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EMERGING ROLES OF PFKFB3 AND CX3CR1 IN THE DNA DAMAGE RESPONSE AND THEIR POTENTIAL AS THERAPEUTIC TARGETS IN CANCER

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Emerging roles of PFKFB3 and CX3CR1 in the DNA damage response and their potential as therapeutic targets in cancer

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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*“De vegades penso que estic anotant el futur.
Em dic que, davant de la incomprensió, en quedin les paraules.
Algú, potser, les podrà entendre.”*

Pol Guasch

POPULAR SCIENCE SUMMARY OF THE THESIS

Human cells replicate all the information gathered in the genome when they divide. To ensure that this information is faithfully maintained, cells have repair mechanisms known as the DNA damage response (DDR) that resolve DNA lesions occurring spontaneously during cells' lifespan. If the DDR machinery is not working properly, high rates of errors and abnormal arrangements in the genome occur, which will eventually be transmitted to daughter cells. This situation is known as genomic instability and contributes to the onset of cancer.

The foundation of many cancer therapies consists of agents that cause severe DNA lesions that cannot be repaired by malignant cells with already high levels of genomic instability. This ultimately results in cell death and reduction of tumor burden. These therapies, although initially being effective, cause undesired side effects to healthy organs of the body.

The research in this thesis has been dedicated to study DNA repair mechanisms specifically used by cancer cells in response to DNA damaging therapies, *i.e.* radio- and chemotherapy. The addition of a second treatment that targets this dependency results in failure to cope with such exerted levels of DNA damage. This therapeutic strategy offers the possibility to increase efficacy and reduce the dosage of DNA damaging agents, limiting toxic side-effects. We focused on the therapeutic targeting of two proteins associated with cancer progression: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), and the fractalkine cellular membrane receptor (CX₃CR1).

In **paper I**, we describe how PFKFB3 accumulates in the nucleus upon ionizing radiation (radiotherapy), together with other DDR factors needed for the repair of DNA double-strand breaks caused by ionizing radiation. We identify that PFKFB3 participates in a repair pathway called homologous recombination and develop a chemical compound, the drug KAN0438757, that targets PFKFB3 and blocks its function. While cancer cells are sensitized to ionizing radiation upon combination with this drug, normal cells are not.

In **paper II**, we study how PFKFB3 alters the repair of DNA lesions caused by platinum-based treatments, namely DNA crosslinks. PFKFB3 enables the recruitment of factors involved in the Fanconi anemia DNA repair pathway, and blocking PFKFB3 with KAN0438757 results in defective resolution of DNA crosslinking lesions during replication of the genome. Targeting PFKFB3 renders cancer cells sensitive to platinum treatments, whereas normal cells are not sensitized.

In **paper III**, we reveal nuclear accumulation of CX₃CR1 upon platinum treatments and under conditions that trigger activation of the Fanconi anemia pathway. Targeting CX₃CR1 with the drug KAND567 results in cancer cells being unable to repair DNA crosslinks and continue to replicate their genome, ultimately reducing cancer cell proliferation.

In **paper IV**, we investigate how CX₃CR1 conveyed signaling alters proliferation of ovarian cancer cells. Using the KAND567 inhibitor, we show that CX₃CR1 is important for survival of cancer cells but not critical for normal cells.

In conclusion, PFKFB3 and CX₃CR1 contribute to the maintenance of genomic stability in cancer cells by novel roles in DNA replication, homologous recombination repair of DNA double-strand breaks, and Fanconi anemia repair of DNA crosslinks. Consequently, specific sensitization of malignant cells to DNA damaging agents occurs upon combination treatment with KAN0438757 or KAND567. This potentiation of classical DNA damaging therapies offers the possibility to design future combination treatments to fight cancer with improved efficacy and reduced harmful effects for the patients.

RESUM CIENTÍFIC I DIVULGATIU DE LA TESI

Les cèl·lules humanes copien tota la informació recollida en el genoma quan es divideixen. Per a assegurar-se que tota aquesta informació genètica es transmet de forma fidedigne, les cèl·lules disposen de varis mecanismes que esmenen lesions de l'ADN produïdes espontàniament. En general, aquests sistemes s'anomenen "mecanismes de reparació de l'ADN". Si aquesta maquinària no funciona correctament, les cèl·lules acaben mostrant elevades taxes d'errors o imprecisions en el genoma, que després es transmetran a les cèl·lules filles. Aquesta situació s'anomena inestabilitat genòmica i contribueix a l'aparició del càncer com a malaltia.

La majoria de teràpies contra el càncer es basen en subministrar agents que causen efectes nocius en l'ADN. Les cèl·lules tumorals que presenten alts nivells d'inestabilitat genòmica són incapaces de reparar aquestes lesions i, per tant, acaben morint degut a l'accumulació de dany en el genoma. Tot i reduir el tamany del tumor, les teràpies contra el càncer causen efectes secundaris no desitjats a la resta d'òrgans del cos.

L'objectiu d'aquesta tesi és estudiar els mecanismes de reparació que fan servir específicament les cèl·lules tumorals quan són exposades a teràpies tradicionals que danyen l'ADN (radioteràpia i quimioteràpia). Aquests tractaments fan que les cèl·lules tumorals activin determinats mecanismes de reparació de l'ADN, i en siguin dependents per a sobreviure. L'addició d'un segon tractament que bloqueja aquesta dependència provoca que les cèl·lules no puguin fer front als alts nivells de dany generats a l'ADN i que morin així més fàcilment. Aquesta estratègia terapèutica s'anomena letalitat sintètica i ofereix la possibilitat d'augmentar l'eficàcia de les teràpies contra el càncer tradicionals, tot reduint-ne els efectes tòxics. La nostra investigació s'ha centrat en dues dianes terapèutiques que s'han associat amb la progressió del càncer: la 6-fosfofructo-2-quinasa/fructosa-2,6-bisfosfatasa 3 (PFKFB3) i el receptor de membrana fractalquina (CX₃CR1).

A l'**article I**, descrivim com la PFKFB3 s'acumula a les lesions de l'ADN degut a la radiació ionitzant de la radioteràpia (que causa la ruptura d' ambdues cadenes de la doble hèlix de l'ADN). La PFKFB3 es localitza conjuntament amb altres factors que s'encarreguen d'esmenar ruptures de doble cadena mitjançant el mecanisme de recombinació homòloga. Desenvolupem un compost químic, el fàrmac KAN0438757, que bloqueja l'activitat de la PFKFB3. Utilitzant aquest fàrmac, realitzem experiments per identificar com l'absència de la PFKFB3 provoca canvis en la reparació de ruptures de doble cadena. És així com identifiquem que les cèl·lules cancerígenes esdevenen més sensibles a la radiació ionitzant quan aquest tractament es combina amb el fàrmac KAN0438757 i, en canvi, les cèl·lules sanes no.

A l'**article II**, estudiem com la PFKFB3 altera la reparació de lesions a l'ADN provocades per compostos basats en platí, un tipus de fàrmacs freqüentment usats en quimioteràpia. Aquestes lesions s'anomenen enllaços covalents entre cadenes d'ADN i es reparen mitjançant el mecanisme de l'anèmia de Fanconi. La PFKFB3 regula el reclutament de factors implicats en l'anèmia de Fanconi. El seu bloqueig amb el fàrmac KAN0438757 fa que les cèl·lules no puguin resoldre els enllaços covalents entre cadenes durant la replicació del genoma. Alhora, en potencia l'efecte tòxic del tractament amb compostos de platí i fa que les cèl·lules cancerígenes esdevinguin més sensibles a aquest tipus de quimioteràpia mentre que les cèl·lules sanes no.

A l'**article III**, revelem que l'acumulació nuclear del receptor CX₃CR1 succeeix com a resposta al tractament amb compostos basats en platí. Quan les cèl·lules cancerígenes són tractades amb KAND567 (un fàrmac que bloqueja el receptor CX₃CR1), són incapaces de reparar enllaços covalents entre cadenes i de replicar l'ADN, fet que acaba aturant la divisió cel·lular. Aquest efecte no s'observa en cèl·lules normals.

A l'**article IV**, investigem com la senyalització desencadenada per l'activació del CX₃CR1 altera la proliferació cel·lular en cèl·lules de càncer d'ovari. Mitjançant la utilització de l'inhibidor KAND567, demostrem que el CX₃CR1 és essencial per a la supervivència de les cèl·lules cancerígenes i que no és important per la proliferació de les cèl·lules normals. A més, el CX₃CR1 regula la replicació de l'ADN per evitar-ne danys i la mort cel·lular programada.

En conclusió, la PFKFB3 i el CX₃CR1 són factors que contribueixen al manteniment de l'estabilitat genòmica del càncer mitjançant funcions fins ara no descrites i que inclouen: la replicació de l'ADN, la reparació homòloga de ruptures de l'ADN de doble cadena, i la reparació dels enllaços covalents entre cadenes d'ADN mitjançant el mecanisme de l'anèmia de Fanconi. Com a conseqüència, la combinació de teràpies que danyen l'ADN amb KAN0438757 o KAND567 dona lloc a una potenciació d'aquestes teràpies clàssiques afectant únicament a les cèl·lules cancerígenes i sense efectes tòxics observables a les cèl·lules sanes. Alhora, ofereix la possibilitat de dissenyar futurs tractaments basats en combinacions de fàrmacs que permetin combatre el càncer amb efectes secundaris reduïts pels pacients.

ABSTRACT

Genomic instability in cancer is exploited therapeutically using DNA damaging therapies that cause irreparable lesions above the threshold of tolerable DNA damage levels. Nevertheless, toxic side effects on healthy tissues limit the therapeutic potential of such therapies. Through the concept of synthetic lethality, therapeutic targeting of factors involved in the DNA damage response (DDR) appears as an attractive strategy to increase the efficacy and improve the therapeutic window of traditional DNA damaging therapies. The aim of this thesis was to study how the metabolic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), and the fractalkine membrane receptor (CX₃CR1) contribute to the maintenance of genome stability and whether they can constitute therapeutic targets in cancer.

In **paper I**, we reveal a hitherto unknown role for PFKFB3 in repair of DNA double-strand breaks (DSBs) upon ionizing radiation (IR). PFKFB3 relocates to IR-induced foci (IRIF) in the nucleus where it colocalizes with homologous recombination (HR) factors. Assembly of PFKFB3 IRIF is dependent on ATM activity and is essential for the recruitment of RAD51 and RPA recombinant mediators, HR activity, and survival upon IR. To interrogate PFKFB3 molecular function, we develop and validate the small molecule inhibitor KAN0438757. Pharmaceutical inhibition of PFKFB3 results in defective HR repair of IR-induced DSBs and impairment of nucleotide incorporation for DNA synthesis by direct interaction with RRM2 at DNA damage sites. Furthermore, targeting PFKFB3 with KAN0438757 sensitizes transformed cells to IR while sparing non-transformed cells.

In **paper II**, we describe a novel role for PFKFB3 in supporting replication-coupled Fanconi anemia (FA) repair of DNA crosslinks, which is essential to resolve stalled replication forks, resume replication, and cell survival. PFKFB3 inhibition provides cancer-selective sensitization to the DNA crosslinkers cisplatin and carboplatin (platinum drugs). This sensitization is not due to a general distortion of glycolysis but appears associated to enhanced PFKFB3 chromatin loading in transformed cells, which is further enriched in platinum resistant cells. Activation of FA pathway upon replication stress results in PFKFB3 recruitment into nuclear foci that depends on ATR activity and FANCM assembly at stalled forks, where PFKFB3 directly interacts with FANCD2 and BLM to ensure FA-mediated repair. Moreover, targeting PFKFB3 increases replication fork stalling and limits fork restart, which results in inability to progress through S phase and fork collapse.

In **paper III**, we introduce an unknown function of CX₃CR1 in promoting FA pathway repair of DNA crosslinks that has functional consequences for the resolution of DNA adducts, DNA replication and survival upon treatment with platinum drugs. Targeting CX₃CR1 by siRNA transfections, lentiviral delivery of shRNA, or with the non-competitive inhibitor KAND567, renders resistant cells hypersensitive to platinum treatments. FA pathway activation under

replication stress conditions triggers CX₃CR1 nuclear localization, which is vital for the assembly of FANCD2 into foci. Suggesting a potential role in fork stabilization, CX₃CR1 inhibition hampers chromatin loading of FANCD2, and its partners FANCI, RAD51 and γ H2AX.

Lastly, in **paper IV** we provide an initial characterization of CX₃CR1 molecular function in survival and proliferation of ovarian cancer cells. KAND567 treatment is cytotoxic in a panel of ovarian cancer cells and in cells derived from an ovarian cancer patient, whereas viability of non-transformed cells is not affected at the same range of concentrations. CX₃CR1 pharmacological inhibition slows down G1 to S phase transition and results in defective DNA replication, accumulation of DNA damage and apoptotic cell death.

Altogether, the present thesis provides evidence for emerging roles of PFKFB3 and CX₃CR1 in the DDR that are critical for cancer cells to maintain genomic stability, and approaches the feasibility of a rational design for a future therapeutic intervention in cancer.

LIST OF SCIENTIFIC PAPERS

- I. Gustafsson, N.M.S., Färnegårdh, K., Bonagas, N., **Huguet Ninou, A.**, Groth, P., Wiita, E., Jönsson, M., Hallberg, K., Lehto, J., Pennisi, R., Martinsson, J., Norström, C., Hollers, J., Schultz, J., Andersson, M., Markova, N., Marttila, P., Kim, B., Norin, M., Olin, T., Helleday, T.
Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination. Nat Commun 9, 3872 (2018).
- II. **Huguet Ninou, A.**, Lehto, J., Chioureas, D., Stigsdotter, H., Schelzig, K., Åkerlund, E., Gudoityte, G., Joneborg, U., Carlson, J., Jonkers, J., Seashore-Ludlow, B., Gustafsson, N.M.S.
PFKFB3 regulates repair of DNA interstrand crosslinks via modulation of the Fanconi Anemia repair pathway. Submitted manuscript.
- III. C Lehto, J., **Huguet Ninou, A.**, Chioureas, D., Jonkers, J., Gustafsson, N.M.S.
Targeting CX3CR1 suppresses the Fanconi Anemia DNA repair pathway and synergizes with platinum. Cancers 13, 1442 (2021).
- IV. Lehto, J., **Huguet Ninou, A.**, Marttila, P., Nordahl, L., Åkerlund, E., Gudoityte, G., Joneborg, U., Carlson, J., Seashore-Ludlow, B., Gustafsson, N.M.S.
Blocking the fractalkine receptor disrupts replication and ovarian cancer cell proliferation. Manuscript.

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LIST OF ABBREVIATIONS

1,3BPG	1,3-Bisphosphoglycerate
2DG	2-Deoxy-d-glucose
2PG	2-Phosphoglycerate
3PG	3-Phosphoglycerate
CldU	5-Chloro-2'-deoxyuridine
EdU	5-Ethynyl-2'-deoxyuridine
IdU	5-Iodo-2'-deoxyuridine
PFK-1	6-phosphofructo-1-kinase
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
APC/C	Anaphase-promoting complex/cyclosome
Alt-NHEJ	Alternative non-homologous end joining
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	Ataxia telangiectasia and Rad3-related -interacting protein
BER	Base excision repair
BRCA1	Breast cancer type 1 susceptibility gene
BRCA2	Breast cancer type 2 susceptibility gene
BLM	DNA2–Bloom syndrome protein
CETSA	Cellular thermal shift assay
CX ₃ CL1	Chemokine (C-X3-C motif) ligand 1, fractalkine
CX ₃ CR1	CX ₃ CL1 receptor, fractalkine receptor
C-NHEJ	Classical non-homologous end joining
CDK	Cyclin dependent kinase
CSK	Cytoskeleton buffer
CtIP	C-terminal-binding protein-interacting protein
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic Acid
DHAP	Dihydroxyacetone phosphate
DR-GFP	Direct repeat green fluorescent protein
BLM	DNA2 bloom syndrome protein
DDR	DNA damage response
DNA-PK	DNA dependent protein kinase
DSBs	DNA double-strand breaks
SSBs	DNA single-strand breaks
LIG4	DNA ligase IV
DSBR	Double-strand break repair
ENO	Enolase
EGFR	Epidermal growth factor receptor
EOC	Epithelial ovarian cancer
ECAR	Extracellular Acidification Rate
ERK	Extracellular signal-regulated kinases

EXO1	Exonuclease 1
FA	Fanconi anemia
FAAP	Fanconi anemia-associated protein
ID2	FANCD2-FANCI complex
MHF	FANCM interacting histone-fold protein
F6P	Fructose-6-phosphate
F1,6P2	Fructose-1,6-bisphosphate
F2,6P2	Fructose-2,6-bisphosphate
ALDO	Fructose-bisphosphate aldolase
FH	Fumarate hydratase
G6P	Glucose 6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GADP	Glyceraldehyde 3-phosphate
GAPDH	Glyceraldehyde phosphate dehydrogenase
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
HK	Hexokinase
HGSOC	High-grade serous ovarian cancer
HER-2	Human epidermal growth factor receptor 2
HR	Homologous recombination
HU	Hydroxyurea
HIF	Hypoxia inducible factor
ICL	Interstrand crosslink
IR	Ionizing radiation
IRIF	Ionizing radiation-induced foci
JAK2	Janus kinase 2
MRE11	Mediator of DNA damage checkpoint protein 1
MCM 2-7	Minichromosome maintenance protein complex
MAPK	Mitogen-activated protein kinases
MMR	Mismatch repair
MMC	Mitomycin C
MRN	MRE11–RAD50–NBS1
PIM2	Murine lymphomas 2
NADH	Nicotinamide Adenine Dinucleotide reduced
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NLS	Nuclear localization sequence
OCR	Oxidative Consumption Rate
PAXX	Paralog of XRCC4 and XLF
PALB2	Partner and localizer of BRCA2
PPP	Pentose phosphate pathway
PGI	Phosphoglucose isomerase
PGAM1	Phosphoglycerate mutase 1
PI3K	Phosphoinositide 3-kinase
PARP	Poly(ADP-ribose)Polymerase
PNKP	Polynucleotide phosphatase/kinase

PCNA	Proliferating cell nuclear antigen
PKA	Protein kinase A
PKB	Protein kinase B
PPi-PFK1	Pyrophosphate-dependent phosphofructokinase-1
PK	Pyruvate kinase
PKM2	Pyruvate kinase 2
PTEN	Phosphatase and tensin homolog
PEP	Phosphoenolpyruvate
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
53BP1	P53-binding protein 1
RPA	Replication protein A
RNR	Ribonucleotide reductase
RRM1	Ribonucleotide reductase M1
RRM2	Ribonucleotide reductase M2
BARD1	RING domain protein 1
SSA	Single-strand annealing
ssDNA	Single-stranded DNA
SDSA	Synthesis-dependent strand annealing
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TPI	Triosephosphate isomerase
USP1	Ubiquitin carboxy-terminal hydrolase 1
UAF1	Ubiquitin carboxy-terminal hydrolase 1-associated factor
XRCC4	X-Ray Repair Cross Complementing 4
XLF	X-Ray Repair Cross Complementing 4-like factor
γ H2AX	Histone H2AX phosphorylated at Ser ¹³⁹

1 INTRODUCTION

1.1 GENOMIC INSTABILITY IN CANCER

1.1.1 The DNA damage response

Through the lifespan of a cell, insults to the DNA occur by endogenous and environmental sources. Endogenous sources of damage arise from reactions inherent in the chemical nature of DNA and from common byproducts of cellular metabolism. These events are estimated to occur as high as 10^5 times per cell on a daily basis¹. Exogenous sources, instead, include xenobiotics and environmental agents such as ultraviolet light (UV) that cause DNA damage. Altogether, these genotoxic insults entail a great potential to cause deleterious outcomes for genomic stability, including the loss of genetic information, mutations, defective chromosome segregation, and impairment of biological processes such as DNA replication and gene transcription^{2,3}. Contemplating such harmful consequences, how do then cells assure transmission of intact genetic information to daughter cells?

To maintain genomic stability, mammalian cells have developed refined mechanisms that detect and resolve DNA lesions, and command cells with deleterious DNA aberrations into programmed cell death. Historically, this integrated signaling network has been coined the DNA damage response (DDR)². Depending on the type of DNA lesion and the cell cycle phase, different DDR pathways are activated to resolve these lesions (Figure 1).

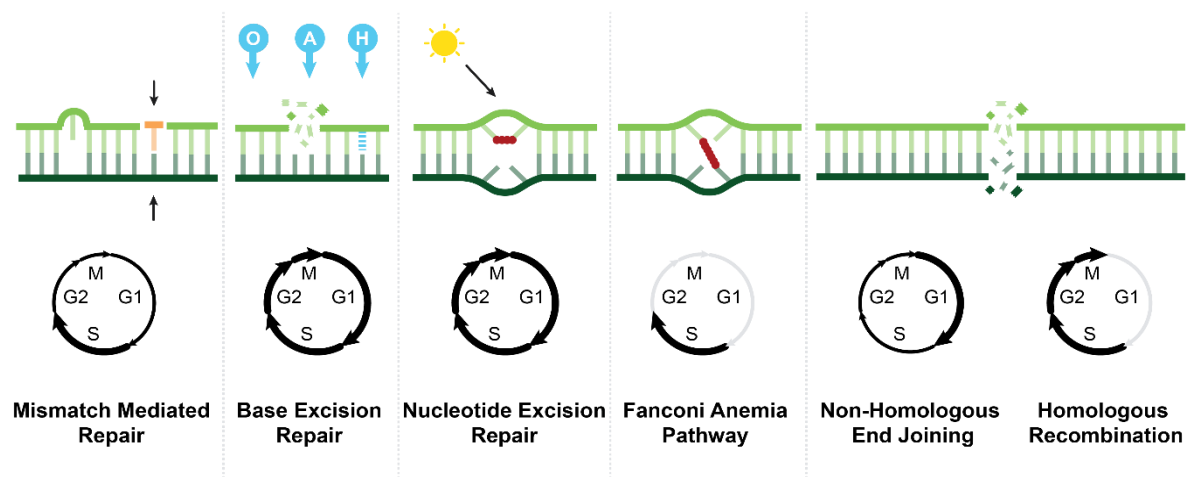


Figure 1. DNA repair pathways are specialized to resolve wide range of DNA lesions. Errors arising during DNA replication such as mismatched base pairs and insertion or deletion loops are repaired by mismatch mediated repair (MMR). Base excision repair (BER) corrects throughout the cell cycle chemically modified DNA bases originated from oxidation (O), alkylation (A), and deamination (H) reactions that lead to DNA single-strand breaks. Nucleotide excision repair (NER) resolves DNA helix-distorting base lesions such as ultraviolet photo-adducts and bulky base adducts. The Fanconi anemia (FA) pathway coordinates the repair of interstrand crosslinks exclusively during the S cell cycle phase as it requires converging replication forks to the damage site. Non-homologous end joining (NHEJ) and homologous recombination (HR) engage in the repair of DNA double-strand breaks. HR requires the availability of sister chromatid as a template and is therefore only active during the S, G2 and M cell cycle phases.

An intricate relationship exists between the DDR network and tumorigenesis. From a genetic perspective, cancer transformation is triggered by activation of oncogenes and inactivation of tumor suppressor genes, inducing high workload for the DDR machinery to ensure faithful DNA replication over the rapid cell proliferation during malignant progression. In contrast to non-transformed tissues, tumors exhibit a constitutive activation of the DNA repair machinery, which at early stages of tumorigenesis provides a protective mechanism triggering cell death⁴. Nevertheless, this chronic signaling constitutes a strong selection pressure towards inactivating mutations of DDR components during tumor development, which enable cells to survive in spite of enhanced mutation rates and persistent genome instability⁵. The fact that constitutive activation of genome maintenance machinery occurs in early-staged tumors and pre-invasive lesions preceding aberrations in the DDR factors led to the realization that DDR acts as an anticancer barrier to prevent tumorigenesis. At the same time, however, it is instrumental for tumor progression since it is selectively advantageous for oncogenotypes that breach this barrier⁶. The perturbation of DNA repair mechanisms and subsequent loss of control over genome integrity entails tumorigenic potential since it confers cancer hallmark capabilities of de-regulated cell proliferation, avoidance of cell cycle controls, and evasion of apoptosis⁷.

DDR factors are intimately related with the genomic instability phenotype observed in most cancer malignancies. By acting as tumor suppressors preventing deleterious DNA aberrations, their loss-of-function results in high rate of mutations and a large number of abnormal chromosomal rearrangements and copy numbers. Indeed, many DDR genes have been clinically documented as cancer-susceptibility genes and thus, designated as risk factors to develop certain types of cancers⁸. This is evident in the case of the hereditary breast–ovarian cancer syndrome, which is defined by the high incidence of breast and ovarian cancers among those families with germline loss-of-function mutations on the homologous recombination repair mediators breast cancer type 1 susceptibility gene (*BRCA1*) and breast cancer type 2 susceptibility genes (*BRCA2*)⁹. Another illustrative case is the Fanconi anemia (FA) disease, which is a recessive genetic disorder characterized by cancer predisposition, most commonly early onset of acute myeloid leukemia, and bone marrow failure due to defects in hematopoietic differentiation¹⁰. The molecular pathogenesis of FA is distinguished by mutations in DDR factors responsible to repair DNA crosslinks, and thus alternations in the so-called FA genes results in hypersensitivity to crosslinking agents and vast genomic instability. Notably, the advent of molecular genetics led to the discovery that many FA genes are, in fact, repair factors such as *BRCA1* and *BRCA2*. Somatic mutations in FA genes appear in up to 40% of all types of cancers¹¹, highlighting its role in malignant transformation and cancer progression.

DDR pathways contain three major elements: DNA lesion sensor proteins, kinases that act as signal transducers orchestrating the signaling response, and downstream effectors that convey DNA repair. Over the cell cycle, the DDR signaling is responsible to coordinate DNA repair with replication and DNA metabolism in order to guarantee genomic stability. This timely coordination takes place through the rapid activation of phosphorylation events initiated by three key signal transducers: ataxia-telangiectasia mutated (ATM), ATM rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK)¹². Each of these kinases interacts with their corresponding DNA binding sensors, which in turn display distinct affinity towards different kinds of DNA lesions. While ATR responds to single-stranded DNA (ssDNA) and phosphorylates the CHK1 checkpoint kinase; ATM is elicited by DNA double strand breaks (DSBs) and, as a response, activates the cell-cycle checkpoint kinase CHK2³. Activated CHK1/CHK2 triggers signaling cascades that convey the slowdown of cell cycle progression and activation of the G1/S, intra-S or G2/M cell-cycle checkpoints in order to prevent cells with damaged DNA to progress to replication or premature mitosis.

Apart from controlling the cell-cycle progression, ATR, ATM and DNA-PK are essential signaling transducers for the repair of the most critical types of DNA damage, namely DNA single-strand breaks (SSBs) and DSBs. Activation of these kinases stimulates the direct recruitment of repair factors to DNA damage sites and thereby activates an extensive network of downstream targets¹³. When repair effector factors do not manage to resolve these breaks, chronic ATM/ATR-mediated signaling results in genomic instability and mitotic catastrophe, ultimately leading to cell death via P53-mediated apoptosis or senescence¹².

1.1.2 Replication fork stability

An essential biological process safeguarded by the DDR network is DNA replication, which requires unperturbed mobility of the replication fork along the DNA template to accurately synthesize a new strand. Despite sophisticated mechanisms to ensure proper genome duplication, replication forks may inevitably encounter endogenous DNA lesions or unstable chromosomal regions, such as common fragile sites, containing sequences difficult to replicate that slow down or even block the replication machinery. Other naturally occurring events, like the collision between replication and transcriptomic machineries or misincorporation of ribonucleotides, constitute challenges to successful replication¹⁴. The delay, stalling or termination of DNA replication caused by these obstacles is known as replication stress.

Replication stress is characterized by long regions of ssDNA due to the uncoupling of DNA helicase-polymerase^{15,16}, premature termination of replication forks, and dissociation of replisome proteins from the DNA (fork collapse)^{17,18}. Eventually, these events generate DSBs lesions and result in genome instability. Transformed cells harbor high levels of replication stress not only because as rapidly dividing cells they are more likely to encounter endogenous

obstacles for DNA replication; but also because DDR factors, whose function is lost during tumorigenesis, constitute mechanisms to stabilize, repair and restart replication forks in order to protect nascent strands¹⁹. Accordingly, comparison of tumor tissues with their normal precursor counterparts illustrates that high levels of replication stress originating genomic instability are associated with malignant transformation²⁰.

To avoid chronic fork stalling, the DDR elicits a response to first stabilize and then restart replication forks¹⁷. The ssDNA generated due to fork stalling is coated by the replication protein A (RPA) complex (consisting of three proteins RPA70, RPA32, RPA14) which protects this unstable DNA intermediate. RPA acts as a replication stress fine tuning sensor as the extent of response elicited by RPA directly depends on the bulk of ssDNA, and thus the amount of the RPA coating molecules²¹. RPA subsequently binds to ATR-interacting protein (ATRIP) to recruit and activate ATR kinase and activate intra-S phase checkpoint via CHK1 phosphorylation²². The intra-S checkpoint activation mediates fork stabilization through various means. One of them is the regulation of deoxyribonucleotide (dNTP) intracellular pool via the activation of ribonucleotide reductase (RNR). The enzyme RNR is a tetramer protein composed by two catalytic subunits (RRM1) and two regulatory subunits (RRM2), and it operates the synthesis of dNTPs from ribonucleotides²³. Simultaneously, the intra-S checkpoint suppresses global origin firing, as well as promotes new origin firing in the proximity of stalled forks to facilitate fork convergence¹⁷. The latter becomes more critical in a context of replication stress, where unscheduled origin firing results in vast levels ssDNA and exhaustion of RPA intracellular pool, which ultimately leads to global replication fork collapse²⁴.

Following RPA fork protection and checkpoint activation, the DNA recombinase RAD51 gets recruited to ssDNA. RAD51 mediates fork regression, a process in which replication forks reverse newly synthesized complementary strands creating a chicken foot-like DNA structure. RAD51-mediated fork reverse provides time for the chromatin loading of DDR factors that protect reversed forks from nucleolytic degradation, resolve replication lesions, and enable replication restart.

Alternatively, proliferating cells can circumvent fork stalling by a traverse mechanism termed translesion synthesis (TLS). This process facilitates bypass of DNA lesions by switching regular high-fidelity DNA polymerases for specialized polymerases, *i.e.* TLS polymerases, which have a large active site that can accommodate nucleotides opposite to DNA lesions. The action of TLS polymerases enables cells to progress to the G2 cell cycle phase. Since they have lower proofreading capacity than replicative polymerases, the resulting daughter cells will often display higher rate of mutations compared to cells where the damage has been repaired²⁵.

1.2 DNA DAMAGE RESPONSE AND CANCER THERAPIES

1.2.1 DNA damaging therapies

On the basis of cancers' hallmark of genomic instability, cornerstones in cancer treatment constitute genotoxic agents that cause extensive DNA damage levels and unbearable replication stress in malignant tumor cells. Current DNA damaging therapies include radiotherapy and chemotherapy (Figure 2). Radiotherapy generates vast levels of DNA damage localized to the tissue where the radiation is directed²⁶. Instead, chemotherapy entails systemic delivery of drugs which target DNA itself or target biological processes associated with DNA replication and cell division²⁷.

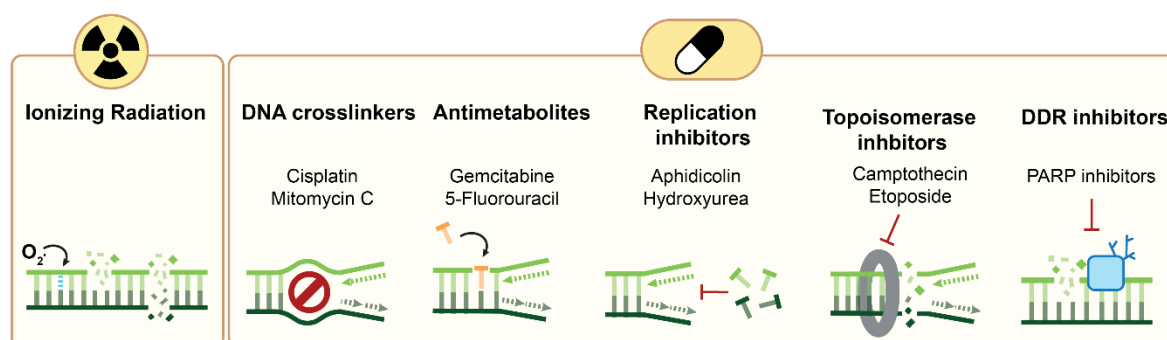


Figure 2. Overview of toxic lesions exerted by DNA damaging therapies used in cancer therapy. Radiation therapy causes oxidative damage to DNA bases, SSBs and DSBs. Chemotherapy includes a wide range of agents. Mono- and bifunctional crosslinkers chemically react with DNA bases producing DNA crosslinks and perturbing DNA helix structure. This bulky adducts cause replication lesions through the S cell cycle phase and eventually result in DSBs. Antimetabolites hinder nucleotide metabolism and DNA synthesis culminating in DNA mismatches and generation of toxic replication lesions. Replication inhibitors trigger replication fork stalling and collapse causing replication lesions and DSBs. Topoisomerase inhibitors provoke topological problems on the DNA double helix resulting in replication lesions, SSBs and DSBs. More recently developed, small molecule inhibitors that target key DDR factors such as Poly (ADP-ribose) polymerase (PARP) inhibitors have emerged as novel therapeutic strategies. PARP inhibitors cause severe impairments on the repair of DNA lesions.

Extensive research over the last 50 years has shown that defects in the DDR network result in large susceptibility to DNA damaging agents such as UV light that may be harmless for cancer patients with proficient DNA damage repair systems²⁸. Considering the mechanism of action of DNA damaging therapeutics (Figure 2), loss-of-function or deletion mutations in DDR genes results in hypersensitivity towards these types of therapies. At the same time, though, amplification or gain-of-function mutations in these genes may confer protective mechanisms to alleviate replication stress and DNA damage. Hence, the proficiency of checkpoint control and DNA damage repair have therapeutic implications because they largely determine the efficacy of DNA damaging treatments and contribute to the development of resistance and tumor regression²⁷. This, in turn, introduces a clinical challenge for those patients carrying defective DDR germline mutations, since the employment of such therapies results in unpredictable toxicity to them^{29,30}.

The current chapter gives an overview of the mechanism of action and the DNA damage responses triggered by two universally used DNA-targeting cancer treatments: ionizing irradiation and platinum-based agents, in order to frame how perturbations in DNA repair systems and checkpoint signaling have an effect on the therapeutic efficacy to these treatments.

1.2.2 Repair of DNA double-strand breaks

Radiation therapy is used in approximately 50% of all cancer patients, making it the most common therapeutic modality used in the clinic²⁶. It is estimated that radiotherapy contributes to cure a rate of 40% of all cancer patients³¹, highlighting its relevance as a successful therapeutic strategy. The most common clinical approach is the external beam radiotherapy, in which high-energy ionizing irradiation (IR) is aimed to the location of the tumor.

The collision of IR high-energy in the tumor tissue generates oxygen free radicals that oxidate DNA molecules resulting in a variety of lesions including DSBs, SSBs and oxidation of DNA bases. The less frequent but most toxic lesions are DSBs, which are provoked in a genome-wide fashion causing a collapse in genomic integrity that activates repair by the DDR network^{32,33,34}. If left unrepaired, IR-induced DSBs result in chromosomal breakage and rearrangements, compromising genomic stability³⁵.

The two pivotal pathways that dominate the repair of DSBs are non-homologous end joining (NHEJ) and homologous recombination (HR) (Figure 3). Whilst NHEJ is a rapid process that involves direct ligation of the two DNA broken ends with minimal dependency on DNA sequence³⁶, the HR pathway requires the availability of homologous sequence as a reference template for repair, and thus is considered to be less error-prone³⁷. As a result, HR is restricted to the S and G2 cell cycle phases, while NHEJ principally can occur throughout the cell cycle.

The NHEJ pathway manages the direct re-ligation of broken DNA ends which can be performed by two different processes: the co-called classical NHEJ (c-NHEJ) and alternative NHEJ (alt-NHEJ).

In c-NHEJ, the nuclear heterodimer Ku70–Ku80 binds to DNA broken ends forming a double-ring structure with high specificity for double strand ends (Figure 3)³⁸. Direct visualization by high-resolution imaging shows that although several dimers can bind to DSB sites, usually a single molecule is loaded to each of the ends³⁹. This DNA binding complex functions as a DSB sensor and orchestrates the recruitment of the DNA-PK kinase, and other DDR-factors such as DNA ligase IV (LIG4), XRCC4, and the scaffolding proteins XRCC4-like factor (XLF) and paralog of XRCC4 and XLF (PAXX)^{37,40}. Together, these factors form a DNA-protein complex that facilitates the close alignment of both DNA ends, known as the synapsis step, and the ligation of broken DNA ends. Moreover, activated DNA-PK phosphorylates Artemis, a

nuclease that manages the transformation of incompatible DNA ends to DNA intermediates suitable for direct ligation⁴¹.

Besides c-NHEJ, alt-NHEJ is a backup mechanism to rejoin DSB ends (Figure 3). Alt-NHEJ occurs when c-NHEJ factors are not engaged and requires the presence of microhomology sequences between strands at the DSB site. In this case, minimal 5' resection of DSB ends is followed by RPA ssDNA coating^{42,43}. RPA is further displaced by DNA polymerase θ that catalyzes the synthesis and ligation of both strands⁴⁴.

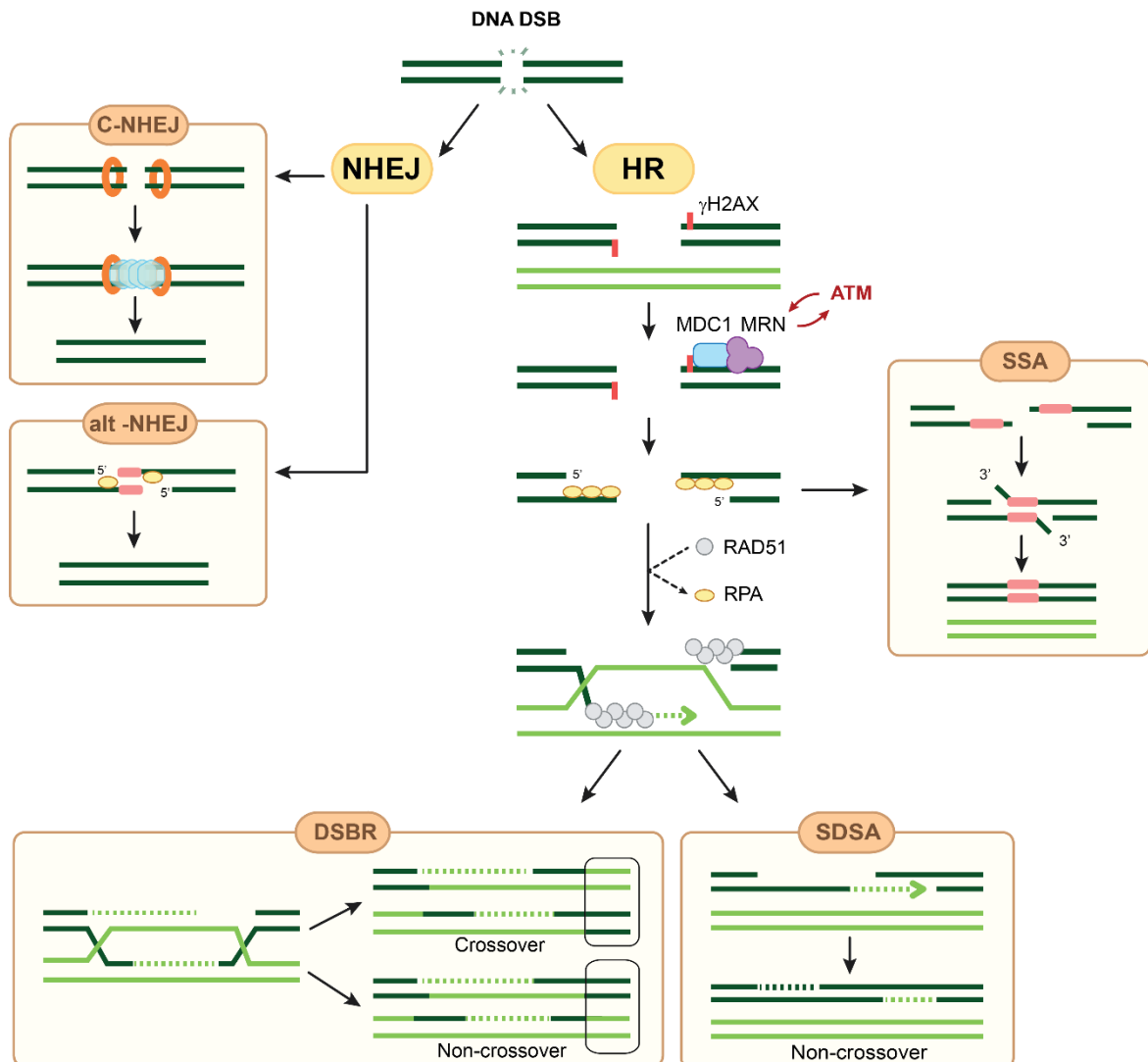


Figure 3. Pathways of DNA double-strand break repair. IR-generated DSBs are repaired via non-homologous end joining (NHEJ) or homologous recombination (HR) repair mechanisms depending on the cell cycle phase. Classical NHEJ mechanism directly joins broken DNA ends, whereas alternative NHEJ requires slight end processing and microhomology sequences in the vicinity of break ends. Homologous recombination is initiated by extensive 5' to 3' DNA end resection, followed by RPA ssDNA coating, displacement of RPA by RAD51 and RAD51 filament formation. DNA strand invasion, homology search and second end capture culminate into two different mechanisms: synthesis-dependent strand annealing (SSDA) and double-strand break repair (DSBR) which after a second end capture can result into two different outcomes cross-over and non-crossover products. Alternatively, DSBs that have undergone extensive end resection and contain homology sequences flanking the ends can be annealed by a RAD52-dependent mechanism termed single-strand annealing (SSA).

Apart from NHEJ repair mechanisms, HR constitutes the other constitutive pathway to resolve IR-induced DSBs (Figure 3). HR repair uses homologous DNA sequences as a template for repair. These sequences can be found in sister chromatids, homologous chromosomes, and non-homologous chromosomes that contain repetitive sequences. The fact that recombinational repair of DSBs displays low mutagenicity rates limiting loss of heterozygosity, suggests that sister chromatids are the preferred correct repair templates⁴⁵.

Following IR-induced DSBs, phosphorylation of the Ser¹³⁹ residue of the histone variant H2AX (γ H2AX) in regions of the chromatin nearby break sites is triggered by either the ATM or DNA-PK kinases⁴⁶. γ H2AX functions as a coordinator of DDR signaling by recruiting the mediator of DNA damage checkpoint protein 1 (MDC1) as a scaffolding protein that physically interacts with MRE11–RAD50–NBS1 (MRN)^{47,48}. The MDC1-MRN complex enables the recruitment and activation of ATM, which in turn phosphorylates MRN, establishing a positive feedback loop to amplify DNA damage signaling initiated by the ATM- γ H2AX axis. Simultaneously, activated ATM phosphorylates the CHK2 transducer conveying G1/S and G2/M cell cycle arrest through phosphorylation of Cdc25A and p53, respectively⁴⁹. These checkpoints remain activated until the damage is repaired. Furthermore, the MDC1-MRN complex recruits the tumor suppressor p53-binding protein 1 (53BP1), a key regulator of NHEJ that protects broken DNA ends from resection⁵⁰.

The next step in HR is the 5' to 3' DNA end resection, generating a ssDNA tail. The ATM-dependent phosphorylation of the MRN complex increases MRN nuclease activity⁵¹. The combined nuclease activities of CtBP-interacting protein (CtIP)⁵² and MRN catalyze a “short-range” resection of the DNA strands. MRN exonuclease activity removes Ku70–Ku80, which physically blocks resection of the DNA ends, and loads the exonuclease 1 (EXO1) and the DNA2–bloom syndrome protein (BLM) to initiate “long-range” resection⁵³. BLM mediates DNA unwinding, whereas EXO1 catalyzes nucleolytic digestion to process DSB ends into 3' single-stranded DNA tails⁵⁴. The recombination mediator BRCA1 modulates the end resection step. When assembled with BRCA1-associated RING domain protein 1 (BARD1), BRCA1 colocalizes with CtIP and MRN to DSB damage sites, and mediates the dephosphorylation of 53BP1 resulting in unprotected DSB ends⁵⁵.

Directly after DNA end resection, the RPA complex binds to the ssDNA generated regions. This binding limits pairing with surrounding ssDNA stretches and also prevents excessive resection, which would be detrimental. The recombination mediator BRCA2 competes with RPA for binding to ssDNA and thus facilitates disassembly of coated RPA. BRCA2-mediated displacement occurs through its interaction with ssDNA, monomers of the DNA recombinase RAD51, and BRCA1-BARD1 complex through the partner and localizer of BRCA2 (PALB2).

As a result, BRCA2 enables the DNA recombinase RAD51 to remove RPA and stimulates RAD51 nucleation, an event in which a small complex of RAD51 monomers bind to ssDNA^{56,57}. After this initial nucleation, RAD51 forms nucleoprotein helical filaments on the ssDNA that constitute essential recombination intermediates for strand invasion and exchange.

The RAD51-ssDNA filaments operate the search for homology sequences for complementary pairing, and subsequent DNA strand invasion of the sister chromatid³⁷. DNA polymerases load to 3' ends of invading strands and synthesize nascent strands using as a template the DNA sequence of the sister chromatid⁵⁸. Several RAD51 recruited recombination mediators are essential to assist displacement, nascent strand synthesis and annealing of the previously resected strand. These factors include, for example, BRCA1–BARD1 proteins which have been shown to facilitate RAD51-mediated base pairing with complementary sequences⁵⁹.

Once the invading strand is completely synthesized based on the donor template sequence, the remaining second end of the DSB must be processed for a complete repair. This can occur via the mechanism termed synthesis-dependent strand annealing (SDSA) (Figure 3)⁶⁰, where the resulting recombinational products are non-crossover because the invaded newly synthesized strand detaches and serves as its own template for the completion of the second DSB end. Alternatively, resolution can occur following the DNA double-strand break repair (DSBR) model (Figure 3)⁶⁰. After the second end capture, DNA synthesis and ligation, the duplex DNA strands can be resolved giving two different recombinational products: either non-crossover product, where the original configuration is kept; or crossover product, where there is exchange of flanking sequences between sister chromatids⁶¹.

RAD51 is the main recombination factor and is essential for strand invasion, homology search and successful HR repair. Alternatively, DSBs can be resolved by a RAD51-independent mechanism known as single-strand annealing (SSA) (Figure 3)⁶². SSA entails the annealing of two DSB ends that are flanked by repeated sequences. Mediated by the action of RAD52, repeated homologous ssDNA regions disclosed during end resection step are annealed, and flapping overhangs are subsequently eliminated. This nucleolytic degradation entails mutagenic potential because it results in the genetic loss of the sequences intervening homologous regions⁶³.

1.2.3 Replication-coupled repair of interstrand crosslinks

Platinum-based drugs, including cisplatin, carboplatin and oxaliplatin, have been widely used in cancer treatment since late 1970s⁶⁴. Nowadays, it is estimated that half of the patients that undergo chemotherapy receive platinum-containing treatment⁶⁵. These drugs constitute first line therapy of testicular and ovarian cancer, malignancies that historically had shown poor outcome for advanced disease and in which platinum-based therapy has led to a significant clinical impact^{66,67}.

The primary cellular target of platinum-based agents is DNA. Once administered, platinum drugs become activated by an aquation reaction. This active form covalently binds with purine bases in the DNA causing irreversible DNA adducts. First, a monofunctional adduct is formed followed by a reaction that result in either: intra-strand crosslinks involving two bases of the same DNA strand, interstrand crosslinks (ICLs) between bases on the complementary strands of the DNA helix, or less commonly protein-DNA adducts^{68,69}. Most of the DNA adducts of cisplatin are intra-strand crosslinks (80-90% of all adducts), whereas ICLs constitute a minority (around 2-5% of all adducts)⁷⁰. Nevertheless, ICLs are thought to be the primary source of platinum cytotoxicity⁷¹. Structurally, platinum-induced ICLs generate a major distortion on the DNA helix with notable unwinding and bending at the site of the crosslink⁷².

Besides platinum, ICLs are also yielded by other types of bifunctional agents. Mitomycin C, an antibiotic produced by *Streptomyces*, reacts with guanine residues generating around 5-14% of ICLs among other adducts, and provokes mild DNA helix distortion⁷³. Upon activation with UV light, psoralen is the bifunctional agent that triggers the highest ICL fraction, up to 40% of all adducts, and disturbs the structure of the DNA resulting in mild unwinding^{72,74}.

ICLs are particularly toxic DNA lesions due to their harmful effect in preventing DNA strand separation. ICL-induced fork stalling blocks essential biological processes that require progression of the replicative machinery alongside DNA strands. The sustained blockage of replication results in DSB formation, mitotic catastrophe and cell death⁶⁴. ICLs trigger clastogenic effects, leading to breakage, loss and rearrangement of chromosomes. Overall, DNA crosslinking agents are regarded as distinguished toxic compounds ranking high in comparative risk assessment studies assessing *in vitro* and *in vivo* genotoxicity⁷⁵.

To preserve genomic stability, replication-coupled ICL repair must ensure not only repair of DNA lesions from both strands, but also replication fork stability and protection. This is achieved by the FA pathway, which coordinates the sequential action of different DDR pathways including nucleotide excision repair (NER), TLS and HR repair (Figure 4)¹¹. The FA pathway is at the heart of ICL replication-coupled repair, as cells from FA patients treated with ICL-inducing agents exhibit cell cycle arrest in late S phase and substantial clastogenic effects⁷⁶. These events ultimately lead to high levels of genomic instability.

The encounter of replication machinery with an ICL triggers fork stalling. This replication fork stalling is an essential requirement for ICL recognition and resolution via FA pathway. First, disassembly of replicative CMG helicase via BRCA1 activation allows the fork to come nearby the ICL site (Figure 4a).

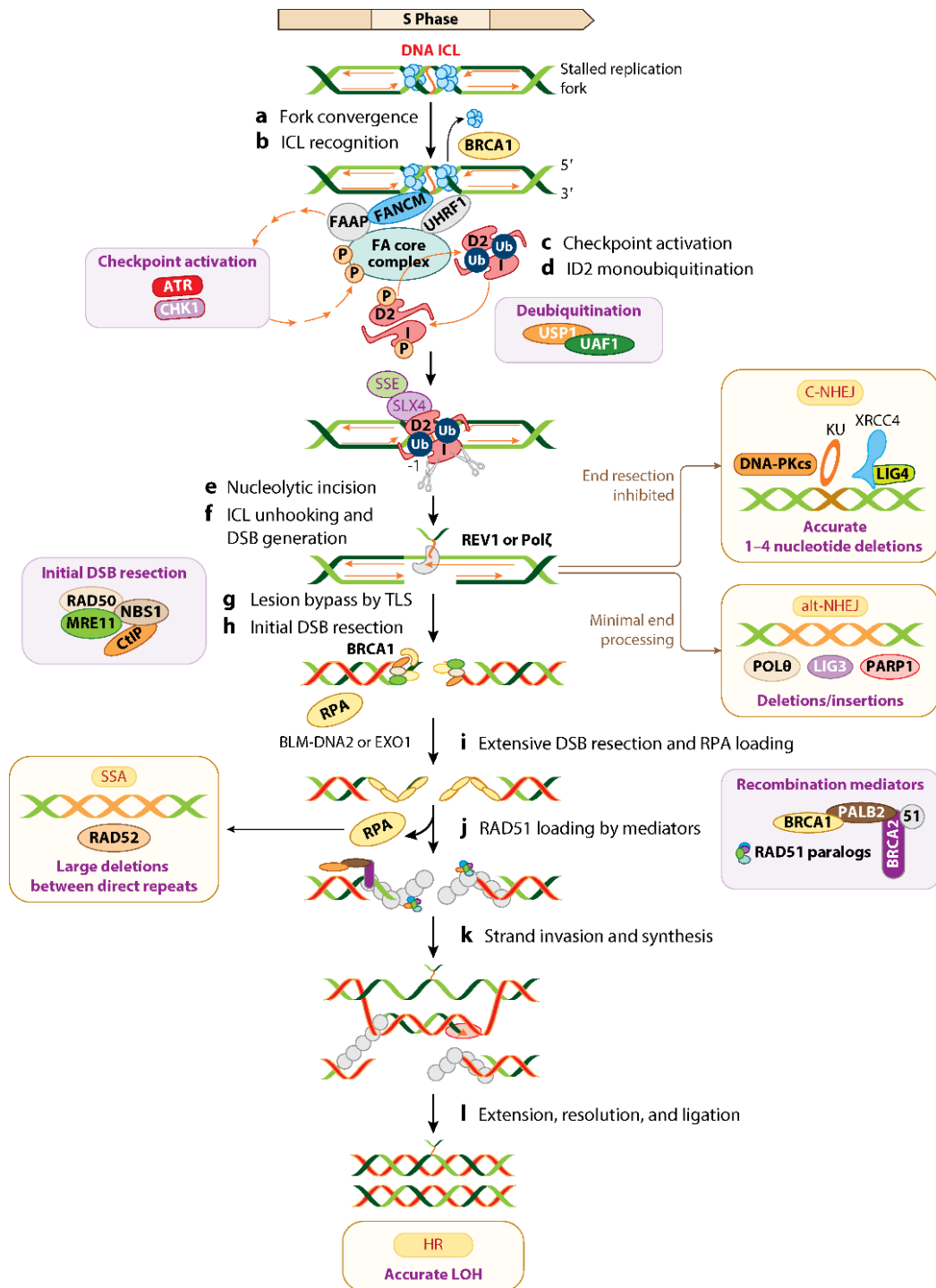


Figure 4. Current model mechanism of replication-coupled ICL repair. Stalled replication forks due to ICL formation are repaired through the action of different pathways: Fanconi anemia (FA), nucleotide excision repair (NER), translesion synthesis (TLS) and homologous recombination (HR). **(a)** Upon ICL generation, the replication fork approaches the crosslink site where it stalls and **(b)** the resulting branched DNA is identified by FANCM sensor protein. ICL recognition triggers **(c)** ATR/CHK1 mediated signaling to convey S cell cycle phase arrest and **(d)** activation of the FANCI-FANCD2 heterodimer (ID2 complex) by monoubiquitylation. **(e)** ID2 recruits NER nucleases that perform nucleolytic incision of one of the strands in the vicinity of the ICL **(f)** generating a DSB with the unhooked strand and its complementary newly synthesized strand. **(g)** The lesion is bypassed by low-fidelity TLS polymerases and **(h)** 5' to 3' end resection leads to initiation of HR pathway repair. Following **(i)** RPA ssDNA loading and **(j)** RAD51 displacement and loading of recombinational mediators, **(k)** homology search takes place and further **(l)** extension, resolution, and ligation of newly synthesized strands. Republished with permission of Annual Reviews, from "The Fanconi Anemia Pathway in Cancer" from Niraj, J. *et al.* 3:457-478 (2018); permission conveyed through Copyright Clearance Center, Inc.

ICLs are identified by the key sensor protein FANCM, whose activity is cell cycle-dependent⁷⁷ (Figure 4b). FANCM displays three main functions. First, it has a structural role enabling the assembly of downstream factors and thus, the loss of FANCM results in impairment or even absence of chromatin loading of downstream FA proteins. Second, via its translocase enzymatic activity FANCM remodels DNA structures at ICL sites essential for subsequent post replicative repair. This DNA remodeling function is supported by FANCM interaction with the BLM complex, which enables the unwinding of DNA strands at sites of stalled replication forks. Last, the fact that FANCM gets hyperphosphorylated upon genotoxic treatments suggests that it has a signal transduction role in response to DNA damage.

Translocation of FANCM to ICL sites requires direct interaction with the BLM complex and phosphorylation by the ATR kinase⁷⁸. Simultaneously, recruited FANCM triggers ATR-mediated activation of cell cycle checkpoint via CHK1 to suppress new origin firing, block the entry into the G2 phase, and avoid subsequent mitotic catastrophe (Figure 4c). The elicitation of this checkpoint requires FANCM-dependent RPA chromatin recruitment for fork protection²¹. Cumulatively, ATR and FANCM form a positive feedback loop, reinforcing the recruitment and activation of each other. Furthermore, the chromatin association of FANCM to ICL sites requires the physical interaction with two proteins: the Fanconi anemia core complex-associated protein 24 (FAAP24)⁷⁹, which recognizes DNA intermediates common in replication fork progression and thereby confers FANCM higher specificity towards these structures; and the FANCM interacting histone-fold proteins 1 and 2 (MHF complex)⁸⁰, which stimulate DNA binding and replication fork remodeling by FANCM.

The FANCM-FAAP24-MHF complex works as a recruitment platform for downstream FA factors that accumulate and colocalize at ICL sites. This is the case of the FA core complex consisting of eight interacting proteins: FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and the associated protein FAAP100⁸¹. The major role of FA core complex is to ubiquitinate the FANCD2-FANCI heterodimer (ID2 complex) (Figure 4d). The binding of ID2 to DNA ICL sites induces a conformational change that exposes buried and inaccessible ubiquitin binding sites of both FANCD2 and FANCI proteins⁸². ATR-mediated phosphorylation of FANCI stabilizes FANCD2 to chromatin, stimulating the ubiquitylation of the heterodimer^{83,84}. However, only the monoubiquitylation of FANCD2 subunit results in a conformational rearrangement of ID2 complex, forming a clamp-like structure promoting the formation of filament arrays alongside the DNA. These ubiquitinated ID2 filaments are thought to stabilize the heterodimers to DNA strands and mediate fork stability⁸⁵. From a functional perspective, chromatin bound ID2 must be ubiquitylated to support ICL repair⁸⁶. Changes in FANCD2 ubiquitin binding site restrict ID2 chromatin binding and sensitize cells to ICL agents⁸⁷, underlining the relevance of this post-translational modification as a key event for FA pathway activation. Ubiquitin carboxy-terminal hydrolase 1 (USP1) together with USP1-

associated factor (UAF1) catalyze ID2 deubiquitylation terminating the pathway signal, albeit the dynamics of this molecular switch are still poorly understood¹¹.

Following ID2 chromatin loading and activation, nucleolytic incision takes place (Figure 4e). Helped by the SLX4 scaffolding protein, the ubiquitinated ID2 complex recruits structure-specific endonucleases from the NER pathway that catalyze DNA incisions flanking the ICL on one strand. After the unhooking (Figure 4f), TLS polymerases execute DNA synthesis of strands opposite to the tethered ICL (Figure 4g).

The unhooking step culminates in the generation of a DSB that can be repaired via HR, classical NHEJ, alternative NHEJ or SSA. It is well established that the preferred mechanism to process this DSB intermediate is HR (Figure 4i-l) since genetic loss of HR factors such as *BRCA1* or *BRCA2* confer hypersensitivity to crosslinking agents^{88,89}, whereas deficiency in NHEJ factors do not⁹⁰. BRCA deficient cells engage Pol θ -mediated alternative NHEJ for successful replication-coupled ICL repair by promoting FANCD2 recruitment in fork stability⁹¹, thus indicating a key role of FANCD2 in pathway choice.

With regards to the resolution via HR (Figure 4i-l), DNA 5' end resection is required for activation of the pathway. The DSB generated by unhooking is susceptible of nucleolytic attack by the DNA nucleases MRE11, CtIP, EXO1 and DNA2, which mediate DNA end resection⁵⁴. Recent evidence suggests that FA factors such as FANCV protect DNA from deleterious strand degradation at this step, yet the exact mechanism is not fully understood¹¹. In summary, HR repair of the DSB intermediate is carried out following DNA end resection, RPA loading, RAD51-mediated strand invasion, homology search and ligation steps as previously explained (see section 1.2.2).

What happens though with the repair of crosslinks in those cells that are not replicating? In this case the absence of replication fork collision poses the question of how ICLs are sensed and recognized. One possibility is that the collision of ICL with RNA polymerase evokes a signal for DNA incision and processing via NER pathway, often called as transcriptional-coupled NER⁸⁹. Upon blockage of transcriptomic machinery, NER resolves ICLs by the action of the endonucleases ERCC1 and XPF. These two endonucleases form a dimer to mediate the incision of one of the strands and removal of damaged nucleotides. Other nucleases such as XPA and XPG are also involved in the incision step; however, it seems that the type of crosslinking agent, and thus the extend of DNA helix distortion, determines the accession and processing of these factors^{89,92}. Once DNA unhooking is done, TLS polymerases mediate DNA lesion bypass thereby contributing in that way to DNA damage tolerance. Sequential action of ERCC-XFP and TLS polymerases in the opposite strand allows the complete resolution of ICLs⁶⁴.

Because the inactivation of genes involved in non-replicative and FA-mediated repair results in synergistic sensitization towards crosslinking agents such as cisplatin and mitomycin C, it has been suggested that these two mechanisms can cooperate to resolve ICLs and are not mutually exclusive⁹². Although transcription-coupled NER occurs across the cell cycle, also during S phase when FA pathway is activated, it often results in minor and incomplete repair of all ICLs. Replication-associated repair is crucial for rapidly dividing cancer cells which harbor high levels of DNA replication stress, whereas non-replicative repair is crucial for cells that barely divide such as stem cells⁸⁹.

Growing body of literature recognizes the critical role of the FA pathway in fork stabilization upon replication stress caused not only by ICL agents, but also by other types of genotoxic agents. For instance, the FA pathway is activated upon exposure to replication inhibitors such as hydroxyurea (HU) and aphidicolin⁹³. HU targets RNR enzyme and severely decreases dNTP intracellular pool, and aphidicolin directly inhibits DNA polymerases. Current knowledge indicates that upon replication stress FANCM engages in fork remodeling via its intrinsic translocase activity⁹⁴, and in fork traverse via BLM helicase interaction⁷⁸. Together with FANCM, recruitment of proliferating cell nuclear antigen (PCNA) and RPA are required to bypass ICLs prior ID2 activation⁹⁵. In contrast to their common function in ICL repair, FANCD2 and FANCI have distinct roles in response to replication stress. Whilst FANCI promotes dormant origin firing under the control of ATR phosphorylation⁹⁶, phosphorylated FANCD2 recruits and cooperates with the BLM helicase to facilitate replication restart of stalled forks while suppressing firing of new replication origins⁹⁷. FANCD2 also restrains DNA synthesis by inhibition of minichromosome maintenance protein complex (MCM 2-7), thereby limiting the generation of ssDNA stretches⁹⁸. In high replication stress conditions, FANCD2 is responsible for fork protection to prevent nucleolytic degradation by binding to ssDNA-RAD51 filaments, likewise BRCA1 and BRCA2 factors^{11,99–101}.

1.2.4 Synthetic lethality

Owing to the high selection pressure in malignant tumor cells, dysfunctional DNA repair pathways arising from aberrant mutations may result in the reliance on alternative DDR pathways for cell survival. Such cancer-specific dependency can be exploited therapeutically by the rationale of synthetic lethality¹⁰². Synthetic lethality originates when a combination of deficiencies of two or more genes leads to cell dismiss, whereas the absence of each single gene function is compatible with cell survival. These deficiencies can derive from loss-of-function mutations, epigenetic alterations, or pharmacological inhibition of the gene product. This concept can be applied to the discovery of targeted therapies. Selective killing of malignant cells with minimal effect on normal cells can be achieved upon inhibiting mechanisms that cancer cells exclusively rely on for functional DNA repair and survival. Nowadays, with the advent of CRISPR-Cas9 genetic screens, identification DDR components

whose loss results in sensitivity towards DNA damaging agents has uncovered new potential synthetic lethal interactions that can be exploited specifically for each type of DNA-damaging therapeutic agent¹⁰³.

An opportunity to improve traditional DNA damaging treatment lies in a more refined understanding of the molecular events in DDR pathways triggered by these agents in cancer cells. Besides gene interactions, combination of drugs targeting DDR pathways may be synthetic lethal when one DNA-damaging agent exposes a vulnerability that can be targeted with a second therapy¹⁰⁴. If this liability displays a cancer-specific behaviour, then rational combination of DDR inhibitors offers the possibility to modulate sensitivity of DNA damaging therapies in a way that treatments are more cytotoxic in tumor tissues compared to non-transformed tissues.

1.2.4.1 PARP inhibitors in ovarian cancer

The most outstanding case of synthetic lethality to date is the discovery of the antitumor activity of poly(ADP-ribose) polymerase (PARP) inhibitors in ovarian cancer patients¹⁰⁵. Ovarian cancer is the most lethal among gynecological cancers with a 5-year overall survival of 46%¹⁰⁶. Epithelial ovarian cancer (EOC) comprises about 95% of all cases, and is in turn classified into different histological subtypes. From these, high-grade serous ovarian cancer (HGSOC) is the most commonly presented in EOC patients, approximately in 75% of cases¹⁰⁷. HGSOC is characterized by extremely aggressive clinical progression, poor prognosis, loss-of-function mutations in the *TP53* gene¹⁰⁸, and frequent low prevalence mutations in genes of the HR pathway, including *BRCA1* and *BRCA2*¹⁰⁷. Cells that harbor loss-of-function mutations in BRCA genes repair DNA lesions by alternative mechanisms that are error-prone such as NHEJ, leading to a remarkably high genomic instability and chromosomal rearrangements, contributing to the pathogenesis of HGSOC. Integrated genomic analyses from The Cancer Genome Atlas show that HR is defective in 51% HGSOC patients, underlining the key role of this DDR pathway in genome maintenance through ovarian tumor progression¹⁰⁹.

Using the synthetic lethality approach, PARP inhibitors have emerged on the basis of the HR-deficient signature of HGSOC¹¹⁰. The loss of *BRCA1* or *BRCA2* tumor suppressors renders tumor cells deficient in HR-based repair of DSBs and thus, more vulnerable to unresolved SSBs that precede to the generation of DSBs. PARP enzymes sensor SSBs and catalyze the synthesis of poly(ADP-ribose) chains (PARylation). These polymers act as signaling molecules to recruit DDR proteins from different DNA repair mechanisms and factors involved in the maintenance of replication fork stability¹¹¹. PARP inhibitors block PARylation and thereby prevent PARP dissociation from the DNA damage sites, trapping PARP on the DNA (see Figure 2, section 1.2.1). The resulting accumulation of unrepaired SSBs eventually transforms into DSBs, which is extremely toxic for cells with HR deficiency. Resolving PARP

trapping and repairing the generated DSBs requires HR-proficient repair for cell survival¹¹². As a consequence, delivering PARP inhibitors to BRCA-deficient patients renders tumour cells completely unable to resolve induced DNA lesions via HR, hence causing selective cell death by accumulation of DNA damage¹¹³.

Olaparib and rucaparib are PARP inhibitors approved for maintenance therapy in patients carrying BRCA mutations in HGSOc^{114–116}. This therapeutic option is of interest since it is calculated that approximately 20% of ovarian cancer patients harbor BRCA germline mutations and exhibit monogenic predisposition in their family lineage to develop ovarian cancer. More recently, prescription of PARP inhibitors have been expanded since clinical trials have shown the effectiveness of niraparib in treating patients irrespectively of HR status^{117–119}.

1.3 PFKFB3: A METABOLIC LIABILITY IN CANCER

1.3.1 Reprogramming energy metabolism in cancer

Reprogramming cellular energetics has been defined as an emerging hallmark of cancer, meaning that metabolic features in tumors are flexible and adaptative to ensure tumor cell fitness⁷. Because of their high growth rates, cancer cells rely in the availability of fundamental building blocks required for the synthesis of macromolecules such as lipids, amino acids and nucleotides. Oncogenic activation drives the adjustment of metabolic pathways in order to meet this liability, enabling uncontrolled cell proliferation and thereby promoting neoplastic transformation¹²⁰.

Metabolic alterations in cancer were first noticed by the scientist Otto Warburg in the early 1920s^{121,122}. Warburg and co-workers observed that tumor cells in culture consumed far more glucose than cells originating from non-transformed tissues. Regardless of the presence of oxygen, glucose was preferably converted to lactate via anaerobic glycolysis over oxidative phosphorylation, in contrast to non-transformed cells (Figure 5). This dependence was later termed as “The Warburg Effect”¹²³ and set the path for the study of cancer metabolism¹²⁴. This observation, however, appeared to be counterintuitive at first glance mainly because anaerobic glycolysis results in extremely lower efficiency in ATP production compared to oxidative phosphorylation¹²⁵. Assuming that cancer cells at a proliferative state have high energy demands, why do they then display such an inefficient energetic metabolism?

The truth is that nowadays it is still not completely understood why cancer cells prefer glycolysis¹²⁶. Metabolic needs in cancer cells exceed the need for ATP molecules, so the urge for building blocks is much higher than the energetic demands¹²⁷. Therefore, one possible reason is that the preference for glycolysis could allow the diversion of metabolic intermediates to biosynthetic pathways in order to supply essential building blocks and meet the metabolic demands of oncogenic-wired cancer cells (Figure 5). The building blocks generated from glycolytic intermediates include hexosamines, glycerol, citrate, nonessential amino acids, ribose sugars and NADPH. Notably, ribose sugars and NADPH are generated via the pentose phosphate pathway (PPP), a parallel pathway to glycolysis, and are substrates for nucleotide and fatty acid synthesis, respectively. Moreover, the yield of NADPH is essential to keep favorable redox homeostasis and prevent excessive levels of oxidative stress in cancer cells¹²⁸. Altogether, increased glycolytic flux results in channeling glucose, the most abundant extracellular nutrient, into anabolic and redox homeostasis mechanisms that enable unrestrained cancer cell growth.

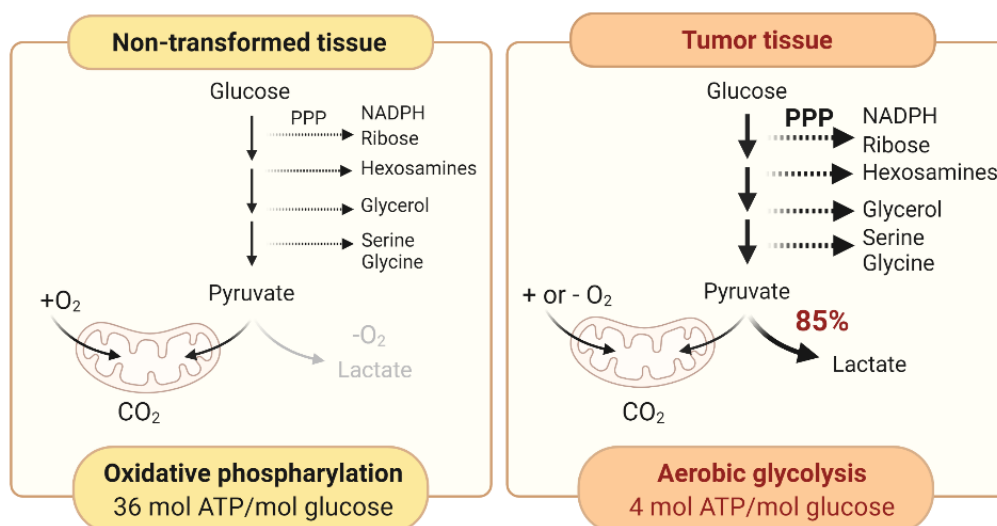


Figure 5. The Warburg Effect. Glucose is metabolized into pyruvate, which can be completely oxidized into CO₂ in the mitochondria via oxidative phosphorylation to produce ATP, or it can be metabolized under hypoxic conditions to lactate via anaerobic glycolysis. This process results in lower energetic yield in comparison to oxidative phosphorylation. The Warburg phenotype provides a supply of anabolic precursors for biosynthetic pathways that enable unrestrained growth in transformed cells. Image generated with BioRender.com from information in refs^{126,127}.

Within a modern understanding of cancer biology, DeBerardinis and colleagues coined the term “convergent properties” to describe those most common metabolic patterns dictated by predominant oncogenotypes¹²⁹. Among other pathways involved in energy metabolism, the Warburg Effect is considered a convergent property that arises from oncogene activation and loss-of-function mutations in tumor suppressor genes. Oncogenic mutations in *MYC*¹³⁰, *KRAS*^{131,132} and *RAS*¹³³ result in a metabolic switch towards anaerobic glycolysis by either transcriptional upregulation or direct enzymatic activation of glycolytic enzymes and glucose transporters. On the other hand, the tumor suppressor p53 counteracts to the glycolytic phenotype and promotes oxidative phosphorylation by, among other mechanisms, regulating the expression of the metabolic enzyme TP53-inducible glycolysis and apoptosis regulator (TIGAR). TIGAR catalyzes the degradation of fructose-2,6-bisphosphate (F-2,6-BP), a glycolysis metabolite that positively controls the glycolytic flux. Therefore, catalytic activity of TIGAR directs the glycolytic pathway into the PPP shunt. As a consequence, loss-of-function of *TP53* gene culminates in losing the inhibitory effect on glycolysis, and thus has been proposed as a tumorigenic mechanism behind the Warburg effect¹³⁴.

Contributing factors of reprogramming cancer cell metabolism are not only aberrant signaling networks emerging from oncogenotypes, but also the metabolic status of the original tissue from which the tumor arise, and the immediate tumor microenvironment¹²⁹. External factors have been described to contribute by selective pressure to adopt a Warburg phenotype, specifically in stressful microenvironments. This is the case of hypoxia, an external factor that accentuates the oncogenic-wired reliance on glycolysis¹³⁵. In tumor hypoxic core, poor-oxygenated levels trigger a cellular response system via the hypoxia inducible factors (HIFs).

HIFs ensue transcriptional upregulation of multiple enzymes of the glycolytic pathway, glucose transporters, and metabolic enzymes that oppose to the shunt of pyruvate into oxidative phosphorylation^{136,137}. The intimate relationship between oxygen availability and glucose metabolism has led to the hypothesis that tumors function as a tissue entity that acts symbiotically in terms of energetic metabolism: with an outer well-oxygenated area where oxidative phosphorylation is predominant, and an hypoxic core where anaerobic glycolysis is preferred¹³⁸.

In conclusion, Warburg's observations made substantial contributions to the early understanding of cancer metabolism which, in turn, have become relevant for cancer diagnostics. The fact that a majority of tumors display substantial glucose uptake *in vivo* is the basis of the current methodology used for identification and monitoring of tumors, the positron emission tomography scanning, which measures the inward flux of the glucose analogue [¹⁸F]fluoro-2-deoxyglucose¹³⁹.

1.3.2 PFKFB3 as an oncogenic regulator of glycolysis

As a catabolic process, glycolysis is controlled by metabolites that match the metabolic demands of the cell to the enzymatic activity of the glycolytic enzymes. One of the enzymes subjected to this control is the phosphofructokinase-1 (PFK-1) enzyme, which catalyzes the first committed and principal rate-limiting step of glycolysis: conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6P2) (Figure 6). The enzymatic activity of PFK-1 is tightly controlled by ATP, ADP, F6P and F-2,6-BP. From these, the metabolite F2,6P2 is the most potent allosteric modulator of PFK-1 activity, increasing PFK-1 affinity towards its substrate F6P constituting a crucial control point of the glycolysis¹⁴⁰.

Intracellular levels of F2,6P2 are tightly controlled by a family of homo-dimeric bifunctional proteins with 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) activity consisting of four different isozymes (PFKFB1-4)¹⁴¹ (Figure 6). These isoenzymes display distinct tissue distribution: PFKFB1 is expressed in the liver and skeletal muscle, PFKFB2 in heart and kidney, PFKFB3 ubiquitously expressed, and PFKFB4 mainly expressed in testes¹⁴². Importantly, whilst PFKFB1 and PFKFB2 are found in non-transformed tissues, PFKFB3 and PFKFB4 are inducible isoform expressed in tumor tissues¹⁴³. Thus far, several lines of evidence have demonstrated that PFKFB3 displays a tumorigenic expression in many malignancies, for instance in high grade astrocytoma¹⁴⁴, head and neck squamous cell carcinoma¹⁴⁵, oral squamous cell carcinoma¹⁴⁶, hepatocellular carcinoma¹⁴⁷, ovarian cancer¹⁴⁸, endometrial cancer¹⁴⁹, breast and colorectal cancer^{150,151}. In the clinical scenario PFKFB3 overexpression predicts worse overall survival and prognosis in hepatocellular carcinoma, colon, breast and neuroblastoma patients^{151–154}. In contrast, overexpression of PFKFB4, which also displays higher levels *in vitro* compared to non-transformed cells, seem

to be associated to favorable prognosis on neuroblastoma, non-differentiated glioblastoma and bladder cancer patients^{153,155,156}. Altogether these evidences suggest opposing contributions for PFKFB3 and PFKFB4 isozymes in malignant progression^{153,157,158}.

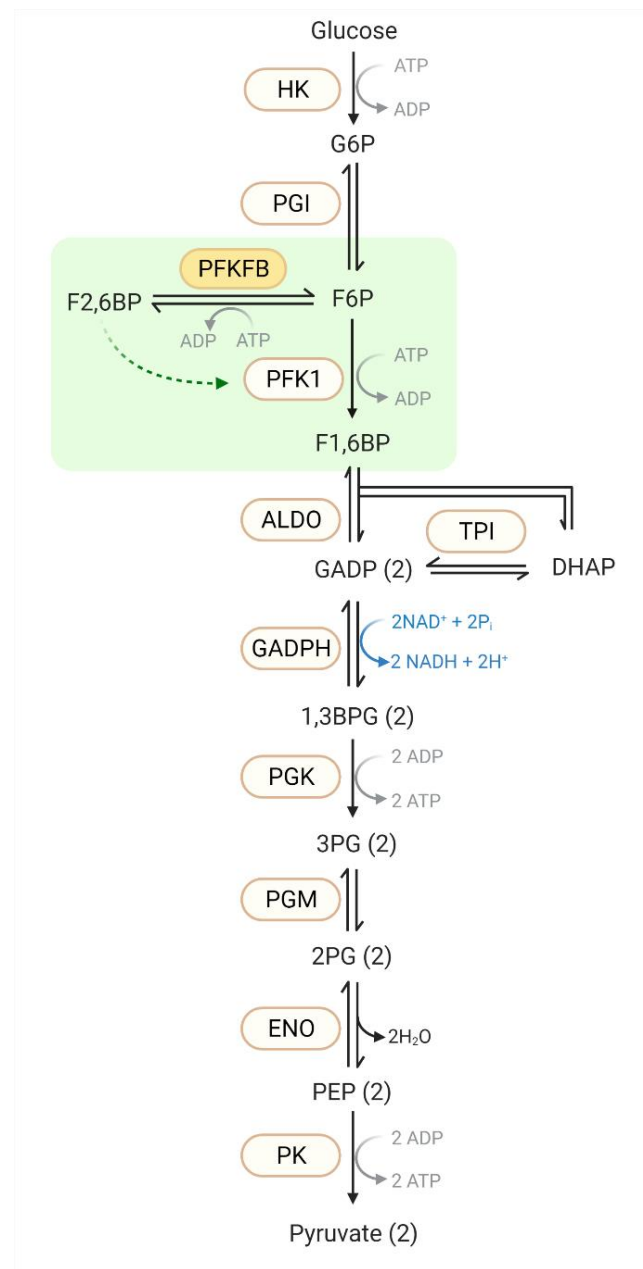


Figure 6. Outline of glycolysis pathway and its regulation by PFKFB enzymes. Glycolysis is the metabolism of glucose into pyruvate by ten sequential steps: five of which constitute the preparatory phase where ATP is spent, and the last five constitute the pay-off phase where ATP is generated. The PFKFB family of enzymes control the glycolytic flux via the production of fructose-2,6-bisphosphate (F-2,6-BP). Abbreviations of the metabolites: glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (F1,6BP), glyceraldehyde 3-phosphate (GADP), dihydroxyacetone phosphate (DHAP), 1,3-bisphosphoglycerate (1,3BPG), 3-phosphoglycerate (3PG), 2-phosphoglycerate (2PG), phosphoenolpyruvate (PEP). Abbreviations of the glycolytic enzymes: hexokinase (HK), phosphoglucose isomerase (PGI), 6-phosphofructo-1-kinase (PFK1), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB), fructose-bisphosphate aldolase (ALDO), triosephosphate isomerase (TPI), glyceraldehyde phosphate dehydrogenase (GADPH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase (ENO), and pyruvate kinase (PK). Image generated with BioRender.com

Besides tissue specificity, the PFKFB family present different enzyme kinetics in relation to the synthesis and degradation of F-2,6-BP. Even though core catalytic domains display a high sequence similarity across PFKFB family members (>85%), regulatory sequence domains at both kinase and phosphatase domains are different, resulting in distinct affinity for the synthesis or degradation of F-2,6-BP¹⁵⁹. Due to a unique β -hairpin structure at the N-terminal that interacts with the phosphatase catalytic domain, PFKFB3 exhibits a distinct conformational rotation that reduces its phosphatase activity¹⁶⁰. As a result of this conformational self-inhibition, PFKFB3 displays a kinase activity greatly predominant over the phosphatase activity (ratio of 700:1), while for the other isozymes the ratio is closer to 1:1. Thereby, PFKFB3 activity derives in higher net production of F2,6P2 compared to other PFKFB family members¹⁶¹.

Oncogenic-wired transformation is tightly associated with the PFKFB3 enzyme. Activation of oncogenic signaling regulates PFKFB3 at post-translational, transcriptional and protein levels. Post-translational modifications by oncogenic signaling directly modulate PFKFB3 enzymatic activity. Kinase-activating phosphorylation at Ser⁴⁶¹ by AKT, protein kinase A (PKA) or AMP-dependent protein kinase (AMPK) increase PFKFB3 kinase-to-phosphatase ratio further to >3000^{162–164}. This post-translational modification has been reported in several cancers including colon, breast and ovarian cancer tissues^{148,152}. Carried out by the oncogene proviral insertion in murine lymphomas 2 (PIM2), the activating Ser⁴⁷⁸ phosphorylation has been recently characterized to promote PFKFB3 kinase activity in breast cancer cells¹⁶⁵. Furthermore, phosphorylation at Tyr¹⁹⁴ by c-Src oncogene has been found in colon adenocarcinomas resulting in increased PFKFB3-mediated glycolysis *in vivo*¹⁶⁶, yet in which extend this post translational modification affects PFKFB3 kinase activity has not been addressed. With regards to transcriptional regulation, PFKFB3 is a transcriptional target of several oncogenic factors including the Ras signaling pathway^{167,168}, and hormonal factors such as progestin¹⁶⁹ and estradiol¹⁷⁰. Activation of PFKFB3 transcription occurs as a response to P53 loss in tumor cells¹⁷¹ and to hypoxic conditions via HIF-1 α stabilization^{163,172}. Janus kinase 2 (JAK2) oncogenic transformation in leukemia cells induces expression of PFKFB3 at both transcriptional and protein levels, suggesting a link between oncogenic tyrosine kinase activity and regulation of PFKFB3¹⁷³. In relation to PFKFB3 protein levels, loss-of-function of *PTEN* compromises the E3 ubiquitin ligase APC/C-Cdh1-mediated protein degradation of PFKFB3 protein¹⁷⁴.

Taken together, PFKFB3 characteristics highlight the key metabolic role of this enzyme in regulating glycolysis in order to provide anabolic precursors and support cell proliferation of transformed cells. Indeed, blocking PFKFB3 *in vitro* in cancer cells using siRNAs results in cell cycle arrest, suppression of cell growth and apoptotic cell death^{175–178}. Aside from experimental data indicating that there is a consensus regarding PFKFB3's supporting role in cancer cell

survival, research carried out in astrocytoma brain cancer cells points in another direction. In this case, PFKFB3 splice variant UBI2K4 did not exhibit a cancer-specific pattern of expression relative to non-transformed brain tissues and its overexpression impeded cell survival¹⁷⁹. Therefore, PFKFB3 function in cancer may be dependent of splice variant, tissue of origin and oncogenic stimuli.

1.3.3 The emerging role of PFKFB3 beyond glycolysis

Unlike the other isoforms which predominantly localize in the cytoplasm where glycolysis takes place, PFKFB3 contains a highly conserved nuclear localization sequence (NLS) in its C-terminal domain. This lysine-rich sequence is required for the import of the protein into the cell nucleus via importin α ⁵¹⁸⁰. It has been reported that acetylation in Lys⁴⁷² impairs NLS recognition, which hinders PFKFB3 nuclear re-localization and leads to its accumulation in the cytoplasm, highlighting the functional significance of this post-translational modification to dictate PFKFB3 cellular localization. This unique feature of the member 3 of the PFKFB family has been puzzling for many years as glycolysis is a metabolic process that takes place in the cytoplasm. Which would then be the nature of PFKFB3 nuclear localization?

The first study that delved into PFKFB3-mediated role in the nucleus was performed by Atsumi *et al.* in 2009, where it was shown that PFKFB3 has the capacity to drive cancer cell proliferation exclusively from its nuclear localization without affecting intracellular glycolysis to any perceptible extent, suggesting nuclear functions beyond glycolysis¹⁸¹. Later on, in purified nuclear extracts, F2,6P2 was reported to activate cyclin-dependent kinases (CDKs) which trigger the degradation of the G1/S cell-cycle checkpoint repressor p27¹⁷⁵. In line with a potential function in controlling cell cycle progression, PFKFB3 interacts with CDK4 that controls the G1/S transition¹⁸².

More recent investigation has revealed the involvement of PFKFB3 in response to cisplatin in cervical cancer cells, as well as in the P53-mediated response to UV damage in primary mouse embryonic fibroblasts^{180,183}. These studies, however, focused on the cytoplasmic role of PFKFB3 in promoting glycolysis as a protective mechanism to these genotoxic agents, yet leaving the nuclear DDR mechanisms unexplored. Additionally, PFKFB3 activity has been linked to platinum tolerance in endometrial cancer cells, which display enhanced phosphorylated PFKFB3(Ser⁴⁶¹) levels compared to sensitive cells¹⁴⁹.

1.3.4 Therapeutic potential of PFKFB3 inhibitors in cancer

Given the tight control over glycolytic flux by F2,6P2, the importance of PFKFB3-mediated glycolysis in supporting cancer cell survival, and the overexpression of PFKFB3 in diverse cancers; PFKFB3 represents a promising therapeutic target in cancer. This has prompted the investigation and development of selective inhibitors targeting PFKFB3 and thus, to date, several potent and selective small molecule inhibitors have been disclosed¹⁸⁴. This thesis

focuses primarily on those small molecule inhibitors with characterized anti-proliferative effects at least *in vitro*.

The first-in-class PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO) was synthesized to target recombinant PFKFB3 using competitive binding experiments with F6P substrate¹⁸⁵. Given its poor pharmacokinetic profile, selectivity and potency, its evaluation for *in vivo* or clinical studies has not been endorsed¹⁸⁴. So far, no co-crystal structure showing 3-PO binding mode have been published. The 3-PO structural analogue PFK15 was further designed to improve potency and selectivity of the compound towards the PFKFB3 isozyme. Moreover, next analogue PFK158 was developed and molecular docking studies identified its binding mode to the ATP binding pocket¹⁸⁴. PFK158 displays increased binding potency, enhanced proapoptotic activity and better pharmacokinetic properties including reduced clearance and longer half-life¹⁴⁸. As a consequence, PFK158 has become the first-in-class PFKFB3 inhibitor to undergo phase I clinical trials for patients with advanced solid malignancies (NCT02044861).

Although both compounds have been suggested to target PFKFB3, recent reports using biochemical assays have revealed that 3-PO and PFK-158 inhibitory effects were similar across PFKFB isoforms¹⁸⁴. Compelling evidence shows that the glycolytic inhibitory effects of 3-PO compound and its analogue are not consequence of PFKFB3 enzymatic activity inhibition. In biochemical assays using purified human recombinant PFKFB3 protein extracts, both compounds have been reported to not inhibit kinase activity at concentrations in which lactate production and glucose consumption was affected in cell-based assays from previous literature^{186,187}. In line with these findings, a study revealed that neither 3-PO nor PFK-158 induced changes in the glycolytic flux at concentrations tested that could be ruled out from general cytotoxicity¹⁸⁸. More recently, isothermal titration calorimetry assays to study PFKFB3-ligand binding affinity determined that 3-PO does not even bind to PFKFB3¹⁸⁹. As a result, Veseli *et al.* (2020) suggested that *in vivo* effects mediated by these compounds could not be consequence of direct PFKFB3 enzymatic activity, but instead a non-specific inhibition of enzymatic reactions of the glycolysis pathway due to intracellular accumulation of lactic acid¹⁸⁹. Nonetheless, both 3PO and PFK-158 small molecule inhibitors have been used extensively in literature in order to evaluate PFKFB3 therapeutic targeting in disease models, as well as to interrogate its biological function in mechanistic studies.

More recently, high throughput screening of the AstraZeneca compound collection led to the identification of AZ26, which targets as well the ATP binding pocket in the kinase domain¹⁸⁷. This small molecule inhibitor has micromolar potency in A549 cells in biochemical assay with regards to reduction on F2,6BP levels, inhibition of PFKFB3 kinase activity, and decrease lactate secretion. AZ67 treatment in neuron cells has been proven cytoprotective against glutamate-mediated excitotoxicity by limiting glycolytic metabolic rewiring, oxidative

damage and mitochondrial stress¹⁸⁶. In line with these observations, delivery of AZ26 alleviated neurological impairment and damage in a brain ischemia and reperfusion murine model. Nevertheless, no reports so far have investigated target engagement across PFKFB isozymes and evaluated its antiproliferative effects in cancer cells.

1.4 CX₃CR1: A KEY PLAYER IN CANCER TRANSFORMATION

1.4.1 Chemokine network

Although being initially characterized as chemoattractants that trigger recruitment of immune cells to inflamed tissues, chemokines are nowadays known to be involved in a myriad of biological processes ranging from embryogenesis to tissue homeostasis. Furthermore, being the inflammatory state of tumors an enabling hallmark of cancer⁷, this family of intercellular signaling proteins and their associated receptors display fundamental roles in promoting tumor growth and metastatic dissemination^{190,191}.

To date, the chemokine network is characterized by 50 different chemokines, small signaling peptides, and 19 chemokine receptors, which are integral plasma membrane G protein-coupled receptors (GPCRs). The chemokine system is promiscuous meaning that is not restricted to single chemokine-receptor interactions and thus, a single chemokine receptor can be stimulated and activated by several chemokines. Additionally, due to the nature of GPCR signal transduction, the chemokine signaling is pleiotropic and triggers multifaceted cellular responses including increased cellular motility, invasion and proliferation¹⁹².

1.4.2 The relevance of CX₃CL1-CX₃CR1 axis in cancer

Among all known chemokines, the C-X₃-C chemokine ligand 1 fractalkine (CX₃CL1) differs from the rest because it binds to a sole receptor, the CX₃CR1. Fractalkine is also characterized for being synthesized as a membrane bound chemokine bearing the most external part of the polypeptide bound to mucin-containing stalk. This structure enables membrane bound CX₃CL1 to form aggregates in the cell membrane¹⁹³ which mediate adhesion with CX₃CR1-expressing cells but do not trigger its signal transduction^{194,195}. On the other hand, the transmembrane CX₃CL1 protein is cleaved by metalloproteinases generating a soluble ligand which harbors the chemotactic function by activating CX₃CR1-mediated signal transduction.

The fractalkine ligand was first detected in the central nervous system more than 20 years ago¹⁹⁶, albeit nowadays it is known to be ubiquitously expressed in blood vessels, heart, lung, kidney, intestines and skeletal muscle¹⁹⁷. With regards to the fractalkine receptor, it is found expressed in microglia¹⁹⁸, osteoclasts¹⁹⁹ and malignant epithelial cells²⁰⁰. Proinflammatory cytokines transactivate CX₃CR1 gene expression²⁰¹, as well as HIF1 α in hypoxic conditions²⁰².

The physiological function of the fractalkine axis is the modulation of the inflammation response. Proinflammatory cytokines trigger CX₃CL1 expression in endothelial cells from activated endothelium, which is fundamental for the chemotaxis and migration of CX₃CR1-expressing subpopulations of leukocytes and monocytes to damaged tissue during inflammatory processes²⁰³. Given that inflammation is tightly linked to the etiology of many diseases, the fractalkine axis engages in inflammatory-driven pathological processes. For

instance, fractalkine axis has been shown to mediate the characteristic microglial activation of neurodegenerative conditions such as amyotrophic lateral sclerosis, Alzheimer and Parkinson in *in vivo* murine models^{204,205}.

Cancer is another example where inflammation is a significant component of the pathogenesis of the disease¹⁹⁰. Research illustrates that the fractalkine signaling is involved in tumor progression through various means, including the stimulation of proliferative signaling of cancer cells, as well as the activation of invasion and metastasis²⁰³. Upon binding of soluble CX₃CL1 to the fractalkine receptor, this GPCR undergoes ligand-specific conformational change allowing the activation of a G_{ai}-protein. This event leads to increased extracellular influx of calcium and activation of a plethora of downstream effector signaling pathways such as the extracellular signal-regulated kinases (ERK)1/2 module of the mitogen-activated protein kinases (MAPK) pathway, and the phosphoinositide 3-kinase (PI3K)-Akt signaling axis^{206–209}, both eliciting proliferative and survival signaling. Additionally, CX₃CL1-mediated phosphorylation of Src leads to the activation of FAK kinase in breast cancer cells²¹⁰ which ultimately triggers cell motility and migration response²¹¹. Termination of the receptor signaling occurs by ligand-induced internalization of CX₃CR1 via β -arrestin recruitment and further mediated endocytosis^{212,213}.

Clinical data shows that the role of the fractalkine axis in cancer dissemination is a two-edged sword. From one side, overexpression of CX₃CR1 or CX₃CL1 has been reported in tumor tissue samples across different malignancies¹⁹⁷, and it has been associated with poor clinical prognosis^{214,215,216,217}. Similar to the chemotaxis of CX₃CR1-positive leucocytes to inflamed endothelium, CX₃CR1-expressing tumor cells that have escaped from primary lesions and are found circulating in the blood vessels migrate and adhere to CX₃CL1-rich epithelium of distant organs promoting metastatic dissemination. This was elegantly illustrated by a study performed by Barbolina *et al.* (2012), in which silencing CX₃CR1 in ovarian carcinoma cells robustly declined the adhesion to peritoneal epithelial cells, the main metastatic target of ovarian carcinoma²¹⁸. Migration occurred in a CX₃CL1-dependent manner, as treatment with blocking antibodies that compete with the ligand reduced migration and adhesion of ovarian cancer cells. This underlying mechanism of metastatic colonization in the peritoneum was later corroborated as downregulation of CX₃CR1 in ovarian carcinoma cells resulted in reduction of metastasis across the peritoneum *in vivo*²¹⁹.

On the other hand, studies in colorectal and breast cohorts provided evidence for a positive correlation between high CX₃CL1 levels and better prognosis^{220,221}. Based on the chemotactic role of fractalkine axis in the migration of tumor infiltrating lymphocytes, recruitment of immune cells to tumor sites would ultimately hamper tumor growth. Recent transcriptomic analysis show that the fractalkine axis exhibits a multifaceted role suggested to be different between cancer subtypes. CX₃CL1 overexpression corresponds with longer survival for lung

adenocarcinoma patients, but it results in worse clinical prognosis for lung squamous cell carcinoma patients²²².

Besides these differences, CX₃CR1-CX₃CL1 signaling has been consistently involved in pathogenesis and metastatic progression of EOC^{200,202,207,216,223}. Whilst CX₃CR1 expression in normal ovarian surface epithelial cells is neglectable, the receptor is found overexpressed in primary and metastatic EOC tumor samples²⁰⁰ and its expression is correlated with disease progression and poor survival, in particular for post-menopausal late-stage diagnosed EOC patients²¹⁶. The fractalkine ligand, instead, is expressed in peritoneal mesothelium²¹⁸, surface epithelium of healthy ovaries and fallopian tubes, and in benign, borderline and malignant EOC tumors²¹⁶. CX₃CL1 levels are correlated with the proliferation index of malignant EOC cells and with the expression factors that stimulate proliferation of ovarian cancer cells²⁰⁷.

1.4.3 Non-canonical roles of fractalkine signaling

Recent transcriptomic data reflect the relevance of CX₃CR1 expression in predicting survival of patients treated with DNA damaging therapies²²⁴. The study by Xie *et al.* (2018) revealed that increased CX₃CR1 levels tightly correlate with shorter overall and progression-free survivals in EOC patients who underwent platinum, gemcitabine, and topotecan therapies²²⁴. Therefore, pointing towards an emerging role of fractalkine signaling in regulating DDR that is significant for tumor burden. In line with this observation, only 10% of CX₃CR1-expressing leucocytes were reported to migrate to fractalkine soluble ligand, suggesting a CX₃CR1 role beyond chemotaxis and adhesion¹⁹⁴.

Suppression of CX₃CR1 expression sensitizes EOC cells to platinum therapy²²⁴. Similarly, CX₃CR1 knockdown synergizes with IR and results to increased levels of IR-induced DSBs and lower clonogenic survival²²⁴. In this case, ablation of the fractalkine receptor impairs DNA repair of DSBs by inhibition of ATM and DNA-PK phosphorylation, as well as CHK1/2 phosphorylation. These effects were attributed to diminished protein levels of RAD50, a component of the upstream MRN complex. Of note, CX₃CR1 gene ablation was performed employing small interfering RNA treatment for 72h, leaving room for off-targeted effects due to considerable long-time suppression of fractalkine signal transduction²²⁵. Although these results reveal a potential requirement of CX₃CR1 protein in DDR, they leave unexplored the functional implications of modulating the fractalkine signaling by pharmacological inhibition.

1.4.4 Fractalkine as a target for therapeutic intervention

Given the comprehensive functional repertoire of CX₃CR1-CX₃CL1 axis, coupled to the successful history of targeting GPCRs in drug development, several attempts have been performed to therapeutically target the fractalkine signaling. To date there are two therapeutic approaches targeting the fractalkine axis that are under clinical development. The first, a humanized monoclonal antibody anti-CX₃CL1 developed by Eisai Pharmaceuticals²²⁶,

Quetmolimab, has a neutralizing effect on the receptor-ligand interaction blocking the signal transduction. Because fractalkine signaling has been involved in the pathogenesis of various chronic inflammatory diseases such as rheumatoid arthritis, this antibody is currently being evaluated in phase II clinical trial for the treatment of rheumatoid arthritis patients (NCT02960438)²²⁷.

The second therapeutic option consists of a small molecule inhibitor targeting CX₃CR1, KAND567 (previously AZD8797), issued by Kancera AB. KAND567 is a non-competitive, allosteric antagonist that decreases bound CX₃CL1 binding affinity to the receptor²¹². When CX₃CL1 is bound to the receptor, the addition of KAND567 inhibitor results in two different outputs by means of signal transduction: at low concentrations KAND567 interaction potentiates β -arrestin recruitment suggesting a potential receptor internalization, and at high concentrations KAND567 completely abolishes CX₃CL1 binding to the receptor and blocks β -arrestin recruitment. KAND567 has become the first-in-class and first-in-human CX₃CR1 small molecule inhibitor to be evaluated ever in clinical trials²²⁸. KAND567 is nowadays under phase II for myocardial infarction indication and phase II for SARS-CoV-2 acute respiratory disease (both indications under EudraCT: 2020-002322-85 trial)²²⁹. Hence, targeting CX₃CR1-CX₃CL1 signaling by KAND567 constitutes a promising strategy for treatment of acute inflammatory diseases.

2 DOCTORAL THESIS

2.1 AIMS OF THE THESIS

The overall purpose of this thesis was to investigate the uncanonical functions of two cancer targets, PFKFB3 and CX₃CR1, in the DNA damage response. The specific aims for each project were:

Paper I: To elucidate the function of PFKFB3 in DSB repair and develop and validate a potent inhibitor for therapeutically targeting PFKFB3.

Paper II: To characterize the molecular mechanism of PFKFB3 in the DNA damage response upon treatment with platinum compounds.

Paper III: To explore CX₃CR1 as a putative target for cancer by interrogating its involvement in DNA repair upon platinum sensitization.

Paper IV: To examine the contribution of CX₃CR1 in proliferation of epithelial ovarian cancer cells.

2.2 METHODOLOGICAL CONSIDERATIONS

2.2.1 Development of KAN0438757

The contradictory data across literature in relation to the 3-PO small molecule inhibitor and its structural analogues^{186–189,230}, together with our own preliminary results indicating that 3-PO does not modulate F2,6P2 product formation in cell-based assays; prompted the development of PFKFB inhibitors that selectively target the isozyme 3. In collaboration with the pharmaceutical company Kancera AB, development of potent small molecule inhibitors for PFKFB3 was performed to further interrogate its biological functions in cancer cells.

Methodologies used for drug development were multidisciplinary and ranged from physicochemical assays, *in vitro* biochemical assays to cell-based assays. The main PFKFB3 inhibition biochemical assay was a kinase luminescent assay (ADP-Glo™ Kinase Assay from Promega). The principle of this method is to monitor ADP left at the reaction mix after incubation of human recombinant PFKFB3 with its substrates ATP and F6B, and the inhibitor probe tested. The kinase reaction is terminated and remaining ATP is depleted. Then, firefly luciferase enzyme is added to the system simultaneously with a reagent that converts ADP to ATP. Luciferase uses ATP to transform a substrate into a product called luciferin generating light, which can be quantified using a luminescent detection system. The amount of remaining ADP left after PFKFB3 enzymatic reaction inversely correlates to the kinase activity inhibition of tested probes. This simple and fast system is routinely used in drug discovery to measure inhibitory effect of small molecules in primary screenings²³¹.

An orthogonal assay used to assess PFKFB3 biochemical activity was the so-called van Schaftingen assay²³². This method is based on the pyrophosphate-dependent phosphofructokinase-1 (PPi-PFK1) from potato tubers. This enzyme is allosterically activated by F-2,6-BP, the PFKFB3's kinase activity product. There is a linear correlation between levels of F-2,6-BP and PPi-PFK1 activity, which can be used to extrapolate the extend of PFKFB3 inhibition. Adding a series of enzymes to the reaction results in the consumption of NADH to generate NAD, which can be quantified spectrophotometrically to indirectly determine levels of F-2,6-BP in the reaction mix. After cell lysis and normalization to equal total protein amount, the van Schaftingen assay was employed in cell lysates to quantify intracellular F-2,6-BP levels. The main advantage of this cell-based assay is that by laying so close to the target, it offers the possibility to assess target engagement in relation to biochemical activity.

The drug discovery approach consisted of a high-throughput screening of 50000 compounds using a luminescence-based kinase activity assay with recombinant human PFKFB3. Series of compounds with μMolar IC_{50} were selected to test for competition binding with ATP in a ligand binding assay. Since ATP-binding pockets are conserved across kinases, the aim was to select those non-ATP competitive molecules that could rule out further cross-reactivity with

kinases. The lead compound KAN0438241 (Figure 7a) showed great selectivity towards PFKFB3 with minimal inhibitory effect in PFKB1, 2 and 4 in biochemical kinase assay. Isothermal titration calorimetry method was performed to ascertain KAN0438241 binding to PFKFB3 and co-crystal structure was solved revealing binding to the F6P substrate pocket of the kinase protein domain (Figure 7b). However, physicochemical properties like cell permeability limited biological evaluation of this compound. In order to improve KAN0438241 cell permeability, an ester version was designed termed KAN0438757 (Figure 6a). This compound is a pro-drug, meaning that it relies on the hydrolysis from physiological esterases to cleave the ester group and generate the active compound. Importantly, on-target effect of these PFKFB3 inhibitors was validated with the van Schaftingen assay in cell extracts to assess F-2,6-BP intracellular levels. The pro-drug showed greater enzymatic inhibition in relation to F-2,6-BP levels in cells, whereas the active compound displayed better potency on kinase enzymatic assays. Viability assays across a panel of cell lines determined μ Molar potency of KAN0438757 with cell dismiss as a readout, and cellular thermal shift assay (CETSA) was used to confirm KAN0438757 target engagement with respect to drug binding.

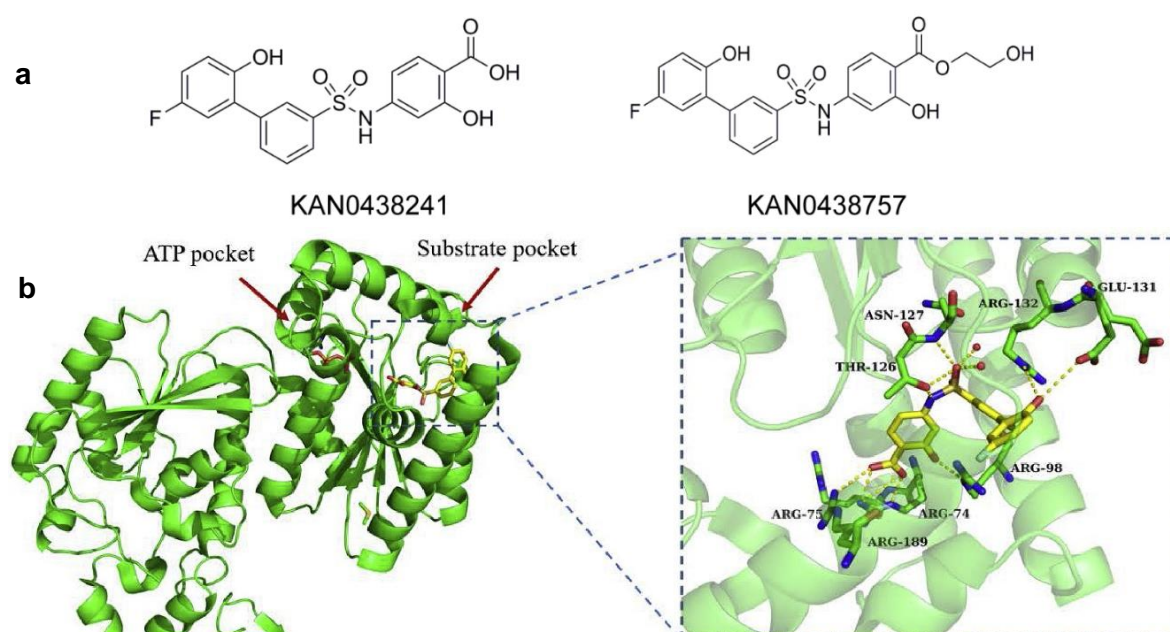


Figure 7. PFKFB3 small molecule inhibitors. (a) Chemical structure of KAN0438241 active compound and KAN0438757 pro-drug. (b) Co-crystal structure of PFKFB3-KAN04438241 complex resolved (PDB: 6ETJ) displaying its binding mode to the substrate pocket located on the kinase domain. Republished with permission from Elsevier, from Wang, Y. *et al.*(2020)¹⁸⁴

Altogether, drug discovery funnel led to the validation of KAN0438757 as a potent and cell-active small molecule inhibitor of PFKFB3. Unlike previously described PFKFB3 inhibitors 3-PO, PFK-158 and AZ26 targeting the ATP binding pocket, KAN compounds bind to F6P substrate pocket. This binding module reduces the probability of off-targeted effects, as has been seen when probing KAN0438757 to a broad panel of 96 kinases. Recent studies characterized specific hydrogen-bonding interactions in F6P binding pocket that drive potency as well as selectivity of KAN compounds towards PFKFB3 (Figure 7b)¹⁸⁴.

2.2.2 Immunofluorescence confocal microscopy

Immunofluorescence confocal microscopy has been the method of choice to examine the assembly of DDR factors at DNA damage sites. This subcellular proteomic technique allowed us to monitor PFKFB3 subcellular relocation, abundance and distribution in the nucleus following DNA damage induction²³³.

In confocal microscopy, a pinhole located in front of the detector blocks out-of-focus emitted light from fluorescently labeled molecules outside the focal plane, which allows good optical resolution in relation to sample depth generating thin optical sections of the sample (approximately of 1µm). These features provide the opportunity to detect with great precision the distribution and colocalization of fluorescent molecules, as well as to quantify signal intensities. Because much of the fluorescent signal is blocked at the pinhole, acquired signal intensities in confocal microscopy are normally dim compared to widefield microscopy. Parameters like depth penetration of the sample, resolution and contrast are considered assets of confocal microscopy technique, yet the amount of time that requires acquisition is a shortcoming²³⁴.

Sample preparation was optimized empirically to generate optimal imaging. Because DDR proteins are recruited to chromatin structures after induction of DNA damage, we performed in situ cell fractionation using cytoskeleton buffer to remove soluble proteins and loosely held nuclear proteins. Sample fixation was performed employing the cross-linking agent formaldehyde, which generates crosslinks in polypeptides and thus preserves nuclear morphology. The incubation time with formaldehyde was optimized to avoid extreme loss of target antigenicity. Sometimes, additional fixation step by precipitation was performed using a mixture of organic solvents methanol and acetone in order to expose epitopes. Cells were permeabilized to promote intranuclear penetration of primary and secondary antibodies.

For immunostaining, coverslips were first blocked and then incubated with the primary antibody overnight at 4°C to improve specificity of the staining. Constituting one of the drawbacks of immunofluorescence, antibody specificity was carefully assessed during the studies. We performed siRNA-mediated knockdown of target proteins to ensure that primary antibodies that were not validated previously in literature recognized proteins of interest. Secondary antibodies with conjugated fluorochromes against the primary antibody epitope were used. Immunofluorescence provides the opportunity to use combinations of fluorochromes to assess more than one protein in each staining which, in turn, allows the examination of protein-protein colocalizations. However, staining combinations were optimized to limit as much as possible the overlap of excitation-emission spectra between fluorochromes because it can be misleading to determine colocalization. Furthermore, cross-reactivity was assessed to ensure that secondary antibodies did not bind to unintended proteins in the fixed samples leading to staining artifacts. Since we were interested in

examining nuclear proteins, we used DAPI as a counterstaining. DAPI is a dye which intercalates between DNA strands. Mounting was performed in order to retain fluorescent signal and avoid fading of intensity. Image acquisition was performed with Zeiss LSM 780 microscope using 63x or 40x oil immersion lens.

Upon IR exposure, DSB repair proteins re-distribute to sub-nuclear structures called foci, which can be recognized by immunofluorescence microscopy as bright speckles all over the nucleus (IR-induced foci, IRIF)^{235,236}. Otherwise, in unperturbed conditions, DDR proteins are microscopically visualized as dim nuclear staining. CellProfiler 3.0 software was used for image-based analysis. We generated pipelines containing a series of processing modules that allowed nuclear segmentation, IRIF identification and quantification from input images²³⁷. In **paper I**, this quantitative automated analysis provided information about the recruitment kinetics of PFKFB3 and HR proteins to IRIF marked by γ H2AX foci. Besides being considered as a surrogate marker of DNA damage, γ H2AX is an essential factor for the generation of IRIF since it alters the chromatin structure surrounding DSBs and promotes the assembly of DNA repair factors colocalizing in the foci²³⁸. Indeed, knockout mice lacking H2AX are hypersensitized to radiation because their cells are unable to form IRIF and recruit HR downstream factors such as 53BP1, Nbs1 and BRCA1²³⁹. For this reason, to assess PFKFB3 assembly to IRIF at DSB sites, we evaluated its colocalization with γ H2AX.

In **paper II** and **paper III**, immunofluorescence confocal microscopy was employed for the analysis of FA pathway activation upon crosslinking agents. In this case, the induction of foci indicated recruitment of DDR proteins to stalled forks and FANCD2 foci formation was used as a *bona fide* marker of FA activation⁸². If ID2 complex is recruited to ICL sites or not, this can be microscopically visualized as a diffuse and dim FANCD2 nuclear staining (Figure 8a-b). Instead, FANCD2 monoubiquitination by FA core complex is strictly required for the formation of distinct FANCD2 foci (Figure 8c)²⁴⁰. This post-translational modification results in a conformational change of ID2 complex structure making it an anchor point for the recruitment of FA downstream factors, and an essential factor for FA pathway activation²⁴¹. To distinguish between soluble and chromatin loaded ID2 complex, subcellular fractionations were performed to isolate within the nuclear compartment the chromatin-bound proteins. In turn, this enrichment of chromatin-bound factors enabled the evaluation of FANCD2 or FANCI ubiquitylation. In conclusion, we combined two subcellular proteomic techniques²³³, imaged-based analysis of FANCD2 foci and subcellular fractionations, to examine the status of FA pathway activation upon treatment with crosslinking agents (Figure 8a-c).

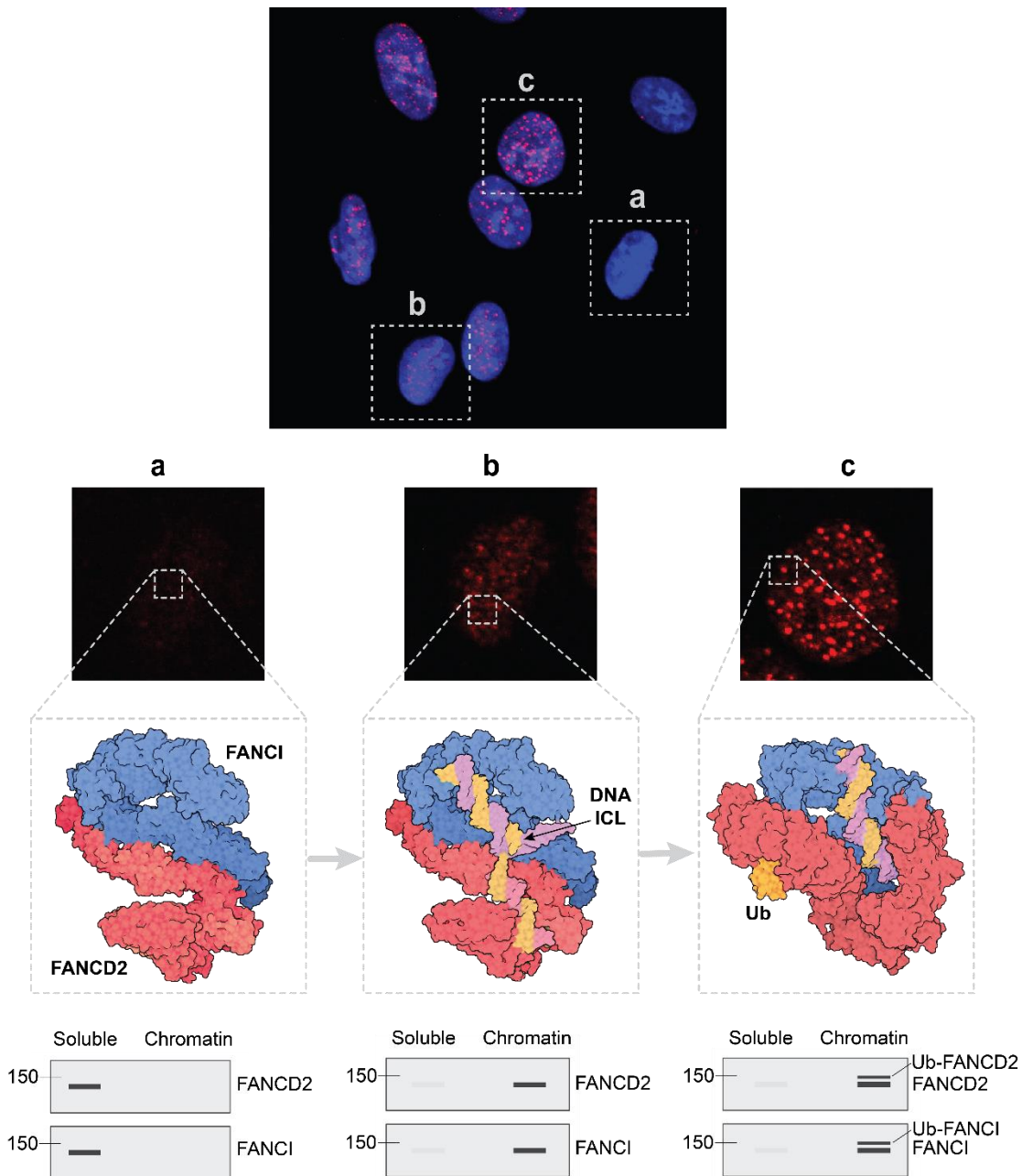


Figure 8. Assessment of FA pathway activation by FANCD2 foci formation and chromatin fractionations. DAPI: blue, FANCD2: red. **(a)** Soluble inactive ID2 complex (PDB: 6VAD) appears as dim FANCD2 nuclear staining under the microscope and it is not retained to chromatin upon fractionation experiments. **(b)** ID2 complex recruited to ICL-induced stalled forks (PDB: 6VAA) exhibits diffuse FANCD2 staining under the microscope but enriched FANCD2 and FANCI chromatin fractions. **(c)** Activated ID2 complex requires FANCD2 mono-ubiquitination (PDB: 6VAE) and can be identified microscopically by bright FANCD2 foci. Chromatin fractions of FANCD2 and FANCI proteins show a shift in the bands due to the ubiquitin tag. The ID2 clamp-like structure promotes formation of ID2 filaments alongside DNA for the stabilization of stalled forks and functions as a recruitment platform for FA factors. Protein-DNA assemblies are available at PDB repository and were originally solved by Wang , R. *et al.* (2020)²⁴¹.

2.2.3 DNA fiber assay

Dynamics of DNA replication in cells can be monitored and quantified with DNA fiber technique²⁴². This assay is based on the incorporation of halogenated nucleoside analogues IdU (5-Iodo-2'-deoxyuridine) and CldU (5-Chloro-2'-deoxyuridine) into nascent DNA strands by the replication machinery. Immunostaining of these analogs enables the visualization of newly

synthesized strands and further imaged-based measurement of track lengths allows assessment of challenged replication fork progression and its directionality.

In this thesis we used DNA fiber spreading technique (Figure 9a). Briefly, cells in culture were sequentially labeled with pre-warmed media containing CldU and IdU for corresponding amount of time (see individual papers). Of note, the concentration of the second analogue was at least 10-fold higher than the first one to ensure that resulting fibers exhibit two clear sections on the labeled new strands. Thus, the IdU analogue was found in excess to displace remaining CldU that was removed through washings steps. After DNA labeling, cells were lysed in spreading buffer and carefully loaded into positively coated lanes of Ibidi slides (ibiTreat μ -slide VI 0.4). Slides were tilted 15° degrees to allow spreading of DNA fibers. Slow spreading of the cell suspension ensured that fibers would not cluster making the analysis step more difficult. DNA spreads were air dried and fixation was performed overnight at 4 °C with a mixture solution of methanol and acetic acid. DNA denaturation was carried out with hydrochloric acid and further washes of PBS to restore pH and limit further fiber acidic degradation. Once blocking was done, immunofluorescent DNA staining was performed with antibodies recognizing CldU (anti-rat) and IdU (anti-mouse). After washing with PBS and fixation with paraformaldehyde, secondary staining was done using antibodies raised against rat or mouse and conjugated with different fluorochromes. Last, addition of mounting media allowed retention of signal from immunostained DNA strands. Images were captured either with confocal Zeiss LSM 780 microscope or NikonTi2 fluorescence microscope.

Approximately 150 forks that did not overlap were measured per condition using the ImageJ software. Data analysis was based on the determination of DNA fiber length (μ m) of unidirectional forks. Track length divided by labeling time was converted to replication fork speed using the conversion factor of 2.59 kilo base pairs/ μ m of nascent DNA, originally described by Jackson, *et al.* (1998)²⁴³.

Different labeling regimes allow for diverse applications of DNA fiber technique to assess specific replication parameters²⁴⁴. In **paper I** we wanted to assess unperturbed replication and how PFKFB3 inhibition could modulate global replication dynamics. In **paper III**, we aimed to assess global defects in replication fork progression by CX₃CR1 inhibition after induction of replication stress. For this reason, in both cases we performed dual-labeling regime with CldU and subsequent IdU after corresponding treatments (Figure 9b). Measurement of CldU and IdU track lengths post treatment provides an indication of how replication dynamics is perturbed by drugs tested, and if there is replication recovery (CldU speed < IdU speed). Instead, in **paper II**, we interrogated whether replication restart was affected upon induction of DNA crosslinks. To this end, we carried out a first pulse of CldU combined with a potent DNA crosslinker Mitomycin C, followed by washout of drugs, and a second pulse of IdU (Figure 9c). With this setup, replication forks that are able to resume DNA synthesis and

bypass crosslinks provide strands containing both CldU and IdU tracks. On those strands, measurement of CldU length is informative of replication fork stalling, whereas IdU length describes dynamics of replication restart of recovered forks. Fraction of tracks only labeled with CldU correspond to terminated forks due to stalling, and single-labeled IdU tracks correlate with new origins fired after drug washout.

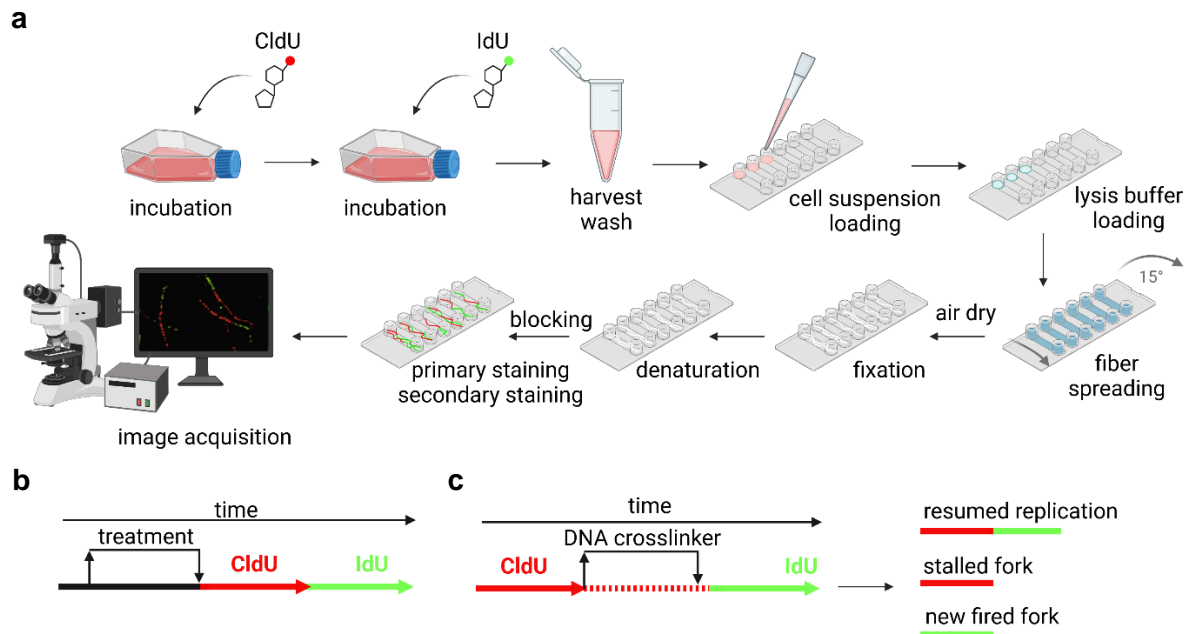


Figure 9. DNA fiber assay. (a) Schematic of assay's methodology. **(b)** Labeling regime to assess challenged replication fork progression after replication stress upon crosslinking agents. **(c)** Labeling regime to analyze recovery of DNA replication after replicative stress. This protocol enables the differentiation of strands derived from restarted forks, newly fired origins, stalled replication forks. Image generated with BioRender.com

2.2.4 Ethical considerations

In this thesis we include *ex vivo* experiments with tumor material derived from HGSOC patients. Written informed consent was obtained from all participants before being included in the study. Obtained tumor-derived cells were used to validate the mechanistic findings in relation to FA pathway activation in **paper II** and provided insights for the proof of concept. In **paper III**, patient cells were employed for translational evaluation of the therapeutic potential of targeting CX3CR1 with KAND567 in epithelial ovarian cancer patients. The studies were conducted in compliance with the ethical requirements from Helsinki II Declaration and approved by the Swedish Ethical Review Authority as described in the materials and methods of corresponding papers.

2.3 RESULTS AND DISCUSSION

2.3.1 Paper I

Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination

High *PFKFB3* mRNA levels have been correlated with clinical radiotherapy resistance in Acute lymphoblastic leukemia patients²⁴⁵ and IR sensitization has been linked to enhanced HR repair capacity in this type of malignancy²⁴⁶. These observations, together with the identification of PFKFB3 as a putative DDR factor in genome-wide siRNA screens^{247,248}, prompted the initial investigation of PFKFB3 role in repair of DSBs.

IR exposure triggered a relocation of PFKFB3 to discernible nuclear foci that colocalized with γ H2AX in IR-induced foci (IRIF) in an ATM-dependent manner. PFKFB3 IRIF appeared with fast kinetics, already at 30min, and in an IR dose-dependent manner suggesting a correlation with the magnitude of DNA damage generated²⁴⁹. Similar recruitment was observed in Ras-transformed fibroblasts, and corroborated that this was not a mere cell line dependent effect. Ablation of PFKFB3 by siRNA impaired IRIF of the HR factors RPA, RAD51 and BRCA1, but not 53BP1 and γ H2AX. This, together with that both γ H2AX and MDC1 transient knockdown abolished PFKFB3 recruitment at DSBs sites, led us to the hypothesis that PFKFB3 operates at the end 3' resection step after DSB sensing. Altogether, the data pointed towards an upstream role of PFKFB3 in the DDR response upon IR.

We next interrogated whether the defective recruitment of HR downstream factors by PFKFB3 siRNA transfections resulted in functional effects for repair and survival. Employing Direct Repeat Green Fluorescent (DR-GFP) assay²⁵⁰ we showed that HR activity was significantly reduced by PFKFB3 knockdown in similar extent as knockdown of the essential recombination mediator RAD51. The decrease in HR capacity was not due to skewed S/G2M cell cycle population, and ultimately resulted in increased residual γ H2AX levels and decreased clonogenic survival.

We further developed the PFKFB3 inhibitor KAN0438757 (see section 2.2.1). Using the KAN0438757 inhibitor in combination with IR, we ascertained that PFKFB3 catalytic activity was required for RPA and RAD51 foci formation, proficient HR activity, recovery from IR-induced cell cycle arrest and long-term cell survival. The impaired recruitment upon PFKFB3 inhibition of recombinational factors that operate post 5' end resection step, RPA and RAD51; together with no change of foci formation in MRN complex proteins, Nbs1 and MRE11; provided evidence that PFKFB3 operates downstream of MDC1. Notably, no differences in total protein levels could explain such differences in chromatin recruitment. In accordance with PFKFB3 being induced by oncogenic transformation¹⁶⁸, sensitivity to IR occurred in cancer

cells and Ras-transformed fibroblasts, while sparing non-transformed cells. Whilst combination of KAN0438757 and IR displayed a great therapeutic window, combination between IR and DDR inhibitors KU55933 (ATM inhibition) and VE-821 (ATR inhibitor) turned out equally cytotoxic to non-transformed as transformed cells. These results, although preliminary, suggest a successful combination therapy that could improve the effectiveness of radiotherapy for patients.

HR-mediated repair requires RAD51-mediated strand invasion and DNA synthesis of complementary strands. Since PFKFB3 kinase activity is a control point for glycolysis and thus modulates the PPP shunt for synthesis of dNTPs, we further investigated the functional consequences of targeting PFKFB3 in HR-dependent repair synthesis. KAN0438757 treatment significantly decreased incorporation of the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) in cells in the G2/M phase post IR. PFKFB3 interacted with and colocalized into IRIF with the RRM2 subunit of the RNR enzyme. RRM2 is known to relocate to the nucleus in response to IR-induced DNA damage to promote local synthesis of dNTPs for DNA repair²⁵¹ and thus, we showed that this role has functional consequences for proficient HR repair. Altogether, we concluded that PFKFB3 supports dNTP incorporation for DSB repair via nuclear recruitment of RRM2, as well as recruitment of HR repair factors RPA and RAD51 operating after end resection step.

In line with the phenotype observed upon DNA repair synthesis, inhibition of PFKFB3 decreased EdU incorporation into the DNA, resulted in shorter DNA fibers, and reduced dNTP intracellular pools. De Oliveira *et al.* (2021) recently observed that among all glycolytic intermediates, KAN0438757 treatment reduces glucose 6-phosphate, which is the metabolite that diverges to PPP to generate precursor molecules of nucleotides¹⁵¹. It is possible, therefore, that decrease of the dNTP pool upon PFKFB3 inhibition is explained by a reduced PPP flux and thus, this effect is due to PFKFB3 cytoplasmic mediated process. Accordingly, the block on DNA replication and proliferation due to PFKFB3 inhibition could be rescued with metabolic supplementation of nucleosides.

Given the role of PFKFB3 nucleoside supply upon replication and DNA repair synthesis, we next delved into a potential function for PFKFB3 in replication stress induced by inhibition of RNR by hydroxyurea (HU). Similar to the IR scenario, PFKFB3 inhibition blocked RPA induction upon HU treatment, suggesting potential lack of ssDNA formation or helicase-polymerase uncoupling at replication forks. PFKFB3 inhibition abolished HU-induced phosphorylation of ATM, ATR, Chk1, H2AX and p53 indicating a situation where replication forks are stalled but no fork collapse or checkpoint activation is triggered.

In conclusion, the major findings of **Paper I** are:

- PFKFB3 is relocated into nuclear IRIF and ATM- γ H2AX-MDC1 pathway stimulates its recruitment to DSBs to establish HR repair.
- Successful development of a novel PFKFB3 small molecule inhibitor KAN0438757.
- PFKFB3 activity coordinates foci recruitment of HR factors downstream the end resection step, as well as the recruitment of RRM2 to support dNTP incorporation at DNA break sites.
- KAN0438757 is a radiosensitizer that renders cancer cells defective in HR repair and DNA repair synthesis upon IR.

2.3.2 Paper II

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

Statistics show that major reductions in EOC mortality have not been yet achieved²⁵². Still nowadays the main prognostic factor for survival is the lack of residual tumor tissue after hysterectomy, highlighting the poor efficacy of adjuvant chemotherapy²⁵³. As a result, platinum resistance is considered incurable in about 75% advanced staged patients. We aimed to apply knowledge from **paper I** to the translational research of gynecologic malignancies with a high unmet medical need. Preliminary findings demonstrated that ovarian cancer cells, including platinum resistant cell lines, are dependent on PFKFB3 enzymatic activity for proliferation (data not shown). The distinct nuclear role of PFKFB3 in HR, together with reported synthetic lethal interactions between platinum compounds and HR deficiency^{103,254}, encouraged us to study PFKFB3 role in the context of platinum treatment.

To this aim, we performed drug combination screenings in viability assays that allowed us to evaluate the synergistic effect of combining KAN0438757 and the platinum-based drugs cisplatin and carboplatin. Using computational aided calculations from the obtained dose-response matrixes²⁵⁵, we identified strong synergies across a panel of EOC cell lines that was cancer-specific, as the combination treatment resulted in additive effect in non-transformed cell lines. Altogether, indicating a promising efficacy to toxicity ratio specific for cancer cells. This could not be achieved with a general glycolysis inhibitor, we tested 2-Deoxy-D-glucose (2DG) in combination with carboplatin which resulted in high synergy scores independently of transformation status. Platinum sensitization in resistant cells was achieved in clonogenic assays upon KAN0438757, and shPFKFB3 knockdown viability experiments validated the observed synergies employing the inhibitor. Altogether, these results corroborate the findings of previous work showing chemosensitization to platinum drugs upon PFKFB3 gene knockdown^{149,256}.

Intrigued by the explicit cancer specific synergies between KAN0438757 and platinum, and by recent research showing that cisplatin promotes glycolysis and thereby proliferation in cervical cancer cells *in vitro*²⁵⁷, we investigated energy metabolism in EOC cells upon platinum exposure to answer whether synergistic effects in cell viability could be due to a general distortion of the glycolysis. Real time analysis of cellular energy metabolism via the measurement of glycolysis (extracellular acidification rate, ECAR) and oxidative phosphorylation (by means of oxygen consumption rates, OCR) displayed no differences upon platinum treatment across a transformation series of immortalized fibroblasts (BJh TERT, SV40 and RAS), yet all showed block of ECAR rates when adding KAN0438757. In line with this data, alterations of glucose levels in the cell media did not affect the sensitivity towards carboplatin in single-agent viability experiments.

Upon assessing PFKFB3 levels in relation to other glycolytic enzymes upstream and downstream of PFKFB3, we surprisingly discovered PFKFB3 to be present in the chromatin fraction. Whereas other glycolytic enzymes were either unchanged or increased on soluble fractions, PFKFB3 chromatin association upon platinum treatment correlated with cancer transformation. Likewise, the extent of chromatin loading of DDR proteins of the FA pathway correlated to transformation status. Altogether, these results suggested that KAN0438757 selective sensitization on cancer cell viability may not be consequence of glycolytic rewiring upon platinum treatments, but to a nuclear-associated mechanism upon ICL-induced DNA damage.

PFKFB3 nuclear relocation and foci formation preceded induction of γ H2AX and RPA foci upon cisplatin and mitomycin C (MMC) exposure. PFKFB3 knockdown cells were sensitized to low dose MMC and HU treatments, known to activate FA repair but not cause massive replication fork collapse⁹³, suggesting a potential role for PFKFB3 in FA repair essential for cancer cell survival. In accordance with FA pathway activation being associated to ICL tolerance and platinum resistance^{258–262}; PFKFB3 chromatin translocation was enriched in platinum-resistant cells compared to sensitive counterparts concomitantly with ICL repair proteins including phosphorylated ATR, the FA factors FANCD2 and FANCI, the fork protector RPA32, the PCNA and its ubiquitinated form PCNAub-164, both required for TLS, and the DNA damage marker γ H2AX. Notably, recruitment of these downstream DDR factors is essential for a successful coordinated repair of ICLs¹¹ and therefore, the enriched recruitment in resistant cells suggests an enhanced DNA damage repair capacity contributing to increased IC₅₀ towards platinum. This, in turn, strengthens the clinical relevance of the used *in vitro* systems to address platinum-resistance in cancer cells^{107,246}. Accordingly, enhanced PFKFB3 chromatin recruitment and faster kinetics into foci were associated with platinum-resistance phenotype and, in turn, suggested a potential role for PFKFB3 in replication associated FA repair.

Since the FA pathway is only active during the S cell cycle phase¹¹, we performed cell synchronization at the G1/S phase border with aphidicolin treatment and release upon crosslinking agents to study the molecular role of PFKFB3 in replicative repair of ICLs. Cells released into cisplatin and MMC displayed increased PFKFB3 foci formation at mid S phase, which correlated with γ H2AX induction. PFKFB3 recruitment was dependent on ATR, FANCM and its own kinase activity. Nevertheless, this recruitment was not dependent on RPA, which apart from its downstream role in HR, engages in ssDNA protection at stalled forks upon FANCM-mediated recruitment to elicit ATR checkpoint signaling²¹. These results indicate that PFKFB3 putative role on replication stress is exclusively dependent on FANCM.

To place PFKFB3 in the replication-coupled repair signaling we investigated recruitment dynamics of FA repair factors upon KAN0438757 cotreatment with ICL inducing agents. To this aim we used chromatin fractionations in cell extracts and in-situ cell fractionation to assess foci formation by confocal microscope. Defective chromatin loading of BLM helicase, its complex partner Topoisomerase III α , RPA and FANCM, FANCD2 and FANCI, PCNA and γ H2AX strongly suggests that PFKFB3 activity is required at the very upstream of the FA pathway. The ID2 complex is required for the unhooking step, DSB generation and its recombinational repair. Thus, the loss of ID2 chromatin association locates PFKFB3 upstream of this nucleolytic incision step indicating an independent role from the HR function described in **paper I**²⁴⁵. Likewise, besides their roles in DNA repair, BRCA1/2 stabilize stalled DNA replication forks and RAD51 mediates fork reversal upon cisplatin treatment^{99,100,263,264}.

Confocal microscopy showed impaired FANCD2 foci formation and γ H2AX foci upon KAN0438757 cotreatment, indicating defective FA activity and impaired DNA damage signaling upon ICLs. Interestingly, co-immunoprecipitation experiments in chromatin fractions revealed interaction between PFKFB3 and BLM, FANCD2 and γ H2AX. Overall, these results point towards a mechanism which nuclear PFKFB3 directly interacts with FANCD2 and BLM at DNA damage sites to trigger assembly into foci and mediate FA repair.

We hypothesized that PFKFB3 might contribute to resolve stalled replication forks by recruiting BLM and FANCD2 to DNA ICL lesions. Hence, we next sought to evaluate the functional consequences of KAN0438757 treatment in relation to fork progression and fork recovery from replication stress induced by ICLs. PFKFB3 inhibition resulted in substantial impairment in DNA replication upon carboplatin treatment that couldn't be rescued upon release. Furthermore, PFKFB3 inhibition upon ICL treatment resulted in increased fork stalling and slowed replication fork restart as assessed in DNA fiber assays, overall indicating a decreased tolerance and compromised fork recovery upon ICL induction. Inhibition of PFKFB3 rendered replicating cells unable to progress through S phase and this cell population accumulated high levels of DNA damage assessed by γ H2AX, probably due to collapsed forks and unresolved

ICLs. Notably, FA patient cells are characterized by a similar phenotype: defective ICL replicative-coupled repair triggers accumulation at late S phase⁷⁶.

Lastly, the formation of FANCD2 foci was used as a surrogate marker of FA repair activity^{240,241} to validate PFKFB3 involvement in FA repair in ovarian cancer patient-derived material. Decreased FANCD2 foci upon KAN0438757 and cisplatin cotreatment was present in those patient cells that were sensitive to ICLs, marked by FANCD2 induction. On the other hand, patient cells irresponsive to cisplatin characterized by no damage induction of FANCD2 foci, showed no impairment of FANCD2 foci formation upon PFKFB3 inhibition, emphasizing the relevance of using experimental systems that reflect tumor characteristics and heterogeneity in clinical response.

From **Paper II** we conclude that:

- Cancer-specific synergy between platinum compounds and PFKFB3 inhibition is not due to rewiring of the glycolysis but due to PFKFB3 chromatin loading upon DNA damage induction through cancer transformation.
- Nuclear recruitment of PFKFB3 is triggered by FA pathway activation and requires FANCM assembly to stalled forks and ATR activity.
- At ICL damage sites, PFKFB3 directly interacts with FANCD2 and BLM to promote replication fork recovery, recruitment of FA factors and ICL repair by the FA pathway.

2.3.3 PFKFB3: General discussion and concluding remarks

2.3.3.1 PFKFB3 inhibition as a targeted therapy in cancer

The findings presented herein position PFKFB3 at the crossroads of oncogenic metabolic reprogramming and DNA repair. Many metabolic enzymes are moonlighting proteins, meaning that they operate in additional mechanisms beyond their canonical catalytical roles²⁶⁵. We provide evidence that PFKFB3 is a moonlighting glycolytic enzyme with regulatory functions in the DDR specifically in cancer cells but not in non-transformed cells. This noncanonical function beyond glycolysis appears associated with PFKFB3 nuclear localization and is significant in the therapeutic context where proficiency of DNA repair mechanisms largely determines efficacy of DNA damaging cancer therapeutics²⁷. Indeed, we have shown that both PFKFB3 inhibition and PFKFB3 knockdown compromise survival to IR and platinum compounds by modulating the DNA damage response of these classical therapeutics, consistent with PFKFB3 being able to drive proliferation without affecting intracellular glycolysis¹⁸¹.

These evidences suggest that employing KAN0438757 small molecule inhibitor may be feasible for therapeutic intervention in oncology, where organ-associated toxicity is the limiting factor for dosage of therapeutics. Notwithstanding, an aspect that remains to be addressed is

the efficacy of KAN0438757 in reducing tumor burden using *in vivo* disease models. *In vivo* studies will provide insights for further KAN0438757 translational developments, not only to assess improved efficacy of combination therapies, but also to ascertain whether a greater therapeutic window can be achieved. So far, a recent study demonstrates that KAN0438757 is well tolerated in mice and does not trigger systemic toxicity, suggesting a favorable safety profile¹⁵¹. In accordance to our results consistently showing a cancer-specific cytotoxic effect when targeting PFKFB3 *in vitro*, KAN0438757 treatment is detrimental for viability and morphology of colorectal carcinoma patient-derived organoids, while preserving normal colon-derived organoids¹⁵¹. In **paper II** we have employed ovarian cancer tumor-derived material to address our mechanistic findings. In this regard, further *ex vivo* viability screening could be done to corroborate the feasibility of PFKFB3 therapeutic targeting in cancer. Additionally, expanding the patient cohort for *ex vivo* studies could, in turn, provide the opportunity for biomarker identification for future patient stratification.

2.3.3.2 Dissecting PFKFB3 glycolytic and moonlighting roles

While genomic instability and reprogramming cellular energetics are both hallmarks of cancer⁷, the interplay between metabolic alterations and DNA repair mechanisms remains poorly characterized²⁶⁶. For example, it has been reported that following a ketogenic diet, characterized by low carbohydrate intake, can promote clinical efficacy of DNA damaging therapies in certain tumor types by tumor starvation^{267,268}. In addition, it has been shown that radiation-induced response entails a transient enhancement of glycolysis in cancer cells, which was reported to directly promote DSB repair by supporting HR and NHEJ pathways²⁶⁹.

With the present body of work, we have dedicated our efforts to dissect PFKFB3 glycolytic role and its noncanonical role in DDR. These two functions are not mutually exclusive per se, and it is plausible that upon cancer transformation they become somehow synergistic to support unrestrained cancer growth. Moreover, each of these roles is not confined to a subcellular compartment, as glycolytic-mediated PFKFB3 control of intracellular dNTP pools in the cytoplasm could have impact on the DNA damage and replication stress response, and it is still unknown whether the formation of F-2,6-BP in the nucleus has functional consequences for DNA repair. Both options could be contemplated as they have been reported for other metabolic enzymes. For instance, enzymatic inhibition of the glycolytic enzyme phosphoglycerate mutase 1 (PGAM1) renders cancer cells defective in HR repair by decreasing intracellular dNTP pools and subsequently triggering p53-mediated CtIP degradation²⁷⁰. Alternatively, the nuclear production of the metabolite fumarate by fumarate hydratase (FH) supports DSB repair via inhibition of histone H3 demethylation, and thus, FH loss has been reported to confer resistance to IR-induced DNA damage^{271,272}.

Two common factors appear to be fundamental for PFKFB3-mediated role in DDR: its catalytic activity and its relocation into clear and distinct nuclear foci colocalizing with DNA damage repair factors. In relation to the first, PFKFB3 is a kinase so it is not unreasonable to hypothesize that it engages in DDR signaling events. The requirement of FANCM phosphorylation by ATR to elicit FA pathway and ATR/CHK1 feedback loop upon DNA damaging conditions^{11,273} is a good illustration of how phosphorylation events are essential to trigger DNA damage response to genotoxic stress. Therefore, a possible participation of PFKFB3 in targeting this factor could explain why KAN0438757 treatment compromises replication fork recovery and impairs ICL repair.

Alternatively, foci colocalization and direct protein interactions seem to be important for the role of PFKFB3 in the DDR as well. So far, we have identified direct binding with RRM2 in the context of IR-induced repair synthesis (**paper I**), and FANCD2, γ H2AX and BLM in chromatin fractions in relation to ICL replicative repair (**paper II**). This is not a unique feature of PFKFB3, as pyruvate kinase 2 (PKM2) is another glycolytic enzyme recently proven to directly interact with the DDR factor CtIP, and by this co-localization support HR repair²⁷⁴. The authors reported that PKM2 ablation leads to defective HR via the loss of CtIP phosphorylation, and allocate this glycolytic enzyme at the 5' end resection step of the HR cascade. With regards to FANCD2, its chromatin loading and assembly at stalled forks requires of γ H2AX-mediated phosphorylation²⁷⁵. It is therefore likely that PFKFB3 interaction with γ H2AX (**paper II**) is required for functional γ H2AX signaling, and thus, PFKFB3 inhibition abolishes such interaction resulting in lack of FANCD2 foci formation. Further studies could shed light to the nature of γ H2AX-PFKFB3 interaction, whether PFKFB3 interacts with γ H2AX via its substrate pocket and consequently, inhibition by KAN0438757 impairs this physical interaction. In relation to BLM, considering that this helicase engages in 5' resection step during HR to initiate long-range incision of DSB ends⁵³, the herein identified BLM-PFKFB3 direct interaction (**paper II**) argues for PFKFB3 intervention at the end resection step via BLM interaction, which could explain loss of downstream RPA and RAD51 recruitment (**paper I**). In this regard, further work should be directed towards the validation of endogenous PFKFB3-BLM protein interaction and its consequences for replication fork recovery upon replication stress and recombinational repair of DSBs upon IR. Orthogonal methods such as proximity ligation assay offer the possibility to confirm protein interactions identified in co-immunoprecipitation experiments²⁷⁶.

In **paper I**, we show that PFKFB3 nuclear localization at IRIF and its role in HR is regulated by ATM kinase activity. Similarly, nuclear relocation of the glycolytic enzyme PKM2 was shown to be ATM-dependent²⁷⁴. PKM2 localization was assessed by means of cell fractionations and fluorescence microscopy, peaking at 30 min post IR like PFKFB3 recruitment kinetics, but no distinct recruitment to IRIF was observed. Based on this and our data, we can infer that ATM controls nuclear relocation of metabolic enzymes with DDR functions. This could then

constitute an additional ATM mechanism to orchestrate DNA damage repair, including the modulation of metabolic routes to provide nucleotides for DNA synthesis repair. Accordingly, Cosentino *et al.* (2011) showed that ATM activates the PPP pathway through direct phosphorylation of the first enzyme of this pathway, glucose-6-phosphate dehydrogenase (G6PD), to promote nucleotide generation for DNA repair synthesis²⁷⁷. Whether PFKFB3 is a target for ATM phosphorylation, like its glycolytic partners PKM2 and G6PD, and whether this putative interaction is required for PFKFB3 role in HR repair, are questions that remain to be addressed.

In conclusion, **paper I** and **paper II** provide new insights on the molecular mechanisms of PFKFB3 in the DNA damage response upon induction of DSBs and DNA crosslinks. Importantly, many questions remain to be answered. For instance, whether the PFKFB3-mediated modulation of DDR is due to alterations of F-2,6-BP product formation in the nucleus, due to phosphorylation events via PFKFB3 kinase activity, or due to PFKFB3 scaffolding functions at DNA damage sites. Future studies employing PFKFB3 constructs with truncated NLS motif, mutations in post-translational modifications residues or enzymatic activity sites, are suitable approaches to further dissect how PFKFB3 subcellular localization and activities contribute to the phenotypes described herein. This, together with metabolic profiling of subcellular fractions, will contribute to a clearer picture of PFKFB3 nuclear versus cytoplasmic functionalities.

2.3.3.3 Understanding the functions of PFKFB3 at the replication fork

Recent studies have challenged the view of that ICL blocks replication forks, instead slowing of replication fork progression by the FA pathway and global fork slowing by ATR has emerged as important events upon replication stress^{278,279}. The fact that PFKFB3 inhibition triggers fork slowdown of both stalled and restarted forks, but not specific for ICL fork stalling during CldU pulse (**paper II**), suggests a possible involvement in global fork slowing; however, an active checkpoint signaling would be expected in this case. The lack of ATR, CHK1 and P53 phosphorylation in PFKFB3 inhibited cells upon HU from **paper I**, together with loss of RPA foci formation and phosphorylated ATR chromatin recruitment in S phase synchronized cells from **paper II**, indicate no elicitation of checkpoint signaling when PFKFB3 is inhibited under high levels of replication stress.

On the other side, the fork stalling phenotype in ICL treated cells upon PFKFB3 inhibition seen in **paper II** could result from abolished interaction with RRM2 reported in **paper I**. RRM2 has been associated not only with DNA synthesis repair upon IR, but also with preventing oncogene-evoked replication stress by fork stabilization. Transgenic mouse carrying extra alleles of *RRM2* gene are protected towards replication stress induced by HU and ATR inhibition treatments²⁸⁰. Notably, inhibition of RRM2 with triapine renders glioblastoma cells

sensitive to PARP inhibitor²⁸¹, which could explain the strong sensitization obtained from the combination between KAN0438757 and PARP inhibitors, but the overall additive effect of KAN0438757 and triapine (data unpublished).

Recruitment of factors that support fork stabilization and protection is essential to maintain genome stability¹⁷. Looking at **paper II**, kinetics of PFKFB3 foci formation precedes recruitment of γ H2AX and RPA, reflecting a specific function of PFKFB3 in replication fork stabilization prior ICL induced DNA damage. Interestingly, a recent study using nascent chromatin capture technique, a method that purifies biotin-labeled DNA from replicating cells and analyzes by quantitative proteomics the replication fork proteome, identified PFKFB3 as an uncharacterized protein with a predicted function in nascent chromatin replication²⁸². Altogether, this combination of findings points towards a function of PFKFB3 in DNA lesion sensing at replication fork sites during DNA synthesis that may be essential to resolve DNA crosslinks that demand replication-coupled repair.

Understanding PFKFB3 molecular function at replication forks would contribute to the assessment of its therapeutic targeting in different contexts of platinum tolerance. Resistance to platinum compounds has been associated with an increased DNA damage repair capacity and resolution of DNA crosslinks²⁸³ as well as glycolytic rewiring of energy metabolism²⁸⁴. Of note, platinum-resistant endometrial cancer cells not only exhibit higher PFKFB3 total levels, but also enhanced phosphorylated PFKFB3(Ser⁴⁶¹)¹⁴⁹, which suggests that PFKFB3 enzymatic activity could contribute to this enhanced DDR phenotype eliciting an effect that ultimately determines sensitivity to ICL inducing agents. In line with this reasoning, PFKFB3 knockdown sensitizes ovarian cancer cells to platinum drugs^{149,256}.

2.3.3.4 Validation of selective inhibitors to assess molecular roles of PFKFB3

The previous lack of specific PFKFB3 small molecule inhibitors prior KAN0438757 development has constituted a problem in the field. Non-selective 3-PO and PKF-158 inhibitors have been widely employed as molecular tools to study uncanonical roles of PFKFB3 in the nucleus, and therefore, results from these studies must be interpreted with caution. For example, although in accordance with our findings, a recent study shows block of RAD51 foci results in defective HR repair in endometrial cancer cells upon PFK-158 inhibition¹⁴⁹. Several studies demonstrate that targeting PFKFB3 with 3-PO or its derivative PFK-158 chemosensitizes cancer cells to DNA damaging agents^{148,149,256,285}. Discrepancies continue to appear in functional studies using these inhibitors. Whilst 3-PO treatment induced G2/M phase arrest in Jurkat cells¹⁸⁵, it resulted in G0/1 phase cell cycle arrest in A375 human melanoma cells²⁸⁶.

Given the fact that isozyme selectivity is an important factor, the employment of unselective inhibitors may be misleading to characterize PFKFB3 role in DDR. An aspect that was not

addressed in this study was the contribution from other members of the PFKFB family to the observed phenotype. Based on the kinase enzymatic activity assays performed in **paper I**, the isozyme PFKFB4 exhibited closest IC₅₀ compared to PFKFB3 (more than 10-fold difference). This result indicates that at the range of concentrations used in our *in vitro* experiments we could expect that a fraction of PFKFB4 isozyme is blocked, perhaps contributing to the phenotype observed upon KAN0438757 treatment. This would ultimately depend on the protein levels of PFKFB4 enzyme in relation to PFKFB3, which has not been addressed in this body of work.

Another source of uncertainty is the employment of stable knockdown cell lines to address mechanistic questions. In hepatocellular carcinoma cells, PFKFB3 stable knockdown leads to more DNA breaks and increases ATM, p21 and Chk1 protein levels resulting in G2/M phase arrest²⁸⁷. This phenotype was attributed to a lack of Akt phosphorylation resulting from PFKFB3 ablation, and a consequent downregulation of ERCC1 expression, a transcriptional target of AKT²⁸⁸. Nevertheless, a note of caution is due here since knockdown experiments were performed in conditions that can lead to G0/G1 cell cycle arrest²⁸⁹. Considering that PFKFB3 expression varies during the cell cycle²⁹⁰, the effects on proliferation and cell cycle distribution derived from prolonged knockdown may result in phenotypes due to secondary effects.

In this body of work, we validated KAN0438757 small molecule inhibitor with proven protein-ligand binding in co-crystal structures, selectivity towards PFKFB3 isozyme across the other members of PFKFB family and in a broad panel of 96 kinases, biochemical inhibitory effects in kinase assays, and intracellular target engagement by inhibition of enzyme's product formation as well as drug's protein binding. We have demonstrated that KAN0438757 blocks glycolysis within minutes in ECAR measurements and it displays a cytotoxic effect on cancer cell viability across multiple cell lines. Additionally, others provided proof of concept of KAN0438757 effectiveness using advanced patient-derived three dimensional *ex vivo* models¹⁵¹. In conclusion, with the development and validation of KAN0438757 small molecule inhibitor, we hope to provide to the scientific community the opportunity to accurately interrogate PFKFB3 molecular functions.

2.3.4 Paper III

Targeting CX₃CR1 Suppresses the Fanconi Anemia DNA Repair Pathway and Synergizes with Platinum

The unmet medical need for EOC patients due to the clinical challenge of platinum resistance²⁵³, together with previous research reporting sensitization to DNA damaging agents in CX₃CR1 knockdown EOC cells via DDR modulation²¹⁹, prompted our interest to assess in a pre-clinical setting whether targeting CX₃CR1 by the small molecule inhibitor KAND567 could be beneficial to improve efficacy of platinum-based treatments.

We performed viability assays to quantify and compare sensitization potential of KAND567 combination with platinum drugs, cisplatin and carboplatin, utilizing a panel of EOC cell lines containing sensitive and resistant cell lines. Transformed EOC cells displayed higher delta scores in synergy viability matrixes compared to the non-transformed cells, and resistant cells were further sensitized to platinum drugs reflected by the further increase in synergy delta scores. Notably, these synergies observed in cell viability assays were validated in long term clonogenic assays, in which KAND567 resulted in dose-dependent reduction of survival. Sensitization to platinum drugs was also observed in gene dosing experiments, as lentiviral delivery of shRNA resulted in decreased growth rates, cell proliferation and long-term survival as assessed by colony formation assays. Importantly, shRNA CX₃CR1 knockdown in platinum resistant cells was not possible because the cells did not survive upon clonal selection, highlighting the essentiality of CX₃CR1 for cell survival upon DDR rewiring to tolerate crosslinks.

Given the synthetic lethality between CX₃CR1 inhibition and platinum treatments, coupled with the replication-associated toxicity of ICLs lesions, we next aimed to evaluate replication impairments upon combination treatment in platinum sensitive-resistant isogenic cell lines. Synchronized cells at the G1/S boundary, pulsed with EdU, and further released into platinum with or without KAND567 revealed an enrichment of cells in S phase that could not proceed to G2/M upon CX₃CR1 inhibition. This phenotype was aggravated in platinum resistant cells which could not resume replication up to 16h post release from the G1/S phase, whereas sensitive cells were able to proceed to next generation G1 at this timepoint. In line with this data, combination of cisplatin and KAND567 significantly decreased fork speed and track length in both sensitive and resistant cells as assessed in DNA fiber analyses. Whilst ICL-induced replication fork stalling was significantly enhanced in platinum sensitive cells, the resistant counterparts showed mild effects probably due to their slower overall cell cycle progression. This observation suggests a fork protection mechanism for tolerance of ICLs associated to the resistant phenotype of our *in vitro* models²⁹¹. Quantification of DNA-cisplatin-induced adducts by flow cytometry revealed that CX₃CR1 inhibition upon platinum treatment resulted in higher residual amount of crosslinks, indicative of defective ICL repair.

Since FA pathway operates in replication-dependent DNA crosslink repair¹¹, our results prompted our interest to assess a potential role of CX₃CR1 in FA repair by assessing its intracellular localization under treatments that activate FA pathway. Alongside FA pathway activation assessed by FANCD2 foci formation we surprisingly observed CX₃CR1 nuclear staining which correlated with FANCD2 induction. Of note, CX₃CR1 did not appear into clear distinct nuclear foci but displayed a pan nuclear pattern, whose specificity was further confirmed by siRNA CX₃CR1 transfections which significantly decreased the nuclear CX₃CR1 staining.

Recognition of the strong correlation between CX₃CR1 and FANCD2 nuclear staining, together with the impairment of cisplatin- and mitomycin-induced FANCD2 foci formation upon CX₃CR1 gene ablation and CX₃CR1 pharmacological inhibition with KAND567; suggested that CX₃CR1 operates in the FA pathway activation to assist in the resolution of stalled replication forks. Hence, we next hypothesized that CX₃CR1 might modulate chromatin association of FA factors required for fork stabilization. Indeed, inhibition of CX₃CR1 resulted not only in reduced chromatin association of ID2 complex, but also reduction of chromatin loading of RAD51, reported as FANCD2 interacting partner at DNA break sites to stabilize replication forks²⁶⁴. Importantly, CX₃CR1 appeared in both soluble and chromatin fractions; however, upon MMC treatments the soluble CX₃CR1 fraction decreased while CX₃CR1 chromatin fraction was enriched. This event was reversed when adding KAND567, leading to enriched soluble CX₃CR1 fraction. Notably, the effects herein described were not due to reduction of total protein levels, as western blots from whole cell extracts confirmed that KAND567 treatment did not modify FANCD2 or FANCI intracellular protein levels. Altogether, these findings present a mechanism in which upon CX₃CR1 inhibition, this receptor remains in the soluble fraction and cannot relocate to chromatin to trigger FANCD2-mediated fork stabilization and ICL resolution.

The main conclusions from **Paper III** are:

- Cancer-specific synthetic lethality between CX₃CR1 inhibition and platinum compounds
- Targeting CX₃CR1 with KAND567 chemosensitizes platinum resistant EOC cells by interference of the FA DNA repair pathway, resulting in slower replication fork progression and incapacity to progress through the S cell cycle phase due to unresolved DNA adducts.
- Activation of FA pathway conveys CX₃CR1 nuclear localization which supports FANCD2 foci formation and recruitment of FA factors.

2.3.5 Paper IV

Blocking the fractalkine receptor disrupts replication and ovarian cancer cell proliferation

Growing body of literature indicates that the fractalkine axis is fundamental for proliferation and survival of tumor cells. Furthermore, CX₃CR1 expression levels have been associated to worse prognosis in EOC patients^{200,227}. Prior to research efforts from **paper III**, we initially sought to explore whether CX₃CR1 was determinant for cancer cell survival in unperturbed conditions by means of cell cycle checkpoint control and apoptosis, DNA replication and repair.

In cell viability assays, KAND567 treatment consistently reduced survival in the micromolar range across a panel of EOC cell lines including cisplatin and carboplatin resistant ones, whereas at same concentrations the viability of non-cancerous cell lines was not affected indicating a potential therapeutic window. Accordingly, KAND567 is well tolerated and exhibits a safe toxicity profile in humans as it has passed clinical phase I with healthy volunteers²²⁹. We employed two systems of silencing CX₃CR1 expression, siRNA transfections and lentiviral delivery of shRNA, which both resulted in decreased growth rates, cell proliferation and long-term survival as assessed by colony formation assays. Additionally, we used tumor-derived cells from one HGSOC patient to further validate therapeutic targeting of CX₃CR1 with KAND567. While the patient-derived cells were slightly responsive to carboplatin, the current ovarian cancer standard of care, cell survival was severely affected by KAND567 treatment. Extending the cohort of patient samples and performing *ex vivo* screening studies will reinforce these preliminary findings and further strengthen the hypothesis that EOC tumors are dependent on fractalkine signaling for proliferation.

Next, assessment of cell cycle profile and replication was performed to initially characterize CX₃CR1 molecular function in EOC survival. Upon KAND567 exposure, dose-dependent reduction of S cell cycle phase subpopulation was observed, which hampered G2/M transition. Of note, the halt of S phase progression preceded accumulation in G0/G1 phase. Evaluation of EdU incorporation using confocal microscopy revealed reduced EdU signal intensity by CX₃CR1 inhibition indicating slowdown of replication, which upon 24h exposure was comparable to DNA replication stress inducers HU or H₂O₂. In addition, percentage of cells actively replicating that managed to incorporate EdU decreased, suggesting replication impairments. Consistent with the decrease in S phase cells upon CX₃CR1 inhibition, phosphorylation of the retinoblastoma protein pRB, which engages in G1/S transition, decreased in western blot analysis of cell lysates together with phosphorylated ATR and RPA. Moreover, reduced phosphorylation of ERK1/2 was shown, in line with being a downstream effector signaling pathway of fractalkine axis. Notably, inhibition of ERK by U0126 inhibitor displayed same phenotype as KAND567 inhibition: reduced activation of ATR, RPA and pRB.

ERK has been shown to modulate ATR activity upon replication stress induced by HU and thus to support S phase damage checkpoint²⁹². These findings point towards that the phenotype observed upon KAND567 inhibition, including diminished ATR activation, delayed G1 to S transition and replication defects, potentially could arise as a consequence of CX₃CR1-mediated ERK action. Furthermore, CX₃CR1 inhibition resulted in dose-dependent DNA damage and apoptosis induction as measured by western blot markers cleaved-PARP and cleaved caspase 3 markers, as well as AnnexinV/PI staining in flow cytometry.

Because of the strong phenotype on impaired DNA replication, we next evaluated effects of CX₃CR1 inhibition with DNA damaging agents that target DNA replication. We first pre-treated cells with carboplatin to induce replicative damage, and then added KAND567 to assess how CX₃CR1 modulates proliferation in EOC cells. Cells treated solely with carboplatin accumulated at S phase due to fork stalling at DNA crosslinks. In line with the findings from **paper III**, the addition of KAND567 resulted in cells unable to undergo G2/M transition. This, together with the reduction of phosphorylated ATR, ATM and CHK2 upon cotreatment, indicates a different CX₃CR1 molecular mechanism from unperturbed conditions.

The major findings of **Paper VI** are:

- Ovarian cancer cells are dependent on fractalkine signaling for proliferation and survival which can be exploited therapeutically with KAND567 treatment.
- In unperturbed conditions, CX₃CR1 inhibition conveys defective DNA replication, impairs RPA-ATR signaling, prevents G1 to S cell cycle progression, induces DNA damage and ultimately leads to apoptotic cell death.

2.3.6 CX₃CR1: General discussion and concluding remarks

2.3.6.1 Molecular tools to characterize the role of CX₃CR1 in the DDR

Transmembrane GPCRs have recently emerged as promising targets to control DDR by acting as DNA damage sensors and evoking effector signaling processes to mitigate this damage²⁹³. In **paper III** and **paper IV**, we provide comprehensive investigation of the implication in DNA damage and repair mechanisms of the chemokine receptor CX₃CR1. As a GPCR, successful pharmacological intervention can be achieved using the KAND567 compound to assess inhibition of a subset of signaling pathways^{212,294}. Until now, however, observations made to ascertain the non-canonical function of fractalkine signaling in DDR were based on comparative studies between knockdown CX₃CR1 cells and normal endogenously expressing cells. Barbolina *et al.* (2018) provided first evidence that reduction of CX₃CR1 expression resulted in sensitization to DNA damaging agents, including IR and cisplatin, by regulating total protein levels of RAD50 by a MYC-dependent mechanism²²⁴. Nevertheless, we could not confirm the RAD50-mediated intervention to resolve DSBs by

CX₃CR1 pharmacological inhibition (data unpublished). Taking into account that CX₃CR1 appears essential for survival of EOC cells, as several of our stable lentiviral shRNA transfections resulted in cell dismissal, a possible explanation for this difference is that prolonged knockdown of CX₃CR1 gene may result into adaptation mechanisms to ensure survival. These rewired mechanisms may make the study of functional phenotypes difficult. It is well established that GPCRs display functional selectivity, as ligand-receptor interactions provoke specific receptor conformational changes that convey different subset of effector signaling pathways, ultimately leading to unique cellular responses depending on ligand interaction²⁹⁴. Therefore, long-term ablation of CX₃CR1 loses this complexity and may result in distinct cellular responses in comparison to pharmacological inhibition.

2.3.6.2 Exploring the molecular mechanism behind CX₃CR1 modulation of the DDR

In **paper III**, we have disclosed that the fractalkine receptor exhibits a nuclear localization using immunofluorescence and chromatin fractionations, thus suggesting transmembrane to nuclear relocation upon damage. The mechanism of this subcellular distribution pattern remains to be elucidated. Nevertheless, it has been demonstrated that it entails functional implications for DNA repair kinetics of FA pathway. **Paper III** raises the possibility that FANCD2 acts as a signal transducer of CX₃CR1 activity as a transmembrane receptor, which is plausible since FA pathway has been linked to GPCR signaling for more than a decade. Larder *et al.* (2006) identified FANCA factor, member of the FA core complex, as a downstream signal transducer of the gonadotropin-releasing hormone receptor (GnRHR)²⁹⁵, whose signaling is required for gonadal function. GnRHR signaling controls FANCA nuclear and cytoplasmic distribution assessed by microscopy and cell fractionations like in our work. As a signal transducer, FANCA was required for transactivation of gonadotropin hormones and GnRHR expression in gonadotropic cells, offering a mechanistic explanation of the characteristic clinical infertility in FA patients. Another illustrative case of nuclear relocation of a transmembrane receptor as a response to DNA damage is the EGFR. Under exposure to IR or cisplatin treatment, EGFR modulates DNA damage repair from its nuclear localization by direct interaction with DNA-PK in mouse fibroblasts²⁹⁶. In this study, co-immunoprecipitation experiments and proximity ligation assay revealed that direct protein interaction was required for DNA-PK kinase activity.

The diffuse nuclear pattern of CX₃CR1 staining after in-situ fractionations in **paper III**, but not clear characteristic foci, suggests a modulation of DDR response by CX₃CR1 via signal transduction. Instead of being a scaffolding factor at DNA damage sites or an interacting partner of DDR factors, fractalkine receptor may convey to signaling pathways that contribute to the maintenance of genomic stability. For instance, ERK1/2 constitutes a signal transducer of fractalkine axis¹⁹⁷ and ERK has a reported regulatory role on ATR activity upon replication stress²⁹². These findings, together with our preliminary observations showing reduced

phosphorylated ERK1/2, ATR and RPA upon CX₃CR1 inhibition, reflect a signaling mechanism that contributes to the crosstalk between survival signaling and genomic stability via CX₃CR1-ERK1/2 signal transduction. Further studies could address how CX₃CR1 pharmacological inhibition modulates MAPK signaling as a stress response pathway to genotoxic agents, and how this signaling events may control cell cycle transitions. On this matter, another effector pathway elicited by fractalkine activation is the Akt signaling axis²⁰⁷, which has been reported essential for G1 cell cycle progression²⁹⁷. Hence, impaired replication phenotype and G1 cell cycle delay by KAND567 treatment in **paper IV** could be explained by reduced Akt signaling events.

Furthermore, it is interesting to note that treatment of KAND567 at concentrations used in our body of work abolishes CX₃CL1 binding to the receptor and blocks β -arrestin²¹². Beta-arrestins are scaffolding proteins that act as intracellular signal transducers per se triggering multiple signaling networks²¹³. Recent reports associate β -arrestin signaling to the modulation of DDR in DNA damaging conditions^{298,299}. In which extend the block of intracellular trafficking of CX₃CR1 by β -arrestin and the lack of signaling conveyed upon receptor internalization has functional consequences for DNA replication and repair, is a matter that could be addressed in the future.

Overall, the precise mechanism of CX₃CR1 modulation of the DDR remains to be elucidated. We have shown for the first time that this transmembrane chemokine receptor appears in the nucleus as a response to DNA damage and validated CX₃CR1 antibody nuclear staining with knockdown experiments. However, current available antibodies that recognize CX₃CR1 are not optimal as they show unspecific bands in western blotting. Hence, further studies could employ methodologies to track receptor localization such as expression of fluorescent tagged-CX₃CR1 or bioluminescent resonance energy transfer-based assays, which are widely used to interrogate GPCR pharmacology, membrane localization and internalization via β -arrestin³⁰⁰. This, together with phosphoproteomic analysis, could prove useful to explore the mechanistic aspect of whether CX₃CR1 modulation of DNA damage repair and replication is a consequence of signaling through MAPK pathways.

2.3.6.3 Tackling platinum resistance by targeting CX₃CR1

The KAND567 small molecule shows good safety and pharmacokinetic profiles since it has passed phase I clinical trials with 100 healthy volunteers. Thereby, it is well qualified for clinical efficacy trials in patients. For this reason, further studies should address the proof of concept of platinum sensitization utilizing *in vivo* models in order to determine the effective dose and select preferred treatment modality for clinical trials.

Inflammatory processes occurring naturally in the ovaries, such as ovulation, have been identified as mechanisms contributing to the pathogenesis and aggressiveness of ovarian

cancer³⁰¹. Furthermore, compelling evidences demonstrate that the fractalkine axis is involved in metastatic progression of EOC^{200,202,207,216,223}. This, together with the herein shown defects on EOC cell survival upon CX₃CR1 targeting, strongly indicates that EOC patients could benefit the most from a putative therapeutic intervention using KAND567. Delivery of KAND567 to EOC patients could not only improve efficacy of platinum-based therapy by reducing tumor burden, but also by mitigating metastatic spreading to the omentum and ultimately improving clinical outcome.

A major clinical challenge nowadays is the development of resistance to platinum neoadjuvant chemotherapy, ultimately contributing to poor survival to EOC³⁰². Owing to the mechanism of action of platinum, the efficacy of this type of DNA damaging agent is influenced by the cancer cells' capacity to resolve DNA crosslinks and alleviate replication fork stress²⁸³. Indeed, enhanced FA pathway activation has been identified as a mechanism of platinum resistance *in vitro*^{261,262} and targeting the FA pathway has been proven to sensitize cancer cells to platinum^{258–260}. Accordingly, elevated expression of genes participating in replication-coupled ICL repair such as *BRCA1*, *FANCA*, *FANG*, *FANCI* and *BLM* have been correlated to clinical platinum resistance in EOC patient cohorts^{303,304}. Based on the findings from **paper III**, in which we show sensitization to platinum compounds upon KAND567 by suppressing FA pathway activation³⁰⁵, therapeutic targeting of CX₃CR1 offers the possibility to tackle the clinical challenge of platinum resistance in ovarian cancer.

2.4 FUTURE PERSPECTIVES

With this body of work, we establish a novel foundation for therapeutic strategies with small molecule inhibitors that target the DDR. State-of-art therapies to cure cancer are formulated from mechanism-guided combinations, resulting in better outcomes to DNA damaging treatments^{306,307}. In this regard, therapeutic targeting of PFKFB3 or CX₃CR1 offers the opportunity for a therapeutic intervention based on combination treatments with DNA damaging therapies. Future studies should be undertaken to explore the potential of combining KAND567 or KAN0438757 with other DNA damaging treatments than herein reported. Such combinations could result in improved efficacy and also reduction of dose-related toxic side effects. Recently developed ATM inhibitors illustrate this point clearly. Because the loss of FA genes such as FANCD2 is synthetic lethal with ATM pharmacological inhibition³⁰⁸, therapeutic targeting of CX₃CR1 or PFKFB3 offers the possibility to render cancer cells FA-deficient and thus theoretically hypersensitive to ATM inhibition.

Tailoring PARP inhibitor treatment to HR deficient patients has been a major improvement for ovarian and breast cancer patients with *BRCA1/2* mutations¹¹⁰. Given its roles in HR repair, PFKFB3 represents a promising target to obtain a BRCAness phenotype that could be relevant to expand the use of PARP inhibitors beyond BRCA mutated tumors. Future work directed to know whether PFKFB3 inhibition influences other types of DSB repair mechanisms such as NHEJ will be key for the rationale of KAN0438757 in this context.

Malignancies that typically exhibit high degree of genomic instability may benefit the most from inhibitors targeting the DDR²⁷. This is the case for HGSOC, in which the therapeutic targeting of PFKFB3 or CX₃CR1 could potentiate DDR deficiencies and render ovarian cancer cells more sensitive to platinum, the standard of care of the disease. Expanding the battery of clinically applicable biomarkers to detect tumors with high degree of genomic instability will contribute to identify those patients who would likely respond to such therapies^{309,310}.

The discovery of synthetic lethal interactions provides the opportunity to target rewired DDR mechanisms to restore sensitization and overcome treatment resistance³¹¹. Employment of different resistant *in vitro* models could provide insight on mechanistic questions to explain KAND567- and KAN0438757-induced sensitization of traditional anti-cancer therapies. For example, besides replication-coupled repair proficiency, backup DNA repair mechanisms involved in the resolution and sensing of platinum adducts have been associated to platinum resistance³¹². In this regard, increased expression of factors from NER and MMR pathways correlate with poor clinical response to platinum-based therapies^{313,314}. Future studies could be directed to evaluate the modulation of these DNA repair mechanisms by CX₃CR1 and PFKFB3 molecular functions and how they contribute to the platinum sensitization phenotype.

The DDR network entails great deal of redundancy as multiplicity of mechanisms engage to ensure protection of the genomic integrity³¹⁵. Another aspect that could be addressed in future studies is the redundancy between CX₃CR1 and PFKFB3 inhibition. Whether KAN0438757 and KAND567 combination is synergistic, or how the monotherapy with one of these compounds renders a DDR liability that can be targeted with the other compound, are both aspects worth to consider. Additionally, CRISPR-Cas9 genetic screens could be employed to identify genetic vulnerabilities among DDR genes predisposing to CX₃CR1 or PFKFB3 inhibitor responses¹⁰³.

In conclusion, the present thesis provides biological insights into CX₃CR1 and PFKFB3 roles in the DDR and in the maintenance of genomic stability under DNA damaging conditions. Furthermore, our work lays the groundwork for future research into combination therapies in cancer targeting these two proteins.

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