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CIP2A IS A CRITICAL DNA DAMAGE RESPONSE PROTEIN THAT DRIVES BASAL-LIKE BREAST CANCER

Srikar Nagelli

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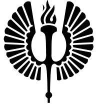
SARJA – SER. D OSA – TOM. 1699 | MEDICA – ODONTOLOGICA | TURKU 2023



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To my family

UNIVERSITY OF TURKU
Faculty of Medicine
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SRIKAR NAGELLI: CIP2A is a critical DNA damage response protein that drives basal-like breast cancer
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ABSTRACT

Basal-like breast cancer (BLBC) is an aggressive and therapeutically most challenging breast cancer subtype, due to a lack of druggable breast specific surface receptors (ER, PR, HER2) or driver mechanisms. BLBC patients are treated mainly with standard chemotherapies but due to poor patient stratification strategies, a vast majority of patients receive aggressive chemotherapy even though some of them could be spared from its unnecessary side effects. Protein Phosphatase 2A (PP2A) is an important serine-threonine tumor suppressor phosphatase and its inactivation is an important requirement for malignant transformation in humans. In this dissertation, we demonstrate the role of PP2A inhibitor protein CIP2A (Cancerous Inhibitor of PP2A) in BLBC.

We identified a novel PP2A-independent role for CIP2A as a non-genetic driver protein and a biomarker of poor prognosis in BLBC. We discovered that CIP2A interacts with DNA damage response protein TopBP1, prevents its recruitment at the site of DNA damage and allows the progression of damaged cells into mitosis. We further established that CIP2A drives BLBC by coordinating the BLBC hallmarks. CIP2A controls the high proliferation activity of MYC and E2F1 by preventing their dephosphorylation by PP2A but also promotes high genomic instability by deregulating the G2/M checkpoint (PP2A-independent role). We also identified that CIP2A is a synthetic lethal target in BRCA mutant BLBCs and established that CIP2A is selectively more important in predicting prognosis of basal-like TNBC patients compared to non basal-like TNBC subtype.

To develop these findings towards clinical utility, we further developed a robust CIP2A transcriptional signature and highlighted several applications of this tool for BLBC patients. The CIP2A signature can predict the aggressivity of breast cancer and it was used to identify drugs and drug combinations that show clinical benefit for the different stratified BLBC subgroups.

Collectively, this dissertation reports the yet uncharacterized role of CIP2A in DNA Damage response (DDR), BLBC tumorigenesis, and its potential use as a personalized medicine strategy for effective clinical management of BLBC.

KEYWORDS: Basal-like breast cancer (BLBC), CIP2A, PP2A, DNA damage response (DDR), transcriptional signature, personalized medicine

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TIIVISTELMÄ

Basaalinen rintasyöpä (BLBC) on hoidollisesti kaikkein haastavin rintasyövän alatyyppi sillä yleensä sitä ei voida hoitaa täsmälääkkeillä. Tämä johtuu solun pintareseptorien tai targetoitavien ajurimekanismien puutteesta. BLBC-potilaita hoidetaan pääasiassa tavanomaisella kemoterapialla mutta koska keinoja potilaskohtaiseen hoidonvalintaan ei ole olemassa, hyvin suuri osa potilaista saa aggressiivista kemoterapiaa, vaikka osa heistä voitaisiin säästää sen sivuvaikutuksilta. Proteiinifosfataasi 2A (PP2A) on tärkeä seriini-treoniini kasvunestäjäfosfataasi ja sen inaktivaatio on edellytys syövän synnylle ihmisellä. Tässä väitöskirjassa olen tutkinut PP2A:ta inhiboivan CIP2A-proteiinin (Cancerous Inhibitor of PP2A) roolia BLBC-syövissä.

Väitöskirjatutkimuksessa osoitin CIP2A:n toimivan sekä ei-geneettisenä ajuriproteiinina että biomarkkerina huonoennusteisessa BLBC:ssä. Erityisen tärkeä oli havainto, että CIP2A on selektiivisesti tärkeämpi määrittämään basaalisen kaltaisten kolmoisnegatiivisten (TNBC) potilaiden ennustetta. Mekanistisesti löysimme CIP2A:n vuorovaikutuksen DNA-vauriovasteproteiini TopBP1:n kanssa. Tämä mekanismi estää TopBP1:sen ohjautumisen DNA:n vauriokohtaan edistäen vaurioituneiden solujen siirtymistä mitoosivaiheeseen. Havaitsimme myös, että CIP2A kykenee koordinoimaan useita BLBC:lle tunnusomaisia mekanismeja. CIP2A kontrolloi onkogeenisten MYC- ja E2F1-transkriptiotekijöiden aktiivisuutta estämällä niiden PP2A-välitteisen defosforylaation, mutta myös edistää genomista instabiliteettia estämällä solusyklin G2/M tarkastuskohtaa (PP2A:sta riippumaton rooli). Havaitsimme myöskin, että CIP2A on synteettisesti letaali terapiakohde BRCA-mutanteissa BLBC:eissä. Työssä kehitin myös CIP2A:n säätelemään geeniekspressioprofiiliin perustuvan signature-työkalun ja osoitin että se ennustaa rintasyövän aggressiivisuutta, ja että sitä voi käyttää niiden lääkkeiden ja lääkeaineyhdistelmien tunnistamiseen, jotka erityisesti toimivat BLBC-alatyypin soluja kohtaan.

Yhteenvetona tässä väitöskirjassa kuvataan aiemmin julkaisematon CIP2A:n rooli DNA-vaurion korjausmekanismissa (DDR), BLBC:n tuumorigeneesissä, ja potentiaalinen käyttö yksilöllistetyn lääketieteen strategiassa, joka tähtää BLBC potilaiden entistä tehokkaampaan kliiniseen hoitoon.

AVAINSANAT: Basaalinen rintasyöpä, CIP2A, PP2A, DNA-vaurion korjausmekanismi, geeniekspressio, yksilöllistetty lääketiede

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Abbreviations

2G-TKIs	2nd generation Tyrosine kinase inhibitors
5FU	5-fluorouracil
9-1-1	RAD9-RAD1-HUS1 complex
AAD	ATR activating domain
alt-EJ	Alternative-End Joining
AML	Acute myeloid leukemia
AP site	Apurinic or apyrimidinic site
ASCO	American Society of Clinical Oncology
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
BER	Base excision repair
BLBC	Basal-like breast cancer
BLM	Bloom syndrome protein
BRCT	BRCA1 C terminal domain
CAP	College of American Pathologists
CIN	Chromosomal instability
CIP2A	Cancerous inhibitor of PP2A
CK	Cytokeratin
CK2	Casein kinase 2
CKBD	Chk1 binding domain
CLL	Chronic lymphocytic leukemia
CMA	Chaperone mediated autophagy
CML	Chronic myeloid leukemia
CPD	Cyclobutane pyrimidine dimers
CtBP	C-terminal Binding protein
CtIP	CtBP interacting protein
DAPk	Death associated protein kinase
DCIS	Ductal carcinoma in situ
DDR	DNA damage response
DFS	Disease free survival
DMBA	7,12-dimethylbenz[a]anthracene
DSB	Double strand break

dsDNA	double stranded DNA
DSP	Dual specificity phosphatase
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ERCC	Excision repair cross complementing
EXO1	Exonuclease 1
FA	Fanconi Anemia
FEN1	Flap endonuclease 1
FFPE	Formalin fixed paraffin embedded
FOXM1	Forkhead box protein M1
GEF	Guanine exchange factor
GG-NER	Global genome nucleotide excision repair
GSEA	Gene set enrichment analysis
HEAT	Huntington/Elongation/A-subunit/TOR
HER2	Human epidermal growth factor receptor 2
HRD	Homologous recombination deficiency
HRR	Homologous recombination repair
hTERT	human telomerase reverse transcriptase
ICL	Interstrand cross links
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
IR	Ionizing radiation
LCIS	Lobular carcinoma in situ
LP-BER	Long patch base excision repair
LT	SV40 Large T antigen
MEF	Mouse embryonic fibroblasts
MMEJ	Microhomology mediated end joining
MRN	MRE-11-RAD50-NBS1 complex
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NOCIVA	Novel CIP2A variant
OGG1	8-oxoguanine glycosylase
OS	Overall survival
OXPHOS	Oxidative phosphorylation
PALB2	Promoter and localizer of BRCA2
PAM50	Prediction Analysis Microarray 50
PARP	poly (ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PI3KK	PI3K kinase related kinases
PLA	Proximity ligation assay

PLK1	Polo-like kinase1
PP2A	Protein phosphatase 2A
PR	Progesterone receptor
PSK	protein serine/threonine kinase
PSP	protein serine/threonine phosphatase
PTK	protein tyrosine kinase
PTM	post translational modification
PTP	protein tyrosine phosphatase
RB	Retinoblastoma
RFC	Replication factor C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
ROTS	Reproducibility optimized test statistic
RPA	Replication protein A
SASP	Senescence associated secretory phenotype
SLiM	Short linear motifs
SMA	Smooth muscle actin
SMAP	Small molecule activators of PP2A
SP-BER	Small patch base excision repair
SSA	Single strand annealing
SSB	Single strand break
ssDNA	single stranded DNA
ST	SV40 small T antigen
STRIPAK	Striatin interacting phosphatase and kinase complex
STRN	Striatin
TC-NER	Transcription coupled- nucleotide excision repair
TDLU	Terminal ductal lobular unit
TKI	Tyrosine kinase inhibitors
TMB	Tumor mutational burden
TME	Tumor microenvironment
TMEJ	Theta mediated end joining
TNBC	Triple negative breast cancer
TopBP1	Topoisomerase IIβ Binding protein 1
UV	Ultraviolet
WHO	World Health Organization
XP	Xeroderma pigmentosum
XRCC	Xray repair cross complementation
Y2H	Yeast two-hybrid

List of Original Publications

This dissertation by Srikar Goud Nagelli is based on the following original publications, which are referred to in the text by their Roman numerals:

I Laine, A.*, Nagelli, S.G.*, Farrington, C., Butt, U., Cvrljevic, A.N., Vainonen, J.P., Feringa, F.M., Grönroos, T.J., Gautam, P., Khan, S., Sihto, H., Qiao, X., Pavic, K., Connolly, D.C., Kronqvist, P., Elo, L.L., Maurer, J., Wennerberg, K., Medema, R.H., Joensuu, H., Peuhu, E., de Visser, K., Narla, G., Westermarck, J. CIP2A Interacts with TopBP1 and Drives Basal-Like Breast Cancer Tumorigenesis. *Cancer Research,* 2021 Aug 15; 81(16):4319-4331.

II Nagelli, S.G., Suomi, T., Elzeneini, E., Huhtaniemi, R., Li, L., Courtney, M., Elo, L.L., Westermarck, J. CIP2A drives DNA damage response (DDR) transcriptional signature in Basal-like Breast Cancer. *Manuscript.*

* These authors contributed equally to the publication I

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1 Introduction

Breast cancer has recently overtaken lung cancer as the most common cancer type globally (Sung et al., 2021). Basal-like breast cancer (BLBC), which constitutes 15% of all breast cancer cases, is a highly aggressive subtype that is relatively common in premenopausal women (Alluri & Newman, 2014; Marra et al., 2020; Toft & Cryns, 2011). The key traits of BLBC are high proliferation activity (owing to elevated MYC, E2F1, Cyclin E, and EGFR activities) and a high degree of genomic instability (due to mutations in TP53, RB, CDKN2A, BRCA1, and deregulated checkpoints) (E. A. Rakha et al., 2008; Badve et al., 2011; Milioli et al., 2017). Majority of the BLBCs belong to the triple-negative subtype and do not express breast specific surface receptors Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2) which can be therapeutically targeted. In the clinic, BLBC and triple negative breast cancer (TNBC) are used interchangeably, as EGFR positivity and basal cytokeratins that help in distinguishing BLBC from TNBC are not assessed routinely (Alluri & Newman, 2014; Burstein et al., 2021). While there are recently approved drugs such as sacituzumab govitecan and immune checkpoint PD-1/PD-L1 inhibitors which can be used in TNBC patients, such targeted therapies are not suitable for all BLBC patients. Breast cancer is one of the most widely sequenced cancer types, and still, the sequencing efforts have not been able to discover druggable driver mechanisms for BLBC (Koboldt et al., 2012; Yin et al., 2020). Since there are no targeted therapies, chemotherapy remains the mainstays of the treatment for this subtype (Denkert et al., 2017; Yin et al., 2020). BLBC is a very heterogeneous disease and two patients presenting with similar histopathological features might have very distinct clinical outcomes. Majority of the metastatic BLBC patients are treated with aggressive chemotherapy comprising multiple drugs used in combination, even though some of them may not require such an aggressive treatment and could be spared from unwanted side effects. Also it has been reported that some of these patients don’t really respond well to these chemotherapy drugs (Perou et al., 2000; E. A. Rakha et al., 2008; Badve et al., 2011; Milioli et al., 2017). Gene expression signatures such as OncotypeDX, MammaPrint have been adopted to clinical practice to determine the risk of recurrence and to identify which patients to treat with

chemotherapy. But such prognostic and predictive biomarker signatures are so far restricted to ER+ breast cancer (luminal subtype) and not applicable for aggressive breast cancer (Alluri & Newman, 2014; Gagan et al., 2020). Therefore, there is a significant unmet clinical need to identify the molecular mechanisms that drive BLBC and to develop improved patient stratification tools for effective clinical management of BLBC.

Protein phosphorylation is the most common post translational modification in the cells (Bilbrough et al., 2022). Though both kinases and phosphatases have an equally important role in regulating the phosphorylation mediated cell signaling and homeostasis (Barber & Rinehart, 2018), there is a huge knowledge gap in our understanding of the role of phosphatases in cancer. Protein Phosphatase 2A (PP2A) is a major tumor suppressor serine/threonine protein phosphatase, whose inhibition is a critical requirement for malignant transformation of human cells (Hahn & Weinberg, 2002; Rangarajan et al., 2004; Sangodkar et al., 2016; Westermarck & Hahn, 2008). Unlike other tumor suppressors, PP2A is rarely mutated in human malignancies, and it is more commonly inactivated by endogenously overexpressed inhibitors (Kauko & Westermarck, 2018). CIP2A (Cancerous Inhibitor of PP2A) is an endogenous inhibitor of PP2A which is overexpressed in several human cancers and its expression has been associated with poor clinical outcomes in almost all the cancer subtypes where it has been studied (Junttila et al., 2007; Khanna & Pimanda, 2016).

In this thesis, I demonstrate that CIP2A plays a critical role in the DNA damage response (DDR) as a novel non-genetic driver of BLBC (publication I). Furthermore, I have also evaluated the potential of CIP2A's transcriptional signature in stratifying BLBC patients for personalized medicine regimens (publication II). These findings provide several indications on how to better manage the challenging BLBC subtype in the clinic. Notably, the newly identified role of CIP2A in DDR opens several research possibilities and points the field in a new direction.

2 Review of the Literature

2.1 Cancer

Cancer is a group of diseases characterized by the uncontrolled division of cells, due to accumulated genetic mutations, that eventually invade normal tissue barriers and spread to other organs (Jeggo et al., 2016; Martínez-Jiménez et al., 2020; Stratton et al., 2009). Cancer is one of the major contributors to decreased life expectancy and is associated with a significant burden on the healthcare ecosystem across the globe (Siegel et al., 2022; Sung et al., 2021). According to the World Health Organization (WHO) estimates, cancer is the first or second common cause of death in individuals below the age of 70 in 113 out of 183 countries studied (Sung et al., 2021).

Cancer clonal evolution is a multi-step process which involves accumulation of a series of mutations that eventually confer selective growth advantage to a single cell clone to proliferate more than its neighbors. Over time, these clones attain more advantageous mutations that lead to development of malignant tumors with diverse genetic characteristics (Greaves & Maley, 2012; Martínez-Jiménez et al., 2020; Stratton et al., 2009; Vogelstein & Kinzler, 1993). Some cancer types such as melanoma and lung cancer display more mutations than average and the larger number of mutations indicate the involvement of external sources such as UV light and cigarette smoke which caused more mutational burden in the premalignant lesions of these tumors (Garraway & Lander, 2013; Vogelstein et al., 2013). Tumor mutational burden (TMB) is defined as the total number of genetic alterations (mutations) in a cancer cell. TMB is measured as number of mutations in a section of a DNA, typically denoted as mutations per mega base of DNA (mut/Mb). High TMB cancers (greater than 10 mut/Mb) are expected to respond better to immune checkpoint inhibitors (Fusco et al., 2021). The number of mutations in a cancer can vary from anywhere between 10-20 to more commonly hundreds or thousands (Greaves & Maley, 2012). A vast majority of these mutations are neutral and do not offer any clonal growth advantage termed as *"passenger mutations"* but few functionally relevant ones that improve the survival fitness of the dividing cells and are implicated in tumorigenesis are termed *"driver mutations*" (Greaves & Maley, 2012; Stratton et al., 2009).

The cancer driver genes can be classified into oncogenes and tumor suppressor genes based on their role in cancer progression. Oncogenes promote cell division,

while tumor suppressors prevent abnormal cell divisions by activating cell cycle arrest or apoptosis when the damaged DNA cannot be repaired. Gain-of-function mutations in the oncogenes and loss-of-function mutations in the tumor suppressor genes together lead to hyperproliferation and evasion of organismal barriers, needed for the development and progression of cancer (Vogelstein & Kinzler, 2004; E. Y. Lee & Muller, 2010; Lyu et al., 2020). When analyzing cancer incidences across different tissues, it is apparent that there is a significant variation in frequency of cancer across tissues, and intriguingly, some tissues with high mutagen exposure exhibit lower cancer rates report less frequency of cancer (Tomasetti & Vogelstein, 2015). Though hereditary and environmental factors can explain this variability to a very small extent, the main reason for this variation can be attributed to the "*bad luck*" random mutations arising from replication errors during normal cell division, and the lifetime risk of cancer in a tissue correlates with the lifetime number of divisions within the stem cells of that organ (Tomasetti et al., 2017; Tomasetti & Vogelstein, 2015). However, this finding was contradicted by Yusuf Hannun and colleagues who reported that the contribution of random mutations and intrinsic factors to the cancer development is less than 30% (Wu et al., 2016).

In summary, to effectively prevent or treat cancer, it is necessary to understand the exact roles of different extrinsic and intrinsic factors in cancer incidence (Goldstein & Patel, 2019). However, the key takeaway from these studies is that some cancer types are driven primarily by mutations caused by environmental factors (such as lung cancer, melanoma, and cervical cancer), and can be prevented by avoiding these environmental exposures. But, for other cancer types where the driving mechanisms are unclear or occur irrespective of the environmental or hereditary predispositions, early diagnosis and intervention are critical lifesaving options (Goldstein & Patel, 2019; Song et al., 2018; Tomasetti et al., 2017).

2.1.1 Hallmarks of cancer

Cancer is a diverse disease encompassing more than 100 distinct tumor types that share common traits at a molecular level. Hanahan and Weinberg defined these common features as "*Hallmarks of cancer*" that malignant cells acquire during the process of tumorigenesis to escape the organismal control and normal cellular restrictions (Hanahan & Weinberg, 2000). The order in which these hallmark traits are acquired may vary a lot from one tumor to another. Also, sometimes a single genetic mutation can lead to the accumulation of multiple traits whereas in other cases multiple genetic events collaborate to acquire one trait. Initially, six hallmarks were proposed that include "sustained proliferative signaling," "deregulation of anti-growth signals," "avoiding programmed cell death", "inducing angiogenesis", "allowing cells to replicate with unlimited potential", and "activation of invasion and

metastases". As the understanding of different cancer mechanisms improved with significant progress in the field, these hallmarks were also refined and updated accordingly (**Figure 1**), once every decade, first in 2011 and then most recently in 2022 (Hanahan & Weinberg, 2011; Hanahan, 2022).

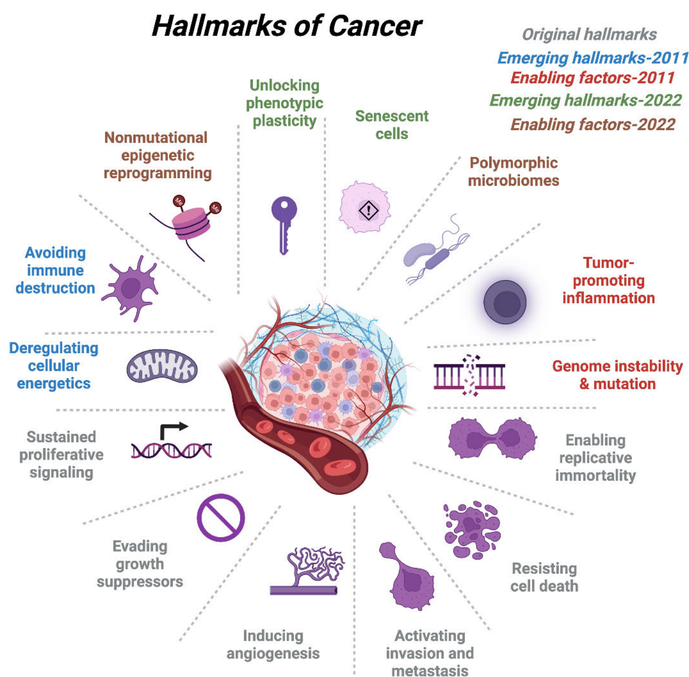

Figure 1: "Hallmarks of Cancer" including the initial hallmarks and updates made in 2011 and 2022, adapted from (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011; Hanahan 2022) made in *Biorender.com*.

In 2011, the hallmarks were revised to include "evasion of immune destruction" and "reprogramming of energy metabolism" as two emerging hallmarks, which were considered characteristic of some tumors if not all. The authors also emphasized the role of "genomic instability" and "tumor-promoting inflammation" as two enabling features that allow and speed up the process of acquiring these traits during tumorigenesis (Hanahan & Weinberg, 2011). Since this dissertation focuses on the importance of DNA damage response (DDR) signaling, and the role of genomic instability in the initiation of tumorigenesis, it is covered in detail in the subsequent chapters. The update in 2011 also highlighted the importance of the stromal cells and the role of tumor microenvironment (TME) in tumorigenesis and therapeutic resistance (Trédan et al., 2007; Schneider et al., 2017). It is now evident that cancer is quite a complex disease and in order to effectively treat and better understand the

course of its progression, a good understanding of not just the biology of individual cancer cells but also the stromal cells and the TME is important. In 2022, Hanahan revised these hallmarks to include “Senescent cells” and “unlocking phenotypic plasticity” as the emerging hallmarks and gave emphasis to the role of “epigenetic changes” and “polymorphic microbiome” as enabling features that aid in the development of other hallmarks (Hanahan, 2022). Some of these new hallmarks are described in detail in the upcoming paragraphs.

Senescence or irreversible cell cycle arrest is an important barrier that cells need to overcome in order to progress into cancer (Gabai et al., 2023). There is growing evidence that senescent cells induce a senescence associated secretory phenotype (SASP) that causes vascular remodeling and immune cell infiltration, that eventually lead to tumor suppression (Muñoz-Espín & Serrano, 2014; Ruscetti et al., 2020; L. Wang et al., 2022). On the other hand, SASP can also induce paracrine signaling to activate angiogenesis and promote growth in adjacent tumor cells, and the elimination of senescent cells has shown to be promising in delaying cancer incidence (Baker et al., 2016; Chaib et al., 2022; L. Wang et al., 2022). Therefore, there is now lot of focus towards developing senolytics (drugs that target and eliminate senescent cells) (Chaib et al., 2022; L. Wang et al., 2022). Since senescence is known to have both pro-tumor and anti-tumor effects, Wang and colleagues propose a one-two-punch strategy of targeting senescence sequentially, first by senescence inducing drugs, followed by senolytic drugs (L. Wang et al., 2017, 2022).

Plasticity is an innate property of stem cells that allows tissues to differentiate during normal development and self-renew during wound healing or tissue regeneration (S. Yuan et al., 2019; Torborg et al., 2022). Cancer cells exploit these features to create a selective advantage, to adjust to an unfavorable environment such as drug treatments and tumor suppression. This plasticity also helps in generating the tumor heterogeneity (Gupta et al., 2019), senescence exit (De Blander et al., 2021) and therapeutic resistance (Torborg et al., 2022). Epithelial to mesenchymal transition (EMT) is a very well-known phenomenon of plasticity which the cancer cells use to switch from an epithelial state to a mesenchymal state, an important requirement for metastasis. The mesenchymal state is associated with upregulation of efflux drug transporters and hence they are resistant to many drugs, but it has been reported that phenotypic plasticity also creates a vulnerability. For example, mesenchymal cells in pancreatic cancer are very resistant to EGFR inhibitors but are sensitive to genotoxic drug gemcitabine, and treatment with gemcitabine can create a selective pressure for a switch to drug sensitive epithelial state, where EGFR inhibitors can be effective again (Collisson et al., 2011; S. Yuan et al., 2019).

For a long time, cancer was thought to be a disease of the genome and predominantly resulting from mutations in driver genes. However, the advances in sequencing capabilities have not been able to succeed in identifying the drivers of metastasis, drug

resistance or progression for many tumor subtypes, indicating that there is a role for non-genetic contributors of tumorigenesis such as epigenetic reprogramming (Koboldt et al., 2012; A. Chatterjee et al., 2018; Nam et al., 2021). Epigenetic reprogramming, due to internal and external stimuli (signals coming from TME), play a role in modifying the chromatin organization and the accessibility of the genome for transcription, driving the progression of cancer (Lau et al., 2017; Gagliano & Brancolini, 2021). The role of epigenetics in tumorigenesis has become more apparent in recent years. There is also growing evidence that the cancer cells can regulate the epigenetic changes in the stromal cells, inducing the production of a pro-tumor TME (Mishra et al., 2018; H. Fan et al., 2020), and overall TME heterogeneity. However, the current challenge is to pinpoint which cancer types are purely driven by non-mutational processes such as epigenetics and which are driven by mutations (Hanahan, 2022).

The microbiome varies a lot between individuals, and it has been reported recently by a study evaluating 1526 human tumors that bacteria resided both within the cancer cells and within the immune cells of the TME. Interestingly, the composition of the microbiome also varied a lot with the tumor type, with breast cancer having the most diverse microbiome (Nejman et al., 2020). It has also been reported that the intra-tumoral microbiome correlates with the disease subtype and can predict the overall prognosis (Sepich-Poore et al., 2021; Park et al., 2022). There is also emerging evidence that the gut microbiota can influence the immune responses, therapeutic responses, and may play a role in tumor promotion or suppression depending on the context. This field of research is still nascent, but it is very important to better understand the multifaceted role of the microbiome to effectively treat cancer (Hanahan, 2022; Park et al., 2022).

To summarize, the process of tumor progression is very complex and heterogeneous and involves the interplay of several components within the tumor but also external to the tumor such as TME and microbiome. The advances in cancer research is expected to unravel more such hallmarks and their role in cancer development in the future years (Hanahan, 2022).

2.1.1.1 Genomic instability as a key hallmark of cancer

Genomic instability occurs when mechanisms that protect the genome from aberrations are compromised, leading to an increased tendency of the genome to acquire mutations (J.-K. Lee et al., 2016; Jeggo et al., 2016). Genomic instability encompasses a wide range of genomic alterations, from small-scale nucleotide changes to large-scale chromosome level structural and numerical defects (known as "*chromosomal instability*" (CIN)) (Abbas et al., 2013; Eischen, 2016). Two distinct hypotheses explain the molecular basis for genomic instability-induced tumorigenesis. The mutator phenotype model suggests that genomic instability is

already present in the precancerous lesions due to the mutations in the gatekeeper genes (such as TP53) driving cancer development (Loeb, 2001; Loeb et al., 2008). This model can explain the molecular basis of hereditary cancers, where germline mutations in genome maintenance genes are present in the individual, and the subsequent loss of the remaining wildtype allele leads to genomic instability and drives tumor development (Negrini et al., 2010). However, since gatekeeper gene mutations are mostly recessive, for mutator phenotype to be valid for sporadic cancers, two alleles of the gene need to be mutated for genomic instability to occur, which by random chance is rare. Also, across several studies, it was observed that genomic instability in 69-97% of sporadic cancer cases was not due to the gatekeeper gene mutations, indicating other mechanisms (Wood et al., 2007; Parsons et al., 2008; Negrini et al., 2010). The oncogene induced DNA replication stress model hypothesizes that oncogene activation induced hyperproliferation causes excessive replication origin firing, leading to replication stress triggered by the stalling and collapse of replication forks, followed by the formation of DNA double strand breaks (DSBs). The constant generation of DSBs produces the genomic instability required to create a selection pressure for additional mutations, which allow the cells to evade apoptosis or senescence and eventually drive tumorigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005; Halazonetis et al., 2008; Kotsantis et al., 2018).

Chromosomal instability (CIN) can result from structural or numerical changes in the chromosomes. Structural changes include chromosomal breaks, translocations, fusions, deletions, or inversions while numerical changes involve loss or gain of one or more chromosomes known as "*aneuploidy*" or gain of whole or part of a genome known as "*tetraploidy*" and "*polyploidy*" (Yao & Dai, 2014; Eischen, 2016; J.-K. Lee et al., 2016). Different types of CIN are shown in **Figure 2**. CIN mostly occurs due to errors in chromosomal segregation during mitosis. It is now evident that almost all cancers have some amount of chromosomal aberrations - structural, numerical or a combination of both (Negrini et al., 2010; Bakhoum & Cantley, 2018; Drews et al., 2022). Telomere dysfunction is another source of CIN. Telomeres are protein complexes that are located at the end of chromosomes and prevent them from being recognized as DSBs, thus blocking fusions or recombination. However, when a break occurs at the chromosome ends, the telomere part of the chromosome can be lost, resulting in incomplete chromosomes that can fuse with other unrepaired DNA fragments (Bolzán, 2020; Maciejowski & de Lange, 2017). Tumor cells can also evolve by failing cytokinesis, leading to polyploidy and creating double the number of centrosomes, which causes chromosomal defects in the subsequent daughter cells (Fujiwara et al., 2005; Lens & Medema, 2019). Cancer evolution for a long period of time was believed to happen gradually by accumulation of mutations, or CIN over a period of time, and for most of the cancer types this is true. However, in 2011, Peter Campbell's group reported a new crisis

event called "*chromothripsis*" in which a single chromosome or a small group of chromosomes is shattered and merged together by error prone non-homologous end joining, causing several concurrent mutations (Stephens et al., 2011; Meyerson & Pellman, 2011). This phenomenon was first identified in a chronic lymphocytic leukemia (CLL) patient sample but analysis of other cancer types estimated that this event occurs in 2-3% of all cancer, and particularly more frequent in osteosarcoma (Stephens et al., 2011). Also, missegregation of chromosomes can lead to small fragments of chromosome to be encapsulated in the nuclear membrane known as "*micronuclei.*" Recently, it was reported that the cytosolic double stranded DNA (dsDNA) released from ruptured micronuclei activates the cGAS-STING and the canonical NF-κB inflammatory pathways, causing immune evasion and distant metastasis (Bakhoum et al., 2018; Bakhoum & Cantley, 2018). It was also found that periods of CIN can accelerate the development of therapeutic resistance and high degrees of CIN correlates with poor response to therapies (Lukow et al., 2021).

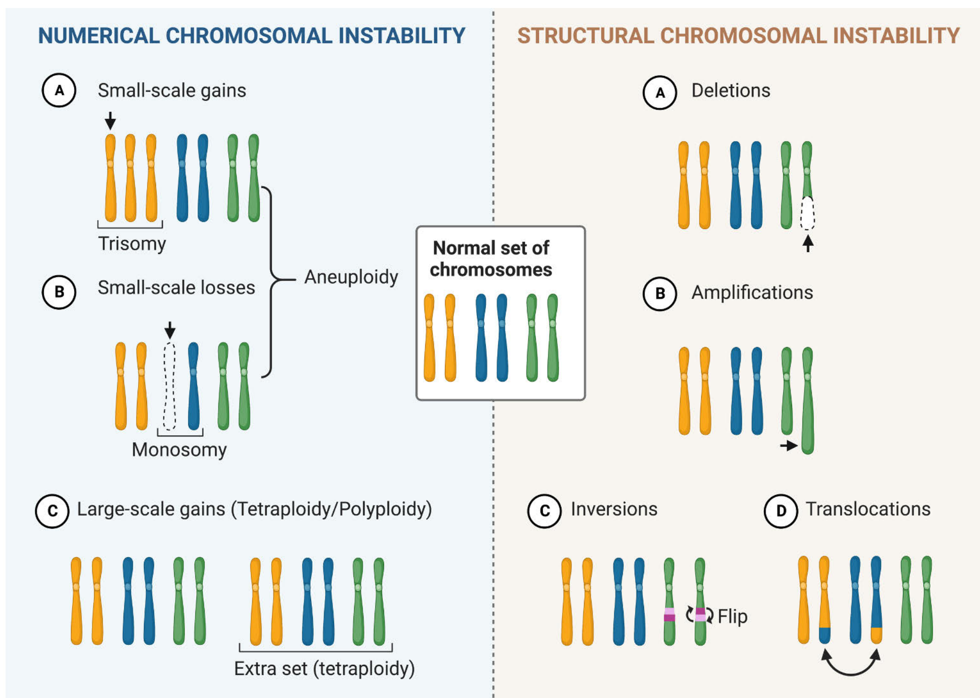

Figure 2: Different types of Chromosomal instability (CIN) created on *Biorender.com.*

2.2 Protein Phosphatase 2A

Post translational modifications (PTMs) are a way of modifying the properties of the protein, either by cleavage or by addition of a modifying group to single or multiple amino acids. PTMs occur in virtually all proteins in the eukaryotes and can transform its activity, localization, turnover or its interactions with other proteins (Mann & Jensen, 2003; Barber & Rinehart, 2018). Protein phosphorylation is the most abundant PTM in the cells (Bilbrough et al., 2022), in which a phosphate group is added in a reversible manner to the protein (Mann & Jensen, 2003). Most commonly the hydroxyl group containing amino acids (serine, threonine, and tyrosine) are phosphorylated but cysteine, lysine, histidine, arginine, aspartic and glutamic acid have also been reported to be phosphorylated at low frequency. The addition of phosphate groups is catalyzed by enzymes called protein kinases and the removal of the phosphate groups is catalyzed by the enzymes called protein phosphatases. (Hunter, 2012). For cellular homeostasis, a balanced and coordinated activity of protein kinases and protein phosphatases is required, and any switch in the balance leads to human diseases such as cancer (Hunter, 1995; Janssens et al., 2005).

The kinases and phosphatases are classified based on the amino acid that they act on. The three broad categories of phosphatases are protein serine/threonine phosphatases (PSPs) that regulate serine and threonine phosphorylations, protein tyrosine phosphatases (PTPs) that regulate tyrosine phosphorylations and dual specificity phosphatases (DSPs) that can regulate both serine/threonine as well as tyrosine phosphorylations (M. J. Chen et al., 2017; Shi, 2009). There are 518 protein kinases and out of these there are 90 protein tyrosine kinases (PTKs) and 428 protein serine/threonine kinases (PSKs) (Fleuren et al., 2016). One would expect that there would be similar number of phosphatases to maintain substrate specificity as the phosphorylation is a reversible reaction. While the number of PTPs (107) roughly match the PTKs, it might seem that the PSPs (~30) are outnumbered by the PSKs, leading to a misconception that they lack substrate specificity (Alonso et al., 2004; Shi, 2009; Sents et al., 2013; M. J. Chen et al., 2017). However, the assembly of PSP holoenzymes is quite complex, and they can form multiple substrate specific PSPs by combining different regulatory subunits with a single catalytic subunit. Hence the number of PSPs is definitely higher than the PSKs in the cell.

Protein Phosphatase 2A (PP2A) accounts for vast majority of the serine/threonine phosphatase activity in eukaryotes. PP2A comprises 1% of the total proteins within the cells but PP2A and PP1 (protein phosphatase 1) are responsible for more than 90% of the serine/threonine phosphatase activity of the cells (Eichhorn et al., 2009). PP2A is a heterotrimeric complex comprising of three subunits – a scaffolding A subunit (also called PR65), regulatory B subunit, and a catalytic C subunit. All the subunits of PP2A exist in multiple isoforms and maybe expressed depending on tissue and cell contexts. The A and C subunits both have two isoforms

represented by α and β, and Aα and Cα isoforms constitute the majority in most of the cells (**Figure 3**) (Eichhorn et al., 2009; Sangodkar et al., 2016). One third of the PP2A enzymes in the cells exist in a dimer form ($PP2A_D$), called the core enzyme comprising of an A subunit and a C subunit and the remaining exists in the trimeric form ($PP2A_T$) (Janssens & Goris, 2001). The scaffolding A subunit consists of 15 tandemly repeated HEAT (Huntington/Elongation/A-subunit/TOR) domains that extend to form a curved horseshoe structure. B subunits bind to the N-terminal 2-8 HEAT domains and the catalytic subunit binds with the C-terminal 11-15 HEAT domains (Groves et al., 1999; Cho & Xu, 2007; Westermarck & Hahn, 2008). The C subunit has two manganese ions which are important for its catalytic activity and interaction with the substrates (Cho & Xu, 2007; Goldberg et al., 1995). Though the Cα and Cβ subunits have 97% similarity in their genetic sequence, it was reported that Cα knockout mice were embryonic lethal indicating that the Cβ subunits do not compensate for the Cα subunit in the cells (Götz et al., 1998).

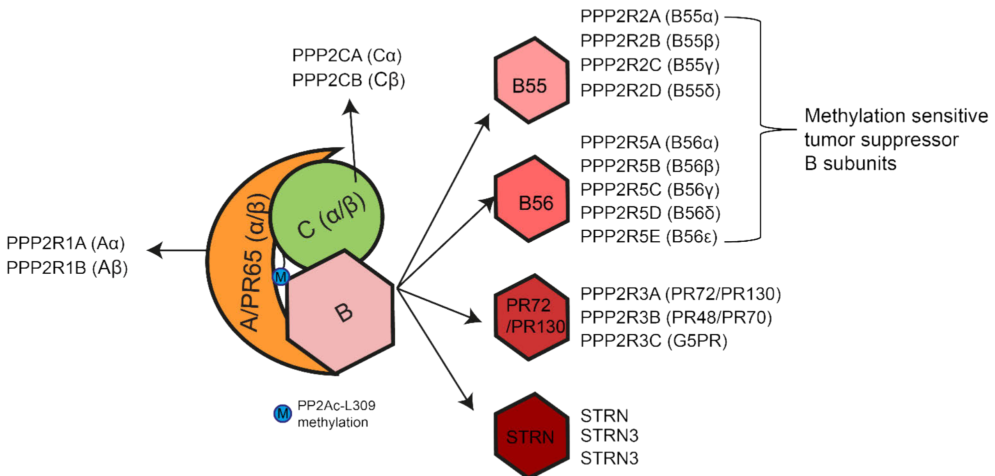

Figure 3: Subunits and gene nomenclatures of the PP2A heterotrimeric complex, adapted from (Westermarck & Neel, 2020).

The substrate specificity, subcellular localizations and finetuned phosphatase activity of PP2A comes from the regulatory subunits it can assemble. PP2A has 4 family of regulatory B subunits (B55, B56, PR72/PR130 or STRN family) and each of the B subunit family also has multiple isoforms encoded by different genes, as represented in the schematic **Figure 3** (Janssens & Goris, 2001; Eichhorn et al., 2009; Seshacharyulu et al., 2013). The B subunit families do not share much sequence similarity between each other except the amino acids at the interaction interface with the A subunit which are conserved (X. Li & Virshup, 2002). All together there are 15 genes which form almost 26 different B subunits when all isoforms and known splice variants are accounted for, and hence PP2A can exist in

atleast 100 different holoenzyme compositions (Janssens & Goris, 2001; Eichhorn et al., 2009; Seshacharyulu et al., 2013). Since there is a possibility of multiple PP2A subunits to be formed within the cell at the same time, the entire holoenzyme assembly process is strictly regulated. It is reported that monomers of C subunits are unstable and get degraded to prevent unnecessary activity (Sents et al., 2013). Also, the C subunits are synthesized in an inactive form, without the metal ions, bound to α4, (another non-canonical subunit of PP2A) which is activated just before the holoenzyme assembly by PTPA (PP2A phosphotyrosyl phosphatase activator) (Fellner et al., 2003; Sents et al., 2013). It has been reported that PTPA deficiency promotes cancer due to the inactive PP2A (Sents et al., 2017). Another form of regulation comes from PTMs (phosphorylation and methylation) of the C subunit. The C subunit C-terminal tail is conserved especially the 304-309 amino acids (TPDYFL). The L309 can undergo methylation which is a reversible reaction regulated by two enzymes LCMT-1 (leucine carboxy methyl transferase-1) which adds the methyl group and PME-1 (PP2A methyl esterase-1) which removes the methyl group. L309 methylation is required for assembly of B55 and B56 family subunits which are also the most widely characterized tumor suppressive PP2A B subunits. The phosphorylation on T304 and Y307 has been reported to inactivate PP2A. While T304 phosphorylation inhibits the B55 family B subunits, the Y307 phosphorylation inhibits both B55 and B56 family B subunits (Janssens et al., 2008; Sents et al., 2013; Sangodkar et al., 2016; Kauko & Westermarck, 2018; Westermarck & Neel, 2020).

Jakob Nilsson's group reported a mechanism of substrate specificity for PP2A-B56 family phosphatases. The B56 family recognizes and binds a short linear motif (SLiM) LxxIxE on its substrates, through which it can dephosphorylate phosphosites in the vicinity of the motif (Hertz et al., 2016). Later, this motif was further expanded to LpSPIxE and it was identified that a serine phosphorylation in the recognition motif is necessary for the PP2A-B56 binding (X. Wang et al., 2016). More recently, it was further elucidated that the substrates also have a basic patch region (positive charge region) which interacts with the acidic patch (negatively charge region) on the B56, and because of this electrostatic force the interaction between the PP2A-B56 and its substrates is strengthened (X. Wang et al., 2020).

2.2.1 Role of PP2A in cancer

The evidence for PP2A's role as a tumor suppressor comes from two historical independent findings **1)** Studies with okadaic acid, a carcinogenic compound found in black sponge was found to inhibit PP2A to promote tumor growth (Suganuma et al., 1988; Fujiki & Suganuma, 1999) **2)** Reports that several tumor promoting DNA viruses such as E4orf4, polyoma small and middle T antigens and simian virus 40

(SV40) small T antigens could replace B subunits of PP2A and inhibit PP2A function (Pallas et al., 1990; Kleinberger & Shenk, 1993; Shtrichman et al., 1999). This was further validated by pioneering work by Robert Weinberg, William Hahn and colleagues who established the rules and differences in malignant transformation of humans and rodents. They found that truly normal (primary) rodent cells can be fully transformed into cancer by just two genetic alterations – activating mutations in RAS and MYC oncogenes while the same does not apply to humans (Hahn & Weinberg, 2002; Land et al., 1983). In human kidney and fibroblast cells, constitutive activation of human telomerase (hTERT) along with the expression of Simian virus 40 (SV40) early region which express the Large T (LT) and the small T (ST) antigens could immortalize the cells, but these cells were fully transformed only after the subsequent introduction of RAS oncogene (Hahn et al., 1999; Hahn & Weinberg, 2002). It was later identified that LT inactivates p53 and retinoblastoma (RB) tumor suppressors (Ali & DeCaprio, 2001) and ST inactivates PP2A by binding to its A subunit or AC dimer but not when ABC trimer is present, indicating that ST has similar properties as B subunits of PP2A (Westermarck & Hahn, 2008; Yang et al., 1991). In summary, the five minimum steps needed for human transformation were identified as - RB, TP53 inactivation and hTERT activation (for immortalization), and further inactivation of PP2A and activation of RAS for transformation of the cells. Later it was identified that there are tissue specific differences in the Ras downstream factors needed for transformation. (Rangarajan et al., 2004; Westermarck & Hahn, 2008). Further it was reported that out of the 11 B subunits tested, only B56α, B56γ and PR72/PR130 were involved in the ST induced cellular transformation (Sablina et al., 2010).

Several years later, now there is plenty of evidence for PP2A as a tumor suppressor phosphatase which comes from inactivation of several cancer promoting proteins such as MYC, E2F1, AKT, DAPk (Kauko & Westermarck, 2018; Fowle et al., 2019). However, unlike other tumor suppressors like p53, Rb and PTEN, PP2A is rarely mutated in human malignancies. PP2A function is inactivated through other non-genetic mechanisms either by PTMs in the C-terminal tail of the C subunit (as discussed before) or more commonly by expression of endogenous inhibitors (Kauko & Westermarck, 2018). Some of the important endogenous inhibitors of PP2A, and their modes of inhibition are listed in the **Table 1**. There are atleast 15 inhibitors of PP2A reported and have distinct modes of PP2A inhibition. The endogenous inhibitors add another layer of substrate and context specific regulation of PP2A activity in the cells, as some of these inhibit specific regulatory subunits and also in specific cell cycle phases, for example ENSA and ARPP19 inhibits PP2A B55α and δ in mitosis (Kauko & Westermarck, 2018).

Table 1: List of well characterized PP2A endogenous inhibitors and their mode of PP2A inhibition

Name of the inhibitor	Mode of PP2A inhibition	References
CIP2A	Inhibits the B56α and γ subunits	(Junttila et al., 2007; J. Wang et al., 2017)
SET	Inhibits C subunit, but recently found to associate with B56 in Gastric cancer	(M. Li et al., 1995; Enjoji et al., 2018)
PME1	Dual effect: promotes demethylation of C-terminal tail L309; Also removes Mn+2 ions form the C subunit to inactivate the PP2A catalytic activity	(Ogris et al., 1999; Xing et al., 2008)
ARPP19	Inhibits the B55α and δ subunits; Isoform ARPP16 inhibits B56α	(Hached et al., 2019; Mochida et al., 2010)
ENSA	Inhibits the B55α and δ subunits	
TIPRL	Binds and inhibits C subunit and mutations in Aα enhance this interaction	(Haesen et al., 2016; Scorsato et al., 2016)

CIP2A- Cancerous inhibitor of PP2A; SET-SET nuclear protein; PME1-PP2A methyl esterase; ARPP19 – cAMP regulated phosphoprotein 19; ENSA- α Endosulfine; TIPRL-TOR signaling pathway regulator like.

PP2A is majorly a tumor suppressive phosphatase but there are some regulatory subunits such as Striatin (STRN) which have an oncogenic role. STRN containing PP2A complexes recruit several proteins and kinases forming a larger complex called Striatin interacting phosphatase and kinase (STRIPAK). It was recently reported that STRIPAK complexes can initiate Hippo signaling and activate YAP by MST1/2 dephosphorylation (R. Chen et al., 2019; Tang et al., 2020; Kurppa & Westermarck, 2020).

2.3 CIP2A

Cancerous inhibitor of Protein Phosphatase 2A (CIP2A), also known as p90 is a 90 KDa protein previously encoded by gene KIAA1524. p90 was first detected in hepatocellular and gastric cancer patient samples, but the function was unknown (Hoo et al., 2002). The true functional relevance of CIP2A in human malignancies was discovered by the Westermarck group in 2007 when it was discovered as an interacting partner of PP2A in tandem affinity purifications with PP2A-A subunit (PR65) (Junttila et al., 2007). Since then, CIP2A has been reported to be overexpressed in several human cancer types, and its expression was associated with poor prognosis in almost all the cancer types in which it was studied, (Vaarala et al., 2010; Côme et al., 2009; Khanna et al., 2009; Böckelman et al., 2011; Lucas et al., 2011; Niemelä et al., 2012; Khanna & Pimanda, 2016).

In breast cancer, CIP2A expression is correlated with aggressive subtypes and higher tumor grade. In a breast cancer cohort consisting of 159 samples, CIP2A expression in tumor grade 2 and 3 samples was much higher than the grade 1 samples (Côme et al., 2009). CIP2A expression was analyzed in the 1028 breast cancer samples from the Finnish registry (Joensuu et al., 2003) by IHC and CIP2A expression was found to be significantly higher in HER2+ and basal-like breast cancer subtypes which are the more aggressive breast cancer subtypes (Niemelä et al., 2012). The same trend was also observed in TCGA breast cancer cohort (n=1028 patients) in which CIP2A expression was higher in basal-like breast cancer followed by HER2+, luminal B, luminal A, and normal like subtypes (publication I, Figure 5C).

CIP2A expression is very low in normal cells except for the testis, but is many-fold higher in cancer cells as seen by IHC staining as well as mRNA expression levels from qPCR (Junttila et al., 2007; Côme et al., 2009). CIP2A knockout mice ($CIP2A^{HOZ}$) generated using genetrap cassette are fully viable and have a normal life span indicating that CIP2A is dispensable in normal tissues. The only exception is that male $CIP2A^{HOZ}$ mice showed defects in spermatogenesis, due to the decreased level of self-renewal protein PLZF (promyelocytic leukemia zinc-finger) in the spermatogonial progenitor cells (SPCs) of the testis (Ventelä et al., 2012).

While CIP2A alone was unable to transform the immortalized mouse embryonic fibroblasts (MEFs), it was able to increase the transformation ability of the Ras-overexpressing cells (Junttila et al., 2007). In immortalized human HEK-TERV cells, which require further PP2A inhibition for complete transformation, CIP2A demonstrated the ability to replace the role of SV40 small T-antigen (ST) in cellular transformation (Junttila et al., 2007). By stabilizing c-Myc, CIP2A overexpression can restore the transformation ability of JNK2 (c-Jun N-terminal Kinase 2) knockout MEFs, which are otherwise defective in Ras-induced transformation. CIP2A was found to be downstream of JNK2 signaling, and its expression was regulated transcriptionally by ATF2 (Activating Transcription factor 2) (Mathiasen et al., 2012).

Previous research has established that PP2A dephosphorylation of serine 62 on MYC marks it for proteasomal degradation (H. K. Arnold & Sears, 2006). When it was initially discovered, the main function of CIP2A as an oncogene was attributed to its PP2A inhibition mediated MYC S62 phosphorylation, thereby increasing its stability (Junttila et al., 2007). However, a recent report published by Welcker et al. has contradicted the previous model that S62 phosphorylation promotes MYC stability. The authors found that S62 phosphorylation actually enhances the binding of FBW7, a MYC ubiquitin ligase, indicating that S62 phosphorylated MYC does not escape the proteasomal degradation as previously thought (Welcker et al., 2022). With this finding coming to light, the exact mechanism of how PP2A inactivation by

CIP2A enhances MYC stability is yet to be elucidated. In gastric cancer cells, MYC regulates the expression of CIP2A mRNA and protein, indicating a positive feedback loop between MYC and CIP2A (Khanna et al., 2009). A microarray based CIP2A transcriptional signature revealed that CIP2A regulates several novel MYC target genes and the gene expression profiles generated by CIP2A silencing could be reversed by depletion of PP2A B55α and B56β subunits. This study also identified a novel MYC independent role of CIP2A in JNK2 signaling. MYC downregulation had no effect on the transwell migration whereas CIP2A silencing decreased JNK2 expression and transwell migration (Niemelä et al., 2012).

CIP2A is amplified and mutated at an extremely low level (from Oncoprint analysis using TCGA pan cancer data in cBioportal https://www.cbioportal.org/). Khanna et al. identified by bisulfite sequencing that though there are many CpG islands close to the CIP2A promoter they are not methylated in normal or cancer cells indicating that CIP2A is not regulated by promoter methylation (Khanna et al., 2011). Both these findings would hint that CIP2A is regulated at a transcriptional level, and this has been confirmed by several following studies. In gastric cancer cells, EGFR and MEK1/2 inhibitors decreased the CIP2A mRNA whereas ERK activator TPA increased the CIP2A mRNA, indicating that the EGFR-MEK pathway might be involved in the transcriptional regulation of CIP2A (Khanna et al., 2011). Further, on mutagenesis of the transcription factor binding sites on the CIP2A promoter, it was identified that EGFR-MEK1/2 regulates CIP2A through the transcription factor ETS1 (Khanna et al., 2011). However, in urogenital cancers, ETS1 and ELK1 were both needed together for CIP2A transcriptional regulation (Pallai et al., 2012). MYC regulates CIP2A transcription but the exact MYC binding site on the CIP2A promoter is not known (Khanna et al., 2009; Khanna & Pimanda, 2016). In gastric cancer, CIP2A was found to be regulated by CHK1 S345 phosphorylation activity which is activated by an upstream DNA damage kinase DNA-PK (Khanna, Kauko, et al., 2013). Recently, it was revealed that CHK1 regulates pSTAT3 which transcriptionally regulates CIP2A (Khanna et al., 2020).

CIP2A is also involved in a positive feedback loop with E2F1 which promotes evasion of senescence. In breast cancer cells, p53 inactivation was found to correlate with CIP2A expression, but the ChIP-Seq analysis revealed that p53 doesn't bind to the CIP2A promoter. Further downstream analysis revealed that p53 and associated p21 downregulate E2F1, which downregulates CIP2A. ChIP-Seq also confirmed that E2F1 indeed binds on the CIP2A promoter and regulates its transcription. Also, it was previously established that PP2A-B55α dephosphorylates E2F1 on serine 364, which is known to be important for its proteasomal degradation. Thus, E2F1 increases CIP2A expression transcriptionally, which inhibits PP2A's dephosphorylation activity on E2F1 S364, further increasing E2F1 stability. In p53 mutant breast cancer cells, depletion of CIP2A was able to rescue the p53 activation

mimicking SA-βGal senescence phenotype, indicating that high activity of E2F1-CIP2A positive feedback loop allows cancer cells to evade senescence (Laine et al., 2013). In TNBCs, CIP2A overexpression was found to mediate AKT S473 dependent phosphorylation of tumor suppressor p27Kip1, which translocates it to the cytoplasm. Also, Myc activation by CIP2A mediates transcriptional repression of p27Kip1 and this combined effect facilitates the progression of TNBCs (H. Liu et al., 2017).

CIP2A is involved in several forms of cancer cell survival or death evasion mechanisms. CIP2A suppresses apoptosis by preventing the dephosphorylation of AKT S473 by PP2A-B55γ in hepatocellular carcinomas (K.-F. Chen et al., 2010). CIP2A prevents the PP2A mediated dephosphorylation of Death Associated Protein kinase (DAPk) at serine 308, which suppresses UNC5H2/Netrin-1 mediated apoptosis (Guenebeaud et al., 2010). CIP2A enhances mTORC1 pathway activity by preventing PP2A mediated dephosphorylation of mTORC1 downstream components RPS6KB1 and EIF4EBP1 which are known to inhibit autophagy. On the contrary mTOR inhibitors such as Rapamycin also promote CIP2A degradation by autophagy (Puustinen et al., 2014; Puustinen & Jäättelä, 2014). It was reported that CIP2A gets degraded by chaperone mediated autophagy (CMA) a type of lysosomal degradation that gets activated in conditions of cellular stress. Depletion of LAMP2A or pharmacological inhibition of CMA by lysosomal inhibitors ammonium chloride and Leupeptine caused upregulation of CIP2A. It has been reported that CMA is defective in several cancer cells, and this is a means to increase levels of CIP2A which promotes MYC stability and tumorigenesis (Gomes et al., 2017).

CIP2A interacts with Polo-like kinase 1 (PLK1) and promotes the progression of cells into mitosis. Also, CIP2A translocates to the nucleus before mitotic entry and is enriched at the spindle poles (J.-S. Kim et al., 2013). In prostate cancer cells, CIP2A causes the proteasomal degradation of a chromosome maintenance protein shugoshin 1 (Sgol1) which destabilizes the cohesion complex, causing premature chromosome segregation and aneuploidy (Pallai et al., 2015). During meiotic maturation in mouse oocytes, CIP2A acts as a scaffold for CEP192 (a centrosomal protein), and recruits Aurora A and PLK1 at the spindle poles. PLK1 phosphorylates CIP2A at S904 and this phosphorylation facilitates the proper organization of microtubule organizing centers (MTOCs) (H. Wang et al., 2017).

CIP2A is also a critical oncoprotein in hematological cancers. In chronic myeloid leukemia (CML), CIP2A promotes MYC and BCR-ABL1 tyrosine kinase activity and is a critical marker that determines progression to blast crisis (Lucas et al., 2011). CML patients with high CIP2A levels treated with second generation tyrosine kinase inhibitors (2G TKIs) showed decreased E2F1 levels and thereby had low risk of progression indicating 2G TKIs maybe a preferential treatment option for high

CIP2A CML patients (Lucas et al., 2015). The first ever splice variant of CIP2A called Novel CIP2A Variant (NOCIVA) was discovered recently. It comprises CIP2A exons 1-13 and a 13 amino acid peptide tail but still retains the ability to bind to PP2A B56α. In both acute myeloid leukemia (AML) and CML, high NOCIVA expression correlates with poor progression. In CML, high NOCIVA expression is linked with resistance to imatinib but not to 2G TKIs nilotinib or dasatinib (Mäkelä et al., 2021).

CIP2A is an attractive cancer drug target because it is overexpressed in several cancer types but dispensable for normal tissues (except testis). Out of the 905 amino acids, a 1-560 amino acid fragment of the CIP2A protein was crystalized recently. The crystal structure revealed that CIP2A exists as a homodimer and interacts with PP2A B56α and B56γ subunits. The dimerization interface was identified as a region between 388-559 amino acids. Interestingly, disrupting the dimerization by single point mutations (such as R522D and L533E) in the dimerization interface led to decreased CIP2A stability and binding. CIP2A stability was also lost when the expression of B56α and B56γ subunits was inhibited by siRNAs. This information is very critical for future drug development efforts (J. Wang et al., 2017). CIP2A has been implicated in drug resistance to several common cancer therapies. CIP2A promotes resistance to doxorubicin by inducing the phosphorylation of AKT which suppresses apoptosis (K.-F. Chen et al., 2010; Y. A. Choi et al., 2011). In HER2 negative breast cancer patients, CIP2A overexpression correlated with poor overall survival specifically in the patient group that received vinorelbine compared to the group that received docetaxel. CIP2A silenced MCF7 cells were more sensitive to E2F1 downregulation on treatment with vinorelbine compared to the non-silenced cells, indicating that CIP2A low cancer is more sensitive to senescence inducing chemotherapy (Laine et al., 2013). CIP2A was reported to be an OCT4 target gene and double positivity in OCT4 and CIP2A was correlated with resistance to radiotherapy in head and neck squamous cell carcinoma (HNSCC) (Ventelä et al., 2015). In mouse intestines, CIP2A was essential for regeneration of intestinal crypt cells from radiation or cisplatin induced DNA damage, indicating that DNA damaging therapies in combination with CIP2A inhibition might have a negative impact in normal cell regeneration and must be used with caution (Myant et al., 2015). In ovarian cancer, ROS (reactive oxygen species) inducing drug APR-246 sensitized the low CIP2A expressing cells, but high CIP2A expressing cells were resistant to this drug due to activation of NF-kB survival signaling. The sensitivity was restored by combining APR-246 with NF-kB inhibitors (Cvrljevic et al., 2022). Recently an endogenous inhibitor of CIP2A was encoded by the long noncoding RNA (lncRNA) LINC00665 was reported. This peptide was named CIP2A-BP (CIP2A binding protein) as it showed ability to bind CIP2A and activate PP2A's B56γ and was downregulated by TGFβ and SMAD signaling in TNBCs (Guo et al.,

2020). Another recent study reported that CIP2A S904 phosphorylation creates a binding site for 14-3-3 regulatory protein, which can be targeted by Fusicoccin-A (Brink et al., 2022).

There have been many compounds reported in the literature, that have been indicated to downregulate CIP2A, but none of these are direct inhibitors but downregulate CIP2A mRNA by inhibiting its transcription factor activity (such as EGFR or DNA-PK or CHK1 inhibitors) or drugs that cause proteasomal degradation of CIP2A (such as celastrol, gambogenic acid) (Soofiyani et al., 2017). Most of these drugs lack mechanistic details and proper testing controls to determine adverse effects. Metformin, a well-known oxidative phosphorylation drug was shown to be downregulating CIP2A, but the exact mechanism is not known (Elgendy et al., 2019). In my dissertation, I identified the role of small molecule activators of PP2A (SMAPs) as CIP2A transcriptional inhibitors and has been presented in publication I (Laine et al., 2021).

2.4 Breast cancer

Breast cancer is the leading cause of cancer incidence across the globe accounting for 2.3 million new cases (11.7% of all cancer cases). Among women, breast cancer is responsible for 1 in 4 new cancer cases, and 1 in 6 cancer deaths (M. Arnold et al., 2022; Sung et al., 2021). If the current trends continue, it is estimated that by 2040, the global mortality rates due to breast cancer will increase to 1 million deaths per year (M. Arnold et al., 2022). As per the WHO's GLOBOCAN database, the incidence of breast cancer is much higher in highly developed countries compared to less developed countries, and this could be attributed to lifestyle factors such as obesity, physical inactivity, or hormonal risk factors such as less breastfeeding, oral contraceptives, later age at first pregnancy or advances in early diagnosis or screening mammography technologies (Sung et al., 2021).

The human mammary gland comprises around six to ten intertwined duct systems, each originating at the nipple and ending at the terminal ductal lobular unit (TDLU). This forms a complex branched network of ducts and lobules (Geddes, 2007; Yoder et al., 2007). The lobules produce the milk which is secreted into the ducts. The epithelium lining the ductal-lobular system have two distinct cell types – the inner luminal epithelial cells which has secretory functions and the surrounding basal or myoepithelial cells which have contractile properties for ejecting the milk (Cristea & Polyak, 2018; Yoder et al., 2007). 95% of breast cancers are carcinomas that form in the epithelial cells lining the ductolobular system. They are termed ductal carcinomas if they originate in the ducts and lobular carcinomas if they originate in the lobules (Lopez-Garcia et al., 2010; Yoder et al., 2007). These carcinomas originate as precancerous lesions which are

confined to the ducts or lobules called Ductal Carcinoma *in situ* (DCIS) or Lobular carcinoma *in situ* (LCIS), respectively. DCIS and LCIS progress into invasive ductal carcinoma (IDC) or Invasive Lobular carcinoma (ILC) when the cancer cells invade the outer myoepithelial cells and enter into the surrounding stroma (Yoder et al., 2007). The DCIS cases were not that easily detected before the implementation of population screening. The challenge with DCIS is that it is difficult to determine which of these lesions would progress to invasive carcinoma and hence, currently all the DCIS patients are treated with breast conserving surgery and radiotherapy or alternatively with mastectomy. However, DCIS remains quiescent, and this overtreatment affects the well-being of the patients (van Seijen et al., 2019). The most common form of invasive breast carcinoma is IDC comprising around 50-80% cases whereas ILC accounts for 10-15% cases. The rest belong to mixed ductal/lobular or other rare histologies (Waks & Winer, 2019).

2.4.1 Intrinsic and molecular subtyping of breast cancer

Breast cancer is a very heterogeneous disease and presents with distinct biological and clinical features (Polyak, 2007; Weigelt & Reis-Filho, 2009; Zardavas et al., 2015). Historically, breast cancer has been classified based the status of the three receptors Estrogen receptor (ER), Progesterone receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) that are found typically on the surface of the breast cancer cells. Based on the receptor expression status they were classified as luminal (ER+/PR+), HER2 amplified (ER- and HER2+), or triple-negative breast cancer (TNBC) (ER-, PR- and HER2-) (Norum et al., 2014; Szymiczek et al., 2021). With the advent of next generation sequencing technologies, a lot of research groups tried to classify this heterogeneous disease into distinct subtypes based on their clinical prognosis. There were majorly five distinct subtypes identified – Luminal A, Luminal B, HER2 amplified, Basal-like breast cancer and normal-like breast cancer (Perou et al., 2000; Sørlie et al., 2001, 2003; Sotiriou et al., 2003). The classification was based on “intrinsic” gene sets that did not vary on repeated testing of the same tumor but can still detect the differences between tumors from other subtypes (Chung et al., 2002; Hu et al., 2006). Hu et al. analyzed all the existing gene signatures at that time and concluded that while there might be differences in gene lists across the signatures, the molecular portraits created by the interplay of these individual genes is preserved (C. Fan et al., 2006; Hu et al., 2006).

On analysis of molecular portraits, basal-like breast cancer (BLBC) group was mainly enriched for keratins 5 and 17, whereas the HER2+ subgroup was highly enriched for genes in the ERBB2 (HER2) amplicon. Normal-like group showed high expression of adipose tissue and other non-epithelial cell markers while also

expressing the basal markers (Sørlie et al., 2001). Since most of these signatures were developed using microarray technology, the samples had to be analyzed from fresh frozen tissue which was a challenge (Toft & Cryns, 2011; Viale, 2012). This, combined with the added time, resources, and expense for undertaking gene expression analysis, has been a major barrier for adopting these signatures (Alluri & Newman, 2014). So immunohistochemical surrogates were developed which can classify the breast cancer to similar intrinsic subtypes. While ER, PR and HER2 markers could identify the patients with luminal and HER2 amplified cases, who can be treated with hormonal or HER2 directed therapies respectively, the major challenge has been identifying BLBCs and distinguishing between luminal A and B subtypes. Though both luminal A and B subtypes comprised of ER associated genes, luminal B had poorer survival. On closer evaluation, luminal B subtype was enriched for proliferation genes such as CCNB1, MKI67 (Ki67) and MYBL2 (Sørlie et al., 2001; Hu et al., 2006). Hence there was a recommendation to use proliferation marker Ki67 in IHC to subclassify the luminal breast cancer into luminal A and B subtypes (Cheang et al., 2009). Cheang et al. proposed the use of ER, PR, HER2, EGFR and cytokeratin 5/6 antibodies in IHC to classify BLBCs (Cheang et al., 2008; Nielsen et al., 2004). Further, statistically defined gene set of 50 genes called PAM50 (Prediction Analysis of Microarray 50) was developed using correlations from microarray and qPCR data, so that it can be used in an easy qPCR format and suitable also for formalin fixed paraffin embedded (FFPE) samples. Interestingly, the PAM50 identified that almost one-third of HER2 amplified intrinsic subtype by PAM50 were not clinically HER2 positive and not detected by IHC and also 6% of clinically HER2 positive tumors were classified as basal-like by PAM50 (Parker et al., 2009). PAM50 is currently approved by US FDA and being administered in a Nanostring format (Wallden et al., 2015). Though the discordance between IHC profiling and gene expression profiles have been acknowledged by the physician experts, it was surprising to see that even in the latest breast cancer treatment guideline updates immunohistochemistry is still considered a superior standard than these signatures for breast cancer classification (Allison et al., 2020). Recently, the 17th St. Gallen international breast cancer conference experts updated the clinicopathologic surrogate definitions for intrinsic breast cancer subtypes. They classified breast cancer into Luminal A, Luminal B, HER2 positive and Triple Negative subtypes as depicted in the **Figure 4**. The group also recommended use of gene-expression signatures for classifying the luminal subtype into luminal A or B depending on the risk of recurrence. Surprisingly, the group also found that staining for basal keratins for classifying TNBCs further into BLBCs was not reproducible for general use and since there was an estimated 80% overlap in the TNBC and BLBCs

they decided to stick to previous triple-negative classification and use it interchangeably for BLBCs (Burstein et al., 2021).

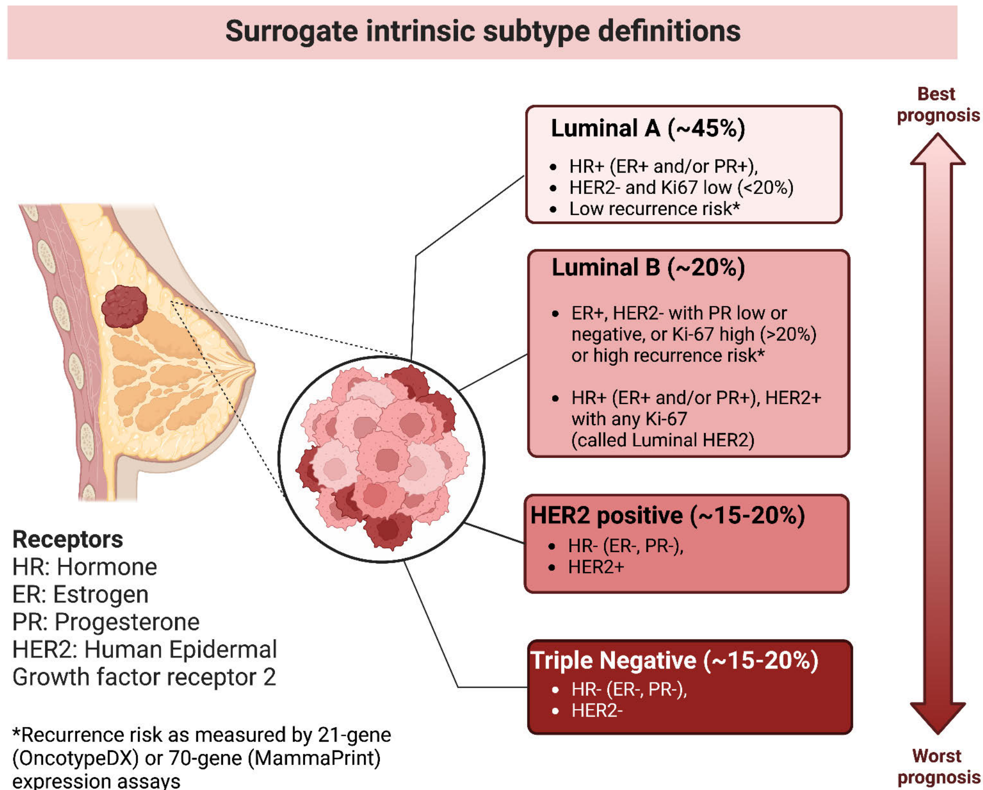

Figure 4: Breast cancer intrinsic classification based on the immunohistology surrogates, as per the 17th St. Gallen International Breast cancer conference, as adapted from (Burstein et al. 2021) created using Biorender.com.

2.4.2 Treatment of breast cancer

The choice of breast cancer treatment is determined based on several factors such as molecular subtype, patient's risk of relapse, tumor size, lymph node involvement, age, menopausal status, and preferences of the patient. In early breast cancer the aim of the treatment is to eliminate tumor from the breast and lymph nodes to prevent metastases whereas in metastatic breast cancer, the goal is to prolong life and overall well-being of the patients (Cardoso et al., 2019; Waks & Winer, 2019; Loibl et al., 2021). In early breast cancer the preferred choice of treatment is breast conserving surgery. If the tumor margins are difficult to determine or if breast conservation is not possible then mastectomy is performed. If the tumors are large, systemic chemotherapy maybe started before the surgery (neoadjuvant chemotherapy) to

shrink the tumor and improve the surgical outcomes. In luminal A subtype, neoadjuvant chemotherapy is given only if the risk of recurrence is high based on the gene-expression signatures. It has been reported that 40% of tumors requiring mastectomy could be converted to breast conserving surgery on treatment with neoadjuvant chemotherapy (Golshan et al., 2016). Patients undergoing breast conserving surgery are treated with post-operative radiotherapy and endocrine therapies (in ER+ breast cancer); or adjuvant chemotherapy (all subtypes); or chemotherapy in combination with anti-HER2 therapies trastuzumab/pertuzumab (for HER2+ breast cancer) (Cardoso et al., 2019).

In metastatic ER+ breast cancer, CDK4/6 inhibitors (palbociclib, ribociclib and abemaciclib) are used in combination with endocrine therapy drugs, and if the disease progresses, they may be treated with mTOR inhibtors in combination with endocrine therapy. If the patients are positive for PIK3CA amplification, they may be treated with PIK3CA inhibitor alpelisib in combination with endocrine therapies, although alpelisib does not currently have reimbursement in Finland. If all the tried lines of targeted treatments fail, or if the patient has risk of organ failure they are started off on chemotherapy (Gennari et al., 2021). In metastatic HER2+ breast cancer, taxane chemotherapy in combination with anti-HER2 therapies such as trastuzumab and pertuzumab is the recommended first line therapy irrespective of the ER status. Approximately after six to eight cycles of chemotherapy, trastuzumab-pertuzumab are given as a maintenance therapy. If the disease is ER+, then endocrine therapies can also be added to the maintenance therapy. The second line treatments in metastatic HER2+ breast cancer are antibody drug conjugates such as trastuzumab-deruxtecan or trastuzumab emtansine (T-DM1) (Gennari et al., 2021; Loibl et al., 2021). Recently a combination with tucatinib, capecitabine and trastuzumab was approved for brain metastases from HER2-positive metastatic breast cancer patients (Murthy et al., 2020).

For metastatic TNBC patients, the first line of treatment is determined based on PD-L1 or BRCA status. In PD-L1+ cases, atezolizumab (PD-L1) inhibitors in combination with albumin bound paclitaxel (nab-paclitaxel) or pembrolizumab (PD-1 inhibitor) in combination with taxane chemotherapy are recommended. In case of BRCA1/2 mutant patients, platinum-based chemotherapy (instead of taxanes), or PARP inhibitors are recommended. In all the other cases, the patients are treated with anthracycline & taxane combination chemotherapy (Loibl et al., 2021). If the disease still progresses, they are treated with sacituzumab govitecan (SG) an antibody drug conjugate that contains an antibody that targets trophoblast cell surface antigen -2 (Trop-2) linked to SN38 a metabolite of irinotecan. In ASCENT study, SG prolonged the overall survival compared to standard chemotherapy (10.9 months vs 4.9 months) (O'Shaughnessy et al., 2021; Carey et al., 2022) and if the

disease further progresses, they are treated with eribulin, vinorelbine or capecitabine chemotherapy (Gennari et al., 2021).

2.4.3 Basal-like breast cancer (BLBC)

2.4.3.1 Hallmarks of BLBC

Basal like breast cancer (BLBC) accounts for 15-20% of breast cancer cases. Contrary to luminal breast cancer, BLBC is more common in premenopausal women and is more prevalent in women with increased parity, an earlier age at menarche, and an earlier age at first pregnancy (Toft & Cryns, 2011). Using IHC, BLBC can be defined by a) No expression of ER, PR and HER2 receptors; b) expression of basal cytokeratins such as CK5/6, CK14 and CK17; c) EGFR positivity or amplification; and d) High Ki67, but there is no standard and internationally accepted consensus definition of BLBC (Banerjee et al., 2006; E. A. Rakha et al., 2008; Badve et al., 2011). Other markers that are sometimes used are positivity for smooth muscle actin (SMA), Vimentin or p63 (Livasy et al., 2006). In the last two decades, advances in next generation sequencing has made enormous strides in our understanding of the molecular portraits of different breast cancer subtypes, especially BLBC (Perou et al., 2000; Sørlie et al., 2001; Sotiriou et al., 2003). BLBCs are characterized by *high degree of genome instability* due to mutation in one or more of p53, PTEN, pRB, BRCA, p16 leading to high number of DNA losses and gains compared with other breast cancer subtypes (E. A. Rakha et al., 2008; Badve et al., 2011; Prat et al., 2015). There is emerging evidence that in many sporadic BLBCs, BRCA1 might be expressed normally but epigenetic or other genetic alterations maybe present in genes associated with BRCA such as RAD50, BLM, ATM or genes of FA pathway which would phenocopy BRCA1/2 mutations, a concept known as "*BRCAness*" (Turner et al., 2004; De Summa et al., 2013; Toft & Cryns, 2011). The other common trait of BLBCs is *high proliferative activity* associated with increased Myc, E2F activity, Cyclin E1 overexpression (Sotiriou et al., 2003; Alles et al., 2009; Milioli et al., 2017) and EGFR expression (or amplification in minority of cases) (Badve et al., 2011; E. Rakha & Reis-Filho, 2009).

TNBC is used as a surrogate for BLBCs in the clinic, and while they may have a lot of similarities and are used interchangeably, they are not the same biologically (Alluri & Newman, 2014). Triple-negative tumors with basal features (BL-TNBCs) have higher mitotic indices and more aggressive progression compared to the triple-negative tumors with non-basal features (10-year survival 56% vs 75.5%) (Rakha et al., 2009). It was reported that BLBCs have better response to neoadjuvant chemotherapy compared to the ER+ breast cancer subtype which could be explained by high mitotic indices (Colleoni et al., 2004). Another study reported that BL-

TNBCs are less responsive to anthracycline chemotherapy compared to non BL-TNBCs (Conforti et al., 2007). Lehmann et al. classified the TNBC tumors based on gene expression profiles into 6 distinct subtypes. The Basal like 1 subgroup were enriched for Cell cycle and DDR genes while the Basal like 2 subgroup was enriched for Growth factor signaling components such as EGF, MET, Wnt/β Catenin (Lehmann et al., 2011).

2.4.3.2 Clinical challenges of BLBC

Breast cancer is genomically one of the most widely characterized cancer subtypes. Despite massive sequencing efforts, there has not been much success in identifying genomic drivers for BLBC (Banerji et al., 2012; Koboldt et al., 2012; Pereira et al., 2016). The search for somatic driver mutations in BLBC thus far only identified TP53 and BRCA1/2 mutations (Koboldt et al., 2012). Although only a tiny percentage of BLBC patients have BRCA mutations, which can be treated with PARP inhibitors, p53 activation therapies have not had much success. Additionally, because p53 is a transcription factor, drugging it has its own set of difficulties (Wallis et al., 2023). In the clinic, triple negative breast cancer (TNBC) is used as a surrogate for BLBC as the EGFR and basal cytokeratin expression required to distinguish BLBC from TNBC are not assessed routinely (Alluri & Newman, 2014; Burstein et al., 2021). Also, since majority of the BLBC tumors are of triple-negative origin, they lack the expression of breast specific surface receptors ER, PR and HER2 which can be targeted (Toft & Cryns, 2011). While immune checkpoint inhibitors and antibody drug conjugates such as sacituzimab govitecan have been approved for use in metastatic TNBC settings, they are not applicable to all BLBCs. Hence one of the major challenges of BLBC has been poor understanding of molecular mechanisms that drive this cancer so that better therapies to target all BLBCs can be developed.

As there are no drivers which can be therapeutically targeted, chemotherapy is the sole choice for many BLBC patients (Yin et al., 2020; Marra et al., 2020). Because BLBC progresses aggressively, even the early diagnosed patients are started off on rigorous polychemotherapy. This is primarily to prevent the risk of distant metastases in these patients. While polychemotherapy decreases the risk of metastases by one-third, it also causes overtreatment. It has been estimated that among the early breast cancer patients that get adjuvant chemotherapy, only 20-30% of the patients receive the benefit, and the others are subjected to unwanted side effects (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2019; Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al., 2012). Though BLBC is highly heterogenous and encompasses multiple diseases, they are all treated uniformly with the same chemotherapy regimen (Marra et al., 2020). Within BLBC, some patients have significant recurrence rates during the first 3-5 years, whilst

others live for more than 10 years, with some patients in the latter category having a better prognosis than luminal breast cancer (Milioli et al., 2017). Existing gene expression profiling techniques such as MammaPrint, PAM50, Oncotype DX etc. have been developed as prognostic and predictive tools and can indicate which patients have high risk of recurrence and thereby advisable to treat with chemotherapy (Alluri & Newman, 2014; Vieira & Schmitt, 2018; Allison et al., 2020). But the challenge is that these diagnostic tools mainly work for ER+ breast cancer patients and not really implemented for aggressive breast cancer (Gagan et al., 2020). To summarize, all the above-mentioned factors indicate that there is a great clinical unmet need for developing better treatment stratification and personalized medicine approaches for BLBC.

2.5 DNA Damage Response (DDR) signaling in cancer

The human genome is subjected to extensive assault each day from a range of endogenous and exogenous sources which cause distinct types of changes in the DNA structure. The eukaryotic cells have evolved a complex integrated and interconnected signaling cascade collectively known as DNA damage response (DDR) which not only detects this damage but also ensures that it is repaired faithfully. The DDR also activates cell cycle checkpoints that arrest cells and provide the necessary time needed to repair the damage before progressing further in the cell cycle. In case the damage is beyond repair, DDR pushes the cells towards senescence or apoptosis. Failure to repair the damage or to repair them incorrectly leads to the formation of mutations. If the mutations occur in genes that are involved in genome maintenance and cell cycle, and thereby compromises the checkpoints, this leads to the accumulation of further mutations causing genomic instability or chromosomal aberrations that eventually lead to cancer (Ciccia & Elledge, 2010; Jackson & Bartek, 2009; Pilié et al., 2019).

2.5.1 Sources of DNA damage

The endogenous DNA damage mainly arises from cellular processes such as replication, metabolism, and inflammatory response. The chemical makeup of the DNA makes it susceptible to spontaneous hydrolysis in an aqueous environment (N. Chatterjee & Walker, 2017). The modifications can occur in DNA by base loss due to depurination or interconversion of bases due to deamination. DNA can also be chemically modified by alkylation or damaged by free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated during cellular metabolism and inflammatory response (Ciccia & Elledge, 2010;

Hoeijmakers, 2009; Kawanishi et al., 2006). During replication, the most common damage that occurs is the incorporation of wrong nucleotides. The external sources of DNA damage include environmental factors like ultraviolet (UV) rays, ionizing radiation (IR, such as X-rays and cosmic rays), and chemical agents like chemotherapeutics and cigarette smoke (Ciccia & Elledge, 2010; Jackson & Bartek, 2009; Tubbs & Nussenzweig, 2017). UV rays cause pyrimidine dimers and other photo products whereas cigarette smoke and platinum-based chemotherapy drugs cause the formation of bulky adducts. IR and other chemotherapeutics cause DNA crosslinks or single strand breaks (SSBs). The SSBs if unrepaired, get converted to double strand breaks (DSBs) which are highly cytotoxic to the cell, and hence require immediate attention (Ciccia & Elledge, 2010; Hoeijmakers, 2009; Tubbs & Nussenzweig, 2017). The DSBs are also considered to be mutagenic as they can form basis for chromosomal translocations (Bunting & Nussenzweig, 2013).

The chemical agents both human-generated and coming from the environment, which cause cancer are termed carcinogens. A carcinogen can be classified as a “genotoxic carcinogen”, if it can cause cancer by directly binding or damaging the DNA or a “non-genotoxic carcinogen” if it causes cancer by indirect mechanisms not related to direct DNA binding or damage (Hayashi, 1992; van Delft et al., 2004). A recent study by Serena Nik-Zainal and colleagues evaluated the mutational signatures by whole genome sequencing of 324 induced pluripotent stem cells (iPSCs) exposed to 79 carcinogens. Intriguingly, each mutagen produced its unique characteristic signature some of which matched the signatures found in human malignancies. The study also revealed mutagens which showed a mixture of signatures. This resource and approach can be used in the future to identify and associate the exact causative carcinogen for a particular cancer type (Kucab et al., 2019).

2.5.2 Mechanisms of DNA repair

Depending on the type of damage, eukaryotes have evolved distinct repair mechanisms and pathways that are non-redundant to a greater extent but at the same time can be compensated by other pathways in case of absence or defects in the optimal pathway (Jackson & Bartek, 2009). Every repair pathway typically comprises four major components – sensors, signal transducers, mediators, and effectors. Sensors are proteins that initiate the DDR by recognizing the aberrant DNA structures. Transducers are master kinases (ATM, ATR, and DNA-PK) that phosphorylate downstream factors and amplify the signal. Effectors are substrates of the kinases and are involved in the downstream cell cycle, DNA replication, repair, and apoptosis process. Mediators are proteins that are involved in the overall DDR

signaling cascade between the sensors and effectors (Harper & Elledge, 2007; Maréchal & Zou, 2013; Lozano et al., 2021). It is estimated that the DDR signaling comprises 450 different proteins and defects in at least 100 of these genes predispose individuals to cancer or other diseases (O'Connor, 2015; Pearl et al., 2015). This dissertation focuses on components of the base-excision repair and double strand break repair pathways, especially homologous recombination repair and hence they are covered in more detail while the rest of the pathways are briefly summarized in the subsequent chapters. The most important DNA damage sources and their repair mechanisms are summarized in the schematic **Figure 5.**

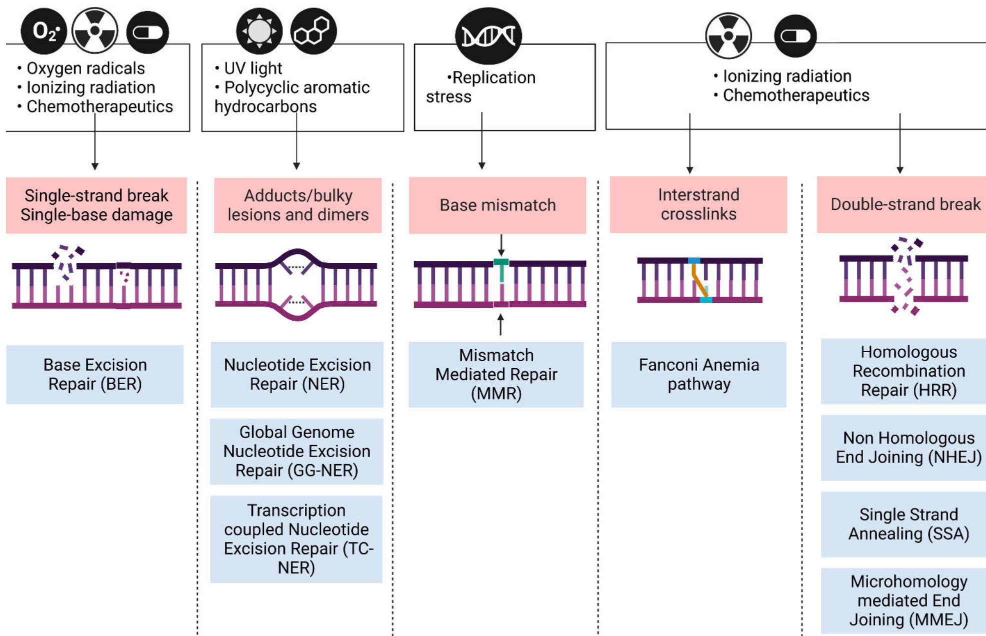

Figure 5: Sources of DNA damage and their repair pathways, created with Biorender.com.

2.5.2.1 Double strand break repair

The double strand breaks (DSBs) are repaired by two major pathways Non-Homologous End Joining (NHEJ) and Homologous Recombination Repair (HRR). There are alternatives to these pathways – alternative End Joining (alt-EJ) and Single Strand Annealing (SSA) that become active in case of defects or unavailability of important factors in these predominant pathways. The NHEJ is pathway is also called canonical/classical NHEJ (c-NHEJ) to distinguish it from the alt-EJ pathways. c-NHEJ is active throughout the cell cycle but predominant in G0/G1. The NHEJ pathway repairs the damage by blunt end ligation and is therefore slightly error

prone/mutagenic because it may cause small deletions of ~1-4 nucleotides. HRR pathway on the other hand requires extra copy of undamaged DNA which can be used as a homology template for accurate repair. It is therefore active only in S/G2 phases of cell cycle (Ceccaldi et al., 2016; Scully et al., 2019). The first step of the DSB repair process is the activation of PI3K-like kinases (PI3KK) ATM (Ataxia Telangiectasia Mutated), ATR (Ataxia Telangiectasia and Rad3 related) and DNA-PKcs (DNA dependent protein kinase catalytic subunit). The breaks generated by ionizing radiation are detected by DNA-PK and ATM kinases, whereas the breaks arising from replication stress or replication fork collapse forming single stranded DNA (ssDNA) are detected by ATR kinase (Shiloh, 2003). On detection of the damage, these kinases get activated by auto-phosphorylation. But in order to prevent promiscuous activation the process is tightly regulated, and each of these kinases need a co-factor protein to be recruited to the site of the damage. Ku80, part of the Ku heterodimer recruits DNA-PK; NBS1 belonging to the MRN (MRE11, RAD50, NBS1) complex recruits ATM; and ATRIP (ATR interacting protein) recruits ATR (Blackford & Jackson, 2017).

c-NHEJ begins by the binding of Ku70-Ku80 heterodimer to the DSBs, which recruits the DNA-PKcs and subsequently activates and recruits other c-NHEJ components to the site of damage such as DNA Ligase IV (LIG4) and its associated scaffolding protein XRCC4 (X-ray repair cross complementing 4) or XRCC4 like factor (XLF) or paralog of XRCC4 and XLF (PAXX). If the DSB creates blunt ends, the Ku70-Ku80 dimer can directly bind to the DSB and ligate the ends with the help of XRCC4-LIG4. If short single strand DNA overhangs are generated, a nuclease Artemis cleaves off the overhangs and Ku dimer binding can get re-established at the damaged site. If the small overhangs are incompatible for end processing or end joining by Artemis or XRCC4-LIG4, then DNA Polymerase μ or λ (POLM, POLL-Pol μ/ λ) maybe involved in an iterative end resection and nucleotide synthesis to generate short sequence of base pairing followed by end ligation (Chang et al., 2017; Scully et al., 2019).

HRR is initiated with recognition of DSB by MRN complex and ATM activation. ATM phosphorylates histone tail H2AX in the vicinity of the DSB (phospho-serine 139 of H2AX known as γ-H2AX). γ-H2AX is a critical requirement for signal amplification and recruitment of downstream DSB repair factors (Bonner et al., 2008; J. Yuan et al., 2010). Mediator of DNA damage Checkpoint protein 1 (MDC1) is first recruited to the damage site via tandem BRCT (BRCA1 C Terminal) domains which recognize the phosphorylation of γ-H2AX. Tandem BRCT domains found on several DDR proteins are evolutionarily conserved and have a phosphopeptide binding capacity. MDC1 also further recruits MRE11-RAD50-NBS1 complex (MRN) and ATM which creates a positive feedback loop that propagates the γ-H2AX to more distal chromatin regions (Stucki & Jackson, 2006). MDC1 recruits

RING E3 ubiquitin ligases RNF8 and RNF168 which work together to extend the ubiquitin chains on the H2A type histones. The H2A ubiquitination cascade creates a binding platform for RAP80 and its associated proteins such as Abraxas. The RAP80 complex is necessary for the recruitment of BRCA1 to the DSBs (Al-Hakim et al., 2010; Bartocci & Denchi, 2013). BRCA1 along with its interacting partner BARD1 ubiquitylate and recruit CtIP (C-terminal binding protein (CtBP) interacting protein) which along with MRE11 regulate short range end resection. CtIP further increases the end resection by regulating BLM (Bloom Syndrome) Helicase, DNA2 Endonuclease, and Exonuclease 1 (EXO1) which leads to the binding of RPA on the ssDNA. PALB2 (Promoter and localizer of BRCA2) and BRCA2 promote the replacement of RPA with RAD51. RAD51 loaded ssDNA can invade the sister chromatid with homologous sequence followed by formation of displacement loops (D-loops), RAD51 disassembly and accurate synthesis of the new strand. (Ceccaldi et al., 2016; Ohta et al., 2011; Scully et al., 2019).

DSBs can also be repaired by SSA, and alt-EJ also known as microhomology mediated end joining (MMEJ). These pathways were initially thought to be a backup to the major NHEJ and HRR pathways without much relevance to the normal physiological situations, but recent literature indicates that these pathways are also active in normal cells (McVey & Lee, 2008). The pathway of choice is determined by the extent of end resection, types of DDR factors available and cell cycle phase (All scenarios and choices depicted in schematic **Figure 6**). c-NHEJ is the pathway of choice in G0/G1 despite its mutagenicity because of its fast kinetics. NHEJ is favored in non-resected DSB ends or if the resected ends are less than 20 nucleotides long (Ceccaldi et al., 2016; Chang et al., 2017). Another form of regulation comes from 53BP1 (p53 binding protein 1), a positive regulator of c-NHEJ whose recruitment to the chromatin inhibits BRCA1 recruitment. Also, 53BP1 along with its interacting partners RIF1 and Shieldin complex protect the DSB ends from end resecting nucleases (Noordermeer et al., 2018). In BRCA mutant cancer DYNLL1 prevents end resection by inhibiting MRE-11 and it was identified that DYNLL1 loss restores HR in BRCA mutant cancer, which has been implicated as a mechanism of resistance for platinum compounds and PARP inhibitors (He et al., 2018). When c-NHEJ is compromised or defective, alt-EJ or MMEJ is preferred which needs short range resection to detect small homologies (microhomologies) that are annealed together. The MMEJ pathway requires a unique polymerase with helicase activity DNA polymerase theta (Polθ, POLQ) along with CtIP and MRN (for short range resection) and regulated by PARP1. Because of involvement of POLQ, this pathway is also termed as TMEJ (Theta mediated End Joining). This pathway has gained a lot of attention in recent years based on the observation that POLQ is upregulated in a lot of human cancers and

associated with poor survival and genetic instability in these cancers (Ceccaldi et al., 2015; Chang et al., 2017; Lemée et al., 2010).

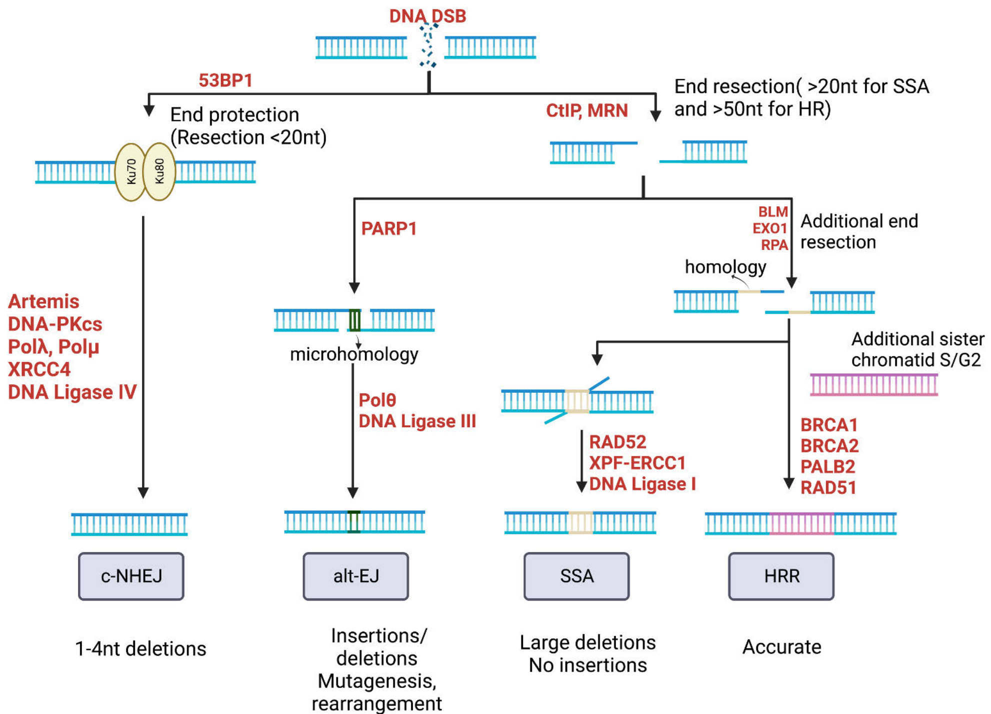

Figure 6: DNA double strand break repair scenarios and pathway choices. Adapted from (Ceccaldi et al. 2016; Chang et al. 2017), made using Biorender.com.

In late S or G2 phases, when there is an extra sister chromatid this favors extensive end resection promoting RAD51 dependent strand invasion and HRR pathway. When RAD51 is defective or other components of the HRR are not available, SSA is preferred. SSA is a RAD-51 independent mechanism which searches for long homology regions and promotes annealing with large deletions. This strand annealing is performed by RAD52 and before ligation, the unannealed non-homologous ssDNA pairs are processed and removed by XPF-ERCC1 (part of the NER pathway) and MSH2-MSH3 (part of the MMR pathway) (Bhargava et al., 2016; Ceccaldi et al., 2016).

2.5.2.2 Other DNA repair pathways and their role in DDR

Base excision repair

Single base damage that is non-helix distorting are repaired by the Base Excision Repair (BER) pathway in which the damaged base is detected and excised by a distinct glycosylase specific to the type of modification (**Figure 7**), which creates an apurinic/apyrimidinic (AP) site (Thompson & Cortez, 2020). The AP sites are very unstable and during the BER, they can sometimes get converted to single strand breaks (SSBs) which recruit the SSB sensor poly (ADP-ribose) polymerase (PARP1/2) proteins at the site of the damage. Due to this, there is a lot of overlap and crosstalk between the SSB repair and BER pathways (Caldecott, 2008). When DNA replication and transcription machinery encounter an AP site, they get stalled and can lead to formation of DSBs and hence these AP sites are considered mutagenic (Thompson & Cortez, 2020). The AP sites are cleaved off by AP endonucleases (APE1/2) and repaired by gap filling DNA synthesis. There are two types of gap filling synthesis in BER- Short Patch Base Excision Repair (SP-BER) in which only 1 nucleotide is repaired or Long Patch Base Excision Repair (LP-BER) in which 2-12 nucleotides can be repaired **(Figure 7)** (Krokan & Bjoras, 2013).

In SP-BER, DNA polymerase β (POLB, Pol β) is recruited to fill the gaps and DNA Ligase 3 (LIG3) seals the nicks. X-ray repair cross-complementing 1 (XRCC1) serves as the scaffolding protein for bringing different repair enzymes to the damage site in SP-BER. LP-BER mainly uses replication proteins for the repair, and it is therefore mainly active in proliferating cells. The gaps are filled by DNA strand displacing activity of DNA polymerase δ or ε (POLD/E, Pol δ/ε) which removes up to 12 nucleotides, generating a flap which is later removed by flap endonuclease1 (FEN1) and sealed by DNA Ligase I (LIG1). The Replication factor C (RFC)-Proliferating Cell Nuclear Antigen (PCNA) complex serve as accessory proteins which recruit POLD/E and LIG1. Also, XRCC1 interacts with PCNA and is necessary for accumulation of RFC-PCNA at SSB sites. SP-BER is the predominant pathway, but the preferred pathway of choice is determined by the cell type, cell cycle phase, type of damage, initial glycosylase and the ATP concentration in the cells (Beard et al., 2019; Krokan & Bjoras, 2013; Petermann, 2003; Thompson & Cortez, 2020).

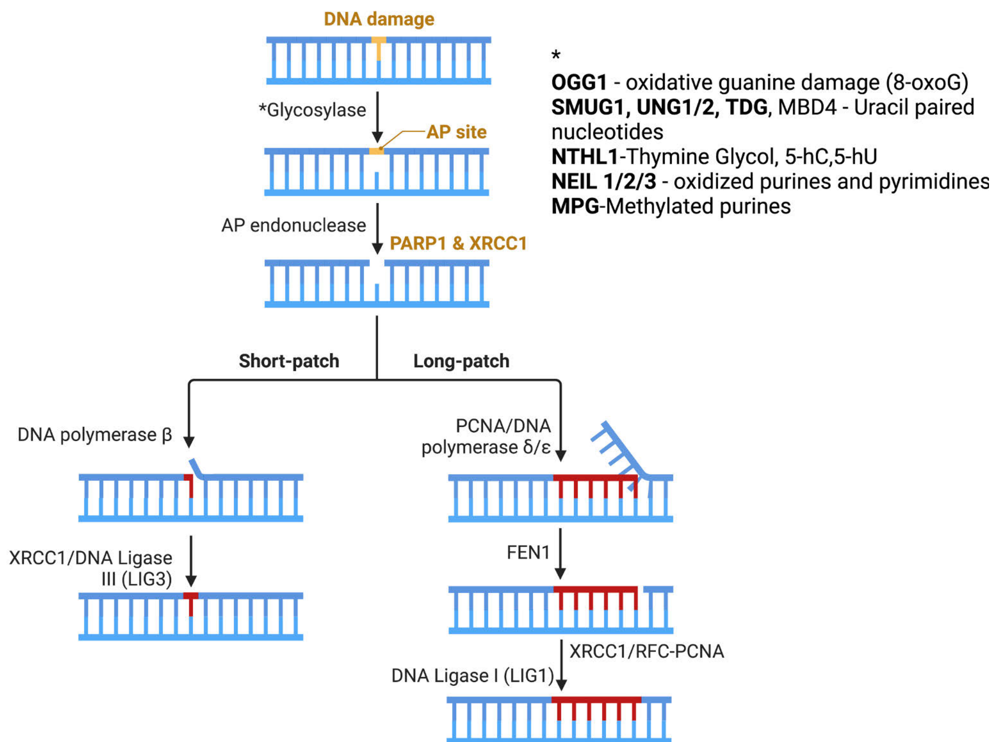

Figure 7: Schematic of the BER along with SP-BER, LP-BER and the functions of the glycosylases involved, adapted from (Krokan & Bjoras, 2013) made with Biorender.com.

Nucleotide excision repair

Helix distorting bulky lesions such as chemical adducts, UV induced pyrimidine dimers or photoproducts, and intra-strand crosslinks are repaired by the Nucleotide Excision Repair (NER). There are two main pathways of NER - Global Genome NER (GG-NER) in which damage is detected throughout the genome and Transcription coupled NER (TC-NER) for large lesions that hinder the transcription machinery during RNA synthesis (D'Souza et al., 2022; Marteijn et al., 2014).

The key proteins involved in the detection and processing of GG-NER (XPA to XPG) are named after *Xeroderma Pigmentosum* (XP), a disorder characterized by extreme sensitivity to sunlight caused by defective NER. Depending on the type of lesion there are two different recognition sensor proteins implicated in GG-NER. The cyclobutane pyrimidine dimers (CPDs) are recognized by UV-DNA Damage binding (UV-DDB) complex- comprising of DDB1 and XPE subunits whereas the photoproducts are recognized by XPC heterotrimer comprising of XPC-RAD23-Centrin2 (CETN2) proteins that continuously probe for helix distorting lesions across the genome. Detection of the bulky lesion triggers the dissociation of RAD23

from the trimer and recruitment of transcription factor IIH (TFIIH) along with XPA at the damage site. XPA verifies the damage whereas the TFIIH comprising of two helicase subunits XPB and XPD unwind the helix. The strand opposite to the damaged strand is coated with Replication Protein A (RPA) to prevent reannealing of the strands. Two incisions are made by ERCC1 (Excision repair cross complementing protein 1)-XPF complex and XPG endonucleases followed by gap-filling DNA synthesis by DNA polymerase and nick sealing by DNA Ligase (D'Souza et al., 2022; Hoeijmakers, 2009; Kusakabe et al., 2019).

Defects in TC-NER leads to *Cockayne's Syndrome* (CS) which causes neuronal dysfunction, growth, and developmental defects and the proteins involved in TC-NER are named after CS, as CSA and CSB. RNA Polymerase II (RNAPII) gets stalled at the bulky lesions. CSB recognizes the stalled RNAPII site and recruits CSA and subsequently TFIIH. The rest of repair process overlaps with GG-NER. The lesion is excised and gap filling DNA synthesis ensues (van den Heuvel et al., 2021).

Mismatch repair

DNA replication is highly faithful, and it is estimated that cells accumulate less than one mutation per genome per cell division (Drake et al., 1998). The most common error that occurs during replication is the incorporation of wrongly paired nucleotides (mismatch) or insertion-deletion loops (IDLs) that arise from slippage of strands at tandemly repeated nucleotide sites (termed microsatellites) (Baretti & Le, 2018). IDLs cause insertions or deletions in the daughter cells if unrepaired. The errors during replication are corrected almost instantaneously by the intrinsic proofreading activity of the DNA polymerases δ or ε. Some errors that escape the proofreading activity are repaired post replication by mismatch repair (MMR). This damage is transient because there is no way to detect the change once the new replication begins and therefore, the most important criterion for efficient MMR is to correct the mismatch or IDLs before the next round of replication. The combined proofreading and MMR activities ensure that the DNA is copied with high fidelity. This is needed to prevent the formation of mutations and degenerative or genetic diseases such as cancer. (Albertson & Preston, 2006; Jiricny, 2013; Kunkel, 2004; Preston et al., 2010). Mismatches and IDLs of up to 2 nucleotides are recognized by MutSα heterodimer (MSH2/MSH6). Larger IDLs are recognized by MutSβ heterodimer (MSH2/MSH3). Subsequently the MutLα heterodimer (MLH1/PMS2) gets recruited and traps MutS dimers at the damage site and introduces irregular strand breaks on the mismatched strand. Exonuclease1 (EXO1) gets directed to these strand breaks, creates an excision and the single strand is coated with RPA. PCNA/RFC, POLD, and LIG1 mediate the gap filling DNA synthesis (N. Chatterjee & Walker, 2017; Jiricny, 2013; Pluciennik et al., 2010). Defects or mutations in the MMR pathway

leads to microsatellite instability (MSI) and Lynch syndrome also known as hereditary nonpolyposis colon cancer (HNPCC) predisposes individuals to ovarian, colorectal and endometrial cancers (Baretti & Le, 2018; Jiricny, 2013).

Fanconi Anemia pathway

Interstrand cross links (ICLs) are lethal to proliferating cells as they cause replication fork stalling, and this is the rationale for using ICL inducing agents such as cisplatin in cancer chemotherapy. The ICLs are repaired by the Fanconi Anaemia (FA) pathway, which is named after the FA syndrome, a rare genetic disorder caused by defects in atleast one of the 14 key proteins of the FA pathway (The genes involved are named starting with FANC). FA causes ICL sensitivity, congenital abnormalities, and elevated risk of hematological and squamous cell cancers. FA pathway has a lot of overlap between HRR and NER. Hence, cancer cells deficient in HRR are also sensitive to ICL agents. ICLs are detected by FANCM which activate the FA core complex which recruit nucleases and polymerases such as XPF-ERCC1 for NER or BRCA1, PALB2 (also known as FANCN), and BRCA2 (also known as FANCD1) required for the HRR (Deans & West, 2011; Jasin & Rothstein, 2013; Nakanishi et al., 2011).

2.5.3 TopBP1 and ATR signaling in DDR

DNA Topoisomerase IIβ binding protein 1 (TopBP1) as the name indicates, was discovered as an interacting partner of DNA topoisomerase IIβ in a Y2H screen (Yamane et al., 1997). TopBP1 knockout mice in which exons 5 and 6 were conditionally deleted are embryonic lethal indicating that it is essential in embryonic development. Also in mice 3T3 cells, depletion of TopBP1 arrested the cells in G1/S and G2/M checkpoint phases (Jeon et al., 2011). Human TopBP1 has 9 BRCT (BRCA1 C terminus) domains designated as BRCT0 to BRCT8, which is a common motif found in several DDR proteins. They are arranged in pairs of tandem BRCTs, each having atleast one phospho binding motif. For example, BRCT1+2, BRCT4+5 and BRCT7+8 each have one or two phospho binding motifs through which interacts with the components of the DDR. BRCT7+8 is called the canonical BRCT pair as they are similar to the BRCT domains on MDC1 or BRCA1, where BRCT7 has the phospho binding ability and BRCT8 creates an extra binding groove which can be important for substrate specific binding. BRCT 3 and BRCT 6 do not have a phospho binding ability while in BRCT1+2, both have a phospho binding capacity indicating that they can both bind to a phosphopeptide ligand independently. There is an ATR activation domain (AAD) between BRCT 6 and 7, and it is necessary for ATR activation (Day et al., 2021; Wardlaw et al., 2014). The main function of TopBP1

has been associated with its role as an interaction hub or scaffold that can bring several required components of the DDR together. It has been linked to functions such as DNA repair, replication, checkpoint activation, ATR signaling, regulation of transcription, and most recently in chromosome segregation through an interaction with CIP2A (Wardlaw et al., 2014; Bagge et al., 2021; Day et al., 2021; Adam et al., 2021; De Marco Zompit et al., 2022). I will be mainly focusing on roles of TopBP1 in ATR signaling, DNA repair and checkpoint activation as these are the main themes of my dissertation. The role of TopBP1-CIP2A complex in chromosome segregation is discussed in section 2.5.5

2.5.3.1 ATR and Chk1 signaling mediated by TopBP1

Research in the last decade has established TopBP1's role mainly as an activator of ATR (Awasthi et al., 2016; Kumagai et al., 2006). ATR is activated in response to the single stranded DNA (ssDNA) which can arise due to replication stress or resection of DNA during different forms of DNA damage (Blackford & Jackson, 2017; Cimprich & Cortez, 2008). Long stretches of ssDNA are immediately coated by Replication protein A (RPA), and this triggers the initiation of ATR signaling and activation of Chk1 (Zou & Elledge, 2003). The ssDNA that are unbound by RPA are very susceptible to DNA breaks. Toledo et al. reported that in the absence of ATR, stalled replication induces genome wide replication origin firing, creating more ssDNA that quickly depletes the levels of the RPA in the cell, creating a catastrophe, where DNA breaks occur at every replication fork. So, the main role of ATR is to guard the genome from the destabilizing replication stress, and to buy enough time for the replenishment of cellular RPA levels (L. Toledo et al., 2017; L. I. Toledo et al., 2013). ATR activation is a multi-step process and involves many accompanying factors (Awasthi et al., 2016). ATR gets recruited to ssDNA loaded RPA along with its interacting partner ATRIP (ATR interacting protein) (Cortez et al., 2001). Simultaneously, RAD17-RFC 2-5 clamp loader complex loads the RAD9-RAD1-HUS1(9-1-1) clamp adjacent to the ssDNA-bound RPA sites. The C-terminal tail of RAD9 is phosphorylated at serine 387 of RAD9 and this phosphorylation interacts with BRCT1+2 domain of TopBP1, and recruits it to the ssDNA site (Delacroix et al., 2007; J. Lee et al., 2007; J. Lee & Dunphy, 2010). However, later it was identified that it is the MRN complex that is actually important for TopBP1 recruitment to the ATR-ssDNA site, but 9-1-1 is essential for its activity. The MRN complex along with 9-1-1 brings ATR closer to the ATR activation domain (AAD) within TopBP1 which stimulates ATR's kinase activity. This fully activates the ATR (Kumagai et al., 2006; J.-H. Choi et al., 2010; Duursma et al., 2013). TopBP1 can also mediate the ATR dependent Chk1 signaling by interacting with 9-1-1 via RHNO1 (Cotta-Ramusino et al., 2011).

ATR also phosphorylates serine 1131 on TopBP1 activating it and further enhancing the ATR kinase activity (Wardlaw et al., 2014). Activated ATR phosphorylates Claspin on its Chk1 binding domain (CKBD), which recruits inactive Chk1 and brings it to the close proximity of ATR, for ATR to phosphorylate Chk1 and activate downstream checkpoint (Smits et al., 2019). Recently, another ATR activator named ETAA1 (Ewing's tumor associated antigen 1) was discovered which gets recruited to the ssDNA-RPA by direct binding. ETAA1 also has an AAD. Though there is not much sequence similarity with the AAD of TopBP1, it is believed to be activating ATR in the same way as TopBP1 (Bass et al., 2016; Blackford & Jackson, 2017; Thada & Cortez, 2021).

2.5.3.2 Role of TopBP1 in DSB repair

ATM requires TopBP1 for the activation of ATR and downstream Chk1 activation. In the presence of DSBs, ATM phosphorylates TopBP1 S1131 to activate TopBP1 which further stimulates ATR and downstream Chk1 activation, indicating a more critical role of TopBP1 during DSB repair than replication stress (Yoo et al., 2007). The first non ATR activation role of TopBP1 was identified when TopBP1 nuclear foci were colocalized with NBS1 on ionizing radiation, indicating that NBS1 might be regulating the recruitment of TopBP1 at the site of DSBs (Morishima et al., 2007). TopBP1 serves as a scaffold and coordinates both BRCA1 mediated pro-end resection and 53BP1 mediated anti-end resection activities during HR. Stabilization of the 53BP1-TopBP1 interaction, decreased the HR mediated repair (Y. Liu et al., 2017). Jiri Bartek group identified that TopBP1 silencing sensitizes tumor cells to PARP inhibitors. TopBP1 silencing also decreased the number of RAD51 foci at the site of DSBs implicating its role in homologous recombination repair. They also found that TopBP1 was not involved in resection of DNA or binding of ssDNA indicating that TopBP1 is involved in processes downstream of RPA loading which replaces the RPA with RAD51 to enable strand invasion and recombination. They discovered that TopBP1 recruits PLK1 to promote S14 phosphorylation of RAD51, which further allows casein kinase 2 (CK2) to bind and phosphorylate T13 of RAD51, and this double phosphorylation is necessary for RAD51 loading and replacement of RPA on the ssDNA (Moudry et al., 2016). Just recently Zhao et al. resolved yet another complex mechanism of RAD51 replacement of RPA mediated by TopBP1. They identified that HIV Tat-specific factor 1 (HTATSF1) recognizes PARylation on RPA by an RNA recognition motif (RRM) on the N terminus, which facilitates its recruitment to the DSBs. HTATSF1 is phosphorylated on S748 by CK2 and this phosphorylation forms a docking site for TopBP1 through BRCT2, which facilitates the BRCA2/PALB2 mediated replacement of RAD51 on RPA. Notably, the HTATSF1 recruitment on DSBs could be dampened by the PARP inhibitors,

indicating that RPA-RAD51 exchange involves a complex PARylation-Phosphorylation signaling cascade (Roychoudhury & Chowdhury, 2022; Zhao et al., 2022).

2.5.4 Targeting the DDR in cancer

One common trait for most cancers compared to the normal cells is that they have defects in one or more of their DDR pathways leading to a greater dependency on the remaining pathways (O'Connor, 2015; Lord & Ashworth, 2017). This creates a vulnerability that can be potentially targeted by using an approach which has been described as synthetic lethality. Synthetic lethality occurs when defects in either of the two genes has no effect due to the compensation by the other gene but the combination of the defects in both the genes leads to death (O'Neil et al., 2017). The most appreciated successful example of synthetic lethality has been that of PARP inhibitors in BRCA1/2 mutant cancers. BRCA mutant cancers have a defective homologous recombination repair pathway (HRR) making them highly reliant on non-homologous end joining (NHEJ) or Base excision repair (BER) pathways which require the activity of PARP1/2 (poly-(ADP-ribose) polymerase) enzymes. Pharmaceutical targeting of PARP1 in these cancers using PARP inhibitors causes synthetic lethality in cancer cells but not in normal cells, which do not have a BRCA mutation (Bryant et al., 2005; Farmer et al., 2005; O'Connor, 2015; Pilié et al., 2019).

The PARP inhibitors have a dual effect. First, the PARP inhibitors compete with NAD+ (which is needed for the catalytic activity of the PARP enzyme) trapping the enzyme on the DNA, preventing the repair of SSBs. Secondly, the "PARP trapping" hinders the progression of the replication machinery converting the unrepaired SSBs to DSBs, which are more fatal for the BRCA mutant cells. While most of the PARP inhibitors currently used in the clinic have similar inhibitory effect on the catalytic activity, the difference in clinical activity comes from their PARP trapping efficiencies (Helleday, 2011; Murai et al., 2012). Similar to PARP trapping, BRCA mutant cancers are generally sensitive to compounds that stall the replication forks, creating DSBs. For example, drugs such as topoisomerase inhibitors and platinum salts trap different DNA repair proteins and stall the replication forks, causing DSBs and hence would cause similar effects as PARP inhibitors (Lord & Ashworth, 2016). There was a recent study which indicated that HR defective tumors switch to oxidative phosphorylation (OXPHOS) to enhance their cellular supply of NAD+, hence they are more sensitive to OXPHOS inhibitors such as Metformin. It was also reported that switching to glycolytic metabolism in these HR defective tumors would also affect the PARP inhibitor sensitivity (Lahiguera et al., 2020). Since the frequency of BRCA1/2 mutations is quite low (1-5%), Davies et al. developed a

signature called HRDetect which can be used to predict the inactivation of genes (both by mutations and promoter methylations) that phenocopy BRCA1/2 mutation or homologous recombination defect – collectively called "BRCAness" and the idea is that these BRCAness cancers (accounting for a larger frequency) would also sensitize to PARP inhibitors (Davies et al., 2017)

2.5.5 Role of PP2A and CIP2A in HR and DDR

PP2A is involved in the phospho regulation of all the PIKK family kinases ATM, ATR and DNA-PK and also the downstream Chk1 and Chk2 kinases (D.-H. Lee & Chowdhury, 2011; Zheng et al., 2015). ATM interacts with the A and C subunit of PP2A and okadaic acid treatment causes the induction of ATM autophosphorylation. Also DNA damage by IR caused dissociation of PP2A from ATM in a phosphorylation dependent manner, indicating that PP2A regulates the ATM phosphorylation (Goodarzi et al., 2004). It was later identified that this phosphorylation is regulated by PP2A-B55α, and loss of B55α, increases ATM phosphorylation and Chk2 activation, causing a G1/S arrest which leads to downregulation of RAD51 and BRCA1, causing a susceptibility to PARP inhibitors (Kalev et al., 2012). Two independent recent studies have further validated these findings. A pooled shRNA screen identified that loss of PP2A-B55α induced sensitization of lung cancer cells to ATR and Chk1 inhibitors (Qiu et al., 2020). Also, activation of PP2A-B55α by loss of FAM122A, causes dephosphorylation and stabilization of WEE1 which induces the resistance to ATR and Chk1 inhibitors (F. Li et al., 2020). PP2A dephosphorylates members of the DNA-PK complex including DNA-PKcs, Ku80 and Ku70 proteins (Douglas et al., 2001). Similarly, PP2A was found to regulate the phosphorylation of ATR in the presence of human immunodeficiency virus Vpr but not in presence of UV or hydroxyurea (G. Li et al., 2007).

PP2A mediates removal of γH2AX from the site of DSB by dephosphorylation, and loss of PP2A leads to inefficient repair and sustained γH2AX foci, which also sensitize the cells to DNA damage (Chowdhury et al., 2005). It was later identified that B56ε containing PP2A complexes are responsible for the dephosphorylation of γH2AX (X. Li et al., 2015). PP2A is also responsible for the attenuation of RPA32 phosphorylation and efficient DNA repair, which allows the cells to progress further in the cell cycle. Loss of PP2A causes hyperphosphorylation of RPA32 and sensitivity to hydroxy urea (HU) (Feng et al., 2009). When DNA is damaged, ATM and ATR kinases phosphorylate BRCA2 at S1106, S1123 and T1128. These phosphorylations promote the binding of PP2A-B56 to BRCA2 via the LxxIxE recognition site. BRCA2-PP2A-B56 complex binding is necessary for the PALB2 dependent replacement of RPA with RAD51 on the end resected DNA during

homologous recombination repair (Ambjørn et al., 2021). PP2A enhances the stability of TopBP1 at the site of DNA damage, by regulating dephosphorylation mediated nuclear localization of deubiquitinase OTUD6A, which prevents TopBP1 interaction with its ubiquitin E3 ligase UBR5 (Zhao et al., 2022).

CIP2A loss sensitizes cells to ATR inhibition and other genotoxins such as camptothecin (Hustedt et al., 2019; Olivieri et al., 2020). Also, CIP2A was among the top 10 genes which were commonly essential for cellular fitness in both BRCA1 KO and BRCA2 KO screens. Also, CIP2A showed synthetic lethality with BRCA1, BRCA2, PALB2 and FANCM in a p53 mutant cell line (Adam et al., 2021). In this thesis, I have identified the novel role of CIP2A as an interaction partner of TopBP1. We have identified a role of CIP2A-TopBP1 in G2/M progression of DNA damaged cells (discussed in detail in the Results and Discussion sections). Ever since, the role of CIP2A-TopBP1 complex has been implicated in maintenance of genome stability and chromosome segregation, as demonstrated by two independent studies. Durocher group found that CIP2A-TopBP1 is involved in the proper chromosomal segregation in cells entering into mitosis with under replicated DNA (Adam et al., 2021). Stucki group found that the CIP2A-TopBP1 is involved in genome stability and proper segregation of chromosomes after DSBs that arise in mitosis. They also indicated that MDC1 is also a part of the complex with CIP2A-TopBP1 that is involved in this process (De Marco Zompit et al., 2022).

3 Aims

BLBC is the most aggressive and challenging breast cancer subtype in the clinic. Firstly, there are no surface markers (ER, PR and HER2) and known driver mechanisms which can be therapeutically targeted (except maximum 5% cases that have BRCA1/2 mutations). Secondly, BLBC and TNBC are almost indistinguishable in the clinic as the IHC markers to separate BLBC from TNBC are not part of the standard guidelines and hence not assessed. All BLBC patients are treated with aggressive chemotherapy though some of these patients might not require such aggressive chemotherapy and could be spared from the side effects. Some patients do not respond well to the chemotherapy, and this could be attributed to poor patient stratification methods. Many gene expression signatures proposed as predictive and prognostic biomarkers are able to identify patients who would benefit from chemotherapy, but they are currently only applicable to ER+ breast cancer patients. There is a need for similar tools for BLBC. Also, there are no diagnostic tools that can be used for tailoring personalized therapies in breast cancer.

The main objectives of my thesis have been to address these clinical unmet needs in the form of two subprojects (listed below), which have also led to two publications included in the thesis.

The specific aims of my dissertation are:

I. To identify novel driver mechanisms that can be therapeutically targeted for BLBC.

II. To develop a CIP2A regulated transcriptional signature that can stratify BLBC patients based on aggressivity and can be used for personalized medicine regimens.

4 Materials and Methods

4.1 Cell culture and transfections (I, II)

4.1.1 Cell lines used (I, II)

Before the start of the study, all the commercial cell lines were bought from DSMZ or ATCC. The cell lines that were obtained from collaborators or present in our lab were authenticated using short tandem repeats (STR) profiling by American Type Culture Collection (ATCC, USA) before utilizing them in the studies. The cell lines were frozen at early passage in liquid nitrogen tanks after the first thaw. All the cell lines were negative on testing periodically for mycoplasma using Mycoplasma Detection Kit (Lonza). The human and mouse cells used in the study are listed in **Table 2** and **Table 3,** respectively.

Table 2: List of human cells used in the study.

Cell line	Source	Identifier	Growth Medium	Used in
CAL-85-1	DSMZ	ACC-440	DMEM + 10%FBS+ 2mM Glutamine+ 1mM Sodium Pyruvate+ 1% Pen/strep	I
HAP1	N/A	From Rene Medema group	Iscove's modified Dulbecco's Medium (IMDM)+ 10% FCS+ 1% Pen/Strep+ 2 mM L-glutamine	I
HCC1143	ATCC	ATCC® CRL-2321™	RPMI 1640+ 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I
HCC1806	ATCC	ATCC® CRL-2335™	RPMI 1640+ 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I
HCC1937	ATCC	ATCC® CRL-2336™	RPMI 1640+10%FBS+ 2mM Glutamine+ 1% Pen/strep	I, II
HCC38	ATCC	ATCC® CRL-2314™	RPMI 1640+10%FBS+ 2mM Glutamine+ 1% Pen/strep	I, II
HCC70	ATCC	ATCC® CRL-2315™	RPMI 1640+ 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I
HDQ-P1	DSMZ	ACC-494	DMEM + 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I
HeLa	ATCC	ATCC® CCL-2™	DMEM + 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I

Cell line	Source	Identifier	Growth Medium	Used in
MDA-MB-231	ATCC	ATCC® HTB-26™	DMEM+ 10%FBS+ 2mM Glutamine+ 1% Pen/strep + 1% Non-Essential Amino Acids (NEAA)	I, II
MDA-MB-436	ATCC	ATCC® HTB-130™	DMEM + 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I, II
MDA-MB-468	ATCC	ATCC® HTB-132™	DMEM + 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I, II
HEK293	ATCC	ATCC® CRL-1573™	DMEM + 10%FBS+ 2mM Glutamine+ 1% Pen/strep	I
MCF10A	ATCC	ATCC® CRL-10317™	DMEM/Nutrient mixture F-12 (1:1) + 4.5 g/L Glucose+ 5% FBS +10 µg/ml human insulin+ 0.5 µg/ml hydrocortisone+ 100 ng/ml cholera toxin+ 20 ng/ml human EGF + 2mM Glutamine +1% Pen/Strep	I
MCF10A-Control	Generated in house	N/A		I
MCF10A-CIP2AOE	Generated in house	N/A		I
BCSC1	Jochen Maurer Lab	(Metzger et al., 2017)	MEBM medium+ 1x B27+1x Amphotericin B+ 4ug/ml Heparin + 35ug/ml Gentamicin+ 20ng/ml human EGF + 20ng/ml human FGF+ 1% Pen/Strep	I
BCSC2	Jochen Maurer Lab			I
BCSC3	Jochen Maurer Lab			I
BCSC4	Jochen Maurer Lab			I
BCSC5	Jochen Maurer Lab			I

Table 3: List of mouse cells used in the study

Name	Source	Identifier	Growth Medium	Used in
MMEC	Isolated from Cip2a WT and KO mice	(Peuhu et al., 2017)	DMEM: F12 + 10%FBS + 2mM Glutamine + 100 IU/ml penicillin, 100mg/ml streptomycin + 5 ug/ml insulin + 1ug/ml Hydrocortisone +10ng/ml murine EGF+ 50ug/ml Gentamycin	I
KB1P	Isolated from K14Cre; Brca1$^{F/F}$; Trp53$^{F/F}$ mice tumors	(X. Liu et al., 2007)	Advanced DMEM: F12 + 10%FBS + 2mM Glutamine + 100 IU/ml penicillin,100mg/ml streptomycin + 5 ug/ml insulin + 5ng/ml murine EGF + 5ng/ml cholera toxin	I
KEP	Isolated from K14Cre; Cdh1$^{F/F}$; Trp53$^{F/F}$ mice tumors	(Derksen et al., 2006)		I
WEA	Isolated from Wap-cre; Cdh1$^{F/F}$; Akt1E17K mice tumors	(Wellenstein et al., 2019)		I

4.1.2 siRNAs and Plasmid DNAs used (I, II)

Table 4: Sequences of siRNA and gRNAs used in the study

Name	Sequence (5'-3') with overhangs	Used in
Non targeting siRNA (siSCR or siCTRL#1 or siCTRL)	CGUACGCGGAAUACUUCGA dTdT	I, II
Non targeting siRNA (siCTRL#2)	UGGUUUACAUGUCGACUAA dTdT	I, II
siCIP2A#1	CUGUGGUUGUGUUUGCACU dTdT	I, II
siCIP2A#2	AAUGCCUUGUCUAGGAUUA dTdT	I, II
siCIP2A#3	CGCAGCAAGUUGAAUCAGA dAdA	I, II
siCHK1	AAGAAAGAGAUCUGUAUCAAU dTdT	I
Non-targeting (NT) control sgRNA Oligo1	caccgACGGAGGCTAAGCGTCGCAA	I
Non-targeting (NT) control sgRNA Oligo2	aaacTTGCGACGCTTAGCCTCCGTc	I
Cip2a#1 sgRNA Oligo1	caccgAATCTGAATAGTGTGCTGTC	I
Cip2a#1 sgRNA Oligo2	aaacGACAGCACACTATTCAGATTc	I
Cip2a#2 sgRNA Oligo1	caccGAGCTAACAATGCCTTGTCT	I
Cip2a#2 sgRNA Oligo2	aaacAGACAAGGCATTGTTAGCTC	I

Table 5: Plasmids and expression vectors used in the study

Plasmid (Length of amino acids)	Backbone	Source:	Used in
FL-TopBP1-GFP (2-1522)	pIRESNeo2	From Niedzwiedz lab	I
T0-GFP: TopBP1-BRCT 0-6+AAD-GFP (ΔBRCT 7/8) (2-1218)	pIRESNeo2	From Niedzwiedz lab	I
T1-GFP: TopBP1-BRCT 0-6-GFP (2-1026)	pIRESNeo2	From Niedzwiedz lab	I
T2-GFP: TopBP1-BRCT 0-5-GFP (2-764)	pIRESNeo2	From Niedzwiedz lab	I
T3-GFP: TopBP1-BRCT 0-3-GFP (2-550)	pIRESNeo2	From Niedzwiedz lab	I
Empty-GFP: (pEGFP-N1)	pEGFP	From Clonetech	I
pcDNA3.1_kozak_CIP2A_1-905_V5His	pcDNA3.1	(J. Wang et al., 2017)	I
pWPI-CIP2A-V5	pWPI	Generated as described in 4.1.4	I
pWPI	pWPI	Addgene (Plasmid #12254)	I
lentiCas9-Blast	pFUGW	Addgene (Plasmid #52962)	I
lentiGuide-Puro	custom	Addgene (Plasmid #52963)	I

4.1.3 Isolation of cells from mouse (I)

Mouse mammary epithelial cells (MMECs) were isolated from *Cip2a*$^{+/+}$(*Cip2a*WT) and *Cip2a*$^{-/-}$ (*Cip2a* KO) mouse and cultured *in vitro* as per the protocol described in (Peuhu et al., 2017). Briefly, fresh mammary glands were pooled from 3-4 mice (~3-4 months old age-matched mice) into ice cold PBS, minced with scalpels and digested with collagenase medium. The epithelial cells were isolated by agitation for 2-3 hours followed by a few iterative centrifugations (1500g, 5 min) until the red blood cells disappear from the pellets. The final colorless pellet is dissociated with Accutase (StemCell Technologies) followed by pipetting up and down to produce single cells which were either cultured for further experiments or taken directly for characterization by flow cytometry analysis. Mouse tumor cell lines KB1P, KEP and WEA were generated from spontaneous mammary tumor producing GEMM models as described in the references listed in **Table 3**. Briefly, cells were isolated by collecting tumors into ice cold PBS and mincing them with scalpels. The lumps were plated out and after 2-3 passages the homogenous epithelial cell morphology appeared, after which the cells were used for further experiments. While KEP and WEA cells were cultured in standard cell culture conditions, KB1P cells were grown in physiological hypoxia conditions (3% O_2)

4.1.4 Cloning and generation of stable cell lines (I)

Human CIP2A containing V5His tag was cloned from pcDNA3.1_kozak_CIP2A_1-905_V5His (Template) into a lentiviral plasmid pWPI (Addgene#12254) as described in I. Briefly, forward primers including restriction sites for SwaI/PacI and reverse primers for V5His tag were used to amplify the CIP2A-V5His region from the template. Phusion Hot Start II High Fidelity PCR Master Mix (F566S Thermo Fisher Scientific) was used. The PCR product and pWPI were digested with FastDigest SmiI (FD1244 Thermo Fisher Scientific) and Fast Digest PacI (FD2204 Thermo Fisher Scientific). The products were ligated with 2.5 units of T4 DNA ligase (EL0011 Thermo Fisher Scientific). The insert was in 3-fold mass excess than the vector. Bacterial transformation was done in DH5α competent cells. Purified plasmid was validated with using PCR and Sanger Sequencing before using it for stable cell line generation. Lentiviral particles were prepared for pWPI (empty vector) and CIP2A-V5 cloned pWPI vectors. MCF10A parental cell line was transduced with the lentiviral particles, single cell sorted based on GFP on a Sony SH800 cell sorter, to generate MCF10A-Control and MCF10A-CIP2AOE cell lines.

4.1.5 Transfections (I, II)

The transfections were performed according to the manufacturer's protocols. For siRNA transfections, Oligofectamine or Lipofectamine RNAiMAX were used (both from Invitrogen). For plasmid transfections, jetPRIME reagent (Polyplus) was used.

4.1.6 Colony growth assays (I, II)

Optimized number of cells were seeded into 12 well plates. For siRNA transfection-based experiments, first the cells were seeded into 6 well plates (Day1), transfected with siRNA (Day2) and then reseeded into 12 well plates (Day 3). The cells were allowed to grow for 7-10 days, with media changes every 2 days. For drug combination testing experiments, drug treatments were done for 72 hours. The cells in the control well were 80% to fully confluent at the conclusion of the experiment. The cell colonies were fixed with cold methanol (stored at -20°C) for 15 minutes, stained with 0.2%crystal violet (in 10% Ethanol) for 10 minutes, and washed a few times with 1X PBS or water. The plates were the dried overnight, scanned and the colony areas were quantified using the Colony Area ImageJ plugin (Guzmán et al., 2014)

4.2 DNA, RNA, and protein analysis (I, II)

4.2.1 Antibodies (I, II)

Table 6: List of primary antibodies used in the study.

Target	Host species	Manufacturer	Identifier	Application & Dilution	Used in
53BP1	Rabbit	Novus Biologicals	NB100-304	IF (1:500)	I
Chk2 (A-12)	Mouse	Santa Cruz Biotechnology	sc-5278	WB (1:100)	I
Chk2 (A-12)	Rabbit	Cell Signaling Technology	6334S	WB (1:1000)	I
CIP2A (2G10-3B5)	Mouse	Santa Cruz Biotechnology	sc-80659	WB (1:500), IF (1:50)	I, II
CIP2A Polyclonal	Rabbit	manufactured inhouse	(Hoo et al., 2002)	IHC & PLA (1:500)	I
c-Myc	Rabbit	Cell Signaling Technology	9402-S	WB (1:1000)	I
c-Myc (9E10)	Mouse	Santa Cruz Biotechnology	sc-40	WB (1:500), IHC (1:250)	I
E2F1(KH95)	Mouse	Santa Cruz Biotechnology	sc-251	WB (1:500)	I

Target	Host species	Manufacturer	Identifier	Application & Dilution	Used in
ER (1D5)	Mouse	Dako	MA5-13191	IHC (1:1000)	I
GAPDH (6C5,4G5)	Mouse	Hytest	5G4	WB (1:10000)	I, II
GAPDH (6C5,4G5)	Mouse	Santa Cruz	sc-32233	WB (1:5000)	I
GFP	Rabbit	Abcam	ab290	WB (1:2500)	I
GFP (B-2)	Mouse	Santa Cruz Biotechnology	sc-9996	WB (1:1000)	I
HER2	Rabbit	Dako	A0485	IHC (1:250)	I
K14 (Poly19053)	Rabbit	BioLegend (previously Covance)	905301	IHC (1:3000)	I
K8 (Keratin, type II/ Cytokeratin 8 TROMA-I)	Rat	Developmental Studies Hybridoma Bank	AB_531826	IHC (1:1000)	I
Ki-67	Rat	eBioscience	14-5698	IHC (1:250)	I
Mouse- CD29-A488 (Itgb1, clone HMβ1-1)	Hamster	BioLegend	102212	FACS (1:50)	I
Mouse CD31-PE/Cy7 (clone MEC 13.3)	Rat	Biolegend	102523	FACS (1:50)	I
Mouse CD45-PE/Cy7 (clone 30-F11)	Rat	Biolegend	103113	FACS (1:100)	I
Mouse TER-119-PE/Cy7	Rat	Biolegend	116221	FACS (1:100)	I
Mouse/Human CD49f -APC (Clone GoH3)	Rat	Biolegend	313615	FACS (1:30)	I
Mouse-CD24-APC (Clone M1/69)	Rat	BD Biosciences	562349	FACS (1:100)	I
Mouse-CD24-FITC (clone M1/69)	Rat	eBioscience	11-0242-81	FACS (1:100)	I
Mouse-CD31-Pacific Blue (Clone 390)	Rat	BioLegend	102422	FACS (1:50)	I
Mouse-CD45-Pacific Blue (clone 30-F11)	Rat	BioLegend	103126	FACS (1:200)	I
Mouse-CD49f-A488 (Itga6, clone GoH3)	Rat	BioLegend	313608	FACS (1:30)	I
Phospho-ATM (Ser1981) (10H11.E12)	Mouse	Cell Signaling Technology	4526S	WB (1:1000)	I
Phospho-ATM (Ser1981) 10H11.E12	Mouse	Rockland antibodies	200-301-400	WB (1:500)	I
Phospho-ATR (Thr1989) (D5K8W)	Rabbit	Cell Signaling Technology	30632S	WB (1:1000)	I
Phospho-Chk1 (Ser317)	Rabbit	Cell Signaling Technology	2344S	WB (1:1000)	I

Target	Host species	Manufacturer	Identifier	Application & Dilution	Used in
Phospho-Chk1 (Ser345) (133D3)	Rabbit	Cell Signaling Technology	2348S	WB (1:1000)	I
Phospho-Chk2 (Thr68) (C13C1)	Rabbit	Cell Signaling Technology	2197S	WB (1:1000)	I
Phospho-cMYC (Ser62)	Rabbit	Abcam	ab51156	WB (1:1000)	I
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit	Cell Signaling Technology	9101S	WB (1:2000)	I
Phospho-E2F1 (Ser364)	Rabbit	Abcam	ab5391	WB (1:500)	I
Phospho-Histone H2AX (Ser139) (20E3)	Rabbit	Cell Signaling Technology	9718S	WB (1:1000)	I
Phospho-Histone H2AX (Ser139) (Clone JBW301)	Mouse	Merck Millipore	05-636	WB (1:1000) IF (1:500)	I
Phospho-Histone H3 (Ser10)	Rabbit	Merck Millipore	06-570	IF (1:1000)	I
PR	Rabbit	Dako	A0098	IHC (1:2000)	I
RAD51(Ab-1)	Rabbit	Merck Millipore	PC130	IF (1:500)	I
TopBP1 (B-7)	Mouse	Santa Cruz Biotechnology	sc-271043	WB (1:500)	I
TopBP1 (Clone 33)	Mouse	BD Biosciences	611874	PLA (1:500)	I
Total ATM	Rabbit	Novus Biologicals	NB100-104SS	WB (1:1000)	I
Total ATM (D2E2)	Rabbit	Cell Signaling Technology	2873S	WB (1:1000)	I
Total ATR	Rabbit	Cell Signaling Technology	2790S	WB (1:1000)	I
Total Chk1 Antibody (G-4)	Mouse	Santa Cruz Biotechnology	sc-8408	WB (1:500)	I
Total Chk1 Antibody (G-4)	Mouse	Cell Signaling Technology	2360S	WB (1:1000)	I
Vinculin (H-10)	Mouse	Santa Cruz Biotechnology	sc-25336	WB (1:1000)	I, II
β-Actin (C4)	Mouse	Santa Cruz Biotechnology	sc-47778	WB (1:3000)	I, II

Table 7: List of secondary antibodies used in the study.

Target	Host species	Manufacturer	Identifier	Application & Dilution	Used in
Anti-Mouse IgG (H+L) Alexa Fluor 488	Goat	Invitrogen	A-11011	IF (1:250-1:1000)	I
Anti-Mouse IgG (H+L) Alexa Fluor 555	Goat	Invitrogen	A-21422	IF (1:250-1:1000)	I
Anti-Mouse IgG (H+L) Alexa Fluor 594	Goat	Invitrogen	A-11005	IF (1:250-1:1000)	I
Anti-Mouse IgG (H+L) HRP	Goat	Jackson	115-035-003	WB (1:5000)	I, II
Anti-Mouse IgG IRDye 680RD	Donkey	Li-COR	926-68072	WB (1:5000)	I, II
Anti-Mouse IgG IRDye 800CW	Donkey	Li-COR	926-32212	WB (1:5000)	I, II
Anti-Mouse IgG VeriBlot for IP conformation specific	Rat	Abcam	ab131368	WB (1:5000)	I
Anti-Mouse-BrightVision Poly-HRP	Goat	VWR	DPVM110 HRP	IHC	I
Anti-Rabbit IgG (H+L) Alexa Fluor 488	Goat	Invitrogen	A-11008	IF (1:250-1:1000)	I
Anti-Rabbit IgG (H+L) Alexa Fluor 555	Goat	Invitrogen	A-21428	IF (1:250-1:1000)	I
Anti-Rabbit IgG (H+L) Alexa Fluor 594	Goat	Invitrogen	A-11012	IF (1:250-1:1000)	I
Anti-Rabbit IgG IRDye 680RD	Donkey	Li-COR	926-68073	WB (1:5000)	I, II
Anti-Rabbit IgG IRDye 800CW	Donkey	Li-COR	926-32213	WB (1:5000)	I, II
Anti-Rabbit IgG, HRP	Donkey	Fisher Scientific	45-000-682	WB (1:2500)	I, II
Anti-Rabbit -Vision+ System- HRP Labelled Polymer	Goat	Dako	K4003	IHC	I
Anti-Rabbit-BrightVision Poly-HRP	Goat	VWR	KDPVR110HRP	IHC	I
Rat on Mouse HRP-polymer Kit	Mouse	Biocare	RT517	IHC	I

4.2.2 Co-Immunoprecipitation experiments (I)

Co-IPs were performed using GFPTrap agarose beads (ChromoTek), using optimized protocol for chromatin bound proteins shared by Prof. Andrew Blackford. Briefly, HEK-393T cells were transfected with GFP-tagged TopBP1 full length or

fragment expressing plasmids in combination with or without V5tagged CIP2A overexpressing plasmid (listed in Table 5) for 48 hours. The cells were lysed using IP lysis buffer (100mM NaCl, 1mM MgCl2, 10% Glycerol, 0.2% Igepal CA-630, 5mM NaF and 50mM Tris, pH 7.5, supplemented with protease and phosphatase inhibitors). 25units/ml benzonase (Cat# 70664-3, Millipore) was added to the lysate and the lysates were rotated on a tube revolver for 20 minutes at 4°C. The salt concentration in lysates was adjusted to 200mM NaCl and 2mM EDTA and further rotated for another 10 minutes. The lysates were centrifuged at a maximum speed for 15 minutes (4°C) and the supernatant was taken for the IP. A small volume (5%) of the lysate was used as inputs and rest was added to the GFPTrap agarose beads (washed 2x with the IP Lysis buffer). The lysate-beads mixture was rotated on a revolver at 4°C for 2.5 hours. GFP-IP complexes were washed 2 times with IP Lysis buffer and eluted with 2X SDS Sample buffer. Protein interactions were evaluated by western blotting of input and IP samples.

4.2.3 Western blotting (I, II)

RIPA Buffer (supplemented with protease and phosphatase inhibitor cocktail) was used to lyse the cells. The protein concentration was quantified using BCA assay kit (Pierce). 30-50ug of total protein processed with 6X SDS sample buffer were loaded onto the 4-20% Mini-PROTEAN TGX Precast protein gels (BioRad). After SDS-PAGE, the proteins were transferred onto nitrocellulose or PVDF membranes using TransBlot turbo transfer system (BioRad). The membranes were blocked with 5% milk in TBS-T for 30 minutes, followed by primary antibody incubation overnight at 4°C, and secondary antibody incubation for 1 or 2 hours at room temperature. The membranes were imaged with Odyssey CLx imaging system for IRDye conjugated secondary antibodies and using ECL based Curix 60 film processor for HRP conjugated secondary antibodies. The list of primary and secondary antibodies used in the dissertation are listed in **Table 6** and **Table 7** respectively.

4.2.4 RNA isolation, Primer sequences and qPCR (I,II)

RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel). RNA was converted into cDNA using Random primers, Recombinant RNAsin ribonuclease inhibitor (cat#N2111, Promega), M-MLV RT RNase (H-) point mutant (cat#M3681, Promega) and dNTP mix (Thermo Fisher). The primers used in the study are listed in **Table 8.** qPCR was run on QuantStudio12K Flex Real-Time PCR System (Thermo Fisher Scientific). GAPDH and ACTB were used as reference genes.

Table 8: Sequences of the primers used in qPCR.

Gene or cDNA	Sequence (5'-3')	Used in
Mouse-*Cip2a*-Fwd	gaacagataaggaaagagttgagca	I
Mouse-*Cip2a*-Rev	accttctaattgagccttgtgc	I
Mouse-*Krt8*-Fwd	agttcgcctccttcattgac	I
Mouse-*Krt8*-Rev	gctgcaacaggctccact	I
Mouse-*Krt14*-Fwd	atcgaggacctgaagagcaa	I
Mouse-*Krt14*-Rev	tcgatctgcaggaggacatt	I
Mouse-*Actb*-Fwd	tggctcctagcaccatgaaga	I
Mouse-*Actb*-Rev	gtggacagtgaggccaggat	I
Mouse-*Gapdh*-Fwd	tgcaccaccaactgcttag	I
Mouse-*Gapdh*-Rev	ggatgcagggatgatgttc	I
Human-*CIP2A*-Fwd1	tgccaggtggagactgttc	I, II
Human-*CIP2A*-Rev1	ggatcctgattcctcttcaaa	I, II
Human-*CIP2A*-Fwd2	ctgccaggtggagactgttc	I, II
Human-*CIP2A*-Rev2	aggatcctgattcctcttcaaa	I, II
Human-*CIP2A*-Fwd3	gaacagataagaaaagagttgagcatt	I, II
Human-*CIP2A*-Rev3	cgaccttctaattgtgcctttt	I, II
Human-*GAPDH*-Fwd	acccactcctccacctttga	I, II
Human-*GAPDH*-Rev	ttgctgtagccaaattcgttgt	I, II
Human-OGG1-Rev-1	tggctcttgtctcctcggta	II
Human-OGG1-Fwd-2	agaaattccaaggtgtgcga	II
Human-OGG1-Rev-2	gatgcgggcgatgttgttg	II
Human-APEX2-Fwd-1	gcagtcaacctggaatgctt	II
Human-APEX2-Rev-1	gccctacatgagaggcagac	II
Human-APEX2-Fwd-2	ggaggtgttccagcccttta	II
Human-APEX2-Rev-2	tgtcagtgcatccctggtca	II
Human-RFC2-Fwd-1	tgagaacgtgttcaaggtctgt	II
Human-RFC2-Rev-1	aggcttcgtcaatgttggca	II
Human-RFC2-Fwd-2	gagaacgtgttcaaggtctgtg	II
Human-RFC2-Rev-2	cagatgccacaagtgagcaag	II
Human-ACTB-Fwd	ccaaccgcgagaagatga	I, II
Human-ACTB-Rev	ccagaggcgtacagggatag	I, II

4.2.5 RNA Sequencing

For RNA sequencing, CIP2A knockdown in the samples was validated using qPCR and samples were submitted to RNA Sequencing at the Finnish Functional Genomics Centre at the Turku Bioscience Centre. The samples were sequenced with Illumina HiSeq2500, using single-end sequencing chemistry library with 50bp length.

4.3 Imaging and Flow cytometry (I)

4.3.1 Ionizing radiation induced foci (IRIF) experiments (I)

MCF10A cells were seeded in a 6-well plate, transfected with siRNAs and trypsinized and reseeded into ibidi chamber slides (ibiTreat#80826). In experiments with Mouse mammary epithelial cells (MMECs), cells from *Cip2a KO* and *Cip2a WT* mice were seeded to the ibidi chamber slides. X-Ray irradiation was done using Faxitron Multirad 350. At different time points post IR treatment, (1 hour, 2 hours and 6 hours), cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT), permeabilized with 1% Triton-X 100 made in 1X PBS for 10 minutes at RT. They were then blocked for 30 minutes at RT with 5% normal goat serum (ab7481, Abcam) in 1X PBS (Blocking buffer). Primary antibodies were incubated at 4°C overnight, and secondary antibodies were incubated for 1 hour at room temperature. The antibodies and their used dilutions are listed in **Table 6 & 7**. The samples were imaged using spinning disk microscopy at 63X magnification and analyzed using cell profiler/ImageJ. The images were background corrected so that the baseline intensities of both the sample groups was identical before the quantification. DAPI nuclear stains were used to mask the nucleus and only the foci that were present in the nucleus were quantified.

4.3.2 Mitotic index experiments (I)

MCF10A cells were seeded in a 6 well plate, transfected with siRNAs and then trypsinized and reseeded into ibidi 8-well chamber slides (ibiTreat #80826). The cells were treated with 10Gy IR and 1 hour later, 100ng/ml nocodazole block for 18 hours. The cells were fixed and stained with p. Ser10.Histone3 antibody following standard immunofluorescence protocols. The samples were imaged using Zeiss Axiovert or Evos-fl microscope at 10X magnification.

4.3.3 Microscopy (I)

For IRIF experiments, 3i CSU-W1 spinning disk confocal microscope with 63X oil immersion objective or LSM780 with 63X objective were used. Z-stack images were taken, and the quantifications were performed on maximum intensity Z-projected images. For mitotic index experiment Zeiss Axiovert or Evos-Fl with 10X objective were used.

4.3.4 Flow cytometry

The freshly isolated MMECs were used for characterizing the basal and luminal proportions of the mammary epithelial cells. The method used was described previously in (Peuhu et al., 2017). Cells were suspended in Tyrodes buffer, and around 0.5 million cells were used per labelling per genotype. Antibody dilutions were made in Tyrodes buffer. Fluorophore conjugated antibodies for two antibody pairs CD24/CD29 or CD24/CD49f were incubated for 30 minutes at 4°C, washed couple of times and then fixed with 4% PFA for 10 minutes at RT. Samples were analyzed using BD LSR Fortessa flow cytometer. For gating, first live and single cells were gated using FSC/SSC. The lineage negative (CD31 negative, CD45 negative) were gated out and CD24 positive cells, representing epithelial cells were gated. Within the epithelial cells, basal cells have CD24 low and CD29 or CD49f high and luminal cells have CD24 high and CD29 low/negative or CD49f low/negative. These parameters were used to determine the proportion of basal and luminal cells. The proportion of luminal and basal cells were quantified using FlowJo software.

4.4 Drug screening experiments

4.4.1 Drugs used in the study (I, II)

Table 9: List of chemical inhibitors used in the study.

Drug name	Mechanism/ Targets	Solvent	Used in
Doxorubicin	Topoisomerase II inhibitor	DMSO	I, II
Gemcitabine	Antimetabolite; Nucleoside analog	DMSO	I, II
Cisplatin	Platinum-based antineoplastic agent	H2O	I
Olaparib	PARP inhibitor	DMSO	I
Epirubicin	Topoisomerase II inhibitor	DMSO	I
Fluorouracil	Antimetabolite	DMSO	I, II
Docetaxel	Mitotic inhibitor, taxane microtubule stabilizer	DMSO	I, II
Paclitaxel	Mitotic inhibitor, taxane microtubule stabilizer	DMSO	I
Eribulin	Mitotic inhibitor, microtubule depolymerizer.	DMSO	I
Talazoparib	PARP1/2 inhibitor	DMSO	I
NZ-1154 (DBK-1154)	small molecule reactivators of PP2A	DMSO	I, II
NZ-1160	small molecule reactivators of PP2A	DMSO	I
DT-061	small molecule reactivators of PP2A	DMSO	I
Thiostrepton	FOXM1 inhibitor	DMSO	II

4.4.2 High throughput drug screening (I)

Drug screening was conducted in collaboration with the Turku Screening Unit, Turku Bioscience Centre, or Finnish Institute of Molecular Medicine (FIMM), University of Helsinki. Compounds in serially diluted concentration range were plated in clear bottom 384 well plates. The drugs were plated using Echo 550 Liquid Handler (Labcyte). Cells were seeded along with CellTox Green (Promega) using MultiFlo FX dispenser (BioTek). The fluorescence (cytotoxicity) and Cell Titer Glo (Promega) luminescence (cell viability) were measured using PheraStar (BMG Labtech) plate reader at 72 hours.

4.4.3 Cell viability experiments (I, II)

Cell viability experiments were done in 96 well format, using WST-1 reagent (Sigma) or Cell Titer Glo (Promega). Optimized cell number of cells were seeded in 96 well plates. Drug treatments were done the following day for 72 hours. In case of combination with CIP2A siRNA, cells were seeded first in 6 well plates, and reseeded to 96 well plates after 24 hours of siRNA transfection. At the end of the treatment time points, WST-1 or Cell Titer Glo reagents were added to the plates. For WST-1, the plates are incubated at 37°C for 2 hours and read using absorbance at 450nm. For Cell Titer Glo, plates are shaken for 5 mins at RT. Plates were read on Biotek Synergy plate reader.

4.4.4 Analysis of drug screening experiments (I, II)

The cell viability raw data was analyzed using BREEZE tool (Potdar et al., 2020) which quantifies the differential sensitivity scores (DSS) for each drug. Greater DSS values indicate more sensitivity. For calculating synergy scores, the raw data of combinations was analyzed using Synergy finder tool (Ianevski et al., 2022) (https://synergyfinder.fimm.fi/). The dose response curves were plotted and IC_{50} were analyzed using GraphPad Prism.

4.5 Mouse experiments (I, II)

4.5.1 DMBA induced carcinogenesis (I)

1mg DMBA (7,12-dimethyl benz[a]anthracene) dissolved in 200µL of corn-oil was dosed to the WT and KO mice by oral gavage once every week for 6 weeks. The mice were monitored twice every week, until development of tumors or other morbidities. When the mice had to be sacrificed, they were autopsied to analyze the

formation of tumors in all the tissues. The premalignant mammary glands were collected 2 weeks after the last DMBA dose. For IHC, tumors and tissues were fixed in formalin whereas for DNA, mRNA and protein analysis samples were snap frozen in liquid nitrogen.

4.5.2 Xenografts (I, II)

The BLBC patient derived xenograft (PDX) study described in Publication I was performed in collaboration with Goutham Narla group at the University of Michigan. Briefly the PDX was generated from a chemotherapy resistant (doxorubicin and cyclophosphamide) patient. The PDX were fragmented and implanted into NOD/scid mice. SMAP treatment (twice a day at 5mg/kg or 15mg/kg) was started when the tumor volume reached 100 mm^3. Tumor volume was measured every two days by caliper measurement. The study was continued until 43 days.

For mammary orthotopic fat pad xenograft study described in publication II, 24 female athymic nude mice (8 weeks old) were randomized into 4 groups of 8 mice. MDA-MB-231 cells were transfected with non-targeted control siRNA (siCTRL) or CIP2A targeted siRNA (siCIP2A) 24 hours prior to the inoculation of the cells into the mice. 2.5 million cells were inoculated orthotopically to the left mammary fat pad of each mouse. Gemcitabine treatment (25mg/kg *i.p.* once every five days*)* was started 2 days after the inoculation of the cells, and tumor volumes were calculated by caliper measurement.

4.6 Bioinformatics analysis (I, II)

4.6.1 Generation of CIP2A transcriptional signature (II)

The RNASeq data was processed in collaboration with the Medical Bioinformatics Centre at the Turku Bioscience Centre. Reproducibility optimized test statistic (ROTS) package (Seyednasrollah et al., 2016; Suomi et al., 2017) was used to generate differentially regulated genes (control vs siCIP2A samples) from the RNA sequencing data. Two weightage factors, siRNA efficiency and CIP2A dependence were used as weights for generating the signature. Log_2 fold changes of CIP2A expression were used as weights for the siRNA efficiency signature and meta-analysis was used to combine ROTS p-values across different cell lines using a weighted Z-test (Zaykin, 2011). In the same way CIP2A dependence indices, calculated from mean colony areas on CIP2A depletion for each cell line were used as weights for CIP2A dependence signature. CIP2A signature was generated by meta-analysis to combine p-values, using a weighted Z test where both the siRNA efficiency signature weights and CIP2A dependence weights are given equal

weightage. To define the signature genes, a meta p-value of 0.01 was used as a cut-off and only genes whose fold changes in each of the cell lines was in the same direction were considered.

4.6.2 Pathway analysis - Reactome and GSEA (I, II)

A ranked list was generated from the gene expression FCs and p-values for each gene. Gene Set Enrichment Analysis (GSEA) was run using default settings of Preranked GSEA and using the Hallmarks gene set from the Molecular Signature Database (MSigDB) (Liberzon et al., 2015). For mouse data, the mouse genes were converted to the equivalent human genes before running the GSEA. The CIP2A transcriptional signature genes were analyzed on Reactome database (https://www.reactome.org) (Gillespie et al., 2022). Reacfoam plots and most significant pathways for differentially regulated genes were identified.

4.6.3 Clinical validation of signature using METABRIC datasets (II)

METABRIC dataset consists of 1980 breast cancer samples from UK and Canada and represented all grades and subtypes of breast cancer. The median age at diagnosis was 61.7 years and maximum follow up time of 351 months (Curtis et al., 2012; Pereira et al., 2016). The mapping of signature genes with the METABRIC patient's expression profiles was done similar to Connectivity Map (CMap). For each patient, the enrichment scores were calculated by evaluating CIP2A signature as geneset in GSEA. The final enrichment score (ES) for each individual was calculated as

$$ES = (ES_{up} - ES_{down})/2$$

where ES_{up} is the enrichment score for upregulated genes of the patient, and ES_{down} down is the enrichment scores for downregulated genes. The p-values were calculated as average of upregulated and downregulated enrichment p-values. Using p-value cutoff of 0.05, all the patients with a positive enrichment score indicate that they are positive for the signature (behaving like CIP2A control samples) and patients whose enrichment scores is negative indicate that have negative signature or behaving similar to CIP2A knockdown samples. The remaining patients were unclassified. Kaplan Meier analysis was done for the stratified individuals using Survival (https://cran.r-project.org/web/packages/survival/index.html) and Survminer (https://cran.r-project.org/web/packages/survminer/index.html) R packages, and overall and relapse free survival were calculated for each stratified group. Log rank test was used to evaluate significance difference between the groups.

4.6.4 Data mining from public datasets (I, II)

Dependency Map (DepMap) (https://depmap.org) was used to analyze co-dependent genes for CIP2A coming from genome wide CRISPR Cas9 drop out screens across 700+ cell lines. The Chronos and CERES essentiality scores for CIP2A from breast cancer cell lines were also downloaded from DepMap (Dempster et al., 2021; Meyers et al., 2017). Broad Institute's *Drug Connectivity Map (CMap)* (accessed at https://clue.io/) was used to identify drugs that elicit similar expression changes as our queried gene lists. This was used to identify drugs that transcriptionally mimic similar changes as CIP2A downregulation (Lamb et al., 2006; Subramanian et al., 2017). *cBioportal* (accessed at https://www.cbioportal.org/) was used for subgroup analysis of the Top100 CIP2A positive signature patients and Top100 CIP2A negative signature patients from METABRIC. Disease free survival of different PAM50 classification subgroups of breast cancer patients from publicly available gene expression dataset GSE21653 (Sabatier et al., 2011) were analyzed using R2-Genomics Analysis and Visualization Platform (Accessed at https://r2.amc.nl) (Koster et al., 2019).

5 Results

5.1 CIP2A drives DMBA-induced mammary tumorigenesis (I)

5.1.1 CIP2A is selectively essential for mammary tumorigenesis (I)

CIP2A overexpression has been observed in several human cancer types and has been associated with a poor prognosis in almost all the malignancies in which it was studied (Khanna & Pimanda, 2016). To determine whether CIP2A plays a more significant role in any specific type of cancer, we generated CIP2A knockout (*Cip2a* KO, *Cip2a*$^{-/-}$) mice and subjected them to chemical carcinogenesis using oral DMBA (7,12-dimethylbenz[a]anthracene) once every week for six weeks (I, Figure 1A). We evaluated the mice until tumors formed. DMBA treatment caused a similar number of mutations in the mammary glands of both *Cip2a* WT and KO mice (I, Figure 1B), but significantly fewer mammary tumors were formed in the *Cip2a* KO mice (I, Figure 1C, D). Notably, both the *Cip2a* WT and KO mice survived for a similar number of days (I, Figure S1A), indicating that the lower number of tumors in the *Cip2a* KO mice was not due to early death or decreased survival. However, there was no difference in the number of tumors formed in other tissues such as skin, stomach, lung, or ovaries between the *Cip2a* genotype (I, Figure 1C, S1B). We validated using two independent *in vivo* models that CIP2A is not essential for tumorigenesis in ovarian and skin cancer. For ovarian cancer, we crossed MISIIR-Tag mice which spontaneously produce high grade ovarian tumors, with *Cip2a* KO and WT mice. Both *Cip2a* WT and KO MISIIR-Tag mice formed the same number of ovarian tumors, as confirmed by PET/CT imaging (I, Figure S1 C,D,E). For skin carcinogenesis, the *Cip2a* WT and KO mice were treated with DMBA/TPA two-stage carcinogenesis, and there was no difference in the skin tumors between the genotypes (I, Figure S1 F). In summary, these results from multiple *in vivo* models, indicate that *Cip2a* is selectively essential for the initiation of mammary tumors.

5.1.2 DMBA treatment induces CIP2A expression which drives BLBC (I)

Previous reports have shown that CIP2A protein expression in normal breast tissue is very low, and its expression increases with the aggressiveness and grade of tumors (Côme et al., 2009). Consistent with this finding, we found that Cip2a mRNA expression was quite low in *Cip2a* WT untreated mammary glands (I, Figure 2A). On analysis of the premalignant mammary glands collected 2 weeks after the last DMBA dose, it was observed that treatment with DMBA induced the Cip2a mRNA by around two-fold. Most importantly, the *Cip2a* levels in the DMBA induced mammary tumor were ten times higher than the normal mammary glands (I, Figure 2A). Since *Cip2a* expression is almost negligible in normal cells, but induced upon DNA damage in premalignant tissues, it fulfills the requirement of a driver and hence we can conclude that *Cip2a* drives mammary tumorigenesis. The mammary gland tumorigenesis with oral DMBA was previously reported to be forming tumors of basaloid origin (J.-S. Kim et al., 2013). We were able to verify that the tumors belonged to the BLBC subtype by immunohistochemistry. The tumors were positive for keratin-14 (K14) and negative for keratin-8 (K8), indicating basal origin. The tumors also had high proliferation and belonged to high grade as confirmed by positive Ki-67 and MYC staining (I, Figure 2B). Also, 7 out of 10 tumors (70%) characterized were triple-negative on IHC staining of ER, PR, and HER2 (I, Figure 2C, S2A) which is similar to the expected proportion of triple-negative subtype within BLBC.

5.1.3 CIP2A does not have a role in normal mammary gland development (I)

To further investigate the mammary glands of both the *Cip2a* WT and KO mice and determine if there were any developmental defects in the mammary glands of KO mice that could be a reason for their inability to form tumors, we isolated mammary epithelial cells from both genotypes and characterized their luminal and basal proportions by flow cytometry, using standard surface markers. Our results showed that there was no difference in the proportion of basal and luminal cells between Cip2a genotypes (I, Figure S2C-E). Additionally, there was no difference in the *Cip2a* expression between the luminal and basal cells of the mammary gland in *Cip2a* WT mice (I, Figure S2F). Although previous studies have reported defects in spermatogenesis in *Cip2a* WT male mice (Ventelä et al., 2012), our Cip2a KO female mice showed no defects and were fully fertile. We also analyzed the mammary gland branching patterns matched to the estrous cycle of the mice and observed no difference in the mammary gland branching morphogenesis between *Cip2a* WT and KO mice (I, Figure S2G). These findings suggest that while Cip2a is

not essential for normal mammary development, its expression is induced upon DMBA treatment and is crucial for the initiation of BLBC tumors.

5.2 Generation of a CIP2A dependent BLBC transcriptional signature (II)

5.2.1 Novel approach to developing transcriptional signature (II)

Given the newly established role of CIP2A as a driver of BLBC, we hypothesized that it might be driving common transcriptional processes in BLBC. To identify these common mechanisms, we used three distinct siRNAs to depleted CIP2A from five BLBC cell lines and evaluated their differential gene expression by RNA-Seq (II, Figure 1A). We selected cell lines from diverse tumor types, intrinsic subtypes, and a mix of BRCA mutation statuses to truly represent the heterogeneity of BLBC disease. Interestingly, the dependence on CIP2A (from the DepMap database) in these cell lines was also quite variable (II, Figure S1A). We used the reproducibility optimized test statistic (ROTS) (Seyednasrollah et al., 2016; Suomi et al., 2017) to evaluate the differential expression of genes between CIP2A knockdown and CIP2A control samples. At p-value cutoffs of 0.01 and 0.05, the only overlapping differentially expressed gene across five cell lines and three siRNAs was CIP2A itself (II, Figure 1B). Using slightly less stringent p-value cutoffs did not make much of a difference as there were 4 and 25 differentially regulated genes at p=0.1 and 0.2 respectively (II, Figure S1B). However, when we evaluated each of the cell lines individually, two cell lines had a small number of differentially expressed genes (II, Figure S1C) indicating that looking for overlap across five cell lines might not be a suitable approach. We found that there was huge variability in the efficacies of the three CIP2A siRNAs used, especially siCIP2A#2, which was the least efficient in all the cell lines used in the study (II, Figure 1C, S1D). We further validated the CIP2A dependence between cell lines using colony growth assays and as predicted by DepMap data, the dependence on CIP2A varied a lot between cell lines (II, Figure 1D).

We rationalized that the lack of overlap in the RNASeq data was due to the variation in siRNA efficiency between siRNAs and CIP2A dependence between the cell lines. Since the samples in which CIP2A depletion is most effective would resemble closely to the transcriptional changes coming from CIP2A inhibition, we performed a weighted Z test (Zaykin, 2011) by giving more weightage to the samples with the most effective CIP2A downregulation and vice-versa. The signature generated with $\log_2$fold-change of CIP2A expression as weights was termed as "*siRNA efficiency signature*", and it consisted of 97 upregulated and 172

downregulated genes (II, Figure 2A). Following a similar rationale, we gave more weightage to cell lines that were most dependent on CIP2A and vice-versa, based on the mean colony area and this generated "*CIP2A dependence signature*" comprising 61 upregulated and 116 downregulated genes (II, Figure 2A). To generate a combined signature that corrects for both CIP2A siRNA efficiency and CIP2A dependency variations, both CIP2A dependence and siRNA efficiency weights were given equal weightage (as shown in schematic II, Figure 1E). The combined "*mean signature*" (or $CIP2A^{signature}$) consisted of 225 genes (78 upregulated and 147 downregulated genes) (II, Figure 2A, B)

To summarize, this novel bioinformatic approach can be used to generate gene signatures for heterogeneous cancer types with varying functional dependencies and especially in scenarios where siRNAs have varying efficacies.

5.2.2 CIP2A signature can be used to identify aggressive breast cancer (II)

We validated the CIP2A signature by using a publicly available breast cancer METABRIC dataset (Curtis et al., 2012) consisting of 1424 patient samples. The gene expression profiles of the METABRIC patients were used to score the patients as $CIP2A^{signature}$ positive (if their transcriptional profiles matched CIP2A control samples) or $CIP2A^{signature}$ negative if their transcriptional profiles matched CIP2A siRNA knockdown samples. If their transcriptional profiles could not be scored positive or negative based on signature, they were considered unclassified. $CIP2A^{signature}$ successfully stratified the METABRIC patients into three distinct groups with notable differences in their overall survival (OS) and relapse-free survival (RFS) (II, Figure 2C). The median survival of the $CIP2A^{signature}$ negative group was more than 100 months longer than that of the $CIP2A^{signature}$ positive group (250 months vs 120 months) indicating a less aggressive disease for the $CIP2A^{signature}$ negative patients' group. We also evaluated the performance of the other signatures (*siRNA efficiency signature*, *CIP2A dependence signature*) in identifying aggressive breast cancer cases from METABRIC and found that the combined signature ($CIP2A^{signature}$) performed much better in terms of the p-value for differences in the prognosis of positive and negative signature patients stratified (II, Figure S2A). Further evaluation of a subgroup of 200 patients consisting of the top 100 most $CIP2A^{signature}$ positive and top 100 $CIP2A^{signature}$ negative patients revealed that the $CIP2A^{signature}$ performs even better in identifying the good and bad prognosis breast cancer cases (II, Figure 2D). Analyzing the characteristics of the top 100 positive and negative signature patients from METABRIC, we found that a higher proportion of the top100 signature positive patients were of ER- subtype and had high MYC amplification indicative of aggressive breast cancer compared to the top 100

signature negative patients, who had mostly ER+ and PIK3CA mutations indicative of better prognosis (II, Figure S2B, C).

5.3 CIP2A interacts with TopBP1 and promotes the G2/M progression of DNA damaged cells (I, II)

5.3.1 A novel role for CIP2A in DDR (I, II)

We collected mammary glands from premalignant *Cip2a* WT and KO mice treated with DMBA after the sixth dose of DMBA and conducted RNA-Seq analysis. We performed a Gene Set Enrichment Analysis (GSEA) to identify pathways that are enriched between *Cip2a* KO samples compared to the *Cip2a* WT mice. CIP2A inhibition has been previously reported to downregulate MYC (Junttila et al., 2007) and E2F1 (Laine et al., 2013) activity, and hence it was not surprising to see the downregulation of MYC and E2F1 target genes as the enriched pathways in GSEA. Interestingly, we discovered a previously unknown G2/M checkpoint function of CIP2A that is crucial in tumor development (I, Figure 2D). Although CIP2A is primarily a cytoplasmic protein, we found a small amount of nuclear CIP2A in premalignant mammary gland cells and DMBA treated *Cip2a* WT tumors indicating a role for nuclear CIP2A in mammary tumorigenesis (I, Figure S2B). We evaluated a genome wide CRISPR/Cas9 dropout screen performed in 739 human cancer cell lines from DepMap (Avana2020 Q1) and assessed the top co-dependent genes with CIP2A across the genome. Strikingly, all of the top 10 co-dependent genes with CIP2A were involved in DNA Damage response (DDR) (I, Figure 3A). CIP2A was the top co-dependent gene for RHNO1, TOPBP1, POLQ, NBN, PARP1 with CIP2A ranking higher in correlation than TOPBP1 for RHNO1 (Cotta-Ramusino et al., 2011) or ATR for TOPBP1 which are *bona fide* DDR effectors involved in ATR signaling (Mordes et al., 2008) (I, Figure 3B). Furthermore, all the top 10 co-dependent CIP2A genes formed a close network, and none of these proteins have been previously reported to be involved in direct interaction with CIP2A (I, Figure 3C). The pathway analysis of the CIP2A co-dependent genes using Reactome database revealed that the key processes regulated by these genes are G2/M, DNA damage checkpoint, and double strand break (DSB) repair related (I, Figure 3D). Among the CIP2A co-dependent genes, TOPBP1 and POLQ were also among the top 25 significantly co-expressed genes with CIP2A (I, Figure 3E, S4A). This correlation was significant also when specifically analyzing BLBC cases (I, Figure S4B).

The CIP2A^{signature} generated from BLBC cell lines (II, Figure 2A, B) was analyzed using the Reactome database to identify the key processes regulated by CIP2A in BLBC. As previously found, the analysis demonstrated that CIP2A

regulates processes related to DNA repair, double strand break repair, and G2/M transition (II, Figure 3A, B and S3A). The pathway analysis also revealed that CIP2A downregulation leads to a defect in Base Excision Repair (BER) associated with OGG1, a glycosylase that detects and removes oxidatively damaged guanines (8-oxodG; also known as 7,8-dihydro-8-oxoguanine) (II, Figure 3A). qPCR validation of CIP2A downregulated MDA-MB-231 cells revealed downregulation of downstream members of BER pathway such as APEX2 and RFC2 (II, Figure 3C). Moreover, BER associated OGG1 was significantly down on CIP2A depletion in 2 out of the 4 BLBC cell lines tested (II, Figure S3B).

In summary, these findings indicate that CIP2A plays a significant role in regulating DNA damage response (DDR) and G2/M signaling in BLBC.

5.3.2 CIP2A dampens accumulation of TopBP1 and RAD51 at the site of DNA damage (I)

During a genome-wide Yeast two-hybrid (Y2H) screening to identify interaction partners of CIP2A, TopBP1 was discovered as a CIP2A interaction partner. Since Y2H only detects direct interactions between two proteins, we presumed that this direct interaction between CIP2A and TopBP1 is independent of PP2A (I, Figure 4A). This interaction was confirmed also using Proximity Ligation Assay (PLA) (I, Figure S5A) and Co-Immunoprecipitations (Co-IPs) with overexpressed GFP-tagged TopBP1 (I, Figure 4B). To pinpoint the exact region of TopBP1 interacting with CIP2A, we overexpressed different GFP tagged truncated TopBP1 mutants and performed Co-IPs with GFPTrap beads. We observed that CIP2A interaction still occurred inTopBP1 missing the ATR activating domain (AAD) and BRCT7/8, and was lost in TopBP1 fragment with BRCT0-5 domains, indicating that the region between BRCT5 and BRCT6 in TopBP1 is crucial for the interaction with CIP2A (I, Figure 4C, D). By mapping the common interaction region of the TopBP1fragments from Y2H screen, the interaction region on TopBP1 was found to be between amino acids 829 to 853, which is between BRCT5 and 6 as validated by Co-IPs (I, Figure 4A).

Furthermore, we discovered that the interaction between CIP2A and TopBP1 was enhanced in the presence of AAD which is required for TopBP1 interaction with ATR and downstream checkpoint activation (I, Figure 4C, D). CIP2A depletion in the non-transformed basal epithelial MCF10A cell line resulted in a pronounced increase in phosphorylated ATR, indicating a direct role of CIP2A in TopBP1 regulated DDR signaling (I, Figure 4E). Additionally, levels of γ-H2AX, which is a target of phosphorylated ATR were increased in CIP2A depleted cells overexpressing AAD containing mutant of ToPBP1 (I, Figure 4F). In CIP2A silenced MCF10A cells, ionizing radiation (IR) caused increased accumulation of

TopBP1 at the site of DNA double strands (DSBs) as seen using ionizing radiation induced foci (IRIF) assay (I, Figure 4G, H). Also, mice mammary epithelial cells (MMECs) from *Cip2a* KO mice formed more RAD51 foci (which is downstream of TopBP1 in the homologous recombination repair pathway) on IR induced DNA damage compared to the MMECs from Cip2a WT mice (I, Figure 4I, J).

In summary, these results indicate that CIP2A interacts with TopBP1 and inhibits the chromatin recruitment of TopBP1 and RAD51 at the site of DNA damage.

5.3.3 CIP2A allows the mitotic progression of DNA-damaged cells (I)

In order to evaluate the role of CIP2A in G2/M checkpoint, we analyzed the mitotic index of DNA damaged MCF10 cells. The MCF10A cells were treated with 10Gy IR for 1 hour, followed by G2/M arrest using nocodazole for 18 hours. On release from the nocodazole block, only cells that have abrogated the checkpoints proceed to mitosis. The percentage of cells entering into mitosis can be evaluated by staining cells positive for Histone H3, phosphoserine 10 (H3. pS10). Compared to the CHK1 silenced samples in which checkpoints are abrogated, CIP2A silencing had a significantly decreased mitotic cells indicating that CIP2A allows the dampening of G2/M checkpoints and progression of the DNA damaged cells into mitosis (I, Figure 1E). In untreated or non-DNA damaged conditions, there was no difference in the mitotic index values indicating the role of CIP2A in G2/M, specifically in DNA damaged cells (I, Figure S1G). We independently validated these results in another HAP1 genetic screen where CIP2A was essential for the progression of cells under repeated IR damage (I, Figure 1F, S1H).

5.4 CIP2A is essential in BRCA mutant BLBCs (I)

5.4.1 CIP2A is a prognostic biomarker for BL-TNBCs (I)

Our aim was to assess whether CIP2A's role in driving BLBC initiation in mice translates to human BLBC. To do this, we analyzed the mRNA expression levels of CIP2A and TopBP1 in the breast cancer cohort of the TCGA dataset. Both CIP2A and TopBP1 expression were correlated with the grade of the tumors, and highest expression was found in BLBC (I, Figure 5A, S6B). We also evaluated the expression in triple-negative (TNBC) and basal like (BL) subtypes of breast cancer and found that CIP2A expression was higher in basal-like subtypes (both BL-TNBC and BL-non TNBC) (I, Figure S6B). Since it was reported previously that TP53 mutant breast cancers drive the expression of CIP2A by p21-E2F1 (Laine et al., 2013), we evaluated the CIP2A expression in TP53 WT and TP53 mutant breast

cancer from a publicly available expression dataset GSE21653. Consistent with our previous understanding, CIP2A expression was enhanced in TP53 mutant breast cancer cases (I, Figure S6C). To investigate the clinical applicability of CIP2A as a prognostic biomarker, we used the FinHer dataset (Joensuu et al., 2009) and other publicly available IHC and gene expression datasets. FinHer consisted of 1010 breast cancer patients (median age 50.9 years) with 89% having axillary node positive cancer and 28.2% of them having more than three metastatic lymph nodes. Tumors immunostained negative for ER, PR, HER2 and positive for EGFR and positive for basal cytokeratin CK5 were classified as basal-like TNBC whereas tumors immunostained negative for ER, PR, HER2, EGFR and CK5 were classified as non basal-like TNBC. High CIP2A was correlated with poor overall and disease-free survival only in Basal like TNBC subtype (BL-TNBCs) from both protein based IHC and mRNA based expression levels (I, Figure 5B, D). In non-basal like TNBC, there was no difference in the survival of patients between CIP2A high and CIP2A low protein or mRNA expression (I, Figure 5C, E). CIP2A also had a prognostic value in TNBC subtype, not selected into basal and non-basal subtypes (I, Figure S6E, H). Notably, we observed no difference in the prognosis of breast cancer patients between CIP2A high and CIP2A low patients when all subtypes were considered or when only ER+ breast cancer cases were filtered. when breast cancer patients were unselected and all the subtypes were taken together, or when only ER+ breast cancer cases were filtered (I, Figure S6 D,F,G).

Next, we analyzed the dependence of CIP2A in human breast cancer cell lines from the DepMap database. We found that out of the top 12 most CIP2A dependent breast cancer cell lines, we found that 8 were of BLBC subtype (I, Figure 5F). Also, 7 out of the 8 top CIP2A dependent cell lines also harbored a BRCA1 or BRCA2 mutation indicating a homologous recombination repair defect (HRD).

5.4.2 CIP2A is a novel synthetic lethal target in BRCA mutant BLBCs (I)

To evaluate the CIP2A dependence on BRCA mutation status, we isolated mouse cancer cell lines from three different genetic mouse models that spontaneously form tumors (KB1P - *Trp53* and *Brca1 mutant,* KEP – *Trp53/E-Cadherin mutant,* and WEA – *E-Cadherin and Akt mutant)* and performed colony growth assays. Only KB1P cells that had *Brca1* and *Trp53* mutant background, indicative of basal-like breast cancer showed synthetic lethality on CRISPR/Cas9 based CIP2A knockout with two different gRNA (I, Figure 5G) whereas, in KEP and WEA cells which are representing Invasive lobular subtype, CIP2A knockout did not affect the survival of these cell lines (I, Figure 5G, S7A). Also, the most CIP2A dependent BLBC cell line in another independent colony growth assay was HCC38 which belonged to p53

mutant and HRD background (due to BRCA1 silencing by promoter methylation and BRCA2 mutant) (I, Figure 5H). We performed RNASeq analysis in CIP2A silenced HCC38 cells and found that CIP2A downregulation caused downregulation of E2F targets, MYC targets and G2M checkpoint pathways similar to our observation in premalignant DMBA treated *Cip2a* KO mammary glands (I, Figure 5I and Figure 2D). This finding was further validated by western blotting. In HCC38 cells, CIP2A silencing caused downregulation of MYC and E2F1 activity as seen by the decrease of phospho serine 62 of MYC and phospho serine 364 of E2F1 (I, Figure 5I).

5.5 Targeting CIP2A-dependent DDR in BLBC (I, II)

5.5.1 CIP2A downregulation sensitizes BLBC cells to different DDR drugs (I, II)

Since BLBC is characterized by high genomic instability, especially due to homologous recombination repair (HR) deficiency, they are highly dependent on other compensatory repair pathways such as BER and NHEJ (Lord & Ashworth, 2017). This creates a vulnerability in these cells which maybe therapeutically targeted. We evaluated standard of care chemotherapy drugs used in the clinic to treat BLBC patients, to see if any of these drugs would get sensitized in BLBC on CIP2A depletion. Among 5-fluorouracil (5FU), docetaxel, doxorubicin and gemcitabine that were tested, gemcitabine was the only drug which showed highest sensitization with CIP2A silencing in BLBCs (II, Figure 4A). In the highly CIP2A dependent cell line HCC38, where CIP2A silencing already is very effective, further addition of gemcitabine increased the sensitivity by 4-fold (IC50 400nM in siCTRL+gemcitabine vs 99nM in siCIP2A+gemcitabine), measured by cell viability assays (II, Figure 4C). This drug combination was further evaluated in an orthotopic *in vivo* model. We inoculated CTRL siRNA and CIP2A targeted siRNA treated MDA-MB-231 cells to the mouse mammary fat pads and evaluated the sensitivity of gemcitabine treatment on the xenograft growth. On Day 14, there was a significant difference in tumor volumes in siCIP2A+gemcitbaine group compared to siCTRL+gemcitabine (II, Figure 4D). On day 29 when the mice were sacrificed at the end of the study, we dissected the tumors and evaluated the tumors and notably the tumors from siCIP2A+gemcitabine group were the smallest and weighed less compared to the vehicle treated group or siCIP2A alone groups (II, Figure 4E, S4A). These effects are notable considering that the CIP2A was downregulated by transient transfection of siRNA one day before inoculation and still provided a very long term CIP2A downregulation (approximately 9 days) (II, Figure S4B). Overall, the drug

gemcitabine was quite well tolerated and there was no significant weight loss in the mice throughout the duration of the study (II, Figure S4C).

Also, CIP2A silencing sensitized the BRCA WT BLBC cell line (MDA-MB-231) to two different PARP inhibitors olaparib and niraparib, indicating that CIP2A silencing might be creating an HRD-like defect in BLBCs which makes them vulnerable to PARP inhibitors which are normally potent and effective only in BRCA mutant cells (I, Figure S7C). Pathway analysis of CIP2A^{signature} revealed "*ATR activation in response to replication stress*" as one of the pathways enriched in CIP2A downregulated cells (II, Figure 3A). We also observed increased ATR activity in CIP2A depleted non transformed MCF10A cells and also BRCA WT cell line MDA-MB-231 (I, Figure 4E,F). So, this gave us a strong rationale for testing ATR inhibitor drugs in combination with CIP2A silencing. We found that CIP2A silencing sensitized BLBC cell lines to ATR inhibitor drug VE-821 by cell viability assays (II, Figure 4B). This was also observed previously in another published study (Hustedt et al., 2019).

5.5.2 SMAPs downregulate CIP2A transcription and have the potential to be used in BLBC (I,II)

Though the interaction between CIP2A and TopBP1 is independent of PP2A, there is also slight evidence that PP2A maybe also involved in newly discovered DDR roles of CIP2A from a recent phosphoproteomics screen, which identified NBN S432 as a PP2A target regulated by CIP2A (Kauko et al., 2020). Since there are no CIP2A inhibitors, we wanted to test the recently developed small molecule activators of PP2A (SMAPs) in BLBCs to evaluate if they had a role in regulating DDR. We tested a panel of BLBCs with two different SMAPs DBK-1154 and DT-061 and found them to be very effective in these cells (I, Figure 6A, S8A). We also tested SMAPs in five patient derived stem-like cell lines of triple-negative subtype. Since these patients already received neoadjuvant chemotherapy, they showed resistance to standard chemotherapy drugs as expected. But interestingly, SMAPs showed great sensitization in all the patient derived cell lines generally resistant to chemotherapies (I, Figure 6B). We independently studied the SMAP DT-061 in another BLBC patient derived xenograft mouse model and saw that DT-061 treatment significantly decreased tumor growth. (I, Figure 6C). Interestingly, we noticed that SMAP treatment downregulated CIP2A in most of the PDX tumors (I, S8C,D). We further validated the role of SMAPs in CIP2A downregulation in patient derived as well as commercial cell lines (I, Figure 6D,E, S8 E). We also evaluated the time course of CIP2A protein and mRNA on treatment with SMAP and found that CIP2A is downregulated at a transcriptional and protein level by SMAP treatment (I, Figure 6F, S8F,G). To understand the exact mechanism of whether SMAP regulates CIP2A

at a protein or transcriptional level, we checked the time course of upstream ERK1/2 and MYC activity in BLBC cells on treatment with SMAP. The time course revealed that SMAP downregulates the ERK1/2 activity which downregulates CIP2A on a transcriptional level. MYC downregulation was observed to be happening after CIP2A downregulation (I, Figure 6G, S8H). Hence, SMAPs can be considered surrogate CIP2A inhibitors.

Next, we wanted to evaluate if SMAPs also induce similar effects of DDR as CIP2A inhibition. SMAP treatment caused a profound increase in γ-H2AX, p-ATR and activation of CHK2 (I, Figure 6H,I, S9A,B). Interestingly, the SMAP effect on CHK2 activation happened before CIP2A downregulation whereas the activities of ATR and γ-H2AX induction, linked to the TopBP1 interaction of CIP2A were seen to be induced only after CIP2A downregulation (I, Figure 6J, Figure S9D,E,F,G,H) indicating that SMAP has other PP2A dependent effects on DDR such as CHK2 activation which is not observed with CIP2A inhibition. It would have been nice to see the kinetics of γ-H2AX induction with more time points between 6 and 24 hours. We noticed that γ-H2AX increases from 3 fold levels at 6 hours to 15 fold levels at 24 hours, but we do not know if the trend is following the CIP2A kinetics or it is exponential or there are fluctuations. It might tell us if the induction of SMAP comes from other PP2A activation effects of SMAP (which we believe might be the case) as the induction of γ-H2AX is quite drastic compared to the CIP2A downregulation kinetics (I, Figure 6J). We noticed that the effect of SMAPs on ATR activation and γ-H2AX are CIP2A dependent as overexpression of CIP2A decreased the γ-H2AX induction (I, Figure 6K). CIP2A overexpression also abrogated the SMAP response in these cells indicating that the SMAP activity is linked to its CIP2A inhibition role (I, Figure 6L).

Since we have demonstrated that CIP2A silencing sensitizes the BLBC cells to gemcitabine, we tested if SMAPs can replace CIP2A siRNA as a therapeutic strategy, and we found that SMAP DBK-1154 in combination with gemcitabine showed a strong synergy in BLBC cells, also at concentrations where single agents alone had no effect in the colony growth (II, Figure 5F).

5.5.3 Thiostrepton can be repurposed as a CIP2A transcriptional inhibitor drug for BLBC (II)

Since there are no inhibitors of CIP2A, we queried the Broad Institute's Drug Connectivity Map (CMap) repository (Lamb et al., 2006; Subramanian et al., 2017) to see if any drugs can be repurposed as CIP2A inhibitors. The list of upregulated and downregulated genes from CIP2A^{signature} (II, Figure 2A,B) was used to obtain a list of drugs that elicit similar transcriptional profiles as CIP2A downregulation from the CMap database (https://clue.io/). The top 10 drugs that mimic the CIP2A

downregulation transcriptome were identified (II, Figure 5A). Out of these, thiostrepton which was #2 on the list was tested in five different BLBC cell lines and was found to be very effective in decreasing the cell viability in these cells (II, Figure 5B). We then wanted to evaluate if this drug can replace CIP2A silencing as CMap predicted, and we saw that Thiostrepton in combination with Gemcitabine showed excellent synergy at concentrations that do not show any effect when each drug is used alone. For example, 80nM of gemcitabine or 80nM of thiostrepton alone does not have any effect on the cell viability but when combined they kill 82% of the cells (II, Figure 5C). The synergy score was calculated using the SynergyFinder tool (Ianevski et al., 2022) (https://synergyfinder.fimm.fi), which was determined as 28.6, and the best synergy window was predicted to be between the concentration range of 40nM to 160nM of Gemcitabine combined with 20nm to 80nM of thiostrepton (II, Figure 5D). We then tested this combination thiostrepton+gemcitabine on colony growth assays and found that the thiostrepton alone or gemcitabine alone at low concentrations do not inhibit the colony formation ability of the cells, but when they are combined, there is a very potent effect. This effect was observed to be irrespective of the CIP2A dependence as we saw the synergistic effect also in HCC1937 cells which are not dependent on CIP2A (II, Figure 5E).

6 Discussion

6.1 CIP2A drives BLBC by coordinating the key hallmarks

Breast cancer is one of the most widely characterized cancer subtypes genetically. Despite massive sequencing efforts, identifying genomic drivers for BLBC has not been very successful (Banerji et al., 2012; Koboldt et al., 2012; Pereira et al., 2016). This dissertation reports the first ever evidence of a non-genetic driver mechanism of BLBC. It is also the first ever reported evidence of a PP2A inhibitor playing a role as a driver for any human cancer.

Gene expression profiling of breast cancer has made it easier to identify specific molecular traits of each breast cancer subtype (Perou et al., 2000; Sørlie et al., 2001; Sotiriou et al., 2003). Some key hallmarks of BLBCs include: **1)** *High genomic instability* due to mutations in TP53 (seen in 85% of the cases), RB (seen in 30% of the cases), or defective DSB repair (for example BRCA1 mutations) (E. A. Rakha et al., 2008; Badve et al., 2011) **2)** *High proliferative activity* associated with increased MYC and E2F activity (Sotiriou et al., 2003; Alles et al., 2009) **3)** EGFR expression (or amplification in minority of cases) (Badve et al., 2011; E. Rakha & Reis-Filho, 2009) and **4)** Increased expression of cell cycle and DDR genes (Lehmann et al., 2011). Notably, all these key hallmarks also have a critical role in promoting the expression of CIP2A. CIP2A is involved in a positive feedback loop with E2F1 and MYC (Laine et al., 2013; Khanna et al., 2009). Additionally, inactivation of p53 leads to increased E2F1 which drives CIP2A expression (Laine et al., 2013). CIP2A expression is also driven by DNA-PK/CHK1 pathway (Khanna, Kauko, et al., 2013) and EGFR-ETS1 pathway (Khanna et al., 2011). We have also observed in our mammary tumorigenesis experiments with DMBA that *Cip2a* expression is induced a lot earlier before the formation of the tumors. One may argue that the CIP2A overexpression induced MYC activity is driving the BLBCs but we have noticed that CIP2A did not have a role in the Myc-dependent DMBA/TPA skin carcinogenesis model (Oskarsson et al., 2006) and hence we postulated that there is involvement of other MYC-independent mechanisms by which CIP2A drives BLBC. The newly identified role of CIP2A in DDR and CIP2A-TopBP1interaction provide the molecular basis for essential role of CIP2A in mitotic progression of DNA damaged cells. So, one plausible explanation is

that DNA damage induced CIP2A overexpression creates the required selective pressure for the progression of premalignant lesions into BLBCs, and as the BLBC hallmark traits are acquired, this creates positive feedback for further increase in the expression of CIP2A. In fact, we have noticed that the expression of *Cip2a* in the BLBC tumors was much higher than in the premalignant mammary gland.

Based on the results of my thesis, it can be observed that different levels of CIP2A expression have varying effects on the homologous repair and G2/M checkpoints as summarized in the schematic **Figure 8**. In normal tissues or unperturbed CIP2A WT cells, CIP2A expression is quite low (CIP2A level can be anywhere between A& B). Treatment with genotoxic stressors such as DMBA and IR induces double strand breaks (DSBs) which recruits TOPBP1 to the site of DSB. In this condition, the DMBA treatment also induces expression of CIP2A as observed in premalignant mammary glands. Here, the low expression of CIP2A does not fully saturate the TopBP1 required

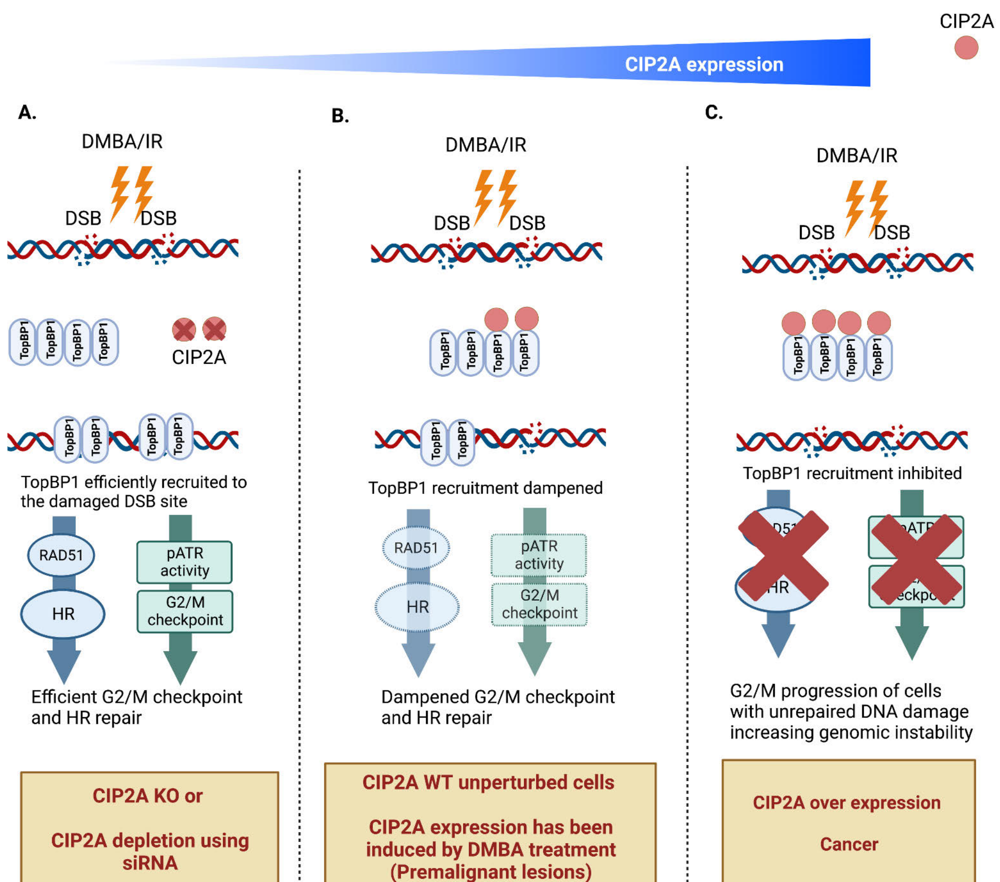

Figure 8: Schematic visualization of DNA repair and G2/M checkpoints at different expression levels of CIP2A in the cells. A) CIP2A KO or CIP2A depleted condition B) Unperturbed CIP2A WT cells and C) CIP2A overexpressed (or cancer) cells.

for complete inhibition of its function, but slightly dampens the G2/M checkpoints and HR repair. In CIP2A knockout or depletion with siRNA (Figure 8A), CIP2A is not present or very low to interact with TopBP1 and therefore the G2/M checkpoints and repair are unperturbed. Therefore, CIP2A knockout mice form less mammary tumors on DMBA treatment. When CIP2A is overexpressed (Figure 8C – note that this hasn't been tested experimentally) I envision that the excessive CIP2A may fully saturate the TopBP1 inhibition, resulting in further defective DNA repair and checkpoints. This could allow the mitotic progression of damaged and unrepaired DNA increasing the genomic instability as seen in cancer cells (Figure 8C).

While the interaction between CIP2A and TopBP1 is independent of PP2A, we have some evidence from a recent phosphoproteomics screen that Nibrin (NBN), which is one of the co-dependent CIP2A genes has a phosphosite on serine 432 whose dephosphorylation by PP2A is inhibited by CIP2A (Kauko et al., 2020). Also, Nibrin is known to interact with TopBP1(Morishima et al., 2007; Yoo et al., 2009). So, while being present in the complex with TopBP1, CIP2A may regulate phosphorylation of other PP2A targets, and this complex phospho-regulation is yet to be evaluated. Also, it would be interesting to gain more insights from the CIP2A structure point of view about which amino acids or regions within CIP2A are associated with the PP2A binding functions and which regions are associated with the PP2A independent DDR functions so that drugs can be developed to selectively inhibit specific functions of CIP2A.

In summary, our data convincingly indicates that CIP2A is at the crossroads of PP2A dependent high proliferative activity, driven by E2F1, MYC, TP53, EGFR and DNA-PK activity, and a PP2A independent TopBP1 interaction, allowing the abrogation of G2/M checkpoints. This coordination is essential for BLBC initiation and progression (**Figure 9**).

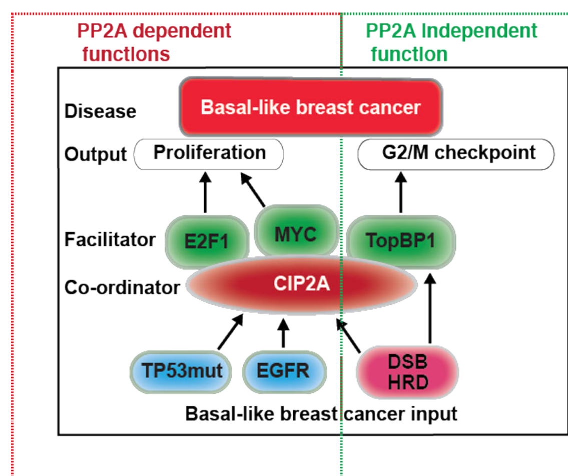

Figure 9: Role of CIP2A as a coordinator of BLBC hallmarks. Adapted from (Laine et al. 2021).

6.2 CIP2A is a critical DDR protein in BLBC

This dissertation has discovered CIP2A's first ever PP2A independent function as a regulator of DNA damage response (DDR). The finding that CIP2A is most co-dependent on a genome-wide level with several well characterized *bona fide* DDR proteins, has sparked up a lot of interest and opened up a new direction of research (I). Some of our novel findings from this dissertation have also been independently validated by two different groups. Daniel Durocher group reported the role of CIP2A-TOPBP1 complex in efficient chromosomal segregation of under replicated DNA. They also reported that CIP2A is essential in BRCA mutated cancer (Adam et al., 2021). Manuel Stucki group reported the role of CIP2A-TopBP1-MDC1 in efficient DNA DSB repair during mitosis (De Marco Zompit et al., 2022). None of these papers have been able to still elucidate the exact molecular mechanism of CIP2A-TOPBP1 complex in their newly identified functions. There are few more preprints which have identified new functions of CIP2A in DDR such as role of CIP2A-TOPBP1-MDC1 complex in repairing DNA damage during Meiosis (Leem et al., 2022) or tethering of shattered chromosomal fragments during mitosis (Trivedi et al., 2022). There are many CIP2A co-dependent genes other than TopBP1 that we recognized from DepMap portal (https://depmap.org/portal/gene/CIP2A) but have not been explored, and trying to understand their role and link with CIP2A might explain the selectivity of CIP2A specifically in BLBC. One such interesting prospect is POLQ, which is involved in micro-homology mediated end joining (MMEJ) an alternative form of NHEJ. It has been reported that HR deficient tumors overexpress and are highly dependent on POLQ (Ceccaldi et al., 2015; Wyatt et al., 2016). The fact that POLQ and CIP2A are co-expressed in BLBC (I, Figure S4B) indicates that it might be closely involved in the processes regulated by CIP2A.

From analysis of the CIP2A signature genes, we identified that CIP2A also has a role in regulating the transcription of several DDR proteins (II) in addition to our reported role as interaction partner of TopBP1 and the phosphoregulation of PP2A targets within the TopBP1-complex (I). We identified a novel role for CIP2A in Base Excision Repair (BER) associated with OGG1, a glycosylase which has a role in recognizing and repairing the oxidative damage of guanines. We recently reported that CIP2A downregulation causes sensitization to ROS-inducing drugs such as APR-246 in ovarian cancer cells (Cvrljevic et al., 2022). Since CIP2A downregulation causes OGG1 associated BER defect, the CIP2A low cells are expected to be extremely vulnerable to oxidative damage (as caused by ROS). So, our results are consistent and, in a way, explain the reason for the sensitization of the CIP2A downregulated cells to ROS inducing drugs. The exact molecular mechanisms of how CIP2A regulates the BER pathways and other DDR targets is yet to be identified.

6.3 Therapeutic targeting of CIP2A-regulated DDR signaling in BLBC

In this dissertation, I evaluated the role of recently developed small molecule activators of PP2A (SMAPs) such as DT-061, DBK-1154 (or DT-1154) and DT-1160 in targeting CIP2A. Since there are no known drugs that directly target CIP2A, we used SMAPs as they are orally bioavailable drugs with a good safety profile and tested across multiple cancer types as single agents and in combination with other inhibitors (Sangodkar et al., 2017; Kauko et al., 2018; McClinch et al., 2018; Merisaari et al., 2020; Allen-Petersen et al., 2019). DT-061 is a molecular glue that binds and selectively activates the PP2A-B56α complexes (Leonard et al., 2020; Westermarck & Neel, 2020), the same complex that CIP2A has been reported to inhibit (J. Wang et al., 2017). We tested SMAPs on a panel of TNBC patient derived stem like cells, which were isolated from the patients who received a few rounds of neoadjuvant chemotherapy and were resistant to the standard chemotherapy regimens (Metzger et al., 2017). The cells showed resistant to standard of care BLBC chemotherapy drugs as expected, but interestingly, were sensitive to SMAPs indicating the potential use of SMAPs in patients not responding to conventional chemotherapy. Furthermore, our experiments revealed a serendipitous finding that SMAPs can downregulate CIP2A on the mRNA and protein level, which was previously unknown (I, Figure 6).

We investigated the kinetics of MYC, ERK, and CIP2A on SMAP treatment as PP2A reactivation by SMAPs is known to downregulate MYC (Allen-Petersen et al., 2019) and ERK activity (Sangodkar et al., 2017), both of which can transcriptionally regulate CIP2A inhibition. Our findings showed that ERK dephosphorylation occurred before CIP2A downregulation, followed by the inhibition of MYC activity. Therefore, SMAPs have a biphasic effect where they first activate the PP2A-B56α complex and then sustain its activation due to CIP2A transcriptional downregulation. While SMAPs showed some discrepancy in the DDR compared to the CIP2A inactivation, our evaluation of CHK2, ATR, and γ-H2AX activity kinetics revealed that CHK2 phosphorylation happened even before the CIP2A downregulation, while ATR activation and γ-H2AX induction began only after CIP2A downregulation. We have used SMAPs as surrogate CIP2A transcriptional inhibitors, but it is important to note that SMAPs also have other CIP2A independent effects associated with direct PP2A activation.

The success of PARP inhibitors has spurred a search for synthetic lethal mechanisms to target different types of cancer. BLBCs are known to exhibit a high degree of genomic instability due to mutations in critical DNA repair pathways, making them highly dependent on alternative pathways. (Ashworth & Lord, 2018; O'Neil et al., 2017). For instance, HR deficient tumors have been shown to rely heavily on NHEJ/MMEJ or BER. Based on CIP2A signature indicating that CIP2A

depletion results in BER defects and downregulation of HR genes, we aimed to identify vulnerabilities that could be targeted. Our hypothesis was that CIP2A silenced cells would be extremely susceptible to DNA damage and replication stress inducing drugs. Accordingly, we screened standard chemotherapy drugs and found that gemcitabine exhibited a strong synergy in CIP2A low cells. Gemcitabine is a prodrug that, once metabolized, produces nucleoside analogues, that incorporate into the DNA and stall the replication machinery, causing single and double strand breaks (de Sousa Cavalcante & Monteiro, 2014; Jones et al., 2014). This could explain why CIP2A low BLBCs are sensitive to gemcitabine. To uncover more targets, high throughput drug screening could be conducted in the future with a more extensive panel of DDR drugs in the CIP2A high and CIP2A low BLBC cells.

We also identified thiostrepton as a repurposed CIP2A transcriptional inhibitor from the Drug Connectivity Map database. Thiostrepton is a natural product and has been FDA approved for topical use as a veterinary antimicrobial agent (T. H. Kim et al., 2019). It has been recently granted an orphan designation for malignant mesothelioma (https://www.ema.europa.eu/). Also, a clinical trial is currently recruiting patients for dose escalation and dose expansion studies to evaluate thiostrepton in malignant pleural effusions and mesotheliomas (https://clinicaltrials.gov/ct2/show/record/NCT05278975). Drug repurposing has benefits that since these drugs have been tested for other clinical indications in humans, and they usually have a good pharmacokinetic and safety profile. Also, developing them for other indication would not involve major costs (Pushpakom et al., 2019). However, when we tested the thiostrepton+gemcitabine combination *in vivo*, the study had to be terminated within 10 days from the start of the dosing because thiostrepton was extremely toxic and drastically affected the wellbeing of the mice (decrease of mice weights in both thiostrepton and thiostrepton+gemcitabine groups (Unused data – **Figure 10**).

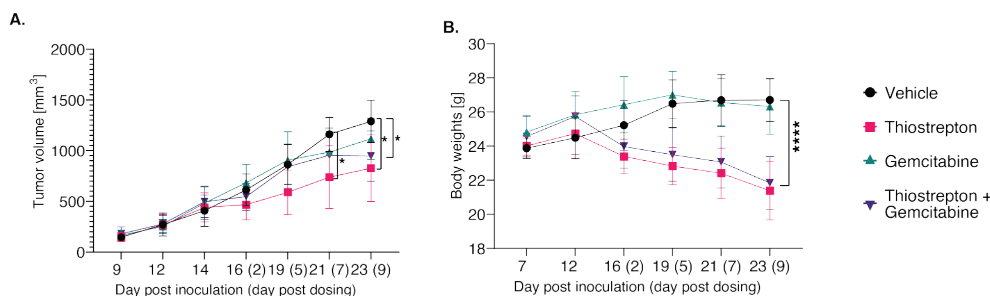

Figure 10: Orthotopic mammary fat pad xenograft experiment in nude mice with MDA-MB-231 cells, to test the Thiostrepton+Gemcitabine combination *in vivo*. Tumor volumes (A) and body weights (B) of the mice in the study. N=8 mice were used for each group (Unused data).

The toxicity of thiostrepton *in vivo* highlights the importance of carefully evaluating the safety and efficacy of repurposed drugs in preclinical and clinical studies before moving forward with their use in patients. While drug repurposing has the potential to accelerate drug development and improve patient outcomes, it is crucial to ensure that the drugs are safe and effective for the intended use. Additionally, it is important to consider potential drug interactions and toxicity when combining drugs, as seen in the case of thiostrepton and gemcitabine. Further studies are needed to identify safer and more effective combinations of drugs for targeting CIP2A in BLBCs.The dosing regimen for thiostrepton+gemcitabine used in this study was adapted from a published study testing thiostrepton in combination with oxaliplatin (Y. Wang et al., 2020). When the tumors were palpable, thiostrepton was dosed at 200mg/kg *i.p.* a day before, on the day and the day after the initiation of gemcitabine chemotherapy (35 mg/kg *i.p.* once every week). It was followed by 40mg/kg maintenance dose of thiostrepton every two days. However, we did not conduct a safety or dose escalation study to identify the effective concentration of thiostrepton in combination with gemcitabine, which could have contributed to the extreme toxicities observed. To ascertain the toxicity of thiostrepton, a careful dose escalation and toxicity study should be conducted before combining it with gemcitabine. Furthermore, there are several other FOXM1 inhibitors such as FDI-6 and RCM-1 that have been reported in the literature (Gormally et al., 2014; Sun et al., 2017) and could be evaluated for their pharmacokinetic, safety and activity profile.

Thiostrepton is a known inhibitor of FOXM1 (Forkhead box M1) (Hegde et al., 2011), a transcription factor that regulates DDR genes and confers resistance to DNA damaging agents (Kwok et al., 2010; Monteiro et al., 2013; Zhang et al., 2012). In the METABRIC study, Curtis and colleagues integrating genome and transcriptomes of 2000 breast cancer samples and classified breast cancer into 10 integrated clusters, with FOXM1being part of the BLBC enriched IntClust10 group. The genes in this group are known to regulate genomic and chromosomal instability (Curtis et al., 2012). Thiostrepton's selection as a top target from the drug repurposing database was particularly insightful because CIP2A signature also regulates genomic instability and DDR functions. We are yet to uncover whether CIP2A regulates DDR through FOXM1, but there is evidence that E2F1 is involved in the activation of FOXM1 and the downstream DDR functions (Bosquet et al., 2021). Since CIP2A is involved in a positive feedback loop with E2F1 (Laine et al., 2013), it is plausible that CIP2A downregulation can lead to downregulation of FOXM1 and DDR via E2F1. The potential link between CIP2A and FOXM1 through E2F1 in regulating DDR is an intriguing avenue for further investigation.

6.4 Clinical relevance of CIP2A in BL-TNBCs

Contrary to the earlier notions that CIP2A is critical for the development of several human cancer types (Khanna, Pimanda, et al., 2013; Khanna & Pimanda, 2016), our results indicate that CIP2A is actually very selective and important only in BLBCs. Additionally, we found that CIP2A plays a selective role as a prognostic biomarker in patient material, where high levels of CIP2A are only correlated with poor prognosis in basal-like TNBCs but not in non-basal like BLBCs, ER+ breast cancer, or breast cancer patients as a whole (I, Figure 5, S6). Furthermore, we discovered that CIP2A is essential in TP53/BRCA mutant BLBCs but is dispensable in p53/ECadherin or ECadherin/AKT mutant cells, which represent invasive lobular carcinoma type. This finding was corroborated in an independent study by Daniel Durocher group who found that CIP2A is synthetic lethal target in p53/BRCA mutant cells (Adam et al., 2021). Additionally, we analyzed the most CIP2A dependent breast cancer cell lines from DepMap and found that most of the highly CIP2A dependent cell lines were also BRCA1/2 mutant.

To summarize, the results of my thesis indicate that CIP2A has the potential to serve as a biomarker for identifying particularly aggressive BL-TNBCs and as a synthetic lethal target in BRCA mutant BLBCs.

6.5 CIP2A transcriptional signature helps in personalized treatment of BLBC

BLBC is a complex and heterogeneous disease, which encompasses several sub diseases. Patients presenting with same histopathological features may have very distinct clinical prognosis or outcomes (Badve et al., 2011; Marra et al., 2020; Toft & Cryns, 2011). With the advances in sequencing technologies, many different gene expression-based classifications of breast cancer have been reported (Perou et al., 2000; Sørlie et al., 2001; Sotiriou et al., 2003; van ’t Veer et al., 2002). Although these classification methods have proven to have a great prognostic and predictive value, their routine utilization in the clinic has been very limited and clinicians have resorted to surrogate pathological markers for subtyping the breast cancer (Prat et al., 2015; Varnier et al., 2021; Weigelt et al., 2011). Despite BLBC being very heterogenous, in the clinic, it is treated uniformly as one single disease with the same chemotherapy (Marra et al., 2020). Since BLBC has an aggressive disease progression, even the early-stage tumors are treated immediately with aggressive polychemotherapy to prevent metastasis. Although this aggressive approach reduces the risk of distant metastases by one-third, it has been estimated that only 20-30% of the early stage breast cancer patients who receive the adjuvant polychemotherapy actually derive the benefit, while everyone is at a risk of toxic side effects (Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) et al., 2012; Early Breast

Cancer Trialists' Collaborative Group (EBCTCG), 2019). Although some of these molecular profiling tools can predict the outcomes and progression of the disease, they are mostly suitable for ER+ breast cancer. In BLBC the clinical decisions on who receives the chemotherapy relies on histopathological factors such as stage and tumor grade (Caldas & Aparicio, 2002; Badve et al., 2011; Varnier et al., 2021) Thus, there is definitely a clinical unmet need for better signatures that can aid in patient stratification and personalized treatment.

The CIP2A signature generated in this dissertation (II) can be used to identify aggressive breast cancer patients. Our results show that the median survival of CIP2A negative signature patients is almost 250 months, compared to 120 months for CIP2A positive signature patients (II, Figure 2C), and hence they are expected to have a less aggressive disease. The signature needs further validation in a clinical trial setting, but there is potential for it to identify patient populations that derive benefit from treating aggressively with chemotherapy and suggest other less aggressive treatments for CIP2A negative signature patients. Therefore, the critical clinical recommendation from my dissertation is to stratify breast cancer patients based on aggressivity and de-escalate aggressive chemotherapy in less aggressive breast cancer cases so that they can be spared from unnecessary side effects. Moreover, this signature needs further improvement, so that it can be utilized routinely in the clinic. The signature currently has 225 genes and in order to have applicability for routine use in the clinic, we need to downsize the signature and validate it, to ensure it behaves the same way as the current version of the signature. The way I envision the signature being applied in the clinic has been outlined in the schematic **Figure 11**. Tumor biopsy samples from breast cancer patients can be processed to run directly on a qPCR plate format or Nanostring format. Based on the expression profile of the signature genes, the patient would be scored as positive, negative, or inconclusive (unclassified). We have also identified drugs such as gemcitabine and ATR inhibitors, that are effective in CIP2A negative signature patients. In CIP2A signature positive patients, we suggest using SMAP or thiostrepton which have been established as CIP2A transcriptional inhibitors in combination with gemcitabine and ATR inhibitors. Using high throughput drug screening, it is possible to identify more drugs which are effective for each of the stratified subgroups in the future.

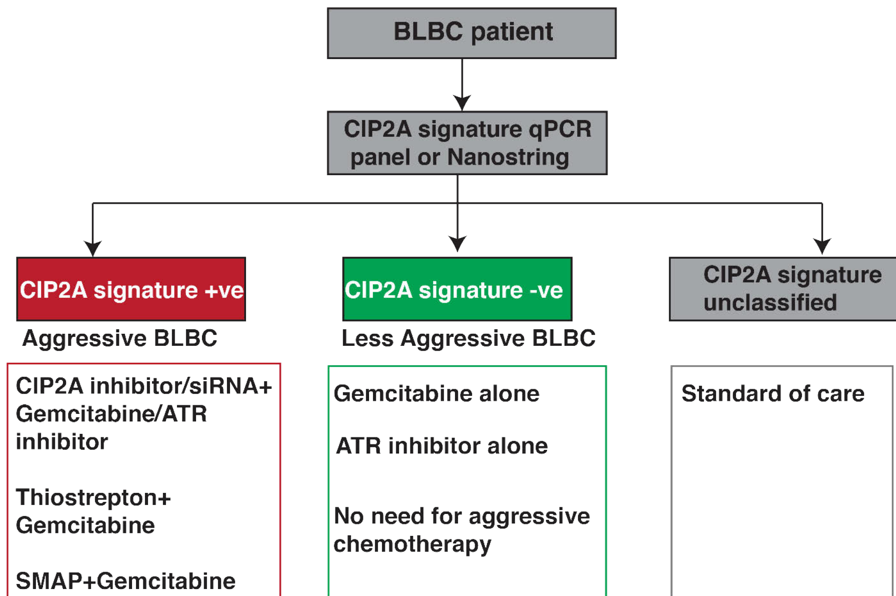

Figure 11: Patient stratification of BLBC using the CIP2A signature. Figure reproduced from (Nagelli et al. *Manuscript*).

Overall, our signature highlights the potential of the CIP2A transcriptional signature to tailor therapies based on the aggressiveness of the patient's disease.

7 Conclusions

The two main objectives of my thesis were to identify driver mechanisms that can be targeted for BLBC and to develop a transcriptional signature that can facilitate patient stratification and personalized treatment for BLBC. We have identified several clues to improve the overall treatment landscape of BLBC patients. The most significant contribution of this thesis is the identification of a novel role of CIP2A as a critical DDR protein which opens new avenues and directions for future research in this topic.

Based on the results of my dissertation the following key conclusions can be made:

1. CIP2A is a novel non-genetic driver protein of BLBC. CIP2A interacts with TopBP1 and inhibits its chromatin recruitment at the site of DNA double strand breaks. CIP2A also promotes the progression of DNA damaged cells into mitosis by dampened G2/M activity.
2. CIP2A coordinates the key hallmarks of BLBC. CIP2A regulates both the PP2A dependent E2F1, MYC hyperproliferation activity and PP2A independent TopBP1 dependent G2/M activity. It is the first endogenous PP2A inhibitor which has been reported to be a driver in any human cancer.
3. CIP2A is a prognostic biomarker for basal-like TNBC and a synthetic lethal target for BRCA mutant BLBCs.
4. CIP2A can be targeted by surrogate CIP2A transcriptional inhibitors such as SMAPs and thiostrepton. SMAPs have a biphasic effect that they directly activate PP2A first, followed by CIP2A downregulation which can prolong the PP2A activation.
5. CIP2A low BLBCs are sensitive to ATR inhibition and gemcitabine whereas CIP2A high BLBCs can be turned sensitive to ATR inhibition or gemcitabine by combining them with SMAP or thiostrepton.
6. CIP2A signature has the potential to be used to stratify BLBC patients based on aggressivity and to tailor personalized treatment regimens.

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