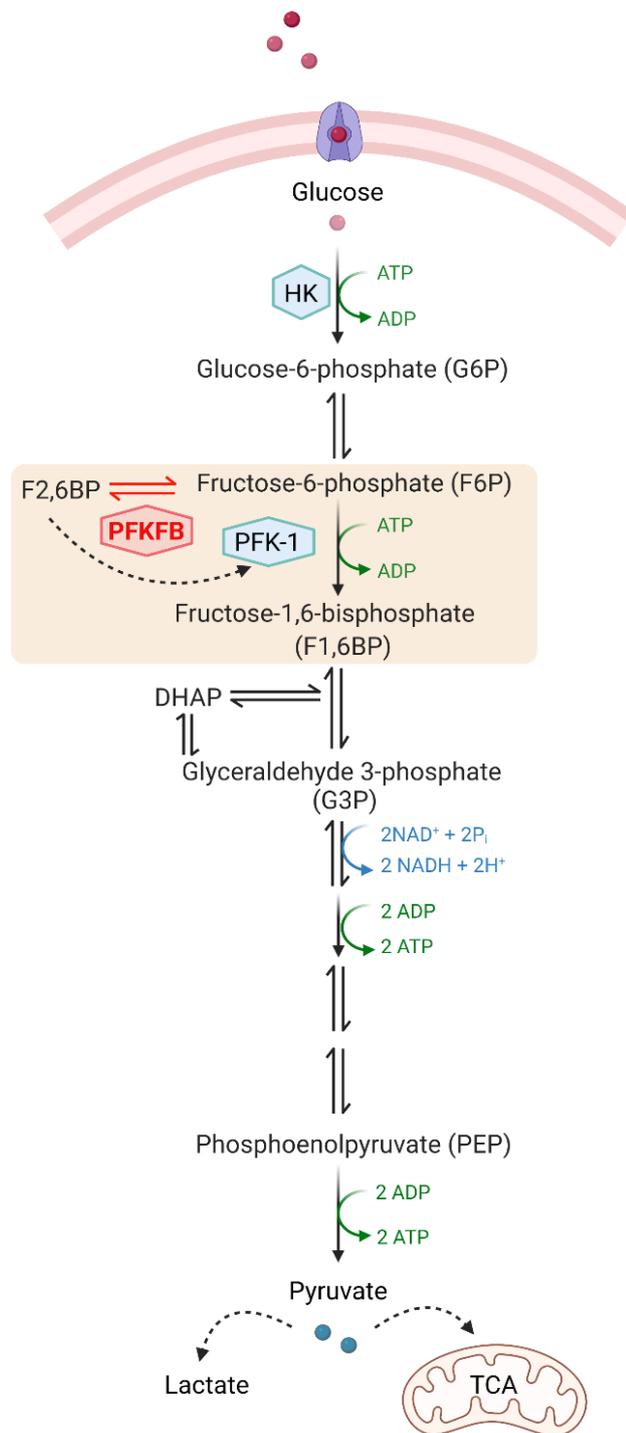


Figure 9. Glycolysis converts glucose to pyruvate to produce energy in the form of ATP and NAD. Fructose-2,6-bisphosphate (F2,6BP) is degraded and synthesized by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) enzymes from fructose-6-phosphate (F6P). F2,6BP activates 6-phosphofructo-1-kinase (PFK-1) enzyme which catalyzes the conversion of F6P to fructose-1,6-bisphosphate (F1,6BP). F1,6BP is converted then to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP) by aldolase. DHAP and G3P can be interconverted. Following this, G3P is converted to pyruvate via further enzymatic steps summarized well by Regina Bailey (2020)¹⁹⁴. Pyruvate can enter Tricarboxylic acid cycle (TCA) in mitochondria or be converted to lactate in the absence of oxygen.

Created with BioRender.com.

1.4.1 PFKFB3 in cancer

PFKFB3 is under regulation of several oncogenes and oncogenic processes¹⁹⁵. Ras signaling, which is one of the major oncogenic pathways connected to cancer transformation, regulates glycolysis via PFKFB3, and the activity of PFKFB3 has been shown to be necessary for *ras*-mediated transformation of cancer cells¹⁹⁶. Notably, in *ras*-transformed mouse lung fibroblasts PFKFB3, but not other PFKFB enzymes, was necessary for the Ras-mediated growth of tumors¹⁹⁶. The link between Ras and PFKFB3 may be mediated by HIF-1 α , as shown in glioblastoma cells¹⁹⁷. In addition, mechanistic target of rapamycin (mTOR) signaling has been shown to upregulate PFKFB3 in acute myeloid leukemia, without affecting the levels of other PFKFB isoforms¹⁹⁸. Furthermore, PFKFBs are upregulated by the overexpression of the *MYC*

oncogene¹⁹⁹ and PFKFB3 can be positively regulated by the estrogen receptor (ER)²⁰⁰ and HER2 signaling¹⁹². The tumor suppressors p53 and Phosphatase and Tensin Homolog (PTEN) suppress the expression of PFKFB3, which supports the evidence of PFKFB3 as a cancer specific modulator of energy metabolism^{201,202}.

PFKFB3 is overexpressed, or involved, in multiple cancers including ovarian cancer²⁰³, breast cancer^{204,205}, pancreatic cancer²⁰⁶, colon cancer^{204,207}, gastric cancer²⁰⁶, lung cancer²⁰⁸, osteosarcoma²⁰⁹, cervical cancer²⁰³, hepatocellular carcinoma²¹⁰, and nasopharyngeal carcinoma²¹¹, among others¹⁹⁵, and its expression correlates with poor prognosis¹⁹⁵. PFKFB3 is often linked to cancer progression via its canonical role in the glycolysis, which has been reported to stimulate for example cancer proliferation, survival, invasion, migration, and angiogenesis¹⁹⁵. However, PFKFB3 also localizes to the nucleus²¹², apart from its role in glycolysis, which has evoked an interest in putative nuclear roles of this enzyme. Yalcin and colleagues first reported that by localizing to the nucleus, PFKFB3 drives cell proliferation via upregulation of CDKs and cyclins without affecting glucose metabolism²¹². Following this, PFKFB3 was seen to regulate p27 via CDK-1 and halt G1/S transition²¹³. During this thesis work, increasing number of reports on the involvement of PFKFB3 in DNA repair have been published and they will be discussed in Chapter 5.2.

1.4.2 PFKFB3 as a therapeutic target

PFKFB3 is a promising target for therapeutic intervention in cancer¹⁹¹ and several small molecule inhibitors targeting PFKFB3 have been developed^{195,214}. 3PO is a widely used PFKFB3 inhibitor used as a tool compound to study the function of PFKFB3²¹⁵. It attenuates tumor growth *in vivo*²¹⁵; however, it does not inhibit the kinase activity of PFKFB3²¹⁶ and was recently shown to not bind PFKFB3 despite inhibiting glycolysis²¹⁷. Moreover, 3PO has poor pharmacokinetic properties, which limits its clinical use²¹⁵. PFK-158 is an improved derivative of 3PO that shows efficacy in preclinical cancer models and it has shown no serious side effects in a phase I study²¹⁸⁻²²², however despite advertised as PFKFB3 inhibitor, it does not target the enzymatic activity of PFKFB3²²³. Furthermore, N4A and YN1 were developed by structure-based design along with the discovery of the crystal structure of PFKFB3, and show inhibition of PFKFB3, suppression of glycolysis, and apoptosis of cancer cells *in vitro* but they are not isoform-selective for PFKFB3²²⁴. Another PFKFB3 inhibitor AZ67, was developed by AstraZeneca; it is a potent PFKFB3 inhibitor but also displays inhibition of PFKFB1 and 2, although with lower potency^{216,223}. Taken together, despite yet unsuccessful, there is a vast interest in developing selective, potent, and safe PFKFB3 inhibitors.

2 RESEARCH AIMS

The overall aim of this thesis was to characterize the role of CX3CR1 and PFKFB3 in the DNA damage response and to evaluate the potential of combining CX3CR1 and PFKFB3 inhibition (by KAND567 and KAN0438757, respectively) with DNA damaging cancer treatments. Specific aims of the papers were:

- I. To investigate the mechanistic role of PFKFB3 in DNA repair following ionizing radiation and to develop a selective PFKFB3 inhibitor (**Paper I**)
- II. To investigate the potential of blocking ovarian cancer cell proliferation by inhibition of CX3CR1 (**Paper II**)
- III. To elucidate the role of CX3CR1 in the DNA damage response upon platinum treatments and assess the synergistic potential of KAND567 with platinum drugs in cancer cells (**Paper III**)
- IV. To assess the role of PFKFB3 in the repair of platinum-induced damage and to evaluate the cancer-specific synergy of KAN0438757 with platinum compounds (**Paper IV**)

3 METHODOLOGICAL APPROACHES

In this chapter the general aspects of some of the key methods applied in this thesis work are presented and discussed. Detailed protocols can be found in the attached research papers.

3.1 DRUG SYNERGY STUDIES

Viability studies assessing drug synergies in this thesis were performed by cell viability measurements based on resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one, sodium). Resazurin is a blue dye that will be enzymatically converted to a highly fluorescent red product, resorufin, in viable cells and can be measured by an emission maximum of 590 nm on a fluorescence plate reader²²⁵. The drawback of this method is that cells that are proliferatively slower will require more time to convert the substance and thus the incubation time must be optimized for each cell line. Moreover, it might be challenging to separate cells that are irreversibly apoptotic from the ones that have merely arrested in cell cycle and slowed down their metabolism, thus a long measurement time point may be needed. Resazurin can also be toxic to the cells itself, which limits long-term measurements²²⁵.

Dose-response matrix viability values for two compounds were used to calculate delta scores (synergy scores) using the Zero Interaction Potency (ZIP) model²²⁶ with Synergy Finder (<https://synergyfinder.fimm.fi>)²²⁷. ZIP model compares the expected value of a drug combination (additive effect with no additional potency) to the observed values to calculate delta scores. The summary synergy score is derived from an average delta score divided by the dose-response matrix values. A synergy score between 0 and 10 indicates that the drug-interaction is likely to be additive and a summary synergy score above 10 indicates likely synergistic effect.

3.2 COLONY-FORMATION ASSAY

Colony-formation assay measures the long-term proliferation capacity of a single cell after drug treatment and drug washout. The idea is to seed cells at so low density that the colonies growing from single cells can be visualized. Colony-formation assay can help to determine if cells are able to continue growing after drug washout, i.e., if the effect of a drug is irreversible on cell survival after certain treatment days. Colony-formation assays can be performed also for example upon siRNA knockdown of a target. One drawback of this method is that it is challenging to seed exactly the same number of cells per well due to the low seeding density which can affect the number of colonies detected in the end of the experiment. Moreover, sometimes cells tend to concentrate in the middle of the wells of the cell culture plates, which makes it hard to separate single-cell colonies. Proper mixing of the cell suspension and careful pipetting of the cells in the wells as well as minimizing the disturbances in the incubator improves the equal distribution of cells in the wells. The distribution of cells and whether they form good visual colonies depends also on the characteristics of the cells as some cells migrate and form elongated shapes that are not optimal for the visualization of the colonies. Moreover,

colony-formation assay requires good attachment of cells on the cell culture plates and thus cannot be performed with suspension cells.

3.3 SUBCELLULAR PROTEIN FRACTIONATION

The fractionation of proteins into soluble and chromatin-bound fractions is a useful technique to study the chromatin association of proteins and complementary to the detection of nuclear damage foci using a confocal microscopy. In this technique protein lysates are first treated with a mild extraction buffer to extract soluble proteins, followed by careful washes of the chromatin-bound fraction to avoid contamination between fractions. In the last step, an enzymatic separation of chromatin-bound proteins is performed to yield the chromatin fraction. For normalization, equal cell numbers were harvested instead of protein quantification which allowed us to compare protein amount between cell lines. As the protocol contains multiple washing and resuspension steps, it is sometimes difficult to obtain equal sample amounts, resulting in experimental failures. Equal amount of protein loading in the immunoblots as well as fraction purity was controlled by histone 3 for chromatin-bound fraction and tubulin for soluble fraction.

Another drawback of this method is that purely membrane bound proteins, such as CX3CR1, will not be included in neither fraction unless internalized to the soluble fraction in endosomes or tightly associated with chromatin. Moreover, band intensities of the following immunoblot of soluble and chromatin-bound fraction are not directly comparable since, due to technical reasons, chromatin-bound fraction is more concentrated in the sample preparation. Nevertheless, since we were mostly interested in the chromatin fraction, using soluble protein fraction as a control, this technique gave us valuable information on the chromatin-association of proteins upon different treatment conditions. Moreover, this technique allows investigation of chromatin-recruitment of proteins that do not have optimal antibodies available for microscopy studies.

3.4 DETECTION OF NUCLEAR REPAIR FOCI BY CONFOCAL MICROSCOPY

The confocal microscopy studies in this thesis, besides measuring total nuclear intensity, focused on detecting nuclear damage foci of DNA repair proteins that are formed at the DNA damage sites²²⁸. By counting the foci numbers per cell and percentage of foci positive cells relative to the vehicle conditions by the CellProfiler software (www.cellprofiler.org), we were able to determine if the foci formation of a certain protein was impaired or induced upon treatment conditions. To visualize the nuclear foci better, *in situ* subcellular fractionation with the cytoskeletal extraction (CSK) buffer was used to remove cytoplasmic and nuclear soluble proteins²²⁹. A limitation in confocal studies is sometimes the lack of antibodies recognizing the endogenous protein or unspecific antibodies. To avoid false positive staining, siRNA mediated knockdown was performed to assure specificity of the antibodies not previously validated for confocal microscopy. Furthermore, antibodies were also used in immunoblot experiments which increases the validity as same results were obtained in two complementary techniques. Furthermore, to avoid species cross-reactivity of secondary antibodies, highly cross-absorbed

antibodies were used in the experiments as well as confirmational single-stainings when necessary.

3.5 GENERATION OF KNOCKDOWN CELL LINES BY LENTIVIRAL TRANSDUCTION

Knockdown cell lines were generated by introducing short hairpin RNA (shRNA) sequences targeting CX3CR1 and PFKFB3 in cancer cells by lentiviral transduction, to validate the findings seen upon CX3CR1 and PFKFB3 inhibition with KAND567 and KAN0438757. A limitation of the method used was that the knockdown of the targets was constitutive. Since constitutive knockdown is present in the cells constantly, it can create a selection pressure for cells that survive without the target (escape cells) which may influence experimental outcomes despite antibiotics selection, whereas inducible knockdown can be switched on at the start of the experiment. Moreover, when knockdown is constitutive, some cells may not survive the knockdown at all if the knockdown is complete and cells depend on the target for survival. Knockdown levels were quantified using quantitative RT-PCR and Western blot, and cells transduced with non-targeting hairpins (scrambled sequences) were used as a control to rule out the general effects of lentiviral transduction on cells.

3.6 DNA FIBER ASSAY

The DNA fiber assay enables the microscopic visualization of DNA replication in molecular level by labeling replicating DNA by one or several different DNA-incorporating dyes²³⁰. The length of these labeled DNA fibers allows us to determine different parameters such as the DNA track length, replication fork speed and replication fork re-start, depending on the protocol applied. The preparation of DNA fibers in this thesis work was performed by “spreading” the DNA on a positively charged microscopic slide which is a fast technique allowing many samples to be processed at the same time and requires less materials compared to alternative techniques. However, the spreading technique yields unaligned DNA fibers, which complicates the analysis of some parameters such as inter-origin distance and new origin firing²³⁰. In DNA spreading technique it is important to select areas for imaging with less DNA crossings which makes the image acquisition more laborious. Alternative methods, DNA “combing” and DNA “stretching”, result in a set of unidirectional DNA fibers which facilitates the analysis of more complicated parameters and make the determination of DNA fork speed more accurate²³⁰.

To improve the throughput of the measurement, cells were synchronized with aphidicolin (**Papers III and IV**) enabling the recording of as much replicating DNA as possible. A drawback of using aphidicolin is that it causes replication stress itself²³¹ which could affect the results. At least 100 unidirectional forks labeled with both 5-Chloro-2'-deoxyuridine (CldU) and 5-Iodo-2'-deoxyuridine (IdU) were measured for every condition using Fiji software²³² and the speed of the replication fork was determined from the length of the DNA fiber by conversion $1 \mu\text{m} = 2.59 \text{ kb}$.

3.7 FLOW CYTOMETRY ANALYSIS OF CELL CYCLE PROGRESSION OF REPLICATING CELLS

In **Paper III** and **IV** we were interested how replicating S phase cells progress in cell cycle upon our treatment conditions. Thus, we used a technique where we first synchronize cells to G1/S boundary and then release them in media containing (5-Ethynyl-2'-deoxyuridine) EdU for 45 min to allow cell cycle to proceed to early S phase simultaneously labeling replicating cells that then can be traced^{233,234}. Performing drug treatments post-EdU labeling allowed us to decipher how cells that have been treated in S phase proceed in cell cycle by fixing the cells at certain time points after treatment. To visualize EdU-labeled cells, Click-iT labeling was performed after fixation with 70 % ethanol by linking fluorescently labeled azide (ATTO 647 azide) to an alkyne group of EdU by copper-catalyzed Click chemistry reaction²³³. In addition, cells were stained with propidium iodide (PI) for the determination of cell cycle distribution of cells. Moreover, labeling of additional markers, such as γ H2AX (**Paper IV**), can be performed in order of to select cells with DNA damage for analysis or to determine in which cell cycle phase DNA damage accumulates.

When pulsing cells with EdU, it is important to use prewarmed cell medium to minimize disturbances in DNA replication caused by temperature changes. When cells are pulsed only 45 min, EdU-mediated toxicity is neglectable, however, as we used cell synchronization with aphidicolin for 24 h to concentrate cells in early-S phase at the time of the drug treatment, cells maybe face replication stress that could potentially slow down their replication and cause “background” with further drug treatments that disturb replication. When setting up the cell gating for flow cytometry cell debris, dead cells (by size) and cell doublets were ruled out from the analysis. However, as live-dead staining was not used, it is possible that some dead cells remained in the analysis. The gating for cell cycle was based on PI intensity histograms after selection of EdU positive cell populations. However, the gate determination is not completely accurate by single PI staining and this could cause small errors in the estimation of the cell cycle distributions that should not however change the conclusion of the results as gating was set as uniformly as possible between different samples.

3.8 ETHICAL CONSIDERATIONS

Paper II and **Paper IV** conducted work with ovarian patient-derived cancer cells which were obtained and processed according to the ethical permits 2016/1197-31/1, 2018/118-32 and 2018/2462-32 approved by the Stockholm Regional Swedish Ethics Review Board. The work conducted with this patient material helped us to confirm that CX3CR1 and PFKFB3 inhibition had a similar effect in clinically relevant cell models to what we had observed in studies with cancer cell lines.

4 RESULTS

4.1 PAPER I

Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination

This paper focused on dissecting the role of PFKFB3 in DNA repair upon ionizing radiation, revealing that PFKFB3 is involved in the repair of DSBs via regulation of HR repair. Immunofluorescence experiments revealed that upon ionizing radiation (IR), PFKFB3 relocates into nuclear foci (IR-induced foci; IRIF), where it co-localizes with the DNA damage marker γ H2AX and, to a lesser extent, with 53BP1, RPA and BRCA1. Furthermore, the PFKFB3 IRIF was dependent on the ATM kinase activity, γ H2AX, MDC1 and the MRN complex, which are all integral components of HR repair. Moreover RAD51, RPA32 and BRCA1 failed to be recruited into IRIF when PFKFB3 was knocked down by siRNA. To provide more evidence on the involvement of PFKFB3 on HR repair, we showed that the HR activity of cells is impaired upon PFKFB3 silencing, utilizing a DR-GFP assay²³⁵. In accordance with decreased HR activity, knockdown of PFKFB3 resulted in delayed G2/M progression upon IR, indicating unrepaired DNA damage, ultimately reducing long-term survival of irradiated cancer cells in colony-formation assays. Altogether this demonstrated that ablation of PFKFB3 disrupts HR repair and sensitizes cancer cells to ionizing radiation.

We further developed a selective small molecule inhibitor that binds in the substrate pocket of PFKFB3. A high throughput screening of 50,000 compounds yielded 105 compounds that bound PFKFB3 in nM or low- μ M concentrations. From these hits, non-ATP competitive compounds were chosen for further development to minimize non-specific activity with other kinases. KAN0438241 was identified as a specific PFKFB3 inhibitor that inhibited PFKFB3 with 20-fold difference in IC₅₀ compared to PFKFB4 and has no effect on the activity of other two PFKFB enzymes. Co-crystal structures demonstrated that KAN0438241 binds in the active site of PFKFB3, similar as the natural substrate fructose-6-phosphate. To improve cell permeability, KAN0438757 was developed, which is an ester of KAN0438241. Upon entering the cell, KAN0438757 is converted to its active metabolite KAN0438241, which is responsible for the inhibitory effect on PFKFB3. In cellular assays, KAN0438757 reduced the production of intracellular F-2,6-P₂ and decreased the viability of several cancer cell lines. KAN0438757 demonstrated intracellular target engagement to PFKFB3 in Cellular Thermal Shift Assay (CETSA®)^{236,237}.

The newly developed PFKFB3 inhibitor KAN0438757 allowed us to investigate if HR repair was dependent on the kinase activity of PFKFB3, as the readily available small molecule PFKFB3 inhibitor 3PO, failed to mimic the effects on DNA repair that we demonstrated by PFKFB3 knockdown. On the contrary to 3PO, inhibition of PFKFB3 by KAN0438757 resulted in the blocked recruitment of RPA and RAD51, as well as PFKFB3 itself, into IRIF without affecting total protein levels. With KAN0438757, we could replicate the effects of PFKFB3 knockdown on the HR activity, G2/M phase cell cycle progression and long-term proliferation

upon IR. Moreover, KAN0438757 resulted in accumulation of DNA damage post IR as demonstrated by the increased γ H2AX IRIF at 24h. Taken together, these results indicated that the kinase activity of PFKFB3 is required for effective HR repair following IR.

Next, we discovered that upon IR, PFKFB3 inhibition decreased nucleotide incorporation into DNA as measured by EdU pulse in the G2/M phase, and PFKFB3 colocalized with the RNR subunit RRM2 in IRIF. We further showed that RRM2 IRIF was dependent on the PFKFB3 activity, that inhibition of RRM2 resulted in reduced HR activity, and that PFKFB3 and RRM2 associated physically, as seen in co-immunoprecipitation experiments. Moreover, PFKFB3 inhibition resulted in a decrease in the total intracellular dNTP pool and stalling of replication forks as measured by DNA fiber assays. The fact that we could rescue the replication speed and cancer cell proliferation by supplying nucleosides supports the hypothesis that PFKFB3-mediated dNTP supply is responsible for the effects seen on DNA replication and repair upon PFKFB3 inhibition.

Finally, when compared to hydroxyurea (HU), which limits the dNTP pool by inhibiting RNR and induces replication stress, PFKFB3 inhibition did not increase RPA or further checkpoint responses. This is in line with our results showing that RPA IRIF are blocked when PFKFB3 is inhibited. Furthermore, PFKFB3 inhibition blocked HU-induced RPA accumulation and checkpoint activation. This demonstrates that replication forks stall upon PFKFB3 inhibition due to decrease in local dNTP supply, but do not collapse and checkpoint response is not activated.

In summary, in this paper we discovered an unexpected role of PFKFB3 in DNA repair, showing for the first time that PFKFB3 co-localizes directly with DNA repair factors in IRIF. The recruitment of PFKFB3 is dependent on MRN complex, ATM, MDC1 and γ H2AX involved in HR repair. At DSB repair sites PFKFB3 activity regulates the local dNTP pool to support repair by the recruitment of RRM2. We conclude that RAD51, BRCA1 and RPA32 recruitment into damage foci occurs downstream of PFKFB3. The regulatory function of PFKFB3 on HR is likely not via its role in glycolysis, as glycolysis occurs in the cytoplasm, and we demonstrate that PFKFB3 is readily recruited to DNA damage foci upon IR, co-localizing with HR repair factors and that loss of PFKFB3 IRIF correlates with impaired HR repair. Importantly, we revealed that PFKFB3 inhibition sensitizes cancer cells to ionizing radiation at doses not affecting non-transformed cells which present a future possibility of using PFKFB3 inhibition as a clinical approach to achieve a greater cytotoxic effect on cancer cells without affecting healthy cells. Importantly, we present a new potent, selective, and specific PFKFB3 inhibitor, KAN0438757, that can be used as a tool to study the molecular functions of PFKFB3 further.

4.2 PAPER II

Blocking the fractalkine receptor disrupts replication and ovarian cancer cell proliferation

In this project we evaluated the effects of our small molecule CX3CR1 inhibitor, KAND567, in blocking ovarian cancer cell viability and further investigated the regulatory role of CX3CR1 on ovarian cancer cell replication. We showed that CX3CR1 inhibition by KAND567 reduced the viability of ovarian cancer cell lines, in short- and long-term viability assays, without affecting non-malignant cells at the same concentrations. The reduced viability was most likely due to the dose-dependent induction of DNA damage and apoptosis following treatment with KAND567, as assessed by western blot markers and flow cytometry. In addition, KAND567 was effective in reducing the viability of platinum resistant ovarian cancer tumor cells. We further investigated the effects of CX3CR1 inhibition on cell cycle progression and replication, showing that cancer cells accumulate in G0/1 and decrease in S and G2/M phases in a dose- and time-dependent manner upon KAND567, indicating a potential slowdown of G1-S transition. Supporting this, serine 780 phosphorylation of the retinoblastoma protein (pRb), which drives cell cycle progression from G1 to S, was blocked when CX3CR1 was inhibited. However, the decrease in S phase occurred prior to significant G1 accumulation.

In line with this, replication of ovarian cancer cells, as measured by EdU incorporation in immunofluorescence experiments, decreased upon KAND567, indicating impairments in replication followed by disturbed G1 to S transition. Furthermore, to assess checkpoint response to replication stress upon CX3CR1 inhibition, we showed by immunofluorescence that CX3CR1 inhibition leads to early reduction in RPA levels. Moreover, reduction of RPA serine 33 phosphorylation, followed by reduced ATR phosphorylation was observed by western blot, indicating impaired checkpoint activation via RPA-ATR axis. Interestingly, when we inhibited ERK signaling, which is one of the possible downstream effectors of CX3CR1, RPA and ATR phosphorylation as well as the phosphorylation of pRb was blocked. However, unlike following CX3CR1 inhibition, cells did not enter apoptosis at the time point and concentration of ERK inhibitor tested, indicated by the lack of cleaved PARP.

A combination treatment of KAND567 and carboplatin or IR revealed that in DNA damage conditions, CX3CR1 inhibition leads to delayed S to G2/M transition, increased DNA damage and increased apoptosis. Consistent with accumulation of cells in S phase, CHK2 was strongly activated in co-treated cells indicating intra-S checkpoint activation in damage conditions. Notably, we observed that the combination treatment of KAND567 and carboplatin was especially effective in platinum resistant ovarian cancer cells although EC50 value for KAND567 as a single treatment did not greatly differ between the platinum resistant and sensitive cell line pairs. Compared to the platinum sensitive cells, the platinum resistant cells displayed a loss in G2/M cell population and induction of DNA damage and apoptosis upon KAND567 in combination with carboplatin compared to carboplatin treatment alone.

To summarize, in this manuscript we show that CX3CR1 is a driver of ovarian cancer cell proliferation and survival, supported by earlier research reports^{136,141,156,164} and clinical survival data^{141,238}. Moreover, by using small molecule inhibitor of CX3CR1, we provide evidence that CX3CR1 supports replication of ovarian cancer cells and potentially regulates G1 to S phase progression in unperturbed conditions. In contrast, when CX3CR1 inhibition is combined with DNA damaging treatments, cells are unable to progress from S phase and display increased DNA damage and apoptosis as assessed by western blot markers. Overall, these results indicate that CX3CR1 has roles in supporting replication and protecting cancer cells against DNA damage and that KAND567 effectively blocks ovarian cancer cell proliferation and sensitizes ovarian cancer cells to DNA damaging agents.

4.3 PAPER III

Targeting CX3CR1 suppresses the Fanconi Anemia DNA repair pathway and synergizes with platinum

Here we investigated further the combination of CX3CR1 inhibition with platinum drugs in cancer cells and the mechanism behind suggested CX3CR1-driven platinum resistance¹⁶⁴. By performing drug synergy studies, we revealed that KAND567 has a synergistic effect with platinum drugs carboplatin and cisplatin on reducing cancer cell survival. High synergy scores were achieved especially in platinum resistant cancer cells compared to platinum sensitive cell lines and the drug combinations did not yield synergistic scores in non-transformed cell lines. To support the inhibitor data, we further demonstrated that siRNA and shRNA mediated knockdown of CX3CR1 sensitized the ovarian cancer cell line A2780 to carboplatin and cisplatin.

As cancer cell resistance upon platinum damage is linked to the FA repair capacity tumors^{239–242}, we hypothesized that the inhibition of CX3CR1 may interfere with this pathway. As the FA pathway regulates the replicative S phase repair, to test our hypothesis, we synchronized platinum sensitive and resistant cancer cells to G1/S border by aphidicolin and pulsed them with EdU during washout to label replicative cells before exposure to cisplatin and KAND567. This allowed us to follow replicating cells upon drug treatment by flow cytometry. When cells were treated with cisplatin and KAND567 they arrested strongly in S phase at 6 h post treatment which was sustained at the 16 h timepoint whereas cisplatin-treated cells progressed throughout the cell cycle. Notably, the effect of CX3CR1 inhibition was especially prominent in the platinum resistant cells which divided and proceeded to G1 in the presence of platinum, but when CX3CR1 was inhibited, a significant percentage of the co-treated cells was still arrested in S phase at the 16 h time point. DNA fiber analyses revealed that the fork speed was reduced in the co-treated cells, indicating slowdown of replication potentially due to unresolved DNA damage. Indeed, when we measured the percentage of DNA-cisplatin adducts after platinum treatment by flow cytometry, we noticed that cancer cells were not able to resolve these adducts effectively when CX3CR1 was inhibited. Notably, this replication slowdown and accumulation of cells in S phase following CX3CR1 inhibition resembles the phenotype of FA cells which,

upon ICL induction, accumulate in late S phase²⁴³, which encouraged us to investigate CX3CR1 further with ICL-inducing agents²⁴⁴.

In following immunofluorescence studies, we investigated the intracellular localization of CX3CR1 in response to treatments that activate the FA pathway²⁴⁴, showing that CX3CR1 localized to the nucleus in response to cisplatin, mitomycin C (MMC) and hydroxyurea. All these drugs activated recruitment of FANCD2 into nuclear foci, consistent with FANCD2 foci being a marker for the FA pathway activation²⁴⁵, and we observed that the nuclear intensity of CX3CR1 and FANCD2 correlated in response to these drugs. Moreover, when CX3CR1 was inhibited or knocked down, FANCD2 nuclear foci formation, in response to cisplatin or MMC, was disturbed. Furthermore, by performing chromatin-fractionations of protein lysates, we showed that the chromatin-recruitment of both FANCD2 and FANCI, as well as the FA pathway downstream factors RAD51 and γ H2AX, was blocked upon CX3CR1 inhibition.

In summary, in this paper we reveal a novel role for CX3CR1 in regulating the FA repair of ICL crosslinks, and that CX3CR1 inhibition by KAND567 sensitizes cancer cells to platinum treatment while sparing non-transformed cells. These findings could partly explain how high CX3CR1 expression can promote platinum resistance in cancer patients, contributing to poor survival rates^{141,150,155}.

4.4 PAPER IV

PFKFB3 regulates repair of DNA interstrand crosslinks via modulation of the Fanconi Anemia repair pathway

As a starting point of this paper, we hypothesized that PFKFB3 inhibition, due to its newly discovered role in DSB repair in **Paper I**, could synergize with DNA damaging treatments. We first revealed that PFKFB3 inhibition by KAN0438757 synergized with platinum in the reduction of cancer cell viability and that platinum resistant cells displayed generally higher synergy scores. Meanwhile KAN0438757 and platinum combination treatments did not have a synergistic effect on non-malignant cells.

In contrast, the glucose analogue 2-deoxy-D-glucose, which competitively inhibits the glucose-6-phosphate production²⁴⁶, did not display a cancer-specific effect in reducing viability. Furthermore, platinum treatment did not induce significant changes in glycolysis as measured by ExtraCellular Acidification Rate (ECAR) and modulating levels of glucose in the cell media do not alter sensitivity to carboplatin, indicating that the synergistic effect between PFKFB3 inhibition and platinum were not due to inhibition of the glycolysis.

Notably, while PFKFB3 inhibition was equally effective in blocking glycolysis in transformed and non-transformed cells, PFKFB3 itself demonstrated a cancer-specific localization to the chromatin following cisplatin treatment together with other DNA repair factors as assessed by chromatin fractionations. Due to the strong synergy of PFKFB3 inhibition with platinum drugs, we wondered if PFKFB3 could be involved in the FA repair of ICLs induced by platinum treatment¹⁸. Thus, we investigated the dynamics of the nuclear PFKFB3 by

immunofluorescence in response to ICL-inducing treatments²⁴⁴, revealing that PFKFB3 accumulated into nuclear foci and this increased in time dependent manner upon both cisplatin and MMC. PFKFB3 foci formation correlated with γ H2AX and RPA foci induction following these treatments. When we compared platinum resistant and sensitive cells, PFKFB3 recruitment to the chromatin, along with several FA pathway factors, was enhanced in resistant cells constantly cultured with a low cisplatin concentration compared to cisplatin sensitive cells receiving a cisplatin pulse. In line with a potential role in the FA pathway, PFKFB3 shRNA mediated knockdown rendered cancer cells sensitive to treatments that activate the FA pathway (cisplatin, carboplatin, MMC and low dose hydroxyurea)²⁴⁴.

We further investigated the mechanistic aspects of PFKFB3 recruitment to nuclear foci upon the FA pathway activation in immunofluorescence experiments and observed that PFKFB3 foci formation upon MMC treatment was dependent on ATR kinase activity and FANCM, but independent of RPA. On the other hand, recruitment of FANCM, FANCD2, BLM, γ H2AX and RPA32 into repair foci upon cisplatin and MMC treatments were blocked when PFKFB3 was inhibited. This data was further supported in chromatin fractionation experiments that allowed us to also assess chromatin-binding of additional factors involved in FA repair. These experiments revealed that PFKFB3 inhibition blocked the recruitment of FANCI, TopIII α , γ H2AX and PCNA. The kinase activity of PFKFB3 was required for its own recruitment into repair foci. Moreover, PFKFB3 physically interacted with FANCD2, BLM and γ H2AX indicating that PFKFB3 has an essential role in the assembly of FA repair factors at the sites of ICL damage.

We next studied the functional consequences of PFKFB3 inhibition in the presence of ICL damage. Given the role of the FA pathway in allowing DNA replication to resume following ICL damage, we were interested to assess effects of PFKFB3 inhibition in replication upon ICL-induction. Accordingly, PFKFB3 inhibition resulted in impaired recovery of replication after cisplatin treatment as measured in EdU incorporation assays. Furthermore, DNA fiber assays revealed a slowdown of fork speed, fork stalling and a reduction of fork restart after MMC and PFKFB3 inhibitor co-treatment, compared to cisplatin or MMC treatments alone. Furthermore, γ H2AX positive S phase cells were not able to progress throughout the cell cycle upon co-treatment whereas cisplatin-treated cells were able to resume their cell cycle progression. This suggested that upon PFKFB3 inhibition, replicating cells are not able to resolve ICLs which results in an accumulation of DNA damage and S phase arrest. Finally, using patient-derived ovarian cancer cells, we confirmed that upon inhibition of PFKFB3, cisplatin-induced FANCD2 foci formation was blocked, strengthening our observations of the regulatory impact of PFKFB3 in FA repair in a clinically relevant cell model. Altogether these findings highlight the importance of PFKFB3 in FA repair and suggests that this function of PFKFB3 might be separate from its role in the glycolysis.

5 DISCUSSION

5.1 CX3CR1

CX3CR1 is a multifunctional regulator of cancer progression¹²⁷. This thesis work investigated the role of CX3CR1 in cancer proliferation and survival, providing evidence that CX3CR1 is involved in DNA replication (**Paper II**) and repair (**Paper III**) in cancer cells. Based on the findings in **Paper III**, we suggest a nuclear role for CX3CR1 that could function independently of the signaling pathways known to be activated downstream of CX3CR1. Besides the results presented in **Papers II** and **III**, another report¹⁶⁴ connecting CX3CR1 to DDR, was published during this thesis work. As summarized in Chapter 1.3.2, Xie et al. show that knockdown of CX3CR1 by siRNA for 72 h results in inhibition of ATM and DNA-PK activation, followed by delay of initial γ H2AX induction, increased DNA damage over time and loss of ovarian cancer cell viability¹⁶⁴. Unlike in our studies with a small molecule inhibitor of CX3CR1, Xie and colleagues reported inhibition of CHK2 phosphorylation and overall reduction in RAD50 levels. However, our assessment of RAD50 total protein levels (unpublished) upon CX3CR1 inhibition, did not reveal any changes in RAD50 overall levels potentially due to differences in cell lines used or the duration of CX3CR1 ablation. In addition, knockdown of CX3CR1 could have a different effect than targeted inhibition of the receptor due to potential off target effects of siRNAs, variable knockdown efficiency and longer time needed for ablation of the protein compared to inhibitor due to possible low turnover of the target.

In **Paper III**, we show that inhibition and knockdown of CX3CR1 blocks the recruitment of FANCD2 following ICL damage. Interestingly, FANCD2 is involved in the repair of DSBs, independently from its role in ICL repair^{247,248}. FANCD2 deletion upon DSB induction leads to slowdown of replication fork progression, increased S phase arrest, impaired recruitment of RAD51 and RPA32, persistent γ H2AX foci and increased genome instability²⁴⁸. Thus, the DNA damage induction upon CX3CR1 knockdown reported by Xie et al. could result from impaired FANCD2 recruitment to DSBs upon IR¹⁶⁴.

Moreover, signaling pathways downstream of CX3CR1 (Figure 8) such as PI3K/Akt, MAPK/ERK, EGFR and β -arrestin pathways are connected to the regulation of cell cycle, survival, replication, and DDR, and are often activated in cancers^{249,250}. For example, activation of Akt signaling in response to cisplatin can be detected in the HGSOV cell line Ovar-3²⁵¹ and inhibition of PI3K/Akt signaling can re-sensitize ovarian cancer cells to cisplatin²⁵². On the other hand, EGFR seems to be an important determinant of radioresistance and EGFR blockers have been shown to improve responses to radiation therapy²⁵³⁻²⁵⁵. There are several possible mechanisms that could explain how these signaling pathways can drive resistance to DNA damaging agents, including involvement in the cell cycle regulation²⁵², crosstalk with DNA repair pathways^{256,257}, inhibition of pro-apoptotic proteins¹⁶⁰ and modulation of tumor microenvironment²⁵⁸, highlighting the various ways survival signaling can modulate the response to platinum and radiation treatments. Thus, part of the CX3CR1-induced effects on

DDR, could be conferred via CX3CR1-mediated signaling. The following chapters will discuss several different mechanisms how CX3CR1 can confer its effects on DDR.

5.1.1 The nuclear role of CX3CR1

The traditional view of GPCR signaling includes receptor activation on the cell surface followed by G protein and β -arrestin-mediated responses in intracellular signaling pathways and eventual termination of the signaling via receptor internalization¹³⁷. However, GPCRs can continue to be active also after internalization and subcellular localization has an important functional role for many GPCRs²⁵⁹. GPCRs are internalized from the membrane to early endosomes in a process involving β -arrestins and other proteins and these endosomes can subsequently fuse into other phospholipid-containing membranes inside the cell¹³⁹. So far more than 30 GPCRs have been reported to localize to the nucleus and the nucleus contains the complete GPCR signaling machinery including G proteins and β -arrestins²⁵⁹. The nucleus contains several intranuclear hydrophobic areas such as nuclear membrane invaginations and nuclear bodies that can harbor GPCRs in addition to the inner and outer nuclear membrane^{139,259}. Interestingly, the CX3CR1 nuclear staining in the confocal microscopy studies in **Paper III** displayed both pan-nuclear but also few concentrated larger foci-like CX3CR1 staining areas in part of the cell population which did not clearly co-localize with the DNA repair factors assessed (data not shown). In contrast to the pan-nuclear staining which increased upon cisplatin and MMC treatments, the larger foci staining was not altered upon these treatments. These structures could be attributed to intranuclear hydrophobic areas favorable to CX3CR1 docking. Another option is that CX3CR1 localizes to centrosomes which have been shown to anchor another GPCR known as the sphingosine 1-phosphate 5 receptor²⁶⁰.

Some GPCRs have been shown to directly associate with the chromatin and be able to regulate gene expression. For example, the coagulation factor II receptor-like 1 (F2r1) translocates from the cell membrane to the nucleus and to the chromatin, where it facilitates the recruitment of a transcription factor to trigger gene expression leading to neovascularization¹³⁸. In addition, the same F2r1 receptor that is found at cell surface triggers a signaling cascade that leads to expression of genes related to vessel maturation¹³⁸, highlighting the separate roles of the receptor supporting complementary functions of the same physiological process. In a similar manner, CX3CR1 could have separate roles in DNA repair and replication via its nuclear role and via CX3CR1-mediated signaling pathways.

Although we could not detect CX3CR1 in distinct DNA repair foci, we discovered that CX3CR1 associated to the chromatin and this was increased upon cisplatin and blocked by KAND567. A limitation of **Paper III** is that although CX3CR1 localization to the nucleus and chromatin was detected, a direct interaction with DDR factors or DNA damage sites was not detected. In follow-up studies, detailed investigation of how CX3CR1 is anchored in the nucleus and how it interacts with chromatin and associates with repair factors should be assessed by co-immunoprecipitations, by epitope-tagged CX3CR1 and by isolation of lipid-containing nuclear compartments. Moreover, the use of bioluminescence resonance energy

transfer (BRET) assays could make it possible to follow the trafficking dynamics and activity of CX3CR1 from the membrane to the nucleus in real-time²⁶¹.

In Paper III we also showed that CX3CR1 translocates to, or redistributes in, the nucleus in response to DNA damaging treatments that activate the FA pathway. This could potentially be achieved by increased CX3CL1 expression and subsequent CX3CR1 activation followed by nuclear translocation or redistribution as several chemokine ligands are upregulated in response to both radiotherapy and platinum treatments, although radiation-induced inflammatory chemokine secretion is better characterized²⁶²⁻²⁶⁵. Another option is that CX3CR1 is internalized by ligand-independent mechanisms in response to platinum. Interestingly, EGFR has been shown to relocate to the nucleus following treatment with cisplatin and IR and bind to DNA-PKcs, possibly in a ligand-independent manner, promoting repair of DNA lesions caused by these agents^{256,266}. In a similar manner, CX3CR1 translocation could modulate DNA repair kinetics following platinum and radiation treatments. Moreover, ROS, created in response to by both platinum and radiation treatments, is an important effector of cell signaling and able to activate a variety of receptors and could therefore perhaps activate CX3CR1²⁶⁷. A third option is that CX3CR1 exhibits constitutive activity²⁶⁸⁻²⁷⁰.

Notably, our preliminary assessment of the effects of CX3CL1 upon platinum treatment indicated that excess CX3CL1 does not increase ovarian cancer survival upon platinum (data not shown), meaning that the function of CX3CR1 upon DNA damage does not require CX3CL1 or that CX3CL1 was already present in adequate quantities and a plateau was reached. Future studies should thoroughly investigate if CX3CL1 knockdown influences CX3CR1-mediated effects on DDR and if CX3CL1 expression or secretion increases upon DNA damage induction. Notably, the fact that KAND567 blocked the CX3CR1-mediated events upon DNA damage further expands the utility of this inhibitor in the case that CX3CR1 activation is found to be ligand-independent.

5.1.2 The MAPK/ERK pathway

Paper II provides evidence that ERK1/2 is phosphorylated in the platinum resistant ovarian cancer cell line A2780Cis and this phosphorylation is blocked by CX3CR1 inhibition. In addition, similar to CX3CR1 inhibition, ERK inhibition blocked the phosphorylation of ATR, RPA, and pRb, suggesting that ERK pathway could partly mediate CX3CR1-dependent responses in the regulation of DDR. This should be however thoroughly investigated by experiments combining CX3CR1 and ERK inhibitors or by cell lines harboring inactive ERK. In addition, cleaved PARP was not induced by ERK inhibition alone which indicates that blocking ERK signaling is not enough to induce apoptosis and inhibition of other CX3CR1-mediated events is required for cells to undergo apoptosis. On the other hand, differences in the phenotypes resulting from CX3CR1 inhibition and ERK inhibition could also depend on the potential different efficacy of the inhibitors used.

Supporting findings in **Papers II and III**, inhibition of MAPK/ERK signaling has been shown to sensitize cancer cells to DNA damaging agents and ERK can regulate both Akt and ATM

activation²⁷¹⁻²⁷⁴. For example, inhibition of MEK in MAPK/ERK pathway has been reported to sensitize ovarian and pancreatic cancer cells to DNA damaging agents via *BRCA2* downregulation²⁷⁵. Reciprocally ERK can be activated by PIKK kinases and ERK activation has been reported for example in response to DSBs and DNA damaging agents such as cisplatin and IR²⁷⁶. At the same time that ERK mediates pro-survival signals, it can also promote apoptosis, and inhibition of ERK1/2 has been reported to prevent cisplatin-induced apoptosis in HeLa cells²⁷⁷, which supports our findings in **Paper II**. It is suggested that ATM activates Akt in response to DSBs, which mediates pro-survival signals, but at the same time activates ERK, which can activate apoptotic pathways²⁷⁶. Depending on the extent of DSB damage, either survival or apoptosis signals dominate. Due to this dual role of ERK in cancer survival and apoptosis inhibition, both ERK and Akt pathway may be required to efficiently stop cancer progression²⁷⁸, a strategy that could be possible via CX3CR1 inhibition.

5.1.3 The PI3K/Akt pathway

The PI3K/Akt/mTOR pathway is the most frequently altered signaling pathway in ovarian cancer and it is associated with poor survival²⁷⁹. Importantly, Akt signaling has been shown to promote ovarian cancer proliferation in a CX3CR1-dependent manner¹⁶¹. Akt activation is associated with platinum^{264,280,281} and radioresistance^{282,283} and its inhibition sensitizes several cancer cell lines to these therapies^{252,284-286}. CX3CR1-induced Akt signaling leads to cell survival for example by regulating the BCL2 associated agonist of cell death (BAD) in non-malignant cells^{121,126,287}. However, Akt is also involved in DNA repair and checkpoint signaling beyond its role in anti-apoptotic pathways²⁸⁸ and is thus an attractive mediator of CX3CR1-induced effects on DDR. Akt phosphorylates DNA-PK to promote NHEJ, and is reciprocally activated by all three PIKKs in response to DNA damage facilitating HR repair and ICL repair²⁸⁹⁻²⁹¹. Inhibition of mTOR, which is a downstream factor of Akt, suppresses FANCD2 expression^{292,293} and mTOR inhibition synergizes with PARP inhibition in *BRCA2*-mutated breast cancer cells²⁹⁴ and sensitizes T-cell lymphoblastic leukemia cells to DNA damaging agents including cisplatin²⁹³. In addition, blocking mTOR leads to potentiation of IR-induced S phase arrest and increase in γ H2AX²⁹⁵ and mTOR inhibition can selectively downregulate factors associated to DDR, cell cycle and survival in platinum-resistant ovarian cancer cells²⁹⁶. Moreover, Akt regulates PCNA ubiquitination in response to UV irradiation, and inhibition of Akt can block the recruitment of TLS polymerases impairing replication forks and conferring synthetic lethality in HR-deficient cells²⁹⁷. Therefore, reduction in Akt signaling could contribute to the DNA repair deficiencies seen upon CX3CR1 inhibition, a hypothesis which could be tested in the future for example in rescue experiments by overexpressing a constitutively active Akt in the presence of KAND567.

Interestingly, it has been reported that the PI3K/Akt pathway regulates G1 progression in A2780 cells by promoting expression of cyclins and that Akt inhibition leads to inhibition of pRb and accumulation of cells in G0/G1²⁹⁸ similar to what was seen upon CX3CR1 inhibition in **Paper II**. **Paper II** reports that CX3CR1 could drive G1 to S progression in A2780 ovarian cancer cells, as CX3CR1-inhibited cells displayed accumulation in G0/G1. On the other hand,

when CX3CR1 inhibition was added subsequently after carboplatin treatment, which first synchronizes the cell population in S phase, S phase arrest and apoptosis were potentiated (**Paper II**). This phenotype was even clearer in **Paper III**, showing that simultaneous treatment of cisplatin and CX3CR1 inhibition in synchronized replicating cells, leads to an increase in S phase arrest. Similarly, Akt inhibition has a protective role in unsynchronized osteosarcoma cells by preventing S phase entry when Akt inhibitor is administered at the same time as cisplatin²⁸⁴. On the contrary, when cells are treated with cisplatin and Akt inhibitor sequentially, Akt inhibition sensitizes cisplatin-treated cells to apoptosis²⁸⁴. This highlights the importance of the correct treatment schedule of drugs in combination treatments. As both platinum and radiation therapy are most toxic for replicating cells^{10,299} and CX3CR1 inhibition blocks S phase repair, treatment regimens that push the cells into S phase prior addition of CX3CR1 inhibitor will maximize the treatment efficacy.

5.1.4 β -arrestins

Along with CX3CR1-induced G protein signaling, β -arrestin-regulated pathways are interesting candidates for CX3CR1-mediated regulatory effects. Besides their role in trafficking and termination of GPCR signaling they work as scaffoldings to elicit multiple signaling pathways such as those also activated by G proteins³⁰⁰. Moreover, β -arrestin-1 can translocate to the nucleus and regulate gene transcription and histone acetylation³⁰¹. In addition, β -arrestins can transactivate receptor tyrosine kinases and promote another round of G protein activation from early endosomes instead of desensitizing the receptor³⁰². The binding of CX3CL1 to CX3CR1 induces β -arrestin recruitment¹⁷⁴ but the consequences of β -arrestin activation in the CX3CL1/CX3CR1 axis have not been well-characterized and they might also be tissue-dependent. As summarized in the introduction, it was shown that KAND567 is partial agonist on CX3CL1-induced β -arrestin recruitment at low concentrations when both CX3CL1 and KAND567 are bound to the receptor but nearly completely blocks β -arrestin recruitment at the concentrations that displace CX3CL1¹⁷⁴. In future studies, it would be interesting to elucidate the potential relevance of the partial agonist functions of KAND567 on cellular responses as well as the β -arrestin mediated effects of CX3CR1 activation by CX3CL1.

In summary, CX3CR1 regulates multiple signaling pathways, and future studies should be directed towards dissecting which CX3CR1-induced signaling pathways are important in the DDR and upon resistance to DNA damaging agents, and to which extend the regulatory effect on DDR is attributed to possible uncanonical roles of CX3CR1. Given that CX3CR1 is an important driver of malignant processes, its inhibition will convey more targeted effects with less toxicity than general kinase inhibitors which interfere with multiple cellular processes as they inhibit many kinases unselectively, instead of one activated by a specific GPCR.

5.1.5 CX3CR1 and replication

Paper II provides evidence that CX3CR1 promotes DNA replication in ovarian cancer cells and CX3CR1 inhibition quickly blocks DNA replication and RPA and ATR phosphorylations. Interestingly, it is suggested that Akt/mTOR signaling controls dNTP synthesis by regulating

the expression *RRM2*^{288,303,304}. Moreover, MEK1/2 inhibition on the ERK signaling pathway suppresses *RRM1* levels via an Akt-dependent feedback loop³⁰⁵ and the combination treatment of the mTOR and ERK inhibitors impairs dNTP synthesis by suppression of both *RRM1* and *RRM2* expression resulting in synergistic reduction of renal cell carcinoma cell viability and tumor growth in a xenograft model³⁰⁶. Based on these findings, blocking CX3CR1 could suppress dNTP synthesis effectively by blocking both Akt/mTOR and ERK and subsequently *RRM1* and *RRM2* expression. Moreover, mTOR inhibition has been shown to prevent PCNA loading to the chromatin which leads to replication stress response via ATR and CHK1 activation³⁰⁷. However, **Paper II** demonstrated that CX3CR1 inhibition blocks phosphorylation of ATR and RPA potentially inhibiting the CHK1-mediated checkpoint response, indicating that upon CX3CR1 inhibition the replication stress response is not activated.

The effects of CX3CR1 inhibition on EdU incorporation and replication speed demonstrated in **Paper II** and **Paper III**, respectively, occur fast within 2 to 4 hours, which makes it worth to consider that the effects on replication could result from the blocked FANCD2 recruitment to the chromatin upon CX3CR1 inhibition (**Paper III**). FANCD2 is required in normal replication to maintain sufficient firing of replication origins³⁰⁸, it facilitates replication of common fragile sites³⁰⁹ and promotes re-start of stalled replication forks also in the absence of ICL induction³¹⁰⁻³¹², thus by blocking FANCD2 recruitment to replication forks, CX3CR1 inhibition could quickly reduce overall replication. Moreover, during high endogenous replication stress of cancer cells, the importance of FANCD is further highlighted as it stabilizes replication forks and its depletion results in spontaneous endogenous damage³¹³. Taken together, block of FANCD2 could be responsible of the impairment of replication and in addition increase replication stress above tolerable threshold to kill cancer cells upon CX3CR1 inhibition without additional DNA damaging agents.

5.2 PFKFB3

5.2.1 KAN0438757 as a selective PFKFB3 inhibitor

Several inhibitors of PFKFB3 have been developed and used in studies aiming to target PFKFB3, but surprisingly, most of them do not target the kinase function of PFKFB3 or are not specific^{216,217,223,224}, which has made it difficult to interpret the results of these studies. For example, **Paper I** demonstrated that 3PO could not mimic the effects seen upon PFKFB3 knockdown and later it has been shown to inhibit glycolysis without binding to PFKFB3²¹⁷. Moreover, the 3PO analogue, PFK-158 was also shown to have no effect on the enzymatic activity of PFKFB3 although it reduces the glycolytic flux and decreases F2,6BP²²³. In **Paper I**, a highly selective PFKFB3 inhibitor, KAN0438757, was developed which impaired the kinase activity of PFKFB3 and demonstrated intracellular target-engagement which was maintained at least 72 h, indicating a long-lasting inhibitory effect. Furthermore, KAN0438757 reduced cancer cell viability in concentrations that rendered non-transformed cells unaffected which encourages its potential as a drug candidate. In addition, the development of KAN0438757 facilitates the research on the function of PFKFB3 as a cancer target, compared

to the investigation of the general function of glycolysis on cancer that was possible with the “older” compounds.

KAN0438757 is used further in **Paper IV** where it synergized effectively with platinum drugs showing a good therapeutic window compared to non-malignant cells. Notably, KAN0438757 is well tolerated in mice without systemic toxic effects which encourages its use in future *in vivo* studies²⁰⁷. However, most of the discoveries in **Paper I** and **IV** were done in a panel of different cancer cell models including pancreatic and ovarian cancer cell lines, patient-derived cells, an osteosarcoma cell line and the BJ transformation series. Therefore, it would be interesting to evaluate the sensitizing effect of KAN0438757 to platinum and radiation both *in vivo* and in a larger panel of patient derived cells for future selection of responding patient subgroups and to evaluate the utility of KAN0438757 as a clinical candidate.

5.2.2 Nuclear and cytoplasmic roles of PFKFB3 in DNA repair and replication

Paper I and **Paper IV** provide evidence on the importance of PFKFB3 in the regulation of DNA repair and replication, showing that inhibition PFKFB3 interferes with HR and FA repair. During these studies, the nuclear role of PFKFB3 was further connected to DNA repair in additional pathways; PFKFB3 was reported to locate to the nucleus in liver cancer where it by interacting with Akt was suggested to upregulate the DNA excision protein ERCC1²¹⁰. In this report, inhibition of PFKFB3 resulted in increased DNA damage and reduction of tumor growth *in vivo*²¹⁰. However, the *in vivo* study was performed with PFK15 compound²²⁰, which is another 3PO derivative that was synthesized in the development series prior to PFK158, and thus the inhibitory effect on tumor growth is likely conveyed via inhibition of glycolysis and not by targeted PFKFB3 inhibition. **Paper I** and **IV** demonstrated for the first time that PFKFB3 associates directly to the chromatin at the DNA damage sites and co-localizes with repair factors, providing evidence that PFKFB3, besides activating signaling cascades that lead to regulation of DNA repair factors, can directly interact with them, and modulate their recruitment to damage sites.

In line with **Paper I** and **IV** revealing an important role for PFKFB3 in functional FA and HR repair, inhibition of PFKFB3 radiosensitized cancer cells and displayed a cancer-specific synergy with platinum. Our findings regarding a key role for PFKFB3 in maintaining genome integrity upon DNA damaging treatments has been supported by findings of other research groups during the course of this thesis work. PFKFB3 has been demonstrated to be activated in response to chemotherapy and to promote resistance to chemo- and radiotherapy in cancer cells, but whether this was due to its nuclear or cytoplasmic role, was unclear in these studies^{203,314,315}. Cisplatin was shown to increase glycolysis in HeLa and about 12 % of the PFKFB3 pool sequestered to the cytoplasm by acetylation in response to cisplatin treatment³¹⁴. These results are in contrast with our findings in **Paper IV** showing that platinum treatments do not induce a preference for glycolysis and that the survival of cancer cells upon platinum treatments was not affected upon altering glucose levels in the cell media which could be due to differences in cell models used in the studies. In support, Yalcin et al. previously discovered

that PFKFB3 can increase cell proliferation without modulating intracellular glucose metabolism¹⁸². In addition, in **Paper IV** PFKFB3 was recruited to the chromatin in response to ICL-inducing treatment in cancer cells. Discrepancies between the interpretations of the findings in these two studies could, apart from different cell types used, be explained by the fact that only 12 % of PFKFB3 was acetylated in HeLa cells in response to cisplatin and that PFKFB3 nuclear foci upon these conditions was not assessed, meaning that although part of the PFKFB3 pool was retained from the nucleus, a major part of PFKFB3 could still have a functional role at the chromatin.

Moreover, chemoresistant ovarian cancer cell lines were demonstrated to have a higher basal rate of glycolysis by another research group, but glycolysis rate in response to treatment with chemotherapeutics was not assessed²⁰³. Furthermore, another report showed that PFKFB3 knockdown sensitized endometrial cancer cells to platinum drugs and increased DNA damage potentially via inhibition of HR caused by suppression of total RAD51 protein levels and Akt/mTOR pathway³¹⁵. In comparison, **Paper I** reported that PFKFB3 inhibition impaired RAD51 foci formation upon IR but not total RAD51 levels indicating that PFKFB3 disables RAD51 recruitment to DNA damage sites without interfering with the total levels of RAD51. Conversely, the report by Xiao and colleagues did not assess nuclear PFKFB3 directly or the foci formation of RAD51³¹⁵. Taken together, the reduction of RAD51 in endometrial cancer cells and the reduction of RAD51 foci demonstrated in **Paper I** upon PFKFB3 ablation are most likely conveyed via separate PFKFB3-dependent mechanisms. Importantly, in **Paper IV** we reveal that PFKFB3 inhibition sensitizes cancer cells to platinum drugs by blocking the initiation step of ICL repair via inhibition of the recruitment of FANCM, FANCD2 and BLM to the damage sites, placing PFKFB3 upstream of the HR repair step. Therefore, the sensitization to cisplatin reported by Xiao et al., could be the result of the novel role of PFKFB3 in ICL repair, instead of the earlier suggested HR repair.

Paper I shows that PFKFB3 recruits RRM2 to the DNA damage sites thus promoting dNTP synthesis and HR repair upon IR. Inhibition of PFKFB3 impaired the recruitment of RAD51, RPA32 and BRCA1 to the damage sites. Interestingly, PFKFB3 colocalized with DSB repair proteins, indicating that PFKFB3 acts as a scaffold for RRM2 to directly provide dNTPs to the DNA damage sites where the need for dNTPs is high, especially in HR repair which requires extensive DNA synthesis^{86,87}. Notably, depletion of another glycolytic enzyme, Phosphoglycerate mutase 1 (PGAM), depletes dNTP pools via its enzymatic activity which leads to increased degradation of CtIP, impaired HR and synergy with PARP inhibitors regardless of functional BRCA1/2³¹⁶.

As it was suggested that Tip60 recruits the RRM1 subunit of RNR complex to the DNA damage³¹⁷, we investigated whether PFKFB3 and Tip60 could coordinate the recruitment of RRM2 and RRM1 in response to DNA damage and whether they are dependent on each other, but could not successfully identify any evidence for this (data not shown). In future studies, it would be interesting to discern, if the regulatory effects of PFKFB3 via dNTP supply extend

to other forms of DNA repair than HR, although the requirement of dNTPs is highest in HR compared to other DNA repair pathways⁸⁷.

In contrast to HR repair upon IR (**Paper I**), we found that γ H2AX foci-formation upon ICL repair was PFKFB3-dependent (**Paper IV**), which supports the hypothesis that PFKFB3 acts at a very upstream step of ICL repair. However, γ H2AX is also required for FANCD2 localization to the damage sites³¹⁸, which indicates that PFKFB3 regulates both these factors by promoting an upstream factor, such as recruitment of BLM. Interestingly, in BLM-deficient cells γ H2AX activation is delayed following replication stress induction, and T99-phosphorylated BLM co-localizes with γ H2AX upon replication damage, indicating that BLM could facilitate H2AX phosphorylation³¹⁹. BLM is essential in promoting the re-start of stalled replication forks upon ICL damage³¹¹, for recruitment of FANCM to stalled forks⁹⁴ and in DNA end-resection in HR repair³²⁰, which could extend the regulatory roles of PFKFB3 in multiple steps of ICL and HR repair depending on whether PFKFB3-controlled BLM regulation is direct or a FA repair-specific event. Future studies should determine which is the critical direct regulatory step of PFKFB3 in the initiation of ICL repair and how much of the effects on ICL repair seen in **Paper IV** can be attributed to the regulatory function of PFKFB3 on dNTP supply and *vice versa*. Finally, due to the central role of RAD51 in HR, several inhibitors and monoclonal antibodies have been developed aiming to block the interaction of RAD51 with chromatin, yet unsuccessfully, partly due to lack of specificity against RAD51³²¹. As PFKFB3 inhibition blocks RAD51 foci formation, targeting PFKFB3 could serve as an effective strategy for RAD51 inhibition.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis work reveals novel roles for CX3CR1 and PFKFB3 in the DDR, promoting cancer cell viability and treatment resistance. **Paper II** shows that CX3CR1 supports cancer cell viability by enhancing DNA replication and **Paper III** reveals that CX3CR1 promotes ICL repair. Furthermore, **Paper I** shows that PFKFB3 has a role in HR repair and in directed supply of nucleotides. In **Paper IV** we discovered that PFKFB3 has a crucial role in the initiation of ICL repair. Importantly, inhibition of these targets by KAND567 and KAN0438757 sensitizes cancer cells to DNA damaging treatments while sparing non-malignant cells. The best-known mechanism of resistance to platinum, and also to PARP inhibitors, is the somatic reversion of the original mutation (such as *BRCA1/2*) that rendered tumors deficient in FA or HR repair and resulted in an initial good response to platinum^{322,323}. Furthermore, the FA pathway activity is enhanced in many platinum resistant tumors^{239–242}. Thus, inhibition of HR and FA repair by KAND567 and KAN0438757 has implications in clinically relevant scenarios. Moreover, both KAND567 and KAN0438757 are well tolerated *in vivo* which encourages their future clinical development for cancer indications^{175,207}. In addition, combining inhibition of both targets could result in a synergistic effect by blocking DNA repair and replication in a more complete manner, a strategy worth exploring further.

CX3CR1 and PFKFB3 have multiple converging functions. **Paper I** and **III** show that PFKFB3 and CX3CR1 can regulate unperturbed DNA replication and **Paper III** and **IV** demonstrate that both targets are involved in ICL repair. Moreover, it was shown that CX3CR1 can also regulate HR repair¹⁶⁴. Inhibition of both targets results in cancer-specific synergies with platinum drugs (**Paper III** and **IV**). An interesting next step would thus be to assess whether inhibition of either target could regulate the recruitment of the other in DNA repair and replication, and whether CX3CR1 and PFKFB3 have redundant or complementary roles in the DDR. Assessment of a possible correlation between CX3CR1 and PFKFB3 expression profiles across cancers could serve as an informative starting point.

Our results showing DNA repair deficiencies upon CX3CR1 and PFKFB3 inhibition open the possibility for additional combination therapies targeting DNA repair. For example, PARP inhibitors have the potential to be effective in combination with CX3CR1 and PFKFB3 inhibition for several reasons. First, as both CX3CR1 and PFKFB3 are involved in HR, by inhibiting these targets PARP-inhibited cells would not be able to repair either SSB or DSB damage efficiently, which could result in similar synthetic lethality as seen in HR-deficient cancers⁶. Secondly, both CX3CR1 and PFKFB3 inhibition could synergize with PARP inhibitors due to PARP trapping, since the FA pathway is involved in removing trapped PARP from the chromatin³². Therefore, by inhibiting the FA pathway, PARP-DNA complexes might not be repaired efficiently, leading to increased replication stress. Moreover, rewiring of the DNA repair pathway can lead to cancer cell resistance to PARP inhibitors. For example, loss of 53BP1 and its effector Shieldin can partially restore HR and promote resistance to PARP inhibitors in *BRCA1*-deficient models^{74,324,325}, thus disabling HR repair by PFKFB3 and CX3CR1 inhibition could circumvent development of PARP resistance by HR restoration.

Furthermore, combining CHK1 or CHK2 inhibitors with CX3CR1 or PFKFB3 inhibition could lead to synergistic responses due to checkpoint override, despite uncompleted repair when combined with another DNA damaging agents. For example, CHK1 inhibition combined with suppression of FA pathway sensitizes lung cancer cells to gemcitabine³²⁶. Moreover, **Paper II** showed that CX3CR1 inhibition by KAND567 alone resulted in phosphorylation of CHK2, showing that inhibition of CHK2 might increase replication stress and DNA damage upon CX3CR1 inhibition. Finally, further drug synergy studies with a larger panel of DDR inhibitors and chemotherapeutic agents combined with KAND567 and KAN0438757 could help to map the type of DNA lesions that CX3CR1 and PFKFB3 assist repairing as well as show the extent of DDR processes they are involved in.

Further studies regarding the functions of CX3CR1 and PFKFB3 in the DDR are needed to pinpoint their value as clinical candidates in cancer therapy, to find rational treatment combinations and to understand which molecular cancer signatures could be best targeted with CX3CR1 and PFKFB3 inhibition. As we move towards personalized medicine, future studies should elucidate which molecular backgrounds of cancer cells make them dependent on CX3CR1 and PFKFB3 for survival during DNA damage and replication stress, and which mechanisms are able to rescue cancer cell viability upon inhibition of these targets. In addition, cancers lacking these proteins might possess targetable DNA repair vulnerabilities. Finally, assessment of the potential of CX3CR1 and PFKFB3 as cancer biomarkers could improve patient stratification and the chance of successful clinical trials, leading to improved treatment options for cancer patients.

7 ACKNOWLEDGEMENTS

I would like to thank everyone who crossed my path and influenced my way here.

My supervisor, **Nina Gustafsson**. Thank you for supervising the project despite the jump to unknown. Thank you for your dedication, for always pushing me and helping me to believe in the project. Without your persistence the project would never have turned out how it did. I have learned enormously during these years working together.

Thomas Olin and **Martin Norin** at Kancera, thank you for choosing me in this project and letting me work as part of Kancera until the end. It has been a great pleasure and of huge value to me to be involved in your projects and activities and it has given me perspective.

Thomas Helleday, thank you for inspirational talks, input as a co-supervisor and for providing an amazing and unique platform to work at!

My co-supervisors **Ali Moshfegh** and **Li-Sophie Rathje**. Ali, thank you for introducing me to various departments in KI and connecting me with people during the first steps of my PhD. Li-Sophie, thank you for the times we worked together and for always offering support.

Ulrika Warpman Berglund, your calm leadership always brought the sense of stability and good environment in the lab, thank you.

Dimitrios Chioureas, you joined the projects at the most crucial time and helped to bring them to the finish line. Thank you for the jokes, scientific talks and for bringing a relaxing atmosphere with your calm attitude. I wish you all the best for the future.

The whole Helleday Lab, past and current members, thank you everyone who helped me along the way whether it was introducing me to new equipment, borrowing reagents, discussing science or experimental setups, or just making me feel good at work. Special mention to **Elisée**, **Ingrid**, **Oliver**, **Therese** and **Carlos**, and the core team members **Mari**, **Lollo**, **Flor**, **Camilla**, **Sabina**, and **Kristina**.

Petra, I wish you the best for the rest of your PhD and after! Thank you for the Finnish talks and being so understanding and helpful.

Miriam, you joined the lab and brought the sunshine with you! Thank you for the positive attitude, supporting words and advice! I am glad we got to meet, and I am sure we will continue being friends.

Linnea, it was great to have you doing your Master's project in the Fractalkine project, thank you for all your efforts! **Saiganesh**, I hope we meet in Turku someday soon. I am looking forward to both of your PhD defenses in few years!

Dimitris Kanellis, it was always nice to see your kind smile around in Scilifelab, thank you for the chats, encouragement, and help.

Ben Lemmens, thanks for patiently helping with the microscope and for always being so kind.

And the whole **SciLifeLab** for creating an interesting and multidisciplinary place to work at.

Everyone who worked at **Kancera** during my time there. Thank you for including me in your discussions and project meetings, I learned a lot! Special thanks to **Carina Norström** for helping me with the cell work in the beginning of my PhD, **Sally Abdelmoaty** and **Kristin Hammer** for nice talks and advice, **Thomas Wehler** for helping me with the computer stuff, as well as **Elisabeth Olsson**, **Charlotta Löfberg**, **Johan Schultz**, **Niclas Brynne**, **Martin Bengtsson**, **Styrbjörn Byström**, **Karl Johan Fasth** and **Jan Vågberg** for being involved in the projects and making a nice atmosphere at Kancera.

I would like to thank the **SYNTRAIN** Marie Curie network: **Anne Vognsen** and all the PIs (Claus Storgaard Sørensen, Dana Branzei, Jiri Bartek, Madalena Tarsounas, Thomas Helleday, Thanos Halazonetis, Vassilis Gourgoulis, Zoi Lygeroy and Jos Jonkers) for creating such a stimulating network to be part of, and the PhD fellows **Nibal**, **Ana**, **Mariana**, **Karolin**, **Luka**, **Timo**, **Florian**, **Georgios**, **Nanda**, **Giacomo**, **Christos**, **Bishoy**, **Anna** and **Aleks**; it was always so much fun to meet around Europe and share stories from our PhD journey.

Mariana, **Ana** and **Catrin**, huge thanks for teaching the lentiviral work, generation of knockdown cell lines and the work with organoids at NKI and for being so helpful in general during my time in Amsterdam. Thanks to **Jos Jonkers** for letting me visit your lab!

The PhD support group, **Anna**, **Aleks**, **Stella** and **Nadilly**: I don't know what I would have done without you. To scratch the surface, thank you for all the lunches shared together and all the messages sent over the years. **Nadilly**, I can't thank you enough for all the help you have given to me during my PhD; your knowledge, attention to detail and diligence set a standard in the lab and your optimized protocols and perfected ELNs are beyond comparison. When in doubt, you will surely know the answer to anything. I am so happy we became friends; I wish you the best for the future and I am so excited for our upcoming reunions across the globe. **Aleks**, thank you for being such a sympathetic friend. I could always trust you with anything I had in my mind and I was sure to feel better afterwards. I am so happy to have been part of important events in your life during this short time and I hope we can share many more memories in the future! **Stella**, thank you for always being the kind realist with factual responses and comforting words to any dilemma at hand. I feel I can always rely on your evaluation, whether it is medical facts or the review of the monthly burger.

Anna, thank you for being the best colleague and friend that I could have wished for my PhD. I could not have imagined that it is possible to work so intensely together and simultaneously be such good friends. Thank you for teaching me many things and never stopping to poke me. You were always there for me whether it was about work or personal matters. Our team-of-two (with matching outfits) made the best out of every situation. Two people are stronger than one, but I think we reached synergy. It would have been much harder without you. Special thanks for your mom and dad for random calls and for rooting for me as well!

Amineh, thank you for your friendship, kindness, sharing troubles and being an example of strength and determination. I can't wait to see you defend!

My Stockholm family, the ones still in Stockholm and the ones already left for further adventures, I would have been very lonely without you and I am so grateful for having you as friends! Thank you for all the dinners, silly jokes, heated discussions, and more great times to come: **Mirco**, you never leave me hungry with your cooking and warm heart, welcoming me to your *deep-cleaned* home at any time (in Stockholm and in Taranto) and for always providing me with some inside knowledge, I still have to make the poem for your defense so I will finish this now; **Shane**, the funniest half-Finn, thank you for your mentorship in career and in life in general, your inception skills are beyond subtle but effective, I am so happy you were able to return to Stockholm quickly and re-join our crew, an extra huge thank you for reviewing parts of my thesis and for insightful comments and discussions; **Alice**, I miss you, you're the sweetest with the shiniest and tiniest backpack, I can't wait to visit you soon in Paris and share many more fun memories; **Nuria**, thank you for introducing me to some great quality beverages and for being the happy one with recognizable laugh from far away; **Alek**, I don't always manage to follow your line of thought but I am sure it's always about something with great intellect; **Gabriele**, the computer genius and editing master, I am so happy everything worked out so well for you; **Marco**, I am convinced you'll achieve all your crazy training goals; **Lorenzo**, Stockholm misses you a lot, I hope to see you soon in Barcelona; **Maria**, you must be one of the most straightforward and genuine people, and I am happy we got to know each other; **Harpa**, I hope to see you and your small family again soon, **Uri**; I am so happy you came to Stockholm and got to share some of our life here, and **Anna**, you are the reason I got integrated into this amazing group of people, so you need to be mentioned here again, I still remember the first time you brought me to MF and I met some of them for the first time, thank you!

Joep, I never could have dreamed of the support you have given me during these past months, and I don't know how I would have kept everything together without you. Thank you for offering a comfortable chair and adequate distraction. Thank you for telling me that everything is going to be alright and for being so patient. Most of all, thank you for reminding me what matters the most in life!

I'd like to thank my friends across the globe, in Finland and in Turku. **Susanna** and **Niina**, I have known you most of my life and you always have special place in my heart no matter how far apart we are physically.

My family, who make sure I have a place to come back to in Finland, thank you! Especially my parents who always boosted my self-esteem since I was little, supported and believed in me. No words can describe how much I owe you. Thank you for always encouraging me and thinking that I can do anything.

8 REFERENCES

1. Weinstein, I. B. & Case, K. The history of cancer research: Introducing an AACR centennial series. *Cancer Res.* **68**, 6861–6862 (2008).
2. Milestones in Cancer Research and Discovery - National Cancer Institute. Available at: <https://www.cancer.gov/research/progress/250-years-milestones>. (Accessed: 19th April 2021)
3. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144**, 646–674 (2011).
4. O'Connor, M. J. Targeting the DNA Damage Response in Cancer. *Molecular Cell* **60**, 547–560 (2015).
5. Jackson, S. P. & Bartek, J. The DNA-damage response in human biology and disease. *Nature* **461**, 1071–1078 (2009).
6. O'Neil, N. J., Bailey, M. L. & Hieter, P. Synthetic lethality and cancer. *Nature Reviews Genetics* **18**, 613–623 (2017).
7. Baldo, B. A. & Pham, N. H. Adverse reactions to targeted and non-targeted chemotherapeutic drugs with emphasis on hypersensitivity responses and the invasive metastatic switch. *Cancer Metastasis Rev.* **32**, 723–761 (2013).
8. Dasari, S. & Bernard Tchounwou, P. Cisplatin in cancer therapy: Molecular mechanisms of action. *European Journal of Pharmacology* **740**, 364–378 (2014).
9. Rabik, C. A. & Dolan, M. E. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treatment Reviews* **33**, 9–23 (2007).
10. Powell, S. & McMillan, T. J. DNA damage and repair following treatment with ionizing radiation. *Radiotherapy and Oncology* **19**, 95–108 (1990).
11. Maier, P., Hartmann, L., Wenz, F. & Herskind, C. Cellular pathways in response to ionizing radiation and their targetability for tumor radiosensitization. *International Journal of Molecular Sciences* **17**, (2016).
12. Vignard, J., Mirey, G. & Salles, B. Ionizing-radiation induced DNA double-strand breaks: A direct and indirect lighting up. *Radiotherapy and Oncology* **108**, 362–369 (2013).
13. Helleday, T., Petermann, E., Lundin, C., Hodgson, B. & Sharma, R. A. DNA repair pathways as targets for cancer therapy. *Nature Reviews Cancer* **8**, 193–204 (2008).
14. Rottenberg, S., Disler, C. & Perego, P. The rediscovery of platinum-based cancer therapy. *Nature Reviews Cancer* **21**, 37–50 (2021).
15. Deans, A. J. & West, S. C. DNA interstrand crosslink repair and cancer. *Nature Reviews Cancer* **11**, 467–480 (2011).
16. Eastman, A. Reevaluation of Interaction of cis-Dichloro(ethylenediamine)platinum(II) with DNA. *Biochemistry* **25**, 3912–3915 (1986).
17. Roberts, J. J. & Friedlos, F. Quantitative estimation of cisplatin-induced DNA interstrand cross-links and their repair in mammalian cells: Relationship to toxicity. *Pharmacol. Ther.* **34**, 215–246 (1987).
18. Wang, L. C. & Gautier, J. The Fanconi anemia pathway and ICL repair: implications for cancer therapy. *Crit. Rev. Biochem. Mol. Biol.* **45**, 424–439 (2010).
19. Dilruba, S. & Kalayda, G. V. Platinum-based drugs: past, present and future. *Cancer Chemotherapy and Pharmacology* **77**, 1103–1124 (2016).
20. Verweij, J. & Pinedo, H. Mitomycin C: mechanism of action, usefulness and limitations. *Anticancer Drugs* **Oct;1**, 5–13 (1990).
21. Warren, A. J., Maccubbin, A. E. & Hamilton, J. W. Detection of Mitomycin C-DNA Adducts in Vivo by ³²P-Postlabeling: Time Course for Formation and Removal of Adducts and Biochemical Modulation. *Cancer Res.* **58**, (1998).
22. Doroshow, J. H. Mitomycin C-enhanced superoxide and hydrogen peroxide formation in rat heart. *J. Pharmacol. Exp. Ther.* **218**, (1981).
23. Baskar, R., Lee, K. A., Yeo, R. & Yeoh, K. W. Cancer and radiation therapy: Current advances and future directions. *International Journal of Medical Sciences* **9**, 193–199 (2012).
24. Begg, A. C., Stewart, F. A. & Vens, C. Strategies to improve radiotherapy with targeted drugs. *Nature Reviews Cancer* **11**, 239–253 (2011).
25. Dobzhansky, T. Genetics of natural populations; recombination and variability in populations of *Drosophila pseudoobscura*. *Genetics* **31**, 269–90 (1946).
26. Bridges, C. B. The origin of variation. *Amer Nat.* 51–63 (1922).
27. Hartwell, L. H., Szankasi, P., Roberts, C. J., Murray, A. W. & Friend, S. H. Integrating genetic approaches into the discovery of anticancer drugs. *Science* **278**, 1064–1068 (1997).
28. Bryant, H. E. *et al.* Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913–917 (2005).
29. Yoshida, K. & Miki, Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Science* **95**, 866–871 (2004).
30. Morales, J. *et al.* Review of poly (ADP-ribose) polymerase (PARP) mechanisms of action and rationale for targeting in cancer and other diseases. *Crit. Rev. Eukaryot. Gene Expr.* **24**, 15–28 (2014).

31. Merchut-Maya, J. M., Bartek, J. & Maya-Mendoza, A. Regulation of replication fork speed: Mechanisms and impact on genomic stability. *DNA Repair* **81**, 102654 (2019).
32. Murai, J. *et al.* Differential trapping of PARP1 and PARP2 by clinical PARP inhibitors. *Cancer Res.* **72**, 5588–5599 (2012).
33. González-Martín, A. *et al.* Niraparib in Patients with Newly Diagnosed Advanced Ovarian Cancer. *N. Engl. J. Med.* **381**, 2391–2402 (2019).
34. Murai, J. Targeting DNA repair and replication stress in the treatment of ovarian cancer. *International Journal of Clinical Oncology* **22**, 619–628 (2017).
35. Boshuizen, J. & Peeper, D. S. Rational Cancer Treatment Combinations: An Urgent Clinical Need. *Mol. Cell* **78**, 1002–1018 (2020).
36. Lazo, J. S. Cutting down the time to identify challenging tumor therapeutic targets and drug combinations using synthetic lethal approaches. *F1000Research* **7**, (2018).
37. Narayan, R. S. *et al.* A cancer drug atlas enables synergistic targeting of independent drug vulnerabilities. *Nat. Commun.* **11**, 1–14 (2020).
38. Tallarida, R. J. Drug Synergism: Its Detection and Applications. *J. Pharmacol. Exp. Ther.* **298**, (2001).
39. Bannister, N. & Broggio, J. Cancer survival by stage at diagnosis for England (experimental statistics): Adults diagnosed 2012, 2013 and 2014 and followed up to 2015. *Office for National Statistics and Public Health England* (2016). Available at: <https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases/bulletins/cancersurvivalbystageatdiagnosisforenglandexperimentalstatistics/adultsdiagnosed20122013and2014andfollowedupto2015>. (Accessed: 26th June 2019)
40. Lheureux, S., Gourley, C., Vergote, I. & Oza, A. M. Epithelial ovarian cancer. *The Lancet* **393**, 1240–1253 (2019).
41. Ferlay, J. *et al.* Global Cancer Observatory: Cancer Today. *Lyon, France: International Agency for Research on Cancer* (2018). Available at: <https://gco.iarc.fr/today>. (Accessed: 26th June 2019)
42. McGuire, W. P. *et al.* Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. *N. Engl. J. Med.* **334**, 1–6 (1996).
43. Prat, J. Ovarian carcinomas: five distinct diseases with different origins, genetic alterations, and clinicopathological features. *Virchows Arch.* **460**, 237–249 (2012).
44. Labidi-Galy, S. I. *et al.* High grade serous ovarian carcinomas originate in the fallopian tube. *Nat. Commun.* **8**, (2017).
45. Risch, H. A. *et al.* Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer. *Am. J. Hum. Genet.* **68**, 700–710 (2001).
46. Malander, S. *et al.* One in 10 ovarian cancer patients carry germ line BRCA1 or BRCA2 mutations: Results of a prospective study in Southern Sweden. *Eur. J. Cancer* **40**, 422–428 (2004).
47. Pal, T. *et al.* BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. *Cancer* **104**, 2807–2816 (2005).
48. Alsop, K. *et al.* BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: A report from the Australian ovarian cancer study group. *J. Clin. Oncol.* **30**, 2654–2663 (2012).
49. Gee, M. E., Faraahi, Z., McCormick, A. & Edmondson, R. J. DNA damage repair in ovarian cancer: Unlocking the heterogeneity. *Journal of Ovarian Research* **11**, (2018).
50. Penner-Goeke, S. *et al.* The temporal dynamics of chromosome instability in ovarian cancer cell lines and primary patient samples. *PLoS Genet.* **13**, e1006707 (2017).
51. Lee, Y. *et al.* A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *J. Pathol.* **211**, 26–35 (2007).
52. Kossai, M., Leary, A., Scoazec, J.-Y. & Genestie, C. Ovarian Cancer: A Heterogeneous Disease. *Pathobiology* **85**, 41–49 (2018).
53. Burger, R. A. *et al.* Incorporation of bevacizumab in the primary treatment of ovarian cancer. *N. Engl. J. Med.* **365**, 2473–2483 (2011).
54. Kaufman, B. *et al.* Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J. Clin. Oncol.* **33**, 244–250 (2015).
55. Kim, G. *et al.* FDA approval summary: Olaparib monotherapy in patients with deleterious germline BRCA-mutated advanced ovarian cancer treated with three or more lines of chemotherapy. *Clin. Cancer Res.* **21**, 4257–4261 (2015).
56. Balasubramaniam, S. *et al.* FDA approval summary: Rucaparib for the treatment of patients with deleterious BRCA mutation-associated advanced ovarian cancer. *Clinical Cancer Research* **23**, 7165–7170 (2017).
57. Ison, G. *et al.* FDA approval summary: Niraparib for the maintenance treatment of patients with recurrent ovarian cancer in response to platinum-based chemotherapy. *Clin. Cancer Res.* **24**, 4066–4071 (2018).
58. Boussios, S. *et al.* Veliparib in ovarian cancer: a new synthetically lethal therapeutic approach. *Investigational New Drugs* **38**, 181–193 (2020).

59. Boussios, S. *et al.* Poly (ADP-Ribose) Polymerase Inhibitors: Talazoparib in Ovarian Cancer and Beyond. *Drugs in R and D* **20**, 55–73 (2020).
60. Bartkova, J. *et al.* DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* **434**, 864–870 (2005).
61. Blackford, A. N. & Jackson, S. P. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. *Molecular Cell* **66**, 801–817 (2017).
62. Khanna, K. K. & Jackson, S. P. DNA double-strand breaks: Signaling, repair and the cancer connection. *Nat. Genet.* **27**, 247–254 (2001).
63. Dueva, R. & Iliakis, G. Replication protein A: a multifunctional protein with roles in DNA replication, repair and beyond. *NAR Cancer* **2**, (2020).
64. Lanz, M. C., Dibitetto, D. & Smolka, M. B. DNA damage kinase signaling: checkpoint and repair at 30 years. *EMBO J.* **38**, e101801 (2019).
65. Parrilla-Castellar, E. R., Arlander, S. J. H. & Karnitz, L. Dial 9-1-1 for DNA damage: The Rad9-Hus1-Rad1 (9-1-1) clamp complex. *DNA Repair* **3**, 1009–1014 (2004).
66. Volkmer, E. & Karnitz, L. M. Human homologs of *Schizosaccharomyces pombe* Rad1, Hus1, and Rad9 form a DNA damage-responsive protein complex. *J. Biol. Chem.* **274**, 567–570 (1999).
67. Yan, S. & Michael, W. M. Cell Cycle TopBP1 and DNA polymerase alpha-mediated recruitment of the 9-1-1 complex to stalled replication forks: Implications for a replication restart-based mechanism for ATR checkpoint activation. *Cell Cycle* **8**, 2877–2884 (2009).
68. Myler, L. R. *et al.* Single-Molecule Imaging Reveals How Mre11-Rad50-Nbs1 Initiates DNA Break Repair. *Mol. Cell* **67**, 891–898 (2017).
69. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S. & Bonner, W. M. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* **273**, 5858–5868 (1998).
70. Thorslund, T. *et al.* Histone H1 couples initiation and amplification of ubiquitin signalling after DNA damage. *Nature* **527**, 389–393 (2015).
71. Fradet-Turcotte, A. *et al.* 53BP1 is a reader of the DNA-damage-induced H2A Lys 15 ubiquitin mark. *Nature* **499**, 50–54 (2013).
72. Gatti, M. *et al.* A novel ubiquitin mark at the N-terminal tail of histone H2As targeted by RNF168 ubiquitin ligase. *Cell Cycle* **11**, 2538–2544 (2012).
73. Mattioli, F. *et al.* RNF168 ubiquitinates K13-15 on H2A/H2AX to drive DNA damage signaling. *Cell* **150**, 1182–1195 (2012).
74. Noordermeer, S. M. *et al.* The shieldin complex mediates 53BP1-dependent DNA repair. *Nature* **560**, 117–121 (2018).
75. Spagnolo, L., Rivera-Calzada, A., Pearl, L. H. & Llorca, O. Three-Dimensional Structure of the Human DNA-PKcs/Ku70/Ku80 Complex Assembled on DNA and Its Implications for DNA DSB Repair. *Mol. Cell* **22**, 511–519 (2006).
76. Daley, J. M. & Sung, P. 53BP1, BRCA1, and the Choice between Recombination and End Joining at DNA Double-Strand Breaks. *Mol. Cell. Biol.* **34**, 1380–1388 (2014).
77. Escribano-Díaz, C. *et al.* A Cell Cycle-Dependent Regulatory Circuit Composed of 53BP1-RIF1 and BRCA1-CtIP Controls DNA Repair Pathway Choice. *Mol. Cell* **49**, 872–883 (2013).
78. Orthwein, A. *et al.* A mechanism for the suppression of homologous recombination in G1 cells. *Nature* **528**, 422–426 (2015).
79. Niraj, J., Färkkilä, A. & D’Andrea, A. D. The fanconi anemia pathway in cancer. *Annual Review of Cancer Biology* **3**, 457–478 (2019).
80. Karanam, K., Kafri, R., Loewer, A. & Lahav, G. Quantitative Live Cell Imaging Reveals a Gradual Shift between DNA Repair Mechanisms and a Maximal Use of HR in Mid S Phase. *Mol. Cell* **47**, 320–329 (2012).
81. Sun, Y., McCorvie, T. J., Yates, L. A. & Zhang, X. Structural basis of homologous recombination. *Cellular and Molecular Life Sciences* **77**, 3–18 (2020).
82. Krejci, L., Altmannova, V., Spirek, M. & Zhao, X. Homologous recombination and its regulation. *Nucleic Acids Research* **40**, 5795–5818 (2012).
83. Sartori, A. A. *et al.* Human CtIP promotes DNA end resection. *Nature* **450**, 509–514 (2007).
84. Powell, S. N. & Kachnic, L. A. Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation. *Oncogene* **22**, 5784–5791 (2003).
85. Spírek, M. *et al.* Human RAD51 rapidly forms intrinsically dynamic nucleoprotein filaments modulated by nucleotide binding state. *Nucleic Acids Res.* **46**, 3967–3980 (2018).
86. Wright, W. D., Shah, S. S. & Heyer, W. D. Homologous recombination and the repair of DNA double-strand breaks. *Journal of Biological Chemistry* **293**, 10524–10535 (2018).
87. Niida, H., Shimada, M., Murakami, H. & Nakanishi, M. Mechanisms of dNTP supply that play an essential role in maintaining genome integrity in eukaryotic cells. *Cancer Science* **101**, 2505–2509 (2010).
88. Auerbach, A. D. Fanconi anemia and its diagnosis. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* **668**, 4–10 (2009).

89. Rodríguez, A. & D'Andrea, A. Fanconi anemia pathway. *Current Biology* **27**, R986–R988 (2017).
90. Long, D. T., Joukov, V., Budzowska, M. & Walter, J. C. BRCA1 promotes unloading of the CMG Helicase from a stalled DNA replication fork. *Mol. Cell* **56**, 174–185 (2014).
91. Zhang, J. *et al.* DNA interstrand cross-link repair requires replication-fork convergence. *Nat. Struct. Mol. Biol.* **22**, 242–247 (2015).
92. Liang, C. C. *et al.* UHRF1 Is a sensor for DNA interstrand crosslinks and recruits FANCD2 to initiate the Fanconi Anemia pathway. *Cell Rep.* **10**, 1947–1956 (2015).
93. Singh, T. R. *et al.* MHF1-MHF2, a Histone-Fold-Containing Protein Complex, Participates in the Fanconi Anemia Pathway via FANCM. *Mol. Cell* **37**, 879–886 (2010).
94. Ling, C. Bloom syndrome complex promotes FANCM recruitment to stalled replication forks and facilitates both repair and traverse of DNA interstrand crosslinks. *Cell Discov.* **2**, 16047 (2016).
95. Huang, M. *et al.* The FANCM/FAAP24 complex is required for the DNA interstrand crosslink-induced checkpoint response. *Mol. Cell* **39**, 259–268 (2010).
96. Ishiai, M. *et al.* FANCI phosphorylation functions as a molecular switch to turn on the Fanconi anemia pathway. *Nat. Struct. Mol. Biol.* **15**, 1138–1146 (2008).
97. Tan, W. *et al.* Monoubiquitination by the human Fanconi Anemia core complex clamps FANCI: FANCD2 on DNA in filamentous arrays. *Elife* **9**, e54128 (2020).
98. Tan, W., van Twest, S., Murphy, V. J. & Deans, A. J. ATR-Mediated FANCI Phosphorylation Regulates Both Ubiquitination and Deubiquitination of FANCD2. *Front. Cell Dev. Biol.* **8**, 2 (2020).
99. Knipscheer, P. *et al.* The fanconi anemia pathway promotes replication-dependent DNA interstrand cross-link repair. *Science (80-.)*. **326**, 1698–1701 (2009).
100. Kim, Y. *et al.* Mutations of the SLX4 gene in Fanconi anemia. *Nature Genetics* **43**, 142–146 (2011).
101. Stoepker, C. *et al.* SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. *Nature Genetics* **43**, 138–141 (2011).
102. Fekairi, S. *et al.* Human SLX4 Is a Holliday Junction Resolvase Subunit that Binds Multiple DNA Repair/Recombination Endonucleases. *Cell* **138**, 78–89 (2009).
103. Roy, U. & Schärer, O. D. Involvement of translesion synthesis DNA polymerases in DNA interstrand crosslink repair. *DNA Repair* **44**, 33–41 (2016).
104. Zhao, F., Kim, W., Kloeber, J. A. & Lou, Z. DNA end resection and its role in DNA replication and DSB repair choice in mammalian cells. *Experimental and Molecular Medicine* **52**, 1705–1714 (2020).
105. Bhargava, R., Onyango, D. O. & Stark, J. M. Regulation of Single-Strand Annealing and its Role in Genome Maintenance. *Trends in Genetics* **32**, 566–575 (2016).
106. Zeman, M. K. & Cimprich, K. A. Causes and consequences of replication stress. *Nature Cell Biology* **16**, 2–9 (2014).
107. Bartkova, J. *et al.* Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* **444**, 633–637 (2006).
108. Gaillard, H., García-Muse, T. & Aguilera, A. Replication stress and cancer. *Nature Reviews Cancer* **15**, 276–280 (2015).
109. Pacek, M. & Walter, J. C. A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. *EMBO J.* **23**, 3667–3676 (2004).
110. Ercilla, A. *et al.* Physiological Tolerance to ssDNA Enables Strand Uncoupling during DNA Replication. *Cell Rep.* **30**, 2416–2429 (2020).
111. Vassin, V. M., Anantha, R. W., Sokolova, E., Kanner, S. & Borowiec, J. A. Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress. *J. Cell Sci.* **122**, 4070–4080 (2009).
112. Ward, I. M. & Chen, J. Histone H2AX Is Phosphorylated in an ATR-dependent Manner in Response to Replicational Stress. *J. Biol. Chem.* **276**, 47759–47762 (2001).
113. Toledo, L., Neelsen, K. J. & Lukas, J. Replication Catastrophe: When a Checkpoint Fails because of Exhaustion. *Molecular Cell* **66**, 735–749 (2017).
114. Toledo, L. I. *et al.* ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* **155**, 1088–1103 (2013).
115. Imai, T. *et al.* Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* **91**, 521–530 (1997).
116. Bazan, J. F. *et al.* A new class of membrane-bound chemokine with a CX3C motif. *Nature* **385**, 640–644 (1997).
117. Pan, Y. *et al.* Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* **387**, 611–617 (1997).
118. Cambien, B. *et al.* Signal transduction pathways involved in soluble fractalkine-induced monocytic cell adhesion. *Blood* **97**, 2031–2037 (2001).
119. Kansra, V., Groves, C., Gutierrez-Ramos, J. C. & Polakiewicz, R. D. Phosphatidylinositol 3-Kinase-dependent Extracellular Calcium Influx Is Essential for CX3CR1-mediated Activation of the Mitogen-activated Protein Kinase Cascade. *J. Biol. Chem.* **276**, 31831–31838 (2001).
120. Brand, S., Sakaguchi, T., Gu, X., Colgan, S. P. & Reinecker, H. C. Fractalkine-mediated signals regulate

- cell-survival and immune-modulatory responses in intestinal epithelial cells. *Gastroenterology* **122**, 166–177 (2002).
121. Chandrasekar, B. *et al.* Fractalkine (CX3CL1) stimulated by nuclear factor κ B (NF- κ B)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. *Biochem. J.* **373**, 547–558 (2003).
 122. Deiva, K. *et al.* Fractalkine reduces N-methyl-d-aspartate-induced calcium flux and apoptosis in human neurons through extracellular signal-regulated kinase activation. *Eur. J. Neurosci.* **20**, 3222–3232 (2004).
 123. White, G. E. *et al.* Fractalkine has anti-apoptotic and proliferative effects on human vascular smooth muscle cells via epidermal growth factor receptor signalling. *Cardiovasc. Res.* **85**, 825–835 (2010).
 124. Tardáguila, M. *et al.* CX3CL1 promotes breast cancer via transactivation of the EGF pathway. *Cancer Res.* **73**, 4461–4473 (2013).
 125. Liu, P. *et al.* CX3CL1/fractalkine enhances prostate cancer spinal metastasis by activating the Src/FAK pathway. *Int. J. Oncol.* **53**, 1544–1556 (2018).
 126. White, G. E. & Greaves, D. R. Fractalkine: a survivor’s guide: chemokines as antiapoptotic mediators. *Arterioscler. Thromb. Vasc. Biol.* **32**, 589–94 (2012).
 127. Korbecki, J. *et al.* Fractalkine/cx3cl1 in neoplastic processes. *International Journal of Molecular Sciences* **21**, 3723 (2020).
 128. Raport, C. J., Schweickart, V. L., Eddy, R. L., Shows, T. B. & Gray, P. W. The orphan G-protein-coupled receptor-encoding gene V28 is closely related to genes for chemokine receptors and is expressed in lymphoid and neural tissues. *Gene* **163**, 295–299 (1995).
 129. White, G. E., McNeill, E., Channon, K. M. & Greaves, D. R. Fractalkine promotes human monocyte survival via a reduction in oxidative stress. *Arterioscler. Thromb. Vasc. Biol.* **34**, 2554–2562 (2014).
 130. Rivas-Fuentes, S., Salgado-Aguayo, A., Arratia-Quijada, J. & Gorocica-Rosete, P. Regulation and biological functions of the CX3CL1-CX3CR1 axis and its relevance in solid cancer: A mini-review. *Journal of Cancer* **12**, 571–583 (2020).
 131. Zhu, W. *et al.* Elevated expression of fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) in the dorsal root ganglia and spinal cord in experimental autoimmune encephalomyelitis: Implications in multiple sclerosis-induced neuropathic pain. *Biomed Res. Int.* **2013**, (2013).
 132. Boag, S. E. *et al.* T lymphocytes and fractalkine contribute to myocardial ischemia/reperfusion injury in patients. *J. Clin. Invest.* **125**, 3063–3076 (2015).
 133. Meucci, O., Fatatis, A., Simen, A. A. & Miller, R. J. Expression of CX3CR1 chemokine receptors on neurons and their role in neuronal survival. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8075–8080 (2000).
 134. Koizumi, K. *et al.* Role of CX3CL1/Fractalkine in Osteoclast Differentiation and Bone Resorption. *J. Immunol.* **183**, 7825–7831 (2009).
 135. Ramos, M. V. *et al.* Interleukin-10 and interferon- γ modulate surface expression of fractalkine-receptor (CX3CR1) via PI3K in monocytes. *Immunology* **129**, 600–609 (2010).
 136. Singh, S. K., Mishra, M. K. & Singh, R. Hypoxia-inducible factor-1 α induces CX3CR1 expression and promotes the epithelial to mesenchymal transition (EMT) in ovarian cancer cells. *J. Ovarian Res.* **12**, 1–10 (2019).
 137. Williams, C. & Hill, S. J. GPCR signaling: understanding the pathway to successful drug discovery. *Methods in molecular biology (Clifton, N.J.)* **552**, 39–50 (2009).
 138. Joyal, J. S. *et al.* Subcellular localization of coagulation factor II receptor-like 1 in neurons governs angiogenesis. *Nat. Med.* **20**, 1165–1173 (2014).
 139. Bhosle, V. K., Rivera, J. C. & Chemtob, S. New insights into mechanisms of nuclear translocation of G-protein coupled receptors. *Small GTPases* **10**, 254–263 (2019).
 140. Pfeil, E. M. *et al.* Heterotrimeric G Protein Subunit G α Is a Master Switch for G β γ -Mediated Calcium Mobilization by Gi-Coupled GPCRs. *Mol. Cell* **80**, 940-954.e6 (2020).
 141. Gurler Main, H. *et al.* Emergent role of the fractalkine axis in dissemination of peritoneal metastasis from epithelial ovarian carcinoma. *Oncogene* **36**, 3025–3036 (2017).
 142. Stout, M. C., Narayan, S., Pillet, E. S., Salvino, J. M. & Campbell, P. M. Inhibition of CX3CR1 reduces cell motility and viability in pancreatic adenocarcinoma epithelial cells. *Biochem. Biophys. Res. Commun.* **495**, 2264–2269 (2018).
 143. Wei, L. M., Cao, S., Yu, W. D., Liu, Y. L. & Wang, J. T. Overexpression of CX3CR1 is associated with cellular metastasis, proliferation and survival in gastric cancer. *Oncol. Rep.* **33**, 615–624 (2015).
 144. Marchica, V. *et al.* Bone marrow CX3CL1/Fractalkine is a new player of the pro-angiogenic microenvironment in multiple myeloma patients. *Cancers (Basel)*. **11**, 321 (2019).
 145. Batool, A., Wang, Y. Q., Hao, X. X., Chen, S. R. & Liu, Y. X. A miR-125b/CSF1-CX3CL1/tumor-associated macrophage recruitment axis controls testicular germ cell tumor growth. *Cell Death Dis.* **9**, (2018).
 146. Wang, Y. *et al.* The M2 polarization of macrophage induced by fractalkine in the endometriotic milieu enhances invasiveness of endometrial stromal cells. *Int. J. Clin. Exp. Pathol.* **7**, 194–203 (2014).
 147. Geismann, C. *et al.* TRAIL/NF- κ B/CX3CL1 mediated onco-immuno crosstalk leading to TRAIL

- resistance of pancreatic cancer cell lines. *Int. J. Mol. Sci.* **19**, 1661 (2018).
148. Ishida, Y. *et al.* Pivotal Involvement of the CX3CL1-CX3CR1 Axis for the Recruitment of M2 Tumor-Associated Macrophages in Skin Carcinogenesis. *J. Invest. Dermatol.* **140**, 1951-1961.e6 (2020).
 149. Erreni, M. *et al.* The Fractalkine-Receptor Axis Improves Human Colorectal Cancer Prognosis by Limiting Tumor Metastatic Dissemination. *J. Immunol.* **196**, 902–914 (2016).
 150. Xu, X. *et al.* High Expression of CX3CL1/CX3CR1 Axis Predicts a Poor Prognosis of Pancreatic Ductal Adenocarcinoma. *J. Gastrointest. Surg.* **16**, 1493–1498 (2012).
 151. Liu, W., Liang, Y., Chan, Q., Jiang, L. & Dong, J. CX3CL1 promotes lung cancer cell migration and invasion via the Src/focal adhesion kinase signaling pathway. *Oncol. Rep.* **41**, 1911–1917 (2019).
 152. Tang, J. *et al.* Upregulation of fractalkine contributes to the proliferative response of prostate cancer cells to hypoxia via promoting the G1/S phase transition. *Mol. Med. Rep.* **12**, 7907–7914 (2015).
 153. Corcione, A., Ferretti, E. & Pistoia, V. CX 3 CL1/fractalkine is a novel regulator of normal and malignant human B cell function. *J. Leukoc. Biol.* **92**, 51–58 (2012).
 154. Erreni, M. *et al.* Human glioblastoma tumours and neural cancer stem cells express the chemokine CX3CL1 and its receptor CX3CR1. *Eur. J. Cancer* **46**, 3383–3392 (2010).
 155. Yao, X. *et al.* Expression of CX3CR1 associates with cellular migration, metastasis, and prognosis in human clear cell renal cell carcinoma. *Urol. Oncol. Semin. Orig. Investig.* **32**, 162–170 (2014).
 156. Kim, M., Rooper, L., Xie, J., Kajdacsy-Balla, A. A. & Barbolina, M. V. Fractalkine Receptor CX3CR1 Is Expressed in Epithelial Ovarian Carcinoma Cells and Required for Motility and Adhesion to Peritoneal Mesothelial Cells. *Mol Cancer Res.* **10**, 11–24 (2012).
 157. Celesti, G. *et al.* Early expression of the fractalkine receptor CX3CR1 in pancreatic carcinogenesis. *Br. J. Cancer* **109**, 2424–2433 (2013).
 158. Matsubara, T., Ono, T., Yamanoi, A., Tachibana, M. & Nagasue, N. Fractalkine-CX3CR1 axis regulates tumor cell cycle and deteriorates prognosis after radical resection for hepatocellular carcinoma. *J. Surg. Oncol.* **95**, 241–249 (2007).
 159. Hyakudomi, M. *et al.* Increased expression of Fractalkine is correlated with a better prognosis and an increased number of both CD8+ T cells and natural killer cells in gastric adenocarcinoma. *Ann. Surg. Oncol.* **15**, 1775–1782 (2008).
 160. Wang, H. *et al.* Fractalkine/CX3CR1 induces apoptosis resistance and proliferation through the activation of the AKT/NF- κ B cascade in pancreatic cancer cells. *Cell Biochem. Funct.* **35**, 315–326 (2017).
 161. Gaudin, F. *et al.* Identification of the Chemokine CX3CL1 as a New Regulator of Malignant Cell Proliferation in Epithelial Ovarian Cancer. *PLoS One* **6**, e21546 (2011).
 162. Ren, H. *et al.* The CX3CL1/CX3CR1 reprograms glucose metabolism through HIF-1 pathway in pancreatic adenocarcinoma. *J. Cell. Biochem.* **114**, 2603–2611 (2013).
 163. Tang, J. *et al.* CX3CL1 increases invasiveness and metastasis by promoting epithelial-to-mesenchymal transition through the TACE/TGF- β /EGFR pathway in hypoxic androgen independent prostate cancer cells. *Oncol. Rep.* **35**, 1153–1162 (2016).
 164. Xie, J., Gurler Main, H., Sacks, J. D., Muralidhar, G. G. & Barbolina, M. V. Regulation of DNA damage repair and lipid uptake by CX3CR1 in epithelial ovarian carcinoma. *Oncogenesis* **7**, 37 (2018).
 165. Tautermann, C. S. GPCR structures in drug design, emerging opportunities with new structures. *Bioorganic and Medicinal Chemistry Letters* **24**, 4073–4079 (2014).
 166. Poeta, V. M., Massara, M., Capucetti, A. & Bonecchi, R. Chemokines and chemokine receptors: New targets for cancer immunotherapy. *Frontiers in Immunology* **10**, 379 (2019).
 167. Anders, H. J., Sayyed, S. A. & Vielhauer, V. Questions about chemokine and chemokine receptor antagonism in renal inflammation. *Nephron - Experimental Nephrology* **114**, e33-338 (2010).
 168. Pervaiz, A. *et al.* CCR5 blockage by maraviroc: a potential therapeutic option for metastatic breast cancer. *Cell. Oncol.* **42**, 93–106 (2019).
 169. Haag, G. M. *et al.* Combined PD-1 inhibition (Pembrolizumab) and CCR5 inhibition (Maraviroc) for the treatment of refractory microsatellite stable (MSS) metastatic colorectal cancer (mCRC): First results of the PICCASSO phase I trial. *J. Clin. Oncol.* **38**, 3010–3010 (2020).
 170. Miao, M., De Clercq, E. & Li, G. Clinical significance of chemokine receptor antagonists. *Expert Opin. Drug Metab. Toxicol.* **16**, 11–30 (2020).
 171. Tanaka, Y. *et al.* Safety, pharmacokinetics, and efficacy of E6011, an antifractalkine monoclonal antibody, in a first-in-patient phase 1/2 study on rheumatoid arthritis. *Mod. Rheumatol.* **28**, 58–65 (2018).
 172. Tanaka, Y. *et al.* Emerging Role of Fractalkine in the Treatment of Rheumatic Diseases. *ImmunoTargets Ther.* **9**, 241–253 (2020).
 173. Karlström, S. *et al.* Substituted 7-amino-5-thio-thiazolo[4,5- d]pyrimidines as potent and selective antagonists of the fractalkine receptor (CX3CR1). *J. Med. Chem.* **56**, 3177–3190 (2013).
 174. Cederblad, L., Rosengren, B., Ryberg, E. & Hermansson, N. O. AZD8797 is an allosteric non-competitive modulator of the human CX3CR1 receptor. *Biochem. J.* **473**, 641–649 (2016).
 175. Kancera provides update on successful phase I study and planned phase II study with KAND567

- treatment following heart attack | Kancera. Available at: https://kancera.com/mfn_news/kancera-provides-update-on-successful-phase-i-study-and-planned-phase-ii-study-with-kand567-treatment-following-heart-attack/. (Accessed: 20th April 2021)
176. Low, S. *et al.* VHH antibody targeting the chemokine receptor CX3CR1 inhibits progression of atherosclerosis. *MAbs* **12**, 1709322 (2020).
 177. Christensen, H. B. *et al.* Applying label-free dynamic mass redistribution assay for studying endogenous FPR1 receptor signalling in human neutrophils. *J. Pharmacol. Toxicol. Methods* **88**, 72–78 (2017).
 178. Wollberg, A. R. *et al.* Pharmacological inhibition of the chemokine receptor CX3CR1 attenuates disease in a chronic-relapsing rat model for multiple sclerosis. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 5409–5414 (2014).
 179. Chen, G. *et al.* A novel CX3CR1 inhibitor AZD8797 facilitates early recovery of rat acute spinal cord injury by inhibiting inflammation and apoptosis. *Int. J. Mol. Med.* **45**, 1373–1384 (2020).
 180. Ho, C. Y. *et al.* CX3CR1-microglia mediates neuroinflammation and blood pressure regulation in the nucleus tractus solitarii of fructose-induced hypertensive rats. *J. Neuroinflammation* **17**, (2020).
 181. Abdelmoaty, S. *et al.* KAND567, the first selective small molecule CX3CR1 antagonist in clinical development, mediates anti-inflammatory cardioprotective effects in rodent models of atherosclerosis and myocardial infarction. *Eur. Heart J.* **40**, ehz746.0080 (2019).
 182. Yalcin, A., Telang, S., Clem, B. & Chesney, J. Regulation of glucose metabolism by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases in cancer. *Experimental and Molecular Pathology* **86**, 174–179 (2009).
 183. Warburg, O. On the origin of cancer cells. *Science (80-)*. **123**, 309–314 (1956).
 184. Heiden, M. G. V., Cantley, L. C. & Thompson, C. B. Understanding the warburg effect: The metabolic requirements of cell proliferation. *Science* **324**, 1029–1033 (2009).
 185. Gatenby, R. A. & Gillies, R. J. Why do cancers have high aerobic glycolysis? *Nature Reviews Cancer* **4**, 891–899 (2004).
 186. DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G. & Thompson, C. B. The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell Metabolism* **7**, 11–20 (2008).
 187. Jones, R. G. & Thompson, C. B. Tumor suppressors and cell metabolism: A recipe for cancer growth. *Genes and Development* **23**, 537–548 (2009).
 188. Rider, M. H. *et al.* 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: Head-to-head with a bifunctional enzyme that controls glycolysis. *Biochemical Journal* **381**, 561–579 (2004).
 189. Van Schaftingen, E., Jett, M. F., Jue, L. & Hers, H. G. Control of liver 6-phosphofructokinase by fructose 2,6-bisphosphate and other effectors. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 3483–3486 (1981).
 190. Bartrons, R. *et al.* Fructose 2,6-bisphosphate in cancer cell metabolism. *Frontiers in Oncology* **8**, 331 (2018).
 191. Shi, L., Pan, H., Liu, Z., Xie, J. & Han, W. Roles of PFKFB3 in cancer. *Signal Transduction and Targeted Therapy* **2**, (2017).
 192. O’Neal, J. *et al.* Inhibition of 6-phosphofructo-2-kinase (PFKFB3) suppresses glucose metabolism and the growth of HER2+ breast cancer. *Breast Cancer Res. Treat.* **160**, 29–40 (2016).
 193. Atsumi, T. *et al.* High Expression of Inducible 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase (iPFK-2; PFKFB3) in Human Cancers. *Cancer Res.* **62**, (2002).
 194. Bailey, R. Glycolysis: The First Stage in Cellular Respiration. *ThoughtCo.* (2020). Available at: <https://www.thoughtco.com/steps-of-glycolysis-373394>. (Accessed: 16th May 2021)
 195. Kotowski, K. *et al.* Role of pfkfb3 and pfkfb4 in cancer: Genetic basis, impact on disease development/progression, and potential as therapeutic targets. *Cancers* **13**, 1–29 (2021).
 196. Telang, S. *et al.* Ras transformation requires metabolic control by 6-phosphofructo-2-kinase. *Oncogene* **25**, 7225–7234 (2006).
 197. Blum, R., Jacob-Hirsch, J., Amariglio, N., Rechavi, G. & Kloog, Y. Ras Inhibition in Glioblastoma Down-regulates Hypoxia-Inducible Factor-1 α , Causing Glycolysis Shutdown and Cell Death. *Cancer Res.* **65**, (2005).
 198. Feng, Y. & Wu, L. mTOR up-regulation of PFKFB3 is essential for acute myeloid leukemia cell survival. *Biochem. Biophys. Res. Commun.* **483**, 897–903 (2017).
 199. Osthus, R. C. *et al.* Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J. Biol. Chem.* **275**, 21797–21800 (2000).
 200. Imbert-Fernandez, Y. *et al.* Estradiol stimulates glucose metabolism via 6-phosphofructo-2-kinase (PFKFB3). *J. Biol. Chem.* **289**, 9440–9448 (2014).
 201. Franklin, D. A. *et al.* P53 coordinates DNA repair with nucleotide synthesis by suppressing PFKFB3 expression and promoting the pentose phosphate pathway. *Sci. Rep.* **6**, (2016).
 202. Cordero-Espinoza, L. & Hagen, T. Increased concentrations of fructose 2,6-bisphosphate contribute to the Warburg effect in phosphatase and tensin homolog (PTEN)-deficient cells. *J. Biol. Chem.* **288**, 36020–36028 (2013).
 203. Mondal, S. *et al.* Therapeutic targeting of PFKFB3 with a novel glycolytic inhibitor PFK158 promotes lipophagy and chemosensitivity in gynecologic cancers. *Int. J. Cancer* **144**, 178–189 (2019).

204. Minchenko, O. H. *et al.* Overexpression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 in the human breast and colon malignant tumors. *Biochimie* **87**, 1005–1010 (2005).
205. Peng, F. *et al.* PFKFB3 is involved in breast cancer proliferation, migration, invasion and angiogenesis. *Int. J. Oncol.* **52**, 945–954 (2018).
206. Minchenko, O. H., Tsuchihara, K., Minchenko, D. O., Bikfalvi, A. & Esumi, H. Mechanisms of regulation of PFKFB expression in pancreatic and gastric cancer cells. *World Journal of Gastroenterology* **20**, 13705–13717 (2014).
207. De Oliveira, T. *et al.* Effects of the novel pfkfb3 inhibitor kan0438757 on colorectal cancer cells and its systemic toxicity evaluation in vivo. *Cancers (Basel)*. **13**, 1–24 (2021).
208. Lypova, N., Telang, S., Chesney, J. & Imbert-Fernandez, Y. Increased 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 activity in response to EGFR signaling contributes to non-small cell lung cancer cell survival. *J. Biol. Chem.* **294**, 10530–10543 (2019).
209. Deng, X. *et al.* Ubiquitin-like protein FAT10 promotes osteosarcoma glycolysis and growth by upregulating PFKFB3 via stabilization of EGFR. *Am. J. Cancer Res.* **10**, 2066–2082 (2020).
210. Shi, W. K. *et al.* PFKFB3 blockade inhibits hepatocellular carcinoma growth by impairing DNA repair through AKT article. *Cell Death Dis.* **9**, (2018).
211. Gu, M. *et al.* PFKFB3 promotes proliferation, migration and angiogenesis in nasopharyngeal carcinoma. *J. Cancer* **8**, 3887–3896 (2017).
212. Yalcin, A. *et al.* Nuclear targeting of 6-phosphofructo-2-kinase (PFKFB3) increases proliferation via cyclin-dependent kinases. *J. Biol. Chem.* **284**, 24223–24232 (2009).
213. Doménech, E. *et al.* AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest. *Nat. Cell Biol.* **17**, 1304–1316 (2015).
214. Bartrons, R. *et al.* The potential utility of PFKFB3 as a therapeutic target. *Expert Opin. Ther. Targets* **22**, 659–674 (2018).
215. Clem, B. *et al.* Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. *Mol. Cancer Ther.* **7**, 110–120 (2008).
216. Boyd, S. *et al.* Structure-based design of potent and selective inhibitors of the metabolic kinase PFKFB3. *J. Med. Chem.* **58**, 3611–3625 (2015).
217. Emini Veseli, B. *et al.* Small molecule 3PO inhibits glycolysis but does not bind to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3). *FEBS Lett.* **594**, 3067–3075 (2020).
218. Telang, S. *et al.* Discovery of a PFKFB3 inhibitor for phase I trial testing that synergizes with the B-Raf inhibitor vemurafenib. *Cancer Metab.* **2**, P14 (2014).
219. Lea, M. A., Guzman, Y. & Desborde, C. Inhibition of Growth by Combined Treatment with Inhibitors of Lactate Dehydrogenase and either Phenformin or Inhibitors of 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase 3. *Anticancer Res.* **36**, (2016).
220. Clem, B. F. *et al.* Targeting 6-phosphofructo-2-kinase (PFKFB3) as a therapeutic strategy against cancer. *Mol. Cancer Ther.* **12**, 1461–1470 (2013).
221. Yi, M. *et al.* 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 and 4: A pair of valves for fine-tuning of glucose metabolism in human cancer. *Molecular Metabolism* **20**, 1–13 (2019).
222. Phase 1 Safety Study of ACT-PFK-158, 2HCl in Patients With Advanced Solid Malignancies. Available at: <https://clinicaltrials.gov/ct2/show/NCT02044861>. (Accessed: 6th May 2021)
223. Burmistrova, O. *et al.* Targeting PFKFB3 alleviates cerebral ischemia-reperfusion injury in mice. *Sci. Rep.* **9**, (2019).
224. Seo, M., Kim, J. Do, Neau, D., Sehgal, I. & Lee, Y. H. Structure-based development of small molecule PFKFB3 inhibitors: A framework for potential cancer therapeutic agents targeting the Warburg effect. *PLoS One* **6**, (2011).
225. Präbst, K., Engelhardt, H., Ringgeler, S. & Hübner, H. Basic colorimetric proliferation assays: MTT, WST, and resazurin. in *Cell Viability Assays. Methods in Molecular Biology* **1601**, 1–17 (Humana Press Inc., 2017).
226. Yadav, B., Wennerberg, K., Aittokallio, T. & Tang, J. Searching for Drug Synergy in Complex Dose-Response Landscapes Using an Interaction Potency Model. *Comput. Struct. Biotechnol. J.* **13**, 504–513 (2015).
227. Ianevski, A., Giri, A. K. & Aittokallio, T. SynergyFinder 2.0: Visual analytics of multi-drug combination synergies. *Nucleic Acids Res.* **48**, W488–W493 (2021).
228. Kuo, L. J. & Yang, L.-X. γ -H2AX - A Novel Biomarker for DNA Double-strand Breaks. *In Vivo (Brooklyn)*. **22**, 305–309 (2008).
229. Sawasdichai, A., Chen, H. T., Hamid, N. A., Jayaraman, P. S. & Gaston, K. In situ subcellular fractionation of Adherent and non-adherent mammalian cells. *J. Vis. Exp.* **41**, 1958 (2010).
230. Quinet, A., Carvajal-Maldonado, D., Lemacon, D. & Vindigni, A. DNA Fiber Analysis: Mind the Gap! in *Methods in Enzymology* **591**, 55–82 (Academic Press Inc., 2017).
231. Vesela, E., Chroma, K., Turi, Z. & Mistrik, M. Common chemical inductors of replication stress: Focus on cell-based studies. *Biomolecules* **7**, (2017).
232. Schindelin, J. *et al.* Fiji: An open-source platform for biological-image analysis. *Nature Methods* **9**, 676–

- 682 (2012).
233. Clarke, S. T., Calderon, V. & Bradford, J. A. Click Chemistry for Analysis of Cell Proliferation in Flow Cytometry. *Curr. Protoc. Cytom.* **82**, 7.49.1-7.49.30 (2017).
 234. Chastain, P. D. *et al.* DNA damage checkpoint responses in the S phase of synchronized diploid human fibroblasts. *Photochem. Photobiol.* **91**, 109–116 (2015).
 235. Pierce, A. J., Johnson, R. D., Thompson, L. H. & Jasin, M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev.* **13**, 2633–2638 (1999).
 236. Molina, D. M. *et al.* Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science (80-.)*. **341**, 84–87 (2013).
 237. Jafari, R. *et al.* The cellular thermal shift assay for evaluating drug target interactions in cells. *Nat. Protoc.* **9**, 2100–2122 (2014).
 238. The Cancer Genome Atlas Program - National Cancer Institute. Available at: <https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>. (Accessed: 6th May 2021)
 239. Ferry, K. V., Hamilton, T. C. & Johnson, S. W. Increased nucleotide excision repair in cisplatin-resistant ovarian cancer cells: Role of ERCC1-XPF. *Biochem. Pharmacol.* **60**, 1305–1313 (2000).
 240. Li, Q. *et al.* Cisplatin induction of ERCC-1 mRNA expression in A2780/CP70 human ovarian cancer cells. *J. Biol. Chem.* **273**, 23419–23425 (1998).
 241. Johnson, S. W. *et al.* Relationship between Platinum-DNA Adduct Formation and Removal and Cisplatin Cytotoxicity in Cisplatin-sensitive and -resistant Human Ovarian Cancer Cells. *Cancer Res.* **54**, (1994).
 242. Wynne, P. *et al.* Enhanced repair of DNA interstrand crosslinking in ovarian cancer cells from patients following treatment with platinum-based chemotherapy. *Br. J. Cancer* **97**, 927–933 (2007).
 243. Akkari, Y. M. N. *et al.* The 4N cell cycle delay in Fanconi anemia reflects growth arrest in late S phase. *Mol. Genet. Metab.* **74**, 403–412 (2001).
 244. Castella, M. *et al.* FANCI Regulates Recruitment of the FA Core Complex at Sites of DNA Damage Independently of FANCD2. *PLoS Genet.* **11**, (2015).
 245. Liang, C. C. *et al.* The FANCD2-FANCI complex is recruited to DNA interstrand crosslinks before monoubiquitination of FANCD2. *Nat. Commun.* **7**, 1–10 (2016).
 246. Pajak, B. *et al.* 2-Deoxy-D-Glucose and its analogs: From diagnostic to therapeutic agents. *International Journal of Molecular Sciences* **21**, (2020).
 247. Federico, M. B. *et al.* Chromosomal Integrity after UV Irradiation Requires FANCD2-Mediated Repair of Double Strand Breaks. *PLoS Genet.* **12**, (2016).
 248. Zhu, J. *et al.* FANCD2 influences replication fork processes and genome stability in response to clustered DSBs. *Cell Cycle* **14**, 1809–1822 (2015).
 249. Leysen, H. *et al.* G protein-coupled receptor systems as crucial regulators of DNA damage response processes. *Int. J. Mol. Sci.* **19**, 2919 (2018).
 250. Meyn, R. E., Munshi, A., Haymach, J. V., Milas, L. & Ang, K. K. Receptor signaling as a regulatory mechanism of DNA repair. *Radiotherapy and Oncology* **92**, 316–322 (2009).
 251. Winograd-Katz, S. E. & Levitzki, A. Cisplatin induces PKB/Akt activation and p38MAPK phosphorylation of the EGF receptor. *Oncogene* **25**, 7381–7390 (2006).
 252. Zhang, D. *et al.* Inhibition of AKT sensitizes chemoresistant ovarian cancer cells to cisplatin by abrogating S and G2/M arrest. *Exp. Mol. Pathol.* **100**, 506–513 (2016).
 253. Bentzen, S. M. *et al.* Epidermal growth factor receptor expression in pretreatment biopsies from head and neck squamous cell carcinoma as a predictive factor for a benefit from accelerated radiation therapy in a randomized controlled trial. *J. Clin. Oncol.* **23**, 5560–5567 (2005).
 254. Krause, M. *et al.* Decreased repopulation as well as increased reoxygenation contribute to the improvement in local control after targeting of the EGFR by C225 during fractionated irradiation. in *Radiotherapy and Oncology* **76**, 162–167 (Radiother Oncol, 2005).
 255. Chinnaiyan, P. *et al.* Mechanisms of enhanced radiation response following epidermal growth factor receptor signaling inhibition by erlotinib (Tarceva). *Cancer Res.* **65**, 3328–3335 (2005).
 256. Dittmann, K. *et al.* Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. *J. Biol. Chem.* **280**, 31182–31189 (2005).
 257. Li, L., Wang, H., Yang, E. S., Arteaga, C. L. & Xia, F. Erlotinib attenuates homologous recombinational repair of chromosomal breaks in human breast cancer cells. *Cancer Res.* **68**, 9141–9146 (2008).
 258. Guo, J. *et al.* Mechanisms of resistance to chemotherapy and radiotherapy in hepatocellular carcinoma. *Translational Cancer Research* **7**, 765–781 (2018).
 259. Joyal, J.-S., Bhosle, V. K. & Chemtob, S. Subcellular G-protein coupled receptor signaling hints at greater therapeutic selectivity. *Expert Opin. Ther. Targets* **19**, 717–721 (2015).
 260. Gillies, L. *et al.* The sphingosine 1-phosphate receptor 5 and sphingosine kinases 1 and 2 are localised in centrosomes: Possible role in regulating cell division. *Cell. Signal.* **21**, 675–684 (2009).
 261. Wright, S. C. *et al.* BRET-based effector membrane translocation assay monitors GPCR-promoted and endocytosis-mediated G_q activation at early endosomes. *Proc. Natl. Acad. Sci.* **118**, e2025846118

- (2021).
262. Matsumura, S. & Demaria, S. Up-regulation of the pro-inflammatory chemokine CXCL16 is a common response of tumor cells to ionizing radiation. *Radiat. Res.* **173**, 418–425 (2010).
 263. Rentoft, M. *et al.* Expression of CXCL10 is associated with response to radiotherapy and overall survival in squamous cell carcinoma of the tongue. *Tumor Biol.* **35**, 4191–4198 (2014).
 264. Zhou, B. *et al.* Cisplatin-induced CCL5 secretion from CAFs promotes cisplatin-resistance in ovarian cancer via regulation of the STAT3 and PI3K/Akt signaling pathways. *Int. J. Oncol.* **48**, 2087–2097 (2016).
 265. Reyes, M. E., de La Fuente, M., Hermoso, M., Ili, C. G. & Brebi, P. Role of CC Chemokines Subfamily in the Platinum Drugs Resistance Promotion in Cancer. *Frontiers in Immunology* **11**, (2020).
 266. Liccardi, G., Hartley, J. A. & Hochhauser, D. EGFR nuclear translocation modulates DNA repair following cisplatin and ionizing radiation treatment. *Cancer Res.* **71**, 1103–1114 (2011).
 267. Zhang, J. *et al.* ROS and ROS-Mediated Cellular Signaling. *Oxidative Medicine and Cellular Longevity* **2016**, (2016).
 268. Ceraudo, E. *et al.* Direct evidence that the GPCR CysLTR2 mutant causative of uveal melanoma is constitutively active with highly biased signaling. *J. Biol. Chem.* **296**, (2021).
 269. Wang, Q. *et al.* Constitutive Activity of Serotonin Receptor 6 Regulates Human Cerebral Organoids Formation and Depression-like Behaviors. *Stem Cell Reports* **16**, 75–88 (2021).
 270. Watkins, L. R. & Orlandi, C. In vitro profiling of orphan G protein coupled receptor (GPCR) constitutive activity. *Br. J. Pharmacol.* bph.15468 (2021). doi:10.1111/bph.15468
 271. Golding, S. E. *et al.* Pro-survival AKT and ERK signaling from EGFR and mutant EGFRvIII enhances DNA double-strand break repair in human glioma cells. *Cancer Biol. Ther.* **8**, 730–738 (2009).
 272. Golding, S. E. *et al.* Extracellular signal-related kinase positively regulates ataxia telangiectasia mutated, homologous recombination repair, and the DNA damage response. *Cancer Res.* **67**, 1046–1053 (2007).
 273. Wei, F., Xie, Y., He, L., Tao, L. & Tang, D. ERK1 and ERK2 kinases activate hydroxyurea-induced S-phase checkpoint in MCF7 cells by mediating ATR activation. *Cell. Signal.* **23**, 259–268 (2011).
 274. Wei, F., Xie, Y., Tao, L. & Tang, D. Both ERK1 and ERK2 kinases promote G2/M arrest in etoposide-treated MCF7 cells by facilitating ATM activation. *Cell. Signal.* **22**, 1783–1789 (2010).
 275. Vena, F. *et al.* MEK inhibition leads to BRCA2 downregulation and sensitization to DNA damaging agents in pancreas and ovarian cancer models. *Oncotarget* **9**, 11592–11603 (2018).
 276. Hawkins, A. J., Golding, S. E., Khalil, A. & Valerie, K. DNA double-strand break - Induced pro-survival signaling. *Radiotherapy and Oncology* **101**, 13–17 (2011).
 277. Wang, X., Martindale, J. L. & Holbrook, N. J. Requirement for ERK activation in cisplatin-induced apoptosis. *J. Biol. Chem.* **275**, 39435–39443 (2000).
 278. Kirkpatrick, D. S. *et al.* Phosphoproteomic characterization of DNA damage response in melanoma cells following MEK/PI3K dual inhibition. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 19426–19431 (2013).
 279. Huang, T. T., Lampert, E. J., Coots, C. & Lee, J. M. Targeting the PI3K pathway and DNA damage response as a therapeutic strategy in ovarian cancer. *Cancer Treatment Reviews* **86**, 102021 (2020).
 280. Fraser, M., Bai, T. & Tsang, B. K. Akt promotes cisplatin resistance in human ovarian cancer cells through inhibition of p53 phosphorylation and nuclear function. *Int. J. Cancer* **122**, 534–546 (2008).
 281. Elsayed, A. M. *et al.* Prkar1b-as2 long noncoding rna promotes tumorigenesis, survival, and chemoresistance via the pi3k/akt/mTOR pathway. *Int. J. Mol. Sci.* **22**, 1–25 (2021).
 282. LOU, M. *et al.* Aberrant methylation of GADD45A is associated with decreased radiosensitivity in cervical cancer through the PI3K/AKT signaling pathway. *Oncol. Lett.* **21**, (2021).
 283. Wan, P. K. T. *et al.* HPV-induced Nurr1 promotes cancer aggressiveness, self-renewal, and radioresistance via ERK and AKT signaling in cervical cancer. *Cancer Lett.* **497**, 14–27 (2021).
 284. Duan, L., Perez, R. E., Hansen, M., Gitelis, S. & Maki, C. G. Increasing cisplatin sensitivity by scheduledependent inhibition of AKT and Chk1. *Cancer Biol. Ther.* **15**, 1600–1612 (2014).
 285. Kim, K. H. *et al.* Gene Expression Profiling Identifies Akt as a Target for Radiosensitization in Gastric Cancer Cells. *Front. Oncol.* **10**, (2020).
 286. Li, X. *et al.* Andrographolide enhanced radiosensitivity by downregulating glycolysis via the inhibition of the PI3K-Akt-mTOR signaling pathway in HCT116 colorectal cancer cells. *J. Int. Med. Res.* **48**, (2020).
 287. Boehme, S. A., Lio, F. M., Maciejewski-Lenoir, D., Bacon, K. B. & Conlon, P. J. The Chemokine Fractalkine Inhibits Fas-Mediated Cell Death of Brain Microglia. *J. Immunol.* **165**, 397–403 (2000).
 288. Wang, Z., Huang, Y. & Zhang, J. Molecularly targeting the PI3K-Akt-mTOR pathway can sensitize cancer cells to radiotherapy and chemotherapy. *Cellular and Molecular Biology Letters* **19**, 233–242 (2014).
 289. Xu, N., Lao, Y., Zhang, Y. & Gillespie, D. A. Akt: A double-edged sword in cell proliferation and genome stability. *J. Oncol.* **2012**, (2012).
 290. Stronach, E. A. *et al.* DNA-PK mediates AKT activation and apoptosis inhibition in clinically acquired platinum resistance. *Neoplasia* **13**, 1069–1080 (2011).
 291. Wang, Q. E. *et al.* P38 MAPK- and Akt-mediated p300 phosphorylation regulates its degradation to

- facilitate nucleotide excision repair. *Nucleic Acids Res.* **41**, 1722–1733 (2013).
292. Shen, C. *et al.* Regulation of FANCD2 by the mTOR pathway contributes to the resistance of cancer cells to DNA double-strand breaks. *Cancer Res.* **73**, 3393–3401 (2013).
 293. Guo, F. *et al.* MTOR kinase inhibitor sensitizes T-cell lymphoblastic leukemia for chemotherapy-induced DNA damage via suppressing FANCD2 expression. *Leukemia* **28**, 203–206 (2014).
 294. El Botty, R. *et al.* Inhibition of mTOR downregulates expression of DNA repair proteins and is highly efficient against BRCA2-mutated breast cancer in combination to PARP inhibition. *Oncotarget* **9**, 29587–29600 (2018).
 295. Silvera, D. *et al.* mTORC1 and -2 Coordinate Transcriptional and Translational Reprogramming in Resistance to DNA Damage and Replicative Stress in Breast Cancer Cells. *Mol. Cell. Biol.* **37**, (2017).
 296. David-West, G., Ertlund, A., Gadi, A. & Schneider, R. J. mTORC1/2 inhibition re-sensitizes platinum-resistant ovarian cancer by disrupting selective translation of DNA damage and survival mRNAs. *Oncotarget* **9**, 33064–33076 (2018).
 297. Villafañez, F. *et al.* AKT inhibition impairs PCNA ubiquitylation and triggers synthetic lethality in homologous recombination-deficient cells submitted to replication stress. *Oncogene* **38**, 4310–4324 (2019).
 298. Gao, N. *et al.* G1, cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. *Am. J. Physiol. - Cell Physiol.* **287**, (2004).
 299. Siddik, Z. H. Cisplatin: Mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22**, 7265–7279 (2003).
 300. Jean-Charles, P. Y., Kaur, S. & Shenoy, S. K. G Protein-Coupled Receptor Signaling Through β -Arrestin-Dependent Mechanisms. *J. Cardiovasc. Pharmacol.* **70**, 142–158 (2017).
 301. Beaulieu, J. M. & Caron, M. G. β -arrestin goes nuclear. *Cell* **123**, 755–757 (2005).
 302. Thomsen, A. R. B. *et al.* GPCR-G Protein- β -Arrestin Super-Complex Mediates Sustained G Protein Signaling. *Cell* **166**, 907–919 (2016).
 303. Shen, C. *et al.* TOR Signaling Is a Determinant of Cell Survival in Response to DNA Damage. *Mol. Cell. Biol.* **27**, 7007–7017 (2007).
 304. He, Z. *et al.* P53 suppresses ribonucleotide reductase via inhibiting mTORC1. *Oncotarget* **8**, 41422–41431 (2017).
 305. Vena, F. *et al.* The MEK1/2 Inhibitor Pimasertib Enhances Gemcitabine Efficacy in Pancreatic Cancer Models by Altering Ribonucleotide Reductase Subunit-1 (RRM1). *Clin. Cancer Res.* **21**, 5563–5577 (2015).
 306. Zou, Y. *et al.* ERK Inhibitor Enhances Everolimus Efficacy through the Attenuation of dNTP Pools in Renal Cell Carcinoma. *Mol. Ther. - Nucleic Acids* **14**, 550–561 (2019).
 307. Wu, X. *et al.* mTOR Signaling Upregulates CDC6 via Suppressing miR-3178 and Promotes the Loading of DNA Replication Helicase. *Sci. Rep.* **9**, 1–8 (2019).
 308. Panneerselvam, J. *et al.* Basal level of FANCD2 monoubiquitination is required for the maintenance of a sufficient number of licensed-replication origins to fire at a normal rate. *Oncotarget* **5**, 1326–1337 (2014).
 309. Madireddy, A. *et al.* FANCD2 Facilitates Replication through Common Fragile Sites. *Mol. Cell* **64**, 388–404 (2016).
 310. Raghunandan, M., Chaudhury, I., Kelich, S. L., Hanenberg, H. & Sobek, A. FANCD2, FANCI and BRCA2 cooperate to promote replication fork recovery independently of the Fanconi Anemia core complex. *Cell Cycle* **14**, 342–353 (2015).
 311. Chaudhury, I., Sareen, A., Raghunandan, M. & Sobek, A. FANCD2 regulates BLM complex functions independently of FANCI to promote replication fork recovery. *Nucleic Acids Res.* **41**, 6444–6459 (2013).
 312. Yeo, J. E., Lee, E. H., Hendrickson, E. A. & Sobek, A. CtIP mediates replication fork recovery in a FANCD2-regulated manner. *Hum. Mol. Genet.* **23**, 3695–3705 (2014).
 313. Tian, Y. *et al.* Constitutive role of the Fanconi anemia D2 gene in the replication stress response. *J. Biol. Chem.* **292**, 20184–20195 (2017).
 314. Li, F. L. *et al.* Acetylation accumulates PFKFB3 in cytoplasm to promote glycolysis and protects cells from cisplatin-induced apoptosis. *Nat. Commun.* **9**, 1–17 (2018).
 315. Xiao, Y. *et al.* Inhibition of PFKFB3 induces cell death and synergistically enhances chemosensitivity in endometrial cancer. *Oncogene* **40**, 1409–1424 (2021).
 316. Qu, J. *et al.* Phosphoglycerate mutase 1 regulates dNTP pool and promotes homologous recombination repair in cancer cells. *J. Cell Biol.* **216**, 409–424 (2017).
 317. Niida, H. *et al.* Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase. *Genes Dev.* **24**, 333–338 (2010).
 318. Bogliolo, M. *et al.* Histone H2AX and Fanconi anemia FANCD2 function in the same pathway to maintain chromosome stability. *EMBO J.* **26**, 1340–1351 (2007).
 319. Rao, V. A. *et al.* Phosphorylation of BLM, Dissociation from Topoisomerase III α , and Colocalization

- with γ -H2AX after Topoisomerase I-Induced Replication Damage. *Mol. Cell. Biol.* **25**, 8925–8937 (2005).
320. Nimonkar, A. V. *et al.* BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev.* **25**, 350–362 (2011).
 321. Grundy, M. K., Buckanovich, R. J. & Bernstein, K. A. Regulation and pharmacological targeting of RAD51 in cancer. *NAR Cancer* **2**, (2020).
 322. Norquist, B. *et al.* Secondary somatic mutations restoring BRCA1/2 predict chemotherapy resistance in hereditary ovarian carcinomas. *J. Clin. Oncol.* **29**, 3008–3015 (2011).
 323. Kondrashova, O. *et al.* Secondary somatic mutations restoring RAD51C and RAD51D associated with acquired resistance to the PARP inhibitor rucaparib in high-grade ovarian carcinoma. *Cancer Discov.* **7**, 984–998 (2017).
 324. Gupta, R. *et al.* DNA Repair Network Analysis Reveals Shieldin as a Key Regulator of NHEJ and PARP Inhibitor Sensitivity. *Cell* **173**, 972-988.e23 (2018).
 325. Jaspers, J. E. *et al.* Loss of 53BP1 causes PARP inhibitor resistance in BRCA1-mutated mouse mammary tumors. *Cancer Discov.* **3**, 68–81 (2013).
 326. Dai, C. H. *et al.* Suppression of the FA pathway combined with CHK1 inhibitor hypersensitize lung cancer cells to gemcitabine. *Sci. Rep.* **7**, 1–12 (2017).